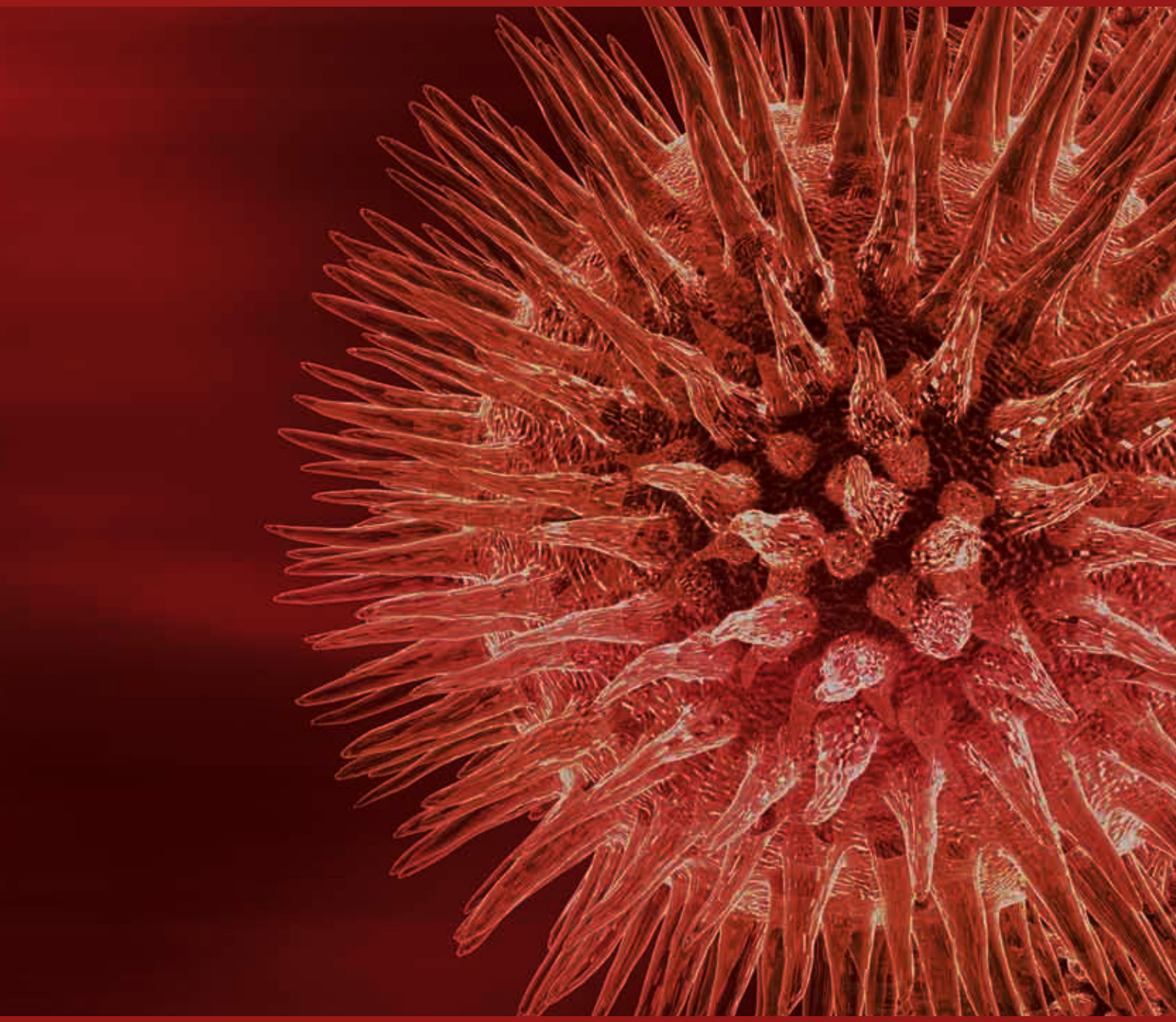
BioMed Research International

Immunology and Cell Biology of Parasitic Diseases 2013

Guest Editors: Luis I. Terrazas, Abhay R. Satoskar, Miriam Rodriguez-Sosa, and Jorge Morales-Montor



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Contents

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Editorial

Immunology and Cell Biology of Parasitic Diseases 2013

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In this, our third, special issue we have chosen 19 reports, including both original and review papers, which represent 60% of the papers submitted to this new special issue. This is a sample that we have maintained the quality of the submissions as an essential requirement to get published in this new era of BioMed Research International (formerly Journal of Biomedicine and Biotechnology).

This special issue was supported mainly by some of the attendants to our V Mexican Immunoparasitology meeting, which has been continuously holding every two years since starting in 2004. This event was created with the expectation to meet Mexican scientist and overseas guest scientists interested in the field of immunoparasitology, given that every time is more difficult to find right places on parasitological congresses as well as in immunological congresses to discuss this interface of knowledge, which has great importance.

Infections by parasites, starvation, insufficient shelter, and lack of clean water sources are the greatest barriers to health in our world's growing population. Several of the parasitic infections that are very common throughout the world frequently occur with mild, obscure symptoms or none at all. It is common for a host to be asymptomatic, which makes more difficult both the diagnostic and the on time treatment. Parasitic infections that are thought to be the most prevalent worldwide include toxoplasmosis, ascariasis, toxocariasis, hookworm disease, amoebiasis, and trichomoniasis; many of them are discussed in this special issue. The scope of this topic could be immense, as there are hundreds of parasitic species that infect humans, and also because parasitic infections are considered as one of the leading causes of high morbidity and mortality in underdeveloped countries, mainly in children and the elderly, affecting the physical and intellectual capabilities of such population and resulting in high expenses in treatment and rehabilitation. Moreover, in recent years the massive migration of people from the south countries, where parasitic diseases are common, towards developed countries fueled that many parasitic infections are now reemerging and generating new health problems at a global level.

The knowledge about parasitic diseases at different levels, such as life cycles, structures, molecular biology, development of new drugs and vaccines, and its ability to escape or modulate different mechanisms of defense of its hosts, is essential to better development of therapies and diagnostics. This special issue addresses many of such issues. We have divided the special issue in two blocks, the first dealing with protozoan parasites and the second one dealing with different helminth infections. In both you will find new and important information related to parasitic diseases.

In our first block (protozoan infections) we started with a very opportune review by A. Vazquez-Mendoza et al. "*Parasitic infections: a role for C-type lectins receptors*" where they describe how important, as well as complicated, is the early recognition of parasites by C-type lectin receptors (CLRs), which are expressed by distinct subsets of dendritic cells and macrophages. They address the question: how are CLRs involved in both pathogen recognition and the

internalization of parasites? They conclude that parasite recognition through different carbohydrates localized on its surface or in the excretory/secretory products are essential to the outcome of the infection, and this seems as a new opportunity area to block the entrance or increase the immune response against them. Next, we have a couple of papers related to amoebiaisis studies: first M. Omaña-Molina et al., propose a new role for the proteases from *Acanthamoeba* in the tissue invasion in "*Reevaluating the role of acanthamoeba proteases in tissue invasion: observation of cytopathogenic mechanisms on MDCK cell monolayers and hamster corneal cells*". As they remark, the most important observation in this study was the fact that proteases apparently do not participate in tissue destruction. By challenging the paradigm, they found that no lysis of corneal tissue was observed as it was previously suggested. Thus, their results support the notion that the invasion and disruption of corneal tissue during acantamoebiasis is performed by the penetration of the amoebae through cell junctions, either by the action of proteases promoting cellular separation but not by their destruction and/or a mechanical effect exerted by amoebae. In the second one H. Aguilar-Diaz et al. used new technology tools to demonstrate that silencing an enzyme in *Entamoeba histolytica* avoids the formation of cysts. This is very important finding since encystment is an essential process in the biological cycle of the human parasite *E. histolytica*; these findings may allow to develop new control strategies against this parasite as stated in "*Silencing of Entamoeba histolytica glucosamine 6-phosphate isomerase by RNA interference inhibits the formation of cyst-like structures*".

Trypanosomatids parasites are a group of protozoan that importantly affects humans. In this area C. Lugo-Caballero et al. in "*Identification of protein complex associated with LYT1 of Trypanosoma cruzi*" identified a protein complex associated with LYT1 in *Trypanosoma cruzi*. This is important because few molecules have been reported participating in the intracellular phase of *T. cruzi* life cycle. Their approach led them to identify the LYT1 interaction profile, thereby providing insights into the molecular mechanisms that contribute to parasite stage development and pathogenesis. In another study, J. A. Díaz-Gandarilla et al. "*PPAR activation induces M1 macrophage polarization via cPLA2-COX-2 inhibition, activating ROS production against Leishmania Mexicana*" showed that PPAR activation induces M1 macrophage polarization via cPLA2-COX-2 inhibition, thus, activating ROS production against *Leishmania mexicana*. By doing a very thorough analysis, they conclude that PPAR agonists used in their work induce M1 macrophages polarization via inhibition of cPLA2 and the increase of aggressive microbicidal activity via reactive oxygen species production. To finalize with the collaborations in this topic, Y. Flores-García et al. "*$CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells induced by rSSP4 derived from T. cruzi amastigotes increase parasitemia in an experimental chagas disease model*" discuss the role in Treg cells induced by rSSP4 derived from *T. cruzi* amastigotes have in increasing parasitemia in an experimental Chagas' disease model. As authors state, currently, there is a considerable controversy on the role of Treg cells during *T. cruzi* infection, the main point being whether these cells play a negative or a positive role. They found that the adoptive transfer of $CD4^+$ $CD25^+$ $FOXP3^+$ T cells from rSSP4- (a recombinant *T. cruzi* amastigote derived protein) reduces cardiac inflammation and prolongs hosts' survival but at the same time, increases blood parasitemia and parasite loads in the heart.

Trichomoniasis is one of the most common sexually transmitted infections in the world; in this issue F. J. Rendón-Gandarilla et al., by studying *Trichomonas vaginalis* cytoadherence, found that the TvLEGU-1, a legumain-like cysteine proteinase, plays a key role in pathogenesis of this parasite.

In our second section, related with helminth infections, several authors discuss relevant issues associated with these widely distributed infections.

B. Faz-López et al. in "*Signal transducer and activator of transcription factor 6 signaling contributes to Control host lung pathology but favors susceptibility against Toxocara canis infection*" found that the signal transducer and activator of transcription factor 6 signaling contributes to control host lung pathology during *T. canis* infection. However, though it helps in the lung, it does favor to increase the susceptibility against this widely distributed parasite. Their findings demonstrate that a Th2-like response induced via STAT6-mediated signaling pathway mediates susceptibility to larval stage of *T. canis*. Furthermore, they also indicate that unlike most gastrointestinal helminths, immunity against larvae of *T. canis* is not mediated by a Th2-dominant response.

Taenia crassiceps is a cestode parasite of rodents (in its larval stage) and canids (in its adult stage) that can also parasitize immunocompromised humans. In the case of the hygiene hypothesis, A. M. Ortiz-Flores et al. in "*Taenia crassiceps infection does not influence the development of experimental rheumatoid arthritis*" found that *T. crassiceps* infection does not influence the development of experimental rheumatoid arthritis. On the same line, A. N. Peón et al. in "*Immunoregulation by Taenia crassiceps and its antigens*" discuss the potential role that antigens from *T. crassiceps* have as immunoregulators. The authors have discovered that in mice and human cells, the whole parasite or its antigens have a strong capacity to induce chronic Th2-type responses that are primarily characterized by high levels of Th2 cytokines, low proliferative responses in lymphocytes, an immature and LPS-tolerogenic profile in dendritic cells, the recruitment of myeloid-derived suppressor cells, and, specially, alternatively activated macrophages. Thus, they thoroughly review the work of others and themselves in regard to the immune-modulation induced by *T. crassiceps* and its antigens and compare their advances in the understanding of this parasitic infection model with the knowledge that has been obtained from other selected models. Moreover, L. I. Terrazas et al. in the paper "*Helminth excreted/secreted antigens repress expression of LPS-induced let-7i but not miR-146a and miR-155 in human dendritic cells*" have found that helminth excreted/secreted antigens of *T. crassiceps* repress the expression of LPS-Induced Let-7i but not miR-146a and miR-155 in human dendritic cells. Since microRNAs have emerged as early key regulators of immune responses due to their influence on immune cells' function and probably the outcome of several infections. Currently, it is largely

unknown if helminth parasites and their antigens modify host microRNAs expression. This let-7i downregulation in dendritic cells constitutes a novel feature of the modulatory activity that helminth-derived antigens exert on their host.

Macrophages are also critically involved in the interaction between *T. crassiceps* and the murine host immune system. Also, a strong gender-associated susceptibility to murine cysticercosis has been reported. Thus, C. Togno-Pierce et al. in "*Sex-associated expression of Co-stimulatory molecules CD80, CD86, and accessory molecules, PDL-1, PDL-2 and MHC-II, in F480+ macrophages during murine cysticercosis*" examined the sex-associated expression of MHC-II, CD80, CD86, PD-L1, and PD-L2 expressed on peritoneal F4/80+ macrophages of BALB/c mice exposed to *T. crassiceps* total extract. They found that female mice recruited higher number of macrophages to the peritoneal cavity than males. Furthermore, macrophages from infected animals showed increased expression of PDL2 and CD80 that was dependent from the host gender. Their findings suggest that macrophage recruitment at early time points during *T. crassiceps* infection is a possible mechanism that underlies the differential sex-associated susceptibility displayed by the mouse gender.

Schistosomiasis, caused by infection with *Schistosoma* species, remains an important parasitic zoonosis. In the design of a better DNA vaccine against the human trematodes species of *Schistosoma*, Y. Cao et al. in "*Gene gun bombardment with DNA-coated golden particles enhanced the protective effect of a DNA vaccine based on thioredoxin glutathione reductase of Schistosoma japonicum*" developed a gene gun bombardment with DNA-coated golden particles that enhanced the protective effect of a DNA vaccine based on a thioredoxin glutathione reductase of *Schistosoma japonicum*, which was very effective when tested in vivo.

In the field of biotechnology, G. Peña et al. in "*In vitro ovicidal and cestocidal effects of toxins from Bacillus thuringiensis on the canine and human parasite Dipylidium caninum*" showed that toxins from *B. thuringiensis* have a strong in vitro ovicidal and cestocidal effects on the parasite *Dipylidium caninum* which causes an important zoonosis in dogs and humans. *B. thuringiensis* is a gram-positive soil-dwelling bacterium that is commonly used as a biological pesticide. Authors remark that this bacterium may also be used for biological control of helminth parasites in domestic animals. They found that proteins of the strain GP526 of *B. thuringiensis* directly act upon *D. caninum* showing ovicidal and cestocidal effects. Thus, *B. thuringiensis* is proposed as a potential biological control agent against this zoonosis.

T. Çelik et al. in the paper "*Toxocara seroprevalence in patients with idiopathic Parkinson's Disease: chance association or coincidence?*" studied specific IgG antibodies against *T. canis* in 50 patients with idiopathic Parkinson and 50 healthy volunteers. They investigated the clinical history of three patients infected with *T. canis*. They also studied specific IgG antibodies against *Toxoplasma gondii* in these groups. Antibodies anti-*Toxocara* were found in 3 idiopathic PD (6%) and antibody titer was not found in control. A patient had history of the presence of dog and current dog ownership. They did not detect any statistically significant association between *T. canis* and IPD. But, we believe that further comprehensive studies are required for understanding whether there is a causal relation between toxocariasis and IPD. In another nonrelated topic to parasitology, but very important to immunobiology, M. G. R. García and F. G. Tamayo in "*The importance of the nurse cells and regulatory cells in the control of T lymphocyte responses*" discuss the Importance of the nurse cells in the control of T lymphocyte responses. The modulatory role that neurotransmitters and hormones play in these interactions is also revised in their contribution. F. Alba-Hurtado and M. A. Muñoz-Guzmán in "*Immune responses associated with resistance to haemonchosis in sheep*" review the immune responses associated with resistance to haemonchosis in sheep. Their contribution examines the actual known on immunological and genetic factors associated with sheep resistance to infection by *Haemonchus contortus*. Interestingly, such resistance appears to be an inheritable genetic trait.

Human neurocysticercosis by *Taenia solium* is considered an emergent severe brain disorder in developing and developed countries. Discovery of new antiparasitic drugs has been recently aimed to restrain differentiation and establishment of the *T. solium* adult tapeworm, for being considered a central node in the disease propagation to both pigs and humans. On regard to new parasiticidal drugs against *T. solium*, R. Hernández-Bello et al. in "*A new parasiticidal compound in T. solium cysticercosis*" reported a new parasiticidal compound. They tested the effect that 16α-bromoepiandrosterone (EpiBr), a dehydroepiandrosterone (DHEA) analogue, had on the cysticerci of *T. solium* in both in vitro and in vivo studies. Authors showed that in vitro treatment of *T. solium* cultures with EpiBr reduced scolex evagination, growth, motility, and viability in a dose- and time-dependent fashion. Administration of EpiBr prior to infection with *T. solium* cysticerci in hamsters reduced the number and size of developed tapeworms in the intestine, compared with controls. These effects were associated with an increase in splenocyte proliferation in infected hamsters. These results leave open the possibility of assessing the potential of this hormonal analogue as a possible antiparasite drug, particularly in cysticercosis and taeniosis. Finally, G. Escobedo et al. in "*Tamoxifen treatment in hamsters induces protection during taeniosis by Taenia solium*" demonstrated that tamoxifen (an anti-oestrogen) treatment in hamsters induces protection during taeniosis by *Taenia solium*. Their results showed that tamoxifen inhibited evagination of *T. solium* cysticerci in a dose-time-dependent manner. In vivo, administration of tamoxifen to hamsters decreased the intestinal establishment of the parasite by 70%, while recovered tapeworms showed an 80% reduction in length, appearing as scolices without strobilar development. Since tamoxifen did not show any significant effect on the proliferation of antigen-specific immune cells, intestinal inflammation, and expression of Th1/Th2 cytokines, in spleen and duodenum, this drug could exert its antiparasite actions by having direct detrimental effects upon the adult tapeworm.

We hope our readers find this third special issue of enticing and enjoy reading contributions by all authors.

Acknowledgments

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Research Article

Identification of Protein Complex Associated with LYT1 of *Trypanosoma cruzi*

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To carry out the intracellular phase of its life cycle, *Trypanosoma cruzi* must infect a host cell. Although a few molecules have been reported to participate in this process, one known protein is LYT1, which promotes lysis under acidic conditions and is involved in parasite infection and development. Alternative transcripts from a single *LYT1* gene generate two proteins with differential functions and compartmentalization. Single-gene products targeted to more than one location can interact with disparate proteins that might affect their function and targeting properties. The aim of this work was to study the LYT1 interaction map using coimmunoprecipitation assays with transgenic parasites expressing LYT1 products fused to GFP. We detected several proteins of sizes from 8 to 150 kDa that bind to LYT1 with different binding strengths. By MS-MS analysis, we identified proteins involved in parasite infectivity (trans-sialidase), development (kDSPs and histones H2A and H2B), and motility and protein traffic (dynein and α- and β-tubulin), as well as protein-protein interactions (TPR-protein and kDSPs) and several hypothetical proteins. Our approach led us to identify the LYT1 interaction profile, thereby providing insights into the molecular mechanisms that contribute to parasite stage development and pathogenesis of *T. cruzi* infection.

1. Introduction

American trypanosomiasis is a disease that is caused by *Trypanosoma cruzi*, an obligate intracellular parasite that infects a variety of mammalian host cells. This disease is endemic in Latin America, where it affects approximately 18 million people, and more than 100 million people are at risk of infection [1]. *T. cruzi* undergoes a complex biphasic life cycle that alternates between two developmental stages in the reduviid beetle vector (i.e., epimastigotes and metacyclic trypomastigotes) and two developmental stages in the mammalian host (i.e., amastigotes and blood trypomastigotes). In the beetle, the flagellated epimastigote proliferates in the midgut before differentiating into the nondividing but infectious metacyclic trypomastigote, which is found in the vector's hindgut. The parasite infects host cells after its introduction into mammalian blood, differentiates into the amastigote, and initiates replication in the cytosol of the infected cell. Ultimately, the amastigotes develop into nondividing bloodstream trypomastigotes, which can either initiate another round of infection or be ingested by the reduviid vector during a blood meal. The life cycle is completed upon the development of epimastigotes from bloodstream trypomastigotes.

Although the infection process of *T. cruzi* was described many years ago, the molecular mechanisms involved remain poorly understood. The parasite infects diverse professional and nonprofessional phagocytes by a process that appears to involve several discrete steps, beginning with the attachment of the parasite to the host cell and followed by its internalization through a parasitophorous vacuole, from which it escapes to multiply freely in the cytosol.

Subsequently, it differentiates into the bloodstream trypomastigote form and is ultimately liberated from the host cell. Although many proteins are undoubtedly important for *T. cruzi* infection, surprisingly few have been identified experimentally. However, one such protein is LYT1, which is a lytic protein that plays a critical role in the parasite infection and stage transition processes [2]. *LYT1* is a single-copy gene that encodes three distinct *LYT1* mRNAs through alternative trans-splicing of the primary transcript, which is differentially regulated during the parasite life cycle. Two transcripts encode full-length LYT1 proteins that contain an N-terminal signal sequence and a nuclear localization sequence, and the third transcript encodes a truncated LYT1 protein lacking the signal sequence and only containing the nuclear localization sequence [3]. *LYT1*-deficient parasites are infection deficient, display accelerated *in vitro* development, and have diminished hemolytic activity in acidic conditions [2]. The differential reconstitution of the two *LYT1* products in null parasites showed that the full form of the protein is localized to the plasma membrane and reverts the infection deficiency phenotype, while the truncated form of the protein is localized in the mitochondrial kinetoflagelar zone and reverts the accelerated *in vitro* stage differentiation phenotype [4]. The differential localization of the full and truncated forms of LYT1 was later confirmed using transgenic parasites that express an exogenous copy of LYT1 fused to EGFP. Furthermore, these studies also revealed that both forms of the LYT1 protein are localized in the nucleus and kinetoplast zone [5].

It is well known that single eukaryotic genes can give rise to proteins that are localized to several subcellular localizations, an event referred to as dual targeting, dual localization, or dual distribution. This event occurs through one of several routes that are based on more than one gene, more than one mRNA from a single gene, or more than one translation initiation on a single mRNA, which can result in different translation products that differ by the presence or absence of specific targeting signals [6]. Repetitious forms of the same protein with identical or nearly identical sequences that are distinctly localized in the cell have been recently called "echoforms" to distinguish them from "isoproteins," which are proteins with the same activity but different amino acid sequences [6]. Proteins that harbor one signal, two separate signals or an overlapping ambiguous signal may also undergo dual distribution in the cell. The mechanism of this dual targeting is driven by the competition or promiscuity of various molecular events that involve protein folding, posttranslational modification, and protein-protein interaction [7].

Subcellular compartments and organelles contain specific proteins that determine their structure and function [7]. Most proteins carry out their functions within a complex network of interactions in which a single component can affect a wide range of other components [8]. If two proteins interact with one another, they usually participate in the same, or related, cellular pathway(s), and clues to the function of a protein can be obtained by determining its interactions with another protein of known function [8, 9]. Therefore, understanding how proteins interact is a significant area of current research.

The dual localization of LYT1 exposes this molecule to different microenvironments and the possibility of interactions with other proteins that could promote different functionality. For this reason, in this work, we began to unravel the LYT1 interaction profile by coimmunoprecipitation assays using stably transfected parasites expressing an exogenous LYT1 protein fused to the enhanced green fluorescent protein (EGFP). The advantage to this *in vivo* approach is that it can be carried out while maintaining intracellular conditions, thereby enabling a better analysis of the LYT1 interaction profile and the possible influence that this could have on the different pathways in which LYT1 is involved.

2. Materials and Methods

2.1. Parasites. Epimastigotes from wild-type (WT) and LYT1s+n-EGFP transgenic *T. cruzi* Cl-Brener strains were maintained in liver infusion tryptose medium (LIT) containing 10% FBS, 0.5% penicillin (10,000 IU)/streptomycin (10 μg), and 0.5% of hemin (5 mg/mL) at 28°C [2]. Mid-log-phase cultures were used in all experiments.

2.2. Construction of pTREXn-LYT1s+n-EGFP and pTREXn-EGFP. The *LYT1s+n* sequence was amplified by PCR with 1 unit of Herculase Taq DNA polymerase (Cat. number 600262-51/Stratagene), the buffer provided by the manufacturer, dNTPs, the *LYT1b* allele DNA as a template (GenBank AF320626) [2], and the specific oligonucleotides LYT11 S [5′-GCG GAA TTC ATG CGG AAG AAA GCC GCA GC-3′/nucleotide (nt) 1–20 of the *LYT1* coding sequence (GenBank AF320626) downstream of the *Eco*RI (nt 4–9) site] and LYT17 AS [5′-GGG GTA CCC CAT CAG CTG CCA GCA TGT TTT C-3′/complementary to nt 1631–1656 of the *LYT1* coding sequence (GenBank AF320626) downstream of the *Kpn*I (nt 3–6) site]. The PCR conditions used were as follows: the enzyme was added to a first cycle of 1 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, 3 min at 72°C, and a final cycle of 7 min at 72°C. The PCR product was digested with *Eco*RI and *Kpn*I, purified by gel electrophoresis, and cloned in the commercial vector pEGFP-N1 (Clontech), which had been previously digested with the corresponding restriction enzymes. The resultant plasmid was digested with *Eco*RI and *Kpn*I and gel purified to obtain the chimera *LYT1s+n-EGFP,* which was subsequently subcloned in pTREXn [10] in the corresponding restriction enzymes sites. The *EGFP* sequence was obtained by the digestion of pEGFP-N1 (Clontech) with *Eco*RI and *Kpn*I and, after gel purification, was subcloned in pTREXn. The resultant plasmids pTREXn-LYT1s+n-EGFP and pTREXn-EGFP (Figure 1) were used for transfection experiments after verifying the correct fusion of *LYT1s+n* to *EGFP* and cloning by sequence analysis.

2.3. Generation of LYT1-Containing Stably Transfected Parasites. Mid-log-phase WT epimastigotes (3×10^8) from the *T. cruzi* CL-Brener strain, resuspended in cold LYT medium, were transfected by electroporation (BTX ECM 830) with

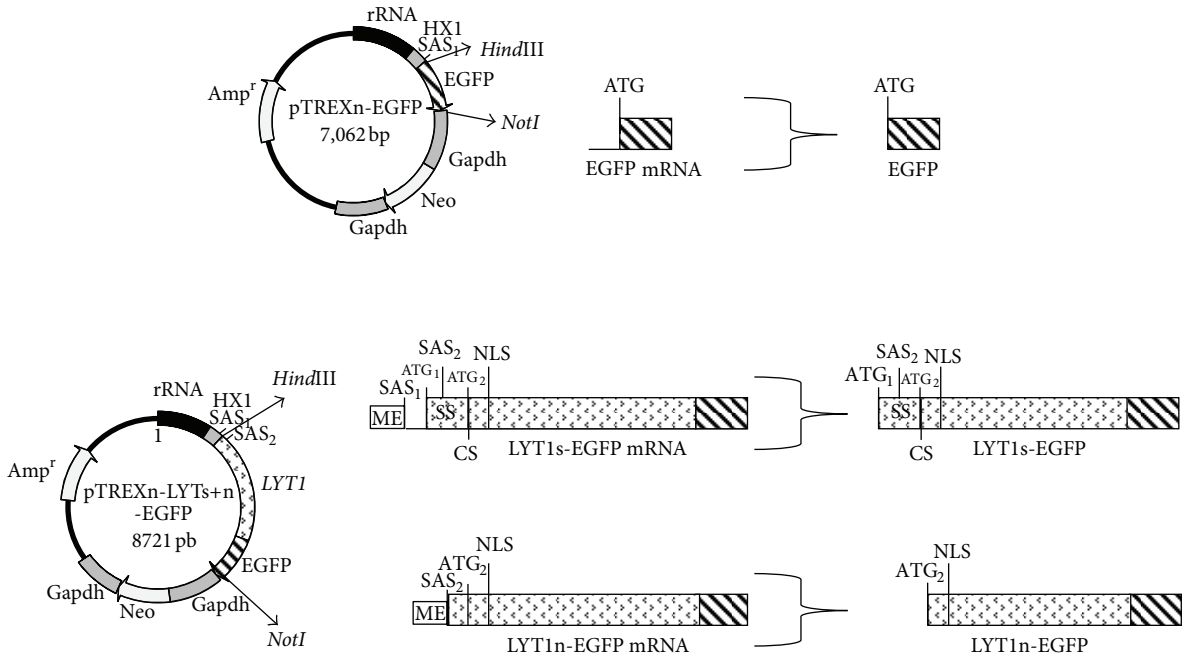

Figure 1: Diagrams representing the pTREXn-EGFP and pTREXn-LYT1s+n-EGFP constructs, transcripts, and protein products (not to scale). The presence and relative position of trans-splicing acceptor sites (SAS_1 and SAS_2), miniexon (ME), translations initiation sites (ATG1 and ATG_2), signal sequence (SS), nuclear localization sequence (NLS), and cleavage site (CS) are shown for the mRNA (EGFP mRNA, LYT1s-EGFP mRNA, and LYT1n-EGFP mRNA) and corresponding proteins (EGFP, LYT1s-EGFP, and LYT1-EGFP). Striped boxes represent EGFP sequences. Arrowheads pointing to filled boxes represent LYT1 sequences. Restriction sites are given. Details of the plasmid construction are described in "Section 2."

100 μg of cesium chloride and purified pTREXn-LYT1s+n-EGFP or pTREXn-EGFP plasmid DNA at 300 volts for 12 ms in 2 mm BTX electroporation cuvettes. After electroporation, the transfected parasites were maintained for 5 min at 4°C and then transferred to fresh complemented LIT medium and incubated at 28°C. After 48 hr, the parasites were exposed to antibiotic selection with 500 g/mL of G418 (Cat. number 10131-035/GIBCO). Once antibiotic-resistant growth cultures were established, fluorescent clonal derivatives were isolated from each population of stably transfected parasites by flow cytometry (FACSVantage, Becton, Dickinson).

2.4. Total Protein Extraction. The parasites (1×10^8) were washed three times with PBS pH 7.2 and then lysed with 500 μL of lysis buffer (50 mM Tris-HCl, pH 7.8, 1% NP40, 5 mM EDTA, 1% SDS, 100 mM $ZnCl_2$, and 1x complete proteinase inhibitor from Roche) at 4°C for 20 min. After two freeze-thaw cycles, the solubilized proteins were quantified by Lowry's technique (Cat. number 500-0114/DC Protein Assay Bio-Rad).

2.5. Western Blotting and Dot Blotting. The same amount of protein (100 μg) was boiled with 1x sample buffer (1% SDS, 10% glycerol, 0.001% pironine, 0.06 M Tris-OH pH 6.8) and loaded into SDS-discontinuous polyacrylamide gel for electrophoresis (PAGE). The proteins were electrotransferred to nitrocellulose membranes (Bio Rad Laboratories), and the quantity of transferred proteins was verified by staining with ponceau/1% acetic acid. The destained membrane was blocked with 6% nonfat milk in 1x PBS and incubated with a 1 : 500 dilution in 6% non-fat milk/1x PBS of the antibody against EGFP (Santa Cruz Biotechnology, Inc./Cat. number SC-9996/monoclonal antibody obtained in mouse) overnight at 4°C. After three washes with 1x PBS, 0.05% tween-20 in 1x PBS, and 1x PBS for 10 min each, the secondary antibody HRP-conjugated anti-mouse IgG (H+L) (Santa Cruz Biotechnology, Inc./Cat. number SC-2380) was added at 1 : 5000 dilution in 6% non-fat milk/1x PBS to detect primary antibodies, for 1 hr at room temperature. The membranes were washed again as indicated before, and positive bands were visualized by chemiluminescence (Cat. number RPN2106 Amersham ECL). For dot blotting, the same amount of protein (10 μg) was directly dripped over nitrocellulose membranes using a mini-fold Bio-Dot (Bio-Rad) and processed as western blots. Antibodies against EGFP (1 : 500 dilution) [Santa Cruz Biotechnology, Inc./Cat. No. SC-9996/monoclonal antibody obtained in mouse] and against the total protein of *T. cruzi* (1 : 1500 dilution) (polyclonal antibody obtained from mice in this work) were used as primary antibodies. The secondary antibody α-mouse IgG (H+L) conjugated to peroxidase [ZYMED LABORATORIES number cat. 81-6520/polyclonal antibody obtained in goat] was used to detect positive bands using 4-chloro-1-naphthol as the peroxidase substrate (Sigma/Cat. number C57804).

2.6. Coimmunoprecipitation Assay. These assays were performed using the Pro-Found coimmunoprecipitation kit (PIERCE 23600) exactly as indicated by the manufacturer. The antibody against EGFP (25 μg) (Santa Cruz Biotechnology, Inc./Cat. No. SC-9996/monoclonal antibody obtained in mouse) was used for all the assays. The antibody against GST tag [ZYMED 13-6700] was used as an unrelated antibody control. To perform the coimmunoprecipitation, 50 μg of total protein was diluted in coupling buffer to reach a final volume of 400 μL, and this mixture was added to the corresponding columns, which were incubated for 8 hr at 4°C using an orbital mixer. Then, the columns were washed six times with 400 μL of coupling buffer, and every wash fraction was collected and analyzed by SDS-PAGE to ensure the absence of protein. Finally, the columns were washed three times with 500 μL of elution buffer to ensure the maximum recuperation of eluted proteins. All assay controls suggested by the manufacturer were simultaneously processed. The eluted fractions and wash fractions were concentrated using MICROCON columns (Millipore 42404 YM3). All the gels containing these controls and samples were silver stained using the kit SILVER-QUEST (Invitrogen LC6070). The stained gels were photographed with a Kodak DS290 digital camera under clear light and analyzed with the Kodak 1D version 3.5.4 software to determine the molecular weight (MW) of each band using the precision plus protein standards (BioRad 161-0374) as MW references. Western blot analysis was performed to verify the samples' integrity.

2.7. Mass Spectrometry Analysis. After 12% sample SDS-PAGE gel separation and silver staining, the protein bands were sent for analysis by MS-MS (Q-TOF) tandem mass spectrometry using an ESIA coupled to a Quadra pole ion trap TANDEM analyzer. Proteins were identified by MASCOT [11] and NCBI-*Blast* [12] software. For putative proteins *ExPASy-Prosite* [13], *ExPASy-ProtParam* [14], *pSORT* [15], and *SMART* [16], software analyses were performed.

3. Results

3.1. Cloning and LYT1s+n-EGFP Stably Transfected Parasite Generation. To identify the proteins that could bind to LYT1, we obtained transgenic parasites expressing LYT1 fused to EGFP. The pTREXn-LYT1s+n-EGFP construct was designed to express the full length and the truncated forms of LYT1 fused to EGFP (Figure 1). This plasmid contains two 3′ AG splice acceptor sites (SAS), one from the HXI vector sequence (SAS_1) [10] and the other from +10 3′ AG of the LYT1 coding sequence (SAS_2), and two ATG (+1/ATG_1 and +85/ATG_2) positions [3]; therefore, both the full-length (LYT1s-EGFP) and the truncated (LYT1n-EGFP) LYT1 proteins fused to EGFP are produced. The construct pTREXn-EGFP was used to express the *EGFP* sequence as a control. To evaluate the presence of EGFP and the LYT1s-EGFP and LYT1n-EGFP chimeras, the constructs described above were transfected into WT epimastigotes of the CL-Brener strain to generate the EGFP and LYT1s+n *T. cruzi* stable lines. To characterize the expression of exogenous EGFP and the LYT1s-EGFP and LYT1n-EGFP protein chimeras, western blot analysis of transfected parasites was performed. As shown in Figure 2(a), the monoclonal antibody against EGFP recognized the EGFP protein and the LYT1s-EGFP and LYT1n-EGFP protein chimeras in transfected parasites. Because the molecular weights of uncleaved (86 kDa) or cleaved (83 kDa) LYT1s-EGFP, and LYT1n-EGFP (83 kDa) are very close, the gel did not resolve separate bands; therefore, a single band was detected in parasites transfected with pTREXn-LYT1s+n-EGFP. Experiments using 6% SDS-PAGE showed the same results (data not shown). As expected, a band of 26 kDa corresponding to the EGFP protein was detected in parasites transfected with pTREXn-EGFP, and no EGFP was observed in WT parasites, demonstrating the specificity of the antibody.

These results indicate that the stable lines expressed the exogenous sequences and demonstrate the successful generation of EGFP and LYT1s+n stably transfected parasite lines.

3.2. Recognition of the Recombinant Protein under Non-denaturing Conditions. Because the coimmunoprecipitation assay would be performed in nondenaturing conditions (Section 3.3), it was necessary to determine whether the antibody against EGFP was also able to recognize the EGFP, LYT1s-EGFP and LYT1n-EGFP exogenous proteins under these conditions. For this determination, a dot-blot assay was performed using total protein extracts of each line of transfected parasites under nondenaturing conditions as described in Section 2. As shown in Figure 2(b), the antibody was able to recognize EGFP and the LYT1s-EGFP and LYT1n-EGFP fusion proteins, indicating that the antibody could be used for the coimmunoprecipitation assays. No signal was observed in extracts from non-transfected parasites, demonstrating the specificity of the antibody (Figure 2(b)). The presence of the total protein extract of non-transfected parasites was confirmed using an antibody against the total protein of WT epimastigotes (Figure 2(b)) and its integrity by SDS-PAGE and Coomassie-blue staining (Figure 2(c)).

3.3. Identification of Proteins Associated with LYT1. To determine whether the LYT1 products interact with other proteins, coimmunoprecipitation assays were performed to establish the *in vivo* protein-protein interactions. Stably transfected parasites and the antibody against the EGFP tag were used for the assays as described in Section 2. As shown in Figure 3, the negative controls using only beads, an unrelated antibody (anti-GST) or quenched beads did not precipitate nonspecific products (Panel (a)). Moreover, as expected, in the control parasites expressing EGFP, we detected a protein of 26 kDa, the molecular weight of EGFP. There were also three other non-specific bands that were eliminated in the analysis for the tested samples (Panel (b)). This result allowed us to be certain that the conditions in which the columns and interaction solutions were prepared were adequate to perform the assays of the samples. When the LYT1s+n stable parasites lines were evaluated, approximately 16 bands ranging from 8 to 150 kDa

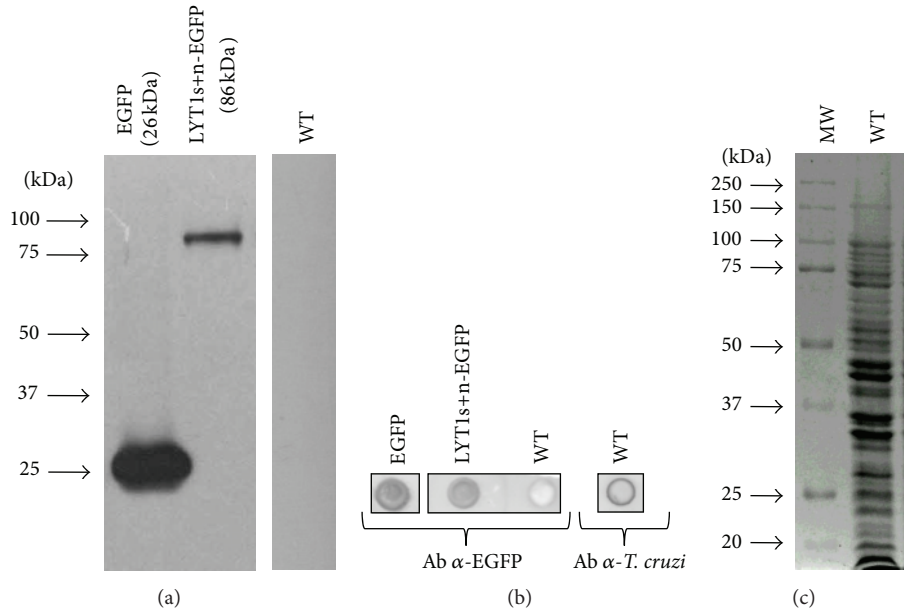

Figure 2: Analysis of EGFP and LYT1s+n-EGFP chimeric proteins expression. (a) Protein extracts of WT parasites and EGFP and LYT1s+n-EGFP transgenic parasites were run on polyacrylamide gels under denaturing conditions and transferred, and the exogenous proteins were revealed using an α-EGFP antibody. (b) Protein extracts of WT parasites and EGFP and LYT1s+n-EGFP transgenic parasites were dotted under native conditions and revealed using an α-EGFP or α-*T. cruzi* antibody. (c) Protein extract of WT parasites analyzed by SDS-PAGE and Coomassie-blue stain.

were coimmunoprecipitated, suggesting that LYT1 interacts with different proteins (Panel (b)).

Because LYT1 has lytic activity and is highly unstable, we determined whether the coimmunoprecipitation bands could be the result of the exogenous LYT1-EGFP protein degradation. Then, the coimmunoprecipitation products were analyzed by western blotting using an anti-EGFP antibody. As shown in Figure 3(c), a single band of approximately 83–86 kDa was observed in the LYT1s+n parasites, and a 26 kDa band was observed in the EGFP parasites, thus demonstrating the sample integrity and confirming the accuracy of the coimmunoprecipitation products.

3.4. Interaction Strength of the Coimmunoprecipitation Products. Once the coimmunoprecipitation pattern of the LYT1s+n stably transfected parasites was determined, we evaluated the interaction strength of the proteins using increasing salt concentrations in the wash buffer. As shown in Figure 4 and Table 1, when 100 mM NaCl was used, the same coimmunoprecipitation pattern was observed. However, when the NaCl concentration was increased to 240 mM, 3 bands (of 66, 93 and 110 kDa) were lost, and 10 more bands (of 10, 12, 15, 21, 31, 35, 43, 45, 59 and 74 kDa) were lost when 290 mM NaCl was used. When the highest salt concentration was used (340 mM), only the 86 kDa band remained, which corresponds to the molecular weight of the exogenous LYT1 chimeric proteins.

These results indicate that LYT1 binds to the various proteins that it interacts with at different affinities.

Table 1: Interaction strength of coimmunoprecipitation products.

Co IP products (kDa)	240 mM (kDa)	290 mM (kDa)	340 mM (kDa)
150	150	150	
110			
93			
86	86	86	86
74	74		
66			
59	59		
45	45		
43	43		
38	38	38	
35	35		
31	31		
29	29	29	
21	21		
15	15		
12	12		
10	10		

The molecular weights of the LYT1 co-immunoprecipitation products (from Figure 3) and under the treatment with increasing salt concentration (240 mM, 290 mN, and 340 mM) in the wash buffer (from Figure 4) were calculated with respect to the molecular weights of standard proteins as described under "Section 2."

3.5. MS-MS Analysis of the Coimmunoprecipitation Products. To determine the identity of the LYT1 interaction profile, the coimmunoprecipitation products were analyzed by MS-MS (Q-TOF) tandem mass spectrometry and *in silico* analysis as

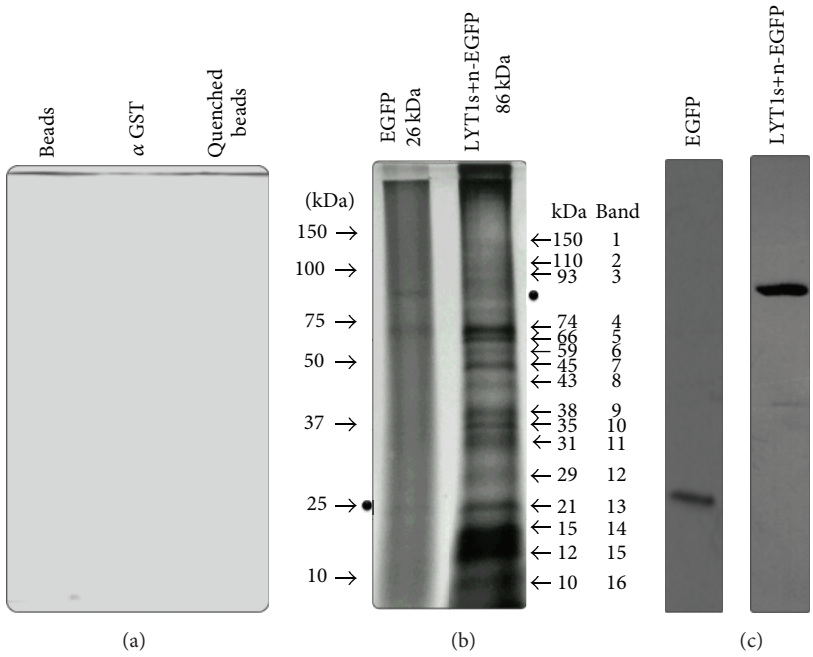

Figure 3: The LYT1 interaction profile. (a) Control gel (beads) composed of the same support material as the coimmunoprecipitation gel but not activated, a nonrelevant antibody (α-GST), and a quenched antibody coupling gel (quenched beads) were used as nonspecific interaction controls. (b) Protein extracts from EGFP and LYT1s+n-EGFP transgenic parasites were processed by immunoprecipitation with an α-EGFP antibody. Molecular weight markers (left of the gel), calculated molecular weights of the interaction products, and band numbers (to the right of the gel) are indicated. The positions of recombinant proteins are indicated with black spots. (c) A western blot containing the EGFP and LYT1s+n-EGFP coimmunoprecipitation products was probed with α-EGFP antibodies. Representative results of three independent experiments are shown.

described in Section 2. As shown in Table 2, we obtained 68 total peptides that corresponded to nine identified proteins: trans-sialidase (TS), kinetoplastid-specific DSPs (kDSPs), histone H2A and histone H2B, α- and β-tubulin, dynein, a tetratricopeptide repeat (TPR) protein (TcC31.24), LYT1, and five other hypothetical proteins with unknown function. The identified proteins are grouped into four functional groups: (1) infection process, (2) transcription, cell cycle, and development, (3) parasite motility and protein traffic and (4) interaction scaffold, which represent 13%, 25%, 19%, and 13% respectively. The remaining 30% corresponds to hypothetical proteins.

These findings reveal that LYT1 interacts with different proteins, thus providing the first LYT1 interaction map for *T. cruzi*.

4. Discussion

To increase the number of functions that a cell can carry out without increasing the number of genes, evolution has produced different solutions. For example, the cell can distribute the products of a single gene to more than one cellular compartment [6], and the proteins can carry out their functions within a complex network of protein interactions that enable them to act in concert [8].

Eukaryotic cells are defined by the existence of subcellular compartments and organelles that contain specific proteins that allow them to regulate their cellular functions. Therefore, understanding how proteins interact is a significant area of study because it can provide insights into the differential functionality of proteins with dual localization.

The dual targeting properties of LYT1 [4, 5] and its multifunctional capacity [2–4] provide an excellent model for the study of protein interaction networks, not only because the full and truncated LYT1 forms are distributed in different organelles [4], but also because the full form of LYT1 exhibits dual localization due to the presence of both a secretion signal and a nuclear localization signal [5]. Consequently, LYT1 products can be exposed to different microenvironments, and interactions with other proteins can modulate its function.

We have identified some potential LYT1-binding proteins by coimmunoprecipitation assays using total protein extracts obtained from parasites that simultaneously express both forms of LYT1 fused to an EGFP tag. Here, the EGFP tag functions as prey, and the antibody against EGFP functions as bait. The advantage of this *in vivo* approach is that it was carried out under intracellular conditions, thereby enabling a better analysis of the physiological roles of LYT1 products. We identified LYT1-binding proteins that participate in the parasite infection process (TS), are related to transcription, cell cycle, and development (histone H2A and histone H2B and kDSPs), are involved in parasite motility and protein traffic (dynein and α- and β-tubulin), and that act as scaffolding proteins (TcC31.24 and kDSPs) that participate in the formation of multiprotein complexes involved in several

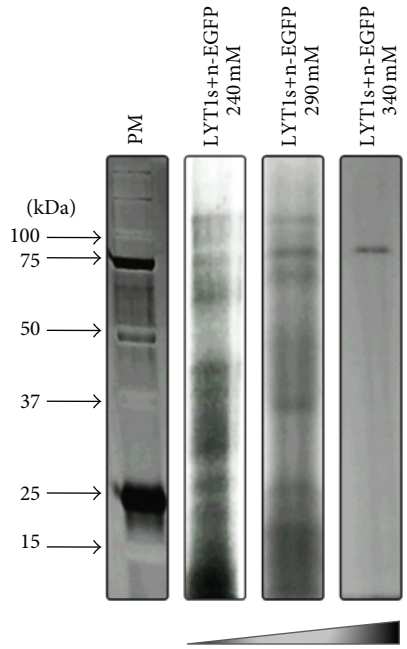

Figure 4: The interacting proteins bind to LYT1 with different affinity levels. To assess the protein-binding properties of the LYT1 interactome, increasing salt concentrations in the interaction wash buffer were used. Representative results of three independent experiments are shown.

aforementioned processes. Additionally, we also found five proteins that are annotated as hypothetical proteins or that have unknown function.

Trans-sialidases (TS) are a family of membrane proteins that transfer sialic acid from the glycoconjugates of the host membrane to terminal beta-galactopyranosyl units present on the surface of the parasite and play a key role in the invasion of the mammalian host cell and immunomodulation of the infected host [17]. The critical role of TS in invasion has been highlighted the fact that invasion was neutralized by human antibodies again TS [18], TS+ parasites were highly invasive and more virulent than TS− or unfractionated parasites [19], and by the observation that high TS expression levels increase the exit of trypomastigotes from the parasitophorous vacuole and their subsequent differentiation into amastigotes [20]. With the results that we have produced so far, we do not know whether the interaction between TS and LYT1 is direct or via scaffolding molecules that are also found in the coimmunoprecipitation products (i.e., TcC31.24).

TcC31.24 contains a TPR motif, which is one of the many repeated motifs that form structural domains in proteins that act as scaffolds in the formation of multiprotein complexes that are involved in numerous cellular processes, such as transcription, cell cycle, protein translocation, protein degradation, and host defense against invading pathogens [21]. As TcC31.24 participates in protein-protein interactions, it is not surprising that it is coimmunoprecipitated with TS, as both molecules are present in the parasite exosomal proteome [22].

Exosomes, originally described in reticulocytes [23], are membrane vesicles that are released into the extracellular milieu by a variety of mammalian cells that play a role in antigen presentation, the transfer of MHC class I- and II-peptide complexes between cells of the immune system, T-cell stimulation, and membrane exchange among cells [24–26]. This type of vesicle has also been described in *T. cruzi*, in which they participate in the release of surface antigens [27]. A preliminary proteomic study of these vesicles reveals that they are rich in TS, gp63, tubulin, kinesin, dynein, HSP 70/90, and TPR hypothetical proteins, among other important proteins for *T. cruzi* virulence [22]. Therefore, TcC31.24 may function as scaffold protein between TS and LYT1, as both proteins are involved in the processes of parasite infection. As the exosomes contain several proteins with a common role in infectivity, we propose that the parasite can regulate the simultaneous secretion of all these proteins. However, future experiments will be necessary to demonstrate the presence of LYT1 in the exosomes and the coordinated secretion of functionally related proteins.

Regarding kDSPs, sequence analysis showed that this protein may be localized to the cytoplasm or the membrane and that it contains the phosphatase conserved domain DSPC: dual specificity phosphatase catalytic domain. Protein phosphatases are conventionally classified according to their substrate preferences, including serine- and threonine-specific phosphatases (STP); tyrosine-specific phosphatases (PTP); dual-specificity phosphatases (DSP) that dephosphorylate phosphoserine, phosphothreonine, and phosphotyrosine substrates; lipid phosphatases (PTEN type and Myotubularins) and the low molecular weight PTP (LMW-PTP). The presence of specific conserved motifs in the catalytic domain as well as additional regulatory or targeting domains allow these types of protein phosphatases to be recognized and classified into different subfamilies [28–30]. Using these criteria, the TriTryp phosphatome has been recently reported, revealing that these organisms have an unusual composition of phosphatases, in which the PTP family is greatly reduced,

Table 2: Proteins identified by MS-MS (Q-TOF) tandem mass spectrometry.

Group	Theoretical MW (kDa)	Protein name	Accession number (TriTrypDB)	Number of identified peptides	Sequence coverage (%)
Infection process (13%)	59	LYT1p	TcCLB.508045.40	4	12%
	85	Trans-sialidase, putative	TcCLB.506331.90	6	8%
Transcription, cell cycle, and development (25%)	13	Histone H2B	Tc00.1047053511635.10	7	21%
	59	LYT1p	TcCLB.508045.40	4	12%
	35	Dual specificity protein phosphatase	TcCLB.504741.170	5	9%
	15	Histone H2A	Tc00.1047053511817.151	5	8%
Parasite motility and protein traffic (19%)	49	β-Tubulin	Tc00.1047053411235.9	4	10%
	50	α-Tubulin, putative	Tc00.1047053411235.9	7	8%
	282	Dynein heavy chain, putative	TcCLB.508815.179	4	3%
Interaction scaffold proteins (13%)	35	Dual specificity protein phosphatase	TcCLB.504741.170	5	9%
	80	TcC31.24	Tc00.1047053506529.460	4	7%
Hypothetical proteins (30%)	70	Hypothetical protein, conserved	TcCLB.506755.10	8	6%
	35	Hypothetical protein, conserved	TcCLB.511301.50	2	5%
	85	Hypothetical protein, conserved	TcCLB.509341.20	2	4%
	90	Hypothetical protein, conserved	TcCLB.506529.460	4	3%
	255	Hypothetical protein, conserved	TcCLB.511511.10	6	2%

whereas the STP family has expanded when compared with human phosphatases. Interestingly, a novel domain architecture was also identified in several phosphatases, and a number of atypical and unique phosphatases were found, suggesting potentially new pathways involving phosphatases [29]. The latter group contains the kDSPs family, which includes the putative phosphatase found in this work. The kDSPs are characterized by considerable divergence from classic DSPs in both their domain organization and sequence features. Although the kDSPs share most of the classic DSP motifs, they are significantly longer than human DSPs and can contain either N- or C-terminal extensions. Some of these extensions contain accessory motifs or domains, including the leucine-rich repeats (LRRs) that are present in scaffolding proteins in signaling pathways [31–33]. The extensions may also contain an ankyrin domain, which is a common protein-protein interaction domain found in proteins involved in transcription initiation, cell-cycle regulation, and signaling [34].

It is well documented that highly specific protein kinases and protein phosphatases control a number of processes, including metabolic pathways, cell-cell communication, cell growth and proliferation, and gene transcription. Furthermore, mutational analysis demonstrated that these proteins have essential roles in the virulence and infection of pathogenic bacteria [35–37]. Although the specific roles of protein phosphatases in unicellular protists, in particular protozoan parasites such as *Trypanosomes* and *Leishmania*, are less well understood, recent work has identified several protein phosphatases and has highlighted the importance of these phosphatases in the regulation of essential developmental aspects of the life cycle of pathogenic kinetoplastids [38–43].

These findings show a correlation between the functions of LYT1 and kDSPs with regard to the parasite infection and stage transition process, so that it is possible to imagine that the presence of both molecules in the coimmunoprecipitation product may have a functional meaning for these processes.

Of the proteins that interact with LYT1 and that are related to transcription, the cell cycle, and development, our analysis detected the histones H2A and H2B. This finding is consistent with the LYT1 nuclear zone localization and the participation of this molecule in the parasite stage differentiation process as a negative regulator [2]. It is unlikely that LYT1 directly interacts with the histone because LYT1 does not contain domains normally associated with a histone-modifying role (e.g., acetyltransferase, methylase, and kinase). Therefore, one possible explanation of this result is the presence of the leucine zipper-like noncanonical domains found in the LYT1 sequence, which may allow its direct interaction with DNA and thus the coimmunoprecipitation of the DNA-binding histone. Another possibility is that LYT1 interacts with a histone-modifying molecule or molecules attached to it, through scaffolding proteins such as TcC31.24 and kDSPs,

and that it could thereby potentially be associated with the nuclear membrane or telomeric regions. In this regard, it is interesting to note that the truncated form of LYT1 has a very similar pattern of localization [5] to that reported for *Leishmania major* telomeres, which are organized in clusters dispersed throughout the nucleus periphery in a speckled pattern [44].

Unlike other trypanosomatids, in which a low proportion of their genomes is stage-regulated [45–47], *T. cruzi* displays stage-regulated control of mRNAs for more than 50% of its genes [48]. The apparent absence of typical promoters in *T. cruzi* has led to several findings that suggest that epigenetic mechanisms play a critical role in gene regulation in this parasite. Therefore, it is not surprising that through its interaction with histones, LYT1 may participate in the *T. cruzi* differentiation process although future experiments are necessary to evaluate such participation.

T. cruzi, as other trypanosomatid flagellate parasites, is characterized by the presence of a cytoskeleton that is responsible for maintaining cellular shape and its modulation among different life cycle stages [49]. Two of the most important cytoskeletal components are α-tubulin and β-tubulin, which polymerize into microtubules that form the parasite subpellicular corset and play an important role in the separation of the basal bodies [50] and the growth of the new flagellum as well as mitosis and cytokinesis [49–51]. The α-tubulin and β-tubulin association with LYT1 found in this work agrees with prior evidence that the secreted proteins, such as LYT1 [4], are exocytosed via the parasite flagellar pocket [52].

The parasite flagellar pocket is a structure formed primarily by microtubules [53] and together with the cytostome, is involved in the *T. cruzi* endocytic and exocytic pathways [53–56]. In these pathways microtubules function as roadways for mechanochemical motor proteins such as kinesin and dynein, using the energy of ATP hydrolysis to transport membrane-bound organelles as well as other structures within the cell, and it has been implicated in vesicular transport to and from the Golgi complex [57].

Kinesin and cytoplasmic dynein have been recognized as the two main microtubule-associated motors, with kinesin involved in the plus-end-directed transport and dynein in the minus-end-directed transport [58]. Therefore, the association of LYT1 with microtubules could result from its interaction with dynein during its transportation to the membrane.

These findings suggest that the association of LYT1 with α-tubulin, β-tubulin, and dynein may more likely result from the LYT1 secretion system rather than an interaction that is necessary for LYT1's function.

Finally, we also found LYT1 peptides in the interaction profile. The presence of these peptides is expected because exogenous LYT1 was used as bait in the coimmunoprecipitation assays. However, the presence of coiled coil sequences in LYT1 supports the possibility that this protein interacts with itself to produce multimers with pore-forming activity. Several intracellular pathogens, including bacteria and protozoa, produce pore-forming proteins (PFPs) to escape from phagosomes and thereby survive within the cell [59–63]. Tc-TOX, a protein functionally related to LYT1, is secreted into the acidic environment of the phagosome, possibly by forming pores in the membrane which contribute to the parasitophorous vacuole disruption [64]; LYT1 shares many molecular and functional characteristics [2–4] with TcTOX [64], for example, both are secreted proteins, are recognized by anti-C9 antibodies, and show hemolytic activity at low pH. Therefore, we believe that it is important to evaluate the possible ability of LYT1 to function as a PFP.

Future experiments also will be necessary to determine the function of proteins classified as hypothetical or with unknown function or homology and to validate the biological significance of the LYT1 interaction with them.

This proteomic approach provides the identification of putative partners of LYT1 from *T. cruzi*. Eventually, these newly identified proteins should be analyzed to complement this *in vivo* study in order to help understand the mechanism involved in not only LYT1 multifunctionality but also the molecular pathogenesis of *T. cruzi* infection and to develop novel approaches of intervention in Chagas disease.

Acknowledgments

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Research Article

PPAR Activation Induces M1 Macrophage Polarization via cPLA$_2$-COX-2 Inhibition, Activating ROS Production against *Leishmania mexicana*

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Defence against *Leishmania* depends upon Th1 inflammatory response and, a major problem in susceptible models, is the turnoff of the leishmanicidal activity of macrophages with IL-10, IL-4, and COX-2 upregulation, as well as immunosuppressive PGE_2, all together inhibiting the respiratory burst. Peroxisome proliferator-activated receptors (PPAR) activation is responsible for macrophages polarization on *Leishmania* susceptible models where microbicide functions are deactivated. In this paper, we demonstrated that, at least for *L. mexicana*, PPAR activation, mainly PPARγ, induced macrophage activation through their polarization towards M1 profile with the increase of microbicide activity against intracellular pathogen *L. mexicana*. PPAR activation induced IL-10 downregulation, whereas the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 remained high. Moreover, PPAR agonists treatment induced the deactivation of cPLA$_2$-COX-2-prostaglandins pathway together with an increase in TLR4 expression, all of whose criteria meet the M1 macrophage profile. Finally, parasite burden, in treated macrophages, was lower than that in infected nontreated macrophages, most probably associated with the increase of respiratory burst in these treated cells. Based on the above data, we conclude that PPAR agonists used in this work induces M1 macrophages polarization via inhibition of cPLA$_2$ and the increase of aggressive microbicidal activity via reactive oxygen species (ROS) production.

1. Introduction

Leishmaniasis is a collection of parasitic diseases caused by two dozens species of protozoa belonging to the genus *Leishmania* and spread by the bite of a sandfly. Two main clinical forms are known: cutaneous leishmaniasis, affecting the skin causing scars and eventually disfiguration, and systemic or visceral leishmaniasis that can lead to fatal complications if untreated [1].

In México, *Leishmania mexicana* is the causative agent of two forms of cutaneous leishmaniasis. Localized cutaneous leishmaniasis (LCL) is characterized by ulcerative skin lesions that develop at the site of the bite of the sandfly; diffuse cutaneous leishmaniasis (DCL), which consists of nonulcerative nodules that spread throughout the skin, leads to severe mutilation because of the invasion of naso- and oropharyngeal mucosa [2, 3]. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors expressed in macrophages, where they control the inflammatory response; there are three isoforms, PPARα, PPARβ/δ, and PPARγ, that exhibit different tissue distribution as well as different ligand specificities [4]. PPARγ promotes the differentiation of monocytes into anti-inflammatory M2 macrophages in humans and mice while the role of PPARβ/δ in this process has been reported only in mice, and no data are available for PPARα [5].

Differential cytokine production is a key feature of polarized macrophages; while Th1 cytokines promote proinflammatory M1 macrophages, Th2 cytokines support an "alternative" anti-inflammatory M2 macrophage phenotype.

Modulation of proinflammatory cytokines by *Leishmania* species *in vitro* and *in vivo* is reported elsewhere [6]. In general, *Leishmania* infections induce tumor necrosis factor (TNF-α) production; interleukin-1β (IL-1β) generation is abrogated by *L. donovani* infection *in vitro* and *in vivo*, whereas it is induced by *L. major* infection. These observations indicate that different species of *Leishmania* can differentially modulate the proinflammatory cytokines. In addition, it is now well documented that these cytokines play a decisive role in the modulation of chemokines, which are recognized for their function in cell recruitment and promotion of the inflammatory reaction [6].

Regarding anti-inflammatory cytokines, recent studies have demonstrated the critical role of IL-10 in susceptibility to cutaneous and visceral leishmaniasis caused by different *Leishmania* species such as *L. major, L. donovani*, *L. mexicana*, and *L. amazonensis*. IL-10 suppresses IFN-γ synthesis by inhibiting accessory cell functions and also can reduce the production of Nitric Oxide (NO) by activated macrophages. IL-10 also downregulates the expression of MHC class I and class II molecules as well as costimulatory B7 molecules on macrophages. Moreover, a recent study has shown that IL-10-deficient BALB/c mice can control infection with *L. major* suggesting that IL-10 plays a key role in mediating the susceptibility and pathogenesis of cutaneous leishmaniasis [7, 8].

Prostaglandins are often associated with anti-inflammatory activities such as inhibition of effector functions of inflammatory cells. These include inhibition of mediator release from macrophages, neutrophils, mast cells, basophils, and lymphocytes; they can also downregulate macrophage functions, particularly, prostaglandin E_2 (PGE_2) [9], which is synthesized throughout the duration of the inflammatory response, largely via the sequential activities of cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase-2 (COX-2; rate-limiting enzyme), and microsomal PGE synthase-1 (mPGES-1). This pleiotropic prostanoid serves as an underlying modulator of inflammation by mediating and modulating cytokine-target gene expression at transcriptional and posttranscriptional/translational levels [10–13].

The $cPLA_2$ and COX-2 promoters contain a PPAR response element (PPRE); thus, PPARγ agonists including anti-inflammatory drugs may affect COX-2 and $cPLA_2$ transcription and expression. Pérez-Santos and Talamás-Rohana [14] demonstrated that indomethacin (INDO) administration induced the intracellular killing of *L. mexicana* parasites in infected BALB/c mice; these results suggest that suppression of PGs by INDO promotes the development of a protective Th1 type response in susceptible mice by enhancement of IL-12, IFN-γ, and NO production.

Classically activated macrophages have a high capacity to present antigens and to produce IL-6, IL-1β, TNF-α, and toxic intermediates (NO and ROS), consequently, orienting the immune system to a polarized type I response. The various life-cycle stages of *Leishmania* have different sensitivities to ROS and elicit different oxidative responses of the macrophage. *Leishmania* protects itself against the macrophage's oxidative burst through the expression of antioxidant enzymes and proteins, as well as actively by the inhibition of NO and ROS production in the macrophage [15].

In the present work, we analyzed the effect of PPAR's agonists during the early-time infection of J774A.1 macrophages with *L. mexicana* and addressed the issue of whether the addition of PPAR agonists to J774A.1 macrophages infected with *L. mexicana* could increase ROS production by polarization of M2 towards M1 macrophages, inhibiting $cPLA_2$ and COX-2 enzymes.

2. Material and Methods

2.1. Antibodies and Reagents. PPARβ/δ and PPARγ antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA); $cPLA_2$, p-$cPLA_2$ (Ser^{505}), COX-2, MR/CD206, and p44/42 MAP kinase (ERK1/2) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); TLR4/CD284 antibody was from IMGENEX (San Diego, CA, USA). Anti-mouse IgG-conjugated horseradish peroxidase was from Pierce Biotechnology, Inc., (Rockford, IL, USA), and anti-rabbit IgG-conjugated horseradish peroxidase was from Zymed Laboratories (San Francisco, CA, USA). PPARβ/δ agonist (GW501516) and PPARγ agonist (GW1929) were obtained from Alexis Biochemicals (ENZO Life Sciences, Inc. Ann Arbor, MI, USA); $cPLA_2$ inhibitor (arachidonyl trifluromethyl ketone (ATK)) was obtained from Cayman Chemical. All D-MEM and RPMI-1640 media were purchased from Gibco-BRL, Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was from PAA Laboratories (GE, Healthcare, UK). All materials for SDS-PAGE were purchased from Bio-Rad. Lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4) and all the other chemicals and biochemicals were from Sigma-Aldrich (St. Louis, MO, USA). Prostaglandin E_1 (PGE_1), prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and deuterated prostaglandins (^2H) were purchased from Cayman Chemical. HPLC grade solvents, glacial acetic acid, acetonitrile, methanol, chloroform and, all the other chemicals were from Sigma-Aldrich. Solid-phase extraction (SPE) cartridges (C18) were purchased from Millipore (Milford, MA, USA).

2.2. Parasites. *Leishmania mexicana* (MHOM/MX/92/UAY-68) promastigotes were grown at 26°C in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES. Promastigotes were used at the stationary phase of growth.

2.3. Cell Culture. Murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, MD, USA) was cultured at 37°C in humidified 5% CO_2/95% air in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated for 24 h before being used for the required assays. For all experiments, cells were grown up to 80–90% confluence, and then the medium was replaced with a fresh

medium, and cells were incubated with *L. mexicana*. Cells were not subjected to more than 20 cell passages.

2.4. Infection of Macrophages. J774A.1 macrophages (1×10^6/well) were cultured in 24-well culture plates. Cells were incubated with promastigotes of *L. mexicana* at a ratio of 20 parasites per macrophage or treated with LPS (1 μg/mL) for the indicated periods, after which noningested promastigotes were washed off with warm D-MEM. Where indicated, cells were also pretreated for 24 h with pharmacological agonists GW501516 (100 nM), GW1929 (600 nM) and for 1 h with ATK (75 μM), prepared in DMSO or ethanol. Vehicle controls were included in each experiment.

2.5. Real-Time PCR Assays. The total mRNA from noninfected, *L. mexicana*-infected, or LPS-stimulated J774A.1 macrophages was extracted with TRIzol reagent (Life Technologies Corporation, USA) according to the manufacturer's instructions. The retrotranscription reaction was performed with the First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Fermentas Life Sciences, USA) in an iCycler Thermal Cycler (Bio-Rad). RT-PCR amplifications were performed as described by Estrada-Figueroa et al. [16]. Reactions were done in a real-time PCR 7500 apparatus (Applied Biosystems) in a final volume of 20 μL using 100 ng of cDNA and 10 μL of TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA). Primers were from Applied Biosystems (TaqMan Gene Expression Assay): TNF-α (Mm00443258_m1), IL-1β (Mm01336189_m1), IL-6 (Mm00446190_m1), COX-2 (PTGS2; Mm00478374_m1), IL-10 (Mm00439614_m1), and β-Actin (Mm00607939_s1), with the following conditions: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. To verify results, each sample was analyzed in quadruplicate. Levels of transcription were normalized to those of β-actin (internal standard) to determine the variability in the amount of cDNA in each sample. With the C_T values obtained, the $2^{-\Delta\Delta C_T}$ method was followed to calculate the level of expression of each cytokine or mediator gene in treated macrophages in comparison with the expression level of the same cytokines or mediators in the nontreated macrophages, according to the formula [17]:

$$\begin{aligned}\Delta\Delta\text{CT} = {} & \left(\text{CT target} - \text{CT}\beta\text{ actin}\right)\ \text{treated} \\ & - \left(\text{CT target} - \text{CT}\beta\text{ actin}\right)\ \text{nontreated.}\end{aligned} \quad (1)$$

2.6. Preparation of Cell Extracts and Western Blot Analysis. Macrophages were pretreated for 24 h with the PPARβ/δ and PPARγ agonists or for 1 h with $cPLA_2$ inhibitor ATK, while some (basal control) were not. After incubation conditions (noninfected, *L. mexicana*-infected, or 2 h LPS-stimulated macrophages, where indicated), cells were quickly washed twice with icecold PBS and lysed by scraping in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM EDTA, 1 mM NaF, and 1 mM Na_3VO_4). Lysates were centrifuged at 10,000 ×g for 15 min at 4°C to yield the whole cell extract. Supernatants were transferred to fresh tubes and stored at −70°C until required. Protein concentration was determined using a BCA protein assay with bovine serum albumin as standard. Equal amounts of total cell lysates (60 μg protein) were solubilized in sample buffer by boiling for 5 min, separated on 10% SDS-PAGE, and then transferred onto a nitrocellulose membrane using a Trans blot system (Bio-Rad). Nitrocellulose membranes were then incubated successively in TBST blocking buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS)) containing 5% skimmed dried milk and 0.05% Tween 20 for 1 h at room temperature, to block nonspecific protein binding. Membranes were incubated overnight at 4°C with a specific anti-PPARβ/δ (1 : 500), anti-PPARγ (1 : 500), anti-phospho-$cPLA_2$ (1 : 500), total $cPLA_2$ (1 : 500), or anti-COX-2 (1 : 500), antibodies in TBST. Membranes were washed with TBST five times for 5 min each and incubated with the appropriate Horseradish peroxidase-conjugated secondary antibody (1 : 1000) for 1 h at room temperature. Blots initially probed with an antibody were stripped by incubation in 50 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS for 30 min at 50°C. Following extensive washing, blots were reprobed with an anti-ERK1/2 antibody (1 : 5000) as a loading control. Immunoreactive proteins were visualized by enhanced chemiluminescence detecting system. Densitometry analyses of immunoblots were performed using Syngene GeneGenius scanning densitometer and software.

2.7. Phagocytic Assays. A flow cytometry-based method was used to study the phagocytic activity of macrophages. J774A.1 macrophages were seeded at 1×10^6 cells/mL per well in 24-well tissue culture plates and incubated at 37°C, 5% CO_2 for 24 h. Macrophages were treated or not with PPAR agonists (24 h) or $cPLA_2$ antagonist (1 h) and incubated with (FITC)-conjugated zymosan A BioParticles (Molecular Probes Europe BV, Leiden, The Netherlands) at 50 particles/cell ratio for 2 h, unless indicated otherwise, or 10 μM CFSE-labeled promastigotes (1 : 10 ratio) for 60 or 120 min at 37°C except control wells. After incubation, excess nonphagocytized promastigotes or particles were removed by washing. Cells were collected in tubes, and phagocytosis was determined by two criteria: (1) the number of phagocytizing cells and (2) the mean fluorescent intensity (MFI) in a FACSCalibur.

2.8. Expression of Mannose Receptor and Toll-Like Receptor 4 (TLR4). Macrophages were pretreated or not for 24 h with the PPARβ/δ and PPARγ agonists or for 1 h with $cPLA_2$ inhibitor ATK. After incubation conditions: noninfected, *L. mexicana*-infected, LPS or zymosan-stimulated macrophages, where it is indicated, cells were quickly washed twice with icecold PBS containing 2% of FBS (FACS buffer) and scraped on FACS buffer; then, cells were spin down, and the supernatant was removed. After that, cells were resuspended in 1 mL of fixer solution and incubated for 1 h at 37°C; then, cells were centrifuged and washed twice with FACS buffer. Nonspecific staining was blocked with 10% PBS-goat serum for 1 h at 37°C (Fc block). After two washes with FACS buffer, the appropriate antibodies were

added, rabbit anti-human MR (1 : 100), and mouse anti-human TLR4 (1 : 100) and incubated for 1 h at 37°C in FACS buffer. After washing the samples twice, they were incubated with the appropriate antibody: goat anti-rabbit IgG (H + L), rhodamine-conjugated antibody (1 : 100; Millipore), donkey anti-mouse IgG (H + L), and Pacific blue-conjugated antibody (1 : 100; Sigma-Aldrich) for 1 h at 37°C. Finally, samples were washed twice and read in a FACSCalibur.

2.9. Oxidative Metabolism. The oxidative metabolism of J774A.1 macrophages was measured by their ability to reduce yellow-colored nitroblue tetrazolium (NBT) to blue formazan, through the production of superoxide anions as described by Nessa et al. [18]. Macrophages were simultaneously incubated with promastigotes and NBT (1 mg/mL in PBS) for the indicated times. To determine if PPAR agonists were able to increase oxidative metabolism, macrophages (2 mL, 1×10^6/mL) were allowed to adhere to coverslips in plastic Petri dishes (35 × 10 mm; Nunclon, Denmark) by incubation at 37°C for 24 h with PPAR agonists or $cPLA_2$ antagonist for 1 h. Then, macrophages were infected with *L. mexicana* promastigotes (1 : 10 ratio). After that, 1 mL of NBT solution was added to the reaction mixture and incubated at 37°C for the indicated times. The reaction was stopped by adding 1 mL of 0.5% HCl, and cells were further stained with fuccina for 30 sec. Then, they were washed three times with PBS, and positive-oxidative burst cells were counted in a light microscope (100 cell/field and six fields/condition). Quantitative production of formazan was determined in 96-well plates [19]; macrophages (1×10^5) were stimulated with PPAR agonists as described above, and then they were infected with promastigotes for the indicated periods and NBT added. At the end of each time, macrophages were washed with 70% methanol in order to remove nonreduced NBT, the produced formazan was dissolved in DMSO and the optical density of the solution was measured in a spectrophotometer (Bio-Rad) at 630 nm wavelength. A Petri dish or a 96-well plate with noninfected macrophages was incubated with NBT and served as control for each type of experiment.

2.10. Prostaglandins Extraction. The following procedure was developed for the separation of eicosanoids from 24-well cell culture plates containing 2 mL of media. Media was collected and centrifuged for 5 min at 10,000 xg to remove cellular debris. Produced eicosanoids were isolated via solid-phase extraction using SPE cartridge C_{18} from Millipore. Columns were prewashed with 2 mL of MeOH followed by 2 mL of H_2O. After applying the sample to the columns, they were washed with 1 mL of 10% MeOH, and prostaglandins were eluted with 1 mL of MeOH. The eluate was dried under vacuum and redissolved in 100 μL of chloroform-MeOH (2 : 1).

2.11. Mass Spectrometry (MS). All MS analyses were performed using an Applied Biosystems 3200 QTRAP hybrid, triple-quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source and operated in MRM mode. For all experiments, the Turbo V ion source was operated in a negative electrospray mode with N_2 gas, and the QTRAP parameters DP, EP, CE, and CXP were set and maximized for each eicosanoid, and all the samples were loaded by direct infusion at 10 μL/min.

2.12. Statistical Analysis. To take into account all values of the kinetics of macrophages infection, a statistical analysis was performed using two-way ANOVA and Bonferroni's multiple comparison tests, using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Differences with $P < 0.05$ were considered significant.

3. Results

3.1. PPAR Agonists Downregulate $cPLA_2$ and COX-2 Expression in J774A.1 Macrophages Infected with Leishmania mexicana Promastigotes. In *L. mexicana*-infected macrophages, the activation of $cPLA_2$ by phosphorylation and the expression of COX-2 are triggered. The activation of these enzymes is considered necessary within the proinflammatory response, whereas PPAR activation is considered as an important part of the anti-inflammatory process, both *in vivo* and *in vitro* [20–22]. To examine the possibility that PPAR agonists could inhibit $cPLA_2$ phosphorylation and COX-2 expression, J774A.1 macrophages were incubated with different PPAR agonists, and then cells were infected with *L. mexicana* promastigotes. The effects of *L. mexicana* promastigotes on the expression of PPAR, COX-2, and $cPLA_2$ phosphorylation in J774A.1 macrophages were examined (Figure 1). Results showed that there were no changes in the expression of PPARs after infection with *L. mexicana* promastigotes of macrophages, whether treated or not with PPAR agonists; however, $cPLA_2$ phosphorylation diminished significantly through the infection when macrophages were treated with PPAR agonists (Figures 1(a) and 1(b)); in addition, COX-2 protein also diminished significantly after infection (Figures 1(a) and 1(c)); COX-2 mRNA expression was strongly upregulated after *L. mexicana* infection (see Figure 1 available in Supplementary Material online at http://dx.doi.org/10.1155/2013/215283), but it was downregulated in a time-dependent manner (Figure 1(d)), when J774A.1 macrophages were treated with PPAR agonists and infected with *L. mexicana* promastigotes.

3.2. PPAR Agonists Downregulate IL-10 and Sustain Proinflammatory Cytokines Expression after Infection. Cytokines and microbial products profoundly and differentially affect the function of mononuclear phagocytes. It is well established that different species of *Leishmania* can differentially modulate important inflammatory response mediators [23]. In order to see how PPAR agonists could modulate the inflammatory response on J774A.1 macrophages infected with *L. mexicana*, we evaluated the transcripts of some inflammatory cytokines and anti-inflammatory IL-10. The capacity of these macrophages to produce TNF-α, IL-1β, IL-6, and IL-10 cytokines in response to *L. mexicana* promastigotes was tested via qRT-PCR (Figure 2). The infection of J774A.1 macrophages resulted in an increase in TNF-α, IL-1β, and IL-6 expression; IL-10 was downregulated during *Leishmania* infection, although its expression recovered compared to

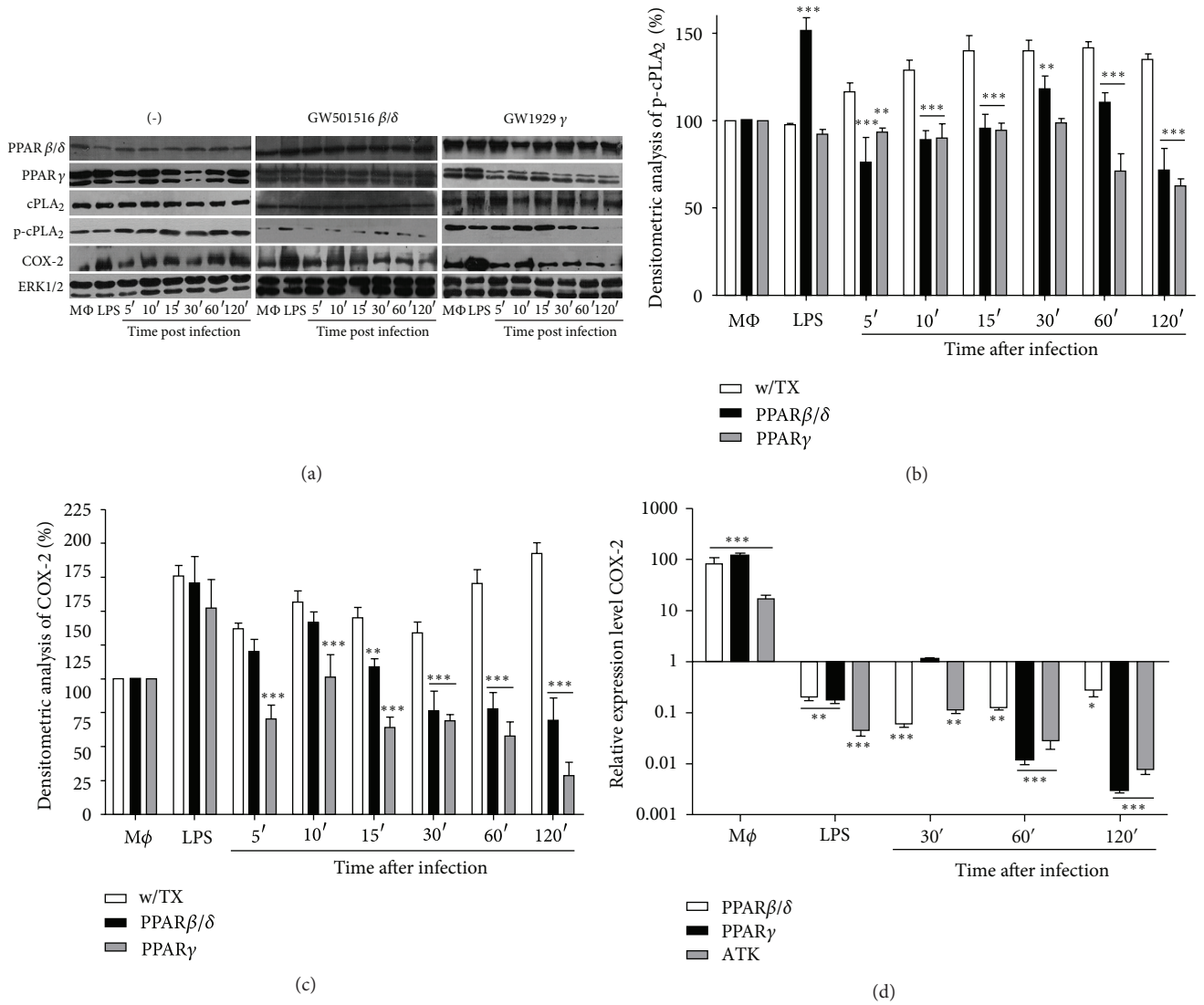

Figure 1: PPAR agonists inhibit $cPLA_2$ phosphorylation and COX-2 expression in *L. mexicana*-infected macrophages. (a) Protein expression for PPARs and COX-2 and $cPLA_2$ phosphorylation levels were evaluated by Western blotting. (b) Densitometry analyses of $cPLA_2$ phosphorylation and (c) COX-2 expression were performed in basal conditions as well as in macrophages treated or not with PPAR agonists. (d) COX-2 mRNA expression was evaluated by qRT-PCR and analyzed by $2^{-\Delta\Delta C_T}$ method. Total ERK1/2 was probed to normalize protein loading. Results are representative of three independent experiments. Graph bars are mean ± SEM of three independent experiments, and statistical analysis was done comparing, for each time, treated versus nontreated macrophages; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

noninfected macrophages (Supplementary Figure 2). However, when noninfected macrophages were previously treated with PPARβ/δ agonist (white bars), proinflammatory IL-6 was upregulated, and its expression was held during infection; TNF-α and IL-1β were overexpressed, but they were downregulated at 30 to 60 min (after infection), and its expression recovered at 120 min (after infection). Moreover, when noninfected macrophages were treated with PPARγ agonist (black bars), TNF-α was upregulated, but its expression was downregulated after infection; overregulation of IL-6 diminished after infection, but it was held equivalent to nontreated macrophages; IL-1β was upregulated, and its expression was held during infection. Anti-inflammatory IL-10 cytokine was downregulated with both PPAR agonists after infection. On the other hand, when $cPLA_2$ was blocked 1 h before the infection by treatment of macrophages with $cPLA_2$ antagonist ATK (gray bars), proinflammatory cytokines TNF-α, IL-1β, and IL-6 were upregulated; furthermore, IL-10 expression was significantly affected when $cPLA_2$ was inhibited. In summary, PPAR agonists and $cPLA_2$ antagonist set down the levels of IL-10; at the same time they upregulated TNF-α, IL-1β, and IL-6 cytokines, or at least they were held overexpressed after infection as occurred with nontreated infected macrophages (Supplementary Figure 2), all together are evidence of a possible M2 to M1 polarization.

3.3. PPAR Activation and $cPLA_2$ Inhibition Induce TLR4 Expression in L. mexicana-Infected Macrophages. Polarized

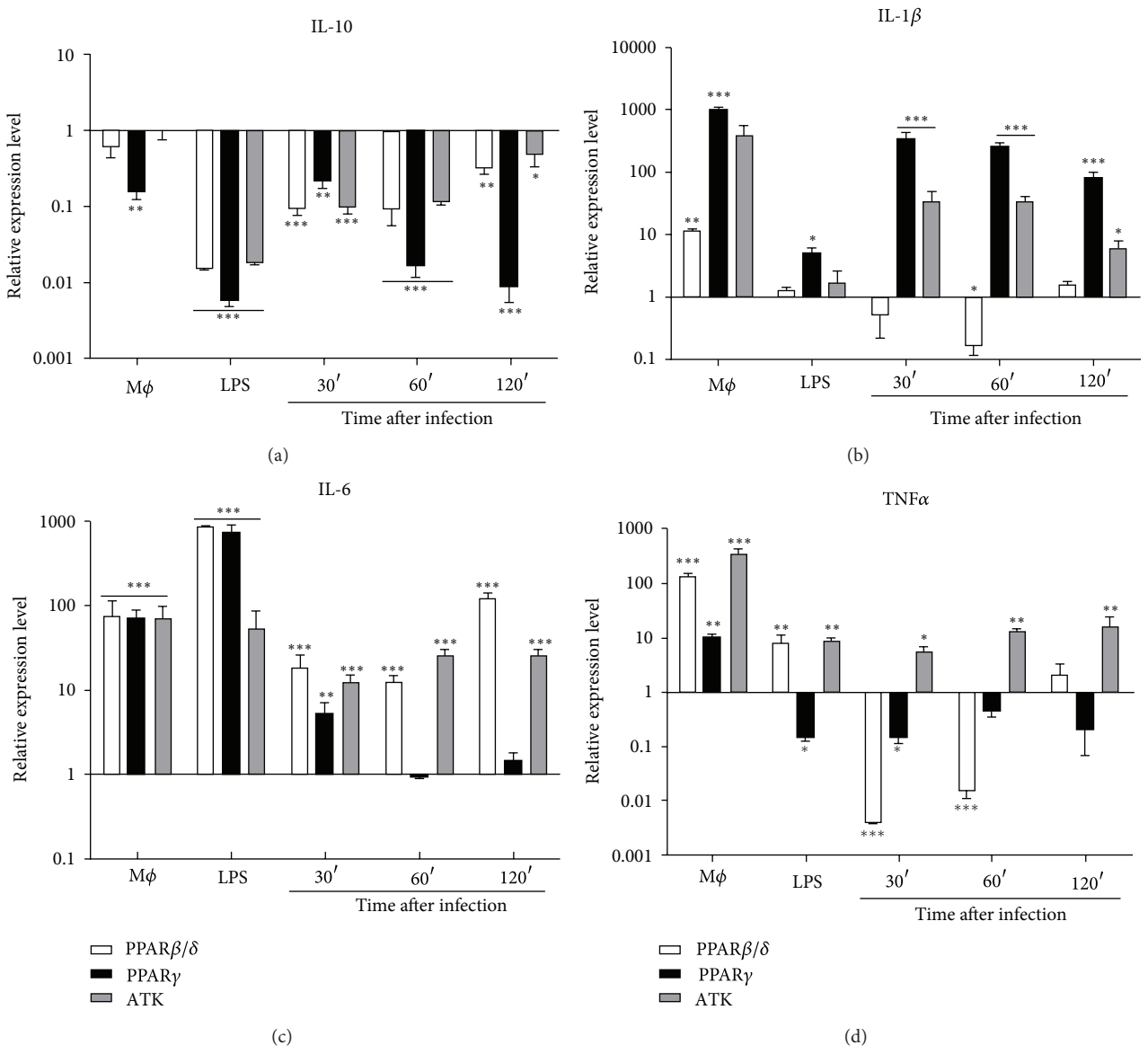

Figure 2: Cytokine determination in *L. mexicana*-infected macrophages. Levels of gene expression for each sample were normalized with β-actin RNA as internal control. Modulation was expressed relative to the untreated control using the $2^{-\Delta\Delta C_T}$ method. The x-axis intercepts the y-axis at "1" to show the increase and the decrease of each cytokine compared to nontreated infected macrophages. Relative expression level for each cytokine was calculated according to $\Delta\Delta C_T = (C_T \text{ test} - C_T \ \beta\text{-actin})$ treated $- (C_T \text{ test} - C_T \ \beta\text{-actin})$ untreated formula [17]. Graph bars are mean ± SEM of three independent experiments, and statistical analysis was done comparing, for each time, treated versus nontreated macrophages; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

macrophages differ in terms of receptor expression, cytokine and chemokine repertoires, and effector function. M1 macrophages exposed to the classic activation signals express receptors such as CD16, CD32, CD64, TLR2, and TLR4, whereas M2 macrophages are characterized by abundant levels of nonopsonic receptors such as the mannose receptor (MR) [24]. In order to investigate if PPAR agonists and $cPLA_2$ antagonist induced macrophage polarization during *L. mexicana* infection, we evaluated MR and TLR4 expression by flow cytometry. Since BALB/c mice macrophages can fully support maturation of alternatively activated macrophages, J774A.1 macrophages were incubated with PPAR agonists and $cPLA_2$ antagonist, and then infected or stimulated with LPS and zymosan; neither infection nor treatments induced MR expression, an M2 receptor classified as an alternative activation marker (Supplementary Figure 3); however, PPAR agonists and $cPLA_2$ antagonist induced an increase of TLR4 expression at 60 and 120 min after infection (Figure 3), an M1 receptor classified as classical activation marker. These results show that PPARβ/δ GW501516 and PPARγ GW1929 agonists and $cPLA_2$ antagonist do not help to keep the M2 polarization profile; instead, PPAR agonists and $cPLA_2$

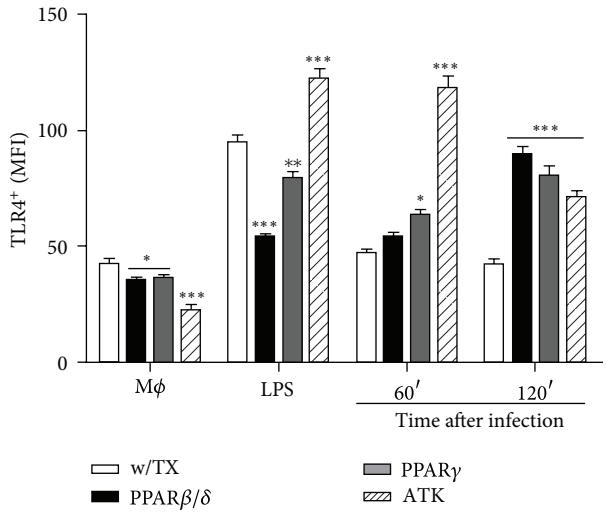

Figure 3: TLR4 expression in *L. mexicana*-infected macrophages. Cells were treated or not with PPAR agonists for 24 h and $cPLA_2$ antagonist for 1 h before infection. TLR4 expression was analyzed by flow cytometry. LPS (2 h) was used as a positive control of induction. Graph bars are mean ± SEM of three independent experiments, and statistical analysis was performed comparing, for each time, treated versus non-treated macrophages; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

antagonist promote the polarization of macrophages toward an M1 profile.

3.4. PPARγ Activation by Agonist and $cPLA_2$ Inhibition Reduce Parasite Burden. PPARγ expression is strongly associated with maturation of M2 macrophages. Gallardo-Soler et al. and Adapala and Chan [25, 26] have demonstrated that PPAR agonists increased intracellular growth of *L. major* in bone-marrow-derived macrophages; moreover, PPARγ agonist, Curcumine, induced PPARγ expression in residential, liver, and spleen macrophages of BALB/c mice. In addition, oral administration of Curcumin further increases PPARα and PPARγ expression, and this increase was associated with a heavier parasite burden. To analyze how PPARβ/δ GW501516 and PPARγ GW1929 agonists and $cPLA_2$ inhibition affected parasite burden in treated macrophages, their phagocytic activity was evaluated through two parameters: the phagocytizing cell percentage and the number of phagocytized zymosan particles or parasites/cell; treated or nontreated macrophages were incubated with FITC-labeled zymosan particles by 2 h or infected with CFSE-labeled promastigotes by 1-2 h (Figure 4). PPARγ GW1929 agonist does not increase the number of zymosan-phagocytizing macrophages, but PPARβ/δ GW501516 agonist and $cPLA_2$ ATK antagonist treatments decreased the number of zymosan-phagocytizing macrophages ($P < 0.05$ and $P < 0.001$, resp.; Figure 4(a) (zymosan)); on the contrary, zymosan particles/cell (mean fluorescence intensity (MFI)) increased significantly with all treatments, GW501516 and GW1929 ($P < 0.001$) and ATK ($P < 0.05$) (Figure 4(b) (zymosan)). This result shows that phagocytic activity *per se* was not affected by treatments. The number of CFSE-labeled parasites-phagocytizing macrophages did not increase by treatments either; instead, parasites-phagocytizing macrophages diminished significantly with GW1929 ($P < 0.01$) and ATK ($P < 0.001$) at 60 min after infection and 2h after infection, GW501516 ($P < 0.01$), GW1929 ($P < 0.001$), and ATK ($P < 0.001$) (Figure 4(a)). Only $cPLA_2$ antagonist (Figure 4(b)) slightly increased the number of parasites/cell ($P < 0.05$) at 60 min after infection, but neither PPARβ/δ nor PPARγ agonists increased it; however, at 120 min after infection PPARβ/δ agonist slightly increased parasite burden ($P < 0.05$), but PPARγ agonist and $cPLA_2$ inhibition decreased parasite burden significantly ($P < 0.001$) (Figure 4(b)). These results together demonstrate that PPAR activation by these agonists and $cPLA_2$ inhibition did not increase parasite load.

3.5. PPAR Activation by Agonists Selectively Regulates Prostaglandin Production in L. mexicana-Infected Macrophages. Prostaglandins are potent ligands of the intracellular PPAR receptors in macrophages, and their binding to PPARα and PPARγ causes macrophage deactivation. Pérez-Santos and Talamás-Rohana [14] have demonstrated that COX-2 inhibition induced leishmanicidal activity by splenocytes [14]. Thus, one possible mechanism of intracellular survival of *Leishmania* is the deactivation of macrophages by prostaglandins produced [27]. In order to investigate if PPAR agonists could modulate inflammatory prostaglandins, macrophages were treated with PPAR agonists or $cPLA_2$ antagonist before the infection to look for PG's metabolites in the conditioned media (Figure 5). PPAR activation and $cPLA_2$ inhibition decreased significantly 6k-$PGF_{1\alpha}$, PGE_1, and $PGF_{1\alpha}$ production (Supplementary Figure 4); however, PPARγ agonist was not able to reduce PGE_2 production which increased significantly after infection ($P < 0.001$) at 60 and 120 min, respectively (Figure 5). PPARβ/δ agonist and $cPLA_2$ antagonist did not increase PGE_2 production; even its production diminished ($P < 0.05$ and $P < 0.001$) at 120 min after infection, respectively; moreover, PPAR agonists and $cPLA_2$ antagonist significantly increased $PGF_{2\alpha}$ production ($P < 0.001$) at 60 and 120 min after infection, respectively. In summary, PPAR agonists and $cPLA_2$ antagonist diminished 6k-$PGF_{1\alpha}$, $PGF_{1\alpha}$, and PGE_1, and neither *Leishmania* infection nor LPS was able to recover their production; however, $PGF_{2\alpha}$ production was increased after infection, and only PPARγ activation increased PGE_2 production.

3.6. PPAR Activation and $cPLA_2$ Inhibition Increase the Oxidative Burst during J774A.1 Macrophages Infection with L. mexicana Promastigotes. Several studies have demonstrated that ROS modulate arachidonic acid metabolism and production of eicosanoids in activated macrophages [28, 29]. In murine and human macrophages, it has been established that the respiratory burst of the cell with production of ROS, such as H_2O_2 and O_2^-, is primarily responsible for parasite control, as these molecules have been reported to be fatal for *Leishmania* promastigotes. We next analyzed the effect of PPAR agonists and $cPLA_2$ antagonist on the oxidative burst induced by *L. mexicana* on J774A.1

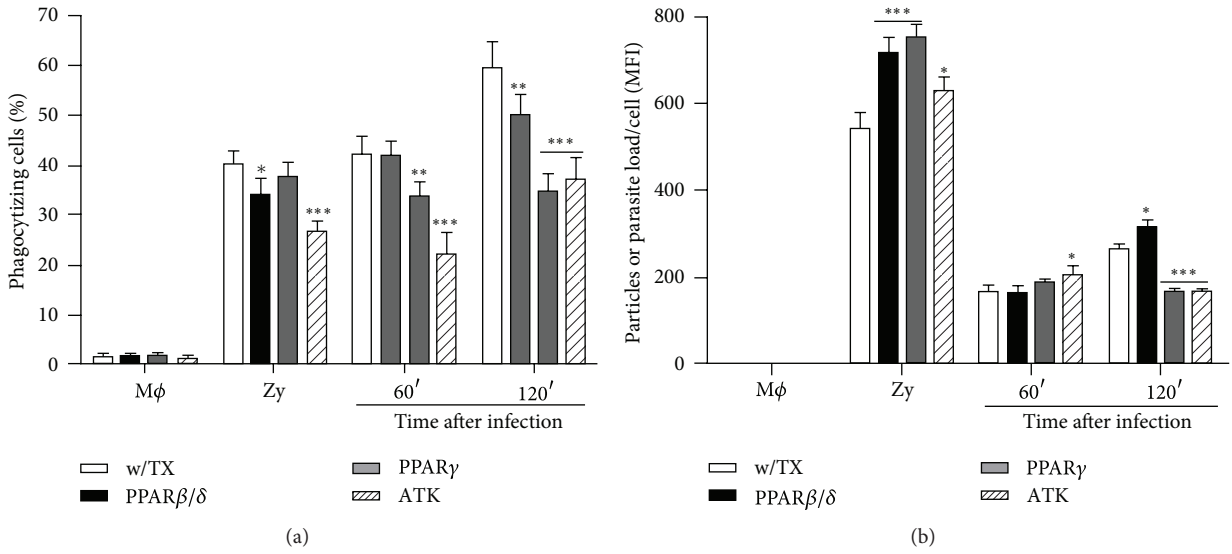

Figure 4: Phagocytic activity of J774A.1 macrophages was determined for zymosan and *L. mexicana* promastigotes uptake; treated or nontreated macrophages were incubated with zymosan-FITC for 2 h or infected with CFSE-promastigotes for 1-2 h. (a) Phagocytizing macrophage percentage. (b) Zymosan particles or parasites/cell (MFI). Graph bars are mean ± SEM of three independent experiments and statistical analysis was done comparing, for each time, treated versus nontreated macrophages; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

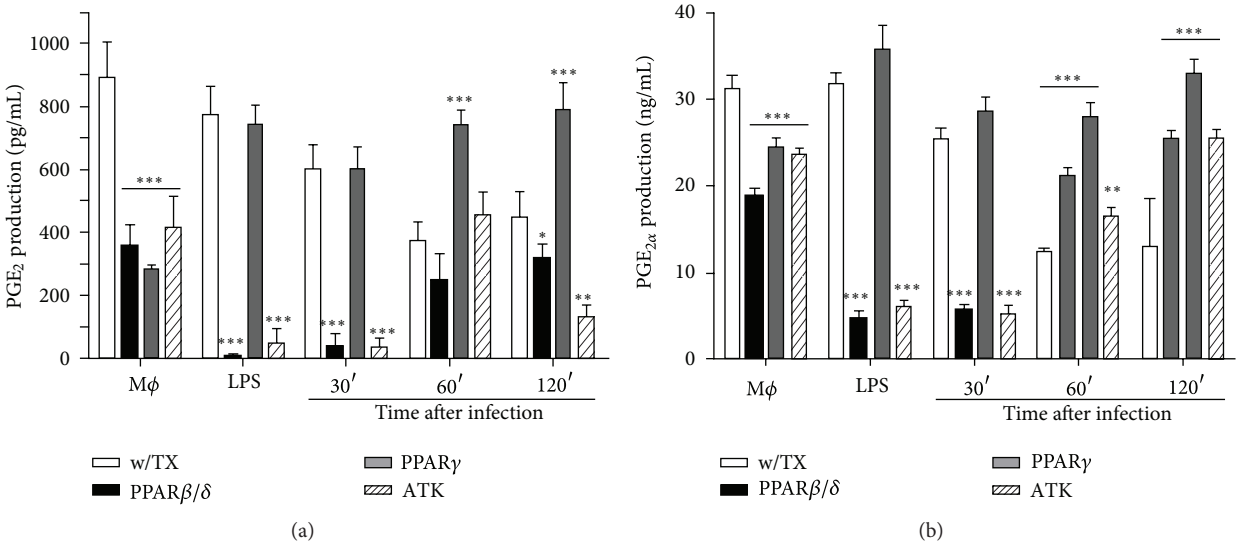

Figure 5: Prostaglandin production by *L. mexicana*-infected macrophages. Prostaglandins were analyzed by MS/MS assay; product scanning experiments were conducted using nitrogen as collision gas, and the collision energy was optimized for individual compounds to generate the most abundant product ions. These product ion spectra were then used to select the precursor-product ion pairs for the development of MRM assays. Deuterium-labeled prostaglandins were used as internal standards for quantitation. Graph bars are mean ± SEM of three independent experiments, and statistical analysis was done comparing, for each time, treated versus nontreated macrophages; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

macrophages (Figure 6). We found that positive macrophages to *L. mexicana*-induced oxidative burst increased from ~34.1 to 58.6% ($P < 0.001$) by PPARγ agonist at 120 min after infection compared to non-treated macrophages, whereas treatment with PPARβ/δ agonist increased from ~34.1 to 40.32% ($P < 0.05$) (Figures 6(a) and 6(b)). PPARβ/δ agonist slightly increased the number of positive oxidative burst macrophages at 120 min after infection, and when the oxidative burst was quantified, it increased, ~1.3-fold at 120 min after infection too ($P < 0.05$) (Figure 6(c)). On the other hand, $cPLA_2$ inhibition increased ~2.51-fold the oxidative burst at 120 min after infection ($P < 0.001$) compared to nontreated macrophages (Figure 6(c)). Finally, PPARγ agonist increased the oxidative burst ~2.52-($P < 0.001$) and ~3.55-fold ($P < 0.001$) at 60 and 120 min after infection, respectively; indicating that PPARγ activation

induces an aggressive oxidative response to intracellular parasites (Figure 6(c)).

4. Discussion

The $cPLA_2$ activation, COX-2 expression, and PG production are positioned at the core of a common regulatory circuit controlling the initiation, magnitude, duration, and resolution of the inflammatory response. During the inflammatory phase, proinflammatory genes expression is controlled at transcriptional, posttranscriptional, and translational levels. According to several reports, in this work we have confirmed that phosphorylated $cPLA_2$ and COX-2 are key enzymes during *Leishmania* infection [14, 23, 30]; in addition, we have shown that PPAR activation by agonists prevents $cPLA_2$ phosphorylation and COX-2, either protein or mRNA expression, during macrophages infection with *L. mexicana*. Previously, Pérez-Santos and Talamás-Rohana [14] showed that COX-2 inhibition increased IL-12 and IFNγ production and induced NO production and parasite killing. Moreover, it has been demonstrated previously that p-$cPLA_2$ activates COX-2 and proinflammatory cytokine genes expression through PPARγ response elements [31]; thus, inhibition of $cPLA_2$ phosphorylation suppresses those genes [32]. In this context, inhibition of $cPLA_2$ phosphorylation with ATK antagonist significantly reduced the mRNA expression of COX-2.

We have demonstrated in this work that PPAR activation by agonists and $cPLA_2$ inhibition by antagonist ATK are able to downregulate IL-10 expression throughout the course of infection with *L. mexicana*. It has been demonstrated that cells from IL-$10^{-/-}$ mice produced more NO, IFNγ, and IL-12 compared with cells from BALB/c mice [8] and IL-$10^{-/-}$ mice which become resistant to infection [7] suggesting that IL-10 increases susceptibility to *L. mexicana* or *L. amazonensis* infection by inhibiting effector cell functions required for parasite killing. IL-10 inhibition after treatments has several consequences. On one hand, it has been demonstrated that it induces the $cPLA_2$-COX-2 pathway; however, results in this work show its downregulation. On the other hand, IL-10 alone or in concert with other molecules activates distinct transcriptional programs that promote the alignment of adaptive responses in a type I or type II direction, as well as by expressing specialized and polarized effector functions [24]. In this case after treatments, infected macrophages did not induce IL-10 expression and remained as classically activated macrophages; this activation program is characterized by TNF-α, IL-1β, and IL-6 expression, these cytokines being responsible of the oxidative burst [15].

Although TNF-α and IL-1β have been shown as detrimental in various pathologies, in this work, they are required to sustain classical macrophage activation combined with a small IL-10 production; several reports have demonstrated that, after *Leishmania* infection, TNF-α and IL-1β induce the phagocytes' NADPH oxidase, whereas IL-10 production inhibits the oxidative stress [15].

The heterogeneity in macrophage phenotypes has given place to its classification as M1 and M2 phenotypes corresponding to classically and alternatively activated macrophages, respectively [33, 34]. M1 macrophages produce high levels of proinflammatory cytokines TNF-α, IL-1, IL-6, IL-23, and ROS; M2 macrophages upregulate scavenger, mannose, and galactose receptors and IL-1 receptor antagonist and downregulate IL-1β and other proinflammatory cytokines [35]. Available information suggests that classically activated M1 macrophages are potent effector cells integrated in Th1 responses, which kill microorganisms and tumor cells and produce copious amounts of proinflammatory cytokines. In this work, we demonstrated that PPAR activation modulates, selectively, different molecules suggesting a macrophage polarization from M2 to M1 profile; among these molecules, we emphasize IL-10 down regulation and upregulation of IL-6, IL-1β, and TNF-α. PPAR activation also diminished $cPLA_2$ phosphorylation and COX-2 expression. This is in agreement with reports showing that the differentiation into classically activated M1 macrophage increases in $cPLA_2$ knockdown cells, whereas the differentiation into alternatively activated M2 macrophage was suppressed by $cPLA_2$-knockdown [36]. These findings suggest that $cPLA_2$ is involved in regulation of macrophage differentiation and macrophage polarization. Polarized macrophages differ in terms of receptor expression. M2 macrophages are characterized by MR (CD206) expression [37], whereas TLR4 expression is associated with M1 macrophages [24, 38]. Our results show that PPAR activation and $cPLA_2$ inhibition significantly increased the TLR4 expression after infection compared to nontreated macrophages, indicating macrophage polarization to M1 profile.

Recent evidence suggests that PPARγ activation may increase the replication of parasites as well as maintain the survival of the host. In particular, PPAR activation has been associated with parasite survival and increase of parasite burden [25, 26, 39, 40]. Flow cytometry analysis revealed that phagocytic activity was not affected by treatments as indicated by zymosan particles assay, and neither PPAR activation nor $cPLA_2$ inhibition increased significantly the percentage of infected macrophages or the parasite burden as other agonists do.

Previous studies have reported that *Leishmania* infection, both *in vitro* and *in vivo*, conducts to PGE_2 production, and it has been postulated that this may favor *Leishmania* persistence and progression [14, 30]. Among prostaglandins analyzed, 6k-$PGF_{1\alpha}$, PGE_1, and $PGF_{1\alpha}$ production was increased after infection, and PPAR agonists and $cPLA_2$ antagonist diminished their production after infection; however, both PGE_2 and $PGF_{2\alpha}$ production was diminished after infection and increased in treated and infected macrophages. This result may seem in conflict with previous data reporting an increase in PGE_2 after infection. This could be explained by the fact that COX-2 enzyme and its principal catalytic product PGE_2 are often equated with inflammation and pathology, a notion fueled primarily by a strong induction of COX-2 expression at sites of inflammation and tissue injury [41]; however, at a later phase, COX-2 promoted resolution by generating an alternate set of reportedly anti-inflammatory prostaglandins through a process now regarded as "eicosanoid class switching." In addition, it has

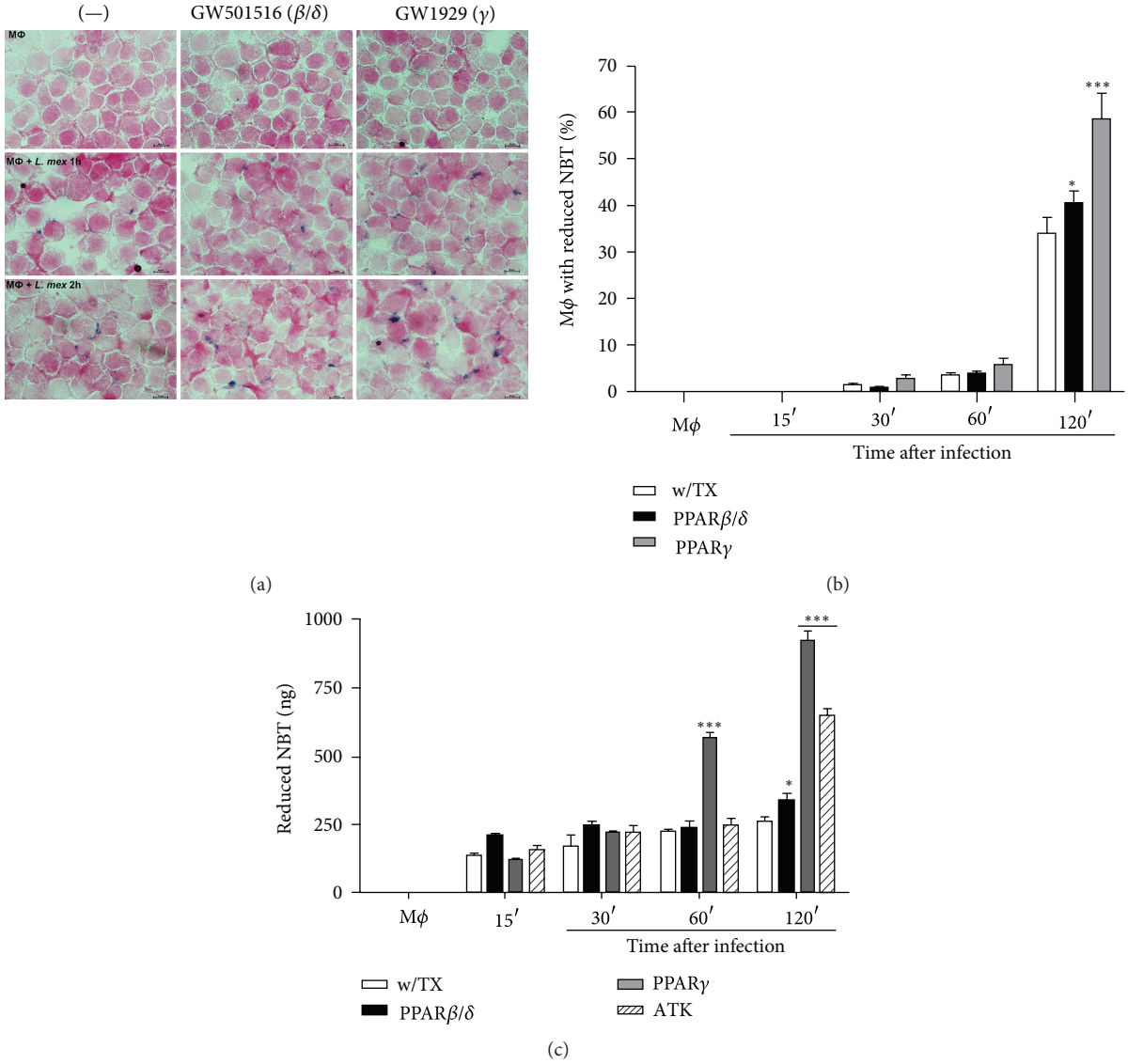

FIGURE 6: Oxidative burst of *L. mexicana*-infected macrophages. Cells were treated with PPAR agonists 24 h before infection, and the oxidative burst was determined by NBT reduction. NBT was added simultaneously with promastigotes. (a) After the indicated times after infection, slides with infected macrophages were washed and stained for 30 min with Fuccina. Microphotographs show positive cells to NBT reduction in comparison with control cells, which were treated or not with agonists in the presence of NBT. (b) The graph shows percentage of cells positive to NBT reduction. (c) Quantitative analysis of NBT reduction of macrophages infected and treated or not with PPAR agonists. Graph bars are mean ± SEM of three independent experiments, and statistical analysis was done comparing, for each time, treated versus nontreated macrophages; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

been demonstrated that PGE_2 can modulate various steps of inflammation; at the beginning it can induce the expression of COX-2; however, as the inflammation progresses and recovering initiates, PGE_2 can also inhibit the expression of this enzyme. Therefore, PGE_2 can exert both proinflammatory and anti-inflammatory effects. Akarasereenont et al. [42] demonstrated that, in HUVEC cells treated with IL-1β, PGE_2 can inhibit COX-2 but not COX-1 protein expression. Therefore, results, suggested that PGE_2 can initiate a negative feedback regulation in the induction of COX-2 elicited by IL-1β in endothelial cells [42]. Based on these results we propose that the expression of IL-1β and TNF-α maintains COX-2 expression in untreated macrophages and as stated by Akarasereenont et al., PGE_2 is able to inhibit COX-2 expression in the presence of these proinflammatory cytokines; together with these results, $cPLA_2$ inhibition also inhibits COX-2 expression via PPARγ [29], and authors have proposed that $cPLA_2$ inhibition can be reverted during M2 to M1 polarization of macrophages [36]. All these results may explain why, during PPARγ activation, PGE_2 production increased during macrophages infection with *L. mexicana*.

In murine and human macrophages, it has been established that the respiratory burst of the cell, with the production of ROS such as H_2O_2 and O_2, is largely responsible for parasite control as these molecules have been reported to be fatal for *Leishmania* promastigotes [43, 44]. It has been demonstrated that *L. donovani* inhibits the respiratory burst in macrophages [45]. In this work, we have shown that PPAR activation, as well as $cPLA_2$ inhibition, increased ROS production by 1-2 folds. It has been demonstrated that long-chain fatty acids increase intercellular ROS synthesis via PPARα, and its inhibitors reduced ROS concentration [46].

The FDA has approved several synthetic PPAR ligands as therapeutic drugs [47]. These PPAR ligands could have a potential use in parasitic diseases. Recently, Serghides et al. [48] have shown that rosiglitazone, a PPARγ agonist, is useful in alleviating cerebral malaria in a murine model [48].

It has been demonstrated that different *Leishmania* species can induce a different profile of cytokines [6, 49], and the enzymes responsible for ROS production are regulated by those cytokines; therefore, treatment against *Leishmania* infection would depend on the infecting species. Thus, cutaneous leishmaniasis caused by *L. major* could be alleviated with PPARα and PPARγ ligands in murine models [26, 39]. In this work, we have demonstrated that PPARβ/δ and mainly PPARγ activation induced macrophage activation through their polarization to M1 profile, with an increase of microbicidal activity against an intracellular pathogen, *L. mexicana*. Based on the above reasons, macrophage polarization from M2 to M1 through PPAR activation in the presence of agonists could be considered as a potential signaling pathway for drug design and eventually to be used as a strategy to control intracellular parasitosis.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

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Research Article

Signal Transducer and Activator of Transcription Factor 6 Signaling Contributes to Control Host Lung Pathology but Favors Susceptibility against *Toxocara canis* Infection

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Using STAT6$^{-/-}$ BALB/c mice, we have analyzed the role of STAT6-induced Th2 response in determining the outcome of experimental toxocariasis caused by embryonated eggs of the helminth parasite *Toxocara canis*. Following *T. canis* infection wild-type BALB/c mice developed a strong Th2-like response, produced high levels of IgG1, IgE, and IL-4, recruited alternatively activated macrophages, and displayed a moderate pathology in the lungs; however, they harbored heavy parasite loads in different tissues. In contrast, similarly infected STAT6$^{-/-}$ BALB/c mice mounted a weak Th2-like response, did not recruit alternatively activated macrophages, displayed a severe pathology in the lungs, but efficiently controlled *T. canis* infection. These findings demonstrate that Th2-like response induced via STAT6-mediated signaling pathway mediates susceptibility to larval stage of *T. canis*. Furthermore, they also indicate that unlike most gastrointestinal helminths, immunity against larvae of *T. canis* is not mediated by a Th2-dominant response.

1. Introduction

Toxocariasis is a helminth infection considered as a zoonosis and is caused by the larvae of *Toxocara canis;* this parasite affects many paratenic hosts including humans. This infection is world-wide distributed mainly because its transmission is associated with domestic dogs. This disease is starting to be considered as a public health problem in Latin America and Asia [1, 2] but has been extended in the last few years to developed countries as shown by increased numbers of cases-report published more frequently [3–5]. Toxocariasis in humans results from accidental ingestion of *Toxocara*-embryonated eggs from excreta in the environment or by direct dog-to-person contact [6, 7]. Although larvae in muscle may be relatively symptom less, those in brain may cause serious neurological disorders [8]; these symptoms are believed to result from inflammation following degeneration of the parasite [9].

In the experimental model of murine toxocariasis, infection of inbred mice with *T. canis* induces a strong Th2-like response similar to that observed following infection with other helminthes such as *Nippostrongylus brasiliensis* and *Trichuris muris* [10–12]. While widely accepted, Th2-type response in clearing helminth infections has limitations [11, 13–17], its role in mediating protection against toxocariasis in paratenic hosts is not clear, mainly because reinfection

in toxocariasis favors parasite survival [18]. Recent advances in the immunobiology of *T. canis* indicate that regulatory mechanisms are raised after infection; mainly T regulatory cells have been involved in limiting pathologic damage by the inflammatory response [19]; however, there are few data regarding on mechanism of protection or susceptibility against such parasite.

Previous studies have found that some extraintestinal larvae from other helminthes such as *Taenia crassiceps and Trichinella spiralis* are apparently eliminated in infected mice by a Th1-mediated inflammatory response during early phase of infection [20–22]. Furthermore, both studies found that STAT6 was involved during early phase of infection in rendering them more susceptible to cysticercosis and trichinellosis, respectively. These findings suggest that while Th2-type response may be involved in mediating resistance on gastrointestinal helminth infections, this pathway may be involved in susceptibility to the larval stages of such parasites. Numerous studies using STAT6$^{-/-}$ mice have shown that STAT6-mediated IL-4/IL-13 signaling pathway is critical for Th2 differentiation [23]. For example, STAT6$^{-/-}$ mice fail to mount a significant Th2 response and cannot control worm burdens following infection with gastrointestinal helminth parasites [17, 24]. Conversely, STAT6$^{-/-}$ mice develop a Th1 like response and control infections caused by intracellular protozoan parasites such as *Leishmania mexicana* and *Trypanosoma cruzi* [25, 26] indicating that STAT6-mediated signaling pathway inhibits development of protective immunity by inhibiting a Th1 development.

The purpose of this study was to determine the role of a Th2-type response induced via STAT6-mediated signaling in the outcome of experimental murine toxocariasis caused by the L2 of the nematode *T. canis*. To approach this question, we compared the course of *T. canis* infection in STAT6$^{-/-}$ BALB/c mice (STAT6$^{-/-}$) with that in the wild-type BALB/c (STAT6$^{+/+}$) mice. In addition, we analyzed both the antibody and cytokine profiles in sera, as well as the phenotype of lung macrophages. Our data demonstrate that Th2-type response induced via STAT6-signaling pathway mediates susceptibility in toxocariasis.

2. Materials and Methods

2.1. Mice. Six-8-week-old male STAT6$^{-/-}$ and STAT6$^{+/+}$ mice in a genetic BALB/c background were originally purchased from The Jackson Laboratory Animal Resources Center (Bar Harbor, Maine, USA) and were maintained in a pathogen-free environment at the FES-Iztacala, U.N.A.M. animal facility in accordance with Institutional and National guidelines.

2.2. Isolation of Larvae and Eggs and Infection Protocol. Adult *T. canis* females' worms were isolated from the intestine of naturally infected puppies (<3 months). Isolation and embryonation of eggs were performed as follows: female worms were dissected and from the uterus eggs were isolated and putted into distilled water; then the mixture was centrifuged two times for 10 min at 2,000 ×g in a solution of NaHCl at 1%. After removal of the supernatant, the sediment was two times washed in distilled water and placed into the solution of formalin at 1% in tissue flasks at 28°C for 1 month with gentle daily agitation until the end of embryonation which was controlled under the microscope.

2.3. Infection. Five hundred larvated eggs were intragastrically administered with a Foley tube to both STAT6$^{-/-}$ and STAT6$^{+/+}$ mice. Infected mice were sacrificed at days 5, 14, and 60 postinfection, and the parasites harvested from different tissues (lung, liver, brain, and muscle) were enumerated as described previously [27].

For histological evaluation of different tissues, animals were euthanized at indicated days. The liver, lung, brain, and muscle were removed and fixed in 4% formalin. Tissue samples were embedded in paraffin, and 5 μm sections were cut on a microtome and stained with hematoxylin and eosin for histological examination.

2.4. Cytokine Measurements. The IL-4, IL-12, and IFN-γ levels were quantified in mouse serum at the indicated point times. Antibody pairs were used according to the manufacturer's instruction (Peprotech México, México, DF).

2.5. Toxocara-Specific Antibody Level and Total IgE. Peripheral blood was collected at the indicated time points after Toxocara infection from tail snips. The blood was centrifuged at 2500 rpm for 10 min, and serum was collected and tested for Toxocara-specific IgG1 and IgG2a in antigen-coated plates (1 μg/mL). After an overnight incubation at 4°C, the plates were washed with PBS supplemented with 0.05% Tween 20 (Sigma, St. Louis, MO, USA) and blocked with PBS supplemented with 1% BSA (US Biological, Swampscott, MA, USA). Serial dilutions (starting from 1 : 100) of the serum samples were added to the plates. The bound antibodies were detected following incubation with HRP-conjugated rat anti-mouse IgG1 or IgG2a (Zymed, San Francisco, CA, USA). The reactions were developed with ABTS solution (Zymed) and read on a microplate reader at 405 nm (Multiskan Ascent, Thermo Labsystems). Results are expressed as the maximal sera dilution (endpoint titer) where OD was detected. Total IgE production was detected by Opt-ELISA from Biolegend.

2.6. Flow Cytometry. It has been previously shown that macrophages recruited by helminth parasites to the site of infection express alternatively activated and suppressive markers, such as mannose receptor (MR), PD1 ligand 1 (PD-L1), and PD-L2. To determine whether *T. canis*-infected mice recruit such population, flow cytometry was performed on lung exudates cells from *T. canis*-infected at different times after infection. Briefly, 5, 14, and 60 days after infection, lungs lavages were aseptically obtained, and 1×10^6 cells were incubated with anti-CD16 and anti-CD32 (Biolegend, San Diego, CA, USA) to block nonspecific antibody binding. The cells were then stained with APC-conjugated anti-F4/80,

FITC-conjugated anti-MR, PE-conjugated anti-PDL1, and PE-conjugated anti-Gr1 (all from Biolegend) and incubated for 30 min. at 4°C in FACS staining buffer (1% FBS, 0.5% sodium azide in PBS). The cells were analyzed using a FACSCalibur and Cell Quest software (Becton Dickinson).

2.7. RT-PCR. RNA was extracted from isolated spleen cells after different day's postinfection using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the isopropanol-chloroform technique. The RNA was quantified, and 5 μg of RNA was reverse transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen). PCR reactions containing 5x PCR Buffer blue, 10 mM dNTP, 40 nM each forward and reverse primers, 1 U Taq DNA polymerase (Sacace Biotechnologies, Italy), and 2 μL of the cDNA were prepared in a 25 μL final volume. The PCR conditions were elsewhere described [28]. Briefly, consisted of an initial denaturation step at 95°C for 5 min; 35 cycles of 95°C for 40 s, the indicated melting temperature for 50 s and 72°C for 40 s; a final extension step at 72°C for 4 min in a thermal cycler (Corbett Research, Australia). The amplified products were mixed with loading buffer containing SYBR green and observed in a 1.5% agarose gel with the Fujifilm FLA 5000 scanner (Fuji, Japan) using the image reader V2.1 software to capture the images.

2.8. Statistical Analysis. Comparisons between wild-type ($STAT6^{+/+}$) and $STAT6^{-/-}$ groups considered in this work were made using student's unpaired t test. $P < 0.05$ was considered significant. The statistical significance of the sera titers were determined by nonparametric tests using Mann-Whitney U-Wilcoxon Rank.

3. Results and Discussion

It is largely accepted that the Th2-like response induced via STAT6-mediated signaling pathway (through IL-4/IL-13 receptors) plays a critical role in mediating protective immunity against most helminthes [12, 17, 28]. For example, STAT6-mediated signaling has been shown to promote protective immunity against gastrointestinal helminthes such as *Trichinella spiralis*, *N. brasiliensis,* and *Hymenolepis diminuta* [12, 17, 29, 30]. However, the role for many molecules associated with the immune response, including STAT6, during infection with *Toxocara canis* is unknown. Here we analyzed the potential role of STAT6 in modulating immunity against this nematode parasite. One of the first organs that *T. canis* larvae reach early after infection is the lungs, in the present study we detected a significant greater number of larvae in $STAT6^{+/+}$ compared to $STAT6^{-/-}$ mice at day 5 after infection, but later both groups displayed comparable parasite burdens at 14 and 60 days postinfection (Figure 1(a)). In contrast, in the liver as early as 5 days pi $STAT6^{-/-}$ displayed lower parasite burdens, and this was more evident after 60 days pi (Figure 1(b)). In a similar way the parasite burdens in the brain were statistically different 60 days pi (Figure 1(c)). Interestingly, as infection progressed, the number of larvae in the muscles increased significantly in $STAT6^{+/+}$ mice as compared to $STAT6^{-/-}$ mice that successfully reduced the number of parasites by day 60 postinfection (Figure 1(d); $^{*}P < 0.01$). Unexpectedly, the lungs from $STAT6^{-/-}$ mice displayed a greater macroscopic damage as we observed an increased number of hemorrhagic spots in such organs (Figure 2(a)) although that numbers of parasites detected in both groups were closely similar at 14 days pi or even lower in $STAT6^{-/-}$ mice at 5 days pi. It is known that *T. canis* infection promotes the recruitment of leukocytes to the lungs generating an acute inflammatory response; here we observed a greater inflammatory infiltration in the lungs of $STAT6^{-/-}$ mice as early as 5 dpi which was maintained higher in these mice until 60 dpi (Figure 2(b)). Moreover, disruption of alveoli was more frequently observed in $STAT6^{-/-}$ mice, as well as a dominant polymorphic cell infiltration (Figure 2(c)). These findings suggest that STAT6-mediated signaling pathway is involved in both susceptibility and pathogenesis during *T. canis* infection in susceptible BALB/c mice.

Previous studies have demonstrated that STAT6-mediated signaling pathway prevents development of protective immunity mainly against intracellular parasites by inhibiting Th1 development [25, 26]. Therefore, we measured levels of Th1-associated IgG2a as well as Th2-associated IgG1 and Total IgE antibodies in $STAT6^{-/-}$ and $STAT6^{+/+}$ mice at different time points following infection with *T. canis*. Additionally, we also compared the cytokine circulating levels from these same mice. Early in the infection, *T. canis*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice displayed minimum and comparable levels of *T. canis* Ag-specific Th1-associated IgG2a antibodies (Figure 3(a)); these data are in line with those previously reported [27], who also found a low production of this subclass of antibody at early times postinfection. However, by day 60 pi $STAT6^{-/-}$ mice displayed significantly higher levels of specific IgG2a antibodies against *Toxocara* antigens (Figure 3(a)). On the other hand, clear differences were observed with the Th2-associated IgG1 production, where $STAT6^{+/+}$ mice displayed significantly higher titers of *Toxocara*-specific IgG1 as compared to $STAT6^{-/-}$ mice since day 14 after infection (Figure 3(b)). Although Th2-associated IgE has been shown to play a role in mediating immunity against certain extraintestinal helminthes, we found that *T. canis*-infected $STAT6^{-/-}$ mice harbored lower parasite burdens despite producing significantly lower levels of IgE as compared to similarly infected $STAT6^{+/+}$ mice, suggesting that IgE may have a limited role in mediating protective immunity against L2 of *T. canis* (Figure 3(c)). Here it is noteworthy that in spite of a higher Th2-associated antibody response in WT mice, these displayed greater susceptibility to *T. canis*. These data agree with those recently reported by [18], who found that after *T. canis* reinfection in BALB/c mice, reinfected mice displayed significantly higher titers of *T. canis*-specific IgG1; moreover, those antibodies showed a greater avidity for *T. canis* antigens. However, re-infected mice displayed a major number of larvae in different tissues [18]. Together with our data, such findings strongly suggest that a humoral immune response is not protective against L2 *T. canis* infection.

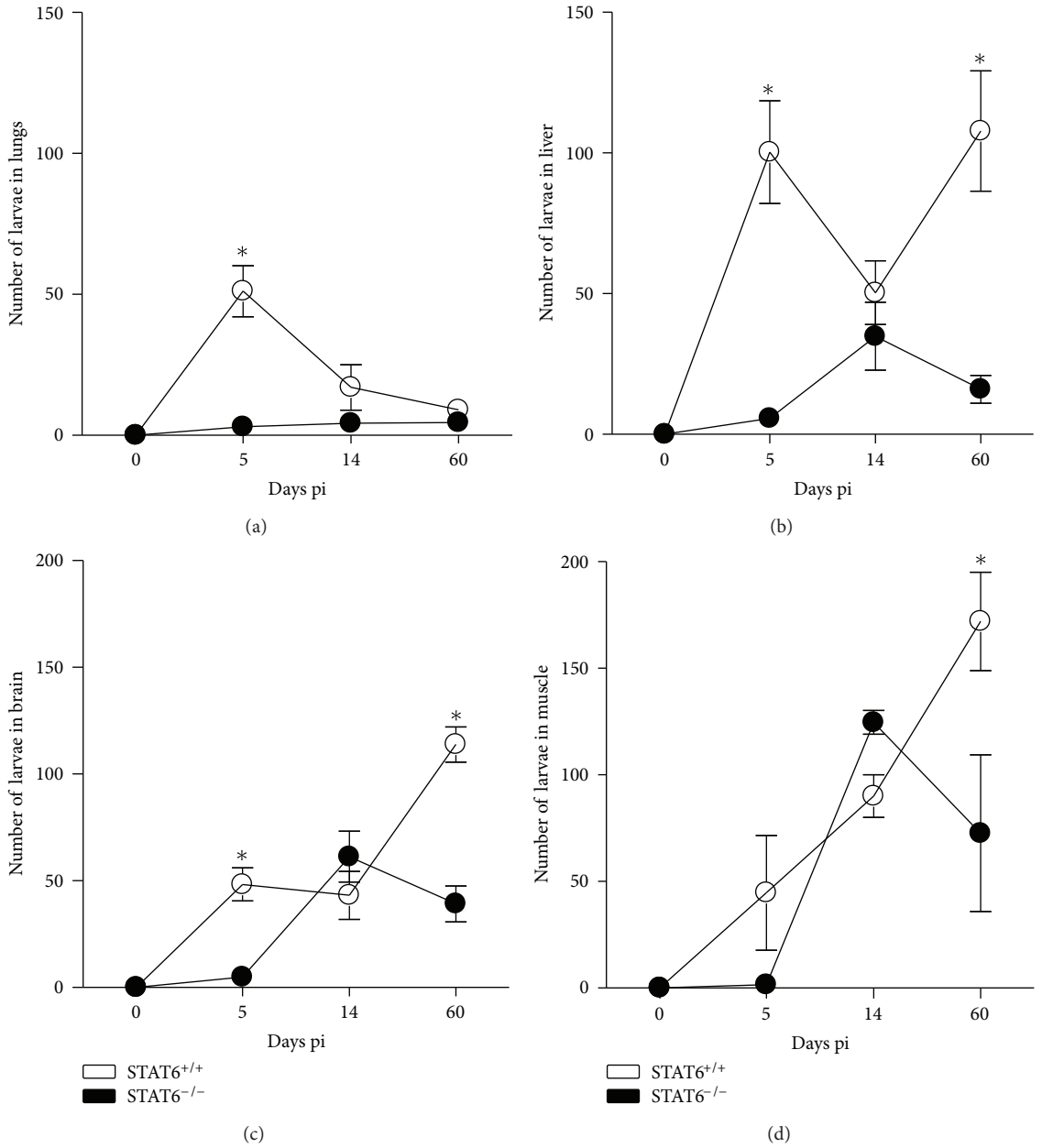

FIGURE 1: STAT6$^{-/-}$ mice efficiently control *Toxocara canis* infection. Course of i.p. *T. canis* infection in STAT6$^{-/-}$ (solid circles) and STAT6$^{+/+}$ (open circles) mice following infection with 500 L2. Data are expressed as the mean ± SE of 4–6 mice per group. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point. Similar results were observed in two independent experiments.

Next we analyzed the cytokine profile in sera that STAT6$^{+/+}$ and STAT6$^{-/-}$ mice displayed during toxocariasis. BALB/c mice at 5 dpi produced significantly more IL-4 and IFN-γ than STAT6$^{-/-}$ mice (Figures 4(a) and 4(b)), whereas the levels of IL-12 were closely similar between groups (Figure 4(c)), indicating that not a clear Th1-type polarization of the immune response was observed in *T. canis*-infected STAT6$^{-/-}$ mice. However, a lack of Th2-type response was confirmed in such mice given the low levels of IgG1 and IgE together with lower systemic levels of IL-4.

To further analyze the immune response, the spleen cells from *T. canis*-infected STAT6$^{+/+}$ and STAT6$^{-/-}$ mice were obtained for RT-PCR analysis of several markers for alternatively activated macrophages (AAM) and some cytokines. While splenocytes from STAT6$^{+/+}$ mice displayed expression of Arginase-1 and Ym-1, both markers for AAM, during early phase of infection, those from STAT6$^{-/-}$ mice did not overexpress such markers (Figure 5(a)). In contrast STAT6$^{-/-}$-infected mice displayed expression of iNOS, a marker for classically activated macrophages, at 14 dpi, whereas STAT6$^{+/+}$

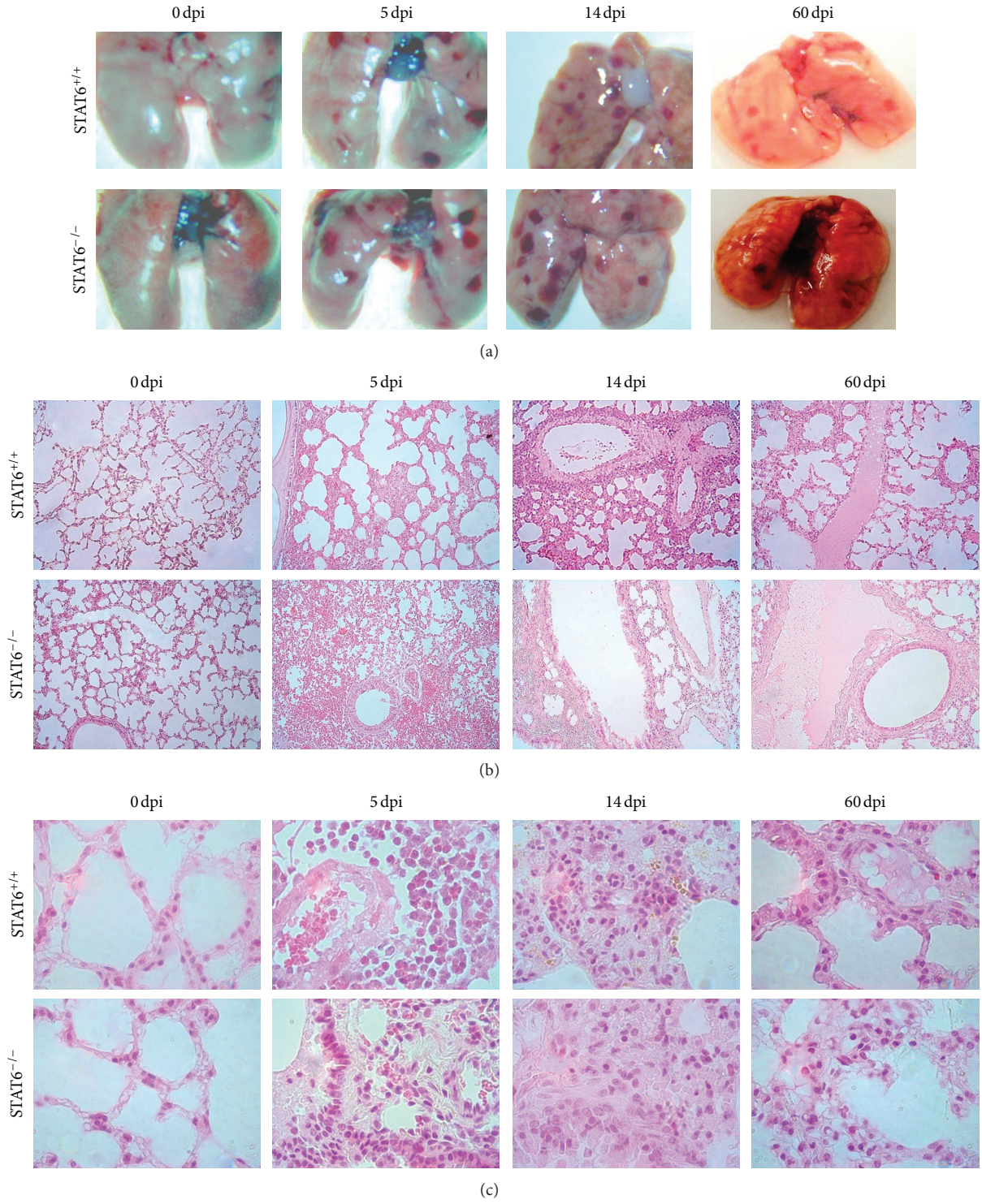

FIGURE 2: STAT6$^{-/-}$ mice display a more severe pathology early in the infection with *T. canis*. (a) Macroscopic appearance of lungs obtained at different time points after oral infection with 500 Larvae of *T. canis*. (b) Lung histology showing airway inflammation in both groups. Magnification 40X. (c) Lung histology, 100X magnification.

mice did not express iNOS (Figure 5(b)). Regarding cytokines we observed a major expression of IL-4 on spleen cells from STAT6$^{+/+}$ than spleen cells from STAT6$^{-/-}$ mice (Figure 5(a)), whereas a similar level of expression was observed for IFN-γ (Figure 5). Thus our data revealed the presence of AAM and IL-4 in the spleens of STAT6$^{+/+}$ mice.

In the last few years, a new cell population has been detected in most of the helminth infections, such population

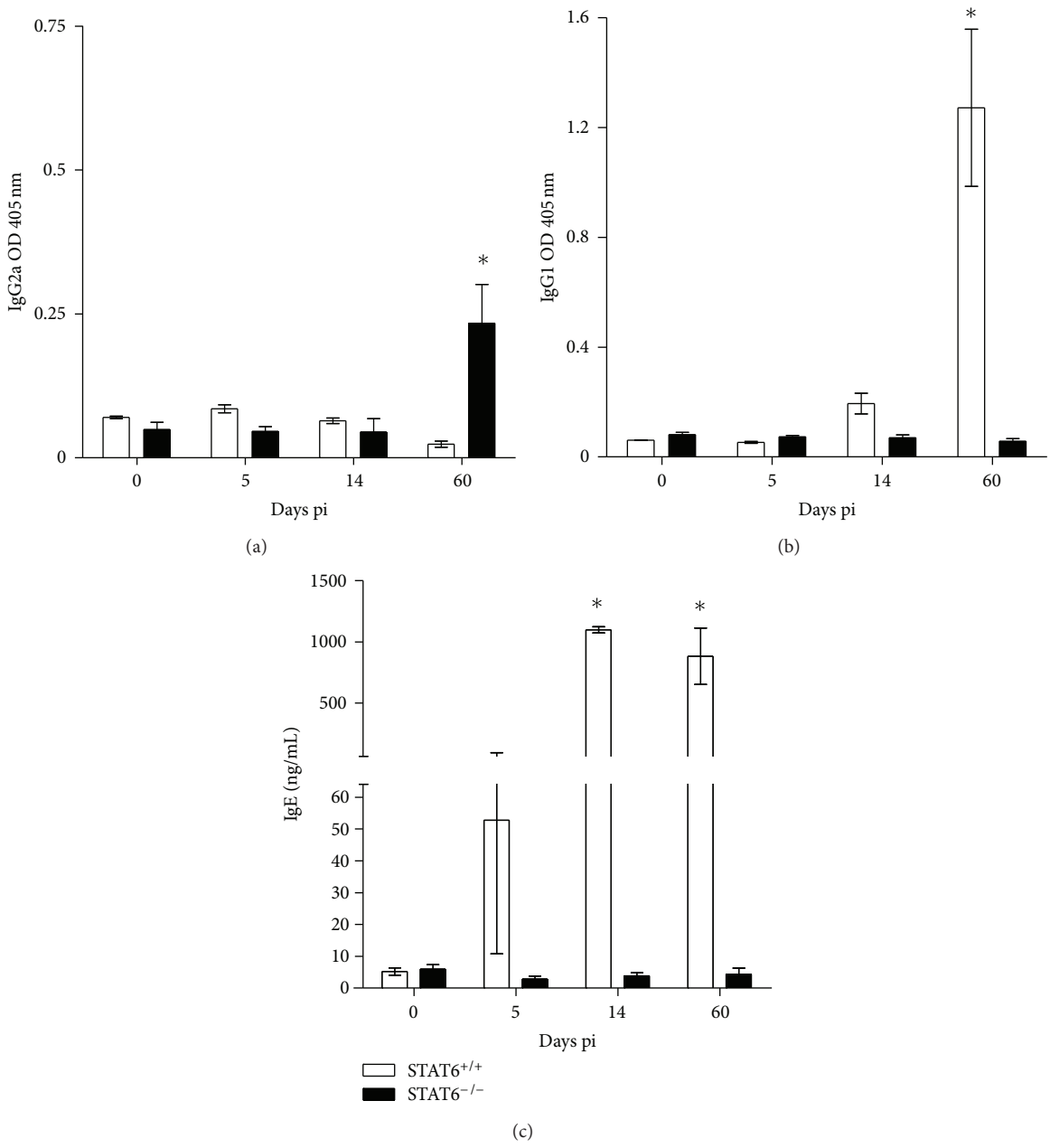

Figure 3: Kinetics of antibody production during *T. canis* infection by STAT6$^{-/-}$ and STAT6$^{+/+}$ mice. (a) Anti-*T. canis* specific IgG2a. (b) Anti-*T. canis* specific IgG1 and (c) Total IgE. Sera were taken from the vein tail of each mouse at time points described. ELISA plates were sensitized with 1 μg/well of soluble extract of *T. canis*. The graphs show the mean ± SE (n = 4–6 animals) and are representative of two independent experiments. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point.

is AAM; the role of these cells appears to be divergent [30] as different authors have demonstrated, for example in gastrointestinal infections by *Nippostrongylus brasiliensis* and *Heligmosomoides bakeri* the presence of AAM is key for worm expulsion [31], whereas in other helminth infections such as *Trichuris muris* and *Hymenolepis diminuta* the presence of AAM was irrelevant [32–34]. In contrast, for schistosomiasis and experimental cysticercosis the presence of AAM appears to be crucial; in the first case, the absence of AAM leads to pathologic disorders in the liver and the hosts die [35, 36], whereas in experimental cysticercosis the presence of AAM leads to susceptibility, given that eliminating AAM with clodronate liposomes helps to clear the infection in otherwise susceptible hosts [37]. According to our knowledge this is the first time that the markers for AAM are reported in *T. canis* infection, but their role is still unknown.

In order to gain knowledge on a possible role for AAM in acute *T. canis* infection, we analyzed the profile of

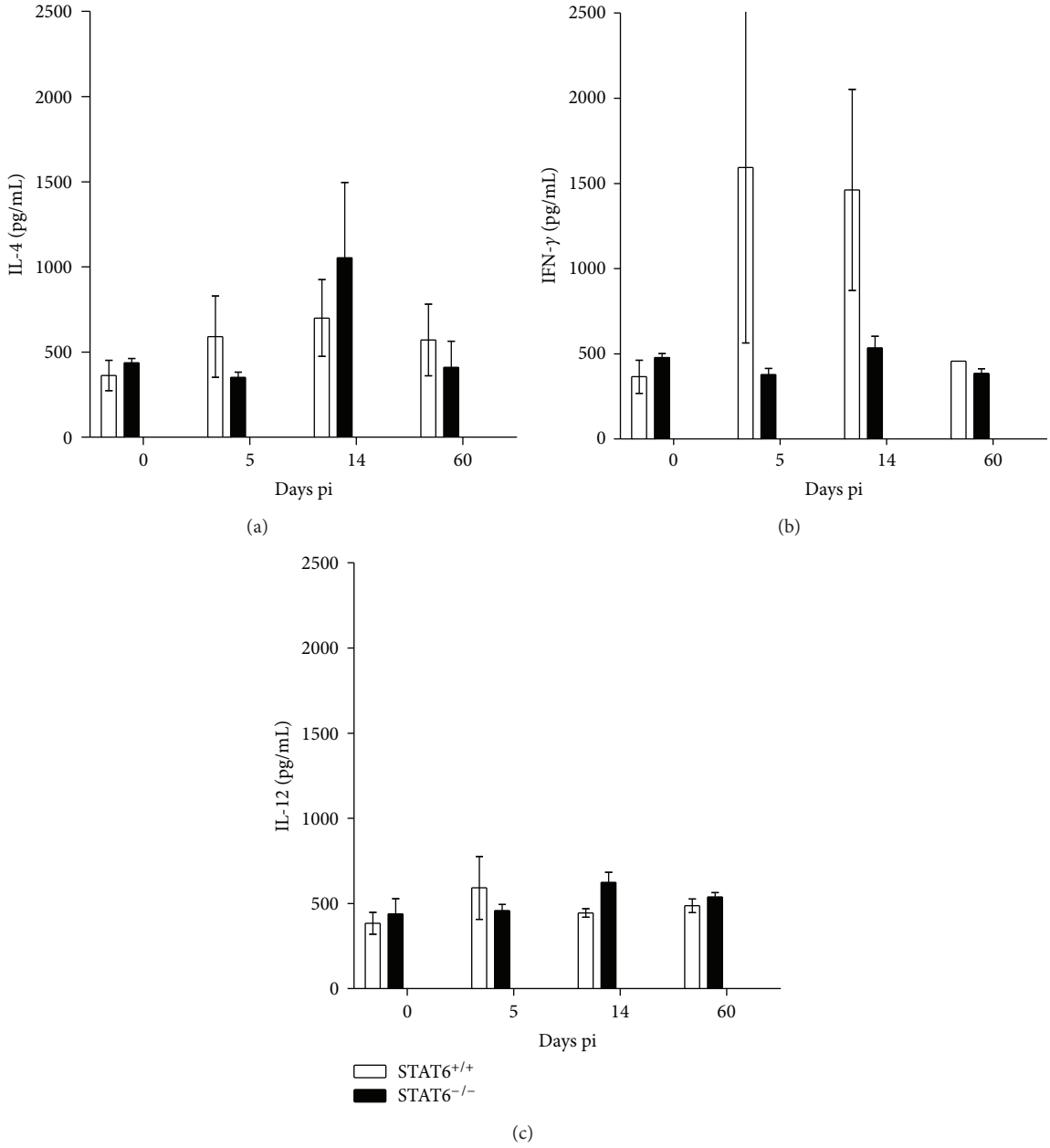

Figure 4: Cytokine profiles from the sera of STAT6$^{-/-}$ and STAT6$^{+/+}$ *T. canis*-infected mice. (a) IL-4 detection. (b) IFN-γ production and (c) IL-12 detection. Data are expressed as the mean ± SE and are representative of three independent experiments, $n = 4$. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point.

macrophages that reach the lungs as early as 5 days pi (time where striking differences in pathology were observed). After 5 dpi lungs were obtained and cut in small pieces, which were further passed through a mesh. Cells were stained for different markers and assayed for cytometry. Stained cells were captured in log, and the region that displayed both high granularity and high size was selected for analysis. As observed in Figure 6(a), lung cells from *T. canis*-infected STAT6$^{+/+}$ mice displayed a greater recruitment of F4/80^{+}MR^{+} cells compared to both naïve mice or *T. canis*-infected STAT6$^{-/-}$ mice; these data suggest a recruitment of AAM, which may participate in tissue repair in the lungs, while in STAT6$^{-/-}$ mice the increase of F4/80^{+}MR^{+} cells was gradual, perhaps because they are unable to mount an efficient Th2 response that may impair the recruitment of AAM in the first days pi, which could be associated with a greatest tissue damage

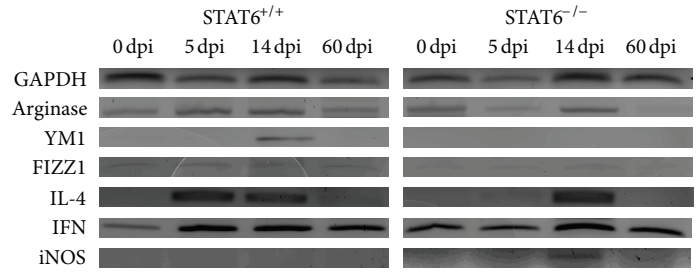

Figure 5: Spleen cells from STAT6$^{-/-}$ and STAT6$^{+/+}$ *T. canis*-infected mice display different levels of transcripts. Spleen cells were harvested at different times after infection and transcript levels of GAPDH, Arginase 1, Ym1, Fizz1, IL-4, IFN-γ, and iNOS were analyzed by RT-PCR. The data shown are from a single mouse and are representative of the findings from three mice examined at each time point.

in the lungs (Figure 2(a)). Interestingly, by day 60 pi this population is increased in both groups, but still more damage prevailed in STAT6$^{-/-}$ mice; this apparent contradiction may be explained just in a time-dependent point of view; it means that AAM early recruited in WT mice had more time to repair the tissue. In contrast, the apparent delay of AAM in reaching the lungs in STAT6$^{-/-}$ mice may need longer time to accomplish their function. An alternate explanation may be the participation of different cell populations for a rapid tissue repair, thus, our observation that another population of cells F4/80^{+}-Gr-1^{+} is also recruited differentially in the lungs of STAT6$^{+/+}$ mice may be indicative that more than one-cell population is involved in tissue repair in this infection (Figure 6(b)). Intriguingly, such F4/80^{+}-Gr-1^{+} took longer to reach the lungs in STAT6$^{-/-}$ mice. However, these cells reach similar levels than in wild-type mice until 60 dpi (Figure 6(b)), if these cells represent a different population of regulatory cells needs further research. On the other hand, the same pattern of recruitment was followed when we analyzed the F4/80^{+}PDL-1^{+} population (Figure 6(c)). All these surface markers have been previously reported associated with macrophages that undergo a distinct activation phenotype in the presence of the Th2 cytokines or helminth infections named AAM [38]. Besides, these AAM have an upregulated expression of arginase-1, RELM-α, and chitinase-like protein Ym1, among other markers. Moreover, although AAM can exhibit antiparasitic activity [31], their most important function in the context of migrating helminth parasites appears to be associated with tissue-repair responses [39, 40]. Taken together these cytometry analyses with those obtained in the macroscopic and microscopic lung analyses, we may associate less tissue damage on *T. canis*-infected STAT6$^{+/+}$ mice with the presence of AAM, and by contrary, we would associate the absence of AAM in *T. canis*-infected STAT6$^{-/-}$ mice with greater lesions and increased lung-cell infiltration. Therefore, we hypothesized that AAM may be involved in mediating protection against helminth-induced immunopathology in the lungs during acute toxocariasis.

Several different reports have shown that Th2 responses are not definitive to kill extraintestinal phases of helminth parasites. For example during muscle infection with *T. spiralis*, BALB/c mice lacking eosinophils displayed similar larval burdens to those of wild-type BALB/c mice [21]. In line with our results, also *T. crassiceps* infection is fully controlled in the absence of STAT6 [22], and more interestingly during experimental neurocysticercosis caused by *Mesocestoides corti* infection STAT6$^{-/-}$ mice displayed a reduction in the number of brain larvae but an increase in clinic neurological symptoms that were associated with lack of AAM [41]. Together all these findings oppose the dogma that Th2-type responses play a critical role in the elimination of all kind of helminthes [42]. These data also suggest that STAT6 pathway may act to limit *Toxocara* larvae-induced immunopathology at least in the lungs.

In conclusion, STAT6$^{-/-}$ BALB/c mice mount a null Th2-like response and efficiently control *T. canis* infection. In contrast, STAT6$^{+/+}$ BALB/c mice develop a predominant Th2-like response that is associated with high levels of IL-4, IgG1, IgE, and AAM and displayed significantly higher parasite burdens in different tissues but interestingly less associated pathology. The findings in our study support the conclusion that STAT6-mediated signaling is critical for the suppression of the immune response that is required for controlling L2 toxocariasis. We postulate that Th2 cytokines may have a dual role during toxocariasis, on one hand may contribute to host susceptibility via STAT6 activation and that neither AAM nor IgE are essential or primarily responsible for eliminating *T. canis* tissue infection, but on the other hand, such response may downregulate the immunopathology induced by *T. canis* larvae in the lungs.

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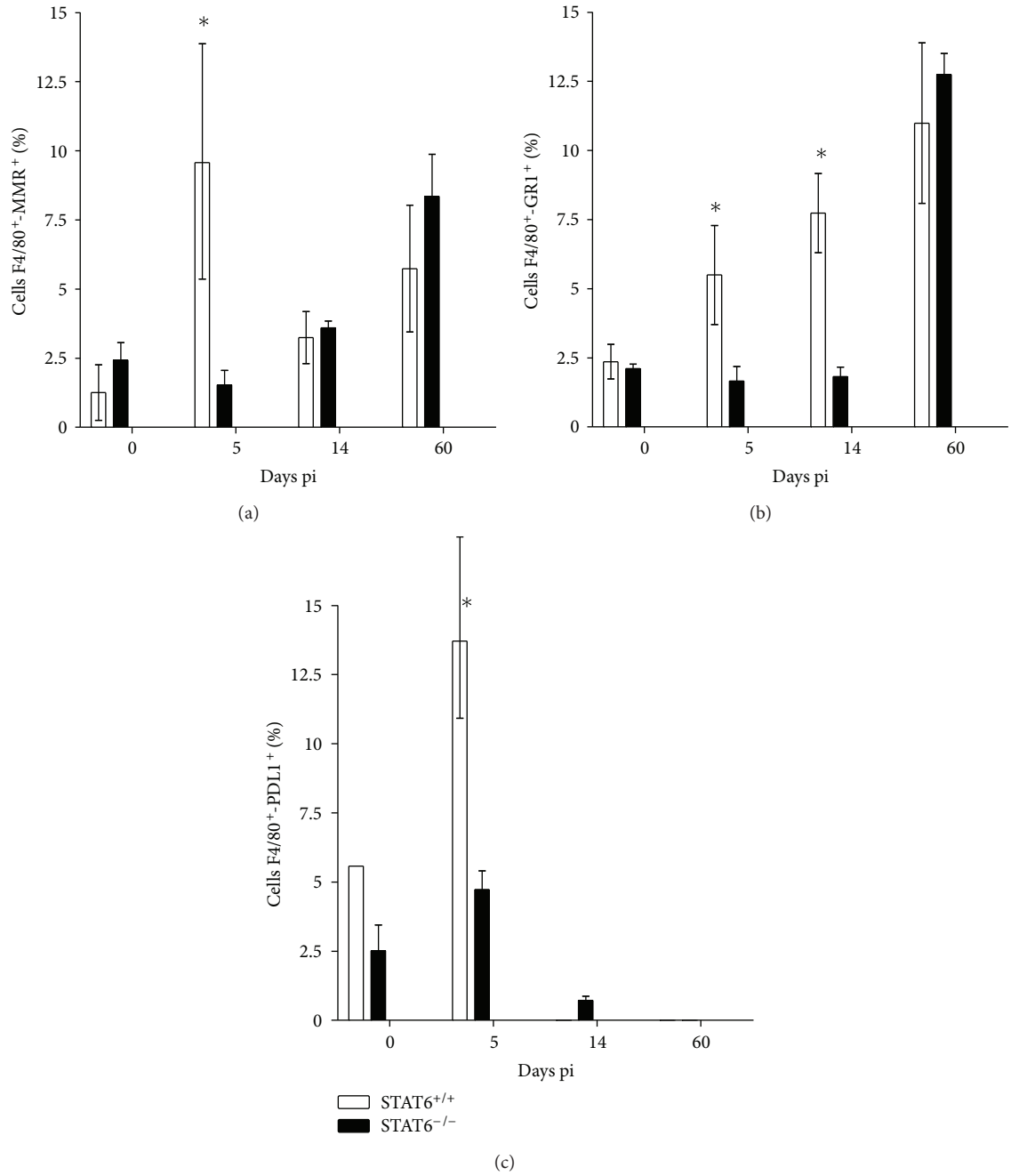

FIGURE 6: Lung macrophages from STAT6$^{-/-}$ and STAT6$^{+/+}$ *T. canis*-infected mice display different phenotypes. Macrophages were obtained at different time points after infection and analyzed by flow cytometry for the detection of different cell markers associated with alternative activation of macrophages. (a) F4/80^{+}MR^{+} lung cells, (b) F4/80^{+}Gr1^{+} lung cells, and (c) F4/80^{+}PD-L1^{+} lung cells, as described in Section 2. Data are expressed as the mean ± SE and are representative of two independent experiments, $n = 4$. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point. ND: not determined.

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Review Article

Parasitic Infections: A Role for C-Type Lectins Receptors

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Antigen-presenting cells (APCs) sense the microenvironment through several types of receptors that recognize pathogen-associated molecular patterns. In particular, C-type lectins receptors (CLRs), which are expressed by distinct subsets of dendritic cells (DCs) and macrophages (MØs), recognize and internalize specific carbohydrate antigens in a Ca^{2+}-dependent manner. The targeting of these receptors is becoming an efficient strategy for parasite recognition. However, relatively little is known about how CLRs are involved in both pathogen recognition and the internalization of parasites. The role of CLRs in parasite infections is an area of considerable interest because this research will impact our understanding of the initiation of innate immune responses, which influences the outcome of specific immune responses. This paper attempts to summarize our understanding of the effects of parasites' interactions with CLRs.

1. Introduction

Lectins are a diverse group of mono- and multivalent proteins and glycoproteins of nonimmune origin that have selective affinity for a carbohydrate or a group of carbohydrates [1]. These proteins are widely distributed in plants, animals, and microorganisms. In animals, lectins have been identified in a great number of cells. Lectins are either embedded in intracellular or cell surface membranes or are present in a soluble form in the plasma. Inside cells, lectins are also found in the cytosol and in the nucleus. Animal lectins play a crucial role in both physiological and pathological processes. Specific interactions between lectins and complex carbohydrates (glycoproteins, glycolipids, polysaccharides, or proteoglycans) are involved in numerous basic phenomena, such as embryonic development, intracellular trafficking, cell-cell and cell-matrix recognition, cell homing, endocytosis, phagocytosis, inflammation, and the metastatic spread of cancer cells (Table 1) [2].

2. Structural Characteristics of C-Type Lectin Receptors

The CLRs constitute a superfamily of more than 1,000 proteins classified into 17 groups based on their phylogeny and domain organization. Most CLRs possess one or more carbohydrate recognition domains (CRDs) or C-type lectin-like domains (CTLDs). The CTLD is a conserved structural motif containing as two protein loops stabilized by two disulfide bridges at the base of each loop. The second loop is more flexible than the first and generally contains the ligand binding site. Most CLRs are membrane-associated receptors that are involved in antigen capture and presentation [25, 26]. Endocytosis mediated by CLRs is guided by their intracellular internalization motifs, whereas some CLRs contain ITIM (immunoreceptor tyrosine-based inhibitory motif)-or ITAM (immunoreceptor tyrosine-based activation motif)-like motifs in their cytoplasmic domains, illustrating the potential immune-suppression or immune-activation functions of these receptors (Figure 1) [27].

Table 1: Summary of structural and functional properties of the lectin family receptors.

Group	Molecules structure	Family members	Ligands	Expression	Function	Reference
C-type	Type-I Type-II (Tm)	MR	Mannose, fucose, and N-acetylglucosamine	MoPh, retina DCs, LCs, Fbls, and kidney	Pathogen recognition, antigen presentation, clearance of endogenous cytopathic molecules, and regulation of circulating hormones	[3–14]
		DC-SIGN	Mannose, ICAM-3	Mesangial cells and CMs, MØ, and DCs	Pathogen recognition, antigen presentation, cell migration, and DC-T-cell interactions	
		SIGNR-1	Zymosan, mannans, and dextran	iDCs spleen MZ, lymph node, and pMØ	Clearance of blood borne antigens	
		Dentin1	β-glucans	DCs, neutrophils, and splenic T cells	Antifungal host defense, induction of TNF-α, and regulation of T-cell proliferation	
		Dectin2	α-mannans	MØ, DCs	Impairment of UV-induced tolerance	
		mMGL1	Gal	MØ, DCs	Internalization and antigen presentation, bind to CD45 to inhibit T cells	
		mMGL2	Sructure Le^x	MØ, DCs	Anti-inflammatory response	
		L-SIGN	Structure $Le^{(a,b,y)}$	Liver sinusoidal endothelial cells	Antigen receptor	
P-type	Type-I (Tm)	CD-MPR CI-MPR	Man-P-GlcNAc Man-6-P	Lysosomal hydrolases	Transport Man-6-P containing acid hydrolases from the Golgi to endosomal/lysosomal compartments	[15–17]
F-type		AAA MsaFBP32	Fucose	Liver and kidney	Modulation of cell functions	[18–22]
I-type	Type-I (Tm)	Siglec-1 Siglec-2 Siglec-4 Siglec-15	Sialic acids with N- and O-linked glycosylations	Myeloid and lymphoid cells	Regulation of cell signaling from leucocytes	[23, 24]
		Siglec-3 10 members humans (3, 5, 6, 7, 8, 9, 10, 11, 14, 16) Rodents Siglec-3, E, F, G, H			Endocytic receptors	

Abbreviations: Tm: transmenbrane; MØ: macrophages; pMØ: peritoneal macrophages; Dcs: dendritic cells; iDCs: immature dendritic cells; MoPh: mononuclear phagocytes; Fbls: fibroblasts; LCs: langerhans cells; CMs: cardiomyocytes; Le^x: Lewis x, a, b, and y structures; Gal: galactose; MR: mannose receptor; DC-SIGN: dendritic cell-specific ICAM-3-grabbing nonintegrin; SIGNR-1: SIGN-related 1; homologe DC-SIGN; mMGL: macrophage galactose type c-lectin; L-SIGN: liver/lymph node-specific ICAM-3 grabbing nonintegrin; CD-MPR: cation-dependent mannose 6-phosphate receptor; CI-MPR: cation-independent mannose 6-phosphate receptor; Man-6-P: mannose 6-phosphate; Man-P-GlcNAc: mannose 6-phisphate N-acetylglucosamine ester; AAA: Anguilla anguilla agglutinin; MsaFBP32: F-lectin present in striped bass (Morone saxatilis).

Based on the primary structure of their CRDs, their folding patterns, and their cation requirements, animal lectins can be classified into several families, including C-, F-, P-, and I-type lectins, galectin, pentraxin, and others (Table 1) [18]. However, the most important molecules from the CLR family include macrophage galactose-type C-type lectin (MGL), dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), the mannose receptor (MR), DEC205, and Dectin-1 (Figure 1).

3. Role of C-Type Lectin Receptors in the Immune Response

The initial recognition of an invading pathogen by antigen-presenting cells APCs, such as macrophages (MØs) or dendritic cells (DCs), is crucial in determining the type of effector T cell that subsequently mediates an immune response [1, 2]. APCs are equipped with highly specialized receptors, including an array of pattern recognition receptors

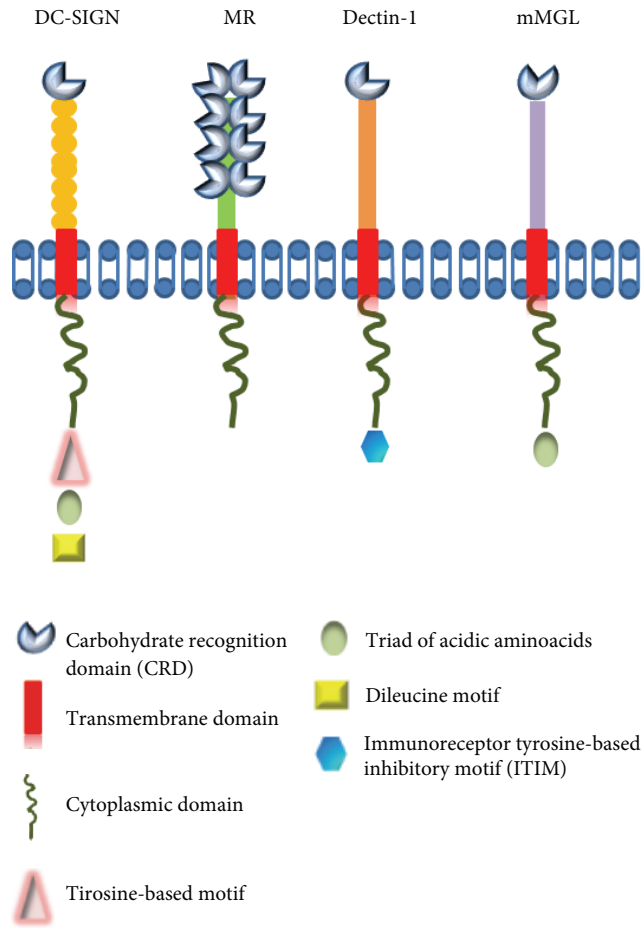

FIGURE 1: Structure of members of the C-type lectin (DC-SIGN, MR, Dectin1, and MGL). These receptors contain one or more carbohydrate-recognition domain (CRD), transmembrane domain, and cytoplasmic domain may contains tyrosine-based motif, triad of acidic amino acids, dileucine motif or immunoreceptor tyrosine-based inhibitory motif.

(PRRs), such as C-type receptors (CLRs) and Toll-like receptors (TLRs). These receptors play an important role in the activation/maturation of APCs upon binding with conserved pathogen structures known as pathogen-associated molecular patterns (PAMPs). In contrast to TLRs, CLRs recognize and internalize specific carbohydrate antigens expressed by pathogens and host tissues in Ca^{2+}-dependent manner [18, 25–27].

Protein-carbohydrate interactions have important roles in two distinct aspects of the immune response. These interactions are involved both in pathogen recognition and in the cellular interactions that lead to pathogen neutralization [28]. Lectin receptors play an important role in the innate immune response by recognizing and binding specific carbohydrate moieties (usually a nonreducing terminal monosaccharide or oligosaccharide) on the surface of potential pathogens through CRDs [1, 2]. CRDs, in combination with other domains, can recognize carbohydrate moieties and induce agglutination, immobilization, complement-mediated opsonization and lysis [25].

In this review, we focus on integral membrane C-type lectins and their participation in the recognition of glycosylated parasite antigens. Despite the presence of a highly conserved domain, C-type lectins are functionally diverse and have been implicated in various processes, including cell adhesion, tissue integration and remodeling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis [3, 29, 30].

The importance of C-type lectins is highlighted by the fact that several pathogens and tumor antigens take advantage of these receptors to escape intracellular degradation and to suppress the generation of an efficient immune response [31]. Several studies have demonstrated that some C-type lectins may function as adhesion, signaling, or antigen-uptake receptors [32–35], and these results are consistent with the fact that some CLRs are present on MØs and DCs, which play a role in the initial step of capturing the antigens containing carbohydrates [36].

Several CLRs have been shown to contribute to the loading of endocytosed antigens on MHC class I and class II, thereby facilitating effective antigen-specific $CD4^+$ and $CD8^+$

T-cell responses [37, 38]. There are evidence that some CLRs (like DC-SING, DC205, and Dectin-1) are able to trigger distinct signaling pathways that modulate cell functions through the expression of specific molecules and cytokines, in most cases promote the antigen presentation and determining the polarizations of T cells [4, 39]. However, most evidence about CLR trigger signaling pathways have emerged using virus, bacterial pathogens, fungus, or peptides. There are not evidence about the interaction of parasites with CLRs and activation of a signaling pathway.

The signaling through MGL is emerging recently, using DCs, has been demonstrated that MGL engagement to anti-MGL antibody or $MUC1_{9Tn}$ triggered the phosphorylation of ERK1,2 and the activation of NF-kB signal promoting DC activation and increase in antigen-specific $CD8^+$ T-cell activation; however, the effects of this activation are strongly dependent on the type of stimulus added to the cells [5].

Moreover, several studies suggest that CLRs may also modulate immune reactions through cross-talk with other receptors, especially TLRs. These results indicate that the outcome of an immune response is determined by the balance between triggering the two receptors families [5, 40]. Many transmembrane C-type lectins belonging to groups II, V, and VI are expressed primarily by myeloid cells. Although many are "orphan" receptors, others have been shown to promote the phagocytosis of nonopsonized microbes and to induce cytokine production in MØs and DCs, leukocytes that play critical roles in innate immunity and in the subsequent modulation of adaptive immune responses [41]. These properties make the C-type lectin family an optimal tool for APCs to target parasites.

4. C-Type Lectins in Parasitic Infection (Table 2)

A number of glycan moieties have been identified in most parasites that potentially bind various CLRs, which act as sensors of the innate immune system.

4.1. Protozoa

4.1.1. Leishmania. The trypanosomatid flagellates of the genus *Leishmania* cause diverse diseases with varying clinical symptoms and underlying pathologies. These diseases include visceral leishmaniasis (Kala-azar), mucocutaneous leishmaniasis, cutaneous leishmaniasis, and post-Kala-azar dermal leishmaniasis (PKDL) [42].

These diseases cause significant morbidity and mortality in the 98 countries or territories, where they are endemic [43]. *Leishmania* have two developmental stages: the promastigote, which is an extracellular flagellated form that is transmitted by insect vectors, and the amastigote, which is an intracellular multiplicative form that multiplies within the phagocytes of the vertebrate host, a process that involves different ligand-receptor systems [44]. The repetitive structure and glycan modifications associated with many *Leishmania* cell surface molecules suggest that these parasites may interact with CLRs, for example, MR and DC-SIGN [6, 45].

Mannose Receptor (MR). MR is a C-type lectin. It is a transmembrane glycoprotein (175 kDa) with eight C-type-lectin-like domains (or carbohydrate-recognition domains, CRDs) that is expressed on the surface of several cell types, such as MØs, DCs, and some epithelial cells. MR mediates the binding and internalization of mannosylated glycoproteins and participates in the endocytosis of different pathogens bearing mannose residues on their surfaces [6, 46, 47].

Previous studies both *in vivo* and *in vitro* have demonstrated the involvement of MR during the recognition and internalization of promastigotes of different *Leishmania* species (*donovani, amazonensis*). Mouse peritoneal MØs infected with *L. donovani* exhibited a decrease in MR activity, with a loss of 50% of original binding activity after 4 days of infection. A possible explanation for this decrease in the expression of MR is the direct correlation with the number of amastigotes within MØs and the recovery of MR activity after the elimination of parasites from MØs after treatment with methotrexate/mL conjugated with bovine serum albumin modified with mannose (Man-BSA) for 3 h [48]. Competition assays with different MR ligands (Man-BSA or D-mannose) revealed an important decrease in the activity of MR, with a loss between 50% to 80% in phagocytic capacity, demonstrating the participation of MR during parasite recognition and the upregulation of MR expression during the initial steps of the infection [6, 35, 48, 49].

A recent study showed that bone marrow-derived macrophages (BMDMs) infected with *L. major* metacyclic promastigotes exhibit TNF-α and IL-12 production levels similar to those in MR-wild-type (MR-WT) mice and MR-knockout (MR-KO) mice. The clinical course of *L. major* and *L. donovani* infections was slightly different with respect to the area covered by lesions between the MR-WT and MR-KO mice at week 7. However, the levels of ulcer healing and the resolution of the lesions were equivalent. Moreover, assays measuring the activation of MAPKs (ERK1/2, p38, and JNK) revealed that MR is not necessary for the inhibition of ERK and p38 activation. In addition, immunohistochemical analysis of cutaneous lesions from MR-KO and MR-WT mice revealed no differences in lesion architecture or cell components. Together, these data suggest that MR is not essential for host resistance against *Leishmania* infections and that either redundant MØ receptors compensate for the lack of MR or MR does not play a role in parasite attachment [45].
Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin (DC-SIGN). Also known as CD209, DC-SIGN is a type II transmembrane CLR that is expressed on DCs and involved in cell-cell interactions through its capacity to bind ICAM-3 and ICAM-2 [50, 61]. This receptor is used by protozoan parasites of the genus *Leishmania*. Previous studies have investigated possible *Leishmania*/DC-SIGN interactions through the use of fluorescence-labeled parasites in combination with blocking agents such as anti-DC-SIGN antibodies and soluble mannan. These studies showed that DC-SIGN is a receptor for the promastigotes and amastigotes of both the visceral (*L. infantum*) and cutaneous (*L. pifanoi*) forms but not for *Leishmania major* metacyclic promastigotes, suggesting that DC-SIGN is a broad *Leishmania* receptor that exhibits variable affinity for distinct infective

Table 2: C-type lectins in parasitic infection.

Parasite	Receptor	Model	*In vivo/in vitro*	Role	Reference
Protozoo					
	MR	BALB/c mice	*in vivo/ in vitro*	Uptake of mannose containing glycoconjugates	[48]
L. donovani	MR	Swiss albino mice	*in vitro*	Binding promastigotes	[36]
	MR	hmDMØ	*in vitro*	Attachment and ingestion promastigotes	[49]
L. amazonensis	MR	Skin Fbls	*in vitro*	Uptake of mannosylated ligands	[6]
L. major	MR	BMDMs MR-KO mice	*in vivo*	Recognizes mannose residues on the surface *Leishmania*, but it's not essential for host defense	[45]
L. pifanoi	DC-SIGN	MDDCs cell line K562	*in vitro*	Binding and internalization of amastigotes	[50]
L. pifanoi *L. infantum*	DC-SIGN	IMDDCs cell lineK562	*in vitro*	Receptor for promastigotes and amastigote infective stages from both visceral and cutaneous leishmaniasis	[51]
T. cruzi *Tulahuen strain*	MR	BALB/c mice Cell line J774 (MØ)	*in vivo/in vitro*	Bind to Cz, increasing MR recycling which leads to arginase activity	[46]
Y and DM strains	MR	CM and MØ	*in vitro*	Adhesion and uptake of parasites	[52]
T. brucei	MGL	C57BL/6 mice BALB/c mice	*in vivo*	Marker of aaMØ	[53]
Nematodes					
T. muris	MR	C57BL/6 MR-KO mice	*in vivo/in vitro*	Recognized components E/S of parasites	[47]
Trematodes					
	MGL	Cell lines SW948, SKBR3, ZR75-1 CHO, BLM, FM3.29 FM6, SK23mel	*in vitro*	Recognized LDN and LDNF glycans	[54]
	MGL	Human DCs	*in vitro*	Internalization of glycolipids of SEA	[55]
	DC-SIGN	Human DCs	*in vitro*	Adhesion to glycolipids of SEA	[7]
S. mansoni	DC-SIGN	Human DCs	*in vitro*	Recognize glycans of SEA	[56]
	L-SIGN	Cell line K562	*in vitro*	Binds to structures $Le^{a,b,y}$ of SEA	[8]
	L-SING	Cell line K562	*in vitro*	Binds and internalization of SEA	[57]
	SIGNR1	BALB/c WT or SIGNR1-KO	*in vivo/in vitro*	Recognize antigens of AWA and SEA	[9]
	Dectin-2	C57BL/6	*in vivo/in vitro*	Binds SEA component	[58]
	MR	C57BL/6 WT or MR-KO	*in vivo/in vitro*	Internalization E/S material by schistosome larvae	[59]
Cestodes *T. crassiceps*	MGL DC-SIGN	Human DCs	*in vitro*	TcES positively modulated the expression of MGL but negatively modulated DC-SIGN	[60]

Abbreviations: MR: mannose receptor; DC-SIGN: dendritic cell-specific ICAM-3 grabbing nonintegrin; SIGNR-1: SIGN-related 1; homologe DC-SIGN; mMGL: macrophage galactose type c-lectin; L-SIGN: liver/lymph node-specific ICAM-3 grabbing nonintegrin; Fbls: fibroblasts; BMDMs: bone marrow-derived macrophages; MDDCs: monocyte-derived dendritic cells; IMDDCs: immature monocyte-derived DCs; MØ: macrophages; CM: cardiomyocyte; Cz: cruzipaina; E/S: excretory/secretory; LDN: [GalNAcβ1–4GlcNAc-R]; LDNF: [GalNAcβ1–4(Fucα1–3)GluNAc-R]; SEA: soluble egg antigens; Le: structures of Lewis; AWA: adult worm antigen; TcES: *Taenia crassiceps* excreted-secreted antigens.

forms and species of the parasite [50, 51]. There is no doubt that these findings are important; however, it remains to be determined whether this recognition influences the immune response to*Leishmaniasis*.

4.1.2. Trypanosoma cruzi. The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), the etiological agent of human Chagas disease, is endemic in Latin America, where 18–20 million of people are infected [62]. Infection leads to an acute phase that may last between 2 and 4 months and is characterized by high numbers of parasites in the bloodstream and tissues. The control of parasite replication leads to chronic, often life-long disease. Most individuals in the chronic phase have a silent, asymptomatic clinical form of Chagas' disease and are classified as indeterminate patients [63]. However, approximately 30% of chronically infected individuals develop a severe clinical form in which digestive and/or cardiac alterations often lead to death [64–67].

During the process of parasite internalization, the interaction between receptors expressed in the host cell and the

parasite is important because these receptors are responsible for recognizing the major antigens of *T. cruzi*. This parasite expresses mucin-like glycoproteins (TcMUCs) on its membrane. These proteins are highly glycosylated glycoconjugates (approximately 60% of their weight is carbohydrates) and are threonine-rich, serine- and proline-rich polyanionic molecules that are anchored to the plasma membrane through glycosylphosphatidylinositol [64, 68, 69].

Furthermore, *T. cruzi* contains a major lysosomal cysteine proteinase called cruzipain (Cz), one of the immunodominant antigens of *T. cruzi*. Cz is a glycoprotein of approximately 52–58 kDa and has both high mannose and complex type-N-linked glycans in the C-terminal domain. It is expressed in all stages of the parasite and is highly immunogenic in humans. Moreover, it has been shown that Cz induces the alternative activation of MØs *in vitro* and upregulates arginase activity. This activation profile was shown to be associated with the functional ability of these cells to promote the intracellular growth of *T. cruzi* [46].

Mannose Receptor (MR). Enzyme binding assays using HRP (horseradish peroxidase) as the mannosylated ligand, which were used to characterize the cardiomyocyte mannose receptor (CM-MR) and its involvement in *T. cruzi* invasion, demonstrated that after the infection of cardiomyocytes (CM) with *T. cruzi*, a considerable reduction in HRP binding was noticed. Binding was almost completely restored by treating the infected cultures with the trypanocidal drug nifurtimox [52]. These results showed that CM-MR participated in the adhesion and uptake of *T. cruzi* by CM.

Another study found that *T. cruzi*-infected MØs preincubated with mannose-bovine serum albumin (Man-BSA, MR specific ligand) exhibited high levels of urea, increased intracellular amastigote growth, the downregulation of JNK and p44/p42 phosphorylation, and an increase in p38 MAPK phosphorylation relative to control cells. In addition, MØs incubated with Cz or Man-BSA exhibited enhanced MR recycling. However, *T. cruzi*-infected peritoneal MØs incubated with an MR-blocking antibody showed reductions in arginase activity and intracellular parasite growth. Moreover, the level of MR on peritoneal cells from *T. cruzi*-infected BALB/c mice at 13 and 15 days after-infection has been evaluated, and flow cytometry analysis revealed an increase in $F4/80^+$ MR^+ cells as the infection progressed. Together, these results showed that the interaction with MR on MØs may be a mechanism by with *T. cruzi* evades the innate immune response both *in vitro* and *in vivo* [46].

4.1.3. Trypanosoma brucei. The protozoan parasite *Trypanosoma brucei (T. brucei)* is the causative agent of the human and animal African trypanosomiasis, which is frequently fatal if not treated. This parasite has a digenetic life cycle, replicating in the alimentary canal of its vector, the tsetse fly, and in the bloodstream of mammals. In the mammalian host, the bloodstream form of *T. brucei* lives and divides extracellularly in the blood, lymph, and interstitial fluids [70, 71]. The bloodstream form of *T. brucei* is rich in galactose-containing glycoproteins, most notably the abundant variant surface glycoprotein (VSG), which protects the parasite from the complement pathway and undergoes antigenic variation to evade specific immune responses [72].

Macrophage Galactose Type C-Lectin (MGL). MGL is a member of the type II family of C-type lectins and has an approximate molecular mass of 42 kDa. MGL is expressed on immature human and mouse DCs and MØs in the skin and lymph nodes [27, 73]. Mice contain two functional copies of the MGL gene, mMGL1 and mMGL2 [74], which are both expressed by dermal DCs and MØs [53, 75], whereas in humans, only one MGL gene is found [76]. mMGL1 and mMGL2 have different carbohydrate specificities: mMGL1 is specific for Lewis X (Le^x) and $Lewis^A$ structures, whereas mMGL2, similar to hMGL, recognizes α/β-GalNAc structures and galactose, including O-linked Tn-antigen, TF-antigen, and core 2 structure [54, 77]. In the skin, MGL is a marker for $CD1a^+$ dermal DCs, a cell type with enhanced ability to stimulate naive T cells relative to other dermal APC subsets.

Raes et al. report that mMGL1 and mMGL2 are induced in peritoneal MØs during *in vivo* infection with *T. brucei*, correlating with a switch from a type I cytokine environment in the early stage of infection to a type II cytokine environment in the late and chronic phases. In addition, it has been demonstrated that the incubation of thioglycolate-elicited peritoneal MØs with IL-4 o IL-13 moderately induced mMGL1 expression and strongly induced mMGL2 expression, but IFN-γ did not [53]. The results presented in this paper suggest that the mMGL1 and mMGL2 receptors are novel markers for type II cytokine-dependent alternatively activated macrophages (aaMØ) both *in vitro* and in the chronic phase of infection with *T. brucei*. These findings are important, but the possible interaction between antigens of *T. brucei* and mMGL remains to be defined, as does the role of mMGL in the immune response.

4.2. Nematodes

4.2.1. Trichuris muris. Several gastrointestinal nematodes have been reported to express ligands for MR on their surface. *Trichuris muris* (*T. muris*) is a natural mouse model of the gastrointestinal nematode parasite *Trichuris trichiura* (*T. trichiura*), one of the most prevalent human helminth infections. Studies of the role of cells in immune responses to *T. muris* and the mechanisms of immune expulsion of these worms from mice have demonstrated that B cells and antibodies are required for resistance to thisparasite. The evasion of the immune response by *T. muris* causes chronic infection, which has the ability to manipulate the host immune system. *T. muris* excretory/secretory (E/S) products from a heterogeneous solution of worm proteins contain substances that have been shown to bear mannose and N-acetylglucosamine residues; therefore, these substances are potential ligands for C-type lectin receptors such as MR [78].

Deschoolmeester et al. showed *in vitro* that MR-KO-derived bone-marrow-derived MØs (BMDMs) expressed similar levels of several cytokines when exposed to *T. muris* E/S. The only difference observed was a reduction in the

production of IL-6 by alternatively activated BMDMs in the absence of MR, and the infection of MR-KO mice revealed the expulsion of *T. muris* with the same kinetics as observed for WT animals and a similar cytokine response in the draining mesenteric lymph nodes. Moreover, there were no differences in MØ recruitment, the ability of MØs to become alternatively activated, goblet cell hyperplasia, or gross crypt pathology during infection. In summary, MR binds to components of *T. muris*, but it is not required for the development of an immune response leading to the expulsion of *T. muris* [47].

4.3. Helminths: Trematodes

4.3.1. Schistosoma mansoni. Parasitic helminths express various carbohydrates containing glycoproteins on their surface and release glycan-rich E/S products that can potentially bind to various CLRs [59]. The parasite helminth *Schistosoma mansoni (S. mansoni)* is the causative agent of the chronic disease schistosomiasis, which is the second most prevalent human parasitic disease, affecting ~300 million people worldwide, particularly in tropical countries [55, 79]. Immunologically, *S. mansoni* infection is dominated by two distinct Th phases: an initial Th1 (IFN-γ) response, which switches to a stronger Th2 (IL-10, IL-5, and IL-13) response [58]. One of the most striking features of schistosomiasis is that the worms are experts in modulating and evading the host immune response, enabling their survival, migration, and development in different host tissues. Schistosomal glycoconjugates (glycoproteins and glycolipids) have shown to play important roles in host-parasite interactions. These glycoconjugates are often developmentally regulated antigens that are expressed during different life cycle stages. Some studies have indicated that Lewis^X antigens Galβ1,4(Fucα1–3)GlcNAc have important roles in host-schistosome interactions. Lewis^X (Le^x) antigens have been found in glycoconjugates from all life cycle stages, including the membrane-bound glycoproteins of adult schistosomes and secreted egg and gut glycoproteins [7].

Macrophage Galactose Type C-Lectin (MGL). Human MGL has an exclusive specificity for terminal GalNAc residues, such as those found in the glycoproteins of the helminth parasite *S. mansoni*, in filoviruses, and in tumor-associated antigens [80].

Binding assays revealed that MGL recognizes both terminal β-GalNAc residues of LDN [GalNAcβ1–4GlcNAc-R] and LDNF [GalNAcβ1–4(Fucα1–3)GlcNAc-R] glycans present in SEA of *S. mansoni*. The specific interaction between MGL and SEA glycoproteins containing LDN and LDNF demonstrates that MGL functions as a pattern recognition receptor for *S. mansoni* [54].

In another study using binding assays and blocking antibodies reported that SEA of *S. mansoni* is internalized by human DCs through MGL. Moreover, the confocal laser scanning microscopy reveals colocalization of SEA with MHC-II in the lysosomal compartments suggests that Ag processing and presentation can occur. Certainly these findings are important, however remains to be answered if this recognition leads to antigen presentation and modulation of the immune response to *S. mansoni* [55].

DC-SIGN. It has been demonstrated that the blockade with monoclonal antibodies against the carbohydrate antigens Le^x and LDNF inhibit the binding of DC-SIGN to soluble egg antigens (SEAs). The glycoproteins several SEAs from different schistosome species (*S. mansoni, S. haematobium, and S. japonicum*) contain ligands for DC-SIGN. It has also been demonstrated that a specific mutation in the carbohydrate-recognition domain (CDR) of DC-SIGN abrogates binding to either SEAs or Le^x [56].

Structural characterization of the glycolipids and the study of cellular binding revealed that DC-SIGN binds to the carbohydrate moieties of glycosphingolipids with Le^x and Le^y structure [Fucα1-2Galβ14(Fucα1–3)GlcNAc] moieties. DC-SIGN recognizes not only the self-glycan ligand Le^x within cercarial glycolipids, but also glycolipids carrying pseudo-Le^y, a nonself-structure that to date has been found within S*chistosome cercarial (S. cercarial)* glycolipids and ES products [7]. These results show that DC-SIGN recognizes Le^x and Le^y antigens present in the SEAs and glycolipids of *S. cercarial*. Thus, DCs likely interact with S*chistosomes* early during infection through this lectin. However, more studies are needed to determine whether the recognition of glycosylated antigens through DC-SIGN is involved in resistance or susceptibility to *S. mansoni* infection *in vivo.*

L-SIGN. Liver/lymph node-specific ICAM-3-grabbing nonintegrin (LSIGN/CD209L/DC-SIGN-R) is a human homolog of DC-SIGN. L-SIGN shares 77% amino acid sequence identity with DC-SIGN and is expressed on liver sinusoidal endothelial cells (LSECs), which function as antigen-presenting cells in the liver [81].

L-SIGN, a highly related homolog of DC-SIGN, can bind both schistosome egg antigens (SEAs) and glycosphingolipids and can mediate the internalization of SEAs. However, binding assays showed that L-SIGN recognizes a glycoprotein fraction different from that recognized by DC-SIGN. It has been demonstrated that L-SIGN does not bind to neoglycoconjugates carrying Le^x but does recognize other fucosylated glycans, that is, Le$^{(a,b\text{ and }y)}$. Other studies have demonstrated that the glycosylation of schistosome antigens plays an important role in immunological process during schistosome infection [8, 57]. Those studies confirmed that L-SIGN recognizes both oligomannosidic N-glycans and multiply fucosylated carbohydrate motifs within SEAs. In addition, these studies demonstrated that L-SIGN can recognize a broad but specific glycan profile.

SIGNR1. Also called CD-209b, is one of the eight mouse homologs of human DC-SIGN and is expressed on particular MØ subsets in the marginal zone of the spleen and the medulla of the lymph nodes and on the peritoneal MØs. SIGNR1 recognizes glycans from different pathogens and has been shown to bind Lewis$^{x/y}$ and Lewis$^{a/b}$-containing carbohydrates [82, 83].

An *in vitro* studyusing cells transfected with SIGNR1 showed that glycans from both SEAs and schistosome worm antigens were bound by SIGNR1 in a dose-dependent manner, demonstrating the ability of SIGNR1 to recognize

and bind to two different stages of the parasite. However, the *in vivo* infection of SIGR1-deficient BALB/c mice (SIGNR-KO) with 25 cercariae of *Schistosoma* revealed that SIGNR1 has no role in primary or secondary pulmonary granuloma induced by schistosome eggs. SIGNR-KO mice exhibited unaltered worm fecundity, and the fecal eggs and the size and eosinophil content of the granulomas surrounding eggs in the liver were comparable, as were the levels of hepatic fibrosis. Moreover, no differences in the cytokine the production by spleen cells were observed. In conclusion, although SIGNR1 can recognize *S. mansoni* antigens *in vitro*, this receptor does not have a functional role *in vivo* during infection [9].

Dectin-2. Dectin-2 is a member of the C-type lectin family and has single complementarity-determining region (CRD). This protein expressed mainly in MØs and DCs. Dectin-2 recognizes α-mannans and transduces the signal through an association with the ITAM-containing Fc receptor γ chain [84, 85].

In vitro restimulation assays using spleen and MLN cells with SEA (20 μg/mL) have demonstrated that SEA associates with Dectin-2 and Fc receptor γ-chain (FcRγ) receptors. Moreover, SEA-mediated IL-1β production was significantly inhibited when BMDCs were pretreated with Dectn-2-specific antibodies or when Dectin-2-deficient BMDCs were used. In contrast, TNF-α production was not impaired. Thus, different components within SEAs mediate different immune reactions. These observations suggest that SEA triggers the Dectin-2 receptor, which couples with FcRγ chain, to activate the Syk-kinase signaling pathway, which controls IL-1β release in an ROS- and potassium efflux-dependent manner, the Nlrp3 inflammasome activation, and IL-1β release. However, even though these findings are important, it is necessary to determine whether this receptor plays a key role during infection *in vivo* [58].

Mannose Receptor (MR). It has been demonstrated that infective larvae of the parasitic helminth *S. mansoni* contain a large number of glycosylated components specific for MR. MR ligands are particularly rich in excretory/secretory (E/S) material released during the transformation of cercariae into schistosomula, a process that is critical for infection of the host. E/S material from carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labeled cercariae showed enhanced binding by Chinese hamster ovary cells (CHO) lines transduced to express MR and by an MØ cell line that overexpresses MR (J774E) relative to the level of binding by WT CHO cells. Conversely, uptake was significantly lower by bone marrow-derived macrophages (BMDM) from MR-KO mice, although these cells were more active as judged by the enhanced proinflammatory cytokine production and CD40 expression. After natural percutaneous infection of MR-KO mice with CFDA-SE-labeled parasites, there were fewer cells in the skin and draining lymph nodes that were CFDA-SE(+) relative to the numbers in WT mice, indicating that there was reduced uptake and presentation of larval parasite antigens. However, the antigen-specific proliferation of skin-draining lymph node cells was significantly enhanced, and these cells secreted markedly elevated levels of IFN-γ but decreased levels of IL-4. These results demonstrated that MR on mononuclear phagocytic cells plays a significant role in internalizing E/S material released by the invasive stages of the parasite, which in turn modulates the production of proinflammatory cytokines. In the absence of MR, antigen-specific $CD4^+$ cells are Th1 biased, suggesting that the ligation of MR by glycosylated E/S material released by schistosome larvae modulates the production of IFN-γ by $CD4^+$ cells [59].

4.4. Helminths: Cestodes

4.4.1. Taenia crassiceps. *Taenia crassiceps (T. crassiceps)* is a tapeworm that is found in wild and domestic animals but does not cause clinical disease in nonimmunocompromised humans. This parasite has been used as an experimental model for cysticercosis [86]. Previous studies demonstrated that soluble antigens from *T. crassiceps* are highly glycosylated and are responsible for Th2 polarization *in vivo* [87, 88]. One study found that the excretory/secretory products of the cestode *T. crassiceps* (TcESs) do not induce the maturation of human DCs, as demonstrated by the lack of increase in the expression levels of CD83, HLA-DR, CD80, and CD86. TcESs enhanced the production of IL-10, positively modulated the expression of mMGL and negatively modulated the expression of DC-SIGN, although the source of these antigens is not a human parasite. These results showed that TcESs induce a tolerogenic-like phenotype in human DCs and modulate the expression of PRRs involved in key functions of DCs such as mMGL and DC-SIGN. This modulation is a possible mechanism used by *T. crassiceps* to modify the phenotype and hence the functions of human DCs, directing the balance toward immune suppression and allowing the survival of this parasite [60].

5. Conclusion

All studies described above demonstrate that CLRs are essential to the recognition of different carbohydrates present on surface or in the excretory/secretory products of different parasites. This recognition can promote the uptake, internalization and processing of parasite antigens that can influence the immune response. However, little is known about the role of CLRs in the immune response to parasitic infections. Future studies are needed to understand the immune mechanisms underlying the interaction of parasite antigens with CLRs.

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Research Article

Silencing of *Entamoeba histolytica* Glucosamine 6-Phosphate Isomerase by RNA Interference Inhibits the Formation of Cyst-Like Structures

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Encystment is an essential process in the biological cycle of the human parasite *Entamoeba histolytica*. In the present study, we evaluated the participation of *E. histolytica* Gln6Pi in the formation of amoeba cyst-like structures by RNA interference assay. Amoeba trophozoites transfected with two Gln6Pi siRNAs reduced the expression of the enzyme in 85%, which was confirmed by western blot using an anti-Gln6Pi antibody. The *E. histolytica* Gln6Pi knockdown with the mix of both siRNAs resulted in the loss of its capacity to form cyst-like structures (CLSs) and develop a chitin wall under hydrogen peroxide treatment, as evidenced by absence of both resistance to detergent treatment and calcofluor staining. Thus, only 5% of treated trophozoites were converted to CLS, from which only 15% were calcofluor stained. These results represent an advance in the understanding of chitin biosynthesis in *E. histolytica* and provide insight into the encystment process in this parasite, which could allow for the developing of new control strategies for this parasite.

1. Introduction

The intestinal protozoan parasite *Entamoeba histolytica,* the causal agent of human amoebiasis around the word, is considered a serious problem of public health mainly in developing countries. Amoebiasis is a major source of morbidity and mortality with estimate of 50 million people infected annually [1]. The *E. histolytica* life cycle includes two biological forms: the trophozoite and the cyst. The cyst is the infective form and has a tetranucleated structure surrounded by a chitin wall highly resistant to adverse environment conditions [2]. In spite of numerous *in vitro* studies focused on obtaining the infective mature forms of *E. histolytica* cysts, no reproducible procedure has yet been developed. However, a recent study by our group achieved induction of cyst-like structures (CLS) with a characteristic chitin thick wall, refringency, multinucleation (even a few of CLS with 4 nuclei) and chromatoid bodies by treatment of trophozoites with hydrogen peroxide in the presence of trace amounts of several metallic dications [3]. The CLS obtained was resistant to lysis by detergents and exposition to environmental conditions for several weeks (unpublished data). These results suggest that encystment pathways can be activated in *E. histolytica* trophozoites when they are exposed to oxidative stress, mainly hydrogen peroxide, as it has been suggested for differentiation in other cells (reviewed in [4]). In this regard, exposure to luminal conditions, such as reactive oxygen species from immune cells or intestinal microbiota, has been suggested to be involved in amoeba encystment [5, 6]. In addition, a role for certain divalent cations as cofactors of enzymes involved in excystment and encystment has also been described [7]. However, the cellular events behind the synthesis of the chitin wall and, in general, the developmental conversion of *E. histolytica*, are still unknown.

The *in vitro* encystment of other protozoans such as *Giardia lamblia* and *E. invadens* is extensively studied (reviewed in [8]). During the encystment of *G. lamblia*, it has been

observed that the filamentous structure of the cyst wall consists of a polymer of *N*-acetylgalactosamine [9]. This polymer is synthesized from endogenous glucose by a biosynthetic pathway where the rate-limiting enzyme is glucosamine-6-phosphate isomerase (Gln6Pi), an enzyme that reversibly isomerizes fructose-6-phosphate to glucosamine-6-phosphate [10]. Accordingly, we also reported that *in vitro* induction of *E. histolytica* CLS is coupled to overexpression of a Gln6Pi, as determined by RT-PCR [3]. However, solid evidence of the functional participation of this enzyme in amoeba encystment was not provided.

RNA interference (RNAi) method is a powerful tool for assessing and manipulating gene function. In this process, a double-stranded RNA (dsRNA) can initiate posttranscriptional sequence-specific silencing of cellular genes by mRNA degradation [11]. The mechanism of RNAi may have evolved as a defense against viruses and transposable elements with dsRNA intermediates [12, 13]. In this process, the small RNA intermediates, short interfering RNAs (siRNAs), result from dsRNA being cleaved at 21 to 23 nucleotide intervals [14] by RNase III-type [15] and are then incorporated into the RNA-induced silencing complex (RISC) and guided to their target mRNA, which is then cleaved by Argonaute proteins [14, 16]. So far we know that the molecular RNAi machinery and siRNAs are present in *E. histolytica* and the silencing of genes in this parasite is relatively common (reviewed in [17, 18]).

In this work, we evaluated the participation of Gln6Pi in the formation of the CLS by RNA interference assay. Our results show that under encystment induction treatment, the Gln6Pi knockdown in trophozoites results in the loss of the capacity of trophozoites to synthesize the chitin wall, and therefore, to transform into a CLS, suggesting a pivotal role for Gln6Pi in *E. histolytica* encystment.

2. Material and Methods

2.1. Parasite Cultures. *E. histolytica* trophozoites, HM-1: IMSS strain, were grown at 37°C in sterile TYI-S-33 medium supplemented with 10% adult bovine serum, 100 U/mL of penicillin, 100 mg/mL of streptomycin sulfate, and 3% of Diamond Vitamins [19].

2.2. Induction of Cyst-Like Structures. The induction of cyst-like structures (CLS) was performed as previously reported [3]. In brief, trophozoites were chilled on ice for 5 min and harvested by centrifugation at 150 ×g for 7 min at 4°C. The cells obtained (1×10^5) were resuspended in 50 mL of fresh TYI-S-33 media in culture flasks and incubated at 37°C. After 72 h, trophozoites in log phase (approximately 5×10^6 cells) were treated with 4 mM of a 30% H_2O_2 solution containing different traces of several dications (cadmium 0.02 ppm, cobalt 0.02 ppm, copper 0.02 ppm, iron 0.1 ppm, nickel 0.02 ppm, lead 0.02 ppm, zinc 0.02 ppm) and other components (free sulfuric acid 40 ppm, chlorine 0.5 ppm, phosphate 5 ppm, and sulfate 2 ppm) (Merck UN 2014, Darmstadt, Germany). The cultures were then incubated at 37°C for 6 h. After treatment, parasites were washed two times with phosphate buffered saline, counted under microscope and resuspended in PBS containing 0.5% sarkosyl, and allowed to sit for 10 min at room temperature. After three washes as above, the detergent-resistant trophozoites were resuspended in PBS and counted again in a microscope. The conversion rate index was estimated as the percentage of parasites that were resistant to sarkosyl and therefore were converted from trophozoites to CLS. Three independent experiments by triplicate were carried out for each analysis.

2.3. Small Interference RNA (siRNA). Design of siRNAs was done by the Ambion Company (TX, USA) using a patented siRNA design algorithm applied to the *E. histolytica Gln6Pi* gene sequence. Design was followed by an *in silico* analysis using the *E. histolytica* genome database in order to avoid matching of the candidate siRNAs with other expressed trophozoite genes. Two 21-nt siRNAs, Si 154-Gln6Pi (5′GGACAUGCAGUAUUAGGAUTT-3′) and Si 229-Gln6Pi (5′GCUGGAGAAGUUUCAUUUATT-3′), were designed and finally purchased from Applied Biosystem (USA). A siRNA with a scrambled sequence, unable to induce degradation in any cellular mRNA, was used as control of specificity (siRNA-A:sc-37007, Santa Cruz Biotechnology, CA, USA).

2.4. RNA Interference Assay. The siRNAs (154-Gln6Pi and 229-Gln6Pi) were used individually and combined at amounts of 5, 10, 20, and 40 μg per culture. The siRNA-A with the scrambled sequence was used at 40 μg per culture following the manufacturer's instructions. Transfection of the trophozoites was performed by soaking as previously reported [17]. In brief, 1×10^5 trophozoites were grown in 6 mL TYI-S33 media in culture flasks. Once reached 50% of confluence (about 5×10^5 cells), cultures were added with the siRNA, individually or mixed (5, 10, 20, and 40 μg of each one), and incubated at 37°C for 16 h. After the incubation, transfected trophozoites were induced to encyst by exposure to the treatment solution (4 mM H_2O_2 + cations, see above) during 6 h. The cells were then extensively washed with PBS pH 7.4 and the conversion rate index (percentage of CLS formed) was determined with 0.5% sarkosyl as described. Morphological evaluation and determination of chitin were carried out by observation under light microscope and calcofluor white staining, respectively.

2.5. Calcofluor White Staining. After the transfection with siRNA and CLS induction, calcofluor white M2R staining was performed (Sigma-Aldrich, USA). The treated cells resistant to detergents (CLS) were washed three times with PBS. Subsequent to centrifugation, pellet samples were placed onto microscope slides and several drops of 0.05% calcofluor white M2R in distilled water were added. The sample was incubated for 10 min and the slide was observed under UV light using a fluorescence microscope.

2.6. Viability Assay. Viability was analyzed by determining the ability of CLS to convert fluorescein diacetate (FDA) to fluorescein. The method was previously used in the infectivity

Table 1: Oligonucleotides and siRNAs used in this work for RT-PCR and interference of Gln6Pi in *E. histolytica*, respectively.

Target gene	Primer name	Sequence (5′ to 3′)
gln6Pi	Gln6PiEh-F	ATGTCATCCACAAACGAAAATATTC
gnl6Pi	Gln6PiEh-R	CAATAGACATGGATTTATCATATC
EhARF	EhARF-F	GTAGGACTTGATGCTGCC
EhARF	EhARF-R	TCACCATTAGTTGCAC
gln6Pi-mRNA	siRNA 154-Gln6Pi	GGACAUGCAGUAUUAGGAUTT
gln6Pi-mRNA	Si 229-Gln6Pi	GCUGGAGAAGUUUCAUUUATT
—	siRNA-A:sc37007	—

Controls for constitutive expression (ARF primers) and specificity of interference (siRNA-A) are also shown.

determination of *G. lamblia* cysts [20]. The viability assays were performed in the cells after the transfection with siRNAs to discard the possibility of toxic effects, as well as after the treatment with 4 mM H_2O_2 and 0.5% sarkosyl. In brief, treated trophozoites were centrifuged, pelleted, and washed three times with PBS pH 7.4. Afterwards, the cells were counted and adjusted to 1×10^6 mL with PBS. Samples of 100 μL were treated with 1.6 μL of FDA stock solution (2.5 μg/μL in acetone; Invitrogen, USA), at room temperature for 8 min. Percentage of viable cells was determined by counting the number of fluorescent cells under a fluorescence microscope with a BP350–460 filter.

2.7. RNA Extraction and RT-PCR. Total RNA was isolated from siRNA-transfected *E. histolytica* trophozoites with TRIZOL (Invitrogen, USA). The level of expression of the *Gln6Pi* gene was determined by RT-PCR using Super Script III One-Step kit (Invitrogen, USA) following the manufacturer's instructions. An amount of 900 ng of total RNA was used for cDNA production and the PCR performed with primers previously designed to target the *E. histolytica* Gln6Pi sequence (TIGR Accession number XM_648225) [3] (Table 1). Amplification of *E. histolytica* mRNA ADP-ribosylation factor was used as control of constitutive expression (ARF; accession number XM_648949) [6, 21] (Table 1). The RT-PCR products were run in 1.5% agarose gels and quantified by densitometry using the Bio-Imaging System MiniBis (Bio-Rad, USA) and the Image J program (Image Processing and Analysis in Java) after ethidium bromide staining.

2.8. Western Blot. The levels of Gln6Pi protein expression in extracts from trophozoites transfected with the siRNAs were determined by western blotting. A total of 15 μg protein extracts prepared with protease inhibitors were run in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Protein extracts from untreated trophozoites and trophozoites transfected with siRNA-A (scrambled sequence) were used as controls of basal expression. The membranes were blocked with 0.3% PBS-Tween-5% BSA solution overnight and probed with a mouse anti-human Gln6Pi polyclonal antibody (GNPDA1, Affinity Bioreagents, USA) diluted 1 : 500 overnight at 4°C. After washing with PBS-Tween, HRP-conjugated anti-mouse IgG A M (Zymed, USA) diluted 1 : 500 was incubated with the membranes during 2 h at room temperature. Antigen-antibody reactions were detected using enhanced chemiluminescence (ECL Plus Western Blotting Detection System; GE Healthcare, UK).

3. Results

3.1. Interference of E. histolytica Gln6Pi Expression with siRNAs. Treatment of *E. histolytica* trophozoites with the two siRNA probes, individually or mixed, at amounts between 5 and 40 μg per 5×10^5 cells did not affect the viability and replication of the parasites (data not shown). As expected, the transfected trophozoites showed decreased expression of Gln6PI RNA in RT-PCR assays when the parasites were treated with either siRNA (data not shown). However, the interference effect was greater when the parasite was treated with the mix of siRNAs. The effect was dose dependent with the mix of 5 μg of each siRNA inhibiting about 24% and the mix of 40 μg inhibiting almost 85% of Gln6Pi expression, when compared to the basal expression of Gln6Pi in untreated trophozoites and trophozoites transfected with the scrambled sequence, where the basal expression of Gln6Pi was similar (Figure 1).

Interference with expression of Gln6Pi was also demonstrated by western blot analysis of protein levels in extracts from interfered trophozoites. As shown in Figure 2, expression of a 37 kDa band corresponding to the molecular weight expected for Gln6Pi decreased depending on the dose of siRNA used. Thus, a mix of 5 μg of each siRNA inhibited Gln6Pi expression in about 47%, whereas a mix of 40 μg of each siRNA blocked completely the expression of Gln6Pi when compared with the basal protein expression of the enzyme. No effect on the expression of Gln6Pi protein was observed with the scrambled sequence (Figure 2).

3.2. Effect of Gln6Pi Knockdown on the Induction of CLS. Once confirmed the downregulation of Gln6Pi expression in the interfered trophozoites, the parasites were subjected to encystment induction by treatment with hydrogen peroxide plus metal dications as indicated. Addition of 4 mM H_2O_2 plus dications for 6 h to parasite cultures at 37°C induced differentiation of trophozoites to CLS at different rates depending on the concentration of siRNA mix used for interference (Table 2). Thus, the conversion rate (defined as the percentage of cells resistant to 0.5% sarkosyl during 10 min) decreased as siRNA concentrations increased, ranging from 27% with 5 μg to 5% with 40 μg of each siRNA per culture. Accordingly,

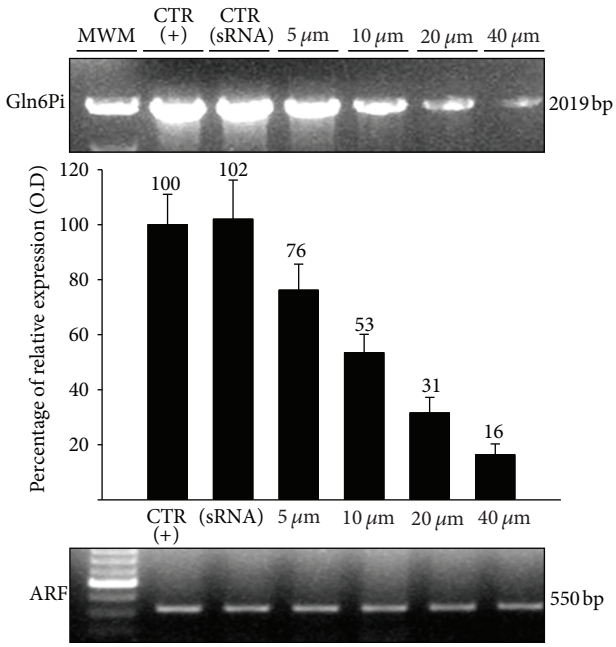

Figure 1: Relative expression of *E. histolytica* mRNA in trophozoites transfected with different amounts of a mix of si 154-Gln6Pi and si 229-Gln6Pi determined by RT-PCR. Trophozoites (about 5×10^5 cells) were transfected with the siRNAs mix at indicated amounts by soaking during 16 h at 37°C. Afterwards, mRNA was extracted and RT-PCR performed using oligonucleotides showed in Table 1. Relative expression was determined by densitometry with respect to the constitutive expression of *E. histolytica* ADP-ribosylating factor (ARF), which was also used as loading control. CTR (+): basal expression of Gln6Pi in trophozoites; CTR (siRNA): trophozoites transfected with scrambled-sequence siRNA-A.

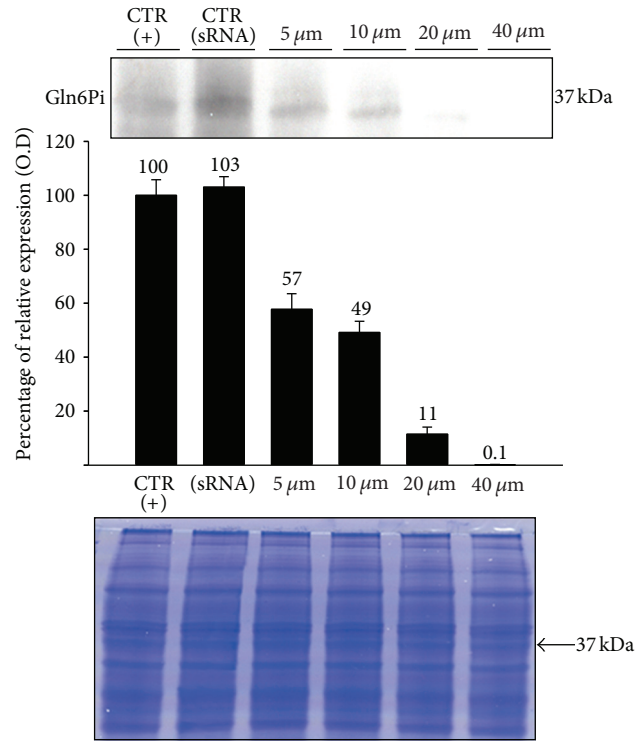

Figure 2: Levels of *E. histolytica* Gln6Pi protein expression in trophozoites transfected with different amounts of a mix of si 154-Gln6Pi and si 229-Gln6Pi determined by western blot. Trophozoites (about 5×10^5 cells) were tranfected with siRNAs mix at indicated amounts by soaking during 16 h at 37°C. Afterwards, total extracts were prepared in the presence of protease inhibitors, run in SDS-PAGE, and transferred to nitrocellulose paper. Gln6Pi (37 kDa) was identified by using a mouse anti-human Gln6Pi polyclonal antibody and revealed using ECL. Relative expression was determined by densitometry taking basal expression of Gln6Pi in untreated trophozoites as 100 percent (CTR (+)). CTR (siRNA): trophozoites transfected with scrambled-sequence siRNA-A. The amount of protein loaded in each lane is shown in a Coomassie stained gel.

viability of parasites determined by FDA staining decreased after sarkosyl treatment from 69% with 5 μg to 12% with 40 μg of each siRNA. As shown in Table 2, viability of trophozoites previous to the sarkosyl treatment was not critically affected by the transfection, ranging between 87, and 95%, suggesting that the transfection procedure is not toxic to the parasite.

3.3. Effect of Gln6Pi Knockdown on Chitin Expression. In agreement with the reduction of cell conversion rates, the increasing concentration of siRNA mix reduced the percentage of cells positive for calcofluor white staining, a disodium salt that detects specifically polysaccharides with β 1-3 and β 1-4 linkages present in cellulose and chitin (Table 2 and Figure 3). As mentioned before, transfection of *E. histolytica* trophozoites with siRNAs did not affect viability, independently of the concentration of siRNA used (Figures 3(a), 3(d), and 3(g)).

Parasites untransfected or transfected with the single si 154-Gln6Pi at 40 μg showed identical percentage of chitin positive cells (conversion rate of around 30%) after CLS induction followed by sarkosyl treatment (Figures 3(b) and 3(e)). This result is in agreement with the scarce effect of a single siRNA on Gln6Pi expression as mentioned above. In contrast, trophozoites transfected with the mix of 40 μg of each siRNA showed a marked reduction in the number of chitin positive cells (Figure 3(h)), in agreement with the knockdown of Gln6Pi expression. In addition, the treated cells did not show morphological and structural features of encystment, such as refringence and multinucleation (data not shown). Noteworthy, most of the chitin positive cells were viable as determined by FDA staining (Figures 3(c), 3(f), and 3(i)). Data on viability after CLS induction, conversion rates, and calcofluor positive CLS in trophozoites transfected with different concentrations of siRNA mix are resumed in Table 2. In general, transfection with the different concentration of siRNA mix had a dose-dependent direct relationship effect on al parameters.

4. Discussion

Encystation is a pivotal process in the life cycle of *E. histolytica* and is indispensable for its transmission. However,

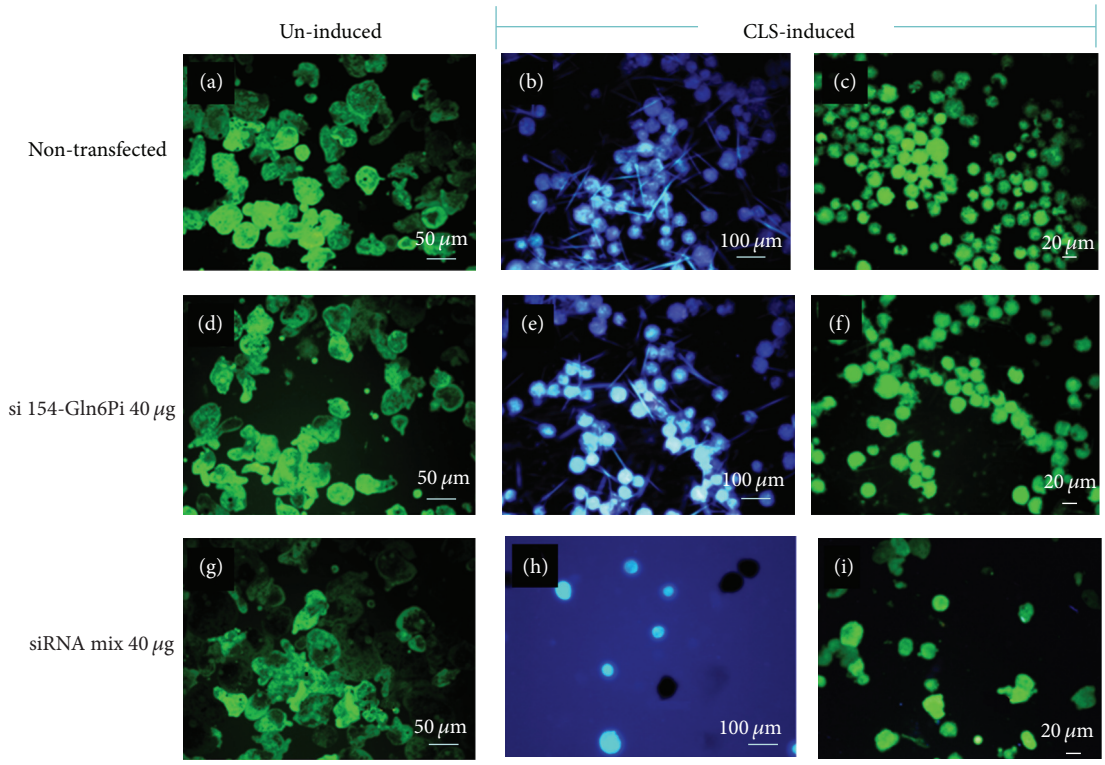

Figure 3: Expression of chitin and viability of Gln6Pi silenced trophozoites after CLS induction and detergent treatment. Untransfected trophozoites or transfected with 40 μg of si 154-Gln6Pi or 40 μg of each si 154-Gln6Pi and si 229-Gln6Pi (siRNA mix 40 ug) were induced to CLS followed by treatment with 0.5% sarkosyl. Cells were stained with FDA for viability before ((a), (d), and (g)) and after ((c), (f), and (i)) CLS induction and detergent treatment. Calcofluor white staining is shown for cells after CLS induction and detergent treatment ((b), (e), and (h)). Viable cells are observed in green and chitin-positive cells are whitish blue under UV light microscopy.

Table 2: Effect of Gln6Pi knockdown by a mix of si 154-Gln6Pi and si 229-Gln6Pi siRNAs on viability, conversion rate and chitin expression of trophozoites induced to CLS.

(siRNA mix)	Interference trophozoites viability (FDA) (% ± S.D.)	Treatment solution[a]/incubation time	Conversion rate (% ± S.D.)[b]	Viability (FDA) (% ± S.D.)	Staining calcofluor white (% ± S.D.)
5 μg	95 ± 3.6	4 mM/6 h	27 ± 4.4	69 ± 5	95 ± 2
10 μg	91 ± 1.5	4 mM/6 h	19 ± 5.3	65 ± 4.5	90 ± 8
20 μg	90 ± 2.3	4 mM/6 h	9 ± 3	36 ± 1.1	65 ± 2
40 μg	87 ± 3	4 mM/6 h	5 ± 2.1	12 ± 2.8	15 ± 4.8

[a]Hydrogen peroxide 30% containing traces of several dications, see Section 2.2.
[b]Three independent experiments were done by triplicate.
Conversion rate: percentage of cells that were resistant to 0.5% sarkosyl.
FDA: fluorescein diacetate.
S.D.: standard deviation.

the luminal stimuli triggering the process and the molecular mechanisms responsible for the stage conversion are still unknown. Recently, we have reported a reproducible *in vitro* treatment to induce cyst-like structures with most of the features of a mature cyst, including multinucleation and a chitin wall, by using a combination of hydrogen peroxide and metallic dications [3]. In the same report, we also gave evidence supporting the association between the over-expression of glucosamine-6-phosphate isomerase (Gln6Pi), the first enzyme in the theoretical route of chitin biosynthesis in amoeba, and the onset of the encystment process assayed by RT-PCR.

In the present work, we analyzed the effect of silencing the expression of *Gln6Pi* gene on encystment of *E. histolytica* trophozoites using synthetic siRNA duplexes and the soaking method, as previously described for gene silencing in this parasite [22, 23]. As expected, transfection of *E. histolytica* trophozoites with a mixture of two siRNA duplexes

during 15 h resulted in the knockdown of Gln6Pi mRNA levels in a dose-dependent manner. The mRNA levels were decreased by 85% whereas the levels of protein expression were decreased by almost 100% when parasites were soaked in a mix of 40 μg of each siRNA. Our results are in agreement with the results of knockdown of γ-tubulin RNA and protein levels in respect of siRNA concentrations used [22]. In contrast to *Giardia lamblia*, where two *Gln6Pi* genes were identified [24], *in silico* analysis carried out by our group using the *E. histolytica* genome database suggests that this parasite may have a single gene coding for a putative Gln6Pi (data not shown). This could explain why a single interference with a mixture of two siRNAs was able to completely block the expression of the enzyme when assayed by WB. However, the exact molecular mechanism of silencing in amoeba is still unknown and waiting for demonstration, though the iRNA machinery and small RNAs are evidently present in *E. histolytica* (reviewed in [17, 18]). In this regard, several studies on *E. histolytica* G3 strain, an *in vitro* obtained strain stably silenced for the amoebapore A gene, have suggested that transcriptional and posttranscriptional mechanisms could be involved in amoeba iRNA silencing [25–28].

The levels of downregulation of Gln6Pi expression correlated with the decrease in the rates of *E. histolytica* encystment under induction treatment, as judged by increased susceptibility to detergent and a proportional decrease in the production of chitin-positive structures and multinucleation. Therefore, the inhibition of the Gln6Pi expression resulted in the inhibition of chitin cyst wall synthesis making the cells induced for encystment susceptible to the detergent treatment quite similar to the uninduced trophozoites. This result is in agreement with our previous proposal of Gln6Pi being the first enzyme of the pathway leading to the synthesis of β-(1,4)-linked N-acetylglucosamine homopolymer, the main constituent of chitin in *E. histolytica*, which in combination with other proteins such as Jessie, Jacob, and the Gal-binding lectin give place to a surface hard cover that confers resistance to harmful environmental agents, facilitating the parasite's survival and dissemination (reviewed in [8]). Thus, in combination with our previous observations [3], the present results suggest that *E. histolytica* possesses a functional metabolic pathway of chitin synthesis similar to Giardia, where the isomerization of fructose-6-phosphate to glucosamine-6-phosphate by Gln6Pi seems to be critical. However, the activities of the other four theoretical enzymes of the pathway [29] were not conducted in this study and it is a matter of further studies in our group.

The results reported here also support the efficiency of hydrogen peroxide plus dications treatment in *in vitro* triggering the encystment process in *E. histolytica* trophozoites. The fact that stage conversion induced by hydrogen peroxide plus dications is inhibited by Gln6Pi interference, suggests that oxidative stress is able to activate, directly or indirectly, the expression of the *Gln6Pi* gene. In this regard, we have evidence of hydrogen peroxide response elements present in the promoter of *Gln6Pi* gene (data not published) that could be involved in its over-expression, in agreement with evidence of multiple genes responsive to oxidative stress having a role in cellular differentiation and many other physiological functions in eukaryotes (reviewed in [4]). However, studies on the infectivity of CLS are necessary in order to demonstrate that they are really mature cysts, and therefore, that interference of Gln6Pi will in fact inhibit the encystment *in vivo*. As mentioned above, studies on the role of the other four enzymes from the theorical pathway of chitin biosynthesis are also necessary and are actually being carried out in our laboratory.

In general, our results suggest that the encystment of *E. histolytica* is dependent on the upregulation of the Gln6Pi enzyme, which controls the onset of chitin synthesis under oxidative stress conditions.

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Research Article

Sex-Associated Expression of Co-Stimulatory Molecules CD80, CD86, and Accessory Molecules, PDL-1, PDL-2 and MHC-II, in F480+ Macrophages during Murine Cysticercosis

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Macrophages are critically involved in the interaction between *T. crassiceps* and the murine host immune system. Also, a strong gender-associated susceptibility to murine cysticercosis has been reported. Here, we examined the sex-associated expression of molecules MHC-II, CD80, CD86, PD-L1, and PD-L2 on peritoneal F4/80^{hi} macrophages of BALB/c mice infected with *Taenia crassiceps*. Peritoneal macrophages from both sexes of mice were exposed to *T. crassiceps* total extract (TcEx). BALB/c Females mice recruit higher number of macrophages to the peritoneum. Macrophages from infected animals show increased expression of PDL2 and CD80 that was dependent from the sex of the host. These findings suggest that macrophage recruitment at early time points during *T. crassiceps* infection is a possible mechanism that underlies the differential sex-associated susceptibility displayed by the mouse gender.

1. Introduction

Gender of the host influences the outcome of many parasitic diseases. For example, in *Leishmania major* infection, female mice mount a strong Th1 response and resolve the infection. In contrast, male mice mount a Th2-dominant response and develop chronic lesions [1]. In other protozoan infections, such as toxoplasmosis, an opposite finding was observed: female mice succumb to *Toxoplasma gondii* infection despite a Th1 response, whereas male mice display resistance and survive for a longer period of time to similar challenges [2].

In helminth infections, the gender of the host also plays an important role in the outcome of the infection by inducing different responses depending on the sex [3, 4]. In contrast to the well-described adaptive immunity against these helminthic infections, the role of macrophages (Mϕs) is still unclear. There have been only limited studies on the macrophage response to helminth-derived antigens and the impact of these responses on the outcome of the infection is not known. Much lesser information exists in relation to the role of sex on the macrophage response to helminth-derived antigens.

A sexual dimorphism exists in the acquired immune response against different pathologies and in many autoimmune diseases, which suggests a linkage between the immune and reproductive endocrine system [5]. Moreover, reciprocal endocrine interactions between host and parasite are a strong factor that has an influence in parasite success [6, 7].

Experimental murine cysticercosis caused by *Taenia crassiceps* [8, 9] is well known as a manageable experimental system which explores the role of biological factors involved

in host susceptibility [10]. Interestingly, in *T. crassiceps* cysticercosis, females of all strains of mice studied sustain larger intensities of infection than males [11]. At the same time, the cellular immune response (Th1) is markedly diminished in both sexes, and the humoral response is enhanced (Th2) [12]. Estradiol is involved in the immunoendocrine regulation of murine *T. crassiceps* cysticercosis as a major protagonist in promoting cysticercus growth by interfering with the thymus dependent cellular immune mechanisms that obstruct parasite growth [13]. Gonadectomy alters this resistance pattern and makes intensities equal in both sexes by increasing that of male mice and diminishing it in female mice [14]. In addition, the hormonal substitution of gonadectomized males and reconstitution of female mice with 17β-estradiol increased parasite loads [13]. Also, specific splenocyte cell proliferation, IL-2, and IFN-γ production were depressed in gonadectomized-parasitized mice of both genders, and after the reconstitution with testosterone or dihydrotestosterone, there was a significant recovery of the splenocyte proliferation and Th1 cytokine production on these animals. On the other hand, mice treated with estradiol were not able to induce these cellular responses [15].

Macrophages are phagocytic cells that are widely distributed on the organism and have an important role in the maintenance of the homeostasis [16]. These cells are involved in T cell activation through antigen presentation by the expression of MHC molecules and costimulatory/inhibitory molecules. It has been demonstrated that the expression of MHC molecules and the expression of costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) could modulate T cell activation and Th1/Th2 polarization during infection and autoimmunity [17, 18]. Programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) have been related to alternate activated phenotype in macrophages induced during *Taenia crassiceps* infection [19]. Macrophages also have a broad participation in the development of the immune response to many pathogens, particularly to helminthes [20] by polarizing T helper (Th) cells activation in Th1 or Th2, and also have a role in tissue remodeling and wound repair [21]. In the context of immunoendocrine communication, it has been shown that sex steroids are able to modulate survival of human macrophages cell lines [22], the recruitment of macrophages to the site of inflammation, and their effector functions [23]. As occurred with other immune cells, the effect of sex steroids on macrophages depends on the concentration, type, and the context in which macrophages are studied [24]. Furthermore, it has been previously established that sex steroid effects on macrophages depend on the expression of the androgen receptor (AR) [25, 26], progesterone receptor (PR) [27], and both types of estrogen receptor (ERα y ERβ) [28].

Since macrophages have been importantly involved in susceptibility/resistance in murine cysticercosis and also can be modulated by sex steroids, we evaluated and compared the response of molecules of early activation of recruited F4/80^{hi} macrophages, such as MHC-II, CD80, CD86, PD-L1, and PD-L2 in both gender infected mice. Our results showed that indeed there is a differential expression of these molecules in female and male mice and that this could partially impact the different sex-associated susceptibility to cysticercosis in mice.

2. Materials and Methods

2.1. Ethics Statement. The Animal Care and Use Committee at the Instituto de Investigaciones Biomédicas evaluated animal care and experimentation practices according to the official Mexican regulations (NOM-062-ZOO-1999) in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH and the Weatherall Report) of the USA. The Ethics Committee of the Instituto de Investigaciones Biomédicas approved this protocol (Permission Number 2009-13).

2.2. Animals and Experimental Infections. Male and female BALB/cAnN (H2-d) inbred mice obtained from Harlan (Mexico City) were used in all experiments. Animals were housed in the animal care facilities at Instituto de Investigaciones Biomédicas, (UNAM), under controlled conditions of temperature and 12 h dark-light cycles with lights on between 0700 and 1900. They were fed Purina Diet 5015 (Purina, St. Louis, MO) and given tap water ad libitum.

2.3. Aantigen Extraction and Infection. Metacestodes of *Taenia crassiceps* of the ORF strain were harvested in aseptic conditions from the peritoneal cavity of female BALB/cAnN mice after 4 months of infection. Metacestodes were washed with cold sterile saline (Solución CS, Laboratorios PISA. S.A. de C.V. [NaCl 0.9%]). *T. crassiceps* soluble extract (TcEx) was prepared in cold aseptic conditions, homogenizing whole metacestodes (30 mL volume) with tree pulses of 60 Hz with a duration of 1 s, by using an ultrasonic homogenizer (Vibracell, SONICS & MATERIALS, Newtown, USA). The homogenates were centrifuged at 20,000 g for 30 min at 4°C, and the supernatants containing saline-soluble antigens were collected and frozen at −20°C until further use. Protein concentration was estimated by Bradford protein kit assay (BioRad). Sex-and age-matched mice were infected by intraperitoneal (ip) injection with 20 small (approximately 2 mm) nonbudding cysticerci/300 μL saline, with 400 μg TcEx in 300 μL saline or 300 μL saline as control. Six days after-injection, animals were sacrificed by inhalation of an overdose of sevoflurane (Sevorane; Abbott) and peritoneal cells were collected for analysis.

2.4. Isolation of Peritoneal Macrophages. Peritoneal exudate cells (PECs) were obtained from saline, TcEx-treated, or 6-day-*T. crassiceps* infected mice (BALB/c male or female) by peritoneal lavage with 7 mL of sterile ice-cold saline (Laboratorios PISA. S.A. de C.V. [NaCl 0.9%]). The cells were washed twice with cold PBS. After two washes, the viable cells were counted by trypan blue exclusion with a Neubauer hemocytometer. Viable cells were counted and adjusted to 1×10^6 cells/mL. Viability was measured by trypan blue exclusion. Routinely viability was around over 95%.

2.5. Analysis of Cell Surface Markers in Macrophages. The surface expression of macrophage markers was analyzed using multicolor flow cytometry. Mϕs were suspended in cold PBS containing 2% FCS and 0.02% NaN_3. The Fc receptors were blocked with anti-mouse CD16/CD32 for 20 min at 4°C. The cells were washed and triple stained with an APC-conjugated mAbs against F4/80, PE-conjugated mAbs against CD86 or PD-L2, PerCP-conjugated IA/IE (MHC-II), PE-Cy5-conjugated CD80 or Biotin-conjugated PD-L1, and PE-Cy5-conjugated Streptavidin. All Abs were purchased from BioLegend (BioLegend, San Diego, CA, USA). A gate including high forward light scatter (FSC)/high side light scatter (SSC) cells was generated and in that gate the different markers were analyzed. The stained cells were captured using a FACsCalibur flow cytometer (Becton Dickinson) and data analyzed with the FlowJo (Tree Star) software. Absolute numbers in all assays were calculated according to the percentage of positive macrophages and the total numbers of PECs.

2.6. Statistical Analysis. The data of the three replications of each experiment were pooled and expressed as their average. The data were analyzed using analysis of variance (ANOVA) with sex (2 levels) and number of experiment (3 levels) as independent variables and as dependent variables: the total number of developed cysticerci and the expression of each molecule. If significant differences between treatments were found by ANOVA, differences between the group means were assessed within each experiment by means of Tukey test using the residual variance estimated by ANOVA to test for significance. Differences were considered significant when $P < 0.05$.

3. Results

In order to determine the role of sex during early infection, mice of both sexes were infected and sacrificed 6 days after-infection. As previously reported [29], at this time point of infection, there is no statistical difference in parasite loads between males and females, though there is a slight trend in males to have less parasites than females (Figure 1). This result is also consistent with the observation that sexual dimorfism begins after the first week of infection in BALB/c mice [30].

To detect the presence of Mϕs and to look for a difference in the number of total Mϕs during early infection, we analyzed the population of PECs recruited to the peritoneal cavity (site of infection) of saline-treated, TcEx, and infected mice of both sexes. Total PECs recruitment in infected male mice is decreased ($P < 0.05$) compared to infected females, while treatment with saline solution or TcEx did not affect the total number of PECs recruited (Figure 2(a)). Since Mϕs have been previously involved in the susceptibility/resistance to murine cysticercosis, we decided to analyze their percentage (Figure 2(b)) and their total number (Figure 2(c)), defined by their high expression of F4/80 ($F4/80^{hi}$). We found no differences in the percentage of $F4/80^{hi}$ cells between sexes (Figure 2(b)), but there was a marked increase in the total number of Mϕs detected in infected females with respect to infected males. This difference was not observed in the other treatments (Figure 2(c)).

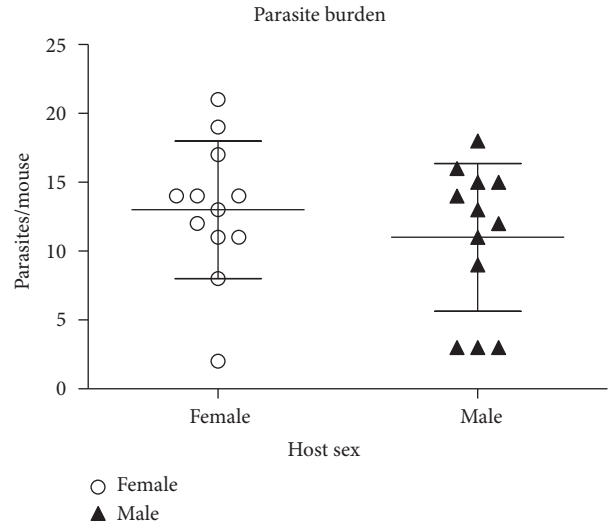

Figure 1: Parasite load obtained of Female (F) and male (M) mice. Data show the number of parasites recovered from the peritoneal cavity at 6 days post-infection. At this time of infection, animals did not show the typical sexual dimorphism of this infection observed in longer infection times. Each point represents individual parasite loads.

To characterize the phenotype of Mϕs recruited of the peritoneal cavity of infected mice of both sexes, we look for the expression of MHC-II (Figure 3), CD80/CD86 (Figure 4), and PD-L1/PD-L2 (Figure 5) by flow cytometry. In Figure 3(a), the percentage of MHC-II+ cells found is depicted. There is no difference associated to treatment or sex, in the percentage of Mϕs expressing these molecule. However, as seen in the total number of PECs of infected female mice, the total number of Mϕs MHC-II+ is also increased (Figure 3(b)). As for the relative mean intensity of the expression of MHC-II (a measure of the amount of the total MHC-II per cell), there is no difference between animals, either by treatment or sex (Figure 3(c)).

In Figure 4, the analysis of the expression of CD80 and CD86 is plotted. There were no differences associated to sex in the percentage of Mϕs expressing CD80 or CD86 (Figures 4(a) and 4(d)). However, there is a marked difference in the total number of CD80+ or CD86+ Mϕs that is observed in infected mice; female mice show an increased number of this population when compared to male mice (Figures 4(b) and 4(e)). We also compared the relative mean intensity (MSR) of the expression of these molecules, in terms to define differences in the coestimulatory ability of these cells. We found no differences between male and female mice in terms of CD80 expression either by treatment or sex (Figure 4(c)). However, CD86 appeared less expressed in male mice than in female mice, even when these data did not show significance (Figure 4(f)).

Finally we look for differences in the expression of PD-L1 and PD-L2 inhibitory molecules. As shown in Figures 5(a)

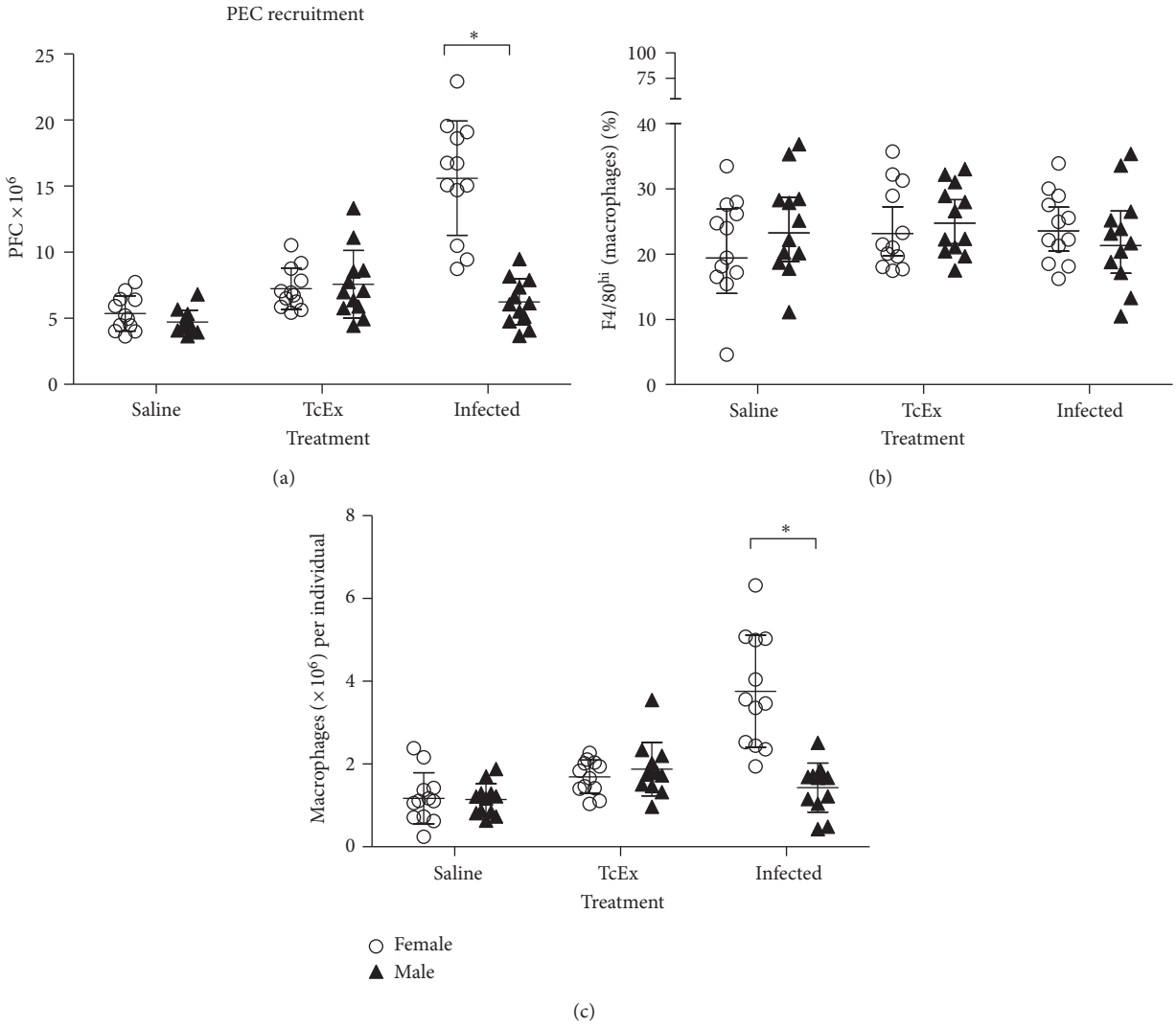

Figure 2: Kinetics of total peritoneal exudate cells (PECs) recovered from the peritoneum after *T. crassiceps* infection. (a) Peritoneal cells were isolated from male and female BALB/c at 6 days after infection with twenty cysticerci. Without any additional stimulation, the cells were processed for flow cytometry and analyzed. (b) Flow cytometry analysis shows that macrophages ($F4/80^{hi}$) are recruited within 6 days p.i. (d.p.i.). (c) Increased numbers are detected per individual associated to sex as infection progresses peritoneal exudate cells.

and 5(d), there were no differences in the percentage of PD-L1 or PD-L2 expressing Mϕs among males and females, but there were differences among treatment: infection induced a higher expression of PD-L1/2 than TcEx. As observed for CD80/86, there were also higher numbers of PD-L1- or PD-L2-positive Mϕs in infected female mice than in infected male mice (Figures 5(b) and 5(e)); but there were no changes in the expression of these molecules on Mϕs (Figures 5(c) and 5(f)).

4. Discussion

Given the reported importance of sex- and pregnancy-associated hormones in the establishment and outcome of parasitic diseases, this is an area of research that is likely to grow. The important role that sex steroids plays during murine cysticercosis has been previously demonstrated in experiments in which gonadectomy, thymectomy, and whole body irradiation showed that both the endocrine and immune systems of the mice were involved in the parasite load differences between the host sexes. Interestingly orchidectomy in male mice raises parasite loads while ovariectomy has the opposite effect; it increased them 3-fold [14]. Thymus hindered parasite reproduction in both sexes but more so in males than in females, thus tending to equalize the number of parasites in thymectomized mice of both sexes [31].

Macrophages play a key role in directing the host immune response to parasites and they can also function as effector cells. The recruitment and activation of macrophages by helminth-derived molecules initiate with the expression of accessory molecules. These immune mediators play crucial roles in the development of immunity against a variety of

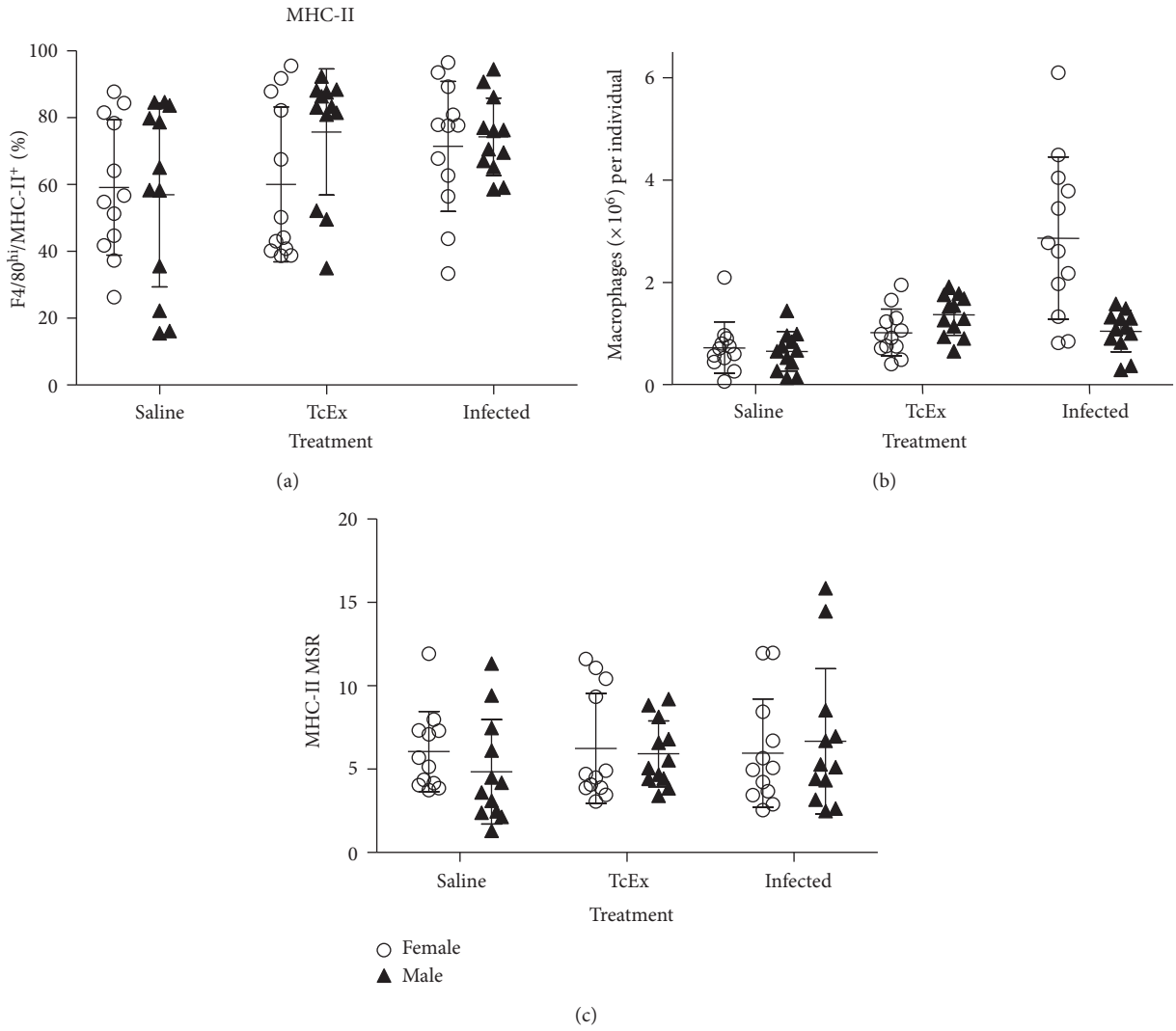

Figure 3: MHC-II characterization in Mϕs recovered from the peritoneum after *T. crassiceps* infection. MHC-II expression was determined on Mϕs (F4/80^{hi}) recruited after 6 days of infection with 20 cysts of *T. crassiceps*. The percentage (a), total numbers (b), and the expression of this molecule (relative mean intensity, MSR) (c) are shown.

pathogens, but their role in helminthic infections is less well understood [32, 33]. In this study, we found an increased number of recruited macrophages from *T. crassiceps*-infected female BALB/c mice in comparison with male mice and expressed MHC-II, the coestimulatory molecules CD80, CD86, and the accessory molecules PD-L1 and PD-L2. However, the major difference that we found was associated to infection, though a clear difference in the number of parasites did not exist. There were more Mϕs in infected females compared to those observed in infected males after similar stimulation. These data are consistent with the susceptible phenotype observed in IL-12 KO mice [34] and suggest a major role for macrophages in cysticercosis. The mechanism underlying the differential expression of MHC-II, CD80, CD86, PD-L1, and PD-L2 in our system remains to be elucidated; however, it may be associated with an impaired intracellular signaling in BALB/c male mice but not in female mice.

The relevance of these observations is highlighted by the finding that macrophages from BALB/c female mice became more rapidly alternatively activated in *T. crassiceps* chronic infection, whereas macrophages from male mice presented a transient and incomplete alternate activation during early infection [35]. Thus, the presence and the persistence of AAMϕ are another striking difference between the susceptible and resistant sex of mice to *T. crassiceps* infection.

In the context of immunoendocrine communication, it has been previously established that macrophages express the androgen receptor (AR) [25, 26], progesterone receptor (PR) [27], and both types of estrogen receptor (ERα y ERβ) [28]. It has been shown that sex steroids are able to modulate survival of human macrophages cell lines [22], the recruitment of

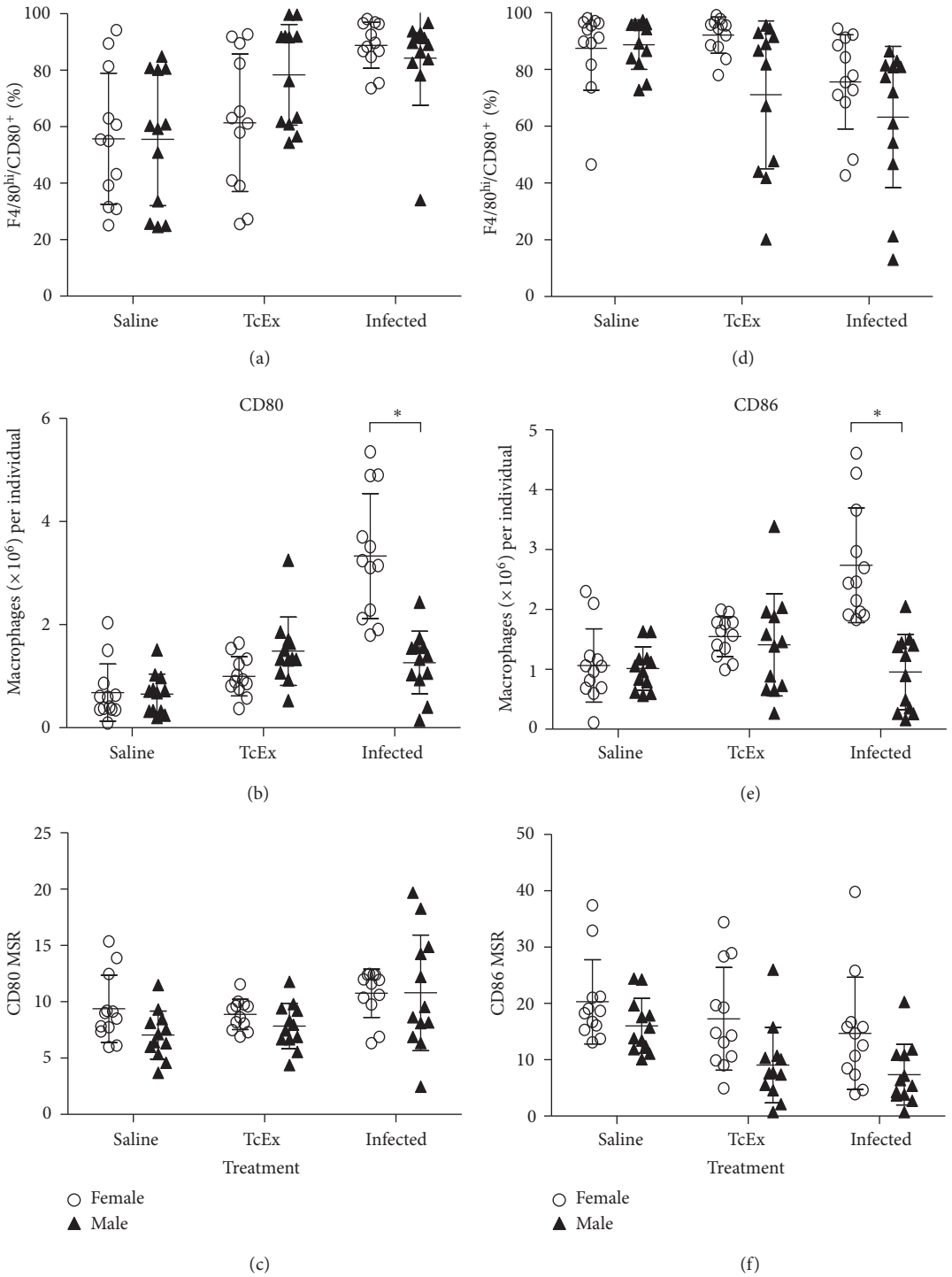

Figure 4: CD80/CD86 characterization in Mϕs recovered from the peritoneum after *T. crassiceps* infection. Costimulatory molecules CD80 and CD86 expression was determined on Mϕs (F4/80^{hi}) recruited after 6 days of infection with 20 cysts of *T. crassiceps*. The percentages (a) and(d), Total numbers (b) *and* (e), and the expression of these molecule (relative mean intensity, MSR) (c) and (f) are shown.

macrophages to the site of inflammation [23], and their effector functions. As occurred with other immune cells, the effect of sex steroids on macrophages depends on the concentration, type, and the context in which macrophages are studied [24].

For instance, in the murine model of incisional wound, gonadectomy of females is associated to an increased inflammation and delay in wound healing. This effect is due to the fact that ovariectomy induces an increase in the secretion of TNF-α and MIF, as well as in the number of infiltrated

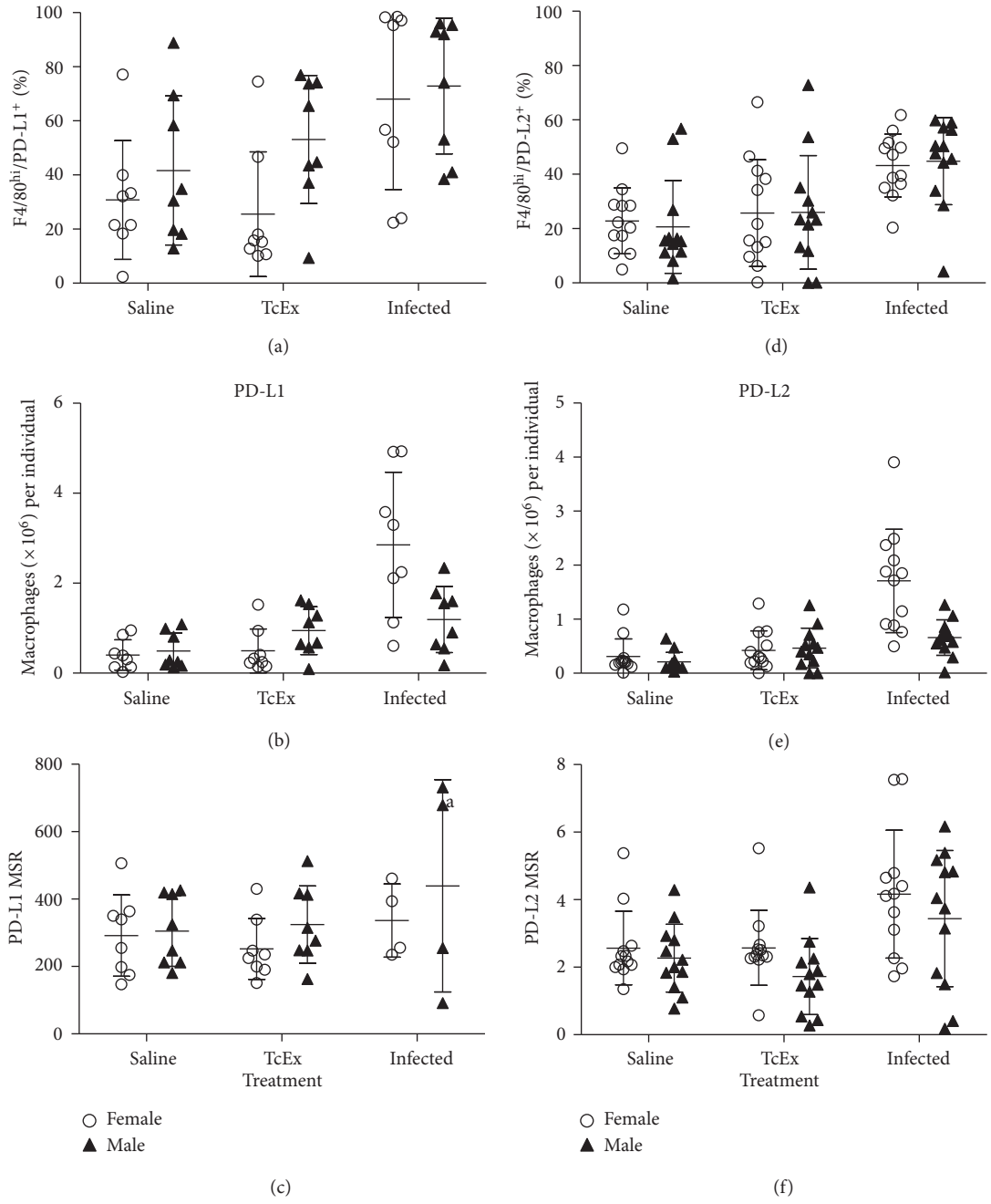

Figure 5: PD-L1/PD-L2 characterization in Mϕs recovered of the peritoneum after *T. crassiceps* infection. Inhibitory molecule, PD-L1, and PD-L2 expression was determined on Mϕs (F4/80^{hi}) recruited after 6 days of infection with 20 cysts of *T. crassiceps*. The percentages (a) and (d), total numbers (b and e), and the expression of these molecules (relative mean intensity, MSR) (c) and (f) are shown.

macrophages at the site of the lesion. Also, the percentage of alternatively activated macrophages is decreased [23, 36]. If castrated females are reconstituted with E2 concentrations observed during estrous, then the production of TNF-α, MIF, and the total number of infiltrated macrophages in the wounds are decreased. However, treatment with physiological levels of progesterone has a modest effect, in comparison to the effect induced by estradiol, on the same parameters studied [23]. Moreover, sex steroides regulate the production of nitric oxide (NO) by macrophages, in a dichotomic way. At low concentrations, E2 stimulates the secretion of NO by LPS-activated macrophages in vitro; however, at high concentrations of E2, there is a decrease of NO [37, 38]. Furthermore, estradiol and to a lesser extent progesterone decrease the activity of the enzyme catalase, a very important modulator of the NO synthesis [39]. As such, these data may represent an important mechanism underlying the immunomodulating effects of sex steroids.

Previously, we showed that during murine cysticercosis, an impressive feminization process is produced in the male host, characterized by an increase in serum estradiol level of 200 times above their normal value, roughly similar to those of normal females, while those of testosterone decreased by 90% relative to controls [29]. These changes in the hormonal milieu of the host equalize the parasite loads between genders. In the same way, progesterone treatment tends to equalize parasite loads in females and males, which suggests that other gonad-associated factors are involved in the control of parasite growth. Therefore, a more intricate strategy of parasite activity has to be considered. Perhaps, high estrogen levels are the main feature of this intriguing puzzle, since, in males, the parasite loads increased more markedly than in females. We suppose that expression of costimulatory molecules early during infection could be differential, and this fact impacts the parasite loads that are different among males and females, late during infection. This hypothesis was tested in this study and found that always females have higher expression of MHC-II, CD80, CD86, PD-L1, and PD-L2 during infection, but not in response to saline or TcEx. Interestingly, estradiol concentrations are higher in infected females early in infection [29].

5. Conclusion

In summary, the results presented here demonstrate that recruitment and expression of MHC-II, CD80, CD86, PD-L1, and PD-L2 in Mϕ of peritoneal cavity in *T. crassiceps* early at infection is associated to the sex of the host, although at the time of infection the number of parasites does not differ between both sexes. Whatever the cysticercosis-relevant "sex steroid target" may prove to be, the fact steroids positively may interfere with the development of protective immune mechanisms against *Taenia crassiceps* cysticerci has an important implication for future vaccine and vaccination trials, among others projections.

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Research Article

Reevaluating the Role of *Acanthamoeba* Proteases in Tissue Invasion: Observation of Cytopathogenic Mechanisms on MDCK Cell Monolayers and Hamster Corneal Cells

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The morphological analysis of the cytopathic effect on MDCK cell monolayers and hamster cornea and qualitative and quantitative analyses of conditioned medium and proteases were evaluated and compared between two strains of *Acanthamoeba* genotype T4. Further than highlighting the biological differences found between both strains, the most important observation in this study was the fact that proteases both in total extracts and in conditioned medium are apparently not determinant in tissue destruction. An interestingly finding was that no lysis of corneal tissue was observed as it was previously suggested. These results, together with previous studies, allow us to conclude that the invasion and disruption of corneal tissue is performed by the penetration of the amoebae through cell junctions, either by the action of proteases promoting cellular separation but not by their destruction and/or a mechanical effect exerted by amoebae. Therefore, contact-dependent mechanisms in *Acanthamoeba* pathogenesis are more relevant than it has been previously considered. This is supported because the phagocytosis of recently detached cells as well as those attached to the corneal epithelium leads to the modification of the cellular architecture facilitating the migration and destruction of deeper layers of the corneal epithelium.

1. Introduction

Free-living amoebae of the genus *Acanthamoeba* are one of the most common amoebae found in a wide variety of habitats, ranging from tropical zones to arctic regions [1]. These amoebae can be found in dust [2], air, soil [3], fresh water, sea water, tap water [4, 5], bottled mineral water [6], and sewage [7].

These opportunistic pathogens have gained medical importance due to their ability to infect the skin, brain, and eye [8–10]. Various species of *Acanthamoeba* genus can cause granulomatous amoebic encephalitis (GAE), which is usually associated with immunocompromised individuals, and they are also the etiological agent of *Acanthamoeba* keratitis (AK), a painful chronic inflammatory disease of the cornea frequently associated with contact lens wearers [11]. Unlike debilitated patients with GAE or cutaneous acanthamebiasis, individuals with AK are generally immunocompetent. Nevertheless, these individuals do not develop protective immunity, and reinfection can occur. In addition, the infection is highly resistant to many antimicrobial agents mainly due to the existence of a cyst stage in these pathogens [12].

At least eight species of *Acanthamoeba* have been implicated in human infections: *A. castellanii* and *A. polyphaga* are the most common isolated species. Molecular classification of *Acanthamoebae* strains has allowed clustering of these

Table 1: Summary of pathogenicity tests of *A. castellanii* and *A. polyphaga* in a murine model according to Culbertson et al. [19].

	% Virulence	Amoebae recovery	Dead postinoculation
A. castellanii	60%	Brain, lung, kidney, liver	5–9 days
A. polyphaga	20%	Brain, lung	7–20 days

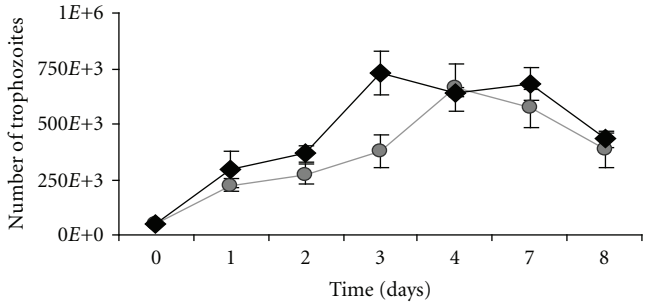


Figure 1: Representative growth curves of the optimal temperature (30°C) for *A. castellanii* (grey circle) in bacto casitone medium and for *A. polyphaga* (black diamond) in PBSG medium. Assays were carried out in triplicate with 98% parasite viability.

pathogens into 17 different genotypes being T4 the most prevalent in environment and clinical cases [13].

Parasitic infections may occur in a sequential manner and are initiated by the adherence of the amoebae to the host cells [14, 15]. Amebic adhesion may be mediated by mannose recognition sites localized in the target cells. The recognition of these surface oligosaccharides by *Acanthamoeba* is mediated by a 136 kDa-mannose-binding protein (MBP) on their surface [16]. After recognition and binding, *Acanthamoeba* cytopathogenicity occurs and may result in phagocytosis or induction of host cell necrotic and apoptotic death.

Recently, an *in vitro* animal model of AK has been implemented which allows the evaluation of the early most evident morphological events that take place in the cornea in the target cells. Moreover, it has also been demonstrated that if *Acanthamoeba* trophozoites are cocultured with isolated hamster and human corneas, the amoebae are able to invade and cause damage to the intact corneal epithelium, without the requirement of a previous corneal abrasion [17, 18].

The role of proteases in these processes has been previously discussed [13]; however, it has been evaluated in cellular monolayers and not directly in the target tissue. For that reason it is important to determine the role of these proteases, the phagocytosis phenomena and the mechanical action that these amoebae exert on the target tissue during the invasion process.

2. Material and Methods

2.1. Amoebae. This study was carried out with two *Acanthamoeba* strains isolated in the association to prevent blindness in Mexico, (Luis Sánchez Bulnes Hospital, Mexico City); *Acanthamoeba polyphaga* was obtained from the contact lens of a patient with AK, and *Acanthamoeba castellanii* was also isolated from the contact lens of a patient that was suffering intense ocular pain. No amoebae were isolated from the corneal scrapes of these patients. Amoebae were preliminarily identified to the species level using the morphological criteria of [20]. Molecular identification of the amoebic strains at the genotype level was carried out as previously

Table 2: Summary of proteolytic activities detected through SDS-PAGE gels copolymerized with gelatin in total extracts (TE) and conditioned medium (CM) from of *A. castellanii* and *A. polyphaga*. The majority of proteases were inhibited 100% with PMSF (1 mM), only two of them were partially inhibited (*).

	Proteases (kDa)	100% inhibition of proteases PMSF (1 mM)
A. castellanii	TE, CM 120	CM 39*
	CM 97	
	TE, CM 66	
	TE, CM 64	
	CM 57	
	CM 55	
	CM 50	
	CM 47	
	CM 45	
	CM 40	
	CM 38	
A. polyphaga	TE, CM 120	
	CM 97	
	CM 66	
	CM 64	
	CM 57	
	TE 55	TE 58*
	CM 50	
	CM 40	
	CM 38	

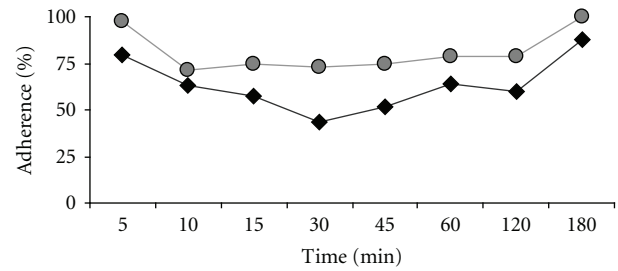


Figure 2: Adherence of *Acanthamoeba* spp. to MDCK cells. Trophozoites (7.5×10^4) of *A. castellanii* (grey circle) and *A. polyphaga* (black diamond) were incubated for different times with confluent MDCK epithelial cell monolayers. Adhesion was evaluated by an ELISA-based assay (absorbance of 7.5×10^4 trophozoites corresponded to 100%). Assays were performed in triplicate.

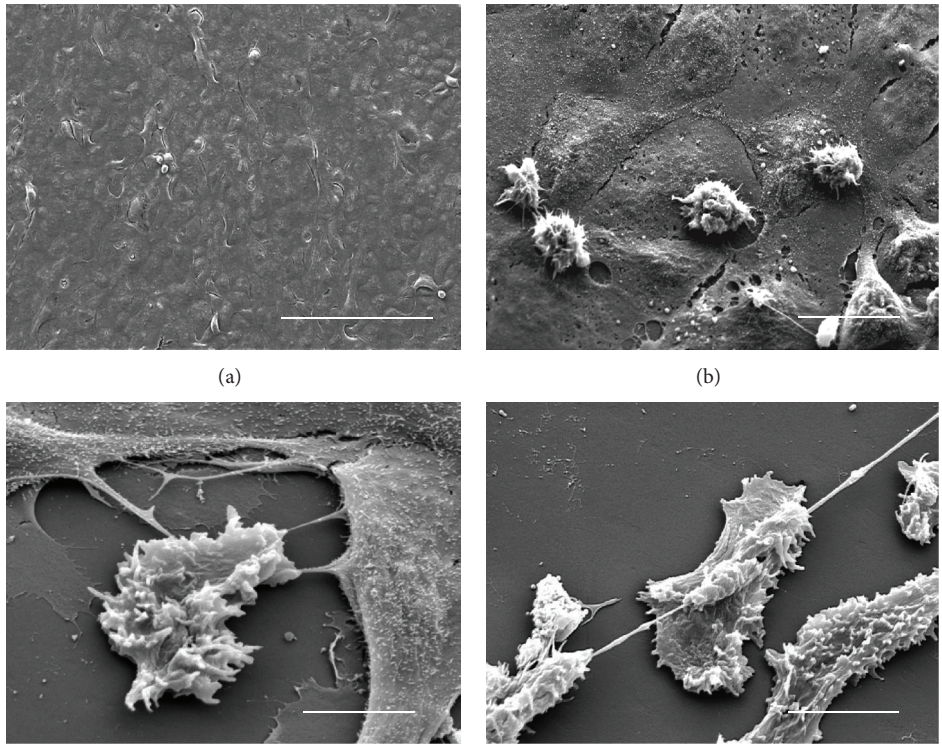


Figure 3: Scanning electron microscopy of the interaction of *A. polyphaga* with MDCK cells. (a) No morphological evidence of damage was observed on control MDCK monolayers incubated for 3 h with 1 : 1 PBSGM : DMEM medium. Bar = 200 μm. (b) After 1 h of coincubation, small number of trophozoites were observed adhered to MDCK cells surface Bar = 20 μm. (c) By 2 h only few areas of monolayer discontinuity were observed, and a small number of trophozoites were located under and over the surface of MDCK cells. Bar = 10 μm. (d) At 3 h of coincubation, scarce areas devoid of cells were observed, in which a trophozoite was seen phagocyting fragments of MDCK cells. Bar = 10 μm.

described by sequencing the diagnostic fragment 3 (DF3) of the 18S rDNA gene of *Acanthamoeba* [20, 21].

2.2. Isolation and Maintenance of Acanthamoeba Strains in Monoxenic Cultures. The techniques used for recovery and maintenance of *Acanthamoebae* from clinical and environmental sources are described elsewhere [22, 23]. Briefly, primary isolation was performed from infected human corneal tissues and contact lenses using 1.5% nonnutrient agar plates seeded with heat-killed *Enterobacter aerogenes*. Subsequent incubation was performed at ambient temperature (22 to 24°C) for up to 10 days. Upon evidence of amoebic growth, clonal cultures were established by transferring a single double-walled cyst to fresh agar medium containing antibiotics (penicillin, 100 mg/mL; streptomycin, 10 mg/mL).

2.3. Axenic Cultures. Monoxenic cultures were selected from areas of profuse amoebic growth. Selected pieces of agar were transferred to axenic culture mediums such as phosphate-biotriptase-serum glucose medium (PBSGM) [24] and 2% Bacto Casitone medium (DIFCO), which are culture media widely used for growth and amoebic development. Both mediums were supplemented with 10% fetal bovine serum (Equitech-bio, Kerville, TX, USA) and 1% of an antibiotics mix (Penicillin-Streptomycin). Trophozoites were incubated in both mediums at 30°C in borosilicate tubes (Pyrex, Mexico). The medium was changed twice daily for 2 days and afterwards once daily for 3 more days. The cultures were considered axenic if no bacterial growth was observed. It is important to emphasize that all trophozoites interactions assays were performed with Bacto Casitone medium free of fetal bovine serum.

2.4. Temperature Tolerance Test. To determine the optimal culture medium and temperature for growth, amoebae were incubated at 30°C, 35°C, and 37°C in borosilicate tubes (Pyrex, Mexico). Assays were carried out simultaneously by placing axenic trophozoites in the culture media mentioned above. Optimal growth was determined by plotting logarithmic growth phase curves (triplicate assays were performed). The viability of the trophozoites was determined using the trypan blue (0.4%) exclusion test.

2.5. Pathogenicity Test. Trophozoites from axenic cultures in the exponential phase of growth (2.5×10^5 parasites/20 μL) were inoculated into the nostrils of 15 male Balb/C mice (3 weeks old), according to Culbertson et al. [19]. A group of five mice was inoculated with culture medium without

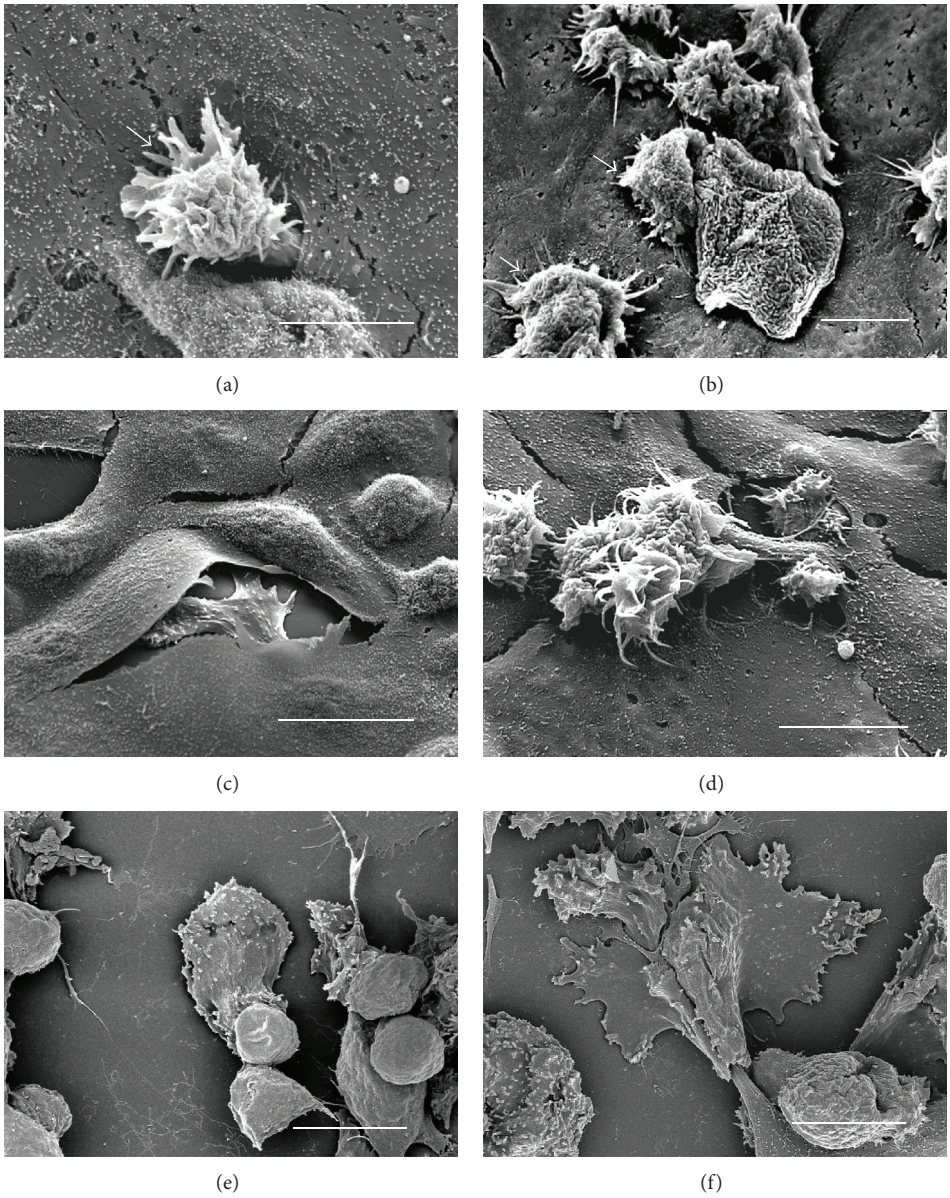
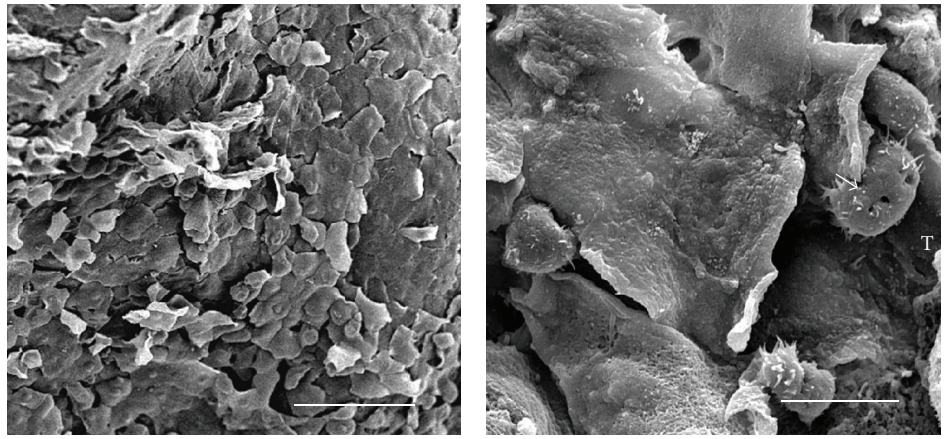


Figure 4: Scanning electron microscopy of the interaction of *A. castellanii* with MDCK epithelial cell monolayer. After 1 h of interaction, alone (a) or in group (b) trophozoites were observed penetrating the MDCK monolayer. Typical acanthopodia were clearly seen (arrows). Bar = 10 μm. (c) In another area one trophozoite penetrated the monolayer and formed a protuberance on the surface. (d) By the second hour, numerous trophozoites were seen adhered to the monolayer apparently phagocyting a MDCK cell. Bar = 10 μm. (e) Clearly damaged areas of the cell monolayer were observed 3 h after interaction. Several regions of the substrate became apparent as a consequence of the detachment and/or ingestion of the MDCK cells by the amoebae. Bar = 20 μm. (f) Trophozoite apparently phagocyting a portion of an MDCK cell. Bar = 20 μm.

amoebae used as control. After 21 days, the surviving mice were sacrificed. The brain, liver, lungs, and kidneys were cultured in agar plates with nonnutritive enriched medium (NNE) to recover the amoebae [24, 25].

2.6. Quantitative Analysis of Trophozoite Adherence to MDCK Cells. Monolayers of epithelial cells of the established MDCK line of canine kidney origin (Madin Darby Canine Kidney) were grown on 25 cm^2 cell culture flasks (Corning Incorporated, NY) in Dulbecco's modified Eagle's medium (Microlab, Mexico). They were supplemented with 10% fetal bovine serum (Gibco, Grand Islands, NY) and antibiotics in a 5% CO_2 atmosphere at 37°C.

Cells were trypsinized and transferred to 96 well plates. Confluent cell monolayers were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h and washed twice with Dulbecco's Phosphate Buffered Saline (DPBS). Trophozoites from both *Acanthamoeba* species (7.5×10^4) were added to the monolayers and incubated at different times (5, 10, 15, 30, 45, 60, 120, and 180 min) at 30°C, and fixed as



(a) (b)

Figure 5: Scanning electron microscopy of the interaction of *A. polyphaga* trophozoites with hamster cornea. In the first hours of coincubation areas of corneal injury were not observed. (a) After 16 h, only few regions of cellular disorganization and loss of the most superficial layers of the cornea were detected. Bar = 100 μm. (b) Trophozoite (T) was found in close relation with epithelial cells (arrow). Bar = 10 μm.

mentioned above. Then samples were washed twice with DPBS-Tween 0.05%. Adhesion was evaluated by an ELISA-based assay; briefly the plates were blocked overnight with 200 μL of 1% casein at 4°C, and plates were washed 5 times with PBS-Tween 0.05% and 100 μL of polyclonal IgG anti-*Acanthamoeba*: *A. castellanii* (1 : 8000) and *A. polyphaga* (1 : 4000) were added and incubated for 2 h at 37°C. Subsequently, plates were washed 5 times with PBS-Tween then 100 μL of a dilution of 1 : 50000 conjugate anti-rabbit IgG was added and incubated for 1 h at 37°C. After that 100 μL of developing solution was added for 10 min; at that moment a mixture of citric acid and 0.1 M dibasic sodium phosphate 0.2 M pH 5, finally 40 mg of orthophenylenediamine and 40 μL hydrogen peroxide 30% were added. The reaction was blocked with 50 μL of H_2SO_4 to 2.5 M. Absorbance was read at 490 nm (Bio-Rad 550) [26].

2.7. Cytopathic Effect on MDCK Cells. MDCK cell monolayers were trypsinized and transferred to round plastic cover slips placed in 24 well styrene plates and kept at 37°C in 5% CO_2; after 24 h cells formed confluent monolayers. Afterwards, cell cultures were incubated at different times (1, 2, and 3 h) in the same conditions in a mixture of amoebae medium and Dulbecco's modified Eagle's medium serum free (Gibco BRL) in equal proportions. *A. castellanii* and *A. polyphaga* trophozoites were added in a 1 : 1 (target cell: amoebae) ratio.

2.8. Scanning Electron Microscopy. Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and dehydrated with increasing concentrations of ethanol. Samples were then critical point-dried with liquid CO_2 using a Samdri 780 apparatus (Tousimis Research Corp.) and coated with a thin layer (30 nm) of gold in an ion-sputtering device (JEOL, JFC-1100). Specimens were examined with a Philips XL30 ESEM scanning electron microscope.

2.9. Interactions with Hamster Cornea. Adult male golden hamsters (*Mesocricetus auratus*) weighing 120 to 130 g were used. Experiments were based on protocol 002/02, approved by the Institutional Animal Care and Use Committee, in accordance with norm-062-Zoo-1999, based on the *Guide for the Care and Use of Laboratory Animals*, published in the Official Journal of the Federation (Mexico) 2001. After anesthesia with sodium pentobarbital (Sedatphorte) at 4.72 mg/100 g of body weight, both corneas were removed leaving a peripheral rim of scleral tissue, as previously described [12]. Corneas were placed in 96 well plates and interacted with 2.5×10^5 trophozoites for different periods of time (30 min, 1, 2, 4, 8, 16, and 24 h). Control corneas were treated in a similar way, but only culture medium was added.

2.10. Interactions of Conditioned Medium with Hamster Cornea. Assays were carried out as described above by using conditioned medium obtained as follows: 2×10^5 trophozoites from a culture in exponential phase of growth were placed in culture flasks containing 5 mL of fresh Bacto Casitone or PBSGM mediums free of fetal bovine serum and incubated at 30°C for 24 h. Trophozoites were then chilled on ice for 10 min and centrifuged. The supernatant was removed, centrifuged at 1500 g, and filtered through 0.22 μm filters (Millex GV Durapore PVDF). Viability of trophozoites was determined using the trypan blue exclusion technique before collecting conditioned medium [27].

2.11. Light and Transmission Electron Microscopy. After coincubation, samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at room temperature, postfixed with 1% osmium tetroxide, and dehydrated with increasing concentrations of ethanol. Samples were transferred to propylene oxide, later on to a mixture of propylene oxide/epoxy resin (1/1) and finally embedded in epoxy resins. Semithin sections (0.5 μm), stained with toluidine blue were examined with an Axiophot photomicroscope

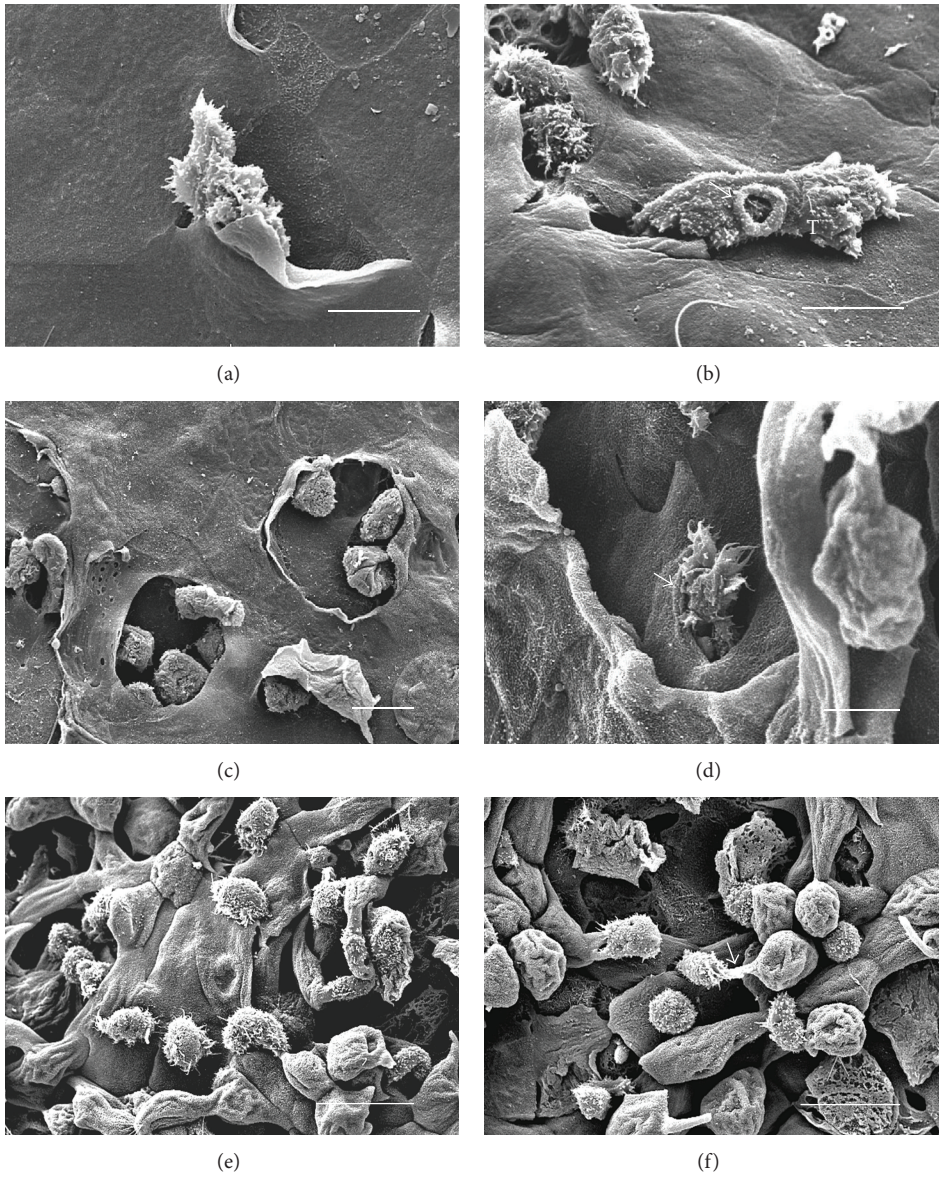


Figure 6: Scanning electron microscopy of the interaction of *A. castellanii* trophozoites with hamster cornea. (a) At 1 h, one trophozoite was observed penetrating the most superficial epithelial cells. Bar = 10 μm. (b) Trophozoite (T) emitting a phagocytic structure (arrow) was a frequent event. Bar = 10 μm. (c) After 2 h of interaction numerous trophozoites were located under the first layer of the corneal epithelium. Bar = 20 μm. (d) By 4 h *A. castellanii* continued migrating towards the deepest layers of the corneal epithelium (arrow). Bar = 10 μm. (e) At 16 h, numerous trophozoites remained adhered to the surface of the epithelial cells that had not been detached. Bar = 20 μm. (f) After 24 h damage to the structure of the cornea is evident. A trophozoite was observed apparently phagocyting an epithelial cell (arrow). Bar = 20 μm.

(Carl Zeiss, Germany). Thin sections previously stained with uranyl acetate and lead citrate were observed in a Morgagni 268 D transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

2.12. Whole-Cell Extracts and Conditioned Medium of A. castellanii and A. polyphaga. *Acanthamoeba* spp. trophozoites in logarithmic phase of growth were chilled at 4°C, centrifuged and washed twice with DPBS. The cells were disrupted by 10 vortex-ice cycles in DPBS. Protein concentration (1×10^5 trophozoites) was quantified by Bio-Rad RC-DC method.

The conditioned media from both species were obtained as follows: 6×10^6 trophozoites from a culture in exponential phase of growth were placed in culture flasks containing 5 mL of fresh Bacto Casitone free of fetal bovine serum and incubated at 30°C for 24 h. The viability of the amoebae was determined using trypan blue exclusion test. Trophozoites were then chilled on ice for 10 min, and centrifuged. The supernatant was removed, centrifuged, and filtered through 0.22 μm filters (Millex GV Durapore PVDF). Total crude

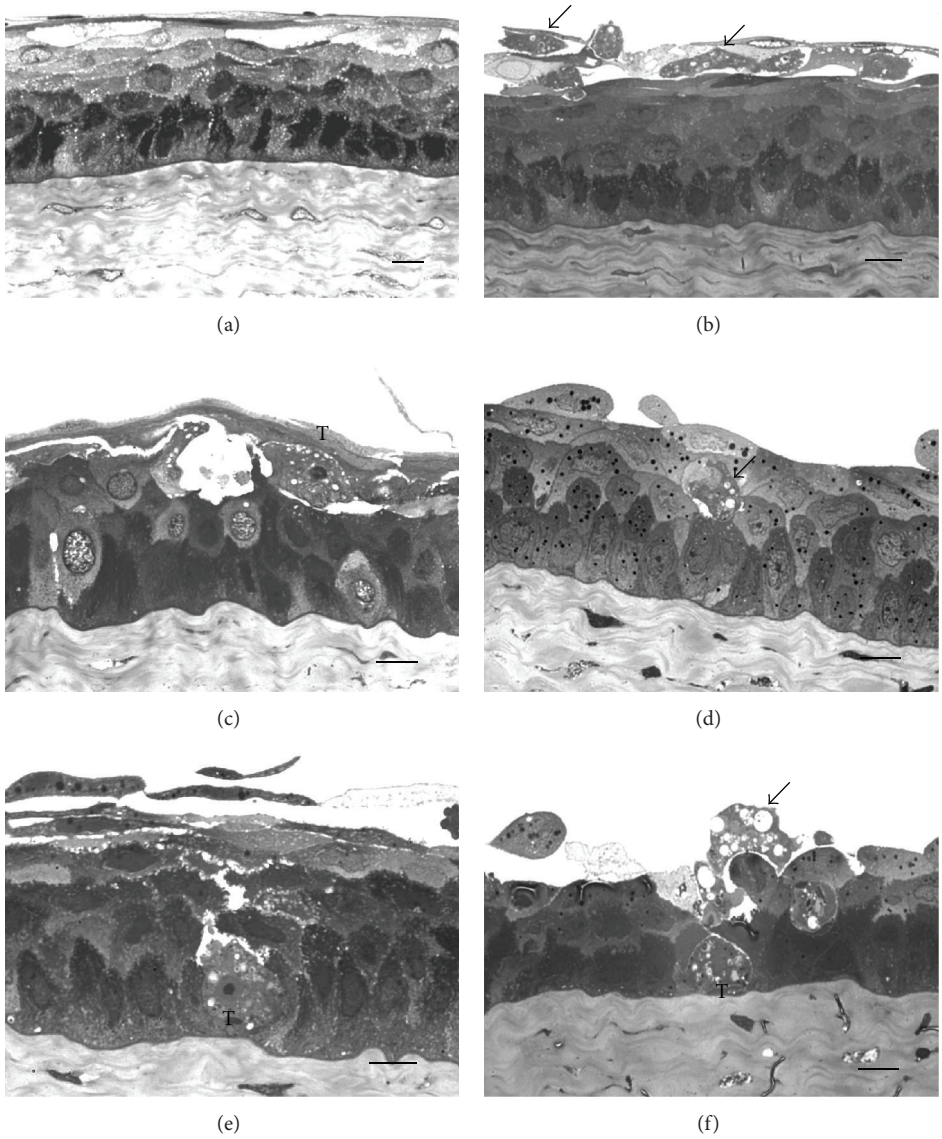


Figure 7: Light microscopy of semithin sections of the interaction of *A. castellanii* trophozoites with hamster cornea. The most representative time points are shown. (a) Control hamster cornea incubated with Bacto Casitone medium for 24 h. No evidence of damage was observed. (b) After 1 h of interaction numerous trophozoites (arrows) were observed penetrating the most superficial layers of hamster's cornea. (c) After 8 h a trophozoite (T) migrated toward wings epithelial cells; part of the corneal epithelium remained, but the normal tissue structure had been lost. (d) At 16 h superficial and some middle (wings) cells of the corneal epithelium had been separated from the rest of the tissue. A trophozoite phagocyting an epithelial cell is shown (arrow). (e) After 24 h of interaction *A. castellanii* trophozoite had migrated toward the basal cells. (f) In other areas of interaction most of the epithelium had been lost. A trophozoite (T) reached the corneal stroma layer while another was seen phagocyting a detached cell (arrow). Bar = 10 μm.

extracts and conditioned media were stored at −70°C until used.

2.13. Protease Inhibitors. The inhibitors concentrations used were as follows: N-ethylmaleimide (NEM, cysteine proteases inhibitor) 20 and 50 mM; ethylenediaminetetraacetic acid (EDTA, metalloproteases inhibitor) 100 and 200 mM; phenylmethylsulfony fluoride (PMSF, serine proteases inhibitor) 1 and 5 mM.

2.14. Substrate Gel Electrophoresis. To detect proteolytic activity in crude extracts and conditioned medium, SDS-PAGE gels were copolymerized with 0.1% (w/v) gelatin as protease substrate.

The electrophoresis was performed at 4°C for 1 h; gels were washed twice with 2.5% triton X-100 solution and incubated at 37°C overnight with 100 mM Tris-OH buffer (pH 7.0) supplemented with 2 mM $CaCl_2$. Gels were stained with 0.5% Coomassie blue R-250 [28].

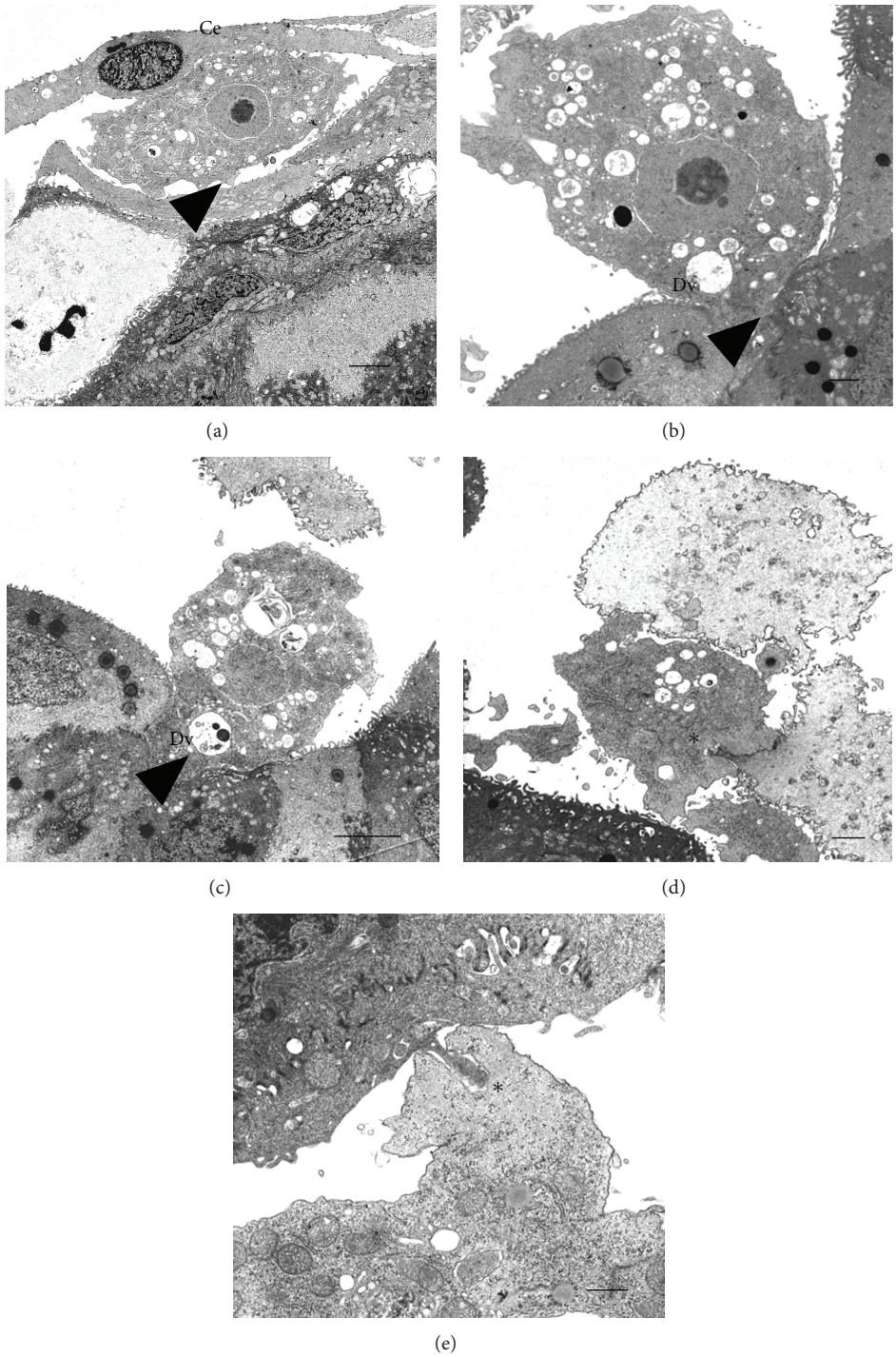


Figure 8: Transmission electron microscopy of the interaction of *A. castellanii* with hamster cornea. (a) After 16 h of interaction a trophozoite migrated and penetrated into middle layer of corneal epithelium (Ce), emitting acanthopodia of different sizes (arrow head). Bar = 3 μm. (b) and (c) By 24 h trophozoites migrated to deeper layers presumably by the emission of cytoplasmic projections (arrow head), which were frequently observed being introduced between the epithelial cells junctions. The amoebae preserved their characteristic morphological features. Some trophozoites shown in the figure contain digestive vacuoles (Dv) of different sizes. (b). Bar = 1 μm; (c). Bar = 3 μm. (d) and (e) Frequently, amoebae emitted amoebostomes (∗) of different sizes with their characteristic fibrogranular cytoplasm, which allowed them to ingest portions of cells. (d). Bar = 1 μm; (e). Bar = 1 μm.

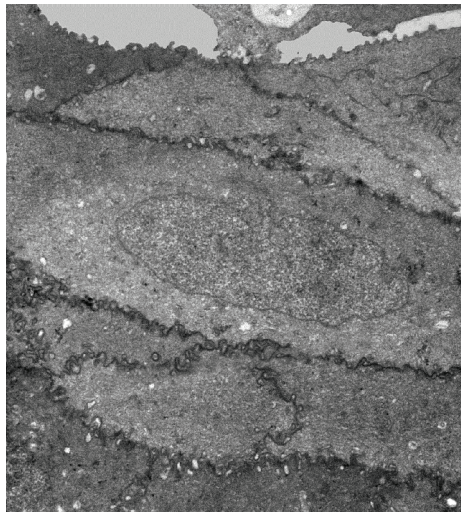


Figure 9: Interaction of conditioned culture medium with hamster cornea. At 6 h after interaction with Bacto Casitone medium, no evidence of damage or cell disorganization was observed; only normal desquamation was detected in scarce zones of corneal epithelial surface.

3. Results

3.1. Axenic Cultures and Optimal Temperature of Growth. Acanthamoeba castellanii grew better in Bacto Casitone medium while the best growth rate for *Acanthamoeba polyphaga* was observed in PBSG medium. The optimal growth temperature for both amoebae was 30°C. *A. polyphaga* reached the exponential phase of growth in 48–72 h, with a homogeneous and abundant number of trophozoites that were able to form mature cysts after several weeks. In contrast, *A. castellanii* reached the exponential phase of growth after 72–96 h of culture. The cultures had a low number of trophozoites and form mature cysts in a few days (Figure 1). After sequencing of the DF3 region of both strains, it was concluded that both amoebae belonged to genotype T4.

3.2. A. castellanii Is Virulent in Animal Model of EAG in Contrast with A. polyphaga. It is only invasive. Mice infected with *A. castellanii* became ill very quickly, as it was manifested by ruffled fur and aimless wandering of the animals, followed by coma and death 5 to 9 days after inoculation. Amoebae were isolated from the brain, liver, lung, and kidney. In contrast, mice infected with *A. polyphaga* showed little evidence of illness. Only 3 mice died between 7 to 20 days after inoculation, and amoebae were recovered only from the brain and lung (Table 1).

3.3. Trophozoites of A. castellanii and A. polyphaga Showed Significant Differences in Their Adhesion Rate to MDCK Cells. Significant differences in the adhesion rate to MDCK cells were observed. Figure 2 shows the percentage of adherence of amoebae to monolayers of MDCK cells at different times of interaction. Adherence of *A. castellanii* was higher at all time when compared to the *A. polyphaga* strain. Roughly 90% of *A. castellanii* trophozoites adhered to the monolayer after 5 min of incubation, while less than 80% of *A. polyphaga* trophozoites were able to adhere to the cells. Nevertheless, adherence in both strains was time dependent, reaching 100% adherence after 180 min.

The obtained data were transformed and analyzed as their natural logarithm as they were not normally distributed (Kolmogorov-Smirnov test). A two-factor analysis of variance (ANOVA) showed significant differences in the adhesion of the two species under study, as well as differences along the interaction time ($P < 0.001$).

3.4. Trophozoites of A. castellanii Individually or in Groups Were Observed Penetrating the MDCK Monolayer Forming Protuberances on It. No morphological evidence of damage was observed in the MDCK control monolayer after 3 h of interaction (Figure 3(a)). At 1 h of interaction only a small number of *A. polyphaga* trophozoites penetrated the monolayer (Figure 3(b)), scarce areas devoid of cells (Figure 3(c)) and trophozoites phagocyting minor fragments of MDCK cells were observed at 3 h (Figure 3(d)). In contrast, from the first hour of interaction, trophozoites of *A. castellanii* individually or in groups were observed penetrating the MDCK monolayer (Figures 4(a) and 4(b)), forming protuberances on it (Figure 4(c)). By the second hour, trophozoites were seen adhered and beneath to the monolayer apparently phagocyting MDCK cells (Figure 4(d)). Several regions of the substrate became apparent as a consequence of the detachment and/or ingestion of the MDCK cells by the amoebae and during all assays parasites apparently ingested whole cells or fragments of detached cells (Figures 4(e) and 4(f)). Intact MDCK cells were observed in close proximity to the damaged areas, suggesting a focal damage of the cell monolayer related to a contact-dependent trophozoite-target cell interaction.

3.5. Structural Analysis of the Interaction Revealed That Both Amoebae Produced Different Degrees of Damage to the Corneal Epithelium. After 16 to 24 h of interaction *A. polyphaga* reached only the most superficial corneal cells (Figure 5(a)). Few trophozoites migrated to wing cells layer phagocytizing detached cells (Figure 5(b)). In contrast, *A. castellanii* invaded and disorganized corneal epithelium by penetrating through the cell junctions into the inner epithelium layers (Figure 6(a)), emitting phagocytic structures (Figure 6(b)). At 2 h, trophozoites were seen beneath the superficial cell layers detaching epithelial cells (Figure 6(c)). From 4 h to 16 h, trophozoites continued migrating towards the deepest layers of corneal tissue (Figure 6(d)), which had lost its original morphology at this stage. Moreover it was not possible to recognize in which region of the epithelium the trophozoites were located due to the damage caused at this stage (Figure 6(e)). After 24 h of interaction, the migration of the trophozoites continued and phagocytosis was constantly observed (Figure 6(f)).

3.6. Semithin Sections of the Squamous Epithelium Show Focal Damage Related to Contact-Dependent A. castellanii Trophozoite-Target Cell Interaction. Semithin sections of the

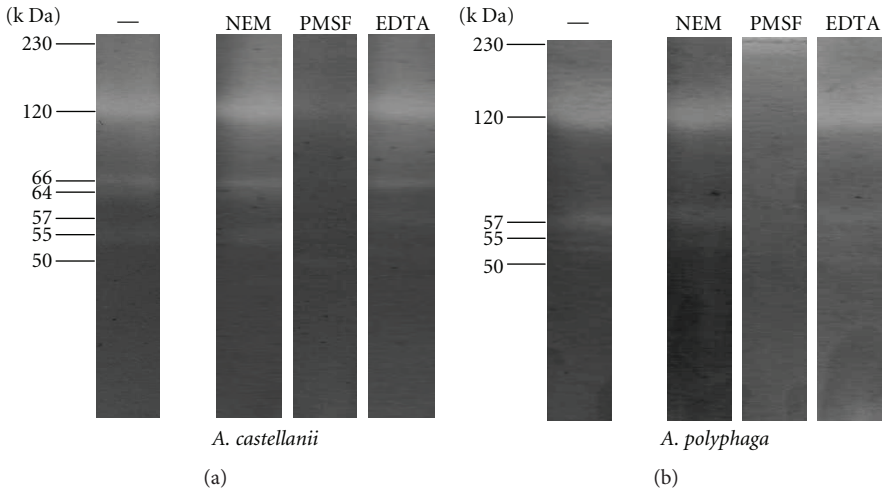


Figure 10: Proteolytic activity profiles from *A. castellanii* and *A. polyphaga*. 1 μg of protein was analyzed from total extracts, which is equivalent to 3.6×10^3 trophozoites. The extracts were obtained from axenic cultures of *A. castellanii* and *A. polyphaga*; these were analyzed on SDS PAGE gels copolymerized with gelatin and incubated in the presence of protease inhibitors with the following concentrations: 20 mM NEM, 100 mM EDTA, and 1 mM PMSF. Protease analysis represents the mean of 5 separate experiments.

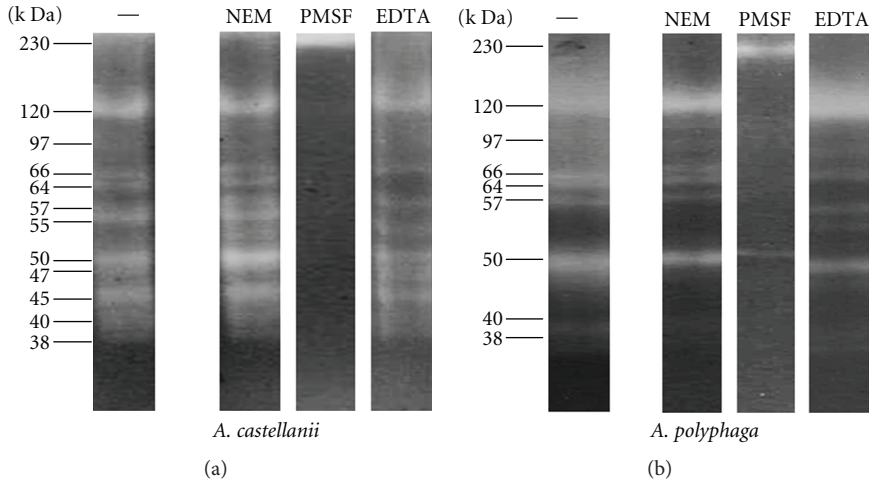


Figure 11: Proteolytic activity of conditioned medium of *A. castellanii* and *A. polyphaga*. SDS-PAGE gels copolymerized with gelatin were incubated independently with the following inhibitors: 20 mM NEM, 100 mM EDTA, and 1 mM PMSF. Protease analysis represents the mean of 5 separate experiments.

squamous epithelium of control corneas incubated in Bacto Casitone culture medium showed a typical morphology as well epithelium incubated with PBSG medium (Figure 7(a)).

During early interaction times, numerous trophozoites were observed penetrating the most superficial corneal epithelial layers (Figure 7(b)). By 8 h trophozoites had migrated toward wings epithelial cells (Figure 7(c)). At 16 and 24 h of interaction *A. castellanii* migrated and injured basal epithelial corneal cells (Figures 7(d) and 7(e)) and stroma layer (Figure 7(f)). Areas in which the amoebae attached and penetrated were morphologically intact suggesting a focal damage related to a contact-dependent trophozoite target cell interaction. Phagocytosis was a recurrent process (Figures 7(d) and 7(f)). No evidence of harm produced by *A. polyphaga* trophozoites was seen at the same time of incubation.

3.7. A. castellanii Invade Tissue with Acanthopods and Phagocyte Epithelial Corneal Cells with Amoebostomes. Invasion by amoebae takes place with cell projections (acanthopodia) which were frequently observed (Figure 8(a)), allowing the passage of amoebae between corneal epithelial cells junctions (Figures 8(b) and 8(c)). Amoebae regularly emitted amoebostomes of different sizes with their characteristic fibrogranular cytoplasm ingesting whole cell or portion of them (Figures 8(d) and 8(e)). Digestive vacuoles were a frequent finding.

3.8. Conditioned Medium Does Not Lyse Hamster Cornea Tissue. Analysis of the conditioned culture medium interaction with hamster cornea at different point times showed that neither Bacto Casitone nor PBSGM conditioned mediums were able of lysing cells of corneal epithelium by itself, since

no evidence of damage or cell disorganization was observed (Figure 9).

3.9. Proteolytic Activity of Whole-Cell Extracts of A. castellanii and A. polyphaga with Conditioned Medium. The profiles of proteolytic activity of extracts from *A. castellanii* and *A. polyphaga* showed the presence of constitutive proteases in both strains (Figure 10). In the case of *A. castellanii* proteases were 64, 66, and 120 kDa molecular weight. In comparison, *A. polyphaga* presents proteolytic activities ranging between 55, 57, and 120 kDa.

Proteolytic activity inhibition tests strongly suggest that both amoebae produce serine proteases.

Analysis of proteolytic activity from the conditioned medium (Figure 11) evidenced the presence of proteases with molecular weight similar to those detected in extracts from trophozoites of both strains. The tests performed with the proteases inhibitors showed again that the main proteases profile belongs to the family of serine proteases, because the PMSF showed a better inhibitory effect. Table 2 summarizes the molecular weights detected in both conditions.

4. Discussion

Acanthamoeba keratitis has been recognized as a significant ocular amoebic infection where use of contact lenses plays an important risk factor [29], as they cause epithelial changes which may reduce the corneal epithelial resistance to microbial invasion [13]. At present, there is little information about differences in biological and cytopathogenic mechanisms between *Acanthamoeba* species, and whether they are related or not to the virulence of each strain. In the course of the analysis of the results, we determined that even though both species in this study were evaluated under the same laboratory conditions and belonged to the same potentially pathogenic genotype (T4); significant differences were found between them. It was observed that *A. castellanii* strain grew slower than *A. polyphaga*. We expected that the fast-growing *A. polyphaga* could facilitate *in vitro* damage in target cells and therefore establish an infection more easily than slow-growing *A. castellanii*, creating enough amoebae to start an infection and therefore growing more rapidly in the host; however, the results showed otherwise. Moreover, significant differences were observed during the tissue invasion of amoebae to mice organs incubated *postmortem*; *A. castellanii* invaded all the evaluated organs (brain, lungs, liver, and kidney) being more virulent and invasive than *A. polyphaga* which was able to invade only brain and lung. These results are in agreement with Levandowsky et al. [30], who reported that pathogenic free-living amoebae migrate more rapidly than nonpathogenic species.

A. castellanii shown to be more efficient during all the evaluated processes since the quantitative and qualitative determination of the adherence rate was constant and higher, correlating with migration and penetration to the MDCK monolayer epithelial cell, mice pathogenicity test, and hamster cornea damage.

Phagocytosis plays an important role in the pathogenic mechanisms of *Acanthamoeba* species, since it is the mechanism of food acquisition during this parasitic phase. The loss of stromal keratocytes due to phagocytosis and apoptosis by *Acanthamoeba* has been previously documented through *in vitro* studies [8, 31].

As observed in this work, *Acanthamoeba* is able to damage intact corneal epithelium phagocytizing not only the keratocytes that are located in deeper layers of corneal tissue, but also the most superficial cells.

The most important issue in this study is that in addition to showing in detail the process of phagocytosis and migration of amoebae within the cell junctions, it was also possible to evaluate the proteases and their participation in the target tissue damage in a similar way as it takes place *in vivo*.

Zymography assays performed in this work confirmed that almost all proteases in trophozoites of *A. polyphaga* and *A. castellanii* were mainly serine proteases. *A. castellanii* produced eleven extracellular proteases and *A. polyphaga* seven with similar molecular weight, which is in accordance with previous studies with T4 strains. These previous studies have evaluated T4 isolates associated with GAE and AK allowing the identification of extracellular serine proteases of 36, 49, and 66 kDa [28], all of them with similar molecular weight to the ones observed for the strains included in this study. Cao et al. [11] reported 55, 97, and 230 kDa serine proteases, which correlates to the 55 y 97 kDa proteases found in this work. Alfieri et al. [32] reported 27, 47, 60, 75, 100, and lower than 110 kDa serine proteases; showing a coincidence with the 47 y 60 kDa proteases found in our strains. It is also important to mention that a 40 kDa serine protease related to genotype T4 [33] was also observed in our strains corroborating their genotypical classification. Even though the presence of proteases was confirmed in both species, *A. castellanii* secrete most of them.

Clarke and Niederkorn [34] suggested that pathophysiology of this infection includes the production of several pathogenic proteases that degrade basement membranes and induce cytolysis of the cellular elements of the cornea, conversely, we consider that the process of cornea invasion differs from the previously proposed, since the observed damage on the target tissue was strongly associated with amoeba contact-dependent mechanism more than damage due to independent mechanisms during amoebae corneal epithelium invasion. As mentioned, amoebae individually or grouped penetrate through the cell junctions and the neighborhood does not reveal any damage. Sometimes even when amoebae penetrate deeper in the corneal epithelium, no evidence of complete destruction of corneal tissue was observed. Besides it was not possible to see how trophozoites got to that site, Garner [8] reported similar images in histological sections of patients. We observed comparable results when evaluating early events of the invasion of *Acanthamoeba* trophozoites when introduced intranasally in a mice model for granulomatous amoebic encephalitis (data not shown). Our results are in agreement with Takaoka-Sugihara et al. [31], who suggested that direct contact with trophozoites, but not with soluble factors, was essential to induce the cytopathic effect on human corneal cells.

Proteolytic activity has been considered as a main source of damage, leaving phagocytosis in a second term as well as the mechanical forces produced by the amoebae during migration through the cells.

The results of this study agree that enzymatic contribution is relevant but it is imperative to emphasize that no evidence of whole cell lysis was found during the interaction assays with the MDCK cells and hamster cornea until the phagocytosis process was detected. The results observed in this study show that conditioned medium may promote the separation between the cells, leaving small spaces between them, which may facilitate the passage of amoebae between cell junctions. Besides we have proved that conditioned medium alone is able to damage only the most superficial human corneal tissue but amoebae invade and phagocyte epithelial cells reaching the Bowman layer of human cornea [17]. Ruqaiyyah and Khan [35], with reference to contact-independent factors of *Acanthamoeba,* have mentioned that these amoebae possess hydrolytic enzymes including elastases, phospholipases, glycosidases and a variety of serine, cysteine and metalloproteases, and ensure that their precise mechanisms of action at the molecular level are only beginning to emerge. We propose that the participation of proteases in this process facilitates passage of amoebas through corneal cells either by proteases facilitating cell separation but not their destruction. The findings suggest that intimate contact with the target tissue is important since trophozoites are able to form cytoplasmic projections between one cell to another in order to migrate and invade deeper layers of the target tissue. It could be possible that the movement of amoebae between the cells exerts a mechanical force that promotes separation and allows migration into the deeper layers of corneal tissue and MDCK cells, which may also explain the loss of the cellular structure (without the loss of integrity or damage to corneal cells). We emphasized that enzymatic activity may play a role in the citolytic mechanisms facilitating the separation of cells and probably not by destroying the tissue as it has been previously suggested.

It has been demonstrated that two species of *Acanthamoeba* genus even belonging to the same genotype showed important differences in their biological and cytopathogenic mechanisms. Takaoka-Sugihara et al. [31] suggested pathophysiological diversity of *Acanthamoeba* within the T4 genotype based in varied cytopathic effect provoked by these amoebae.

Amoebic pathogenicity may be an intrinsic characteristic and *Acanthamoeba* infection could be a correlation between amoebic features such as growth temperature, adhesion, phagocytosis, proteolytic activity, and mechanical effect during the invasion process as well as the host conditions.

5. Conclusion

Further than showing differences found between amoebae, the most important remark in this study is that the presence of proteases in both, total extracts and in conditioned medium are not determinative in tissue destruction. In view of our results, together with previous studies, we suggest that the invasion and disruption of corneal tissue is performed by the penetration of the amoebae through cell junctions either by the action of proteases only promoting cellular separation but not their destruction and/or a mechanical effect exerted by amoebae. Phagocytosis of recently detached cells as those attached to the corneal epithelium, leads to the modification of its architecture, facilitating the migration and destruction of deeper layers of the corneal epithelium suggesting that the contact-dependent activity is an important pathogenic mechanism of *Acanthamoeba castellanii* and *Acanthamoeba polyphaga*.

Acknowledgments

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Research Article

The TvLEGU-1, a Legumain-Like Cysteine Proteinase, Plays a Key Role in *Trichomonas vaginalis* Cytoadherence

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The goal of this paper was to characterize a *Trichomonas vaginalis* cysteine proteinase (CP) legumain-1 (TvLEGU-1) and determine its potential role as a virulence factor during *T. vaginalis* infection. A 30-kDa band, which migrates in three protein spots (pI~6.3, ~ 6.5, and ~6.7) with a different type and level of phosphorylation, was identified as TvLEGU-1 by one- and two-dimensional Western blot (WB) assays, using a protease-rich trichomonad extract and polyclonal antibodies produced against the recombinant TvLEGU-1 (anti-TvLEGU-1r). Its identification was confirmed by mass spectrometry. Immunofluorescence, cell binding, and WB assays showed that TvLEGU-1 is upregulated by iron at the protein level, localized on the trichomonad surface and in lysosomes and Golgi complex, bound to the surface of HeLa cells, and was found in vaginal secretions. Additionally, the IgG and Fab fractions of the anti-TvLEGU-1r antibody inhibited trichomonal cytoadherence up to 45%. Moreover, the Aza-Peptidyl Michael Acceptor that inhibited legumain proteolytic activity in live parasites also reduced levels of trichomonal cytoadherence up to 80%. In conclusion, our data show that the proteolytic activity of TvLEGU-1 is necessary for trichomonal adherence. Thus, TvLEGU-1 is a novel virulence factor upregulated by iron. This is the first report that a legumain-like CP plays a role in a pathogen cytoadherence.

1. Introduction

Trichomoniasis is one of the most common sexually transmitted infections worldwide caused by *Trichomonas vaginalis* [1]. Trichomonal adherence to host cells is a multifactorial process where adhesins and proteinases play important roles [2–8]. Proteinases are abundant in *T. vaginalis,* being reported more than 400 distinct proteinase genes in the draft of its genome. Up to 220 correspond to the cysteine type (CP) [9], but only 23 CPs have been detected by two-dimensional (2D) substrate gel electrophoresis [10], less were identified by recent proteomic studies [11–15], and only few CP genes have been cloned and characterized [15–22]. These gene products show homology to cathepsin L-like peptidases, which belong to the papain-like CP family of clan CA and to the legumain-like CP family of clan CD [23, 24].

The thiol proteinases of this parasitic protozoan have been implicated in a variety of biological events including nutrient acquisition [25], immune evasion [26, 27], and virulence [1, 4, 5, 7, 8, 22, 28–35]. The expression of some of these CPs is regulated by environmental factors such as pH and the redox state [1], polyamines [31], and iron [18, 20, 21, 30, 32, 35].

Iron is an essential nutrient for growth, metabolism, and virulence of *T. vaginalis* [36]. The environment of the human vagina, especially its nutrients and the iron concentration, is constantly changing throughout the menstrual cycle.

T. vaginalis may respond to varying iron concentrations by differential gene expression through poorly understood mechanisms [20, 21, 37] in order to survive, grow, and colonize the vaginal hostile environment.

We previously reported that some of the CPs of the 30-kDa region are involved in cytoadherence [4, 5, 7]. This region is formed by at least six spots with proteolytic activity that correspond to two distinct CP families: the papain-like family of clan CA, represented by four spots with pI between 4.5 and 5.5, and the legumain-like family of clan CD, represented by two spots with pI 6.3 and 6.5 [38] that are differentially regulated by iron at the transcript and proteolytic activity levels [21].

Among the ten legumain-like CP genes reported in the draft of the *T. vaginalis* genome [9], we have cloned and sequenced two cDNAs coding for the TvLEGU-1 and TvLEGU-2 precursor proteinases of 42.8- and 47.2-kDa. These CPs were classified within the asparaginyl endopeptidase (AE) subfamily of the family C13, belonging to the clan CD [38]. The family C13 of peptidases includes two distinct subfamilies with different functions, the glycosylphosphatidylinositol (GPI): protein transamidase and the asparaginyl endopeptidase. Interestingly, TvLEGU-1 and TvLEGU-2 share ~30% amino acid identity with the AE subfamily and ~26% with the GPI: protein transamidase subfamily [38]. We also showed that the amount of TvLEGU-1 transcript is positively regulated by iron, whereas the TvLEGU-2 mRNA is not affected by it [21]. Additionally, TvLEGU-1 is one of the most immunogenic proteinases detected by trichomoniasis patient sera [15].

Thus, the main goal of this work was to identify, characterize, and determine the function of TvLEGU-1. Our data show that TvLEGU-1 is a surface proteinase upregulated by iron, with affinity to the surface of HeLa cells that plays a major role in trichomonal cytoadherence. Hence, TvLEGU-1 is a novel virulence factor of *T. vaginalis* that is also released in vaginal secretions during infection.

2. Materials and Methods

2.1. Parasites and HeLa Cell Cultures. The fresh clinical *T. vaginalis* isolate CNCD 147 [7, 15, 29] was used in this study. Parasites were kept in culture at 37°C up to two weeks by daily passage in trypticase-yeast extract-maltose (TYM) medium [39] supplemented with 10% heat-inactivated horse serum (HIHS) (TYM-HIHS), containing ~20 μM iron [36]. Parasites in the logarithmic phase were grown either in iron-rich or in iron-depleted medium by the addition into the culture medium of 250 μM ferrous ammonium sulfate or 150 μM 2-2 dipyridyl (Sigma Co., St Louis, MO, USA) an iron-chelator, respectively, as previously reported [30]. HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% HIHS at 37°C for 48 h in a 5% CO_2 atmosphere to obtain confluent cell monolayers [6].

2.2. Generation of Antiserum against Recombinant TvLEGU-1. Rabbits were subcutaneously inoculated four times at two-week intervals with 0.3 mg of the affinity-purified TvLEGU-1r protein [15] in the presence of Freund's complete adjuvant (Gibco) for the first immunization. Booster injections were given in Freund's incomplete adjuvant (Gibco). The immune serum (anti-TvLEGU-1r) was obtained seven days after the last immunization [40]. This antiserum was used in western blot (WB) analysis, indirect immunofluorescence, and cytoadherence inhibition assays. Preimmune (PI) serum was obtained before the immunization schedule began and was used as a negative control in all the experiments with antibodies.

2.3. Papain Fragmentation of IgG to Fab. To obtain the Fab fragment, 0.5 mg/mL purified IgGs from the anti-TvLEGU-1r or PI serum [40, 41] in PBS pH 8.0 were digested with 0.2 mg/mL papain in digestion buffer (PBS pH 8.0 containing 0.02 M cysteine and 0.02 M EDTA) at 37°C for 6 h. The reaction was stopped with 0.3 M iodoacetamide in PBS pH 8.0. After digestion, samples were dialyzed in PBS pH 7.0 for 18 h at 4°C and incubated with protein A agarose during 2 h to eliminate the Fc fraction and recover the unbound Fab fragment [40].

2.4. Two-Dimensional Gel Electrophoresis (2DE). The 2DE for protease-rich extracts was performed as recently described [22]. Briefly, for the first dimension, supernatant from lysed parasites (6×10^7 cells/mL equivalent to 500 μg protein) in rehydration solution (Bio-Rad) was loaded onto a 7 cm Ready immobilized pH gradient (IPG) strips (linear pH gradient 4–7; Bio-Rad). IPG strips were actively rehydrated for 16 h at 4°C. Isoelectric focusing (IEF) of proteins was performed in three steps: 250 V for 20 min, 4 000 V for 3 h, and a gradual increase up to 10 000 V-h. For reduction and alkylation, strips were equilibrated in buffer I and II (Bio-Rad) for 10 min at room temperature each. Proteins were resolved by SDS-PAGE using 12% polyacrylamide gels, silver-stained, or transferred onto nitrocellulose (NC) membrane for WB detection. Gels and NC membranes were documented using the ChemiDoc-XRS (Bio-Rad) and analyzed using the Quantity One software (Bio-Rad). A tridimensional analysis using the PD Quest (Bio-Rad) and Melanie software was also performed for differentially expressed proteins. Three independent protein preparations were done, each obtained from an independent parasite culture, and similar results were observed.

2.5. Proteinase Identification. Identification of protein spots was performed at the Protein Unit of the Columbia University (NY, USA) as before [15]. Protein spots of interest were manually excised from silver-stained gels, distained, and prepared for in-gel digestion with trypsin. Resulting peptides were analyzed by MALDI-TOF mass spectrometry (MS) peptide mass mapping method on a Voyager DE pro-mass spectrometer in the linear mode (Applied Biosystems). Peptide masses were searched against the National Center for

Table 1: Peptides identified by MALDI-TOF-MS analysis of the three TvLEGU-1 protein spots of *Trichomonas vaginalis* detected by the anti-TvLEGU-1r antibody.

Peptide number[a]	Position[b] (aa)	Number in the sequence[c]	m/z (av)[d]	Spot 1[e]	Spot 2[e]	Spot 3[e]	Amino acid sequence[f]
1	13–27	I	1749.9186	−	+	+	FAVLIAGSNDFYNYR
2	28–41	II	1734.9719	+	+	+	HQADIFNMYQQLVK
3	41–66	III	2774.0025	−	+	+	GFDDQHITMMAYDDIALSSENPFR
4	42–66	IV	2930.1882	−	+	+	RGFDDQHITMMAYDDIALSSENPFR
5	75–84	V	1101.2126	−	+	+	HVNIYPGSSK
6	85–105	VI	2399.6528	−	+	+	INYAHNSVTADQFYTVLTTLK
7	144–151	VII	911.4059	+	+	−	AFDTMEAK
8	157–174	VIII	1994.1426	−	+	+	LFFGIEACYSGSVAAVFR
9	157–176	IX	2193.5226	+	+	+	LFFGIEACYSGSVAAVFRAK
10	232–248	X	1931.1107	−	+	+	AQTTGSHVCYYGDVNMK

[a] Consecutive number assigned to the identified peptides. [b] Position in amino acids (aa) residues of the identified peptides (start-end) in the aa sequence of the *T. vaginalis* TvLEGU-1 [38]. [c] Arbitrary nomenclature used to describe the ten identified peptides in *T. vaginalis* TvLEGU-1 (see Supplementary Figure 1S in Supplementary Material available online at doi:10.1155/2012/561979). [d] Peptide mass average m/z (av) identified by MALDI-TOF-MS after tryptic digestion of the three protein spots obtained from 2DE of protease-rich extracts from *T. vaginalis* grown in normal iron conditions (Figure 1). [e] Presence (+) or absence (−) of the peptides identified by MALDI-TOF-MS in the three TvLEGU-1 protein spots analyzed. [f] Amino acid sequence of the peptides obtained from a theoretical tryptic digestion of the deduced aa sequence of TvLEGU-1 [38] with identical masses to the experimental one (Supplementary Figure 1S).

Table 2: Densitometric analysis of the three TvLEGU-1 protein spots observed in silver-stained gels and WB NC membranes from parasites grown in high and low iron concentrations.

Spot number	Pixel intensity of silver stained spots		Pixel intensity of WB spots	
	High iron[a]	Low iron[b]	High iron[c]	Low iron[d]
1	292685.95	83473.87	93367.94	29862.21
2	637882.72	416358.36	296207.75	245016.26
3	1781948.40	1171066.01	842341.15	794637.06

[a] Densitometry values of the spots detected in the silver stained gel in high iron condition (H) of Figure 2(A). [b] Densitometry values of the spots detected in the silver stained gel in low iron condition (L) of Figure 2(A). [c] Densitometry values of the spots detected in the WB NC membranes in high iron condition (H) of Figure 2(A). [d] Densitometry values of the spots detected in the WB NC membranes in low iron condition (L) of Figure 2(A).

Biotechnology nonredundant database (NCBInr) using the MASCOT program (http://matrixscience.com/).

2.6. Western Blot Analysis (WB). Total trichomonad proteins, proteinase-rich extracts, proteins obtained after cell-binding assays from (2×10^7) parasites grown in iron-rich medium, obtained as before [7], and TCA-precipitated proteins present in vaginal washes (VWs) (100 μL) from patients with vaginitis (Table 2) were separated by SDS-PAGE using 10% polyacrylamide gels. Duplicated gels were transferred onto NC membranes for WB. The TvLEGU-1 proteinase was immunodetected with the anti-TvLEGU-1r rabbit serum (at 1 : 1000 to 1 : 40000 dilutions). As a quantity control, a monoclonal antibody against α-tubulin (Zymed Laboratories, South San Francisco, CA) (at 1 : 100 dilution) was used. WB was developed by chemiluminescence using the ECL-Plus kit (Amersham Co., Arlington Heights, IL, USA) and SuperSignal Femto Maximum Sensitivity Substrate kit (Pierce) and documented using the ChemiDoc-XRS (Bio-Rad).

To determine the presence of phosphorylations on TvLEGU-1, anti-phospho-Ser, -Tyr, and -Thr monoclonal antibodies at 1 : 500 dilution (Zymed) were used in 2DE-WB assays over NC membranes containing protease-rich extracts and developed by chemiluminescence. These experiments were performed at least three times with similar results.

2.7. In Vitro Secretion Kinetic Assay. The *in vitro* secretion assay was performed as previously described [22, 33]. Briefly, after 18 h of growth in iron-rich conditions, parasites were harvested, washed three times with PBS pH 7.0, and suspended in PBS-0.5% maltose at 1×10^6 cells/mL parasite density. Parasites were incubated for 15, 30, 60, and 90 min at 37°C, collected by centrifugation at 700 g, and supernatants were analyzed directly by substrate-gel electrophoresis and by WB after TCA-precipitation. The viability of trichomonads was assessed by trypan blue exclusion throughout the assay.

2.8. Indirect Immunofluorescence Assay. For confocal microscopy, parasites grown in iron-rich conditions were fixed with 4% paraformaldehyde for 1 h at 37°C, washed with PBS, and half of them were treated with 50 mM NH_4Cl/PBS pH 7.0 for 10 min, washed with PBS, and with 1 N HCL for 1 h and permeabilized with 0.2% Triton X-100 for 10 min. The other half was used as nonpermeabilized parasites. Permeabilized and nonpermeabilized parasites were blocked with 1% fetal bovine serum for 15 min and with 0.2 M glycine for 1 h at room temperature. Then,

trichomonads were incubated for 18 h at 4°C, with the anti-TvLEGU-1r or PI serum used as a negative control, both at 1 : 1 000 dilution. Parasites were incubated with the secondary antibody, fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulins (Pierce) at 1 : 200 dilution for 1 h at 37°C, washed, mounted with Vectashield mounting solution (Vector Laboratories), and visualized by confocal microscopy with a Leica LSM-SPC-5 Mo inverted confocal microscope fitted with HCXPLapo lambda blue 63 × 1.4 oil immersion lens. Time series were captured and processed using the confocal LAS AF software (Leica). Also, live HeLa cells were incubated with 10 μg/mL TvLEGU-1r or supernatant from an *in vitro* secretion assay for 30 min at 37°C, washed with PBS, fixed, blocked, and treated with antibodies as the parasites described above for immunofluorescence assays.

For lysosomal colocalization assays, the acidic compartments of *T. vaginalis* were stained with 1 μM LysoTracker RED DND-99 (Invitrogen) for 12 h at 37°C in TYM medium supplemented with 10% heat-inactivated horse serum. After that, parasites were processed for indirect immunofluorescence with the anti-TvLEGU-1r antibody as described in the previous paragraph.

For immunogold labeling assays, parasites were fixed overnight at room temperature in 0.5% glutaraldehyde, 4% formaldehyde in 0.1 M cacodylate buffer. Afterwards, cells were dehydrated in ethanol and embedded in Unicryl. Ultrathin sections were harvested on 300 mesh nickel grids. The samples were washed and incubated with 50 mM ammonium chloride for 30 min in order to quench free aldehyde groups. The sections were incubated in a series of blocking solutions (PBS containing 1% bovine albumin (BSA), 3% PBS/BSA, and 0.2% Tween-20, pH 8.0) for 10 min on each step. Cells were incubated with the anti-TvLEGU-1r antibody at 1 : 50 dilution, overnight. After several washes in 1% PBS/BSA, the sections were incubated with 10 nm gold-labeled goat anti-rabbit IgG (BB International, UK). As control some samples were incubated only with the secondary antibody. Finally, sections were stained with 5% uranyl acetate and 1% lead citrate and then observed with a JEOL 1210 transmission electron microscope.

2.9. Cell-Binding Assay for Proteinases. To detect the affinity of the native and recombinant TvLEGU-1 proteins to the surface of host cells, we performed cell-binding assays as previously described [7]. Briefly, a clarified detergent extract from 2×10^7 parasites or 25 μg of TvLEGU-1r was incubated for 18 h at 4°C with 1×10^6 glutaraldehyde-fixed HeLa cells. The native and recombinant TvLEGU-1 proteins bound to the surface of fixed-HeLa cells were eluted with Laemmli sample buffer for 20 min at 37°C. The released proteins were analyzed by SDS-PAGE and blotted onto NC for WB detection with the anti-TvLEGU-1r antibody.

2.10. Cytoadherence Inhibition Assay. Cytoadherence inhibition assays were performed over confluent HeLa cell monolayers on 96-well microtiter plates as previously described [7, 42]. Briefly, [^{3}H]-thymidine-labeled parasites were incubated for 30 min at 4°C with 0, 50, and 100 μg/mL IgG or Fab fraction from the anti-TvLEGU-1r or PI serum before interaction with HeLa cell monolayers.

The cytoadherence inhibition assays with proteinase inhibitors were performed over confluent live HeLa cell monolayers on 12 mm coverslips as recently described [41]. Briefly, cell monolayers (5×10^5 cells/coverslip) were incubated with live parasites (1×10^6 cells/well) previously labeled with 25 mM CellTracker Blue CMAC (Molecular Probes) in serum-free DMEM-TYM (2 : 1) medium and incubated at 37°C for 30 min and 5% CO_2. For inhibition experiments before interaction with HeLa cell monolayers labeled parasites were incubated for 20 min at 4°C with different CP inhibitors (1 mM TLCK, 0.2 mM leupeptin, or 0.18 mM E-64; all purchased from Sigma) used as controls. The Aza-Peptidyl Michael Acceptor (Mu-Ala-Ala-AAsn-CH=CH–CON, kindly donated by Dr. James Powers), a specific inhibitor for legumains [43], was also used at 5, 10, and 50 μM. After interaction with CP inhibitors parasites were washed and added to HeLa cell monolayers. After the interaction, the coverslips were washed with warm PBS, fixed with 4% paraformaldehyde, and mounted on slides. Each condition was performed in triplicate, and ten fields with a 40x magnification were analyzed per coverslip. Fluorescent parasites adhered to host cells (in blue) were counted using an Eclipse 80i epifluorescence microscope (Nikon) and the NIS-Elements BR 2.1 software (Nikon) (Table 3). The experiment was repeated at least two independent times with similar results.

2.11. Measurement of the Proteolytic Activity of Live Parasites. To detect the proteolytic activity of live trichomonads, 2.5×10^5 parasites were incubated for 20 min at 4°C with 1 mM TLCK, 0.2 mM leupeptin, 0.18 mM E-64, or 50 μM Aza-Peptidyl Michael Acceptor [43]. The activity was measured with two CPs substrates (Z-Phe-Arg-AMC for papains; and Cbz-Ala-Ala-AAsn-AMC for legumains). Release of free 7-amino-4-methylcoumarin (AMC) was measured by emission at excitation wavelengths of 355 and 460 nm, respectively, in a luminometer (BioTek) using a Gen5 2.0 Data Analysis Software. The linear regression of the substrate hydrolysis curves was used to calculate initial velocities. The experiment was repeated at least three independent times with similar results. The viability of trichomonads was assessed by trypan blue exclusion throughout the assay.

2.12. Statistical Analysis. The statistically significant difference between means was determined by analysis of variance (ANOVA) using GraphPad Prism 5.0. The data were analyzed by one-way ANOVA using the Bonferroni method comparing all pairs of columns ($P < 0.001$) for Figures 7(A), 7(B), 8(B), 8(C), and 8(D). The scores showing statistical significance are indicated in the figures with asterisks. The corresponding P values are indicated in the figure legends.

Table 3: The average number of *T. vaginalis* parasites attached to HeLa cell monolayers per coverslip with or without treatment using different proteinase inhibitors (Figure 8(B)).

Treatment	Parasites attached	Inhibition (%)
None[a]	1450	0.0 ± 13.9176
MA (5 μM)[b]	742	46.33 ± 7.7764
MA (10 μM)	556	60.78 ± 5.6311
MA (50 μM)	301	77.23 ± 5.2907
TLCK (1 mM)	622	57.05 ± 6.6416
Leupeptin (0.2 mM)	898	36.79 ± 6.1359
E-64 (0.18 mM)	662	50.16 ± 8.1047

[a]None corresponds to the control parasites without treatment with CP inhibitors. The number of parasites attached to the HeLa cell monolayer was taken as 100% adherence for comparative purpose. [b]MA corresponds to the Aza-Peptidyl Michael Acceptor, a legumain-specific inhibitor. These differences were statistically significant with a $P < 0.001$ (Figure 8(B)).

3. Results

3.1. Identification of the TvLEGU-1 Proteinase. To identify the TvLEGU-1 CP in *T. vaginalis* proteinase-rich extracts polyclonal antibodies were produced against the recombinant TvLEGU-1 protein (anti-TvLEGU-1r) previously cloned and expressed in *Escherichia coli* [15]. By WB assays, the anti-TvLEGU-1r antibody reacted with the recombinant TvLEGU-1 protein used as antigen (Figure 1(A)). It also recognized a 30-kDa band in *T. vaginalis* protease-rich trichomonad extracts and two bands of 30- and 20-kDa in *T. vaginalis* total protein extracts of parasites grown in normal iron conditions. A light band of 60-kDa was also observed (Figure 1(B)). As expected, the PI serum used as a negative control had no reaction (Figure 1).

To confirm the identity of the protein spots corresponding to TvLEGU-1 in a protease-rich trichomonad extract (dubbed "active degradome") [15], parasites grown in normal iron conditions were analyzed by 2DE and WB assays. The anti-TvLEGU-1r antibody recognized three spots in the 30-kDa region with pI ~6.3, ~6.5, and ~6.7 (Figure 1(C)). These protein spots were identified by MALDI-TOF MS analysis as TvLEGU-1 proteins; ten of the 25 peptides obtained by tryptic digestion of the three spots had identical masses to TvLEGU-1 peptides. The MS analysis showed that protein spots 1, 2, and 3 were identified with MASCOT scores of 72, 85, and 150, respectively, and sequence coverage of 14, 33, and 31%, respectively; reinforcing their identification as part of the TvLEGU-1 protein (Figure 1; Table 1; Supplementary Figure 1S).

3.2. TvLEGU-1 Is Phosphorylated and Upregulated by Iron. To investigate whether phosphorylation could be an explanation for the three protein spots with the same size but distinct pI identified as TvLEGU-1 in 2DE WB assays, protease-rich extracts from parasites grown in normal iron concentrations were analyzed by 2DE WB assays with antiphospho-Ser, -Thr, and -Tyr antibodies. Figure 1(D) shows that while none of the protein spots have phosphorylation in Ser residues, the protein spot 1 did not show either in Thr or Tyr residues, the protein spots 2 and 3 showed in Thr, and only spot 3 in Tyr residues. Interestingly, the intensity of the protein spot 3 was greater with the anti-Tyr than with the anti-Thr antibody (Figures 1(D) and 1(E)). Thus, the three protein spots of TvLEGU-1 are isoforms with distinct type and degree of phosphorylation, as was predicted in its amino acid sequence [38].

To check the effect of iron at the protein expression level of TvLEGU-1, protease-rich extracts from parasites grown in iron-rich and iron-depleted conditions were analyzed by silver-stained 2DE and by 2DE WB. A densitometric analysis was also performed (Table 2). Figure 2 shows that the three protein spots in the 30-kDa region are present in both iron conditions but protein spot 1 with less intensity in iron-depleted than in iron-rich parasites (Figure 2(A)). These differences are well appreciated in the densitometric analysis (Figure 2(B), Table 2). Furthermore, the surface localization of TvLEGU-1 in nonpermeabilized parasites grown in different iron concentrations was explored. The data show that TvLEGU-1 surface localization is positively modulated by iron (Figure 3). Only a light surface localization of TvLEGU-1 was observed in iron-depleted parasites. These data together with the 2D WB results suggest that the major differences of this protein due to the iron concentration could be observed at its surface localization. Therefore, the rest of the experiments involving the anti-TvLEGU-1r antibody were performed with parasites grown in iron-rich conditions, except when indicated.

3.3. TvLEGU-1 Is Localized in the Cytoplasm and on the Surface of T. vaginalis. To explore the total real distribution of TvLEGU-1 in trichomonads immunofluorescence assays were performed using the anti-TvLEGU-1r antibody with fixed nonpermeabilized or permeabilized parasites grown in iron-rich condition and analyzed by confocal microscopy. Figure 4 shows that fluorescence with the anti-TvLEGU-1r antibody (in green) was detected on the surface, colocalizing with the membrane marker (Dil, in red; panels e–h) and in the cytoplasm of trichomonad parasites (panels i–l) as compared with the PI serum used as a negative control (panels a–d). A very interesting labeling in the Golgi complex and vesicles that could be lysosomes was observed in the cytoplasmic localization of TvLEGU-1.

To explore the hypothesis that TvLEGU-1 is also a lysosomal CP, we performed colocalization assays using

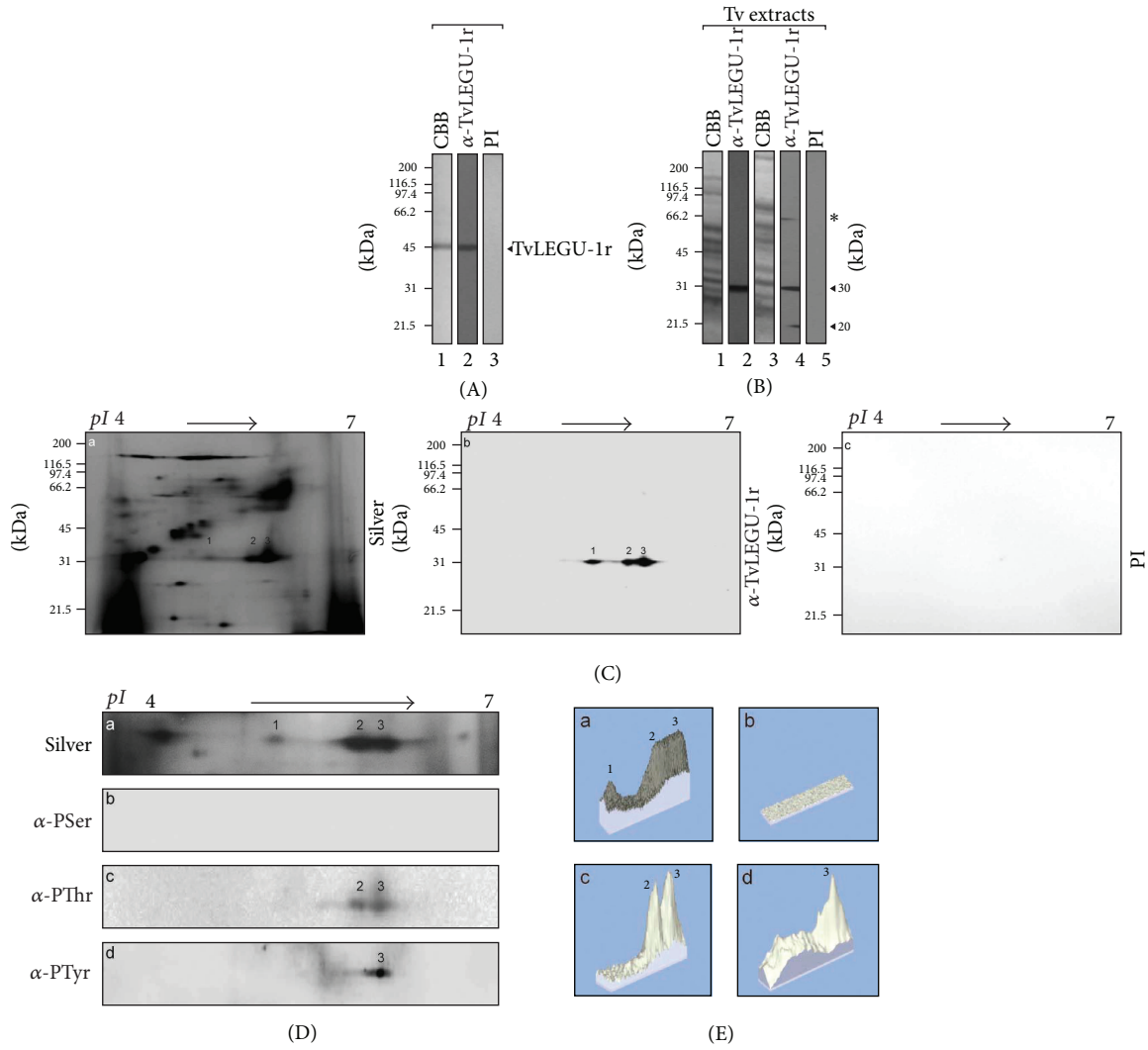

Figure 1: Recognition of TvLEGU-1 by the anti-TvLEGU-1r antibody in total protein extracts and protease-rich extracts of *T. vaginalis*. (A) Coomassie brilliant blue-stained purified recombinant TvLEGU-1r protein (CBB; lane 1). WB assays of TvLEGU-1r incubated with the anti-TvLEGU-1r antibody (lane 2) or with the PI serum (lane 3) used as a negative control, both at 1 : 40000 dilution. Arrowhead points to the recombinant protein band TvLEGU-1r (~46-kDa). kDa, molecular weight markers in kilodaltons (Bio-Rad). (B) Coomassie brilliant blue-stained protein patterns of trichomonad protease-rich (lane 1) or total protein extracts (lane 3); WB assays of duplicated gels transferred onto NC membranes containing trichomonad protease-rich (lane 2) or total protein extracts (lane 4) incubated with the anti-TvLEGU-1r antibody, or with the PI serum (lane 5) used as a negative control, both at 1 : 20000 dilution. Arrowheads show the position of the native TvLEGU-1 (~30- and ~20-kDa) proteins. Asterisk shows a light protein band of ~60-kDa also detected by the anti-TvLEGU-1r antibody. kDa, molecular weight markers in kilodaltons (Bio-Rad). (C) Silver-stained 2DE protease-rich extracts from parasites grown in regular medium (panel a). WB of duplicate gels transferred onto NC membranes incubated with the anti-TvLEGU-1r antibody (panel b) or with the PI (panel c) serum both at 1 : 5000 dilution. Numbers 1–3 show the position of the TvLEGU-1 protein spots. (D) Differential phosphorylation of TvLEGU-1. 2DE WB assays from duplicate protease-rich extracts from part (C) separated by SDS-PAGE were (a) silver-stained or transferred onto NC membranes and incubated with the (b) antiphosphoserine (α-PSer), (c) antiphosphothreonine (α-PThr), and (d) antiphosphotyrosine (α-PTyr) monoclonal antibodies. pI, direction of IEF using IPG Ready-strips (linear gradient of pH 4–7; Bio-Rad). Numbers show the position of the TvLEGU-1 spots. (E) Landscape representation of the densitometric analysis (PDQuest and Melanie software) of the protein spots corresponding to TvLEGU-1. Numbers 1–3 show the position of the TvLEGU-1 spots.

LysoTracker as a lysosomal marker in addition to the anti-TvLEGU-1r antibody. Figure 5 shows that indeed TvLEGU-1 colocalized (~60%) with the lysosomal marker in iron-rich parasites, as could be expected for legumain-like CPs (Figure 5(A)). Moreover, immunogold localization assays confirmed the cytoplasmic localization of TvLEGU-1 in vacuoles/lysosomes containing degrading material and in the Golgi complex (Figure 5(B)), suggesting that this is an excreted/secreted proteinase. These data suggest that TvLEGU-1 could have multiple functions that will depend on its cellular localization possible modulated by the iron concentrations.

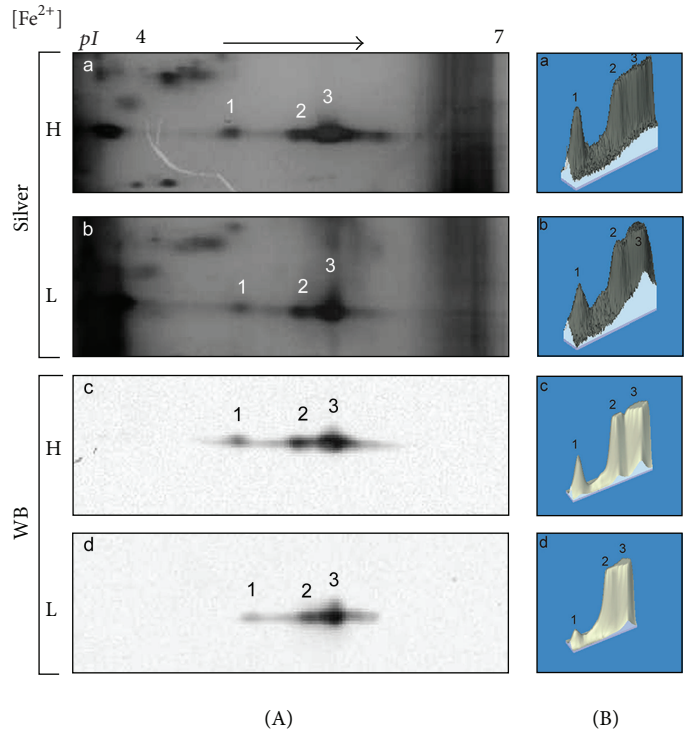

Figure 2: Iron effect on the protein expression of TvLEGU-1. (A) Silver-stained 2DE pattern of protease-rich extracts, corresponding to the 30-kDa region from (a) parasites grown in iron-rich (H) or (b) iron-depleted (L) conditions. (c) and (d) 2DE WB assays of duplicated gels (a) and (b) transferred onto NC membranes and incubated with the anti-TvLEGU-1r antibody (1 : 5000 dilution). pI, direction of IEF using IPG Ready-strips (linear gradient of pH 4–7; Bio-Rad). Numbers 1–3 show the position of the distinct TvLEGU-1 protein spots. (B) Landscape representation of the densitometric analysis of the three TvLEGU-1 protein spots of (a) and (b) silver-stained gels and (c) and (d) WB shown in (A) was carried out with the Melanie and PDQuest (Bio-Rad) programs. Numbers 1–3 correspond to the protein spots of TvLEGU-1.

3.4. TvLEGU-1 Binds to the Surface of HeLa Cells. To determine whether TvLEGU-1 binds to the surface of HeLa cells, cell-binding and WB assays were performed with protease-rich extracts from parasites grown in iron-rich medium and fixed HeLa cells. WB assays showed that the anti-TvLEGU-1r antibody reacted with the trichomonad 30-kDa band that bound to fixed HeLa cells (Figure 6(A)), suggesting the presence of TvLEGU-1. This was confirmed in a cell-binding assay using the recombinant TvLEGU-1 protein that also bound to the surface of fixed HeLa cells (Figure 6(B)), whereas the bovine serum albumin (BSA) used as a negative control did not bind as expected (Figure 6(B)). Additionally, TvLEGU-1r was recognized by the antinative CP30 antibody [7] in WB assays (Figure 6(C)). Together, these data show that TvLEGU-1 is one of the CP30 proteinases that interact with the surface of HeLa cells [7].

Furthermore, to confirm it, immunofluorescence assays using fixed and live HeLa cells incubated with the TvLEGU-1r protein and the anti-TvLEGU-1r antibody were performed. Confocal microscopy images showed that indeed TvLEGU-1r bound to the surface of fixed and live HeLa cells, whereas HeLa cells directly incubated with the anti-TvLEGU-1r antibody used as a negative control had no reaction as expected (Figures 6(D) and 6(E)).

3.5. TvLEGU-1 Participates in T. vaginalis Cytoadherence. To study the role of TvLEGU-1 in trichomonal adherence, we performed adherence inhibition assays over HeLa cell monolayers by preincubating [^{3}H]-thymidine-labeled iron-rich parasites with varied concentrations of the anti-TvLEGU-1r IgG or Fab fractions. Figure 7 shows that the anti-TvLEGU-1r antibody inhibited the levels of *T. vaginalis* adherence to HeLa cell monolayers in a concentration-dependent manner. A maximum inhibition of ~45%, using 100 μg/mL of IgGs or Fab fractions, was observed. IgGs or Fab fractions from PI serum used as a negative control did not affect trichomonal cytoadherence. These results illustrate that TvLEGU-1 is a virulence factor that plays a role in cellular attachment as one of the 30-kDa CPs required for trichomonal adherence [7].

3.6. TvLEGU-1 Proteolytic Activity Is Necessary for T vaginalis Cytoadherence. To determine whether TvLEGU-1 proteolytic activity was required for cellular attachment, live nonradioactive-labeled parasites [41] were treated with distinct CP inhibitors (TLCK, leupeptin, or E-64) or with increasing concentrations (0, 5, 10, and 50 μg/mL) of a specific legumain inhibitor the Aza-Peptidyl Michael Acceptor (Mu-Ala-Ala-AAsn-CH=CH–CON) [43] before interaction with live HeLa cell monolayers. Figures 8(A) and 8(B)

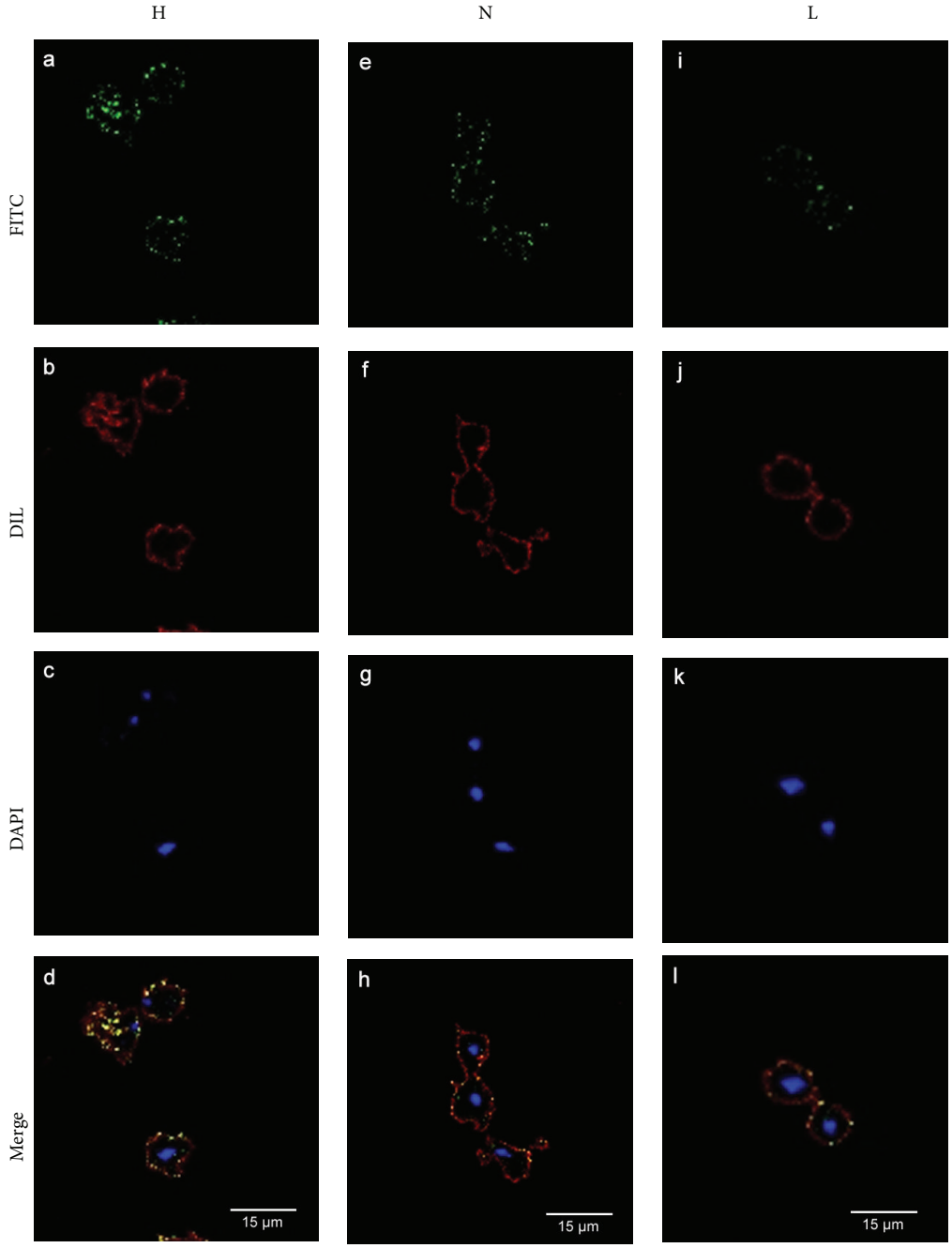

Figure 3: The TvLEGU-1 surface localization on *T. vaginalis* is affected by iron. Parasites grown in iron-rich (H; a, b, c, and d), normal (N; e, f, g, and h), and iron-depleted (L; i, j, k, and l) conditions, fixed and nonpermeabilized were incubated with the anti-TvLEGU-1r antibody (1 : 100 dilution). Anti-rabbit IgG-FITC (in green) was used as a secondary antibody (1 : 100 dilution) (a, e, and i). Parasite membranes were labeled with DIL (in red; b, f, and j). Nuclei were labeled with DAPI (in blue; c, g, and k). Merge (d, h, and l) in yellow indicates colocalization. Bars: 15 μm (d, h, and l).

show that the specific legumain inhibitor decreased the levels of *T. vaginalis* adherence to HeLa cell monolayers in a concentration-dependent manner up to ~80%, whereas TLCK, leupeptin, and E-64 inhibited ~60, ~40, and ~50%, respectively. The average number of parasites without treatment attached to HeLa cells per coverslip was higher than the number of parasites treated with inhibitors (Table 3), and these differences were statistically significant $P < 0.001$ (Figure 8(B)). These results suggest that both legumain and papain-like CP proteolytic activities are necessary for trichomonal cytoadherence, especially the legumain-like activity. These data are consistent with previous reports [4].

To check the effect of these inhibitors over the CP proteolytic activity of live parasites fluorescent substrates for

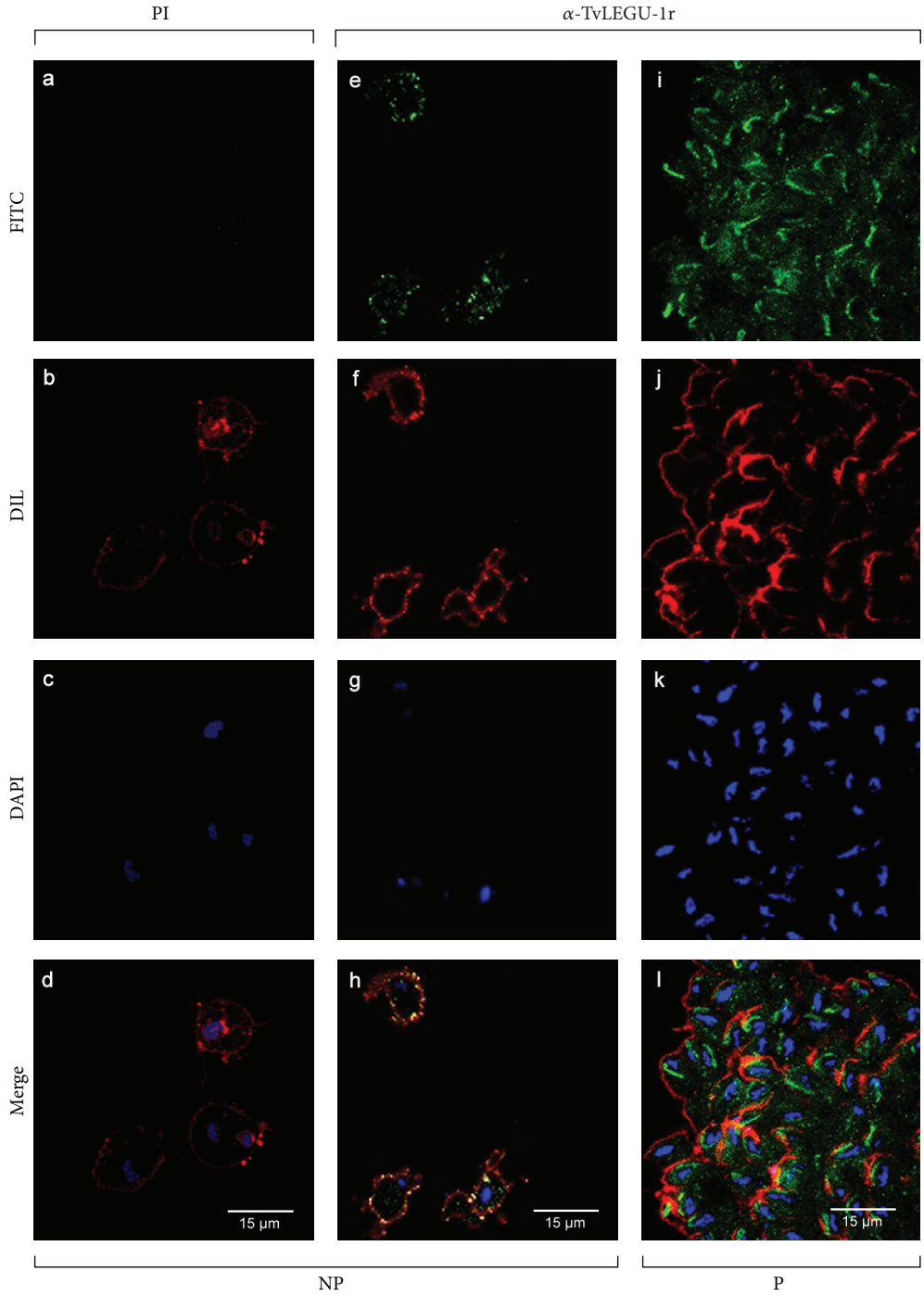

Figure 4: The TvLEGU-1 protein is localized on the surface and cytoplasm of *T. vaginalis*. Parasites grown in iron-rich conditions, fixed nonpermeabilized (NP) were incubated with the PI serum, (1 : 100 dilution) as a negative control (a, b, c, and d). Nonpermeabilized (NP; e, f, g, and h) and permeabilized (P; i, j, k, and l) parasites were incubated with the anti-TvLEGU-1r antibody (1 : 100 dilution). Anti-rabbit IgG-FITC (in green) was used as a secondary antibody (1 : 100 dilution) (a, e, and i). Parasite membranes were labeled with DIL (in red; b, f, and j). Nuclei were labeled with DAPI (in blue; c, g, and k). Merge (d, h, and l) in yellow indicates colocalization. Bars: 15 μm (d, h, and l).

papain-like (Z-Phe-Arg-AMC) and legumain-like (Cbz-Ala-Ala-AAsn-AMC) CPs were used. Live untreated parasites used as control showed proteolytic activity for both substrates, which were taken as 100% activity. Parasites treated with the specific legumain inhibitor (Aza-Peptidyl Michael Acceptor) abolished the legumain-like proteolytic activity (Figure 8(C)) and has no effect on the papain-like proteolytic activity (Figure 8(D)). TLCK, a potent inhibitor of papain-like and legumain-like CPs, greatly reduced both proteolytic activities (~80% and ~95%, resp.) of treated parasites as

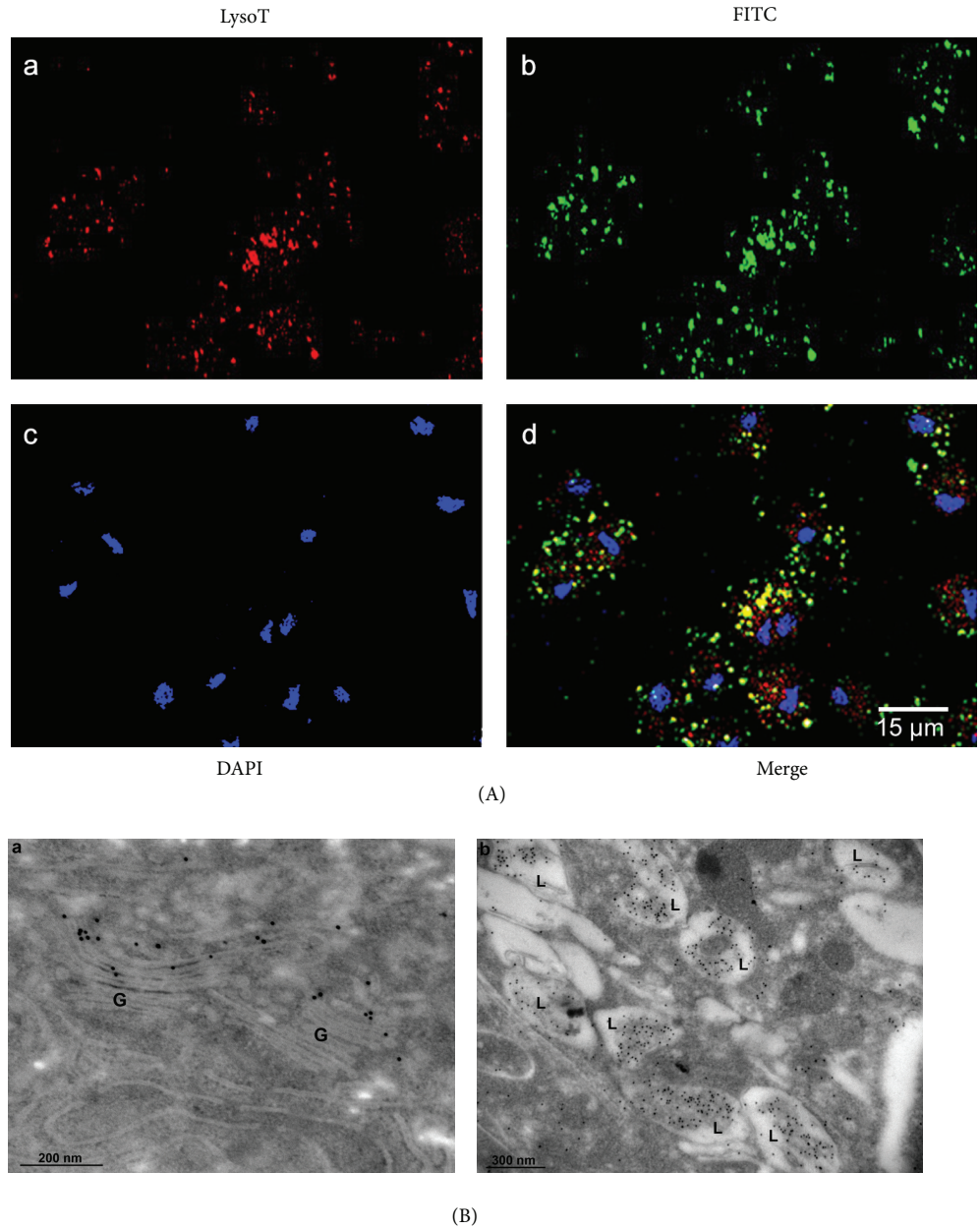

Figure 5: TvLEGU-1 is also localized in lysosomes and Golgi complex of *T. vaginalis*. (A) Parasites grown in iron-rich and labeled with LysoTracker were fixed, blocked, and incubated with the anti-TvLEGU-1r antibody (1 : 100 dilution) and a secondary antibody coupled to FITC stained in green; lysosomes were stained in red with LysoTracker, LysoT; and nuclei in blue with DAPI. The samples were analyzed by confocal microscopy. (B) Immunogold labeling of thin cryosections of parasites using the anti-TvLEGU-1r antibody at 1 : 100 dilution. The samples were analyzed by transmission electron microscopy. The labeling is observed on (a) Golgi complex (G) and (b) vesicles similar to lysosomes (L). Bars: 200 nm and 300 nm, respectively.

expected (Figures 8(C) and 8(D)). E-64 and leupeptin, potent inhibitors of papain-like CPs, greatly reduced the papain-like proteolytic activity (between ~80 to ~90%) of treated parasites (Figure 8(D)) and had a minimal or no effect on the legumain-like proteolytic activity (Figure 8(C)) of live parasites. Therefore, both types of CP proteolytic activity are present in live parasites and are necessary for trichomonal adherence to host cells (Figure 8).

3.7. The TvLEGU-1 Proteinase Is Expressed during Infection and Is Present in Vaginal Secretions of Patients with Trichomoniasis. To investigate the relevance of TvLEGU-1 during trichomonal infection, we analyzed vaginal washes from vaginitis patients with [Tv (+)] or without [Tv (−)] *T. vaginalis* (Table 4) for the presence of TvLEGU-1 by TCA-precipitation and WB assays using the anti-TvLEGU-1r antibody. Figure 9 shows that the anti-TvLEGU-1r antibody

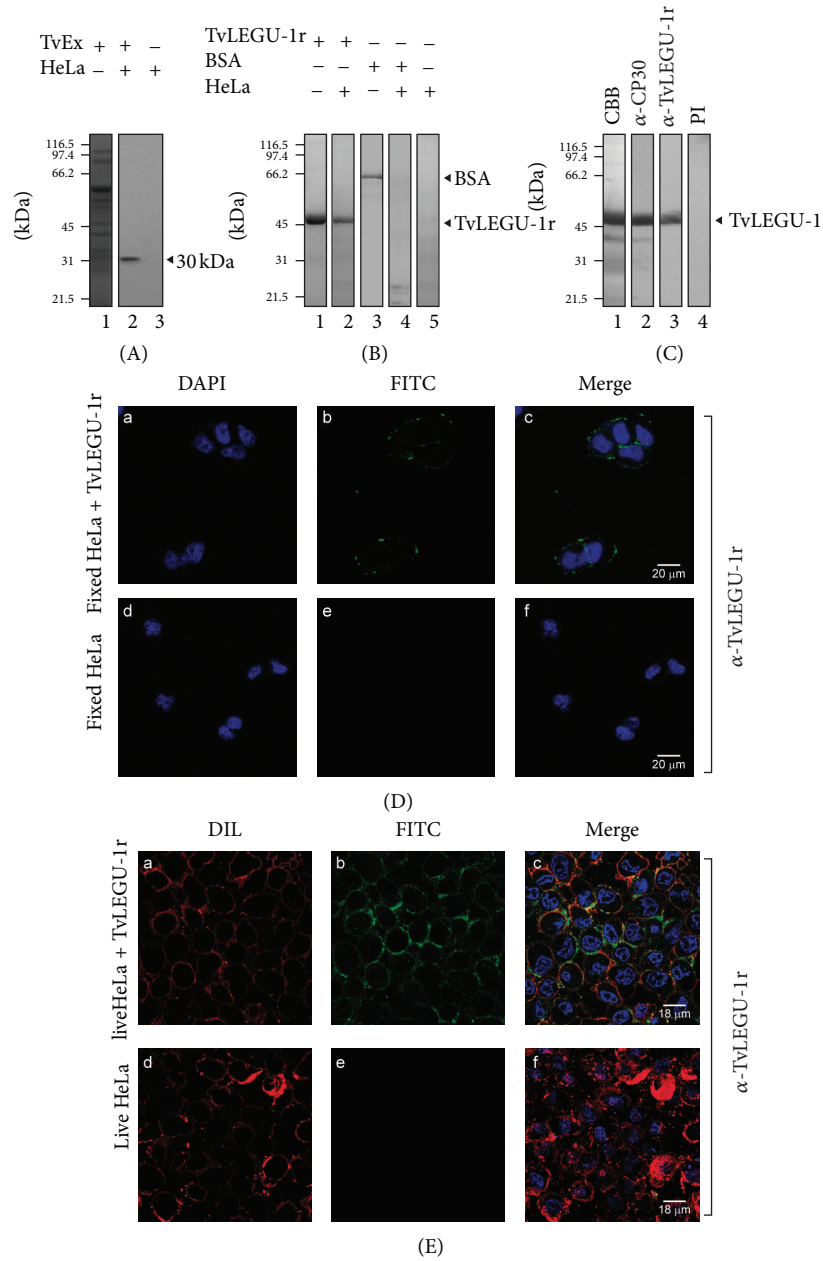

Figure 6: TvLEGU-1 binds to the surface of fixed and live HeLa cells. (A) Coomassie brilliant blue-stained protease-rich extracts from parasites grown in iron-rich conditions (lane 1). WB assay of eluted proteinases after cell-binding assay of protease-rich extracts with fixed HeLa cells (lane 2) or mock HeLa cells (lane 3) incubated with the anti-TvLEGU-1r antibody (1 : 1 000 dilution). (B) The TvLEGU-1r protein interacts with fixed HeLa cells. Coomassie brilliant blue-stained TvLEGU-1r protein (lane 1) and TvLEGU-1r protein eluted after cell-binding assays with fixed HeLa cells (lane 2). Coomassie brilliant blue-stained bovine serum albumin (BSA) (lane 3) and BSA eluted after cell-binding assays with fixed HeLa cells (lane 4) used as a specificity control; mock experiment with fixed HeLa cells (lane 5). (C) Recognition of TvLEGU-1r by the anti-CP30 antibody. Coomassie brilliant blue-stained TvLEGU-1r (lane 1); WB assay of the TvLEGU-1r protein incubated with the anti-CP30 (1 : 5 000 dilution) [7] or the anti-TvLEGU-1r antibody (1 : 10000 dilution), or PI serum (1 : 1 0000 dilution) used as a negative control (lanes 2, 3, and 4, resp.); kDa, molecular weight markers in kilodaltons. Protein bands were visualized by SDS-PAGE on 10% polyacrylamide gels. Arrowheads show the position of TvLEGU-1 (A), BSA (B), or TvLEGU-1r ((B) and (C)) proteins. (D) Confocal microscopy images after immunofluorescence assays using the anti-TvLEGU-1r antibody with fixed HeLa cells incubated with the TvLEGU-1r protein (a, b, and c). Fixed HeLa cells directly incubated with the anti-TvLEGU-1r antibody were used as negative controls. Conjugated anti-rabbit IgG-FITC was used as a secondary antibody (1 : 100 dilution) (b and e). Nuclei stained with DAPI (a and d). Merge and bars: 20 μm (c and f). (E) Confocal microscopy images after immunofluorescence assays using the anti-TvLEGU-1r antibody with live HeLa cells incubated with the TvLEGU-1r protein (a, b, and c). Live HeLa cells were directly incubated with the anti-TvLEGU-1r antibody and used as negative controls (d, e, and f). Conjugated anti-rabbit IgG-FITC was used as a secondary antibody (1 : 100 dilution) (b and e). Parasite membranes were labeled with DIL (a and d). Nuclei labeled with DAPI, merge, and bars: 18 μm (c and f).

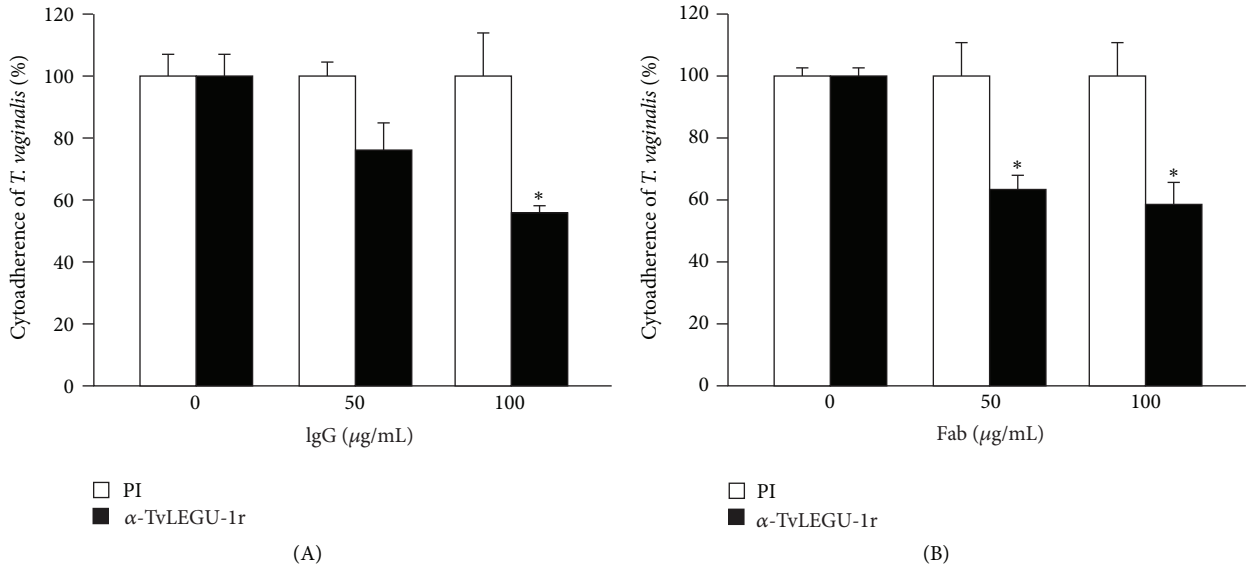

Figure 7: TvLEGU-1 participates in *T. vaginalis* cytoadherence. For cytoadherence inhibition experiments, [^{3}H]-thymidine-labeled iron-rich parasites (1×10^6 cell/mL) were incubated for 20 min at 4°C with different concentrations (0, 50, and 100 μg/mL) of the IgG (A) and Fab (B) fractions from the anti-TvLEGU-1r or PI serum before interaction with HeLa cell monolayers. (A) Cytoadherence inhibition with IgG fractions from the anti-TvLEGU-1r (black bar) or PI (white bar) serum. (B) Cytoadherence inhibition with Fab fractions from the anti-TvLEGU-1r (black bar) or PI (white bar) serum. Each bar is the mean of the percentage of cytoadherence of triplicate samples; error bars represent the standard deviations of three experiments in triplicate with similar results. $^*P < 0.05$ is the significance of the difference between 100 μg/mL IgG fractions of the control PI serum and the anti-TvLEGU-1r serum. $^*P < 0.001$ is the significance of the difference between 50 or 100 μg/mL Fab fractions of the control PI serum and the anti- TvLEGU-1r serum.

detected the presence of protein bands that ranged from 35- to ~30-kDa in Tv (+) VWs (Figure 9(A)), but none in those Tv (−) with other vaginitis (Figure 9(B)), used as negative controls. These data illustrate that the TvLEGU-1 is expressed and might be released during infection.

To confirm this, *in vitro* secretion kinetic assays were performed (0, 15, 30, 60, and 90 min). Zymograms of the supernatants showed a 30-kDa band with proteolytic activity released through time (Figure 9(C)) from parasites that exhibited ~95 to ~99% viability measured by trypan blue exclusion assays. WB assays of the TCA-precipitated supernatants using the anti-TvLEGU-1r antibody confirmed the presence of TvLEGU-1 among the released proteins and its amount increased through time. The anti-α-tubulin antibody used as a negative control gave no reaction as expected, suggesting that no significant parasite lysis occurred.

Moreover, immunofluorescence assays were also performed using the anti-TvLEGU-1r antibody with cells obtained from Tv (+) VWs and with live HeLa cells incubated with TvLEGU-1-containing parasite supernatants from the *in vitro* secretion assays (Figure 9(C)). The confocal microscopy images showed that endogenous TvLEGU-1 decorates the surface of live HeLa cells and cells obtained from Tv (+) VWs (Figure 9(D)). These data show that TvLEGU-1 is part of the excretion/secretion products from live trichomonads that also bound to the surface of cells present in vaginal secretions (Figure 9).

4. Discussion

Numerous thiol-proteinases, including cathepsin L- and legumain-like proteinases, are encoded in the *T. vaginalis* genome. However, few have been characterized at either the molecular, biochemical, or functional level. Understanding the role of CPs, especially those of the legumain-like type in this parasite, is relevant, as they are known to be involved in numerous biological processes including the host-parasite interplay.

In this study, we identified and characterized one of the ten legumain-like proteinases described in the draft of the *T. vaginalis* genome sequence [9], TvLEGU-1 [38], that showed multiple localizations, in the lysosomes and Golgi complex when in the cytoplasm and also at the parasite surface in the presence of iron (Figures 3–5). We confirmed its positive iron regulation [21] at the protein level (Figure 2) and surface localization (Figures 3 and 4). Moreover, we demonstrated its role in trichomonal adherence (Figures 7 and 8) and its presence in vaginal secretions during trichomonal infection (Figure 9).

The three protein spots in the 30-kDa region, with distinct pI identified by 2DE WB and MS as TvLEGU-1 (Figure 1, Table 1), are in agreement with the protein spots identified as TvLEGU-1 in the trichomonad active degradome [15]. These represent isoforms with a different type and level of phosphorylation (Figure 1(D)) as previously suggested [38]. These findings are consistent with

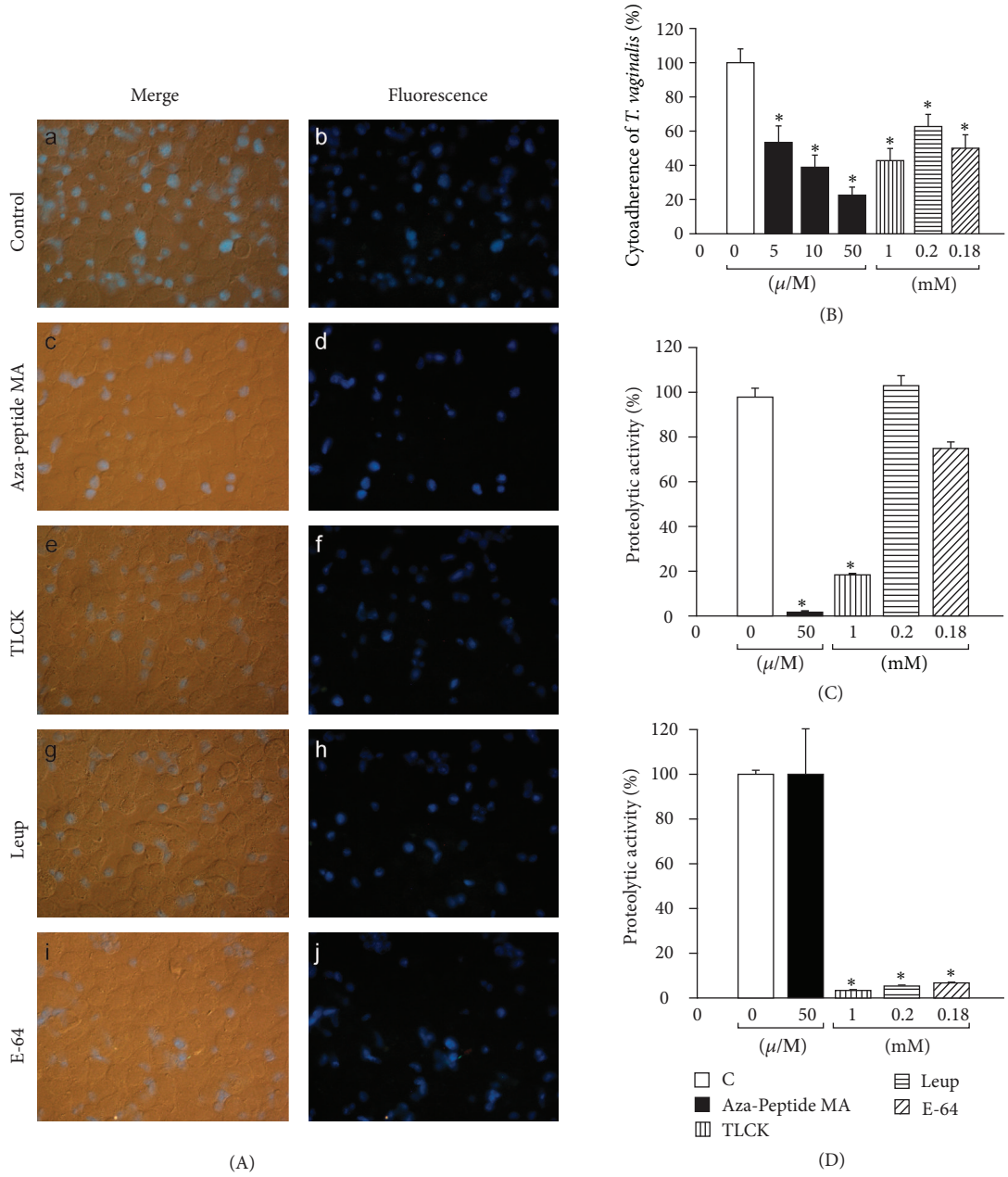

Figure 8: Effect of distinct CP inhibitors in trichomonal cytoadherence ((A) and (B)) and proteolytic activity of live parasites ((C) and (D)). (A) Fluorescence microscopy of a representative cytoadherence inhibition assay (over live HeLa cell monolayers) of fluorescence-labeled parasites pretreated with different CP inhibitors. Panels a and b show parasites without treatment (100% adherence). Panels c and d correspond to parasites treated with 50 μM legumain inhibitor Aza-Peptide Michael Acceptor (Mu-Ala-Ala-AAsn-CH=CH-CON). Panels e and f show parasites treated with 1 mM TLCK. Panels g and h, parasites treated with 0.2 mM Leupeptin (Leup). Panels i and j, parasites treated with 0.18 mM E-64. (B) Data from the fluorescent parasites of the cytoadherence inhibition assay show the percentage of *T. vaginalis* bound to HeLa cell monolayers in the absence (0, used as a control) or presence of distinct concentrations of CP inhibitors described in (A). Each bar is the mean of the percentage of triplicate samples; error bars represent the standard deviations of two experiments in triplicate with similar results. $^*P < 0.001$ is the significance of the difference between the control and the distinct treatments. (C) Proteolytic activity of live trichomonads over legumain substrate (Cbz-Ala-Ala-AAsn-AMC). Live parasites were incubated with the same inhibitors previously described in (A), and the released fluorescence from the legumain substrate was measured in a fluorometer. Each bar is the mean of the percentage of triplicate samples; error bars represent the standard deviations of three experiments in triplicate with similar results. $^*P < 0.001$ is the significance of the difference between the control and the distinct treatments. (D) Proteolytic activity of live trichomonads over papain substrate (Z-Phe-Arg-AMC). Live parasites were incubated with the same inhibitors previously described in (A), and the released fluorescence from the papain substrate was measured in a fluorometer. Each bar is the mean of the percentage of triplicate samples; error bars represent the standard deviations of three experiments in triplicate with similar results. $^*P < 0.001$ is the significance of the difference between the control and the distinct treatments.

Table 4: Characteristics of the biological samples from patients with vaginitis.

Sample number[a]	Clinical diagnosis[b]	*T. vaginalis*[c]		Other microorganisms[d]
		Wet mount	InPouchTv	
HGM483	+	−	+	*Corynebacterium* sp., coagulase-negative *Staphylococcus* sp.
HGM315	+	+	+	*Lactobacillus* sp., coagulase-negative *Staphylococcus* sp.
HGM295	+	+	+	+[e]
HGM225	+	+	+	Coagulase-negative *Staphylococcus* sp.
HGM124	+	+	+	*Corynebacterium* sp.
HGM114	+	+	+	+[e]
HGM9	−	−	−	*Candida* sp.
HGM39	−	−	−	*Corynebacterium* sp.
HGM47	−	−	−	*Candida* sp.
HGM48	−	−	−	*Corynebacterium* sp.
HGM67	−	−	−	*Candida* sp., *Corynebacterium* sp.
HGM331	−	−	−	Coagulase-negative *Staphylococcus* sp., *Gardnerella vaginalis, Lactobacillus* sp.

[a] Biological samples obtained from Laboratorio Central del Hospital General de México (HGM). [b] Clinical diagnosis of cervicovaginitis in all patients. [c] Presence of *T. vaginalis* detected by direct wet mount microscopic observation and by *in vitro* culture with the InPouchTv system. [d] Presence of bacteria in the wet mount that were identified by a microbiological analysis. [e] Presence of bacteria in the wet mount that were not identified by a microbiological analysis as the rest of the samples.

legumains from other organisms such as the Cs-legumain from *Clonorchis sinensis* with a similar pattern in 2DE gels [44], which is also phosphorylated.

Interestingly, the anti-TvLEGU-1r antibody showed a different recognition in the WB assays depending of the protein preparation. This could be due to the presence of fewer amount of protein in the protease-rich extract corresponding to the different protein bands that the antibody was unable to detect them. However, previous data showed that a 60-kDa protein spot was also observed in the 2DE gels from protease-rich extracts, and it was identified as part of the TvLEGU-1 by MS [15]. At this point, we do not have an explanation to this high molecular size TvLEGU-1 protein. It is something that needs to be studied further. Although the 20-kDa protein was not observed nor identified by MS in the protease-rich extract 2DE gels [15], we can speculate that this protein could represent a processing stage of TvLEGU-1 or a degradation product. Additionally, we could not discard that these proteins are the intracellular forms of TvLEGU-1. It is also something that deserves further investigation to help to understand the way this protein is processed, activated, localized, and showed different functions that could be modulated by the iron concentrations in the microenvironment.

TvLEGU-1 is one of the 30-kDa CPs localized on the surface of iron-rich trichomonad parasites (Figure 3) that also bound to the surface of HeLa cells (Figures 6 and 9). These are properties consistent with the proteolytic activity necessary for trichomonal cytoadherence [4, 5, 7]. As expected, the antibodies against the recombinant TvLEGU-1r inhibited trichomonal adherence in a similar range (Figure 7) as the anti-CP30 antibody [7]. Additionally, *T. vaginalis* cytoadherence reduction by the specific legumain inhibitor supports that the TvLEGU-1 proteolytic activity may play a major role in the host-parasite interaction during trichomonal adherence (Figure 8). Therefore, this finding is consistent and corroborates our previous observations that the CP30 proteolytic activity is necessary for trichomonal adherence [4, 5, 7] and a legumain-like CP, TvLEGU-1, is part of it. Additionally, TvLEGU-1 is positively regulated by iron at the protein level (Figure 2) and surface localization (Figures 3 and 4), similar to the iron upregulation of the trichomonad adhesins [6, 41, 42, 45]. This behavior could be expected for molecules that participate in the same trichomonal virulence property, cytoadherence [4, 6, 7, 45]. Interestingly, the lack of surface recognition of the anti-TvLEGU-1r antibody in the Triton X-100-permeabilized parasites could be explained based on previous reports. These demonstrate that nonionic detergents, such as Triton X-100, redistribute and solubilize phospholipid anchored proteins, even in previously fixed cells. Detergents such as Triton X-100 also have significant adverse affects on the immunochemical analysis of gangliosides and GPI-anchored proteins [46, 47]. Consistent with this explanation recent unpublished data suggest that TvLEGU-1, lacking transmembrane domains [38], is on the parasite membrane through a putative phospholipid anchor (work in progress).

The localization of the TvLEGU-1 in different compartments, particularly in lysosomes, suggests the typical role for TvLEGU-1, participating in the lysosomal degradation of food [48–50] or internal organelles during an autophagy process for remodelling of the cellular components [51] or in the parasite metabolism. This is in addition to its new role as a virulence factor involved in cytoadherence, as has been demonstrated in here, supporting previous data [6, 41, 42]. Moreover, the localization of TvLEGU-1 in the Golgi complex suggests that this CP undergoes part of its processing and maturation steps in this organelle as occurs with other legumains. Commonly, these proteinases are translated as a preproform, transferred through the Golgi complex as the proform of legumain with a molecular mass of 56-kDa,

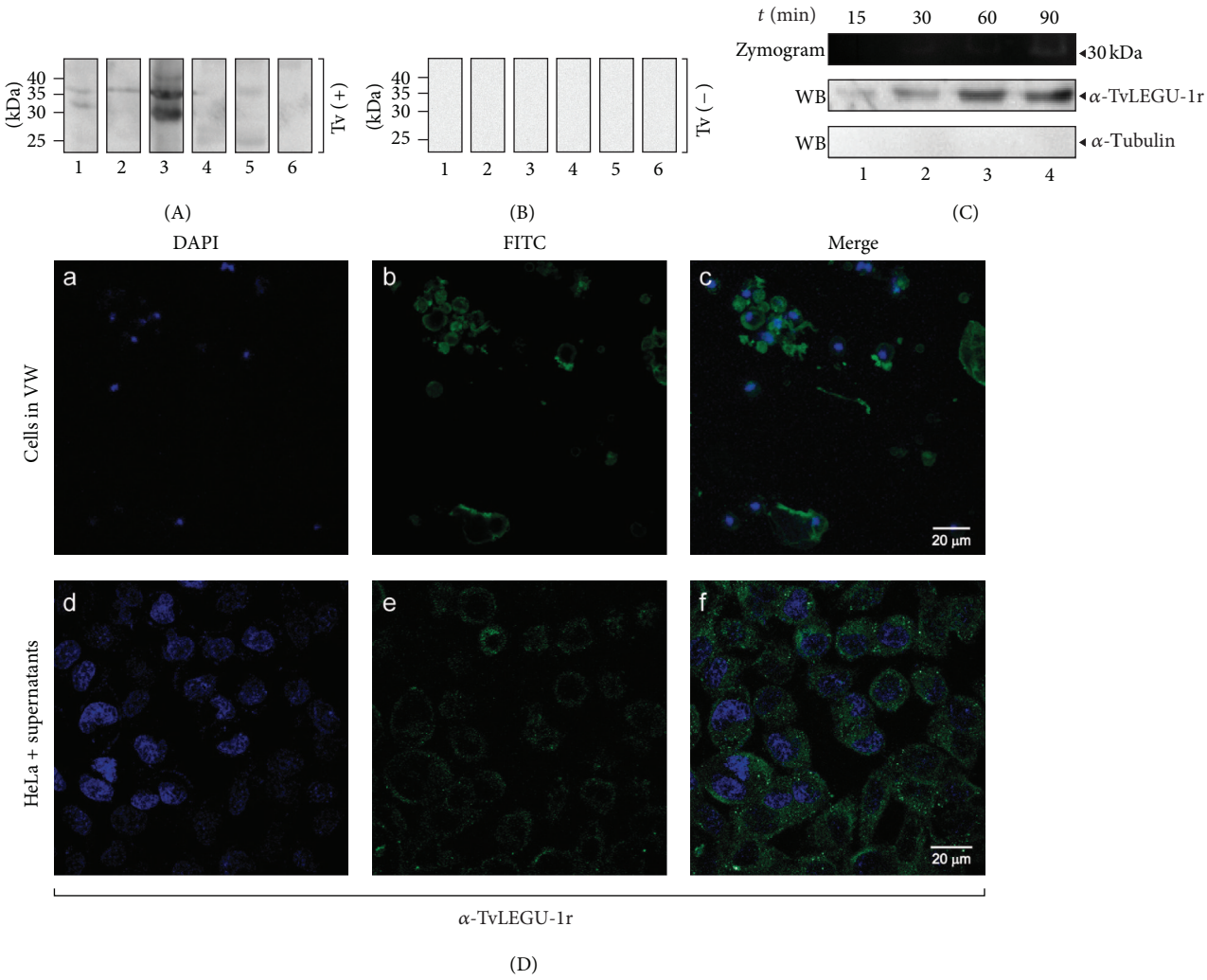

Figure 9: Presence of TvLEGU-1 in vaginal secretions and in *in vitro* secretion assays. (A) WB assays of TCA-precipitated proteins present in VWs from *T. vaginalis* positive culture patients [Tv (+)] (lanes 1–6) incubated with the anti-TvLEGU-1r antibody. (B) WB assays of TCA-precipitated proteins from people with other vaginitis [Tv (−)] used as negative controls (lanes 1–6) incubated with the anti-TvLEGU-1r antibody. (C) Zymogram and WB assays of the proteins present in the *in vitro* secretion products obtained from metabolically active parasites (1×10^6 cells/mL) that were incubated in PBS-0.5% maltose at 37°C for 15, 30, 60, and 90 min (lanes 1–4, resp.). NC membranes containing the TCA-precipitated *in vitro* secretion products incubated with the anti-TvLEGU-1r antibody (1 : 10,000) or the anti-α-tubulin antibody (1 : 1000) used as a negative control. (D) Confocal microscopy images of fixed cells obtained from vaginal washes (VWs) and from live HeLa cell. (a, b, and c) Cells from VWs of patients with trichomoniasis confirmed by *in vitro* culture [Tv (+)] directly incubated with the anti-TvLEGU-1r antibody. (d, e, and f) Live HeLa cells incubated with supernatants from the *in vitro* secretion assays in which TvLEGU-1 is present (C) and with the anti-TvLEGU-1r antibody. Conjugated anti-rabbit IgG-FITC was used as a secondary antibody (1 : 100 dilution) (b and e). Nuclei labeled with DAPI (a and d); merge, and bars: 20 μm (c and f).

and localized in late endocytic compartments as the mature enzyme with a molecular mass of 46-kDa [52], as occurs in lysosomal cysteine proteinase [53].

The legumain CPs, which belong to the clan CD, are distinct from those of all other clans (with regards to their amino acid sequence, tertiary structure fold, protein substrates, effect of inhibitors, and biological functions). Their discovery has led to a reassessment of the relevance of roles played by CPs in parasitic protozoa [23, 24]. Moreover, legumains have greater specificity in their functions than the clan CA enzymes. Thus, the fact that legumain-like CPs have a very restricted type of substrates [48], as compared with the papain-like CPs, which are very promiscuous [23, 24], suggests that this could be one of the major proteolytic activities necessary for trichomonal adherence to host cells [4, 5, 7]. We can speculate that this type of CPs such as TvLEGU-1 will unmask the surface of *T. vaginalis* by degrading the host proteins covering the adhesins, as previously suggested [4, 5, 54]. However, it is important to mention that as shown here the proteolytic activity of CPs of both clans, CA and CD, is necessary for trichomonal cytoadherence (Figure 8). One explanation is that both types of CPs could

be necessary for directly processing the same protein targets such as the host proteins that cover the trichomonad surface [4, 5, 54]. Another possible explanation is through a similar mechanism as the one described for hemoglobinolysis of parasitic organisms such as apicomplexan and nematodes, where the participation of several proteinases is necessary in a cascade of proteolytic activation. A clan CD cysteine peptidase of the legumain type is implicated in the first step activating proteinases of clan CA directly involved in hemoglobin degradation [55–57].

Therefore, we could speculate that in *T. vaginalis* an activation cascade could also be occurring to uncover the parasite surface, in which TvLEGU-1 will participate in the first step activating the papain-like CPs involved in host protein degradation. Once trichomonad CPs digest the proteinaceous cover on the *T. vaginalis* surface, by any of the two proposed pathways, the adhesins will then be free to interact with the host cell receptors for attachment [6, 41, 42]. Thus, further studies will be required to determine whether TvLEGU-1 directly participates in host protein degradation or in the first step of a putative activation pathway that will degrade particular host proteins as a prerequisite for cytoadherence. It also will be relevant to identify the substrate proteins for TvLEGU-1. Further work is needed to examine these questions.

It is relevant to mention that the recombinant protein TvLEGU-1r obtained in this study did not have proteolytic activity and could not be activated (data not shown) for biochemical and direct functional assays, using the same reported conditions [58]. This could be due to the fact that the *tvlegu-1* gene was expressed in bacteria and lacked the sequence coding the first 10 aa residues of the N-terminal region, which may be required for its correct folding and activation. In spite of that, the recombinant TvLEGU-1 interacted with fixed HeLa cell, as the native protein did, suggesting that, in TvLEGU-1, the cell-binding and catalytic domains are different and could function separately. Moreover, identification of TvLEGU-1 in vaginal washes from women with active trichomoniasis is consistent with the presence of the CP30 proteolytic activity [7] in trichomoniasis symptomatic patients [7, 59], suggesting that, during infection, *T. vaginalis* releases several CPs, including TvLEGU-1, which is highly immunogenic [15]. Interestingly, we observed several bands (35- to ~30-kDa) specifically recognized by the anti-TvLEGU-1r antibody in the Tv (+) VWs analyzed, suggesting the presence of different processing states of TvLEGU-1 during infection. Further work is needed to determine the processing steps of TvLEGU-1.

5. Conclusion

In this work, we have demonstrated that indeed CP proteolytic activity is necessary for trichomonal adherence to host epithelial cells that is consistent and corroborates our previous observations. One of these CPs is the TvLEGU-1, a legumain-like CP that is located in lysosomes, Golgi complex, and at the parasite surface in the presence of iron and shows different levels of phosphorylation. It will be interesting to identify the particular substrates for this CP, in addition to determine the phosphorylation or dephosphorylation effect on the proteolytic activity and its impact on cytoadherence. This CP was also found in vaginal secretions of patients with trichomoniasis, supporting its potential as biomarker. This work is the first paper that shows that a legumain-like CP plays a role in a pathogen cytoadherence.

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Research Article

Gene Gun Bombardment with DNA-Coated Golden Particles Enhanced the Protective Effect of a DNA Vaccine Based on Thioredoxin Glutathione Reductase of Schistosoma japonicum

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Schistosomiasis, caused by infection with *Schistosoma* species, remains an important parasitic zoonosis. Thioredoxin glutathione reductase of *Schistosoma japonicum* (SjTGR) plays an important role in the development of the parasite and for its survival. Here we present a recombinant plasmid DNA vaccine, pVAX1/SjTGR, to estimate its protection against *S. japonicum* in BALB/c mice. The DNA vaccine administrated by particle bombardment induced higher protection than by intramuscular injection. All animals vaccinated with pVAX1/SjTGR developed significant specific anti-SjTGR antibodies than control groups. Moreover, animals immunized by gene gun exhibited a splenocyte proliferative response, with an increase in IFN-γ and IL-4. The recombinant plasmid administrated by gene gun achieved a medium protective efficacy of 27.83–38.83% ($P < 0.01$) of worm reduction and 40.38–44.51% ($P < 0.01$) of liver egg count reduction. It suggests that different modes of administering a DNA vaccine can influence the protective efficacy induced by the vaccine. Interestingly, from the enzymatic activity results, we found that worms obtained from pVAX1/SjTGR-vaccinated animals expressed lower enzymatic activity than the control group and the antibodies weakened the enzymatic activity of SjTGR *in vitro*, too. It implies that the high-level antibodies may contribute to the protective effects.

1. Introduction

Schistosomiasis is an important disease distributed in many parts of the world, most of which are the places with poor sanitation or irrigation areas, and it is estimated that 779 million people are at risk of schistosomiasis [1]. *S. japonicum* is the most difficult form of schistosomiasis to control among the 5 *Schistosoma* species which infect humans [2–4]. Schistosomiasis is a chronic and debilitating disease [5, 6] which is always accompanied by emaciation and anemia, and even death.

Over the past decades of years, the Chinese government has implemented several control programs, including community-based praziquantel chemotherapy [7], health education, improved sanitation, environmental modification, and snail control. However, schistosomiasis remains an important public health concern in China. As snail control [8] is always difficult to achieve, and praziquantel has no effect on reinfection [9, 10], the disease is difficult to control. Therefore, a complementary approach to integrate chemotherapy, vaccination for example, is needed.

Since the 20th century, scientists have been trying to develop an effect vaccine against *S. japonicum* for field use [11–13], mainly for yellow cattle and water buffalos. Through decades of efforts, several kinds of vaccines have been developed, including cercariae-attenuated vaccines [14], natural or recombinant protein vaccines, nucleic acid vaccines, and multivalent affiliate vaccines. Currently, DNA vaccines have received increased attention and are considered advantageous compared to other vaccine preparations [15, 16], for low cost and easy preparation. However,

the mode of delivering a DNA vaccine can influence the effect induced by the vaccine [17].

The schistosome tegument is a single syncytium that covers the surface of the parasite body [18]. Although there remains many unresolved questions in relation to the structure and function of the tegument, the dynamic host-interactive layer tegument is believed to involve in nutrient uptake, immune evasion and modulation, sensory reception, and signal transduction, and is important from a vaccine perspective [19–22]. A number of described vaccine candidates are membrane proteins [23, 24], muscle proteins [25], and enzymes [26–28]. Thioredoxin glutathione reductase of *S. japonicum* (SjTGR) is also a tegument antigen mainly distributed in the tegument of adult worms [29]. Adult schistosome worms, which reside in the hepatic portal system, are exposed to reactive oxygen compounds from metabolism and the host immune response. In eukaryocyte, two major systems, the thioredoxin (Trx) system and the glutathione (GSH) system, exist to detoxify reactive oxygen species (ROS). However, it has been proved that there are no separate Trx reductase and GSH reductase enzymes in *S. japonicum*, instead of the linked thioredoxin-glutathione system (TGR) [30]. As such, this union enzyme, thioredoxin glutathione reductase, is thought to be an attractive vaccine antigen candidate.

In this paper, a recombinant DNA plasmid was constructed containing a complete open reading fragment of SjTGR and immunized with two different modes, particle bombardment, and needle inoculation to evaluate the ability to protect BALB/c mice against *S. japonicum* challenge and explore the conceivable immune protective mechanism.

2. Material and Methods

2.1. Experimental Mice and Parasites. Male BALB/c mice, 6–8 weeks old, were purchased from Slac Animal Laboratory (Shanghai, China). The freshwater snail, *Oncomelania hupensis*, was maintained in the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Cercariae were collected by exposing infected snails to light and the number and viability of cercariae were determined under a light microscope before challenge. Animal care and all procedures involving animals were conducted according to the principles of Shanghai Veterinary Research Institute for the Care and Use of Laboratory Animals.

Specific anti-SjTGR serum was collected from BALB/c mice thrice immunized with recombinant protein SjTGR.

2.2. Construction of Recombinant Plasmid DNA. The eukaryotic expression plasmid, pVAX1, which contains the strong cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal, was used as the vector. The entire SjTGR open reading fragment was amplified from the *S. japonicum* adult worm cDNA library, with primers: 5′-CGC*GGATCC*ATGCCTCCGATTGAT-3′ and 5′-GC*CTCGAG*TCAGCAACCGGTTACC-3′ and subcloned into cleaved pVAX1 to construct the recombinant expression plasmid, pVAX1/SjTGR.

The recombinant plasmid was sequenced to ensure the insert sequence was cloned correctly. Then, the expression plasmid was transferred into DH5α, a type of competent cell, for large-scale preparation and purification using a Qiagen Plasmid Maxi Kit (Qiagen), followed by the manufacturer's protocol.

2.3. Expression in 293T Cells. Transfections of plasmid (pVAX1/SjTGR, pVAX1) were done by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol to detect gene expression in 293T cells. One day before transfection, cells were plated into a 6-well plate in 2 mL/well of growth medium without antibiotics so that the cells will be 90%–95% confluent when transfection was performed. For each well, 5 μL Lipofectamine 2000 and 10 μg DNA were mixed gently and incubated for 20 min at room temperature. Then, the complex was volume to 2 mL with Opti-MEM and added to the monolayer of cells in each well.

After incubating cells at 37°C in a 5%CO_2 incubator for 48 h, the monolayer cells were fixed with 80% ethanol, and thrice washed with PBS-0.05%/Tween-20 (PBST). Specific anti-SjTGR serum was added to each well and incubated at 37°C for 2 h, then thrice washed with PBST. After that, Cy3-labeled goat anti-mouse antibodies (Beyotime) were used as secondary antibodies at a dilution of 1 : 5000 and added to each well. After 1 h incubation at 37°C keeping in dark, the plates were thrice washed again. Finally, the potential protein in the cells was detected using a converted fluorescence microscope. The tests were assayed in triplicate.

2.4. Immunization with the Helios Gene Gun System. Cartridges were prepared prior to the day of the experiments, followed by general methods [31]. First, the amount of DNA and gold required for each transformation was calculated. The DNA loading ratio (DLR) used was 5 μg DNA/mg gold, and the microcarrier loading quantity (MLQ) was 0.5 mg/cartridge. For the duration of producing bullets, polyvinylpyrrolidone (PVP) (Sigma) served as an adhesive. At higher discharge pressures from the nitrogen source, DNA mixed with nanolevel gold particle was coated in the walls of the tubes (BioRad) and is referred to as bullet. Similarly, at higher discharge pressures from the helium source, murine abdominal epidermis was bombarded with the Helios gene gun system (BioRad). The optimum pressure for mice was determined to occur at 600 psi.

2.5. Immunization Schedule and Challenge Infection. Two mice vaccinations were carried out in this study. In trail 1, fifty-male BALB/c mice were randomly divided into 5 groups (10 each group, pVAX1/i.m., pVAX1/SjTGR/i.m., pVAX1/g.g., pVAX1/SjTGR/g.g., PBS). All mice were given 2 intramuscular immunizations 3 weeks apart called prime-boost inoculation by two different modes, particle bombardment and needle inoculation. In trail 2, forty-five male BALB/c mice were randomly divided into 3 groups (15 each group, pVAX1/g.g., pVAX1/SjTGR/g.g., and PBS) and immunized with a gene gun with the same schedule. Ten days after each immunization in the trail 2, blood from

each animal was collected. Serums were separated and stored at −20°C for antibody assays and cytokine detections. Two weeks later, mice in each group of the two trails were infected with 40 ± 2 *S. japonicum* cercaria and sacrificed 6 weeks after challenge and blood was collected. The total worm and liver egg burden was determined (Figure 1).

2.6. Detection of Specific Antibodies in Serum by Enzyme-Linked Immunosorbent Assay (ELISA). In trail 2, the levels of specific IgG antibodies against SjTGR were detected by ELISA following standard methods [32]. A 96-well flat-bottomed plate was coated with recombinant protein SjTGR at 4°C overnight (1 μg/well), thrice washed with PBS-0.05%/Tween-20 (PBST), blocked with 150 μL/well of PBST-1.5% (m/v) normal bovine serum albumin (BSA) for 2 h at room temperature (25°C), then thrice washed with PBST. The serum samples collected in the previous section were diluted with PBST in 1 : 100, added to the plate (100 μL/well), incubated at 37°C for 2 h, and thrice washed again with PBST. Horseradish peroxidase labeled goat anti-mouse IgG, IgG1, and IgG2a (BD Pharmingen) were used as secondary antibodies at a dilution of 1 : 5000 and added at 100 μL/well. After a 1 h incubation at 37°C, the plates were thrice washed and the substrate, 3,3′5,5′-tetramethyl benzidine dihydrochloride (TMB), was added (100 μL/well). The plates were incubated for 10 min at room temperature in the dark and the reaction was stopped using 2 M H_2SO_4 (50 μL/well). All of the samples were assayed in triplicate. The results were detected in a microplate reader (BioTek) and the absorbance was measured at 450 nm.

2.7. Calculation of the Percentage of CD4+ and CD8+ Cells and Cytokine Determination by Flow Cytometry. Five mice in each group in trail 2 were sacrificed 2 weeks after the booster immunization and splenocytes were collected. 1 mL RPMI 1640 medium (Gbico) with 10% FBS was added to each spleen and grinded. After grinding, 1.5 mL FACS lysing solution (BD Pharmingen) was added to the cells, and thrice washed by centrifuging at 3000 g for 5 min at 4°C. The cells were adjusted to 10^7/mL and cultured overnight and the next day stimulated with 2.5 μL (1 μL/mL) PMA (Sigma) 2 μL (1 μg/mL) Ionomycin (Sigma) and 3.4 μL Monensin (eBioscience), for 6 h at 37°C in a 5% CO_2 incubator. Then, 0.25 μg PE-labeled Cy5 CD3 ε and 0.25 μg PE-labeled Cy7 CD8 α (BD Pharmingen) were added to each sample, and incubated at 25°C for 20 min at dark, thrice washed as usual. Then 1 mL dyeing buffer was used to wash the cells for three times as usual. After that, 0.5 mL cell-fixed liquid was added to each sample for 20 min and washed thrice as usual. Cells were resuspended with 100 μL permeabilization for another 20 min. Then for each sample, 0.25 μg PE-labeled anti-IL-4 antibodies and 0.25 μg FITC-labeled anti-IFN-γ antibodies (BioLegend) were added and thrice washed as usual. Finally, 0.6 mL cell staining buffer was added to resuspend the cells, and a flow cytometry system (Beckman) was used to detect the interferon-gamma (IFN-γ) and interleukin (IL)-4 levels. The criteria for this study were set according to the blank measurement. The ratio of CD4+ and CD8+ T cells in total cells was examined and the rates of T cells producing IFN-γ or IL-4 were reported.

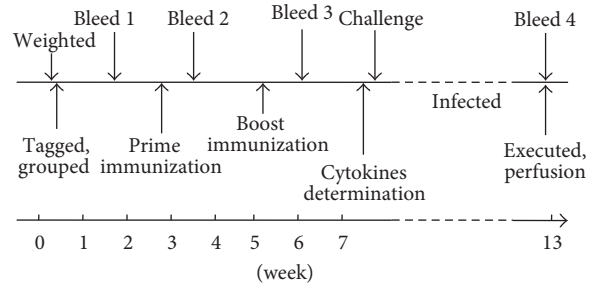


Figure 1: Immunization schedule and challenge infection of animals.

2.8. Count of Worm and Liver Egg Burden. Forty-two days after challenge, all mice (10 in each group) were euthanized in the two independent trails, and worms were collected by perfusion from the hepatic portal system then counted.

To determine the liver egg burden, each mouse liver were weighed, homogenized, and digested for approximately 1 h at 56°C with 10 mL 10% NaOH. The suspensions were agitated, and 1-mL aliquots, collected from the middle of each tube, were transferred into 1.5-mL Eppendorf tubes and spun at a low speed to sediment the particles. Pellets were then resuspended in 200 μL of PBS and the egg counts were determined under a microscopy.

Reductions in the parasite burden were calculated as follows: worm reduction rate (%) = ((average number of recovered worms of control group-average number of recovered worms of experimental group)/average number of recovered worms of control group) × 100; and egg reduction rate (%) = ((average number of eggs/g liver in control group-average number of eggs/g liver in experimental group)/average number of eggs/g liver of control group) × 100.

2.9. Enzyme Activity Analysis by Thioredoxin Reductase Assay Kit. The enzyme activity analysis was referenced to Han [29]. Six-week-old worms were collected in trail 2 and stored at −80°C with Dulbecco's phosphate buffered saline and protease inhibitor cocktail (Sigma) at a ratio of 1 : 1000. Then, all of the worms (50 worms in each group) were grinded with Ready Prep Mini Grinders (BioRad) on ice and all the samples were cracked thoroughly by freezing and thawing three times. The thawed lysates were centrifuged at 10000 ×g for 20 min at 4°C and the supernatants containing thioredoxin glutathione reductase were used to detect the enzymatic activity in each individual group.

The enzyme activity was assessed using a Thioredoxin Reductase Assay Kit (Sigma) for an easy and simple colorimetric assay [33]. It is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm. Components were added to a cuvette with a final volume of 1 mL, and 30 μL of DTNB in DMSO (100 mM) was added immediately before

detection with a Thermo NanoDrop ND-2000C (Thermo). The enzymatic kinetic program was set as follows using the spectrophotometer: delay = 120 sec, interval = 10 sec, and number of readings = 6. During the test, an inhibitor solution for specific inhibition of mammalian thioredoxin reductase contained in the kit was used, to determine the reduction of DTNB due only to thioredoxin reductase activity of SjTGR. All of the samples were detected in triplicate independently. And the data was calculated as the following computational formula:

$$\text{Unit/mL} = \frac{\Delta A_{412}/\text{min (thioredoxin reductase)} \times \text{dil} \times \text{vol}}{\text{enzol}}, \quad (1)$$

where, ΔA_{412}/min (thioredoxine reductase) = [ΔA_{412}/min (sample) $-\Delta A_{412}$/min (sample + inhibitors)], dil = sample dilution factor, vol = volume of reaction in mL, and enzol = volume of enzyme in mL.

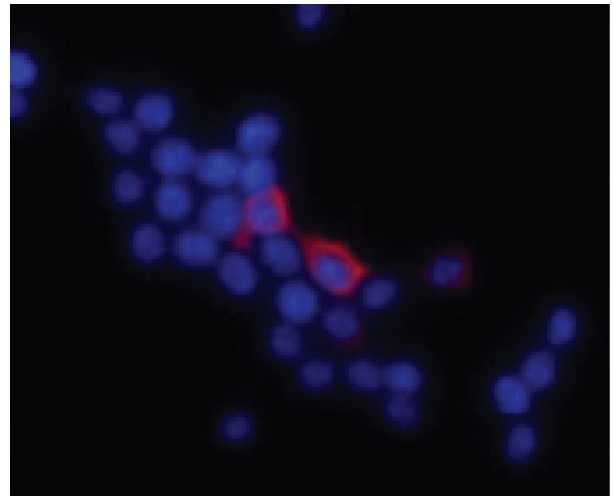
An *in vitro* test was carried out to evaluate the weakened effect of specific anti-SjTGR serum to thioredoxin reductase. Forty-two-day-old worms were carefully collected and soluble adult worm antigen preparations (SWAPs) were extracted using the above methods. Anti-SjTGR and normal mouse sera were added to SWAPs (1 mg/mL) at a ratio of 1 : 1, and both of the mixtures were incubated at room temperature for 2 h. The enzymatic activity of SjTGR was then detected with the methods described above.

2.10. Statistical Analysis by SPSS. All data were compared by analysis of variance (ANOVA) and Student's t-test using SPSS v.12.0 software. P values < 0.05 were considered statistically significant.

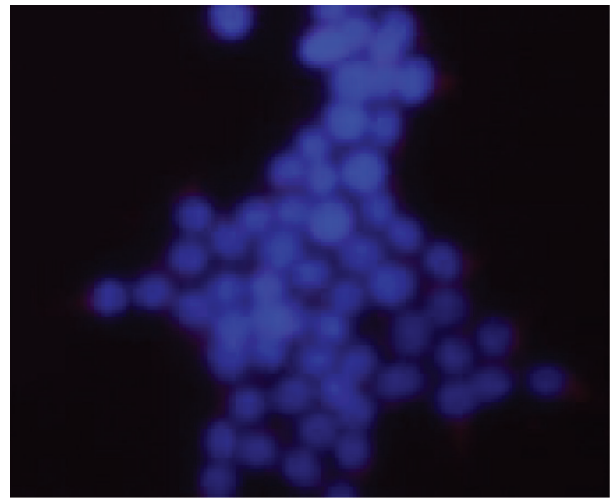
3. Results

3.1. Transient Expression of Recombinant Plasmid in 293T Cells. The SjTGR entire open reading frame amplified by PCR with special primers was subcloned into the plasmid pVAX1 with T4 DNA ligase and was confirmed correct by restriction enzyme digestion and sequencing. Then, the recombinant plasmids were transiently transfected into 293T cells.

Forty-eight hours after transfection of recombinant plasmid pVAX1/SjTGR, the cells were fixed to detect the expression of the protein of interest. Red fluorescence was observed on the cells transferred with pVAX1/SjTGR (Figure 2(a)), but not on those transferred with pVAX1 vector DNA alone (Figure 2(b)).



(a)



(b)

Figure 2: Protein expression of SjTGR in 293T cells. (a) Red fluorescence was observed on the 293T cells after pVAX1/SjTGR plasmid transfection; (b) negative control transfected with naked pVAX1 plasmid alone, and no fluorescence was observed.

3.2. Evaluation of Protective Efficacy Induced by pVAX1/SjTGR. The total worm burden and eggs per gram (EPG) in each group, as well as the percent reduction in the worm burden and EPG in the vaccinated group compared with PBS control group are summarized in Table 1. In trail 1, animals administrated with particle bombardment induced better protective efficacy. Mice vaccinated with pVAX1/SjTGR by bombarding murine epidermis with the Helios gene gun system resulted in a significant worm burden reduction of 27.83% ($P < 0.01$) and 38.83% ($P < 0.01$) compared to the PBS control group in two independent trails, respectively. And no protection was observed in pVAX1/SjTGR/i.m. group as well as in two pVAX1 vaccinated groups. Mice in the vaccinated group by gene gun resulted in a significant liver egg burden reduction of 40.38% ($P < 0.01$) and 44.51% ($P < 0.01$) to the blank control group, in trail 1 and 2, respectively. Significant egg reduction also observed in pVAX1/SjTGR/i.m. group, but not in pVAX1 vaccinated groups.

3.3. Antibody Assay. Total IgG antibodies and its subtypes of IgG1 and IgG2a were detected by ELISA in trail 2, as described. SjTGR-specific IgG antibody was detected 10 days after immunization, significantly increased after boost vaccination, in the pVAX1/SjTGR immunized mice, and the

Table 1: Protective efficacy induced by pVAX1/SjTGR in mice.

Groups	Worm reduction		Egg reduction	
	Worm burden (mean ± SE)	Percent reduction in worm burden (%)[a]	Liver egg per gram (EPG) (mean ± SE)	Percent reduction in liver egg count (%)[b]
Trail 1				
pVAX1/SjTGR/g.g. ($n = 10$)[c]	15.6 ± 8.86*	27.83	31591.3 ± 14647.42*	40.38
pVAX1/g.g. ($n = 10$)	23.3 ± 6.85	−8.03	42995.7 ± 11730.94	18.86
pVAX1/SjTGR/i.m. ($n = 10$)	24.6 ± 12.18	−14.12	25558.7 ± 15171.82*	51.77
pVAX1/i.m. ($n = 10$)	23.7 ± 5	−8.09	57686.6 ± 20951.58	−8.86
PBS ($n = 10$)	21.6 ± 3.94	—	52989.6 ± 19448.42	—
Trail 2				
pVAX1/SjTGR/g.g. ($n = 10$)	12.6 ± 7.17*	38.83	33326.5 ± 6875.44*	44.51
pVAX1/g.g. ($n = 10$)	21.6 ± 8.67	−4.85	65934.8 ± 16702.02	−9.79
PBS ($n = 10$)	20.6 ± 4.90	—	60055.1 ± 19211.27	—

Differences were significant at $P < 0.05$ (*); data was presented in 95% confidence interval.
[a]Percent reduction was determined using total worms in immunized group compared to PBS group.
[b]Percent reduction was calculated using liver egg burden in immunized group compared to PBS group.
[c]The number of animals in each group when perfusion.

specific antibody had no obvious change in mice that received pVAX1 vector DNA or PBS only (Figure 3(a)).

Both SjTGR-specific IgG1 (Figure 3(b)) and IgG2a antibodies (Figure 3(c)) increased significantly after the booster immunization with pVAX1/SjTGR, and the level of IgG1 was higher than IgG2a; the IgG1/IgG2a ratio was significantly increased after boost immunization (Figure 3(d)). No significant changes were noted in the two control groups in specific IgG1 and IgG2a antibody levels. We also found that different inoculation modes can induce different levels of antibodies, and the level of specific IgG antibody induced by recombinant plasmids pVAX1/SjTGR is significantly higher when delivered by gene gun than that by i.m. (Figure 4).

3.4. T Cell Subsets and Cytokine Determination. After the last immunization, the splenic lymphocytes of animals from each group in trail 2 were collected. Grinded cells were cultured, stimulated, stained by fluorescent-labeled antibodies, and detected with a flow cytometry system (FMC). In this paper, particle bombardment immunization pushs T cells forward to CD4+ T cells (Table 2). And the percent of cells producing IL-4 (Figure 5) or IFN-γ (Figure 6) in the pVAX1/SjTGR-vaccinated group were significantly increased compared to those in pVAX1 or PBS group.

3.5. Activation Changes of Enzyme. SWAPs were extracted from worms in each individual group and enzymatic activity was detected. The detectable products of substrate were increased with incubation time. With same amount of SWAPs, the thioredoxin reductase (TR) enzymatic activity in the worms from pVAX1/SjTGR-immunized group expressed much lower enzymatic activity than that of the pVAX1 or PBS control groups (Figure 7).

Furthermore, SWAPs treated with anti-SjTGR antibodies expressed a lower TR enzymatic activity than untreated SWAPs, and the enzyme activity was not affected after incubating with normal mouse serum, indicating that anti-SjTGR antibodies influenced the thioredoxin reductase activity of SjTGR to catalyze DNTB into NTB, which can be measured at 412 nm (Figure 8).

4. Discussion

Thioredoxin glutathione reductase of *Schistosome japonicum* is a membrane protein of about 65 kDa, which is considered as a promising vaccine candidate antigen based on its immunogenicity [29] and its important role in parasite metabolism as a vital enzyme to balance redox equilibrium [34]. In the current study, we focused on evaluation of a recombinant plasmid pVAX1/SjTGR as a DNA vaccine based on SjTGR against schistosomiasis japonicum with two different modes. And pVAX1 was chosen as the vaccination regimen because it is mentioned by FDA before (http://www.fda.gov/ohrms/dockets/ac/00/transcripts/3664t1_a.pdf).

The main methods of delivery plasmid DNA into animals include intramuscular injection and intradermal delivery into skin by a gene gun system [35]. And the administration mode of delivering a DNA vaccine can influence the type of immune response [17] and somehow influence the result of immunoprotective efficacy. In this study, mice vaccinated with pVAX1/SjTGR by bombarding murine epidermis with the Helios gene gun system (BioRad) elicited a much stronger IgG antibody response specific for SjTGR by ELISA (Figure 3(a)), and achieved a prominent reduction of worm (27.83%, 38.83%, $P < 0.01$, Table 1) and liver EPG (40.38%, 44.51%, $P < 0.01$, Table 1), which was considered as a high-efficiency

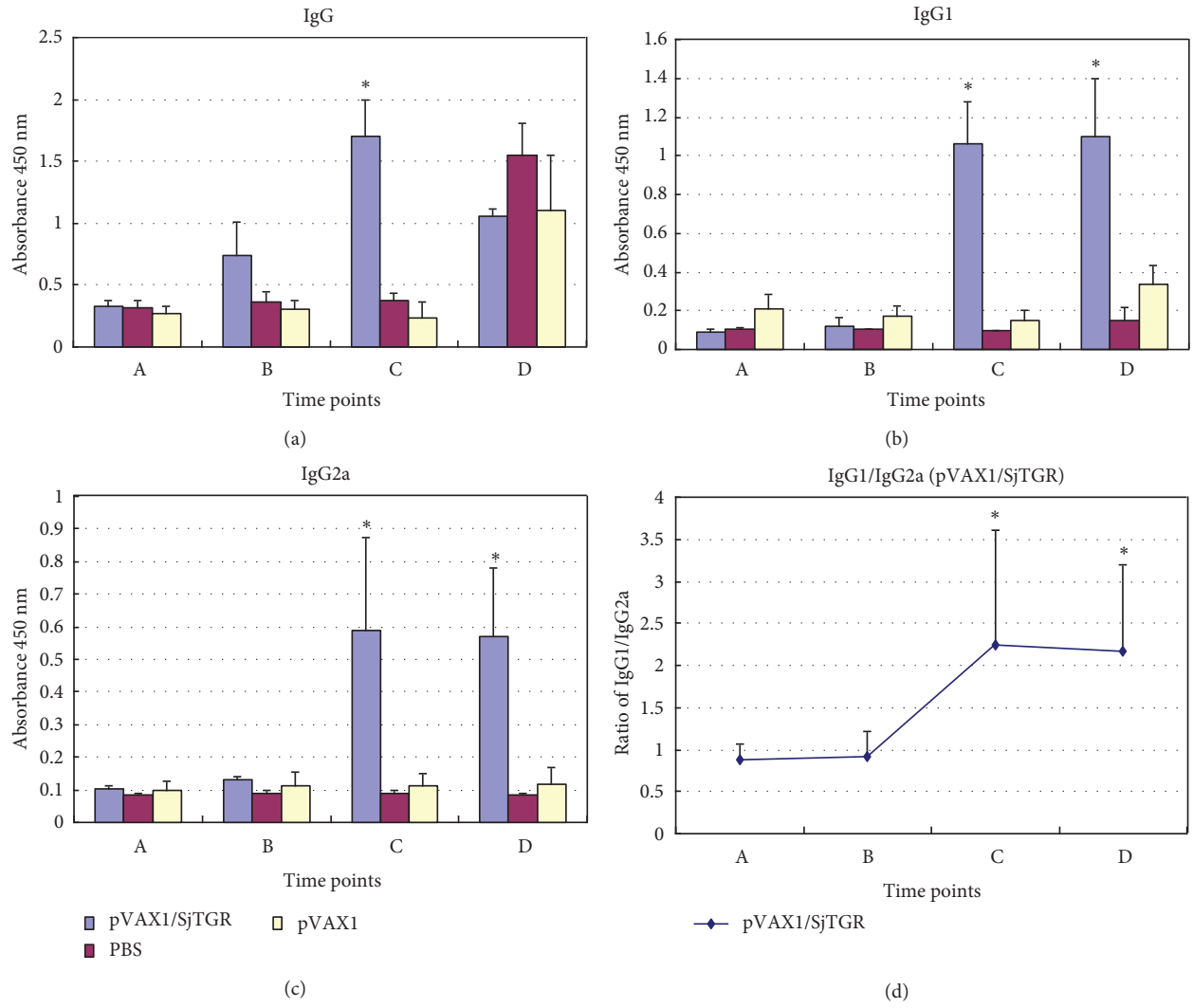


Figure 3: Levels of antibodies of mice immunized with gene gun in each group by ELISA. Figures (a), (b), and (c) display detection of specific IgG, IgG1, and IgG2a antibodies, respectively. Figure (d) displays the ratio of IgG1-to-IgG2a in pVAX1/SjTGR group. A, B, and C indicated before vaccination, 10 d after prime vaccination, and 10 d after boost vaccination. D indicates that 42 d after challenged with cercariae. The results are presented as mean ± SD for each group (pVAX1/SjTGR, pVAX1, PBS). The asterisk (*) indicates significantly increased antibody levels of serum collected from pVAX1/SjTGR compared with both pVAX1 and PBS control.

method for less cost (2.5 μg/mouse), compared with needle injection (20 μg/mouse). Yoshida et al. compared the two methods in reproducible induction of specific immune responses and found that gene gun DNA delivery appeared to bring about highly reproducible and reliable results while the results obtained by intramuscular inoculation vary significantly [36]. They also thought that intramuscular injection appears to favor Th1 responses, while gene gun prefers to promote Th2 responses. And our results are like theirs to some extent.

The DNA vaccine transferred into cells by gene gun bombardment with golden particles under a special-high pressure (600 psi) will be assimilated by professional antigen-presenting cells or some other cells, which can induce humoral and cellular immunity [37]. DNA-coated golden particles delivery by gene gun predominantly produces IgG1 and induces Th2-type responses [17]. In our experiment, the dose of DNA plasmid that used in particle bombardment is 8-fold less than needle injection. But the former received much better protection. And it is considered that the CpG motif provided by the vector pVAX1 can influence the immune response, too [38].

In this paper, mice immunized pVAX1/SjTGR by particle delivery induced both anti-IgG1 and IgG2a antibodies increasing. The antibodies induced by SjTGR DNA constructs were dominantly IgG1 type which is proved to be efficient in complement fixation and with cytophilic property in antibody-dependent cell-mediated cytotoxicity [39], and the result is the same with Da'dara et al. [40], who stated that gene gun immunization resulted in significantly higher

Table 2: T cell subsets after boost immunization by gene gun.

Groups	T cell subsets		Description (ratio)
	CD4+ T cells (%)	CD8+ T cells (%)	
pVAX1/SjTGR	20.49	7.40	2.77 : 1
	19.04	7.26	2.54 : 1
	20.84	7.49	2.78 : 1
pVAX1	14.23	7.30	1.95 : 1
	16.19	7.54	2.15 : 1
	14.78	7.26	2.04 : 1
PBS	13.92	7.89	1.74 : 1
	16.42	8.72	1.88 : 1

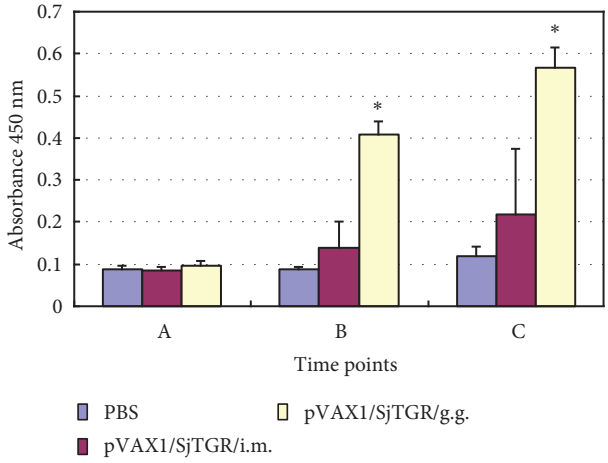


Figure 4: Levels of antibodies of mice immunized with two different modes by ELISA. A, B and C indicated that before vaccination, 10d after prime vaccination and 10d after boost vaccination. The asterisk (*) indicates significantly increased antibody levels compared with PBS control.

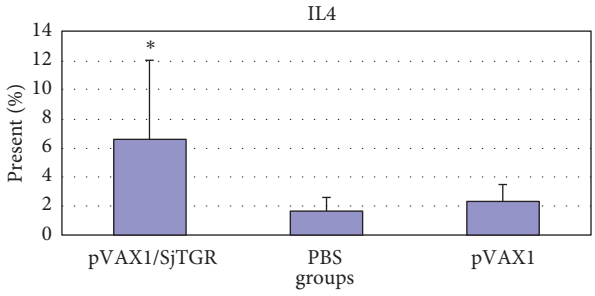


Figure 5: The level of IL-4 in each group. The asterisk (*) indicates the significant increase of the level of IL-4 in pVAX1/SjTGR-immunized group compared with PBS and pVAX1 group ($P < 0.05$).

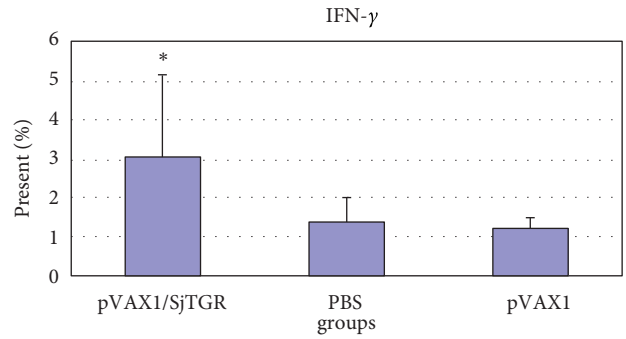


Figure 6: The level of IFN-γ in each group. The asterisk (*) indicates the significant increase of the level of IFN-γ in pVAX1/SjTGR-immunized group compared with PBS and pVAX1 group ($P < 0.05$).

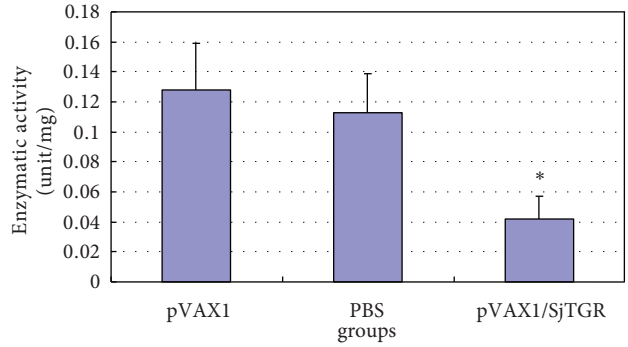


Figure 7: Enzymatic activity detection of thioredoxin reductase of SjTGR in each immunization group. The results are presented as mean ± SD after three independent tests. The asterisk (*) indicates the significant decrease of the enzymatic activity of SjTGR in pVAX1/SjTGR-immunized group compared with PBS and pVAX1 group ($P < 0.05$).

levels of IgG1. And from the FMC results, we found that the ratio of CD4+ T cells/CD8+ T cells was promoted after boost immunization by gene gun, from the normal ratio (2 : 1) to about 2.7 : 1 (Table 2). The cell surface antigen CD4 is the receptor of major histocompatibility complex-II (MHCII), which is located on the surface of antigen presenting cells (APCs), such as dendritic cells (DCs). The activated CD4+ T cells work as three different subsets depending on their different functions. One of them is helper T cell (Th), which induced humoral and cellular immune responses by means of two kinds of Th cells. The Th1 cells mediate cytokine producing with the signal of IFN-γ level increasing, known as Th1 response, while the Th2 cells secrete IL-4 and other cytokines,

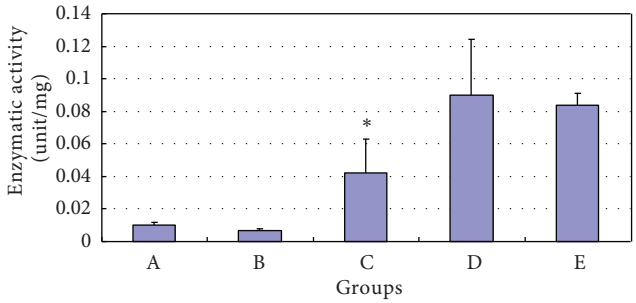


Figure 8: The TR enzymatic activity of SWAP after serum-treatment. The results are presented as mean ± SD after three independent tests. A, B, C, D, and E indicated that anti-SjTGR serum, normal mouse serum, anti-SjTGR serum-treated SWAP, normal mouse serum-treated SWAP, and untreated SWAP. The asterisk (*) indicates the significant decrease of the enzymatic activity of SWAP after serum treatment compared with normal SWAP and SWAP treated with normal serum ($P < 0.05$).

named Th2 response [37, 41]. On the other hand, Th cells can activate B cells and regulate their differentiation and antibody producing. IL-4 can activate B lymphocytes to produce IgG1 subtype antibodies, which is beneficial to transform between antibody subtypes and enhances Th2-type immune responses [41, 42]. And mice immunized with the recombinant plasmid by gene gun elicited both Th1-type cytokine IFN-γ and Th2-type cytokine IL-4 secreting augment, suggesting that mice-inoculated pVAX1/SjTGR plasmid induced mixed Th1/Th2 immune responses. Sawant [43] suggested that IL-4 may be more appropriate as a genetic adjuvant than IFN-γ for ND (Newcastle) DNA vaccine. One strategy to improve DNA vaccine-induced immune responses is the utility of cytokine cDNA as a molecular adjuvant [44]. Coimmunization of these cytokine molecular cassettes is an effective method to modulate the direction of the immune responses (humoral or cellular immune response).

In addition, SWAPs were prepared to determine SjTGR thioredoxin reductase activity *in vitro*. SWAPs of worms from immunized animals expressed a much lower enzymatic activity in catalyzing DTNB into TNB (Figure 7) and SWAPs treated with specific anti-SjTGR serum displayed lower enzyme activity compared to untreated SWAP (Figure 8). This may be an important evidence to explain reduction of worms and eggs. We speculate that the complex of specific anti-SjTGR antibodies and the interest enzyme lead to the changes of spatial structure of SjTGR, which may be a good competitive inhibitor of interaction between the enzyme and substrate. Consequently, the development progress of worms may be blocked partially, which is responsible for the protective effect against schistosomiasis. On the other hand, the DNA vaccine inoculated by golden-particle bombardment induced much higher antibody level (Figure 4), which is probably beneficial to the protection.

In this study we investigated the protective ability of pVAX1/SjTGR as a DNA vaccine by inoculating with a gene gun system. We found that animals immunized with pVAX1/SjTGR induced both humoral and cellular immunity. And the enzymatic activity of SjTGR was weakened to some extent by high-titer antibodies. Based on our work, SjTGR may be considered as a prospective vaccine candidate antigen against schistosomiasis in a BALB/c mouse model.

Acknowledgments

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Research Article

In Vitro Ovicidal and Cestocidal Effects of Toxins from *Bacillus thuringiensis* on the Canine and Human Parasite *Dipylidium caninum*

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Bacillus thuringiensis is a gram-positive soil-dwelling bacterium that is commonly used as a biological pesticide. This bacterium may also be used for biological control of helminth parasites in domestic animals. In this study, we evaluated the possible ovicidal and cestocidal effects of a total protein extract of *B. thuringiensis* native strains on the zoonotic cestode parasite of dogs, *Dipylidium caninum* (*D. caninum*). Dose and time response curves were determined by coincubating *B. thuringiensis* proteins at concentration ranging from 100 to 1000 μg/mL along with 4000 egg capsules of *D. caninum*. Egg viability was evaluated using the trypan blue exclusion test. The lethal concentration of toxins on eggs was 600 μg/ml, and the best incubation time to produce this effect was 3 h. In the adult stage, the motility and the thickness of the tegument were used as indicators of damage. The motility was inhibited by 100% after 8 hours of culture compared to the control group, while the thickness of the cestode was reduced by 34%. Conclusively, proteins of the strain GP526 of *B. thuringiensis* directly act upon *D. caninum* showing ovicidal and cestocidal effects. Thus, *B. thuringiensis* is proposed as a potential biological control agent against this zoonosis.

1. Introduction

Bacillus thuringiensis (*B. thuringiensis*) is a gram-positive bacterium occurring naturally in the soil and on plants. This bacterium produces proteins used for biological control against several agriculture pests and for some mosquito vectors of human diseases [1–3]. The proteins are classified into the crystal (Cry), and cytolytic (Cyt), vegetative insecticidal protein, and S-layers families; the mechanism of action of specific Cry proteins against agriculture pests has been characterized in detail [3]. *B. thuringiensis* has the advantage of being innocuous for humans, domestic animals and plants, and their proteins are highly biodegradable [4], making it a suitable and viable option to perform biological control on the parasites that infect mammals [2]. Certain proteins have been used in goats and ewes infected with

Haemonchus contortus, resulting in effective control against larval and adult stages of this parasite [5, 6]. Furthermore, four strains of *B. thuringiensis*, referred to as GP123, GP138, GP130, and GP140, exhibited toxicity against *Rhipicephalus (Boophilus) microplus,* an ectoparasite that affects cattle [7]. It was also observed that the Cry5B protein of *B. thuringiensis* is effective *in vitro* and *in vivo* against the human nematode *Ancylostoma ceylanicum*. The effect was achieved by induction of a reduction in the number of eggs that is produced by the adult female and also decreased the development of the larval stage of the worm [8]. In a related study, the same protein administered in mice had antihelminthic activity against *Heligmosomoides bakeri* [9]. To date, there are no previous reports on the use of *B. thuringiensis* against cestodes. However, based on the described antihelminthic activity and its safety features, we propose in this study the bacterium's possible use against dipylidiasis, the parasitic disease caused by the cestode *D. caninum*, which is a zoonotic disease of public health importance that involve fleas as intermediate hosts and both humans and dogs as carriers of the adult parasite [10, 11]. The frequency of *D. caninum* in dogs worldwide varies from 0.1 to 44% [12–14]. In Mexico, higher frequencies, ranging from 54.7 to 60% have been reported [15–17]. This disease in dogs has been traditionally treated with drugs intended primarily to kill tapeworms, for example, praziquantel, pyrantel, and oxantel [18], however, there are parasites that had developed resistance to a number of these drugs [19]. Furthermore, these drugs may induce side effects, such as vomiting, nausea, headache, and hepatomegaly [20]. Particularly in the case of praziquantel, genotoxic potential has been established [21], which suggests the need to find other effective drugs that are safe and inexpensive. Taking into consideration this information, the aim of the present study was to explore the role of *B. thuringiensis* toxins against the adult cestode and egg capsules of *D. caninum* evaluating its *in vitro* effects on eggs development and survival and adult worm tegument thickness, which are key processes in the maintenance of the infectious cycle in dogs and humans. The *in vitro* effect of *B. thuringiensis* toxins on *D. caninum* was studied through pharmacological (dose and time effect) and microscopical (morphological studies) approaches to define the mechanisms of *B. thuringiensis* toxins' actions in the parasite. Our results demonstrated the ovicidal and cestocidal effects of *B. thuringiensis* toxins and promise a new therapeutic agent against *D. caninum*.

2. Materials and Methods

2.1. Parasites. Adult tapeworms of *D. caninum* were obtained from the intestine of infected dogs, which were humanely euthanized at the Canine Control Center in Tláhuac, México City. The method was previously evaluated by the University Animal Care and Use Committee to ensure compliance with international regulations and guidelines. After a lengthwise slitting, each intestine was inspected in search of the cestode in the lumen. The adult cestodes of *D. caninum* were identified based on the macroscopic appearance of proglottids [22]. The viability of parasites was determined by direct observation of the cestode under the microscope, counting as viable the parasites that exhibited full motion during 1 minute of observation [8].

2.2. Strain and Protein Recovery. The strain GP526 of *B. thuringiensis* used in this study belongs to the collection of the Vegetal Parasitology Laboratory, Center of Biological Research, University of Morelos, Mexico. The GP526 strain was isolated from a cyst of the phytoparasitic nematode *Meloidogyne* sp. (Tylenchida: Heteroderidae). This strain was grown using solid medium Luria-Bertani (LB) until complete sporulation (72 h at 28°C) and preserved in 60% of glycerol at 4°C until protein recovery. Crystal inclusions and spores were observed using an optical phase-contrast microscope and recovered using a bacteriological loop and suspended in sterile water. Protease inhibitor, (PMSF) 0.1 mM, was added to the suspension to avoid protein degradation [7], and total protein was quantified by the Bradford technique [23].

2.3. Egg Capsules Recovery. From each cestode, 2–4 gravid proglottids were chosen and analyzed using a microscope. The chosen proglottids were dissected, and the eggs were obtained and maintained at 4°C in 0.9% NaCl supplemented with antibiotics (penicillin and streptomycin, Sigma-Aldrich, St. Louis, MO, USA). The viability of eggs was measured according to Wang and cols. [24]. Briefly, egg hatching was induced using a 0.4% sodium hypochlorite solution. Viability was evaluated microscopically in eggs using the trypan blue exclusion test.

2.4. Determination of LD50 y LT50 on Eggs. To calculate the median lethal dose (LD50) and median lethal time (LT50), dose-response and time-response curves were performed using concentrations of GP526 total protein. Each test was performed in quadruplicate and used 1000 eggs/mL in a final volume of 4 mL of culture medium. The ovicidal effect of the protein was quantified at intervals of 30 minutes during a 4-hour period and analyzed morphologically for the integrity of capsule eggs and viability using an optical microscope (40X objective lens). This analysis was performed by evaluating 10 microscope fields.

2.5. Determination of LD50 and LT50 on Adult Parasites. Six concentrations of GP526 protein, in the range of 0.25 to 10 mg/mL, were evaluated. For each of the concentrations tested we used a petri dish containing 15 mL of RPMI 1640 and 42 cestodes incubated at 37°C. A control group was administered with distilled water and PMSF (vehicle), and another with the commercial multispectrum intestinal wormer drug Pfizer Canex (oxantel embonate 543 mg/pyrantel embonate 143 mg/praziquantel 50 mg). The viability was determined by direct microscopic observation of parasite motility prior to the treatments and after the different times or doses of incubation. Each test was carried out in triplicate.

2.6. Morphological Study. Segments of viable adult *D. caninum* were obtained and immediately fixed in 10%

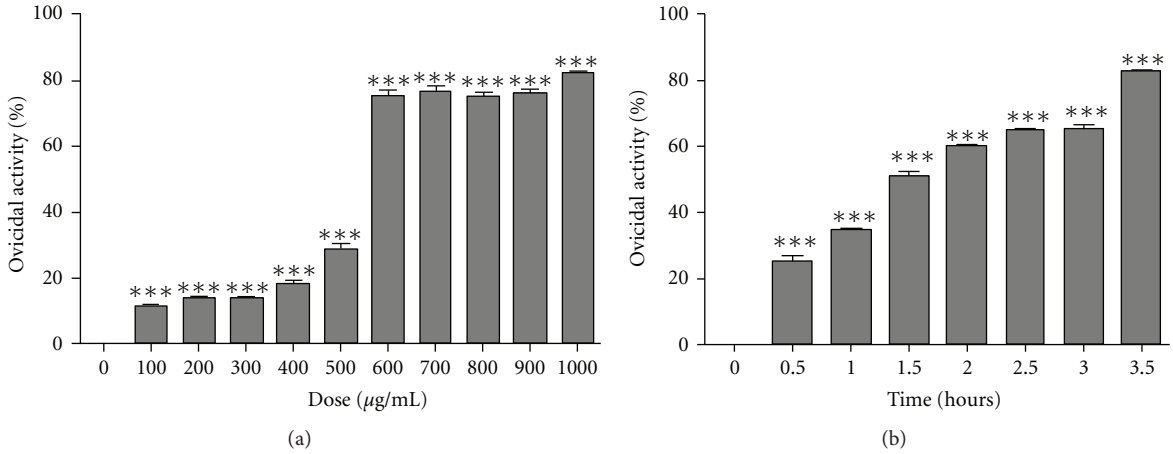

Figure 1: *B. thuringiensis* induces ovicidal effect on *D. caninum*. The ovicidal effect of *B. thuringiensis* is observed in a concentration-independent pattern (a) and maintained over time (b). In dose-response curves (panel (a)), *D. caninum* eggs treated with vehicle are referred as concentration zero. Data are represented as the mean ± SD. $^{***}P < 0.05$ compared to control.

paraformaldehyde during 72 h at 4°C [25]. The fixed segments were embedded in paraffin, and semiserial histological sections from 6 to 7 μm were obtained. Sections were stained with hematoxylin-eosin to observe the general histological structure and measure the thickness of the integument. Ten microscope fields utilizing a 40X objective lens were evaluated in each section, performing 5 measurements of the tegument thickness for each of the fields and photodocumenting the teguments with an image analyzer [26].

2.7. Statistical Analysis. Concentration-response and time-response curves were estimated from six independent experiments; each experiment was performed with 1000 eggs/mL and 42 adults, freshly extracted from infected donor dogs. Each experiment was replicated in 24 different wells. The response variable used in statistical analysis was the sum of morphological integrity of capsule eggs and viability in the 24 wells with each treatment and time of exposure of the experiments. Data from the six replications of each experiment were pooled and expressed as the mean ± S.D. Data were analyzed using either Student's *t*-test or one-way ANOVA and subsequently with Dunnett's Multiple Comparison Test, depending on the experimental design. The motility data were analyzed using a one-way ANOVA test followed by a Tukey-Kramer test. Data concerning tegument thickness was analyzed using a nonparametrical ANOVA and Dunn's test. Differences were considered to be statistically significant with $P < 0.05$.

3. Results

A dose- and time-dependent ovicidal effect of GP526 total protein was found, as shown in Figure 1. A clear 75% ovicidal effect is observed at 600 μg/mL of protein after 3.5 h after incubation. A higher dose (800 μg/mL), showed the same effect (75.5%) at 3.5 h, and the optimal effectiveness under our experimental conditions was of 82.75% using 1000 μg/mL of protein after 3.5 h of culture. In Figure 2, a panel of micrographs indicates morphological changes induced by *in vitro* treatment of eggs with 600 μg/mL of GP526 protein extract after 3.5 hours. The protein is capable of lysing the ovigerous capsule, the composition of which is enriched with polysaccharides and glycoproteins; next, lysis of the egg surface containing the exacant embryo is observed, inducing hatching and subsequently causing their lysis and dissolution.

Table 1: Percentage of motility in *D. caninum* after incubation with different dose of GP26 strain proteins.

Experimental group	Time after incubation	
	12 hours	18 hours
Control (vehicle)	99.2 ± 0.80	98.4 ± 0.80
0.25 mg/mL GP526	88.13 ± 4.12	88.13 ± 4.12
0.5 mg/mL GP526	88.13 ± 4.12	88.13 ± 4.12
1 mg/mL GP526	85 ± 0.80	81.8 ± 2.11
1.5 mg/mL GP526	81.8 ± 2.11*	81.8 ± 2.11*
2.0 mg/mL GP526	77.0 ± 2.09**	77.0 ± 2.09**
10.0 mg/mL GP526	0**	0**
Oxantel/Pirantel/Praziquantel	0**	0**

Significant differences among groups were assessed by Tukey-Kramer test ($^{*}P < 0.01$; $^{**}P < 0.001$). Three repetitions were performed using a total of 126 adult worms per group.

To determine the effect of the GP526 proteins in the adult worms, we tested random doses of 0.25, 0.5, 1.0, 1.5, 2.0, and 10.0 mg/mL on the motility of the parasite, and to determine if proteins of the bacteria were more or equally effective than commercial drugs, we used the commercial drug oxantel/pirantel/praziquantel as a positive control. We observed a 12% of reduction in motility in *D. caninum* using 0.25 mg/mL of GP526, and this percentage was decreased up to zero incubating with 10.0 mg/mL at 12 hours in culture (Table 1).

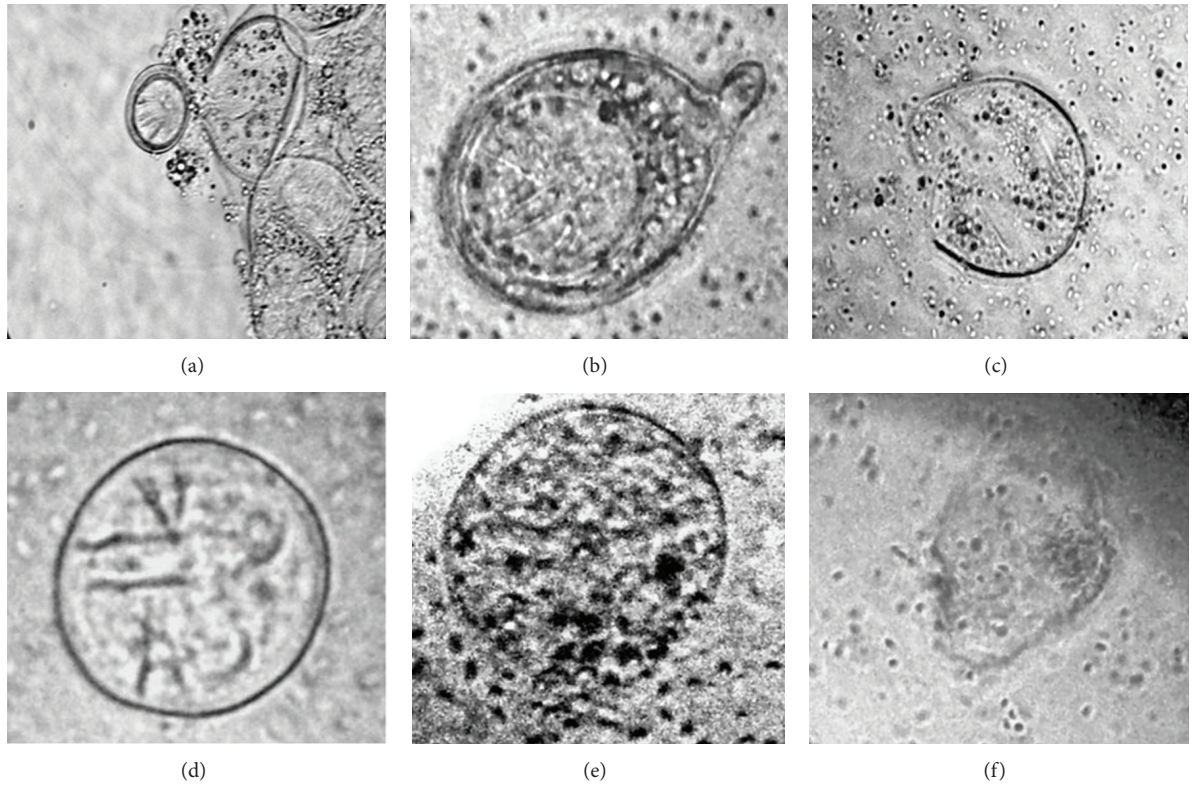

Figure 2: Lethal effect of the GP526 protein of *B. thuringiensis* on eggs of *D. caninum* (a). Lysis of the ovigerous capsule. (b) Fracture of the egg shell. (d) Exit of the exacant embryo. ((e) and (f)) Initial and final phases of the destruction of the exacant embryo.

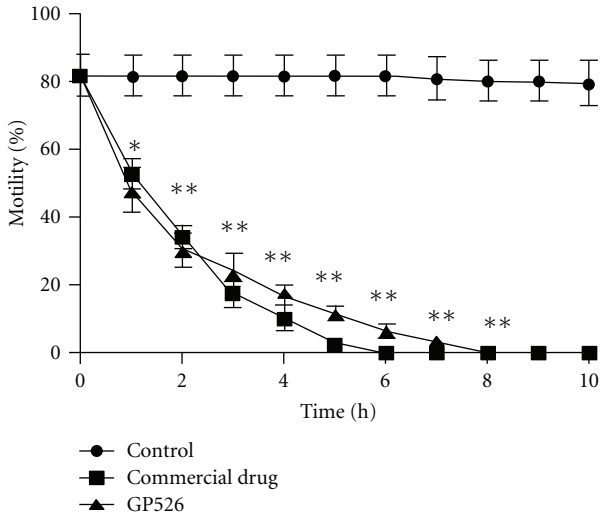

Figure 3: Total inhibition of motility in the adult cestode *D. caninum*. Total inhibition was reached by *in vitro* coincubation of the strain GP526 (10 mg/mL) of *B. thuringiensis* toxins and adult worms of *D. caninum*. *Significantly different from control group using a Tukey-Kramer test ($^{*}P < 0.01$; $^{**}P < 0.001$).

In Figure 3, we show the time-dependent effect of 10.0 mg/mL of GP526 or the commercial drug on the adult cestode. In the control group treated with oxantel/pirantel/praziquantel, we observed that GP526 induced an inhibition of 50% in the mobility starting at the first hour of treatment, and this effect was increased up to 70% of inhibition after two hours, reaching a total inhibition after six hours of culture. Interestingly, a notably similar effect was observed with GP526 treatment. To determine the mechanisms by which GP526 affected the adult worm motility, we evaluated the possible tissue damage induced by *B. thuringiensis*, analyzing histological sections of *D. Caninum* adults. The thickness of the tegument was studied demonstrating that the strain GP526 was able to reduce by 34% the thickness of the adult cestode tegument (from 17.85 ± 0.35 to 11.79 ± 0.41) at 8 h after incubation with a concentration of 10 mg/mL ($P < 0.001$) (Figure 4). It is interesting to note that the effect of oxantel/pirantel/praziquantel was stronger because it inhibited the tegument thickness by 42% (from 17.85 ± 0.35 to 10.27 ± 0.22).

4. Discussion

In this study, we found an ovicidal and cestocidal effect of the strain GP526 of *B. thuringiensis* on *D. caninum*. It could be useful as a biological control method to interrupt the parasite's life cycle, destroying the egg and preventing the flea from becoming infected, thus mitigating the infection of dogs or humans. It should be noted that there is no commercial drug that has shown ovicidal effect on parasites, particularly in tapeworms. This aspect could also be important in preventing autoreinfection, and for this

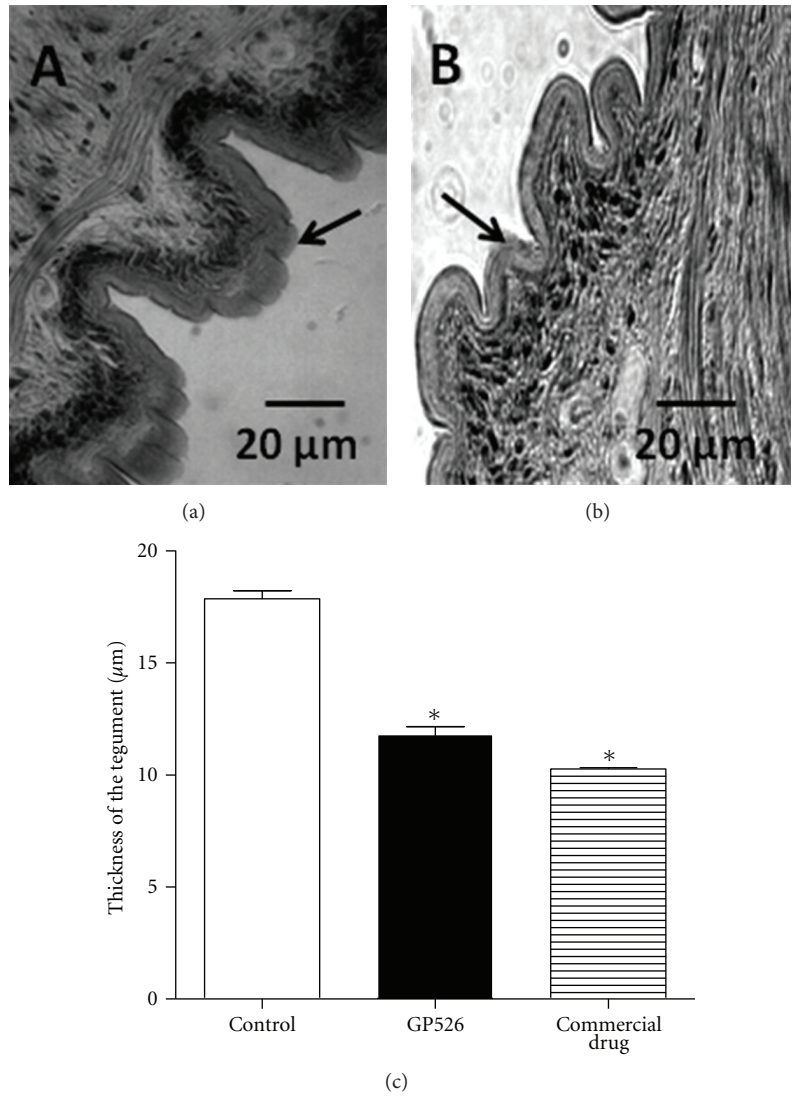

Figure 4: Reduction in the thickness of the tegument of *D. caninum* adult worms. Adult cestode untreated (a) or treated (b) with 10 mg for 8 hours post-incubation (40X). The strain GP526 of *B. thuringiensis* reduced the thickness tegument of the adult cestode *D. caninum* by 34% (c) (Dunn's test $^{*}P < 0.001$).

reason, considerable efforts are being undertaken in the search for drugs with ovicidal effects. Previous studies using the fungus *Pochonia chlamydosporia,* report that after 15 days of *in vitro* after incubation on *D. caninum* eggs, the fungus affected only 49.2% of egg survival [27]. However, using the *B. thuringiensis* GP526 strain, eggs were killed in a shorter period of time, showing greater effectiveness relative to the fungus. In the case of the fungus *P. chlamydosporia*, it has been proposed that the ovicidal effect is based on enzymatic activity, but with respect to *B. Thuringiensis,* the mechanism of action is unknown. Furthermore, we observed that the strain GP526 of *B. thuringiensis* inhibited 100% of the adult cestode motility *in vitro*. Although there are no reports of *B. thuringiensis* effectiveness against tapeworms, it has been reported that six species of nematodes were found to be susceptible to proteins of *B. thuringienis* with the result of decreased motility and growth [28]. In another nematode, such as *Haemonchus contortus*, which affects small ruminants, it has been possible to inhibit the motility after two days of incubation with a concentration of 110 μg/mg of body weight [5]. This concentration ratio is lower than the one observed in this work, wherein 100% of the motility of the adult cestode of *D. caninum* was inhibited (10 mg/mL of strain GP526). However, in our study, the inhibition of motility is faster, being recorded in only 8 h after incubation. The 100% effectiveness to inhibit motility in this study can be attributed to the way in which the cestodes have to nourish [29], unlike the nematodes. Cestodes feed throughout the tegument, meaning that the absorption surface of the protein may be higher, and because of the higher dose absorbed, the lethal time is shorter. It is important to take into account that at 10 mg/mL, the strain GP526 inhibited the motility of the cestode in a similar way to that of the commercial drug (oxantel/pirantel/praziquantel).

Biological control is the conscious use of living beneficial organisms, called natural enemies, for the control of pests [30]. Virtually all helminths, ecto- and endoparasites, have some natural enemies. Managing these natural enemies can effectively control parasite helminths [31, 32]. Often, the use of commercial drugs or other practices can create resistance to the drug; however, natural enemies are more difficult to manage by the parasite. Often, some of the most effective natural enemies of an organism are those that have coevolved with it in its native habitat. Natural enemies of parasites, particularly worms, must be carefully screened under rigid conditions to be certain that (1) they will provide benefit in controlling the target parasite, (2) they will not become parasites themselves, and (3) they do not harbor their own natural enemies that might interfere with their effectiveness or that of other natural enemies. The use of certain microbial insecticides (such as those containing *Bacillus thuringiensis*) is an example of inundation [33–35]. *Bacillus thuringiensis* is commonly used for controlling European corn borer larvae [36]. There is no doubt that well-researched applications of natural enemies can be highly effective. This approach includes the use of microbial insecticides, as well as many specific uses of predators of parasites and parasitic insects. Most likely, the common thread that exists with "failures" in the use of biological control of parasitic worms is a lack of knowledge. This ignorance encompasses both a lack of research needed to make recommendations for successful implementation and the user's lack of knowledge regarding the biology of parasites, their natural enemies, and their environment, all of which are crucial to successful biological control.

5. Conclusions

Taking into consideration our findings, *B. thuringiensis* may have a dual therapeutic effect: egg removal and inhibiting the motility of the adult cestode and the integrity of its tegument. Although the commercial drug shows a greater effect on the thinning of the tegument of *D. caninum,* the drug has no effect on the eggs. Furthermore, *B. thuringiensis* has the advantage of being harmless to the treated animal and does not have the adverse effects related to the trade drug [2], which makes *B. thuringiensis* a potential candidate for use as antihelminthic in carrier animals. Future studies will be needed *in vivo* and at the tegument level to explain the molecular mechanisms of action by which proteins of strain GP526 acts against the adult cestode and eggs of *D. caninum*. Consideration of the biological and ecological needs of natural enemies is critical for the success of any biological control effort. While there are innumerable practices in the production system that may benefit or harm the natural enemies that researchers are seeking to manage, understanding the biology and life cycles of the desired species to conserve is the first step to achieving the best results.

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Research Article

Taenia crassiceps Infection Does Not Influence the Development of Experimental Rheumatoid Arthritis

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It was previously reported by our group that infection with *Taenia crassiceps* reduces incidence and severity of inflammatory and autoimmune experimental diseases like type 1 diabetes and experimental autoimmune encephalomyelitis. In this research, we set out to study whether infection with *T. crassiceps* would affect the development of experimental rheumatoid arthritis (RA). We found that mice infected with the parasite and induced with experimental RA showed similar clinical scores as the noninfected experimental RA group; systemic cytokines were not affected while anti-CII Abs were higher in the infected group. Histological evaluation showed damage in both infected and noninfected experimental RA-induced groups and although some surface molecules such as PDL-2 and MR which are associated with immunomodulatory mechanisms were upregulated in the infected and RA-induced group as compared to the noninfected RA group, they did not exert any changes in the outcome of experimental RA. Thus, we determined that infection with *T. crassiceps* does not influence the outcome of experimental RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease with an estimate prevalence of 0.5%–1% around the world; it is characterized by inflammation and tenderness of the joints, which could lead to physical incapacity and even premature death due to complications. RA is an autoimmune disease, although the immunopathogenesis of RA is very complex to define due to the fact that a definitive cause of the disease has not yet been elucidated. Furthermore, the genetic background, exposure to environmental factors, and infectious agents contribute to the susceptibility, making it a multifactorial disease [1, 2].

Different signaling and effector pathways take place in the synovium in RA, and as a result a cascade of pathophysiological events leads to the progressive destruction of the joint. In normal conditions the synovium is acellular, and the membrane is mainly composed of macrophages and synovial fibroblast that surround all of the joints, but in RA the synovial membrane is infiltrated by macrophages, synovial fibroblasts, T cells, and B cells. Chronic inflammation and the hyperplastic synovial membrane lead to articular destruction. Macrophages and synovial fibroblast are important sources of proinflammatory cytokines like TNF-α and IL-1β; these cytokines are involved in various mechanisms that perpetuate the inflammatory milieu [3], as they up regulate NFκB which in turns trigger the release of more pro-inflammatory cytokines and chemokines (e.g., IL-6, IL-8); in addition, these cytokines induce the expression of matrix degrading enzymes, the metalloproteinases (MMP) MMP1, MMP3, MMP8, MMP13, and MMP14 [4]. Furthermore TNF-α and IL-1β also upregulate (receptor activator of nuclear factor-κB (RANK) ligand RANKL), which is necessary for the differentiation of osteoclasts; these cells are responsible for bone

remodeling, but in RA the constant up regulation of RANKL leads to bone destruction. The involvement of T cells in RA has been very clear, since T cells have been found in RA patients synovium [5]; classically RA as well as other autoimmune diseases was classified as being one of a Th-1 phenotype based on the cytokine milieu found in experimental models of RA [2]. But with the recent discovery of IL-17 producing Th17 cells, new evidence seems to implicate even more this phenotype that may synergize with TNF-α and IL-1β, although we cannot rule out the participation of Th1 cells [6].

Most of the insight that have been gained in the development in the field of immunology have been made possible by the use of experimental murine models; the gold standard model for the induction of RA has been the collagen induced arthritis (CIA) model, that is based on the induction of type II collagen (CII) antibodies by immunization of DBA/1 mice with heterologous CII in Complete Freund's Adjuvant (CFA), with onset of the disease around day 40–50 postinoculation of the CII, resulting in infiltrating and hyperplasic synovium with erosion of bone and cartilage. Among other models of experimental RA, one that is able to break self-tolerance by inducing CII Abs production without the immunization of CII, is an experimental model of adoptive T cell transfer, in which DO11.10 $CD4^+$ T cells (which specifically recognize a small ovalbumin peptide, $OVA_{323–339}$) are skewed to a Th-1 phenotype by IL-12 and after culture are i.v. transferred followed by immunization of OVA in CFA and challenged with heat aggregated OVA (HAO). This experimental model is characterized by synovial infiltration and CII antibody production [7].

In the last two decades there has been a marked increase in inflammatory autoimmune diseases including multiple sclerosis (MS), type 1 diabetes (T1D), inflammatory bowel diseases (IBD: chron's disease and ulcerative colitis), rheumatoid arthritis (RA), as well as allergy, especially in developed countries. The observation that early and constant contact with pathogens reduced allergy was introduced by David Strachan, which led to the now known hygiene hypothesis, which was later modified to include autoimmune diseases [8]. The increase in hygiene, use of antibiotics, and less contact with infectious agents like helminthes and their products appear to be factors in the raise of allergic and autoimmune diseases [9]. Helminth parasites are known to modulate exacerbated immune responses; evidence of this comes from the observations that the incidence of allergy and autoimmune diseases is lower in underdeveloped countries where contact with helminths is constant [9].

The growing reports on experimental models of helminth-based therapy for relieving symptoms of inflammatory autoimmune diseases have showed promising results [10], recently our group has proven that infection with *Taenia crassiceps* reduced incidence and severity to T1D [11] and experimental autoimmune encephalomyelitis (EAE, a model for MS) [12], and protection was associated with the immune-regulatory mechanisms induced by the parasite. Thus, the focus of this research was to characterize the immune response induced by the infection with *T. crassiceps* on the outcome of experimental RA.

2. Material and Methods

2.1. Mice. Six-to seven-week-old male BALB/cAnN mice were purchased from Harlan Laboratories (Mexico) and were used as adoptive transfer recipients. Ten-to twelve-week-old male DO11.10 BALB/c TCR Tg mice that contain $CD4^+$ T cells expressing a TCR that recognizes the chicken $OVA_{323–339}$ peptide complexed with the MHC class II molecule $I\text{-}A^d$ (detected by the clonotypic mAb KJ1.26) were obtained from The Jackson Laboratories (Bar Harbor, Maine USA); all animals were housed in a pathogen-free environment at the FES-Iztacala, U.N.A.M. animal facility in accordance with Institutional and National guidelines.

2.2. Parasites and Infection. Metacestodes of *Taenia crassiceps* were harvested in sterile conditions from the peritoneal cavity of female BALB/c mice after 2–4 months of infection. The cysticerci were washed four times in sterile phosphate buffered saline 1X (PBS 1X; 0.15 M NaCL, 0.01 M sodium phosphate buffer, pH 7.2) and used for mouse infection. Mice were infected with an intraperitoneal (i.p.) injection of 20 small, nonbudding cysticerci of *T. crassiceps* suspended in 0.3 mL of sterile PBS 1X.

2.3. Induction of Experimental RA. After DO11.10 BALB/c TCR Tg mice were humanly sacrificed, spleens were extracted and $CD4^+$ T cells were purified by positive selection with anti-$CD4^+$ mAbs bound by anti-IgG MACS beads (Militenyi Biotec, Auburn, CA), according to manufacturer's instructions. APCs were obtained from the peritoneal cavity and cultured for 2 h in supplemented RPMI medium, then washed twice with RPMI to discard nonadherent cells from the plates (Costar, MA. USA). Th1 cell differentiation was carried out culturing 2×10^5/mL $CD4^+$ T cells with 2×10^6/mL APC, 0.3 μM $OVA_{323–339}$, and 5 ng/mL IL-12 (Peprotech Rocky Hill, NJ, USA) and 10 μg/mL anti-IL-4 mAb. After 72 hours of culture, cells were washed and harvested for cell transfer. A total of 2×10^6 DO11.10 $CD4^+$ cells was injected i.v. via the tail vein into BALB/c recipient mice, and control mice received sterile PBS 1X only. One day after adoptive transfer, mice were immunized (subcutaneous, s.c.) with 100 μg of OVA in CFA [7].

2.4. Assessment of Experimental RA. Ten days after immunization with OVA in CFA, all animals were injected s.c. close to both rear ankle joints with 100 μg of HAO. Mice were monitored for sign of arthritis, and disease was scored based on erythema and swelling in each paw on a scale of 0–3, giving a maximum score of 6 per mouse. Paw thickness was measured for seven days with a dial caliper (Mitutoyo, Japan).

2.5. Histology. Mice were sacrificed, and hind paws were removed and fixed in paraformaldehyde. Tissue was, then, processed and embedded in paraffin; 7 μm sections were cut for histological analysis and stained with H&E.

2.6. Anticollagen Specific IgG1 and IgG2 Abs Detection. Briefly, 96 well microplates (Nunc, Denmark) were coated with CII

diluted in 0.1 M de $NaHCO_3$ (pH 9.6) to a final concentration of 10 μg/mL; 100 μL of the solution was added to each well and stored overnight at 4°C, then plate were washed twice in PBS 1X 0.05% Tween 20 (USB Corporation, USA, washing buffer). Plates were blocked in PBS 1X in 1% BSA (blocking buffer) by adding 200 μL of the solution to each well and incubated 1 h at 37°C, then washed three times in washing buffer. Serum samples were diluted in series starting at 1 : 50 in blocking buffer, and 100 μL of the diluted samples was added to the microplate and incubated 1 h at 37°C, then washed four times in washing buffer. Peroxidase conjugated antimouse IgG1 and IgG2a (Zymed, USA) was diluted at a concentration of 1 : 1000 in blocking buffer, 100 μL were added to each well, and they were incubated 45 minutes at 37°C. Microplates were washed four times in washing buffer, 100 μL of hydrogen peroxide (3% H_2O_2) in 11 mL of 2,2′-azino-bis(3-ethilbenzthiazoline-6-sulphonic acid) was prepared, and 100 μL of the solution was added as substrate to each well; OD values were measured at 405 nm using the Multiskan Ascent Thermo Lab Systems (AIE, USA) microplate reader.

2.7. Cytokine Detection. Peripheral blood was collected from tail snips at day 2, 5, and 15 postchallenge with HAO. Serum cytokine IL-1β, IFN-γ and IL-4 levels were measured by sandwich ELISA using commercial kits purchased from PeproTech (Mexico) following manufacturer instructions. Briefly, captured antibody 2 μg/mL was diluted in 10 mL of PBS 1X, 100 μL was added to each well in 96 well Maxisorb microplate (Nunc, Denmark), and was incubated overnight at 4°C. Microplates were then washed twice with washing buffer, and plates were then blocked by adding 200 μL of blocking buffer to each well and incubated for 2 h at 37°C. Plates were then washed three times in washing buffer. Recombinant mouse cytokines were used for standard curves starting at a concentration of 50 ng to 0.01 ng. Serum samples were diluted at a concentration of 1 : 5 in 100 μL of blocking buffer, plates were incubated two hours at 37°C. Plates were washed four times in washing buffer, biotinylated antibody final concentration 1 μg/mL was diluted in blocking buffer, and 100 μL of the solution was added to each well, plates were incubated one hour at 37°C, then washed four times in washing buffer. Avidin (PeproTech Inc, USA) was diluted 1 : 4000 in blocking buffer; 100 μL of the solution was added to each well, and the plates were incubated 30 minutes at 37°C and then washed five times in washing buffer. 100 μL of the same substrate was used as above. OD values were measured at 405 nm using the Multiskan Ascent Thermo Lab Systems (AIE, USA) microplate reader.

2.8. Isolation of Peritoneal Macrophages. After mice were sacrificed at the end of the experiment peritoneal exudates cells (PEC's) were obtained using 5 mL of ice cold sterile PBS 1X, and the red blood cells were lysed by resuspending the cells in Boyle's solution. Following two washes, the viable cells were counted by trypan blue exclusion with a Neubauer hemocytometer. PECs were adjusted 1×10^6/mL in modified RPMI medium.

2.9. Antigen Specific Stimulation. Spleens were removed 15 days post-HAO injection, and total cells were counted by trypan blue exclusion and adjusted to 3×10^6 and then plated to a final concentration of 3×10^5 cells/well. Then, cells were cultured with 50 μg/mL of OVA peptide for 72 h, supernatants were then collected and stored until cytokine determination.

2.10. Analysis of Cell Surface Markers in Macrophages. The Fc receptors in peritoneal macrophages were blocked with anti-mouse CD16/CD32 (Biolegend, CA, USA) and then stained with an FITC-conjugated monoclonal antibody against F4/80, PE-conjugated antibodies against PD-L1 and PD-L2, and APC-conjugated antimannose receptor antibody (all obtained from Biolegend). The stained cells were analyzed on a FACs Calibur flow cytometer using Cell Quest software (Becton Dickinson).

3. Results

3.1. Th1 Polarization Was Induced in the DO11.10 $CD4^+$ T Cells. $CD4^+$ T cells from spleens of DO11.10 mice were purified by positive selection with anti-$CD4^+$ mAbs (97.88% were $CD4^+$, Figure 1(a)) and 91.88% were $KJ1.26^+$ $CD4^+$ (Figure 1(b)), and so more than 90% of the $CD4^+$ cells were positive for the OVA TCR. Th1 polarization was then induced in these purified cells by coculture of DO11.10 $CD4^+$ T cells and macrophages plus IL-12 and $OVA_{323–339}$ peptide. IFN-γ levels were measured in the supernatants to verify the phenotype, and cells stimulated with OVA plus IL-12 produced significantly higher levels of IFN-γ as compared to the cells that received no stimuli (Figure 1(c)). Therefore, DO11.10 $CD4^+$ T cells were polarized to a Th1 phenotype.

3.2. Assessment of Experimental Arthritis. Experimental RA was induced by the transfer of Th1-type DO11.10 $CD4^+$ T cells in recipient BALB/c mice (RA), and after 6 weeks of infection with *T. crassiceps* (Tc + RA), control mice received unpolarized cells (Control), as well as the infected group (Tc); all groups were immunized with OVA in CFA followed by a periarticular injection near the ankle joints with HAO in both rear paws. RA was then evaluated in the four experimental groups based on erythema and swelling in each paw on a scale of 0–3, giving a maximum score of 6 per mouse for seven days. The not-transferred DO11.10 $CD4^+$ T cell Control and Tc groups showed minimum clinical scores compared to the transferred RA and Tc + RA groups. The RA-induced group reached a peak in clinical signs around day 3 with a clinical score around 3 (Figure 2(a)); thereafter, clinical signs were reduced. Similarly, infected RA-induced mice (Tc + RA) reached a peak between days 2–4 with a maximum score of around 2. Tc+ RA group had slightly lower clinical scores than uninfected RA group, but differences were not significant among the two groups. Significant differences were found among the RA versus Control group on days 2–5, as well as with Tc + RA versus Tc group on days 2–4. Furthermore, measurements of paw thickness showed similar results within groups (Figure 2(b)), again the Control and Tc groups

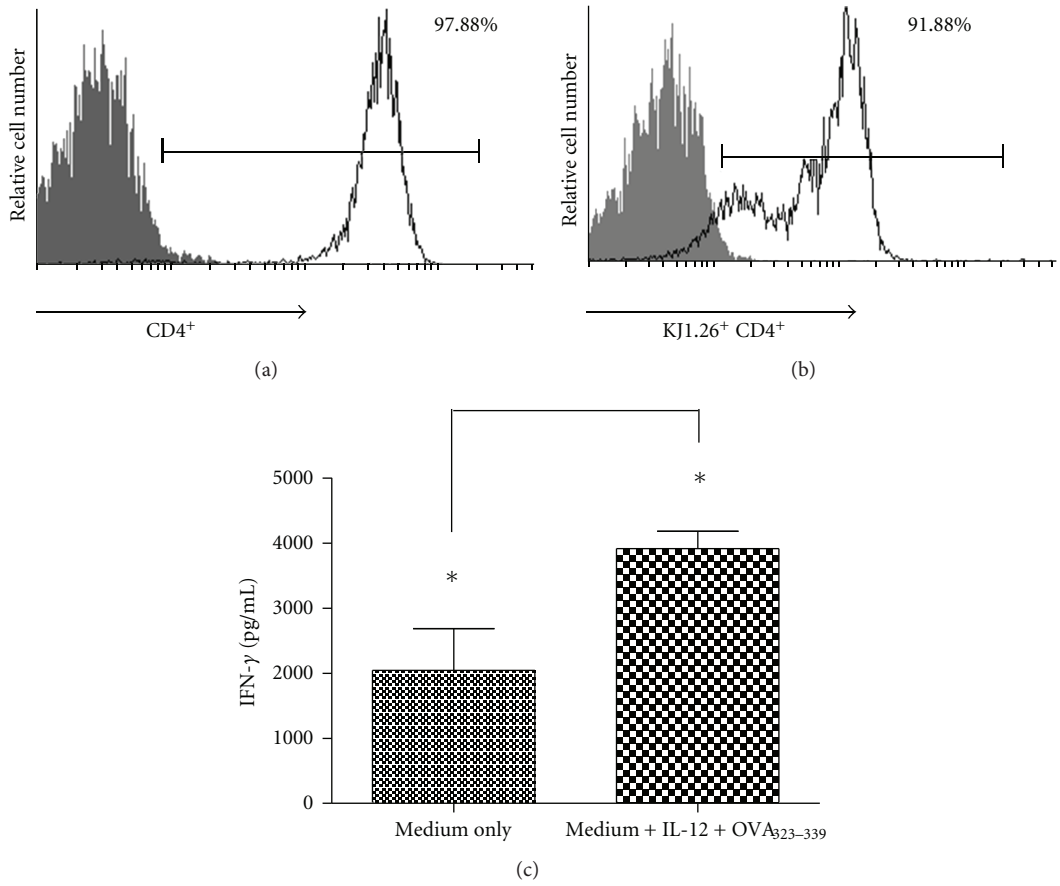

Figure 1: KJ1.26$^+$ CD4$^+$T cell purification and Th1 polarization of DO11.10 cells. CD4$^+$ T cells from spleens of DO11.10 BALB/c TCR Tg mice were purified: (a) 97.88% were CD4$^+$ and (b) 91.88% were KJ1.26$^+$ CD4$^+$. Th1 polarization, then, was induced in purified cells, (c) IFN-γ levels were measured in the supernatant of cocultures of DO11.10 CD4$^+$ T cells, and macrophages, IL-12, and $OVA_{323–339}$ peptide were used. $^*P < 0.05$, T-test.

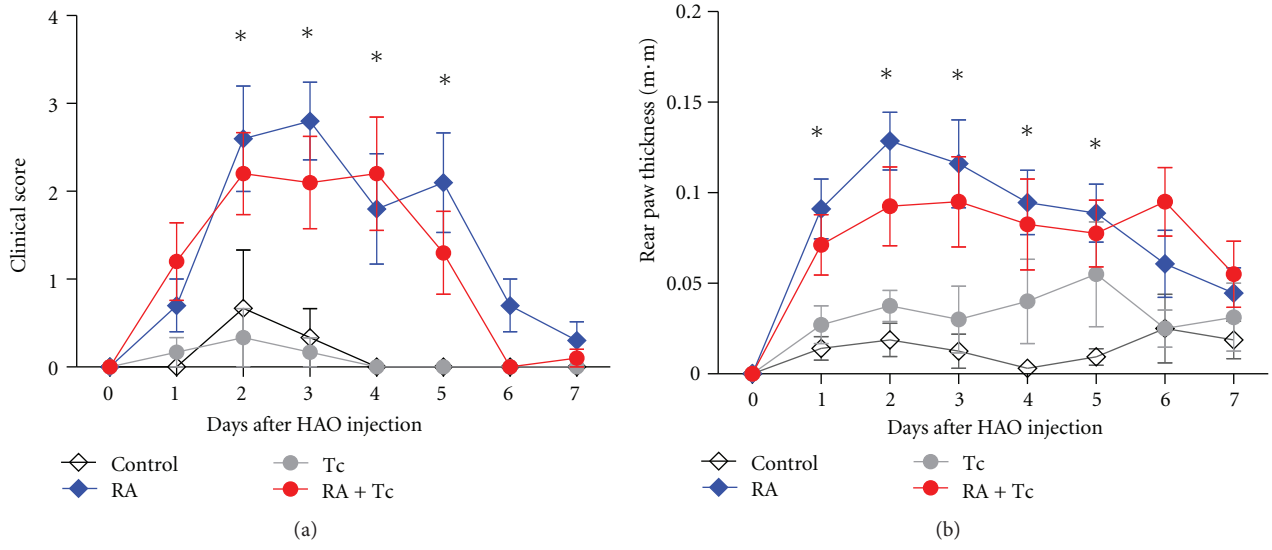

Figure 2: Clinical assessment of experimental arthritis. (a) Clinical score was evaluated based on erythema and swelling in each paw on a scale of 0–3, giving a maximum score of 6 per mouse. (b) Paw thickness was measured on both rear paws and was added to obtain an average measure for each mouse; the measures were taken seven days after HAO challenge. Statistical analysis, two-way ANOVA, $^*P < 0.05$.

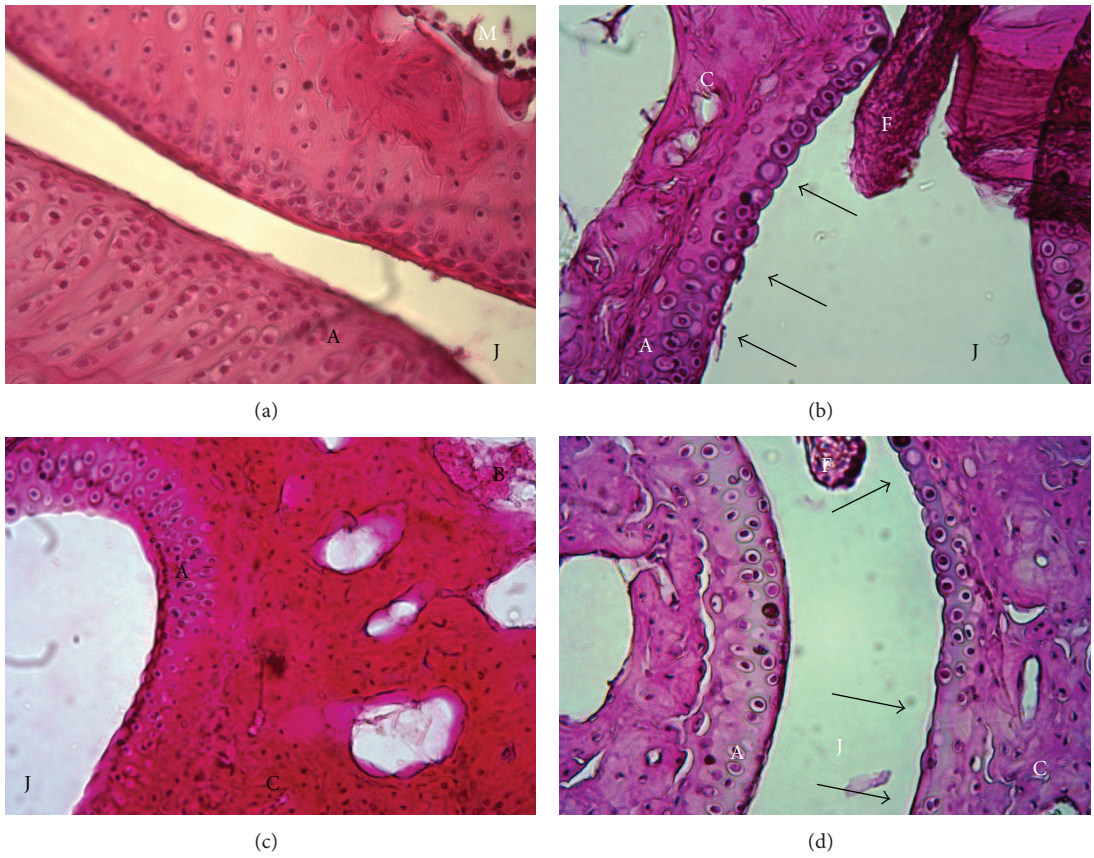

Figure 3: Histological evaluation. H and E staining was performed on sections of the distal phalanges joints. (a) Control; (b) RA; (c) Tc; (d) Tc + RA. Arrows show empty chondrocytes as well as isogenic bodies on cartilage. Magnification showed is 40X. J: joint cavity, A: articular cartilage, F: fibrous joint capsule, B: bone, C: calcified cartilage, and M: bone marrow. Arrows show empty chondrocytes as well as isogenic bodies on cartilage.

displayed minimum paw thickness. The RA group showed greater paw swelling than the Tc + RA group, which seem to have a slight tendency to decrease, but was not enough to show statistically significant differences between these groups. In contrast, the Control group showed significant differences with both transferred groups, on days 1–5 with the RA group and on days 2–4 with the Tc + RA group. These similar results obtained from the clinical scores and measurements of the paw thickness showed that infection with *T. crassiceps* does not seem to influence the outcome of experimental RA.

3.3. Histological Evaluation. After experimental RA was induced, 15 days post-HAO injection, all groups of mice were sacrificed; hind limbs were extracted, embedded in paraffin and sections of the distal phalanges joint were stained with H and E. The Control group (Figure 3(a)) and the Tc group (Figure 3(c)) showed normal arrangement of the joint cartilage, unlike the RA group (Figure 3(b)) which displayed empty chondrocytes which are characteristics of cartilage erosion; isogenic bodies were also found which are also commonly found in RA histology due to the fact that chondrocytes are in division trying to replace the loss of these cells in the cartilage of the joints; the same histological observations were seen in the Tc+RA group as well (Figure 3(d)). Since we found the same type of damage in the Tc + RA group as in the noninfected RA group, these results also indicated that infection with the parasite did not seem to modulate experimental RA.

3.4. Infected- and Experimental RA-Induced Mice Produce Higher Levels of Anti-CII IgG1 and IgG2a Antibodies. Anti-CII antibodies are pathogenic determinants of arthritis, therefore, IgG1 and IgG2a Abs were measured; the Control group produced irrelevant levels of anti-CII IgG1 and IgG2 Abs (Figures 4(a) and 4(b)). Interestingly, both infected groups Tc and Tc + RA produced higher levels of anti-CII IgG1 Abs even than the RA group. A similar finding was observed with anti-CII IgG2a. Labeling experiments have showed that many helminths possess a surface rich in collagen [13], so our data showing higher levels of anticollagen antibodies in the infected groups may be associated with this feature of the helminths surface. Significant differences were also observed in the IgG1 antibodies between the RA versus Tc + RA in the 1 : 10 dilution, whereas in the Control versus Tc + RA groups the difference was observed also in 1 : 20 dilution. In the IgG2a Abs Control versus RA and Control versus Tc + RA showed significant differences in the

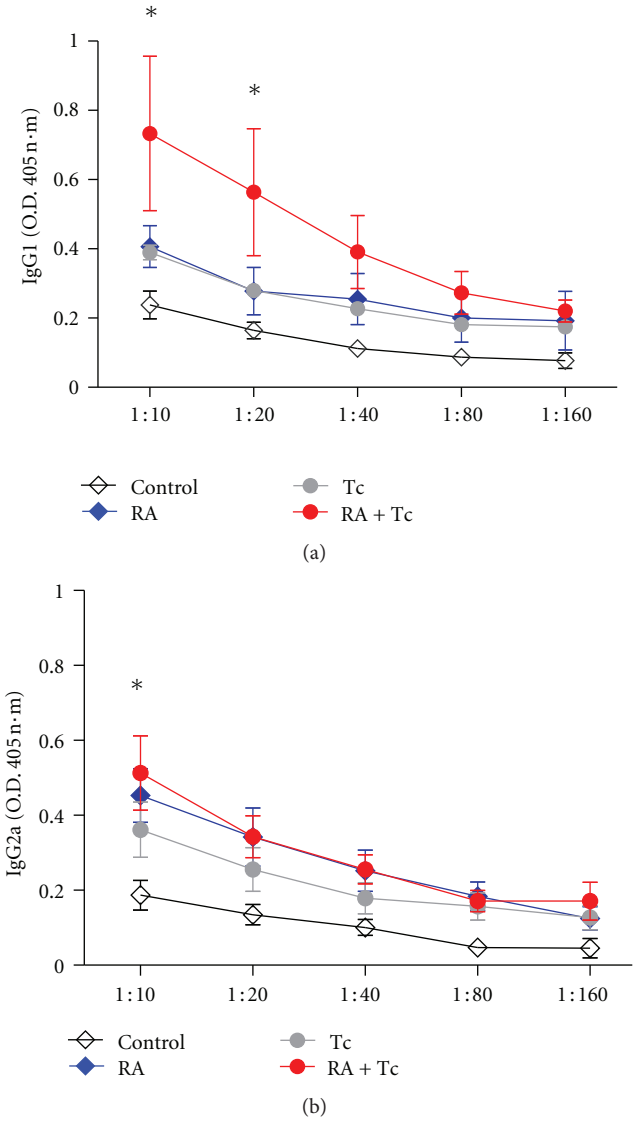

FIGURE 4: Infected and RA-induced mice produce higher levels of serum anti-CII IgG1 and IgG2 antibodies. Levels of (a) anti-CII IgG1; and (b) IgG2a Abs were measured in all of the groups sera were obtained 15 days post-HAO injection. Significant differences were observed in the IgG1 Abs between the RA versus Tc + RA and Tc versus Tc + RA in 1 : 10 dilution and Control versus Tc + RA in the 1 : 10 and 1 : 20 dilutions. In the IgG2a Abs Control versus RA and Control versus Tc + RA showed significant differences in the 1 : 10 dilution. Statistical analysis, two-way ANOVA, $^{*}P < 0.05$.

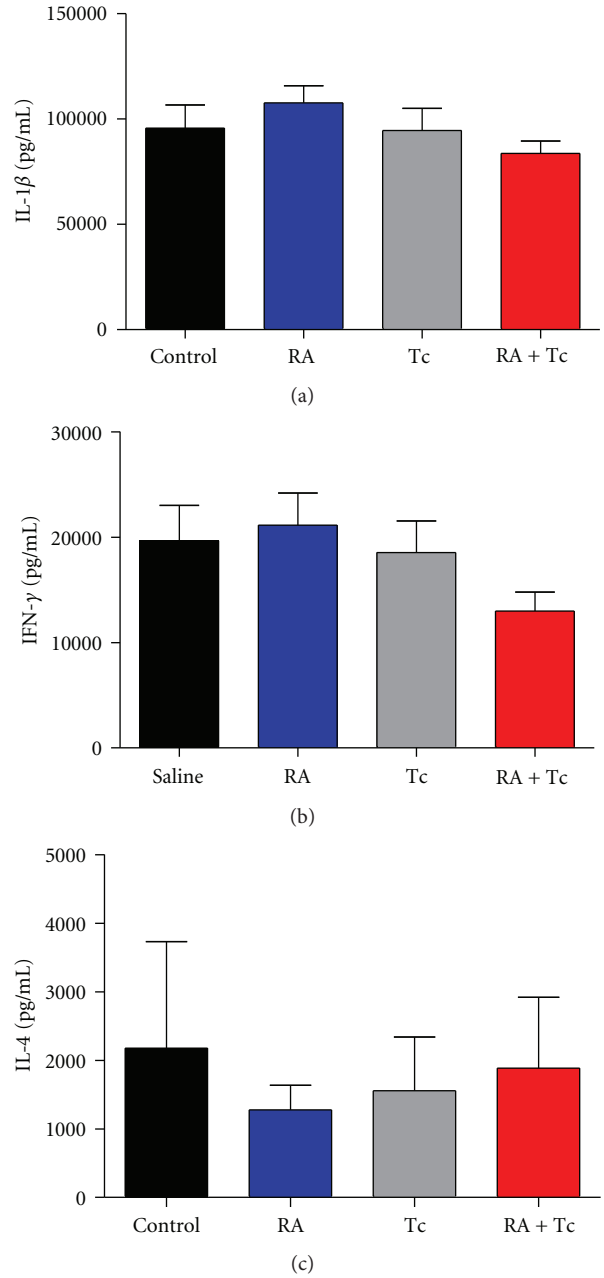

FIGURE 5: Infection with *T. crassiceps* does not modify systemic serum cytokine levels. Sera were obtained 5 days post-HAO injection, IL-1β, IFN-γ, and IL-4 cytokine levels were measured. No significant differences were observed within the groups.

1 : 10 dilution. These results are also in agreement with the data from the clinical score and measurements, since again infection with *T. crassiceps* did not reduce anti-CII IgG1 and IgG2a Abs, on the contrary both specific anti-CII antibodies were augmented.

3.5. Infection with T. crassiceps Does Not Modify Systemic Serum Cytokine Levels. Sera from all experimental groups were obtained 5 days post-HAO injection; Th1-type cytokines IL-1β (Figure 5(a)), IFN-γ (Figure 5(b)) and Th2-type cytokine IL-4 (Figure 5(c)) levels were measured. We did not observe significant differences within the groups. Although we did observe a tendency to decrease the levels in the pro-inflammatory cytokines IL-1β and IFN-γ in the infected groups and even more in the Tc + RA group, however, such differences did not reach significant values. Also the Tc + RA group displayed higher levels of IL-4 than Tc group; thus, this effect could be attributed to the parasite. The Control group had higher levels of IL-1β and IFN-γ than expected; this could have been the result of the strong Th1-type response

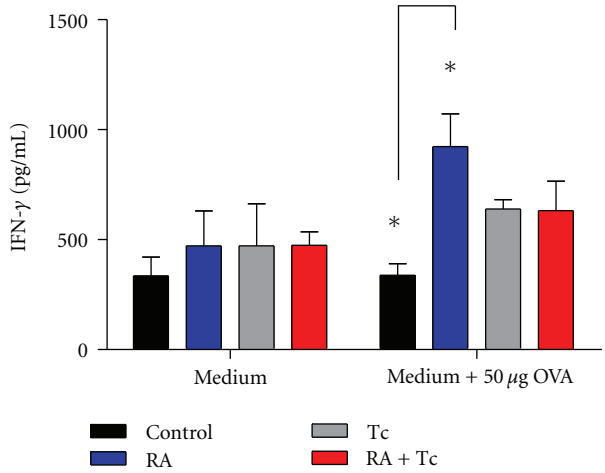

FIGURE 6: Antigen specific IFN-γ production. Spleens were extracted and cultured with medium alone or with OVA to determine IFN-γ levels. Significant differences were only observed in the Control versus RA group after stimulation. Statistical analysis, two-way ANOVA, $^{*}P < 0.05$.

induced by the immunization with OVA in CFA followed by the injection of the joints with HAO.

3.6. Antigen Specific IFN-γ Production. In order to determine if the antigen specific response was modified by the presence of *T. crassiceps* infection, total splenocytes were extracted and cultured with medium alone or stimulated with OVA to determine IFN-γ levels in the supernatants. Significant differences were observed in the Control versus RA group after stimulation with OVA, as expected after RA induction with OVA mice produced significant amounts of the Th1 cytokine (Figure 6). In agreement with previous works [14], *T. crassiceps* infection suppresses the antigen specific response of $CD4^{+}$cells; thus, culture of splenocytes from *T. crassiceps*-infected groups did not produced significant amounts of IFN-γ after they were stimulated with OVA peptide.

3.7. AAM Surface Markers Are Upregulated in the RA-induced T. Crassiceps-Infected Mice. Flow cytometry was performed in order to analyze the presence of AAM during RA in mice-bearing *T. crassiceps*. MFI was analyzed on the AAM markers PD-L1 (Figure 7(a)), PD-L2 (Figure 7(b)), and MR (Figure 7(c)), and we found that the Tc + RA group had significantly increased expression of PDL-2 and MR on AAM than the noninfected groups; but in comparison with the Tc group, the differences were not statistically significant, although in both cases again it seems to be a pattern to increase in the Tc + RA group in contrast to the Tc group. AAM are known for their suppressive capacity; PD-ligands down-regulate T cell proliferation through direct contact with their receptor PD-1, usually expressed in T-activated cells [14]. The higher expression of PDL-2 and MR in Tc + RA could be an effect of the immunomodulatory mechanisms turned on for this parasite to down regulate the inflammatory response from the RA.

4. Discussion

In recent years there has been development of the now-called "helminth therapy" which basically states that infection with helminths or their products could potentially alleviate and improve inflammatory autoimmune diseases, due to the fact that helminths induce Th2 type phenotype that is able to antagonize the Th1 type phenotype of inflammatory autoimmune diseases as well as induce a series of regulatory cells such as AAMs, Tregs, and myeloid suppressor cells [10]. Proof of this comes from epidemiological observations, clinical studies as well as by experimental models and recent reviews [15].

Our group has recently demonstrated that infection with *T. crassiceps* reduces the incidence and severity to T1D [11], EAE [12], and colitis (unpublished observations) in murine models, and such protection has been associated with immunoregulatory mechanisms induced by this parasite. In contrast, the results showed by this study indicated that infection with *T. crassiceps* metacestodes does not alter the outcome of experimental RA.

Our data revealed that quantification of IgG2a anti-CII antibodies which are used as an indication of RA, showed, as expected, in the Contol group almost no production of anti-CII Abs, and the RA group did show significant amounts of antibodies in accordance with the disease, but unexpectedly the Tc + RA group had higher levels of IgG2a Abs compared to the RA group; apparently this could be due to the composition of the parasite itself [13] since the helminth own collagen could be interfering with the actual levels of CII antibodies due to the disease; this is an interesting fact that has not been reported before. Also the level of pro-inflammatory systemic cytokines showed similar results as the assessment of arthritis, where a tendency to decrease in the Tc + RA group was observed, but the other groups had similar amounts of IL-1β and IFN-γ; this could be due to the strong effects of the immunization with CFA plus OVA as well as the challenge injection with HAO. Infection with *T. crassiceps* increased expression of PDL-2 and MR which are markers of AAM in the Tc + RA compared to the RA non-infected group; it has been showed that infection with this parasite increased expression of PDL-1 and PDL-2 on AAM [14]; these ligands are involved in the PDL's pathway which is responsible for the inhibition of proliferative response of T cells in a contact-dependent manner. Thus, the increase of PDL-2 expression could be an attempt to inhibit the inflammatory response from arthritis, but, in this case the parasite immune modulation is just not enough to overcome the intense inflammatory response of the experimental RA.

The gold standard model for the induction of experimental RA has been the collagen-induced arthritis (CIA) model, with onset of the disease around day 40–50 after inoculation of the CII, resulting in infiltrating and hyperplasic synovium, erosion of bone and cartilage, thus, displaying a very similar pathology as in humans [16]. Amelioration of CIA has been proven successful in infection with *Schistosoma mansoni* [17] and *S. japonicum* where is stage-dependent [18]; in both cases proinflammatory cytokines were down-regulated in infected mice as compared to noninfected. On the other

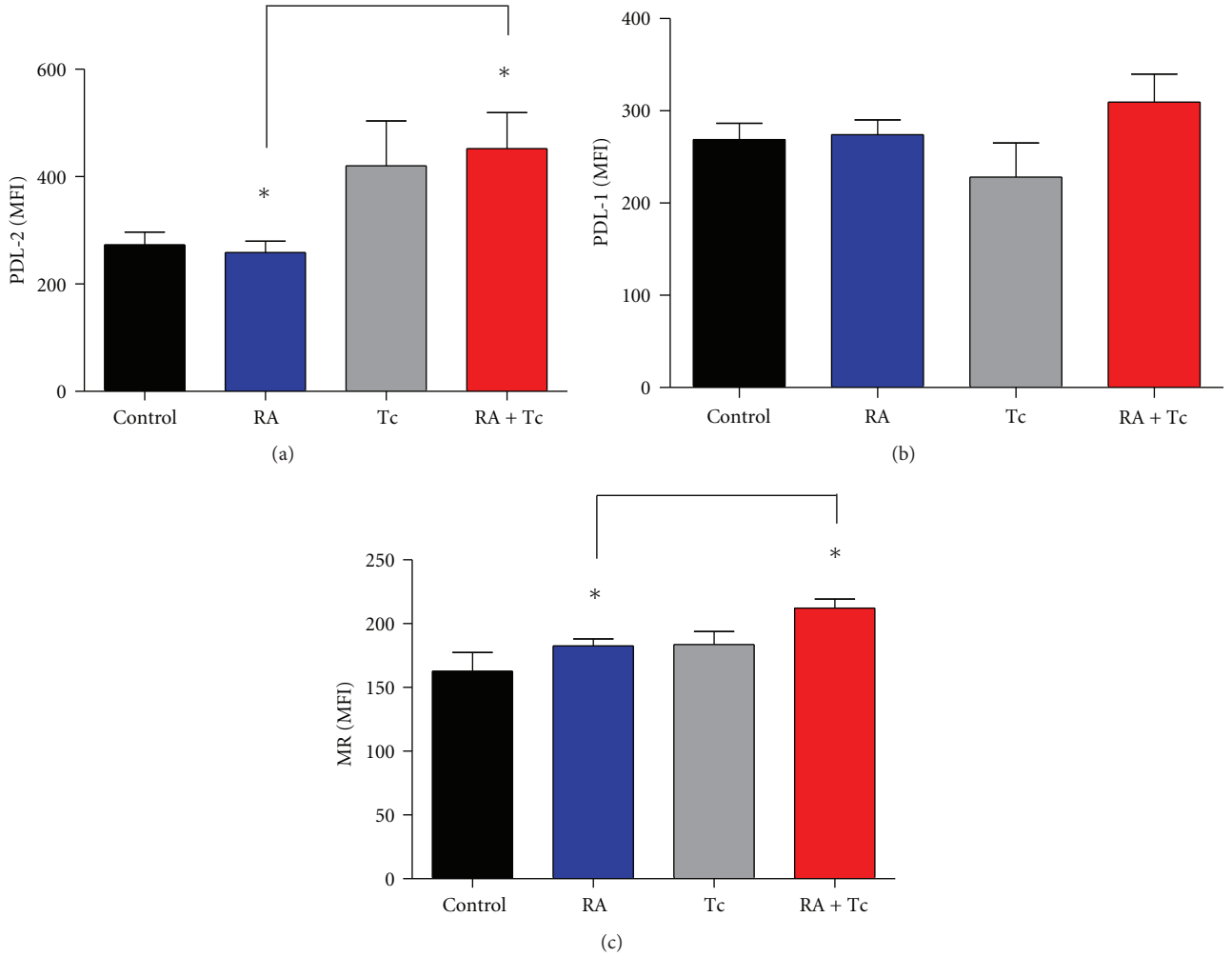

Figure 7: Flow cytometry analysis for alternatively activated macrophage (AAM) surface markers. AAM markers are up-regulated in the experimental rheumatoid arthritis induced on *T. crassiceps*-infected mice. Analysis of medium fluorescence intensity (MFI) on AAM markers PD-L1, PD-L2, and MMR was done. Statistical analysis, one-way ANOVA, $^{*}P < 0.05$.

hand, Osada and Kanazawa tried schistosome worm antigens (SWAP) or egg antigens (SEA), and either of them had effects on CIA [15]. Spontaneous arthritis is also another model used for studying this inflammatory disease; here it has been demonstrated that a gastrointestinal helminth such as *Nippostrongylus brasiliensis* [19] was able to down-modulate experimental arthritis. However, it is clear that one can expect that not all the helminth infections as well as the same helminth parasite would be able to cure or ameliorate every single inflammatory disease; in line with this idea, there has been occasions where infection with helminth parasites can turn out a whole range of possibilities as is the case in infection with *Hymenolepis diminuta*, where the infection with this parasite has augmented oxazolone-induced colitis in mice [20]; also this infection did not show any effects in acetic acid-induced ulceration in rats [21]; but, interestingly, infection with *H. diminuta* can be protected in dinitrobenzene sulfonic acid (DNBS)-induced colitis [22]. Therefore, even when infection is carried out with the same parasite, the outcome of the diseases can greatly vary, as in the case of *H. diminuta* even when in theory the same disease was being reproduced, although different methods of induction of the disease were used the results reflected an ample spectrum of possibilities. Moreover, another disease where any effect of a helminth infection was not detected was in EAE developed in mice carrying a gastrointestinal infection with *Strongyloides venezuelensis* [23]. Taken together all these data accumulated on helminth therapy, we suggest that more detailed studies are necessary before "generalize" that any single helminth parasite or their derivatives would be useful for any inflammatory or autoimmune disease.

5. Conclusions

Based on the different outcomes of the many studies on "helminth therapy" and even though our group has previously proven that infection with *T. crassiceps* reduces incidence and severity of other inflammatory and autoimmune diseases, it is feasible that *T. crassiceps,* or any other helminth

infection, does not affect the development of some disorders like experimental RA since most of inflammatory or autoimmune diseases have many different components involved that may be not affected by these parasites. Thus, in the context of the potential use of helminths or their molecules as a way to treat or improve the outcome of inflammatory or autoimmune diseases, it deserves larger and deeper studies before giving the title of a "close reality" to the use of such strategy.

Acknowledgments

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Review Article

Immunoregulation by *Taenia crassiceps* and Its Antigens

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Taenia crassiceps is a cestode parasite of rodents (in its larval stage) and canids (in its adult stage) that can also parasitize immunocompromised humans. We have studied the immune response elicited by this helminth and its antigens in mice and human cells, and have discovered that they have a strong capacity to induce chronic Th2-type responses that are primarily characterized by high levels of Th2 cytokines, low proliferative responses in lymphocytes, an immature and LPS-tolerogenic profile in dendritic cells, the recruitment of myeloid-derived suppressor cells and, specially, alternatively activated macrophages. We also have utilized the immunoregulatory capabilities of this helminth to successfully modulate autoimmune responses and the outcome of other infectious diseases. In the present paper, we review the work of others and ourselves with regard to the immune response induced by *T. crassiceps* and its antigens, and we compare the advances in our understanding of this parasitic infection model with the knowledge that has been obtained from other selected models.

1. Introduction

Helminth parasites have developed complex and versatile mechanisms to evade the immune responses of their hosts, utilizing immunoregulatory strategies to avoid immune effector mechanisms. In general, these processes are necessary for the parasites to complete their long life cycles [1] and/or to favor host survival [2]. Despite their great evolutionary divergence and variety of stages, life cycles, and pathogenic and invasive mechanisms, helminths have developed similar strategies and induce strikingly similar immune responses, which have been called "stereotypical Th2-type immune responses." However, there are differences in the immune responses evoked by distinct helminths, mainly with regard to leukocyte involvement and the roles of these cells [3].

The stereotypical Th2 response induced by helminth parasites is characterized by the secretion of high levels of anti-inflammatory cytokines such as interleukin-6 (IL-6), IL-9, IL-10, IL-25, IL-33, and transforming growth factor-β (TGF-β), but the main cytokines are IL-4 and IL-13 [4]. As a consequence and/or origin of this cytokine secretion, there are alterations in leukocyte recruitment and activation, such as high levels of CD4+ T lymphocytes differentiated into Th2 and T regulatory (Treg) subsets, the recruitment and activation of immunoglobulin G1 (IgG1)- and IgE-producing B cells, eosinophilia, basophilia, and mastocytocis [4, 5]. Interestingly, an immature dendritic cell (iDC) phenotype with a Th2-driving ability and huge populations of alternatively activated macrophages (AAMs) with the ability to suppress lymphocyte proliferation can also be found within this response [3, 5, 6]. Furthermore, another characteristic of Th2 responses is the suppression of the immune response to bystander antigens, which may compromise the effectiveness of vaccination [7] and alter the immune response to several other antigens, even autoantigens.

It is commonly accepted that most of these changes in leukocyte phenotype and activation, as well as in the induction of the inflammatory milieu, are dependent upon the ability of the parasite to excrete/secrete antigens with immunoregulatory properties [8–12]. Many research teams [13–15], including ours [16, 17], have used these Th2 responses elicited by helminths and their antigens to control autoimmune disease development as well as to alter the outcome of other infectious diseases [18].

2. The Immune Response to Experimental *T. crassiceps* Infection: Th1/Th2 Balance and Susceptibility

T. crassiceps is a helminth parasite (class Cestoda) that can be found in its adult form within the small intestine of canids, whereas the main larval stage (metacestode) can be found in the muscles, peritoneal, and pleural cavity of rodents. *T. crassiceps* metacestodes can also parasitize immunocompromised human patients with cancer [19], human immunodeficiency virus and hepatitis C virus [20]. In addition, this parasite can infect perfectly healthy patients, although only one case has been reported [21]. An interesting feature of *T. crassiceps* is its ability, or evolutive advantage, to reproduce asexually through budding at the larval stage. This characteristic permits the larval stage to maintain and colonize its hosts for long periods of time; thus, after the intraperitoneal inoculation of a few parasites (10 to 20 metacestodes), hosts can harbor hundreds of parasites 6–8 weeks later. This feature has been useful for maintaining the parasite at the larval stage in the laboratory via passage from mouse to mouse through intraperitoneal injections, and these animals are also important sources of antigens that have been utilized for immunodiagnostic tests for cysticercosis [22]. Additionally, the fact that the larval stage of the parasite is innocuous for humans is important; although its macroscopic size facilitates the accumulation of an acute parasite burden, the parasite does not kill the host and is able to cause chronic infections with a minimum amount of damage in mice. Furthermore, the results are very reproducible. All of these features confer many advantages on this model for laboratory work and even for the development of vaccine strategies [23].

Early studies on the immune response against this parasite were performed in the late 1970s and early 1980s by Siebert and Good [24, 25]. This work mainly focused on the humoral immune response against *T. crassiceps* and found that antibodies anti-*T. crassiceps* cannot be correlated with cytotoxic effects or tegument degradation. Later, following the definition of the dichotomous Th1 and Th2 responses, a new series of investigations were conducted by different groups. Most of these studies coincided with the general knowledge that, during the acute stage, murine infection with this parasite leads to the induction of a transient Th1 proinflammatory immune response with high serum levels of gamma interferon (IFN-γ), nitric oxide (NO), and IgG2a that lasts for the first 2-3 weeks and then is replaced by a dominant Th2-type response rich in IL-4/IL-13, as well as IgG1 and IgE antibodies that last for at least two months (Figure 1) [26]. Later findings demonstrated that spleen cells from *T. crassiceps*-infected mice were refractory to polyclonal stimuli such as Concanavalin A and anti-CD3 [27], indicating that infection has a clear modulatory effect on the hosts immune system. Our next studies, conducted in the late 1990s, sought to block the cytokines involved in immune regulation *in vivo* early during infection, such as IFN-γ, IL-4, and IL-10, or inject IFN-γ plus IL-2 to support our idea that a Th1 response was efficacious at eliminating the larval stage of *T. crassiceps*. Early blockade of IFN-γ with specific antibodies in the first

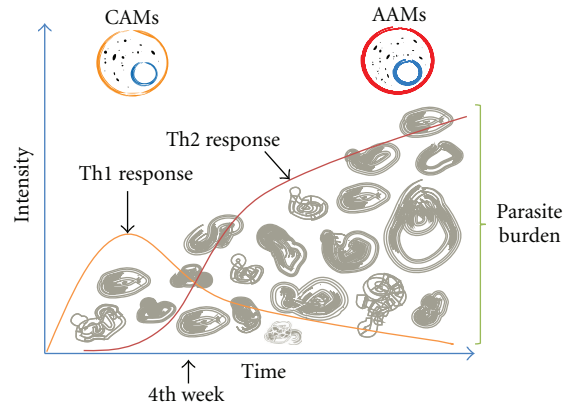

Figure 1: The initial Th1 response to *T. crassiceps* is rapidly replaced by a Th2 response by the third or fourth week after infection. This shift is accompanied by a change in macrophage phenotype, as the early CAM population is replaced by a dominant AAM population. Moreover, an increase in parasite burden can be observed when the AAM population and the dominant Th2 response are established.

week of infection greatly favored the establishment of the parasite. In contrast, the injection of recombinant murine IFN-γ plus IL-2 at the time point improved resistance to the infection, whereas the blockade of IL-10 or IL-4 had little effect on parasite loads [28].

Our proposal that a Th1 response was involved in eliminating a helminth infection was not widely accepted for several more years. Early in the 2000s, confirmatory experiments were performed with knockout mice to show that susceptibility to the larval stage of *T. crassiceps* is dependent upon signal transducer and activator of transcription-6 (STAT6) signaling, a key transcription factor involved in Th2 lymphocyte differentiation and alternative macrophage activation [29]. Conversely, resistance to this parasite was shown to be dependent on the IL-12/STAT4 signaling axis [30, 31], which is the main inducer of Th1 immunity. Thus, a Th2-type response was found to be associated with susceptibility to helminth infection, whereas a Th1-type response (dependent on STAT4) was shown to be clearly involved in protection against *T. crassiceps* (Figure 1). Moreover, when the immune responses in susceptible (BALB/c) and resistant (C57BL/6) mouse strains were compared, it was found that the Th1 immune responses mounted by C57BL/6 mice are stronger than those of susceptible mice [32]. These data together sustain the notion that susceptibility to the *T. crassiceps* metacestode is dependent upon a Th2 immune response, while resistance depends on the adequate and rapid development of a proinflammatory response.

One of the most interesting findings from a separate series of studies was the fact that, in parallel to the shift from a Th1-type towards a Th2-type response, a distinct population of macrophages emerges; these macrophages display low IL-1β, IL-12, and nitric oxide (NO) secretion but express high levels of arginase-1 (Arg1), Ym1, resistin-like molecule-alpha (RELMα), macrophage mannose receptor (MMR), and interleukin-4 receptor alpha (IL-4Rα). This population has a poor ability to induce antigen-specific proliferative

responses in T cells and a Th2 driving ability [33] and is now recognized as AAMs (Figure 2); importantly, AAMs have been reported to be present during most helminth infections [3]. Interestingly, the immune polarization toward a Th2 profile and the establishment of an AAM population are accompanied by an increase in parasite burden during experimental murine cysticercosis caused by *T. crassiceps* [29] (Figure 1). These findings suggest that the parasite itself may be the main impetus for this tolerogenic response.

3. Alternatively Activated Macrophages and Their Role in *T. crassiceps* Susceptibility and Immunoregulation

Two main macrophage phenotypes have been described according to the inflammatory stimuli that induce their activation. Classically activated macrophages (CAMs) are activated through toll-like receptor (TLR) stimulation with bacterial-, virus- and protozoan-derived molecules such as lipopolysaccharide (LPS) and peptidoglycan as well as IFN-γ, tumor necrosis factor-alpha (TNF-α) and IL-1β, which are secreted during inflammatory responses. CAMs show enhanced phagocytic, microbicidal, and Th1/Th17-driving abilities and consequently have an important role in immunity to intracellular pathogens. They typically express inducible nitric oxide synthase (iNOS), which is the main enzyme involved in NO production, and they also secrete proinflammatory cytokines such as IL-1β, IL-12, IL-23, and TNF-α [38]. In contrast, AAMs are induced mainly by IL-4 and IL-13 [39] stimulation through IL-4Rα [40], causing the activation and nuclear translocation of STAT6 [41]. Additionally, several helminth antigens have been proven to induce the alternative activation of macrophages independently of IL-4 stimuli [9–11, 42].

AAMs may secrete high levels of IL-10 and TGF-β but low levels of proinflammatory cytokines and express the enzyme Arg-1, which competes with higher affinity than iNOS for the common substrate L-arginine and produces urea, polyamines, and L-ornithine. AAMs also express YM-1, RELMα, programmed death-ligand 1 (PD-L1), and PD-L2 [3] and play a role in several aspects of the immune response, such as lymphocyte Th2 differentiation [33, 43], recruitment of IL-4-producing eosinophils [44], and, primarily, induction of low proliferative T cell responses [45, 46] (Figure 2).

In recent years, it has been demonstrated that AAMs are a common cell population induced during diverse helminth infections [3, 6], in which they have been shown to display diverse roles in host survival [2] as well as resistance to this type of infection [47] or as part of the wound healing machinery [2]. However, the role for AAMs in the *T. crassiceps* cysticercosis model is quite different.

The notion that IFN-γ [28] and STAT6 deficiency [29] correlate with resistance to infection led us to investigate the role of CAMs and AAMs in the immune response and susceptibility to *T. crassiceps*. Interestingly, macrophages from infected resistant mice displayed a greater ability to induce T cell proliferative responses, secreted pro-inflammatory cytokines and produced more NO, thereby displaying a classical activation phenotype, while macrophages from a susceptible mouse strain did the opposite and displayed an alternative phenotype [32]. We believe that macrophages that are recruited or polarized to become AAMs during *T. crassiceps* infection have been one of the most characterized during helminth infections. These AAMs show an increased expression or production of Arg-1, Ym-1, RELM-α, TREM-2, SLAM, MMR, mMGL, OX40-ligand, MHC-II, CD23, CCR5, IFN-γR, IL-4Rα, TLR4, PD-L1, PD-L2, PGE2, IL-10, and IL-6. In contrast, these AAMs have a low production or expression of iNOS, IL-12, IL-15, IL-18, IL-23, IL-1β, TNF-α, MIF, and NO [30, 32, 33, 45]. Thus, a total of 28 different molecules have been identified as altered in macrophages during experimental cysticercosis. Importantly, these molecules are intimately bound to the modulation of the immune response. Therefore, the study of such a cell population has become essential to understand helminth immunology.

To gain insights into the role of macrophages in facilitating or clearing *T. crassiceps* infection, we developed new experimental strategies. The treatment of STAT6 KO mice, which develop CAMs and are highly resistant to infection, with an iNOS inhibitor *in vivo* rendered these mice susceptible to *T. crassiceps* infection [52]. Similarly, deficiency in TLR2, which helps to induce pro-inflammatory responses in mice that are otherwise genetically resistant, rendered them highly susceptible to helminth infection [53]. In contrast, the early depletion of AAMs with clodronate-loaded liposomes in susceptible BALB/c mice reduced parasite loads by 90% [54]. Together, these data demonstrate that AAMs, a cell population that plays a key role in immunomodulation during *T. crassiceps* infection, may also be implicated in susceptibility to this parasite, while CAMs appear to be related to resistance.

The mechanism by which AAMs mediate susceptibility to this cestode is not currently well known, but it may be the inhibition of NO production through the expression of Arg-1 [47, 52], the release of prostaglandin E2, which also has immune-modulatory properties [55], or their suppressive capacity over lymphocyte proliferation [45]. Regardless, it is clear that the presence of CAMs is an important factor that contributes to host resistance. Several major findings support this idea, including the discovery that strains of mice that are resistant to *T. crassiceps* infection do not develop AAMs [32]; for example, C57BL/6 mice challenged with a similar number of metacestodes as BALB/c mice develop CAMs, but if STAT4-KO mice on the same resistant genetic background are similarly challenged, they have huge parasite burdens and develop AAMs [30]. In contrast, mice with a susceptible genetic background, such as BALB/c, but lacking the STAT6 gene became highly resistant to infection and do not develop AAMs. Instead, they recruit CAMs that highly produce NO, TNF-α, and IL-12 [29]. Moreover, as we stated above, the *in vivo* inhibition of iNOS was shown to induce susceptibility in STAT6-KO mice [52]. Thus, the activation state of macrophages plays a critical role in the outcome of helminth infection.

Additionally, both low proliferative responses and low lymphocyte counts in tissues near the parasite may be

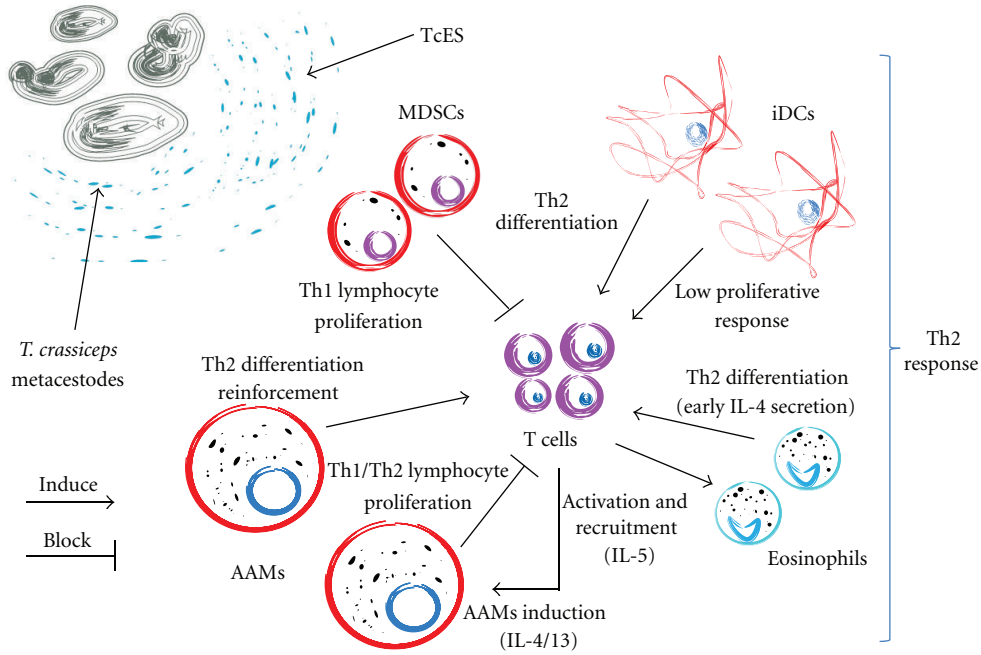

Figure 2: With our information to date, we propose a model of Th2 induction in which the excreted/secreted antigens of *T. crassiceps* (TcES) recruit MDSC populations to downmodulate the early Th1 response, while iDCs and eosinophils recruited by these antigens induce Th2 differentiation. AAMs reinforce Th2 lymphocyte differentiation while limiting their proliferation. Together, these changes favor a dominant Th2 response.

important factors in susceptibility to this infection, as it has been shown that there are many apoptotic lymphocytes surrounding viable metacestodes in *T. solium*-infected pigs [56] and during *T. crassiceps* infection can be seen a lower lymphocyte proliferative response in susceptible mice strains than in resistant ones [27]. These findings are in line with our observations, in which we have found that AAMs induced by *T. crassiceps* can suppress the proliferative responses of naive T cells stimulated with anti-CD3/CD28 antibodies *in vitro* [45]. As we had previously detected high levels of PD-L1 and PD-L2 expression in AAMs recruited during *T. crassiceps* infection, we hypothesized that the Programmed death-1/Programmed death-Ligands (PD-1/PD-Ls) pathway may be involved in such inhibition. Thus, transwell assays and *in vitro* blockade of PD-L1 or PD-L2 were found to reverse the suppressive activity of these AAMs. Moreover, AAMs induced by *T. crassiceps* infection were also demonstrated to suppress the specific response of $CD4^+$ DO11•10 cells to OVA peptide stimulation when unpolarized macrophages were used as antigen presenting cells. Again, in this assay, the blockade of the PD-1/PD-L's pathway reestablished the peptide-specific proliferative response of $CD4^+$ DO11•10 cells [45]. Therefore, AAMs can participate as a third party suppressive cell. This idea was confirmed with a different set of experiments, in which we demonstrated that the presence of AAMs in a DC-mediated mixed lymphocyte reaction was sufficient to inhibit the response of $CD4^+$ cells from a different genetic background. Mechanistically, AAMs recruited during chronic *T. crassiceps* infection are able to suppress immunological events mediated through distinct molecular pathways that may induce strong proinflammatory responses (Figure 2). We also demonstrated that the PD-1/PD-L pathway participates in modulating the anti-*Taenia*-specific cell proliferative response. However, whether these T cells exposed to AAMs undergo anergy and/or apoptosis and the *in vivo* significance of the PD-1/PD-L pathway in susceptibility to *T. crassiceps* are currently unknown, and further research is needed to resolve these questions.

4. Immunoregulation by *T. crassiceps* Antigens

It is commonly accepted that the inhibition of proinflammatory responses and the induction of Th2 immunity during helminth infections are dependent upon the parasite's ability to excrete/secrete antigens with immunoregulatory properties that have important effects on myeloid-derived suppressor cells (MDSCs), eosinophil and basophil recruitment, DC maturation impairment, alternative macrophage activation, impaired lymphocyte proliferative responses, and, in some cases, Treg induction [8–12, 57, 58]. The first *in vivo* evidence for these conclusions is that the pharmacological treatment of helminth-infected patients can trigger proinflammatory responses [59] and that much experimental data have been obtained indicating that the inoculation of helminth antigens alone has the ability to induce such immunoregulatory effects, as reviewed in [12, 58]. Thus, it has been largely accepted that the *in vivo* injection of some helminth-derived antigens is able to mimic some of the immune features induced by these parasitic infections,

but the mechanisms, putative receptors, and intracellular signaling pathways involved in these effects still have yet to be recognized [60]. Pioneering studies by the group led by Donald Harn at Harvard University have demonstrated that the main Th2-inducing activity of injected soluble egg antigen (SEA) from *Schistosoma mansoni* is dependent on the intact structure of carbohydrates in the antigen [61]. Thus, it was hypothesized that glycoproteins are essential for Th2 induction during schistosomiasis. This idea was rapidly adopted by several "helminth immunologists," who corroborated many of the Harn's group findings using different sources of helminth antigens, such as *Brugia, Echinococcus, Ascaris, Caenorhabditis, Hymenolepis* [62] and, of course, *Taenia*. In this area, our team has also evaluated the effects of the *in vivo* inoculation of antigens derived from *T. crassiceps* metacestodes. The injection of a soluble extract of these larvae can rapidly (18 h post-inoculation) recruit a $CD11b^+F4/80^+Gr1^+$ cell population, consisting of what are now called myeloid-derived suppressor cells (MDSCs), which possess a strong capacity to inhibit proliferative responses in activated lymphocytes and may have an important role in inhibiting the initial Th1 response to this parasite (Figure 2) [63]. Interestingly, when *T. crassiceps* antigens are treated with sodium metaperiodate to alter glycan structures, these antigens lose the ability to recruit MDSCs, indicating a critical role for glycoproteins in modulating the immune response to this parasite. Further research has demonstrated that glycans present in *T. crassiceps* excreted/secreted (TcES) molecules are also important in modulating DC maturation [64].

DC maturation involves the upregulation of several costimulatory molecules that play important roles in antigen presentation and T cell activation, such as CD40, CD80, CD86 and major histocompatibility complex II (MHCII), as well as proinflammatory cytokines such as IL-12 and IL-18. A fully mature DC is capable to induce T cell activation, proliferation, and differentiation into the Th1 phenotype, whereas an iDC drives Th2 differentiation and induces an impaired proliferative response in T cells [71]. Recently, we have found that the *in vitro* exposure of murine [64] and human [72] DCs to TcES impairs their maturation. DCs also become refractory to stimulation with different TLR ligands and thereby produce low levels of pro-inflammatory cytokines such as IL-12, IL-15, and TNF-α. Importantly, when these DCs exposed to TcES are used as antigen presenting cells, they are able to induce the Th2 differentiation of naïve $CD4^+$ T cells (Figure 2). Moreover, all these effects of TcES are glycan-dependent [64]. Interestingly, other research groups in this field have found many similarities in the responses of monocyte-derived dendritic cells following exposure to glycoproteins derived from *Echinococcus granulosus, E. multilocularis* [73, 74], egg carbohydrate antigens from *S. mansoni* [75], or larval carbohydrate antigen from the nematode *Trichostrongylid* [76].

Moreover, *in vivo* assays have also demonstrated that carbohydrates in helminth-derived antigens are essential to bias Th2-type responses to bystander antigens, and thus glycoproteins from SEA and from *T. crassiceps* coinjected with the unrelated proteins human serum albumin and ovalbumin, respectively, into mice were shown to induce strong Th2 responses to these antigens. However, when glycan structures were altered, the Th2 polarization effect of the helminth antigens was eliminated [8, 61]. Thus, it is clear that host immune responses to helminth parasitic diseases or to bystander antigens are modulated by helminth-expressed glycans, and therefore most of these effects must be mediated by carbohydrate recognition receptors. It is important to keep in mind that the chemical composition of helminth antigens varies greatly among species, but the most common types are proteases, protease inhibitors, cytokine/chemokine homologs, antioxidant enzymes, lectins, and other carbohydrates [12]. Consequently, other receptors may also be involved in recognizing such diverse molecules. Likewise, it is critical to elucidate the protein, glycan, and lipid composition of helminth-derived molecules with immunomodulatory ability.

5. The Therapeutic Potential of *T. crassiceps*

The last two decades have witnessed a dramatic increase in the number of new cases of inflammatory diseases in developed countries, while, at the same time, the hygiene conditions in these countries have greatly improved, leading to a reduction in the prevalence of different bacterial or parasitic infections, including helminth infections [77]. Taken together, these observations led to the postulation of the hygiene hypothesis, which states that in the absence of intense infections that modulate host immunity to a Th2-type response (such as during helminth infections), the immune system then tends to present exaggerated Th1 inflammatory responses directed against microbial antigens or even autoantigens, thus leading to autoimmunity [57]. Although the contribution of genetic factors in the development of these diseases is evident, epidemiological [77–79] and experimental [80] evidence suggests that environmental factors can also be involved in the etiology of autoimmunity.

The main experimental evidence supporting the hygiene hypothesis came from studies in which helminth-infected mice were able to successfully control type 1 diabetes (T1D) [17, 51], experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) [13, 16], inflammatory bowel disease (IBD) [81], and rheumatoid arthritis (RA) [66]. More importantly, some studies conducted with parasite-derived antigens showed their ability to improve the outcomes of these diseases [14, 51, 82, 83]. Helminth therapy and its likely benefits have started to be applied in humans; the treatment of patients with the eggs of the nonhuman parasite *Trichuris suis*, which is related to the human parasite *T. trichuria*, has been shown to moderately improve the outcome of MS [84], Crohn's disease [85], and, at a lower level, ulcerative colitis [85]. Because treatment with living organisms can generate adverse or side effects [84], treatment with helminth-derived immunomodulators is a very promising alternative [58].

Based on the notion that *T. crassiceps* induces strong anti-inflammatory and long-lasting Th2 responses, characterized by high systemic levels of IL-4, IL-10, and IL-13 as well

as the recruitment of different regulatory cell populations such as AAMs, MDSCs, and iDCs accompanied by low T cell proliferative responses and the induction of low NO, IL-1β, IL-12, IL-15, IL-18, IL-23, TNF-α, and IFN-γ levels that may block pathologic inflammation, we investigated the role of this infection in the modulation and outcome of experimental autoimmune diseases such as EAE, rheumatoid arthritis, and T1D.

Recent findings in our laboratory show that the preinfection (8 weeks) of mice with *T. crassiceps* metacestodes can reduce the incidence of EAE by 50% and reduce the severity score of the disease (1 out of 5) in sick animals. This effect was accompanied by high systemic levels of IL-4, IL-10, IgG1, and IgE and low levels of IFN-γ, TNF-α, IL-17, and IgG2a, as well as a reduced inflammatory infiltrate into the spinal cord. Importantly, we could find AAMs with strong suppressive activity over lymphocyte proliferation and a reduced number of $CD3^+$ cells entering in the brain [16]. Other populations, such as $CD4^+/CD25^+/FoxP3^+$ Tregs, have been associated with the secretion of high levels of IL-10, thereby suppressing Th1, Th2, and Th17 responses. Tregs are likely to exert this regulatory effect on autoimmune diseases, but we have not been able to find Tregs in the brain, spleen, mesenteric lymph nodes, or peritoneal cavity of *T. crassiceps*-infected mice from susceptible or resistant strains [16, 17]. Strikingly, an examination of the brains and spleens of *T. crassiceps*-infected EAE mice using flow cytometry and rtPCR failed to reveal a significant increase in $CD4^+/CD25^+/FoxP3^+$ Treg cells [16].

Other research groups have shown that infection with *S. mansoni* [13], *Fasciola hepatica* [35], and *Trichinella pseudospiralis* [34] can regulate the incidence and/or severity of EAE, whereas other parasites such as *Strongyloides venezuelensis* [36] and *T. spiralis* [37, 86] did not significantly affect EAE development. Furthermore, the cytokines generally associated with the downregulation of EAE are IL-4, IL-5, and IL-10 as well as low IFN-γ, TNF-α, and IL-17, but none of these other models are associated with AAMs as possible key players in the regulation of such diseases; instead, Th2 $CD4^+$ T cells are a common hallmark of EAE regulation (Table 1).

Importantly, *Hymenolepis nana, T. trichuria, Ascaris lumbricoides, Strongyloides stercolaris,* and *Enterobius vermicularis*-infected MS human patients showed a significantly lower number of disease exacerbations, brain damage, and variation in disability scores during a 4.5-year-follow up study. This protective effect was correlated with high eosinophilia, IgE titers and $IL\text{-}10^+/TGF\text{-}\beta^+$ Treg cell induction as well as low IL-12 and IFN-γ secreting cells [87], partially resembling the observations in the animal models.

Additionally, we have shown that the preinfection of mice with *T. crassiceps* can reduce the incidence of T1D induced by streptozotocin (STZ) up to 50%, mainly by lowering blood glucose levels to below 200 mg/dL, leukocyte infiltration into the pancreas and, in consequence, the degree of insulitis. Such effects last for at least 6 weeks after induction of T1D. These protective effects were associated with high systemic levels of IL-4, a reduction in TNF-α circulating levels, and the induction of AAM populations; however, analysis of the spleens did not show increased populations of Treg cells [16]. To our knowledge, only one work regarding STZ-induced diabetes and helminth-induced immunoregulation has been published in addition to ours; in this paper, it was shown that *S. mansoni* infection could reduce T1D incidence and pancreatic cell infiltration, but the authors did not suggest which cell populations may be involved in the modulation of this disease [48] (Table 2).

Although other works examining the regulation of diabetes by helminth infections were primarily performed in less aggressive and slower models, such as nonobese diabetic (NOD) mice, there are several similarities and differences compared to our observations in the *T. crassiceps* model. Strikingly, it was shown that *Heligmosomoides polygyrus* infection during the early weeks of life can decrease the incidence of T1D in a mechanism dependent upon AAMs but not Tregs [15], but it has also been shown that infection with *S. mansoni* can significantly reduce the incidence of diabetes and pancreatic damage in a scenario where AAMs together with Tregs play an important role in the regulation of the disease [49]. By contrast, the infection of mice with *Litomosoides sigmodontis* promotes protection and reduced insulitis that is dependent on increased Treg populations and Th2 induction [51], while T1D incidence and blood glucose modulation in the *Trichinella spiralis* model are mainly regulated by Th2 cells [50]. Together, these studies indicate that multiple pathways are involved in the modulation of experimental T1D by helminths, but some similarities can be found regarding regulatory leucocyte populations and cytokines (Table 2).

Despite the strong regulatory activity of *T. crassiceps* in EAE and T1D, the infection with this parasite was shown to be unable to modify the outcome of experimental RA, given that 100% of infected animals developed medium clinical scores [65]. Strikingly, the pre-infection of mice with other helminths such as *Syphacia oblevata* [67] and *Hymenolepis diminuta* [70] can reduce the incidence [67] and severity [67, 70] of experimental RA. Additionally, the pre-infection of mice with *S. mansoni* [68] and *S. japonicum* [66, 69] can ameliorate RA in other models. In all of these models, the downregulation of IgG2a anti-collagen antibodies and the induction of high levels of IL-4 and IL-10 appear to be important in limiting RA progression and these effects were not achieved by *T. crassiceps* infection in this model (Table 3).

The main mechanisms involved in the abrogation of EAE and T1D with *T. crassiceps* infection may be IL-4 and IL-10 secretion as well as the induction of anergy in lymphocytes, as it has been shown that these diseases are dependent upon autoreactive lymphocyte proliferation [88, 89] and commitment to Th1 and Th17 subsets [90]. We therefore hypothesize that AAMs are the main cell population involved in tolerance induction because we have shown that they have a strong suppressive ability over lymphocyte proliferation [45] while also having the capacity to drive Th2 responses [33]. iDC populations may also be involved in this phenomenon due to their strong Th2-driving abilities, but further *in vivo* investigation is needed to confirm this hypothesis. Also, it would be important to research on the role of eosinophils [87] and B cells [91] in autoimmune disease regulation as these cells have been associated with MS

Table 1: Parasite helminths involved in the regulation of EAE.

Helminth	Autoimmune disease model	Incidence	Clinical score of sick animals[¶]	Onset delay (days)	Associated leukocyte populations	Cytokines involved	Ref.
T. crassiceps	MOG-induced EAE	≈50%	0.5/5	1	AAMs, Th2 cells	High IL-4/10; low IFNγ, TNFα, IL-17	[16]
T. pseudospiralis	MOG-induced EAE	≈67%	1.5/5	11	Th2 cells	High IL-4/5/10; low IL-1β/6/17 IFNγ and TNFα	[34]
S. mansoni	MOG-induced EAE	≈57%	1.5/5	≈2	Th2 cells	High IL-4/5/10; low IL-12, IFNγ, TNFα	[13]
F. hepatica	MOG-induced EAE	N.S.	1/5	2	Th2 cells, Tregs, iDCs, AAMs, eosinophils and MDSCs	High TGFβ; low IFNγ and IL-17	[35]
S. venezuelensis	MBP-induced EAE	100%	3/5	0	Non	N.S.	[36]
T. spiralis	SCTH-induced EAE	100%	2/4	≈1	Th2 cells, Tregs	High IL-4/10; low IL-17 and IFNγ	[37]

MOG: myelin oligodendrocyte protein; MBP: myelin basic protein; SCTH: spinal cord tissue homogenate; N.S.: not specified; [¶] see original references, as disease severity is differentially evaluated between authors.

Table 2: Helminth regulation of type 1 diabetes.

Helminth	Autoimmune disease model	Incidence	Insulitis	Blood glucose level	Associated leukocyte populations	Cytokines involved	Ref.
T. crassiceps	T1D/MLD-STZ	≈50%	0%	≈200 mg/dL	AAMs, Th2 cells	High IL-4; low TNF-α	[17]
S. mansoni	T1D/MLD-STZ	N.S.	Relatively less infiltration and injury	≈200 mg/dL	Th2 cells	High IL-4/10/5; low IFN-γ	[48]
S. mansoni	T1D/NOD mice	10–30%	N.S.	<150 mg/dL	Th2 cells, eosinophils	High IL-4	[49]
T. spiralis	T1D/NOD mice	10%	N.S.	<200 mg/dL	Th2 cells	High IL-4	[50]
L. sigmodontis	T1D/NOD mice	0%[¶]	≈70%	≤230 mg/dL	AAMs, Th2 cells, Tregs	High IL-4/5	[51]
H. polygyrus	T1D/NOD mice	0%	≈20%	<200 mg/dL	AAMs, Th2 cells	High IL-4/13/10; low IFN-γ/IL-17	[15]

T1D: type 1 diabetes; MLD-STZ: multiple low doses of streptozotocin; NOD: nonobese diabetic; N.S.: not specified; [¶] incidence defined as mice with blood glucose levels greater than 230 mg/dL, while in the other models this was defined as blood glucose levels greater than 200 mg/dL.

regulation in humans and, at least eosinophils, are strongly and rapidly recruited by *T. crassiceps* infection [54] (Figure 3).

The absence of Treg induction during *Taenia*-induced immunomodulation of these autoimmune disease models reinforces the idea that AAMs and iDCs may play a central role in the induction of tolerance (Figure 3), but further investigation is needed to confirm the role of these cell populations in disease regulation [12, 15, 78, 79]. Additionally, we have not yet shown whether *T. crassiceps* infection can act both as a prophylactic and as a therapy option, and, more importantly, we have not yet investigated whether TcES may regulate the outcome of these diseases, which is one of the ultimate goals of our team.

6. *T. crassiceps* Immunoregulation: Fibrosis and Bystander Suppression

It is commonly accepted that Th2 cytokines such as IL-4/13 and TGF-β induce fibrosis, which might be useful in wound healing but in other instances might be pathogenic as well [92, 93]. Moreover, AAMs have been proposed to induce fibrosis, mainly through the overexpression of Arg1, which may contribute to collagen deposition in the extracellular matrix [94, 95]. In fact, it has been shown that *T. crassiceps* infection can induce liver fibrosis in association with alternatively activated Kupffer cells and therefore exacerbate tetrachloride-induced liver damage [96]. Moreover, several epidemiological studies show that parasite-parasite coinfections are common in developing countries, with children being the most susceptible group [97–99]. Furthermore, experimental data show that helminths can modify the host immune response and alter immunity to other parasites. For example, *Litomosoides sigmodontis* infection can alter the development of a secondary infection such as *Leishmania major*, increasing susceptibility to the second parasite [100]. Similarly, it has been shown that preinfection with *T. crassiceps* modifies the immune response to *Trypanosoma cruzi* [101], *Leishmania major,* and *L. mexicana* [18], increasing susceptibility to these infections as well as tissue and organ damage resulting from the downmodulation of Th1 immunity and classical macrophage activation, which are both associated with resistance to these protozoan parasites.

Table 3: Helminth regulation of rheumatoid arthritis.

Helminth involved	Autoimmune model	Incidence	Clinical score[¶]	Histopathology	Humoral immunity	Cytokines	Leukocytes	Ref.
T. crassiceps	CFA monoarthritis	100%	2/4	Similar damage in the infected group	High IgG1 and IgG2a	Low IFN-γ and IL-4	AAMs	[65]
S. japonicum[*]	DBA.1 mice	60%	≈3/9	Ameliorated sinovial hyperplasia, mononuclear cell infiltration, and angiogenesis	Low IgG2a; high IgG1	High IL-4/10; low IL-1β/6 TNF-α, IFN-γ	Th2 cells, Tregs	[66]
S. oblevata	CFA monoarthritis	21%	1.45/4	N.S.	N.S.	N.S.	N.S.	[67]
S. mansoni	DBA.1 mice	0%	0/6	N.S.	Low IgG1 and IgG2a	High IL-4/10; low IFN-γ	Tregs	[68]
S. japonicum[**]	DBA.1	N.S.	≈1/9	No synovial hyperplasia, inflammatory infiltrate, or bon/cartilage destruction	Low IgG2a	High IL-4/10 RANKL; low IL-17-α, IFN-γ, TNF-α	No Tregs	[69]
H. diminuta	CFA monoarthritis	N.S.	N.S.	Any knee swelling	N.S.	High IL-4/10; low TNF-α, IL-12p40	AAMs, Th2 and B2 cells, less granulocyte infiltration	[70]

CFA: complete Freund's adjuvant; [*]two-week preinfected mice; [**]seven-week preinfected mice; N.S.: not specified. [¶]see original references, as disease severity is differentially evaluated between models.

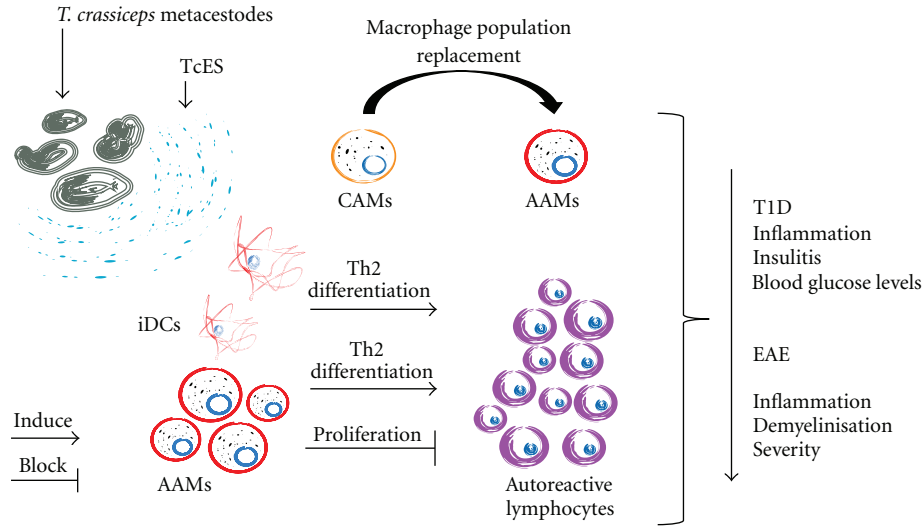

Figure 3: Based on our information to date, it is possible that iDCs recruited by TcES can prime Th2 differentiation, while AAMs may reinforce this activation and block pathogenic lymphocyte proliferation. Additionally, a shift from pathology-inducing CAMs to a protective AAM population can be seen, and all these changes together may protect mice from autoimmunity.

T. crassiceps infection can also negatively modulate the outcome of viral infections; an enhanced susceptibility to vaccinia virus via the suppression of cytotoxic T cell responses in mice infected with this helminth has been shown [102]. Moreover, the stimulation of mice with CpG, a bacteria- and virus-derived agonist of TLR9, can augment protective immunity to the cestode [103], opening the possibility for a cross regulation of susceptibility between virus and *T. crassiceps* when coinfection exists. This possibility can be extended to bacterial, protozoan, helminth, and fungal coinfections, given the discovery that TLR2 is involved in mediating resistance to this parasite [53].

7. Concluding Remarks

As with other helminths, infection with the cestode *T. crassiceps* induces strong and long-lasting Th2-polarized immune responses, and high systemic levels of IL-4, IL-5, IL-10, IL-13, IgG1, and IgE as well as low NO, IL-1β, IL-12, IL-15, IL-18, IL-23, TNF-α, and IFN-γ serum concentrations are achieved. These changes in cytokine secretion are accompanied, induced, and/or regulated by AAMs, MDSC, eosinophil and iDC populations with suppressor and Th2-driving abilities. Thus, the characteristics of the immune response to this parasite can be coopted to regulate the outcome of autoimmune diseases. In fact, we have successfully used the immune response to this parasite to regulate EAE and T1D incidence and severity. Despite these benefits, *T. crassiceps* immunoregulation has some drawbacks, such as the fact that infection with this cestode can exacerbate fibrosis and protozoa infections. Moreover, we have seen that several *T. crassiceps* antigens can mimic the effects of parasite infection, making them promising Th2 adjuvants or anti-inflammatory biocompounds that may be used in autoimmune or inflammatory disease regulation while avoiding the pathogenic side effects of infection with the live parasite. Further investigation is needed to uncover the role of TcES in the regulation or amelioration of inflammatory diseases and, in particular, the mechanisms it utilizes to modulate the immune response towards a distinct regulatory profile.

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Research Article

Helminth Excreted/Secreted Antigens Repress Expression of LPS-Induced Let-7i but Not miR-146a and miR-155 in Human Dendritic Cells

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MicroRNAs have emerged as key regulators of immune responses. They influence immune cells' function and probably the outcome of several infections. Currently, it is largely unknown if helminth parasites and their antigens modify host microRNAs expression. The aim of this study was to explore if excreted/secreted antigens of *Taenia crassiceps* regulate LPS-induced miRNAs expression in human Dendritic Cells. We found that these antigens repressed LPS-let-7i induction but not mir-146a or mir-155 and this correlates with a diminished inflammatory response. This let-7i downregulation in Dendritic Cells constitutes a novel feature of the modulatory activity that helminth-derived antigens exert on their host.

1. Introduction

MicroRNAs (miRNAs) are small (~23 nucleotides) noncoding RNAs that negatively regulate protein-coding gene expression mainly via down-regulation of mRNA levels and/or translational suppression [1]. In the last years, several reports have linked miRNAs to multiple and essential functions in the immune system. These molecules have emerged as important regulators of the development and differentiation of T and B lymphocytes and dendritic cells (DCs) [2–4], as modulators of inflammation [5], of balance between Th1 and Th2 responses [6], and of antibody production [7] amongst other functions. Besides their physiological role, miRNAs participate in pathological aspects of the immune response; for example, these molecules may be important mediators in cancer and autoimmunity [8] but participate as well in the control of viral infections [9]. The response of host miRNAs in parasitic infections is largely unknown. The few reports that have addressed the influence of parasites upon host-derived miRNAs have focused on protozoan infections [10, 11] while the role of helminth parasites and their antigens as possible modulators of such miRNAs is widely unexplored.

Dendritic Cells (DCs) dictate immune responses through the different signals derived from them such as secreted cytokines, chemokines, and also costimulatory molecules. Once an immature DC faces stimuli, either exogenous or endogenous, this cell will undergo an intracellular process that ultimately will render it capability of supporting the different types of immune responses [13]. DCs exposed to lipopolysaccharide (LPS) are cells that suffer a typical maturation process with secretion of inflammatory cytokines and

chemokines mainly IL-12, TNF, IL-6, RANTES, MCP-1 among others, and an upregulation of certain membrane molecules. Altogether, these changes confer DCs with the capability to induce the appropriate adaptive immune responses [14, 15]. Recently, it has been described that some miRNAs are upregulated and may participate in these dendritic cell's maturation/activation events [16, 17]. Specifically, miR-146a, miR-155, and let-7i are microRNAs that are modulated positively when DCs are exposed to maturation agents including LPS, TNF, and IFN-γ [18, 19] and seem to be related to the activation events triggered in DCs by such stimuli including the expression of costimulatory molecules, secretion of proinflammatory cytokines, and even induction of apoptosis [16–18]. Although there is one report showing that chronic ascariasis and trichuriasis modify the expression of miRNA let-7d in peripheral-blood mononuclear cells (PBMCs) [20], the impact of helminthes and their antigens on DCs-derived miRNAs and its possible consequences for the function of these cells has not been addressed to date.

We recently demonstrated that some properties of human DCs can be affected by their exposure to the excreted/secreted antigens derived from the cysticerci of *Taenia crassiceps* (TcES) [12]. These cells were characterized by an immature phenotype with low expression of the molecules CD80, CD86, and CD83 and were capable of secreting the regulatory cytokine IL-10 but not any of the inflammatory cytokines tested. More importantly, DCs exposed to TcES showed impaired maturation/activation to subsequent LPS stimulation, where both expression of costimulation molecules and secretion of proinflammatory cytokines were significantly diminished, indicating the anti-inflammatory effects of TcES upon this cell type [12]. Since TcES affects events related with the activation of DCs by LPS, here we explored if these parasite antigens modify the expression in human DCs of the microRNAs miR-146a, miR-155, and let7i, which, as stated earlier, seem to play a critical role in the classical maturation and activation processes induced by LPS in these cells.

2. Methods

2.1. Taenia crassiceps Excreted/Secreted Antigens. Metacestodes of *Taenia crassiceps* were harvested from the peritoneal cavity of female Balb/c mice after 2–4 months of infection. This was done under a laminar flow chamber and sterile 1X PBS was used. The cysticerci were washed four times with 1X PBS and maintained in culture in 1X PBS at 37°C for 24 h. TcES were recovered from the supernatant and centrifuged for 10 min at 1000 g using LPS-free filters. This fraction was concentrated using 50 kDa Amicon Ultra Filter (Millipore). Concentrations of different lots range between 400 and 920 ug/mL. Samples were stored at −70°C until further use.

2.2. Monocyte-Derived Dendritic Cells. Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of 12 healthy blood donors from the Instituto Nacional de Cardiología Ignacio Chávez's Blood Bank. Informed consent was obtained for the use of blood samples according to the declaration of Helsinki and the local scientific and ethics committees approved the protocol. PBMCs were isolated by Ficoll-gradient centrifugation (GE Healthcare), analyzed in a Coulter AcT for cellular types (Beckman Coulter), and 3×10^6 monocytes were left to adhere in 6-well culture plates for 2 h. After this period, nonadherent cells were washed away and adherent cells were cultured in RPMI medium supplemented with 10% SFB and penicillin/streptomycin in presence of 400 U/mL of IL-4 and 800 U/mL of GM-CSF during 6 days with replacement of medium and cytokines at day 3. At day 6 nonadherent cells were recovered and placed for 24 h in fresh medium. At this point we determined by flow cytometry the percentage of CD11c+ cells and for all experiments this was ≥80%. Cells were challenged with 20 ug/mL TcES, 1 ug/mL LPS, or a combination of them for 3 or 24 h. Control cells received RPMI.

2.3. QRT-PCR for Mature MicroRNAs. To asses mature microRNA expression, we used two-step qRT-PCR, with TaqMan microRNA assays (Applied Biosystems). For microRNA cDNA synthesis, a 15 μL reaction volume, composed of 1.5 μL of Buffer (10x), 0.15 μL 100 mM dNTPs (100 mM), 1.0 μL reverse transcriptase, 0.19 μL RNAse inhibitor (20 U/μL), and 3.0 μL of each specific microRNA primer, was mixed with 10 ng of total RNA. RT reaction was incubated 30 minutes of 16°C, 30 minutes of 42°C, and 5 minutes of 85°C. Duplicate real-time PCR reactions were performed in a Roche LigthCycler 2.0. Reaction mix was composed of 1x LigthCycler TaqMan Master (Roche), 1X of each specific microRNA probe, and 2.5 uL of specific microRNA cDNA (diluted 1 : 3). These were followed by 10 minutes 95°C for preincubation, 40 cycles of amplification program consisting of 95°C 10 sec, 60°C 40 sec, and 72°C 5 sec (fluorescence acquisition). To asses possible bias for reference gene selection U6 and let7a were used as reference genes, and relative quantification was calculated by the formula 2-(Cp microRNA target—Cp U6 or let7a). All microRNA assays were tested for reproducibility and linearity (PCR efficiency was between 1.9 and 2.0 for all assays).

2.4. Quantification of Cytokine Production. Supernatants were recovered after 3 h and 24 h stimulation period and production of the cytokines TNF, IL-6, IL-12, IL-10 (Peprotech), and MCP-1 (R&D) was measured by ELISA kits in the supernatants of DCs cultures. For the analysis of cytokine mRNAs levels cells were recovered from cultures 3 h after stimulation.

2.5. Statistics. Comparisons were performed by the Mann-Whitney Wilcoxon test for cytokine comparisons and Kruskal-Wallis and Dunn's *post hoc* test for miRNAs expression. Significance was set on a P value <0.05. All analyses were performed with the GraphPad Prism v. 5 statistical software.

3. Results and Discussion

3.1. TcES Dampen the Inflammatory Activity of LPS in Human DCs. LPS activation of DCs induces their maturation and secretion of pro-inflammatory cytokines and chemokines

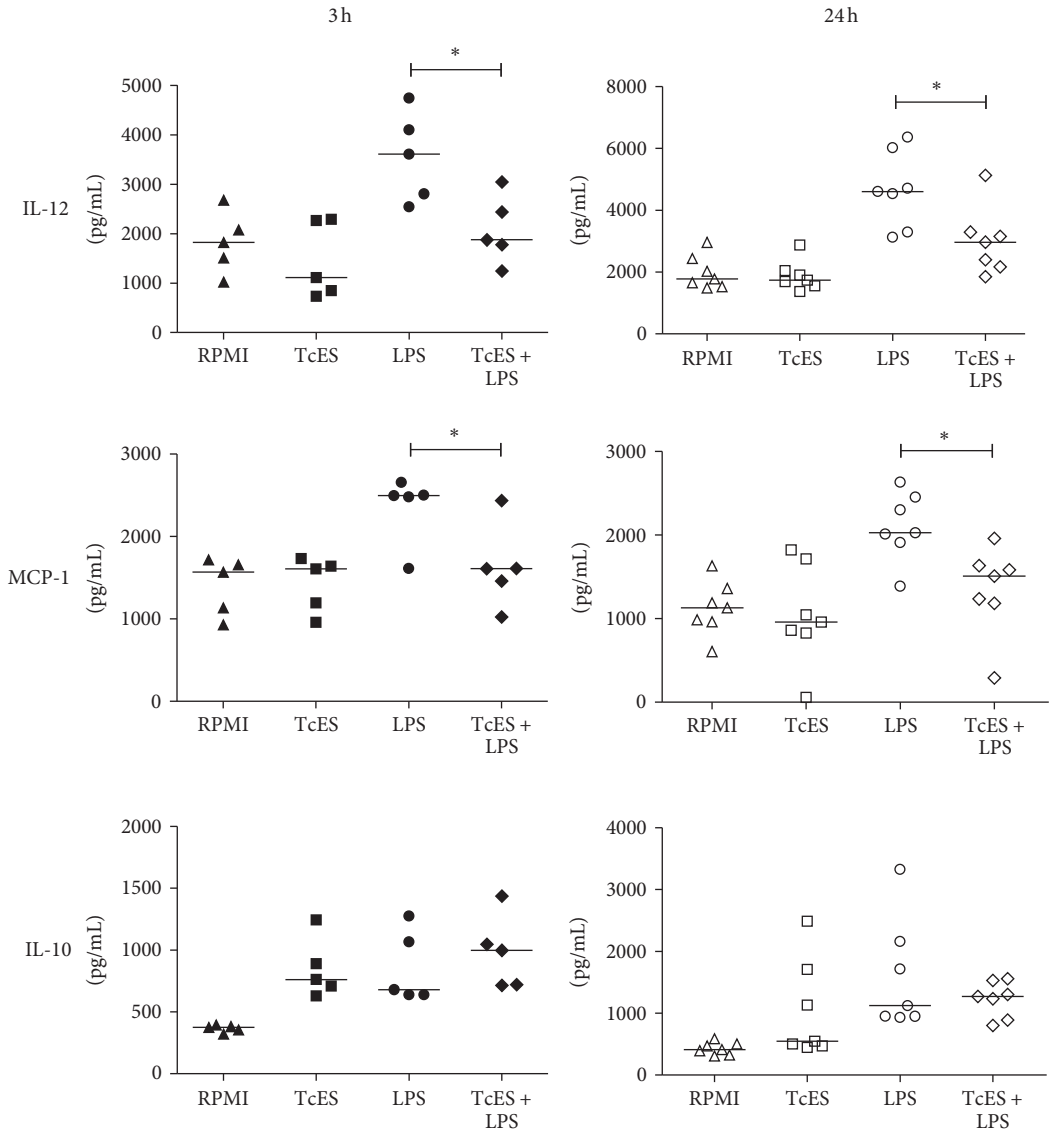

Figure 1: TcES modify the secretion of cytokines by human DCs activated with LPS. Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of 12 healthy blood donors. Informed consent was obtained for the use of blood samples according to the declaration of Helsinki and the local scientific and ethics committees approved the protocol. Monocyte-derived DCs were cultured for six days in the presence of IL-4 and GM-CSF [12]. Cells were challenged with 20 ug/mL TcES, 1 ug/mL LPS, or a combination of them for 3 h or 24 h. Control cells received RPMI. Cells and supernatants were collected and cytokine response was measured by ELISA kits (Peprotech and R&D). The data is shown as scattered plots and medians of twelve independent experiments ($n = 5$ for 3 h and $n = 7$ for 24 h). Comparisons were performed by the Mann-Whitney test. $^{*}P < 0.05$ LPS *versus* LPS/TcES. All analyses were performed with the GraphPad Prism v. 5 statistical software.

such as IL-12, TNF, IL-6, MCP-1 among others [11, 13]. We demonstrated previously that TcES does not induce secretion of the pro-inflammatory cytokines IL-12, IL-1β, TNF, or IL-6 but contrary it upregulates the secretion of IL-10. More importantly, these cysticerci-derived antigens interfered with the LPS-activation of DCs, downmodulating their maturation, and reversing the secretion of all the pro-inflammatory cytokines tested but not the one of regulatory cytokine IL-10 [12].

We tested if the phenomenon of modulation of TcES upon LPS-induced cytokine response was observed in the monocyte-derived DCs of the donors used for this study. At day six of culture, DCs received TcES, LPS, or a combination of both for 3 h and 24 h, and supernatants were recovered. As expected, we found that unlike LPS, TcES did not induce secretion of the inflammatory molecules IL-12 and MCP-1 (Figure 1) but instead, these antigens were capable of downmodulating such response to LPS both at 3 h or 24 h after stimulation (Figure 1). Interestingly, secretion of the regulatory cytokine IL-10 was induced similarly by TcES, LPS, and the combination of both (Figure 1). Altogether, these results indicate that these helminth antigens promote human DCs

to acquire an anti-inflammatory phenotype while dampening the inflammatory response induced by LPS. These observations allowed us to establish that the cells tested for miRNAs expression were indeed consistently modulated by TcES as reported previously by our group [12].

3.2. TcES Do Not Modify Expression of Mir-146 and Mir-155 but Downregulate Expression of Let-7i. miRNAs control expression of genes through mRNA degradation or translational suppression [1]. The miRNAs mir-146a, mir-155, and let-7i have been reported to play a role in the induction of maturation and activation of DCs [14–17]. Currently, it is unknown if helminth parasites or their antigens exert a modulatory activity on these host miRNAs. Since in a previous work we found that TcES affects the maturation and secretion of pro-inflammatory cytokines in DCs activated with LPS [12], we explored if these antigens affect the expression of the LPS-induced mir-146a, mir-155, and let-7i in human DCs.

qRT-PCR was performed in independent DCs culture experiments of nine healthy donors ($n = 4$ for 3 h and $n = 5$ for 24 h) and two reference genes (*u6* and *let-7a*) were used to establish fold-induction of miRNAs. As already described by others [18, 19], we found that at 24 h after stimulation of DCs, LPS upregulated the expression of mature mir-146a, mir-155, and let7i when compared to cells receiving medium alone (Figure 2). Exposure of DCs to TcES alone did not induce an upregulation on the three selected miRNAs (Figure 2) but when these cells were exposed to the combination of TcES plus LPS we observed a contrasting response. Prior to such combined stimuli, we found no significant differences in the expression of mir-146a and mir-155 (Figures 2(a) and 2(b)), indicating that TcES do not modulate the expression of these two miRNAs in human DCs activated by LPS. However, when we assessed the influence of such antigens over the LPS-induced expression of let-7i, the upregulation of this miRNA was no longer observed: statistical difference was found between LPS and LPS/TcES whereas none was found between LPS/TcES and RPMI (Figure 2(c)). At 3 h after stimulation, the expression of none of the selected miRNAs was statistically significant between groups (Figure 2). Results with the reference gene *U6* were found similar to those with let-7a (data not shown). Altogether, these data show that the helminth-derived antigens TcES importantly interfere with the LPS-induced expression of let-7i in human DCs and suggest that such antigens prevent the upregulation of this miRNA rather that diminishing it after a prior augmentation. TcES appear to exert a fine modulatory activity upon host miRNAs since even when mir-146a and mir-155 have also been related to the LPS-induced response in DCs, only let-7i was affected by such antigens. Thus, this is the first evidence showing that antigens derived from a helminth parasite, indeed, possess modulatory activities over the expression of miRNAs in human DCs.

The lethal-7 (let-7) family of miRNAs has been linked to important aspects of the immune response, such as inflammation, autoimmunity, and host-pathogen interactions [21, 22]. In particular, the expression of its member let-7i seems to be affected by some protozoans like *Cryptosporidium parvum* and *Plasmodium berghei* and in turn possibly affecting the immune response against them [23, 24]. More importantly, let-7i is up-regulated in DCs in response to LPS and participates in the induction of their maturation and secretion of proinflammatory cytokines such as IL-12 [17, 18]. Interestingly, secretion of IL-10 is only favored when let-7i is inhibited [17], indicating the inflammatory activity that this miRNA possesses on DCs activated by LPS. Here we show that TcES downmodulate the LPS-induced expression of let-7i in human DCs in parallel to a diminished inflammatory cytokine/chemokine profile and a normal secretion of IL-10. Thus, it is likely that the previously reported impaired maturation and the repeatedly observed anti-inflammatory phenotype of DCs exposed to TcES can be due to the repressing effects that these antigens possess upon let-7i. Moreover, it has been shown that one of the targets of let-7i is the suppressor of cytokine signaling 1 (SOCS1) [17], a molecule that has been related to the downregulation of DC maturation and secretion of the pro-inflammatory cytokines IL-12, IL-6, and IFN-γ [25, 26]. Even when we did not assess the expression of SOCS1 in human DCs exposed to TcES, we hypothesize that the downregulation of let-7i by these antigens may in turn affect the levels of SOCS1 regulating in this manner the maturation and the inflammatory cytokine profile of such DCs. The mechanisms involved in the modulation of TcES on let-7i expression in human DCs are currently unknown. Interestingly, O'Hara and colleagues showed that *C. parvum* parasite decreases let-7i expression by promoting the formation of a NF-κB p50-C/EBPβ silencer complex in cholangiocytes [27]. If this is the mechanism involved in the repression of let-7i expression by the helminth antigens used in our study remains to be determined.

In conclusion, TcES are helminth-derived antigens that exert modulatory effects upon the expression of the LPS-induced miRNA let-7i. It is possible that this downmodulation underlies the previously reported dampening of maturation in human DCs activated with LPS and the diminished secretion of pro-inflammatory cytokines observed again in this study. To our knowledge, this is the first report that assesses the possible regulatory actions of helminth antigens upon host miRNAs. The downregulation of let-7i by TcES opens a new route in the study of modulation of the host response by *T. crassiceps* and possibly other helminths. This study establishes the basis for the implement of functional studies where silencing and overexpression of miRNAs may allow us to elucidate the complex mechanisms involved in the regulatory activities that helminth antigens possess over the immune system.

Authors' Contribution

The two authors L. I. Terrazas and F. Sánchez-Muñoz contributed equally to this work.

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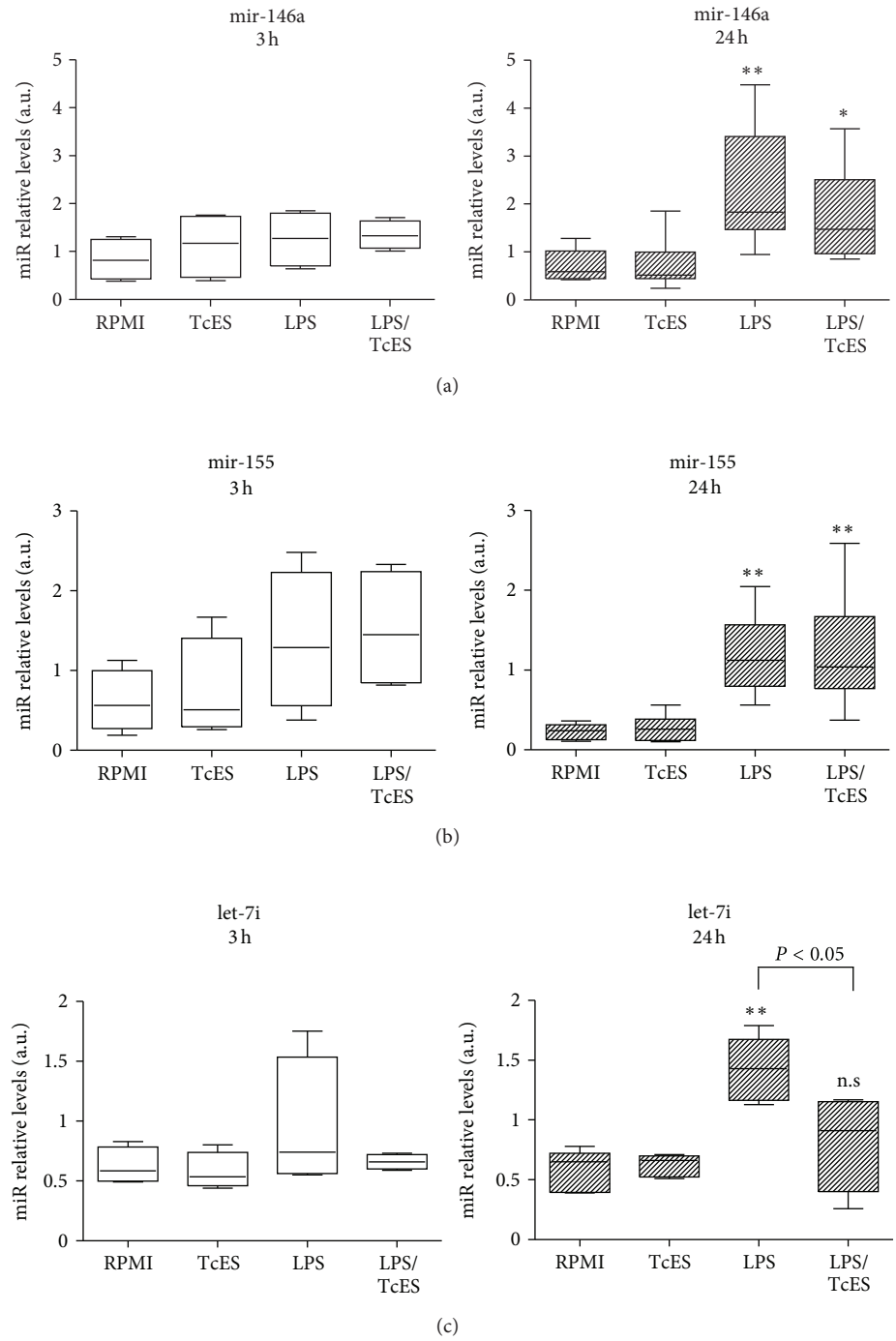

FIGURE 2: Expression of miRNAs mir-146a, mir-155, and let-7i in human DCs activated with LPS and exposed to excreted/secreted *T. crassiceps* antigens. The miRNAs expression was evaluated after stimulation of human DCs with 20 ug/mL TcES, 1 ug/mL LPS, or a combination of them for 3 or 24 h. Control cells received RPMI. Determination of miRNAs relative levels by qRT-PCR was conducted using mature miRNAs specific TaqMan assays in cultures from nine independent experiments (4 donors for 3 h and 5 donors for 24 h). The gene *let-7a* was used as reference gene and relative quantification was calculated by the formula $2^{-(\text{CT targetmiRNAs}-\text{CT reference})}$. All miRNAs assays were tested for reproducibility and linearity (PCR efficiency was between 1.9 and 2.0 for all assays). The data is shown as boxplot, horizontal line denotes the median value, box encompasses the upper and lower quartiles and the whiskers, and the minimum and maximum data value. The relative expression values were analyzed using the nonparametric Kruskal-Wallis test and Dunn's *post hoc* comparisons. For (a) y (b) $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* RPMI control group were deemed significant. For (c) $^{*}P < 0.05$ for LPS *versus* LPS/TcES and n.s. for LPS/TcES *versus* RPMI control group. All analyses were performed with the GraphPad Prism v. 5 statistical software.

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Research Article

Toxocara Seroprevalence in Patients with Idiopathic Parkinson's Disease: Chance Association or Coincidence?

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Most cases of idiopathic Parkinson disease (IPD) are believed to be due to a combination of genetic and environmental factors. The purpose of this study is to investigate the relationship between toxocariasis and Parkinson disease (PD). Patients were selected from people who were admitted to the Movement Disorders Branch, Neurology Department of Elazığ University Faculty of Medicine Elazığ, Turkey. We studied specific IgG antibodies against *Toxocara canis* (*T. canis*) in 50 patients with idiopathic Parkinson and 50 healthy volunteers. We investigated the clinical history of three patients infected with *T. canis*. We also studied specific IgG antibodies against *Toxoplasma gondii* in these groups. Antibodies anti-*Toxocara canis* were found in 3 idiopathic PD (6%) (P = 0.121) and antibody titer was not found in control. A patient had history of the presence of dog in current dog ownership. We did not detect any statistically significant association between *T. canis* and IPD. But, we believe that further comprehensive studies are required for understanding whether there is a causal relation between toxocariasis and PD. We didn't find possible association between *Toxoplasma gondii* and IPD (P = 0.617).

1. Introduction

The idiopathic form of Parkinson disease (IPD) is one of the most common neurodegenerative disorders. It is mainly characterized by a progressive and massive loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta* (SNpc), which leads to several clinical motor symptoms such as akinesia, rigidity, and resting tremor. The molecular pathways leading to these concomitant clinical alterations remain obscure, but it is believed that it may result from environmental factors, genetic causes, or a combination of the two [1]. The frequency of contact with infection agents in patients with IPD and the relationship between infections and clinical findings or pathogenesis of IPD are unclear. In relation to this, a few studies in the literature have drawn attention to the possible role of infectious agents in the pathogenesis of IPD [2–5].

Toxocariasis is caused by ingestion of the eggs of *Toxocara canis* (*T. canis*), a roundworm in dogs, puppies, and cats [6]. Human who ingest *T. canis* eggs may remain unaffected or may manifest a mono- or multisystem disease with symptoms arising from larval invasion of different organs. Though neurological manifestations of *T. canis* larvae are rare, toxocariasis remains an important differential diagnosis of various neurological disorders [6, 7]. Neurological manifestations of toxocariasis have documented both the central and the peripheral nervous system. Dementia, behavioral disturbances, meningoencephalitis, myelitis, cerebral vasculitis, epilepsy, optic neuritis, radiculitis, affection of cranial nerves, lower motor neuron disease, and musculoskletal involvement have all been associated with neurotoxocariasis [6–12]. However, a possible relationship between *T. canis* infection and IPD has not been reported in the literature, but a few studies suggested a possible change in the neurotransmitter levels such as GABA, dopamine, serotonin, and monoamines in *Toxocara*-infected animals [13, 14]. This observation drew our attention to a possible link between PD and *T. canis* infection. In order to further explore this association, we

Table 1: Comparison of demographic and clinical characteristics between groups.

Parameters	Patients (n: 50)	Controls (n: 50)	P
Gender			
Male	35 (50.7)	34 (49.3)	$P = 0.824$
Female	15 (48.4)	16 (51.6)	
Ages (years)	75.0 ± 73.92	64.4 ± 10.61	$P = 0.21$
Duration of IPD (years)	4.73 ± 3.57 (min: 1–max: 17)	—	—
Positive for *Toxocara canis* IgG antibodies	3 (6%)	0 (0%)	$P = 0.121$
Positive for *Toxoplasma gondii* IgG antibodies	9 (18.0%)	11 (22.0%)	$P = 0.617$

evaluated the *Toxocara* antibodies in patient with IPD and compared the results with those of control group.

2. Materials and Methods

2.1. Patients and Evaluation. The patients were diagnosed with IPD according to the United Kingdom Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria in the Neurology Outpatient Clinic of the Medical Faculty of Elazığ University. All patients were randomly selected from the database of the neurology department. Control group were randomly selected among the healthy people who underwent a complete neurological examination to exclude the presence of neurological disorders and were matched to the case patients by age and gender. Demographic characteristics and information concerning factors possibly associated with *T. canis* infection were evaluated by a structured interview. We recorded data as follows: age, gender, duration of IPD, the presence of dog in the house, and clinic symptoms (dementia, epilepsy headache, fever, chronic urticaria, etc.). The study was performed in accordance with the Helsinki Declaration. All subjects signed informed consent after receiving a detailed explanation of the study procedures.

2.2. Serological Detection of Toxocara Canis Infection. Five milliliters blood samples were obtained from the cubital vein of both the patients and controls. The sera were separated from whole blood shortly after collection and was stored at −20°C until the analysis. The sera were examined by ELISA *T. canis* IgG test (NOVATEC, Immundiagnostica GmbH). In the next step, in order to detect possible involvement of *Toxoplasma gondii* in patients, the sera were also examined by ELISA *Toxoplasma gondii* IgG test (Cobas Core, Roche, Germany).

2.3. Statistical Assessment. Statistical evaluations were performed by using the SPSS 11.5 programme. For metric variables mean ± standard deviation (minimum-maximum), for categorical variables frequency (percentage) was used as descriptive statistics. Student t-test was used to compare mean ages of groups. chi-square test was used to determine whether there was a difference between groups in terms of *T. canis* positivity. $P < 0.05$ was considered as statistically significant.

3. Results

Fifty patients with IPD and fifty controls were enrolled in the study. The mean age of patients was 65.6 ± 10.2 (range: 43–81). Of the 50 patients, 32 (64%) were males and 18 (36%) were females. The mean duration of IPD was 5 ± 4 years (range: 1–17). The mean age of controls was (65.6 ± 30.4). Among the 50 controls, 29 (58%) were males and 21 (32%) were females. There were no statistically significant differences among the patients and controls subjects with respect to age and gender ($P = 1.000$ and 0.818, resp.). While 3 out of 50 (6%) cases with IPD were positive for anti-*T. canis* IgG antibodies, all control subjects were negative. There were also no statistically significant differences between the rates of positivity between the IPD patient group and the control group ($P = 0.121$). In 9 (18%) of the 50 patients, and in 11 (22%) of 50 control group, anti-*Toxoplasma gondii* antibodies were detected ($P = 0.617$). Sociodemographic characteristics and clinical characteristics among groups are given in Table 1.

3.1. Presentation of the Patients with Positive Toxocara Antibodies

Patient 1. A 78-year-old male had a ten-year history of IPD. He had no history of presence of dog in the house and clinic symptoms of toxocariasis.

Patient 2. A 46-year-old male reported a history of IPD for two years. He had a dog in the house. But the patient reported no clinic symptoms of toxocariasis.

Patient 3. A 76-year-old male had an eight-year history of IPD. He had neither the presence of dog in the house nor clinic symptoms of toxocariasis.

Toxoplasma gondii IgG antibodies were negative in all patients.

4. Discussion

In this study, we investigated the *T. canis* IgG antibodies in patients diagnosed with IPD. Although *Toxocara* IgG antibodies were found in 3 IPD (6%), all control subjects were negative. IPD is an age-related neurodegenerative disorder that is characterized by a slow and progressive degeneration of DA neurons in the SNpc combined with intracytoplasmic proteinaceous inclusions known as Lewy bodies. Despite an extensive research, the exact cause of IPD is not completely

known, but important clues point at the involvement of some combination of genetic and environmental factors [5]. Several potential the pathogenic mechanisms of neuronal degeneration in IPD have been shown, including oxidative stress, neuroinflammatory processes, mitochondrial abnormalities, excitatory amino acids, a rise in intracytoplasmic free calcium, cytokines, and apoptotic processes. Recent studies have revealed an essential role for neuroinflammation that is initiated and driven by activated microglial and infiltrated peripheral immune cells and their neurotoxic products (such as proinflammatory cytokines, reactive oxygen species, and nitric oxide) in the pathogenesis of IPD [15]. Epidemiologic studies suggest that a number of environmental factors may increase the risk of developing IPD. These include exposure to well water drinking, farming, pesticides exposure (paraquat, organophosphates, and rotenone), herbicides, metals (manganese, copper, mercury, lead, iron, zinc, and aluminum), wood pulp mills, rural residence, diet, head trauma, and infections [5, 15]. Parkinsonian states have been described following viral encephalitis due to measles, Japanese B virus, Western equine virus, polio, epstein-barr virus, cytomegalovirus, influenza A virus, and human immunodeficiency virus disease [5].

More recently, Miman et al. found *Toxoplasma gondii* IgG antibodies in IPD higher than controls. They speculated that there might be a possible association between *Toxoplasma gondii* and IPD and *Toxoplasma* infection may be involved in the pathogenic mechanisms of IPD [4]. But, our both studies did not detect any statistically significant association between *T. gondii* and PD [3].

The clinical presentations of toxocariasis have been documented both the central nervous system and the peripheral nervous system in the literature since 1951. More recently, the biochemical and immunopathological alterations in the brain in experimental neurotoxocariasis model have also been shown [13, 16]. Othman et al. demonstrated significant increase in gene expression of proinflammatory cytokines and nitric oxide in the brains of *Toxocara*-infected mice especially in the chronic stage. Proinflammatory cytokines can be neuroprotective, but with sustained or overstimulated increase, they will have deleterious effects on the neurons [14]. They found also significant changes in neurotransmitter profile. They reported that *T. canis* infected mice demonstrated significantly decreased GABA, dopamine, monoamines, and serotonin levels compared with uninfected mice. However, norepinephrine levels were higher in infected mice in this study.

We could not find any published study which has evaluated the seroprevelance of toxocariasis in IPD patients. Besides, there are a few studies in Turkey about seroprevalence *Toxocara*. These studies show that the seroprevalence of toxocariasis varies between 2.6% and 51% in our country [17–23]. All of these studies except two reports have been done in childhood. One of these studies, Kaplan et al. showed that *T. canis* seroprevalence was 2.6% in healthy individuals in Elazığ, an urban region in Turkey [17]. Another study has shown that the seroprevalence was 6% in students at veterinary college and 10% in people exposed to dogs [18].

We found seroprevelance of *Toxocara* as 7.1% in patients with IPD and 0% in the controls. Despite the high anti-*T. canis* seropositivity found in our IPD patients, we did not detect any statistically significant differences between the rates of positivity between the IPD patient group and the control group. The relationship between anti-*T. canis* seropositivity and IPD may be chance association in these cases. On the other hand, the rate of anti-*T. canis* seropositivity in our patients increased nearly by a threefold when compared with study performed from Elazığ in healthy individuals. Furthermore, experimental models of neurotoxocariasis have also shown increased neuroinflammatory mechanisms and significant changes in neurotransmitter profile which all of neurotransmitters also play mainly role in IPD.

5. Conclusion

The present study suggest the possibility that the presence of the *Toxocara* IgG antibodies in patients diagnosed with IPD may extend beyond the chance. The pathogenic mechanisms involved in IPD remain incompletely understood. Based on the concept that IPD seems to be a multifactorial disease, genetic factors, oxidative stress, and neuroinflammatory processes have been implicated in its pathogenesis [24]. We believe that further comprehensive studies are required for understanding whether there is a causal relation between toxocariasis and IPD and/or the presence of the *Toxocara* IgG antibodies may be the possibility risk factor for IPD.

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Research Article

$CD4^+CD25^+FOXP3^+$ Treg Cells Induced by rSSP4 Derived from *T. cruzi* Amastigotes Increase Parasitemia in an Experimental Chagas Disease Model

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Currently, there is a considerable controversy over the participation of Treg cells during *Trypanosoma cruzi* infection, the main point being whether these cells play a negative or a positive role. In this work, we found that the adoptive transfer of $CD4^+CD25^+FOXP3^+$ T cells from rSSP4- (a recombinant *Trypanosoma cruzi* amastigote derived protein, previously shown to have immunomodulatory properties on macrophages) immunized BALB/c donors into syngenic recipients simultaneously with *T. cruzi* challenge reduces cardiac inflammation and prolongs hosts' survival but increases blood parasitemia and parasite loads in the heart. These $CD4^+CD25^+FOXP3^+$ Treg cells from immunized mice have a relatively TGF-β-dependent suppressive activity on $CD4^+$ T cells. Therefore, regulatory $CD4^+CD25^+$ T cells play a positive role in the development of acute *T. cruzi* infection by inducing immunosuppressive activity that controls early cardiac inflammation during acute Chagas disease, prolonging survival, but at the same time promoting parasite growth.

1. Introduction

Trypanosoma cruzi is an intracellular protozoan parasite transmitted through the feces of blood-sucking insect vectors (*Triatoma*) and causes Chagas disease [1]. Intracellular amastigotes are responsible for the persistence of *T. cruzi* infection and induce inflammatory tissue damage in organs such as the heart, esophagus, and colon [2]. Currently, there is a considerable controversy over the participation of Treg cells during *Trypanosoma cruzi* infection, the main point being whether these cells play a negative or a positive role. Cytokines produced in response to infection with *T. cruzi* largely determine the immunopathology and susceptibility to disease. IL-10 and TGF-β both are differentiation factors of Treg cells. TGF-β production decreases elimination of parasites by macrophages (MΦs), associated with exacerbation of disease [3]. Similarly, IL-10 has also been associated with susceptibility to *T. cruzi* infection [4, 5] by blocking the production of IFN-γ by mouse spleen cells and inhibiting some IFN-γ-induced MΦ killing of intracellular *T. cruzi* [6, 7].

Parasites actively secrete or express molecules including parasite-derived proteins, lipids, and glycoconjugates that have potent effects on the immune system [8]. Newly transformed amastigotes, both intracellular and extracellular, express a major surface glycoprotein (SSP4) bound to the plasma membrane by a GPI anchor [9]. The gene that codifies for this protein was cloned [10], and rSSP4 was shown to be a modulator of the immune response, inducing high levels of IgG1, IgG2a and IgG2b isotypes, and the expression of iNOS and production of NO by MΦs [11]. Moreover, rSSP4 was also able to induce the mRNA for

IL-1α, IL-6, IL-12, IFN-γ, and TNF-α cytokines in normal mice, and IL-10 in immunized mice [11], suggesting that TcSSP4 may be involved in modulating T cell populations during *T. cruzi* infection.

The goal of this study was to evaluate the role of antigen-specific induced $CD4^+CD25^+$ T cells during Chagas disease, either controlling or exacerbating infection by *T. cruzi*. Results show that indeed rSSP4 induced expansion of $CD4^+CD25^+FOXP3^+$ T cells that exacerbate Chagas disease by promoting parasite proliferation during acute *T. cruzi* infection. These $CD4^+CD25^+FOXP3^+$ Treg cells have a partially TGF-β-dependent suppressive activity on $CD4^+$ T cells, indicating that these Treg cells play a positive role in the development of acute *T. cruzi* infection by inducing immunosuppressive activity.

2. Materials and Methods

2.1. Mice. Ten-week-old BALB/c mice from CICUAL (CINVESTAV, Mexico) were used. Mice were housed in a controlled microenvironment at the animal facility at CINVESTAV and managed according to institutional animal care guidelines.

2.2. Antibodies. Antibodies used in this work were APC-Cy7-anti-mouse CD4 (Cat. number 552051), purified anti-TGF-β (Cat. number 555052) and anti-IL-10, anti-TGF-β and anti-IFN-γ optEIA sets, from BD Bioscience (San Jose, CA, USA); APC-anti-mouse CD25 (Cat. number 17-0521), FITC or PE-anti-mouse FOXP3 (Cat. number 72-5775), PE-anti-mouse CD14 (Cat. number 12-0141), PE-anti-mouse CD19 (12-0193), from eBioscience (San Diego, CA, USA); purified anti-IL-10 (Cat. No. 505012) from Biolegend (San Diego, CA, USA).

2.3. Purification of Recombinant SSP4 (rSSP4). *TcSSP4*, the gene that codifies for *T. cruzi* amastigote-specific surface antigen, was cloned in the EcoR1 site of the expression vector pMAL-C2, resulting in the plasmid pMAL-TcSSP4. *E. coli* DH5-α was transformed with this plasmid to obtain the fusion protein MBP::SSP4 (rSSP4) [10, 11]. rSSP4 and MBP were purified by amylase affinity chromatography; after purification, material was analyzed by 10% SDS-PAGE on which a 127 kDa protein corresponding to rSSP4 and a 43 kDa protein corresponding to MBP were observed, respectively (data not shown). In all experiments, purified MPB protein was included in restimulation conditions as a control, and no significant effects were observed with this protein; MBP alone did not induce $CD4^+CD25^+FOXP3^+$ neither cytokines, as did the recombinant protein (data not shown).

2.4. Mice Immunization Protocol. Ten-week-old female BALB/c mice were divided into two groups, 3 mice per group. One group was treated with PBS (NIM), and the other one was immunized with rSSP4 protein (IM) once a week for 3 weeks (10 μg per dose per mouse by intraperitoneal route). The number of repetitions of experiments is indicated in figure legends.

2.5. Flow Cytometry Analysis. Spleen cells from immunized and nonimmunized mice cultured for 72 h were stained, according to the desired cell markers, with one or more of the following antibodies: PE-anti-mouse CD19, PE-anti-mouse CD14, PE-anti-mouse CD4, APC-anti-mouse CD25, or FITC-anti-mouse FOXP3, according to the manufacture's protocol. In brief, nonspecific staining was blocked with anti-CD16/CD32 mAb (Fc block from eBioscience), and cells were incubated with the appropriate antibodies for 30 min on ice and washed twice with PBS containing 2% fetal bovine serum. For FOXP3 staining, cells were fixed/permeabilized for 45 min using a FOXP3 kit from eBioscience. Cells were then washed with 1X permeabilization buffer and stained with FITC-anti-FOXP3. Analysis of intracellular FOXP3 was performed according to the manufacturer's instructions. Briefly, spleenocytes were resuspended at 2×10^6 cells/mL in complete medium or the conditions mentioned above for 72 h. Cells were stained with PE-Cy5.5-anti-CD4 and APC-anti-CD25, then cells were simultaneously fixed and permeabilized with Fix/Perm Buffer (eBioscience), and intracellular staining with PE-anti-FOXP3 mAb was developed. Cellular population analyses were performed with a FACS Calibur Becton Dickinson Cytometer (San Diego CA, USA) by acquiring 1×10^5 events (gated by forward and side scatter properties; in the case of intracellular staining, these parameters were adjusted accordingly) and analyzed using Summit Software (Beckmann Coulter; Brea, CA, USA).

2.6. Isolation of $CD4^+$ and $CD4^+CD25^+$ T Cells. Freshly isolated spleen $CD4^+$ and $CD4^+CD25^+$ T cells from immunized mice were purified by positive selection by flow cell sorting. First, $CD4^+$ and $CD4^+CD25^+$ T cells were stained with labeled antibodies APC-Cy7-anti-mouse CD4 and APC-anti-mouse CD25 for 30 min. Cell suspensions were passed through a high speed flow cytometer MoFlo from Beckman Coulter. Positively selected $CD4^+$ and $CD4^+CD25^+$ cells were found to be more than 95% pure on FACS analysis.

2.7. T. Cruzi Challenge of Naïve, Immunized, and Adoptively Transferred Mice. $CD4^+CD25^+$ T cells were isolated from the spleens of naïve or rSSP4-immunized BALB/c mice as described previously. Nonprimed or rSSP4-primed $CD4^+CD25^+$ T cells (1×10^6) were adoptively transferred into five-weeks-old female BALB/c mice through the tail vein. These mice were immediately infected with blood trypomastigotes of *Trypanosoma cruzi* (8×10^4, H8 Yucatan strain), by intraperitoneal inoculation; naïve and immunized mice (3-4 animals) were infected following the same protocol. Parasitemia and survival rates were calculated. Naïve infected mice and nonprimed $CD4^+CD25^+$ T cells-transferred mice were used as controls.

2.8. Inflammatory Infiltrates and Amastigote Nests in the Cardiac Parenchyma. Areas of inflammation and nests

of amastigotes were manually selected from photomicrographs, using the image software Image J (available at http://rsb.info.nih.gov/ij/index.html). Selected areas were quantified as pixels numbers, and determination of the relative area, corresponding to inflammation or amastigote nests, was obtained by dividing the area of interest into the total number of pixels and multiplied by 100. Analyses were performed in four different sections of the same heart.

2.9. T Cell Proliferation Assays. For *in vitro* proliferation, spleens from nonimmunized and immunized mice were excised aseptically 15 days after the third rSSP4 immunization, and cells were cultured in flat-bottom 24 – (2×10^6) or 96 – (0.8×10^6) well plates (Costar) in complete D-MEM medium for cytokine determination and proliferation, respectively. Cells were stimulated with Concanavalin A (ConA) (6 μg/mL), MBP (5 μg/mL), rSSP4 (10 μg/mL), or in medium alone. Proliferation was measured after 72 h by [*methyl*-^{3}H]TdR (Amersham) incorporation (1 μCi per well). Cells were harvested onto glass filters, placed in scintillation fluid, and counted in a Beckman scintillation counter. For cytokines measurements by ELISA, supernatants from 24 well plates were recovered, and to discard contamination with endotoxins, cells were induced to proliferate in the presence of Polymixin B (100 U/mL).

2.10. Suppression Assays. Suppression assays were performed as described by Gavin et al. [12]. Briefly, $CD4^+CD25^-$ T cells (5×10^4), $CD4^+CD25^+$ T cells (titrating amounts) or a combination of the two populations were stimulated for 72 h with 1×10^5 APCs (12 Gy irradiated spleen cells from nonimmunized mice). This was done in the presence of anti-CD3 (25 μg/mL) plus anti-CD28 (2 μg/mL) and rSSP4 (10 μg/mL) in 96 well plates; in all conditions, cells were pulsed with 1 μCi/well of [*methyl*-^{3}H]TdR (Amersham) for the final 16 h. Results are presented as mean±SD cpm values of triplicate wells. These experiments were also developed in the presence of anti-IL-10 (5 and 10 μg/mL) or anti-TGF-β (5 μg/mL) neutralizing antibodies. Viability of anti-IL-10 and anti-TGF-β antibodies was confirmed by western blot (data not shown).

2.11. Analysis of TGF-β mRNA Levels by RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) from spleen cells cultured in 24 well plates with different treatments for 24 h. RNA (5 μg) was transcribed to cDNA with oligonucleotides ($poly(dT)_{16}$), and SuperScript II reverse transcriptase, and PCR was performed with primers for TGF-β (sense; GCCCTGGATACCAACTATTGC, antisense; TCAGCTGCACTTGCAGGAGTAGCG) [13] and GAPDH sequences (as internal control; sense, CCTTCATTGACCTCAACTAC, antisense, GGAAGGCCATGCCAGTGAGA). Each PCR cycle consisted of a denaturation step (95°C, 1 min), an annealing step (65°C, 30 sec), and an elongation step (72°C, 30 sec). DNA was amplified for 30 cycles in a Bio-Rad Thermocycler. PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide.

2.12. Cytokines ELISA. IL-10, TGF-β, and IFN-γ were quantified by ELISA (BD optEIATM ELISA Kit) in culture supernatant of cells under different conditions of restimulation, as described above, according to the manufacture's protocol. Briefly, 96 well flat bottom plates were coated with capture antibody (dilution 1/250 for IL-10 and TGF-β, and 1/2,000 for IFN-γ), blocked with 10% PBS-FCS, washed three times, and incubated with the antigen for 2 h. After washing the plates, they were incubated with detection antibody coupled to avidin-HRP (horseradish peroxidase, 1/250 dilution); after several washes, substrate solution was added and the reaction was stopped after 30 min with 2 N H_2SO_4. Plates were read at 450 nm using a microplate reader (Bio-Rad model 680).

2.13. Statistical Analysis. Analyses were performed using GraphPad Prism version 5.0 software or Sigma Plot 10.0. Differences were considered statistically significant when a P value of less than 0.05 was obtained by Student's t or square Chi test.

3. Results

3.1. Mice Receiving rSSP4-Primed $CD4^+CD25^+$ T Cells but Not Mice Receiving Nonprimed $CD4^+CD25^+$ T Cells Show Exacerbation of Acute T. cruzi Infection. To evaluate the role of regulatory T cells and specifically rSSP4-induced $CD4^+CD25^+FOXP3^+$ T cells during the acute phase of *T. cruzi* infection, $CD4^+CD25^+$ T cells (more than 96% pure) were purified from rSSP4-treated and from nontreated mice and transferred to naïve BALB/c mice just prior to *T. cruzi* challenge. *T. cruzi*-infected recipient mice receiving $CD4^+CD25^+$ T cells from rSSP4-immunized donors developed significantly less severe cardiac inflammation (+) (Figure 1(a), right panels; Figure 1(c)) but higher heart parasite loads (►)(Figure 1(a), right panels; Figure 1(b)) and higher blood parasitemia (○) compared to controls (◊) (Figure 1(d)). Interestingly, mice receiving rSSP4-primed $CD4^+CD25^+$ T cells (○) also survived longer than controls (◊) (Figure 1(e)). On the contrary, when mice were transferred with $CD4^+CD25^+$ Treg cells from naïve mice, blood parasitemia showed the same level as control mice (data not shown). When cardiac tissue was examined for the presence of amastigote nests and inflammatory foci (Figure 1(a), third panel from left to right), they showed the same appearance as that seen with control mice (Figure 1(a), left panels), that is, there were few amastigote nests, always surrounded by inflammation, and, in general, inflammation was more accentuated in these two conditions. These results indicate that rSSP4-induced $CD4^+CD25^+$ T cells although control immunopathology, they promote parasite proliferation during acute *T. cruzi* infection. Moreover, rSSP4 immunized mice (Figure 1(a), second panels from left to right) showed a similar behavior after *T. cruzi* infection in terms of cardiac inflammation (+) and parasite load (►) (Figures 1(a)–1(c)), as well as in survival rate and blood parasitemia (data not shown). Natural Treg cells present in naïve mice or natural Treg cells that came from nonimmunized mice and were

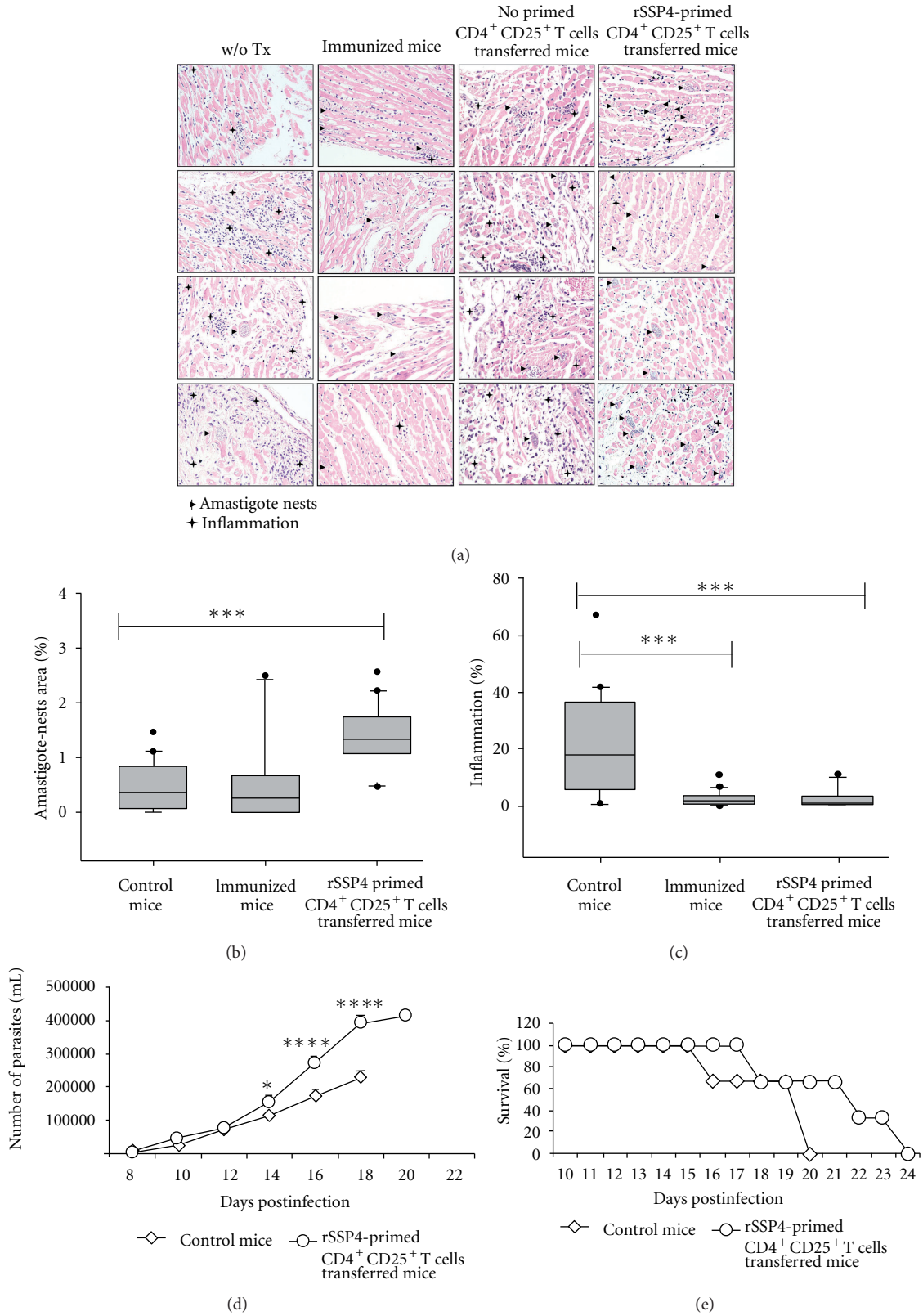

FIGURE 1: Mice receiving rSSP4-primed $CD4^+CD25^+$ T cells but not mice receiving nonprimed $CD4^+CD25^+$ Tcells show exacerbation of acute *T. cruzi* infection and prolonged survival rate. (a) Histological examination by hematoxylin-eosin staining of cardiac tissue from untreated (w/o Tx), rSSP4 immunized, nonprimed- or rSSP4-primed-$CD4^+CD25^+$ T cells-transferred mice. Amastigotes nests are shown by arrow heads and inflammation foci with +. Magnification 40x. (b) Amastigotes nests area between different groups is calculated using Image J program. Graphs represent the average measure of 20 fields. (c) Percentage of inflammation foci was calculated considering the whole area in 20 fields using J program. Statistical analysis was done using Sigma Plot 10.0. (d) Parasitemia levels measured by direct parasite counting from blood samples. (e) Survival rate of infected mice. For (d) and (e), results are the average of three independent experiments; statistical analysis was done with GraphPad Prism 5.0.

adoptively transferred to naïve mice were unable to control inflammation and/or to promote parasite growth.

3.2. Spleen $CD4^+CD25^+FOXP3^+$ T Cells Are Induced Upon Restimulation with rSSP4. Because we found that $CD4^+CD25^+FOXP3^+$ T cells induced by immunization with rSSP4 promoted the development of Chagas disease, we wanted to see whether these regulatory T cells were antigen specific. Flow cytometric analysis revealed that rSSP4-stimulated spleen cells from rSSP4-immunized mice contained significantly higher percentage of $CD4^+CD25^+FOXP3^+$ T cells as compared to similarly stimulated spleen cells from nonimmunized mice (14.06% and 0.17%, resp.) (Figure 2, right panels). No significant difference was noted in percentage of $CD4^+CD25^+FOXP3^+$ regulatory T cells, in spleen cells from rSSP4-immunized or nonimmunized mice in the absence of stimulation (Figure 2, left panels), or in the presence of MBP (data not shown). These results indicate that rSSP4 promotes *in vitro* induction of $FOXP3^+$ regulatory T cell population in an antigen-specific manner, because in the absence of stimuli or in the presence of MBP, which is part of the rSSP4, the proportion of T reg cells remained low.

3.3. rSSP4 Immunization Induced $CD4^+CD25^+$ T Cells with Suppressive Function In Vitro . A characteristic feature of regulatory T cells is their ability to inhibit cell proliferation of effector T cells, and once we evaluated the role of rSSP4-induced $CD4^+CD25^+FOXP3^+$ T cells *in vivo*, we proceeded to perform suppression assays in order to confirm, *in vitro*, their suppressive capacity. $CD4^+CD25^+$ T cells induced after rSSP4 immunization exert a suppressor function on naïve $CD4^+CD25^-$ T cells. Freshly isolated spleen $CD4^+CD25^+$ T cells from immunized mice showed suppressive activity over *in vitro* activated $CD4^+$ T cells, such that their activity increased with increasing numbers of Treg cells. Clearly a suppressive activity could be observed at the ratio of 1 : 4 (Treg : Teff), (Figure 3(a)). $CD4^+CD25^+$ T cells from nonimmunized mice showed a weaker suppressive activity (data not shown). $CD4^+CD25^+$ T cells used for suppressive assays were analyzed to determine whether they also express $FOXP3^+$. Around 75% of these cells were positive for the presence of FOXP3 (Figure 3(b)). These results show that $CD4^+CD25^+FOXP3^+$ T cells induced in rSSP4 immunized mice exhibit a strong suppressor activity.

3.4. Anti-TGF-β Antibodies Partially Inhibit Suppressor Activity of rSSP4-Primed $CD4^+CD25^+$ T Cells. In order to understand the mechanism of Treg cells suppression and confirm or deny the role of IL-10 and TGF-β as suppressor cytokines under these experimental conditions, we developed further experiments assessing the role of IL-10 and TGF-β and their suppressive function using anti-IL-10 or anti-TGF-β neutralizing antibodies as previously described [14]. Blockade of IL-10 using anti-IL-10 Ab (5 μg/mL) had minimal or no effect on suppressive activity of rSSP4-primed $CD4^+CD25^+$ T cells (Figure 3(c)); a higher concentration of anti-IL-10 Ab (10 μg/mL) did not affect either (data not shown). On the other hand, neutralization of TGF-β partially blocked suppressive activity of these cells as indicated by higher proliferation of T effector cells cocultured with rSSP4-primed $CD4^+$ $CD25^+$ T cells in the presence of anti-TGF-β antibodies (5 μg/mL) indicating that anti-TGF-β restores $CD4^+$ T effectors cells proliferation (Figure 3(d)). Taken together, these findings suggest that suppressor activity of rSSP4-induced $CD4^+$ $CD25^+$ T cells is TGF-β but not IL-10 dependent (Figures 3(c) and 3(d)).

3.5. Immunization with rSSP4 Induces TGF-β mRNA Expression and TGF-β, IL-10, and IFN-γ Production. TGF-β and IL-10 have been implicated in the pathogenesis of *T. cruzi* infection, and high levels of both cytokines are usually associated with regulatory T cell differentiation [15, 16]. Once we observed the suppressive properties of Treg cells both *in vivo* and *in vitro*, we continue examining the role of rSSP4 on the immune response and continue to look for the presence of different cytokines, responsible of Treg cells differentiation and of their functions. High levels of TGF-β mRNA expression were found in cultured cells and the presence of the protein both in serum of rSSP4 immunized mice, and in culture supernatant of restimulated cells from immunized animals. A high level of TGF-β mRNA expression was found in immunized mice in comparison with mice that were not immunized, independently of the stimulation condition, suggesting that this immunomodulatory cytokine is induced by the amastigote-specific antigen SSP4 *in vivo* (Figure 4(a)). Furthermore, sera from rSSP4-immunized mice contained significantly higher levels of TGF-β compared to culture supernatant from rSSP4-stimulated T cells (Figure 4(b)). Because none of the other restimulation conditions induced TGF-β, these results show that rSSP4 is a potent inducer of this immunosuppressive cytokine. Proinflammatory cytokines such as IFN-γ contribute to host resistance against *T. cruzi,* whereas anti-inflammatory cytokine IL-10 has been implicated in mediating susceptibility; therefore, we examined the effect of rSSP4 on Th1/Th2 cytokines production by spleen cells. rSSP4-stimulated spleen cells from rSSP4-immunized mice produced significantly more IL-10 than similarly stimulated spleen cells from nonimmunized control mice. This difference between immunized and nonimmunized mice was not found upon mitogenic stimulation with ConA (Figure 4(c)). IL-10 production was also measured in cell culture supernatant from cells grown in the presence of polymyxin B; no differences in IL-10 production between presence and absence of polymyxin B were found indicating that LPS was not responsible for the increase in IL-10 production (data no shown). These data support previous observations where rSSP4 immunization increased levels of IL-10 mRNA expression in BALB/c mice [11].

With respect to IFN-γ, a key Th1 cytokine, differences were also observed under the assay conditions. As in the case of IL-10, there were no differences between the two groups when cells were stimulated with ConA. However, this cytokine was found in greater amounts in supernatant of spleen cells from immunized mice, restimulated with rSSP4 (Figure 4(d)).

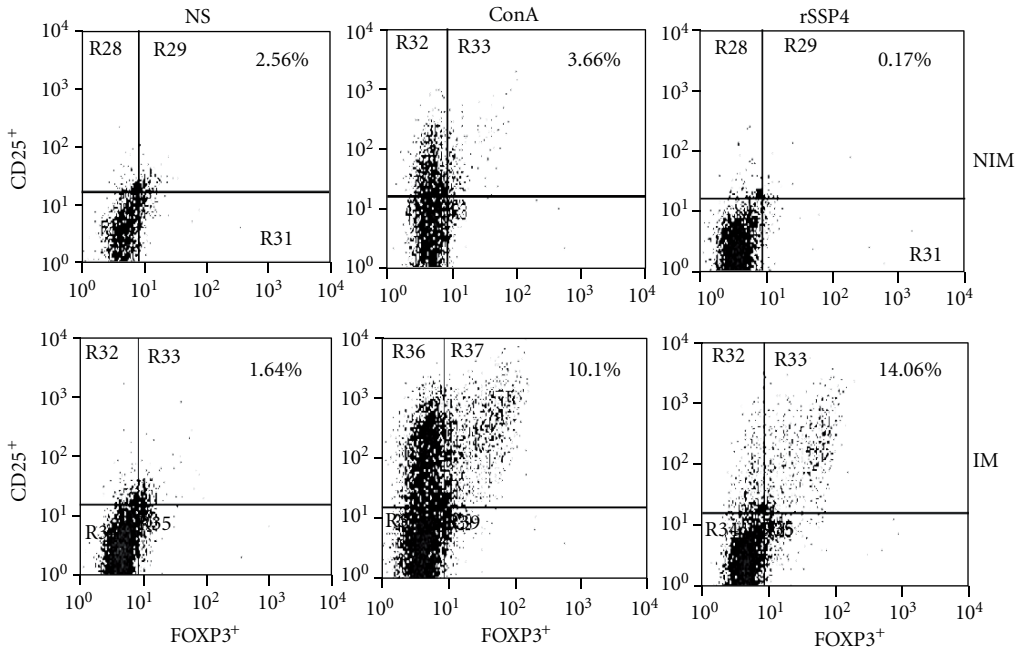

Figure 2: Spleen $CD4^+CD25^+FOXP3^+$ T cells are rSSP4-specific. Mice were i.p. immunized with 10 μg/mouse of rSSP4 or administered PBS. After three immunizations, spleen cells were stimulated *in vitro* with ConA, rSSP4, or nonstimulated for 72 h, and surface expression of CD25 and intracellular expression of FOXP3 were measured in spleen cells from both nonimmunized and immunized mice. Shown cells are $CD4^+$ lymphocytes. Results are representative of four independent experiments.

4. Discussion

The results presented in this study indicate that $CD4^+$ $CD25^+$ regulatory T cells, induced by rSSP4, suppress cardiac pathology and prolong host survival during acute *T. cruzi* infection in a specific way. Still they contribute to disease progression by promoting peripheral blood parasitemia and cardiac parasite growth. In this study, we showed that rSSP4-primed $CD4^+CD25^+$ regulatory T cells play a decisive immunoregulatory function by decreasing inflammation and increasing survival and parasitemia in immunized and rSSP4-primed $CD4^+CD25^+$ T cells-transferred mice. Based on data from suppression assays, most probably, Treg cells exert their suppressive activity over $CD4^+$ T cells in a partial TGF-β dependent mechanism. Even though immunized mice and transferred mice showed higher cardiac and blood parasitemia, they also showed better survival and reduced cardiac inflammation; these effects were not observed in nonimmunized mice or in mice transferred with naïve mice-derived $CD4^+CD25^+$ T. In these two groups, the same level of parasitemia and the same level of cardiac inflammation were observed. These results support our hypothesis that regulatory T cells induced by rSSP4 are antigen-specific.

Furthermore, results clearly show that *T. cruzi* amastigote stage-specific antigen SSP4 induces high levels of TGF-β and expansion of $CD4^+CD25^+FOXP3^+$ T cells. These cells mediate suppression of effector T cells, via a TGF-β-dependent but IL-10 independent pathway. These results suggest that the high levels of IL-10 produced after rSSP4 immunization could be crucial for the differentiation process of $CD4^+CD25^+FOXP3^+$ cells, but not for their suppressive mechanism. It is important to mention that rSSP4 induced an antigen-specific immune response, based on the fact that ConA, MPB, or absence of stimuli do not induce this population. Moreover, the induction of Treg cells does not occur by immunization with other *T. cruzi*-derived recombinant antigens [17].

To survive an infection requires that the host generates a controlled immune response that recognizes and eliminates the invading pathogen, while limiting collateral damage to self-tissues that may result from a vigorous immune response [18]. At the time of their first encounter with their host, parasites might modulate the immune response by actively secreting or expressing molecules with potent effects on the immune system [8, 18]. A large variety of modulatory parasite-derived proteins, lipids, and glycoconjugates has been described [10]. In the *T. cruzi* protozoan parasite, specifically in the amastigote stage, a surface glycoprotein named SSP4 was described by Andrews et al. [9]. This study found that newly transformed amastigotes, both intracellular and extracellular, express SSP4 that is bound to the plasma membrane by a GPI anchor. We had previously reported that SSP4 induces mRNA expression of pro- and anti-inflammatory cytokines from macrophages *in vitro* [11]. In the present study, we have extended the findings on the immunomodulatory function of *T. cruzi*-derived SSP4 showing that rSSP4 can induce the expansion of regulatory T cells during *T. cruzi* infection, accompanied by TGF-β, IL-10, and IFN-γ production.

Several pathogens have been reported to induce the expansion of Treg cell populations [16], including naturally

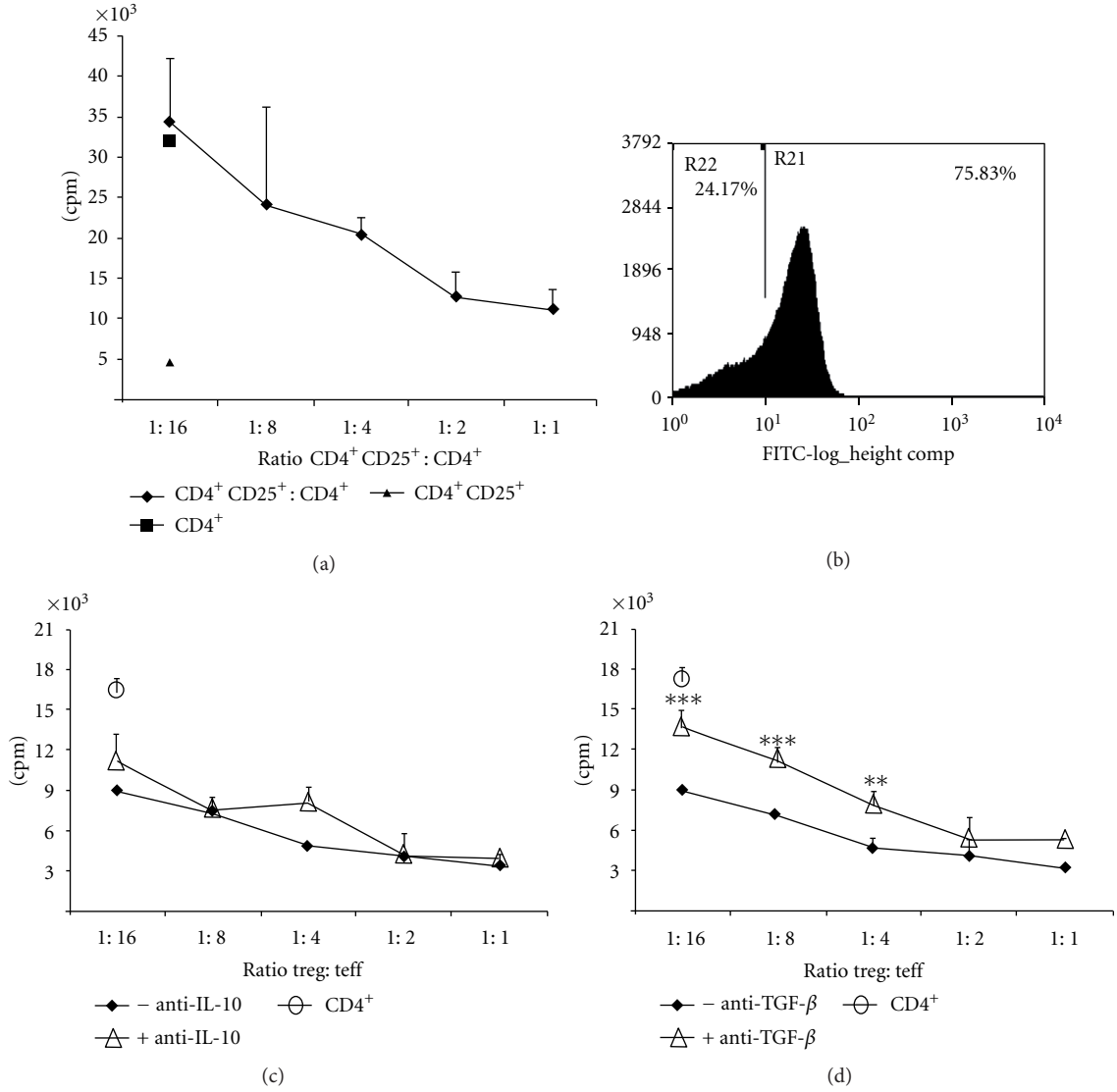

Figure 3: rSSP4 immunization induced $CD4^+CD25^+$ T cells with suppressive function *in vitro*. (a) A total of 5×10^4 $CD4^+CD25^-$ T cells (from naïve mice) and 1×10^5 APCs (from nonimmunized mice) were cultured alone or in combination with titer quantities of rSSP4-primed $CD4^+CD25^+$ T cells in the presence of anti-CD3 and anti-CD28 antibodies plus antigen. Proliferation was quantified by ^{3}H-Thymidine incorporation during the last 16 h of culture. (b) An aliquot of rSSP4-primed $CD4^+CD25^+$ sorted T cells was tested for FOXP3 expression. Results shown are representative of three independent experiments (c) and (d). Anti-TGF-β antibodies partially inhibit suppressor activity of $CD4^+CD25^+$ T cells. A total of 5×10^4 $CD4^+$ T cells (from immunized mice) and 1×10^5 APCs (from nonimmunized mice) were cultured alone or in combination with titer quantities of rSSP4-primed $CD4^+CD25^+$ T cells in the presence of anti-CD3 and anti-CD28 antibodies plus antigen in the presence of 5 μg/mL of neutralizing anti-IL-10 (c) or anti-TGF-β (d) antibodies. Proliferation was quantified by ^{3}H-Thymidine incorporation during the last 16 h of culture. Results are the average of three independent experiments done in duplicates.

occurring $FOXP3^+$ Treg and induced Treg cells, including Tr1 [19] and Th3 cells [15]. Although $CD4^+CD25^+$ T cells have been identified as critical regulators of immune response during infections caused by different protozoa [20], their role in regulating the outcome of *T. cruzi* infection is not clear. A previous research by Kotner and Tarleton reported that depletion of regulatory T cells prior to *T. cruzi* challenge had no effect on the outcome of acute *T. cruzi* infection caused by a Brazilian strain [1]. In contrast, Mariano et al. (2008) found that $CD4^+CD25^+GITR^+FOXP3^+$ T cells migrate to the heart after *T. cruzi* challenge and that the administration of anti-CD25 or anti-GITR Ab resulted in increased mortality during infection [21]. In addition, this study found that anti-GITR treatment was associated with increased TNF-α production and myocarditis as well as tissue parasitemia. In this work, we found that *in vivo* exposure of passively transferred or immunized animals to SSP4 antigen, expressed by the amastigote stage, or *in vitro* by restimulation with rSSP4, induces the conversion of Treg cells $CD25^+FOXP3^+$. These cells were able to inhibit proliferation of effector $CD4^+$

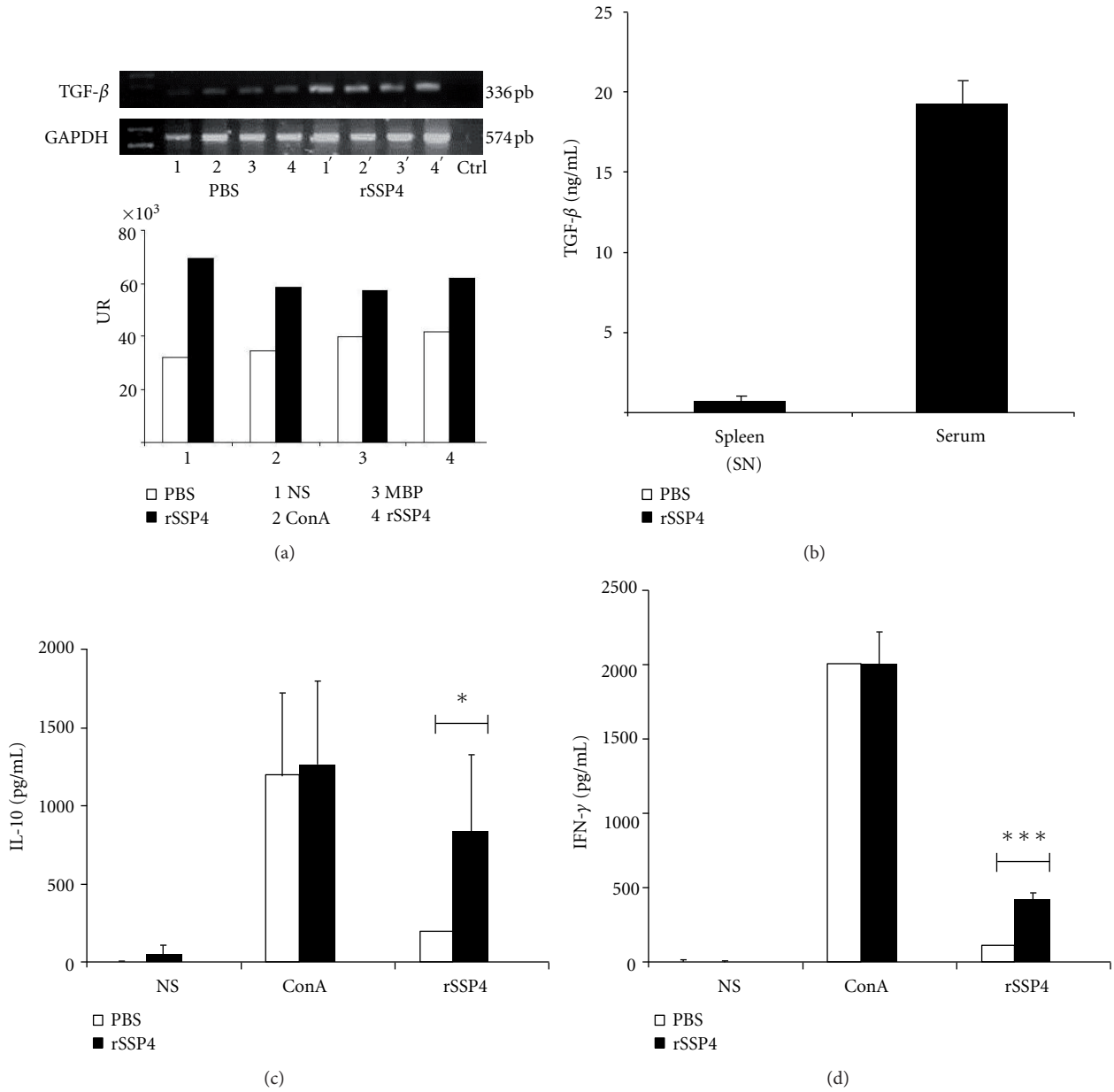

Figure 4: Immunization with rSSP4 induces TGF-β mRNA expression and TGF-β, IL-10, and IFN-γ production. RNA from 24 h cultured spleen cells was extracted and used to determine TGF-β expression by RT-PCR, and culture supernatants were harvested and assessed for cytokines production by ELISA ($n = 3$). (a) TGF-β mRNA expression by wild type spleen cells (upper panel); densitometric analysis of TGF-β mRNA expression (lower panel). Culture supernatants were harvested after 72 h of culture and assessed for TGF-β, IL-10, and IFN-γ production by ELISA. (b) TGF-β production was measured in culture supernatants (SNs) of spleen cells from rSSP4 immunized mice under rSSP4 restimulation condition and in sera from immunized mice. Graphs show values in pg/mL (mean ± SD). (c) and (d) IL-10 and IFN-γ production, respectively, by wild type mice in immunized (full bars) and nonimmunized mice (open bars) under different conditions: NS (non-stimulated), ConA, or rSSP4. Results shown are the average of at least six independent experiments done in triplicate. Differences between groups are considered significant at P less than 0.05 and are represented by $^{*}P < 0.05$, and $^{***}P < 0.001$ in a Student t-test.

T cells *in vitro* and to promote peripheral blood and heart parasitemia *in vivo*. Sun et al. (2012) reported the induction of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells by rSj16, a recombinant protein derived from a protein present in the secretions of *Schistosoma japonicum* [22].

The role of IL-10 as an immunoregulatory cytokine in infection has been documented primarily in the context of chronic infections. IL-10 can suppress immune responses (either Th1 or Th2 cells) towards many pathogens in experimental models. The four major T-cell sources of IL-10 are T-helper type 2 (Th2) cells, subsets of regulatory T cells designated Tr1, Th1, and Th17 cells [23]. Nevertheless, cells such as macrophages, B cells, NK cells, and $CD8^{+}$ T cells, which are involved in determining the outcome of *T. cruzi*

infection, also produce IL-10. We examined different cell types, such as B cells, MΦs, NK, and T cells, as potential sources of rSSP4-induced IL-10. We found that at least for the rSSP4-antigen and under restimulation conditions, IL-10 is produced by $CD4^+CD25^+$ cells (data not shown). This is perhaps not surprising, since a recent study from our laboratory found that rSSP4 induces a population of IL-10/IFN-γ $CD4^+$ double producers T cells [24], which have also been identified as a major source of IL-10 during infections such as leishmaniasis [25].

Taken together, these findings suggest that regulatory CD4+ T cells are involved in progression and pathogenesis of experimental *T. cruzi* infection. Indeed, clinical studies by Vitelli-Avelar et al. (2008), Fiuza et al. (2009), and de Araújo et al. (2011, 2012) found that individuals in the indeterminate clinical form of the disease have a higher frequency of $CD4^+CD25^{high}$ T cells population secreting IL-10 and expressing FOXP3, indicating that the balance between regulatory and effector T cells might be a critical determinant of disease progression in Chagas disease [26–29].

5. Conclusion

In conclusion, *T. cruzi* amastigote stage-specific protein SSP4 enhances production of TFG-β and IL-10 and induces expansion of $CD4^+$ regulatory T cells in susceptible BALB/c mice. These cells suppress proliferation of effector $CD4^+$ cells by an IL-10 independent mechanism but a partially TGF-β-dependent mechanism. In addition, $CD4^+CD25^+$ regulatory T cells from rSSP4 treated mice suppress cardiac inflammation and prolong survival, but promote peripheral blood and heart parasitemia when transferred into syngenic recipients just prior to *T. cruzi* challenge. These findings suggest that *T. cruzi* amastigote stage-specific protein SSP4 could contribute to immune evasion and establishment of chronic infection, by inducing expansion of disease exacerbating $CD4^+C25^+$ regulatory T cells during acute *T. cruzi* infection.

Abbreviations

rSSP4: amastigote stage-specific recombinant protein;
MΦs: macrophages.

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Review Article

The Importance of the Nurse Cells and Regulatory Cells in the Control of T Lymphocyte Responses

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T lymphocytes from the immune system are bone marrow-derived cells whose development and activities are carefully supervised by two sets of accessory cells. In the thymus, the immature young T lymphocytes are engulfed by epithelial "nurse cells" and retained in vacuoles, where most of them (95%) are negatively selected and removed when they have an incomplete development or express high affinity autoreactive receptors. The mature T lymphocytes that survive to this selection process leave the thymus and are controlled in the periphery by another subpopulation of accessory cells called "regulatory cells," which reduce any excessive immune response and the risk of collateral injuries to healthy tissues. By different times and procedures, nurse cells and regulatory cells control both the development and the functions of T lymphocyte subpopulations. Disorders in the T lymphocytes development and migration have been observed in some parasitic diseases, which disrupt the thymic microenvironment of nurse cells. In other cases, parasites stimulate rather than depress the functions of regulatory T cells decreasing T-mediated host damages. This paper is a short review regarding some features of these accessory cells and their main interactions with T immature and mature lymphocytes. The modulatory role that neurotransmitters and hormones play in these interactions is also revised.

1. Background

Lymphocytes are cells that express receptors that can recognize foreign antigens and activate inflammatory reactions in their surroundings to eliminate them. In this way, lymphocytes provide specific adaptive immunity in all vertebrates. These cells are classified into two main subpopulations, B and T, which recirculate inside the peripheral blood and lymph vessels distributed through the entire body, but lymphocytes can also migrate across the high endothelial cells of venous capillaries and home to different organs where foreign antigens are located. When the lymphocyte's receptors recognize them, the lymphoid cells proliferate and form clones that start a wide set of specific defensive humoral and cellular responses to eliminate microorganisms and infected or malignant cells [1]. In this paper we will refer only to T lymphocytes.

The development of T lymphocytes in the thymus and the control of their functions in the periphery are mainly controlled by two special cell populations named "nurse cells" and T regulatory cells. Early in their development, immature T lymphocyte precursors migrate inside the thymus gland and are engulfed by epithelial "thymic nurse cells" (TNCs) [2], which stimulate their development and simultaneously remove most of the defective or self-reactive T lymphocytes. Once the remaining mature T cells leave the thymus, they are subjected to a second control by "regulatory cells" that can inhibit their excessive or dangerous responses [3].

The thymic nurse cells eliminate within their vacuoles high affinity autoreactive and poorly developed T lymphocytes, thus preventing subsequent autoimmune reactions and diseases. The peripheral regulatory cells instead, only reduce the functions of circulating T lymphocytes. While the nursing work kills and negatively selects defective T lymphocytes [4], the regulatory work only suppresses T lymphocyte-mediated inflammatory reactions, supporting the immune tolerance

and dampening hypersensitivity responses [5]. The normal modulator activity of nurse cells and regulatory cells can be also disrupted in the course of diverse chronic infectious diseases, particularly those with a parasitic etiology.

1.1. T Lymphocytes Migration. T lymphocyte progenitors enter the thymus via the bloodstream by using integrins, selectins and chemokines during periodically receptive times that are spatially and temporally regulated. Once intrathymic niches are saturated with T cell progenitors, no other new T cell progenitors are allowed to enter until the former move on and leave the niches empty [6]. T cell development in the thymus is more active from fetal to perinatal stages and declines with aging [7].

The intrathymic route of immature T lymphocytes and their eventual development involve an ordered and regulated movement of their progenitors that follow chemokine gradients and interact with adhesion molecules such as integrins, P-selectin, neuropilin-1, and semaphorin-3A [8] through thymic cell networks until they are in the care of nurse cells [9].

Acquiring functional T cell receptors and coreceptors and recognizing self or non-self antigens are the first decisive steps in the life of immature T lymphocytes in the thymus. In the course of their intrathymic migration, T lymphocyte precursors engage their bidirectional interactions with TNCs. Initially they are named double negative (DN) cells, since they do not express neither the CD4 nor CD8 coreceptors. The development of these immature lymphocytes consists of four stages (DN1, DN2, DN3, and DN4) according to the expression of the CD4 and CD8 coreceptors [10] on the membrane surface. As the migration goes on, T lymphocytes begin the expression of their receptors to recognize antigens called T cell receptors (TCR).

In the DN3 stage of their development, immature T lymphocytes begin the expression of a pre-T cell receptor. Signaling pathways through the intracellular domain of the Notch1 transmembrane protein [11] and others [12] control the recombination and rearrangement of the V(D)J gene segments of the α, β or γ, δ chains of these pre-TCRs. Furthermore, the expression of receptors to recognize antigens in immature T cells is influenced by endogenous cytokines [13], which also modulate the migration of immature lymphocytes through thymic epithelial cells and their subsequent selection.

Once their maturation begins, T lymphocytes produce interferon gamma (INF-γ), which controls the expression of the fibronectin and laminin receptors on the thymic epithelial cells (TECs), modulates thymocyte adhesion to thymic epithelial cells, induces the expression of the human leukocyte antigen DR in human thymic epithelial cells, and finally is also involved in thymocyte selection and their subsequent release from the TNC vacuoles [14].

Lymphocyte stimulation by TNC promotes the expression of T cell receptors to recognize antigens and CD4 and CD8 coreceptors even though lymphocytes have not yet completed their development. Maturing lymphocytes must pass through an intrathymic selective process to avoid apoptosis. Lymphocytes stay alive and leave the thymus only after proving that their receptors do not recognize self-antigens with high affinity. To this purpose, the anatomical integrity of the thymus and the efficient function of their nurse cells to perform the first selective and dangerous event in the life of T lymphocytes are necessary.

Different exogenous molecules including hormones such as oxytocin, neurotensin, insulin growth factor 2 (IGF-2), vasopressin [15], glucocorticoids [16], androgens [17], and estrogens [18] and neurotransmitters produced by the autonomic nervous system [19] such as acetylcholine [20], histamine, and serotonin [21] also influence the migration of T lymphocytes by promoting the expression of adhesive molecules.

1.2. Thymic Involution. The competence of the immune system depends on thymic abilities to support developing T lymphocytes and eliminate high affinity autoreactive cells. However, thymic activities do not remain constant throughout life. In fact, the size and cellularity of the thymus undergo a physiological decline almost immediately after birth. In adolescence, when the production of sexual hormones increases, thymic involution accelerates and the number of thymocytes gradually decreases at a rate of 3–5% per year; in adulthood, the number of thymocytes continues its decline at a rate of 1% per year [22]. Furthermore, transitory increases in the production of steroid hormones reduce thymic cellularity during pregnancy [23], and the thymus reduces both its volume and thymocyte numbers [24] during chronic stress. These changes influence the functions of nurse cells and regulatory cells, thus affecting the number and competence of different T lymphocyte subpopulations that flow into the peripheral circulation.

Studies on thymus-hormone interactions have shown that thymic involution and T lymphocyte deficiency of aged male rats could be reversed by reducing testosterone levels through surgical castration [25] or by administering the antiestrogenic agent tamoxifen [26]. The elevation in sex hormone levels is associated with the natural involution of the thymus gland since testosterone induces the apoptosis of $CD4^+CD8^+$ double-positive developing thymocytes [27] via the tumor necrosis factor-alpha (TNF-α).

Besides, despite the relation between pregnancy and thymic involution [28], other authors [29] have shown that elevated progesterone levels can increase the number and functions of peripheral thymus-derived T cells. Pregnancy and estrogen treatments improve Treg cell functions and enhance the expression of their Forkhead-winged helix box p transcription factor 3 (Foxp3) [30]; other authors [31] have reported similar effects after using glucocorticoids in asthmatic patients. Results suggest that hormones and other factors do not always reduce the T lymphocyte number and functions. Although apoptosis decreases the T cell activities during aging [32], thymic involution is also associated with an increase in Treg cell numbers in peripheral blood [33, 34]. Other studies have shown that inhibiting Transforming Growth Factor-β (TGF-β) signaling in thymic epithelial cells slows thymic involution [35] demonstrating that not only elevated Treg numbers but also increased Treg functions are

associated with thymic involution. Therefore, physiological or pathological thymic involution affects the development of different T lymphocyte subpopulations at different ages.

On the other hand, the thymus gland and its different cell subpopulations are seriously injured by diverse parasites, which can induce sparse cellular apoptosis, tissue damage, or thymus involution, affecting the development, proliferation, migration, and the export to the periphery of T lymphocytes, and depressing the cellular immune response. In addition, parasites change the thymic microenvironment by increasing the intrathymic chemokine production and inflammatory cytokines production as well as extracellular matrix components [36].

For instance, in murine Chagas' disease model, the thymus is affected by severe thymocyte depletion mediated by corticosterone and excessive production of TNF-α associated with the export of immature $CD4^+CD8^+$ double-positive (DP) cells to the periphery [37]. Similar changes and thymic hypoplasia can be observed in experimental infections of mice with *Plasmodium berghei*. The parasites cause apoptosis of $CD4^+CD8^+$ thymocytes, exit of both these DP cells and DN thymocytes to mesenteric lymph nodes, as well as changes in the cortical and medullary limits of the thymus [38].

1.3. Care and Surveillance of Immature T Lymphocytes. Cells specialized in binding to other cells to assist them in their development are called "nurse cells." Several types of cells in the body fit this definition. In particular, skeletal muscle cells in people infected with *Trichinella spiralis* are a classic example of nurse cells. Infected muscle cells retain the larvae of *Trichinella* in a cytoplasmic capsule, providing them with a protective envelope and nutrients from the host [39]. Thus, skeletal muscle "nurse cells" protect the *Trichinella* larvae from recognition, attack, or elimination by immune cells [40].

Some macrophages from the bone marrow also function as "nurse cells" secreting factors that stimulate the growth and development of just formed immature erythrocytes and absorbing their nuclei to enhance their oxygen transport [41]. Other cells with similar nursing functions are the bone marrow-derived fibroblastic stromal cells that infiltrate the synovial tissues of patients with rheumatoid arthritis (RA). Fibroblastic stromal cells contribute to inflammation and bone damage in RA by secreting cytokines and chemokines that trigger both the accumulation and activation of lymphocytes and monocytes in synovium [42]. Mesenchymal stromal cells and bone marrow-derived fibroblastic stromal cells in the synovium also promote antibody production and the survival of B cells, which contribute to synovium damage. Furthermore, Sertoli cells of the testes have been called "nurse cells" because they provide nutrients and growth factors to developing germ cells; therefore, impairment in Sertoli cell differentiation reduces spermatogenesis and testicular size [43].

The best known "nurse cells" are a subpopulation of epithelial cells of the thymus called thymic nurse cells that endocytose newly arrived immature T lymphocyte progenitors and sequester them in vacuoles named caveoles. Within these vesicles, the intercellular adhesion molecule 1 (ICAM-1) from TNCs interacts with lymphocyte function-associated antigen 1 (LFA-1) on thymocytes and activates signals necessary for lymphoid cell maturation [44]; other adhesion molecules enhance the selection of self-reactive T lymphocytes. Once these processes are completed, TNCs allow mature T lymphocytes to leave their cytoplasmic vacuoles and move into peripheral circulation. Defective thymocytes and autoreactive immature T lymphocytes that fail positive selection die by apoptosis inside nurse cells and are phagocytized by thymic resident macrophages [45].

Thus, the main function of thymic "nurse cells" seems to support the development and survival of healthy T cells and enhance the elimination of aberrant or damaged T cells. The protective role of TNC contrasts with the *Trichinella spiralis* nurse cells' deleterious functions, which promote invasive ability and survival of parasites and host's tissue damage.

1.4. Thymic Nurse Cells. Nurse cells of the thymus were discovered in mice thirty years ago [2], but later they had also been isolated from humans, rats, pigs, fish, ewes, frogs and chickens [46]. Nurse cells in the thymus are epithelial cells that temporally bind and internalize immature T lymphocyte progenitors in specialized vesicles [47] to help in their development, maturation, and selection [48]. Although thymic nurse cells are not easily studied *in vitro*, nurse cell lines are available by transforming nurse cells with the SV40 virus [49].

Thymic nurse cells are the major epithelial component in the thymus microenvironment and one of the major cell populations involved in monitoring the immature T lymphocytes' access to antigens that can stimulate or suppress their functions. However, the main function of TNCs is to participate in the positive and negative selection of immature T lymphocytes to tolerate self-antigens and eliminate foreign antigens [50]. Furthermore, the interaction between TNCs and immature T lymphocytes is necessary for their viability during their triple positive stage of intrathymic development [51].

Thymic nurse cells bind and internalize 50–200 immature $TCR^-CD4^-CD8^-$ thymocytes in specialized cytoplasmic vesicles called caveoles, which are formed from the invaginations of the TNC plasma membrane [52]. A fine and detailed description of the internalization process of immature thymocytes into TNCs had been shown in Hendrix et al. [51].

Nurse cells of the thymus were initially described as multicellular complexes expressing cytokeratin 5 (K5) and/or cytokeratin 8 (K8) in their cytoskeleton [14] with a different location in the thymus. TNCs can express lysosomal-specific molecules and different proteases involved in the peptide/major histocompatibility complex (MHC) molecules—MHC class I and MHC class II—presentation. In human TNCs, laminin 211 has been detected [53], which is essential for the binding of these epithelial cells to immature T lymphocytes and in releasing T lymphocytes from the TNC vacuoles. Some TNCs also express the epithelial stem cell phenotype-associated transcription factor Trp-63 [51]

and are K5+K8+ cells, suggesting that TNCs also possess different stages of development.

TNCs also express gap junctions formed by connexin 43 [54] and several P2Z purinergic receptors [55]. Both proteins are necessary in the communication of TNCs with each other, and in propagating calcium waves between neighboring nurse cells. TNCs secrete the hormone thymulin, which is necessary for the production of the Th1 cytokines interleukin-2 (IL-2) and interferon-gamma (INF-γ) in the thymus; they can also secrete IL-18, which helps in the development of fetal thymic $CD11b^+$ dendritic cells and is also necessary for the induction of self-tolerance [56].

Furthermore, when TNCs are seeded and cultured on microplates, they produce the E_2 and I_2 prostaglandins [57] suggesting that TNCs may provide all these and other messages to immature T lymphocytes at specific developmental stages. Changes in the production of these endogenous and exogenous molecules can affect the balance on the events that modulates thymic microenvironment in which the development of T lymphocytes occurs.

On the other hand, TNCs are controlled by ligands and receptors from the endocrine and the nervous systems, since thymic epithelial cells express receptors for diverse hormones and neurotransmitters. Thymic nurse cells produce somatostatin, serotonin, gastrin [58], and the growth hormone [59] whose expression modulates the proliferation of T lymphocytes and thymic nurse cells and stimulates the secretion of thymic hormones, cytokines, chemokines, and extracellular matrix proteins in the thymic microenvironment, thus increasing thymocyte traffic inside and outside the thymus.

Nurse cells of the thymus also express some components of the cholinergic system [60] and acetylcholine. *In vitro* studies, using rat thymic epithelial cells [61] have demonstrated that adenosine triphosphate or noradrenaline induces the release of IL-6, a cytokine involved in thymocyte proliferation and differentiation. Besides, primary cultures of human and murine thymic epithelial cells produce thymulin [62] in response to beta-endorphin and leu-enkephalin added to the culture.

1.5. Distribution of Nurse Cells in the Thymus. In the thymus gland, nurse cells are distributed in both the cortical and the medullary zones of thymus lobules. In accordance with their location in the thymus, these populations are known as cortical thymic nurse cells (cTNCs) and medullary thymic nurse cells (mTNCs), respectively. These two TNC types share a common embryonic origin, but they exert different functions [63]. Some nurse cells are found within the corticomedullary interphase and express both K5 and K8, however.

cTNCs located in the subcapsular region of the thymus contain cytokeratin 8 in their cytoskeleton and engulf viable, immature, double negative ($CD4^-CD8^-$) thymocytes within TNC vacuoles. These cortex thymic nurse cells contain up to 200 immature thymocytes [64] inside caveoles. The inner membrane of caveoles expresses adhesion molecules called caveolins, intercellular adhesion molecule 1 (ICAM-1) and MHC class I and MHC class II molecules, by which cTNCs actively participate in the positive and negative selection of thymocytes [65]. Nurse cells of the thymus cortex also express several proteases such as cathepsin L, thymus-specific serine protease (TSSP) and the multicatalytic protease complex located in thymoproteosomes; all of them are involved in the positive selection of T lymphocytes after the cTNCs display unique self-antigens loaded onto MHC class I molecules [66].

Within cTNCs caveoles, the immature double-negative T lymphocytes transiently express CD25, the α chain of the IL-2 receptor [67] and begin to express $CD4^+$ and $CD8^+$ at low levels. Upon the induction of IL-7 and delta-Notch ligands, the DN T lymphocytes rearrange first the β chain and then the α chain of their TCR genes. Afterwards, they proliferate and maturate to the $\alpha\beta TCR^{high}$ double-positive $CD4^+CD8^+$ stage [66] via an intermediate $CD4^-CD8^+$ single-positive, semimature stage. Thus, results suggest that cTNCs are involved in $\alpha\beta$T cell receptor-mediated positive selection.

In opposition to cortex thymic nurse cells, medullary thymic nurse cells contain semimature T lymphocytes within their caveoles, whose internal membrane surface expresses cytokeratin 5 (K5), and tissue-specific and tissue-restricted peptides. Peripheral self-antigens gain access to the thymus by both the blood supply and by differentiated dendritic cells that migrate from the periphery to the thymus [68]. Medullary TNCs also express the tissue-restricted antigens, insulin and thyroglobulin, the XC-chemokine ligand 1(XCL1), and the CCL19 and CCL21 chemokines to attract dendritic cells and positive selected thymocytes from the thymic cortex, respectively [69, 70]. The expression of these self-antigens in TNC is partially regulated by the transcription factor autoimmune regulator (AIRE), which also regulates tolerance in periphery [71].

Intravacuolar interactions of positively selected immature T lymphocytes with all these tissue-restricted peptides are decisive steps for establishing self-tolerance. For this reason, the main functions of medullary thymic epithelial nurse cells are associated with the negative selection of autoreactive T lymphocytes and the establishment of a self-tolerant T cell repertory.

In addition, medullary thymic epithelial cells that express tissue-specific self-antigens indirectly participate in the generation of natural Tregs [72] with the help of dendritic cells. Simultaneously, mTNCs induce the development of T-regulatory cells to compensate the incomplete presentation of self-antigens in the thymus [73].

1.6. The Positive and Negative Selection of Thymocytes. The selection of thymocytes in the thymus is an MHC-TCR-restricted process associated with the recognition of self-antigens. This process is necessary to complete the maturation of T lymphocytes (positive selection) or to eliminate autoreactive T lymphocytes by apoptosis (negative selection). Interactions between cortical thymic nurse cells and semimature T lymphocytes through the affinities of the MHC/peptide-TCR$\alpha\beta$ define the further fate of lymphoid cells. Strong affinities with self-antigens trigger the deletion of immature T lymphocytes, minimal affinities lead to death by neglect and intermediate affinities promote positive selection

by survival and differentiation of $CD4^+CD8^+$ double-positive thymocytes to either the $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive linage [74].

In the thymic cortex, semimature T lymphocytes use their new TCRs to recognize self- and non-self antigens associated with MHC molecules on TNCs or dendritic interdigitating cells. $CD4^+CD8^+$ double-positive semimature T lymphocytes inside cTNC that recognize self-antigens with low avidity through their $\alpha\beta$T cell receptor (TCR) are positively selected [66], as well as those DP semimature T lymphocytes that produce low intensity activating signals through their TCR [10]. Thus, the main function of cortex TNC appears to support the positive selection of $CD4^+$ or $CD8^+$ T cells [75].

Positive selected $CD4^+$ or $CD8^+$ T lymphocytes are viable semimature cells transitorily contained in TNC vacuoles, where they express the $Qa2^{low}CD62L^{low}HSA^{high}CD69^{high}CD24^{high}$ phenotype, which is susceptible to apoptosis [76]. As T lymphocytes differentiate and express the $Qa2^{high}CD62L^{high}HSA^{low}CD69^{low}CD24^{low}$ new phenotype, they are transformed in mature cells refractory to apoptosis and are released from the thymus to start their own immunological functions [10]. In addition, when immature T lymphocytes from TNCs have been cultured, they show a decrease in their Bcl-2 expression [77]. This result suggests that they may be less susceptible to apoptosis because of the protection conferred by TNCs [78].

In the neonatal period, T lymphocytes positively selected (maybe 2%–5%) receive a survival signal, express receptors (CCR7 and CCR9) for CC chemokines and move into the thymic medulla [79], although the CCR7 is not necessary for their emigration from the thymus in adulthood. During their migration into thymus medulla, immature T lymphocytes acquire a single-positive (SP) phenotype either the $CD4^-CD8^+$ or the $CD4^+CD8^-$, both of which are dependent on the recognition of MHC class I or MHC class II molecules by their TCRs [80]. Semimature double-positive cells are developed by stronger or sustained signals from TCRs through the small GTPase RasGRP1, and the kinase ERK yielding $CD4^+$ SP thymocytes; meanwhile weaker or transient signals on TCRs produce $CD8^+$ SP thymocytes.

When high affinity TCRs of developing T lymphocytes recognize self-antigens/self-MHC complex in TNC vacuoles or on sparse thymic dendritic cell surface, they do not leave the TNC caveolae [45] and die by apoptosis inside cortical thymic nurse cells or survive and then leave the thymus but into a state of unresponsiveness or anergy.

In this way, immature T cells make their first immunological synapses with MHC molecules when they are inside TNC caveoles. The recognition of TNC-derived MHC molecules is a critical event for both the positive and negative selection of immature T lymphocytes upon expression of their $\alpha\beta$TCR [81]. Antibodies specific for either MHC class I or MHC class II molecules reduce the release of viable T lymphocytes from TNCs to the culture medium [78], thus suggesting that TNCs rescue immature T lymphocytes from apoptosis through the TCR-MHC interaction.

1.7. The Kiss of Death. Although TNCs can protect some immature T lymphocytes from apoptosis [77], the lymphoid cells confined within TNC caveoles are killed in most cases. The synapse between the internal membrane of the TNC caveolae and the TCR on the membrane surface of immature T lymphocytes acts as a kiss of death for autoreactive cells. After the high-affinity TCR-mediated recognition of self-antigens expressed on TNC caveoles, autoreactive thymocytes remain arrested within the nurse cells and are eliminated there by apoptosis. The event is called negative selection.

Thymic epithelial cell-mediated apoptosis needs cell priming, and 98% of the $CD4^+CD8^+$ double-positive immature T lymphocytes undergo apoptosis since they are more sensitive to the BAD, PUMA, and HRK-1 apoptotic sensitizer proteins [82] than are single-positive $CD4^+$ or $CD8^+$ cells. The epithelial thymic cell-mediated apoptosis of double-positive immature T lymphocytes is also induced by nitric oxide [83], which synergizes with glucocorticoids and activates cathepsin B and caspase-3, -8, and -9 [84].

On the other hand, adenosine also induces apoptosis into the thymus. *In vitro* studies have demonstrated that the apoptosis of mouse autoreactive immature T lymphocytes requires both high doses of adenosine produced by macrophages and adenosine A2 receptors expressed in immature lymphoid cells. The activation of A2 receptors by adenosine induces a Bcl-2-mediated apoptotic process that needs the proapoptotic protein Bim. In humans, thymocyte apoptosis also involves the $Ca2^+$-dependent induction of the transcription factor Nur77, a member of the steroid/thyroid hormone receptor superfamily [85] that binds to the promoters of the Fas ligand, TNF-related apoptosis-inducing ligand, and NDG-1 and -2 apoptosis-inducer proteins.

Once apoptosis is induced in autoreactive immature T lymphocytes, the epithelial TNCs recognize them via the scavenger receptor B1, a high-density lipoprotein receptor and a phosphatidylserine receptor and then the thymocytes undergoing apoptosis are retained within TNC caveoles. There, apoptotic cell residues are eliminated by lysosomal enzymes from TNCs [86], resident thymic cortex macrophages, or peripherally-recruited macrophages that arrive at the thymus when thymocyte apoptosis is increased [87]. Electron microscopy images from the cortex of the human thymus have shown that macrophages surround TNCs [88]. Macrophages have also been revealed within the vacuoles of TNCs [89], where macrophages move in and out rapidly. In this way, the physiological elimination of autoreactive thymocytes is performed and prevents the further development of autoimmune diseases.

The fatal kiss between the thymic nurse cells and their protected immature lymphocytes is a physiological event that has critical consequences for the development of self-tolerance in the immune system [77]. Self-antigens presented by mTNCs are essential for the negative selection of dangerous autoreactive maturing lymphocytes that must die in the thymic nursery [48]. Immune tolerance for self-antigens can be disrupted by defective elimination of autoreactive thymocytes that have a deficient expression of the proapoptotic protein Bim [90], mutations on the tyrosine kinase ZAP-70

signaling [91] or the depressed expression of the Mer tyrosine kinase [92]. These disorders have been associated with the deficient elimination of autoreactive cells by negative selection [47] resulting in the induction of autoimmune diseases.

The negative selection of autoreactive lymphocytes does fail however, since the deletion of these defective cells is incomplete, and some autoreactive T lymphocytes evade the kiss of death in the TNC microenvironment [93] and move into the peripheral blood circulation. In general, these autoreactive cells do not induce autoimmune diseases cause they are controlled by T regulatory (Treg) cells, which suppress peripheral T lymphocyte-mediated responses against self-antigens [94]. Nevertheless, the loss of the homeostasis between TNC and Treg cell functions can result in the excessive output of autoreactive T lymphocytes or defects in the suppression of T cells reactivity.

1.8. The Regulatory T Cells. Once T lymphocytes mature and leave the thymus, their surveillance and defensive functions begin to be controlled by other accessory cells called regulatory T (Treg) lymphocytes. The natural Treg cells are thymus-derived T lymphocytes, which modulate many aspects of the normal immune responses, suppressing inflammation, hypersensitivity, or autoimmune mechanisms. However, not all Treg cells develop in the thymus gland [95].

Moreover, not all T regulatory cells exert a suppressor role since Tregs subpopulations with a proinflammatory function have been reported [96]. Tregs are specifically activated by antigens although their effector function is antigen nonspecific [97]. Besides, the self or non-self antigen binding specificity of the Treg lymphocyte through their TCR does not influence their selective process within the thymus gland when immature Treg cells are under the influence of cytokines, hormones, and neurotransmitters. In addition, Treg lymphocytes exert their suppressive functions on other immune cells [98] by releasing cytokines, through cell-cell contact, or by inducing cytotoxicity or anergy in antigen presenting cells [99].

Treg cells developed in the thymus are referred to as "natural" Treg (nTreg) cells, whereas "adaptive" or "induced" Treg (iTreg) cells are either naïve peripheral $Foxp3^{-}CD4^{+}$ T lymphocytes or $Foxp3^{-}CD8^{+}$ T lymphocytes, which develop in the periphery upon subimmunogenic antigen presentation during chronic inflammation or normal homeostasis of the gut after T lymphocytes recognize foreign antigens [100, 101]. The origen and location of Treg cells in the body is depicted in Figure 1.

Natural Treg cells are long-lived cells that comprise a very small subpopulation of thymus-derived $CD4^{+}$ T cells (5–10%). They are produced as a result of the high-affinity TCR-self peptide : MHC class II molecule interactions between maturing thymocytes and TNCs requiring also of external stimuli such as IL-2, CD80 and CD86. Natural Tregs constitutively express the alpha chain of the IL-2 receptor (CD25), need TGF-β and IL-2 for their maduration and are neither phenotypic nor functionally homogeneous [102]. Natural human Tregs (70%) express the Helios transcription factor, which regulates both Foxp3 expression and regulatory T cell activity [103], and possess the $Foxp3^{+}Helios^{+}$ phenotype. Notwithstanding, $Foxp3^{+}Helios^{-}$ Tregs can be expanded *in vitro* into $Foxp3^{+}Helios^{+}$ T regulatory cells by adding a DNA oligonucleotide [87].

On the other hand, induced Tregs may play an important role in the immune tolerance of foreign antigens such as those derived from commensal bacteria in the intestine [104], and their functions are essential and complementary to the regulatory function of nTregs. Natural or induced T regulatory cells have different origins and specificities and exert their functions through distinct mechanisms. An excellent review regarding the role of self-reactivity as the decisive factor in Treg development in the thymus has just been published [105].

Other Treg subsets have been proposed, such as the Type 1 regulatory T cells (Tr1) and T-helper-3 (Th3) lymphocytes. The former are T cells, which produce TGF-β and IL-10 upon antigen exposure and specific tolerogenic conditions, and the latter produces TGF-β upon intestinal tolerance [106]. Accordingly, Tregs are stimulated by different agents and possess different properties.

1.9. The Heterogeneity of Treg Cells. The Treg subpopulation contains suppressor and effector/memory cells that also control tumors and pathogens [101]. The same as all T cells, natural T regulatory cells are long-lived and migrate from the thymus into the periphery and secondary lymph tissues, where they also balance self-tolerance and autoimmunity. Previously, Coutinho et al. [107] have proposed that Tregs are selected in the thymus upon high-affinity recognition of self-ligands in cortex thymic epithelial stromal cells.

All Tregs acquire their specific phenotype and functions when they upregulate the expression of Foxp3, which expression requires Signal Transducer and Activator of Transcription-5 (STAT-5) activation driven by IL-2 [108]. However, it is worth keeping in mind that epithelial and other subtype of cells such as cancer cells can also express the Foxp3 protein [109]. Moreover, Treg cells are modulated by cytokines released by cells that express Toll-like receptors such as TLR-2 and TLR-4, as has been reported in patients with atopic dermatitis [110] and hepatoma cell lines [111], in a context that has not yet been fully explored.

In the thymus, Tregs are thymocytes that develop from $Foxp3^{-}CD4^{+}CD25^{+}$ cells and appear more frequently during the transition of the late $CD4^{+}CD8^{+}$ DP stage to the final $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ SP stage [112]. T regulatory cells also express the $CD45RB^{high}$ or $CD45RB^{low}$, $CD38^{+}$ or $CD38^{-}$, $CD69^{+}$ or $CD69^{-}$, and $CD62L^{high}$ or $CD62^{low}$ membrane markers [113]. However, there are $CD4^{+}CD25^{-}$ T cells possessing regulatory functions. Other surface markers expressed in Tregs are $CD127^{low}$, HLA-DR, CD103, CD39, Neuropilin-1 (Nrp-1), and Tumor Necrosis Factor receptor family-related members such as the Glucocorticoid-induced TNF receptor (GITR/TNFRSF18), OX-40, and CD137.

The activated $CD8^{+}CD25^{+}$ natural Treg lymphocytes are thymus-derived cells that share phenotypic and functional characteristics of the $CD4^{+}$ Tregs, since they also express the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4),

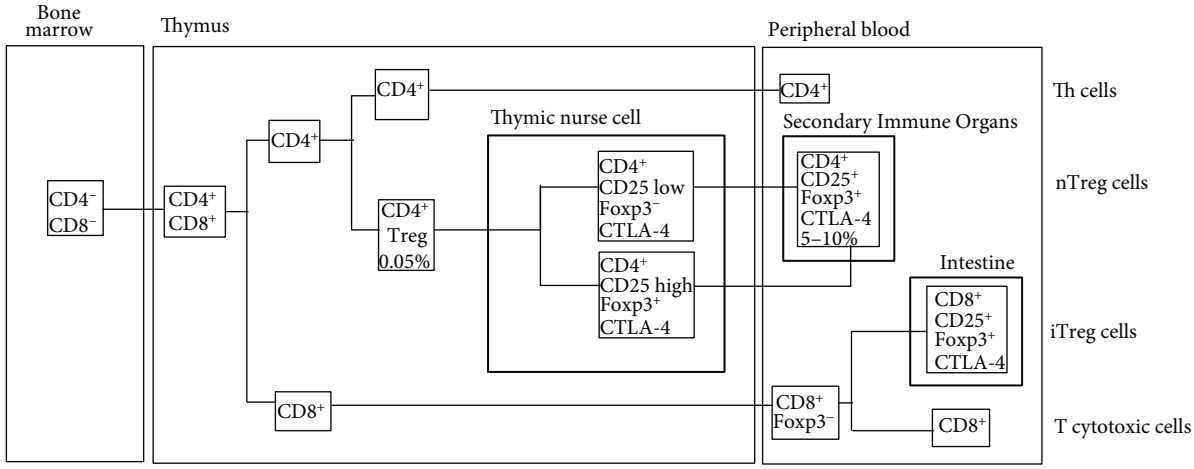

Figure 1: The intra- and extrathymic origin of T regulatory (Treg) lymphocytes. The bone marrow-derived pre-T lymphocytes arrive at the thymus microenvironment as double negative $CD4^-CD8^-$ cells, which are engulfed by thymic nurse cells, where they mature to $CD4^+$ or $CD8^+$ lymphocytes or are negatively selected. A reduced proportion (0.05%) of the $CD4^+$ lymphocytes become regulatory cells by expressing CD25, Foxp3, CTLA-4, and other molecules. When this subpopulation of natural T regulatory (nTreg) lymphocytes is mature, they are released from the medullary thymic nurse cells, leave the thymus, and go into the blood and peripheral lymphoid organs where they release IL-10 and TGF-β suppressor cytokines that downmodulate the functions of other cells from the immune system. A different subpopulation of T regulatory cells can be experimentally induced from $CD8^+$ cytotoxic lymphocytes located outside the thymus. These lymphocytes exert an *in vitro* suppressor activity through IL-10, IL-4 , and TGF-β; they are called inducible T regulatory (iTreg) lymphocytes and have been found as infiltrating cells with an effective antitumor activity.

the Glucocorticoid-induced Tumor Necrosis Factor receptor (GIRT), and the Transforming Growth Factor-β1 [98, 114]. They comprise less than 1% of $CD8^+$ lymphocytes.

In addition, diverse $CD8^+$ Tregs subtypes exists, which are naturally produced or induced with cytokines such as IL-4, IL-10, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), IL-2, INF-γ and TGF-β, viral antigens, xenogeneic antigen presenting cells, allogeneic stimulation, non-antigen specific stimulation, cocultures with monocytes, cocultures with LPS-stimulated dendritic cells (DCs), and plasmacytoid DC from tumor ascites [98] among others.

Surprisingly, a fraction (10–15%) of $CD25^+$ natural Treg cells never express or lose the transcription factor $Foxp3^+$ when they proliferate in a T cell deficient environment [108]. Some of these Treg-derived $Foxp3^-$ T cells exert an effector T helper function while others maintain their ability of expressing Foxp3 upon activation [115], showing that T regulatory cells possess plasticity. The $Foxp3^+$ T regulatory cells can also functionally differentiate to control the T_H1, T_H2, and T_H17 cell response [116] by changing the expression of the T_H lineage-specific transcription factors T-bet, Interferon Regulatory Factor 4 (IRF-4), and Orphan Nuclear Receptor γt (RORγt), respectively.

Different cells and molecules are involved in the Treg development in the thymus. Accordingly, T cell expression and signaling, the expression and signaling of CD11a/CD18, CD28, and CD40L on thymocytes, ICAM-1 and CD80, CD86, and CD40 expressed on thymic stromal cells [3] control Tregs development and the selection of their heterogeneous subpopulations [105]. The expression of MHC class II molecules on medullary TEC also promotes Treg development [75]. Tregs develop if the MHC-TCR affinity is high [111], although the affinity for the development of the $CD4^+CD25^+$ thymocytes is different from that of $CD4^+CD25^-$ thymocytes. Dendritic cells induce the differentiation of T regulatory cells [117]. TGF-β is needed for the Foxp3 expression in Treg cells [108], and glucocorticoids stimulate Treg activity [118]. Tregs need B-lymphocytes to survive and proliferate on the periphery [113]. In addition, IL-2, IL-7, and IL-15 are required for the peripheral maintenance of Tregs, and they are also probably needed for the survival of immature Tregs in the thymus medulla [112].

1.10. Blood Levels and Functions of the Treg Cells. Once Treg cells have been developed, their numbers can be increased or decreased by diverse diseases or physiological conditions, which can influence their suppressor functions according to the quality of the required immune response. In addition, the number and functions of Tregs cells are necessary in controlling autoimmunity since depletion of $CD4^+CD25^+$ T cells in mice, mutations in the Foxp3 gene, or environmental agents affecting Treg cells cause or predispose to autoimmunity [116] because the lack in balancing the activity of self-reactive T cells produced in the thymus. Thus, $CD4^+$ Tregs or $CD8^+$ Tregs appear impaired in number and/or function in diverse autoimmune diseases [98] such as lupus erythematous, autoimmune diabetes, rheumatoid arthritis, myasthenia gravis, multiple sclerosis, allergy, inflammatory bowel disease, hepatitis C, herpes simplex, HIV infections, and cancer. However, Treg lymphocytes are frequently expanded in physiological conditions such as aging [119] and pregnancy [120]. An overview regarding the role of the Treg cells in controling infection, inflammation and the function of other T lymphocytes are depicted in Figure 2.

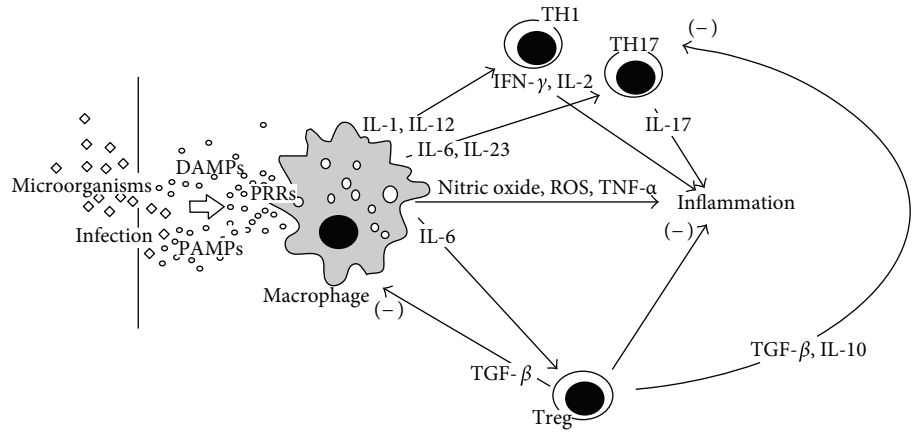

Figure 2: A defensive inflammatory response starts after the pattern recognition receptors (PRRs) of macrophages and dendritic cells are stimulated by both pathogen-associated molecular patterns (PAMPs) released by microorganisms and damage-associated molecular patterns (DAMPs) from injured tissues. As a consequence, diverse signaling pathways increase both the production of proinflammatory cytokines and the release of free radicals during the cellular respiratory burst. The evolution of the inflammatory response is modulated by various subpopulations of cells including T lymphocytes. The proinflammatory T lymphocytes (Th1 and Th17) mainly release IL-2, IL-17, and IFN-γ, and the antiinflammatory lymphocytes (Treg) release TGF-β and IL-10. The effective modulatory work of Treg cells gradually slows down the progression of the inflammatory responses and reduces any possible risk of autoimmunity, allergies, or other chronic diseases.

Experimental models of parasitic infection with *Strongyloides ratti* [121] and *Trichuris muris* [122] increase the number of Foxp3$^+$ Treg lymphocytes suppressing the protective immune response and probably reducing the parasite-induced damage in the host. Similar results have been observed during experimental acute or chronic malaria infection by *Plasmodium chabaudi* in C57BL/6 mice [123] and in filaria-infected non-obese diabetic mice [124].

Other different studies have shown that there is also an inverse relationship between the peripheral nonphysiological increase in Treg numbers and thymic involution [33]. The numbers of Treg cells in the blood increases as a consequence of thymic involution and lymphopenia, as can be observed in children with the Down syndrome [125]. In opposition, low numbers of circulating Treg cells have been associated with the onset of allergic and autoimmune responses [126]. Other authors have reported that the proportion of Treg cells increases in the spleen and lymph nodes of mice with experimentally induced arthritis [127].

Although deficient T lymphocyte-mediated immune responses are usually associated with elevated amounts of Treg cells in the blood, the increased production of proinflammatory cytokines such as IL-17 and IL-18 and the elevated number of Th17 lymphocytes are frequently associated to decreased numbers of Treg lymphocytes in the blood and lymphoid organs [128].

In support of the role for Treg cells in the prevention of immunopathology, it has been reported that the experimental depletion of Foxp3$^+$ Treg cells reduces the control of the inflammatory immune responses, increases the frequency of autoimmune reactions due to TCR-mediated self-antigen recognition and enhances the development of a scurfy-like disease in mice, which die at 3-4 weeks of age [129]. In contrast, Foxp3$^+$ Treg cells from mice deficient in CD5, a negative regulator of TCR signaling increases the suppressive activity of their Treg cells [97].

Furthermore, the presence of cellular markers of Treg cells in the microenvironment of thymic nurse cells [71] suggests a relationship between autoimmunity and disorders in the intra-TNC maturation of Treg cells. Incomplete Treg cell development in TNCs can result in defective Treg cell suppressor activity and consequently in the development of autoimmune reactions or diseases. For example, nonobese diabetic (NOD) mice are diabetic because of a defect in their antigen presenting cells to activate Treg cells. NOD mice also have Treg cells, which are defective in their regulatory function and possess lower percentages of CD4$^+$CD25$^+$ T regulatory cells than NOD mice that never develop diabetes [130].

The extremely complex relationship between Treg cells and autoimmunity is a function not only of the defective suppressor activities of peripheral Treg cells, however. Neurotransmitters and sex hormones have recently been added to the long list of modulators of the Treg cell function. Thus, *in vitro* experiments show that pituitary adenylyl cyclase-activating polypeptide (PACAP) [131], vasoactive intestinal peptide [132], and nicotine [133] increase the suppressor activity of Treg cells. Furthermore, studies of cultured fluorescence-activated-sorted Tregs from pregnant women and peripheral blood mononuclear cells from non-pregnant women have revealed that progesterone and 17 β-estradiol especially decrease the expression of Foxp3 in CD4^{dim}CD25^{high} Treg cells [134] as well as their cell numbers, although these Tregs cells maintain their regulatory function. In contrast, other authors have shown that oestrogen enhances the frequency of Treg cells and reduce the production of IL-17 in a mouse model of multiple sclerosis [135].

The suppressive control of T lymphocyte responses is a complex event in which the major inductors are Treg cells modulated by numerous factors coming from the immune system or not.

1.11. Regulating the Regulators. Since immune system responses are regulated by Treg cells and MHC class II-expressing nurse epithelial cells from the thymus regulate the development of Treg cells [75, 136], a question arises: what regulates both the development and the functions of thymic nurse cells? To answer this question, most researchers have focused their attention outside the thymus gland, specifically on the endocrine [137] and the nervous [138, 139] systems.

Understanding the interactions between the nervous and immune systems has considerably increased [140] in the last three decades, and a great deal of studies have shown that these interactions are possible because immune and nervous cells share receptors for several neurotransmitters and cytokines [139]. Moreover, the innervation of the thymus gland, bone marrow and all secondary organs of the immune system by the autonomous nervous system are essential for the functioning of the immune system [140].

Accordingly, the connective tissue of the thymus contains non-myelinated nerves forming a lattice over their surface [141]. These nerves contain calcitonin gene-related peptide (CGRP), noradrenaline, substance P (SP), vasointestinal peptide (VIP), and neuropeptide Y (NPY) [142–148]; other nerves have acetylcholinesterase, which can affect the thymocyte development. The direct effect of GABA, Histamine, NPY, SP, VIP, and CGRP on the proliferation of rat thymic epithelial cells was reported years ago [149]. The P2Z receptors, a kind of purinergic receptor, were later demonstrated to exist in thymic epithelial nurse cells [150]. In addition, catecholamines (adrenaline, noradrenaline, and dopamine) from the sympathetic nervous system have a main role in controlling lymphocyte development and immunomodulation [151].

Since the thymus is innervated by the sympathetic nervous system through norepinephrine [141], sympathectomy of adult rats results in a reduction of thymus weight, decreased intrathymic cellularity and increased T cell apoptosis [143] by affecting noradrenergic, vasointestinal peptide, acetylcholine and CGRP nervous fibers in the thymus. In contrast, other authors [144] have shown that the peripheral administration of 6-hydroxydopamine (6-OHDA) increased the numbers of $CD4^+Foxp3^+$ Tregs in the spleen and lymph nodes in a TGF-β-dependent manner without affecting their regulatory function or the frequency of all $CD4^+$ and $CD8^+$ T cells.

On the other hand, *in vitro* experiments have confirmed that cultured thymic epithelial cells express both the α, β, and ε subunits of the acetylcholine receptor similar to those shown in muscle cells [20] and functional $\beta1$ and $\beta2$ mRNA to express adrenoreceptors, activated by adrenaline and noradrenaline [143]. Besides, the adrenergic agonist carbachol inhibits apoptosis of DP thymocytes by TECs [146]. Furthermore, immunohistochemistry assays have shown that epithelial cells of the rat thymus are close to catecholaminergic nerves controlled by dopamine [147].

A possible role for acetylcholine has been also proposed in the mutual interplay between immature lymphocytes and thymic epithelial cells because the TE750 thymic epithelial cell line and primary thymic epithelial cell cultures have shown *in vitro* the expression of acetylcholine, the $\alpha3$, $\alpha5$, and $\beta4$ mRNA subunits of one cholinergic receptor and choline acetyltransferase [60]. Outside the thymus gland, the histamine released by bone marrow-derived mast cells inhibits the *in vitro* suppressor function of Treg lymphocytes and downregulate the expression of CD25 and Foxp3 markers of $CD4^+CD25^+$ cells [152] via the histamine 1 receptor expressed by these cells.

Other studies have revealed that the levels of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) are elevated in the thymus during the immune response [153] as compared with other glands. Epithelial cells in thymic medulla expresses the glutamate decarboxylase-67 isoform, which predominantly synthesizes GABA in the central nervous system [154], and the thymus also expresses high GABA-transaminase activity after IL-1 stimulation [155]. Nevertheless, the expression of components of the GABAergic system or GABA receptor subunits has not been demonstrated in thymic epithelial cells. We have found evidence for a GABAergic system in mouse peritoneal macrophages [156], but the presence of a similar system in intrathymic macrophages has not been addressed.

Regarding the role of GABA on the thymus and lymphocytes, Tyurenkov et al. [157] demonstrated that baclofen, a GABA-B receptor agonist, restores thymus weight and thymocyte numbers after experimental immunosuppression. Notwithstanding its inhibitory effect on the cells from the nervous system, GABA increases the proliferation of rat thymic epithelial cells cultured *in vitro* [149], whilst its oral administration inhibits both diabetes development in type 2 diabetic mice [158] and inflammation in fat diet-fed mice [159] by increasing the frequency of their Tregs cells. These two later *in vivo* results suggest that GABA administration may be a useful tool that enhance the effects of the conventional therapy for preventing type 1 diabetes and other T lymphocyte-mediate autoimmune diseases in mice, although the role of GABA in the TNC-Treg cells interaction remains unexplored.

Thymic nurse cells are the main engaged cells in the development and selection of immature T lymphocyte in the thymus. Although relationships between TNCs and the nervous system have been emerging in the last decades, their data are scanty. Even more limited are the studies regarding the relationships between neurotransmitters or neuropeptides and the density of their receptors expressed on immune cell subsets [160]. Further studies are needed to unveil the existing interactions between the nervous system and both TNC and Tregs cells completely. Understanding the mechanisms by which the vegetative nervous system regulate the TNC functions and development through neurotransmitters and neuropeptides may be helpful in controlling autoimmune diseases, transplants, inflammation, and allergy.

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Review Article

Immune Responses Associated with Resistance to Haemonchosis in Sheep

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This paper examines the known immunological and genetic factors associated with sheep resistance to infection by *Haemonchus contortus*. Such resistance is an inheritable genetic trait (h^2, 0.22–0.63) associated with certain sheep breeds. Resistant sheep do not completely reject the disease; they only harbor fewer parasites than susceptible sheep and therefore have a lower fecal egg count. Protective immune response to haemonchosis is an expression of genetic resistance. Genes associated with resistance and susceptibility are described. Genetically resistant sheep have nonspecific mechanisms that block the initial colonization by *Haemonchus contortus* larvae. These sheep also have an efficacious Th2 type response (e.g., increases in blood and tissue eosinophils, specific IgE class antibodies, mast cells, IL-5, IL-13, and TNFα) that protects them against the infection; in contrast, susceptible sheep do not efficiently establish this type of immune response. Finally, the main reported antigens of *H. contortus* were reviewed.

1. Introduction

Gastroenteric verminosis is a disease with a great economic impact on sheep farms located in humid areas including tropical and subtropical regions of the world [1]. In Australia, losses due to this disease have been estimated at more than 400 million dollars (USD) per year; treatments in Kenya, South Africa, and India cost up to 26, 46, and 103 million USD, respectively [2]. Due to its ubiquity and virulence, *Haemonchus contortus* is the most important gastroenteric nematode of sheep in many regions of the world. It is a blood-sucking parasite of the abomasum that causes a disease known as haemonchosis [3, 4].

Haemonchosis is acquired by ingesting pasture contaminated with the third stage larvae (L3) of *H. contortus*. L3 penetrates the abomasal glands, where they molt into L4. The presence of larvae induces abomasal gland hyperplasia, inflammatory cell infiltration, and the substitution of wall cells secreting HCl with young nonsecreting cells. Consequently, the abomasal pH increases, which in turn reduces the transformation of pepsinogen to pepsin, reduces protein digestion, increases mucosa permeability, and increases the loss of endogenous proteins in the abomasum. Adult parasites are found in the abomasum lumen, and they are voracious hematophagous parasites, daily consuming 0.05 mL of host blood per worm [5]. The negative effects of haemonchosis on the biological and economic efficiency of sheep herds include malnutrition, low feed conversion, anemia, loss of appetite, low fertility indices, and in certain cases the death of young animals [3, 6].

Parasite control is based almost entirely on the administration of anthelmintic chemical compounds. Unfortunately, one of the problems generated by the massive, and indiscriminate use of anthelmintic products is the increasing resistance to these drugs, and this situation has huge consequences in those countries where sheep production is one of the main economic activities [7–9]. Together with the anthelmintic resistance problem, there is a trend toward the reduction of drug residues in human food and in the environment, which mandates that antiparasitic control strategies must not depend on chemicals. Among some of the proposed strategies are the development of specific vaccines against gastroenteric nematodes and the use of animal genotypes that are resistant to parasite infections.

2. Resistance and Resilience

Nematode resistance includes the initiation and maintenance of a host response that prevents, reduces, or clears parasitic infection [10, 11]. Resistant animals do not completely reject the disease, but they have a lower parasitic load than susceptible animals, as measured by fewer eggs in their feces. This resistance is based on the immunological capabilities of each individual when challenged with parasitoses [12].

Resilience is the capacity of an animal to compensate for the negative effects of parasitism by the maintenance of productive parameters [13]. Sheep in general show simultaneously high resistance and resilience to haemonchosis. Some breeds have moderate or low resistance with relatively high resilience, allowing them to have productivity similar to those that are naturally resistant [14].

3. Breeds Susceptible and Resistant to Haemonchosis

Differences between sheep breeds in their susceptibility to infection by abomasum-inhabiting nematodes were first reported by Stewart et al. [15], who described higher resistance to *Ostertagia circumcincta* (currently *Teladorsagia circumcincta*) in Romney Marsh lambs compared with lambs of the Rambouillet, Shropshire, Southdown, and Hampshire breeds and their crosses. Ross et al. [16] reported the first evidence for heritable resistance to haemonchosis in sheep. Subsequently, it has been shown that some sheep breeds are more resistant to gastroenteric nematodes than others. Table 1 lists selected comparisons between breeds and the parameters of susceptibility or resistance that were measured. Additionally, there are individual differences within breeds [17].

The resistance of some breeds can be explained by their place of origin. In general, resistant breeds were selected from areas where the climate favors the growth of gastroenteric nematode larvae in the environment, such that selection for certain productive parameters over several generations affected an indirect selection for nematode resistance. In fact, native breeds that have prospered despite unfavorable environmental conditions, poor zootechnical management, and no anthelmintic treatments are more resistant than highly productive breeds selected in areas with optimal health and zootechnical management [14].

There are several ways to assess genetic resistance to gastroenteric nematodes. The most common method is the fecal egg count (FEC), which has intrinsic limitations because the number of eggs in feces is not necessarily correlated with the host's parasite load [18]. Low or reduced FEC has been used as a parameter for sheep selection in Australia [19, 20] and New Zealand [21]. The most trusted method to measure a sheep breed's resistance to gastroenteric nematodes is to count the total parasites (larvae and adults) in the gastrointestinal tract of the assessed sheep. Because this method can only be performed at necropsy, it is not useful for the genetic selection of sheep [10, 22].

The use of haemonchosis-resistant sheep breeds has been proposed as a way to control the spread of drug-resistant strains of *H. contortus*. However, many of these breeds do not have the productive indices of other breeds; instead, some researchers are trying to select sheep for high resistance from productive breeds such as Merino and Romney, this resistance is a characteristic that is inherited by their descendants [19, 20]. The hereditability (h^2) of FEC varies between 0.22 and 0.63, indicating that selection for resistance or against susceptibility using this parameter can be moderately useful [23, 24]. Genetic markers associated with resistance could also be used to select sheep within a breed. There are many ongoing studies of resistance-associated genetic markers and some preliminary results. Alleles OMHC1-188 and OLADRB2-282 of the major histocompatibility complex (MHC) [25] and several quantitative trait loci (QTL) that contain diverse significant loci, such as the IFNγ locus in chromosome 3 [26, 27], have been associated with FEC reduction. Furthermore, some genes associated with the early inflammatory response including those encoding toll-like receptors (TLR2, 4 and 9) or involved with free radical production (DUOX1 and NOS2 A) are more abundantly expressed in lambs that are resistant to *H. contortus* and *Trichostrongylus colubriformis* infections [28].

4. Immune Response in Ovine Haemonchosis

The immunological mechanisms by which sheep have or acquire resistance to haemonchosis are not very clear [50, 53]; this resistance is an individual characteristic that has been associated with age, breed, and previous exposure to the parasite (infection or reinfection).

Both innate and adaptive immunities protect the host from *H. contortus* infection. Clearance of the nematode in immunized sheep requires several events, including the activation of nonspecific defense mechanisms, the recognition of parasitic somatic and excretion/secretion antigens, and the initiation of an appropriate acquired response [54].

4.1. Nonspecific Response Mechanisms to Haemonchosis. *H. contortus* larvae must inhabit an appropriate gastrointestinal niche that nourishes their development and growth and protects them from mechanical (peristaltic movement) and chemical (abomasum mucus) host barriers. Parasite colonization of the host abomasum initially depends on the motility of the larvae and the parasite load. Some host individuals, after sensitization via previous infections, can modify the microenvironmental conditions of the niche to expel the parasite [55].

Complement fixation is one of the first innate responses to *H. contortus* infection. Several studies demonstrated that helminths activate the alternate complement pathway and bind some molecules (opsonins) on their surface [56]. After larvae activate complement, vasoactive and chemotactic peptides (C3a and C5a) are generated, and these peptides mobilize eosinophils to the area of infection independently of specific mechanisms (CD4+ and IL-5). At the same time, *H. contortus* secretes chemoattractants for eosinophils and neutrophils, which reinforce the inflammatory response [57]. The thymus-independent increase in tissue eosinophils is an

Table 1: Comparative studies of susceptibility/resistance to *Haemonchus contortus* or gastrointestinal nematodes between sheep breeds.

Resistant breed	Susceptible breed	Parasite	Infection	Evaluated parameters	References
Florida Native	Saint Croix and crossbred Dorset × Rambouillet	*H. contortus*	AI	Par, Hem, Immunol, Hist	[29]
Saint Croix	Dorper	*H. contortus*	NI	Par, Bioch, Immunol, Hist	[30]
Florida Native	Suffolk and Rambouillet	*H. contortus*	NI y AI	Par, Hem, Bioch, Immunol	[31, 32]
Red Maasai	Blackheaded Somali, Dorper and Romney Marsh	GINs	NI	Par, Hem	[1]
Castellana	Nil	*H. contortus*	AI	Par, Immunol	[33]
Florida Native and Pelibuey	—	GINs	NI	Par, Hem, LW	[34]
Rhön	Nil	*Trichostrongylus spp.*	NI	Par, Hem , Bioch	[35]
German Merino	Rhön	*H. contortus*	AI	Par, Hem, Immunol	[36]
Sabi	Dorper	GINs	NI	Par, Hem, LW	[37]
Blackbelly	INRA 401	*H. contortus* and *Trichostrongylus colubriformis*	AI	Par	[38]
Crossbred Saint Croix × Blackbelly	Crossbred Dorset (50%), Rambouillet (25%) Finnsheep (25%)	GINs	AI y NI	Par	[6]
Katahdin and crossbred Blackbelly × Saint Croix	Dorper and Dorset	*H. contortus*	AI	Par, Hem, LW	[39]
Gulf Coast Native	Suffolk	GINs	NI	Par, Hem	[40]
Saint Croix	Katahdin and Dorper	GINs	NI y AI	Par, Hem, LW	[41]
Saint Croix	Katahdin and Suffolk	GINs	NI	Par, Hem, LW	[42]
Crioula Lanada of Brasil	Corriedale	*H. contortus*	NIT	Par, Bioch, Hem, Hist	[11]
Red Maasai	Dorper	*H. contortus*	AI	Par, Hem, LW, MP	[43]
Santa Ines	Ile de France	*H. contortus*	AI	Par, Hem, Bioch, Hist, Immunol, LW	[44]
Santa Ines	Suffolk and Ile de France	GINs	NI	Par, Hem, Hist, Immunol	[45]

Table 1: Continued.

Resistant breed	Susceptible breed	Parasite	Infection	Evaluated parameters	References
Texel	Suffolk	GINs	NI	Par, Hist	[46]
Blackbelly	Columbia	*H. contortus*	AI	Par, Hem, Hist, Immunol	[47]
Blackbelly	INRA 401	*H. contortus*	AI	Par, Hist, Immunol	[48]
Lohi	Thalli and Kachhi	*H. contortus*	AI y NI	Par, Hem, Bioch, Hist, LW	[49]
Criolla Native to the Central Mexican Plateau	Suffolk	*H. contortus*	AI	Par, Hem, FT, LW	[14]
Gulf Coast Native	Suffolk	*H. contortus*	AI	Par, Immunol	[50]
Santa Ines	Ile de France	*H. contortus*	AI	Par. Immunol	[51]
Canaria Hair	Canaria	*H. contortus*	AI	Par.	[52]

NI: natural infection; AI: artificial infection; GINs: gastrointestinal nematodes; Par: parasitological (fecal eggs count, worm burden, etc.); Hem: hematological (packed cell volume, blood eosinophils, etc.); Immunol: immunological (antibodies); Hist: cellular count in abomasum (eosinophils, leucocytes, mast cells, etc.); Bioch: biochemical (serum protein, albumin, etc); LW: live weight; MP: mortality percentage FT: FAMACHA test.

important innate response in which complement activation mediates the cytotoxicity of eosinophils against larvae in early infection stages in the absence of specific antibodies.

When rodents are used as experimental models for gastrointestinal helminths, the quick elimination of parasites during the first infection is associated with inflammation induced by the alternate complement pathway and mediated by mast cells and eosinophils [56]. In contrast to rodent models, efficient elimination of nematode larvae in ruminants generally requires repeated infections [58].

Expulsion of *H. contortus* larvae in sheep can be immediate or delayed. Immediate expulsion occurs when larvae are attacked by tissue mast cells and a special type of intraepithelial mast cells (globule leucocytes) before the larvae enter their niche (abomasum gland). Similar to murine experimental models, other important mechanisms in the immediate expulsion from sheep are hypermotility, gastric hypersecretion, and hyperplasia of calciform cells with the subsequent increase in mucus production [55, 58]. These mechanisms may explain why some sheep breeds or genetically resistant genotypes counteract infection during its early stages.

MacKinnon et al. [59] found that resistant and susceptible sheep breeds exhibited differential gene expression that was associated with an nonspecific response to *H. contortus*. At 3 days after infection (PI) with *H. contortus*, resistant sheep had reduced expression of genes associated with blood coagulation and higher expression of genes involved in the inhibition of coagulants, tissue repair and restructuring, blood vessel formation, and cell migration in the abomasum and abomasal lymph node. At day 27 PI, resistant sheep had higher expression of genes associated with intestinal motility, inflammatory response, cell differentiation and proliferation, and the reduction of apoptosis.

Ghrelin is a growth hormone peptide (28 amino acids) of the stomach and is the endogenous ligand for GH secretagogue receptor [60]. It also stimulates appetite, regulates homeostasis of energy metabolism, and contributes to the modulation of the inflammatory response [61, 62]. Experimental infection with *H. contortus* in susceptible lambs reduces the expression of the ghrelin gene in abomasum and decreases the protein in plasma; in contrast, ghrelin gene expression and protein plasma content increase in resistant lambs [63]. Ghrelin reduction is most likely associated with appetite suppression and downregulation of the prolonged inflammatory response in susceptible lambs.

Immediate expulsion of the parasite is also associated with the presence of histamine and leukotrienes in the abomasum mucus, which inhibit the motility of nematode larvae *in vitro*. When challenged with the parasite, sheep immunized with *H. contortus* or *Trichostrongylus colubriformis* have a higher number of mast cells and globule leukocytes in the abomasum mucosa, and these cells have higher secretion of leukotrienes and factors that inhibit larvae migration [64]. High concentrations of histamine in the abomasal mucosa of sheep that are resistant to haemonchosis aid parasite expulsion by promoting abomasal hypersecretion and hypermotility, which are detrimental to the fecundity and motility of the worm [65]. Furthermore, histamine facilitates the translocation of plasma proteins including humoral antibodies into the lumen of the abomasum [55].

Delayed expulsion of *H. contortus* larvae occurs when a specific immune response is mounted against the larvae in the abomasum glands. This action is regulated by CD4+ T lymphocytes, IgA and IgE antibodies, antibody-dependent eosinophil cytotoxicity, and the classic complement pathway [58].

Tissue and blood eosinophils are increased during both the specific and nonspecific responses against gastrointestinal nematodes. The activation of the alternate pathway and degranulation of mast cells cause the increase and nonspecific degranulation of tissue eosinophils which is independent of IL-5. In addition to recruiting eosinophils to the abomasum wall, complement promotes eosinophil cytotoxicity against *H. contortus* larvae [66]. Infection with *Oestrus ovis* or inoculation with *Taenia hydatigena* larvae extracts induces eosinophilia in the abomasum and promotes resistance to haemonchosis in sheep [67, 68].

Eosinophil degranulation releases major basic protein, cationic proteins, and peroxidase, which are cytotoxic to helminths. Lipid mediators such as leukotrienes, prostaglandin E2, platelet aggregation factor, and lipoxins are secreted, and these molecules promote increases in permeability, mucus secretion, chemotaxis, and coagulation. Eosinophils also produce cytokines IL4 and IL10, suggesting that these cells have a regulatory function in the immune response [69].

The exact role of $\gamma\delta$ T lymphocytes is unclear. These cells have been associated with resistance to haemonchosis [4, 70], but it is unknown whether they are involved in resistance and/or immunity or if their presence is only a secondary effect *H. contortus* infection.

4.2. H. contortus Antigens . During the infection of sheep, *H. contortus* progresses through various life cycle stages (L3, L4, L5, and adult), among which there are differences in surface molecule expression. Some antigens specific to L3 and L4 are not expressed during the adult stage [71]. Quick changes in surface antigens make an effective adaptive response difficult in the initial stages of infection; therefore, each developmental stage is immunologically a different organism [54]. Thus, the larval antibody response does not cross-react with the adult stage.

Hidden antigens from the *H. contortus* intestine have been used to elicit a Th2-type response and the production of host serum antibodies, which are subsequently ingested when nematodes feed on the host's blood. The ingested antibodies recognize the nematode's intestinal antigens and alter its digestion [72]. The best-characterized and most effective intestinal antigens are the enzyme complexes H11 and H-gal-GP. The first is a family of microsomal aminopeptidases, and the latter is an aspartyl protease and metalloprotease complex. Together, these antigens, which have been obtained directly from adult worms, provide substantial protection against natural infection by *H. contortus* in sheep [73–75]. Immunization with H-gal-GP results in the production of host antibodies that inhibit the hemoglobinase activity of the endogenous enzyme, leading to *H. contortus* malnutrition

due to decreased blood digestion [76]. However, the induced protection is short lived, and the difficulties of large-scale production of immunogens limit their commercial development. Sheep immunized with the same recombinant antigens expressed in *Escherichia coli* and insect intestinal cells have been unsuccessful to be protected from infection [75, 77].

Other antigens have been evaluated as immunogens. Molina et al. [78] showed that immunization with cysteine protease-enriched protein fractions obtained from adult *H. contortus* worms protected sheep and goats against experimental infection with the parasite. The 70–83 kDa surface antigens obtained from exsheathed larvae, and the 15 and 24 kDa excretion/secretion antigens produce some degree of protection [79, 80]. Infection with different nematodes induces the abomasal and intestinal production of IgG antibodies against a carbohydrate larval antigen (CarLA) present on the surface of various strongylid nematodes. Incubation of exsheathed *Trichostrongylus colubriformis* larvae with these antibodies inhibited their implantation in the small intestine. However, the incubation of exsheathed *H. contortus* larvae with these antibodies did not have an effect on their implantation in abomasum [81].

Haemonchosis resistance has been associated with alleles of the ovine MHC (OMHC1-188) and with certain surface molecules of ovine leukocytes (OLADRB2-282), suggesting that the mechanisms of antigen presentation differ between breeds [25, 82]. Some dendritic cells can internalize antigens homologous to those of *H. contortus* [83], so the specific response to *H. contortus* may be induced by the dendritic cell-mediated presentation of parasite antigens to helper T lymphocytes. Eosinophils also function as antigen-presenting cells, particularly in the case of helminthic infections. Eosinophils exposed to *Strongyloides stercoralis* antigens had increased expression of CD69, CD86, and MHC class II similar to dendritic cell controls, these eosinophils transformed *in vitro* naïve CD4+ lymphocytes to IL-5-producing CD4+ Th2 cells [84].

4.3. Antibodies and Resistance to Haemonchosis. Natural and experimental infections with *H. contortus* induce the production of specific antibodies. The serum antibody response has been widely studied although results have been variable. While some studies show an association between serum IgG levels and resistance [47], others found an association with infection but not with resistance [33, 45]. Abomasum antibodies are more important than serum antibodies in the protection against gastroenteric nematodes. High specific IgA levels in the abomasal mucus decrease the fertility and length of *Teladorsagia circumcincta*, which is another abomasum nematode of sheep [85]. There is a negative correlation between the amount of specific IgA in abomasum mucus and the parasite burden in *H. contortus* infections [45].

A typical characteristic of helminthic infections is the induction of specific IgE, which results from a Th2-type response. IgE induces antibody-dependent cytotoxicity in eosinophils, mast cells, and macrophages. An increase in local IgE levels has been associated with resistance to gastroenteric nematodes in sheep and goats [86–88]. In *in vitro* assays, this immunoglobulin recognizes nematode surface allergens and directs eosinophils and mast cells to attack the parasite cuticle [48]. These functions are mediated by a high affinity IgE receptor present on the surface of these cells (FcεRI). A surface epitope of *H. contortus* has a $\alpha 1 \rightarrow 3$-fuc domain that is recognized by IgE. This epitope was previously found in other helminths, plants, and some arthropods, and it has been associated with the induction of Th2-type responses and allergic processes [89].

Infection produces an increase in antibody-producing plasma cells, mainly of the IgA isotype [90]. Because the number of these cells is similar in susceptible and resistant sheep breeds experimentally infected with *H. contortus*, they have not been associated with resistance [91]. Activated B lymphocytes (CD45R+) are also increased during *H. contortus* infection [4, 58].

4.4. Immune Response Cells Associated with Resistance. Inoculation with *H. contortus* larvae induces T lymphocyte proliferation and the subsequent enlargement of abomasal lymph nodes and an increase in CD4+ lymphocytes in the abomasum wall and peripheral blood [70, 92, 93].

In experimental infections, CD4+ lymphocytes are required for inducing immunity in ovine haemonchosis. Neutralization of CD4+ lymphocytes by monoclonal antibodies negates *H. contortus* immunity and increases the parasite burden in sheep resistant to infection. This neutralization also suppresses mucosa mast cell hyperplasia, eosinophil infiltration of the abomasum, and the development of humoral memory response [94–96]. In contrast, the presence or absence of CD8+ lymphocytes seems to have no effect on resistance [58, 91].

Depending on the activation stimulus, murine helper CD4+ T lymphocytes differentiate into two cell types with different cytokine production profiles. Type 1 T lymphocytes (Th1), characterized by the production of IFNγ and IL-2 among others, constitute the cellular response and protect against intracellular parasites such as *Leishmania sp*. and *Toxoplasma gondii*. The Type 2 response (Th2), characterized by the production of IL-4, IL-5, and IL-10, is part of the humoral response and associated with the presence of helminths. The Th1 and Th2 responses are antagonistic to each other. The Th1 response inhibits the Th2 response through IL-10 [97]. The polarization of the Th1-Th2 response observed in mice and humans has not been demonstrated in ruminants, but it has been possible to establish the existence of a differentiated response associated with IL-5, eosinophils, mast cells, IgG1, and IgE in sheep resistant to haemonchosis [98]. There is also evidence that effector mechanisms of the Th2 type response are involved in immunity against *H. contortus* [50, 91].

It appears that susceptibility and resistance to haemonchosis depend on the type of immune response mounted against the parasite. CD4+ lymphocytes increase during experimental infection of both susceptible and resistant sheep. Thus, both groups respond to the presence of the parasite but do so in different manners. Compared with resistant sheep, susceptible sheep produce relatively more IFNγ and less parasite-specific serum antibodies, blood eosinophils, and abomasum eosinophils [98]; therefore susceptibility is

most likely associated with a Th1 type response [47, 50], while resistance includes a Th2 type response. A differential response has also been observed in different abomasum regions. Muñoz-Guzmán et al. [91] found that resistant lambs experimentally infected with *H. contortus* had a Th2 type response (increase of eosinophils and CD4+ lymphocytes) in their abomasal pyloric region, and this response was not observed in the fundus region of the same lambs or in any abomasal regions of susceptible lambs.

Other studies suggest that there is a Th1/Th2 dichotomy in sheep infected with gastroenteric nematodes. Gill et al. [98] studied the levels of IFNγ and IL-5 produced *in vitro* by abomasum lymphocytes stimulated with *H. contortus* antigens. Lymphocytes obtained from uninfected resistant sheep produced quantities of each cytokine similar to susceptible sheep, but lymphocytes obtained from infected resistant sheep produced less IFNγ and more IL-5 than lymphocytes obtained from susceptible sheep. These studies indicate that protection is mainly due to a Th2-type response.

Genetic studies confirm the aforementioned observations. Pernthaner et al. [99] showed that resistant sheep expresss the genes for IL-5, IL-13, and TNFα and do not express those of IL-4, IL-10, and IFNγ. Andronicos et al. [100] showed that, after the initial infection, there were no differences in cxcl10 gene (regulator of IFNγ) expression in the abomasum mucosa of lambs susceptible and resistant to haemonchosis. In subsequent infections susceptible lambs overexpressed this gene, which most likely made them incapable of establishing a protective Th2-type response. A similar effect was reported in mice, where overexpression of *cxcl10* decreased clearance of *Trichuris muris* infection in susceptible mice [101].

An essential factor modulating the type of response is the age at the time of infection. Lambs that are three to six months old have fewer CD4+ lymphocytes in the abomasum wall related to diminished immune response against *H. contortus* [102]. In contrast, a greater number of $\gamma\delta$ T lymphocytes have been observed in the abomasum wall of young sheep [103]. Bovine $\gamma\delta$ T lymphocytes stimulated with concanavalin A produced IL-2, IFNγ, and TNFα [104]. If the same pattern of cytokines is produced by $\gamma\delta$ T lymphocytes of young sheep, they would mount Th1 type response. While this hypothesis could explain the high susceptibility of young lambs to infection, it requires the support of further studies.

In the first infection with *H. contortus*, the abomasum lymphocytes of susceptible sheep breeds do not produce cytokines associated with a Th2 response, but, in later infections, the production of these cytokines increases [58]. While these sheep do not reach the levels of resistance of genetically resistant sheep, the increased production of Th2 cytokines could contribute to the increased resistance to *H. contortus* in adult sheep of susceptible breeds.

5. Conclusions

Resistance to haemonchosis is an inheritable genetic characteristic associated with some sheep breeds. The immune response that protects against *H. contortus* is the expression of this genetic resistance. Genetically resistant sheep have innate defense mechanisms that prevent their colonization by larvae during their first infection. Additionally, they establish a Th2 type immune response in the abomasum mucosa that protects them from infection, but susceptible sheep do not efficiently establish this type of immune response. Finally, the immune response and the associated resistance can be modified by the type of antigen that is recognized and by such factors as age, nutrition, and the number of infections.

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Research Article

A New Parasiticidal Compound in *T. solium* Cysticercosis

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The effect of 16α-bromoepiandrosterone (EpiBr), a dehydroepiandrosterone (DHEA) analogue, was tested on the cysticerci of *Taenia solium*, both *in vitro* and *in vivo*. *In vitro* treatment of *T. solium* cultures with EpiBr reduced scolex evagination, growth, motility, and viability in dose- and time-dependent fashions. Administration of EpiBr prior to infection with *T. solium* cysticerci in hamsters reduced the number and size of developed taenias in the intestine, compared with controls. These effects were associated to an increase in splenocyte proliferation in infected hamsters. These results leave open the possibility of assessing the potential of this hormonal analogue as a possible antiparasite drug, particularly in cysticercosis and taeniosis.

1. Introduction

Cysticercosis, caused by the metacestode stage of *Taenia solium*, is a serious health and veterinary problem in many developing countries [1–3]. In humans, *T. solium* cysticerci cause neurocysticercosis, which affects ~50 million people worldwide, and it has been considered as an emergent disease in the United States [4]. *T. solium* also infects pigs, its intermediate host, leading to major economic losses [5, 6].

When humans ingest undercooked contaminated pork meat, the adult worm develops in the small intestine. After two months of asymptomatic infection, this tapeworm starts producing thousands of eggs, that, once released with the stools, can contaminate the environment, infecting pigs (rapidly differentiating into cysticerci mainly in the muscle) and humans (where most severe symptoms are observed due to the presence of cysticerci in the brain) [1, 7].

Thus, maintenance of the parasite's life cycle depends on the adult tapeworm development [8]. In fact, even in communities which do not rear or consume pigs, human neurocysticercosis can be found, because of the presence of a tapeworm carrier [9, 10]. Furthermore, tapeworm development in turn depends on scolex evagination, the initial step through which a single cysticercus becomes an adult parasite with capability of producing infective eggs [11].

Dehydroepiandrosterone (DHEA) is a steroid hormone produced from cholesterol by the adrenal glands, the gonads, adipose tissue, and the brain. It is the most abundant hormone in the human body. In humans and in mammals generally (except for rodents), DHEA is the dominant steroid hormone and precursor of sex steroids and has proved to be an important molecule in the immune responses that could drive resistance against a variety of infections [12, 13]. These infections include intracellular parasites such as *Cryptosporidium parvum* [14] and *Plasmodium falciparum* [15] as well as extracellular parasites such as *E. histolytica* [16], *Schistosoma mansoni* [17], and *Taenia crassiceps* [18], among others. 16-bromoepiandrosterone (EpiBr), a DHEA analogue without significant androgenic activity and also known as HE2000, has shown activity in a number of infectious disease settings, including tuberculosis [19],

feline immunodeficiency virus viremia [20], malaria [21], cysticercosis by *T. crassiceps,* and amoebiasis by *E. histolytica* [16]. As it can be seen, direct effects of sex steroids upon helminth parasites (cestodes, nematodes, and trematodes) and protozoan parasites are not unusual. In fact, previous results suggest that these pathogens not only are directly affected by adrenal hormones, but they have also developed several strategies to exploit the host's endocrine microenvironment [22, 23], which include degradation of host proteins as an alternative source of amino acids [24], development of parasitic-sex steroid receptors [25, 26], and cross-activation of signal transduction pathways [27, 28].

Taking into consideration this information, the aim of the present study was to explore whether EpiBr has direct *in vitro* and *in vivo* modulating effects on *T. solium*. Our results suggest that EpiBr treatment may be used as a new therapeutic approach against natural cysticercosis and taeniosis.

2. Materials and Methods

2.1. Ethics Statement. Animal care and experimentation practices at the Instituto de Investigaciones Biomédicas are frequently evaluated by the Institute's Animal Care and Use Committee, according to the official Mexican regulations (NOM-062-ZOO-1999). Mexican regulations are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH and The Weatherall Report) of the USA, to ensure compliance with established international regulations and guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto de Investigaciones Biomédicas (Permit number 2009-16). Pigs sacrifice to obtain parasites was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. All animals were maintained in a common room under controlled temperature and a 14 h dark/10 h light cycle in the animal facility of the Biological Sciences Building at the Institute of Biomedical Research (IIB).

2.2. Obtention of Parasites. T. solium cysticerci were dissected from the muscle of infected pigs, which were euthanized at the Veterinary School of the Universidad Nacional Autónoma de México. The method was previously evaluated by the University Animal Care and Use Committee to ensure compliance with international regulations and guidelines. The fibrous capsule that surrounds each parasite was carefully separated with the use of a dissection microscope. Once dissected, cysticerci were placed in tubes containing sterile PBS (1X) supplemented with 100 U/mL of antibiotics fungizone (Gibco, Grand Island, NY) [29]. Samples were centrifuged for 10 min, at 800 g at 4°C, and the supernatant was discarded. Pellets containing cysticerci were incubated in Dulbecco's Modified Eagle Medium (DMEM) without fetal serum supplementation (Gibco, BRL, Rockville, MD). They were then washed by centrifugation 3 times for 10 min at 800 g with DMEM. After the final wash, viable parasites (complete and translucent cystic structures) were counted using a binocular microscope.

2.3. In Vitro EpiBr Assays. Culture grade 16α-bromoepiandrosterone (EpiBr) was obtained from Hollis-Eden Pharmaceuticals. EpiBr was dissolved in corn oil to the desired stock concentration and sterilized by passage through a 0.2 μm millipore filter. For *in vitro* tests against *T. solium* cysticerci, hormone analogue was dissolved in corn oil and AIM-V (free of calf serum and other hormones) culture medium to the desired stock concentration and sterilized by passage through a 0.2 μm millipore filter. The experimental design was as follows: using a 24-well culture plate, six wells were used for untreated controls, six wells were supplemented with the vehicle in which EpiBr was diluted, and six wells were treated with different concentrations of EpiBr. The analogue concentrations used were based on the DHEA concentrations of serum levels found in humans and other species and were chosen to approximate analogue levels *in vivo*. The analogue was administered every other day, based on the time that DHEA (the natural hormone) remains circulating before being metabolised and filtered in the kidney. This assures that there is no toxic effect of the analogue, and the circulating levels are proximate to what we want. Our concentrations were calculated to resemble those observed *in vitro* and going from lower to higher levels. However, we converted the doses to concentrations, in order to be in line with most of the studies that use hormones *in vitro* and report those doses in terms of concentration as micromolar. So, based on these facts, we choose analogue doses to be used in both experimental systems. Concentrations of EpiBr were randomised across the plates. Control cysticerci were treated with the solvent in which EpiBr was diluted, so that a constant volume of solvent (2 mL) was added to each well. Scolex evagination and worm length were daily determined in all cultured cysticerci using an inverted microscope (Olympus, MO21, Tokyo) at 10x and 20x magnification. Worm length was considered as the millimetric addition of scolex, neck, and strobila. Immediately, the new cultures (2 mL in glass tubes) were added with 50 μL of corn oil diluted in medium (control) or 50 μL of several concentrations of EpiBr ranging from 0.1 to 50 μg/mL. Samples of 100 μL were collected from the same tube with a particular dose at 24, 48, and 72 h by ice chilling for 5 min and centrifugation at 150 g for 5 min at 4°C.

2.4. In Vivo Infections with T. solium. T. solium cysticerci were selected according to the macroscopic criteria reported by León-Cabrera and coworkers [30]. Briefly, parasites were dissected from muscle of naturally infected pigs, which were euthanized at the Veterinary School of the Universidad Nacional Autónoma de México, under consent of the University Animal Care and Use Committee to ensure compliance with international regulations and guidelines. The fibrous capsule surrounding each parasite was carefully separated under a dissection microscope. Once separated, cysticerci were placed in tubes containing sterile PBS (1X) supplemented with 100 U/mL of antibiotics fungizone (Gibco,

Grand Island, NY). Samples were centrifuged at 800 g at 4°C for 10 min, and the supernatant was discarded. Pellets containing cysticerci were placed in Dulbecco's Modified Eagle Medium (DMEM, Gibco, BRL, Rockville, MD) without fetal serum supplementation. Then, they were washed and centrifuged 3 times at 800 g at 4°C for 10 min. After the final wash, complete and translucent reddish cysticerci were incubated on 6-well culture plates containing DMEM medium with 25% pig fresh bile supplementation for infectivity test. When the evagination rate was higher than 90%, then parasites were used for subsequent oral infections.

2.5. EpiBr Administration. Twenty male golden hamsters (*Mesocricetus auratus*) of 140–160 gr, aging between 8 and 10 weeks, were subcutaneously administered with 2 mg/Kg body weight of EpiBr. Ten were infected with *T. solium* as previously described. Each single dose of EpiBr was diluted in 0.4 mL of saline solution (0.9% NaCl, Baker). Control infected animals ($n = 10$ for *T. solium* infections) received 0.4 mL of saline solution as vehicle. A stress-related additional control group ($n = 10$ for *T. solium* infections) was included in our experiments, which consisted in ten sham injection animals. Hormone analogue and vehicle administration was carried out each other day during four weeks, in order to maintain the same hormonal serum concentration for the entirely time of the experiment. Our results were obtained from two independent experiments performed in similar conditions. Animals were fed with Purine Diet 5015 (Purine, St. Louis, MO) and water *ad libitum* during all the experiment. During animal necropsy, the entire small intestine and liver were dissected and placed on a Petri dish containing sterile PBS (1X) (Sigma-Aldrich, USA). Using a stereoscopic microscope, the lumen of all small intestines was carefully exposed by making a longitudinal cut using sterile dissection scissors. Then, duodenum-anchored parasites were counted and measured with a calibrator. Blood samples were individually collected from all animal groups for posterior serum analysis. Ileum attachment zones where *T. solium* scolices were located were placed in 4% paraformaldehyde (J. T. Baker, México), or Trizol reagent (Invitrogen, Carlsbad, CA) for posterior analysis. Immediately after necropsy, spleen weight was individually recorded. Spleen and liver samples from all animal groups were individually obtained and placed in RPMI (Gibco, BRL, Rockville, MD) supplemented with 10% fetal calf serum (Gibco, BRL, Rockville, MD), or 4% paraformaldehyde (J. T. Baker, México), or Trizol reagent (Invitrogen, Carlsbad, CA) for posterior analysis.

2.6. Cell Culture and Lymphoid Proliferation. Total leukocytes and red blood cells were extracted from spleen and mesenteric lymph nodes of all animal groups. After single washing with ACK Lysing Buffer (Invitrogen, USA), total leukocytes were recovered and cultured in 96-well sterile plates (1×10^4 cells/well) containing serum-free RPMI medium (Gibco-BRL), at 37°C in humidified 5% CO_2 atmosphere for 72 h. After this time, cultured leukocytes from spleen and mesenteric lymph nodes of all animals were exposed to 15 μg/well of freshly extracted *T. solium* total antigen during 48 h. Twenty-four hours before the end of the experiment, 20 μL of AlamarBlue reagent (Biosource International) were added to each culture well. Then, culture plates were frozen at −30°C under darkness and the absorbance was quantified at 570 and 600 nm, using a microplate reader. The 570–600 nm lecture coefficient was employed to assess proliferation index.

2.7. Experimental Design and Statistical Analysis. We used a two factorial experiment. Independent variables were (1) treatment (two levels: EpiBr or vehicle); and (2) infection (two levels: Yes, No). The dependent variable was the number and size of parasites. Two *in vivo* experiments were performed, and data were analysed using one-way analysis of variance (ANOVA). When performed, post hoc individual contrasts of group means to test for significant differences were carried out using *t*-tests. Hormone dose-response time curves were estimated in three independent experiments performed with freshly isolated *T. solium* cysticerci. EpiBr was tested at five different doses; each dose was run in triplicate. Differences between groups were estimated by the ANOVA test. Post hoc analysis used was the *t*-test to examine for significant differences. Differences were considered significant when $P < 0.01$. The software Prism 2.01 (GraphPad Software Inc.) was used to calculate probability values.

3. Results

3.1. In Vitro Effect on T. solium Cysticerci. When *T. solium* cysticerci were *in vitro* exposed to EpiBr, a decrease in the scolex evagination was observed in all treated parasites compared to control groups (Figure 1(a)). However, this evagination-inhibiting effect mediated by EpiBr was independent of the tested concentrations (Figure 1(a)). Concomitantly, the evagination-inhibiting effect of EpiBr (0.25 μM) was maintained through all 10 days of *in vitro* culture, reaching its highest response on the third day of culture, in relation to control and vehicle parasites (Figure 1(b)). It is important to mention that viability of evaginated cysticerci was verified daily by means of worm motility in the culture plate, which was constant through all days of *in vitro* culture. Injured parasites were recognized by a progressive internal disorganization: development of opaque areas in the tegument and loss of translucence of the vesicle.

EpiBr also affected *in vitro* worm growth. From the lowest concentration (0.1 μM), EpiBr inhibited worm length on day 10 (measured as the addition of scolex, neck, and strobila of the developing parasite) with respect to the control group and reached a plateau (Figure 2(a)). In addition, the *T. solium* worm gradually decreased up in response to 0.5 μM of EpiBr (Figure 2(b)). Differentiated worms in absence of hormones or vehicle stimulus had a spontaneous development, reaching their maximum length (8.5 mm) at 3 day in culture. Once again, in the presence of EpiBr, no worm differentiation was observed with 2.0 μM along all the time of *in vitro* culture.

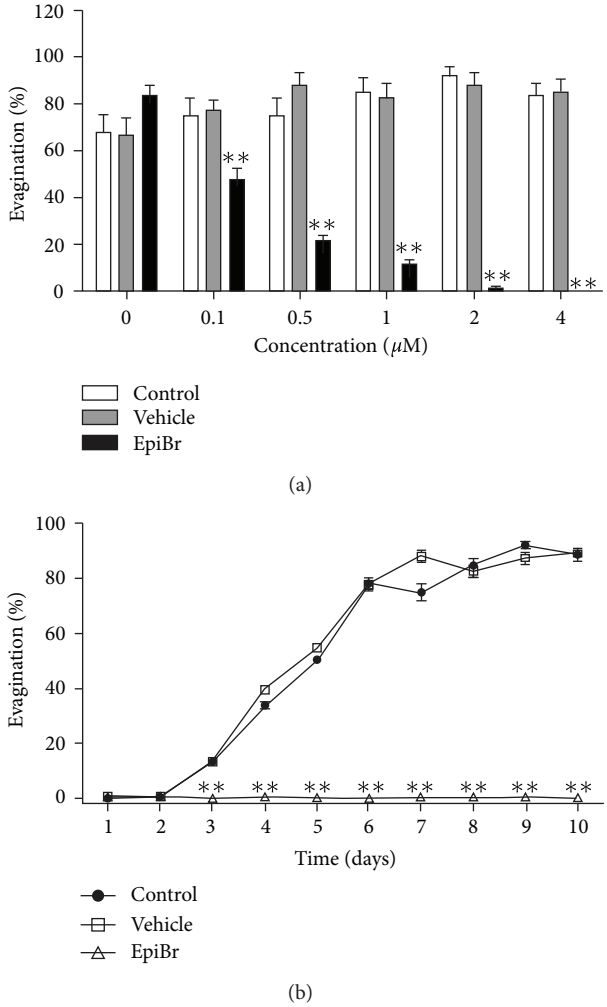

Figure 1: EpiBr decreased scolex evagination of *Taenia solium* in a concentration-dependent pattern (a) maintained along the time (b). In concentration-response curves (panel (a)), cysticerci treated with vehicle are referred to as concentration zero. Data are represented as mean +/− SD. $^{**}P < 0.05$.

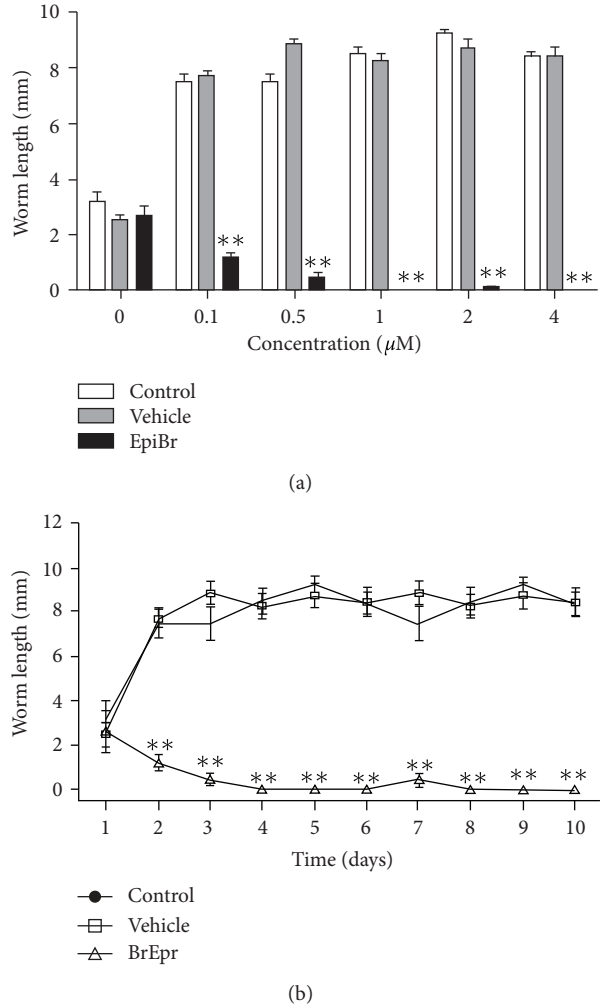

Figure 2: EpiBr decreases worm growth in a concentration-dependent pattern (a), reaching its maximum effect at 4 days of *in vitro* during the whole culture time (b). EpiBr-treated parasites were motile and undamaged on the culture plate. Worm length was considered as the addition (mm) of scolex, neck, and strobila. In the concentration-response curves (panel (a)), cysticerci treated with vehicle are referred to as concentration zero. Data are represented as mean +/− SD. $^{**}P < 0.05$.

3.2. In Vivo Effect on T. solium Infection. After 15 days after infection, EpiBr treatment significantly reduced the number of intestinally anchored *T. solium* tapeworms by 80–87% (Figure 3(a)). Vehicle-treated and control-infected hamsters showed between three and four viable parasites (Figure 3(a)). It is important to remark that all found tapeworms were strongly attached to the duodenum zone. As expected, tapeworms from vehicle-treated and control-infected hamsters grew up more than 8.8 ± 1.6 mm (Figure 3(b)). In contrast, parasite from EpiBr-treated hamsters did not develop more than 1.7 ± 0.3 mm in length (Figure 3(b)), showing besides poorly differentiated scolices. Thus, steady concentrations of EpiBr exerted a protective role against the *T. solium* intestinal infection, diminishing both the number of attached parasites and their development.

To assess the possible mechanism involved in EpiBr protective actions during infection, spleens from all animal groups were weighed and splenic leukocytes assayed for antigen-specific proliferation (Figure 4). Significant differences were observed among the spleen weight from EpiBr-treated hamsters, compared with vehicle-treated animals and control-infected hamsters (Figure 4(a)), where hamsters exposed to EpiBr increase spleen weight with respect to their infected control littermates. Furthermore, *in vivo* EpiBr treatment clearly increased proliferation *in vitro* of *T. solium* antigen-specific leukocytes by 3.5-fold compared to both infected control groups (Figure 4(b)). This result suggests that EpiBr should protect hamsters from *T. solium* infection through promotion of a local mucosal antiparasite immune response.

4. Discussion

It has been reported that exogenous DHEA administration upregulates the immune system, specifically the cellular

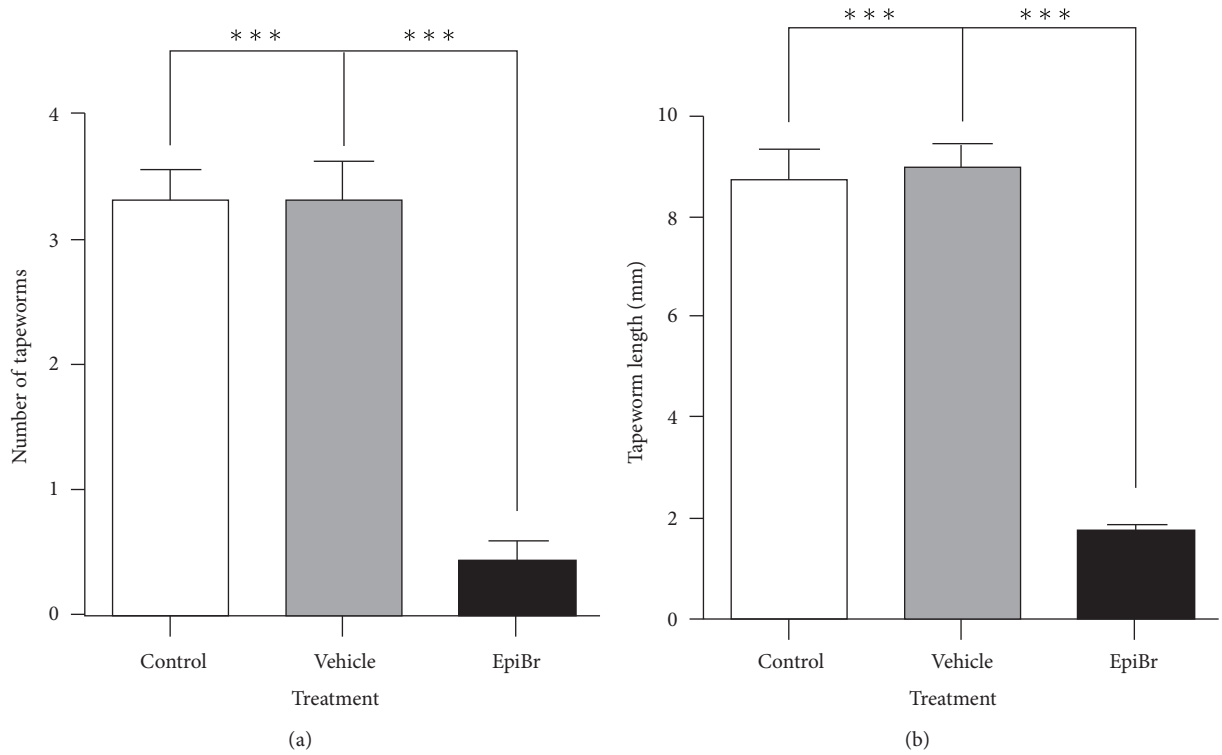

Figure 3: EpiBr decreases both parasite load and tapeworm length in *T. solium* cysticerci orally infected hamsters. (a) Administration of BrEpi significantly diminished the number of intestinally attached adult tapeworms by 80–87%, with respect to both control and vehicle infected groups. (b) Parasites exposed to constant concentrations of BrEpi showed total length reduction of fourfold. These parasites seemed as undifferentiated scolices with no develop of neck and strobila, compared to those tapeworms from control and vehicle groups with well-differentiated structures. Tapeworm length was determined as the longitudinal sum of scolex, neck, and strobila. Nonmanipulated infected hamsters were denominated as control, meanwhile comparative lines represent significant differences when $P < 0.05$. Results are presented as mean +/− standard deviation.

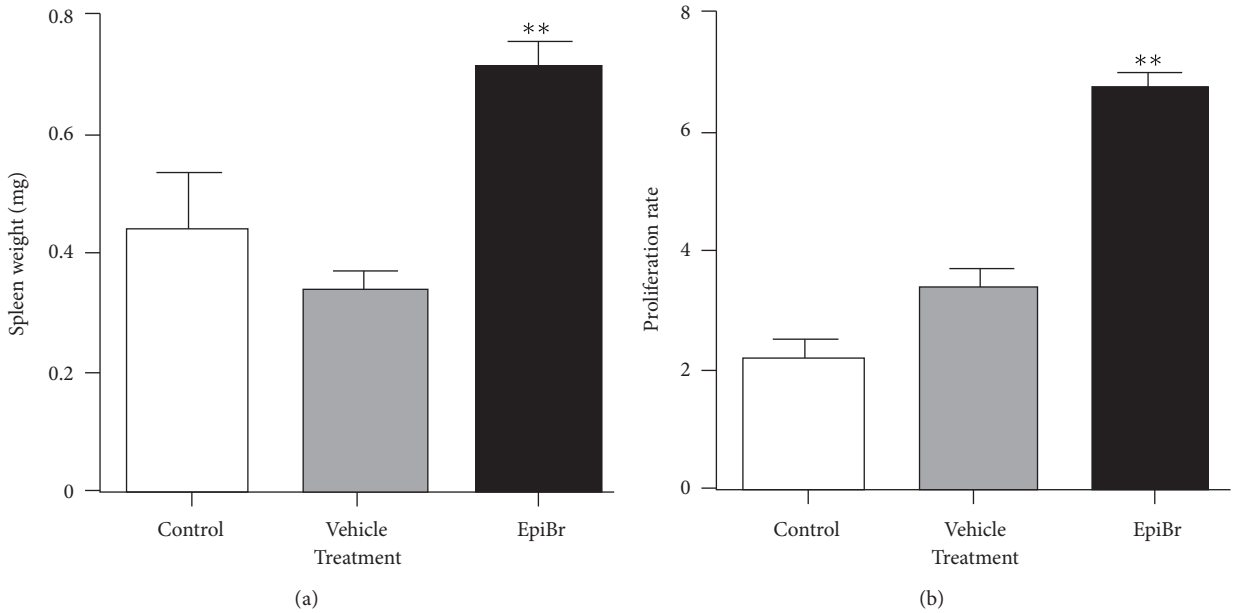

Figure 4: EpiBr administration increases proliferation rate of parasite specific leukocytes from spleen. (a) EpiBr-treated animals showed a tendency to increase spleen weight, with respect to control- and vehicle-treated animals. (b) Spleen leukocytes from EpiBr-treated, vehicle, and control infected hamsters were *in vitro* cultured in presence of *T. solium* total antigen. As a consequence of the *in vivo* exposition to EpiBr, the leukocyte proliferation index in presence of parasitic antigen was augmented by 3.5- and 2.7-fold, compared to immune cells from both control- and vehicle-treated hamsters, respectively. Results are presented as mean +/− standard deviation. $^{**}P < 0.05$ versus the other groups.

immune response, by increasing the natural killer cell number and function [13]. Our previous findings do not support this notion, since IL-2 mRNA levels do not change in response to DHEA treatment. The lack of effect of DHEA on cytokine mRNA but its dramatic effect *in vivo* on parasite load and parasite reproduction and *in vitro* on survival supports the hypothesis that DHEA analogue exerts its protective properties via direct effects on the parasite. To the best of our knowledge, this effect is consistent with the known effects of DHEA on the survival of other parasites, both metazoan [17, 31] and protozoan [16].

For instance, it has been suggested that in human schistosomiasis, DHEA is the cause of the puberty-associated drop in susceptibility [32]. This idea has been reinforced by experiments in which treatment of mice with the bloodstream form of DHEA, DHEA-S (DHEA-sulfate), protected them from infection with *S. mansoni* [17] and mice against *T. crassiceps* infection [18].

Other findings in mice of a decrease in DHEA levels as infection progresses agree with previous results in a *S. mansoni*-baboon model, in which baboons with primary infections showed decreasing levels of DHEA as the infection progressed, compared with uninfected and reexposed baboons [31].

The protective effect of DHEA has also been demonstrated in parasitic infections like *T. cruzi*, where increase the levels of lytic antibodies and to reduce *T. cruzi* parasitemia in rats [33] or in the protozoan parasite *C. parvum*, where significantly reduced both the shedding of fecal oocysts and parasite colonisation of the ileum [34, 35]. On the other hand, 16-bromoepiandrosterone (EpiBr), a DHEA analogue without significant androgenic activity and also known as HE2000, has activity in a number of infectious disease settings [16, 18–20]. Here, we explored whether EpiBr has direct *in vitro* and *in vivo* modulating effects on *T. solium* and the immunomodulating effects on *E. histolytica* reproduction, growth, viability, and infectivity.

The *in vitro* DHEA treatment of *E. histolytica* trophozoites also reduced the growth and viability of this parasite. The effects of DHEA were associated with the inhibition of G6PD activity [16]. Also, DHEA is known to exert antimalarial protection, via the enhanced opsonisation and phagocytosis of rings, the early forms of this parasite [36, 37]. Our results confirm and extend the notion that DHEA is a strong parasiticidal agent, since *in vitro* EpiBr treatment of *T. solium* remarkably reduced the reproduction rate and viability of cysticerci. Also, in our present experiments, the effects of EpiBr significantly reduced the parasite burden in males. Finally, our results support and extend the notion that EpiBr is a potentially useful treatment against a large variety of parasitic diseases. The fact that EpiBr interferes with the development of *T. solium* cysticerci may be applied to the development of future therapeutic protocols against this parasite that affects pigs and humans.

DHEA is a hormone able to inhibit the *in vitro* growth of numerous parasites, including *T. crassiceps*, a murine cestode, and *E. histolytica*, the causal agent of amoebiasis in human [16, 18]. Such results, in addition to the benefits discovered for this hormone on neuroprotection, anxiety, depression, schizophrenia, dementia, some neoplasias such as breast cancer, diabetes, and numerous inflammatory disorders [38–40], have raised discussion about the possibility of using the DHEA analogue, EpiBr, for therapeutic purposes, including the control of parasitic diseases. Thus, analogs lacking androgenic activity but maintaining the other biological activities of the natural steroid have been designed. In this study, we provide evidence demonstrating the ability of the analog EpiBr for inhibiting the establishment and growth of *T. solium*. *In vitro*, the effect of the analogue on the parasite was observed in several parameters of cell and organic functionality, affecting the establishment and growth and survival in general. These effects were similar to those previously reported by our group regarding the *in vitro* effect of DHEA on both *Taenia crassiceps* and *Entamoeba histolytica* [16–18]. Our results are in agreement with similar inhibitory effect shown for the analog on the proliferation of *P. falciparum* and *P. berghei* parasites *in vitro* [21].

The *in vitro* inhibitory effects of EpiBr on *T. solium* were also extended to the *in vivo* infections. Thus, treatment with the analog decreased the *T. solium* cysticerci evagination and growth up to 80–87% in hamsters.

Since EpiBr enhanced splenocyte proliferation, we suggest that its *in vivo* protective activity was likely due, at least in part, to a possible immunomodulatory effect on the host by EpiBr. The latter theory could be in fact involved as EpiBr has anti-inflammatory properties and it is also able to induce cellular immunity that may aid the control of infections [19, 41, 42]. Our results *in vivo* are also in agreement with previous studies supporting a potential pharmacological use of EpiBr to treat infections such as tuberculosis, AIDS, and malaria [19, 21, 41, 42]. Thus, administration of intramuscular HE2000 in patients with *P. falciparum* malaria resulted in a 50% reduction of parasitemia with notably improve in symptomatology and mild adverse events [43].

5. Conclusion

We have demonstrated that EpiBr is a promising new compound that can be used in cysticercosis, taeniosis. The results of this study leave open the possibility of assessing the potential of this analog of DHEA as an antiparasitic drug, and in particular against human/pig cysticercosis, taeniosis, major health public problems by parasites in developing countries.

Acknowledgments

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Research Article

Tamoxifen Treatment in Hamsters Induces Protection during Taeniosis by *Taenia solium*

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Human neurocysticercosis by *Taenia solium* is considered an emergent severe brain disorder in developing and developed countries. Discovery of new antiparasitic drugs has been recently aimed to restrain differentiation and establishment of the *T. solium* adult tapeworm, for being considered a central node in the disease propagation to both pigs and humans. Tamoxifen is an antiestrogenic drug with cysticidal action on *Taenia crassiceps*, a close relative of *T. solium*. Thus, we evaluated the effect of tamoxifen on the *in vitro* evagination and the *in vivo* establishment of *T. solium*. *In vitro*, tamoxifen inhibited evagination of *T. solium* cysticerci in a dose-time dependent manner. *In vivo*, administration of tamoxifen to hamsters decreased the intestinal establishment of the parasite by 70%, while recovered tapeworms showed an 80% reduction in length, appearing as scolices without strobilar development. Since tamoxifen did not show any significant effect on the proliferation of antigen-specific immune cells, intestinal inflammation, and expression of Th1/Th2 cytokines in spleen and duodenum, this drug could exert its antiparasite actions by having direct detrimental effects upon the adult tapeworm. These results demonstrate that tamoxifen exhibits a strong cysticidal and antitaeniasic effect on *T. solium* that should be further explored in humans and livestock.

1. Introduction

Human neurocysticercosis by *Taenia solium* is considered a serious brain disorder in developing countries [1], with an alarmingly increased number of new cases in developed industrialized nations [2]. Neurocysticercosis has been recently recognized as a major neglected disease in endemic communities of Latin America, with prevalence estimates of infection of 15% for the Mexican population, whereas it increases to 23% and 38% in Ecuador and Honduras, respectively [3]. Furthermore, it has been estimated that around 0.45–1.35 million cases of epilepsy are attributable to neurocysticercosis in those countries, which may directly increase morbidity and mortality rates associated with this parasite infection [3].

The parasite life cycle takes place in both pigs and humans [4]. In this way, pigs develop the intermediate larvae stage of *T. solium*, while the definitive adult tapeworm is found in the human being [5]. After a subject ingests undercooked contaminated pork meat, the *T. solium* larvae starts to differentiate into an adult tapeworm with the ability to establish at the human bowel [5]. Once this tapeworm has developed gravid mature proglottids, thousands of eggs are released with the stools into the environment, where they will be capable to infect free-ranging boars, maintaining the parasite life cycle [1, 5]. In parallel, neurocysticercosis can be acquired by humans once they have been accidentally exposed to stools containing *T. solium* eggs [6]. Thus, the *T. solium* intestinal tapeworm carrier is considered as the central node in the propagation of the disease for both organisms [7].

For this reason, discovery of new anti-taeniosic drugs should be aimed to restrain differentiation, establishment, and egg production of the *T. solium* adult intestinal tapeworm.

Tamoxifen is a competitive antagonist of the estrogen receptor that has been widely used for treating breast cancer in premenopausal women and gynaecomastia in men receiving hormonal therapy for prostatic carcinoma [8, 9]. Interestingly, the use of this antiestrogenic drug has also proved to be effective against several protozoan parasites, including *Leishmania major*, *L. braziliensis*, *L. chagasi*, *L. amazonensis*, and *Trypanosoma cruzi* [10–13]. Nevertheless, the tamoxifen effect upon helminth cestode parasites has been exclusively studied for the case of *Taenia crassiceps*, the causal agent of experimental murine cysticercosis [14]. Actually, tamoxifen inhibits *T. crassiceps* proliferation and viability *in vitro* [15], whereas it induces protection against the infection *in vivo*, through reducing parasite load by 80% [16]. Since *T. crassiceps* has a very close phylogenetic relationship with *T. solium* [17, 18], we hypothesize that the use of tamoxifen could also exhibit detrimental actions upon the latter one.

Thus, we evaluated the effect of tamoxifen on *Taenia solium*, focusing on several important aspects of the adult tapeworm stage, including differentiation from cysticercus to worm on *in vitro* cultures, and establishment of the intestinal tapeworm using the hamster model for experimental taeniosis *in vivo*. Our results demonstrate that tamoxifen totally inhibits the *in vitro* evagination of the *Taenia solium* larvae in a dose-response manner, while it also reduces the intestinal establishment of tapeworms by 70%, without affecting the host immune response. This paper could contribute to the search and design of novel therapeutic agents for the control of cysticercosis and taeniosis in livestock and humans.

2. Materials and Methods

2.1. Ethic Statement. Animal care and experimentation practices at the Instituto de Investigaciones Biomédicas are frequently evaluated by the Institute's Animal Care and Use Committee, in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH and The Weatherall Report) of the USA, to ensure compliance with established international regulations and guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto de Investigaciones Biomédicas, at the Universidad Nacional Autónoma de México (Permit Number: 2009-16). Pigs sacrifice to obtain parasites was performed under sodium pentobarbital anesthesia, and all efforts were made in order to minimize suffering.

2.2. Parasites. *T. solium* cysticerci were selected according to the main criteria previously reported by León-Cabrera and coworkers [19]. Briefly, parasites were dissected from the muscle of naturally infected pigs, which were previously euthanized at the Veterinary School of the Universidad Nacional Autónoma de México, under consent of the University Animal Care and Use Committee to ensure compliance with international regulations and guidelines. The fibrous capsule surrounding each cysticercus was carefully separated under a dissection microscope. Once separated, cysticerci were placed in tubes containing sterile PBS (1X) supplemented with 100 U/mL of penicillin-streptomycin-fungizone (Gibco, Grand Island, NY). The tubes were then centrifuged at 1200 rpm/4°C for 10 min and the supernatant was discarded. Cysticerci were then placed in Dulbecco's modified medium (DMEM, Gibco, BRL, Rockville, MD) without fetal calf serum (FCS) supplementation. After this, parasites were washed and centrifuged 3 times at 1200 rpm/4°C for 10 min using DMEM. Afterward, complete and translucent reddish cysticerci were incubated on 6-well culture plates containing DMEM medium supplemented with 25% pig fresh bile for infectivity test. When the evagination rate was higher than 90%, cysticerci were used for subsequent oral infections.

2.3. Tamoxifen Concentration-Time Response Curves. All of the *in vitro* cultures were performed using FCS-free DMEM with 100 U/mL of penicillin-streptomycin-fungizone (Gibco, Grand Island, NY). Culture grade tamoxifen was obtained from Sigma (Sigma-Aldrich, USA), and dissolved in ethanol (J. T. Baker) to the desired stock concentration. Stock solutions were sterilized by passage through a 0.2 μM millipore filter and used for culture media supplementation. For concentration-response curves, the experimental design was as follows: 20 parasites equally divided into five culture wells were incubated in presence of 0.01 μM tamoxifen-0.06% ethanol for 20 days; 20 parasites equally divided into five culture wells were incubated in presence of 0.05 μM tamoxifen-0.06% ethanol for 20 days; 20 parasites equally divided into five culture wells were incubated in presence of 0.5 μM tamoxifen-0.06% ethanol for 20 days; finally, 20 parasites equally divided into five culture wells were incubated in presence of 1 μM tamoxifen-0.06% ethanol for 20 days. Control parasites were incubated either in presence of 0.06% ethanol or in absence of this solvent for the same time. For time-response curves, cysticerci were incubated in presence of increasing doses of tamoxifen (0.01 μM, 0.05 μM, 0.5 μM, and 1 μM) for 20 days. Both concentration and time-response experiments were daily inspected for scolex evagination and worm growth using an inverted microscope at 4 and 10X magnification (Olympus, MO21, Tokyo). Worm growth was considered as the millimeter sum of scolex, neck, and strobila, as we previously reported [20]. Cultures were performed under 5% CO_2 at 37°C, replacing the supplemented culture media every 24 hours during the entire time of the experiments.

2.4. Tamoxifen In Vivo Administration. Ten female golden hamsters (*Mesocricetus auratus*) of 140–160 g, aging between 8 and 10 weeks, were subcutaneously administered with 1 mg/Kg body weight tamoxifen (Sigma-Aldrich, USA). Each single dose of tamoxifen was diluted in saline solution (0.9% NaCl, J. T. Baker) containing 0.06% ethanol. Two different groups of control animals were used in all of our experiments, as follows: the vehicle group consisted in ten animals subcutaneously administered with saline solution containing

Table 1: Primers used for amplification of hamster-specific genes. Primer sequences were designed based on hamster-specific gene sequences reported in the Gene databank, NCBI, NIH. Primer sequence as well as molecular weight expected of the PCR product is shown.

Primer definition	Primer sequence	Molecular weight of the PCR product (bp)
IL-4 Forward	5′-CCAGGTCACAGAAAAAGGGA-3′	247
IL-4 Reverse	5′-CGTGGACTCATTCACATTGC-3′	
IL-6 Forward	5′-CAACAAGTCGGAGGTTTGGT-3′	302
IL-6 Reverse	5′-AGGGTTTTGATGGTGCTCTG-3′	
IL-10 Forward	5′-CTGACTCCTTACTGCAGGACT-3′	267
IL-10 Reverse	5′-TGAAGACGCCTTTCTCTTGG-3′	
IL-12 Forward	5′-CTCTGAGCCACTCACGA-3′	167
IL-12 Reverse	5′-GTCAGTGCTGATTGCA-3′	
IFN-γ Forward	5′-CAAAAGGCTGGTGACACAAA-3′	326
IFN-γ Reverse	5′-TTCTTGTTGGGACGATTTCC-3′	
TNF-α Forward	5′-GGGAAGAGAAGTTCCCCAAC-3′	229
TNF-α Reverse	5′-TAAACCAGGTACAGCCCGTC-3′	
18S Forward	5′-CGCGGTTCTATTTTGTTGGT-3′	219
18S Reverse	5′-AGTCGGCATCGTTTATGGTC-3′	

bp: base pairs.

0.06% ethanol; the control group consisted in using ten non-manipulated animals in order to dismiss a possible effect of manipulation-induced stress on the results. Tamoxifen and vehicle administration was carried out each other day for 4 weeks, in order to maintain a constant serum concentration for the entire time of the experiment. Animals were fed with Purine Diet 5015 (Purine, St. Louis, MO) and water *ad libitum*.

2.5. Oral Infection Experiments. Two weeks after the beginning of the drug administration, tamoxifen, vehicle, and control animals were orally infected with four viable *T. solium* cysticerci, according to previous reports [19, 20]. All of the animals were euthanized 15 days postinfection, using a CO_2-saturated chamber. During animal necropsy, the entire small intestine was dissected and placed on a Petri dish containing sterile PBS (1X) (Sigma-Aldrich, USA). Under a stereoscopic microscope, the lumen of the small intestine was carefully exposed by making a longitudinal cut using a sterile dissection scissor. Duodenum-anchored parasites were then counted and measured with a calibrator. Scolex-associated duodenal tissue was placed in 4% paraformaldehyde (J. T. Baker, México), or Trizol reagent (Invitrogen, Carlsbad, California) for posterior analysis. Immediately after necropsy, mesenteric lymph nodes and spleen were dissected from all of the euthanized animals and placed in RPMI medium-10% FCS (Gibco, BRL, Rockville, MD), or Trizol reagent (Invitrogen, Carlsbad, California), respectively.

2.6. Cell Culture and Lymphoid Proliferation. Total leukocytes and red blood cells were individually extracted from the mesenteric lymph nodes of all of the animals. After a single washing with ACK Lysing Buffer (Invitrogen, USA), total leukocytes were recovered and cultured in 96-well sterile plates (1×10^4 cells/well) containing RPMI medium-10% FCS (Gibco-BRL, Rockville, MD), at 37°C in humidified 5% CO_2 atmosphere for 72 hours. After this time, cultured leukocytes were exposed to 15 μg/well of freshly extracted *T. solium* total antigen during 48 hours. Twenty-four hours before the end of the experiment, 20 μL of AlamarBlue reagent (Biosource International) were added to each culture well. Culture plates were then frozen at −30°C under dark-ness, and the absorbance was quantified at 570 and 600 nm, using a microplate reader (MultiSkan Ascent, Thermo Scientific). The 570–600 nm lecture coefficient was employed to assess the proliferation index.

2.7. Cytokine Expression. Spleen and scolex-associated duodenal tissue were placed in Trizol reagent (Invitrogen, Carlsbad, California). Total RNA extraction was as follows: both tissues were separately disrupted in Trizol reagent (1 mL/0.1 g tissue) and 0.2 mL of chloroform was added per mL of Trizol. The aqueous phase was recovered after 15 min of centrifugation at 13000 rpm, and treated with a same volume of isopropyl alcohol for RNA precipitation. After 15 min of centrifugation at 13000 rpm, the RNA pellet was washed with 75% ethanol and dissolved in RNAase-free water. RNA concentration was determined by absorbance at 260 nm, and its purity was verified after electrophoresis on 1.0% denaturing agarose gel in presence of 2.2 M formaldehyde. Immediately after, total RNA samples were reverse-transcribed by using the M-MLV Retrotranscriptase system and dT primer (Invitrogen, USA). cDNA was then used for specific PCR amplification of IL-4, IL-6, IL-10, IL-12, IFNγ, and TNF-α, using hamster-specific primers (Table 1) and TaqDNA polymerase in a semiquantitative system (Biotecnologías Universitarias, UNAM, México). Briefly, the 50 μL PCR reaction included 10 μL of previously synthesized cDNA, 5 μL of 10X PCR-buffer (Perkin-Elmer, USA), 1 mM MgCl, 0.2 mM of each dNTP, 0.05 μM of each primer, and 2.5 units of TaqDNA polymerase (Biotecnologias Universitarias, Mexico). After an initial denaturation step at 95°C for 5 min, temperature cycling was as follows: 95°C for 30 s, from 51°C

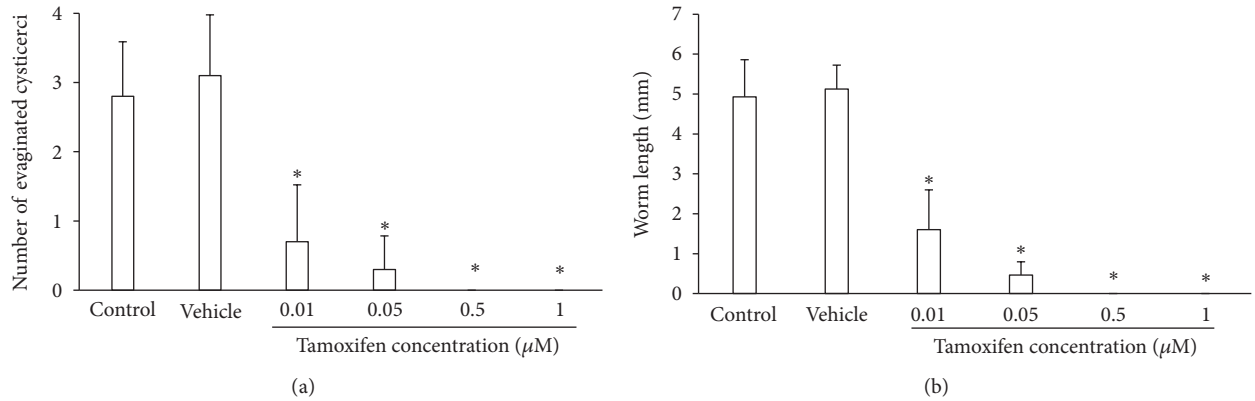

Figure 1: Tamoxifen inhibits the *in vitro* evagination and development of *Taenia solium* cysticerci in a concentration-dependent manner. (a) Concentration-response curves for evaluating the *in vitro* effect of tamoxifen on the evagination of *T. solium* cysticerci in culture. (b) Concentration-response curves for evaluating the effect of tamoxifen on the growth of *in vitro* differentiated *T. solium* worms. Control = parasites cultured in FCS-free DMEM; Vehicle = parasites cultured in FCS-free DMEM containing 0.06% ethanol. Tamoxifen was dissolved in 0.06% ethanol to the desired stock concentration. Total accumulative results at twentieth day of *in vitro* culture are shown. Data were pooled from two independent experiments using cysticerci obtained from two different pigs. Results are presented as mean ± standard deviation. Differences were considered significant when $P < 0.05$. **Significant differences concerning control groups.

to 62°C (depending on the primer sequence) for 45 s, and 72°C for 45 s during 35 cycles. An extra extension step was completed at 72°C/10 min for each run. The 50 μL of the PCR reaction was electrophoresed on a 2% agarose gel and stained with ethidium bromide in the presence of a 100 bp ladder as molecular weight marker (Gibco, BRL, NY). The relative expression rate of each amplified gene was obtained by optical density analysis (OD), using the 18S-ribosomal RNA as constitutive control of expression.

2.8. Histological Examination of Inflammatory Infiltrate. It has been previously reported that hormone-associated factors are able to induce an intestinal inflammatory response associated with *T. solium* tapeworm elimination [21]. We then analyzed a possible tamoxifen-induced intestinal inflammatory response related to control of the parasite load. Scolex-associated duodenal samples from all of the animals were placed in 4% paraformaldehyde for 2 weeks (J. T. Baker, México). After this time, all of the tissues were embedded in paraffin for being posterior cross-sectioned in thin 4 μM slices, by using a microtome (Microtome Olympus Cut 4060, USA). Sections were stained with hematoxylin-eosin for evaluating the inflammatory infiltrate degree on each sample, considered as number of polymorphonuclear leukocytes per ten microvilli, using an optical microscope at 40 and 100x magnification (Nikon Microphot-FXA Microscope).

2.9. Statistical Analysis. The *in vitro* and *in vivo* assays were performed in two independent experimental series. Data were pooled and analyzed as mean ± standard deviation using the GraphPad Prism 5 software. After evaluation of the normal distribution of data by means of the Shapiro-Wilk test, one-way analysis of variance (ANOVA), and the Tukey *post-hoc* test were performed to determine significant differences among groups. Differences were considered significant when $P < 0.05$.

3. Results

Tamoxifen exhibited a strong cysticidal effect on *Taenia solium* larvae *in vitro*. As compared with controls, the use of 0.01 μM tamoxifen decreased parasite evagination by 80%, while increasing concentrations of this antiestrogenic drug totally inhibited differentiation of *in vitro* cultured larvae, reaching a plateau at 0.5 μM after 20 days (Figure 1(a)). Furthermore, the worm length showed a 70% reduction in response to 0.01 μM tamoxifen, whereas no parasite development was observed since 0.5 μM tamoxifen as compared with controls (Figure 1(b)).

Control cysticerci displayed a spontaneous evagination after two days of *in vitro* culture, reaching a plateau at eighteen day (Figure 2(a)). On the contrary, parasites exposed to 0.01 μM tamoxifen started to differentiate after eight days in culture, whereas 0.05 μM tamoxifen delayed this process by double of the time when compared with controls (Figure 2(a)). The *T. solium* scolex evagination was not observed in parasites exposed to 0.5 and 1 μM tamoxifen after 20 days of *in vitro* culture (Figure 2(a)). Similarly, *in vitro* differentiated worms reached a 4.96 ± 0.93 mm length under control con-ditions, while cysticerci differentiated in presence of the lowest tamoxifen concentration showed a 1.86 ± 0.65 mm maximum length (Figure 2(b)). Once again, increasing concentrations of tamoxifen induced a significant delay in the parasite development onset, accompanied by a progressive diminution in the growth of *in vitro* differentiated worms (Figure 2(b)). Notably, since no difference between control groups were observed, we assume that addition of

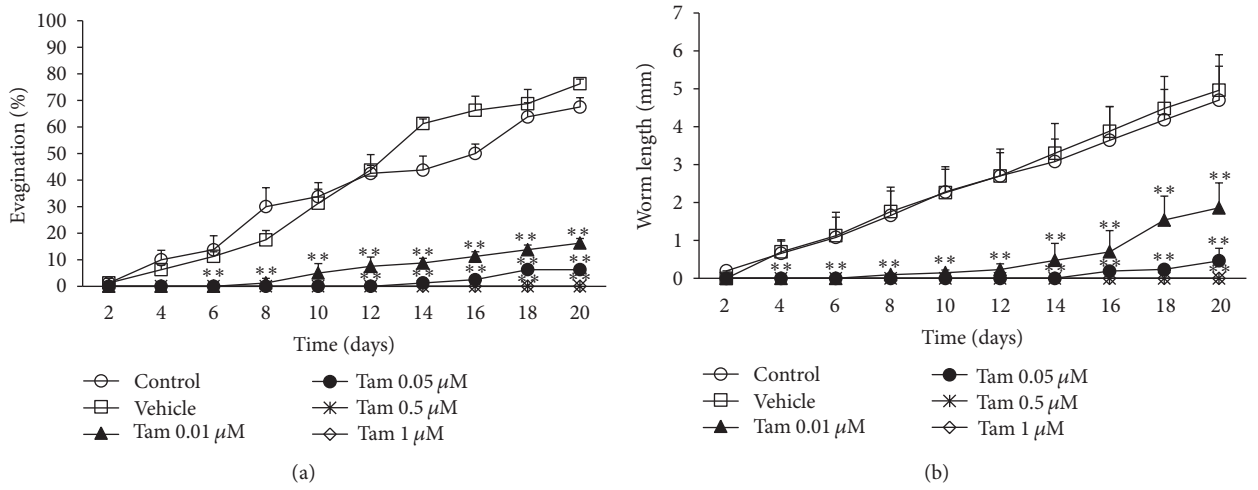

Figure 2: Tamoxifen inhibits the *in vitro* evagination and development of *Taenia solium* cysticerci in a time-dependent manner. (a) Time-response curves for evaluating the *in vitro* effect of tamoxifen on the evagination of *T. solium* cysticerci after 20 days in culture. (b) Time-response curves for evaluating the effect of tamoxifen on the growth of *in vitro* differentiated *T. solium* worms after 20 days in culture. Control = parasites cultured in FCS-free DMEM; Vehicle = parasites cultured in FCS-free DMEM containing 0.06% ethanol. Tamoxifen was dissolved in 0.06% ethanol to the desired stock concentration (Tam). Data were pooled from two independent experiments using cysticerci obtained from two different pigs. Results are presented as mean ± standard deviation. Differences were considered significant when $P < 0.05$. **Significant differences concerning control groups.

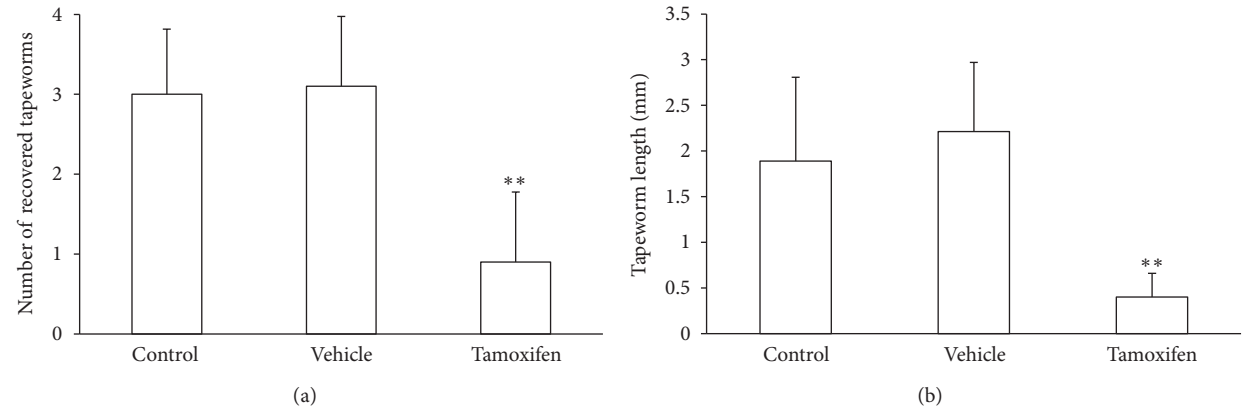

Figure 3: Tamoxifen impairs the *in vivo* establishment of *Taenia solium*. Hamsters were subcutaneously treated with 1 mg/Kg body weight tamoxifen and orally infected using four viable *T. solium* cysticerci each. Control = Nonmanipulated animals, infected with four viable cysticerci each; Vehicle = animals subcutaneously treated with 0.06% ethanol-saline solution, infected with four viable cysticerci each. (a) Evaluation of the number of duodenum-anchored tapeworms at day fifteen post-infection. (b) Assessment of the length of recovered tapeworms at day fifteen post-infection. Data were pooled from two independent experiments using ten animals per group in each experimental series and cysticerci obtained from two different pigs. Results are presented as mean ± standard deviation. Differences were considered significant when $P < 0.05$. **Significant differences concerning control groups.

0.06% ethanol to the culture media had no significant effects on *T. solium* scolex evagination and worm growth *in vitro* (Figures 1 and 2).

In vivo, tamoxifen exerted a protective effect against the *T. solium* intestinal infection, diminishing parasite load and development. In fact, hamsters treated with this anti-estrogenic drug exhibited a significant 70% reduction in the number of duodenum-anchored *T. solium* tapeworms, as compared to controls (Figure 3(a)). Furthermore, while vehicle-treated and control animals had between 3 and 4 viable tapeworms associated to the host duodenal mucosa, tamoxifen-treated hamsters showed no more than 1 or 2 poorly developed parasites (Figure 3(a)). Indeed, tapeworms from both control groups reached a maximum length of 2.21 ± 0.75 mm (Figure 3(b)), exhibiting well differentiated rostellum, suckers, and strobila (data not shown). In contrast, parasites from tamoxifen-treated hamsters did not grow up more than 0.42 ± 0.25 mm in length (Figure 3(b)), frequently appearing as scolices without strobilar development.

In order to determine a possible mechanism through which tamoxifen could exert its protective role during the experimental taeniosis in hamsters, total leukocytes from mesenteric lymph nodes were assayed for antigen-specific proliferation (Figure 4). Interestingly, there were no significant differences in the lymphoid proliferation rate between tamoxifen-treated animals and controls (Figure 4).

As intestinal inflammation has been related to parasite elimination, we decided to evaluate whether tamoxifen administration is able to induce recruiting of inflammatory cells into the host duodenal mucosa (Figure 5). The duodenal tissue from tamoxifen-treated and control hamsters showed well defined intestinal microvilli on the mucosa, accompanied by a scant inflammatory infiltrate probably associated with parasite attachment (Figure 5). No significant differences in the percent of infiltrated neutrophils, eosinophils, and basophils into the intestinal mucosa of tamoxifen-treated, vehicle-treated, and control animals were observed (Figure 5).

It has been previously reported that hormone-associated factors can stimulate cytokine expression which in turn is associated with *T. solium* tapeworm elimination. We then studied whether tamoxifen treatment could promote an immunostimulatory effect through inducing cytokine expression at the local and systemic levels. Locally at the duodenum, it was a clear expression of IL-4, IL-12, IFN-γ, and TNF-α in vehicle-treated and control hamsters (Figure 6). Nevertheless, expression of these cytokines was no significantly changed concerning tamoxifen-treated animals (Figure 6). Systemically at the spleen, the cytokine expression pattern was similar to that observed in the duodenum, characterized by high mRNA levels of IL-4 and IL-12, besides IFN-γ and TNF-α (Figure 6). However, once again there were not significant differences in the spleen cytokine expression between tamoxifen-treated and control animals (Figure 6).

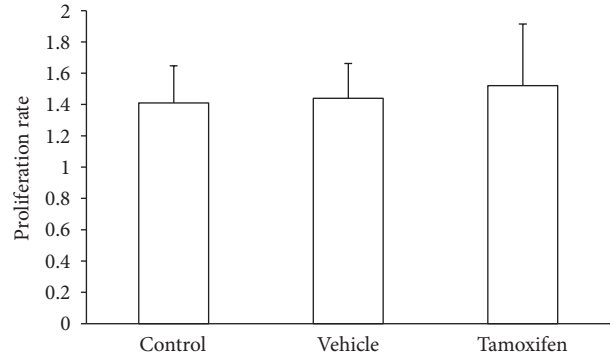

Figure 4: Evaluation of the proliferation rate of antigen-specific immune cells. Total leukocytes from mesenteric lymph nodes of tamoxifen-treated, vehicle-treated, and control hamsters were separately cultured in triplicate in presence of 15 μg/well of *T. solium* total antigen. Proliferation rate was estimated after 48 hours under described conditions. No significant differences in the leukocyte proliferation rate were observed among experimental groups. Control = Nonmanipulated animals, infected with four viable cysticerci each; Vehicle = animals subcutaneously treated with 0.06% ethanol-saline solution, infected with four viable cysticerci each; Tamoxi-fen = animals subcutaneously treated with 1 mg/Kg BW tamoxifen. Data were pooled from two independent experiments using ten ani-mals per group in each experimental series and cysticerci obtained from two different pigs. Data are expressed as mean ± standard deviation. Differences were considered significant when $P < 0.05$.

4. Discussion

To our knowledge, this study describes for the first time the effect of tamoxifen upon the *in vitro* evagination and the *in vivo* establishment of *Taenia solium*. Conventional drugs against intestinal taeniosis (such as albendazole, praziquantel, or niclosamide) exhibit numerous side effects in humans, as well as induction of drug-resistant parasite strains. Besides those inconvenient, these antihelminthic drugs have shown to be only effective as therapeutic agents but not in prophylactic schemes. Taking also into consideration that the adult tapeworm carrier has been now recognized as the central node in the maintaining of the disease dissemination to both humans and pigs [1, 2, 7], several research groups have then focused on designing new drugs and vaccines in order to prevent the intestinal establishment of *T. solium*, as a promissory strategy for interrupting the parasite life cycle and possibly the infection [19, 21, 22]. In this sense, the S3PVac synthetic peptide vaccine protects hamsters orally exposed to *T. solium* cysticerci by 74% [22], whereas the use of *T. solium*-derived recombinant proteins seems to confer around 40–100% protection [19]. Our research group recently reported that administration of progesterone to infected hamsters is able to diminish the adult tapeworm establishment by 80% [21]. However, effectiveness of synthetic or recombinant vaccines is known to be dependent on host-associated factors such as host's sex and age, as well as parasite-associated factors including cysticerci size, morphological aspect, and genetic background [19, 23]. Similarly, hormonal therapy with progesterone exhibits controversial results, inducing protection *in vivo* but stimulating parasite evagination and growth *in vitro* [20, 21]. Interestingly, our results suggest that low concentrations of tamoxifen exhibit a strong cysticidal effect upon *T. solium* cysticerci in culture, while administration of this antiestrogenic drug protects hamsters against the intestinal tapeworm establishment. Thus, tamoxifen seems to show consistent results *in vitro* and *in vivo*, which suggests that a possible future antiparasite therapy could not only be restricted to treat the adult tapeworm carrier, but also be extended to pigs in order to diminish the *T. solium* metacestode's viability and differentiation capacity. An additional interesting issue that should be taken into consideration in designing more effective strategies against the adult stage of *T. solium*, is a combinatorial therapy using immunogenic molecules and low doses of tamoxifen. In this sense, the combined use of vaccines with hormone-associated factors has previously shown major results against virus and bacterial infections [24, 25]. We thus considered that such a combinatorial therapy against *T. solium* could improve the protective responses reported to date.

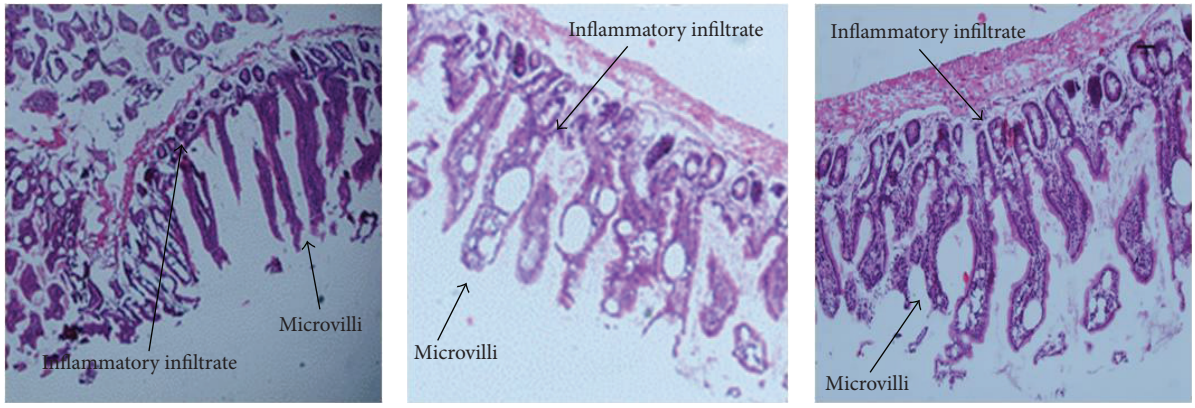

Figure 5: Histological assessment of the duodenal inflammatory infiltrate associated with the *Taenia solium* intestinal infection. Scolex-associated duodenal samples from tamoxifen-treated (c), vehicle-treated (b), and control (a) hamsters were stained with hematoxylin-eosin. The inflammatory infiltrate degree was considered as the number of polymorphonuclear leukocytes per ten microvilli. No significant differences in the inflammatory level of the intestinal mucosa were observed among experimental groups. Data were pooled from two independent experiments using ten animals per group in each experimental series and cysticerci obtained from two different pigs. Differences were considered significant when $P < 0.05$.

An intriguing question is the possible mechanism through which tamoxifen restricts the *T. solium in vivo* establishment. It has been widely described that hormone-associated factors are able to enhance the host immune response during a parasite infection, as it is well known for murine strongyloidiasis, experimental cysticercosis, trypanosomiasis in rats, murine trichuriasis, and trichinosis in guinea pigs, among many others [21, 26–29]. For the specific case of experimental taeniosis in hamsters, it has been previously reported that an intestinal inflammatory response accompanied by a local expression of Th1 and Th2 cytokines are involved in parasite elimination [21, 30]. However, our data suggest that although tamoxifen induces a strong restrictive response against the *T. solium* adult tapeworm, this effect does not seem to be through recruiting inflammatory cells into the intestinal mucosa, or stimulating the local or systemic expression of IL-4, IL-12, IFN-γ, and TNF-α. Furthermore, our research group recently showed that proliferation of antigen-specific immune cells could be stimulated by hormone-associated factors and involved in the eradication of *T. solium* [21]. Nevertheless, tamoxifen administration did not have a significant effect on the proliferation of antigen-specific immune cells. In this sense, as we mentioned, a previous study demonstrated that tamoxifen exerts a strong protective effect against experimental cysticercosis in mice by two main mechanisms: induction of the IL-2 expression, and by having direct detrimental effects upon *Taenia crassiceps* viability and reproduction [16]. It has been also reported that tamoxifen is able to directly diminish viability of all life cycle stages of *Trypanosma cruzi* at micromolar concentrations [13]. In a similar way, *Leishmania braziliensis* and *L. chagasi* intracellular amastigotes considerably decrease their viability in response to the *in vitro* treatment with tamoxifen [11]. Since neither humoral immunity nor the cellular response associated with *T. solium* elimination increase in response to tamoxifen treatment, and considering that this drug is able to directly decrease viability in protozoa and helminth parasites, it is then possible that tamoxifen effects described in this paper could not be mediated by the hamster's immune system, but through having direct detrimental actions upon the adult tapeworm of the parasite. This possibility seems to be plausible since the study of the *T. solium* genome sequences revealed the presence of hormone response genes [31], and it has been previously reported that helminth parasites are able to respond to host-derived hormonal factors [15, 32–34]. Additionally, it has been previously described that tamoxifen increases synthesis of nitric oxide (NO) in fibroblasts, and bone marrow-derived macrophages [12, 35]. Thus, in order to elucidate a possible alternative mechanism through which tamoxifen could exert its antitaeniasic properties, it is convenient to assess whether tamoxifen treatment in *T. solium*-infected hamsters is capable of increasing NO release, evaluating the ability of reactive nitrogen species against helminth parasites such as *T. solium*. However, such an intriguing hypothesis and questions require further experimental investigation.

In here, we have described a new cysticidal action of tamoxifen on the helminth cestode *T. solium*. Since collateral effects of high tamoxifen doses have been largely documented in clinical trials, the use of low doses of this drug as a short-term therapy for treating taeniasic individuals may be a novel alternative approach for disrupting the *T. solium* life cycle with minimal secondary effects for the host. Another promissory strategy for some poor communities involves administration of tamoxifen to rural free-ranging pigs for a short period of time, in order to diminish cysticerci viability and potential differentiation into an adult tapeworm in the human being. Collectively, these results could open an interesting window in the discovery of new therapeutic properties of old drugs for the treatment of parasite diseases in humans and livestock.

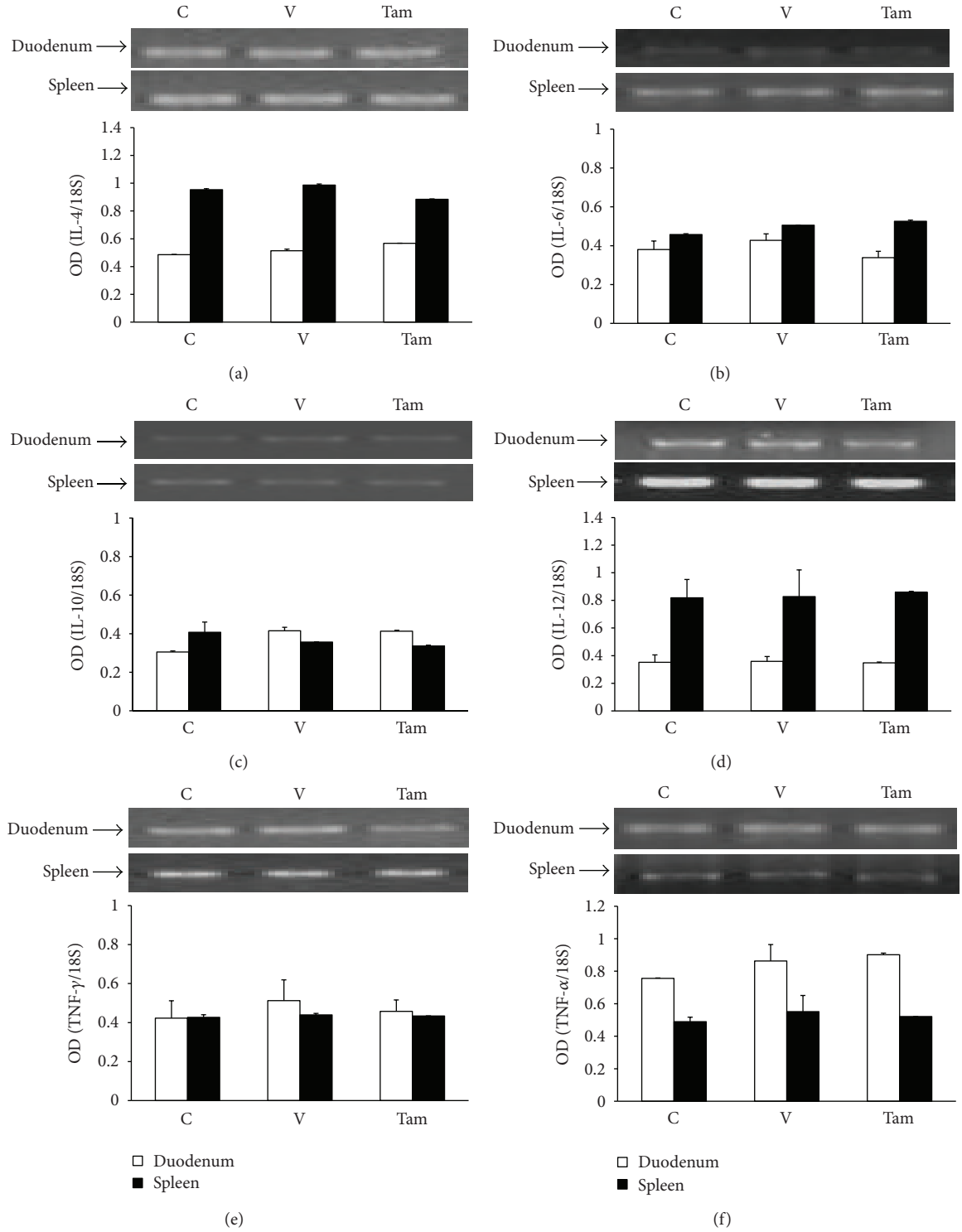

Figure 6: Expression levels of Th1 (a), Th2 (b), proinflammatory (c), and anti-inflammatory cytokines (d) associated with the *Taenia solium* intestinal infection. Cytokine expression in duodenum and spleen tissue samples from tamoxifen-treated, vehicle-treated, and control hamsters was analyzed. An increase in the expression of IL-4, IL-12, IFN-γ, and TNF-α was strongly associated with the *Taenia solium* intestinal infection. However, no significant changes in this cytokine expression pattern were observed among experimental groups. Control = Nonmanipulated animals, infected with four viable cysticerci each; Vehicle = animals subcutaneously treated with 0.06% ethanol-saline solution, infected with four viable cysticerci each; Tam = animals subcutaneously treated with 1 mg/Kg BW tamoxifen, infected with four viable cysticerci each. Data were pooled from two independent experiments using ten animals per group in each experimental series and cysticerci obtained from two different pigs. Differences were considered significant when $P < 0.05$.

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