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ORIGINAL PAPER

Control of neutrophil influx during peritonitis by transcriptional cross-regulation of chemokine CXCL1 by IL-17 and IFN-γ

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Abstract

Neutrophil infiltration is a hallmark of peritoneal inflammation, but mechanisms regulating neutrophil recruitment in patients with peritoneal dialysis (PD)-related peritonitis are not fully defined. We examined 104 samples of PD effluent collected during acute peritonitis for correspondence between a broad range of soluble parameters and neutrophil counts. We observed an association between peritoneal IL-17 and neutrophil levels. This relationship was evident in effluent samples with low but not high IFN-γ levels, suggesting a differential effect of IFN-γ concentration on neutrophil infiltration. Surprisingly, there was no association of neutrophil numbers with the level of CXCL1, a key IL-17-induced neutrophil chemoattractant. We investigated therefore the production of CXCL1 by human peritoneal mesothelial cells (HPMCs) under *in vitro* conditions mimicking clinical peritonitis. Stimulation of HPMCs with IL-17 increased CXCL1 production through induction of transcription factor SP1 and activation of the SP1-binding region of the *CXCL1* promoter. These effects were amplified by TNFα. In contrast, IFN-γ dose-dependently suppressed IL-17-induced SP1 activation and CXCL1 production through a transcriptional mechanism involving STAT1. The SP1-mediated induction of CXCL1 was also observed in HPMCs exposed to PD effluent collected during peritonitis and containing IL-17 and TNFα, but not IFN-γ. Supplementation of the effluent with IFN-γ led to a dose-dependent activation of STAT1 and a resultant inhibition of SP1-induced *CXCL1* expression. Transmesothelial migration of neutrophils *in vitro* increased upon stimulation of HPMCs with IL-17 and was reduced by IFN-γ. In addition, HPMCs were capable of binding CXCL1 at their apical cell surface. These observations indicate that changes in relative peritoneal concentrations of IL-17 and IFN-γ can differently engage SP1–STAT1, impacting on mesothelial cell transcription of *CXCL1*, whose release and binding to HPMC surface may determine optimal neutrophil recruitment and retention during peritonitis.

Keywords: mesothelial cells; CXCL1; IL-17; IFN-γ; peritonitis; peritoneal dialysis

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No conflicts of interest were declared.

Introduction

Peritonitis continues to represent a common complication of peritoneal dialysis (PD) [1], which can lead to treatment failure. An early phase of peritonitis involves a massive neutrophil infiltration that is driven by local secretion of neutrophil-specific chemoattractants. These include chemokines, such as CXC-chemokine ligand 1 (CXCL1), derived from the peritoneal mesothelium. Human peritoneal mesothelial cells (HPMCs) respond

rapidly to classic pro-inflammatory cytokines IL-1β and TNFα by secreting huge quantities of CXCL1 [2]. More recently, IL-17 has been identified as another inducer of neutrophil-mediated responses [3]. We have previously demonstrated that IL-17 strongly stimulates HPMCs to produce CXCL1 *in vitro* and promotes CXCL1-mediated neutrophil trafficking in mice [4]. However, the mechanisms by which IL-17 controls the magnitude of chemokine production and neutrophil infiltration during peritonitis in humans are not fully defined.

IL-17 originates largely from T_H17 and γδ T cells, but other cell types may also contribute [5,6]. Whilst IL-17 is virtually undetectable in a healthy human peritoneum, it appears in peritoneal biopsies from patients undergoing PD [7]. The expression of IL-17 correlates with the duration of PD treatment and the extent of peritoneal inflammation and fibrosis. The concentration of IL-17 in the dialysis effluent increases variably at the onset of peritonitis [8] and declines when the infection resolves [9]. Interestingly, IL-17 levels seem to be higher in patients with a rapid response to antibiotic treatment compared with those with a delayed response [9]. This may indicate that local IL-17 contributes to the peritoneal host defence. In this respect, IL-17 seems to be crucially involved in the immunity against staphylococci [10]. Peritoneal inoculation of *Staphylococcus aureus* in mice leads to rapid recruitment of IL-17-producing cells and expansion of memory γδ T cells, which produce high levels of IL-17 promptly after re-infection with *S. aureus* [11]. The IL-17 responses to staphylococci may be initiated by the activation of Toll-like receptor 2 (TLR2), resulting in the production of cytokines which stimulate IL-17-expressing cells [10].

On the other hand, excessive accumulation of IL-17 precedes the formation of adhesions and abscesses after peritoneal infection or surgical injury in mice [12,13]. These complications can be prevented by blocking either IL-17 or the receptor CXCR2 for neutrophil-specific chemokines, including CXCL1 [13]. In the context of PD, daily injections of PD fluids in mice lead to the infiltration of the peritoneum by T_H17 and γδ T cells and increased peritoneal IL-17 [7,14].

The magnitude of IL-17-induced CXCL1 expression is regulated by transcriptional and post-transcriptional mechanisms [15]. The post-transcriptional control is related predominantly to stabilisation of *CXCL1* mRNA [16,17]. The mechanism of transcriptional *CXCL1* activation is less clear [15,18]. This occurs partly through activation of the transcription factor NF-κB [19,20]. However, the involvement of other transcription factors is also possible [15].

IFN-γ is a key mediator of T_H1 cell responses, but it is also derived from γδ T cells [21]. Moreover, T_H17 cells show developmental plasticity that allows them to express IFN-γ in addition to IL-17 [22]. In the peritoneum, IFN-γ acts jointly with IL-6 and contributes both to the recruitment and to the clearance of neutrophils [23]. Early during peritonitis, IFN-γ ensures the generation of CXC chemokines at the level necessary for effective neutrophil recruitment. At later stages, IFN-γ controls neutrophil removal by down-regulating CXC chemokine production [24,25].

The functions of IFN-γ are mediated by direct activation of target genes but also by cross-regulation of responses to other cytokines [26]. It is increasingly clear that the interaction between IFN-γ and IL-17 is important for the effective host response to infection. While the suppressive effect of IFN-γ on T_H17 cell differentiation is well recognised [27], little is known about the effect of IFN-γ on effector functions of IL-17. Considering that the interplay between IFN-γ and IL-17 depends strictly on the pathophysiological context and is less understood in humans than in mice, we set out to investigate whether IL-17 and IFN-γ interact to modulate neutrophil influx during PD and exert transcriptional control over expression of *CXCL1* in human mesothelium.

Materials and methods

Materials

Unless stated otherwise, all chemicals were from Sigma-Aldrich (St Louis, MO, USA) and culture plastics were Falcon® from Corning Life Sciences (Tewksbury, MA, USA). Human recombinant IL-17, IFN-γ, TNFα, and CXCL1 were from R&D Systems (Bio-Techne; Wiesbaden, Germany). The specific activity of IFN-γ was 2×10^4 WHO standard units per 1 μg protein (1 U/ml = 50 pg/ml).

Patient samples

Samples of PD effluent (104 samples; 68 male and 36 female samples) were obtained from separate infective episodes from 87 patients (30 female patients and 57 male patients). The samples were collected in the course of the 'Patient Immune Responses to Infection in PD' (PERIT-PD) study, registered on the UK Clinical Research Network Portfolio (No 11838). The study was approved by the South East Wales Local Ethics Committee (04WSE04/27) and all patients provided written informed consent. Subjects positive for HIV or hepatitis C were excluded from the study. All samples were from the first day of presenting at hospital with a cloudy bag, before commencing antibiotic treatment. The patient characteristics are summarised in supplementary material, Table S1.

An exemplary peritoneal effluent used in the *in vitro* experiments was from a patient with *Enterobacter cloacae* peritonitis. Cytokine concentrations in the effluent were determined using Quantikine ELISA kits (R&D Systems, Bio-Techne; Wiesbaden, Germany), and the recorded levels were IL-17, 4 pg/ml; IFN-γ, undetectable; and TNFα, 231 pg/ml.

Cells from peritoneal effluents were analysed as previously described [28] and as detailed in supplementary material, Supplementary material and methods.

Mesothelial cell culture

HPMCs were cultured as previously described [29]. For the experiments, cells were rendered quiescent by serum deprivation for 24 h and then stimulated as specified in the figure legends. All experiments were performed with cells from the third passage and not later, to minimise the number of senescent cells, as this may affect the level of CXCL1 released [30].

Immunoassays

CXCL1 concentration in cell culture supernatants was measured using a DuoSet Immunoassay Kit (R&D Systems, Bio-Techne; Wiesbaden, Germany). Binding of recombinant CXCL1 to HPMCs was assessed by an immunoassay according to van Gemst *et al* [31]. CXCL1 and other soluble parameters in cell-free peritoneal effluents were measured as previously described [28]. Details of these immunoassays and CXCL1 binding experiments are provided in supplementary material, Supplementary material and methods.

Gene expression analysis

Expression of target genes relative to β2-microglobulin (*B2M*) mRNA was assessed using reverse transcription and quantitative PCR (RT-qPCR), as described elsewhere [29]. The data are presented as a fold-change over control levels in untreated cells. PCR conditions are specified in supplementary material, Supplementary materials and methods. PCR primer sequences are listed in supplementary material, Table S2.

DNA constructs and reporter plasmids

Genomic DNA from HPMCs was isolated with IsolRNA Lysis Reagent (5 Prime, Hamburg, Germany) and used to generate progressive *CXCL1* luciferase plasmid constructs (pLuc 1700, pLuc 1120, pLuc 620, and pLuc 150) through PCR amplification with appropriate primer pairs. The Infusion Cloning Kit (Takara Bio, Mountain View, CA, USA) was used with the pGL4.10 vector backbone to create the luciferase reporter constructs. The length of the promoter segments was verified by restriction digestion and sequencing (LGC Genomics, Berlin, Germany). To target specific binding sites within the *CXCL1* promoter, the desired sequence was inserted into the *CXCL1* construct using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs, Frankfurt, Germany), as detailed in supplementary material, Supplementary materials and methods.

Transfection studies

Transient transfection and luciferase assays were performed as previously described [29] using the siRNA Transfection Reagent (Santa Cruz Biotechnology, Heidelberg, Germany). The siRNAs used are listed in supplementary material, Supplementary materials and methods.

Computational analysis of the *CXCL1* promoter

The human *CXCL1* promoter region −519 to −51 (GenBank NC_000004.12) was analysed using the PROMO virtual laboratory (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) for the presence and location of potential transcription factor binding sites.

Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP)

Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Darmstadt, Germany). Formaldehyde cross-linking and ChIP were performed using a ChIP-IT High Sensitivity Kit (Active Motif, Carlsbad, CA, USA). The conditions of EMSA and ChIP are summarised in supplementary material, Supplementary material and methods.

Western blotting

Cell extracts were prepared as previously described [32], electrophoresed on sodium dodecyl sulphate-polyacrylamide gels, and immune-blotted using antibodies listed in the supplementary material, Table S3. The bands obtained were visualised and analysed using an Enhanced Chemiluminescence Detection System (Thermo Scientific) and ImageJ 1.43 software (National Institutes of Health, Bethesda, MD, USA).

Neutrophil transmigration assay

Neutrophil migration was measured using a Transwell assay, as previously described [33]. Migration was assessed in response to conditioned medium from HPMCs or through HPMC monolayers established on Transwells [34] as specified in the supplementary material, Supplementary materials and methods.

Statistics

All patients' data were processed in R software (version 3.5.0), using base packages and VGAM, tidyverse and stringr. Data with missing values were excluded from the study. To compute the threshold for the effect of IFN-γ expression on IL-17 activity *in vivo*, R^2 values of IL-17 versus neutrophil numbers were calculated for all possible IFN-γ thresholds. A Loess curve was fitted to the relationship between R^2 and IFN-γ, and local maxima were found for the data below and above the threshold. By repeating the analysis with random sets of 100 samples, a clear maximum in combined R^2 was seen when the threshold was between 23.8 and 25.4 pg/ml IFN-γ. This was averaged to 24.5 pg/ml for use in further analyses.

All other analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). The data were analysed with the *t*-test or repeated measures analysis of variance for paired data.

Results were expressed as mean ± SD. Differences with a P value less than 0.05 were considered significant.

Results

Association between neutrophil influx during peritonitis and intraperitoneal IL-17 and IFN-γ levels

First, we examined whether there existed a correspondence between peritoneal levels of IL-17 and/or IFN-γ and neutrophil numbers. These were measured in 104 samples of dialysate effluent from patients with acute peritonitis. As expected, neutrophils were increased in the culture-positive samples, but no distinction could be made between neutrophil numbers in Gram-positive and Gram-negative bacterially infected patients (Figure 1A). Similarly, IL-17 was lower in culture-negative than in culture-positive samples (Figure 1B). The distributions of IL-17 levels were very broad in both Gram-positive and Gram-negative infections, and were not distinguishable between the two. In contrast, the distributions of IFN-γ levels were largely the same in patients with culture-negative and culture-positive peritonitis, with only a longer high-expressing tail in Gram-positive infections (Figure 1C). When IL-17 and neutrophil numbers were plotted, IL-17 showed a moderate but highly significant association with neutrophil levels (Figure 1E). IFN-γ showed a weak association that could be fitted with a quadratic equation (Figure 1F), suggesting a differential effect of low and high IFN-γ concentrations. Modelling neutrophil numbers with respect to interactions between IFN-γ and IL-17 showed that including interaction terms with IFN-γ improved the fit. Indeed, if samples were separated into groups expressing low (< 24.5 pg/ml) or high (≥ 24.5 pg/ml) IFN-γ levels, it could be seen that IL-17 had a significant association with neutrophil numbers in samples with low IFN-γ but no association with neutrophil numbers in the presence of higher IFN-γ concentrations (Figure 1G). This analysis suggested that low IFN-γ levels allowed for the expression of the IL-17 phenotype with respect to increasing neutrophil influx. Interestingly, the pattern of expression of IL-17 and its effect on neutrophil numbers were paralleled by those of IL-12p40 (a member of the IL-17/IL-23 axis) and IL-16 (supplementary material, Figure S1A,B). This was in contrast to mediators derived primarily from neutrophils [matrix metalloproteinase (MMP)-8, neutrophil gelatinase-associated lipocalin (NGAL), myeloperoxidase], which were tonically up-regulated independently of IFN-γ levels (supplementary material, Figure S1C–E), and also to a wide range of chemokines and cytokines unrelated to the IL-17 pathway (such as IL-2,IL-12p70, and TNFα), which showed no correspondence to neutrophil levels (supplementary material, Figure S1F–H). Surprisingly, there was also no appreciable association of neutrophil numbers with a key neutrophil-attracting chemokine, CXCL1 (Figure 1D,H).

Effect of IL-17 and IFN-γ on CXCL1 secretion and expression by HPMCs

To understand the observed paradox, we analysed the interaction between IL-17 and IFN-γ with respect to

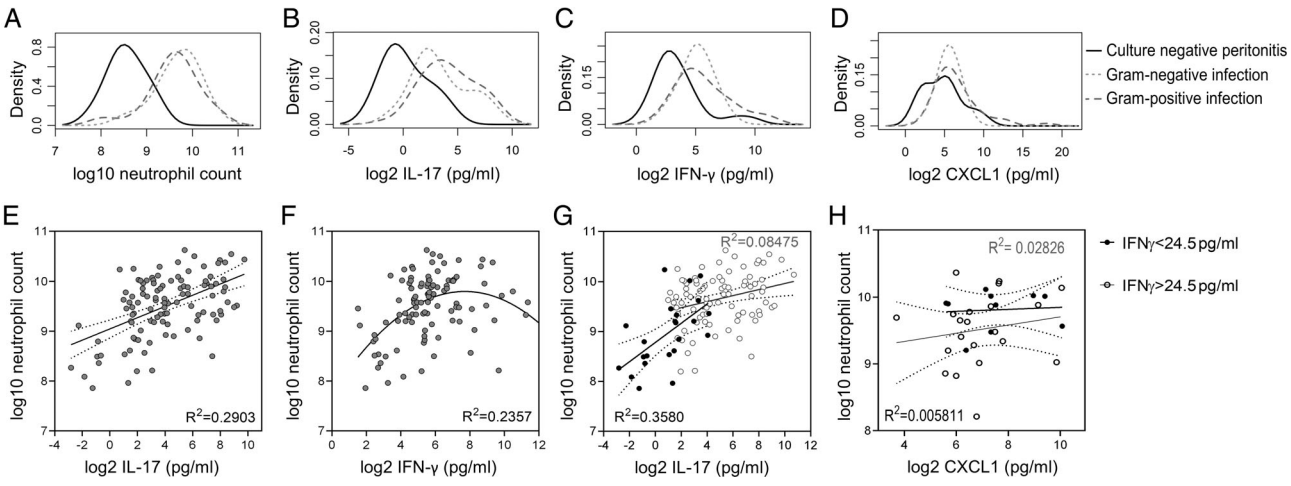

Figure 1. Associations between neutrophil numbers during peritonitis and intraperitoneal levels of IL-17, IFN-γ, and CXCL1. (A–C) Distributions of neutrophils, and IL-17 and IFN-γ expression in peritoneal effluent from patients with peritonitis. (A) Neutrophil count in total peritoneal fluid from patients. (B–D) Concentrations of IL-17 (B), IFN-γ (C), and CXCL1 (D) in peritoneal fluid. Neutrophils were defined and counted using flow cytometry on the basis of forward and side scatter and expression of CD15. The graphs are density plots, with the y axis representing the likelihood of a sample having a particular x value, for data averaged using weights across a small interval to produce a smoothed curve. (E, F) Association of IL-17 and IFN-γ levels with neutrophil numbers. (E) IL-17 or (F) IFN-γ concentrations were compared with overall neutrophil counts in peritonitis samples and either linear or quadratic regressions fitted to the log data. (G) Effect of IFN-γ concentrations on the relationship between IL-17 as neutrophil numbers. Regression of samples in which IFN-γ was below 24.5 pg/ml (filled circles) as compared with those above 24.5 pg/ml (open circles) showed that a relationship between IL-17 concentration and neutrophil numbers existed below but not above the threshold. (H) Comparison of CXCL1 concentrations and neutrophil numbers. The samples were analysed using the IFN-γ threshold as above.

CXCL1 production by HPMCs *in vitro*. Exposure of HPMCs to IL-17 resulted in a time- and dose-dependent increase in CXCL1 secretion. This increase was significantly reduced by IFN-γ (Figure 2A,B). The half-maximal inhibitory concentration of IFN-γ (IC_{50}) for this effect was 280 ± 95 pg/ml. Anti-IFN-γ antibodies, but not control IgG, abolished the inhibitory activity of IFN-γ, confirming the specificity of its effect towards CXCL1 (Figure 2C). A similar inhibition of IL-17 activity by IFN-γ was also observed at the mRNA level (Figure 2D).

Effect of IL-17 and IFN-γ on activation of the *CXCL1* gene promoter

Exposure of HPMCs transiently transfected with *CXCL1* luciferase reporter gene constructs to IL-17 strongly increased activity of the full-length *CXCL1* promoter (Figure 2E). IFN-γ alone exerted no effect on promoter activity, but it reduced the activity induced by IL-17. Truncation of successive *CXCL1* promoter fragments revealed that IL-17 activity was mediated within a promoter region spanning positions −519 to −51 (Figure 2F). *In silico* analysis determined that this region contained high affinity binding sites for the transcription factors specificity protein-1 (SP1) and signal transducer and activator of transcription-1 (STAT1). The STAT1-binding site mutation did not affect the activation of the *CXCL1* promoter by IL-17, but prevented IFN-γ from inhibiting IL-17-induced activity (Figure 2G). On the other hand, the level of *SP1* mRNA increased significantly in response to IL-17 and decreased after the addition of IFN-γ (Figure 2H).

Effect of IL-17 and IFN-γ on activation of the transcription factor SP1

Nuclear extracts from IL-17-treated HPMCs formed a DNA–protein complex with a consensus oligonucleotide for SP1 binding that encompassed positions −227 to −250 of the *CXCL1* promoter (Figure 3A). The specificity of SP1 binding was confirmed by EMSA with the anti-SP1 antibody, which dose-dependently supershifted the DNA–protein complex (Figure 3B). Another EMSA showed that IFN-γ alone had no effect on SP1 binding; however, it completely abolished the effect of IL-17 (Figure 3C). ChIP using the PCR-amplified fragment of the *CXCL1* promoter and HPMC-derived chromatin precipitated with an SP1 antibody confirmed that IL-17 stimulated SP1 binding to the *CXCL1* promoter (Figure 3D) and that this effect was eliminated by IFN-γ (Figure 3E).

The functional role of SP1 for *CXCL1* induction was also confirmed by stimulating HPMCs with IL-17 in the presence of *SP1*-targeting siRNA, which led to inhibition of *CXCL1* mRNA (Figure 3F).

Role of IFN-γ–STAT1 signalling in modulation of IL-17-induced *CXCL1* expression

Whilst the above experiments demonstrated the role of SP1 in the induction of *CXCL1* by IL-17, they did not fully elucidate the mechanism of modulatory activity of IFN-γ. Experiments using site-directed mutagenesis (see above) suggested that this effect occurred through STAT1. Indeed, the addition of IFN-γ to IL-17-stimulated HPMCs resulted in a significant up-regulation of STAT1 protein by a mechanism that was blocked by *STAT1*-specific RNA interference (Figure 4A). When HPMCs stimulated with IL-17 + IFN-γ were treated with *STAT1* siRNA, the ability of IFN-γ to down-regulate *SP1* and *CXCL1* expression was lost (Figure 4B,C). ChIP analysis confirmed that exposure to IFN-γ led to an association of STAT1 with the *CXCL1* promoter DNA (Figure 4D).

Effect of TNFα on IL-17-induced SP1-mediated CXCL1 expression

We previously demonstrated that TNFα amplified IL-17-induced CXCL1 release [4]. Here, we analysed whether this effect involved SP1. The addition of TNFα to IL-17-treated HPMCs led to a significant increase in both *SP1* and *CXCL1* mRNAs, but only a marginal induction of *STAT1*. Administration of IFN-γ to IL-17 + TNFα-treated HPMCs resulted in a significant up-regulation of *STAT1* and a dose-dependent decrease in *SP1* and *CXCL1* expression (supplementary material, Figure S2A–C).

Role of SP1- and STAT1-mediated pathways in dialysate-induced *CXCL1* expression during peritonitis

To mimic the conditions of clinical PD, HPMCs were exposed to the effluent obtained from a model PD patient presenting with peritonitis. We asked whether such an effluent could induce *CXCL1* in HPMCs through the newly identified mechanisms. To this end, the effluent containing IL-17, but no IFN-γ, was used, as it was hypothesised that it would show an uninhibited effect towards CXCL1. Indeed, exposure of HPMCs to this effluent resulted in an increase in both *CXCL1* and *SP1* mRNAs (Figure 5A,B), and the increased *CXCL1* expression was reduced with *SP1* siRNA (Figure 5C). Although this inhibitory effect confirmed the contribution of SP1 to *CXCL1* induction, its magnitude was surprising given that the level of IL-17 in this particular effluent was rather low. We hypothesised that IL-17 synergised with TNFα, whose concentration in the effluent was quite substantial. Indeed, the stimulatory effect of the effluent on *CXCL1* and *SP1* expression was significantly reduced by antibodies neutralising IL-17 and TNFα (Figure 5D,E). As IFN-γ was not detected in the effluent, we spiked it with increasing doses of exogenous IFN-γ. The addition of IFN-γ resulted in a significant decrease in effluent-induced *CXCL1* and *SP1* expression (Figure 5F,G). This effect was associated with a marked dose-dependent induction of *STAT1* by IFN-γ (Figure 5H).

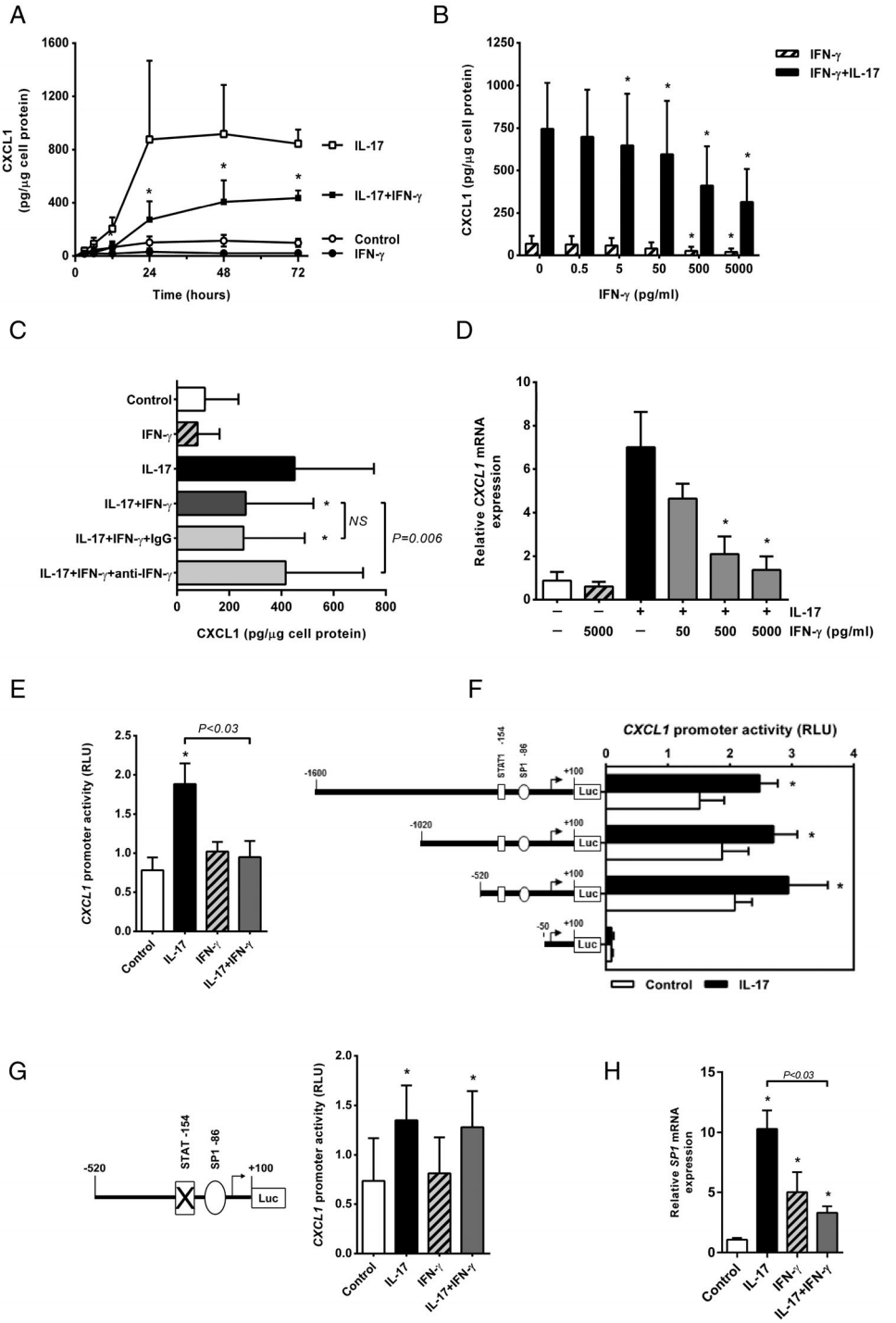

Figure 2. Effect of IL-17 and IFN-γ on CXCL1 secretion and activation of the *CXCL1* gene promoter expression by in HPMCs. (A) Kinetics of CXCL1 secretion in response to IL-17 (50ng/ml), IFN-γ (1.25ng/ml) or combination thereof. *$p < 0.05$ for comparison IL-17 versus IL-17±IFN-γ at each time point ($n = 5$). (B) Dose effect of IFN-γ on basal or IL-17-induced (50ng/ml) CXCL1 release over 24h ($n = 8$). *$p < 0.05$ versus basal or IL-17 control values. (C) Effect of IFN-γ neutralisation on its inhibitory activity towards IL-17-induced CXCL1 secretion. Cells were incubated with IL-17 (50ng/ml) and IFN-γ (1.25ng/ml) in the presence of IFN-γ-specific or control antibodies (at 10μg/ml) for 24h. *$p < 0.05$ versus cells treated with IL-17 alone ($n = 6$). (D) Effect of IL-17 and IFN-γ on *CXCL1* mRNA expression. Cells were stimulated for 24h with IL-17 (100ng/ml) and IFN-γ at the doses indicated. *$p < 0.05$ versus untreated cells ($n = 4$). (E–G) Cells were transiently transfected with *CXCL1* promoter constructs and then stimulated with IL-17 (100ng/ml) and/or IFN-γ (5ng/ml) for 6h as indicated. Luciferase activity was expressed as relative light units (RLU). (E) Activity of 1700bp-length *CXCL1* promoter after stimulation with IL-17 and/or IFN-γ. *$p < 0.05$ versus unstimulated controls ($n = 4$). (F) Effect of progressive 5′-deletions of the *CXCL1* promoter on its responsiveness to IL-17. *$p < 0.05$ versus unstimulated controls ($n = 4$). (G) Effect of site-directed mutagenesis in the STAT1-binding site of the *CXCL1* promoter on its activity after stimulation with IL-17 and/or IFN-γ. *$p < 0.05$ versus unstimulated control ($n = 5$). (H) *SP1* mRNA expression after stimulation with IL-17 and/or IFN-γ for 3h. *$p < 0.05$ versus unstimulated controls ($n = 4$).

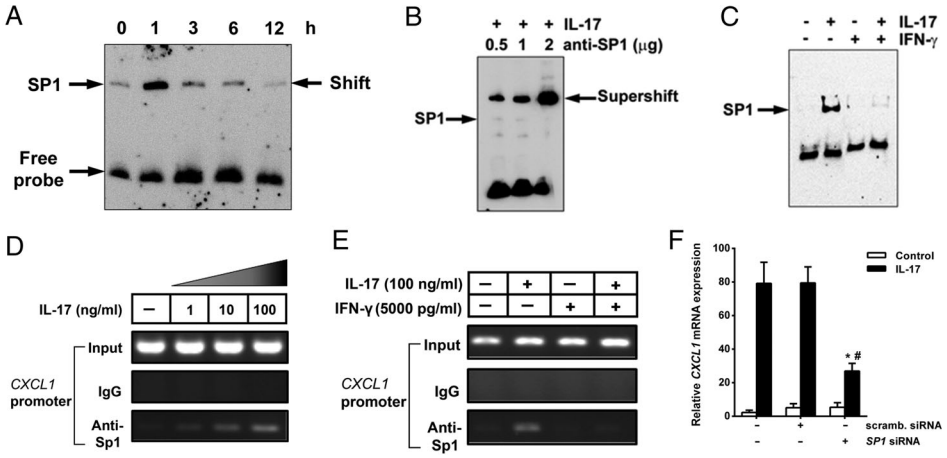

Figure 3. Identification of SP1 as a transcriptional mediator of *CXCL1* promoter induction by IL-17. (A–C) Nuclear fractions from cells stimulated with IL-17 (100 ng/ml) and/or IFN-γ (5 ng/ml) were analysed using EMSA and a consensus oligonucleotide probe for SP1. (A) Nuclear extracts were obtained from cells stimulated with IL-17 for various times. (B) Nuclear extracts were isolated 6 h after stimulation with IL-17 and subjected to EMSA in the presence of increasing concentrations of SP1-specific antibody (resulting in a supershift). (C) EMSA was performed with nuclear extracts from cells stimulated with IL-17 ± IFN-γ for 6 h. (D, E) After stimulation with IL-17 (100 ng/ml) and/or IFN-γ (5 ng/ml) for 6 h, chromatin was isolated and precipitated using an SP1 antibody, and the DNA bound was identified by PCR. (F) Cells were transiently transfected with 10 nM of either SP1-specific siRNA or scrambled siRNA, stimulated with IL-17 (100 ng/ml) for 24 h, and then assessed for *CXCL1* mRNA expression. $p < 0.05$ versus cells stimulated with IL-17 (*) in the absence of siRNAs or ($^{\#}$) in the presence of scrambled siRNA ($n = 4$).

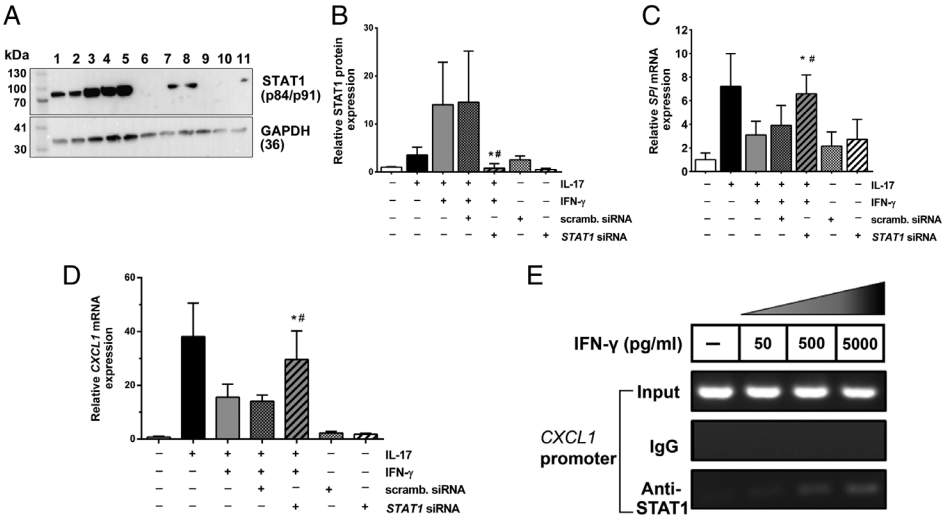

Figure 4. Effect of STAT1 on IFN-γ-modulated CXCL1 induction. (A–D) Cells were transiently transfected with 10 nM of either STAT1-specific siRNA or scrambled siRNA control, stimulated with IL-17 (100 ng/ml) and/or IFN-γ (5 ng/ml) for 24 h, and then assessed by western blotting for (A, B) STAT1 protein expression and by RT-qPCR for (C) *SP1*, and (D) *CXCL1* mRNA expression. $p < 0.05$ versus cells stimulated with IL-17 + IFN-γ (*) in the absence of siRNAs or ($^{\#}$) in the presence of scrambled siRNA ($n = 6$). (E) Dose effect of IFN-γ on STAT1 binding to *CXCL1* promoter, as assessed by ChIP. In A, a representative immunoblot is shown; in B, immunoblot data quantified from six independent experiments.

Effect of HPMC exposure to IL-17 and IFN-γ on neutrophil migration

To see whether HPMCs exposed to IL-17 ± IFN-γ modulate migration of neutrophils, we first assessed the effect of medium conditioned by HPMCs. The samples from IL-17-treated HPMCs produced a strong migratory response in neutrophils in a Transwell assay (Figure 6A). Surprisingly, the effect of medium from HPMCs treated with IL-17 ± IFN-γ was not different from that of IL-17 alone. We then assessed neutrophil transmigration through HPMC monolayers seeded on Transwells and stimulated with IL-17 ± IFN-γ. IL-17 induced neutrophil migration also in this setting; however, the addition of IFN-γ significantly reduced the effect (Figure 6B). This suggested that the presence of HPMCs played a key role in controlling leukocyte migration. Since it has recently been

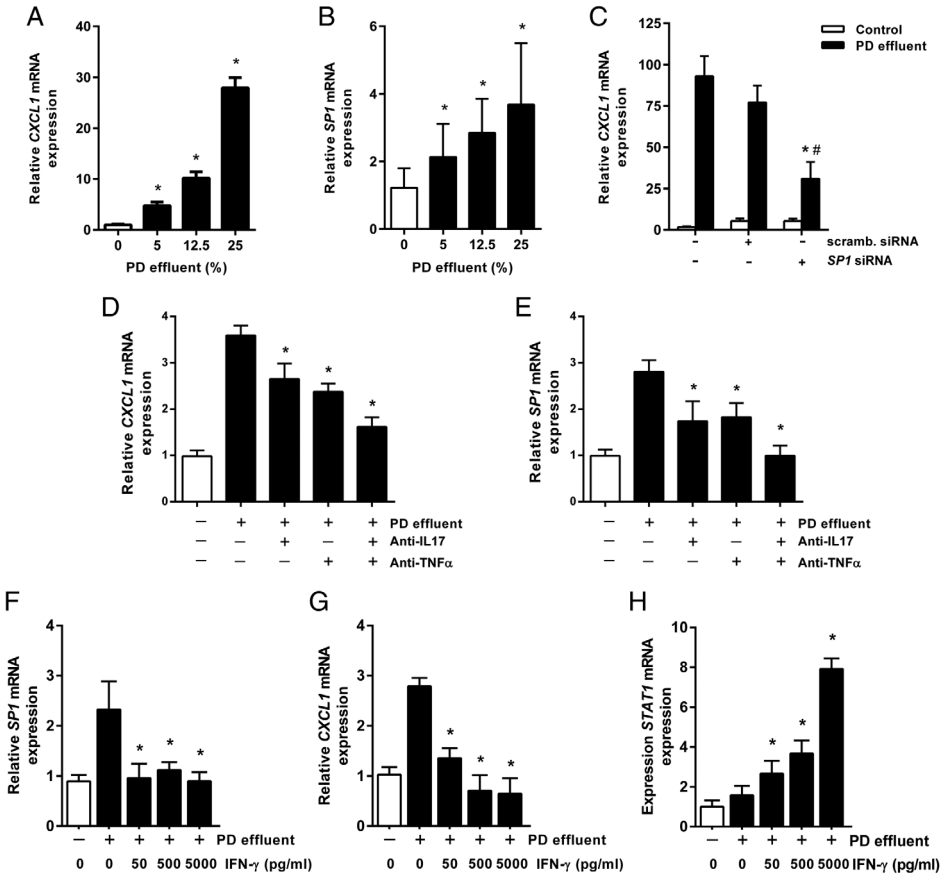

Figure 5. Contribution of SP1 and STAT1 to CXCL1 induction in HPMCs during peritonitis. (A, B) Cells were treated for 24 h with increasing doses of exemplary PD effluent drained during peritonitis and analysed for mRNA expression of (A) *CXCL1* and (B) *SP1*. *$p < 0.05$ versus untreated controls ($n = 4$). (C) Cells were transiently transfected with 10 nM *SP1*-specific siRNA or scrambled (scramb.) siRNA and then exposed for 24 h to peritoneal effluent (25% v/v) from a patient with peritonitis and assessed for *CXCL1* mRNA expression. $p < 0.05$ versus cells treated with the dialysate (*) in the absence of siRNAs or (#) in the presence of scrambled siRNA ($n = 4$). (D–H) Cells were exposed to peritonitis PD effluent (25% v/v) for 24 h in the presence or absence of (D, E) antibodies specific for TNFα (10 μg/ml) and IL-17 (0.5 μg/ml) or (F-H) IFN-γ (50–5000 pg/ml). Cells were then assessed for mRNA expression of (D, G) *CXCL1*, (E, F) *SP1* or (H) *STAT1*. *$p < 0.05$ versus cells treated with the dialysate in the absence of (D, E) antibodies or (G-H) IFN-γ ($n = 4$).

demonstrated that neutrophil migration during peritonitis in mice depends on the expression of CXCL1 over the mesothelial surface [35], we tested whether CXCL1 could bind to HPMCs. After the exposure of HPMCs to recombinant CXCL1, the presence of CXCL1 was detected on the HPMC surface by immunoassay (Figure 6C).

Discussion

Having observed that the number of neutrophils infiltrating the peritoneum during peritonitis associated with IL-17, we analysed the mechanism by which IL-17 induced a neutrophil-attracting chemokine, CXCL1, in HPMCs. We identified the transcription factor SP1 as being pivotal to this process, which is a novel finding since SP1 has not previously been linked with IL-17-induced genes. Many IL-17 target genes are thought to be activated through NF-κB, as their promoters are often enriched in NF-κB binding sites [18]. While IL-17 can activate NF-κB in HPMCs [36], our study demonstrates that the effect of IL-17 on *CXCL1* transcription is ultimately mediated by SP1.

Although SP1 is ubiquitously expressed, its transcriptional activity differs considerably across the tissues [37]. Except for the finding that SP1 is required for *CXCL1* transcription in retinal and melanoma cells [38], there were no other reports showing the involvement of SP1 in IL-17-induced responses and the *CXCL1* promoter regulation. Thus, it remains to be determined whether our observation represents a general phenomenon or is an example of tissue-specific regulation.

The SP1-mediated activity of IL-17 could be dampened by IFN-γ through a mechanism involving STAT1. This is an interesting finding since the interactions between STAT1 and SP1 are poorly recognised. There are only a few reports on SP1–STAT1 co-operation, which turned out to be necessary for full transcriptional gene activation [39–43]. That STAT1 can curtail rather than amplify SP1-driven *CXCL1* transcription reveals a novel aspect of transcriptional control. STAT1 deficiency in mice was found to increase *CXCL1* expression and neutrophil infiltration, which could be suppressed by

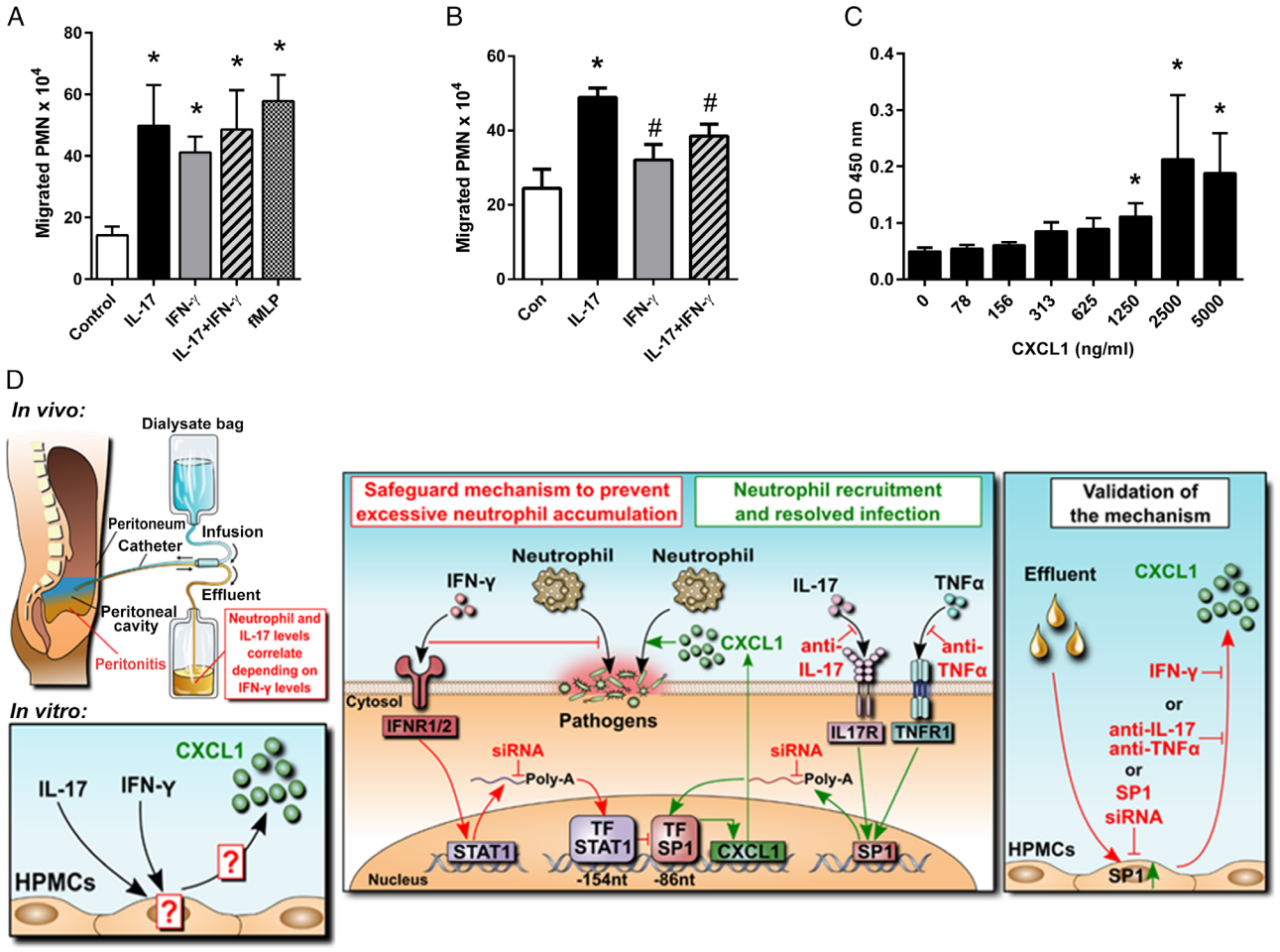

Figure 6. (A, B) Effect of HPMC exposure to IL-17 and IFN-γ on neutrophil migration. (A) Effect of conditioned media from HPMCs on neutrophil migration. HPMCs were stimulated with IL-17 (100 ng/ml), IFN-γ (5 ng/ml) or IL-17 ± IFN-γ (n = 3). The conditioned media were collected after 24 h, diluted 1:1 (v/v) in plain medium, and applied to the lower chambers of the Transwell systems. fMLP (10 nM) was used as a positive control. *$p < 0.05$ versus medium from unstimulated cells. (B) Neutrophil migration through HPMC monolayers. HPMCs were established on the underside of Transwells and stimulated as above for 24 h (n = 4). For both A and B, neutrophils (1.5×10^6) were loaded into the upper Transwell chamber and cells that had migrated to the lower chamber were quantified using a standard curve prepared by determining myeloperoxidase activity in the lysates of known numbers of neutrophils. *$p < 0.05$ versus untreated cells; #$p < 0.05$ versus IL-17-stumulated cells (n = 4). (C) CXCL1 binding to HPMCs *in vitro*. HPMCs were exposed to serial dilutions of recombinant CXCL1 for 30 min and CXCL1 binding was detected by immunoassays. *$p < 0.05$ versus untreated cells (n = 5). (D) Implications of the present study. Neutrophil counts in the dialysate fluid from PD patients with acute peritonitis associate with IL-17 levels depending on the concentration of IFN-γ. They do not, however, correlate with the levels of CXCL1, a key mesothelial cell-derived IL-17-induced neutrophil chemoattractant. Thus, we sought to determine how IL-17 and IFN-γ interact to modulate CXCL1 production by HPMCs. IL-17 initiates rapid transcription and translation of SP1, a transcription factor essential for the production of CXCL1. This process is amplified by TNFα but inhibited by IFN-γ operating through the STAT1 pathway. Following the stimulation of HPMCs by IL-17 and IFN-γ, the CXCL1 is released and cell-bound, which modulates accordingly transmesothelial migration of neutrophils. The effluent collected during peritonitis contains IL-17 and TNFα and is capable of stimulating HPMCs to produce CXCL1 through SP1 activation. Supplementing the effluent with IFN-γ, adding antibodies against IL-17 and TNFα, or the introduction of siRNA targeting *SP1* mRNA can suppress SP1 and inhibit CXCL1 induction.

blocking IL-17 [44]. In humans, the gain-of-function *STAT1* mutations inhibited the development of IL-17-producing T cells and impaired IL-17 immunity [45]. Introduction of the human *STAT1* mutation into mice decreased the number of IL-17-producing cells upon *Candida albicans* infection [46]. Moreover, the death receptor Fas was demonstrated to promote T_H17 cell development by preventing STAT1 activation [47]. Indeed, some *FAS* polymorphisms affected the interactions between SP1 and STAT1 and their recruitment to the *FAS* promoter [43].

IL-17 is particularly important for mucocutaneous host defence [15,48]. Our observations suggest that IL-17 may play a similar role in the serosal membranes. By inducing CXCL1 production in HPMCs and amplifying the effects of TNFα during peritonitis, IL-17 may facilitate prompt neutrophil recruitment and effective bacterial clearance. In turn, IFN-γ may act as a safeguard mechanism protecting against excessive neutrophil infiltration. Consequently, the relative concentrations of IL-17 and IFN-γ may underlie the course of infection. In this respect, it has been determined that different bacteria induce distinct 'immune fingerprints' of soluble mediators in the peritoneal effluent [28] and may thus create a specific microenvironment promoting differentiation of either IL-17- or IFN-γ-producing cells. Involvement

of IL-17 in the regulation of neutrophil influx during peritonitis is supported by the fact that the same pattern of interaction with IFN-γ was observed for other cytokines of the IL-17 axis but not for members of other signalling pathways.

It will be interesting to examine whether the interplay between SP1 and STAT seen in HPMCs occurs in conditions other than PD. It has been reported that impaired IFN-γ signalling exacerbated IL-17-mediated neutrophil infiltration of joints in murine arthritis [49], and that IFN-γ prevented excessive IL-17-mediated neutrophil accumulation in tuberculosis [50]. In addition, IFN-γ priming of macrophages was found to decrease, possibly through a STAT1-dependent mechanism, expression of several IL-17-inducible genes, including *CXCL1* [51].

Immune responses to environmental threats are driven by subpopulations of T_H cells and aim to restore an equilibrium, which protects tissues from damaging inflammation [52]. In contrast, an unbalanced response may lead to inflammatory pathologies. According to this paradigm, our demonstration of IFN-γ down-regulating IL-17-induced CXCL1 production can be viewed as a mechanism controlling neutrophil recruitment and ensuring resolution of inflammation. On the other hand, excessive STAT1-mediated IFN-γ signalling may impair the host's ability to mount an IL-17-driven response to effectively clear infection. In the context of PD, it has been demonstrated that repeat episodes of acute peritonitis lead to expansion of T_H1 cells, which outnumber IL-17-producing cells [53]. Importantly, the exaggerated STAT1 signalling that is associated with such repeat peritoneal inflammation has been demonstrated to underlie the development of peritoneal fibrosis [54].

Interestingly, the suppressive effect of IFN-γ on IL-17-induced *CXCL1* expression in HPMCs *in vitro* did not translate into the corresponding pattern of neutrophil migration in response to conditioned medium from HPMCs stimulated with IL-17 ± IFN-γ. However, it became apparent when neutrophils migrated across the monolayer of stimulated HPMCs. This suggested that not only the released chemokines but also the presence of HPMCs contributed to leukocyte trafficking. In this regard, it has recently been demonstrated that intraperitoneal neutrophil migration during peritonitis occurs predominantly in the omental immune cell clusters (known as milky spots), which are covered by specialised mesothelial cells secreting and retaining CXCL1 [35]. This enables neutrophils to form dense aggregates on the mesothelial surface and trap invading microorganisms. Since our HPMC isolates were of omental origin, it was possible that they could retain on their surfaces some of the CXCL1 produced. Indeed, we showed that HPMCs were capable of binding CXCL1, possibly through cell surface glycosaminoglycans, whose nature remains to be established [31]. Such a scenario would explain the absence of a straightforward association between neutrophil counts and CXCL1 concentration in the dialysate. In fact, blocking CXCL1 was found to reduce the number of neutrophils entering the omentum but did not affect those in the peritoneal fluid in a zymosan model of peritonitis [35].

In conclusion, IL-17 and IFN-γ cross-react through a novel mechanism involving SP-1 and STAT1 to tailor CXCL1 production by the peritoneal mesothelium (Figure 6D). This mechanism may be of clinical relevance as dysregulated CXCL1 release may lead to either excessive or ineffective neutrophil recruitment during peritonitis.

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Author contributions statement

JW and AJ designed and supervised the study. RAC, LC, QL, HZ, GM, EK, and JW carried out experiments. AKM, DZ, and DJF collected peritoneal effluent from peritoneal dialysis patients. GP, PD, and ME analysed the peritoneal effluents. SMC performed statistical analysis of peritonitis sample data. RK supervised and analysed the neutrophil migration experiments. JKM made the graphics. RAC, DD, KUE, AJ, and JW analysed the data, and drafted and revised the paper. All the authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Correlations of cytokine concentrations with neutrophil counts

Figure S2. Effect of IFN-γ on TNFα-amplified *CXCL1* induction

Table S1. Patient characteristics

Table S2. PCR primers used

Table S3. Antibodies used throughout the study