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IL-17 Stimulates Intraperitoneal Neutrophil Infiltration Through the Release of GROα Chemokine from Mesothelial Cells

Janusz Witowski,† Krzysztof Pawlaczyk,* Andrzej Breborowicz,* Axel Scheuren,† Malgorzata Kuzlan-Pawlaczyk,* Justyna Wisniewska,* Alicja Polubinska,* Helmut Friess,‡ Gerhard M. Gahl,‡ Ulrich Frei,† and Achim Jörres†

IL-17 is a newly discovered cytokine implicated in the regulation of hemopoiesis and inflammation. Because IL-17 production is restricted to activated T lymphocytes, the effects exerted by IL-17 may help one to understand the contribution of T cells to the inflammatory response. We investigated the role of IL-17 in leukocyte recruitment into the peritoneal cavity. Leukocyte infiltration in vivo was assessed in BALB/Cj mice. Effects of IL-17 on chemokine generation in vitro were examined in human peritoneal mesothelial cells (HPMC). Administration of IL-17 i.p. resulted in a selective recruitment of neutrophils into the peritoneum and increased levels of KC chemokine (murine homologue of human growth-related oncogene α (GROα)). Pretreatment with anti-KC Ab significantly reduced the IL-17-driven neutrophil accumulation. Primary cultures of HPMC expressed IL-17 receptor mRNA. Exposure of HPMC to IL-17 led to a dose- and time-dependent induction of GROα mRNA and protein. Combination of IL-17 together with TNF-α resulted in an increased stability of GROα mRNA and synergistic release of GROα protein. Anti-IL-17 Ab blocked the effects of IL-17 in vitro and in vivo. IL-17 is capable of selectively recruiting neutrophils into the peritoneal cavity via the release of neutrophil-specific chemokines from the peritoneal mesothelium.

Peritoneal macrophages are commonly viewed as the first line of defense against invading microorganisms (1, 2). The role of other leukocyte populations in peritoneal immunity is, however, less understood. In healthy individuals, 5–10% of peritoneal leukocytes are lymphocytes, with the vast majority belonging to the T lineage (3, 4). It has been suggested that the specific peritoneal microenvironment may affect T cell selection in the peritoneum (5). As compared with PBLs, the peritoneal CD4+CD8+ ratio is inverted (4–7), and within these subsets ~70–90% cells exhibit the CD45RO+ phenotype of memory cells (8). The substantial proportion of peritoneal T cells have also been found to express either CD8+αα isoform or RAG-1 mRNA transcripts (5, 8). The presence of these traits is believed to reflect thymus-independent T cell differentiation (9) and may support the concept of the peritoneal lymphoid tissue as an intestinal thymus (10, 11). A broad spectrum of effects exerted by lymphocyte-derived mediator IFN-γ (12) points to a significant role of lymphocytes in the inflammatory response. In this respect, increased levels of IFN-γ have been detected in the inflamed peritoneum, and this rise has been clearly attributed to peritoneal lymphocytes (13).

IL-17 is a newly identified T cell-specific cytokine (14). The human form of IL-17 is a ~20-kDa glycoprotein of 155 aa, the sequence of which exhibits a close homology to both cytotoxic T lymphocyte-associated Ag-8 (CTLA-8) and the open reading frame 13 of T-lymphotropic Herpesvirus saimiri (HVS-13) (15, 16). Expression of IL-17 has been detected almost exclusively in activated CD4+ and CD8+ T lymphocytes (predominantly of the memory CD45RO+ subset) (15, 17–19). In sharp contrast, the specific IL-17R is widely distributed in most tissues and cell lines (20, 21). Accumulating evidence suggests that IL-17 may be an important mediator of the hemopoietic system (14, 22). It has been found to stimulate the production of IL-6, G-CSF, and LIF (17, 23, 24) cytokines with a known impact on hemopoietic progenitors. Moreover, the soluble form of IL-17R protein has been shown to inhibit mitogen-induced proliferation and IL-2 production in murine T cells (21). In contrast, IL-17 also appears to be involved in the inflammatory reaction. The expression of several genes associated with inflammation, including IL-1β, TNF-α, IL-6, IL-8, cyclooxygenase-2, NO synthase, and stromelysin, is up-regulated after stimulation with IL-17 (17, 23, 25–29). In addition, increased production of the complement component C3 in response to IL-17 has been detected in renal proximal tubule cells (28).

In this study, we set out to investigate the potential role of IL-17 in the peritoneal inflammatory response. We demonstrate that IL-17 selectively recruits neutrophils into the peritoneal cavity and that this effect is likely to be mediated via the release of neutrophil-specific chemokines from the peritoneal mesothelium.

Materials and Methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany). Tissue culture plastics were from...
Falcon Becton Dickinson (Heidelberg, Germany). Recombinant human and murine cytokines and anti-cytokine Abs were obtained from R&D Systems (Wiesbaden, Germany). According to the manufacturer, the endotoxin concentration in the above materials was <0.1 ng/μg protein as measured by Limulus amebocyte lysate assay. All cytokine preparations were batched, stored at −70°C, and freshly thawed for each experiment. All media and buffers were of tissue culture grade with an endotoxin concentration of <0.1 ng/ml.

**Animal studies**

All in vivo experiments were performed using male BALB/C inbred mice weighing 25–30 g. BALB/C/J/NIMP mice were obtained from the Institute of Occupational Medicine (Lodz, Poland) and housed under 12-h light/dark cycles with free access to standard chow and water. All studies were performed according to the guidelines of the Committee for Animal Studies at University Medical School (Poznan, Poland). Recombinant mouse IL-17 (rmIL-17) was diluted in sterile endotoxin-free PBS (Dulbecco’s PBS, PAA Laboratories, Linz, Austria) and administered i.p. at a dose of 0.5 μg/mouse in a total volume of 500 μl. The concentration of IL-17 to be applied was determined in preliminary dose–response experiments which showed that 0.5 μg IL-17 consistently produced a significant influx of neutrophils (data not shown). Control animals received an equivalent volume of PBS alone. For the procedure the animals were placed under brief ether anesthesia and then allowed to recover. In all in vivo experiments, the animals were first given i.p. injection of either monoclonal anti-mouse KC neutralizing Ab (0.1 mg/200 μl PBS) or PBS alone, and after 15 min they received i.p. either IL-17 (0.5 μg/mouse in 200 μl PBS) or PBS alone. In an additional set of experiments, recombinant mouse KC was administered i.p. in 500 μl PBS. At designated time points, the animals were euthanized, sacrificed by bleeding, and injected i.p. with 2.5 ml PBS containing 3 mM EDTA (PBS/EDTA) (30). The peritoneal cavity was then opened, and the lavage fluid was carefully collected. Cell pellets were resuspended in PBS/EDTA, and total cell counts were determined in a hemocytometer using Türk’s solution. Differential cell counting was performed on cytospin preparations stained with May-Grünewald-Giemsa using a QCA staining kit (Quimica Clinica Aplicada, Amposta, Spain). Aliquots of cell-free peritoneal lavage fluids and sera were stored at −70°C until further analysis for cytokines.

**Peritoneal mesothelial cell culture**

Human peritoneal mesothelial cells (HPMC) were isolated from the specimens of omentum obtained from consenting patients undergoing elective abdominal surgery. Cells were isolated and characterized as described in detail elsewhere (31, 32). Cells were propagated in Earle’s buffered M199 culture medium (Seromed, Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Life Technologies, Egg-stein, Germany). Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere.

All experiments were performed using cells derived from at least six separate donors, and from the first or second passage to minimize the number of senescent cells which appeared from the third passage onwards (31).

**Effect of IL-17 on the production of growth-related oncogene product α (GROα) by human peritoneal mesothelial cells**

HPMC were grown to confluence and rendered quiescent by serum deprivation for 48 h before stimulation. Preliminary experiments had demonstrated that under these conditions cells could be maintained for at least up to 120 h without any significant loss of viability (as assessed by intracellular ATP concentrations). HPMC were exposed to recombinant human IL-17 at doses ranging from 0.01 to 100 ng/ml. In some experiments, cells were exposed to IL-17 in the presence or absence of TNF-α. In the inhibition studies, HPMC were pretreated with transcription (actinomycin D) or translation (cycloheximide) inhibitors for 45 and 120 min, respectively, and then stimulated with IL-17. The doses of inhibitors used did not impair cell viability. In separate experiments, IL-17 preparations were first preincubated with either anti-human IL-17 polyclonal neutralizing Ab or the equivalent dose of control IgG of the same class, and then applied to HPMC cultures.

At designated time intervals, the cell supernatants were removed, centrifuged at 12,000 × g to remove any cellular debris, and stored at −70°C until assayed. Cell monolayers were washed with PBS and solubilized with 0.1 N NaOH. Total cellular protein was then analyzed using Bradford method with Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Munich, Germany) and BSA as the standard. Repeated cell counts revealed that 1 μg HPMC protein corresponded to (mean ± SD) 2.1 ± 1.0 × 106 cells (n = 16). All data for GROα secretion were expressed as picograms per microgram cellular protein.

**Cytokine measurements**

Concentrations of mouse KC and macrophage inflammatory protein-2 (MIP-2) in serum and peritoneal lavage fluid were determined using Quantikine Mouse Immunoassays (R&D Systems) with sensitivities of 2 pg/ml for KC and 1.5 pg/ml for MIP-2. GROα levels in supernatants from HPMC cultures were measured using the Quantikine Human GROα Immunoassay (R&D Systems) with a sensitivity of 5.0 pg/ml.

**RNA isolation and analysis**

Total RNA from HPMC cultures was extracted with the RNA Isolator (Genosys Biotechnologies, Cambridge, U.K.) and purified according to the manufacturer’s protocol. Expression of IL-17R and of IL-17-induced GROα mRNA was assessed using reverse transcription-PCR or Northern blot analysis as described below.

**Reverse transcription and PCR**

One microgram of total RNA was reverse transcribed into cDNA with random hexamer primers, as previously described (33). PCR amplification was performed in a total volume of 50 μl consisting of 2 μl reverse transcription product and 48 μl of the reaction master mix. The master mix contained 36.25 μl H2O, 2.5 μl sense and antisense primers (20 μM each), 4 μl dNTPs, 5 μl 10× PCR buffer (100 mM Tris, 500 mM KCl, 15 mM MgCl2, 0.01% gelatin), and 0.25 μl Taq polymerase (1.25 U. AmpliTaq; Perkin-Elmer Cetus, Westerfield, Germany). The amplification was conducted on the Perkin-Elmer 480 Thermocycler (Perkin-Elmer Cetus, Applied Biosystems). Specific oligonucleotide primer pairs were synthesized by TIB MolBiol SyntheseLabor (Berlin, Germany). The primer sequences were as shown in Table I.

The reaction for β-actin and GROα primers began with a 3-min denaturation step at 94°C and was followed by 27 (GROα), 30 (β-actin), or 33 (β-actin) cycles of denaturation at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final cycle was 94°C for 40 s and 60°C for 10 min. Preliminary experiments had determined that under these conditions PCR products were generated during the exponential phase of amplification. The protocol of PCR amplification with primers for IL-17R was similar except that annealing temperature was 60°C, samples were amplified for 35 cycles, and the final extension was at 72°C for 2 min. PCR products were separated by electrophoresis in 3% agarose gels (FMC Bioproducts, Biodyzgn Diagnostic, Hess Oldendorf, Germany), stained with ethidium bromide (1 μg/ml) and visualized under UV transillumination. Expression of target mRNAs was assessed by comparison with the expression of the “housekeeping” genes of α- or β-actin in the same sample. The bands corresponding to the intended products were analyzed using Scanpack 14.1A27 software (Biometra, Göttingen, Germany).

**Northern blot analysis**

Twenty micrograms total RNA were size-fractionated on 1.2% agarose gel and stained with ethidium bromide for verification of RNA integrity and loading equivalence (38–40). The RNA was electrotransferred onto nylon membranes (Gene Screen, DuPont, Boston, MA) and cross-linked by UV irradiation. The filters were then prehybridized, hybridized, and washed under conditions appropriate for the 32P-labeled GROα and 75 cDNA probes, as previously described in detail (38–40). Hybridization of the membranes was conducted at 42°C for 4–8 h at 0.2 × SSC containing a buffer consisting of 30% formamide, 1% SDS, 0.75 M NaCl, 5 mM EDTA, 5× Denhardt’s solution, 100 μg/ml salmon sperm DNA, 10% dextran sulfate, and 50 mM sodium phosphate buffer, pH 7.4. Hybridization was conducted at 42°C for 18 h with either 106 cpm/ml of the 32P-labeled GROα probe or 103 cpm/ml of the 32P-labeled 75 cDNA probe. After the hybridization, the blots were washed under stringent conditions with two rinses in 2× SSC at 50°C and three washes (20 min each) in 0.2× SSC/2% SDS at 55°C. Blots were then exposed to Fuji x-ray films with intensifying screens (DuPont) at −80°C, and the intensity of the radiographic bands

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3 Abbreviations used in this paper: HPMC, human peritoneal mesothelial cells; GROα, growth-related oncogene α; MIP-2, macrophage inflammatory protein-2; PMN, polymorphonuclear leukocyte.
was quantified by video image analysis (Image-Pro plus, Media Cybernetics, Silver Spring, MD), as previously reported (38, 41). The ratio between GROα and corresponding 7S signal was calculated for each sample.

**Probe for Northern blot analysis**

GROα DNA probe used in Northern blot analysis consisted of a 231-bp fragment corresponding to positions 452–682 of the GROα mRNA. The probe was designed using nucleotide sequence of the GROα gene as published by Baker et al. (42). The fragment was cloned by reverse transcribing human normal pancreas RNA, amplifying the cDNA obtained by PCR, and ligating the amplicon into the pGEM-T Easy Vector (Promega, Biotechnology, Madison, WI). The 7S DNA probe consisted of a 212-bp fragment of the 7S RNA which was cloned as described above. This probe was used to verify equivalent RNA loading in the Northern blot experiments (38, 40). Authenticity of GROα and 7S fragments was confirmed by sequencing the dye terminator method (ABI 373A, Perkin-Elmer, Rotkreuz, Switzerland). For Northern blot analysis, the GROα and 7S DNA probes were radiolabeled with [α-32P]dCTP (DuPont International, Georgsdorf, Switzerland) using a random primer labeling system (NEN Life Science Products, Boston, MA).

**Stability of IL-17-induced GROα mRNA**

The inherent stability of mesothelial cell GROα mRNA was assessed by measuring the rate of GROα mRNA degradation in the presence of the transcription inhibitor actinomycin D. HPMC were stimulated with IL-17 (50 ng/ml) in the presence or absence of TNF-α (1 ng/ml) for 2 h. After that, cells were washed and pulsed with actinomycin D (5 µg/ml). At defined time intervals, the total RNA was extracted, reverse transcribed into cDNA, and PCR amplified for GROα and α-actin as described above.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 3.00 software (GraphPad Software, San Diego, CA). Multiple comparisons of paired data were made with nonparametric repeated measures ANOVA with Friedman modification. Unpaired data derived from animal studies were analyzed using Mann-Whitney U or Kruskal-Wallis tests, when appropriate. A p value of <0.05 was considered significant. All data are presented as means ± SEM.

**Results**

**IL-17 induces i.p. neutrophil infiltration**

Injection of IL-17 i.p. in experimental animals resulted in a time-dependent increase in the total number of cells in the peritoneal cavity (Fig. 1). The maximal effect was observed 4 h after the administration of IL-17 when the accumulation of cells was 2.6-fold above the numbers detected in control mice. Differential cell counting revealed that this increase could be accounted for by a substantial rise in the number of polymorphonuclear neutrophils (PMN, Table II, Fig. 2). In untreated mice (no i.p. injections, lavage only, n = 9), PMN constituted merely 1.1 ± 0.4% of the peritoneal cell population. In control animals, the procedure of i.p. injection and/or PBS itself produced a small nonspecific increase in the number of PMN. However, the influx of PMN triggered by IL-17 was significantly above these background levels (data not shown). During the time frame studied, the absolute number of cells from other populations did not differ significantly from those detected in control and untreated animals (Table II). Administration of IL-17 together with anti-IL-17 neutralizing Ab reduced the specific IL-17-mediated PMN influx by 61.2 ± 9.5% within 4 h.

**IL-17 stimulates i.p. generation of KC chemokine**

Administration of IL-17 produced a massive increase in the i.p. concentrations of KC chemokine, a murine analogue of human GROα (Ref. 43 and Fig. 2). The level of KC increased rapidly within 1 h and by 4 h returned to basal values. Comparison of KC concentrations in sera and lavage fluids indicated that KC released in response to IL-17 was of the local i.p. origin. At the 1-h time point, the mean concentration of KC in the lavage fluid was 3401 ± 592 pg/ml compared with 264 ± 50 pg/ml in serum (n = 6). The mean serum KC level in untreated mice was 150 ± 48 pg/ml (n = 8) and corresponded to the values detected by the manufacturer of the mouse KC immunoassay (R&D Systems). In addition, IL-17 triggered a rapid i.p. release of neutrophil chemoattractant MIP-2. Within 1 h, MIP-2 concentration in the lavage fluid rose from 14 ± 5 pg/ml to 1250 ± 116 pg/ml, compared with plasma levels of 45 ± 6 pg/ml (n = 6–9).

**Neutralization of KC reduces IL-17-stimulated neutrophil recruitment**

The observation that IL-17-induced PMN influx was preceded by a rapid increase in KC levels (Fig. 2) suggested that the effect could have been mediated by KC, a powerful neutrophil chemoattractant (43). Treatment i.p. with recombinant KC resulted in a dose-dependent and selective increase in PMN recruitment (data not shown). The dose of 0.5 µg KC/animal produced a 7.5 ± 0.7-fold increase in PMN accumulation within 4 h. Furthermore, administration of anti-KC neutralizing Ab before IL-17 injection reduced the IL-17-specific PMN infiltration within 4 h by 67.2 ± 7.9%, although it had no significant effect on basal PMN influx triggered by the injection of PBS vehicle (Fig. 3).

**Peritoneal mesothelial cells express IL-17R**

RT-PCR analysis using primers specific for either the extracellular or intracellular domain of IL-17R revealed that HPMC expressed IL-17R mRNA. Constitutive expression of IL-17R gene transcripts was detected in all primary cultures of HPMC examined (Fig. 4).

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**Table I. Primer sequences for specific oligonucleotide primer pairs**

<table>
<thead>
<tr>
<th>Primer Pairs</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROα</td>
<td>F: 5′-ACTCAAGAATGGCCGAAAG-3′ R: 5′-TGGCATGTTGCAGGCTCTT-3′</td>
<td>468</td>
<td>34</td>
</tr>
<tr>
<td>IL-17R extracellular domain</td>
<td>F: 5′-CTAAACTGCGAGCTCAAGAAT-3′ R: 5′-ATGACACCATCACCCCAAAC-3′</td>
<td>833</td>
<td>20</td>
</tr>
<tr>
<td>IL-17R intracellular domain</td>
<td>F: 5′-ATGGACAGGTTCGAGGAG-3′ R: 5′-TTCCAGATGCCGGTTC-3′</td>
<td>276</td>
<td>35</td>
</tr>
<tr>
<td>α-Actin</td>
<td>F: 5′-GGAGCAGTATGCTTCGAGGAGGTTCC-3′ R: 5′-CTCTGGAGTGAGGCTC-3′</td>
<td>204</td>
<td>36</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: 5′-ATCCCCCAAAGGTCTCAAAA-3′ R: 5′-CTGGGCGCATCTCTTAG-3′</td>
<td>147</td>
<td>37</td>
</tr>
</tbody>
</table>

* F, forward; R, reverse.
### IL-17 stimulates GROα production by peritoneal mesothelial cells

HPMC released GROα constitutively. Exposure of HPMC to a recombinant form of human IL-17 resulted in a time- and dose-dependent increase in GROα generation. With IL-17 at a dose of 50 ng/ml, this increase became significant above control levels after 6 h of incubation and was followed by large increments during the next 18 h and a plateau by 24 h (Fig. 5). Statistically significant increase in GROα secretion was achieved with IL-17 at a dose of 1 ng/ml and above (Fig. 6). Administration of IL-17 in the presence of anti-IL-17 neutralizing Ab reduced IL-17-stimulated but not in control Ab of the same class did not affect the stimulatory activity of IL-17 (882.0 ± 115.0 pg/µg cell protein). In contrast, control Ab of the same class did not affect the stimulatory activity of IL-17 (882.0 ± 115.0 pg/µg cell protein).

**Transcription and translation inhibitors reduce IL-17-stimulated GROα release**

Preexposure of HPMC to actinomycin D for 45 min at 37°C resulted in a dose-dependent decrease in IL-17-stimulated but not in constitutive GROα secretion. Maximal inhibition was achieved with the dose of 1 µg/ml, which reduced IL-17-driven GROα release by 90.1 ± 6.8% (687.3 ± 116.8 vs 68.1 ± 46.8 pg/µg cell protein) to the level detected in unstimulated cells (n = 5, p < 0.01). Generation of GROα in HPMC stimulated with IL-17 could also be inhibited by cycloheximide. At the highest nontoxic dose of cycloheximide tested (50 µg/ml) GROα release was reduced by 51.4 ± 6.1% (747.3 ± 105.4 vs 363.1 ± 45.5 pg/µg cell protein, n = 7, p < 0.01).

**Exposure to IL-17 induces GROα mRNA in peritoneal mesothelial cells**

Stimulation of HPMC with IL-17 induced a time- and dose-dependent accumulation of GROα mRNA as demonstrated by Northern blot analysis (Fig. 7). Unstimulated cells expressed very faint signals for GROα mRNA. After treatment with IL-17, the GROα mRNA expression was rapidly up-regulated within 1 h. Increased expression of GROα mRNA was detected in cells stimulated with a dose of IL-17 as low as 0.1 ng/ml.

**TNF-α superinduces IL-17-driven GROα synthesis by stabilizing GROα mRNA**

Exposure of HPMC to TNF-α increased GROα release in a time- and dose-dependent manner (data not shown). Combination of TNF-α together with IL-17 triggered GROα production above the levels generated by each stimulus alone and significantly above the calculated additive value (Fig. 8). This synergistic effect became

### Table II. Changes in number of cells in the peritoneal cavity following the administration of IL-17

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Neutrophils (x 10^3/animal)</th>
<th>Macrophages</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
<th>Mast cells</th>
<th>Eosinophils</th>
<th>Mesothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>68 ± 28</td>
<td>1955 ± 294</td>
<td>265 ± 197</td>
<td>3167 ± 403</td>
<td>77 ± 22</td>
<td>0</td>
<td>102 ± 96</td>
</tr>
<tr>
<td>1</td>
<td>616 ± 153</td>
<td>1009 ± 89</td>
<td>160 ± 80</td>
<td>2834 ± 489</td>
<td>40 ± 20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4625 ± 637***</td>
<td>1531 ± 469</td>
<td>288 ± 157</td>
<td>2570 ± 418</td>
<td>99 ± 41</td>
<td>0</td>
<td>8 ± 7</td>
</tr>
<tr>
<td>4</td>
<td>5147 ± 593***</td>
<td>2256 ± 373</td>
<td>746 ± 318</td>
<td>2902 ± 409</td>
<td>26 ± 15</td>
<td>0</td>
<td>34 ± 24</td>
</tr>
<tr>
<td>8</td>
<td>4446 ± 1038**</td>
<td>1064 ± 449</td>
<td>386 ± 177</td>
<td>2954 ± 789</td>
<td>44 ± 36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>1584 ± 385</td>
<td>1663 ± 269</td>
<td>808 ± 345</td>
<td>4090 ± 904</td>
<td>95 ± 36</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mice were injected i.p. with 0.5 µg rmIL-17. At designated time intervals, the peritoneal lavage and differential cell counting were performed as described in Materials and Methods. Data were derived from 6–9 animals for each time point and were expressed as absolute numbers of cells in the peritoneum. Asterisks represent a statistically significant difference compared with values obtained before the injection of IL-17.
evident when TNF-α (1 ng/ml) was combined with 0.1 ng/ml IL-17, and maximal synergy was obtained at the highest dose of IL-17 tested (100 ng/ml). GROα release under these conditions was 2.2 ± 0.2-fold above the predicted additive value. Actinomycin D chase experiments revealed that in HPMC treated with a combination of IL-17 and TNF-α the degradation of GROα mRNA was delayed compared with cells treated with IL-17 alone (Fig. 9).

Discussion

Increasing evidence suggests that IL-17, acting either directly or indirectly, may significantly affect neutrophil maturation and function. Initial observations came from Fossiez et al. (17), who had found that IL-17-treated fibroblasts produced G-CSF and were capable of promoting differentiation of CD34⁺ hemopoietic progenitors toward neutrophils. Subsequently, Schwarzenberger et al. demonstrated that adenovirus-mediated overexpression of IL-17 in mice resulted in a massive peripheral neutrophilia associated with increased levels of G-CSF and marked stimulation of splenic granulopoiesis (44). Additional experiments revealed that in several cell systems IL-17 induced the release of neutrophil-specific chemokines (17, 29, 35, 45). With the present study, we extend these observations and demonstrate that IL-17 possesses a significant potential to recruit neutrophils into the peritoneum.

Administration of IL-17 i.p. resulted in a massive and selective influx of neutrophils. Neutrophil population appeared to have been targeted specifically because the trafficking of other leukocyte subsets was not significantly affected. Similar effects of IL-17 were observed in the rat airways where intratracheal IL-17 instillation induced selective accumulation of neutrophils in the bronchoalveolar lavage fluid (45, 46). The specificity of these effects was confirmed by the inhibition with anti-IL-17 neutralizing Ab. The fact that this inhibition was incomplete (~60%) could be explained by the limited neutralizing capacity of the Ab under in vivo conditions in comparison with the dose of IL-17 used.
Because IL-17 has been shown to have no direct effect on neutrophil chemotaxis in vitro (45), we hypothesized that PMN accumulation in response to IL-17 could have been mediated by the induction of chemokines. Analysis of the peritoneal fluid revealed that IL-17 induced a rapid rise in i.p. levels of KC which preceded the influx of PMN. The similar time course of KC induction has been observed in various models of murine peritonitis (30, 47). KC is a chemokine with a powerful chemotactic activity toward neutrophils (48) and, indeed, the i.p. injection of recombinant KC triggered a massive accumulation of PMN in the peritoneal cavity. Furthermore, when IL-17-receiving animals were pretreated with anti-KC neutralizing Ab, the IL-17-mediated PMN recruitment was reduced by >60%. Again, the magnitude of inhibition observed under these conditions could be influenced by the neutralizing capacity of the Ab used. However, it is also possible that the remaining chemotactic activity could be attributed to other chemokines induced by IL-17. Indeed, we found that IL-17 also produced a significant rise in i.p. MIP-2 levels. In this respect, Laan et al. (45) have convincingly documented the role of MIP-2 as a mediator of IL-17-induced leukocyte trafficking in the rat, and Walley et al. (49) have demonstrated the importance of MIP-2 in the pathogenesis of cecal ligation and puncture model of peritonitis. MIP-2 is a chemokine with no exact human homologue but closely related to either IL-8 (50) or GROα (43, 51).

The observation that the levels of KC in the peritoneum were much higher than those detected in serum suggested the local origin of KC. One possible source could be the peritoneal macrophage; however, in a recent study Ajuebor et al. (47) have demonstrated that in an LPS model of peritonitis in mice the removal of peritoneal macrophages and monocytes did not diminish the peritoneal generation of KC. We have therefore concentrated on peritoneal mesothelial cells. The peritoneal mesothelium is a recognized source of chemotactic activity in the peritoneum (32, 52–54), and it has been demonstrated that on appropriate stimulation human mesothelial cells are capable of generating GROα, a homologue of KC (55). The presence of IL-17R mRNA in HPMC was confirmed by RT-PCR and confirmed the ubiquitous nature of IL-17R distribution (30, 21). Exposure of quiescent HPMC to IL-17 led to a significant time- and dose-dependent increase in the secretion of GROα. The range of IL-17 doses that triggered this effect corresponded to those that had been shown to stimulate the release of cytokines in other in vitro systems (17, 25, 26, 29, 35, 45, 56). The IL-17-driven GROα release could be inhibited in a dose-dependent manner by the pretreatment of HPMC with both transcription and translation inhibitors which suggested that IL-17 stimulated de novo GROα synthesis. Northern blot analysis confirmed that exposure of HPMC to IL-17 resulted in a rapid up-regulation of GROα mRNA. Using a rat intestinal epithelial cell line, Awane et al. (29) have recently analyzed the IL-17-activated

**FIGURE 7.** Expression of GROα mRNA in HPMC treated with IL-17. Quiescent HPMC were exposed to IL-17 (50 ng/ml) for the time periods indicated. Total RNA was extracted and subjected to Northern blot hybridization with probes for GROα and 7S, as described in Materials and Methods. After densitometric analysis, the data were expressed as GROα/7S ratios.

**FIGURE 8.** Effect of combined IL-17 and TNF-α stimulation on GROα production by HPMC. Quiescent cells were exposed to control medium or TNF-α (1 ng/ml) in the presence of increasing doses of IL-17 (0.01–100 ng/ml). After a 24 h incubation, the supernatants were assayed for GROα. Data were derived from seven experiments with HPMC isolated from different donors.

**FIGURE 9.** Effect of TNF-α on the stability of IL-17-induced GROα mRNA. Quiescent HPMC were stimulated with either IL-17 (50 ng/ml; ○) or the combination of IL-17 (50 ng/ml) and TNF-α (1 ng/ml; ●) for 2 h and then treated with actinomycin D (5 μg/ml). Total RNA was extracted at the time points indicated and analyzed by RT-PCR. PCR products were separated on 3% agarose gels stained with ethidium bromide (A). After densitometric analysis, the GROα/actin ratios were calculated, and the results were expressed as a percentage of the value obtained at time zero (B).
signal transduction pathway leading to the induction of CINC, a C-X-C chemokine related to human GROα. In this system, IL-17 has been shown to induce the activity of NF-κB-dependent CINC promoter via the pathway regulated by TNFR-associated factor-6 and NF-κB-inducing kinase. IL-17-induced NF-κB activity has also been demonstrated in macrophages (25), chondrocytes (26), and fibroblasts (21).

We have found that IL-17-induced generation of GROα could be synergistically augmented in the presence of TNF-α. This effect was at least partially related to the stabilization of GRO mRNA. Because IL-17 has been demonstrated to stimulate the release of TNF-α from macrophages (25), one may imagine that by acting simultaneously on mesothelial cell and macrophages in the peritoneal cavity in vivo lymphocyte-derived IL-17 amplifies the generation of GROα and increases the transperitoneal chemotactic gradient for neutrophils. In this respect, it has been demonstrated that truncated form of GROα, which acts as a C-X-C chemokine receptor antagonist, is capable of inhibiting leukocyte recruitment into the peritoneal cavity (57). In other cell systems, IL-17 has been shown to synergize with TNF-α in the production of IL-8 (35, 45), IL-6, and GM-CSF (17). The potential synergy between IL-17 and TNF-α under in vivo conditions is currently being investigated.

The exact role of IL-17 in human pathology remains to be determined. Available data suggest that IL-17 may promote cartilage destruction in various forms of arthritis (23, 26, 56, 58, 59) and mediate alloimmune reactivity and organ allograft rejection (28, 60). Our findings demonstrate that IL-17 may act as a potent and selective inducer of neutrophil chemotaxis. In the setting of the peritoneal cavity, this effect appears to be mediated through the stimulation of GROα release from peritoneal mesothelial cells. These properties of IL-17 provide further evidence that IL-17 may have a significant role to play in the peritoneal inflammatory response. Our findings also add to the understanding of mesothelial cell biology and point to the importance of lymphocyte-mesothelial interactions in peritonitis.

References


