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

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<table>
<thead>
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<th>Abbreviations:</th>
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<td>BALF</td>
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<td>cDCs</td>
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<td>COPD</td>
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<td>CS</td>
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<tr>
<td>DCs</td>
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<td>Flt3L</td>
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<td>mDC</td>
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Abstract

The recruitment and activation of inflammatory cells into the respiratory system is considered a crucial feature in the pathophysiology of chronic obstructive pulmonary disease (COPD). Since dendritic cells (DCs) have a pivotal role in the onset and regulation of immune responses, we investigated the effect of modulating of DC subsets on airway inflammation by acute CS exposure.

CS-exposed mice (5 days) were treated with Flt3L (fms-like tyrosine kinase 3 ligand) and 120g8 antibody to increase total DC numbers and deplete plasmacytoid DCs (pDCs), respectively. Flt3L treatment decreased the number of inflammatory cells in the BALF of the smoke-exposed mice and increased these in lung tissue. DC modulation reduced IL-17 and increased IL-10 levels, which may be responsible for the suppression of the BALF cells. Furthermore, depletion of pDCs led to increased infiltration of alveolar macrophages while restricting the presence of CD103+ DCs.

This study suggests that DC subsets may differentially and compartment-dependent influence the inflammation induced by CS. pDC may play a role in preventing the pathogenesis of cigarette smoke by inhibiting the alveolar macrophage migration to lung and increasing CD103+ DCs at inflammatory sites to avoid extensive lung tissue damage.

Keywords: COPD, Dendritic cells, Inflammation, Cigarette smoke
Introduction

Cigarette smoking is the main risk factor for the development of inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) (16, 23, 37). The limited knowledge on disease mechanism hampers an effective treatment of disease. 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 which 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 responses to acute CS exposure. Previously, we reported that modulation of 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), but the contribution of DC subsets to the immunologic response in COPD is not well understood (17, 19).

Two major DC subsets in the mouse are myeloid/conventional DC (mDC/cDC) and plasmacytoid DC (pDC) with distinct roles in the regulation of T cell-mediated adaptive immunity. Further, 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 as compared with CD11b$^{hi}$ 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 (CSE), 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 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.
Materials and methods

Animals and groups of study

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

Cigarette smoke exposure

Mice were subjected to whole-body cigarette smoke or air (sham) twice daily for five consecutive days as follows: day 1: 6 cigarettes (~30 min exposure); day 2-5: 10 cigarettes (~50 min exposure) (See Fig. 1A).

Treatments-Smoke Combination protocols:

Flt3L treatment

Mice were treated s.c. (at the nape of the neck) with 10µg of human Flt3L (eBioscience) or as a control with serum albumin (0.01 % in PBS) daily for 10 consecutive days [31] and combined with cigarette smoke exposure from day 6 (See Fig. 1B, I).

120g8 treatment

Mice were injected i.p. with 200 µg of pDC-selective depleting 120g8 Abs (provided by Bioceros BV) or as a control with 200 µg of isotype-matched rat IgG (Sigma-Aldrich). Mice were either injected for 4 consecutive days starting 1 day before smoke exposure (2) (See Fig. 1B, II).
**Flt3L plus 120g8 Abs treatment**

Mice were injected either with daily Flt3L (10µg, s.c.) for 10 consecutive days and with 120g8 (i.p. 200 µg) from day 5 for 4 consecutive days starting 1 day before smoke exposure. Control mice received injections of rat IgG and serum albumin (See Fig. 1B, III).

**Bronchoalveolar lavage Fluid (BALF) and cytospins**

16 hours after the last smoke or air exposure, mice were sacrificed 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, CA, USA).

**Flow cytometry analysis**

Lung and mediastinal lymph nodes (mLN) were removed and digested as described previously [28]. Mononuclear cells were analyzed on a FACSCantoII flow cytometer (BD Biosciences), and data processed with FACSDiva software (v6.1.2). All staining reactions were performed at 4°C and dead cells were excluded using 7-AAD viability staining. The following antibodies were used for flow cytometry analysis: FITC-conjugated anti-CD11c; phycoerythrin (PE)-conjugated anti-CD103 or anti-CD86; PE-Cy5.5 conjugated anti-CD8; PE-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–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. 2B 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 five days of smoke exposure compared with the control groups but did not change significantly across the treatments (appendix figure 1). Treatments with 120g8 and/or FLt3L had no effect on cellular composition in non-smoked 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 DCs 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. 3A). pDC were found increased in lung tissue after CS exposure (Fig. 3B). 120g8 significantly suppressed the percentage of pDCs in both the CS-exposed and CS-exposed+Flt3L-treated animals (Figure 3B). 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. 3C and D).

Modulation of DCs with Flt3L, 120g8 or both enhanced the neutrophil and macrophage infiltration significantly in the lungs compared to smoke-exposed mice, but no difference between the different treatment conditions was observed (Fig. 3C and D).

**Effect of DC subset modulation on the release of proinflammatory mediators in BAL fluid**

CS is a strong inflammatory stimulus that induces the release of proinflammatory mediators in the BALF (32). To investigate whether cellular changes induced by DC subset modulated the cytokine production, proinflammatory mediators were measured in BAL fluid. After five days, cigarette smoke exposure significantly enhanced the level of KC, IL-1β, IL-6, IL-10, IL-12, IL-17 and MIP-1α in BAL fluid compared to control mice (Fig 4, Table 2). Flt3L even further promoted the production of KC, IL-6, IL-12, MIP-1α (Table 2) and IL-10 (Fig. 4A), and diminished IL-17 (Fig. 4B). It is notable that combined treatment, Flt3L+120g8, restored IL-10 levels to smoke exposed mice and IL-17 levels to air exposed mice (Fig. 4A and B).

**pDCs depletion significantly increases CD11c+/CD11bhi alveolar macrophages 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. 5A) within lung parenchyma compared to smoke-exposed and air-exposed mice. Furthermore, treatment with 120g8 decreased the trafficking of CD103⁺ CD11b⁺ migratory DCs within lung parenchyma compared to smoke-exposed mice (Fig. 5B).
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 decrease 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 dendritic cell 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 bio-marker of inflammation and involved in 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 cigarette smoke 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. 4) suggesting that pDC may be linked to IL-17 production in smoke exposed animals. IL-17 is a
pro-inflammatory cytokine, mainly by stimulating mediator production such as IL-8 (equivalent to 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 into 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 suppression of 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 cigarette smoke in early stage by inhibiting the alveolar macrophage migration to lung and increasing CD103⁺ DCs at inflammatory sites to avoid extensive lung tissue damage.


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Table 1. Animal groups of study

<table>
<thead>
<tr>
<th>Control (Air-Exposed)</th>
<th>Smoke exposed</th>
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<tr>
<td>PBS</td>
<td>*</td>
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<tr>
<td>120g8 or (Rat IgG) = pDCs</td>
<td>*</td>
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<tr>
<td>Flt3L or (Serum albumin) = DC subsets</td>
<td>*</td>
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<tr>
<td>Flt3L + 120g8 or (Serum albumin/Rat IgG) = mDCs</td>
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BALB/c mice were divided in two main groups of smoke-exposed and control (air-exposed) with each group further divided into subgroups. n= 6 mice per group.

Table 2. Effect of DC subset modulation on cigarette smoke-induced cytokines and chemokines in BAL fluid

<table>
<thead>
<tr>
<th>BALF cytokine (pg/ml)</th>
<th>Control</th>
<th>120g8</th>
<th>Flt3L</th>
<th>Flt3L+120g8</th>
<th>Iso-Fit3L+120g8</th>
<th>Smoke</th>
<th>Smoke-120g8</th>
<th>Smoke-Fit3L</th>
<th>Smoke-Iso Fit3L+120g8</th>
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<tr>
<td>KC</td>
<td>13.2±1.8</td>
<td>9.17±1.3</td>
<td>7.15±0.4</td>
<td>7.68±0.6</td>
<td>5.67±0.85</td>
<td>101.6±7.2 *</td>
<td>279±38.7 **^</td>
<td>870.1±88 *^</td>
<td>594.7±63.5 *^</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>2.88±0.1</td>
<td>3.04±0.1</td>
<td>2.7±0.13</td>
<td>3.0±0.17</td>
<td>2.30±0.10</td>
<td>69.92±6.5 *</td>
<td>69.2±2.47 *</td>
<td>96.6±4.06 *</td>
<td>85.49±2.7 *</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12.0±1.3</td>
<td>10.38±1.1</td>
<td>10.13±0.9</td>
<td>10.14±0.7</td>
<td>15.2±1.2</td>
<td>68.72±5.5 **</td>
<td>51.26±4 ***</td>
<td>75.96±5.52 ***</td>
<td>56.47±5 **</td>
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<tr>
<td>IL-6</td>
<td>0.04±0.0</td>
<td>0.03±0.01</td>
<td>0.07±0.01</td>
<td>0.06±0.0</td>
<td>0.04±0.01</td>
<td>1.64±0.21 ***</td>
<td>2.88±0.64 ***</td>
<td>4.47±0.61 ***</td>
<td>3.60±0.6 **</td>
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<tr>
<td>IL-12</td>
<td>3.48±0.4</td>
<td>2.53±0.24</td>
<td>3.79±0.17</td>
<td>2.43±0.19</td>
<td>3.37±0.67</td>
<td>5.75±0.26 **</td>
<td>6.18±0.50 **</td>
<td>8.17±0.36 **</td>
<td>6.66±0.4 ***</td>
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The cyto/chemokine profile in BAL fluid measured by Bio-plex analysis. Values are expressed as mean ± S.E.M, n= 4-6 animals per group *P<0.05, **P< 0.01 and ***P< 0.01 significantly different compared to control, # P<0.05 and ##P< 0.01 significantly different compared to smoke-exposed mice and ^ P<0.05 significantly different compared to smoke-Fit3L.
Figures legend:

Fig.1. Cigarette smoke exposure and treatment-smoke combination protocols. Smoke exposure protocol (A): BALB/c mice were exposed to cigarette smoke or air for 5 days (n=6 mice/group). Combination protocols (B): Combination protocol I: Mice were injected with human Flt3L or serum albumin for 10 consecutive days to expand DC subsets combined with cigarette smoke or air exposure (n=6 mice/group). Combination protocol II: Mice were treated with monoclonal 120g8 Ab or control rat IgG for 4 consecutive days to deplete pDCs combined with cigarette smoke or air exposure (n=6 mice/group). Combination protocol III: Mice were either injected with daily Flt3L or serum albumin for 10 consecutive days and with 120g8 or control rat IgG for 4 consecutive days to expand mDCs, combined with cigarette smoke or air exposure (n=6 mice/group). S: time of analysis after sacrificing the animals.

Fig.2. DC subsets modulation suppresses the inflammatory cell infiltration induced by cigarette smoke in BAL fluid. Mice were treated with 120g8 or FLt3L to modulate DC subtypes combined with cigarette smoke exposure or sham (air only). After five days smoke exposure, the total and differential cell counts were analyzed in BAL fluid. Total number of BAL fluid cells (A), BAL neutrophils (B), and macrophages (C). n= 4-6 animals per group. Values are expressed as mean ± S.E.M. Significant differences are indicated by asterisks (*P<0.05 and**P<0.01).
Fig. 3. DC subsets 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 (upper panel) and bar graphs (lower panel) 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) Ly-6G⁺ neutrophils and (D) CD11c⁺ MHCII⁺ F4/80⁺ alveolar macrophages. At least five animals were analyzed per group and data show one representative experiment of three. Values are expressed as mean ± S.E.M. Significant differences are indicated by asterisks (*P<0.05, **P<0.01 and ***P<0.001).

Fig. 4. Effects of DC subset modulation on release of proinflammatory mediators in BAL fluid. The cytokine profile was measured in BAL fluid by Bioplex method after DC subtypes modulation combined with cigarette smoke exposure. Data presents in figure 4 shows the level of IL-10, and IL-17 in BAL fluid. n= 4-6 animals per group. Values are expressed as mean ± S.E.M. Significant differences are indicated by asterisks (*P<0.05 and **P<0.01).
Fig.5. pDC depletion significantly enhances CD11c^+/CD11b^{hi} positive alveolar macrophages and decreases the CD103^+ CD11b^- 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 (upper panel) and bars (lower panel) show percentages of total CD11c^+ CD11b^{hi} alveolar macrophages and Cd11c^{hi} CD11b^-CD103^+ migratory DCs in lungs. At least five animals were analyzed per group and data show one representative experiment of three. Numbers indicate percentages of cells within the gated leukocyte population (using forward and side scatter). Values are expressed as mean ± S.E.M. Significant differences are indicated by asterisks (*P<0.05 and **P<0.01).
Figure 1

A

6 Cigarettes

Day

1 2 3 4 5

Smoke/Air

10 Cigarettes per day

16h

B

I

Flt3L/ Serum albumin (0.01 % in PBS)

Day

1 2 3 4 5 6 7 8 9 10

Smoke/Air

16h

II

120g8 / Rat IgG

Day

1 2 3 4 5 6 7 8 9 10

Smoke/Air

16h

III

Flt3L/ Serum albumin (0.01 % in PBS)

Day

1 2 3 4 5 6 7 8 9 10

Smoke/Air

16h
Figure 2
Figure 5

A

B

Lung CD11c+ CD11bhi %

Lung CD103+ CD11b- %
Figure 1

Lung and MLN CD4/CD8 T cell

Figure 2

Lung and MLN CD4/CD8 T cell