Airway dendritic cell phenotypes in inflammatory diseases of the human lung

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Running head: Dendritic cell phenotypes in lung diseases

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ABSTRACT

Airway dendritic cells (DCs) are key regulators of pulmonary immune responses. However, there is limited information on the characteristics of airway DCs in human lung diseases.

Plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) were analysed using four-color flow cytometry in bronchoalveolar lavage fluid (BALF) of non-smoking controls, and of patients with sarcoidosis, idiopathic pulmonary fibrosis (IPF) and pneumonia (in the presence or absence of immunosuppression).

Compared to controls, immunocompetent patients with pneumonia displayed strongly enhanced pDC counts in BALF. In contrast, pDC counts in BALF of immunocompromised patients with pneumonia were even lower than in controls. This discrepancy was not explained by a different chemotactic milieu in the airways: all patients with pneumonia were characterised by strongly increased concentrations of the pDC attracting chemokine CXCL10 in BALF. Patients with IPF were characterised by normal percentages of DC subtypes, but less mature (CD83-positive) mDCs. Patients with sarcoidosis displayed a unique increase in CD1a-negative mDCs in the airways. In addition, there was an altered expression of co-stimulatory molecules (increased CD80 and decreased CD86 expression) on mDCs in patients with sarcoidosis.

Our data suggest that inflammatory diseases of the human lung are associated with a differential phenotype and recruitment of airway DCs.
INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) with a unique capacity to initiate and modulate specific immune responses [1]. In the lung, they form a highly sensitive sentinel network within and around the airways. By continuously reporting antigenic information from the airways to pulmonary lymph nodes, they are capable of upregulating or downregulating immune responses in the lung [2]. Human DCs are identified by the abundant expression of major histocompatibility complex class II (HLA-DR) and the absence of lymphocyte, natural killer cell, monocyte, and granulocyte lineage markers. They are currently classified into two developmentally and functionally distinct subsets: CD11c-positive myeloid DCs (mDCs) and CD123-positive plasmacytoid DCs (pDCs) [2]. CD1a-positive DCs have been identified as a subgroup of mDCs in human bronchoalveolar lavage fluid (BALF)[3]. These CD1a-positive DCs appear to differ from CD1a-negative mDCs in their cytokine profile and their capacity to direct T-cell differentiation [4, 5].

Based on animal models, it has been postulated that airway DCs play a key role in diseases such as asthma [6]. However, there is limited information on the role of DCs in the human lung. Currently available studies described DCs in patients with asthma [3, 7-9], bronchiolitis [10] and chronic obstructive pulmonary disease (COPD)[11]. In contrast, information on airway DCs in other inflammatory diseases of the human lung, and their relationship to healthy controls, are lacking. There are only reports describing the presence of DCs in lymph nodes of patients with sarcoidosis [12] and in lung parenchyma of patients with interstitial pneumonias [13, 14]. In patients with asthma, we have recently established a comprehensive flow cytometric method to quantify and characterise DCs in human BALF [3]. By using this method, the present study describes for the first time the phenotype of airway DCs in patients with sarcoidosis, idiopathic pulmonary fibrosis (IPF), pneumonia and healthy controls.
METHODS

Participants
The BALFs of 106 consecutive patients who were referred to our department for suspected sarcoidosis, interstitial lung disease or pneumonia were examined. Of these patients, 35 patients fulfilled our criteria for sarcoidosis, IPF or pneumonia, and were included in the study. Patients were diagnosed as having sarcoidosis if they displayed typical clinical and radiological signs (stage I or II) of sarcoidosis, no evidence in transbronchial lung biopsies or BALF for tuberculosis or other pulmonary diseases, and an enhanced percentage of lymphocytes (> 20%) in BALF with a CD4/CD8 ratio > 3. Patients were diagnosed as having IPF based on the ATS/ERS consensus criteria [15]: 1. exclusion of other causes of pulmonary fibrosis, 2. restriction and impaired gas exchange in pulmonary function tests, 3. bibasilar reticular abnormalities on high-resolution computed tomography (HRCT) scans compatible with usual interstitial pneumonitis (UIP), 4. transbronchial lung biopsy and BALF showing no features to support an alternative diagnosis. Patients were diagnosed as having pneumonia based on the following criteria: 1. onset of dyspnoea and/or cough within the last 14 days prior to bronchoscopy, 2. radiological and clinical signs suggesting pneumonia, 3. enhanced serum levels of c-reactive protein (> 5 µg/ml). Immunosuppression was defined as the presence of chemotherapy-induced neutropenia (< 0.5 x 10⁶ neutrophils per ml blood) and/or treatment with immunosuppressive agents (corticosteroids, cyclosporine or mycophenolate mofetil). As controls, twelve healthy non-smoking volunteers (inclusion criteria: 1. no history of smoking, 2. no history of respiratory diseases, 3. no regular medication) were recruited. All participants gave their written informed consent. The study was approved by the local ethics committee of Rostock (Germany).
**Bronchoalveolar lavage**

Bronchoalveolar lavage was performed using flexible bronchoscopes (Olympus, Hamburg, Germany). The bronchoscope was wedged into a subsegment of the right middle lobe (in controls, and patients with sarcoidosis or IPF) or an affected lobe (in patients with pneumonia), and a total of 100 ml prewarmed sterile saline was instilled. The BALF was recovered by gentle aspiration.

**Flow cytometry and enzyme-linked immunosorbent assay (ELISA)**

BALF samples were filtered through a two layer sterile gauze into sterile plastic vials, and centrifuged at 4°C and 500 x g for 10 min. Supernatants were aliquoted for ELISA measurements, and the cells resuspended in phosphate buffered saline. Differential cell counts in BALF were determined as described [3]. Freshly isolated BALF cells were analysed using four-color flow cytometry as described [3], using the antibody panel detailed in Table 1. The specific gating of DCs in BALF is detailed for each of the diseases and for the controls in Fig. 1. Surface molecules were quantified in histogram-plots using appropriate isotype control antibodies to discriminate between specific and non-specific antibody staining (data not shown). CCL20 and CXCL10 concentrations were measured in BALF supernatants using ELISA according to the instructions of the manufacturer (R&D Systems, Minneapolis, USA).

**Statistical analysis**

Data were analysed using SPSS (Chicago, IL, USA). Most parameters were non-normally distributed. Therefore, correlation analysis was performed using the Spearman’s correlation coefficient, and the comparison of BALF parameters between the groups was performed using the Mann Whitney U test. Probability values of p < 0.05 were regarded as significant. The percentage of CD1a-negative mDCs was calculated by subtracting the percentage of CD1a-positive from the percentage of CD11c-positive DCs.
RESULTS

**Patient characteristics**

The number, age and gender of the participants, as well as characteristics of the BALF are detailed in Table 2. There was a positive smoking history in one patient with sarcoidosis (former smoker), 4 patients with IPF (2 former and 2 current smokers), 2 patients of the pneumonia subgroup with IS (former smokers), and 2 patients of the pneumonia subgroup without IS (current smokers). None of the patients with a positive smoking history had evidence of COPD. Subgroup analysis showed that the observed differences in DC characteristics between controls and patients were not influenced by the smoking history of the patients (data not shown). Of the 10 patients with sarcoidosis, six patients (60%) had stage I disease, and 4 patients (40%) stage II. Transbronchial biopsies yielded noncaseating epitheloid cell granulomas in 7 patients (70%). No differences were observed between stage I and stage II regarding the differential cell counts or the DC characteristics in patients with sarcoidosis (data not shown). In all patients with IPF, transbronchial biopsies did not yield specific findings. In one patient, an open lung biopsy was performed which confirmed the diagnosis of UIP.

Of the 17 patients with pneumonia, 10 patients fulfilled our criteria for immunosuppression. All of the patients with immunosuppression received prednisolone directly before or during the onset of the symptoms. Two patients received immunosuppressive therapy for renal transplants (triple therapy with prednisolone, cyclosporine and mycophenolate mofetil in both cases), the other 8 patients were treated for the following haematological disorders: multiple myeloma (n = 2), acute or chronic myeloid leukemia (n = 3), acute or chronic lymphoblastic leukemia (n = 2), and B-cell non-Hodgkin lymphoma (n = 1). Four of the haematological
patients suffered from neutropenia following aggressive chemotherapy (which included prednisolone), the other four haematological patients were treated with prednisolone and cyclosporine after allogenic stem cell transplantation and did not suffer from neutropenia. In all immunocompetent patients with pneumonia (n = 7), there was circumstantial evidence for a bacterial origin of the infection: bronchial secretions were purulent, and the patients responded promptly to the antibiotic treatment. Microbiological evaluation of the BALF of this group yielded positive results in 3 out of 7 cases (including mycoplasma pneumoniae, chlamydia pneumoniae, legionella pneumophila). Patients with immunosuppression displayed bronchopneumonia or atypical infiltrates. Microbiological evaluation of the BALF of this group yielded positive results in 7 out of 10 cases (including pseudomonas aeruginosa, aspergillus fumigatus, candida albicans, pneumocystis jiroveci).

**DC subsets in BALF**

CD1a-positive DCs co-expressed CD11c in all BALFs studied (data not shown). Total pDC and mDC percentages did not differ between patients with sarcoidosis and healthy controls. However, there was a significant increase of CD1a-negative mDCs in patients with sarcoidosis, as compared to controls (Table 3, Fig. 2). DC subsets in BALF were not significantly different between patients with IPF and controls (Table 3, Fig. 2). Immunocompetent patients with pneumonia were characterised by a unique increase in BALF pDCs, as reflected by a strongly enhanced pDC:mDC ratio. In contrast, immunocompromised patients with pneumonia displayed a decrease in BALF pDCs, as compared to controls (Table 3, Fig. 2). The decrease in BALF mDCs in immunosuppressed patients was attributable to a decrease in the CD1a-positive subset (Fig. 2).

**DC surface markers**
Surface markers on BALF DCs of the controls are shown in Table 4. The markers BDCA-3 and BDCA-4 were not specific for mDC or pDCs in BALF, neither in controls (Table 4) nor in any of the patients’ groups (data not shown). CD80, CD86 and CD83 were weakly expressed on pDCs of controls (Table 4) and patients (data not shown). They could not be reliably quantified due to the low number of pDCs in most patients and controls. In patients, surface marker analysis included CD40, CD80, CD83, CD86 on pDCs and mDCs, and for BDCA-1 and BDCA-3 on mDCs. However, surface molecules could not be reliably analysed in the BALF of the immunosuppressed group, due to the very low numbers of pDCs and mDCs in most patients. Therefore, these data were omitted in the analyses. The expression of CD40 (on pDCs and mDCs), and of BDCA-3 on mDCs did not differ between the analysable groups (data not shown). Patients with sarcoidosis displayed an increased expression of CD80 on mDCs. In contrast, CD83 and CD86 were significantly decreased on mDCs (Fig. 3). IPF was characterised by a decreased expression of CD83, but a normal expression of CD80, CD86 and BDCA-1 on mDCs (Fig. 3). Pneumonia without immunosuppression was associated with a remarkably normal expression of DC surface molecules (Fig. 3).

**Chemokines in BALF**

To elucidate the mechanisms of DC recruitment, we measured concentrations of the DC attracting chemokines CCL20 and CXCL10 in BALF supernatants of the controls and of the patients (Fig. 4). Both chemokines were not detectable in BALF supernatants of controls. CCL20 was increased in patients with IPF, and in patients with pneumonia (with and without immunosuppression). There was no significant difference in CCL20 levels between patients with IPF and patients with pneumonia (Fig. 4). In contrast, the chemokine CXCL10 was specifically and strongly elevated in patients with pneumonia. Of note, this increase of CXCL10 concentrations was not significantly influenced by immunosuppression (Fig. 4). However, only in immunocompetent patients with pneumonia, but not in those with
immunosuppression, there was trend to a positive correlation between CXCL10 concentrations and pDC percentages in BALF (r = 0.63, p = 0.12).

DISCUSSION

This study is the first to describe the distribution and surface marker expression of airway DCs in patients with sarcoidosis, IPF and pneumonia, and to compare these findings with healthy controls. Using a comprehensive flow cytometric method to analyse DC subsets in human BALF [3], we identified a differential phenotype of airway DCs in these diseases. In addition, we found a differential expression of DC attracting chemokines in the airways. Thus, our study provides new insights into the characteristics and the recruitment of airway DCs in inflammatory diseases of the human lung. Information on additional surface molecules expressed by airway DCs, which are important to further characterise their function, could not be obtained in the present study due to the low DC counts in most BALF samples. Subsequent studies are, therefore, needed to reveal functional properties of these DCs.

Current knowledge on the pathophysiology of pulmonary DCs is largely derived from animal studies [2]. Although there were recent advances in the characterisation of DCs in human lung homogenates [16, 17] and in human lung parenchyma [18-20], the specific features of human airway DCs are still poorly understood. Currently available studies on airway DCs in human lung diseases were performed in patients with asthma, bronchiolitis and COPD [3, 8, 10, 11]. In the present study, we analysed the occurrence and the phenotype of airway DCs in patients with sarcoidosis, IPF and pneumonia, and compared these findings with healthy volunteers. Some of the patients (25%), but none of the controls, had a positive smoking history which might have affected the comparability between the groups. However, subgroup analyses suggested that the observed differences in DC phenotypes were not attributable to smoking.
In patients with asthma, we have recently demonstrated that all CD1a-positive DC in human BALF co-express CD11c, and concluded that CD1a-positive DCs represent a subset of human mDCs [3]. In addition, we were able to show that two blood dendritic cell antigens (BDCA-3 and BDCA-4), which are specific for mDCs (BDCA-3) or pDCs (BDCA-4) in human blood [21], are not specific for the respective subsets in human BALF [3]. In the controls and patients of the present study, the co-expression of CD11c on all CD1a-positive DCs and the non-specific expression of BDCA-3 and BDCA-4 were similar to the previous data [3]. Thus, our observations in healthy volunteers and patients with sarcoidosis, IPF and pneumonia confirm three general findings from our previous study with asthma patients: (1) CD1a-positive DCs represent a subset of airway mDCs, (2) BDCA-3 is also expressed by airway pDCs and thus not a specific marker for airway mDCs, and (3) BDCA-4 is also expressed by airway mDCs and thus not a specific marker for airway pDCs.

Accordingly, mDCs in human airways can be divided into CD1a-positive and CD1a-negative mDCs. This classification is substantiated by evidence suggesting a markedly different cytokine profile and T-cell stimulatory capacity of CD1a-positive and CD1a-negative monocyte-derived DCs [5]. A study by van Haarst and colleagues from 1996 postulated that CD1a-positive and CD1a-negative accessory cells represent functionally different entities in human BALF, although the described population of CD1a-negative cells probably contained both DCs and monocytes [4]. Therefore, functional analyses of purified CD1a-positive and CD1a-negative DCs in human BALF are still needed to really establish the roles of these DCs in human airways. Nevertheless, the study by van Haarst and colleagues showed that CD1a-negative accessory cells in human BALF are potent producers of the cytokines Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumor necrosis factor alpha (TNF-alpha), whereas CD1a-positive cells are not [4]. In patients with sarcoidosis, we observed a specific increase of CD1a-negative mDCs within the airways. Interestingly, sarcoidosis is associated with an
enhanced production of IL-1, IL-6 and TNF-alpha [22, 23]. Thus, the observed increase in CD1a-negative mDC might contribute to this cytokine profile. The hypothesis that CD1a-negative mDC are involved in the pathogenesis of sarcoidosis is supported by immunohistochemical data showing that all DCs in human epitheloid cell granulomas (both in tumor-related sarcoid reactions and in granulomas of patients with sarcoidosis) are CD1a-negative [24]. It is of note that alveolar macrophages of patients with sarcoidosis can express co-stimulatory molecules (such as CD86 and CD80) which are nearly absent on macrophages of healthy controls. Given the expression of HLA-DR on alveolar macrophages, these data suggest that alveolar macrophages can acquire antigen presenting capacities in patients with sarcoidosis [25]. Therefore, the relative contribution of DCs and macrophages to the pathogenesis of sarcoidosis remains to be elucidated [26]. Notably, we observed that airway DCs in sarcoidosis express more CD80 than CD86 whereas macrophages in sarcoidosis were previously reported to express more CD86 than CD80 [25]. These findings point to different antigen presenting properties of airway DCs and alveolar macrophages in sarcoidosis.

Evidence from animal models suggests that mDCs are potent inducers of pulmonary immune responses [27], whereas pDCs inhibit these responses [28]. The resulting concept that mDCs and pDCs are antagonistic players, with pro-inflammatory properties of mDCs and tolerogenic properties of pDCs [29], has not yet been substantiated in humans. Recent reports demonstrate that acute upper respiratory tract infections [30] and acute allergic airway inflammation [3, 31] are associated with a strong influx of pDCs into human airways. This is in keeping with our observation that immunocompetent patients with pneumonia displayed a marked increase of airway pDCs. In contrast, inflammatory disorders with a more chronic course such as sarcoidosis or IPF were characterised by normal pDC numbers in the airways. Thus, human pDCs appear to be particularly attracted to sites of acute inflammation.
There are several potential stimuli which might account for this phenomenon. On one hand, human pDCs can be recruited and activated by viral or bacterial antigens [32]. In our study, immunocompetent patients with pneumonia had evidence of a bacterial origin of the disease. In addition, a concomitant viral infection cannot be excluded in our patients. Thus, the strong increase in pDCs might be due to a release of bacterial and viral antigens in the airways. On the other hand, the strong influx of pDCs into the airways could also be due to an endobronchial release of pDC attracting chemokines. In fact, we found a strong and specific elevation of the chemokine CXCL10 in the airways of patients with pneumonia. CXCL10, a ligand of the chemokine receptor CXCR3 which is strongly expressed on human pDCs, has been described as a crucial chemotactic factor for pDCs [33, 34]. Thus, increased endobronchial CXCL10 concentrations could recruit circulating pDCs into the airways. Of note, several studies demonstrated a strong influx of pDCs into the airways in the absence of an infection [3, 31]. It is, therefore, conceivable that the secretion of chemokines such as CXCL10, rather than the release of bacterial or viral antigens, is primarily responsible for pDC recruitment into the airways.

Another noteworthy result of our study is the strong decrease of airway pDCs and CD1a-positive mDCs in immunocompromised patients with pneumonia. Of note, these patients displayed endobronchial concentrations of CCL20, a potent chemotactic factor for mDCs [11], and CXCL10, a potent chemotactic factor for pDCs [33, 34], which were similar to those observed in immunocompetent patients with pneumonia. These data suggest that immunocompromised patients with pneumonia do not lack the stimuli which attract DCs into the airways. Thus, it is more likely that immunosuppression impairs the survival of the DCs or the ability of the DCs to respond to chemotactic stimuli. Of note, there was a large heterogeneity in the underlying diseases in immunocompromised patients, but a treatment with prednisolone in 100% of the cases. Thus, the effects observed in the immunosuppressed
group appear to be related to the treatment, rather than to the underlying disease. This is in line with reports demonstrating that immunosuppressive agents such as corticosteroids impact on the number and maturation of airway DCs [8, 35]. The finding that pDCs were more affected than mDCs might be due the fact that pDCs are more susceptible to immunosuppressive therapies than mDCs [36]. Taken together, our data suggest that immunocompromised patients with pneumonia are characterised by very low pDC counts in the airways, and that this phenomenon is not due to a decrease in pneumonia-associated chemotactic stimuli. Given the crucial role of pDCs for innate and adaptive defense mechanisms in the lung [32], it might be speculated that a reduced capacity to accumulate pDCs in the airways contributes to an increased susceptibility for respiratory tract infections in immunosuppressed patients.

In conclusion, the present study provides evidence that inflammatory diseases of the human lung are associated with a differential phenotype and recruitment of airway DCs. Studies are warranted to define the role of these DCs in the pathogenesis of human lung diseases.
ACKNOWLEDGEMENT

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REFERENCES


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Table 1. Antibodies used for four-color flow cytometry
Abbreviations denote: Blood Dendritic Cell Antigen (BDCA), Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC), Peridinin chlorophyll protein (PerCP).

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<th>P</th>
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Table 2. Characteristics of the participants and of the BALF

The table displays characteristics of the participants and of the BALF of the following groups: controls (C), patients with sarcoidosis (S), idiopathic pulmonary fibrosis (IPF), pneumonia without immunosuppression (P) and pneumonia during immunosuppression (PI). Leukocyte subpopulations in BALF are shown as % of all leukocytes. Except for the number of subjects and the gender distribution (male / female), all parameters are displayed as median values (range).
Table 3. DC subsets in BALF

The table displays the concentration of BALF DC subsets in controls (C), patients with sarcoidosis (S), idiopathic pulmonary fibrosis (IPF), pneumonia without immunosuppression (P) and pneumonia during immunosuppression (PI). Plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) are displayed as % of all BALF cells and as cells per ml BALF. All parameters are displayed as median values (range). pDC:mDC ratio denotes: quotient of the cell counts per ml BALF of pDCs and mDCs. CD1a:mDC ratio denotes: quotient of the cell counts per ml BALF of CD1a-positive DCs and mDCs.
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<th>Surface Molecule</th>
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<td>BDCA-2</td>
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<td>BDCA-3</td>
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Table 4. Surface molecules on DCs in BALF from healthy controls

The table displays the percentages (median values and ranges) of marker-positive mDCs and pDCs in BALF from the control group. The results of ten subjects are presented (the presented surface molecules were not analysed in 2 subjects of the control group). Numbers of pDCs were too low to reliably quantify the expression of the weakly expressed molecules CD80, CD83, and CD86 (n.a. denotes not analysable).
FIGURE LEGENDS

Figure 1. Gating of DC subsets in BALF

Total BALF cells were identified in a FSC/SSC-plot (first column) and lin$^{neg/dim}$ cells were gated (second column). Among lin$^{neg/dim}$ cells, pDCs were identified by CD123 and HLA-DR co-expression (third column), mDCs by CD11c and HLA-DR co-expression (fourth column) and CD1a-positive DCs by CD1a and HLA-DR co-expression (fifth column). Shown are: controls (C), patients with sarcoidosis (S), idiopathic pulmonary fibrosis (IPF), pneumonia without immunosuppression (P) and pneumonia during immunosuppression (PI).
Figure 2. DC subsets in BALF

Shown are the percentages (*left column*) and cell counts per ml (*right column*) in BALF, of pDCs (*first row*), total mDCs (*second row*), CD1a-positive mDCs (*third row*) and CD1a-negative mDCs (*fourth row*) of the following groups: controls (C), patients with sarcoidosis (S), idiopathic pulmonary fibrosis (IPF), pneumonia without immunosuppression (P) and pneumonia during immunosuppression (PI). Boxplot graphs (*white*: controls, *grey*: patients) display the median (line within the box), interquartile range (edges of the box) and the range of all values less distant than 1.5 interquartile ranges from the upper or lower quartile (vertical lines). Significant differences to the control group (p < 0.05) are marked with an asterisk.
Lommatzsch et al., Fig. 2
Figure 3. Surface molecules on mDCs in BALF

This figure shows the expression of the markers CD80, CD83, CD86 and BDCA-1 on BALF mDCs (% marker-positive mDCs among all mDCs) of controls (C), patients with sarcoidosis (S), idiopathic pulmonary fibrosis (IPF), and pneumonia without immunosupression (P). Boxplot details are described in Fig. 2. Significant differences to the control group (p < 0.05) are marked with an asterisk.
Lommatzsch et al., Fig. 3
Figure 4. Chemokine concentrations in BALF

Shown are the concentrations of CCL20 and CXCL10 in BALF of controls (C), patients with sarcoidosis (S), idiopathic pulmonary fibrosis (IPF), pneumonia without immunosupression (P) and pneumonia during immunosuppression (PI). Boxplot details are described in Fig. 2. Significant differences to the control group (p < 0.05) are marked with an asterisk. The abbreviation n.s. denotes not significant.
Lommatzsch et al., Fig. 4