Genetic and Phenotypic Dissection of Smoke Induced Emphysema in Mouse

by

Salomon Martin CORNEJO PERALES

Division of Experimental Medicine, Department of Medicine

Meakins-Christie Laboratories

McGill University, Montreal

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Contributions of Authors:

The candidate performed all the research included in this thesis. Contributions of other authors to this work are described below:

From Dr. Manuel COSIO at the Meakins-Christie Laboratories, McGill University.

In **Chapter II**, all the experiments described inhere chapter, were performed by CORNEJO PERALES, Salomon M.

******* All the studies were conducted under the supervision of Dr. Manuel Cosio and Co-supervision of Dr. Danuta Radzioch**

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Abbreviations and Definitions

ACC	Animal care committee
ADRB2	Adrenergic, beta-2-, receptor, surface
AM	Alveolar macrophage
AP	Activator protein
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
CCR	Chemokine (C-C motif) receptor
CLUSFAVOR	Cluster and factor analysis with varimax orthogonal rotation
COPD	Chronic obstructive pulmonary disease
COB	Chronic obstructive bronchitis
CT	Threshold cycle
CTP	Cytidine 5'-triphosphate
CXCR	Chemokine (C-X-C motif) receptor
ECP	Eosinophil cationic protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ENA	Epithelial neutrophil activating protein
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)
EPAS	Endothelial PAS domain protein
EPO	Eosinophil peroxidase
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GO	Gene ontology
GRO	Growth related oncogene
GST	Glutathione S-transferase
HIV	Human immunodeficiency virus
HMOX1	Heme oxygenase (decycling) 1
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IP-10	Interferon-γ induced protein of 10 kDa
I-TAC	Interferon-inducible T cell γ chemoattractant
LM	Mean linear intercept
LT	Leukotriene
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon-γ
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinases
MSLN	Mesothelin
MUC	Mucin
NADPH	Nicotinamine adenine dinucleotide phosphate

NCF4	Neutrophil cytosolic factor 4
NE	Neutrophil elastase
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PEEP	Positive end-expiratory pressure
PG	Prostaglandin
PHS	Prostaglandin H synthase
PMN	Polymorphonuclear cell
QTL	Quantitative trait loci
RAC2	RAS-related C3 botulinum substrate 2
RANTES	Released by activated normal T-cells expressed and secreted
ROS	Reactive oxygen species
SE	Smoke-exposed
SLPI	Secretory leukoprotease inhibitor
SOD	Superoxide dismutase
SP	Surfactant protein
SPP	Secreted phosphoprotein
S100a8	S100 calcium binding protein A8 (calgranulin A)
S100a9	S100 calcium binding protein A9 (calgranulin B)
TBARS	Thiobarbituric acid-reactive substances
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
UTP	Uridine 5'-triphosphate
VEGF	Vascular endothelial growth factor
VEGF2	Vascular endothelial growth factor receptor 2

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a composite of conditions that include an abnormal inflammatory response and emphysema. Cigarette smoking is the main risk factor for developing COPD and mouse models are widely used in the study of smoking induced emphysema. The C57BL/6 mouse strain develops airspace enlargement after chronic smoke exposure, with no change in lung mechanics. The broad objective of this thesis is to try to identify in mice, candidate genes that cause a difference in susceptibility to the development of the disease and to better understand the inflammatory process that occurs in response to smoke exposure.

A detailed introduction to COPD and proposed mechanisms of its pathophysiology are presented in Chapter I.

Chapter II consists of a manuscript containing data comparing the genetic expression profiles and inflammation in lung tissue of mice (C57BL/6) chronically exposed to cigarette smoke, with age-paired controls. The findings presented in Chapter II are discussed in greater detailed in Chapter III.

RÉSUMÉ

La maladie pulmonaire obstructive chronique (MPOC) est caractérisée, entre autre, par une réponse inflammatoire anormale et de l'emphysème. Le tabagisme est le principale facteur de risque de développement de la MPOC et le modèle animal le plus fréquemment utilisé pour l'étude de cette maladie est la souris, plus spécifiquement la souche C57BL/6. Cette dernièr est intéressante puisque, suite à l'exposition à la fumée de cigarette, les espaces aériens des poumons des souris C57BL /6 augment en volume mais leur fonction pulmonaire demeure inchangée. L'objectif principal de cette thèse est d'identifier, chez la souris, des gènes susceptibles d'influencer le développement de la maladie et de mieux comprendre le processus inflammatoire en reponse à l'exposition à la fumée de cigarette.

Le premier chapitre consiste en une introduction détaillée de la MPOC et de ses mécanismes pathophysiologiques probables.

Le deuxième chapitre présente un manuscrit comparant l'expression génétique et l'inflammation pulmonaire chez les souris (C57BL/6) exposées de manière chronique à la fumée de cigarette et celles du groupe contrôle. Finalement, les résultats présentés au chapitre II sont discutés en détail au dernier chapitre.

CHAPTER I.

I.1. – General introduction

Chronic obstructive pulmonary disease (COPD) has been defined as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases" (1). COPD is a composite of conditions that include chronic obstructive bronchitis (COB), obstruction of the small airways, and emphysema. Emphysema is the enlargement of airspaces and destruction of lung parenchyma, which lead to loss of lung elasticity and closure of small airways (2). Furthermore, most COPD patients also present mucus hypersecretion, which is not necessarily accompanied by airflow limitation, but that may cause obstruction of the peripheral airways according to some studies (3).

COPD was the sixth leading cause of death worldwide in 1990, and according to the Global Burden of Disease Studies it will be the third most common cause of death in the world by the year 2020 (4-6). The COPD outburst is a global phenomenon, affecting the industrialized and developing worlds. A significant increase in cigarette smoking and environmental pollution in developing countries, are the main reasons for the rise in COPD cases (7). In countries like the USA, the number of affected individuals is estimated to be around 14 million and in 1995 this condition resulted in 92 thousand deaths (8;9). Similarly, in the UK in the last decade, there has been an increase in COPD diagnosis and hospital admissions due to this disease (10;11). These terrible predictions, together with growing health care costs have caused a boost of interest in COPD research,

in order to try to understand the cellular and molecular mechanisms responsible for the development of the disease, as well as a search for novel therapies (12).

Even though it is widely accepted that cigarette smoking is the main environmental determinant of COPD, several other factors like latent adenoviral infections, childhood respiratory infections, air pollution, and occupational exposures have been suggested as possible players (13;14). Furthermore, some studies have shown that diet and other factors during *in utero* and adolescent lung development might be important in the subsequent predisposition to obstructive lung disease (15-18). Although the importance of all these factors is likely to be small compared to cigarette smoking, they could interact with smoking causing an increase in the risk of COPD (9;19). Nowadays, in westernised societies more than 90% of patients develop COPD primarily due to cigarette smoking (20). However, only 15-20% of heavy smokers develop COPD, suggesting that other factors play a key role in determining which cigarette smokers are at risk of developing airflow obstruction (21).

The forced expiratory volume in 1s (FEV_1) declines normally at a rate of 20-30 ml/year in adult non-smokers. However, in most smokers a rate of decline of 30-45 ml/year is observed, and in a subset of smokers susceptible to the development of COPD this rate of decline can be as high as 80-100 ml/year (9) (Figure 1).

Among smokers, the propensity to develop COPD is only weakly correlated to the amount of cigarettes smoked, and only 15% of the variability in FEV_1 is accounted for by smoking history (9;22). These facts suggest that genetic determinants are crucial for determining susceptibility.

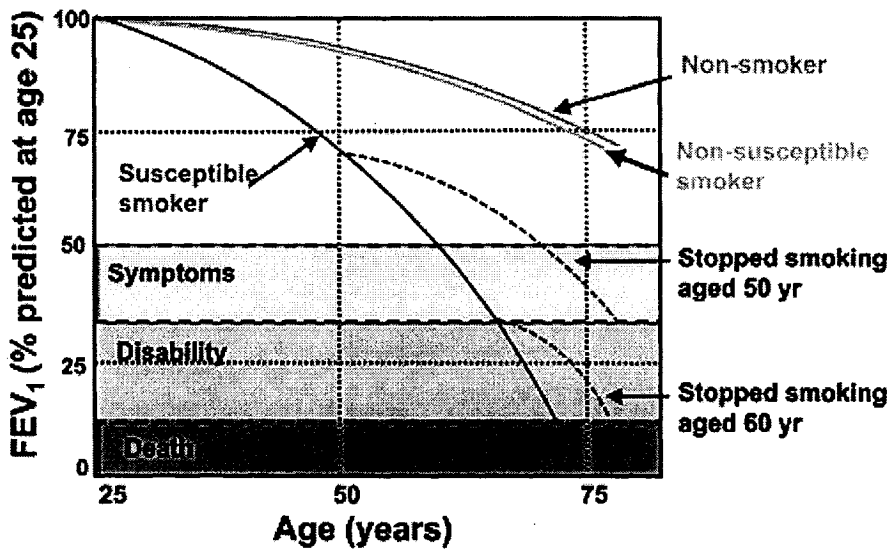


Figure 1.1 Annual decline of lung function. Smokers susceptible to the development of COPD show an accelerated annual decline in FEV_1. Reproduced from Barnes (2004) (22).

For the most part, candidate gene and linkage analysis studies for COPD have given conflicting results, probably due to differences in study populations and disease phenotypes (23). In the past, the use of animal models has been proven useful in overcoming some of the problems encountered in studies using human subjects. Recently, it has been shown that different mouse strains vary in their susceptibility to develop COPD or at least emphysema, after been exposed to cigarette smoke (24).

The main objective of this thesis is to try to identify in mice, candidate genes that cause a difference in susceptibility to the disease. Furthermore, even though inflammation is a key component of COPD, little is known about the behaviour of the inflammatory process over time after smoke exposure. Thus, the kinetics of inflammation was studied in one mouse strains in an attempt to better understand this process.

I.2. – The genetics of COPD

As a complex disease, COPD can have influences from genetic factors, environmental determinants, and genotype-environment interactions. Even though cigarette smoking is the main risk factor for developing the disease, familial clustering studies of patients with early-onset COPD suggest that genes are also important (25). Furthermore, it has been shown that genetic factors influence variations in pulmonary function (26). However, the identification of individual genes responsible for determining susceptibility to the disease has been challenging and so far the only proven genetic cause for developing COPD is α_1-antitrypsin deficiency.

I.2.1. – Alpha-1 antitrypsin

The Z allele of the α_1-antitrypsin gene, which encodes for a serine protease inhibitor, is the main genetic factor in the development of emphysema. Patients homozygotes for the Z allele (PI ZZ) show levels of the α_1-antitrypsin protein that are 10-15% of that produced by the normal M allele, due to a blockage in the final stage of processing of the protein in the liver (27;28). A molecular interaction between the reactive centre loop of one molecule and the gap in the A-sheet of another, cause the polymerization of the Z antitrypsin, inhibiting its release and causing its accumulation in the endoplasmic reticulum of hepatocytes (29). PI ZZ smokers are not only predisposed to develop emphysema at an earlier age than non-smoking PI ZZ individuals, but they also show a greater rate of decline in FEV_1 than never smokers or ex-smokers(30-32). On the other hand, even though heterozygotes (PI MZ) have plasma levels of the α_1-antitrypsin protein that are around 65% of normal, they show no clear increased risk of lung damage (9;33). However, severe α_1-antitrypsin deficiency is a rare occurrence and it only explains 1-2% of all COPD cases, which suggests of the existence of other genetic factors involved in the susceptibility of smokers to develop chronic airflow obstruction.

I.2.2. – Other candidate genes

Recently there has been an increased interest in conducting linkage analysis studies for COPD and suggestive linkage for airflow obstruction, chronic bronchitis and several spirometrical parameters have been found (34-37). Even though the results are only suggestive, they can be used as a guide for the selection of candidate genes needed for association studies.

Several candidate genes that might predispose smokers to COPD have been assessed in case-control studies. The destruction of the lung parenchyma observed in COPD patients and the development of the disease due to a deficiency in α_1-antitrypsin, suggested that other antiproteases could be important. Alpha 1-antichymotrypsin, a plasma serine protease inhibitor, has been studied in this context and several polymorphisms have been found. Two point mutations, which cause a change in the amino acid sequence of this gene (227Pro→Ala and 55Leu→Pro) have been associated with COPD (38;39). Poller *et al.* found an association between COPD and the point mutation that causes the change of proline for alanine at position 227 on the α_1-antichymotrypsin protein. However, this study was not replicated by two other groups (40;41). On the other hand, it has been shown that the 55Leu→Pro mutation causes a conformational change that favours the spontaneous polymerization of the protein, rendering it unable to exit hepatocytes and leading to low levels of circulating α_1-antichymotrypsin (42). Nevertheless, this variant is very rare and this deficiency is unlikely to account for a large proportion of COPD cases.

A deregulation in the expression of matrix metalloproteinases (MMPs) has been suggested as a possible mechanism for the pathogenesis of emphysema. MMPs are potent proteinases capable of degrading the extracellular matrix components and studies in animal models show that they can cause airspace enlargement (43). Minematsu *et al.* found an association between a functional polymorphism (C→T) of MMP-9 at position -1562 and the development of emphysema in a Japanese population (44). Another group failed to find an association between MMP-9 polymorphisms and the rate of decline in lung function in smokers, but found one with the MMP-1 (G–1607GG) and MMP-12

(357Asn→Ser) haplotypes (45). These findings need further confirmation and other polymorphisms in the various members of the MMP family have yet to be studied.

Tumour necrosis factor-α (TNF-α), which is a cytokine that has a crucial role in the inflammatory response, is one more gene that has been assessed by case control studies. Around 10% of the population carry a variant of this gene, known as tumour necrosis factor 2, which is the result of a transition (G→A) at position -308 in the 5' promoter region of this gene, and which leads to a two fold increase in TNF-α plasma levels (46). Huang *et al.* found that this polymorphism was more common among COPD patients than controls matched for age, sex and smoking history, in a Taiwanese population (47). However, opposite results have been found by two other groups which have studied this polymorphism in Italian and British populations (48;49).

Beta2-adrenoreceptor (ADRB2) is a key player in the regulation of smooth muscle tone and several polymorphisms in the coding region of the gene have been identified and associated with asthma severity, airway hyperresponsiveness, and level of lung function (50-53). Ho *et al.* found that the 16Arg→Gly polymorphism was less frequent in COPD patients than in healthy controls from a Chinese population (54). However, another group found no association between this polymorphism and the rate of decline of lung function, bronchial hyperresponsiveness or bronchodilator response in smokers (55).

Another gene that has been studied is the one that encodes for the microsomal epoxide hydrolase (EPHX1), which is a metabolizing enzyme important in the detoxification of exogenous chemicals (56). Cigarette smoke contains around 4,700 chemical compounds and each puff carries ~10^{14} radicals, which have the capacity to

damage the lung (57;58). Therefore, smokers with defects in the detoxification process could be more susceptible to the development of emphysema. Smith and Harrison found that a greater proportion of Caucasian subjects with COPD had a slow epoxide hydrolase activity compared to healthy controls (59). However, these results were not reproduced by Yim *et al.* in a Korean population (60).

Glutathione S-transferases (GSTs), another group of detoxifying enzymes, have been studied due to their possible protective role in the development of emphysema and lung cancer. A null genotype of the GSTM1 gene, which is normally expressed in the lung and the liver, occurs in between 30-50% of humans, depending on the ethnic origin of the individual (61). The deletion of this gene has been associated with emphysema in lung cancer patients, as well as with severe chronic bronchitis in heavy smokers (62;63). However, in a Korean population there was no association between GSTM1 polymorphisms and COPD (60).

Oxidant injury is believed to be another factor contributing to the development of emphysema. Heme oxygenase-1 (HMOX1) catalyzes the first and rate-limiting step in the oxidative degradation of heme to biliverdin (64). HMOX1 has been shown to be induced mainly by heme, but also by cytokines, heavy metals, endotoxin and hormones(65-68); and it has been found to protect cells from oxidant injury (69). Yamada *et al.* found an association between a microsatellite polymorphism $(GT)_n$ within the HMOX1 promoter and emphysema in Japanese smokers (70). The authors show that the proportion of allelic frequencies and genotypes with $\geq$30 GT repeats was significantly larger in emphysema patients than in smokers without the disease. Even though it is biologically reasonable

that HMOX1 is associated with COPD, more studies are needed in order to confirm these results.

I.3. – COPD: A complex inflammatory disease

Cigarette smoke triggers an inflammatory response, which is believed to be crucial for the development of COPD. The chronic inflammation observed in the lungs of smokers who develop the disease is thought to be responsible for the remodelling and narrowing of the small airways and emphysema. Furthermore, studies have shown that the progression of the disease is correlated to the increase in inflammation in the lung parenchyma and airways, in a process that involves both innate and adaptive immune cells (71). As a complex inflammatory disease, multiple inflammatory cells and mediators have been implicated in the development of COPD, but their relationship with each other and the specific sequence in which they appear and persist in the lung is still not completely understood (22) (Figure 2).

I.3.1. – Cellular players

For many years, researchers proposed the protease/antiprotease imbalance hypothesis based on the findings that individuals with α_1-antitrypsin deficiency develop early onset emphysema and that the instillation of elastolytic enzymes produced emphysema in laboratory animals (72). Furthermore, the increased neutrophil and macrophage numbers in the lungs of COPD patients, led researchers believe that these cells were the key mediators of the lung destruction observed in COPD. However, this hypothesis has been constantly challenged due to an inability to correlate neutrophil

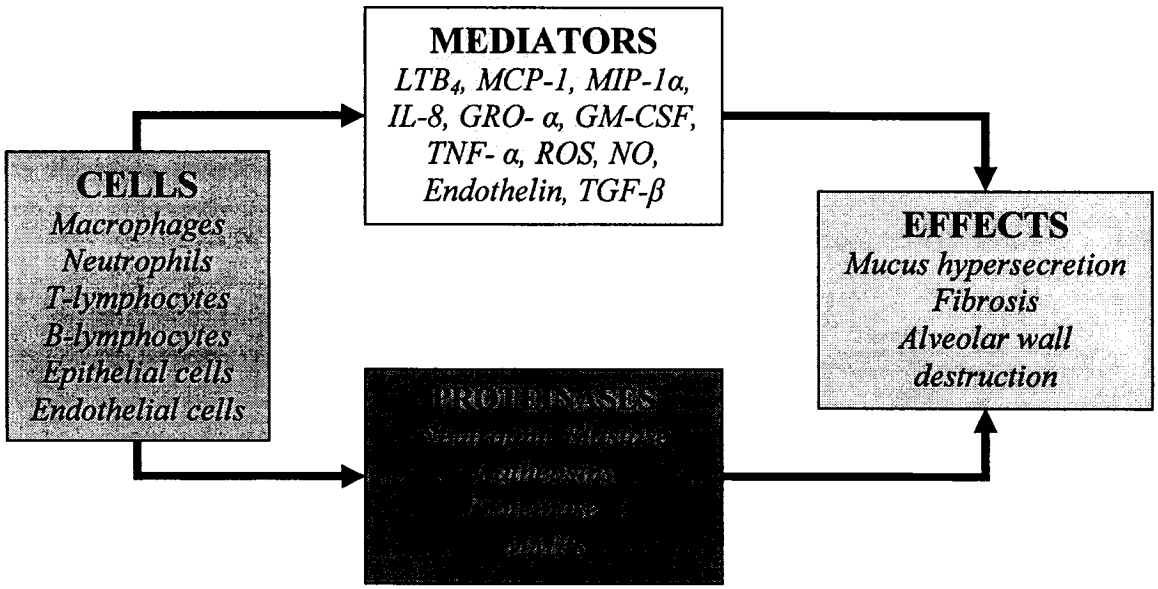


Figure 1.2 Complexity of the inflammatory reaction in COPD. Inflammation in this pathology involves multiple activated inflammatory and structural cells, which can release several factors that can prolong and amplify inflammation, as well as cause tissue destruction. Abbrev: LTB4, leukotriene B4; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; GRO, growth related oncogene; GM-CSF, granulocyte macrophage colony stimulating factor; TNF, tumour necrosis factor; ROS, reactive oxygen species; NO, nitric oxide; TGF, transforming growth factor; MMP, matrix metalloproteinases. Modified and adapted from Barnes (2003) (2).

numbers to the extent of emphysema and by the findings that the lungs of COPD patients have a significant T-cell infiltration that is related to the degree of lung destruction (73;74). Overall, studies have shown abnormal numbers of $CD4^+$ and $CD8^+$ T-cells, as well as macrophages, neutrophils, and B lymphocytes (13;71;74-76). These findings have made researchers hypothesize that COPD could have an autoimmune component, which originates in the lung induced by cigarette smoke (77-79).

I.3.1.1. – Neutrophils

Studies have shown the presence of abnormally elevated numbers of neutrophils in the sputum and bronchoalveolar lavage (BAL) fluid of patients with airway obstruction (80-82). However, when the lung parenchyma and airways are analysed, neutrophils are only slightly increased (74). These findings could be explained by the fact that neutrophils have the ability to transit fast through the interstitial space, making their passage through the lungs and airways a rapid event (2;83). Even though it is not clear what exact role neutrophils play in the development of COPD, it has been shown that circulating levels of this inflammatory cell correlate with the decline in FEV_1 (84). Animal studies have demonstrated that neutrophils are rapidly recruited to the airways in response to cigarette smoke and other irritants (85). Moreover, cigarette smoke stimulates the production and release of neutrophils from the bone marrow, and some of its components, like nicotine, can act as chemotactic factors for these inflammatory cells (86;87). Furthermore, smoking may cause a reduction in the rate of neutrophils clearance from the lungs (88).

Neutrophils have the capacity to induce alveolar breakdown by the secretion of serine proteases, like neutrophil elastase (NE), cathepsin G, and proteinase-3, at the same time as MMP-8 and MMP-9 (7). In addition, these serine proteases can potently stimulate mucus production, and studies show a direct correlation between the quantities of sputum expectorated in a day and the levels of NE in the secretion (83). The recruitment of neutrophils to the lungs is a complex sequence of events, which include the adherence to the vascular endothelium, via adhesion molecules like E-selectin, which has been proven to be up-regulated on bronchial vessels of COPD patients (89). After adhesion, neutrophils continue their migration into the respiratory tract following chemotactic factors, like interleukin 8 (IL-8), epithelial neutrophil activating protein 78 (ENA-78), leukotriene B_4 (LTB_4), and growth related oncogene-α (GRO-α), all of which are elevated in COPD (90;91). Once in the lung, neutrophils have the ability to cause tissue damage by releasing proteases and oxidants. Even though, not much is known about neutrophil survival and apoptosis in COPD, studies have shown that smoke stimulates the survival of this inflammatory cell in the respiratory tract, probably mediated by granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) (86;92).

Even though neutrophils have the potential of causing tissue destruction, reports from multiple groups show that there is not a significant increase in the numbers of this cell type in the lungs of COPD patients (76;82;93). In fact, Finkelstein *et al.* demonstrated a negative correlation between the number of

neutrophils and the degree of alveolar wall destruction (74). Moreover, a high degree of elastolysis is not observed in other pathologies, like cystic fibrosis, in which levels of neutrophils are more elevated than in COPD (22). These findings suggest that there are other players in the development of emphysema and that neutrophils instead of contributing to it are probably a consequence of the disease.

I.3.1.2. – Macrophages

Macrophages are key players in pulmonary host defence, by reacting to endogenous and exogenous stimuli and producing factors that can alter the behaviour of neighbouring cells (94). Smoking can cause more than a fivefold increase in the number of cells recovered in BAL fluid, of which at least 95% are macrophages (95). Moreover, elevated numbers of this type of inflammatory cell have been observed not only in BAL fluid, but also in the lung parenchyma and airways, as well as sputum of COPD patients (2). An analysis done by Retamales *et al.* showed a 25-fold increase in the number of macrophages present in the tissue and airspaces of emphysematous lungs, compared to normal smokers (13). Several studies have shown the existence of macrophage concentration at sites of alveolar destruction in emphysema patients and a positive correlation between the number of macrophages and the extent of the disease (74;96). Due to their predominant presence in the lungs and to their ability to account for most of the known characteristics of COPD, it is believed that macrophages have a crucial role in the pathogenesis of this disease (95).

The importance of macrophages in the development of emphysema has been further reinforced by studies using animal models. Ofulue and Ko depleted the lungs of cigarette smoke-exposed rats from either macrophages or neutrophils, and observed that in the absence of neutrophils airspace enlargement due to smoke exposure was not prevented. However, when macrophages were not present, rats exposed to cigarette smoke were protected from lung destruction, suggesting that the smoke-induced elastin breakdown might be macrophage dependent (97).

Macrophages can release inflammatory mediators like TNF-α, IL-8, LTB_4, and reactive oxygen species, as well as elastolytic enzymes like MMP-1, MMP-2, MMP-9, MMP-12, cathepsin K, L and S and neutrophil elastase from endocytosed neutrophils (7;43;98;99). Cell culture studies have shown that alveolar macrophages from COPD patients produce more inflammatory mediators and have greater elastolytic activity (98;100). Moreover, the transcription factor nuclear factor-κB (NF-κB), which controls most of the inflammatory mediators that are up-regulated, is activated in these cells (101).

Two possible scenarios could explain the elevated numbers of macrophages in the lungs of COPD patients; they could be the result of enhanced recruitment of monocytes from the circulation or from increased survival and proliferation in the lungs (92). Monocyte chemotactic protein-1 (MCP-1) and GRO-α, two chemoattractants for monocytes, are increased in the sputum and BAL fluid of patients with COPD (91;102). Similarly, there is an overexpression

of the anti-apoptotic protein Bcl-X_L and $p21^{CIP/WAF1}$ in macrophages from smokers, suggesting that these cells might have prolonged survival (2;103).

I.3.1.3. – T-lymphocytes

For years, researchers debated upon the importance of macrophages and neutrophils in the pathogenesis of COPD, neglecting the possible crucial roles that other cells could play. It was not until Finkelstein *et al.* reported increased numbers of T-cells in patients with COPD that the significance of these cells was made obvious (74). This group showed a correlation between the number of T-lymphocytes/mm^3 and the extent of the disease. Studies followed confirming the existence of elevated numbers of T-cells in the airways and lung parenchyma of COPD patients, with a predominance of $CD8^+$ cells (13;71;75;76;104). There is increasing evidence that most of the T-lymphocytes found in COPD airways are of the Tc1 and Th1 subtypes, producing interferon-γ (IFN-γ), a potent macrophage activating cytokine (105;106). The elevated numbers of $CD4^+$ T-cells, suggest that they might play a role in the inflammatory process. $CD4^+$ cells are necessary for the priming of $CD8^+$ cytotoxic T-lymphocyte responses and for the survival of these cells, suggesting a linkage between the elevated numbers of $CD4^+$ T-cells and the $CD8^+$ cell infiltrate found in smokers (107).

The presence of T-lymphocytes in the lungs of COPD patients, suggest that they play an active role in the development of the disease because normally these cells when not activated would not remain in the lung for a prolonged period of time. In order for T-cells to migrate to the lungs and proliferate, they need to be

activated by antigen presenting cells (APC), in a process in which they recognize an antigen which is being produced in this organ. It has been proposed that the initial inflammatory response composed of macrophages and neutrophils produces lung injury, resulting in the creation of cross-reactive neoantigens or the release of sequestered autoantigens (107). Once in the lung, T-cells could start producing cytokines, like IFN- γ, which would lead to an amplification of the inflammatory response by recruiting and activating macrophages (108). Interestingly, there is a correlation between the number of T-cells and macrophages in the lungs of smokers (74). Furthermore, activated macrophages secrete cytokines like IL-12, which promotes the differentiation of T-lymphocytes into the Tc1 and Th1 subsets (107). In this way, the T-cell infiltration could be responsible for the amplification and perpetuation of inflammation in the lungs of COPD patients.

Saetta *et al.* showed that the T-lymphocytes present in the lungs of COPD patients overexpress CXCR3, a receptor activated by the chemokines: monokine induced by interferon-γ (MIG), interferon-γ induced protein of 10 kDa (IP-10) and interferon-inducible T cell γ chemoattractant (I-TAC) (105). Furthermore, this group also demonstrated that there is increased expression of IP-10 in the bronchiolar epithelium of smokers with COPD, but not in the epithelial cells of normal smokers or non-smoking controls.

The exact function of the increased $CD8^+$ cells in the pathogenesis of COPD is still unknown. However, it is clear that these cells can have a cytotoxic effect on epithelial cells, causing cytolysis and apoptosis through the release of granzymes, perforins and TNF-α (78;109). COPD patients have been reported to

have elevated concentrations of perforins in their sputum, as well as increased apoptosis of their alveolar cells, which correlates with an increase in the number of $CD8^+$ cells (75;110).

I.3.1.4. – Epithelial cells

In response to external stimuli, like allergens and cigarette smoke, epithelial cells can modify their normal defence mechanisms, such as the production of oxygen and nitrogen species, mucus secretion and the influx of inflammatory cells (111). Furthermore, it is widely documented that the epithelium is a source of inflammatory mediators in lung pathologies like asthma, suggesting a possible involvement in COPD. Studies have shown that cigarette smoke activates epithelial cells to produce IL-8, TNF-α, IL-1β, and GM-CSF (112;113). Moreover, de Boer *et al.* found increased mRNA and protein expression of MCP-1 in epithelial cells and its receptor CCR2 in macrophages of COPD patients, suggesting that epithelial cells play a role in the recruitment of macrophages to the airway epithelium (114).

Alveolar cell survival is dependant on vascular endothelial growth factor (VEGF), and studies in rats have shown that blockage of VEGF receptor 2 (VEGF2) cause apoptosis of alveolar cells leading to airspace enlargement (115). Other than forming part of the innate defence system, by secreting defensins, epithelial cells also play a role in tissue repair processes and adaptive immune responses (22). Epithelial cells have the ability to produce secretory leukoprotease inhibitor (SLPI), which is not only an antiprotease but also induces the

proliferation of these cells (116). Gompertz *et al.* found lower concentrations of SLPI in the sputum of COPD patients with more frequent exacerbations (117).

I.3.1.5 – Other cellular players

Due to their widespread localization in the lungs and its key role in the integration of the innate and adaptive immune responses, dendritic cells are believed to be important in the reaction of the lungs to noxious agents (92). Dendritic cells have the capacity to activate a large number of immune cells, like neutrophils, macrophages, T- and B- lymphocytes (118). Exposure to cigarette smoke leads to increases in the number of dendritic cells found in mice lungs (119;120). Furthermore, several groups have reported an accumulation of these cells in the airways and lung parenchyma of smokers (121;122). However the exact mechanisms how smoke triggers an activation of the immune system is still not clear and more studies are needed in order to determine the relevance of dendritic cells in this process.

Even though the role of eosinophils is well established in asthma, their involvement in the pathophysiology of COPD is still unknown. There have been conflicting results in studies looking at the eosinophilic profile in airways and BAL during stable COPD (20). Keatings and Barnes found elevated concentrations of eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) in the sputum of COPD patients (123). However, there was an absence of eosinophils, suggesting that these cells might have degranulated. On the other hand, Saetta *et al.* have reported the existence of eosinophilia during

exacerbations of COPD (124;125). The data available indicates that these cells might be primordially important during periods of exacerbation.

I.3.2. – Molecular mediators

COPD is a complex inflammatory disease, in which multiple inflammatory and structural cells are believed to take part. Furthermore, this wide spectrum of cells produces a variety of interactive mediators, like cytokines, chemokines, growth factors and lipids, amongst others.

I.3.2.1 – Cytokines

Due to the inflammatory nature of COPD it is not surprising that multiple cytokines have been implicated in the pathology of this disease (99). Analyses of the sputum of COPD patients have shown increased levels of soluble TNF receptors and TNF-α, especially during exacerbations (81;126;127). Furthermore, in a group of COPD patients who show weight loss, the levels of TNF-α in the circulation are elevated and peripheral blood monocytes show increase production of this cytokine, suggesting its involvement in cachexia (128). TNF-α, which can be produced by macrophages, T-lymphocytes, mast and epithelial cells, activates NF-κB, that switches on the transcription of chemokines and proteases (2). Additionally, TNF-α increases the expression of intracellular adhesion molecule 1 (ICAM-1), which is augmented in the serum and lavage of COPD patients (129). Muscle wasting is a characteristic seen in COPD and TNF-α has been shown to be a potent inhibitor of myogenesis via the activation of the NF-κB pathway (130).

Moreover, studies using a knockout mouse model for the TNF receptor, suggest that TNF might be responsible for ~70% of the smoke-induced emphysema seen in mice (131).

A second potent proinflammatory cytokine that might be involved in COPD is IL-1β. This cytokine induces the release of neutrophils from the bone marrow, as well as the secretion of a variety of cytokines, some of which are IL-1, IL-6, IL-8, released by activated normal T-cells expressed and secreted (RANTES), GM-CSF, IFN-γ, and TNF from different inflammatory cells (99). IL-1β activates alveolar macrophages leading to the release of MMP-9 in a dose dependent manner, and this effect is increased in alveolar macrophages from COPD patients (132). Similarly, this cytokine also induces the expression of other MMPs, including MMP-2, MMP-3 and MMP-13 (133). Cigarette smoke causes an increase in the release of IL-1β from epithelial cells of COPD patients *in vitro*, but this effect is not seen in smokers with normal lung function (134). Lucey *et al.* compared the response to elastase intratracheal instillation between IL-1β receptor knockout mice, TNF-α receptor deficient mice and mice deficient of both receptors. After the end of the treatment, mice with the double deficiency had significantly lower emphysema compared to the other groups, suggesting that both TNF-α and IL-1β are important in the development of COPD (135).

Levels of IL-6 are elevated in the BAL fluid of smokers as well as in the exhaled breath condensate, induced sputum and plasma of COPD patients, especially during exacerbations (136-140). This cytokine can be produced by monocytes, macrophages, T- and B-lymphocytes, fibroblasts and epithelial cells,

and acts as a T- and B- cell growth factor (99). However its precise role in the pathogenesis of COPD is not certain.

Other interleukins studied in the context of COPD are IL-9 and IL-13, amongst others. T-cells from bronchial biopsies of COPD patients show increased expression of IL-9 (141). Even though the exact relevance of IL-9 in the disease is not known, this cytokine induces mucus cell metaplasia by increasing the expression of MUC5AC, suggesting a possible role in mucus plugging observed in some COPD patients (142). Bronchial biopsies of smokers with mucus hypersecretion show elevated levels of IL-13 compared to normal smokers (143). Furthermore, van der Pouw Kraan *et al.* have shown an association between a polymorphism (C→T) at position -1055 in the promoter region of the IL-13 gene and COPD (144). In the mouse, overexpression of this cytokine leads to increased production of MMP-2, MMP-9, MMP-12, MMP-13, and MMP-14 and cathepsins B, S, L, H, and K, resulting in emphysema (145). A study by the same group showed a similar phenotype when IFN-γ was overexpressed (146). Subjects with COPD show a lung infiltration of Tc1 and Th1 cells, which produce IFN-γ, as well as increased levels of Th1 lymphocytes in the peripheral blood (106;147). IFN-γ has the potential of perpetuating inflammation by activating macrophages and epithelial cells, which secrete CXCR3 chemoattractants, leading to further increases in T-cells (78).

GM-CSF is a cytokine produced by a wide spectrum of inflammatory and structural cells, including T-lymphocytes, macrophages, airway smooth muscle and epithelial cells (99). BAL fluid of COPD patients show increased levels of

this cytokine and a greater increase is seen during exacerbations (148). GM-CSF is involved in the priming and survival of inflammatory cells like neutrophils and eosinophils (22;149). Furthermore, it induces macrophages to produce and secrete other cytokines, like IL-1 and TNF-α (99).

I.3.2.2. – Chemokines

Chemokines are a class of cytokines which are usually small (7-10kDa) secreted proteins involved in the trafficking and recruitment of leukocytes into tissues (150). They produce their biological effects by interacting with specific G-protein coupled receptors in their target cells, and mediate cellular processes like proliferation, differentiation and survival of these cells (22;151). Multiple chemokines have been now implicated in the inflammatory process of COPD (152).

IL-8, a CXC chemokine, is a potent neutrophil chemoattractant and activator, promoting exocytosis and release of enzymes and proteins from cell granules (99). It is produced, upon stimulation with other cytokines, in several cells, including macrophages, neutrophils, epithelial and endothelial cells (153). Levels of this chemokine in the sputum of subjects with COPD are increased and they correlate with the severity of the disease (81;154). Similarly, IL-8 levels are elevated in BAL fluid of smokers with emphysema but not in normal smokers (90). Furthermore, IL-8 has chemotactic activity towards $CD8^+$ T-lymphocytes and B-cells, possibly explaining the high numbers of these cells seen in the lungs of COPD patients (99;155). The exact source of IL-8 in COPD is not known, but

several studies have shown that airway epithelial cells and alveolar macrophages from COPD patients have increased basal secretion of this chemokine (156;157). In addition, during acute exacerbations, airway epithelial cells up-regulate the expression of CXCR2, one of the IL-8 receptors, and levels of its mRNA correlate with the numbers of neutrophils in bronchial biopsies of subjects with COPD (158).

Another CXC chemokine that has been involved in COPD is GRO-α. This chemokine is produced by airway epithelial cells and alveolar macrophages, upon stimulation with cytokines (22). Like IL-8, GRO-α has high affinity for the CXCR2 receptor, through which it activates monocytes, neutrophils and basophils (159). Induced sputum and BAL fluid from COPD patients have elevated levels of GRO-α, and BAL leukocytes from smokers secrete more of this chemokine after stimulation (91;160). Furthermore, GRO-α has a greater chemotactic effect on monocytes from COPD patients, than from normal subjects, possibly explaining the accumulation of alveolar macrophages observed in COPD lungs (13;161).

Epithelial cells are believed to be involved in the development of inflammation by producing cytokines and chemokines like the epithelial cell-derived neutrophil-activating peptide-78 (ENA-78). Morrison *et al.* showed that after stimulation, BAL cells from smokers secrete significantly more ENA-78 than cells from non-smokers (160). Furthermore, during exacerbations, there is an up-regulation of the ENA-78 gene in epithelial cells (158). However, there is no

difference between ENA-78 levels in the BAL fluid of COPD patients and normal smokers (90).

MCP-1 is a CC-chemokine that attracts and activates monocytes, macrophages, mast and T-cells via the CCR2 receptor (162). This chemokine is expressed in macrophages, epithelial and endothelial cells, as well as T-lymphocytes, and its expression can be induced by cytokines like TNF-α and IL-1β (114;163). The sputum, BAL fluid and lungs of COPD patients have increased levels of MCP-1 (91;102;114). In mice, intratracheal instillation of this chemokine leads to a macrophage infiltration of the lungs and magnifies smoke-induced emphysema (164).

CXC3 chemokines, like IP-10, MIG and I-TAC, activate CXCR3, a receptor that is overexpressed in T-cells of the peripheral airways of COPD patients (105). Furthermore, the lungs of these patients show increased expression of IP-10, possibly explaining the accumulation of $CD8^+$ T-cells observed their lungs (105;165). IP-10 can be produced by a variety of cells, including alveolar macrophages, epithelial cells and airway smooth muscle cells, giving them the potential to attract $CD8^+$ T-lymphocytes (105;165;166). IFN-γ can induce the production of IP-10 and Mig from multiple cells (22). In this was, by producing IFN-γ and leading to the subsequent recruitment of more T-cells, Tc1 cells in the lungs of COPD patients could be responsible for the perpetuation of inflammation (78).

Even though eosinophils are not the most predominant inflammatory cell in the lungs of stable COPD patients, their numbers increase significantly during

exacerbations (124). Therefore, chemoattractants for eosinophils, such as RANTES and eotaxin, might play an important role in these periods of the disease. RANTES mRNA levels are increased in epithelial and subepithelial cells and correlate with increased numbers of eosinophils in the lungs of patients with chronic bronchitis exacerbations (167).

I.3.2.3. – Growth factors

COPD is characterized by dramatic structural changes in the lungs and growth factors could play a significant role in the pathogenesis of the disease by been involved in airway repair and remodelling processes (99). The three main growth factors that have been implicated in COPD are: transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF).

TGF-β1 regulates the synthesis of extracellular matrix proteins like collagen and fibronectin, as well as the proliferation and differentiation of cells (168). Furthermore, it can act as a chemotactic factor for monocytes, mast cells and T-lymphocytes (169;170). TGF-β1 is normally expressed in the bronchial epithelium, smooth muscle and alveolar macrophages (171). mRNA levels of this growth factor are increased in the epithelial cells of the small airways of smokers, and correlates with smoking history and the degree of small airway obstruction (172). Furthermore, de Boer *et al.* found increased mRNA and protein levels of TGF-β1 in bronchiolar and alveolar epithelium of COPD lungs, and these levels correlated with the number of macrophages present in the lungs (168). Monocytes

from the peripheral blood of COPD patients have been shown to have increased production of TGF-β (173).

Alveolar macrophages also synthesize TGF-α, which as EGF, can activate the EGF receptor (EGFR) (22). Activation of EGFR leads to increased expression of the mucin genes, mucus production and goblet cell metaplasia (174). Cigarette smoke causes the shedding of TGF-α from the epithelial cell surface, leading to the binding and activation of EGFR (175). This suggests that EGF and EGFR might be responsible for the mucus plugging present in some COPD cases.

VEGF is a growth factor that is involved in angiogenesis, cell survival and vascular permeability. Furthermore, it can act as a chemoattractant for vascular smooth muscle cells, polymorphonuclear cells, and monocytes (176). It is believed that VEGF modulates the functional and structural changes seen in the pulmonary circulation in COPD (22). Santos *et al.* found increased expression of VEGF in the pulmonary vascular arteries of smokers and mild COPD patients, but not in subjects with severe disease (177). The authors suggest that VEGF might be responsible for the vessel remodelling observe in COPD primordially during the early stages of the disease. Chronic treatment of rats with a VEGF receptor blocker leads to septal cell apoptosis and air space enlargement (115). Similarly, VEGF levels are increased in the induced sputum of chronic bronchitis patients and correlate with airflow limitation. However, in patients with emphysema, decreased concentrations of VEGF are observed and these are associated with airflow limitation and degree of alveolar destruction (178).

I.3.2.4. – Proteases

The protease/antiprotease hypothesis for the development of COPD was proposed long time ago after the findings that patients with α_1-antitrypsin deficiency develop emphysema and that the use of proteases leads to airspace enlargement in animal models. Even though the initial hypothesis focused on neutrophil elastase as the main mediator of destruction, it is likely that other proteases and the interactions between proteases and other mediators play more important roles in the pathogenesis of the disease (72).

I.3.2.4.1. – Serine proteases

Neutrophil elastase (NE) is a powerful serine protease 218 amino acids long, that has the ability to break most of the extracellular matrix protein components and proteins of the complement cascade (179). The reactive site of NE is composed of the catalytic triad Hisp-Asp-Ser, which after a proton transfer becomes highly nucleophilic, capable of attacking the peptide bonds in the target substrate (180). NE is synthesized by myelocytic precursor cells in the bone marrow and the NE gene gets turned off before neutrophils leave this tissue (181). NE is stored in azurophilic granules in the cytoplasm of neutrophils from which it is secreted upon stimulation (182). Intratracheal instillation of NE leads to emphysema in hamsters and the use of a NE inhibitor reduces airspace enlargement in smoke-exposed guinea pigs (183;184). Furthermore, Shapiro *et al.* have shown that NE $^{-/-}$ mice exposed to smoke have impaired recruitment of monocytes and neutrophils to the lungs and do not develop emphysema (180).

BAL fluid from COPD patients have increased concentrations of the complex formed by NE and its inhibitor, α_1-antitrypsin (185). Moreover, Betsuyaku *et al.* found that levels of this complex in BAL fluid correlate with the rate of decline in FEV_1 (186).

Even though proteolysis is the main function of NE, there are other roles that this serine protease could play in the development of COPD. Cell culture experiments show that bronchial epithelial cells synthesize more IL-8, a neutrophil chemoattractant, in response to NE, suggesting a possible role of this protease in the perpetuation of inflammation (187). Additionally, it causes goblet cell degranulation, promoting mucus hypersecretion, which can be the origin of severe cough, airway plugging, and further inflammation (188).

Primary granules of neutrophils also contain two other serine proteases, cathepsin G and proteinase 3 (43). As NE, these two proteases also promote mucus hypersecretion and might be implicated in the pathogenesis of COPD (189;190).

I.3.2.4.2. – Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteinases that can be classified based on their target substrates as collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and –9), stromelysins (MMP-3, -10 and -11), elastases (MMP-7 and -12) and membrane associated MMPs (MT-MMPs, MMP-14, -15, -16, and -17) (191). MMPs are mainly secreted by macrophages, neutrophils and pulmonary epithelial cells when activated in

response to different mediators (192). These proteinases have the ability to degrade all the components of the extracellular matrix but their proteolytic activity can also play an important role in immunity. They can cleave cytokines and chemokines, regulating their gradients consequently affecting the migration of inflammatory cells (193;194). Furthermore, they can regulate other processes, including cellular proliferation, apoptosis, and intercellular communications (195).

Studies using mice have demonstrated that overexpression of human MMP-1 in the lungs of these animals leads to spontaneous airspace enlargement (196). Furthermore, mice deficient in MMP-12 are protected from smoke-induced emphysema (164). Similarly, Leco *et al.* showed that lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3) develop spontaneously emphysema-like lesions (197). The studies using animal models demonstrate that MMPs have the capacity to disturb the normal lung architecture and cause airspace enlargement. However, studies in subjects with COPD suggest that the repertoire of MMPs important in the pathogenesis of the disease might be different in humans than in murine models (191).

Concentrations of MMP-1 and MMP-9 are elevated in the BAL fluid of COPD patients, and this is probably a reflection of the observed increased expression of these proteinases in alveolar macrophages of these subjects (198;199). Immunohistochemical analyses have also shown increased expression of MMP-1, MMP-2, MMP-8, MMP-9, and -14 in COPD lungs (200;201). Furthermore, Kang *et al.* found a correlation between the concentration of MMP-9 and smoking history as well as with FEV_1 (202). A study found that the main

sources of MMP-1 in COPD lungs are type II pneumocytes, suggesting that these cells might also play a role in lung destruction (203). There have been conflicting results about the expression of MMP-12 in COPD lungs. Although MMP-12 seems to be important in mouse models, in humans MMP-9 appears to be the main MMP (191).

I.3.2.4.3. – Cysteine proteases

Lysosomal cysteine proteases (Cathepsins) are a group of proteases that are synthesized as inactive precursors and require the removal of the N-terminal propeptide in order to become functional (204). Even though their main function is the degradation of proteins in the lysosome, they have also been implicated in other processes, including apoptosis, extracellular matrix remodeling, prohormone processing, and MHC class II responses (205). Mice overexpressing IFN-γ show increased levels of cathepsins B, D, H, L and S in their lungs and develop emphysema (146). Moreover, treatment with cathepsin inhibitors decreases the inflammation and airspace enlargement seen in mice overexpressing IL-13 (145). BAL fluid from smokers with emphysema has increased concentrations of cathepsin L compared to normal smokers (206). Furthermore, macrophages from COPD patients have a greater cysteine elastase activity upon stimulation (98).

I.3.2.5. – Lipid mediators

Lipid mediators, such as prostaglandins and leukotrienes, could play an important role in the pathogenesis of emphysema. Exhaled breath condensate of

COPD patients show increased concentrations of prostaglandin (PG)E_2, $PGF_{2\alpha}$ and LTB_4 (207). Prostaglandin H synthase (PHS) is an enzyme that performs the rate limited step in the biochemical reaction that generates prostaglandins (208). Macrophages from COPD patients have increased expression of PHS-2, one of the two PHS isoforms, compared to controls (209). PGE_1 induces mucous cell development and mucin gene synthesis in mice airway epithelium (210). Furthermore, PGE_2 stimulates mucus secretion and expression of the mucin gene MUC5AC in human airway epithelial cells (211).

LTB_4 is a potent neutrophil chemoattractant that has been shown to be increased in the exhaled breath condensate of subjects with COPD, with further increases during exacerbations (207;212). Similarly, increased LTB_4 concentrations have been reported in plasma and induced sputum of these patients (213;214). The importance that LTB_4 plays in the development of COPD is not clear, but it has been found LTB_4 can regulate the recruitment of T-lymphocytes to inflamed tissues (215).

I.3.2.6. – Oxidative stress

There is increasing evidence that oxidative stress, which results from an oxidant/antioxidant imbalance, plays an important role in the injury and inflammatory processes in COPD (216). Activated inflammatory and structural cells from COPD lungs generate reactive oxygen species (ROS) (92). Superoxide anions ($O_2^{\cdot-}$) are produced by reduced nicotinamine adenine dinucleotide phosphate (NADPH) oxidase. $O_2^{\cdot-}$ and H_2O_2 react in the presence of Fe^{++} to

generate a hydroxyl radical (·OH) (217). Macrophages from BAL fluid of smokers have increased intracellular iron concentrations and release it in greater quantities that those of nonsmokers (218;219). It is believed that cigarette smoke is the main contributor of iron, since each cigarette contains approximately 0.042μg of this metal (217). Due to its high reactivity, iron is usually bound to compounds like ferritin, but cigarette smoke causes iron release and this could contribute to the increased oxidative burden observed in the lungs of smokers (220). Furthermore, cigarette itself can contribute significantly to the presence of high levels of oxidants, because it contains ~10^{14} radicals per puff, as well as ~500ppm of nitric oxide (NO) (58;221). Additionally, cigarette smoke increases the activity of NO synthase and NO can react with $O_2^{\cdot-}$ to produce peroxynitrates (217). BAL fluid of COPD patients contain increased levels of xanthine oxidase, a hydrolase that produces superoxide (222).

In the normal lung, levels of oxidants are counteracted by multiple endogenous antioxidant mechanisms (223). The three main pulmonary antioxidants are catalase, glutathione peroxidase, which inactivates H_2O_2, and superoxide dismutase (SOD), which degrades $O_2^{\cdot-}$ (217). Even though it is fairly accepted that cigarette smoke causes oxidative stress, its effect on antioxidant expression is yet not very clear. Taylor *et al.* found a deficiency in plasma antioxidant activity and this correlated with an abnormal mean FEV_1/FVC ratio (224). Erythrocytes from smokers have a decreased glutathione peroxidase activity and show greater tendency to peroxidize compared to those of nonsmokers (225). Similar results and an additional reduction is SOD activity

were found by Jendryczko *et al.* in erythrocytes from children of smoking parents (226). There is a decrease in plasma levels of vitamin C, an extracellular antioxidant, in smokers and this correlates with the degree of lipid peroxidation (227). On the other hand, in smoke-exposed rats there is an increase of antioxidants, including SOD, catalase and glutathione peroxidase, but these increases are ineffective in protecting the lungs from the detrimental effects of cigarette smoke (228).

Measurements of biomarkers in COPD patients confirm the existence of oxidative stress and suggest a possible involvement of it in the pathogenesis of COPD. H_2O_2 levels are elevated in exhaled breath condensate of COPD patients, and they further increase during acute exacerbations (229). Increased levels of thiobarbituric acid-reactive substances (TBARS), a marker of lipid peroxidation, were also found (230). Moreover, Montuschi *et al.* showed that there is an increase in 8-isoprostane, a product of the peroxidation of arachidonic acid, in the exhaled breath condensate of COPD patients, and this was not dependent on smoking status (231). Malondialdehyde, another lipid peroxidation marker, was also increased in exhaled breath condensate of COPD patients compared to normal smokers (232).

Increased ROS in the lungs could have multiple important effects which may possibly contribute to the development of COPD. ROS, especially H_2O_2, are known apoptotic inducers of epithelial and endothelial cells (22). Also, oxidation inactivates antiproteases, like α_1-antitrypsin, and this may result in increased proteolysis (233). Oxidants induce mucus secretion in rodent airway epithelium,

suggesting a possible link between oxidative stress and mucus hypersecretion observed in COPD (234). More importantly, oxidative stress could amplify the inflammatory response in COPD by activating transcription factors such as NF-κB and activator protein 1 (AP-1), which switch on the transcription of various inflammatory genes (92). Furthermore, Moodie *et al.* demonstrated that H_2O_2 enhances the acetylation of histone proteins and reduces histone deacetylase activity, leading to chromatin remodelling and increased transcription of inflammatory genes (235).

I.4. – Animal models for COPD

The use of animal models has had great impact on research and it has helped to expand our knowledge on many medical conditions. Gross *et al.* developed the first animal model of emphysema by inducing the disease via the instillation of papain into the lungs of rats (236). This model was one of the pillars upon which the protease/antiprotease hypothesis for COPD was developed. Since then different animals, including rats, dogs, monkeys, sheep, guinea pigs and especially mice, have been used in the study of COPD (237).

I.4.1. – Mouse model

The mouse offers unique advantages over other species when used as an animal model. There is a vast knowledge about mouse biology, as well as great availability of molecular probes to study this species. Furthermore, the mouse genome has been sequenced and it has great homology with the human genome. Moreover, knock-out and

transgenic animals can be produced, permitting the study of specific genes. Additionally, the mouse has short reproductive cycles, large litter sizes and housing is relatively inexpensive, allowing researchers to have significant number of animals for experiments fairly easy. However, even though most biological processes are conserved within mammals, there are important differences between mice and humans (238). Mice are obligate nose breathers with no extensive cilia, no goblet cells and no respiratory bronchioles (239). Despite these differences, research using the mouse model can provide valuable data that can improve our understanding of pulmonary pathologies, such as COPD.

There are several inbred strains of mice that develop spontaneous airspace enlargement (240). The study of these strains could increase our knowledge about lung development, and processes of lung damage and repair, but they might not necessarily correlate to the pathophysiology of COPD. Emphysema has also been induced in mice by intrapulmonary instillation of proteinases and toxic chemicals, as well as by exposure to cigarette smoke. Finally, several transgenic and knock-out mice that develop emphysema have been produced (241).

Overexpression of human MMP-1 in the lungs of transgenic mice results in airspace enlargement (196). However, it is not clear if the emphysema-like phenotype observed in these animals is a result of a disruption of normal alveogenesis or breakdown of mature alveolar walls. Similarly, Hoyle *et al.* showed that overexpression of platelet-derived growth factor B (PDGF-B) leads to airspace enlargement, but this is probably a consequence of an interference with normal lung development since this phenotype was not only observed in adults but also in neonatal mice (242). Transgenic mice with

increased pulmonary expression of other genes, including TNF-α, IL-11 and IL-6 have also been shown to have altered lung development (237).

Knock-out mice are produced in order to study the effects of the disruption of expression of specific genes. Wert *et al.* showed that ablation of the surfactant protein D (SP-D) leads to progressive pulmonary emphysema after 3 weeks of age, which was characterized by inflammation and increased MMP-12 and MMP-9 activities (243). Similarly, TIMP-3 null mice show spontaneous airspace enlargement after 2 weeks of age, with elevated MMP activity but no inflammatory response in the lungs (197). As with the transgenic cases, the emphysema seen in these two knock-out models is very likely to have resulted from developmental abnormalities.

In order to overcome the problem of abnormal lung development and to clarify the role of specific genes in the pathogenesis of emphysema, several groups have used induced transgenic expression. Zheng *et al.* showed that inducible expression of IL-13 leads to emphysema, accompanied with mucus metaplasia, inflammation and increased expression of various MMPs and cathepsins (145). In the same way, induced overexpression of IFN-γ in the lungs of transgenic mice causes airspace enlargement, with increased macrophage, neutrophil and lymphocyte numbers, as well as altered expression of proteases and antiproteases (146).

The most common way of inducing emphysema in mice is exposing the animals to exogenous agents, like tissue-degrading enzymes and cigarette smoke. Tracheal instillation of several proteases, including papain, human neutrophil elastase and porcine pancreatic elastase, lead to the development of emphysema in mice (244). Lucey *et al.* demonstrated that mice deficient in both TNF-α type 1 receptor and IL-β type 1 receptor

were protected from emphysema induced by porcine pancreatic elastase (135). Similarly, MMP-12 null mice do not develop macrophage infiltration after smoke exposure and are protected from emphysema (164). Shapiro *et al.* have shown that NE $^{-/-}$ mice exposed to cigarette smoke have impared lung recruitment of neutrophils and monocytes and according to the authors the absence of this elastase provides significant protection (59%) against the development of emphysema (180).

It has been previously shown by Guerassimov *et al.* that smoke-induced emphysema is strain dependent in mice (24). This group selected five inbred strains of mice based on their major histocompatibility complex (MHC) haplotypes and chronically exposed the animals to cigarette smoke. The rationale behind the selection of the strains was that COPD has an autoimmune component and that differences in the ability of MHC molecules to present autoantigenic peptides to naïve cells is an important determinant of susceptibility to these kind of diseases (24;245). After exposing mice to two cigarettes per day five days a week for a period of six months, it was found that each of the five strains had dramatically different susceptibilities to the development of emphysema. Animals were phenotyped by measuring the lung mechanical properties, the inflammatory response (inflammatory cell counts and cytokine expression), and the mean linear intercept (Lm), which is an index of airspace. The authors classified the NZWLac/J strain as "resistant" because after chronic smoke exposure it does not develop an inflammatory response, and there are no changes in lung elastance or Lm. Furthermore, a down-regulation of a series of pro-inflammatory cytokines, including TNF-α, IL-10, MIP-1α and MIP-1β, is observed. On the other hand, the AKR/J strain ("super-susceptible") showed significant inflammatory cell infiltration of the lungs (Table 1.1),

Table 1.1 Cellular inflammatory response after chronic smoke exposure

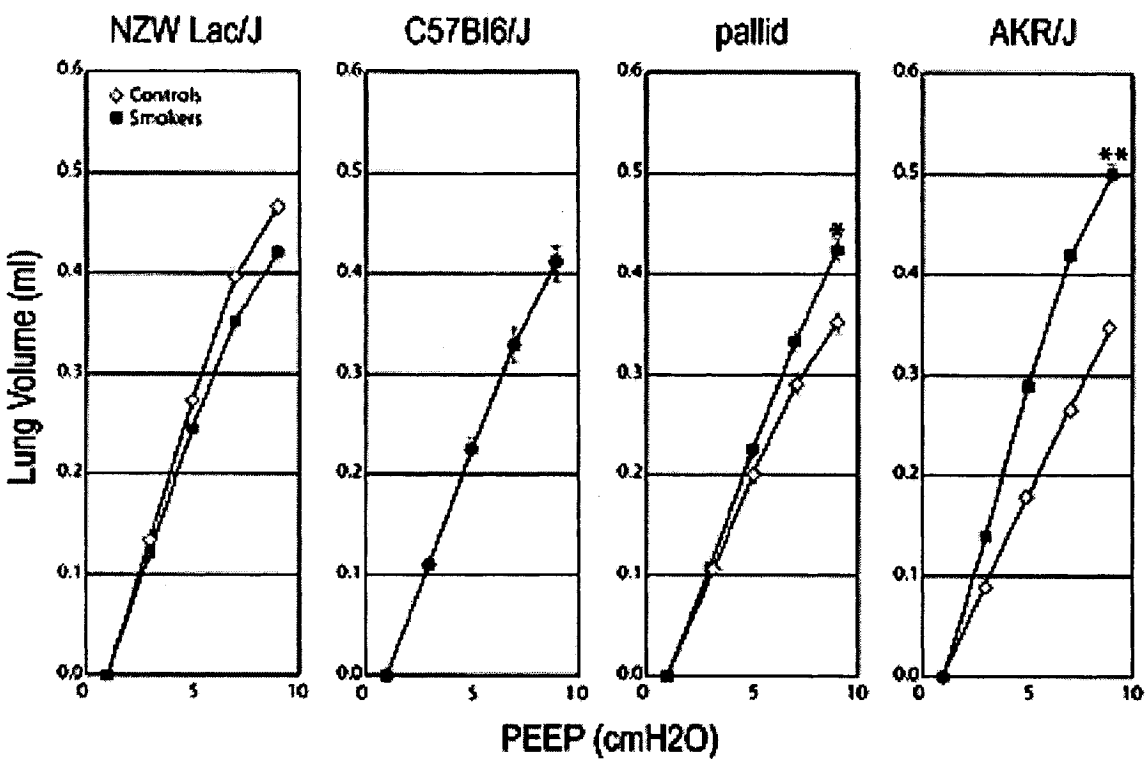
	PMN %		AM %		CD8%		CD4%		gd % *	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NZW										
NS*	12.77	3.76	1.15	0.76	1.68	1.39	4.7	1.47	0	0
S*	9.77	1.94	2.91	1.04	1.78	1.03	5.32	1.91	0.3	0.16
(t ; p)	1.73	0.11	3.35	0.007	0.14	0.89	0.63	0.54	4.59	0.001
C57										
NS	4.95	2.18	7.14	3.4	1.9	0.95	3	1.59	0.4	0.66
S	5.76	2.27	3.14	2.54	0.62	0.19	2.1	0.72	0.11	0.09
(t ; p)	0.63	0.54	2.31	0.04	3.24	0.009	1.26	0.24	1.07	0.31
AKR										
NS	7.66	3.34	1.84	0.85	1.9	0.76	1.98	0.9	0.07	0.16
S	13.47	2.54	9.27	2.49	3.1	1.3	3.98	1.35	0.63	0.28
(t ; p)	3.39	0.007	6.92	0.0004	1.95	0.05	3.02	0.01	4.26	0.002

Definition of Abbreviations: AM = alveolar macrophages; gd = gamma delta T-cells; p = statistical probability; PMN = polymorphonuclear cells; t = t statistic. * Percentage of inflammatory cells over total cells in the alveolar wall. Modified and adapted from Guerassimov *et al.* (2004) (24).

decreased lung elastance and enlargement of the airspaces. Moreover, 6 cytokines, including TNF-α, IL-12p35, IL-12p40, IL-10, MIP-1α and MIP-1β, showed a significant up-regulation after chronic smoke exposure. Interestingly, the C57BL/6J strain ("semi-susceptible") exhibited a small but significant airspace enlargement. However, the authors failed to observe an inflammatory response in the lungs of these mice and the lung mechanical properties were unaltered after 6 months of smoke exposure (Figure1.3).

The importance of this study lies in the fact that it showed that mice have different susceptibilities to the development of the disease, just like humans. Further analysis of each strain would be helpful in identifying factors that could alter the susceptibility of individuals to emphysema. This thesis consists of a deeper analysis of one of the strains, the C57BL/6, with the main objective of identifying key candidate genes and their products that might be involved in the airspace enlargement, one of the landmarks of the pathogenesis of COPD, observed in this strain of mice.

Figure 1.3 Partial pressure-volume (PV) curves between 3- and 9-cm H_2O pressure. PV curves for the "resistant" (NZWLac/J), "semi-susceptible" (C57BL/6J), and "super-susceptible" (AKR/J) strains are shown. In addition, PV curves for the pallid strain are shown for comparison. PEEP = positive end-expiratory pressure. Reproduced from Guerassimov *et al.* (2004) (24).


NZW Lac/J
C57Bl6/J
pallid
AKR/J
Controls
Smokers
Lung Volume (ml)
PEEP (cmH2O)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
*
**

Preface

The introduction described up-to-date information on the pathophysiology of COPD, as well as molecular mechanisms involved in the development of the disease that have been previously proposed. Nevertheless, our current understanding of COPD is still limited. The use of animal models has somehow resolved some of the problems encountered in human-based studies for complex diseases. Several mouse models for emphysema have been developed and our laboratory has previously shown that smoke-induced emphysema in mice is strain dependent. The C57BL/6 strain was classified as mildly susceptible because there is a small but significant increase of airspace after 6 months of smoke exposure. However, there was an absence of an inflammatory response in the lungs, as well as no change in lung mechanics. COPD represents a complex condition and the study of this strain allowed us to focus on one of the important phenotypes of the disease – airspace enlargement or emphysema. The following chapter presents a study that takes a closer look at the inflammatory kinetics of the disease. Furthermore, using microarray technology several candidate genes that could explain the complex phenotype of this mouse strain after smoke exposure were identified.

CHAPTER II.

TIME-COURSE INFLAMMATORY AND GENETIC PROFILING OF C57BL/6 MICE AFTER CHRONIC SMOKE EXPOSURE

based on manuscript to be submitted to Physiological Genomics Journal. Data presented orally at the American Thoracic Society International Conference

Cornejo Perales, S.M., *Radzioch D. and M.G. Cosio*

II.1. – Abstract

Chronic obstructive pulmonary disease (COPD) susceptibility depends on the interaction of several genes and it is characterized by several phenotypes including airspace enlargement or emphysema, changes in lung mechanics and inflammation. The most important factor for the development of COPD is cigarette smoke, but only 15-20% of smokers develop the disease, suggesting the existence of a genetic predisposition. Mice models are widely used in the study of smoking induced emphysema and data from our laboratory has shown that the development of this disease in cigarette smoke-exposed mice is strain dependent. The C57BL/6 strain of mice is commonly used as a model of smoke induced emphysema. This strain develops airspace enlargement after chronic smoke exposure (6 months), with no change in lung mechanics, rendering it suitable for studying the basis of alveolar enlargement induced by smoke that represents one of the important features of COPD. Microarray technology was used to compare the genetic expression profiles in lung tissue of mice (C57BL/6) chronically exposed (6 months) to

cigarette smoke, with age-paired controls. A series of genes involved in the activation, proliferation, maturation, as well as the migration of inflammatory cells were down-regulated, potentially explaining the absence of inflammation in the lungs of smoke-exposed C57BL/6 mice after 6 months of smoke exposure. In this study, we also investigated the changes in inflammatory cell populations and cytokine expression in the lung over time. An initial inflammatory response was observed, but it was completely suppressed by 2 months of smoke-exposure. Overall, these data provide clues explaining the mechanism of airspace enlargement in chronically smoke-exposed mice and the molecular basis explaining the absence of lung inflammation in this particular strain, even if the mice are chronically exposed to smoke, shedding light on which gene expression might be promoting emphysema and which genes might play a protective role in the development of the disease.

II.2. – Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible and that is associated with an abnormal inflammatory response in the lungs to noxious particles and gases (1). COPD is a composite of conditions that includes bronchiolitis, emphysema, and chronic bronchitis (20). Even though it is widely accepted that the main risk factor for developing the disease is cigarette smoking, the cellular and molecular mechanisms involved in its pathogenesis are not fully understood. Moreover, only 15-20% of heavy smokers develop COPD, which suggests that genetic factors play a key role in the susceptibility to acquire the disease (21). In a study by Silverman *et al.*, it was found that the heritability of FEV_1

in subjects with severe early onset COPD was 35% (34). The only proven genetic risk factor for the development of COPD is α_1-antitrypsin deficiency. However, this abnormality accounts for only 1-2% of all COPD cases (9). Therefore, it is very likely that there are other genes, which could act epistatically, and alter the susceptibility of individuals to the disease.

Although individual case-control studies have suggested that some candidate genes, like tumour necrosis factor α (TNF- α) or α-antichymotrypsin, might be associated with COPD, most of these findings were not corroborated in subsequent studies (40;47;48;246). The inconsistency in the results is probably due to differences in study populations, including the number of patients analyzed, and population stratification, like differences in race and age, between cases and controls (9).

Mice have been used successfully as animal models in an attempt to overcome some of the problems encountered in human-based studies. Emphysema has been previously studied in different strains of mice that develop spontaneous airspace enlargement or by the administration of exogenous agents, like proteinases or cigarette smoke (237). Some mouse strains exposed to cigarette smoke seem to develop low-grade chronic inflammation and lung destruction, similar to what is seen in humans and our laboratory has recently shown that smoke induced emphysema in mice is strain dependent (24;240). One of the strains analyzed was the C57BL/6, widely used as a model of cigarette-induced emphysema and in gene knock-out technology. This strain was classified as mildly susceptible because even though there was airspace enlargement, there was an absence of an inflammatory response in the lungs, as well as no change in lung mechanics after 6 months of smoke exposure (24). This semi-susceptible strain does

not develop all the characteristics of "true emphysema" i.e. airspace enlargement with a pulmonary function abnormality (loss of elastic recoil) and lung inflammation, that the suceptibles pallid and AKR/J strains do. The study of the C57BL/6 response has the potential interest of allowing the investigation of factors predisposing to airspace enlargement without inflammation, while also investigating the possible mechanisms regulating the inflammatory response secondary to cigarette-smoke exposure.

The objectives of this paper were to study the progression of the inflammatory response following smoke-exposure in the C57BL/6 strain, and to identify possible candidate genes that could explain the complex phenotype of this strain after smoke exposure.

Our results suggest the importance of a genetic response which suppressing the inflammatory process, leads to the control of the disease, with mild increases in airspace in the C57BL/6, as compared to the full emphysema other more susceptible strains develop.

II.3. – Materials and Methods

II.3.1. – Animal treatment

Male C57BL/6 mice were purchased (Charles River Laboratories, St. Constant, PQ, Canada) at two months of age and kept in our animal facility for a month before starting their exposure to smoke. Mice were then exposed to the smoke of two standard research non-filtered cigarettes (2R1) per day, for five days a week, using a nose-only smoking apparatus (Cigarette Laboratory at the Tobacco and Health Research Institute,

University of Kentucky, Lexington, KY). The animals were divided into groups, which were smoke-exposed for different time periods (two days, one week, two months and six months) and their respective controls. After each period of treatment the animals were sacrificed, the lung was perfused and removed for analysis.

All experimental procedures were conducted in accordance with the Canadian Council on Animal Care and with the approval of the Animal Care Committee (ACC) of McGill University, Montreal, QC, Canada.

II.3.2. – Microarray experiment

A microarray was used to obtain the profile of mRNA expression in the lung of controls and mice exposed to 6 months of cigarette smoke. Total RNA was isolated from the whole right lung using TRIzol (Invitrogen, Burlington, ON, Canada), according to the manufacturer's instructions. The quality of the RNA was assessed using electrophoresis in 2.2 M formaldehyde 2% agarose gel stained with ethidium bromide. The RNA samples were used for microarray only when they were of high quality and integrity.

Three mRNA samples from different mice were selected in each of the two experimental sets (C57BL/6 control, C57BL/6 smoke) and subjected to MG-U74Av2® microarray (Affymetrix Inc., Santa Clara, CA). Briefly, 20 μg of total RNA was reverse-transcribed into cDNA. The single-stranded cDNA was then converted into double-stranded cDNA with T4 DNA polymerase. After phenol/chloroform extraction and ethanol precipitation, the cDNA was incubated with biotinylated UTP and CTP and T7 RNA polymerase to obtain biotin-labelled cRNA. The cRNA was treated with heat and Mg^{++} to be fragmented. The fragmented cRNA was then hybridized to the array for 16

hours at 45°C. The array was washed and stained using the fluidics station and the image was scanned with a Hewlett Packard Gene Array scanner. The hybridization assays and data collection were performed at the McGill University and Genome Quebec Innovation Centre (Montreal, QC) and analyzed using Microarray Suite 5.0® (Affymetrix Inc., Santa Clara, CA) according to the instructions provided by the company.

II.3.3. – Gene expression analysis

The gene expression pattern of 6 arrays was compared by hierarchical clustering. Cluster analysis was performed for 111 genes that met the criteria using CLUSFAVOR (CLUSter and Factor Analysis with Varimax Orthogonal Rotation) (http://condor.bcm.tmc.edu/genepi/clusfavor.html) with the centroid method (247). Distance between the arrays was measured as Euclidean distance.

The gene expressions in the 3 chips in each set of mice were compared by ANOVA using Kensington Discovery Edition® (InforSense Ltd., London, United Kingdom). Gene expressions were considered to be significantly different when 1) ANOVA and following post-hoc tests in 6 chips gave a p-value less than 0.05 2) when comparing two sets, more than two arrays had "present" call values in either set, and 3) there was more than a 50% difference between the two sets.

In order to confirm the ANOVA analysis we performed an alternative analysis of the Affymetrix package using the RMA processing method suggested by Bioconductor (software project for analysis of genomic data: www.bioconductor.org). With this method "perfect match" probe intensities are corrected by using a global model for the distribution of probe intensities. All the genes were then statistically analyzed by a

nonparametric permutation procedure for 2 samples. Each pair wise comparisons of the 2 experimental groups were done using 3 chips per group. A significance value of 0.05 was obtained only when there was no overlap in the values between each of the two groups being compared. We found a coincidence of 85.47% between the 2 methods, a result that validates the use of the ANOVA method for the interpretation of the results.

The array data of the two experimental sets (C57BL/6 control and C57BL/6 smoke) were then compared according to gene ontology (GO). Gene ontology analysis was carried out using GENMAPP~MAPPFinder (http://www.genmapp.org/) in all 12488 genes (248). GO terms were considered to be significantly changed if 1) more than 2 genes changed in each group, and 2) the Z score was larger than 2.

II.3.4. – Validation of gene expression analysis using real-time polymerase chain reaction

The mRNA expressions were also quantified using the real-time reverse transcription polymerase chain reaction (RT)-PCR method. Four genes whose expressions were changed by smoke in the microarray experiments were selected. Primers were designed for those selected genes (Table 2.3). Total RNA was treated with DNase I and reverse transcribed into cDNA using the DNA-free kit (Ambion Inc., Austin, TX). The cDNA was amplified in the MX4000 system (Stratagene) using the brilliant SYBR Green QPCR kit (Stratagene, Cedar Creek, TX) according to the manufacture's instruction. The amount of cDNA was calculated based on the threshold cycle (C_T) value and standardized by the amount of the house-keeping gene using the $2^{-\Delta\Delta C}{}_T$ method:

$$\Delta\Delta C_T = (C_{T,Target} - C_{,GAPDH})_{Time\ x} - (C_{T,Target} - C_{,GAPDH})_{Time\ 0}$$

where the "target" represents the gene of interest tested, as previously described (249). Each gene expression was standardized by the expression of a house-keeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The melting curve and agarose gel electrophoresis analyses, was performed to confirm that a single product of expected length was amplified.

II.3.5. – Bronchoalveolar lavage

Mice were sacrificed twenty four hours after smoke exposure by pentobarbital overdose. The heart was perfused in an attempt to eliminate all cells in the lung vasculature and a 20-gauge catheter inserted in the trachea. The lung was lavaged 3 times with 1ml of ice-cold saline and the saline lavage was centrifuged at 1200 rpm at 4°C for 10 minutes. The supernatants were decanted and the cell pellets were resuspended in 100µl of cold saline. 10µl of the cell suspension were mixed with 90µl of Trypan blue and total cell counts were done using a hemacytometer. Cytospins were done with 100µl of bronchoalveolar lavage. Slides were air-dried and fixed in acetone-methanol and stained with hematoxylin and eosin for differential cell counts.

II.3.6. – Ribonuclease protection analysis of lung gene expression

Total RNA was isolated using a total RNA isolation reagent (TRIZOL®) and mRNA expression of cytokines (TNF-α, IL-12p35, IL-12p40, IL-10, MIP-1β, MIP-1α, MIP-2, IP-10, IL-6, and IFN-γ) as well as two housekeeping genes (GAPDH and L32) was measured using a Multi-Probe RNase Protection Assay System (BD Biosciences

Pharmingen, San Diego, CA). Briefly, following overnight hybridization of multiprobe to target mRNA (20μg) in solution, free probe and single-stranded RNA were digested by RNase treatment. Protected probes were then purified and resolved by electrophoresis on a 5% acrylamide gel. Detection was done by autoradiography and optical densities of mRNA bands were quantified with ImagePro Plus software (Media Cybernetics Inc.). Signals were normalized to the housekeeping gene GAPDH as a loading control. All values are expressed in arbitrary units.

II.4. - Results

II.4.1. – Lung morphology after chronic smoke exposure

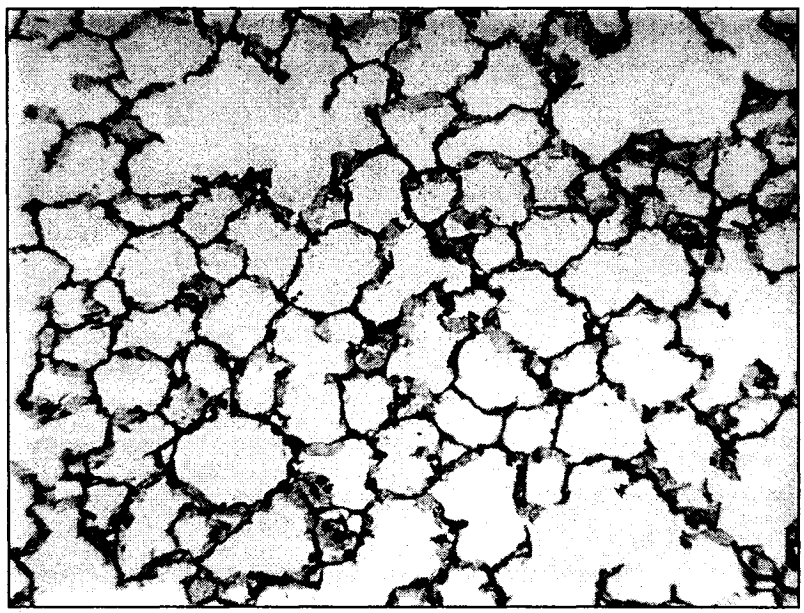
Analysis of the lung morphology of the C57BL/6 strain showed obvious airspace enlargement after 6 months of smoke exposure (Figure 2.1).

II.4.2. – Genes differentially expressed between control and smoke-exposed C57BL/6 mice

Tables 2.1 and 2.2 summarize the set of candidate genes that were significantly different between control and smoke-exposed mice. The list of genes was generated on microarray data from lung mRNA of C57BL/6 mice that were analyzed by Affymetrix Analysis Microarray Suite MAS 5 software and confirmed by a nonparametric permutation comparison. A total of 111 genes were differentially expressed after smoke exposure; 77 genes were down-regulated in the smoke-exposed mice compared to the control mice and 34 were up-regulated. Figure 2.2 shows the results of two-way hierarchical clustering. The control (CTR) and smoke-exposed (SE) samples are properly arranged by the algorithm.

Figure 2.1 Lung morphology after smoke exposure. Lung photomicrographs (X250) of control and smoking mice after 6 months of exposure. CTR = control; SE = smoke exposed.

CTR



SE

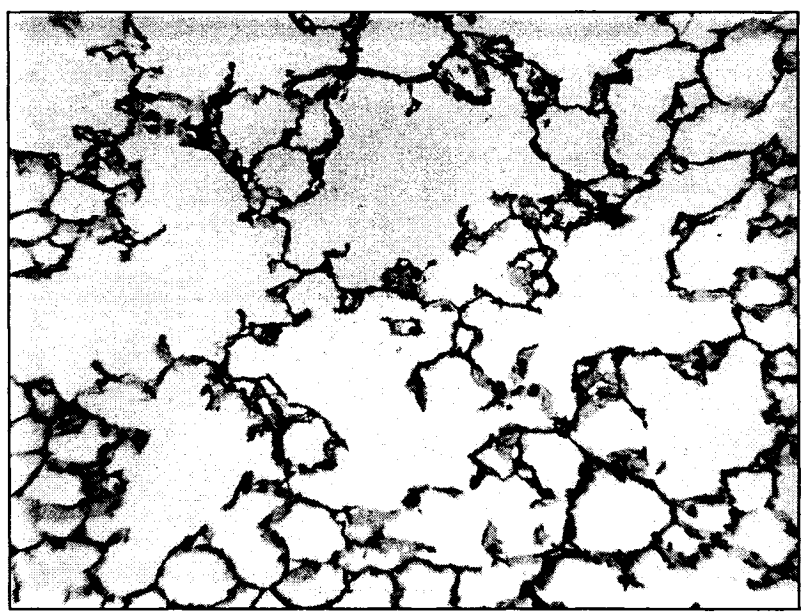


Table 2.1 Genes differentially expressed between smoke-exposed and age-matched control C57BL/6 mice

Over-expressed Genes in SE	Fold difference	P value *	Affymetrix probe†
Mesothelin (Msln)	2.85	2.50E-02	102953_at
Uroplakin 3B (Upk3b)	2.83	1.75E-02	104512_at
Resistin (Retn)	2.69	1.40E-02	102366_at
Guanosine diphosphate (GDP) Dissociation inhibitor 2 (Gdi2)	2.60	4.55E-02	94642_at
Solute carrier family 16 (monocarboxylic acid transporters), member 1 (Slc16a1)	2.55	1.63E-02	101588_at
Adipsin (And)	2.38	4.18E-02	99671_at
Basonuclin 1 (Bnc1)	2.29	1.87E-02	93371_at
Desmocollin 2 (Dsc2)	2.28	2.86E-02	103506_f_at
Secreted modular calcium binding protein 2 (Smoc2)	1.94	2.73E-02	96926_at
Annexin A2 (Anxa2)	1.86	1.65E-02	100566_at
Inter alpha-trypsin inhibitor, heavy chain 4 (Itih4)	1.83	2.37E-03	98467_at
Clusterin (Clu)	1.82	7.34E-04	161294_f_at
Histocompatibility 2, Q region locus 1 (H2Q1)	1.76	2.84E-02	92222_f_at
Prion protein (Prnp)	1.74	2.32E-02	100606_at
DnaJ (Hsp40) homolog, subfamily B, member 3 (Dnajb3)	1.74	3.36E-04	97480_f_at
Bleomycin hydrolase (Blmh)	1.71	1.95E-02	162221_i_at
Scm-like with four mbt domains 2 (Sfmbt2)	1.70	4.43E-02	100706_f_at
Wilms tumor homolog (Wt1)	1.69	2.36E-02	93856_at
Cathepsin K (Ctsk)	1.67	5.12E-03	160406_at
Chitinase 3-like 3 (Chi3l3)	1.64	4.84E-03	92694_at
REST corepressor 1 (Rcor1)	1.64	2.61E-02	161471_f_at
Immediate early response 3 (Ier3)	1.62	3.16E-02	161281_f_at
P450 (cytochrome) oxidoreductase (Por)	1.61	8.27E-03	161668_f_at
Hairy and enhancer of split 1, (Drosophila) (Hes1)	1.58	4.14E-02	160887_at
Phospholipase D3 (Pld3)	1.57	1.19E-02	100607_at
Protease, serine, 12 neurotrypsin, (motopsin) (Prss12)	1.56	4.18E-02	93953_at
Growth arrest specific 1 (Gas1)	1.56	8.08E-03	94813_at
Karyopherin (importin) beta 3 (Kpnb3)	1.53	1.27E-03	161757_f_at
Transcription factor 23 (Tcf23)	1.53	2.43E-03	162332_f_at
Ubiquitin-conjugating enzyme 8 (Ube2l6)	1.52	1.71E-02	98597_at
Zinc finger protein 289 (Zfp289)	1.52	1.38E-02	99644_at

Under-expressed Genes in SE			
Zinc finger protein 503 (Zfp503)	4.73	8.93E-03	161741_r_at
Matrix metalloproteinase 9 (Mmp9)	4.58	3.13E-02	99957_at
S100 calcium binding protein A9 (calgranulin B)	4.35	3.80E-02	103887_at
S100 calcium binding protein A8 (calgranulin A)	3.76	4.01E-02	103448_at
Integrin alpha M (Itgam)	3.15	3.03E-02	98828_at
Vesicle-associated membrane protein, associated protein A (33 kDa) (Vapa)	2.88	4.78E-02	161516_r_at
Phospholipase A2 group VII (platelet-activating factor acetylhydrolase, plasma) (Pla2g7)	2.82	4.75E-02	101923_at
Myocyte enhancer factor 2A (Mef2a)	2.71	2.37E-04	94133_r_at
Shugoshin-like 2 (S. pombe) (Sgol2)	2.16	1.74E-02	95369_at
Selectin, lymphocyte (Sell)	2.08	3.95E-02	102838_at
Protein tyrosine phosphatase, receptor Type, C (Ptprc)	2.08	2.77E-02	101298_g_at (2)
G-protein coupled receptor 25 (Gpr65)	2.07	4.84E-02	96553_at
B-cell linker (Blnk)	2.06	3.85E-03	100772_g_at
Immunoglobulin heavy chain 6 (heavy Chain of IgM) (Igh6)	2.05	4.51E-05	92740_at (4)
Sialophorin (Spn)	2.04	1.64E-02	99436_at
Mitochondrial ribosomal protein L46 (Mrpl46)	2.01	6.13E-03	104567_at
Secreted phosphoprotein 1 (Spp1)	1.94	2.98E-02	97519_at
Proline-serine-threonine phosphatase-interacting protein 1 (Pstpip1)	1.90	1.81E-03	103946_at
Formin-like (Fmnl1)	1.89	1.89E-03	92483_g_at
Spi-B transcription factor (Spi-1/PU.1 related) (Spib)	1.88	4.95E-03	93657_at
Mitogen-activated protein kinase kinase kinase 6 (Map3k6)	1.88	1.52E-02	92276_at
Pre-B lymphocyte gene 3 (Vpreb3)	1.87	1.16E-02	93957_at
Neutrophil cytosolic factor 4 (Ncf4)	1.87	1.89E-02	103662_at
T-complex-associated testis expressed 2 (Tcte2)	1.87	1.84E-02	103541_at
UDP-glucose ceramide glucosyltransferase (Ugcg)	1.86	1.81E-03	94197_at
Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (Slc11a1)	1.86	2.67E-02	96562_at
CD72 antigen (Cd72)	1.84	6.62E-03	101878_at
AT rich interactive domain 3A (Bright like) (Arid3a)	1.84	3.90E-02	103496_at

Complement factor H-related protein (Cfh)	1.83	9.32E-03	94993_f_at
Glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1 (glutamate binding) (Grina)	1.82	9.37E-03	99160_s_at
Membrane-spanning 4-domains, subfamily A, member 2 (Ms4a2)	1.81	5.77E-03	99446_at
Potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (Kcnma1)	1.81	2.96E-02	97759_at
Chemokine (C-X-C) receptor 4 (Cxcr4)	1.80	4.82E-02	102794_at
Polypyrimidine tract binding protein 2 (Ptbp2)	1.79	3.65E-02	160374_r_at
Dedicator of cyto-kinesis 2 (Dock2)	1.79	3.91E-03	103462_at
Integrin beta 2-like (Itgb2l)	1.79	2.88E-02	94171_at
Killer cell lectin-like receptor subfamily A, member 13 (Klra12)	1.79	4.01E-02	100387_f_at
Homeo box D1 (Hoxd1)	1.79	3.09E-02	98820_g_at
RAS-related C3 botulinum substrate 2 (Rac2)	1.76	4.23E-02	103579_at
Endothelial PAS domain protein 1 (Epas1)	1.75	3.07E-02	102698_at
Repetin (Rptn)	1.75	6.35E-03	94732_at
Immunoglobulin-associated alpha (Cd79a)	1.73	4.98E-03	102778_at
POU domain, class 2, associating factor 1 (Pou2af1)	1.70	5.27E-03	93915_at
Paired box gene 6 (pax6)	1.70	1.39E-02	92271_at
Intracisternal A particles	1.68	1.52E-02	93909_f_at
Period homolog (Drosophila) (Per1)	1.67	2.61E-02	93619_at
Interferon inducible GTPase 1 (Iigp1)	1.65	2.98E-02	103963_f_at
Tumor necrosis factor receptor superfamily, member 26 (Tnfrsf26)	1.65	1.86E-03	101524_at
Guanylate cyclase 1, soluble, beta 3 (Gucy1b3)	1.64	1.56E-02	93954_at
Integrin alpha 8 (Itga8)	1.63	1.24E-04	100313_at
Serine (or cysteine) proteinase inhibitor, Clade B, member 6c (Serpinb6c)	1.62	1.30E-02	103306_at
Serine protease inhibitor 13	1.62	5.74E-03	161115_r_at
Ring finger protein 125 (Rnf125)	1.60	3.59E-02	99823_r_at
Trefoil factor 1 (Tff1)	1.60	1.78E-03	160300_at
Visinin-like 1 (Vsnl1)	1.59	2.95E-02	92995_at
CD19 antigen (Cd19)	1.59	1.33E-04	99945_at
Interferon, alpha-inducible protein (G1p2)	1.57	3.91E-02	161511_f_at
Paired-Ig-like receptor B (Pira6)	1.57	3.80E-02	98003_at
Serine/threonine kinase 3 (Ste20, yeast homolog) (Stk3)	1.55	4.47E-02	98775_at
Importin 4 (Ipo4)	1.54	2.92E-02	161250_at

Vitronectin (Vtn)	1.52	2.69E-02	98549_at
ARP1 actin-related protein 1 homolog A (yeast) (Actr1a)	1.52	1.32E-02	97714_r_at
Immunoglobulin-associated beta (Cd79b)	1.52	6.22E-04	161012_at
Cytoplasmic tyrosine kinase, Dscr28C related (Drosophila) (Tec)	1.52	2.55E-02	103539_at
Glucose-6-phosphate dehydrogenase 2 (G6pd2)	1.52	1.19E-03	101294_g_at
Zinc finger protein, subfamily 1A, 1 (Ikaros) (Znfn1a1)	1.52	1.09E-02	102293_at
Coagulation factor II (thrombin) receptor (F2r)	1.52	2.39E-02	92267_at
Growth associated protein 43 (Gap43)	1.51	3.00E-02	102389_s_at
Signal sequence receptor, gamma (Ssr3)	1.51	3.57E-02	104249_g_at

Definition of Abbreviations: SE = smoke exposed mice

* Statistically significant at $p<0.05$

† In parentheses: total number of probes for that gene differentially expressed between SE and C C57BL/6 mice; the probe with the highest fold difference is presented in the table

Table 2.2 Genes without known-functions differentially expressed between smoke-exposed and age-matched control C57BL/6 mice

Over-expressed Genes in SE	Fold difference	P value *	Affymetrix probe
expressed sequence C81072	1.99	9.33E-03	96487_at
RIKEN cDNA 2510029B14 gene	1.58	3.12E-02	93551_at
RIKEN cDNA 4930467B06 gene	1.51	1.06E-03	103663_at
Under-expressed Genes in SE			
"ESTs, Moderately similar to LY6A MOUSE LYMPHOCYTE ANTIGEN LY-6A.2/LY-6E.1 PRECURSOR [M.musculus]"	3.81	3.96E-02	101820_at
"ESTs, Weakly similar to S26689 hypothetical protein hc1 - mouse [M.musculus]"	2.29	9.52E-03	95890_r_at
"DNA segment, Chr 7, ERATO Doi 183, expressed"	1.75	1.57E-04	95872_at
expressed sequence C80816	1.71	2.84E-02	104256_at
RIKEN cDNA 1300007C21 gene	1.61	3.96E-04	103562_f_at
expressed sequence AW108224	1.58	3.66E-06	160173_at
ESTs	1.54	4.81E-02	93204_r_at
RIKEN cDNA 1300007C21 gene	1.52	4.93E-03	160799_at

Definition of Abbreviations: SE = smoke exposed mice

* Statistically significant at $p<0.05$

Figure 2.2 Cluster analysis of microarray experiments in controls (CTR) and smoke-exposed (SMOKE) mice. Differentially expressed genes were selected and subjected to cluster analysis. Each column represents the gene expression of one mouse. Data in the same row represent that of the same gene. All the controls and smoke-exposed mice are grouped together.


Color based on sorted raw data
Distance function: Euclidean distance
4.05
136.36
382.07
1407.3429000.39
CTR
SMOKE

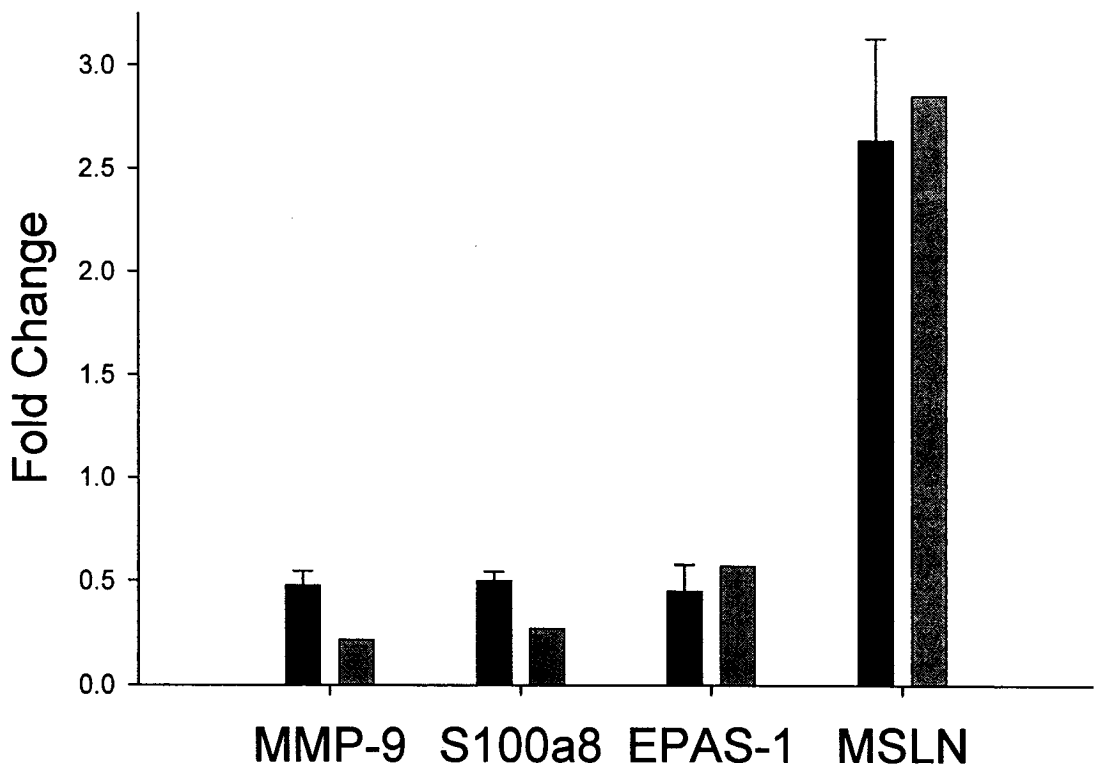
II.4.3. – Validation of gene expression differences found by microarray data

In order to validate the microarray data, the expression of 4 candidate genes were analyzed using quantitative real-time-RT-PCR as described in the Materials and Methods section. Table 2.3 lists the primer sequences for the selected candidate genes. MMP-9 was selected because of its known importance in human COPD. S100a8 and Msln genes were selected because of their previously reported role in the inflammatory process and potential role in the development of emphysema. Finally, Epas1 has been shown to be involved in the regulation of antioxidant enzymes, a process believed to be crucial in the response to reactive oxygen species present in cigarette smoke. Figure 2.3 shows the fold change from controls according to real-time-RT-PCR and microarray data. As it can be seen in this figure, changes observed by the microarray were confirmed by the real-time-RT-PCR.

Table 2.3 Primer sequences for the validation of selected candidate genes

Gene Name	Sense	Anti-sense
Mmp-9	5'-TTCTTCTCTGGACGTCAAAT-3'	5'-CCTAGACCCAACTTATCCAG-3'
S100a8	5'-CCATGCCCTCTACAAGAATGAC-3'	5'-CTACTCCTTGTGGCTGTCTTTG-3'
Epas-1	5'-CTCCTGTCCTCAGTCTGCTCTG-3'	5'-CACCACAGCAATGAAACCCTCC-3'
Msln	5'ACGGGATGCAGAACAGAAAGCC-3'	5'-TGGTGCCATCTACACAAGCCTC-3'

Figure 2.3 Validation of the difference in mRNA expression between control and smoke-exposed mice for selected genes. Lung mRNA abundance was determined by quantitative real-time-RT-PCR. Fold change difference in lung gene expression between smoke-exposed mice compared to age-matched controls according to real-time-RT-PCR (black bars) and microarray (grey bars) are shown.


Fold Change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MMP-9
S100a8
EPAS-1
MSLN

II.4.4. – Gene ontology classification

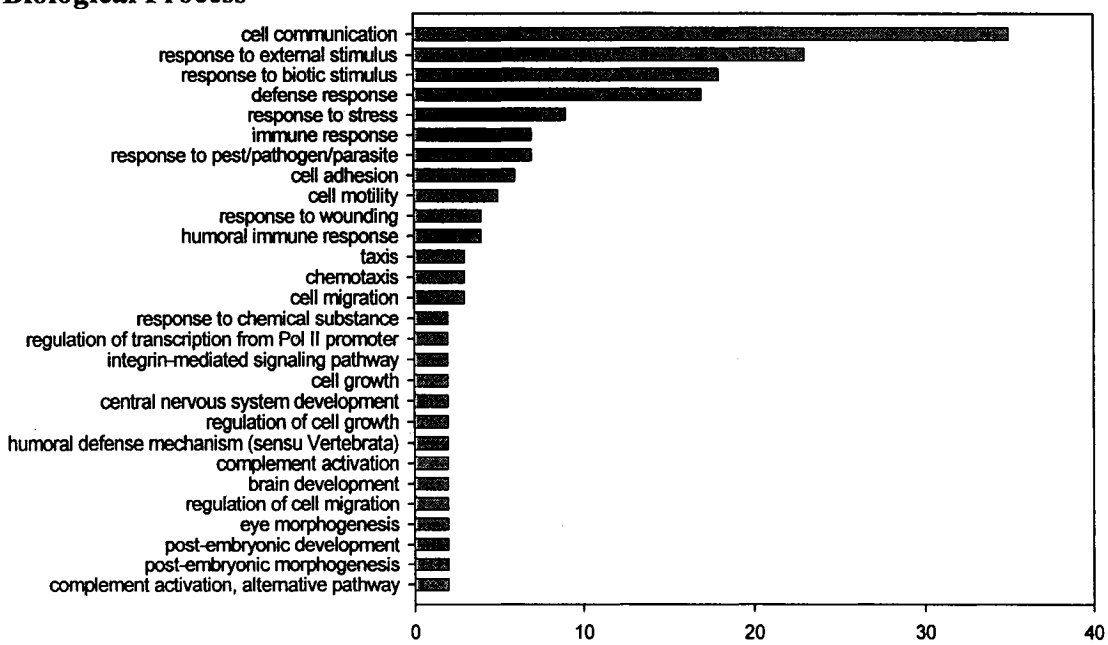
The GENMAPP and MAPPFinder softwares were used in our microarray data in order to classify genes according to gene ontology (GO). This analysis enables us to identify which specific processes might be important in the lung disease phenotype. The software separates the genes into three main categories (Biological Processes, Molecular Function and Cellular component), which are further divided into sub-classes. Figure 2.4 shows the functions that were significantly affected by the exposure to cigarette smoke in C57BL/6 mice. Significance was obtained as explained in the Materials and Methods section.

Most genes differentially expressed between control and smoke-exposed mice fell into the biological processes category. According to our analysis, 28 biological processes categories were significantly affected and they are illustrated on Figure 2.4, panel A. We found 23 genes in the response to external stimulus and 17 genes show to be important in the defence response. Interestingly, we found 7 genes shown to be involved in the regulation of immune response, regulating lymphocyte activation, proliferation, antigen processing and immune cell chemotaxis. Nine genes involved in the response to stress, 3 in cell migration and 2 in complement activation were also identified.

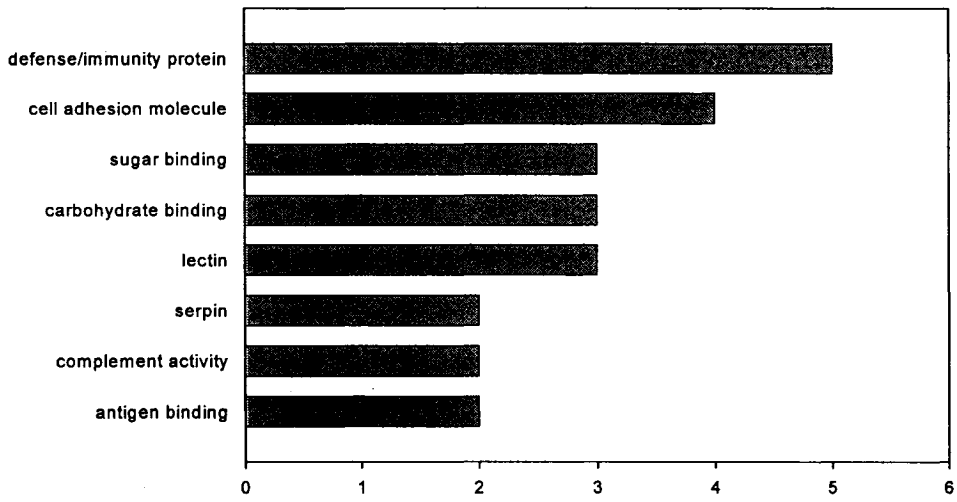
The molecular function category includes genes, whose products regulate cell function at the molecular level, like catalysis or ligand binding. In this category, 8 functions were significantly changed (Figure 2.4, panel B). Two of the identified gene products seem to play important role in antigen binding and five of them are proteins involved in host defence against pathogens and immunity. Two identified

Figure 2.4 Gene ontology-based analysis; effect of cigarette smoke on the gene expression in the lungs of mice. Panels show significantly changed functions (≥2 genes changed in each group and Z score ≥ 2) in each category: Biological Process (A), Molecular Function (B), and Cellular Component (C).

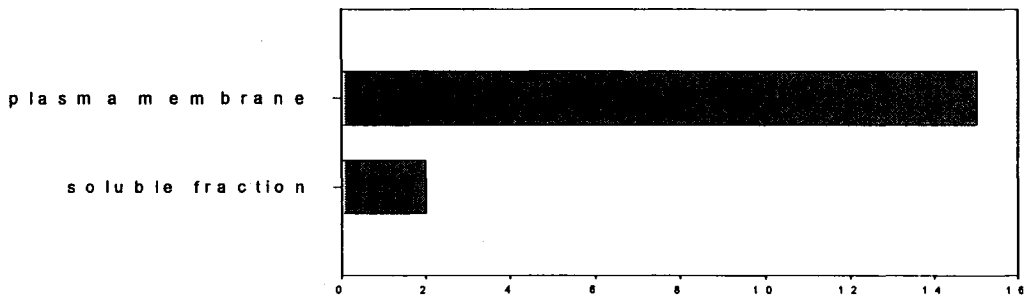
A. Biological Process



B. Molecular Function



C. Cellular Component



genes encode proteins with complement activities and four others encode cell adhesion molecules.

As shown in Figure 2.4, panel C, the cellular component includes genes whose products constitute structural cell components and macromolecular complexes. We have identified fifteen genes differentially expressed between control and smoke-exposed mice that encode proteins localized in the plasma membrane, including two that constitute part of the B-cell receptor complex.

II.4.5. – Inflammatory response

Table 2.4 shows the inflammatory response in the lung of C57BL/6 mice after 2, 7 and 60 days of exposure to cigarette smoke. Our data show that, as soon as 2 days following smoke exposure, a clear inflammatory response develops in the lungs, with a significant increase in the amount of polymorphonuclear cells ($p < 0.0001$), alveolar macrophages ($p = 0.001$), as well as lymphocytes ($p = 0.016$) as compared to non-exposed to smoke controls. By day 7, most of the increase in the amount of inflammatory cells diminishes. At day 7 only alveolar macrophage numbers were still augmented in smoke-exposed mice compared to controls ($p = 0.004$). After 2 months of exposure to smoke, we found no signs of an inflammatory response in the smoke-induced lungs in C57BL/6 mice.

Table 2.4 Number of inflammatory cells in units in bronchoalveolar lavage ($x10^4$/ml) at four different time points after smoke exposure

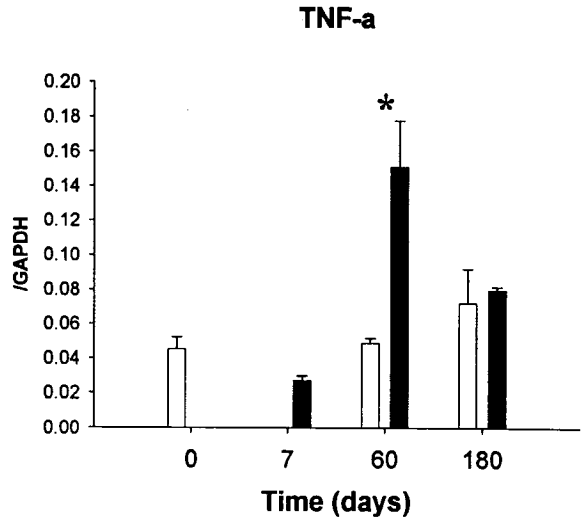
	PMN		AM		Lymphocytes	
	Mean	SD	Mean	SD	Mean	SD
0 day control	0.05	0.004	2.93	1.24	0.15	0.01
2 days	0.15	0.005 *	18.50	3.08 *	0.28	0.05 *
7 days	0.02	0.020	15.72	3.59 *	0.06	0.07
60 days	0.02	0.003	4.45	2.96	0.04	0.03

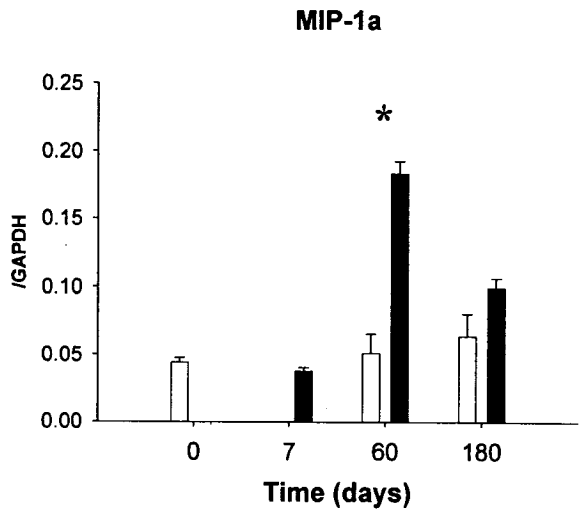
Definition of Abbreviations: AM = alveolar macrophages; PMN = polymorphonuclear cells. * Statistically significant at $p < 0.05$, compared with controls

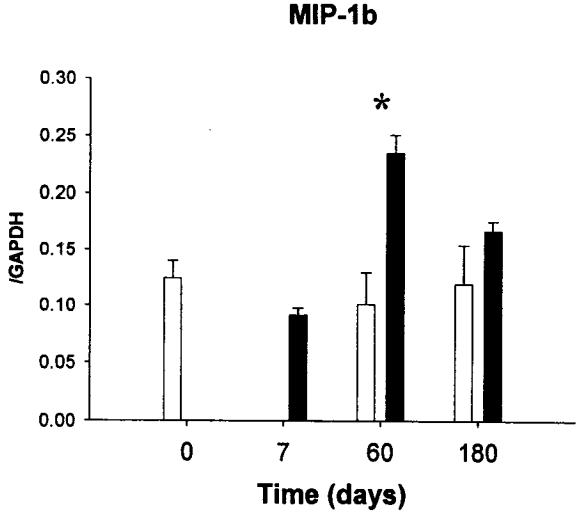
II.4.6. – Cytokine expression

The kinetic analysis of gene expression in lungs from C57BL/6 mice exposed to smoke for various periods of time (0, 7, 60, 180 days) and their controls is seen in Figure 2.5. We analyzed 10 different inflammation-associated genes by using a multi-probe RNase protection assay system and we found a significant increase in TNF-α ($p = 0.02$), MIP-1α ($p = 0.001$) and MIP-1β ($p = 0.013$) mRNA expression in smoke-exposed mice compared to controls at 2 months post-initial exposure, but not at earlier time points. After 6 months of smoke-exposure all cytokine mRNA levels (TNF-α, MIP-1α, MIP-1β, IL-12p35, IL-12p40, IL-10, MIP-2, IP-10, IL-6) did not differ significantly from controls.

Figure 2.5 Comparison of relative abundance of cytokine mRNA over time between controls (open bars) and smoke exposed mice (solid bars) as measured by RNase protection assay. Control animals were analysed at 0, 60, and 180 days after beginning of experiment. Experimental mice were analysed at 7, 60 and 180 days after beginning of smoke exposure. MIP = macrophage inflammatory protein; TNF-a = tumour necrosis factor α. Values are means ± SE. * Statistical significant at $p < 0.05$, compared with controls.


TNF-a
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
/GAPDH
*
0
7
60
180
Time (days)


MIP-1a
0.25
0.20
0.15
0.10
0.05
0.00
/GAPDH
*
0
7
60
180
Time (days)


MIP-1b
0.30
0.25
0.20
0.15
0.10
0.05
0.00
/GAPDH
*
0
7
60
180
Time (days)

II.5. - Discussion

After 6 months of smoke-exposure, the C57BL/6 strain develops airspace enlargement (Figure 2.1) with a ~15% increase in the mean linear intercept, but without any changes in lung mechanics (24;250). The genetic response to smoke-exposure showed a predominant suppression of a variety of genes, mainly inflammatory genes. Of interest, the cellular and molecular inflammatory response appears to be suppressed after 2 months of exposure, in concordance with the genetic response.

As indicated by the ontologies affected, smoke-exposure had an important effect in the lungs of these animals (Figure 2.4) and the majority of the ontologies were related to the response to external and biotic stimulus and defence and immune responses, both cellular and humoral, including complement. Cell communication, mobility, adhesion, and integrin signalling were the other groups of functions highly represented in the response to smoke. Thirty six percent of the biological and molecular functions were defence/immune and 21% were related to cellular activity.

By using microarray technology we were able to identify genes whose expression were altered in response to six months of exposure to cigarette smoke. Tables 2.1 and 2.2 show the 111 genes that were differentially expressed between controls and smoke-exposed mice. Analysis of these genes showed that 69% (77 genes) were down-regulated, while 31% were up-regulated relative to the control animals, a somehow surprising finding. Forty percent of the genes had inflammatory/immune functions both humoral and cellular, and 81% of these genes were down-regulated after smoke-exposure, thus suppressing the inflammatory response, as it can be seen by out analysis of the inflammatory cells and mediators. Genes controlling inflammatory functions including

factors like PAF, complements, neutrophils, macrophages, dendritic cells, B-cells, T-cells, and antigen presentation were mostly down-regulated by smoke-exposure.

Some of the genes affected by smoking deserve special attention. S100a8 (calgranulin A) and S100a9 (calgranulin B) were two of the genes that were most significantly down-regulated. The products of these two genes are known to form a complex called calprotectin, which is believed to play a regulatory role in inflammatory processes by exerting an effect on the survival and/or growth of cells involved in inflammation (251). The production of calprotectin has not only been detected in neutrophils and macrophages but also in epithelial cells (252). A large number of inflammatory states, like Crohn's disease, cystic fibrosis and human immunodeficiency virus (HIV) infection, are characterized by increased level of calprotectin in the blood (253-255).

Secreted phosphoprotein 1 or osteopontin (Spp1) was another of the genes down-regulated. Recent studies suggest that Spp1 regulates basic intracellular mechanisms necessary for the migration of macrophages to inflammatory sites (256). Moreover, it is believed to play a role in lung pathologies, as it was found to have increased expression in fibrotic lungs compared to controls (257). Recently, Woodruff *et al.* found that Spp1 was highly induced in macrophages of smokers and that its increased expression correlated with lung function impairment (258). More interestingly, it has been shown by *in vitro* studies, that Spp1 has the ability to enhance the production of MMP-1, MMP-2, and MMP-9 in smooth muscle and tumour cell lines, possibly linking the down-regulation in Spp1 with the observed decreased expression of matrix metalloproteinases in our study (259;260).

Genes controlling integrins and cell adhesion were mostly down-regulated by smoke-exposure. Of special interest is the down-regulation of L-selectin, which is known to be involved in the migration of cytotoxic T-cells into inflamed tissues (261). Moreover, Hamaguchi *et al.* have shown that L-selectin plays a critical role in pulmonary fibrosis by mediating the accumulation of leukocytes, which regulate the production of proinflammatory cytokines (262).

Besides lung inflammation, proteinases-mediated lung injury is considered an important mechanism of emphysema production. Over the past years, evidence of the possible involvement of matrix metalloproteinases (MMPs) in COPD has increased exponentially (263). Increased levels of MMP-1, MMP-8, and MMP-9 have been reported in sputum from COPD patients (264). Moreover, Ohnishi *et al.* have reported increased elastolytic activity of MMP-9 in the lung parenchyma of patients with emphysema (201). However, the role of MMP-9 in the development of emphysema by cigarette smoke has been controversial in mice. MMP-9$^{-/-}$ mice are not protected from cigarette smoke induced emphysema, whereas MMP12$^{-/-}$ are (164;265). Interestingly, based on the results generated using our microarray analysis, MMP-9 was one of the genes most significantly down-regulated in response to cigarette smoke in the C57BL/6 mice. It is possible that the lower expression of this matrix metalloproteinase might be associated with the lesser airspace enlargement that develops after six months of smoke-exposure in this strain in comparison to other more susceptible strains. On the other hand, 2 other proteinases, a serine proteinase Prss12 and cathepsin K (Ctsk) were up-regulated after smoke exposure. Neurotrypsin (Prss12) is a trypsin-like proteinase associated with brain function and although it is produced in the lung and bone marrow, has never been

associated with any lung pathology. Cathepsin K, the most potent mammalian elastase, is a lysosomal cysteine proteinase found in monocytes and fibroblasts, and might play an important role in the remodelling of fibrous tissue in the lung (205). $Ctsk^{-/-}$ mice exposed to bleomycin develop more fibrosis that wild-type controls, suggesting the potential role in collagen destruction by this cathepsin (266). However, the role of this proteinase in emphysema has not been described.

The dramatic inhibition of a large number of genes playing important roles in the regulation of immune and inflammatory processes in the smoke-exposed mice is in agreement with our previous findings. This strain, as well as the NZWLac/J strain, shows a wide down-regulation of inflammatory genes and does not develop an inflammatory reaction after 6 months of smoke-exposure (24;250). Inflammation is a significant component of human COPD, and the study of the regulation of this process could be of importance to understand the kinetics of the disease.

The significance of the down-regulation of a variety of inflammatory and transduction genes is validated by the paucity of the inflammatory cells and mediators' response after chronic smoke-exposure. As shown in Table 2.1, an acute inflammatory response, composed of macrophages, neutrophils and lymphocytes, developed in C57BL/6 mice after 2 days of cigarette smoke exposure. However, after 1 week of treatment only macrophages were increased in BAL. Furthermore, after 2 and 6 months of daily exposure to smoke, inflammation in the lungs was not detectable in BAL or lung parenchyma (24;250).

We also investigated the kinetics of the expression of inflammatory cytokines after smoke exposure. After one week of smoke-exposure the expression of all tested

cytokines (TNF-α, IL-12p35, IL-12p40, IL-10, MIP-1β, MIP-1α, MIP-2, IP-10, IL-6, and IFN-γ) were at control levels but at 2 months of exposure a significant increase in TNF-α, MIP-1α and MIP-1β could be observed. However, after 6 months of daily smoke exposure, there was no difference in the expression of all the tested cytokines between control and smoke-exposed mice, further confirming our microchip data, since these genes were not seen differentially expressed by this method either.

Our data suggests that in the C57BL/6 mouse strain, protective mechanisms are turned on to abort the inflammatory response to cigarette smoke, which prevents the development of severe lung damage observed in susceptible strains, such as AKR/J.

In contrast to the C57BL/6, after 6 months of smoke-exposure, the "super-susceptible" AKR/J strain developed "true emphysema", airspace enlargement with decreased elastic recoil and a large inflammatory response comprised of macrophages, polymorphonuclear cells and lymphocytes (24). Furthermore, an increased expression of inflammatory cytokines, including MIP-1α, MIP-1β, and TNF-α, along with an important genetic pro-inflammatory response was observed. On the other hand, the 'resistant' NZWLac/J strain showed no airspace enlargement and minimal inflammatory reaction after chronic smoke-exposure, with a significant decrease of the same cytokines, and like the C57BL/6, an important genetic response directed toward the suppression of inflammation.

However, in spite of lack of pronounced inflammation, the C57BL/6 strain develops a significant airspace enlargement, ~15% compared to 38% in the "super-susceptible" AKR/J, and without any major changes in the lung architecture (24;250). Takubo *et al.* clearly showed the differences in the pattern of airspace enlargement

developed after smoke exposure by the C57BL/6 in comparison with the pallid strain, which develop emphysema along with inflammation and changes of recoil (250).

In summary, the development of airspace enlargement and emphysema after smoke exposure in mice seems to be a complexed multifactorial process in which oxidative stress, proteinases, apoptosis and an inflammatory reaction may play important roles. In our animal models (NZWLac/J, AKR/J and C57BL6), the outcome to cigarette smoke exposure is most likely genetically determined. Perhaps in the C57BL/6 the airspace enlargement, possibly induced by oxidative stress and proteinases at the early stages of smoke-exposure, does not progress to more severe alveolar breakdown with losses of recoil (emphysema), as seen in the AKR/J, because of the lack of an important inflammatory response.

CHAPTER III.

III.1. – General discussion and conclusions

It is widely accepted that the main environmental determinant of COPD is cigarette smoking. However, only a minor percentage of heavy smokers develop the disease, suggesting that other factors are important in determining which smokers are at risk. Familial clustering studies have provided strong evidence about the involvement of genes in affecting pulmonary function as well as in the development of COPD (25;26). Our laboratory has developed a mouse model for cigarette smoke-induced emphysema, in an attempt to better understand the basis for the susceptibility and/or resistance to the development of this disease.

As it has been previously mentioned, the C57BL/6 is a mouse strain that develops airspace enlargement after 6 months of smoke-exposure. However, the morphological change in the lung of these mice is not accompanied by an inflammatory response and altered lung mechanics. Gene expression analysis suggest that after smoke exposure this strain down-regulates a series of inflammatory genes, which probably prevents a major inflammatory response and inhibits the more severe and functionally evident lung damage observed in other more susceptible strains. As mentioned in Chapter II, the data showed down-regulation of genes shown to be important in inflammatory processes like the calgranulins A and B, osteopontin and L-selectin, after 6 months of smoke exposure. The identification of these genes is important, because their regulation could be central to inhibit the major inflammation necessary to develop the full disease. A closer study of

these candidate genes and their regulation could elucidate their involvement in the development of emphysema.

Inflammation is a crucial and complex component of COPD, therefore the kinetics of the inflammatory process over time was studied in the C57BL/6 mouse strain. Interestingly, an initial inflammatory response develops in the lungs of these mice after 2 days of smoke exposure but it is rapidly controlled and by day 7 only alveolar macrophages are still elevated in BAL of smoke-exposed mice (Table 2.4). Furthermore, no signs of an inflammatory response in BAL of C57BL/6 mice are detected after 2 months of exposure to smoke. Similarly, after 6 months of smoke exposure, no difference in lung cellularity is found between control and smoke-exposed C57BL/6 mice (24;250). However, even though inflammation is controlled this mouse strain develops some airspace enlargement (Figure 2.1). It is possible that the initial inflammation developed in response to cigarette smoke causes a suttle matrix damage which becomes apparent as the animal grows older and the lung is exposed to more mechanical stress. Studies done by Wright *et al.* in guinea pigs have shown that smoke-induced lung damage can be partially (~45%) prevented if the animals exposed are treated with a NE inhibitor for the entire 6 months of exposure (184). However, if the treatment starts 4 moths after the beginning of smoke exposure, the animals are not protected from emphysema, suggesting that the processes that lead to the development of the disease start early on in response to smoke. In the case of the C57BL/6 mouse strain, it is possible that the mild morphological changes observed in the lung after chronic smoke exposure are a direct result of the initial insult and the control of the inflammatory process prevents the development of functional significant "real emphysema".

In an attempt to better understand the regulation of the inflammatory process, the expression of cytokines over time was studied. No difference between controls and smoke-exposed animals was found initially. However, after 2 months of treatment a significant increase in TNF-α, MIP-1α and MIP-1β could be observed. Similarly to what is observed in the cellular profile, the overexpression of these cytokines was controlled and after 6 months post-initial exposure to cigarette smoke, there was no difference in the expression of all the tested cytokines between control and smoke-exposed mice.

MIP-1α and MIP-1β are CC chemokines produced by several different cell types, including macrophages, lymphocytes, but also by fibroblasts, dendritic and epithelial cells (267). Since we have assessed the expression of cytokine mRNA at the level of whole lung tissue, it is difficult to know which cells are responsible for the up-regulation of MIP-1α and MIP-1β. It is possible that the increased expression of these two chemokines occurred in a type of cell which numbers were not increased. Furthermore, the up-regulation in TNF-α could be a direct result in increased levels of MIP-1α. It is known that MIP-1α, functions in vivo as an autocrine activator, enhancing the production of TNF-α (268). Another possible explanation could be that the increase in the levels of MIP-1α and MIP-1β highlighted the beginning of a secondary inflammatory wave that happened between 2 and 6 months of smoke exposure.

These findings in conjunction with our previous results in the expression of cytokines in two other strains of mice, AKR/J ('super-susceptible') and NZW/Lac/J ('resistant'), suggest that the regulation of MIP-1α, MIP-1β, and TNF-α might be important in the resolution of inflammation and the development of the disease (24). After 6 months of smoke-exposure the 'super-susceptible' AKR/J developed airspace

enlargement, and a large inflammatory response comprised of macrophages, polymorphonuclear cells and lymphocytes. Furthermore, there was an increased expression of a series of cytokines, including MIP-1α, MIP-1β and TNF-α. On the other hand, the 'resistant' NZW/Lac/J showed no airspace enlargement and no inflammatory reaction after chronic smoke-exposure. Interestingly, a significant decreased expression of the same cytokines was observed.

CC-chemokines have been identified as important mediators in the pathogenesis of immune-mediated diseases such as myocarditis, suggesting that they might be key players of tissue injury in the production of emphysema (269). The observed correlation between the expression of these three chemokines and the degree of lung tissue damage cannot be mere coincidence, but more studies are needed to clarify what the exact role of these chemokines is in the development of emphysema.

Proteinases are believed to be important in the remodelling processes of the pathological lung. Various proteinases have been suggested to play a role in emphysema and in this study smoke exposure causes the change in expression of several of them. MMPs are a large family of zinc-dependent proteinases which have the ability to degrade all the components of the extracellular matrix. Furthermore, their proteolytic activity has been shown to play an important role in immunity, by regulating cytokine and chemokine gradients and consequently affecting the migration of inflammatory cells (193;194). It was interesting to find that MMP-9 was one of the genes most significantly down-regulated in response to smoke exposure in C57BL/6 mice. This suggests that the down-regulation of this MMP might be linked to the lesser airspace enlargement observed in these mice in comparison to other more susceptible strains, like the AKR/J. However,

C57BL/6 mice do develop some degree of airspace enlargement and this might be associated with the observed increased expression of two other proteinases, neurotrypsin and cathepsin K. Neither of these proteinases has been previously implicated in emphysema, making this an interesting finding.

Neurotrypsin (Prss12) is a proteinase mainly produced in the nervous system but it is also expressed in the bone marrow and lungs. Cathepsin K is a potent elastase which cleaves type I and II collagen at multiple sites within the triple helix and at the end of the molecules (270). Studies have mainly focused on its role in bone turnover but it has been recently suggested that it might play an important role in the remodelling of fibrous tissue in the lung (266). It is of interest to see the up-regulation of this proteinase at the same time as a decreased expression of MMP-9 since bone resorption studies have suggested that they might have compensatory roles (271). The results from this study suggest that this same compensatory mechanism might be occurring in the lung and it might explain why even when MMP-9 is inhibited, airspace enlargement occurs in response to smoke-exposure.

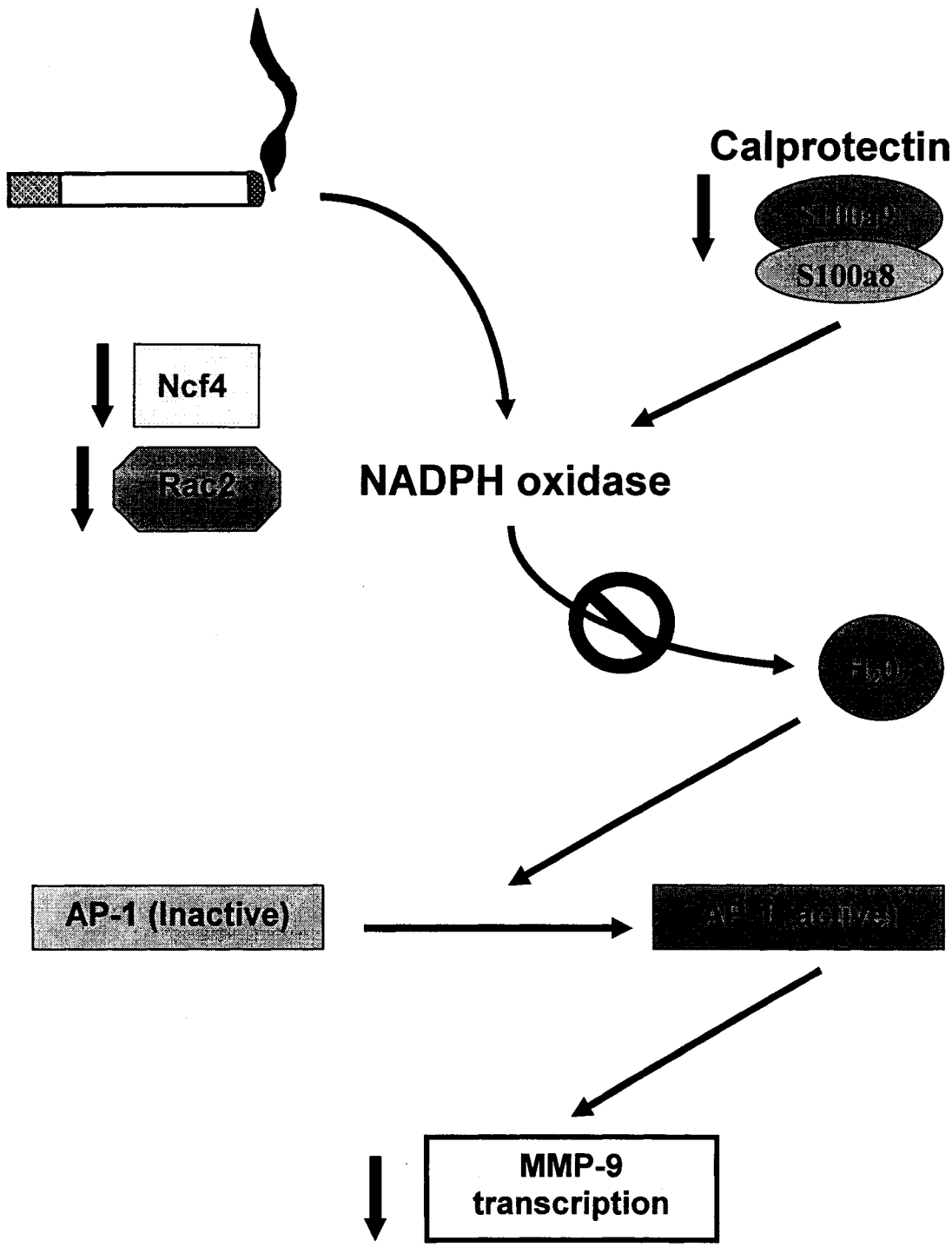
A possible explanation for the down-regulation of MMP-9 is the decreased activation of AP-1, a hydrogen peroxide (H_2O_2)-sensitive transcription factor (272). A large number of MMPs, including MMP-9 and MMP-12, have AP-1 binding sites in their promoters and their transcription is somehow regulated by this factor (273). *In vitro* studies have shown that cigarette smoke condensate induces the expression of MMP-12 through an H_2O_2-dependant pathway involving NADPH oxidase and AP-1 (274). Interestingly, several genes that encode for essential components of the NADPH oxidase system, as well as genes that regulate its activity are down-regulated in the C57BL/6

mice exposed to cigarette smoke (Figure 3.1). NADPH oxidase activation is dependant on the translocation to the membrane of its cytosolic components, including Ncf4 and Rac2, two genes that are down-regulated in the smoke exposed C57BL/6 mice (275).

Moreover, it has been recently shown that NADPH oxidase is highly activated by calprotectin, a complex of S100a8 and S100a9, genes which are also down-regulated in the C57BL/6 smoke-exposed mice (276). The decreased expression of these genes could have led to a reduced activation of the NADPH oxidase system, causing a low production of H_2O_2 and lower activation of the transcription factor AP-1, possibly altering the expression of MMP-9. However, more detailed studies are needed to verify if this is true and to clarify what is the exact role of each of the proteinases whose expression is altered in the development of emphysema.

Our laboratory has also conducted a study, which I have been part of, looking at the gene expression of mouse strains "resistant" (NZWLac/J) and "super-susceptible" (AKR/J) to the development of emphysema after chronic smoke exposure. Using the same techniques as described in the previous chapter, it was found that there was a striking difference in the response to smoke between both strains. In the NZWLac/J, chronic smoke-exposure induced a genetic response leading to the suppression of lung inflammation and up-regulation of anti-oxidant genes, similar to what is seen in the C57BL/6 strain. The response to smoke observed in the resistant strain included the down-regulation of pro-inflammatory genes, including lysophospholipases, immunoglobulins and complement, as well as genes that directly affect the function of neutrophils, macrophages, dendritic cells, and B and T lymphocytes. Moreover, the NZWLac/J strain showed increased expression of a series of anti-inflammatory genes

Figure 3.1 Proposed mechanism of inhibition of MMP-9 transcription in the C57BL/6 smoke-exposed mice. Under normal conditions cigarette smoke activates NADPH oxidase, leading to increase H_2O_2 production. C57BL/6 mice exposed to cigarette smoke down-regulate the expression of components (Ncf4 and Rac2) and activators (S100a8 and S100a9) of the NADPH oxidase system, leading to decreased levels of active AP-1 and possibly decreased transcription of the MMP-9 gene.

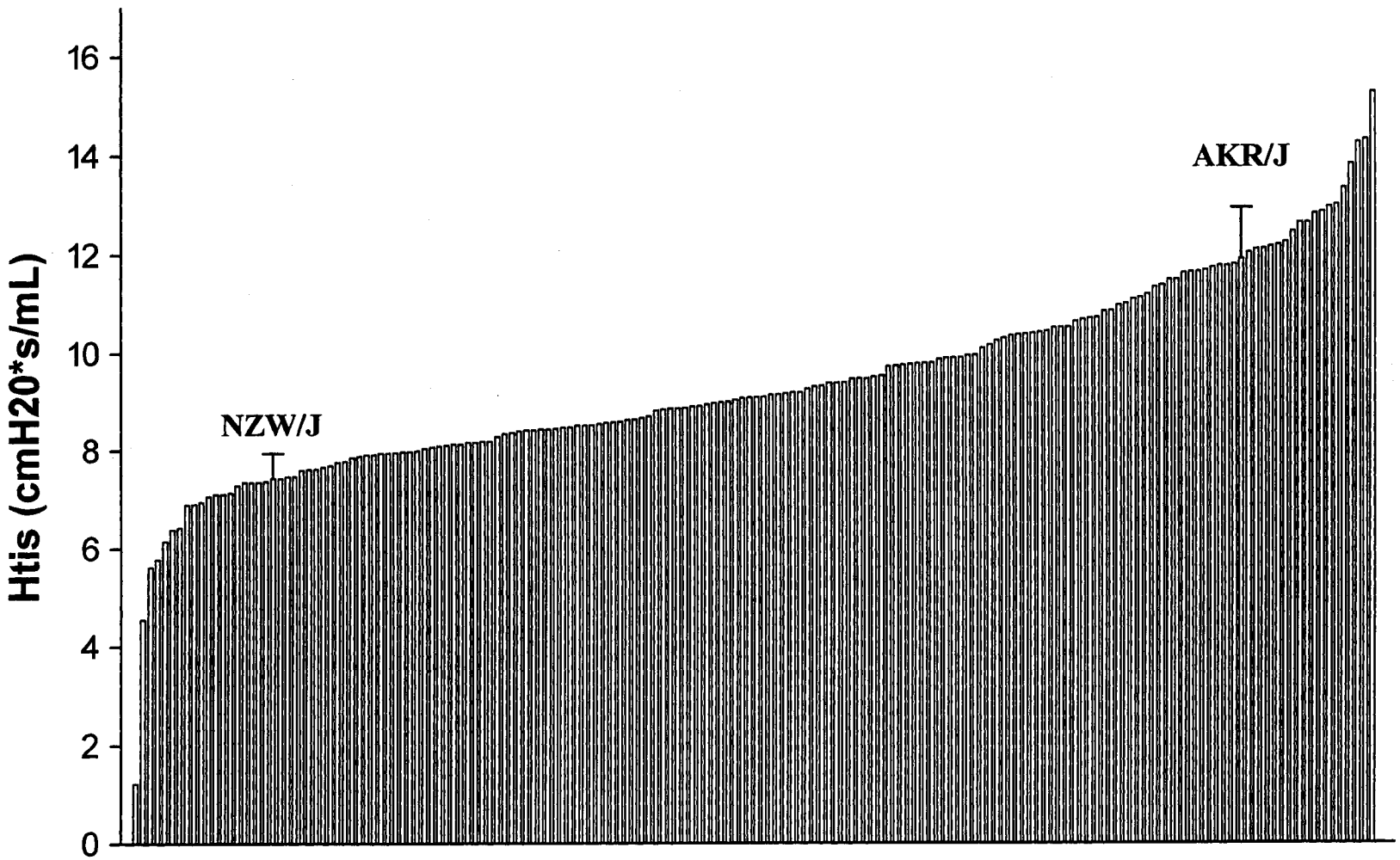

Calprotectin
S100a8
Ncf4
Rac2
NADPH oxidase
AP-1 (Inactive)
MMP-9
transcription

after chronic smoke exposure. The study of the genomic response to cigarette smoking in this resistant strain could be helpful in the understanding of the progression of the inflammatory process following smoke exposure and how it can be controlled. In contrast, the AKR/J strain response to smoke was characterized by enhanced expression of genes of innate and adaptive immunity and apoptosis, possibly explained the development of emphysema.

It is important to understand that our studies look at the gene expression of mouse strains with different susceptibilities to smoke-induced emphysema at one single time-point. It would be of interest to observe the changes in gene expression over time in the different strains in order to better understand the early response to smoke in each of them, and how this could affect the gene regulation at later time points. Furthermore, this kind of study would permit us to possibly pin point individual genes whose early regulation is important in order for the "resistant" strains not to develop the disease.

The identification of strains with extreme opposite phenotypes after chronic smoke exposure allowed us to start a quantitive trait loci (QTL) study, which I have been in charged of. An F2 generation has been developed between the cross of the NZW/J (resistant) and the AKR/J (super-susceptible) strains. More than 250 F2 mice have been exposed to smoke for a period of 6 months and they have been phenotyped by assessing their lung mechanical properties, as well as Lm. These animals have a wide range of phenotypes and individuals with more extreme phenotypes than the parental strains have been observed (Figure 3.2). Genotyping of these mice is currently in progress, and results from the QTL study could be very promising. This work will allow us to identify regions of the genome that are linked to the susceptibility of the development of emphysema

Figure 3.1 Elastance (Htis) at 3-cm H_2O positive end-expiratory pressure (PEEP) in F2 mice after 6 months of cigarette smoke exposure. Values for AKR/J and NZW/J mice are means of eight animals ± SD.


Htis (cmH20*s/mL)
0
2
4
6
8
10
12
14
16
NZW/J
AKR/J

in the mice model, which could advance our understanding of the susceptibility to the development of emphysema in humans. Genes present in these regions will then be identified and possible candidate genes based on their function and/or expression regulation will be studied.

The present thesis highlights the multifactorial and complex mechanisms that take part in the development of emphysema. Furthermore, it raises the question of what does airspace enlargement mean in mice and whether the phenotype observed in the C57BL/6 strain is "real emphysema" or mere airspace enlargement. These morphological changes (airspace enlargement) in the lungs of mice are readibly inducible and this has questioned the validity of some mouse models for human emphysema (277). We believe that a good model of emphysema by definition requires the presence of an abnormal pulmonary function and probably an inflammatory response, including T-cells, as seen in the human disease. The study of the genetic profile of the C57BL/6 strain of mice helped us to identify possible candidate genes that could explain the mechanism of airspace enlargement and inhibition of inflammation observed in this strain when chronically exposed to cigarette smoke. Furthermore, a time-course study of inflammation was presented, allowing us to understand better how the inflammatory process is resolved in this strain. The suggested follow-up studies, as well as the culmination of the QTL study, will provide us with valuable data that could possibly help us elucidate the molecular and cellular mechanisms responsible for the development of emphysema.

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Appendices