Pathology - Research and Practice 215 (2019) 97–105

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

The evaluation of inflammatory, anti-inflammatory and regulatory factors contributing to the pathogenesis of COPD in airways

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ARTICLE INFO

Keywords:
Cytokines
COPD
Lung
Inflammation
Immunohistochemistry

ABSTRACT

Introduction: Chronic obstructive pulmonary disease (COPD) is a progressive chronic disease leading to obstructive lung airways and airflow limitations. The background of COPD is extensive cytopathology and histopathology orchestrated by mostly chronic inflammation with the local release of inflammatory, anti-inflammatory and regulatory mediators, as well as further remodeling and shaping of local architecture. Inflammatory mechanisms are provided by complex intercellular signalling networks and regulation of locally occurring immune responses.

Material and methods: In this study, lung tissue specimens obtained from 33 COPD patients and 49 control patients were analysed. Tissue samples were examined by hematoxylin and eosin staining. Immunoreactive cells positive for interleukin (IL)-1α (IL-1α), IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and tumour necrosis factor-α (TNF-α) were detected by an immunohistochemistry (IHC) method.

Results: We evaluated overall higher numbers of IL-7, IL-8 and IL-10 (mostly from few (0/+) to almost abundance (++++)) and overall less numbers of IL-1α and IL-6 (mostly from no positive (0) to numerous to abundance (+++/++++)) immunoreactive cells in airway epithelium and connective tissue of COPD affected lung. Furthermore, we evaluated statistically significant ($P < 0.05$) higher numbers of immunoreactive cells located in control group airway epithelium for IL-4, IL-6, IL-7, IL-10, and IL-12 compared to mucosal and submucosal connective tissue. Moreover, in COPD group airway epithelium for IL-1α, IL-4, IL-6, IL-7, IL-8, and IL-10. We found no statistically significant difference between the numbers of IL-12 and TNF-α immunoreactive cells in airway epithelium and connective tissue of COPD affected lung. In comparison with the control group, we found statistically significant ($P < 0.05$) higher numbers of immunoreactive cells positive for all examined markers in COPD group.

Conclusions: Increased numbers of IL-1α, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and TNF-α immunoreactive cells highlight the local significance of these markers in COPD pathogenesis. Moreover, the pattern with dominance of immunoreactive cells in COPD affected airway epithelium over connective tissue is highlighting the essentials of epithelium in inflammatory signalling.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of mortality and morbidity in respiratory and general medicine, moreover, COPD is defined as worldwide health issue [1]. Exposure to cigarette smoke and environment pollution promotes oxidative stress and initiates intercellular signalling pathways that further manage the augmentation of various mediator release (like cytokines) directly from airway epithelium being a barrier to an outer environment. These mechanisms promote inflammation, cell injury and apoptosis leading to development of COPD [2]. Complex intercellular signalling networks, interrelations and wide distribution of various cytokines cause a wide spectrum of mechanisms leading to airway obstruction. In COPD, widespread chronic inflammation with prominent immune cell infiltration, as well as fibrosis and wall remodeling of diverse degree, impairment of mucociliary clearance mechanisms, mucus hypersecretion and emphysema highly dominates; moreover, all contributes to prominent luminal narrowing of bronchial structures [3].

Chronic inflammation of COPD affected lung tissue is achieved by an activation of various immune cells, as well as airway epithelial cells and fibroblasts [4]. Moreover, this may point to these cells mirroring the ontogenetic background and shaping architecture in the

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https://doi.org/10.1016/j.prp.2018.10.029
Received 15 August 2018; Received in revised form 15 October 2018; Accepted 26 October 2018
0344-0338/

microscopic environment of lung tissue, where the cell cross-talk and complex network of signalling molecules takes place [5].

Signalling mediators like interleukin (IL)-8 and tumour necrosis factor alpha (TNF-α) are expressed and released in airway tissue by various cells orchestrating the inflammatory processes in COPD [6]. Moreover, the pathogenesis of COPD is associated with the local appearance and distribution of various cytokines IL-1α [7], IL-4 [8], IL-6, IL-7 [9], IL-8 [10], IL-10 [11], IL-12 [12] and TNF-α [13], which have been investigated to affect the immune cell profile, engagement of non-immune cells (epithelial cells, fibroblasts), overall molecular mechanisms and signalling patterns influencing the pathological and clinical outcomes in COPD affected lung.

The IL-1 family has been described to operate autoimmune, profibrotic, mitogen, both inflammatory and anti-inflammatory functions. IL-1α itself is a pro-inflammatory cytokine with an effect on immune response initiation and amplification, cell recruitment and functional support. IL-1α is an important inflammatory mediator in COPD with bronchial epithelium being one of the most prominent sources in lungs [14].

Cytokine IL-4 acts in allergic immune responses due to an allergen exposure maintaining a key mediator role in intercellular signalling network. IL-4 also affects mucosal connective tissue fibroblasts and promotes further local fibrosis [15]. In COPD, increased mucus production has been associated with an increased release of cytokine IL-4 from immune and non-immune cells [16].

Of cytokines with controversially different functions due to the local environment, IL-6 possesses pro-inflammatory, anti-inflammatory and regenerative functions [17], as well as participates in fibrosis through the direct and indirect mechanisms on connective tissue fibroblasts [15]. Neutrophilic inflammation – one of the leading pathogenesis mechanisms in COPD – is associated with the increased IL-6 signalling [18].

Cytokine IL-7 is an immunoregulatory cytokine with pleiotropic functions that increases repopulation and generation of lymphoid cell lineage, regulates T lymphocyte overall development, maintenance and homeostasis while working as a lymphoid regenerative factor [19]. IL7R gene network (including IL-7) was associated with COPD and the rate of ageing, where higher expression of IL7R gene network was indicating an increased prevalence of COPD [20], however, no actual findings of IL-7 have been evaluated in COPD, moreover, in particular tissue compartment.

Cytokine IL-8 is one of the most active and widespread biological molecules maintaining pro-inflammatory, chemoattractant and initiating roles of immune responses. IL-8 is a cytokine with tissue specific chemotactic and paracrine properties, moreover, IL-8 is a chemoattractant and mediator for immune cells [6]. IL-8 is also associated with an increase of mucus production and remodeling processes in airways affecting [6,16].

Cytokine IL-10 is a cytokine with mostly anti-inflammatory properties. IL-10 is thought to achieve immune suppression on cellular immunity while having stimulating effect on humoral immunity responses. IL-10 inhibits the production of IL-4, IL-6, IL-8, IL-12, TNF-α by various immune cells (Th1 and Th2 cells, mononuclear phagocytes, natural killer cells), but no particular outcome of these cytokines altogether has been evaluated [21].

Cytokine IL-12 is predominantly pro-inflammatory/pro-stimulatory cytokine. IL-12 is produced by dendrite cells, macrophages and B cells [22]. IL-12 is upregulated in lung fibroblasts after the smoke exposure in COPD. Following increase in the expression of collagens and transforming growth factor (TGF)-beta may be evaluated leading to extensive airway remodelling, therefore pointing out high significance of IL-12 in connective tissue [23].

TNF-α is a pleiotropic cytokine maintaining numerous functions being mostly pro-inflammatory cytokine. TNF-α is produced not only by immune cells (macrophages, lymphocytes, natural killer cells, mast cells, dendritic cells), but also by stromal cells (fibroblasts) [24] and airway epithelial cells [25]. Pro-inflammatory cytokine TNF-α amplifies inflammatory responses in COPD by affecting the release of other cytokines [26].

With an interest of respiratory epithelium managing the outline barrier functions in the lung environment, an obvious importance is cytokine presence. Cytokines possibly produced in respiratory epithelium maintain cross-talk with cells in subepithelial connective tissue and initiate various cell and tissue changes in COPD affected bronchial wall. Cytokines shape signalling of local immunity and provide wide intercellular communication networks, therefore their levels are highly promoting cytokine regulation in COPD affected airways.

Thus, the aim of this study was to determine the appearance and relative distribution of various cytokines in COPD affected lung tissue material in comparison with the normal control group.

2. Materials and methods

2.1. Patients

In patient group, COPD affected lung tissue specimens were obtained during fibrobronchoscopy from 33 patients aged from 53 to 85. The tissue material was obtained from large airways. The diagnosis of stable COPD was assessed by clinical criteria, physical examination and during bronchoscopy. COPD patients with objectively determined acute exacerbations, severe respiratory conditions, pulmonary and respiratory failure were excluded from this study. COPD patients with any history of acute or severe chronic pathology and treated with medications conflicting the possible results of this study were excluded. In control group, we evaluated lung tissue material obtained during a *post mortem* autopsy of large airways from forty-nine healthy control subjects aged 9 to 95 years. Among the diagnoses of the control subjects, mostly sudden cardiac death (sudden cardiac arrest), intentional self-harm (suicide) and unintentional major injury due to trauma (vehicle crash, traffic collision) were dated. Control group tissue samples with medical data records and histopathological findings conflicting the possible results were excluded.

All authors hereby declare that all performances were examined and approved by the appropriate ethics committee and were therefore implemented in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. This study was approved by the Ethical Committee of Pauls Stradins Clinical University Hospital dated January 23, 2013.

2.2. Routine histological analysis

Approximately 1 cm^3 sized lung tissue specimens were obtained during fiberoptic bronchoscopy and autopsy. Specimens were then fixed in 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer with pH 7.2. Tissue specimens were rinsed in Tyrode's solution (136.9 mM NaCl, 2.68 nM KCl, 1.8 mM CaCl2·2H2O, 1.05 mmol/l MgCl2·6H2O, 11.9 mM NaHCO3, 0.42 mM NaH2PO4·H2O, 5.5 mM glucose in distilled water) for 12 h. The following scheme of dehydration procedure was performed: 70% ethanol (ethanol I) for 30 min, 80% ethanol (ethanol II) for 1–2 h, 96% ethanol (ethanol III) for 3–4 h, 96% ethanol (ethanol IV) for 24 h. Tissue specimens were cleared using two xylene baths (xylene I and xylene II) for 1 h total. In upcoming procedure, tissue specimens were infiltrated using two sequential paraffin wax baths with paraffin I for 1 h and paraffin II 2 h. Lung tissue sections were cut using a rotation microtome (*Leica RM2245; Leica Biosystems Richmond Inc., Richmond, VA*) at 5 μm thickness. Sections were mounted on the glass slides, then deparaffinised in xylene and rehydrated through graded series (70–96%) of ethanol. Tissue sections were stained with hematoxylin (*05-M06002, Mayer's Bio Optica Milano S.p.A., Milano, Italy*) and eosin (*05-B10003, Mayer's Bio Optica Milano S.p.A., Milano, Italy*) [27].

2.3. Immunohistochemistry

Lung tissue sections of 49 control subjects and 33 COPD subjects were used to detect pro-inflammatory, anti-inflammatory and regulatory markers by using the biotin-streptavidin immunohistochemistry (IHC) method [28]. Lung tissue sections were deparaffinised with xylene and washed in ethanol and distilled water. Then tissue sections were washed for 10 min in a wash buffer (tri-buffered saline; TRIS buffer *(15-M106; Bio-Optica, Milano, Italy)*). The following procedure was to insert tissue sections in EDTA boiling buffer (EDTA (pH 9.0) buffer (*T0103; Diapath, Martinengo BG, Italy*)) within the use of microwave for 5 min. Tissue sections were cooled down and then washed twice for 5 min in wash buffer (TRIS buffer). For the blockage of endogenous peroxidase, tissue sections were placed in 3% peroxide (H_2O_2) for 10 min and then washed twice for 5 min in wash buffer. The following chemical agent *Antibody Diluent* (*ab64211; Abcam, Burlingame, CA*) was used to dilute all antibodies used in this study. For the detection of antibodies acquired from mouse or rabbit, lung tissue specimens were incubated for 10 min at room temperature with *HiDef DetectionTM reaction amplifier (954D-31; Sigma-Aldrich, Sigma-Aldrich, Rocklin, CA)*. All tissue samples in this study were incubated with primary antibodies for 1 h. Further washing in wash buffer (TRIS buffer) for three times of 5 min each time was performed. In a continuing procedure, incubation for 10 min with *HiDef DetectionTM HRP polymer marker* (*code-954D-32; Sigma-Aldrich, Rocklin, CA*) was managed at room temperature. For the detection of antibodies acquired from goat, *ImmunoCruzTM ABC staining system* (*sc-2018, Santa Cruz Biotechnology, Inc., USA*) was used. Tissue sections were incubated with blocking serum in TRIS buffer ar room temperature for 1 h. Tissue section incubation with primary antibody was performed for 1 h at room temperature. After the application of wash buffer for three times of 5 min each time, tissue sections were incubated with secondary biotinylated goat Ig for 30 min. The same procedure protocol was performed to incubate tissue sections for the tertiary antibody. These procedures were followed by washing the tissue sections in a wash buffer for 5 min and processing with *DAB Substrate Kit* (*code-957D-30; Sigma-Aldrich, Rocklin, CA*) for 10 min to obtain immunoreactive structure staining in brown color. Samples were then rinsed in distilled water and stained with haematoxylin (*05-M06002, Mayer's Bio Optica Milano S.p.A., Milano, Italy*) within an appropriate procedure.

We used mouse antibodies for the detection of IL-1α (*sc-9983*, diluted 1:50, *Santa Cruz Biotechnology, Inc., USA*) and IL-6 (*sc-130326*, diluted 1:50, *Santa Cruz Biotechnology, Inc., CA, USA*); rabbit antibodies for IL-4 (*orb10908*, diluted 1:100, *Biorbyt, Cambridge, UK*), IL-7 (*orb48420*, diluted 1:100, *Biorbyt, Cambridge, UK*), IL-10 (*P22301*, diluted 1:400, *Nordic BioSite, Sweden*), IL-12 (orb10894, diluted 1:100, *Biorbyt, Cambridge, UK*), TNF-α (*ab6671*, diluted 1:100, *Abcam, Burlingame, CA, USA*); goat antibody for IL-8 (*sc-1269*, diluted 1:50, *Santa Cruz Biotechnology, Inc., CA, USA*) by using the biotin-streptavidin immunohistochemistry method [28].

In parallel sections to investigated ones, primary antibody was replaced with chemical agent *Antibody Diluent* (*ab64211; Abcam, Burlingame, CA*) to dilute all antibodies. These sections were used as negative control. For each series of tissue sections, positive controls were prepared.

The samples were examined using bright field microscopy with a *Leica DC 300F* camera microscope (*Leica DM500RB, Leica Biosystems Richmond Inc., Richmond, VA*) for conventional histology and photography. Acquired images were analysed using *Image Pro Plus 6.0* software (*Media Cybernetics, Silver Spring, MD*).

2.4. Quantification of immunoreactive cells

In our study, we examined the appearance and local distribution of marker-containing immunoreactive cells by semi-quantitative grading method [29,30]. The following tissue compartments of large bronchus were evaluated: airway epithelium, mucosal and submucosal connective tissue. The following scale of the semi-quantitative method was used, counting the immunoreactive (positive) structures seen in the visual field: 0 – no positive structures (0%), 0/+ – occasional positive structures (12.5%), + – few positive structures (25%), +/+ + – few to a moderate number of positive structures (37.5%), + + – moderate number of positive structures (50%), + +/+ + + – moderate number of to numerous positive structures (62.5%), + + + – numerous positive structures (75%), + + +/+ + + + – numerous to abundant positive structures (87.5%), + + + + – abundance of positive structures (100%) observed in three random visual fields by magnification level X400 (ocular X10, objective X40) [30].

2.5. Data statistical analysis

To perform the statistical analysis, we used non-parametric statistical methods. All the acquired data were ranked as ordinal values, where no positive cells (0) seen in visual field of bright field microscopy were ranked with the value of 0, occasional positive cells (0/+) were ranked with the value of 0.5, few positive cells (+) were ranked with the value 1.0 and continuing to the value of 4.0.

The Wilcoxon matched pairs Signed Rank Test [31] was conducted to determine whether there was a difference in the number of immunoreactive cells of one examined cytokine in two different tissue types, as well as to evaluate the positive ranks, negative ranks and ties.

The Mann-Whitney *U* Test [32] was conducted to determine a difference in the number of positive structures of each examined marker within particular lung tissue compartment in control and COPD groups.

Spearman's Rank Order Correlation [33] as a nonparametric measure of statistical dependence was performed to determine the relation between the numbers of immunoreactive cells in airway epithelium and connective tissue of COPD group. The correlation coefficient, r_s (Spearman's rho), with calculated values of 0.00–0.30, is regarded as negligible correlation, 0.30–0.50 as low, 0.50–0.70 as moderate, 0.70–0.90 as strong and 0.90–1.00 as very strong correlation [34].

The statistical analysis was performed using the statistical program *SPSS Statistics*, version 23.0 (*IBM Company, Chicago, USA*). In all the statistical analyses, two-tailed P values < 0.05 were considered statistically significant.

3. Results

3.1. Findings of routine histological analysis

In routine histological analysis with haematoxylin and eosin, COPD affected lung tissue presented evidences of various degrees of chronic inflammation and tissue remodeling in all examined COPD patients. Goblet cell hyperplasia (Fig. 1A), bronchial epithelial squamous metaplasia, subepithelial basement membrane thickening, airway fibrosis with thick and prominent connective tissue collagen fibre bundles, bronchial gland hypertrophy and hyperplasia (Fig. 1B), bronchial microvasculature remodeling, smooth muscle cell hyperplasia and hypertrophy, remarkable inflammatory cell infiltration in airway mucosa and submucosa, and glandular tissue were overall evaluated. Various numbers of dust-containing alveolar macrophages were found almost in all COPD affected lung tissue. Goblet cell hyperplasia of COPD affected lung was evaluated as a unique landscape in respiratory epithelium covering the wall of a large-calibre bronchus.

3.2. Findings of immunohistochemistry

The numbers of IL-1α immunoreactive cells in control group varied from no positive cells (0 or 0%) to few to moderate number (+/+ + or 37.5%) (Fig. 1C), whereas in COPD group the number of IL-1α immunoreactive cells (Fig. 1D) varied from few positive cells (0/+ or 12.5%) to numerous and abundance (+ + +/+ + + + or 87.5%) of

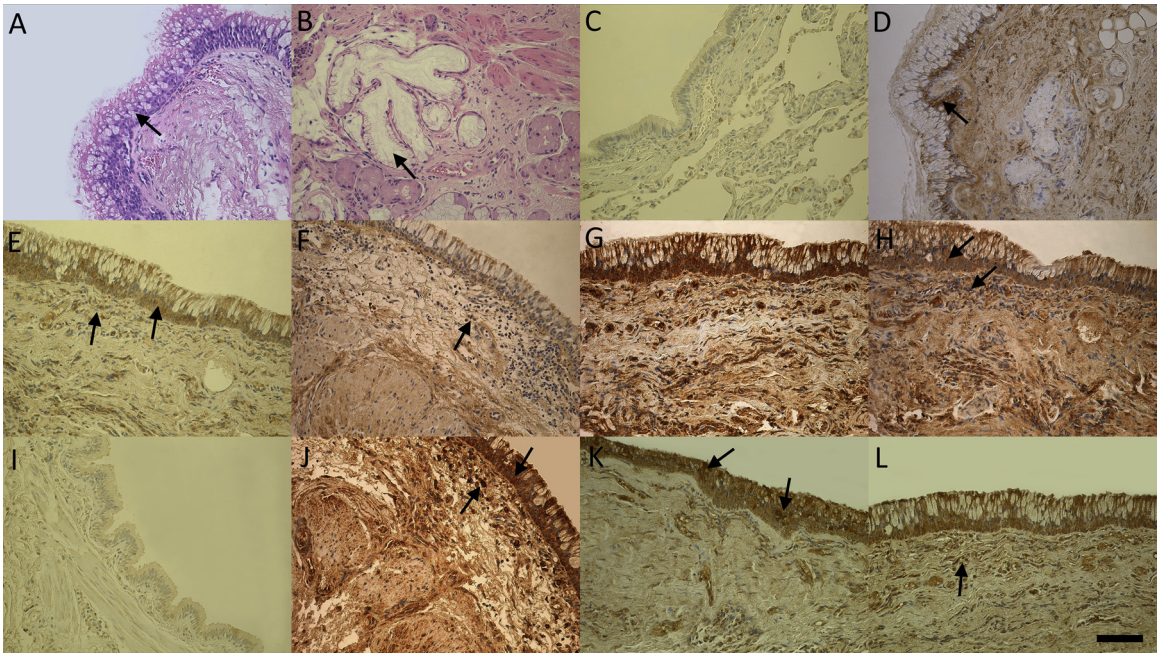

Fig. 1. Appearance of IL-1α, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and TNF-α immunoreactive cells by biotin-streptavidin immunohistochemistry (IHC) method. (A) Goblet cell hyperplasia (*arrow*) in 64 years old male (*COPD*). Hematoxylin and eosin, X200. (B) Bronchial gland hyperplasia and hypertrophy (*arrow*), smooth muscle cell hyperplasia in 60 years old male (*COPD*). Hematoxylin and eosin, X200. (C) Almost no positive (0) IL-1α containing epithelial cells and few (+) IL-1α positive fibroblasts in 81 years old female bronchus (*Control*). IL-1α IHC, X200. (D) Few to moderate number (+/++) IL-1α immunoreactive cells in bronchial epithelium (*arrow*) and connective tissue of 78 years old male bronchus (*COPD*). IL-1α IHC, X200. (E) Moderate to numerous number (++/+++) IL-4 containing cells in bronchial epithelium and connective tissue (*arrows*) of 78 years old male (*COPD*); goblet cell hyperplasia. IL-4 IHC, X200. (F) Few (+) IL-6 containing cells in bronchial epithelium and connective tissue (*arrows*) of 56 years old male (*COPD*). IL-6 IHC, X200. (G) Almost abundance (++++) of IL-7 containing cells in epithelium and connective tissue of 68 years old male (*COPD*). IL-7 IHC, X200. (H) Numerous to abundance (+++/++++) cells in bronchial epithelium and numerous (+++) cells in connective tissue (*arrows*) of 61 years old male (*COPD*). IL-8 IHC, X200. (I) Occasional (0/+) IL-10 immunoreactive cells in bronchial epithelium and connective tissue of 81 years old female (*Control*). IL-10 IHC, X200. (*J*) Almost abundance (++++) of IL-10 positive cells in bronchial epithelium and numerous (+++) IL-10 positive cells in connective tissue (*arrows*) of 60 years old male (*COPD*). IL-10 IHC, X200. (K) Numerous (+++) IL-12 immunoreactive cells in airway epithelium (*arrows*) and moderate to numerous (++/+++) IL-12 immunoreactive cells in connective tissue of 60 years old male (*COPD*). IL-12 IHC, X200. (L) Numerous (+++) TNF-α immunoreactive cells in airway epithelium and numerous to abundance (+++/++++) TNF-α immunoreactive cells in connective tissue (*arrow*) of 60 years old male (*COPD*). TNF-α IHC, X200. Scale bar 50 μm.

positive cells (Table 1).

Appearance and distribution of IL-4 in control group lung tissue marked a range from no positive cells (0 or 0%) to numerous (+++ or 75%) immunoreactive cells. In COPD group, IL-4 immunoreactive cells were evaluated in a range from few positive cells (0/+ or 12.5%) to numerous and abundance (+++/++++ or 87.5%) (Fig. 1E).

Cytokine IL-6 findings in normal control group presented a variance with no positive (0 or 0%) to numerous (+++ or 75%) of immunoreactive cells in the wall of large-calibre bronchus. In COPD affected lung tissue, appearance and distribution of IL-6 immunoreactive cells were evaluated ranging from no positive (0 or 0%) to numerous and abundance (+++/++++ or 87.5%) immunoreactive cells (Fig. 1F).

The numbers of IL-7 immunoreactive cells in control group varied from no positive IL-7-containing cells (0 or 0%) to moderate to numerous number (++/+++ or 62.5%), whereas in COPD, we found a range of IL-7-containing cells from few to moderate numbers (+/++ or 37.5%) to almost abundance (++++ or 100%) (Fig. 1G).

Cytokine IL-8 immunoreactive cells were graded with values from no positive (0 or 0%) to moderate number (++ or 50%) in control group. Occasional (0/+ or 12.5%) to almost abundance (++++ or 100%) of IL-8 immunoreactive cells was determined in COPD affected lung tissue (Fig. 1H).

Anti-inflammatory cytokine IL-10 immunoreactive cells were evaluated from no positive (0 or 0%) to numerous (+++ or 75%) in control group (Fig. 1I). Few (0/+ or 12.5%) to almost abundance (+++ or 100%) numbers of IL-10 immunoreactive cells were determined in COPD group lung tissue (Fig. 1J).

Appearance and distribution of IL-12 immunoreactive cells in control group marked a variance from no positive cells (0 or 0%) to moderate to numerous (++/+++ or 62.5%) positive cells. Numbers of IL-12 immunoreactive cells were evaluated ranging from few (0/+ or 12.5%) to numerous to abundance (+++/++++ or 87.5%) in COPD group (Fig. 1L).

The numbers of TNF-α immunoreactive cells in control group were evaluated with a range from no positive (0 or 0%) to mostly few to moderate number (+/++ or 37.5%) positive cells. In COPD, few (0/+ or 12.5%) to numerous to abundance (+++/++++ or 87.5%) numbers of TNF-α immunoreactive cells were observed (Fig. 1K).

Overall, higher numbers of IL-1α, IL-4, IL-6, IL-7, IL-8, and IL-10 immunoreactive cells were also evaluated in bronchial airway epithelium of COPD group. Similar semi-quantitative grading scores were found for the IL-12 and TNF-α immunoreactive cells in bronchial airway epithelium and mucosal and submucosal connective tissue of COPD group.

3.3. Findings of data statistical analysis

The Wilcoxon matched pairs Signed Rank Test determined no statistically significant difference between the numbers of IL-1α immunoreactive cells in bronchial airway epithelium and connective tissue ($Z = -0.378$, $P = 0.706$) of control group. In COPD group, statistically significant more pronounced findings of IL-1α were detected in bronchial airway epithelium compared to the findings of IL-1α in connective tissue ($Z = -3.784$, $P < 0.0001$) (Fig. 2).

Furthermore, in the lung tissue of control and COPD groups, the

Table 1
Appearance and distribution of IL-1α, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and TNF-α immunoreactive cells by semi-quantitative grading in the lung tissue of control group and COPD group.

		Control (N = 49)		COPD (N = 33)	
		Mdn (Q_2) IQR (Q_3 – Q_1) [95% CI]			
IL-1α	Airway epithelium	**0.5**	1.0 [0.0, 1.0]	**2.5**	2.0 [1.0, 3.0]
	Connective tissue	**0.5**	1.0 [0.0, 1.0]	**1.0**	1.5 [0.5, 2.0]
IL-4	Airway epithelium	**1.5**	1.5 [0.5, 2.0]	**3.0**	1.5 [2.0, 3.5]
	Connective tissue	**0.5**	0.5 [0.0, 0.5]	**1.5**	1.5 [1.0, 2.5]
IL-6	Airway epithelium	**1.0**	1.5 [1.0, 2.5]	**2.25**	2.0 [1.0, 3.0]
	Connective tissue	**0.5**	0.5 [0.0, 0.5]	**0.5**	0.75 [0.5, 1.25]
IL-7	Airway epithelium	**1.5**	1.0 [1.0, 2.0]	**3.5**	1.0 [3.0, 4.0]
	Connective tissue	**0.5**	1.0 [0.0, 1.0]	**2.75**	0.75 [2.25, 3.0]
IL-8	Airway epithelium	**0.5**	0.5 [0.5, 1.0]	**3.25**	1.25 [2.5, 3.75]
	Connective tissue	**1.0**	1.0 [0.5, 1.5]	**2.0**	1.0 [1.5, 2.5]
IL-10	Airway epithelium	**1.5**	1.0 [1.0, 2.0]	**3.5**	1.0 [3.0, 4.0]
	Connective tissue	**1.0**	0.5 [0.5, 1.0]	**2.5**	1.0 [2.0, 3.0]
IL-12	Airway epithelium	**1.5**	0.75 [1.0, 1.75]	**2.5**	1.5 1.5, 3.0]
	Connective tissue	**0.5**	0.75 [0.25, 1.0]	**2.0**	1.5 [1.5, 3.0]
TNF-α	Airway epithelium	**0.25**	0.5 [0.0, 0.5]	**2.0**	0.75 [1.75, 2.5]
	Connective tissue	**0.5**	1.0 [0.0, 1.0]	**2.0**	1.5 [1.5, 3.0]

Semi-quantitative grading scores are displayed with rank values. “Control” – Control group, “COPD” – COPD group, “N” – Number of the study subjects, “IL” – Interleukin-1α, -4, -6, -7, -8, -10, -12, “TNF-α” – Tumor necrosis factor alpha, “Airway epithelium” – Immunoreactive cells in bronchial airway epithelium, “Connective tissue” – Immunoreactive cells in mucosal and submucosal connective tissue, “Mdn” – Median value, “IQR” – interquartile range, “Q_1” – 1st Quartile (25th percentile value), “Q_2” – 2nd Quartile (50th percentile value), “Q_3” – 3rd Quartile (75th percentile value), “95% CI” – 95% Confidence interval (with Lower and Upper Bounds).

numbers of IL-4, IL-6, IL-7, IL-10 immunoreactive cells were graded higher in bronchial airway epithelium compared to mucosal and submucosal connective tissue. Moreover, statistically significant difference was calculated within the findings of cytokines IL-4 ($Z = -4.832$, $P < 0.0001$ (control); $Z = -4.239$, $P < 0.0001$ (COPD)), IL-6 ($Z = -4.632$, $P < 0.0001$ (control); $Z = -4.278$, $P < 0.0001$ (COPD)), IL-7 ($Z = -4.518$, $P < 0.0001$ (control); $Z = -4.461$, $P < 0.0001$(COPD)), and IL-10 ($Z = -4.081$, $P < 0.0001$ (control); $Z = -3.260$, $P = 0.001$ (COPD)).

In control group, we found statistically significant higher numbers of IL-8 immunoreactive cells in connective tissue in comparison with airway epithelium ($Z = -2.8863$, $P < 0.004$). In COPD group, statistically significant higher numbers of IL-8 immunoreactive cells were determined in airway epithelium ($Z = -4.296$, $P < 0001$).

In control group, we found statistically significant higher numbers of IL-12 immunoreactive cells in airway epithelium compared to mucosal and submucosal connective tissue ($Z = -4.626$, $P < 0.0001$). In COPD group, no statistically significant difference was estimated within both tissue compartments analysed ($Z = -0.528$, $P = 0.598$).

No statistically significant difference was observed in the numbers of TNF-α immunoreactive cells in bronchial airway epithelium and mucosal and submucosal connective tissue of control group tissue ($Z = -0.753$, $P = 0.452$) and COPD group tissue ($Z = -0.209$, $P = 0.835$).

The Mann-Whitney U Test determined statistically significant higher numbers of all examined markers IL-1α, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and TNF-α immunoreactive cells in bronchial airway epithelium of COPD affected lung in comparison with control group (Table 1).

We found statistically significant ($P < 0.05$) positive moderate ($0.50 < r_s < 0.70$) correlations between the numbers of IL-6 and the numbers of IL-1α ($r_s = 0.526$, $N = 31$, $P = 0.002$), IL-8 ($r_s = 0.528$, $N = 32$, $P = 0.002$), and IL-10 ($r_s = 0.609$, $N = 32$, $P < 0.0001$) immunoreactive cells in bronchial airway epithelium indicating mutual dependence of these factors.

Statistically significant ($P < 0.05$) positive moderate correlations were evaluated between the numbers of IL-7 immunoreactive cells and IL-8 ($r_s = 0.514$, $N = 32$, $P = 0.003$) and IL-10 ($r_s = 0.549$, $N = 32$, $P = 0.001$) immunoreactive cells in airway epithelium; also low ($0.3 < r_s < 0.5$) correlations were evaluated between the numbers of IL-7 immunoreactive cells and IL-1α ($r_s = 0.387$, $N = 31$, $P = 0.032$) and IL-4 ($r_s = 0.367$, $N = 32$, $P = 0.039$) immunoreactive cells in airway epithelium of COPD affected lung.

Investigating relationship of IL-12 and other cytokines evaluated in this study, we observed single statistically significant ($P < 0.05$) negative moderate correlation with immunoreactivity of IL-8 in airway epithelium ($r_s = -0.471$, $N = 32$, $P = 0.007$), as well as between the findings of IL-12 and IL-8 ($r_s = 0.549$, $N = 32$, $P = 0.001$) in connective tissue of COPD affected lung.

4. Discussion

In this study, we found an evidence of increased immunoreactive cell numbers positive for all evaluated inflammatory, anti-inflammatory and regulatory factors in COPD compared to control group. Numerous studies state, that COPD is associated with various cytokine gene and their networks, as well as with increased expression of IL-1, IL-1R, IL-4, IL-6, IL-7, IL-8, IL-8R, IL-12, TNF-α [35,36]. COPD is studied to be associated with Th1 inflammatory cytokines IL-1, IL-8, IL-12, and TNF-α, as well as Th2 inflammatory cytokines IL-6 and IL-10 [37].

Moreover, statistically significant higher numbers were evaluated in airway epithelium for IL-1α, IL-4, IL-6, IL-7, IL-8, and IL-10, whereas immunoreactive cell numbers positive for IL-12 and TNF-α were graded similarly in both airway epithelium and connective tissue in COPD. Ongoing smoke exposure may affect the release of various cytokines within airway epithelial cells in COPD affected lung tissue; moreover, COPD phenotype of airway epithelial cells have been stated [10]. Airway epithelial cells have been associated with the barrier function forming an interactive surface between inhaled air with complex chemical composition (e.g. cigarette smoke exposure) above and tissue beneath. With further signalling responses, airway epithelial cells have become an important member of an innate and adaptive lung immunity [6]. In COPD, airway epithelial cells may be an important source for the release of various inflammation-associated mediators. Airway epithelial cells are activated by and further release various cytokines (e.g. IL-1α, IL-4, IL-6, and IL-10) [38,39]. Furthermore, epithelial cells produce inflammatory, anti-inflammatory and regulatory mediators as IL-1, IL-6, IL-8, TNF-α in a response to different stimuli [4,8].

In our study, we found mostly occasional (0/+) IL-1α immunoreactive cells in control group, whereas mostly few (+) and moderate to numerous (++/+++) numbers of immunoreactive cells were evaluated in connective tissue and airway epithelium of COPD, respectively. Statistically significant ($P < 0.05$) more pronounced numbers of IL-1α immunoreactive cells were found in bronchial airway epithelium of COPD affected lung tissue compared to the healthy control group. Airway epithelial cells are studied to be a source of cytokine IL-1α [40], where IL-1α precursor is present in airway epithelium. With the release of IL-1α by epithelial cells, a whole cascade of cytokine release is initiated [14]. In human subjects, IL-1α was significantly increased in overall lung tissue and induced sputum of COPD patients compared with never-smokers. These findings suggest IL-1α should be considered as an important mediator in cigarette smoke-associated inflammation in COPD [41]. Hereby IL-1α may be associated with an initiation, maintenance and amplification of complex inflammatory responses in COPD with more highlighted role of airway epithelium.

Mostly few to moderate (+/++) and numerous (+++) IL-4 immunoreactive cells were found in mucosal and submucosal connective tissue and bronchial airway epithelium of COPD group, respectively.

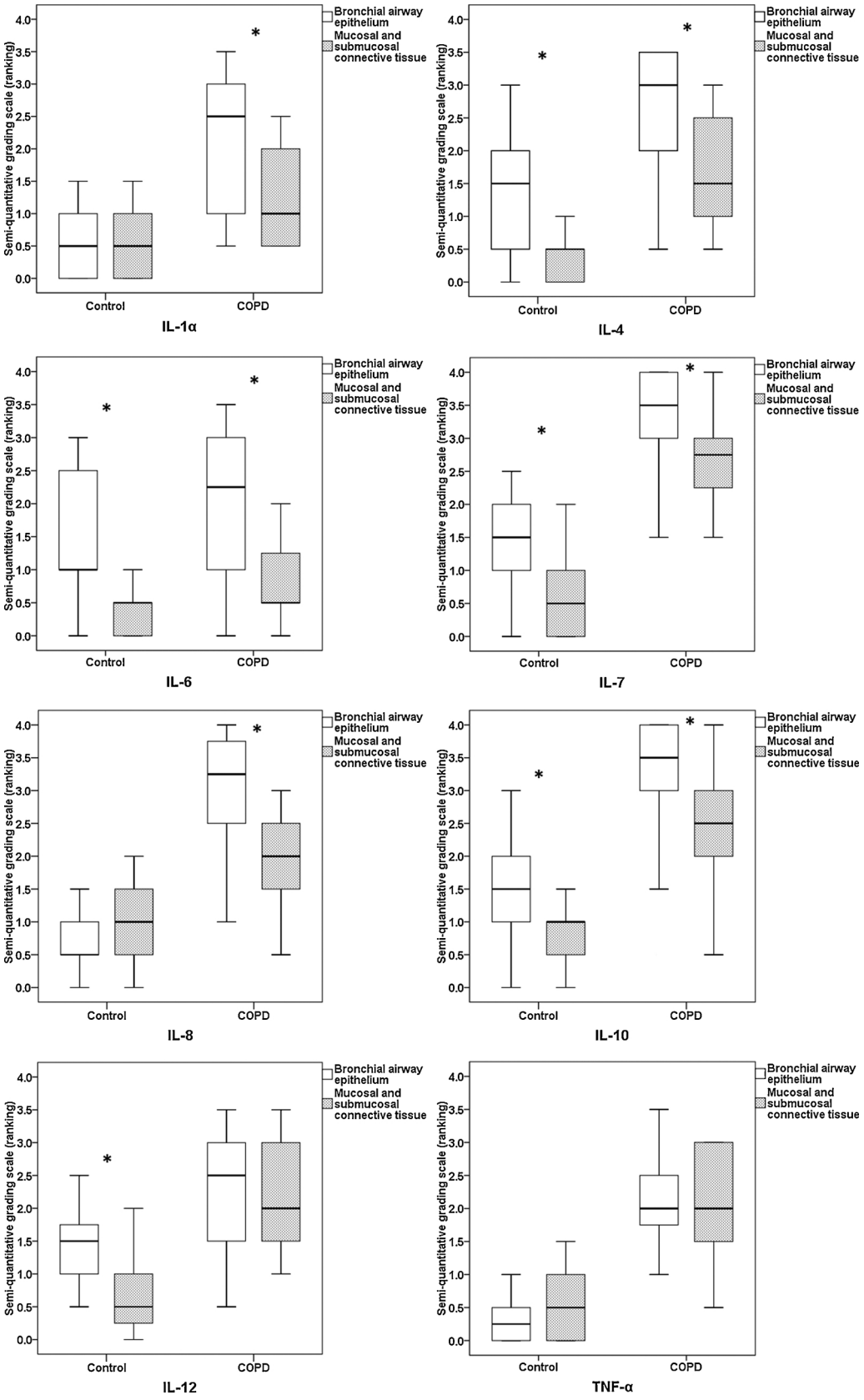

Fig. 2. Evaluation of IL-1α, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and TNF-α immunoreactive cells by semi-quantitative grading in the lung tissue of control group and COPD group. Semi-quantitative grading scores are displayed with rank values. "0″ – no positive (0) immunoreactive cells, "0.5" – rare (0/+), "1.0" – few (+), "1.5" – few to a moderate (+/++), "2.0" – moderate (++), "2.5" – moderate to numerous (++/+++), "3.0" – numerous (+++), "3.5" – numerous to abundance (+++/++++), "4.0" – abundance (++++) of immunoreactive cells. "Control" – Control group, "COPD" – COPD group, "*" – statistically significant difference ($P < 0.05$) by the Wilcoxon matched pairs Signed Rank Test.

Statistically significant higher numbers of immunoreactive cells positive for IL-4 were observed in epithelium of both control and COPD groups; moreover, more pronounced IL-4 findings were associated with COPD compared to control group. Within the findings observed by routine histological analysis in our study, goblet cell hyperplasia, bronchial gland hypertrophy and hyperplasia, smooth muscle cell hyperplasia and hypertrophy, as well as fibrosis must be pointed out. Apart from the suggestive role of IL-4 in allergic immune responses

[42], the signalling of IL-4 is related also with COPD pathogenesis, where numerous immune cells show secretory properties of IL-4. Plasma cells associated to mucus producing glands express IL-4 and these likely promote mucus hypersecretion in chronic bronchitis and COPD [43]. Furthermore, CD8 + T cells produce IL-4 leading to mucus hypersecretion occurring as characteristic of chronic bronchitis [44]. Mast cells in airway tissue produce IL-4 that influences T cell responses, mucus gland hyperplasia, smooth muscle cell hypertrophy and hyperplasia, thus indicating the significance of IL-4 in COPD [16]. Considering this in account, we may propose IL-4 is associated with the various degrees of histopathological findings in COPD with more pronounced source in epithelium.

Although we found statistically significant ($P < 0.05$) differences in the findings of IL-6 immunoreactive cells between airway epithelium and connective tissue, as well as between control and COPD group, still similar ranges of semi-quantitative data for IL-6 were observed (Table 1, Fig. 2) possessing a broad spectrum and variability of results. Compared to healthy subjects and asthma patients, cytokine IL-6 level is increased in COPD [45]. IL-6 is a pleiotropic cytokine with multiple functions, moreover, cytokine IL-6 role is highly dependent on the presence of other mediators as IL-6 may achieve controversial anti-inflammatory and pro-inflammatory functions due to the local signalling environment [3]. Numerous inflammatory effects of IL-6 are described in lungs, where effects of prominent T cell infiltration, increased mucus secretion, mast cell proliferation and up-regulation are determined. Moreover, IL-6 signalling mediates pro-inflammatory and immunomodulatory role of various immune cells [17]. IL-6 production is markedly enhanced by TNF-α in human lung epithelial cells and fibroblasts indicating its inflammatory role within the presence of TNF-α [46]. IL-6 is selectively associated with clinical outcomes of COPD, moreover, IL-6 is rather an indicative for the possible damage of airway epithelium [47]. In our study, statistically significant ($P < 0.05$) moderate ($|r_s| = 0.5–0.7$) correlations between the numbers of IL-6 immunoreactive cells and numbers of immunoreactive cells positive for inflammatory cytokines (IL-1α, IL-8) and anti-inflammatory cytokines (IL-10) in airway epithelium must be emphasized. Thus we may suggest IL-6 is associated with an equilibrium of inflammatory and anti-inflammatory signalling, moreover, within an engagement of airway epithelium in COPD.

Occasional (0/+) to few to moderate (+/++) numbers of IL-7 immunoreactivity were found in airway epithelium and connective tissue of control group, respectively. Numerous (+++) and numerous to abundance (+++/++++) IL-7 immunoreactive cells were found in COPD affected lung tissue. IL-7 is studied for its regulatory functions for T cell maintenance with the source of various immune and non-immune cells [19]. Cytokine IL-7 production sites have been detected in various immunity-associated cells, as well as endothelial cells, smooth mucle cells and fibroblasts. IL-7 may be associated with various survival patterns shared by multiple cell types focusing on the avoidance from cell death and affecting cell proliferation, thus providing protective role [48]. In submucosa of stable COPD and healthy smokers, the number of IL-7 immunoreactive cells was higher than of control non-smokers. Contrary to our data, no significant differences of IL-7 expression in bronchial epithelium were evaluated in patients with mild/moderate and severe COPD compared to healthy smoking and non-smoking control biopsies [49]. We found statistically significant ($P < 0.05$) moderate ($|r_s| = 0.5–0.7$) correlations between the numbers of IL-7 immunoreactive cells and IL-8 and IL-10 immunoreactivity in airway epithelium, as well as low (($|r_s| = 0.3–0.5$) correlation with IL-1α and IL-4 immunoreactivity. Since other evidence on the presence of IL-7 in COPD affected lung tissue is poor, increased IL-7 in airway epithelium is suggested to represent regulatory role.

We found mostly occasional (0/+) to few (+) IL-8 immunoreactive cells of control group airway epithelium and connective tissue, whereas data of COPD group marked moderate number (++) to numerous to abundance (+++/++++) of IL-8 immunoreactive cells found in airway connective tissue and epithelium, respectively. Notably, higher numbers of IL-8 were evaluated in airway epithelium of COPD group. Cytokine IL-8 participates in neutrophilic inflammation, where marked increase of IL-8 is estimated that further correlates with an increase of neutrophils [26]. One of the major sources for inflammatory cytokine IL-8 is lung airway epithelium [6,10], that promotes the findings of IL-8 immunoreactivity in airway epithelium presented by our study. Within other studies, normal human bronchial/tracheal epithelial cells showed an increased IL-8 production in response to onsite cigarette smoke, whereas COPD affected human bronchial/tracheal epithelial cells had decreased IL-8 production indicating airway epithelial cell impaired capacity to respond to continuous and ongoing cigarette smoke [10]. When compared to non-smoking and even smoking control group, IL-8 release is determined to be higher in COPD patients [50]. The findings of IL-8 in our study supports the role of IL-8 in inflammation associated with COPD, moreover, dependent of airway epithelium.

Overall few (+) to few to moderate (+/++) numbers of IL-10 immunoreactive cells were evaluated in control group; moreover, moderate to numerous (++/++++) up to numerous to abundance (+++/++++) of IL-10 immunoreactive cells were evaluated in COPD group. For both groups, more pronounced findings were observed in airway epithelium. IL-10 is a strong anti-inflammatory cytokine, where the release of IL-10 maintain suppressive effect on inflammatory processes [51]. IL-10 modulates neutrophilic inflammation induced by cigarette smoke by being an endogenous suppressor of airway neutrophilic inflammation [52]. The findings of increased anti-inflammatory cytokine IL-10 point to its protective role to balance inflammation and tissue damage that follows the pattern of inflammatory cytokines produced mostly by airway epithelium.

No statistically significant difference was observed between the immunoreactive cells in airway epithelium and connective tissue of both control and COPD group. However, we observed more pronounced findings of TNF-α in COPD group compared to control group. Pro-inflammatory cytokine TNF-α released by airway epithelial cells and macrophages initiates effector phase of innate immune responses [51]. Furthermore, TNF-α induces the release of other pro-inflammatory cytokines (e.g. IL-8 from immune cells, airway epithelial cells and connective tissue fibroblasts) having a wide range of pro-inflammatory properties [6,46]. Due to the stimulation with cytokines (e.g. IL-6, IL-8, TNF-α), various immune cells participate in ongoing inflammatory responses with further remodeling of airway wall [53]. In the lungs of COPD animal model, IL-6 and TNF-α showed an increased expression when compared to normal control [54]. High levels of IL-8 and TNF-α in lung tissue of COPD patients may indicate exaggerated inflammatory responses. In the inflammatory environment, pro-inflammatory cytokine TNF-α escapes physiological suppressive control by high levels of anti-inflammatory cytokine IL-10 in bronchial tissue [55]. All findings may suggest the widespread distribution of increased TNFα regardless of a certain location.

In COPD group, we found mostly moderate to numerous (++/+++) numbers of cells immunoreactive for IL-12. No statistically significant difference was estimated between the numbers of IL-12 immunoreactive cells in airway epithelium and connective tissue of COPD affected lung tissue. Importantly, exposure to cigarette smoke induces more extensive cytokine IL-12 local release in lung tissue [56]. Cigarette smoke exposure may initiate local production of IL-12 derived from airway epithelium, dendritic cells and tissue macrophages [57]. We found statistically significant ($P < 0.05$) moderate ($|r_s| = 0.5–0.7$) correlations between the numbers of IL-12 immunoreactive cells and IL-8 immunoreactivity in airway epithelium and connective tissue. Hereby we suggest IL-12 may be associated with inflammatory cytokine IL-8, therefore pro-inflammatory role may be accepted for IL-12 in COPD. Interestingly, an increase of IL-8 may stabilize inflammatory responses by decreased IL-12 (determined by findings of negative correlation), however, only in airway epithelium.

Numerous biomarkers from investigations of non-invasive COPD

samples like sputum, bronchoalveolar lavage (BAL) and blood plasma have been evaluated in most of the studies, however, the local findings of expressed signalling molecules are limited due to the invasive nature of an acquirance of airway biopsies and tissue samples [1]. Moreover, histopathological findings are independent of factors affecting non-invasive samples. Hereby local findings of the airway wall provide valuable information about tissue structural damage. This approach provides wider investigation and the relevance of biomarkers in the pathogenesis of COPD.

5. Conclusions

Increased numbers of IL-1α, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and TNF-α immunoreactive cells suggest the extensive presence and local significance of these markers in COPD pathogensis. Increased surge in the numbers of IL-1α and IL-8 immunoreactive cells within bronchial airway epithelium may promote the location-dependent signalling important for the possible initiation and maintenance of COPD. When compared to control group, stable increase of the numbers of IL-4, IL-6, IL-7, and IL-10 immunoreactive cells in both COPD affected bronchial airway epithelium and underlying connective tissue may excel pattern of widely distributed and more pronounced signalling of these factors to shape inflammatory responses. Moreover, the pattern with dominance of immunoreactive cells in COPD affected airway epithelium over connective tissue is highlighting the essentials of epithelium in inflammatory signalling. Homogenous increase of the numbers of IL-12 and TNF-α immunoreactive cells regardless of location may indicate co-dependent balance of the release of these mediators in COPD affected lung tissue.

Conflict of interest statement

The authors state that there are no conflicts of interest regarding the publication of this article.

Author contributions

This work was conducted as a collaboration between all the authors. All authors read and approved the final manuscript.

Acknowledgment

None.

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