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Diesel Exhaust Particles Stimulate Adaptive Immunity by Acting on Pulmonary Dendritic Cells

Sharen Provoost,* Tania Maes,* Monique A. M. Willart,[†] Guy F. Joos,* Bart N. Lambrecht,[†] and Kurt G. Tournay*

Particulate matter, such as diesel exhaust particles (DEPs), modulate adaptive immune responses in the lung; however, their mechanism of action remains largely unclear. Pulmonary dendritic cells (DCs) are crucial mediators in regulating immune responses. We hypothesized that the immunomodulatory effects of DEPs are caused by alteration of DC function. To test this, we instilled mice with DEPs and examined the pulmonary DC recruitment and maturation, their migration to the mediastinal lymph node (MLN), and the subsequent T cell response. We demonstrated that exposure to DEPs increased DC numbers in the bronchoalveolar lavage and the lungs and that DEPs increased the maturation status of these DCs. DEP exposure also enhanced the DC migration to the MLN. Moreover, we showed that DEPs themselves were transported to the MLN in a CCR7- and DC-dependent manner. This resulted in an enhanced T cell recruitment and effector differentiation in the MLN. These data suggest that DEP inhalation modulates immune responses in the lung via stimulation of DC function. *The Journal of Immunology*, 2010, 184: 426–432.

Diesel exhaust particles (DEPs) are a major component of ambient particulate matter and are associated with various health effects. In both animal models and humans, DEP exposure has been shown to modulate innate and adaptive immune responses in the lung (1, 2). DEP inhalation also induces sensitization to Ags and aggravates allergic airway responses (1–3). However, the mechanism of action of these immune reactions remains unclear.

Dendritic cells (DCs) are crucial in regulating immune responses and play an important role in both the induction and maintenance of asthma (4). Pulmonary DCs reside near the epithelium in an immature state, where they continuously sample the airway lumen and are specialized in Ag uptake. Different subsets of DCs can be found in the lung interstitium, each with a functional specialization (5, 6): conventional dendritic cells (cDCs), subdivided in CD11b⁺ and CD11b[−] cDCs, and plasmacytoid dendritic cells (pDCs). Upon the recognition of danger signals, DCs upregulate costimulatory molecules such as CD86 and migrate to the mediastinal lymph nodes (MLNs) (4). This migration is mediated by chemokine signals acting on chemokine receptor CCR7 (7, 8). Once arrived in the MLN, DCs present the Ags to naive T cells and induce a polarized T cell response (4). The outcome of this response depends on the context in which an

Ag is encountered by DCs and is determined by the presence of danger signals in the lung, such as damage-associated molecular patterns released by stressed or damaged cells (9).

Given their central location in the airways, DCs could come in close contact with the inhaled DEPs or with inflammatory mediators released upon DEP inhalation. One could therefore speculate that DEP exposure has an impact on several aspects of the DC biology. Indeed, *in vitro* data demonstrated that DEP exposure induces DC maturation (10, 11) via epithelial cell-derived mediators (12, 13) and that DEP-exposed DCs induce a Th2 polarization (10, 13–15).

In this study, we examined the consequences of DEP exposure on the key functions of the DC *in vivo*. We characterized the innate immune response upon DEP instillation and examined DC recruitment and maturation to the lung, DC migration to the MLN, and the subsequent T cell response.

Materials and Methods

Mice

Female BALB/c mice (6–8-wk-old) were purchased from Harlan Breeders (Zeist, The Netherlands). OVA-TCR transgenic mice (DO11.10) were bred at the Ghent University Hospital. Female CCR7 knockout (KO) and C57BL/6 wild types (WTs) (6–8-wk-old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The ethics committee for animal experimentation of Ghent University approved all *in vivo* manipulations.

DEP instillation

DEP (SRM 2975) was purchased from the National Institute for Standards and Technology (NIST; Gaithersburg, MD) and was derived from an industrial diesel-powered forklift. The relevance of this SRM 2975 and the comparison with diesel from an automobile engine has been assessed by others (16, 17). DEPs were suspended in sterile saline containing 0.05% Tween 80 (Invitrogen, Ghent, Belgium) to a final concentration of 0.2 mg/ml (a 10- μ g DEP dose, used for the DEP-induced innate immune and DC responses in the lung) or 2 mg/ml (a 100- μ g DEP dose, used for the mechanistic studies and DEP-induced responses in the MLN). Mice were instilled at days 1, 4, and 7 via oropharyngeal aspiration. Briefly, anesthetized mice (i.p. ketamine [80 mg/kg; Ketamine 1000 CEVA; Ceva Sante Animale, Brussels, Belgium] – xylazine [8 mg/kg; Rompun 2%; Bayer AG, Leverkusen, Germany]) were held vertically, and 50 μ l of suspension 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 i.p. pentobarbital (Sanofi-Ceva, Paris, France).

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; BM, bone marrow; cDC, conventional dendritic cell; DC, dendritic cell; DEP, diesel exhaust particle; KO, knockout; LN, lymph node; MHC-II, MHC class II; MLN, mediastinal lymph node; pDC, plasmacytoid dendritic cell; WT, wild type.

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Bronchoalveolar lavage

A tracheal cannula was inserted, and bronchoalveolar lavage (BAL) was performed by instillation of $3 \times 300 \mu\text{l}$ HBSS without Ca^{2+} or Mg^{2+} (Pasteur, Brussels, Belgium) supplemented with 1% BSA (for protein analysis) and $6 \times 500 \mu\text{l}$ HBSS without Ca^{2+} or Mg^{2+} supplemented with 0.6 mM sodium EDTA (for maximal recovery of BAL cells). The lavage fractions were pooled and resuspended in HBSS. Total cell counts were performed in a Bürker chamber. Differential cell counts were performed on cytospin preparations stained with May-Grünwald (Sigma-Aldrich, St. Louis, MO) Giemsa (VWR, Leuven, Belgium) using standard morphologic criteria.

Lung and MLN single-cell suspensions

Pulmonary circulation was rinsed with saline/EDTA to remove the intravascular pool of cells. As described previously (18), organs were minced and incubated in digestion medium (RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 0.05 mM 2-ME [all Life Technologies, Brussels, Belgium], 100 U/ml penicillin – 100 $\mu\text{g}/\text{ml}$ 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 Mannheim, Brussels, Belgium]) for 45 min at 37°C and 5% CO_2 . RBCs were lysed using ammonium chloride buffer. Cell counting was performed with a Z2 Coulter counter (Beckman Coulter, Fullerton, CA).

Flow cytometry

All staining procedures were performed in PBS without Ca^{2+} or Mg^{2+} containing 5 mM EDTA and 1% BSA (Dade Behring, Eschborn, Germany). To minimize nonspecific bindings, single-cell suspensions were preincubated with anti-CD16/CD32 (clone 2.4G2). Cells were labeled with combinations of CD11c (HL3), MHC class II (MHC-II) (M5/114.15.2), CD86 (GL-1), CD80 (16-10A1), CD40 (3/23), CD11b (M1/70), Ly6C (AL-21), Ly6G (1A8) (all BD Pharmingen, San Diego, CA), F4/80 (BM8), CCR7 (4B12), and DO11.10 TCR (KJ1-26) (all eBioscience, San Diego, CA). 7-Aminoactinomycin D (BD Pharmingen) was used for dead cell exclusion. Data acquisition was performed on a FACSCalibur flow cytometer running CellQuest software (BD Biosciences, San Jose, CA). For the study of the lung DC subsets and maturation, cells were labeled with CD11c (N418) (Invitrogen), CD86 (GL-1), CD45 (30-F11), MHC-II (M5/114.15.2) (all BD Pharmingen), CD11b (M1/70), F4/80 (BM8), and anti-siglec-H (eBio440c) (all eBioscience). Fixable live/dead marker in aqua (Invitrogen) was used for dead cell exclusion. Data acquisition was performed on a FACS Aria flow cytometer running FACSDiva software (BD Biosciences). FlowJo software (Tree Star, Ashland, OR) was used for data analysis. Cell sorting of DCs was performed on a FACS Aria flow cytometer.

Histology

The MLN, axillary lymph node (LN), and spleen were fixed in 4% paraformaldehyde. Organs were embedded in paraffin, and $3\text{-}\mu\text{m}$ transversal sections were cut and stained with H&E (Sigma-Aldrich). MLN sections were subjected to an immunological CD3/B220 double staining: in the first step, MLN sections were stained with anti-CD3 (Dako A0452), followed by a biotinylated Ab. Slides were incubated with streptavidin HRP and colored diaminobenzidine (all Dako, Glostrup, Denmark). In the second step, MLN sections were stained with anti-B220-biotin (RA3-6B2) (BD Pharmingen). Slides were incubated with streptavidin alkaline phosphatase (Dako) and colored with Vector Blue (Vector Laboratories, Peterborough, U.K.).

Generation of bone marrow-derived DCs

Using a standard protocol (19), bone marrow (BM) cells were cultured for 9 d in DC culture medium (RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 20 ng/ml GM-CSF (granted from Prof. K. Thielemans, Vrije Universiteit Brussel). Twenty-four hours before harvesting, BM-derived DCs were exposed to 10 $\mu\text{g}/\text{ml}$ DEPs. Cells were analyzed for the CD40 (3/23), CD80 (16-10A1), and CD86 (GL-1) expression on CD11c⁺ (HL3) MHC-II⁺ (M5/114.15.2) (BD Pharmingen) DCs or used for the CCR7 reconstitution experiment.

DEP translocation mechanism

CCR7 KO and C57BL/6 WT mice were instilled with 100 μg DEPs on day 1. On day 3, MLNs were digested and enriched for CD11c⁺ cells by CD11c microbeads (N418; Miltenyi Biotec, Bergisch Gladbach, Germany). The number of DEP⁺ cells within the enriched CD11c⁺ cells population was scored on cytospin preparations stained with May-Grünwald Giemsa. For the CCR7 reconstitution experiment, CCR7 KO mice were instilled with

5×10^6 DEP-exposed WT BM-derived DCs (described above) on day 1. On day 3, MLNs were digested and processed as described above.

DC migration

Control and 100- μg DEP-treated mice were instilled with 350 μg FITC-conjugated OVA (Molecular Probes, Ghent, Belgium) diluted in 50 μl sterile PBS on day 9. Zero, 12, 24, 48, 72, and 168 h later, the MLNs were digested and analyzed for the FITC⁺ signal in CD11c⁺ (HL3) MHC-II⁺ (M5/114.15.2) (BD Pharmingen) DCs.

T cell proliferation and cytokine production

LN and spleen single-cell suspensions from DO11.10 mice were labeled with 10 μM CFSE (Molecular Probes) at 37°C for 10 min, as described previously (20). Control and DEP-treated mice were i.v. injected with 1×10^7 OVA-specific DO11.10 T cells diluted in 200 μl RPMI 1640 medium on day 8. On day 9, mice were instilled with 350 μg OVA (Worthington Biochemical) diluted in 50 μl sterile PBS. Four days later, MLNs were digested. For the T cell proliferation experiment, MLNs were analyzed for the CFSE division profile in the CD4⁺ (GK1.5) (BD Pharmingen) DO11.10 TCR⁺ (KJ1-26) (eBioscience) OVA-specific T cells. The CFSE content was calculated as $\sum n_i/2^i$, where n_i = the number of cells in the i^{th} division peak, as described previously (20). For the cytokine production experiment, MLNs (2×10^5 cells/well in triplicate) were cultured in RPMI 1640 medium (supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, and 100 U/ml penicillin – 100 $\mu\text{g}/\text{ml}$ streptomycin) and 100 $\mu\text{g}/\text{ml}$ OVA in round-bottom 96-well plates (BD Biosciences). Four days later, supernatant was harvested for cytokine measurement.

Protein measurements

BAL MCP-1, keratinocyte-derived chemokine, and MIP-3 α levels, and MLN supernatant IL-4, IL-13, IL-10, and IFN- γ levels were measured by using commercially available ELISA kits (R&D Systems).

Statistical analysis

Statistical analysis was performed with SPSS for windows, version 16.0 (SPSS, Chicago, IL). Groups were compared using nonparametric tests (Kruskal-Wallis, Mann-Whitney U tests) following standard statistical criteria. Reported values were expressed as mean \pm SEM. Values of $p < 0.05$ were regarded as significant.

Results

DEPs induce an innate immune response and increase the number of BAL and pulmonary DCs

DCs are crucial in regulating immune responses, and we hypothesized that the effects of DEP are mediated through the DCs. First, we characterized the innate immune and DC response upon DEP instillation (Fig. 1A). DEP exposure increased the total BAL leukocytes dose dependently. This increase was due to a recruitment of neutrophils (CD11b⁺, Ly6C⁺, Ly6G⁺, and F4/80⁺ cells; Fig. 1B) and to increased numbers of inflammatory monocytes (CD11b⁺, Ly6C⁺, Ly6G⁺, and F4/80⁺ cells, Fig. 1C) and DCs (low autofluorescent, CD45⁺, CD11c⁺, MHC-II⁺ cells; Fig. 1D). This DEP-induced cellular response was paralleled with increased BAL chemokine levels of KC (Fig. 1E), MCP-1 (Fig. 1F), and MIP-3 α (Fig. 1G).

In digested lungs (from which the large conducting airways were dissected), DEP exposure also increased the number of neutrophils and inflammatory monocytes (data not shown). Different DC subsets are characterized in the pulmonary tissue (5, 6). Exposure to DEPs increased both lung CD11b⁺ (low autofluorescent, CD45⁺, CD11c⁺, MHC-II⁺, and CD11b⁺ cells; Fig. 1H) and CD11b[−] (low autofluorescent, CD45⁺, CD11c⁺, MHC-II⁺, and CD11b[−] cells; Fig. 1I) DCs dose dependently. In contrast, the amount of pDC (low autofluorescent, CD45⁺ CD11b[−], MHC-II^{int}, CD11b^{int}, and SiglecH⁺ cells; Fig. 1J) did not change after DEP instillation.

Because DCs continuously sample the airway lumen, we examined whether they also phagocytosed DEPs. Therefore, we sorted BAL DCs and pulmonary DCs (low autofluorescent, CD45⁺, CD11c⁺, and MHC-II⁺ cells) from control and DEP-exposed mice.

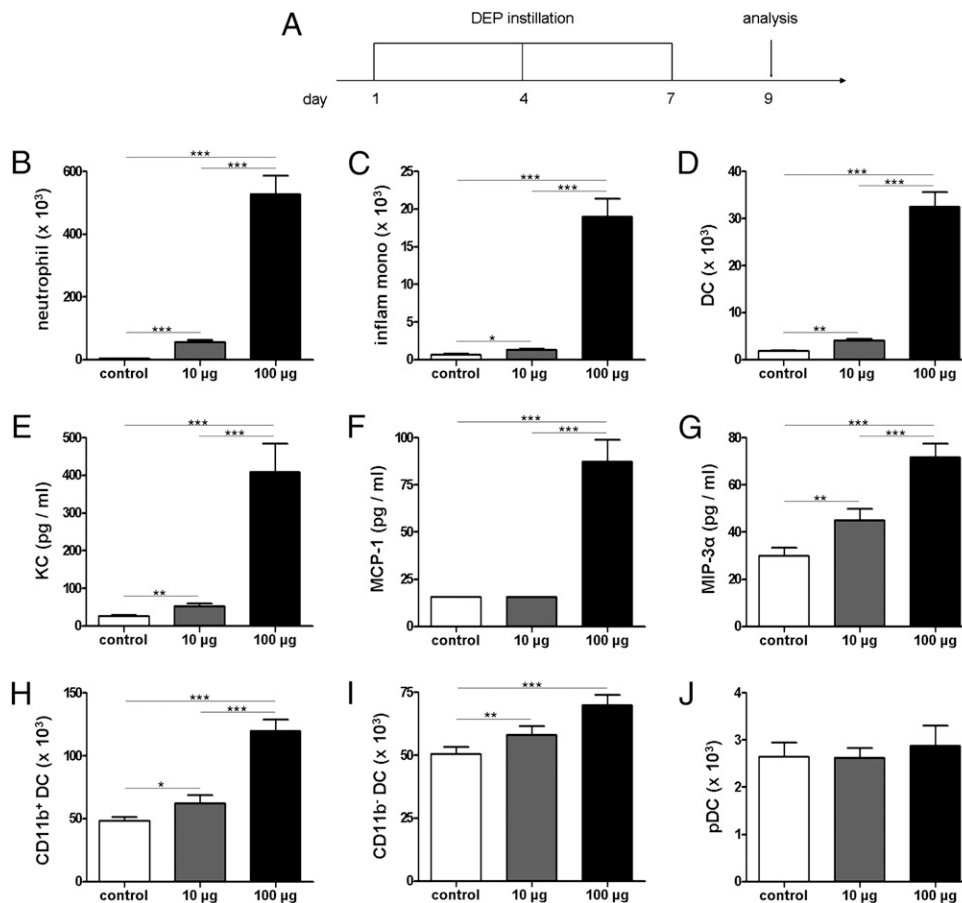


FIGURE 1. Innate immune and DC response upon DEP exposure. *A*, Mice were instilled with saline (□), 10 µg DEPs (gray bar), and 100 µg DEPs (■). *B–D*, Cell differentiation in BAL. CD11b⁺Ly6C⁺Ly6G⁺F480[−] neutrophils (*B*), CD11b⁺Ly6C⁺Ly6G[−]F4/80⁺ monocytes (*C*), and low autofluorescent CD45⁺CD11c⁺MHC-II⁺ DCs (*D*) were determined by flow cytometry. DEPs did not change the autofluorescent characteristics of DCs (data not shown). *E–G*, Chemokine levels in BAL. KC (*E*), MCP-1 (*F*), and MIP-3α (*G*) protein levels were determined by ELISA. *H–J*, Lung DC subset numbers. Low autofluorescent CD45⁺CD11c⁺MHC-II⁺CD11b⁺ DCs (*H*), low autofluorescent CD45⁺CD11c⁺MHC-II⁺CD11b[−] DCs (*I*), and low autofluorescent CD45⁺CD11b[−]CD11c^{int}MHC-II^{int}SiglecH⁺ pDCs (*J*) were determined by flow cytometry. Results are expressed as means ± SEM. *n* = 9 mice/group; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

DEPs were detectable inside vacuoles of both BAL (Fig. 2*B*) and lung DCs (Fig. 2*D*).

DEPs induce DC maturation indirectly

Depending on the maturation level, DCs initiate immune responses (4). We examined whether exposure to DEPs induced DC maturation. DEP instillation upregulated the maturation marker CD86 on BAL DCs and on both CD11b⁺ (Fig. 3*A*) and CD11b[−] pulmonary DCs (Fig. 3*B*). In contrast, no CD86 upregulation was detected on pulmonary pDCs (Fig. 3*C*). Next, we studied whether DEPs induced the DC maturation directly. For this purpose, BM-derived DCs were exposed to DEPs. We detected no CD40 (Fig. 3*D*), CD80 (Fig. 3*E*), and CD86 (Fig. 3*F*) upregulation upon DEP exposure in vitro.

DEPs translocate to the MLN

Upon necropsy, we found that the MLN were enlarged and that they had a gray appearance upon DEP instillation. This suggested that DEPs were translocated to the MLN. As shown in Fig. 4*A*, we demonstrated DEP in the MLN tissue with light microscopy. In contrast, no DEPs were detected in the nondraining LN (Fig. 4*B*) or spleen (Fig. 4*C*). In the MLN, DEPs accumulated in the T cell zone (Fig. 4*D*). To analyze whether DEPs were engulfed in the cellular compartment of the MLN, cytospin preparations were made. We demonstrated DEP⁺ cells in the MLN, with a DC-like morphology, that cluster with lymphocytes (Fig. 4*E*). To confirm

that the DEP⁺ cells were DCs, we sorted DCs (CD11c⁺ and MHC-II⁺ cells) of the MLN. Fig. 4*F* shows DEPs inside vacuoles of these sorted DCs.

DEPs are transported to the MLN in a CCR7-dependent manner

To examine whether the observed DEP translocation to the MLN is cell-mediated or is due to a passive leakage into the lymph vessels, we exposed CCR7 KO and WT mice to DEPs. In contrast with the WT mice, we were unable to detect any DEPs in the MLN of CCR7 KO mice (Fig. 5*A*). To exclude that this lack of DEP translocation was because of structural abnormalities potentially present in the CCR7 KO mice, we instilled WT DEP-loaded BM-derived DCs in CCR7 KO mice and examined the MLN for the presence of DEPs. WT DEP-loaded BM-derived DCs were detected in the MLN of CCR7 KO mice (Fig. 5*B*).

DEPs enhance the Ag-induced DC migration to the MLN

We next examined whether DEPs accelerated or enhanced the lung DC migration to the MLN. Control and DEP-treated mice were instilled with OVA-FITC, and the FITC⁺ DCs (CD11c⁺ and MHC-II⁺ cells) in the MLN were analyzed at various time points (21). The DC migration to the MLN peaked 24–48 h after Ag challenge. DEP exposure significantly enhanced the number of migrating OVA-FITC⁺ DCs (Fig. 6*A*). Because the chemokine receptor CCR7 regulates the

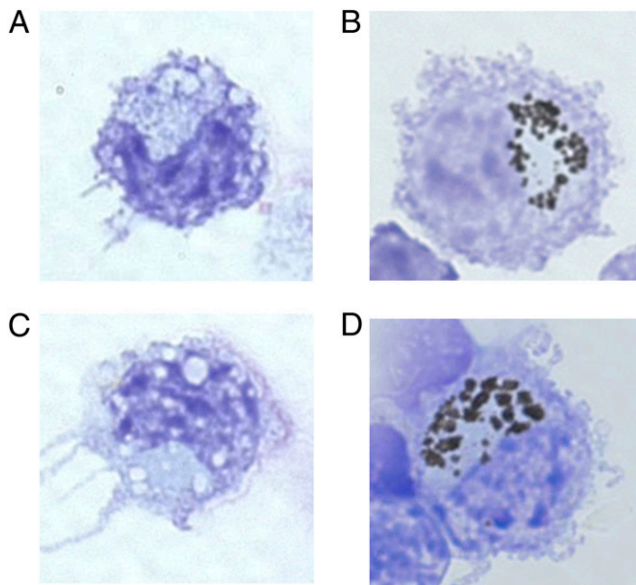


FIGURE 2. Phagocytosis of DEPs by DCs. *A, B*, BAL cytopsin preparations, stained with May-Grünwald Giemsa, of low autofluorescent CD45⁺ CD11c⁺MHC-II⁺ DCs sorted by flow cytometry of control (*A*) and 100 µg DEP (*B*)-treated mice. *C, D*, Lung cytopsin preparations, stained with May-Grünwald Giemsa, of low autofluorescent CD45⁺CD11c⁺MHC-II⁺ DCs sorted by flow cytometry of control (*C*) and 100 µg DEP (*D*)-treated mice.

migration of pulmonary DCs to the MLN (7, 22), we studied whether DEPs induced CCR7 expression on DCs. As shown in Fig 6*B*, DEP exposure upregulated the CCR7 expression on BAL DCs.

DEPs induce a T cell response in the MLN

Once arrived in the MLN, DCs induce the proliferation and differentiation of naive T cells. We evaluated whether DEP exposure increased the *in vivo* T cell proliferation in the MLN. For this

purpose, control and DEP-treated mice were *i.v.* injected with CFSE-labeled, OVA-specific T cells on day 8 and instilled with OVA on day 9. Four days later, the accumulation of OVA-specific T cells and CFSE staining profiles was analyzed in the MLN. Exposure to DEPs increased the number of OVA-specific T cells in the MLN (Fig. 7*A*). Shown in Fig. 7*B* is a representative histogram of the CFSE staining profiles of OVA-specific T cells. DEP exposure increased the number of T cells entering into division, without affecting the number of cell divisions. This was due to an increased recruitment of naive T cells to the MLN, as measured by the increased CFSE content (Fig. 7*C*). No OVA-specific T cell response was detected in the nondraining LN (data not shown).

We also examined whether DEPs increased the differentiation of T cells into effector cells. Control and DEP-treated mice were *i.v.* injected with OVA-specific T cells on day 8 and instilled with OVA on day 9. Four days later, MLNs were cultured with OVA and analyzed for cytokine production. Exposure to DEPs increased the levels of IL-4, IL-13, IL-10, and IFN-γ in the MLN (Table I).

Discussion

Environmental pollutants like DEPs are regarded as irritants, causing or enhancing inflammatory reactions in the lung; however, their mechanism of action remains unclear (1). In recent years, it has become clear that pulmonary DCs are crucial mediators in regulating immune responses in the lung and that these DCs bridge the innate and adaptive immune response. In asthma, for example, DCs are important in both the induction and maintenance of the disease (4). We hypothesized that the adverse effects of DEPs are caused by modulation of pulmonary DCs.

In agreement with previously published data, we found that administration of DEP induced a dose-dependent innate immune response in the lung (1, 2). DEP exposure increased the recruitment of not only neutrophils but also of monocytes and DCs in the BAL compartment. In accordance with these cellular events, DEP

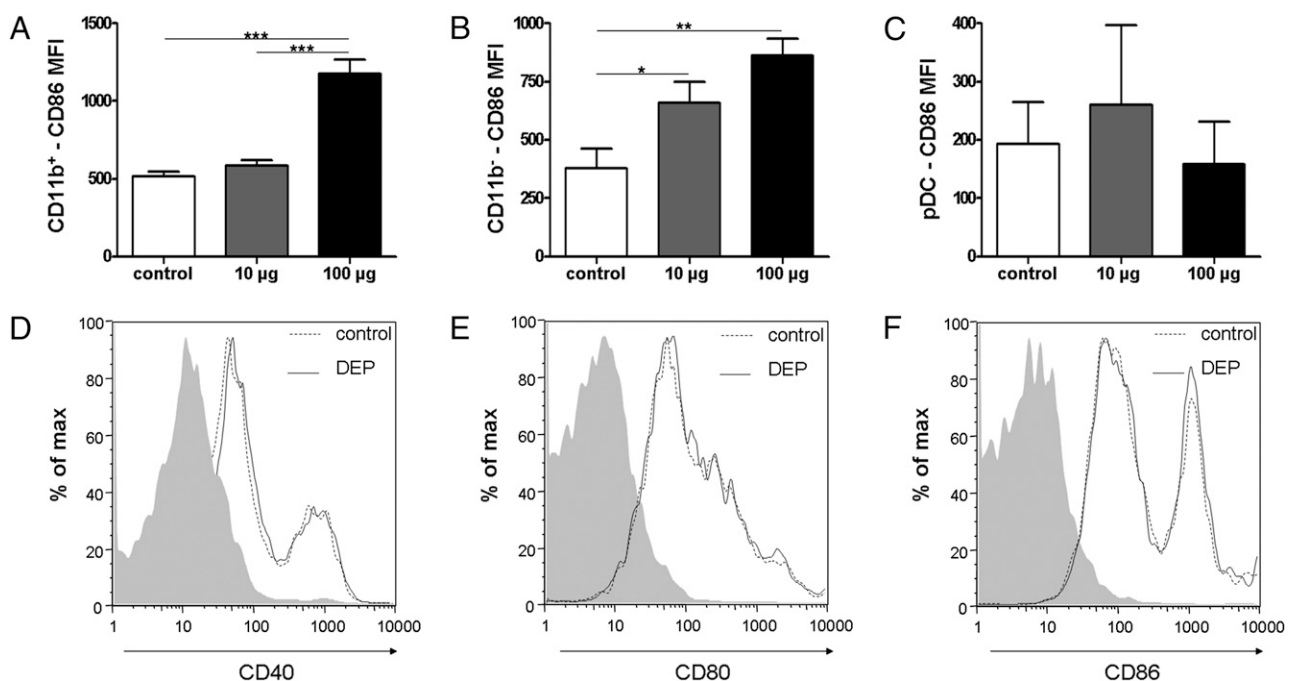
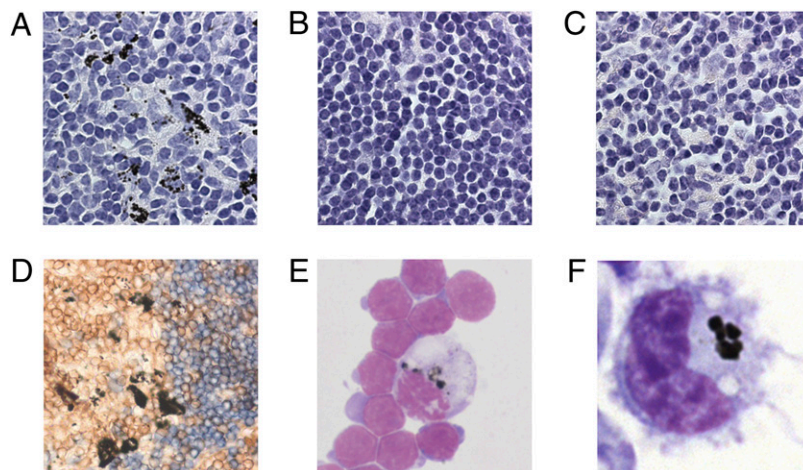


FIGURE 3. DC maturation upon DEP exposure. *A–C*, Expression of the CD86 costimulatory molecule on lung DC subsets of control (□), 10 µg DEP (gray bar), and 100 µg DEP (■)-treated mice. Mean fluorescence intensity (MFI) of CD86 on CD11b⁺ DCs (*A*), CD11b⁻ DCs (*B*), and pDCs (*C*) was determined by flow cytometry. Results are expressed as means ± SEM. *n* = 7–8 mice/group; **p* < 0.05; ***p* < 0.01; ****p* < 0.001. *D–F*, Expression of costimulatory molecules on BM-derived DCs. BM-derived DCs were exposed to saline (dotted line) and 10 µg/ml DEPs (full line). Twenty-four hours later, the CD40 (*D*), CD80 (*E*), and CD86 (*F*) expression on CD11c⁺MHC-II⁺ BM-derived DCs was determined by flow cytometry. Gray full histogram represents isotype control.

FIGURE 4. DEP translocation toward the lymphoid tissue. A–C, Photomicrographs of H&E-stained MLN (A), nondraining axillary LN (B), and spleen (C) of 100 μ g DEP-treated mice (magnification $\times 630$). D, Photomicrograph of CD3 (brown) – B220 (blue)-stained MLN of 100 μ g DEP-treated mice (magnification $\times 630$). E, MLN cytospin preparation, stained with May-Grünwald Giemsa, of 100 μ g DEP-treated mice. F, MLN cytospin preparation, stained with May-Grünwald Giemsa, of low autofluorescent CD11c⁺ MHC-II⁺ DCs sorted by flow cytometry of 100 μ g DEP-treated mice.



exposure elevated the BAL levels of the monocyte and the DC attracting chemokines MCP-1 (CCL2) and MIP-3 α (CCL20). Production of MIP-3 α occurs when human bronchial epithelial cells are stimulated with ambient particulate matter (23). Upon inflammation, we and others previously demonstrated that monocytes and DCs accumulate in the lung in a CCR2- and CCR6- (receptors for MCP-1 and MIP-3 α) dependent manner (24, 25). Our data suggest that these chemokine receptors were also involved in the DEP-induced immune response.

In the pulmonary tissue, different DC subsets can be found, based on anatomical location and function (5, 6): cDCs, subdivided in CD11b⁺ and CD11b⁻ cDCs, and pDCs. CD11b⁻ cDCs are located adjacent to the epithelium and extend their dendrites between epithelial cells to sample the airway lumen (26), whereas CD11b⁺ cDCs are located underneath the epithelium and pick up Ag that has passed the basal membrane. CD11b⁺ cDCs were shown to be strong producers of inflammatory chemokines, and in allergen-challenged mice, CD11b⁻ cDCs were shown to produce the highest amounts of Th2 cell-attracting chemokines (27). In this study, we showed that DEP exposure increased the lung CD11b⁻ cDCs and greatly increased the CD11b⁺ cDCs. However, upon inflammation, another lung DC subset has been described that derived from the Ly6C⁺ monocytes, namely the inflammatory CD11c⁺CD11b⁺ Ly6C⁺ DCs (6). As these inflammatory DCs closely resemble the CD11b⁺ cDC, they possibly contaminated our CD11b⁺ cDC population. Whereas cDCs are important in inducing sensitization, pDCs are involved in the induction of tolerance and depletion of pDCs during exposure toward harmless Ags is previously shown to induce sensitization (28). In our study, DEPs did not affect lung

pDC numbers. This suggests that exposure to DEPs could shift the cDC/pDC balance favoring sensitization, thereby providing one possible explanation for the adjuvant activity of DEPs observed in many experimental models (2).

Besides altering balances in cDCs over pDCs, other factors can favor sensitization. Depending on their maturation level, cDCs initiate immune responses (full maturation) or induce tolerance (limited maturation) (29). We demonstrated that exposure to DEPs upregulated the CD86 expression on BAL and pulmonary DCs. Because this costimulatory molecule is involved in the priming of naive T cells into Th2 cells and in the subsequent development of allergic airway inflammation (30), this could represent another possible mechanism how DEPs exert their adjuvant effects. There exists some controversy about the ability of DEPs to activate DCs directly or indirectly. Because we found that DCs phagocytosed DEPs, we hypothesized this could directly induce DC maturation. However, exposure of BM-derived DCs to DEPs did not lead to maturation. This is in line with observations in human monocyte-derived DCs, in which DEP induce DC maturation through release of the growth factors GM-CSF and thymic stromal lymphopoietin by epithelial cells (12, 13). Other DC-activating cytokines that are produced by the epithelium include IL-1 β , IL-6, and TNF- α (4). Upon exposure to DEP, the levels of these cytokines in BAL increased (data not shown), suggesting that these could also play a role in the indirect DC maturation.

One remarkable finding upon DEP exposure was that the MLN enlarged and got a gray color. This suggested that DEP translocated to the MLN. We confirmed the presence of DEPs in the MLN by microscopy and showed that DEPs translocated to the T cell zone.

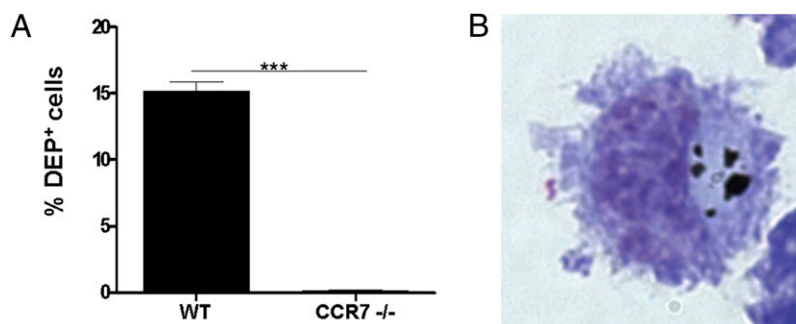


FIGURE 5. Mechanism of DEP translocation toward the MLN. A, Role of CCR7 in DEP translocation. WT (■) and CCR7 KO (□) mice were instilled with 100 μ g DEP on day 1. On day 3, MLNs were digested and enriched for CD11c⁺ cells by magnetic bead separation. Cytospin preparations were made, and DEP⁺ cells were calculated. Results are expressed as means \pm SEM. $n = 8$ mice/group; *** $p < 0.001$. B, CCR7 reconstitution experiment. WT DEP-loaded, BM-derived DCs (5×10^6) were instilled in CCR7 KO mice on day 1. On day 3, MLNs were digested and enriched for CD11c⁺ cells by magnetic bead separation. Cytospin preparations, stained with May-Grünwald Giemsa, were made and analyzed for DEP⁺ cells.

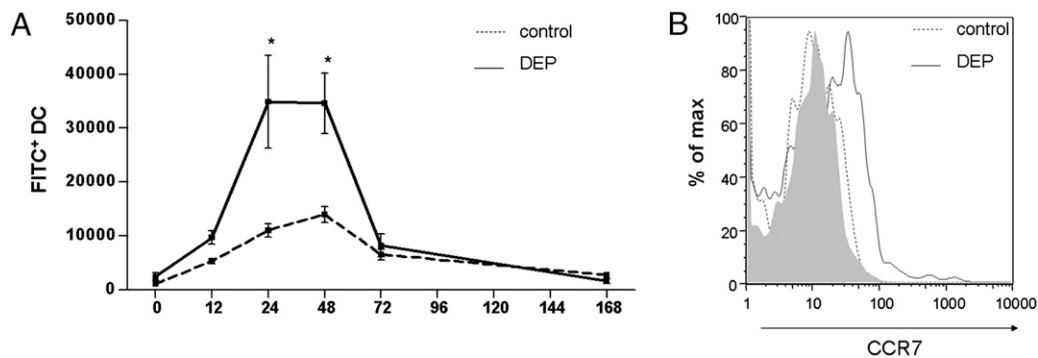


FIGURE 6. DC migration to the MLN upon DEP exposure. *A*, Kinetics of lung DC migration to the MLN. Control (dotted line) and 100 μ g DEP (full line)-treated mice were instilled with 350 μ g OVA-FITC on day 9. At various time points after instillation, MLNs were digested, and the FITC⁺ signal in CD11c⁺MHC-II⁺ DCs was determined by flow cytometry. Results are expressed as means \pm SEM. $n = 5$ –7 mice/group; $*p < 0.05$. *B*, CCR7 expression on BAL DCs. Mice were instilled with saline (dotted line) and 100 μ g DEPs (full line). CCR7 expression on low autofluorescent CD11c⁺ DCs was determined by flow cytometry. Gray full histogram represents isotype control.

Moreover, we detected DEPs within the mediastinal DCs. In humans, particulate matter has also been observed within biopsies of bronchial lymph nodes (31). Because this DEP translocation to the mediastinal DC population is a relevant finding to explain the effects upon inhalation, we investigated the DEP translocation mechanism. Inhaled Ags can reach the MLN by several mechanisms (4–6); however, the fate of inhaled particulate matter remains unknown. Most evidence suggests that inhaled Ags are taken up by lung DCs, which migrate in a CCR7- and CCR8-dependent manner to the MLN. As we found that pulmonary DCs phagocytosed DEPs; this seemed the most obvious mechanism of DEP translocation. However, the epithelial tight junction barrier can also act as a molecular sieve that allows passive leakage into the afferent lymph vessels and Ags sampled in this manner gain access to the resident mediastinal DCs (4). To make things even more complex, both scenarios can come into play. In this study, we showed that the DEP translocation toward the MLN is completely CCR7 mediated, because we detected no DEPs in the MLN of CCR7 KO mice. The structural organization of the lymphoid organs is aberrant in the CCR7 KO mice (32), and one could therefore argue that the absence of DEPs in the MLN of CCR7 KO mice was due to structural deficiencies rather than representing a true CCR7-dependent DC transport mechanism. This was, however, unlikely, as we demonstrated that adoptive transfer of DEP-loaded WT, BM-derived DCs to the airways of CCR7 KO mice restored the translocation of DEPs to the MLN.

Ag transport to the MLN is an important step in the initiation of an adaptive immune response. Changes in Ag handling upon DEP

exposure could therefore also explain the adjuvant or aggravating effects of pollutants. Our data showed that DEP instillation greatly enhanced the DC-mediated transport of inhaled proteins toward the MLN. We further showed that exposure to DEPs upregulated the CCR7 expression, an important chemokine receptor involved in the migration of lung DCs to lymphoid tissues (7, 22), on BAL DCs. These findings are in parallel with what is observed upon cigarette smoke exposure (33). An alternative explanation for these increased Ag titres in the MLN could be that DEPs increased the airway epithelial permeability. However, in an *in vitro* system, DEPs did not alter the permeability of human bronchial epithelial cells (34).

As DEPs were present in the MLN and as DEP exposure increased the migration of OVA-loaded DCs to the MLN, we investigated whether exposure to DEPs also enhanced the T cell proliferation and differentiation into effector cells. This could then explain the adjuvant effects of DEP exposure in models published before (2), and *in vitro* observations indeed suggest that DEP-exposed DCs increase the T cell proliferation (12, 13). To our surprise, we observed comparable T cell proliferation in control and DEP-treated mice. However, T cells from DEP-treated mice produced higher levels of IL-4, IL-13, IL-10, and IFN- γ cytokines. Several *in vivo* studies showed that adoptively transferred TCR transgenic T cells can proliferate in response to a harmless Ag without differentiation to an effector function (30, 35). In these experiments, T cells then fail to differentiate because Ags are presented by immature DCs (35). The fact that we observed increased effector Th1 and 2 differentiation strongly suggests that DEP-exposed DCs were mature cells

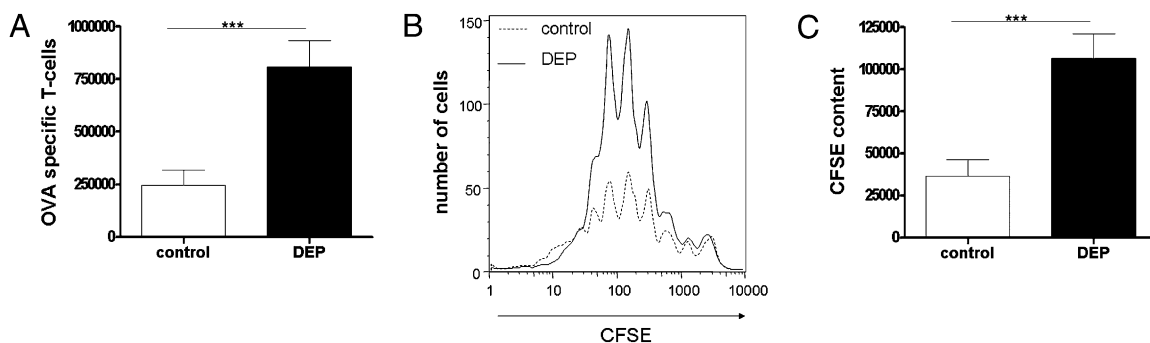


FIGURE 7. T cell response in the MLN upon DEP exposure. Control and 100 μ g DEP-treated mice were i.v. injected with 10×10^6 OVA-specific T cells on day 8. On day 9, mice were instilled with 350 μ g OVA. Four days later, MLNs were digested, and the accumulation and CFSE division profiles of DO11.10 TCR⁺CD4⁺ T cells were determined by flow cytometry. *A*, DO11.10 TCR⁺CD4⁺ T cells in control (\square) and 100 μ g DEP (\blacksquare)-treated mice. *B*, Representative histogram of CFSE division profiles of DO11.10 TCR⁺CD4⁺ T cells in control (dotted line) and 100 μ g DEP (full line)-treated mice. *C*, CFSE content (calculated as described in *Materials and Methods*) in control (\square) and 100 μ g DEP (\blacksquare)-treated mice. Results are expressed as means \pm SEM. $n = 5$ mice/group; *** $p < 0.001$.

Table I. Cytokine production in MLN upon DEP exposure

	Control	DEP
IL-4 (pg/ml)	1.7 ± 0.68	14.46 ± 3.74*
IL-13 (pg/ml)	274.26 ± 106	916.5 ± 181.67 [†]
IL-10 (pg/ml)	11.75 ± 3.65	96.2 ± 6.1 [†]
IFN-γ (pg/ml)	43.15 ± 11.69	111.46 ± 16.92*

Control and 100 µg DEP-treated mice were i.v. injected with 10×10^6 OVA-specific T cells on day 8. On day 9, mice were intratracheally instilled with 350 µg OVA. Four days later, MLNs were digested and cultured in medium plus 100 µg/ml OVA. Four days later, IL-4, IL-13, IL-10, and IFN-γ protein levels in the supernatant were determined by ELISA. Results are expressed as means ± SEM. $n = 7-8$ mice/group.

* $p < 0.005$; [†] $p < 0.01$.

that prevented the induction of abortive T cells. Although we did not study the maturation state of migrated DEP-loaded DCs in the MLN, we observed increased expression of the costimulatory molecule CD86 on lung DCs in DEP-exposed mice. In addition, we showed an increase in the CFSE content of adoptively transferred OVA-specific, TCR transgenic T cells as well as an increase in OVA-specific T cells in the MLN of DEP-exposed mice. As an increase in CFSE content means that there is recruitment of these cells to the MLN (20), we speculate that DEP-exposed DCs could also be a prominent source of T cell attractive chemokines like CCL19, CCL21, and CCL17, CCL22 in the MLN.

In conclusion, our data demonstrated that DEP exposure profoundly affects the biology of pulmonary DCs. We showed that DEP exposure induced DC recruitment and maturation and that DEPs increased the DC migration toward the MLN. We further demonstrated that DEPs themselves were transported to the MLN in a CCR-7 and DC-dependent manner and that DEP exposure increased the accumulation of Ag-specific T cells and their differentiation into effector T cells. Taken together, these findings suggest a mechanism by which DEPs can modulate immune responses and can act as an adjuvant to coadministered protein Ags.

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Disclosures

The authors have no financial conflicts of interest.

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