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Impact of cigarette smoke exposure on host-bacterial pathogen interactions

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Abstract

The human respiratory tract from individuals with a normal lung function maintains a fine-tuned balance, asymptomatically colonized by the normal microbiota in the upper airways and sterile in the lower tract. This equilibrium may be disrupted by the exposure to insults such as cigarette smoke. In the respiratory tract, the complex and noxious nature of inhaled cigarette smoke alters host-microorganism interaction dynamics at all anatomical levels, causing infections in many cases. Moreover, continuous exposure to cigarette smoke itself causes deleterious effects on the host which can trigger the development of chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD) and lung cancer. COPD is an irreversible airflow obstruction associated to emphysema, fibrosis, mucus hypersecretion and persistent colonization of the lower airways by opportunistic pathogens. COPD patients keep a stable but progressively worsening condition, and suffer periodic exacerbations caused, in most cases, by infections. Although smoking and smoking associated diseases are associated to high risk of infections, most therapies aim to reduce inflammatory parameters, but not necessarily take into account the presence of persistent colonizers. The effect of cigarette smoke on host-pathogen interaction dynamics in the respiratory tract, together with current and novel therapies is discussed.

Effect of cigarette smoke exposure on the human respiratory tract

General features of the human respiratory tract

The human upper respiratory tract is colonized since birth by the respiratory microbiota. Colonizers are commensal microorganisms and/or opportunistic pathogens [1]. Microorganisms encompassing the human respiratory microbiota are highly adapted to the host, and examples of co-evolution have been described for several human restricted opportunistic pathogens such as *Neisseria meningitidis*, *N. gonorrhoeae* and *Moraxella catarrhalis* [2]. Differently, the lower respiratory tract of individuals with normal lung function is sterile. Maintenance of lung sterility is physiologically relevant given that the lung is the region where the gas exchange takes place. Physical, anatomical and mechanical barriers including nasal hair, coughing and the mucociliary escalator constitute a first line of defense, avoiding the arrival of microorganisms to the lower tract. The mucociliary escalator is a layer of hydration above the lung tissue, which combined with mucus and the cilia present on the respiratory epithelium, is a trapping and removal system for foreign particles and invading pathogens [3]. Alveolar epithelium consists of type I and II pneumocytes. Type I pneumocytes display an anatomical function; by covering 95% of the alveolar surface, they generate a thin barrier between the alveolar space and the blood vessels. Type II, although covering 5% of the alveolar surface, are more abundant in number than type I pneumocytes [4]. Pneumocytes play crucial roles in lung defense: (i) maintenance of a low lung surface tension, stopping the surfaces for gas exchange sticking together by synthesis, secretion and reabsorption of pulmonary surfactant; (ii) transport of water and sodium; (iii) metabolism of xenobiotic compounds; (iii) lung regeneration; (iv) recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs); (v) secretion of antimicrobial peptides; (vi) secretion of cytokines and

chemokines which orchestrate host inflammatory responses; (vii) generation of a barrier for pathogen entry by tight junction formation between epithelial cells [5,6,7,8,9]. Alveolar macrophages are lung resident professional phagocytes, responsible for eliminating microorganisms by phagocytosis and phagolysosomal processing. Alveolar macrophages also secrete inflammatory mediators directing, when necessary, neutrophil recruitment from the bloodstream to the alveolar space [10]. Moreover, airway cells produce a repertoire of soluble molecules which are essential players in microbial clearance of the lower tract. Those molecules, present in the aqueous fluid on the surface of the respiratory tract, include the complement system, antimicrobial peptides, lysozyme, lactoferrin, the secretory leukoprotease inhibitor (SLPI), and SP-A and SP-D surfactant proteins [11].

The fine equilibrium orchestrated to guarantee alveolar sterility is altered upon continuous host exposure to noxious particles and gases present in the environment. In this review, we will focus in the deleterious effect of continuous exposure to tobacco smoking and in the impact of such a noxious agent in the respiratory microbiota.

Cigarettes and tobacco smoke: features and components

A cigarette consists of a blend of tobaccos surrounded by a paper with a defined specification. Most cigarettes are filter tipped and tip ventilated. Tip ventilation means that mainstream smoke is diluted with a defined amount of air during a puff. The tobacco blend, the cigarette paper, the type and efficiency of the filter, and the degree of tip ventilation determine the chemical composition of cigarette smoke. When cigarettes are smoked, a complex mixture is inhaled into the respiratory system. During the sequence from lighting a cigarette to inhaling a puff of smoke, various overlapping chemical, physical and physiological phenomena occur, i.e. burning, pyrolysis,

pyrosynthesis, distillation, sublimation and condensation processes [12]. Tobacco smoke is an aerosol consisting of solid/liquid droplets (particulate- ("tar") phase) in a gaseous phase. Approximately 4700 different substances have been identified in fresh tobacco smoke. These include neutral gases, carbon oxides, nitrogen oxides, amides, imides, lactames, carboxylic acids, lactones, esters, aldehydes, ketones, alcohols, phenols, amines, volatiles N-nitrosamines, N-heterocycles, hydrocarbons, nitriles, anhydrides, carbohydrates, ethers, nitro-compounds, metals and short-/long-living radicals. The quantity of the components in mainstream smoke of a single cigarette ranges from mg (water, carbon monoxide, carbon dioxide, nicotine) to pg levels (heterocyclic amines and heavy metals) [12]. Inhaled particulate matter (PM) is deposited in the respiratory tract depending on the particle size, with larger particles deposited in the upper and larger airways and smaller particles penetrating deep into the alveolar spaces. Ineffective clearance of this PM causes particle retention in lung tissues, resulting in a chronic, low-grade inflammation that may be important in the progression of chronic lung diseases associated to long term smoking [13]. In addition to chemicals, it has been documented the presence of microorganisms in cigarettes. All tobacco is cured, during which time there is a rapid growth of diverse bacteria and fungi, and accumulation of microbial toxins. Mesophilic bacteria have been found in both fresh and cured tobacco leaves. A range of additional bacteria and fungi have been found in minor amounts; moreover, storing cigarettes at high humidity results in elevated levels of fungi in the cigarette tobacco, leading to increased ergosterol concentrations in the smoke [14]. In addition, the bacterial metagenome of a cigarette-based study revealed fifteen different classes of bacteria and a broad range of potential pathogens (*Acinetobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Klebsiella*, *Pseudomonas aeruginosa*, *Serratia*, *Campylobacter*, *Enterococcus*, *Proteus* and

Staphylococcus) [15,16]. The risk of infection with potential pathogens by inhaling the mainstream smoke is currently unknown.

Pathologies associated to tobacco smoking: an overview

Smoking tobacco causes up to 90% of all lung cancers and is a significant risk factor for stroke and heart attacks. Smoking is also recognized as a risk factor for a variety of respiratory tract and systemic infections in children and adults, including common cold, influenza, pneumonia and tuberculosis [17]. Importantly, smoking is the leading risk factor for chronic obstructive pulmonary disease (COPD). COPD is characterized by a slowly progressive and irreversible airflow obstruction, loss of lung tissue leading to emphysema, and remodeling of tissue (fibrosis), both of which contribute to further lung function decline, reduced quality of life and high mortality [3,18]. Changes in the immune system, triggered by noxious particles and gases present in the tobacco smoke, lead to an inflammatory cellular infiltrate and to a pronounced and chronic lung inflammation. This in turn leads to other pathological changes including chronic obstructive bronchitis with fibrosis and obstruction of small airways, emphysema with enlargement of airspaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways [19,20]. Tobacco smoke also leads to lung infections by pathogenic bacteria and viruses, which are key triggers of the acute worsening of COPD called exacerbation [21]. Exacerbations are an additional major factor in the morbidity and mortality caused by COPD, and the major healthcare costs associated with the disease [22,23,24].

Molecular and cellular mechanisms associated to COPD progression

The effect of cigarette smoke on airways immunity has been extensively characterized in COPD [19,25] (summarized in Fig. 1). COPD progression is associated with the accumulation of inflammatory mucous exudates in the lumen and infiltration of the wall by innate and adaptative inflammatory immune cells; these changes are coupled to a repair and remodeling process that ultimately thickens the airways walls [26]. An additional consequence of long term smoking is the persistent colonization of the lower respiratory tract by opportunistic pathogens, which often has an amplification effect and contributes to the progression of the disease [27,28].

Cigarette smoke has deleterious effects on the mucociliary system by promoting a decrease of the ciliary beating frequency, denudation of the ciliary epithelium, increase in the number of globet cells, submucosal gland hypertrophy and squamous cell metaplasia [29]. Cigarette smoke also damages the epithelial junctions, due to a significant down regulation of genes involved in the formation of tight junctions such as occludin, ZO1 and claudin-1, which leads to a decrease of the epithelial transepithelial resistance, correlated to an increase of epithelial permeability [30,31].

Cigarette smoke activates the respiratory epithelium to produce inflammatory mediators (TNF-α, IL-1β, GM-CSF, IL-8, leukotriene B_4-LTB_4), responsible for activating and/or recruiting alveolar macrophages and neutrophils. Several studies have shown that there is an increase in the total number of neutrophils, macrophages and T lymphocytes in lung parenchyma and peripheral and central airways of COPD patients [20,32]. Epithelial cells in the small airways also secrete TGF-β, which induces local fibrosis [19]. It is well-known that cigarette smoke induces epithelial cell death, which also amplifies the on-going inflammatory response [19]. Regarding the impact of cigarette smoke on the production of antimicrobial molecules by airway epithelial cells, the expression of the antimicrobial peptide hBD-2 in brushed bronchial epithelial cells from

COPD patients has been found to be lower than in tissues from healthy subjects [33]; in addition, significantly decreased levels of SP-A and SP-D surfactant proteins have been observed in smokers, compared to non-smokers [34]. Alveolar macrophages activated by cigarette smoke secrete a repertoire of inflammatory mediators, some of them (IL-8, GRO-α, LTB_4, MCP-1) being neutrophil chemoattractants [19]. Alveolar macrophages show an increase in the respiratory burst and release elastolytic enzymes, including matrix metalloproteinases (MMPs) and cathepsins K, L, S. Those enzymes, MMP-9 in particular, contribute to alveolar emphysema by enhancing the effects of elastase released by neutrophils [19]. Even though the inflammatory response of smokers is clearly different to that of non-smokers, the effect of cigarette smoke on the expression of TLR2, TLR4 and CD14 on alveolar macrophages and monocytes in response to their ligands is currently unclear [35,36]. Neutrophils, recruited due to the elevated levels of chemoattractants released by epithelial cells and macrophages, show an increase in the respiratory burst and secrete serine proteases (neutrophil elastase, cathepsin G, proteinase 3, MMP-8 and MMP-9) due to degranulation. The tripeptide proline-glycine-proline PGP (and the N-acetylated-PGP form) is a selective neutrophil chemoattractant generated from extracellular matrix proteins through enzymatic reactions catalysed by MMP-8 and MMP-9. Leukotriene A4 hydrolase (LTB_4H), produced by neutrophils and epithelial cells has a dual function. It generates LTB_4 and it has aminopeptidase activity, thus inactivating PGP, which contributes to resolve neutrophilic inflammation in acute lung infections once the pathogen disappears. Smoke inhibits LTA_4H aminopeptidase activity and stabilizes PGP through acetylation; in this way, neutrophil migration into the lung increases, leading to persistent inflammation [37]. Cigarette smoke exposure also results in a suppression of neutrophil caspase-3-like activity, which ultimately impairs its phagocytic activity [38]. Importantly, cigarette smoke exposure causes an

impairment of both alveolar macrophage and neutrophil phagocytic activity [39,40,41,42].

Oxidative stress is an imbalance that occurs when reactive oxygen species (ROS) cannot be controlled by antioxidant defense mechanisms (enzymatic defense mechanisms are catalase, superoxide dismutase, glutathione peroxidase, etc.; non-enzymatic defense mechanisms are glutathione-GSH, ascorbate, urate, etc.) and results in harmful effects [19]. Oxidative stress plays a key role in the patho-physiology of smoking associated diseases [43,44,45]. ROS from cigarette smoke itself (the gas phase is estimated to contain over 10^{15} free radicals [46], and those produced by inflammatory cells (alveolar macrophages and neutrophils respiratory burst induced by cigarette smoke), result in inflammatory and destructive damaging effects [43]. These effects include: (i) an overall increase in proteases activity leading to emphysema; (ii) amplification of the inflammatory response due to ROS-induced activation of NF-κB, resulting in increased secretion of IL-8 and TNF-α and subsequent neutrophil recruitment; (iii) steroid resistance (see later); (iv) increased oxidation of arachidonic acid leading to the production of isoprostanes, which trigger bronchoconstriction and plasma exudation; (v) activation of TACE (TGF-α converting enzyme), which promotes the shedding of TGF-α and the activation of the epidermal growth factor receptor (EGFR), resulting in the increased expression of mucin (*MUC5AC* and *MUCB*) genes and the differentiation of mucus-secreting cells [19]. Differentiation of globet cells via EGFR activation and mucus secretion are also stimulated by IL-13 [47]. The excess production of mucus contributes to the occlusion of the small airways in COPD. Independently, ROS also activate JNK by Src, triggering *MUC5AC* expression in an EGFR independent manner [48].

Cigarette smoke has also an impact in the host adaptative immunity. Smoking has been shown to reduce serum levels of immunoglobulins in humans [49,50]. Moreover, there is an increase in the total number of T lymphocytes in lung parenchyma and peripheral and central airways of COPD patients, more prominent for CD8+ cells [19,51]. These patients show an increase of mature dendritic cells (DC) in the peripheral airways, and DC from smokers display an increased expression of CD80 and CD86 [52]; it is likely that material in the lung of smokers is taken up by these cells and presented by DC-MHC I to CD8+ lymphocytes. Once activated by antigen-bearing DC, T cells may access to the lung parenchyma by means of their tissue specific chemokine receptors [20]. Indeed, T cells in peripheral airways of COPD patients show increased expression of CXCR3, preferably CD8+ cells. The ligands for CXCR3 (CXCL9, 10 and 11) are expressed by bronchial epithelial, airway smooth muscle cells and alveolar macrophages, which would contribute to CD8+ cell accumulation [19,53]. CD8+ cytotoxic T cells abundance in the lung from COPD patients correlates with the degree of airflow obstruction and emphysema; CD8+ cells cause alveolar epithelial cell death through the release of perforin and granzyme A and B [20,54]. CD4+ T cells are also found in large numbers in the airways and parenchyma of COPD patients, where they express STAT4, IFN-γ and Th1 cytokines, contributing to transendothelial migration of inflammatory cells to the airways; such a recruitment progresses as COPD worsens [20]. However, cigarette smoke suppresses Th1-mediated immune response to gram-negative bacterial infections by interfering MyD88/IRAK signaling, thereby reducing LPS-induced TLR4 expression; this may contribute to explain the increased susceptibility to bacterial infections in COPD [55].

Effect of cigarette smoke exposure on bacterial infections

Continuous exposure to cigarette smoke has been associated to changes in the composition of the nasopharynx's microbial community. The microflora from smoker´s nasopharynx contains larger proportions of opportunistic pathogens (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. pyogenes*) than never smokers which, in turn, mainly contain α-hemolytic streptococci, *Peptostreptococcus spp*., *Prevotella spp.* [56]. Interestingly, smoking cessation is associated with a reversion to the microflora found in never-smokers, thereby suggesting that cigarette smoke does indeed favor colonization by pathogens [57]. Supporting this notion, cigarette smoke enhances bacterial attachment to epithelial cells and promotes changes in virulence by modifying bacterial gene expression [58,59,60].

Cigarette smoke affects the upper airways. Tobacco smoke is a risk factor for periodontitis [61,62,63], being a more severe disease in smokers than in never-smokers [62,64]. Tobacco smoke promotes colonization of the sub-gingival space by opportunistic pathogens such as *Porphyromonas gingivalis, Campylobacter rectus, Prevotella intermedia, Tannerella forsythia, Treponema denticola* and *Fusobacterium nucleatum* [63,65,66,67]. Smoking cessation correlates with a decrease of periodontal pathogens prevalence [65,68]. *P. gingivalis* is the causative agent of chronic periodontitis; when bacteria are exposed to cigarette smoke, it has been observed an increased expression of the bacterial fimbrial protein FimA upon smoke exposure, which could abrogate bacteria triggered inflammatory responses and promote biofilm formation and bacterial adherence to the airway epithelium [58]. Cigarette smoke also promotes changes in sinosanal microbiota, driving the formation of reversible robust biofilms maybe involved in bacterial recalcitrant persistence in the nasal cavity [69]. Tobacco smoking is related to an increase in the occurrence and severity of acute infections by bacterial pathogens [61,70]. Moreover, second hand smoke causes a wide

range of diseases in children. Parental smoking increases *S. pneumoniae* infant carriage in general, and carriage of serotypes included in the conjugate 7-valent vaccine in particular [71]. Parental smoking also increases the risk of meningococcal meningitis [72,73], otitis media [1], and lower respiratory tract infection in infants younger than two years [74].

The "vicious circle hypothesis"

An additional consequence of cigarette smoke exposure is the persistent colonization of the lower respiratory tract by opportunistic microbial pathogens. Such a chronic microbial colonization contributes to COPD progression, by further amplifying the inflammatory processes previously described. The so called "vicious circle hypothesis" was proposed to explain how chronic bacterial colonization of the lower airways in smokers can perpetuate inflammation and contribute to the progression of smoking associated diseases (Fig. 2) [28,75]. Central to this hypothesis is the notion that once pathogens have gained a foothold in the lower respiratory tract due to smoking-triggered impairment of the mucociliary clearance, they persist by further blocking mucociliary clearance [28,75]. Cigarette smoke also up-regulates mucus production, impairs epithelial elastic properties, down-regulates the levels of IgA, and affects the phagocytic activity of professional phagocytes [19,41]. Together, these alterations facilitate bacterial colonization of the lower respiratory tract, associated to an exacerbation of the inflammatory response due to the recognition of PAMPs. Both bacterial products and bacterially produced epithelial damage contribute to the impairment of host immunity, further allowing the access of microorganisms to the lower respiratory tract in an endless loop, ultimately translated in high chronic inflammation and persistent microbial colonization of the lungs [75]. This endless loop

is known as “vicious circle” [28,75]. Microorganisms frequently isolated from the lower respiratory tract of smokers and of persistently colonized patients are nontypable *Haemophilus influenzae* (NTHi), *M. catharralis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. The most frequently isolated pathogen, and the one responsible for a significant percentage of exacerbation episodes in COPD is NTHi [76,77].

Effect of smoking on NTHi, M. catharralis*,* S. pneumoniae *and* P. aeruginosa *infections*

H. influenzae is a member of the human respiratory microflora located mainly in the oro- and nasopharynx. It colonizes 40-80% of healthy individuals, with a frequency of carriage higher in children than in adults [1,78]. Transmission occurs by aerosols or by direct contact with mucosal surfaces. *H. influenzae* carriers are simultaneously colonized with multiple strains in continuous renewal, mainly nontypable (non-capsulated) [79,80]. *H. influenzae* is endowed with molecular strategies to adapt to the host, evade predation, and compete or coexist with other bacteria from the same or different species such as *Staphylococcus aureus* and *S. pneumoniae* [81]. Simultaneous presence of *H. influenzae* and *S. pneumoniae* in the upper respiratory tract triggers a synergistic inflammation, resulting in neutrophil recruitment to the respiratory mucosa [82]. Such a neutrophil recruitment leads to a selective killing of complement-opsonized *S. pneumoniae*. Co-colonisation by *S. pneumoniae* and *H. influenzae* provides a stimulus (the *H. influenzae* peptidoglycan) to induce neutrophil and complement-mediated clearance of *S. pneumoniae* from the mucosal surface in a Nod-1 dependent manner [83,84]. *H. influenzae* co-colonisation seems to favor the selection of opsonophagocytosis-resistant *S. pneumoniae* capsule serotypes. Thus, competition with *H. influenzae* during their commensal state turns pneumococci into more virulent

populations, which may account for further development of invasive disease [85]. Although cigarette smoke does not seem to alter NTHi viability [41], host cell exposure to this irritant reduces bacterial invasion of respiratory epithelial cells (P. Morey, unpublished) and alveolar macrophages phagocytic ability [39,40,41,42]. Normally, alveolar macrophages efficiently phagocyte and degrade NTHi by phago-lysosomal fusion. Cigarette smoke dramatically impairs bacterial ingestion, but not the ingestion of inert particles. PI3K signaling including Akt phosphorylation is required for NTHi phagocytosis by alveolar macrophages. Cell exposure to cigarette smoke diminishes phospho-Akt levels, which may account for the observed phagocytic deficiency; same observations were made by using immortalized macrophages and macrophages from bronchoalveolar lavage (BAL) from both smokers and COPD patients, compared to macrophages from never-smokers [41]. The levels of lipopolysaccharide-binding protein (LBP) and CD14 are higher in BAL from smokers and COPD patients than from never-smokers [86]. Furthermore, cigarette smoke induces the expression of LBP and CD14 by airway epithelial cells. Both proteins inhibit NTHi-dependent secretion of IL-8 and both NF-κB and p38 MAPK signaling pathways, but they increase NTHi entry in epithelial cells [86]. Given that NTHi can reside inside a late endosome-like compartment [87], LBP and CD14 may indeed contribute to NTHi colonization by favoring bacterial location inside a subcellular niche. Regarding adaptative immune cells, the main lymphocyte subsets shown to proliferate in response to NTHi stimulation are CD8+ and natural killer [88]; in terms of CD4+ memory T cell responses, NTHi-specific CD4+ memory T cells had memory phenotype with moderate to high CD27 and CCR7 expression, and circulated at low frequency in the peripheral blood of both healthy individuals and COPD patients [89].

Community-acquired pneumonia (CAP) is a major cause of hospitalization and provokes high mortality rates. *S. pneumoniae* is the most commonly isolated pathogen from CAP patients [90]. Smoking is a substantial risk factor for pneumococcal pneumonia, especially in patients with COPD [91,92], and for invasive pneumococcal disease [93]. Smoke also seems to exacerbate the impairment in mucociliary clearance of *S. pneumoniae* induced by the ingestion of ethanol [94]. Cigarette smoke has been shown to prevent pneumococci complement-mediated phagocytosis by alveolar macrophages, while the ingestion of unopsonized bacteria or IgG-coated microspheres is not affected, thus impairing pulmonary bacterial clearance [95].

M. catarrhalis causes about 10% of exacerbations in COPD and also colonises the lower airways of stable patients. Analysis of a collection of inflammatory parameters in sputum samples from a cohort of COPD patients before and after *M. catarrhalis* acquisition revealed a significant increase in IL-8, TNF-α and neutrophil elastase levels after infection [96]. An independent study detected *M. catarrhalis* specific Th1 cells in BAL fluid of COPD infected patients [97]. Moreover, cigarette smoke showed to decrease *M. catarrhalis*-induced hBD-2 antimicrobial peptide expression and PGE_2 induction, and increased this bacterial load on bronchial epithelium from smokers [33].

P. aeruginosa is another pathogen frequently isolated from pneumonia patients. Exposure to cigarette smoke increases host inflammation and decreases the rate of *P. aeruginosa* clearance [98]. The mechanism for the increased susceptibility to *P. aeruginosa* infection may be related to the fact that cigarette smoke decreases the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [99]. Epidemiological studies point that COPD patients are usually infected with one *P. aeruginosa* clone that remains in the lung for years, without evidence of interpatient transmission; during the chronic infection, the pathogen evolves towards an increased

mutation rate, increased antibiotic resistance, and reduced production of proteases, coexisting different morphotypes, and with patterns of infection and evolution that resemble those observed in cystic fibrosis [100].

Smokers are also more likely to suffer legionnaire's disease [101] and tuberculosis [102]. Tobacco smoke leads to loss of weight and an increased mortality by impairing $CD4^+$ T lymphocytes response to *Mycobacterium tuberculosis*, which is a key factor for macrophage IFN-γ-dependent activation and subsequent killing of intracellular *M. tuberculosis* [103]. Finally, *Mycoplasma pneumoniae* is another common pathogen in COPD patients [104]. As a smoking consequence, the lung tries to maintain the redox environment by mounting and maintaining high levels of GSH and gluthatione reductase (GR) (GSH adaptative response). *M. pneumoniae* infection interferes with this lung adaptative response to cigarette smoking, causing oxidative stress, which may contribute to the progression of the chronic disease [105].

Impact of anti-inflammatory therapies on bacterial respiratory infections

Given that inflammation is a main feature of smoking associated diseases, the control of both chronic and acute inflammation associated to exacerbations is a main issue in the treatment of these patients. COPD treatments are generally palliative, such as oxygen-therapy, bronchodilators, mucolytic agents and antibiotics. The use of anti-inflammatory agents is also a usual practice in these patients; an extensively used therapy is based in corticosteroids [106,107]. Considering that the upper (and frequently the lower) airways of patients receiving anti-inflammatory therapy are likely to be colonized, the effect of corticoids on pathogen-host interaction and/or microbial clearance should be taken into account. Exogenous blockage of the host inflammatory response orchestrated to face an infection could be detrimental for the host. Indeed,

although glucocorticoid (dexamethasone in particular) treatment of cultured cells upon infection by *S. pneumoniae*, *Neisseria meningitidis* or *Aspergillus fumigatus* has been shown to be effective in terms of inflammation reduction [108,109], adverse effects of steroid therapy on resistance to infection have been reported [110]. As an example, dexamethasone seems to impair *P. aeruginosa* clearance by suppressing iNOS expression and peroxynitrite production [111]. Independently, dexamethasone attenuates NTHi-induced NF-κB activation, but also synergistically enhances NTHi-induced TLR2 expression via specific up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38 MAPK. Glucocorticoid-mediated inhibition of NTHi-induced *MUC5A* expression also occurs via MKP-1 dependent inhibition of p38 MAPK [112,113,114,115].

Airway epithelium exposure to cigarette smoke does not modify NTHi adhesion to host cell surface, independently of the presence of dexamethasone. However, epithelial exposure to cigarette smoke reduces NTHi invasion of host cells, and this impairment is restored to normal levels when cigarette smoked cells are simultaneously treated with dexamethasone (P. Morey, unpublished). Differently, cigarette smoke-mediated impairment of alveolar macrophage ability to phagocyte NTHi is not restored when cells are simultaneously treated with dexamethasone [41]. The gluococorticoid fluticasone propionate seems to reduce the invasion of airway epithelial cells by *S. pneumoniae* [116].

These observations, together with the fact that the use of inhaled corticoids in COPD increases the risk of hospitalization for pneumonia [117], support the notion that corticosteroids may facilitate infections, despite their efficacy on reducing smoking-associated inflammation. In addition, there is evidence indicating that exposition to cigarette smoke may limit the efficiency of corticosteroids to attenuate the transcription

of inflammatory genes by affecting the balance between histone acetyltransferases (HAT) and histone deacetylases (HDAC) [118] (Fig 3).

Therefore, alternative treatments become compulsory. Although several novel possibilities are available and others are at different stages of clinical trials [107,119,120,121], it should be noted that in most cases there is no information on their impact on host-pathogen interaction. Even more, this important aspect is hardly considered as an outcome in the on-going clinical trials. Antioxidants and inhibitors of inducible nitric oxide synthase (iNOS) may be effective through inhibiting the generation of peroxynitrite. Available antioxidants are vitamins C and E and N-acetylcysteine; selective iNOS inhibitors and peroxynitrite scavengers are under development [118]. The HDAC activator theophylline [122] and the therapeutic inhibition of PI3K [123] have been proved able to reverse the steroid resistance induced by cigarette smoke. Other therapies are (i) long acting bronchodilators (long acting β2 agonist salmeterol; long acting anticholinergic tiotropium); mediator antagonists (inhibitors of LTB_4, IL-8, TNF-α or EGFR); (iii) protease inhibitors (endogenous antiproteases such as α1-antitrypsin, SLPI, elafin, cystatins, or small molecule inhibitors); (iv) novel anti-inflammatory treatments (inhibitors of phosphodiesterase 4, p38 MAPKinase, NF-kB or PI3K; resveratrol) [119,121]. Salmeterol has been shown to contribute to the protection of the airway epithelial barrier against *P. aeruginosa* [124]. The combination of salmeterol and fluticasone propionate has been shown to attenuate the inflammatory response of human airway epithelial cells infected with *Staphylococcus aureus* [125]. Although salmeterol also seems to protect the respiratory epithelium against *H. influenzae*-induced damage [126], *in vivo* data point that inhalation of this bronchodilator may negatively influence an effective clearance of NTHi from the murine respiratory tract [127]. Differently, resveratrol has been shown to

ameliorate *Serratia marcescens* induced acute pneumonia in rats [128], to inhibit swarming and virulence factor expression in *Proteus mirabilis* [129], to be a potential candidate against various *Helicobacter pylori* related gastric pathogenic processes [130], and to selectively inhibit *Neisseria gonorrhoeae* and *N. meningitidis* [131]. Finally, the increase of eukaryotic cAMP levels by adenylate cyclase activation could have a benefit in the treatment of NTHi infections by reducing bacterial invasion of epithelial cells (unpublished data). Same observations have been made for urinary tract infections caused by uropathogenic *E. coli* [132]. The PDE4 inhibitor rolipram has also shown to be effective in preventing *P. aeruginosa*-induced epithelial damage [133]. Opposite, PDE4 inhibition seems to impair host defense to *K. pneumoniae* infection in the pneumonia mouse model [134]. Altogether, these observations reinforce the notion that caution should be taken to extrapolate the findings obtained with one pathogen to infections caused by different microorganisms.

Final remarks

Alterations of the normal respiratory microflora caused by host exposure to external factors such as smoking have an undoubted impact in the host health and constitute a risk factor for chronic respiratory diseases and respiratory infections. Understanding the nature of host-pathogen dynamics is essential for the development of effective therapies, but the modulation of those dynamics by the host exposure to environmental agents should also be considered. Moreover, the therapies focused on the treatment of chronic respiratory diseases should also take into account the microbial component, if any, of the chronic disease, given that such therapies may influence, positive or negatively, on pathogen clearance and therefore, on the progression of the chronic disease. In conclusion, we would like to put forward the notion that, before approval by competent

authorities, any treatment likely to be taken by chronically colonized patients should be assessed in terms of its potential impact on host-pathogen dynamics, by testing a panel of relevant pathogens, and preferably including *in vitro* and *in vivo* approaches.

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Figure legends

Figure 1. Molecular mechanisms involved in COPD progression. Three major host alterations characteristic of COPD progression, fibrosis, emphysema and mucus hypersecretion, are shown in orange. A fourth characteristic of COPD progression, lower airways colonization by opportunistic pathogens, is shown in green. Microbial persistency is relevant because it greatly contributes to deleterious amplification of COPD features. Main host cell players in COPD patient airways, epithelial cells, alveolar macrophages (AM), neutrophils and CD8+ lymphocytes, are shown. Cigarette smoke activates epithelial cells to produce inflammatory mediators, activating and/or recruiting AMs and neutrophils. Epithelial cells also secrete the local fibrosis inducer TGF-β. Chemokines produced by epithelial cells and AMs activate CD8+ lymphocytes, which release the emphysema mediators perforin and granzymes. AMs secrete neutrophil chemoattractants and release proteases. MMP-9 activates the fibrosis inducer TGF-β and causes elastolysis, directly of by α1-AT inactivation. Proteases produced

AMs and neutrophils promote emphysema. Together with proteases, neutrophils secrete inflammatory mediators and granule content. Neutrophil chemoattractants are produced directly by AMs and epithelial cells, and by enzymatic activity of the AM protease MMP-9. COPD patient airways display oxidative stress. Cigarette smoke itself contains high levels of ROS and both AMs and neutrophils increase their respiratory burst in response to cigarette smoke. High concentration of oxygen and nitrogen reactive species have multiple consequences: (i) decreased antiprotease defenses and proteolysis; (ii) activation of NF-κB and neutrophil recruitment; (iii) steroid resistance; (iv) increased isoprostanes production; (v) mucus hypersecretion.

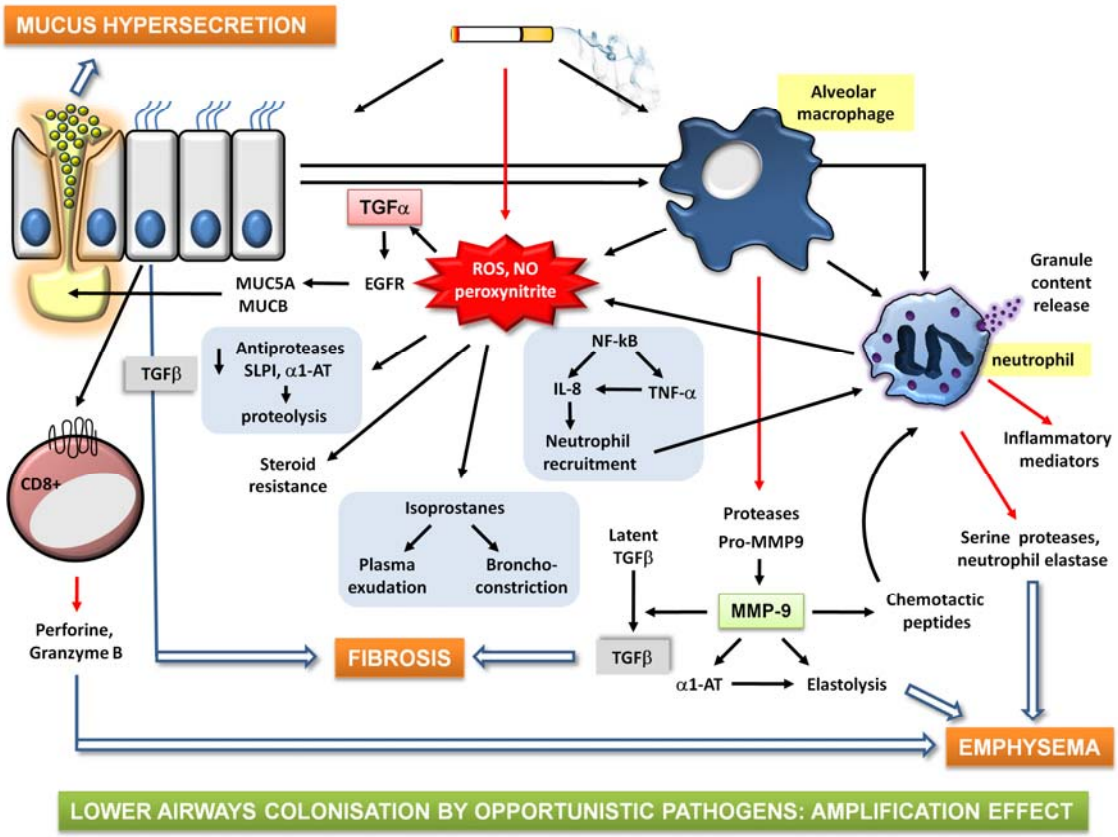


Figure 2. Vicious circle hypothesis in COPD progression. Cigarette smoke is an external insult which damages the respiratory tract immunity, allowing the lower respiratory tract colonization by microorganisms. Such a colonization is a starting point

for a cyclic sequence of events which progressively contribute to a high level of chronic inflammation, tissue damage and fibrosis, together with persistent bacterial infection of the lower airways. Altogether, these processes continuously contribute to the non-reversible progression of the chronic respiratory disease.

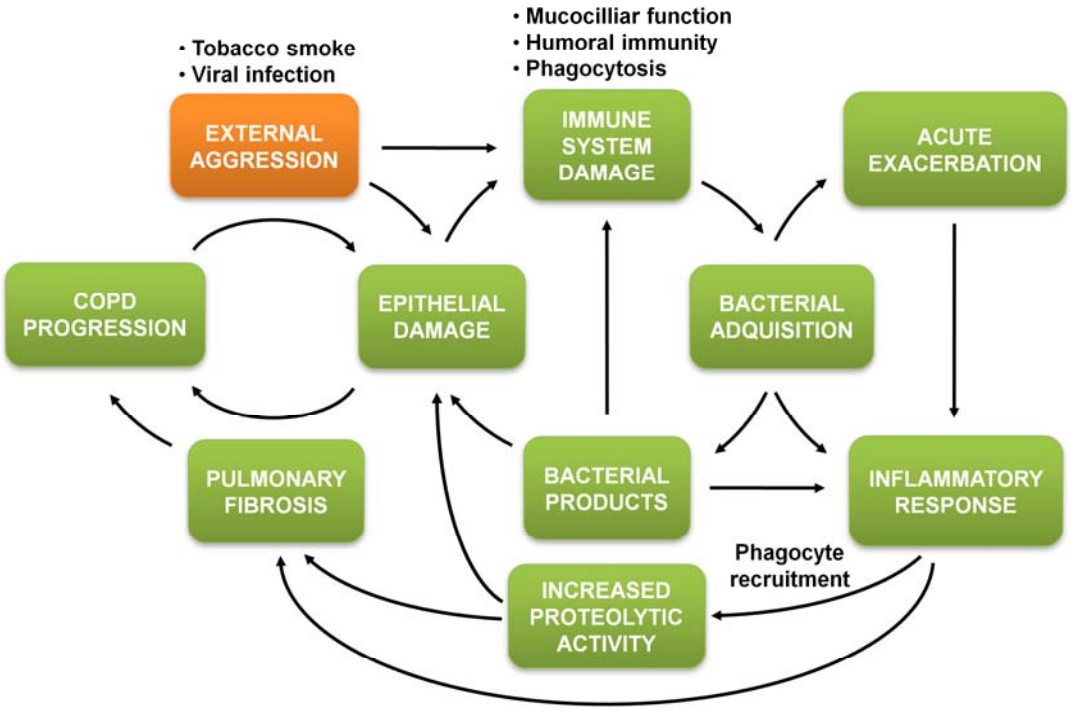
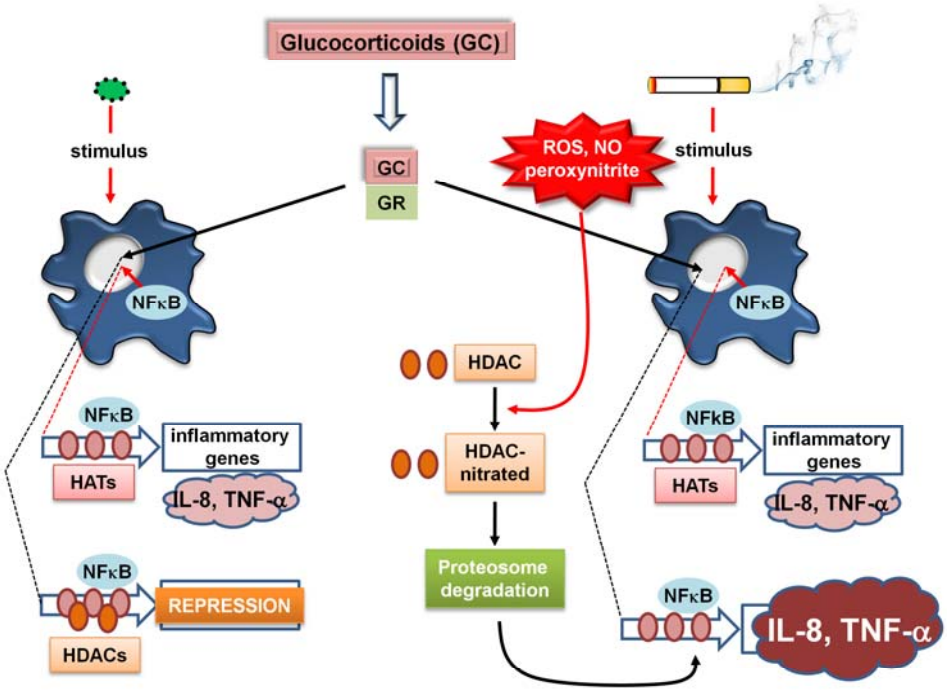


Figure 3. Cigarette smoke and host insensitivity to corticoids. HDAC reduction caused by cigarette smoke may account for an amplification of the inflammatory response (right), and for insensitivity to the anti-inflammatory effect of corticoids (left). Cigarette smoke activates NFκB in alveolar macrophages (right). Gene expression is activated by HAT-mediated core histone acetylation; histone acetylation of inflammatory gene promoters activated by NFκB is increased in COPD. The increase in acetylation is due to a reduction of HDACs. HDACs reverse histone acetylation and switch off gene transcription. HDACs are inactivated by oxidative and nitrative stress.

Oxidative and nitrative stress leads to the formation of peroxynitrite, which nitrates HDAC, leading to its degradation, resulting in low HDAC levels and, subsequently, in an amplification of the inflammatory response. HDAC reduction by cigarette smoke induced oxidative stress impairs the response to corticoids. Corticoids bind glucocorticoid receptor (GR) and recruit HDAC to activated inflammatory genes; by reversing the acetylation of those genes, their transcription is switched off and the inflammation is reduced (left).



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