The Journal of Immunology

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J Immunol 2003; 170:3273-3278; ;
doi: 10.4049/jimmunol.170.6.3273
http://www.jimmunol.org/content/170/6/3273

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Monocytes Are Potent Facilitators of Alveolar Neutrophil Emigration During Lung Inflammation: Role of the CCL2-CCR2 Axis[1]

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Coordinated neutrophil and monocyte recruitment is a characteristic feature of acute lung inflammatory responses. We investigated the role of monocyte chemotactic protein-1 (CCL2, JE) and the chemokine receptor CCR2 in regulating alveolar leukocyte traffic. Groups of wild-type (WT) mice, CCR2-deficient mice, lethally irradiated CCR2-deficient and WT mice that were reciprocally bone marrow transplanted (chimeric CCR2 deficient and WT, respectively), chimeric CCR2-deficient mice with an enriched $CCR2^+$ alveolar macrophage population, and CCR2-deficient mice transfused with $CCR2^+$ mononuclear cells were treated with intratracheal CCL2 and/or *Escherichia coli* endotoxin. Our data show that alveolar monocyte recruitment is strictly dependent on CCR2. LPS-induced neutrophil migration to the lungs is CCR2 independent. However, when CCR2-bearing blood monocytes are present, alveolar neutrophil accumulation is accelerated and drastically amplified. We suggest that this hitherto unrecognized cooperativity between monocytes and neutrophils contributes to the strong, coordinated leukocyte efflux in lung inflammation. *The Journal of Immunology*, **2003, 170: 3273–3278.**

Local tissue inflammatory responses to microbial challenge are characterized by early neutrophil attraction and subsequent prolonged monocyte accumulation. Although chemokine release and cell surface display of complementary leukocyte and endothelial/epithelial adhesion molecules are centrally involved in these processes (1–3), the underlying mechanism(s) that shapes the kinetics and extent of neutrophil and monocyte recruitment is unclear (1, 4). In recent investigations in which we used a mouse model of acute lung inflammation provoked by intratracheal application of combined LPS and CCL2 to mimic elevated intra-alveolar levels of CCL2 observed in septic adult respiratory distress syndrome patients (3), the successive waves of early alveolar neutrophil and delayed alveolar monocyte recruitment were resolved in detail (5, 6). It was not surprising that induced monocyte recruitment to the lungs would be blocked by treatment with Abs against CCR2 (7), the major receptor for the potent monocyte chemoattractant CCL2, or in mice deficient in CCR2 (8). However, it was completely unexpected that the accumulation of neutrophils, which do not express CCR2, depended on the activity of CCR2. To define the CCR2-bearing cells that promote neutrophil accumulation in lungs instilled with CCL2 and LPS, we used bone marrow transplantation and adoptive transfer of mononuclear cells to generate chimeric mice with disparate expression of CCR2 on circulating cells vs sessile lung cells. The ability to distinguish between CCR2-bearing monocytes and alveolar macrophages allowed us to assemble additional evidence that blood-borne monocytes are the facilitators of neutrophil recruitment in this model of inflammation. The strong synergistic contribution of monocytes to the burst of neutrophil emigration broadens the scope of cellular communication that underlies pulmonary inflammatory responses and has implications for anti-inflammatory therapeutic strategies aimed at interfering with the CCL2-CCR2 axis.

Materials and Methods

Animals

CCR2-deficient mice were generated on a mixed C57BL/6 × 129/Ola genetic background by targeted disruption of the CCR2 gene, as described previously (8). The disrupted CCR2 gene was backcrossed for six generations to wild-type (WT)[3] BALB/c mice. Parent and offspring $CCR2^{-/-}$ mice on the BALB/c background were bred under specific pathogen-free conditions. WT control animals were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals 8–12 wk old and between 18 and 21 g were used for the described experiments. This animal study was approved by the local government committee.

Reagents

The red fluorescent dye PKH26-PCL and diluent B solution were purchased from commercial sources (Zynaxis, Malvern, CA; Sigma-Aldrich, Deisenhofen, Germany). Murine JE/monocyte chemotactic protein-1/CCL2 was purchased as a recombinant protein preparation from R&D Systems (Wiesbaden, Germany). Rat anti-mouse Gr-1 mAb (clone RB6-8C5) was obtained from BD PharMingen (Wiesbaden, Germany), and *Escherichia coli* LPS (O111:B4) was purchased from Sigma-Aldrich.

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Received for publication September 17, 2002. Accepted for publication January 9, 2003.

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[1] This study has been supported by the German Research Foundation, Grant SFB 547, "Cardiopulmonary Vascular System."

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[3] Abbreviations used in this paper: WT, wild type; PMN, polymorphonuclear neutrophil; rAM, resident alveolar macrophage.

0022-1767/03/$02.00

Table I. *Treatment regimen and CCR2 expression profile of experimental groups*

Groups	Treatment Regimen	Designation and CCR2 Expression Profile[a]
1) WT	None	WT (CCR2-positive circulation + CCR2-positive periphery)
2) CCR2 knockout	None	CCR2 deficient (CCR2-deficient circulation + CCR2-deficient periphery)
3) CCR2 knockout, WT transplanted	Lethal irradiation, reconstitution with WT bone marrow cells	Chimeric CCR2 deficient (CCR2-positive circulation + CCR2-deficient periphery)
4) WT, CCR2 knockout transplanted	Lethal irradiation, reconstitution with CCR2 knockout bone marrow cells	Chimeric WT (CCR2-deficient circulation + CCR2-positive periphery)
5) CCR2 knockout, WT transplanted with alveolar macrophage repopulation	See No. 3, plus treatment with liposomal clodronate	Chimeric CCR2 deficient/**rAM** $CCR2^{+/+}$
6) CCR2 knockout with WT mononuclear cell transfusion	Transfusion of purified WT peripheral blood mononuclear cells into CCR2-deficient mice	CCR2 deficient/PB-Mo $CCR2^{+/+}$

[a] CCRZ expression profile is indicated in parentheses.

Isolation of bone marrow cells and transplantation into recipient animals

Bone marrow cells were isolated under sterile conditions from the tibias and femurs of sex-matched, syngeneic WT and CCR2-deficient donor mice. Single cell suspensions were carefully prepared from the bone marrow isolates and filtered through 70- and 40-μm nylon meshes (BD Biosciences, Heidelberg, Germany) to remove residual cell aggregates. The cells were washed in Leibovitz's L15 medium (Invitrogen, Eggenstein, Germany) before transplantation. Recipient WT and CCR2-deficient mice received 12 Gy of total body irradiation using a ^{60}Co source. To reduce gastrointestinal toxicity, the irradiation was applied in two doses separated by a 3-h interval. For transplantation, the lethally irradiated recipients were sedated with ketamine and slowly infused via the lateral tail veins with donor marrow cells suspended in Leibovitz's L15 medium (1×10^7 bone marrow cells/mouse). The recipient chimeric animals were then housed under specific pathogen-free conditions for at least 3–4 wk with free access to autoclaved food and water.

Preparation of liposome-encapsulated dichloromethylene-diphosphonate

Liposomal encapsulation of clodronate was done, as recently outlined in detail (9). Briefly, 8 mg of cholesterol was added to 86 mg of egg phosphatidylcholine, and the chloroform phase evaporated under helium. Removal of the chloroform phase was performed under low vacuum in a speedvac Savant concentrator. The clodronate solution was made by dissolving 1.2 g of dichloromethylene diphosphonic acid in 5 ml of sterile PBS. Five milliliters of the clodronate solution was added to the liposomes and mixed thoroughly. Empty liposomes were made by the addition of sterile PBS alone. This solution was sonicated and ultracentrifuged at $10{,}000 \times g$ for 1 h at 4°C. The liposomal pellets were then removed and resuspended in PBS, followed by ultracentrifugation at $10{,}000 \times g$ for 1 h at 4°C. Subsequently, liposomes were resuspended in 5 ml of sterile PBS, stored at 4°C, and used within 48 h. The final concentration of the liposomal clodronate suspension was 5 mg/ml.

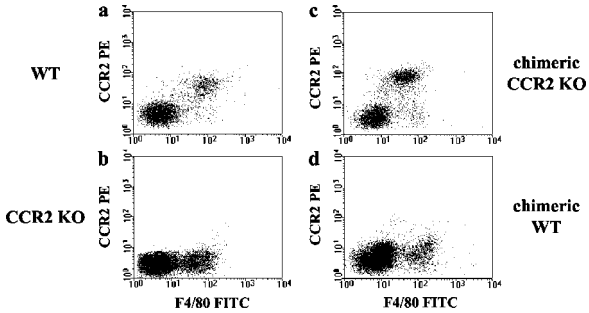
Treatment protocols

Alveolar neutrophil and monocyte recruitment profiles were evaluated in four treatment groups, as summarized in Table I. These groups included WT mice, CCR2-deficient mice, chimeric WT mice (lethally irradiated WT mice reconstituted with bone marrow cells from CCR2-deficient mice), and chimeric CCR2-deficient mice (lethally irradiated CCR2-deficient mice reconstituted with WT bone marrow cells). Mice were challenged by intratracheal instillation of CCL2 (50 μg/mouse), LPS (10 ng/mouse), or the combination of CCL2 (50 μg/mouse) and LPS (10 ng/mouse) for various times (0, 6, 12, 24, and 48 h), according to protocols previously described in detail (2, 5, 10).

In some experiments, chimeric CCR2-deficient mice were treated with liposomal clodronate (100 μl/mouse) within 1 wk after lethal irradiation and bone marrow transplantation to deplete the resident alveolar macrophage population (9). Subsequently, mice were allowed to rest for 4 wk to allow complete repopulation of the alveolar air space with $CCR2^+$ donor alveolar macrophages (resident alveolar macrophage (rAM) counts before clodronate treatment, 4, 7 ± 0, 3×10^5 cells; 24 h after clodronate treatment, 4, 9 ± 0, 5×10^4 cells; 4 wk after clodronate treatment, 4, 4 ± 0, 4×10^5 cells; $n = 5$ each). Subsequently, mice received intratracheal instillations of combined CCL2 and LPS for 12, 24, or 48 h.

Adoptive mononuclear cell transfer

WT mice were sedated with ketamine and given an i.p. injection of complement-fixing anti-Gr-1 mAb for induction of transient neutropenia, as described previously (9). After 24 h, neutropenic mice were sacrificed with isoflurane, and whole blood was collected in EDTA. RBCs were lysed by



Treatment groups	% CCR2 expressing, F4/80+ monocytes (mean ± SEM)
Wild-type	98.5 ± 1.5
CCR2 KO	0.5 ± 0.1
chimeric CCR2 KO	95.5 ± 4.5
chimeric wild-type	3.5 ± 1.5

FIGURE 1. Flow cytometric CCR2 expression profiling of peripheral blood monocytes collected from the various treatment groups. Monocytes contained in EDTA-anticoagulated blood collected from WT mice (*a*), CCR2-deficient mice (*b*), chimeric CCR2-deficient (*c*), or chimeric WT mice (*d*) were subjected to dual color flow cytometric analysis of F4/80 and CCR2 expression, according to the protocol described in *Materials and Methods*. Dual color dot plots show a homogeneous F4/80 expression (increased FITC fluorescence 1 emission; F4/80 FITC; *x*-axis) and CCR2 expression (increased PE fluorescence 2 emission; CCR2-PE; *y*-axis) on circulating monocytes collected from WT mice (*a*) or chimeric CCR2-deficient mice (*c*), whereas monocytes collected from CCR2-deficient mice (*b*) and chimeric WT mice (*d*) showed a homogeneous F4/80, but no CCR2 expression. The table below shows the mean percentage of values of CCR2-expressing, F4/80-positive blood monocytes of the various treatment groups ($n = 5$ per group).

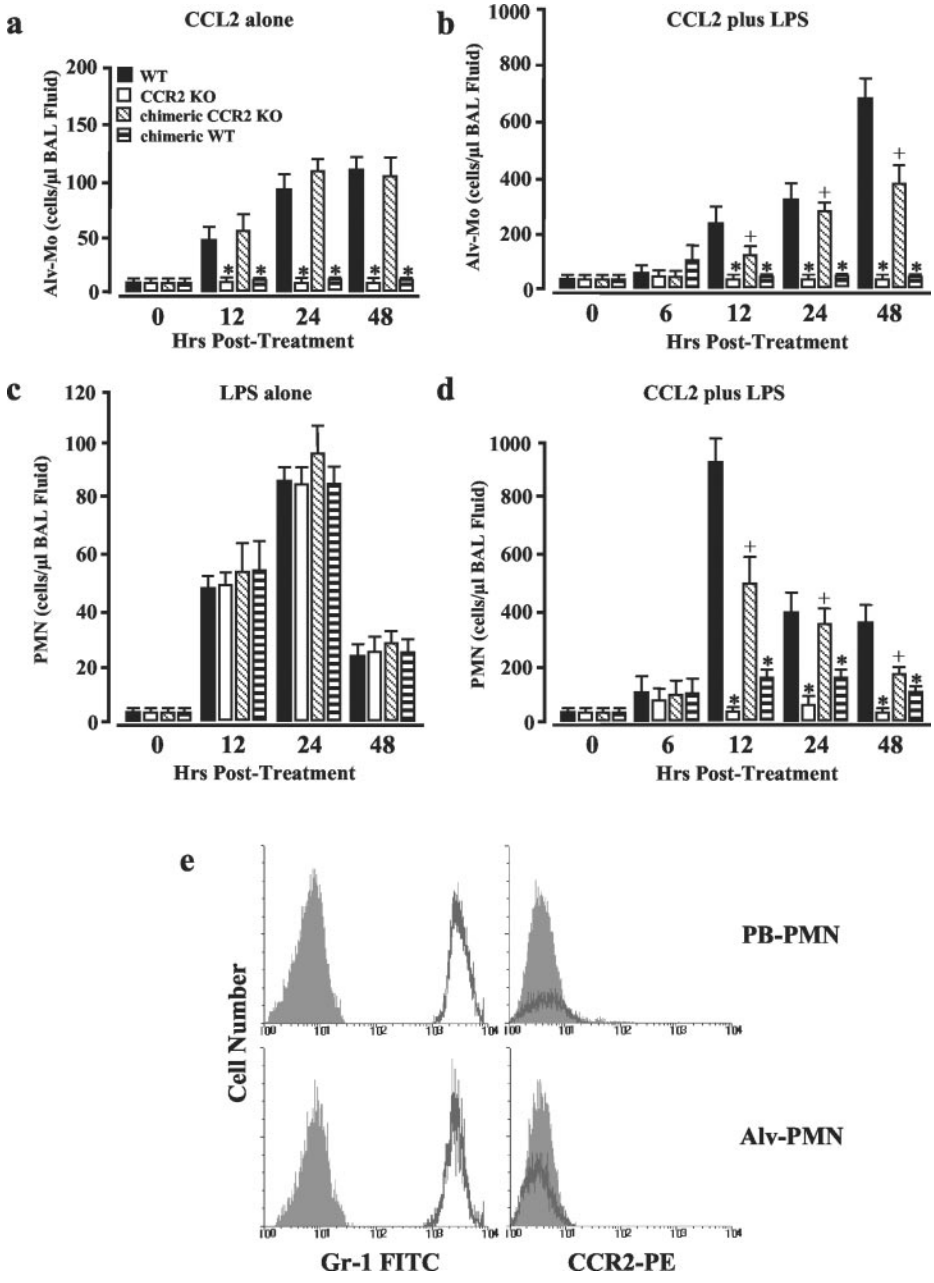


FIGURE 2. The coordinated alveolar neutrophil and monocyte traffic is linked to CCR2 expression by cells of the hemopoietic system. *a* and *b*, Alveolar monocyte accumulation in WT mice, CCR2-deficient mice, chimeric CCR2-deficient, or chimeric WT mice in response to CCL2 (*a*) or combined CCL2 plus LPS (*b*). *c* and *d*, Alveolar neutrophil accumulation in WT mice, CCR2-deficient mice, chimeric CCR2-deficient, or chimeric WT mice in response to LPS (*c*) or combined CCL2 plus LPS (*d*). $*$, $p < 0.05$ vs WT; $+$, $p < 0.05$ vs CCR2 knockout (KO); mean $\pm$ SEM ($n = 5$), Mann-Whitney *U* test. *e*, Dual color flow cytometric profiling of Gr-1 FITC and CCR2 PE expression by circulating (PB-PMN) or alveolar recruited PMN (Alv-PMN) collected from CCL2 plus LPS-challenged (24 h) WT mice. *Left histogram column*, Shows Gr-1 expression (white histogram of increased fluorescence 1 emission; Gr-1 FITC) by PB-PMN and Alv-PMN. Note that CCR2 expression of both circulating and alveolar recruited neutrophils (solid lines in *right histogram column*) is indistinguishable from negative controls (gray histograms).

two exposures of 5 min each to NH_4Cl solution. The resulting population of mononuclear cells was verified to be depleted (<2%) of polymorphonuclear neutrophils (PMN) by examination of Pappenheim-stained cytocentrifuge preparations and by flow cytometry using purified anti-Gr-1 mAb and secondary FITC-labeled anti-rat IgG. Dual color flow cytometric analysis of mononuclear cell preparations revealed lack of highly CD11c-positive dendritic cells (11) in the F4/80-positive and CD3-positive subpopulations. Ficoll-based methods were not used for mononuclear cell preparation to increase recovery and to avoid Ficoll-mediated activation of mononuclear cells. Approximately 1.5×10^7 mononuclear cells were transfused into sedated CCR2-deficient mice via lateral tail veins. Control animals received mononuclear cell preparations isolated from neutropenic CCR2-deficient mice. Fifteen minutes after transfusion, mice were anesthetized and received intratracheal instillation of CCL2 and LPS.

Isolation of peripheral blood leukocytes and alveolar macrophages

Mice were sacrificed with an overdose of isoflurane (Forene; Abbott, Wiesbaden, Germany). Isolation of peripheral blood leukocytes and bronchoalveolar lavage for the differentiation and quantification of resident alveolar macrophages, alveolar recruited neutrophils, and alveolar recruited monocytes was performed, as previously described (2, 5, 10).

Immunofluorescence

Single color immunofluorescence analysis was used to assess expression of the monocyte/macrophage marker F4/80 and the CCR2 receptor on the surface of resident alveolar macrophages from untreated or liposomal clodronate-treated chimeric CCR2-deficient mice (9). Briefly, cells were preincubated on ice in flexible microtiter plates with Fc-Block (10 μl; BD Biosciences) for blockade of Fc_{IgG} receptors. For negative controls, the cells were incubated with isotype-matched control IgG (BD PharMingen). Cells were incubated with either anti-F4/80 mAb (Serotec, Oxford, U.K.) or anti-CCR2 mAb (7), washed three times, and incubated on ice with biotinylated $F(ab')_2$ for 30 min. Cells were then washed, and PE-conjugated streptavidin (BD Biosciences) was added to the wells for 15 min on ice in the dark.

Dual color immunofluorescence was used to simultaneously analyze CCR2 expression on the cell surface of $F4/80^+$ peripheral blood monocytes from mice in the various treatment groups. Blood leukocytes were incubated with anti-CCR2 mAb, washed three times, incubated with secondary

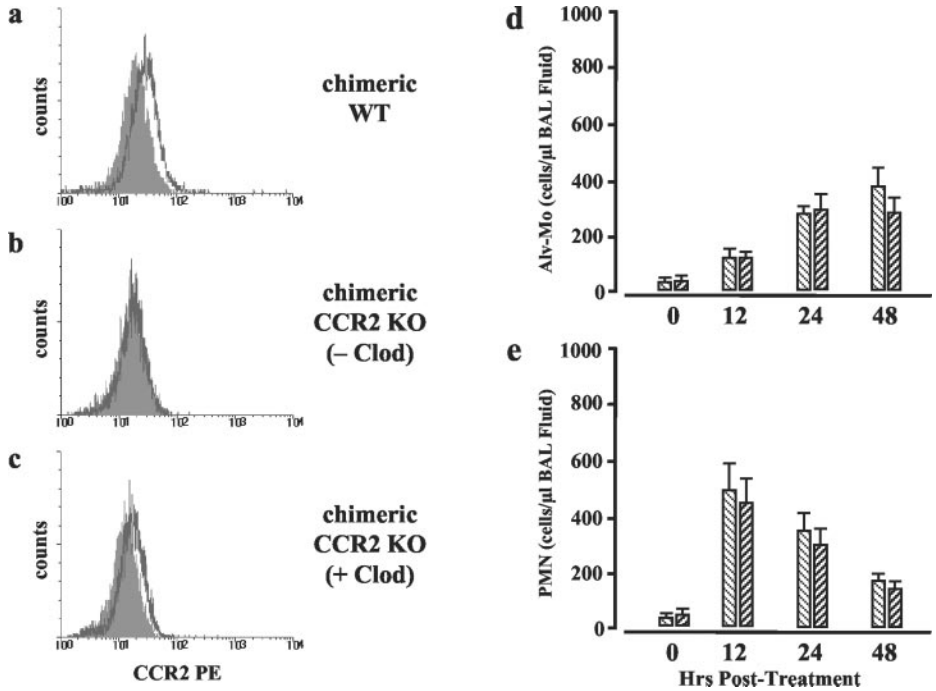


FIGURE 3. CCR2 expression analysis of alveolar macrophages and corresponding lung leukocyte recruitment profiles of liposomal clodronate-pretreated chimeric mice. Resident alveolar macrophages recovered from the lungs of chimeric WT mice (*a*) or chimeric CCR2-deficient mice without (*b*) or with clodronate pretreatment (*c*) were subjected to flow cytometric CCR2 expression analysis, as outlined in *Materials and Methods*. Open histograms in *a–c* show CCR2-PE fluorescence characteristics by rAM of the respective treatment groups, as indicated. Solid histograms of low fluorescence intensity in *a–c* show negative controls. The *left histogram bars* shown in *d* and *e* represent the alveolar monocyte and neutrophil accumulation in nonclodronate-treated chimeric CCR2-deficient mice challenged with CCL2 and LPS for various time points (0, 12, 24, and 48 h), whereas the *right histogram bars* in *d* and *e* represent the alveolar monocyte and neutrophil traffic in clodronate-pretreated chimeric CCR2-deficient mice challenged with combined CCL2 plus LPS for various time points (0, 12, 24, and 48 h).

biotinylated $F(ab')_2$ for 30 min on ice, followed by incubation of cells with PE-conjugated streptavidin and FITC-conjugated anti-F4/80 mAb. Negative controls were incubated with isotype-matched control IgG (BD PharMingen). After 15 min, cells were washed twice and analyzed by flow cytometry.

Flow cytometry

All samples were analyzed on a FACStar^{Plus} flow cytometer (BD Biosciences) equipped with an argon ion laser operating at 488-nm excitation wavelength and a laser output of 200 mW. The optical system of the flow cytometer was daily adjusted, using standardized fluorescent Calibrite beads (BD Biosciences).

CCR2 or F4/80 expression on resident alveolar macrophages was detected by single color flow cytometry by gating on forward scatter vs side scatter characteristics, followed by analysis of F4/80 or CCR2 in the fluorescence 2 channel (F488/575), as described previously (9).

For dual color flow cytometry of CCR2 expression on F4/80$^+$ peripheral blood monocytes, leukocytes were gated on forward scatter vs side scatter, and F4/80 and CCR2 expression were analyzed in the fluorescence 1 channel (F4/80-FITC; *x*-axis; F488/535; log scale) vs fluorescence 2 channel (CCR2-PE; *y*-axis; F488/575; log scale).

Statistics

The study data are expressed as mean ± SEM. Significant differences between treatment groups were estimated by Mann-Whitney *U* test. Differences were assumed to be significant when p values were <0.05.

Results and Discussion

To discriminate between the role of blood-borne monocytes and lung resident cells in pulmonary inflammation induced by CCL2 and LPS, we used WT and CCR2-deficient mice and a series of chimeric mice generated by bone marrow transplantation and adoptive cell transfer, as summarized in Table I. This approach was validated by using a rat anti-mouse CCR2 mAb (7) to monitor cell surface CCR2 expression in the various chimeric animals. Expression of CCR2 was easily detected on F4/80$^+$ blood monocytes from untreated WT mice, but was absent from monocytes from CCR2-deficient mice (Fig. 1, *a* and *b*). F4/80$^+$ monocytes isolated from irradiated CCR2-deficient mice reconstituted with WT bone marrow showed homogeneous expression of CCR2 (Fig. 1*c*). F4/80$^+$ monocytes collected from irradiated WT mice reconstituted with bone marrow from CCR2-deficient mice lacked cell surface expression of CCR2 (Fig. 1*d*).

We then analyzed leukocyte trafficking in WT, CCR2-deficient, chimeric CCR2-deficient, and chimeric WT mice treated with CCL2 alone or with CCL2 plus LPS. WT mice treated with CCL2 alone showed prolonged alveolar monocyte recruitment with peak accumulation observed at 48 h posttreatment (Fig. 2*a*). In contrast, CCR2-deficient mice completely lacked CCL2-driven alveolar monocyte recruitment during the 48-h observation period. However, reconstitution of CCR2-deficient mice with WT bone marrow fully restored alveolar monocyte accumulation in response to CCL2 with the number of monocytes in the lavage fluid virtually identical with the number found in WT mice. Chimeric WT mice lacked a detectable increase in alveolar monocyte accumulation upon CCL2 challenge (Figs. 1*d* and 2*a*). Similar results were obtained after combined CCL2 and LPS challenge of the different groups of mice. WT mice, but not CCR2-deficient mice challenged with CCL2 and LPS showed enhanced alveolar monocyte accumulation that peaked at 48 h (Fig. 2*b*). Monocyte recruitment was rescued in CCR2-deficient mice by transplantation of WT bone marrow, but was lost in WT mice after transplantation of bone marrow from CCR2-deficient animals (Fig. 2*b*). These data indicate that the direct interaction between circulating CCR2-bearing monocytes and the alveolar-intravascular CCL2 gradient is the main driving force for alveolar monocyte accumulation in this model.

In contrast to the sharp differences in monocyte recruitment exhibited by the four experimental groups after treatment with either CCL2 alone or CCL2 plus LPS, all four groups showed identical levels of neutrophil accumulation in response to LPS alone (Fig. 2*c*). It can be concluded from this result that CCR2, either on resident alveolar macrophages or circulating monocytes, does not play a role in the neutrophil response to LPS alone. However, the addition of CCL2 to the LPS treatment protocol drastically raised neutrophil accumulation in WT mice when cell numbers were harvested and counted at 12 h (Fig. 2*d*). This dramatic increase was

almost completely attenuated in CCR2-deficient mice and in chimeric WT mice, but was restored in chimeric CCR2-deficient mice, most notably at the 12- and 24-h time points (Fig. 2*d*). Because CCR2 is not expressed on circulating neutrophils nor on alveolar recruited neutrophils from WT mice (Fig. 2*e*), the observed defects in neutrophil accumulation in CCR2-deficient mice or chimeric WT mice are not a direct effect of neutrophil CCR2 deficiency. The data support the idea that another bone marrow-derived leukocyte subpopulation(s) is necessary to restore neutrophil influx.

Recent flow cytometric and histological studies revealed that CCR2 is expressed on resident alveolar macrophages (9). It is possible that these cells are actively involved in the initiation and overall inflammatory response to combined CCL2 and LPS treatment (9). To investigate the role of resident alveolar macrophages in pulmonary inflammation, chimeric CCR2-deficient mice were treated with liposomal clodronate to repopulate the alveolar air spaces with CCR2-bearing macrophages (Fig. 3, *a–c*). Interestingly, the alveolar neutrophil and monocyte recruitment profiles observed in clodronate-pretreated chimeric CCR2-deficient mice were identical with the profiles obtained from chimeric CCR2-deficient mice (Fig. 3, *d* and *e*). These results offer additional evidence that CCR2 expression on resident alveolar macrophages does not contribute to overall pulmonary leukocyte trafficking in response to combined CCL2 and LPS treatment, whereas CCR2 on circulating monocytes is essential for leukocyte trafficking in this model.

Because WT and chimeric WT mice are expected to possess similar extrahemopoietic CCR2 expression profiles (see Table I), the initial inflammatory response provoked by intra-alveolar instillation of CCL2 and LPS should be similar in both experimental groups. Indeed, both WT and chimeric WT mice showed a comparable TNF-α release profile in response to CCL2 and LPS. Relative to baseline TNF-α levels of <30 pg/ml in untreated mice, after 6 h of stimulation with CCL2 and LPS, WT mice had bronchoalveolar lavage TNF-α levels of 3150 ± 660 pg/ml ($n = 5$) and chimeric WT mice had TNF-α levels of 2910 ± 430 pg/ml ($n = 5$). Thus, the different neutrophil migration patterns are most likely not related to differences in the alveolar milieu of secondary inflammatory mediators, but are dependent on CCR2 expression on leukocytes in the downstream intravascular compartment.

To test further this central hypothesis, we investigated whether partial reconstitution of $CCR2^+$ cells in CCR2-deficient mice might be sufficient to restore the alveolar neutrophil trafficking in response to CCL2 and LPS. Mixtures of donor WT and CCR2-deficient bone marrow cells were prepared at the ratios of 50:50 and 25:75 and transplanted into irradiated recipient CCR2-deficient mice. Analysis of CCR2 expression on circulating monocytes isolated from chimeric mice 4 wk after transplantation showed proportional levels of $CCR2^+$ cells (Fig. 4). Interestingly, a threshold of 25% $CCR2^+$ circulating monocytes was sufficient to fully restore alveolar neutrophil recruitment (Fig. 4*c*). In contrast, the number of monocytes recruited into lungs challenged with CCL2 and LPS was strictly dependent on the percentage of reconstituted $CCR2^+$ monocytes in the circulation of the chimeric CCR2-deficient mice (Fig. 4*d*).

Finally, direct transfusion of $CCR2^+$ vs $CCR2^-$ mononuclear cells was used as an alternative to bone marrow transplantation. Transfusion of CCR2-deficient mice with mononuclear cells from WT, but not from CCR2-deficient mice restored alveolar neutrophil accumulation in response to CCL2 and LPS (Fig. 4*e*), again demonstrating that the cellular component of WT mice promoting alveolar neutrophil accumulation is contained within the mononuclear cell fraction of peripheral blood. A relevant contribution by

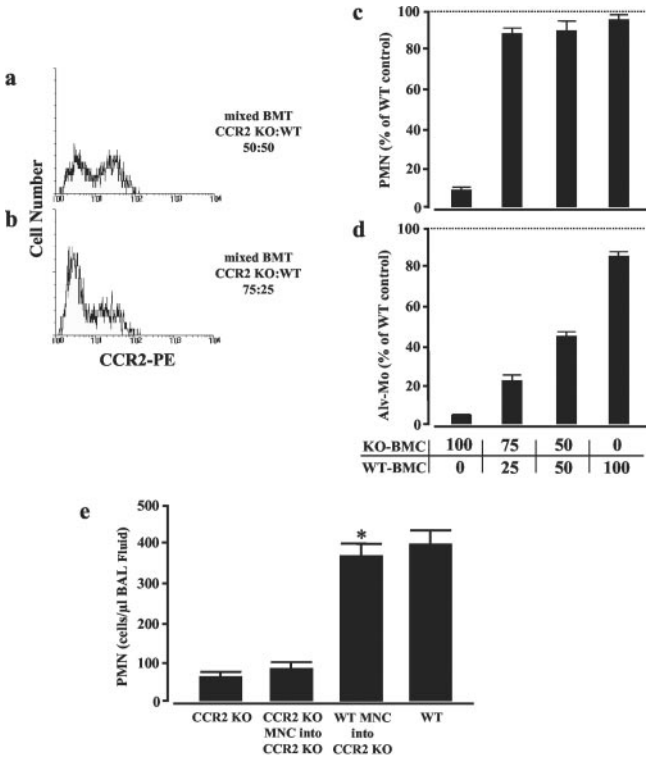


FIGURE 4. The CCR2-expressing cell required for a coordinated alveolar neutrophil traffic is bone marrow derived and contained within the mononuclear cell fraction of peripheral blood. *a* and *b*, Flow cytometric distribution pattern of CCR2 expression by circulating monocytes collected from lethally irradiated CCR2-deficient mice transplanted with defined mixtures of CCR2-deficient and WT bone marrow cells, indicating successful generation of mixed CCR2-deficient/WT chimeric mice (*a*, mixed BMT of 50:50 CCR2-deficient:WT bone marrow cells; *b*, mixed BMT of 75:25 CCR2-deficient:WT bone marrow cells). *c* and *d*, A threshold value of 25% CCR2-expressing monocytes in the blood of mixed CCR2-deficient/WT chimeric mice is sufficient to fully restore the alveolar neutrophil traffic at 24-h postcombined CCL2 plus LPS challenge (*c*), while the monocyte recruitment proportionally increases with numbers of CCR2-positive cells (*d*). *e*, Restoration of alveolar neutrophil recruitment to combined CCL2 plus LPS challenge in CCR2-deficient mice receiving transfusions of purified WT mononuclear cells. MNC, mononuclear cell. *, $p < 0.05$ vs CCR2 knockout (KO) MNC transfusion (mean $\pm$ SEM, $n = 4$).

the minor fraction of cotransfused $CCR2^+$ lymphocytes is unlikely, as these cells do not significantly migrate into the alveolar compartment during the observation period. Recent tracking studies using PKH26-labeled donor mononuclear cells transfused into recipient WT mice revealed recruitment of circulating donor mononuclear cells into the lungs of recipient mice upon intratracheal application of CCL2 (10). Ongoing studies will clarify whether translocation of circulating wild-type donor cells into the lungs of recipient CCR2-deficient mice is associated with increased induction of neutrophil chemoattractants as driving force for the observed increase in alveolar neutrophil accumulation in CCR2-deficient mice upon CCL2 and LPS challenge.

In conclusion, our results provide a detailed scenario of alveolar leukocyte trafficking under inflammatory conditions in vivo. Extravasation of monocytes is regulated by an alveolar intravascular gradient of CCL2, and there is a direct correlation between the percentage of WT bone marrow cells used for reconstitution and the number of circulating CCR2-expressing monocytes that accumulate in the lungs. There is a low level of neutrophil trafficking to the lungs in response to low-dose LPS treatment alone. This

neutrophil response is independent of CCL2, but may be dependent on other mediators derived from alveolar macrophages, such as macrophage-inflammatory protein-2 (12). Upon alveolar co-challenge with CCL2 and LPS, the speed and magnitude of neutrophil influx into the alveolar compartment are dramatically increased. This substantial amplification of the neutrophil response is dependent on a facilitator function of blood-borne $CCR2^+$ mononuclear cells. $CCR2^+$ resident alveolar macrophages do not appear to play a role. The potent effect of $CCR2^+$ monocytes on neutrophil accumulation is demonstrated by the finding that reconstitution of CCR2-deficient mice with bone marrow cells that contained only 25% WT cells was sufficient for the full enhancement effect. It is not yet clear whether the $CCR2^+$ mononuclear cells fulfill the facilitator function from the intravascular side of the blood-air barrier or whether premigration or comigration of monocytes with neutrophils is required. The reduced lung neutrophil responses previously noted in WT mice treated with anti-CCL2 Abs and challenged with *Cryptococcus neoformans* (13) and in CCR2-deficient mice challenged with *Aspergillus fumigatus* conidia (14) are consistent with our model of interdependent neutrophil and monocyte trafficking during pulmonary inflammation. This concept may offer new perspectives for therapeutic interventions, considering the fact that the regulation of alveolar neutrophil trafficking is a critical element of infectious diseases, such as pneumonia, and autoimmune diseases, such as idiopathic lung fibrosis.

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