Alternative activation of macrophages in human peritoneum: implications for peritoneal fibrosis

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Abstract

Background. Depending on the cytokine microenvironment, macrophages (Mφ) can adopt a proinflammatory (M1) or a profibrotic (M2) phenotype characterized by the expression of cell surface proteins such as CD206 and CD163 and soluble factors such as CC chemokine ligand 18 (CCL18). A key role for Mφ in fibrosis has been observed in diverse organ settings. We studied the Mφ population in a human model of peritoneal dialysis in which continuous stress due to dialysis fluids and recurrent peritonitis represent a risk for peritoneal membrane dysfunction reflected as ultrafiltration failure (UFF) and peritoneal fibrosis.

Methods. We used flow cytometry and quantitative reverse transcription–polymerase chain reaction to analyse the phenotype of peritoneal effluent Mφ and tested their ability to stimulate the proliferation of human fibroblasts. Mφ from non-infected patients were compared with those from patients with active peritonitis. Cytokine production was evaluated by enzyme-linked immunosorbent assay (ELISA) in spent dialysates and cell culture supernatants.

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Results. CD206+ and CD163+ M2 were found within peritoneal effluents by flow cytometry analysis, with increased frequencies of CD163+ cells during peritonitis (P = 0.003). TGFβ1, MMP9 and CCL18 messenger RNA (mRNA) levels in peritoneal macrophages (pMφ) were similar to those found in M2 cells differentiated in vitro. The ability of pMφ to stimulate fibroblast proliferation correlated with CCL18 mRNA levels (r = 0.924, P = 0.016). CCL18 production by pMφ was confirmed by immunostaining of cytospin samples and ELISA. Moreover, CCL18 effluent concentrations correlated with decreased peritoneal function, which was evaluated as dialysate to plasma ratio of creatinine (r = 0.724, P < 0.0001), and were significantly higher in patients with UFF (P = 0.0025) and in those who later developed sclerosing peritonitis (P = 0.024).

Conclusions. M2 may participate in human peritoneal fibrosis through the stimulation of fibroblast cell growth and CCL18 production as high concentrations of CCL18 are associated with functional deficiency and fibrosis of the peritoneal membrane.

Keywords: CCL18; macrophages; M2; peritoneal dialysis; peritoneum

Introduction

Macrophages (Mφ) are key players in tissue homeostasis. They not only present antigens to lymphocytes but also play an important role in the resolution of inflammation and wound healing [1]. Depending on the tissue microenvironment, Mφ can differentiate into distinct subsets that present specialized and polarized functions. In this regard, the functions of Mφ activated by microbial products such as lipopolysaccharide (LPS) in a T helper (Th) 1 cytokine microenvironment, and referred to as classically activated Mφ, have been extensively documented. Recently, it was recognized that Mφ exposed to cytokines generated by Th2 cells exert an alternative activation programme. The initial concept of alternatively activated Mφ as cells activated in the presence of interleukin (IL)-4 or IL-13 [2] was broadened to consider the phenotype of Mφ activated by immunocomplexes, transforming growth factor (TGF)-β, IL-10 and glucocorticoids or by the capture of apoptotic cells [3–6]. Mirroring the Th1/Th2 nomenclature, polarized Mφ are referred to as M1 (classical activation) and M2 (alternatively activated profibrotic/proangiogenic Mφ) [7] (Supplementary Figure S1). M2 are characterized by cell surface expression of the macrophage mannose receptor (MMR)/CD206 [2], CD163 [8], CD23 [9] and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)/CD209 [10] and the synthesis of TGF-β [11], thymus and activation-regulated chemokine/CC chemokine ligand 17 (CCL17) [12] and alternative macrophage activation-associated CC chemokine-1 (AMAC-1)/CCL18 [13]. However, specific phenotypes may vary depending on the nature of the stimulus [14]. M2 cells are key players in tissue remodelling, angiogenesis and fibrogenesis [1, 15–17]. Additionally, the M2-specific chemokine AMAC-1/CCL18 (also known as pulmonary and activation-regulated chemokine, PARC, and DC-CK1) has recently been associated with lung fibrosis [18, 19]. In vivo, M1 and M2 polarized cells are regarded as two extremes of a continuum of functional stages of Mφ since heterogeneity and plasticity are hallmark features of this cell lineage [20]. M2 are found in vivo during parasite infections [21], lung fibrosis [22] and scarring [23] and in the decidua [24, 25] and tumours [26].

Although M2 Mφ have been associated with profibrotic and proangiogenic functions, most reports are related to murine models. In humans, most studies concerning macrophage involvement in fibrosis are related to alveolar Mφ. We explored the polarization status of Mφ in a human model of peritoneal dialysis (PD). The use of PD as an alternative to haemodialysis is limited by the peritoneal membrane’s capacity to perform long-term diffusive and/or convective transport [27, 28] and whose worst functional consequence is ultrafiltration failure (UFF), which restricts technique continuity. The morphological and functional UFF substrate is the disappearance of the mesothelial layer and fibrosis [29]. Epithelial-to-mesenchymal transition of mesothelial cells has been identified as a key process leading to peritoneal fibrosis [30–32]. Nonetheless, any cell involved in fibrosis stimulation is a potential inducer of UFF. Since Th2 immunodeprivation has been reported in PD patients [33], we hypothesized that a subpopulation of M2 peritoneal macrophages (pMφ) could be part of the cellular constellation that intervenes in peritoneal fibrosis in long-term PD patients. Accordingly, we characterized the Mφ population ex vivo from peritoneal effluent drained by patients. We extended the study to patients with peritonitis episodes, which are clinically related to an earlier appearance of UFF associated with fibrosis [27, 29]. The results suggest that CCL18-producing M2 are present within the peritoneum and may contribute to peritoneal fibrosis in long-term PD patients.

Materials and methods

Patient samples

Patients (47 men and 27 women aged between 21 and 84 years) were recruited from the peritoneal dialysis unit (Hospital La Paz, Madrid, Spain). Among them, 15 men and 5 women developed one or more peritonitis episodes during the study. Diagnosis of PD-associated peritonitis was based on the presence of abdominal pain, cloudy PD effluent with a leucocyte count > 100 cells/μl and a positive microbiological culture. Healthy skin biopsies were obtained at the time of catheter insertion or removal. Encapsulating peritoneal sclerosis (EPS) was defined as a syndrome generally demonstrated after PD withdrawal and characterized by intra-peritoneal adhesions that compromise intestinal motility. This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Institutional Review Board of Hospital La Paz. Informed consent was provided by all patients.

Cell isolation

Cells were isolated from PD effluents by centrifugation at 500 g for 15 min at 4°C. Peripheral blood mononuclear cells (PBMCs) from control donors were prepared by Ficol-Hypaque (Amer sham Pharmacia Biotech, Sweden) density gradient centrifugation. Monocytes or pMφ were obtained from healthy donor PBMCs or PD effluent concentrates, respectively. Positive selection was performed by magnetic isolation using CD14 microbeads (Miltenyi Biotech GmbH, Germany). Purity of fractions was at least 95%, as assessed by flow cytometry.

CD163+ pMφ were isolated from effluent concentrates by labelling with an anti-CD163 monoclonal antibody (mAb; BD Pharmingen) and anti-mouse immunoglobulin (IgG) microbeads (Miltenyi Biotech GmbH). Flow cytometry analysis of the resulting population revealed that >90% of the cells were CD163+CD14+. CD163+CD14+ Mφ were purified from the resulting CD163+ fraction with CD14 microbeads as described above. CD66b+ neutrophils were purified from effluent cells by labelling with CD66b-fluorescein...
isothiocyanate (FITC) mAbs (BD) and anti-FITC microbeads (Miltenyi Biotech GmbH). Peritoneal CD3⁺ lymphocytes were purified with CD3 microbeads (Miltenyi Biotech GmbH). Monocyte-derived M2 cells (Mo-M2) were generated from CD14⁺ peripheral blood monocytes as described [34]. Briefly, purified monocytes were incubated in RPMI_1640 medium with 5% heat-inactivated autologous serum and 100 U/mL IL-4 (Peprotech, UK) for 96 h with medium replacement at 48 h.

Flow cytometry

One hundred and thirty-two non-infected peritoneal effluent samples were collected from a total of 68 PD patients at different times of dialysis. Cell surface markers were also analysed in 56 samples from 20 patients with active peritonitis (Days 1–3; cell count > 100 cells/μL). After harvesting, the mAbs were used for flow cytometry analysis of surface molecules on Mo: anti-CD-SIGN/CD209 (MR-1; kindly donated by Dr A. L. Corbi), CD16 (3G8; Beckman Coulter, Immunotech, France), Human leukocyte antigen (HLA)-DRs, anti-MMRI/CD206, CD86-Pe, CD83-Pe, CD23-Pe, CD1αe-PE (BD Pharmingen), CD11c-FITC, CD11b-FITC and CD14-FITC (Miltenyi Biotech GmbH). Cells were pre-incubated with 50 μg/mL human IgG to prevent binding to immunoglobulin Fc receptors, then unconjugated antibodies were added for 30 min at 4°C, washed and stained with allophycocyanin-anti-mouse Ig (Invitrogen, Molecular Probes, OR). Normal mouse serum was added to block unbound reagent, and phycoerythrin (PE) and FITC-conjugated mAbs were added and incubated for 20 min at 4°C. Cells were washed and analysed by three-colour flow cytometry in a FACSCalibur using CellQuest Pro software (BD Biosciences). For analysis of Mo, gates were set on mononuclear cells according to forward and side light scatter criteria, and CD14⁺ cells within this gate were further analysed (Supplementary Figure S2).

Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche, Germany) and 1 μg was reverse transcribed with random hexanucleotides and avian myeloblastosis virus reverse transcriptase (Promega) in a final volume of 20 μL for 1 h at 42°C. Primers used for polymerase chain reaction (PCR) amplification are shown in Table 1. For quantitative analysis of messenger RNA (mRNA) content, 1-μL aliquots of the resulting complementary DNA were PCR amplified in a Light Cycler (Roche) with FastStart DNA Master SYBR Green I (Roche). Standard curves for target mRNA expression were generated by amplifying 10-fold serial dilutions of known quantities of the specific PCR products. Quantification of target gene expression was obtained using Light Cycler system software. Relative units estimated from the quantification represent the ratio between specific mRNA molecules and B2M mRNA molecules in each sample.

Macrophage-fibroblast co-cultures and assay for fibroblast proliferation

The human lung fibroblast cell line, IMR90 [35], and primary human fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS). Primary human skin fibroblasts were isolated through trypsinization of skin biopsies [36]. Peritoneal fibroblasts were obtained upon collagenase digestion of omentum [36]. Peritoneal fibroblasts were isolated through trypsinization of skin biopsies [36]. Peritoneal ultrafiltration (UF) capacity was considered abnormally low when it was <400 mL of negative balance after a 3.86% glucose exchange with a dwell time of 4 h. Urea and creatinine mass transfer coefficients (umMTC and Cr-MTC, respectively) were calculated using a standard method (Pyle–Popovich method). Type I peritoneal membrane UFF was defined as a Cr-MTC >11 mL/min and an UF <400 mL/h [27, 39].

Cytospin and immunostaining

For cytospin preparations, peritoneal effluent cells were applied to slides at 10⁶ cells/slide. They were then fixed in 4% paraformaldehyde at room temperature. Afterwards, sequential blocking was performed with 0.03% hydrogen peroxide in methanol and 10% normal goat serum/1% bovine serum albumin/0.1% saponin in Tris-buffered saline. Biotinylated goat anti-human CCL18 (R&D Systems) was added and incubated overnight at 4°C. The sections were washed three times with phosphate-buffered saline and the signal was developed with peroxidase-conjugated streptavidin (R&D Systems) and 3,3'-diaminobenzidine. Slides were counterstained with haematoxylin prior to visualization.

Statistical analysis

The statistical analysis was performed using GraphPad Prism (San Diego, CA). The non-parametric t-test was used to analyse differences of mean values and correlation was assessed using Spearman’s correlation tests with P-values. P-values of <0.05 were considered statistically significant.

Table 1. Primer Sequences and Predicted Products for the PCR Reactions

<table>
<thead>
<tr>
<th>Primer source</th>
<th>Primer sequence</th>
<th>Predicted products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFB1</td>
<td>Sense: 5'-GGTGGAACCCACAC AACGAAACT-3' Antisense: 5'-AAATCC CTCCTCAGGCTCACCCAAACT-3'</td>
<td>305</td>
</tr>
<tr>
<td>CCL18</td>
<td>Sense: 5'-ACAAAGAGCTCT GCTGCCTC-3' Antisense: 5'-CCCCAC TTCTTATGGGTCTA-3'</td>
<td>192</td>
</tr>
<tr>
<td>MMP9</td>
<td>Sense: 5'-GGCCAGAGGAAATCT TGGTAC-3' Antisense: 5'-GAGTAG TTTTGAGCTACTGGACG-3'</td>
<td>255</td>
</tr>
<tr>
<td>B2M</td>
<td>Sense: 5'-CCAGCAGAGAATGGAA TACGGTGTTTCTACG-3' Antisense: 5'-ATGTCG TGTTTACATGTCCTC-3'</td>
<td>300</td>
</tr>
</tbody>
</table>
Results

Peritoneal effluent Mϕ express M2 cell surface markers

As a first step in the characterization of pMϕ, we used flow cytometry to analyse the cell surface expression of several macrophage-specific receptors. Peripheral blood monocytes were treated with IL-4 to obtain Mo-M2 [34] and analysed in the same way for comparative purposes. The expression of CD206 (MMR), CD163, CD23, CD209 (DC-SIGN), CD1a, CD86 and CD16 was analysed in CD14+ cells from peritoneal effluents. Among them, CD23, CD209, CD206 and CD163 have been reported to be induced in M2 Mϕ. CD11b, CD11c and HLA-DR were also analysed as hallmarks of the mononuclear phagocyte lineage. CD83 expression was studied to discern CD83+CD209+ dendritic cells (DCs) from CD83–CD209+ M2. Similar levels of CD11b, HLA-DR, CD86 and CD11c were found in most peritoneal samples. However, variability was found within the CD14+ population regarding the expression of CD23, CD16, CD1c and CD209, and a high degree of heterogeneity was found in CD163 and CD206 expression. As shown in Supplementary Figure S3, Mo-M2 up-regulated the expression of CD23 and down-regulated CD16 as previously described [3, 5, 9]. Samples with negligible levels of CD16 and/or CD23 and samples with most CD14+ cells expressing variable levels of both receptors were found in PD fluid from stable patients as well as in peritonitis effluents. A bimodal distribution of CD16 expression was found in some patients, with only a subpopulation of Mϕ expressing high levels of CD16. On the other hand, CD23 expression was found to be unimodal with variable expression levels in different donors. While similar expression of CD11c was found in the various samples, CD1a levels varied in donors. Small percentages of CD209+CD83+ cells corresponding to DCs were found in some samples; however, subpopulations of CD209+CD83– cells were also found, which is consistent with the presence of M2 cells in the peritoneum. CD163 and CD206 expressions have been described as characteristic features of different subpopulations of M2 [14]. Multiple expression patterns were found for these two membrane receptors, with double-negative, double-positive and single-positive populations. Expression of CD163 and CD206 was detected in uninfected as well as in peritonitis samples (mean percentage of CD163+ pMϕ 41.81 ± 22.61%, range 0.9–98.22%, and mean percentage of CD206+ pMϕ 18.16 ± 12.97%, range 0.0–57.5%, in uninfected samples). Nonetheless, increased frequencies of the CD163+CD14+ subpopulation were found in Mϕ from patients with active peritonitis, which paralleled the rise in absolute numbers of Mϕ infiltrating the peritoneum (Supplementary Figure S4). As a result, the overall number of CD163+ pMϕ was transiently increased in the early days of infection (Figure 1A and Supplementary Figure S4). However, interindividual and intra-individual heterogeneities were found in regard to the percentage of CD14+ cells expressing various markers (Supplementary Figure S3 and Tables S1 and S2). In light of the inter- and intra-individual variability in cell surface expression, we considered each effluent to

Fig. 1. Expression of membrane receptors in CD14+ pMϕ. (A) Kinetics of receptor expression in pMϕ during acute peritonitis. Data are represented as percentages of CD14+ cells expressing CD163 (black squares), DC-SIGN/CD209 (open triangles), MMR/CD206 (crosses) and CD16 (open circles) as analysed by flow cytometry in a representative patient 10 months before the onset (~10 mo) and on Days 1, 2, 3, 4 and 7 (D1–D7) during peritonitis. (B) Expression of CD163 and CD206 membrane receptors in CD14+ pMϕ. The percentage of CD14+ cells expressing each receptor was analysed by flow cytometry in CD14+ gated cells from peritoneal effluents. Box plots represent frequencies of each receptor analysed. The horizontal middle line represents the median, the top and bottom lines represent the quartile values and the T bars represent the maximum and minimum values. Statistical analysis was performed using the non-parametrical t-test for independent samples to compare the mean percentages of expression of each receptor in non-infected (n = 117, white boxes) and peritonitis samples (n = 49, grey boxes).

Fig. 2. qRT-PCR analysis of gene expression in CD14+ purified pMϕ. CD14+ Mϕ were purified from peritoneal effluents and total RNA was isolated. Samples from non-infected patients (n = 6, light grey bars) and from patients with active peritonitis (n = 8, dark grey bars) were analysed. Gene expression levels were also measured in peripheral blood CD14+ purified monocytes (Mo, n = 3, white bars) and in Mo-M2 (n = 3, black bars) for comparative purposes. Results are shown as relative units (RU) of specific mRNA molecules normalized to B2M mRNA molecules in each sample. Mean values and standard deviation of mRNA levels in the four groups are shown. Statistical analyses were performed by Kruskal–Wallis (#) and Mann–Whitney U tests (*) to compare expression levels. # and * statistically significant (P < 0.05).
be an independent sample. Statistical analysis showed a significant increase in the percentage of CD14$^+$ expressing CD163 and a lower expression of CD206 during active peritonitis (number of cells/ul/C21 $\times 10^3$), as compared to uninfected samples (>1 month without peritonitis episodes) (Figure 1B).

Gene expression of TGFB1, MMP9 and CCL18 in pMφ

Differential activation of Mφ induces the expression of certain cytokines or chemokines characteristic of M1 or M2. While tumour necrosis factor $\alpha$ has typically been associated with classical activation of Mφ, TGF-$\beta$ is a characteristic of M2 [3], and TGF-$\beta$1 is the cytokine most intensively linked to the development of fibrosis [17]. An increase in TGF-$\beta$1 activity has been reported in peritonitis effluents from PD patients [40]. However, in Mφ, the main level of control is the activation of latent TGF-$\beta$1, a process that is catalysed by several agents including MMP-9 [11, 41]. In addition, MMP9 gene expression is stimulated by TGF-$\beta$1 in Mφ [42]. We measured the mRNA levels of TGFB1, MMP9 and CCL18 in purified CD14$^+$ pMφ from non-infected patients and those suffering peritonitis using qRT–PCR and compared them to those induced in Mo-M2. As shown in Figure 2, no statistically significant differences were found in TGFB1 mRNA levels between groups. In contrast, MMP9 gene
expression was significantly induced in Mo-M2. MMP9 gene expression was also detected in uninfected pMφ and was significantly increased in peritonitis-derived macrophage samples. CCL18 mean mRNA levels in non-infected and peritonitis samples were significantly higher than those found in peripheral blood monocytes and were similar to those induced by IL-4. TGFBI, MMP9 and CCL18 gene expression data show that pMφ resemble M2 either during peritonitis or under steady-state conditions. No significant association was found between gene expression levels and cell surface markers in these samples (Supplementary Figure S5A).

M2 and pMφ stimulate the proliferation of primary human fibroblasts

It has been previously published that Mo-M2 stimulated the proliferation of WI-38 fibroblasts [34]. Similarly, we found that Mo-M2 were able to stimulate the proliferation of the fibroblast cell line IMR90 (Supplementary Figure S6A). Purified pMφ were also able to stimulate the proliferation of IMR90 cells above basal proliferation to levels similar to those induced by Mo-M2 (Figure 3A). Moreover, pMφ either from non-infected or from peritonitis effluents, as well as Mo-M2 were able to stimulate the proliferation of primary fibroblasts (Figure 3B and Supplementary Figure S6B). Although highly purified CD14+ populations were used for these assays, proliferation of a minimal residual amount of lymphocytes could be activated in allogenic cultures and may account for the incorporation of [3H]-thymidine. To overcome this issue, assays were performed in which pMφ from stable PD patients and from patients with active peritonitis were able to stimulate the proliferation of autologous fibroblasts (Figure 3C). Moreover, pMφ were able to stimulate cell growth of human primary peritoneal fibroblasts (Figure 3D). Mo-M2 were mainly CD206+CD163-, while CD206+CD163+ cells were the most abundant Mφ population during peritonitis (see Supplementary Figures S3B and S4B). To study whether CD206+CD163+ pMφ can stimulate fibroblast proliferation, CD163+CD14+ pMφ were purified from a patient with peritonitis and negligible levels of CD206+CD163+ cells. As shown in Figure 4, CD163+CD14+ pMφ stimulated the proliferation of human primary fibroblasts.

**Fig. 5.** CCL18 expression by pMφ is related to the stimulation of fibroblast cell growth. (A) Correlation analysis of fibroblast proliferation and mRNA levels of TGFBI, MMP9 and CCL18 in purified pMφ. The stimulation index achieved in co-cultures of pMφ with primary allogenic fibroblasts was plotted against the log of specific mRNA levels in each purified pMφ sample, and a regression analysis was performed. A trend line was inserted. Correlation coefficients and P-values were estimated by the Spearman rank correlation test. (B) pMφ showing higher production of CCL18 protein levels are more efficient stimulators of fibroblast cell growth. Primary human skin fibroblasts were co-cultivated or not with CD14+ purified allogenic pMφ from two different donors. Six replicated samples were tested for each condition. Stimulation index was calculated as above. CCL18 protein levels were stimulated in 48-h culture supernatants from the same CD14+ populations. (C, D) Recombinant CCL18 stimulates the proliferation of primary fibroblasts. Primary skin fibroblasts from two different donors (C) and primary peritoneal fibroblasts (D) were cultured in the presence or absence of human recombinant CCL18 during 4 days. Six replicated samples were tested for each condition. The proliferation of fibroblasts was measured by [3H]-thymidine incorporation. Data are expressed as mean c.p.m. values ± SEM. Mann–Whitney U test was used for statistical analysis.
Six of the pMφ samples tested in primary fibroblast proliferation assays were also used for the qRT–PCR determinations shown in Figure 2. Regression analysis showed that the ability of these pMφ to stimulate the proliferation of allogenic primary fibroblasts was associated with CCL18 mRNA levels (Figure 5A). While we did not find any association between gene expression levels and cell surface phenotypes, a significant association was found between the expression of CD206 and the stimulation of primary fibroblast cell growth (Supplementary Figure S5B). On the contrary, when the same analysis was performed to analyse the stimulation of IMR90 cells, we found a better association with the expression of CD163. This result may reflect that a higher number of peritonitis-derived samples were used in these assays (see Figure 3A) and the higher expression of CD163 in pMφ during peritonitis (see Figure 1). Furthermore, the ability of pMφ to stimulate fibroblast proliferation was associated to the production of CCL18 as populations secreting low amounts of the chemokine were unable to stimulate cell growth (Figure 5B). Moreover, the addition of human recombinant CCL18 to cell cultures was also able to stimulate the proliferation of primary human fibroblasts (Figure 5C and D).

TGF-β1 and phagocytosis of apoptotic cells induce expression of CCL18

TGF-β1 and phagocytosis of apoptotic cells induce an M2 phenotype in human Mφ [14]. However, the expression of CCL18 in M2 induced by these stimuli has not been explored. As shown in Figure 6A, TGF-β1 induced the expression of CCL18 in monocytes at levels similar to those induced by IL-4. Co-cultures of Mφ with UV-irradiated apoptotic Jurkat cells and with non-irradiated viable Jurkat cells were also established. Gene expression of CCL18 was greatly enhanced by co-culture with apoptotic cells (Figure 6B). These results indicate that M2 induced by these two stimuli are also able to express high levels of CCL18.

CCL18 is mainly produced by pMφ in peritoneal effluent cell populations and secretion increases during peritonitis episodes

To confirm CCL18 protein production by pMφ, cytospin slides were prepared from peritonitis effluent cell samples and stained with anti-CCL18-specific antibodies. Strong staining was detected primarily in mononuclear cells (Figure 7A). Moreover, CCL18 was secreted by purified pMφ and was barely detected in culture supernatants from isolated CD66b+ peritoneal neutrophils from the same effluent samples (Figure 7B and C). The secretion of CCL18 was lower in pMφ from uninfected patients that did not suffer from previous peritonitis when compared to secretion by pMφ during or after acute peritonitis (Fig 7D). Secretion of CCL18 was also measured in peritoneal effluent purified CD3+ and CD14+ CD3- subpopulations with negative results (Supplementary Figure S3), thus confirming pMφ as the main cellular source of CCL18 in peritoneal effluents.

CCL18 protein levels are associated with poor UF capability and peritoneal fibrosis

Since CCL18 has been associated with fibrosis in various systems and peritoneal fibrosis is related to UF, peritoneal permeability was determined in 25 patients as dialysate to plasma ratio (D/P) of creatinine and the concentration of CCL18 was simultaneously measured in peritoneal effluents. CCL18 protein levels correlated with D/P of creatinine. These data were consistent with significantly increased levels of CCL18 in patients with UF. Moreover, CCL18 concentration was found to be further increased in effluents from those patients that later developed EPS. On the other hand, no relationship was found with IFN-γ levels measured in the same samples. IL-10 levels were also determined in the same samples since IL-10 is also secreted by M2 cells. Although IL-10 levels correlated with the D/P of creatinine, protein concentrations were not significantly higher in patients with UF or who were prone to develop EPS (Figure 8). The strong association of CCL18 protein levels with peritoneal function and development of EPS was maintained when protein levels were normalized according to dialysate volume (Supplementary Figure S8).

Discussion

Fibrosis is the end result of chronic inflammatory reactions. A subpopulation of Mφ that appears secondarily to inflammation is widely involved in subsequent fibrotic processes. One of the main features of Mφ is their phenotypic and functional heterogeneity as Mφ display a high degree of plasticity and different phenotypes may develop according

Fig. 6. TGF-β1 and apoptotic cell intake stimulate CCL18 gene expression. (A) CD14+ purified peripheral blood monocytes were cultured 96 h in RPMI plus 10% autologous serum in the presence or absence of 100 U/mL recombinant human IL-4 or 2.5 ng/mL TGF-β1. Cells were collected and mRNA content was estimated by qRT–PCR. (B) CD14+ purified peripheral blood monocytes were cultured 96 h in RPMI plus 10% autologous serum to induce Mφ maturation, and either Jurkat cells or apoptotic UV-irradiated Jurkat cells were added to Mφ and cultured for an additional period of 24 h. After washing, Mφ were harvested, total RNA was purified and CCL18 mRNA content was estimated by qRT–PCR. RNA from healthy and UV-irradiated Jurkat cells was included in the analysis for comparative purposes. Results are expressed as relative units (RU) representing the ratio between CCL18 mRNA molecules and B2M mRNA molecules in each sample. Data are representative of two independent experiments with similar results.
to environmental cues. The M\(_2\) subpopulation involved in fibrosis has been defined as alternatively activated or M2. M2 cells are characterized by their ability to promote matrix deposition, tissue remodelling and angiogenesis [3, 5, 7, 14, 17]. In fact, M2 are more closely related to reparative processes than to defence ability. In this sense, a relationship between fibrosis and recurrent peritonitis in end-stage renal disease patients treated with PD has been established [27, 29]. In this study, we detected M2 cells within the peritoneum of PD-treated patients, as defined by the expression of cell surface receptors CD206 and CD163, soluble factors and functional capabilities. In addition, we identified a relationship between CCL18 (a hallmark of human M2) produced by pM\(_2\) and the loss of peritoneal function associated with peritoneal membrane fibrosis.

During acute bacterial peritonitis, large quantities of neutrophils are recruited to the peritoneum. As neutrophil numbers decrease during the following days, these cells are replaced by an influx of mononuclear phagocytes. It is thus tempting to speculate that the clearance of apoptotic neutrophils by infiltrating M\(_2\) may induce the expansion of an M2 subpopulation, characterized by the expression of CD163, and TGF-\(\beta\)1. In this sense, induction of CD163 expression has been reported upon ingestion of apoptotic bodies [6], and pM\(_2\) have been shown to display high phagocytic activity [43]. Moreover, the phagocytosis of apoptotic cells by M\(_2\) stimulated with LPS has been shown to induce the expression of IL-10 [44]. Increased effluent levels of IL-10 have been found during peritonitis episodes [45] and may also participate in the up-regulation of CD163.

Increased TGF-\(\beta\) protein levels have also been reported in PD effluents during peritonitis [40]. Quantitative analysis showed a trend towards increased TGFB\(_1\) mRNA levels in CD14\(^{+}\) effluent cells, particularly in peritonitis-derived M\(_2\). Nevertheless, as most cell lineages are able to synthesize TGF-\(\beta\), other cell types in the peritoneum may account for the increased levels found in peritonitis. Moreover, the regulation of TGF-\(\beta\) activity is mainly post-transcriptional, and multiple factors may activate latent TGF-\(\beta\). Among

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**Fig. 7.** CCL18 protein production by peritoneal effluent M\(_2\). (A) Cytospin preparations were prepared from peritonitis-derived effluent cell samples and stained with a CCL18-specific goat antiserum. Non-immune serum was used as a negative background control (a). (B) Flow cytometry analysis of purified CD14\(^{+}\) M\(_2\) and CD66b\(^{+}\) neutrophils from peritonitis-derived peritoneal effluents. (C) Concentration of CCL18 in 24 and 48-h culture supernatants of purified CD14\(^{+}\) M\(_2\) and CD66b\(^{+}\) neutrophils from two patients with active peritonitis. Mean values and standard deviation of triplicate cultures are shown. (D) Concentration of CCL18 in 48 h culture supernatants of purified CD14\(^{+}\) M\(_2\) from uninfected patients and three patients with peritonitis at different time points. Secretion by peripheral monocytes from a healthy donor is shown for comparative purposes. Mean values and standard deviation of triplicate cultures are shown.
them, we found increased expression of *MMP9* in CD14<sup>+</sup> pMφ during peritonitis, which may contribute to the increased TGF-β1 activity found in peritonitis effluents.

*CCL18* mRNA was detected in purified CD14<sup>+</sup> pMφ populations at levels comparable to those found in M2 generated *in vitro*. *CCL18* expression is induced by IL-4, IL-13, IL-10 and glucocorticoids. [13, 34, 46, 47] Furthermore, our data demonstrate that TGF-β1 and phagocytosis of apoptotic cells are also able to induce CCL18 expression in human monocyte-derived Mφ, which further supports CCL18 expression in M2. We detected similar mean CCL18 mRNA levels in purified Mφ from non-infected and peritonitis samples. Nonetheless, protein levels were higher in culture supernatants from peritonitis-derived Mφ, which is in agreement with previous reports, suggesting a complex regulation of CCL18 protein synthesis and secretion (revised in [48]).

Moreover, inasmuch as the total number of Mφ increases during peritonitis episodes, the amount of CCL18 (and other fibrogenic factors) secreted by these cells would increase. Although *CCL18* was reported as expressed exclusively by cells of the mononuclear phagocyte system, it has been detected in polymorphonuclear leukocytes from rheumatoid arthritis patients [49]. However, we did not detect expression in peritoneal neutrophils or other CD14<sup>+</sup> subpopulations within peritonitis-derived effluents.

In summary, our data showing expression of CD163, CD206 and CCL18 suggest that the microenvironment within the peritoneal cavity supports alternative macrophage activation. CCL18 expression by pMφ and its correlation with the capacity of pMφ to induce fibroblast proliferation and with peritoneal membrane loss of function suggest that this M2-derived chemokine, which is involved in the development of lung fibrosis [18, 19, 22, 50], may also participate in peritoneal fibrosis. In this sense, it is noteworthy that we found the highest concentration of CCL18 in effluents from patients who later developed EPS. In this sense, our results...
confirm recently reported data [51] and identify the pMφ as the cellular source of CCL18 within the peritoneum. Continuous stress due to dialysis fluids and infection may promote the infiltration of cells that would be activated towards an M2 phenotype and may contribute to the fibrotic processes leading to UFF in long-term PD patients through the secretion of TGF-β, CCL18, MMP-9 and other fibrogenic factors. In conclusion, our study supports a role for M2 cells and M2-derived products, with a special significance of CCL18, in a human model of peritoneal fibrosis. Further research is needed to precisely delineate the contribution of various Mφ-derived factors to peritoneal membrane fibrosis.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. The authors have no conflicts of interest to declare. The results presented in this paper have not been published previously in whole or part, except in abstract format.

References

10. Puig-Kröger A, Serrano-Gómez D, Caparrós E et al. Regulated expression of the pathogen receptor dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin in THP-1 hu-
Alternatively activated peritoneal macrophages

40. Mlambo NC, Hylander B, Brauner A. Increased levels of transforming growth factor beta 1 and basic fibroblast growth factor in patients on CAPD: a study during non-infected steady state and peritonitis. *Inflammation* 1999; 23: 131–139

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