**Control of neutrophil influx during peritonitis by transcriptional cross-regulation of chemokine CXCL1 by IL-17 and IFN-γ**Catar *et al. J Pathol* DOI: 10.1002/path.5438

**Supplementary materials and methods**

Reference numbers refer to the main text list

**Cellular analysis of patient samples**

Cells from cloudy peritoneal effluents were analysed as described previously [28]. Cells were acquired on an eight-color FACSCanto II (BD Biosciences, San Diego, CA, USA) and analysed using FlowJo 10.1 (TreeStar, Ashland, OR, USA), using monoclonal antibodies against CD3 (SK7) and CD15 (HI98 or HIM1) from BD Biosciences; and anti-CD14 (61D3) from eBioscience (San Diego, CA, USA); together with appropriate isotype controls. Neutrophils were identified based on their appearance in side scatter and forward scatter area/height and exclusion of live/dead staining (Fixable Aqua; Invitrogen, Carlsbad, CA, USA), as well as positive staining for CD15 and absence of staining for CD3 and CD14.

**Measurement of soluble biomarkers in peritoneal effluent**

Cell-free peritoneal effluents were analysed as described previously [28] using a SECTOR Imager 6000 (Meso Scale Discovery, Rockville, MD, USA) and the V-PLEX Human Cytokine 30-Plex Kit to measure levels of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IFN-γ, TNF-α, TNF-β, GM-CSF and VEGF, CCL2, CCL3, CCL4, CCL11, CCL13, CCL17, CCL22, CCL26, CXCL8 and CXCL10; ultrasensitive single-plex assays for soluble IL-6 receptor (sIL-6R) and IL-18 (Meso Scale Discovery); and a customer-made single-plex assay for IL-22 using capture (MAB7822) and biotinylated detection antibodies (BAM7821) and recombinant human IL-22 from R&D Systems (Wiesbaden, Germany). Conventional ELISA kits were used to measure TGF-β, total MMP-8, total MMP-9 and surfactant protein D (SPD) (R&D Systems); calprotectin (Hycult, Plymouth Meeting, PA, USA); and CCL2 (BD Biosciences). Human neutrophil elastase (HNE) was measured using a B.I.T.S. ELISA kit (Mologic, Bedford, UK). Active HNE was measured using the fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-AMC (Bachem, Bubendorf, Switzerland); active MMP was measured using the fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (Enzo Life Sciences, Farmingdale, NY, USA). Total protein was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Measurements that were below or above the detection limit were replaced by the lowest and highest detectable values for each biomarker, respectively.

**PCR conditions**

The expression of target genes was assessed using reverse transcription and quantitative PCR (RT-qPCR). Total RNA was extracted using a PerfectPure RNA Cultured Cell Kit (5 Prime, Hamburg, Germany), reverse transcribed into cDNA with random hexamer primers, and amplified using real-time qPCR on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). The reaction was carried out in 14 μl reaction volumes containing 2 μl of cDNA (20 ng), specific sense and anti-sense primers (250 nM each), and 7 μl Power SYBR Green PCR Master Mix (Applied Biosystems). PCR primers were synthesized by TIB Molbiol (Berlin, Germany). Sequences of primers are shown in supplementary material, Table S2.

**Transfections**

Transfections with siRNAs were performed using the siRNA Transfection Reagent and siRNAs for STAT1 (sc-44123), SP1 (sc-29487), or with scrambled siRNA control (sc-37007), as per manufacturer’s instructions (all materials from Santa Cruz Biotechnology, Heidelberg, Germany).

**EMSA**

Oligonucleotide probes were labelled using a Biotin 3' End DNA Labeling Kit (Thermo Scientific, Darmstadt, Germany). The probe for *SP1* used in EMSA was as follows: 5’-GTGGACCCCCACACCCCACCCGCA -3’ (the corresponding region of the *CXCL1* promoter was −227 to −250). Each binding mixture (20 µl) contained 5 µg nuclear extract, 20 fmol labelled double-stranded probe, 1 µg poly-dI/dC, and 2 µl 10 x reaction buffer and was incubated at room temperature for 30 min. The protein-DNA complexes were then analyzed by electrophoresis in 6% non-denaturing polyacrylamide gels and visualized using a LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Darmstadt, Germany).

**ChIP**

ChIP was performed using a ChIP-IT High Sensitivity Kit (Active Motif, Carlsbad, CA, USA) following the manufacturer’s instructions. In brief, HPMCs were fixed for 10 min with Complete Cell Fixation Solution containing 37% formaldehyde then sonicated to generate 500–800 bp DNA fragments. Immunoprecipitation was performed using protein G agarose beads and 4µg of the appropriate antibody (all from Santa Cruz, Heidelberg, Germany): anti-SP1 (sc-420), anti-STAT1 (sc-271661) or R&D Systems control antibody (1-001-A). The chromatin extracts were incubated with the antibodies at 4°C overnight with mild shaking and ChIP DNA was eluted according to the manufacturer’s instructions. PCR amplifications were performed using the primers listed in supplementary material, Table S2.

**SP1 sites in the *CXCL1* promoter (sequence for EMSA)**

-519 CTCCAGCCACAAATCCGAGACACAACGCTCTTCCTCCAAAGAGGTCGCGCCTTCTCTGTGGTGGTTCTCAGGGATCCGCCCCAGCTCCTTCTCCGTTCCCAGCCCCACACACTGGGATCACCAGGCACCCAAGATCCCACCTCTCAGGTGGTATCTTCAGCGCAGGCTGCCACTCAGCCCCCCTCCAGGGATCTGGGGCAGAAGGCGAATATCCCAGAGTCTCAGAGTCCACAGGAGTTACTCTGAAGGGCGAGGCGCGGGCTGCATCAGTGGACCCCCACACCCCACCCGCACCCCAAGCGCTCCACCCTGGGGGCGGGGCCGTCGCCTTCCTTCCGGACTCGGGATCGATCTGGAACTCCGGGAATTTCCCTGGCCCGGGGGCTCCGGGCTTTCCAGCCCCAACCATGCATAAAAGGGGTTCGCGGATCTCGGAGAGCCACAGAGCCCGGGCCGCAGGCACCTCCTCGCCAGCTCTTCCGCTCCTCTCACAGCCGCCAGACCCGCCTGCTGAGCCCCatggcccgcgctgctctctccgccgcccccagcaatccccggctcctgcgagtggcactgctgctcctgctcctggtagccgctggccggcgcgcagcag +100

SP1 GTGGACCCCCACACCCCACCCGCA

The nucleotides written in CAPITAL LETTERS above correspond to *CXCL1* promoter sequence -519 to 0. Nucleotides written in lower case letters correspond to *CXCL1* gene sequence +1 to +100. The sequence highlighted in green was used as a synthetic oligonucleotide for SP1 EMSA experiments.

**SP1 and STAT1 sites in the *CXCL1* promoter (sequence for ChIP)**

Forward primer

GAAGGCGAATATCCCAGAGTCTCAGAGTCCACAGGAGTTACTCTGAAGGGCGAGGCGCGGGCTGCATCAGTGGACCCCCACACCCCACCCGCACCCCAAG

SP1 motif

CGCTCCACCCTGGGGGCGGGGCCGTCGCCTTCCTTCCGGACTCGGGATCGATCTGGAACTCCGGGAATTTCCCTGGCCCGGGGGCTCCGGGCTTTCCAGC

STAT1 motif

CCCAACCATGCATAAAAGGGGTTCGCGGATCTCGGAGAGCCACAGAGCCC

Reverse primer

**Site-directed mutagenesis.**

To target the STAT1 binding site at positions -144 to –153 (ATTTCCCTGG) within the *CXCL1* promoter, the desired sequence (GCCCAAACTT) was inserted into the pLuc 620 construct using the Q5 Site–Directed Mutagenesis Kit (New England BioLabs, Frankfurt, Germany) with forward primer -148- aacttCCCGGGGGCTCCGGGCTT -126 and reverse primer -180- tgggcTCCCGGAGTTCCAGATCGATCCCGAG -149.

**Neutrophil migration**

Neutrophil migration was measured using a Transwell assay, essentially as previously described by Jerke *et al* [33]. Migration was assessed in response to conditioned medium from HPMCs or through HPMC monolayers established on Transwells as described by Deng *et al* [34]. In brief, 105 HPMCs were seeded on the underside of a 24-well PET Transwell (3 μm pore membrane) for 4 h at 37°C in 5% CO2. Transwells were then inverted, placed in a 24-well plate. and stimulated with IL-17 (100 ng/ml) ± IFN-γ (5 ng/ml) for 24 h. The upper chamber was loaded with 1.5 x 106 purified neutrophils (ca. 95% pure) and their migration to the lower well was assessed after 90 min. Cells that had migrated to the lower well were quantified using a standard curve with known numbers of neutrophils (with determination of myeloperoxidase activity after cell lysis using ABTS substrate). fMLP at 10 nM was used as a positive control.

**CXCL1 binding**

Confluent HPMCs in 96-well plates were washed gently with PBS and then exposed to serial dilutions (78–5000 ng/ml) of recombinant CXCL1 in 1% BSA in PBS (pH 7.3) for 30 min at room temperature. After that the exposure media were removed and bound CXCL1 was detected using biotinylated anti-CXCL1 (R&D Systems; BAF275; 100 ng/ml) followed by incubation with streptavidin-peroxidase conjugate and substrate solution of H2O2 and tetramethylbenzidine. The reaction was stopped after 20 min and the OD was read at 450 nm.