CCR2 Mediates Homeostatic and Inflammatory Release of Gr1^{high} Monocytes from the Bone Marrow, but Is Dispensable for Bladder Infiltration in Bacterial Urinary Tract Infection[1]

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CCR2 is thought to recruit monocytes to sites of infection. Two subpopulations of murine blood monocytes differing in Gr1 and CCR2 expression have been described. The exact role of CCR2 in migration of CCR2^{low}Gr1^{low} and CCR2^{high}Gr1^{high} monocytes into nonlymphoid tissue is controversial. In this study, we have addressed this question in a murine model of bacterial urinary tract infection. Only Gr1^{high} monocytes were recruited into the infected bladder. CCR2 deficiency reduced their frequency in this organ, indicating a requirement of this chemokine receptor. Importantly, CCR2-deficient mice also showed reduced Gr1^{high} monocyte numbers in the blood, but not in the bone marrow (BM), indicating that CCR2 acted at the step of monocyte release into the circulation. The same was found also in noninfected mice, indicating a further involvement of CCR2 in steady-state BM egress. An additional requirement of CCR2 in monocyte recruitment from the blood into the bladder was excluded by tracking particle-labeled endogenous monocytes and by adoptive transfer of BM-derived monocyte subsets. These findings demonstrate that CCR2 governs homeostatic and infection-triggered release of Gr1^{high} monocytes from the BM into the blood but is dispensable for recruitment into a nonlymphoid tissue. ***The Journal of Immunology,*** **2008, 181: 5579–5586.**

Monocytes and macrophages are characterized by a lack of lymphocyte markers and by expression of CD11b and CD14 in humans and of CD11b, CD115 (M-CSF receptor), and F4/80 in mice (1–5). Their precursors are released from the bone marrow (BM)[3] (1, 5, 6) into the bloodstream as nondividing monocytes. Murine monocytes can be subdivided by their expression of Gr1 and of the chemokine receptors CCR2 and CX_3CR1 (1). Gr1^{high}CCR2^{high}CX_3CR1^{low} monocytes are relatively large, short-lived, and actively enter inflamed tissue (1, 2). Gr1^{low}CCR2^{low}CX_3CR1^{high} monocytes are smaller and less granular, patrol blood vessels (7, 8), and home also to noninfected tissues (1, 2). It has been shown that these monocytes can develop from Gr1^{high} monocytes in the blood (9). Both subsets can give rise not only to macrophages but also to dendritic cells (DC) (2, 3, 8–10).

The role of chemokine receptors in regulating monocyte migration is under intense investigation (11). CXCR4 retains their precursors in the BM when its ligand SDF-1 is available (12, 13). CCR2 is required for monocyte recruitment into inflamed or infected tissue, as evidenced by a major reduction of infiltrating monocytes in CCR2$^{-/-}$ mice (14–16) and aggravated infection (17, 18). This has been particularly well studied in infection with *Listeria monocytogenes*, a Gram-positive rod that targets the spleen (15, 17–19). Likewise, inflammatory diseases involving aberrant monocyte recruitment were attenuated in CCR2$^{-/-}$ mice (20–22). Gr1^{high} inflammatory monocytes, which are particularly well recruited to sites of infection, express high levels of CCR2 (1, 17). Thus, it has been concluded that CCR2 mediates entry of monocytes into infected tissue. This conclusion is supported by several in vitro studies demonstrating CCR2-mediated attachment of monocytes to inflamed endothelial cells (23, 24) and local upregulation of the CCR2 ligand MCP-1 in inflammatory conditions (25). Several inhibitors of CCR2 or its ligand MCP-1 have been developed and are currently being tried in various diseases such as rheumatoid arthritis, multiple sclerosis, atherosclerosis, asthma, or in graft-vs-host diseases (20, 25–28).

A recent study has challenged this view by demonstrating that CCR2$^{-/-}$ mice infected with *Listeria monocytogenes* showed diminished monocyte numbers in the spleen and in the circulation (17). It was proposed that CCR2 mediates egress of BM progenitors into the circulation rather than into infected tissues (17, 29). Studies testing this hypothesis in infection models other than listeriosis are missing. In particular, immigration into nonlymphoid tissues, where the endothelium needs

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Received for publication June 4, 2008. Accepted for publication August 16, 2008.

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[1] This work was supported by the Deutsche Forschungsgemeinschaft (Grants Ku1063/4, Ku1063/5, Ku1063/6, and Ta434/2-1).

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[3] Abbreviations used in this paper: BM, bone marrow; UPEC, uropathogenic *Escherichia coli*; UTI, urinary tract infection; PMN, polymorphonuclear neutrophilic granulocyte; DC, dendritic cell; clo-lip, clodronate-loaded liposome; Lx, latex.

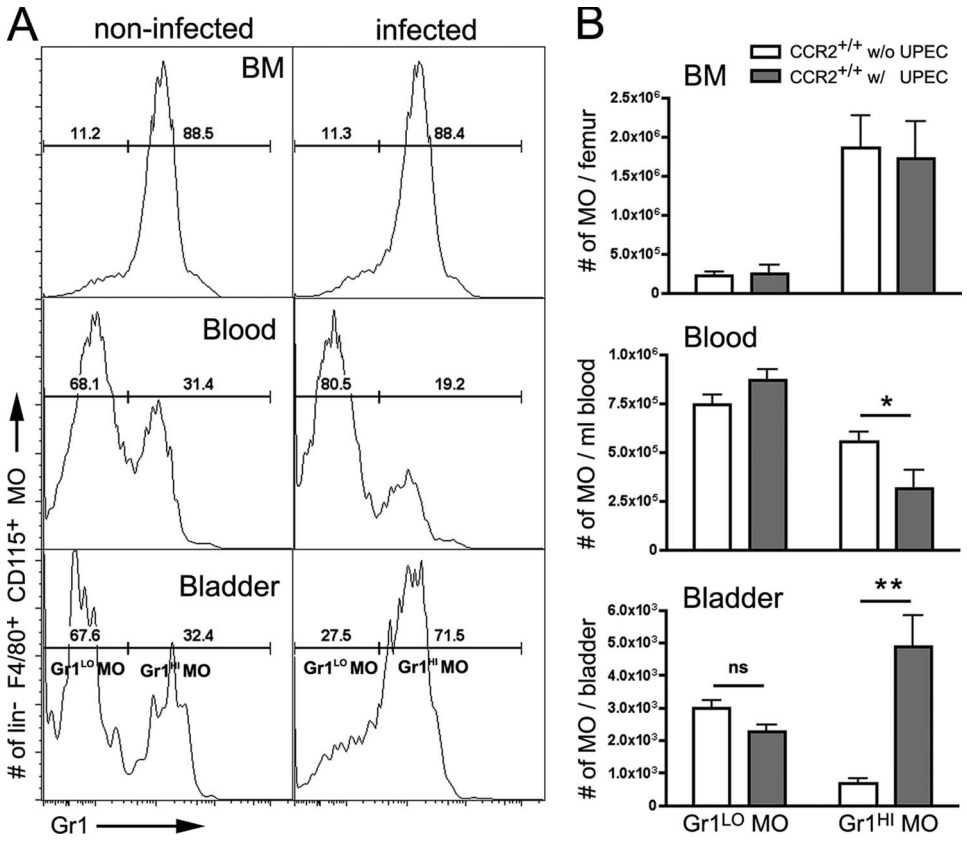

FIGURE 1. $Gr1^{high}$, but not $Gr1^{low}$ monocytes are recruited from the blood into the infected bladder. A total of 14 h after transurethral instillation of C57BL/6 mice with UPECs (infected) or PBS (noninfected), cells from the BM, the blood and the bladder were isolated and $CD115^+F4/80^+Lin^-$ (CD3, B220, NK1.1) monocytes were analyzed for Gr1 expression. *A*, Histograms of typical Gr1 expression by these cells in the respective organs. *B*, Mean ± SD of the numbers of $Gr1^{low}$ and $Gr1^{high}$ monocytes in the BM, blood, and bladder of noninfected (white bars) and infected (gray bars) mice (n = 3–4 mice per group). Data are representative of three individual experiments. MO, Monocyte.

to be crossed by monocytes, has not been examined yet. In arteriosclerosis, a role of CCR2 in monocyte infiltration into inflamed vessel walls has been reported (29). However, it has recently been reported that $CCR2^{low}Gr1^{low}$ monocytes were rapidly recruited in bacterial peritonitis, suggesting that CCR2-independent recruitment pathways can operate (7). The exact role of CCR2 in monocyte migration remains to be clarified.

Urinary tract infections (UTI), such as cystitis and pyelonephritis, are among the most prevalent infections and account for considerable morbidity and medical costs (30). Most of these infections are caused by invasive Gram-negative uropathogenic *Escherichia coli* (UPEC) that ascend through the urinary tract (31, 32). Polymorphonuclear neutrophilic granulocytes (PMN), monocytes, and DC are rapidly recruited to the inflamed bladder (33), but only the former two cell types contribute to the early innate defense (33). Although observations in other infection models suggest that CCR2 may recruit monocytes also in UTI, we recently found that this chemokine receptor was not essential for bacterial clearance in mice (33). It is unknown whether this chemokine receptor is required for monocyte recruitment in UTI and, if so, whether it operates at the BM/blood or at the blood/bladder barrier and whether it affects $Gr1^{low}$ and/or $Gr1^{high}$ monocytes. In this study, we have addressed these open questions using a murine model of UTI.

Materials and Methods

Mice and reagents

$CCR2^{-/-}$ mice had been backcrossed >10 times to the C57BL/6 background (14, 33) and were bred and kept under specific pathogen-free conditions. Animal experiments had been approved by the government and local animal ethics reviewing boards. Unless indicated otherwise, all reagents were obtained from Sigma-Aldrich.

UPEC and UTI model

The UPEC strain 536 (O6:K15:H31) originating from a UTI patient was used for infections. UPEC were cultured overnight under static conditions in LB medium, harvested by centrifugation at 1200 g for 5′ and resuspended in PBS to a concentration of 1×10^{10} CFU per ml. Anesthetized female mice of 8–10 wk of age were infected by transurethral inoculation of 5×10^8 *E. coli* 536 (0.05 ml) into the bladder using a soft polyethylene catheter (outer diameter 0.6 mm; BD Biosciences). Expression of the virulence factor FimH on UPECs, which is crucial for invasion into urothelial cells (34), was confirmed by mannose-sensitive hemagglutination of guinea pig erythrocytes (35) and by microscopic detection of UPEC within urothelial cells (data not shown). In ~20% of infected mice, UPEC could be grown also from kidney homogenate, but the number of CFU was ~1000-fold lower compared with a homogenate of the extensively rinsed bladder.

Isolation and analysis of leukocytes

The digestion protocol for isolation of nonlymphoid leukocytes (36, 37) was modified to isolate leukocytes from the bladder. In brief, bladders were sliced with a scalpel and digested for 30 min at 37°C with 0.5 mg/ml collagenase and 100 μg/ml DNase I in RPMI 1640 medium (Invitrogen) containing 0.5% heat-inactivated FCS (PAA Laboratories) and 20 mM HEPES. Cell suspensions were filtered through 100-μm nylon mesh and washed with $Ca2^+$ and $Mg2^+$-free HBSS containing 10 mM EDTA, 0.1% BSA, and 20 mM HEPES.

Whole blood was subjected to red cell lysis buffer and washed twice in DMEM medium (Invitrogen) containing 0.02% NaN_3, 2 mM EDTA, and 2% FCS. BM cells were obtained by flushing the femur with HBSS medium containing 0.1% BSA and 5 mM EDTA and then treated like blood cells.

The numbers of viable cells was determined by trypan blue staining. Fc receptors were blocked with mouse serum. Titrated amounts of the following labeled Abs from BD Pharmingen were used for staining of 1×10^6 cell samples: anti Gr-1 (RB6-8C5), anti CD11c (HL-3), anti F4/80-biotin (CI:A3-1) from Serotec, and anti CD115 (AFS98) from eBioscience. Monocytes and macrophages were identified as $CD11b^+$, $F4/80^+CD115^+$ cells deficient in expression of the lymphoid lineage (Lin) markers CD19, NK1.1, and CD3ε (1). PMN were identified as $F4/80^-Gr1^+SSC^{high}$ cells. Flow cytometry was performed on a LSR II

cytometer (BD Biosciences) and results were analyzed using Flow Jo software (Tristar). To calculate absolute cell numbers, 10×10^4 10 μm PerCP Cy5.5-labeled micro beads (BD Biosciences) were added to the measured tubes.

Depletion and labeling of blood monocytes

Clodronate-liposomes and control PBS-liposomes were prepared as described earlier (38). Blood monocytes were eliminated by i.v. injection of 250 μl clodronate-liposome suspension into the lateral tail vein. Blood monocytes were labeled by injection of 0.5-μm FITC-conjugated (yellow gold) plain beads (2.5% solid microspheres; Polysciences) (39). These beads were diluted 1/25 in PBS, and 250 μl of this suspension was injected i.v. for labeling of $Gr1^{low}$ monocytes. To label $Gr1^{high}$ monocytes, 250 μl of liposomes containing clodronate were i.v. injected, followed by 250 μl of fluorescent beads i.v. 16–18 h later (39).

BM transplantation

Recipient mice were irradiated with 9 Gy and reconstituted with 5×10^6 donor BM cells. For mixed BM chimeras, 50% of CCR2-deficient (CD45.2) and 50% of wild-type (CD45.1) BM were injected i.v.

Adoptive transfer of BM monocytes

BM cells from $CCR2^{-/-}$ and $CCR2^{+/+}$ mice were isolated as described above. For enrichment of BM monocytes, cell suspensions were incubated with anti Ly6G (clone 1A8; BD Biosciences) labeled to biotin using a commercial kit (Pierce) followed by incubation with MACS anti-biotin, anti-CD19, anti-CD8, and anti-CD4 beads (Miltenyi Biotec). After negative selection, the cell suspension comprised 75–85% of $Gr1^{high}$ and 5–10% $Gr1^{low}F4/80^+CD115^+$ monocytes. These cells were labeled with 10 μM CMTMR (Molecular Probes), and 2×10^6 $CCR2^{-/-}$ and $CCR2^{+/+}$ cells (ratio 1:1) were infused into infected mice. To exclude contaminations of bladder monocytes with blood cells, recipient mice were thoroughly perfused before analysis.

TNF-α ELISA

Bladders were homogenized in the presence of the protease inhibitors Complete Mini (Roche) in 2 ml PBS. Supernatants after centrifugation, as well as serum samples, were analyzed by a TNF-α ELISA kit (R&D Systems).

Statistical analysis

Prism (GraphPad) was used for statistical analysis. The unpaired Student's *t* test was used to compare groups; a *p*-value of less than 0.05 was considered significant. In all figures, * indicates *p*-value <0.05, ** $p < 0.01$, and *** $p < 0.001$.

Results

$Gr1^{high}$, but not $Gr1^{low}$, monocytes are recruited from the blood into the infected bladder

Blood monocytes can be subdivided into $Gr1^{low}CCR2^{low}$ and $Gr1^{high}CCR2^{high}$ subsets (1). We studied the mechanisms governing migration of these subsets in bacterial infection, using a murine model of UTI, induced by transurethral instillation of UPEC (33). The BM contained considerably more $Gr1^{high}$ than $Gr1^{low}$ monocytes both in noninfected and infected mice (Fig. 1) (17). In noninfected control mice, $Gr1^{low}$ monocytes were more frequent in the blood and the bladder (Fig. 1). A total of 14 h after UTI induction, $Gr1^{high}$ monocytes had decreased in the blood and increased the bladder, whereas numbers of $Gr1^{low}$ monocytes were not significantly altered (Fig. 1). These findings showed that only $Gr1^{high}$, but not $Gr1^{low}$, monocytes were recruited to the bladder in UTI.

CCR2 deficiency reduces $Gr1^{high}$ monocytes in the infected and noninfected bladder and also in the blood

CCR2 has been proposed to mediate recruitment of monocytes to sites of infection (14, 15, 17, 18). Since $Gr1^{high}$ monocytes have been shown to express more CCR2 (1), we investigated whether their preferential recruitment to the infected bladder (Fig. 1) was mediated by CCR2. Indeed, $Gr1^{high}$ monocytes

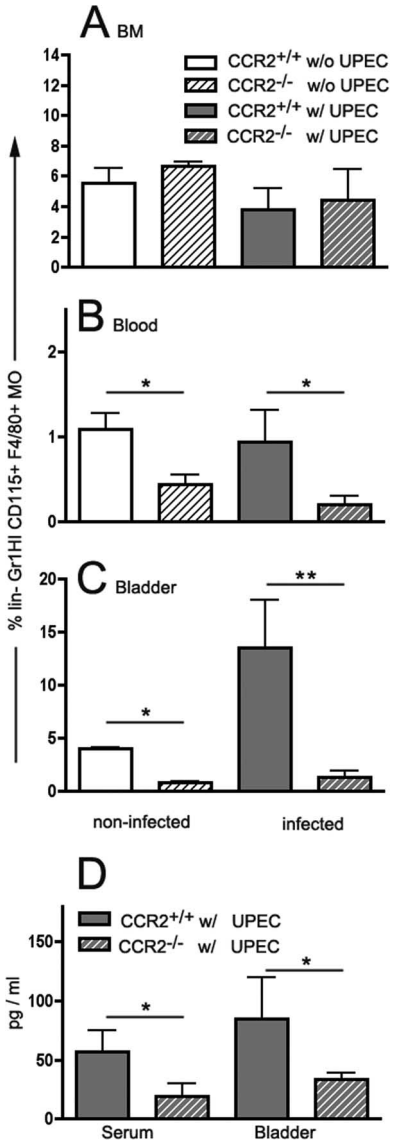

FIGURE 2. CCR2 deficiency reduces $Gr1^{high}$ monocytes in the infected and noninfected bladder, and also in the blood. $CCR2^{+/+}$ (not striped) and $CCR2^{-/-}$ (striped) mice were infected with UPECs. After 14 h, cells from the BM (*A*), the blood (*B*), and the bladders (*C*) of infected (gray) and noninfected (white) controls were isolated and stained for $CD115^+F4/80^+Lin^-$ (CD3, B220, NK1.1) and Gr1 monocytes. *D*, TNF-α concentrations in serum and bladder homogenate of infected $CCR2^{+/+}$ and $CCR2^{-/-}$ mice. Shown are the mean $\pm$ SD of groups of five mice (three mice in *D*). Results are representative of three individual experiments in *A–C*, and two in *D*. MO, Monocyte.

were less abundant in infected bladders of $CCR2^{-/-}$ mice (Fig. 2*C*). These cells were less frequent also in noninfected $CCR2^{-/-}$ mice as compared with noninfected wild-type controls (Fig. 2*C*), indicating an additional role for CCR2 in homeostasis.

Importantly, CCR2 deficiency reduced $Gr1^{high}$ monocytes also in the blood (Fig. 2*B*), whereas no significant differences were seen in the BM of CCR2-competent and -deficient mice (Fig. 2*A*). These observations raised the possibility that CCR2 acted at the step of $Gr1^{high}$ monocyte release from the BM, as recently suggested (17, 29). This interpretation was further corroborated by lower levels of the effector cytokine TNF-α in the serum and bladder homogenate of CCR2-deficient mice (Fig. 2*D*), consistent with a reduction of monocyte functionality.

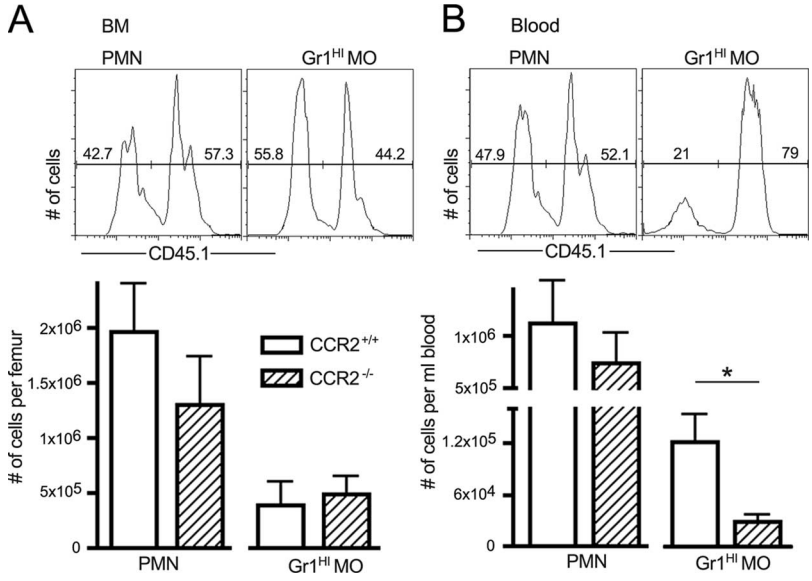

FIGURE 3. Homeostatic release of Gr1^{high} monocytes into the blood is also controlled by CCR2. Irradiated CCR2$^{+/+}$ (CD45.2) mice were reconstituted with a 1:1 ratio of BM cells from CCR2$^{+/+}$ (CD45.1) and from CCR2$^{-/-}$ (CD45.2) mice. After 6 wk, the number of Lin$^-$CD115$^-$F4/80$^-$Ly6G$^+$Gr1$^+$ PMN and Lin$^-$CD115$^+$F4/80$^+$Gr1^{high} monocytes were determined in the BM (*A*) and the blood (*B*). Shown are the mean ± SD of cell numbers in groups of six mice. Results are representative of two individual experiments.

Homeostatic release of Gr1^{high} monocytes into the blood is also controlled by CCR2

To further examine the effect of CCR2 on Gr1^{high} monocyte release from the BM in the steady state (Fig. 2), we reconstituted lethally irradiated CCR2$^{+/+}$ mice with a 1:1 mixture of BM from CCR2$^{+/+}$ CD45.1 and from CCR2$^{-/-}$ CD45.2 mice. After 6 wk, we determined the numbers of Gr1^{high} monocytes and those of PMN, which served as a CCR2-independent

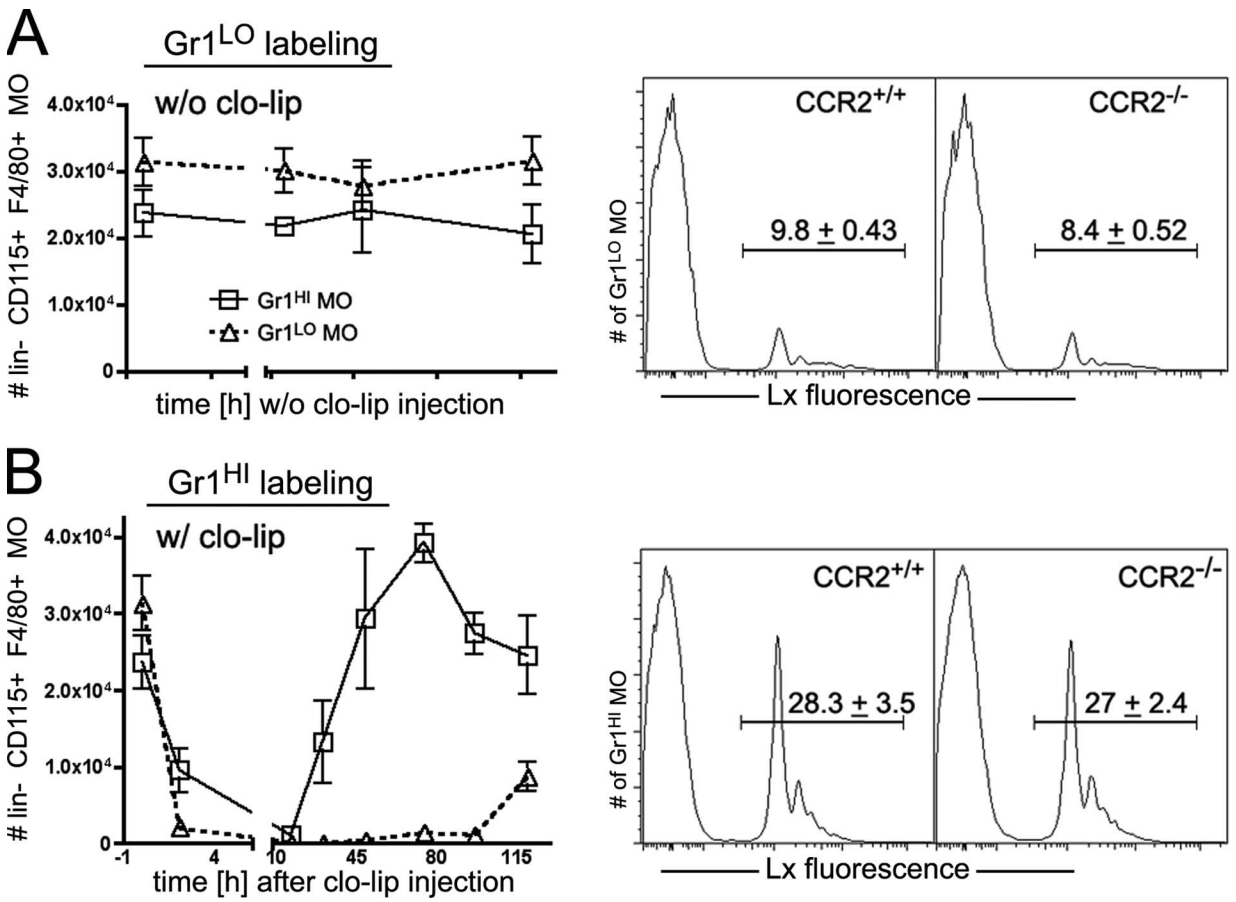

FIGURE 4. CCR2 deficiency does not affect labeling of Gr1^{low} or Gr1^{high} monocytes in the blood. *A*, Efficiency of labeling Gr1^{low} monocytes. Lx-FITC (Lx) beads were injected i.v. into CCR2$^{+/+}$ and CCR2$^{-/-}$ mice. At various time points, the blood was analyzed for Gr1^{low} (triangles) and Gr1^{high} monocytes (squares) per ml blood. The *right panel* shows bead uptake after 2 days. *B*, Efficiency of labeling Gr1^{high} monocytes. The *left panel* shows Gr1^{low} (triangles) and Gr1^{high} monocytes (squares) blood cell numbers at various time points after i.v. injection of clo-lips (w/clo-lip). Lx beads were injected i.v. 18 h after clo-lip treatment. After 2 days, the blood was analyzed for Lx-positive monocytes. Shown are mean ± SD of groups of three mice. Results are representative of two individual experiments.

control cell population. In the BM from one femur, CCR2 deficiency neither affected the absolute numbers of monocytes and PMN, nor the ratio between $CCR2^{+/+}$ ($CD45.1^{+}$) and $CCR2^{-/-}$ (CD45.2) cells, indicating that entry of the injected cells into the recipient BM was independent of CCR2 (Fig. 3*A*). In contrast, $Gr1^{high}$ monocytes in the blood were significantly reduced when they lacked CCR2, as compared with wild-type cells (Fig. 3*B*). The numbers of PMN in the blood were not affected by CCR2 (Fig. 3*B*). These data confirmed that not only inflammatory but also homeostatic release of $Gr1^{high}$ monocytes into the blood stream depended on CCR2.

Applicability of latex (Lx) bead labeling for in vivo tracking of $Gr1^{low}$ and $Gr1^{high}$ monocytes in $CCR2^{-/-}$ mice

The requirement of CCR2 for egress of monocytes from the BM (Figs. 2 and 3) did not exclude an additional role in mediating entry into the bladder. To investigate this possibility, we used a recently developed technique that allows selective in vivo labeling of either $Gr1^{high}$ or $Gr1^{low}$ monocytes. $Gr1^{low}$ monocytes in the blood can be labeled by i.v. injection of FITC fluorescent Lx beads, which are spontaneously phagocytosed by these cells (39). Such phagocytosis was not affected by CCR2 expression, since $Gr1^{low}$ blood monocytes of $CCR2^{+/+}$ and $CCR2^{-/-}$ mice showed comparable uptake of one or, less frequently, several beads, resulting in labeling of 6–10% of these cells for 1–5 days (Fig. 4*A*). Preferential labeling of $Gr1^{high}$ monocytes was conducted by injection of Lx beads in the absence of $Gr1^{low}$ monocytes. This could be achieved by i.v. injection of clodronate-loaded liposomes (clo-lip) (39), which efficiently depleted both monocyte subsets in the blood within less than 10 h (Fig. 4*B*). The $Gr1^{high}$ monocyte subset re-emerged after 24 h, whereas $Gr1^{low}$ monocytes remained absent for 5–7 days (Fig. 4*B*). $Gr1^{high}$ monocytes could be labeled by injecting Lx beads 18 h after depletion with clo-lip (Fig. 4*B*), resulting in efficient labeling of 25–30% of $Gr1^{high}$ monocyte at 66 h after depletion, both in $CCR2^{+/+}$ and $CCR2^{-/-}$ mice (Fig. 4*B*). At this time point, $Gr1^{low}$ monocytes in fact had not yet re-appeared (Fig. 4*B*). These findings demonstrated the applicability of the monocyte-subset-specific Lx bead labeling technique (39) in $CCR2^{-/-}$ and $CCR2^{+/+}$ mice.

CCR2 is dispensable for recruitment of in vivo-labeled monocyte subsets into the bladder

To investigate the role of CCR2 in monocyte immigration into the infected bladder, we infected $CCR2^{+/+}$ and $CCR2^{-/-}$ mice with UPEC 2 days after labeling $Gr1^{high}$ (Fig. 5*B*) or, as a control, $Gr1^{low}$ (Fig. 5*A*) monocytes as described above. Another day thereafter, $Gr1^{low}$ monocytes had been efficiently labeled in the blood (Fig. 5*A*), but very few labeled cells could be recovered from bladders of infected and noninfected animals (Fig. 5*A*), confirming that this monocyte subset was recruited very slowly to the infected and noninfected bladder. This result also implied that cells recovered from the bladder were not contaminating blood cells because, in this case, their abundance should have been similar to that in the blood. Loss of CCR2 did not affect this low-level recruitment (Fig. 5*A*), as expected for this $CCR2^{low}$ $Gr1^{low}$ monocyte subset.

Labeled $Gr1^{high}$ monocytes efficiently entered the infected bladder and reached proportions comparable to the blood (Fig. 5*B*). Importantly, CCR2 deficiency reduced their immigration into the bladder neither in the steady state, nor in infection (Fig. 5*B*), indicating that this chemokine receptor was dispensable for recruitment of $Gr1^{high}$ monocytes in both situations. Furthermore, if CCR2 would mediate $Gr1^{high}$ monocyte recruitment into the bladder, then blood levels of CCR2-competent cells should have been lower than those of CCR2-deficient ones.

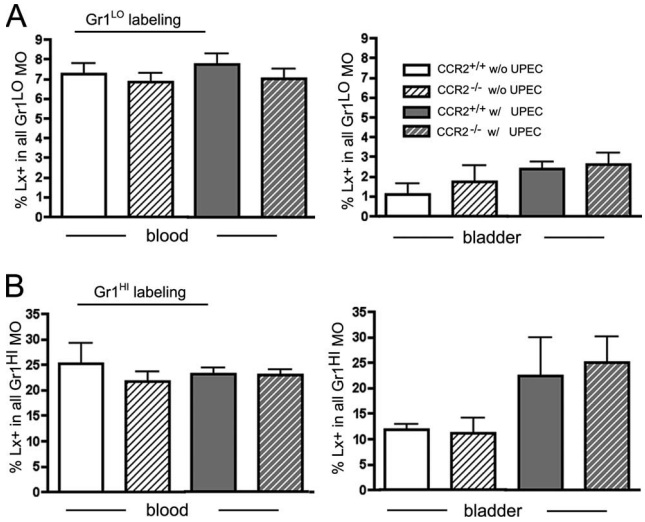

FIGURE 5. CCR2 is dispensable for recruitment of in vivo labeled monocytes into the bladder. Blood $Gr1^{low}$ (*A*) or $Gr1^{high}$ (*B*) monocytes in $CCR2^{+/+}$ (not striped) and $CCR2^{-/-}$ (striped) mice were labeled with fluorescent Lx beads. Two days later, UPECs were instilled intravesically. After further 24 h, blood (*left panels*) and thoroughly perfused bladders (*right panels*) of infected (gray) and noninfected (white) mice were analyzed for Lx-labeled $Gr1^{low}$ and $Gr1^{high}$ monocytes. Shown are the mean ± SD of groups of five mice. Results are representative of three individual experiments.

CCR2 is dispensable for recruitment of adoptively transferred monocytes into the bladder

To confirm the interpretations above with a different approach, we cotransferred $CCR2^{+/+}$ and $CCR2^{-/-}$ BM monocytes into recipient mice with UTI, and subsequently determined the ratio between these subsets in BM, blood, and bladder. To this end, a 1:1 mixture of CD45.1 $CCR2^{+/+}$ and CD45.2 $CCR2^{-/-}$ monocytes was labeled with CMTMR and then transferred. Fig. 6*A* shows an analysis of this mixture.

One hour after transfer, the ratio between $CCR2^{+/+}$ and $CCR2^{-/-}$monocytes in the BM, the blood, and the bladder was determined (Fig. 6*B*; analysis of the bladder shown as example in Fig. 6*C*). Some transferred $Gr1^{low}$ and $Gr1^{high}$ monocytes were detected at this early time point in the BM at 1:1 ratio (Fig. 6*B*), verifying our previous finding that entry of monocytes into the BM was CCR2 independent. Although the proportion of $Gr1^{high}$ monocytes in the BM and blood was somewhat lower than before transfer (79 or 80 vs 84% before transfer), their proportion in the bladder was increased (87 vs 84%), consistent with selective and rapid recruitment of $Gr1^{high}$ monocytes to the site of infection. Importantly, $CCR2^{+/+}$ and $CCR2^{-/-}$ monocytes were similarly frequent in the infected bladder, and this was true for both $Gr1^{high}$ and $Gr1^{low}$ monocytes (Fig. 6, *B* and *C*), confirming that monocyte entry into this organ did not require CCR2.

Discussion

The traditional view of CCR2 function has recently been challenged by experiments suggesting that this chemokine receptor may affect monocyte entry into infected tissues only indirectly by promoting monocyte release from the BM (17). These findings were obtained in splenic listeriosis and, therefore, did not exclude an additional role of CCR2 in monocyte entry into nonlymphoid tissues, where these cells have to cross other endothelial barriers to leave the blood. A role of CCR2 in such recruitment is supported by in vitro studies showing CCR2-dependent monocyte attachment to inflamed endothelium (23,

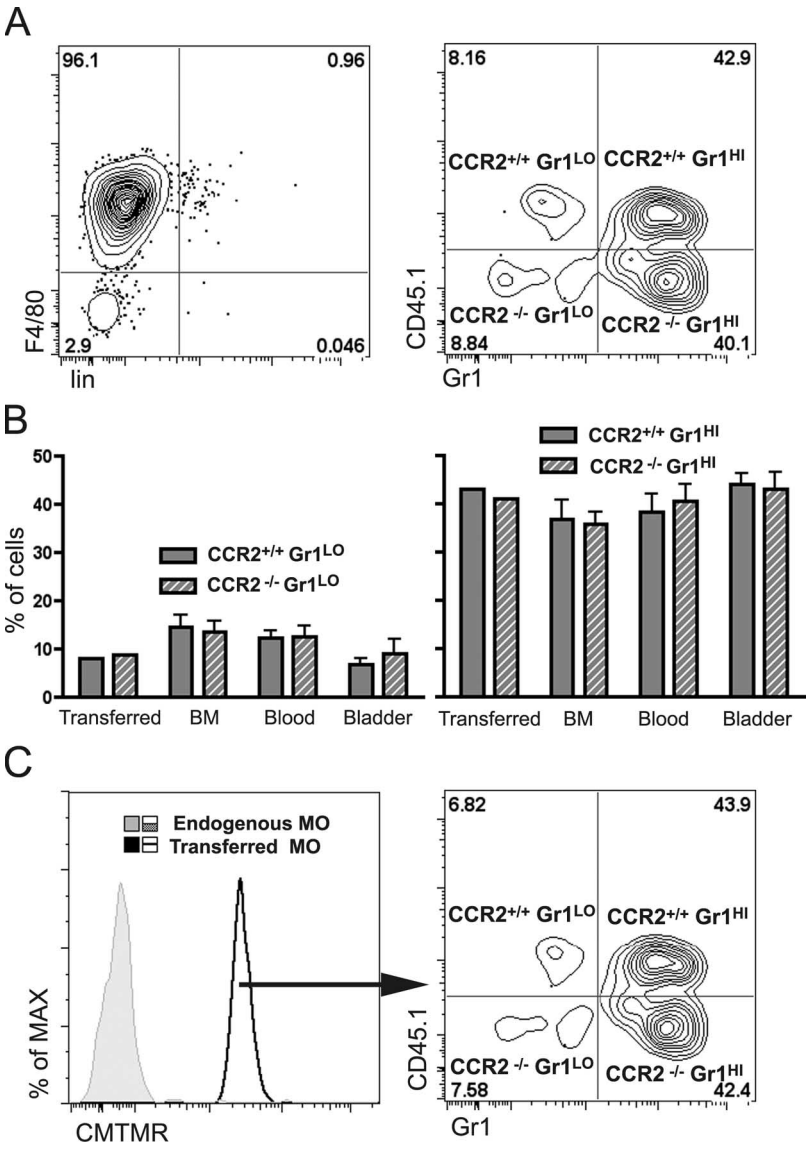

FIGURE 6. CCR2 is dispensable for entry of adoptively transferred monocytes into the bladder. Monocytes were prepared from CCR2$^{+/+}$ (CD45.1^{+}) and CCR2$^{-/-}$ (CD45.1^{-}) BM by negative selection of CD4^{+}, CD8^{+}, CD19^{+}, and Ly6G^{+} cells. Both populations were mixed at a 1:1 ratio, labeled with CMTMR and transferred into C57BL/6 mice infected with UPEC 3 h before. *A*, Characterization of the transferred mix by F4/80 and Lin, and by CD45 and Gr1 expression. *B*, Then, 1 h after adoptive transfer, CMTMR^{+} cells in the BM, the blood, and in the bladder were analyzed for CD45 and Gr1 expression. The bars represents the relative proportions of CCR2$^{+/+}$ (CD45.1^{+}) and CCR2$^{-/-}$ (CD45.1^{-}) expressing high or low levels of Gr1. *C*, Analysis in the bladder is given as an example. Shown are the mean ± SD of groups of four mice. Results are representative of two individual experiments.

24). We addressed this question in UTI, one of the most prevalent bacterial infections. We found that only Gr1^{high} monocytes, which express high levels of CCR2, accumulated in the infected organs, consistent with the proposed role of this subset in inflammation (1). The absolute numbers of Gr1^{high} monocytes that disappeared from the blood were lower than their increase in the bladder, which may be due to their recruitment into other tissues, to their death in the bladder, or to their urinary loss by leukocyturia. Gr1^{low} monocytes are usually not assumed to show CCR2 dependency, since they express little CCR2 levels in the blood (1). Consistently, these cells did not accumulate in the infected bladder. This result somewhat differs from observations in *Listeria monocytogenes* peritonitis, where Gr1^{low} monocytes recently were reported to be recruited into the peritoneal cavity, although at a much lower frequency than Gr1^{high} monocytes (7). The absence of recruited Gr1^{low} monocytes in our UTI model may be due to differences between the immune responses against Gram-negative UPEC and Gram-positive *Listeria*. Alternatively, it may relate to the distinct infection sites studied.

Importantly, we found that CCR2$^{-/-}$ mice displayed reduced abundance of Gr1^{high} monocytes in the steady-state, both in the blood and in noninfected tissue (17). This finding is consistent with previous observations in CCR2$^{-/-}$ mice, and provides formal proof of a homeostatic role of CCR2. In infected CCR2-deficient mice, this reduction was far more pronounced in the bladder. Importantly, this was not only true at the site of infection in the bladder, but also in the blood, and was detectable also on the functional level of a monocyte/macrophage effector molecule, TNF-α. Furthermore, several experimental approaches did not reveal any evidence that Gr1^{high} monocytes use CCR2 to enter the inflamed bladder. Instead, Gr1^{high} monocyte homeostasis and "availability" in cases of infection was primarily regulated on the level of egress from the BM compartment. Therefore, the CCR2 ligand, MCP-1 most likely reached the BM via the blood stream, which is supported by the finding that MCP-1 levels were increased not only in infected organs, but also in the blood stream (40). An alternative, not mutually exclusive explanation for CCR2 signaling in the BM could be local MCP-1 production in this site, as recently suggested (41). Such local production might facilitate monocytes egress from the BM also in noninfected mice. In contrast to previous observations (17), we did not observe accumulation of Gr1^{high} monocytes in the BM of CCR2-deficient mice. Possibly, the BM niche could not accommodate more monocyte precursors when CCR2 was lacking.

Of note, there is experimental evidence for a role of CCR2 in $Gr1^{high}$ monocyte recruitment at the level of egress from the BM compartment in other noninfectious inflammatory conditions, namely atherosclerosis. By dissecting effects of different chemokines for monocyte subset recruitment, several studies independently highlighted regulation of atherosclerosis-associated $Gr1^{high}$ monocytosis via CCR2 and its ligand MCP-1 (29, 42, 43). The degree of $Gr1^{high}$ monocyte levels in the blood directly correlated with the accumulation of macrophages in plaques (29, 42, 43). However, especially in atherosclerosis, $Gr1^{high}$ monocytes may also additionally use CCR2 at the level of transendothelial migration into the intima of chronically inflamed blood vessels (43).

Our finding of CCR2-dependent monocyte egress of the BM does not exclude that CCR2 may play a role in monocyte entry into arteriosclerotic large arteries, nor into tissues other than the bladder. Nevertheless, the present results warrant reassessment of previous in vivo infection studies and of in vitro studies that support CCR2-dependent endothelial attachment. Such studies usually used human umbilical vein cells or ex vivo cultured endothelial cells isolated from large vessels, which may not generally reflect endothelial functions in the entire body or in capillaries. Also, isolated BM monocytes from wild-type or $CCR2^{-/-}$ mice might differ in maturation or activation, either related to intrinsic differentiation properties or to ex vivo isolation procedures.

In summary, our study demonstrates that the chemokine receptor CCR2 is required for release of $Gr1^{high}$ monocytes into the blood, both in homeostasis and infection, but is dispensable for recruitment into the infected bladder. Although the mechanisms underlying such recruitment remain to be elucidated, these findings question therapeutic attempts to treat inflammatory diseases in nonlymphoid tissues by local CCR2 blockade (20, 25–28). Systemic administration that reaches the BM may be effective, but the concomitant general reduction of blood monocytes might result in increased susceptibility to circulating pathogens.

Acknowledgments

We thank W. A. Kuziel for $CCR2^{-/-}$ mice and Steffen Jung for reading the paper. We acknowledge support by the Flow Cytometry Core Facility of the Institute of Molecular Medicine and Experimental Immunology and by the House for Experimental Therapy.

Disclosures

The authors have no financial conflict of interest.

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