Dendritic cells inversely regulate airway inflammation in cigarette smoke-exposed mice

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Cigarette smoking is the main risk factor for the development of inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) (16, 23, 30). The limited knowledge on disease mechanism hampers an effective treatment of COPD. Thus, detailed understanding of the mechanism underlying the inflammatory processes induced by cigarette smoke (CS) may lead to better therapeutic approaches in COPD.

Cigarette smoking causes several well-established features such as airway dysfunction, mucus hypersecretion, and immune alterations that contribute to COPD (4, 7). Inflammatory cells from both the innate and the adaptive immune system, together with their mediators, participate in the inflammatory response presumed to play a role in the pathogenesis of COPD (3, 38). Alveolar macrophages, airway epithelial cells, and the rapid influx of neutrophils are thought to be involved in the immune response to acute CS exposure. Previously, we reported that modulation of dendritic cell (DC) subsets by pharmacological agents in a chronic model of smoke-exposed mice alters the CS-induced lung emphysema (18). Pulmonary DCs are rare and considered to be major effector cells in the immune system, playing a critical role in the pathogenesis of (allergic) airway disease (5, 24, 34). Lung DCs are ideally situated close to alveolar epithelium; thus, these cells might be critical to the initiation and suppression of lung immune response to CS exposure.

Various studies suggest a role for DCs in the CS-induced inflammatory responses (2, 13, 31, 35), but the contribution of DC subsets to the immunological response in COPD is not well understood (17, 19).

Two major DC subsets in the mouse are myeloid/conventional DC (cDC) and plasmacytoid DC (pDC) with distinct roles in the regulation of T cell-mediated adaptive immunity. Furthermore, lung CD11c+ DCs can be divided into two major migratory subsets, based on their expression levels of the CD103 and CD11b receptors. The biological function of these subsets is beginning to unfold: antigen uptake of CD103+ cells is lower compared with CD11bhi cells, and the latter population migrates faster to the draining lymph nodes (LNs) (8, 9, 12, 17, 23, 36).

In vitro studies, using bone marrow/monocyte-derived DCs exposed to varying doses of nicotine and cigarette smoke extract, and in vivo studies, in CS-exposed animals, reported conflicting data related to the number and function of pulmonary DCs (1, 6, 22, 25, 26, 27). Moreover, treatment with fms-like tyrosine kinase 3 ligand (Flt3L) leading to a drastic increase in functionally active DCs and the pDC depleting antibody 120g8 have been used to investigate the role of DC subsets in mice (3, 29). In this regard, the modulation of these DC subsets may influence adaptive immune responses by modulation of CD4+ and CD8+ T cells.

In this study, we aimed to examine whether acute inflammation responses induced by CS could be affected by modulating the DC subsets in the lungs.

Glossary

- BALF: Bronchoalveolar lavage fluid
- cDCs: Conventional dendritic cells
- COPD: Chronic obstructive pulmonary disease
- CS: Cigarette smoke
- DCs: Dendritic cells
- Flt3L: Fms-like tyrosine kinase 3 ligand
- LN: Lymph node
- LPS: Lipopolysaccharide
- mDC: Myeloid dendritic cell
- pDC: Plasmacytoid dendritic cell

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MATERIALS AND METHODS

Animals and Groups of Study

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories. All in vivo manipulations were approved by the Animal Ethical Committee of the Utrecht University (Utrecht, Netherlands). Mice were divided into two main groups of smoke-exposed and control (air exposed) with each group further divided into subgroups (see Table 1).

CS Exposure

Mice were subjected to whole body CS or air (sham) two times daily for five consecutive days as follows: day 1, 6 cigarettes (~30 min exposure); days 2–5, 10 cigarettes (~50 min exposure) (see Fig. 1A).

Treatments-Smoke Combination Protocols

Flt3L treatment. Mice were treated subcutaneously (at the nape of the neck) with 10 μg of human Flt3L (eBioscience) or as a control with serum albumin (0.01% in PBS) daily for 10 consecutive days (11) and combined with CS exposure from day 6 (see Fig. 1, B, combination protocol I).

120g8 Treatment. Mice were injected intraperitoneally with 200 μg of pDC-selective depleting 120g8 Abs (provided by Bioceros) or as a control with 200 μg of isotype-matched rat IgG (Sigma-Aldrich). Mice were injected for four consecutive days starting 1 day before smoke exposure (2) (see Fig. 1B, combination protocol II).

Flt3L plus 120g8 Abs treatment. Mice were injected either with daily Flt3L (10 μg sc) for 10 consecutive days and with 120g8 (200 μg ip) from day 5 for four consecutive days starting 1 day before smoke exposure. Control mice received injections of rat IgG and serum albumin (see Fig. 1B, combination protocol III).

Bronchoalveolar Lavage Fluid and Cytospins

After the last smoke or air exposure (16 h), mice were killed, and bronchoalveolar lavage (BAL) was performed. Cytospins were prepared from BAL to count and differentiate cellular composition. BAL supernatant was used for cytokine analysis by a Bio-plex (Invitrogen, Biosource).

Flow Cytometry Analysis

Lung and mediastinal lymph nodes were removed and digested as described previously (6). Mononuclear cells were analyzed on a FACSCantoII flow cytometer (BD Biosciences), and data were processed with FACSDiva software (v6.1.2). All staining reactions were performed at 4°C, and dead cells were excluded using 7-aminoacti-

Table 1. Animal groups of study

<table>
<thead>
<tr>
<th>Groups of Study</th>
<th>Control (Air-Exposed)</th>
<th>Smoke Exposed</th>
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<tbody>
<tr>
<td>PBS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>120g8 (or rat IgG) = ↓ pDCs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flt3L (or serum albumin) = ↑ DC subsets</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flt3L + 120g8 (or serum albumin/rat IgG) = ↑ mDCs</td>
<td>+</td>
<td>+</td>
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pDCs, plasmacytoid dendritic cells; Flt3L, fms-like tyrosine kinase 3 ligand; ↑, increase; ↓, decrease. BALB/c mice were divided in two main groups of smoke-exposed and control (air exposed) with each group further divided into subgroups (see Table 1).

Fig. 1. Cigarette smoke exposure and treatment-smoke combination protocols. A: smoke exposure protocol. BALB/c mice were exposed to cigarette smoke or air (sham) two times daily for five consecutive days as follows: day 1, 6 cigarettes (~30 min exposure); days 2–5, 10 cigarettes (~50 min exposure) (see Fig. 1A).
nomycin D viability staining. The following antibodies were used for flow cytometry analysis: FITC-conjugated anti-CD11c; phycoerythrin (PE)-conjugated anti-CD103 or anti-CD86; PE- and Cy5.5-conjugated anti-CD8; PE- and Cy7-conjugated anti-CD11b or anti-Ly6G or anti-F4/80; APC-conjugated anti-major histocompatibility complex (MHC) class II or anti-mPDCA-1; APC- and eFluor780-conjugated anti-mCD45R (B220) or anti-CD4. All antibodies were purchased from eBioscience or BD Biosciences (San Diego, CA).

Data Analysis and Statistics

Differences between groups were analyzed by using one-way ANOVA with a Bonferroni post hoc test or unpaired Student’s t-test using GraphPad (version 5). \( P < 0.05 \) was considered significant.

RESULTS

DC Subset Modulation Suppresses the Early Stages of Smoke-Induced Inflammation

The effects of selective expansion/depletion of DCs in smoke-induced airway inflammation, the airway, and alveolar lumen (by BAL fluid) of air- or smoke-exposed mice were studied. Acute smoke exposure led to a significant increase in the total number of inflammatory cells in BALF compared with the control group (Fig. 2A). Differential cell analysis revealed that this increase was due to the accumulation of neutrophils and macrophages (Fig. 2, B and C). The CS-induced accumulation of BAL cells (Fig. 2A), neutrophils (Fig. 2B), and macrophages (Fig. 2C) was significantly reduced after Flt3L treatment. Interestingly, depletion of pDCs by 120g8 in the Flt3L group restored the influx of macrophages again in the CS-exposed mice (Fig. 2C). The number of lymphocytes was also increased after 5 days of smoke exposure compared with the control groups but did not change significantly across the treatments (Fig. 3). Treatments with 120g8 and/or Flt3L had no effect on cellular composition in nonsmoked control mice.

DC Subset Modulation Increases the Early Accumulation of Inflammatory Cells in the Lung Tissue

Next, we investigated whether the selective expansion/depletion of DC subsets affected the smoke-induced inflammation in lung tissue. Therefore, we first confirmed the presence of cDCs and pDCs in lung tissue using flow cytometry (29) (Fig. 4A). pDC were found increased in lung tissue after CS exposure (Fig. 4B). 120g8 significantly suppressed the percentage of pDCs in both the CS-exposed and CS-exposed + Flt3L-treated animals (Fig. 4B). Furthermore, flow cytometric analysis of lung homogenates demonstrated that CS exposure induced a neutrophilic inflammation and increased the number of macrophages in the lung tissue (Fig. 4, C and D). Modulation of DCs with Flt3L, 120g8, or both enhanced the neutrophil and macrophage infiltration significantly in the lungs compared with smoke-exposed mice, but no difference between the different treatment conditions was observed (Fig. 4, C and D).

![Fig. 2. DC subset modulation suppresses the inflammatory cell infiltration induced by cigarette smoke in bronchoalveolar lavage fluid (BALF). Mice were treated with 120g8 or Flt3L to modulate DC subtypes combined with cigarette smoke exposure or sham (air only). After 5 days of smoke exposure, the total and differential cell counts were analyzed in BALF. A–C total number of BALF cells (A), bronchoalveolar lavage (BAL) neutrophils (B), and macrophages (C); n = 4–6 animals/group. Values are expressed as means ± SE. Significant differences are indicated by asterisks (*P < 0.05 and **P < 0.01).](#)

![Fig. 3. Effect of DC subset modulation on lymphocyte counts in BAL fluid. Mice were treated with 120g8 or Flt3L to modulate DC subtypes combined with cigarette smoke exposure or sham (air only). After 5 days smoke exposure, the lymphocyte counts were analyzed in BALF; n = 4–6 animals/group.](#)
Fig. 4. DC subset modulation enhances inflammatory cells in lung of smoke-exposed mice. Single-cell suspensions of the total lung were analyzed by flow cytometry in sham- treated/smoke-exposed mice. Representative dot plots (top) and bar graphs (bottom) indicate percentages of cells within the gated leukocyte population (using forward and side scatter). A and B: CD11c⁺, CD11b⁺, B220⁺/PDCA-1⁺ pDC, and CD11c⁺/CD11b⁺ cDC (mDC). C and D: Ly-6G⁺ neutrophils (C) and CD11c⁺ MHCIIm⁰ F4/80⁺ alveolar macrophages (D). At least 5 animals were analyzed per group, and data show 1 representative experiment of 3. Values are expressed as means ± SE. Significant differences are indicated by asterisks (*P < 0.05, **P < 0.01, and ***P < 0.001).
Effect of DC Subset Modulation on the Release of Proinflammatory Mediators in Bronchoalveolar Lavage Fluid

CS is a strong inflammatory stimulus that induces the release of proinflammatory mediators in the bronchoalveolar lavage fluid (BALF) (32). To investigate whether cellular changes induced by the DC subset modulated cytokine production, proinflammatory mediators were measured in BALF. After 5 days, CS exposure significantly enhanced the level of KC, IL1-β, IL-6, IL-10, IL-12, IL-17, and MIP-1α in BALF compared with control mice (Fig. 5 and Table 2). Flt3L even further promoted the production of KC, IL-6, IL-12, MIP-1α (Table 2), and IL-10 (Fig. 5A) and diminished IL-17 (Fig. 5B). It is notable that combined treatment of Flt3L + 120g8 restored IL-10 levels to smoke-exposed mice and IL-17 levels to air-exposed mice (Fig. 5, A and B).

pDC Depletion Significantly Increases CD11c⁺/CD11bhi Alveolar Macrophages and Decreases CD103⁺ DCs Within Lung Parenchyma

Flow cytometric analysis of lung homogenate samples showed that depletion of pDCs by 120g8 antibody significantly increased the numbers of CD11c⁺CD11bhi alveolar macrophages (Fig. 6A) within lung parenchyma compared with smoke-exposed and air-exposed mice. Furthermore, treatment with 120g8 decreased the trafficking of CD103⁺ CD11b⁻ migratory DCs within lung parenchyma compared with smoke-exposed mice (Fig. 6B).

DISCUSSION

The outcome of pulmonary antigen exposure is regulated by the balance of alveolar macrophages and pulmonary DCs (20). In this study, we show that modulation of DC subsets by Flt3L or 120g8 during CS exposure decreases the number of inflammatory cells in BALF but not in lung tissue. pDC depletion significantly increased the migration of alveolar macrophages with high expression of CD11b to the lung parenchyma during acute smoke exposure. Furthermore, we show that the number of migratory CD103⁺ DCs within the lung parenchyma decreases by pDC depletion.

Expression of CD11b by alveolar macrophages is functionally crucial for entry of these cells or their precursors into the lungs during inflammation but not for pulmonary DCs (20), while CD103⁺ DCs are the migratory DC subsets in lungs required for a cytotoxic T lymphocyte response, and they appear selectively specialized to engulf apoptotic cells and transport to LNs (14, 28). Lung fibroblasts regulate DC trafficking, and IL-1β plays a critical role in this process (21). Our study demonstrates that pDC modulation significantly decreases IL-1β production. This cytokine is frequently used as a biomarker of inflammation and is involved in the inflammatory process by recruitment and retention of inflammatory cells and also DC subsets (40). Indeed, human pDC were shown to promote and modify Th17 cell differentiation and function by release of IL-1β (39). This may point to a possible role of pDCs in the recruitment of the inflammatory cells by IL-1β production during CS exposure.

CS-exposed mice have significantly increased IL-17 levels in BALF, which was shown decreased by DC modulation. Depletion of the pDC subset in Flt3L-treated animals with 120g8 resulted in complete reduction of the smoke-induced IL-17 production to control levels (Fig. 5), suggesting that pDC may be linked to IL-17 production in smoke-exposed animals. IL-17 is a proinflammatory cytokine, mainly by stimulating mediator production such as IL-8 (equivalent KC in mice), IL-6, and growth factors at the site of inflammation, promoting neutrophil and macrophage recruitment (10). It is notable that the modulation of DC subsets in the current study diminished the IL-17 level in BALF but not in serum (smoke: 12.5 ± 2.1 pg/ml, smoke-120g8: 28.54 ± 3.2 pg/ml). Moreover, in contrast to BALF, the DC modulation significantly enhanced the distribution of macrophages and neutrophils in lung tissue. Whether this distribution of neutrophils and macrophages in lungs is dependent on systemic levels of IL-17 needs further research.

In summary, besides acting as antigen-presenting cells, DCs could play a role in activating and suppressing inflammatory...
responses by releasing cytokines in acute lung inflammation. Our study suggests that DCs may be involved in early leukocyte accumulation at the site of inflammation induced by CS. pDC could play a role in preventing the pathogenesis of CS in the early stage by inhibiting the alveolar macrophage migration to lung and increasing CD103+ DCs at inflammatory sites to avoid extensive lung tissue damage.

AUTHOR CONTRIBUTIONS


REFERENCES

Fig. 6. pDC depletion significantly enhances CD11c<sup>+</sup>/CD11b<sup>hi</sup> positive alveolar macrophages and decreases the CD103<sup>+</sup> CD11b<sup>-</sup> migratory DCs within lung parenchyma. Single-cell suspensions of total lung were analyzed by flow cytometry in sham-treated and smoke-exposed mice. Representative dot plots, histogram (top), and bars (bottom) show percentages of total CD11c<sup>+</sup> CD11b<sup>hi</sup> alveolar macrophages and CD11c<sup>+</sup> CD11b<sup>-</sup> CD103<sup>+</sup> migratory DCs in lungs. At least 5 animals were analyzed per group, and data show 1 representative experiment of 3. Nos. indicate percentage of cells within the gated leukocyte population (using forward and side scatter). Values are expressed as means ± SE. Significant differences are indicated by asterisks (*p < 0.05 and **p < 0.01).


