Differential switching to IgG and IgA in active smoking COPD patients and healthy controls

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Abstract

Several studies have demonstrated the presence of B-cell follicles and autoantibodies in COPD. It is unclear against which antigens this B-cell response is directed and whether it contributes to development or worsening of disease.

We assessed different B-cell subsets in blood and lung tissue from COPD patients and controls and compared differences in B-cell responsiveness to stimulation with lung specific antigens.

Active smoking induced an adaptive immune response with relatively high levels of (class switched) memory B cells in blood and IgG memory B cells in the lung. COPD smokers showed more switching to IgG, whereas healthy smokers switched more to IgA. COPD patients had higher levels of memory B cells in the lung and stimulation with lung specific antigens induced higher numbers of anti-decorin antibody producing cells in COPD patients compared to healthy controls.

Differential switching to IgG and IgA indicates that the adaptive immune response to smoke differs between COPD patients and healthy controls. A higher level of memory B cells in lungs of COPD patients may reflect an antigen specific immune response, which could be directed against decorin as suggested by the induction of anti-decorin antibody producing cells in response to antigen specific stimulation in COPD patients.

**Key words;** cigarette smoke, immune response, lung tissue, memory B cells, decorin

**Word count;** 200
Introduction

Chronic obstructive pulmonary disease (COPD) is a pulmonary disease characterized by a chronic inflammatory response in the lungs that is reflected by an influx of cells of the innate immune system, i.e. neutrophils and macrophages as well as cells of the adaptive immune system; CD4 and CD8 T cells and B cells [1,2]. The exact mechanisms underlying this chronic inflammatory response in COPD have not been elucidated yet. Since a few years there are increasing indications of an antigen specific, and possibly, autoimmune response in COPD. Richmond et al demonstrated that the presence of lymphoid follicles, described as bronchus associated lymphoid tissue (BALT), was more prominent in smokers than never smokers [3]. More recently, Hogg et al reported the presence of lymphoid follicles in the lungs of COPD patients and showed a relation with disease severity [4]. Furthermore, we demonstrated the presence of oligoclonal B cells with ongoing mutations in these follicles [5], indicating the presence of a specific B-cell response. Several papers have now confirmed the presence of B-cell follicles in COPD [6-10], some also in relation with disease severity. Noteworthy are the findings of Polverino et al, who showed the presence of B-cell activating factor of TNF family (BAFF) in these follicles [8], which is a marker of B-cell survival and activation and has been associated with autoimmune diseases [11,12]. In addition to B cells, oligoclonal CD4 T cells have also been demonstrated in lung tissue of severe COPD patients [13] as well as an antigen specific Th1 response against lung elastin [14]. In line with this, Motz et al recently demonstrated that chronic cigarette smoke exposure can generate T cells in mice, which are capable of inducing a COPD like pathology upon transfer in naïve recipients lacking functional T and B cells [15]. To our knowledge this study of Motz et al is the first functional study demonstrating smoke induced autoreactivity with COPD like pathology in mice.
With respect to the autoantigens that may drive autoimmunity, autoantibodies against Hep 2 epithelial cells [16,17] bronchial epithelial cells [16], endothelial cells [16,18], elastin [14], cytokeratin 18 [19] and several immunogenic peptides [20] have been demonstrated in patients with COPD. It should be noted however, that we [21] and others [22-24] could not demonstrate this anti-elastin autoantibody response in COPD. Recent data demonstrating that long term cigarette smoke exposure can induce antibodies in mice which can induce COPD like pathology upon transfer into naïve recipients supports a possible pathogenetic role for autoantibodies in COPD [25].

Interestingly, cigarette smoking has been demonstrated as a risk factor for the development of several autoimmune disorders [26], including rheumatoid arthritis (RA) [27], systemic lupus erythematosus (SLE) [28], primary biliary cirrhosis (PBC) [29] and multiple sclerosis (MS) [30]. Moreover, a recent study showed that patients with several autoimmune disorders such as RA, SLE, Graves’ disease, PBC, polymyalgia rheumatica and psoriasis, had an increased risk to develop COPD [31]. Together, these findings implicate a role for smoking in autoimmune pathogenesis.

Although there is convincing evidence for the presence of B-cell follicles and autoantibodies in COPD, it is still unclear to which antigens these B cells respond and whether this response is pathologic in humans and contributes to the development or worsening of COPD.

We investigated whether B cells from COPD patients and healthy individuals respond differently to stimulation with lung-specific antigens with respect to plasma cell differentiation and antibody production. Given the previous results of Lee et al [14], our own recent data regarding autoantibodies against elastin, collagen and decorin [21,32], and our previous findings regarding a decreased presence of decorin in COPD [33,34] we chose elastin, collagen and decorin as lung-specific antigens in this study.
Since we previously demonstrated that current smoking can have a pronounced effect on the adaptive immune response [35], equal numbers of smoking, ex-smoking and never smoking subjects were included in this study. As a follow up to our previous findings [35], we now analyzed different B-cell subsets in lung tissue from COPD patients and non-COPD controls as well as analyzing IgG expression on memory B cells.
Materials and Methods

For detailed description of the material and methods see online data supplement.

Subjects

For the isolation of leukocytes from blood, 23 COPD patients with post-bronchodilator FEV\(_1\)/FVC < 70%, and FEV\(_1\) < 80% predicted were included, as were 36 healthy individuals without pulmonary symptoms, and with FEV\(_1\)/FVC > 70%, and FEV\(_1\) > 90% predicted. All were male, > 40 years, and had no major co-morbidities and a negative skin prick test or Phadiatop. Exacerbations or the use of corticosteroids in the past 6 weeks were not allowed. Smokers and ex-smokers (no smoking last year) had to have ≥ 10 packyears. Approval was obtained from the local medical ethics committee and participants gave written informed consent.

For the isolation of leukocytes from lung tissue, 14 COPD patients and 9 non-COPD controls were included. Lung tissue was derived from patients undergoing surgery for lung transplantation or pulmonary carcinoma or from donor lungs. The study protocol was consistent with national ethical and professional guidelines ("Code of Conduct; Dutch Federation of Biomedical Scientific Societies"; http://www.federa.org).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated and used for flow cytometry analysis, immunocytochemical staining on cytospins, and stimulation experiments (see figure E1, online repository). Lung tissue single cell leukocytes suspensions were freshly isolated using a slightly adapted protocol as described before in the mouse (see online repository) [32,36].
**Flow cytometry analysis**

One million PBMCs or lung leukocytes were stained with CD20-Alexa fluor700, CD27-APCCy7, CD38-PECy7, CD138-APC, IgM-Biotin + Strept-PE, IgG-PECy5, CD3-FITC, CD14-FITC, and CD16-FITC. Fluorescent staining of the cells was measured on a LSR-II flowcytometer and data were analyzed using FlowJo Software. First, lymphocytes were gated based on cell size (forward scatter) and density (side scatter). This population was used as input population for the analyses. Subsequently, cells with expression of CD3, CD14 and CD16 were excluded from the analysis to leave out T cells, monocytes, macrophages and granulocytes. Next, the different B-cell subsets were distinguished based on the expression of CD20, CD27, IgM and IgG. Total B cells were analyzed based on CD20 expression, and total memory B cells were analyzed based on co-expression of CD20 and CD27 (Figure E2A online repository). Within the total CD20 population, class-switched memory B cells were classified as CD27 positive and IgM negative (Figure E2A online repository). Within the memory B-cell population, IgG positive memory B cells, IgM positive memory B cells and memory B cells negative for IgG and IgM could be distinguished (Figure E2C online repository).

**PBMC stimulation**

Two million PBMCs from COPD patients and healthy controls were stimulated for 6 days with lung elastin, lung collagen (both 1μg/ml, Elastin Products, Owensville, USA) and recombinant human decorin (1μg/ml, R&D Systems, Minneapolis, USA) in combination with a mixture of IL-10 (50ng/ml), IL-4 (10ng/ml, both Peprotech, Rocky Hill, USA), IL-2 (1.25 pg/ml, R&D Systems) and anti-CD40 (5μg/ml, eBioscience)(cytokine mix) to stimulate plasma cell differentiation and Ig production (will be referred to as ECM + cytokine mix)
stimulation). A double dose of this cytokine mix served as positive control (will be referred to as cytokine mix stimulation) and no stimulants as negative control.

**Immunocytochemistry**

IgA, IgG and IgE expressing B cells and plasma cells were determined in cytopspins from PBMCs or stimulated cells using immunocytochemistry. Per cytopsin, 1000 cells were counted and expressed as percentage positive cells.

**IgG, IgA and IgM ELISA**

IgA levels were determined using an ELISA kit (Bethyl laboratories). For detection of IgM and IgG, plates were coated with anti-human Ig (Southern Biotech (SBA), Birmingham, USA), followed by incubation with the culture supernatants and alkaline phosphatase conjugated anti-human IgG or IgM. Staining was visualized using p-nitrophenyl phosphate substrate.

**Antigen specific ELISPOT analysis**

Ninety six wells filter plates were coated with lung elastin, lung collagen or recombinant human decorin and incubated overnight with $0.5 \times 10^6$ stimulated cells per well. The following day, plates were incubated with rabbit-anti-human IgG-biotin followed by peroxidase labelled streptavidin. Spots were visualized with AEC substrate and counted using an automated reader.

**Statistical analyses**

Multiple linear regressions were used to determine whether COPD, current smoking or their combination affected the different parameters from the PBMC experiments. Only when
significant effects of COPD or current smoking were found or a significant interaction was present between COPD and current smoking, additional Mann Whitney U (MWU) tests were used for specific post-hoc analyses. For the lung tissue experiments, MWU tests were used to compare COPD patients with non-COPD controls and current smokers with ex- and never smokers. A value of p<0.05 was considered significant.
Results

Participant characteristics

The characteristics of the subjects included for the PBMC and lung tissue analyses are shown in Table 1. COPD patients included in the PBMC analyses were older than the healthy individuals and had more packyears smoked when compared to healthy current and ex-smokers.

B cell subsets in peripheral blood

As we demonstrated previously [35], the percentage of total B cells and memory B cells was lower in COPD patients compared to healthy controls and the percentages of memory B cells and class switched memory B cells were higher in current smokers compared to the ex- and never smokers (Figure E3 online repository).

In the former study[35], class switched memory B cells were identified as being CD27 positive and IgM negative, which gives no information on actual isotype switching. Therefore, we now also analyzed IgG expression on memory B cells. The percentage of IgG positive memory B cells was higher in COPD smokers compared to COPD ex-smokers (Figure 1, MWU; p=0.036). This effect of current smoking was not present in the healthy controls. When analyzed with linear regression analyses a significant positive interaction was found between current smoking and COPD (p=0.008), which means that the effect of current smoking is different in COPD patients compared to healthy controls. In contrast to IgG, there was a lower percentage of IgM memory B cells in COPD smokers compared to COPD ex-smokers (Figure 1, MWU; p=0.003) and again this effect of current smoking was not present in healthy controls. Linear regression analysis for IgM memory B cells showed a negative interaction between current smoking and COPD (p=0.038). There was a higher percentage of
IgG and IgM negative memory B cells (and thus positive for IgA or IgE) in current smokers compared to ex- and never smokers, irrespective of COPD (Figure 1, smoke effect p=0.007). Because the effect of current smoking on IgG memory B cells was different between COPD patients and healthy controls, while the percentage of class switched memory B cells was higher in both COPD smokers and healthy smokers, we anticipated that there could be difference in the effect of current smoking in COPD patients and healthy controls for the percentage of IgA or IgE memory B cells. Therefore, we assessed percentages of IgA and IgE positive cells using immunocytochemistry on the same cells as used in the flowcytometry analyses. We found a higher percentage of IgA positive B cells in current smokers compared to ex- and never smokers (Figure 2, Smoke effect; p=0.003). This effect of current smoking was mainly driven by the significant difference between healthy smokers and healthy never smokers (MWU p= 0.039), because there was no difference between COPD smokers and COPD ex-smokers (MWU p=0.1). Additionally, COPD patients had a lower percentage of IgA positive B cells than healthy controls (COPD effect; p=0.022). COPD patients also had a lower percentage of IgE positive B cells than healthy controls (Figure E4 online repository, COPD effect p=0.001), yet without an effect of current smoking on IgE positive B cells.

**Antibody production by PBMCs**

To study the response of B cells to stimulation with lung specific antigens, PBMCs were cultured for 6 days with a mixture of extracellular matrix proteins (ECM) and cytokine mix. Without stimulation, PBMCs from current smokers produced higher IgA levels than PBMCs from ex- and never smokers (smoke effect p= 0.02). This effect of current smoking was mainly caused by higher IgA levels in healthy smokers compared to healthy never smokers (Figure 3, MWU p=0.01), because there was no difference in IgA levels between COPD
smokers and COPD ex-smokers (MWU p=0.3). After stimulation with ECM + cytokine mix or cytokine mix alone, the effect of current smoking on IgA production was more pronounced than without stimulation (Figure 3, smoke effect p=0.001 (ECM), p=0.002 (cytokine mix)). PBMCs from healthy smokers produced higher levels of IgA compared to healthy ex-smokers (MWU p=0.007 (ECM), p=0.01 (cytokine mix)) and healthy never smokers (MWU p=0.01 (ECM), p=0.01 (cytokine mix)). For COPD smokers there was a trend for higher IgA levels compared to COPD ex-smokers (MWU p=0.06 (ECM), p=0.09 (cytokine mix)).

Without stimulation, PBMCs from current smokers produced lower levels of IgG than ex-and never smokers (Figure E5A online repository, Smoke effect p=0.027). This effect was mainly caused by the lower levels of IgG in healthy current smokers compared to healthy ex-and never smokers. After stimulation with ECM + cytokine mix or cytokine mix alone, there was no effect of current smoking or COPD on IgG production (Figure E5A online repository). There were trends towards lower IgM production by PMBCs from COPD patients compared to healthy controls for all culture conditions (Figure E5B online repository, COPD effect p=0.065 (no stimulation), p=0.06 (ECM), p=0.05 (cytokine mix)).

**Plasma cell differentiation of PBMCs**

Stimulation with cytokine mix resulted in more IgA plasma cells in current smokers than in ex-and never smokers (Figure E6 online repository, Smoke effect; p=0.034). This effect was not present after stimulation with ECM + cytokine mix.

After stimulation with ECM + cytokine mix or cytokine mix alone, less IgG plasma cells were present in COPD patients compared to healthy controls (Figure E6 online repository, COPD effect; p=0.038 (ECM), p=0.017 (cytokine mix)).
**Induction of antigen-specific antibody producing cells**

Stimulation with ECM + cytokine mix resulted in more anti-decorin IgG antibody producing cells in COPD patients compared to healthy controls (Figure 4, MWU p=0.03, COPD effect p=0.08). This difference was not present after stimulation with cytokine mix alone and there were no differences in anti-elastin or anti-collagen antibody producing cells (data not shown).

**B-cell subsets in lung tissue**

To investigate whether our findings regarding circulating B cells in blood are a good reflection of the situation in the lung, we investigated the presence of the same B-cell subsets in lung tissue from COPD patients and non-COPD controls.

COPD patients had higher percentages of memory B cells in lung tissue than non-COPD controls (Figure 5, MWU p=0.035). There were no differences in the percentages of total B cells, class switched memory B cells and IgG positive memory B cells between COPD patients and non-COPD controls (Figure 5). When investigating effects of current smoking on B-cell subsets in lung tissue, we found a higher percentage of IgG memory B cells in current smokers than ex- and never smokers (Figure 6, MWU p= 0.001). There was no effect of current smoking on the percentages of total B cells, (class switched) memory B cells and IgM memory B cells (Figure 6).
Discussion

This study showed that active smoking induces an ongoing adaptive immune response that is reflected by relatively high levels of (class switched) memory B cells in peripheral blood and higher percentages of IgG memory B cells in the lung. In addition, COPD smokers showed more switching to IgG in peripheral blood, while healthy smokers switched more to IgA. Furthermore, COPD patients had higher levels of memory B cells in lung tissue than non-COPD controls and after stimulation with lung specific extracellular matrix proteins, higher numbers of anti-decorin IgG antibody producing cells were present in PBMCs derived from COPD patients when compared to healthy controls.

The increase in IgA positive B cells in peripheral blood of current smokers was consistent with higher levels of IgA production by PBMCs from current smokers, and higher numbers of IgA plasma cells in response to cytokine mix stimulation in current smokers. The effect of current smoking on IgG memory B cells in peripheral blood of COPD patients was not consistent with our findings regarding IgG production and differentiation to IgG plasma cells in vitro. However, it was very similar to what we found in lung tissue, suggesting that in this respect the peripheral blood compartment is a good reflection of the lung compartment.

Although we had relatively low numbers of currently smoking COPD patients and non-COPD controls in the lung tissue analyses, we demonstrated a clear increase in IgG memory B cells in current smokers. Due to the low numbers of current smokers it was not possible to perform separate analyses for COPD patients and non-COPD controls. Therefore we could not test whether a similar difference in IgG response to smoking existed in COPD and control lung tissue, as found in peripheral blood. Nevertheless, given the higher percentage of total memory B cells in lung tissue of COPD patients, it is likely that the total number of IgG memory B cells will be highest in lung tissue from COPD smokers. The higher levels of memory B cells in lung tissue from COPD patients compared to non-COPD controls
correspond with previous immunohistochemical studies demonstrating higher numbers of B cells and B-cell follicles in COPD [4,37] and provide further support for an antigen specific immune response in the lungs of COPD patients.

Our findings regarding the higher numbers of anti-decorin IgG antibody producing cells in stimulated PBMCs from COPD patients are in line with these findings and may point out decorin as potential antigen. Although the differences in anti-decorin antibody producing cells were not very large, we suppose the effect is specific because the difference was only present after stimulation with lung specific ECM and was only present for decorin. Decorin is an important matrix protein in the lung, which is known to interact with fibrillar collagens contributing to collagen fiber network formation [38,39] and its capacity to bind transforming growth factor β neutralizing its pro fibrotic actions [40,41]. Loss of decorin in the lung may contribute to loosening of the collagen fiber network and higher levels of active transforming growth factor β. As we have shown that decorin is consistently reduced in presence and production in COPD [33,34,42] it will be of interest to evaluate whether a humoral auto-immune response may be involved. The next step is to also perform ELISPOT analysis on lung tissue to find out whether these anti-decorin IgG antibody producing cells are also prominent in the lungs of COPD patients.

The observation that COPD smokers express more IgG and healthy smokers more IgA is novel and intriguing and suggests that the smoke-induced adaptive immune response is different in COPD patients and healthy controls. Regarding our findings in healthy smokers, switching to IgA is typical for mucosal immune responses [43] and it can be envisaged that the high levels of circulating IgA positive B cells and the higher IgA production, as found in response to ex vivo stimulation in current smokers, is a direct reflection of the ongoing immune triggering of the airways by smoking. The observation that this effect is only present in the current smokers and not in ex-smokers supports this.
Regarding our findings in COPD patients, isotype switching to IgG can have several causes. It can be a reflection of immune responses against bacterial or viral infections, but more exciting is the possibility of a specific (auto) immune responses against neo-antigens. These neo-antigens may be continuously induced by (long-term) smoking in the damaged COPD lung and to a lesser extent in a healthy smoker’s lung [5,44]. Recent findings of Kirkham et al, demonstrating that carbonyl modification of proteins by exposure to chronic oxidative stress, such as cigarette smoking, can trigger an antibody mediated immune response [45], support this theory. Interestingly, as smoking was already implicated to play a role in autoimmune pathogenesis [26,31], our current results suggest that smoking may also be a risk factor of an autoimmune component in the development of COPD.

In conclusion, we showed that active smoking induces relatively high levels of (class switched) memory B cells in peripheral blood and increased percentages of IgG memory B cells in the lung. The smoke-induced adaptive immune response in blood is different in COPD patients and healthy controls, i.e. healthy smokers shift towards an IgA response, whereas when also COPD is present, this response is shifted more towards IgG. The smoke-induced IgA response is likely the result of a mucosal immune response, evoked by the constant triggering of the airways in smokers. The IgG response in COPD smokers may reflect a specific (auto) immune response against smoke-induced neo-antigens or a specific immune response against microbial pathogens. Additionally, we found a higher level of memory B cells in lungs of COPD patients, which may reflect an antigen specific immune response that could be directed against decorin.

These findings are novel and may be of clinical relevance given the profound effects of active smoking on the adaptive immune system, in particular given the different effect of smoking in COPD patients. Moreover, we provide further support for the presence of an antigen specific immune response in COPD.
Acknowledgements

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Figure legends

Figure 1. Memory B cells in peripheral blood

Percentages of IgG memory B cells, IgM memory B cells, and memory B cells negative for IgG and IgM in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols) are shown. The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05.
**Figure 2. IgA positive cells in peripheral blood**

Percentages of IgA positive cells in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols) are shown left. The results of the Mann Whitney U tests are depicted in the figure. * indicates that p< 0.05. A representative picture of cells stained positive for IgA (red staining) is shown on the right.

**Figure 3. IgA antibody levels in supernatant**

Total IgA antibody levels in supernatants from PBMCs derived from COPD patients (closed symbols) and healthy individuals (open symbols), which were cultured for 6 days without stimulation (left), with ECM + cytokine mix stimulation (middle) or cytokine mix stimulation (right) are shown. The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05.
Figure 4. Anti-decorin IgG antibody producing cells.

The numbers of anti-decorin IgG antibody producing cells in PBMCs derived from COPD patients (closed symbols) and healthy individuals (open symbols), which were cultured for 6 days with ECM + cytokine mix stimulation are shown for all the subgroups (left) and for COPD compared to healthy controls (right). The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05.

Figure 5. B cell subsets in lung tissue; COPD vs non-COPD

Percentages of total B cells, memory B cells, class switched memory B cells and IgG memory B cells in lung tissue are shown for COPD patients (closed symbols) and non-COPD controls (open symbols). The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05.
B cells in lung tissue

% CD20 of lymphocytes

COPD    non-COPD

Memory B cells in lung tissue

% CD20CD27 of lymphocytes

COPD    non-COPD

Class switched memory B cells in lung tissue

% CD27+IgM- of total B cells

COPD    non-COPD

IgG positive memory B cells in lung tissue

% IgG of CD20CD27

COPD    non-COPD
Figure 6. B cell subsets in lung tissue; *current smokers vs ex- and never smokers*

Percentages of total B cells, memory B cells, IgG memory B cells and IgM memory B cells in lung tissue are shown for current smokers (closed symbols) and ex- and never smokers (open symbols). The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05.

Table 1; Participant characteristics

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<tr>
<th>Smoking status</th>
<th>Subjects</th>
<th>Age</th>
<th>Packyears</th>
<th>FEV1 % pred.</th>
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<td>12</td>
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<td>39 (28-75) *</td>
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Never smoker 12 58 (52-74) 0 110 (94-129)

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<td></td>
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<td></td>
<td>25 (16-77)</td>
<td>103 (90-116)$</td>
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Median (range) is depicted. FEV$_1$ = Forced expiratory volume in 1 second. CS = current smoker, ES = ex-smoker, NS = never smoker, UNK = smoking status unknown. * COPD patients versus healthy controls p<0.05.

$^*$Packyears were unknown for 3 COPD patients and 4 non-COPD controls. $^{55}$ Lung function was unknown for the two donor longs.