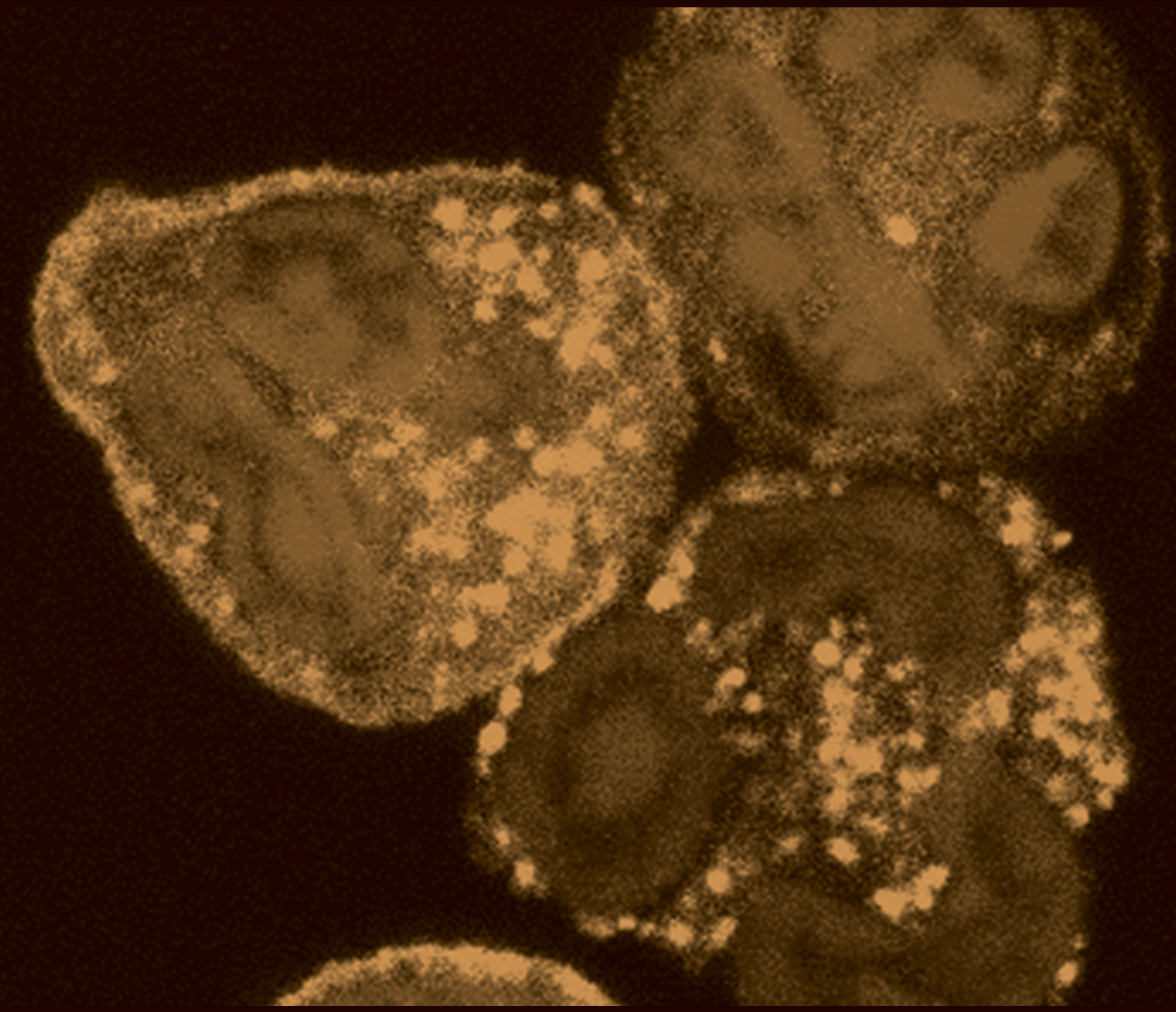
Mediators of Inflammation

Peritoneal Infection and Inflammation

Guest Editors: Markus Wörnle, Nicholas Topley, Christoph Aufricht, and Wolfgang Neuhofer



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Editorial Board

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Editorial

Peritoneal Infection and Inflammation

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Peritonitis is a major complication of various disease entities in internal medicine, surgery, and gynecology and, despite treatment advances, remains associated with unacceptably high morbidity and mortality. Causes of peritonitis are manifold including infectious, autoimmune, or chemical processes. A precise knowledge of inflammatory mechanisms in the initiation and establishment of peritoneal inflammation is of pivotal importance for the prevention and treatment of peritonitis.

This special issue aims to bring the molecular mechanisms of inflammatory processes in the peritoneal cavity into focus, regardless of the organ system or the specific syndrome they result from.

In this special issue of *Mediators of Inflammation*, we are pleased to present to the reader a series of special features written by designated experts in the field.

Local production of proinflammatory mediators at sites of tissue and cell injury within the peritoneal membrane plays a critical role in the propagation of peritoneal inflammation. In addition to infectious causes, various forms of peritoneal damage, induced by surgical trauma and peritoneal dialysis, compromise peritoneal integrity and can ultimately lead to tissue damage, fibrosis, adhesions, or even in the most severe cases the development of encapsulating peritoneal sclerosis. Peritoneal mesothelial cells appear to play a major role in these processes as they are capable of expressing a raft of proinflammatory and immunemodulatory mediators that either directly mediate fibrotic remodelling or attract immune cells to the peritoneal cavity or participate in immune activation. Therapeutic modulation of this sequence of events could provide novel avenues to attenuate peritoneal inflammation and its resulting tissue damage. The present special issue presents novel data on this topic, particularly underscoring the potential impact of the peroxisome proliferator-activated receptor-gamma (PPAR-γ) in regulating peritoneal inflammation and fibrosis. Furthermore, a novel PEGylated Toll-like receptor 7 (TLR7) ligand might be of great value for treatment of mast-cell-mediated neutrophilic inflammation.

M. W. J. A. Fieren describes the regulatory role of cytokines, macrophages, and leukocytes during local inflammatory processes in the peritoneal cavity. These processes are of special interest in patients on peritoneal dialysis treatment. A successful peritoneal dialysis treatment requires an intact peritoneal membrane. Treatment can be complicated by infectious or noninfectious sterile peritonitis due to the use of nonphysiologic dialysis solutions. S. Yung and T. M. Chan present an overview of pathophysiological changes to the peritoneal membrane during peritonitis related to peritoneal dialysis. G. Baroni et al. focus on structural changes during peritoneal dialysis-related inflammation. Moreover, experimental data presented in this special issue point to a novel role of the osmosensitive transcription factor NFAT5 in osmolality-induced expression of chemokines in human mesothelial cells. This is of special interest because peritoneal dialysis solutions are usually hypertonic. A rare but fatal complication of peritoneal dialysis treatment is sclerosing peritonitis; in their review article, M. Merkle and M. Wörnle

overview causes and treatment options, a complication which can also be seen after kidney or liver transplantation.

Peritoneal adhesions frequently occur after abdominal surgery. Patients with this complication present with abdominal and pelvic pain or intestinal mechanical obstruction. In this special issue, C. Di Filippo et al. provide evidence for a pivotal role of the ubiquitin-proteasome system in the formation of experimental postsurgical peritoneal adhesions. Furthermore, the authors show a possible therapeutic strategy to prevent adhesion formation by blocking the ubiquitin-proteasome system. W. Kessler et al. present novel data on the particular importance of the vagus nerve for the pathophysiology of peritonitis. According to their data, a functioning vagus nerve has a significant modulating influence on mortality in an animal model of polymicrobial sepsis.

In this special issue, T. Kelkka et al. present data in arthritis and peritonitis mice models, where overexpression of SOD3 results in anti-inflammatory effects that are largely independent of NOX2-mediated oxidative bursts. The anti-inflammatory action of extracellular superoxide dismutase (SOD3) is mediated by the dismutation of superoxide into hydrogen peroxide. To analyze whether SOD3 can regulate inflammation in the absence of functional NOX2 complex, T. Kelkka et al. used an elegant design, comparing anti-inflammatory effects of overexpression of SOD3 in $Ncf1^{-/-}$ mice with a deficient oxidative burst with that in wild-type mice. The relevance of these findings will need verification in the clinical setting; however, SOD3 is an important redox regulatory enzyme, and polymorphisms in SOD3 gene are associated with COPD, coronary artery disease, myocardial infarction, and acute lung injury.

We would like to thank all contributors and reviewers for their support to this special issue and feel sure that readers will learn much about this important topic.

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Research Article

NFAT5 Contributes to Osmolality-Induced MCP-1 Expression in Mesothelial Cells

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Increased expression of the C-C chemokine monocyte chemoattractant protein-1 (MCP-1) in mesothelial cells in response to high glucose concentrations and/or high osmolality plays a crucial role in the development of peritoneal fibrosis during continuous ambulatory peritoneal dialysis (CAPD). Recent studies suggest that in kidney cells osmolality-induced MCP-1 upregulation is mediated by the osmosensitive transcription factor, nuclear factor of activated T cells 5 (NFAT5). The present study addressed the question of whether activation of NFAT5 by hyperosmolality, as present in PD fluids, contributes to MCP-1 expression in the mesothelial cell line Met5A. Hyperosmolality, induced by addition of glucose, NaCl, or mannitol to the growth medium, increased NFAT5 activity and stimulated MCP-1 expression in Met5A cells. siRNA-mediated knockdown of NFAT5 attenuated osmolality-induced MCP-1 upregulation substantially. Hyperosmolality also induced activation of nuclear factor-κB (NF-κB). Accordingly, pharmacological inhibition of NF-κB significantly decreased osmolality-induced MCP-1 expression. Taken together, these results indicate that high osmolalities activate the transcription factor NFAT5 in mesothelial cells. NFAT5 in turn upregulates MCP-1, likely in combination with NF-κB, and thus may participate in the development of peritoneal fibrosis during CAPD.

1. Introduction

Peritoneal dialysis (PD) is a well-established and effective renal replacement therapy that is employed regularly in patients suffering from end-stage chronic kidney disease. The long-time efficiency of PD is limited by the bio-incompatibility of the currently used PD fluids (PDF) [1]. The latter induces severe pathophysiological changes in the peritoneal membrane, such as fibrosis and angiogenesis, which eventually are responsible for the functional failure of continuous ambulatory peritoneal dialysis (CAPD) [2, 3]. Conventional PDFs are characterized by high concentrations of glucose degradation products (GDPs), an unphysiological low pH, and high osmolalities [4]. In the last two decades significant efforts have been undertaken to improve the biocompatibility of PDFs by minimizing the formation of GDP during heat sterilization [2, 4] and by establishing a more physiological pH [5]. Although the physicochemical properties have been improved, efficient ultrafiltration across the peritoneal membrane requires supraphysiological osmolalities in the range of 380–510 mosm/kg H_2O, depending on the respective PDF. Accordingly, peritoneal mesothelial cells are exposed to local osmotic stress for several hours during PD.

The assumption that local osmotic stress contributes to the bio-incompatibility of PDFs is supported by the notion that even with the use of novel PDF, with a nearly physiological pH and low content of GDP, the mesothelium produces large amounts of established markers of peritoneal damage [6]. Proinflammatory mediators such as TGF-β1 or MCP-1 are synthesized in peritoneal mesothelial cells not only in response to glucose but also in response to osmotic stress [7].

The exposure of mesothelial cells to local osmotic stress during PD suggests an activation of the osmosensitive transcription factor NFAT5 (nuclear factor of activated T cells 5; also known as TonEBP or OREBP) in peritoneal mesothelial cells. NFAT5 was originally identified in collecting duct cells of the renal medulla [8], which are exposed to interstitial osmolalities severalfold higher compared to plasma osmolality (up to 1.200 mosm/kg H_2O) during antidiuresis. In renal

cells NFAT5 regulates the expression of various genes necessary for an efficient urinary concentration, for example, AQP-2 or UT-A, as well as genes required for the adaptation to high osmolalities, for example, aldose reductase or HSP70 [9]. NFAT5-regulated genes contain tonicity enhancer (TonE) elements in their promoter region, to which upon activation NFAT5 binds and stimulates the transcriptional machinery.

Recent studies suggest that under various pathophysiological conditions NFAT5 is activated by local osmotic stress and stimulates the expression of proinflammatory cytokines [10, 11], probably in cooperation with NF-κB [12]. In particular, upregulation of MCP-1 in renal tubular epithelial cells exposed to osmotic stress has been shown to be NFAT5 dependent [12, 13]. The C-C chemokine MCP-1 is a potent chemoattractant for circulating T cells and macrophages/monocytes [14] and plays a key role in the recruitment of these cells to the peritoneal cavity [15]. Besides its chemoattractant activity, MCP-1 stimulates expression of adhesion molecules and proinflammatory cytokines in monocytes [16] and induces calcium flux and the respiratory burst [17]. In fibroblasts, MCP-1 may increase synthesis of collagen and TGF-β1 [18], another key factor for the remodelling of peritoneal tissue. Pathophysiological upregulation of MCP-1 expression contributes to fibrotic lesions in the lung [19], the liver [20], and the kidney [21]. Since MCP-1 is also involved in the development of peritoneal damage [22–27], the aim of the present study was to determine whether activation of NFAT5 in response to osmotic stress, as present during PD, contributes to enhanced expression of MCP-1 in peritoneal mesothelial cells.

2. Methods

2.1. Materials. The NF-κB inhibitor Bay 11-7082 was obtained from Sigma (Deisenhofen, Germany). Anti-NFAT5 antibody weas from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-actin antibody was from Sigma; anti-p65 and anti-phospho-p65 (Ser536) and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Cell Signaling (Beverly, MA, USA); anti-histone H1 was from Millipore (Billerica, MA, USA). Unless otherwise indicated, other reagents were purchased from Biomol (Hamburg, Germany), Biozol (Eching, Germany), Carl Roth (Karlsruhe, Germany), or Sigma.

2.2. Cell Culture. Immortalized human mesothelial cells (Met5A, ATCC CRL-9444) were cultured in M199 culture medium supplemented with 4 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), with 10% fetal bovine serum (Biochrom, Berlin, Germany), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere (95% air/5% CO_2). Cells were grown in 24-well plates to confluency. For experiments, medium osmolality was increased by addition of glucose or, as osmotic controls, NaCl or mannitol.

2.3. qRT-PCR Analysis. For determination of MCP-1 and β-Actin mRNA expression levels, the total RNA from Met5A cells was prepared by adding TRIFAST Reagent (PEQLAB, Erlangen, Germany). The primers (Metabion, Martinsried, Germany) used in this experiment were MCP-1_fw: 5′-AGT CTC TGC CGC CCT TCT-3′; MCP-1_rev: 5′-GTG ACT GGG GCA TTG ATT G-3′; actin_fw: 5′-CCA ACC GCG AGA AGA TGA-3′; actin_rev: 5′-CCA GAG GCG TAC AGG GAT AG-3′. Experiments were carried out on a Roche LightCycler 480, using the SensiMix SYBR One-Step Kit (Bioline, Luckenwalde, Germany) according to the manufacturer's recommendations. Specificity of PCR product formation was confirmed by monitoring melting point analysis and by agarose gel electrophoresis.

2.4. Immunoblot Analysis. Aliquots (5–30 μg protein) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Nonspecific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T) at room temperature for 1 h. Samples were incubated with primary antibodies in PBS-T containing 5% nonfat dry milk over night at 4°C. Subsequently, the blots were washed 3 times with PBS-T for 5 min each, and the membranes then incubated with appropriate secondary antibody at room temperature for 1 h in PBS-T containing 5% nonfat dry milk. After washing with PBS-T 3 times for 5 min each, immunocomplexes were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.5. Preparation of Cytosolic and Nuclear Extracts. Subcellular extracts were prepared with the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's recommendations, with broad specificity protease inhibitor cocktail (Sigma) added at 1 : 100 (v/v).

2.6. MCP-1 Measurement. Concentration of MCP-1 in the cell culture supernatant was determined using a specific ELISA kit (PeproTech, Hamburg, Germany) according to the manufacturer's recommendations.

2.7. Reporter Gene Assays. Activation of transcription factors NFAT5 or NF-κB in response to hyperosmolality was assessed using the secreted alkaline phosphatase system (SEAP), with reporter constructs in which the SEAP open reading frame is under control of the respective transacting elements. pNF-κB-SEAP (Clontech, Heidelberg, Germany) contains four copies of the κB response element; pSEAP-TonE contains two TonE sites [28]. For transfection, Met5A cells were grown to ~80% confluency, trypsinated, washed in PBS, and 10^6 cells were finally resuspended in 200 μL modified HBS electroporation buffer (0.5% HEPES, 1% glucose, 0.5% Ficoll, 5 mM NaCl, 135 mM KCl, 2 mM $MgCl_2$, pH 7.4) together with 10 μg of the respective reporter vector. Electroporation was performed using a Gene Pulser X cell electroporation system (Bio-Rad, Hercules, CA, USA) at 150 V and 950 μF (exponential decay pulse) in a 2 mm cuvette, and the cells were subsequently seeded immediately in 96-well plates. After growing to confluency, the cells were treated as indicated and

SEAP activity in the medium determined as described before [29].

2.8. NFAT5 Transactivation Assay. NFAT5 transactivation activity was determined using the GAL4 binary assay as initially described by Ferraris et al. [30]. pGAL4-TonEBP-TAD contains the yeast GAL4 DNA-binding domain fused in frame to the transactivation domain (TAD) of NFAT5 (amino acids 548-1531; kindly provided by Dr. J. Ferraris, National Institutes of Health, Bethesda, MD, USA). pFR-SEAP (Agilent Technologies, Santa Clara, CA, USA) contains five tandem repeats of the GAL4 binding site upstream of a minimal promoter and the SEAP gene. Briefly, 10^6 cells were electroporated with 10 μg pGAL4-TonEBP-TAD and 10 μg pFR-SEAP as described above. After growing to confluency, the cells were treated as indicated and SEAP activity in the medium determined as described before [29].

2.9. NFAT5 Knockdown. Accell SMARTpool siRNA construct for knockdown of NFAT5 or Accell nontargeting siRNA (no. 2) were obtained from Thermo Fisher Scientific (Epsom, UK). Knockdown in Met5A cells was performed according to the manufacturer's instructions. The concentration of siRNA constructs was 1 μM in Accell delivery medium, containing 2% FCS. Cells were incubated for 5 days, and knock-down efficiency was determined by qRT-PCR and by western blot analysis.

2.10. Statistical Analyses. Data are expressed as means ± SEM. The significance of differences between the means was assessed by Student's t-test. $P < 0.05$ was regarded as significant. All experiments were performed at least three times, and representative results are shown.

3. Results

3.1. Osmolality-Induced Upregulation of MCP-1 Expression in Met5A Cells. The effect of medium osmolality on MCP-1 secretion of Met5A cells was tested by measurement of MCP-1 concentration in the cell culture supernatants. The cells were incubated in isosmotic (300 mosm/kg H_2O) or hyperosmotic (400 mosm/kg H_2O) medium. The medium osmolality was elevated by the addition of glucose. To distinguish between glucose-specific effects and osmolality-induced effects, mannitol or NaCl was used as osmotic controls. Samples of cell culture supernatant were taken at various times between 2 and 24 h. Under isosmotic conditions constitutive MCP-1 secretion could be observed: MCP-1 concentration in the cell culture supernatant rose from 25 ± 8 pg/mL after 2 h to 220 ± 43 pg/mL after 24 h (Figure 1(a)). This constitutive MCP-1 secretion was significantly enhanced under hyperosmotic conditions. The strongest effect was observed when medium osmolality was elevated by addition of glucose: MCP-1 concentration reached 760 ± 26 pg/mL after 24 h. The effects of mannitol (480 ± 67 pg/mL after 24 h) and NaCl (390 ± 36 pg/mL after 24 h) were less pronounced than that of glucose but were still significantly increased compared to the isosmotic control.

An osmolality-induced increase of MCP-1 expression was also observed at the mRNA level (Figure 1(b)). Surprisingly, at all tested times NaCl (rather than glucose) had the strongest effect on MCP-1 mRNA abundance, while on the protein level glucose had the stronger effect (see above), probably indicating that glucose, and not osmolality per se, also stimulates posttranscriptional and/or posttranslational mechanisms, thus further enhancing MCP-1 secretion.

3.2. Osmolality-Induced Activation of NFAT5 in Met5A Cells. In kidney cells, hyperosmolality stimulates overall NFAT5 activity by (i) increased NFAT5 expression, (ii) increased NFAT5 translocation into the nucleus, and (iii) activation of the NFAT5 transactivation domain. Accordingly, hyperosmolality elevated NFAT5 expression also in Met5A cells, at both the protein and mRNA levels (Figures 2(a)–2(c)). The most prominent effect was observed when medium osmolality was raised by addition of NaCl; glucose and mannitol also caused a robust increase of NFAT5 abundance. Additionally, hyperosmolality increased translocation of NFAT5 from the cytoplasm into the nucleus (Figures 2(d) and 2(e)) and activity of the NFAT5 transactivation domain (Figure 2(f)).

Activation of NFAT5 in Met5A cells by hyperosmolality was assayed using a TonE-driven reporter vector. Medium osmolalities were elevated to 325–550 mosm/kg H_2O by addition of glucose, mannitol, or NaCl, and Met5A cells, transfected transiently with the reporter construct, were incubated for 24 h. Raising the medium osmolality increased NFAT5 activity approximately 2-3 fold (Figure 2(g)). For glucose and mannitol, NFAT5 activity reached a maximum at a final osmolality of 400 mosm/kg H_2O, for NaCl at 450 mosm/kg H_2O. At even higher osmolalities, NFAT5 activity declined.

3.3. Osmolality-Induced MCP-1 Upregulation Is Decreased by NFAT5 Knockdown. To evaluate the role of NFAT5 in osmolality-induced MCP-1 expression, NFAT5 was knocked down using a siRNA approach. Knock-down efficiency, as tested by immunoblotting (Figure 3(a)), was at ~80% compared with control cells transfected with a scrambled siRNA. As expected, hyperosmolality, induced either by glucose or NaCl addition, significantly increased MCP-1 both in the cell culture supernatant (Figure 3(b)) and at the mRNA level (Figure 3(c)) of control cells. Knockdown of NFAT5 largely attenuated osmolality-induced increase in MCP-1 expression (Figures 3(b) and 3(c)), indicating a central role for NFAT5 in this process.

3.4. Role of NF-κB in Osmolality-Induced MCP-1 Expression. Another transcription factor probably involved in upregulation of MCP-1 during hyperosmotic stress is NF-κB [27]. In Met5A cells transfected transiently with a κB-driven reporter vector, hyperosmolality, induced by addition of NaCl or glucose, significantly increased reporter activity (Figure 4(a)). Accordingly, phosphorylation of the p65 subunit was enhanced significantly under these conditions (Figure 4(b)), indicating that hyperosmolality activates NF-κB. Next, NF-κB in Met5A cells was inhibited by treatment with the pharmacological inhibitor Bay 11-7082. NF-κB inhibition not only

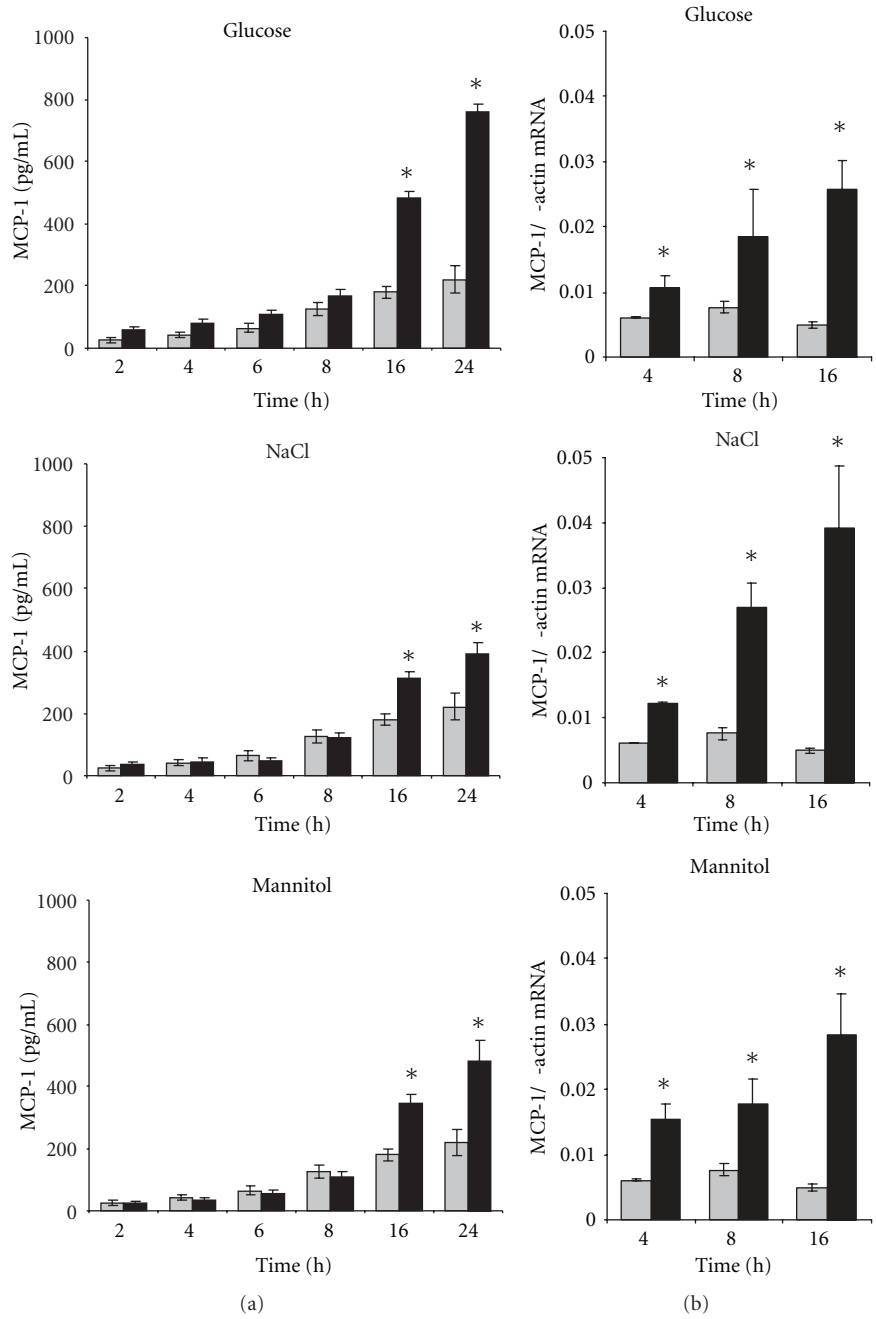

Figure 1: Osmolality-induced MCP-1 expression. (a) For determination of MCP-1 secretion, confluent Met5A cells were incubated in isosmotic medium (gray column; 300 mosm/kg H_2O) or were exposed to hyperosmotic medium (black column; 400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose, NaCl, or mannitol as indicated. At the indicated times, medium samples were collected and the concentration of MCP-1 in the cell culture supernatant determined by ELISA as described in Section 2. Means ± SEM for $n = 4$ per point; $^*P < 0.05$ versus isosmotic control. (b) For determination of MCP-1 transcription, confluent Met5A cells remained in isosmotic medium (gray column; 300 mosm/kg H_2O) or were exposed to hyperosmotic medium (black column; 400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose, NaCl, or mannitol as indicated. At the indicated time points, RNA was extracted from the cells and the abundance of MCP-1 mRNA transcript was determined by qRT-PCR as described in Section 2. Relative MCP-1 mRNA abundance was normalized to that of β-actin to correct for differences in RNA input. Data are means ± SEM for $n = 4$ per point; $^*P < 0.05$ versus isosmotic control.

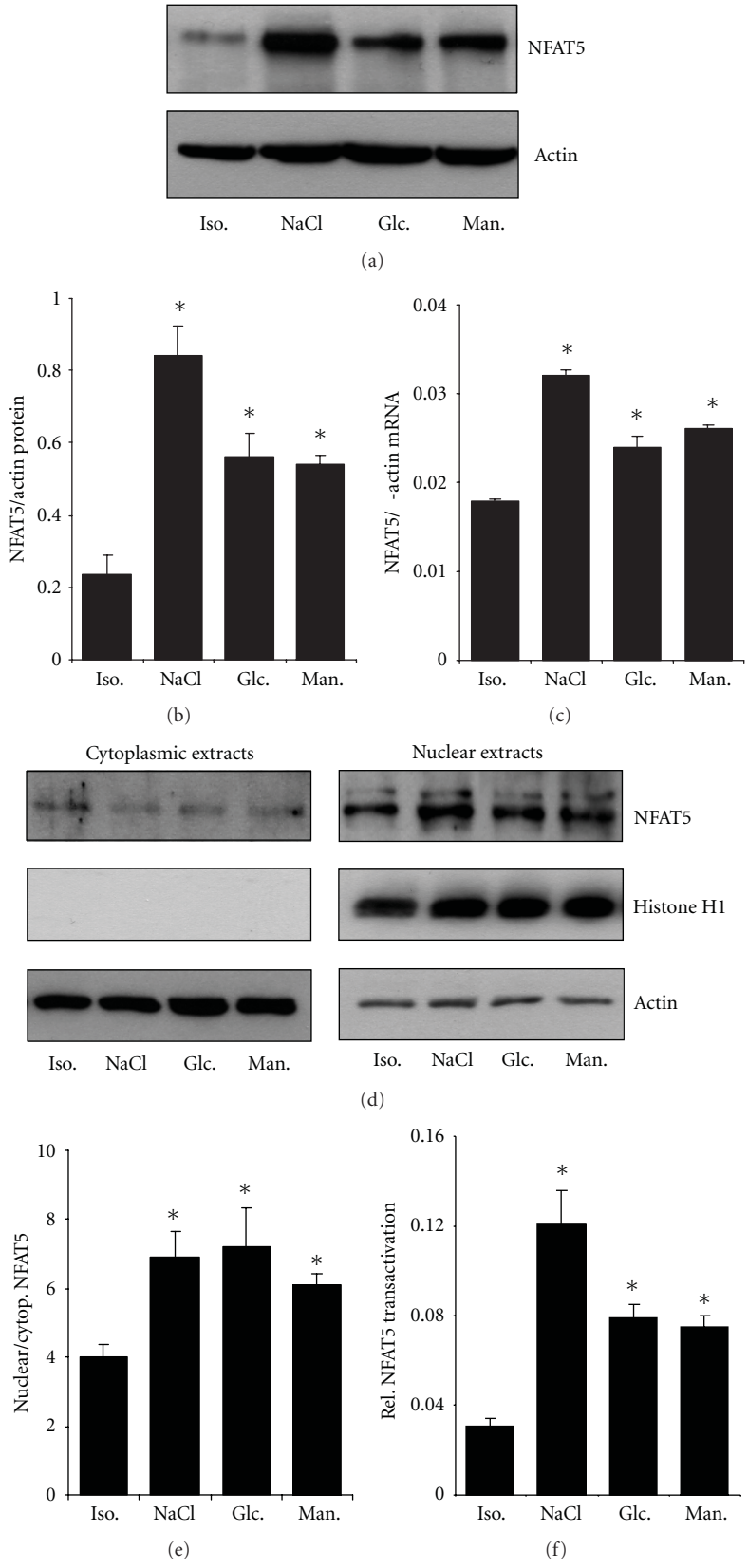

Figure 2: Continued.

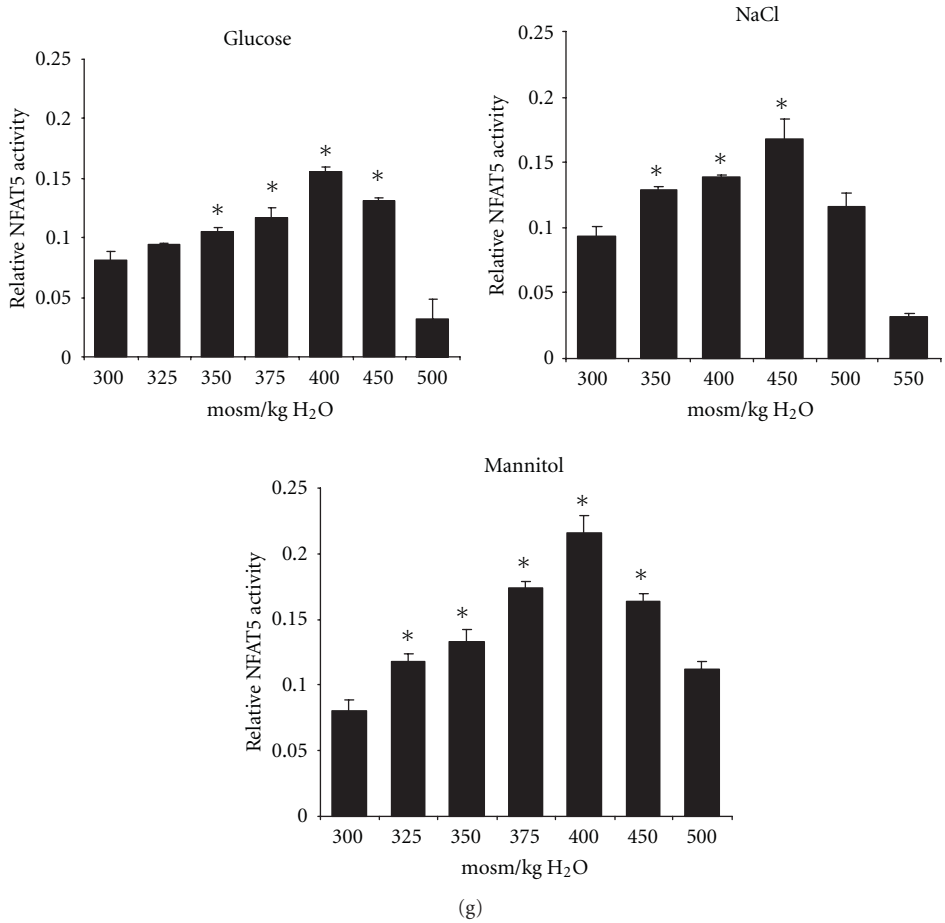

Figure 2: Expression and activation of NFAT5 in Met5A cells. Met5A cells were kept in isosmotic medium (300 mosm/kg H_2O) or were exposed to hyperosmotic medium (400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose, NaCl, or mannitol as indicated. (a) Cells were incubated for 24 h and subsequently processed for immunoblotting as described in Section 2. To demonstrate comparable protein loading, the blots were also probed for actin. A representative blot from 3 independent experiments is shown. (b) Relative NFAT5 protein abundance was quantified by densitometric analysis of immunoblots and normalized to that of actin to correct for differences in protein loading. Means ± SEM for $n = 3$; $^*P < 0.05$ versus isosmotic control. (c) Cells were incubated for 16 h. Thereafter, RNA was extracted and the abundance of MCP-1 mRNA transcript determined by qRT-PCR as described in Section 2. Relative MCP-1 mRNA abundance was normalized to that of β-actin to correct for differences in RNA input. Means ± SEM for $n = 3$ per point; $^*P < 0.05$ versus isosmotic control. (d) Cells were incubated for 1 h, and subsequently cytoplasmic and nuclear extracts prepared and processed for immunoblotting as described in Section 2. To demonstrate purity of extracts and comparable protein loading, the blots were also probed for histone H1 and actin. A representative blot from 4 independent experiments is shown. (e) Relative NFAT5 nuclear versus cytoplasmic abundance was quantified by densitometric analysis of immunoblots. Means ± SEM for $n = 3$; $^*P < 0.05$ versus isosmotic control. (f) Activity of the transactivation domain of NFAT5 during osmotic stress. Met5A cells were cotransfected with a vector encoding the fusion protein GAL4dbd-TonEBP-TAD (amino acids 548-1541 of NFAT5 fused to the yeast GAL4 DNA binding domain) together with the reporter vector pFR-SEAP. Cells were kept in isosmotic medium (300 mosm/kg H_2O) or were exposed to hyperosmotic medium (400 mosm/kg H_2O). After 48 h, SEAP activity was measured as described in Section 2. Means ± SEM for $n = 3$; $^*P < 0.05$ versus isosmotic control. (g) Met5A cells were transiently transfected with a reporter construct in which the SEAP gene is under control of two TonE sites. Cells were kept in isosmotic (300 mosm/kg H_2O) medium or were exposed to hyperosmotic medium, with osmolalities between 325 and 550 mosm/kg H_2O as indicated. After 24 h, SEAP activity was measured as described in Section 2. Means ± SEM for $n = 4$; $^*P < 0.05$ versus isosmotic control.

abolished MCP-1 expression under hyperosmotic conditions but also significantly reduced constitutive MCP-1 expression under isosmotic conditions (Figures 4(c) and 4(d)).

4. Discussion

Although the biocompatibility of PD solutions has been improved during the recent years by the use of bicarbonate rather than lactate buffers and by limiting excessive GDP formation, hyperosmolality of PDF is required for effective ultrafiltration into the dialysate. The latter is in most cases achieved by the addition of glucose to osmolalities of 380–510 mosm/kg H_2O. Such high glucose concentrations induce the expression of proinflammatory mediators in mesothelial cells that in turn promote serious long-term complications such as progressive peritoneal fibrosis or even sclerosing

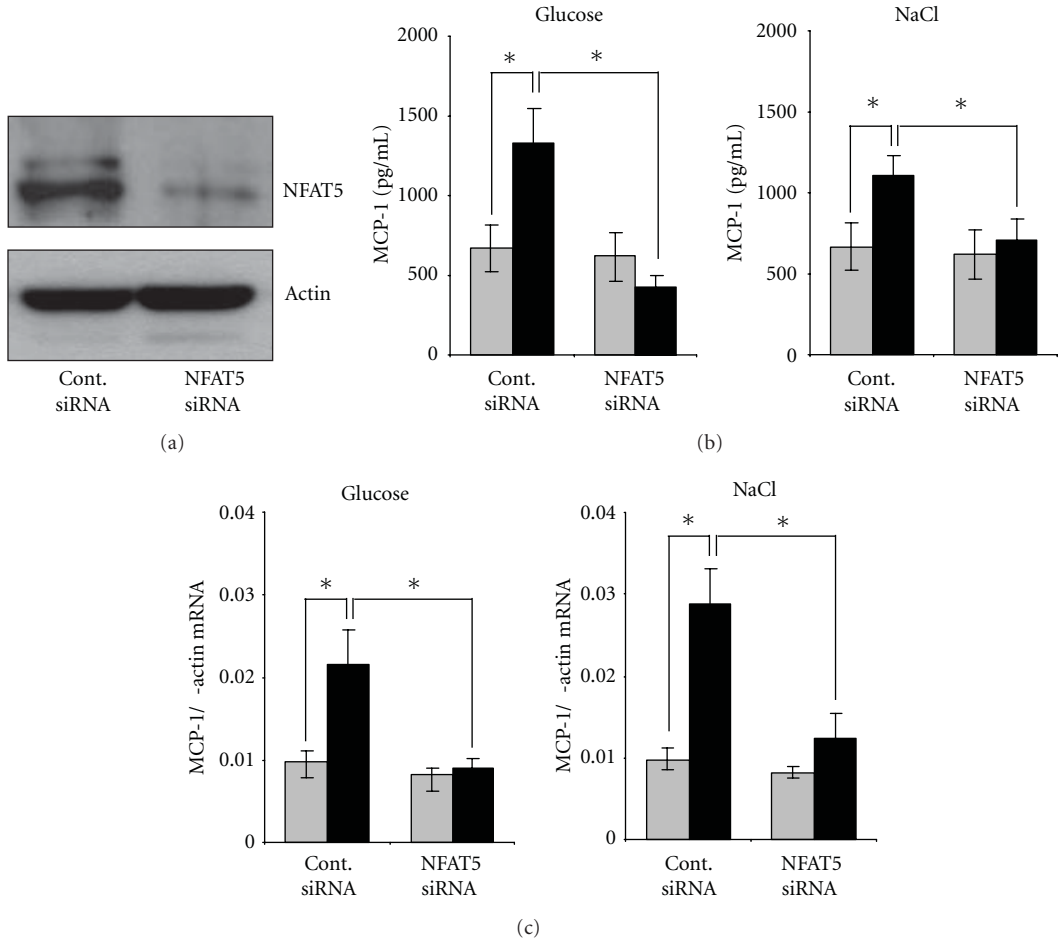

Figure 3: NFAT5-knockdown attenuates osmolality-induced MCP-1 expression. Met5A cells were transfected with siRNA constructs for NFAT5 or with nontargeting siRNA as control as indicated. Cells were kept in isosmotic medium (gray column; 300 mosm/kg H_2O) or were exposed to hyperosmotic medium (black column; 400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose or NaCl as indicated, and cells were incubated for 24 h. (a) To demonstrate efficiency of NFAT5 knockdown, cells were processed for immunoblotting as described in Section 2. To demonstrate comparable protein loading, the blots were also probed for actin. (b) For determination of MCP-1 secretion, medium samples were collected and the concentration of MCP-1 in the cell culture supernatant was determined by ELISA as described in Section 2. Means ± SEM for $n = 4$ per point; $^*P < 0.05$. (c) For determination of MCP-1 transcription, RNA was extracted from the cells and the abundance of MCP-1 mRNA transcript was determined by qRT-PCR as described in Section 2. Relative MCP-1 mRNA abundance was normalized to that of β-actin to correct for differences in RNA input. Means ± SEM for $n = 4$ per point; $^*P < 0.05$.

peritonitis. Particularly, the expression of TGF-β1 and MCP-1 is induced in mesothelial cells in response to high glucose concentrations or high osmolality, respectively [7, 27]. The C-C chemokine MCP-1 recruits monocytes and CD8 T lymphocytes to the peritoneal cavity, where these cells secrete a variety of cytokines and growth factors, which induce or aggravate damage of the peritoneal membrane. In the present study, we provide evidence that the osmosensitive transcription factor NFAT5 contributes to osmolality-induced MCP-1 expression in the mesothelial cell line Met5A. To distinguish between glucose-specific effects and osmolality-induced effects, experiments were carried out not only with glucose but also with NaCl or mannitol as osmotic controls. Generally, activation of NFAT5 and upregulation of MCP-1 were induced by all three agents, indicating that MCP-1 expression is largely induced in response to hyperosmotic stress. However, some differences between NaCl-induced and glucose-induced MCP-1 expression could be observed and are discussed below. siRNA-mediated knockdown of NFAT5 attenuated the osmolality-induced activation of MCP-1 expression, clearly demonstrating the important role for NFAT5 in this context. The regulation of MCP-1 expression under hyperosmotic conditions by NFAT5 has been recently described in rat and human kidney cells [12, 13]. At least one TonE element at position −199 bp to −186 bp upstream from the transcriptional start site has been identified in the MCP-1 regulatory region, to which NFAT5 binds in response to osmotic stress [13]. Activation of NFAT5 under these conditions probably depends on the MAP kinases p38 and ERK1/2 [12, 13].

NFAT5 is reportedly regulated by various mechanisms in response to osmotic stress in kidney cells: by increased

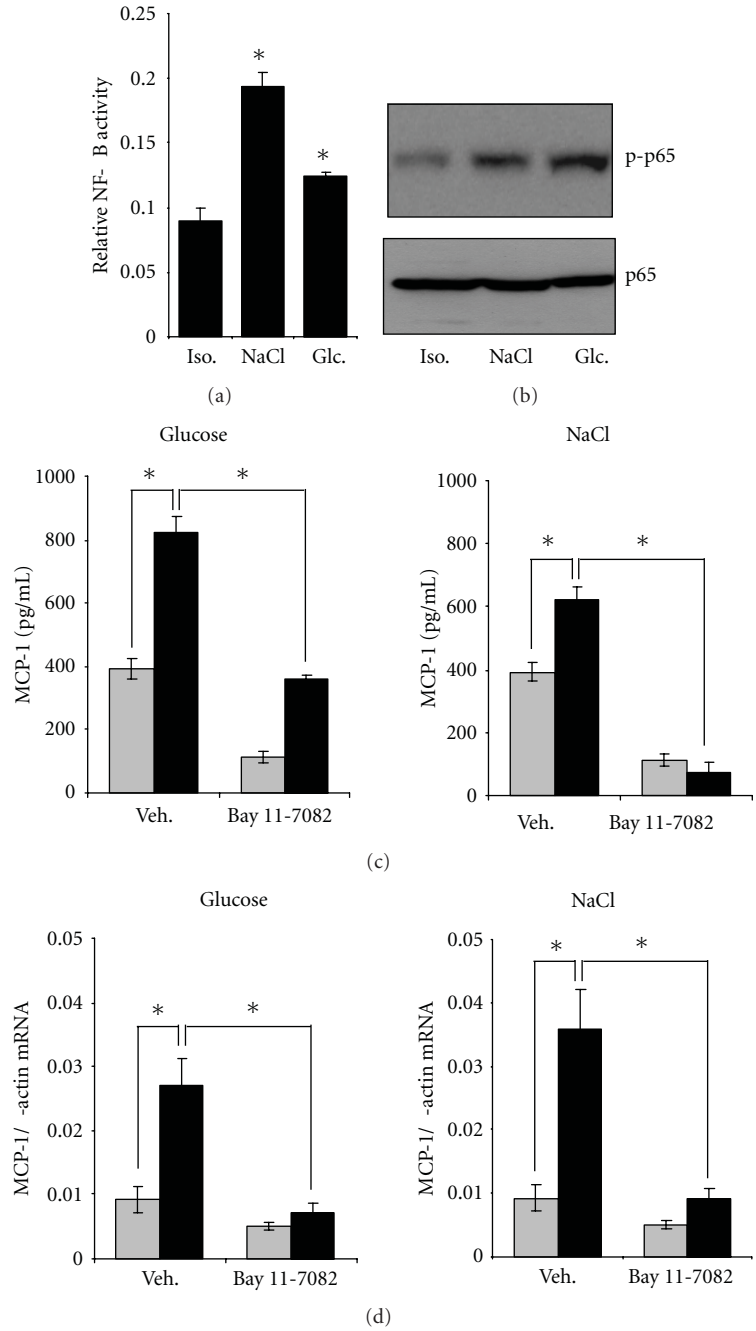

Figure 4: Role of NF-κB in osmolality-induced MCP-1 expression. (a) Activation of NF-κB by osmolality. Met5A cells were transiently transfected with a reporter construct in which the SEAP gene is under control of κB sites. Cells were kept in isosmotic (300 mosm/kg H_2O) medium or were exposed to hyperosmotic medium (400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose or NaCl. After 24 h, SEAP activity was measured as described in Section 2. Means ± SEM for $n = 4$; $^{\#}P < 0.05$ versus isosmotic control. (b) Phosphorylation of the p65 subunit by osmolality. Met5A cells were kept in isosmotic medium (300 mosm/kg H_2O) or were exposed to hyperosmotic medium (400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose or NaCl. After 16 h, cells were processed for immunoblotting as described in Section 2. Abundance of phosphorylated p65 or whole p65 was tested using specific antibodies. A representative blot of three independent experiments is shown. (c) MCP-1 secretion. Met5A cells were preincubated for 1 h with the NF-κB inhibitor Bay 11-7082 (5 μM) or with vehicle DMSO only. Cells were kept in isosmotic (gray column; 300 mosm/kg H_2O) medium or were exposed to hyperosmotic medium (black column; 400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose or NaCl. After 24 h, medium samples were collected and the concentration of MCP-1 in the cell culture supernatant was determined by ELISA as described in Section 2. Means ± SEM for $n = 4$ per point; $^{*}P < 0.05$. (d) MCP-1 transcription. Met5A cells were preincubated for 1 h with the NF-κB inhibitor Bay 11-7082 (5 μM) or with vehicle DMSO only. Cells were kept in isosmotic (gray column; 300 mosm/kg H_2O) medium or were exposed to hyperosmotic medium (black column; 400 mosm/kg H_2O). Medium osmolality was elevated by addition of glucose or NaCl. After 24 h, RNA was extracted from the cells and the abundance of MCP-1 mRNA transcript was determined by qRT-PCR as described in Section 2. Relative MCP-1 mRNA abundance was normalized to that of β-actin to correct for differences in RNA input. Means ± SEM for $n = 4$ per point; $^{*}P < 0.05$.

NFAT5 expression probably due to stabilization of NFAT5 mRNA [31], by increased activity of the transactivation domain within the c-terminal portion of NFAT5 [30, 32], and by enhanced translocation from the cytoplasm to the nucleus [33, 34]. Accordingly, hyperosmolality induced a significant upregulation of NFAT5 expression, increased nuclear translocation, and an increased activity of the transactivation domain also in Met5A cells.

We demonstrated activation of NFAT5 activity in response to hyperosmolality in Met5A cells using a TonE-driven reporter construct. Generally, the induction of NFAT5 activity by hyperosmolality in Met5A cells is relatively weak compared to kidney cells. With the same TonE-driven reporter construct, we observed an approximately 20-fold induction of NFAT5 activity in kidney cells [28], while in Met5A cells an approximately 2-fold induction occurred. Notably, maximal NFAT5 activation could be observed at about 400–450 mosm/kg H_2O and declined rapidly at higher osmolalities, while in kidney cells maximal NFAT5 activation is achieved at osmolalities of >500 mosm/kg H_2O. In kidney cells, the nonreceptor tyrosine kinase focal adhesion kinase (FAK) is a positive regulator of NFAT5 activity under hyperosmotic conditions [35]; in mesothelial cells, high osmolalities, and especially high glucose concentrations, inhibit FAK [36], which may account for the decreasing NFAT5 activity at osmolalities >450 mosm/kg H_2O. Furthermore, the decreased NFAT5 activity may reflect a general decrease in cellular activity and perhaps cellular damage of mesothelial cells in response to high osmolalities [37]. In contrast, kidney cells have evolved effective mechanisms to maintain cellular activity even during hyperosmolality [38].

Met5A cells showed significant constitutive MCP-1 expression under isosmotic conditions. This basal expression appears to be largely independent of NFAT5, as NFAT5 knockdown had marginal effects on MCP-1 abundance at both the mRNA and protein levels under isosmotic conditions. In contrast, the pharmacological NF-κB inhibitor Bay 11-7082 significantly reduced not only hyperosmotic-induced MCP-1 expression but also basal expression under isosmotic conditions, indicating that basal NF-κB activity under isosmotic conditions is necessary for basal MCP-1 expression. Involvement of NF-κB in regulation of osmolality-induced MCP-1 expression in mesothelial cells has been shown previously [27]. In Met5A cells, hyperosmolality activated a κB-driven reporter vector, especially in response to NaCl but also, to a lesser extent, in response to glucose. Accordingly, hyperosmolality induced phosphorylation of the p65 subunit of NF-κB. A cooperation of NFAT5 with NF-κB during osmolality-induced expression of cytokines has been already demonstrated in kidney cells [12]. In this study, the authors propose a model in which direct interaction of NFAT5 with the p65 subunit of NF-κB increases binding of NF-κB to κB sites around -2470 bp and -2440 bp in the promoter region of MCP-1 and enhances NF-κB transcriptional activity under hypertonic conditions. However, the identification of a TonE site in the MCP-1 promoter region provides evidence that NFAT5 may stimulate MCP-1 expression by two different mechanisms: first, by direct binding to the TonE site around -190 bp and activation of the transcriptional machinery; second, by interaction with NF-κB to enhance DNA binding and transcriptional activity of NF-κB.

Interestingly, there were some differences in MCP-1 upregulation depending on whether medium osmolality was elevated by NaCl or glucose. Addition of NaCl to the medium had a stronger effect on NFAT5 abundance and activity and also on NF-κB activity. Accordingly, MCP-1 mRNA abundance was more robustly induced by addition of NaCl compared to glucose. In contrast, the abundance of MCP-1 protein in cell culture supernatants was significantly more enhanced by glucose than by NaCl, indicating that post-transcriptional and/or posttranslational mechanisms further stimulate MCP-1 accumulation in the presence of glucose. A possible mechanism which may account for this observation could be protein glycosylation. MCP-1 can be modified by O-glycosylation and sialylation [39, 40], and this glycosylation enhances MCP-1 protein stability [41]. Since high glucose concentrations can enhance protein O-glycosylation [42, 43], it is conceivable that enhanced MCP-1 O-glycosylation contributes to the observed increased MCP-1 concentrations in cell culture supernatants under these conditions. However, since O-glycosylation of MCP-1 was not assessed in this study, this possibility remains to be established.

As mentioned above, hyperosmolality activated NFAT5 in mesothelial cells, regardless of whether glucose, NaCl, or mannitol was used as the osmotic agent. This is not surprising since NFAT5 activation by these compounds has been shown before in various cell lines [13, 44, 45]. Several studies indicate that replacement of glucose as osmotic agent by icodextrin or amino acids may improve biocompatibility of PD fluids [46–48]. For future studies, it may be interesting to test the effects of icodextrin- or L-carnitine-induced hyperosmolality on NFAT5 activation and MCP-1 expression in mesothelial cells.

In T cells, NFAT5 has also been identified as a positive regulator for the expression of the proinflammatory cytokines TNF-α and lymphotoxin-β (LT-β) in response to osmotic stress [49]. Since especially TNF-α is an important mediator of pathological alterations of the peritoneal membrane during CAPD [50], we also analyzed osmolality-induced expression of TNF-α and LT-β in Met5A cells. However, we could not detect significant expression of TNF-α or LT-β either under isosmotic or hyperosmotic conditions (data not shown). This is in accordance with the assumption that, during CAPD, TNF-α is synthesized preferentially by monocytes/macrophages [51] rather than mesothelial cells.

The gene encoding the chaperone HSP70 is another NFAT5-regulated gene [52]. Upregulation of HSP70 in response to PDF has been demonstrated in *in vitro* and *in vivo* models of PD [53, 54] and confers increased resistance to PDF toxicity to mesothelial cells [55]. Knockdown of NFAT5 in Met5A cells also decreased HSP70 expression (data not shown), indicating that hyperosmolality-induced NFAT5 activity may also have an important role for cytoprotection of mesothelial cells during CAPD.

5. Conclusions

Taken together, the present study indicates that the transcription factor NFAT5 is activated in response to high osmolalities in mesothelial cells and that this activation contributes to increased expression of MCP-1, probably in collaboration with NF-κB.

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Review Article

Pathophysiological Changes to the Peritoneal Membrane during PD-Related Peritonitis: The Role of Mesothelial Cells

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The success of peritoneal dialysis (PD) is dependent on the structural and functional integrity of the peritoneal membrane. The mesothelium lines the peritoneal membrane and is the first line of defense against chemical and/or bacterial insult. Peritonitis remains a major complication of PD and is a predominant cause of technique failure, morbidity and mortality amongst PD patients. With appropriate antibiotic treatment, peritonitis resolves without further complications, but in some PD patients excessive peritoneal inflammatory responses lead to mesothelial cell exfoliation and thickening of the submesothelium, resulting in peritoneal fibrosis and sclerosis. The detrimental changes in the peritoneal membrane structure and function correlate with the number and severity of peritonitis episodes and the need for catheter removal. There is evidence that despite clinical resolution of peritonitis, increased levels of inflammatory and fibrotic mediators may persist in the peritoneal cavity, signifying persistent injury to the mesothelial cells. This review will describe the structural and functional changes that occur in the peritoneal membrane during peritonitis and how mesothelial cells contribute to these changes and respond to infection. The latter part of the review discusses the potential of mesothelial cell transplantation and genetic manipulation in the preservation of the peritoneal membrane.

1. Introduction

Peritoneal dialysis (PD) is an effective and affordable form of renal replacement therapy that is presently used by approximately 11% of the total global dialysis population [1]. Although PD has greatly improved the quality of life in patients with end-stage renal disease, a major disadvantage of this treatment is that PD solutions are bioincompatible and provoke peritoneal inflammation and mesothelial cell injury [2–5]. Furthermore, peritonitis is a major complication of PD and remains the single most important cause of technique failure and subsequent transfer to hemodialysis. It contributes to severe abdominal pain, hospitalization, catheter removal, and increased morbidity and mortality in PD patients. The mortality risk ascribed to PD-related peritonitis is 18% in the United States and >16% in Hong Kong [6, 7]. Peritonitis is characterized by turbidity in the dialysate effluent, abdominal pain, the presence of a white blood cell count of more than 100,000 cells/mL, of which 50% of the white blood cells are polymorphonuclear neutrophil cells, and a positive culture [8, 9]. Although the etiology of the bacteria is a determinant of morbidity and mortality in PD patients [10–12], studies have also demonstrated that peritoneal inflammation, age, residual renal function, malnutrition, and comorbidity can affect the outcome of the patient [13].

The majority of peritonitis episodes are due to a single microorganism [14, 15]. In contrast to surgical peritonitis, about half of these infections arise from Gram-positive bacteria [15], which originate from the patients' own nasopharyngeal or skin flora [16, 17]. With improvements in connection technology and better sterile techniques, Gram-positive peritonitis has gradually declined. Polymicrobial infection that involves more than one Gram-positive bacteria would suggest touch contamination or catheter infection, whereas polymicrobial Gram-negative bacteria would suggest perforation of the bowel [9, 18]. Gram-positive microorganisms that induce PD-related peritonitis

include *Staphylococcus aureus* (*S. aureus*) and coagulase-negative *Staphylococcus* (CNS), that is, *S. epidermidis*, while *Pseudomonas* species, *Escherichia coli (E. coli)*, *Klebsiella* species, and *Acinetobacter* species account for the majority of Gram-negative peritonitis [19].

Peritonitis causes severe injury to mesothelial cells, specialized epithelial cells that line the peritoneal membrane and play a key role in peritoneal homeostasis, peritoneal host defense, and maintenance of the peritoneal membrane structure [2]. Compelling evidence has demonstrated that the constant exposure of the peritoneal membrane to bio-incompatible PD solutions induces peritoneal inflammation, exfoliation of mesothelial cells and structural changes to the peritoneal membrane resulting in the progressive loss of peritoneal functions and unfavorable outcome [3, 20–23]. These changes are exacerbated by peritonitis [24, 25]. We and others have demonstrated that following clinical resolution of peritonitis dialysate levels of inflammatory and fibrotic mediators remain elevated compared to preperitonitis levels [26–28], which would prolong peritoneal inflammation and mesothelial cell injury. This review will provide a brief overview of the structure and functions of mesothelial cells and how they regulate and/or contribute to peritoneal inflammation and structural changes to the peritoneal membrane during PD and peritonitis. The last section of this review will discuss the potential therapeutic interventions that may be employed to preserve the dialytic potential of the peritoneal membrane.

2. Peritoneal Mesothelial Cells

Although previously considered to function simply as a lubricating, nonadhesive surface to facilitate intracoelomic movement, there is now compelling evidence to show that peritoneal mesothelial cells are not inactive cells but play essential roles in peritoneal homeostasis, fluid and solute transport across the peritoneal membrane, peritoneal inflammation, and tissue repair [29–32]. Mesothelial cells synthesize a myriad of growth factors, cytokines, proteases, matrix proteins, and proteoglycans that contribute to the function of the peritoneal membrane [33–40].

Although mesothelial cells originate from the mesoderm, they possess many features of epithelial cells. Such features include the acquisition of a polygonal, cobblestone appearance, becoming polarized upon cell-cell contact and resting upon a basement membrane [29, 30]. Epithelial and mesothelial cells are also endowed with microvilli and the expression of the intermediate filament protein cytokeratin [29, 41–43]. Mesenchymal characteristics of mesothelial cells include vimentin, desmin, and α-smooth muscle actin expression and the acquisition of a fibroblastic phenotype following epithelial-to-mesenchymal transdifferentiation (EMT) [41, 44–46]. The ability of mesothelial cells to undergo phenotypic changes under physiological and pathological conditions underscores the plasticity property of these cells. Mesothelial cells are connected by intercellular junctions comprising tight junctions, gap junctions, adherens junctions, and desmosomes that contribute to the establishment and maintenance of a continuous mesothelial monolayer [47–49]. Reduced expression of adherens junctions during inflammatory processes is associated with a breakdown of cell-cell communication and cell-matrix interaction resulting in the denudation of the mesothelium, a process that is often observed during PD and peritonitis [50, 51]. Mesothelial cells also express E-cadherin, a cardinal feature of epithelial monolayers [52, 53]. E-cadherin is a calcium-dependent transmembrane glycoprotein localized in adherens junctions in the basolateral membrane and bestows upon mesothelial cells their apico-basolateral polarity [53, 54]. A loss of E-cadherin at the intercellular junctions is strongly associated with epithelial dedifferentiation and EMT, with the appearance of Snail, a zinc finger transcription factor that is critical for the initiation of EMT [55, 56]. Snail has been implicated in E-cadherin repression through its ability to bind to components in the promoter region of E-cadherin [57, 58]. Long-term PD and peritonitis have been shown to induce Snail expression and EMT in mesothelial cells, mediated in part through increased TGF-β1 bioactivation and the interaction of advanced glycation end products (AGEs) with its receptor RAGE [55, 59, 60].

The luminal surface of mesothelial cells contains numerous microvilli and occasional cilia that serve to increase the peritoneal surface area for transport of solutes across the peritoneal cavity. Microvilli entrap water and serous exudates, which protect the delicate surface of mesothelial cells from frictional damage [29]. Microvilli permit mesothelial cells to sense and respond to their microenvironment and also entrap bacteria thereby preventing infection. A reduction in the number of microvilli on mesothelial cells would therefore have a profound effect on peritoneal function and their ability to fend off bacterial infection. The density of microvilli on regenerating mesothelial cells may vary and is dependent on the anionic charge of the glycocalyx [43]. The glycocalyx is a thin film of fluid that is found on the surface of mesothelial cells. It is composed of lipoproteins, phospholipids, proteoglycans, and hyaluronan and serves to lubricate the peritoneal viscera and protect the mesothelial surface from abrasions and adhesions. The glycocalyx also plays an important role in cell-cell contact, tissue hydration, regulation of inflammation, tissue remodeling, and flow of nutrients and growth factors across the peritoneal membrane [61]. The integrity of the glycocalyx is in part attributed to the presence of negatively charged proteoglycans and hyaluronan [62, 63].

Mesothelial cells provide the first line of defense against chemical or bacterial insult to the peritoneal membrane. It is therefore crucial that following injury and denudation restoration of the mesothelium occurs promptly and is not hindered. Numerous mechanisms have been proposed for mesothelial replenishment and these include centripedal migration of mesothelial cells, exfoliation of healthy mesothelial cells from neighboring sites which settle on the denuded area, free-floating reserve cells, submesothelial and bone-marrow-derived precursor cells, and macrophage transformation [29, 64–71]. It is noteworthy that these mechanisms have been identified *in vitro* and experimental systems and their relevance in the clinical setting remain to

be defined. We have demonstrated that following mechanical denudation of cultured mesothelial cells, repopulation of the monolayer is mediated through the induction of EMT in mesothelial cells at the leading edge of the wound and the migration of these cells into the denuded area [72, 73]. Increased *de novo* synthesis and subsequent deposition of hyaluronan and matrix proteins into the extracellular milieu act as a substratum that allows mesothelial cells to attach and migrate into the denuded area [72, 73]. Once the mesothelial monolayer is reestablished and cell-cell contact restored, cells resume their epithelial morphology. Although the process through which mesothelial cells revert back to their epithelial morphology has not been explored, it is likely that it is achieved by mesenchymal-to-epithelial transdifferentiation.

During peritoneal homeostasis, a fine balance exists between mesothelial injury and regeneration. Mesothelial cells are most susceptible to injury and if repopulation of the monolayer is compromised following long-term PD or recurrent episodes of peritonitis, in the absence of its protective mesothelial covering, the interstitium will initiate reparative processes that may overcompensate resulting in peritoneal fibrosis and sclerosis.

3. Changes to the Peritoneal Membrane during PD and Peritonitis

3.1. The Normal Peritoneal Membrane. The peritoneum is a delicate, continuous, and translucent membrane that lines the peritoneal cavity [74, 75]. It is composed of a monolayer of mesothelial cells resting upon a thin basement membrane, underneath which is the submesothelium comprising interwoven bundles of collagen fibres, intermittent fibroblasts, and blood vessels [74, 76]. The thickness of the submesothelium is quite variably in different sections along the peritoneum, and movement of molecules through the submesothelium is governed not only by its thickness but also by the molecular weight, charge, and shape of the molecule [76]. An in-depth discussion on the ultrastructure of the peritoneum is outside the scoop of this review and readers are referred to an excellent review by Gotloib [76].

3.2. Alterations in the Peritoneal Membrane during PD and Peritonitis. Many patients on long-term PD exhibit reduplication of the mesothelial and endothelial basement membranes, increased synthesis and deposition of matrix proteins within the submesothelium, and progressive subendothelial hyalinization, with narrowing or obliteration of the vascular lumen [3, 74, 75, 77]. Vascular and interstitial changes become more apparent with progressive use of PD, thereby demonstrating a temporal relationship between peritoneal fibrosis, vasculopathy, and time on PD [3]. Peritoneal fibrosis is detected in 50% and 80% of PD patients within one and two years, respectively, on PD [78–80]. With regards to mesothelial cells, independent researchers have demonstrated significant changes to these cells following their exposure to PD, which include cell activation, cell hypertrophy, increased vacuolation, partial or complete loss of microvilli, dissolution of cell-cell contacts, and alterations in the number of endoplasmic reticulum and micropinocytotic vesicles [3, 74, 75, 81]. Some degree of mesothelial denudation is invariably observed in PD patients and is associated with thickening of the submesothelium and vasculopathy [3]. What happens to mesothelial cells following their exfoliation is currently unclear. Do they represent degenerative cells that are destined for removal from the peritoneal cavity by phagocytosis or are they still viable, even though in suspension, and are able to maintain a functional role within the peritoneum? The ability to culture mesothelial cells from dialysis effluent would indicate that these detached cells are viable. The phenotypes of these cells is diverse and consist of cells with a normal epithelial morphology, large senescent cells containing multi-nuclei and multivacuoles, and cells with a fibroblastic phenotype [59].

Peritoneal specimens obtained from PD patients with peritonitis show more pronounced degenerative changes in the mesothelium and exfoliation of mesothelial cells is more prominent. In areas where mesothelial cells are still apparent, changes observed are similar to those mentioned above with a loss of microvilli and cell-cell contact [81, 82]. Peritonitis also induces the loss of the underlying basement membrane and promotes extensive interstitial fibrosis attributed to increased synthesis of matrix proteins and a concomitant loss of decorin [3, 38, 77, 83]. Acute infiltration of inflammatory cell into the submesothelium is also noted, which may account at least in part, to the expansion of the interstitial. These observations have been confirmed in animal models of experimental peritonitis [76, 84–91].

Whilst animal studies have contributed significantly to our understanding of peritoneal inflammation and injury induced by PD and peritonitis, one must also be aware of the limitations of these models. Numerous PD studies are conducted in animals that are not uremic, whereas in other studies animal models of PD-related peritonitis are conducted in animals that are not infused with PD solution. Given that peritoneal host defense mechanisms are impaired in PD patients attributed to the constant exposure of PD fluids [92], can mechanistic findings obtained in non-PD models of peritonitis be extrapolated to the clinical scenario? Even in animal models of PD-associated peritonitis, structural changes and the time that such changes occur do not replicate those observed in PD patients. In this respect, daily infusion of glucose-based PD fluid into rats for 4 weeks following an initial exposure of LPS to mimic Gram-negative peritonitis resulted in submesothelial thickening and an increase in the density and number of blood vessels [93]. However, denudation of the mesothelium and vasculopathy were not detected in this rat model of PD-related peritonitis, a finding that is often observed in PD patients with or without peritonitis [3, 81]. An excellent review by Mortier et al. summarizes the advantages and disadvantages of known experimental models of PD [94].

Vascular changes in the peritoneal membrane are commonly observed in PD patients. It has been suggested that changes in blood vessel density may directly affect the functional attributes of the peritoneal membrane. Mateijsen et al. observed an increase in blood vessel density, capillary

dilation, and vessel wall thickening within the submesothelium of PD patients with peritoneal sclerosis when compared to controls [95]. Pathological alterations in the vasculature of peritoneal specimens obtained from uremic non-PD patients as well as PD patients include hyalinization of the blood vessels, vasculopathy, and submesothelial thickening [3, 77]. These abnormalities are more prominent in patients who have used PD for more than 6 years and are associated with the deterioration of peritoneal function [3, 77]. Peritoneal vascular changes in PD patients resemble alterations in the microvasculature of diabetic patients that include deposition of matrix proteins within the arterial wall and media of arterioles and reduplication of the capillary basement membrane [76, 96].

There is emerging evidence that increased synthesis of VEGF may at least in part contribute to neoangiogenesis and increased vasodilation and vessel permeability in PD patients [97, 98]. Invariably, these vascular changes are associated with the deposition of AGEs in the vessel wall, which accumulate with progressive use of PD [98]. Increased peritoneal expression and dialysate levels of VEGF are associated with increased permeability of small solutes and a loss of ultrafiltration [98–102], the increase in VEGF a result of local synthesis by peritoneal vascular endothelial and mesothelial cells [103–107]. Aroeira et al. noted that mesothelial cells isolated from dialysate effluent with a fibroblastic phenotype, (i.e., cells that have undergone EMT) synthesized significantly more VEGF than their epithelial counterpart [106]. Patients whose PD effluent contained mesothelial cells that had undergone EMT demonstrated higher serum levels of VEGF compared to levels detected in patients with mesothelial cells of normal morphology, which correlated with peritoneal transport rates [106]. The observation that these fibroblastic cells stained for cytokeratin confirms their mesothelial origin, which colocalized with VEGF expression in the submesothelium [106]. This study thus underscores the importance of mesothelial cells in the synthesis of VEGF and their contribution to vascular changes during PD. The observation that capillary tube formation in human umbilical vein endothelial cells (HUVEC) can be induced by supernatant obtained from RAGE-stimulated mesothelial cells or the coculture of RAGE-stimulated mesothelial cells with HUVEC suggests direct communication or cross-talk between mesothelial cells and endothelial cells *in vivo* and substantiates the contributing role of the former cell type in mediating neoangiogenesis and vascular changes [108].

Preliminary studies by Szeto et al. showed that VEGF levels are further increased at the onset of peritonitis in PD patients and these levels correlate with the degree of diminished ultrafiltration [109]. In an LPS-induced rat model of Gram-negative peritonitis, Pawlaczyk et al. demonstrated that the infusion of LPS at various concentrations together with PD solution increased dialysis effluent concentrations of VEGF in a dose-dependent manner [110] but its role in mediating changes in the peritoneal vasculature was not investigated.

The causal relationship between peritonitis and functional changes of the peritoneal membrane is controversial. Whilst some studies have demonstrated a causal relationship between peritonitis rate and peritoneal dysfunction, others have failed to find any correlation [24, 111–115]. These discrepancies may be related to the limited patient number, short period of followup, insufficient longitudinal studies, and grouping Gram-positive and Gram-negative complicating peritonitis together. Ates et al. assessed the degree of peritoneal function remaining in 18 PD patients over the course of 24 weeks following onset of infection. These researchers demonstrated that one episode of peritonitis was insufficient to induce permanent changes in peritoneal transport properties although full recovery of ultrafiltration was not achieved following the resolution of peritonitis [116]. In another study, data obtained from Davies et al. confirmed that one episode of peritonitis is not sufficient to have any significant effect on peritoneal function, whereas recurrent peritonitis that occurred in close proximity and the severity of peritoneal inflammation exacerbated and accelerated solute transport and the loss of ultrafiltration in PD patients [24]. It is noteworthy that since the structural and functional properties of the peritoneal membrane vary significantly between patients, their response to peritonitis, even towards the same pathogen, can differ considerably.

3.3. Role of Anionic Sites in the Peritoneal Membrane during PD and Peritonitis. Accumulation of plasma proteins in the peritoneal cavity and their subsequent loss from the patient following the exchange of PD fluid is often observed during peritonitis [85, 117]. It can perhaps be likened to proteinuria whereby a loss of heparan sulfate proteoglycans, such as perlecan or agrin, in the glomerular basement membrane (GBM) results in the increased permeability of the GBM to anionic macromolecules such as albumin [118, 119]. Proteoglycans are anionic macromolecules that comprise a core protein to which one or more glycosaminoglycan chain(s) is/are attached [120]. Glycosaminoglycan chains are classified as heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronan depending on their disaccharide units. With the exception of hyaluronan, all glycosaminoglycan chains are attached to a protein core and are endowed with sulfate groups that bestow up these macromolecules a high net negative charge that contributes to their biological properties and interactions with cytokines, chemokines, and growth factors [121, 122]. Perlecan and agrin are the most characterized heparan sulfate proteoglycans in the GBM, which contribute to the structural integrity of the kidney and restrict the passage of albumin and other proteins out of the glomerular capillaries into the urinary space [118, 119]. Through their ability to sequester chemokines, heparan sulfate proteoglycans can also regulate lymphocyte recruitment during tissue injury [123]. Therefore, changes in the expression of proteoglycans in any given tissue will have a profound effect on both their structural and functional property.

Gotloib et al. provided evidence that anionic sites exist in the normal peritoneum and are found within the mesothelial glycocalyx, subendothelium, and along the basement membrane [124–126]. In an experimental model of septic peritonitis whereby rats were administered live *E. coli* by intraperitoneal injection, a significant reduction in anionic

sites at these locations was observed, which was accompanied by increased transperitoneal passage of proteins [85]. Although the nature of these anionic sites was not further investigated by these researchers, it is possible that perlecan may contribute at least in part to the anionic staining. We have demonstrated that perlecan expression is predominately observed within the mesothelium and underlying basement membrane in peritoneal specimens obtained from new PD patients [38]. Mesothelial expression of perlecan decreased with increasing duration on PD with a concomitant increase in the submesothelium [38]. The functional role of perlecan in the mesothelium remains to be fully elucidated, but it is possible that perlecan plays a critical role in preserving the structural and functional integrity of the peritoneum and maintenance of the selective charge barrier of the peritoneal membrane. Emerging evidence suggests that heparan sulfate proteoglycans may possess angiogenic properties [122, 127]. Through their N-terminal, heparan sulfate proteoglycans may stimulate angiogenesis through their ability to bind growth factors such as VEGF, bFGF, and PDGF and presenting them in a biologically active form to their cognate receptors [127]. With regards to the observed increase in perlecan expression in the submesothelium in PD patients, it is plausible to suggest that it may play a role in peritoneal angiogenesis although further studies are warranted to confirm this. Whether the expression of perlecan in the submesothelium is further increased following peritonitis remains to be determined.

4. Peritoneal Inflammation

Inflammation is the body's adaptive response to remove the inciting insult and restore homeostasis to the tissue. Given the molecular heterogeneity of bacteria pathogens, it is quite remarkable that efficient and coordinated recognition strategies have evolved to deal with bacterial infection, and this is based primarily on the ability of the host to detect molecular patterns that are unique to bacteria. If the epithelial barrier is breached, the pathogen is eliminated by the innate immunity, followed by the adaptive host immune processes. Innate immunity consists of various preexisting, rapidly mobilized cells that include the immune cells, that is, neutrophils, macrophages, mast cells, eosinophils and natural killer cells, and resident cells. These cells express a number of pattern recognition receptors such as the toll-like receptors (TLRs) that are activated by microbial components resulting in complement activation and the release of proinflammatory mediators such as cytokines, chemokines, nitric oxide, prostaglandin, acute phase proteins, and antimicrobial peptides. Cells of the innate immune system subsequently activate the adaptive immune system, which initiate the maturation of dendritic cells and recruitment of T and B cells [128]. Under normal circumstances, cell infiltration is initiated within minutes of detecting a bacterial insult and through a coordinated series of cellular and humoral events [129], the inciting insult is removed with a systematic return of the normal physiological functions of the tissue within days. With successful resolution of inflammatory processes, the extent of tissue damage is limited. If the inciting insult persists, chronic inflammation ensues, which may persist for weeks, months, or even years.

4.1. The Role of Infiltrating Cells in Peritoneal Inflammation. Before the processes of bacterial-induced peritoneal inflammation and the involvement of infiltrating and mesothelial cells in these processes are discussed, one must remember that the peritoneal host defense system of PD patients is already compromised. *In vitro* and *ex vivo* studies have demonstrated that PD fluids have a marked effect on macrophage functions. In this respect, PD fluid has been shown to induce cytokine secretion and inhibit the respiratory burst and phagocytotic property of peritoneal macrophages [130, 131]. Furthermore, McGregor et al. demonstrated that peritoneal macrophages became increasing immature with increasing time on PD, which was accompanied by an increase in cytokine release [130]. Components of PD solution have also been shown to alter leukocyte functions and are cytotoxic to these immune cells [132, 133]. Daily use of PD not only dilutes the number of macrophages and leukocytes and concentration of opsonins in the peritoneal cavity of PD patients, but these cells and components are lost from the peritoneal cavity after each PD exchange, further compromising peritoneal host defense.

How does onset of infection affect the inflammatory processes and the structural and functional properties of the peritoneum when on-going peritoneal inflammation is already present? Is it possible to dissect out subtle changes that occur within the peritoneum consequent to peritonitis from those that have already arisen from the use of long-term PD? Whilst it is impractical to obtain frequent peritoneal biopsies from PD patients to monitor the morphological changes within the peritoneal membrane with time on PD and during episodes of infection, we rely on the measurement of surrogate markers in dialysate effluent that may provide limited insight in the inflammatory processes that occur within the peritoneum. The use of transgenic mouse studies has provided us with intricate details of the roles of inflammatory and fibrotic mediators and their ligands synthesized by immune and resident cells, in inflammatory and reparative processes of the peritoneum [134–136].

The initial recognition of an infection in the peritoneum is mediated by peritoneal macrophages and mast cells, triggered by TLR activation, which results in the release of various vasoactive substances such as prostaglandins and histamines. This results in vasodilatation and increased permeability of the peritoneal blood vessels leading to increased synthesis of complement, immunoglobulins, opsins, fibrin, and clotting factors [25]. Peritoneal macrophages also synthesize various inflammatory mediators that include IL-1β, TNF-α, IFN-γ, and TGF-β1, which in turn mediate the induction of chemokine secretion. The main effect of these mediators is to elicit the recruitment of polymorphonuclear neutrophils that are normally restricted to the blood vessels to the site of injury [129]. These neutrophils are activated either by direct contact with the invading pathogen or through the actions of the cytokines and chemokines secreted by resident mesothelial cells. In order to eradicate

the pathogen, neutrophils release the toxic content of their granules which include reactive oxygen and nitrogen species, cathepsin G, and elastase [137]. These potent effectors do not discriminate between pathogens and host cells and therefore injury to the peritoneal membrane is unavoidable. Neutrophils are subsequently progressively cleared from the peritoneal cavity by apoptosis and are replaced by a population of monocytes/macrophages and leukocytes. Ingestion of neutrophils by macrophages results in the release of TGF-β1, its mode of action now becoming anti-inflammatory where it assists in the reparative processes [138]. Repopulation of resident macrophages in the peritoneal cavity following the resolution of peritoneal inflammation has recently been shown to be through local proliferation [139].

The temporal switch in immune cell population is pivotal for the clearance of infection and resolution of inflammation, mediated in part through IL-6, a cytokine known to prevent the accumulation of neutrophils. Hurst et al. demonstrated that leukocyte recruitment into the peritoneal cavity is mediated by the interaction of soluble IL-6R and IL-6, shed from infiltrating neutrophils and mesothelial cells, respectively, which in turn induce chemokine expression essential for lymphocyte recruitment [140]. Over the last decade or so, our understanding of the mechanisms through which neutrophils are recruited from the circulation, migrate across the submesothelium into the peritoneal cavity, and the sequential change in the population of infiltrating cells to initiate peritoneal inflammation and resolution has increased significantly through a series of in-depth *in vitro, ex vivo,* and experimental studies [134–136, 141–144].

4.2. The Role of Mesothelial Cells in Peritoneal Inflammation. Both infiltrating and resident peritoneal cells play critical roles in the initiation and amplification of peritoneal inflammation during PD and peritonitis through their ability to synthesize chemotactic and proinflammatory mediators [4, 33–35]. Whilst the roles of infiltrating cells in peritoneal inflammation have been described above, the next section will focus on the resident mesothelial cells and their contribution to cellular infiltration and initiation and resolution of peritoneal inflammation.

The initiation phase is characterized by the activation of resident macrophages and mesothelial cells by the invading microorganism or its secreted products. The initiation phase is followed by the amplification phase, in which mesothelial cells play a predominant role. Mesothelial cells are activated by proinflammatory cytokines, such as TNF-α and IL-1β derived from peritoneal macrophages [25]. Stimulation of mesothelial cells by TNF-α or IL-1β induces IL-8 production, a potent chemoattractant that mediates leukocyte migration from the circulation to the peritoneal cavity [145, 146]. Mesothelial cells constitutively express adhesion molecules, such as ICAM-1, VCAM, and PECAM, which are required for leukocyte adherence and migration across the mesothelium [147–149], a multistep process that is dependent on the establishment of a chemotactic gradient across the mesothelium, increased mesothelial expression of ICAM-1, and specific adhesive interactions between the leukocytes and the endothelium [146, 150].

CD40 is a member of the TNF family of receptors and is expressed on the membrane of activated $CD4^+$ T-cells. Its activation contributes to increased chemokine and cytokine secretion during inflammatory processes and binding to its ligand initiates mononuclear cell infiltration during peritonitis [143]. Basok et al. demonstrated that mesothelial cells also express CD40 [151]. Activation of CD40 on mesothelial cells by proinflammatory cytokines induced IL-15 secretion, a T-cell growth factor and activator [152]. The presence of CD40 on mesothelial cells may suggest that these cells play an important role in T-cell regulated inflammatory response during peritonitis.

In the previous section, the importance of macrophages in the initiation and resolution of peritonitis was highlighted. Once the pathogen is eliminated, resolution of peritoneal inflammation may begin. Rapid and effective clearance of macrophages dictates the duration of peritoneal inflammation and may be an important determinant of chronic peritoneal inflammation. Unlike neutrophils which are removed by apoptosis, macrophage clearance is through emigration into the draining lymphatics [153]. Recently, Bellingan et al. demonstrated in a murine model of peritonitis that macrophages adhered specifically to the peritoneal mesothelium through VLA-4 and VLA-5 and this interaction was RGD sensitive [154]. The adhesion of macrophages to the mesothelium was localized to areas overlying the draining lymphatics, was adhesion molecule dependent, and the rate of emigration was controlled by the level of macrophage activation [154]. Mesothelial-macrophage interactions are therefore prerequisite for the removal of macrophages from the peritoneal cavity and for the resolution of peritoneal inflammation.

We and others have demonstrated that increased levels of cytokines and growth factors may persist in the peritoneal cavity despite clinical resolution of peritonitis [26–28], which will prolong injury to the mesothelial cells. This will initiate the fibrogenic phase within the peritoneum, which is followed by the destruction phase. Overproduction of matrix proteins in the peritoneum will result in peritoneal fibrosis and sclerosis and invariably lead to cessation of treatment. Table 1 summarizes the detrimental processes that occur during each phase.

5. The Role of Defensins during Peritonitis and Peritoneal Inflammation

Numerous host proteins have been shown to possess antimicrobial activity. Many are constitutively expressed by resi-dent cells and stored in secretory granules, whereas others are induced upon proinflammatory stimuli. Defensins are a group of antimicrobial peptides that are produced by mesothelial cells and cells of the innate immune system in response to bacterial infection [155, 156]. These peptides are activated in the presence of bacteria and act by disrupting the lipid membranes of bacteria. In order to avoid elimination from the host, bacteria have developed mechanisms that allow them to utilize components of the host cells to enhance their virulence. In this respect, pathogens have been shown

Table 1: Induction of peritoneal fibrosis.

Phases of peritoneal fibrosis	Events that occur in the peritoneum during each phase
Induction phase	Release of chemokines by mesothelial cells (i) Infiltration of mononuclear cells (ii) Release of profibrotic mediators (iii) Activation of resident cells (mesothelial cells and fibroblasts)
Fibrogenic phase	Increased synthesis and deposition of matrix Continued secretion of profibrogenic mediators by infiltrating cells
Peritoneal destruction phase	Cessation of primary inflammatory stimulus Secretion of profibrotic cytokines by mesothelial cells Autocrine proliferation of fibroblasts and myofibroblasts EMT Submesothelial thickening Vasculopathy

to exploit cell surface proteoglycans, which have a high net negative charge, to neutralize the antimicrobial actions of cationic defensins. Schmidtchen et al. demonstrated that exogenous dermatan sulfate and heparan sulfate glycosaminoglycans are able to bind α-defensin, which fully neutralized its bactericidal activity against *P. aeruginosa, E. faecalis,* and *S. pyogenes* [157]. These researchers further demonstrated that through the actions of their proteinases, these pathogens have the capacity to utilize and degrade host-derived proteoglycans to release anionic glycosaminoglycan chains that bind and neutralize the actions of defensins [157]. Syndecan-1 is a cell surface heparan sulfate proteoglycan that is synthesized by fibroblasts, mesothelial cells, airway epithelial cells, and intestinal epithelial cells [158–160]. In an animal model of *S. aureus* corneal infection, Hayashida et al. noted that *S. aureus* induced shedding of the syndecan-1 ectodomain, which resulted in the inhibition of the innate immune mechanism and the inability of neutrophils to eradicate *S. aureus* [161]. The observation that syndecan-1 knockout mice were resistant to *S. aureus* infection underscores the importance of syndecan-1 shedding as a pathogenic mechanism that mediates virulence of *S. aureus* [161]. *P. aeruginosa* is also able to release the ectodomain of syndecan-1 from the cell surface of mouse mammary epithelial cells, lung epithelial cells, and fibroblasts using LasA, a zinc metalloendopeptidase [162], and this has been implicated as a pathogenic mechanism that permits *P. aeruginosa* to mediate tissue injury in the lung and cornea. Given that *S. aureus* and *P. aeruginosa* are common pathogens that induce peritonitis in PD patients, it is possible that both microorganisms implement the shedding of syndecan-1 ectodomain from the mesothelium to promote their pathogenesis during peritonitis. In this regard, our preliminary studies have demonstrated increased levels of glycosaminoglycans in dialysis effluent obtained from patients with peritonitis. It is also plausible to suggest that this mechanism may also account in part for the loss of anionic sites from the mesothelium in experimental models of peritonitis.

Mesothelial cells are a major contributor of defensin production in the peritoneum but their antimicrobial function role in the peritoneum remains to be fully elucidated. Denudation of mesothelial cells from the peritoneal membrane during PD and peritonitis would suggest a concomitant loss of defensin production, but thus far, this does not appear to have any impact on the incidence of peritonitis [163]. In addition to their antimicrobial activity, defensins are thought to possess chemoattractant properties for immature dendritic cells and indirectly contribute to leukocyte infiltration by activating resident cells to secrete proinflammatory chemokines and cytokines [164–166]. It is therefore possible that defensins may contribute to both the innate and adaptive immune responses in the peritoneum although further studies are warranted to confirm this.

6. Effect of Peritonitis on Mesothelial Cells

There is compelling evidence to show that mesothelial cells play an essential role in the orchestration of peritoneal responses during inflammation and peritonitis. Changes to the structural and functional integrity of the mesothelium during PD and infection will therefore have a profound effect on how peritonitis is resolved. In order to delineate the mechanisms through which mesothelial cells regulate peritoneal inflammation and infection, and the underlying mechanisms through which peritonitis can modulate the structural and functional integrity of the mesothelium, the establishment of a reproducible model that can mimic the *in vivo* environment is essential to allow researchers to perform experiments in a controlled manner. Mesothelial cells isolated from omental specimens possess identical biochemical and morphological characteristics to those identified in peritoneal mesothelial stem cells. Therefore, cultured mesothelial cells provide a relevant tool to study the underlying mechanisms through which pathogens alter the structural and functional properties of the mesothelium. It is noteworthy that mesothelial cells isolated from mature

donors have an inflammatory phenotype even in the absence of any stimuli [167] and therefore it is imperative when assessing inflammatory processes that one can distinguish between changes induced by the inciting stimulus and that by the age of the cells.

Previous studies have shown that different causative microorganisms of peritonitis are associated with distinct clinical outcomes and therefore should not be considered comparable in terms of outcome [10]. Troidle et al. reported that patients with Gram-positive peritonitis fared better on PD compared to patients with Gram-negative peritonitis since the latter was associated with a greater need for hospitalization and catheter removal, and higher incidences of relapse and mortality [10]. It is therefore conceivable that Gram-positive and Gram-negative bacteria will have distinct effects on the mesothelium and therefore one should not ideally collate data from Gram-positive and Gram-negative bacteria together.

$\gamma\delta$ T-cells constitute approximately 0.5–5% of the total human peripheral blood T-cell population and are activated by small nonpeptide phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) [168]. HMB-PP is produced predominantly in Gram-negative bacteria [169]. HMB-PP-dependent cross-talk between Vγ9/Vv2 T-cells and autologous monocytes has recently been shown to drive the induction of chemokine and cytokine secretion and induce the differentiation of monocytes to inflammatory dendritic cells [169]. It is thus possible that HMB-PP may contribute to the observed difference between the severity of peritoneal inflammation in Gram-positive and Gram-negative bacteria although further studies are warranted to confirm this.

6.1. Toll-Like Receptors and Nucleotide-Binding Oligomerization Domain- (Nod-) Like Receptors. TLR play important roles in the initial recognition of bacterial, viral, and fungal components in the host defense system, and ten TLRs have been identified thus far. Independent researchers have demonstrated that both human and murine mesothelial cells constitutively express mRNA for TLR1-6, whilst TLR7-10 are barely detectable [170, 171]. TLR4 recognizes LPS, a major component of the outer membrane of Gram-negative bacteria. In an attempt to delineate the biological role of TLR4 during infection, Kato et al. administered LPS to C3H/HeN and C3H/HeJ mice by intraperitoneal injection, the latter strain being hyposensitive to LPS due to a point mutation in the TLR4 gene and investigated its effect on inflammatory processes. The observation that NFκB activation, induction of MCP-1 and MIP-2 secretion, and recruitment of leukocytes into the peritoneal cavity was dependent on TLR4 highlights its importance in peritoneal inflammation [170]. Recently, Colmont et al. demonstrated that human peritoneal mesothelial cells are able to respond to Gram-positive and Gram-negative bacterial ligands through TLR2 and TLR5, respectively [171]. Unlike murine mesothelial cells, human mesothelial cells demonstrated a lack of TLR4 responsiveness to LPS [171]. Although the functional consequences of these interactions remain to be determined, given that Gram-positive and Gram-negative bacteria induce different clinical outcomes in PD patients, is it possible that their recognition by mesothelial cells through distinct TLR subsets may induce distinct inflammatory processes within the peritoneum and which may explain, at least in part, the observed differences in clinical outcome?

Sensing of bacterial pathogens by mesothelial cells may also be mediated by nucleotide-binding oligomerization domain- (Nod-) like receptors [172]. Whilst TLR mediates the recognition of bacterial components either at the cell surface or in endosomes, Nod-like receptors induce the innate immune system through cytosolic recognition of bacterial constituents [173]. Recently, in transgenic animal studies, Park et al. demonstrated that Nod-1 and Nod-2 can regulate chemokine and antibacterial innate immune responses in mesothelial cells through the kinase RICK/RIP2 pathway, which mediated the downstream activation of NF-κB and MAPK pathways [172]. The ability of mesothelial cells to respond to bacterial components to initiate inflammatory responses highlights their pivotal role in peritoneal host defense.

6.2. Induction of Inflammatory and Fibrotic Mediators. Peritonitis has been shown to induce local production of various inflammatory and fibrotic mediators in mesothelial cells. Although the list of mediators is ever increasing, we will focus on some mediators that have been suggested to possess dual roles in inflammation and fibrosis. Hyaluronan is a negatively charged, linear polysaccharide that is widely distributed in epithelial, connective, and neural tissues [174, 175]. In normal tissues, hyaluronan is synthesized as a macromolecule with a molecular weight in excess of 10^6 Da [176]. Despite its simple structure, hyaluronan is a multifaceted molecule that contributes to the structural integrity of tissues, maintains water balance, and possesses anti-inflammatory properties [177, 178]. High-molecular-weight hyaluronan undergoes steady-state turnover and its degradation into small, nonbiologically active fragments is rapidly removed from the body by the liver. In chronic inflammation, elevated serum levels of hyaluronan and its deposition at sites of injury have been observed. Fragmentation of extracellular matrix (ECM) components often occurs during tissue injury and these fragments possess functional properties that are distinct from their parent molecule [178]. Removal of ECM fragments from the tissue is therefore vital for the resolution of tissue injury. Independent researchers have suggested that hyaluronan fragments may deposit in inflamed tissues consequent to their *de novo* synthesis or through the depolymerization of native hyaluronan following increased activity of hyaluronidase or reactive oxygen species [178–180]. Unlike native hyaluronan, hyaluronan fragments have been shown to induce multiple signaling cascades and increase cell proliferation, cytokine secretion, MMP activity, and matrix protein synthesis in murine models of lung disease or cultured mesothelial cells, keratinocytes, macrophages, and dendritic cells [178, 181–187]. Proinflammatory cytokines and profibrotic growth factors have been shown to increase synthesis of both high- and low-molecular-weight hyaluronan in various cell types

[188–191]. Studies have also demonstrated that TLR-2, TLR-4 and nod-like receptors can detect low molecular weight hyaluronan, and through these interactions are able to initiate inflammatory responses in an animal model of lung injury, whereas over-expression of high molecular weight hyaluronan was shown to maintain epithelial cell integrity and promote recovery [192].

Hyaluronan is a surrogate marker of inflammation. We and others have demonstrated that low levels of hyaluronan can be detected in dialysis effluent obtained from noninfected PD patients, and these levels are significantly increased during peritonitis [193, 194]. Our observation that dialysate hyaluronan levels are almost 2- and 10-folds higher that the corresponding serum levels in noninfected and infected PD patients, respectively, suggests that hyaluronan is synthesized locally [193]. We have demonstrated that cultured mesothelial cells can synthesize hyaluronan in abundance, of which 90% is secreted into their culture medium. The observation that the hydrodynamic size of hyaluronan synthesized by mesothelial cells is identical to that of hyaluronan detected in PD fluid, together with our observation that infected PD fluids can induce *de novo* synthesis of hyaluronan in these cells, provides evidence that mesothelial cells contribute to the increased local synthesis of hyaluronan during PD and peritonitis [193]. The molecular weight of hyaluronan present on the surface of mesothelial cells is higher than the secreted form, suggesting partial depolymerization of the parent molecule as it is released from the plasma membrane. Once released, hyaluronan appears stable and does not undergo further fragmentation in the peritoneal cavity during chronic PD or peritonitis [193]. Increased synthesis of hyaluronan in mesothelial cell during peritonitis is attributed to their induction by proinflammatory cytokines and growth factors, in particular, IL-1β IL-6, TNF-α, TGF-β1, and PDGF [188, 194]. The inability to detect hyaluronan fragments in spent infected and noninfected dialysate corroborates previous reports that low-molecular-weight hyaluronan is rarely observed in injured tissue *in vivo* [195]. It is noteworthy that studies detailing hyaluronan fragments as inflammatory mediators predominantly stem from *in vitro* studies [178, 183, 184, 196–199] and thus their existence in injured tissues and clinical relevance remains to be defined.

We have demonstrated that increased hyaluronan levels can induce EMT in mesothelial cells under physiological conditions, which is essential for cell migration during wound healing and remesothelialization [73]. Once the mesothelial monolayer is restored, hyaluronan levels are reduced [73]. A sustained increase in hyaluronan levels within the peritoneum during peritonitis would imply prolonged activation of mesothelial cells thereby preventing mesenchymal-to-epithelial transdifferentiation and their ability to revert back to their epithelial morphology. The acquisition of a migratory and invasive phenotype allows mesothelial cells to adopt a more fibrogenic characteristic whereby synthesis of MMPs that degrade the underlying basement membrane is increased, thus permitting the migration of transdifferentiated mesothelial cells into the submesothelium [59]. In this respect, Fukudome et al. demonstrated increased MMP-9 activity in dialysis effluent obtained from PD patients with peritonitis [200]. Transdifferentiated mesothelial cells have been shown to contribute to the thickening of the submesothelium and subsequent peritoneal fibrosis [55, 201]. Their migration into the submesothelium may also contribute to the denudation of the mesothelium, although this warrants further investigation.

Apart from hyaluronan, mesothelial cells also synthesize and secrete TGF-β1, IL-1β, IL-6, and TNF-α [26, 33–35, 55, 202] and their levels are increased during peritonitis. These peptides have also been shown to induce EMT in mesothelial cells and further augment peritoneal inflammation and fibrosis [55, 84, 93, 203–205]. Failure to restore the mesothelial monolayer is associated with unfavorable structural and functional changes to the peritoneal membrane of PD patients [3]. The role of TGF-β1 in the pathogenesis of peritoneal fibrosis is well documented [55, 205]. TGF-β1 also possesses an anti-inflammatory property but its ability to regulate peritoneal inflammation has been less characterized. In an animal model of peritonitis induced by *E. Coli*, Wang et al. noted a transient increase in TGF-β1 in the peritoneum that was associated with the activation of TGF-β1 and NFκB signaling pathways, increased secretion of TNF-α, and impaired peritoneal function [206]. Resolution of peritonitis was observed after 7 days without progressing to peritoneal fibrosis. In rats whereby TGF-β1 signaling pathways were block by genetic manipulation, *E. coli*-induced peritonitis exacerbated peritoneal inflammation as demonstrated by increased infiltration of leukocytes and further induction of inflammatory signaling pathways and secretion of TNF-α [206]. These data would suggest that TGF-β1 may also exert a protective, anti-inflammatory activity on the peritoneum during peritonitis. The role of TGF-β1 in immune tolerance and in particular in the inhibition of T-cell mediated immunopathology was first demonstrated over 2 decades ago in TGF-β1, deficient mice, which developed an early and fatal multifocal inflammatory disease [207, 208]. TGF-β1 can induce T-reg cell differentiation, but in the presence of IL-6, TGF-β1 induction of T-reg cells is inhibited [209]. TGF-β1 together with IL-6 has been shown to induce Th17 cells, a subset of T helper cells that have been implicated in autoimmune disease [209, 210]. Th17 cells have also been shown to synthesis IL-17 A, IL-17F, and IL-22 following infection, and these cytokines are involved in the recruitment and activation of neutrophils and tissue homeostasis [211]. The mechanisms that dictate whether TGF-β1 should follow an anti-inflammatory or profibrotic pathway remain to be determined.

HGF is a growth factor that has antifibrotic and profibrotic properties depending on the cell type. It has been shown to attenuate renal fibrosis by suppressing the actions of TGF-β1, slow the progression of diabetic nephropathy in db/db mice, and ameliorate podocyte injury and proteinuria in a murine model of chronic progressive glomerular disease [212–215]. On the other end of the spectrum, HGF has also been shown to induce cell proliferation and EMT in endothelial cells and hepatocytes [216, 217]. Rampino et al.

demonstrated that HGF induced cell proliferation, EMT, and collagen synthesis in mesothelial cells, thus indicating a pro-fibrotic role for HGF in the mesothelium. These researchers also observed an increase in the levels of HGF in dialysis effluent obtained from patients with peritonitis when compared to levels detected in non-infected PD fluid. This would indicate that HGF may contribute to the denudation of the mesothelium and increase fibrogenesis during peritonitis [218].

Angiotensin II is a potent vasoactive peptide that plays a critical role during renal fibrosis and peritoneal injury [219, 220]. Its levels are increased during peritonitis and angiotensin II has been shown to induce ERK1/2 and p38 MAPK activation and fibronectin synthesis in mesothelial cells, thereby contributing to peritoneal inflammation and fibrosis, respectively [221].

6.3. Alterations in the Fibrinolytic Cascade. Mesothelial cells play a critical role in maintaining the balance between fibrin accumulation and degradation through the expression of plasminogen activators, namely, tissue-type plaminogen activator (tPA) and urokinase plaminogen activator (uPA), and their specific inhibitor plasminogen activator inhibitor type 1 (PAI-1) [222]. Depending on the fibrinolytic capacity of mesothelial cells during peritonitis, fibrin may be lysed, which promotes healing, or deposited within the peritoneal structure where they induce fibroblast proliferation and collagen deposition, which inevitably results in peritoneal fibrosis [223, 224]. Most often that not, during peritoneal inflammation and peritonitis, a peritoneum devoid of its mesothelium and therefore devoid of its fibrinolytic mechanism is accompanied by the accumulation of fibrin within the peritoneum. If it is not removed, the fibrin will be replaced by granulation tissue, which in turn will be substituted by dense fibrous matter [225]. Studies have demonstrated that *S. aureus* complicating peritonitis can contribute to peritoneal fibrosis by their ability to produce a self-protecting coagulase that initiates the clotting of plasma and generation of a thrombin-like substance that permits the conversion of fibrinogen to fibrin [223, 225]. *In vitro* studies have shown that upon stimulation with proinflammatory mediators, such as IL-1α and TNF-α, tPA synthesis is inhibited in mesothelial cells, which is accompanied by an increase in PAI-1 synthesis [226, 227]. Furthermore, TGF-β1 has been shown to increase gene and protein expression of PAI-1 in cultured mesothelial cells, which enhanced fibrin deposition [222]. These cytokines are all increased in the peritoneum during peritoneal inflammation and peritonitis and may thus impede fibrin degradation *in vivo*. Recently, Haslinger et al. provided evidence that simvastatin could abrogate the suppressive effect of TNF-α on tPA synthesis in cultured mesothelial cells [228], but in the clinical setting, it is unlikely to be of much benefit if the mesothelium is already denudated.

6.4. Injury to Mesothelial Cells. The mesothelium plays an essential role in peritoneal homeostasis and host defense against infection. Prolonged use of PD and recurrent episodes of peritonitis result in the denudation of the mesothelium [3, 229]. In contrast to noninfected PD, where exfoliated mesothelial cells still remain viable in the peritoneal cavity, and which may have the potential to reestablish the mesothelium, emerging evidence has shown that invading pathogens such as *S. aureus* can induce caspase-independent mesothelial cell death [230]. Haslinger-Löffler et al. investigated the ability of various laboratory strains of *S. aureus* and *S. epidermidis* to induce mesothelial cell death. These researchers demonstrated that only *S. aureus* with an invasive and hemolytic phenotype induced mesothelial cell death, whereas none of the strains of *S. epidermidis* demonstrated any cytotoxic effect on mesothelial cells [230]. *S. aureus* has developed a number of mechanisms that allow the pathogen to adhere to the host cell, which is essential for progression of infection. Adhesion of *S. aureus* to components of the host cell is mediated by adhesins [231]. Fibronectin has been shown to mediate *S. aureus* attachment and subsequent invasion in mesothelial cells, an observation also noted in endothelial cells [231, 232]. In an intact, polarized mesothelium, fibronectin is normally localized to the basolateral cell membrane and therefore not readily available from the luminal aspect of the cell. In the context of PD and peritonitis, it is plausible to suggest that due to a compromised mesothelium with reduced synthesis of tight junctions, extracellular fibronectin may be exposed and accessible for *S. aureus* to bind, although this warrants further investigation. We have previously demonstrated that fibronectin is also present on the surface of mesothelial cells [40], which may also contribute to the binding and invasion of *S. aureus*. The molecular mechanism of *S. aureus* invasion bears remarkable similarities to complement-enhanced phagocytosis mediated by β_2-integrins in macrophages and neutrophil granulocytes [231]. The pathogen is engulfed by pseudopodia in a time-, dose-, and temperature-dependent manner [233] and can be located in vacuoles within mesothelial cells without being digested. This is dependent on the strain of bacteria, for example, *S. aureus* Cowan I can remain in the host cell without inducing any visible signs of cell injury, whilst other strains (*S. aureus* ST 239) can induce cell and nuclear shrinkage, vacuolization, and chromatin condensation [230]. Studies have demonstrated that at low concentrations of bacteria, cell death may be mediated by caspase activation and apoptosis, whereas higher concentrations result in necrotic cell death [234]. Haslinger-Löffler et al. demonstrated that *S. aureus* with a hemolytic phenotype mediated mesothelial cell death through necrosis [230]. Increased levels of TNF-α and Fas ligand during peritoneal inflammation and peritonitis may also induce apoptosis in mesothelial cells [235]. In our preliminary studies, we have demonstrated that Gram-negative bacteria such as *P. aeruginosa, K. pneumoniae* and *E. coli* induced denudation of the mesothelial monolayer and cell lysis more prominently that Gram-positive species (Yung and Chan, unpublished data). If cell death through apoptosis or necrosis supersedes cell proliferation, the replenishment of mesothelial cells will be insufficient, which will initiate subsequent peritoneal fibrosis.

7. Potential Role of Mesothelial Cell Transplantation and Gene Therapy in Peritoneal Preservation

It is without doubt that mesothelial cells play a crucial role in numerous cell processes in the peritoneum and a loss of mesothelial cells is accompanied by impairment of the structural and functional integrity of the peritoneal membrane. The omentum is a highly vascularized tissue that has been used in reconstructive surgery over the past two decades [236–238]. How the omentum facilitates the healing process remains to be fully defined, but it has been suggested that mesothelial cells may secrete growth factors at sites of injury or are themselves incorporated into the tissue. In order to preserve the dialytic efficacy of the peritoneum during PD and peritonitis, due to encouraging results obtained with the use of the omentum in reconstructive surgery, it is perhaps possible to transplant cultured mesothelial cells into the peritoneum once the structure of the peritoneal membrane is compromised. Independent researchers have suggested that genetic engineering may offer a novel therapeutic strategy, whereby omental specimens from predialysis patients are removed at the time of catheter implantation and mesothelial cells isolated and stored frozen until required [93, 239]. *Ex vivo* gene therapy may potentially bestow upon the peritoneal membrane an increased healing property or replenish proteins crucial for the maintenance of the mesothelium, which are lost during PD. Following peritonitis episodes when denudation of the mesothelium is prominently observed, genetically modified mesothelial cells may be infused into the peritoneal cavity through the catheter, allowed to settle on the denuded tissue and repopulate the peritoneal membrane. This stimulating concept is timely since it is currently impossible to completely dialyzed patients with PD solutions free of glucose and mesothelial injury will inevitable always emerge. In reality, is such a technique feasible in PD patients? Although mesothelial transplantation in an animal model of peritonitis has provided us with some encouraging results, it was also accompanied by deranged changes to the structure of the peritoneum with induction of inflammatory processes and activation of the peritoneum [240], which rather alarmingly this technique was specially intended to prevent. Before clinical trials can even be considered, it is essential that we determine the mechanisms by which transplanted mesothelial cells are activated in order to devise approaches to inhibit such activation.

In an in-depth morphologic study of the peritoneal membrane, Williams et al. observed that of the peritoneal biopsies analyzed, mesothelial denudation was noted in 18.1% of specimens obtained from predialysis and hemodialysis patients, and in specimens that presented with an intact mesothelium, the cells assumed a reactive state [3, 229]. Although the mechanisms that results in mesothelial denudation in predialysis and haemodialysis patients have yet to be fully identified, given that chronic inflammation is a common feature of patients with end-stage renal failure [241–245], it is possible that local ischemia, uremia, and systemic inflammation may result in increased levels of proinflammatory mediators and growth factors within the peritoneum, possibly derived from the circulation or local production, which induce cell detachment. These mediators include TNF-α, IL-1β, and TGF-β1, peptides known to induce cell detachment in mesothelial cells [201]. Plasma levels of AGE are elevated in patients with chronic renal disease [246] and these may also play a role in mesothelial cell denudation although further studies are warranted to confirm this. In predialysis diabetic patients, it is also possible that increased tissue levels of TGF-β1 may contribute to mesothelial cell detachment. Is it therefore possible to obtain adequate quantities of mesothelial cells from uremic patients to store for future transplantation? Given that mesothelial cells in culture have a defined life-span and enter senescence after the second to third passage, is it possible to collect sufficient mesothelial cells that maintain their polygonal morphology without a loss of their proliferative potential? Growth factors may be added to maintain their proliferative capability but this may also increase their fibrogenic potential. Accumulating evidence suggests that mesothelial progenitor cells exist, which may be harvested to assist in the repair and regeneration of the denuded mesothelium [247].

Much of the structural changes that are observed in the peritoneal membrane are induced by the bioincompatible nature of PD fluids. The use of PD solutions with alternative osmotic agents or partial or complete replacement of lactate-buffer with bicarbonate may be more beneficial in preserving the structural and functional integrity of the peritoneal membrane. A recent study demonstrated that PD patients using bicarbonate-buffered, neutral pH PD solutions showed a reduction in the frequency of peritonitis compared to conventional glucose-based, lactate buffered PD fluids [248], but these data have yet to be reproduced.

Although peritonitis exacerbates structural changes to the peritoneal membrane, the initial insult to the peritoneal membrane is the bioincompatible nature of PD fluids resulting in the induction of peritoneal inflammation. In an attempt to halt or even reverse peritoneal injury during PD and peritonitis, is it feasible to rest the peritoneum? Zhe et al. demonstrated that overnight peritoneal rest can improve ultrafiltration capacity in stable PD patients who had been on PD for more than 3 months [249]. In a separate study, Rodrigues et al. observed a recovery in ultrafiltration following peritoneal rest in 8 of 12 PD patients who had developed hyperpermeability [250]. In experimental models of PD, a period of 4–12 weeks of peritoneal resting was associated with a marked reduction in peritoneal fibrosis and angiogenesis, and complete remesothelialization of the peritoneal membrane [251, 252].

8. Conclusions

Despite considerable improvement in PD over the past 3 decades, peritonitis remains one of the major complications of PD and is an important cause technique failure and unfavorable clinical outcomes. A frequent cause of peritonitis is contamination at the time of exchange with Gram-positive bacteria that originate from the skin flora. Numerous studies have highlighted the critical role of *Staphylococcus* species in mediating mesothelial cell injury, denudation, and cell

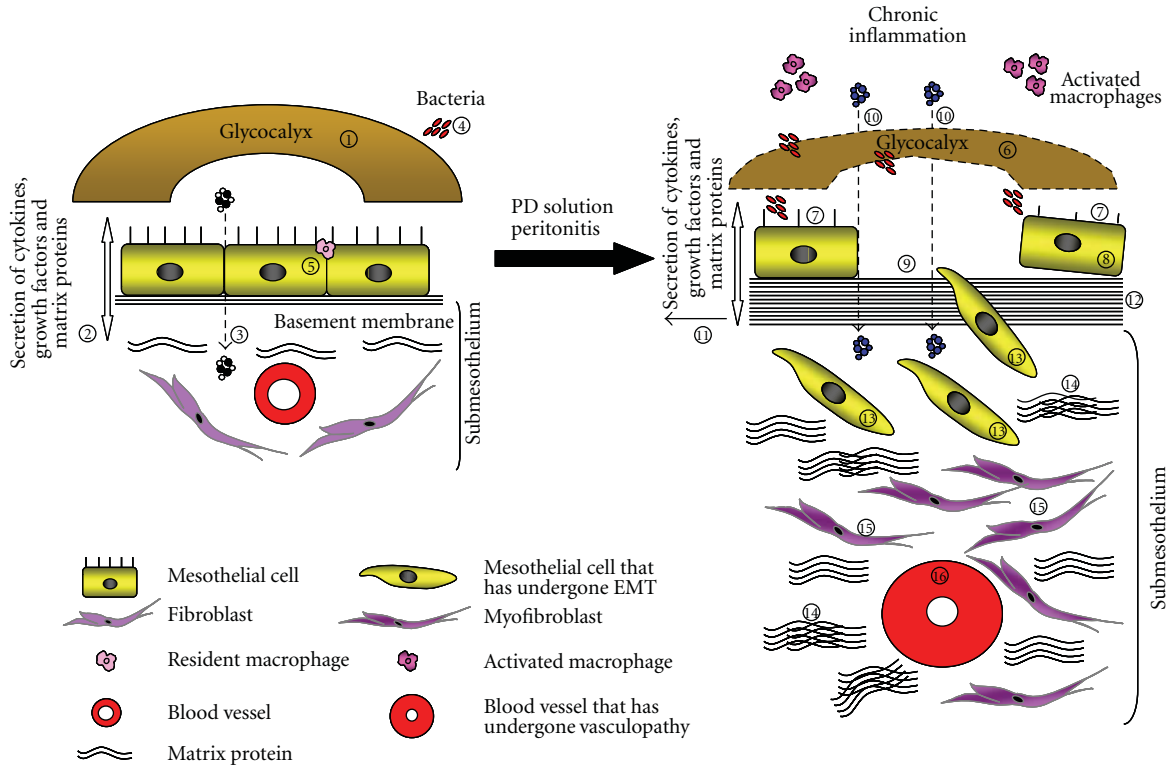

Figure 1: Mesothelial cells line the peritoneal membrane and play a crucial role in peritoneal homeostasis. Their apical surface is endowed with a glycocalyx that provides a protective barrier against abrasion, and a slippery, nonadhesive surface for intracoelomic movement (1). Through their ability to synthesize various cytokines, growth factors, and matrix protein components, mesothelial cells actively participate in tissue repair and induction and resolution of peritoneal inflammation (2). Synthesis of matrix proteins by mesothelial cells may be incorporated into the underlying basement membrane on which mesothelial cells adhere to. Mesothelial cells facilitate in the transport of fluids and solutes across the peritoneal membrane (3), are the first line of defense against bacterial peritonitis (4), and can maintain a chemotactic gradient to assist in leukocyte infiltration (5) during peritoneal inflammation. The submesothelium contains sparse fibroblasts, collagen fibrils and capillaries. Changes to the structural integrity of the peritoneal membrane are invariably observed in PD patients. Constant exposure of the peritoneum to PD fluids, together with peritonitis, results in a reduction of the glycocalyx volume and a concomitant loss of anionic charge in the glycocalyx (6). Alterations in the anionic charge of the peritoneum can result in the reduction in the length and density of microvilli on the surface of mesothelial cells (7). Chronic exposure to PD fluid and peritonitis can induce detachment of mesothelial cells from their underlying basement membrane (8) resulting in partial (9) or complete denudation of the mesothelium. A loss of cell-cell interaction between mesothelial cells permits PD fluid to enter into the submesothelium (10). Increased synthesis of proinflammatory cytokines and matrix proteins is observed following the activation of infiltrating and resident peritoneal cells (11), leading to morphological changes such as reduplication of the basement membrane (12), induction of EMT in mesothelial cells, a breakdown of the basement membrane and their migration into the submesothelium (13). Transdifferentiated mesothelial cells have a greater fibrogenic potential and thus contribute to the deposition of matrix proteins and fibrin in the submesothelium (14), which if not controlled will lead to thickening of the submesothelium and ultimately peritoneal fibrosis and sclerosis. A loss of the protective mesothelium allows PD fluid and toxins released by bacteria to induce the activation of peritoneal fibroblasts (15), hyalinization of blood vessels, and vasculopathy (16). Such detrimental changes to the peritoneal membrane will significantly suppress the dialytic potential of the peritoneal membrane, which will invariably lead to the cessation of treatment.

death, which leads to increased fibrin and matrix protein accumulation, and ultimately peritoneal fibrosis. Repeated episodes of peritonitis will aggravate these processes and accelerate catheter removal and technique failure. Gram-negative complicating peritonitis is less common that Gram-positive infections but is associated with higher rates of death, hospitalization, and transfer to hemodialysis compared to Gram-positive peritonitis [9]. Our preliminary studies have demonstrated that mesothelial cell denudation is more pronounced when they are exposed to dialysis effluent from PD patients with Gram-negative peritonitis, attributed in part to the higher dialysate levels of proinflammatory cytokines compared to Gram-positive peritonitis [27]. One should therefore bear in mind that different species of microorganisms induce distinct changes to the mesothelium and submesothelium and therefore should not be grouped as one. Furthermore, for experimental and *in vitro* studies simulating PD-related peritonitis, it is noteworthy that subtle structural and regulatory changes to laboratory-based bacteria may result in alterations in their invasiveness and cellular behavior, which are not observed in the clinical setting.

With a greater understanding of the underlying mechanisms through which different species of microbes can modulate mesothelial cell function and their attachment to the peritoneal membrane during peritonitis, it is envisaged that in time we may devise novel therapeutic interventions to preserve the structural and functional integrity of the peritoneum and thereby improve patient survival on PD. Figure 1 is a schematic diagram that highlights our current knowledge of how PD and peritonitis may affect the structural integrity of the peritoneal membrane.

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Review Article

Sclerosing Peritonitis: A Rare but Fatal Complication of Peritoneal Inflammation

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Sclerosing peritonitis is a rare form of peritoneal inflammation with an often fatal outcome. The major risk factor of sclerosing peritonitis is peritoneal dialysis treatment but it can also occur following renal or liver transplantation or be associated with certain drug treatment. This article gives an overview of reasons and treatment options for sclerosing peritonitis and shows a summery of current literature about sclerosing peritonitis.

Sclerosing peritonitis (SP) is a rare form of peritoneal inflammation which involves both the visceral and the parietal surfaces of the abdominal cavity. SP is characterized by a fibrous thickening of the peritoneum and is reported to complicate peritoneal dialysis, certain drugs, and infectious peritonitis. SP was first described in 1907 [1], with the term "abdominal cocoon" being in use since 1978 [2].

Due to the low incidence and the relatively slow disease progress, there is no valuable data with respect to the comparative incidence of SP related to dialysis versus SP without any known risk factor [3, 4]. Published data indicate prevalence rates between 0.54% and 0.9% [5–7]. The etiology of SP remains unknown and presumably is multifactorial [3, 4]. There is no distinct relationship between the renal disease leading to end stage renal failure and the development of SP.

The major risk factor for SP is peritoneal dialysis treatment (PD) [8, 9]. Indeed, the utilization of the peritoneal cavity for dialysis therapy is associated with an increased risk of structural and functional damage to the peritoneal membrane. PD-related risk factors such as duration of therapy, poor biocompatibility of dialysis solutions, and peritonitis are considered to be important for the development of SP, with the duration of PD being the most relevant single factor. SP usually occurs in patients receiving PD for more than 4 or 5 years and the incidence of SP in patients on PD treatment for less than 2 years is very low [6, 7]. Nonphysiologic dialysis solutions may induce a chronic sterile inflammation in the peritoneal cavity with upregulation of several cytokines resulting in collagen synthesis by mesothelial cells and fibroblasts. Moreover, the high concentrations of glucose and lactate as much as the low pH of the dialysis solutions and bioincompatible substances directly damage the peritoneal membrane. Alike bacterial or fungal peritonitis, they lead to the loss of mesothelium and a decline in fibrinolytic capacity of the peritoneal membrane, and might thereby contribute to the development of SP [7]. The largest observational study published by Kim et al. [6] included 4.290 PD patients followed from 1981 to 2002, 34 of whom developed SP, corresponding to an overall prevalence of 0.79%. The male to female ratio in these patients was 1 : 1, their median age was 44.5 years (range 19–66 years) and the median duration of PD treatment until the diagnosis of SP was 64 months (range 9–144 months), with 23 of 34 patients (68%) having been on PD for more than 4 years. Remarkably, 27 of the 34 SP patients (79%) had a medical history of peritonitis, including two cases of fungal peritonitis, with a median of 6 and at most 15 episodes of PD-related peritonitis ultimately leading to catheter removal. 18 patients were diagnosed by clinical and radiological methods, the remaining 16 were diagnosed

surgically. 11 patients were treated by laparotomy with excision of the sclerosed peritoneum. The overall mortality in SP patients was 24%, the mortality in patients who had undergone surgical treatment was 43%.

Betablockers are also supposed to contribute to the development of SP [9], which has most commonly described in association with practolol, but also metoprolol, propranolol and atenolol [10, 11]. The pathomechanism is not entirely clear, but probably relates to the inhibition of surfactant release by betablockers [9]. Clinical studies found a history of corresponding medication in many patients with SP, yet the factual significance of this drug class for the development of SP could not be clarified [6]. Antibiotics used for treatment of peritonitis are as well discussed to be a risk factor for SP [12].

Clinical signs of patients developing SP while on peritoneal dialysis therapy are ultrafiltration and clearance failure. Furthermore, small bowel obstruction due to encapsulation, adhesions, and mural fibrosis with anorexia, nausea, and vomiting is frequently observed and ultimately entails malnutrition (Table 1) [9]. Following termination of PD treatment, patients with SP often develop ascites [13]. Histopathologic examination reveals fibrosis of the entire peritoneum with substantial thickening. This differs from simple sclerosis, which is also found in patients with ascites or peritoneal dialysis and is usually not associated with a clinically relevant disease progress [14].

There are some reports of SP as late sequelae of PD, typically occurring in patients who had previously undergone successful renal transplantation [15]. Adamidis et al. reported a case of a former PD patient who presented 2 years after renal transplantation with abdominal discomfort, vomiting, and malnutrition due to SP. Despite the initial conservative treatment, for persistence of symptoms he underwent surgical treatment later on and recovered without any further complication [16]. Bowers et al. reported 3 cases of SP in former, meanwhile successfully transplanted PD patients. Each of these patients suffered from a mechanical small bowel obstruction secondary to a densely fibrosing and encasing peel of reactive tissue visibly different from the usual postoperative adhesions [17]. Morrow et al. reported the case of a 55-year-old woman with end-stage renal disease secondary to systemic lupus erythematodes who had received two renal transplants within 15 years; between the two transplantations she had been on PD for 5 years. Following her second successful kidney transplant, she presented with persistent nausea and vomiting and was diagnosed to have SP by CT scan. Due to failure of conservative management she underwent exploratory laparotomy with extensive lysis of adhesions and the postoperative course was complicated by intolerance to feedings. 3 months later, a second laparotomy had to be performed due to obstruction of the proximal jejunum. Intraoperative findings were similar to the prior ones. Extensive lysis of adhesions and Billroth II gastrojejunostomy bypass is done, and the patient recovered tolerating a regular diet [15]. Table 2 summarizes the publications relating to patients with SP following renal transplantation, the respective therapy, and patient survival. From the sporadic case reports, however, no preferential association with determinate immunosuppressive regimens could be inferred nor be corroborated by any other evidence. Anyway, even though being extremely rare, SP has to be included in the differential diagnosis of every renal transplant patient with prior PD treatment who presents with unexplained malnutrition and symptoms of abdominal obstruction.

Table 1: Clinical characteristic of sclerosing peritonitis (SP).

Abdominal pain
Nausea
Vomiting
Weight loss
Loss of ultrafiltraion (in PD patients)
Blood-stained dialysate (in PD patients)

Table 2: Sclerosing peritonitis after kidney transplantation.

	No. of cases	Therapy	Survival : death
Bowers et al. Clin transplant 1994	3	Surgery	3 : 0
Morrow et al. Dig Dis Sci 2011	1	Surgery	1 : 0
Adamidis et al. Ren Fail 2011	1	Surgery	1 : 0

SP has been also described in association with other diseases and as an idiopathic form [18]. The idiopathic form predominantly occurs in adolescent females and a possible relationship to retrograde menstruation has been discussed [19, 20]. SP has also been reported in patients with ventriculoperitoneal shunts [21]. Moreover, patients with end-stage liver disease waiting for liver transplantation are at a higher risk for developing SP. With continuous peritoneal irritation from ascites and recurrent spontaneous bacterial peritonitis, these patients suffer from two independent conditions identified as risk factors for SP. SP has also been reported in patients after liver transplantation. The typical symptoms are abdominal pain, refractory ascites, bowel obstruction, and malnutrition [22–24]. In a large prospective study including 1.800 liver transplant recipients, Maguire et al. reported on 5 patients aged 16 to 57 years who developed SP after liver transplantation. None of these patients had a peritoneal-venous shunt or had undergone peritoneal dialysis. All 5 patients developed fever as early as 66 ± 21 hours posttransplant, with confirmation of bacterial peritonitis in two patients and additional symptoms including epigastric discomfort and intermittent vomiting occurring 12 ± 10 days later. While abdominal CT consistently showed marked ascites confined to definite areas of the abdomen, ultrasound and intestinal contrast studies were not diagnostic. All patients underwent an early second laparotomy with removal of an abdominal cocoon membrane. 4 of 5 patients survived without long-term sequelae [22]. Mekeel et al. reported on 3 patients suffering from SP after liver transplantation, namely, two 42- and 62-year-old males with end-stage liver disease due to hepatitis C infection without significant other past

Table 3: Sclerosing peritonitis after liver transplantation.

	No. of cases	Therapy	Survival : death
Maguire et al. American Journal of Surgery 2001	5	Surgery	4 : 1
Lin et al. World Journal Gastroenterol 2005	1	Surgery	1 : 0
Mekeel et al. Liver Transplantation 2009	3	Surgery	0 : 3

medical or surgical history and a 59-year-old alcoholic. All 3 patients had massive refractory ascites with episodes of spontaneous bacterial peritonitis prior to transplantation. Two patients had evidence of a fibrous peel already at the time of transplantation. Postoperatively, all 3 patients continued to have refractory ascites and episodes of peritonitis, along with partial small bowel obstructions, abdominal pain, and malnutrition. Beyond that, in two patients a constriction of the graft, including biliary as well as inferior vena cava and outflow obstruction occurred [23]. Lin et al. reported on a patient with hepatitis-B-related hepatocellular carcinoma and a prior peritoneal-venous shunt who developed SP with small bowel obstruction two weeks after liver transplantation [24]. Accordingly, not only SP apparently occurs rather early in recipients of liver transplants, but its diagnosis proves to be difficult. Table 3 summarizes the publications relating to patients with SP following liver transplantation, the respective therapy, and patient survival.

At last, SP has been observed in children who had previously undergone intestinal transplantation; it is characterized by progressive serositis as well as fibrous changes of the intestinal allograft mesentery and serosa with progressive contraction culminating in intestinal obstruction. The native intestinal tract is always spared. The causes of SP associated with intestinal transplantation are not completely understood. No distinct risk factors as cold ischemia time or severity of rejection could be identified. In an adult patient, sclerosing mesenteritis ascribed to a vascular form of antibody-mediated rejection has been reported after a combined liver-intestine transplantation; therefore, chronic rejection may be considered as a trigger for the development of SP in intestinal transplant recipients. Macedo et al. retrospectively reviewed the medical records of 121 children who underwent intestinal transplantation between 1990 and 2003; in this cohort, three children (2.4%) suffered from distal ileal obstruction of the allograft intestine secondary to SP. The indication for their intestinal transplantation was intestinal failure secondary to gastroschisis in two patients and midgut volvulus in one patient. All patients had become independent of total parenteral nutrition and were asymptomatic until the diagnosis of SP. The mean time to presentation with symptoms of SP was 6.6 years (range 5.3–8 years) from intestinal transplantation. Laparotomy was performed in all three patients and showed serositis, dense fibrous adhesions, and a contracted mesentery. In all patients adhesiolysis and segmental resection of the distal ileum were performed. Two patients died 2.5 and 3, respectively, years after transplantation [25].

The diagnosis of SP is made clinically, radiographically, or by laparotomy. Characteristic radiographic findings can be visualized on small bowel follow-through series or CT imaging. CT scans typically show small bowel congregated in the center of the abdomen, thickened peritoneum, or large locular fluid collections. In addition, a delayed transit of contrast can be observed on fluoroscopy [15, 26–28].

There is no expert agreement on whether the treatment of choice should be surgical or conservative. As obvious from the low incidence of SP, there are no respective clinical studies comparing the different therapeutical approaches, and experience is limited to case reports. Treatment of PD patients with SP includes cessation of PD therapy and conversion to hemodialysis. Bowel rest and total parenteral nutrition seem to alleviate symptoms substantially. Medical treatment regimens based on corticosteroids and methotrexate have been recommended [29, 30]; for its known effects on other fibrotic diseases, including retroperitoneal fibrosis and desmoid tumors, tamoxifen has been adopted as therapeutic agent in SP as well [31]. However, there are minimal data supporting a positive effect of any of the cited medical treatment options. Surgical treatment is exclusively recommended for patients suffering from intestinal obstruction [6]. The mortality of SP has been reported as high as 24% and may attain 60% in patients who is managed operatively [6] due to perioperative complications like anastomosis insufficiency, intraabdominal infections, and enterocutaneous fistulas.

In conclusion, SP is a rare but serious complication affecting mainly patients on PD treatment. SP is a potentially late sequela of PD and can be found as well in patients after liver or intestinal transplantation without a history of PD; hence, it should be included in the differential diagnosis of every case of unexplained malnutrition and abdominal obstruction, especially in patients after solid organ transplantation.

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Review Article

Inflammation and the Peritoneal Membrane: Causes and Impact on Structure and Function during Peritoneal Dialysis

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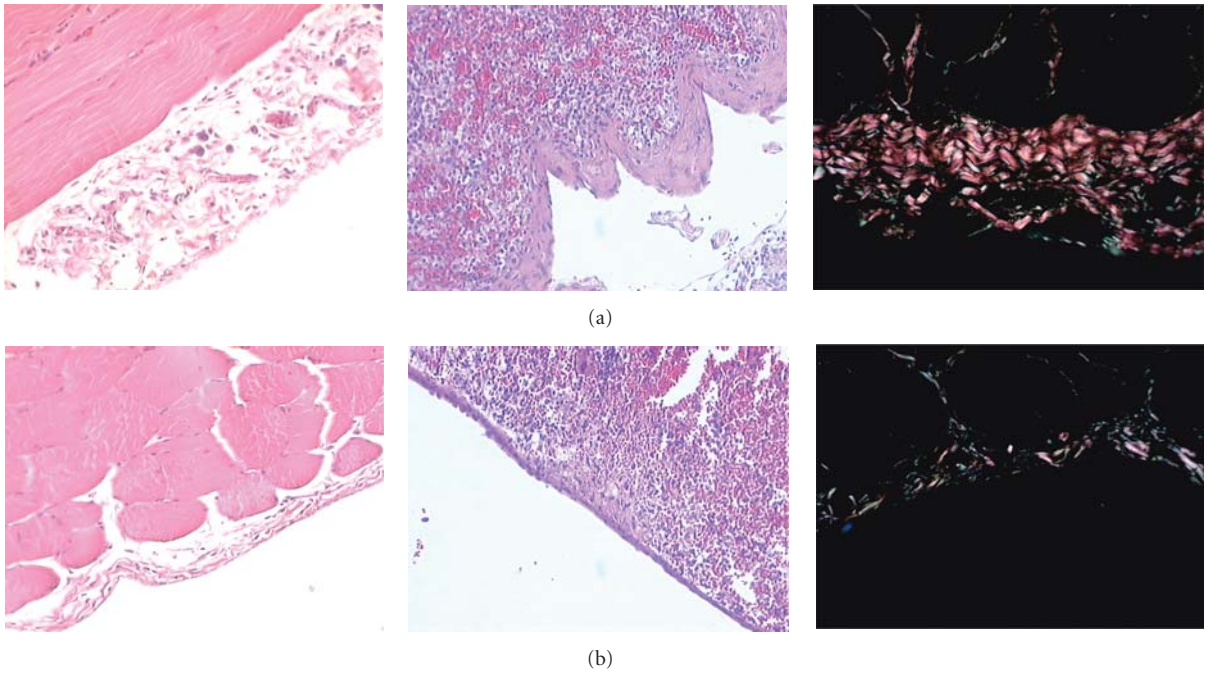
Peritoneal dialysis therapy has increased in popularity since the end of the 1970s. This method provides a patient survival rate equivalent to hemodialysis and better preservation of residual renal function. However, technique failure by peritonitis, and ultrafiltration failure, which is a multifactorial complication that can affect up to 40% of patients after 3 years of therapy. Encapsulant peritoneal sclerosis is an extreme and potentially fatal manifestation. Causes of inflammation in peritoneal dialysis range from traditional factors to those related to chronic kidney disease *per se*, as well as from the peritoneal dialysis treatment, including the peritoneal dialysis catheter, dialysis solution, and infectious peritonitis. Peritoneal inflammation generated causes significant structural alterations including: thickening and cubic transformation of mesothelial cells, fibrin deposition, fibrous capsule formation, perivascular bleeding, and interstitial fibrosis. Structural alterations of the peritoneal membrane described above result in clinical and functional changes. One of these clinical manifestations is ultrafiltration failure and can occur in up to 30% of patients on PD after five years of treatment. An understanding of the mechanisms involved in peritoneal inflammation is fundamental to improve patient survival and provide a better quality of life.

1. Introduction

Peritoneal dialysis (PD) therapy has increased in popularity since the end of the 1970s. The method was developed as an alternative to hemodialysis (HD) presenting a patient survival rate equivalent to HD and better preservation of residual renal function. However, technique failure remains high, resulting in frequent modality changes. Currently, the two principal causes of technique failure in order of importance are (a) peritonitis, this important medical problem can also represent nearly 16% of the causes of death; (b) ultrafiltration failure, a multifactorial complication that can affect up to 40% of patients after 3 years of therapy [1].

The peritoneal membrane is composed of different cell types with varying functions. Peritonitis as well as contact with bioincompatible solutions have deleterious effects on the membrane. These proinflammatory stimuli can induce lymphokine secretion by macrophages, which in turn, activate fibroblasts. Fibroblast activation has been associated with structural alterations in the peritoneal membrane of varying intensity. These alterations can be seen in Figure 1 which was extracted from a submitted study of our group. In this prospective controlled study in 20 nonuremic Wistar rats, peritoneal fibrosis occurs after exposure to glucose-based PD solutions and regardless the use of simvastatin.

Encapsulant peritoneal sclerosis (EPS) is an extreme and potentially fatal manifestation. EPS is a clinical syndrome that leads to persistent or recurrent intestinal obstruction, with or without inflammatory parameters of peritoneal thickening, sclerosis, calcification, and encapsulation, and can be inferred by clinical symptoms and radiology, but confirmed only by direct visualization with laparotomy [2, 3]. Incidence of EPS is heterogenous and has been



(a)

(b)

Figure 1: Typical alterations in the peritoneal membrane in an experimental model of hypertonic dialysate infusion (a) and the impact of oral statin use during 8 weeks of followup (b).

reported to vary from 6 to 20% in eight years depending on the region.

2. Causes of Inflammation in PD

Causes of inflammation in peritoneal dialysis range from traditional factors to those related to chronic kidney disease *per se* as well as from the peritoneal dialysis treatment itself.

Uremia is a factor present in all PD patients and generates an inflammatory state causing stress on the peritoneum due to the formation of carbonyl products. It accelerates the formation of advanced glycation end products (AGEs) that induces an upregulation of the receptors of advanced glycation end products (RAGE) [4]. Dialysis decreases the impact of uremia, however, does not remove it completely.

The peritoneal dialysis catheter is the first proinflammatory factor associated to PD with which the patient comes into contact. After implantation in the peritoneum, the catheter can induce an inflammatory reaction as was demonstrated by Flessner et al. [5]. In addition, the catheter can occasionally be the site of bacterial biofilm formation.

Initial therapy introduces the second inflammatory factor associated with PD: dialysis solution. Several PD solutions are available on the market today, and all are, to varying degrees, associated with peritoneal inflammation. Such inflammation is generated by several characteristics of these solutions, varying from low pH, presence of lactate, hyperosmolality, increased glucose concentration, presence of glucose degradation products (GDP) and advanced glycation end products (AGEs), and icodextrin metabolites, among others [6, 7].

Currently available glucose-based PD solutions present concentrations varying between 1.5 and 4.25% of glucose. The glucose load offered daily by a traditional PD prescription usually ranges from 120 g to 400 g.

The majority of PD solutions prescribed today markedly acidify pH to nearly 5.7 in approximately 2 to 3 minutes. This pH decreases viability of neutrophils and mesothelial cells, thus decreasing cytokine production and phagocytosis cap-acity. Lactate is utilized as a buffer in the majority of solutions. Its bioincompatibility with the peritoneal membrane is well known as well as its capacity to stimulate the production of fibroblast growth factors contributing to peritoneal fibrosis [8].

The association of icodextrin with EPS development is controversial. Some studies have associated the osmotic agent with EPS development [7], while others have shown it to be distinct, confirming its safety even with long-term utilization [6]. The relative rarity of the disease makes a definitive conclusion difficult. Even experimental studies with rats addressing this question are compromised by the increased α-amylase activity in these animals. The presence of this enzyme in plasma and in the peritoneal cavity provokes a rapid drop in peritoneal icodextrin concentration [9].

Chronic exposure to high glucose load in traditional PD solution induces significant inflammation of the peritoneal membrane. These solutions induce several proinflammatory factors such as PGA [10], vascular endothelial growth factors (VEGFs), fibroblast growth factor (TGF-β1), AGEs, and *upregulation* of RAGEs. Together, these factors contribute to the occurrence of neoangiogenesis and mesothelial fibrosis [11]. Glucose degradation products (GDPs), such

as methylglyoxal, glyoxal, and 3-deoxyglucosone generated during the heat sterilization process, increase inflammation by inducing oxidative stress, which thus causes damage to mesothelial cells and leads to apoptosis and mesothelial denudation [12].

Substituting traditional solutions for more biocompatible solutions was recently associated with reduced membrane alterations [13]. It has been suggested for some years that the pathway of transforming growth factor β1/Smad plays a part in the development of peritoneal fibrosis. High glucose concentration in PD solutions is related to the activation of this pathway. The relationship between Smad2 and VEGF expression has also been reported. The latter is recognized as playing a role in angiogenesis, a histological characteristic that allows for differentiation from simple peritoneal fibrosis to EPS [14].

The endothelial system is another known factor with potent profibrotic characteristics and plays a role in the development of peritoneal fibrosis. This system can be activated by two receptors, endothelial receptors A and B. However, endothelial receptor B apparently does not play a role in peritoneal membrane thickening in experimental studies inducing deficiency of endothelial receptor B.

Finally, and of extreme importance, infectious peritonitis is an obvious cause of peritoneal inflammation and is associated with EPS development. Gram-positive organisms remain as the more prevalent peritonitis agents over the past decades representing up to 60% of cases followed by gram-negative organisms. However, the prevalence of peritonitis due gram-negative organisms is growing fast with the development of efficient strategies to control gram-positive infections. Despite all efforts made over the past decades, it still represents the most important cause of treatment discontinuation.

In sum, all the above-mentioned factors contribute to the release of proinflammatory cytokines such as interleukin 1β (IL 1β), tumor necrosis factor (TNF-α), IL-6, and IL-18. Structural lesions as a result of this process will be addressed below.

3. Structural Consequences of Inflammation of PD

Peritoneal inflammation generated by PD causes significant structural alterations in the peritoneum. These alterations, when severe, can trigger encapsulant peritoneal sclerosis [12]. Mesothelial exposure to PD solution in rats increased cytoplasm in these cells [15]. Thickening and cubic transformation of mesothelial cells occurs and is more accentuated in the parietal peritoneum [16]. Human peritoneal mesothelial cells (HPMCs) also suffer structural alterations and prominent transdifferentiation of HPMC to myofibroblasts occurs [17].

Histological alterations of the peritoneal membrane observed in EPS cases are nonspecific and are masked by the alterations commonly observed in patients with ultrafiltration failure and infectious peritonitis over the long term [18]. The most common findings are fibrin deposition, fibrous capsule formation, perivascular bleeding, interstitial fibrosis, and the presence of tissue granulation with vascular proliferation. Submesothelial tissue thickening also occurs with an increase in deposition of mesothelial conjunctive tissue [19, 20]. Fibrosis is characterized by the accumulation of extracellular matrix (ECM), resulting in disequilibrium between synthesis and degradation. Expression of collagen types 1 and 3 is significantly increased [21] as well as collagen type 4 [10]. Mesothelial cell denudation has also been described [22]. With respect to neoangiogenisis, we observed an arteriole diabetiform alteration and subendothelial hyalinosis of the venules [23].

4. Functional Consequence of Inflammation in PD

Structural alterations of the peritoneal membrane described above result in clinical and functional changes. One of these clinical manifestations is ultrafiltration (UF) failure and can occur in up to 30% of patients on PD after five years of treatment [1]. One of the presentations of UF failure occurs due to the increase in pores in the peritoneal membrane, which in turn accelerates small-solute transport dissipating the osmotic gradient necessary to maintain adequate fluid balance. This increase in vascular surface is observed in conjunction with an increase in density of interstitial fibers. These findings help justify the increase in transport of small molecules, while the alterations in the UF coefficient are only moderate [24]. In addition to UF failure, clinical manifestations such as severe malnutrition, subocclusion or intestinal occlusion, and ascites suggest the presence of EPS even after discontinuation of PD.

Prescribing more hypertonic glucose solutions is a common strategy to counter this drop in UF, primarily where there is no available icodextrin. This intensifies and perpetuates inflammatory disturbances, with a direct impact on dialysis adequacy and fluid balance. The final consequence is the inevitable transfer to HD. Despite all damage to the peritoneal membrane with therapies performed today, large observational studies have shown an important evolution in PD patient survival when compared to HD over the past years [25].

5. Conclusion

PD initiation increases inflammatory stimuli for the chronic kidney patient such as the presence of the peritoneal catheter, use of bioincompatible solutions, and possible infectious peritonitis. Together, these factors generate structural and physiological alterations of the peritoneal membrane. These manifestations are frequently observed and can range from difficulties in obtaining an adequate fluid balance until the dreaded encapsulant peritoneal sclerosis. Nevertheless, patient survival in PD is similar to that of HD. An understanding of the mechanisms involved in peritoneal inflammation is fundamental for the development of new strategies. This knowledge can provide not only a better technique survival, but also improvements in patient survival and a better quality of life.

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Research Article

The Role of the Vagus Nerve: Modulation of the Inflammatory Reaction in Murine Polymicrobial Sepsis

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The particular importance of the vagus nerve for the pathophysiology of peritonitis becomes more and more apparent. In this work we provide evidence for the vagal modulation of inflammation in the murine model of colon ascendens stent peritonitis (CASP). Vagotomy significantly increases mortality in polymicrobial sepsis. This effect is not accounted for by the dilatation of gastric volume following vagotomy. As the stimulation of cholinergic receptors by nicotine has no therapeutic effect, the lack of nicotine is also not the reason for the reduced survival rate. In fact, increased septic mortality is a consequence of the absent modulating influence of the vagus nerve on the immune system: we detected significantly elevated serum corticosterone levels in vagotomised mice 24 h following CASP and a decreased *ex vivo* TNF-alpha secretion of Kupffer cells upon stimulation with LPS. In conclusion, the vagus nerve has a modulating influence in polymicrobial sepsis by attenuating the immune dysregulation.

1. Introduction

Peritonitis and subsequent sepsis remain a severe problem in the surgical field. The immune response of a septic organism is mediated by the adaptive and the innate immune system. Beside "classical" mechanisms like humoral and paracrine regulation or direct cell-to-cell interaction, the autonomic nervous system seems to be critically involved. Neural circuits that directly control certain immune reactions, also known as the "inflammatory reflex", were identified [1–3]. Within these neural circuits, the vagus nerve plays an important role as it mediates afferent effects triggered by inflammatory mediators and also interacts with visceral organs by efferent activity.

During sepsis, the vagus nerve is essential for balancing anti- and pro-inflammation. Experimental vagotomy results in hyperinflammation and can lead to increased mortality [4–7]. Several studies showed that animals deficient in vagus nerve activity are more sensitive to inflammatory challenges like endotoxemia, sepsis, pancreatitis [8] and hypovolemic shock. This may be due to the uncontrolled hyperinflammation which is mirrored by a critically increased release of proinflammatory cytokines [1, 6, 7, 9].

An efferent vagal connection to adrenal glands is established [10–12]. However, there is still an incomplete understanding of the potential role of vagal nerve in context of adrenal gland derived glucocorticoids. It is known that adrenal gland function is essential for the outcome of patients with sepsis [13–15]. Nevertheless, glucocorticoid therapy in sepsis, based on their ability to induce IL-10 elevation und TNF-alpha decrease, was controversially discussed [16–18]. Additionally, vagal activity seems to be involved in fever regulation [19, 20], apoptosis [21, 22], and regeneration

of hepatocytes [23]. The liver and its resident macrophages, the Kupffer cells (KCs), are considered to play a crucial role in the course of sepsis [24]. An anatomical link between the vagus nerve and the liver is well described: the hepatic branch of the vagus nerve [25, 26]. KCs produce many kinds of soluble mediators such as cyokines, especially IL-10 and TNF-alpha, prostanoids, proteases, and oxygen radicals [27, 28]. 80–90% of them are located in the liver [27]. A vagal modulation of the cytokine release by Kupffer cells in inflammation is presumed, but poorly understood [29].

The aim of this study was to further investigate the role of the vagus nerve in the course of peritonitis. Therefore, the colon ascendens stent peritonitis (CASP) was used. In contrast to models of LPS shock, CASP is a model of polymicrobial abdominal sepsis, that mirrors a common course of systemic infection in surgical intensive care patients [30–32]. The influence of subdiaphragmal vagotomy on corticosterone release by adrenal glands and KCs function after CASP were examined. Additionally, the potential therapeutic effect of nicotine as an unspecific agonist of nicotinic acetylcholine receptors (nACHRs) during peritonitis was analyzed.

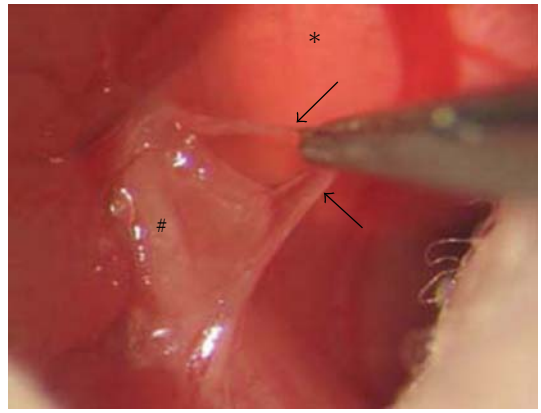

Figure 1: Surgical dissection of the vagus nerve using a microscope (40-fold magnification): the abdominal wall was opened through a transverse incision. Esophagus (#) and diaphragm (∗) were exposed and the ventral branch of the vagal nerve (arrows) was dissected. The dorsal branch of the vagus nerve was excised in the same way.

2. Methods

2.1. Mice. For all experiments, 8- to 12-week-old female C57BL/6 mice purchased from Charles River (Sulzfeld, Germany) (weight 20–25 g) were used. Prior to surgery, mice were kept for at least 2 weeks in the animal facility to recover from transport. All experimental procedures were performed according to German animal safety regulations. For all surgical procedures, Avertin (Sigma-Aldrich Chemie, Taufkirchen) anaesthesia was used.

2.2. CASP Surgery. The surgical procedure for CASP was performed as previously described [30, 31]. After disinfection of the abdomen, the ascending colon was identified and a prepared catheter (16 gauge, Venflon; BOC, Ohmeda, Sweden) was implanted in the antimesenteric wall of the ascending colon. To ensure intraluminal positioning of the stent, stool was milked from the ascending colon into the stent. Afterwards, 0.5 mL of sterile saline solution was flushed into the peritoneal cavity before closure of the abdominal walls (single layer; 4/0 Polyester, Catgut, Markneukirchen, Germany).

2.3. Vagotomy. Upper abdominal wall was opened through a transverse incision. Esophagus was exposed by carefully keeping costal arc, liver, and stomach out of sight. Further preparation was done using a surgical microscope (40-times magnification, Leica M651, Bensheim, Germany). The ventral branch of the vagal nerve was exposed and about 3 mm were excised (see Figure 1). After its passage through the diaphragm, the esophagus was mobilized on its hepatic side and lifted. The dorsal branch of the vagal nerve was exposed and about 3–5 mm were resected. After fluid resuscitation (0.5 mL of sterile saline solution), the abdominal wall was closed (one layer; 4/0 Polyester, Catgut, Germany). For control purposes, sham operations without transsection of the vagal nerve were performed.

2.4. Implantation of Osmotic Pumps. Alzet osmotic pumps (Modell 1002, Alzet, Cupertino, USA) where filled with nicotine dissolved in 0.9% NaCl according to manufacturer instructions. With a liberation volume of 0.20–0.22 μL/h nicotine concentrations were adapted to the individual weight of each animal to ensure the expected liberation rate of 1, 1.5, and 3 mg/kg bodyweight (BW)/h. Control groups received osmotic pumps filled with 0.9% NaCl. After a short incision in the neck region of the mice, the primed pumps were placed in a subcutaneous pouch. Thereafter, the incision was closed (4/0 Polyester, Catgut, Germany).

2.5. Isolation of Kupffer Cells. The isolation procedure was modified from Valatas et al. [33]. All steps were performed with sterile solutions at pH = 7.4. The portal vein was identified, punctured with a 22-gauge cannula and irrigated by 50 mL pretempered HBSS. To allow the irrigation solution to evade the venous system, the inferior vena cava was incised right above its bifurcation. Afterwards, the liver was perfused with pronase E 0.4% for enzymatic digestion of hepatocytes and connective tissue and disintegration of cell junctions. A second enzyme mixture containing collagenase 0.0143% and DNAse 0.0014% was applied to dissolve the extracellular matrix and released DNA. The liver was transferred onto a petri dish, the capsule was cut and the parenchyma carefully fragmented before a third enzymatic digestion (using pronase E 0.1% and DNAse 0.01%) was performed under constant agitation. The cell suspension was pushed through a nylon mesh to remove larger cell aggregates. Subsequent steps were conducted with 4°C media to abate cell attachment on plastic surfaces.

A step of differential centrifugation followed (10 min, 380 g, 4°C) to wash out the residual enzymatic solution,

DNA, and cell debris. The supernatant was discarded, the pellet resuspended and density gradient centrifugation was performed to separate parenchymal from nonparenchymal cells. Iodixanol (OptiPrep) was applied as seperation medium. HBSS and OptiPrep were added to the cell suspension to gain 4 mL of a 11.7% OptiPrep solution which was bedded on a density cushion of 4 mL 17.6% OptiPrep. An additional 4 mL of HBSS was used as a top finishing. Density centrifugation was carried out at 1400 g and 4°C for 17 min. The resulting layer of mainly non-parenchymal cells on top of the 11.7% OptiPrep cushion was carefully removed and transferred into RPMI+ medium followed by another centrifugation step (10 min, 380 g, 4°C). The pellet was resuspended with 1 mL RPMI+.

Total number of non-parenchymal cells was assed using the Neubauer chamber. Adding the appropriate amount of RPMI+ a final cell concentration of 5×10^4/mL was generated. For cell culture a 96-well culture plate was used and 200 μL of cell suspension was added. Kupffer cell function was shown by phagocytosis of fluorescing latex beads (3 μm Fluoresbrite). Kupffer cell purity was analysed by cell adherence to glass slides and subsequent immunofluorescence staining with FITC-conjugated anti-F4/80 antibody. We reached purity of 79 percent. Cells were kept at 37°C and 5% CO_2-atmosphere. After 24 hours of incubation, cell media and all nonadherent cells were removed by thorough washing. All subsequent stimulation experiments were performed using FCS-free media.

2.6. Stimulation of Kupffer Cells. Kupffer cells were cultured in 96-well plates containing 1×10^4 cells per well in 200 μL cell culture medium (RPMI without FCS). After 24 hours, medium was changed and cells were stimulated with LPS (*E. coli*, Sigma-Aldrich Chemie, Taufkirchen) at concentrations of 0.1 μg/mL, 1 μg/mL and 10 μg/mL (same volume of medium in each well) dissolved in 1×PBS. After 24 hours, the supernatant was transferred to 1.5 mL Eppendorf tubes and centrifuged (10 min; 16100 g; 4°C; Centrifuge 5415R, Eppendorf, Germany). Cytokines/chemokines were analysed using a commercial available kit (BD cytometric bead array mouse inflammation kit, BD bioscience, Heidelberg, Germany).

2.7. MRI Imaging and Analysis. MRI was modified from Partecke et al. [34]. For all MRI studies, anaesthesia had to be carried out using isoflurane (1%–1.5%). The depth of anaesthesia was monitored by the breathing rate (about 40 breaths per minute). MRI sequences were triggered by breathing rate. To reduce the influence of bowel motility in all MRI examinations, mice were kept *nil per os* (NPO) for at least 4 hours before starting MRI scans. For MRI scans, all mice were placed in a supine position. Mice were scanned in a high-field 7.0 Tesla MRI scanner for small animals (Bruker, ClinScan, 7.0 Tesla, 290 mTesla/m gradient strength, Bruker, Ettlingen, Germany). MRI scans were performed in a whole mouse body coil (Bruker, Ettlingen, Germany) using a T2-TSE (turbo spin echo) sequence. For size and volume assessment, we used high resolution coronary and axial T2-weighted images (coronary plane: TR (repetition time): ca. 1200 ms; TE (echo time): 41.0 ms; FA (flip angle): 180°; FoV (field of view): 42 mm × 42 mm; matrix: 240 × 320; 24 slices of 0,7 mm per slice, acquisition time: ca. 15 min; axial plane: TR: ca. 1250 ms; TE: 41.0 ms; FA: 180°; FoV: 40 mm × 40 mm; matrix: 240 × 320; 24 slices of 0,7 mm per slice, acquisition time: ca. 10 min). Generated images were analyzed using MIPAV (medical imaging processing and visualisation, National Institutes of Health, Bethesda, MD, USA) and Image J (Image Processing and Analysis in Java, National Institutes of Health). By defining regions of interest (ROI) on each slice, the software was able to calculate volumes and diameters. This was finally done by a complex algorithm using all image inherent information including thickness of slices, resolution as well as size of ROIs.

2.8. Serum Corticosterone Levels. For the detection of serum corticosterone levels, we used a commercially available ELISA-kit (Corticosterone (Rat/Mouse) Elisa, DRG Instruments GmbH, Marburg, Germany) following customers instructions. Serum was separated by centrifugation of whole blood (10 min; 16100 g; Centrifuge 5415R, Eppendorf, Germany).

2.9. Survival Analysis. Survival of animals was observed for 240 hours after CASP induction.

2.10. Statistical Methods. Statistical analysis was performed using GraphPad Prism for Windows software (GraphPad Software, San Diego, CA, USA). Statistical differences in survival rates were assessed using log-rank test. Results from cytokine levels were analyzed using the two-tailed Mann-Whitney U test for nonparametric probes. A significance level of 0.05 was applied for all calculations.

3. Results

3.1. Vagotomy Increases the Mortality in Polymicrobial Sepsis. To analyse the influence of the vagus nerve on the mortality in polymicrobial sepsis, we compared the survival rate of mice in the following surgical procedures: CASP, vagotomy (VGX), CASP in combination with vagotomy. Sham-operation was performed in the control group. Sham-surgery as well as vagotomy did not change the survival rate of mice as both were 100% (Figure 2, $n = 10$ per group). Comparable to our recent data, the induction of a septic peritonitis by CASP significantly decreased the survival rate to 63.6% ($P = 0.025$, $n = 33$). The survival of the vagotomised CASP group (VGX + CASP, $n = 33$) was significantly decreased further to 35.3% ($P = 0.048$). Thus, the intact vagus function is essential for the survival in polymicrobial sepsis, whereas vagotomy in absence of a septic focus does not affect the survival rate.

3.2. The Effect of Enlarged Gastric Volume by Vagotomy Is Independent of the Presence of Sepsis. By visualization of the stomach in small animal 7-Tesla-MRI, we confirmed that vagotomy results in an increased gastric volume. Figure 3(a) displays the regular empty gastric volume (193 ± 9 mm^3) in

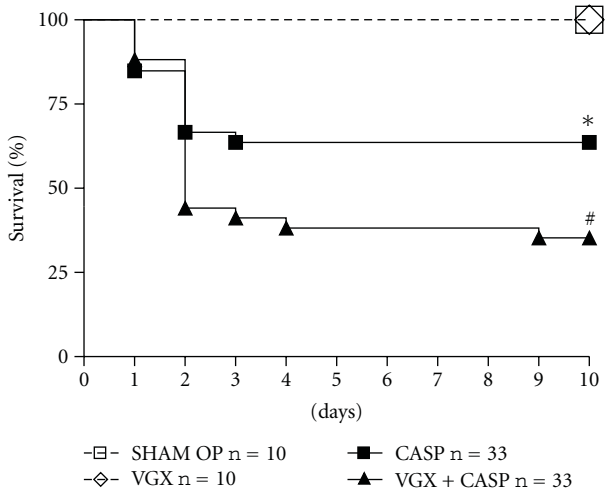

Figure 2: The mortality in polymicrobial sepsis (CASP) is significantly increased compared to sham-operation (laparotomy without CASP or vagotomy) by 63.6% versus 100% ($^*P = 0.025$, $n = 10$ and 33, resp.). The survival of the vagotomised CASP group (VGX + CASP, $n = 33$) is significantly decreased further to 35.3% (CASP versus VGX + CASP: $^{\#}P = 0.048$). Vagotomy itself ($n = 10$) does not affect the survival rate (100%).

untreated animals. Seven days following vagotomy, the volume was extended to $1064 \pm 71.7\ mm^3$. This difference is significant as shown in panel c ($n = 6$, $P < 0.0001$). In polymicrobial sepsis, the gastric volume was detected with $320.8 \pm 41.64\ mm^3$, which is a significant increase to the control group ($P < 0.05$) but a decrease as compared with the volume following the vagotomy procedure ($P < 0.0001$). Induction of peritonitis in vagotomised mice results in an enlarged gastric volume of $836.7 \pm 151\ mm^3$ at 24 hours postoperative. Vagotomy increases the gastric volume in the presence as well as in the absence of sepsis. These data suggest that the dilated intestine and the resulting ileus may not be the reason for the increased mortality in vagotomised septic mice, as these findings are also observed in septic mice (100% survival).

3.3. The Stimulation of Nicotinic Acetylcholine Receptors Has No Effect on the Survival in Polymicrobial Sepsis. The influence of a continuous application of nicotine on the survival in sepsis was analysed following implantation of osmotic pumps subcutaneously before induction of CASP. By this method, a detectable and dose-dependent serum level of cotinine, a metabolite of nicotine, can be reached (Figure 4(a)). The survival rate in septic peritonitis during continuous administration of nicotine in different bodyweight (BW)-adapted dosages was compared: 1 mg/kgBW/h, 1.5 mg/kgBW/h, and 3 mg/kgBW/h. In the control group the osmotic pumps released NaCl 0.9%, and the survival rate was detected by 9.67% (Figure 4(b), $n = 31$). A dosage of 1 mg/kgBW/h correlates with a survival of 20% ($n = 10$), 1.5 mg/kgBW/h ($n = 32$) leads to a survival of 21.85% and 3 mg/kgBW/h to a survival of 12.5% ($n = 8$). These data suggest that a systemic application of the unspecific nicotinic acetylcholine receptor agonist nicotine has no protective effect on the outcome of septic peritonitis.

3.4. Serum Corticosterone Levels in Sepsis Are Significantly Elevated in Vagotomised Mice. Due to the central role assigned to adrenal glands in peritonitis [13–15, 35], we focused on the influence of the vagus nerve on the adrenal gland function in polymicrobial sepsis (Figure 5). Vagotomy in the nonseptic organism did not influence the corticosterone level (205.9 ± 56.75 ng/mL) as compared to a control group. In polymicrobial sepsis, the serum corticosterone was detected with 159.4 ± 53.24 ng/mL. In contrast, 24 h following CASP in vagotomised mice the corticosterone level is significantly increased up to 975.4 ± 261.9 ng/mL ($^*P = 0.031$, $n = 5$). This indicates that the serum levels of corticosterone in sepsis are modulated by the vagus nerve.

3.5. The Vagus Nerve Has a Stimulating Effect on the EX Vivo Cytokine Release of Kupffer Cells. Kupffer cells were isolated seven days following vagotomy. The levels of TNF-α were detected in the cell culture supernatant. Kupffer cells isolated from untreated animals served as control. The basal TNF-α release of Kupffer cells *ex vivo* was significantly decreased as compared with the control group (164.7 ± 40.9 pg/mL versus 61.1 ± 4.4 pg/mL, $^*P = 0.04$). Furthermore, we stimulated Kupffer cells *ex vivo* with 1 μg/mL lipopolysaccharide from *E. coli* (LPS). In the septic organism, there was a significantly decreased TNF-α release by stimulated Kupffer cells from vagotomised mice (2960 ± 513.1 pg/mL) when compared to mice with an intact vagus nerve (5746 ± 292.5 pg/mL, $^{***}P = 0.0002$, $n = 10$ per group). These data substantiate the hypothesis that the vagus nerve has an immunological influence on Kupffer cell function.

4. Discussion

The present study underlines the crucial role of the vagus nerve for the survival in septic peritonitis. Impaired vagal function results in increased mortality rates in inflammatory animal models (CASP) [7]. Stimulation of nervus vagus function ameliorates survival as described for different animal models like CLP, LPS application, or i.p. *E. coli* injection [1, 6, 36]. Additionally, the vagus nerve has strong influence on the intestinal tonus: increased ileus incidence is described following vagotomy [37], whereas vagus stimulation attenuates the postoperative ileus [38].

Surgical trauma or peritoneal inflammation can result in paralysis and consecutive ileus, too. It could therefore be possible that the ileus triggered by vagotomy is the critical factor of decreased survival rate in CASP following vagotomy. Vagotomy itself causes an increased pylorus tone with delayed gastric emptying (DGE) as we could describe by measuring gastric volumes in MRI scans (Figure 3(a)). Vagotomy without induction of sepsis significantly enlarged gastric volume when compared to CASP mice. In combination of both, Vagotomy and CASP, the vagotomy effect seems to stimulate ileus by sepsis. Our data on postoperative mortality suggest an unchanged survival rate in vagus

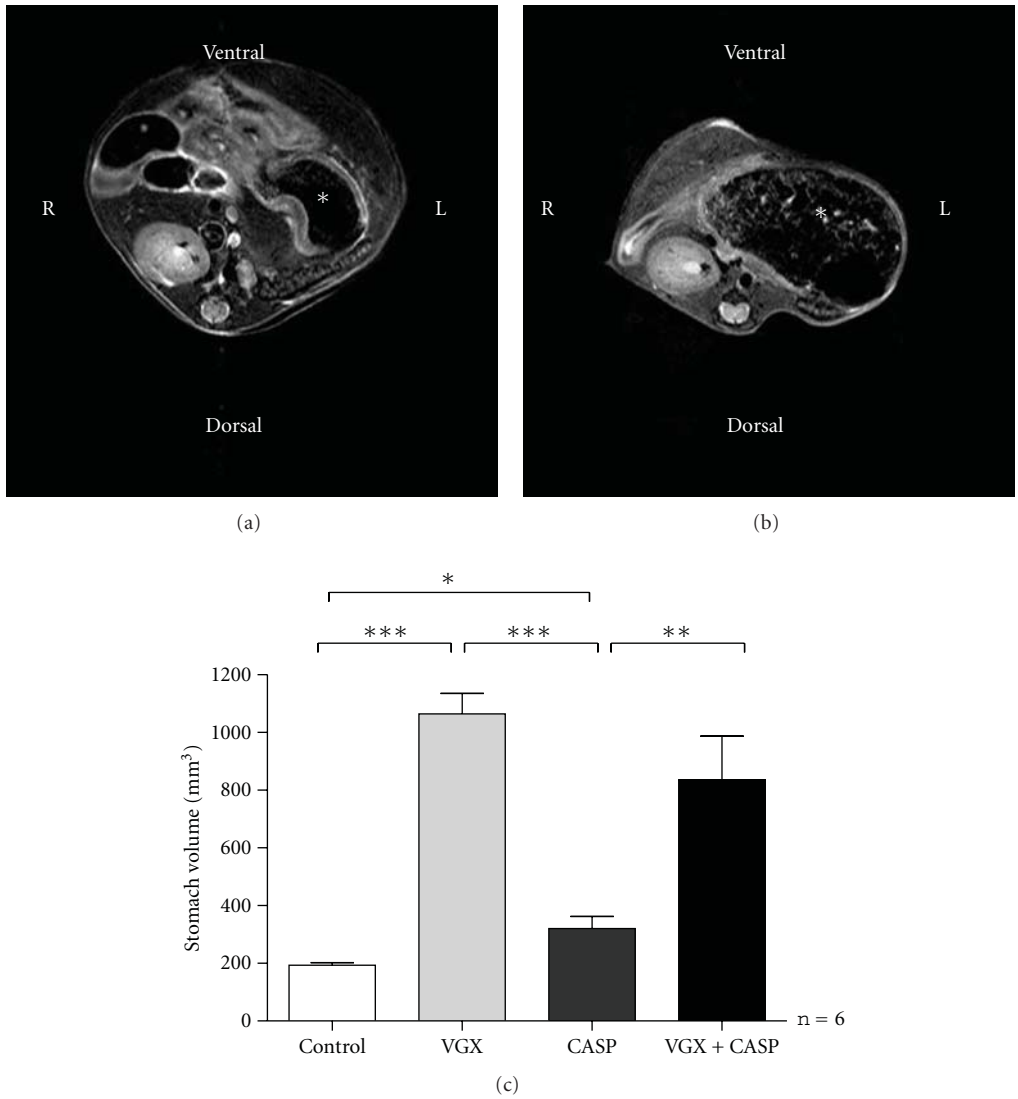

Figure 3: Images of MRI of the upper abdomen of mice. The asterisk identifies the stomach. Panel a shows the sectional image of an untreated mouse with normal gastric volume (a). Panel b displays the representative scan of a mouse with increased gastric volume after CASP and vagotomy (b). Values of gastric volume are shown in panel c. CASP alone led to a slight but significant increase of gastric volume. In both vagotomy groups, a strong dilatation of the stomach was measured that was independent from the presence of sepsis (c). ($n = 6$ per group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$).

dissected animals (Figure 2). Surgical sectioning of the vagus nerve per se is performed in procedures like gastrectomy or oesophagectomy and itself does not result in elevated mortality rates [39–42]. Therefore, vagal impairment is responsible for marked DGE and ileus but not for higher mortality.

Vagotomy results in an attenuated release of nicotine in efferent signaling. The role of nicotine in sepsis, especially its possible therapeutic effect, and stimulation of the vagus nerve were subject of several studies: The et al. described reduced experimental postoperative Ileus [43] using a central stimulus for the cholinergic pathway. Other studies define better survival in CLP and LPS models using nicotine as an unspecific stimulator of nACHR [1, 36]. In our experiments, we decided for continuous nicotine administration by subcutaneously implanted osmotic pumps, since in our opinion permanent administration ensures sufficient serum levels, especially due to the short half life of nicotine in C57Bl/6 mice (about 9 minutes) [44]. We reached adequate doses as demonstrated by the serum level of the nicotine metabolite cotinine (Figure 4(a)). We observed no change in survival rates by administration of nicotine (Figure 4(b)). This observation may attribute to our CASP model which in contrast to CLP or LPS models is associated with a very high bacterial load [30, 32]. Our finding of worse survival correlates with Westerloo et al. who administered living *E. coli* i.p. in mice [6]. They also detected even decreased survival after oral nicotine substitution. Action potentials transmitted in the vagus nerve lead to release of acetylcholine that blocks cytokine production by cells-expressing

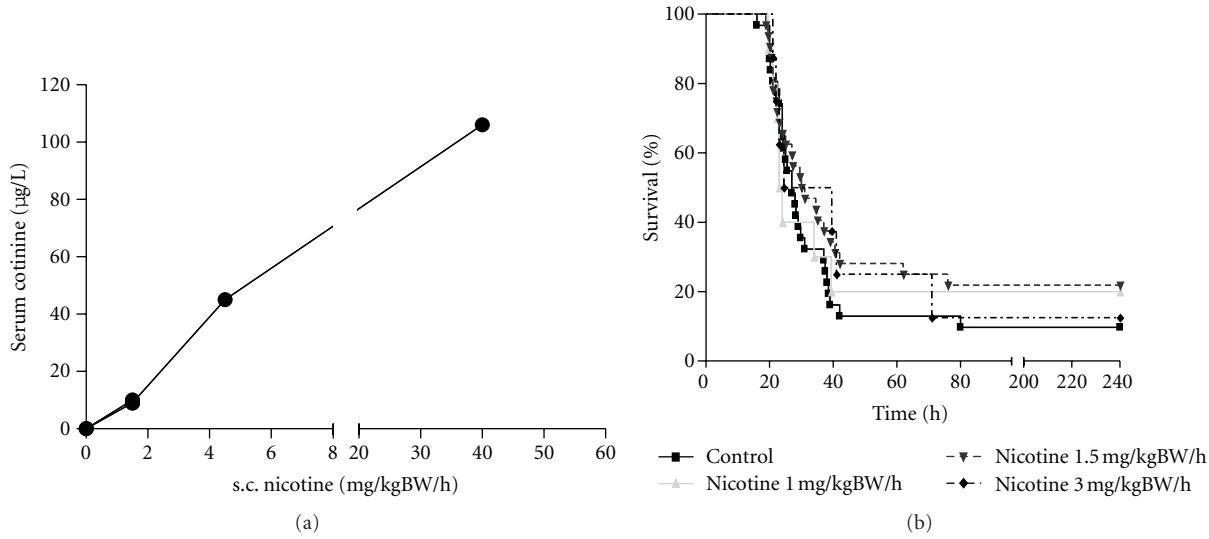

Figure 4: (a) To verify the therapeutic dose of nicotine in the murine serum, we analysed the serum cotinine level as this correlates with the amount of nicotine dose applicated by osmotic pumps. Application of nicotine in a dose of 1.5 mg/kgBW/h results in a serum cotinine level of 9.24 μg/l ($n = 4$). 4.5 mg/kgBW/h nicotine leads to a cotinine serum concentration of 45 μg/l ($n = 1$) and 40 mg/kgBW/h nicotine induces a cotinine serum concentration of 106 μg/l ($n = 1$). Serum samples were analysed 18 hours following CASP. (b) The survival in CASP is not altered by continuous nicotine administration through subcutaneously implanted osmotic pumps. A nicotine exposition of 1 mg/kg bw per hour correlates with a survival rate of 20% ($n = 10$), 1.5 mg/kgBW/h ($n = 32$) nicotine leads to a survival rate of 21.85% and 3 mg/kgBW/h nicotine correlates with a survival rate of 12,5% ($n = 8$). In the control group, NaCl was applied where the survival rate was detected with 9.76% ($n = 31$).

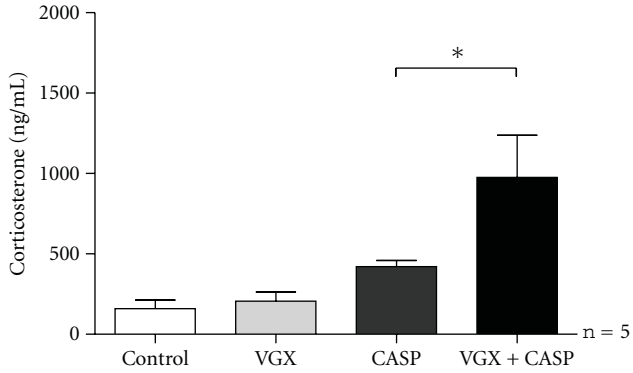

Figure 5: The serum levels of corticosterone in sepsis are modulated by the vagus nerve. Vagotomy in the nonseptic organism does not influence the corticosterone level (205.9 ± 56.75 ng/mL). In polymicrobial sepsis, serum corticosterone is detected with 159.4 ± 53.24 ng/mL. In contrast, 24 h following CASP in vagotomised mice the corticosterone level is significantly increased up to 975.4 ± 261.9 ng/mL (*$P = 0.031$, Mann-Whitney, $n = 5$).

acetylcholine receptors. In particular, the efferent vagus can inhibit inflammation via interaction between acetylcholine and $\alpha 7$ subunit of cholinergic receptors [45]. Signal transduction by the nicotinic $\alpha 7$ cholinergic receptor subunit is the regulator of intracellular signals that control cytokine transcription and translation. Neutrophils expressing several nicotinic receptors, including the $\alpha 7$ cholinergic receptor [23], and stimulation of these receptors have been shown to inhibit neutrophil migration by a mechanism that involves inhibition of adhesion molecule expression on both the endothelial surface and neutrophils [23]. Mice deficient in $\alpha 7$ cholinergic subunit have an optimized bacterial clearance caused by a faster recruitment of neutrophils [46]. As the early recruitment of neutrophils to the site of an infection is considered important for an adequate antibacterial defense our present results are consistent with reports showing that nicotine (which stimulates $\alpha 7$ receptors) facilitates the growth and dissemination of *E. coli* after intraperitoneal infection [6]–the protective effect of nicotine administration seems to be not potent enough in case of massive living pathogen load.

Another advice for higher mortality in polymicrobial sepsis are the significantly increased corticosterone levels in vagotomized mice (Figure 5). Especially human cortisol has several anti-inflammatory and immunosuppressive effects, that is, reduced TNF-alpha, increased IL-10-concentrations and apoptosis of mature T-lymphocytes [16, 47]. Adrenal insufficiency is frequently diagnosed in critical ill patients with sepsis [48] and it is associated with a high mortality rate [13–15]. A glucocorticoide administration during human sepsis was a discussed controversial [17, 18]. In rodents cortisol is replaced by corticosterone because of lack of C17-hydroxylase function [49, 50], in many ways [51]. We found increased corticosterone levels in septic animals that had undergone subdiaphragmal vagotomy seven days before CASP procedure (Figure 5). CASP mice with intact vagus nerve had a moderate but not significant elevation of serum corticosterone, whereas vagotomy alone had no effect on serum corticosterone levels.

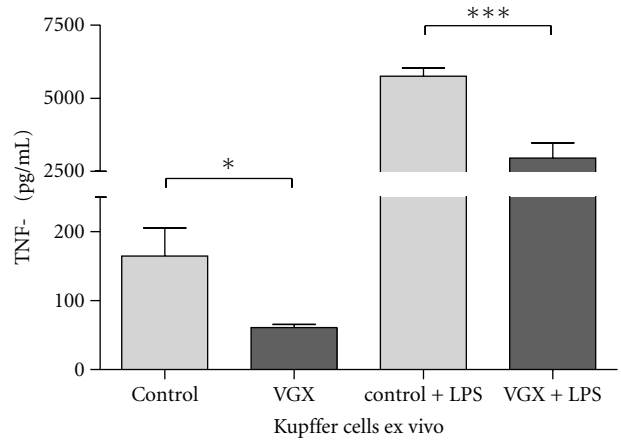

Figure 6: The vagus nerve has a stimulating effect on kupffer cells. Kupffer cells were isolated from control mice (column "control") and from vagotomised mice without sepsis (column "VGX"). The basal TNF release of kupffer cells *ex vivo* is significantly decreased if mice were vagotomised 7 days before cell isolation (164.7 ± 40.9 pg/mL versus 61.1 ± 4.4 pg/mL, $^{*}P = 0.04$). In addition, we stimulated the cells *ex vivo* with 1 μg/mL lipopolysaccharide from *E.coli* (LPS). In the septic organism, there is a significantly decreased TNF-α release by stimulated kupffer cells from vagotomised mice (2960 ± 513.1 pg/mL) when compared to mice with an intact nervus vagus (5746 ± 292.5 pg/mL, $^{***}P = 0.0002$, $n = 10$ per group).

To compensate for vagotomy, the endocrine axis not only seems to develop a stronger countereffect, as reflected by the corticosterone response [52]: Our data support the hypothesis of a relevant connection between vagus nerve and adrenal glands [10–12, 53] and suggest a vagal regulative function concerning hypoinflammation during sepsis via corticosterone.

In the course of sepsis immune system alternates between hyper- and hypo-inflammation. Increased proinflammatory TNF-levels and compensatory anti-inflammation with increased IL-10 levels contribute to immune paralysis status [54–56]. This effect seems to be dependent of vital pathogens, corticosterone responses were not affected in LPS models evaluating effects of vagotomy by Hansen et al. [57] or Gaykema et al. [58].

Furthermore, our data from in vitro experiments with Kupffer cells (KCs) indicate in the same line. KCs taken from vagotomized mice secreted reduced TNF-alpha amount after LPS stimulus (Figure 6).

Kupffer-cells are tissue macrophages located in the liver [27]. They liberate cytokines like TNF-alpha, IL-1, IL-6, and IL-10 and chemokines such as MCP-1 during inflammation [28]. Ikeda et al. stimulated Kupffer cells with acetylcholine and detected increased IL-6 secretion rates [23]. In our model, we performed subdiaphragmal vagotomy to dissect the established anatomical link by the hepatic branch of the vagus nerve [25, 26]. Our data correlate with Ikedas studies on cytokine release: *ex vivo* LPS stimulation induces a high level of TNF-alpha secretion. This LPS effect is attenuated in KCs isolated from animals that had undergone vagotomy seven days before (Figure 6). Basal rate (without LPS) of TNF-alpha secretion is significantly lower in vagotomy group. This underlines that the vagus nerve has a stimulating effect on the Kupffer cell activity. Following vagotomy, these macrophages are inhibited in their immunologic function, so the organism is impaired in its ability to cope with the septic situation. Their sensitivity seems to be downregulated resulting in less release of proinflammatory cytokines.

In summary, past studies have shown that the vagus nerve controls the immune response of hyperinflammation. Yet, our studies also suggest that the vagus nerve controls both stimulation as well as inhibition of inflammatory responses to severe bacterial threats. A high load of pathogens has unmasked the effects of the vagus nerve on states of hypoinflammation in our CASP model. Future studies will show the impact of further therapeutic modulation.

5. Conclusion

The vagal nerve plays an important role during peritonitis and leads to increased sepsis mortality after vagotomy. A stimulation of cholinergic receptors by nicotine has no therapeutic effect. Increased septic mortality seems to be a consequence of the absent modulating influence of the vagus nerve on the immune system. To underline this hypothesis we detect significantly elevated serum corticosterone levels in vagotomised mice 24 h following CASP and a decreased *ex vivo* TNF-alpha secretion of Kupffer cells upon stimulation with LPS. The recent study suggests that the vagus nerve controls both stimulation as well as inhibition of inflammatory responses to severe bacterial threats.

Author's Contribution

W. Kessler and S. Diedrich contributed equally to this paper.

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Research Article

Superoxide Dismutase 3 Limits Collagen-Induced Arthritis in the Absence of Phagocyte Oxidative Burst

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Extracellular superoxide dismutase (SOD3), an enzyme mediating dismutation of superoxide into hydrogen peroxide, has been shown to reduce inflammation by inhibiting macrophage migration into injured tissues. In inflamed tissues, superoxide is produced by the phagocytic NOX2 complex, which consists of the catalytic subunit NOX2 and several regulatory subunits (e.g., NCF1). To analyze whether SOD3 can regulate inflammation in the absence of functional NOX2 complex, we injected an adenoviral vector overexpressing SOD3 directly into the arthritic paws of *Ncf1*$^{*/*}$ mice with collagen-induced arthritis. SOD3 reduced arthritis severity in both oxidative burst-deficient *Ncf1*$^{*/*}$ mice and also in wild-type mice. The NOX2 complex independent anti-inflammatory effect of SOD3 was further characterized in peritonitis, and SOD3 was found to reduce macrophage infiltration independently of NOX2 complex functionality. We conclude that the SOD3-mediated anti-inflammatory effect on arthritis and peritonitis operates independently of NOX2 complex derived oxidative burst.

1. Introduction

Extracellular superoxide dismutase (SOD3) is an enzyme known to catalyze dismutation of the highly reactive, superoxide anion into longer-lived and more stable hydrogen peroxide [1]. The consequences of SOD3 action in the cells reach beyond the antioxidative functions, as it has been shown to downregulate inflammation [2], stimulate cell proliferation during tissue injury recovery [3], and to counteract apoptosis [4, 5] by affecting cytokine production, cell signal transduction, and expression of survival-related genes.

The anti-inflammatory properties of SOD3 have been studied in models of pulmonary disease and peritonitis [2, 6]. Previous work with collagen-induced arthritis (CIA) suggests that both genetic transfer of the SOD3 gene [7] as well as a small molecular SOD mimetic have the ability to ameliorate arthritis [8]. The arthritis ameliorating effect of SOD3 was later confirmed using SOD3 knock-out mice [9]. These results were explained on the basis that SOD3 acts as an antioxidant and catalyses dismutation of superoxide into hydrogen peroxide, thus reducing inflammation-induced oxidative stress and restoring the oxidant balance in the arthritic joints [10, 11]. This explanation, however, is difficult to reconcile with the finding that animals naturally deficient in the induced oxidative burst in fact develop more severe arthritis [12, 13].

The most potent producer of superoxide, the substrate for SOD3, is the well-characterized phagocytic NOX2 complex. In inflamed tissues NOX2 complex produces massive amounts of superoxide upon activation in a process called phagocyte oxidative burst. In addition to NOX2, superoxide is produced from various other cellular sources, such as from the mitochondria during cellular respiration and by other members of the NOX enzyme family [14]. However, it

should be noted that during inflammation these superoxide producers are not nearly as efficient superoxide producers as the NOX2 complex.

In the current work we studied the role of SOD3 in collagen-induced arthritis (CIA) to understand whether the therapeutic effect of SOD3 on arthritis operates through attenuating the biological effects of the induced oxidative burst produced by the NOX2 complex. To avoid artifacts introduced by chemical inhibitors of NOX2 complex [14–16] we used mice that genetically lack functional NOX2 complex. Thus we used wild-type ($Ncf1^{+/+}$) and *Ncf1* mutated ($Ncf1^{*/*}$) mice on B10.Q background. These strains differ at only one SNP in the *Ncf1* gene, which makes the $Ncf1^{*/*}$ strain unable to produce oxidative burst [13]. The mutated mouse is more susceptible to induced arthritis due to hyperactivated T cells [13], and also increased susceptibility to thioglycollate peritonitis has been reported in the *Ncf1* knockout mouse [17]. Our results confirm the previously documented anti-inflammatory role of SOD3 and additionally, for the first time, we show that it can downregulate both CIA and peritonitis even in the absence of functional NOX2 complex and phagocyte oxidative burst.

2. Materials and Methods

2.1. Mice. The previously described $Ncf1^{m1J}$ (protein also called *p47phox*) mouse [18], which carries a point mutation globally and completely abolishing NOX2 complex derived ROS production, has been backcrossed onto the B10.Q background [13] and shown to contain only the causative mutation using a 10 k SNP typing chip. The mice were housed under specific pathogen-free conditions in climate-controlled environment and fed standard rodent chow and water ad libitum at Turku University Central Animal Facility. All experimental mice were sex- and age-matched, treatment groups were blinded, and experimental groups were mixed in cages in all experiments. The experiments were performed in accordance with the national and EU guidelines and the study was approved by the Oulu section of the national Animal Experiment Board (Eläinkoelautakunta, ELLA) with ethical approval numbers ESLH-2008-02873, ESLH-2008-07941, and ESAVI-0000497/041003/2011.

2.2. Viral Expression Vectors Used in the Study. Replication deficient adenoviral E1-partially-E3-deleted AdBglII vectors (developed from serotype Ad5) expressing rabbit SOD3 (Ade-SOD3) or bacterial β-galactosidase lacZ (Ade-LacZ) [4] were used in both in vitro and in vivo experiments.

2.3. Collagen-Induced Arthritis. Collagen-induced arthritis (CIA) was induced under isoflurane anesthesia by injecting 100 μg rat type II collagen (purified from chondrosarcoma) [19] emulsified in complete Freund's adjuvant intradermally at the base of the tail. Arthritis was boosted day 19 with 50 μg rat type II collagen emulsified in incomplete Freund's adjuvant intradermally at the base of the tail. Disease development was evaluated macroscopically three times a week before the booster immunization and daily after the boost [20]. One point was given for each swollen toe or joint and five points for a swollen ankle, each paw having the maximum of fifteen points.

In the arthritis experiments Ade-SOD3, Ade-LacZ (vector control), and PBS (injection control) were injected locally in the left front paw in 25 μL injection volume containing 2,5 $\times$ 10^8 PFU virus. Injections were performed right after the booster immunization during the same anesthesia at day 19, before the onset of clinically apparent arthritis.

2.4. Peritonitis. Peritonitis experiments were performed as described in [2]. Briefly, mice were pretreated i.p. with 0.5 $\times$ 10^9 PFU Ade-SOD3, Ade-LacZ, or PBS three days before peritonitis induction with 5% proteose peptone (BD Difco, Sparks, MD, USA) and 10 ng IL-1β (R&D Systems, Minneapolis, MN, USA) in 1 mL PBS. After 18 hours peritoneal infiltrating cells were collected with 10 mL ice-cold RPMI cell culture medium. Cells from the peritoneal lavage were counted and cytocentrifuged, slides were stained with Reastain Diff-Quick (Reagena, Toivala, Finland), and differential counting was performed under a standard light microscope. Experiments were pooled and the total cell numbers are presented as percentual increase from the PBS injection control. All peritonitis results were normalized to adjust the vector control group (Ade-LacZ) mean to 100.

2.5. Cell Culture and In Vitro Extracellular Oxidative Burst. COS-7 cells stably expressing all the essential components of the NOX2 complex, namely, Cybb (gp91phox), Cyba (p22phox), Ncf2 (p67phox), and Ncf1 (p47phox) were provided by Dr. Mary C. Dinauer, Indiana University, USA [21]. Cells were cultured in Dulbecco's complete medium (Gibco), 10% fetal calf serum, and penicillin-streptomycin (Invitrogen, Paisley UK).

Extracellular superoxide production was quantified two days after transduction (MOI 4) with adenoviral constructs (Ade-SOD3 and Ade-LacZ) or medium control directly on the 96-well cell culture plate using an isoluminol-enhanced chemiluminescence method [22, 23]. Briefly, the cells were washed with PBS, and 100 μL of isoluminol buffer was added in each well. Isoluminol buffer contained isoluminol (10 μg/mL, Sigma-Aldrich) and horse radish peroxidase-type II (4 U/mL, Sigma-Aldrich) dissolved in PBS with PMA (200 ng/mL, dissolved in DMSO, Sigma-Aldrich) and data collection was initiated immediately and followed at 37°C as produced luminescence signal (Tecan Infinite M200, Tecan, Männedorf, Switzerland) for 30 minutes. DMSO vehicle controls represent the nonstimulated background values. Representative data from the 15-min time point is reported.

2.6. In Vitro Intracellular Oxidative Burst. Red blood cells were lysed from heparinized whole blood with hypotonic lysis buffer and leukocytes were surface stained with APC conjugated anti-Gr-1 (RB6-8C5) and eFluor 450 conjugated anti-CD11b (M1/70) antibodies (eBioscience). Cells were suspended in high-glucose D-MEM (Gibco) with antibiotics without FCS and incubated for 10 min at 37°C with 3 μM dihydro-rhodamine 123 (DHR-123; Molecular Probes and

Invitrogen Life Technologies) followed by 20 min activation at 37°C with 200 ng/mL PMA (Sigma-Aldrich). After oxidation by ROS, DHR-123 emits fluorescence upon excitation with the blue laser. The cells were washed into PBS and acquired on LSR II flow cytometer equipped with FACS Diva software (BD Biosciences). Live cells were gated on the cell type, and geometric means of respective populations were analyzed with Flowing Software (Cell Imaging Core, University of Turku).

2.7. In Vivo Oxidative Burst. Isoflurane anesthetized animals with equal arthritis scores were injected intraperitoneally with 20 mg/kg L-012 probe (Wako Chemicals, Germany) dissolved in PBS [24]. The luminescent signal was detected with IVIS 50 bioluminescent system (Xenogen, USA) that consists of an anesthesia unit built in a light tight chamber equipped with a CCD camera. Image acquisition and analysis were performed with Living Image software (Xenogen).

2.8. Detection of Ade-SOD3 from the Injected Paws by RT-PCR. RNA was isolated from the treated paws collected d25 according to the manufacturer's instructions with TRI Reagent (Sigma-Aldrich). Glass homogenizers were used to homogenize and separate the soft tissue from the bone. The isolated RNA was DNAse treated with deoxyribonuclease I (Fermentas) in the presence of Ribolock RNase Inhibitor (Fermentas). The RNA was used for reverse transcription reaction performed with Revert-Aid M-MULV (Fermentas). The acquired cDNA was subjected to Q-PCR with Ade-SOD3 (fw: GTG TGC TCC TGC CTG CTC, rev: CTG CTC CAC CGT GTC TGA G) and β-actin specific primers (fw: CTA AGG CCA ACC GTG AAA AG, rev: ACC AGA GGC ATA CAG GGA CA), and the gene expression level was analyzed using SYBR Green PCR Master Mix (Applied Biosystems), iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad), and the iCycler (version 3.1) software. Ade-SOD signal was normalized against β-actin expression levels and the results are reported as fold change from the Ade-LacZ group mean denoted as 1.

2.9. Statistics. Quantitative data are expressed as ± SEM. Statistical significance was analyzed by using two-tailed Student's t-test or if more than two groups were analyzed one-way ANOVA with LSD post hoc analysis was run using IBM SPSS Statistics 19 software (SPSS Inc.), $P < 0.05$ is considered as statistically significant.

3. Results

3.1. Ade-SOD3 Produced Enzymatically Active SOD3 In Vitro. Adenoviral SOD3 gene delivery reduced the superoxide predominant ROS signal 37% compared to the Ade-LacZ control virus when investigated two days after transduction with Ade-SOD3 and Ade-LacZ viruses (Figure 1).

3.2. SOD3 Downregulated Arthritis. Mice started to develop mild, clinically apparent arthritis at day 20 and made a full response, mean arthritis scores reaching 7 (out of the maximum of 15) in Ade-Laz treated and 3 points in Ade-SOD3-treated mice some days later. The mean disease score in the treated paws was lower in the Ade-SOD3-treated group when compared to the Ade-LacZ-injected control group, and the difference reached statistical significance at d26 (Figure 2(a)). The treatment effect was only seen in the treated paw, while there were no differences between the treatment groups in sum scores of the three untreated paws (see Supplementary Figure 1(a) in supplementary material available online at doi: 10.1155/2012/730469) highlighting the local character of the used gene therapy vector expressing SOD3.

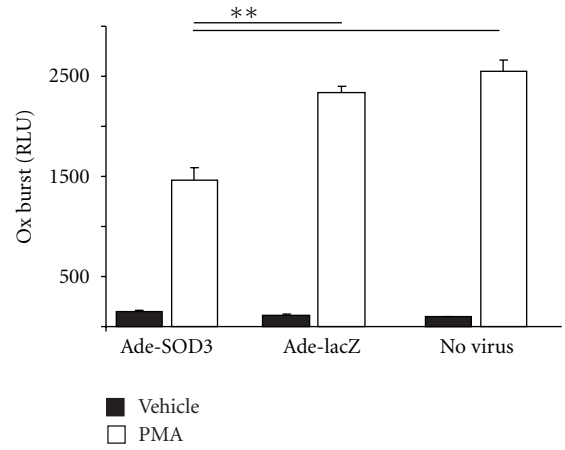

Figure 1: The amount of extracellular ROS is reduced by SOD3. Adenoviral vector coding for SOD3 (Ade-SOD3) significantly reduced the amount of extracellular ROS produced by COS cells expressing all functional components of the NOX2 complex, when compared to the Ade-LacZ control virus.

Both virus vector treated groups showed elevated arthritis scores in the treated paws when compared to the PBS-injected control paws in the injection control group. This difference is illustrated in Figure 2(b), where Ade-LacZ-treated paws are shown to have larger increase in arthritis score than the Ade-SOD3-treated paws.

3.3. SOD3 Downregulated Arthritis in the Absence of Functional NOX2 Complex. Starting from d20 the *Ncf1*$^{*/*}$ mice started to develop arthritis with significantly higher mean score than the wild-type mice. Interestingly, the mutated mice without functional NOX2 complex derived superoxide production also responded to Ade-SOD3 treatment. Ade-SOD3-treated paws of the *Ncf1*$^{*/*}$ mice had significantly lower mean disease score than the Ade-LacZ-treated vector control paws at days 24 and 25 after arthritis induction. Similarly to the wild-type mice, the difference in the mean arthritis score in NOX2 complex deficient mice was observed when SOD3 was highly expressed from the adenoviral vector (Figure 3(a)) [4]. No differences were observed between the treatment groups in the sum scores of the untreated paws (Supplementary Figure 1(b)) again supporting the local character of the immunomodulatory effect of Ade-SOD3 treatment.

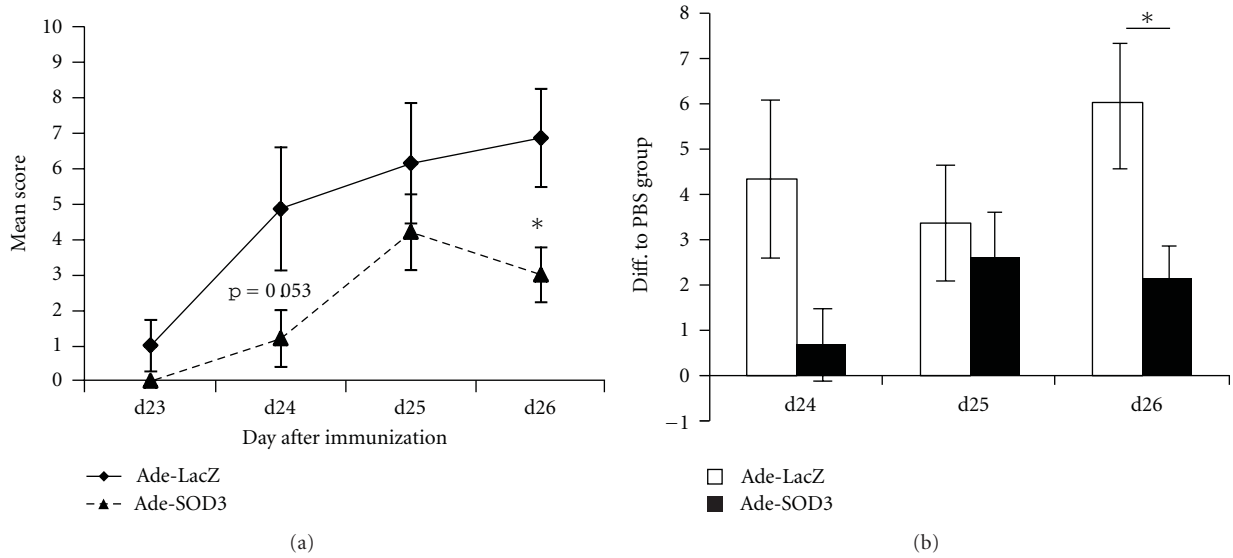

Figure 2: SOD3 downregulated arthritis in wild-type B10.Q mice. Adenovirally expressed SOD3 reduced arthritis severity in the treated paws (a) in wild-type B10.Q mice (Ade-SOD3 $n = 6$, Ade-lacZ $n = 7$). When compared to the PBS-treated injection control ($n = 8$) group, Ade-SOD3-treated mice had less increase in the arthritis score in the treated paw than the Ade-LacZ-treated control mice (b).

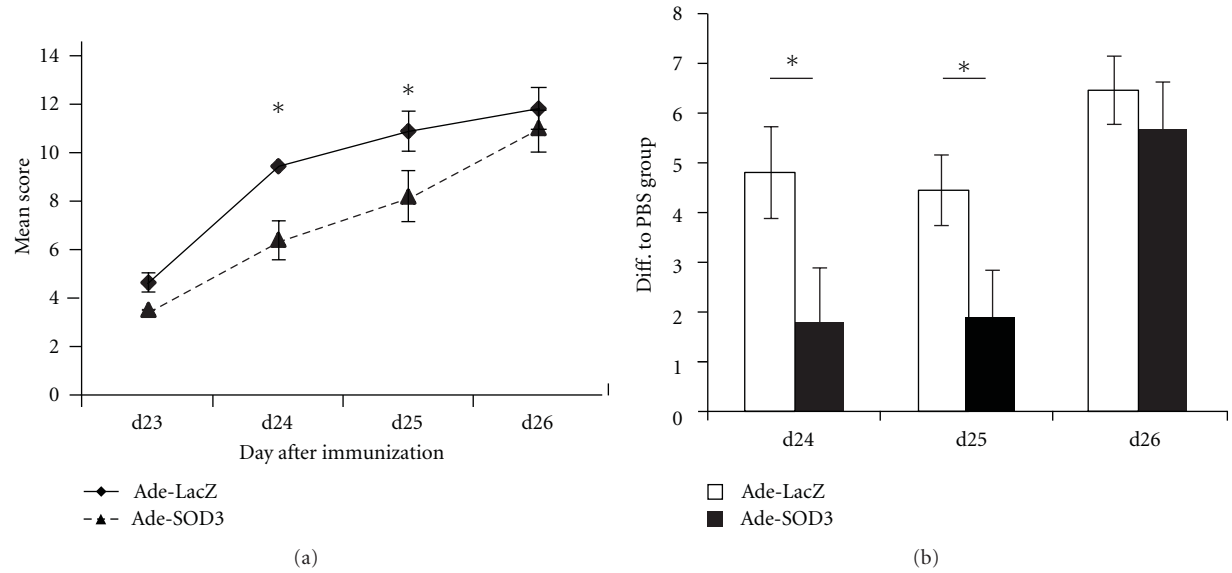

Figure 3: SOD3 downregulated arthritis in the absence of functional NOX2 complex. Arthritis severity was significantly reduced in Ade-SOD3-treated paws of mice lacking functional oxidative burst when compared to the control vector-treated paws (a). Shown is pooled data from two independent experiments (Ade-SOD3 $n = 22$, Ade-lacZ $n = 23$). The difference in arthritis score in the treated paw between the PBS-treated control mice ($n = 10$) and Ade-SOD3-treated mice was smaller than the difference between PBS-treated and Ade-LacZ-treated mice at days 24 and 25 (b).

Similarly to the wild-type mice, Ade-SOD3 treatment in *Ncf1*$^{*/*}$ paws induced significantly smaller difference between the virus-treated and PBS-injected paws than Ade-LacZ virus injection (significant d24 and d25 after immunization), again confirming the arthritis limiting effect of SOD3 (Figure 3(b)). The experiment was repeated and as the experiments were well reproduced, data from both experiments was combined for analysis.

3.4. SOD3 Limited Peritonitis Both in the Presence and Absence of Phagocyte Oxidative Burst. SOD3 expression significantly reduced the number of peritoneal infiltrating cells in proteose peptone and IL-1β induced peritonitis in wild type mice (Figure 4(a)). The decrease in infiltrating leukocytes was mainly due to a lowered number of infiltrating macrophages (Figure 4(b)), which well corresponds with the macrophage phase of peritonitis taking place three days

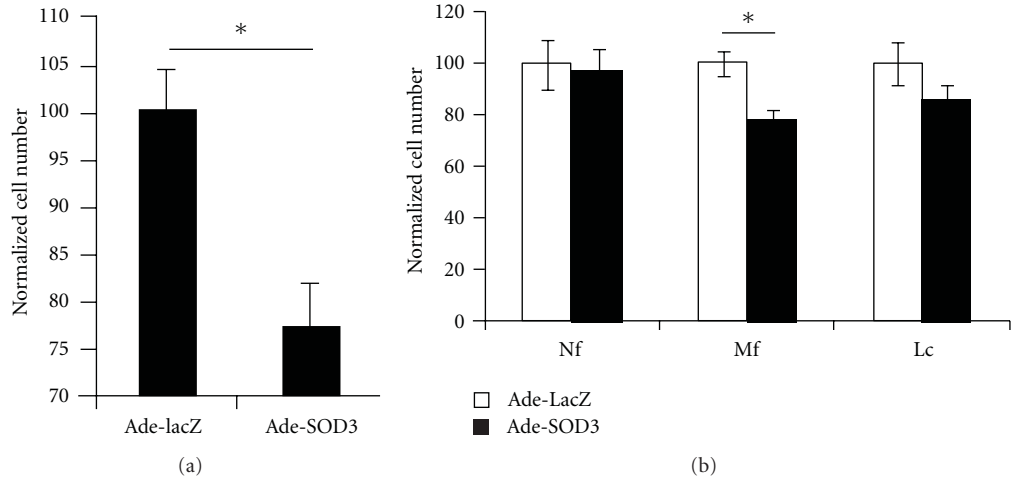

Figure 4: Peritonitis is limited by SOD3. Ade-SOD3-treated mice had less peritoneal infiltrating cells after peritonitis induction with proteose peptone and IL-1β than Ade-LacZ-treated control mice (a) ($n = 5$). The reduction in cell number was mainly due to diminished peritoneal macrophage population (b) (Nf = neutrophil, Mf = macrophage, Lc = lymphocyte).

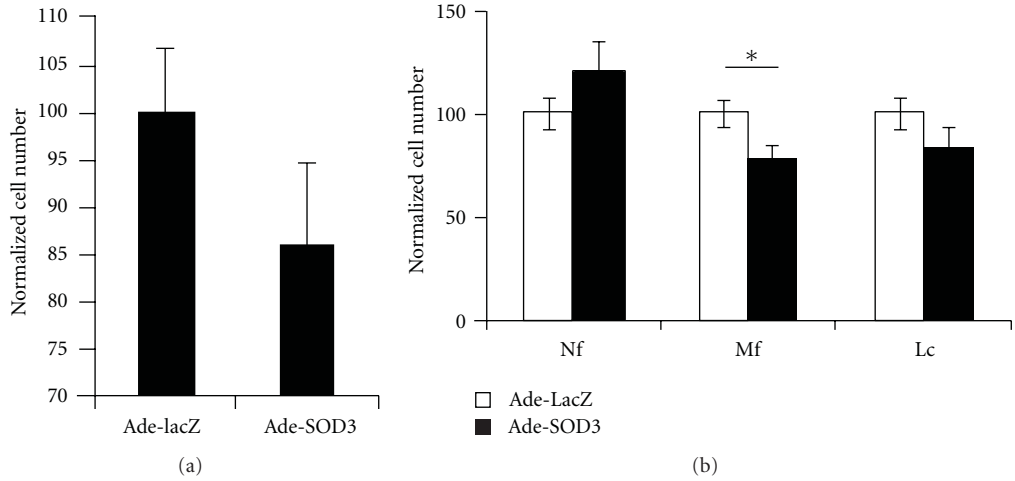

Figure 5: Peritonitis is limited by SOD3 in the absence of functional NOX2 complex. Similarly to wild-type mice, Ade-SOD3-treated *Ncf1*$^{*/*}$ mice had less peritoneal infiltrating macrophages than the Ade-LacZ-treated vector controls in the proteose peptone and IL-1β-induced peritonitis model (a). Normalized total cell numbers (b) show significant decrease in infiltrating macrophage population. Shown is pooled data from two independent experiments ($n = 10$) (Nf = neutrophil, Mf = macrophage, Lc = lymphocyte).

after virus injection and 18 hours after peritonitis induction. Both virus-treated groups had more infiltrating cells than the PBS-injected control mice.

Similarly as in the wild-type mice Ade-SOD3 was shown to reduce the number of infiltrating cells in the peritoneal cavity of *Ncf1*$^{*/*}$ mice when compared to Ade-LacZ-treated mice. The difference was due to a significantly lowered number of infiltrating macrophages in the Ade-SOD3-treated mice (Figures 5(a) and 5(b)).

3.5. Ncf1$^{*/*}$ *Mice Lack Oxidative Burst In Vivo and In Vitro.* In the wild-type mouse both severely inflamed paws (right hind leg and right front paw) emitted strong ROS-induced L-012 luminescence signal, while in spite of the severe inflammation, no luminescent signal was detected from the *Ncf1*$^{*/*}$ mice (severe arthritis with arthritis score of 15 in both front paws and milder symptoms in the left hind leg) (Figure 6(a)). The intraperitoneal L-012 injection site emitted similar background signal in both genotypes.

Monocytes (CD11b-POS, Gr-1-LO, or Gr-1-NEG) and granulocytes (Gr-1-HI, CD11b-POS) from *Ncf1*$^{*/*}$ mice were unable to generate efficient ROS production upon PMA stimulation, while phagocytes from the wild-type animals responded to PMA stimulation and induced significant increase in DHR-123 derived fluorescence signal detected by flow cytometry (Figures 6(c) and 6(d)).

3.6. Ade-SOD3 Expression In Vivo. Quantitative RT-PCR revealed a A 5.5-fold (FC, fold change) expression of adenovirally produced SOD3 mRNA in the Ade-SOD3-treated joints

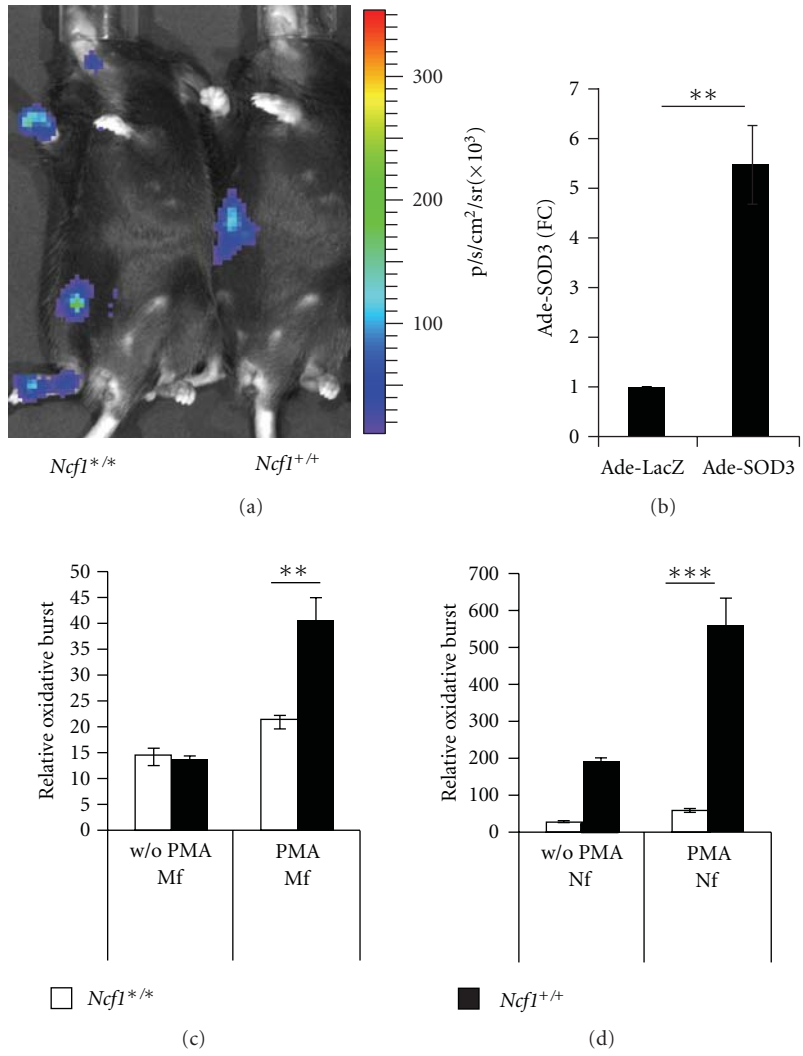

Figure 6: No oxidative burst was detected from the *Ncf1* mutated mice. In vivo imaging with the ROS sensitive L-012 probe revealed a bright luminescent signal from the inflamed wild-type paws with CIA, while no signal was detected from *Ncf1*$^{*/*}$ mice with comparable arthritis severity (a). Imaging was reproduced in two independent experiments with similar results. Expression of SOD3 by Ade-SOD3 expression vector in the treated paws was confirmed by Q-RT-PCR (n = 7/group) (b). Ex vivo oxidative burst was measured from phagocytes from *Ncf1*$^{*/*}$ (n = 5) and wild-type mice (n = 6) by using DHR-123 assay with and without (w/o) PMA stimulation. Neither macrophages (c) nor neutrophils (d) from the *Ncf1*$^{*/*}$ mice were able to exert a measureable oxidative burst.

collected at d25 when compared to the Ade-LacZ-treated vector control paws (Figure 6(b)).

4. Discussion

SOD3 is an enzyme that has been shown to give rise to therapeutic responses in damaged tissues such as reduced ischemia-reperfusion injury [25], arthritis [7], peritonitis [2], hind limb injury [3], and lung injury [6] models. These tissue healing promoting and anti-inflammatory effects induced by SOD3 are accompanied by reduced macrophage infiltration [2], inhibition of oxidative fragmentation of the extracellular matrix matrix [26], decreased apoptosis [4, 5], and enhanced cell proliferation [27]. The beneficial effects of SOD3 are mostly explained by its antioxidant properties and reduction of oxidative stress in the injured tissues. However, in this report we show that overexpression of SOD3 downregulated inflammation even in the absence of phagocyte oxidative burst, thus highlighting the capacity of SOD3 to affect cellular processes independently of NOX2 complex' superoxide production.

The adenoviral gene expression system used in this work reaches its maximal expression around three days after the injection, after which the vector is eliminated by the immune system and the expression of the transgene slowly decreases to undetectable levels 14 days after the initial injection [4]. We confirmed the presence of virally delivered SOD3 mRNA in the paws d25 and thus confirmed that the decrease in arthritis severity coincided with substantial adenovirus driven SOD3 expression. Similarly, pretreating the mice three

days before induction of peritonitis allowed us to analyze the effect of SOD3 during substantial SOD3 expression in the peritoneal cavity.

The percentual treatment effect in both inflammation models was comparable with the effect previously obtained with transgenic SOD3 overexpression in pulmonary emphysema [26]. Similarly, the degree of SOD3-induced macrophage infiltration inhibition in peritonitis was similar as reported previously [2]. Even though SOD3 is an important local regulator of the acute inflammatory reaction, it is obvious that there are a number of other factors affecting inflammation severity in vivo.

The physiological function of SOD3 is to dismutate extracellular superoxide into hydrogen peroxide. When the NOX2 complex is not functional, there are still a number of other enzymes and enzyme complexes producing superoxide in the inflamed tissue. NOX1 has been reported to worsen hyperoxia-induced acute lung injury in mice [28], and NOX4 has been suggested to stimulate microglial IL-6 expression [29] and to hamper neurodegeneration after poststroke ischemia reperfusion injury [30]. However, only NOX2 is abundantly expressed on phagocytes and thus recruited to inflammatory foci. Other NOX family members are expressed on other cell types than phagocytes and their ROS production is not upregulated during inflammation.

In addition to the NOX family enzymes, superoxide is also produced during mitochondrial respiration at levels corresponding to the general metabolic rate in the tissue. Mitochondrial superoxide has also been linked to TLR2/4 signaling [31] and is also suggested as a pathologic mechanism in tissue injury [32]. All these superoxide generating processes, however, cannot compensate for the massive production of ROS by the NOX2 complex during inflammation as the arthritic paws of *Ncf1*$^{*/*}$ mice emitted no detectable luminescence signal when probed with L-012 dye (Figure 6(a)). L-012 reacts with any radical to produce light and cannot be used to distinguish between superoxide and hydrogen peroxide in vivo.

As a tool to study the NOX2 complex dependency of SOD3, we used *Ncf1* mutated mice, in which a splice site point mutation abolishes the production of NCF1 protein leading to complete loss of NOX2 complex derived oxidative burst. In line with previous reports, arthritis severity was significantly higher in *Ncf1*$^{*/*}$ animals when compared to B10.Q wild-type mice [13]. Mutated mice also developed arthritis quicker after the booster allowing us to work with a more homogenous and extremely well reproducible disease model. Better arthritis synchronization in the mutated mouse model together with larger treatment groups resulted in less variability and increased statistical power in data analysis. In proteose peptone-induced peritonitis we observed no difference between the genotypes. The use of *Ncf1* mutant mice allowed us to avoid pit falls associated with the use of chemical NOX2 complex inhibitors such as DPI and apocynin, which are not specific for the NOX2 complex, do not provide full suppression of superoxide production and additionally profoundly affect various other cellular processes [14–16].

In both arthritis and peritonitis, adenoviral gene expression vectors locally enhanced inflammation. The arthritis enhancing effect was restricted to the treated paw, as the virus-injected groups did not differ from the PBS-injected control group when their nontreated paws were analyzed. This is in line with previous reports where intra-articular injections of adenoviral gene expression vectors have been shown to induce increased paw swelling and elevated levels of inflammation mediators [33, 34]. Intravenous injection route has not given rise to enhanced arthritis [35–37], but has triggered liver inflammation; liver being the primary target of systemically administered adenoviral vectors [35]. The immunogenicity of the adenoviral gene expression systems is well documented in the literature [38–40].

ROS regulate a number of physiological and disease-related pathways in humans. SOD3 polymorphisms are associated with COPD [41], coronary artery disease, myocardial infarction [42] as well as acute lung injury and related mortality [43]. Additionally, SOD3 has been reported to be downregulated in thyroid cancer tumors [44]. Both SOD3 and *Ncf1* are highly conserved ROS regulators [45]. There is also genetic [46] and functional [47] evidence linking *Ncf1* to human diseases.

5. Conclusions

We report that SOD3 limits inflammation in CIA and peritonitis both in the presence and in the absence of phagocyte oxidative burst. The anti-inflammatory function of SOD3 is not compromised by the lack of functionality of the NOX2 complex as both *Ncf1*$^{+/+}$ and *Ncf1*$^{*/*}$ mice develop milder inflammation when treated with SOD3. Thus, we conclude that the anti-inflammatory effect of SOD3 is not dependent on superoxide produced by the NOX2 complex derived phagocyte oxidative burst and thus acts via other signaling pathways.

Acknowledgments

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Review Article

The Local Inflammatory Responses to Infection of the Peritoneal Cavity in Humans: Their Regulation by Cytokines, Macrophages, and Other Leukocytes

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Studies on infection-induced inflammatory reactions in humans rely largely on findings in the blood compartment. Peritoneal leukocytes from patients treated with peritoneal dialysis offer a unique opportunity to study in humans the inflammatory responses taking place at the site of infection. Compared with peritoneal macrophages (pMϕ) from uninfected patients, pMϕ from infected patients display *ex vivo* an upregulation and downregulation of proinflammatory and anti-inflammatory mediators, respectively. Pro-IL-1β processing and secretion rather than synthesis proves to be increased in pMϕ from infectious peritonitis suggesting up-regulation of caspase-1 *in vivo*. A crosstalk between pMϕ, $\gamma\delta$ T cells, and neutrophils has been found to be involved in augmented TNFα expression and production during infection. The recent finding in experimental studies that alternatively activated macrophages (Mϕ2) increase by proliferation rather than recruitment may have significant implications for the understanding and treatment of chronic inflammatory conditions such as encapsulating peritoneal sclerosis (EPS).

1. Introduction

Continuous ambulatory peritoneal dialysis (CAPD) was introduced in 1978 as a new treatment modality for patients with end-stage renal failure. In CAPD, after infusion of typically 2 litres of dialysis fluid via a catheter into the peritoneal cavity, retained metabolites diffuse from the blood to the peritoneal cavity during a dwell time of 4 to 8 hours, after which the dialysis fluid is drained and replaced with fresh dialysis fluid. In this way, the patient exchanges 3–5 times a day dialysis fluid. A major complication of CAPD is peritonitis caused by contamination by microorganisms that can enter the peritoneal cavity via infusion of dialysis fluid during the exchange, or by spreading of an infection from the skin and tissue around the catheter to the peritoneal cavity, or from the intestines [1]. In the early years, an episode of peritonitis occurred on average one time per 8 treatment months, but since the nineties the frequency was substantially reduced to one time every 24 months due to novel connections of the infusion systems. These so-called "flush-before-fill" systems reduce the risk of peritonitis during the exchange of dialysis fluids, which is caused especially by coagulase negative Staphylococci and other gram positive microorganisms. Infectious peritonitis is characterized by abdominal pain and turbid drained dialysate (peritoneal effluent) due to an increased number of leukocytes more than 50% of which are neutrophilic PMN's. Peritonitis is almost invariably revealed by opalescence of dialysate, which is noticed by patients when the leucocyte count is greater than 100/mm^3. The majority of peritonitis episodes can be treated successfully with the intraperitoneal administration of antibiotics while continuing CAPD.

Infectious peritonitis in CAPD patients has been shown to provide a unique opportunity to study the inflammatory reactions in humans at the site of inflammation by studying cellular players including macrophages, lymphocytes, granulocytes, and mesothelial cells as well as soluble mediators present in peritoneal effluent [2–4]. In this paper, various studies are reviewed that are conducted in the past few decades on this topic with emphasis on the role of

macrophages (Mϕ) and cytokines. The findings will be put in the context of new insights that developed the past decade in the biology of Mϕ and cytokines. Studying leukocytes from an inflammatory environment can make a valuable contribution to a better understanding of inflammatory reactions in humans.

2. Macrophages, Heterogeneity versus Polarization

Tissue Mϕ are derived from circulating blood monocytes, which in turn arise from their bone marrow precursors. These cells together make up the mononuclear phagocyte system, as described by van Furth and Cohn [5]. After monocytes have entered the tissues to become Mϕ, they have the potential to acquire a variety of different functional attributes depending on signals they receive from the environment. Thus, the mononuclear phagocyte system consists of a heterogeneous and highly versatile, multipotential cell population. The differentiation and activation to diverse functions in the tissues are governed by the presence of regulatory signals in the environment and occur in several distinct steps [6]. In the past decade, a new view on Mϕ differentiation and activation has been developed. *In vitro* two types of Mϕ are distinguished: Classically activated Mϕ display a proinflammatory profile, induced by IFN-γ or LPS, whereas alternatively activated Mϕ express anti-inflammatory and tissue repair properties induced by IL-4 or IL-13 [7–11]. IFN-γ is a prototypical Th-1 cell secretory product, while IL-4 and IL-13 are produced by Th-2 cells and Mϕ. Classically and alternatively activated Mϕ are also named as Mϕ1 and Mϕ2, mirroring the Th-1 and Th-2 polarization, respectively. Type 1 and type 2 inflammation represent ancient innate pathways with fundamentally different purposes. Type 1 promotes killing of microbial pathogens and intracellular parasites and is involved in tissue destruction and tumor resistance. Type 2 participates in tissue repair and controls infection with macroparasites through encapsulation. Mϕ1 typically show a high expression of the cytokines IL-12, IL-23, TNFα, IL-1β, and Mϕ1 chemokines and are efficient producers of reactive oxygen and nitrogen intermediates, whereas IL-10 production is low. In contrast, in Mϕ2 expression of IL-12, IL-23, TNFα, and IL-1β is low, whereas expression of IL-10, IL-1ra, TGFβ, Mϕ2 chemokines and scavenger, mannose and galactose receptors is high. In experimental *in vivo* studies, it has been found that a subset of patrolling, circulating monocytes, which may correspond to human CD16+ monocytes, are rapidly recruited to the peritoneal cavity, peaking at 2 hours after infection with Listeria monocytogenes, when PMN is only beginning to enter the peritoneal cavity [12]. After 1 and 2 hours after infection these mononuclear phagocytes produce TNFα and show an upregulated expression of genes coding for IL-1 and various chemokines and pattern recognition receptors such as toll-like receptors (TLRs). Notably, the production of TNFα and IL-1β is transient and turns off at 8 hours, whereas these mononuclear phagocytes turn on, at 2 and 8 hours, in genes involved in tissue remodeling. A different subset of conventional monocytes arrive later and give rise to inflammatory dendritic cells (DCs) and Mϕ1 macrophages [8, 12]. In a recent experimental study, it was found that both resident and recruited Mϕ can be alternatively activated and be driven to proliferate *in situ* by a Th-2 environment *in vivo*, implying that there is neither a specific precursor for Mϕ2 nor is proliferative capacity restricted by lineage [13]. While the paradigm of macrophage dichotomy is well established, employing it as a rigid scheme could bring about a risk of oversimplification. Thus, Mϕ can reversibly shift their functional phenotype through a multitude of patterns in response to changes in cytokine environment, as illustrated in Figure 1 [14]. In humans, arginase, which is considered to be characteristic of alternatively activated macrophages, is not expressed prominently IL-4-induced Mϕ2 macrophages [15]. Furthermore, during the resolution phase of experimental inflammation a Mϕ phenotype with properties of both Mϕ1 and Mϕ2 could be distinguished [16].

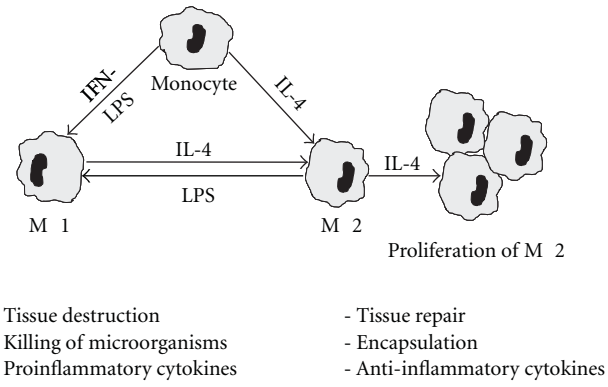


Figure 1: Simplified model of the paradigm of polarized macrophage activation. Monocytes are either classically (Mϕ1) or alternatively (Mϕ2) activated. Substances such as IFN-γ and LPS induce classical activation while IL-4, IL-13, and TGFβ cause alternative activation. Mϕ1 macrophages typically produce high levels of proinflammatory proteins including TNFα, IL-1β, IL-12, and IL-23 as well as reactive oxygen and nitrogen intermediates. Mϕ2 produce high levels of the anti-inflammatory cytokines IL-10 and IL-1ra and show a high expression of scavenger and mannose receptors. Under the influence of IL-4, Mϕ2 can accumulate in tissues by local proliferation rather than by recruitment. Either functional phenotype can shift to another depending on microenvironmental influences, speaking against rigid interpretation of the dichotomy between the macrophage phenotypes.

3. Peritoneal Macrophage (pMϕ) from CAPD Patients

Approximately 1–40 millions of leukocytes can be collected from peritoneal effluent after a dwell time of 6–8 hours. The yield decreases in the course of CAPD treatment. In uninfected patient, the leukocyte population was found to be composed of 85% mononuclear phagocytes by nonspecific esterase staining, while >75% of each cell population was HLA-DR+. Six percent were neutrophilic

and/or eosinophilic PMNs [17, 18]. Using flow cytometric analysis for surface markers, about 40% of the peritoneal cells were identified as lymphocytes [19]. Various subsets are distinguished in peritoneal lymphocytes including B cells and various subsets of T cells. Two to six percent of peritoneal cells can be characterized as DC's, which are differentiated in the peritoneal cavity from monocyte-derived CD14+ cells [20, 21]. Peritoneal CD14+ cells were characterized as Mϕ2 macrophages on the basis of a CD163+CD16− phenotype, a high capacity for phagocytosis and production of high amounts of IL-10, sharing these properties with *in vitro* polarized Mϕ2 [19]. In contrast, the production of substantial amounts of IL-6, as found in this study, is a property of Mϕ1 rather than Mϕ2. The CAPD cell population is continually renewed and is exposed to dialysis fluids with an unphysiological composition and to the dialysis catheter. Yet, in many respects the macrophages from CAPD patient bear resemblance to those from healthy women undergoing laparoscopy [17, 18]. Compared with such cells from rats, CAPD pMϕ resemble starch-elicited rather than resident cells [22].

When a peritoneal infection becomes clinically manifest, there is a sharp, up to 100 fold increase in peritoneal leukocytes, 50–90% of which are neutrophils. Also the number of pMϕ, dendritic cells and various subsets of lymphocytes show a marked increase, including $\gamma\delta$ T cells [20, 23].This minisubset is rapidly recruited to the inflammatory site and responds to the microbial molecule HMB-PP that is found in various species—30% to 50% of peritonitis episodes is caused by HMB-PP+ microbes—and is released when microorganisms are killed by other leukocytes including neutrophils [24]. By interaction of $\gamma\delta$ T cells with mononuclear phagocytes, the inflammatory reaction is amplified. Already one to two days before the infection becomes clinically manifest, an increased number of pMϕ and neutrophils is found [25]. Following appropriate antibiotic treatment, the mononuclear cells and especially the neutrophils show a sharp drop in the next few days, resulting in a relative increase of pMϕ and lymphocytes. While on the first day of the peritonitis pMϕ outnumber lymphocytes, in the resolution phase the macrophages/lymphocytes ratio is reversed [26]. Using flow cytometry, pMϕ from infected patients displayed an increased expression and production of selected Mϕ2-associated cell surface markers (CD163+) and chemokines (CCL18), respectively, but expression of the Mϕ2-associated mannose receptor CD206+ was lower in peritonitis pMϕ. Gene expression of TGF-β1, metalloproteinase 9 (MMP9), and CCL18 in pMϕ from infected and uninfected patients were similar [27].

4. Cytokines in CAPD during Infectious Peritonitis

The pro-inflammatory cytokines IL-1β, TNFα, and IL-6 play a key role in the inflammatory response. By exerting their pleiotropic effects in an autocrine, paracrine, and endocrine fashion, these cytokines are able to orchestrate the inflammatory responses. Although they can be produced by various cells, macrophages are the prototypical cell source. PMϕ from CAPD patients collected during infectious peritonitis, showed a marked increase in the secretion of TNFα and IL-1β as compared with macrophages from infection free patients, when they were stimulated *ex vivo* with LPS [28, 29]. In contrast, unstimulated pMϕ secreted similar amounts of TNFα and IL-1β *ex vivo* in pMϕ from patients with and without infection. These findings are in line with the paradigm of stepwise activation of Mϕ. On the other hand, the *ex vivo* secretion of the anti-inflammatory IL-10 was decreased in peritonitis macrophages, in line with a pro-inflammatory phenotype [30]. In the effluent from patients with infectious peritonitis, as compared with uninfected patients, increased levels of various pro-inflammatory cytokines were found, including IL-1β, IL-8, TNFα, IL-6, and IFNγ [26, 31–35]. Remarkably, also levels of anti-inflammatory cytokines for example, TGFβ and IL-1ra were elevated [26, 32, 36]. It should be noted that in addition to Mϕ and other leukocytes, mesothelial cells may also contribute substantially to the production of various cytokines including IL-6 and IL-8 [37, 38].

We investigated at which level the increased capability of peritonitis pMϕ to secrete IL-1β after *ex vivo* stimulation with LPS occurs, using ELISA's specific to the 32 kDa, biologically inactive pro-IL-1β and the mature 17 kDa, bioactive IL-1β [39]. Pro-IL-1β processing and subsequent release of mature IL-1β (mIL-1β) rather than its production were found to be increased in peritonitis pMϕ (Figures 2(a), 2(b), and 2(c)), suggesting increased caspase-1 activity. Caspase-1 is present in the cell as the bioinactive procaspase-1 to become a bioactive cysteine protease after autocleavage. In the last decade, the understanding of the molecular mechanisms behind caspase-1 activation has been significantly increased. Briefly, NOD-like receptors (NLRs), present in the cytosol, recognize microbial molecules leading to oligomerization of NLRs and along with recruited procaspase-1 and other proteins, to the forming of multiprotein inflammasome complexes [40]. This results in auto-cleavage and activation of caspase-1, whereupon pro-IL-1β is cleaved and mIL-1β is released by an unconventional, poorly understood, mechanism as IL-1β lacks a signal peptide [41]. Microbial ligands induce transcription of pro-IL-1β and inflammasome components by activation of the transmembrane TLRs. Taken together, in the setting of our study increased caspase-1 activation might be postulated as priming mechanism *in vivo*. Interestingly, in a study using high-density oligonucleotide microarrays to investigate the transcriptional profile induced in human monocytes by IL-13, one the most striking findings, besides a variety of other characteristic genetic markers of alternatively activated macrophages, was downregulation of caspase-1 and changes in other components of the IL-1 system such as up-regulation of IL-1ra [15]. The LPS-inducible caspase-1 activity was also found to be reduced, resulting in a decrease in pro-IL-1β processing. Further studies are needed to reveal which molecular mechanisms account for the increased IL-1β processing and export in peritonitis pMϕ. We also found that LPS stimulated not only pro-IL-1β production but also release of mIL-1β in a dose-dependent fashion, suggesting

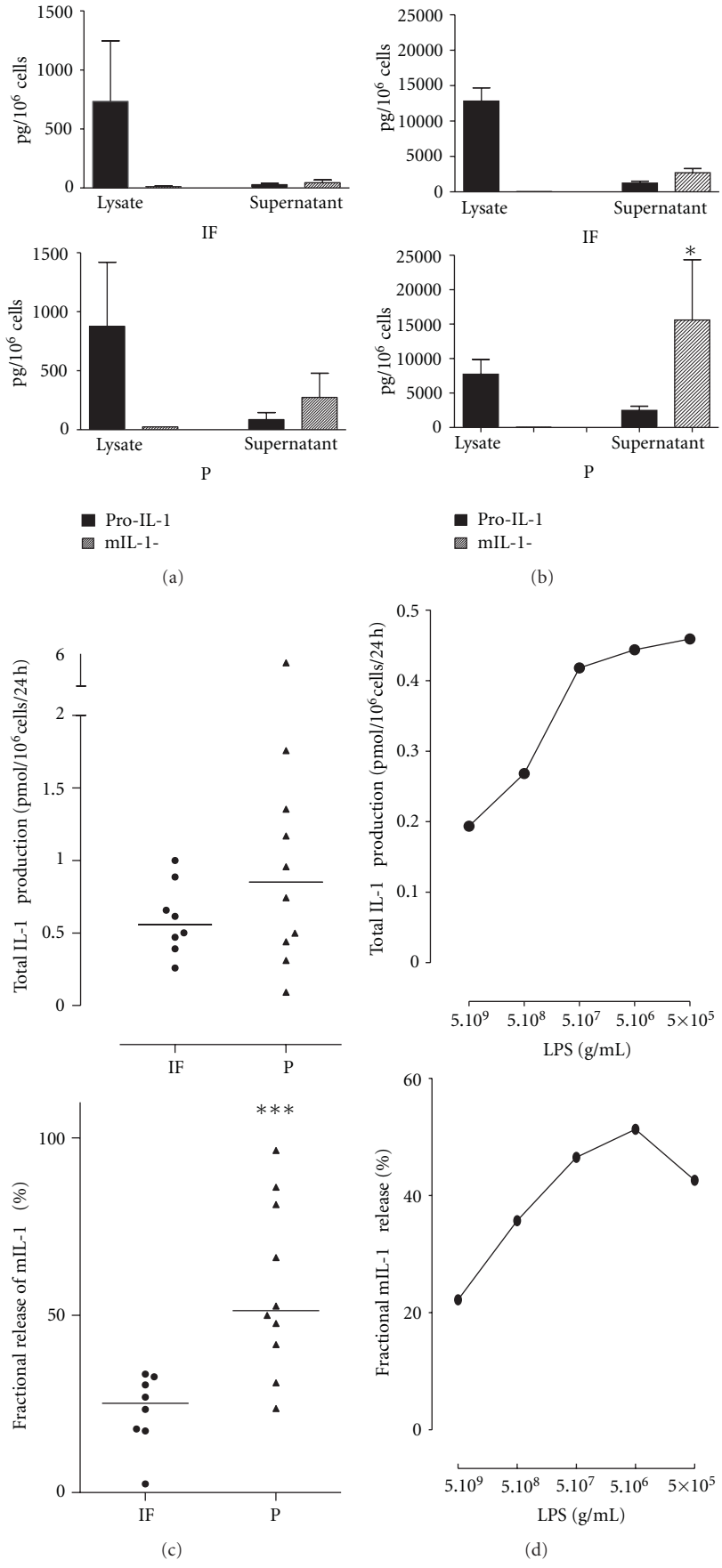
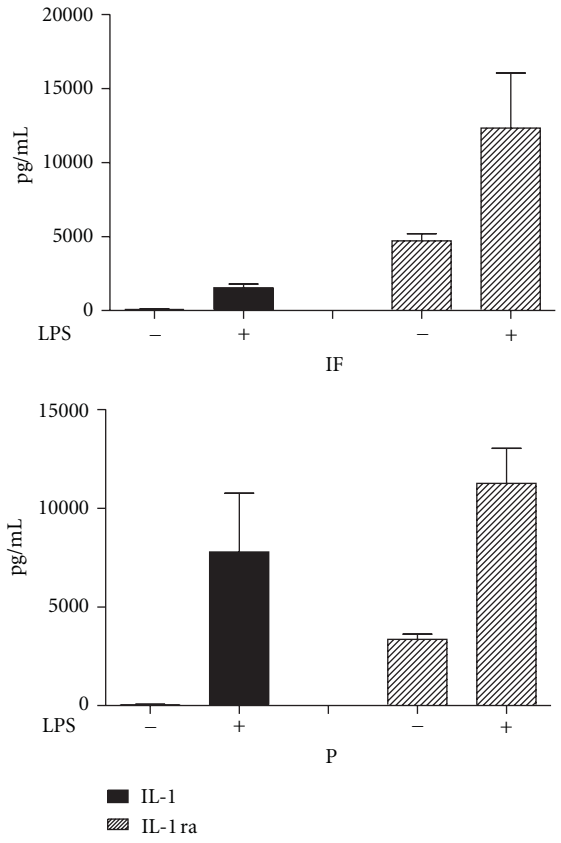


Figure 2: Continued.



(e)

Figure 2: Peritoneal macrophages (pMϕ) were isolated from CAPD patients who were at least 3 months infection free (IF), or during an episode of infectious peritonitis (P), prior to the start of treatment with antibiotics. 1.10^6 Cells were incubated *ex vivo* during 24 h in medium or 5 μ/mL of LPS. Cytokines were determined in supernatants or cell lysates using ELISA. (a) Pro-IL-1β production and processing and mIL-1β release. Quantities of pro-IL-1β (black bars) and mature IL-1β (mIL-1β) (white bars), expressed in pg/10^6 cells/24 h, using ELISA specific for either form of IL-1β (CISTRON^R), in supernatants and cell lysates from pMϕ isolated during IF-period ($n = 8$) or P ($n = 10$) and incubated in medium alone (a) or 5 μ/mL of LPS (b). Values are expressed as (M ± SEM). mIL-1β levels in supernatants from LPS-stimulated pMϕs were significantly higher when cells were isolated during P ($P < 0.05$), while pro-IL-1β in cell lysates was decreased ($P \approx 0.05$). (c) total IL-1β production (= pro-IL-1β + mIL-1β in supernatants + cell lysates) expressed in pmol/10^6 cells/24 h for each IF-period (circles) and P episode (triangles) in LPS stimulated pMϕ. Difference between total IL-1β production from IF and P pMϕ was not statistically significant. fraction of total IL-1β that is released in supernatant as mIL-1β, expressed as percentage. Fractional release from peritonitis pMϕ was significantly higher ($P < 0.005$). (d) total IL-1β production and fractional mIL-1β release in response to increasing doses of LPS in pMϕ isolated during 3 episodes of peritonitis, expressed in pmol/10^6 cells/24 h. Total IL-1β production increased in a dose-dependent fashion. Within the range of 5.10^{-9}–5.10^{-6} g/mL, LPS induced a dose-related increase in fractional IL-1β release. (e) release of IL-1β and IL-1ra in supernatants from infection-free ($n = 15$) and peritonitis pMϕ ($n = 8$) stimulated with and without 5 μg/mL of LPS. Substantial amounts of IL-1ra were released in unstimulated cells. LPS-stimulated peritonitis pMϕ released similar quantities of IL-1β and IL-1ra.

a stimulating effect of LPS on caspase-1 activity. (Figure 2(d)) PMϕ displayed a rather high constitutive production of IL-1ra that further increased by stimulation with LPS, with pMϕ from infection-free and peritonitis patients releasing similar amounts (Figure 2(e)). It has been reported that a 10–500 fold molecular excess of IL-1ra is required to obtain 50% inhibition of IL-1 biological effects *in vitro* [42]. In our study, similar amounts of IL-1ra and IL-1β were released in LPS-stimulated peritonitis pMϕ implying a virtually unimpeded secreted IL-1 bioactivity. There was no production of the bioactive form of IL-12 (Figure 3(a)). The secretion of the anti-inflammatory cytokine IL-10 by LPS-stimulated peritonitis pMϕ was significantly reduced (Figure 3(b)). However, IL-10 levels in peritoneal effluent were higher during peritonitis. The large increase in macrophages and other leukocytes during peritonitis, probably accounts for the discrepancy in the direction of the changes of IL-10 and other anti-inflammatory cytokines between macrophage cultures and peritoneal effluents. Absorption of pro- and anti-inflammatory cytokines from the infectious inflammatory site might offer in part an explanation for the discrepancy in the blood compartment

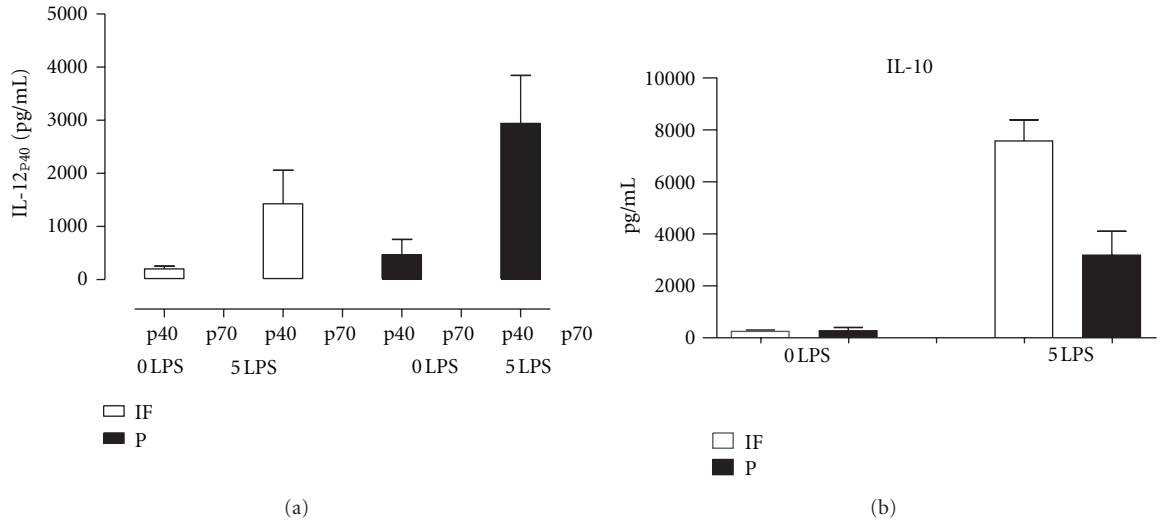


Figure 3: (a) Patients and methods as in Figure 1. *Ex vivo* IL-12_{p40} release is stimulated by LPS. Difference between pMϕ from an infection-free period ($n = 8$) and an episode of peritonitis ($n = 8$), was statistically not significant. Using an ELISA specific to the bioactive, heterodimeric IL-12_{p70}, no active IL-12 was detectable in supernatants from uninfected patients ($n = 5$) nor in those with peritonitis ($n = 5$), whether or not the cells were stimulated with LPS. Consistent with this finding, virtually no IL-12_{p35} mRNA was expressed (data not shown). All peritonitis episodes were caused by gram positive bacteria. (b) *ex vivo* IL-10 release from pMϕ from infected patients ($n = 8$) is decreased as compared with pMϕ from an infection free environment ($n = 8$), ($P < 0.01$). IL-10 levels in peritoneal effluents from peritonitis were higher compared with infection-free effluents, 235 pg/mL and 25 pg/mL, respectively (data not shown in figure).

between higher levels of circulating pro-inflammatory cytokines and a decreased capacity of blood monocytes to secrete TNFα and IL-1β as found in patients with sepsis. Compartmentalization of the inflammatory response is a key feature of the sepsis syndrome [43].

PMϕ from infected patients have also an increased capability to release TNFα [29]. PGE_2 has been found to have strongly inhibitory effects on LPS-stimulated TNFα release, almost eliminating the actions of LPS in a clearly dose-related fashion, whereas cyclooxygenase inhibition caused an increase in TNFα release [44]. The PGE_2-induced down-regulation, which was similar for pMϕ from an infectious or infection-free environment, is probably brought about via elevation of intracellular cAMP levels. Moreover, it has been found that peritonitis macrophages have suppressed cAMP levels and a diminished release of prostaglandins compared to uninfected macrophages [45, 46]. Similarly, *ex vivo* stimulation of pMϕ from uninfected patients with *Staphylococcus epidermidis* induced a marked decrease of cyclooxygenase products [47]. Prostaglandins are known for their pro-inflammatory effects, notably on the vascular components of inflammatory reactions, but in various settings these short-lived and locally acting substances have proved to possess anti-inflammatory properties as well. Recently it was reported, that, using low-dose and high-dose zymosan induced peritonitis as a model for self-limiting, resolving inflammation, and a more protracted response leading to systemic inflammation, respectively, pMϕ from either environment displayed distinct characteristics [16]. PMϕ from the protracted peritonitis had a typical Mϕ1 phenotype, while those from the resolving inflammation had characteristics of both Mϕ1 and Mϕ2 and were named as resolving macrophages (rMϕ). These rMϕ, as compared with Mϕ1, released *ex vivo* fewer pro-inflammatory cytokines, including TNFα, IL-1β, and IL-12 but more IL-10 and PGD_2. The expression of COX 2, iNOS, and intracellular cAMP contents were also increased. Elevating cAMP levels by cAMP analoga transformed Mϕ1 to rϕM, whereas cAMP inhibitors converted rMϕ to Mϕ1. These findings demonstrate that cAMP plays a central role in the regulation of Mϕ phenotype. In addition, it has been found that cyclooxygenase inhibition improved bacterial killing and resistance to infection in mice and humans, confirming the important role of cAMP. Interestingly, COX 1 rather than COX 2 turned out to be the predominant form that is active during infection [48]. Similarly, phagocytosis of apoptotic cells by Mϕ proved to inhibit the production of several mediators such as IL-1β, TNFα, and IL-10, but it increased the production of TGF-β1, PGE_2, and PAF [49]. The latter mediators induced suppression of LPS-stimulated cytokine production by such Mϕ. In contrast, indomethacin restored the inhibition of cytokines and inhibited TGF-β1 production by phagocytosing Mϕ. These findings show that PGE_2 along with TGF-β1 and PAF plays an actively suppressing role in the shift from a pro-inflammatory to a more anti-inflammatory phenotype in Mϕ that have ingested apoptotic cells.

5. Conclusions and Future Perspectives

Compared with pMϕ from uninfected CAPD patients, pMϕ from an infected peritoneal cavity display *ex vivo* an upregulation of production and secretion of pro-inflammatory cytokines and a downregulation of anti-inflammatory mediators. In terms of polarized macrophage activation, these

findings show that during infectious peritonitis the pMϕ population is on average shifted to a Mϕ1 phenotype. In the above-mentioned studies, the cells were collected when the first signs and symptoms of peritonitis became manifest, that is, before antibiotic treatment was started. Following successful treatment, signs and symptoms improve within a few days. *Ex vivo* studies with effluents could also provide an unique opportunity to follow up human pMϕ and other leukocytes during the resolution phase, set in motion after antibiotics have brought about reduction and elimination of microbes. What changes do pMϕ and other leukocytes undergo in the recovery phase during the shift from Mϕ1 to a more typical Mϕ2 profile? What is the time course and how long do Mϕ1 features persist? Using current techniques including transcriptional profiling, proteomics and flow cytometry, a better understanding of the regulation of infection-induced inflammatory reactions in humans may be achieved. The findings of the comparative studies on cytokine release from pMϕ from an infection-free and infectious environment are in line with the postulate that *in vivo* Mϕ1 and Mϕ2 are extremes of a wide spectrum of phenotypes. Yet, the fact that Mϕ2 may increase by local proliferation rather than by recruitment, as recently found in experimental studies, may have important implications for the way we look at the pathogenesis and therapy of chronic inflammatory disorders, if this interesting discovery also applies in humans [13, 50]. Severe fibrosis and neoangiogenesis of the peritoneum are the histological hallmarks of encapsulating peritoneal sclerosis (EPS), a rare but serious complication of long-term CAPD [51–56]. Etiology and pathogenesis are incompletely understood, but EPS may be conceived as an extreme example of type 2 inflammation. Histological studies and *ex vivo* studies of pMϕ from peritoneal effluents, assuming they are representative of peritoneal tissue Mϕ, may help to gain a better understanding of this complication.

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Research Article

Involvement of the Ubiquitin-Proteasome System in the Formation of Experimental Postsurgical Peritoneal Adhesions

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We investigated the Ubiquitin-Proteasome System (UPS), major nonlysosomal intracellular protein degradation system, in the genesis of experimental postsurgical peritoneal adhesions. We assayed the levels of UPS within the adhered tissue along with the development of peritoneal adhesions and used the specific UPS inhibitor bortezomib in order to assess the effect of the UPS blockade on the peritoneal adhesions. We found a number of severe postsurgical peritoneal adhesions at day 5 after surgery increasing until day 10. In the adhered tissue an increased values of ubiquitin and the 20S proteasome subunit, NFkB, IL-6, TNF-α and decreased values of IkB-beta were found. In contrast, bortezomib-treated rats showed a decreased number of peritoneal adhesions, decreased values of ubiquitin and the 20S proteasome, NFkB, IL-6, TNF-α, and increased levels of IkB-beta in the adhered peritoneal tissue. The UPS system, therefore, is primarily involved in the formation of post-surgical peritoneal adhesions in rats.

1. Introduction

Postsurgical adhesions are polymorphic clinical pictures ranging from abdominal and pelvic pain to intestinal mechanical occlusion. They are due to stable lesions scarring, the adhesions. Intraperitoneal adhesions are connective bridges between adjacent portions of the peritoneum [1], their formation occurs in approximately 90% of patients undergoing abdominal surgery, and it is an important reason of postoperative morbidity and mortality [2].

Adhesions formation is initiated by the trauma surgery, and it is supported by a number of factors including fibrin, thromboplastin, mesothelial cells, fibroblasts, collagen fibrils, and the development of a local inflammatory process, among others [3].

Until a few years ago to prevent the formation of adhesions, the focus was mainly on the procedures to be implemented during surgery. It is worth mentioning that there is considerable consensus that laparoscopic surgery is associated with the reduced development of adhesions compared to open surgery in the international arena [4, 5].

A surgical trauma stimulates the release of several chemical mediators of inflammation, including IL-1, IL-6, and TNF-α whose involvement in the inflammatory process, that underlies the formation of adhesions, has been documented in many studies [6].

The transforming growth factor beta-1 (TGF-β1) and transforming growth factor beta-3 (TGF-β3) appear to be involved in the process too. They are released after hypoxia generated by tissue injury during surgery [7].

In recent years numerous studies have been aimed at a likely pharmacological prevention of postsurgical adhesion formation. For example, some authors proposed the use of statins as they are considered as being able to increase the peritoneal fibrinolysis [8]. Other authors have suggested that interferon gamma is a possible therapeutic target to prevent the formation of adhesions, due to its crucial role in the differential regulation of PAI-1 and t-PA, which are involved in this process [9]. However other ones suggest the use of antitack agents such as carboxymethylcellulose and hyaluronic acid resorbable membranes. Finally it is also alleged the possible use of propofol [10].

In this wide scenario of hypotheses we want to include the centrality of the inflammatory component in the unfolding process of adhesions; we hypothesized that the Ubiquitin-Proteasome System (UPS), the major nonlysosomal intracellular protein degradation system in eukaryotic cells and which is capable of inducing inflammation [11–14], may play a major role in the formation of post-surgical peritoneal adhesions. We hypothesized that the activation of this system during the surgery can be suggestive of the beginning of inflammatory events that in cascade lead to adhesions formation. So, in this experimental study on rats we have evaluated whether changes in UPS levels and activity into the adhered tissue occur along with the development of post-surgical peritoneal adhesions, and then we used the specific UPS inhibitor bortezomib [15–17] in order to ascertain the role that the UPS may have in the peritoneal adhesions formation.

2. Methods

2.1. Surgical Procedure. All experimental procedures and protocols used in this study were reviewed and approved by the Special Ethics Commission at the 2nd University of Naples.

The surgery was performed as previously described [4]. Male Sprague-Dawley rats ($n = 15$) were marked with a pencil as 1 to 15, anesthetized with urethane (1.2 g/kg ip), subjected to midline laparotomy. A sample of parietal peritoneal tissue was taken and an enterotomy was performed at the level of the ileum. The surgical incision was sutured with absorbable surgical wire 4/0 in order to induce an inflammatory peritoneal insult. All of the surgical procedure was then ended by a nonabsorbable suture, and the rats were placed in the recovering room for awakening. Five days after the surgery the rats were subjected to another laparotomy, had a new tissue sample taken, and were assayed for the development of peritoneal adhesions by means of qualitative and quantitative evaluation. A score from 1 to 6 was established, and it was given as follows: 1 to the presence of poor and lapse adhesions in a limited peritoneal zone; 2 to the presence of poor adhesions in an extended zone; 3 to the presence of several lapse adhesions into the peritoneum; 4 to the presence of localized dense adhesions; 5 to the presence of extended dense adhesions with access to peritoneal cavity; 6 to the presence of extended dense adhesions and impossible access to peritoneal cavity. The same procedure was repeated after 10 days from the first surgery, having particular attention to keep always the same rat numbering over the time course considered. A surgeon blinded for the procedure and different from the one who performed the surgery scored the adhesion. Biopsies of peritoneal tissue were snap-frozen and used to determine: TNF-alpha, IL6, NFkB, and activity of the UPS system.

2.1.1. Experimental Groups. The study was conducted on male Sprague-Dawley rats (4-month old and weighing 200 g) induced with only the surgical procedure ($n = 15$) or treated with bortezomib + surgical procedure ($n = 15$). This latter was first treated with bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, UK) by intravenous injection (0.05 mg/kg) given 1 hour before surgery, and a second injection was given 1 hour after the surgical procedure (total dose of 0.1 mg/kg) according to our previous experience in other setting [4].

There were no significant side effects after injections.

2.1.2. Biochemical Parameters Assessed. The tissue samples taken during the surgery were homogenized in 50 mM Tris-HCl (pH 7.2) containing leptin 1 μM, pepsatin A 1 μM, and phenyl methyl sulfonyl fluoride 200 μM and centrifuged for 10 min at 10,000 $\times$g at 4°C.

200 μL of homogenate were used to determine total protein according to the Bradford's method. The levels of ubiquitin, IL-6, and TNF-alpha were quantified by a commercial colorimetric ELISA kit (R&D Systems, USA). For the quantitative measurement of the 20S proteasome, a specific SDS activation kit (Boston Biochem, USA) was used following the instructions of the manufacturer.

2.1.3. Statistics. Data are presented as mean $\pm$ SE. Continuous variables were compared among the groups of rats with one-way analysis of variance (ANOVA) for normally distributed data and Kruskal-Wallis test for nonnormally distributed data. When differences were found among the groups, Bonferroni correction was used to make pairwise comparisons. A $P < 0.05$ was considered statistically significant. All calculations were performed using the SPSS2 software.

3. Results

Starting from day 5 from surgery there was a development of peritoneal adhesions within the rats. After 10 days from the surgery 7 control rats were scored 6 for the severity of the adhesions, 4 rats were scored 5, and 4 control rats were scored 1 for the presence of poor and lapse adhesions in a limited peritoneal zone. An increase in the levels of ubiquitin and the 20S proteasome within the adhered tissue was observed in parallel with the development of peritoneal adhesions. For both the markers this increase was significant ($P < 0.01$ versus day 0) already 5 days after surgery (Figure 1). A further increase in the levels of ubiquitin and the 20S proteasome was observed in the adhered peritoneal tissue after 10 days of surgery ($P < 0.01$ versus day 0) (Figure 1).

The qualitative evaluation by the peritoneal adhesions score indicates a substantial decrease of adhesions in the group of rats treated with bortezomib (e.g., 4 rats were scored 6 and 2 rats were scored 5) after 10 days of surgery if compared with the control group same day (Figure 2). This was accompinied by a significant reduction of the levels of both ubiquitin and proteasome 20S ($P < 0.01$ versus day 0) in the peritoneal tissue 5 days after surgery. The reduction was found to be significant even 10 days after surgical procedure ($P < 0.01$ versus day 0) (Figure 3). At this time point there was a significant correlation between the levels of ubiquitin/proteasome 20S and the adhesion score assigned to the rats after bortezomib treatment (Figure 4).

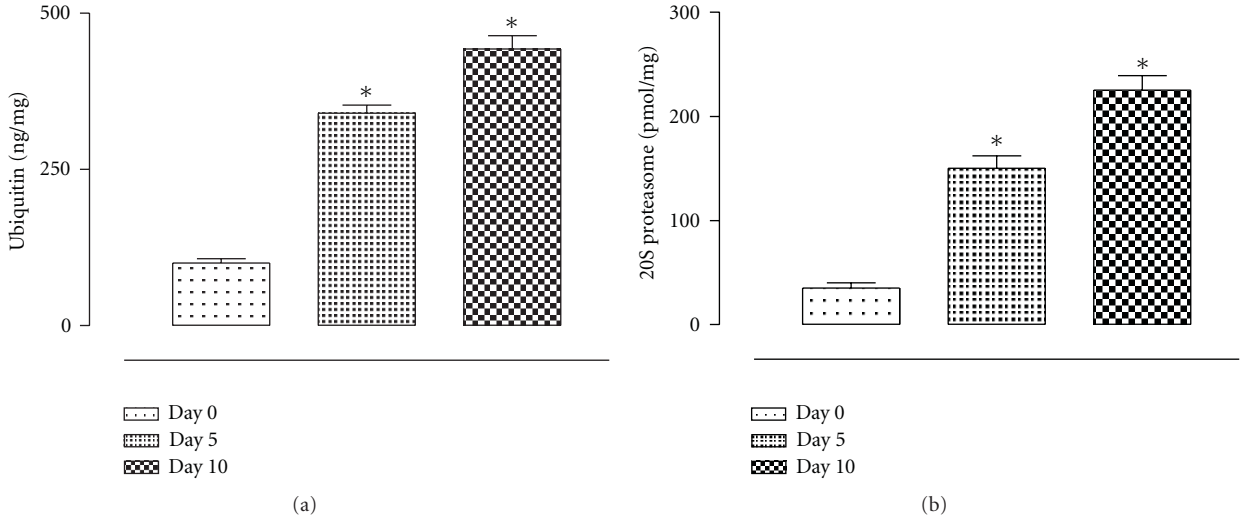

Figure 1: Levels of ubiquitin and 20S proteasome in the tissues from rats during peritoneal adhesions development at time 0, 5, and 10 days from the surgery in the control group. The differences from day 0 are considered with $^*P < 0.01$.

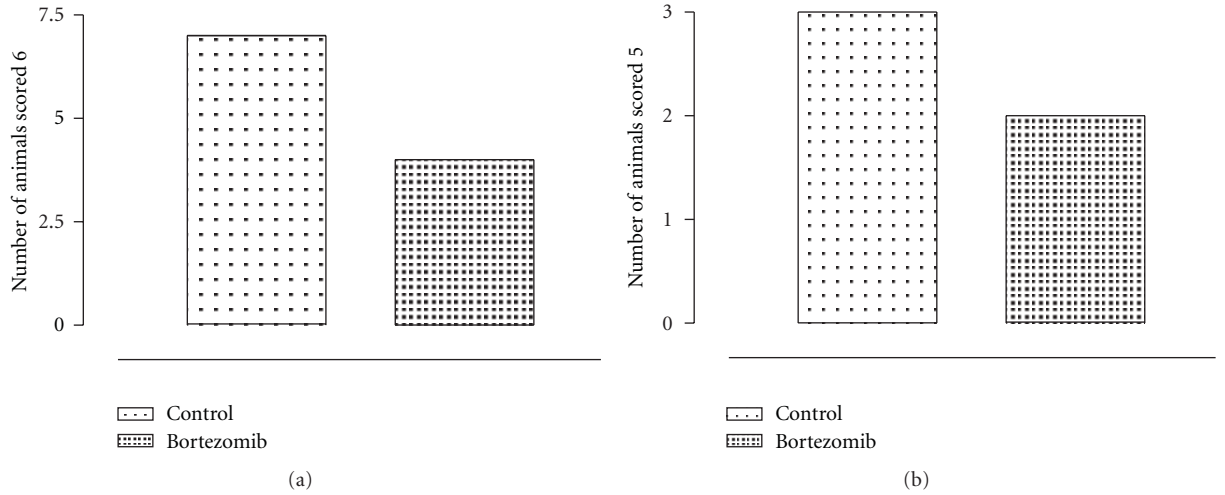

Figure 2: Quantitative evaluation by the peritoneal adhesions score indicating a substantial decrease of adhesions in the group of rats treated with bortezomib after 10 days of surgery if compared with the control group same day.

In the rat adhered peritoneal tissue the TNF-alpha, IL-6, p50, and p65 (subunit of NFkB) levels were significantly increased after 5 and 10 days after surgery (Figure 5). In contrast, in the same tissue the levels of the IkB-beta both at day 5 and at day 10 ($P < 0.01$ versus day 0) were seen decreased (Figure 6).

Treatment with bortezomib resulted in reduced levels of TNF-alpha and IL-6, p50, and p65 in both time day 5 and time day 10 ($P < 0.01$ versus control group with adhesions) (Figure 5), together with a significant increase in levels of IkB-beta ($P < 0.01$ versus control group) (Figure 6).

4. Discussion

In a previous research this group found that post-surgical peritoneal adhesions occur in rats when they are subject to laparatomy and enterotomy of the ileum [4]. These adhesions are strictly related to the time from the surgery and have severity depending on the inflammatory response occurring within the peritoneal specimens, which deteriors the peritoneal matrix. During this phase, leukocytes, cytokines, chemokines, and cell adhesion molecules alters the initial tissutal equilibrium predisposing them to the formation of adhesions [4].

In the present study it is shown that a key role in the tissutal alterations that follows a surgical intervention and development of peritoneal adhesions is assumed by the Ubiquitin-Proteasome System (UPS). This study demonstrates that the activity of this system, measured on biopsies of the adhered tissue, relates to the severity of the adhesion; animals that show high levels of UPS activity also have severe adhesions, whilst animals that show low levels of UPS have moderate adhesions.

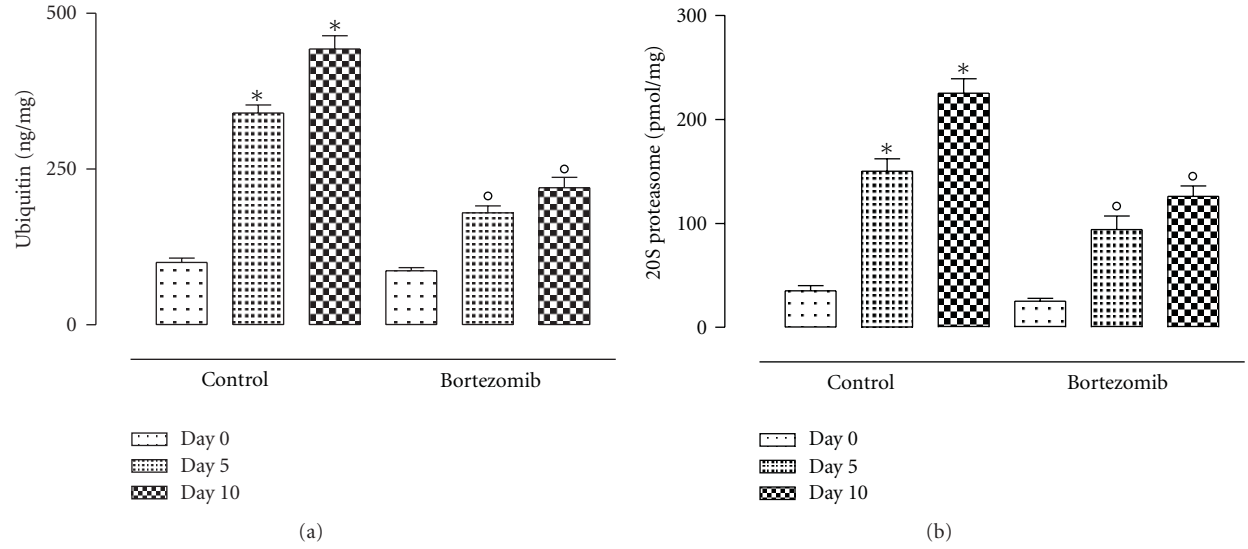

Figure 3: Levels (ng/mg tissue) of ubiquitin and 20S proteasome in the tissues from rats during peritoneal adhesions development. At time 0, 5, and 10 in the group treated with bortezomib by intravenous injection (0.05 mg/kg) given 1 hour before surgery, a second injection was given 1 hour after the surgical procedure (total dose of 0.1 mg/kg) and in the control group. The differences from day 0 are considered with $^{*}P < 0.01$, and the differences from the control group at the same day are considered as $^{\circ}P < 0.01$.

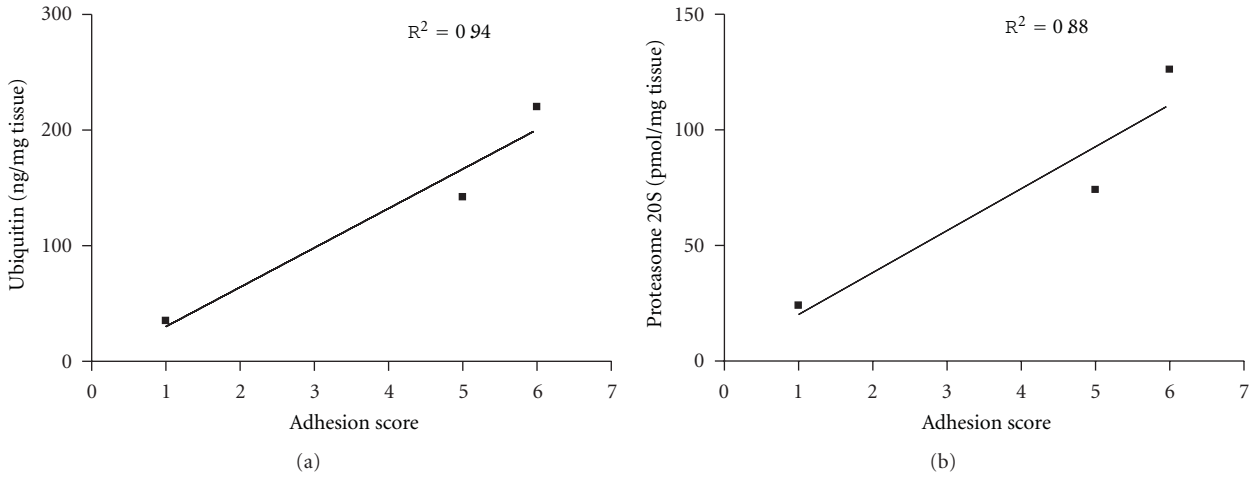

Figure 4: Correlation between the adhesion score and the local levels of ubiquitin and protesome 20S 10 days after the surgery.

UPS, generally known as the major pathway for nonlysosomal intracellular protein degradation in eukaryotic cells, was discovered in the eighties by the pioneering work of Goldberg, Hershko, and their collaborators, using reticulocyte lysates [18–20]. The UPS usually degrade proteins in two steps. First, the substrate is covalently modified by addition of a polyubiquitin chain, through an enzymatic cascade that involves three classes of factors: E1, the ubiquitin-(Ub-) activating enzyme [21], E2, a member of the family of Ub-carriers [22], and E3, a member of the very large family (several hundred members) of the so-called Ub-ligases [23], which specifically recognizes and recruits the substrate of the ubiquitylation reaction. Second, the ubiquitylated protein is usually addressed to and degraded by the 26S proteasome, a giant multisubunit and multicatalytic protease [24]. Due to its multiple roles, the proteasome is essential in eukaryotes and its dysfunction has deleterious effects for the cell or the organism as a whole. In humans, UPS deregulation has been implicated in a number of pathologies such as cancer, autoimmune diseases, neurodegenerative diseases, or viral infections. As a consequence, the proteasome is seen as a potential therapeutic target in many pathologies [25], including inflammation [26] and possibly now peritoneal adhesions.

In the present study is also shown that post-operative adhesions are associated with decreased levels of the inhibitory protein for the transcriptor factor nuclear factor kappa B (NF-kB), the IkB within the adhered tissues, indicating that one other component in the generation of adhesion is the activated NF-kB pathway, a central transcription factor

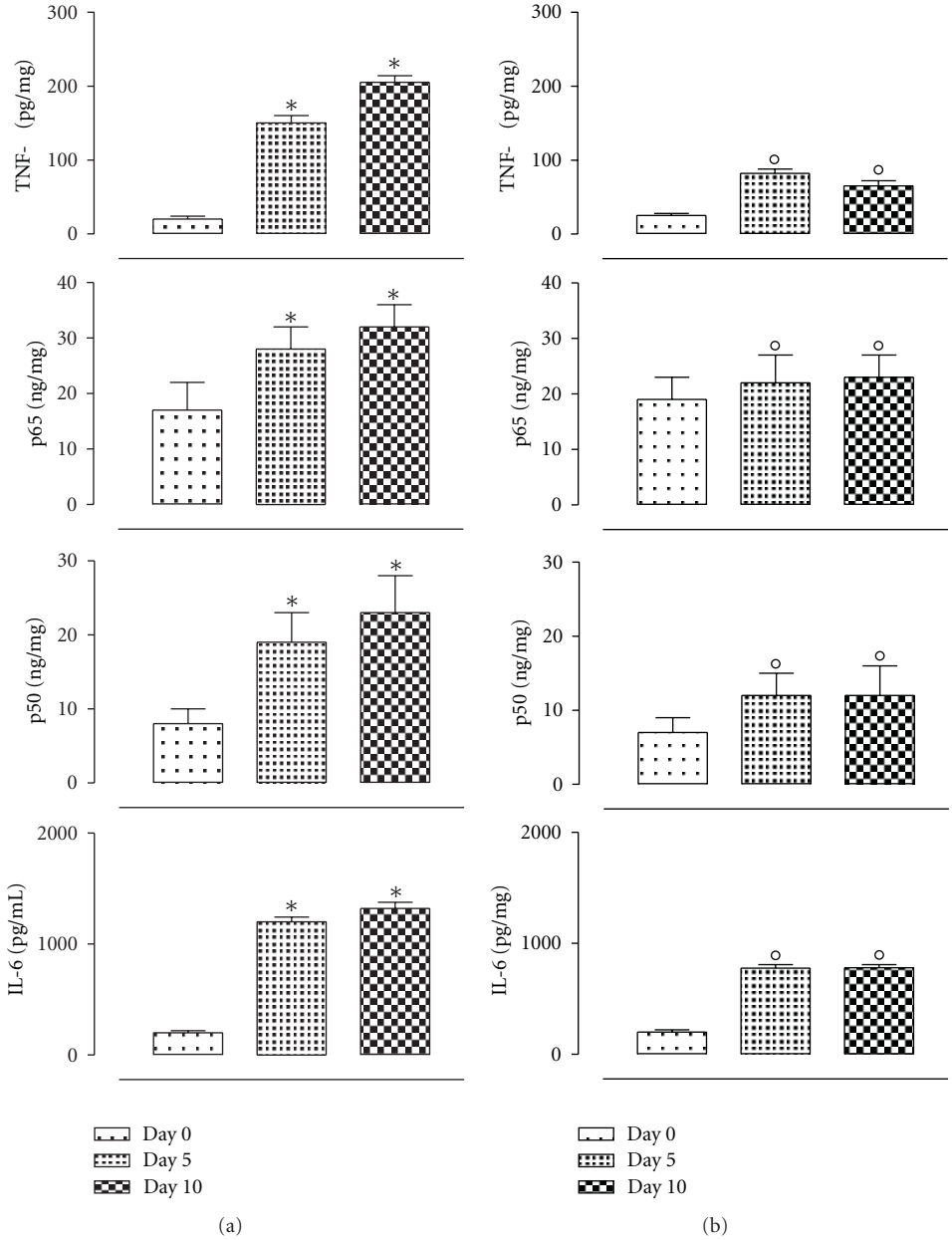

Figure 5: Levels (ng/mg tissue) of TNF-alpha, p50, p65, and IL-6 within the tissues from rats during peritoneal adhesions development at time 0, 5, and 10 days from the peritoneal surgery. The rats were treated or not with bortezomib by intravenous injection (0.05 mg/kg) given 1 hour before surgery, and a second injection was given 1 hour after the surgical procedure (total dose of 0.1 mg/kg). The differences from day 0 are considered with $^{*}P < 0.01$, and the differences from the control group at the same day are considered as $^{\circ}P < 0.01$.

that regulates inflammatory genes. Activated NF-kB pathway leads tissue deregulation and favours cell proliferation in many circumstances [27–29]. Interestingly, the UPS is required for activation of NF-kB by degradation of its inhibitory IkB proteins [30]. NFkB is normally bound to IkB in the cytosol; this binding prevents its movement into the nucleus [31]. Various cellular stimuli, such as inflammatory stimuli, induce ubiquitination of phosphorylated IkBs and subsequent degradation by the proteasome [32, 33]. Degradation of IkBs results in unmasking of the nuclear localization signal of NF-kB dimers, which subsequently translocates to the nucleus, where it induces the transcription of proinflammatory cytokines like IL-6 and TNF-α that play a central role in tissue injury [34]. Here we would suggest that a surgical insult may induce degradation of IkBs via ubiquitin-proteasome overactivity, thus enhancing NFkB activation. This in turn enhances the inflammatory potential of the injured tissue, worsening the peritoneal recovery. A contention is based on the fact that the levels of UPS, IkB, TNF-alpha, and IL-6 measured on adhesions biopsies seem to be related to the severity of the adhesion. Animals that show moderate levels of UPS, TNF-alpha, and IL-6 have also

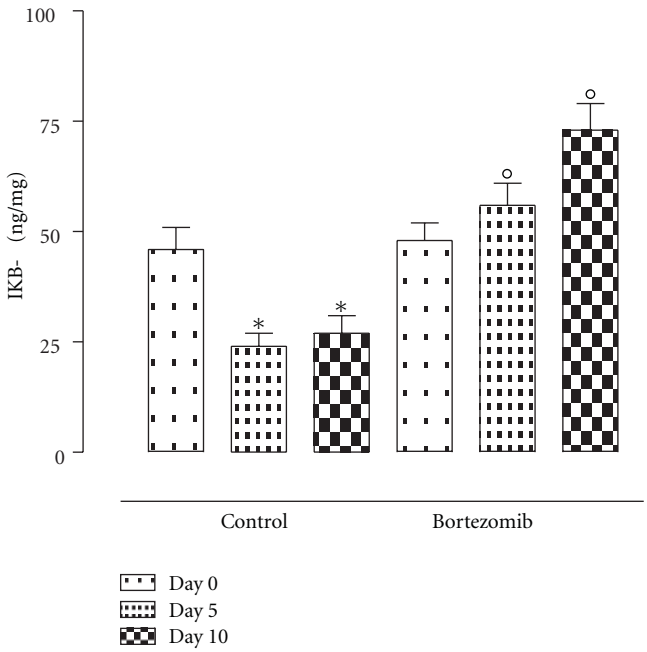

Figure 6: IKB-β levels (ng/mg tissue) within the tissues from rats during peritoneal adhesions development. Rats at time 0, 5, and 10 days from the surgery and treated or not with bortezomib. The differences from day 0 are considered with $^{*}P < 0.01$, and the differences from the control group at the same day are considered as $^{\circ}P < 0.01$.

less severe adhesions than those with higher levels of these same mediators.

Proof of concept for a central role of UPS in peritoneal adhesions is given by the protection from the adhesion formation achieved with the treatment of injured rats with bortezomib. This drug, initially proposed as anticancer drug [35], is a specific inhibitor of the UPS activity and exerts inhibition of the inflammation associated to the transcription factor NFκB, through stabilization of its inhibitor IκB [36], since NFκB is bound to its inhibitor IκB in the cytoplasm and is activated by proteasomal degradation of IκB. Inhibition of proteasome activity prevents degradation of IκB and subsequent activation and translocation of NFκB to the nucleus to activate downstream pathways [36]. Bortezomib here is able to reduce the number of rats showing adhesions and the score assigned to the adhesions, and as consequence of UPS inhibition, bortezomib also reduced the burden of inflammation occurring in the adhered tissue. At the same time point, in fact, rats treated with bortezomib had the lowest level of ubiquitin and proteasome 20S activity, NF-kB activation, inflammatory cells, and cytokine levels associated with the reduced adhesions formation.

In conclusion, post-surgical peritoneal adhesions formation is associated with local increased UPS system activity which is primarily involved in the formation of post-surgical peritoneal adhesions in rats and with a local inflammatory factors boost.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Activation of Peroxisome Proliferator-Activated Receptor-Gamma by Glitazones Reduces the Expression and Release of Monocyte Chemoattractant Protein-1 in Human Mesothelial Cells

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Human peritoneal mesothelial cells (MC) play an important role in inflammatory processes of the peritoneal cavity by producing various cytokines and chemokines, such as monocyte chemoattractant protein-1 (MCP-1). The present study was designed to assess the effect of the peroxisome proliferator-activated receptor-gamma- (PPARγ-) activator rosiglitazone on the mesothelial MCP-1 expression and release. Primary cultures of MC were obtained from omental tissue. MCP-1 antigen concentrations were measured in the cell supernatant by ELISA and MCP-1 mRNA levels by real-time polymerase chain reaction. The presence of PPARγ on MC was assayed in a Western Blot analysis. MC constitutively express PPARγ. Activation of this receptor via rosiglitazone (0,1–10 μmol/L) resulted in significantly reduced amounts of mesothelial MCP-1 release as well as MCP-1 mRNA. The use of the PPARγ inhibitor GW-9662 could completely prevent the rosiglitazone effects. Rosiglitazone was also effective in reducing TNFα-induced enhanced secretion of MCP-1. Our findings indicate that glitazones are effective in reducing constitutive and TNFα-stimulated mesothelial MCP-1 mRNA expression and release.

1. Introduction

Mesothelial cells (MC), which form the innermost monolayer of the peritoneal cavity, are critical in morphological and functional alterations of the peritoneal membrane in patients who undergo peritoneal dialysis (PD). They are a major source of intraperitoneal monocyte chemoattractant protein-1 (MCP-1) which is a chemokine that has been reported to play a key role in the recruitment of monocytes toward the peritoneal cavity [1]. Monocytes in turn contribute to peritoneal fibrosis by producing various cytokines and growth factors [2] that induce cell proliferation and extracellular matrix production in mesothelial cells and fibroblasts [3, 4].

Thiazolidinediones (TZD) are a novel group of antidiabetic agents that act via the activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ), a nuclear hormone receptor. PPAR-γ regulates a variety of metabolic pathways as a transcription factor [5]. Therefore, TZD like rosiglitazone are not only capable of increasing the insulin sensitivity in peripheral organs (as e.g., adipose tissue) and thus lowering blood glucose levels in diabetic patients; they also possess anti-inflammatory capacities in certain circumstances as they decrease the expression of inflammatory proteins like iNOS and MMP9 in macrophages [6, 7]. A reduced expression of MCP-1 due to TZD treatment has been shown for diverse cell types as, for example, lung epithelial [8], endothelial [9], and mesangial cells [10]. In this context, the present study was designed to investigate the presence of PPAR-γ on human peritoneal MC and to characterize the effect of PPAR-γ activation by TZD on mesothelial MCP-1 mRNA transcription and release.

2. Materials and Methods

2.1. Materials. Medium M199 and newborn calf serum were obtained from Gibco BRL (Eggenstein, Germany); tissue-culture plates came from Costar (Cambridge, Massachusetts, USA). Human serum was prepared from freshly collected blood of healthy donors and stored at –20°C. Fibronectin from human serum, trypsin, and TNFα were purchased from Boehringer (Mannheim, Germany), collagenase type II from Worthington (Freehold, NY, USA).

Monoclonal antibodies against cytokeratins 8 and 18 as well as vimentin were a gift from Dr. G. van Muijen (University of Nijmegen, The Netherlands). Antibody against PPAR-γ was from Santa Cruz Biotechnology (Santa Cruz, California, USA). MTT was from Sigma-Aldrich (St. Louis, MO, USA). Rosiglitazone was from Molekula (Nienburg/Weser, Germany), and GW-9662 was purchased from Cayman chemical (Ann Arbor, Michigan, USA).

2.2. Cell Culture Experiments. MC were isolated from the omental tissue of consenting patients undergoing elective surgery, as described previously [11]. The patients were free from peritonitis or peritoneal carcinosis. Cells were grown in fibronectin-coated dishes in M199 medium supplemented with 25 mmol/L HEPES (pH 7.3), 2 mmol/L glutamine, 10% (v/v) human serum and 10% (v/v) newborn calf serum (heat inactivated), penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C under 5% CO^2/95% air atmosphere. The medium was replaced every 2 to 3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1 : 3. The cells were purely MC, as assessed by their uniform cobblestone appearance at confluence, by the absence of von Willebrand factor, and by their uniform positive staining for cytokeratins 8 and 18 as well as for vimentin. For the experiments, confluent cultures were used at the second or third passage, and cells were refed with 2% human serum 24 hours before the experiment. Conditioned media were obtained by incubating cells in 2 cm^2 dishes at 37°C with 0.5 mL serum-free M199 containing the appropriate concentration of the test compound. Serum-free medium M199 served as a control. In coincubation experiments with TNFα, cells were preincubated for 24 hours with rosiglitazone, and then TNFα was added. In experiments using a PPAR-γ blocker, cells were preincubated with GW-9662 for 24 hours. Supernatants were centrifuged 5 minutes at 2000 ×g to remove cells and cellular debris, and the samples were frozen at –20°C until use. All experiments were done with cells from 3 to 6 individual donors and were measured in triplicate.

2.3. Western Blot Analysis. Cultured human mesothelial cells were harvested with lysis buffer (50 mM Tris-HCl pH7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, and 1 mM Na3VO4, Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany)). Extracted proteins were boiled in loading buffer for 30 min, resolved by 8% SDS-PAGE under reducing conditions, and transferred to an Immobilon-P membrane (Millipore, Eschborn, Germany). The membrane was blocked with 3% skim milk, incubated in a 1 : 1000 dilution of a rabbit polyclonal IgG-antibody against human PPARγ-1 (SC-7196, Santa Cruz Biotechnology, Heidelberg, Germany) over night, and rinsed with PBS containing 0.1% Tween 20. Immune complexes were visualized using enhanced chemoluminescence (ECL, Amersham Biosciences, Freiburg, Germany). Human breast carcinoma cell lysate protein extract served as positive control for PPARγ-1 detection.

2.4. MTT Assay. Human mesothelial cells (30×10^3/100 μL medium) were cultured in 96-well microtiter plates for 24 h under standard conditions to yield firmly attached and stably growing cells. After discarding supernatants, 50 μL of medium M199 containing rosiglitazone in concentrations of 1 and 10 μg/L or medium M199 as a control was added to the cells and incubated for 48 h. Then 50 μL of a 1 mg/mL solution of (3,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) MTT (SIGMA-ALDRICH, Taufkirchen, Germany no. M2128) was added. After 3 h incubation at 37°C, formazan crystals were dissolved by addition of 100 μL isopropanol and 0.04 N HCl. Absorbance was then measured at 590 nm using a GENios plus TECAN ELISA reader. For each experiment at least 6 wells were analyzed per experimental condition.

2.5. MCP-1 Assay. MCP-1 antigen levels [pg/10^5 cells] were measured by Quantikine human MCP-1 immunoassay from R&D Systems (Minneapolis, MN, USA). Diluted aliquots of the cell supernatants were assayed without prior purification.

2.6. RNA Isolation and Real-Time Quantitative RT-PCR. Total RNA was extracted from cells using silica gel columns (RNeasy, Qiagen, Hilden, Germany). 2 μg of isolated total RNA underwent random hexamer-primed reverse transcription for one hour at 42°C using a modified Molony murine leukaemia virus (MMLV) reverse transcriptase (Superscript, Life Technologies, Karlsruhe, Germany). Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed on a Taq-Man ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems, Darmstadt, Germany). Thermal cycler conditions contained holds for two minutes at 50°C and ten minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and one minute at 60°C. Message expression was calculated following the $\Delta\Delta$Ct procedure [12]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (rRNA) served as the reference housekeeping gene. Controls consisting of H_2O or samples that were not reverse transcribed were negative for the target and reference. Sequences with following gene bank accession numbers served for the design of the predeveloped Taq Man assay reagents (PDAR) or primers and probe, purchased from Applied Biosystems (Darmstadt, Germany): X14768 (human MCP-1/CCL2) M33197 (human GAPDH) and X03205.1 (human18s-rRNA).

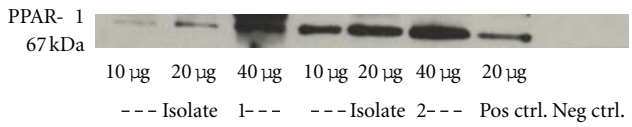

Figure 1: Western Blot analysis of PPARγ-1 in two different isolates o unstimulated mesothelial cells. Total protein was extracted from unstimulated peritoneal mesothelial cells or human breast carcinoma cells (positive control) and were analysed using the Western Blot technique. The amount of total protein loaded is indicated. Pos. ctrl: positive control; neg. ctrl: negative control.

2.7. Statistical Analysis of the Data. Data are given as mean ± SD. Statistical analysis was performed using the ANOVA analysis. A P value < 0.05 was considered to indicate statistically differences.

3. Results

3.1. Detection of Constitutive PPAR-γ Expression in MC. We evaluated the presence of PPAR-γ1 on human MC via Western Blot. Analysis of the extracted total protein of unstimulated MC showed a single band at 67 kDa as has been described for PPAR-γ1 and was comparable to the recommended positive control (protein from human breast carcinoma cells) (Figure 1).

3.2. Effect of Rosiglitazone on the Secretion of MCP-1 in Unstimulated MC. Confluent unstimulated MC were incubated with increasing concentrations of rosiglitazone (0.1–10 μmol/L) for 48 hours. This resulted in a concentration-dependent decrease of MCP-1 protein levels in the cell culture supernatants. Rosiglitazone treatment reached statistical significancy at each employed concentration: A concentration of 0.1 μmol/L reduced the MCP-1 level to 13200 pg/10^5 cells versus control 18200 pg/10^5 cells ($P = 0.013$) whereas a concentration of 1 μmol/L reduced MCP-1 levels to 12700 pg/10^5 cells ($P = 0.001$) and a concentration of 10 μmol/L to 9400 pg/10^5 cells ($P < 0.001$) (Figure 2(a)). Rosiglitazone treatment in the shown concentrations did not effect cell viability as assessed in trypan blue staining or cell proliferation as assessed in an MTT assay (Figure 3).

3.3. Effect of Rosiglitazone on the MCP-1 mRNA Levels in Unstimulated MC. The incubation of MC with 10 μmol/L rosiglitazone resulted in a marked decrease of MCP-1 mRNA after a 4 hours' dwell time (0.09 versus 1) (Figure 2(b)). A longer incubation time (8 hours) and the use of another housekeeping gene (GAPDH) resulted in comparable findings (data not shown).

3.4. Impact of PPAR-γ Inhibition. In order to investigate whether the effect of TZD on the mesothelial MCP-1 release is dependent on the TZD property to activate PPAR-γ, MC were preincubated with the PPAR-γ inhibitor GW-9662 (10 μmol/L) or control media for 24 hours. Afterwards, cells were incubated with control media, rosiglitazone 10 μmol/l, GW-9662 10 μmol/L, or the combination of GW-9662 and rosiglitazone for 8 hours. In accordance with longer incubation times (Figure 2), rosiglitazone treatment led to a significant decrease in MCP-1 protein (1700 pg/10^5 cells versus control: 2660 pg/10^5 cells; $P = 0.028$). GW-9662 did not have an effect on MCP-1 secretion and resulted in similar MCP-1 protein levels compared to control. The blocking of PPAR-γ by administration of GW-9662 before a rosiglitazone application could completely prevent the rosiglitazone-induced attenuation in MCP-1 release (2870 pg/10^5 cells versus 1700 pg/10^5 cells; $P = 0.039$) (Figure 4(a)).

Corresponding results could be found on the transcriptional level: whilst rosiglitazone markedly reduced MCP-1 mRNA levels (0.28 versus 1), PPAR-γ blockade by GW-9662 resulted in considerably higher MCP-1 mRNA levels (0.62). GW-9662 alone had no effect on the MC MCP-1 mRNA production, as the administration resulted in a MCP-1 mRNA level comparable to the control (0.86) (Figure 4(b)).

3.5. Effect of Rosiglitazone on TNFα-Induced Enhanced Mesothelial MCP-1 Release. The incubation of MC with TNFα (100 U/mL) resulted in a drastic increase in mesothelial MCP-1 secretion (67500 pg/10^5 cells versus control 4000 pg/10^5 cells). Prior incubation with rosiglitazone 10 μmol/L and subsequent coincubation resulted in a one-third reduction in mesothelial MCP-1 levels (43000 pg/10^5 cells versus 67500 pg/10^5 cells) (Figure 5).

4. Discussion

MC are supposed to be critical in the pathogenesis of complications following PD treatment. By producing profibrotic [13, 14] and neoangiogenetic factors [15], they contribute to peritoneal fibrosis. In addition, MC are a major source of intraperitoneal MCP-1 and thus account for the recruitment of monocytes toward the peritoneal cavity [1]. Apart from an inflammatory reaction, this invasion contributes to peritoneal fibrosis by producing various cytokines and growth factors [2]. MCP-1 can be found in markedly elevated concentration in the dialysate of PD patients during and after episodes of peritonitis [16, 17]. IL-1β, IFNγ, and TNFα as well as high glucose concentrations (due to high osmolality and the polyol pathway) are found to increase the mesothelial MCP-1 synthesis rate [18, 19]. TZD are activators of PPAR-γ. This receptor is a member of the nuclear receptor family that includes 48 human transcription factors regulated by direct binding of steroid and thyroid hormones, vitamins, lipid metabolites, and xenobiotics [20]. By differential promotor usage and splicing two isoforms are generated: PPAR-γ1 which can be found on a variety of cell types and PPAR-γ2, which has an additional 30 amino acids at its N-terminal end and is expressed specifically in adipocytes [21]. We could now demonstrate the constitutive expression of PPAR-γ1 on human MC. In PD patients, there are some experiences with TZD: Lin et al. described that rosiglitazone improved glucose metabolism in nondiabetic uremic patients on CAPD [22], and Wong et al. found reduced insulin requirement and C-reactive protein levels in

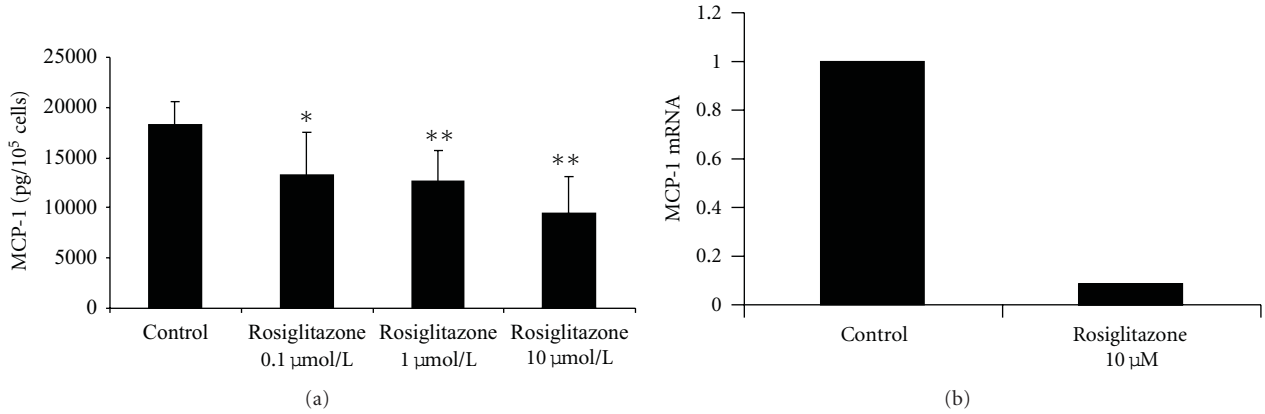

Figure 2: (a) Effect of rosiglitazone on mesothelial MCP-1 release. MC were stimulated with rosiglitazone in the given concentrations for 48 hours. MCP-1 antigen was measured in the cell culture supernatants using ELISA technique. *indicates a P value < 0.05; **indicates a P value < 0.01 ($n = 6$). (b) Effect of rosiglitazone on mesothelial MCP-1 mRNA levels. MC were incubated with rosiglitazone 10 μM for 4 hours. Total RNA was extracted and analysed via RT-PCR. MCP-1 mRNA levels were adjusted to the housekeeper rRNA and are expressed as relative to the control. The figure is a representative of three independent experiments.

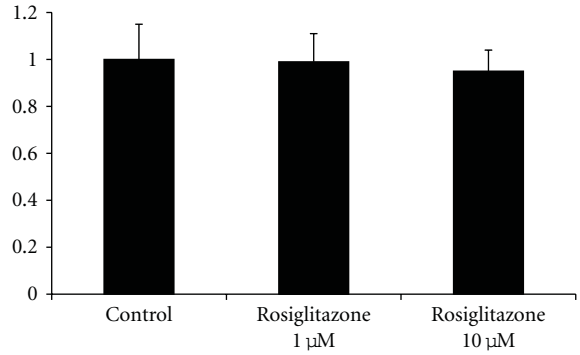

Figure 3: Effect of rosiglitazone on mesothelial cell viability and proliferation. MC were stimulated with rosiglitazone in the given concentrations for 48 hours, and then a MTT test was performed as described in the Methods section. Results are described in relation to the control.

type 2 diabetic patients receiving peritoneal dialysis [23]. In the present study, we could demonstrate that TZD are able to reduce the constitutive MCP-1 release in MC by PPAR-γ stimulation. Furthermore, rosiglitazone was able to attenuate enhanced MCP-1 secretion resulting from a stimulation with the proinflammatory cytokine TNFα. Peng et al. found a decrease in high glucose concentration-induced mesothelial production of TGFβ, collagen I, and fibronection secretion after treatment with troglitazone [14]. Some in vivo animal model studies point toward positive TZD effects on the peritoneal membrane in PD or peritoneal inflammation: Yao et al. found maintained peritoneal morphology and increased ultrafiltration after intraperitoneal administration of rosiglitazone in comparison to commercial PD solution alone [24]. Sandoval et al. described a reduction in the accumulation of AGEs as well as reduced fibrosis and angiogenesis resulting in an improved peritoneal function [25]. In accordance with our findings, Hornung et al. were able to demonstrate that the intraperitoneal administration of ciglitazone was able to significantly reduce the number of invading peritoneal macrophages following a thioglycollate-induced peritoneal inflammation [26]. PPAR-γ stimulation in MC may be a promising possibility in the attempt to minimize long-term PD complications. However, potential negative effects of the TZD (the only commercially available PPAR-γ activators at present) such as their ability to cause edema and their negative cardiovascular risk profile [27] should be considered critically.

5. Conclusions

PPAR-γ1 protein is expressed on human peritoneal MC. Its activation via rosiglitazone decreases the mesothelial release and mRNA expression of MCP-1 and attenuates the TNFα-induced enhancement in MCP-1 release in these cells. Therefore, the PPAR-γ1 receptor may be a therapeutic target to ameliorate peritoneal inflammation and long-time

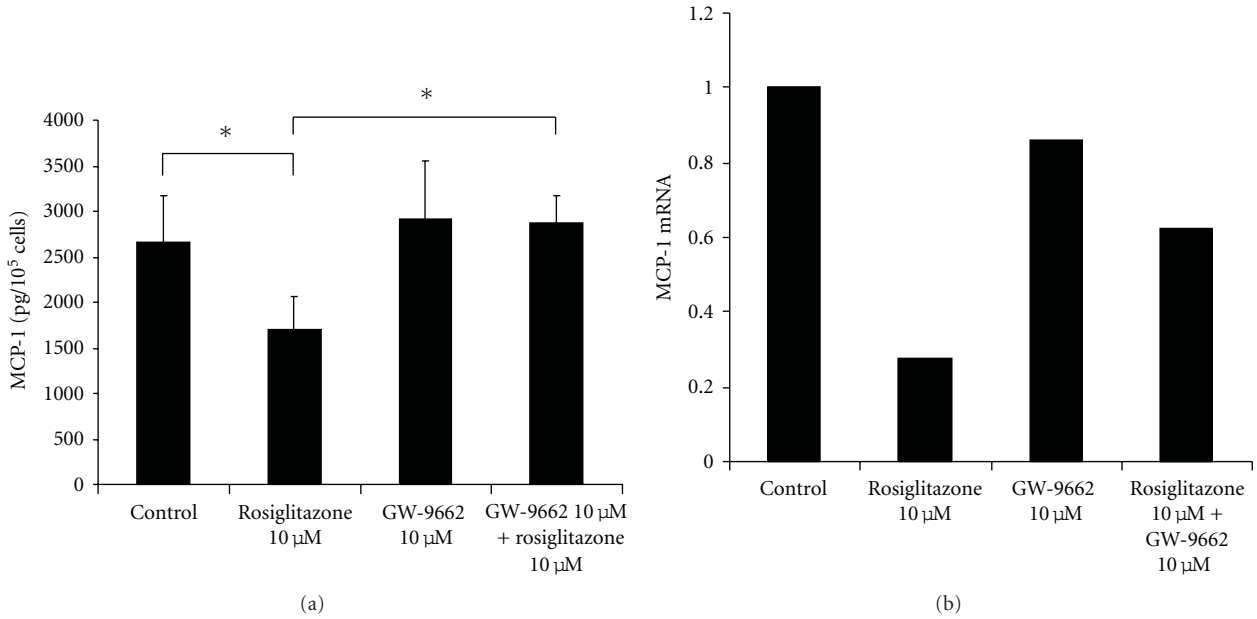

Figure 4: (a) PPAR-γ-dependency of the rosiglitazone effect on mesothelial MCP-1 release. MC were preincubated with GW-9662 10 μM for 24 hours (or with control media) and then incubated with the given conditions for 8 hours. Cell supernatants were examined by ELISA. *indicates a P value < 0.05 ($n = 5$). (b) PPAR-γ-dependency of the rosiglitazone effect on mesothelial MCP-1 mRNA expression. MC were preincubated with GW-9662 10 μM for 24 hours (or with control media) and then incubated with the given conditions for 8 hours. Total RNA was extracted and examined by RT-PCR. The figure shows a representative experiment out of three independent ones.

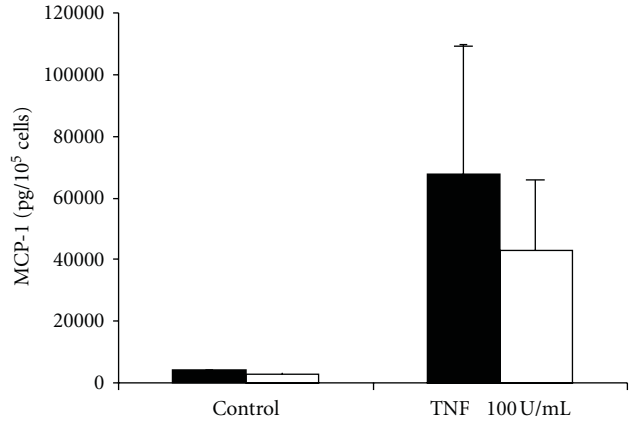

Figure 5: Effect of rosiglitazone on TNFα-induced enhancement of mesothelial MCP-1 secretion. MC were preincubated with rosiglitazone 10 μM (white bars) or with control medium (black bars) for 24 hours. Afterwards, the culture media was replaced with media containing rosiglitazone 10 μM (white bars) or not (black bars) to the given conditions. After an incubation time of 8 hours, culture supernatants were analysed by ELISA ($n = 3$).

survival of the peritoneal membrane in PD. However, potential negative systemic effects of the TZD have to be considered critically.

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Research Article

Mast Cell-Mediated Inhibition of Abdominal Neutrophil Inflammation by a PEGylated TLR7 Ligand

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Although the mechanisms for sustained chemokine gradients and recurring cell infiltration in sterile peritonitis have not been elucidated, toll-like receptors (TLRs) have been implicated. To abate the deleterious recruitment of neutrophils in sterile inflammation, we repeatedly administered a TLR7 ligand that hyposensitized to TLR7 and receptors that converged on the MyD88-signaling intermediary and reduced cellular infiltration in murine autoimmune models of multiple sclerosis and arthritis. To reduce potential adverse effects, a polyethylene glycol polymer was covalently attached to the parent compound (Tolerimod1). The proinflammatory potency of Tolerimod1 was 10-fold less than the unconjugated TLR7 ligand, and Tolerimod1 reduced neutrophil recruitment in chemically induced peritonitis and colitis. The effects of Tolerimod1 were mediated by the radioresistant cells in radiation chimeric mice and by mast cells in reconstituted mast cell-deficient mice ($Kit^{W\text{-}sh}$). Although the Tolerimod1 had weak proinflammatory agonist activity, it effectively reduced neutrophil recruitment in sterile peritoneal inflammation.

1. Introduction

The inflammatory response is a major host defense mechanism to prevent infection or to repair injury [1]. However, in the context of sterile inflammation the perpetual recruitment of neutrophils into the local environment can lead to deleterious sequelae from proteases and other products released from their granules [2]. The early events of inflammation include increased vascular permeability and enhanced immune cell mobility to allow cells and proteins to access the site of inflammation [3]. Chronicity is established when sustained levels of chemokines attract the influx of neutrophils and monocytes. Early disruption of this recurring cycle and reduction of neutrophil recruitment could suppress subsequent infiltration of other types of immune cells and could then prevent tissue damage [4].

The myeloid differentiation primary response gene 88- (MyD88) signaling pathway has been implicated in perpetuating the inflammatory response in experimental peritonitis and colitis. This pathway is shared by the TLRs except TLR3, which uses the MyD88-independent TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway [5]. In a previous study, we demonstrated that chronic administration of low doses of a synthetic TLR7 ligand (9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine; designated here as 1V136) provided pharmacological suppression of the MyD88-signaling pathway and subsequently reduced the severity of inflammation in mouse models of autoimmunity [6].

We have synthesized modifications of 1V136 to study the effects on potency and alter the pharmacodynamics to reduce potential toxicities [7]. One compound that was modified with a 6 unit PEG moiety had reduced ability to induce inflammatory cytokines *in vitro* and *in vivo* and was designated Tolerimod1 [7]. TLR-mediated signals can be ultimately protective in models of acute intestinal inflammation and considered to be therapeutic targets [8, 9]. Hence Tolerimod1 was selected to test our hyposensitization strategy to limit inflammatory recruitment in mouse models of peritonitis and inflammatory bowel disease.

In the present study, the hyposensitization effect of repeated dosage with the parent compound 1V136 was confirmed in the murine acute dextran sodium sulfate (DSS)-colitis model. The anti-inflammatory effects of Tolerimod1 were also tested in the DSS-colitis and thioglycolate (TG)-elicited peritonitis models. The PEGylated compound was equivalent to the parent compound in its ability to reduce peritoneal inflammation and DSS-colitis. Radiation chimeras using wild-type (WT) and $Tlr7^{-/-}$ mice indicated that this effect was mediated by the radioresistant cells in the recipient and not by the transferred bone marrow cells. In addition mast cell-deficient mice ($Kit^{W\text{-}sh}$) were relatively refractory to the therapeutic treatment. However, the treatment response was recapitulated in mice that had been reconstituted with bone marrow cells enriched for mast cells. These results implicate mast cells as a primary effector cell for Tolerimod1 activity *in vivo*.

2. Materials and Methods

2.1. Animals. C57BL/6 (wild type, WT) and $Kit^{W\text{-}sh}$/HNihr-JaeBsmJ mice were purchased from the Jackson Laboratories (Bar Harbor, MA, USA). $Tlr7^{-/-}$ mice were a gift from Dr. Akira (Osaka University, Japan) and backcrossed for 10 generations onto the C57BL/6 background at University of California, San Diego (UCSD). Bone marrow chimeras were generated by injecting 10^7 bone marrow cells intravenously into whole-body irradiated (950 cGy) recipient mice [6]. $Kit^{W\text{-}sh}$ mice were engrafted with mast cells by intravenous (i.v.) injection of 10^6 mast cells as previously described [10]. All animal experiments were approved by the UCSD Institutional Animal Care and Use Committee.

2.2. Reagents. Dextran sodium sulfate (DSS) was purchased from Sigma (St Louis, MO). Thioglycolate (TG) medium, PBS, RPMI 1640 medium, and DMEM were purchased from Invitrogen (Carlsbad, CA, USA). RPMI 1640 and DMEM were supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. 1V136 (unconjugated TLR7 ligand) and PEGylated TLR7 ligand (Tolerimod1, TMX-302, described as compound 1b in [7]) were prepared in our laboratory as previously described [7, 11]. 1V136 and Tolerimod1 were dissolved in DMSO as a 100 mM stock solution and kept at -20°C until use. Endotoxin levels in all reagents were measured using Endosafe (Charles River laboratory, Wilmington, MA, USA). Endotoxin levels of the compounds were <0.05 EU/μmol.

2.3. Compound Activity Tests. The RAW264.7 mouse monocyte macrophage cell line was obtained from ATCC (Rockville, MD, USA) and cultured in DMEM (Irvine Scientific, Irvine, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin/100 μg/mL streptomycin). Primary bone marrow-derived dendritic cells (BMDCs) were prepared from C57BL/6 mice as described [12]. The cells (1×10^6/mL) were incubated with the indicated compound for 18 hours at 37°C, 5% CO_2, and culture supernatants were collected. IL-6, IL-10, IL-1β, TNFα, and KC were measured from the supernatants or sera by ELISA (BD Bioscience, or eBioscience, San Diego CA, USA). Minimum detection levels of these factors ranged from 5 to 15 pg/mL.

2.4. Induction of DSS-Colitis. WT mice were given 2% (wt/vol) DSS dissolved in sterilized, distilled water *ad libitum* for 7 days. Mice were injected subcutaneously (subcutaneously) with daily doses of 1V136 (150 nmol), Tolerimod1 (200 nmol) or vehicle on days 0 to 7. On day 8, mice were sacrificed and disease activity index (DAI; the combined score of weight loss and intestinal bleeding) was determined as described previously [13].

2.5. Induction of TG-Induced Peritonitis. Mice were injected s.c. with a single dose (day-1 or -3) or three daily doses of 1V136 (150 nmol) or Tolerimod1 (200 nmol). On day 0, mice were intraperitoneally (i.p.) injected with 2 mL 4% TG medium. Peritoneal exudates were collected at 3 h with 3 mL cold PBS. Total cell number was determined by counting with a hematocytometer. Peritoneal cells were cyto-centrifuged and stained with Wright-Giemsa to determine differential leukocyte counts.

2.6. Histological Analysis. Colons were fixed in buffered formalin and embedded in paraffin. Five μm sections were cut and stained with hematoxylin and eosin (H&E). The sections were stained for myeloperoxidase (MPO) using rabbit anti-MPO antibody (Abcam, Cambridge, MA) and horseradish peroxidase-(HRP) conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) by the Histology and Immunohistochemistry Shared Resources at the Moores UCSD Cancer Center.

2.7. Myeloperoxidase (MPO) Assay. MPO activity (kinetic assay) was performed as previously described with minor modifications [14]. Briefly, colon tissues were homogenized in 0.5% hexadecyltrimethyl-ammonium bromide (Sigma) in 50 mM phosphate buffer, pH 6.0. The homogenate was sonicated for 10 seconds, frozen and thawed 3 times, and then centrifuged for 15 minutes. Protein concentration in the supernatants was quantified by bicinchoninic acid assay (Thermo Scientific Pierce). The supernatants were diluted 1 : 30 in reaction buffer (0.68 mM O-dianisidine, 50 mM potassium phosphate buffer, pH 6.0, 29 mM H_2O_2), and MPO activity was measured as the absorbance at 460 nm during the first 2 minutes, and expressed as OD per minute per mg of protein.

2.8. Assessment of Vascular Permeability. Briefly, mice were intravenously injected with 200 μL 0.625% Evans blue solution 10 min before TG injection. After two hours peritoneal lavage was performed using 2 mL cold PBS, and absorbance at 620 nm was measured.

2.9. Statistical Analysis. The data are represented as means ± standard errors of the mean (SEM). The Mann-Whitney test was used to compare two groups, and one-way ANOVA with Dunnett's *post hoc* test was used for multiple comparisons

to a control group using Prism 4 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Repeated Treatment with a TLR7 Ligand-Reduced Disease Severity in DSS-Colitis. We previously demonstrated that the repeated administration of a TLR7 ligand ameliorated joint inflammation in the K/BxN serum-transferred arthritis model and reduced inflammatory cell influx in the EAE model [6]. To examine this effect on sterile abdominal inflammation, we tested this treatment regimen in the DSS-colitis model. Mice were treated daily with 150 nmol s.c. 1V136 (unconjugated TLR7 ligand) starting on day 0 until the end of the experiment (Figure 1(a)). This was the lowest s.c. dosage that was effective when administered s.c. in the K/BxN serum transferred arthritis model [6]. Mice treated with daily 1V136 exhibited significantly lower DAI ($P = 0.005$, Figure 1(b)) and less body weight loss ($P = 0.005$, Figure 1(c)). Decreased disease severity was concordant with decreased MPO activity in the colons of 1V136-treated mice (Figure 1(d)). Histological examination showed fewer ulcerative lesions and less inflammatory cell infiltration in the colons of 1V136-treated mice compared to vehicle-treated mice (Figure 1(e)).

3.2. Tolerimod1 Reduced Neutrophil Recruitment in TG-Elicited Peritonitis. In a previous study, we observed that a higher dose (500 nmol) of 1V136 administered intraperitoneally or intranasally resulted in TLR7-dependent adverse effects [10]. To limit these adverse effects, we prepared several modified forms of the parent compound and tested them *in vitro* and *in vivo* for chemokine and cytokine release [7]. Amongst these compounds, one containing a 6-unit PEG chain, was a weakly active TLR7 agonist (Tolerimod1). Dose titration studies are shown with a murine macrophage cell line, RAW264.7 cells, and primary murine BMDCs stimulated with Tolerimod1 and 1V136 for cytokine release. Tolerimod1 ($EC50_{RAW} = 4.8\,\mu M$ and $EC50_{BMDC} = 3.9\,\mu M$) was 10 times less potent than 1V136 ($EC50_{RAW} = 0.37\,\mu M$ and $EC50_{BMDC} = 0.3\,\mu M$) (Figures 2(a) and 2(b)).

The unconjugated parent TLR7 ligand (1V136) reduced MPO-positive cell infiltration in DSS-colitis (Figure 1(d)), indicating that 1V136 treatments attenuated neutrophil recruitment into the colon. To further examine the mechanism of Tolerimod1 effects on neutrophil recruitment, Tolerimod1 was used in the TG-elicited peritonitis model (Figures 2(c) and 2(d)). Mice received three daily treatments (days-3, -2, and -1), or a single treatment on day-1 or day-3 of Tolerimod1, and peritonitis was induced on day 0. Tolerimod1 treatment reduced overall cell infiltration (Figure 2(c)), primarily neutrophil influx (Figure 2(d)) into the peritoneal cavity. In addition mice that were pretreated with daily s.c. (200 nmol) or oral (200 nmol) Tolerimod1 for three days and then were injected with TG intraperitoneally had a significant reduction in total neutrophil count after 3 h ($2.33 \pm 0.33 \times 10^{6*}$, and $2.39 \pm 0.38 \times 10^{6*}$, respectively, compared to $4.60 \pm 0.51 \times 10^6$ in the vehicle control; $^*P < 0.05$ by one-way ANOVA). However, the number of infiltrating monocytes after 3 days daily s.c. (200 nmol) of pretreatment was not significantly reduced ($2.43 \pm 0.30 \times 10^6$ versus $3.1 \pm 0.5 \times 10^6$ in the vehicle control).

3.3. Reduction in Peritoneal Neutrophil Influx Was Associated with Reduced Local Chemokine Levels. The immune cell recruitment is influenced by numerous factors, such as local chemokine secretion and vascular permeability. Hence, we examined the levels of chemokines and cytokines in sera and peritoneal lavages 3 h after TG peritonitis induction. Tolerimod1 treatment significantly reduced KC levels in the lavage (Figure 3(a)), but IL-1β levels were not reduced (Figure 3(b)). In contrast, serum levels of KC and IL-6 were not influenced by Tolerimod1 treatments (Figures 3(c) and 3(d)). IL-1β, TNFα, and IL-10 in the sera and peritoneal lavage and IL-6 in the peritoneal lavage were below detectable levels (data not shown). Tolerimod1 treatment did not alter the vascular permeability as measured by Evans blue dye extravasation (Figure 3(e)).

3.4. Radioresistant Mast Cells Were Involved in Anti-Inflammatory Effects of Tolerimod1. Since Tolerimod1 treatment influences local chemokine levels, but not systemic levels of chemokines or cytokines, we thought it important to identify the cell types in the local tissue involved in the effects of Tolerimod1. Tolerimod1 treatment in $Tlr7^{-/-}$ mice did not reduce TG-elicited recruitment of inflammatory cells (Figures 4(a) and 4(b)), indicating that the treatment effects of Tolerimod1 were TLR7 dependent. To further test the involvement of hematopoietic cells in the effects of Tolerimod1 treatment, $Tlr7^{-/-}$ and WT (C57BL/6) radiation bone marrow chimeric mice were generated. Interestingly, reduced peritoneal neutrophil recruitment after TG injection was observed in the WT donor $\rightarrow$ $Tlr7^{-/-}$ recipients, but not in $Tlr7^{-/-}$ donor $\rightarrow$ WT recipients (Figures 4(c) and 4(d)), suggesting that the radioresistant cell population was predominantly mediating the anti-inflammatory effects of Tolerimod1.

Mast cells are relatively radioresistant and involved in many inflammatory processes associated with an increase in vascular permeability [15–17]. To examine the potential role of mast cells, mast cell-deficient $Kit^{W\text{-}sh}$ mice were treated with Tolerimod1 for 3 days, and peritonitis was induced. Tolerimod1 treatment did not reduce neutrophil infiltration into the peritoneum of $Kit^{W\text{-}sh}$ mice (Figure 4(e)). Furthermore, the response to Tolerimod1 treatment in $Kit^{W\text{-}sh}$ mice was restored by reconstitution of WT mast cells into $Kit^{W\text{-}sh}$ mice (Figure 4(e)). Of note is the relative neutrophilia in $Kit^{W\text{-}sh}$ mice, which appeared as a trend in the higher neutrophil recruitment in the mast cell replete mice [18].

3.5. Tolerimod1 Reduced Severity of DSS-Colitis. Repeated administration of unconjugated TLR7 ligand, 1V136, reduced neutrophil inflammation in DSS-colitis (Figure 1). Our data indicate that the inhibitory effect of Tolerimod1 on neutrophil infiltration was at least in part mediated by mast cells, which are known to be involved in intestinal inflammation in DSS-colitis [19]. Hence, we treated DSS-colitis mice with Tolerimod1 s.c. or orally with daily Tolerimod1 for

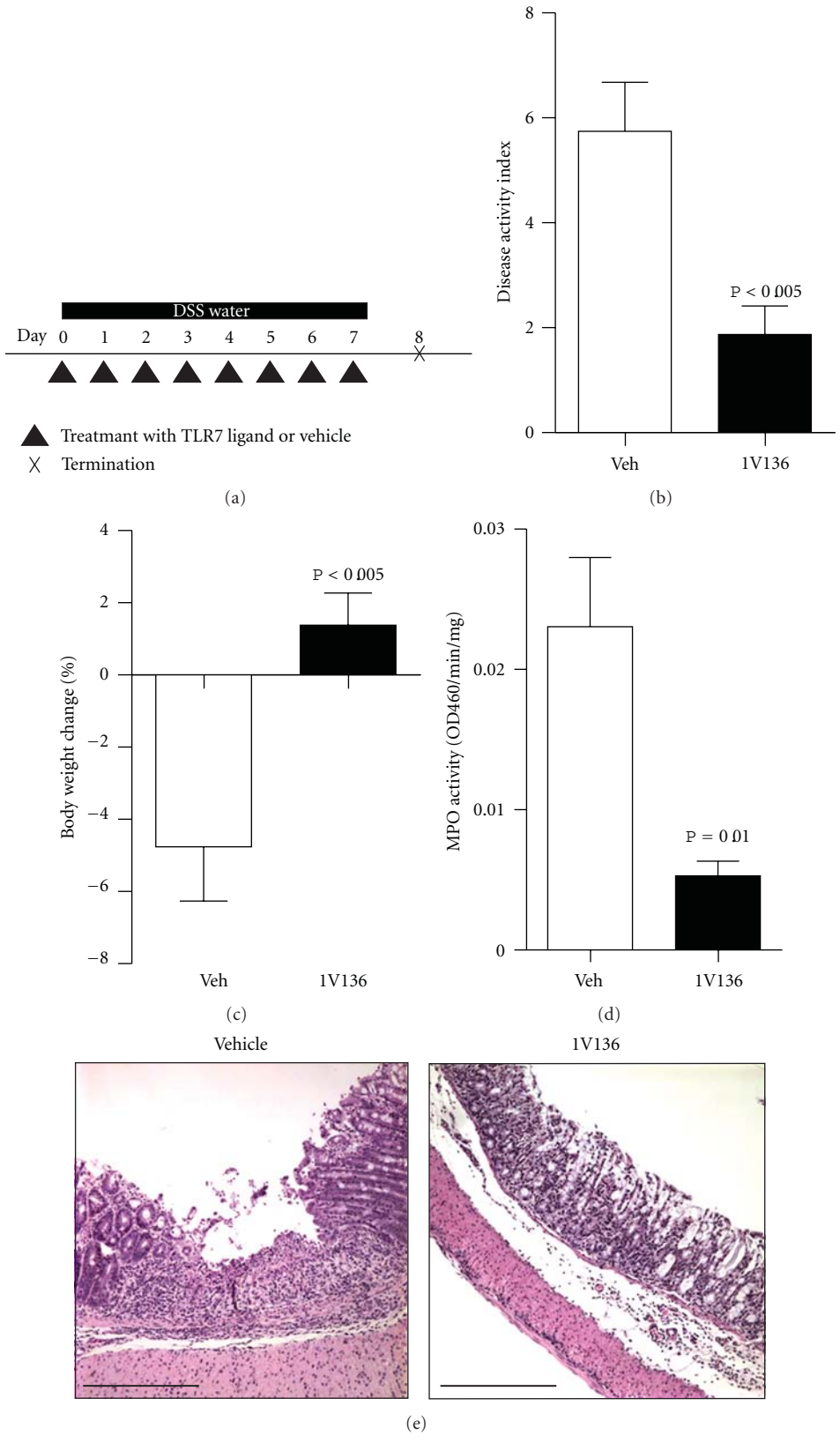

Figure 1: TLR7 ligand hyposensitization reduces the inflammation of DSS-induced experimental colitis. (a) Experimental protocol of induction of DSS-induced colitis and treatment regimen. WT mice ($n = 5$) received 2% DSS for 7 days. Mice were s.c. treated with 150 nmol 1V136 or vehicle. (b) Disease activity index, (c) percent body weight changes, and (d) MPO activity of colonic tissue was determined as described in Section 2. Data are expressed as means ± SEM and are representative of 2 independent experiments. *Denotes $P < 0.05$ by Mann-Whitney test. (d) Examples of colons of vehicle- or 1V136-treated colitis mice that were removed and prepared for histological examination. Original magnification is ×100. Calibration bar: 100 μm.

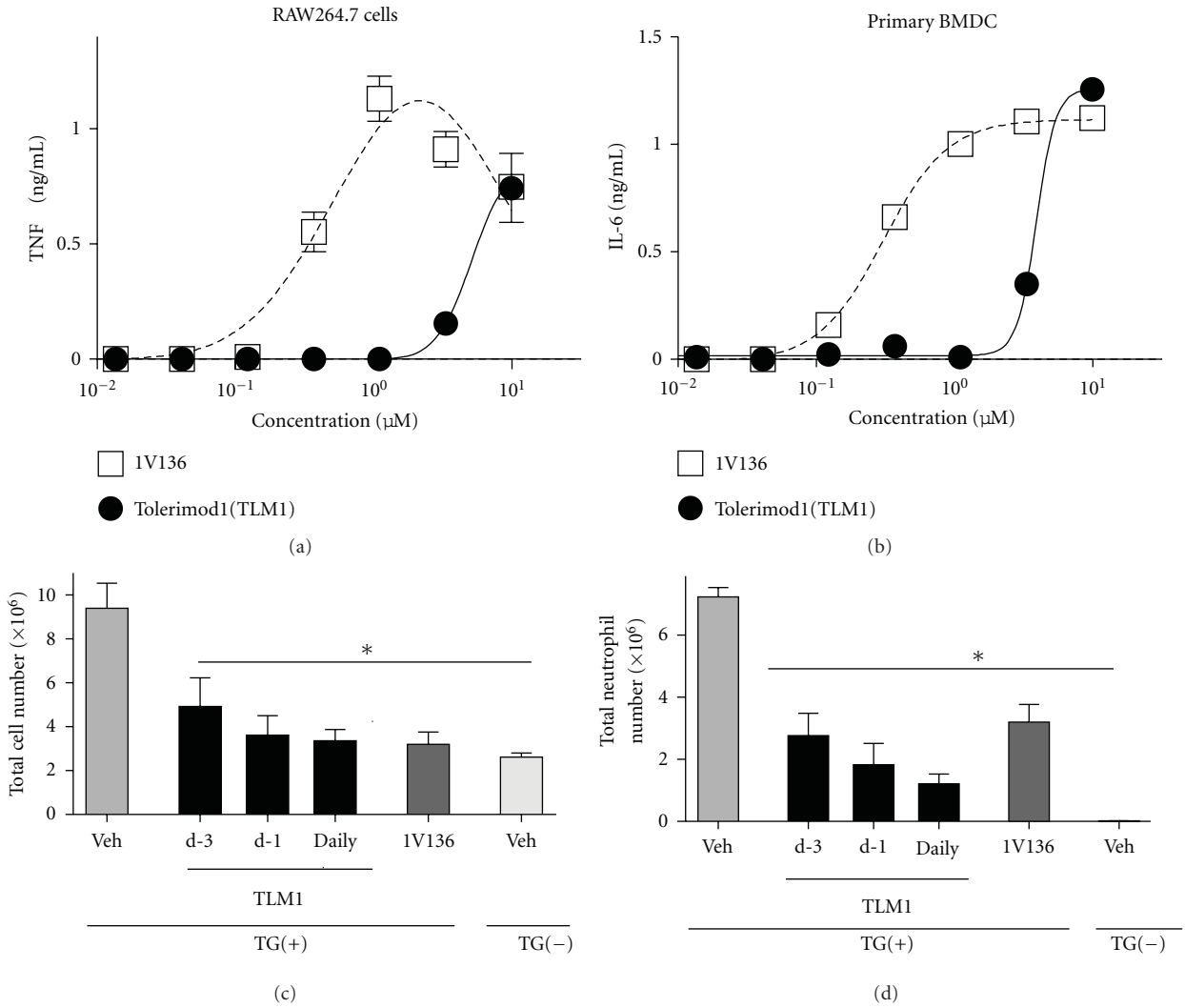

Figure 2: PEGylated TLR7 ligand reduced neutrophil recruitment in TG-elicited peritonitis. RAW264.7 (a) and primary BMDC (b) were treated 18 h with Tolerimod1 (TLM1), and TNFα and IL-6 in the supernatants were measured by ELISA. (c and d) WT mice (n = 7 to 10 per group) were treated daily for three days (-3, -2, and -1) or one day (-3 or -1 day) of s.c. 200 nmol Tolerimod1 (TLM1). Peritonitis was induced on day 0. Total cell number (c) and total neutrophil number (d) recovered in the peritoneal lavage were measured. Data shown are mean ± SEM and are pooled 2 independent experiments that showed similar trends. *Denotes $P < 0.05$ by one way ANOVA with Dunnett's *post hoc* testing compared to the vehicle-treated group.

a week (Figure 1(a)). Both s.c. and oral administration of Tolerimod1 reduced the DAI and prevented body weight loss (Figures 5(a) and 5(b)). Histologic examination revealed a reduction in inflammatory cell infiltration in the lamina propria (Figure 5(c)). There was also a notable reduction in staining for MPO-positive neutrophils (Figure 5(c)).

4. Discussion

Neutrophils are the first responders in acute injury and inflammation. During the early phase of inflammation, neutrophils migrate from blood vessels to the site of inflammation. In the tissue, neutrophils can then mediate damage by releasing the contents of their granules and further amplifying inflammatory processes [1]. We previously demonstrated that repeated administration of an unconjugated TLR7 ligand (1V136) reduced joint inflammation in the neutrophil-dependent K/BxN serum transfer arthritis model [6]. In the current study, we tested the anti-inflammatory effects of TLR7 ligands in the TG peritonitis and DSS-colitis models. Neutrophil-associated inflammation was markedly attenuated by oral or subcutaneous administration of doses of the parent compound, 1V136, or a PEG-modified version, Tolerimod1 [7]. Although proinflammatory potencies of PEGylated TLR7 ligand (Tolerimod1) was 10 times less than unconjugated TLR7 ligand, it retained anti-inflammatory properties in DSS-colitis as well as TG-elicited peritonitis models.

Mucosal administration of 1V136 resulted in hypothermia and caused anorexic behavior that was associated with TNFα release [10]. Hence, we hypothesized that the adverse effects could be attenuated by reducing the proinflammatory

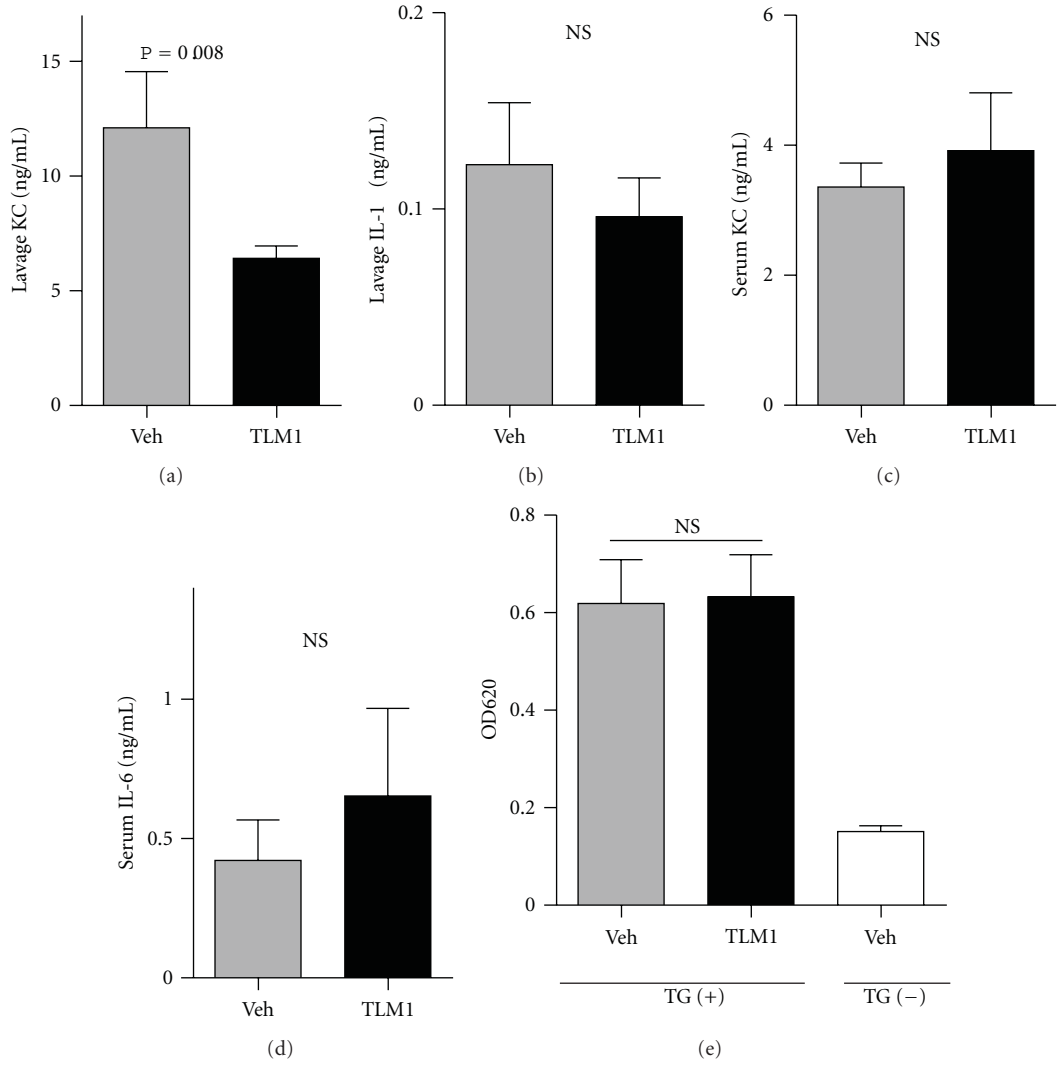

Figure 3: Tolerimod1 treatment reduced local chemokine release but had no effect on systemic proinflammatory cytokine levels. TG-elicited peritonitis was induced in Tolerimod1 (TLM1) or vehicle (veh)-treated mice (n = 8–10 per group). Sera and peritoneal lavage were harvested 3 h after TG injection. Levels of KC (a) and IL-1β (b) in the lavage and KC (c) and IL-6 (d) in sera were measured by ELISA. IL-1β, TNFα, and IL-10 in the sera or lavage, or IL-6 in the lavage, were below detection levels. (e) Vascular permeability was measured by Evans blue assay. Peritoneal lavage was collected 2 h after TG injection. Data shown are mean ± SEM and are pooled from 2 independent experiments that showed similar trends. P values are compared between vehicle (veh)- and Tolerimod1 (TLM1)-treated group by Mann-Whitney test. NS indicates not significant.

potency of the drug. Among the PEGylated derivatives of 1V136 that we prepared, we found that a conjugate with a 6-unit PEG chain (Tolerimod1 in this study) had minimal TLR7-dependent proinflammatory activities [7, 11]. We, therefore, selected this conjugate to study for anti-inflammatory applications. Tolerimod1 treatment reduced neutrophil inflammation in both DSS-induced colitis, despite our initial concern that Tolerimod1 would not be able to retain anti-inflammatory properties due to its low agonistic activity.

In the DSS-colitis model, treatment with TLR7 ligands reduced MPO activity in the colon, indicating that the treatment resulted in reduction of neutrophil recruitment. We, therefore, used TG-elicited peritonitis, a widely used model of sterile inflammation to measure the migratory function of neutrophils [20–23], and to evaluate the treatment effects of Tolerimod1 on neutrophil recruitment. Although this model does not fully represent neutrophilic inflammation in humans, it can provide mechanistic insights into this hyposensitization treatment. The current study demonstrated that the beneficial effects of Tolerimod1 are TLR7 dependent. In addition, the reduced acute neutrophil accumulation in the peritoneal cavity was accompanied by a reduction in KC, a potent neutrophil chemoattractant. These results suggested that a weak TLR7 agonist could influence neutrophil-mediated inflammation by reducing the chemokine recruitment to the site of inflammation.

Although we studied sterile inflammation, clinical peritonitis is usually associated with infections. However, human

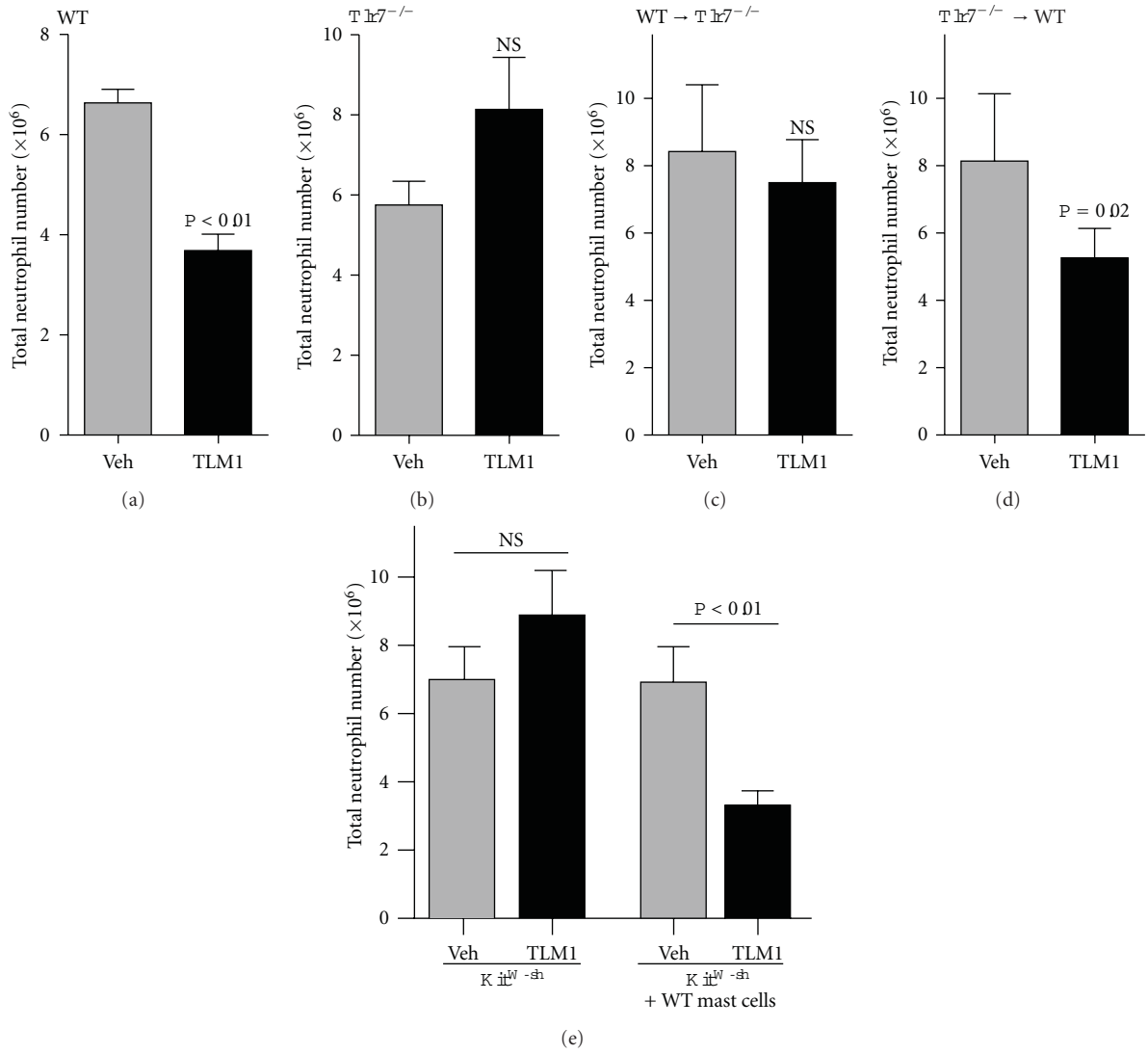

Figure 4: Radioresistant mast cells were involved in suppression of neutrophil recruitment by Tolerimod1 treatment. (a) WT, (b) $Tlr7^{-/-}$, or chimeric mice [WT → $Tlr7^{-/-}$ (c), and $Tlr7^{-/-}$ → WT (d)] were treated with 200 nmol Tolerimod1 (TLM1) s.c. for 3 days and peritonitis was induced with TG ($n = 4$ to 8 per group). (e) Mast cell-deficient $Kit^{W\text{-}sh}$ mice or $Kit^{W\text{-}sh}$ mice reconstituted with WT mast cells were treated with 200 nmol Tolerimod1 (TLM1) s.c. for 3 days, and peritonitis was induced with i.p. TG. The peritoneal cells were recovered by lavage after 3 hours and quantitated. Data shown are mean ± SEM and are pooled from 2 independent experiments that showed similar trends. *P* values are from comparisons of vehicle (veh)- and Tolerimod1-treated groups by Mann-Whitney test.

responses such as in dialysis-associated peritonitis are also in part TLR mediated. A murine model that mimics the progression of a bacterial peritonitis by injecting lyophilized cell-free supernatant prepared from *Staphylococcus epidermidis* (termed "SES") has been developed [24]. TLR2 plays a predominant role in mediating the proinflammatory effects of SES on human cells [25] and soluble TLR2-attenuated inflammation in the SES peritonitis model [26]. Although we did not formally test this model, we previously demonstrated that TLR7 ligand administration does demonstrate "cross tolerance" for TLR2 and does not reduce host defense in a murine infectious disease model [6]. Hence it is possible that intervention with a weak TLR7 agonist could prove beneficial in other TLR-dependent models of peritonitis.

The MyD88 pathway is involved in chemokine expression in macrophage/dendritic cells [27], and chemokine receptor expression on neutrophils [28]. In murine models of myocardial injury and aseptic brain injury, neutrophil recruitment to these sites was severely impaired in MyD88-deficient mice [28, 29]. Also, neutrophil mobility was shown to depend on radiosensitive cells in bone marrow chimeras in these models of injury [28]. Independently, we reported that repeated injection of an unconjugated TLR7 ligand (1V136) induced refractoriness to subsequent activation of the MyD88 signaling pathway and that radiosensitive hematopoietic cells were involved in this process [6]. Hence, we thought that hematopoietic cells also contributed in the Tolerimod1 effects. Unexpectedly, experiments reported here using radiation bone

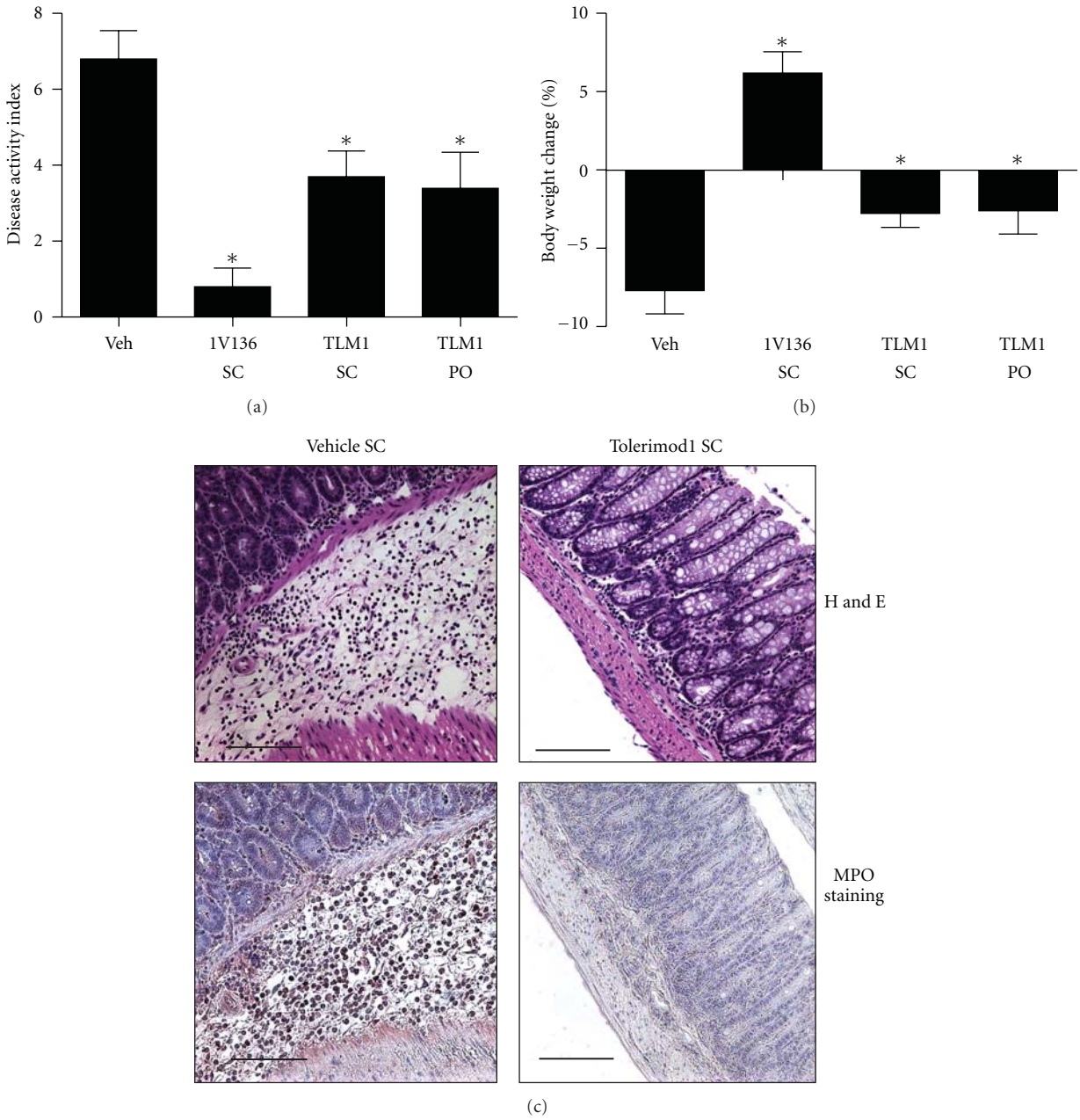

Figure 5: Tolerimod1 reduced the severity of DSS-induced colitis (a and b) DSS-colitis mice ($n = 12$ per group) were treated with 200 nmol Tolerimod1 subcutaneously (SC) or orally (PO) for 7 days. Disease activity index (a) and body weight loss (b) were evaluated. (c) The colons isolated from mice treated s.c. with vehicle or Tolerimod1 were stained with hematoxylin or eosin, or immunostained for MPO. Data shown are mean ± SEM and are pooled from 2 independent experiments that showed similar trends. *Denotes $P < 0.05$ by one-way ANOVA with Dunnett's *post hoc* testing compared to the vehicle-treated group.

marrow chimeric mice, and mast cell-deficient $Kit^{W\text{-}sh}$ mice showed that radioresistant cells and mast cells were involved in the suppressive effects of Tolerimod1. Although mast cells are derived from bone marrow cells, they are relatively resistant to radiation-induced cell death [30]. The anti-inflammatory effects of Tolerimod1 were diminished in mast-deficient $Kit^{W\text{-}sh}$ mice and were partially restored in $Kit^{W\text{-}sh}$ mice engrafted with WT mast cells, indicating that mast cells played a significant role in the reduction of neutrophil recruitment in the TG peritonitis model.

Mast cells have also been suggested to play a proinflammatory role in models of colitis [31]. DSS-colitis was less severe in mast cell-deficient mice or rats [19, 32]. Interestingly, mast cells and their mediators contributed in early vascular permeability seen in inflammation [33] and specifically in TG-induced peritonitis [34]. Our results indicated that

there is no difference in vascular permeability, suggesting that the treatment did not influence the release of mediators involved in vascular permeability by mast cells.

TLR7 expression by host cells is enhanced in inflamed tissues [35]. Therefore, a therapeutic strategy to target TLR7 would be more directed toward inflamed, rather than normal tissues. In addition, tissue or mucosal mast cells also express TLR7 [36, 37]. We demonstrated in this study that a weakly potent TLR7 ligand could reduce severity of acute neutrophilic inflammation in a mast cell-dependent manner. The effect did not involve radiosensitive immune cells, suggesting that less immunosuppressive adverse effects might occur. Our findings indicated that Tolerimod1 treatment reduced chemokine levels locally in the peritoneal lavage fluid, but not systemically. Tolerimod1 was orally active, reduced local peritoneal, and colonic inflammation, without causing a systemic inflammatory cytokine storm.

5. Conclusion

In conclusion, a PEGylated TLR7 ligand (Tolerimod1) exhibited less proinflammatory potency than the parent compound. Tolerimod1 reduced neutrophil inflammation in murine models of experimental colitis and peritonitis. The treatment effects of Tolerimod1 were mediated by radioresistant cells, including mast cells. Tolerimod1 could be a new candidate anti-inflammatory drug with potentially minimal systemic adverse effects.

Abbreviations

TLR:	Toll like receptors
MyD88:	Myeloid differentiation primary response gene (88)
DSS:	Dextran sodium sulfate
TG:	Thioglycolate
BMDCs:	Bone marrow derived dendritic cells
IL-1β :	Interleukin1-β
TNFα :	Tumor necrosis factor α
NFκB:	Nuclear factor kappa B
KC:	Keratinocyte chemoattractant.

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