Suppression of pro-inflammatory T-cell responses by human mesothelial cells

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ABSTRACT

Background. Human γδ T cells reactive to the microbial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) contribute to acute inflammatory responses. We have previously shown that peritoneal dialysis (PD)-associated infections with HMB-PP producing bacteria are characterized by locally elevated γδ T-cell frequencies and poorer clinical outcome compared with HMB-PP negative infections, implying that γδ T cells may be of diagnostic, prognostic and therapeutic value in acute disease. The regulation by local tissue cells of these potentially detrimental γδ T-cell responses remains to be investigated.

Methods. Freshly isolated γδ or αβ T cells were cultured with primary mesothelial cells derived from omental tissue, or with mesothelial cell-conditioned medium. Stimulation of cytokine production and proliferation by peripheral T cells in response to HMB-PP or CD3/CD28 beads was assessed by flow cytometry.

Results. Resting mesothelial cells were potent suppressors of pro-inflammatory γδ T cells as well as CD4+ and CD8+ αβ T cells.
cells. The suppression of γδ T-cell responses was mediated through soluble factors released by primary mesothelial cells and could be counteracted by SB-431542, a selective inhibitor of TGF-β and activin signalling. Recombinant TGF-β1 but not activin-A mimicked the mesothelial cell-mediated suppression of γ8 T-cell responses to HMB-PP.

**Conclusions.** The present findings indicate an important regulatory function of mesothelial cells in the peritoneal cavity by dampening pro-inflammatory T-cell responses, which may help preserve the tissue integrity of the peritoneal membrane in the steady state and possibly during the resolution of acute inflammation.

**INTRODUCTION**

Peritoneal inflammation remains a major cause of technique failure in peritoneal dialysis (PD) patients and is strongly associated with progressive membrane fibrosis and ultrafiltration failure [1]. Consequently, there is considerable interest in understanding the basic processes that regulate peritoneal immune responses in the steady state and during infection [2, 3]. The peritoneal membrane is lined with a monolayer of mesothelial cells, which play an integral role in peritoneal homeostasis, solute transport and tissue repair as well as in inflammation, by producing growth factors, cytokines, chemokines, proteases, extracellular matrix proteins and proteoglycans [4]. In this respect, one of the key molecules released by mesothelial cells is TGF-β1, a potent pro-fibrotic cytokine driving epithelial-mesenchymal transition on one hand and a classical anti-inflammatory suppressor of overshoooting immune responses on the other hand [5–8]. Of note, TGF-β1 is readily produced by resting mesothelial cells, but expression can be up-regulated by osmotic or glucose stress during inflammation. As a result, TGF-β1 can be detected at low levels in the peritoneal effluent of stable non-infected PD patients and increases considerably in acute infection [9, 10].

Vγ9/Vδ2+ γ8 T cells constitute a minor unconventional T-cell population that is found only in humans and higher primates. Vγ9/Vδ2 T cells occupy a unique niche in sensing invading pathogens, with an extraordinary sensitivity for certain bacteria based on the selective responsiveness to the microbial isoprenoid precursor (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an essential metabolite in many microorganisms [11, 12]. Pathogens recognized by Vγ9/Vδ2 T cells include the causative agents of cholera, diphtheria, dysentery, syphilis and tuberculosis as well as malaria, diseases which continue to be major threats to global health [13]. HMB-PP species also comprise the majority of highly problematic pathogens causing hospital-acquired infections associated with emerging multidrug resistance [14]. We have previously shown that PD-associated infections caused by HMB-PP+ bacteria are associated with locally elevated Vγ9/Vδ2 T-cell frequencies and poorer clinical outcome compared with HMB-PP− infections [15], implying that γδ T cells may be of diagnostic, prognostic and therapeutic value in acute disease. γδ T cells represent a sizeable proportion of peritoneal leukocytes in the effluent of stable non-infected PD patients [15, 16]. Given their capacity to rapidly produce pro-inflammatory cytokines such as IFN-γ and TNF-α in a T-cell receptor-dependent and independent manner [17, 18], Vγ9/Vδ2 T cells may thus contribute to subclinical inflammation, acute tissue damage and progressive membrane fibrosis if not counter-regulated appropriately.

Recent studies have identified a number of mechanisms by which Vγ9/Vδ2 T-cell responses can be inhibited, primarily in a tumour immunotherapy setting. In this respect, mesenchymal stem cells have been shown to suppress Vγ9/Vδ2 T-cell responses via the COX-2-dependent production of prostaglandin E2 (PGE2) that acts on EP2 and EP4 inhibitory receptors expressed by Vγ9/Vδ2 T cells [19, 20]. Similarly, TGF-β inhibits proliferation and cytokine production by Vγ9/Vδ2 T cells in response to infected target cells [21] and to soluble HMB-PP analogues [22]. The interplay between human γδ T cells and mesothelial cells has not been addressed thus far. We here provide evidence that resting mesothelial cells are potent suppressors of Vγ9/Vδ2 T-cell cytokine production and proliferation in the presence of HMB-PP. This inhibition is mediated by soluble factors released by primary mesothelial cells and involves the TGF-β superfamily signalling pathway.

**METHODS**

**Ethics statement**

This study was conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines (Bro Taf Health Authority, Wales). Tissue sampling was approved by the South East Wales Local Ethics Committee under reference numbers 04WSE04/27 (PD effluent), 96/1730 (omentum) and 08/WSE04/17 (peripheral blood and surplus tissues). All patients and healthy volunteers provided written informed consent for the collection of samples and their subsequent analysis.

**Isolation and culture of mesothelial cells**

Human mesothelial cells were isolated by trypsic digest of omental tissue from patients undergoing abdominal surgery [23, 24] and cultured in M-199 medium (Invitrogen) supplemented with 10% FCS (Invitrogen). Prior to experimentation, mesothelial cell monolayers were growth arrested for 48 h in serum-free M-199 medium. Mesothelial cell culture supernatants were obtained by incubating resting mesothelial cells for 20 h in complete RPMI-1640 (10^5 cells in 200 μL/well in a 96-well plate), rendered cell-free by centrifugation and stored at −80°C until further use. For exosome-depletion experiments, mesothelial cell culture supernatants were subjected to serial centrifugation steps: 2000g, 20 min; 10 000 g, 20 min; 100 000 g, 1 h; 200 000 g, 1 h (Optima-Max ultracentrifuge, Beckman Coulter). Culture supernatants were tested for the presence of soluble TGF-β1 using a Dynex MRX II reader and an ELISA kit from eBioscience.
RESULTS

γδ T cells from different anatomical locations differ in their anti-microbial responsiveness

We recently identified human γδ T cells in the peritoneal effluent of stable and infected PD patients [15, 16]. Here, the proportion of Vγ9/Vδ2+ γδ T cells within the whole T-cell population was low but clearly detectable across individual stable PD patients and comparable in size to that of Vγ9/Vδ2 T cells in peripheral blood and omentum (Figure 1A) as well as in tonsils, appendices and colorectal biopsies (data not shown). In functional assays, Vγ9/Vδ2 T cells from both blood and PD effluent responded to HMB-PP as assessed by intracellular staining for the pro-inflammatory cytokines IFN-γ and TNF-α. However, we noted that the responses by peritoneal γδ T cells were significantly lower than those by their peripheral counterparts (Figure 1B).

Figure 1: HMB-PP responsiveness of Vγ9/Vδ2 T cells from different anatomical locations. (A) Flow cytometric analysis of Vγ9/Vδ2 T cells in the peripheral blood of healthy volunteers (n = 5), in omental tissues of patients undergoing abdominal surgery (n = 5), and in the peritoneal effluent of stable PD patients (n = 13). Numbers show mean frequency ± SEM of Vγ9/Vδ2 T cells within the total T-cell population. (B) Responsiveness of peripheral (n = 3) and peritoneal (n = 9) Vγ9/Vδ2 T cells to overnight stimulation with 100 nM HMB-PP, shown as a proportion of cytokine-producing cells after intracellular staining. Each symbol represents an individual donor, horizontal lines show the mean values. Statistical differences between groups were analysed using unpaired t-tests, within groups using paired t-tests.
Mesothelial cells inhibit anti-microbial γδ T-cell responses

PD effluent contains considerable numbers of detached but viable mesothelial cells [5]. We therefore speculated that an interaction with mesothelial cells during the culture period might contribute to the impaired γδ T-cell responses in peritoneal effluent and reflect a possible inhibitory influence of the mesothelium on local immune cells in the peritoneal cavity. We, therefore, investigated the immunomodulatory function of primary mesothelial cells in more detail. Peripheral γδ T cells cultured alone or co-cultured with autologous monocytes responded readily to overnight stimulation with HMB-PP, as judged by their potential to produce IFN-γ and TNF-α (Figure 2A). In contrast, co-culture of γδ T cells with allogeneic mesothelial cells derived from fresh omental tissues abrogated the HMB-PP stimulated production of IFN-γ and TNF-α completely (Figure 2A). CFSE dilution assays demonstrated that mesothelial cells did not provide any accessory help for γδ T cells as opposed to the strong γδ T-cell proliferation observed in the presence of autologous monocytes (Figure 2B). Of note, triple co-cultures consisting of γδ T cells, monocytes and mesothelial cells resulted in a drastically reduced proliferation compared with γδ T-cell-monocyte cocultures in the absence of mesothelial cells, indicating that the suppressive capacity of mesothelial cells is potent enough to effectively abrogate the accessory effect provided by monocytes (Figure 2B).

Soluble factors released by resting mesothelial cells inhibit pro-inflammatory responses by γδ and αβ T cells

To determine whether the inhibition of γδ T-cell responses was mediated through soluble factors, we repeated the above stimulation assays using culture supernatants of resting mesothelial cells. At a dilution of 1:2 (50%), mesothelial cell-conditioned medium strongly inhibited the HMB-PP induced expression of IFN-γ and TNF-α by γδ T cells (Figure 3A) as well as γδ T-cell proliferation (Figure 4A). This inhibitory effect on γδ T-cell proliferation was dose-dependent and still detectable at dilutions of 1:8 (12.5%) (data not shown). Of note, we detected a similarly pronounced inhibition of αβ T-cell responses. This was the case for both peripheral CD4+ and CD8+ T cells stimulated with anti-CD3/CD28 beads in the presence of 50% mesothelial cell-conditioned medium (Figure 3B).

The inhibitory activity of mesothelial cells cannot be rescued by exosome depletion

Exosomes play a potent role in driving cell–cell communication including suppression of T-cell responses via surface expressed CD39, CD73 and TGF-β [28–30]. We, therefore,
evaluated whether exosome-bound molecules play a role in the suppressive activity of mesothelial cells and subjected mesothelial cell supernatants to serial centrifugation steps. These experiments demonstrated that even 1 h centrifugation at 200,000 g—conditions under which all exosomes would have been pelleted—did not rescue the inhibitory activity on γδ T cells (data not shown). Taken together, our findings indicate that resting mesothelial cells release soluble factors into the supernatant which inhibit γδ and αβ T-cell responses.

SB-431542 rescues the inhibitory effect of mesothelial cells on γδ T cells

To identify the soluble mediator(s) responsible for the inhibition of T-cell responses by resting mesothelial cells, we tested a range of blocking reagents, including SB-431542. SB-431542 is a selective inhibitor of TGF-β and activin signalling via activin receptor-like kinases (ALK)-4, ALK-5 and ALK-7 [31, 32]. As shown in Figure 4A, SB-431542 effectively rescued the HMB-PP-driven proliferation of γδ T cells in the presence of mesothelial cell-conditioned medium. Moreover, SB-431542 almost completely rescued the HMB-PP-stimulated production of IFN-γ and TNF-α of γδ T cells in the presence of mesothelial cell supernatant (Figure 4B).

Recombinant TGF-β1 but not activin-A mimics the mesothelial cell-mediated inhibition of γδ T-cell responses

The above data suggested that factors signalling via ALK-4, ALK-5 or ALK-7 play a role in the suppressive activity of mesothelial cells. We, therefore, tested the effects of recombinant TGF-β1 and activin-A on γδ T-cell activation and proliferation. Of note, TGF-β1 but not activin-A mimicked the mesothelial cell-mediated inhibition of γδ T-cell responses to HMB-PP, implying that the active compound released by mesothelial cells might be TGF-β1 (Figure 4C). This conclusion is supported by the fact that we failed to see any rescue of the inhibitory activity using human follistatin, a potent antagonist of activin-A (data not shown).

DISCUSSION

Mesothelial cells participate in peritoneal homeostasis and the control of inflammation through the release of both pro- and anti-inflammatory mediators. We previously identified a role of mesothelial cells in orchestrating peritoneal response during inflammation and infection by secreting a range of pro-inflammatory factors such as IL-6, CXCL8 and CCL2 upon exposure to bacterial products and inflammatory signals.
function. These data are in accordance with a recent study in mice suggesting that both CD45^+ and CD45^- omental cells may exert suppressive effects on murine CD4^+ effector T cells [34].

The suppressive activity of mesothelial cells was susceptible to inhibition with SB-431542 and thus implied a crucial role of the TGF-β superfamily, a large family of structurally related regulatory proteins with complex downstream signalling pathways. TGF-β superfamily members can be divided into distinct subgroups: the TGF-β family, the activins, the bone morphogenetic proteins and nodal. All these ligands bind to constitutively active type II receptors that subsequently activate downstream type I receptors, seven of which have been identified so far (ALK-1 to ALK-7) [35, 36]. SB-431542 acts as a competitive ATP-binding site kinase inhibitor and is a selective inhibitor for ALK-4, ALK-5 and ALK-7 [31]. The fact that in the present study SB-431542 rescued the suppressive activity of mesothelial cell supernatant, suggested that TGF-β and/or activin-A might be the main inhibitory compound secreted by mesothelial cells. Activin-A is a pleiotropic cytokine that plays an important role in fundamental biological processes, such as development and tissue repair. An increasing body of evidence shows that activin-A exerts both pro- and anti-inflammatory effects depending on the target cell type and the prevailing cytokine milieu, including an inhibition of tumour-suppressive epidermal γδ T cells in the mouse [37]. Notably, the biological effects of activin-A are controlled through interaction with its physiological antagonist, follistatin [36]. Here, recombinant follistatin did not have any significant rescue effect on the inhibitory activity of mesothelial cells on γδ T cells, nor did recombinant activin-A inhibit γδ T cells, arguing against a major role of mesothelial cell-derived activin-A in the suppression of T-cell responses. While we were unable to detect consistent levels of TGF-β1 in the mesothelial cell culture supernatants above the background of culture medium alone, previous investigators estimated the amounts of TGF-β1 released by primary mesothelial cells to be ∼10–100 pg per 10^5 cells [6–8]; such levels would correspond to <30 pg/mL. TGF-β1 in our own cultures and would thus be at or below the detection limit of the ELISA kit used. Of note, previous reports described a TGF-β1-dependent inhibition of immune cell functions by tumour cells and tissue cells at comparably low concentrations [38–40].

We recently showed that the peritoneal cavity hosts a population of resident CD4^+ and CD8^+ effector/memory T cells capable of rapidly mounting a pro-inflammatory response characterized by IFN-γ production [26]. Of note, our present findings demonstrate that in addition to their inhibition of γδ T-cell responses, mesothelial cells exert a similar suppressive effect on human CD4^+ and CD8^+ αβ T cells, suggesting a general homoeostatic function of mesothelial cells in the regulation of peritoneal immunity. Future work will now address the complex changes in the interaction between mesothelial cells and resident T cells during episodes of acute infection, when rapid production of inflammatory chemokines by mesothelial cells and local immune cells
in response to microbial signals leads to pronounced recruitment of neutrophils, monocytes/macrophages and lymphocytes [15, 24, 41, 42]. It is conceivable that the mesothelium plays an important role in regulating these acute inflammatory responses and contributes to the resolution from infection, thereby limiting inflammation-related tissue damage in PD patients.

**ACKNOWLEDGMENTS**

We are grateful to all patients for participating in this study, and the surgeons and nurses for their cooperation. We also thank Hassan Jomaa for providing synthetic HMB-PP; Aled Clayton, Ian Humphreys and Phil Taylor for sharing reagents; Joanne Welton for help with the exosome depletion; Raj Bansal for tissue analyses and Derek Doherty and Donald Fraser for the stimulating discussion.

**FUNDING**

This research was supported in part by Welsh Assembly Government grant #08AP002 from Baxter Healthcare.

**CONFLICT OF INTEREST STATEMENT**

None declared.

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Received for publication: 11.9.2012; Accepted in revised form: 17.12.2012

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