

# Airway inflammation in healthy smokers

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Cigarette smoking is a risk factor associated with lung cancer and many other neoplasms of various organs, coronary artery disease and numerous vascular disorders, Chronic Obstructive Pulmonary Disease (COPD) and other types of lung diseases [1-3]. Much work has been done on the *in vitro* and *ex vivo* carcinogenic effects of tobacco smoking while the effects of smoking on innate and adaptive immune function have been studied to a lesser degree. Recent data suggests that cigarette smoke alters the functions of the immune system and increases susceptibility to viral and bacterial infections [4-6].

In the respiratory system along the last years many studies have depicted the changes induced by smoke on various aspects of local immunoregulation and inflammation in patients with various stages of COPD. Less attention has been paid to the changes induced by cigarette smoking on the vast category of individuals defined as "healthy smokers". This is a population outnumbering COPD patients, as it is estimated that COPD develops in a minority of smokers. "Healthy smokers" data is usually gathered to compare different aspects of airway inflammation with those obtained from patients with COPD with a history of tobacco smoking. The rationale for making this comparison is that the two populations, healthy smokers and COPD patients, were (or are still) exposed to similar burden of tobacco smoke during their life, yet only those who develop a progressive and non reversible airflow limitation over the years may be called patients, as they have COPD.

From the COPD point of view this comparison holds its value, as we try to find the "Holy Grail" in the pathogenesis of COPD: the particular aspect or pattern of immunodisregulation that leads to disease only in a fraction of smokers.

From the point of view of control individuals who smoke and yet have normal spirometry, this comparison is somehow unfair and may be misleading. They may appear to be healthy, i.e. individuals who do not have all the changes in local inflammation and immunity as seen in COPD patients. Therefore, they may appear as individuals who are in fact healthier than the smokers who develop COPD, as they can cope better than COPD patients with the noxious effects of cigarette smoke.

However, when compared with normal individuals who never smoked, the assumption that control smokers are healthy is much less obvious. We believe that in this field a better comprehension of the changes associated and induced by cigarette smoking on the airway inflammation and immunity may help us to redefine, at least from a biological point of view, the real status of "healthy smokers" for these individuals.

In the present paper we will review the effects of cigarette smoke on innate and adaptive immune function looking mainly at *in vitro* data and to the inflammatory changes induced *in vivo* by cigarette smoke in the bronchial mucosal tissue and bronchoalveolar lavage (BAL) of healthy smokers.

## Cigarette smoke

Cigarette smoke contains more than 45,000 chemicals, which have toxic, mutagenic and carcinogenic effects. The major components of smoke include nicotine, tar, ammonia, carbon monoxide, carbon dioxide, formaldehyde, acrolein, benzopyrenes, hydroxyquinone, nitrogen oxides, cadmium [7, 8]. Many of these substances are known to be carcinogenic and toxic to the cells. Tar and nicotine have shown to have immunosuppressive properties by affecting the innate immune response of the host [7, 8]. Exposure to cigarette smoke may cause T cell unresponsiveness [9]. Nicotine impairs antigen mediated signal transduction in lymphocytes and induces a state of T cell anergy [10, 11]. In addition, nicotine modulates the production of inflammatory cytokines by alveolar macrophages [12]. An *in vitro* study showed that a post-infection treatment of alveolar macrophages with nicotine increased the replication of bacteria in the macrophages and downregulated the macrophage-production of Interleukin (IL)-6, IL-12 and Tumour Necrosis Factor (TNF) $\alpha$  [13]. Furthermore, the authors demonstrated that nicotine induced suppression of antimicrobial activity was mediated by nicotinic acetylcholine receptors [13]. Cigarette smoke extracts also alters the function of immune cells. An inhibitory effect of tar was reported for *in vitro* production of IL-1 $\beta$ , IL-2, Interferon (IFN) $\gamma$  and TNF $\alpha$ . A phenolic compound, hydroxyquinolone,

and catechol inhibited lymphocyte proliferation [14]. Acrolein inhibited the expression of IL-8 and human beta defensin-2 in sinonasal epithelial cells [15].

### Effects of smoking on epithelium, alveolar macrophages, neutrophils and lymphocytes

Cigarette smoke has deleterious effects on the ciliary epithelium. Varying degrees of denudation of ciliary epithelium, an increase in the number of goblet cells, submucosal gland hypertrophy and squamous cell metaplasia [16, 17] have been reported. Cigarette smoke impairs bacterial clearance leading to increased inflammation related to epithelial damage [18]. In a mouse model of infected lung, addition of cigarette smoke increased the inflammatory response due to cytokines, chemokines and myeloperoxidase (MPO) as compared to sham exposed animals [18]. Measurements of transepithelial electrical resistance in response to cigarette smoke, decreased with an increase of albumin influx indicating a loss in barrier function of the epithelial layer, suggesting that cigarette smoke damages the epithelial junctions making it more permeable to macromolecules [19].

Macrophages are responsible for the presentation of antigen to the immunocompetent cells and alveolar macrophages are key phagocytic cells in the normal lung. Exposure to cigarette smoke changes the macrophage phenotype [20], reducing significantly its phagocytic function and the capacity to clear the inflammatory cells and debris from the lung [21, 22]. In particular, the ability of the phagosomes and lysosomes to fuse is defective in macrophages from smokers compared to non-smokers [23]. Alveolar macrophages also secrete inflammatory mediators, oxidants, proteases in response to cigarette smoke extracts. Furthermore, alveolar macrophages from smokers show a decreased expression of Toll-like receptor (TLR)2, TLR4 and Cluster Denomination (CD)14 compared to alveolar macrophages from non-smokers [24, 25].

Exposure to cigarette smoke leads to an influx of macrophage and neutrophils in the airways. *In vitro* treatment of neutrophils with cigarette smoke extract results in a high suppression of neutrophil caspase-3-like activity leading to impaired phagocytosis [26]. Acrolein and nicotine also have similar effects on neutrophil's phagocytosis [27]. Other neutrophil functions such as chemotaxis, glycolytic activity, cellular adhesion and arachidonic acid metabolism are impaired by cigarette smoke [27]. These effects render these cells less effective against bacterial infections and have a potential for damaging surrounding tissues.

T cells activate alveolar macrophages to produce matrix metalloproteinase (MMP) 12, an elastin degrading enzyme that has been linked to emphysema [28]. CD8+ lymphocytes are required for inflammation and tissue destruction in smoke induced emphysema [29] and increased levels of CD8+ cells have been reported in central and peripheral airways of COPD patients [30]. Studies in mice have shown that chronic exposure to cigarette smoke induces an

oligoclonal T cell (CD8+ and CD4+) expansion [31]. Cigarette smoke also promotes the retention of virus-specific CD8+ memory effector T cells but to weaken their defensive ability [32]. Cigarette smoke may promote a reduction in the breadth of the TCR repertoire, contributing, by this way, to the increased susceptibility to viral infections that is observed in smokers [33].

### Inflammatory alterations in bronchial biopsies of healthy smokers and healthy non smokers

The analysis of bronchial biopsies of healthy smokers with normal lung function, when compared with healthy non-smokers with normal lung function, matched for age and sex, showed signs of tissue inflammation in smokers [30]. A slight increase of CD3+ and CD8+ lymphocytes was associated to smoking habit [30]. Inducible NO synthase (iNOS) and endothelial NO synthase (eNOS), the two functional isoforms of NO synthase involved in the release of endogenous NO in the bronchial tissue, are both increased in smokers [34], suggesting an increased NO consumption also in smokers with normal lung function, not related, however to increased protein nitration, documented by the increased nitrotyrosine immunorexpression, as observed in severe COPD patients [34]. We also reported increased epithelial levels of CX-Chemokine ligand (CXCL)6 (granulocyte chemotactic protein (GCP)-2 and of its receptor, CXCR1, in the submucosa of smokers, compared to non-smoking subjects [35]. Human GCP-2 induces increased Ca<sup>2+</sup> intracellular concentrations and chemotactic responses in CXCR1 transfected cells. It also induces activation of neutrophils and can contribute to detrimental tissue damage [36]. Increased levels of infiltrating IL-17A+ cells are reported in smokers [37], suggesting again a role in inducing neutrophil migration and activation in association with smoking habit. Nuclear factor- $\kappa$ B (NF- $\kappa$ B), the transcription factor involved in the regulation of nuclear transcription of a number of cytokines and mediators, is increased in the epithelium of smokers [38] as well as its nuclear expression in the submucosa of the same subjects [38], suggesting again an increased inflammatory response in these subjects. Interestingly, total, cytoplasm and nuclear expression of the transcription factor signal transducer and activator of transcription (STAT)4, together with IFN $\gamma$ , was similarly expressed in smokers and non-smoking subjects [39], suggesting a modest activating process of T lymphocytes producing IFN $\gamma$  in these subjects. This data clearly shows the presence of an ongoing inflammatory process in the bronchi of smokers with normal lung function. However, this inflammatory process seems to be under control, since the effects of iNOS and eNOS increase become dangerous only in COPD patients showing a parallel detrimental increase of nitrotyrosine in their bronchial tissue. Also the increase of p65NF- $\kappa$ B is not accompanied by the parallel increase of IFN $\gamma$ , seen in COPD patients with worsening disease, suggesting again that the inflammatory response, even though present,

remains at least in part, under control in healthy smokers with normal lung function.

### BAL

Through the use of BAL we were able to widen our knowledge on the inflammatory and immune pathways in the respiratory system. It is considered that BAL samples are representative of the molecular and cellular processes ongoing into the lower part of the respiratory tree and also into the alveolar spaces. Not surprisingly data obtained from BAL do not exactly reflect those from bronchial biopsies (representative of the large airways).

The literature data on the "normal values" for BAL in smokers and non-smokers dates back many years, as BAL was developed in the late seventies, but it contains rather large data sets and thus it is considered reliable [40]. Typically smokers have increased numbers of total BAL cells, with a shift in the proportions of different cell types, as compared with normal non-smokers. Increased percentages of alveolar macrophages, of neutrophils and/or eosinophils and decreased percentages of BAL lymphocytes are usually reported. For lymphocytes, a shift in CD4/CD8 ratio with a decrease of the ratio due to over representation of CD8+ cells has also been reported [41]. Macrophages recovered by BAL in a normal smoker are usually loaded with carbon particles [40].

Many molecules and mediators were studied and measured in the BAL samples from "healthy smokers". Some of these molecules are increased and/or show enhanced biochemical or biological activity in smokers vs. non-smokers. Our group showed some years ago that, compared with control non-smokers, control smokers had higher levels of monocyte chemoattractant protein-1 (MCP-1) in BAL, an increase that is comparable to what observed in BAL from COPD patients [42]. IL-8 and IL-1 $\beta$  levels in BAL were also increased in smokers as compared with non-smokers in a dose (i.e. pack/year)-dependent manner [43]. Other molecules mediating tissue damage as MPO, Human Neutrophil Lipocalin (HNL), MMP9 and 12 were demonstrated to have either increased levels and/or enhanced activities in samples from BAL of smokers as compared with non-smokers [44-46].

In contrast, other markers had been shown to be decreased in BAL from smokers when compared with non-smokers. One example is Vascular endothelial growth factor (VEGF) $\alpha$  [47]. This is probably related to a destructive activity of cigarette smoke compounds on the complex between VEGF $\alpha$  and its receptor. Similar detrimental effects of tobacco smoke are the cause of the reduction of Superoxide Dismutase (SOD) and of alpha-1 antitrypsin activity in BAL from smokers vs. non-smokers [48, 49].

### Conclusions

The analysis of the available data on the changes observed in many different aspects of airway immune homeostasis, protease/antiprotease balance and pathways leading to inflammatory processes

shows that individuals who smoke present an impressive number of abnormal molecular and cellular parameters in their bronchi and lung. Further investigations are needed to discriminate between pathologic and non-detrimental, well controlled, inflammatory pathways in different categories of smokers. However, the presence of a normal spirometry in individuals who smoke and as a consequence show the above described abnormalities does not seem to us sufficient to classify them as "healthy smokers".

In clinical practice, individuals who are symptomatic for chronic cough and sputum production are considered to have chronic bronchitis (CB). This diagnosis relies only on symptoms, not on spirometry, as this can be normal in patients with CB. In this context, previous COPD Guidelines indicated that individuals who smoke and are symptomatic (cough and excessive sputum production) may be considered at risk for the development of COPD or even already stage 0 COPD patients.

However the data above reported refers also to individuals who smoke and declare to have no respiratory symptoms, in addition to show normal spirometric values. Also this category of individuals should be regarded not as a healthy population but as a diseased one.

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