CXCL5 Drives Neutrophil Recruitment in T\textsubscript{H}17-Mediated GN


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ABSTRACT

Neutrophil trafficking to sites of inflammation is essential for the defense against bacterial and fungal infections, but also contributes to tissue damage in T\textsubscript{H}17-mediated autoimmunity. This process is regulated by chemokines, which often show an overlapping expression pattern and function in pathogen- and autoimmunity-induced inflammatory reactions. Using a murine model of crescentic GN, we show that the pathogenic T\textsubscript{H}17/IL-17 immune response induces chemokine (C-X-C motif) ligand 5 (CXCL5) expression in kidney tubular cells, which recruits destructive neutrophils that contribute to renal tissue injury. By contrast, CXCL5 was dispensable for neutrophil recruitment and effective bacterial clearance in a murine model of acute bacterial pyelonephritis. In line with these findings, CXCL5 expression was highly upregulated in the kidneys of patients with ANCA-associated crescentic GN as opposed to patients with acute bacterial pyelonephritis. Our data therefore identify CXCL5 as a potential therapeutic target for the restriction of pathogenic neutrophil infiltration in T\textsubscript{H}17-mediated autoimmune diseases while leaving intact the neutrophil function in protective immunity against invading pathogens.


Neutrophils are the most abundant type of leukocytes in the blood and form an indispensable part of the innate immune system. Their trafficking into peripheral tissues is pivotal in the defense against invading bacterial and fungal pathogens.\textsuperscript{1} To ensure that neutrophils reach the sites of tissue injury, their recruitment is regulated by the local expression of chemoattractants, including chemokines. However, the infiltration of neutrophils also significantly contributes to end-organ damage in autoimmune diseases mediated by T helper (T\textsubscript{H}) cell T\textsubscript{H}17,\textsuperscript{2} including human and experimental crescentic GN.\textsuperscript{3,4}

Chemokines are a large family of small (8–12 kD) secreted proteins that are identified as attractants of different types of leukocytes, including neutrophils, to sites of infection and inflammation.\textsuperscript{5} They are produced locally in tissues and act through interaction with specific G protein–coupled receptors that are predominantly expressed on leukocytes. Neutrophil infiltration is mainly mediated by chemokines that have a glutamate-leucine-arginine motif (ELR\textsuperscript{7} chemokines). In humans, there are seven ELR chemokine ligands with a C-X-C motif (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14).

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CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7, which all bind to the murine CXCR2. Interestingly, previous reports show that IL-1β, IL-6, IL-8, IL-17A, the master effector cytokine of T<sub>H</sub>17 cells, induces the expression of the ELR<sup>+</sup> chemokines CXCL1, CXCL2, and CXCL5, and CXCL7, which act via the chemokine receptor CXCR2, and thereby might drive the recruitment of pathogenic neutrophils in autoimmunity. The development of a therapeutic strategy targeting ELR<sup>+</sup> neutrophil-attracting chemokines or their receptors is complicated by an often overlapping expression pattern and function of these molecules in pathogen- and autoimmune-induced inflammatory reactions.

Here we describe for the first time a nonredundant function of the chemokines CXCL1 and CXCL5 in murine models of crescentic GN and acute bacterial pyelonephritis. CXCL1 mediated early glomerular neutrophil recruitment in the non–T cell–dependent initiation phase of GN, whereas CXCL5 was responsible for the infiltration of pathogenic neutrophils into sites of inflammation in later T<sub>H</sub>17–dependent phases of the disease. Of note, CXCL5 did not affect neutrophil infiltration and bacterial clearance in a murine model of acute bacterial pyelonephritis, one of the most prevalent kidney infections in humans. These findings suggest that CXCL5 has a unique function in the trafficking of neutrophils in T<sub>H</sub>17 cell–mediated autoimmunity, but not in the innate immune response. CXCL5 therefore represents an attractive therapeutic target for the restriction of pathogenic neutrophil infiltration in T<sub>H</sub>17-driven autoimmune diseases without affecting the vital functions of neutrophils in the defense against acute bacterial infections.

RESULTS

Time- and Compartment-Specific Infiltration of Neutrophils in Murine Crescentic GN

Nephrototoxic nephritis (NTN) is a well-characterized model of murine crescentic GN, which is induced by the injection of sheep antiserum raised against kidney cortical components. During the early heterologous phase of the disease, the deposited antibodies result in glomerular complement activation and neutrophil recruitment, which cause substantial glomerular injury and renal dysfunction. An adaptive immune response against the foreign sheep protein develops in the subsequent autologous phase (starting from days 3 to 5), resulting in the activation of nephritogenic CD<sup>+</sup> T<sub>H</sub>17 and T<sub>H</sub>1 cells in lymphatic organs. First, T<sub>H</sub>17 cells and, subsequently, T<sub>H</sub>1 cells migrate into the kidney and promote renal tissue injury. The role of neutrophils in the T cell–mediated phase (starting from day 5) is largely unknown. We therefore assessed the time course of renal neutrophil infiltration using immunohistochemical staining for the neutrophil marker Gr1 (Ly6C/Ly6G) (Figure 1A). In the early stage of nephritis (until day 3), neutrophils were mainly found in the glomerulus (Figure 1, A and B). The infiltration of neutrophils into the tubulointerstitial area started at day 5, peaked around day 10, and then declined (Figure 1, A and B). This demonstrates a previously unknown time- and compartment-specific recruitment of neutrophils into the kidney.

CXCL1 and CXCL5 Have Unique Functions in the Recruitment of Neutrophils in Crescentic GN

One important prerequisite for the infiltration of neutrophils into sites of inflammation is the expression of ELR<sup>+</sup> chemokines, namely CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7, which act via the chemokine receptor CXCR2. Quantitative RT-PCR analysis of the renal cortex revealed that CXCL1, CXCL2, and CXCL3 mRNA expression was strongly upregulated in the early stages of nephritis (12 and 24 hours) (Figure 1C). Renal CXCL7 expression was low throughout the disease course (data not shown). CXCL1 expression rapidly declined to the baseline level by day 3. By contrast, CXCL5 mRNA expression was not increased at early time points but markedly increased from day 5 onward, with maximum expression levels at day 10 (detectable after approximately 25 PCR cycles). Chemokines can be modified post-translationally by proteolytic cleavage in order to achieve the active form and stored intracellular, therefore, a discrepancy between local mRNA level and protein level/activity might occur. However, our attempts to quantify renal CXCL5 protein levels in nephritic mice by ELISA failed because of high background signals when using CXCL5 knockout (KO) mice as negative controls (data not shown).

Renal mRNA expression of CXCR2 was characterized by a bimodal course reaching two maxima after 24 hours and 10 days (Figure 1C). In line with this, glomerular and tubulointerstitial neutrophil recruitment was reduced in nephritic CXCR2<sup>−/−</sup> mice at days 3 and 14 (Figure 1D). This showed that CXCR2 drives both the “early” and “late” waves of neutrophil recruitment.

To elucidate whether the expression patterns of CXCL1 and CXCL5 might result in different functions of these chemokines in the disease course, we performed early (until day 3) and late (days 9 to 14) in vivo neutralization with specific anti-CXCL1 or anti-CXCL5<sup>14</sup> antibodies. In line with their temporal expression profile, neutralization of CXCL1 resulted in ameliorated glomerular neutrophil infiltration at day 3, whereas anti-CXCL5 antibody treatment at day 9 resulted in significantly reduced tubulointerstitial neutrophil recruitment at day 14 (Figure 1E).

Accordingly, renal FACS analysis revealed a reduction in CD4<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in nephritic CXCL5<sup>−/−</sup> mice at day 14 (Figure 1, F and G), but not at day 3 (Supplemental Figure 1A). Likewise, tubulointerstitial neutrophil accumulation was significantly decreased in nephritic CXCL5<sup>−/−</sup> mice at day 14 (Figure 1H), but not at day 3 (Supplemental Figure 1B). Immunofluorescence staining revealed that neutrophils in nephritic CXCL5<sup>−/−</sup> mice had a reduced capacity to exit...
the peritubular capillaries and to enter the tubulointerstitial space (Figure 1I).

By comparison, the infiltration of T cells and macrophages/dendritic cells was not significantly affected by CXCL5 deficiency or anti-CXCL5 treatment (Supplemental Figure 2, A–H, J). Furthermore, neutrophil, monocyte, CD4\(^+\), and CD8\(^+\) T cell abundance in the blood, spleen, and kidney under homeostatic and nephritic conditions was not significant different between wild-type (WT) and CXCL5\(^{-/-}\) mice (Supplemental Figure 3). In addition, kidney morphology and function were not affected by CXCL5 deficiency under basal conditions (Supplemental Figure 4). This indicates that reduced neutrophil abundance in the kidney of nephritic CXCL5\(^{-/-}\) animals was not a consequence of a developmental defect in the hematopoietic system or the kidney, but rather represents reduced recruitment to the inflamed tissue in CXCL5\(^{-/-}\) mice.

To analyze the role of CXCL1 in the early infiltration of neutrophils into the kidney using a second, independent approach, we induced NTN in WT and CXCL1-deficient mice. In accordance with our anti-CXCL1 neutralization data, neutrophil recruitment was reduced in nephritic CXCL1\(^{-/-}\) mice at day 3, thus underscoring the importance of this chemokine in the early course of nephritis (Figure 1, J and K).

**Figure 1.** CXCL1 and CXCL5 have unique functions in the time- and compartment-specific infiltration of neutrophils in crescentic GN. (A) Immunohistochemistry of kidney sections stained for the neutrophil marker GR1 at indicated time points after nephritis induction. Inserts demonstrate the polymorphonuclear morphology of GR1\(^+\) cells (neutrophils). (B) Quantification of tubulointerstitial (per low-power field [lpf]) and glomerular (per glomerular cross-section [gcs]) PMN infiltration in the course of nephritis \((n=3–6\) per time point). (C) RT-PCR analysis of renal CXCL1, CXCL2, CXCL3, CXCL5, and CXCR2 mRNA expression \((n=4–6\) per time point). (D) Quantification of tubulointerstitial and glomerular PMNs in nephritic WT and CXCR2\(^{-/-}\) mice at day 3 or day 14 after induction of the disease \((n=3–5\) per time point). (E) Quantification of tubulointerstitial and glomerular PMNs in nephritic WT mice at day 3 or 14 of nephritis after administration of anti-CXCL1, anti-CXCL5, or isotype rat-lgG2B antibodies \((n=3–5\) per time point). (F) FACS analysis of renal leukocytes from nephritic WT and CXCL5\(^{-/-}\) mice stained for the PMN marker Ly6G at day 14. Plots are representative of three independent experiments. (G and H) Quantification of neutrophils from FACS analysis (G) and quantification of tubulointerstitial PMNs from GR1 stained kidney sections (H) in nephritic WT and CXCL5\(^{-/-}\) mice 14 days after nephritis induction. (I) Immunofluorescence staining of nephritic kidney section (day 14) of WT and CXCL5\(^{-/-}\) mice using anti-CD31 (green/endothelial cells), anti-Ly6G (red/PMNs), and collagen type (blue/basement membrane). (J and K) Quantification of renal neutrophils by FACS analysis (J) and quantification of glomerular PMNs from GR1 stained kidney sections (K) in nephritic WT and CXCL1\(^{-/-}\) mice 3 days after nephritis induction. Symbols/bars represent means±SDs. *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\). Original magnification, ×200. Con, control.
Kidney Tubular Cells Produce CXCL5 in Late Crescentic GN

To localize renal CXCL5 expression, we performed in situ hybridization experiments. CXCL5 expression was clearly detectable in tubular cells 14 days after nephritis induction, but not at day 3 or in control mice (Figure 2A).

Replacement of the CXCL5 gene by a β-galactosidase (β-Gal) gene in CXCL5-deficient mice enabled us to indirectly assess the renal CXCL5 protein expression by β-Gal detection. Nuclear β-Gal staining was observed exclusively in tissues collected from nephritic CXCL5<sup>−/−</sup> mice (Figure 2B). In line with the CXCL5 mRNA expression pattern, β-Gal–positive cells were detected in the tubular epithelium of nephritic mice (Figure 2B).

Quantitative RT-PCR analysis of glomerular and tubulointerstitial tissue samples isolated from nephritic mice using laser microdissection at days 3 and 14 after induction of NTN revealed that CXCL5 mRNA expression was predominantly upregulated at day 14 in the tubulointerstitial compartment, as indicated by the tubulointerstitial to glomerular expression ratio (Figure 2C). CXCL1 mRNA was mainly expressed in glomeruli at day 3, whereas glomerular and tubulointerstitial expression were at control levels at day 14 (Figure 2C).

To study whether CXCL5 is mainly produced by renal tissue cells, we generated bone marrow (BM) chimeras using WT and CXCL5<sup>−/−</sup> mice as donors (Figure 2D). Independent of the BM transplant used, 14 days after induction of nephritis, high CXCL5 mRNA expression levels were only present in animals with WT renal tissue cells. An approximately 500-fold increase in CXCL5 mRNA expression was found in both WT recipient mice transplanted with WT BM or CXCL5<sup>−/−</sup> BM compared with control mice. By contrast, CXCL5 mRNA expression in CXCL5<sup>−/−</sup> mice receiving WT BM or CXCL5<sup>−/−</sup> BM was not elevated (Figure 2D). As control, we used WT mice carrying the CD45.2 allele as recipients. Six weeks after transplantation, >90% of blood leukocytes, >98% of BM leukocytes, and >90% of splenic leukocytes expressed the phenotypic marker of the transplanted BM (n=6).

The TH17/IL-17 Immune Response Mediates Renal CXCL5 Expression in Crescentic GN

The striking temporal association between CXCL5 expression, tubulointerstitial neutrophil infiltration (Figure 1, A–H), and the recruitment of CD4<sup>+</sup> T<sub>h</sub>17 cells into the kidney at day 10 (Figure 3A, Supplemental Figure 5) suggest a functional relationship. To determine whether IL-17A drives CXCL5 expression in vivo, we induced nephritis in IL-17A<sup>−/−</sup> mice. WT animals showed strong upregulation of CXCL5 mRNA at day 10 after nephritis induction, whereas IL-17A<sup>−/−</sup> mice displayed reduced upregulation (WT versus IL-17A<sup>−/−</sup>, P<0.01) (Figure 3B). By contrast, CXCL1 and CXCL2
expression was not significantly altered in nephritic IL-17A−/− mice (Figure 3B).

IL-17A and TNF-α Synergistically Induce CXCL5 Expression in Mouse Tubular Cells

Given the high renal expression of CXCL5, predominantly in tubular cells (Figure 2, A and B), we investigated whether IL-17A drives CXCL5 expression in kidney tubular cells. Because the biologic effects of IL-17A are mediated by activation of the IL-17 receptors A and C, we demonstrated the presence of these receptors in mouse tubular cells by RT-PCR (Supplemental Figure 6A). Next, we demonstrated that IL-17A induces CXCL5 mRNA and protein production in tubular cells in a dose-dependent manner (Supplemental Figure 6B), whereas IL-17F and IFN-γ had no effect (Supplemental Figure 6C).

The signal transduction pathway mediated by IL-17A is still incompletely characterized but involves NF-κB and extracellular-signal regulated kinase (ERK) activation. Therefore, it is of interest that the combination of IL-17A and TNF-α synergistically amplified the RNA expression and protein secretion of CXCL5 (Figure 3, C and D), and synergistically induced ERK activation but not NF-κB signaling in tubular cells (Supplemental Figure 6D). Although the in vivo importance of this finding remains to be elucidated, the time kinetic of renal IL-17A (Figure 3A) and TNF-α expression in NTN (Supplemental Figure 6E) is consistent with CXCL5 formation in the inflamed kidney.

Tubulointerstitial Infiltration of CXCR2+ Neutrophils Promotes Renal Tissue Injury

To investigate the functional importance of the observed tubulointerstitial neutrophil recruitment, we depleted neutrophils in nephritic mice from days 9 to 14 by using a Ly6G-specific mAb16 (Supplemental Figure 7). Neutrophil depletion significantly reduced tubulointerstitial tissue injury and to a lesser degree glomerular crescent formation, demonstrating the pathogenic role of neutrophils during the T17 cell–mediated phase of the disease (Figure 4, A and B). Levels of BUN, a marker inversely correlated with renal function, were slightly but not significantly reduced (Figure 4B).

Next, we showed that CXCR2-deficient mice developed less severe nephritis, in terms of renal tissue damage and BUN levels at day 14 (Figure 4C). This is in line with reduced neutrophil infiltration in nephritic CXCR2−/− mice (Figure 1D). The recruitment of CD3+ T cells and mononuclear phagocytes was not significantly affected by CXCR2 deficiency (Supplemental Figure 2I). Mouse anti-sheep globulin–specific IgG, subclass IgG1, subclass IgG2a, and IgG2b titers were unaffected in nephritic CXCR2−/− mice compared with the WT group (Supplemental Figure 8F).

Targeting of CXCL5 Reduces Renal Tissue Damage in the T Cell–Mediated Stage of Crescentic GN

To investigate whether CXCL5 contributes to renal tissue injury, nephritis was induced in CXCL5−/− and WT mice. At day 14, CXCL5−/− mice showed significantly less tubulointerstitial and glomerular injury compared with controls (Figure 4, D and E). In addition, BUN levels were reduced in nephritic CXCL5−/− mice compared with nephritic WT mice at day 14 (P<0.001; Figure 4E). Albuminuria was upregulated in all nephritic groups, but was not significantly different between nephritic WT and CXCL5 KO mice at day 14 (urine albumin/creatinine ratio: WT control [n=6], 0.1±0.1; WT NTN [n=8], 15.8±4.7; CXCL5−/− NTN [n=10], 9.7±8.9).

Treatment of nephritic mice with a neutralizing anti-CXCL5 antibody at day 9 significantly reduced renal tissue injury at day 14 (Figure 4F). The more pronounced protection of nephritic CXCL5−/− mice compared with anti-CXCL5–treated animals, in terms of BUN levels, is likely the result of a complete
absence of CXCL5 in the KO mice and the timely restricted CXCL5 neutralization from days 9 to 14. The beneficial effect of neutrophil and CXCL5 targeting on glomerular injury might reflect reduced renal PMN infiltration.

By contrast, the treatment of nephritic mice with a neutralizing anti-CXCL1 antibody at day 9 (nephritic controls received IgG2a isotype control antibodies) had no effect on the clinical outcome of the disease at day 14 (Figure 4G).

Serum titers of anti-sheep IgG, IgG1, IgG2a/c, and IgG2b antibodies directed against the nephritogenic antigen and semi-quantitative scoring of glomerular sheep and mouse IgG deposition revealed no differences between nephritic WT and CXCL5−/− mice (Supplemental Figure 8, A–E). Moreover, IL-17 and IFN-γ production of sheep IgG-stimulated splenocytes is not impaired in nephritic CXCL5 KO mice compared with nephritic WT mice at day 14 (Supplemental Figure 9), indicating that the absence of CXCL5 did not impair the systemic immune response against the nephritogenic antigen.

CXCL5 Is Dispensable for Neutrophil Recruitment and Bacterial Clearance in a Murine Model of Acute Bacterial Pyelonephritis

We next studied the role of CXCL5 in neutrophil trafficking and function in a well established model of acute bacterial pyelo-

Figure 4. The CXCL5/CXCR2 axis promotes renal tissue injury. (A) Representative photographs of PAS-stained kidney sections of mice ± neutrophil depletion (from days 9 to 14) at day 14 after induction of nephritis. (B) Quantification of glomerular crescent formation, tubulointerstitial damage, and BUN levels at day 14. (C) Quantification of renal tissue damage and renal function assessed by BUN levels at day 14 in WT and CXCR2−/− mice. (D) Representative photographs (PAS staining) of the tubulointerstitial compartment of control, nephritic WT, and nephritic CXCL5−/− mice at day 14. (E) Quantification of glomerular crescent formation, tubulointerstitial damage, BUN levels, and renal neutrophil recruitment 14 days after disease induction. (F and G) Quantification of glomerular crescent formation, tubulointerstitial damage, BUN levels, and neutrophil infiltration in animals treated with anti-CXCL5 (or IgG2B isotype antibody) (F) and anti-CXCL1 (or IgG2A isotype antibody) (G) 14 days after disease induction. Bars represent means ±SDs. *P<0.05; **P<0.01; ***P<0.001. Original magnification, ×200 in A; ×400 in D. Con, control; PAS, periodic acid–Schiff.
nephritis. This model is induced by transurethral instillation of the uropathogenic *Escherichia coli* strain 536 (UPEC), which invades the kidney and is subsequently cleared by recruited neutrophils. A massive infiltration of neutrophils into the kidney (assessed by immunohistochemistry and flow cytometry) was detected in WT mice 24 hours after UPEC infection (Figure 5, A and B). Of note, neutrophil recruitment was not affected by CXCL5 deficiency, whereas neutrophils were strongly reduced in infected CXCR2-deficient mice. In line with the pivotal role of neutrophils in bacterial clearance, the numbers of CFUs in the kidneys were significantly higher in CXCR2−/− mice compared with WT and CXCL5−/− animals (Figure 5C). Renal expression of CXCL1, and to a lesser degree of CXCL2, was markedly enhanced, whereas CXCL5 was only slightly upregulated 24 hours after infection (Figure 5D).

**Renal CXCL5 Expression in Patients with ANCA-Associated GN and Acute Bacterial Pyelonephritis**

We isolated RNA from renal biopsies and performed real-time PCR to analyze the renal expression of neutrophil-attracting chemokines in patients with acute ANCA-associated GN, the most common form of crescentic GN in humans, and in patients with acute bacterial pyelonephritis. Although neutrophil recruitment is common to ANCA-associated GN but even stronger in acute pyelonephritis (Figure 6A), upregulation of renal tissue CXCL5 expression was more profound in ANCA-associated GN (1013-fold; Figure 6B) compared with acute pyelonephritis (16-fold; Figure 6B). Similar results were obtained for CXCL6, which is a close homolog to human CXCL5. These results suggest a predominant role of CXCL5 in human autoimmune disease, but not in acute bacterial infection. Table 1 provides the clinical characteristics of the included patients.

**DISCUSSION**

In recent years, a large number of studies have established the crucial role of Th17 cells in the pathogenesis of autoimmune diseases, including proliferative and crescentic GN. Consequently, the findings from animal models have been translated into clinical practice, and IL-17A and related cytokines have been successfully targeted for treatment of psoriasis and Crohn’s disease.

However, the mechanisms by which the Th17 immunity directly contributes to end-organ damage still remain to be
fully elucidated. T_{H}17 cells produce a variety of proinflammatory cytokines, including IL-17A, IL-17F, IL-22, TNF-α, and GM-CSF, which drive the recruitment of neutrophils to sites of inflammation and thereby promote tissue injury by mechanisms that are thus far incompletely understood. Recent studies indicate that ELR⁺ chemokines play a pivotal role in this process and might therefore represent an attractive target for the prevention of neutrophil-induced tissue injury.25–27 This approach is complicated by an often overlapping/redundant expression pattern and function of chemokines in autoimmune and infectious disease.5 Targeting of chemokines in dant expression pattern and function of chemokines in auto-

It is generally thought that IL-17A, the major effector cytokine of the T_{H}17 immune response, induces the expression of all neutrophil-attracting ELR⁺ chemokines in a redundant manner.25,26,33 In murine crescentic GN, however, we unexpectedly found that the expression of CXCL1 and CXCL2 was upregulated until day 3 and then declined even before infiltration of the first T_{H}17 cells. By striking contrast, CXCL5 expression peaked 10 days after induction of the disease and was predominantly expressed by resident tubular cells when the renal T_{H}17 immune response had reached its maximum level. In line with this, IL-17A resulted in a strong upregulation of CXCL5 mRNA and protein expression in murine tubular epithelial cells in vivo, which is further enhanced in the presence of TNF-α, potentially as a consequence of synergistic activation of the ERK signaling pathway. This finding is in accordance with a recent report by Liu et al. showing that CXCL5 mRNA is induced by IL-17A in alveolar epithelial cells.26 To further support our hypothesis that IL-17A (produced by renal T_{H}17 cells) is directly involved in the induction of CXCL5 expression in tubular cells in vivo, the generation of mice that are deficient in the IL-17 receptors A and C or in the IL-17 receptor adaptor protein connection to IkB kinase and stress-activated protein kinases,34 specifically in renal tubular cells, would be of great interest.

IL-17A promoted renal CXCL5 expression and neutrophil infiltration in NTN, whereas CXCL1 and CXCL2 expression was IL-17A independent. These data further point to non-redundant roles of CXCR2 ligands in the attraction of neutrophils to the kidney, most likely resulting from the temporal and spatial segregation of their expression. CXCL1 and CXCL5 in vivo neutralization experiments indeed confirmed that early glomerular neutrophil migration is mainly driven by CXCL1, whereas the second wave of interstitial neutrophil infiltration is strongly dependent on IL-17A–induced CXCL5. Our finding is consistent with a recent study in a model of moderate airway inflammation in CXCL5−/− mice showing that neutrophil infiltration into the inflamed tissue was dependent on CXCL5.25 In contrast with a study by Mei et al. that indicates that enterocyte-derived CXCL5 in the gut regulates local IL-17A levels and contributes to CXCR2-dependent neutrophil homeostasis,35 our study did not show any

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Clinical characteristics of patients with acute ANCA-associated GN (patients 1–8) and acute pyelonephritis (patients 9–14) at the time of renal biopsy. pANCA, perinuclear ANCA; cANCA, cytoplasmic ANCA; PR3, proteinase 3; MPO, myeloperoxidase; CyP, cyionate.
indication of a role for CXCL5 in the kidney under noninflammatory conditions. The time-dependent role of CXCL5 in GN is further emphasized by the finding that CXCL5−/− mice and anti-CXCL5–treated WT animals displayed an ameliorated disease course at day 14, but not at day 3.

To determine whether the blockade of CXCL5 and CXCR2 generally interferes with neutrophil infiltration, we induced acute bacterial pyelonephritis in WT, CXCL5−/−, and CXCR2−/− mice. In this model, the antibacterial defense relies on the rapid recruitment of neutrophils. As reported, neutrophil trafficking and bacterial clearance critically depend on CXCR2, which is most likely activated by locally expressed CXCL1 and CXCL2,17,36–39 making CXCR2 an unfavorable candidate for therapeutic targeting in autoimmunity. By contrast, CXCL5 deficiency did not affect early neutrophil infiltration and bacterial clearance in this model of acute bacterial pyelonephritis.

A recent study in a murine model of ANCA-associated GN demonstrated that IL-17A–producing T cell–derived IL-17A effector cells directly induce renal inflammation by effector responses involving neutrophils.40 Moreover, the first studies in human ANCA-associated GN suggest a prominent role for T cell IL-17 responses in this form of crescentic GN.4,41 To examine whether our findings are relevant to human disease, we assessed the renal CXCL5 mRNA expression in patients with acute ANCA-associated GN and acute bacterial pyelonephritis, which are both characterized by neutrophil recruitment. In line with the results from our animal models, CXCL5 expression was much higher in renal biopsy specimens from patients with autoimmune-mediated ANCA-associated GN compared with those with acute bacterial pyelonephritis. Interestingly, van der Veen et al. recently reported an increased renal CXCL5 expression in a murine model of anti-myeloperoxidase IgG/LPS-induced crescentic GN, further supporting the role of CXCL5 in autoimmune-mediated ANCA disease.42 The similar expression pattern of CXCL1, CXCL2, and CXCL5 during the course of the disease (assessed at two time points) might be a consequence of the coapplication of LPS in this model.

In conclusion, our study provides the first evidence for a previously unknown nonredundant effector mechanism of the T cell–derived IL-17A–, CXCL5–, and CXCR2–bearing neutrophils that promote renal tissue injury. By contrast, CXCL5 seems to be dispensable for maintaining neutrophil-mediated innate immune surveillance. However, the potential renal functional benefit of an intervention to target CXCL5 in established crescentic GN remains to be fully elucidated.

**CONCISE METHODS**

**Animals**

CXCR2−/− and IFN-γ−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17A–deficient mice were provided by Toichiro Iwakura (University of Tokyo, Tokyo, Japan), CXCL5–deficient mice were provided by the Max Plank Institute for Infection Biology (Berlin, Germany) and CXCL1–deficient mice were provided by S. Lira (Mount Sinai Hospital, New York, NY). All KO mice were backcrossed to the C57BL/6 background for at least 10 generations. All mice were raised under specific pathogen-free conditions. Animal experiments were performed according to national animal care and ethical guidelines, and were approved by local ethical committees.

**Induction of NTN and Functional Studies**

NTN was induced in male mice aged 8 to 10 weeks by intraperitoneal injection of 0.6 ml of nephrototoxic sheep serum per mouse, as previously described.39 Controls were injected intraperitoneally with an equal amount of nonspecific sheep IgG. Urinary albumin excretion was determined by standard ELISA analysis (Mice-Albumin Kit; Bethyl, Montgomery, TX), whereas urinary creatinine and serum BUN were measured using standard laboratory methods. For neutralization/depletion experiments, anti–Ly6G-Ab (clone IA8),16 anti–CXCL1 Ab (clone 48415; R&D Systems, Minneapolis, MN),14 or anti-CXCL5 Ab (clone 61905; Leinco Technologies, St. Louis, MO)14 was used at 100 μg per mouse (intraperitoneally). Nephritic control mice in interventional studies received 100 μg rat IgG2A (clone 54447; R&D Systems) or rat IgG2B (clone 61905; Leinco Technologies) antibodies intraperitoneally. Anti-CXCL1 and anti-CXCL5 antibodies were given either the day before NTN induction in the case of early neutralization studies (analysis was performed at day 3) or at day 9 in the late stages of the interventional experiments (analysis was performed at day 14).

**Acute Bacterial Pyelonephritis Model**

Female mice aged 8 to 12 weeks were infected by transurethral instillation of 1×10⁹ UPEC. Three hours later, the procedure was repeated to induce pyelonephritis. Twenty-four hours after infection, the number of ascended bacteria was quantified by scoring CFUs after overnight culture of kidney collagenase digest as previously described.17

**Real-Time RT-PCR Analyses**

Total RNA of the renal cortex was prepared according to standard laboratory methods. RNA from microdissected tissues was isolated using the RNA Nano prep kit (PALM, Bernried, Germany) and CXCL1–deficient mice were purchased by the Max Plank Institute for Infection Biology (Berlin, Germany) and CXCL1–deficient mice were provided by S. Lira (Mount Sinai Hospital, New York, NY). All KO mice were backcrossed to the C57BL/6 background for at least 10 generations. All mice were raised under specific pathogen-free conditions. Animal experiments were performed according to national animal care and ethical guidelines, and were approved by local ethical committees.

**In Situ Hybridization**

In situ hybridization was performed as described.45 In brief, the CXCL5 cRNA probe was labeled with α[³⁵S]UTP (1250 Ci/mmol; PerkinElmer) of subcloned cDNA corresponding to nucleotides 208–532 of cDNA sequence NM_009141.2. In situ hybridization was performed on 16-μm cryosections using the ³⁵S-labeled antisense and sense RNA probes. Sections were dipped into Kodak NTB nuclear track emulsion and exposed for 3 weeks; after development, sections were stained with Mayer’s hemalun.

**Morphologic Analyses**

Glomerular crescent formation was assessed in 30 glomeruli per mouse in a blinded fashion in paraffin sections stained with periodic acid–Schiff.
acid–Schiff.\textsuperscript{45} To assess tubulointerstitial injury, photographs of four nonoverlapping cortical areas from kidney sections stained with periodic acid–Schiff were taken per mouse and counted in low-magnification fields (×200). The interstitial area was then determined by superimposing the photographs with a grid containing 40 points and by counting the matches of points with interstitial tissue (excluding glomeruli, blood vessels, and tubules) in a blinded fashion. Dividing the positive matches of four photographs by 160 provided the percentage of positive tissue equal to the interstitial injury score.\textsuperscript{11,45}

For immunohistochemical stainings, paraffin-embedded sections were deparaffinized, rehydrated, and stained with the following antibodies: GR-1 (1:50, Ly6 G/C, NIMP-R14; Hycult Biotech, Uden, The Netherlands), CD3 (1:1000, A0452; Dako, Germany), F4/80 (1:400, BM8; BMA, Germany), MAC-2 (1:1000, M3/38; Cedarlane, ON, Canada), sheep IgG/mouse IgG (both Jackson Immunoresearch Laboratories), and proteinase 3 to detect PMN in humans. Antigen retrieval was performed by incubation with proteinase type XXIV (5 mg/ml; Sigma-Aldrich, St. Louis, MO) for 15 minutes at 37°C (F4/80, GR1, sheep IgG/mouse IgG) or by microwave antigen retrieval in citrate buffer (DAKO 52367), pH 6.1, for 25 minutes (CD3, MAC-2). Nuclear β-Gal was revealed after protease-mediated antigen retrieval using a polyclonal antibody (Europa Bioproducts, Cambridge, UK). Slides were counterstained with wheat germ agglutinin (Vector, Auckland, New Zealand) and daq 5 (Molecular Probes, Eugene, OR). Tissue sections were developed with the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA). Glomerular GR1\textsuperscript{+}, MAC-2\textsuperscript{+}, and CD3\textsuperscript{+} cells in 30 glomerular cross-sections and tubulointerstitial F4/80\textsuperscript{+} and CD3\textsuperscript{+} cells in 30 high-power fields (×400) per kidney were counted in a blinded manner. To quantify tubulointerstitial GR1\textsuperscript{+} cells, at least 20 low-power fields (×200) were counted. All slides were evaluated under an Axioskop light microscopy (Zeiss, Jena, Germany) and photographed with an Axioscam HRc (Zeiss) or by confocal microscopy with a LSM 510 meta microscope using the LSM Jena, Germany) and photographed with an Axiocam HRc (Zeiss) or by confocal microscopy with a LSM 510 meta microscope using the LSM Jena, Germany).

Culture of Mouse Kidney Epithelial Tubular Cells and Stimulation
Mouse kidney tubular cells\textsuperscript{47} were cultured in DMEM with 3% FCS (Gibco, Eggenstein, Germany) and stimulated with varying concentrations of IL-17A, IL-17F, TNF-α, and IFN-γ, as indicated (all from Pepro Tech, Hamburg, Germany). CXCL5 mRNA expression levels were analyzed after 4 hours of incubation. Protein levels were determined after 8 and 24 hours in the supernatants by specific CXCL5 ELISA (R&D Systems). Antibodies to detect NF-κB p65 (D14E12) and phospho-NF-κB p65 (93H1) as well as ERK and phospho-ERK (D13.14.4E) in immunoblots were from Cell Signaling Technology (Beverly, MA).

BM Transplantation
Male CXCL5\textsuperscript{−/−} or WT mice aged 5 to 6 weeks received 9.5 Gy total body irradiation. Each recipient mouse (WT or CXCL5\textsuperscript{−/−}) was intravenously injected with 5×10\textsuperscript{6} BM cells (WT or CXCL5\textsuperscript{−/−}) within 6 hours of irradiation.\textsuperscript{48} The efficiency of BM replacement was assessed using congenic CD45 mouse strains (CD45.1 and CD45.2). After irradiation and transplantation, >90% of circulating leukocytes, >98% of BM leukocytes, and >90% of splenic leukocytes expressed the phenotypic marker of the transplanted BM (n=6; data not shown). NTN was induced 4 weeks after BM transplantation.

RNA Isolation and RT-PCR Analysis in Human Patients
RNA was isolated with the RNA Micro Kit (Roche) from paraffin-embedded renal specimens of patients with ANCA-associated GN or acute bacterial pyelonephritis. Chemokine expression was analyzed by RT-PCR. Baseline kidney allograft biopsies before transplantation (without significant pathology) served as controls. All samples were run in duplicate and were normalized to 18S ribosomal RNA to account for small RNA and cDNA variability. Human analysis was approved by the local ethics committee (PV3162).

Statistical Analyses
Data were expressed as the means±SDs. All statistical analyses were performed with the SPSS package and GraphPad Prism 5 software. \textit{P}<0.05 was considered statistically significant. The \textit{t} test was used for comparison between two groups. In the case of three or more groups, one-way ANOVA was used, followed by a post hoc analysis with Bonferroni’s test for multiple comparisons.

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Supplemental Figure 1: Early neutrophil recruitment in WT and CXCL5−/− mice
(A) FACS-based quantification of PMN from the kidney of nephritic wild-type, nephritic CXCL5−/− and wild-type control mice at day 3 after induction of nephritis. (B) Immunohistochemical quantification of renal PMN infiltration into the tubulointerstitial (per low power field, lpf) and glomerular compartment (per glomerular cross section, gcs) of nephritic WT and CXCL5−/− mice (day 3) using the PMN marker GR1.
Supplemental Figure 2: Renal leukocyte infiltration

Paraffin-embedded sections (2 µm) were stained with the following antibodies: F4/80 (BM8, BMA, Germany), MAC-2 (M3/38; Cedarlane, Ontario, Canada) and CD3 (A0452, Dako, Germany). Representative immunohistochemical photographs and quantification of renal leukocyte infiltration in nephritic wild-type, nephritic CXCL5−/− and WT control mice at day 14 after induction of nephritis (lpf = low power field; gcs= glomerular cross section) using the macrophage markers F4/80 (A, B) MAC2 (C, D) and the T-cell marker CD3 (E-H). Original magnification x200 for the tubulointerstitial compartment (A, E) and X400 for the glomerulus (C, G). F4/80, MAC-2 and CD3 positive cells were also quantified in CXCR2−/− (I) and anti-CXCL5 or IgG2B isotype antibody (J) experiments.
Supplemental Figure 3: Immune cell composition in WT and CXCL5−/− mice
FACS-based quantification of leukocyte subpopulations in the blood (A), in the spleen (B) and the kidney (C) of WT and CXCL5−/− mice at day 14 after induction of NTN. Untreated WT and CXCL5−/− mice served as controls. PMNs were identified as CD45+, CD11b+, Ly6G+, Ly6cint cells. Blood and bone marrow monocytes were identified as CD45+, CD11b−, Ly6G−, Ly6cint cells. Kidney mononuclear phagocytes were identified as CD45+, CD11b−, Ly6G−, F4/80+ cells. CD4+ T cells were identified as CD45+, CD3+, CD4+ cells and CD8+ T cells were identified as CD45+, CD3+, CD8+ cells. (n=4-5 per group, bars represent means ± SD).
Supplemental Figure 4: Renal phenotype of CXCL5−/− mice
Renal phenotype and function of wild-type and CXCL5−/− mice under homeostatic conditions. (A) PAS staining revealed no histologic differences between the knockout and wild-type groups. To analyze possible minimal morphologic abnormalities in the glomerular filtration barrier, we performed electron microscopy. As shown in B, no pathologic findings were detectable in both groups in terms of glomerular basement membrane morphology, endothelial cell structure, and podocyte foot process morphology. (C+D) Functional analysis of CXCL5-deficient and wild-type mice demonstrated identical urinary albumin / creatinine ratio (ACR) and blood urea nitrogen (BUN) levels. (n=4 per group, bars represent means ± SD).
Supplemental Figure 5: Flow cytometry of renal T\(_{\text{H}17}\) cells
Flow cytometric analyses of renal CD\(^3\) T cells isolated from controls and nephritic kidneys at days 3 and 10. Cells were re-stimulated with PMA/ionomycin and intracellularly stained for IL-17A. IL-17-expression by CD4\(^+\) T cells peaked at day 10 and was hardly detectable in controls and at day 3. In contrast CD4\(^-\)CD3\(^+\)IL-17\(^+\) cells (such as \(\gamma\delta\) T cells, see Turner and Krebs et al. JASN, 23: 1486-1495, 2012) were present in control and nephritic mice at day 3. FACS-plots are representative of each point of time as indicated.
Supplemental Figure 6: IL-17A induces CXCL5 expression in kidney tubular cells

(A) Murine epithelial tubular cells express IL-17 receptors IL-17RA and IL-17RC as measured by PCR from cultured cells. (B) IL-17A induces dose-dependent CXCL5 expression in cultured tubular cells. (C) IL-17A but not IFN-γ or IL-17F induced CXCL5 in cultured tubular cells as measured by RT-PCR (after 4 hours of stimulation) and ELISA from the supernatant (after 24 hours). (D) Lysates of stimulated cells were immunoblotted with monoclonal antibodies to phospho-ERK1/2 (upper left) or total ERK1/2 (lower) as well as phospho-p65 and p65 (NF-κB subunit p65, right panel) and β-actin as loading control. (E) Quantitative PCR from renal cortex shows TNF-α expression in the kidney in the course of nephrotoxic nephritis. Bars represent means ± SD (**P < 0.001).
Supplemental Figure 7: Anti-Ly6G-antibody depletes neutrophils in vivo

Flow cytometric analysis of leukocytes in the kidney (A) and blood (B) of nephritic wild-type mice after administration of PMN depleting rat-anti-Ly6G antibody or IgG isotype. Administration of anti-Ly6G was performed every 48 hours for 3 times, starting at day 9 after induction of the disease. Analysis of leukocytes was performed at day 14. Plots are gated on CD45+ and CD11b+ myelo-monocytic leukocytes and were representative for 3 independent experiments. FACS-based quantification of PMN and myelo-monocytic leukocyte subpopulations from the kidney (C) and the blood (D) of nephritic WT mice after administration of PMN depleting anti-Ly6G antibody or IgG Isotype.
Supplemental Figure 8: Humoral immune response
(A) Quantification of mouse anti-sheep specific IgG subclasses present in the serum of nephritic WT or CXCL5−/− mice and non-nephritic WT control mice measured by ELISA. (B, D) Representative immunohistochemical stainings for glomerular deposition of mouse IgG (B) and sheep IgG (D) in nephritic wild-type and CXCL5−/− mice 14 days after NTN induction (original magnification x400). (C) Quantification of glomerular deposition of mouse IgG (mIgG) and sheep IgG (sIgG) (E) in kidney sections of controls, nephritic wild-type, and nephritic CXCL5-deficient mice. (F) Quantification of mouse anti-sheep specific IgG subclasses present in the serum of nephritic WT or CXCR2−/− mice and non-nephritic WT control mice measured by ELISA. Bars represent means ± SD (* P < 0.05).
Supplemental Figure 9: Splenocyte supernatants
IL-17A and IFN-γ measurement by ELISA from supernatants of cultured splenocytes from nephritic CXCL5-deficient, nephritic wild-type, and control mice after stimulation with sheep IgG.