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Bronchial inflammation biomarker patterns link humoral immunodeficiency with bronchiectasis-related small airway dysfunction

Ulrich M. Zissler[1] | Aljoscha Thron[2] | Jonas Eckrich[3] | Shahrzad Bakhtiar[4] | Ralf Schubert[2] | Stefan Zielen[2]

[1]Center of Allergy & Environment (ZAUM), Technical University of Munich and Helmholtz Center Munich, German Research Center for Environmental Health, Germany, Member of the German Center for Lung Research (DZL), Munich, Germany

[2]Division of Allergology, Pulmonology and Cystic Fibrosis, Department for Children and Adolescents, Goethe University, Frankfurt, Germany

[3]Department of Otorhinolaryngology, University Medical Center Bonn (UKB), Bonn, Germany

[4]Division for Stem Cell Transplantation, Immunology and Intensive Care Unit, Department for Children and Adolescents, Goethe University, Frankfurt, Germany

Correspondence
Ulrich M. Zissler, Center of Allergy & Environment (ZAUM), Technical University and Helmholtz Center Munich, Biedersteiner Str. 29, 80802 Munich, Germany.
Email: ulrich.zissler@tum.de

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Abstract

Background: The progression of chronic destructive lung disease in patients with humoral immunodeficiency (ID) and concomitant development of bronchiectasis is difficult to prevent. Lung function tests in these patients typically show bronchial obstruction of the small airways in combination with increased air trapping in the distal airways, which is consistent with small airway dysfunction.

Objective: The objective was to assess the grade of chronic lower airway inflammation and small airway dysfunction from induced sputum and the corresponding local pro-inflammatory mediator pattern to discriminate patients affected by bronchiectasis-related Small Airway Dysfunction (SAD).

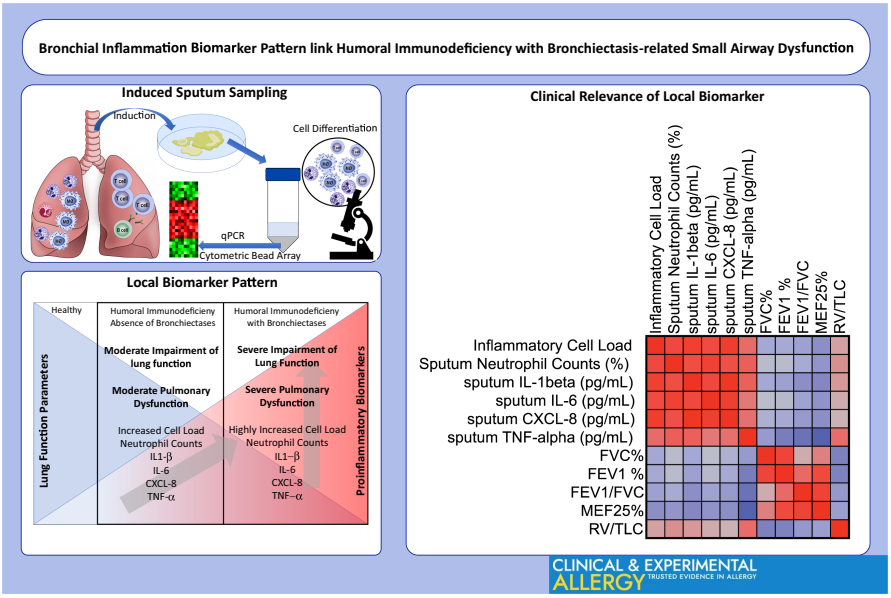
Methods: In a prospective design, 22 patients with ID (14 CVID, 3 XLA, 3 hyper-IgM syndrome, 1 hyper-IgE syndrome and low IgG levels due to treatment with rituximab and 1 SCID after BMT and persistent humoral defect) and 21 healthy controls were examined. Lung function, Fraction Expiratory Nitric Oxide (FeNO) and pro-inflammatory cytokine levels were compared in subsets of patients with (ID + BE) and without bronchiectasis (ID) pre-stratified using high-resolution computed tomography (HRCT) scans and control subjects.

Results: Analysis of induced sputum showed significantly increased total cell counts and severe neutrophilic inflammation in ID. The concomitant SAD revealed higher total cell numbers compared to ID. Bronchial inflammation in ID is clearly mirrored by pro-inflammatory mediators IL-1β, IL-6 and CXCL-8, whilst TNF-α revealed a correlation with lung function parameters altered in the context of bronchiectasis-related Small Airway Dysfunction.

Conclusions: In spite of immunoglobulin substitution, bronchial inflammation was dominated by neutrophils and was highly increased in patients with ID + BE. Notably, the pro-inflammatory cytokines in patients with ID were significantly increased in induced sputum. The context-dependent cytokine pattern in relation to the presence

of concomitant bronchiectasis associated with SAD in ID patients could be helpful in delimiting ID patient subgroups and individualizing therapeutic approaches.

KEYWORDS
antibody deficiency syndrome, biomarker, bronchiectasis, CVID, induced sputum, primary immodeficiency



GRAPHICAL ABSTRACT

The minimally invasive technique of induced sputum can reveal the intrapulmonal cellular and humoral inflammatory pattern in patients with humoral immunodeficiency associated with lung function parameters. This is mirrored by gene regulation and secretion of proinflammatory secreted mediators IL-1β, IL-6, CXCL-8, and TNF-α, which were induced in lower airway cells along with local neutrophil counts. In addition, bronchiectasis-related airway dysfunction was associated with higher levels of proinflammatory cell load and increased levels of proinflammatory secreted mediators IL-1β, IL-6, CXCL-8, and TNF-α.

1 | INTRODUCTION

Patients with severe humoral immunodeficiency (ID) and related disorders of antibody production suffer from recurrent bacterial infections of the respiratory tract such as sinusitis, otitis media, bronchitis or pneumonia.[1] According to the current state of knowledge, more than half of CVID patients suffer from obstructive pulmonary disease in the sense of chronic bronchitis and bullous lesions, of which 40% develop a restrictive ventilation disorder.[2] However, the obstructive component of lung disease in ID shows a clear longitudinal progression as in heavy smokers with a higher annual decline in FEV1.[3] In general, pulmonary dysfunction is associated with a sharp increase in morbidity up to lung transplantation.[4] In addition, a simultaneous increased incidence of lymphoid interstitial pneumonia (LIP), granulomatous diseases and pulmonary lymphomas has been described.[5] Granulomatous diseases with associated interstitial lung manifestations affect approximately 10% of all CVID patients and cause an increasing deterioration in lung function parameters.[6] Unfortunately, the diagnosis of an immune disease is made too late in many patients, so that chronic lung damage with bronchiectasis has already occurred. The therapy of choice is the regular parenteral or subcutaneous substitution of immunoglobulins (IG) to reduce the susceptibility to infection and the quality of life of the patient.[7–9] Despite treatment with IG and clearly improved quality of life, almost 100% of ID patients suffer from chronic mucosal infections

Key messages

- Induced sputum can reveal intrapulmonal cellular and humoral inflammatory patterns in patients with humoral immunodeficiency associated with lung function parameters.
- Pro-inflammatory mediators were induced in lower airways along with neutrophil counts.
- Bronchiectasis-related airway dysfunction was associated with higher levels of inflammatory cell load and increased levels of pro-inflammatory secreted mediators IL-1β, IL-6, CXCL-8 and TNF-α.

(chronic rhinosinusitis and bronchitis), even with early diagnosis and start of therapy.[10]

Typically, pulmonary disease manifests itself in the small airways in ID patients. The small airways in "Bronchiectasis-related Small Airway Dysfunction" (SAD) are defined as bronchioles with a diameter of <2 mm and play a decisive role in the pathogenesis of ID as well as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). Histologically, neutrophilic inflammation with increasing obliteration and fibrosis of the small airways characterizes SAD.[11] The assessment of the lung function shows a characteristic flow limitation with hyperinflation, especially in the small airways, which is generally not reversible.[12]

The triggers for the structural remodelling processes in the lungs of ID patients are recurrent infections and the chronic colonization of the airways with pathogens, especially in the small airways. The typical spectrum of pathogens in ID patients with bronchiectasis includes *Pneumococci*,[13] *Haemophilus*,[13] *Staphylococcus*[14] and, after years of illness, also *Pseudomonas aeruginosa*.[15]

Recurrent sinopulmonary infections result in chronic obstructive and restrictive ventilation disorders or a corresponding mixed picture that can be evaluated by measuring lung function.[16] Little is known about the role of the small airways in lung function in ID. Only a few previously published data included the maximum expiratory flow rate (MEF) in the assessment to diagnose existing or incipient obliteration in SAD.[17] It has been shown that MEF is only of limited informative value in advanced SAD with restricted FEV1, but in some cases, it can be used to assess early forms with normal FEV1.[11] In addition to bronchial obstruction, the obliteration of the small airways leads to increased hyperinflation ("air trapping"). In body plethysmography, this can be expressed in increased values for total lung capacity (TLC), residual volume (RV) and functional residual capacity (FRC). This also applies to relative pulmonary hyperinflation (RV/TLC), in which RV increases at the expense of TLC. Normally, RV in healthy controls is largely determined by the static properties of the chest wall. Apart from that, dynamic factors can also influence the RV. As the diameter of the bronchioles decreases, bronchial obstruction first occurs with subsequent hyperinflation.[18] High-resolution computed tomography (HRCT) is used as gold standard for detecting the smallest structural changes in the peripheral.[19] However, since the risk of radiation-induced damage and tumours is increased in patients with CVID due to increased chromosomal radiosensitivity, particularly, strict indication and monitoring biomarkers are required.[20]

The development of immune dysregulation in CVID is accompanied by known risk factors such as low frequencies of naïve CD4 T cells and IgA deficiency.[21–23] Whilst these factors pose a long-term risk for the development of clinical complications, they do not provide any information about the current state of inflammation or the involvement of bronchiectasis in CVID. Therefore, they are less suitable for monitoring short-term disease progression or therapeutic response. For these purposes, the use of local, non-invasive biomarkers for other pro-inflammatory diseases is finding their way into clinical practice.[24]

To identify specific bronchial biomarkers that might reflect an immune imbalance in CVID, we used induced sputum samples from a CVID cohort in this study. Our strategy was to evaluate the potential of local inflammatory biomarkers in stratifying patients with immune imbalance from one in order to identify discriminatory biomarkers and signalling pathways that underlie the immune imbalance in CVID.

2 | METHODS

2.1 | Study population

In the present cross-sectional cohort study, we examined 22 patients (14 CVID, 3 XLA, 3 hyper-IgM syndrome, 1 hyper-IgE syndrome and low IgG levels due to treatment with rituximab and 1 SCID after BMT and persistent humoral defect) with primary humoral immunodeficiencies and intravenous immunoglobulin (IVIG) therapy as well as 21 healthy control subjects. Patients were potentially influenced by recent infectious state. Patients with immunodeficiency (ID) were stratified according to affection by bronchiectasis (BE) based on HRCTs of the chest, which, with one exception, were not older than 2 years. The diagnosis regarding the prevalence of bronchiectasis categorized in yes and no considering the latest findings was reported by local department of radiology. The patients were further classified as ID ($n = 9$) or ID + BE ($n = 13$). Details of patient characteristics are provided in Table 1. The study was registered at www.clinicaltrials.gov (NCT01359384). Each participant gave a written, informed declaration of consent. The study was approved by the ethics committee of the Medical School of the Goethe University Frankfurt/Main (84/11).

2.2 | Lung function

Lung functions and spirometry were performed on all subjects using devices from VIASYS Healthcare GmbH (Höchberg, Germany). Forced vital capacity (FVC), one-second capacity (FEV1), Tiffeneau index (FEV1/VC), maximum expiratory flow rate (MEF25), residual volume (RV) and functional residual capacity (RV/TLC) were measured. As part of the lung function examination, a reversibility test with 400 μg salbutamol was also performed in all patients.

2.3 | Exhaled NO

The measurement of nitrogen monoxide (NO) in the exhaled air was determined using the NIOX® Nitric Oxide Monitoring System (Aerocrine, Solna, Sweden) based on chemiluminescence analysis. Patients inhaled filtered NO-free air until full lung capacity was reached. The patients then exhaled continuously at a constant flow rate of 0.05 L/s for 10 s in total. The fractionated exhaled Nitric Oxide (eNO) value was calculated by determining a plateau value using the ATS (American Thoracic Society) criteria,[25] which was measured on the day of the study visit.

TABLE 1 Patients' characteristics and sputum cell distribution

	Healthy controls ($n = 21$)	ID patients in total ($n = 22$)	ID w/o BE ($n = 9$)	ID with BE ($n = 13$)
Age (years)	16.1 (7.8–27.5)	23.8 (10.0–61.0)	27.0 (12.3–61.0)	21.8 (10.0–54.4)
Sex (m/f)	11/10	12/10	5/4	7/6
Duration of substitution (years)	n.d.	14.5 (1.0–23.0)	13.0 (2.0–17.0)	15.0 (1.0–23.0)
Trough IgG level (mg/dL)	n.d.	831 (433–1122)	834 (433–1122)	828 (440–945)
FVC (%)	101.2 (83.4–113.6)	99.7 (49.3–126.1)	110.0 (85.5–126.1)	93.1 (49.3–122.9)
FEV1 (%)	104.5 (83.8–114.1)	94.9 (38.2–129.7)	116.0 (76.3–129.7)	85.8 (38.2–116.3)
FEV1/FVC ratio	86.5 (75.9–96.2)	81.5 (57.0–99.7)	85.4 (72.1–93.7)	76.4 (57.0–102.6)
MEF25 (%)	91.6 (49.1–175.1)	61.4 (16.8–127.4)	88.8 (34.2–127.4)	47.8 (16.8–102.6)
RV (%)	91.4 (27.1–221.3)	116.3 (67.8–199.4)	102.2 (67.8–123.9)	116.3 (70.1–199.4)
RV/TLC (%)	84.9 (13.8–131.3)	105.5 (57.5–194.3)	97.5 (57.5–144.1)	110.5 (70.6–194.3)
eNO (ppb)	17.9 (6.9–61.0)	12.9 (5.7–30.5)	14.3 (5.7–30.5)	11.5 (6.0–26.9)
Inflammatory cell load ($\times 10^4$ cell/mL sputum)	22.5 (6.5–153.0)	114.0 (21.0–1125.0)	45.5 (21.0–162.0)	252.5 (25.0–1125.0)
Alveolar macrophages (%)	97.0 (62.8–100.0)	38.0 (9.0–87.0)	60.0 (17.0–87.0)	28.0 (6.0–69.0)
Neutrophils (%)	1.8 (0.0–32.0)	60.5 (11.0–90.0)	38.0 (11.0–82.0)	68.0 (29.0–90.0)
Lymphocytes (%)	1.9 (0.0–9.0)	1.5 (0.0–12.0)	1.0 (1.0–3.0)	2.0 (0.0–12.0)

Note: All values are expressed as median (range) if not otherwise indicated.

2.4 | Serum analysis

Blood was drawn from the patients and controls, and blood cell counts, CRP and immunoglobulin levels of IgG, IgA and IgM were determined in the serum. Immediately prior to regular intravenous immunoglobulin therapy, blood was drawn to determine the global minimum IgG level.

2.5 | Sputum induction and processing

The participants first inhaled 400-µg salbutamol and consecutively nebulized hypertonic saline in increasing concentrations of 3%, 4% and 5% NaCl every 7 min according to previous publications.[26,27] During this procedure, participants cleaned their noses and rinsed their mouths to reduce squamous epithelial cells in the samples. The sputum was processed within 1 h of collection. The selected sputum plugs, which contained as little saliva as possible, were placed in a weighed Eppendorf tube and processed with 4× weight/volume of 0.1% DTT working solution (Merck, Darmstadt, Germany). Then, 2× weight/volume of phosphate-buffered saline (PBS) was added. Samples were filtered through a 70-µm mesh and centrifuged for 10 min at 790 × *g* without brake to remove the cells. The resulting cell-free supernatants were stored at −80°C for additional analysis using a protein assay.

2.5.1 | Identification of cell populations by differential cell count

The cell suspension was centrifuged in a Labofuge 400 cytospin column (Heraeus, Germany) at 44 × *g* for 6 min. The slides produced were air-dried, fixed with methanol for 5 min and stained using the Pappenheim method.[28,29] Specimens with a content of less than 15% of squamous epithelial cells were considered adequate. Three investigators blinded to the subject classification read at least 400 non-squamous cells on each sputum slide. Neutrophils, lymphocytes, eosinophils, basophils and macrophages were calculated as proportions of the total cell counts (for details see Table 1). The degree of bronchial inflammation was mirrored by the sputum inflammatory cell load, defined as cells per mL of sputum.[29]

2.6 | Microbiological examinations

Immediately after obtaining induced sputum specimen, a microbiological smear was taken from each sample and sent to the Institute for Medical Microbiology and Hospital Hygiene at the University of Frankfurt for examination for pathogens.

2.6.1 | RNA isolation and RNA quality assessment

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion (Qiagen) in order to avoid DNA contaminations. RNA quantification was performed by ultraviolet–visible spectrophotometry (Nanodrop Technologies, Wilmington, DE); the RNA quality index (RQI) was measured using the Experion Bioanalyzer (Bio-Rad, Hercules, CA, USA). Samples with a RQI value above 7.5 were used for qRT-PCR analysis.

2.7 | Real-time quantitative PCR

After quality assessment, isolated total RNA was subjected to reverse transcription using an iScript Reverse Transcriptase Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's

instructions. Real-time PCR profiles were visualized using the commercially available QuantiTect SYBR-Green I Master Mix (Qiagen, Hilden, Germany) and Qiagen Human Primer Assays (Qiagen, Hilden, Germany) specific for *GAPDH*, *IL1β*, *IL6*, *IL8*, *TNFα*, *IFNγ* and *IL10*, quantified by the Eppendorf realplex S PCR System (Eppendorf AG, Hamburg, Germany). The amount of *IL1β*, *IL6*, *IL8*, *TNFα*, *IFNγ* and *IL10* mRNA expression were normalized with endogenous control *GAPDH* and the relative quantification and calculation of range of confidence was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (relative gene expression).[30]

2.8 | Cytometric bead array

The CBA® technology (BD, Heidelberg, Germany) was performed for the cytokines IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α in induced sputum samples as described before.[11,31] The lower limits of detection (LLODs) for the individual cytokines were: IL-1β: 2.30 pg/mL; IL-6: 1.60 pg/mL; IL-8: 1.20 pg/mL; IL-10: 11.20 pg/mL; IFN-γ: 1.80 pg/mL and TNF-α: 0.07 pg/mL.

2.9 | Statistical approaches

All experimental procedures and analyses in this exploratory study were conducted by blinded research staff. Two groups were compared using a two-tailed Mann–Whitney test. Since the data points were not normally distributed, non-parametric statistical test was chosen. Data are shown in parenthesis as median (range) throughout the result section, unless otherwise indicated. Statistically significant differences are depicted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. Correlations between two parameters were determined using two-tailed Spearman rank correlation. The statistics program GraphPad Prism 8.0 was used for calculations.

3 | RESULTS

To understand the pathological consequences and local mechanisms of airway inflammation in immunodeficiency, we analysed sputum cells from patients with a diagnosis of immunodeficiency (ID), partly with bronchiectasis (ID + BE; Tables 1 and 2) and from healthy controls, and discovered a previously unknown pro-inflammatory fingerprint in sputum cells. In patients with ID, no difference was observed for the lung function parameters FEV1, FVC, FEV1/FVC and MEF25, whilst a slightly but not significant increase can be stated for RV and RV/TCL (Figure 1A–F). In contrast, ID patients with bronchiectasis (ID + BE) showed significant differences to ID without BE for FEV1, FVC, FEV1/FVC and MEF25 (Figure 1A–D). In addition, ID with BE also showed a significant decrease in FEV1, FEV1/FVC and MEF compared to healthy control subjects (Figure 1A, C and D). Interestingly, a significant increase could also be determined for RV/TLC, which reveals air trapping (Figure 1F). Interestingly, ID patients with BE (11.5 ppb, 5.7–30.5) showed decreased FeNO levels compared to ID patients without BE but only significantly compared to healthy controls (17.0 ppb, 6.0–61.0; $p < 0.05$; Figure 1G).

A significantly higher sputum cell load was generally observed in ID patients (114.0×10^4, 21–1125) compared to healthy controls (22.5×10^4, 6.5–153; $p < 0.001$; Figure 1H). When ID patients were subdivided into groups in regards to BE, there was also a significant difference between the two patient groups with a higher inflammatory cell load in BE-affected ID patients (252.5×10^4, 25–1125) compared to ID patients without BE (45.5×10^4, 21–162; $p < 0.01$) as well as healthy controls ($p < 0.001$).

In addition, the cytological differentiation of the inflammatory cell distribution analysis revealed an increased number of neutrophil granulocytes in the lower airways of ID patients in general, however when segregated into subjects with and without with (68.0%, 29.0–90.0; $p < 0.001$) or without (38.0%, 11.0–82.0; $p < 0.001$) BE affection showed increased neutrophil numbers compared to healthy controls (1.8%, 0.0–31.0; Figure 1I).

Furthermore, pathogens such as *Haemophilus influenzae* and *parainfluenza* ($n = 4$), *Staphylococcus aureus* ($n = 1$), *Moraxella catarrhalis* ($n = 1$) and *Serratia marcescens* ($n = 1$) were detected in ID + BE patients in 7 of 13 cases (53%) compared to patients without BE in 1 out of 9 cases (11%; *Staphylococcus aureus*). The serum CRP levels of ID patients were below the inflammatory threshold of 0.26 mg/dL.

3.1 | Local inflammation in ID

To assess local inflammation in patients with ID, induced sputum was generated successfully from all patients and healthy controls. The sputum cells were then examined for pro-inflammatory biomarkers on the gene expression level and on the secreted protein level. The analysis of the pro-inflammatory cytokines IL-1beta, IL-6, CXCL-8 and TNF-alpha revealed significant differences both at the transcriptome and at the protein level (Figure 2). In general, a significant 8.40-fold (0.23–133.80) upregulation of IL-1beta gene expression levels was observed in ID patients compared to healthy controls (1.00, 0.31–5.35; $p < 0.0001$; Figure 2A), which was confirmed at the protein level in sputum supernatants (ID: 1150 pg/mL, 162.0–8466.0; controls: 147.0 pg/mL, 56.0–3511.0; $p < 0.0001$; Figure 2G). In addition, IL-6 showed a 1.43-fold upregulation (0.09–11.79) in ID patients compared to healthy subjects (1.00, 0.05–2.26; $p < 0.05$; Figure 2B), which was also reflected in the secreted protein levels in the sputum supernatants (ID: 274.5 pg/mL, 0.0–3410.0; Controls: 0.00 pg/mL, 0.0–604.0; $p < 0.0001$; Figure 2H). The gene expression of the pro-inflammatory mediator CXCL-8 also showed significant differences between all ID patients (8.48-fold, 0.44–108.70) and healthy controls (1.00, 0.11–7.73; $p < 0.0001$; Figure 2C), which were also strongly regulated at the protein level in sputum supernatants of ID patients (13794.0 pg/mL, 1691.0–47967.0) and healthy control subjects (1743.0, 529.0–49129.0; $p < 0.0001$; Figure 2I).

TABLE 2 Sputum biomarker on protein level and gene expression level

Analysed biomarker	Healthy controls ($n = 21$)	CVID patients ($n = 22$)	*P*-value
Interleukin1beta gene expression (fold change)	1.00 (0.31–5.66)	8.40 (0.23–144.8)	<.0001
Secreted Interleukin-1beta (pg/mL)	147.5 (56.0–3511.0)	1150.0 (162.0–84655.0)	<.0001
Interleukin6 gene expression (fold change)	1.00 (0.05–2.31)	1.43 (0.09–11.88)	.0123
Secreted Interleukin-6 (pg/mL)	0.0	274.5 (0.00–3410.0)	<.0001
CXCL8 gene expression (fold change)	1.00 (0.11–7.84)	8.48 (0.44–109.1)	<.0001
Secreted CXCL-8 (pg/mL)	1743.0 (529.0–49658.0)	13749.0 (1691.0–49658.0)	<.0001
Interleukin10 gene expression (fold change)	1.00 (0.25–53.45)	2.02 (0.19–20.68)	.0504
Secreted Interleukin-10 (pg/mL)	33.5 (0.0–54.0)	45.0 (0.0–115.0)	.0240
TNF-alpha gene expression (fold change)	1.00 (0.02–6.06)	2.07 (0.41–13.64)	.0031
Secreted TNF-alpha (pg/mL)	0.0	45.0 (0.0–2497.0)	<.0001
Interferon-gamma gene expression (fold change)	1.00 (0.04–31.34)	2.69 (0.76–75.06)	.0275
Secreted Interferon-gamma (pg/mL)	116.0 (0.0–174.0)	158.0 (0.0–645.0)	.0179

In addition, for TNF-alpha, differential expression on gene expression levels was detected in ID patients (2.07-fold, 0.31–13.23) compared to healthy controls (1.00, 0.02–6.04; $p = 0.0031$; Figure 2D). These differences were confirmed in the sputum secretome in ID patients (45.0 pg/mL, 0.0–2497.0) compared to the control group (0.0 pg/mL, 0.0; $p < 0.0001$; Figure 2J).

In addition, IFN-gamma gene expression and protein levels were determined in ID patients and healthy controls. In ID patients, IFN-gamma was upregulated on average 2.69-fold (2.69-fold, 0.76–74.30) compared to healthy subjects (1.00, 0.04–31.30; $p = 0.0275$; Figure 2E). At the protein level, a significant increase was also measured (ID: 158.0 pg/mL, 0.0–645.0) and controls (116.0 pg/mL, 0.0–174.0; $p = 0.0178$; Figure 2K).

Finally, the sputum expression of the anti-inflammatory mediator IL-10 was measured. For the IL-10 gene expression, a 2.02-fold (0.19–20.49) increased gene expression was found in ID patients compared to healthy controls (1.00, 0.25–53.20; 45.0 pg/mL, 0.0–115.0), which could be confirmed at the protein level (ID: 45.0 pg/mL, 0.0–115.0; controls: 33.5 pg/mL, 0.0–54.0; $p = 0.0240$; Figure 2L).

3.2 | Role of bronchiectasis in ID

In order to assess the local inflammation in patients with ID with BE, the patients were further divided into ID patients with (ID + BE) and without bronchiectasis (ID). When separated according to BE, it became apparent that (sputum) neutrophil counts were substantially increased in patients (68.0%, 29.0–90.0; $p < 0.001$) compared to ID patients (38.0%, 11.0–82.0; $p < 0.001$; Figure 1I). Further, the analysis for the pro-inflammatory cytokines IL-1beta, IL-6, CXCL-8 and TNF-alpha revealed significant differences both at the gene expression level and at the protein level (Figure 3).

In subgroups of ID patients, a significant 10.8-fold (0.23–133.80) up-regulation of IL-1beta gene expression levels was observed (Figure 3A) in ID + BE compared to ID (3.10-fold, 1.10–139.10; $p < 0.01$), which was consistently detectable at the protein level in sputum supernatants (ID + BE: 3034.0 pg/mL, 237.0–84654.0; ID: 338.7 pg/mL, 162.1–1703.0; $p < 0.01$). When comparing ID + BE with healthy controls, a significant difference was visible not only in gene expression of sputum cells, but also in secreted sputum protein levels of IL-1beta (147.5 pg/mL, 56.0–3511.0; $p < 0.001$; Figure 3F). This difference, although not in this amplitude at the excreted protein level, became visible in BE-unaffected patients compared to healthy controls ($p < 0.05$).

In addition, IL-6 was upregulated 2.20-fold in ID patients (0.1–187.40) compared to healthy subjects (1.00, 0.05–2.26; $p < 0.05$), which was also reflected in the secreted protein levels in the sputum supernatants (ID: 274.5 pg/mL, 0.0–3410.0; controls: 0.00 pg/mL, 0.0–604.0; $p < 0.0001$; Figure 3B&G). In contrast, in patients without BE, a significant increase in IL-6 was only seen at the excreted sputum protein level (330.4 pg/mL, 0.0–3410.0; $p < 0.001$) compared to healthy subjects. A difference became visible between the two patient subgroups, but it did not reach statistical significance.

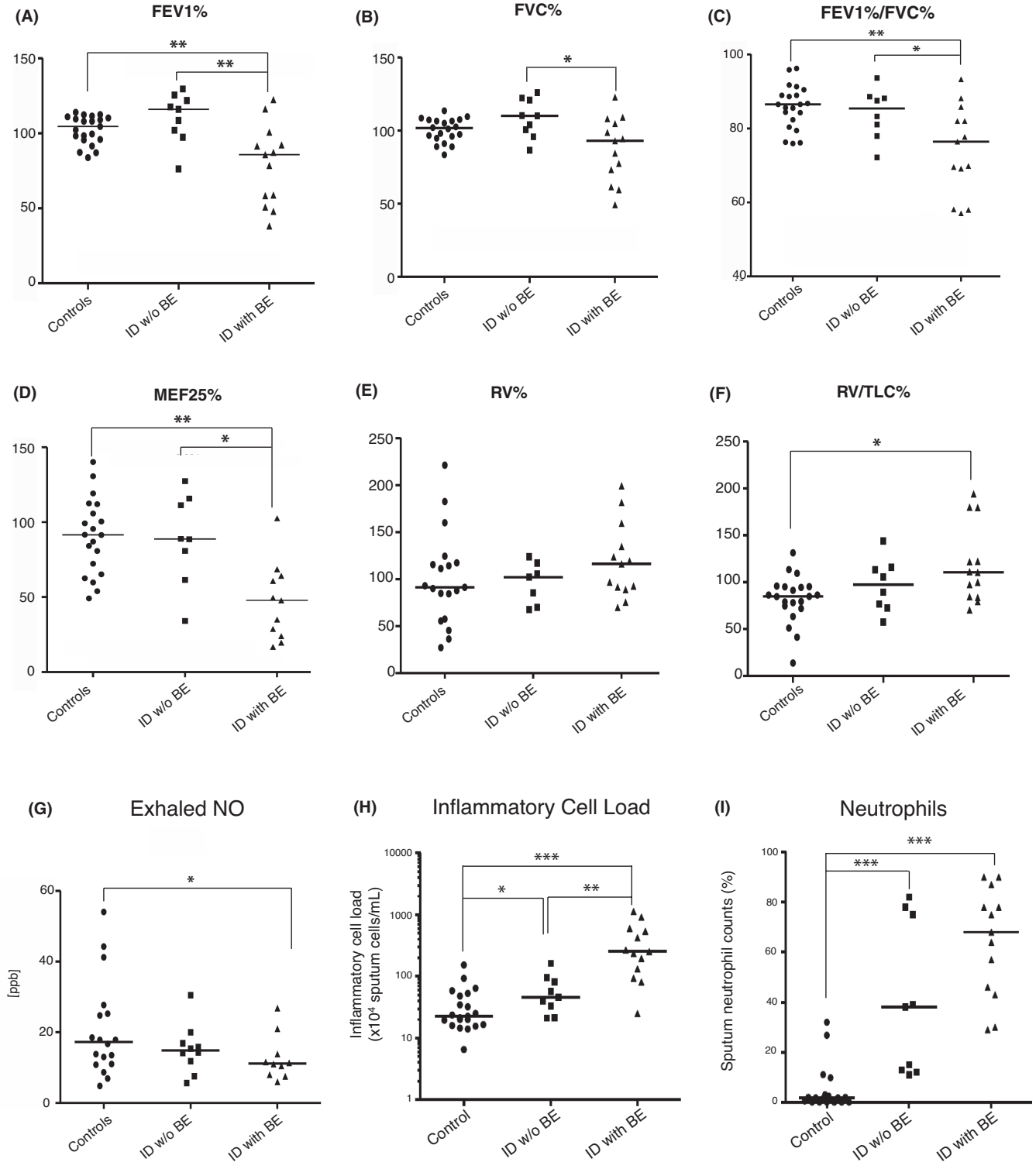
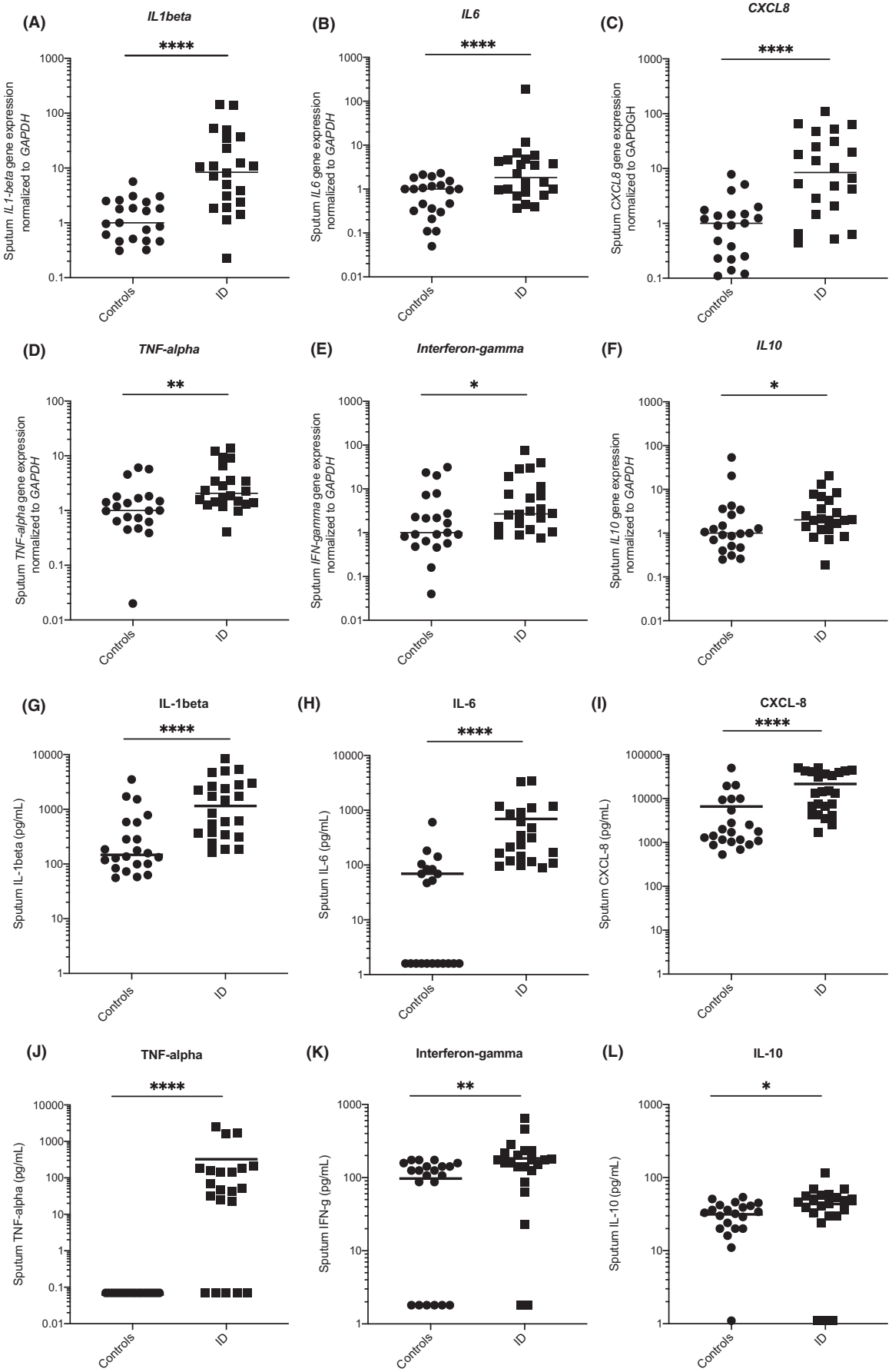


FIGURE 1 Lung function parameters, inflammatory sputum cell load and sputum neutrophil counts of controls and ID patients without or with bronchiectasis affection. Data are shown as medians (range). The non-parametric Mann–Whitney test was used to compare controls with patients with ID patients and ID patients affected from bronchiectasis (ID + BE)

FIGURE 2 Expression and protein levels of excreted mediators. Gene expression levels and protein levels for IL-1beta, IL-6, CXCL-8, TNF-alpha and *IFNγ* were detected in cells and supernatants of induced sputum samples of ID patients and healthy control subjects. The amounts of *IL1β*, *IL6*, *IL8*, *TNFα* and *IFN-γ*mRNA expression were normalized with endogenous control *GAPDH* and the relative quantification and calculation of range of confidence was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (relative gene expression)


(A)
IL1beta
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Sputum IL-1-beta gene expression normalized to GAPDH
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Controls
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IL6
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Sputum IL6 gene expression normalized to GAPDH
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CXCL8
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Sputum CXCL8 gene expression normalized to GAPDGH
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(D)
TNF-alpha
**
Sputum TNF-alpha gene expression normalized to GAPDH
100
10
1
0.1
0.01
Controls
ID
(E)
Interferon-gamma
*
Sputum IFN-gamma gene expression normalized to GAPDH
1000
100
10
1
0.1
0.01
Controls
ID
(F)
IL10
*
Sputum IL10 gene expression normalized to GAPDH
1000
100
10
1
0.1
0.01
Controls
ID
(G)
IL-1beta
****
Sputum IL-1beta (pg/mL)
10000
1000
100
10
1
Controls
ID
(H)
IL-6
****
Sputum IL-6 (pg/mL)
10000
1000
100
10
1
Controls
ID
(I)
CXCL-8
****
Sputum CXCL-8 (pg/mL)
100000
10000
1000
100
10
1
Controls
ID
(J)
TNF-alpha
****
Sputum TNF-alpha (pg/mL)
10000
1000
100
10
1
0.1
0.01
Controls
ID
(K)
Interferon-gamma
**
Sputum IFN-g (pg/mL)
1000
100
10
1
Controls
ID
(L)
IL-10
*
Sputum IL-10 (pg/mL)
1000
100
10
1
Controls
ID

In the subgrouping of the patient entities, the gene expression of the pro-inflammatory mediator CXCL-8 also showed significant differences between ID patients with BE affection (18.10-fold, 0.40–65.80; $p < 0.001$) and patients without BE (3.1-fold, 0.50–109.10; $p < 0.05$) compared to healthy controls (1.00, 0.11–7.73; Figure 3C). These differences were also reflected in the levels of excreted CXCL-8, which revealed a strong increase at the protein level in sputum supernatants of BE-affected (39236.0 pg/mL, 6781.0–49657.0; $p < 0.001$) and BE-unaffected ID patients (4254.4 pg/mL, 1691.0–14678.0; $p < 0.05$) compared to healthy control subjects (1743.0, 529.0–49129.0; $p < 0.0001$; Figure 3H).

TNF-alpha also showed differential gene expression in subgrouped ID patients. BE-affected (2.70-fold, 0.40–9.50; $p < 0.05$) and BE-unaffected (1.60-fold, 1.00–13.60; $p < 0.05$) ID patients showed an increase in the inflammatory response compared to healthy controls (1.00, 0.02–6.10; Figure 3D). This also applied to the excreted protein levels for BE-affected (157.3 pg/mL, 0.0–2497.0; $p < 0.001$) and BE-unaffected (32.0 pg/mL, 0.0–141.2; $p < 0.001$) ID patients, which showed an increased inflammatory response at the gene expression level compared to healthy controls (0.0 pg/mL, 0.0; Figure 3I).

Finally, whilst IFN-gamma only showed an increasing trend on gene expression level in ID patients compared to healthy controls, the excreted sputum protein levels reflected this trend in a significant manner in BE-affected (173.5 pg/mL, 0.0–645.3; $p < 0.01$) as well as BE-unaffected patients (173.5 pg/mL, 0.0–457.3; $p < 0.05$) compared to healthy controls subjects (116.0 pg/mL, 0.0–174.0; Figure 3E,J).

3.3 | Dependency of pulmonary manifestations on disturbed immune cell profiles

To investigate the relevance of differences in sputum cell composition, inflammatory cell load in sputum samples and cytokine/chemokine profiles from ID patients, we correlated lung function parameters with markers in the induced sputum samples from patients and healthy controls (Table S1). The inflammatory cell load in sputum samples correlated significantly with FEV1, MEF25, RV and RV/TLC. Interestingly, the proportions of sputum neutrophils only revealed an impact on MEF25, RV and RV/TLC but not on FEV1. The strongest effects of excreted pro-inflammatory mediators were found between TNF-alpha and FEV1 ($r = -0.5349$, $p < 0.05$), TNF-alpha and MEF25 ($r = -0.6495$, $p < 0.001$), TNF-alpha and Tiffeneau-Index ($r = -0.4638$, $p < 0.05$) and TNF-alpha and RV/TLC ($r = 0.5302$, $p < 0.001$). In contrast, at the gene expression level, the strongest correlations were detected between IL-1beta and RV ($r = 0.442$; $p < 0.01$), CXCL-8 and RV ($r = 0.455$; $p < 0.01$), IL-1beta and RV/TLC ($r = 0.317$; $p < 0.05$) and CXCL-8 and RV/TLC ($r = 0.319$; $p < 0.05$).

However, recognizing the impact of the inflammatory cell load and neutrophil granulocytes, we correlated the pro-inflammatory mediators with these two parameters. As expected, there were strong correlations between the inflammatory cell load and the excreted sputum levels of IL-1beta, IL-6, CXCL-8 and TNF-alpha (Figure 4; Tables S1 and S2). In addition, a strong dependence of the neutrophil numbers in the sputum on the levels of IL-1beta, IL-6, CXCL-8 and TNF-alpha was observed (Figure 4; Tables S1 and S2).

4 | DISCUSSION

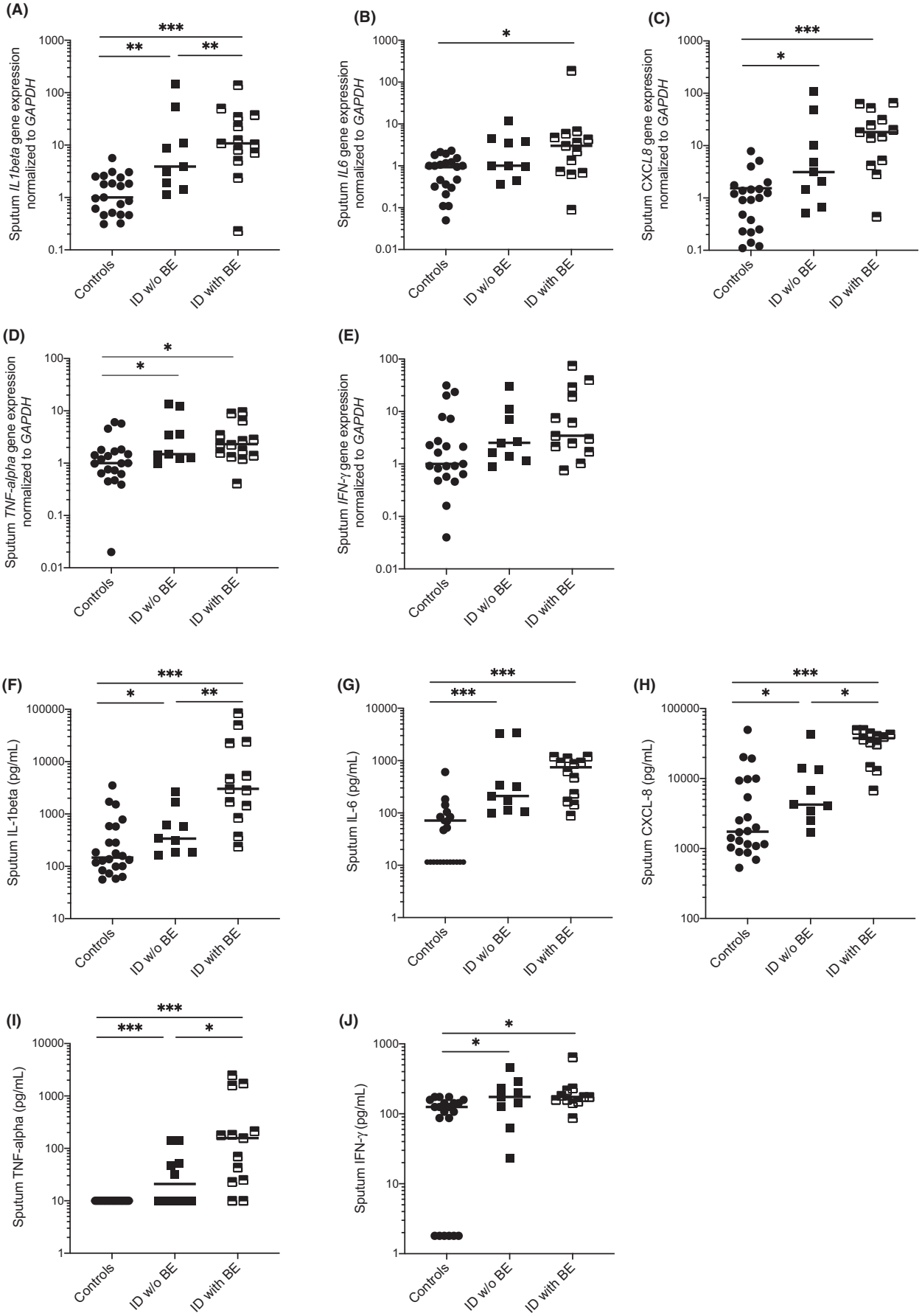
This study identified local biomarkers mirroring immune imbalance in humoral ID. These local sputum-derived biomarkers reveal the potential to depict the ongoing immune imbalance. This is the first study that takes advantage of induced sputum as a surrogate parameter elucidating ID and a window to related lung pathology like a snapshot of the inflammatory status quo in lower airways.[32]

Despite substitution therapy with immunoglobulins and the resulting clearly improved quality of life, patients with ID suffer from chronic airway infections, even if the diagnosis and IVIG treatment was carried out early.[20,33]

Recurrent sinopulmonary infections and progression of the chronically destructive lung disease with a concomitant development of bronchiectasis can hardly be prevented.[16,34] Since bronchiectasis often marks the end-point and is mostly part of the more severe course of the disease, the subgroups were distinguished on the basis of the involvement of bronchiectasis in order to identify possible differences in the type of inflammation.

In the present study, the course of immunodeficiencies was reviewed according to the pulmonary function test. Fifty-nine percent of the patients showed abnormal lung function values, despite adequate IgG levels. Bronchiectasis was detected in 13 of 22 patients, although not all of them revealed abnormal pulmonary function values. The extent to which the pulmonary function examination is suitable to detect small bronchiectatic changes was investigated in a previously published study, which examined bronchiectasis diagnosed with HRCT in 43 children.[35] The described mild bronchiectatic changes in the HRCT could not be reliably detected by the pulmonary function examination; however, this study was limited to FEV1 and FVC and, in contrast to the present study, did not include lung function values such as MEF25 and RV/TLC. Two-third of our patients with bronchiectasis showed severe limitations in FVC and FEV1, whilst one-third

FIGURE 3 Expression and protein levels of excreted mediators in subgroups of ID patients. Gene expression levels and protein levels for IL-1beta, IL-6, CXCL-8, IFN-γ and TNF-alpha were detected in cells and supernatants of induced sputum samples of ID patients with or without bronchiectasis and healthy control subjects. The amounts of *IL1β*, *IL6*, *IL8*, *TNFα* and *IFNγ* mRNA expression were normalized with endogenous control *GAPDH* and the relative quantification and calculation of range of confidence was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (relative gene expression)


(A)
Sputum IL1beta gene expression normalized to GAPDH
1000
100
10
1
0.1
***
**
**
Controls
ID w/o BE
ID with BE
(B)
Sputum IL6 gene expression normalized to GAPDH
1000
100
10
1
0.1
0.01
*
Controls
ID w/o BE
ID with BE
(C)
Sputum CXCL8 gene expression normalized to GAPDH
1000
100
10
1
0.1
***
*
Controls
ID w/o BE
ID with BE
(D)
Sputum TNF-alpha gene expression normalized to GAPDH
100
10
1
0.1
0.01
*
*
Controls
ID w/o BE
ID with BE
(E)
Sputum IFN-γ gene expression normalized to GAPDH
100
10
1
0.1
0.01
Controls
ID w/o BE
ID with BE
(F)
Sputum IL-1beta (pg/mL)
100000
10000
1000
100
10
***
*
**
Controls
ID w/o BE
ID with BE
(G)
Sputum IL-6 (pg/mL)
10000
1000
100
10
1
***
***
Controls
ID w/o BE
ID with BE
(H)
Sputum CXCL-8 (pg/mL)
100000
10000
1000
100
***
*
*
Controls
ID w/o BE
ID with BE
(I)
Sputum TNF-alpha (pg/mL)
10000
1000
100
10
1
***
***
*
Controls
ID w/o BE
ID with BE
(J)
Sputum IFN-γ (pg/mL)
1000
100
10
1
*
*
Controls
ID w/o BE
ID with BE

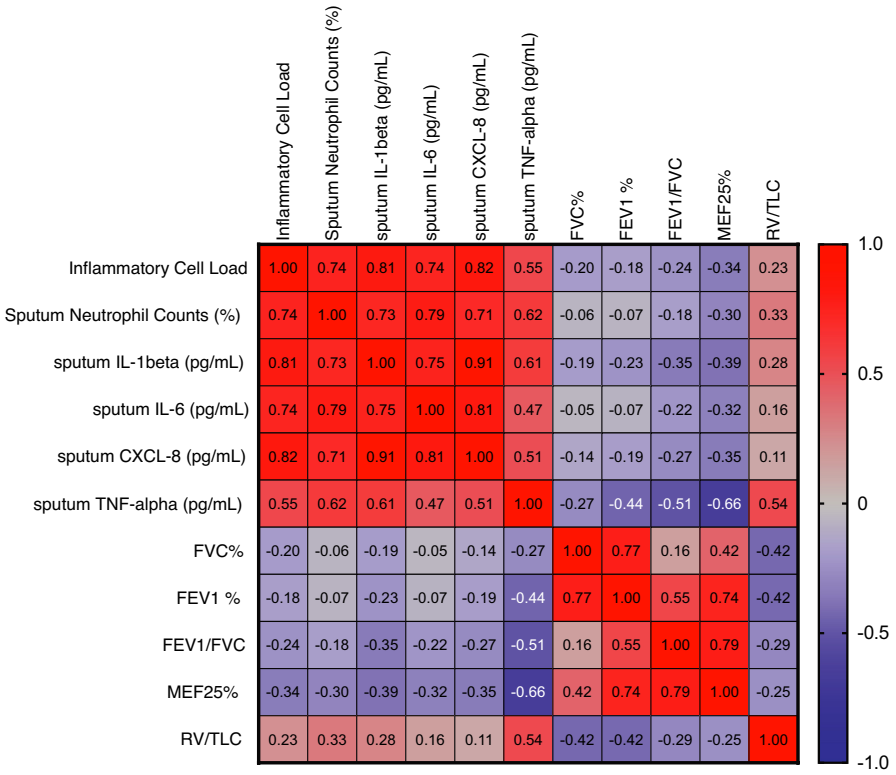


FIGURE 4 Correlation matrix for pro-inflammatory mediators, local cellular components, and lung function parameters. Correlation analysis was performed for inflammatory cell load or neutrophils with gene expression levels and protein levels for IL-1beta, IL-6, CXCL-8 and TNF-alpha

showed normal values in this regard. As it was not possible to reveal all bronchiectasis-related changes on the basis of FEV1 and FVC, the present observations are consistent with previously published data.[35] Patients with concomitant bronchiectasis but normal FEV1 and FVC, however, showed either a MEF25 below 50% or a significantly increased inflammatory cell load in the sputum (leucocyte number per mL sputum)[28,29] accompanied by high neutrophil numbers.

Previously published studies revealed that lung disease in ID patients, similar to patients suffering from CF, originates from the small airways.[36] Due to the flow limitation in the small airways in ID, lung function diagnostics characteristically exhibit a reduced MEF25 value in combination with an increased RV or RV/TLC due to the consecutively increased air retention.[37] Measurements of RV and RV/TLC in body plethysmography may reveal certain pathologies of the small airways, such as the degree of hyperinflation, more reliably. Both parameters increase with severe flow limitation in the distal airways.[18,38,39] Since patients suffering from ID showed significant flow limitations of the large and small airways, a reversibility testing was performed in all patients. As expected, 95% of our patients showed no reversibility in the reversibility test with 400-µg salbutamol confirming an irreversible airway obstruction.

As expected, these findings were confirmed for ID with bronchiectasis by the present study, which showed significantly reduced MEF25 values in the lung function analysis and a significantly increased RV.[40]

Previous studies observed a correlation between increased IL-8 (CXCL8) concentrations and neutrophil inflammation in chronic inflammatory airway diseases.[41,42] We were able to confirm this with the present study at the protein and gene level, as biomarker pattern revealed clear differences towards a pro-inflammatory phenotype in ID patients. Further, the pro-inflammatory phenotype was indicated by increased sputum cytokine and chemokine levels for *IL1beta*, *IL6*, *CXCL8*, *TNF-alpha* and *Interferon-gamma* not only on transcriptome level, but also on excreted protein levels. It was obvious from these results that this interaction between high CXCL-8 concentrations and an accumulation of neutrophils also has an impact on the lung function of the patients. In the context of our study, this was particularly present in parameters associated with bronchiectasis-related small airway dysfunction. As the pro-inflammatory mediators IL1beta, CXCL8 and TNF-alpha showed different expression and secretion levels in ID patients compared to healthy subjects and ID patient with or without BE, these parameters could be used a discriminating biomarker for bronchiectasis in ID patients. In contrast, even if revealing increased levels in ID patients compared to healthy controls, IL-6 at least on gene expression does not seem to be a useful biomarker for bronchiectasis in ID patients. Further, excreted sputum IL6 levels also revealed differences between ID patients and controls, however not between ID subgroups. Different cell types such as Th2 cells, B cells and DCs are expressing IL-6, which could take the role as a plasma cell growth factor [71,74]. It was previously shown that ID patients expressed higher amounts of IL1beta and IL6, which were abolished as response to pneumococci vaccination.[43] Moreover, significantly increased levels of TNF-alpha on transcriptome and excreted protein levels were observed. The role of TNF in immunodeficiency was discussed controversially. Our findings concord with the trend of our finding were confirmed in

previous publications revealing increased peripheral TNF levels.[44] Large amounts of TNF-alpha as response secondary to infections may be explained as an inappropriate inflammatory reaction of the T helper cells and macrophages. Increased levels of TNF-α were shown to be associated to a wide variety of inflammatory diseases such as arthritis, diabetes and Crohn's disease. Expression of TNF-alpha induces mediator causal for tissue necrosis or apoptosis, e.g. collagenases, proteases and reactive oxygen species.[45] Increased TNF-α can further up-regulate the transcription factors *NF-κB* and *AP-1*, which mediate the expression of downstream TNF-α-responsive genes. Further, the TNF-α promoter contains *NF-κB* and *AP-1* binding sites; TNF-α can positively promote its own synthesis, leading to the persistence of inflammation and airway tissue damage.[46] In contrast, levels of anti-inflammatory IL-10 were significantly increased in patients suffering from ID. Whilst some publications did not observe increased levels of IL-10 in ID patients,[47,48] another study found also significantly increased IL-10 levels in serum of ID patients along with an increased immunity towards a Th2 phenotype.[49] Our finding of increased IL-10 levels could be a hint for compensatory mechanisms for returning to a homeostatic state, which could be in opposition to IL-1beta, IL-6, IL-8 and IFN-γ-mediated inflammation. Also, a component in these mechanisms driving the pulmonary inflammation in ID could be IL-10-producing immune cells such as regulatory B cells.[50] However, these mechanisms need to be further elucidated in a future study resolving the ID-related inflammation on a single cell level. Moreover, anti-inflammatory mechanisms should be investigated in the context of ID in future studies.[28,51,52]

In conclusion, the upregulation of IL1beta and CXCL8 in immune dysregulation in ID may indicate a bronchiectasis state. Not only inflammatory, but also functional T cell exhaustion could play a role reported by previous studies.[53,54] This is hallmarked by diminished effector function and sustained expression of inhibitory receptors, such as PD-1, CTLA-4, TIM-3 and LAG-3.[55] Therefore, the use of not only one but a mixture of representative pro-inflammatory, local mediators could be used to determine the involvement of bronchiectasis in ID patients along lung function parameters covering small airway obstruction. Further, the inflammatory cell load as well as the presence of increased neutrophil numbers could hint the presence of bronchiectasis.

A limitation of the present study is the heterogeneity of ID patients, which are not a uniform group, but different clinical pictures. Different degrees of severity are included in the present study, which are 14 CVID, 3 XLA, 3 hyper-IgM syndrome, 1 hyper-IgE syndrome and low IgG levels due to treatment with rituximab and 1 SCID after BMT and persistent humoral defect. Regardless of this disadvantage, the patient group is well defined by the complete humoral immunoglobulin deficiency and the patient group is representative. Due to the heterogeneous nature of CVID, the interpretation about the cytokines situation in CVID could be impeded.

The strength of this study was the standardized execution of the induction of sputum induction and processing and the confidence of a cytokine profile on transcriptome and protein level. The ID without the presence of a BE seems to be associated with an early stadium of the disease, as the inflammatory cell, and cytokine load was increased compared to healthy controls, however diminished compared to ID + BE. Thus, the presence of bronchiectasis in immunodeficiency patients could hint for disease progression, as ID + BE patients showed increased bronchial inflammation and decreased lung function compared to healthy subjects and ID patients without BE.

4.1 | Conclusions

The small airway dysfunction was not in the focus for a long time, as it is difficult to capture using imaging and functional methods. Persistent inflammatory processes in the small airways lead to functional changes and structural remodelling processes. Bronchial inflammation was dominated by neutrophils despite immunoglobulin substitution and was highly increased in ID patients with bronchiectasis. The degree of bronchial inflammation was mirrored by the sputum inflammatory cell load (cells per mL of sputum). Notably, the pro-inflammatory mediators IL-1beta, IL-6, CXCL-8, IFN-γ and TNF-α were significantly increased in patients with ID in induced sputum mirroring the bronchial inflammation in this disease condition. In particular, TNF-α could serve as biomarker to demark the involvement of bronchiectasis in ID patients. The context-dependent cytokine pattern with respect to the presence of concomitant bronchiectasis in ID patients could be helpful to delineate ID patient subgroups and to individualize therapeutic approaches, however further validation is required in future studies.

CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTION

Study design U.M.Z, R.S., S.Z.; sampling U.M.Z, A.T., J.E., R.S., S.Z; conduction of experiments U.M.Z, A.T., J.E., R.S.; data collection U.M.Z, A.T., J.E., R.S.; data analysis U.M.Z, A.T., J.E., R.S.; data interpretation U.M.Z, A.T., J.E., S.B., R.S., S.Z; literature search U.M.Z, A.T., J.E., S.B., R.S., S.Z; writing U.M.Z, A.T., J.E., S.B., R.S., S.Z.

DATA AVAILABILITY STATEMENT

All data are accessible on request from the authors.

ORCID

Ulrich M. Zissler https://orcid.org/0000-0003-4826-3419

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SUPPORTING INFORMATION

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