Cell

Crosstalk between Sentinel and Helper Macrophages Permits Neutrophil Migration into Infected Uroepithelium

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http://dx.doi.org/10.1016/j.cell.2014.01.006

SUMMARY

The phagocytes of the innate immune system, macrophages and neutrophils, contribute to antibacterial defense, but their functional specialization and cooperation is unclear. Here, we report that three distinct phagocyte subsets play highly coordinated roles in bacterial urinary tract infection. Ly6C$^-$ macrophages acted as tissue-resident sentinels that attracted circulating neutrophils and Ly6C$^+$ macrophages. Such Ly6C$^+$ macrophages played a previously undescribed helper role: once recruited to the site of infection, they produced the cytokine TNF, which caused Ly6C$^-$ macrophages to secrete CXCL2. This chemokine activated matrix metalloproteinase-9 in neutrophils, allowing their entry into the uroepithelium to combat the bacteria. In summary, the sentinel macrophages elicit the powerful antibacterial functions of neutrophils only after confirmation by the helper macrophages, reminiscent of the licensing role of helper T cells in antiviral adaptive immunity. These findings identify helper macrophages and TNF as critical regulators in innate immunity against bacterial infections in epithelia.

INTRODUCTION

Phagocytes, such as neutrophils and macrophages, play an essential role in the innate cellular immune response against bacterial infections. Neutrophils are particularly important; they clear bacteria by phagocytosis or kill them by secreting toxic compounds (Amulic et al., 2012). Neutrophils are bone-marrow-derived, circulate through the blood, and enter infected tissues by penetrating the vascular endothelium (Borregaard, 2010; Kolaczkowska and Kubes, 2013; Nathan, 2006). Such endothelial neutrophil migration requires ligands of the chemokine receptors CXCR1 (Fan et al., 2007) and CXCR2 (Cacalano et al., 1994), which include CXCL1, CXCL2, and CXCL6 (previously designated CXCL5) (Ley et al., 2007; Soehnlein and Lindbom, 2010). The mechanisms underlying endothelial migration have been studied extensively (Chin and Parkos, 2006; Ley et al., 2007; Nathan, 2006; Nourshargh et al., 2010; Voisin et al., 2010; Wang et al., 2006), whereas those guiding neutrophils within tissues toward microbes are less well known.

Of particular importance is neutrophil migration into tissue epithelia, the interfaces of barrier organs like the urinary tract to the outside world, which represent a preferential entry site for bacteria (Anderson et al., 2003). To intercept them, neutrophils need to traverse the epithelial basement membrane. This basement membrane is composed primarily of collagen IV (Chin and Parkos, 2006; Ichiyasu et al., 2004; LeBleu et al., 2007), which can be cleaved by metallomatrix proteinases (MMPs). MMPs are involved in infectious and malignant diseases of the urinary tract (Szarvas et al., 2011; Tan and Liu, 2012). Besides MMPs, chemokines are involved in crossing these membranes (Lämmermann et al., 2008; Shulman et al., 2009). Furthermore, in vitro studies suggest that some MMPs are regulated by chemokines (Chabot et al., 2006; Chakrabarti and Patel, 2005; Luca et al., 1997) and vice versa (Van den Steen et al., 2000). However, the role of chemokines, MMPs, and their

interaction for epithelial neutrophil migration in vivo are incompletely understood.

Macrophages are an important source of neutrophil-attracting chemokines in bacterial infections, such as CXCL1 and CXCL2 (Benoit et al., 2008; Kopydlowski et al., 1999; Mantovani et al., 2004; Mosser and Edwards, 2008). Macrophages possess intrinsic antibacterial activity, but this seems to be less critical for bacterial clearance than neutrophil effector functions (Dale et al., 2008; Kantari et al., 2008). After bacteria are cleared, macrophages phagocytize apoptotic neutrophils to promote resolution of inflammation (Bratton and Henson, 2011; Soehnlein and Lindbom, 2010). It is assumed that macrophages and neutrophils cooperate during infections (Kolaczkowska and Kubes, 2013; Soehnlein and Lindbom, 2010), but a coherent model on how they communicate and share the anti-infectious tasks is presently lacking.

Virtually all tissues are populated by a dense network of tissue-resident macrophages, which have been proposed to partially originate from embryonic progenitors (Ginhoux et al., 2010; Schulz et al., 2012). In infections, the bone marrow releases in a CCL2/CCR2-dependent fashion inflammatory monocytes, which differentiate into further macrophages or into dendritic cells within tissues (Serbina and Pamer, 2006). Inflammatory macrophages can be distinguished from tissue-resident macrophages by the marker Ly6C, until it is downregulated after 1–2 days (Landsman et al., 2007; Plantinga et al., 2013). Little is known about the functional specializations of inflammatory Ly6C$^+$ and resident Ly6C$^-$ macrophages.

Macrophages produce proinflammatory cytokines during bacterial infections, and, among these, tumor necrosis factor (TNF) is particularly important (Belge et al., 2002; Engel et al., 2008; Medzhitov, 2007; Mosser and Edwards, 2008). Also neutrophils and epithelia cells can produce TNF (Roulis et al., 2011; Theilgaard-Mönch et al., 2005), but it is unclear whether the cellular source of TNF impacts bacterial clearance. TNF is involved also in immune-mediated diseases, such as rheumatoid arthritis and inflammatory bowel disease, that respond well to TNF blockade therapy (Kountouras et al., 2005; Mpofu et al., 2005; Targan et al., 1997; Wright et al., 2010). However, bacterial infections often relapse during such inhibition, especially tuberculosis (Flynn et al., 1995; Keane et al., 2001) and the incidence of bacterial infections of the lung and the urinary tract infections is increased (Dixon et al., 2006; Listing et al., 2005; Strangfeld and Listing, 2006; Winthrop et al., 2009; Wright et al., 2010). This is often explained by the ability of TNF to stimulate phagocytosis and ROS production by neutrophils. The exact antibacterial role of TNF is incompletely understood.

Bacterial infections of the urinary tract (UTI) are among the most prevalent infections and affect more than 25% of the population in developed countries, especially young females (Foxman, 2002). They mainly result from uropathogenic *E. coli* (UPEC) ascending through the urethra into the bladder, where they first invade the uroepithelium (Mulvey et al., 1998). UPEC can persist intracellularly, and cause relapsing infections (Martinez et al., 2000), which is associated with an increased risk for bladder cancer (Parker et al., 2004). Their ascension into the kidney causes pyelonephritis, which can progress to renal failure (Kurts et al., 2013; Svensson et al., 2011). The defense against UTI depends on neutrophils (Frendéus et al., 2000). These are attracted by CXCR2 ligands, which mediate both endothelial and epithelial migration (Godaly et al., 1997; Ley et al., 2007). The exact identity and the source of these ligands is unclear. The macrophage inhibitory factor (MIF) was recently described as a further CXCR2 ligand (Bernhagen et al., 2007; Santos et al., 2011). Its function in UTI is unclear. Also the roles of macrophages and of TNF in UTI are unknown.

Here, we studied the interplay of neutrophils and macrophages in a murine model of UTI of the bladder. We discovered that tissue-resident Ly6C$^-$ and proinflammatory Ly6C$^+$ macrophages play distinct roles in antibacterial immunity. Their TNF-mediated crosstalk regulates neutrophil migration into the infected uroepithelium and facilitates optimal antibacterial defense. We propose a model of tri-cellular crosstalk in innate cellular immunity.

RESULTS

Kinetics and Location of Neutrophils and Macrophages in Urinary Tract Infection

We studied the roles of macrophages and neutrophils in a mouse model of UTI induced by transuretheral instillation of UPECs. Bladders of uninfected mice mostly contained resident Ly6C$^-$ macrophages (Figures 1A and S1A available online). Already at 2 hr after infection, the numbers of neutrophils and Ly6C$^+$ macrophages increased significantly within the infected bladder, whereas numbers of Ly6C$^-$ macrophages remained largely constant during the course of infection (Figure 1B). Maximal numbers of neutrophils and Ly6C$^+$ macrophages were reached after 24 hr (Figure 1B). Hardly any of these cell types proliferated in the bladder (Figure S1B), suggesting that their rapid accumulation was due to recruitment from the circulation. Mainly neutrophils migrated into the uroepithelium (Figures 1C and 1D).

To study the importance of these phagocyte types for bacterial clearance, we determined uptake of UPEC expressing recombinant GFP. Almost 90% of the phagocytosed UPECs were detected within neutrophils 6 hr after infection (Figure 1E), supporting their central role in the defense against UTI (Frendéus et al., 2000). Ly6C$^-$ macrophages took up hardly any UPEC, and only some Ly6C$^+$ macrophages did so after 24 hr, suggesting regulatory roles.

Distinct Functions of Ly6C$^+$ and Ly6C$^-$ Macrophages in UTI

We speculated that Ly6C$^-$ or Ly6C$^+$ macrophages might regulate the antibacterial defense by producing chemokines that attract the neutrophils (Soehnlein and Lindbom, 2010). Interestingly mainly the Ly6C$^-$ macrophages produced the neutrophil attractors MIF, CXCL1, CXCL2, and CXCL6 (Figures 2A and S2A). Ly6C$^+$ macrophages hardly produced chemokines, but instead they were the main producers of TNF (Figures 2A and S2A), which is generally important in bacterial infections (Nathan, 2006). These observations suggested that Ly6C$^-$ and Ly6C$^+$ macrophages might play distinct functions in UTI.

At this point, we wondered how well the Ly6C marker discriminates between these macrophages, since previous studies

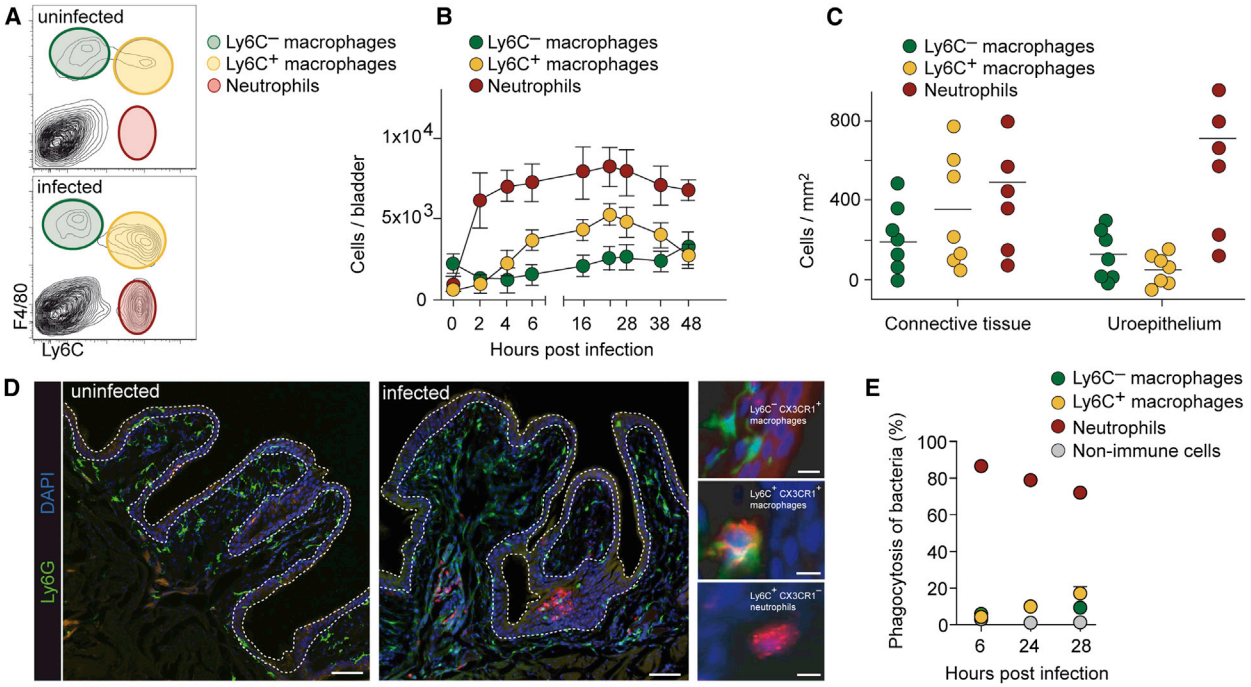

Figure 1. Recruitment and Positioning of Macrophages and Neutrophils in Bacterial UTI

(A) Flow-cytometric analysis of bladder homogenates of uninfected and infected mice (concatenated plots of five mice) 1 day after infection. Neutrophils (red), Ly6C$^+$ (yellow) and Ly6C$^-$ (green) macrophages were distinguished by their expression of Ly6C and F4/80 as indicated.

(B) Numbers of neutrophils, Ly6C$^+$ and Ly6C$^-$ macrophages in single-cell suspensions of bladders determined by flow cytometry.

(C) Number of neutrophils, Ly6C$^+$ and Ly6C$^-$ macrophages in bladder cryosections 24 hr after infection as shown in (D).

(D) Localization of neutrophils within the epithelium 1 day after infection. Cryosections from bladders of uninfected and infected *Cx$_3$cr1*$^{+/GFP}$ mice, which express green fluorescent protein (GFP) in macrophages, were stained for Ly6C (red) and for cellular nuclei (DAPI-blue). The white dashed line highlights the uroepithelial border and the white bar indicates 200 μm. The small images show representative stainings of the three phagocyte types. The white bar indicates 10 μm.

(E) Contribution of neutrophils, macrophages and nonimmune cells (CD45$^-$ cells) to phagocytosis of GFP-expressing UPECs, calculated by the formula ((mean fluorescence intensity of GFP$^+$ cells in UPEC-GFP infected mice − mean fluorescence intensity of GFP$^+$ cells in UPEC infected mice) × number of UPEC-GFP$^+$ cells).

Data are means ± SEM and represent five (A and B) and three (C, D, and E) independent experiments in groups of five to seven mice. See also Figure S1.

showed that Ly6C$^+$ monocytes downregulate this molecule after entering tissues (Landsman et al., 2007; Plantinga et al., 2013). When we transferred Ly6C$^+$ monocytes, all of them expressed Ly6C in the infected bladder after 6 hr, almost 90% after 24 hr, and 70% after 48 hr (Figure S2B). To determine whether Ly6C$^-$ macrophages upregulate this marker in UTI, we infected *Ccr2*$^{-/-}$ mice, which lack Ly6C$^+$ macrophages (Serbina and Pamer, 2006), and mice treated with clodronate liposomes, which selectively depletes Ly6C$^+$ macrophages, but neither Ly6C$^-$ macrophages (Figure S2C) nor neutrophils (Figure 2B). In both cases, no Ly6C$^+$ macrophages were detected in the bladder 6 hr after infection (Figure S2D). Thus, Ly6C expression was mostly stable during the first 24 hr of the antibacterial immune response studied here, supporting the notion that Ly6C$^-$ and Ly6C$^+$ macrophages were functionally distinct macrophage types.

We next determined the roles of the chemokines produced by Ly6C$^-$ macrophages by blocking them with neutralizing antibodies. CXCL1 was as critical for neutrophil recruitment as its receptor CXCR2 (Figure 2B). MIF contributed to such recruitment, whereas CXCL2 and CXCL6 were not required (Figure 2B). Ly6C$^+$ macrophage numbers were substantially reduced in *Ccr2*$^{-/-}$ mice (Figure S2D), whose ligand CCL2 was produced by Ly6C$^-$ macrophages as well (Figures 2A, S2A and S2E). CCL2 production by these macrophages was independent of the presence of Ly6C$^+$ macrophages (Figure S2F), supporting the central role of resident Ly6C$^-$ macrophages in the recruitment of circulating phagocytes. Bacterial clearance of the bladder was impaired in the absence of neutrophils (αCXCL1, αMIF, and *Cxcr2*$^{-/-}$ mice), the main antibacterial effectors (Figure 2C) (Frendéus et al., 2000). Notably, it was also impaired in mice lacking or depleted of Ly6C$^+$ macrophages (Figure 2C). In summary, Ly6C$^-$ macrophages produced the chemokines that attracted neutrophils and Ly6C$^+$ macrophages into the infected bladder, and both recruited phagocyte types were important for antibacterial defense.

TNF from Ly6C$^+$ Macrophages Permits Transepithelial Migration of Neutrophils

The importance of Ly6C$^+$ macrophages was astonishing given that these cells hardly contributed to bacterial phagocytosis within the first day (Figure 1E). We speculated that their main

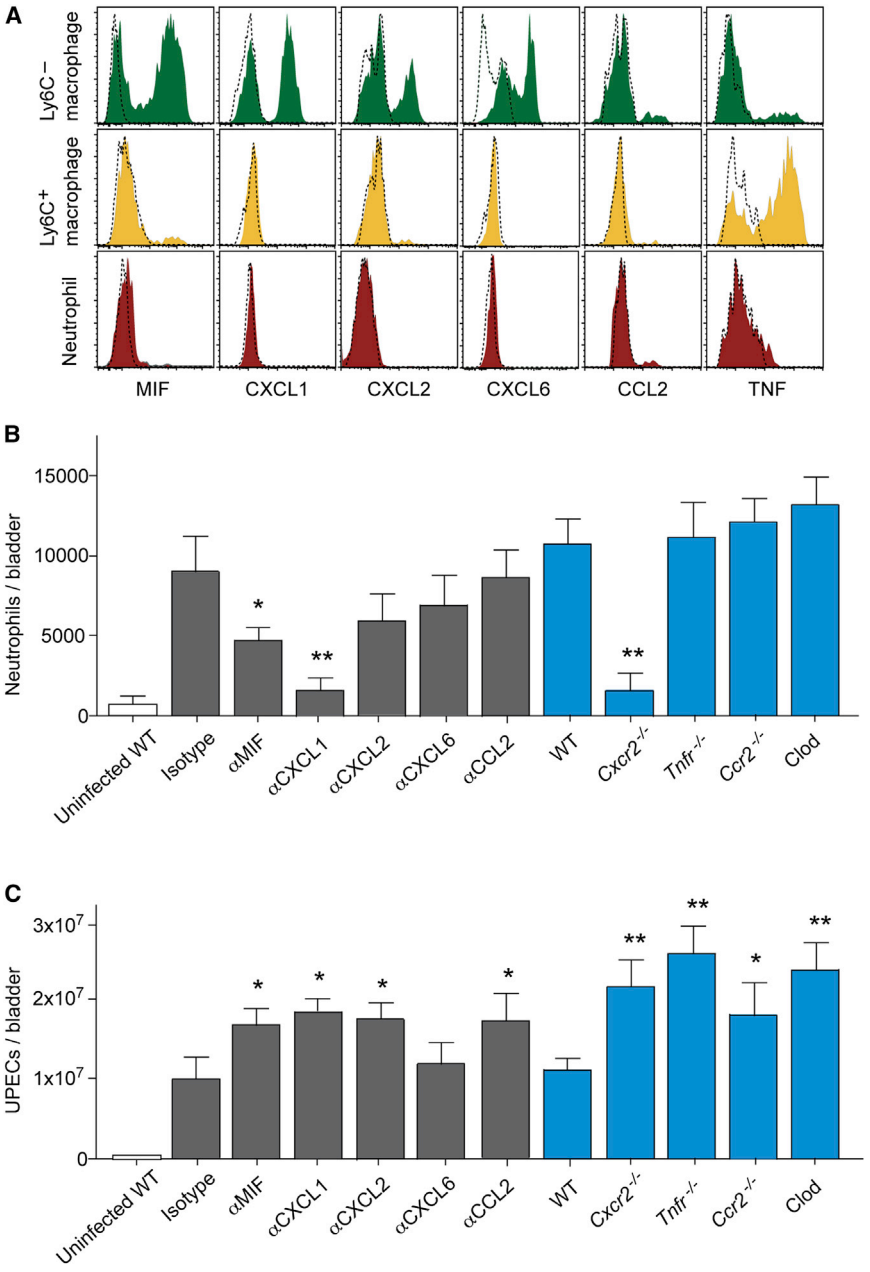

Figure 2. Chemokines Produced by Ly6C$^-$ Macrophages Recruit Neutrophils into the Infected Bladder

(A) Intracellular flow-cytometric staining of Ly6C$^-$ (green) and Ly6C$^+$ (yellow) macrophages and neutrophils (red) 6 hr after infection for immune regulatory molecules (solid line) and the respective isotype control (dashed line).

(B and C) Intravesical numbers of neutrophils (B) and UPECs (C) 6 hr after infection and treatment with antibodies (gray) or in knockout animals (blue). Clod = Clodronate liposomes.

Data are means ± SEM and represent three experiments in groups of three to five mice. *$p < 0.05$; **$p < 0.01$. See also Figure S2.

product, TNF might be involved (Figure 2A). Although this cytokine is generally important in bacterial infections (Nathan, 2006), its role in UTI is unknown.

When we infected *Tnfr*$^{-/-}$ mice deficient for both TNF receptors, the bacterial load in their bladders was much higher than in wild-type controls (Figure 2C). In addition, the number of infected uroepithelial cells, which indicates persistence of UTI, was increased both in *Tnfr*$^{-/-}$ mice and in the absence of the TNF-producing Ly6C$^+$ macrophages (Figure 3A). Moreover, throughout a period of 6 weeks, more cystitis relapses of higher severity occurred (Figure 3B) and more mice developed pyelonephritis (Figure 3C). Thus, TNF is important to limit UTI.

To clarify the role of TNF in UTI, we first asked whether it activates neutrophil effector functions. However, the in vitro ability of neutrophils to phagocytose UPEC and their antibacterial capacity were independent of TNF (Figure S3). The discrepancy between the requirement for TNF in vivo (Figures 2C and 3) and in vitro (Figure S3) suggested an effect on neutrophil migration. However, neutrophil recruitment into the whole bladder was not impaired in *Tnfr*$^{-/-}$ mice (Figure 2B). Histological analysis revealed that despite their ability to enter the bladder, neutrophils failed to migrate into the bladder uroepithelium in *Tnfr*$^{-/-}$ mice (Figures 4A to 4C), the site from where UPEC enter. Fewer neutrophils were detected also in the urine of *Tnfr*$^{-/-}$ mice and in mice depleted of TNF-producing Ly6C+ macrophages (Figure 4D), consistent with their inability to enter and transmigrate through the uroepithelium into the bladder lumen.

When we reconstituted infected *Tnf*$^{-/-}$ mice either with recombinant TNF (Figure 4E) or with TNF-producing Ly6C$^+$ macrophages (Figures 4E to 4G), neutrophils were able to enter the uroepithelium, verifying that Ly6C$^+$ macrophages can permit epithelial neutrophil migration through TNF.

TNF Induces CXCL2 in Ly6C$^-$ Macrophages

To determine whether migration of neutrophils into the uroepithelium required TNF to directly act on neutrophils, we adoptively transferred TNFR-competent and -deficient neutrophils into infected wild-type mice. TNFR-deficient neutrophils entered the bladder and its uroepithelium of infected wild-type mice as efficiently as TNFR-competent neutrophils (Figures 5A and 5B), indicating that TNFR on neutrophils was dispensable for both endothelial and epithelial migration. This differed from the situation in mice ubiquitously lacking TNFR, where endothelial

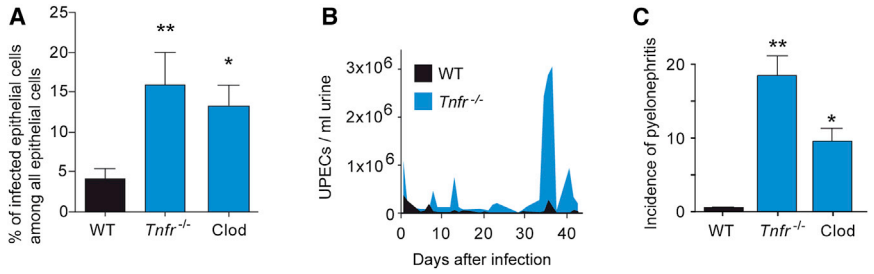

Figure 3. Aggravated Infection in Mice Lacking TNF Receptor or TNF-Producing Ly6C$^+$ Macrophages

(A) Mice were infected with GFP-expressing UPECs and the percentage of infected GFP$^+$ CD45-negative epithelial cells were determined one day after infection.

(B) Relapsing infections in wild-type (WT) and *Tnfr*$^{-/-}$ mice analyzed by counting the number of UPECs within the urine.

(C) Incidence of pyelonephritis one day after infection in percent.

Data are means ± SEM and represent three independent experiments in groups of three to five mice. *$p < 0.05$; **$p < 0.01$.

(Figure 2B), but not epithelial migration (Figures 4A to 4C), was intact. We concluded that TNF acted on TNFR$^+$ cells other than neutrophils.

To identify this cell, we generated bone marrow chimeras in which hematopoietic and/or nonhematopoietic cells lacked TNFR. When we infected these chimeras, transepithelial neutrophil migration required TNFR expression only by bone-marrow-derived cells, but not by tissue cells, such as stroma or epithelial cells (Figures 5C and 5D).

To clarify which bone-marrow-derived cell responded to TNF, we examined several molecules known to affect neutrophil migration and asked which were regulated in a TNF-dependent manner. We found that CXCR2, CXCL1, MIF, GM-CSF, IFNγ, IL-1a, IL-4, IL-5, L-6, IL-17, CCL2, CCL3, CCL4, CCL5, CCL7, CD11b, CD47, CD49b, and ICAM-1 (Figures 6A and S4) were similarly expressed in bladders of infected *Tnfr*$^{-/-}$ and wild-type mice. Only CXCL2 was markedly reduced in *Tnfr*$^{-/-}$ mice (Figure 6A). This chemokine was required for the defense against UTI (Figure 2C), but not for neutrophil recruitment into the whole bladder (Figures 2B and 5A). However, like TNF (Figures 4A to 4C), CXCL2 is required for uroepithelial neutrophil migration (Hang et al., 1999), suggesting a causal connection. Indeed, secretion of CXCL2 was strongly reduced in the infected bladders of *Tnf*$^{-/-}$ and *Tnfr*$^{-/-}$ mice, and of mice depleted of the main TNF producers, Ly6C$^+$ macrophages (Figure 6A), indicating that TNF induced CXCL2. The cellular source of CXCL2 according to our flow cytometric analysis were Ly6C$^-$ macrophages (Figures 2A and S2A). But as nonhematopoietic cells often escape flow cytometry, we also analyzed infected bladders by immunofluorescence microscopy. We failed to detect CXCL2 in uroepithelial cells at 3 hr after infection (Figures 6B and S5A), consistent with previous observations (Hang et al., 1999). By contrast, bladder macrophages stained brightly for intracellular CXCL2, not only in infected but also in uninfected animals (Figures 6B and S5B), indicating the existence of preformed intracellular pools. Macrophages also contained preformed CCL2 (Figures 6C and S5C), which can attract Ly6C$^+$ macrophages. The rapid release of preformed pools can explain how these chemokines could be released within a few hours after urinary tract infection (Figure S5D). As only Ly6C$^-$ macrophages produced CXCL2 in the infected bladder (Figures 2A and S2A), we concluded that TNF acted only on Ly6C$^-$ macrophages, and not on neutrophils.

These results suggested that supplying CXCL2 should restore the in vivo migratory defect of neutrophils in the absence of TNF. To test this hypothesis, we transurethrally inoculated CXCL2 into the bladder of infected *Tnf*$^{-/-}$ mice. Indeed, under these conditions neutrophils entered the uroepithelium (Figures 6D, 6E, and S5E), confirming that CXCL2 acted downstream of TNF.

CXCL2 Permits Epithelial Neutrophil Migration by Inducing MMP-9

Next, we asked how CXCL2 enabled neutrophils to enter the uroepithelium. Epithelial basement membranes are comprised mainly of collagen IV, which is degraded by metallomatrix proteinase 9 (MMP-9) (Van den Steen et al., 2002), and CXCR2 ligands have been shown to induce MMP-9. Histological sections of infected *Mmp-9*$^{-/-}$ mice showed that neutrophils accumulated underneath the uroepithelium and failed to enter it (Figures 7A and S6A) or to transmigrate into the urine (Figure 7B). Furthermore, *Mmp-9*$^{-/-}$ mice showed a markedly higher bacterial load in the urine (Figure 7C), indicating that MMP-9 is also required for defense against UTI. When we stained bladder cells for MMP-9 expression, almost 99% of the MMP-9$^+$ cells were neutrophils (Figure S6B). To test whether neutrophil-intrinsic MMP-9 was important, we transferred MMP-9-competent neutrophils into infected *Mmp-9*$^{-/-}$ mice, and MMP-9-deficient neutrophils into infected MMP-9-competent mice. Only MMP-9-competent neutrophils were detected within the uroepithelium (Figures 7D and S6C) indicating that neutrophil-intrinsic MMP-9 was necessary and sufficient for epithelial migration and explaining its requirement for antibacterial defense.

The migratory defect of MMP-9-deficient neutrophils was analogous to that in *Tnfr*$^{-/-}$ mice (Figures 4A to 4D), suggesting a causal connection between TNF and MMP-9. We tested this by measuring secretion of MMP-9 by neutrophils in *Tnfr*$^{-/-}$ and wild-type mice by zymography (Vandooren et al., 2013). Indeed, this secretion was reduced by 70%–80% in *Tnfr*$^{-/-}$ and *Tnf*$^{-/-}$ mice (Figures 7E and S7A), whereas secretion of MMP-2 was unaffected (Figure S7B). A similar reduction was seen in mice depleted of Ly6C$^+$ macrophages, the main TNF producers (Figures 7E and S7A), indicating that TNF from Ly6C$^+$ macrophages caused the secretion of MMP-9. However, our finding that TNFR expression by neutrophils was dispensable for transepithelial migration (Figure 5B) implied that a downstream mediator, such as CXCL2, induced the secretion of

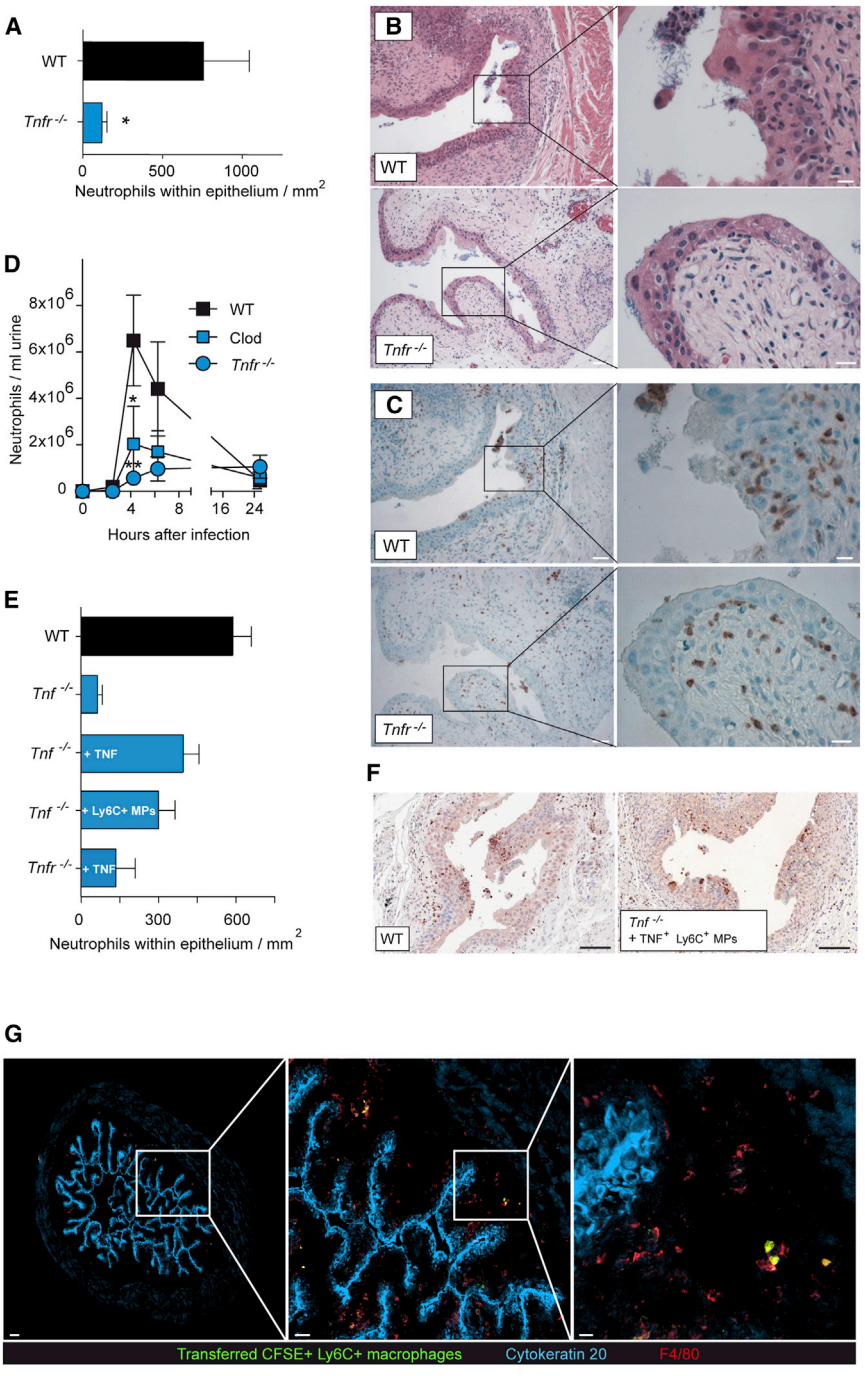

Figure 4. TNF Enables Neutrophils to Enter the Uroepithelium

(A–C) Analysis of transepithelial migration by histological enumeration of neutrophils 1 day after infection. Consecutive bladder sections of wild-type (WT) or *Tnfr*$^{-/-}$ mice were either stained with HE (B) or Ly6G (C, brown), which specifically reveals neutrophils. The right panels show 4-fold magnifications of the inlay highlighted in the left column with a black box. White bars are 100 μm (left) or 25 μm (right). Quantitative analysis in (A).

(D) Kinetics of neutrophil numbers in the urine of WT or *Tnfr*$^{-/-}$ mice or after depleting Ly6C$^+$ macrophages with clodronate liposomes (Clod) determined by flow cytometry.

(E–G) Restored neutrophil migration into the uroepithelium of *Tnf*$^{-/-}$ mice either 24 hr after i.v. transfer of 5 × 10^6 CFSE-labeled Ly6C$^+$ monocytes (E–G) or after injection of 50 pg TNF into the bladder (E). Neutrophil localization was revealed by myeloperoxidase staining of paraffin sections 1 day after infection. The transferred CFSE-labeled Ly6C$^+$ monocytes (green) were counterstained for F4/80 (red) and the uroepithelium for cytokeratin 20 (blue). The bar graph indicates 300 μm (F), 100 μm (G, left), 30 μm (G, middle), 10 μm (G, right). Quantitative analysis in (E).

Data are means ± SEM and represent four (A–D) and two (E–G) independent experiments in groups of five to eight mice. *p < 0.05; **p < 0.01, ***p < 0.001. See also Figure S3.

DISCUSSION

Taken together, our findings documented the following sequence of events during the first 4 hr of the phagocyte response against UTI: (1) resident Ly6C$^-$ macrophages sensed the infection and produced chemokines that recruited neutrophils and Ly6C$^+$ macrophages, (2) recruited Ly6C$^+$ macrophages produced TNF in response to the infection, (3) TNF induced the release of preformed CXCL2 by Ly6C$^-$ macrophages, (4) CXCL2 caused MMP-9 secretion by neutrophils, which allowed these cells to cross the epithelial basement membrane in order to combat the infection.

These results demonstrated that the antibacterial neutrophil response is coordinated by two macrophage subsets with distinct tasks: the Ly6C$^-$ macrophages acted as tissue-resident sentinels and attracted circulating phagocytes by chemokines. The Ly6C$^+$ macrophages did not directly participate in bacterial elimination but instead played a hitherto unknown helper role: in response to the infection, they licensed the Ly6C$^-$ macrophages to send neutrophils into the frontline of infection, the uroepithelium.

The reliance on cues from helper cells is reminiscent of adaptive immunity. For example, dendritic cells can cross-prime

MMP-9 in neutrophils. We tested this by stimulating neutrophils in vitro with the different chemokines and measuring MMP-9 secretion. CXCL2 increased MMP-9 levels in wild-type neutrophils in a concentration-dependent manner, whereas neither CXCL1, CXCL6, MIF, CCL2, nor TNF was able to do so (Figure 7F). Furthermore, CXCL2, but not TNF, stimulated TNFR-deficient neutrophils to secrete MMP-9 (Figure S7C), confirming that the downstream mediator CXCL2, and not TNF, activated neutrophils to release MMP-9.

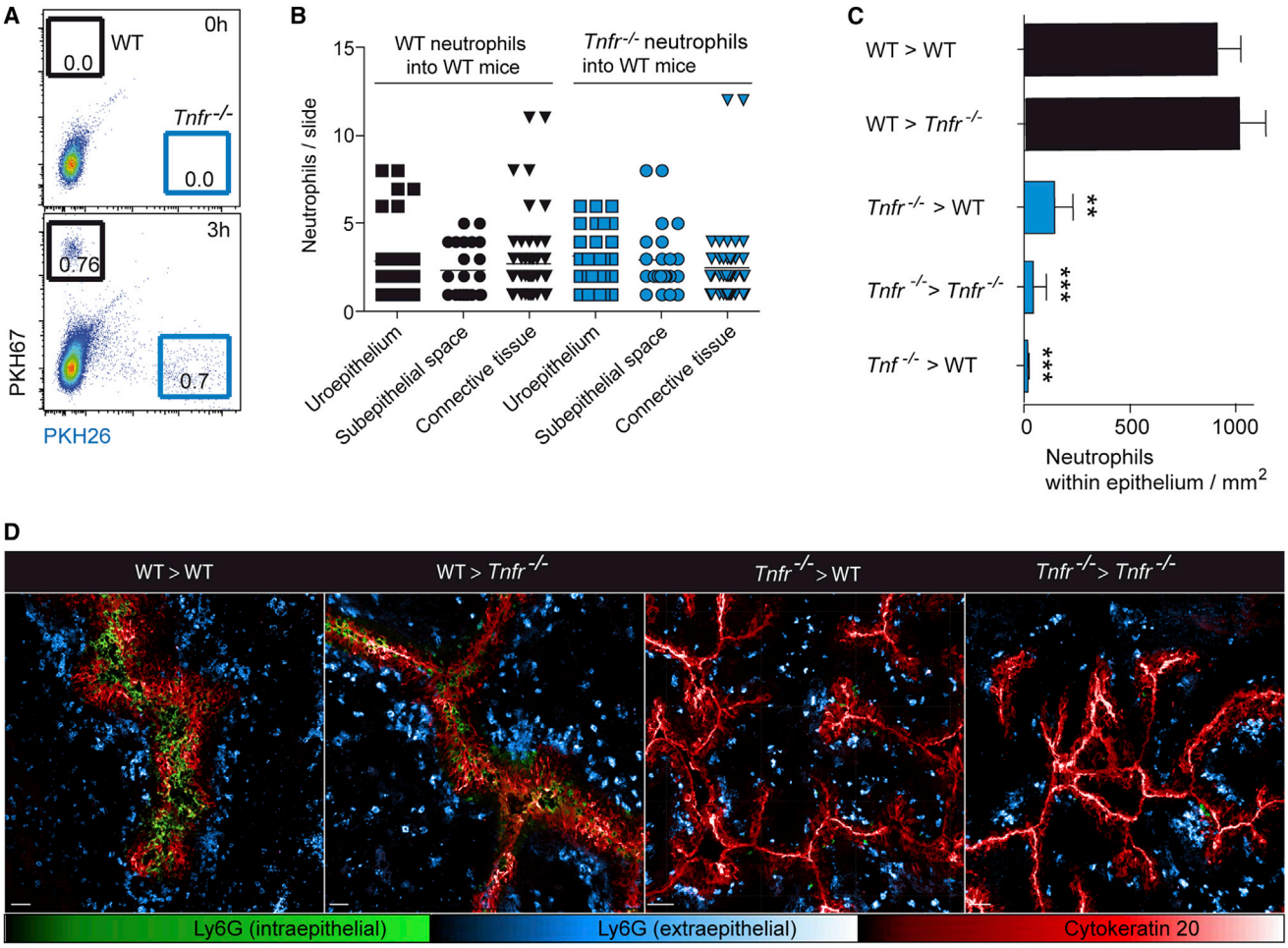

Figure 5. Transepithelial Neutrophil Migration Requires TNFR Only on Bone-Marrow-Derived Cells

(A) Flow cytometric analysis of TNF-dependent transendothelial migration by transferring $3x10^6$ PKH26-labeled *Tnfr*$^{-/-}$ neutrophils together with the same number of PKH67-labeled WT neutrophils into infected WT animals.

(B) Bone-marrow-derived neutrophils were labeled with CFSE and adoptively transferred into infected mice. One day later the fluorescent neutrophils were localized in bladder cryosections stained for Ly6G.

(C and D) Irradiated *Tnfr*$^{-/-}$ and wild-type (WT) were transplanted with bone marrow from *TNF*$^{-/-}$, *Tnfr*$^{-/-}$ or WT mice. One day after infection, the localization of neutrophils was analyzed by immunofluorescence staining of bladder cryosections for Ly6G. The uroepithelium was labeled by cytokeratin 20 staining (red). Ly6G$^+$ cells outside the uroepithelium are displayed as pseudocolor in blue, and those inside the uroepithelium in green. White bars are 100 μm. Quantitative analysis is given in (C).

Data are means ± SEM and represent two (A and B) and three (C and D) independent experiments in groups of five to eight mice. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

cytotoxic CD8$^+$ T lymphocytes (CTL) against viral infections only after having been licensed by CD4$^+$ T helper cells or NKT cells (Castellino et al., 2006; Semmling et al., 2010; Smith et al., 2004). This involves the secretion of chemokines that bind CCR5 or CCR4, respectively, which attract the CTL toward the licensed DCs. Later, in infected tissues, the crosstalk between T helper cells and dendritic cells results in the production of CXCR3-specific chemokines that direct CTL toward virus-infected cells (Nakanishi et al., 2009). Our findings reveal an analogous role for the crosstalk between Ly6C$^-$ and Ly6C$^+$ macrophages in guiding neutrophils within bacterial infected tissues through coordinated chemokine production.

The chemokines that bind CXCR2 are often considered redundant. We found that Ly6C$^-$ macrophages produced several such chemokines, which played distinct chemotactic roles in regulating neutrophil migration in UTI: CXCL1, and to a lower extent MIF, caused endothelial migration, whereas CXCL2 by Ly6C$^-$ macrophages played a nonredundant role in epithelial migration. Diverse signaling events by different ligands for the same receptor has been referred to as biased agonism and the underlying mechanisms are under intense investigation (Reiter et al., 2012). Biased agonism has been described for CCR5, CCR7, and CXCR3 ligands (Kenakin et al., 2012; Kohout et al., 2004; Scholten et al., 2012). We have observed that CXCL2 differs from other CXCR2 ligands by modulating phosphorylation of mitogen-activated protein kinase p38, but neither of Akt, ERK1/2 nor of JNK in neutrophils (D.R.E. and K.L., unpublished data), demonstrating biased agonism for CXCR2 ligands. p38

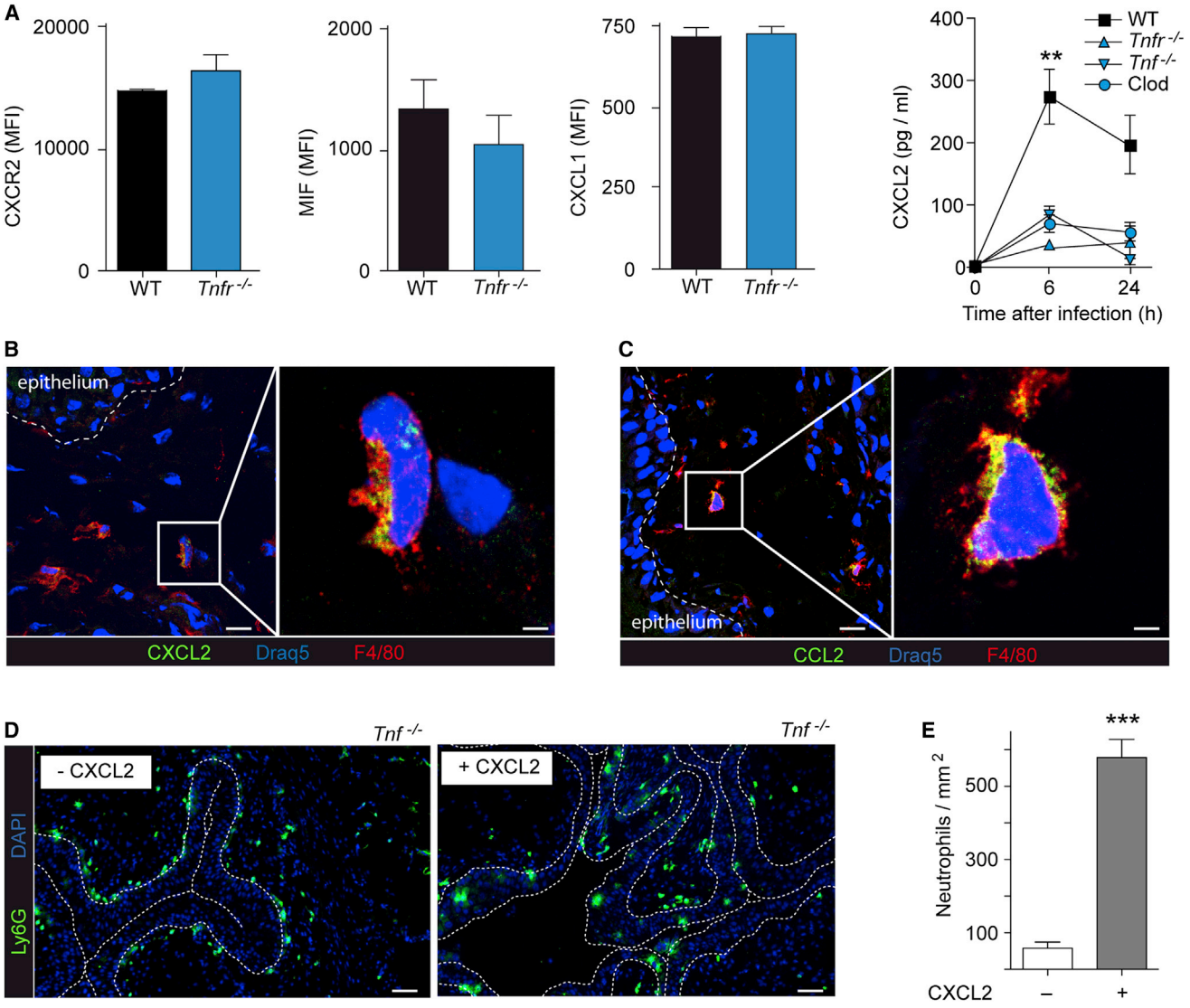

Figure 6. TNF-Dependent CXCL2 Enables Neutrophils to Enter the Uroepithelium

(A) Expression of CXCR2, MIF, CXCL1 and CXCL2 expression in WT and *Tnfr* $^{-/-}$ mice 1 day after infection (First three bar graphs; MFI = Mean Fluorescence Intensity). CXCL2 expression was determined by ELISA in bladder homogenates of wild-type (WT), *Tnfr* $^{-/-}$, *Tnf* $^{-/-}$, and clodronate-liposome-treated animals (Clod) 6 and 24 hr after infection.

(B and C) Staining of cryosections of 3 hr infected bladders for macrophages by F4/80 (red) and CXCL2 (B) or CCL2 (C) in green. Nuclei were counterstained with DRAQ5 (blue). The white bar indicates 20 μm (left) or 4 μm (right).

(D) Restored migration of neutrophils into the uroepithelium by injection of CXCL2 into the bladder (+ CXCL2, right) into *Tnf* $^{-/-}$ mice infected one day before (Detailed quantitative analysis is shown in Figure S5E). The white dashed line highlights the uroepithelial border and the white bar indicates 100 μm.

(E) Quantitative analysis of intraepithelial neutrophils as shown in (D).

Data in (A) and (E) are means ± SEM and represent three independent experiments in groups of five to eight mice. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See also Figures S4 and S5.

has been shown to induce neutrophil MMP-9 secretion in some (Dumitru et al., 2012), but not in other in vitro studies (Chakrabarti and Patel, 2005). Future studies are required to clarify whether CXCL2 modifies p38 signaling to induce MMP-9 secretion and epithelial neutrophil migration in vivo.

Epithelial immune cell migration is less well understood than endothelial migration (Kolaczkowska and Kubes, 2013; Ley et al., 2007; Nourshargh et al., 2010), and our knowledge about epithelial migration mostly stems from in vitro experiments, which do not mimic all aspects of the in vivo situation, especially barriers formed by basement membranes. The ability of CXCL2 to induce the secretion of MMP-9 (Chakrabarti and Patel, 2005) in the immediate vicinity of the epithelial basement membrane explains how neutrophils can penetrate collagen IV-rich epithelial basement barriers. These findings identify CXCL2 and MMP-9 as TNF-regulated gatekeepers for epithelial neutrophil migration.

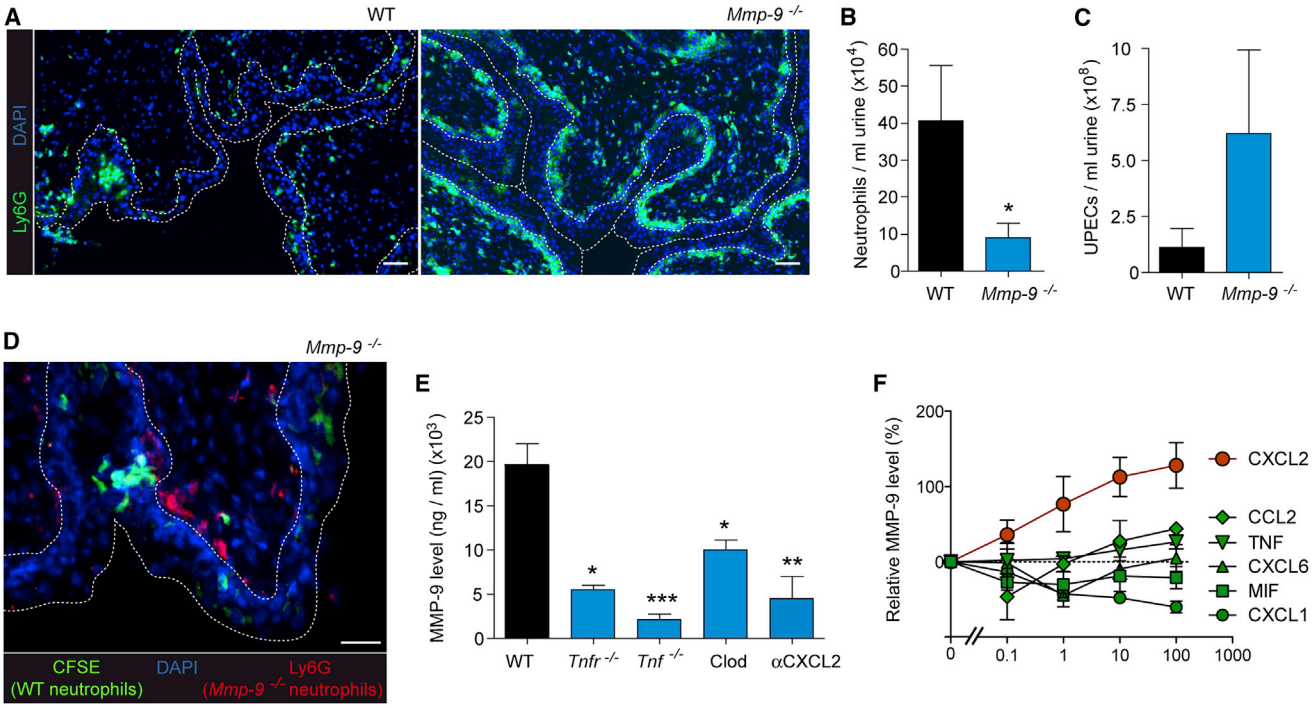

Figure 7. CXCL2-Induced MMP-9 Secretion Mediates the Entry of Neutrophils into the Uroepithelium

(A) Positioning of neutrophils in bladder sections of wild-type (WT) and *Mmp-9*$^{-/-}$ mice one day after infection, revealed by staining with Ly6G Alexa488 (green). The white dashed line highlights the uroepithelial border and the white bar indicates 200 μm (left) and 100 μm (right). (Detailed quantitative analysis is shown in Figure S6A).

(B and C) Reduced numbers of neutrophils (B) and increased bacterial load (C) in the urine of *Mmp-9*$^{-/-}$ mice over WT controls 1 day after infection.

(D) In contrast to endogenous neutrophils (stained with the red antibody Ly6G Alexa568) in *Mmp-9*$^{-/-}$ mice, adoptively transferred MMP-9-competent neutrophils stained with CFSE (green) can enter the infected uroepithelium 1 day after infection. The white bar indicates 40 μm.

(E) Analysis of MMP-9 levels by zymography (blots in Figure S7A) in one day infected murine bladders of WT, *Tnf*$^{-/-}$, *Tnfr*$^{-/-}$, Clodronate Liposome and anti-CXCL2 treated mice.

(F) Relative MMP-9 secretion of bone-marrow-derived neutrophils 6 hr after stimulation by the indicated chemokine concentrations, determined by zymography. Data are means ± SEM and represent three independent experiments in groups of five to eight mice. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figures S6 and S7.

TNF is produced by recruited immune cells not only in UTI, but also in infections of other barrier organs, e.g., the lung (Chen et al., 2013) or the intestine (Platt and Mowat, 2008), consistent with its role as a licensing factor. Interestingly, TNF was produced by CCR2-dependent Ly6C^{+} DCs in listeriosis of the spleen (Serbina et al., 2003), whereas patrolling Ly6C^{-} monocytes recruited from the blood through CX_3CR1 did so in listeria peritonitis (Auffray et al., 2007), suggesting that immune cells other than Ly6C^{+} macrophages may play a helper role in different organ and/or pathogen contexts.

Consistent with its well-known central role in antibacterial defense (Nathan, 2006), pharmacologic TNF blockade in patients with rheumatoid arthritis (Dixon et al., 2006) or inflammatory bowel disease often causes relapses of tuberculosis infection (Flynn et al., 1995). Furthermore, such blockade is correlated with a higher incidence of pneumonia and, notably, of UTI (Strangfeld and Listing, 2006). Our findings in *Tnfr*$^{-/-}$ mice can explain the latter association. Furthermore, these mice also showed more intraepithelial bacterial communities, which underlie the frequent relapses of bacterial cystitis. Our findings show that TNF is required for curtailing these communities. It remains to be seen whether CXCL2 induction is important also in other TNF-dependent bacterial infections, for example listeriosis (Pfeffer et al., 1993) or tuberculosis (Flynn et al., 1995).

The ability of intravesically applied CXCL2 to restore transepithelial migration of TNF-deficient neutrophils might represent a new therapeutic concept in patients with relapsing UTI, especially after treatment with TNF blockers. Intravesical CXCL2 instillation might help eliminating bacteria and prevent their spreading into the kidney. Topical CXCL2 provision might also be effective in other infections that exacerbate during TNF inhibitor therapy.

In conclusion, our study demonstrates that the innate immune response against bacterial infections requires the coordinated interaction between three phagocyte subsets with distinct tasks: (1) Ly6C^{-} macrophages as sentinels/coordinators, (2) Ly6C^{+} macrophages as helpers/advisors, (3) neutrophils as antibacterial effectors. The sentinels request a second opinion from the helpers, before unleashing the antibacterial effector cells into epithelial tissue. This mechanism may be relevant for barrier organs with an epithelial interface to the outside world, like the urogenital tract. The reliance on the coordination between

several immune cells may serve to reduce the risk of inadequate neutrophil responses that cause immunopathology.

EXPERIMENTAL PROCEDURES

Mice

Female mice between 8 and 12 weeks of age were maintained in specific pathogen-free condition and used in accordance with local governmental review boards (Bezirksregierung Köln, from 2008 Landesamt für Natur, Umwelt und Verbraucherschutz NRW in Recklinghausen, Germany). (See also Extended Experimental Procedures).

Reagents

$Ly6C^+$ macrophages were depleted by intravenous injection of 250 μl clodronate-liposome suspension into the lateral tail vein 6 hr prior to infection. For blocking experiments, 100 μg of the antibodies were intravenous injected into wild-type mice 1 hr prior to infection (See also Extended Experimental Procedures). To restore neutrophil migration in $Tnf^{-/-}$ mice, 50pg TNF or 2.5 μg murine recombinant CXCL2 (AbD Serotec) was inoculated 1 hr before infection, 2, 4, 24, and 27 hr after infection and mice were sacrificed 30 hr after infection.

UPEC and UTI Model

The uropathogenic *E. coli* (UPEC) strain 536 (O6:K15:H31) originating from a patient suffering from urinary tract infection was used for induction of bladder infection (Berger et al., 1982). The generation of GFP-expressing UPECs (Engel et al., 2006) and the murine urinary tract infection model were described previously (Engel et al., 2008; Tittel et al., 2013; Tittel et al., 2011; Tittel et al., 2012). (See also Extended Experimental Procedures)

Flow Cytometry

Single-cell suspensions were generated by digestion with 1 mg/ml collagenase IV (Sigma) and 100 μg/ml DNaseI (Sigma) in FCS-containing RPMI for 30 min at 37°C under mild stirring. Fc receptors were blocked and cells were then incubated with titrated amount of antibodies (See the complete list of antibodies in the Extended Experimental Procedures). We performed flow cytometry on a FACSCanto II, LSR II and on a Fortessa (BD Biosciences) and analyzed the data with Flow-Jo software (Tristar).

Histology

Immunofluorescence microscopy in cryosections: we embedded bladders into Tissue-Tec (Sakura) and immediately froze it at −80°C. Frozen blocks were cut into 5 μm sections and mounted on poly-L-lysin-coated glass slides (Menzel) and fixed them with Aceton. Immunohistochemistry in paraffin-sections: Bladders were fixed in 10% IHC Zinc Fixative (BD Bioscience). Afterward the tissue was embedded in paraffin, cut into 5 μm sections and mounted on lysine-coated glass slides (Menzel). The sections were stained with hematoxylin and eosin or with the neutrophil-specific antibody Ly6G.

We analyzed slides using the CellR software (Olympus), the AIM and ZEN software (Zeiss) and IMARIS (Bitplane) on an IX71 microscope (Olympus), on a laser scanning confocal microscope LSM 510 Meta (Zeiss) and on a LSM 780 (Zeiss). A complete list of antibodies is provided in the Extended Experimental Procedures.

Bone Marrow Chimeras and Adoptive Transfer of Leukocytes

Bone marrow chimeric animals were generated by irradiating mice with 9Gy and reconstitution with 5×10^6 donor BM cells injected into the tail vein. To adoptively transfer bone-marrow-derived macrophages and neutrophils, a MACS isolation procedure was established, which is described in detail in the Extended Experimental Procedures.

Chemokine and Cytokine Analysis

The levels of CXCL2, MIF, CXCL1, CXCL5, CCL2, and CXCL2 in bladder homogenates were measured by ELISA (MM200, DY1978, MKC00B, MX000, MJE00, MM200 R&D Systems) according to the manufacturer's protocol. The expression of chemokines and cytokines in the infected bladder was determined using the Th1/Th2 (BMS820FFRTU, eBioscience) and the chemokine Flow Cytomix Multiplex Kit (BMS821FF, eBioscience).

In Vitro Assays

Neutrophils were isolated as described in the Extended Experimental Procedures and cultured in RPMI 1640 media containing 1% FCS. To analyze the MMP-9 levels, cells were stimulated with 10 ng/ml TNF (eBioscience) and/or 10 ng/ml CXCL2 (AbD Serotec) for 6 hr. Afterward the supernatants were analyzed for MMP-9 level by zymography. (See also Extended Experimental Procedures).

Analysis of MMP-9 Activity by Zymography

Zymograms were performed as described previously (Vandooren et al., 2013). Briefly, proteins were separated on an SDS-gel containing 0.1% gelatin. After different incubation procedures (See Extended Experimental Procedures) the gel was stained with Coomassie and digitally analyzed using a Canon LIDE scanner. The bands were quantified by densitometry using ImageJ software. Alternatively, MMP9 activity was measured with the Biotrack MMP9 activity assay (RPN2634, GE Health Care).

Statistical Analysis

Results are expressed as mean ± s.e.m, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Comparisons were made using nonparametric Mann-Whitney or Kruskal-Wallis test for comparing two or more groups, respectively, in combination with Dunn's posttest using Graphpad Software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.01.006.

AUTHOR CONTRIBUTION

M.S., C.W., L.F. and D.R.E. designed and performed the main experiments. S.G., A.D., S.T., J-M.P., N.J.M., C.M-S, K.L, T.Q., and M.F. performed further experiments. G.B., M.J.L., G.O., J.B., R.B., U.P., W. Kolanus, H.-J. G., N.G., W. Kastenmüller, and P.A.K. contributed essential analytic tools and edited the manuscript. C.K. and D.R.E conceived and supervised the study, interpreted the results and wrote the paper. All authors discussed and interpreted the results.

ACKNOWLEDGMENTS

The authors thank Simon Teteris and Katharina Hochheiser for helpful suggestions, Juliane Maurer, Christina Ohliger, Tanja Töpfer, Chrystel Llanto, Andreas Dolf, Peter Wurst, Greet Thijs, Manfred Dewor, and Hongqi Lue for technical assistance and H. Bluethmann for $Tnfr^{-/-}$ mice. We acknowledge support by the Central Animal Facilities of the Medical Faculty Bonn and the Flow-Cytometry Core Facility. This work was supported by intramural Bonfor fellowships to D.R.E and C.W., the Deutsche Forschungsgemeinschaft (Grants En984/1-1, KFO228, SFB704, SFBTR57, Excellence Cluster ImmunoSensation, Gottfried Wilhelm Leibniz-Price to C.K.), the European Union 7th Framework Program (FP7/2007-2013) grant agreement n° 261382 (INTRICATE), NIH AI042310 and the Geconcerteerde Onderzoeksacties (GOA 2013/014) at the KU Leuven.

Received: June 28, 2013
Revised: October 21, 2013
Accepted: January 6, 2014
Published: January 30, 2014

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