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Expression and regulation of the chemokine CXCL16 in Crohn's disease and models of intestinal inflammation

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Abstract

Background/Aims—CXCL16 mediates adhesion and phagocytosis of both Gram-negative and Gram-positive bacteria and is a strong chemoattractant for CXCR6+ T cells. In this study, we determined the so far unknown expression and signal transduction of the novel CXCL16-CXCR6 chemokine-ligand receptor system in intestinal inflammation *in vivo* and *in vitro*.

Methods—CXCL16 mRNA was measured by quantitative PCR in human colonic biopsies of patients with Crohn's disease (CD) as well as in the TNFΔARE mouse model of ileitis and in murine cytomegalovirus (MCMV)-induced colitis. CXCL16 serum levels were analyzed by ELISA. CXCL16-induced signal transduction was analyzed in IEC with phospho-specific antibodies for MAP kinases and Akt.

Results—We found an inverse expression pattern of CXCL16 and CXCR6 with highest CXCL16 mRNA levels in the proximal murine small intestine and highest CXCR6 mRNA expression in the distal colon. CXCL16 and CXCR6 mRNA were expressed in colorectal cancer (CRC)-derived IEC lines. CRC-expressed CXCR6 was functional as demonstrated by CXCL16-induced MAP kinase and Akt activation. Intestinal CXCL16 expression was elevated in the TNFΔARE mouse model of ileitis and in MCMV-induced colitis ($p<0.05$) and in the sera and colons of patients with CD ($p<0.05$), where its expression correlated highly with CXCR6 and IL-8 levels ($r=0.85$ and 0.89, respectively).

Conclusion—CRC-derived IEC express the functional CXCL16 receptor CXCR6. CXCL16 mRNA and protein expression is up-regulated in intestinal inflammation *in vitro* and in CD patients, suggesting an important role for this chemokine in intestinal inflammation.

Keywords

Crohn's disease; ulcerative colitis; inflammatory bowel disease; chemokine; CXCL16; CXCR6; mouse model; intestinal inflammation; intestinal epithelial cells; cytokine; signaling

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Introduction

Intestinal epithelial cells (IEC) are not a passive barrier as previously assumed, they also act as sensitive indicators of infection that initiate defense responses against noninvasive as well as invasive organisms via production of chemoattractants [1]. Epithelial cells have been found to express both chemokines and chemokine receptors [2–7] which are differentially upregulated in uninflamed intestine and during inflammation. The combined cellular functions of IEC and classical immune cells co-ordinate the recruitment and activation of leukocytes at sites of intestinal injury, inflammation and wound repair via the expression of adhesion molecules and chemokines [1, 8, 9]. In addition to their function as chemoattractant for leukocytes, chemokines were recently identified as potential regulators of intestinal epithelial cells [2–4, 6, 7]. As we recently demonstrated, chemokines such as CXCL12 or CCL20 are able to induce IEC migration and proliferation leading to increased cell restitution [7, 10]. Furthermore, chemokines can induce proinflammatory responses [5, 7] in IEC and they play a role in neoangiogenesis [11]. However, the mechanisms of chemokine receptor signaling and discovery of the functional role of chemokines and their receptors in the control of the intestinal epithelial immune function are outstanding questions for the full understanding of the role of chemokines in the intestine.

Recently, a new chemokine ligand receptor pair (CXCL16-CXCR6) was identified and added to the growing family of chemokines. CXCL16 is a unique chemokine with characteristics of CC chemokines and a structure similar to that of fractalkine in having a transmembrane region and a chemokine domain suspended by a mucin-like stalk [12]. CXCL16 is expressed on the surface of antigen presenting cells (APCs), including subsets of CD19+ B cells and CD14+ monocytes/macrophages [12]. Membrane-bound CXCL16 mediates adhesion and phagocytosis of both Gram-negative and Gram-positive bacteria [13] while soluble CXCL16 is a strong chemoattractant for CXCR6+ T cells [14, 15]. Importantly, anti-CXCL16 antibodies, which suppress chemotactic activity of CXCL16, significantly inhibit bacterial phagocytosis by human APCs [13]. Therefore, CXCL16 may play an important role in facilitating uptake of various pathogens.

CXCL16 binds to the chemokine receptor CXCR6 [12, 16]. CXCR6 was first recognized as receptor for simian and human immunodeficiency viruses [17]. This chemokine receptor was originally cloned as an orphan receptor by three independent groups who assigned three different names to it: STRL33 (seven transmembrane receptor-like from clone 33), Bonzo, and TYMSTR (T lymphocyte-expressed seven-transmembrane domain receptor) [17–19]. In mice, CXCR6 is expressed by subsets of CD4+, CD8+ and natural killer T cells, whereas in humans CXCR6 is expressed by small subsets of Th1 or T-cytotoxic 1 (Tc1) cells, establishing CXCR6 as a differential marker of polarized type 1 T cells [20]. CXCR6+ T cells are dramatically enriched among T cells at sites of tissue inflammation, such as rheumatoid joints and inflamed livers [20]. CXCR6 mRNA expression has been detected primarily in lymphoid tissue such as spleen and thymus [17] but also in the small intestine and the colon [17, 21, 22]. So far, little is known about CXCR6 expression in intestinal epithelial cells. In one study, CXCR6 expression has been demonstrated mainly in normal epithelial cells of the colon, but not or only at low levels in cancerous tissue [22]. However, the signal transduction and biological functions of this chemokine receptor system in IEC and its role in intestinal inflammation are unknown. We therefore analyzed the expression of the CXCL16-CXCR6 chemokine-ligand receptor system in *in vitro* and *in vivo* models of intestinal inflammation including its expression in sera and colonic biopsies of IBD patients. Furthermore, we determined if CXCR6 is similar to other chemokine receptors such as CXCR4 [2, 3, 6], CCR6 [7] and CX3CR1 [4] expressed in IEC suggesting functions for this chemokine system in the maintenance of the intestinal epithelial barrier. In addition, we analyzed the CXCR6 mediated signal transduction in IEC.

Patients and Methods

Reagents

Specific polyclonal antibodies to phosphorylated ERK-1/2 (Thr183/Tyr185) were obtained from Promega (Madison, WI); antibodies against phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser473), SAPK/JNK, p38 and Akt and the MEK-1 inhibitor PD98059 were purchased from New England Biolabs (Beverly, MA). ERK-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Human CXCR6 and CXCL16 antibodies and recombinant human and mouse CXCL16 were from R&D Systems (Minneapolis, MN), and PI3 kinase inhibitor wortmannin was from Sigma (St. Louis, MO). Recombinant human TNF-α, IL-1β and IFN-γ were from R&D Systems. LPS preparation was purchased from Sigma-Aldrich (Taufkirchen, Germany) which was derived from *E. coli* (serotype 026:B6) by phenol extraction.

Cell culture

The human colorectal cancer-derived IEC lines T84, SW480, Caco-2, HT-29 and the murine colorectal cancer cell line CMT93 were obtained from American Type Culture Collection (Rockville, MD). While T84 cells were grown in Dulbecco's modified Eagle medium/F-12 (Cellgro, Mediatech Inc., Herndon, VA), the other cell lines were grown in Dulbecco's modified Eagle medium (Cellgro) with 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated FCS (Sigma, St. Louis, MO) in a humidified 5% CO_2 atmosphere at 37°C. For stimulation experiments with CXCL16, cells were starved overnight in serum-free medium.

Gel electrophoresis and immunoblotting

Total protein was isolated by solubilizing cells in lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 μg/mL aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and phosphatase inhibitors (400 mM sodium orthovanadate and 4 mM NaF) and passing the lysates six times through a 21G needle. After 30 minutes on ice, lysates were cleared by centrifugation at 10,000 × *g* for 20 minutes. Cytosolic and membrane protein fractions were isolated as previously described [4, 23]. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described [24].

Immunohistochemistry

Intestinal biopsy specimens were taken during diagnostic endoscopy after informed consent. Immunohistochemistry was performed on 3 μm sections following standard protocols. In brief, endogenous peroxidases were blocked with H_2O_2 after deparaffinisation and demasking of antigens, Following incubation with 10% normal serum and avidin and biotin, slides were incubated with the primary antibody overnight at 4°C. Detection was performed with an biotin-coupled secondary antibody and HRP streptavidin using 3′-diaminobenzidine (DAB) as peroxidase substrate. Slides were counterstained with haematoxylin. Immunohistochemical analysis of CXCR6 and CXCL16 expression in the IEC cell line HT-29 was performed adopting a previously established staining protocol using FITC-conjugated anti-mouse and anti-goat secondary antibodies (Sigma, Taufkirchen, Germany) and Hoechst 33342 (Sigma) staining [6, 24]. In negative controls, cells were stained omitting the primary antibody.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free™-Kit, Ambion, Austin, TX) to remove potential genomic DNA contaminants. The following conditions were used for all PCRs: 35 cycles of denaturing at 95 °C for 1 min, annealing temperature for 30 sec, extension at 72 °C for 1 min. The following primers were used: human CXCL16: forward 5′-GCA GCG TCA CTG GAA GTT GTT AT-3′, reverse 5′-TGC GGT GAG GAT GAA GAT GAT GA-3′, human CXCR6: forward 5′-CAG GCA TCC ATG AAT GGG TGT-3′, reverse 5′-CAA GGC CTA TAA CTG GAA CAT GCT G-3′, murine CXCL16: forward 5′-AAA CAT TTG CCT CAA GCC AGT-3′, reverse 5′-GTT TCT CAT TTG CCT CAG CCT-3′, murine CXCR6: forward 5′-TGT ACG ATG GGC ACT ACG A-3′, reverse 5′-GTG AGA GAG GCA GCC GAT A-3′. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Quantitative PCR

Real-time PCR was performed with a Rotorgene RG-3000 cycler (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR Kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers were designed according to published sequences, and the following primer pairs were used: human CXCL16: forward 5′-GAG CTC ACT CGT CCC AAT GAA-3′, reverse 5′-TCA GGC CCA ACT GCC AGA-3′; human beta-actin: forward 5′-GCC AAC CGC GAG AAG ATG A-3′, reverse 5′-CAT CAC GAT GCC AGT GGT A-3′; human interleukin-8 (IL-8): forward 5′-CCA GGA AGA AAC CAC CGG-A-3′, reverse 5′-GAA-ATC-AGG-GCT-GCC-AAG-3′ (MWG-Biotech, Ebersberg, Germany). murine CXCL16: forward 5′-AGC GCA AAG AGT GTG GAA CT-3′, reverse 5′-GGT TGG GTG TGC TCT TTG TT-3′; murine TNF-α: forward 5′-CCC CAA AGG GAT GAG AAG TT-3′, reverse 5′-CAC TTG GTG GTT TGC TAC GA-3′; murine GAPDH: forward 5′-CGT CCC GTA GAC AAA ATG GT-3′, reverse 5′-TCT CCA TGG TGG TGA AGA CA-3′. CXCL16 expression was normalized to beta-actin or GAPDH expression in the respective cDNA preparations.

Northern blotting

Northern Blot analysis was performed as previously described [4]. Mouse CXCR6 and CXCL16 cDNA were generated by RT-PCR using primers as listed above. For generation of GAPDH cDNA, previously published primers were used [25].

Enzyme-linked immunosorbent assay (ELISA)

For the quantification of CXCL16 in serum samples of IBD patients and healthy controls as well as in cell culture supernatants, human CXCL16 Quantikine Elisa Kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions.

Isolation of primary ileal epithelial cells from heterozygous TNFΔARE mice

Heterozygous TNFΔARE/+ mice (a generous gift from Dr. G. Kollias; Biomedical Sciences Research Centre "Alexander Fleming", Varkiza, Greece), which gradually develop chronic inflammation in the ileum from moderate to severe levels at 8 and 18 weeks of age [26], and wild-type TNF+/+ mice (wt) were killed at the age of 8 and 18 weeks. Primary IEC from the ileal epithelium of wt and TNFΔARE/wt mice were purified as previously described [27, 28].

Murine cytomegalovirus (MCMV) infection in vivo

C57BL/6 mice were infected i.v. with 1×10^6 pfu murine cytomegaly virus (MCMV) of the Smith strain [29] in PBS as previously described [30]. Control mice received an injection of PBS

only. After 45 h, mice were euthanized by CO_2 asphyxiation. Total RNA of the colon was isolated using Trizol reagent.

Colonic biopsy and serum sampling

Colonic biopsies were collected after written, informed consent from patients with Crohn's disease undergoing diagnostic colonoscopy in the IBD center of the University Hospital Munich-Grosshadern (Germany). Four biopsies were collected from each patient: two from macroscopically non-inflamed sites and two from macroscopically inflamed mucosa (for location of biopsy sampling see Table 1). CXCL16, CXCR6 and IL-8 mRNA levels were determined by quantitative RT-PCR and were normalized to β-actin expression. Serum samples were collected after written, informed consent from IBD patients as well as from healthy, unrelated controls. For analysis, active IBD was defined as Crohn's disease activity index (CDAI) >150 or colitis activity index (CAI>4); remission of disease was defined as CDAI<150 or CAI<4.

Statistics

Fisher's exact test or χ^2 test was used, where appropriate, for comparison between categorical variables. Student's t-test was applied for quantitative variables. All tests were two-tailed and p-values < 0.05 were considered as significant. Statistical analyses were performed using the SAS 8.2 software for Windows.

Results

CXCR6 and CXCL16 mRNA are expressed in the murine and human intestine

First, we determined the organ-specific mRNA expression of CXCR6 and CXCL16 analyzing murine tissues derived from BALB/c mice by Northern blot. CXCL16 mRNA expression was detected as a major 1.8 kb and a 2.5 kb transcript in small and large intestine, kidney, spleen and liver (Fig. 1A). While CXCR6 mRNA expression was highest in the ileum, cecum and colon, CXCL16 mRNA expression was highest in the duodenum and jejunum but still detectable in the colon demonstrating a segment specific CXCL16 expression pattern inverse to that of CXCR6 (Fig. 1A). CXCL16 and CXCR6 mRNA were expressed in the human colorectal cancer derived IEC lines (T84, HT-29, Caco-2, SW480), as well as in the murine colorectal cancer cell line CMT93 (Fig. 1B). Anti-CXCR6 antibodies detected CXCR6 in the membrane fraction of the IEC line HT-29 (Fig. 1C) which was consistent with the high CXCR6 expression in the membrane of these cells in the immunohistochemical analysis (Fig. 1D; negative control Fig. 1E). In addition, we demonstrated by Western blot analysis (Fig. 1F) and immunohistochemistry (Fig. 1G) CXCL16 protein expression predominantly in the membrane fraction and to a lesser degree in the cytosol of the IEC line HT-29 (negative control: Fig. 1H). Similar results for CXCR6 and CXCL16 protein expression were obtained in SW480 cells (data not shown).

CXCL16 induces MAP kinase and Akt phosphorylation in IEC

We next investigated whether CXCR6 is functionally active in IEC by analyzing signaling pathways in the murine IEC line CMT93 after stimulation with murine CXCL16. As shown in Fig. 2A/B, CXCL16 increased MEK-1 dependent ERK-1/2 phosphorylation in CMT93 cells, independent of PI3 kinase activity. Since phosphorylation of p38, SAPK/JNK kinases and Akt has been shown in response of chemokine receptor dependent signalling [4, 6, 7], we assessed the phosphorylation of these kinases following CXCL16 stimulation. CXCL16 induced weak phosphorylation of SAPK/JNK kinases (Fig. 2C) and only very weakly p38 phosphorylation (Fig. 2D). Moreover, CXCR6 activation resulted in PI3-kinase dependent and partly MEK-1 dependent phosphorylation of Akt (Fig. 2E, 2F). Similar results for the

activation of ERK and Akt kinases were obtained in the human colorectal cancer-derived cell line HT-29 following stimulation with 100 ng/mL human CXCL16 (Fig. 2G, H).

CXCL16 mRNA and protein expression in human IEC is under the control of inflammatory signals

Given the important role of chemokines in intestinal inflammation, we analyzed whether proinflammatory cytokines and LPS up-regulate CXCR6 and CXCL16 mRNA expression in human IEC. The human colorectal cancer cell line HT-29 was stimulated with either TNF-α (50 ng/mL), IL-1β (10 ng/mL), IFN-γ (1000 U/mL) or LPS (1 μg/mL). As shown in Fig. 3A, TNF-α up-regulated CXCL16 mRNA expression up to 5.1-fold in HT-29 cells, while CXCR6 expression remained unchanged (data not shown). Similar to TNF-α, IL-1β and IFN-γ stimulated CXCL16 mRNA expression up to 4.2 and 6.2-fold, respectively (Fig. 3A) while LPS had no effect on CXCL16 expression. Having demonstrated that CXCL16 mRNA levels are regulated by proinflammatory cytokines, we next analyzed the modulation of CXCL16 protein release in the cell culture supernatants of HT-29 during proinflammatory stimulation. As demonstrated in figure 3B, CXCL16 protein levels were below the ELISA's detection threshold of 156 pg/ml during the first 4 hours of stimulation. Corresponding to the increase of CXCL16 mRNA after stimulation with proinflammatory cytokines, CXCL16 protein concentration rose following TNF-α, IL-1β and IFN-γ stimulation. TNF-α showed the strongest effect with detectable CXCL16 protein after 8 hours and reaching the highest concentration after 24 hours of stimulation (Fig. 3B). LPS had only a marginal, delayed effect on CXCL16 protein levels with levels slightly above the detection threshold after 24 hours (Fig. 3B).

CXCL16 mRNA expression is up-regulated in murine models of ileitis and colitis in vivo

We next examined CXCL16 expression in two murine models of intestinal inflammation *in vivo*. We isolated epithelial cells from the small intestine and the colon of wild-type (wt) mice and of heterozygous TNFΔARE mice, which develop chronic ileitis [26]. In these mice, we demonstrated that CXCL16 mRNA expression in ileal epithelial cells was significantly increased 3.5-fold in 18 week old mice compared to wt mice of the same age ($p<0.05$, Fig. 3C), while no significant change was observed in 8 week old mice (data not shown). Additionally, we measured TNF-α mRNA expression which was 7.0-fold higher in TNFΔARE mice than in wt mice ($p<0.05$) proving that the TNFΔARE mice had severe intestinal inflammation (data not shown). Similar to our results in BALB/c mice (Fig. 1A), in the combined group of TNFΔARE and wt mice (n=18), CXCL16 expression was 3.1-fold higher in epithelial cells derived from the small intestine (ileum and jejunum) than in colonic epithelial cells ($p=0.025$; data not shown).

Next, we analyzed colonic CXCL16 mRNA expression in intestinal inflammation following viral infection *in vivo* using the murine model of MCMV infection. C57BL/6 mice were infected with 10^6 pfu MCMV of the Smith strain [29]. Forty-five hours after infection, the colon was removed and mRNA was isolated. The TNF-α mRNA expression, which was used as a marker of inflammation, was 7.9-fold increased in comparison to uninfected control mice ($p<0.05$). Accordingly, CXCL16 mRNA levels were upregulated 1.8-fold in the colon of infected mice compared to non-infected mice ($p=0.03$; Fig. 3D).

CXCL16 mRNA expression is increased in the inflamed colonic mucosa of CD patients

Following the analysis in murine models of intestinal inflammation, we compared CXCL16 mRNA expression levels in biopsy samples taken from 20 different sites of 10 CD patients with endoscopically (macroscopic) inflamed colonic mucosa with those of endoscopically non-inflamed colonic mucosa taken from 20 different sites of the same 10 patients (total number of biopsies: n=40). IL-8 expression, which was used as a control marker for

inflammation, was significantly increased (6.9-fold, $p< 0.05$) in the inflamed biopsy samples compared to the biopsies taken from non-inflamed areas. The increase in IL-8 mRNA expression ranged from 1.4 up to 31.1-fold compared to the non-inflamed tissues as determined by qPCR (data not shown). Similarly, CXCL16 mRNA expression levels were higher in biopsy samples from inflamed mucosa when compared with non-inflamed lesions (increase between 1.3 and 16.3-fold; Table 1). Moreover, the increase of CXCL16 mRNA levels correlated highly with the increase of IL-8 mRNA expression levels (r=0.89), demonstrating its association with intestinal inflammation in CD patients. CXCR6 expression was also slightly higher (1.8-fold) in inflamed in comparison to uninflamed biopsies (Table 1). CXCR6 mRNA expression correlated highly with that of CXCL16 in the respective biopsies (correlation coefficient of r=0.85).

Mucosa-infiltrating immune cells are a major source of intestinal CXCL16 expression

To determine CXCL16 protein expression *in situ*, we next performed immunohistochemical staining for CXCL16 in intestinal biopsies from patients with Crohn's disease comparing uninflamed and inflamed tissue of the same patients. CXCL16 abundance was higher in inflamed tissue in comparison to uninflamed tissue of the same patients (Figure 4). However, this was mainly a result of the increased mucosal infiltration with immune cells expressing high levels of CXCL16 rather than an upregulation of CXCL16 expression in IEC (Figure 4).

CXCL16 serum levels are increased in active CD

We next analyzed CXCL16 protein expression in sera of another group of IBD patients. Compared to a healthy control population (n=30), the CXCL16 serum levels were significantly higher ($p<0.005$) in the 47 IBD patients but without a significant difference between 30 CD patients and 17 patients with UC (4.07 vs. 3.95 ng/mL, Fig. 5A). High CXCL16 serum levels were detected particularly in a subgroup of 13 patients with active IBD (Crohn's disease activity index, CDAI>150 or colitis activity index, CAI>4, respectively) compared to patients in remission (CDAI<150 or CAI<4; n=9) and healthy controls (4.35 vs. 3.50 ng/mL CXCL16; p=0.04; data not shown). In patients with active IBD, CXCL16 serum levels correlated with CRP serum levels (r=0.485, data not shown). However, in a subgroup analysis of this patient group, increased CXCL16 expression was detected only among patients with active CD (CDAI >150, n=8) but not in patients with active UC (CAI >4, n=5, data not shown). CXCL16 levels in patients with active CD were significantly higher compared to the control population (p=0.02, Fig. 5B), while CXCL16 serum levels in CD patients in remission (CDAI < 150) were not significantly different from the control population (p=0.29, Fig. 5B).

Discussion

IEC form an active barrier and are sensitive indicators of infection that initiate defense responses based on their capacity to express both chemokines and chemokine receptors. We [4–7, 24, 31] and others [2, 32] have identified chemokines and their receptors expressed on IEC as key regulators involved in the inflammatory response of the gastrointestinal mucosa which therefore potentially play an important role in the pathogenesis of IBD. However, the exact mechanisms of chemokine receptor signaling and their specific functions in the intestinal epithelium are still largely unknown.

In this study, we provide evidence that the CXCL16-CXCR6 chemokine ligand receptor system is expressed in the intestinal mucosa and plays a significant role in both intestinal inflammation and in human IBD. Our results demonstrate that CXCL16 as well as its receptor CXCR6 are expressed in IEC lines. For the first time, this study provides evidence

that IEC-expressed CXCR6 is functional: CXCL16 predominantly activated the MEK-ERK MAP kinase signaling pathway which has also been shown to be the major signaling pathway of other chemokine receptors such as CCR6, CXCR4 and CX3CR1 [4, 6, 7, 33]. However, in contrast to CX3CR1 signaling [4], CXCR6 activation also resulted in increased Akt phosphorylation levels and – less strongly – increased phosphorylation of SAPK/JNK and p38 MAP kinases. Recent studies focusing on these signaling pathways demonstrated that SAPK/JNK and p38 MAP kinases are activated in CD [34, 35], and that inhibition of SAPK/JNK and p38 activation resulted in significant clinical benefit and rapid endoscopic ulcer healing [35].

The CXCL16-CXCR6 chemokine-chemokine receptor system may also contribute to the integrity of the intestinal barrier but also to cancer metastasis given that all IEC analyzed in our study were colorectal cancer-derived and showed CXCR6 expression. This is in line with recent studies demonstrating CXCR6 expression in colorectal carcinoma cells [22] as well as in other cancerous epithelial cells such as prostate carcinoma cells [36, 37] or melanoma [38], thereby contributing to cell migration and invasive tumour growth. Studies in breast cancer cells demonstrated that soluble CXCL16 chemokine enhances cell proliferation via CXCR6 activation, while transmembrane-bound CXCL16 reduces proliferation [39]. However, if similar opposite effects of soluble and transmembrane CXCL16 apply also to colorectal cancers needs further investigation. Moreover, a very recent study demonstrated that CXCL16 and its receptor CXCR6 are markers and promoters of inflammation-associated cancers [40].

Here, we showed that CXCL16 gene expression in IEC is stimulated by treatment with the proinflammatory cytokines TNF-α, IL-1β or IFN-γ suggesting a role for this cytokine in inflammatory processes. The increase in CXCL16 mRNA expression is followed by an accumulation of secreted CXCL16 protein in the cell culture supernatants. These results are in agreement with a previous study in which stimulation of endothelial cells with TNF-α and IFN-γ leads to increased CXCL16 protein levels [41].

In addition, we demonstrated increased intestinal CXCL16 expression in murine *in vivo* models of intestinal inflammation including TNFΔARE mice and MCMV infected mice. TNFΔARE mice are characterized by an overexpression of TNF-α and develop a chronic transmural ileitis with characteristics of human CD [26]. Interestingly, CXCL16 expression is increased in the ileum of these mice compared to wt control mice. Similarly, colonic CXCL16 expression is also increased in MCMV infected mice compared to uninfected mice. Recent studies demonstrated up-regulation of CXCL16 in herpes simplex virus type 1 (HSV-1) infection [42] and an influence of CXCL16 on the nature and specificity of CpG-induced immune activation [43], suggesting a role for this chemokine in the immune response following viral infection.

Analogous to the murine models of intestinal inflammation, we observed in CD patients an increase in colonic CXCL16 mRNA expression up to 16.3-fold when comparing biopsies from uninflamed and inflamed tissue of the same patients. Further immunohistochemical analysis revealed that infiltrating immune cells were the main producers of CXCL16 in the inflamed tissue areas. This is in concordance with a recent study which describes B cells, $CD14^+$ monocytes and macrophages as the primary source of CXCL16 production [12]. Another study by Hase et al. revealed that in mice CXCL16 is also expressed by specialized IECs in the follicle-associated epithelium (FAE) covering the Peyer's patches [21] which are located in the small intestine. However, for our immunohistochemical analysis only biopsies from human colonic tissue but not from the small intestine were available. Further analysis is needed to show if CXCL16 is also expressed in human FAE.

Next, we measured increased CXCL16 protein expression in the serum of patients with CD suggesting not only a local but also systemic function of this chemokine in CD. Our data are in accordance with a recent study demonstrating higher CXCL16 expression in CD patients [44]. In contrast, CXCL16 serum levels in our UC patients were not significantly different to the control group. Our subgroup analysis showed that CXCL16 serum levels are particularly high in patients with active CD (CDAI>150) pointing to an important role of CXCL16 in intestinal inflammation in CD patients. This hypothesis is supported by our recent data demonstrating an association of a CXCL16 polymorphism (p.Ala181Val) with the phenotypic characteristics and the severity of CD [45] but not with UC [45].

In summary, this is the first comprehensive analysis of the CXCL16-CXCR6 chemokine ligand receptor system in intestinal inflammation. We have shown that the chemokine receptor CXCR6 is expressed by IEC. Upon stimulation with CXCL16, several distinct signaling pathways including ERK-MAP kinases and Akt are activated. Proinflammatory stimuli such as IL-1β, IFN-γ and TNF-α increase CXCL16 mRNA and protein expression. Similarly, CXCL16 mRNA expression is increased in murine intestinal inflammation and human IBD. We therefore hypothesize that CXCL16 plays an important role in regulating mucosal innate and adaptive immune responses. Further functional studies are necessary to define exact roles of this novel chemokine system in the intestine; however, the chemoattractation of CXCR6+ T cells during intestinal inflammation through highly expressed CXCL16 protein seems to be another important function in human IBD.

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Abbreviations

APC	antigen-presenting cell
CAI	colitis activity index
CDAI	Crohn's disease activity index
CD	Crohn's disease
CpG	cytosine-phospho-guanine
ERK	extracellular signal–regulated kinase
FAE	follicle associated epithelium
FCS	fetal calf serum

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein-kinase
MCMV	murine cytomegalovirus
MEK	mitogen-activated protein kinase kinase
PI	phosphatidylinositol
SAPK/JNK	stress-activated protein kinase/c-Jun-N-terminal kinase
TNF	tumor necrosis factor
UC	ulcerative colitis
vs	versus
wt	wild-type

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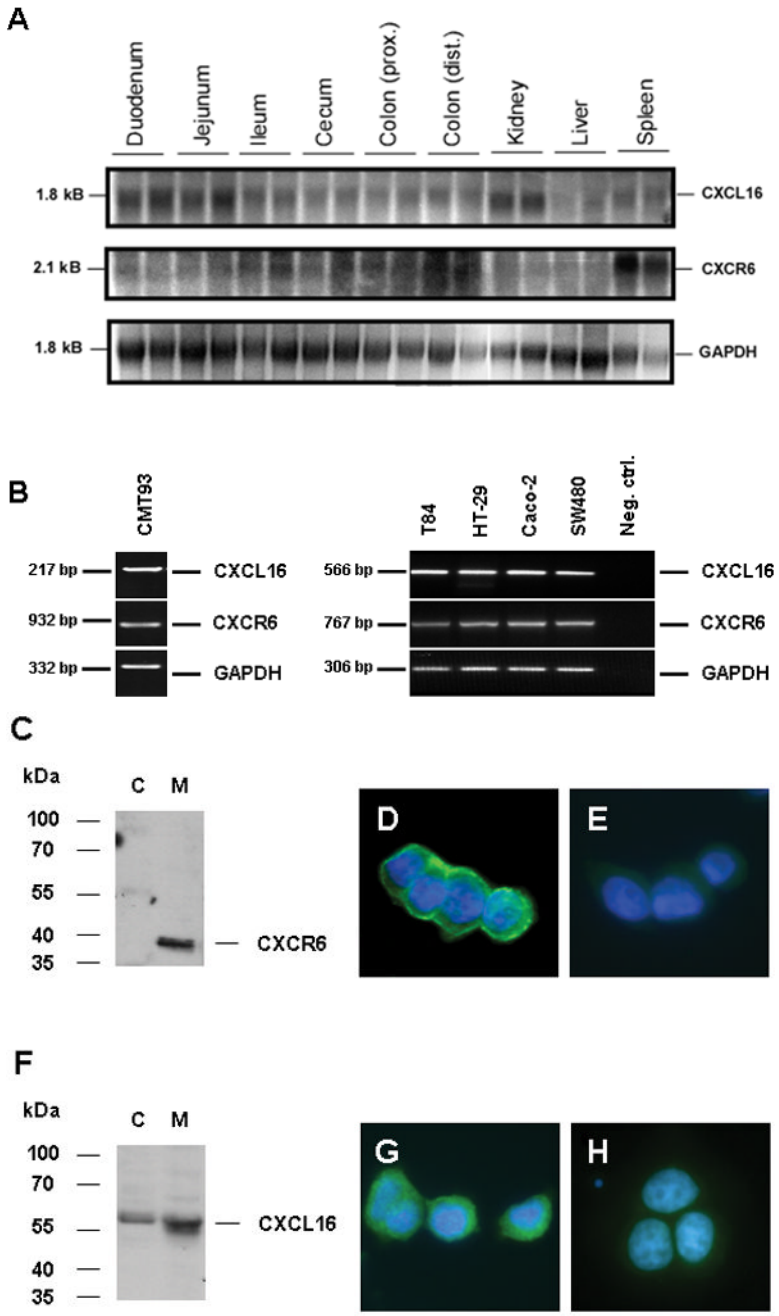

Figure 1. IEC express CXCR6 and CXCL16

(A) Northern Blot analysis of mRNA derived from murine tissue of two wt BALB/c mice as indicated. Note the inverse segment-specific expression of CXCL16 and CXCR6 mRNA in the murine gastrointestinal tract with high CXCL16 expression in the small intestine and high CXCR6 expression in the colon. **(B)** CXCL16 and CXCR6 mRNA expression in human IEC lines and the murine IEC line CMT93 were analyzed by RT-PCR. **(C)** Western blot analysis of CXCR6 protein expression in cytosolic *(C)* and membrane *(M)* protein fractions of HT-29 cells. **(D)** Immunocytochemical staining reveals a membrane associated expression of CXCR6 in HT-29 cells (anti-CXCR6 antibody detected with a FITC conjugated secondary antibody, nucleus: stained with Hoechst 33342). **(E)** No CXCR6

specific staining was obtained using the secondary antibody (FITC conjugated) only. **(F)** Western blot analysis of CXCL16 protein expression in cytosolic *(C)* and membrane *(M)* protein fractions of HT-29 cells. **(G)** Immunocytochemical staining with a CXCL16 specific antibody in HT-29 cells. **(H)** No CXCL16 specific staining was detected using the secondary antibody only.

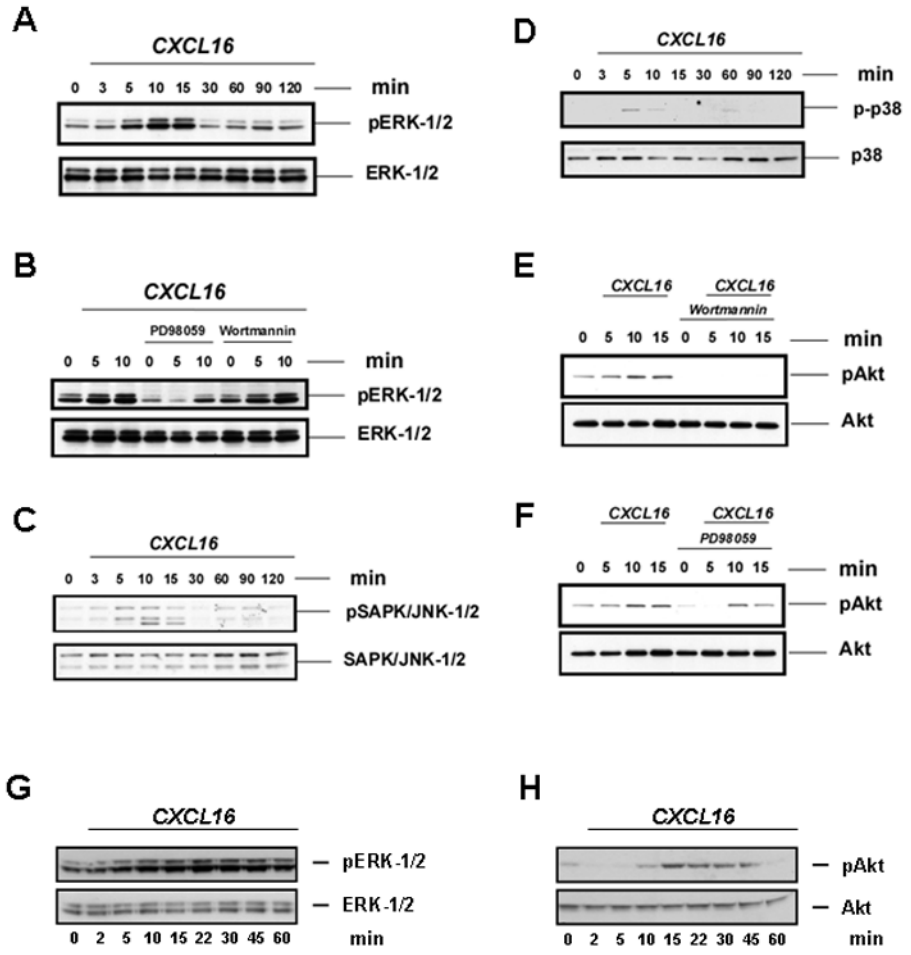

Figure 2. CXCL16 activates MAP kinases and Akt in IEC

(A) Detection of phosphorylated ERK-1/2 in CMT93 IEC lines after CXCL16 stimulation (100 ng/mL). **(B)** Phospho-ERK1/2 activation by CXCL16 in CMT93 cells pretreated with the MEK-1 inhibitor PD98059 (5 μmol/L) and the PI3 kinase inhibitor wortmannin (200 nmol/L). **(C)** Phosphorylated SAPK/JNK kinases in CMT93 cells after CXCL16 stimulation (100 ng/mL). **(D)** CXCL16 stimulation of CMT93 cells resulted also in very weak phosphorylation of p38. **(E)** Phosphorylated Akt in CXCL16 stimulated CMT93 cells (including wortmannin pretreated cells) and in **(F)** PD98059 pretreated cells. **(G)** ERK-1/2 MAP kinases are activated in HT-29 cells following CXCL16 stimulation. **(H)** CXCL16 leads to Akt phosphorylation in HT-29 cells. For all signaling experiments (Fig. 2A–H), one representative experiment of three performed is shown.

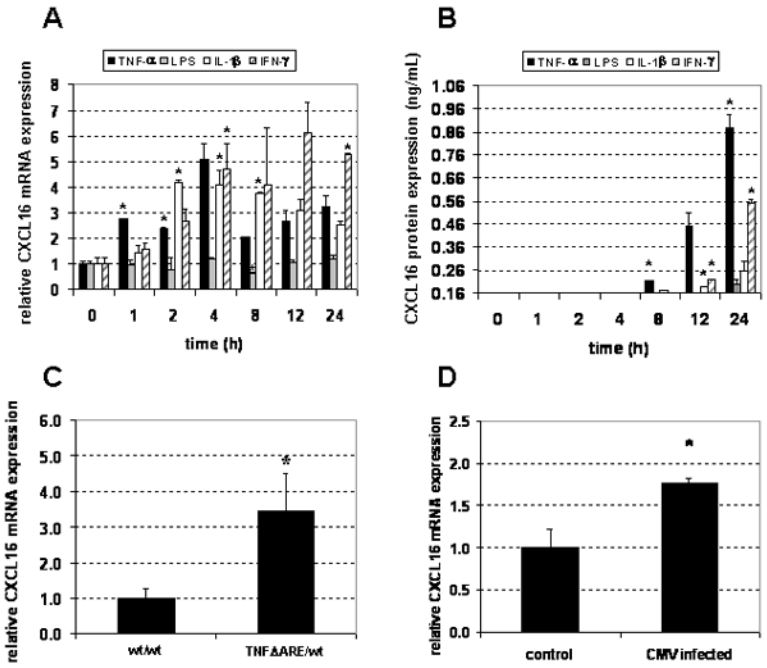

Figure 3. CXCL16 gene expression is upregulated in intestinal inflammation

(A) The proinflammatory cytokines TNF-α (50 μg/mL), IL-1β (10 μg/mL) and IFN-γ (1000 U/mL) significantly increase expression of CXCL16 mRNA in HT-29 cells as determined by quantitative PCR (*$p<0.005$ vs. t=0h) while LPS (1 μg/mL) has no stimulating effect. **(B)** Similarly, we measured soluble CXCL16 protein concentration by ELISA cell culture supernatants of HT-29 cells during cytokine stimulation (*$p<0.05$ vs. t = 0 h). Note the corresponding but delayed kinetics of CXCL16 protein release in comparison to the CXCL16 mRNA increase. The detection limit of the ELISA was 156 pg/mL **(C)** CXCL16 mRNA expression is increased 3.5-fold in ileal epithelial cells from TNFΔARE heterozygous mice (n=9) compared to wt mice (n=9) as determined by quantitative PCR (*$p<0.05$ vs. wt). Additionally, there was a higher CXCL16 expression in ileal and jejunal epithelial cells compared to colonic epithelial cells (data not shown). **(D)** CXCL16 mRNA expression is increased in colonic tissue from 1×10^6 pfu MCMV i.v. infected C57BL/6 mice (n=11) in comparison to PBS injected C57BL/6 control mice (n=4, * $p=0.03$ vs. controls).

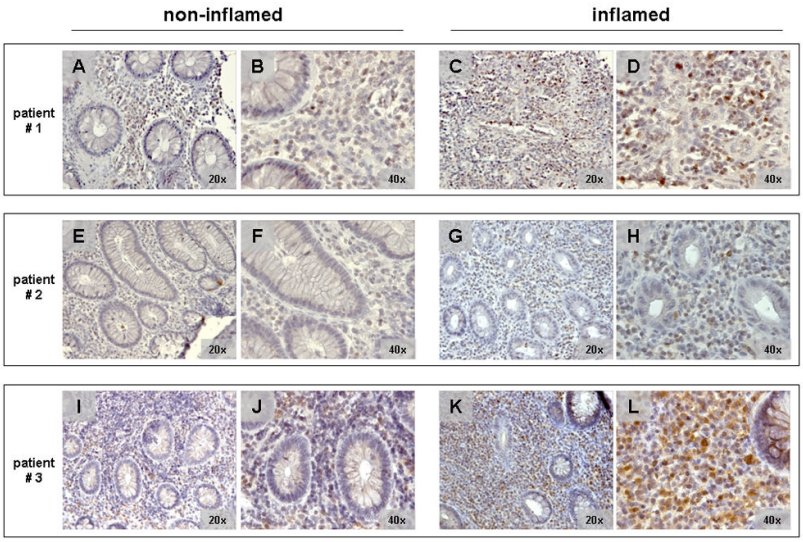

Figure 4. CXCL16 expression is increased in inflamed intestinal tissue of patients with Crohn's disease

Immunohistochemical analysis of biopsies from three different CD patients (A–D: patient 1, E–H: patient 2, I–L: patient 3) reveals higher mucosal infiltration with CXCL16-expressing immune cells in inflamed regions (C/D; G/H; K/L) in comparison to uninflamed tissue (A/B; E/F; I/J). 20×, 40×: magnification.

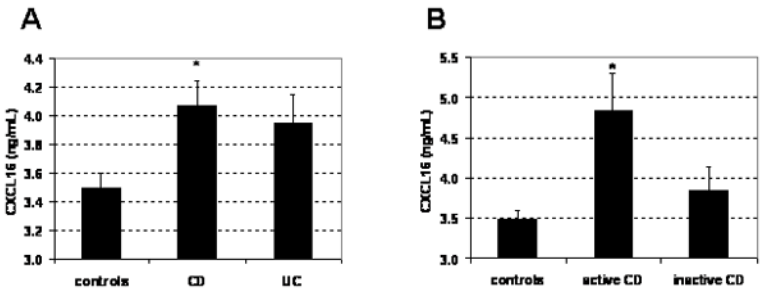

Figure 5. CXCL16 serum protein concentration is increased in active Crohn's disease

(A) In the sera of patients with Crohn's disease (n=30), significantly higher CXCL16 serum protein levels were detected in comparison to healthy controls (n=30; * $p<0.01$ vs. controls; UC: n=17; $p=0.057$ vs. controls). **(B)** CXCL16 expression was particularly high in CD patients with active disease (CDAI >150; * $p=0.02$ vs. controls).

Table 1

CXCL16 and CXCR6 mRNA expression in inflamed and non-inflamed colonic lesions from patients with CD determined by quantitative RT-PCR and normalized to beta-actin expression levels. The current medical therapy during biopsy sampling and the anatomic site, from which the samples were taken, are given for all patients.

Pat. #	CXCR6 mRNA expression (fold increase) inflamed vs. non-inflamed	CXCL16 mRNA expression (fold increase) inflamed vs. non-inflamed	Anatomic site of biopsy sampling		Current Medication
			non-inflamed	inflamed	
1 *	1.7	1.3	cecum *	terminal ileum *	mesalazine, corticosteroids
2 *	5.1	16.3	cecum*	ileocecal valve*	MTX
3 *	n.d.	1.7	cecum *	terminal ileum *	AZA, IFX
4	1.0	2.5	descending colon	descending colon	mesalazine, AZA
5	3.1	2.1	cecum	cecum	AZA
6 *	0.7	2.0	ascending colon*	terminal ileum*	AZA
7	1.2	2.3	descending colon	descending colon	no medication
8	0.5	1.7	transverse colon	transverse colon	AZA
9 *	1.4	4.2	cecum*	terminal ileum*	AZA, corticosteroids
10	1.4	1.9	ascending colon	ascending colon	AZA
Average ± SEM	1.8 ± 1.5	3.6 ± 1.3			

While it was intended to take biopsies from inflamed and non-inflamed lesions in the same intestinal segment, in patients with severe inflammation biopsies from neighbouring sites were taken that are indicated by asterisk (*).

Abbreviations: AZA, azathioprine; IFX, infliximab; MTX, methotrexate