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Crosstalk between sentinel and helper macrophages permits neutrophil migration into infected uroepithelium

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Abstract

Neutrophils are potent immune effectors against bacterial infections. Macrophages are important in infections as effectors and regulators, but their exact roles, phenotypic characterization and their relation to neutrophils is incompletely understood. Here we report in a model of bacterial urinary tract infection, one of the most prevalent bacterial infections that tissue-resident $Ly6C^{-}$ macrophages recruited circulating neutrophils and inflammatory $Ly6C^{+}$ macrophages through chemokines. Neutrophils were primarily recruited through ligands of the chemokine receptor CXCR2, in particular by CXCL1 and less by macrophage migration inhibitory factor (MIF), but not through CXCL5 and CXCL2. Neutrophils, but not $Ly6C^{+}$ macrophages, cleared the bacteria by phagocytosis. $Ly6C^{+}$ macrophages instead performed a regulatory function: in response to the infection, they produced the cytokine tumor necrosis factor (TNF), which in turn caused the resident macrophages to secrete CXCL2. This chemokine induced the secretion of matrix

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Author Contributions

M.S., C.W., L.F. and D.R.E. designed and performed the experiments. S.G. A.D., S.T., T.Q., and M.F. performed further experiments. U.P., H.-J. G., W.K. G.O, J.B., H.-J. G., P.A.K. and R.B. 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 results.

metalloproteinase-9 (MMP-9) in neutrophils and allowed these cells to degrade the uroepithelial basement membrane, in order to enter the uroepithelium, the mucosal interface from where the bacteria invade the bladder. Thus, the phagocyte response against bacteria is a highly coordinated event, in which Ly6C^{-} macrophages act as sentinels and Ly6C^{+} macrophages as innate helper cells. In analogy with T helper cells (Th), we propose to name these helper macrophages (Ph) as they provide a second signal on whether to unleash the principal effector phagocytes, the neutrophils. This cellular triage may prevent 'false-positive' immune responses. The role of TNF as innate 'licensing' factor contributes to its central role in antibacterial immunity.

Keywords

Macrophages; chemokines; bacterial infection; neutrophils; matrix metalloproteinase; MIF; immunologic help

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There are two types of phagocytes: macrophages and neutrophils. Both cell types are critical for the defense against bacterial infections and form partnerships, but the exact nature of their collaboration is incompletely understood[1]. Neutrophils are bone marrow-derived, circulate through the blood and must be recruited into infected or inflamed tissues[1b]. They are attracted by ligands of the chemokine receptor CXCR1 or CXCR2, which include CXCL1, CXCL2, CXCL5[1c] and macrophage inhibitory receptor (MIF)[xy]. By contrast, all organs harbor a dense network of resident macrophages, which has been proposed to partially originate from yolk sac progenitors[2,2b]. In infections, the bone marrow produces inflammatory monocytes, which travel through the blood and emigrate to sites of infection, where they differentiate into macrophages[3]. The functions of inflammatory macrophages are thought to overlap with those of resident macrophages.

Urinary tract infections (UTI) are highly prevalent, affecting more than 25% of the population in developed countries, especially young females[4,5]. They mainly result from uropathogenic *E. coli* (UPEC) ascending through the urethra into the bladder, where they invade the bladder uroepithelium[6]. UPECs can persist intracellularly and cause relapsing infections[7]. In humans, their ascension into the kidney causes pyelonephritis that may progress to renal failure[8]. The defense against UTI depends on TLR/MyD88 signaling[9], the CXC chemokine receptors CXCR1 and CXCR2[1c] and on neutrophils[10], which phagocytose the UPEC. The role of macrophages in UTI is unclear.

Here we studied the roles of macrophages and of neutrophils in a mouse model of UTI induced by transureteral instillation of UPECs. Bladders of uninfected mice contained only resident macrophages defined by expression of the marker F4/80 and by the lack of Ly6C (Fig. 1a, Supplementary Fig. 1). Already 2 hours after infection, F4/80^{-} Ly6C^{+} neutrophils started infiltrating the bladder and F4/80^{+} Ly6C^{+} inflammatory macrophages followed at 6 hours after infection (Fig. 1b). Ly6C^{-} macrophage numbers remained largely constant during the course of infection (Fig. 1b). Histological analysis demonstrated neutrophils, and some Ly6C^{-} macrophages within the uroepithelium, whereas Ly6C^{+} macrophages were confined to the lamina propria (Fig. 1c, d). When we infected mice with UPECs expressing recombinant green fluorescent protein (GFP) and determined bacterial uptake by flow

cytometry, almost 90% of the phagocytosed UPECs were detected within neutrophils (Fig. 1e), consistent with their known critical role in UTI[10].

We reasoned that most likely the bladder-resident Ly6C$^-$ macrophages recruited the circulating phagocytes. However, proving this hypothesis was difficult, because no techniques exist to selectively remove Ly6C$^-$ macrophages, and because vesical Ly6C$^-$ macrophages are not targeted by general macrophage depletion techniques like clodronate liposomes (Supplementary Fig. 2). Instead, we decided to block the chemokines that Ly6C$^-$ macrophages would predictably use to recruit inflammatory phagocytes. We first identified these mediators by intracellular flow cytometry. At 6 hours after infection, only Ly6C$^-$ macrophages produced the neutrophil attractors MIF, CXCL1, CXCL2 and CXCL5 (Fig. 2a, b). When we blocked these chemokines with neutralizing antibodies, CXCL1 was a critical chemokine and similarly important as CXCR2 for neutrophil recruitment, whereas CXCL2 and CXCL5 played minor roles (Fig. 2c). As a further control, Ly6C$^+$ macrophage numbers were substantially reduced in *Ccr2*-deficient mice (Fig. 2d), whose ligand CCL2 was produced by Ly6C$^-$ macrophages as well (Fig. 2a,b). Also neutrophils produced some CCL2 (Fig. 2a,b) and consistently, Ly6C$^+$ macrophage numbers were reduced also in *Cxcr2*-deficient mice (Fig. 2d). Bacterial clearance of the bladder was impaired in the absence of CXCL1, CXCL2, MIF, CXCR2, CCR2 and after depletion of Ly6C$^+$ macrophages by clodronate-liposomes, but not after blocking CXCL5 (Fig. 2e). Thus, Ly6C$^-$ macrophages rapidly produced chemokines that attracted neutrophils and Ly6C$^+$ macrophages into the infected bladder, and both cell types were important for antibacterial defense.

The importance of Ly6C$^+$ macrophages was astonishing given that these cells hardly contributed to bacterial phagocytosis (Fig. 1e). We noted that these cells were the main producers of tumor necrosis factor (TNF) (Fig. 2a, b), a cytokine considered generally important in bacterial infections[11]. Several TNF functions have been reported in other models, for example inducing neutrophil degranulation[12], phagocytosis[12,13] and bactericidal activity[13]. The role of TNF in UTI is unknown. When we infected *Tnfr*-knockout (KO) mice deficient for both TNF receptors, the bacterial load in the bladder was much higher than in wild-type controls (Fig. 2e). Also the number of infected uroepithelial cells, which indicates persistence of UTI, was severely increased in *Tnfr*-KO mice and in the absence of the TNF-producing Ly6C$^+$ macrophages (Fig. 2f). Moreover, over a period of 6 weeks, more relapses of higher severity occurred (Fig. 2g). But strikingly, the absolute number of neutrophils, was not reduced in *Tnfr*-KO mice (Fig. 2c), suggesting that the antibacterial function of TNF is distinct from recruiting neutrophils.

Since TNF was reported to activate neutrophils[11], we first hypothesized that it might activate their antibacterial effector mechanisms. However, *Tnfr*-deficient neutrophils still phagocytosed UPECs *in vitro* (Supplementary Fig. 3) and maintained activity of the antibacterial effector molecules elastase and myeloid peroxidase (Supplementary Fig. 3). Moreover, adding TNF did not enhance the bactericidal activity of neutrophils (Supplementary Fig. 3). These findings argued against TNF controlling neutrophil activity. Given that TNF was important for antibacterial activity only *in vivo* but not *in vitro*, we next hypothesized that it might impact neutrophil positioning within the bladder. Indeed, histology revealed that neutrophils were excluded from the uroepithelium of *Tnfr*-KO mice,

the location from where UPECs enter (Fig. 3a–c and Supplementary Fig. 4). Less neutrophils were detected also in the urine of *Tnfr*-KO mice and after depleting the TNF-producing Ly6C$^+$ macrophages (Fig. 3d), consistent with their inability to enter and transmigrate through the uroepithelium into the bladder lumen. When we reconstituted *Tnf*-KO mice with the main TNF producers, Ly6C$^+$ macrophages (Supplementary Fig. 4), neutrophils did enter the uroepithelium. This is a verification that these cells permitted epithelial migration of neutrophils.

We next asked whether such migration required TNF to directly act on neutrophils, by cotransferring neutrophils from *Tnfr*-deficient and –competent mice into infected wild-type mice. However, *Tnfr*-KO neutrophils entered the bladder as efficiently as the controls, and appeared in similar numbers in the uroepithelium (Supplementary Fig. 5), indicating that TNF acted on TNFR$^+$ cells other than neutrophils. To identify this cell, we first generated bone marrow chimeras in which hematopoietic cells lacked either the TNFR or TNF (Supplementary Fig 6). Transepithelial neutrophil migration required TNFR expression and TNF secretion by bone marrow-derived cells (Fig. 3e and Supplementary Fig. 6). Therefore, we next examined whether the expression of molecules known to affect neutrophil migration are expressed 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, ICAM-1 (Fig. 4a, b, c and Supplementary Fig. 7) were similarly expressed in infected *Tnfr*-KO and wild-type mice. Only CXCL2 was markedly reduced in *Tnfr*-KO mice (Fig. 4d). This is consistent with a previous report that this chemokine is required for uroepithelial neutrophil migration[14]. As only Ly6C$^-$ macrophages produced CXCL2 in the infected bladder (Fig. 2a, b), we concluded that TNF acted on these macrophages, causing these to produce CXCL2. If so, then the absence of TNF or of its source, the Ly6C$^+$ macrophages, should abrogate CXCL2 production. Indeed, this chemokine was almost undetectable in the infected bladder after depleting Ly6C$^+$ macrophages and in *Tnf* or *Tnfr*-deficient mice (Supplementary Fig. 8). Furthermore, transurethral inoculation of CXCL2 into the bladder of infected *Tnf*-KO mice restored the ability of neutrophils to enter the uroepithelium (Fig. 4e and Supplementary Fig. 9), confirming that CXCL2 can induce such migration and that it acted downstream of TNF. At later time points, CXCL2 might also be produced by neutrophils, consistent with previous reports[15]. Our findings at early time points suggest this might represent an autocrine feedback loop induced after neutrophils sensed CXCL2 from Ly6C$^-$ macrophages.

Next, we asked how CXCL2 enabled neutrophils to enter the uroepithelium. Epithelial basement membranes are comprised mainly of collagen IV, which is first cleaved in two fragments by e.g. neutrophil collagenase or matrix metalloproteinase-8 (MMP-8)[15b]. Thereafter, this denatured collagen is further cleaved by gelatinase B/MMP-9[15c,16]. The role of MMP-9 in UTI is unknown. *Mmp-9*-KO mice showed a markedly higher bacterial load in the urine (Fig. 4f), indicating that MMP-9 is required for defense against UTI. Histological sections of infected *Mmp-9*-KO mice showed that neutrophils accumulated underneath the uroepithelium and failed to enter it (Fig. 4g and Supplementary Fig. 10) or to transmigrate into the bladder lumen (Fig. 4h). When we stained bladder cells for MMP-9 expression, more than 90% of the MMP-9$^+$ cells were neutrophils (Supplementary Fig. 11). To test

whether neutrophil-intrinsic MMP-9 was important, we transferred MMP-9-competent neutrophils into infected *Mmp-9*-KO mice. Only competent neutrophils were detected within the uroepithelium, but not the endogenous *Mmp-9*-KO neutrophils (Fig. 4i and Supplementary Fig. 12), confirming that neutrophil-intrinsic MMP-9 was required for crossing the uroepithelial basement membranes.

The migratory defect of neutrophils lacking MMP-9 was analogous to that in TNFR-KO (Fig. 3a, b), suggesting a direct or indirect causal connection between TNF and MMP-9. We tested this by measuring MMP-9 protein levels in neutrophils in TNFR-KO and wild-type mice. By ELISA for MMP-9 we only observed a trend, but no significant effect of TNF (Supplementary Fig. 13). We corroborated these findings by in gel zymography analysis[16b]. In line with the above observations MMP-9 levels were reduced by 70–80% in TNFR-KO and TNF-KO animals (Fig. 4j,k). A similar reduction was seen in mice depleted of $Ly6C^+$ macrophages, the main TNF producers (Fig. 4j,k), indicating that TNF from $Ly6C^+$ macrophages caused the induction of MMP-9. However, our finding that TNFR expression by neutrophils was dispensable for transepithelial migration (Supplementary Fig. 5) implied that a downstream mediator, such as CXCL2, induced the secretion of MMP-9 in neutrophils. We tested this by stimulating neutrophils directly with CXCL2 and measuring their gelatin zymolytic level[16b]. CXCL2 increased MMP-9 levels in wild-type and TNFR-KO, but not in *Cxcr2*-KO neutrophils (Supplementary Fig. 14). By contrast, TNF was unable to induce MMP-9 in neutrophils (Supplementary Fig. 14). This confirmed that CXCL2, and not TNF, activated neutrophils to release MMP-9. To test for *in vivo* relevance in UTI, we inhibited CXCL2 in infected mice with a blocking antibody. This reduced MMP-9 levels in the bladder substantially (Fig. 4j, k) and aggravated UTI (Fig. 2e). By contrast, levels of MMP-2, which has also been implicated in gelatin degradation[16], was not regulated by TNF, $Ly6C^+$ macrophages or by CXCL2 (Supplementary Fig. 15). Thus, TNF-induced CXCL2 specifically regulated MMP9 in UTI.

Taken together, our findings document the following sequence of events (Supplementary Fig. 16): 1) resident $Ly6C^-$ macrophages sense the infection and produce chemokines including CXCL1, MIF and CCL2, which recruit neutrophils and $Ly6C^+$ macrophages; 2) recruited $Ly6C^+$ macrophages produce TNF in response to the infection, 3) TNF induces CXCL2 production by $Ly6C^-$ macrophages, 4) CXCL2 causes MMP-9 secretion in neutrophils, which allows these cells to cross the epithelial basement membrane in order to combat the infection.

These results demonstrate that the antibacterial neutrophil response is coordinated by two macrophage subsets with distinct tasks: The $Ly6C^-$ macrophages act as tissue-resident sentinels and attract circulating phagocytes with chemokines. The $Ly6C^+$ macrophages do not directly participate in bacterial elimination, but instead play a hitherto unknown regulatory role. In response to the infection, they license the $Ly6C^-$ macrophages to send neutrophils into the frontline of infection, the uroepithelium. The reliance on cues from helper cells is known from adaptive immunity. For example, dendritic cells can cross-prime cytotoxic $CD8^+$ T lymphocytes (CTL) against viral infections only after having been licensed by $CD4^+$ T helper cells or NKT cells[17–19]. This causes secretion of chemokines binding CCR5 or CCR4, respectively, which attract the CTL towards 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 towards virus-infected cells[20]. Our findings reveal an analogous role for the crosstalk between Ly6C$^-$ and Ly6C$^+$ macrophages in guiding neutrophils within bacterial infected tissues by coordinated chemokine production.

The chemokines that bind CXCR2 are often considered redundant. 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 played a non-redundant role in epithelial migration[14]. Epithelial immune cell migration is less well understood than endothelial migration[21,22]. In endothelial migration towards chemotactic cues, MMP-9 has been shown to be a determinant[22b] already long ago. 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 MMP-9, which assists in collagen IV degradation explains how neutrophil can penetrate such barriers. These findings identify MMP-9 as a CXCL2-regulated gate-keeper for tissue barriers.

The factor by which Ly6C$^+$ macrophages licensed Ly6C$^-$ macrophages for CXCL2 production was TNF, a cytokine well known to be critical in bacterial infections[11]. Consequently, pharmacological TNF blockade in patients with rheumatoid arthritis or inflammatory bowel disease causes relapses of *mycobacterium tuberculosis* infection[23], and is correlated with a higher incidence of pneumonia, and, notably, also of UTI[24–28]. Our findings in TNF-deficient mice can explain the latter association. Furthermore, these mice also showed more intraepithelial bacterial communities, which underlie the frequent relapses of bacterial cystitis, and more infectious relapses. Our findings show that TNF is required for their control. It remains to be seen whether CXCL2-induction is important also in other TNF-dependent bacterial infections, for example listeriosis[29] or tuberculosis[23].

The ability of intravesically applied CXCL2 to restore transepithelial migration of TNF-deficient neutrophils suggests therapeutic opportunities in patients with relapsing UTI, especially after treatment with TNF blockers. The topical application of chemokines has recently been shown to improve local CTL responses against genital herpes virus[30]. Accordingly, 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 a coordinated triage 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. The reliance on the agreement between several immune cells can reduce the likelihood of inducing ‘false-positive’ immune responses and immunopathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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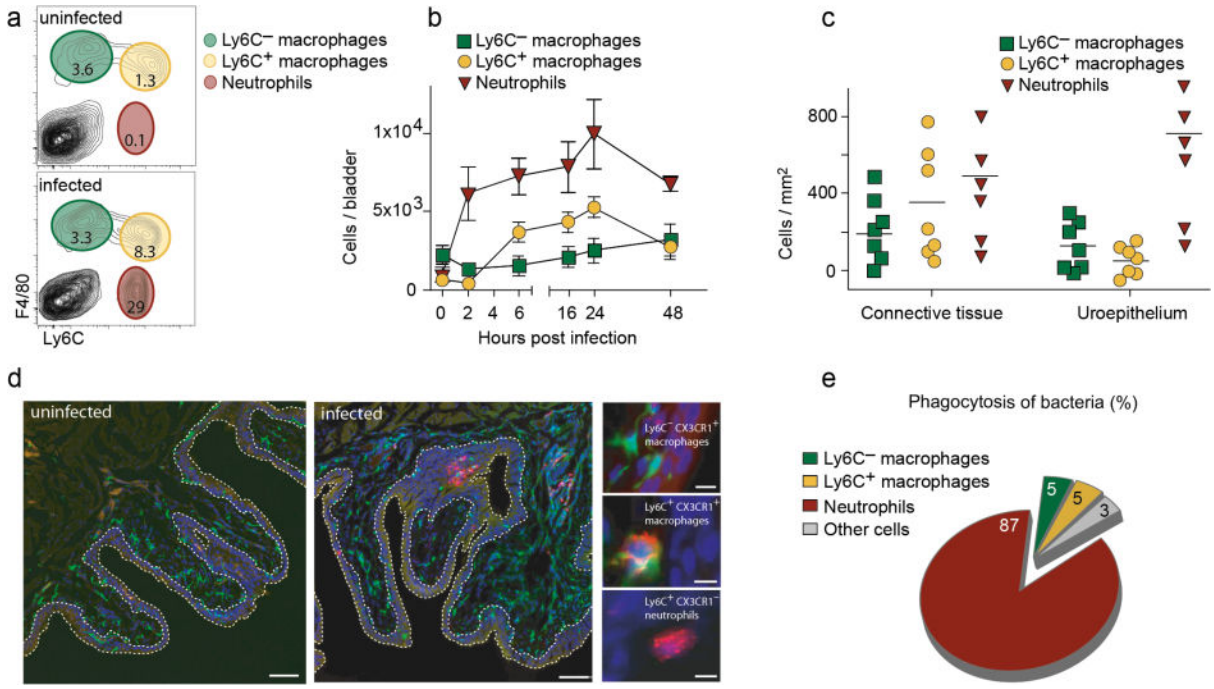

Figure 1. Recruitment and positioning of macrophages and neutrophils in bacterial UTI

a, Flow cytometry analysis of bladder homogenates of uninfected and infected mice 24 hours 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**, Numbers of neutrophils, Ly6C$^+$ and Ly6C$^-$ macrophages in bladder cryosections 24 hours after infection. **d**, Cryosections from bladders of uninfected and infected $CX_3CR1^{+/GFP}$ mice, which express green fluorescent protein (GFP) in macrophages[31], were stained for Ly6C (red) and for cellular nuclei (DAPI-blue). The white dashed line highlights the uroepithelial border. The small images show representative stainings of the three phagocyte types. **e**, Contribution of phagocyte subsets 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 ± s.e.m. and represent five (**a, b, e**) and three (**c, d**) independent experiments in groups of five mice.

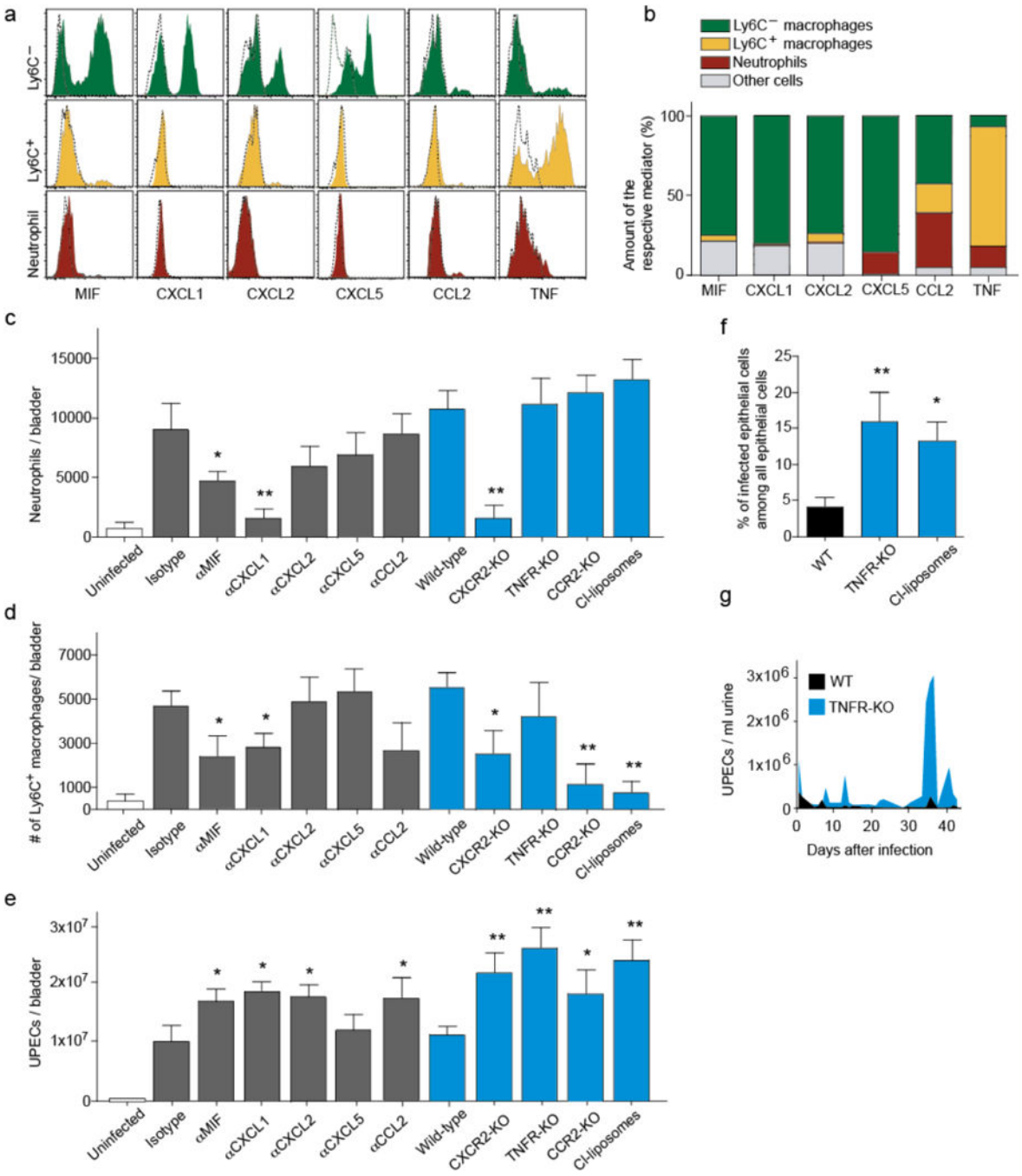

Figure 2. Chemokines produced by Ly6C^{-} macrophages recruit Ly6C^{+} macrophages and neutrophils into the infected bladder

a, Intracellular flow-cytometric staining of Ly6C^{-} (green) and Ly6C^{+} (yellow) macrophages and neutrophils (red) 6 hours after infection for immune regulatory molecules (solid line) and the respective isotype control (dashed line). **b**, Contribution of phagocyte subsets to the total production of immune regulatory molecules, calculated using the formula ((mean fluorescence intensity of antibody^{+} cells – mean fluorescence intensity of isotype^{+} cells) × number of cells positive for the molecule)) and giving the proportion within the total

contribution of all bladder cells. **c–e**, Intravesical numbers of neutrophils (**c**), Ly6C$^+$ macrophages (**d**) and UPECs (**e**) 6h after infection in mice treated with the isotype control or antibodies against the immune regulatory molecule (grey) and in wild-type controls or in deficient animals (blue). **f**, Mice were infected with GFP-expressing UPECs and the percentage of infected GFP$^+$ epithelial cells were determined. **g**, Relapsing infections in wild-type (WT) and TNFR-KO mice analyzed by counting the number of UPECs within the urine. Data are means ± s.e.m. and represent three independent experiments in groups of three to five mice. *P < 0.05; **P < 0.01.

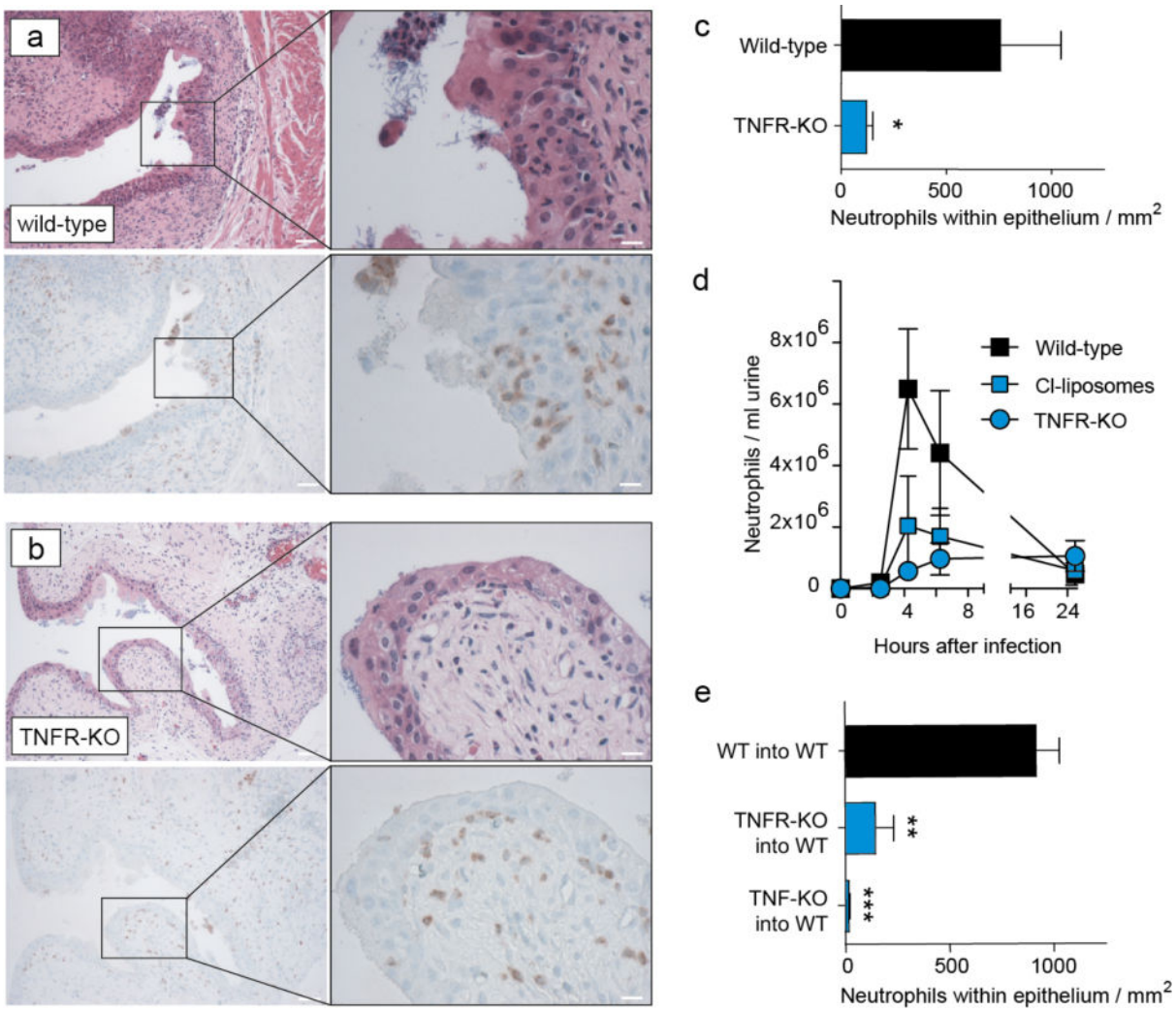

Figure 3. TNF from Ly6C$^+$ macrophages enables neutrophils to enter the uroepithelium

a, b, Presence of neutrophils in the urothelium in consecutive bladder sections one day after infecting wild-type (**a**) or TNFR-KO (**b**) mice, identified by HE (upper row) or Ly6G (brown staining in the lower panel row) staining. The right panels show fivefold magnifications of the inlay highlighted in the left column with a black box. White lines are 50μm (left) or 10μm (right). **c**, Quantitative analysis of the histology shown in (**a**) and (**b**). **d**, Kinetics of neutrophil numbers in the urine of wild-type or TNFR-KO mice or after depleting Ly6C$^+$ macrophages with clodronate liposomes (Cl-liposomes) determined by flow-cytometry. **e**, Neutrophil numbers in bladder sections 1 day after infection in bone marrow chimeric mice expressing TNF or TNFR in all cells (WT into WT) or only in non-hematopoietic cells (TNFR-KO into WT, TNF into WT, respectively). Data are means ± s.e.m. and represent four (**a–d**) and three (**e**) independent experiments in groups of five to eight mice. *P < 0.05; **P < 0.01, ***P < 0.01.

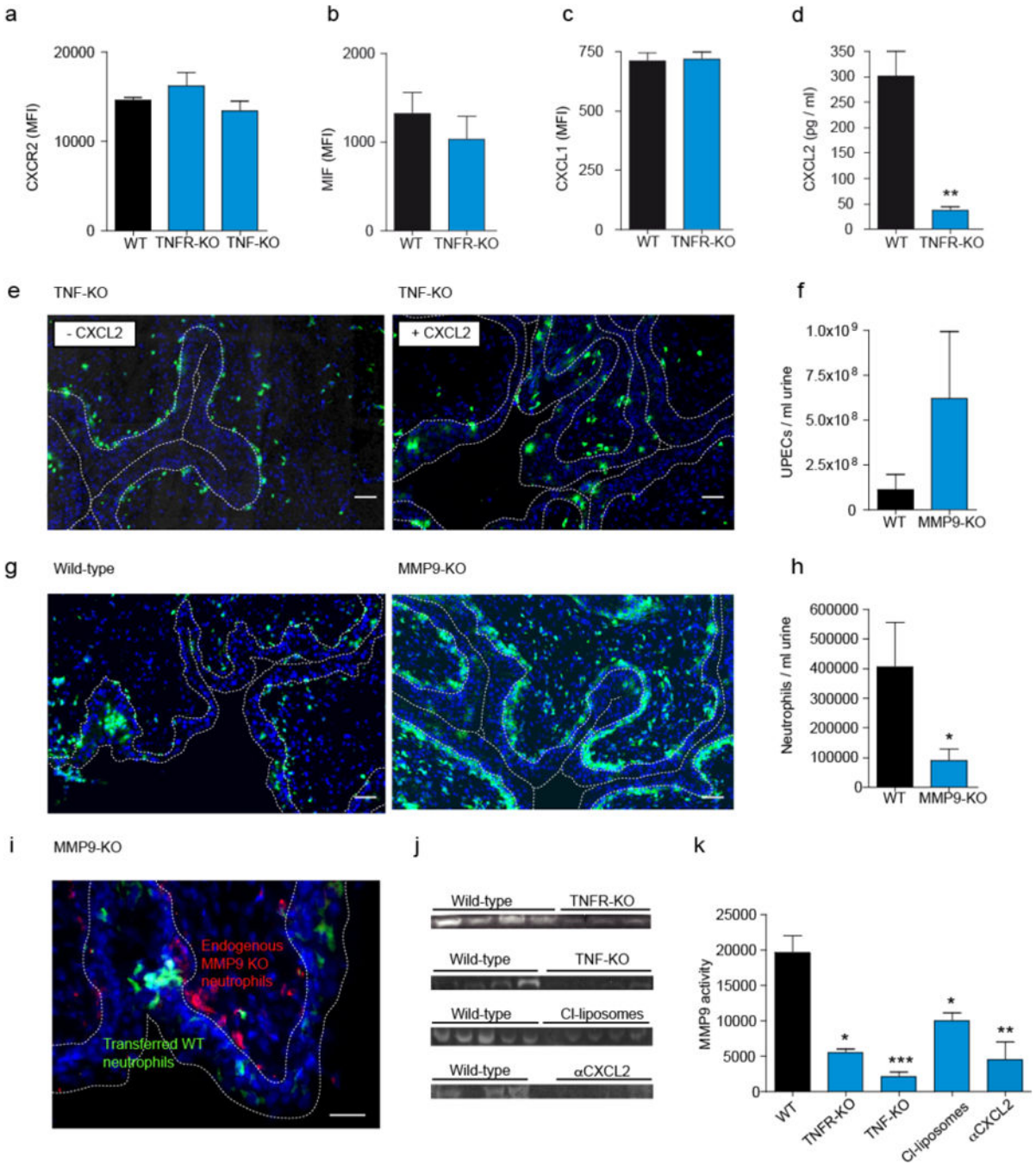

Figure 4. TNF induces CXCL2 in Ly6C$^-$ macrophages, which activates MMP9 in neutrophils

a–d, Expression of CXCR2 by neutrophils (**a**), and of MIF (**b**), CXCL1 (**c**) and CXCL2 (**d**) by Ly6C$^-$ macrophages in wild-type (WT), TNFR- or TNF-KO mice, determined by flow-cytometry (MFI=Mean fluorescence intensity) (**a–c**) or ELISA (**d**). **e,g**, Positioning of neutrophils in bladder sections of, wild-type TNF- (**e**) or MMP9-KO (**g**) mice one after infection, revealed by staining with Ly6G Alexa488 (green). The white dashed line highlights the uroepithelial border. **f,h**, Numbers of UPECs (**f**) and neutrophils (**h**) in the urine of WT and MMP9 KO mice. **i**, Inability of endogenous neutrophils in MMP-9-KO

mice, stained with red Ly6G Alexa568, to enter the infected uroepithelium, in contrast to adoptively transferred MMP-9-competent neutrophils stained with CFSE (green). **j,k**, MMP-9 levels in bladder homogenates one day after infection determined by zymography (**j**) and numerically displayed after analysis by densitometry (**k**). Data are means ± s.e.m. and represent 3 independent experiments in groups of 5–8 mice. *P < 0.05; **P < 0.01; ***P < 0.001.