UNIVERSITÀ
DEGLI STUDI
DI PALERMO

Dottorato di Ricerca internazionale in BIOMEDICINA E NEUROSCIENZE
Indirizzo: Medicina Sperimentale e Molecolare
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IL-17A induces chromatin remodeling promoting IL-8 and TSLP release in bronchial epithelial cells. Effect of Tiotropium.

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TRIENNIO 2013-2015

Introduction

The chronic obstructive pulmonary disease (COPD) is defined as a:

... common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients.

COPD involves chronic inflammation of the peripheral airways and lung parenchyma, which leads to progressive narrowing of the airways and shortness of breath. This inflammation is resistant to treatment with corticosteroids and there are currently no safe and effective alternative anti-inflammatory treatments (1).

Asthma and chronic obstructive pulmonary disease (COPD) are both very common and their incidence is increasing globally, placing an increasing burden on health services in industrialized and developing countries. Both diseases are characterized by airway obstruction, which is variable and reversible in asthma but is progressive and largely irreversible in COPD. In both diseases, there is chronic inflammation of the respiratory tract, which is mediated by the increased expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors. In both diseases there are acute episodes or exacerbations, when the intensity of this inflammation increases. The similarity between these airway diseases prompted the suggestion in the 1960s that asthma and COPD are different forms of a common disease (chronic obstructive lung disease) (Barnes 2008).

The main types of COPD are the development of small-airway obstruction and emphysema, which can occur alone or together, but which both involve progressive airflow limitation and are usually caused by tobacco smoke. The differences in inflammation between asthma and COPD are linked to differences in the immunological mechanisms that underlie these two diseases. The appreciation that similar immune mechanisms are involved in both asthma and COPD has important implications for the development of new therapies for these troublesome diseases.

Inflammation is present in the lungs, particularly the small airways, of all people who smoke. This normal protective response to the inhaled toxins is amplified in

COPD, leading to tissue destruction, impairment of the defence mechanisms that limit such destruction, and disruption of the repair mechanisms. In general, the inflammatory and structural changes in the airways increase with disease severity and persist even after smoking cessation. Besides inflammation, two other processes are involved in the pathogenesis of COPD—an imbalance between proteases and antiproteases and an imbalance between oxidants and antioxidants (oxidative stress) in the lungs.

There is evidence for familial susceptibility in both COPD and lung cancer. This familial susceptibility appears to be linked and not just associated with the common consumption of cigarettes; implying that the underlying genetic predisposition to both diseases may be the same or reflect the link between the immune system, inflammation and cancer. Linkage studies have implicated regions in chromosome 6 as being linked to both diseases. Furthermore, GWAS studies in large COPD and lung cancer cohorts have found the same risk loci including *CHRNA3* and *CHRNA5* SNPs (15q) and regions at 4q31 (*HHIP*), 4q24 (*FAM13A*) and 5q (*HTR4*). Nicotine addiction may explain the overlap in risk loci between lung cancer, smoking behavior and COPD. Epithelial to mesenchymal transition (EMT) and inflammation are pathogenic features of COPD and lung cancer and the rs7326277TT genotype in *VEGFR1*, which promotes inflammation, EMT and tumor growth, is a susceptible locus for both COPD and lung cancer. Several studies have demonstrated that polymorphisms in the anti-inflammatory gene *IL10* are associated with increased rates of lung and other cancer. (Durham AL 2015).

Increased production (or activity) of proteases and inactivation (or reduced production) of antiproteases results in imbalance. Cigarette smoke, and inflammation itself, produce oxidative stress, which primes several inflammatory cells to release a combination of proteases and inactivates several antiproteases by oxidation. The main proteases involved are those produced by neutrophils (including the serine proteases elastase, cathepsin G, and protease 3) and macrophages (cysteine proteases and cathepsins E, A, L, and S), and various matrix metalloproteases (MMP-8, MMP-9, and MMP-12). The main antiproteases involved in the pathogenesis of emphysema include α_1 antitrypsin, secretory leucoprotease inhibitor, and tissue inhibitors of metalloproteases.

The oxidative burden is increased in COPD. Sources of oxidants include cigarette smoke and reactive oxygen and nitrogen species released from inflammatory cells. This creates an imbalance in oxidants and antioxidants of oxidative stress. Many markers of oxidative stress are increased in stable COPD and are further increased in exacerbations. Oxidative stress can lead to inactivation of antiproteases or stimulation of mucous production. It can also amplify inflammation by enhancing transcription factor activation (such as nuclear factor κB) and hence gene expression of pro-inflammatory mediators.

Mucous hypersecretion results in a chronic productive cough. This is characteristic of chronic bronchitis but not necessarily associated with airflow obstruction, and not all patients with COPD have symptomatic mucous hypersecretion. The hypersecretion is due to squamous metaplasia, increased numbers of goblet cells, and increased size of bronchial submucosal glands in response to chronic irritation by noxious particles and gases. Ciliary dysfunction

is due to squamous metaplasia of epithelial cells and results in an abnormal mucociliary escalator and difficulty in expectorating.

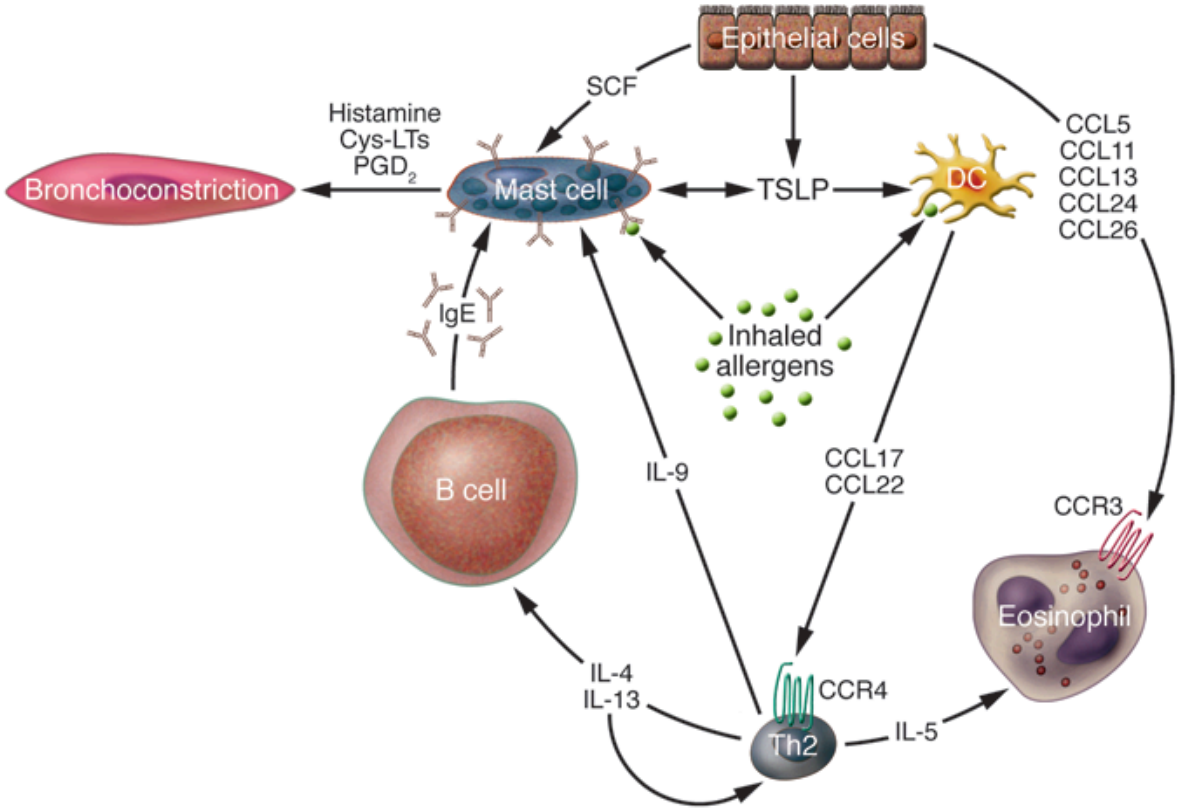
The main site of airflow obstruction occurs in the small conducting airways that are < 2 mm in diameter. This is because of inflammation and narrowing (airway remodelling) and inflammatory exudates in the small airways. Other factors contributing to airflow obstruction include loss of the lung elastic recoil (due to destruction of alveolar walls) and destruction of alveolar support (from alveolar attachments).

The airway obstruction progressively traps air during expiration, resulting in hyperinflation at rest and dynamic hyperinflation during exercise. Hyperinflation reduces the inspiratory capacity and therefore the functional residual capacity during exercise. These features result in breathlessness and limited exercise capacity typical of COPD. The airflow obstruction in COPD is best measured by spirometry and is a prerequisite for its diagnosis.

Gas exchange abnormalities occurs in advanced disease and are characterised by arterial hypoxaemia with or without hypercapnia. An abnormal distribution of ventilation: perfusion ratios due to the anatomical changes found in COPD is the main mechanism for abnormal gas exchange. The extent of impairment of diffusing capacity for carbon monoxide per litre of alveolar volume correlates well with the severity of emphysema (Mac Nee, 2006).

There are many differences between mild asthma and COPD in the type of inflammation that occurs in the lungs, with a different range of inflammatory cells and mediators being implicated. However, many of the cytokines and chemokines that are secreted in both asthma and COPD are regulated by the transcription factor nuclear factor-κB (NF-κB), which is activated in airway epithelial cells and macrophages in both diseases, and may have an important role in amplifying airway inflammation (Barnes 2008).

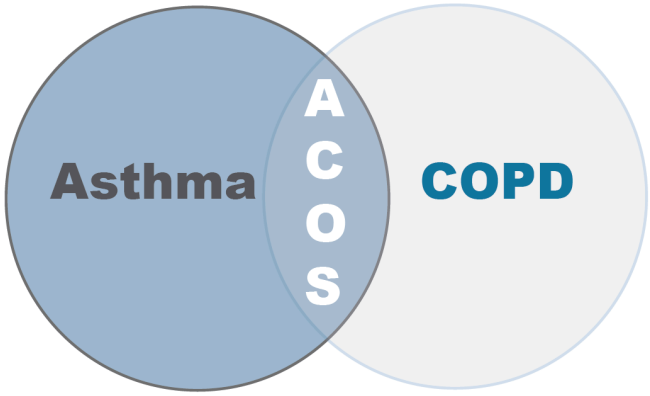
Approximately 10% of patients with COPD have a reversibility of bronchoconstriction, showing greater than 12% improvement in lung function as assessed by forced expiratory volume in 1 second (FEV1), and therefore behave more like asthmatics. Furthermore, compared with most patients with COPD, these patients more frequently have eosinophils in their sputum, an increase in exhaled nitric oxide and respond better to corticosteroid treatment, all of which are characteristic features of asthma. It therefore seems likely that these patients have concomitant asthma and COPD (Barnes 2008).



Epithelial cells are activated by cigarette smoke and other inhaled irritants, such as biomass fuel smoke, to produce inflammatory mediators, including tumor necrosis factor (TNF) alpha, interleukin (IL)-1 beta, IL-6, granulocyte-macrophage colony–stimulating factor (GM-CSF), and CXCL8 (IL-8). Epithelial cells in small airways may also be an important source of transforming growth factor (TGF) beta which then induces local fibrosis. Vascular endothelial growth factor (VEGF) seems to be necessary to maintain alveolar cell integrity, and blockade of VEGF receptors (VEGFR2) in rats induces apoptosis of alveolar cells and an emphysemalike disorder. Airway epithelial cells are also important in defense of the airways, with mucus production from goblet cells, and secretion of antioxidants, antiproteases, and defensins. It is possible that cigarette smoke and

other noxious agents may impair these responses of the airway epithelium, increasing susceptibility to infection. The airway epithelium in chronic bronchitis and COPD often shows squamous metaplasia, which may result from increased proliferation of basal airway epithelial cells but the nature of the growth factors involved in epithelial cell proliferation, cell cycle, and differentiation in COPD are not yet certain. Epithelial growth factor receptors (EGFR) show increased expression in airway epithelial cells of patients with COPD and can contribute to basal cell proliferation, resulting in squamous metaplasia and an increased risk of bronchial carcinoma (Barnes 2014).

Overlap Syndrome



Asthma and COPD are two different diseases.

Asthma-chronic obstructive pulmonary disease overlap syndrome (ACOS) is a loosely-defined clinical entity referring to patients who exhibit characteristics of both asthma and chronic obstructive pulmonary disease (COPD).

It has been well recognized that specific phenotypes exist in patients with COPD and asthma. It has been shown that patients with ACOS have increased reversibility of airflow, more exacerbations and more severe dyspnoea. Identifying patients with ACOS seems therefore relevant for the management of their disease.

ACOS has been defined as two clinical phenotypes: asthma with partially reversible airflow obstruction, with or without emphysema or reduced carbon monoxide diffusion capacity (DLCO) to less than 80% predicted; and COPD with emphysema accompanied by reversible or partially reversible airflow obstruction with or without environmental allergies or reduced DLCO. A document from Global initiative for chronic obstructive lung disease (GOLD) and GINA described ACOS as persistent airflow limitation with several features usually associated with asthma and several features usually associated with COPD

[GINA-GOLD, 2015]. When a patient has similar numbers of features of asthma and COPD, the diagnosis of ACOS could be considered (Slats A. 2015).

The estimated prevalence of ACOS ranges from 12.1% to 55.2% among patients with COPD and 13.3%–61.0% among patients with asthma alone. In the general population of Italy, the prevalence of ACOS was 4.5% in the 65–84 age group.

In COPD, eosinophilic inflammation has been shown during exacerbations both in induced sputum as well as in bronchial biopsies. In addition, blood eosinophilia was associated with increased mortality. Patients with COPD and eosinophilic inflammation during exacerbation usually have increased concentrations of peripheral eosinophils in stable disease as well. Some studies identified a set of airway epithelial genes that are altered in asthma, and in some current and former smokers with COPD. This 'Th2 gene signature', associated with asthma-like inflammation, is also expressed in COPD and is associated in those patients with eosinophilic inflammation, reversibility and favourable corticosteroid response as reflected by more improvement in hyperinflation. Improvement in hyperinflation in COPD is important since it is related to dyspnoea and exercise tolerance.

Airway wall remodelling refers to alterations in the distinct aspects of the airway wall, that is, mucosal oedema, airway smooth muscle hypertrophy and hyperplasia, and thickening of basal membrane. This leads to airway wall thickening and altered airway mechanics. Airway remodelling occurs throughout the whole respiratory tract, including small airways. Both in asthma and COPD

there is evidence of airway wall thickening and remodelling, however the degree of changes in specific structures of the airway wall differ between asthma and COPD. In lots of studies, increased bronchial wall thickening has been demonstrated on high-resolution computed tomography scans in patients with overlap syndromes compared with COPD alone, but has not been compared in patients with asthma alone. In a small group of nonsmoking patients with asthma, a decrease in lung elastic recoil was demonstrated, with microscopic centrilobular emphysema in three autopsied patients. However, there are no data yet on whether the specific structures of airway remodelling differ in overlap syndrome compared with asthma and COPD alone.

Data on medical treatment of patients with ACOS is rare because these patients has been systematically excluded from both COPD and asthma pharmacological trials.

Understanding the reasons for differences in prevalence estimates of ACOS across the literature can help guide decision making on the most appropriate criteria for defining ACOS and aid investigators in designing future ACOS clinical studies aimed at effective treatment.

Cigarette smoke

Cigarette smoking is the main risk factor in COPD and results in the imbalance of oxidant and antioxidant and increased airway inflammation in alveolar macrophages.

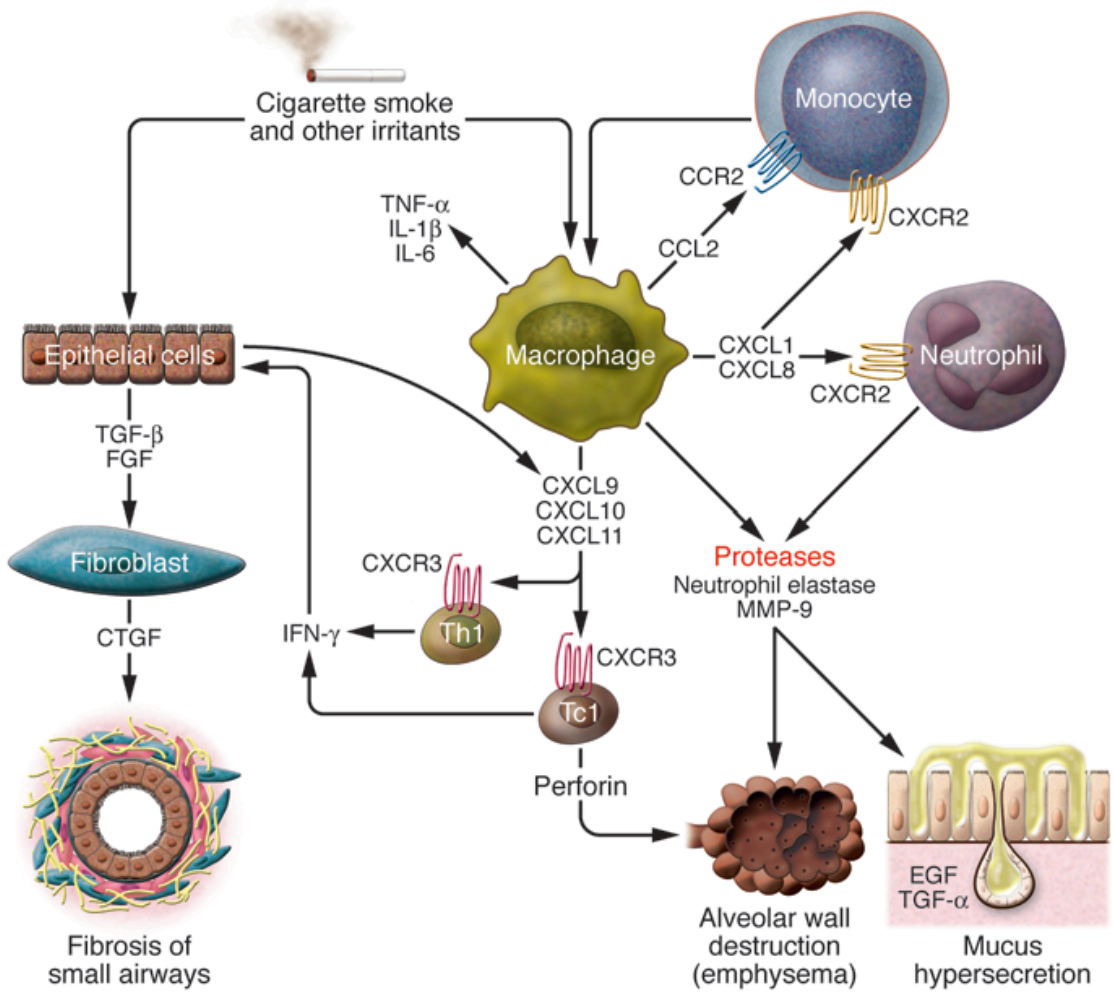
Chronic airway inflammation is an archetypal feature of COPD, and increased oxidative stress has been suggested to be responsible for triggering inflammatory events observed within the lungs of smokers and COPD patients.

The majority of smokers know that smoking is harmful to their health; and it is estimated that 50 % of smokers with COPD are amenable to smoking cessation support. However, results from smoking cessation intervention studies comparing smokers with and without COPD have yielded ambiguous results. Higher, equal and lower smoking cessation rates in smokers with COPD were found compared to smokers without COPD. Nevertheless, the prevalence of smoking in patients with COPD is still high and exceeds the rate of smoking in the general population. A large population-based study showed that the prevalence of smoking currently was 35 % among patients with COPD compared with 22 % among patients without COPD. This implies that when patients get a diagnosis of COPD many still continue smoking even though quitting is their best treatment option. Their chances of quitting might partly be reduced because of a higher level of tobacco addiction and susceptibility to develop depressive symptoms. Therefore, smokers

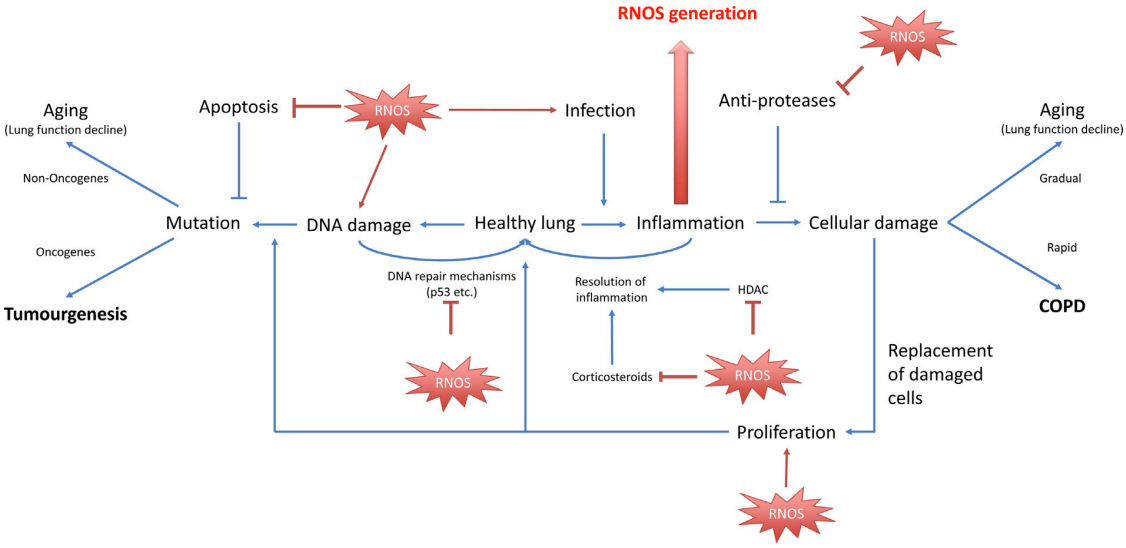
with COPD seem to be a special subgroup of smokers that have a more urgent need to quit smoking, but might find it more difficult to do so.

Although the precise mechanisms behind the pathogenesis of COPD are yet to be fully dissected, the current hypothesis suggests that cigarette smoke causes airway inflammation by activating macrophages, neutrophils, and T lymphocytes, which release proteases and reactive oxygen species (ROS) leading to cellular injury. As a consequence, chronic inflammatory processes are triggered that lead to small airway obstruction. An estimated 10 to 15 % of all smokers develop clinically significant airflow obstruction (van Eerd EA 2015).

An increased oxidant burden in smokers may be derived from the fact that cigarette smoke contains an estimated 1017 oxidants/free radicals and 4,700 chemical compounds, including reactive aldehydes (carbonyls) and quinones, per puff. Many of these are relatively long-lived, such as tar-semiquinone, which can generate hydroxyl radicals (OH) and H2O2 by the Fenton reaction. One consequence of this increased oxidative stress is activation of redox-sensitive transcription factors, such as NF-κB and activator protein-1 (AP-1), which are critical to transcription of proinflammatory genes (IL-8, IL-6, and TNF-α) (Yang SR. 2006).



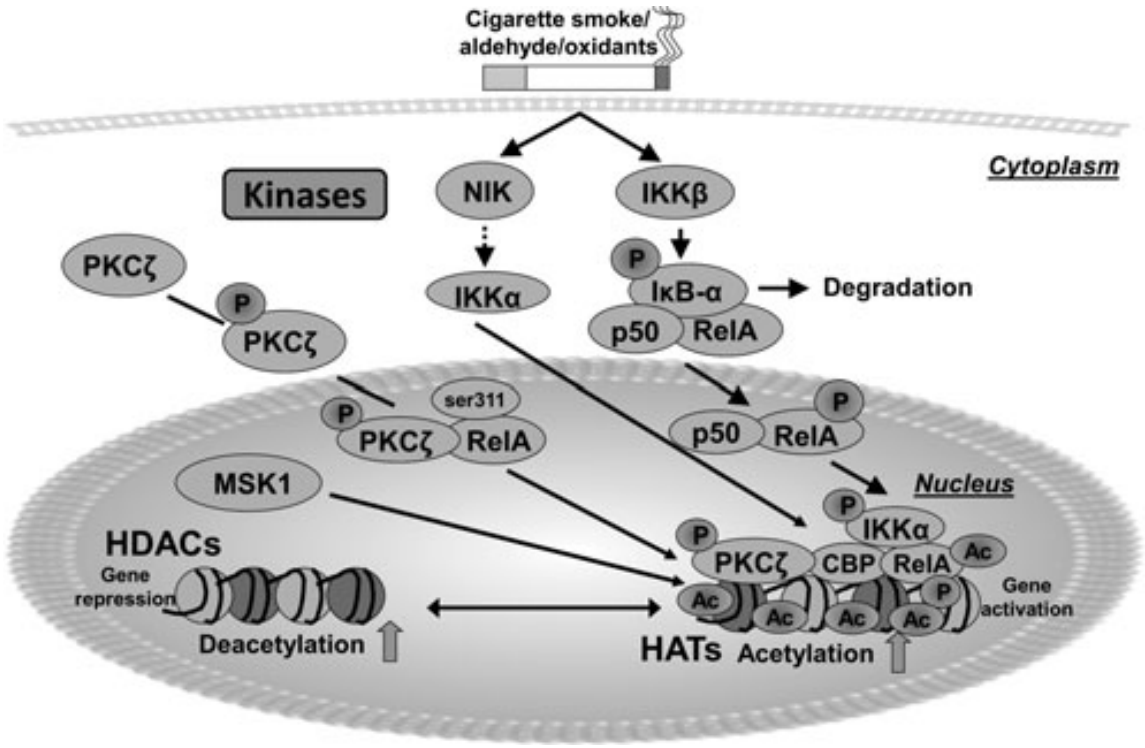
In addition to exogenous reactive nitrogen and oxygen species (RNOS), mitochondrial respiration is a major source of RNOS generation and mitochondrial dysfunction is present in many cancers. RNOS damage cells through a number of mechanisms including DNA damage (especially mitochondrial DNA) lipid peroxidation, oxidation of amino acids and oxidation of inorganic enzyme co-factors (Durham AL 2015).



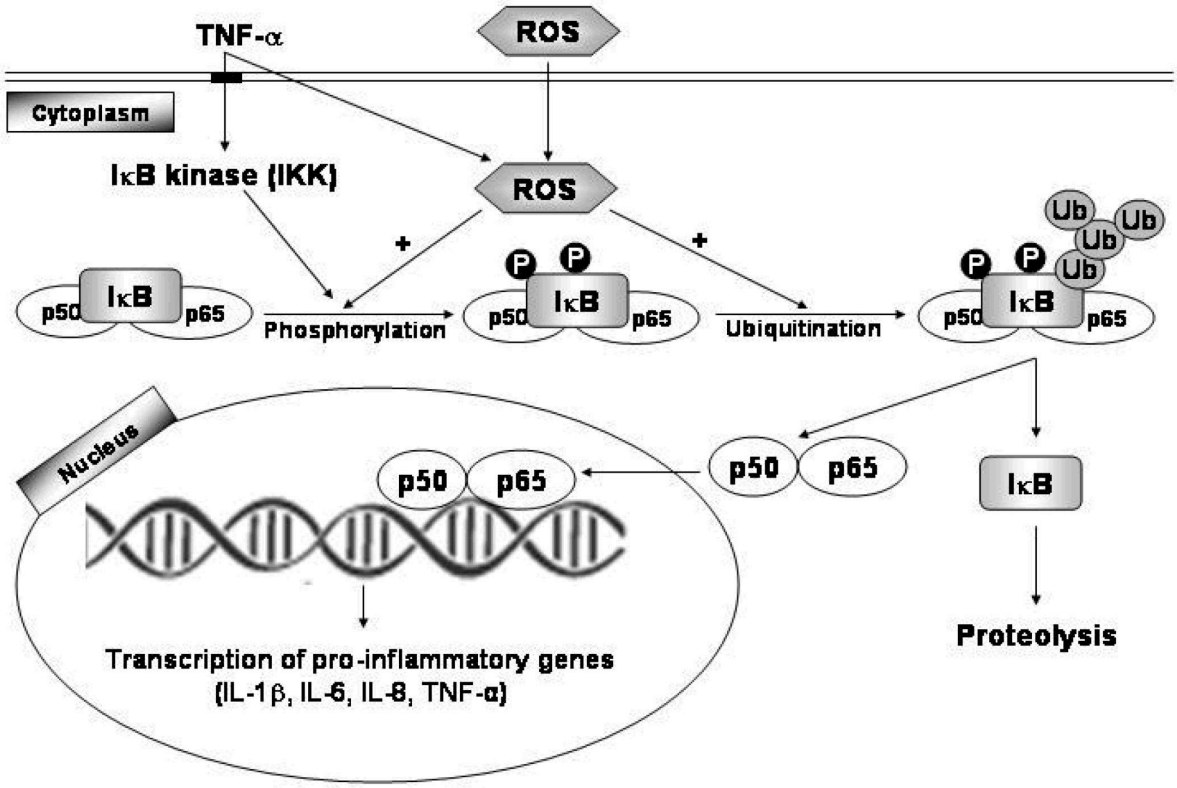
Oxidative Stress

In the airways, oxidative stress, inflammation and protease-antiprotease imbalance represents the classic pathologic triad associated with chronic obstructive pulmonary disease (COPD) (Barnes 2014).

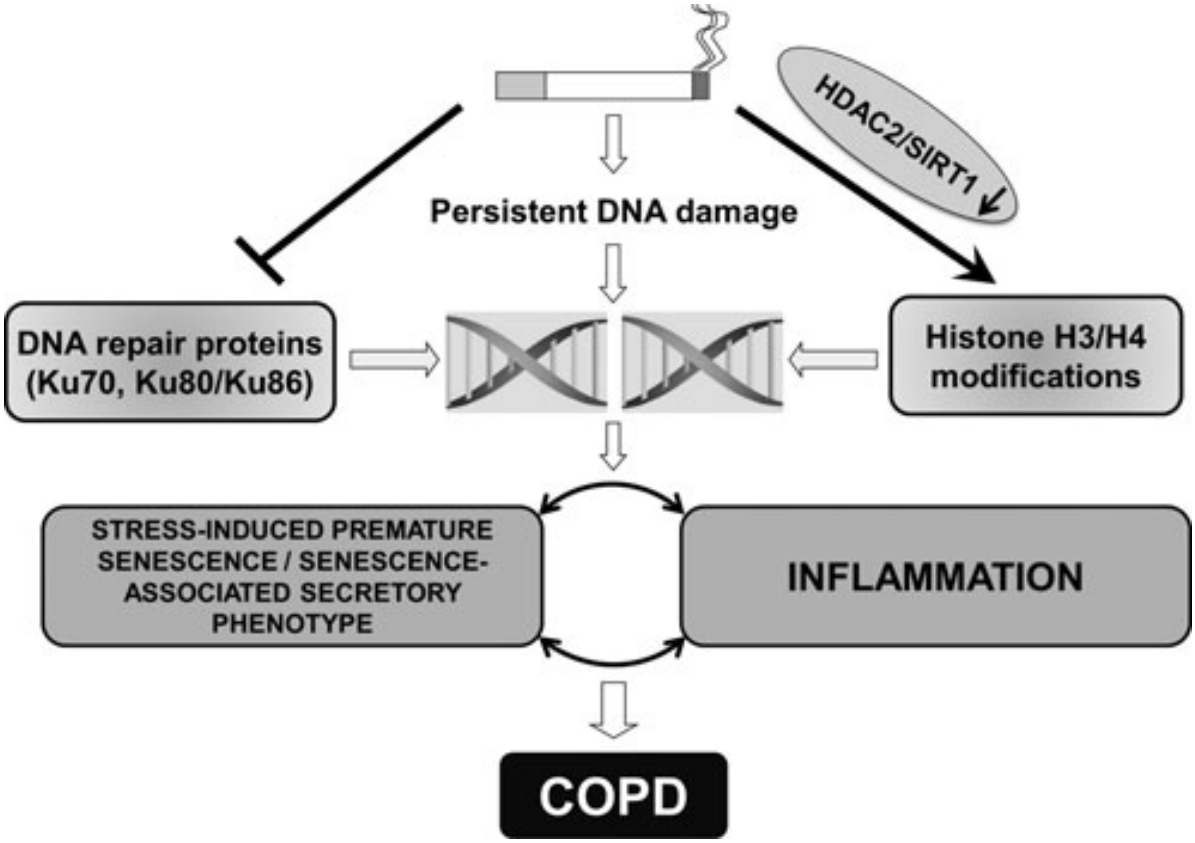
Oxidative stress has been implicated in cell and tissue damage associated with many chronic inflammatory lung diseases such as asthma, COPD, idiopathic pulmonary fibrosis (IPF) and adult respiratory distress syndrome (ARDS). Increased ROS production has been directly connected to oxidation of protein, DNA, and lipids that may cause direct lung injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species.



The pathogenesis of many forms of lung injury has implicated peroxidative breakdown of polyunsaturated fatty acids due to the effects on membrane function, inactivation of membranebound receptors and enzymes, and increased tissue permeability. There is increasing evidence that aldehydes, generated endogenously during the process of lipid peroxidation, are involved in many of the pathophysiological events associated with oxidative stress in cells and tissues. In addition to their cytotoxic properties, lipid peroxides are increasingly recognized as being important in signal transduction for a number of important events in the inflammatory response of the lungs.



ROS can alter the remodeling of extracellular matrix, apoptosis and mitochondrial respiration, cell proliferation, maintenance of surfactant and the antiprotease screen, effective alveolar repair responses and immune modulation in the lung. ROS secreted by phagocytes that have been recruited to sites of inflammation, are a major cause of the cell and tissue damage associated with many chronic inflammatory lung diseases such as asthma, COPD, IPF and ARDS. All these diseases involve the recruitment of immune and inflammatory cells to the lungs. These cells are activated and produce mediators of inflammation including ROS and cytokines, such as the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α).



The response of a cell or organism to an increase in ROS normally involves the activation of numerous intracellular signaling pathways. These cytosolic pathways can regulate a host of transcriptional changes that allow the cell to respond appropriately to the perceived oxidative stress. In addition to the regulation achieved by classical cytosolic signaling pathways, such as the family of mitogen-activated protein kinases, evidence suggests that certain transcription factors can directly or indirectly alter their activity, depending on cellular redox conditions (Rajendrasozhan S., 2008) .

RNOS stimulates the production of inflammatory mediators either directly or indirectly. Cells directly detect RNOS via the ROS receptor/proto-oncogene

ROS1. ROS1 activates the phosphoinositide 3-kinase (PI3K)-mTOR signaling pathway and other proteins related to cell differentiation, proliferation, growth and survival including AKT1, MAPK1, MAPK3, IRS1 and PLCG2. Furthermore, ROS activates NF-κB thereby upregulating the expression of numerous immune and inflammatory genes.

RNOS can alter protein structure and function by modifying amino acid residues, inducing protein dimerization, and interacting with Fe-S moieties or other metal complexes. In COPD these post-translational mechanisms include the nitration of histone deacetylase (HDAC) 2, leading to its inactivation and degradation resulting in the prolonged inflammatory phenotype seen in patients. Additionally, RNOS can modify various proteins, rendering them auto-antigenic (i.e., immunoinflammatory). Therefore oxidative stress can be a key cause of both proliferation (lung cancer) and inflammation (COPD) in the lungs (Durham AL 2015).

Inflammatory mediators

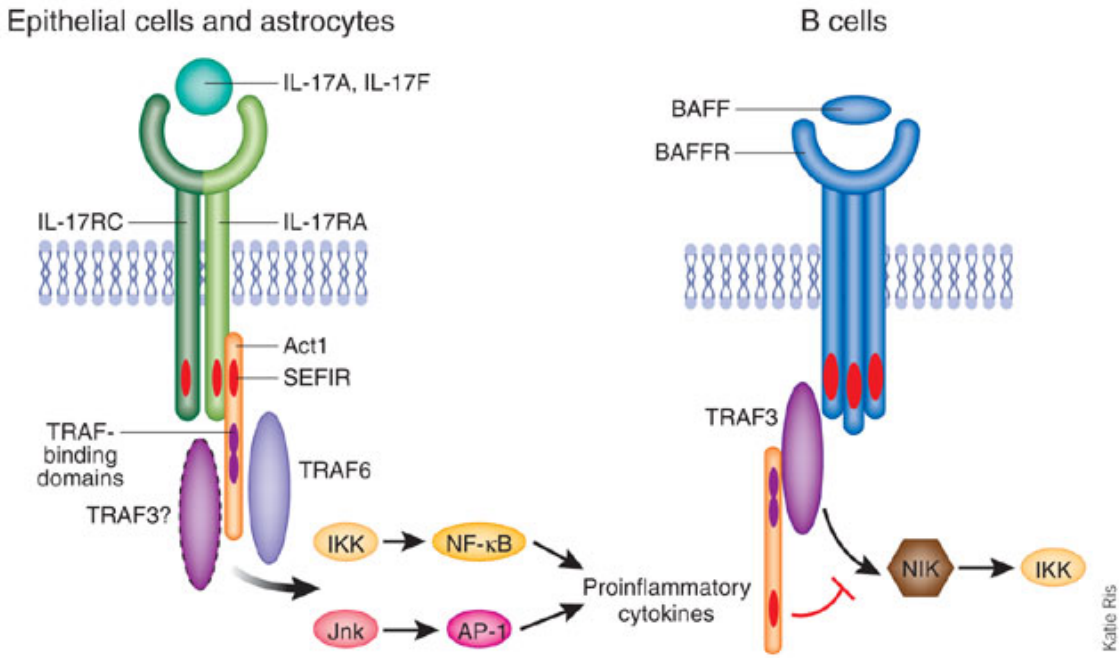
The degree of inflammation in patients with COPD increases as the disease progresses, with increased numbers of neutrophils, macrophages and lymphocytes (Hogg, J. C. *et al.* 2004)

Chronic inhalation of irritants (for example, cigarette smoke, air pollutants…) initially activates pattern recognition receptors such as Toll-like receptors (TLRs) (Freeman, 2013), (Nadigel, 2011). This leads to the activation of an innate immune response, with increased numbers of neutrophils and macrophages as well as the activation of airway epithelial cells and mucus secretion. In later stages of the disease the adaptive immune response is activated, with increased numbers of T-and B lymphocytes, which may be organized into lymphoid follicles, and this involves an increase in the number and activation of dendritic cells (Vassallo, R. *et al.* 2010). The numbers of CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T helper 1 (TH1) cells are also increased, as well as CD4+ TH17 cells, which may further amplify neutrophilic inflammation (Majo, J. *et al* 2001; Di Stefano, A. *et al 2009;* Pridgeon, C. *et al. 2011)* .

IL-17 A

Interleukin (IL)-17A is increasingly recognized as an important regulator of cellular immunity and is conventionally considered to arise predominantly from the 'Th17' (T helper 17 cells)-specific subset of Th cells (CD4 cells), which is phenotypically and functionally distinct from Th-1 and Th-2 cells and from T regulatory (Treg) cells (Park H., 2005).

Most current evidence supports IL-17A as having an important role as a pro-inflammatory cytokine uniquely positioned at the interface of innate and adaptive immunity (Ouyang, W_2008). IL-17A induces the release of secondary pro-inflammatory chemokines and growth factors in most epithelial and mesenchymal cells leading, in turn, to the recruitment and accumulation of neutrophils [Laan, M 1999]. Specifically, it is known that IL-17A stimulates the production of C-X-C chemokines (such as chemokine (C-X-C motif) ligand 8, CXCL8), granulocyte-chemotactic protein-2 and growth-stimulatory cytokines such as granulocyte colony-stimulating factor (G-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Prause, O 2003; Jones, C 2002). IL-17A, in particular, promotes neutrophil activity locally in inflamed tissues as judged by increased activity of myeloperoxidase, neutrophil elastase and matrix metalloproteinase (MMP)-9 after local administration of recombinant IL-17 protein (Prause, O. 2004; Ivanov, S., 2007).



IL-17A has also been implicated in lung diseases including asthma. IL-17A promotes recruitment and survival of airway macrophages during allergen-induced airway inflammation (Sergejeva, S ; 2005). IL-17A is increased in asthmatic BALF, sputum and blood (Molet, S., 2001; Chakir, J., 2003) and increased immunoreactivity for IL-17A in the asthmatic airway submucosa is associated with impaired lung function (Chakir, J., 2003). In IL-17A knockout (KO) mice, the allergen-induced airway hyper-reactivity to methacholine is significantly reduced (Nakae, S 2002). Systemic blockade of IL-17A also inhibited the allergen-induced accumulation of neutrophils in the airway (Hellings, P.W., 2003). Moreover, IL-17A levels correlate with neutrophil counts in the sputum of moderate to severe asthmatics (Bullens, D.M., 2006); in addition, IL-17 induces epithelial cells to release CXCL8, a chemokine that is important for the attraction of neutrophils (Kim V 2008).

Serum IL-17A levels are increased in patients with stable COPD compared to healthy smokers and nonsmokers, increase with COPD stage, and are inversely correlated with predicted forced expiratory volume in 1 second (FEV_1) percentage. IL-17^+ neutrophils are present in induced sputum from patients with stable COPD, but it remains unclear whether the sputum levels of IL-17A are increased in patients with stable COPD. There is a significant increase in the number of IL-$17A^+$ immunoreactive cells in the bronchial submucosa of mild or moderate and severe COPD patients compared to control nonsmokers, and in the peripheral lungs of stable COPD patients compared to smokers with normal lung function and nonsmoking subjects (Caramori G.; 2014).

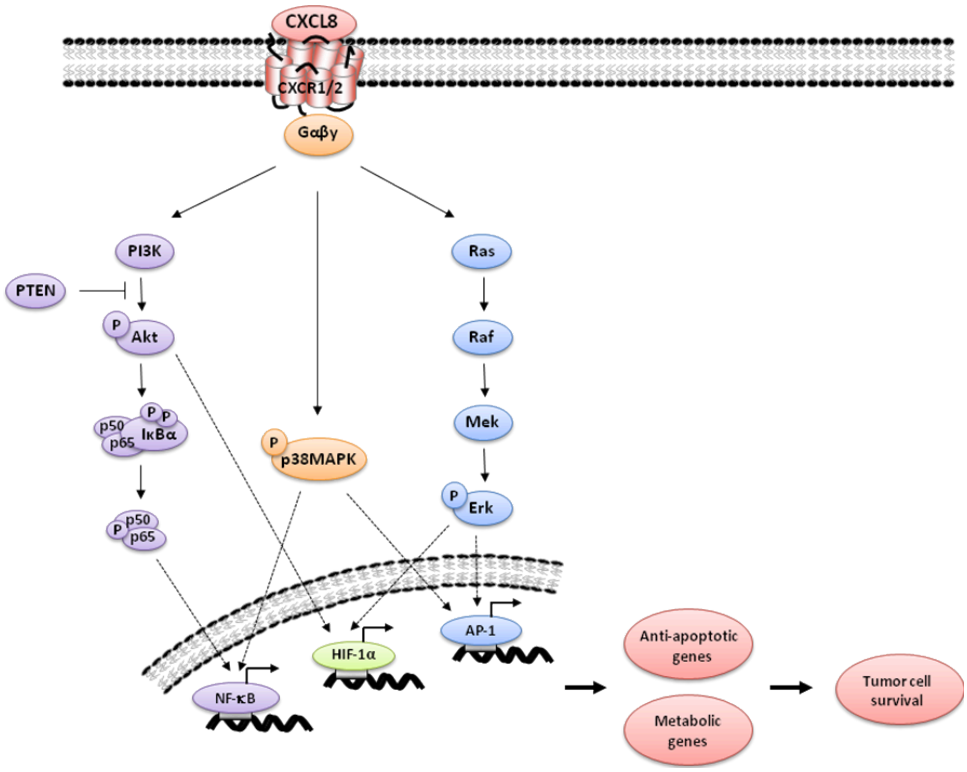
IL-8

Neutrophil accumulation in the airways of patients with COPD is driven by increased release of cytokines exerting a chemotactic effect on these cells. Among them, an important role may be played by tumour necrosis factor α (TNF-α) and interleukin 8 (IL-8 or CXCL 8). In addition, TNF-α and IL-8 levels are increased in the airways of patients with COPD, suggesting that these mediators may play an important role in the pathogenesis of the disease (Profita M., 2003).

CXCL8 levels are markedly elevated in the sputum of patients with stable COPD, and are correlated with disease severity; blocking antibodies to CXCL8 and related chemokines inhibits certain types of neutrophilic inflammation in experimental animals. The neutralization of CXCL8 with a blocking antibody

significantly reduces the neutrophil chemotactic activity of sputum from patients with stable COPD; however, this reduction is only partial, indicating that other neutrophil chemotactic factors, such as leukotriene B_4 and the activated complement factor C5a, are also involved. However, CXCL8 plays a major role in neutrophil chemotaxis caused by alveolar macrophage-derived conditioned media, and this is most effectively inhibited by dual antagonism of CXCR1 and CXCR2 receptors (Caramori G.; 2014)

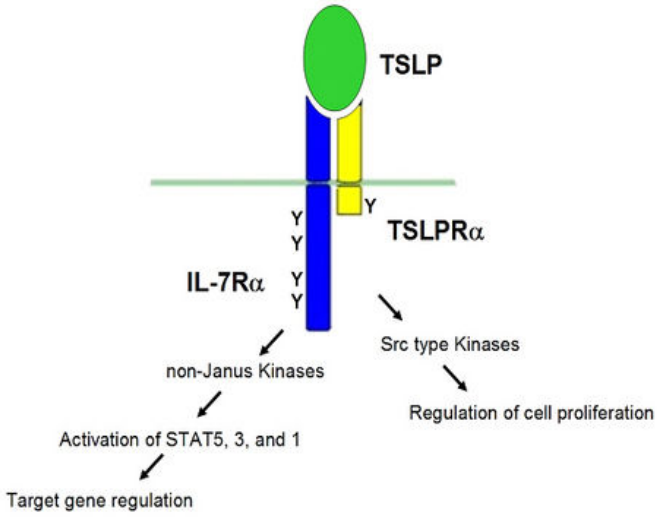
IKKa and IKKb are activated differently in asthmatic patients in comparison with patients with COPD and HSs and affected differently by the therapy (Gagliardo et al. (JACI 2011). These findings suggest that although IKKa and IKKb are both involved in the synthesis of IL-8, different proinflammatory circumstances might induce a different upstream regulation of the IKK/NF-kB pathway, associated with different pharmacologic response in patients with asthma and COPD. High levels of p-IKKa protein were present in patients with COPD and HSs, whereas they were present only in 4 asthmatic patients. The kinase activity assay demonstrated that IKKa activity was significantly higher in patients with COPD than in asthmatic patients and control subjects.



A number of recent studies have examined the effect of IL-17A on IL-8 secretion in airway epithelial cells and airway smooth muscle cells. Furthermore, it was observed that IL-17A induces epigenetic changes which in turn diminish the ability of glucocorticosteroids (GC) to inhibit IL-8 production from human bronchial epithelial cells (Albano G.D. 2013)

TSLP

TSLP is a cytokine of the IL-7 family, is produced mainly by stromal cells, including mast cells, and is involved in the activation, expansion, and survival of T lymphocytes and dendritic cells. TSLP expression in the airway epithelium is inducible through a NF-κB-dependent pathway in airway epithelium. Its action is mediated by a heterodimeric receptor composed of IL-7Rα and TSLP receptor (TSLPR). Some functions of TSLP and its receptor overlap that of IL-7 and its receptor, despite signaling predominantly through signal transducer and activator of transcription (STAT)-5 at variance with IL-7Rα, and thus this represents an alternative pathway to the IL-7/IL-7Rα axis. In human airway smooth-muscle cells, TSLPR signaling is mainly mediated by STAT3. In vitro TSLP and TSLP-R expression in human airway smooth-muscle cells is increased after chronic exposure to cigarette smoke extract, and TSLP is a mediator of cross talk between airway smooth-muscle and mast cells. TSLP and TSLP-R-blocking antibodies neutralize the increased contraction of airway smooth-muscle cells induced by cigarette smoke extract, suggesting a role for this pathway in bronchoconstriction.



TSLP has also been implicated in the induction of glucocorticoid resistance in Th cells during airway inflammation by controlling the phosphorylation of STAT5. In addition, TSLP may amplify alternatively activated airway macrophage polarization and chemokine production. An increased number of cells expressing TSLP mRNA has been reported in the bronchi of stable COPD patients and control smokers with normal lung function, and increased TSLP immunostaining has been shown in the smooth muscle of patients with stable COPD compared to nonsmoking subjects. Blocking antibodies have been developed, but there have been no studies on COPD so far (Caramori 2014).

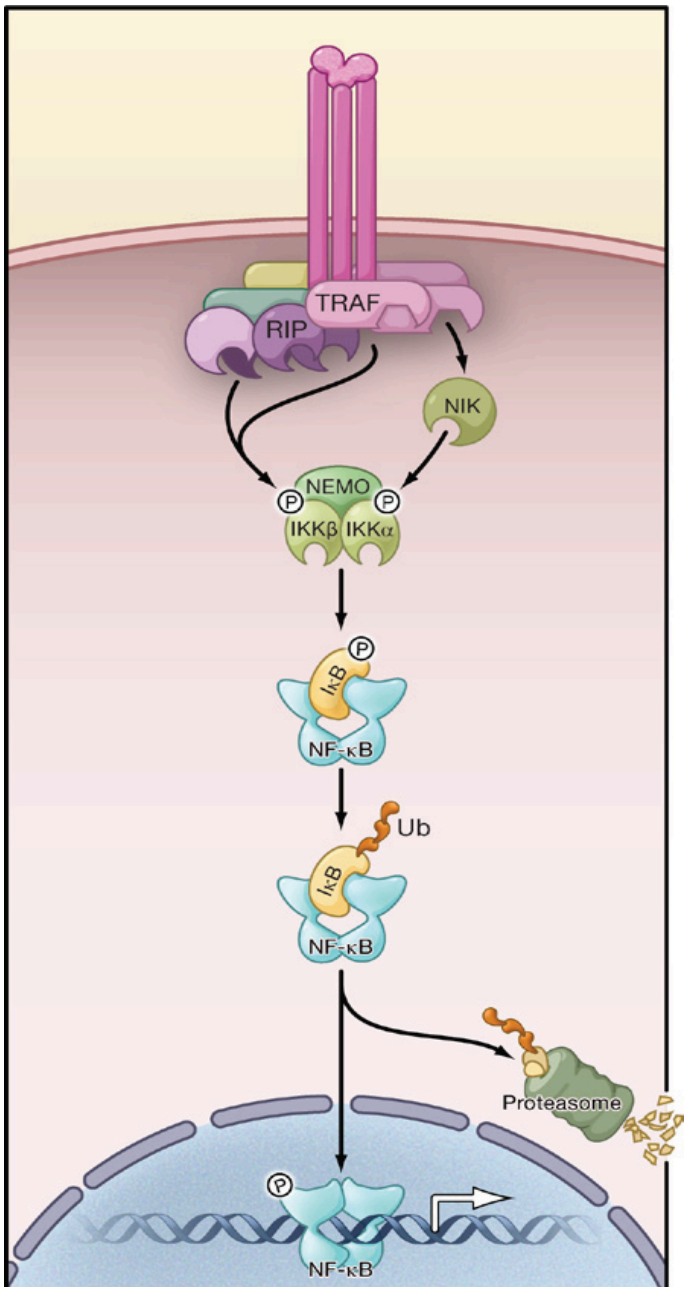
The current view is that NF-κB signalling is essential for epithelial production of TSLP. Based in part on mouse studies in which TSLP has been overproduced or its gene knocked out, respectively, TSLP has emerged as a ‘master switch’ of Th2-type inflammation. Studies involving human cells have generated additional features compatible with involvement of TSLP in further aspects of COPD

immunopathogenesis. Thus, given an appropriate micro milieu, TSLP itself, or TSLP through priming of dendritic cells, may expand and proliferate cytotoxic T cells, which may be important in causing tissue destruction in COPD. TSLP may also induce generation of cytokines and cells potentially involved in development of bronchi-associated lymphatic tissue and autoimmunity, which are topical facets of severe COPD. These intriguing actions of TSLP, together with its increased occurrence in COPD lungs and its overproduction by virally stimulated bronchial epithelial cells from asthmatic and COPD donors, underpin the hypothesis that TSLP may contribute to exacerbations and development of severe asthma and COPD (Brandelius A., 2009).

NF-κB

Nuclear Factor kappa B (NF-κB) is a transcription factor that regulates the expression of numerous genes involved in cell survival, apoptosis, and inflammation; it regulates the expression of multiple pro-inflammation genes and is thus a key player in maintaining immune system homeostasis.

The basic scheme of NF-κB signaling consists of a series of positive and negative regulatory elements. Inducing stimuli trigger IKK activation leading to phosphorylation, ubiquitination, and degradation of IκB proteins. Released NF-κB dimers are further activated through various posttranslational modifications and translocate to the nucleus where they bind to specific DNA sequences and promote transcription of target genes. In its most



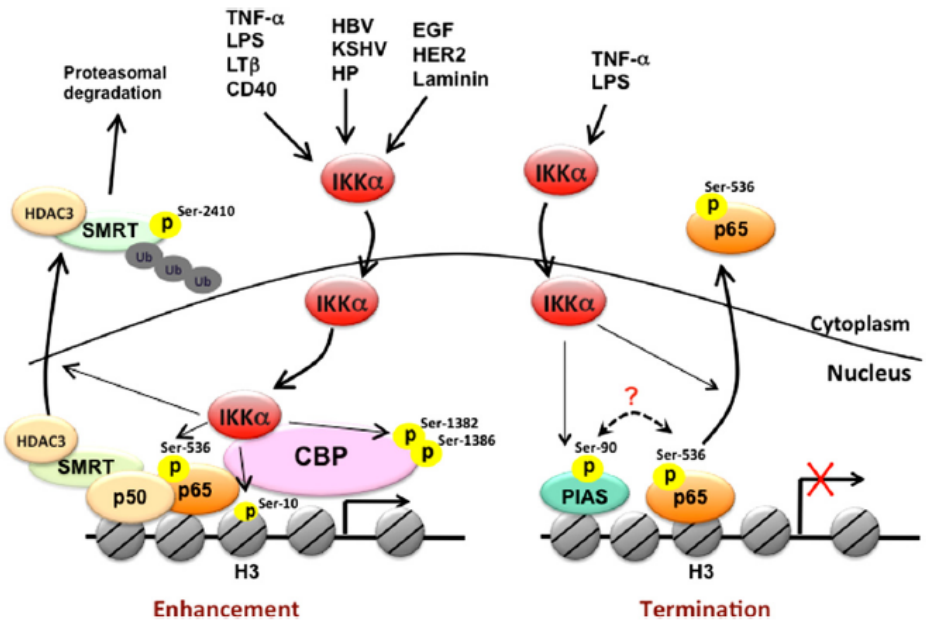
basic form, therefore, the pathway consists of receptor and receptor proximal signaling adaptor molecules; the IKK complex; IκB proteins; and NF-κB dimers.

The NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB, encoded by NFKB1, NFKB2, RELA, REL, and RELB, respectively, which share an N-terminal Rel homology domain (RHD) responsible for DNA binding and homo- and heterodimerization. NF-κB dimers bind to κB sites within the promoters/enhancers of target genes and regulate transcription through the recruitment of coactivators and corepressors. The transcription activation domain (TAD) necessary for the positive regulation of gene expression is present only in p65, c-Rel, and RelB. As they lack TADs, p50 and p52 may repress transcription unless associated with a TAD-containing NF-κB family member or other proteins capable of coactivator recruitment. Constitutive binding of p50 or p52 homodimers to κB sites on NF-κB-responsive promoters may thus act to check NF-κB transactivation until displaced by transcriptionally competent NF-κB dimers (Hayden MS . 2008).

The established model of IκB function posits that IκBα retains NF-κB dimers in the cytoplasm, thereby preventing their nuclear translocation and subsequent DNA binding; however, the situation is actually more complex. The crystal structure of IκBα bound to the p65/ p50 heterodimer reveals that the IκBα protein masks only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains exposed. The exposed NLS of p50 coupled with nuclear export sequences (NES) in IκBα and p65 leads to constant shuttling of IκBα/NF-κB

complexes between the nucleus and the cytoplasm, despite steadystate localization that appears almost exclusively cytosolic.

IKKα and IKKβ are both able to phosphorylate multiple members of the IκB family, although with differing specificities. For example, both IKKα and IKKβ phosphorylate IκBα at Ser32 and Ser36 and IκBβ at Ser19 and Ser23; however, IKKα is less efficient and consequently cannot complement IKKβ knockout cells. Furthermore, both IKKα and IKKβ prefer IκBα to IκBβ, which is consistent with the difference in IκBα and β degradation kinetics—IκBβ degradation is significantly slower than IκBα in most canonical pathways in which both are degraded . IκBα bound to NF-κB is thought to be a preferred substrate to free IκBα (Hayden MS . 2008).



NF-kB is activated in the airways of asthmatic patients and patients with COPD (Gagliardo 2011) , it is activated through phosphorylation and degradation of IkB by IkB kinase (IKK) complex that in turn lead to nuclear translocation of NFkB and subsequent transcription of NF-kB–dependent genes. Phosphorylation of IkB proteins is accomplished by IKKa and IKKb (Hacker 2006).

Many studies have indicated that IKKα can be detected in both the cytoplasm and the nucleus whereas IKKβ is detected predominantly in the cytoplasm, The observation of nuclear/cytoplasm shuttling of IKKα led to the discovery of the first nuclear role of IKKα in phosphorylating histone H3, which results in NF-κB-mediated gene expression. Many studies provided an explanation of why IKKα is dispensable for IκBα degradation but remains essential for NF-κB-dependent transcription. Aside from nuclear regulation of NF-κB dependent gene transcription through chromatin modification in response to pro-inflammatory stimuli, nuclear IKKα also functions in apoptosis, cell cycle, and tumor progression in colorectal, breast, pancreatic, gastric, osteosarcoma, and prostate cancers (Anest V, 2003; Yamamoto Y 2003).

Epigenetic mechanisms

Many lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, interstitial lung disease and acute respiratory distress syndrome, involve inflammation, with the coordinate expression of multiple

inflammatory genes in the lungs. These inflammatory genes code for the expression of cytokines, chemokines, enzymes that synthesize inflammatory mediators, inflammatory mediator receptors and adhesion molecules, resulting in a regulated influx and activation of inflammatory cells and stimulation of resident structural cells. Many of these inflammatory genes are regulated by proinflammatory transcription factors, including nuclear factor kappaB (NF-κB) and activator protein (AP)-1. These transcription factors orchestrate, amplify and perpetuate the inflammatory response and form the molecular basis of chronic inflammation. The term epigenetics; as currently used, refers to a change in gene expression which is heritable but that does not involve any change in DNA sequence.

In response to injury, gene expression can be modified through epigenetic mechanisms, consisting in any modification of the genome or of gene expression not resulting from alteration in DNA nucleotide sequence (Hagood JS 2013).

Post-translational modifications of histones play an important role in epigenetic regulation of gene expression, and thus have critical effects on environment-mediated chronic lung diseases such as COPD and asthma . Since histones are post-translationally modified during disease progression, the identification of these patterns as well as the altered activity of the enzymes that ‘write’ and ‘erase’ these marks are important mechanisms for the understanding of human diseases. The most intensively studied modifications are histone acetylation and methylation which through the action of specific enzymes form marks that allow

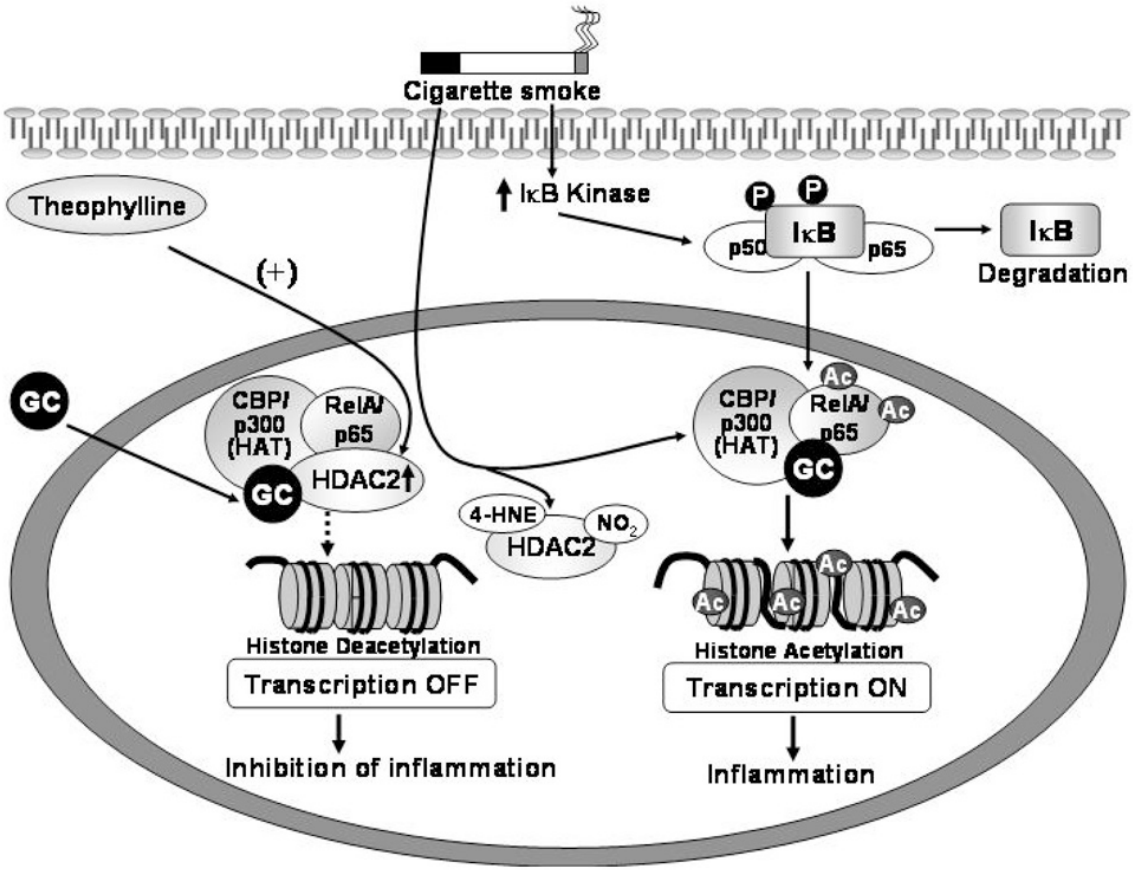
‘readers’ of these marks to remodel chromatin producing the open chromatin structure associated with active gene transcription or a closed repressive chromatin state linked to a lack of active transcription. (Mortaz E., 2011)

Acetylation, deacetylation, methylation and other chromatin histone modification, can regulate genes transcription through promoters accessibility. Transcriptionally active, “open” euchromatin generally has hyperacetylated and hypomethylated histones, whereas more inactive heterochromatin tends to be hypoacetylated and hypermethylated. In addition to acetylation and methylation, which have been extensively studied (Campos El, 2009), histone can be phosphorylated, nitrated, ubiquitynilated, and SUMOylated.

Histone acetyltransferases (HATs, including p300- CREB binding protein [CBP]-associated factor, p300/CBP, and GCN5) and histone deacetylases (HDACs e.g., HDAC2 and SIRTUIN1 [SIRT1]) maintain the balance between histone acetylation and deacetylation. HATs affect the binding of DNA sequence-specific transcription factors and, subsequently, recruit coactivators or corepressors on gene-specific regions to form either coactivator or corepressor complexes (Wang H, 2001). It is well known that transcriptional coactivators possess intrinsic HAT (CBP/p300) and HDAC activities, suggesting that histone acetylation and deacetylation play a causal role in regulating gene transcription. It is generally accepted that increase in histone acetylation leads to increased gene transcription, while histone hypoacetylation is linked to decreased gene transcription . Histone

acetylation by HATs and deacetylation by HDACs are also linked to cell-cycle progression, proliferation, senescence, DNA repair, and recombination events, as well as proin- flammatory gene transcription, which can be affected by oxidative stress and redox signaling (Sundar IK. 2013)

Histone deacetylation by histone deacetylases (HDACs) removes acetyl moieties from lysine residues of histones, causing rewinding of DNA and hence silencing gene transcription. The HDAC family of enzymes has been reported to have 17 isoforms, each differentially expressed and regulated in different cell types (De Ruijter AJ 2003).



HDACs not only cause the inhibition of gene transcription but also directly affect the nuclear binding of transcription factors such as NF-κB. It was recently

reported that HDACs 1 and 2 play a key role in the regulation of cell proliferation and corticosteroid-mediated inhibition of proinflammatory mediator expression (Ito K 2000; Sambucetti LC 1999).

Several reports have shown that HDACs 1–3 can also be associated with inactive RelA/p65 and play a role in the regulation of NF-κB-mediated gene transcription. Thus, changes in HDAC activity associated with RelA/p65 can enhance or repress NF-κB mediated gene expression.

Among several types of HDACs, HDAC2 is well characterized and reported to play a role in the regulation of inflammation and has been implicated in the dysregulation in smokers and patients with COPD. HDAC2 expression and activity is decreased in smokers, COPD subjects, and mild asthma patients and that there is a good correlation between cytokine production and HDAC activity in alveolar macrophages from smokers and non-smokers. Decreased levels/activities of HDAC2, i.e. disruption of the acetylation: deacetylation balance, may lead to sustained transcription of pro-inflammatory gene controlled by NF-κB, resulting in a chronic inflammatory response. Cigarette smoke extract-mediated reduction in HDAC2 was associated with increased RelA/p65, and indicated RelA/p65 interacts with HDAC2 and RelA/p65 becomes available or retained in the nucleus for pro-inflammatory gene transcription when HDAC2 is decreased. In addition, it's been demonstrated that in HDAC3, but not HDAC1, interact with RelA/p65, suggesting an important role of specific HDACs such as HDAC2 and HDAC3 in regulation of NF-κB signaling pathway particularly in response to cigarette smoke. The binding of NF-κB to HDAC2 presumably reflects the recruitment of HDAC2 to specific NF-κB target promoters. The

domains of HDAC2 and NF-κB that interact remain to be identified. It is unlikely that this interaction involves the DNA binding domain of NF-κB, because trichostatin A (TSA) treatment did not significantly interfere with DNA binding. HDAC2 has also been implicated in the anti-inflammatory effects of corticosteroids as ligand bound steroid receptors recruit HDAC2 to promoters of pro-inflammatory genes leading to deacetylation and transcriptional repression. However, a small proportion of severe asthmatics who smoke and severe COPD patients exhibit unresponsiveness towards high doses of oral corticosteroids. The current hypothesis indicates that cigarette smoke-induced oxidative stress alters the basal HDAC2 response via post-translational modifications and an induced net loss of HDAC2. The absence of HDAC2 or the presence of a defective HDAC2 protein may thus explain the abnormal inflammatory response and corticosteroid inefficiency in patients with COPD. Decreased HDAC2 protein expression and deacetylase activity observed in the macrophage of COPD patients is implicated in an impaired ability of dexamethasone to inhibit basal cytokine release in alveolar macrophages from COPD patients compared to casual smokers.

Many studies have focused on establishing a link between decreased HDAC2 expression and increased susceptibility to persistent inflammation, less is known on how HDAC2 expression is regulated in response to cigarette smoke-induced oxidative stress (Rajendrasozhan S., 2008) .

Increased acetylation of lysine (K) residues on histone H3 (K9, K14, K27) and H4 (K5, K8, K12) results in neutralization of positive charge on histone tails and facilitates access to transcription factors (Chung S. 2011)

Therapy

Asthma is usually highly responsive to corticosteroid therapy and inhaled corticosteroids have become the mainstay of disease management. Corticosteroids suppress inflammation by inducing the recruitment of the nuclear enzyme histone deacetylase 2 (HDAC2) to multiple activated inflammatory genes, which leads to deacetylation of the hyperacetylated genes, thereby suppressing inflammation.

By contrast, patients with COPD respond poorly to corticosteroid treatment, and even high doses of inhaled or oral corticosteroids fail to suppress inflammation. This appears to be related to decreased activity and expression of HDAC2 in the inflammatory cells and peripheral lungs of COPD patients. This is the result of increased oxidative and nitrative stress, which together generate peroxynitrite that nitrates tyrosine residues in HDAC2, impairing enzyme activity and decreasing expression. The poor response to corticosteroid treatment seen in patients with severe asthma, in asthmatics who smoke and during acute exacerbations may also reflect a reduction in HDAC2 protein levels and function, as oxidative and nitrative stress are also increased in these situations. So, patients with severe asthma have a relative corticosteroid resistance, and this is linked to impaired

HDAC2 function. Reversal of corticosteroid resistance may therefore be a useful therapeutic strategy in the future for patients with COPD and severe asthma.

The objective of pharmacological treatment of chronic obstructive pulmonary disease (COPD) is to prevent and control symptoms, reduce the frequency and severity of exacerbations, and improve general health status and exercise tolerance. None of the classes of drugs currently used in the treatment of COPD are able to modify the progressive decline in lung function which is the hallmark of this disease. Smoking cessation is currently the only intervention which has been shown to reduce the progression of COPD (GICOPD 2001). To achieve this objective, behavioral therapy and pharmacological treatment such as the administration of bupropion (an antidepressant), and nicotine replacement therapy have proved useful. However, it is important to try to control symptoms of COPD with pharmacological treatment using the following general proposals (GICOPD 2001):

1) There should be a stepwise increase in treatment, according to the severity of the disease. The step-down approach used in the chronic treatment of asthma is not applicable to COPD.

2) Treatment needs to be chronic and maintained at the same level for long periods of time, unless significant side effects or exacerbations occur.

3) Since individual patient response to the pharmacological treatment is variable, it is important to monitor pharmacological treatment closely and, if necessary, adjust it frequently. Drugs currently recommended for the treatment of COPD are:

1) Bronchodilators (selective β2 -agonists, anticholinergic antimuscarinic agents and methylxanthines);

2) glucocorticoids;

3) other types of medication (vaccines, antibiotics, α1- antitrypsin augmentation therapy, mucolytic agents, antioxidants, immunoregulators, antitussives and vasodilators). These drugs will be presented in the order in which they would normally be prescribed for the treatment of patients with COPD, based on the level of severity of the disease (Montuschi P. 2006).

Long-Acting β-Agonists

During its development, salmeterol was found to have some antiinflammatory actions, specifically, inhibition of the release of proinflammatory cytokines from neutrophils in vitro. Subsequently, studies have indicated a potential for inhibition of an experimental inflammatory challenge in humans. Salmeterol reduces the frequency of acute exacerbations in COPD, but other than this and its well-established action in augmenting the effects of inhaled corticosteroids, there is little evidence that salmeterol itself has clinically relevant anti-inflammatory effects. Similarly, the ultra-long-acting β-agonist, indacaterol, has shown trends toward reductions in acute exacerbations, although at dosages above those approved in the United States.

Tiotropium Bromide

Tiotropium bromide, an anticholinergic agent, has also been shown to have anti-inflammatory potential. Many cells in proinflammatory pathways have abundant cholinergic receptors that are accessible to anticholinergic agents, suggesting the potential for inhibiting the release of nonneuronal paracrine mediators of inflammation by anticholinergic agents. There is accumulating evidence that tiotropium, in particular, can have such actions both in vitro, and in vivo. A recent study of the inhibitory effect of tiotropium on the release of inflammatory markers from lipopolysaccharide-stimulated human airway epithelial cells and fibroblasts in vitro showed that IL-8 and NF-κB and other markers were substantially reduced. Similarly, an in vivo study of lipopolysaccharide-induced lung inflammation in guinea pigs showed that cellular proliferation and collagen deposition in the lungs were abrogated by tiotropium administration. Of particular interest is that all the previously mentioned effects of tiotropium were achieved by concentrations that were at or below the therapeutic plasma level following clinical inhalation in humans, and therefore were consistent with a relevant clinical effect. (Novel Antiinflammatory Therapies for COPD Nicholas J. Gross, MD, PhD, FCCP 2012)

Glucocorticoid resistance

Glucocorticosteroids are the most effective anti-inflammatory treatments available for many inflammatory and immune disease, including asthma, rheumatoid arthritis, inflammatory bowel disease, and autoimmune disease. However, a few patients with these disease show a poor or absent response even to high doses of glucocorticoids. Other inflammatory disease, such as chronic obstructive pulmonary disease, interstitial pulmonary fibrosis, acute respiratory distresses syndrome, and cystic fibrosis, seem to be largely glucocorticoid resistant. Since chronic inflammatory disease are widespread and their prevalence is rising, glucocorticoid resistance or insensitivity represents an important barrier to effective treatment and accounts for substantial health care spending (Barnes, 2009).

In a small proportion of patients with glucocorticoid-resistant asthma, glucocorticoid receptors translocate normally to the nucleus after dexamethasone exposure, but do not acetylate Lys5; thus, transactivation of genes does not occur. These patients show a poor response to high-dose inhaled corticosteroids, but have fewer adverse events than patients with glucocorticoid resistance. This finding is because side-effects are mediated via GREs. Recruitment of HDAC2 to activated inflammatory genes is a major mechanism of gene repression by glucocorticoids. HDAC2 activity and expression is reduced in patients with some diseases that respond poorly to glucocorticoid treatment. For example, HDAC2 expression and activity are very low in alveolar macrophages, airways, and peripheral lung in patients with COPD. Similarly, low HDAC2 expression has

been found in PBMCs and alveolar macrophages from patients with refractory asthma and in the airways of asthmatic patients who smoke. Overexpression of HDAC2 (by use of a plasmid vector) in bronchoalveolar macrophages from patients with glucocorticoid-resistant COPD restored glucocorticoid sensitivity to the level seen in controls. Whether HDAC2 expression is also reduced in glucocorticoid-resistant patients with other inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, has not yet been investigated; however, HDAC2 and SWI/SNF expression is reduced in glucocorticoid-resistant adenocarcinomas of patients with Cushing's disease. The mechanisms for the inactivation of HDAC2 in COPD are now being studied; oxidative and nitrative stress might inhibit HDAC2. Since oxidative stress is frequently found in patients with severe and glucocorticoid-resistant inflammatory diseases, it might be an important mechanism of glucocorticoid resistance (Barnes, 2009).

A novel strategy for the treatment of steroid-resistant/severe asthma and COPD is reversal of steroid resistance by interfering with the pathways that cause it. Understanding these pathways has highlighted several therapeutic targets, such as p38MAPK. Perhaps the most attractive target is HDAC2 because restoration of HDAC2 with a plasmid vector has been shown to restore steroid responsiveness in macrophages from patients with COPD that are normally resistant.

It is now well established that adding long-acting b2-agonists (LABAs) to ICSs improves asthma control to a greater degree than increasing the dose of ICS. Accumulating evidence suggests that LABAs might enhance the function of corticosteroids through increasing nuclear translocation of the GR, which increases the anti-inflammatory effects of corticosteroids. This suggests that LABAs might be able to overcome steroid resistance in asthmatic patients when this is due to reduced nuclear translocation of the GR, as discussed above. LABAs reverse the increased GR phosphorylation that is found in PBMCs of some patients with severe asthma. The GR phosphorylation at Ser226 induced in PBMCs by IL-2 plus IL-4 stimulation is reversed by the LABA formoterol though inhibition of JNK1 and p38MAPKg. The effects of formoterol might be mediated by activation of the phosphatase protein phosphatase A2, which reverses the phosphorylation of the GR and JNK1. However, this effect of formoterol is not inhibited by b2-receptor antagonists and is seen in cell-free systems, suggesting a receptorindependent effect. LABAs might also reverse steroid resistance induced by oxidative stress through a different mechanism involving inhibition of PI3Kd. Interestingly, both formoterol and salmeterol restore steroid responsiveness in PBMCs from patients with severe asthma, whereas only formoterol has this effect in cells from patients with COPD, suggesting that the full agonist effect of formoterol compared with the partial agonist effect of salmeterol might be necessary to reverse steroid resistance when there is a greater degree of oxidative stress. LABAs also enhance the steroid-induced increase in MKP-1 expression, thereby more effectively inhibiting p38MAPK and JNK, an effect that is mediated through protein kinase A (Barnes 2013).

There is an unmet need for safe and effective antiinflammatory treatments for COPD, but it has proved difficult to develop such drugs, despite the discovery of several logical targets. Blocking individual cytokines with blocking antibodies or blocking chemokine receptors has so far proved to be disappointing in clinical studies. Broadspectrum antiinflammatory treatments, such as PDE4 and proinflammatory kinase inhibitors, have often been poorly tolerated with side effects that limit the dose that can be used. For example, a PDE4 inhibitor, roflumilast, has significant antiinflammatory effects in COPD cells and animal models of COPD but the dose in patients with COPD is limited by side effects, so the therapeutic benefit is marginal. It may be necessary to develop potent inhaled drugs in order to reduce systemic exposure and side effects, but it has proved difficult to discover inhibitors with high local potency that are retained within the lungs. If systemic inflammation is derived from peripheral lung inflammation, inhaled antiinflammatory treatments should reduce systemic inflammation and may therefore reduce or treat comorbidities (Barnes 2014).

Aims of the study

This study hypothesises that the IL-17A present in the induced sputum supernatant from COPD patients (or the human recombinant IL-17A), induces chromatin remodeling promoting IL-8 and TSLP release in bronchial epithelial cells.

My idea is that the increased levels of IL-17A might alter the IL-8 and TSLP production due to

a markedly reduction of the nuclear HDAC2 expression and activity;

a new nuclear activity of Ikkalpha, that throught acetylation of Histone H3 (Lisin 9), can "open" the chromatin and increase the expression of proinflammatory genes;

Finally we hypothesises that Tiotropium, an anticholinergic drug usually used in the treatment of COPD, might be effective in the control of the above mentioned activity generated by Induced sputum of COPD patients and hrIL-17A involving the related production of IL-8 and TSLP.

Materials and Methods

Patients

We recruited three groups of subjects: healthy asymptomatic non-smoking subjects with normal lung function (HC) (n=14), symptomatic smokers with normal lung function (HS) (n=10), and COPD (n=16). The diagnosis of COPD and the assessment of its severity were defined and classified according to the criteria reported by the Global Initiative for Obstructive Lung Disease (GOLD) guidelines for COPD management (GOLD stage ≥I). COPD subjects with exacerbations within 1-month prior to the study were excluded. Patients with COPD had a smoking history of 10 pack years or more.

All COPD patients were in stable conditions. All COPD patients who had routine chest X-rays and computed tomographic scans that showed obvious emphysema were excluded. All patients were characterized with respect to gender, age, smoking history, COPD symptoms, comorbidity, and current history of treatment. Exclusion criteria included the following: other systemic diseases, chronic bronchitis, chronic spontaneous, sputum production, other lung diseases, upper and lower respiratory tract infections, treatment with glucocorticoids or anticholinergics within 3 months before the study and treatment with long acting beta adrenergic agonists 15 days before the study.

The local Ethics Committee approved the study, and participating subjects gave their informed consent.

Reversibility test to bronchodilator was performed to exclude an asthmatic component, and the increase in forced expiratory volume in the 1st second (FEV1) after salbutamol was lower than 12% and 200 ml compared with basal values, in all COPD subjects.

Sputum induction and processing

Sputum induction and processing were performed according to the method of the plugs. Briefly after the collection of the sputum, the selected plugs were diluted with 4 volumes of phosphate-buffered saline (PBS 1X; Gibco). The resulting suspension was vortexed for 30 s and then centrifuged at 1000 g for 20 min. The induced sputum supernatants (ISs) were then aspirated and frozen at – 80 C in separate aliquots for the subsequent biochemical analyses. The cells obtained from IS were then cytocentrifuged (Cytospin 2; Shandon, Runcorn, United Kingdom) and stained with May–Grunwald–Giemsa. For differential cell counts, 2 independent investigators who counted at least 400 cells per slide read the slides blindly.

Measurement of IL-17A, IL-8 and TSLP

The levels of IL-17A were measured in ISs using a commercial available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems. Inc, MN, USA). The lower limit of detection was 15 pg/ml. The levels of IL-8 were determined in ISs and in 16HBE supernatants, using commercial ELISA kits (R&D Systems. Inc,

MN, USA), according to the manufactures' specifications. The lower detection limit for IL-8 was <5 pg/ml. The levels of TSLP were determinated in ISs using a commercial available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems. Inc, MN, USA). The lower limit of detection was <5 pg/ml.

Epithelial cell cultures

The SV40 large T antigen-transformed 16HBE cell line (16HBE) was used for these studies. 16HBE is a cell line that retains the differentiated morphology and function of normal airway epithelial cells. The cells represent a clonal diploid (2n=6) cell line isolated from human lung. 16HBE cells were cultured as adherent monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated (56° C, 30 min) fetal bovine serum (FBS), 1% MEM (non-essential amino acids, Euroclone), 2 mM L-glutamine and gentamicin 250 μg/ml at 37° C in a humidified 5% CO_2 atmosphere. Evidences showed that 16HBE are similar to primary normal human bronchial epithelial (NHBE) cells (Lonza, Brussels, Belgium), and bronchial epithelial cells (BECs) from bronchial brushings concerning the response to inflammatory stimuli and antiinflammatory drugs.

Bronchial epithelial cells stimulation

16HBE cells (180,000 cells/well) were plated in standard six-well culture plates in MEM 10% FCS and grown to confluence (70–80%). After 1 h in 1 ml of MEM 1% FCS, the 16HBE cells were stimulated with ISs (20%) from HC (n=6), HS (n=6), and COPD (n=6) subjects or with recombinant human (rh) IL-17A (20 ng/ml) (n=6). Furthermore, ISs from COPD patients with the IL-17 concentrations closest to the median of the values were selected to stimulate 16HBE. 200 μl of ISs were incubated with an anti IL-17A Ab for 1 h at 37° C to neutralize the specific activity before the stimulation of 16HBE (n=6). To determine the effects of anticholinergic bronchodilator compounds on IL-17A activity, Tiotropium Spiriva® (100 nM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) was added to 16HBE 30 minutes before the stimulation with ISs from COPD (n=6) or with rhIL-17A (20 ng/ml) (n=6). 16HBE were stimulated for 24 hrs to test IL-8 and TSLP by ELISA and Real time PCR, for 4 hrs to test HDAC2 and Ac-His H3 (k9,) and for 2 hrs to test IKKα.

Total and cytoplasmic/nuclear protein extraction

16HBE were washed with cold PBS and lysed in a buffer containing 10 mmol/L Tris-HCl (PH 7.4), 50 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40;

phosphatase inhibitors consisted of 20 mmol/L β-glycerophosphate, 0.3 mmol/L Na_3VO_4, and 1 mmol/L Benzamidine (ICN Biochemicals, Inc, Aurora, Ohio); and protease inhibitors consisted of complete protease inhibitor cocktail (Roche). Nuclear extracts were obtained by NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) that provides for efficient cell lysis and extraction of separate cytoplasmic and nuclear protein fractions by centrifugation. 25–30 μg of lysate was then denatured under reducing conditions by boiling for 3 min in 50 mM Tris-HCl (pH 6.8), 1% SDS, 2%β-mercaptoethanol, and 0.01% bromophenol blue. Total and cytoplasmic/nuclear protein extracts were analyzed by western blot.

Western Blot Analysis

Total and nuclear proteins were separated by SDS-PAGE and transferred by electrophoresis onto Immobilon-P membranes (Millipore, Bedford, MA). After transfer, the membranes were blocked overnight at room temperature in PBS containing 3% BSA and 0.5% Tween 20 and then incubated for 1 h at room temperature with the primary Abs. After washing, the blot was incubated for 45 min with the appropriate horseradish peroxidase conjugated secondary Ab; bound Ab was detected using the ECL chemiluminescence detection system (Amersham-Pharmacia, Biotech), according to the manufacturer's instructions. Membranes were stripped and reprobed with housekeeping proteins β-actin or glyceraldehyde-3-phosphate dehydrogenase Abs to normalize differences in protein loading. Autoradiographic films were scanned by densitometry and analyzed using the

NIH Image/Gel Plotting analysis program (National Institutes of Health, Bethesda, MD). Results were normalized and expressed as ratio of the tested protein band intensity with β-actin.

Antibodies

Rabbit anti-human IKKα and Anti-acetyl-Histone H3, were obtained from Millipore (Ca) and used diluted 1:500. Mouse anti-human histone deacetylase (HDAC) 2, mouse anti-human IL-8 (B-2) and rabbit anti-human IL-17 (H-132) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, Ca) and diluted 1:100. Anti rabbit TSLP was obtained from ProSci incorporated (Ca). Finally, mouse monoclonal anti β-Actin Ab was obtained from Sigma (St. Louis, MO) and used diluted 1:20000.

HDAC activity

Cells were stimulated for 4 hrs with ISs from HC, HS, and COPD. Protein levels were determined using a BCA kit (Thermo Scientific, Rockford, IL, USA). HDAC activity was assessed in nuclear extracts by a fluorometric HDAC activity assay (BioVision, Mountainview, CA, USA) performed according to the manufacturer's instructions and expressed as fluorimetric units (F.U.) normalized to μg of protein.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) of IL-8 and TSLP

Total RNA was extracted from 16HBE cells with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and was reverse-transcribed into cDNA, using M-MLV-RT and oligo(dT) primer (Invitrogen). Quantitative real-time PCR of IL-8 transcript was carried out on StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for IL-8 Hs00174103m1, TSLP Hs00263639m1, Assays on Demand, Applied Biosystems). IL-8and TSLP genes expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control gene. Relative quantitation of gene expression was carried out with the comparative C_T method ($2^{-\Delta\Delta Ct}$) and was plotted as fold-change compared to untreated cells chosen as the reference sample.

Silencing

To confirm that IKK-α is involved in HDAC2 translocation and Histon H3 acetylation, we tested the effect of IKK-α silencing in human bronchial epithelial cells using specific siRNA transfection. 16HBE cells were plated in six well tissue culture plates and grown in medium containing 10% FBS without antibiotics until

60 to 80% confluence. IKK-α siRNA (10 μM; Santa Cruz Biotechnology, Inc.) was then added to 100 μl of siRNA transfection medium, and the reaction was performed according to the manufacturer's instructions until complete cell transfection (7 hrs at 37°C). For optimal siRNA transfection efficiency, siRNA (10 μM; Santa Cruz Biotechnology, Inc.) containing a scrambled sequence, that did not lead to the specific degradation of any known cellular mRNA, was used to control non-specific effects. Finally, cells were stimulated with ISs from COPD (20%) patients for 24 hrs and total and nuclear protein were extracted. The silencing efficacy of IKK-α RNA interference was assessed by Western blot analysis.

Co-immunoprecipitation

16HBE cells were washed with cold PBS 1X before lysing in mild protein lysis buffer (50 mM Tris · HCl, 150 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40) with protease and phosphatase inhibitors. The cell lysate was precleaned with protein A agarose beads (Protein A/GPlus-Agarose, Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently incubated overnight with a rabbit Anti-acetyl-Histone H3 antibody (pull-down). Protein A agarose beads were added and incubated for 1 h at 4 °C. The immunoprecipitates (IP) were washed and boiled in 2 × SDS sample buffer for 5 min, centrifuged and cell lysates separated on 10%

SDS/PAGE gels for Western blot using a rabbit anti-human IKKα Ab. Non immunized IgG was applied as the pull-down control to confirm the binding specificity. Total protein without IP was run at the same time as control.

Results

Table 1: Demographic characteristics of patients.

	HC (n=14)	HS (n=10)	COPD (n=16)	p value		
				HC *vs* HS	HC vs COPD	HS vs COPD
Age, yr	65 ± 7	61.5 ± 8	62 ± 12	ns	ns	ns
Gender, Male/Female	7/7	6/4	7/9	-	-	-
FEV1, % predicted	108.6 ± 12.6	105.2 ± 10	58.7 ± 24.5	ns	<0.006	<0.03
FVC, % predicted	109.2 ± 16.7	109.1 ± 15	72.6 ± 19.2	ns	<0.04	<0.04
FEV1/ FVC (%)	93.6 ± 3.4	89.3 ± 4.9	64.4 ± 9.0	ns	<0.002	<0.02
Smoking, pack/yr	0	60 ± 18.0	63 ± 18.0	<0.001	<0.001	ns

Data are presented as **mean ± SD.** Abbreviations: HC = healthy asymptomatic nonsmoking subjects with normal lung function; HS = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease; FEV1 = forced expiratory volume in one second; FVC = forced vital capacity. Statistical analysis were calculated using Kruskal-Wallis followed by Bonferroni Dunn correction for multiple comparison.

Table 2: Total and differential cell count from induced sputum

	HC (n=14)	HS (n=10)	COPD (n=16)	p Value		
				HC vs HS	HC vs COPD	HS vs COPD
Macrophages (%)	81.5 (67.8-89.4)	51.9 (47.1-68.1)	24.6 (12.4-52.2)	<0.02	<0.003	ns
Neutrophils (%)	19.7 (10-29.1)	46.1 (30.2-48.2)	72.8 (42.2-85.5)	<0.01	<0.002	ns
Lymphocytes (%)	0.6 (0-1.2)	0.7 (0.4-1.2)	1.2 (0-1.6)	ns	ns	ns
Eosinophils (%)	0.1 (0-0.7)	0.9 (0-1.4)	0.8 (0.2-2.4)	ns	ns	ns
Epithelial cells (%)	0.7 (0.5-2.2)	0.5 (0-0.8)	0.4 (0-1.7)	ns	ns	ns
Total cells (10^6/g IS)	3.8 (2.2-7.6)	4.3 (3.6-6)	6.6 (3-15.9)	ns	ns	ns

Results are expressed as median (25th to 75th percentiles). Abbreviations: HC = healthy asymptomatic nonsmoking subjects with normal lung function; HS = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease; ns = not significant. Statistical analysis were calculated using Kruskal-Wallis followed by Bonferroni Dunn correction for multiple comparison.

Demographic characteristic of patients and differential cell counts of IS.

The patient characteristics are summarized in Table 1. The results of the differential cell counts of induced sputum samples showed a significant increase in the number of cells in HS and COPD subjects. In HS, this increase reflected an increase in the number of both macrophages and neutrophils, whereas in COPD subjects neutrophils showed a large increase with the number of macrophages being significantly lower than in HS and in HC. Eosinophils resulted significantly

higher in COPD subjects than in HC, whereas lymphocytes did not show significant differences among the three study groups (Table 2).

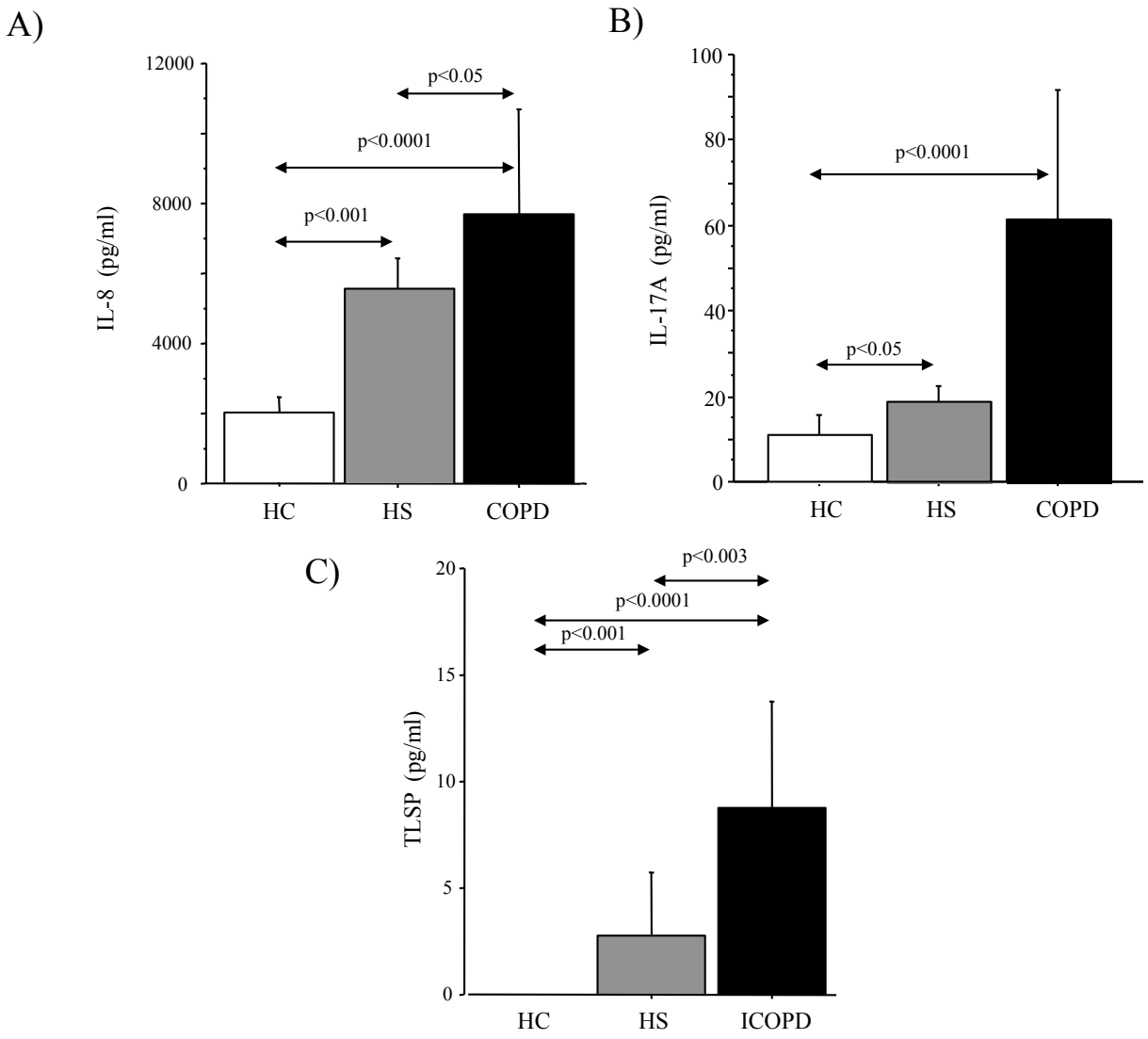


Figure 1

Levels of IL-17A, TSLP and IL-8 in ISs.

IL-8 and TSLP concentrations showed significantly higher levels in ISs from HS subjects and from COPD patients compared to ISs from HC subjects; and in ISs from COPD patients compared to ISs from HS subjects (Figure 1 A and C). Furthermore, IL-17A concentrations were significantly higher in ISs from COPD patients and from HS subjects than in HC (Figure 1 B).

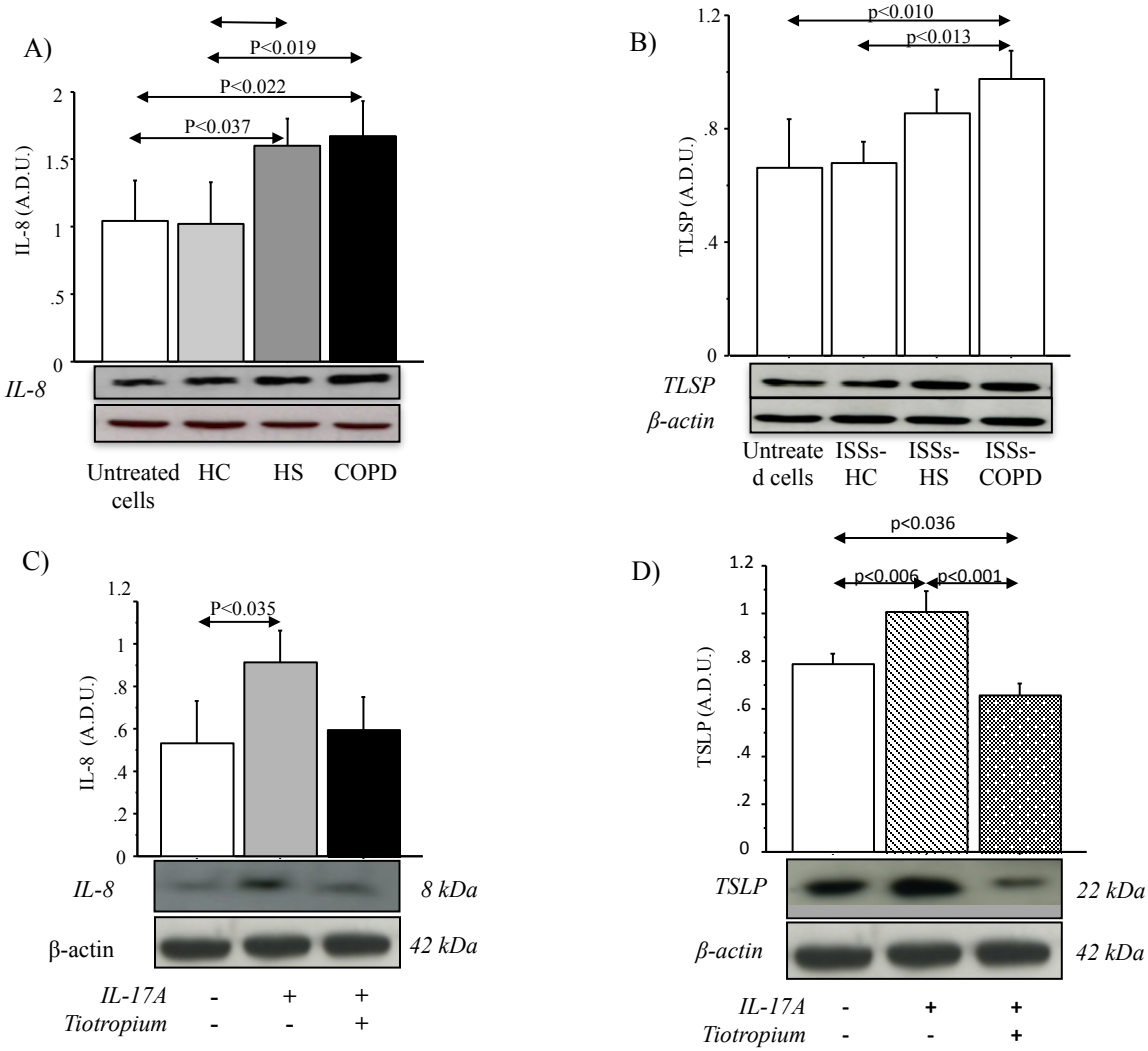


Figure 2

Effect of ISs on IL-8 and TSLP release in 16HBE cells

Protein extract from 16HBE showed a significant increase of IL-8 and TSLP proteins expression when the cells were stimulated with ISs from COPD patients and from HS subjects compared to HC (Figure 2, A and B). 16HBE stimulated with Tiotropium (100 nM) or with ISs from COPD patients pretreated with an anti IL-17A Ab significantly reduced the expression of IL-8 and TSLP compared to 16HBE stimulated with ISs from COPD patients (Figure 2, C and D).

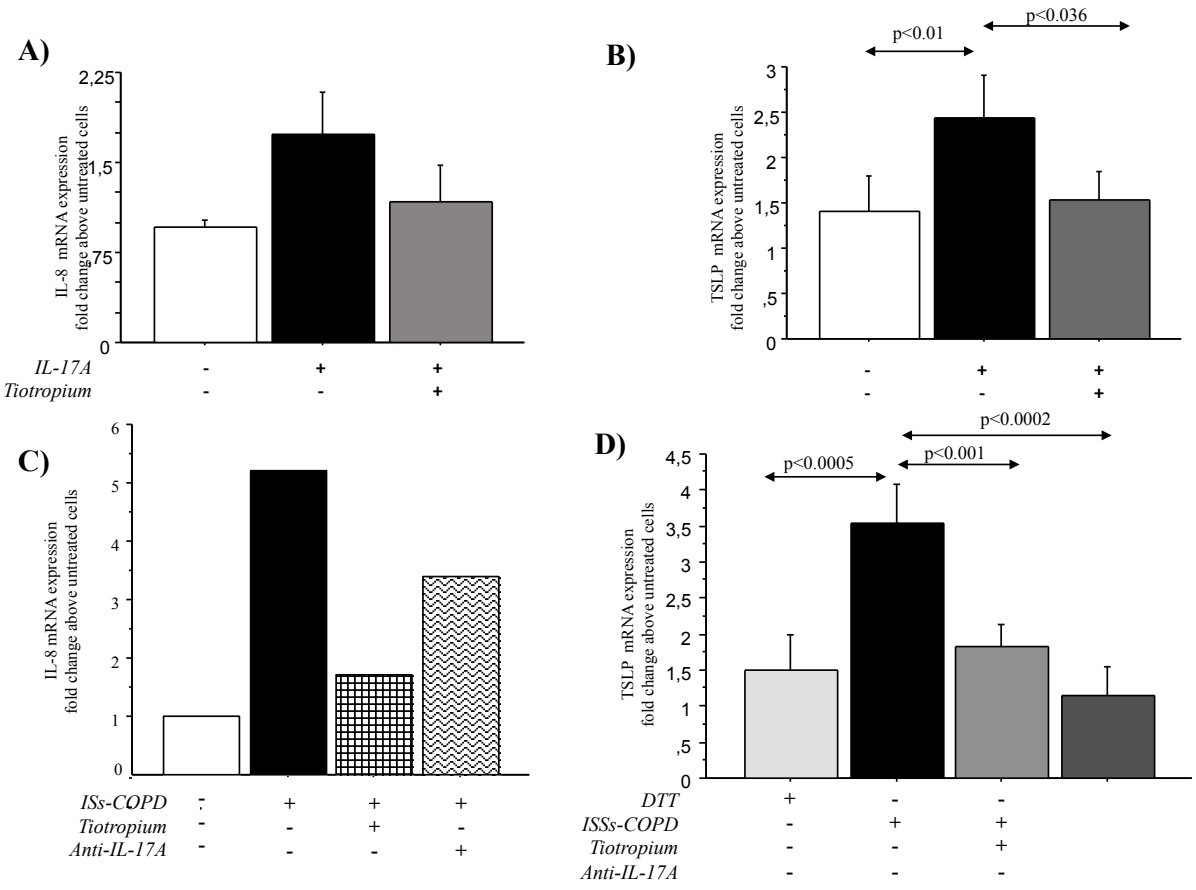


Figure 3

IL-8/ TSLP mRNA levels in bronchial epithelial cells stimulated with IL-17A and ISs of COPD patients. Effect of Tiotropium.

RT-PCR analysis of IL-8 and TSLP mRNA showed that 16HBE stimulated with hrIL-17A (fig.3, A and B) or with ISs from COPD showed higher levels of mRNA expression (as indicated by the lower number of amplification cycles required) compared to untreated cells or to 16HBE pretreated with Tiotropium (100 nM) or to 16HBE stimulated with ISs from COPD treated with anti IL-17A (Figure 3, C and D)

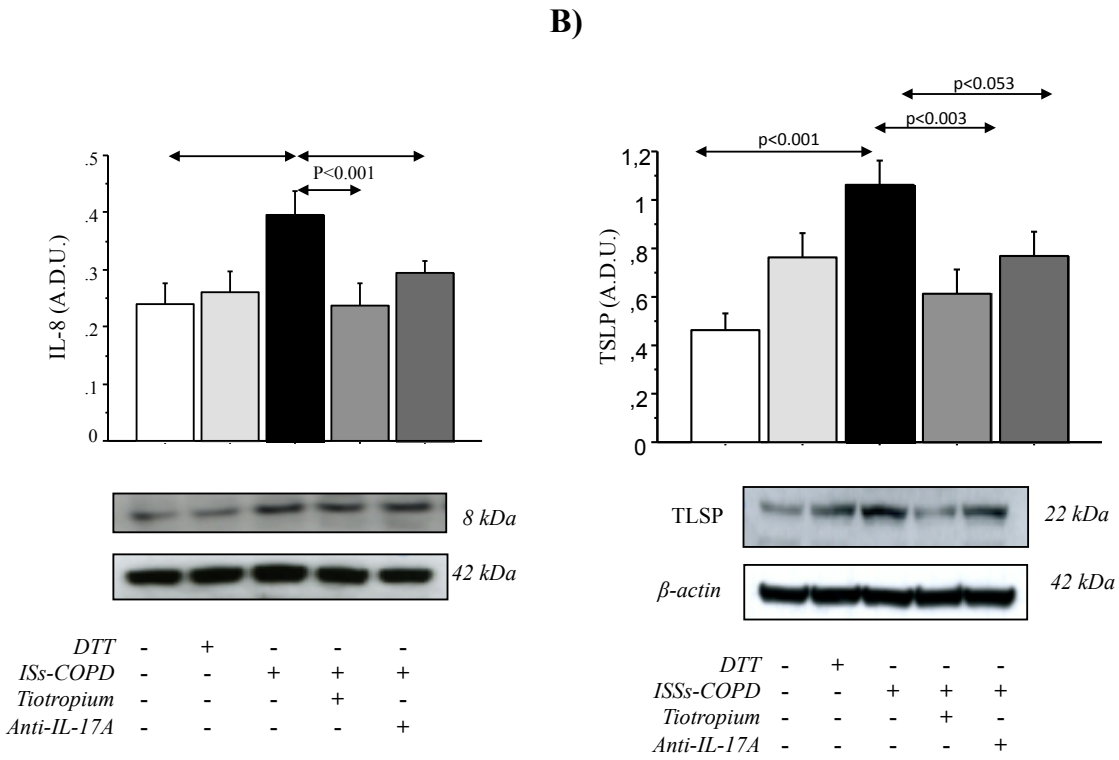


Figure 4

ISs of COPD induce IL-8 / TSLP proteis productions. Effect of Tiotropium.

The stimulation of 16HBE with ISs of COPD significantly increased the production of IL-8 and TSLP in cell protein extract compared to untreated cells. The preincubation of the 16HBE with Tiotropium or anti-IL-17 A, significantly reduced the effect of ISs on the IL-8 and TSLP production (Figure 4)

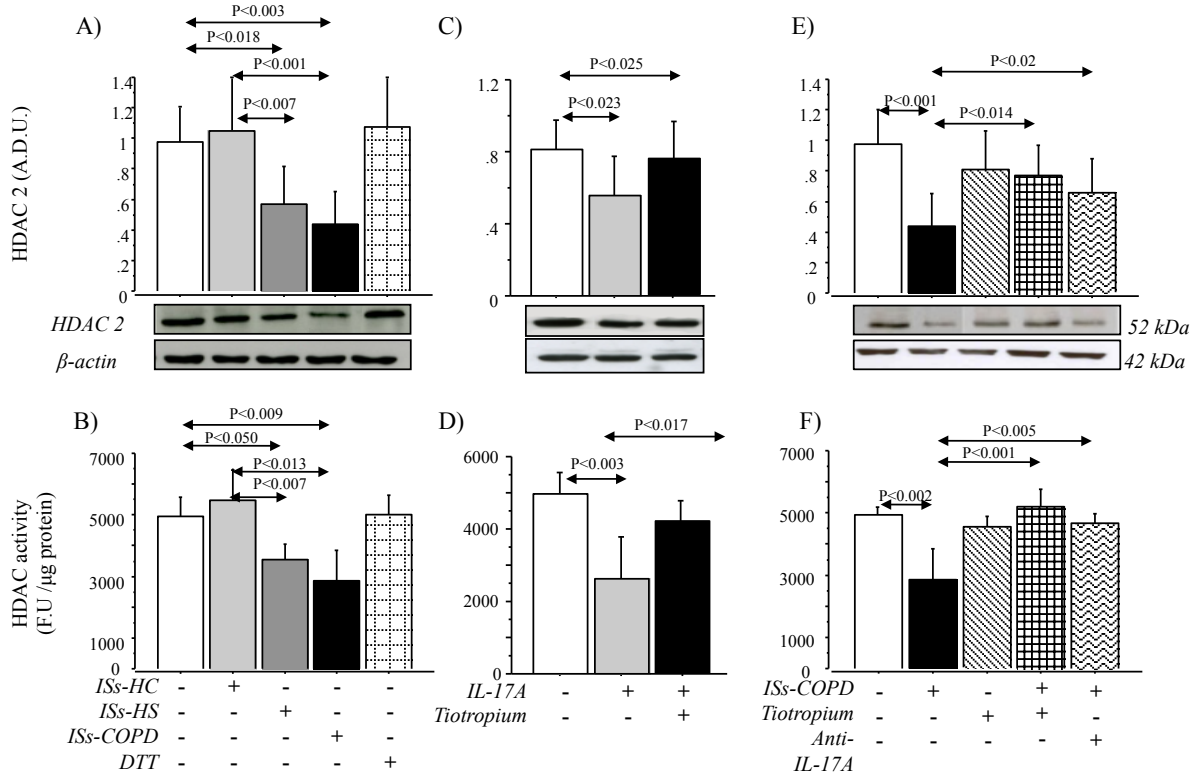


Figure 5

Chromatin remodelling after ISs treatment

ISs from COPD patients and HS subjects, significantly decreased the levels of nuclear HDAC2 protein obtained by western blot analysis (Figure 5, A) as well as the levels of nuclear HDAC activity obtained by fluorometric assay (Figure 5, B) in 16HBE compared to untreated cells. Furthermore, ISs from COPD patients and HS subjects significantly decreased the levels of nuclear HDAC2 protein in 16HBE compared to the cells stimulated with ISs from HC (Figure 5, A). Accordingly, the levels of nuclear HDAC activity (Figure 5, B) significantly decreased in 16HBE stimulated with ISs from COPD patients and HS subjects compared to the cells stimulated with ISs from HC. Additionally, we showed that the treatment of the 16HBE with Tiotropium (100 nM) significantly reduced HDAC2 translocation from the cytoplasm to the nucleus as well as HDAC activity in the cells stimulated with ISs from COPD patients compared to the cells treated with COPD ISs alone. Furthermore, we showed that the stimulation of the cells with ISs from COPD patients, pretreated with an anti-IL-17A antibody, significantly increased HDAC2 translocation from the cytoplasm to the nucleus and HDAC activity in 16HBE compared to the cells treated with ISs from COPD alone (Figure 5, E and F). Finally, we detected that the nuclear levels of HDAC2 expression and activity was significantly reduced in 16HBE stimulated with rhIL-17A compared to untreated cells. The preincubation of the cells with Tiotropium significantly reduced the effect of rhIL-17A on the nuclear levels of HDAC2 expression and activity (Figure 5, C and D).

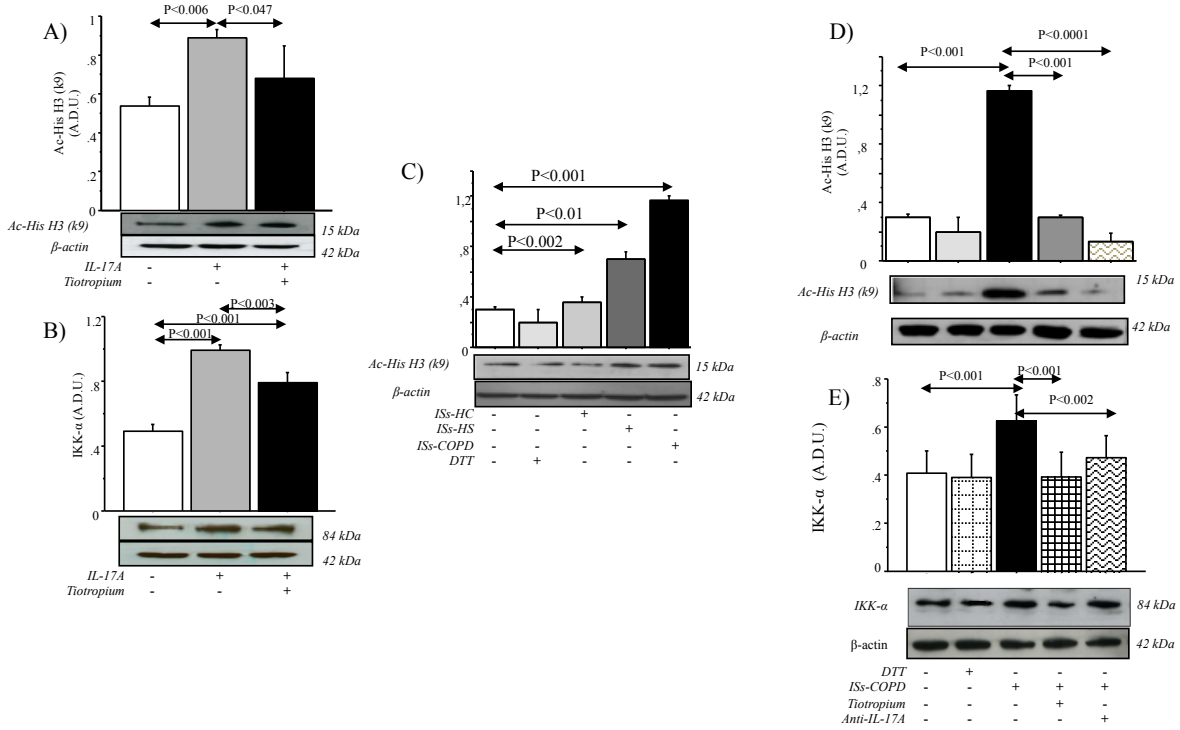


figure 6

Levels of Histone H3 acetylation and IKKα into the nucleus

The analysis of nuclear protein lysates, showed a statistically significant increase of the Ac-His H3 (k9) when the cells were treated with ISs from HS and from COPD patients compared to untreated cells and compared to the cells stimulated with ISs from HC subjects. 16HBE stimulated with Tiotropium or with ISs from COPD patients treated with an anti-IL-17A Ab, statistical significantly restored the nuclear levels of Ac-His H3 (k9) compared to the cells treated with ISs from

COPD patients (Figure 6, C). These findings support an opposite tendency between Ac-His H3 (k9) and HDAC2 at the nuclear levels.

The nuclear IKKα protein level is higher in the cells stimulated with ISs from COPD patients compared with untreated 16HBE. Furthermore, the pretreatment of the cells with Tiotropium (100 nM) significantly reduced the translocation of nuclear IKKα protein level compared to the cells stimulated with ISs from COPD. Furthermore, the stimulation of 16HBE with ISs from COPD patients treated with an anti-IL-17A antibody, significantly reduced the levels of nuclear IKKα compared to the 16HBE treated with ISs from COPD patients (Figure 6, B). Finally, we showed that the nuclear levels of Ac-His H3 (k9) and IKKα were significantly increased in 16HBE stimulated with rhIL-17A compared to untreated cells. The preincubation of the cells with Tiotropium significantly reduced the effect of rhIL-17A on the nuclear levels of Ac- His H3 and IKKα (Figure 6, A).

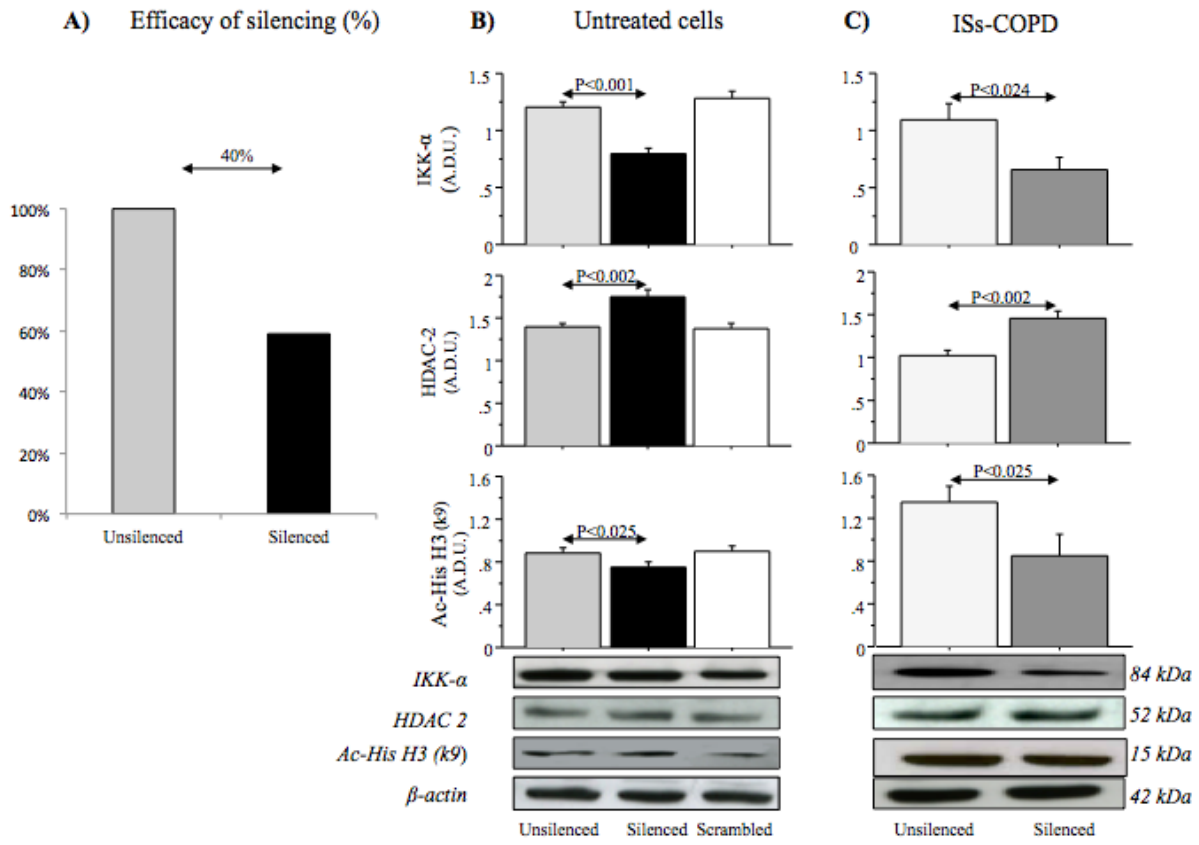


Figure 7

Effect of IKK alpha silencing on HDAC 2, Histone H3 and IL-8/TSLP expression.

Temporary transfection of 16HBE cells with IKKα siRNA caused a statistical significant decrease in the synthesis of IKKα protein in 16HBE compared with unsilenced cells. The silencing efficacy of the RNA interference for IKKα was 40±3.6% (Figure 7, A). The IKKα silencing showed a statistically significant increased HDAC2 protein expression in 16HBE compared to unsilenced cells. Furthermore, Histone H3 ac (k9) protein expression significantly decreased in IKKα silenced cells compared to untreated. Finally the treatment of 16HBE with

scrambled siRNA sequence do not affect IKK alpha, HDAC 2 and Histone H3 compared to unsilenced 16HBE (Figure 7, B). The silencing of IKKα protein significantly increased HDAC2 protein and significantly reduced Ac-His H3 (k9) protein expression in 16HBE stimulated with 20% ISs of COPD patients compared to unsilenced stimulated cells (Figure 7, C).

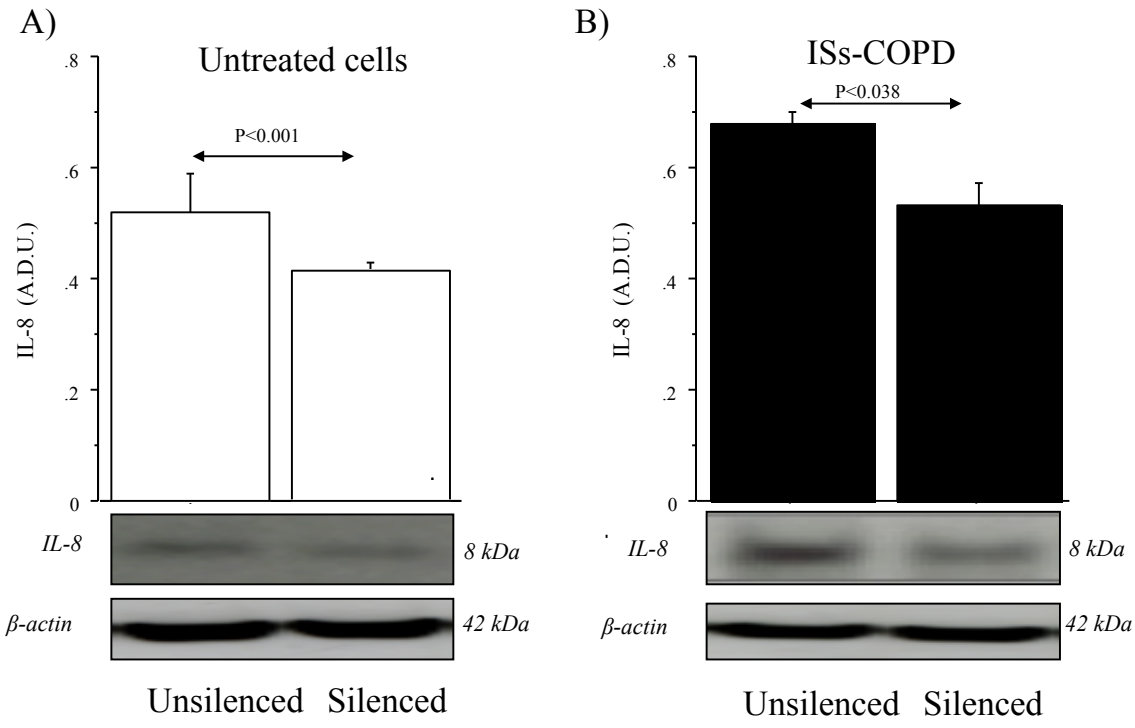


Figure 8

IL-8 protein expression in Ikkalpha silenced cells

IL-8 protein expression showed lower levels in cell lysates from untreated 16HBE silenced for IKKα protein compared to unsilenced cells ($p<0.001$) (Figure 8, A) as well as in 16HBE silenced stimulated for 24 hours with COPD ISs and compared to unsilenced cells stimulated with ISs ($p<0.038$) (Figure 8, B).

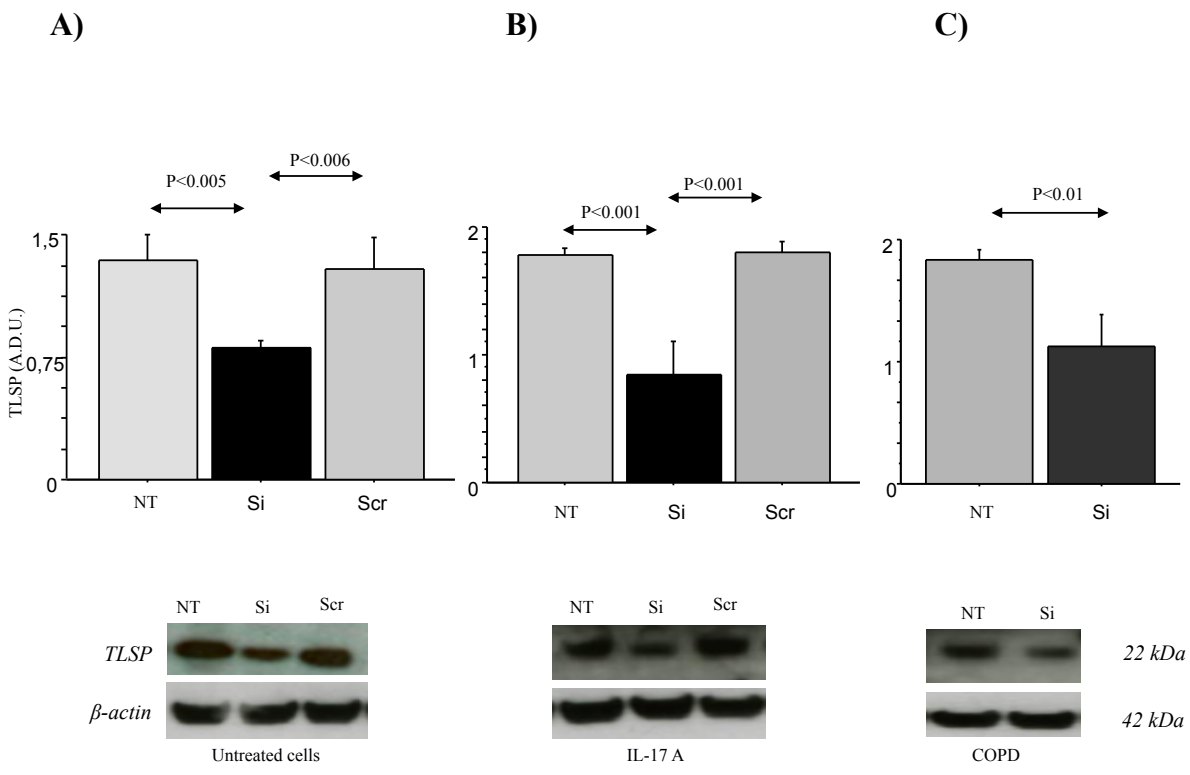


Figure 9

TSLP protein expression in 16HBE silenced for IKKα

TSLP protein expression showed lower levels in cell lysate from untreated 16HBE silencd for IKKα protein compared to unsilenced cells, as well as 16HBE silenced stimulated for 24 hours with IL-17A 20 ng/ml or with COPD ISs and compared to unsilenced cells stimulated with IL-17A or Iss (Figure 9)

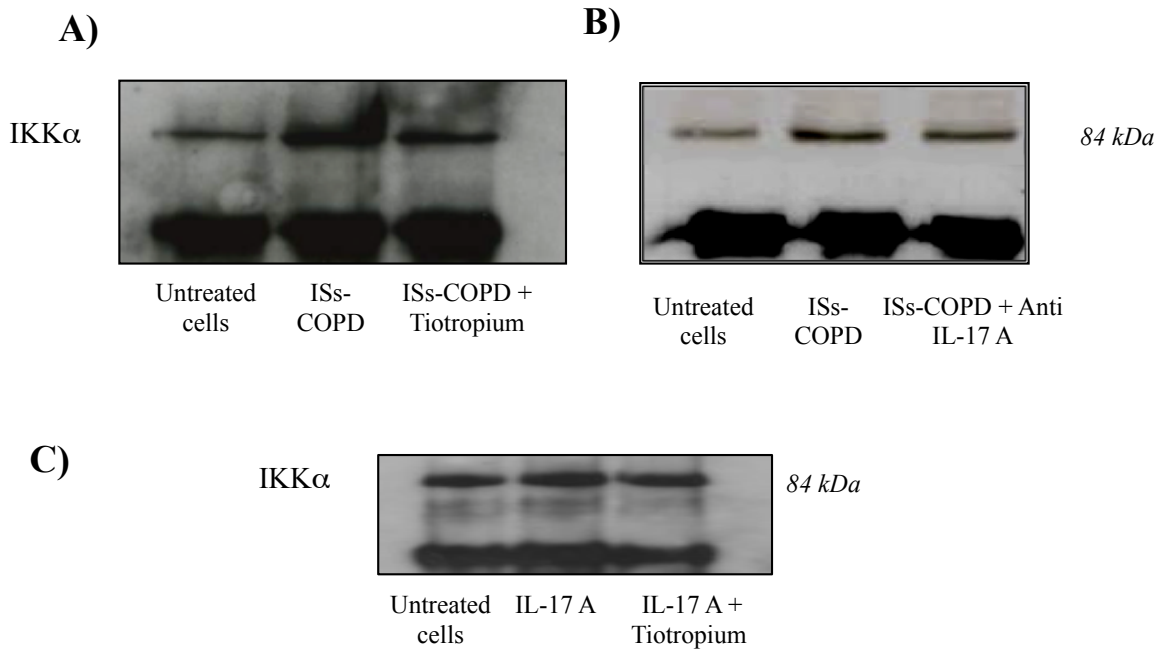


Figure 10

Co-immunoprecipitation His H3/IKKa

We next evaluated the interaction between Ac-His H3 (k9) and IKKα proteins using co-immunoprecipitation. We found that ISs from COPD patients or rhIL-

17A significantly induced an increase of cross coupling between IKKα and Ac-His H3 (k9) compared to untreated 16HBE. The preincubation of the 16HBE with Tiotropium or the depletion of IL-17A in ISs significantly reduced the effect of stimulation with ISs from COPD patients (Figure 10). These findings might suggest a role of IKKα in the mechanism of His H3 acetylation in K9 involving pro-inflammatory IL-8/TSLP genes expression in 16HBE.

Discussion

In this study we described, using an in vitro model obtained by induced sputum samples and bronchial epithelial cell line 16HBE, the immunological link between the levels of IL-17A, IL-8 and TSLP measured in the induced sputum supernatants from COPD patients. Particularly, we showed that IL-17A generated molecular mechanisms of chromatin remodeling, through HDAC2 translocation and Histone H3 acetylation by IKKα activity. This molecular activity of IL-17A promoted IL-8 and TSLP production in bronchial epithelial cells. Finally, we provided evidences that Tiotropium (non-selective anticholinergic drug blocking muscarinic receptors) was able to counteract the proinflammatory activity of IL-17A.

Acting as a physical barrier, the lung epithelium regulates lung fluid balance, modulates metabolism and clearance of inhaled agents, and secretes numerous mediators, several of which recruit and activate inflammatory cells in response to injury.

Dysregulation of airway epithelial cell function may contribute to the pathogenesis of major lung diseases such as COPD. Th17 immunity and the related cytokines such as IL-17A are involved in both innate and adaptive aspects of airway immunity, representing a crucial crosstalk between the immune system and structural cells. It has been reported that IL-17A levels are increased in submucosal biopsy specimens from the large airways of patients with COPD compared to control subjects. These data support the involvement of IL-17A in the airway inflammation of COPD patients promoting, alone or in combination

with other cytokines, the production of IL-6, IL-8, and ICAM-1 in primary epithelial cells.

Environmental factors such as Toll- like receptors ligands, viruses, microbes, allergen sources, cigarette smoke and proinflammatory citokines trigger TSLP production. An increased number of cells expressing TSLP mRNA has been reported in the bronchi of stable COPD patients and control smokers with normal lung function, and increased TSLP immunostaining has been shown in the smooth muscle of patients.

We detected higher levels of IL-17, IL-8 and TSLP in the samples of ISs from COPD and healthy smokers than in control subjects. Accordingly with previous study, these findings suggest a link between IL-17A and IL-8 in the airways of smoking COPD patients supporting the crosstalk between the cells of the immune system producing IL-17A and activated epithelial cells producing IL-8 observed during innate and adaptative immunity of the airways.

In vitro studies have shown that differentiated cell culture is an invaluable model in understanding the physiological properties of the human airway epithelium. Much of our knowledge from the interactions between environmental and inflammatory stimuli, and the airway epithelium has been derived extensively from in vitro cell culture models using transformed cells 16HBE. Accordingly, we studied the effect of ISs from COPD, HS and HC on IL-8 and TSLP production in 16HBE. The analysis of protein and mRNA clearly demonstrated that ISs from COPD and HS are able to increase the IL-8 and TSLP production rather than HC in 16HBE. Although the levels of IL-8 are not reflective of the levels of IL-17A since we did not identified a positive correlation between the two cytokines in ISs

from COPD, we observed that the depletion of IL-17A in ISs from COPD patients reduced the levels of IL-8 and TSLP mRNA transcripts and protein productions in 16HBE. These findings might support the role of IL-17A in the epithelial cells activation during the inflammatory process of COPD. However, we underline that the anti-IL-17A treatment had different levels of inhibition on IL-8/TSLP mRNA and IL-8/TSLP proteins probably for a different time of mRNA stability and proteins half-live.

The inflammatory pattern that occurs in COPD, with increased numbers of neutrophils and increased amounts of IL-8 and tumor-necrosis factor, increased oxidative stress and a poor response to corticosteroids. Anticholinergic drugs including Tiotropium, currently used for the treatment of COPD, block the activation of airway secretory cells and smooth muscle and so, theoretically, may reduce vagal tone and mucus secretion in COPD facilitating cough-induced mucus clearance. However although Tiotropium reduces exacerbation frequency in COPD, this effect does not appear to be due to a reduction in airway or systemic inflammation. Therefore, many studies suggest novel pharmacological strategies using Tiotropium as anti-inflammatory and anti-remodeling drugs in COPD. In vitro, it was observed that Tiotropium is able to control IL-8 and TSLP release from bronchial epithelial cells. Furthermore, IL-17A induces epigenetic changes, which in turn diminishes the ability of glucocorticosteroids (GC) to inhibit IL-8 production from human bronchial epithelial cells. In this study, we showed that the pretreatment of 16HBE with Tiotropium might be able to control the production of IL-8 and TSLP in term of protein and mRNA generated by IL-17A and by proinflammatory mediators present in ISs from COPD. In this

scenario, the growing understanding of the epithelium and its interactions with inflammatory cells obtained by our findings might open new pharmacological perspectives to treat the epithelial dysregulation associated with Th17 immunity during inflammatory lung conditions.

In light of these observations, we speculate that blockade of IL-17A downstream involving the pretreatment of epithelial cells with Tiotropium, might represent a new strategy for therapeutic intervention in GC-insensitive airway inflammation of COPD patients. Furthermore we suggest that, since the inflammatory components present in the airways of COPD patients are able to promote a deregulation of muscarinic AChReceptors expression and muscarinic AChReceptors activation in bronchial epithelial cells, this action might be exercised by IL-17A present in the ISs from COPD. This observation might justify the antinflammatory role of Tiotropium in our in vitro model of 16HBE. However, further studies might be necessary to clarify this concept.

The HDAC family of enzymes has been reported to have 17 isoforms, each differentially expressed and regulated in different cell types playing a role in the regulation of cell proliferation and inflammatory responses. IL-17A induces GC insensitivity, probably mediated by PI3K activation and subsequent reduction of HDAC2 activity, in airway epithelium. Our findings showed that ISs from COPD patients, rather than ISs from HC subjects or untreated cells, generated a reduction of HDAC2 translocation from cytoplasm to the nucleus in 16HBE cells. The depletion of IL-17A with anti IL-17A antibody in ISs from COPD patients or the pretreatment of 16HBE with Tiotropium restored the nuclear levels of HDAC2 in

the cells stimulated with ISs from COPD patients. Our findings might suggest that anticholinergic drugs exert an anti-inflammatory role on IL-17 activity controlling chromatin remodeling that involved the release of inflammatory cytokines such as IL-8 and TSLP. However, further study might be necessary to clarify whether the specific inhibition of HDAC2 activity reduces the levels of IL-8 and TSLP production in 16HBE stimulated with ISs and IL-17A.

The acetylation status of histones is controlled by the opposing actions of two classes of enzymes: histone acetyltransferases (HATs), which transfer acetyl groups to lysine residues within the N-terminal tails of core histones, and histone deacetylases (HDACs), which remove the acetyl groups. The acetylation status of histones influences chromatin conformation and affects the accessibility of transcription factors and effector proteins to the DNA, thereby modifying gene expression. It is known that inflammation can induce a chromatin remodeling through different molecules; the acetylation of Histone H3 in lysin 9 is a modification that facilitates the access to the promoter of the transcription complex. The kinase IKKα has a cytoplasmatic and nuclear function. The shuttling into the nucleus is crucial for his function as chromatin kinase and acetylase inducing specific modification of Histones. This implies a different expression of genes in response to an inflammatory stimulus. Cigarette smoke/TNFα-induced acetylation of histone H3 and inflammation through differential activation of IKKα in human lung epithelial cells. In this study, we showed that 16HBE cells treated with ISs from COPD patients increased the nuclear levels of IKK-α and Ac-H3 k9 compared to untreated cells. The pretreatment of 16HBE with Tiotropium or the depletion of IL-17A in ISs from

COPD patients restored the basal levels of IKKα and Ac-H3 k9 as well as the basal levels of IL-8 and TSLP production. These findings suggest that IL-17A and inflammation present in the airways of COPD patients might generate IL-8 and TSLP production trough chromatin remodeling mechanisms involving the IKKα mediated acetylation of histone H3 in the gene promoter of IL-8 and TSLP in bronchial epithelial cells. The anticholinergic Tiotropium might act as antinflammatory drug controlling these mechanisms. Furthermore we found that IKKα silencing promoted the increase of nuclear levels of HADAC2, a reduction of the Ac H3 (k9) and of IL-8 and TSLP synthesis in 16HBE treated with ISs from COPD patients compared to unsilenced cells. Finally, the cellular extract from 16HBE (co-*immunoprecipitated* with anti Ac-His H3 (k9) and revealed with anti-IKKα) showed higher levels of IKKα in 16HBE stimulated with ISs from COPD patients or IL-17 A than in the cells pretreated with Tiotropium or than in the cells stimulated with ISs depleted of IL-17A. All together, these findings suggest that IL-17A present in the airways of COPD patients might be able to generate IKKα activation that in turn is able to control acetylation of Ac-His H3 (k9). Finally, our results suggest that the anticholinergic drug might exert its antinflammatory role controlling molecular mechanism involving IKKα activation generated by IL-17A and the related synthesis of IL-8 and TSLP in bronchial epithelial cells.

Conclusions

In conclusion, we demonstrate the anti-inflammatory effects of an anticholinergic drugs on IL-17A mediated chromatin remodeling mechanisms promoting IL-8 and TSLP release in bronchial epithelial cells. These findings might suggest the use of Tiotropium as an useful alternative therapy to control IL-17A induced glucocorticoid insensitivity in human bronchial epithelial cells during COPD.

The study of Induced sputum could be an important instrument in the personalized therapy of the chronic disease. With these different approach, the therapeutic strategy could be more targeted and specific for any patients, reducing costs and optimizing drug administrations.

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