Cytokines and chemokines in systemic and urinary tract infection by Escherichia coli

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Chapter 6

CXC chemokine receptor 2 (CXCR2) contributes to host defense in murine urinary tract infection.

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CXC chemokines have been implicated in the recruitment of neutrophils to sites of infection. To determine the role of CXC chemokines in the host response to urinary tract infection (UTI), female mice were treated with an antibody against the sole CXC chemokine receptor in the mouse, CXCR2, prior to intravesical inoculation with *Escherichia coli*. Anti-CXCR2 prevented the influx of neutrophils in urine and kidneys. The absence of a neutrophil response only temporarily impaired the clearance of bacteria from the urinary tract, as indicated by 100- and 1000-fold more *E. coli* colony forming units in urine and kidneys of anti-CXCR2 treated mice at 24 hours, but not at 48 hours after the infection. UTI induced increases in the renal concentrations of the CXCR2 ligands macrophage inflammatory protein 2 and keratinocyte, which were not influenced by anti-CXCR2 administration. CXC chemokines play an important role in the development of a local inflammatory response to UTI.
Introduction

Urinary tract infections (UTIs) are responsible for as many as 8 million visits to physicians a year in the United States alone [1]. They comprise entities such as pyelonephritis, cystitis and asymptomatic bacteruria. *Escherichia coli* is the most common pathogen, causing 85 percent of community-acquired UTIs [2] and 50 percent of UTIs in hospitalized patients [3]. Furthermore, the urinary tract is identified as the most common source of bacteremia [4-6].

Chemokines are a group of small chemotactic proteins. They play an important role in the host response to bacterial infections by attracting leukocytes to the site of infection [7, 8]. CXC chemokines represent a subfamily of chemokines, that can be further divided into two subclasses based on the presence or absence of a three amino acid motif termed ELR (glutamic acid – leucine – arginine). ELR positive CXC chemokines are chemotactic for neutrophils and include interleukin (IL)-8, epithelial cell-derived neutrophil-activating protein (ENA)-78, growth-related oncogene (GRO)-α, -β and -γ in humans, and keratinocyte (KC) and macrophage inflammatory protein (MIP)-2 in mice [9]. UTI is associated with an influx of neutrophils in the urinary tract. Several lines of evidence indicate that ELR positive CXC chemokines are involved in this characteristic inflammatory response. First, high concentrations of IL-8, ENA-78 and GRO-α were detected in the urine of patients with UTI [10-13]. Second, infected urine was chemotactic for neutrophils, and the chemotactic activity of this urine could be inhibited by neutralizing antibodies against either IL-8, ENA-78 or GRO-α [11, 13]. Third, treatment of mice with experimental UTI with an anti-MIP-2 antibody resulted in a reduction of neutrophil numbers in urine [14].

In humans, two receptors for ELR positive CXC chemokines have been identified on the surface of granulocytes, the CXC chemokine receptor – 1 and - 2 (CXCR1 and CXCR2) [15, 16]. CXCR2 is a promiscuous chemokine receptor, which binds all ELR positive CXC chemokines [17]. Mice lack CXCR1, and CXCR2 exclusively mediates granulocyte responses to ELR positive CXC chemokines in this species [18]. In the present study we sought to determine the importance of CXCR2 in neutrophil recruitment.
and antibacterial host defense in a murine model of ascending UTI with *E. coli*.

**Materials and Methods**

**Mice.**

Female BALB/c mice were obtained from Harlan Spague Dawley Inc. (Horst, the Netherlands). 10-week old mice were used in all experiments.

**Induction of urinary tract infection.**

*Escherichia coli* 1677, isolated from a patient with an acute febrile urinary tract infection, was a generous donation from Dr W.J. Hopkins (University of Wisconsin Medical School, Madison, WI). This strain has been used to induce pyelonephritis in mice previously, and has virulence characteristics that include type 1 and P fimbriae, hemolysin, aerobactin, and the O6 serotype [19-21]. Bacteria were cultured for 12 h at 37°C in 5% CO₂ in trypticase soy broth. This suspension was diluted 1:100 in fresh medium and grown for 4 h to midlogarithmic phase. *E. coli* were harvested by centrifugation at 1500 g for 10 min. and washed three times in sterile 0.9% saline. Bacteria were resuspended in saline at a concentration of $10^{10}$ CFU/ml as determined by plating 10-fold serial dilutions of the suspensions on blood agar plates. To confirm that none of the mice had UTI prior to the experiments, urine was collected by applying pressure in the abdominal area and cultured on blood agar plates. For inoculation with *E. coli*, mice were anesthetized by administration of FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H₂O; of this mixture 7.0 ml/kg intraperitoneally). To minimize reflux of the inoculum, the bladder was emptied prior to infection by applying pressure to the abdominal area. For instillation of the bacteria a radiopaque catheter with a diameter of 0.55 mm (Abbott B.V. Amstelveen, Netherlands) was introduced into the bladder, and 100 μl of bacterial suspension ($10^9$ CFU) was inoculated. Two hours before inoculation, mice received either goat anti-mouse CXCR2 antiserum [22-24] or normal goat serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands), both intraperitoneally in a volume of 500 μl.
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Antiserum directed against murine CXCR2 was produced by immunizing goats with a peptide containing the ligand-binding sequence Met-Gly-Glu-Phe-Lys-Val-Asp-Lys-Phe-Asn-Ile-Glu-Asp-Phe-Phe-Ser-Gly. This protein sequence has been shown to contain the ligand-binding portion of the CXCR2 receptor [25], and has been used previously to block mouse CXCR2 in vivo [22-24].

Histologic examination.

Kidneys were harvested at 6, 24 and 48 h after infection, fixed in 4% formalin and embedded in paraffin. 4 µm sections were stained with hematoxylin and eosin, and analyzed by a pathologist who was blinded for groups. The degree of inflammation over the total area of each section was graded on a semi-quantitative scale of 0 to 5+ [19].

Determination of bacterial outgrowth.

At 6, 24 and 48 h after infection, urine was collected as described above. Mice were anesthetized by FFM, and sacrificed by cervical dislocation. Blood was collected in tubes containing EDTA. From each mouse the left kidney and part of the right kidney were homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK), that was carefully cleaned and desinfected with 70% alcohol after each homogenization. Serial 10-fold dilutions were made in sterile saline and 15 µl volumes of urine, blood, and homogenized kidneys were plated onto blood agar plates which were incubated at 37°C at 5% CO2. CFUs were counted after 16 h. The remaining part of the right kidney was used for histologic examination.

Preparation of kidney tissue for chemokine measurements.

Kidney homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed.
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Assays.

MIP-2 and KC levels were measured by ELISA according to the instructions of the manufacturer. All reagents were obtained from R&D Systems, Abingdon, United Kingdom. Detection limits were: 20 pg/ml (MIP-2) and 25 pg/ml (KC).

Statistical analysis.

Data are expressed as mean ± SE unless stated otherwise. Differences between groups were analyzed by Mann-Whitney U test. The presence of leukocytes in urine was analyzed in time by oneway analysis of variance (ANOVA), followed by Dunnett’s test. P < 0.05 was considered to represent a statistically significant difference.

Results

Defective neutrophil recruitment in anti-CXCR2 treated mice.

None of the mice had leukocyturia prior to the experiment. To determine the role of CXCR2 in the recruitment of neutrophils to the urinary tract during UTI, mice pretreated with either anti-CXCR2 or preimmune serum were inoculated intravesically with *E. coli* via the urethra. In control mice, UTI with *E. coli* resulted in a transient increase in the number of granulocytes in urine, peaking at 6 h (380 ± 170 cells/mm³; P < .05) (Figure 1). Thereafter, neutrophil numbers decreased, and at 48 h after infection very few neutrophils remained in the urine. In sharp contrast, mice treated with anti-CXCR2 demonstrated no influx of neutrophils in urine at any time point after intravesical inoculation with *E. coli* (P < .05 vs mice treated with preimmune serum at t=6 and 24 h). Histopathologic examination of kidneys revealed a profound reduction in neutrophil recruitment to the site of the infection in mice treated with anti-CXCR2 (Figure 2). Six hours after *E. coli* inoculation, 30% of control mice and 40% of anti-CXCR2 treated mice showed multifocal abscesses localized in the medulla but also in the cortex (grade 3-4). However the type of inflammatory reaction was strikingly different in both groups. Indeed, abscesses in control mice were predominantly composed of neutrophils
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**Figure 1.** Anti-CXCR2 treatment inhibits the influx of neutrophils to urine during experimental UTI. UTI was induced by intravesical administration of *E. coli* at t=0; 2 hours before inoculation mice received an intraperitoneal injection of either anti-mouse CXCR2 antiserum (black bars) or preimmune serum (open bars). Data are mean ± SE. * P < .05 versus anti-CXCR2.

with few lymphocytes and little tubular necrosis. In contrast, mice treated with anti-CXCR2 presented large necrotic areas with almost no influx of neutrophils. These findings were even more evident 24 hours after

**Figure 2.** Anti-CXCR2 treatment results in the absence of neutrophilic infiltrates in infected kidneys. At 24 hours after *E.coli* inoculation, control mice present small abscesses predominantly composed of granulocytes in renal parenchyma (A, arrow; x40). Anti-CXCR2 injected mice develop large necrotic areas in the renal cortex with little inflammatory infiltrates (B, arrow; x40).
pyelonephritis. Two days after inoculation, no signs of pyelonephritis could be observed in the majority of mice (70% in both groups).

Enhanced bacterial outgrowth in anti-CXCR2 treated mice.

Prior to induction of UTI, all mice had negative urine cultures. To determine the role of CXCR2 in antibacterial host defense during UTI, the bacterial outgrowth in urine, kidneys and blood of mice with blocked and functioning CXCR2 was compared at 6, 24 and 48 hours after intravesical inoculation with *E. coli* (Figure 3). At 6 hours postinfection, mice treated with anti-CXCR2 or preimmune serum had similar bacterial counts in urine and kidneys. However, at 24 hours, mice pretreated with anti-CXCR2 had significantly more *E. coli* CFUs in urine and kidneys than mice injected with control serum (Figure 3). Anti-CXCR2 treatment is associated with a transient increase in bacterial loads in urine and kidneys. UTI was induced by intravesical administration of *E. coli* at t=0; 2 hours before inoculation mice received an intraperitoneal injection of either anti-mouse CXCR2 antiserum (black dots) or preimmune serum (open dots). Horizontal lines represent medians. N.S. = nonsignificant.
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with control antiserum (both P < .05). The relatively impaired clearance of *E. coli* from the urinary tract of anti-CXCR2 treated mice was temporary, i.e. at 48 hours the recovery of *E. coli* from urine and kidneys of mice from both treatment groups was similar again. Blood cultures were positive for *E. coli* in 20 percent of mice treated with anti-CXCR2 and 25 percent of control mice at 6 h postinfection. At 24 h, these numbers were 10 and 8 percent, respectively, while at 48 h post inoculation none of the mice had bacteremia in either group.

*Release of the CXCR2 ligands MIP-2 and KC.*

In order to further assess the role of CXCR2 in the pathogenesis of UTI, we determined local and systemic levels of the CXCR2 ligands MIP-2 and KC. UTI with *E. coli* elicited increases in MIP-2 and KC concentrations in kidney

![Graphs showing MIP-2 and KC concentrations in kidney and plasma](image)

**Figure 4.** MIP-2 and KC concentrations in kidneys and plasma during experimental UTI. UTI was induced by intravesical administration of *E. coli* at t=0; 2 hours before inoculation mice received an intraperitoneal injection of either antiamouse CXCR2 antiserum (black bars) or preimmune serum (open bars). Data are mean ± SE. *P < .05 versus anti-CXCR2.
and blood, peaking 6-24 h after the infection (all $P < .05$ vs 0 h, except for plasma MIP-2, nonsignificant) (Figure 4). Anti-CXCR2 treatment did not influence renal MIP-2 and KC concentrations (Figure 4). In plasma, anti-CXCR2 treatment was associated with higher MIP-2 and KC levels than those measured after treatment with the control antibody (both $P < .05$ at 6 and 24 h for MIP-2 and at 24 h for KC) (Figure 4).

**Discussion**

Neutrophils constitute an important component of early host defense against bacterial infection. UTI results in a rapid appearance of neutrophils in urine, a response that at least in part is mediated by effects of locally produced ELR positive CXC chemokines on specific neutrophil receptors [13, 14, 26]. The present study examined the role of CXCR2, the sole receptor that is responsive to ELR positive CXC chemokines in the mouse, in ascending UTI. Administration of a blocking CXCR2 antibody was found to virtually completely prevent the recruitment of neutrophils to the urinary tract, as reflected by the absence of leukocyturia and the lack of neutrophilic infiltrates in kidneys. The clearance of *E. coli* from the urinary tract was impaired only transiently, with 100- and 1000-fold higher bacterial loads in urine and kidneys respectively at 24 hours, but not at 48 hours after the bacterial inoculation. Furthermore, anti-CXCR2 treatment was not associated with an enhanced dissemination of the infection, considering that blood cultures were positive in a similarly small subset of anti-CXCR2 treated and control mice, and only at early time points. These data suggest that although CXCR2 is essential for the attraction of neutrophils to urinary tract, this chemokine receptor does not have a major impact on host defense during UTI.

The role of neutrophils and chemokines in experimental UTI has been studied in previous investigations. Systemic depletion of neutrophils by administration of a monoclonal antibody specifically targeting neutrophils and eosinophils, was associated with a complete absence of neutrophil influx in urine during murine UTI, and an increased outgrowth of bacteria in kidneys and bladders at 24 hours after the intravesical inoculation with *E. coli*; the numbers of bacteria recovered from the urinary tract at other time points during the infection were not reported in that study [27].
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Treatment of mice with an antibody directed against MIP-2, an important ELR positive CXC chemokine and CXCR2 ligand in the mouse, resulted in a reduced neutrophil influx in urine [14]. Interestingly, in anti-MIP-2 treated mice neutrophil recruitment to kidneys remained intact, with neutrophils accumulating on the tissue side of the pelvic urothelium, which is suggestive for a role of MIP-2 in neutrophil migration across the urothelium into urine [14]. This finding contrasts with our observations in anti-CXCR2 treated mice, which demonstrated a lack of neutrophils accumulation in infected renal tissue, indicating that CXCR2 ligands different from MIP-2 may be involved in influx of neutrophils in renal tissue during UTI. Notably, a similar difference between effects of anti-MIP-2 and anti-CXCR2 antibodies was reported in a mouse model of pulmonary *Nocardi a asteroides* infection, in which blocking of CXCR2 strongly inhibited neutrophil influx in lungs concurrently facilitating bacterial outgrowth, whereas anti-MIP-2 was without effect [22]. Moreover and in accordance, anti-CXCR2 treatment had a much larger negative influence on host defense against pulmonary infections with *Aspergillus fumigatus* or *Pseudomonas aeruginosa* than treatment with anti-MIP-2 [23, 24]. We previously showed that different ELR positive CXC chemokines, i.e. IL-8, GROα and ENA-78, all contribute to the chemotactic activity of urine derived from urosepsis patients on neutrophils, further supporting the notion that multiple CXCR ligands may play a role in host defense against UTI [13]. In murine UTI, anti-MIP-2 treatment did not hamper the clearance of *E. coli* from kidneys [14]. Together, these data suggest that neutrophils that accumulate under the urothelium due to a reduced capacity to cross the epithelial barrier remain able to protect the host against outgrowth of bacteria in the urinary tract [14], whereas an overall reduced recruitment of neutrophils to the kidneys results in a reduced ability to eliminate bacteria [27 and the present study].

While our investigation was in progress, Frendeus et al. reported a study in which UTI was induced in CXCR2 knockout mice [28]. These mice were found to have a strongly reduced influx of neutrophils in urine and to be unable to control local bacterial outgrowth, resulting in bacteremia and symptoms of severe disease. The effect of CXCR2 deficiency on the neutrophil content of renal tissue was not reported. Our results with an anti-CXCR2 antibody differ from those obtained with CXCR2 knockout mice with respect to the impact on the clearance of bacteria from the
urinary tract, i.e. anti-CXCR2 treatment was associated with transiently increased bacterial loads in urine and kidneys, and did not result in dissemination of the infection. It is unlikely that an incomplete blockade of CXCR2 by the antibody explains this discrepancy, considering that anti-CXCR2 virtually completely prevented neutrophil influx in kidneys and urine, and considering that the same antibody used in the same amount also profoundly diminished neutrophil recruitment to the pulmonary compartment during lung infections with different pathogens [22-24]. Knockout mice may differ from wild type mice not only with respect to the product of the deleted gene. Hereditary deficiency of a protein may result in compensatory changes that have little to do with the original defect. Indeed, CXCR2 knockout mice demonstrate lymphadenopathy resulting from an increase in B cells, and splenomegaly, resulting from an increase in metamyelocytes, band and mature neutrophils [18]. The number of neutrophils in peripheral blood of CXCR2 knockout mice is twelve-fold higher than that in normal wild type littermates [18], whereas anti-CXCR2 treatment does not influence peripheral neutrophil counts [22-24]. One cannot exclude that these secondary alterations in CXCR2 deficient mice influence their behavior during experimental UTI. In addition, differences in the E. coli strains used in the study by Frendeus et al. and in the present investigation may have played role in the different effects on bacterial outgrowth. The strain used in the previous investigation, E. coli 1177 of serotype 01:K1:H7, expresses P and type 1 fimbriae, but is hemolysin negative. Our strain was of the O6 serotype, also expresses P and type 1 fimbriae, but is hemolysin positive [19-21]. It should be noted, however, that in normal control mice both strains induced a self-limiting disease with transient leukocyturia with spontaneous clearance of the infection [19-21, 27, 28].

UTI was associated with an elevation of the CXCR2 ligands MIP-2 and KC within the urinary tract, confirming an earlier report [14]. These increases coincided with peak granulocyte influx in urine. Anti-CXCR2 treatment did not influence renal concentrations of MIP-2 and KC. In plasma, MIP-2 and KC concentrations were higher in anti-CXCR2 treated mice. The explanation for this finding is not certain, although elevated plasma levels of a ligand in the absence of its receptor have been described previously [29, 30], suggesting that at least some receptors may exert a negative feedback effect on the production of ligand.
The pathogenesis of UTI is multifactorial. Host factors such as the short female urethra and the proximity of the urethral meatus to the rectum, and bacterial factors such as the presence of fimbriae and the production of hemolysin are considered to contribute significantly to the susceptibility to UTI [31]. We here demonstrate that in the mouse CXCR2 played an essential role in the recruitment of neutrophils to the urinary tract during experimental UTI, and that the absence of neutrophils at the site of the infection only temporarily reduced host resistance. Further studies are warranted to examine the individual contribution of different CXCR2 ligands in the inflammatory response to UTI.
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