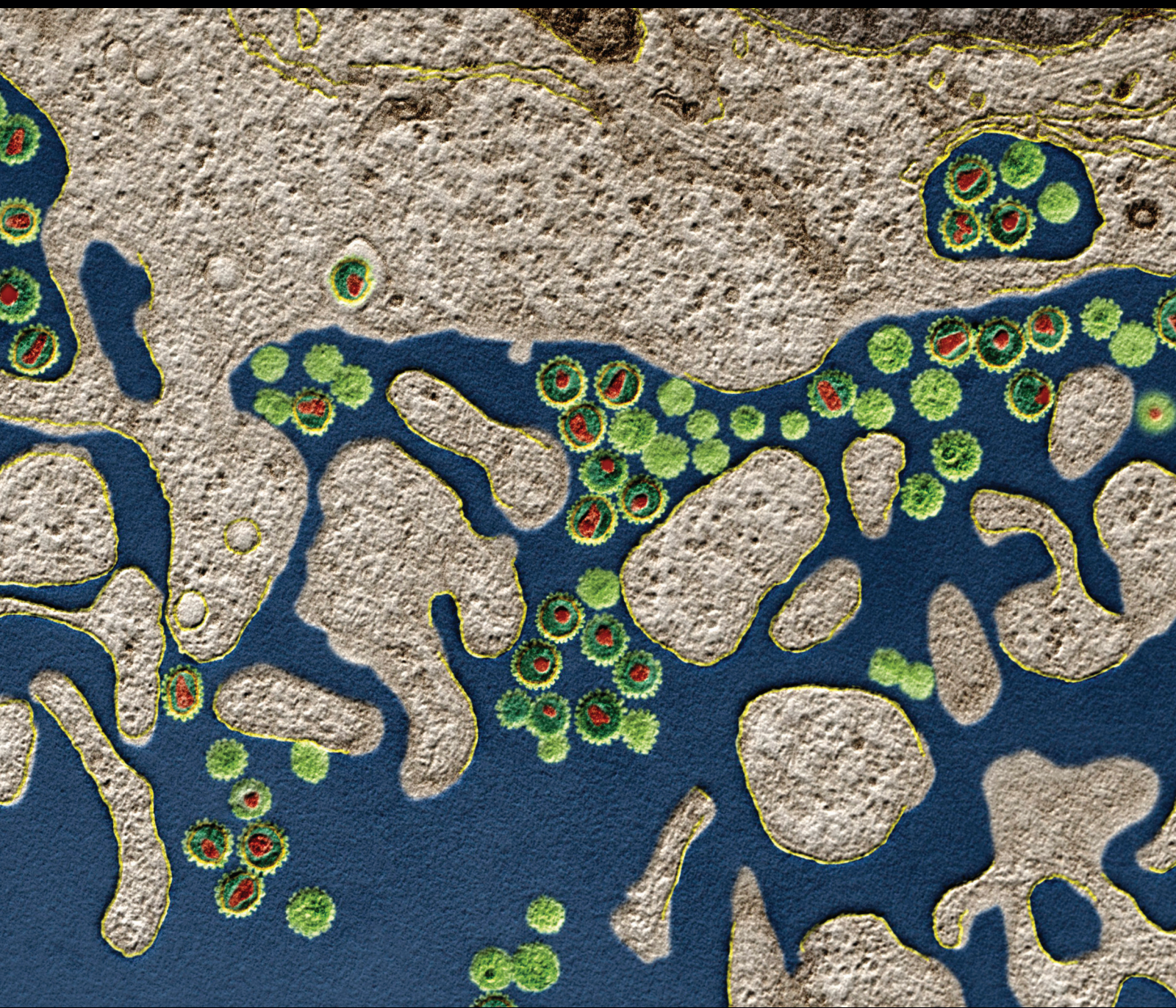
Journal of Immunology Research

T-Regulatory Cells in Health and Disease

Special Issue Editor in Chief: Eyad Elkord
Guest Editor: Varun S. Nair



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Editorial

T-Regulatory Cells in Health and Disease

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Different mechanisms of the immune system are pivotal to discriminate self-antigens from non-self antigens in order to maintain tolerance and induce protective immunity against foreign antigens. T regulatory cells (Tregs) are essential for peripheral tolerance but they contribute to the immunopathogenesis of different diseases including autoimmunity, allergy, inflammation, graft rejection, and cancer.

We are pleased to introduce this special issue, bringing 11 contributions from different research groups. These articles advance our understanding on the role and function of Tregs in different pathological conditions.

Several studies reported that Tregs are induced and/or expanded in the tumor microenvironment, where they can suppress antitumor immune responses and contribute to tumor progression and poor prognosis [1–4]. In this issue, an article by M. Niedźwiecki et al. reported a statistically higher level of $FoxP3^+$ Tregs in the bone marrow than in peripheral blood of a group of 42 children with acute lymphoblastic leukemia, which might be favorable for the development of leukemic bone marrow at early stages. This higher level of Tregs in bone marrow could be a potential risk factor for poor prognosis of hematological malignancies. In another article addressing the importance of Tregs in hematological malignancies, M. Delia et al. investigated the percentage of Tregs in the diagnostic bone marrow aspirates (dBMA) and their correlation with response to chemotherapy and survival in 23 acute myeloid leukemia (AML) patients. They reported that higher Treg numbers in dBMA predicted better response and survival of AML patients. This study suggests a prognostic role for Tregs in AML patients receiving intensive chemotherapy. In the following review article, N. Hosaka discussed the role of Tregs in tumor-bearing mice treated with allo-hematopoietic stem cell transplantation plus thymus transplantation. This review highlights the importance of Tregs in the enhanced graft-versus-tumor effect and reduction of graft-versus-host disease, leading to a better outcome and longer survival.

Forkhead box P3 (FoxP3) is the master transcription factor for Treg development and function [5]. Mutations in FoxP3 lead to substantial decrease in Tregs, which results in severe autoimmune disorders [6]. In this special issue, five articles give us more insights into the importance of Tregs in autoimmune disorders. H. Keino et al. present an interesting and comprehensive review on the ocular immune privilege associated with eye-derived Tregs. The authors provided a robust background on the molecular mechanisms responsible for the development and maintenance of ocular immune privilege by Tregs. Clearly, further understanding of the ocular immune privilege associated with Tregs could offer a new approach to therapeutic interventions for ocular autoimmunity. Next review article by D. Calzada et al. reviewed different mechanisms of Tregs involved in allergy and allergen tolerance. The authors provided an update on the function of Tregs in allergic diseases and the potential use of Tregs as novel therapeutic approaches. The next review article by D. Vdovenko and U. Eriksson focused on the role of Tregs in myocarditis. The authors concluded that different $CD4^+$ T effector cells including Th1 and Th17 have

a critical role in myocarditis, and Tregs have a specific role to limit disease progression. However, understanding the specific roles of T cell subpopulations at different stages of the disease progression is critical for the development of successful therapeutic strategies. Next, T. Chen et al. showed that in the peripheral blood of asthmatic patients, apart from $CD25^+FOXP3^+$ canonical Tregs, there is a distinct population of Tregs which express a higher level of GATA3. Additionally, authors showed that in patients, both the percentage and immunosuppressive function of canonical Tregs were highly impaired due to the elevated expression of these Th2-like Tregs. However, the role of $GATA3^+$/$FOXP3^+$ Th2-like Tregs is still not fully disclosed. In another article, M. Vitales-Noyola et al. assessed the influence of low versus high sodium intake on different immunological parameters, especially on Tregs and Th17 subsets, in patients with rheumatic arthritis (RA) and systemic lupus erythematosus (SLE). The authors concluded that the level of sodium intake is not associated with different immune parameters in healthy donors or patients with SLE or RA.

The last group of these articles is categorized under transplantation biology. The role of Tregs in transplantation is to create tolerance and suppress graft-versus-host disease. N. Pilat et al. described the effect of CTLA4Ig on induction and suppressive function of mouse-induced Tregs (iTregs) in *in vitro* culture. CTLA4Ig has been approved for the treatment of autoimmune diseases and transplant rejection. The authors reported that the costimulation blocker significantly improves the generation and suppressive function of iTregs. Moreover, these iTregs could be a better choice for graft survival over calcineurin-based immunosuppressive regimens. Next, M. Vieyra-Lobato et al. comprehensively described the role of $CD8^+$ Tregs in various disease aspects. This review discussed different surface markers including Treg signatures and cytokines in several diseases including autoimmunity, cancer, and graft-versus-host disease as a strategy in their prevention, monitoring, and cure. In another article, T. E. C. Kieffer et al. showed that pregnancies with male fetuses more often lead to pregnancy complications such as preterm birth and preeclampsia. The authors suggested that in male fetuses, there exist a Y chromosome-associated pathophysiology consisting of lower FOXP3, IFN-γ, and IL-6 mRNA expression, leading to a high complication risk during pregnancy, which was absent in females. This study advances our current knowledge in reproductive immunology regarding the immunologic differences between male and female fetuses with a divergent pathophysiology outcome.

In summary, a considerable progress has been made to understand both genetic and phenotypic changes acquired in Tregs at different disease aspects. We hope that the articles in this special issue improve our understanding on the role and function of different Treg subsets in various disease settings. Further underlying mechanisms behind the phenotypic and functional changes acquired by Tregs in various diseases should be revealed, which could provide insights to target them and enhance different therapeutic strategies.

Acknowledgments

We are grateful for all authors for their contributions to this special issue and reviewers for their valuable help. We hope that this issue will be a valuable resource for researchers in the field of Tregs.

Eyad Elkord
Varun Sasidharan Nair

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Review Article

Description of CD8$^+$ Regulatory T Lymphocytes and Their Specific Intervention in Graft-versus-Host and Infectious Diseases, Autoimmunity, and Cancer

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Gershon and Kondo described CD8$^+$ Treg lymphocytes as the first ones with regulating activity due to their tolerance ability to foreign antigens and their capacity to inhibit the proliferation of other lymphocytes. Regardless, CD8$^+$ Treg lymphocytes have not been fully described—unlike CD4$^+$ Treg lymphocytes—because of their low numbers in blood and the lack of specific and accurate population markers. Still, these lymphocytes have been studied for the past 30 years, even after finding difficulties during investigations. As a result, studies have identified markers that define their subpopulations. This review is focused on the expression of cell membrane markers as CD25, CD122, CD103, CTLA-4, CD39, CD73, LAG-3, and FasL as well as soluble molecules such as FoxP3, IFN-γ, IL-10, TGF-β, IL-34, and IL-35, in addition to the lack of expression of cell activation markers such as CD28, CD127 CD45RC, and CD49d. This work also underlines the importance of identifying some of these markers in infections with several pathogens, autoimmunity, cancer, and graft-versus-host disease as a strategy in their prevention, monitoring, and cure.

1. Introduction

In general, CD8$^+$ Treg lymphocytes have been characterized as a heterogeneous population consisting of lymphoid cells that express certain surface markers depending on their inhibition activity and the microenvironment they are found in [1].

In 1970, Gershon and Kondo described CD8$^+$ Treg lymphocytes for the first time when they published the results of experiments using mice. The study described a population of lymphocytes from bone marrow responsible for tolerance. These cells were originally called "suppressor T lymphocytes." In their work, the researchers proved the cross-reactivity of related antigens by immunizing mice, first using sheep erythrocytes and then horse erythrocytes. The treatment induced tolerance to horse red blood cells in mice that had been immunized with high levels of sheep red blood cells. This tolerance was proven to be mediated by thymic cells [2]. They later proved the regulatory role of peripheral thymocytes, specifically those located in the spleen [3]. The study of these cells was further developed in 2007 under the concept of CD8$^+$ Treg cells in the context of some viral infections and development of some tumors. These works established the indirect importance of IFN-γ in the induction of their regulatory activity through molecules as indoleamine 2,3-dioxygenase (IDO) [4].

It is currently known that CD8$^+$ Treg lymphocytes have an inhibitory effect through soluble factors or cell-cell contact. In murine and human models, different works have described a number of regulatory mechanisms mediated by

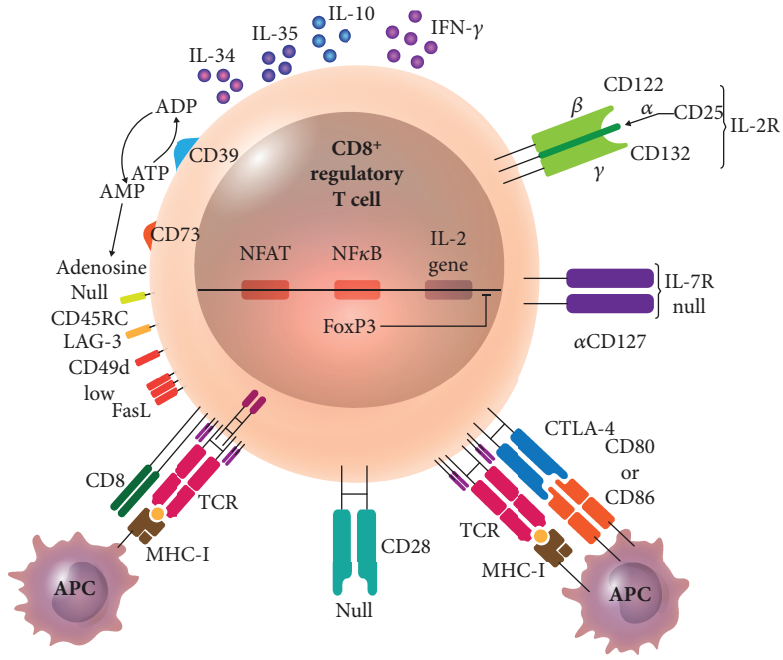

Figure 1: $CD8^+$ Treg lymphocyte. $CD8^+$ Treg lymphocytes have different suppression mechanisms of cell activation and survival, using their own molecule expression: IL-2 receptor α-chain (CD25), IL-2 and IL-15 receptor β-chains (CD122), inhibitory receptor CD152 or CTLA-4, ectoenzymes CD39 and CD73 degrading ATP to ADP (CD39) and AMP to adenosine (CD73), an MHC-II-binding molecule called LAG-3 (lymphocyte activation gene-3), and the apoptosis-inducing molecule FasL. This T cell subset expresses low or absent costimulatory receptor CD28 and the IL-7 receptor α-chain (CD127), the cellular activation molecule CD45RC, and the integrin CD49d and releases cytokines as IL-10, IL-34, IL35, and IFN-γ; transcription factor FoxP3 inhibits IL-2 gene transcription. APC: antigen-presenting cell; CTLA-4: cytotoxic T-lymphocyte antigen 4.

$CD8^+$ Treg lymphocytes: (a) direct death of target cell [5, 6], (b) negative signaling through CTLA-4 or PD1 when interacting with the antigen-presenting cell [7], and (c) release of immunosuppressive cytokines as IL-10 and TGF-β [8, 9]. The suppressor effect is evident when $CD8^+$ Treg lymphocytes are able to inhibit the proliferation of effector $CD4^+$ and $CD8^+$ effector T lymphocytes [10]. The immunosuppressive effect of $CD8^+$ Treg lymphocytes is likely to be beneficial by reducing the severity of the inflammatory response present during the development of the graft-versus-host disease (GVHD) or autoimmune diseases. On the other hand, it would be beneficial to decrease the $CD8^+$ Treg population in diseases such as cancer or infections where they participate in the evasion of the immune response. Proving this effect would shed light on its application as preventive or healing cell therapy.

The expression of surface molecules acting as cell markers helps to phenotypically identify $CD8^+$ Treg lymphocytes. Phenotypic markers include the high expression of the IL-2 receptor α-chain CD25 and expression of CD122 (IL-2 and IL-15 receptor β-chains), adhesion molecule CD103, ectoenzymes CD39 and CD73, the inhibition receptor CD152 or CTLA-4 (cytotoxic T lymphocyte-associated molecule-4), an MHC-II-binding molecule called LAG-3, and the apoptosis-inducing molecule FasL. The soluble molecules that $CD8^+$ Treg lymphocytes can express are FoxP3, IFN-γ, IL-10, IL-34, and IL-35. The absence of activation markers is also studied when looking for $CD8^+$ Treg lymphocytes. The costimulation molecule CD28, the IL-7 receptor α-chain (known as CD127), the cell activation molecule CD45RC, and the integrin CD49d are absent or show low expression [10] (Figure 1).

1.1. Surface Markers of $CD8^+$ Treg Lymphocytes. The overexpression of CD25, widely described in $CD4^+$ Treg lymphocytes, indicates the presence of a regulatory activity, inhibiting the proliferation of effector lymphocytes in competition for IL-2. Given the high expression of CD25 in the membrane of Treg lymphocytes, the latter obtain most of the cytokine, leaving effector T lymphocytes without the supply of this growth factor. For its part, marker CD25 is commonly sought together with transcription factor FoxP3 [11]. In $CD8^+$ Treg lymphocytes, it is unclear whether CD25 subtracts IL-2 from the medium as a regulatory mechanism. However, $CD8^+CD25^+$ Tregs are present in both human and mouse and are very sensitive to IL-2 to proliferate compared to T effectors and capable of inhibiting the proliferation of effector T cells [12].

On the other hand, a subset of $CD8^+CD122^+$ Treg lymphocytes in mice has been observed to be efficient in the suppression of allogeneic, autoimmune, and antitumor responses. Additionally, $CD8^+CD122^+$ T cells express large amounts of IL-15 receptor α-chain (IL-15RA). The β- (CD122) and γ- (CD132) chains are overexpressive and common for CD25 and IL-15Rα; however, CD25 is absent in those cells. Therefore, the distinctive molecule is CD122 and not CD25. That is why these lymphocytes consume IL-15 to proliferate and not IL-2 [13]. The expression of CD122 is associated with memory lymphocytes [13, 14].

Because nonregulatory memory lymphocytes can also express CD122, the presence of PD-1 is evaluated to confirm that it is $CD8^+CD122^+$ Treg [15]. Apparently, TCR-MHC-I is a mechanism of interaction between these lymphocytes and the target cells [16], and $CD8^+CD122^+$ cells regulate through IL-10 [17].

When $CD8^+CD122^+$ T cells are eliminated from mice, there is a growth of specific tumor T cells and infiltration of effector/memory T cells in the tumor [15, 18]. In mice, marker CD122 is exclusive of $CD8^+$ Treg lymphocytes and is absent from $CD4^+CD25^+$ Treg lymphocytes [19]. CD122 works as an IL-15 receptor, which promotes survival and proliferation of $CD8^+$ Treg lymphocytes, so that the transfer of $CD8^+CD122^+$ T lymphocytes, along with the administration of recombinant IL-15, promotes its regulatory activity, extending the survival of mice after pancreas transplant [18]. Indeed, in mice, CD122 has made a difference between $CD4^+$ and $CD8^+$ Treg lymphocytes since, as it has been already stated, the first often express high levels of CD25 while the latter exhibit elevated levels of CD122. For their part, $CD8^+CD122^+$ Treg lymphocytes are related to the success of allogeneic transplant via the induction of apoptosis among alloeffector T lymphocytes and thus inhibiting transplant rejection [20].

In mice, $CD8^+CD122^+$ T cells are comparable with $CD8^+CXCR3^+$ T cells in humans since they release IL-10 and suppress IFN-γ production by $CD8^+CXCR3^-$ effector T cells [21].

Also known as LFA-1, CD103 is an adhesion molecule present in T lymphocytes bound to E-cadherin from the parenchymal epithelial tissue or mucous membranes. This molecule promotes retention of Treg lymphocytes in such tissues in areas expressing E-cadherin where the regulation of immune response is needed. This is highly useful to identify $CD8^+$ Treg lymphocyte subpopulations according to their location [22]. It must be considered that molecule CD103 does not provide an exclusive regulatory function to $CD8^+$ Treg lymphocytes given that $CD8^+$ effector T lymphocytes also express it [23, 24].

Ectoenzymes CD39 and CD73 are found on the cell surface of lymphocytes and other cell lines. While CD39 produces ADP and AMP via ATP dephosphorylation, CD73 catabolizes AMP to produce adenosine, which inhibits T lymphocyte response and has an anti-inflammatory effect. The regulatory activity of adenosine starts after it is bound to any of its four receptors: A1, A2A, A2B, and A3. Its effect is greater when bound to receptor A2A. Even though the pathway through which adenosine signals when it is bound to its receptor, *in vitro* studies have found that CD73 inhibits the proliferation of effector T lymphocytes in mice; such effects have been proven in $CD4^+$ Treg lymphocytes. Because these markers were later found in human $CD8^+$ Treg lymphocytes, they are considered therapeutic targets in therapy against cancer [25–27].

Cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152) blocks the production of IL-2, the expression of IL-2R, and the cell cycle of activated T lymphocytes [28]. CTLA-4 antagonizes CD28 and prevents CD28-CD80/CD86 interaction like an inhibition mechanism [29]. Also, when there is CTLA-4 engagement, the membrane-proximal region of the CTLA-4 cytoplasmic domain delivers a tyrosine-independent signal that inhibits T cell activation, another inhibition mechanism by CTLA-4 [30–32]. Recent works propose a different CTLA-4 suppressor mechanism that involves the capture and depletion of its ligands, CD80 and CD86, from antigen-presenting cells by transendocytosis. During the process, CD80/CD86 are transferred into CTLA-4-expressing cells. Therefore, not only does CTLA-4 uptake its ligands and internalize them but also is likely to degrade them [33–35]. A reduced costimulation in T lymphocytes also reduces positive signals between them and antigen-presenting cells that promote the maturation of the latter. This event occurs in the infiltration of T cells in some types of cancer [28, 36, 37]. The subpopulations of Treg $CD8^+CTLA\text{-}4^+$ suppress the immune response against tumor, inhibiting the proliferation of effector T lymphocytes, where they can participate in the regulatory mechanism of IL-35 [38] and are also able to inhibit dependent allogeneic responses [39].

For its part, LAG-3 (lymphocyte activation gene 3) is a molecule with a similar structure to CD4. Because of this similarity, it competitively binds to MHC-II molecules with higher affinity than CD4. When it binds to MHC-II in antigen-presenting cells, it signals in a negative way, unlike CD4 does [40–42]. Therefore, LAG-3 interacts with the TCR-CD3 complex and inhibits its signaling [43]. The interaction between LAG-3 and MHC-II inhibits the activation and proliferation of $CD4^+$ and $CD8^+$ T cells and the production of cytokines from a Th1 subset [44]. This immune system suppression molecule acts against tumors by blocking them with an antibody, restoring the immune response [45]. Finally, its presence in regulatory cells can decrease the severity of autoimmune diseases [46].

FasL is a molecule involved in the induction of apoptosis of a target cell, a mechanism used by $CD8^+$ Treg lymphocytes to kill effector T cells in a direct cytotoxicity. For this regulatory mechanism to work, it is essential that the Treg lymphocyte and the target cell express FasL and Fas, respectively [47, 48].

The characterization of $CD8^+$ Treg lymphocytes via the detection of membrane molecules that identify them should be complemented with the research on soluble molecules they express.

1.2. Soluble Molecules. FoxP3 is an intracellular DNA-binding protein that prevents transcription and probably involves the direct repression of NF-AT-mediated transcription [4, 49, 50]. It was initially described in scurfy mice that do not express FoxP3. Studies found that $CD4^+$ T lymphocytes in scurfy mice were chronically activated, expressing high levels of several activation markers and cytokines ex vivo. This lymphocyte hyperactivation phenotype was refractory to inhibition with a number of drugs, specifically immunosuppressants cyclosporine A and rapamycin [51]. The phenotype of these mutant mice is similar to the one observed in CTLA-4-deficient mice, indicating that FoxP3 is an important regulator of T lymphocyte activation [52]. In $CD4^+$ Treg lymphocytes, FoxP3

is a suppression marker of cell activation and thus used as identification marker. For $CD8^+$ Treg lymphocytes, the role of FoxP3 is not so clear since it is only expressed in less than 5% of $CD8^+$ T lymphocytes [53]. However, populations of $CD8^+$ Treg cells expressing FoxP3 are important immune-suppressors during chronic or asymptomatic infections caused by suboptimal amounts of the infectious agent [54]. They also play this role during GVHD and skin transplantation [55].

The proinflammatory cytokine IFN-γ polarizes Th1 immune cell response and has been observed to play another role in $CD8^+$ Treg lymphocytes. When producing IFN-γ, these lymphocytes induce IDO production by dendritic and endothelial cells [4, 56]. This enzyme is responsible for catabolizing tryptophan amino acid. This amino acid is essential to lymphocyte proliferation after activation; therefore, the presence of IDO leads to a decrease in circulating tryptophan levels, restricting the proliferation of activated effector lymphocytes [57]. The single nucleotide polymorphisms (SNPs) of the IDO enzyme are related to autoimmune diseases such as systemic sclerosis [58].

The anti-inflammatory cytokine IL-10 is considered a characteristic molecule of $CD4^+$ Treg lymphocytes. Besides mediating the anti-inflammatory regulatory action, it is added to cultures in order to induce $CD4^+$ Treg lymphocytes *in vitro*. A similar observation has been made in $CD8^+$ Treg lymphocytes, further proving that IL-10 is an evidence of the regulatory function of these cells [59]. For example, IL-10 produced by $CD8^+$ Treg lymphocytes inhibits $CD4^+$, Th1, and Th2 cell proliferation [60, 61]. In addition, IL-10 suppresses the cytotoxic activity of cytotoxic T lymphocytes by the reduction of MHC-I expression in target cells [62].

A Treg-specific cytokine, IL-34, has an immunosuppressive function and is involved in the maturation of immunoregulatory macrophages during immunological tolerance processes as pregnancy and the inhibition of rejection in solid organ transplantation [63, 64]. The cytokine carries out its regulatory function when it is recognized through the Fms receptor, which it shares with the macrophage colony-stimulating factor (M-CSF) [65]. Additionally, IL-34 has been found to be involved in the regulation of several subpopulations of tissue resident macrophages, including Langerhans cells and microglia [66].

Another cytokine, IL-35, inhibits the maturation of dendritic cells and the proliferation of $CD4^+$ and $CD8^+$ T cells and the Th1 polarization of $CD4^+$ T lymphocytes [67]. Specifically, this cytokine is involved in the suppressive role of $CD8^+$ Treg cells in tumors, synergizing with CTLA-4 and avoiding the potentiation of an antitumor immune response [38].

TGF-β (transforming growth factor) is an immunoregulatory cytokine that can be expressed in 3 isoforms: TGF-β1, TGF-β2, and TGF-β3, depending on the tissue and the stage of development. It promotes $CD8^+$ effector T lymphocyte apoptosis through SMAD-2 signaling and the upregulation of proapoptotic protein Bim [68]. The TGF-β-producing $CD8^+$ Tregs are able to suppress autoimmune responses very efficiently [69, 70]. It is known that TGF-β acts on antigen-presenting cells as dendritic cells decreasing the expression of costimulation and MHC-I molecules and effector T lymphocytes by inhibiting their proliferation. This has been a mechanism described in the evasion of the antitumor immune response [71, 72].

1.3. Absence of Activation Molecules. As previously stated, $CD8^+$ Treg lymphocytes are characterized by the presence of molecules used as markers to detect and identify these cells. However, it should also be considered that $CD8^+$ Treg lymphocytes lack the expression of certain molecules associated to activation and are present in effector T cells. In mice and human, marker CD28 is scarcely expressed in the thymus and has a reduced expression in peripheral blood cells with anti-inflammatory cytokine production; therefore, it is considered that some subpopulations of $CD8^+$ Treg could be $CD28^{low}$ [1]. The absence of CD28 in human T lymphocytes correlates with two biological events: cell senescence [73, 74] and extended exposure to antigens [75]. Because of that, there is an increase in $CD8^+CD28^-$ T lymphocyte population during chronic inflammatory processes and in elderly subjects. These cells are produced from $CD8^+$ T lymphocytes that have repeated antigen stimulation [76]. This explains the fact that elderly subjects show higher concentrations of these lymphocytes. $CD8^+$ T lymphocytes are also unable to proliferate once they are induced to differentiate into $CD28^-$ cells [77, 78]. They express regulation molecules that are present in $CD4^+$ Treg lymphocytes as CD39, CTLA-4, and CD25. In addition, studies have proven that they are able to inhibit effector $CD4^+$ and $CD8^+$ effector T lymphocytes. These lymphocytes are considered Treg and able to inhibit a Th1-type response [10, 79–81].

The surface marker CD127 (IL-7 receptor α-chain) is also absent from $CD8^+$ Treg lymphocytes, recovering its levels of expression in effector and memory cells [82–85] but not in $FoxP3^+$ and those that are likely to be regulatory [85]. There is evidence that CD127 is absent from $CD8^+$ Treg lymphocytes. This was proven *in vitro* when differentiating naïve $CD8^+$ T lymphocyte with TGF-β and IL-2 and obtaining lymphocytes with suppressor action expressing $CD127^-CD25^{hi}FoxP3^{hi}$ markers [86]; however, these lymphocytes are not so helpful. In humans and mice, $CD4^+$ and $CD8^+$ Treg lymphocytes expressing $CD25^{+-}$ $FoxP3^+$ exhibit low concentrations or absence of CD127, unlike effector T cells. This difference is more evident in humans [12].

A T cell activation marker, CD45RC, is absent or found at low concentrations in $CD8^+$ Treg lymphocytes involved in solid organ transplant acceptance by IL-34 production [4]. The isoform of CD45, CD45RC, is a transmembrane protein-tyrosine phosphatase that belongs to the Src kinase family. It is essential to signal transduction after T cell receptor activation and is present in rats, mice, and humans [4, 87–92].

Finally, CD49d is a surface molecule expressed at low levels in $CD8^+$ Treg lymphocytes. Although the role these lymphocytes play remains unclear, one of their subpopulations can induce apoptosis in activated T lymphocytes through FasL-Fas interactions [48].

Table 1: Phenotypes of $CD8^+$ Treg lymphocyte populations and their role in different pathologies.

Pathology	Agent/condition	Phenotype of $CD8^+$ regulatory T lymphocytes	Exert suppressive action	Model	References
Infection	Mycobacteria	$CD25^+FoxP3^+CD39^+$	Inhibit Th1 lymphocyte proliferation	Human	[93, 94]
	HIV	$CD28^-CD127^{lo}CD39^+$	Inhibit mononuclear cell proliferation	Human	[97]
	Epstein-Barr virus	$FoxP3^+$	Inhibit $CD4^+$ T lymphocyte proliferation and produce IL-10	Human	[98]
Autoimmune disease	EAE	$CD28^-$	Reduce amount of IFN-γ produced by $CD4^+$ T lymphocytes	Mouse	[101]
	EAE	$CD122^+$	Inhibit characteristic IL-7 production of inflammatory process during EAE; inhibit $CD4^+$ T lymphocyte proliferation	Mouse	[102]
	Multiple sclerosis	$CD8^+CD28^-CD39^+CD127^-$	Inhibit proliferation		[103, 104]
	SLE	$FoxP3^+$	Regulate by TGF-β	Human	[70]
	SLE	$CD25^+FoxP3^+$	Suppress production of autoantibodies	Human	[105]
	Primary biliary cirrhosis	$CD28^-CD39^+CD127^-$	Suppress proliferation	Human	[106]
Cancer	Colorectal cancer	$CD25^+FoxP3^+$	Inhibit $CD4^+CD25^-$ T lymphocyte and Th1 cytokine production	Human	[110]
	Prostate cancer	$CD25^+FoxP3^+$	Inhibit naïve T lymphocyte proliferation	Human	[111]
	Inoculation with tumor cell lines	$CD39^+Tim\text{-}3^+PD\text{-}1^+LAG\text{-}3^+$	Exert cytotoxic activity	Mouse	[112]
GVHD	Allogeneic cells	$LAG\text{-}3^+FoxP3^+CTLA\text{-}4^+$	Suppress allogeneic response via CTLA-4	Human	[39]
	Allogeneic cells	$CD25^+CTLA\text{-}4^+FoxP3^+$	Inhibit cell proliferation and release of cytokines as IL-1α, IL-17a, IFN-γ, and TNF-α	Human	[115]
	Allogeneic cells	$CD28^-$	Inhibit $CD4^+$ T lymphocyte proliferation	Human	[10]
	Allogeneic cells	$CD25^+FoxP3^+$	Inhibit allogeneic response	Human	[123]
	Allogeneic cells	$FoxP3^+$	Inhibit $CD4^+$ and $CD8^+$ T lymphocyte proliferation and CD40, CD80, and CD86 expression in CD	Mouse	[125, 126]

GVHD: graft-versus-host disease; HIV: human immunodeficiency virus; EAE: experimental autoimmune encephalomyelitis.

1.4. Participation of $CD8^+$ Treg Lymphocytes in Infection, Autoimmunity, Cancer, and GVHD. Membrane, intracellular, and secretory originating molecules from cells previously mentioned have allowed for the characterization and identification of Treg lymphocytes. Additionally, such molecules confer a suppressant activity upon the activation of other cell populations. In literature, $CD8^+$ Treg lymphocytes have been described as key elements in a number of pathologies, including infectious and autoimmune diseases, cancer, and GVHD (Table 1).

1.5. $CD8^+$ Treg Lymphocytes in Infectious Diseases. In infectious diseases, $CD8^+$ Treg lymphocytes reduce immune response against pathogens, which is beneficial to prevent tissue damage caused by an exacerbated response. In contrast, it can also participate in the evasion of host immune response against the pathogen. As an example, the mycobacteria have coexisted with humans for a long time, as *M. tuberculosis*. These bacteria possess different evasion strategies, like the capacity to induce suppressant activity of the immune response mediated by $CD8^+CD25^+FoxP3^+CD39^+$ Treg lymphocytes. These lymphocytes, found at higher levels during mycobacteriosis, are able to suppress the proliferation of Th1 (proinflammatory type 1 T helper cells) that produces IFN-γ, necessary to activate other cells against mycobacteria. In addition, the measurement of IFN-γ has been used in the diagnosis and monitoring of patients. It has recently been observed that vaccination with bacilli Calmette-Guérin induces an increase in $CD8^+$ Treg lymphocyte population, which has been related to the low protective action of the vaccine against *M. tuberculosis* [93, 94] (Figure 2).

In individuals coinfected with hepatitis C and human immunodeficiency viruses, the TGF-β produced by $CD8^+$ Treg lymphocytes reduces the levels of hepatitis C virus-specific effector T lymphocytes. This effect is reversed by blocking TGF-β and IL-10 produced by Tregs [95]. Additionally, it has been reported that, during HIV infection, the levels of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg lymphocytes are increased with respect to those found in healthy subjects; CD73 is less abundant [96]. The levels are reduced after administering the antiretroviral treatment to the patients. The Tregs observed in HIV patients are antigen-specific and inhibit the proliferation of peripheral-blood lymphocytes. These observations suggest that the suppressant activity of Treg lymphocytes is one of the factors affecting the immune function in HIV patients [97] (Figure 2).

Although the cytomegalovirus can coexist with the human in a subclinical way, it is of great importance in the

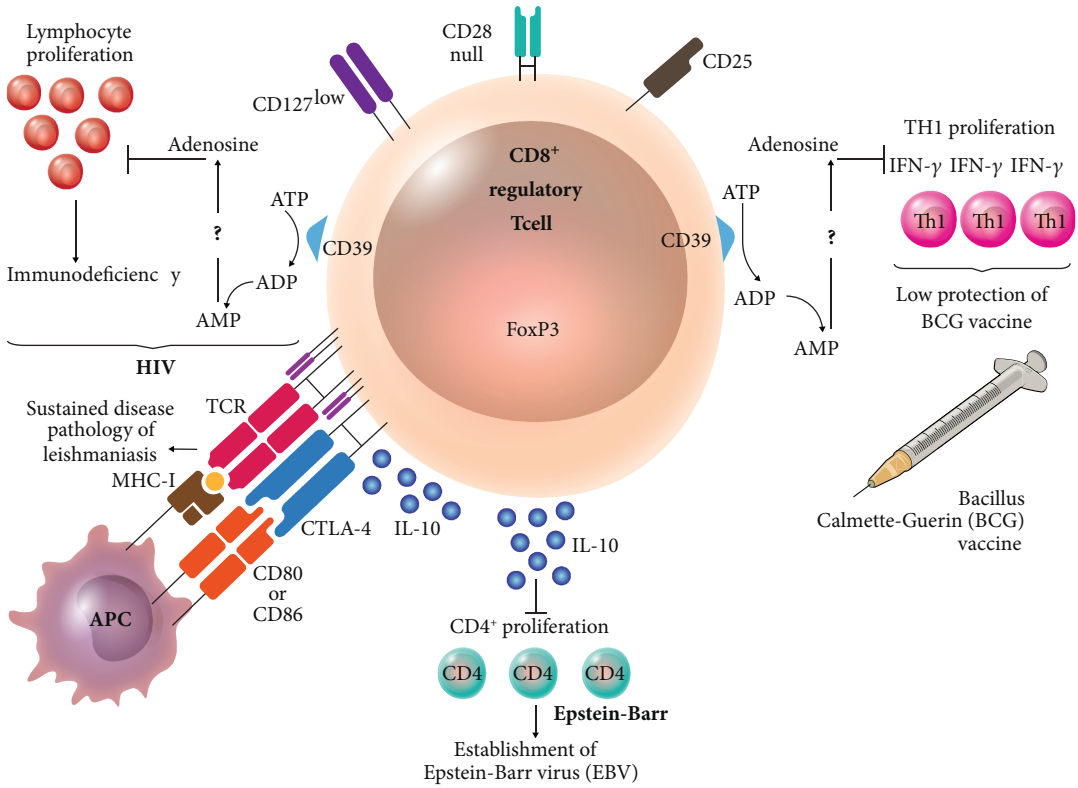

Figure 2: Participation of $CD8^+$ Treg lymphocytes in infectious diseases. In an infection with human immunodeficiency virus (HIV), $CD8^+$ T lymphocyte has a phenotype $CD28^-CD127^{lo}CD39^+$ inhibiting lymphocyte proliferation, which is probably related to the immunodeficiency shown during the disease. In a parasitic infection as leishmaniasis, the persistence of the parasite partly depends on the existence of $CD8^+$ Treg lymphocytes expressing CTLA-4 and producing IL-10, which results in the prevalence of the disease. During immunosuppression situations, there is an increase in the population of IL-10-producing $CD8^+FoxP3^+$ Treg lymphocytes that inhibit $CD4^+$ T cell proliferation, promoting infection by Epstein-Barr virus. The low protection of bacillus Calmette-Guérin vaccine is attributed factors as $CD8^+CD25^+CD39^+$ Treg lymphocytes that inhibit the proliferation of $CD4^+$ T lymphocytes producing Th1 cytokines as IFN-γ, necessary to activate other cell lines against mycobacteria.

production of $CD8^+$ T lymphocyte arrays in adult age. This is because studies in adults have found that cytomegalovirus epitope-specific $CD8^+$ T lymphocytes constitute a high percentage (33%, approximately) of the total $CD8^+$ T lymphocytes, which might compromise the response against other pathogens [75]. A high ratio of these lymphocytes is probably $CD28^-$, given that, as it was mentioned before, the absence of CD28 indicates senescence and repeated stimulation with persistent antigens.

Also, $CD8^+$ Treg lymphocytes are key to the infection process in transplant patients who are under immunosuppressant conditions due to conditioning chemotherapy previous to transplant and subsequent treatment with immunosuppressants to prevent transplant rejection and GVHD. The levels of IL-10-producing $CD8^+$ Treg lymphocytes in transplant patients are higher than those in healthy subjects, which agrees and seems to be associated to the presence of opportunistic pathogens as the Epstein-Barr virus, caused by the inhibition of effector $CD4^+$ T lymphocyte proliferation [98] (Figure 2).

In parasitic infections, $CD8^+$ regulatory T lymphocytes have been found in visceral leishmaniasis patients who express CTLA-4 and produce IL-10 [99] (Figure 2). When dermal sequelae are caused by *Leishmania donovani* infection, the percentage of $CD8^+CD28^-$T lymphocytes is increased and only restored after treatment [100].

1.6. $CD8^+$ Treg Lymphocytes in Autoimmune Diseases. As $CD4^+$ Tregs, $CD8^+$ Treg lymphocyte show reduced levels and function in autoimmune disease patients. In mouse experimental autoimmune encephalomyelitis (EAE) studies, it has been observed that $CD8^+CD28^-$ Treg lymphocytes reduce levels of IFN-γ produced by myelin oligodendrocyte glycoprotein-specific $CD4^+$ T lymphocytes. In consequence, the expression of costimulatory molecules in antigen-presenting cells interacting with $CD4^+$ T lymphocytes is reduced [101]. In this autoimmunity model, there is also a $CD8^+CD122^+$ regulatory T lymphocyte population. This cell population inhibits IL-17, typical of inflammatory process during EAE, and proliferation of $CD4^+$ T lymphocytes [102] (Figure 3).

Multiple sclerosis in humans, comparable to EAE in mice, is a disease in which lymphocytes exhibit immune deregulation that is shown as chronic persistent inflammatory response [103]. In that sense, IFN-β treatment modulates the immune system, reducing autoreactive T cell clones and increasing $CD8^+CD25^+CD28^-$ Treg lymphocytes together with plasmacytoid dendritic cells. Treatment

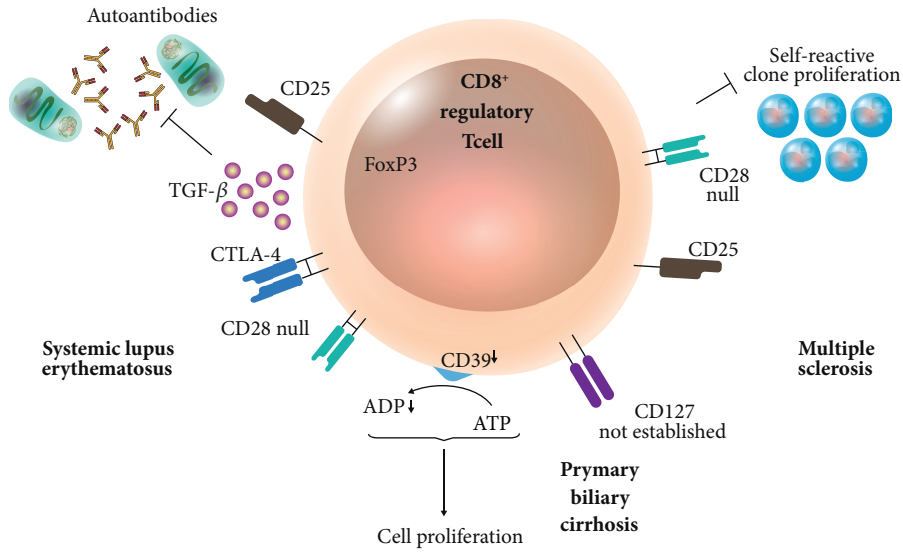

Figure 3: Participation of $CD8^+$ Treg lymphocytes in autoimmune diseases. During SLE, antibody production is eliminated thanks to the suppressive activity of TGF-β-producing $CD8^+CD25^+FoxP3^+$ Treg lymphocytes, which are induced after an autologous transplant of hematopoietic progenitor cells, achieving the remission of the disease. On the other hand, during primary biliary cirrhosis, $CD8^+CD28^-$ T lymphocytes show decreased CD39 expression and fail to inhibit cell proliferation, promoting the severity of this autoimmune disease. In human multiple sclerosis, therapy with IFN-β regulates the immune system by reducing autoreactive T cell clones and increases $CD8^+CD25^+CD28^-$ regulatory T cells. SLE: systemic lupus erythematosus.

with IFN-β is highly promising: its use could reduce the activity of the disease [104].

The autologous transplant of hematopoietic progenitor cells in refractory disease systemic lupus erythematosus (SLE) has proven to be highly effective, achieving the remission of the disease. This fact is directly related to the restoration of the $CD8^+FoxP3^+$ Treg lymphocyte population characterized by CD103, PD-1, PD-L1, and CTLA-4 expression. In this case, the function of $CD8^+$ Treg lymphocytes on target cells depends on cell-cell contact and TGF-β production by regulatory lymphocytes [70]. In addition, $CD8^+CD25^+FoxP3^+$ regulatory T lymphocytes have been found to be able of suppressing autoantibody production [105] (Figure 3).

Primary biliary cirrhosis is another autoimmune disease that affects humans. In this disease, $CD8^+$ Treg lymphocytes express low CD39 and high CD127, a condition that does not change even after culturing the lymphocytes with IL-10. Additionally, the lymphocytes show a deficient suppressant function [106] (Figure 3).

1.7. $CD8^+$ Treg Lymphocytes in Cancer. Immune response has been well documented to be altered in cancer. It has been established that antitumoral immune response is avoided by different types of cancer, including kidney, bladder, and colorectal cancer. Antitumoral evasion has been associated to $CD8^+CD28^-CD127^{lo}CD39^+$ lymphocytes [107] (Figure 4). Such lymphocytes can be produced in tumor tissue thanks to the cytokines produced by tumor cells as GCS-F and IL-10. Furthermore, regulatory lymphocytes can be attracted to the tumor because it releases chemokines as CCL2 and CCL22, highly attractive to regulatory lymphocytes expressing specific CCR2 and CCR4. Also, $CD8^+CD28^-$ Treg lymphocytes directly correlate with tumor diagnosis: the higher the concentration of lymphocytes, the worse the diagnosis and vice versa [108]. $CD8^+CD28^-$ T lymphocytes are found at higher levels in advanced stages of non-small-cell lung cancer, maintaining the increase up to the resection of the tumor when there is a decrease in the concentration and the prognosis for the patient is favorable. However, these lymphocytes have yet to be functionally evaluated to confirm whether they were regulatory [109]. In colorectal cancer patients, studies have successfully isolated $CD8^+$-$CD25^+FoxP3^+$ Treg lymphocytes directly from a tumor. The immunosuppressant phenotype of those lymphocytes is characterized by CTLA-4 expression and TGF-β production. They inhibit $CD4^+CD25^-$ T lymphocyte proliferation ex vivo and suppress Th1 cytokine production in themselves [110]. Therefore, these Treg lymphocytes contribute to immune response evasion against tumor and progression of the disease in consequence. In prostate cancer patients, studies have found tumor-infiltrating regulatory lymphocytes with the same phenotype ($CD8^+CD25^+FoxP3^+$) as the one observed in lymphocytes of colorectal cancer patients. These cells are able to inhibit naïve T lymphocyte proliferation. However, the regulatory activity of these lymphocytes can be reverted by exposing them to TLR-8 ligands as poly-G2. Therefore, the possibility that the manipulation of the TLR-8 signaling pathway can revert immunosuppression mediated by Treg lymphocytes and use it as a therapeutic strategy against cancer is promising [111] (Figure 4). In mice, $CD8^+$ Treg lymphocytes have been found as well in cancer induced by inoculation with tumor cell lines. Furthermore, the population $CD8^+CD39^+Tim\text{-}3^+PD\text{-}1^+LAG\text{-}3^+$ has been found to be tumor-infiltrating, produces low levels of IL-2 and TNF, and has a high cytotoxic potential evaluated

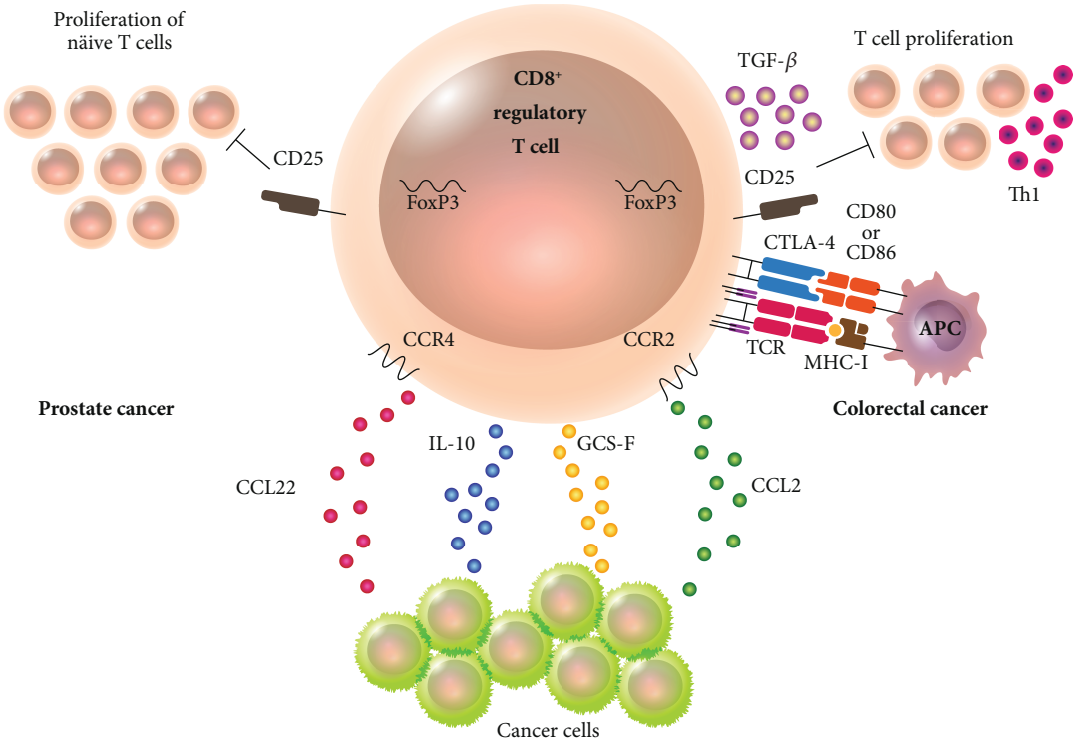

FIGURE 4: Participation of $CD8^+$ Treg lymphocytes in cancer. Regulatory T lymphocytes often aid in the evasion of the immune system by the cancer cell. Specifically, $CD8^+$ Tregs can be induced in tumor tissue due to the presence of cytokines as GCS-F and IL-10. They can also be attracted to the tumor after the latter releases the chemokines CCL2 and CCL22 that attract regulatory lymphocytes expressing CCR2 and CCR4. In colorectal cancer, $CD8^+CD25^+FoxP3^+$ Treg lymphocytes with an immunosuppressive phenotype characterized by expressing CTLA-4 and TGF-β inhibit Th1 lymphocyte proliferation. In prostate cancer, $CD8^+CD25^+FoxP3^+$ Treg lymphocytes have been found to share markers with colorectal cancer and can inhibit naïve T lymphocyte proliferation.

by granzyme B activity and CD107a mobilization. The expression of CD39 in Treg lymphocytes is created by a recognition of the TCR pathway and promoted by IL-6 and IL-27, which are present in the microenvironment surrounding the tumor. The manipulation of the microenvironment, as well as some therapeutic strategy whose target molecule is CD39, might reduce the evasion of the immune system promoted by Treg lymphocytes and improve the immune response against cancer [112].

1.8. $CD8^+$ Treg Lymphocytes in Graft-versus-Host Disease. $CD8^+$ Treg lymphocytes have been described in solid organ transplant and bone marrow transplant as well, which is currently used as hematopoietic stem cell transplantation. In solid organ transplantation, $CD8^+$ Treg lymphocytes reduce the risk of transplant rejection in the host by creating host tolerance towards the received tissue or organ [47, 113]. An inverse situation occurs in hematopoietic stem cell transplantation: $CD8^+$ Treg lymphocytes participate in the tolerance of donor cells towards the host's tissues. In addition to undergoing ablation of their bone marrow, the host is immunosuppressed by the pharmacological treatment received prior to the transplant and is therefore susceptible to attacks by the immune system cells of the donor. In this situation, the available Treg lymphocytes reduce the risk of GVHD, decreasing the intensity of the damage caused by the donor's cells (Figure 5). As a beneficial collateral effect on the host, a graft-versus-tumor can occur mediated by donor cell, lowering the risk of primary disease relapse. The immunosuppressant effect of Treg cells that prevent GVHD apparently does not compromise the effect of graft-versus-tumor [114, 115]. Still, $CD8^+$ Treg lymphocytes are not always found in sufficient quantities, which seems to predispose the patient to GVHD.

Because the inherent immune response to the disease is proinflammatory, the pharmacotherapy given to patients against the illness includes strong immunosuppressants that jeopardize the patient's health since they can lead to infections and/or primary disease relapse. Although the immune response of effector T lymphocytes in the graft versus leukemic cells of the host is needed to prevent relapse, an exacerbated immune response, along with a reduced number of Treg lymphocytes, might cause the death of the host by triggering severe GVHD [116].

This disease causes severe damage in a number of organs, including tissues such as skin, liver, and gastrointestinal tract. It is triggered when immunocompetent donor cells recognize the host cells as foreign and its onset depends on three factors: (1) infused donor cells must be immunocompetent; (2) the host must have antigens absent in the graft; and (3) the host must be unable to generate a response against the graft [117].

Then, why is GVHD generated? It is well known that the main reason of graft rejection in solid organ transplant

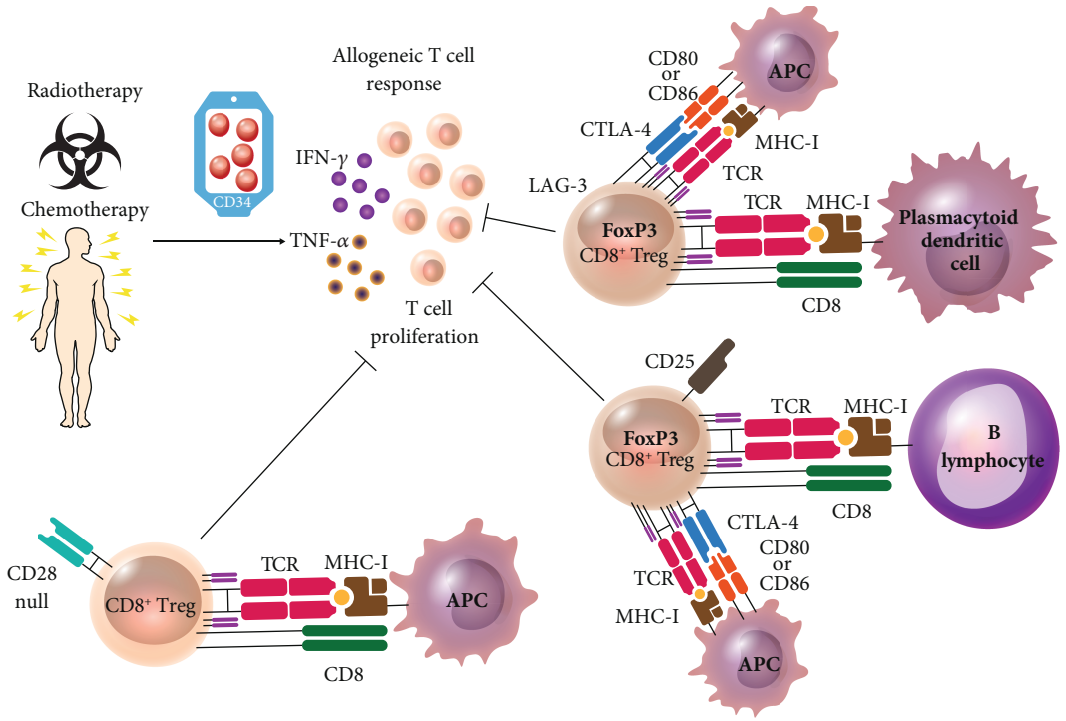

Figure 5: Participation of $CD8^+$ Treg lymphocytes in GVHD. After the remission of an oncohematologic disease, patients are treated with chemotherapy and radiotherapy. Later, they receive a hematopoietic stem cell graft from an HLA-compatible donor, at risk of developing GVHD that is characterized by being proinflammatory and producing IFN-γ and TNF-α. This response can be stopped by $CD8^+$ Treg lymphocytes. When they encounter a plasmacytoid dendritic cell, lymphocytes are activated; they acquire phenotype LAG-3^+FoxP3^+CTLA-4^+ and are able to suppress T lymphocyte allogeneic response via CTLA-4. If they are activated by a B lymphocyte, the $CD8^+$ Tregs will express $CD25^+$CTLA-4^+FoxP3^+, which suppresses cell proliferation and release of proinflammatory cytokines. The $CD8^+CD28^-$, a regulatory T cell subpopulation, play a critical role in in vitro and in posttransplantation allogeneic response. They can be generated by in vitro interaction with allogeneic peripheral blood mononuclear cells. Epitope presented in MHC-I is an alopeptide (allogeneic peptide) in all three Treg phenotypes.

patients (as in kidney transplant) is high incompatibility between donor and host in HLA histocompatibility. Despite HLA compatibility between donor and host for HLA cells expressing high polymorphism is sought in hematopoietic stem cell transplantation, there may be differences in the HLA showing lower polymorphism that they are not studied routinely. Therefore, foreign antigen recognition after transplant by donor cells is latent and can trigger GVHD [118]. In addition to these risk factors, we must also consider non-HLA genes. An example is that some polymorphisms have been identified in regulatory sequences of genes associated to NK cell KIR receptors. Ligands of KIR receptors are class I HLA molecules. In consequence, the absence of the correct ligands for KIR receptors during hematopoietic stem cell transplanting can lead to cytotoxic activity of the donor NK cells. This can be beneficial to the patient because primary disease relapse is avoided; however, the severity of GVHD is increased as well [119]. Simultaneously, other factors have been related to the development of the disease. Some of them are the source of hematopoietic stem cells (the risk of GVHD is higher when peripheral blood mobilized with growth factors to induce the exit of stem cells is transfused than when bone marrow is transfused), the patient's age (higher risk is associated to older ages), and conditioning of the host with chemotherapy and/or radiotherapy and prophylaxis [117]. These risk factors place GVHD as one of the main causes of failure in hematopoietic stem cell allogeneic transplantation. Nearly 60% of the transplant patients at the Centro Médico Nacional "La Raza" of the Instituto Mexicano del Seguro Social in Mexico City suffer GVHD (unpublished data).

Some hypotheses consider $CD8^+$ Treg lymphocytes as responsible for tolerance in the first days after hematopoietic stem cell transplant. This is because, after the transplant, the first T lymphocytes to be present in the peripheral blood are $CD8^+$, followed by $CD4^+$ lymphocytes in a later stage [120]. Furthermore, recent studies show that when higher concentrations of $CD8^+$ T lymphocytes are found in the graft, the possibility of primary disease relapse is reduced without increasing the risk of GVHD. Still, these lymphocytes were not characterized beyond the expression of molecule CD8 on their surface [121].

In GVHD, $CD8^+$ Treg lymphocytes have been identified as antigen-specific that are activated when they encounter foreign antigens; that is, they are alloreactive. Their activation is triggered by the encounter of an antigen-presenting cell, like a dendritic cell or a B lymphocyte. In humans, lymphocytes are activated when they encounter a plasmacytoid dendritic cell and acquire a LAG-3^+FoxP3^+CTLA-4^+ phenotype. These cells are able to suppress the allogeneic response of T lymphocytes via CTLA-4 [39]. If the activating cell is a B

lymphocyte, the phenotype acquired by the $CD8^+$ Treg lymphocyte will be $CD25^+CTLA\text{-}4^+FoxP3^+$. This phenotype suppresses cell proliferation and release of proinflammatory cytokines as IL-1β, IL-2, IL-17a, IFN-γ, and TNF-α by autologous peripheral blood mononuclear cells; CTLA-4 is the molecule with the most involvement in this suppressant function [115] (Figure 5). During the follow-up after a year, a different population of $CD8^+CD28^-$ Treg lymphocytes was observed to be increased and constant *in vivo* in patients that were infused with allogeneic donor cells, using B7-blocking reagents like CTLA-4-Ig that inhibit CD28-B7 together with CTLA-4-B7 interactions as immunosuppressive agent. All the patients survived without showing GVHD [10]. After an allogeneic hematopoietic stem cell transplant, $CD8^+$CD28- T lymphocytes are found in increased percentage in the patient (Figure 5). These lymphocytes are antigen-specific for tumors related to leukemia patients in remission. Additionally, their proliferation and degranulation are stopped and they become senescent with short telomeres [122].

In human *in vitro* experiments in which the allogeneic condition occurring in a transplant was simulated, $CD8^{+-}CD25^-$ T lymphocytes of a donor were incubated together with dendritic cells of a different donor. This culture yielded $CD8^+CD25^+FoxP3^+$ Treg lymphocytes that were able to inhibit the allogeneic immune response without affecting the one against the cytomegalovirus, a risk of infection among patients transplanted with hematopoietic stem cells [123]. Another study found that the $CD8^+$ cells found in higher concentrations in patients without GVHD expressed $FoxP3^+$, unlike GVHD patients. The latter exhibited higher levels of IFN-γ-producing Tc1 and IL-17-producing Tc17 lymphocytes [124].

In mice, $CD8^+FoxP3^+$ lymphocytes are the most relevant population and are sufficient to decrease the severity of GVHD [125, 126]. These mouse lymphocytes express the transcription factor FoxP3 and GITR, CD62L, CD28, and CTLA-4 molecules. They produce lower levels of IL-10 and IL-17 and higher concentrations of IFN-γ. Additionally, they inhibit $CD4^+$ and $CD8^+$ T lymphocyte proliferation and expression of costimulatory CD40, CD80, and CD86 molecules during antigenic presentation by dendritic cells [126].

Although the direction of the immune response during graft rejection is inverse to the one present during GVHD, it is also caused by an exacerbated immune response. According to evidence, this response can be controlled by $CD8^+$ Treg lymphocytes. In that regard, different subpopulations of $CD8^+$ Treg lymphocytes have been described in solid organ transplantation. For instance, the human kidney is not rejected when the percentage of $CD8^+CD28^-$ and $CD4^{+-}CD25^+FoxP3^+$ Treg lymphocytes increases during the first six months after the transplant [127].

On the other hand, $CD8^+CD122^+PD\text{-}1^+$ Treg lymphocytes reduced rejection to skin graft in mice. These lymphocytes exert a regulatory activity independently from FasL-Fas and thus promote effector $CD3^+$ T lymphocyte apoptosis. The inhibition of effector T lymphocyte proliferation depended on IL-10 [47].

A $CD8^+$ Treg lymphocyte subpopulation recently described in rats is specific for at least two allogeneic class II MHC peptides in a heart transplant model [128]. This subpopulation shows a low expression or absence of CD45RC ($CD45RC^{lo/-}$) [63, 128]. These lymphocytes exert a regulatory action through IL-34 that they produce. This cytokine acts generating regulatory macrophages from monocytes, promoting in turn the suppressor activity of $CD8^{+-}CD45RC^{low}$ T lymphocytes. Apparently, $CD8^+CD45RC^{lo/-}$ lymphocytes have a regulatory activity only when they are the result of blocking the interaction CD40–CD40L ($CD8^+$CD40lg) since they produce more IL-34 than naïve $CD8^+CD45RC^{lo/-}$ lymphocytes (spleen), which are positive to FoxP3. Their regulatory activity can be proven by their ability to inhibit effector $CD4^+CD25^-$ T lymphocyte proliferation, which was induced by IL-34 in a dose-dependent manner. When *in vivo*, these lymphocytes extended the acceptance of the allograft while the production of antibodies against the graft was inhibited [63]. This might constitute a therapeutic strategy to reduce the fatality of acute GVHD in humans, as proven by the use of human anti-CD45RC antibodies in humanized mice [129].

Those $CD8^+$ Treg lymphocyte populations that mediate solid organ transplant rejection in GVHD are likely to play a key role in decreasing acuteness of GVHD and promoting the graft-versus-tumor effect.

1.9. Concluding Remarks. Although $CD8^+$ lymphocytes are described to have an immunosuppressive action, $CD4^+$ lymphocytes have been more thoroughly characterized, becoming the model to describe $CD8^+$ Treg lymphocytes. No exclusive markers have been described for any of these regulatory lymphocyte populations. For this reason, more than one criterion has been employed to characterize and identify them. The three requisites that must be met to identify $CD8^+$ Treg lymphocytes are as follows: (1) they must express more than one marker indicating regulation. (2) They must produce anti-inflammatory cytokines as IL-10 and/or TGF-β, and (3) they must inhibit the proliferation of $CD4^+$ and/or $CD8^+$ effector T lymphocytes. Although FoxP3 is a less abundant marker for $CD8^+$ Treg lymphocytes when compared against $CD4^+$ Treg, it is relevant to $CD8^+$ Treg identification.

The markers that have been described are useful to group Treg lymphocytes in different subpopulations according to their characteristics, location, or role in a pathology. In order to be certain of a subpopulation taking part in GVHD regulation, studies should choose the population with the highest number of markers. This would improve the specificity, but populations showing all the markers would be very small. Working with a reduced and insufficient quantity of $CD8^+$ Treg lymphocytes would be inconvenient. If the aim is to find an abundant and regulatory population, it would probably be best to look for subpopulation $CD8^+CD28^-$ and check its regulatory activity, seeking anti-inflammatory cytokine production and proliferation inhibition. A thorough characterization is important given that a $CD8^+CD28^-$ T lymphocyte population might also contain effector lymphocytes [130, 131]. In general, if we were to look for $CD8^+$ Treg

lymphocytes specific of a pathology, we would resort to the information provided, as shown in Table 1.

The benefits of $CD8^+$ Treg lymphocyte participation vary between the pathologies in which the cells play a role. In infectious diseases, it is desirable for the lymphocytes to counter the exacerbated inflammation produces as a response to the microorganism to prevent damage in own tissue. However, an increased participation of regulatory cells might contribute to the pathogen's evasion of the immune response generated by the host and the consequent persistence of the parasite. As shown in Figure 2, $CD8^+$ Treg lymphocytes that express ectoenzyme CD39 produce adenosine, which suppresses immune response against two agents: one viral and one bacterial. On the other hand, $CD8^+$ Treg lymphocytes allow for the establishment of a parasitic and a viral agent, through IL-10.

Although they are present in some autoimmune diseases and show a presumptive regulatory phenotype, lymphocytes express their regulatory molecules at low levels. In consequence, the molecules are not effective to inhibit lymphocytes and innate immune response cells, responsible for triggered autoimmune inflammatory response. However, effector $CD8^+$ Treg lymphocytes inhibiting autoantibody production have been identified in systemic lupus erythematosus (Figure 3).

In cancer progression, $CD8^+$ Treg lymphocytes exhibit higher levels and seem to be a tumor-mediated immunosuppressive strategy. They are attracted to the tumor and their permanence is promoted thanks to the evasion of the immune response that might eradicate cancer cells (Figure 4).

Finally, two events occur after an allogeneic hematopoietic stem cell transplant. The first one is GVHD, which can be exhibited in four stages, according to its severity (being 4 the most severe stage). On the other hand, there is the desired graft-versus-tumor effect, in which a strong participation of $CD8^+$ Treg lymphocytes is not convenient since it would allow for the reestablishment of the primary disease.

Some *in vitro* studies have obtained $CD8^+CD28^-$ Treg lymphocytes by stimulation of the microenvironment of the cells after an allogeneic transplant, inducing alloanergized $CD8^+$ Treg cells. Furthermore, these same markers have been found in increased lymphocyte populations of transplant patients induced to tolerance with belatacept, an immunosuppressant from a fusion molecule bound to CTLA-4. These data define this as one of the ideal cell populations to be studied in allogeneic hematopoietic stem cell transplantation [10]. However, this is not the only $CD8^+$ Treg lymphocyte subpopulation involved in the modulation of the immune response in GVHD. Those $CD8^+$ Treg lymphocytes with CTLA-4-mediated suppressor activity that are induced by B lymphocytes and plasmacytoid dendritic cells are effective against an allogeneic response (Figure 5).

The study of $CD8^+$ Treg cells is not yet complete. A detailed analysis of their identification, regulation mechanisms, and ways of induction, among other events, will allow researchers to know the proportion of $CD8^+$ Treg and $CD4^+$ effector lymphocytes. This will allow for a cell therapy to prevent and cure infectious and autoimmune diseases as well as cancer and GVHD.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Analysis of Sodium Chloride Intake and Treg/Th17 Lymphocytes in Healthy Individuals and Patients with Rheumatoid Arthritis or Systemic Lupus Erythematosus

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We assessed different immune parameters in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) with low (LSI) and high (HSI) sodium intake. Thirty-eight patients with RA, thirty-seven with SLE, and twenty-eight healthy subjects were studied and classified as LSI or HSI. Levels and suppressive function of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD69^+Foxp3^-$ Treg cells were determined by flow cytometry in blood samples. Levels and *in vitro* differentiation of Th17 cells were also assessed. Similar levels of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD69^+Foxp3^-$ Treg cells were observed in LSI and HSI patients or controls. However, a positive correlation was detected between sodium intake and levels of $CD4^+CD25^+Foxp3^+$ Treg cells in SLE and a negative association between $CD4^+CD69^+Foxp3^-$ Treg cells and sodium intake in RA. No other significant associations were detected, including disease activity and sodium intake. Moreover, the suppressor activity of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD69^+Foxp3^-$ Treg cells was similar in LSI and HSI patients or controls. The levels and *in vitro* differentiation of Th17 cells were also similar in LSI and HSI individuals. Our results suggest that, in the population studied (Mexican mestizo), the level of sodium intake is not apparently associated with different relevant immune parameters in healthy subjects or patients with SLE or RA.

1. Introduction

Human autoimmune diseases are complex disorders that arise from the interactions between polygenic risk factors, environmental influences, and defects in immune regulatory mechanisms. Rheumatoid arthritis (RA) is an autoimmune chronic disease characterized, among others, by pain, inflammation, and destruction of diarthrodial joints, resulting in functional disability [1]. Systemic lupus erythematosus (SLE) is an autoimmune condition characterized by the synthesis of many different autoantibodies, deposition of immune complexes, and inflammation of several tissues and organs [2]. In both conditions, aberrations in the immune modulatory mechanisms, including the number and function of T regulatory (Treg) cells have been described [3–5].

Treg cells are able to inhibit the activation and proliferation of effector lymphocytes, and their activity has an important role in the pathogenesis of autoimmune and chronic inflammatory diseases [6]. Several Treg cell subsets have been characterized, including the $CD4^+CD25^{high}$ natural nTreg lymphocytes expressing the forkhead box P3 transcription factor (Foxp3), which exert their inhibitory effect through

different mechanisms, including the synthesis of regulatory cytokines, including the transforming growth factor-β (TGF-β) and interleukins-10 and -35 (IL-10 and IL-35, resp.) [6]. $CD4^+Foxp3^+$ Treg cells may differentiate in the thymus (tTreg cells), in the peripheral lymphoid tissues (pTreg cells), or *in vitro* (induced or iTreg cells) [6]. Different data indicate the relevant role of these $CD4^+Foxp3^+$ Treg cells in the pathogenesis of autoimmune diseases, including the congenital deficiency of Foxp3, which results in the IPEX syndrome (immune dysregulation, autoimmune polyendocrinopathy, and inflammatory enteropathy) [7]. In addition, alterations in the number and function of $CD4^+Foxp3^+$ Treg cells have been reported in most autoimmune diseases as well as in other immune-mediated conditions [4–6, 8, 9]. Accordingly, abnormal number and function of $CD4^+Foxp3^+$ Treg cells have been reported in RA and SLE, and it has been proposed that the autoimmune responses observed in these patients could be controlled by the clinical use of Treg cells to treat these conditions [4–6].

Other regulatory lymphocyte subsets have been described, including Tr1, Tr35, and Th3 lymphocytes [10, 11]. In addition, we and others have detected a subset of T cells that show a constitutive expression of CD69 (with a $CD4^+$-$CD69^+Foxp3^-TGF\text{-}\beta^+IL\text{-}10^+$ phenotype and a variable expression of CD25) and that exerts a relevant *in vitro* suppressive effect on the activation of autologous effector T cells [12, 13]. These $CD69^+$ Treg cells also show an abnormal number and function in patients with different conditions, including autoimmune thyroid diseases, SLE, and liver carcinoma [14–16]. Moreover, it has been described that $CD4^+NKG2D^+$ T cells with regulatory activity and primed to produce IL-10 are detected in normal individuals and that patients with juvenile-onset SLE show increased levels of these lymphocytes [17]. In addition, we have observed that a fraction of this cell subset corresponds to $CD4^+CD69^+$ Treg lymphocytes [13] and that patients with autoimmune thyroid disease or SLE show abnormal numbers and function of these cells [14, 15].

T helper 17 (Th17) lymphocytes are mainly characterized by the synthesis of IL-17A, IL-17F, and IL-22, and their differentiation is induced by a set of cytokines, including IL-1β, IL-6, and IL-23. It has been described that Th17 cells are clearly involved in the pathogenesis of and the inflammatory phenomenon observed in different conditions, including RA, Crohn's disease, and multiple sclerosis (MS) [18, 19]. Accordingly, the cytokines that induce this cell subset as well as the cytokines synthesized by them have become therapeutic targets for the treatment of several inflammatory immune-mediated conditions [20].

It has been shown that high-salt (NaCl) concentrations favor the differentiation of Th17 lymphocytes, both *in vivo* and *in vitro* [21]. Accordingly, a high-salt intake (HSI) is significantly associated with the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [22]. Likewise, it has been reported that those MS patients with HSI show a most severe form of the disease compared to patients with low-salt intake (LSI) [22]. Similar findings have been observed in MRL/lpr mice, an animal model of SLE [23]. In addition, Scrivo et al. observed that a low-sodium dietary regimen is associated with a significant reduction of $CD4^+CD45RA^-Foxp3^{low}$ lymphocytes in patients with RA as well as with a reduction in Th17 cells with an enhancement in $Foxp3^+$ Treg cells in patients with SLE [24]. The mechanisms involved in the induction of Th17 cells by high-NaCl concentrations have been characterized. Thus, it has been reported that high concentrations of sodium induce the activation of the p38 protein kinase and the nuclear factor NFAT5 as well as the synthesis and activation of the serum and glucocorticoid-regulated kinase 1 or SGK1; this kinase in turn induces the expression of the receptor for IL-23 and thus favors the differentiation of Th17 cells and the release of proinflammatory cytokines [25]. Moreover, it has been reported that high-sodium concentrations interfere with the differentiation and function of $CD4^+Foxp3^+$ Treg cells [26] that favors the differentiation and activation of proinflammatory M1-type macrophages [27]. Overall, these data have strongly suggested that high-salt intake is causally associated with the pathogenesis of different chronic inflammatory conditions, through different mechanisms.

In order to further assess the role of NaCl intake in the pathogenesis of inflammatory immune-mediated diseases, we decided to analyze different clinical and laboratory parameters in healthy controls and patients with SLE and RA, classified as LSI and HSI. Our results suggest that the level of sodium intake does not seem to be significantly associated with different relevant immune parameters in either healthy subjects or patients with the immune-mediated diseases analyzed.

2. Materials and Methods

2.1. Aim and Design. The aim of this study was to explore the possible association between the level of salt intake and different immune parameters in healthy individuals and patients with SLE and RA. A cross-sectional observational and experimental study was carried out. This study was done at the Research Center for Health Sciences and Biomedicine, UASLP, and the Regional Unit of Rheumatology and Osteoporosis, Hospital Central Dr. Ignacio Morones Prieto, San Luis Potosí, SLP, México.

2.2. Individuals. Thirty-eight patients with RA (36 females and 2 males), according to the criteria of the American College of Rheumatology, and a mean age of 42.6 ± 10.5 years were included in the study (Table 1). According to the DAS28 index, at the time of the study, twenty patients had low activity ($DAS28 \leq 4$) and eighteen had high activity ($DAS28 > 4$). Twenty-eight patients were receiving prednisone (2.5 to 5.0 mg/day) and/or disease-modifying antirheumatic drugs (methotrexate 7.5 to 20.0 mg/week and/or sulfasalazine 1.0 to 3.0 g/day) at the time of study; thus, seven patients were under methotrexate monotherapy; four were receiving a combination of methotrexate, prednisone, and sulfasalazine; sixteen methotrexate and prednisone; and one patient was under sulfasalazine monotherapy. No patients under therapy with biological agents were included in the study. Ten patients were untreated at the time of study.

Table 1: Main clinical and laboratory characteristics of patients with rheumatoid arthritis.

Number (female/male)	38 (36/2)
Mean age (range)	42.5 ± 10.5 years (19–61)
Disease duration (range)	5.4 ± 4.7 years (0–10.5)
DAS28 > 4 (%)	18/38 (47%)
Therapy*	
Prednisone (dose range)	20/38 (2.5–5.0 mg/day)
Methotrexate (dose range)	27/38 (7.5–20.0 mg/week)
Sulfasalazine (dose range)	5/38 (1.0–3.0 g/day)
Biological agents	0/38
Untreated patients (%)	10/38 (26%)
Anticitrullinated peptide antibodies	37/38
Salt intake	
Low (range)	8/38 (1.7–4.9 g/day)
High (range)	30/38 (5.0–13.5 g/day)

*Most treated patients were receiving two or more drugs.

Table 2: Main clinical and laboratory characteristics of patients with systemic lupus erythematosus.

Number (female/male)	37 (34/3)
Mean age (range)	36.5 ± 15.5 years (18.3–52.5)
Disease duration (range)	8.3 ± 6.7 years (0.4–16.3)
MEX-SLEDAI > 4.0	28/37 (75.6%)
Therapy*	
Prednisone(dose range)	24/37 (2.5–7.5 mg/day)
Methotrexate (dose range)	26/37 (10.0–15.0 mg/week)
Azathioprine (dose range)	9/37 (50.0–100.0 mg/day)
Biological agents	0/37
Untreated patients (%)	9/31 (29%)
Antinuclear antibodies (%)	37/37 (100%)
Low complement levels	25/37 (68%)
Salt intake	
Low (range)	6/37 (1.8–4.6 g/day)
High (range)	31/37 (5.0–15.1)

*Most treated patients were receiving two or more drugs.

Thirty-seven patients with SLE, according to the classification criteria of the American College of Rheumatology, were also studied (Table 2). Thirty-four patients were females and three males, with a mean age of 36.5 ± 15.5 years. According to the MEX-SLEDAI score, 75% of patients had a moderate-to-severe active disease and 25% were in remission. Twenty-eight patients were receiving prednisone (2.5 to 7.5 mg/day) and/or immunosuppressive drugs (methotrexate 10.0 to 15.0 mg/week and/or azathioprine 50–100 mg/day); thus, four patients were under methotrexate monotherapy; fifteen were receiving methotrexate and prednisone; seven methotrexate, prednisone, and azathioprine; and two prednisone and azathioprine. Nine patients were untreated at the time of study, and no patients with evidence of renal failure or receiving biological agents were studied. Twenty-eight healthy subjects (twenty-four females and four males) with a mean age of 36.3 ± 12.1 years were also studied. This study was approved by the Bioethical Committee of the Hospital Central Dr. Ignacio Morones Prieto, and all individuals included in it signed an informed consent.

2.3. Estimation of Sodium Chloride Intake. Salt intake was estimated through two different parameters, the measurement of sodium excretion by urine and a written questionnaire. In the first case, NaCl intake was calculated through 24-hour urinary sodium excretion, by using the following formula: NaCl = Na (g/day) × 100/39.3 [28]. In addition, a written questionnaire of alimentary habits, validated by the World Health Organization (WHO) and the Pan-American Health Organization (PAHO), was employed [29, 30]. The latter one was adapted (by including three additional foods and by considering the salt added to the foods with the salt shaker during the meal) for the population of our study (Mexican mestizos), and the results were compared with those obtained using the urinary sodium excretion. According to these instruments, the individuals that had been included in the study were subsequently classified as having LSI (less than 5.0 g/day of NaCl) or HSI (5.0 or more g/day of NaCl). Those individuals with a discordance greater than 3.0 g/day of salt intake between the values obtained with the questionnaire and sodium excretion were excluded from the study.

2.4. Flow Cytometry Analysis. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare Life Sciences, Issaquah, WA) density-gradient centrifugation, and cellular viability was evaluated by trypan blue stain. When indicated, cells were incubated in RPMI-1640 culture medium (Gibco by Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, glutamine (2.0 mM), and penicillin (100 u/ml)/streptomycin (100 μg/ml) for 4 hours at 37°C with 5% CO_2. Brefeldin (10 μg/ml) was also added. Then, cells were washed and stained with the following monoclonal antibodies (mAbs): CD4/FITC or CD4/APC-Cy7 (BioLegend, San Diego, CA), CD25/APC-Cy7 (BD Biosciences, San Jose, CA), NKG2D/FITC (eBioscience), anti-LAP/PerCp-Cy5.5 (BioLegend), and CD69/APC (eBioscience). Then, cells were fixed and permeabilized with the Foxp3 Fix/Perm kit (eBioscience) and additionally stained with mAbs against IL-10 (PE) (BioLegend) and Foxp3 (PE-Cy7) (eBioscience). Thus, as previously reported [13], when $CD69^+$ Treg cells were analyzed, the following strategy was employed: 1st dot plot: CD4/FITC and CD25/APC-Cy7; 2nd dot plot: LAP/PerCp-Cy5 and CD69/APC; and 3rd dot plot: Foxp3/PE-Cy7 and IL-10/PE. In the case of $CD69^+NKG2D^+$ Treg cells, the analysis strategy was as follows: 1st dot plot: CD4/APC-Cy7 and NKG2D/FITC; 2nd dot plot: LAP/PerCp-Cy5 and CD69/APC; and 3rd dot plot: Foxp3/PE-Cy7 and IL-10/PE. Moreover, other cells were stained with CD4/FITC (eBioscience) and fixed with p-formaldehyde 4% and permeabilized with saponin 0.1% and stained with mAbs against IL-17/PE (eBioscience) and IL-22/APC (eBioscience).

Data were acquired in FACSCanto II flow cytometer (Becton Dickinson) and analyzed using FlowJo version 10 (Tree Star). According to these analyses, $Foxp3^+$ Treg cells corresponded to $CD4^+CD25^{high}Foxp3^+$ lymphocytes, $CD69^+$ Treg cells to $CD4^+CD25^{var}CD69^+LAP^+IL\text{-}10^+Foxp3^-$ lymphocytes, $CD69^+NKG2D^+$ Treg cells to $CD4^+NKG2D^+CD69^{+-}LAP^+IL\text{-}10^+Foxp3^-$, and Th17 cells to $CD4^+IL\text{-}17A^+IL\text{-}22^+$ lymphocytes. In some cases, $CD4^+NKG2D^+$ double-positive cells were also analyzed, which correspond to a fraction of $CD69^+NKG2D^+$ Treg cells and to a subset of $CD4^+$ lymphocytes with no regulatory activity [17].

2.5. Functional Analysis of $CD25^+$ and $CD69^+$ Treg Cells. The suppressive function of $CD69^+$ Treg lymphocytes was assessed by an assay of inhibition of cell activation [31]. In these assays, one aliquot of PBMC was depleted from $CD69^+$ lymphocytes by magnetic negative selection using a MACS cell separation system (Miltenyi Biotec Inc., St Louis, MO). Briefly, PBMC were incubated with an anti-CD69 mAb (eBioscience) for 30 minutes, washed and then incubated with paramagnetic rat anti-mouse IgG MicroBeads (Miltenyi Biotec), and washed again. Afterwards, cells were poured onto MS columns (Miltenyi Biotec), and CD69-negative cells were recovered. Then, these CD69-depleted cells as well as an aliquot of whole PBMC were stimulated by culturing in 24-well plates (Costar) precoated for 1.5 h with an anti-CD3 (OKT3 clone, 5.0 μg/ml) and an anti-CD28 (clone 28.2, 5.0 μg/ml) mAb at 37°C, 5% CO_2. Finally, cells were incubated in RPMI-1640 medium supplemented with 10% FBS, glutamine, and penicillin/streptomycin for 7 hours at 37°C with 5% CO_2. At starting the cell culture, an anti-CD40L/PE mAb (BD Biosciences) was added, and at the end of incubation, cells were washed and analyzed for CD40L expression in a FACSCanto II flow cytometer (Becton Dickinson) and data processed with the FlowJo software (Tree Star). In the case of assays for $CD4^+CD25^{high}Foxp3^+$ cell activity, we employed the same procedure but using an anti-CD25 mAb (eBioscience) instead of an anti-CD69. In both cases, the suppressor activity was calculated by comparing the percent of $CD40L^+$ cells in the two aliquots of cells, as follows: %inhibition = 100 − [(%$CD40L^+$ cells in whole PBMC)/(%$CD40L^+$ cell cultures depleted from $CD69^+$ cells) 100].

2.6. In Vitro Differentiation of Th17 Cells. PBMC from patients or healthy controls were activated with plate-bound anti-CD3/CD28 (5.0 μg/ml) and grown in IMDM culture medium supplemented with 10% fetal bovine serum, glutamine (2.0 mM), and penicillin (100 u/ml)/streptomycin (100 μg/ml) and 5% CO_2 at 37°C. These cells were induced to differentiate or not towards the Th17 lineage by adding 8 ng/ml TGF-β, 10 ng/ml IL-1β, 50 ng/ml IL-6, 10 ng/ml IL-23, and 10 μg/ml anti-IL-4 mAb for 6 days. Three hours before its harvesting, cells were restimulated with a leukocyte activation cocktail with GolgiPlug (BD Pharmingen). Finally, cells were collected, washed, and analyzed for the presence of $CD4^+IL\text{-}17A^+$ lymphocytes, as stated above.

2.7. Quantification of Cytokines. Cell cultures similar to those employed to test the suppressive function of Treg cells on T cell activation were run but in the absence of an anti-CD40L mAb and incubated for 24 h. At the end of the incubation, cell-free supernatants were obtained and the levels of the indicated cytokines were determined by flow cytometry using a Cytometric Bead Array (BD Biosciences). The following cytokines were analyzed: IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A. Data analysis was performed by using the FCAP Array Software v3.0 (BD Biosciences).

2.8. Statistical Analysis. Data with normal distribution were presented as the arithmetic mean and SD, and data with a non-Gaussian distribution were presented as the median and interquartile range (Q_1–Q_3). Analysis of 2 groups was performed with the Mann–Whitney U test and comparisons among 3 groups with the Kruskal-Wallis sum-rank test. Data were analyzed using the Graph Pad Prism 5 software, and p values < 0.05 were considered as significant.

3. Results

As stated in the Materials and Methods, the salt intake was estimated in the individuals included in this study and they were classified as LSI (less than 5.0 g/day of NaCl) and HSI (5.0 g/day of NaCl or more). As shown in Figure 1, sodium excretion (mEq/L) and sodium intake (g/day) were similar in the three groups studied. In addition, when the proportions of LSI and HSI individuals in each group were compared, no significant differences were observed. However, the percent of individuals with LSI tended to be higher in the healthy control group ($p = 0.060$ compared to SLE, Fisher exact test, Figure 1(c)).

As it has been previously reported, when we analyzed the levels of $CD4^+CD25^{high}Foxp3+$ Treg cells, significant lower levels of these lymphocytes were observed in patients with SLE or RA compared to healthy controls, both in the case of LSI or HSI individuals ($p < 0.05$ in all cases, Figure 2(a)). However, we did not detect significant differences when LSI and HSI individuals were compared, in either healthy subjects or patients with SLE or RA ($p > 0.05$ in all cases, Figure 2(a)). In the case of $CD69^+$ Treg cells, significant enhanced levels of these lymphocytes ($CD4^+CD25^{var}CD69^{+-}LAP^+IL\text{-}10^+Foxp3^-$) were observed in SLE and RA patients compared to controls, both in the case of LSI or HSI individuals ($p < 0.05$ in all cases, Figure 2(b)). Nevertheless, as in the case of $Foxp3^+$ Treg cells, similar levels of $CD69^+$ Treg lymphocytes were observed when LSI and HSI were compared, in the three groups studied ($p > 0.05$ in all cases, Figure 2(b)). Likewise, no significant differences were detected between LSI and HSI individuals in the case of the $CD69^+NKG2D^+$ Treg cell subset levels, in all groups studied ($p > 0.05$ in all cases, Figure 2(c)). Moreover, although increased levels of peripheral blood Th17 cells were observed in patients with SLE or RA compared to healthy controls ($p < 0.05$ in all cases, Figure 2(d)), similar levels of this helper cell subset were observed when LSI and HSI individuals were compared, in the three groups studied ($p > 0.05$ in all cases, Figure 2(d)).

When an association analysis was performed, a significant negative correlation between NaCl intake and the

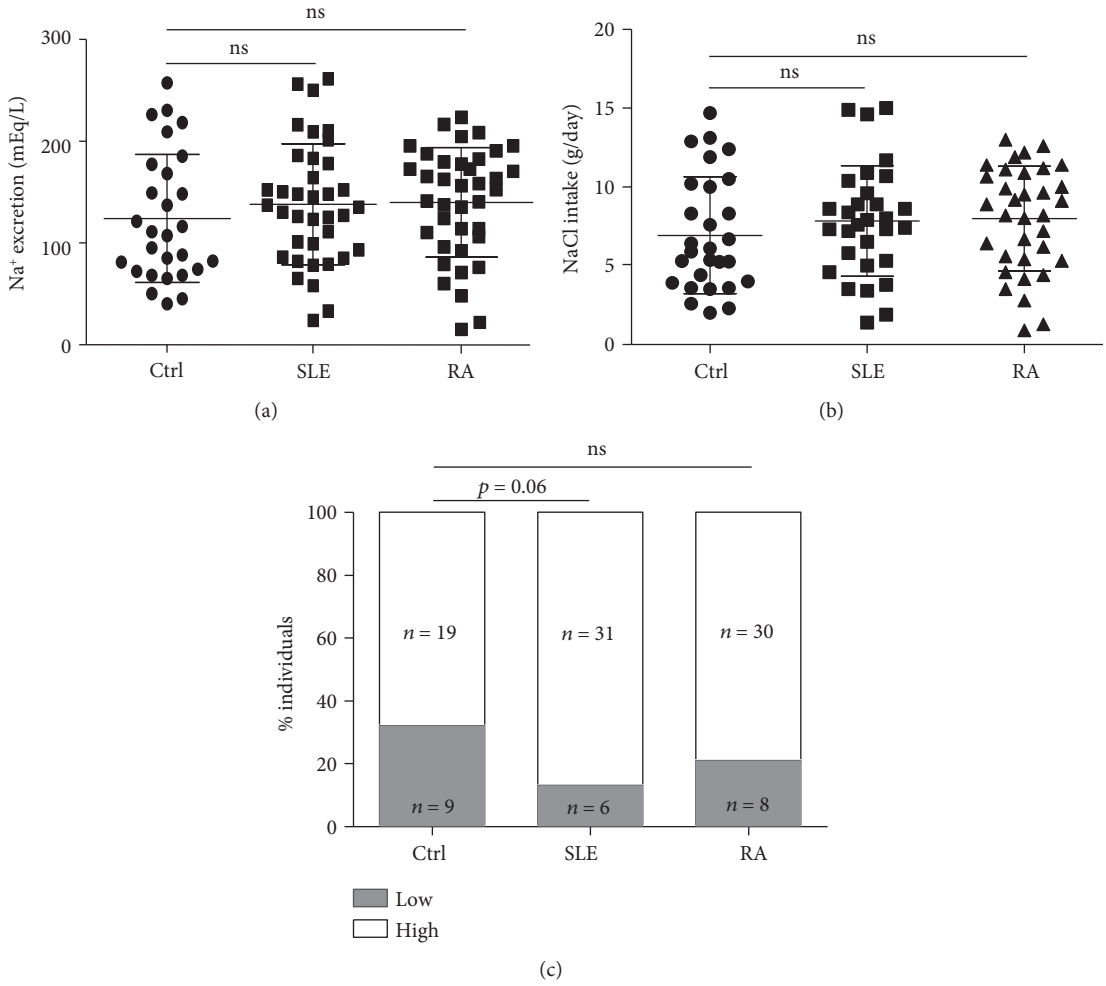

Figure 1: Estimation of salt intake in controls and patients. (a, b) Sodium excretion and salt intake was estimated in healthy controls (Ctrl) and patients with SLE or RA, as stated in the Materials and Methods. No significant differences were observed among the three groups. Horizontal lines correspond to the arithmetic mean and SD. (c) Proportions of individuals with low (less than 5.0 g/day of NaCl) and high (5.0 g/day of NaCl or more) salt intake in healthy controls (Ctrl) and patients with SLE and RA. ns: nonsignificant.

percent of $CD69^+$ Treg cells was observed in patients with RA ($r = -0.37$, $p = 0.046$, Figure 3(a)). Furthermore, a significant negative association between NaCl intake and the levels of $CD4^+NKG2D^+$ cells was observed in patients with SLE ($r = -0.55$, $p = 0.02$, Figure 3(b)). A similar result was observed in the case of $CD69^+NKG2D^+$ Treg lymphocytes ($r = -0.56$, $p = 0.016$, Figure 3(c)). Conversely, the levels of $Foxp3^+$ Treg cells tended to be positively associated with NaCl intake in patients with SLE; however, a no significant value of p was obtained in this case ($r = 0.04$, $p = 0.08$, Figure 3(d)). Likewise, no apparent association was detected between the levels of Th17 cells and NaCl intake in the three groups studied ($p > 0.05$ in all cases, data not shown). Furthermore, LSI and HSI patients with SLE or RA showed similar levels of disease activity, according to the SLEDAI and DAS28 indices, respectively ($p > 0.05$ in both cases, Figures 3(e) and 3(f)). Moreover, an additional analysis showed no apparent association between the therapies that the patients were receiving and the different parameters measured in the study (data not shown). Accordingly, when these immune parameters were compared in treated and untreated patients (with SLE or RA), no significant differences were observed (data not shown).

We then analyzed the function of two subsets of Treg lymphocytes. As shown in Figure 4(a), the function of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD69^+$ Treg cells was assessed through assays of inhibition of lymphocyte activation (expression of CD40L) and suppression of cytokine release. In the former assay, we observed that patients with SLE showed a significant diminution in the suppressor activity of $CD4^+CD25^+Foxp3^+$ cells compared to healthy controls ($p < 0.05$, Figure 4(b)). A similar trend was observed in the case of RA, but in this case, a significant difference was not reached (Figure 4(b)). Moreover, when the activity of these Treg cells was compared in LSI and HSI individuals, we did not detect significant differences in any group studied ($p > 0.05$ in all cases, Figure 4(b)). As shown in Figure 4(c), similar results were observed for the activity of $CD4^+CD69^+$ Treg cells, with no significant differences between LSI and HSI individuals, in either healthy controls or patients with RA or SLE ($p > 0.05$). Accordingly, when we analyzed the suppression of cytokine release by Treg cells,

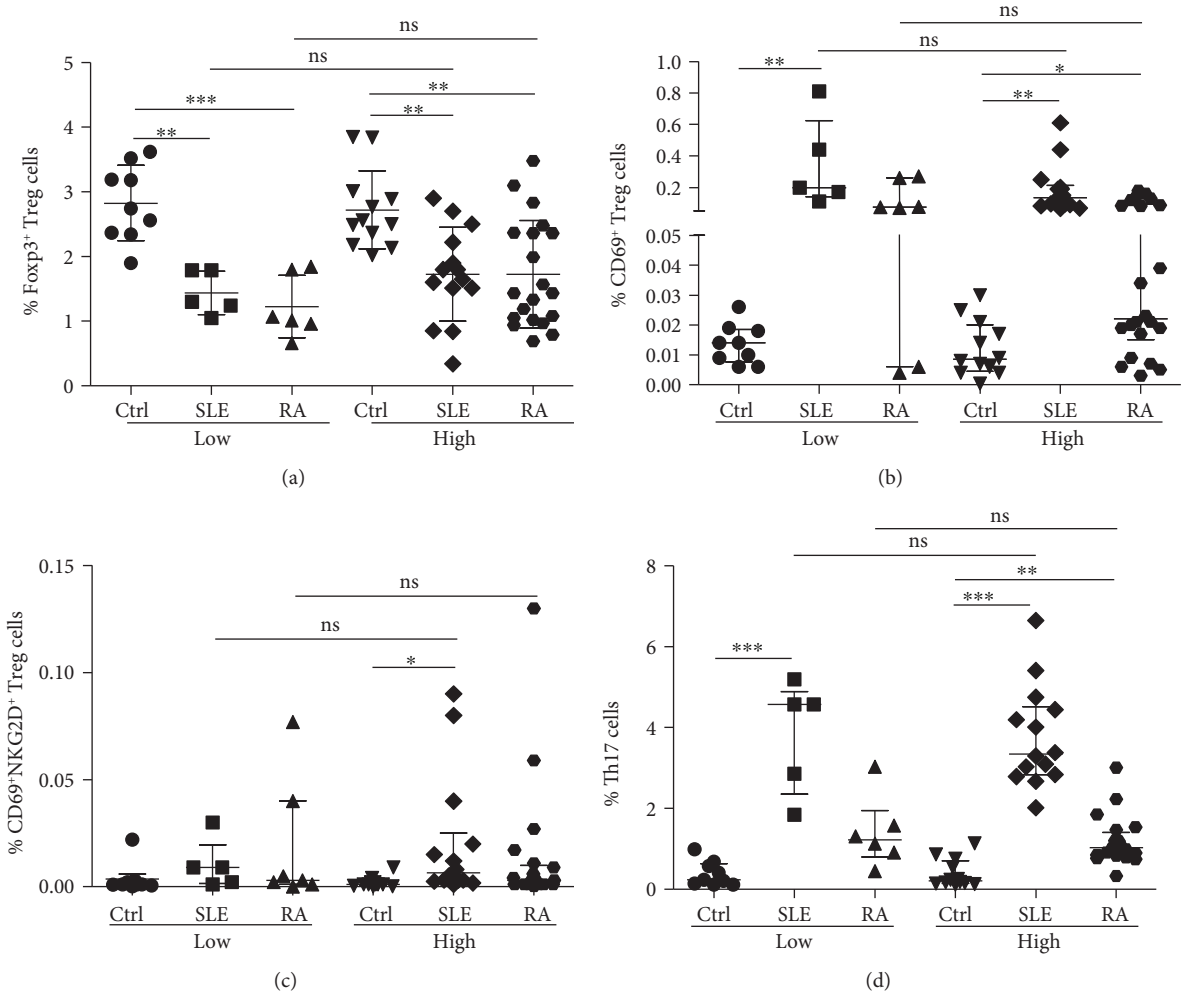

Figure 2: Levels of Treg and Th17 cells in the peripheral blood from healthy subjects and patients with SLE or RA with low- and high-salt intake. The frequency of $Foxp3^+$ and $CD69^+$ Treg cells and Th17 lymphocytes was determined by multiparametric flow cytometry analysis in blood samples from healthy controls and patients with SLE or RA, with low- or high-salt intake. (a) Percent of $CD4^+CD25^{high}Foxp3^+$ cells. (b) Frequency of $CD4^+CD25^{var}CD69^+LAP^+IL\text{-}10^+Foxp3^-$ Treg cells. (c) Percent of $CD4^+NKG2D^+CD69^+LAP^+IL\text{-}10^+Foxp3^-$ Treg cells. (d) Frequency of $CD4^+IL\text{-}17^+$ lymphocytes. All percentages are referred to total lymphocytes. Data correspond to the arithmetic mean and SD (a) or the median and IQR (b–d). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns: nonsignificant.

we observed similar levels of inhibition in LSI and HSI individuals, in the three groups studied and for all cytokines analyzed (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A) (Figures 4(d)–4(f) and data not shown).

We also analyzed the *in vitro* differentiation of CD4 T naïve cells into Th17 lymphocytes in healthy individuals and patients with RA or SLE with low- and high-sodium intake. Although in these assays we only observed a modest (but significant) differentiation of Th17 cells, we observed that similar levels of these cells were generated in LSI and HSI individuals, in the three groups studied ($p > 0.05$ in all cases, Figure 5 and data not shown).

Finally, since we only performed a single determination of NaCl excretion to all individuals included in the study, we decided to further analyze our results after the exclusion of those patients and controls with sodium intake levels near to the cutoff value (5.0 g/day) employed by us. Under such conditions, five patients with RA, four with SLE, and five controls, with daily sodium intake between 4.0 and 6.0 g, were excluded. These analyses showed similar results, with no significant differences between these LSI (<4.0 g/day) and HSI (>6.0 g/day) groups, regarding the levels of Foxp3+ and CD69+ Treg cells, their suppressive activity, and the number and *in vitro* differentiation of Th17 lymphocytes (data not shown). In addition, we also analyzed the results of this study after classifying healthy controls and patients by tertiles of sodium intake. In this case, similar results were obtained, with no significant differences among the three categories of salt intake (low, high, and very high) in the three groups studied (data not shown).

4. Discussion

Different factors determine the loss of immune tolerance to self-antigens and the appearance of chronic inflammatory autoimmune disease, mainly genetic and environmental

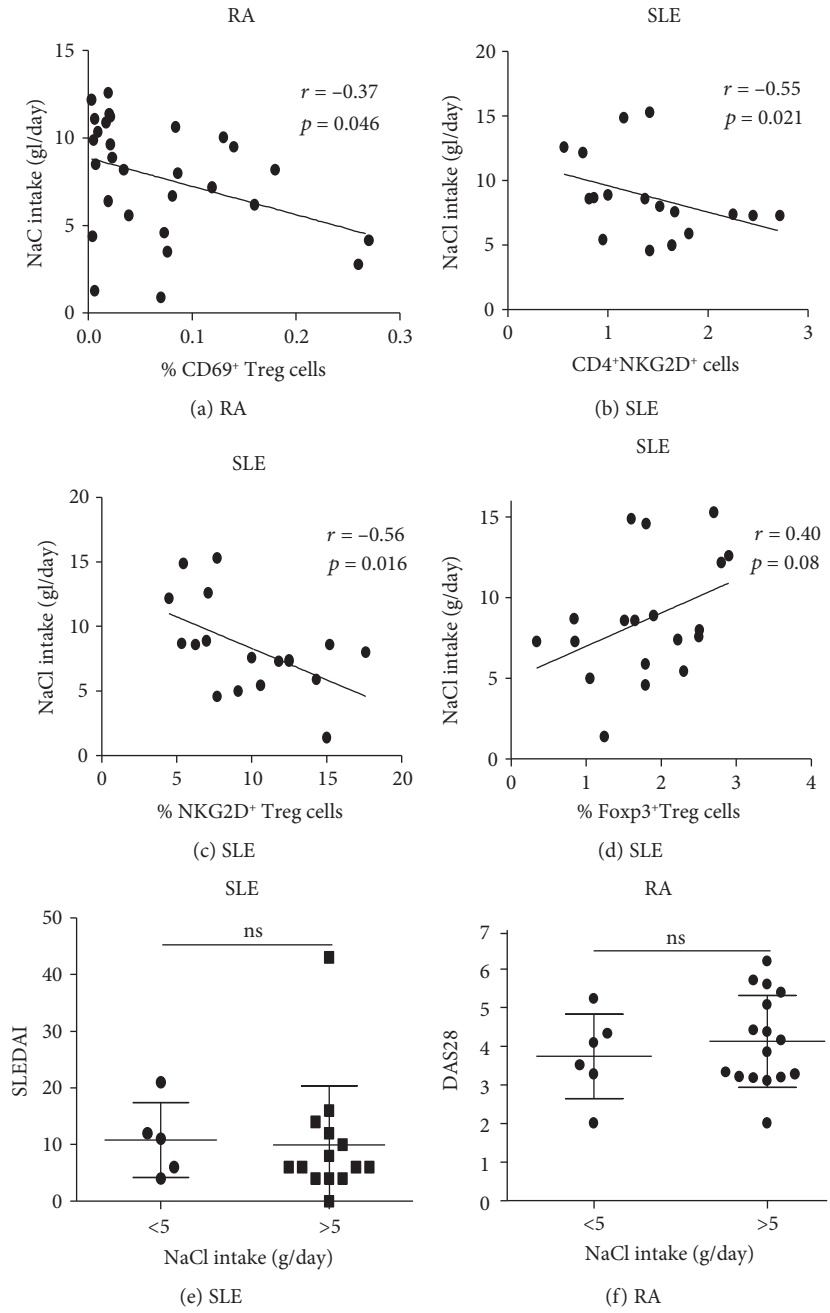

Figure 3: Correlation analysis between salt intake and clinical and laboratory parameters in patients with SLE and RA. Patients with RA showed a significant negative correlation between salt intake and % of $CD69^+$ Treg cells ($CD4^+CD25^{var}CD69^+LAP^+IL\text{-}10^+Foxp3^-$) (a), whereas in patients with SLE, a significant negative association of salt intake and the levels of $CD4^+NKG2D^+$ (b) or $NKG2D^+$ Treg cells ($CD4^+NKG2D^+CD69^+LAP^+IL\text{-}10^+Foxp3^-$) (c) was observed. Although in SLE patients salt intake tended to be associated with the % of $Foxp3^+$ Treg cells, the value of r was nonsignificant (d). Both patients with SLE (e) and RA (f) showed similar levels of disease activity in those cases with low- and high-salt intake. ns: nonsignificant. (e, f) Data correspond to the arithmetic mean and SD.

factors as well as abnormalities in the immune regulatory mechanisms and, very likely, the gut microbiota [1–6]. In recent years, different very interesting reports have indicated the important influence of sodium concentration and salt intake on the innate and adaptive immune responses. Thus, it has been described that high-sodium concentrations (that can be detected in the interstitial space of different tissues and organs, including the lymph nodes, skin, and renal medulla) are able to induce the differentiation of Th17 cells [21], which are involved in the pathogenesis of several inflammatory immune-mediated diseases, including RA and SLE [32]. In addition, it has been shown that high-NaCl concentrations favor the differentiation of proinflammatory type M1 macrophages, which also have a key role in the pathogenesis of the tissue damage associated to autoimmunity [27]. Furthermore, it has been observed that sodium

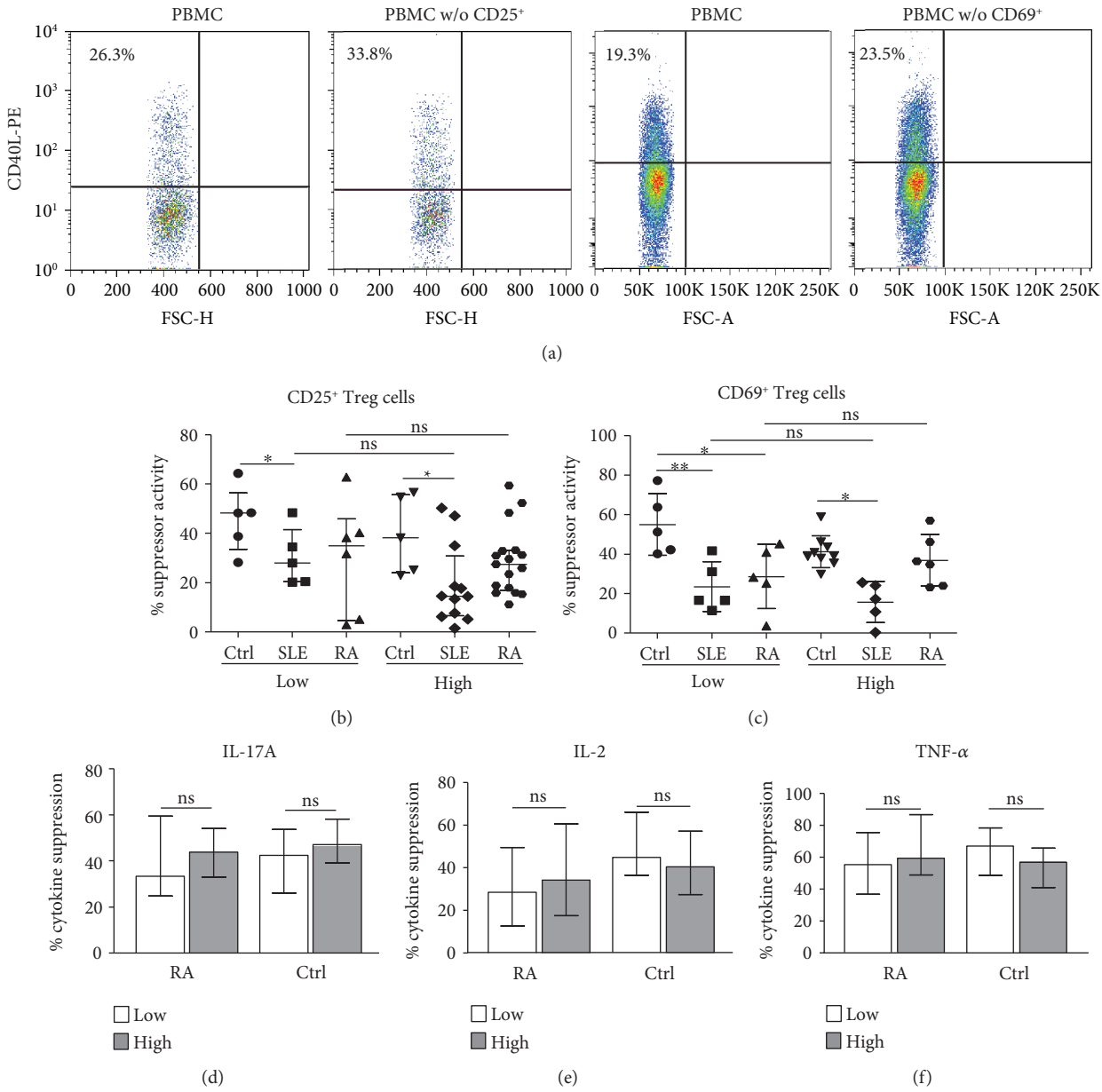

Figure 4: Functional analysis of Treg lymphocytes in healthy controls and patients with SLE or RA, with low- and high-salt intake. PBMC were depleted or not of $CD69^+$ or $CD25^+$ cells, stimulated through CD3/CD28 for 7 h, and then, the expression of CD40L was assessed by flow cytometry, as indicated in the Materials and Methods. (a) Flow cytometry dot plots showing the expression of CD40L in unfractionated and Treg cell-depleted PBMC stimulated through CD3/CD28. These data correspond to cells from a HSI healthy control. (b) Suppressor activity (calculated as stated in the Materials and Methods) of $CD25^+$ Treg cells in healthy controls (Ctrl) and patients with SLE or RA, with low- or high-sodium intake. (c) Suppressor activity of $CD69^+$ Treg cells in healthy controls (Ctrl) and patients with SLE or RA, with low- or high-sodium intake. (d–f) In separate experiments, PBMC were depleted or not of $CD69^+$ or $CD25^+$ cells and stimulated through CD3/CD28 for 24 h, and then, the levels of the indicated cytokines were analyzed in cell supernatants as stated in the Materials and Methods. Data from patients with RA and healthy controls, with low- and high-sodium intake, are shown. $^*p < 0.05$; $^{**}p < 0.01$; ns: nonsignificant. Data correspond to the median and IQR.

chloride inhibits the suppressive activity of $Foxp3^+$ Treg lymphocytes [26]. Accordingly, it has been proposed that high-salt intake may have a relevant role in the induction and maintenance of autoimmune conditions, including RA, SLE, and MS [25, 33, 34].

In apparent contrast with all the above information, we have observed that healthy individuals as well as patients with SLE or RA, with low- and high-salt intake, show similar levels of $Foxp3^+$ and $CD69^+$ Treg cells in the peripheral blood. In addition, no significant differences were detected in the suppressive activity of these Treg cell subsets in those individuals with low and high dietary sodium. Similar results were obtained regarding the proportion of Th17 cells in the peripheral blood of the three groups studied as well as in

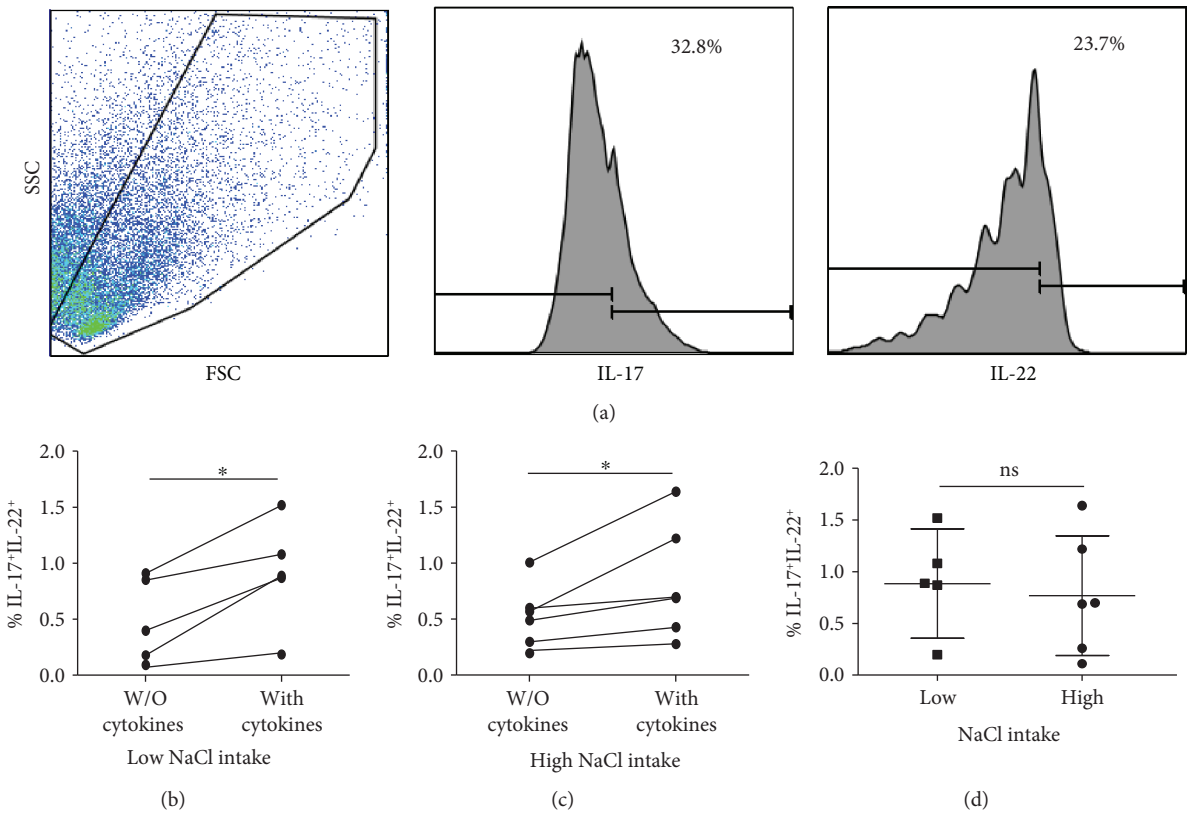

Figure 5: *In vitro* differentiation of Th17 cells in healthy controls and patients with RA, with low- and high-salt intake. PBMC were cultured for seven days in the presence or not of a cytokine cocktail to induce the differentiation of Th17 cells, as described in the Materials and Methods. (a) Flow cytometry analysis of $CD4^+IL\text{-}17A^+IL\text{-}22^+$ cells. A representative dot plot (forward versus side scatter) and histograms of IL-17 and IL-22 expression are shown. Data correspond to a patient with RA with high-salt intake. (b, c) Percent of Th17 cells in PBMC cultures with the addition or not of a cytokine cocktail that induces the differentiation of these cells. Data correspond to patients with RA with low- and high-salt intake, as indicated. (d) Comparison of the percent of Th17 cells induced *in vitro* in PBMC from RA patients with low- and high-salt intake. Data correspond to the arithmetic mean and SD. $^*p < 0.05$.

the assays of the *in vitro* differentiation of these lymphocytes. Finally, no apparent association was observed between disease activity and the level of sodium intake either in SLE or in RA patients.

Although we do not have a conclusive explanation for the apparent contradiction between our results and the previous reports regarding the effects of high-sodium concentration on Treg cell function and Th17 lymphocyte differentiation, it is worth mentioning that the laboratory parameters studied by us had not been analyzed in previous studies, comparing LSI and HSI subgroups of individuals. In this regard, the interesting work of Hernandez et al., which demonstrates the inhibitory effect of high-sodium concentrations on the activity of human Foxp3+ Treg cells [26], was performed in a xenogenic graft-versus-host disease model. Moreover, in the study of Yi et al., showing that low-salt diet is associated with a diminished production of proinflammatory cytokines [34], no *in vitro* experiments of cytokine release were performed. Likewise, in the report of Wen et al., the induction of IL-17 by HSI in healthy subjects (and its reversion by potassium supplementation) was assessed by measuring the mRNA levels of this cytokine in PBMC and the protein in plasma, with no *in vitro* assays of cytokine synthesis [35]. Therefore, it is possible that the apparent contradiction between our results and previous reports on the effect of HSI on Treg and Th17 cells in humans might be due to methodological differences. In this regard, although our results may not be considered conclusive, we think that this study further supports that the possible role of HSI on the pathogenesis of both SLE and RA could be rather complex. In this regard, it is of interest the study of Kuek et al., which detected an apparent lack of association between the level of sodium intake and the development of RA in nonsmoker individuals [32]. Accordingly, it has been reported that high-sodium consumption is significantly associated with the presence of anticitrullinated peptide antibodies only among smoker patients with RA [36]. Likewise, although in the MRL/lpr mice model of SLE HSI increases the severity of nephritis [23], this effect is not observed in the female NZBWF1 mice model of SLE [37]. In addition, in the recent study of Scrivo et al., a nonsignificant effect of a low-sodium dietary regimen on Th17 and $Foxp3^+$ Treg cell levels was observed in patients with RA (however, significant changes in the proportions of these cell subsets were detected in patients with SLE) [24]. Accordingly, different authors have commented that it could be premature to state, at this time, that dietary salt influences autoimmune disease in humans [25, 38, 39]. In this regard, it is worth mentioning that there are also controversial results

in the case of the possible association between sodium intake (or urinary excretion) and cardiovascular events in normotensive individuals [40, 41]. However, in the case of multiple sclerosis, there are additional data that support the association between HSI and disease severity [27, 42].

Different factors may influence the results obtained in this pilot study. The first one is the relatively small proportion of individuals, in the three groups studied, with LSI. This was due to the study design, which stated that patients and controls should be consecutively recruited, and then the salt intake should be determined. Other factors may correspond to the genetic background of the population studied (Mexican mestizo), the diet, the degree of sun exposure and vitamin D levels, and so on. In any case, we consider that the data of this cross-sectional pilot study strongly suggest that, in order to further define the precise role of salt intake in the pathogenesis of SLE and RA, it would be very convenient to perform a cohort study with a great number of patients with a serial analysis of different clinical and laboratory parameters.

In conclusion, data of this cross-sectional pilot study suggest that the degree of salt intake does not seem to be significantly associated with the levels and function of at least two Treg cell subsets and the proportion of Th17 lymphocytes in the peripheral blood from healthy subjects or patients with SLE or RA. Since these data do not support the putative role of sodium intake on the pathogenesis of immune-mediated inflammatory diseases, we consider that it would be very convenient to confirm these results through a longitudinal study with a great number of individuals.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Clinical Study

Impact of Bone Marrow Aspirate Tregs on the Response Rate of Younger Newly Diagnosed Acute Myeloid Leukemia Patients

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Acute myeloid leukemia (AML) is widely considered a distinct clinical entity with a well-defined molecular and genetics-based prognosis. Particularly in a younger patient, the therapeutic approach depends largely on diagnostic risk stratification, which has an impact on the outcome after therapy. We added Treg evaluation to the usual molecular and cytogenetics profile in the AML younger patients' diagnostic bone marrow aspirate (dBMA) in order to search for any correlation between Tregs and overall response (OR) as well as survival (OS) rates. We studied 23 AML young patients, all treated with standard induction chemotherapy: OR (complete remission (CR) + CR incomplete (CRi)) was documented in 10 of 23 patients (44%); there were two partial responder patients. The optimal dBMA Treg cut-off value for predicting response to treatment (≥21/μL) was obtained by ROC curve analysis. However, in multivariate analysis, apart from the expected impact of the molecular/cytogenetic risk ($p = 0.049$) and NPM mutation ($p = 0.001$), dBMA Tregs ≥ 21/μL was not correlated with OR. Actually, higher dBMA Tregs were associated with the good intermediate molecular/cytogenetic risk group ($p = 0.02$), whose median OS was confirmed to be better as compared with that of the poor risk group (18 versus 5 months, $p = 0.05$) and equal to the dBMA Tregs ≥ 21/μL group (5 versus 5 months, $p = 0.902$), respectively. The possible prognostic value of such an immunological player as BMA Tregs in the diagnostic and successive phases of AML needs to be confirmed in larger patient numbers.

1. Introduction

Considerable progress has been made in understanding the pathogenesis of acute myeloid leukemia (AML) [1] and in the development of diagnostic assays [2], including European Leukemia Net (ELN) 2017 updated therapies [3]. But although AML subgroups have distinct prognoses and different therapeutic needs [2], the therapeutic approach remains based on induction chemotherapy followed by allogeneic stem cell transplantation (allo-HSCT) in the case of poor prognosis AML subgroups [4]. In this scenario, the contribution of an immunological player such as T regulatory cells (Tregs), evaluated in diagnostic bone marrow aspirate (dBMA), might suggest novel insights that could be useful in terms of prognosis and outcome.

In fact, while in solid tumors, the role of Tregs seems to be associated with tumor escape from immunosurveillance and, consequently, a worse outcome [5, 6], in AML; its action is still not fully understood [7]. Actually, in selected lymphomas, higher Tregs seem to be associated with a better outcome [8], while they have shown conflicting results in terms of a worse [9, 10] or better prognosis [11] for AML patients. In fact, in allo-HSCT, the ability of Tregs to suppress the function of other T cells and accordingly, to limit the immune response, to regulate immune homeostasis, and to maintain self-tolerance, has recently been reviewed [12] and correlated with beneficial effects on the outcome of AML patients after allo-HSCT [13]. Here, we have applied our previous experience regarding the effects of Treg graft contents on immunological recovery [14] to dBMA results obtained in AML patients. In particular, the aim of our preliminary study was to investigate the role of AML diagnostic phase-Tregs in terms of a possible prognostic impact on the overall response (OR) and outcome.

2. Materials and Methods

2.1. Patients. We prospectively analyzed newly diagnosed AML patients (<65 years) treated at our institution between March 2016 and March 2018. Patients gave written informed consent to the collection of personal data in accordance with the Declaration of Helsinki and Italian laws. The study included 23 AML patients (13 males and 10 females, median age 55 years, range 20–65). Fluorescence in situ hybridization for molecular rearrangements was performed on bone marrow samples, as previously reported [15]. According to cytogenetic-molecular risk stratification [16], 3 (13%) patients were assigned to the favorable, 12 the intermediate (52%), and 8 (35%) the adverse prognosis group. Molecular evaluation (i.e., NPM, FLT3, and CEBPA) was performed in all cases: NPM1 (A or B mutation) and FLT3 mutations (ITD or D835) were positive in 6 (26%) and 5 (22%) patients, respectively. There were no CEBPA-positive cases. Median values of white blood cells (WBC) were 18430/μL and of dBMA Tregs 21/μL. The Treg study population, together with the B and NK cell distribution in BMA, is summarized in Table 1. All patients underwent induction chemotherapy (i.e., "3 + 7") with cytarabine 100 mg/mq, intravenously, on days 1 to 7 and an anthracycline [daunorubicin 60 mg/mq on days 1 to 3 or mitoxantrone 10 mg/mq on days 1 to 3] and thereafter intermediate dose cytarabine for consolidation (up to 2 cycles) [3] or at higher doses (FLAG-Ida for all patients) as a bridge to allotransplantation [17, 18] for nonresponder patients. AML response was evaluated according to the ELN 2017 [3].

2.2. Flow Cytometry. To determine the percentage and the absolute count of CD3 and CD4 T cell subsets, 50 μL of whole marrow blood was stained with CD45 PerCP-Cy™5.5, CD3 FITC, CD4 PE-Cy7™, CD8 APC-Cy7, CD16 and CD56 PE, and CD19 APC monoclonal antibodies (MoAbs) (BD Multitest 6-color TBNK) in a calibrated number of fluorescent beads (Trucount, BD Pharmingen). For Treg identification, 100 μL of marrow blood was incubated with a lyophilised pellet of CD45RA FITC, CD25 PE, CD127 PerCP-Cy 5.5, HLA-DR PE-CY™7, CD39 APC, and CD4 APC-H7 MoAbs (BD Pharmingen). Samples were processed according to the manufacturer's guidelines and acquired on a DB FACSCanto II Flow Cytometer. The absolute number (cells/μL) of positive cells was calculated by comparing cellular events to bead events using BD FACSCanto clinical software (version 3).

2.3. Treg Populations. BMA Tregs we found were the following (Table 1):

(i) CD4+/CD127^{low}/CD25^{high}

(ii) CD4+/CD45RA-/CD25^{high}/CD127^{low}

(iii) CD4+/CD45RA-/CD127^{low}/CD25^{high}/DR+/39+

The population we studied in our analysis was the CD4+/CD45RA-/CD25^{high}/CD127^{low} group (Figure 1).

Table 1: Bone marrow aspirate T/NK/B cell distribution.

	Median value/μL	Range
CD3+	1350	4587–504
CD4+	721	64–1715
Tregs CD4+/CD127^{low}/CD25^{high}	56	6–201
Tregs CD4+/CD45RA-/CD127^{low}/CD25^{high}*	**21**	**2–82**
Tregs CD4+/CD45RA-/CD127^{low}/CD25^{high}/DR+/39+	*5*	1–27
CD8+	370	56–1338
CD16+/56+	225	90–1536
CD19+	265	0–2560

CD16+/CD56+: NK; CD19+: B cells. *Study population.

2.4. Statistical Analysis. The Mann–Whitney rank sum test was used to compare absolute cell counts while chi-square or Fisher's exact test (2-tailed) was performed to compare proportions. The comparison of the dBMA population with the posttreatment group was performed with the paired t-test or Wilcoxon signed-rank test as appropriate. The variables analyzed for a correlation with OR were age, WBC, and integrated molecular-cytogenetic risk; the NPM mutation; the FLT3 ITD or D835 mutation; NPM^{mut}FLT3^{wt}Normal Karyotype; and dBMA Tregs and de novo versus secondary AML. The variables analyzed for the correlation with Tregs were integrated molecular-cytogenetic risk; the NPM mutation; the FLT3 ITD or D835 mutation; and de novo versus secondary AML and WBC. Covariates in the multivariate logistic regression models were chosen by stepwise-with-backward elimination variable selection procedures. The discriminatory power of the dBMA Tregs value to predict response was assessed by estimating the area under the ROC curve (AUC). The optimal cut-off was determined by maximizing both sensitivity and specificity, computed at the optimal cut-off, as reported along with the 95% confidence intervals. Overall survival (OS) curves were plotted with the Kaplan-Meier method and compared by log-rank test, censoring patients (6 out of 23) at allotransplantation.

The significance was defined as a p value of <0.05.

3. Results

OR (complete remission (CR) + CR incomplete (CRi)) was documented in 10 of 23 patients (44%). There were two partial responder patients. The variables impacting on OR are reported in Table 2.

Figure 2 shows a dBMA CD4-lymphocyte correlation with dBMA Tregs ($r = 0.7$, $p < 0.001$), the association between OR and higher dBMA Tregs ($p = 0.024$), the optimal dBMA Tregs cut-off value for predicting response to treatment (≥21/μL, AUC = 0.78, $p = 0.02$), and accordingly, OS patient stratification according to dBMA Tregs ($p = 0.03$).

The BMA population modifications after treatment (i.e., at day 28 after the start of "3 + 7") are summarized in Table 3.

The dBMA Treg ≥ 21/μL correlation with integrated molecular-cytogenetic risk; the NPM mutation; the FLT3

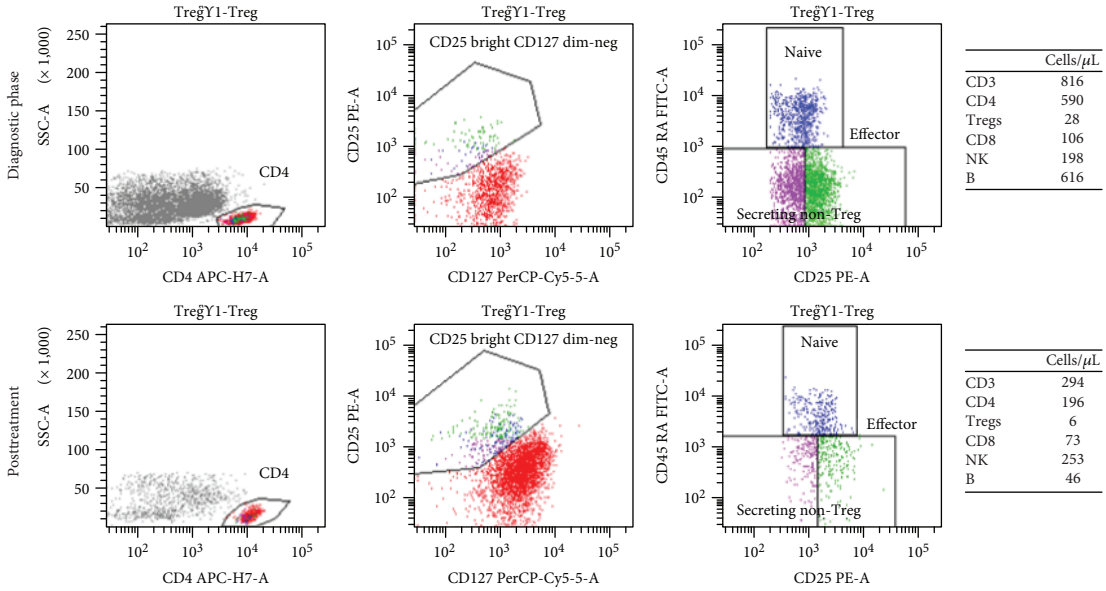

Figure 1: Flow cytometry plot in a responder patient: diagnostic and posttreatment evaluation (the T/B/NK population is summarized in the grid).

ITD or D835 mutation; and de novo versus secondary AML and WBC are summarized in Table 4: the molecular-cytogenetic risk association ($p = 0.020$) was demonstrated.

The responder and nonresponder patients' mean dBMA Tregs after treatment are reported in Figure 3.

The whole group median OS was 18 months. OS according to Tregs and molecular-cytogenetic risk is reported in Figure 4 (median OS in dBMA Tregs $\geq 21/\mu$L and the low-intermediate risk group, 18 versus 18 months, $p = 0.902$; median OS in the high and low-intermediate risk-group, 5 versus 18 months, $p = 0.05$, all pairwise multiple comparison procedures, Holm-Sidak method).

4. Discussion

The possible role of Tregs in terms of their effects on the prognosis in the AML setting has been amply hypothesized [9–11] but is still not clearly understood. The key point in the present investigation was to restrict the analysis to as homogeneous a patient's group as possible, limiting any pre- and posttreatment confounding factors. Hence, we analyzed dBMA Tregs from younger newly diagnosed AML patients, all treated with the "3 + 7" regimen according to a sequential enrollment procedure.

Primarily, we were interested in evaluating whether our study population was similar to the one reported in the literature in regard to the higher Treg frequencies in dBMA that we chose to analyze. In fact, it is well known that Tregs increase not only in the peripheral blood [9, 10, 19–21] but also in the bone marrow, where they seem to be higher and also more immunosuppressive [10, 19]. Of note, studying the same population CD4+/CD25^{high}/CD127^{low}, Shenghui et al. [10] reported frequencies of 11.8 (% of CD4+ T cells); our relative dBMA frequencies were similar: 10 (median value, 56/μL, Table 1).

Table 2: Factors affecting overall response.

	Response		
	Yes $n = 10$	No $n = 13$	p
Age			
Years, median value	52	56	ns
WBC			
WBC/μL, median value	9565	18430	ns
Molecular/cytogenetic group[@], *n (%)*			**0.027**[b]; **0.049**[d]
Poor	1 (10)	7 (54)	
Intermediate	6 (60)	6 (46)	
Good	3 (30)	0 (0)	
NPM/FLT3, n (%)			
NPM^{mut}	6 (60)	0 (0)	**0.002**[c]; **0.001**[d]
NPM^{wt}	4 (40)	13 (100)	
FLT3 ITD+ or D835+	1 (10)	4 (31)	ns
FLT3^{wt}	9 (90)	9 (69)	
NPM^{mut}/FLT3^{wt}/NK	3 (30)	0 (0)	ns
No (NPM^{mut}/FLT3^{wt}/Nk)	7 (70)	13 (100)	
dBMA Tregs[#], *n (%)*			**0.036**[c]; ns[d]
<21/μL	2 (20)	9 (69)	
≥21/μL	8 (80)	4 (31)	
De novo AML, n (%)			**0.046**[c]; ns[d]
Yes	10 (100)	8 (61)	
No	0 (0)	5 (39)	

[@]According to ELN 2010 [ITD allelic ratio not performed]. [b]Chi-square test. [c]Fisher exact test. [d]Multivariate stepwise-backward elimination procedure. Bold values are statistically significant ($p < 0.05$). WBC: white blood cells; dBMA Tregs: diagnostic bone marrow aspirate T regulatory cells; NK: normal karyotype; AML: acute myeloid leukemia.

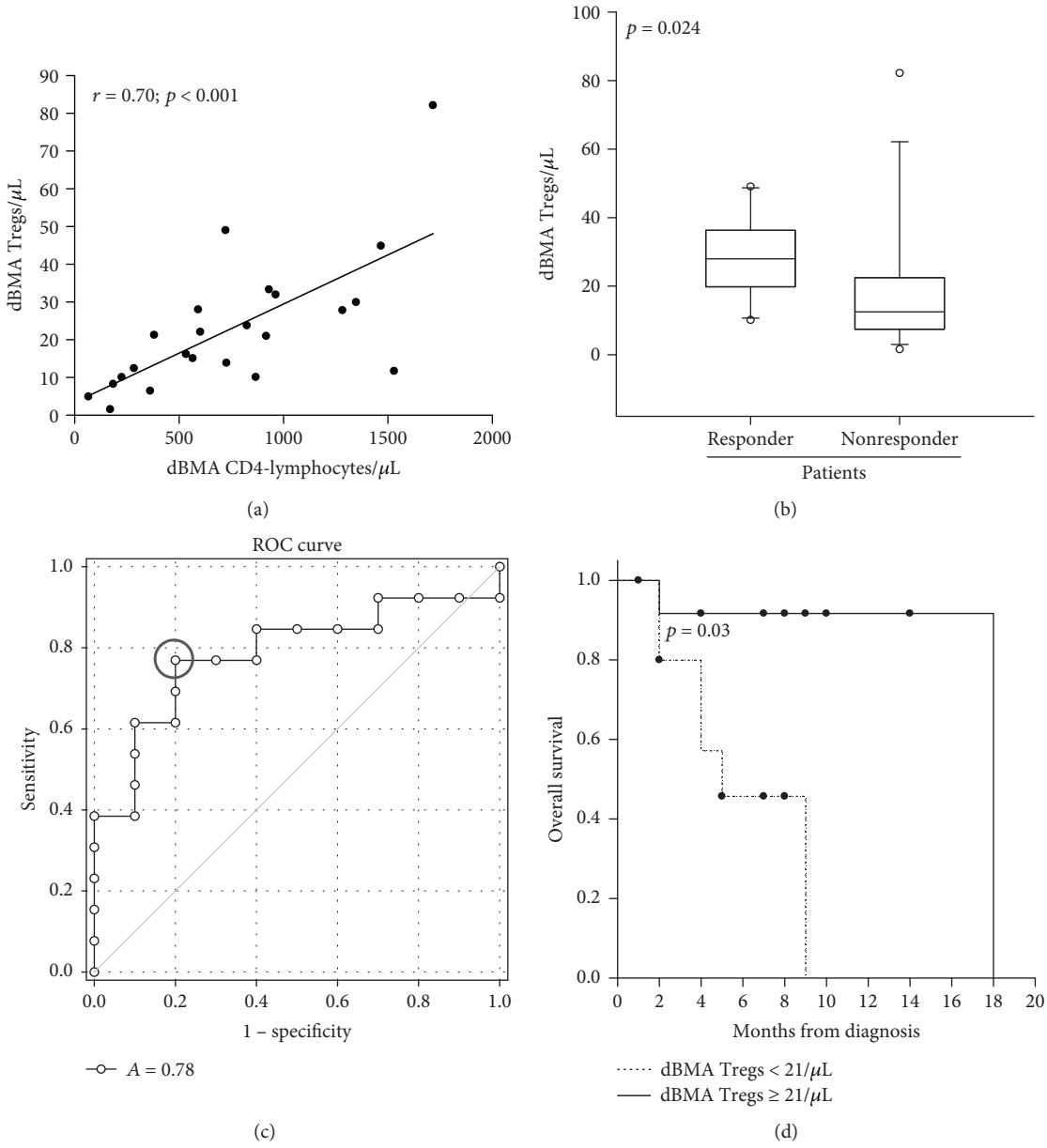

Figure 2: (a) Diagnostic bone marrow aspirate (dBMA) CD4-lymphocyte correlation with Tregs ($r = 0.70$, $p < 0.001$). (b) Median dBMA Treg value in responder and nonresponder patients (28 versus 13/μL, Mann–Whitney rank sum test, $p = 0.024$). (c) ROC curve: AUC analysis (AUC, 0.80; 95% CI, 0.5902–0.9791, $p = 0.02$), 21/μL, optimal dBMA Treg cut-off value for predicting response to treatment, yielding 77% sensitivity (95% CI, 53% to 92%) and 80% specificity (95% CI, 44% to 97%). (d) Median OS in ≥21/μL and <21/μL dBMA Tregs (18 versus 5 months, log-rank test, $p = 0.03$).

Given the possible expansion [22, 23] and bearing in mind the immunosuppressive weight of dBMA Tregs (CD4+/CD45RA-/CD25^{high}/CD127^{low}) which finally favor AML cells [7, 19], we investigated the possible correlation of dBMA Tregs with the outcome and obtained the ROC curve optimal cut-off value to predict a better OR (≥21/μL, Figure 2(c)) and OS (Figure 2(d)). Consequently, at univariate analysis, dBMA Tregs (as both continuous (Figure 2(b)) and categorical variables (Table 2) were correlated with OR as well as NPM mutation, molecular-cytogenetic risk, and *de novo* AML (Table 2). However, while the impact of Tregs on OR was not confirmed at multivariate analysis (Table 2) but molecular-cytogenetic risk remained, we found a correlation between Tregs and the factors known to have an impact on AML outcome [1] (Table 4).

Therefore, our data might appear conflicting with the observations showing diagnostic Tregs to be correlated with poor prognosis in both the peripheral blood [9, 10] and the bone marrow [10], while on the contrary, in our study population, the higher number of Tregs benefit was due to their association with the good-intermediate molecular-cytogenetics risk group and not to the Treg value itself

Table 3: Bone marrow aspirate T, NK, and B population in diagnosis and after treatment phase.

	Diagnosis	After treatment	p
CD3+/μL, *mean value (mv)*	1350	960	0.125[a]
CD4+/μL, *mv*	721	481	0.116[a]
CD8+/μL, *mv*	370	373	0.870[a]
Tregs/μL, *mv*	21	12	**0.038[a]**
CD16+/56+, *median value (med v)*	225	256	0.742[b]
CD19+/μL, *med v*	265	12	**0.008[b]**

[a]Paired t-test. [b]Wilcoxon signed-rank test. CD16+/56+: NK; CD19+: B cells. Bold values are statistically significant ($p < 0.05$).

Table 4: Correlation between dBMA Tregs and AML-related prognostic factors.

	Tregs < 21/μL $n = 11$	Tregs ≥ 21/μL $n = 12$	p
Molecular/cytogenetic group[@], n (%)			**0.020[b]**
Poor	7 (64)	1 (8)	
Intermediate	3 (27)	9 (75)	
Good	1 (9)	2 (17)	
NPM/FLT3, n (%)			
NPM^{mut}	2 (18)	4 (33)	0.64[c]
NPM^{wt}	9 (82)	8 (67)	
FLT3 ITD+ or D835+	3 (27)	2 (17)	0.64[c]
FLT3^{wt}	8 (73)	10 (83)	
WBC count			0.99[c]
<100 × 10e3/μL	10 (91)	10 (83)	
≥100 × 10e3/μL	1(9)	2 (17)	
De novo AML, n (%)			0.155[c]
Yes	7 (64)	11 (92)	
No	4 (36)	1 (8)	

[@]According to ELN 2010 [16] [ITD allelic ratio not performed]. [b]Chi-square test. [c]Fisher exact test. Bold value is statistically significant ($p < 0.05$).

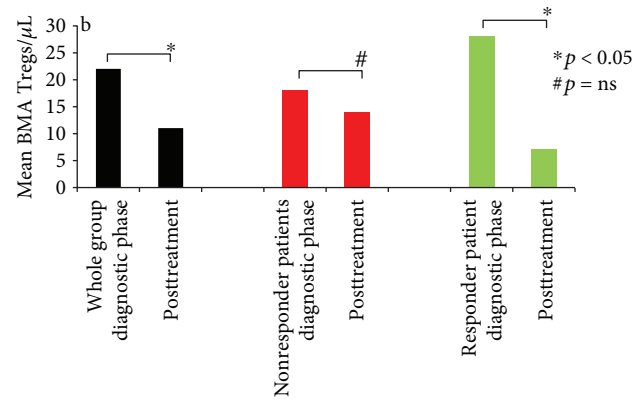

Figure 3: After treatment, mean dBMA Tregs in the whole (black), responder (green), and nonresponder (red) patient groups, respectively (22 versus 12/μL, paired t-test, $^{*}p = 0.038$, 28 versus 7/μL, Wilcoxon signed-rank test $^{*}p = 0.030$; 18 versus 14/μL, paired t-test, $^{\#}p = 0.32$).

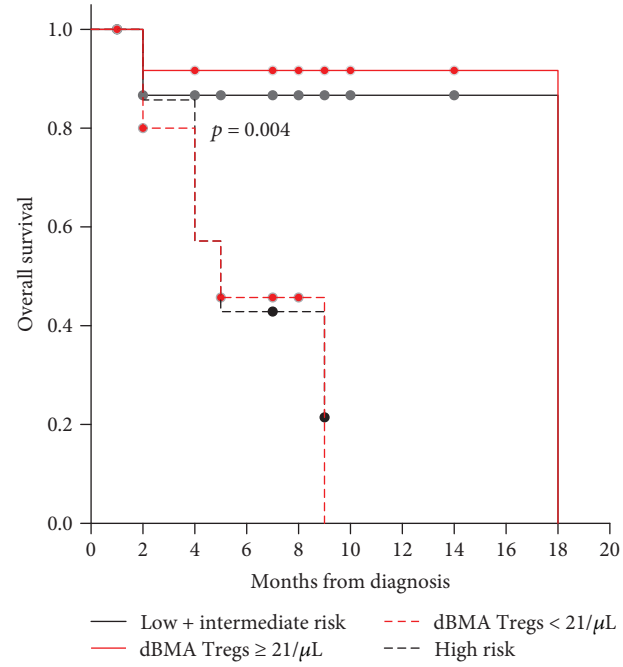

Figure 4: Median OS patients in Tregs < 21/μL; Tregs ≥ 21/μL; poor and low-intermediate risk group, respectively (5, 18, 5, and 18 months; log-rank test; $p = 0.004$).

(Table 2, Figure 4). Of note, Szczepanski et al. [9] did not demonstrate any correlation between Treg levels and the cytogenetic subgroup, although their study population was the peripheral one and the prognostic stratification referred to karyotypic analysis [24] and not to the integrated molecular-cytogenetics stratification we used [3, 16]. Of course, the reason why, in our study population, dBMA Tregs were higher in AML with a better prognosis risk remains to be elucidated and study of a larger patient cohort will be needed to confirm or dismiss the association.

All the same, in the peripheral blood, it has been already shown [20] that treatment-induced lymphopenia is not a random process and susceptibility to intensive chemotherapy differs between T cells subsets (i.e., CD4, CD8, and Tregs). In fact, although in the bone marrow we confirmed that CD4+ and CD8+ T cell levels did not differ after treatment, a statistically significant difference was documented for Tregs (Table 3). Of note, in responder patients, Tregs statistically decreased (Figure 3), confirming the correlation with OR and suggesting their possible utility to monitor response after treatment. In fact, albeit in peripheral blood Tregs, a secondary response to inflammation caused by induction chemotherapy and to cytokine secretion has already been reported to justify the expansion of Tregs after treatment in CR patients [9]. Obviously, in this regard, a longer monitoring time might clarify the correlation between the Treg levels and the risk of AML relapse.

On the other hand, a stereotyped immunologic response to chemotherapy in patients with AML that is independent of the AML etiology, cytogenetics, or molecular characteristics was also proposed [21] to understand the increase of peripheral Tregs during treatment. Analyzing BMA Tregs, we did not find this (Figure 3). Moreover, in the early phases after induction (i.e., day 17), another group [11] supposed that Tregs might also foster the recovery of normal hematopoietic cells, thus promoting higher CR rates and a better OS.

5. Conclusions

It is widely recognized [7] that BMA Tregs are higher in, and act at, the neoplastic AML site (i.e., bone marrow) primarily favoring leukemia growth but, taken together, our data, while confirming this point, might suggest that their final effect is probably due to both disease-associated and chemotherapy-induced modifications. Moreover, several studies [9, 10, 19–21] suggest a prognostic impact of Tregs in AML patients receiving intensive chemotherapy, but Tregs persistence after treatment [11, 21] and in CR patients [9] might even sustain opposite mechanisms such as recognition of leukemia-associated antigens (as supposed in the allo-setting) [25] on the one hand and leukemia-relapse promotion on the other. In this regard, sequential measurements (i.e., diagnostic, day 14, and recovery phase, after obtaining CR and during consolidation) of Tregs in a larger number of patients are needed to confirm the prognostic value of BMA Tregs in the diagnostic phase of AML and to monitor their value after treatment and to clarify any correlation with leukemia relapse.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

There are no conflicts of interest to report.

Authors' Contributions

Mario Delia wrote the paper and compiled and analyzed the data. Anna Mestice and Claudia Brunetti performed the flow cytometry. Paola Carluccio and Francesco Albano collected the data. Mario Delia and Giorgina Specchia designed the study.

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Review Article

Role of Regulatory T Cells in Tumor-Bearing Mice Treated with Allo-Hematopoietic Stem Cell Transplantation Plus Thymus Transplantation

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We recently developed a new allogeneic hematopoietic stem cell transplantation method (allo-HSCT) combined with thymus transplantation (TT) from the same donor (allo-HSCT + TT). This method induces elevated T cell function with mild graft-versus-host disease (GVHD) in comparison to conventional HSCT alone and HSCT + donor lymphocyte infusion (DLI). This new method is effective against several intractable diseases, including malignant tumors, for which conventional treatments are ineffective. Regulatory T (T_{reg}) cells play an important role in the enhanced graft-versus-tumor (GVT) effect and reduction of GVHD, thus leading to longer survival. Replacement and reduction of elevated T_{reg} cells by donor-derived allo-T_{reg} cells from the transplanted thymus may play one of crucial roles in the effect. This review discusses the role of T_{reg} cells in a tumor-bearing mouse model treated with allo-HSCT + TT.

1. Introduction

We recently developed a new allogeneic hematopoietic stem cell transplantation method (allo-HSCT) in conjunction with thymus transplantation (TT) from the same donor (allo-HSCT + TT) [1–11]. This method results in elevated T cell function with mild graft-versus-host disease (GVHD) compared to HSCT alone or HSCT + donor lymphocyte infusion (HSCT + DLI) [8]. The mechanism underlying these effects involves $CD4^+$ $FoxP3^+$ regulatory T (T_{reg}) cells, which suppress immune activity and prevent autoimmunity and GVHD [12, 13]. The percentages of these cells in $CD4^+$ T cells are intermediate between HSCT alone and HSCT + DLI, while the opposite is true for the percentage of $CD4^+$ $FoxP3^-$ effector T (T_{eff}) cells. There are two main ways of producing T_{reg} cells—that is, from the thymus (as naturally occurring T_{reg}, nT_{reg}) and from peripheral cells (inducible T_{reg}, iT_{reg}) [14, 15]. We observed that not only the number of T cells but also the quantity of T cell receptor rearrangement excision circles (TREC) [8], which reflect production of T cells from the thymus, are increased in HSCT + TT. Although we did not purify the T_{eff} and T_{reg} cells in TREC analysis, we suggest that both naive cells are produced from the transplanted thymus and move to the periphery because of fundamentally similar mechanisms of them for those cells [16].

This method showed efficacy against several intractable diseases and conditions, such as autoimmune diseases in aging and radioresistant hosts [2, 3], exposure to supralethal irradiation [4], multiple-organ transplantation from different donors [5], type 2 diabetes mellitus [6], low hematopoietic stem cell (HSC) number or low dose of irradiation [7], and malignant tumors, including leukemia [8–11]. Malignant tumor-bearing mice treated with allo-HSCT + TT showed a strong graft-versus-tumor (GVT) effect but weak GVHD compared with HSCT alone and HSCT + DLI. These effects may involve replacement and reduction of the elevated T_{reg} cells by allo-T_{reg} cells.

The regulation of T_{reg} cells was suggested to be one mechanism of action of immunotherapy for cancer, and this has

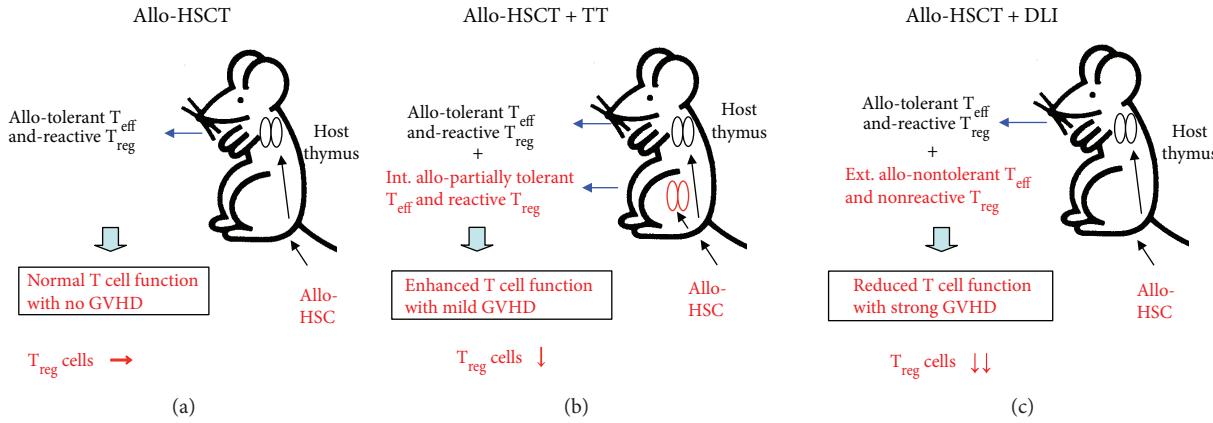

Figure 1: Theory of allo-HSCT + TT. In the case of conventional allo-HSCT (a), allo-T_{eff} and T_{reg} cells develop, are tolerated, and react in the host thymus. No GVHD occurs. The proportion of T_{reg} cells is comparable to that in normal mice. In the case of allo-HSCT + DLI (c), allo-nontolerant T_{eff} and nonreactive T_{reg} cells are externally supplied, and strong GVHD is induced with reduction of T cell function. The proportion of T_{reg} cells is markedly decreased. In the case of allo-HSCT + TT, the allo-T_{eff} and T_{reg} cells develop internally in the allo-transplanted thymus. The T cells show partial tolerance and reaction with the host, and only mild GVHD occurs with elevation of T cell function (b). The proportion of T_{reg} cells decreases slightly. Figure 1 is modified from Hosaka [1], under the Creative Commons Attribution License/public domain.

been examined in clinical trials [17]. It may also be applicable under allo-HSCT + TT. We review and discuss the utility of T_{reg} cells for treatment of cancer.

2. Main Text

2.1. Review

2.1.1. Theory of HSCT + TT with T_{reg} Cells. First, we present the theory of allo-HSCT + TT [1, 8]. This method makes use of intra-bone marrow-bone marrow transplantation (IBM-BMT) for HSCT, which involves the direct injection of HSC into the bone marrow cavity, and results in superior engraftment of donor cells and reduced incidence of GVHD with mesenchymal stem cells (MSC) [18–20]. In the case of conventional allo-HSCT, allo-HSC are transplanted into the host, and allo-T cells develop in the host thymus (Figure 1(a)). The T_{eff} cells induce tolerance toward the host with thymic antigen-presenting cells (APC) and/or epithelial cells (TEC) [21]. Host-reactive T_{reg} cells are also reacted with host thymic dendritic cells (DC) [22]. Neither T cell type induces apparent GVHD, and the proportion of T_{reg} cells is comparable to that in normal mice. In contrast, nontolerant allo-T_{eff} and nonreactive T_{reg} cells are externally supplied in the case of HSCT + DLI, resulting in strong GVHD (Figure 1(c)). As this results in expansion of T_{eff} cells and little proliferation of T_{reg} cells, the proportion of T_{reg} cells is markedly reduced. In HSCT + TT (Figure 1(b)), allo-T_{eff} and T_{reg} cells develop internally from the transplanted allo-thymus in the host. The T_{eff} and T_{reg} cells are partially tolerant and reactive to the host, which was suggested to show a low response in mixed lymphocyte reaction, resulting in low GVHD [8]. Under these conditions, most allo-T_{eff} cells derived from the transplanted thymus are in the naïve state and may not expand well to host antigens. The T_{reg} cells also suppress activation of naïve cells by deprivation of activation signals [23]. Therefore, T_{reg} cells may play a role in allo-HSCT + TT. Nonetheless, the degree of inhibition may be insufficient, leading to mild GVHD with a slight decrease in the proportion of T_{reg} cells.

2.1.2. Effects of Allo-HSCT + TT in Tumor-Bearing Mice and the Dynamics of T_{reg} Cells. Next, we describe the effects of HSCT + TT in tumor-bearing mice and the dynamics of T_{reg} cells (Table 1). Non-tumor-bearing mice without treatment, or those treated with HSCT alone, with HSCT + adult thymus (AT) transplantation, or with HSCT + DLI, were used as representative non-tumor-bearing controls (Figure 1, group 1). The tumor-bearing mice showed an increase in T_{reg} cell number with inducible T_{reg} cells [24]. Treatment with HSCT in the early phase of tumor progression (group 2a) resulted in a reduction in the proportion of T_{reg} cells among $CD4^+$ T cells, although they were still elevated compared with non-tumor-bearing mice. In HSCT + AT treatment, the proportion of T_{reg} cells decreased further and was comparable to the level in non-tumor-bearing mice. The mice showed the longest survival with strong GVT effects and mild GVH effects.

The functions of the transplanted thymus from AT, newborn thymus (NT), and fetal thymus (FT) in mice treated with HSCT were compared (group 2b, c), as the functions differ between ages. The proportion of T_{reg} cells did not change with any type of HSCT alone or HSCT + TT, and all of the HSCT + TT mice showed strong GVT and longer survival compared to nontreated controls or those treated with HSCT alone. However, the GVT effects in HSCT + NT or FT transplantation were greater than those of HSCT + AT transplantation, and the survival was longest in HSCT + NT transplantation. These animals showed the highest levels of IFNγ and effector memory (EM) T cells and the lowest numbers of myeloid suppressor cells [10].

Table 1: Effects of T_{reg} cells in tumor-bearing mice treated with allo-HSCT + TT

Group	TT	Comparison of T_{reg} cells*				Effect of HSCT + TT[$]	Ref.
		No treated[#]	HSCT	HSCT + TT	HSCT + DLI		
1. No tumor	AT		→	↓	↓↓	aGVH, elevated T cell function	[1, 8]
2a. Early tumor	AT	↑↑	↑	→	ND	GVT[a], LS[a],	[8, 10]
2b. Same as above	NT	↑↑	↑	→	ND	GVT[b], LS[b], IFNγ[b], EM T cells[b], and MS cells[c]	[10]
2c. Same as above	FT	↑↑	↑	→	ND	GVT[b], LS[d]	[10]
3. Advanced tumor	FT	↑↑↑	↑↑	↑	ND	Inhibition of metastasis, LS	[9]
4. Leukemia	AT	ND	→	↓	↓↓	GVL, aGVH, and LS	[11]

*% of $FoxP3^+$ cells in $CD4^+$ T cells compared with non-tumor-bearing mice: no change, →: mild increase, ↑; moderate increase, ↑↑; strong increase, ↑↑↑; slight decrease, ↓; moderate decrease, ↓↓. [#]Host-derived cells. [$]Compared with HSCT and/or HSCT + DLI in the same group. [a]3rd in group 2, [b]1st in group 2, [c]lowest in group 2, [d]2nd in group 2. AT: adult thymus; NT: newborn thymus; FT: fetal thymus; aGVH: attenuated graft-versus-host; GVT: graft-versus-tumor; LS: longest survival; EM: effector memory; MS: myeloid suppressor; GVL: graft-versus-leukemia; ND: not determined.

In advanced tumors (group 3), nontreated tumor-bearing mice showed marked elevation of T_{reg} cell number. HSCT + TT reduced the T_{reg} cell number to a greater extent than did HSCT alone and inhibited lung metastasis leading to the longest survival, although the T_{reg} cell level did not decrease to normal and there was no significant regression of the primary tumor [9].

The results with regard to T_{reg} cells in leukemia-bearing mice in group 4 were similar to those of non-tumor-bearing controls (group 1). The T_{reg} cell number in the HSCT + AT transplantation group was intermediate between those of HSCT alone and HSCT + DLI, and HSCT + AT transplantation yielded the longest survival with the greatest graft-versus-leukemia (GVL) effect and attenuated GVHD [11].

2.2. Discussion. HSCT + TT is a valuable method for treatment of cancer, and T_{reg} cells play a crucial role in mediating the effects of this method. As shown in Figure 2, T_{reg} cell number was elevated in untreated hosts bearing tumors and increased with tumor progression (Figure 2(a)). Tumor cells produce TGFβ, which induces iT_{reg} cells leading to inhibition of immune reaction against cancer [25, 26]. Allo-HSCT alone showed a mild GVT effect by allo-reaction with a slight reduction in T_{reg} cell number compared to syngeneic HSCT [9] (Figure 2(b)). Additional transplantation of thymus grafts showed a further GVT effect with further reduction in T_{reg} cell number (Figure 2(c)). The level of GVT was comparable to that from HSCT + DLI leading to long survival, although animals treated with HSCT + DLI showed higher GVHD and shorter survival with lower T_{reg} cell number (Figure 2(d)). These findings were consistent with an important role of T_{reg} cells in inducing strong GVT effects and mild GVH effects in HSCT + TT [27].

Thymic function is known to differ according to age [28–30]. Therefore, we next performed comparisons between fetal, newborn, and adult thymic grafts. Although the proportion of T_{reg} cells was the same in all of these groups, NT showed the best effect with regard to GVT and survival. This may have been related to its strong reduction of myeloid suppressor cells, which inhibit immune activity [31, 32], and elevated production of effector memory T cells and IFNγ [10]. Although the detailed mechanism is not yet clear, it is possible that NT shows the highest function of T cell production among the thymus grafts [4].

Mice bearing advanced tumors showed further elevation of T_{reg} cell level. Therefore, the level was not normalized by HSCT + TT, and the primary tumor did not show significant regression. Nonetheless, they showed inhibition of metastasis and long-term survival, suggesting that this method is still effective with regard to GVT on newly developed tumor cells.

Although mice bearing leukemia showed similar results, those treated with either HSCT + TT or DLI showed long survival with complete remission of tumor cells by donor-derived cells. Therefore, the latter may have reduced production of T_{reg} cells from the tumor and/or thymus graft leading to greater GVHD than the former.

Some of our data were based on conversion of T_{eff} cells to T_{reg} cells in the tumor microenvironment [24, 25]. However, a recent study involving analysis of TCR repertoires in a mouse model using chemical carcinogen-induced fibrosarcoma showed that such conversion does not occur [33]. Although the reason for the discrepancy remains unclear, it is possible that the properties of the tumors were different between the studies. Generally, cancers develop with gradual accumulation of gene mutations and express cancer antigens accompanied by immune reactions involving T_{eff}/T_{reg} interaction and/or conversion. The specific carcinogen-induced tumor is unknown to be the same condition. Further studies are required using several cancer models developed with different mechanisms.

The mechanism underlying the production of T_{reg}/T_{eff} cells from allo-TT has not been clarified under cancer-bearing conditions. As shown in Figure 3, the host antigen comes into direct contact with the transplanted thymus from renal capsules, in which it is translated, whereas cancer antigens are relatively isolated from and do not come into direct contact with the transplanted thymus, as tumor cells were transplanted subcutaneously into the backs of the experimental animals [8–10]. This may lead to host antigen-specific T_{reg} cells being superior to cancer antigens for inducing thymic DC in the transplanted thymus, likewise tolerance with intrathymical administration [34], which may result in inhibition of GVHD, but not GVT.

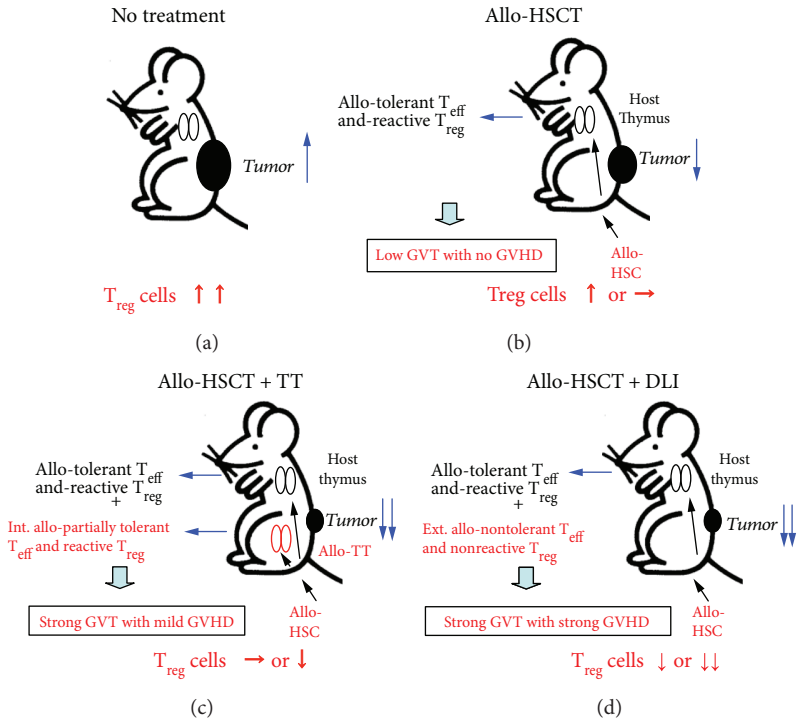

FIGURE 2: Theory of allo-HSCT + TT for tumors. Untreated controls bearing tumor tissue showed increased numbers of T_{reg} cells, including iT_{reg} cells, with tumor progression (a). In the case of conventional allo-HSCT (b), the allo-T_{eff} and T_{reg} cells develop with tolerance and reactivity to the host in the thymus. A low GVT effect is then induced with no/minimal GVHD. Mild tumor regression is induced compared with untreated controls (a). The proportion of T_{reg} cells still increases slightly or is at the normal level. In the case of allo-HSCT + DLI (d), nontolerant and nonreactive allo-T_{eff} and T_{reg} cells are supplied externally, and a strong GVT effect occurs with strong GVHD. The proportion of T_{reg} cells decreases either slightly or markedly. In the case of allo-HSCT + TT (c), internally allo-partially tolerant T_{eff} and reactive T_{reg} are induced from the transplanted thymus. As a result, strong GVT occurs with mild GVHD. The proportion of T_{reg} cells decreases slightly or is at the normal level.

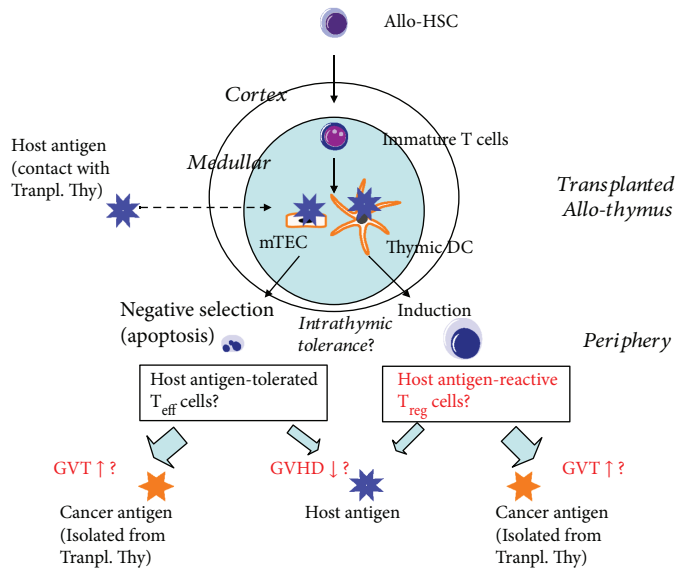

FIGURE 3: Hypothesis for immune regulation of malignant tumor by allo-HSCT + TT. The host antigen comes into direct contact with the transplanted thymus, whereas cancer antigens are relatively isolated from the transplanted thymus. Therefore, host antigen-specific T_{reg} cells may be induced intrathymically to a greater extent than those specific to cancer antigens with thymic DC. Conversely, T_{eff} cells are tolerant toward host antigens, but not cancer antigens with medullary TEC (mTEC), yielding the same results. This may result in strong GVT with mild GVHD. Figure 3 is modified from Hosaka [1], under the Creative Commons Attribution License/public domain.

Conversely, T_{eff} cells are tolerant toward host antigens, but not cancer antigens, yielding the same results.

The regulation of T_{reg} cells is regarded as a suitable target for immune therapy in human cancers and has been the subject of several clinical trials. The main purpose is effective deletion of T_{reg} cells to enhance immune function against tumor cells [35]. Treatment with antibodies for cytotoxic T lymphocyte-associated antigen (CTLA) 4, which is expressed constitutively in T_{reg} cells, or CC chemokine receptor (CCR) 4, which is expressed in activated T_{reg} cells, has been shown to have beneficial effects against melanoma, renal cell carcinoma, and bladder cancer [36–39]. Administration of metronomic cyclophosphamide, which reduces highly proliferative T_{reg} cells, has beneficial effects on advanced cancers and metastatic breast cancers [40, 41]. As a side effect, blockade of immune checkpoints, such as CTLA-4 and programmed death (PD) 1, may induce serous autoimmune diseases [42–44]. In such cases, targeted depletion of tumor-infiltrating T_{reg} cells has been suggested [45]. In addition, it is also important to assess susceptibility to autoimmune diseases in patients with human lymphocyte antigen (HLA) haplotype and monitoring the number of T_{reg} cells.

Although allo-HSCT + TT shows the opposite immune reaction to these reports, the basic theory and points of note are similar. Although allo-T cells can induce GVT, the method using the above antibodies may be applicable in cases when the immune reaction is insufficient. In contrast, if the reaction is too strong with GVHD as autoimmune disease, iT_{reg} can be induced in vivo and/or ex vivo by treatment with IL-10 and/or TGFβ and subsequently transferred to the host [46, 47].

Taken together, allo-HSCT + TT is effective for treatment of malignant tumors, and T_{reg} cells may play one of crucial roles in the regulation. Among TT from various ages, NT showed the best functionality. Therefore, regenerative thymus tissue would be better than surgically obtained tissue. With recent progress in engineering for thymus regeneration [48–52], HSCT + TT may be useful as next-generation therapy for treatment of human cancer with control of T_{reg} cells.

3. Conclusions

T_{reg} cells play a crucial role in allo-HSCT + TT for treatment of malignant tumors. Additional control and regulation of T_{reg} cells may lead to better results, and this method may be applicable to human cancer.

Abbreviations

allo-HSCT: Allogeneic hematopoietic stem cell transplantation
TT: Thymus transplantation
AT: Adult thymus
DLI: Donor lymphocyte infusion
FT: Fetal thymus
NT: Newborn thymus
GVHD: Graft-versus-host disease
GVT: Graft versus tumor
GVL: Graft-versus-leukemia
HSCT: Hematopoietic stem cell transplantation
T_{eff}: Effector T cell
T_{reg}: Regulatory T cell
nT_{reg}: Naturally occurring T_{reg}
iT_{reg}: Inducible T_{reg}
DC: Dendritic cell
TEC: Thymic epithelial cell.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this article.

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Research Article

CTLA4Ig Improves Murine iTreg Induction via TGFβ and Suppressor Function *In Vitro*

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Blockade of the CD28:CD80/86 costimulatory pathway has been shown to be potent in blocking T cell activation *in vitro* and *in vivo*. The costimulation blocker CTLA4Ig has been approved for the treatment of autoimmune diseases and transplant rejection. The therapeutic application of regulatory T cells (Tregs) has recently gained much attention for its potential of improving allograft survival. However, neither costimulation blockade with CTLA4Ig nor Treg therapy induces robust tolerance on its own. Combining CTLA4Ig with Treg therapy would be an attractive approach for minimizing immunosuppression or for possibly achieving tolerance. However, since the CD28 pathway is more complex than initially thought, the question arose whether blocking CD80/86 would inadvertently impact immunological tolerance by interfering with Treg generation and function. We therefore wanted to investigate the compatibility of CTLA4Ig with regulatory T cells by evaluating direct effects of CTLA4Ig on murine Treg generation and function *in vitro*. For generation of polyclonal-induced Tregs, we utilized an APC-free *in vitro* system and added titrated doses of CTLA4Ig at different time points. Phenotypical characterization by flow cytometry and functional characterization in suppressor assays did not reveal negative effects by CTLA4Ig. The costimulation blocker CTLA4Ig does not impair but rather improves murine iTreg generation and suppressor function *in vitro*.

1. Introduction

In order to exert a proper T cell immune response, the T cell needs at least two signals, namely, an antigen-specific signal via the T cell receptor (TCR) and a costimulatory signal provided by a number of specialized cell surface receptors [1]. One of the best studied costimulatory pathways is the CD28:B7 pathway, mediated by the binding of CD28, which is expressed on T cells, to B7 molecules (CD80 and CD86), expressed on antigen-presenting cells (APCs). Costimulation via CD28 induces proliferation, survival, and cytokine production, whereas lack of CD28 signaling following TCR ligation induces classical T cell anergy [2]. Physiologically, T cell activation leads to the upregulation of the negative costimulatory molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which binds B7 molecules with higher affinity and avidity, thereby providing a negative feedback mechanism, which prevents further CD28 signaling [3]. Additionally, CTLA4 is constitutively expressed on regulatory T cells (Tregs) being critical for suppressor function [4] and overall immune homeostasis [5]. As direct CD28 blockade is difficult to achieve, the fusion protein CTLA4 immunoglobulin (CTLA4Ig) was developed as an alternative strategy to indirectly block CD28 ligation [6], at that time still unaware of the importance of CTLA4 signaling. In light of the protolerogeneic functions of CTLA4, the therapeutic use of CTLA4Ig in tolerance protocols was put into question [7], as it prevents not only ligation of CD28 but also ligation of CTLA4, which is critical for Treg function [4].

$CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) are critical mediators of self-tolerance [8] and have been shown to prevent autoimmunity and to induce (transplantation) tolerance in numerous experimental animal models [9–11]. Therefore, Tregs would be promising candidates for the intentional induction of transplantation tolerance or as part of calcineurin inhibitor- (CNI-) sparing immunosuppressive regimens,

as chronic use of CNIs—still being the backbone of current immunosuppressive regimens—is associated with substantial side effects, including profound nephrotoxicity. Furthermore, previous studies already demonstrated that CNIs inhibit Treg function and have markedly negative effects on Tregs [12], including decreased FoxP3 expression and demethylation status and subsequent impaired suppressive capacity [13].

Whereas CTLA4Ig has proven great potency in tolerance induction in various mouse models in combination with anti-CD40L [14], donor-specific transfusion (DST) [15], or bone marrow transplantation [16–18], it was less effective in nonhuman primate studies. However, its immunosuppressive efficacy, combined with the absence of renal toxicity, maximized its clinical relevance and enabled its successful use in clinical transplantation [19].

The combination of CTLA4Ig treatment and Treg cellular therapy seems an attractive approach for studies of minimization or even withdrawal of chronic immunosuppressive therapy. Several studies have investigated the effect of CTLA4Ig on Treg survival and potential effects on Treg function utilizing *in vitro* and *in vivo* models [20–22]; however, results are still inconclusive and conflictive. While most studies focused mainly on the effect on thymus-derived Tregs (tTregs), we used an in vitro model of transforming growth factor beta- (TGF-) induced Tregs (iTregs), which have been shown to be potent in the suppression of alloresponse in a mixed chimerism model for tolerance induction *in vivo* [17, 23]. In this study, we show that iTreg induction and suppressive potential of Tregs are not impaired by the presence of CTLA4Ig, therefore adding another piece of puzzle to the complex relationship between costimulation blockade of the CD28/CTLA4/B7 pathway and its effect on the different subsets of Tregs. Indeed, we provide evidence that the presence of CTLA4Ig rather enhances TGFβ-mediated conversion towards a suppressive phenotype, indicated by expression of Treg-specific markers and suppressive function *in vitro*.

2. Materials and Methods

2.1. Animals. Female C57BL/6 (B6, H-2^b) and BALB/C (H-2^d) mice were purchased from Charles River Laboratories (Sulzfeld, Germany), housed under specific pathogen-free conditions, and used at 6 to 12 weeks of age. All experiments were approved by the local review board of the Medical University of Vienna and the Austrian Federal Ministry of Science, Research and Economy and were performed in accordance with the national and international guidelines of laboratory animal care.

2.2. Generation of Tregs. Tregs were generated as described previously [17]. Shortly, cells were isolated from spleen and lymph nodes of naïve B6 mice. For iTreg generation, CD4$^+$ cells were isolated (L3T4 microbeads, Miltenyi Biotec) and cultured for 6 days (144 h) in precoated 24-well plates (100 μg/ml anti-CD3 (145-2C11), 10 μg/ml anti-CD28 (37.51); BD Pharmingen) in the presence of 100 U/ml IL-2 (Sigma) and 5 ng/ml rhTGFβ (R&D Systems) [24]. Human CTLA4Ig (abatacept, purchased from Bristol-Myers-Squibb) was added at different concentrations (low dose, LD 40 μg/ml; high dose, HD 200 μg/ml) for the length of culture or for the last 24 h of culture (HD 200 μg/ml). Due to intentionally introduced mutations to achieve higher avidity for human B7 molecules, belatacept lost effective binding capacity for murine B7; therefore, only abatacept is used in the current study [25]. Living cells were counted at indicated time points using CASY System (Innovatis). Purity of MACS-sorted populations was >90%. At the end of culture, the Treg-enriched cell populations were used for subsequent cell culture assays without additional sorting steps [17].

2.3. Antibodies and Flow Cytometric Analysis. Multicolor flow cytometric analysis of Tregs was performed as described previously [17]. Monoclonal antibodies (mAbs) with specificity against CD4 (RM4-4), CD25 (7D4), CD62L (Mel-14), and CTLA4 (UC10-4F10-11) were used. For intracellular staining, FoxP3 (FJK-16s) Staining Kit (eBioscience) was used according to the manufacturer's protocol. PI was used for dead cell exclusion when appropriate. Surface staining was performed according to standard procedures, and flow cytometric analysis was done on Coulter Cytomics FC500 using CXP software (Coulter, Austria) for acquisition and analysis.

2.4. In Vitro Suppression Assays. In vitro suppression assays were performed as described in detail previously [17, 26]. Briefly, 4×10^5 responder splenocytes (B6) were cocultured in triplicates with decreasing numbers of iTregs (4×10^5, 2×10^5, and 8×10^4 for a ratio of 1 : 1, 2 : 1, and 5 : 1 (responder cells versus Tregs)), in the presence of 4×10^5 irradiated (30 Gy) allogeneic splenocytes (BALB/C). Alternatively, responder cells were stimulated polyclonally with anti-CD3 (clone 145-2C11 at 5 μg/ml). Freshly isolated CD4$^+$ cells cultured without recombinant human (rh) TGFβ were used as control. After 72 h of incubation, cells were pulsed with [3H]-thymidine (Amersham, Biosciences, UK) for 18 h. Incorporated radioactivity was measured using scintillation fluid in a β-counter. Stimulation indices (SI) were calculated in relation to medium controls. Results represent averaged data of triplets from pooled animals.

2.5. T Cell Proliferation Assay. CD4 T cells were isolated from spleen and lymph nodes of B6 mice and enriched via magnetic bead-based positive selection (CD4 L3T4 microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany). MACS-sorted cells had a purity > 95%. 4 × 105 CD4 T cells (B6) were cultured in triplicates in the presence or absence of CTLA4Ig (low dose, LD 40 μg/ml; high dose, HD 200 μg/ml) with or without high-dose IL-2 (1000 U, Sigma). Cells were polyclonally stimulated with anti-CD3 (clone 145-2C11 at 5 μg/ml) for 72 h and pulsed with [3H]-thymidine (Amersham, Biosciences, UK) for 18 h as described for suppressor assays.

2.6. Cytokine Analysis. IL-10 and IL-17A were measured by enzyme-linked immunoabsorbent assays (ELISA). Supernatant of *in vitro* cultures was harvested at different time points and stored at −80°C until analysis. ELISA kits were used according to the manufacturer's protocol (eBioscience, San Diego, CA). Plates were measured at 450/595 nm using a VICTOR plate reader (PerkinElmer).

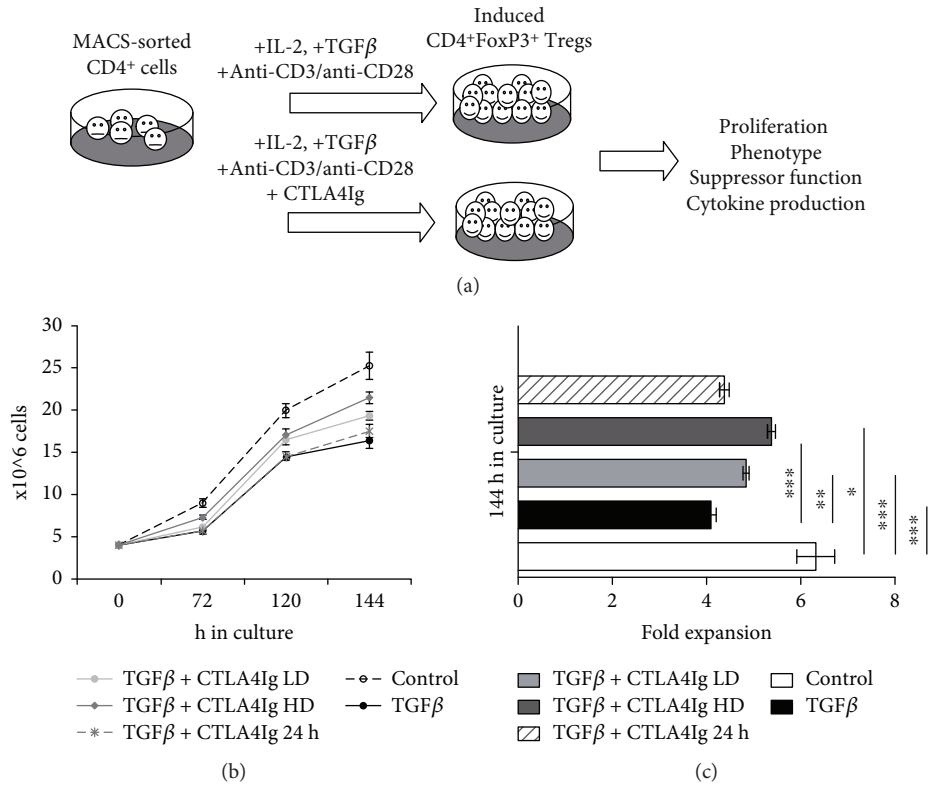


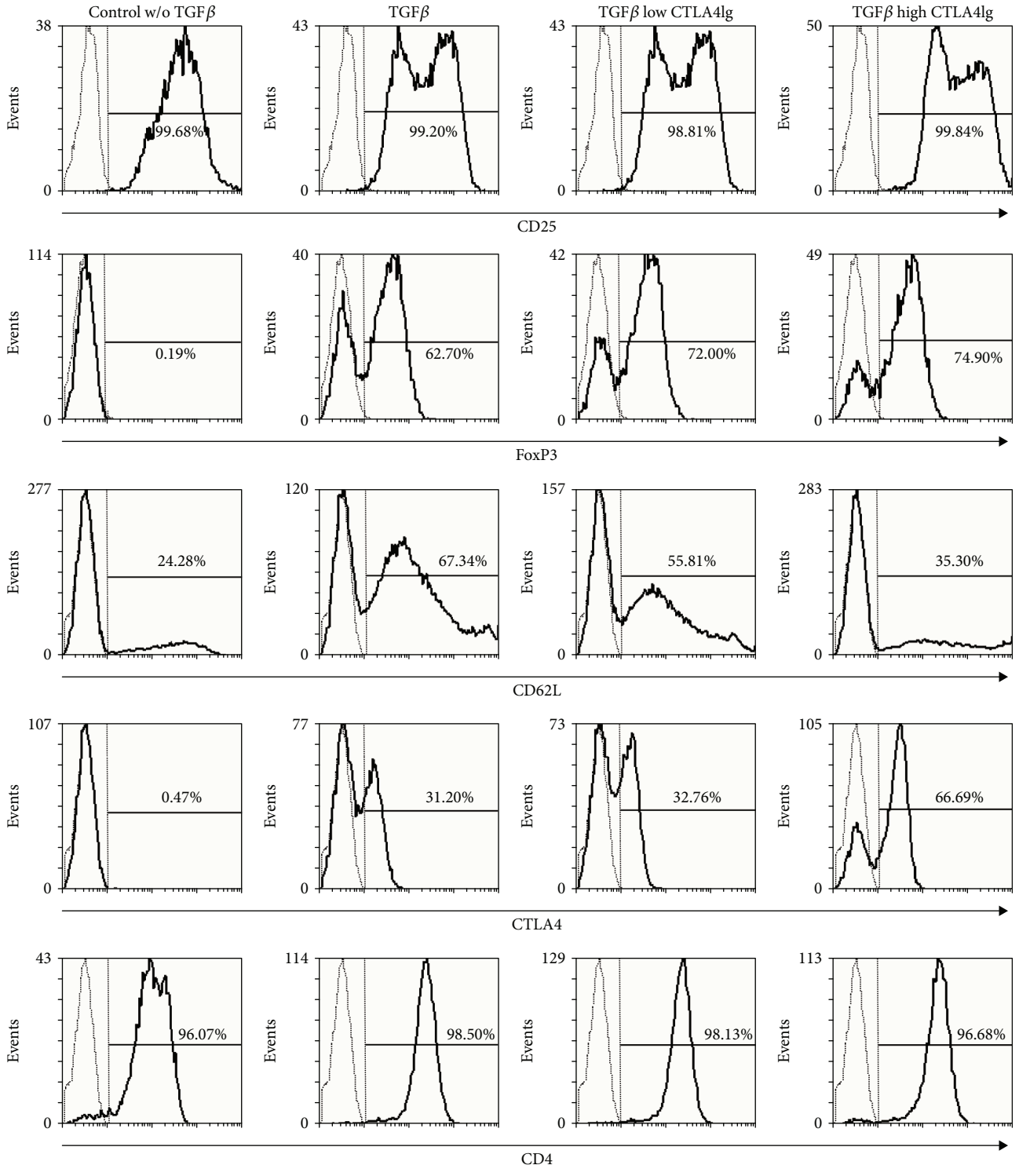
Figure 1: CTLA4Ig does not inhibit Treg proliferation *in vitro*. (a) Schematic illustration of Treg induction *in vitro* culture is shown. (b) Proliferation curve showing mean cell numbers for different culture conditions (all groups were stimulated with anti-CD3/CD28 in the presence of IL2) over time and (c) fold expansion after 144 h in culture are shown. Cells were plated in quadruplicates; control indicates CD4 T cells stimulated with anti-CD3/CD28 in the presence of IL2; results are representative for 3 independent experiments. Error bars represent standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$.

2.7. Statistics. A two-sided Student's t-test with unequal variances was used to compare results and SI values between groups. A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. CTLA4Ig Does Not Impair Proliferation of T Cells in the Presence of TGFβ. For addressing the specific question, whether CTLA4Ig interferes with Treg induction via TGFbeta (TGFb/TGFβ), we used an *in vitro* model for the generation of induced Tregs (iTregs) that were previously shown to generate potent Treg populations which have been successfully used as cell therapy in a model of chimerism-induced transplantation tolerance [17, 23]. Moreover, it has been proposed that in vitro generation of iTregs via TGFβ mimics the in vivo development of adaptive Tregs [27]. We added different amounts of CTLA4Ig to the Treg induction culture (schematic experimental approach outlined in Figure 1(a)), mimicking the therapeutic serum concentration observed in nonhuman primate renal transplantation (~30 μg/ml serum levels → 40 μg/ml chosen for low dose) [25]. These data also served as basis for the clinical studies leading to the approval of belatacept in human renal transplantation [19, 28, 29], strengthening the importance of this study for clinical translation. CTLA4Ig was added either at the beginning of *in vitro* Treg induction culture or 24 h before cells were harvested and used for further analysis. Net Proliferation of total CD4+ T cells was reduced when TGFβ was added, which is consistent with previous findings. Importantly, CTLA4Ig had no detrimental effect on cell proliferation in the presence of TGFβ (Figures 1(b) and 1(c)), whereas in the absence of TGFβ, the same concentration of CTLA4Ig is sufficient to block T cell proliferation almost completely (data not shown and [14]). Moreover, we observed significantly increased proliferation in the presence of CTLA4Ig in a dose-dependent manner.

3.2. Induction of Regulatory Phenotype In Vitro Is Not Impaired by CTLA4Ig. Consistent with literature [24] and our previous results, TGFβ induced a regulatory phenotype, indicated by de novo FoxP3 expression in the majority of $CD4^+$ cells and upregulation of Treg-associated markers CD25, CD62L, and CTLA4 (Figures 2(a)–2(c)). The proportion of FoxP3-expressing cells, namely, $CD4^+CD25^{+-}$ $FoxP3^+$ Tregs, was significantly higher in cultures containing TGFβ, irrespective of the additional presence of CTLA4Ig (Figure 2(a)). Low-dose treatment with CTLA4Ig led to a significant increase in the percentage of $CD4^+CD25^+FoxP3^+$ Tregs (Figure 2(b)), but there was no considerable effect on the expression of CD62L or CTLA4 (Figure 2(c)), which are both considered to be important for *in vivo* Treg function and are considered to be important surface markers of Tregs [4, 30]. High doses of CTLA4Ig on the other hand led to a



(a)

Figure 2: Continued.

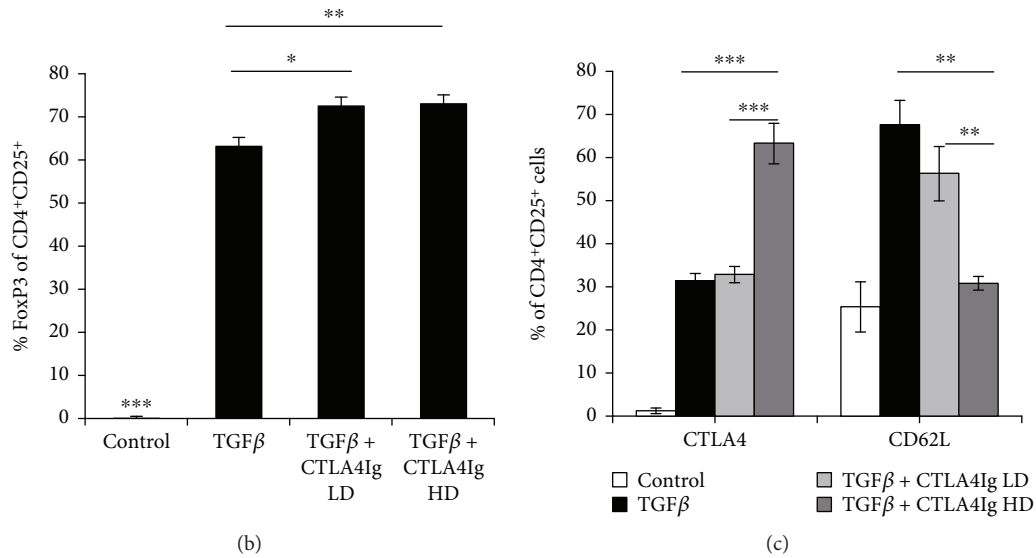


Figure 2: CTLA4Ig enhances the proportion of induced Tregs *in vitro*. (a) Representative histograms of Treg markers are shown for different culture conditions (gated on total leucocytes). $CD4^+CD25^+$ T cells were analyzed (b) for the expression of FoxP3 (indicating induction of regulatory phenotype) by intracellular FACS staining after 6 days of in vitro culture ± CTLA4Ig and (c) Treg-associated markers CTLA4 and CD62L, which were analyzed and compared between groups. Cells were plated in triplicates for each culture condition. Data are representative for 3 independent experiments; control indicates CD4 T cells stimulated with anti-CD3/CD28 in the presence of IL2. Error bars represent standard deviation. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$.

significant increase in CTLA4 expression but also a significant decrease of CD62L expression (Figure 2(c)). Thus, the presence of CTLA4Ig does not impair but rather promotes induction of regulatory phenotype via TGFβ and the expression of FoxP3.

3.3. Tregs Induced via TGFβ In Vitro in the Presence of CTLA4Ig Are Not Impaired in Suppressor Function. To test the suppressive potential of iTregs generated in the presence of CTLA4Ig, we performed coculture assays to determine their potential to suppress proliferation of naïve cells in response to allogeneic or polyclonal stimulation. Titrated numbers of in vitro induced iTregs ± CTLA4Ig were added to MLRs in which unseparated B6 responder splenocytes were stimulated with irradiated Balb/c cells (Figure 3(a)). We could show that iTregs induced in the presence of varying doses of CTLA4Ig suppressed T cell proliferation in response to alloantigen in a dose-dependent manner. In comparison to control iTregs, LD CTLA4Ig Tregs showed increased potential for suppression at all cell doses tested.

Next, we wanted to determine the potential of Treg to suppress polyclonal activation after T cell stimulation with anti-CD3. We could show that iTregs induced in the presence of CTLA4Ig were able to suppress T cell proliferation similar to iTreg controls (Figure 3(b)).

These findings imply that the presence of CTLA4Ig during Treg generation has no negative effect on the suppressor function of *in vitro* induced iTregs. Interestingly, there is a trend towards increased suppressor function by Tregs generated in the presence of CTLA4Ig in response to allogeneic rather than polyclonal stimulation.

3.4. CTLA4Ig Preserves the Ability to Produce IL-10 and Prevents Conversion to IL-17-Producing Cells. Several reports have demonstrated that TGFβ-induced iTregs can redifferentiate into FoxP3-negative conventional T cells upon restimulation in the absence of TGFβ, which suppresses Th1 and Th2 differentiation [31]. Moreover, differentiation into IL-17-producing Th17 cells is not inhibited by the presence of TGFβ and intermediate differentiation stage $IL17^+FoxP3^+$ T cells have been described [32]. We therefore aimed to determine whether the presence of CTLA4Ig affects the cytokine profile, especially the regulatory cytokine IL-10 and the inflammatory cytokine IL-17 (Figure 4(a) and 4(b)). The presence of CTLA4Ig during Treg generation did neither impair production of anti-inflammatory nor enhance production of proinflammatory cytokine IL-17, as determined by ELISA.

3.5. CTLA4Ig Suppresses T Cell Proliferation in the Absence of Antigen-Presenting Cells. The CTLA4Ig concentrations used in iTreg induction experiments have been previously shown to inhibit alloresponses *in vitro* by binding on B7 on APCs and therefore preventing T cell activation via CD28 [33]. However, little is known about the effect of CTLA4Ig on T cells as it is commonly assumed that B7 expression is restricted to APCs and activated T cells can also express B7 [34]. When we used CTLA4Ig in a polyclonal, APC-free proliferation assay, we revealed a dose-dependent inhibition of CD4 T cells; notably, this effect was impeded by high doses of IL-2 (Figure 5).

Taken together, these findings indicate that costimulation blocker CTLA4Ig does not negatively impact TGFβ-mediated conversion of Tregs in terms of proliferation,

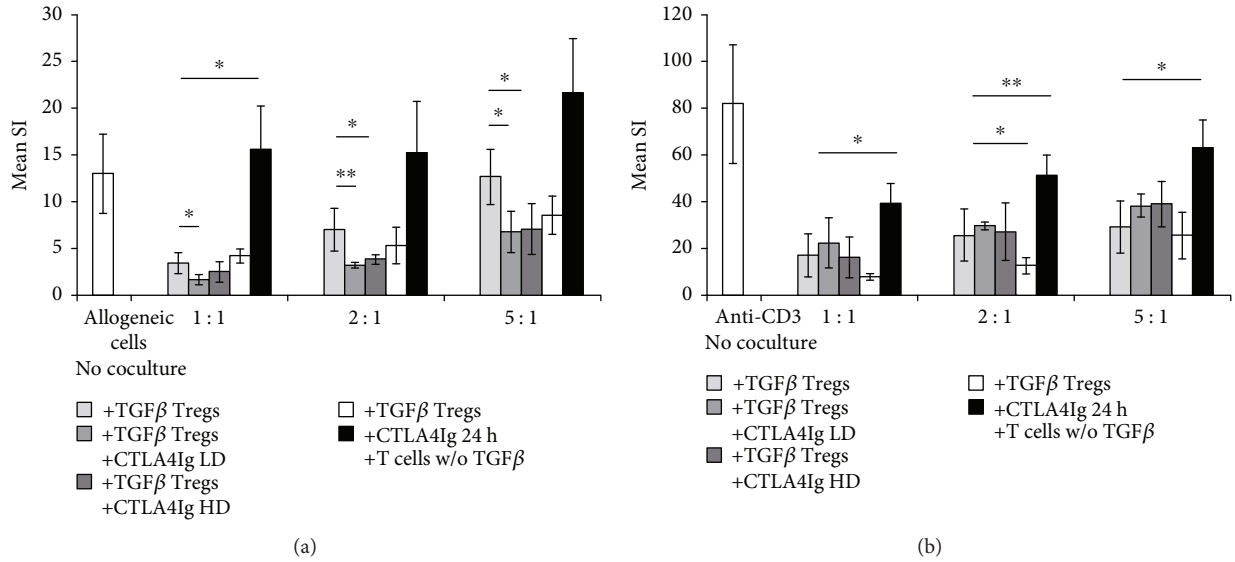


Figure 3: Suppressive potential of *in vitro* induced iTregs is not impaired by CTLA4Ig. For *in vitro* suppressor assays, titrated numbers of Treg-enriched cells (after cultivation with TGF$\beta \pm$ CTLA4Ig; Teff : Tregs) were added to 4×10^6 naïve B6 responder cells (responder cells: Tregs). Responder cells were stimulated with (a) 4×10^6 fully allogeneic BALB/C stimulator cells (irradiated) or (b) by polyclonal stimulation with plate-bound anti-CD3. Stimulation indices (SI; calculated in at least triplicates divided by pooled medium controls) of coculture suppression by Tregs induced in the presence of CTLA4Ig were compared to TGFβ Treg controls. Results are representative for 3 independent experiments; error bars indicate standard deviation. $^*p < 0.05$ and $^{**}p < 0.01$ in comparison to TGFβ Tregs w/o CTLA4Ig.

FoxP3 expression, phenotype, *in vitro* suppressive capacity, or cytokine profile. In the experiments shown herein, we observed a positive effect on Treg conversion and suppressive capacity by the presence of CTLA4Ig, suggesting a possible interaction with B7 molecules expressed on T cells.

4. Discussion

Adaptive peripheral $CD4^+CD25^+FoxP3^+$ Tregs (pTregs) can be deliberately generated from $CD4^+CD25$ conventional T cells *in vivo* under conditions including the presence of suboptimal antigen concentration or antigen delivery via nonimmunogenic methods such as oral or intravenous injection, peptide pumps, or antibody-mediated DC targeting in the absence of adjuvants [35]. In this study, we tried to mimic pTreg generation under defined experimental conditions in an APC-free system in order to directly evaluate a possible impact of the costimulation blocker CTLA4Ig. Although there are substantial differences between *in vitro* induced iTregs and *in vivo* induced pTregs, we think that this study adds valuable mechanistic knowledge regarding a possible negative role of CTLA4Ig during Treg conversion.

The expression of B7 molecules is not only exclusively restricted to APCs but may also occur on T cells upon activation [34, 36]. The role of B7 on APCs has been thoroughly studied while their role for T cells remains largely unknown. Taylor et al. showed that B7 expression by T cells is essential for downregulating immune responses through CTLA4 [37]. In line with this, B7 knockout T cells are resistant to Treg-mediated suppression via the CTLA4 pathway [38]. Moreover, it has been reported that CTLA4Ig inhibits T cell proliferation in a purified CD4 T cell proliferation assay upon stimulation with anti-CD3 [39]. This observation suggests that CTLA4Ig either inhibits T-T cell interactions via the B7-CD28 pathway or induces a negative stimulus in the T cell. However, the short cytoplasmic tails of B7.1 and B7.2 question the latter assumption [40]. Considering that T cells do provide costimulatory help to each other, it seems conceivable that CTLA4Ig covers B7 molecules on T cells and thereby increases the available targets for the anti-CD28 antibody in the *in vitro* iTreg generation system. Costimulation via antibody cross-linking induces a supraphysiological signal which could hypothetically explain improved iTreg induction in the presence of CTLA4Ig [41].

Numerous reports have tried to uncover the relationship between Treg and CTLA4Ig after the introduction of the first rationally designed selective T cell costimulation blocker in the clinics. Initially designed for treatment of autoimmune diseases (abatacept; approved for rheumatoid arthritis in 2005), it was mutated to induce higher avidity binding—especially for CD86—for the prophylaxis of organ rejection (belatacept; approved for renal transplantation in 2011). Although CTLA4Ig was initially envisioned to induce tolerance towards solid organ allografts by selective T cell costimulation blockade, which was intended to lead to anergy and tolerance, concerns arose whether it has a potentially detrimental impact on Tregs. Recently, it has been shown that Tregs depend on CD28 signaling during development in the thymus [42]; however, this might be a concern for tTreg rather than pTreg development. Other data suggest that post maturational CD28 signaling is important for Treg function [43] which was demonstrated by the use of a Treg-specific CD28 conditional knockout mouse. Although these are vital data for the understanding of the CD28/CTLA4/B7 pathway, it does not exactly mimic the situation under CTLA4Ig treatment. Recently, it was postulated that CD28

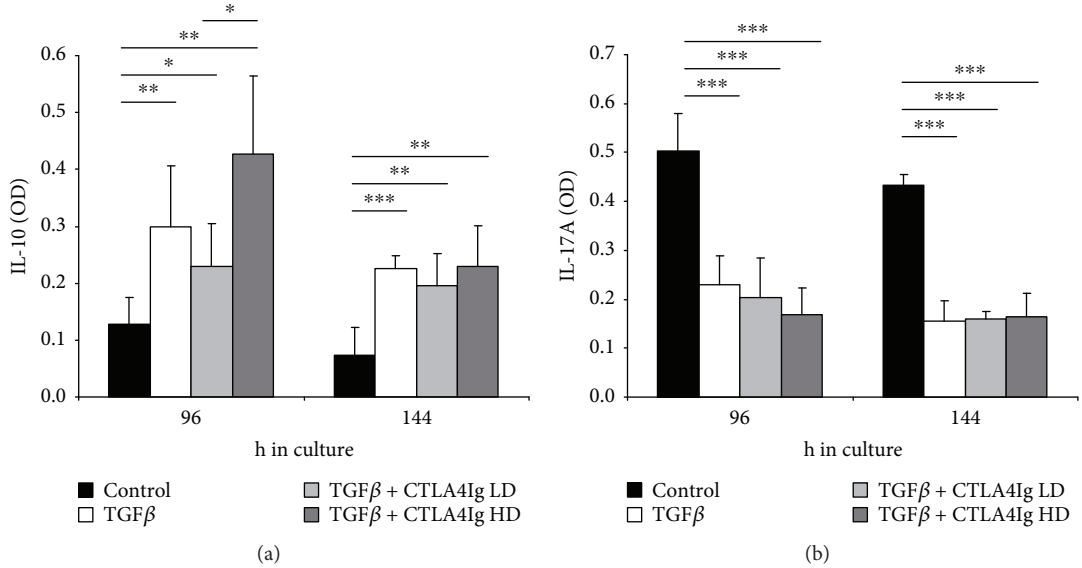


Figure 4: The presence of CTLA4Ig does not interfere with cytokine release *in vitro*. Supernatant of in vitro Treg induction cultures was collected at different time points and analyzed for the change in cytokine expression. (a) $CD4^+$ T cell cultures cultivated in the presence of TGFβ showed significant increase in the production of suppressive cytokine IL-10 and (b) a significant decrease in the production of inflammatory cytokine IL-17A. Data were obtained from cells cultivated in triplicates and are representative for 3 independent experiments; control indicates CD4 T cells stimulated with anti-CD3/CD28 in the presence of IL2. Mean values for optical density (OD) are shown; error bars indicate standard deviation. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$.

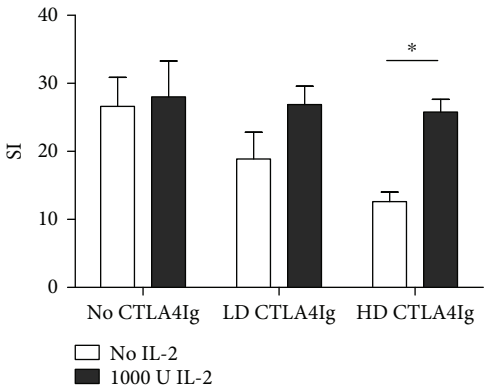


Figure 5: CTLA4Ig directly inhibits CD4 T cell proliferation *in vitro*. For proliferation assays, 4×10^6 T naïve B6 responder cells were stimulated with plate-bound anti-CD3 in the presence or absence of high levels of IL-2. CTLA4Ig was added to T cell cultures at different doses. Stimulation indices (SI; calculated in at least triplicates divided by pooled medium controls) were compared to controls without costimulation blockade. Results are representative for 3 independent experiments; error bars indicate standard deviation. $^*p < 0.01$.

signaling is the main driver behind Treg proliferation but CTLA4:CD80/CD86 interactions are also needed to control homeostatic proliferation [44].

Although the main function of CTLA4 *in vivo* is thought to be T cell extrinsic, there are multiple proposed mechanisms about additional cell intrinsic functions [40]. Uncovering of cell intrinsic functions is complicated by the fact that ligation by CTLA4-specific antibodies might not reflect physiologic balance of CTLA4/CD28 engagement with its natural ligands; nonetheless, several negative signaling pathways to intrinsically inhibit T cells proliferation have been identified. Although several negative signaling scenarios induced by anti-CTLA4 antibodies have been described, no cell intrinsic signals driven by natural ligands have been confirmed [45]; thus, it is rather unlikely that CTLA4Ig causes negative effects by inhibiting CTLA4 signaling.

Several studies intended to evaluate Tregs in transplant patients under belatacept treatment; however, concomitant immunosuppressive regimen complicates interpretation of these results. Whereas some groups reported no short- or long-term effects on Treg numbers and function when compared to treatment with CNIs [46, 47], others reported a decrease in Treg and FoxP3 mRNA levels [48]. The only conclusion from clinical experience with CD28 blockade via CTLA4Ig, which could be agreed on, was the fact that induction of tolerance with CTLA4Ig and current concomitant regimens was unlikely [13, 49]. In mouse models, on the other hand, CTLA4Ig treatment seems to be able to favor regulatory mechanisms in order to induce an operational tolerant state. When we examined the effect of costimulation blockade via CTLA4Ig on Tregs in a dose-dependent murine heart transplantation model, we found that although Treg numbers were initially decreased, they normalized under long-term treatment with CTLA4Ig and that there is a synergy between CTLA4Ig and Tregs when CTLA4Ig is given at nonsaturating doses [20]. Moreover, CTLA4Ig and Treg cell transfer act synergistically in an irradiation-free mixed chimerism model, which is strongly dependent on intragraft regulation [50]. *In vitro* studies have also shown

immunomodulatory potency for CTLA4Ig by preservation of tTregs [22], promotion of Treg conversion [51], and inhibition of effector responses via a Treg/TGFβ-dependent pathway [52]. On the other hand, some studies demonstrated that CTLA4Ig interferes with tolerance by the inhibition of Treg expansion [53, 54], suggesting that there is a complex relationship between CTLA4Ig treatment and Tregs and a better understanding is warranted before synergy between them can be predicted in a specific model. Another theory coming from autoimmune research, which is underlined by several reports, suggests that anergy (as induced by costimulation blockade) is an intermediate between auto-/alloreactive T cells that eventually become Tregs [55]. This is in line with the infectious tolerance model, which was proposed by Kendal and Waldmann [56].

Here, we have shown that CTLA4Ig does not negatively impact Treg conversion via TGFβ *in vitro*, which in our opinion is of major relevance as it mimics the generation of allospecific pTregs in the periphery. Clinical data and murine studies suggest that in long-term kidney transplant patients, indirect allospecific T cells mainly contribute to late graft rejection [13, 57, 58]. As tTregs and pTregs are generally believed to represent distinct TCR repertoires, several reports have suggested a division of labor between those subsets [59]. It has been suggested that while tTregs mainly participate in the inhibition of T cell trafficking in the allograft, pTregs primarily prevent T cell priming by acting on APCs [60]. Our data clearly demonstrate that the presence of CTLA4Ig does not interfere with Treg conversion or proliferation *in vitro*. More importantly, Treg suppressive capacity as well as cytokine production is not impaired even with high doses of the costimulation blocker.

In summary, data from clinical trials using belatacept instead of CNIs show that both immunosuppressive regimens lead to a (transient) decrease of Tregs and impaired suppressor function. Nevertheless, impairment of Tregs is not worse under belatacept treatment, which results in better patient and graft survival [29, 61, 62], making it favorable over CNI-based immunosuppressive regimens.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

There is no conflict of interest related to this paper.

Acknowledgments

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Review Article

Regulatory Role of CD4$^+$ T Cells in Myocarditis

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Myocarditis is an important cause of heart failure in young patients. Autoreactive, most often, infection-triggered CD4$^+$ T cells were confirmed to be critical for myocarditis induction. Due to a defect in clonal deletion of heart-reactive CD4$^+$ T cells in the thymus of mice and humans, significant numbers of heart-specific autoreactive CD4$^+$ T cells circulate in the blood. Normally, regulatory T cells maintain peripheral tolerance and prevent spontaneous myocarditis development. In the presence of tissue damage and innate immune activation, however, activated self-antigen-loaded dendritic cells promote CD4$^+$ effector T cell expansion and myocarditis. So far, a direct pathogenic role has been described for both activated Th17 and Th1 effector CD4$^+$ T cell subsets, though Th1 effector T cell-derived interferon-gamma was shown to limit myocarditis severity and prevent transition to inflammatory dilated cardiomyopathy. Interestingly, recent observations point out that various CD4$^+$ T cell subsets demonstrate high plasticity in maintaining immune homeostasis and modulating disease phenotypes in myocarditis. These subsets include Th1 and Th17 effector cells and regulatory T cells, despite the fact that there are still sparse and controversial data on the specific role of FOXP3-expressing Treg in myocarditis. Understanding the specific roles of these T cell populations at different stages of the disease progression might provide a key for the development of successful therapeutic strategies.

1. Introduction

Myocarditis represents a polymorphic, frequently infection-triggered, and immune-mediated inflammation of the heart muscle [1]. Most often, it resolves spontaneously, but in susceptible individuals, it can progress to a chronic stage, which finally results in pathological cardiac remodelling. Pathological remodelling includes tissue fibrosis, hypertrophy, and apoptosis of cardiomyocytes and results in a phenotype of dilated heart chambers with impaired contractility (inflammatory dilated cardiomyopathy (iDCM)). Patients with iDCM develop heart failure with high mortality [2]. In children, myocarditis leads to cardiomyopathy in 46% of affected individuals [3], and up to 20% of sudden death cases in young adults have been reported to be due to myocarditis [4]. Diagnostic gold standard is myocardial biopsy, despite a lack of sensitivity, mainly due to sampling error [2, 5]. Nevertheless, appropriate histological, immunohistochemical, and molecular biological workup of sufficient numbers of heart biopsies greatly improved diagnostic accuracy and allows meanwhile not only a morphological classification but also detection of replicating viral genomes in the heart [6, 7].

Viral infections are the most frequent cause of myocarditis along with some bacteria, and protozoa. Moreover, toxins, vaccines, and several drugs, as well as systemic autoimmune diseases, can also trigger heart-specific autoimmunity and inflammation [8]. Following tissue damage of any cause, the release of cardiac self-antigens and activation of scavenging self-antigen-presenting dendritic cells in draining lymph nodes may result in a breakdown of heart-specific tolerance triggering production of heart-specific autoantibodies, autoreactive CD4$^+$ T cell expansion, and autoimmunity [9, 10]. Various intracellular cardiac peptides, surface receptors, and mitochondrial antigens had been reported as markers of cardiac injury [11], but not all of them are heart specific or promote autoimmunity. Autoantibodies to both cardiac troponin T and I had been detected in sera of mice and men, but only immunization with troponin I led to myocarditis in mice [12, 13]. Autoantibodies to beta1-adrenoceptors

had been shown to promote dilated cardiomyopathy in rodents [14, 15] and are associated with adverse outcome in patients with dilated cardiomyopathy [16, 17] or Chagas heart disease [18]. Patients with dilated cardiomyopathy also demonstrate increased serum levels of autoantibodies to M(2) muscarinic acetylcholine receptor. In mice, adoptive transfer of M(2) muscarinic acetylcholine receptor-specific splenocytes induces myocarditis, with T cell infiltrations in the heart and a dilated cardiomyopathy-like phenotype [19]. Epitopes of the alpha-myosin heavy chain (α-MyHC) peptide are heart specific, highly immunogenic in various animal models, and associated with autoantibodies and T cell-mediated myocarditis both in mice and humans [20–23].

$CD4^+$ T cells were defined as main drivers of heart-specific autoimmunity in myocarditis [24–27]. Expansion of heart-specific effector $CD4^+$ T cells is facilitated in humans and mice due to a high frequency of circulating naïve α-MyHC-specific $CD4^+$ T cells. The high frequency of α-MyHC-specific $CD4^+$ T cells is a result of defective negative selection in the thymus. In fact, transcripts of *Myh6*, the gene encoding murine α-isoform of myosin heavy chain, are absent in mouse medullary thymic epithelial cells. Humans also do not express α-MyHC in mTECs. Accordingly, patients with inflammatory cardiomyopathy demonstrate increased T cell responses against α-MyHC [28]. Taken together, a natural gap in negative selection of α-MyHC-specific $CD4^+$ T cells can explain susceptibility to heart-specific autoimmunity in the context of tissue damage, self-antigen release, or exposure to pathogen-derived molecules mimicking cardiac proteins [29].

Effector $CD4^+$ T cells (Teff) were reported to be critical for myocarditis development in patients and animal models [24, 30]. Starting from their naïve form, $CD4^+$ T cells differentiate into either mature effector or regulatory cell populations with distinct functions [31, 32]. Aside from $CD4^+$ T cell subsets including regulatory T cells (Treg), several other cell types can exert a regulatory suppressive function in myocarditis development. Such cells include bone marrow-derived progenitor cells, $CD8^+$ T cells, monocytes/alternatively activated macrophages, or dendritic cells [33–37]. Regulatory T cells, expressing forkhead box P3T (FOXP3), suppress effector cells and maintain immune homeostasis and tolerance in various autoimmune disease models, but their role in myocarditis is still debatable [38–41]. Importantly, there is functional polymorphism and high plasticity in all the different T cell subpopulations [42, 43]. In fact, the regulatory role of the different T cell subtypes in myocarditis highly depends on the stage of disease and on a complex and not yet understood interaction between different inflammatory heart infiltrating and heart resident cell types. IFN-γ-producing Th1 effector T cells can convert to suppressor cells [44]. Vice versa, Treg are also able to produce proinflammatory cytokines under certain conditions [45]. In fact, dual IL-17-producing $FOXP3^+$ regulatory T cells may play a critical role in controlling inflammatory balance in humans [46]. Whether these observations are also valid in the context of cardiac inflammatory diseases is not known, however. In this review, we will focus specifically on the regulatory role of different $CD4^+$ T cell subtypes in general in the context of myocarditis and its progression to inflammatory dilated cardiomyopathy. Our current knowledge largely bases on mouse and rat models of viral and experimental myocarditis, as well as from observational studies on patients with myocarditis or inflammatory dilated cardiomyopathy.

2. $CD4^+$ T Cells as Critical Mediators of Heart-Specific Autoimmunity

Autoimmune mechanisms play an important role in myocarditis development and in its progression to inflammatory dilated cardiomyopathy. In patients and mice with myocarditis, heart-specific autoantibodies can be detected [5, 11]. The role of these autoantibodies for disease induction and progression, however, is still largely speculative [47]. In patients with acute myocarditis, biopsies demonstrate accumulation of T cells and macrophages, as well as other inflammatory cells in close contact to injured cardiomyocytes [48, 49]. Many studies, most of them based on mouse models, indicate an exclusive role for $CD4^+$ T cells in myocarditis development and progression. Susceptible mouse strains develop myocarditis after viral, especially coxsackievirus B3 (CVB3), infection [50], as well as upon injections of α-MyHC peptide together with complete Freund's adjuvant [51] or activated *in vitro* α-MyHC-loaded bone marrow-derived dendritic cells [9]. Transgenic mice carrying a $CD4^+$ T cell receptor specific to cardiac myosin spontaneously develop myocarditis progressing to lethal-dilated cardiomyopathy [52]. In all of these mouse models, myocarditis is associated with a marked α-MyHC-reactive effector T helper (Th) cell response. These cells are directly pathogenic, because adoptive transfer of heart-specific $CD4^+$ T cells can induce myocarditis in irradiated recipients, SCID, or $Rag2^{-/-}$ mice [24].

3. T Cell Maturation: Where Is the Breach?

Random recombination in the generation of the diversity of the T cell receptor (TCR) repertoire is an important evolutionary mechanism allowing T cells to specifically recognize and eliminate a large variety of foreign antigens. However, it also harbours potential danger of generating self-reactive clones. Under normal healthy conditions, there are two distinct stages of central and peripheral tolerance, which prevent autoimmunity during the development and activation of T cells. Central tolerance is based on clonal deletion and clonal diversion and is responsible for extracting self-reactive lymphocytes in the thymus [53]. Positively selected for their ability to recognise MHC complexes, thymocytes migrate to the thymic medulla and undergo a process of negative selection. Bearing strongly self-reactive TCRs, cells respond to self-peptide-MHC complexes on medullary thymic epithelial cells and receive apoptotic signals. The autoimmune regulator (AIRE) protein has been shown to play a major role in expression of self-tissue-specific epitopes in these complexes [54]. Humans and mice with compromised or absent AIRE suffer from variable severe autoimmunity in almost all their organs [55]. Just recently, Fezf2, another transcription factor, was introduced to directly

regulate various tissue-restricted antigen genes in mTECs independent of AIRE [56]. The key mechanisms however are still largely unknown. Meaningfully, and as mentioned above, the expression of α-MyHC is missing in both, humans and mouse mTECs, leading to a defect in negative selection of alpha-myosin heavy chain-specific $CD4^+$ T cells in the thymus putting them at risk for autoimmune myocarditis development in the presence of self-antigen release and innate immune activation (Figure 1).

$CD45^+MHCII^+$ bone marrow-derived antigen-presenting cells (APC) constantly process heart-specific epitopes in the heart [57]. This observation was made in many rodent strains, including some, which are not susceptible to viral or immune-mediated myocarditis. Obviously, presentation of cardiac antigens alone is not sufficient for activation and expansion of Teff and myocarditis development. Activation of $CD4^+$ T cells requires not only interaction with a cognate antigen expressed on the MHC class II molecule but also costimulatory signals, such as those mediated by CD28 ligation [58]. In the absence of a local inflammatory milieu, DCs do not express sufficient amounts of costimulatory B7 family molecules and are supposed to play a tolerogenic role. $CD4^+$ T cells interacting with MHC peptide without costimulatory signal undergo anergy, repression of TCR signalling, and IL-2 production [59].

Surface APC molecules programmed death 1 receptor (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) play an important role in T cell anergy. Deficiency in PD-1 or CTLA-4 leads to impaired peripheral tolerance and enhanced T cell activation [60]. In fact, mice deficient for CTLA-4, PD-1, or its ligand demonstrate elevated numbers of effector T cells and develop severe autoimmune myocarditis and DCM [61–64]. By maintaining signalling through these molecules, DCs mediate the peripheral conversion of naive T cells to Treg. Acute inflammatory processes in the heart, on the other hand, result in upregulation of MHC II peptide complexes as well as costimulatory molecules on the surface of DCs and enhance migration of DC to the draining lymph nodes, where they interact with circulating T cells. This leads to a breakdown of peripheral tolerance and differentiation of naïve T cells into an effector phenotype (Figure 1).

4. The Role of Treg in Myocarditis

Since first identified and described as suppressive "regulatory" T cells [65], Treg were intensively studied [66]. Regulatory $CD4^+CD25^+$ T cells represent a specific T cell population responsible for immune homeostasis and tolerance. Their frequencies in the circulation can widely differ depending on the conditions or stage of disease [67]. Treg express FOXP3 transcription factor, which is essential for active suppression of autoimmunity [68]. As other T cells, Treg mainly develop in the thymus, but can also develop in the periphery. Treg suppress autoimmune Teff populations as well as APCs involved in priming and activation via different cell-cell contact-dependent and contact-independent mechanisms. Treg produce inhibitory cytokines such as transforming growth factor beta (TGF-β) and IL-10 or express surface molecules with immunosuppressive properties such as CTLA-4 or glucocorticoid-induced tumor necrosis factor receptor (GITR) modulating immune processes [69–71]. Expansion of regulatory cells is an important mechanism to control autoimmunity. In mouse and rat models of experimental autoimmune myocarditis, EAM numbers of Treg conversely correlated with disease severity. Moreover, the proliferation capacity and inhibitory activity of Treg increased in animals immunized for EAM induction [72, 73]. Adoptive transfer of $CD4^+$ T cells depleted from highly efficient glucocorticoid-induced TNFR family-related gene/protein-expressing Treg resulted in more severe myocarditis in T cell-deficient BALB/c nude mice [74]. Furthermore, adoptive transfer of Treg protected mice from CVB3-induced myocarditis [75] and from progression to cardiomyopathy, if injected after clearance of the acute virus infection [76, 77].

Differences in numbers of circulating Treg explain variations in the susceptibility of different mouse strains to EAM. Comparison of A.SW and B10.S mouse strains sharing the same MHC haplotype showed that development of severe disease in A.SW mice correlated with a lower relative frequency of Treg among the total $CD4^+$ T cell count, compared to resistant B10.S animals [26]. Moreover, gender differences in myocarditis development were linked to differences in circulating Treg. Mice with increased estradiol levels, for example, increased numbers of Treg upon immunization and are less susceptible to CVB3-induced myocarditis [78]. Monocytic myeloid-derived cells from female but not male mice promoted expansion of $CD4^+IL\text{-}10^+$ Treg [36]. Furthermore, IL-10 producing Treg transferred to immunized Lewis rats efficiently suppressed myocarditis induction [79]. A decrease in IL-10 production and Treg numbers was also observed in α-MyHC/CFA-immunized mice after endothelin receptor blockade and resulted in exacerbated EAM [80]. IL-10 efficiently drives the generation of Treg [81] while its immunosuppressive effect includes decreasing MHC II complexes and B7 family costimulatory molecules on the APC surface [82–84]. IL-37 mediated activation of Treg, and IL-10 production downregulates the expression of Th17-related cytokines IL-6 and IL-17 and ameliorates CVB3-induced viral myocarditis [85]. IL-35, on the other hand, was shown not only to have suppressive activities [86] but also to convert naive T cells into a regulatory phenotype [87].

TGF-β directly suppresses self-reactive cells, as shown in models of experimental mouse colitis [88] and encephalitis [89], and protects mice against coxsackievirus-induced myocarditis [75]. Moreover, TGF-β launches a paracrine positive feedback loop converting naïve into regulatory $CD4^+$ T cells [90]. TGF-β, however, was shown to promote disease and adverse cardiac remodelling during later stages of myocarditis: TGF-β-mediated Wnt secretion promoted myofibroblast differentiation and myocardial fibrosis in EAM [91], while treatments targeting TGF-β prevented fibrosis and heart failure [92–94].

Human CTLA4 haploinsufficiency results in serious dysregulation in T and B lymphocyte homeostasis and specifically affects $FOXP3^+$ Treg cells [95]. CTLA-4 as a high-affinity receptor interacts with CD80/CD86 signalling

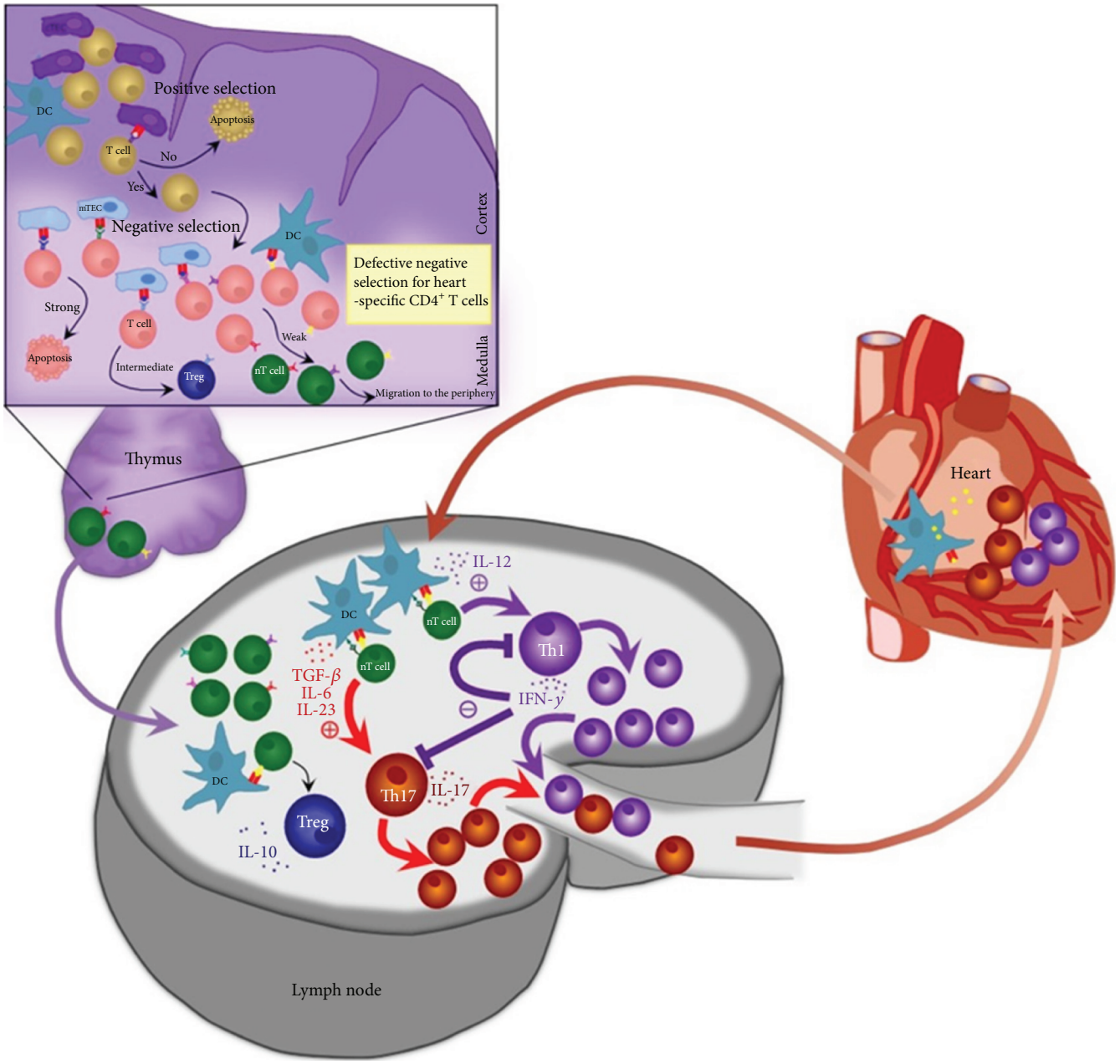

Figure 1: Role of $CD4^+$ T cells in myocarditis. Break of central tolerance: $CD4^+$ T cells undergo maturation and selection in the thymus. Due to a defect in negative selection, α-MyHC-specific $CD4^+$ T cells do not undergo anergy or apoptosis and are released to the periphery. Break of peripheral tolerance: Inflammation results in activation of α-MyHC-loaded DCs which upregulate MHC II-peptide complexes as well as costimulatory molecules on the surface and migrate to the draining lymph nodes, where they interact with circulating T cells. Activated through the TCR meeting cognate peptide and upon costimulation with CD28, naive heart-specific T cells differentiate to effector T cells entering the heart.

[96], causes elimination of these molecules via transendocytosis [97], and suppresses IL-2—a major T cell survival and expansion factor [98–100]. Adenovirus vector-mediated CTLA4Ig gene transfer in mice with EAM leads to downregulation of CTLA-4 and B7-2 proteins but upregulation of Treg, expression of FOXP3 and TGF-β mRNA, and alleviation of myocarditis [73]. Patients with Chagas heart disease demonstrate increased frequencies of suppressive IL-6^+, IFN-γ^+, TNF-α^+, and CTLA-4^+ Treg cells but a rather small $FOXP3^+CTLA\text{-}4^+$ Treg cell population [101, 102]. Reduction of CTLA-4 levels in $CD4^+$ T cells following disruption of T cell Ig mucin signalling during the innate immune response results in decreased Treg populations and increased inflammation in the heart [103]. A direct cytolytic effect of Treg is due to a granzyme B-dependent, perforin-independent mechanism [104] which allows them to eliminate target effector cells.

Interestingly, some observations demonstrate that early activation of Treg might be associated with exacerbation of

CVB3-induced myocarditis [105]. Other viral myocarditis models, however, demonstrate the ability of Treg to decrease virus-induced inflammation and to limit tissue damage associated with viral infection [106]. Thrombospondin-2, for example, protected against cardiac dysfunction in acute CVB3-induced viral myocarditis via activation of anti-inflammatory Treg [107]. Valproic acid was suggested as a promising drug in the therapy of viral myocarditis increasing the percentage of Treg cells and decreasing the percentage of splenic Th17 [108]. Moreover, an approach modulating Th17/Treg immune responses by inhibition of microRNA-155 resulted in a simultaneous decrease of both Th17 and Treg and reduced disease severity. These observations, however, suggest that improvement of EAM mainly resulted from the repressed Th17 response [109]. In Chagas myocarditis, granulocyte colony-stimulating factor administration promoted Treg recruitment and reduced cardiac inflammation and fibrosis [110]. In contrast, endogenous administration of $CD4^+CD25^+$ regulatory T cells during *Trypanosoma cruzi* infection was not at all protective in another study. Depletion of Treg via anti-CD25 monoclonal antibodies neither worsened nor improved the outcome of *Trypanosoma cruzi* infection [111].

Attenuation of acute cardiac inflammation by Treg seems to prevent progression of myocarditis to iDCM in humans [112, 113]. Patients with low responder T cell susceptibility to the suppressive function of regulatory T cells demonstrated progression of DCM [114], and an increase of Treg frequency after immunoadsorption therapy improved cardiac function in iDCM patients [115]. In modulating inflammatory responses and inhibiting proinflammatory cytokines, Treg also ameliorate adverse cardiac remodelling after myocardial infarction [116, 117]. Decreased frequencies of circulating Treg in patients negatively correlate with pro-inflammatory cytokines, such as IL-6, and are associated with a significantly higher incidence of recurrent hospitalization for worsening heart failure [118]. In addition, cell therapy with regulatory T cells prevents chronic rejection of heart allografts in a mouse model of mixed chimerism [119] and enhances mesenchymal stem cell survival and proliferation upon cotransplantation into ischemic myocardium in Yorkshire pigs [120].

5. Regulatory Role of $CD4^+$ T Effector Cells in Progression of Myocarditis to iDCM

Several observations support a role for $CD4^+$ T cells as major drivers of autoimmune myocarditis development [72, 121]. During myocarditis induction, various inflammatory cell subsets infiltrate the heart and produce proinflammatory cytokines, which create an amplification loop enhancing disease progression [72]. The crucial role of self-reactive $CD4^+$ T cells in myocarditis induction is well described [10], although mechanisms remain still poorly understood. It is established that IL-17-producing Th17 cells play a major role in initiation and development of myocarditis [122]. Though both Th1 and Th17 cooperate in disease progression and transition to iDCM [52], it was claimed that IFN-γ and IL-17 have antagonistic functions in myocarditis and inflammatory cardiomyopathy. Immunosuppressive strategies are beneficial for some patients with iDCM and myocarditis, without evidence on actively replicating viruses in heart biopsies [2]. Thus, elimination of Teff and their proinflammatory cytokines appears as a promising therapeutic strategy. Nevertheless, some contradictory findings have also been reported. It was shown recently that T cells—Treg, Th1, and Th17 in particular—possess great capacity to plasticity and are able to change their function and phenotype depending on the local milieu in tissue and lymph nodes. $CD4^+$ T cells often coexpress more than one specific cytokine [123]. Th17 cells, for example, often produce IL-17 and IFN-γ [124]. In fact, in a model of experimental autoimmune encephalomyelitis (EAE), it was shown that IL-23-induced IL-17-producing Th17 demonstrated plasticity, that is, the capacity to change their cytokine production profiles in different inflammatory settings. Using a reporter mouse strain designed to fate map cells that have activated IL-17A, Hirota et al. demonstrated that former Th17 cells produced almost exclusively IFN-γ and other proinflammatory cytokines in the spinal cord [125]. Another study of effector cell plasticity underlines the nonstability of the $IL\text{-}17^+/IFN\text{-}\gamma^+$ population and further differentiation to IL-17 or IFN-γ single-producing cells [126]. Both Th1 and Th17 undergo active expansion in autoimmune myocarditis, and the balance between these populations may strongly influence disease phenotype and outcome. It was observed that α-MyHC/CFA-immunized IFN-γ- and IFN-γR-deficient mice develop more severe and persistent myocarditis [127, 128], suggesting a protective regulatory role of IFN-γ in this disease model. While in wild-type mice inflammatory infiltrates largely subside within few days after the peak of disease, IFN-γR-deficient show ongoing expansion of autoreactive $CD4^+$ T cells, persistent inflammatory infiltrates, and enlarged, functionally impaired hearts with impaired nitric oxide production [128]. It was then confirmed that IFN-γ signalling is crucial for NO production by inducible nitric oxide synthase (NOS) 2 in tumor necrosis factor-α and NOS2-producing dendritic cells, which limit expansion of Teff and cardiac inflammation [33]. In fact, the progressive disease course in IFN-γR-deficient mice was associated with enhanced IL-17 release from heart-infiltrating Th17 cells. The EAM model also demonstrated that IL-17 recruits $CD11b^+$ monocytes confining disease progression in an IFN-γ-dependent manner [129]. Moreover, IFN-γ signalling was crucial for prevention of EAM by vaccination of mice with FMS-like tyrosine kinase 3 ligand pretreated, α-MyHC-loaded splenic $CD8\alpha^+$ DCs. In this experimental approach, DC vaccination enhanced the Th1 response, which was considered to negatively regulate expansion of Th17 effector cell expansion [130]. In line with these findings, IFN-γ-deficient mice also showed severely impaired systolic and diastolic functions and heart failure [131].

In a mouse model of adenovirus 1 infection-mediated myocarditis, depletion of IFN-γ during the acute phase of disease did not affect viral replication, but reduced cardiac inflammation protecting from remodeling and hypertrophy [132]. High IFN-γ levels correlated with cardiac damage and dysfunction in an autoimmune myocarditis model enhanced by purinergic receptor P2X7 deficiency [133]. Mice

lacking Regnase-1 and Roquin, RNA-binding proteins that are essential for degradation of inflammatory mRNAs, demonstrated increased expression of IFN-γ, but not IL-17, and suffered from severe inflammation and fibrosis in their hearts [134]. Dampening IFN-γ overexpression by Ebi3, a compartment of IL-27, prevented *T. cruzi*-induced myocarditis in mice [135]. Thus, although some studies indicate a protective role of IFN-γ as a negative regulator of Teff responses, the same cytokine can also contribute to myocardial inflammation and pathological remodeling.

Recent findings indeed suggest that Teff may play a dual role in myocarditis progression. IL-17 increases myocarditis severity during the acute inflammatory stage [31, 136]. In contrast, it was observed in a *T. cruzi* infection model that anti-mouse IL-17 antibody increased myocarditis severity and mortality [137]. IL-17 signalling via IL-17RA mediated recruitment of IL-10-producing neutrophils, which in turn protect from the development of fatal cardiomyopathy in this model [138]. In line with these findings, it has been shown that in human Chagas disease patients, low frequencies of IL-17-producing T cells correlate with more severe symptoms and cardiac dysfunction [139]. A link between Th17 and Treg has also been shown in a model of viral myocarditis. Neutralization of IL-17 in mice, with an anti-mouse IL-17Ab, resulted in a decrease in Treg counts and T reg cytokines (TGF-β, IL-10) [140]. In patients with inflammatory dilated cardiomyopathy, IL-17 seems essential for the transition of myocarditis to iDCM, but serum levels of IL-17 normalize within one year after the diagnosis, whereas cytokines like IL-6 and TGF-β remain permanently increased in these patients [141, 142]. Moreover, low serum concentrations of IL-17 were associated with a worse prognosis for patients after acute myocardial infarction [143].

Mice immunized with pcDNA3-hM2, a DNA plasmid carrying the entire muscarinic acetylcholine receptor M2 (M2AChR) cDNA sequence, develop anti-M2AChR-associated DCM mimicking the human cardiomyopathy phenotype. In this DCM model, mice lacking P2×7 receptors produced lower amounts of IL-17 and higher amounts of IFN-γ and showed more severe cardiac dysfunction at later stages of disease [133]. Finally, it was shown that mice lacking both cytokines, IL-17 and IFN-γ, simultaneously developed rapidly fatal EAM [144]. In line with these findings, unpublished observations from a group also point to a different role of IFN-γ and IL-17 in the development of cardiac fibrosis following acute myocarditis.

6. Outlook

Myocarditis development and its progression to iDCM are a very complex process. $CD4^+$ T cells are key players in the maintenance of peripheral tolerance, are critical for disease induction, are involved in the progression of acute inflammation to a chronic process of pathological remodelling, and may be part of negative feedback loops confining unlimited heart-specific autoreactive T cell expansion. So far, the delicate interplay between distinct $CD4^+$ T cell subsets such as Treg, Th1, and Th17 cells has only been partly deciphered.

Further studies in animal models, as well as in human tissue samples, will be required to fully understand the specific role of all different $CD4^+$ T cell subsets in myocarditis. Nevertheless, these mechanistic insights are a critical requirement for the development of novel therapeutic concepts and vaccination strategies.

Abbreviations

AIRE: Autoimmune regulator
APC: Antigen-presenting cells
cDNA: Complementary DNA
CFA: Complete Freund's adjuvant
CTLA-4: Cytotoxic T lymphocyte antigen-4
CVB3: Coxsackievirus B3
DC: Dendritic cells
EAE: Experimental autoimmune encephalomyelitis
EAM: Experimental autoimmune myocarditis
FOXP3: Forkhead box P3
GITR: Glucocorticoid-induced tumor necrosis factor receptor
(i) DCM: (Inflammatory) dilated cardiomyopathy
IFN-γ: Interferon-γ
IL: Interleukin
ILR: Interleukin receptor
M2AChR: Muscarinic acetylcholine receptor M2
MHC II: Major histocompatibility complex II
mTEC: Medullary thymic epithelial cells
α-MyHC: Alpha-myosin heavy chain
NO: Nitric oxide
NOS: Nitric oxide synthase
PD-1: Programmed death 1 receptor
TCR: T cell receptor
Teff: Effector T cells
TGF-β: Transforming growth factor beta
Th: T helper cells
TNF: Tumor necrosis factor
Treg: Regulatory T cells.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

The Imbalance of FOXP3/GATA3 in Regulatory T Cells from the Peripheral Blood of Asthmatic Patients

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Background. Treg cells play an important role in the pathogenic progress of asthma. *Objective*. To address the alterations of Treg cells in asthma. *Methods*. Proliferation-and function-associated markers of Treg cells along with the percentage of Treg cells producing some cytokine from asthmatics and healthy subjects were analyzed by flow cytometry. Besides, the expressions of USP21 and PIM2 in Treg cells were measured by cell immunochemistry after Treg cells were sorted. *Results*. Treg cells from asthmatic patients showed lower proliferation activity and were more likely to be apoptotic. These cells expressed lower levels of GITR, CTLA-4, Nrp-1, and IL-10 compared to those from the healthy control. Th2-like Treg cells increased in asthmatic patients, while the percentage of IFN-r$^+$ Treg cells was similar between two groups. Moreover, the percentage of IL-4$^+$ Treg cells is related to the asthma control. Treg cells from asthmatic patients expressed more FOXP3 as well as GATA3; the expression level of GATA3 negatively correlated with FEV1%pred. Increased expressions of USP21 and PIM2 in Treg cells from asthmatic patients were found. *Conclusion*. Treg cells decreased in asthmatic patients, with an impaired immunosupression function and a Th2-like phenotype, which may be due to overexpression of GATA3 and FOXP3, regulated by USP21 and PIM2, respectively.

1. Introduction

Asthma is a heterogeneous disease of the lung and the airway characterized by chronic inflammation, airway hyperresponsiveness (AHR), and tissue remodeling [1]. The prevalence of this disease has markedly increased over the past several decades and has now become one of the major global health problems affecting approximately 300 million people worldwide [2]. Asthma pathogenesis involves multiple cell types of innate and adaptive immunity [3]. A large body of data provided evidence that activated T helper type 2 (Th2) cells played a central role through producing cytokines such as IL-4, IL-5, and IL-13 [4, 5]. Moreover, accumulating evidences of the important effect of regulatory T (Treg) cells in the mechanism of asthma have been replicated in numerous studies.

Treg cells were initially described as a population of CD4$^+$T cells expressing the IL-2 receptor α chain (CD25) and CD45RB, able to protect mice from developing autoimmune diseases [6]. Further studies revealed that Treg cells also play an important role in other diseases, such as asthma. Mice deficient in Treg cells exhibit allergic inflammation within mucosal sites, specifically leading to pathology characteristic of asthma [7]. On the other hand, administration of galectin-9 attenuated the inflammation of *Dermatophagoides farinae*-induced chronic asthma in mice by expanding Treg cells and enhancing transforming growth factor-beta (TGF-β) signaling [8]. Our previous study showed that the percentage of Treg cells was significantly lower in the peripheral blood of patients with moderate to severe allergic asthma than in patients with mild asthma or the control group [4]. However, further studies are needed to investigate the other

alterations of Treg cell in patients with asthma besides cell number.

Forkhead box P3 (FOXP3), the specific transcription factor, plays a necessary and sufficient role in the development and function of Treg cells [9]. Other transcription factors were needed to coordinate with Foxp3 to weaken the immunological effect of effector T cells [10]. Among these transcription factors, GATA3 is crucial for the function of Treg cells in limiting Th2-type inflammatory responses, which indicates that GATA3 in Treg cells may be relevant to the pathogen of asthma.

GATA3 and FOXP3 can be regulated by different mechanisms. In the terms of GATA3, E3 deubiquitinase ubiquitin-specific peptidase 21 (USP21) can upregulate the expression of GATA3 in Treg cells [11]. USP21 belongs to the deubiquitinase family, which opposes the function of E3 ubiquitin ligases [12]. In previous studies, Zhang has revealed that USP21 interacted with GATA3 to promote its stability via deubiquitination and the knockdown of USP21 resulted in the downregulation of GATA3 protein levels in Treg cells [11]. What is more, in the recent published article, it was revealed that PIM2, a kind of serine/threonine kinases, could phosphorylate the Foxp3 N-terminal domain, thus negatively regulating Treg cell suppressive function by influencing the Foxp3 level and expression of Treg cell-associated surface markers [13]. Most importantly, the mRNA levels of USP21 and PIM2 were upregulated in the Treg cells of asthma patients [11]. So we hypothesized that there might be a change in the expressions of USP21 and PIM2 of Treg cells.

2. Methods

2.1. Subjects. The population consisted of patients with asthma aged between 16 and 65 years from Ruijin Hospital (Shanghai, China). The diagnosis of asthma was based on the GINA guidelines. Subjects had received a physical examination, spirometry, and asthma control assessment (ACQ-7 questionnaire). According to the GINA guideline, a score of 0.0–0.75was classified as well-controlled asthma and >0.75 as partly/poorly uncontrolled asthma. Subjects were excluded if they had experienced an asthma exacerbation in the previous four weeks or a respiratory infection in the previous a week. All subjects gave their written informed consent before participation, and this study was approved by the ethics committee of the hospital.

2.2. Reagents. The culture medium used was X-VIVO media (Lonza, USA) supplemented with 10% human AB serum, 1% GlutaMAX (Invitrogen, USA), 1% sodium pyruvate (Invitrogen, USA), and 1% penicillin/streptomycin (Invitrogen, USA). Fluorochrome-conjugated anti-CD4 was from BioLegend (USA). Fluorochrome-conjugated anti-Ki67, CTLA4, and GITR were from eBioscience (USA). Fluorochrome-conjugated anti-CD45RA, TGF-β, IL-10, IFN-γ, IL-4, IL-5, IL-13, FOXP3, and GATA3 were from BD (USA). PE Annexin V Apoptosis Detection Kit I, Cytofix/Cytoperm Kit, Pharmingen™ Leukocyte Activation Cocktail with BD GolgiPlug™, and Leukocyte Activation Cocktail with BD GolgiPlug were also from BD (USA). Anti-Human CD25 PerCP-Cyanine5.5 was from eBioscience (USA), and anti-Human CD25-PE was from BD (USA). Human Neuropilin-1 PerCP MAb was from R&D (USA). Anti-Human USP21 was purchased from Sigma-Aldrich Co. (USA), and anti-Human PIM2 was from Santa Cruz (USA); the secondary antibodies were from Sigma-Aldrich Co. (USA). Recombinant human cytokine IL-2 was purchased from R&D, and rIL-4 and TGF-β were from PeproTech (USA). Ficoll-Paque PLUS was purchased from GE Healthcare (UK). Human IL-10 ELISA Kit and Human TGF-β1 ELISA Kit were from RayBiotech (USA). Anti-CD3/CD8 Dynabeads were purchased from Invitrogen (USA).

2.3. Peripheral Blood Mononuclear Cell (PBMC) Isolation. Sixteen milliliters of peripheral blood was obtained in a sodium heparin vacuum tube. PBMCs were isolated by Ficoll-Paque PLUS according to the manufacturer's instruction; the cells isolated were divided into several shares for the flow cytometry analysis.

2.4. Flow Cytometry Analysis. Treg cells were identified as anti-CD4-positive and anti-CD25-positive. Where indicated, additional markers were evaluated using anti-Human Ki-67 PerCP-eFluor® 710, human Neuropilin-1 PerCP MAb, anti-Human CD152 (CTLA-4) PE, HU FOXP3 APC, and Gata3 PE. Before intracellular staining, Cytofix/Cytoperm Kit and Pharmingen Leukocyte Activation Cocktail with BD GolgiPlug were used for cell fixation and permeation.

PE Annexin V Apoptosis Detection Kit I was used to identify the apoptosis of Treg cells. For intracellular cytokine production, PBMCs were stimulated with Leukocyte Activation Cocktail with BD GolgiPlug for 3 hours before staining. Cytofix/Cytoperm Kit and Pharmingen Leukocyte Activation Cocktail with BD GolgiPlug were used for intracellular cytokine production. Intracellular staining was performed using APC Rat anti-Human IL-4, PE Rat anti-Human IL-5, APC Rat anti-Human IL-13, PE Mouse anti-Human IFN-γ, APC Rat anti-Human IL-10, and PE Mouse anti-Human TGF-β1.

Samples were detected with a FACS flow cytometer, and acquired data were analyzed with FlowJo software.

2.5. Treg Cells and Naïve T Cell Isolation. Fifty milliliters of peripheral blood was obtained in a sodium heparin vacuum tube, and PBMCs were isolated according to the above mentioned. PBMCs were stained with FITC anti-human CD4 antibody, anti-CD25-PE, and anti-CD45RA-APC at 4°C for 30 minutes in the dark, then $CD4^+CD25^+CD45RA^-$Treg and $CD4^+CD25^-CD45RA^+$ naïve T cells were isolated from PBMC by a BD FACSAria II sorter. $CD4^+CD25^+CD45RA^-$Treg cells were prepared for the analysis of the expressions of USP21 and PIM2. $CD4^+CD25^-CD45RA^+$ naïve T cells were cultured in vitro for the analysis of the ability to develop Treg cells.

2.6. The Expression of USP21 and PIM2 in Treg Cells. Freshly sorted $CD4^+CD25^+CD45RA^-$Treg cells were swung to the slide by StatSpin CytoFuge 12. Then, the cells in the slides were fixed in 4% paraformaldehyde for 10 minutes and permeabilized with 1% Triton-100. After being washed with

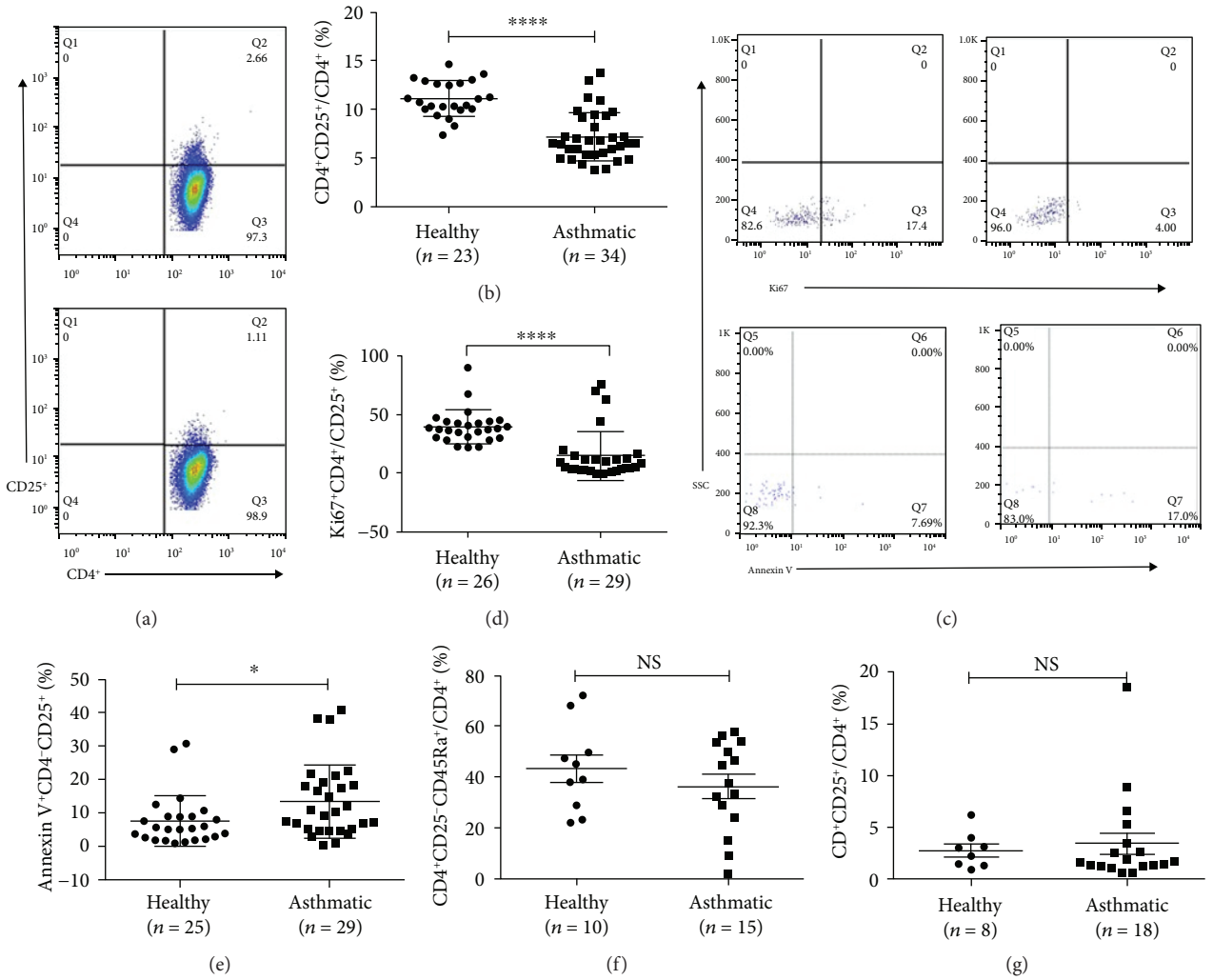

FIGURE 1: Deficiency of Treg cells in patients with asthma. (a) Representative examples of flow cytometry analysis of $CD4^+CD25^+$ Treg cells in healthy and asthmatic subjects. (b) Frequencies of $CD4^+CD25^+$ Treg cells in $CD4^+$ T cell subsets in healthy and asthmatic groups. (c) Representative examples of flow cytometry analysis of $Ki67^+$ and Annexin V^+ cells of $CD4^+CD25^+$ Treg cells in healthy and asthmatic subjects. (d) Frequencies of Ki67 and Annexin V (e) production in healthy and asthmatic subjects. (f) The percentage of $CD4^+CD25^-CD45RA^+$ T cells of $CD4^+$ T cell subsets in healthy and asthmatic groups. (g) Percentage of Treg cells induced from naïve T cells of healthy and asthmatic groups in Treg cell polarity. The graph shows means ± sem. $^*p < 0.05$ and $^{****}p < 0.0001$. NS: no significance.

PBS, samples were incubated with anti-Human USP21 or anti-Human PIM2 in 4°C overnight. After three washings with PBS, cells were incubated with secondary antibodies for 2 hours at room temperature. Then, USP21 or PIM2 was stained with DAB Color Developing Reagent Kit, and nuclei were stained with hematoxylin. These samples were examined on a Nikon Eclipse 50i microscope (Nikon, Japan), and the images were analyzed with Image-Pro Plus 6.0.

2.7. TGF-Beta-Mediated In Vitro Treg Cell Induction. Sorted $CD4^+CD25^-CD45RA^+$ naïve T cells were cultured with anti-CD3/CD8 Dynabeads at a cell-to-bead ratio of 1 : 3 in X-VIVO media supplemented with 10% human AB serum, 1% GlutaMAX, 1% sodium pyruvate, 1% penicillin/streptomycin, recombinant TGF-β (5 ng/ml), retinoic acid (10 nM), and a gradient of recombinant IL-4 (0, 0.625 ng/ml, 1.25 ng/ml, 2.5 ng/ml, 5 ng/ml, and 10 ng/ml, resp.). After 3 days, the $CD4^+CD25^+$ Treg cells were analyzed by flow cytometry.

2.8. Statistical Analysis. Data were graphed and analyzed by Prism 6 software (GraphPad). Statistical significance between two groups was determined by two-tailed Student's *t*-test. Pearson test was used for the analysis of correlation. *P* values less than 0.05 was considered statistically significant.

3. Results

3.1. Deficiency of Treg Cells in Patients with Asthma. Consistent with the previous studies, the percentage of Treg cells in the asthmatic group was lower than that in healthy group ((7.24 ± 2.49)% versus (11.13 ± 1.82)%, $p < 0.01$, Figures 1(a) and 1(b)). To further investigate the reason of the decreasing number of Treg cells, we analyzed the expression of Ki67

in Treg cells, and we found that Treg cells from the asthmatic group expressed less Ki67 than those from the healthy group ((15.04 ± 20.91)% versus (39.78 ± 14.22)%, $p < 0.05$, Figures 1(c) and 1(d)), which meant decreased proliferation of these cells. The same samples also showed evidence of increased apoptosis in the asthmatic group compared to healthy ones ((13.52 ± 11.0)% versus (7.62 ± 7.63)%, $p < 0.05$), as revealed by Annexin V staining (Figures 1(c) and 1(e)). In vitro, differentiation of $CD4^+CD25^-CD45RA^+$ naïve T cells into Treg cells was the same in both groups (healthy versus asthmatic (43.54 ± 17.11)% versus (36.60 ± 17.82)%, $p > 0.05$, Figure 1(f)). The culture of naïve T cells in Treg cell polarity showed that these cells in two groups had the same ability to differentiate into $CD4^+CD25^+$ Treg cells (healthy versus asthmatic (2.78 ± 1.72)% versus (3.46 ± 4.37)%, $p > 0.05$, Figure 1(g)).

3.2. Defective Function of Treg Cells in Immunosuppression. To establish whether Treg cells from patients with asthma are functionally competent, we investigated the expressions of Neuropilin-1 (Nrp-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR), which are related to the suppressive function of Treg cells. And results showed that all those markers were expressed in a lower level in the asthmatic group than those in the healthy group (healthy versus asthmatic group (8.30 ± 4.53)% versus (4.31 ± 2.49)%, $p < 0.01$; (17.81 ± 6.06)% versus (9.89 ± 6.52)%, $p < 0.01$; and (18.55 ± 10.38)% versus (9.20 ± 5.70)%, $p < 0.01$, respectively, Figures 2(a)–2(d)). Moreover, in agreement with the decreased level of IL-10 in the plasma (the data not shown), the percentage of $IL\text{-}10^+$ Treg cells was decreased (healthy versus asthmatic group (8.28 ± 7.56)% versus (1.41 ± 1.20)%, $p < 0.01$, Figure 2(e)), while the TGF-β^+ Treg cell percentage was similar between two groups ((13.46 ± 6.92)% versus (14.31 ± 8.80)%, $p > 0.05$, Figure 2(f)).

3.3. Increased Number of Producing Th2-Cytokine Treg Cells. Some researchers have found that Treg cells are unstable in vivo, and these unstable Treg cells play roles in the pathogenesis of these diseases [14]. For this reason, the expressions of Th2 cytokines, IL-4, IL-5, and IL-13, and Th1 cytokine IFN-γ were analyzed. As shown in Figure 3, the fraction of $IL\text{-}4^+$, $IL\text{-}5^+$, and $IL\text{-}13^+$ Treg cells increased significantly in the asthmatic group (healthy versus asthmatic group, (4.25 ± 3.26)% versus (7.20 ± 4.58)%, $p < 0.05$; (7.77 ± 13.00) versus (25.92 ± 22.72), $p < 0.01$; and (8.323 ± 10.04)% versus (17.14 ± 9.81)%, $p < 0.01$, respectively,), but no difference in IFN-γ^+ Treg cells between two groups (healthy versus asthmatic group, (4.29 ± 2.71)% versus (4.02 ± 3.98)%, $p > 0.05$) (Figure 4). According to patients' clinical symptoms and lung function, they were divided into two groups, the well-controlled and partly/poorly controlled group. We found that Treg cells from partly/poorly controlled asthma patients expressed more IL-4 than those from well-controlled asthma patients ((8.36 ± 5.09)% versus (5.12 ± 3.01)%, $p < 0.05$, Figure 4(a)). However, Treg cells from partly/poorly controlled asthma patients expressed similar levels of CTLA4, GITR, FOXP3, and GATA3, compared to those from well-controlled asthma patients (Figures 4(b)–4(e)). To clarify the role of IL-4 in the development of Treg cells from naïve T cells, we cultured naïve T cells with the concentration gradient of IL-4 and found that IL-4 failed to prevent naive $CD4^+$ T cell differentiation into Treg cells (Figure 4(f)).

3.4. Enhanced Expression of Specific Transcription Factors, Especially GATA3 in Treg Cells from Asthmatic Patients. In that some specific transcription factors play a decisional role in the development and maintenance of T cells, we checked the expression level of FOXP3 and GATA3 in Treg cells. Results showed that the expressions of FOXP3 (healthy versus asthmatic group, (89.81 ± 2.36)% versus (93.82 ± 2.93)%, $p < 0.01$, Figures 5(a) and 5(b)) and GATA3 (healthy versus asthmatic group, (5.37 ± 1.59)% versus (9.40 ± 5.31)%, $p < 0.01$, Figures 5(a), 5(c)) in Treg cells were increased in the asthmatic group. Interestingly, the increase in GATA3 expression was more obvious than in Foxp3 expression, leading to a decreased FOXP3/GATA3 ratio in the asthmatic group compared to the healthy group ((16.81 ± 17.13)% versus (18.34 ± 6.23)%, $p < 0.01$, Figure 5(d)). And we also found that FEV1%pred of patients with asthma was correlated with the percentage of $GATA3^+$ Treg cells (Figure 5(e)).

3.5. Increased Expression of USP21 and PIM2 in Treg Cells from Asthmatic Patients. Previous studies showed that both USP21 and PIM2 are regulators of GATA3 and FOXP3 expression, respectively, in Treg cells, so we measured the expressions of USP21 and PIM2 in Treg cells from patients with asthma and healthy subjects. Cell immunochemistry showed that Treg cells in the asthmatic group expressed more USP21 and PIM2 than Treg did cells in the healthy group (healthy versus asthmatic group, IOD value, (390.2 ± 586.2) versus (2557 ± 2698), $p < 0.01$; (183.5 ± 106.3) versus (1935 ± 1775), $p > 0.01$, respectively, Figure 6).

4. Discussion

We investigated the alterations of Treg cells in the peripheral blood from patients with asthma. Multiple studies have verified that asthma is a complicated process characterized by type 2 inflammatory reactions involving the coordination of innate and adaptive immune responses [15]. Treg cells can maintain the balance of immune self-tolerance and homeostasis via limiting aberrant or excessive inflammation [16, 17], and they can regulate the effector function of all T helper cells [18], including Th2 cells, which play a central role in the pathogenic development of asthma. Our previous study has demonstrated that there is an increased ratio of Th2/Treg cells in patients with moderate to severe asthma, which suggests that Th2/Treg imbalance has an important role in asthma [4]; however, the alteration of the function besides the number of Treg cells in asthma patients needs to be further investigated.

In this study, we found that the percentage of Treg cells from asthmatic patients markedly decreased accompanied by decreased proliferation and increased apoptosis, which gave us an appropriate explanation of the decreased

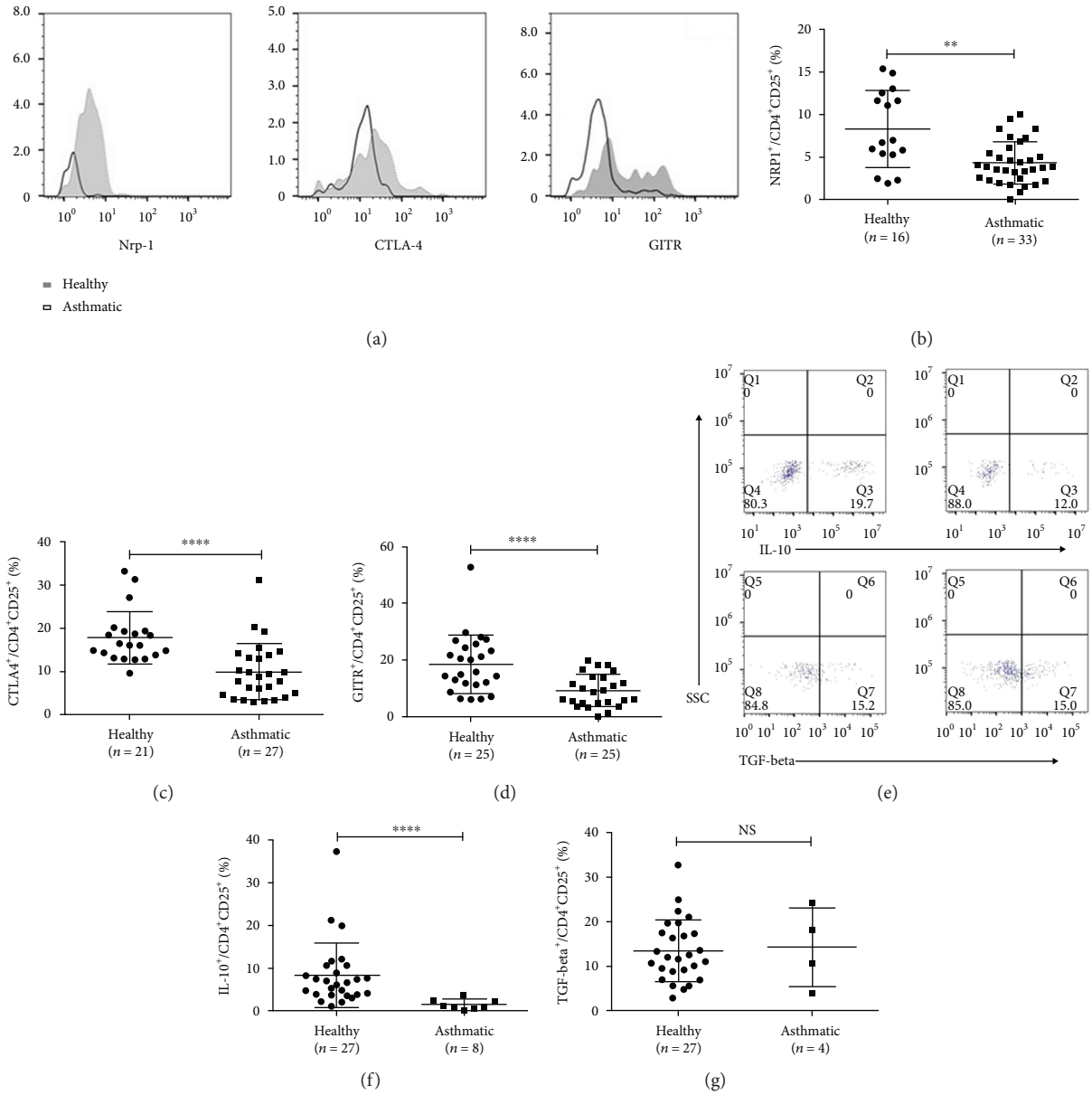

Figure 2: Defective function of Treg cells in immune-suppression. (a) Representative examples of flow cytometry analysis of Nrp-1, CTLA-4, and GITR expression by $CD4^+CD25^+$ Treg cells in healthy and asthmatic subjects. (b, c, d) Frequencies of Nrp-1 CTLA-4 and GITR expressed in healthy and asthmatic subjects. (e) Representative examples of flow cytometry analysis of IL-10 and TGF-beta production by $CD4^+CD25^+$ Treg cells in healthy and asthmatic subjects. (f, g) Frequencies of IL-10 and TGF-beta production in healthy and asthmatic subjects. The graph shows means ± sem. $^{**}p < 0.01$ and $^{****}p < 0.0001$. NS: no significance.

percentage of Treg cells in the asthmatic group. What is more, the same percentage of $CD4^+$ naïve T cells and Treg cells induced in the Treg polarity indicated that there was no difference in the differentiation of naïve T cells between asthmatic and healthy subjects. Therefore, to a great extent, the decreasing percentage of Treg cell in the patients with asthma may result from the decreased proliferation and increased apoptosis.

The change takes place not only in the number of Treg cells in asthmatic patients but also in their functions. Shevach concluded that Treg cells could secrete suppressor cytokines (IL-10 and TGF-β) that can directly inhibit the function of responder T cells and myeloid cells [19]. In addition, CTLA-4 on Treg cells can downregulate or prevent the upregulation of CD80 and CD86, the major costimulatory molecules on antigen-presenting cells [19]. Meanwhile, Nrp-1 can promote long interactions between Treg cells and immature DCs and restrict access of the effector cells to antigen-presenting cells so as to suppress the proliferation mediated by Treg cells when the responder T cells are stimulated with low concentrations of antigen [19]. All those molecules are of great importance in the function of Treg cells, so the expressions of these markers were analyzed to evaluate the function of Treg cells, and we found that Treg cells from

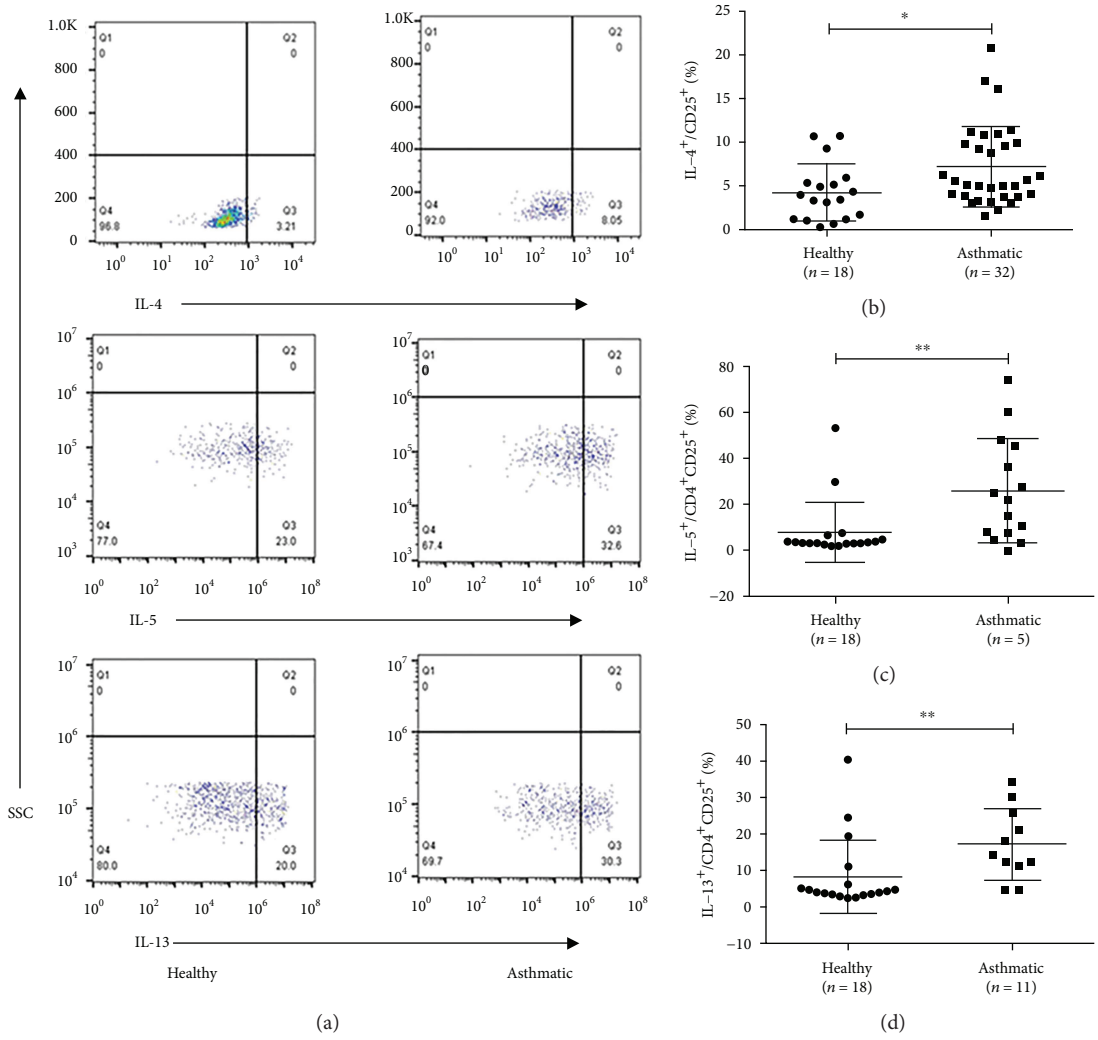

Figure 3: Increasing number of producing Th2-cytokine Treg cells. (a) Representative examples of flow cytometry analysis of IL-4, IL-5, and IL-13 production by $CD4^+CD25^+$ Treg cells in healthy and asthmatic subjects. (b, c, d) Frequencies of IL-4, IL-5, and IL-13 production in healthy and asthmatic subjects. The graph shows means ± sem. $^*p < 0.05$ and $^{**}p < 0.01$. NS: no significance.

patients with asthma expressed less IL-10, Nrp1, and CTLA-4, which indicated the defective function of Treg cells in these patients. GITR (TNFRSF18/CD357/AITR) is a cell surface receptor constitutively expressed at high levels on Treg cells and at low levels on naïve and memory T cells [20]. Previous studies have proved that GITR is a crucial player in Treg differentiation and explanation [21]. While high GITR expression is clearly a marker for Treg cells, GITR has also been demonstrated to lead to FOXP3 loss, inhibit the expansion and suppressive activity of Treg cells, and promote Teff resistance to Treg suppression [20, 22, 23], which means excess expression of GITR may be a clue of impaired function of Treg cells. In a recent study, GITR single-positive cells (GITRsp, $CD4^+CD25^{low/-}Foxp3^{low/-}GITR^+$) have been found that can express high levels of CTLA4, produce much more IL-10, and have regulatory activity, meaning that GITRsp cells might play a role in decreasing T cell activation/proliferation and controlling autoimmune disease [24]. In our study, we found that Treg cells in the asthmatic group expressed less GITR than those in healthy group. This decreased level of GITR expression may not be explained by exiting theories. Herein, more experiments will be needed to investigate the role of GITR in the pathogen of asthma.

Since changes have taken place in the number and function of Treg cells in asthmatic subjects, the underlying mechanism needed to be verified next. FOXP3, an X-linked transcription factor, is highly and specifically expressed in Treg cells [25]. As the specific transcriptional factor of Treg cells, FOXP3 is the absolute need in the development and function of Treg cells [9, 26]. Besides, other transcriptional factors are also needed for the immune-suppressive function of Treg cells [10]. Among them, GATA3 plays an indispensible role in Treg cell function. Previous study has revealed that the low expression of Foxp3 seems to account for a degree of GATA3 upregulation by some mechanism that favors nTreg-to-Th2 conversion [27]; what is more, after depleting GATA3 in Treg cells of mice, these Treg cells expressed reduced amounts of Foxp3 and were enhanced in the ability to produce inflammatory cytokines, which contributed to the inflammatory disorder in mice [26]. Wang revealed that

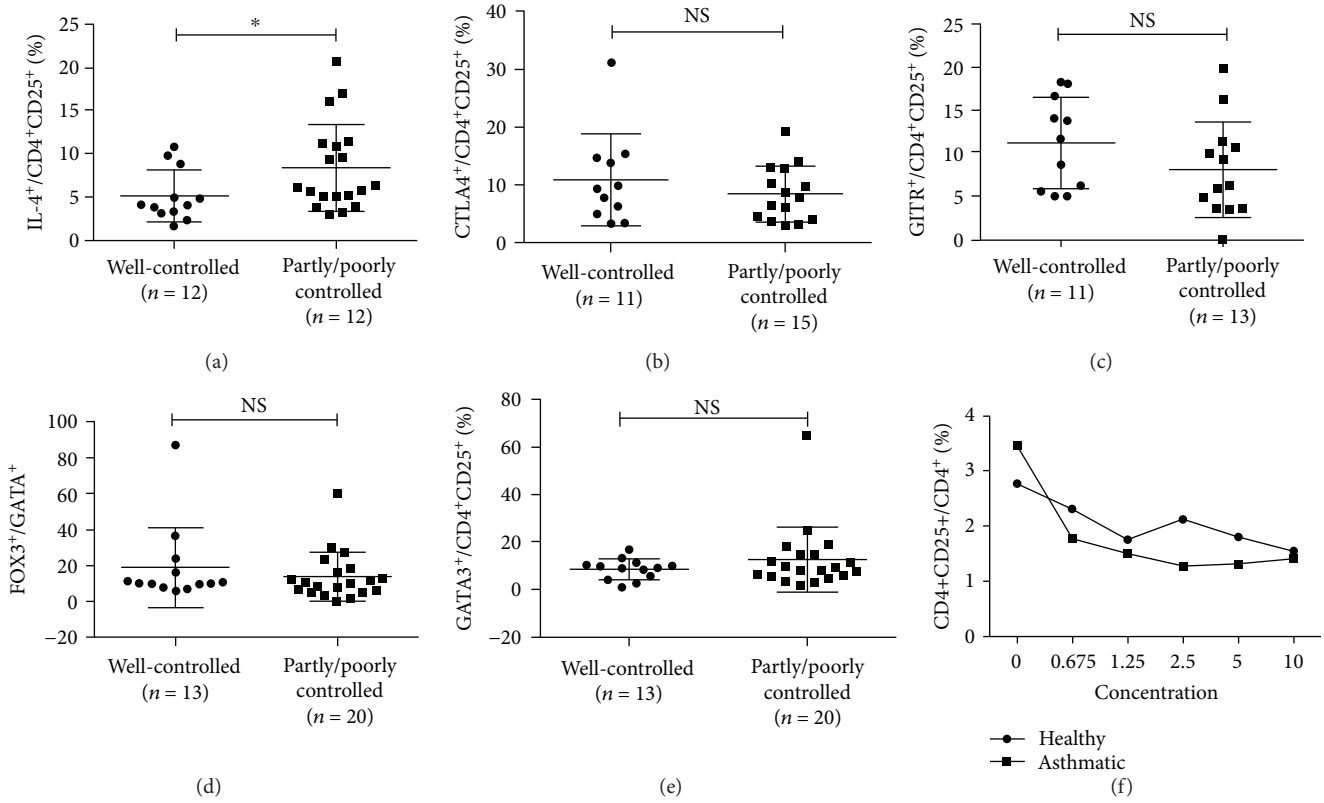

Figure 4: The expression of various markers in Treg cells in well-controlled and partly/poorly controlled groups. (a, c, d, e, f) Frequencies of IL-4, CTLA-4, GITR, FOXP3, and GATA3 expressing in well-controlled and partly/poorly controlled groups. (b) Effect of different IL-4 concentrations on TGF-beta induction of Treg cells from naïve T cells of healthy ($n = 8$) and asthmatic ($n = 18$) groups. The graph shows means ± sem. $^*p < 0.05$. NS: no significance.

GATA3 can bind to CNS2 of the *foxp3 locus* and deletion of GATA3 specifically in Treg cells resulted in an inflammatory syndrome in mice that could be ascribed to defective function of Treg cells [25]. In addition, GATA3-deficient Treg cells expressed reduced amounts of Foxp3 [25]. On the contrary, the upregulation of GATA3 in Treg cells led to the secretion of IL-4 in Treg cells, even the conversion of Treg cells to Th2 cells [28]. In 2012, Rudra et al. have revealed that Foxp3 interacts with GATA3 in Treg cells by biochemical and mass-spectrometric analysis; meanwhile, they also verified that Foxp3 and GATA3 reciprocally increased the expression of each other at least in part through direct binding to the corresponding genetic loci [27]. Based on these experiments, we hypothesized that it was the balance of FOXP3 and GATA3 in Treg cells that could ensure its exerting immuno-suppressive effects. Therefore, we analyzed the expression of FOXP3 and GATA3 from healthy and asthmatic subjects, and we found that both FOXP3 and GATA3 expressed in a higher level in the asthmatic group; however, Treg cells expressed more GATA3 in the asthmatic group, because the ratio of FOXP3 and GATA3 decreased in this group. In other words, there was an imbalance of FOXP3 and GATA3 in Treg cells from asthmatic subjects.

As to the importance of FOXP3 and GATA3 in Treg cells, the regulation of them was focused on in our experiments. Preciously, our collaborating laboratory has verified that USP21 can colocalize and interact with GATA3 and the role of USP21 on GATA3 in FOXP3-expressing cells is certified. Overexpression of USP21 can rescue GATA3 from its degradation so as to stabilize the expression of GATA3 [11]; RT-PCR showed that the mRNA of USP21 is upregulated in the Treg cells of asthma patients. In our experiment, the increasing level of USP21 in Treg cells was shown, which is consistent with the upregulation of GATA3 in Treg cells. Deng's study proved that PIM2 is highly expressed in human Treg cells and phosphorylates FOXP3 in vitro and vivo; beyond that, knockout of PIM2 in vivo enhanced Treg cell suppressive function and stability through altered expression of FOXP3 [13]. Our previous experiment also proved that PIM2 was essential for airway inflammation and airway hyperreactivity [29]. In accordance with the above research, we found the up-expression of PIM2 and the impaired function of Treg cells in asthmatic patients. The correlation analysis showed there was no correlation between GATA3 and USP21 or FOXP3 and PIM2 (the data was not shown). We thought the explanation was that USP21 or PIM2 may regulate the expression in a complicated way rather than in a linear relationship and it is possible that other molecules participate in the regulation of Treg cells in the pathogen of asthma.

As the expression of FOXP3 can suppress the expression of downstream genes, such as Il-4, Il-5, and Il-13, on the other hand, the increased GATA3 can also promote the secretion of Th2-type cytokines [9]. We testified if the change

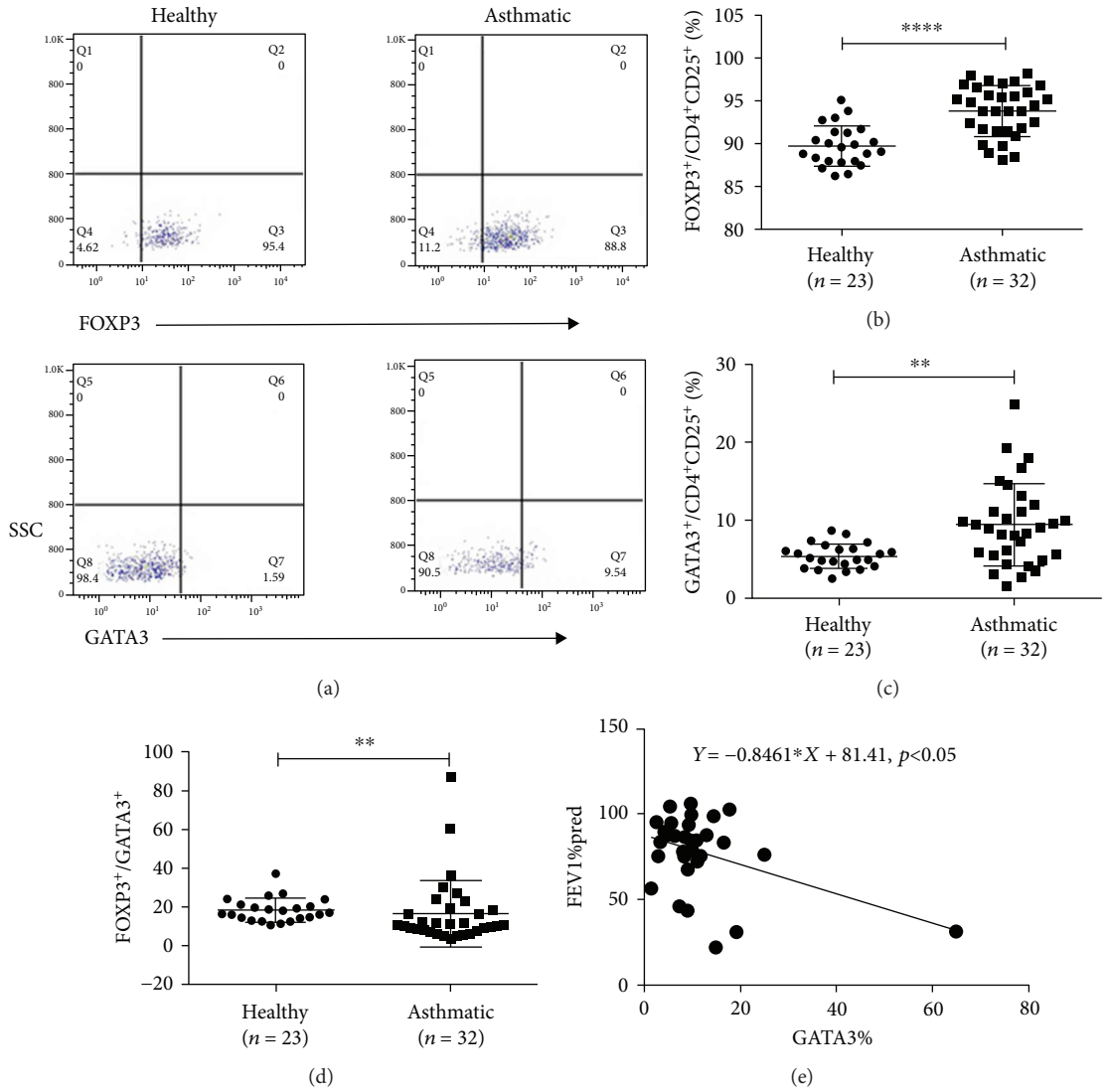

Figure 5: Enhancing expression of specific transcription factors, especially GATA3 in Treg cells from asthmatic patients. (a) Representative examples of flow cytometry analysis of FOXP3 and GATA3 expression by $CD4^+CD25^+$ Treg cells in healthy and asthmatic subjects. (b, c). Frequencies of FOXP3 and GATA3 expressed in healthy and asthmatic subjects. (d) The ratio of FOXP3 and GATA3 expressed in healthy and asthmatic subjects. (e) The correlation of the percentage of $CD4^+CD25^+GATA3^+$ Treg cells with FEV1%pred. The graph shows means ± sem. $^*p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$. NS: no significance.

of transcription factors leads to the change of the expression of cytokines downstream. In our experiment, Treg cells isolated from patients with asthma expressed higher levels of Th2-type cytokines, such as IL-4, IL-5, and IL-13, rather than IFN-γ, the Th1-type cytokine. The increased expression of GATA3 and decreased FOXP3 relatively give us a hint for the higher level of Th2 cells. On the other hand, our experiment also proved that Treg cells were unstable and it may be pathogenic in asthmatic patients. However, the ability of IL-4-, IL-5-, or IL-13-producing Treg cells in the development of asthma needs to be further investigated.

To explore the differences among asthmatic patients in different conditions, these subjects were divided into the well-controlled group and partly/uncontrolled group according to the ACQ-7 questionnaire, and it was found that IL-4 producing Treg cells were increased in the partly/poorly controlled group rather than in the well-controlled group, which means IL-4 may play a direct role in the development of asthma and affect the treatment effect of asthma. Since the percentage of Treg cells was significantly lower in the peripheral blood of patients with moderate to severe asthma than in patients with mild asthma [4], we designed different concentrations of IL-4 to imitate the different stages of asthma and to explore if IL-4 could affect the differentiation of Treg cells. We cultured naïve T cells in the culture polarity of Treg cell plus the concentration gradient of IL-4, and there was no significant difference of Treg differentiation in the different concentration of IL-4. In consideration of the important role of pathogenic Treg cells, how IL-4 produced by Treg cells individually can affect the pathogen of asthma needs more experiments. Besides, there were no significant differences of the expression of marker such as CTLA-4, GITR, FOXP3,

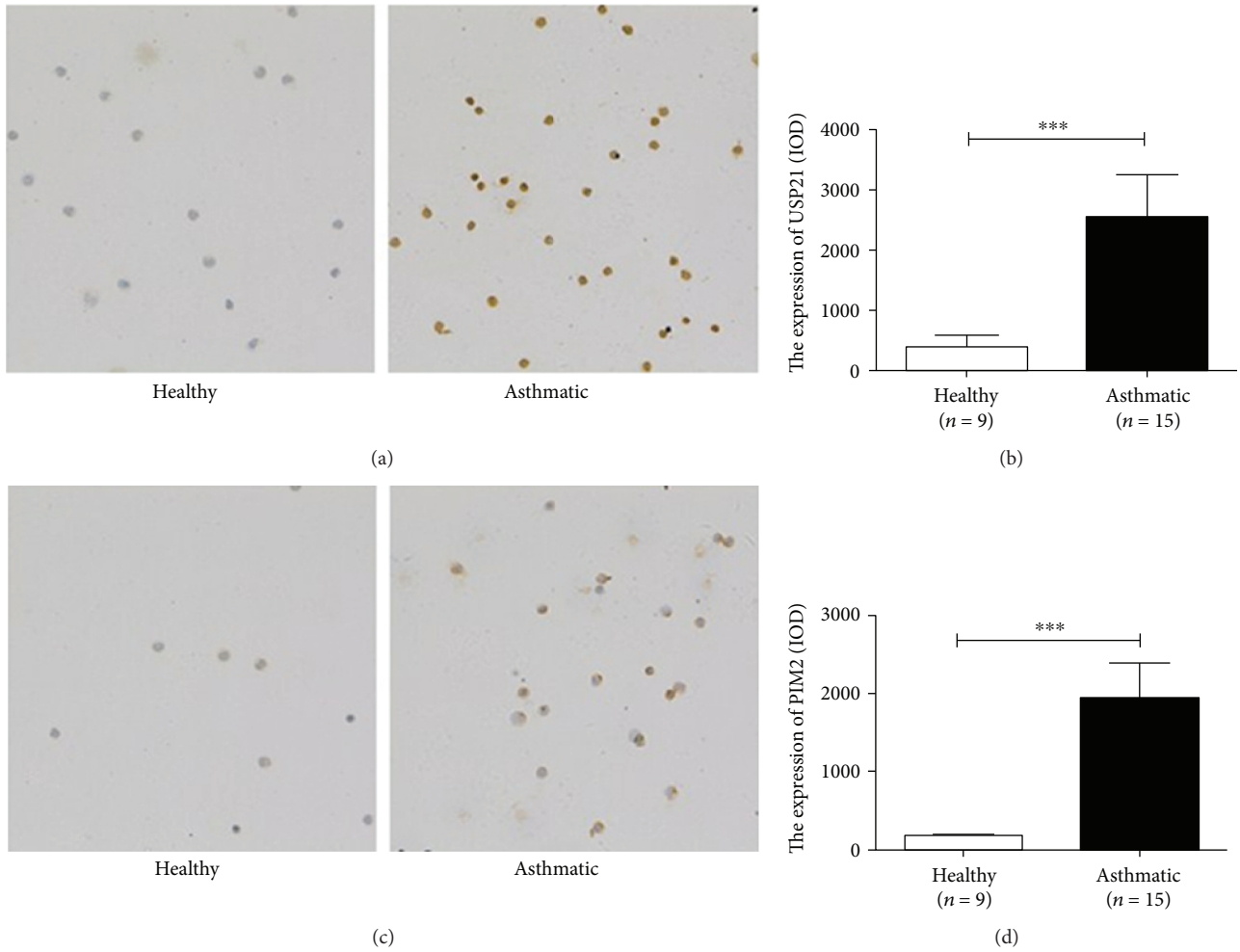

Figure 6: Increasing expression of USP21 and PIM2 in Treg cells from asthmatic patients. (a, c) USP21 and PIM2 staining in $CD4^+CD25^+$ Treg cells (revealed in brown, 200x). (b, d) The expression level of USP21 and PIM2 in $CD4^+CD25^+$ Treg cells from healthy ($n = 9$) and asthmatic ($n = 15$) subjects. The graph shows means ± sem. $^{***}p < 0.001$. NS: no significance.

and GATA3. It may give us the explanation that asthma is a complicated and programming pathology so that it is unreasonable to divide them explicitly.

In summary, multifaceted changes of Treg cells have taken place in the process of asthma. The imbalance of FOXP3 and GATA3 placed an important role in the function of Treg cells, which thus led to the pathogenic alteration of Treg cells, releasing more Th2-type cytokines, such as IL-4, IL-5, and IL-13. USP21 and PIM2 may exert an important role in the process by regulating GATA3 of Treg cells. However, more evidences need to be presented for the role of USP21 and PIM2 in the regulation of GATA3 and FOXP3.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

None of the authors have any potential financial conflict of interest related to this manuscript.

Authors' Contributions

Tiantian Chen and Xiaoxia Hou contributed equally to this work.

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Review Article

Immunological Mechanisms in Allergic Diseases and Allergen Tolerance: The Role of Treg Cells

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The immune system regulates itself to establish an appropriate immune response to potentially harmful pathogens while tolerating harmless environmental antigens and self-antigens. A central role in this balance is played by regulatory T cells (Tregs) through various ways of actions. By means of molecule secretion and cell-cell contact mechanisms, Tregs may have the capacity to modulate effector T cells and suppress the action of proinflammatory cytokines across a broad range of cell types. As a result, abnormal regulatory T cell function has been pointed as a main cause in the development of allergic diseases, a major public health problem in industrialized countries, with a high socioeconomic impact. This prevalence and impact have created an international interest in improving the allergy diagnosis and therapy. Additionally, research has sought to gain a better understanding of the molecular mechanisms underlining this kind of disease, in order to a better management. At this respect, the role of Treg cells is one of the most promising areas of research, mainly because of their potential use as new immunotherapeutical approaches. Therefore, the aim of this review is to update the existing knowledge of the role of Tregs in this pathology deepening in their implication in allergen-specific therapy (AIT).

1. Introduction: Current Knowledge about Treg Cells

The immune system (IS) requires tight control to protect the organism from exaggerated stimulatory signals triggered by harmless antigens, such as self-antigens and environmental substances. Depending on the nature of the antigen, an imbalance in the regulatory mechanisms of the IS can lead to autoimmune disorders or allergic diseases in genetically predisposed subjects [1, 2].

The induction of a tolerant state in peripheral T cells represents a key step in healthy immune responses to antigens. The first hypothesis to explain the break of this tolerance was based on the dichotomy between Th1 and Th2 lymphocytes. Years later, the hygiene hypothesis suggested that the lack of early childhood exposure to infectious agents and parasites could increase the risk of the susceptibility to suppress the correct development of the IS [3, 4].

Currently, there are several evidences that peripheral T cell tolerance is involved in the regulation of the IS. This regulation is characterized by functional inactivation of the cells in contact with the antigen, which in turn eliminates both the proliferative response and cytokine secretion. Several T cell subtypes with immunosuppressive function have been widely studied, and these are generically named regulatory T cells (Tregs) [5, 6]. Tregs suppress inflammation by upregulating immunosuppressive molecules and inhibiting the cells' tissue homing.

Numerous studies have identified Tregs as important immunoregulators in many inflammatory and autoimmune conditions including asthma, multiple esclerosis, and type I diabetes [7]. Additionally, Tregs are phenotypic and functionally specialized according to tissue localization, disease state, activation, and differentiation status [8–11] and are able to play different roles in disease and health [12, 13]. For these reasons, Tregs have been extensively studied and treated as a promising potential therapeutic tool in different types of diseases [14, 15].

Though this review focuses on the function of the Tregs, it is important to keep in mind that these cells do not work in

isolation. In fact, there exists a complex regulatory T cell system, which includes several populations of immunosuppressive cells as myeloid-derived suppressor cells (MDSCs), regulatory B cells (Bregs), regulatory $\gamma\delta$ T cells ($\gamma\delta$-*T*regs), and immunosuppressive plasmocytes (ISPC), a regulatory subset of ILCs (innate lymphoid cells) and so on with connected functions that work together [14].

1.1. Treg Cell Types. Different types of Tregs have been described [6]. These can be classified into two main categories according to their origin: natural Tregs with thymic origin, which mediate tolerance to self-antigens, and peripheral or induced Tregs, which are derived from a pool of naïve conventional $CD4^+$ T cells after exposure to antigens and in the presence of TGF-β [16] and regulate the response to nonself antigens [17]. Both of these Treg subsets play a key function in the maintenance of peripheral tolerance, but due to the nonoverlapping T cell receptor (TCR) repertoires, their actions are directed at different antigens. Although currently, there are no exclusive markers for Tregs, both types express FOXP3, a member of the forkhead or winged helix family of transcriptor factors. Indeed, FOXP3 was proposed as a master switch for Treg development and function in mice and humans [18–21]. FOXP3 controls several cell lineages and develops the differentiation of $CD4^+CD25^+$ $FOXP3^+$Tregs, the most physiologically significant subtype of these cells [22].

Although the most widely studied regulatory T cells are those with FOXP3, there are also populations of Tregs that do not express FOXP3. These include three main kinds of T cells: Tr1 cells, a population activated in the periphery after antigenic stimulation in the presence of IL-10 and which express the surface markers LAG-3 (lymphocyte-activation gene 3) and CD49b in the face of absent FOXP3 and CD25 expression; Th3 cells, which are also differentiated in the periphery and these Tregs mediate the cell suppression by secreting the cytokine TGF-β; and finally, $CD8^+$ Tregs [23], described as antigen-specific memory T cells with Treg properties, which may regulate immune responsiveness by production of IL-10, TGF-β1, and IFNγ though the exact mechanisms underlying this suppression are still largely unknown.

1.2. The Central Role of FOXP3. The relevance of FOXP3 in humans was recognized after the discovery of its implication in X-linked immune dysregulation, polyendocrinopathy syndrome (IPEX). IPEX is characterized by a high incidence of autoimmune and allergic diseases, including early-onset diabetes mellitus and other endocrinopathies, enteropathies, and diseases caused by severe allergic inflammation such as eczema, food allergies, and eosinophil-mediated inflammation [22, 24]. Patients with this pathology present mutations in FOXP3 with low levels of circulating Tregs. Indeed, it has been demonstrated that some single nucleotide polymorphisms (SNPs) in the *FOXP3* gene are associated with higher susceptibility to develop allergies [25, 26] and other immune diseases [27, 28]. Given the importance of FOXP3 in the control of the immune response, the factors which in turn control *FOXP3* expression have become a topic of interest. In fact, peripheral Tregs are known to be less stable than nTregs and under different inflammatory conditions can lose FOXP3 expression (ex-Treg) and adopt various T-helper-cell-like phenotypes. Epigenetic modifications, which can target histones (by acetylation) or DNA directly (by methylation in CpG motifs in noncoding regions in the *FOXP3* locus) could regulate the gene expression profile [24, 29]. One explanation for the lack of stability of pTregs was the methylation status of the conserved noncoding region 2 (CNS2) of the *Foxp3* gene. This locus, which acts to maintain Treg lineage identity under inflammatory conditions, is known to be stably hypomethylated in nTreg whereas it is incompletely demethylated in pTregs [30].

In addition to the transcriptional control of the *Foxp3* gene, the stability of FOXP3 expression is also determined at the posttranscriptional level. For example, Tregs respond to stress signals elicited by proinflammatory cytokines and lipopolysaccharides by degrading FOXP3 protein to then acquiring a T-effector-cell-like phenotype [31–33].

1.3. Mechanisms Involved in the Modulation of FOXP3 and Treg Cells. In view of the clinical relevance of this gene, elucidating the main mechanisms involved in the regulation of FOXP3 expression and Treg function could be very useful in the effort to control immune-dysregulated disorders and in understanding the physiological role in health and disease of this Treg instability. Thus, an increasing number of studies have been published in recent years to describe the different molecular mechanisms related to FOXP3 instability, in order to determine the expression of transcription factors and receptors that enable the suppressive functions of Tregs to operate in inflammatory and noninflammatory conditions. Examples of such researches include the description in Tregs of a positive feedback between USP21 (an E3 deubiquitinase), GATA3 (an essential and sufficient transcription factor for the polarization and function of the Th2 cell lineage), and FOXP3, to promote FOXP3 expression and thus modulate Treg activity [34]. This last report proposed that after TCR stimulation, FOXP3 upregulates USP21 transcription. USP21 could act as a GATA3 deubiquitinase and to stabilize GATA3. This mechanism may contribute to the upregulation of GATA3 expression in Tregs and can be strengthened in a FOXP3-dependent matter. The study also highlighted the possibility that GATA3 could recruit USP21 to the FOXP3 complex to prevent FOXP3 degradation. This work proposes that in Tregs, USP21, GATA3, and FOXP3 may form a positive loop to promote FOXP3 expression and thus modulate Treg activity. Years later [35], it was described how USP21 stabilizes the FOXP3 protein by mediating its deubiquitination and controls Treg lineage stability *in vivo*.

Another regulator proposed was SOCS2, a suppressor of cytokine signaling (SOCS) proteins, which inhibits the development of Th2 cells and allergic immune responses. It was described as being highly expressed in pTregs and a requisite for the stable expression of Foxp3 in these Tregs, *in vitro* and *in vivo*, but with no effect on nTreg development or function. Interestingly, pTreg stability was induced by SOCS2 downregulating IL-4 signaling [36].

Recently, MALT1, a nuclear factor-κB signaling mediator mucosa-associated lymphoid tissue lymphoma translocation protein 1, has been described as an important novel regulator of nTregs and pTregs [37]. The report describes a dual role of MALT1, citing it as crucial for nTreg development and thus important for central tolerance and also in the periphery, where MALT1 determines the threshold for the differentiation of naïve T cells into functional pTregs. Although MALT1 had no influence on the suppressive function of pTregs, it was seen to be able to limit further induction of pTregs at sites of inflammation by downregulating Toll-like-receptor (TLR) 2 expression. In this respect, the influence of TLR signaling on Tregs is quite controversial but it is generally accepted that TLR2, 4, or 5 engagements can enhance Treg cell function, survival, and/or proliferation [38–41].

Interestingly, it has been demonstrated that Helios (a member of the ikaros family of transcription factors) expression by Tregs is key to supporting their suppressive functions and phenotypic stability during inflammation [42].

On the other hand, Treg functions can also be regulated by endogenous danger signals, or alarmins, which are released by epithelial cells at the mucosal barrier. One such alarmin is interleukin- (IL-) 33, a cytokine that is released from epithelial and endothelial cells at barrier surfaces upon tissue stress or damage and that was primarily implicated in the induction of Th2-type immune responses. More recently [43], however, their pleitropic role has been demonstrated as this cytokine is able to mediate immunosuppression and tissue repair by activating Tregs and promoting M2 macrophage polarization.

Finally, another important modulator of tolerance is the microbiota. It has been suggested that the microbiota can promote tolerant or proinflammatory cell subtypes. This aspect has been extensively studied in the context of allergic diseases [44, 45]. The release of microbiotal products can interact directly with immune cells and their innate receptors. Therefore, proallergic or protolerant bacterial species can affect the development of Tregs and Th2 subtypes, thereby increasing the production of IL-10 or IL-4 and IL-13, respectively.

1.4. General Mechanisms of Treg Action. The main role of all Treg subsets is to maintain the integrity of the organism by avoiding excessive immune responses thereby preserving a state of tolerance to innocuous substances through the secretion of soluble factors and by direct contact (cell-to-cell). A variety of molecules has been found to be involved in Treg-mediated suppressive activities. The main mechanisms of Treg action include regulatory cytokine production such as IL-10, IL-35, and TGF-β; the metabolic disruption mechanisms: CD25, cAMP, histamin receptor 2, adenosine receptor 2, CD39, and CD73; mechanisms with targets in DCs such as cytotoxic T lymphocyte antigen-4 (CTLA-4), program death-1 (PD-1), and cytolysis mechanism (granzymes A and B) [14, 46, 47].

As detailed above, it has been described how the suppressive functions of Tregs are induced under inflammatory conditions by the specific expression of receptors and transcription factors. By way of example, GATA3 expression by Tregs is triggered by TCR activation and is required to maintain FOXP3 expression and allow accumulation of Tregs at inflamed sites [48]. GATA3 expression in Tregs appears to be essential in limiting Tregs producing effector cytokines within inflamed tissues [17].

Recently, it has also been described that Tregs secrete microRNAs which could be implicated in inhibiting T effector cells, thus opening a new area of interest in Treg-mediated suppressive mechanisms [23].

To summarize, although the complex functioning of the regulatory network is not well known, Tregs are able to maintain tolerance by multiple mechanisms. Figure 1 summarizes the main roles proposed for Tregs. These cells could act at the initiation of adequate specific antigenic immune response, promoting tolerogenic DC phenotypes, inhibiting the inflammatory ones [49]. It has also been demonstrated that the correct capacity of DCs to induce a tolerogenic response depends on the particular subsets, maturation stages, and several exogenous signals such as microbiota, histamine, adenosine, flavonoids, vitamin D3 metabolites, or retinoic acid [46]. Suppression of DCs appears to be mediated through CTLA-4 (constitutively expressed in Tregs, as a negative costimulatory molecule which is essential to their suppressive functions), LAG-3 (lymphocyte-activation gene 3), and LFA-1 (leukocyte function-associated antigen-1). Tregs also act directly on DCs by decreasing the surface expression of CD80/CD86 and blocking the allergen-specific Th2 cell immune response. In addition, Tregs could inhibit Th development: Tregs suppress the activation process of Th2 cells, reducing the secretion of inflammatory cytokines such as IL-4, IL-5, IL-9, and IL-13 and in Th1 cells, by inhibiting INF-γ secretion, Th17 cell response (IL-17-secreting cells) and Th22, which predominantly produce IL-22 [23]. It has been demonstrated that peripherally induced Tregs suppress the group 2 innate lymphoid (ILC-2) response and its inflammatory cytokines, IL-5 and IL-13 [23]. In addition, Tregs may control effector cells by inhibiting the maturation and degranulation of basophils and mast cells, thus reducing the expression of FcεRI and the degranulation via OX40-OX40L interactions. Tregs also prevent the infiltration of eosinophils and T cells into damaged tissue. Also, Tregs could interact with resident tissue cells by preventing damage and contributing to tissue remodeling [46]. For this reason, Tregs are involved in the reduction of local inflammation and contribute to the repair of damaged tissue [50–52].

Tregs also could modulate the humoral immune response. These cells may directly inhibit the progress of antigen-specific B cells, reducing the production of immunoglobulin (Ig) type E (IgE) and increasing the levels of anti-inflammatory immunoglobulin such as IgG4 [53]. This is another point justifying Tregs as excellent candidates for regulating allergic diseases.

2. Allergic Response: An Imbalance of Regulatory Mechanisms

Regulation of IS a general process that allows inflammation to be attenuated. Defective immunosuppressive mechanisms

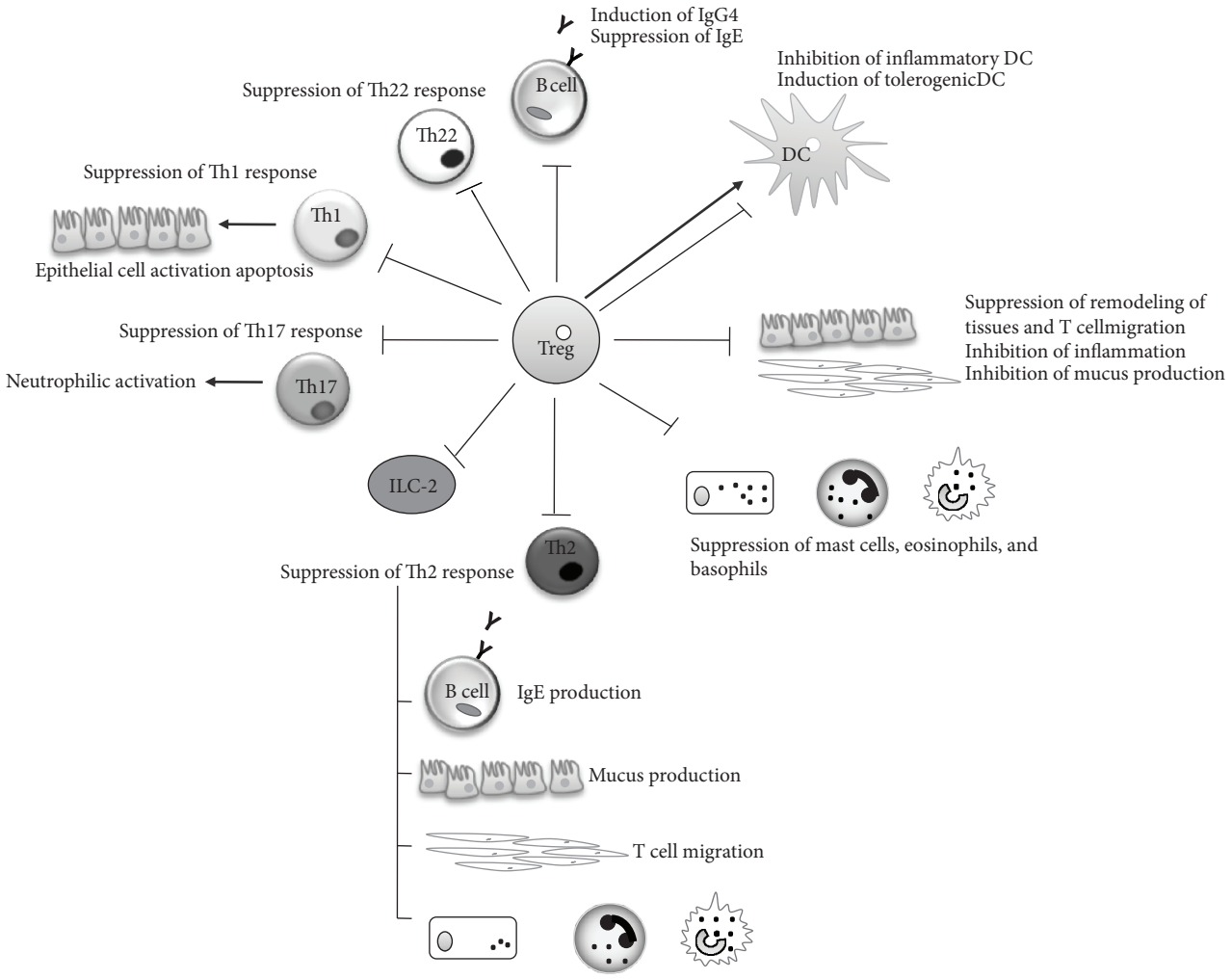

Figure 1: Main roles proposed to Tregs. Regulatory T cells could mediate the healthy immune response through different modes of action. They are able to suppress the inflammatory dendritic cells (DCs), inhibit the activation of effector T cells (Th1, Th2, Th22, and Th17) and type 2 innate lymphoid cells (ILC-2), block the secretion of inflammatory antibodies by antigen-specific B cells, and inhibit the activation of basophils, mast cells, and eosinophils.

by Tregs could explain the development of allergic reactions. Allergic diseases are highly complex adverse reactions of the IS against various innocuous substances. Although the population is continuously exposed to a wide range of allergens, not everyone develops this kind of disease. The reasons why some individuals suffer from allergic diseases while others do not are far from clear. The pathophysiology of allergic diseases is complex and may be influenced by many factors, including genetic susceptibility as well as aspects of the microenvironment, such as allergen dose and route of exposure. In this sense, clinical manifestations depend on the nature of the allergen and the part of the organism affected. The most common symptoms of allergic diseases include allergic rhinoconjunctivitis, allergic asthma, atopic dermatitis, food allergy, and anaphylaxis [54].

In the allergic response, the IS must recognize the pathogenic stimuli and induce a vigorous immune response. Sensitization to a specific antigen is a prerequisite: specific regions of antigens called epitopes are recognized by naïve T and B lymphocytes. First, the allergens are recognized and presented to naïve T cells by specific major histocompatibility complex (MHC) class II antigens expressed on the surface of antigen-presenting cells (APC). T cell activation induces the differentiation and expansion of T helper type 2 (Th2) cells. The key cytokines responsible for the allergic response include interleukin- (IL-) 4, IL-5, and IL-13, as well as innate lymphoid (ILC-2) cells which may amplify and maintain local Th2-driven allergic inflammation by secreting Th2 cytokines, particularly IL-5 and IL-13 [55]. These ILs act on B cells, promoting immunoglobulin (Ig) class switching to Ig type E (IgE). Allergen-specific IgE antibodies bind to high-affinity receptors for IgE (FcεRI) expressed on mast cells and basophils. Repeated exposure to the allergen causes the cross-linking of FcεRI-bound IgE, stimulating the release of histamine and other mediators responsible for the immediate symptoms of allergic disease. The late phase of an

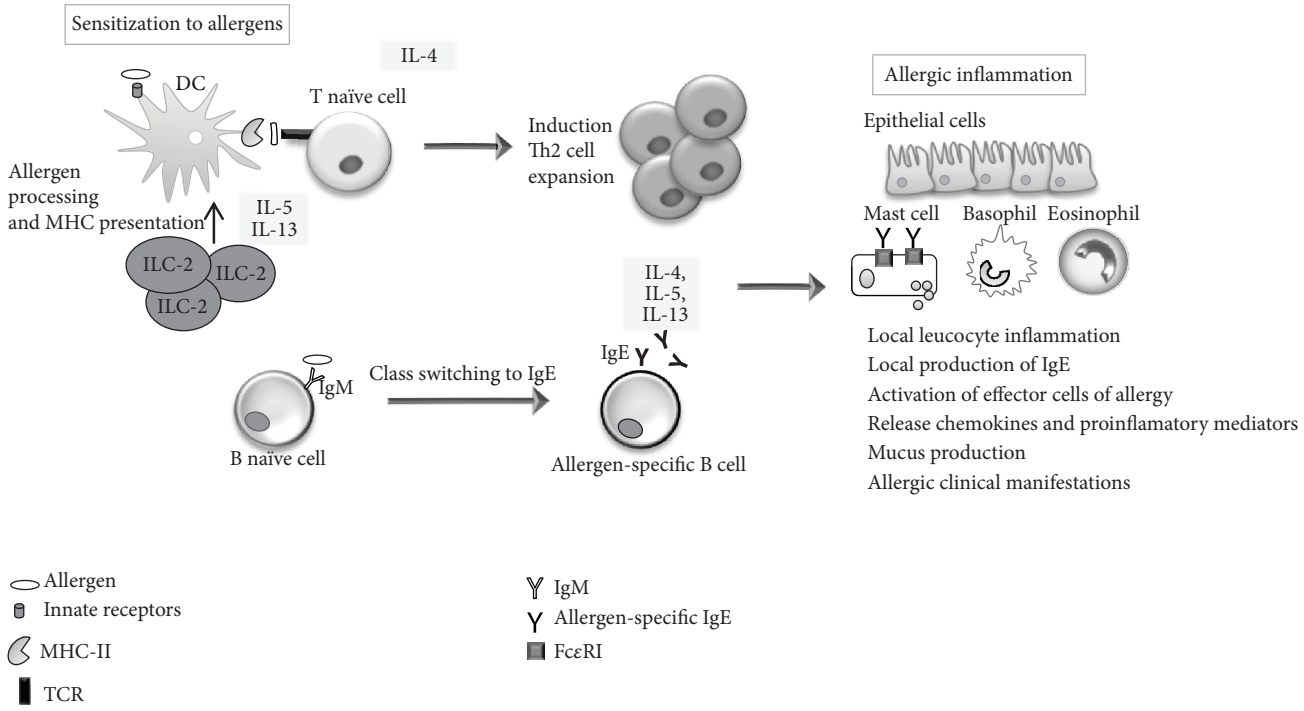

Figure 2: Mechanisms involved in allergic reactions. Sensitization to a specific antigen is a prerequisite for the onset of allergic diseases. Differentiation and expansion to Th2 cell subtypes lead to the production of inflammatory cytokines (IL-4, IL-5, and IL-13). They drive immunoglobulin E (IgE) class-switch in B cells and the recruitment and activation of proinflammatory cells (i.e., eosinophils and mast cells) in mucosal target organs. These activations contribute to the development of the inflammation and the symptoms of allergic disease.

allergic reaction occurs 6–12 hours after allergen exposure, when allergen-specific cells are reactivated and expanded locally. Effector cells (i.e., mast cells, basophils, and specifically, eosinophils) release additional inflammatory mediators and cytokines, perpetuating the proinflammatory response. This phase is responsible for the symptoms of allergic diseases, and continuous exposure to the allergen causes disease chronicity (Figure 2) [1, 56].

Currently, the prevalence of allergic diseases has increased substantially, with allergies affecting up to 25% of the population in industrialized societies and constitute a major public health problem with a high socioeconomic impact [46]. Although allergic symptoms can often be suppressed using anti-inflammatory drugs, allergen-specific immunotherapy (AIT) remains as the only treatment that can cure allergic diseases. AIT has been applied since 1911 and was reported first by De Martinis et al. [57]. Their assays proved that subcutaneous injection of grass pollen extract modulated the course of the disease, and more importantly, these effects remained for more than a year after the conclusion of treatment. The method used by the two researchers was accepted by the scientific community, leading to an increase in this type of studies in subsequent years. Nowadays, AIT is a routine part of clinical practice in allergy [57].

3. Induction of Peripheral Tolerance in Allergy by AIT

AIT is considered a therapeutic vaccine because it uses the patient IS to establish tolerance against specific antigens, providing clinical efficacy with a long-term benefit. However, although the molecular mechanisms involved in the AIT response have been extensively studied, they have not been elucidated in their entirety [58–60].

AIT is believed to promote the absence of proinflammatory signals influenced by the maturation of dendritic cells (DC) into a tolerogenic response [61], thus inducing major changes in allergen-specific T cell subsets: it leads to a down-regulation of the Th2 response with a shift towards the Th1 profile. Allergen-specific T cells with a regulatory phenotype are also promoted, and their presence is associated with an increase in the suppressive cytokines such as IL-10 and TGF-β [53, 62–64]. Through this fact, AIT plays an essential role in changing antibody isotypes. Many studies have shown a decrease of allergen-specific IgE antibodies (inflammatory response) in serum associated with high levels of the allergen-specific IgG4 antibody, a noninflammatory or protective immunoglobulin, in the context of allergic response [44]. IgG4 secreted by regulatory B cells [65] could act as a blocking antibody in competition with the antigen-binding IgE present on the surface of mast cells and basophils, which would limit activation and degranulation [66]. AIT and this promoting of Tregs also suppress allergic inflammation induced by mast cells, basophils, and eosinophils. The recruitment of these cells to the site of allergen exposure and their ability to release mediators are reduced in treated patients, decreasing the inflammatory response of tissues [63]. Due to these capacities, AIT reduces the allergic symptoms in a significant fraction of treated individuals and improves their quality of life (Figure 3) [54].

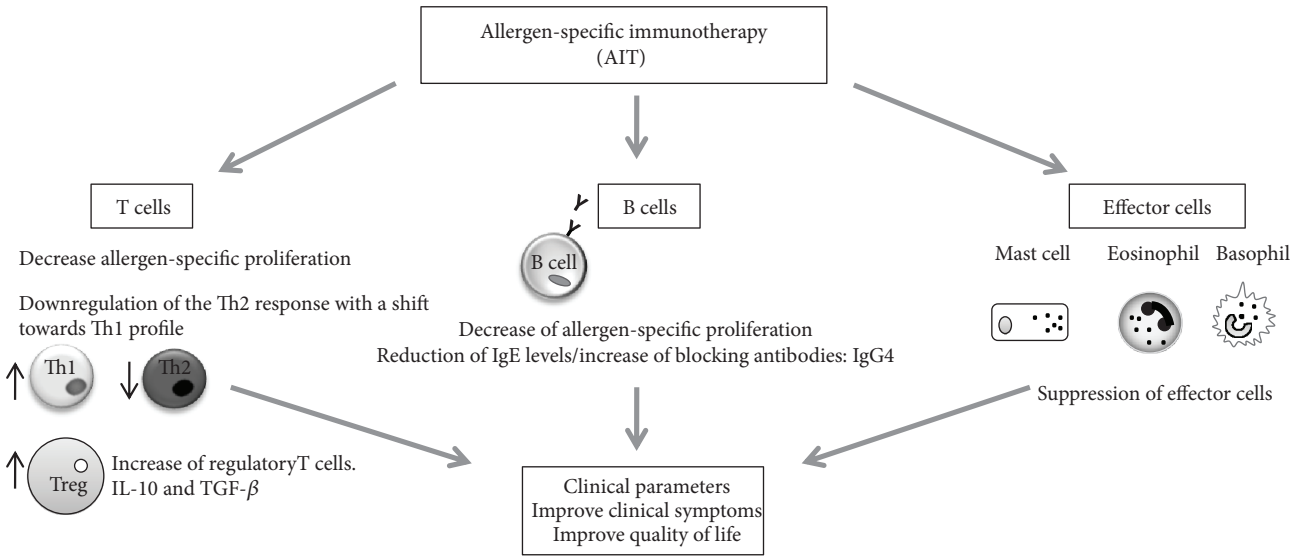

Figure 3: Effects of AIT. AIT induces a state of tolerance based on the increase of Treg functions and the inhibition of the Th2 cell response. The production of blocking antibodies and the suppression of mast cells, basophils, and eosinophils also contribute to the restoration of the appropriate immune response to allergens, by ameliorating the chronic inflammation that occurs in allergic diseases.

4. Regulatory Cytokines in Allergen Tolerance and Specific Immunotherapy

One of the most important mechanisms of Tregs is the secretion of some soluble mediators, such as suppressive cytokines (IL-10, IL-35, and TGF-β). Their importance in allergen tolerance and their modulation with AIT have been widely studied.

4.1. Cytokine 10 (IL-10). This cytokine is formed by two subunits of 178 amino acids [67, 68]. It is synthesized and secreted by a wide range of cell types, including Th cells, monocytes, macrophages, and dendritic cells [69, 70].

Its receptor is formed by two chains: IL10-R1, expressed only in target cells (T, B, NK cells, monocytes, mast cells, and dendritic cells) and IL10-R2, expressed ubiquitously. The junction ligand-receptor produces signaling by phosphorylation of some proteins that induce the activation of signal transducer and activator of transcription 3 (STAT3), which induce the expression of suppressor of cytokine signaling 3 (SOCS3) and several preapoptotic genes, as well as the inhibition in the production of proinflammatory cytokines such as TNF-α.

Due to these activations, IL-10 has broad immunosuppressive and anti-inflammatory capacities. It is a potent inhibitor of proinflammatory cytokine production and their receptors. It inhibits several molecules involved in the antigen presentation to dampen $TCD4^+$ cell activation.

IL-10 produced by Tregs plays an essential role in protecting the host from exaggerated inflammatory responses to pathogens as well as autoimmune diseases. In addition, its implication in tolerogenic and allergic responses has been extensively studied and the protective role it plays nowadays is well-established. It has been demonstrated that dendritic cells from the respiratory system of healthy controls express higher levels of IL-10 than allergic patients (with rhinitis and asthma) [71, 72]. IL-10 modulates the activity of numerous cells involved in allergic diseases, mast cells, Th2 cells, and eosinophils. Several researchers have shown that Treg-specific deletion of IL-10 promoted allergic inflammation [73] suggesting that Treg-derived IL-10 plays a "privileged," nonredundant role in the induction of immune tolerance in allergic airway inflammation. High levels of allergens, as cat allergens, induce IL-10 production by Tr1 cells associated with the secretion of IgG4 and amelioration of clinical symptoms [74]. Also, the exposure to high doses of bee venom in beekeepers has demonstrated a natural mechanism of immune tolerance owing to the expansion of IL-10 secreting Tr1 cells [75]. Furthermore, numerous clinical trials have reported that specific treatments for allergies increase IL-10 levels [76–78].

4.2. Tumor Growth Factor-β (TGF-β). TGF-β is a member of a complex superfamily, being TGF-β1 the most widely studied member [79, 80].

TGF-β is a pleiotropic cytokine required for the maintenance of peripheral tolerance. TGF-β regulates lymphocyte homeostasis by inhibiting Th2 and Th1 cell responses as well as the conversion of *naïve* T cells into peripheral regulatory T cells by the stimulation of FOXP3 expression in a paracrine feedback loop, which promotes the generation of $CD4^+$ $CD25^+$ Tregs able to inhibit allergic airway disease [81–83].

TGF-β also inhibits macrophage proliferation and function, inhibits the secretion of antibodies by B cells, and also blocks the expression of FcεRI in mast cells. In damaged tissues, TGF-β regulates airway inflammatory response, inducing fibrosis [84].

4.3. Cytokine 35 (IL-35). Il-35 is a newly discovered member of the IL-12 family. It is a heterodimer composed of a subunit of IL-12 (p35, IL-12α) and the Epstein-Barr virus-induced gene 3 (EBI3) [85]. While the other IL-12 family members

(IL-13, IL-23, and IL-27) are considered proinflammatory cytokines, IL-35 is secreted by Tregs and was identified as an anti-inflammatory and suppressive cytokine [8, 22] in an inducible manner [86].

Tregs deficient in Ebi3 or IL-12p35 are functionally defective *in vitro* and *in vivo* [17]. Several findings lend support to the importance of these Tregs in some respiratory diseases. Recent findings suggest that expression of IL-35 is abnormal in asthma and plays an important role in the pathogenesis of allergy diseases [87]. It has also been demonstrated how people with asthma and chronic obstructive pulmonary disease (COPD) have low levels of this cytokine and after sublingual immunotherapy, IL35 serum levels increased, being this increase associated with an improvement in clinical symptoms.

5. MicroRNAs as a New Mechanism of Suppression by Tregs

MicroRNAs are short noncoding RNA molecules which have emerged as important regulators of immune response. MicroRNAs are fundamental in Treg development and function. miR-155, miR-15b/16, miR-24, and miR-29a were described as important players in Treg differentiation and maintenance [23, 88].

Furthermore, this mechanism for cell communication mediated by exosomes can inhibit the action of T effector cells. Specifically, Tregs released Let7d to Th1 cells regulating Th1 response [89] and miR-21 in the control of Th2 inflammation. Therefore, Tregs are able to capture and deliver different miRNAs and other substances to different cells at different times, depending on the specific situation, to control Treg function and development status [90].

6. Conclusions and Future Perspective

The immune system requires correct functioning and fine balance that it are controlled by the development and maintenance of a complex network of regulatory mechanisms, where regulatory cells play essential roles. By gaining a fuller understanding of the heterogeneity of Treg populations and the appropriate suppressive function system in inflammatory conditions, we may be able to devise novel therapeutic approaches to inhibit this kind of disease. For this reason, we have reviewed the general mechanisms of Tregs and the immunologic mechanisms involved in allergy and allergen tolerance, where Tregs could act as the nucleus in enforcing *healthy* immune responses to allergens. Tregs are capable of suppressing conventional T cells, APCs, and B cells by molecule secretion and cell-cell contact mechanisms.

Recovery of the correct immune tolerance response in inflammatory diseases such as allergy is an attractive target for immunotherapy, and Tregs could play a main role in this pursuit as new therapy tools. However, one important aspect that should be studied in depth is Th reprogramming of Tregs in allergic diseases. Nowadays, a dynamic view of Tregs is emerging in allergic diseases by which Tregs are seen as playing a central and determining role, not only in tolerance induction but also, when destabilized and reprogrammed, in mediating disease pathogenesis, severity, and chronicity [44]. New findings on the pathways and mechanisms implicated in this regard could provide unique tools to control Treg function to treat allergic diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

$CD4^+CD25^{high}CD127^{low/-}FoxP_3^+$ Regulatory T Cell Subpopulations in the Bone Marrow and Peripheral Blood of Children with ALL: Brief Report

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$CD4^+CD25^{high}CD127^{low/-}FoxP_3^+$ regulatory T cells (Tregs) are currently under extensive investigation in childhood acute lymphoblastic leukemia (ALL) and in other human cancers. Usually, Treg cells maintain the immune cell homeostasis. This small subset of T cells has been, in fact, considered to be involved in the pathogenesis of autoimmune diseases and progression of acute and chronic leukemias. However, whether Treg dysregulation in CLL and ALL plays a key role or it rather represents a simple epiphenomenon is still a matter of debate. Treg cells have been proposed as a prognostic indicator of the clinical course of the disease and might also be used for targeted immune therapy. Our study revealed statistically higher percentage of Treg cells in the bone marrow than in peripheral blood in the group of 42 children with acute lymphoblastic leukemia. By analyzing Treg subpopulations, it was shown that only memory Tregs in contact with leukemic antigens showed statistically significant differences. We noticed a low negative correlation between Treg cells in the bone marrow and the percentage of blasts ($R = -0.36$) as well as a moderate correlation between Treg cells in the bone marrow and Hb level ($R = +0.41$) in peripheral blood before therapy. The number of peripheral blood blasts on day 8th correlates negatively ($R = -0.36$) with Tregs. Furthermore, statistical analysis revealed low negative correlation between the number of Tregs in the bone marrow and the minimal residual disease measured on day 15th, the percentage of blasts in the bone marrow and leukocytosis after 15 days of chemotherapy. These results indicate the influence of Tregs on the final therapeutic effect.

1. Introduction

Acute lymphoblastic leukemia (ALL), the most common childhood cancer, is a heterogeneous disease that occurs due to the malignant clonal proliferation of lymphoid progenitors [1]. The clinical symptoms of the disease and the ultimate therapeutic effect depend on the biological characteristics of the tumor cell. Another very important factor in curing cancer is an efficient immune system. Despite intensive research on the effects of various elements of the immune system on the cancer development, there is still little knowledge about it.

Normal cells in the environment of cancer cells are currently under intensive investigation. Residual nonmalignant T cells and B cells are in permanent cell-to-cell contact with lymphoblasts and are involved in active immune responses [2].

Regulatory lymphocytes constitute a very interesting subpopulation of cells of the human immune system. A growing interest in their biological properties has occurred recently and clinicians have wondered whether they can be also used in the battle against cancer [3, 4].

Recent papers have demonstrated elevated number of Tregs in lung, breast, pancreatic, ovarian, melanoma, digestive

Table 1: Patient characteristics ($n = 42$).

Age	1–5 years = 26	6–11 years = 10	12–18 years = 6
Gender	Male = 21	Female = 21	
Immunophenotype	B cell = 40	T cell = 2	
Protocol of therapy	ALL IC BFM 2002: 1	ALL IC BFM 2009: 41	
Risk group	SR: 5	IR: 25	HR: 12
CNS involvement	Positive: 2	Negative: 40	
EFS	Relapse: 2	Death: 2 (after relapse: 0)	Live in first remission: 38
Steroid sensitivity*	Good: 35	Poor: 6	
BM on day 15*	M1: 28	M2: 9	M3: 4
BM on day 33*	M1: 38	M2: 1	M3: 2

*One child died before the 8th day of the treatment, so we were not able to assess the sensitivity to steroids and the response to treatment on the 15th and 33rd days of chemotherapy.

system cancers, CLL, T cell ALL, and B cell NHL [1, 5, 6]. This concerns both peripheral blood and cancer tissue, where a neoplastic proliferation is accompanied by higher than the usual number of regulatory lymphocytes. In some subtypes of cancer, the differences in the percentage may affect the response to chemotherapy and thus the prognosis of a disease.

It was demonstrated previously that elevated percentages and increased suppressor properties of Treg cells are observed even after achieving a remission and after completing the treatment of AML [6]. This might indicate that Tregs are resistant to chemotherapy and could facilitate a relapse of AML.

Previous research showed that the number of Treg cells may be either elevated or reduced in Hodgkin disease and mature B cell lymphoma. Similarly, a prognosis may be either favorable or adverse [7]. It is known for example that the percentage of Treg cells is higher among patients suffering from CML than among healthy volunteers [8]. This level correlates with an advancement of the disease, the percentage of B cells in peripheral blood, and the level of LDH. Some papers even state that Treg cells may control a neoplasmatic growth [9]. The next interesting issue is a connection between Treg cells and ALL among patients in the developmental age. This group of leukemias are characterized by a separate biology, clinical picture, and first of all—different prognosis.

In our study, we investigated a population of $CD4^{+-}CD25^{high}CD127^{low/-}FoxP_3^{+}$ regulatory T cells in the bone marrow and peripheral blood of children with acute lymphoblastic leukemia treated in the Department of Pediatrics, Hematology and Oncology, Medical University of Gdansk in 2011–2016.

Due to the small number of publications concerning the influence of Tregs on the prognosis in acute childhood leukemias and investigating the percentage of these cells in the bone marrow and peripheral blood of ALL children, a following research was undertaken to understand these relationships better.

Of particular interest to us was the influence of a higher percentage of Tregs in the peripheral blood/bone marrow of patients with acute leukemia on the early and late therapeutic effect, which was reported in the literature [10, 11].

In addition, it was decided to perform an initial assessment of the relationship between biological characteristics of leukemia and Tregs. By assessing the correlation between the number of Tregs and such parameters as hemoglobin, platelets, leukocytosis, or the percentage of blasts in the peripheral blood and bone marrow at the moment of diagnosis, it was decided to verify the preliminary hypothesis which assumes the connections between Tregs and the stage of the cancer process and prognosis. The elevated percentage of Treg cells in the bone marrow observed earlier by some authors in comparison to peripheral blood requires verification due to the use of a narrow panel of antibodies to assess the population of cells of interest. To effectively and reliably count Treg cells in the analyzed material, antibodies were used to identify cells with the $CD4^{+}CD25^{high}CD127^{low/-}FoxP_3$ phenotype, which identifies the T-line regulatory cells in the most accurate way [4].

In case the relationships described earlier in the literature were confirmed, it would be quite advisable to search for therapeutic methods interfering with the immune system through manipulations on Treg cells [9].

An interesting question was also whether the increased percentage of Tregs in the peripheral blood and/or bone marrow is also observed in children with cancers other than leukemia [12].

In summary, Tregs are a potential target of immunotherapy but this hypothesis requires further, intensive investigation of the properties of relationships between regulatory and cancer cells. This could contribute to the improvement of a prognosis with simultaneous reduction of toxic chemotherapy [9].

2. Methods

2.1. Patients and Treatment. The bone marrow and peripheral blood were obtained at diagnosis from 42 patients with acute lymphoblastic leukemia treated according to the BFM SG Protocol ALL-IC BFM 2002 ($n = 1$) and Protocol ALL IC-BFM 2009 ($n = 41$).

All clinical data concerning patients are summarized in Table 1.

This study was approved by the Medical University of Gdansk Ethical Board, and informed consent was obtained from patients and/or their legal guardians.

A response to the steroid therapy was checked in the peripheral blood on day 8, a remission in the bone marrow was checked by the flow cytometry on day 15 and 33. Patients were divided to SR, IR, and HR risk groups according to the protocol rules.

2.2. Control Group. In the control group, 10 bone marrow samples (2 ml) and 46 peripheral blood samples (5 ml) were tested. For ethical reasons, the bone marrow was obtained only from the children requiring a diagnostic bone marrow biopsy to exclude bone marrow involvement by a cancerous disease or to exclude leukemia. In the control group, the following diagnoses were noted: Wilms tumor ($n = 9$), neuroblastoma ($n = 9$), RMS ($n = 2$), Hodgkin disease ($n = 11$), CNS tumor ($n = 5$), anemia ($n = 3$), and lymphadenopathy ($n = 7$).

The analysis involved 24 girls and 22 boys from 1 to 16 years of age.

2.3. Regulatory T Cell Immunophenotyping by Multicolor Flow Cytometry. Only freshly obtained samples were processed up to 24 hours from collection. Briefly, lymphocytes were isolated using density gradient media Lymphoprep (STEMCELL Technologies, Canada) and EDTA bone marrow or peripheral blood samples. Lymphocytes were then stained with the use of CD127 FITC (clone HL-7R-M21), CD25 PE (clone 2A3), CD4 PerCP (clone SK3), CD3 V450 (clone UCHT1), CD45RA PEcy7 (clone L48), and CD62L Alexa Fluor750 (clone Dreg-56). All of the antibodies were obtained from BD Bioscience, USA, except for CD62L from Life Technologies, USA. Permeabilization was done with the use of Foxp3 Staining Buffer Set Kit (eBioscience, USA), while for intracellular staining, $FoxP_3$ APC was used (clone PCH 101, eBioscience, USA). The readout was done with BD FACSCANTO II (BD Bioscience, USA) and 100.000 of cells were acquired.

A representative example of Treg subpopulations gating is given in Figure 1.

2.4. Gating Strategy. First, singlets were identified according to FSC area to height signal distribution (A). Then lymphocytes (B) and CD3+/CD4+ T lymphocytes were gated (C). Next, regulatory T cells were identified as CD4+/$FoxP_3$ double-positive T cells (D), as well as CD127low/CD25+ T cells (E). To get the best overlay between CD127low/CD25 + and $FoxP_3$, Treg gate was put to get minimum 90% of cells in that were $FoxP_3$ positive (F). Then another gate was plotted to identify naïve Tregs as CD45RA+/CD62L+ and memory Tregs as CD45RA−/CD62+ T lymphocytes (G).

2.5. Statistical Analysis. Clinical data, laboratory findings, and family history of the disease were collected in the medical database constructed in Microsoft Excel software for Windows 10 (Microsoft). Data were analyzed using Statistica software version 7.1 for Windows (StatSoft Inc. 2005). Shapiro-Wilk test was used to estimate either normal or abnormal spread of analyzed variables. Depending on the spread of variable, nonparametric Mann–Whitney U test, ANOVA Kruskal-Wallis test, Wilcoxon test, ANOVA Friedman test, and parametric Student's t-test were used. Chi-square test and estimation of the correlation (R Spearman, Pearson) were used for statistical analysis of some variables. Significance level was $p < 0.05$.

3. Results

In our study, for the first time, the percentage of individual subpopulations of regulatory T cells (Tregs) among CD3+CD4+ lymphocytes in the bone marrow and peripheral blood of children suffering from acute lymphoblastic leukemia were determined (Table 2).

3.1. Regulatory T Cell in the Bone Marrow and Peripheral Blood at Diagnosis of Childhood ALL

3.1.1. Comparison of Treg Number in the Bone Marrow and Peripheral Blood in the Group of Children with Acute Lymphoblastic Leukemia (Figure 2). Percentages of regulatory T cells were significantly higher in the bone marrow (9.59+\−3.58) as compared to the peripheral blood (7.81+\−2.73) ($p = 0.002$) (Figure 2). This was not demonstrated in the group of children with diagnosis different than acute leukemia (solid tumors, healthy children) ($p = 0.83$), but the size of this group was quite small. The observations above might be caused by the natural tendency of Treg cells to accumulate in the bone marrow in a higher percentage than in the peripheral blood.

3.1.2. Comparison of Treg Level in the Bone Marrow/Peripheral Blood among Children with Acute Lymphoblastic Leukemia versus Solid Tumors/Healthy Children. When the percentage of Tregs in the bone marrow was measured, the analysis showed no statistically relevant differences between children suffering from ALL and those diagnosed with solid tumor/anemias/lymphadenopathy or even healthy ones. But when the peripheral blood was taken under investigation, there was statistically higher percentage of Tregs among children in the control group in comparison to pediatric patients diagnosed with ALL (Figure 3). Due to the small size of the control group and recognized diseases that may affect the percentage of Treg cells in the bone marrow and peripheral blood, the obtained results are not very reliable. However, the statistically significant differences obtained indicate clearly the need to repeat the analysis among patients with solid tumors.

3.1.3. Statistical Analysis of Selected Treg Subpopulation in Analyzed Population of ALL Children. Memory Treg proportion in the bone marrow of children with ALL was statistically higher than the percentage in peripheral blood ($p = 0.006$) (Figure 4), while percentages of natural naïve Tregs ($p = 0.63$) and induced Tregs ($p = 0.26$) did not differ between blood and bone marrow.

3.1.4. Risk Factors and Biological Characteristic of Cancer Cell versus Tregs Subpopulation in Group of 42 Children with ALL. To identify prognostically relevant parameters, we analyzed

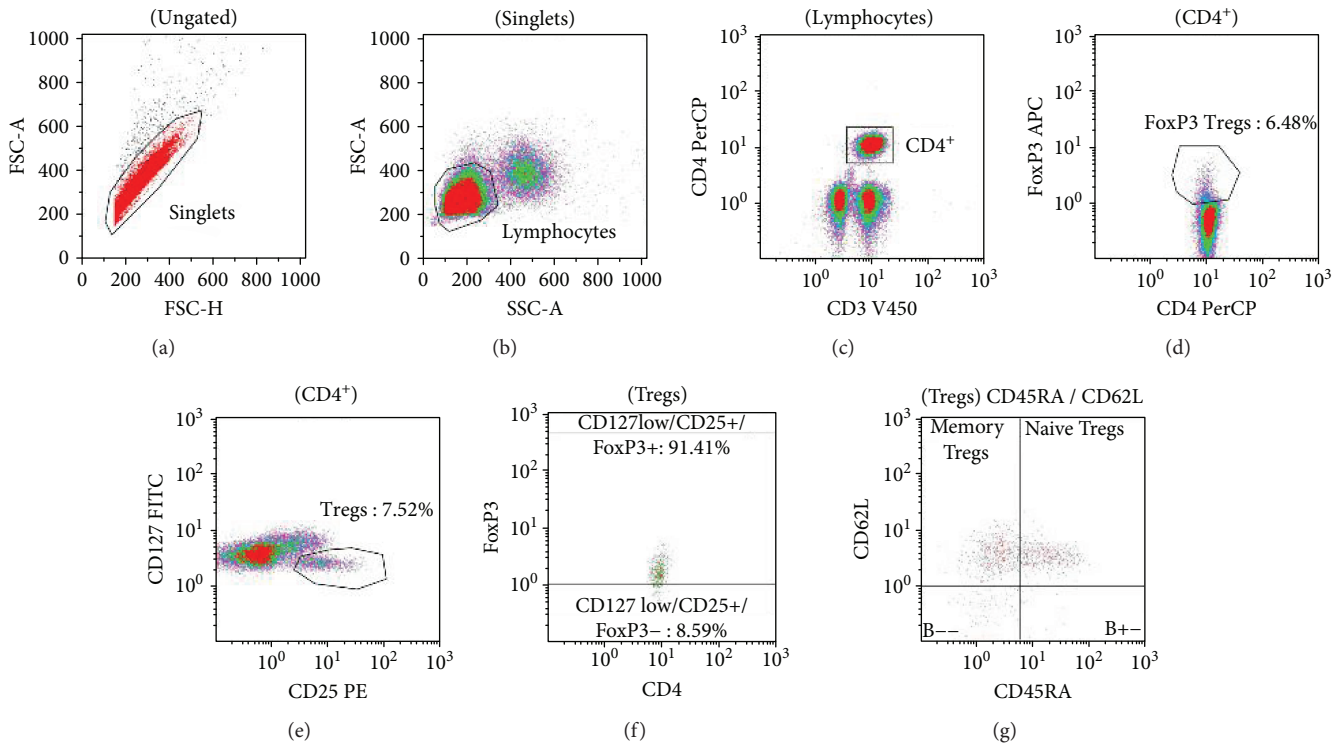

Figure 1

Table 2: Distribution of tested parameters in the bone marrow and peripheral blood in children at diagnosis of ALL.

Tested parameters	Average (%)	Min-max (%)	(Event count) min-max	SD
Treg BM	9.59	2.23–19.03	3528–9913	3.58
Treg PB	7.81	3.33–13.36	2183–6942	2.73
Natural Treg BM	5.39	1.00–16.40	1574–7391	3.80
Natural Treg PB	3.85	0.70–12.00	867–5855	2.38
Natural naive Treg BM	3.86	0.10–8.20	569–4003	1.98
Natural naive Treg PB	3.80	0.90–9.50	1161–5814	2.14

correlation between Treg subpopulations and ALL well-known risk factors and a response to treatment. The risk factors and disease parameters analyzed in our study were gender, age, leukocytosis, and blastosis in the peripheral blood, bone marrow blast count at diagnosis, CNS status (M1; M2; M3 according to Protocol ALLIC 2009), and initial qualification to the risk group (SR, IR, and HR).

The response parameters analyzed in our study were blasts' sensitivity for prednizon (number of blasts on the 8th day of therapy), the percentage of blasts in the bone marrow on day 15 and 33 (<5% (M1 status) or 5% to 20% (M2) or >20% (M3)), and white blood cell count in peripheral blood on day 15 and 33.

Statistical analysis revealed a few interesting observations. A low negative correlation was noticed between Treg cells in the bone marrow and the percentage of blasts in

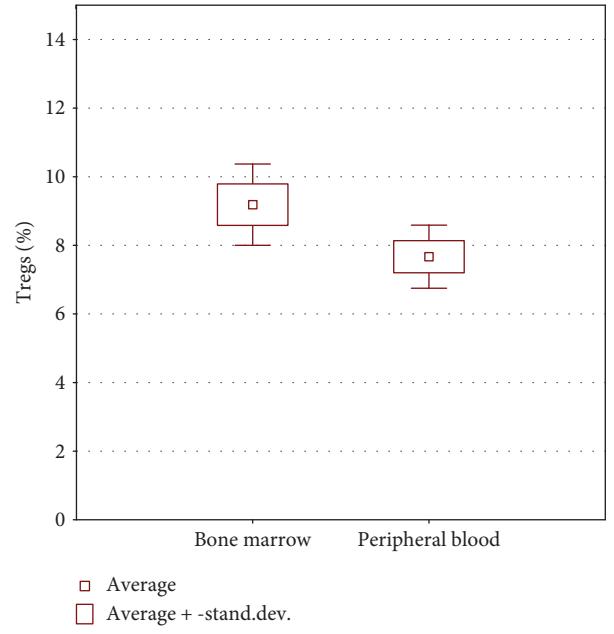

Figure 2: Bone marrow and peripheral blood percentage of Tregs in CD4+ population of cells among children with ALL.

peripheral blood (Figure 5; $R = -0.36$) as well as a moderate correlation between the first one and Hb level in PB before therapy (Figure 6; $R = +0.41$). At the same time, peripheral blood blasts level on day 8 correlates negatively low with the number of Tregs in BM at the moment of diagnosis (Figure 7; $R = -0.36$).

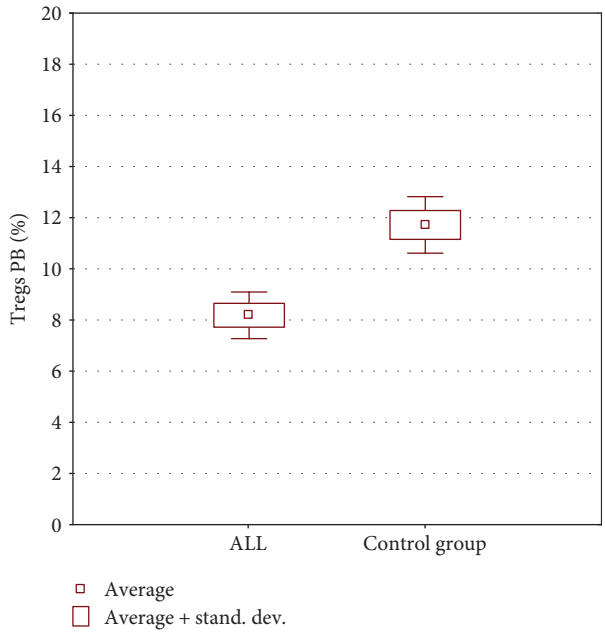

Figure 3: Treg level in peripheral blood among children with ALL versus control group.

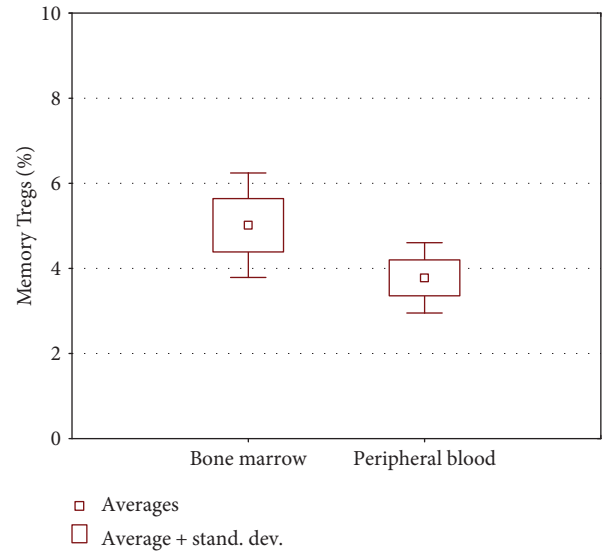

Figure 4: Statistical analysis of selected Treg subpopulations in analyzed population of ALL children.

3.1.5. Answer for the Chemotherapy according to Protocol BFM ALLIC 2009. Statistical analysis revealed a low negative correlation between the level of Tregs in the BM and the minimal residual disease measured on day 15 (MRD 15; $R = -0.24$), as well as the percentage of blasts in the bone marrow ($R = -0.24$) and leukocytosis ($R = -0.2$) after 15 days of chemotherapy.

4. Discussion

ALL is one of the most common childhood cancer with favorable prognosis. Less than 20% of children with acute lymphoblastic leukemia have unfavourable prognosis and suffer from a relapse, resistant ALL, or serious complication of the chemotherapy [13]. For children with recurrent or refractory leukemia, new therapeutic options based on molecular biology and immunological therapy must be found to avoid serious and mortal complications caused by a high-dose chemo- and radiotherapy [14].

Treg cells play a key role in human immunological reaction towards the neoplasmatic cells in the organism. An increased number of Treg cells was noticed in many solid tumors, for example, breast, colon, and lung tumors [7]. In most of the hematological malignances, D'Arena et al. in 2011 noticed that Treg cells numbers were elevated in peripheral blood and correlated with the stage of a disease and the prognosis [15].

Generally in human cancers, higher percentages of Tregs predict worse immunological reaction to the viral infection and cancer antigens. However, the role of Treg cells in the pathogenesis of ALL and AML is still unclear [6].

Tregs are defined on the basis of combined expression of CD4, CD25, $FoxP_3$, low expression of the CD127, and $CD4^+CD25^{high}CD127^{low/-}FoxP_3^+$ regulatory T cell phenotype is the most appropriate one. This sensitive and reliable phenotype was used to determine the percentage of these cells in the bone marrow and/or peripheral blood of children with ALL.

So far, no data has been reported on Treg cell number in the bone marrow among children with acute leukemias. Similarly, very limited evidence is reported about Treg cells in children leukemias and the influence of their number on the prognosis and their correlation with already known risk factors. Lustfeld et al. suggest that elevated proportions of CD4+ T cells among residual bone marrow T cells in ALL is associated with favorable early responses [10]. Several other authors have come to similar conclusions, among others Szczepanski et al. [6] in AML or Idris et al. [1] in ALL.

Our analyses confirm these observations and indirectly indicate a correlation between the percentage of Tregs and prognosis in pediatric ALL. The correlations between all risk factors and hematological parameters of ALL patients and peripheral blood and bone marrow Treg number were analyzed. Probably due to the small size of the group, it was possible to detect only the low negative correlation ($R = -0.36$) between bone marrow Tregs (%) and the number of blasts in peripheral blood after 8 days of steroid therapy. As it is known, blastosis after eight days of steroid therapy is one of the most important prognostic factors in acute lymphoblastic leukemia and often correlates with the response to chemotherapy after 15 and 33 days of intensive treatment.

Thus, the correlation between blasts' sensitivity to steroids and the percentage of Tregs in the bone marrow of children with ALL leukemia is most likely the evidence of the prognostic significance of Tregs for the prognosis of cure. However, if the thesis above would be too daring, then undoubtedly Tregs have at least a significant influence on the response to administered steroids.

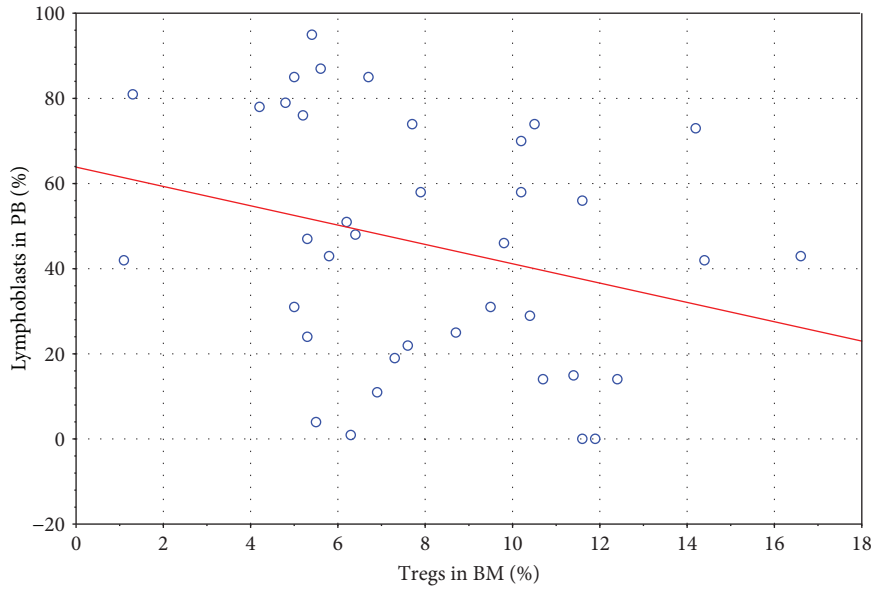

Figure 5: Correlation between the percentage of Tregs in BM and percentage of blasts in PB at the moment of diagnosis ($R = -0.36$).

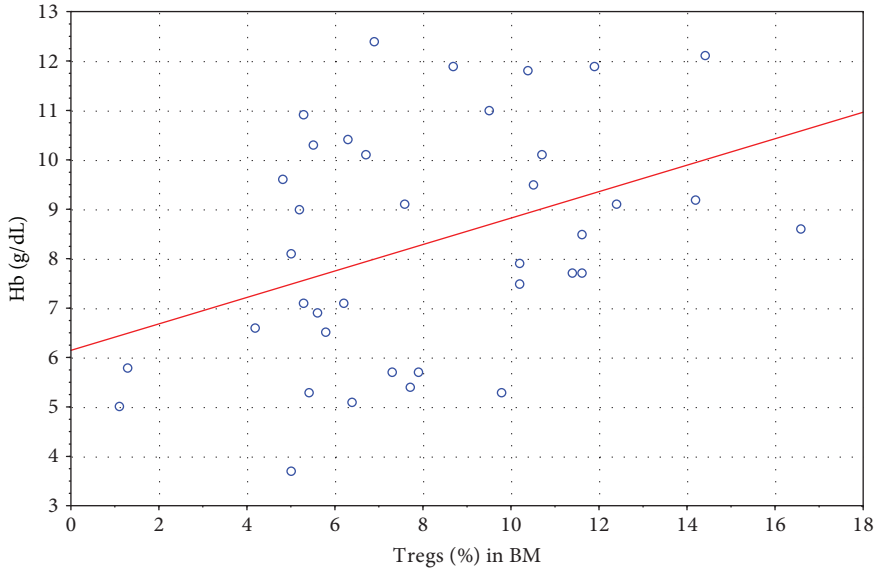

Figure 6: Correlation between the percentage of Tregs in BM at the time of diagnosis with the hemoglobin level ($R = +0.41$).

It is therefore reasonable to assume that by interfering with immune regulatory system, there might be a possibility to influence the effectiveness of the therapy used, which would consequently lead to the reduction of the dose of therapy without affecting the final therapeutic answer [16]. The lack of correlation between regulatory cells and MRD on day 15, as well as the rate of blasts on 15 and 33 days is primarily associated with the abolition of Treg level as a response to treatment with the appropriately selected chemotherapy. Therefore, the most important prognostic factor in acute childhood leukemia is an appropriately selected risk group for intensive chemotherapy.

At the time of diagnosis, Treg level in the bone marrow also showed a low negative correlation ($R = -0.36$) with tumor cell levels in peripheral blood. The latter is an important prognostic factor in ALL. The percentage of blasts found in blood at diagnosis is indirectly indicative when it comes to severity and malignancy of the cancer. Vigorè et al. [17] discovered the dependence of the percentage of Tregs during the progression of cancer on the presence of metastatic changes in various types of solid tumors. Statistically, a higher percentage of regulatory cells in the peripheral blood was detected among patients with advanced cancer than those with no metastatic changes. This may indicate the inhibitory effect of Tregs on the effector anticancer arm of the immune system.

Initially, the most important aim of our study was to confirm the observation of an increased percentage of Treg cells

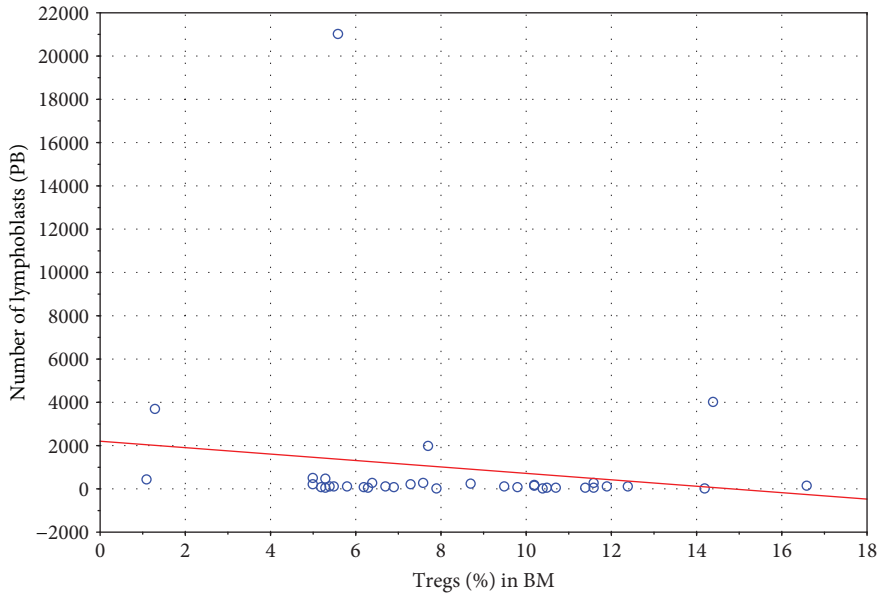

Figure 7: Correlation between blasts level on the 8th day of steroid therapy in PB with the percentage of Tregs in BM at the moment of diagnosis ($R = -0.36$).

in the bone marrow and/or peripheral blood of patients with acute leukemia made by other researchers [1, 6, 11, 13, 18].

The tumor microenvironment, especially suppression of tumor-associated antigen-reactive lymphocytes, is an important factor in the development and progression of cancer [19]. Recent evidence suggests that the cellular composition of the tumor microenvironment, particularly the quantity of the tumor-infiltrating Tregs, can significantly modify the clinical outcome in hematologic malignancies, particularly in some subtypes of lymphomas. Tregs are one of the most interesting populations of immunologically competent cells engaged in fighting cancers [14, 20, 21]. So far, more evidence indicates that regulatory lymphocytes migrate to some particular sites in need of immune regulation [22].

Our study revealed statistically higher percentage of Treg cells in the bone marrow than in peripheral blood in the group of 42 children with acute lymphoblastic leukemia. This fact is a very interesting discovery in the context of described infiltration of regulatory lymphocytes into the neoplasmatic tissue in some types of tumors and hematological malignancies. In case of leukemia, such tissue is bone marrow. A similar relationship in the group of patients diagnosed with other condition than acute lymphoblastic leukemia has not been discovered. Hence, there is our interest in these cells in terms of risk factors and biological features of leukemia cells.

It is not known for sure whether the elevated Tregs in the bone marrow is the response to hematological malignancies or the cause of a developing cancer. It is also unclear whether the percentage of regulatory cells correlates with the recognized prognostic factors in acute lymphoblastic leukemia in children. It was only noted that one study on the murine models showed a correlation between the progression of cancer and the migration of regulatory cells into the tumor tissue [22, 23]. On the other hand, in certain cancer, such as colorectal carcinoma, Tregs suppress bacteria-driven inflammation which promotes carcinogenesis [24, 25]. In this situation, Treg level is an important risk factor. According to some researchers, the tumor-infiltrating, immune-competent regulatory lymphocytes have a great impact on the prognosis. Thus, they might be a very interesting therapeutic option and they need to be determined for each type of cancer separately [15, 16]. Undoubtedly, further research must evaluate this issue.

Another cause of elevated number of lymphatic regulatory cells in the bone marrow of children with acute leukemia may be their natural tendency to accumulate in this tissue [26]. According to some authors, the higher percentage of Treg cells in the bone marrow compared to peripheral blood is natural and is associated with factor CXCL12 [26]. Bone marrow strongly expresses functional stromal-derived factor (CXCL12), the ligand for CXCR4. CXCR4/CXCL12 signals are crucial for Treg homeostasis in the bone marrow and are responsible for the observed effect both in the sick and healthy bone marrow [26].

The question remains whether the increased percentage of Treg cells in the bone marrow compared to peripheral blood in children with ALL results from the physiological tendency of Tregs to accumulate in the bone marrow or from the direct contact of immunocompetent cells with blasts.

The study led us to accept the thesis that a higher proportion of Tregs in the bone marrow of children with acute leukemia is due to the interaction of leukemic cells with Tregs. It is less likely that the accumulation of Treg cells in the bone marrow in a such high percentage is a physiological phenomenon.

Interestingly, it seems that there are no statistically significant differences in the proportion of Tregs between BM and PB in patients who suffer from tumors other than ALL [21, 26, 27].

Detailed analysis of Treg subsets showed very interesting features associated with ALL. The bone marrow of children with ALL was infiltrated by a higher percentage of memory Tregs than the peripheral blood. There was no difference in the number of naive Tregs in both peripheral blood and the bone marrow.

Memory Tregs arise after contact with their own antigen [28]. Undoubtedly leukemia cells express on their surface and cytoplasm antigens identical to the antigens found in the healthy cells. Therefore, in our opinion, the population which is observed is natural regulatory lymphocytes that have been formed after the contact of naive regulatory cells with antigens present on leukemic and normal cells.

The site of this transformation is probably the bone marrow as the percentage of memory Tregs was the highest there. Alternatively, memory Tregs are formed at the periphery and traffic to the sites with high expression of their cognate antigen, such as the leukemic bone marrow.

Most likely, Tregs accumulate there to exert the suppressive effect on the proliferating leukemic blasts. Unfortunately, the increasing percentage of Tregs suppresses also the immune system, which tries to fight a developing tumor. This is probably the reason why the increased percentage of Tregs is seen at the periphery at the very advanced stages of ALL (not analyzed here), while it might be favorable in leukemic bone marrow at early stage of the disease.

Hence, some manipulation on Tregs might be considered as a part of the treatment of hematological malignancies [19, 29, 30].

5. Conclusion

Regulatory T lymphocytes are group of cells that might play important role in the development of cancerous diseases including acute leukemia in children. Their elevated bone marrow and peripheral blood rate among children diagnosed with ALL might be linked to the development of the disease. Manipulations involving Tregs might represent an interesting therapeutic option and may be used to enhance the effect of antitumor chemotherapy. Larger studies are now warranted to validate these findings and determine their clinical implications.

Abbreviations

Tregs: Regulatory T cells
BM: Bone marrow
PB: Peripheral blood
Hb: Hemoglobin
ALL: Acute lymphoblastic leukemia
CLL: Chronic lymphoid leukemia
NHL: Non-Hodgkin lymphoma
AML: Acute myeloid leukemia
RMS: Rhabdomyosarcoma
LDH: Lactate dehydrogenase
BFM SG: Berlin-Frankfurt-Munster study group
SR: Standard risk (group)
IR: Intermediate risk (group)
HR: High risk (group)
EFS: Event-free survival
MRD: Minimal residual disease
CNS: Central nervous system
CD: Cluster differentiation antigen
FoxP3: Forkhead box P3
CXCL12: Stromal cell-derived factor 1
CXCL4: Platelet factor 4.

Data Availability

The statistical analysis (as Excel and Statistica files) used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Lower *FOXP3* mRNA Expression in First-Trimester Decidual Tissue from Uncomplicated Term Pregnancies with a Male Fetus

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Pregnancies with a male fetus are associated with higher risks of pregnancy complications through maladaptation of the maternal immune system. The pathophysiology of this phenomenon is unknown. A possible pathway could be a fetal sex-dependent maternal immune response, since males have a Y chromosome encoding specific allogenic proteins, possibly contributing to a different response and higher complication risks. To analyze whether fetal sex affects mRNA expression of maternal immune genes in early pregnancy, real-time PCR quantification was performed in the decidual tissue from primigravid pregnancies ($n = 20$) between 10 and 12 weeks with uncomplicated term outcomes. Early-pregnancy decidual mRNA expression of the regulatory T-cell marker, *FOXP3*, was sixfold lower ($p < 0.01$) in pregnancies with a male fetus compared to pregnancies with a female fetus. Additionally, mRNA expression of *IFNγ* was sixfold ($p < 0.05$) lower in pregnancies with a male fetus. The present data imply maternal immunologic differences between pregnancies with male and female fetuses which could be involved in different pregnancy pathophysiologic outcomes. Moreover, this study indicates that researchers in reproductive immunology should always consider fetal sex bias.

1. Introduction

Pregnancies with a male fetus have a higher incidence of preterm birth, gestational diabetes mellitus, and preeclampsia [1–4]. The pathophysiology of this phenomenon is unknown. Since these complications of pregnancy are associated with maladaptation of the maternal immune system [5], a possible pathway could lie in a fetal sex-dependent maternal immune response. An explanation might be found in the Y chromosome in males which encodes specific allogenic proteins, possibly contributing to a different maternal immune response when a male fetus is carried.

A number of studies have shown that the maternal immune system develops a fetus-specific immune response [6–8]. Moreover, studies demonstrated fetal sex-specific cytokine levels in maternal peripheral blood during and after pregnancy [9–11]. Additionally, fetal sex was found to affect cytokine expression in placental tissue in asthmatic pregnant women [12, 13]. Until now, differences in the maternal immune response between pregnancies with male or female fetuses were only shown in the peripheral blood and postpartum placental tissue. Whether a fetal sex-specific immune response is elicited at the fetal-maternal interface already in early pregnancy is unknown.

T-regulatory cells (Tregs) are of particular interest in complicated as well as uncomplicated pregnancies because of their immunosuppressive properties [14]. Tregs skew the proinflammatory T-helper 1 response to the more tolerating T-helper 2 response cells [14]. Adequate function and optimal numbers of Tregs are essential for normal implantation and pregnancy outcome, and a lack of adequate Treg numbers is associated with adverse pregnancy outcomes such as

preeclampsia, implantation failure, and infertility [14–16]. The transcription factor forkhead box protein P3 (FOXP3) has been identified as the immunosuppressive protein and marker for Tregs [17]. Whether modulation of the Treg population is dependent on fetal sex and whether Tregs play a role in the etiology of higher rates of pregnancy complications in pregnancies with a male fetus is unknown.

Besides Tregs, other immune cells such as macrophages contribute to tolerance in early pregnancy by shifting towards a more tolerating M2 phenotype and by releasing cytokines which contribute to implantation and tissue remodeling [18, 19]. Fetal sex-specific differences in activation and cytokine profiles of macrophages in placental tissue were found in a mouse study [20]. Early-pregnancy alterations in mRNA expression of macrophage-associated genes in pregnancies with complicated outcomes were demonstrated [21]. However, to our knowledge, neither human studies nor early-pregnancy studies concerning sex-specific macrophages have been performed.

T-lymphocytes and macrophages secrete cytokines that contribute to either a proinflammatory or an anti-inflammatory environment. Both pro- and anti-inflammatory cytokines play a role in implantation, placentation, and pregnancy success [22]. Whereas IL1b and interferon-γ (IFNγ) secretion at the fetal-maternal interface seems beneficial for successful implantation [23], increased levels of IL6 at term are associated with preterm delivery and neonatal morbidity [24, 25]. Presumably, the timing and amount of secretion determine whether a cytokine at a certain stage of pregnancy is beneficial for pregnancy maintenance.

The aim of this study is to analyze fetal sex-dependent differences in mRNA expression of maternal *FOXP3*, macrophage, and other immune-associated gene parameters at the fetal-maternal interface in early pregnancies that developed uneventfully. Hence, the unique first-trimester decidual tissue from ongoing human pregnancies with known uncomplicated term outcomes was studied.

2. Methods

First-trimester decidual tissue was obtained from surplus tissue at vaginally sampled chorionic villus sampling (CVS), between 10 and 12 weeks of gestation for maternal age (over 36 years of age at 18 weeks of gestation) screening for related risk of aneuploidy following the protocol from Huisman et al. [26]. Karyotype analysis was performed for all samples, and the karyogram appeared normal for all fetuses. Immediately after sampling, the decidual tissue was microscopically separated from the villi to minimize trophoblast contamination. Subsequently, samples were stored until further analysis following the protocol from Huisman et al. [26].

Patients were informed that otherwise discarded material could be used for research according to the "Guideline Good Use" by the FMWV committee (Federation of Medical Scientific Associations). Follow-up of pregnancies was available by questionnaires postpartum. Patients on medication, with a history of smoking, diabetes mellitus, or other comorbidities were excluded from the study.

Table 1: Characteristics of patient groups.

	Pregnancies with a female fetus ($n = 8$)	Pregnancies with a male fetus ($n = 8$)
At CVS		
Maternal age (years)	37.4 ± 0.81	39.5 ± 0.46
Gestational age (weeks)	10.97 ± 0.22	10.79 ± 0.19
Gravidity	1	1
Parity	0	0
At delivery		
Gestational age (weeks)	40.5 ± 0.55	41.0 ± 0.60
Birth weight (grams)	3588 ± 120.42	3444 ± 149.41

Mean ± SEM: chorionic villus sampling; CVS: characteristics were compared between groups using Mann–Whitney *U* test with Bonferroni multiple comparison corrections.

Decidual tissues from 20 uncomplicated primiparous pregnancies were randomly selected (10 boys and 10 girls) (see Table 1). All women participating in this study were truly primigravid, did not undergo assisted reproductive techniques, and did not take any medication apart from folic acid. Based on NanoDrop quantity analysis, 4 samples were excluded (2 boys, 2 girls). RNA was isolated and purified; QIAzol lysis reagent (Qiagen, USA) was added, and samples were homogenized using a TissueLyser (Qiagen) (2 minutes, 50 Hertz). Thereafter, RNA was isolated using RNAeasy plus mini-kit (Qiagen). cDNA was reverse transcribed using Superscript-II Reverse Transcriptase kit (Invitrogen, USA). Three housekeeper genes (HPRT, GAPDH, and ACTB) were analyzed. HPRT was the most consistent in all samples and was therefore used for analysis. mRNA expression of *TBX21* (T-helper 1 (Th1) response), *GATA3* (T-helper 2 (Th2) response), *RORC* (T-helper 17 (Th17 response), *FOXP3* (Treg marker), *Interleukin 6 (IL6)*, *IL1b*, *interferon-γ (IFNγ)*, *CD68* (macrophage), *IRF5* (M1 macrophages), and *MRC1* (M2 macrophages) was analyzed using TaqMan On-Demand-Gene-Expression Assays (Thermo Fisher, USA).

PCR reactions were performed in triplicates in a volume of 10 μL consisting of 14 ng RNA, Mastermix (Thermo Fisher, USA), and RNA free water. Runs were performed on a ViiA7 Real-time PCR System (Thermo Fisher, USA), and mRNA data were normalized to *HPRT* mRNA expression using $2^{-\Delta Ct}$. Undetectable cycle threshold (Ct) values (>40) were analyzed as maximum Ct value (40). Outliers were excluded using Grubb's test. For analysis, GraphPad Prism version 5.04 for Microsoft Windows (GraphPad Software, USA) was used. Differences between the groups were evaluated using Mann–Whitney *U* test with Bonferroni multiple comparison corrections. p values < 0.05 were considered statistically significant.

3. Results and Discussion

In pregnancies with a male fetus, there was a sixfold significantly lower mRNA expression of *FOXP3* ($p < 0.01$) compared to pregnancies with a female fetus (see Figure 1). In

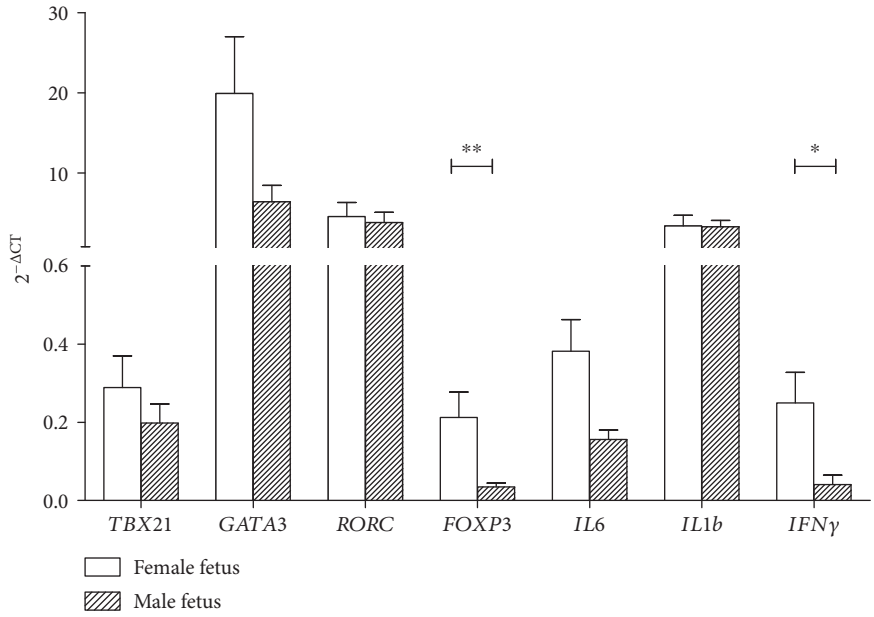

Figure 1: mRNA expression of T-lymphocyte markers and cytokines in the first-trimester human decidual tissue. Data are mean ± SEM mRNA target gene expression normalized to housekeeper gene *HPRT*, in the decidual tissue from pregnancies with a female fetus (open bars, $n = 8$) and pregnancies with a male fetus (black bars, $n = 8$). Comparison between groups was evaluated using Mann–Whitney U test with Bonferroni multiple comparison corrections; $^{*}p < 0.05$, $^{**}p < 0.01$.

both human and murine studies, it has been shown that Tregs play a role in healthy implantation and placental development in early pregnancy [27, 28]. The lower expression of *FOXP3* in the first-trimester decidual tissue from pregnancies with a male fetus could imply an inferior maternal immune tolerance in early pregnancies with a male fetus possibly contributing to a higher risk of pregnancy complications [14].

An explanation for the fetal sex-specific difference in mRNA expression of *FOXP3* could be the presence of the Y chromosome in males, which encodes minor histocompatibility antigens (HY) [29]. HY antigens are expressed in the first-trimester placental tissue and can be recognized by maternal T-lymphocytes eliciting an HY-specific immune response [11, 30, 31]. Only a limited number of studies are performed on the ability of fetal HY antigens to induce or suppress maternal *FOXP3* mRNA expression and Treg cells [32, 33]. Kahn et al. showed that HY induces an HY-specific Treg population that contributes to tolerance in mice; however, no comparison with HY absent pregnancies (solely female fetuses) was made. Therefore, no conclusions of the effects of fetal sex on *FOXP3* induction can be made [33]. Our results show that mRNA expression of the Treg marker *FOXP3* is affected by fetal sex; however, more research is necessary to clarify the role of HY in the difference of mRNA expression between pregnancies with a male and a female fetus.

In addition, significantly higher mRNA expression of *IFNγ* ($p < 0.05$) was found in pregnancies with a female fetus (see Figure 1). Many studies have associated increased IFNγ in different tissues with pregnancy complications such as preeclampsia [34]. Therefore, this finding could appear contradictory to the hypothesis in which male fetuses have a less favorable maternal immune environment in pregnancy. However, the proinflammatory cytokine, which is encoded by the *IFNγ* gene, has also been shown to be favorable for pregnancy [22, 35, 36]. Especially in early pregnancy, IFNγ has been demonstrated to be important [22, 35, 36]. IFNγ plays a role in implantation, placentation, and continuation of pregnancy [22, 35, 36]. Higher decidual IFNγ at term was associated with preeclampsia; however, women delivering preterm had lower IFNγ at midgestation compared to women delivering at term [34, 37, 38]. These data imply that IFNγ synthesis is beneficial in early pregnancy to midgestation and is unfavorable for pregnancy success in the third trimester [36]. Furthermore, IFNγ has been shown to be indispensable for the conversion of non-Treg cells into Treg cells [37, 38]. Since we found higher mRNA expression of both *IFNγ* and *FOXP3*, it could be postulated that during early pregnancy, the higher expression of *IFNγ* is necessary for a robust implantation and placentation and that the possible compensatory higher expression of *FOXP3* mRNA is necessary to dampen the effect of proinflammatory cytokines for successful pregnancy outcome. Herewith, the differences found in this study support the hypothesis that a difference in maternal immune response depending on fetal sex plays a role in the different incidences of pregnancy complications between pregnancies with a male or a female fetus. Our findings are in line with previous studies which showed altered fetal sex-specific cytokine levels in peripheral maternal blood during pregnancy and in placental tissue after delivery [9, 10, 12, 13].

In this study, no fetal sex-specific differences in mRNA expression of macrophage markers were found (see Figure 2). Macrophage mRNA expression is possibly not influenced by fetal sex at this early stage of pregnancy, as in an obesity mouse model where a fetal sex-specific difference

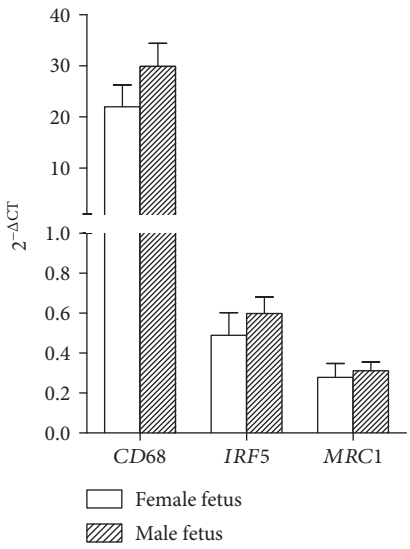

Figure 2: mRNA expression of macrophage markers in the first-trimester human decidual tissue. Data are mean ± SEM mRNA target gene expression normalized to housekeeper gene *HPRT*, in the decidual tissue from pregnancies with a female fetus (open bars, $n = 8$) and pregnancies with a male fetus (black bars, $n = 8$). Comparison between groups was evaluated using Mann–Whitney *U* test with Bonferroni multiple comparison corrections.

in macrophage activation was seen in late pregnancy but not in early-pregnancy placental tissue [20]. Or, alternatively, our sample size is too small to detect these changes.

This study uses the first-trimester decidual tissue from pregnancies with uncomplicated outcomes, which is unique and almost unobtainable. With the current knowledge on risks of pregnancy complications caused by CVS and the availability of alternative techniques [39], nowadays, CVS is not routinely performed anymore. The first-trimester decidual tissue used in this study is therefore highly appreciated and a unique possibility that enabled analysis of immune parameters in early pregnancies with known outcome. To limit the risks of pregnancy complications, the tissue volume taken with CVS was reduced to the smallest amount. The remaining tissue volume after diagnostic tests was only sufficient for PCR analysis of the genes shown and no further experiments could be performed. Therefore, actual protein synthesis and cell quantification were not investigated in this study. However, the differences in mRNA expression shown in this study do imply differences in the maternal immune response between pregnancies with a male and a female fetus. Further research is necessary to elucidate whether the different mRNA expression found does coincide with protein expression and the immune environment in early pregnancy.

In general, despite growing evidence showing the effects of fetal sex on the maternal immune response, still, most studies performed in reproductive research do not consider a fetal sex bias blurring their results. As this study shows that the maternal immune response differs depending on fetal sex, we propose that fetal sex differences between groups should always be considered.

4. Conclusions

In summary, this study shows fetal sex-specific differences in mRNA expression of maternal immune factors in the first-trimester decidual tissue. Lower mRNA expression of *FOXP3* and the proinflammatory cytokine encoding gene *IFNγ* was found in uncomplicated pregnancies with a male fetus compared to pregnancies with a female fetus. In the first-trimester decidual tissue studied, no differences for mRNA expression of macrophage markers were found.

These findings imply a fetal sex-dependent maternal immune response, which could be involved in the pathophysiology responsible for the higher incidence of adverse pregnancy outcomes in pregnancies with a male fetus. Moreover, this study supports that reproductive immunology research should always consider fetal sex bias.

Data Availability

The datasets used to support this study are currently being used for further research on the topic. Access to the data will be considered upon request by contacting the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Tom E.C. Kieffer and Anne Laskewitz contributed equally to this work.

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Review Article

Immune Privilege and Eye-Derived T-Regulatory Cells

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Certain cellular components of the eye, such as neural retina, are unable to regenerate and replicate after destructive inflammation. Ocular immune privilege provides the eye with immune protection against intraocular inflammation in order to minimize the risk to vision integrity. The eye and immune system use strategies to maintain the ocular immune privilege by regulating the innate and adaptive immune response, which includes immunological ignorance, peripheral tolerance to eye-derived antigens, and intraocular immunosuppressive microenvironment. In this review, we summarize current knowledge regarding the molecular mechanism responsible for the development and maintenance of ocular immune privilege via regulatory T cells (Tregs), which are generated by the anterior chamber-associated immune deviation (ACAID), and ocular resident cells including corneal endothelial (CE) cells, ocular pigment epithelial (PE) cells, and aqueous humor. Furthermore, we examined the therapeutic potential of Tregs generated by RPE cells that express transforming growth factor beta (TGF-β), cytotoxic T lymphocyte-associated antigen-2 alpha (CTLA-2α), and retinoic acid for autoimmune uveoretinitis and evaluated a new strategy using human RPE-induced Tregs for clinical application in inflammatory ocular disease. We believe that a better understanding of the ocular immune privilege associated with Tregs might offer a new approach with regard to therapeutic interventions for ocular autoimmunity.

1. Introduction

The microenvironment in the eye is both immunosuppressive and anti-inflammatory in nature. This immunosuppressive property by ocular resident cells/tissues is referred to as immune privilege. This phenomenon helps prevent extensive damage caused by infiltrating inflammatory cells that would otherwise lead to blindness. The eye expresses an extensive array of mechanisms through which innate and adaptive immune cells can be regulated, thereby avoiding blindness as a consequence of intraocular inflammation [1–3]. The immunosuppressive mechanisms that have been revealed to date include a microenvironment in the eye, for example, ocular fluids, blood-retina barriers, and ocular resident parenchymal cells. Ocular fluids, which include aqueous humor and vitreous fluids, have anti-inflammatory properties [4–6]. Some ocular resident cells create a blood-retina barrier to limit the ingress of blood cells, while ocular parenchymal cells express the CD95 ligand (CD95L/Fas ligand) that triggers apoptosis of inflammatory cells [7]. In these ocular immune privilege cells, retinal pigment epithelial (RPE) cells contribute to the immune privilege property of the eye. RPE cells form tight junctions and create the blood-retina barriers. Moreover, RPE cells constitutively express immunosuppressive molecules and secrete soluble immunomodulatory factors that are capable of mediating immunogenic inflammation [8, 9]. These mechanisms make it possible for the eye to regulate the intraocular innate and adaptive inflammatory response and accept transplanted tissue grafts for extended periods. In contrast, conventional body sites summarily reject such grafts. This review focuses on the development and maintenance of the immunosuppressive intraocular microenvironment formed via the generation of regulatory T cells (Tregs) by anterior

chamber-associated immune deviation (ACAID), and ocular resident cells, which include corneal endothelial (CE) cells, ocular pigment epithelial (PE) cells, and aqueous humor. This review also evaluated the therapeutic potential of Tregs as powerful immunosuppressive cells that can be used for active noninfectious uveitis and corneal allograft transplantation.

2. Generation of Tregs in Eye-Derived Tolerance

To achieve immune privilege, the eye uses several different strategies to prevent and regulate sight-destroying inflammation in the eye [1, 10]. One of the strategies is the induction of the peripheral tolerance of eye-derived antigens referred to as ACAID [1, 11]. Antigenic materials in the anterior chamber generate a systemic immune response that retains primed, clonally expanded cytotoxic T-cell precursors and B cells secreting large concentrations of IgG_1, which is a non-complement-fixing antibody. On the other hand, ACAID inhibits $CD4^+$ Th1 and Th2 cells and B cells secreting complement-fixing antibodies [1, 2, 12–16]. The spleens of mice that receive antigen in the anterior chamber acquire three types of antigen-specific Tregs that mediate ACAID [17–19]. One of these populations consists of $CD4^+$ T cells, which are known as the "afferent regulators," as these $CD4^+$ T cells are able to suppress the initial activation and differentiation of naïve T cells into Th1 effector cells. The second population consists of $CD8^+$ T cells, which are known as "efferent regulators," as these $CD8^+$ T cells inhibit the expression of Th1 immune responses such as delayed hypersensitivity. The third population consists of $CD8^+$ T cells that inhibit B cells from switching to the IgG isotype that fixes the complement. Efferent $CD8^+$ Tregs in ACAID act in the periphery, including in the eye, whereas afferent $CD4^+$ Tregs act in the secondary lymphoid organs [11, 20]. In ACAID, eye-derived antigen presenting cells (APCs) induce the expansion of tolerogenic B cells in order to induce antigen-specific Tregs [21] and invariant natural killer T cells, which are additionally required for the generation of ACAID [22]. Furthermore, Hare et al. have also demonstrated that the anterior chamber injection of bovine interphotoreceptor retinoid-binding protein (IRBP) impaired the development of IRBP-specific delayed hypersensitivity and prevented the expression of experimental autoimmune uveoretinitis (EAU). This model of human uveitis can be induced by immunization of susceptible animals with a retinal antigen such as IRBP [23–25]. Moreover, the adoptive transfer of spleen cells obtained from mice that received IRBP to the anterior chamber suppressed and eliminated already established intraocular inflammation, which suggests that IRBP-specific, ACAID-inducing Tregs act on the efferent limb of the immune response [23]. A recent study has also shown that retinal antigen-pulsed tolerogenic APCs (ACAID-genic APCs) suppressed ongoing EAU by inducing $CD8^+$ Tregs that, in turn, suppressed the effector activity of IRBP-specific T cells [26]. Thus, ACAID via antigen-specific Tregs suppresses IRBP-induced autoimmune uveoretinitis.

3. Generation of Tregs by an Immunosuppressive Intraocular Microenvironment That Includes Corneal Endothelium, Aqueous Humor, and Pigment Epithelial Cells

There is growing evidence that ocular resident cells, which include CE cells and PE cells, can contribute to the development and maintenance of the immunosuppressive intraocular microenvironment via the generation of Tregs [8]. In addition to the ocular PE cells, the eye also contains resident myeloid cell populations such as macrophages and microglial cells. However, most of the macrophages are restricted to the cornea and uveal tract, where they are responsible for maintaining homeostasis by removing debris and dead cells. Microglial cells also play important roles in retinal development/homeostasis and can mediate local neuroinflammatory reactions [27, 28].

Tregs induced by ocular PE cells, which constitutively express the transcription factor Foxp3, are indispensable for immune tolerance and homeostasis, as they suppress excessive immune responses that are harmful to the host [29]. Since Tregs have been involved in a series of pathologic processes associated with autoimmune disease and cancer [30, 31], $Foxp3^+$ Tregs as well as Tregs in ACAID have been considered to be the key regulators in ocular immune privilege. In the following section, we describe the molecular mechanisms that underlie the Treg induction by ocular resident cells, in addition to evaluating the therapeutic potential of CE and PE-induced Tregs in helping to maintain the ocular immune privilege.

3.1. Strategy for Generation of Tregs by Ocular Resident Cells. We performed *in vitro* experiments to investigate whether cultured ocular resident cells, including CE, iris PE, ciliary body PE, and retinal PE (RPE) cells, would have the capacity to convert activated T cells into Tregs [8]. To generate Tregs *in vitro*, naïve $CD4^+$ or $CD8^+$ T cells obtained from C57BL/6 mice were cocultured with ocular PE cells in the presence of anti-CD3 antibody. T cells exposed to CE or PE cells were harvested, x-irradiated, and used as regulators (PE-induced Tregs). $CD4^+$ T cells obtained from C57BL/6 mice were used as responder T cells. The responder T cells and PE-induced Tregs were then cocultured in the presence of anti-CD3 antibody in order to evaluate whether PE-induced Tregs suppressed the proliferation and cytokine production of the responder T cells. If there was suppression of the responder T cell activation, this would confirm that there was induction of Tregs by the ocular resident cells. The molecular mechanism underlying the generation of Tregs differs in accordance with the microenvironment of the ocular resident cells.

3.2. CE Cell-Induced Tregs. CE cells are part of the inner surface of the anterior chamber of the eye and come in contact with the aqueous humor. Human CE cells contribute to local immune tolerance in the human eye, as activated T cells exposed to CE cells fail to acquire effector T-cell function [32–34]. In addition, it has been reported that murine CE cells constitutively express various immunomodulatory molecules such as the Fas ligand, programmed death-ligand 1

(PD-L1/CD274), and glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand, which leads to apoptosis of the effector T cells [35–37]. We have previously demonstrated that cultured human CE cells suppressed the activation of $CD4^+$ Th1 cells in a cell contact-dependent manner via an interaction between the PD-1 and PD-L1 costimulatory molecules *in vitro* [34]. Subsequently, we then investigated whether human CE cells were capable of inhibiting T cells and generating Tregs *in vitro*. Cultured human CE cells produced enhanced membrane-bound active transforming growth factor beta 2 (TGF-β2) and suppressed activation of $CD8^+$ T cells via a membrane-bound form of TGF-β [38]. Furthermore, cultured CE cells converted $CD8^+$ T cells into Tregs via their membrane-bound active TGF-β. In addition, CE cell-induced $CD8^+$ Tregs expressed both $CD25^{high}$ and Foxp3 and suppressed activation of bystander effector T cells [38].

In a further experiment, we also examined whether murine CE cells have the capacity to generate Tregs. $CD4^+$ T cells exposed to cultured murine CE cells expressed both $CD25^{high}$ and Foxp3, with these T cells suppressing the activation of the bystander target T cells, which indicates that cultured murine CE cells have the capacity to generate Tregs [39]. Moreover, cytotoxic T lymphocyte-associated antigen-2 alpha (CTLA-2α: cathepsin L inhibitor), which is expressed on murine CE cells, promoted Tregs through TGF-β signaling [39]. Taken together, these findings suggest that cultured CE cells expressing TGF-β and CTLA-2α promote the generation of CD4/$CD8^+$ Tregs that are able to suppress bystander effector T cells, thereby helping to maintain the immunosuppressive intraocular microenvironment.

3.3. Aqueous Humor-Induced Tregs. The aqueous humor participates in the local defense system of the eye and protects the intraocular tissue from immunogenic inflammation [6]. The aqueous humor contains immunosuppressive factors such as α-melanocyte-stimulating hormone (α-MSH), vasoactive intestinal peptide, and TGF-β2 [6]. It has been reported that the aqueous humor is capable of inducing Tregs via α-MSH and TGF-β2 [40, 41]. Furthermore, it has been reported that the aqueous humor obtained from rats recovering from monophasic EAU was able to enhance the regulatory function of ocular Tregs in recurrent EAU [42]. A recent study additionally showed that the aqueous humor promoted the conversion of naïve T cells into $Foxp3^+$ Tregs, while TGF-β and retinoic acid had a synergistic effect on the Treg conversion mediated by the aqueous humor [43].

3.4. Ocular PE Cell-Induced Tregs. Ocular PE cells of the iris, ciliary body, and retina have been identified as important participants in creating and maintaining ocular immune privilege [8, 10, 44]. Iris PE cells have the capacity to suppress anti-CD3-driven activation of primed or naïve T cells [44]. We have previously shown that cultured iris PE cells suppressed TCR-driven T-cell activation *in vitro* through direct cell contact in which the B7-2 (CD86) expressed by the iris PE cells interacted with CTLA-4 on the responding T cells [45]. B7-2^+ iris PE cells in the presence of anti-CD3 agonistic antibody supported selective activation of CTLA-4^+CD8^+ T cells that express their own B7-2 and secreted enhanced amounts of active TGF-β, leading to the global suppression of entire T-cell populations, including $CD4^+$ T cells [46].

Subsequently, we then examined whether TGF-β was necessary for this process. Our study showed that both the iris PE and T cells exposed to iris PE cells were able to: (1) upregulate their TGF-β and TGF-β receptor genes, (2) convert the latent TGF-β they produced into the active form, and (3) use membrane-bound or soluble TGF-β to suppress bystander T cells. This demonstrated that both iris PE cells and B7-2^+CTLA-4^+CD8^+ iris PE-induced Tregs produce enhanced amounts of active TGF-β, with the membrane-bound form of TGF-β used to suppress T-cell activation [47]. Furthermore, iris PE cells promoted the generation of $Foxp3^+CD8^+CD25^+$ Tregs with cell contact via the B7-2/CTLA-4 interactions [48, 49]. In addition, iris PE-induced $CD8^+$ Tregs greatly expressed PD-L1 costimulatory molecules and suppressed the activation of bystander Th1 cells that express PD-1 costimulatory receptor via a contact-dependent mechanism [50]. A previous study clearly demonstrated that thrombospondin-1 (TSP-1) binds and activates TGF-β [51]. Furthermore, iris PE cells generated $CD8^+$ Tregs via TSP-1 and iris PE-induced $CD8^+$ Tregs suppressed activation of bystander T cells via TSP-1 [52]. Taken together, these results strongly suggest that iris PE cell-induced $CD8^+$ Tregs play a role in maintaining immune privilege in the anterior segment of the eye (Figure 1).

Previous studies have shown that the subretinal space is also an immune privileged site and that RPE cells act as immune privilege tissue [53, 54]. Moreover, RPE cells play pivotal roles in helping to maintain immune privilege in the subretinal space [3]. RPE cells have been shown to secrete soluble factors including TGF-β, TSP-1, and PGE_2, which are mediators that alter the innate and adaptive immune responses [55–57]. Depending upon the inflammatory conditions, RPE cells are able to inhibit activated T cells that are regulated by the levels of the MHC class II expression [58]. Moreover, under the presence of inflammatory cytokines such as IL-17 and IFN-γ, RPE cells also highly express PD-L1, which can lead to suppression of the pathogenic activity of IRBP-specific T cells that induce EAU [59].

We have also reported that unlike for the iris PE cells, the RPE and ciliary body PE cells can suppress bystander T cells through inhibitory soluble factors and that the soluble form of the active TGF-β1/2 produced by the RPE and ciliary body PE cells demonstrated an immunosuppressive effect on the bystander T cells [56]. Subsequently, we then investigated whether RPE cell-exposed T cells could become Tregs *in vitro* and if the soluble form of TGF-β produced by the cultured RPE cells could convert T cells into Tregs. Our results showed that cultured RPE cells converted $CD4^+$ T cells into Tregs in the presence of CTLA-2α [60]. RPE cells constitutively expressed CTLA-2α (cathepsin L inhibitor), which promoted the induction of Tregs, and $CD4^+$ T cells exposed to RPE cells predominantly expressed $CD25^+$ and Foxp3 [60]. Furthermore, recombinant CTLA-2α promoted the development of $CD4^+$, $CD25^+Foxp3^+$ Tregs through TGF-β signaling *in vitro*, with these Tregs producing high

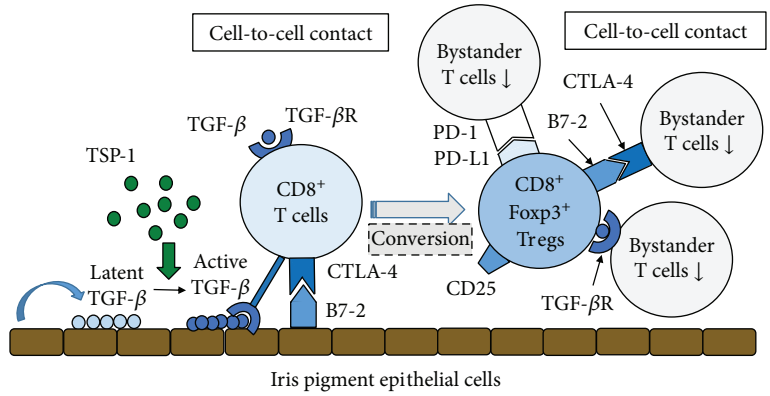

FIGURE 1: Molecular mechanism underlying the generation of regulatory T cells (Tregs) by murine iris pigment epithelial (PE) cells. Cultured iris PE cells suppress anti-CD3-driven T cell activation *in vitro* by direct cell contact in which B7-2 (CD86) expressed by iris PE cells interacts with cytotoxic T-lymphocyte antigen-4 (CTLA-4) on responding T cells. Furthermore, cultured iris PE cells expressing B7-2 induce the activation of CTLA-4$^+$CD8$^+$ T cells that express their own B7-2 and secrete enhanced amounts of active transforming growth factor beta (TGF-β), leading to the global suppression of entire T-cell populations including CD4$^+$ T cells. Both iris PE cells and T cells exposed to iris PE cells upregulate their TGF-β and TGF-β receptor (TGF-βR) genes and suppress bystander T cells using membrane-bound or soluble TGF-β. In addition, iris PE cell-induced Foxp3$^+$CD8$^+$CD25$^+$ Tregs suppress bystander T cells through cell contact via B7-2/CTLA-4 and/or programmed cell death- (PD-) 1/PD-L1 interactions. Thrombospondin-1 (TSP-1) produced from iris PE cells greatly contributes to the conversion of TGF-β from latent form to active form.

levels of TGF-β [60]. These findings demonstrated that RPE cell-induced Tregs participated in the establishment of immune tolerance in the posterior segment of the eye (Figure 2). Our recent study also showed that RPE cells that produced retinoic acid and cultured RPE cells from vitamin A-deficient mice were unable to induce Foxp3$^+$ Tregs [61]. These data are compatible with previous studies that have shown that the conversion of naïve T cells into Foxp3$^+$ Tregs in the eye required TGF-β and retinoic acid [43, 61]. Thus, overall, these findings indicate that TGF-β and retinoic acid interact to induce Tregs for immunological regulation in the eye (Figure 2).

4. Immunomodulation of Uveitis by Tregs

Thymus-derived naturally occurring Tregs play an essential role in preventing autoimmune disease, with depletion of the naturally occurring Tregs leading to multiorgan autoimmune disease [29, 30]. Indeed, depletion of CD4$^+$CD25$^+$ T cells before immunization has been shown to exacerbate the murine EAU model of human uveitis [62]. A recent study reported that retinal antigen-specific Foxp3$^+$ Tregs play a role in the natural resolution of EAU and the maintenance of remission [63]. Conversely, there is growing evidence that administration of Tregs can effectively suppress uveitis in mice. Antigen-specific Tregs generated by α-MSH and TGF-β2 have also been shown to suppress EAU [64]. In addition, lipopolysaccharide-activated dendritic cell-induced CD4$^+$CD25$^+$Foxp3$^+$ Tregs inhibit CD4$^+$CD25$^-$ effector T cells, and when adoptively transferred, these Tregs suppress EAU [65]. Moreover, intravenous administration of antigen-specific Tregs has the capacity to control uveitis in mice [66]. In addition, an intravitreous injection of preactivated polyclonal Tregs was also shown to suppress uveitis in mice [67]. In our own study, we also demonstrated that the adoptive transfer of CD4$^+$CD25$^+$ natural Tregs ameliorated the development of EAU [68]. However, the ability to prepare large numbers of Tregs for adoptive transfer and stable expression of Foxp3 *in vivo* remains problematic.

Since retinoic acid has been reported to contribute to high and stable Foxp3 expression via the retinoic acid receptor in the presence of TGF-β [69], we investigated whether retinoic acid has the capacity to expand Tregs and ameliorate the development of EAU. The results of our study demonstrated that retinoic acid promoted the generation of CD4$^+$Foxp3$^+$ Tregs in the presence of TGF-β, with systemic administration of retinoic acid during the induction phase reducing the clinical score of EAU [70, 71]. Furthermore, oral administration of a novel synthetic retinoic acid, Am80, not only increased the frequency of Tregs in draining lymph nodes in mice with EAU but also suppressed the Th1/Th17 response [71]. Am80 is more stable to light, heat, and oxidation than retinoic acid, and Am80 is clinically available in Japan for the treatment of relapsed acute promyelocytic leukemia. Thus, systemic administration of retinoid may not only have the potential to promote the expansion of Tregs *in vivo*, but it appears that it may also have therapeutic possibilities. In addition, since a previous report demonstrated that TGF-β levels were significantly elevated in the aqueous humor from EAU eyes [72], it is conceivable that the expression of Foxp3 on intraocular T cells in Am80-treated mice may be increased, with expansion of Foxp3$^+$ Tregs possibly contributing to the amelioration of murine EAU.

Stabilization of Foxp3 expression is necessary for the generation and maintenance of highly suppressive Tregs *in vivo* for clinical use. Presently, various reagents and drugs, such as rapamycin, IL-2, and retinoic acid, have been reported to stabilize Foxp3 expression [73]. Furthermore, epigenetic modification of Foxp3 expression may be required in order to generate stable Tregs for clinical application [74].

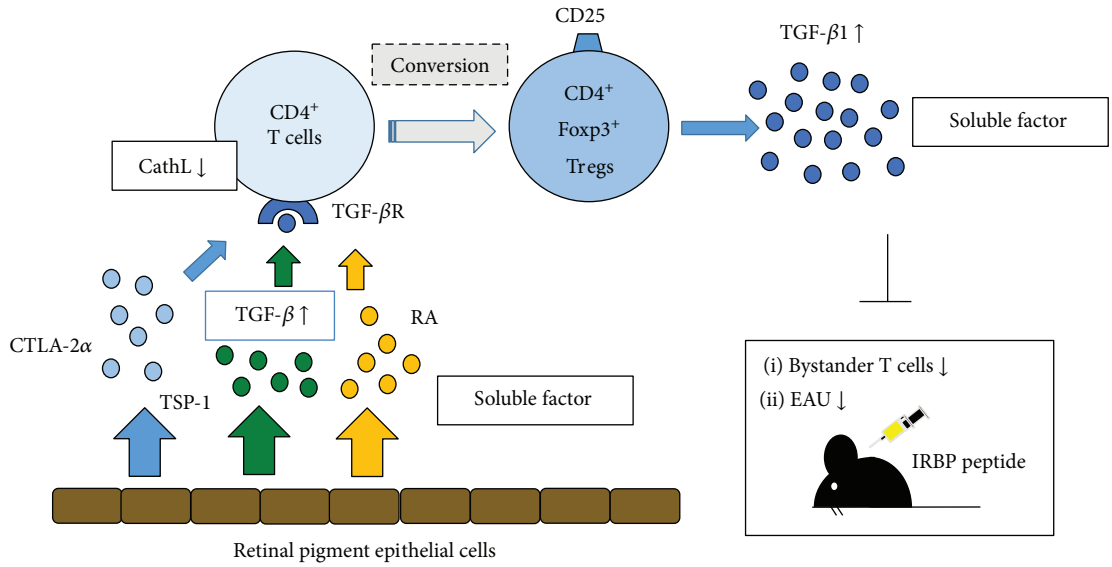

Figure 2: Molecular mechanism underlying the generation of regulatory T cells (Tregs) by murine retinal pigment epithelial (RPE) cells. RPE cells constitutively express cytotoxic T lymphocyte-associated antigen 2 alpha (CTLA-2α), a cathepsin L (CathL) inhibitor, which promotes the induction of Tregs. In addition, $CD4^+$ T cells exposed to RPE cells predominantly express CD25 and Foxp3. CTLA-2α, thrombospondin-1 (TSP-1), and retinoic acid promote the development of $CD4^+CD25^+$ $Foxp3^+$ Tregs by transforming growth factor beta (TGF-β) signaling *in vitro*. These Tregs produce high levels of TGF-β and suppress bystander T cells and experimental autoimmune uveoretinitis (EAU) induced by retinal antigen interphotoreceptor retinoid-binding protein (IRBP).

As described above, we demonstrated that recombinant CTLA-2α (rCTLA-2α) derived from RPE cells has the capacity to generate Tregs through the promotion of TGF-β production [60]. Indeed, rCTLA-2α-treated mice had a high population of $Foxp3^+$ Tregs compared with $CD4^+$ T cells from control EAU mice [75]. Furthermore, the severity of EAU was significantly reduced in rCTLA-2α-treated mice and cathepsin L-deficient mice as compared with wild type mice. Thus, these findings suggest that CTLA-2α secreted from RPE cells converts intraocular effector T cells into $Foxp3^+$ T cells that then acquire regulatory functions and lead to the amelioration of ocular inflammation [75].

We next assessed the ability of murine RPE cell-induced Tregs to suppress EAU in mice through the use of adoptive transfer. Our data revealed that the administration of RPE cell-induced Tregs that greatly expressed Foxp3 were able to suppress ocular inflammation in mice with EAU [76]. Moreover, the retinal antigen-specific cytokine response (IFN-γ and IL-17) was reduced when intraocular T cells were cocultured with RPE cell-induced Tregs *in vitro* [76]. These findings suggest that RPE cell-induced Tregs might possibly have a therapeutic potential for the treatment of autoimmune uveoretinitis.

Another recent challenge encountered with Treg therapy was reported while using a murine ocular inflammatory model, which included both antigen-specific and nonantigen-specific murine disease models [67]. In the antigen-specific model, TCR-hemagglutinin (HA) transgenic mice and HA-specific effector T cells were used to induce uveitis in mice in which HA is constitutively expressed in the retina. The authors found that Treg transplantation in the systemic circulation significantly suppressed local ocular inflammation. Moreover, polyclonal Tregs that expanded ex vivo also significantly improved ocular inflammation when these Tregs were injected locally, that is, intravitreally. Other recent investigations have additionally shown that several regulatory molecules including IL-22, aryl hydrocarbon receptor, and CD73/adenosine contribute to the generation of Tregs/regulatory mesenchymal stem cells to control EAU in mice [77–79]. These murine study findings support the concept of Treg therapy for ocular inflammation and are the foundation for further human clinical trials.

5. Ocular Surface Disease and Tregs

Dry eye disease (DED) is one of the major ocular surface inflammatory disorders [80, 81]. It is well known that activation and infiltration of pathogenic immune cells, primarily $CD4^+$ T cells, contribute to the development of ocular surface inflammation in DED [82–84]. Increased IL-17 and IFN-γ have been observed in both clinical and experimental DED [85–89]. Recent studies have demonstrated that Th17 cells are the principal effectors actively mediating DED [90, 91]. In fact, Chauhan et al. reported that while Treg frequencies remained unchanged, there was a marked decrease in their potential to suppress the effector Th17 cells in a mouse model of DED. This suggests that dysfunction of Tregs can be presumed to be one of the major causes in ocular anterior segment inflammation such as DED [90, 92]. It has also been reported that *in vitro*-expanded $Foxp3^+$ Tregs maintain a normal phenotype and are capable of suppressing immune-mediated ocular surface inflammation in animal models, with *in vitro*-expanded Tregs able to more efficiently reduce tear cytokine levels and conjunctival cellular infiltration compared to freshly isolated Tregs [93]. Antigen specificity is one of important factors required for Tregs in order to more

effectively regulate the pathogenic inflammatory cells. Presently, while the specific autoantigen responsible for the induction of dry eye disease has yet to be identified, it has been suggested that α-fodrin might be a candidate autoantigen in primary Sjögren's syndrome [94]. Identification of a specific autoantigen in DED could potentially lead to the generation of antigen-specific Tregs that ultimately could become a promising therapy for immune-mediated ocular surface inflammation.

6. Corneal Transplantation and Tregs

The three fundamental factors that contribute to corneal allograft survival are (1) blocking the induction of the immune response against allograft antigens, (2) generation of Tregs that can suppress the destructive alloimmune reaction, and (3) induction of apoptosis of inflammatory cells at the graft/host interface [95]. Long-term corneal allograft survival leads to an antigen-specific suppression of the delayed type hypersensitivity immune response and resembles the suppression of the delayed type hypersensitivity that is observed in ACAID [96]. Cunnusamy et al. have reported that there are two different Tregs that can promote corneal allograft survival. These include (1) $CD4^+CD25^+$ Tregs induced by the corneal allograft act at the efferent arm of the immune response in order to suppress the delayed type hypersensitivity and (2) $CD8^+$ Tregs induced by anterior chamber injection of alloantigens to suppress the efferent phase of the immune response [95]. Furthermore, it has also been demonstrated that the levels of Foxp3 expression in Tregs from corneal allograft acceptors were significantly higher compared to that seen in Tregs from the corneal allograft rejectors, which suggests that dysfunction of Tregs can be presumed to be one of the major causes of corneal allograft rejection [97]. Moreover, Tregs of allograft acceptors during adoptive transfers were reported to significantly increase the allograft survival rate [97]. In addition, it was also shown that the presence of allospecific Tregs in graft recipients primarily suppressed the induction of alloimmunity in the regional draining lymph nodes rather than suppressing the effector phase of the immune response in the periphery [97]. Hori et al. have shown that the expression of the glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand (GITRL) in the cornea led to the local expansion of $Foxp3^+CD4^+CD25^+$ Tregs, thereby contributing to the immune privilege status for the corneal allografts [98]. As previously described, we have demonstrated that cultured CE cells expressing TGF-β and CTLA-2α promote the generation of $CD4/CD8^+$ Tregs that are able to suppress the bystander effector T cells [39]. Taken together, these findings suggest that cell therapy performed when using Tregs may potentially be able to promote corneal allograft survival during transplantation. However, other recent evidence has shown that there is an increased risk of corneal allograft rejection in mice with allergic conjunctivitis and impaired function of the peripherally induced regulatory T cells in hosts who were at a high risk of graft rejection [99–101]. In fact, increases in corneal graft rejection were found in hosts reported to have previous ocular allergies during routine clinical practice examinations due to allergic inflammatory responses [102, 103]. A recent study demonstrated that systemic treatment of high-risk recipient mice with low-dose IL-2 led to an expansion and improved suppressive function of Tregs, reduced leukocyte infiltration of the graft, and promotion of corneal allograft survival [104]. Further studies that help to better clarify the mechanism of the generation and function of Tregs in corneal allograft transplantation will hopefully lead to the promotion of ocular immune privilege and survival of the corneal allograft in hosts with inflamed or vascularized recipient beds after Treg-based therapy.

7. Current Concept and Strategy of Treg Therapy in Humans

Adoptive transfer of Tregs in humans has been examined and tested in order to treat systemic autoimmune diseases or posttransplant-related complications [105, 106]. These pathologic states are partly caused by the dysfunction of Tregs or due to the relative inferior activity of Tregs to effector T cells. Restoration or reinforcing immune regulation by Tregs is the primary aim of the treatment. Furthermore, there is clear evidence for a relationship between the dysfunction of Tregs and autoimmune disease onset. The Foxp3 gene is mutated in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome. Thus, $Foxp3^+$ Tregs are thoroughly absent throughout the whole body, which can cause fatal autoimmunity leading to death during the early stages of life if hematopoietic stem cell transplantation is not performed [107, 108]. These examples demonstrate that Treg deficiency or relative dominant proinflammatory cytokines are in fact related to the autoimmune disease onset. Consequently, we believe that adoptive transfer of Tregs into affected patients will be a promising strategy against this pathological inflammation.

In order to achieve new therapeutic strategies for clinical application, the critical issues that need to be addressed include the following: (1) human Treg phenotypes need to be characterized in detail in order for clinical application, (2) techniques need to be standardized for isolating and expanding Tregs in order to avoid contamination, and (3) a method for delivering Tregs into patients will need to be established. With regard to the first issue, appropriate characteristics of Tregs will not be identical for each disease. Presently, the use of antigen-specific Tregs is an ideal choice for cases in which the target antigen is already known. However, the causative self-antigen remains unknown in most autoimmune diseases. Furthermore, it is practically impossible to cover all antigen repertoires in autoimmune diseases. Therefore, a more realistic idea for addressing this issue would be to utilize polyclonal non-antigen-specific Tregs, which may suppress inflammation in a bystander manner. Although polyclonal Tregs may have a relatively broad suppressive function, the effectiveness of polyclonal Tregs is still unclear and could potentially differ for individual organs and disorders.

For the second issue, there are several potential sources of Tregs. Autologous peripheral blood is a straightforward

choice, as it is easy to collect. In addition, allogeneic umbilical cord blood, preferably HLA matched, is a favorable alternative choice [109]. Moreover, in the case of graft-versus-host disease (GVHD), allogeneic donor-derived Tregs are also usable material. Regardless of the Treg source, the next critical technical step is the sorting of the polyclonal Tregs according to the characteristic surface markers. $CD4^+CD25^+$ selection is the most common method. Although Foxp3 is the most Treg-specific marker, it can only be detected by permeabilization, which, unfortunately, causes cell death. Therefore, this procedure cannot be used for the purpose of selection. In addition to $CD4^+CD25^+$ selection, cells are commonly sorted according to $CD127^{low}$ expression for further purification [110].

Human $Foxp3^+$ Tregs have recently been categorized based on CD25 and CD45RA expression, with $CD25^{low}CD45RA^+$ expression indicating resting Tregs, $CD25^{low}CD45RA^-$ expression indicating nonsuppressive Tregs, and $CD25^{high}CD45RA^-$ expression indicating active Tregs. However, for these treatments, it has been suggested that $CD25^{high}CD45RA^-$ Tregs might be the best population to use [111, 112]. After sorting the specific populations, cell expansion is essential because the numbers of circulatory Tregs are relatively small (up to 5–7% of $CD4^+$ T cells) [110].

Costimulation with anti-CD3/CD28 and IL-2 stimulation is a popular technique among the general expansion protocols [106]. This protocol enables expansion by a few hundredfold at most. However, contamination with T cells other than Tregs is unpreventable to some extent following this massive expansion. While the acceptable amount of contamination for clinical use remains uncertain, it may be dependent on the target disease. From this point of view, the use of umbilical cord blood-derived T cells, which constitute naïve cells, may be advantageous since natural Tregs are used [113], thereby avoiding contamination of the memory effector T cells in the injected cells.

For the third issue, there are many options for delivering expanded cells. Systemic injection via peripheral circulation is common, while local administration is also possible in some organs. However, careful attention should be paid to potential infusion reactions that could occur following administration via blood circulation. Even so, the eye is one of the best target organs for local administration. Inflammatory disease in the eye, such as uveitis, is the next challenge for targeted Treg therapy [76].

7.1. Application of Tregs in Treatment of Ocular Inflammation. Since the cause of noninfectious uveitis is diverse, most disorders can be treated or well controlled with immunosuppression. As a result, noninfectious uveitis can be viewed as an autoimmune disease of the eye. Systemic or topical administration of steroids has long been used as major immunosuppressive therapies for ocular inflammation. In addition to steroids, immunosuppressive agents or recently introduced monoclonal antibodies against inflammatory cytokines are also frequently administered in these patients [114]. In uveitis, proinflammatory cytokines from pathological T cells play central roles in the inflammation [115, 116]. We previously reported the decreased frequency of peripheral Tregs in patients with active uveitis such as Behçet's disease [117]. In healthy individuals, organ homeostasis is maintained by central and local tolerance [118]. As previously mentioned, the eye is one of the major immune privileged sites [10], where ocular PE cells play a central role in developing local tolerance [9]. The breakdown of immune tolerance leads to unfavorable autologous antigen-specific attacks against organs by the effector T cells. Failure in immune tolerance is partly due to Treg dysfunction and/or dominant effector T cell activity. Since noninfectious uveitis is considered an autoimmune disease, it is logical to assume that adoptive transplantation of Tregs should inhibit ocular inflammation. Thus, restoration of Treg function or artificial transfer of Tregs into noninfectious uveitis patients is likely to be a promising therapeutic choice for treating this disease.

Based on the therapeutic effect of Tregs in animal models of autoimmune uveitis [67], a phase I/II clinical trial has been started in Europe in patients with severe bilateral uveitis who are refractory to standard treatments and presented with a low visual acuity [119, 120]. The objective of this trial is to evaluate the safety of an intravitreal injection of ex vivo-activated polyclonal Tregs in patients with refractory and end-stage noninfectious uveitis. The result of this ongoing clinical trial and further studies on the safety and efficacy of Tregs should provide valuable information for the application of Tregs in patients with refractory uveitis.

7.2. Establishment of Tregs by Ocular Microenvironment. Similar to our previous murine studies, human ocular PE cells have been shown to have immunosuppressive functions, which form the immune privilege in the eye [121]. Primary cultured human iris PE cells are able to suppress the activation of bystander responder T cells *in vitro* [122]. Human iris PE cells suppress cell proliferation and cytokine production by responder T cells via direct cell-to-cell contact in a TGF-β-dependent manner. Furthermore, responder T cells are not only conventional autogenic activated T cells but also allogeneic activated T cells or T cell clones that have been established from uveitis patients [122]. In addition, human CE cells have an immunoregulatory function equivalent to that of human iris PE cells [38]. Interestingly, human CE cells can inhibit activated $PD\text{-}1^+$ helper T cells via the PD-L1–PD-1 interaction [34], while activated T cells are suppressed via membrane-bound TGF-β [38]. Thus, human iris PE cells and CE cells cooperatively create immune privilege in the anterior chamber.

Focusing on the posterior ocular segment, human RPE cells show potent regulatory function in ocular inflammation as well. Inflammatory cells in the retina, where cells cannot freely move, do not always come in direct contact with RPE cells. However, RPE cells can regulate inflammation by secreting soluble inhibitory molecules or generating Tregs [9]. Similar to the results of previous murine studies, human RPE cells have shown a great ability to generate Tregs *in vitro* [121]. RPE-induced Tregs strongly suppress cytokine production and proliferation of intraocular T-cell clones derived from active uveitis patients. $CD4^+$ T cells express CD25 and Foxp3 after culture with RPE supernatants,

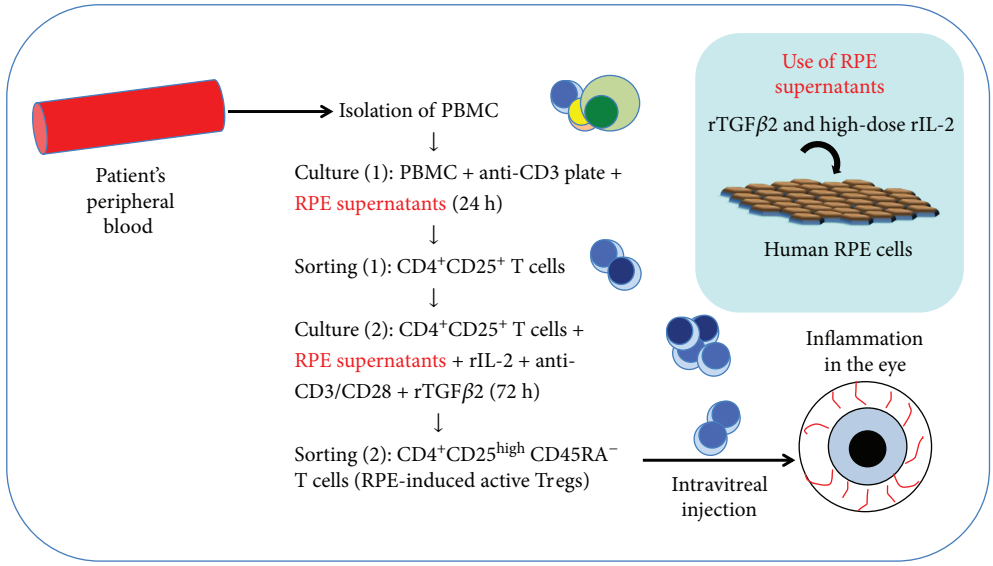

Figure 3: Regulatory T-cell (Treg) therapy in ocular disease: the original source of Tregs is the patient's peripheral blood. Following isolation of peripheral blood mononuclear cells (PBMCs) from the blood, PBMCs are cultured on anti-CD3-coated plates with RPE supernatants for 24 h. RPE supernatants are collected from culture media of human RPE cell lines with transforming growth factor beta 2 (TGF-β2) and high-dose interleukin 2 (IL-2). $CD4^+CD25^+$ T cells are first selected from cultured PBMCs. Sorted $CD4^+CD25^+$ T cells are then recultured with RPE supernatants together with recombinant IL-2 (rIL-2) and anti-CD3/CD28 antibodies for 72 h. In the final sort, $CD4^+CD25^{high}CD45RA^-$ T cells are collected, which are best suited for intravitreal injection into uveitis patients.

especially TGF-β2-pretreated RPE cells. The suppressive mechanism of human RPE-induced Tregs is mediated in a TGF-β-dependent manner, similar to that observed for murine RPE-induced Tregs. Based on these results, practical application of RPE-induced Tregs for treating uveitis and transplantation of a retina/RPE graft in patients with retinal degeneration appears to be a logical approach. However, for future clinical applications of Tregs in inflammatory ocular diseases and retina/RPE transplantation, there needs to be further optimization of the establishing and expanding of Tregs.

Based on these previous studies, we subsequently developed a method that could be used to more selectively and efficiently obtain RPE-induced Tregs (Figure 3). With this method, PBMCs are first cultured with recombinant TGF-β2-pretreated RPE supernatant on an anti-CD3-coated plate. $CD4^+CD25^+$ T cells are then sorted and recultured together with high-dose recombinant IL-2, antihuman CD3/CD28 antibodies, and TGF-β2 for 3 days. Using this method, it is possible to produce a large amount of $CD25^{high}CD45RA^-$ active Tregs that highly express Foxp3, CTLA-4 (CD152), and tumor necrosis factor receptor superfamily 18 (TNFRSF18). Furthermore, these RPE-induced Tregs secrete large amounts of suppressive cytokines TGF-β1 and IL-10 and suppress bystander target Th1 cells or Th17 cells [76].

8. Conclusions and Future Directions

Although CE and RPE cells are responsible for maintaining the homeostasis of the microenvironment of the eye, they also have unique anti-inflammatory and immunogenic roles in inflammation. Both ocular resident mesenchymal cells and peripheral tolerance of ACAID actively contribute to the regulation of immune responses via the generation of Tregs. These eye-specific Tregs have the therapeutic potential for not only autoimmune uveoretinitis but also promoting allograft survival after transplantation. At present, the therapeutic potential of Tregs in humans has been both examined and tested in order to treat systemic autoimmune diseases or posttransplant-related complications. However, further studies will be required in order to establish Treg therapy for active noninfectious uveitis. In addition, a better understanding of the molecular mechanism that regulates ocular immune privilege may lead to an effective therapeutic strategy that can be used to target individual patients with refractory uveitis.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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