

Monocyte-derived dendritic cell recruitment and allergic T_H2 responses after exposure to diesel particles are CCR2 dependent

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Background: The inhalation of diesel exhaust particles (DEPs) is associated with increased sensitization toward inhaled allergens. Dendritic cells (DCs) are important mediators in immune regulation. We previously showed that the inhalation of DEPs increased the accumulation of DCs in the lung and enhanced the T_H2 response in the mediastinal lymph node. **Objective:** We hypothesized that CC chemokine receptors CCR2, CCR5, and CCR6 critically mediate the DC recruitment upon exposure to DEPs and that these CC chemokine receptors are important in the DEP-induced T_H2 response. **Methods:** We exposed CCR2 knockout, CCR5 knockout, CCR6 knockout, and wild-type mice to DEPs and examined the pulmonary monocyte and DC accumulation. By an adoptive transfer experiment, we assessed the direct involvement of CCR2 and CCR6 in the recruitment of blood monocytes toward the lung upon exposure to DEPs. We also examined the T_H2 cytokine production in the mediastinal lymph nodes of DEP-exposed CCR2 knockout and CCR6 knockout mice. **Results:** We observed that the DEP-induced monocyte and monocyte-derived DC recruitment was completely abolished in CCR2 knockout mice. CCR6 knockout mice also showed impaired monocyte recruitment upon exposure to DEPs. In contrast, monocyte and DC recruitment was comparable between DEP-exposed wild-type and CCR5 knockout mice. The impaired monocyte-derived DC recruitment in DEP-exposed CCR2 knockout, not CCR6 knockout, mice resulted in an abolished T_H2 response in the mediastinal lymph node. **Conclusion:** These data suggest that monocyte-derived DCs, recruited in a CCR2-dependent manner, are critical in inducing T_H2 responses upon inhalation of DEPs. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: CCR2, CCR5, CCR6, dendritic cell, diesel exhaust particles, monocyte

Particulate matter inhalation is associated with several adverse health effects. Diesel exhaust particles (DEPs) are a major component of this ambient particulate matter. Diverse mouse models and human studies have demonstrated that inhalation of DEPs increases T_H2 sensitization to co-inhaled allergens and that exposure to DEPs exacerbates allergic airway responses.¹

Dendritic cells (DCs) are important in bridging the innate and adaptive immunity.² In allergic asthma, DCs are crucial in the sensitization to inhaled allergens.³⁻⁵ Pulmonary DCs reside near the epithelium as immature cells and continuously sample the lumen for inhaled antigens. Upon danger signals, DCs mature and migrate to the mediastinal lymph node (LN), where they induce a polarized T_H response.² We previously showed that exposure to DEPs induces the accumulation and maturation of DCs in the lung. Exposure to DEPs also increases the migration of DCs to the mediastinal LN and enhances the subsequent T-cell response. We suggested that this DC modulation upon inhalation of DEPs represented a mechanism how particulate matter induces T_H2 sensitization to co-inhaled allergens.⁶

Monocyte and DC recruitment to tissues in response to inflammation is mediated through the interaction of specific surface receptors on these cells with various chemokines that are released in the tissue. For the pulmonary tissue, there is no unique chemokine receptor that mediates DC recruitment and depending on the inflammatory stimulus or the model, various chemokine ligand-chemokine receptor interactions appear to be implicated. Upon cigarette smoke, for example, decreased lung DC accumulation was observed in CC chemokine receptors (CCRs) CCR5 knockout and CCR6 knockout mice,^{7,8} while during allergic airway inflammation, CCR2, but not CCR5 or CCR6, mediated pulmonary DC recruitment.⁹

In response to DEPs, however, the mechanism of monocyte and DC recruitment is unstudied. We and others previously showed that instillation of DEPs is associated with increased CC chemokine ligands (CCLs), including monocyte chemotactic protein 1 (MCP-1)/CCL2, RANTES/CCL5, and macrophage inflammatory protein 3 alpha (MIP-3α)/CCL20,^{6,10} which could suggest an involvement of the respective CCL/CCR interactions in the DEP-induced monocyte and DC recruitment.

In this study, we examined the relative contribution of CCR2, CCR5, and CCR6—the receptors of CCL2, CCL5, and CCL20, respectively—to the monocyte and DC recruitment and T_H2 sensitization in response to DEPs.

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Abbreviations used

BALF: Bronchoalveolar lavage fluid
 CCL: CC chemokine ligand
 CCR: CC chemokine receptor
 CFSE: Carboxyfluorescein diacetate succinimidyl ester
 DCs: Dendritic cells
 DEPs: Diesel exhaust particles
 LN: Lymph node
 MCP-1: Monocyte chemoattractant protein 1
 MIP-3 α : Macrophage inflammatory protein 3 alpha
 OVA: Ovalbumin
 WT: Wild type

METHODS**Mice**

CCR2 knockout mice, aged 6 to 8 weeks, were obtained from Taconic (Hudson, NY).¹¹ C57BL/6 wild-type (WT) mice, aged 6 to 8 weeks, were obtained from Harlan Laboratories (Horst, The Netherlands). CCR5 knockout mice,¹² CCR6 knockout mice, and C57BL/6 WT mice, aged 6 to 8 weeks, were obtained from the Jackson Laboratory (Bar Harbor, Me). OT-II TCR transgenic mice were obtained from Bart N. Lambrecht (Laboratory of Immunoregulation and Mucosal Immunology, Department of Respiratory Medicine, Ghent, Belgium). All mice were bred in the animal facility of the Ghent University. All *in vivo* manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Instillation of DEPs

DEPs (standard reference material 2975) were obtained from the National Institute for Standards and Technology (Gaithersburg, Md) and were derived from an industrial diesel-powered forklift. The relevance of standard reference material 2975 and its comparison with diesel from an automobile engine have been assessed by others.^{13,14} Previously, we compared various doses of DEPs ranging from 10 to 100 μ g, relevant to the human situation.⁶ Since the effects were more pronounced with 100 μ g of DEPs, this dose was used for the mechanistic studies in the current paper. We suspended the DEPs in saline containing 0.05% Tween 80 (Sigma-Aldrich, St Louis, Mo) to a concentration of 2 mg/mL and instilled the mice at days 1, 4, and 7. For instillation, anesthetized mice (intraperitoneal ketamine [70 mg/kg; Ketamine 1000 CEVA, Ceva Sante Animale, Libourne, France]–xylazine [7 mg/kg; Rompun 2%, Bayer, Leverkusen, Germany]) were held vertically and 50 μ L of saline or DEP solution was pipetted just above their vocal cords. By grasping the tongue, the mice were prevented from swallowing. On day 9, the animals were sacrificed by a lethal dose of intraperitoneal pentobarbital (Ceva Sante Animale).

Bronchoalveolar lavage

A tracheal cannula was inserted and bronchoalveolar lavage fluid (BALF) was recuperated by the instillation of 3×300 μ L HBSS without Ca^{2+} or Mg^{2+} (BioWittaker, Lonza, Basel, Switzerland) supplemented with 1% BSA (for protein measurement; Sigma-Aldrich) and 6×500 μ L HBSS without Ca^{2+} or Mg^{2+} supplemented with 0.6 mM sodium EDTA (Sigma-Aldrich). The lavage fractions were pooled and resuspended in HBSS. Total cell counts were performed in a Bürcker chamber.

Lung and LN single-cell suspensions

The pulmonary circulation was rinsed with saline/EDTA to remove the intravascular pool of cells. Organs were minced and incubated in digestion medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol [Gibco; Invitrogen, Paisley, United Kingdom], 100 U/mL penicillin–100 μ g/mL streptomycin [Sigma-Aldrich], 1 mg/mL collagenase type 2 [Worthington Biochemical, Lakewood, NY], and 0.02 mg/mL DNase I [grade II from bovine pancreas; Boehringer Ingelheim,

Ingelheim, Germany]) for 45 minutes at 37°C and 5% CO_2 . Red blood cells were lysed by using ammonium chloride buffer. Cell counting was performed with a Z2 coulter counter (Beckman Coulter, Fullerton, Calif).

Flow cytometry

All staining procedures were performed in PBS without Ca^{2+} or Mg^{2+} (BioWittaker, Lonza) containing 5 mM EDTA and 1% BSA. To minimize nonspecific bindings, single-cell suspensions were preincubated with anti-CD16/CD32 (clone 2.4G2). Cells were labeled with combinations of CD11c (HL3), MHCII (2G9), CD11b (M1/70), Ly6C (AL-21), and Ly6G (1A8) (all from BD Pharmingen, San Diego, Calif). Data acquisition was performed on a FACSCalibur flow cytometer running CELLQuest software (BD Biosciences, San Jose, Calif). FlowJo software (TreeStar, Inc, Ashland, Ore) was used for data analysis.

Bone marrow adoptive transfer experiment

Bone marrow cells were isolated from the femur and tibia from CCR2 knockout, CCR6 knockout, and WT mice. Red blood cells were lysed by using ammonium chloride buffer. Cells were labeled with 2 μ M 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen) at 37°C for 10 minutes, as previously described.¹⁵ Cells were analyzed with flow cytometry to adjust the cell suspension to 75×10^6 CD11b⁺ cells/mL. Next, we injected 200 μ L cells intravenously in WT recipient mice, which were instilled with 100 μ g of DEPs at the time of adoptive transfer. Forty-eight hours later, lung single-cell suspensions were made and CFSE⁺ CD11b⁺ monocytes were analyzed by using flow cytometry. This adapted protocol was sufficient to induce CCL expression in the BALF, and pulmonary monocyte and DC subset recruitment (data not shown).

T-cell response in mediastinal LN

On day 8, saline- and DEP-treated mice were intravenously injected with 10×10^6 spleen cells from OT-II mice. Mice were instilled with 350 μ g of ovalbumin (OVA; Worthington Biochemical) in sterile PBS on day 9. Four days later, mediastinal LNs were digested. A total of 2×10^5 cells (in triplicate) were cultured in RPMI 1640 medium (supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol, 100 U/mL penicillin–100 μ g/mL streptomycin) and 100 μ g/mL of OVA in round-bottom 96-well plates (BD Bioscience). Four days later, the supernatant was harvested for cytokine measurement.

Cytokine and chemokine measurement

MCP-1 (CCL2), RANTES (CCL5), MIP-3 α (CCL20), and IL-4, IL-10, and IL-13 concentrations in the BALF or mediastinal LN culture supernatant were measured by using Quantikine ELISA Kits (R&D Systems, Abingdon, United Kingdom).

RT-PCR analysis

RNA from the lung tissue was extracted by using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Expression of CCR2, CCR5, and CCR6 relative to hypoxanthine guanine phosphoribosyltransferase mRNA was analyzed by using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif). RT-PCR was performed starting from 10 ng total RNA, using a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland). Reverse transcription was performed by murine leukemia virus reverse transcriptase (Applied Biosystems) at 48°C for 30 minutes. Amplification conditions were as follows: 10 minutes at 95°C and 45 cycles of 10 seconds at 95°C and 15 seconds at 60°C.

Statistical analysis

Statistical analysis was performed with SPSS, version 17.0 (SPSS, Chicago, Ill). Groups were compared by using nonparametric tests (Kruskal-Wallis and Mann-Whitney *U*) according to standard statistical criteria.

Reported values were expressed as mean $\pm$ SEM. *P* values of $<.05$ were regarded as significant.

RESULTS

Exposure to DEPs increases pulmonary monocyte and DC subsets

Recruitment of monocytes and DC subsets is crucial in regulating pulmonary immune responses.² We characterized the monocyte and DC subset accumulation in the lung interstitium (accessible by enzymatic digestion) and the alveolar space (accessible by the BALF) upon exposure to saline and DEPs. We observed that monocytes (characterized as CD11c[−]CD11b⁺Ly6C⁺Ly6G[−] cells) accumulate in the lung interstitium and the alveolar space in response to DEPs in WT mice (Fig 1, A and B).

In the lung interstitium, 2 major DC populations are described: conventional DCs (cDCs; characterized as CD11c⁺ low autofluorescent MHCII⁺ cells) and plasmacytoid DCs (pDCs; gated as CD11c^{med} low autofluorescent CD11b[−]SiglecH⁺ cells). Lung cDCs are further subdivided into CD11b[−] cDCs and CD11b⁺ cDCs.¹⁶ Exposure to DEPs increased both the CD11b[−] cDC subset and the CD11b⁺ cDC subset (Fig 1, C and D, respectively). Lung pDCs did not change in response to DEPs (data not shown; Provoost et al⁶). Upon pulmonary inflammation, such as virus infection and fungal infection, an additional monocyte-derived DC subset is recruited: the inflammatory DC subset (characterized as CD11c⁺ low autofluorescent CD11b⁺Ly6C⁺ cells).^{17–19} Upon exposure to DEPs, inflammatory DCs were increased in the lung interstitium (Fig 1, E). DCs in the alveolar space are CD11c⁺ low autofluorescent MHCII⁺ cells and are also increased upon exposure to DEPs (Fig 1, F).

Exposure to DEPs induces pulmonary CCL and CCR expression

CCL/CCR interactions are important in monocyte and DC subset recruitment toward the lung. We assessed whether exposure to DEPs induces CCL proteins by using ELISA. Exposure to DEPs increased MCP-1 (CCL2, Fig 2, A), RANTES (CCL5, Fig 2, B), and MIP-3 α (CCL20, Fig 2, C) concentrations in the BALF of WT mice. By using qualitative RT-PCR, we next examined whether exposure to DEPs also induces CCR expression. Exposure to DEPs increased CCR2 and CCR5 mRNA expression in total lung tissue (Fig 2, D and E, respectively). CCR6 mRNA expression tended to increase upon exposure to DEPs (Fig 2, F); however, this was less pronounced than CCR2 and CCR5 mRNA expression.

DEP-induced pulmonary monocyte and DC subsets in CCR2 knockout, CCR5 knockout, and CCR6 knockout mice

To determine the contribution of CCR2, CCR5, and CCR6 to the DEP-induced monocyte and DC subset accumulation, we exposed CCR2 knockout, CCR5 knockout, CCR6 knockout, and WT mice to saline or 100 μ g of DEPs. Upon exposure to DEPs, there was—in contrast to WT mice—no monocyte accumulation in the lung interstitium and the alveolar space of CCR2 knockout mice (Fig 3, A and B). Also, DEP-induced lung CD11b⁺ cDCs (Fig 3, D), inflammatory DCs (Fig 3, E), and alveolar DCs (Fig 3, F) were markedly reduced in CCR2 knockout mice. DEP-induced CD11b[−] cDC accumulation in the lung was comparable

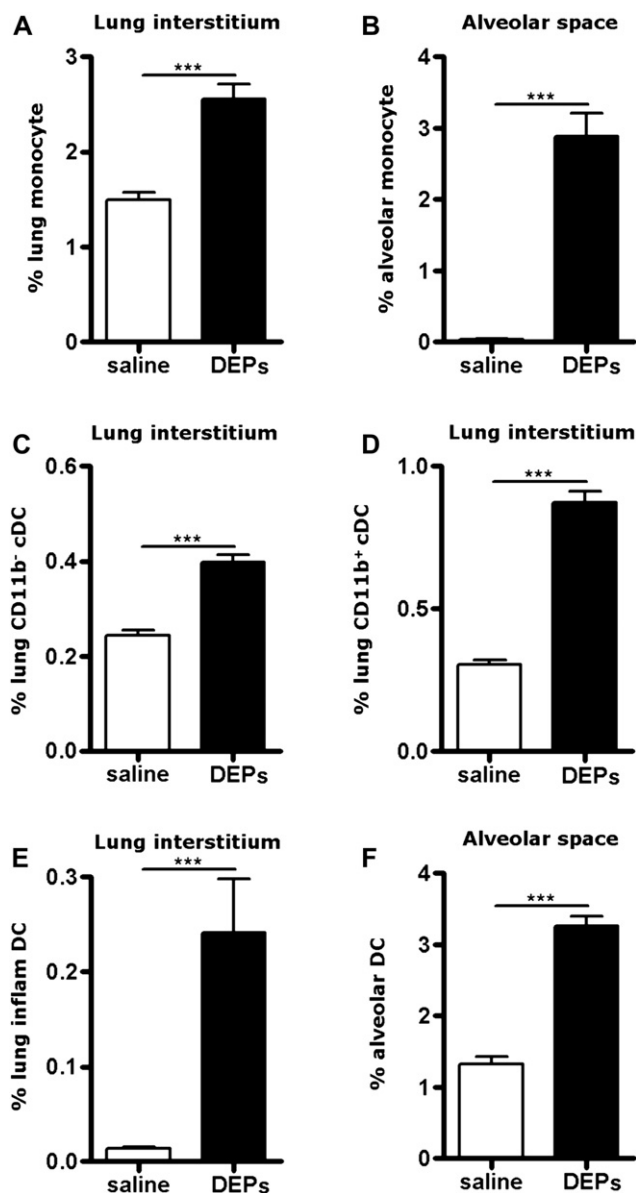


FIG 1. WT mice were exposed to saline (white bars) or 100 μ g of DEPs (black bars). Monocytes in the lung interstitium (A) and the alveolar space (B) were determined by using flow cytometry. C–F, Lung CD11b[−] cDCs (Fig 1, C), CD11b⁺ cDCs (Fig 1, D), inflammatory DCs (Fig 1, E), and alveolar DCs (Fig 1, F) were also determined by using flow cytometry. Results are expressed as mean $\pm$ SEM. *n* = 8 mice per group. ****P* < .005.

between CCR2 knockout and WT mice (Fig 3, C). Upon saline exposure, lung monocytes were severely reduced in CCR2 knockout mice (Fig 3, A). CCR2 knockout mice also showed a baseline reduction in lung CD11b⁺ cDCs (Fig 3, D).

In contrast to the CCR2 knockout mice, we found that the DEP-induced pulmonary monocyte and DC subset accumulation was unaffected by CCR5 deficiency (data not shown).

DEP-induced monocyte accumulation was absent in the lung interstitium of CCR6 knockout mice (Fig 4, A). Alveolar monocytes and lung inflammatory DCs were also reduced in CCR6 knockout mice (Fig 4, B and E, respectively). In contrast, lung cDC subsets and alveolar DCs were similar between DEP-exposed CCR6 knockout and WT mice (Fig 4, C, D, and F).

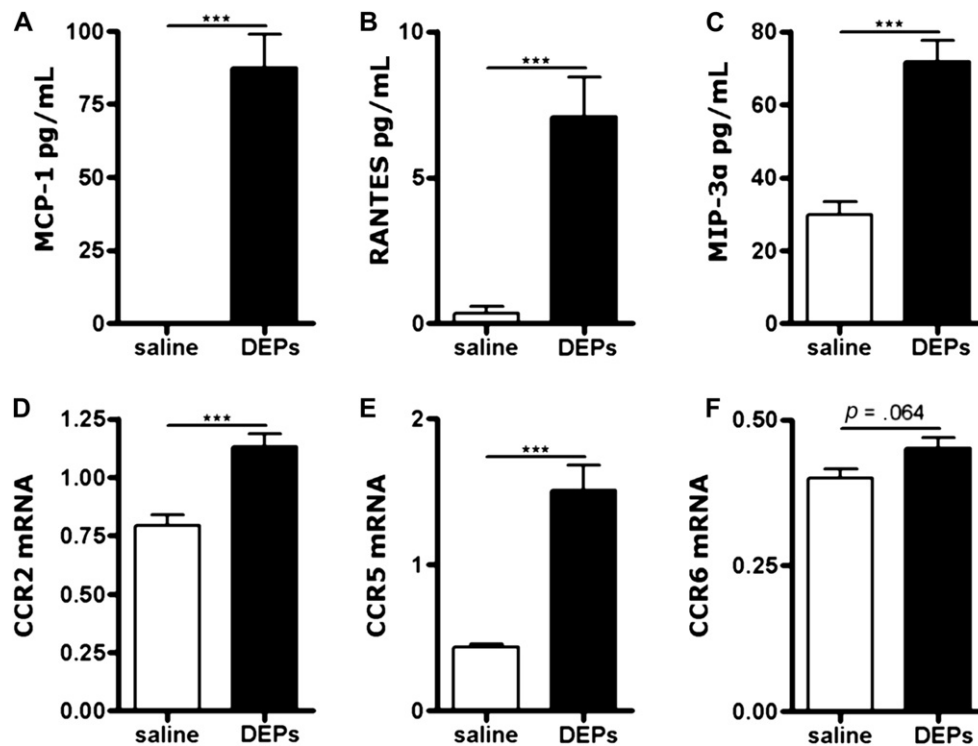


FIG 2. WT mice were exposed to saline (white bars) or 100 μ g of DEPs (black bars). **A-C**, MCP-1 (Fig 2, A), RANTES (Fig 2, B), and MIP-3 α (Fig 2, C) protein levels in the BALF were determined by using ELISA. **D-F**, CCR2 mRNA (Fig 2, D), CCR5 mRNA (Fig 2, E), and CCR6 mRNA (Fig 2, F) expression in the lung tissue was determined by using RT-PCR. Results are expressed as mean $\pm$ SEM. $n = 8$ mice per group. *** $P < .005$.

DEP-induced neutrophils and macrophages were similar between WT, CCR2 knockout, CCR5 knockout, and CCR6 knockout mice (Table I).

DEP-induced monocyte recruitment toward the lung is CCR2 and CCR6 dependent

Since the pulmonary monocyte numbers were severely reduced in saline-exposed CCR2 knockout mice, we evaluated the number of monocytes in the blood at baseline conditions. As described previously,^{20,21} naive CCR2 knockout mice had a marked reduction in blood monocytes (Fig 5, A). One could therefore speculate that the reduced lung monocyte accumulation upon exposure to DEPs originate from this defect in blood monocytes. However, the impaired monocyte accumulation upon exposure to DEPs can also originate from a CCR2-dependent blood-to-lung monocyte migration. To investigate the functional role of CCR2 in the blood-to-lung migration directly, bone marrow monocytes from WT and CCR2 knockout mice were labeled with CFSE and intravenously injected in WT mice. The recipients were subsequently exposed to 100 μ g of DEPs, and 48 hours later, CFSE⁺ monocytes in the pulmonary tissue were assessed. We observed decreased CCR2^{-/-} monocyte recruitment to the lung compared with CCR2^{+/+} monocyte recruitment (Fig 5, B), indicating that CCR2 is necessary in the DEP-induced lung monocyte recruitment.

In contrast to blood monocyte numbers at baseline in CCR2 knockout mice, blood monocyte numbers at baseline were similar in CCR6 knockout and WT mice (Fig 5, A). However, since we observed reduced monocyte accumulation in DEP-exposed

CCR6 knockout mice, we also wanted to examine the DEP-induced blood-to-lung monocyte migration more directly in the CCR6 knockout mice. As shown in Fig 5, C, the recruitment of CCR6^{-/-} monocytes to the lung was reduced compared with the recruitment of CCR6^{+/+} monocytes.

Attenuated lung monocyte and DC recruitment in DEP-exposed CCR2 knockout mice is associated with a decreased T_H2 response in mediastinal LNs

Previously, we and others showed that inhalation of DEPs was associated with an increased T_H2 response in the mediastinal LNs.^{1,6} Since DCs are important in inducing T_H2 responses, we next examined the DEP-induced T_H2 response in the mediastinal LNs of CCR2 and CCR6 knockout mice. For this purpose, CCR2 knockout, CCR6 knockout, and WT mice were exposed to saline or DEPs. On day 8, mice were injected with OVA-specific T cells and instilled with OVA on day 9. Four days later, mediastinal LNs were harvested, cultured with OVA, and analyzed for T_H2 cytokine production. We observed increased IL-4, IL-10, and IL-13 cytokine levels in the mediastinal LNs of WT and CCR6 knockout mice when these were exposed to DEPs. T_H2 cytokine production in CCR2 knockout mice, however, was greatly decreased (Fig 6, A-C).

DISCUSSION

Environmental pollutants such as DEPs are regarded as irritants that enhance allergic airway responses.¹ Here, we show that exposure to DEPs induces MCP-1 and MIP-3 α in

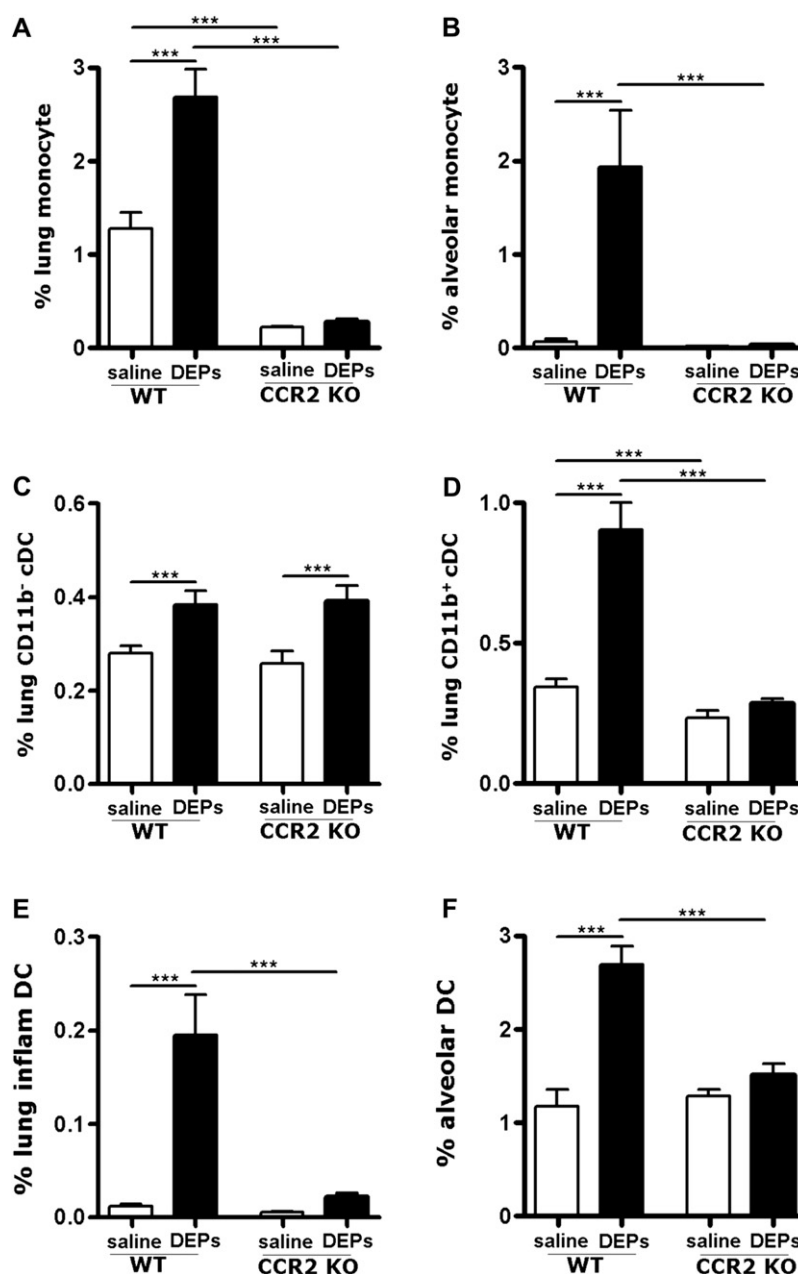


FIG 3. WT mice and CCR2 knockout mice were exposed to saline (white bars) or 100 µg of DEPs (black bars). Monocytes in the lung interstitium (A) and the alveolar space (B) were determined by using flow cytometry. C-F, Lung CD11b⁺ cDCs (Fig 3, C), CD11b⁺ cDCs (Fig 3, D), inflammatory DCs (Fig 3, E), and alveolar DCs (Fig 3, F) were determined by using flow cytometry. Results are expressed as mean ± SEM. n = 7-8 mice per group. ***P < .005.

the lung, which is associated with the accumulation of monocytes and DC subsets in a CCR2- and CCR6-dependent manner. We also demonstrate that the impaired monocyte-derived DC recruitment in CCR2 knockout mice is associated with an abolished T_H2 cytokine production upon inhalation of DEPs.

Lung epithelial cells are in close association with the DCs and represent the first line of defense against inhaled particles. Exposure to DEPs can activate the epithelium to produce chemokines that attract monocytes and DC precursors from the blood. Epithelial stimulation with particulate matter and

DEPs, for example, was shown to induce CC chemokine expression, including MCP-1, RANTES, and MIP-3α, *in vitro*.²²⁻²⁴ In healthy volunteers, challenge with DEPs enhanced the expression of CCR2 and CCR5 ligands in nasal washes.¹⁰ Corresponding with this, we show here that exposure to DEPs is associated with increased CC chemokines MCP-1, RANTES, and MIP-3α in the lungs. Specifically, we found increased CCR2, CCR5, and CCR6 mRNA expression in the lung tissue upon exposure to DEPs, which suggests a role for these CCL/CCR axes in the DEP-induced lung monocyte and DC subset recruitment.

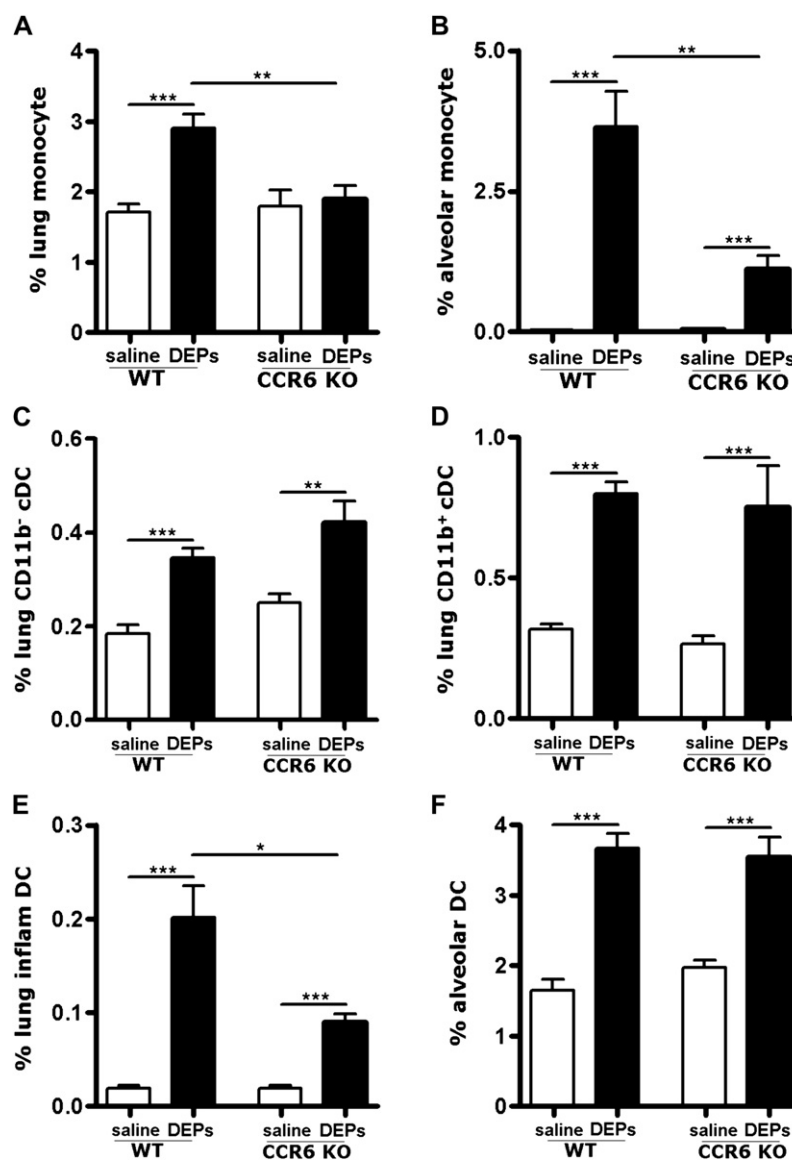


FIG 4. WT mice and CCR6 knockout mice were exposed to saline (white bars) or 100 μ g of DEPs (black bars). Monocytes in the lung interstitium (A) and the alveolar space (B) were determined by using flow cytometry. C-F, Lung CD11b⁺ cDCs (Fig 4, C), CD11b⁺ cDCs (Fig 4, D), inflammatory DCs (Fig 4, E), and alveolar DCs (Fig 4, F) were determined by using flow cytometry. Results are expressed as mean $\pm$ SEM. $n = 7-8$ mice per group. * $P < .05$, ** $P < .01$, *** $P < .005$.

Circulating inflammatory (CD11b⁺Ly6C^{hi}CCR2⁺) monocytes are recruited toward tissues in response to various inflammatory stimuli.²⁵ We observed that exposure to DEPs increased the inflammatory monocytes in the alveolar space and the lung interstitium and that this accumulation was severely impaired in CCR2 knockout mice. A CCR2-dependent inflammatory monocyte recruitment has also been demonstrated in response to other inflammatory and pathogenic stimuli.^{17,26} Depending on the inflammatory stimulus and model, however, several other CCRs were also described to be implicated in the monocyte recruitment toward (lung) tissues. CCR5, for example, mediates monocyte/DC accumulation upon cigarette smoke, and this could suggest a role for this chemokine receptor in the cell recruitment upon irritants.⁷ However, we found that CCR5 was not involved in inflammatory monocyte recruitment upon inhalation of DEPs.

Also, MIP-3 α /CCR6 signaling can be implicated in monocyte migration.²⁷ For instance, in the pulmonary tissue, CCR6 knockout mice were shown to have reduced monocyte accumulation upon fungal infection.²⁸ We found an impaired inflammatory monocyte accumulation in CCR6 knockout mice when these were exposed to DEPs. These findings suggest a role for CCR2 and CCR6 in inflammatory monocyte accumulation upon inhalation of DEPs.

Since CCR2 is implicated in the release of inflammatory monocytes from the bone marrow into the blood,²⁰ CCR2 knockout mice lack circulating inflammatory monocytes.^{20,21} One could therefore argue that the reduced monocyte accumulation in the lungs of DEP-exposed CCR2 knockout mice represented the lack in circulating monocytes rather than representing an involvement of CCR2 in the migration of monocytes from blood toward lung tissue. In the literature, some controversy exists about

TABLE I. Alveolar macrophages and neutrophils after exposure to DEP

	Alveolar macrophages (%)		Alveolar neutrophils (%)	
	Saline	DEPs	Saline	DEPs
CCR2 ^{+/+}	63.74 ± 6.93	24.46 ± 2.36*	0.57 ± 0.15	16.31 ± 1.07*
CCR2 ^{-/-}	80.44 ± 2.15	25.55 ± 2.87*	0.19 ± 0.06	19.52 ± 1.92*
CCR5 ^{+/+}	77.24 ± 3.88	16.00 ± 2.03*	0.26 ± 0.03	11.77 ± 0.85*
CCR5 ^{-/-}	80.20 ± 1.61	23.05 ± 1.51*	0.25 ± 0.06	12.19 ± 1.02*
CCR6 ^{+/+}	77.44 ± 3.04	19.90 ± 2.11*	0.59 ± 0.44	16.90 ± 1.18*
CCR6 ^{-/-}	84.23 ± 1.41	16.96 ± 1.30*	0.10 ± 0.02	18.91 ± 2.80*

WT mice, CCR2 knockout mice, CCR5 knockout mice, and CCR6 knockout mice were exposed to saline or 100 µg of DEPs. Alveolar macrophages (CD11c⁺ high autofluorescent MHCII⁺) and neutrophils (CD11c⁻CD11b⁺Ly6C⁺Ly6G⁺) were determined by using flow cytometry. Results are expressed as mean ± SEM. n = 7-8 mice per group.

**P* < .05 compared with appropriate saline-exposed mice.

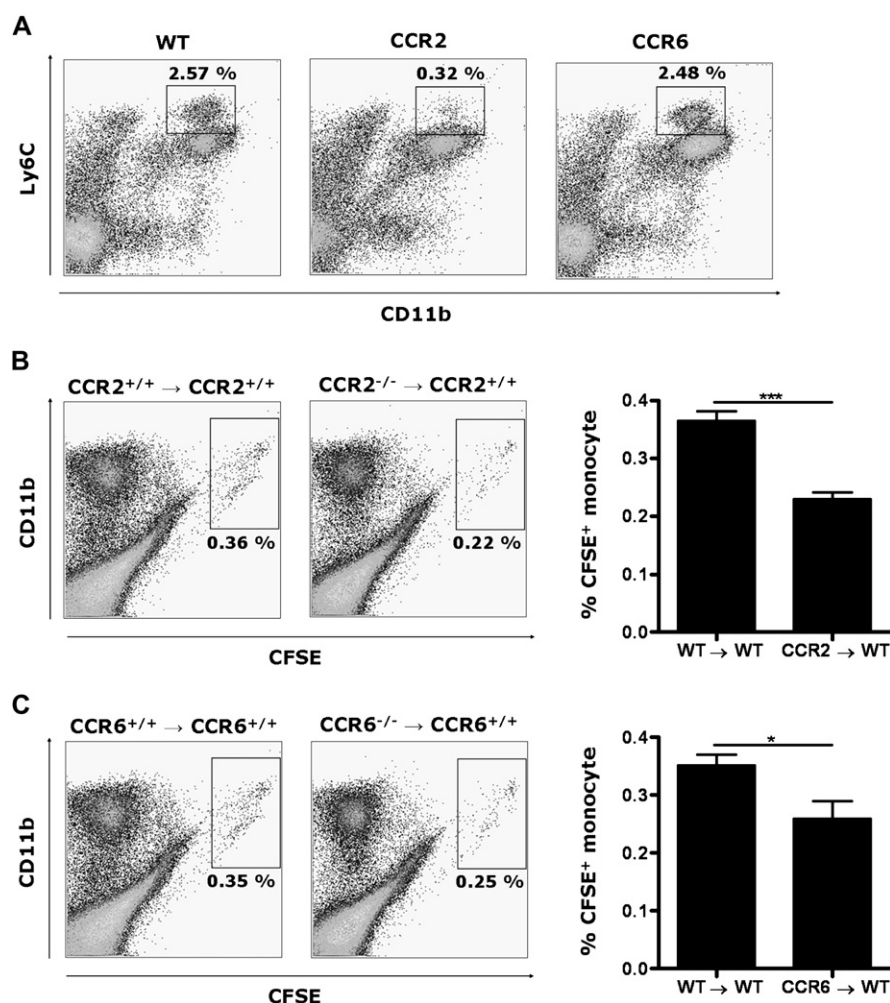


FIG 5. **A**, Representative dot plots of baseline circulating CD11b⁺Ly6C⁺ monocytes in WT, CCR2 knockout, and CCR6 knockout mice. **B**, Bone marrow cells from WT mice and CCR2 knockout mice were labeled with CFSE and intravenously injected into DEP-treated WT mice. Forty-eight hours later, CFSE⁺CD11b⁺ monocytes in the lung tissue were determined by using flow cytometry. **C**, Bone marrow cells from WT mice and CCR6 knockout mice were labeled with CFSE and intravenously injected into DEP-treated WT mice. Forty-eight hours later, CFSE⁺CD11b⁺ monocytes in the lung tissue were determined by using flow cytometry. Numbers on graphs represent mean percentage monocytes. Results are expressed as mean ± SEM (Fig 5, C). n = 7-8 mice per group. **P* < .05, ****P* < .005.

this: upon bacterial infection, it is demonstrated that CCR2 is not required for the migration of monocytes from blood to spleen,²⁰ whereas in a thioglycolate model, CCR2 was shown to be necessary for the migration from blood to the peritoneal cavity.²¹ To

examine the contribution of CCR2 following inhalation of DEPs directly, adoptive transfer of CFSE-labeled bone marrow cells was done. We observed that CCR2^{+/+} monocytes were more efficiently recruited than CCR2^{-/-} monocytes toward the

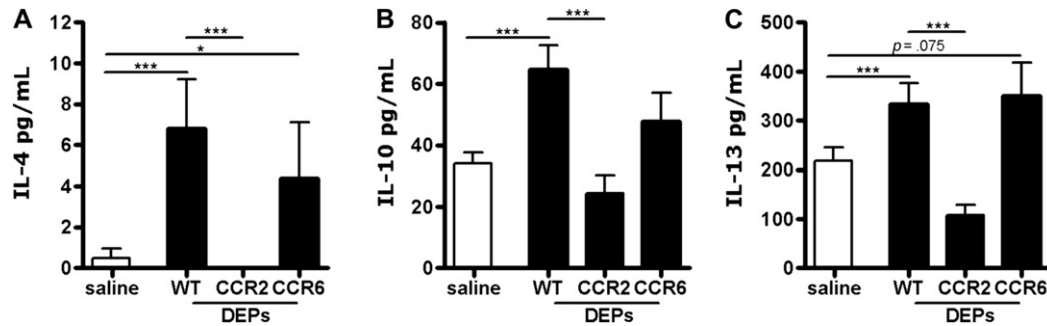


FIG 6. WT mice, CCR2 knockout mice, and CCR6 knockout mice were exposed to saline (white bars) or 100 μ g of DEPs (black bars). On day 8, mice were intravenously injected with 10×10^6 OT-II cells and instilled with 350 μ g OVA on day 9. Four days later, mediastinal LNs were digested and cultured in medium plus 100 μ g/mL OVA. **A-C**, IL-4 (Fig 6, A), IL-10 (Fig 6, B), and IL-13 (Fig 6, C) protein levels in mediastinal LN supernatant were determined by using ELISA. Results are expressed as mean $\pm$ SEM. $n = 7-8$ mice per group; saline groups were pooled to make graphs more clear (there were no statistical differences between saline groups); * $P < .05$, *** $P < .005$.

lung. CCR6 knockout mice had no monocytopenia, but they also showed reduced monocytes upon exposure to DEPs. Therefore, we followed the same strategy to directly investigate the involvement of CCR6 in the monocyte migration from blood to the lung tissue. CCR6^{-/-} monocytes were less efficiently recruited to the lung than their WT counterparts. Together, these findings show an important role for CCR2 and CCR6 in the DEP-induced monocyte migration from blood toward the lung.

Upon inflammatory stimuli, monocytes recruited to the lung rapidly upregulate CD11c and become inflammatory DCs. These inflammatory DCs, however, express Ly6C as a permanent indication of their monocytic origin.¹⁶ Exposure to DEPs increased the inflammatory DC subset in the lung. Corresponding with the DEP-induced monocytes, we found that lung inflammatory DCs were absent or reduced in DEP-exposed CCR2 and CCR6 knockout mice. Besides the inflammatory DC subset, monocytes can also give rise to CD11b⁺ cDCs.^{25,29,30} We observed no CD11b⁺ cDC accumulation in DEP-exposed CCR2 knockout mice, which suggests that DEP-recruited CD11b⁺ cDCs also derive from inflammatory monocytes. In line with our observation, it was previously shown during fungal infection that inflammatory monocytes differentiate into lung CD11b⁺ cDCs.^{18,19} Of interest, these monocyte-derived DCs are important in T_H2 sensitization.⁵ In CCR6 knockout mice, DEP-induced CD11b⁺ cDCs were not decreased, despite reduced inflammatory monocytes in the lung. Possibly, this can be explained because the reduction in monocytes and inflammatory DCs in CCR6 knockout mice was only marginal, compared with the observation in CCR2 knockout mice.

In contrast to inflammatory DCs and CD11b⁺ cDCs, the CD11b⁻ cDC subset exclusively derives from a Flt3⁺ cDC precursor, which is not related to monocytes.³¹ We observed similar CD11b⁻ cDC numbers in DEP-exposed CCR2 knockout, CCR5 knockout, and CCR6 knockout mice. A tolerogenic role has been suggested for these CD11b⁻ cDCs¹⁶; however, the role of these cells in the inflammatory response after exposure to DEPs remains unclear. DCs that populate the pulmonary tissue under homeostatic conditions are also thought to be derived from the Flt3⁺ cDC precursor. This could explain why we detected no baseline deficiency in DCs in the CCR2 knockout mice whereas the monocytes were severely reduced at baseline in these mice.

Monocyte-derived DCs are crucial in inducing T_H2 airway inflammation toward inhaled house-dust mite.⁵ Diverse studies

have demonstrated that inhalation of DEPs is associated with increased sensitization to co-inhaled allergens.¹ Previously, we also reported that exposure to DEPs increased the T_H-cell response in the mediastinal LNs.⁶ Since DEP-exposed CCR2 knockout and CCR6 knockout mice showed a reduction in monocytes and monocyte-derived DCs, we were interested in the consequences on the T_H-cell response. We found that CCR2 knockout mice, but not CCR6 knockout mice, failed to induce 2 T_H2 cytokines in response to DEPs. This finding suggests that inflammatory monocytes and monocyte-derived DCs, recruited upon CCR2 signaling, may play a role in the DEP-induced adaptive immune responses and sensitization to co-inhaled allergens.

Exposure to DEPs induces chemokines, including MCP-1 and MIP-3 α from the epithelium. We suggest that in response to these chemokines, circulating inflammatory monocytes were recruited to the lung tissue in a CCR2-dependent, and to a lesser extent, CCR6-dependent, manner. We demonstrate that DEP-induced monocytes can then become inflammatory and CD11b⁺ cDCs. Furthermore, we suggest that the monocyte-derived DCs, recruited in a CCR2-dependent manner, are critical in the DEP-induced T_H2 response.

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Clinical implications: This study provides evidence that inhalation of diesel particles triggers the immune system in a very specific (CCR2-dependent) way, thereby facilitating allergic sensitization.

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