**Plasmacytoid Dendritic Cells in Pulmonary Lymphoid Follicles of Patients with COPD**

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## ABSTRACT

*Rationale*: Plasmacytoid dendritic cells (pDC) are professional antigen presenting cells with antiviral and tolerogenic capabilities. Viral infections and auto-immunity are proposed as

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important mechanisms in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD).

*Aim of the study:* To quantify BDCA2+ pDC in lungs of subjects with or without COPD by immunohistochemistry and flowcytometry, combined with the investigation of the influence of cigarette smoke extract (CSE) on the function of pDC in vitro.

*Results:* pDC were mainly located in lymphoid follicles, compatible with their expression of lymphoid homing chemokine receptors CXCR3 and CXCR4. pDC accumulated in the lymphoid follicles and in lung digests of patients with mild to moderate COPD compared to smokers without airflow limitation and patients with COPD GOLD stage III-IV. Exposing maturing pDC of healthy subjects to CSE in vitro revealed an attenuation of the expression of co-stimulatory molecules and impaired interferon alpha production. Maturing pDC from patients with COPD produced higher levels of TNF-alpha and IL-8 compared to pDC from healthy subjects.

*Conclusions:* CSE significantly impairs the antiviral function of pDC. In COPD, a GOLD-stage dependent accumulation of pDC in lymphoid follicles is present, combined with an enhanced production of TNF-alpha and IL-8 by maturing pDC.

**Key words:** Airway inflammation, Chronic Obstructive Pulmonary Disease, Cigarette smoke, Dendritic cell maturation, Lymphoid follicle, Plasmacytoid dendritic cells.

**INTRODUCTION**

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disease of the airways and lung parenchyma, inducing substantial morbidity and mortality worldwide (1).

The inflammatory process causes narrowing of the small airways (obstructive bronchiolitis), leading to an airflow limitation that is not fully reversible. In addition, there is a destruction of the alveolar parenchyma (emphysema), resulting in impaired gas exchange and reduced elastic recoil of the lung. In the Western countries, cigarette smoking is by far the most important risk factor for developing COPD (2). The exact pathogenetic mechanisms, driving the ongoing inflammation despite smoking cessation still remain to be elucidated. Several pathogenetic entities such as low grade bacterial and viral infections, auto-immune responses against changed epitopes and genetic predispositions have been proposed in this respect (3).

The inflammatory process in COPD comprehends both the innate immune response (with epithelial activation and infiltration of neutrophils and macrophages) as well as the adaptive immune response (with influx of cytotoxic CD8+ T cells, CD4+ T helper cells and B cells). In addition, increased numbers of lymphoid follicles are found in the lungs of patients with COPD (4;5).

Dendritic cells (DC) are professional antigen presenting cells from hematopoietic origin, linking these innate and adaptive immune responses. Using specific receptors, DC sense for danger signals while sampling their environment for antigens. DC process the antigen, present it on Major Histocompability Class II and I molecules and integrate this information with the sensed danger signals by upregulating costimulatory molecules and producing specific cytokines. DC then form an immunological synapse by meeting naïve lymphocytes, directing the proliferation of antigen-specific T cells and thus orchestrating the adaptive immune response (6).

In general, two major distinct subsets of DC are known: myeloid dendritic cells and plasmacytoid dendritic cells (7). pDC represent a unique population of professional antigen presenting cells with a plasmacell-like morphology and a unique surface receptor phenotype, capable of producing large amounts of type I interferons in response to viruses and nucleic

acid-containing complexes from the host, sensed through Toll-Like Receptors (TLR) 7 and 9 (8;9). Apart from this innate antiviral defense function, pDCs play a crucial role in maintaining tolerance by expressing the enzyme indoleamine 2,3 dioxygenase (IDO) which induces T cell death by depleting the amino-acid tryptophane (10). In addition, through upregulating Inducible T cell Costimulator Ligand (ICOS-L), pDC are able to generate regulatory T cells (11). Taken together, evidence suggests that pDC play an important role in peripheral tolerance and in antiviral defense mechanisms.

We and others described the presence of mDC and pDC in human lungs, using flowcytometry on single cell suspensions of digested human lung tissue and in broncho-alveolar lavage fluid (BAL) (12-15). Recently, more evidence became available on the different subsets of myeloid DC, their function and role in the pathogenesis of respiratory diseases such as COPD (16-19). However, until now, the exact location of pDC in human lung was unknown. In this study, we were interested in identifying pDC in the small airways. Moreover, considering the important immunological role of pDC, we hypothesized that in smokers and in patients with COPD, pDC could be altered in number and function, contributing to impaired antiviral defense and / or loss of tolerance, both alleged mechanistic concepts in the pathogenesis of COPD (20).

This study describes for the fist time the distribution of pDC in the small airways of human lungs, highlighting the concentration of pDC in lymphoid follicles. In addition, there is a significant accumulation of pDC in lymphoid follicles of patients with mild to moderate COPD compared to smokers without airflow limitation. Finally, we found an important impact of cigarette smoke extract (CSE) in vitro on the innate and adaptive functions of pDC.

**MATERIALS AND METHODS**

**Lung tissue**

Tissue was obtained from surgical lung resection specimens of patients diagnosed with solitary pulmonary lesions at the Ghent University Hospital. Lung tissue at maximum distance from the pulmonary lesion and without signs of retro-obstructive pneumonia or tumour invasion was collected by a pathologist. None of the patients operated for malignancy were treated with neo-adjuvant chemotherapy. Lung tissue from end-stage COPD was obtained from explant lungs from patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium) or lung volume reduction surgery (Maastricht University Medical Centre, Maastricht, The Netherlands). All patients signed informed consent prior to surgery and were interviewed about their smoking habits and medication use. COPD diagnosis and severity was defined using pre-operative spirometry according to the GOLD classification (2). This study was approved by the Medical Ethical Committee of the Ghent University Hospital, University Hospital Gasthuisberg Leuven and the Maastricht University Medical Centre.

**Histology**

Cryosections were incubated with anti - Blood Dendritic Cell Antigen 2 (BDCA-2, CD303) monoclonal antibody (clone AC141, Miltenyi Biotec, Bergisch Gladbach, Germany). Langerin (CD207) immunohistochemical staining was performed as described previously (16). Details on the immunohistochemical stainings are provided in the online data supplement.

**Image analysis**

pDC in small airways were quantified using a computerized image analysis system (Axiocam, Axioskop II mot + KS400, Zeiss, Oberkochen, Germany). Airways without cartilage that had

a perimeter of the basement membrane of less than 6000 μm were selected for analysis (21).
Lymphoid follicles near small airways were identified in hematoxylin-stained sections at
100x magnification as an aggregate of contiguous mononuclear cells (22). In adjacent
sections, follicles with a B cell zone were identified by CD3/CD20 double staining. Follicle
boundaries were delineated by tracing the perimeter and the area was calculated using Image J
software (NIH, Bethesda, MD). The number of BDCA-2 positive cells was counted within
these follicles. The observers (GRVP and SV) were blinded for clinical data. More
information is available in the online supplement.

**Flowcytometry**

Resection specimens were processed as described previously to obtain single cell suspensions
of pulmonary mononuclear cells (12). Monoclonal antibodies and equipment used are
presented in the online supplement.

**In vitro pDC culture**

CSE was prepared as described previously (23). pDC were isolated from fresh blood of
healthy non-smoking volunteers and of patients with COPD. All patients with COPD were
ex-smokers and had a spirometry compatible with GOLD stage II. None of the patients used
systemic corticosteroids. Subjects were free from exacerbation or infection during the 2
months prior to the study. All participants signed informed consent prior to the study. pDC
were isolated by negative selection using Plasmacytoid Dendritic Cell Isolation Kit
MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

PDC were incubated with 3 μg/ml CpG-oligodeoxynucleotides (2216 CpG-type A) or
10μg/ml imiquimod-R837 (Invivogen, San Diego, CA) in the presence of absence of CSE
during 18 hours at 37°C under a humidified atmosphere with 5% $CO_2$.

Interferon alpha concentration in the supernatant was measured using a sandwich ELISA (PBL Interferon Source, NJ, USA). Other cytokines were measured using cytometric bead array human inflammation kit. The expression of maturation markers by pDC was analyzed by flowcytometry. Antibodies used are described in the online supplement.

**Statistical analysis.**

For the immunohistochemical study, statistical analysis was carried out in SPSS 16.0 (SPSS inc. Chicago, IL, USA). When evaluating differences in continuous variables between multiple independent groups, the Kruskal-Wallis test was used. Where values of probability were < 0.05, selected pairs of groups were investigated by the Mann-Whitney U test. Spearman rank test was used to examine correlations. P values < 0.05 were considered significant. For the in vitro study, statistical analysis was carried out in Graph Pad Prism (Graph Pad software Inc. San Diego, CA, USA). Relative expression of maturation markers was analyzed using the one sample t -test. Other comparisons were investigated by the paired and unpaired student T test.

**RESULTS**

**Identification of plasmacytoid dendritic cells in small airways of human lungs.**

Using immunohistochemical staining for the specific marker BDCA-2, pDC were detected at low numbers in the small airways of human lungs (Figure 1). Appropriate isotype control staining was performed on human airways and tonsil in order to assure specificity (shown in Figure E1 in the online depository). pDC were mainly found in the adventitia of the small

airways and to a limited extent in the lamina propria and the epithelium. Interestingly, pDC in small airways were often located in lymphoid follicles (Figure 1 B). The localization of the pDC in these lymphoid follicles was examined by CD3/CD20 double staining in adjacent sections (figure E2 in the online supplement). Quantification revealed that 72.1% (standard deviation: 33.1%) of the pDC in lymphoid follicles were located in the T cell zone.

**Pulmonary plasmacytoid dendritic cells express CXCR3 and CXCR4.**

In order to investigate the lymphoid homing potential of pulmonary pDC, the expression of lymphoid homing chemokine receptors CXCR3, CXCR4 and CCR7 was assessed by flowcytometry on labeled single cell suspensions of digested human lung tissue (Figure 2) using the previously described gating strategy (12). Pulmonary pDC showed high expression of CXCR3 and CXCR4. Importantly, the expression of CCR7 was very low in these pDC. When comparing the expression of these chemokine receptors between smokers without airflow limitation (n=3) and patients with COPD (n=2), a trend towards higher expression of CXCR3 was observed in patients with COPD (Figure 2F).

**Quantification of plasmacytoid dendritic cells in small airways in patients with COPD and subjects without COPD.**

The characteristics of the study population are shown in table 1. A total number of 74 subjects was investigated. The population consisted of never smokers (n=10), smokers without COPD (n=22), COPD GOLD stage I-II (n=28) and COPD GOLD stage III-IV (n=14). Patients with mild to moderate COPD did not use inhaled or systemic corticosteroids. Quantification of the number of pDC in the total airway wall, epithelium, lamina propria or adventitia of the small airways, excluding areas with lymphoid follicles, revealed no significant differences between the study groups (Figure 3A and Figure E3 in the online

supplement).  In addition, there were no differences between current and ex-smoking subjects, both with or without airflow limitation (data not shown).

There were no significant correlations between the number of pDC and the number of langerin positive myeloid DC in the total airway wall.  However, when subdividing the subjects into the different study groups according to GOLD stage, a significant positive correlation between the number of pDC and langerin positive myeloid DC in the total airway wall emerged ($r_s$:0.64; p=0.004) in patients with COPD GOLD stage I.  Interestingly, this positive correlation was lost in patients with COPD GOLD stage III-IV. (Figure E4 in the online data supplement)

**Quantification of plasmacytoid dendritic cells in lymphoid follicles.**

In the same study population, small airways with lymphoid follicles were identified.   The percentage of airways with a lymphoid follicle was significantly higher in COPD GOLD stage III-IV compared to the COPD GOLD stage I-II (p=0.02) and tended to be higher in COPD GOLD stage III-IV compared to never smokers (p=0.06) (Figure 3B).

The number of pDC was significantly higher in lymphoid follicles of mild to moderate COPD patients compared to smokers without airflow limitation and compared to patients with severe to very severe COPD. (p= 0.004 and p= 0.04 respectively) (figure 3C).   There were no significant differences between never smokers and smokers without COPD.   Importantly, when pDC were quantified in follicles with a confirmed B cell zone in the adjacent section (which comprehend 30% of all follicles), a similar trend towards accumulation of pDC was observed in patients with mild-moderate COPD (Figure E5 in the online data supplement). Representative cryosections for the different study groups are shown in Figure 4.

**Quantification of plasmacytoid dendritic cells in lung digests.**

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In a second independent study population, pDC were quantified by flowcytometry. pDC were identified in mononuclear single cell suspensions of human lung digests as BDCA-2 positive cells within the low autofluorescent, CD3 negative, CD19 negative gate. The characteristics of the study population are shown in table 2.

The number of pDC in the single cell suspensions of COPD patients (GOLD stage I-II) was significantly higher compared to smokers without airflow limitation (p=0.02 ) (Figure 3D).

**Cigarette smoke extract suppresses maturation-associated co-stimulatory molecule expression of pDC in vitro**.

Human Blood derived "untouched" pDC cells were exposed to a range of concentrations of CSE to determine the effect on cellular viability. CSE concentrations from 2% had a detrimental effect on cellular viability, as shown in Figure E6 in the online data supplement.

Blood derived pDCs were matured by adding imiquimod (a TLR-7 agonist) or CpG oligonucleotides (a TLR-9 agonist) in the presence or absence of cigarette smoke extract (0.5 or 1%). Toll like receptor 7 and 9 stimulation was used to induce maturational response in CSE exposed pDC. There was an attenuation of the maturational response in cigarette smoke exposed pDC with a significantly impaired expression of CD83 in CpG/CSE stimulated pDC and of CD80, CD83 and CD86 in imiquimod/CSE stimulated pDC of healthy non-smoking subjects. A similar reduced maturational response due to cigarette smoke extract was observed in pDC of patients with COPD. (Figure 5) and (figure E7 in the online data supplement).

**Cigarette smoke extract alters CpG-induced cytokine production by pDC in vitro.**

Plasmacytoid dendritic cells were stimulated with CpG oligonucleotides in the presence or absence of CSE (1%) (Figure 6 A-C). CSE attenuated the production of interferon alpha by

CpG, both in healthy subjects and in patients with COPD. Remarkably, there was a significantly lower production of interferon alpha by CSE-exposed pDC of patients with COPD compared to pDC of healthy subjects. The production of TNF alpha and IL-8 already tended to be higher at baseline in pDC of patients with COPD. The CpG induced production of IL-8 and TNF alpha was increased by CSE in healthy subjects, but was not significantly altered by CSE in patients with COPD.

**Cigarette smoke extract alters imiquimod-induced cytokine production by pDC in vitro.**

In imiquimod-stimulated pDC, CSE induced a marked decrease in TNF-alpha production in both pDC from healthy subjects, as in pDC from patients with COPD. (Figure 6 D-F). We observed significantly higher levels of TNF-alpha and IL-8 produced by pDC of patients with COPD compared to pDC of healthy subjects.

There were no differences in production of IL-6, IL-10, IL-12 or IL-1beta in CpG or imiquimod treated pDCs under the influence of 1% CSE (data not shown) .

**DISCUSSION**

This study identified for the first time the presence of plasmacytoid dendritic cells in small airways of human lungs and highlighted their presence in lymphoid follicles, compatible with the expression of lymphoid homing chemokine receptors CXCR3 and CXCR4 on these cells. In addition, we showed a significant accumulation of pDC in lymphoid follicles and lung digests of patients with mild-moderate COPD. We also demonstrated a marked influence of CSE on pDC function in vitro and showed that pDC of patients with COPD are capable of producing higher levels of TNF-alpha and IL-8.

Although the presence of pDC in human lungs is well established by studies using single cell suspensions of digested lung tissue or by analyzing BAL fluid, the exact distribution and localization of pDCs in small airways was unknown until now. In the past, Masten et al used immunohistochemical staining for CD123 on human lung tissue to identify pDC, but this marker is non-specific as it is also expressed by other cells such as myeloid dendritic cells, macrophages and granulocytes (13). Using the specific marker BDCA-2, pDC were identified at low numbers in the mucosal surfaces of the small airways and were often found in lymphoid follicles. This predominant lymphoid localization is parallel to the described distribution of pDC in other peripheral tissues such as nasal mucosa and synovium (24-26). Indeed, blood pDCs are known to home directly from the blood circulation towards these lymphoid tissues via high endothelial venules under the influence of CXCR3 and CXCR4 ligands. Interestingly, a limited number of pDC was found outside these mucosa-associated lymphoid tissue structures in both individuals with and without COPD, indicating that pDC can also migrate to peripheral non-lymphoid tissue.

Pulmonary BDCA-2 positive pDC are mainly considered immature as shown previously on flow cytometric analysis of single cell suspensions of digested human lung tissue (12). Upon maturation, pDC downregulate BDCA-2, but retain the expression of CD123 (27). In addition, our data show a low expression of the chemokine receptor CCR7, which is generally upregulated by DC during their maturation process. Freeman et al (17) showed recently that BDCA-2 positive pDC can express higher levels of maturation markers, especially in COPD (17). This suggests that the BDCA-2 positive cell population consists of immature and maturing pDC, whereas mature pDC could become BDCA-2 negative.

When quantifying the number of pDC in the small airways of patients with COPD and subjects without airflow limitation, we found a significant accumulation of these cells in lymphoid follicles of patients with mild and moderate COPD compared to smokers without COPD. Importantly, these findings were confirmed by the flowcytometric quantification of pDC in human lung digests of an independent study population. As lymphoid follicles harbor the majority of pDC in the lungs, it is conceivable that the results of the flowcytometric study of lung digests are mirrored in the quantification of pDC in lymphoid follicles. The accumulation of pDC in lymphoid follicles is compatible with the observed increase of CXCR3 expression on pulmonary pDC of patients with COPD in our study and with the recently published observations of increased follicular expression of CXCR3 in patients with COPD (22).

In contrast, we found no differences in pDC numbers between never smokers and smokers without airflow limitation. These results are in accordance with the findings in BAL fluid after acute smoke exposure (28) and indicate that smoking as such does not influence the number of pDC in the airways and lymphoid follicles.

As immature pDC are present in the mucosal areas of the lung, they can be directly influenced by the effects of cigarette smoke. We therefore incubated pDC with CSE, mimicking mainsteam cigarette smoke exposure. This resulted in a blunted maturation response of these cells when imitating viral infection by using the TLR-9 agonist CpG oligonucleotides or the TLR-7 agonist Imiquimod. These findings are in accordance with the previously published observations of impaired maturation of CSE exposed myeloid DC (23).

Moreover, we found an altered cytokine production during the maturation process with a reduction of the interferon alpha response by CSE in TLR-9 stimulated pDC. This blunted interferon alpha response is both present in pDC of healthy subjects and in pDC of patients

with COPD. This finding is important as interferon alpha production is the cardinal innate feature of pDC and a cornerstone of early antiviral defense. A blunted type-I interferon response combined with impaired maturational capabilities of pDC due to cigarette smoking could contribute to the increased susceptibility of smokers to viral infections (29) and the development of low grade infections in patients with COPD, stimulating to the ongoing pathogenic inflammatory process (30). In addition, CSE induced an increase of IL-8 production in maturing pDC of healthy subjects, which could contribute to the increased influx of neutrophils in smokers. In maturing pDC of patients with COPD, a significantly higher production of IL-8 and TNF-alpha is present compared to maturing pDC of healthy subjects. In the case of IL-8, CSE can not further augment this production, whereas CSE decreases the production of TNF alpha in pDC of patients with COPD. As increased maturation status of pDC has been described in COPD (17), this enhanced production of TNF-alpha and IL-8 by pDC could also be present in vivo, contributing to the inflammatory process in COPD.

Importantly, our in vitro data confirm and extend the recently published in vitro study by Mortaz et al, showing impaired interferon alpha response and increased IL-8 production in CpG stimulated CSE exposed pDC of healthy subjects (31).


The exact role of the accumulating pDC in lymphoid follicles of patients with mild-moderate COPD remains to be elucidated. One could speculate that this increased number of pDC reflects an influx of these cells as a compensatory mechanism which tries to dampen the enhanced immune response in COPD, as pDC are known for their tolerogenic properties. Accordingly, a recent publication showed an accumulation of regulatory CD4+Foxp3+ T cells in lymphoid follicles of patients with moderate COPD compared to smokers and non smokers without COPD (32). PDC could also play an important role in lymphoid neogenesis and the

homeostasis of tertiary lymphoid structures in the lung (33;34). In addition to the increased influx of pDCs, a reduced DC maturational process in COPD, as suggested by Tsoumakidou et al (19), could contribute to the accumulation of immature BDCA-2 positive DCs in lymphoid follicles of patients with COPD.

Importantly, in the severest stages of COPD, the number of BDCA-2 positive DCs was at the same level as in the individuals without airflow limitation.  This could reflect a relative depletion of pDC, as often seen in chronic low grade viral infections (35).  An increased maturational response of pDC in severe COPD, - as suggested by Freeman et al (17) – could also explain the lower numbers of BDCA-2 positive pDC.  Finally, the use of inhaled and systemic corticosteroids by patients with severe and very severe COPD could contribute to the reduced number of pDC in this study, as these drugs are known to induce apoptosis in circulating and tissue-resident pDC (36-39).  This lower number of pDC in lymphoid follicles of patients with very severe COPD treated with inhaled steroids could be linked to the observed association of increased risk of pneumonia and the use of inhaled corticosteroids in patients with COPD (40).

A reduction in pDC numbers could lead to a state of "loss of tolerance", which can be linked to a drop in regulatory T cell numbers and the occurrence of auto-immune processes in the severest stages of COPD  (41).  In addition, an imbalance between myeloid DC and plasmacytoid DC in the lungs could cause a derailment in the control of inflammation. Indeed, in COPD GOLD stage I, we observed a significant positive correlation between the number of langerin positive myeloid DC and the number of pDC in the total airway wall. However, this positive correlation was completely lost in COPD GOLD stage III-IV.  This suggests  that langerin positive myeloid DC (accumulating in small airways of patients with COPD and stimulating T-helper 1 and T- helper 17 responses) (20) dominate over the tolerogenic pDC.

There are several aspects that strengthen this study. First, the immunohistochemical study addressed for the first time the number of pDCs in human lungs of a large population, using a specific pDC marker, covering the different severities of COPD and comparing them to individuals without airflow limitation. Descriptive quantitative ex-vivo data were supplemented with in vitro functional testing, highlighting the influence of cigarette smoke extract on pDC function.

There are however several limitations which should be addressed. First, tissue samples for the immunohistochemical and flowcytometric studies were mainly obtained from patients operated for a lung tumor. Theoretically, this tumor could influence the number and phenotype of the plasmacytoid dendritic cells. However, tissue samples were obtained at a maximal distance from this lesion, minimizing its effect.

Secondly, quantification of immunohistochemical stainings for pDC in small airways and follicles revealed rather low numbers of these cells. This might have an impact on the sensitivity for detecting differences in pDC numbers between study groups. However, the immunohistochemical study data were confirmed by flowcytometric quantification of pDC in an independent population. In addition, our data are in line with a recent flowcytometric study on lung digests by Freeman et al, showing a tendency towards higher pDC numbers in patients with moderate COPD (17).

Finally, the in vitro experiments with CSE only investigated the direct influence of smoke on the pDC. However, the indirect effect of cigarette smoke (through mediators released by smoke-exposed epithelium, inflammatory and structural cells) can be of great influence on the function of these antigen presenting cells.

**CONCLUSIONS**

Cigarette smoking does not influence the number of pDC in the human lungs. However, pDC are functionally altered by cigarette smoke extract, resulting in impaired antiviral defense mechanisms. This observation directly links pDC function to the increased susceptibility of smokers to viral infections.

pDC are present in the small airways of healthy subjects and patients with COPD. In COPD, maturing pDC can produce higher levels of TNF-alpha and IL-8, contributing to enhanced influx of neutrophils and the activation of macrophages in the lungs of patients with COPD. Finally, pulmonary plasmacytoid dendritic cells, through the enhanced expression of the lymphoid homing chemokine receptor CXCR3, accumulate significantly in lymphoid follicles of patients with mild/moderate COPD. However, in the severest stages of COPD, the number of pDC is reduced, which could be compatible with an impaired control on the adaptive immune response and a loss of tolerance in end-stage COPD.

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## Tables

| TABLE 1: CHARACTERISTICS OF STUDY POPULATION A (immunohistochemical study) | | | | | |
|---|---|---|---|---|---|
| | Never Smokers | Smokers without | COPD I-II | COPD III-IV | statistic |

|  | COPD | | | | |
|---|---|---|---|---|---|
| n | 10 | 22 | 28 | 14 | |
| Sex ratio (M/F) | 3 / 7 | 17 / 5 | 27 / 1 | 6 / 8 | $p < 0.001$ * |
| Age (years) | 56.3 (11.2) | 59.9 (10.6) | 63.3 (9.0) | 58.8 (5.4) | $p = 0.17$ |
| Smoking history (packyear) | 0 | 33.0 (26.2) | 45.9 (22.4) | 30.7 (16.0) | $p < 0.001$ * |
| Smoking status (Current/Ex) | N.A. | 12 / 10 | 19/9 | 3 / 11 | $p = 0.02$ * |
| FEV$_1$ (%pred) | 100.6 (12.1) | 103.2 (17.8) | 80.9 (11.0) | 29.9 (10.4) | $p < 0.001$ * |
| FEV$_1$/FVC (%) | 77.3 (7.3) | 77.8 (5.2) | 60.1 (6.0) | 40.4 (11.6) | $p < 0.001$ * |
| LABA (Y/N) | 0 / 10 | 0 /22 | 2 / 26 | 12 / 2 | $p < 0.001$ * |
| Inhaled corticosteroids (Y/N) | 0 / 10 | 0 / 22 | 0 / 28 | 12 / 2 | $p < 0.001$ * |
| Inhaled corticosteroid dose (µg BDP/24h) | 0 | 0 | 0 | 1353.9 (659.1) | $p < 0.001$ * |
| Systemic corticosteroids (Y/N) | 0/10 | 0/22 | 0/28 | 9 / 5 | $p < 0.001$ * |
| Systemic corticosteroid dose (mg prednisolon /24h) | 0 | 0 | 0 | 4.6 (4.1) | $p < 0.001$ * |

Data are expressed as mean (standard deviation); FEV$_1$: forced expiratory volume in one second; FVC: forced vital capacity; LABA : long acting β2 agonist; BDP: Beclomethasone diproprionate.

Statistic indicates p value generated by Kruskal-Wallis test for continuous variables and Fisher exact test for categorical variables. (*) indicates significant p value.

| TABLE 2: CHARACTERISTICS OF STUDY POPULATION B (flowcytometric study) | | | | |
|---|---|---|---|---|
|  | Never Smokers | Smokers without COPD | COPD I-II | statistic |
| n | 4 | 11 | 16 | |
| Sex ratio (M/F) | 1/3 | 9/2 | 16/0 | p=0.001 |
| Age (years) | 55.8 (13.7) | 62.4 (8.9) | 66.8 (9.3) | NS |
| Smoking history (packyear) | 0 | 34.2 (15.7) | 37.2 (20.5) | p=0.007 |
| Smoking status (Current/Ex) | N.A. | 6/5 | 8/8 | NS |
| FEV$_1$ (% pred) | 102.5 (11.3) | 107.2 (21.5) | 74.8 (12.4) | p=0.001 |
| FEV$_1$/FVC (%) | 78.8 (4.1) | 76.2 (7.8) | 61.4 (5.7) | p<0.001 |
| LABA (Y/N) | 0/4 | 0/11 | 4/12 | NS |
| Inhaled corticosteroids (Y/N) | 0/4 | 0/11 | 1/15 | NS |
| Systemic corticosteroids (Y/N) | 0/4 | 0/11 | 0/16 | NS |

Data are expressed as mean (standard deviation); FEV$_1$: forced expiratory volume in one second; FVC: forced vital capacity; LABA : long acting β2 agonist;

Statistic indicates p value generated by Kruskal-Wallis test for continuous variables and Fisher exact test for categorical variables. (*) indicates significant p value. NS: not significant: p value > 0.05

**FIGURE LEGENDS**

**Figure 1: Identification of plasmacytoid dendritic cells (pDC) in small airways.**

A: Cryosection of human lung (smoker without airflow limitation) was incubated with anti-BDCA-2 antibody, stained with new fuchsin (red color) and counterstained with hematoxylin. pDC are located in the adventitia (black arrows) and concentrated in the lymphoid follicle (white arrows). (original magnification 100x).

B: Triple staining of a cryosection of human lung tissue for CD3 (blue color: indicating the T cell zone), CD20 (red-pink color, indicating the B cell zone) and BDCA-2 (brown color, indicating plasmacytoid dendritic cells). Plasmacytoid dendritic cells (arrows) are mainly found in the T cell zone of lymphoid follicles.
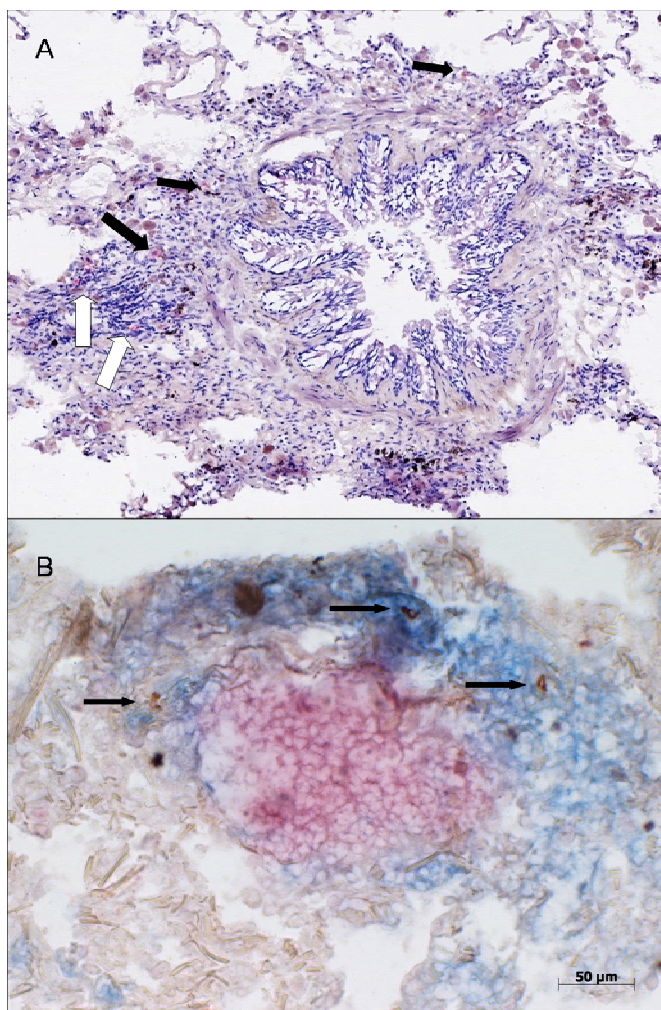
**Figure 2: Chemokine receptor expression on pulmonary plasmacytoid dendritic cells (pDC).** Pulmonary pDC were identified by flowcytometry on single cell suspensions of enzymatically digested human lung tissue. (A) & (B): gating strategy for identification of pDC: first, lineage negative, CD11c negative and low autofluorescent cells were selected (A), then gating focused on HLA-DR and BDCA-2 double positive cells (B). Within these pDC, expression of lymphoid homing chemokine receptors CXCR3 (C), CXCR4 (D) and CCR7 (E) was evaluated (red histograms). Blue histograms represent isotype control staining. Data are from a patient with COPD.

(F-H): Comparison of the chemokine receptor expression on pulmonary pDC between smokers without COPD (n=3) and patients with COPD (n=2). Data are represented as mean + standard error of the mean.
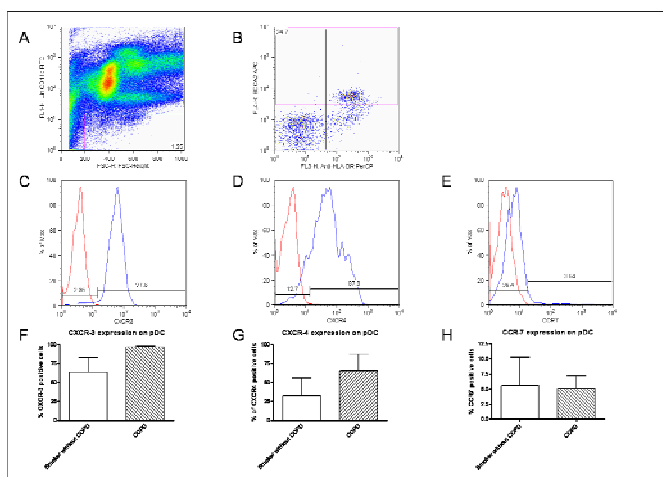


**Figure 3: Quantification of plasmacytoid dendritic cells in small airways, in small airway-associated lymphoid follicles and in human lung digests** (A) BDCA-2 positive plasmacytoid dendritic cells, identified by immunohistochemical staining, were quantified in the small airway wall (excluding the area of lymphoid follicles) of never smokers, smokers without COPD, COPD GOLD stage I-II and COPD GOLD stage III-IV. Horizontal line represents the median for each group. (B) Quantification of lymphoid follicles: the mean

number (+standard error of the mean) of the percentage of small airways containing a lymphoid follicle is shown per group. Significant differences (p<0.05) between groups are indicated by full lines and an asterisk (*). Dashed line indicates p value = 0.09. (C) BDCA-2 positive plasmacytoid dendritic cells, identified by immunohistochemical staining, were quantified in the lymphoid follicles of never smokers, smokers without COPD, COPD GOLD stage I-II and COPD GOLD stage III-IV. Full lines indicate significant differences between groups (p<0.05) and dashed line indicates p value=0.08.

(D) Plasmacytoid dendritic cells were quantified by flow cytometry in mononuclear single cell suspensions of digested human lung tissue in an independent study population. pDC were identified as BDCA-2 positive cells. Quantification is expressed as the percentage of the low autofluorescent, CD3 negative, CD19 negative population. Horizontal lines indicate mean values. Significant p value is shown by an asterisk (*).
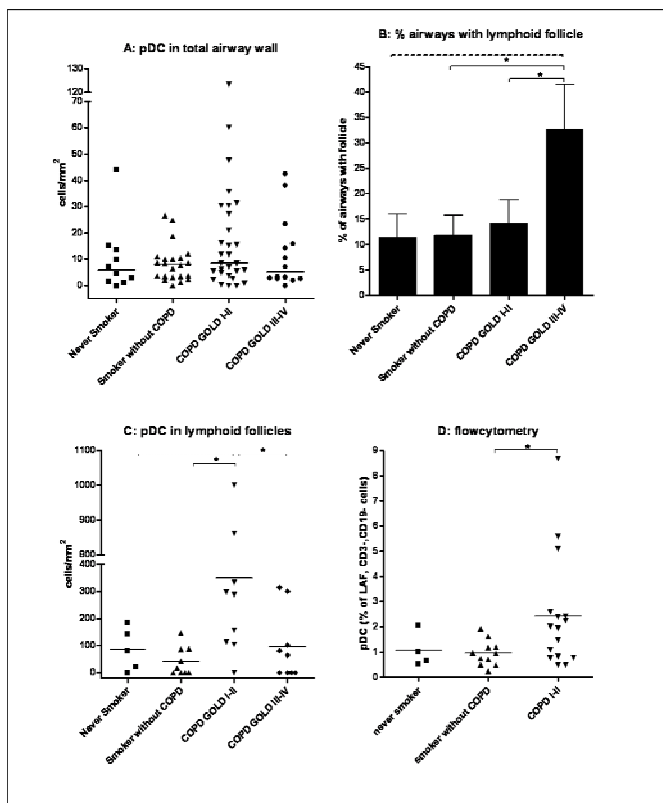
**Figure 4: Plasmacytoid dendritic cells in lymphoid follicles.** Immunohistochemical staining for BDCA-2 on cryosections of human lungs, focusing on lymphoid follicles. (A) Never smoker, (B) Smoker without COPD, (C) COPD GOLD stage I, (D) COPD GOLD stage IV. (original magnification 200x).
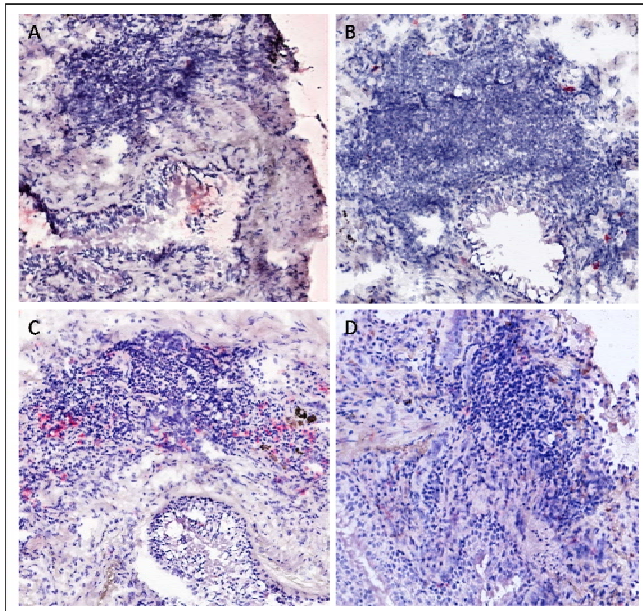


**Figure 5: Cigarette smoke extract (CSE) impairs plasmacytoid dendritic cell maturation.** Blood derived plasmacytoid dendritic cells were exposed during 18 h to cigarette smoke extract and a maturation stimulus. Expression of maturation markers on viable pDC was evaluated by flowcytometry. Mean (+SEM) upregulation of the maturation marker in 0.5% CSE (grey bar) and 1% CSE (black bar) environment is depicted relative to the maturation response in absence of CSE. Dashed bars show the relative maturation of CpG or Imiquimod stimulated pDC of patients with COPD in a 1% CSE environment .
(A) with CpG oligonucleotides as maturation stimulus. (B) with imiquimod (IMQ) as maturation stimulus. Significant differences $p < 0.05$ are marked with an asterisk (*). Data are representative for respectively 6 and 5 independent experiments. CSE: cigarette smoke extract; NS: non smoker (healthy); COPD: chronic obstructive pulmonary disease.
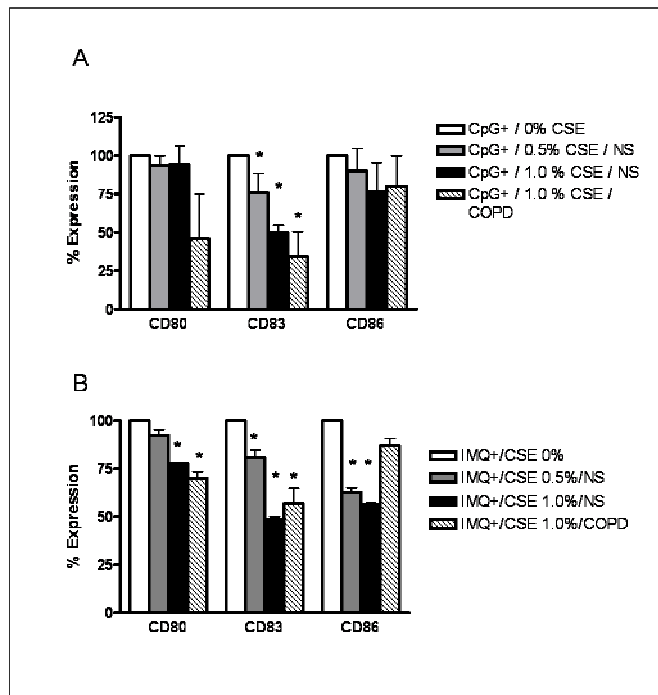
**Figure 6: Cigarette smoke extract (CSE) alters cytokine production of plasmacytoid dendritic (pDC) cells in response to CpG oligonucleotides or imiquimod.** Blood pDC of healthy subjects and patients with COPD were incubated during 18 hours with (+) or without (-) CpG oligonucleotides or imiquimod (IMQ) in the presence or absence of cigarette smoke extract (1%). Interferon alpha (IFNa) (A&D), Tumor Necrosis Factor alpha (TNFa) (B&E) and interleukin-8 (IL-8) (C&F) concentrations were measured in the culture supernatant. Bars represent mean + standard error of the mean. Significant differences ($p<0.05$) are marked with a full line and with an asterisk (*). p values between 0.05 en 0.08 are marked with a dashed line. Data represent 6 (for CpG) and 5 (for IMQ) independent experiments.