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Regulation of proteostasis and proteasome function by cigarette smoke

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Rome, 26.07.2021 Alessandra Mossina

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LIST OF ABBREVIATIONS

19S	19S proteasome activator
20S	20S proteasome core particle
26S	proteasome core particle in association with one 19S activator
30S	proteasome core particle in association with two 19S activators
ABP	Activity-Based Probe
ALP	Autophagy-Lysosome Pathway
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
CP	Core Particle
COPD	Chronic Obstructive Pulmonary Disease
cTEC	cortical Thymic Epithelial Cell
C-L	Caspase-Like
CTL	Cytotoxic T Lymphocytes (also known as cytotoxic T cell or activated $CD8^+$ T cell)
CT-L	Chymotrypsin-Like
CS	Cigarette Smoke
CSE	Cigarette Smoke Extract
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
FEV	Forced Expiratory Volume
FVC	Forced Vital Capacity
GOLD	Global initiative for Chronic Obstructive Lung Disease
IFN-γ	Interferon-γ
iBALT	inducible Bronchus-Associated Lymphoid Tissue
IL	Interleukin

LMP2	Low molecular Mass Polypeptide 2
LMP7	Low molecular Mass Polypeptide 7
MECL-1	Multicatalytic Endopeptidase Complex Subunit 1
MHC	Major Histocompatibility Complex
mRNA	messenger Ribonucleic Acid
mTEC	medullary Thymic Epithelial Cell
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PTM	Post-Translational Modification
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SILAC	Stable Isotope Labeling of Amino acids in Cell culture
SPARC	Secreted Protein Acidic and Rich in Cysteine
TAP	Transporter associated with Antigen Processing
TCR	T Cell Receptor
TFG-β	Transforming Growth Factor-β
T-L	Trypsin-Like
TNF-α	Tumor Necrosis Factor-α
UPR	Unfolded Protein Response
UPS	Ubiquitin-Proteasome System

PUBLICATIONS INCLUDED IN THIS THESIS

Peer-reviewed Publications

Cigarette smoke alters the secretome of lung epithelial cells

Alessandra Mossina*, Christina Lukas*, Juliane Merl-Pham, Franziska E. Uhl, Kathrin Mutze, Andrea Schamberger, Claudia Staab-Weijnitz, Jie Jia, Ali Ö. Yildirim, Melanie Königshoff, Stefanie M. Hauck, Oliver Eickelberg and Silke Meiners

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Proteomics 2017 Jan;17(1-2). doi: 10.1002/pmic.201600243.

Impairment of immunoproteasome function by cigarette smoke and in COPD

Ilona E. Kammerl*, Angela Dann*, **Alessandra Mossina**, Dorothee Brech, Christina Lukas, Oliver Vosyka, Petra Nathan, Thomas M. Conlon, Darcy E. Wagner, Hermen S. Overkleeft, Antje Prasse, Ivan O. Rosas, Tobias Straub, Susanne Krauss-Etschmann, Melanie Königshoff, Gerhard Preissler, Hauke Winter, Michael Lindner, Rudolf Hatz, Jürgen Behr, Katharina Heinzelmann, Ali Ö. Yildirim, Elfriede Noessner, Oliver Eickelberg, Silke Meiners

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is a chronic lung disease characterized by an irreversible and progressive airflow limitation associated with an exaggerated inflammatory response of the lung. Lungs of COPD patients show an abnormal infiltration of innate and adaptive immune cells. In particular, the increased amount of $CD8^+$ T cells has been shown to correlate with lung tissue injury and disease severity. COPD pathogenesis progresses with acute exacerbations, which refer to periods of worsening of respiratory capacities often due to pathogen infections of the lungs. COPD is currently one of the main causes of mortality worldwide and its burden is projected to increase, predicting that in ten years COPD will be the third leading cause of death. Cigarette smoke is the most important risk factor for the development of COPD. Cigarette smoke causes oxidative stress in the lung leading to repetitive challenges of the cellular protein homeostasis machinery and to adaptive immune responses.

Protein homeostasis includes all cellular processes of synthesis, maturation, folding and degradation that occur during the life cycle of proteins and is responsible for maintaining proteome stability. The proteasome is the major proteolytic machinery in the cell and is responsible for the degradation of short-lived, but also misfolded and/or damaged proteins, therefore protecting the cell from proteotoxic stress. The proteasome trims intracellular proteins into small peptides that are eventually loaded into MHC class I grooves for presentation to the immune system via binding with $CD8^+$ T cell receptors. Furthermore, the immunoproteasome is a type of proteasome induced during infections and particularly specialized in enhancing antigen presentation by producing peptides that efficiently bind MHC class I molecules.

The first publication included in this thesis (Mossina *et al.* 2017) investigated the effects of acute cigarette smoke exposure *in vitro* on the proteome of alveolar lung epithelial cells. We observed an impaired proteome stability that consisted mostly of downregulation of secreted cellular proteins. In particular, we identified altered regulation of cellular proteins involved in extracellular matrix organization and wound-healing responses. This acute response of the lung epithelium to cigarette smoke could lead to a dysregulated lung epithelium secretome which may contribute to tissue destruction and remodeling as observed in COPD patients.

The second publication of this thesis (Kammerl *et al.* 2016) studied the effects of cigarette smoke on immunoproteasome function *in vitro* in murine immune cells and *in vivo* in mice and in COPD patients. We observed reduced immunoproteasome mRNA levels in BAL cells and in isolated macrophages from COPD patients. Immunoproteasome activity was severely impaired, both in COPD lung tissue and *in vitro* in murine immune cells exposed to cigarette smoke extract. Most importantly, in murine immune cells, the decline in immunoproteasome activity was associated with reduced presentation of an immunoproteasome-dependent MHC class I epitope. The use of activity-based probes enabled us to monitor the ratio between single immunoproteasome active subunits and their standard proteasome counterpart. We observed that in isolated macrophages of mice that had been acutely exposed to cigarette smoke the activity shifted from immuno- to standard proteasome after 10 days of smoke exposure. Presentation of an immunoproteasome-

dependent MHC class I epitope similarly followed the course of immunoproteasome activity, with a major impairment at day 10. Such dysfunction in MHC class I antigen presentation could contribute to the impaired clearance of pathogens in COPD lungs driving disease exacerbations.

Taken together, our results show that cigarette smoke at non-toxic doses affects proteome stability and composition. We observed, particularly, that a dysfunctional proteome is a major response of the lung epithelium to acute cigarette smoke exposure. As an important player in proteome stability and antigen presentation, the immunoproteasome is as well affected by cigarette smoke. Reduced immunoproteasome activity and diminished presentation of an immunoproteasome-dependent MHC class I epitope establish a causal link between cigarette smoke and the altered immune system response that may also apply to COPD pathogenesis. In particular, alterations in the antigen presentation process may contribute to increase susceptibility to virus-induced exacerbations finally resulting in autoimmune responses.

ZUSAMMENFASSUNG

Bei der chronisch obstruktiven pulmonalen Erkrankung (COPD) handelt es sich um eine chronische Lungenkrankheit, die charakterisiert ist durch eine progressive und irreversible Verminderung der Sauerstoff-Zufuhr. COPD ist derzeit eine der Haupt-Todesursachen weltweit. Vorhergesagt wird eine weitere Zunahme von COPD Fällen in den kommenden 10 Jahren, so dass voraussichtlich COPD-bedingte Todesfälle auf Platz 3 aller Todesursachen weltweit steigen werden. COPD ist assoziiert mit einer erhöhten inflammatorischen Immunantwort der Lungen. So zeigt das Lungengewebe von COPD Patienten eine vermehrte Infiltration von Immunzellen der adaptiven sowie angeborenen Immunantwort. Insbesondere die erhöhte Anzahl von CD8 positiven T-Zellen im COPD Lungengewebe korreliert mit dem Schweregrad der Erkrankung und dem Ausmaß der Lungenschädigung. Eine wesentliche Verschlechterung des Krankheitszustands bei COPD Patienten wird bei sogenannten Exazerbationen beobachtet, welche häufig mit einer viralen oder bakteriellen Infektion einhergehen. Zigarettenrauch ist einer der Haupt-Risikofaktoren für die Entstehung von COPD und verursacht eine oxidative Schädigung des Lungengewebes, was zu einer Dysregulation der Proteinhomöostase in der Zelle und zu einer veränderten adaptativen Immunantwort beiträgt und somit die Pathogenese der COPD maßgeblich beeinflusst.

Der Begriff Proteinhomöostase umfasst alle zellulären Prozesse von der Proteinsynthese, über die korrekte Proteinfaltung bis hin zum Proteinabbau in der Zelle und trägt damit wesentlich zur Funktion der Gesamtheit aller Proteine in der Zelle – dem Proteom – bei. Das Proteasom ist eine der Haupt-Proteinabbau Maschinerien der Zelle und spaltet alte und geschädigte Proteine in kleine Proteinfragmente, sogenannte Peptide. Damit trägt das Proteasom zentral zur Proteinqualitätskontrolle und dem Erhalt der zellulären Proteinphomöostase bei und schützt die Zelle vor Stress. Darüberhinaus werden die durch das Proteasom generierten Peptide zum Teil auf MHC Klasse I Molekülen auf der Zelloberfläche dem Immunsystem präsentiert und definieren das „Selbst“ der Zelle. CD8 positive T-Zellen erkennen fremde MHC Klasse I Antigene, wie sie zum Beispiel bei einer Virusinfektion durch den proteasomalen Abbau viraler Proteine entstehen, und können dann die virus-infizierte Zelle abtöten. Eine besondere Form des Proteasoms – das Immunoproteasom – ist darauf spezialisiert, diese antigenen Peptide effizient herzustellen, und trägt damit zu einer optimierten Immunantwort bei intrazellulären Infektionen bei.

In der ersten Publikation dieser Arbeit wurde der *in vitro* Effekt von Zigarettenrauch auf das Proteom von Lungenepithelzellen untersucht (Mossina et al., 2017). Wir konnten eine veränderte Proteomstabilität beobachten, einhergehend mit einer verminderten Sekretion extrazellulärer Proteine. Insbesondere beobachteten wir eine reduzierte Freisetzung von Proteinen, welche die extrazelluläre Matrix und Wundheilungsprozesse regulieren. Diese akute Antwort des Lungenepithels auf Zigarettenrauch könnte zum pathologischen Gewebeumbau beitragen wie bei COPD Patienten beobachtet.

Die zweite Publikation in dieser Dissertation (Kammerl et al., 2016) untersucht den Effekt von Zigarettenrauch auf die Funktion des Immunoproteasoms *in vitro*, *in vivo* und in Lungengewebe

von COPD Patienten. Die Expression des Immunoproteasoms zeigte sich deutlich vermindert in Immunzellen der Lunge, die aus der bronchoalveolären Lavage von COPD Patienten isoliert worden waren. Die Aktivität des Immunoproteasoms war sowohl im COPD Lungengewebe wie auch nach *in vitro* Exposition von Immunzellen mit Zigarettenrauch-Extrakt signifikant reduziert. Diese verminderte Aktivität des Immunoproteasoms ging einher mit einer reduzierten MHC Klasse I Antigenpräsentation eines Immunoproteasom-spezifischen Epitops. Durch die Verwendung von activity-based probes (ABP) konnten wir die verschiedenen Standard- und Immunoproteasom-spezifischen Aktivitäten des Proteasoms quantifizieren und beobachteten hier eine Verschiebung der Aktivität in Lungenimmunzellen in Richtung Standardproteasom-Untereinheiten nach 10 tägiger Zigarettenrauch Exposition *in vivo.* Diese verminderte Immunoproteasomaktivität war begleitet von einer reduzierten MHC Klasse I Antigenpräsentation. Unsere Daten deuten somit darauf hin, dass die durch Zigarettenrauch-induzierte Hemmung des Immunoproteasoms in Immunzellen der Lunge zu einer Veränderung der MHC Klasse I Antigenpräsentation beiträgt, welche bei Virusinfektionen der Lunge zu einer gestörten Immunantwort und zur Exazerbation der COPD führen könnte.

Zusammengefasst lässt sich sagen, dass wir in dieser Arbeit zeigen konnten, das Zigarettenrauch bereits bei nicht-toxischen Konzentrationen zu einer veränderten Stabilität und Komposition des zellulären Proteoms führt und insbesondere eine akute Antwort des Lungenepithels mit einer veränderten Proteinsekretion auslöst. Das Immunoproteasom, ein zentraler Regulator der Proteomstabilität und MHC Klasse I Antigenpräsentation, wird durch Zigarettenrauch gehemmt, was mit einer veränderten adaptiven MHC Klasse I vermittelten Immunantwort einher geht. Diese Fehlfunktion trägt potentiell zu einer deregulierten Immunfunktion bei COPD Patienten bei und führt möglicherweise zu einer erhöhten Suszeptibilität dieser Patienten gegenüber viralen Infektionen, die zu Exazerbationen und zur Progression der COPD beitragen könnten.

1 INTRODUCTION

1.1 Pathogenesis of chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is an umbrella term used to describe chronic lung diseases that cause progressive airflow limitation associated with an abnormal inflammatory response of the large airways and mucus hypersecretion (chronic bronchitis), wall thickening in the small airways together with tissue damage and disturbed repair (emphysema)[1,2]. These features of COPD result in patients' chronic cough and impaired lung function, which normally worsen over years, remarkably decreasing the quality of life, with the possibility of leading to death. COPD is not curable. Medical treatments available nowadays can only relieve symptoms and slow down disease progression, reducing the risk of death[1]. The main risk for COPD is cigarette smoke (including second-hand exposure). However, other risk factors could increase the possibility of COPD development in non-smokers. These include maternal smoking, intrauterine growth retardation, frequent childhood respiratory infections, history of pulmonary tuberculosis, indoor and outdoor air pollution, occupational exposure and genetic susceptibility[1]. In most patients, COPD is associated with significant concomitant diseases (comorbidities, such as lung cancer, cardiovascular disease, osteoporosis, muscle weakness, and depression/anxiety) which increase its morbidity and mortality[3]. Moreover, COPD pathogenesis evolves and worsens with the disease exacerbations, described as periods of acute worsening of respiratory symptoms (exaggerated inflammatory processes, increased mucus production, marked air trapping and subsequent hyperinflation) that result in additional therapy.

1.1.1 The burden of COPD

COPD kills around 3 million people per year. The Global Burden of Disease Study 2015 estimated about 174 million cases of COPD, defining it as one of the main leading cause of morbidity and mortality worldwide[4]. It is currently the fourth leading cause of death, but its prevalence and burden are predicted to increase due to the high exposure to tobacco smoking and air pollutants and to the aging population. COPD is therefore projected to become the third leading cause by 2030[5]. Most studies classify COPD patients according to the "Global Initiative for Chronic Obstructive Lung Disease" (GOLD) definition of chronic airflow obstruction. Based on the GOLD criteria, COPD is diagnosed when FEV_1/FVC (also called Tiffeneau index or FEV_1%) is below 70%, after administration of a bronchodilator. FEV_1 (Forced Expiratory Volume) is the expired volume of air in one second measured after maximal inspiration, while FVC (Forced Vital Capacity) is the total expired volume of air measured in the same respiratory maneuver from

which the FEV_1 is obtained[6]. According to the GOLD definition, COPD patients are divided into four stages (I-IV) on the basis of severity of airflow obstruction (mild, FEV_1 >80% predicted, moderate FEV_1 50-80% predicted, severe FEV_1 30-50% predicted, and very severe FEV_1 <30% predicted). Despite the high number of COPD cases documented in these studies, the global burden of COPD might be underestimated as surveys suggest that COPD is strongly underdiagnosed especially when determining factors are younger age, never smoking, lower level of education, absence of reported symptoms and very moderate severity of airflow limitation[3,7]. Currently, 60-85% of patients with a mild form of the disease are thought to remain undiagnosed[1]. Although COPD has been often diagnosed in men, prevalence in women is rising. Both the increase in tobacco smoking among women and the higher risk to indoor air pollution in low-income countries contribute to the escalating number of women diagnosed with COPD. In fact, studies suggest that cigarette smoking may contribute only to half of the documented COPD cases[8]. Other risk factors, such as exposure to indoor smoke and particles from biomass fuel, have substantially increased in the past years. Currently, inhalation of indoor particulate matter pollution is estimated to contribute for 35% of COPD cases in low-income and middle-income countries[3].

1.1.2 Pathomechanisms of COPD

The broad spectrum of clinical phenotypes in COPD reflects the complexity and the heterogeneity of this disease. The main clinical characteristic that affects all COPD patients is an airflow limitation that is not fully reversible. Such airflow limitation is due to a remodeling of the small airways (thickening of airway walls) and to an emphysematous lung parenchyma destruction, which causes the loss of the elastic recoil force that drives air out of the lungs[3,9]. The airway obstruction impedes proper lung emptying through expiration, trapping air in the lung and causing hyperinflation, which in turn reduces the inspiratory capacity, altogether resulting in breathlessness and limited exercise capacity typical of COPD[9].

Tobacco smoke remains the main cause of COPD worldwide. During the process of tobacco smoking, the lungs are constantly exposed to more than 4500 compounds contained in cigarette smoke (CS). Beside nicotine, heavy metals, carcinogens and toxins in general, tobacco smoke contains highly reactive oxidants (e.g. α,β-unsaturated aldehydes, reactive oxygen species (ROS) such as superoxide, nitric oxide and peroxyl organic free radicals) that cause lipid peroxidation[2,9,10]. Nowadays there is considerable evidence that cigarette smoke causes a shift of the oxidant-antioxidant balance in favor of oxidants[11]. This process, known as oxidative stress, involves in particular the airway epithelium, being the first line of defense in direct contact with the environment. The reactive compounds present in cigarette smoke can interact with and damage lipids, proteins, DNA, and organelles of the lung epithelial cells, causing direct injury and possible post-translational modifications (acetylation, nitrosylation, carbonylation). Intracellular ROS can cause, at high concentration, structural changes, such as lipid peroxidation, DNA strand breaks

and changes in enzymatic activities. At lower levels, ROS decrease cell proliferation and induce apoptosis and necrosis[12]. Beside epithelial cells, also alveolar lining fluid, local macrophages and pulmonary fibroblasts are primary targets for ROS activities. In turn, alveolar macrophages, lung neutrophils and fibroblasts may also become a second source of ROS, especially after stimulation with inflammatory cytokines[13] .

Tobacco oxidants not only have a detrimental effect on lung cells, but they can also damage through direct or indirect pathways components of the lung extracellular matrix (such as elastin and collagen) and interfere with their synthesis and repair[9,14,15]. This altered biogenesis and repair mechanisms is hypothesized to be one of the causes of the destruction of the connective tissue observed in the emphysematous lung. Moreover, breakdown of the connective tissue components might also be due to an imbalance created in the protease-antiprotease system of the lung induced by harmful substances of cigarette smoke[9,16]. In fact, noxious particles and irritants present in cigarette smoke attract inflammatory cells able to produce large amounts of proteases (e.g. protease 3, elastase and various matrix metalloproteases) that destroy connective tissue components. This results in the production of ECM (extracellular matrix) fragments that may act as chemokines attracting further inflammatory cells and perpetuating inflammation even after smoking cessation[12]. As a result, imbalanced protease-antiprotease promotes proteolysis potentially leading to the development of emphysema[9,12].

In the lung affected by COPD, the ECM is degraded but also airflow limitation is observed in response to exposure to tobacco particles and gases. Such reduced airflow is due to the inflammation of the epithelium and of the submucosal glands that provoke increased mucus production by the augmented numbers of goblet cells. Mucus hypersecretion together with reduced mucociliary clearance leads to airways obstruction and to alteration of the surface tension of the epithelial lining fluid, causing the facilitation of airways closure[9,17]. These processes lead to a clinical condition defined as chronic bronchitis characterized by increased cough and sputum production. Severely increased dyspnea, cough and mucus production are also the clinical manifestation of acute exacerbations[14]. This acute aggravation of the respiratory capacities can occasionally occur in COPD patients and force them to be hospitalized. Susceptibility to exacerbation is defined by background inflammation in the lung tissue, status of the immune system, comorbidities, and presence of infectious pathogens. Respiratory tract infections, bacterial or viral, are actually responsible for driving the exacerbation process in more than half of the cases[12].

Of note, the unbalanced proteolysis that contributes to the development of emphysema takes place extracellularly. On the other hand, smoke also affects proteolytic processes within the cell[18,19]. Indeed, damaged or misfolded proteins can accumulate upon exposure to ROS and toxins of the cigarette smoke thereby challenging cellular protein homeostasis and protein quality control mechanisms.

1.2 Protein homeostasis and the proteasome system

1.2.1 Quality control and protein homeostasis

Cellular integrity relies on the correct folding of its proteins in order to maintain the cellular processes that are necessary to preserve normal physiology protecting the cell and organism from disease pathology. Protein folding, its maintenance and repair in case of misfolding is the task of protein homeostasis[20]. More specifically, protein homeostasis (also called proteostasis) refers to the integrated biological pathways that affect the levels of biogenesis, conformational stability, trafficking and degradation of protein within the cell. Proteostasis maintenance is necessary for preservation of a functional proteome and therefore for proper cell viability and growth, resistance to environmental stress and to homeostasis perturbations caused by pathogens[21,22]. Failure to maintain protein homeostasis results in diseases associated with protein misfolding, development of protein aggregates and improper protein degradation[23,24]. Indeed, mechanisms by which protein homeostasis is ensured include, above all, protein stabilization (folding) and protein degradation (proteolysis) (Fig. 1). These mechanisms, altogether defined as posttranslational quality control, are modulated by specific chaperones and proteases[22]. Molecular chaperones can help in the initial folding of proteins through the binding to exposed hydrophobic domains on proteins, therefore preventing the possible formation of insoluble protein aggregates. Subsequent ATP-triggered release from the chaperone promotes folding into the functional conformation[25].

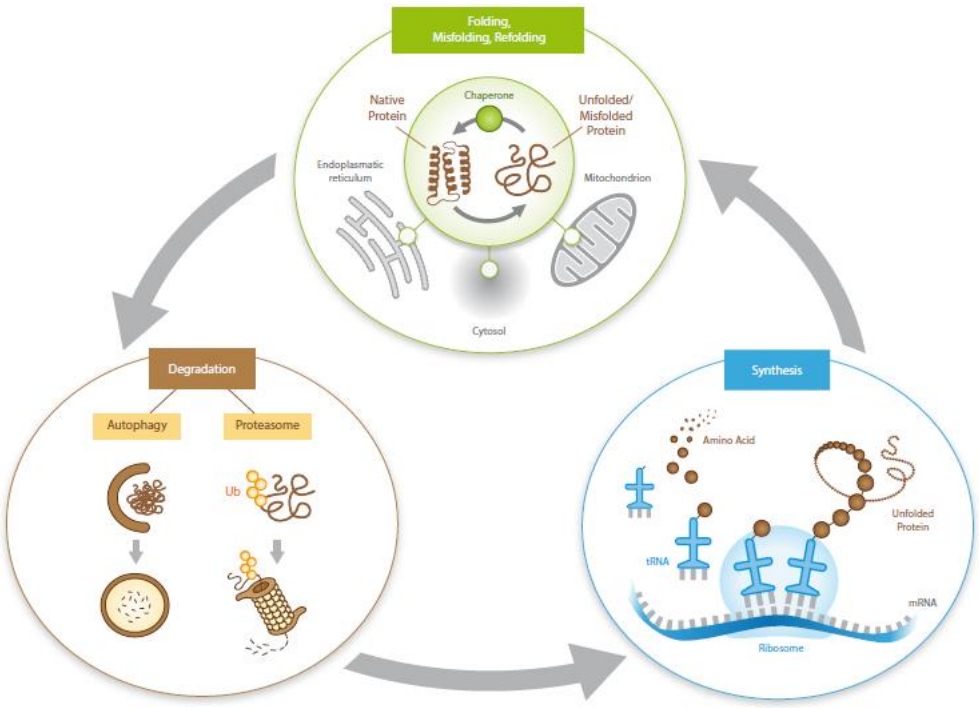

Figure 1 – Protein homeostasis. Protein homeostasis includes all cellular processes of synthesis, maturation, folding and degradation that occur during the life cycle of proteins. After protein synthesis or protein misfolding, exposed hydrophobic regions can be bound by chaperones that help the protein to reach its native confirmation in the cytosol as well as in other cellular compartments, such as endoplasmic reticulum or mitochondria. If proper folding is not possible, the protein is returned into the pool of non-native proteins, perhaps able to rebind to another chaperone. Sustained protein misfolding and/or inability of reaching the proper functional conformation induces protein degradation, mostly through the ubiquitin proteasome pathway. When both the chaperone and the proteasome systems fail, misfolded or partially folded proteins will aggregate into insoluble and nonfunctional inclusions that are removed through autophagy. Degradation products obtained after autophagy or proteasomal degradation are recycled for synthesis of new proteins[26].

When correct folding fails and upon irreparable protein damage, the process of degradation is initiated. Two major protein-degradation systems have evolved in the cell: the ubiquitin-proteasome system (UPS) for degradation of specific and soluble proteins, and the autophagy-lysosome pathway (ALP) for clearance of protein aggregates[22,27]. Autophagy (which literally means “self-eating”) involves sequestration of substrates into double-membraned vesicles and subsequent fusion with the lysosome, where the cargo is degraded by specific hydrolases[21]. While proteolysis of large and insoluble inclusions as well as of damaged and unwanted organelles relies on autophagy, the degradation of specific and soluble proteins is carried out by the ubiquitin-proteasome system.

If the amount of proteins that have to be degraded exceeds the proteolytic capacity of the cell, damaged or misfolded proteins accumulate. An increased load of misfolded proteins in the endoplasmic reticulum (ER) induces the so-called “ER stress”. In general, ER stress can be induced by oxidants, a decrease in ER calcium, or hypoxia, all of which impair protein folding in the lumen of the ER[28]. Upon ER stress the cells evolve an adaptive response named the “unfolded protein response (UPR)”, which consist of a series of transcriptional, translational and post-translational events, whose aim is to slow down protein synthesis on one hand, and on the other to increase protein folding and/or degradation[29,30]. The various processes of the UPR are meant to reverse the ER stress. If ER stress is chronic or severe, the UPR activates signaling pathways that will promote cell apoptosis[29–31].

1.2.2 The ubiquitin proteasome system.

One of the main proteolytic systems involved in protein homeostasis is the ubiquitin-proteasome system. The proteasome is the major regulatory protein complex for “regulated proteolysis of short-lived proteins” (i.e. proteins that control cell cycle, cell differentiation, DNA repair, stress response, gene expression and apoptosis), but also for degradation of misfolded or damaged proteins[32,33]. The proteasome is an ATP-dependent protease complex that recognizes its targets by the presence of a covalently linked chain of at least four ubiquitin molecules

(polyubiquitination) and binds them through one of its ubiquitin receptors[33]. It is a 2.5 MDa multisubunit complex, consisting of a catalytic core (i.e. 20S proteasome or core particle, CP) and two terminal regulatory sub-complexes (i.e. 19S proteasome or regulatory particle, RP)[34]. The 19S RP binds one or both ends of the CP to form the 26S (RP-CP) or 30S (RP-CP-RP) proteasome, respectively. Beside the regulatory particle 19S, four other alternative regulatory particles are known: PA28αβ, PA28γ, PA200, and PI31. Similar to the 19S, these regulators can bind to one or both sides of the 20S CP[35]. The regulatory particles are important for the modulation of substrate specificity, its entry into the CP and turnover rate[35].

The CP is a barrel-shaped structure formed by 28 subunits, arranged into four rings. Each ring consists of seven distinct α and β subunits, organized in a α_7-β_7-β_7-α_7 configuration[32]. Three β-type subunits of each inner ring (β1, β2, and β5) have catalytically active threonine residues with different peptide cleavage specificity: β1 preferring to cleave after acidic residues (caspase-like activity, C-L), β2 after tryptic residues (trypsin-like activity, T-L) and β5 after hydrophobic residues (chymotrypsin-like activity, CT-L)[33]. Through these three distinct catalytic activities, the proteasome degrades proteins into small peptides that range in size from 4 to 20 amino acids[36]. Some of these peptides are further degraded into amino acids by cytosolic peptidases, some others will instead be trimmed to 8-11 residues for binding to major histocompatibility complex (MHC) class I molecules that will transport the peptide to the plasma membrane and present it to the immune system through binding to the T cell receptor (TCR)[37,38]. In immune cells and upon interferon-γ (IFN-γ) induction, the three constitutively active subunits are substituted by three alternative catalytic proteasome subunits, also called immunosubunits: the low molecular mass polypetides 2 and 7 (LMP2 or β1i and LMP7 or β5i), and the multicatalytic endopeptidase complex subunit 1 (MECL-1 or β2i) (Fig. 2)[35,37,39]. After expression, immunosubunits are preferentially incorporated into freshly synthesized 20S core particles giving rise to a new type of proteasome known as the immunoproteasome[37,39]. The replacement of the β1 subunit with the β1i immunosubunits enhances the chymotrypsin-like activity of the immunoproteasome, enforcing the generation of peptides with hydrophobic C-terminal residues, more efficient at binding MHC class I molecules[39]. In addition to constitutive and immunoproteasomes, mixed proteasome have been described (Fig. 2)[35,40]. Each of these intermediate-type proteasomes, consisting partially of constitutive and partially of immunosubunits, exhibit slightly different enzymatic activities, increasing the variety of the peptide pool produced by the proteasome[40]. Finally, in cortical thymic epithelial cells (cTECs) another type of proteasome is expressed. The t20S thymoproteasome contains the two immunosubunits LMP2 and MECL-1 and a thymus-specific catalytic subunit (β5t). Thymoproteasomes are important for positive selection of T cells.

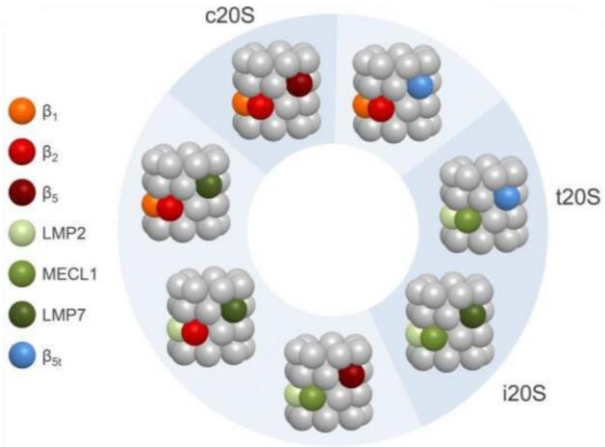

Figure 2 – Variety of proteasome subpopulations. The catalytic subunits β1, β2, and β5 of the constitutive 20S CP (c20S) can be

replaced in response to inflammatory signals, by the immunosubunits LMP2, MECL-1, and LMP7, respectively, to form the immunoproteasome (i20S). In cortical thymic epithelial cells, the catalytic subunit β5t (thymus-specific) together with LMP2 and MECL-1 can assemble the thymoproteasome (t20S)[35].

1.2.3 Function of immunoproteasome and antigen presentation in shaping the immune response

Cell surface MHC class I molecules present antigenic peptides to the immune system. The generation of these peptides requires a multi-step process that includes the degradation of proteins by the proteasome and further trimming by aminopeptidases into peptide fragments of an appropriate size (8-11 residues in length) for transport through the transporter associated with antigen processing (TAP) into the ER (Fig. 3)[38]. Once in the ER, the peptides bind into the groove of MHC class I molecules, which are then transported through the Golgi and finally to the plasma membrane where they present the epitopes for binding to TCR.

Although standard proteasomes are able to generate MHC class I epitopes, immunoproteasomes generate antigenic peptides with improved binding capacity using alternative cleavage sites. This leads to the production of a set of peptides qualitatively more prone in adapting at the TAP-dependent transport and at the MHC class I requirements for stable binding[37,39]. Immunoproteasomes not only have a role in generating MHC class I ligands that are more efficient for cytotoxic T cell (also known as cytotoxic T lymphocyte, CTL, or activated $CD8^+$ T cell) stimulation, but can shape the immune response also by influencing T cell differentiation[41].

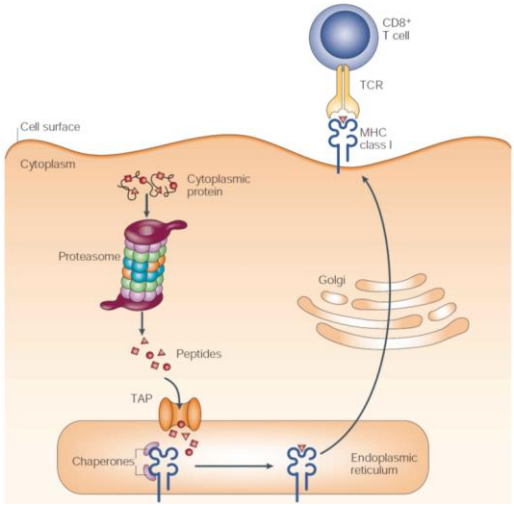

Figure 3 – MHC class I antigen presentation. Soluble proteins are degraded by the proteasome into peptides that are then transported into the ER lumen by the transporter for antigen processing (TAP). In the ER, MHC class I-peptide binding is required for stabilization of the MHC class I molecules and following release from the ER and transport to the plasma membrane. Once at the plasma membrane, the complex MHC class I-peptide can bind the T-cell receptor of $CD8^+$ T cells and eventually induce proper immune response[38].

In fact, immunoproteasomes have an important role in peptide presentation in the thymus for shaping the TCR repertoire. As previously mentioned, the thymoproteasome (an immunoproteasome-like type of proteasome with a special β5t subunit) has a function in the positive selection of T cells that takes place in the cortex of the thymus. T cells are at first positively selected for the ability to recognize self-MHC class I molecules[37]. Such selection is based on a weak interaction between TCR and MHC molecules that bind self-peptides. In this

context, β5t-containing proteasomes inefficiently produce peptides with hydrophobic C-termini, leading to a weak binding to MHC class I molecules and therefore a faster TCR off-rate necessary for T cell survival and commitment to either $CD4^+$ or $CD8^+$ T cell lineage[41,42]. On the other hand, the immunoproteasome expressed in the medullary thymic epithelial cells (mTECs) is responsible for the production of self-peptides that bind strongly to MHC class I molecules allowing the negative selection, thanks to which T cells that recognize autoantigens through a strong TCR-MHC binding are eliminated[37,41]. Once selected, the naïve $CD8^+$ T cells migrate to lymph nodes where they wait to be activated by antigen presenting cells (APCs). Professional APCs include dendritic cells and macrophages and are essential for presentation of viral or bacterial antigens to naïve T cells and for induction of an effective adaptive immune response. APCs mainly expresses immunoproteasomes and are able to engulf infected apoptotic or necrotic cells at the site of infection[37]. After processing foreign proteins via the immunoproteasome, APCs will then mature, present on the surface viral or bacterial antigens bound to MHC class I molecules and travel to the draining lymph node. Here, the APCs will activate $CD8^+$ T cells, through the binding MHC-TCR and with the help of co-stimulatory molecules. Once activated in CTLs, the $CD8^+$ T cells will clonally expand and move back to the site of infection where, once they recognize the foreign antigen that evoked their activation, they will exert a specific cytotoxic immune response. To this purpose, infected cells upregulate the immunoproteasome via IFN-γ at the site of infection in order to stimulate proteolysis and increase the chance of presenting the proper foreign antigen needed for the binding with a specific CTL that will start an immune response aimed at killing the infected cells and at limiting pathogen replication and diffusion[37,39,43]. Immunoproteasome therefore are extremely important in shaping the immune response first by contributing to the process of negative T cell selection, and secondly by mounting an adaptive response upon viral or bacterial infection.

1.3 Tobacco smoke is the main risk factor for COPD

Worldwide, the most commonly encountered risk factor for COPD development remains tobacco smoking (which counts for about 37% of the global burden)[44], although occupational, outdoor and indoor air pollution are more and more becoming other important risk factors[8]. Despite the continuous efforts made to reduce cigarette smoke exposure, more than one billion people continue to smoke. About 50% of them will develop COPD, and 1-5% will develop a smoking-related malignancy (i.e. lung cancer)[10]. Furthermore, cigarette smoke increases the risk for respiratory infections and stimulates and worsens other lung diseases (i.e. asthma) in individuals who are exposed to second-hand smoke[10].

1.3.1 Effects of tobacco smoke on protein homeostasis

Proteostasis can be significantly challenged in response to pathogens encounter and to environmental stressors such as smoking[26,45]. Cigarette smoke can alter protein homeostasis at different levels, including protein synthesis, folding, function, aggregation and degradation. Lung cell proteostasis has to face constantly the exposure of the respiratory epithelium to the noxious particles of cigarette smoke. The excessive amounts of reactive oxygen species, carcinogens and free radicals present in cigarette smoke are known to trigger oxidant stress in lung cells. Repetitive oxidative stress challenge on cellular proteostasis might cause modification and aggregation of proteins that cannot be degraded by the UPS[46–48]. At the same time, protein aggregates might overwhelm and dysregulate the autophagy-lysosomal degradation pathway[49], thereby inducing ER stress. At least two studies have identified accumulation of insoluble ubiquitinated proteins *in vivo* in COPD lung tissue and mice lungs exposed to cigarette smoke[18,50]. Moreover, two *in vitro* studies from our group and Somborac-Bacura *et al.* observed that exposure of A549 alveolar lung epithelial cells to the extract or to the gas phase of cigarette smoke, respectively, caused an accumulation of polyubiquitinated proteins both in the soluble and insoluble cellular fractions[50,51]. Similar findings have been demonstrated in alveolar macrophages exposed to cigarette smoke extract or in alveolar macrophages isolated from smokers[52] and *in vivo* in the lungs of cigarette smoke-exposed mice[50]. These results suggest a decrease in the cellular protein degradation capacity as a possible cause of the accumulation of soluble and insoluble aggregates of ubiquitinated proteins. Indeed, studies from our group and others showed a decreased proteasome function in alveolar lung epithelial cells upon exposure to cigarette smoke extract[50,51]. Similar results were observed also in bronchial epithelial cells and *in vivo* in mice lungs exposed to cigarette smoke[50]. Yamada and colleagues identified a possible role of the decreased proteasomal activity in the pathogenesis of COPD. In their mouse model with reduced proteasomal CT-L activity, they observed accelerated development of cigarette smoke-induced pulmonary emphysema[53]. It has been suggested that inhibition of the proteasome and the accumulation of misfolded ubiquitinated proteins upon exposure to CS is directly linked to the formation of protein aggregates in cultured airway epithelial cells and to the accumulation of autophagosomes in smokers' alveolar macrophages[47,52]. These studies show that cigarette smoke affects not only the UPS, but also the ALP. Cigarette smoke also has been associated with increased cytosolic free calcium, suggesting the possibility that depletion of ER calcium may contribute to the development of ER stress[28].

When misfolded proteins accumulate inside the ER, the UPR activates a series of transcriptional and translational events, whose final purpose is i) to lower the rate of protein synthesis and enhance folding capacity ii) to promote disposal of irreversibly misfolded proteins iii) to trigger cell apoptosis if ER stress cannot be reversed[28,31]. In this respect, it has been proposed that cigarette smoke not only raises the load of misfolded proteins in the ER, but concomitantly may affect the ER folding and protein degradation capacity by directly targeting pathways and proteins involved in protein homeostasis, thereby triggering a UPR. Kenche and colleagues showed that a variety

of cigarette smoke components modifies and thereby affects the enzymatic activity of protein disulfide isomerase, an essential ER chaperone needed for proper protein folding[54]. Additional studies in alveolar epithelial cells, bronchial epithelial cells, and lung fibroblasts showed that cigarette smoke exposure induces the activation of the UPR-dependent PERK signaling pathway, and the overexpression of several UPR proteins (such us the regulators BiP and eIF2α, the chaperone GRP78, and the transcription factors ATF4 and Nrf2)[55–57]. Similar results were obtained analyzing human lung from smokers, where the upregulation of the chaperones GRP78, calnexin, calreticulin, and PDI has been observed[30,55]. For all these emerging evidence, an imbalanced protein homeostasis is hypothesized to contribute to the development of COPD. In this perspective and according to Bouchecareilh and colleagues, challenges to the protein folding are initiating events for the onset of COPD (Fig. 4)[45]. At first, ROS from cigarette smoke cause damage to cytosolic and ER-resident proteins, activating the UPS, the ALP, and ultimately the unfolding-protein response, further challenged by the declined function of both protein degradation machineries. If exposure to cigarette smoke persists over years, the chronic activation of proteostasis responses may result in oxidative inflammation, cellular death, protein aggregation and cellular dysfunction; all characteristics typical for COPD[24]. Repetitive challenges to the protein homeostasis system might lead to the decline of the proteostatic capacity and ultimately to its failure during normal aging[24,45].

Importantly, protein homeostasis, through the process of autophagy and antigen presentation, interacts closely with the immune system which also plays an important role in the onset of COPD[37,39,58].

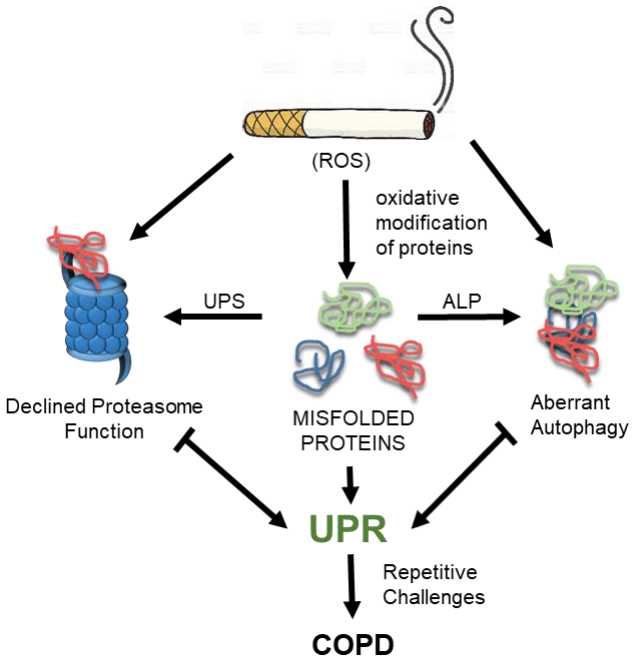

Figure 4 – Cigarette smoke affects protein homeostasis. The highly reactive compounds of cigarette smoke cause modification of proteins that have to be degraded via the UPS or via the ALP in case of formation of protein aggregates. Cigarette smoke affects directly both degradation pathways, finally leading to accumulation of misfolded proteins, ER stress and ultimately to the UPR. Repetitive challenges to the proteostatic machinery might in the end cause its failure and the onset of COPD.

1.3.2 Effects of tobacco smoke on the immune system

On the pathological level, COPD is considered a progressive immunological disorder, where an abnormal inflammatory response perpetuates even after smoking cessation, causing constant

tissue damage and impaired repair[59]. Several observations show that cigarette smoke affects both the innate and adaptive immune response. Tobacco smoke activates the innate immune system by triggering pattern recognition receptors (PRRs) (e.g.: Toll-like receptors-4 and -2) of alveolar macrophages, dendritic cells, and epithelial cells, through the release of endogenous intracellular molecules (such as proteins, DNA, ATP) from stressed or dying cells[59]. The recognition of such molecules, also called damage-associated molecular patterns (DAMPs) by PRRs induces the effector phase of innate immune responses. This consists in the release of cytokines (such as TNFα, IL-1, and -8) by epithelial cells and alveolar macrophages and in the infiltration of the mucosa, submucosa and glandular tissue by inflammatory cells (such as macrophages and neutrophils). Activated neutrophils and macrophages cause lung tissue destruction through the release of proteases and matrix metalloproteinases and the production of ROS and inflammatory mediators[59,60]. Besides neutrophils and macrophage recruitment, the presence of DAMPs and PAMPs (pathogen-associated molecular patterns released from incoming pathogens) leads to the maturation of immature dendritic cells that normally reside in the lungs. The number of cells of the adaptive immune system (namely $CD4^+$ and $CD8^+$ T cells, but also B cells) is increased in the lungs of patients with COPD[61,62], emphasizing how the adaptive immune response is involved in the airway remodeling and pathogenesis of COPD. So far the predominant cell present in COPD lungs is the $CD8^+$ T cell, which has been shown to correlate with the degree of tissue injury and disease severity[63,64]. As described by Cosio et al., the development of lung destruction in COPD might be facilitated by mechanisms of apoptosis mediated by $CD8^+$ T cells[62]. Moreover, studies in mouse models of COPD have shown that $CD8^+$ T cells accumulate in the lungs of mice chronically exposed to cigarette smoke and persists for six months after smoke cessation[65]. Mouse models have also been used to prove the importance of T cells in COPD development. Maeno and colleagues showed how $CD8^+$ T cell-deficient mice were protected from the development of emphysema upon long-term exposure of cigarette smoke[66]. Recently, it has also been demonstrated that T cells, acquired from mice exposed to cigarette smoke, have the capacity of transferring emphysematous changes to unexposed mice[67]. In this study, co-transfer of $CD8^+$ and $CD4^+$ T cells was required and the process was antigen recognition-dependent, suggesting that COPD mechanisms might be driven also by an autoimmune component. In fact, it has been proposed that the persistence of a progressive pulmonary inflammation even after smoking cessation might be the consequence of a breakdown in self-tolerance rising from the tissue injury caused by tobacco smoke[60]. Relevant to the autoimmune hypothesis in COPD is the growing evidence for a role of autoreactive T and B cells. The presence of iBALT (inducible bronchus-associated lymphoid tissue) points to an adaptive immune response against specific antigens promoting autoimmune reactions that trigger chronic inflammation[3,62,64]. iBALT is a tertiary lymphoid tissue that forms in the lung after pulmonary inflammation and consists of lymphoid aggregates with resident T cells, B cells, and dendritic cells[68,69]. The formation of iBALT is associated with persistent exposure to antigens which causes chronic inflammation noted even years after smoking cessation[64,68,70]. Such antigens can arise both from direct damage of the epithelium, from extracellular matrix degradation products, and

from modified proteins upon exposure to cigarette smoke[3,59]. So far, several autoantibodies have been identified in COPD patients and in mouse models, including antibodies directed against elastin, epithelial cell antigens and carbonylated proteins, possibly modified upon cigarette smoke exposure[61–75]. Moreover, the absence of viral and bacterial products in the lymphoid aggregates suggests that the priming and clonal expansion of T and B cells is triggered by lung antigens[62]. Nonetheless, viral and bacterial infections bring a strong contribution to the development of COPD. Such infections, in fact, not only are regarded as the main cause of acute exacerbations in COPD, but they are important in amplifying and perpetuating the inflammatory process[59,62].

1.4 Objectives

The main risk factor for the development of COPD is cigarette smoke, which exposes the lung to persistent oxidative stress, thereby inducing adaptive immune response, ER stress and imbalanced protein homeostasis.

Considering the above mentioned studies, it is clear that cigarette smoke has an effect on the proteome, directly through oxidative modifications of the proteins, or indirectly through inhibition of proteasome functions and alteration of the degradation machinery in general. The effects of cigarette smoke will be most pronounced in the pulmonary epithelium, the first physical and protective barrier of the lung. In this context the first objective of this thesis is to investigate how proteome complexity is affected upon exposure to cigarette smoke. We used an *in vitro* model of human alveolar cells acutely exposed to cigarette smoke and cellular fractionation coupled to label-free quantitative mass spectrometry to investigate the effect of cigarette smoke on the proteome of the lung epithelium[76].

While maintaining protein homeostasis, the proteasome is concomitantly involved in shaping adaptive immune responses through the generation of MHC class I antigens. In particular, a specialized type of proteasome, the immunoproteasome, produces specific peptides in order to improve MHC class I antigen presentation. Antigen presentation plays a pivotal role in defending the lung from viral and bacterial infections that contribute to acute exacerbations in COPD patients. The second objective of this thesis is to characterize the impact of cigarette smoke on proteasome-dependent MHC class I antigen presentation and in COPD. To this aim we used *in vitro* and *in vivo* smoke exposure models to analyze immunoproteasome-specific MHC class I antigen presentation in lung immune cells and spleen. Immunoproteasome expression and activity was also investigated *in vivo* in bronchoalveolar lavage and lungs of COPD patients and in blood-derived macrophages exposed *in vitro* to cigarette smoke extract.

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2 RESULTS: MANUSCRIPTS ORIGINATING FROM THIS THESIS

2.1 Cigarette smoke alters the secretome of lung epithelial cells

Alessandra Mossina*, Christina Lukas*, Juliane Merl-Pham, Franziska E. Uhl, Kathrin Mutze, Andrea Schamberger, Claudia Staab-Weijnitz, Jie Jia, Ali Ö. Yildirim, Melanie Königshoff, Stefanie M. Hauck, Oliver Eickelberg and Silke Meiners

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RESEARCH ARTICLE

Cigarette smoke alters the secretome of lung epithelial cells

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Cigarette smoke is the most relevant risk factor for the development of lung cancer and chronic obstructive pulmonary disease. Many of its more than 4500 chemicals are highly reactive, thereby altering protein structure and function. Here, we used subcellular fractionation coupled to label-free quantitative MS to globally assess alterations in the proteome of different compartments of lung epithelial cells upon exposure to cigarette smoke extract. Proteomic profiling of the human alveolar derived cell line A549 revealed the most pronounced changes within the cellular secretome with preferential downregulation of proteins involved in wound healing and extracellular matrix organization. In particular, secretion of secreted protein acidic and rich in cysteine, a matricellular protein that functions in tissue response to injury, was consistently diminished by cigarette smoke extract in various pulmonary epithelial cell lines and primary cells of human and mouse origin as well as in mouse ex vivo lung tissue cultures. Our study reveals a previously unrecognized acute response of lung epithelial cells to cigarette smoke that includes altered secretion of proteins involved in extracellular matrix organization and wound healing. This may contribute to sustained alterations in tissue remodeling as observed in lung cancer and chronic obstructive pulmonary disease.

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Abbreviations: **COPD**, chronic obstructive pulmonary disease; **CSE**, cigarette smoke extract; **ECM**, extracellular matrix; **HBEC**, human bronchial epithelial cell; **MMP2**, matrix metalloproteinase-2; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PGRN**, progranulin; **SPARC**, secreted protein acidic and rich in cysteine; **TGF**, transforming growth factor; **TIMP**, tissue inhibitor of metalloproteinases

1 Introduction

According to the World Health Organization (2015), around 6 million deaths are attributable annually to tobacco-related diseases [1]. Tobacco smoking is the most relevant risk factor for a variety of lung diseases, including lung cancer and chronic obstructive pulmonary disease (COPD). Cigarette smoke contains more than 4500 chemicals, many of which are free radicals that act strongly oxidizing, pro-inflammatory, and carcinogenic [2, 3]. These effects are most pronounced

*These authors contributed equally to this work.

Significance of the study

According to the World Health Organization, around 6 million deaths/year are attributable to tobacco-related diseases among them lung cancer and chronic obstructive pulmonary disease. More than 4500 reactive compounds are found in cigarette smoke, which potentially alter protein structure, abundance, and function. Here, we analyzed the proteomic changes induced by cigarette smoke in different compartments of lung epithelial cells. Subcellular fractionation coupled to label-free quantitative MS revealed that the most pronounced changes in lung alveolar cells are observed within the cellular secretome. In particular, we identified differential regulation of several proteins involved in wound-healing responses and extracellular matrix organization, among them secreted protein acidic and rich in cysteine, a matricellular protein that functions in tissue response to injury. A similar but distinct response was observed in bronchial epithelial cells. This acute response of lung epithelial cells to cigarette smoke may contribute to the sustained alterations in tissue remodeling as observed in lung cancer and chronic obstructive pulmonary disease.

in the pulmonary epithelium, the first barrier of the lung. Cigarette smoke easily interacts with DNA, lipids, and proteins and modifies them. In particular, cigarette smoke affects expression and/or posttranslational modifications of proteins, thereby altering their structure, abundance, and function [3, 4].

Several proteomic studies have been applied to human lung tissues and cells in order to identify and quantify protein alterations caused by exposure to cigarette smoke [3]. Most of these studies involved comparative 2D gel electrophoresis coupled with MS. Some studies analyzed sputum, bronchoalveolar lavage or epithelial lining fluids of nonsmokers, healthy smokers, and COPD subjects identifying alterations in mucin proteins and peptidase regulators [5], differential regulation of proteins involved in tumor growth and invasion [6], or in oxidative and inflammatory responses [5–9]. Proteomic analysis of lung tissue from nonsmokers, current smokers, and ex-smokers revealed cigarette smoke mediated induction of an unfolded protein response [10]. Although several proteomic studies have been conducted on pulmonary human fibroblasts exposed to cigarette smoke extract (CSE) [11] and on bronchial airway epithelium from current and never smokers [12], little is known on the effect of cigarette smoke on alveolar epithelial cells.

In this study, we investigated the effects of cigarette smoke on the proteome of A549 human alveolar epithelial cells by performing cellular fractionation coupled with label-free quantitative MS (i.e. LC-MS/MS). Subcellular fractionation enabled detection of proteins of low abundance, thus allowing for improved sensitivity and identification of proteins that would otherwise be difficult to quantify in a complex cellular extract. Moreover, cellular fractionation also provides unique data on compartment-specific alterations of protein expression. This information can be particularly valuable as it allows identifying proteins that may shuttle between one subcellular compartment and the other upon exposure of cells to cigarette smoke.

2 Materials and methods

2.1 Cell culture

A549 (human adenocarcinoma cell line) and MLE12 (SV40-immortalized mouse alveolar cell line) cell lines were obtained from ATCC (Manassas, USA). A549 were maintained in DMEM (Life Technologies, Carlsbad, USA), MLE12 in RPMI (Life Technologies), and 16HBE14o$^-$ human bronchial epithelial cells (HBECs) were cultivated in minimum essential medium (MEM). Media was supplemented with 10% FBS (PAA Laboratories, Cölbe, Germany) and 100 U/mL penicillin/streptomycin (Life Technologies). Primary HBECs and isolated mouse airways were obtained and cultivated as described previously [13, 14]. For cell fractionation and MS analysis, phenol red free media without FBS was used. Primary mouse alveolar type II cells were isolated from C57BL6/N mice (Charles River Laboratories, Sulzfeld, Germany) as previously described [15]. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were repeated several times to obtain independent biological replicates.

2.2 Preparation of CSE

Stocks of CSE were prepared by bubbling smoke from six 3R4F research-grade cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) through 100 mL of phenol-red free cell culture media as described (see Supporting Information, [16]).

2.3 Preparation of three-dimensional ex vivo lung tissue cultures and exposure to CSE

Three-dimensional ex vivo lung tissue cultures were prepared from mouse lung tissue as published [17] and detailed in the Supporting Information.

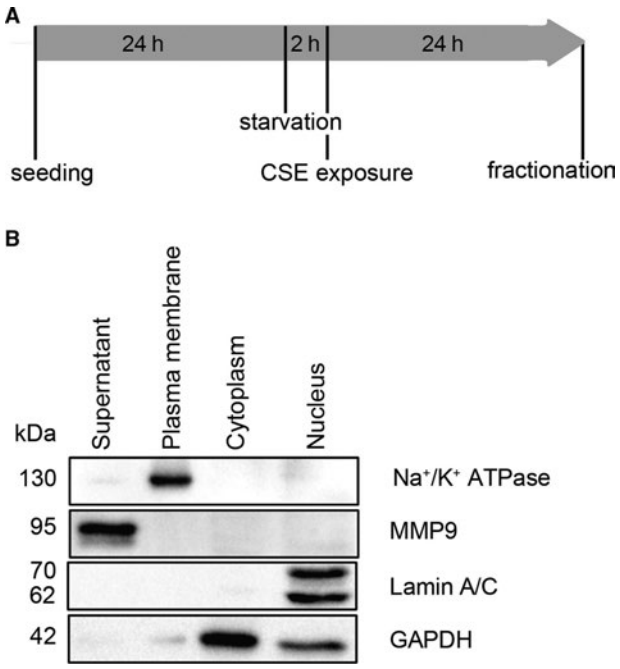

Figure 1. Subcellular fractionation of A549 lung epithelial cells exposed to cigarette smoke extract. (A) Experimental setup. (B) Western blot analysis of cell compartment specific proteins.

2.4 Cytotoxicity and proliferation assays

Cytotoxicity of CSE was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16] and proliferation using the BrdU assay (both Roche Diagnostics, Mannheim, Germany) [18].

2.5 Cell fractionation

Triplicates for each sample of A549, 16HBE14o$^-$, and MLE12 cells were exposed to 50, 25, or 10% CSE, respectively, for 24 h or to normal media. For each fraction, we used 2–4 million cells 24 h after seeding, which were prestarved for 2 h. Cells were exposed for additional 24 h to serum-free CSE-containing (treated) or normal media (control) (Fig. 1A). Fractionation was carried out as previously described [19]. Briefly, cell supernatants were collected, filtered, and concentrated with Vivaspin 6 concentrators (Sartorius, Göttingen, Germany). Cells were washed and surface proteins were labeled with biotin. After lysis, cells were scraped and cytosolic and nuclear proteins were separated through differential centrifugation while surface proteins were pulled down with streptavidin beads.

2.6 Mass spectrometry

Protein concentration of the different fractions was measured with Pierce BCA protein assay (Thermo Fisher Scientific, Schwerte, Germany). Secreted, nuclear, and cytoplasmic proteins were digested with Lys-C and trypsin with a filter-aided sample preparation procedure as previously described [19, 20] and subjected to proteomic analysis. Biotinylated and affinity-enriched surface proteins were digested with trypsin and PNGaseF directly on the streptavidin beads. LC-MS/MS analysis was performed on an Ultimate 3000 nano-RSLC coupled to a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific) as described [21, 22]. The acquired spectra were loaded to Progenesis LC-MS software (version 2.5; Nonlinear Dynamics) for label-free quantification and analyzed as published [21, 22]. MS/MS spectra were used for identification with Mascot (version 2.3; Matrixscience) as previously described [19] using organism-specific proteins databases from Ensembl (Ensembl mouse, release 75, 51 765 sequences; Ensembl human, release 69, 96 556 sequences). Search parameters used were: 10 ppm peptide mass tolerance and 0.6 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation was set as fixed modification, methionine oxidation, and asparagine or glutamine deamidation were allowed as variable modifications. A Mascot-integrated decoy database search calculated an average false discovery of $<1\%$ when searches were performed with a mascot percolator score cut-off of 15 and an appropriate significance threshold p. After inverse hyperbolic transformation (arcsinh function), normalized abundances of proteins were used for statistical analysis by Student's t-test in order to identify proteins, which were significantly altered after CSE treatment ($p < 0.05$).

2.7 GO cellular component enrichment analysis

The list of proteins identified in each fraction was subjected to GO enrichment analysis using STRAP software [23].

2.8 Network analysis

Fifty-five significantly different and at least twofold altered proteins after CSE treatment were fed into the Genomatix GePS software (http://www.genomatix.de/index.html). The interaction filter was set to "validated regulatory level" and the generated network was extended with five proteins with most frequent co-citation (gray).

2.9 Antibodies, Western blot, and quantitative real-time RT-PCR

Details are provided in the Supporting Information.

2.10 Statistical analysis

Details on the statistical analyses used are given in the respective figure legends. Statistical analysis was performed using the GraphPad Prism software (version 5.00; GraphPad Software, LaJolla, CA, USA).

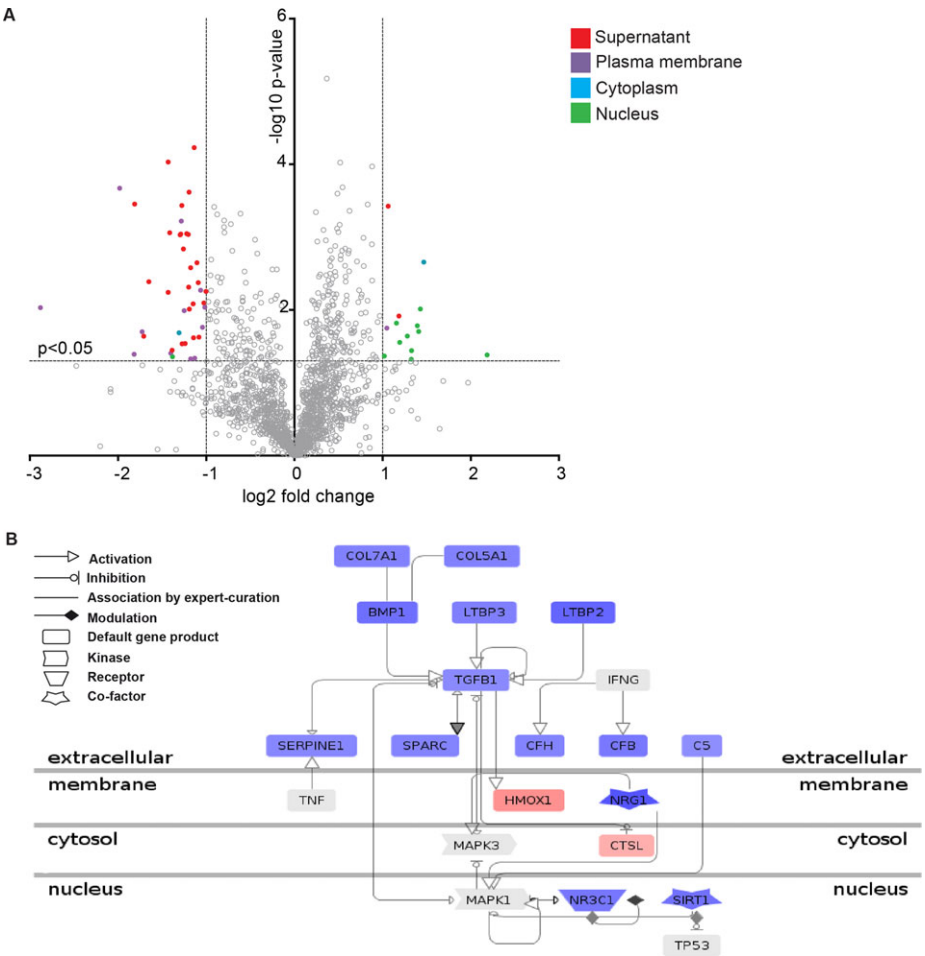

Figure 2. Cigarette smoke extract alters the cellular secretome. (A) Volcano plot showing the A549 proteome exposed to 50% CSE for 24 h. Colored dots indicate proteins that were significantly ($p < 0.05$) altered by at least twofold. Gray dots represent proteins whose expression was not significantly altered and/or was less than twofold regulated compared to control, $n = 3$. (B) Genomatix GePS network analysis of CSE-regulated proteins: proteins downregulated (blue), proteins upregulated (red), proteins used to extend the network according to frequent co-citation (gray). Unconnected proteins were discarded.

3 Results

3.1 Fractionation of the A549 proteome allowed identification of more than 2500 proteins

To investigate the effect of CSE on protein expression, A549 cells were exposed for 24 h to serum-free media containing 50% CSE (Fig. 1A). While metabolic activity and proliferation were reduced, morphology of the cells was not grossly altered indicating that this dose was well tolerated by A549 cells (Supporting Information Fig. E1). To obtain large coverage of the A549 proteome, we performed subcellular fractionation prior to MS analysis into four main compartments: cellular supernatant, plasma membrane, cytoplasm, and nucleus. Efficient enrichment of compartment-specific proteins was confirmed by Western blot analysis of cell compartment specific markers such as the integral membrane protein Na^+/K^+ ATPase, the secretory matrix metalloproteinase-9, nuclear lamin A/C, and cytoplasmic glyceraldehyde 3-phosphate dehydrogenase (Fig. 1B). For each fraction, we performed LC-MS/MS analysis and subsequent label-free quantification resulting in the identification of 2715 proteins (Supporting Information Table E1). GO cellular component enrichment analysis revealed that each fraction was clearly enriched for proteins of the respective cellular compartment.

3.2 CSE predominantly alters protein expression in the secretome of A549 cells

We next identified proteins that were differentially regulated by CSE. For that, we considered only proteins that were unambiguously identified by at least two unique peptides (1839 proteins, Supporting Information Table E1). Fifty-five of them were significantly ($p < 0.05$) regulated by CSE with a minimum of twofold change compared to controls (Fig. 2A and Supporting Information Table E2), 41 of these were downregulated. Remarkably, the majority of CSE-regulated proteins (29 of 55) belonged to the cellular secretome (Fig. 2A, red dots). Most of these proteins are involved in the organization of the ECM such as fibrillin and collagens, proteins of the transforming growth factor β (TGF-β) superfamily (BMP1, LTBP2, LTBP3, and TGFB1), the ECM glycoproteins EGF containing fibulin-like extracellular matrix protein 1, and members of the secreted protein acidic and rich in cysteine (SPARC) family (SPARC and SPOCK1). Subsequent network

analysis using the Genomatix Pathway System Software identified concerted downregulation of proteins involved in TGF-β signaling, wound-healing responses, as well as interferon-γ and tumor necrosis factor responsive proteins (Fig. 2B). We did not observe any significant changes in the subcellular distribution of these proteins in response to CSE exposure. These data strongly indicate that nontoxic smoke exposure of lung epithelial cells to CSE has an acute inhibitory effect on the cellular secretome with reduced collagen expression and impaired wound-healing responses.

3.3 CSE downregulates proteins involved in wound healing and ECM organization in alveolar epithelial cells

As A549 cells are human alveolar adenocarcinoma cells, we next confirmed CSE-mediated regulation of the secretome using a noncarcinoma lung alveolar epithelial cell line. For that we analyzed the secretome of mouse alveolar epithelial MLE12 cells by LC-MS/MS after exposure to CSE for 24 h. CSE doses were adjusted according to the increased sensitivity of these cells to CSE as determined by MTT assay (Supporting Information Fig. E2) [15]. We identified and quantified the abundance of more than 100 proteins that were differentially regulated upon CSE exposure (Supporting Information Table E4). Importantly, we again observed predominant downregulation of proteins involved in ECM organization and wound-healing responses such as bone morphogenetic proteins, latent TGF-β binding proteins, and SPARC; thus, confirming our data obtained with A549 cells.

Several of the ECM organizing proteins that were downregulated in both A549 and MLE12 cells were found to be also regulated on the mRNA level in MLE12 alveolar cells: mRNA expression of BMP1, progranulin (PGRN), and SPARC were dose-dependently reduced with 10 and 25% CSE, while LTBP3 was not downregulated on the mRNA level (Fig. 3A). In addition, we observed concerted downregulation of several other well-known ECM molecules and organizing cytokines, i.e. fibronectin and TGF-β2 and 3, while collagen Iα1 and collagen IVα1 as well as TGF-β1 were not altered (Fig. 3A). Downregulation of fibronectin was confirmed on the protein level by 1.6-fold in our proteomics data of MLE12 cells.

For further validation of our proteomic data, we chose two newly identified CSE-responsive target proteins: PGRN, which functions as a wound-healing mediator in tissue regeneration [24, 25], and SPARC, a major ECM organizing protein [26, 27]. In our proteomic profiling, CSE exposure reduced PGRN expression by fourfold in the secretome of MLE12 cells (Supporting Information Table E4). Using a specific ELISA for PGRN, we confirmed dose-dependent downregulation of secreted PGRN in cellular supernatants of MLE12 cells by CSE (Fig. 3B). Furthermore, the matricellular protein SPARC was downregulated both in A549 and MLE12 proteomic profiling by about twofold (Supporting Information Tables E2 and E4). Western blot analysis confirmed reduction of SPARC protein levels in supernatants of MLE12 cells by about 65% with 25% CSE (Fig. 3C). Of note, downregulation of SPARC and also fibronectin was even more pronounced when fresh medium was added to the MLE12 cells for recovery (Fig. 4A and B), indicating that the observed transcriptional regulation of wound-healing mediators effectively relays the acute effects of CSE to sustained ECM matrix regulation.

We further confirmed reduced secretion of wound-healing mediators by CSE in supernatants of primary mouse alveolar type II cells: PGRN ELISA and Western blot analysis for SPARC and fibronectin revealed significant downregulation of these mediators also in primary alveolar epithelial cells (Fig. 4C and Supporting Information Fig. E2 for MTT assay). These data clearly indicate that acute exposure to cigarette

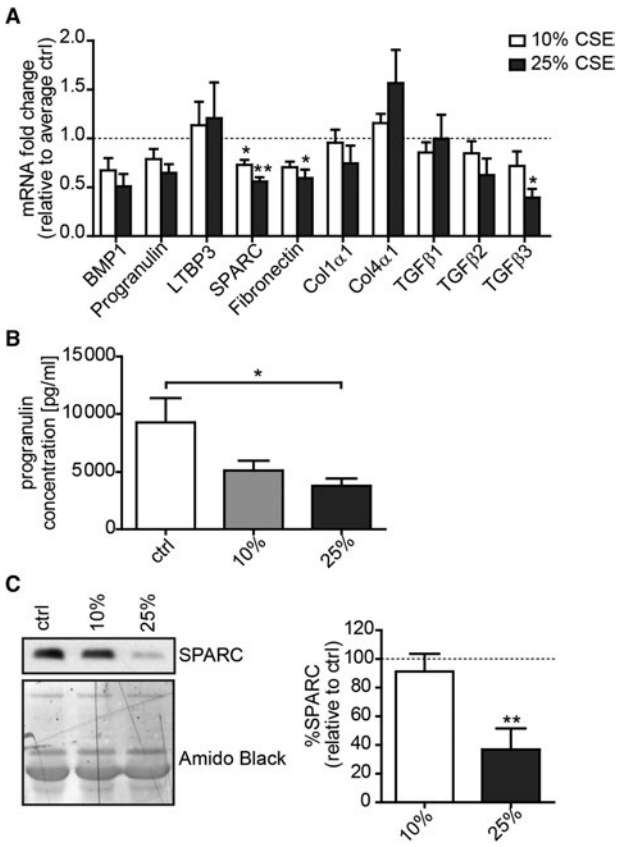

Figure 3. Validation of the dose-dependent effects of CSE on selected ECM organizers and wound-healing mediators. (A) Dose-dependent effects of CSE (10 or 25%) on mRNA expression in MLE12 cells after 24 h, normalized to housekeeper and controls (ctrl), $n = 3$, mean ± SEM. (B) ELISA-based detection of secreted progranulin in supernatants of MLE12 cells exposed to 10 or 25% CSE for 24 h, $n = 4$, mean ± SEM. (C) Western blot analysis and quantification of SPARC in MLE12 supernatants after exposure to 10 or 25% CSE for 24 h ($n = 4$); mean ± SEM. Amidoblack staining confirmed equal protein loading of cell culture supernatants. One-way ANOVA with Dunnett's multiple comparison test was used.

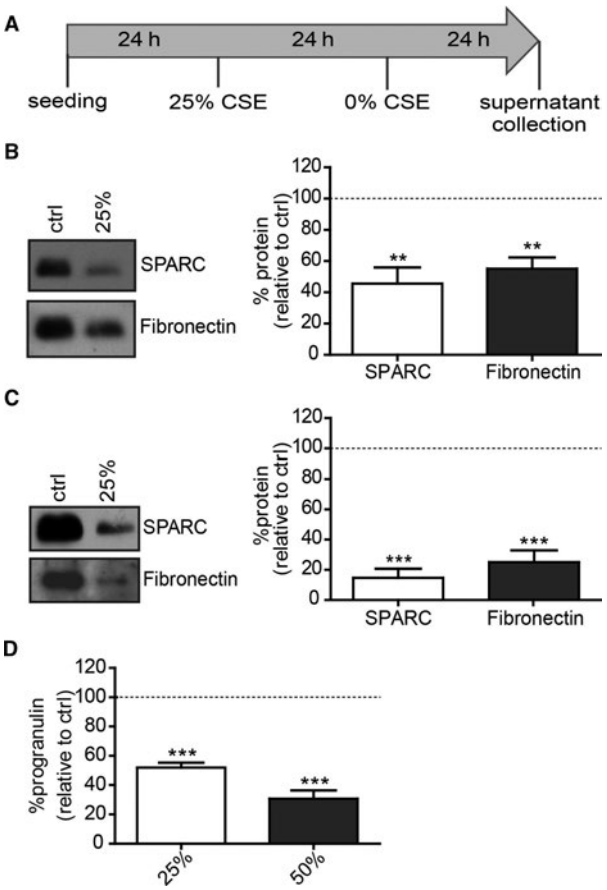

Figure 4. CSE-mediated downregulation of SPARC, fibronectin, and progranulin in primary mouse ATII cells. (A) Scheme of MLE12 recovery experiment. (B) Western blot analysis and quantification of SPARC and fibronectin after recovery ($n = 3$); mean ± SEM. (C) Representative Western blots and relative to control (ctrl) quantification of SPARC and fibronectin protein expression in primary mouse alveolar type II cells (pmATII) supernatants after 24-h exposure to 25% CSE; $n = 3$, mean ± SEM. (D) ELISA-based detection of progranulin in supernatants of pmATII exposed to 25% CSE for 24 h, $n = 3$, mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was used.

smoke causes an altered alveolar epithelial response that involves ECM remodeling and tissue injury mechanisms.

3.4 Acute CSE exposure alters expression of distinct ECM organizers in bronchial epithelial cells

We also performed CSE exposure of a HBEC line, i.e. the 16HBE14o$^-$ cells, and subsequent LC-MS/MS analysis of supernatants to investigate whether CSE induces a similar response in bronchial epithelial cells. Twenty-four hours exposure of 16HBE14o$^-$ cells to nontoxic doses of 10% CSE (Supporting Information Fig. E2) induced only minor changes in the supernatant with a total of 22 proteins more than twofold differentially regulated (Supporting Information Table E3). Among them were only two proteins with distinct functions in ECM remodeling, i.e. matrix metalloproteinase-2 (MMP2) and the tissue inhibitor of metalloproteinases (TIMP) metallopeptidase inhibitor 1. Notably, there was only a minor overlap of CSE-regulated proteins in 16HBE14o$^-$ cells with those of A549 and MLE12 cells including vascular endothelial growth factor A, some cadherin, and TIMP members. While TIMP1 and MMP2 were increased about twofold by CSE in 16HBE14o$^-$ cells, TIMP2 and MMP2 were markedly downregulated in A549 and MLE12 cells. As these data suggested a differential responsiveness of bronchial epithelial cells to CSE compared to alveolar cells, we exposed primary HBECs to nontoxic doses of CSE for 24 h to validate this assumption [13, 14]. On the RNA level, expression of several ECM organizing molecules such as SPARC, fibronectin, collagen Iα1 and IVα1, as well as TGF-β2 and PGRN was downregulated by CSE (Supporting Information Fig. E3A). Total protein levels of fibronectin were also downregulated, although not significantly, while SPARC levels were maintained as determined by Western blotting (Supporting Information Fig. E3B). These data indicate that bronchial epithelial cells also downregulate several ECM organizers in response to CSE in a similar but distinct way compared to alveolar epithelial cells.

3.5 Acute CSE exposure downregulates ECM organizers and wound-healing mediators in ex vivo lung tissues

To study the effect of cigarette smoke in a more physiologically relevant setting, we used isolated airways from mice and exposed them to 10% CSE for 24 h. RNA expression of both SPARC and fibronectin was significantly downregulated by CSE exposure (Fig. 5A). Moreover, we exposed mouse three-dimensional ex vivo lung tissue cultures to 5% CSE. Very similar to our previous results, we observed predominant downregulation of several wound-healing mediators and ECM organizing molecules on the mRNA level (Fig. 5B). In particular, SPARC, fibronectin, collagen IVα1, and TGF-β1 were downregulated. Reduced expression of SPARC was also observed on the protein level (Fig. 5C). These results clearly confirm an acute effect of CSE on matrix organizing molecules and mediators of wound healing in the pulmonary epithelium. We did not, however, detect any significant alteration in SPARC and fibronectin levels in the lungs of mice that had been smoked for 10 days possibly due to the low basal SPARC expression in alveolar epithelial cells of the lung (Supporting Information Fig. E4).

4 Discussion

In the present study, we used subcellular fractionation coupled to label-free quantitative MS to identify compartment-specific changes in the composition of the proteome of lung

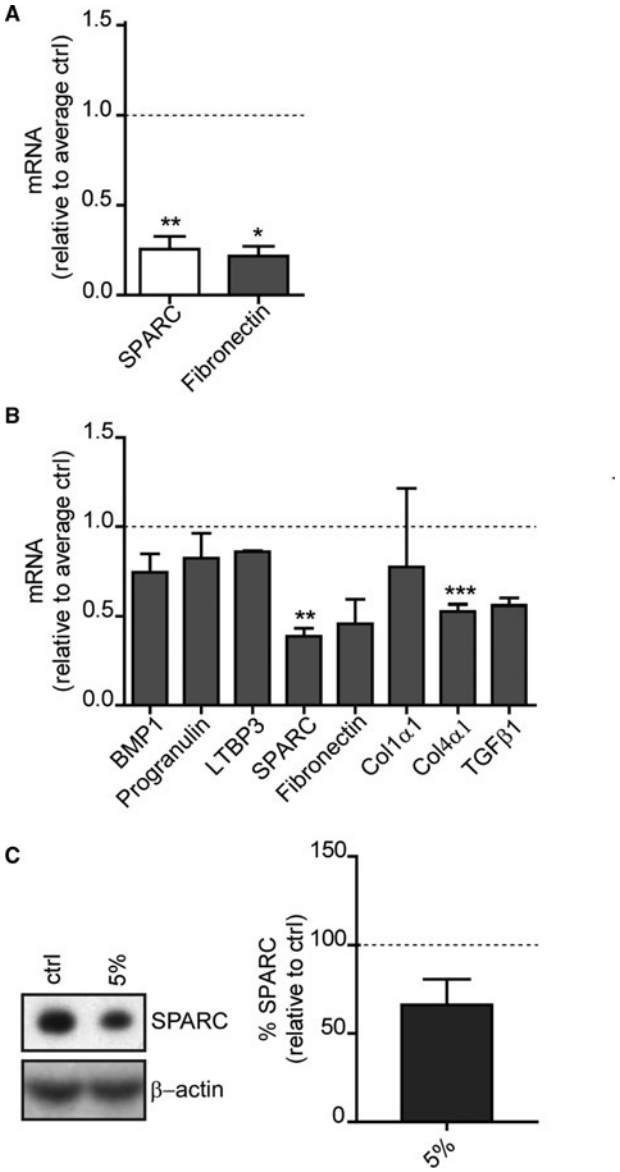

Figure 5. Regulation of ECM organizers and wound-healing mediators in ex vivo mouse lung tissue. (A) RT-qPCR analysis of isolated mouse airways treated with 10% CSE for 24 h ($n = 3$, unpaired *t*-test). (B) RT-qPCR analysis of 5% CSE-treated three-dimensional ex vivo lung tissue cultures (3D-LTC) after 24 h ($n = 3$, unpaired *t*-test). (C) Representative Western blot and relative (to control and β-actin housekeeper) quantification of SPARC in 3D-LTC after exposure to 5% CSE for 72 h with $n = 7$; mean ± SEM, unpaired *t*-test.

epithelial cells in response to acute and nontoxic CSE exposure. This approach resulted in the identification of more than 2500 proteins. Compared to other shotgun proteomic studies of lung cells or tissues, our fractionation approach thus increased total proteome coverage by at least threefold [7, 9, 10, 12]. It also allowed us to identify the secretome as the cellular compartment with the most prominent changes in protein composition: while exposure of lung epithelial cells to CSE did not influence overall subcellular distribution of proteins, we observed distinct changes in the abundance levels of secreted proteins in the supernatant fraction. Other proteomic studies that analyzed epithelial lining fluids and bronchoalveolar lavages upon cigarette smoke exposure did not detect similar changes [7–9, 28]. This might be due to the generally low abundance of these proteins and the possibility of directional secretion as suggested recently by a comprehensive proteomic study of the secretome of human bronchial cells [29]. Gene enrichment analysis of differentially expressed proteins in the secretome identified the genes involved in ECM organization as mostly regulated. All proteins in this pathway were downregulated including several mediators of the TGF-β signaling pathway, ECM proteins and regulators thereof. Concerted downregulation of TGF-β signaling in response to cigarette smoke was confirmed for the immortalized mouse alveolar epithelial MLE12 cells on the mRNA and protein level. This finding, however, is in contrast to other studies, which observed increased secretion of TGF-β in alveolar and small airways epithelial cells from smokers and COPD patients [30, 31]. Our data obtained with alveolar and bronchial epithelial cells of different origin, however, agree with previous observations that cigarette smoke can inhibit the capacity of HBECs to release TGF-β [32]. Contrasting findings might be due to the different in vitro settings used for the exposure to cigarette smoke. Importantly, our results support the hypothesis that cigarette smoke inhibits wound repair in lung epithelial cells [32, 33]. In particular, we observed smoke-mediated downregulation of PGRN, a central wound-healing mediator in tissue regeneration [24, 25], in the secretome of both MLE12 and primary mouse ATII cells. As a wound-related growth factor, PGRN promotes the granulation phase of wound healing and supports vascularization and formation of a fibronectin scaffold necessary for subsequent collagen deposition [24]. Another protein that was downregulated in lung epithelial cells in response to cigarette smoke is SPARC. SPARC is a matricellular protein that binds several resident proteins of the ECM and alters the activity of extracellular proteases and growth factors. It thereby participates in the assembly and organization of ECM and is essential for proper wound-healing responses [26, 34]. Indeed, SPARC is highly expressed during development and at sites of injury and disease where tissues undergo constant repair and remodeling [34, 35]. SPARC also alters ECM organization by reducing levels of fibronectin via outside–in signaling [36]. In our study, both SPARC and fibronectin were significantly downregulated by acute cigarette smoke exposure in vitro in alveolar and bronchial epithelial cells as well as in ex vivo lung tissue. As we did not observe any pronounced changes in SPARC expression in mice that had been exposed to cigarette smoke for 10 days, it is well feasible that the observed changes in vitro might be initially counteracted in vivo by adaptive changes. Upon chronic exposure to cigarette smoke, however, altered secretion of ECM organizers may contribute

to pathogenic lung tissue remodeling as observed in COPD patients.

Taken together, our results show that lung epithelial cells acutely respond to nontoxic doses of cigarette smoke by impaired secretion of key factors for wound healing and tissue remodeling. This acute response of the pulmonary epithelium to cigarette smoke may contribute to the detrimental tissue damage observed in COPD patients. Indeed, it has been suggested that tissue destruction in COPD patients is a consequence of the inadequate capacity of damaged cells of the lung to successfully repair lung tissue and maintain lung structure [32, 33, 37].

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The authors have declared no conflict of interest.

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2.1.1 Supplementary information

Supplementary information for the manuscript

Cigarette smoke alters the secretome of lung epithelial cells

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SUPPLEMENT METHODS

Preparation of Cigarette Smoke Extract (CSE): CSE was sterile filtered, aliquoted, and stored at -20 °C for further use. For the MLE12 cells, 6 cigarettes were smoked by bubbling smoke through 100 mL of RPMI phenol free medium (Life Technologies). Both stocks were considered as 100% CSE. For cell treatment, CSE stocks were serially diluted to the stated concentrations with full media without FBS. Control cells were maintained in a different incubator of cells exposed to CSE in order to avoid interaction with cigarette smoke volatile components.

Primary murine alveolar epithelial type II (pmATII) cell isolation and culture: pmATII cells were isolated from C57BL6/N mice (Charles River Laboratories, Sulzfeld, Germany) as previously described [1]. After cell attachment, pmATII cells were cultured in DMEM supplemented with 10% FBS (PAA Laboratories), 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies), 3.6 mg/ml glucose (Applichem, Darmstadt, Germany), and 10 mM HEPES (PAA Laboratories). Cells were cultured up to 5 days at 37 °C in a humidified atmosphere containing 5% CO2. Purity of pmATII cells was assessed as described in Chen et al., [2]. Treatment medium for pmATII cells consisted of FBS-free DMEM supplemented with 1% penicillin/streptomycin, with or without 25% CSE.

Preparation of three-dimensional ex vivo lung tissue cultures (3D-LTCs) and exposure to CSE: 3D-LTCs were prepared as previously described [3]. In short, healthy mice were anaesthetized and intubated. Lungs were flushed via the pulmonary artery and infiltrated with warm, low gelling temperature agarose. The lungs were excised and cooled on ice in cultivation medium before lobes were separated and cut with a vibratome to a thickness of 300 µm. The 3D-LTCs were cultivated in DMEM-F12 medium supplemented with 0.1% fetal calf serum (FCS), antibiotics, and antimycotics. Individual 3D-LTCs were cultivated at 37°C at humidified conditions containing 5% (volume/volume) CO2 in 24-well plates under submerged conditions with changes of medium every other day. For CSE exposure 3D-LTCs were incubated with DMEM-F12 medium supplemented with 0.1% FCS, antibiotics, antimycotics, and 5% CSE. Slices exposed to CSE were kept in a different incubator than control slices in order to avoid interaction with CSE volatile components.

GO Cellular Component enrichment analysis: The list of proteins identified in each fraction was subjected to GO enrichment analysis using STRAP software [4]. In order to better visualize fractionation efficiency, some GO Cellular Component subcategories were manually rearranged to the four main categories used for the fractionation (supernatant, plasma membrane, cytoplasm and nucleus). In particular, concerning all those proteins that were annotated under the GO category “Other” the following rules were applied: the GO category “Other" was not taken in

consideration if the protein was annotated also under another category, the protein was not considered for the enrichment analysis if “Other” was the unique annotation.

Antibodies and Western blot: Anti-collagen antibody was obtained from Rockland Immunochemicals (Limerick, PA, USA). Anti-fibronectin and anti-LaminA/C were purchased from Santa Cruz Biotechnology (Dallas, USA). Anti-MMP9 was obtained from Millipore (Billerica, USA) and anti-Na+/K+ -ATPase from Santa Cruz Biotechnology. Anti-GAPDH and anti-SPARC antibodies were purchased from Cell Signaling (Danvers, USA) and anti-β-actin from Sigma-Aldrich (St. Louis, USA).

For Western blot analysis, 4 x 106 cells were seeded per well in 10 cm cell culture plates. 24h after seeding, cells were treated with different concentration of CSE. After 24h, cell supernatants were collected, filtered, and concentrated with Vivaspin 6 concentrators (Sartorius). Finally, the concentrated supernatant was supplemented with protease inhibitor cocktail (CompleteTM, Roche) and protein content was determined using the Pierce BCA protein assay kit (Thermo Scientific).

For Western blot analysis of murine lungs, frozen whole lung tissue was homogenized using the Micro-Dismembrator (Sartorius) and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonident P-40, 0.5% sodium deoxicholate, 0.1% SDS), supplemented with protease inhibitor cocktail. Protein content was then measured using the Pierce BCA protein assay kit (Thermo Scientific).

Western blot analysis was performed as previously described [5], using 10% SDS gels and antibody dilutions according to manufacturer´s instructions.

Quantitative real-time RT-PCR: Total RNA isolation, reverse transcription and quantitative PCR were performed as previously described [6]. The following gene-specific primers were used: forward PGRN: 5´-TCCTGCTTCCAGATGTCAGA-3´, reverse PGRN: 5´-CATCGTGTGTGAACCAGGTC-3´, forward SPARC: 5´- AAACATGGCAAGGTGTGTGA-3´, reverse SPARC: 5´-AAGTGGCAGGAAGAGTCGAA-3´, forward FN: 5´-GTGTAGCACAACTTCCAATTACGAA-3´, reverse FN: 5´- GGAATTTCCGCCTCGAGTCT-3´.

Mice smoke exposure experiments: For smoke exposure experiments, BALB/cAnNCrl females were used (Charles Rivers Laboratories, Sulzfeld, Germany). Mice were smoked for 50 minutes once daily for 10 consecutive days. The smoke of 10 3R4F research-grade cigarettes (Tobacco and Health Research Institute, University of Kentucky) without filter was drawn into the exposure chamber using a peristaltic pump for each 50 min exposure cycle. Immediately after the last smoke exposure, mice were euthanized and samples were prepared. For immunohistochemistry, non-lavaged lungs were fixed with 4 % paraformaldehyde and embedded in paraffin. All animal procedures were conducted according to international guidelines and with approval of the Bavarian Animal Research Authority in Germany.

Immunohistochemistry: Immunohistochemistry was performed as previously described [7]. SPARC specific antibody (Cell Signaling) was used according to manufacturer´s instructions.

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SUPPLEMENT FIGURES

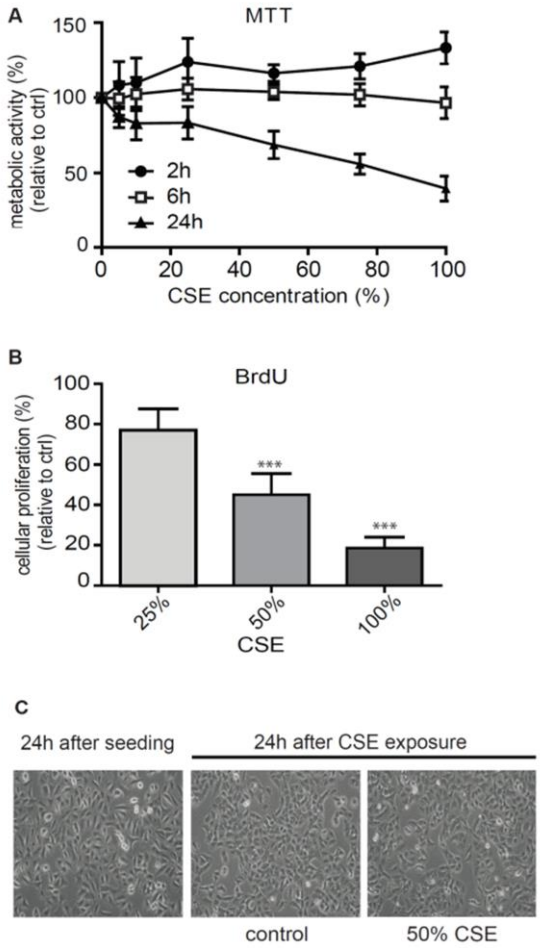

FIGURE E1 – Exposure of A549 lung epithelial cells to cigarette smoke extract. Metabolic activity of A549 cells exposed to different concentrations of CSE at different time points (n=3); mean ± SEM, two-way ANOVA and Bonferroni post-test (A). BrdU cell proliferation activity of A549 cells exposed to CSE for 24 h (n=4); mean ± SEM, one-way ANOVA and Dunnett´s post-test (B) Light microscopy pictures of A549 cells before and after 24h exposure to 50% CSE (C).

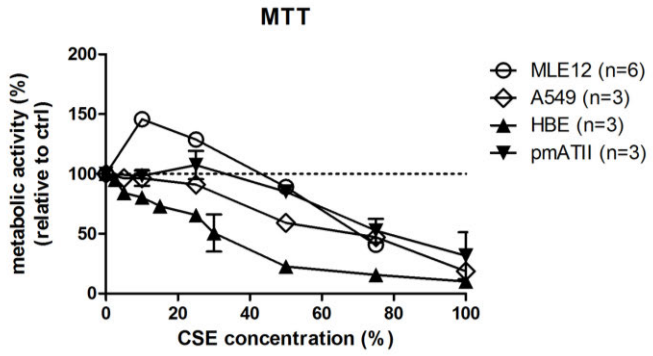

FIGURE E2 – Comparative analysis of the dose dependent effects of cigarette smoke extract on metabolic activity of A549, MLE12, pmATII and 16HBE14o^{-} cells. Cells were exposed to varying doses of CSE for 24 h with n=4 for A549, n=6 for ML12, n=3 for primary murine ATII cells (pmATII) and n=3 for 16HBE14o- cells and metabolic activity was assessed as a measure of cell viability.

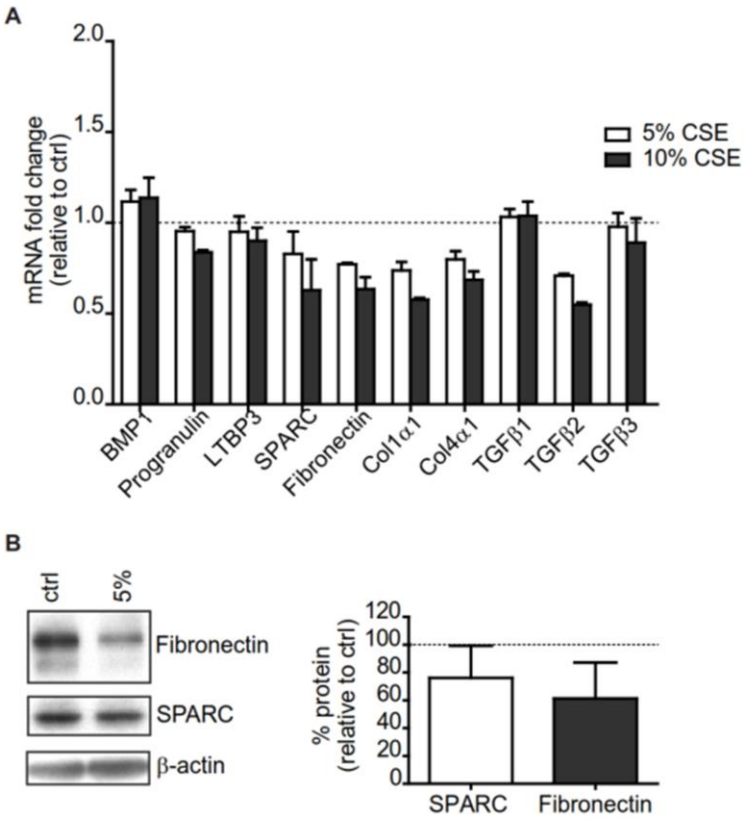

FIGURE E3 – Regulation of ECM organizers in primary human bronchial epithelial cells. (A) RT-qPCR analysis of HBECs treated with 5 or 10% of CSE for 24h with n=2. (B) Representative Western blot and relative (to control and β-actin housekeeper) quantification of SPARC and fibronectin expression in HBECs after exposure to 5% CSE for 24h with n=3; mean ± SEM, unpaired t-test.

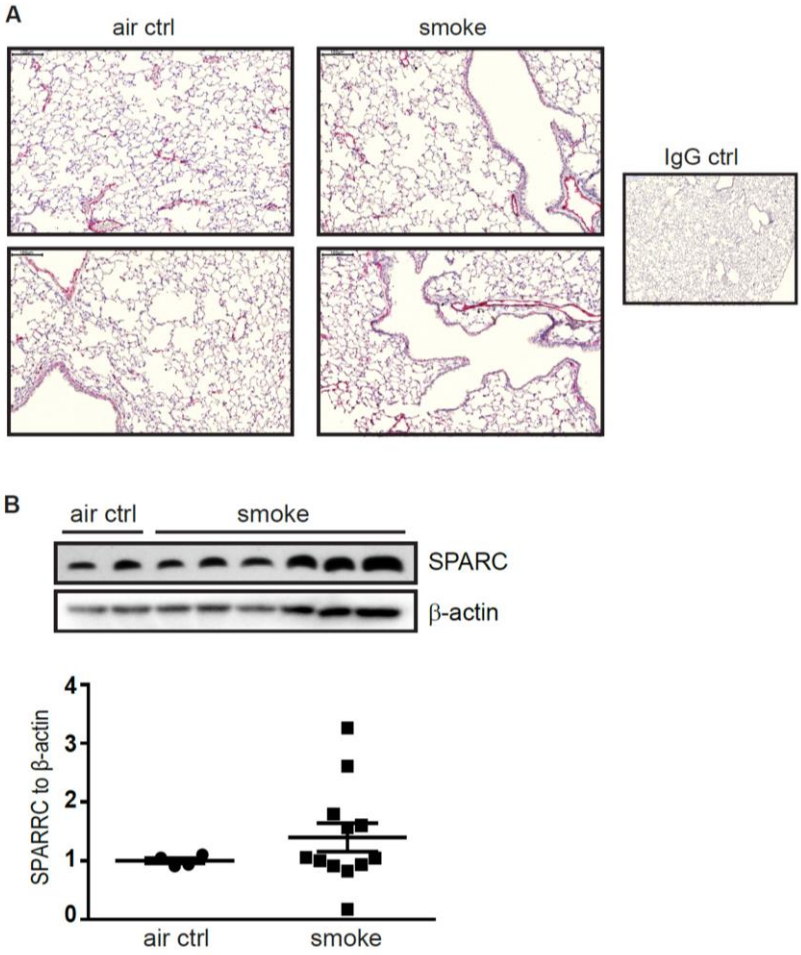

FIGURE E4 – Exposure of mice to cigarette smoke. Female mice were exposed to cigarette smoke for 10 days. Upon harvesting, lungs were perfused, fixed and paraffin-embedded for immunohistological analysis of SPARC expression (A). Pictures show two representative stainings for SPARC in lung sections of mice exposed to air (air ctrl) or cigarette smoke. An IgG antibody was used as a negative control (IgG ctrl) to control for specific staining of SPARC. (B) Western blot analysis of lung homogenates of air control (air ctrl) (n=4) and smoke exposed mice (n=12) for SPARC. Pictures show representative blots with relative quantification of SPARC protein expression normalized to expression of the housekeeping gene β-actin; mean ± SEM, *t*-test.

2.1.2 Author's contribution

Alessandra Mossina	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. 1A, 4A, E1A/C, E2); A549 subcellular fractionation and mass spectrometry data analysis (fig 1A/B, 2A/B, Table E1); Western Blot (fig. 3C, 4B); Western Blot analysis (fig. 3C, 4B/C, 5C, E3B, E4B); lung section staining (fig E4A); study design; preparation and editing of figures and manuscript.
Christina Lukas	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. E1B, E2); quantitative real-time RT-PCR (fig. 3A, 5A/B, E3A); ELISA (fig. 3B, 4D); Western Blot (fig. 4C, 5C, E3B, E4B); preparation and editing of figures and manuscript.
Juliane Merl-Pham	mass spectrometry experiments and mass spectrometry data analysis (fig 2A/B, Table E1); preparation and editing of figures and manuscript.
Franziska E. Uhl	preparation of mouse three-dimensional ex-vivo lung tissue cultures and exposure to cigarette smoke extract (fig 5A/B/C), editing of manuscript.
Kathrin Mutze	isolation of primary mouse alveolar type II cells (fig. 4C/D, E2), editing of manuscript.
Andrea Schamberger	in vitro exposure of primary bronchial epithelial cells to cigarette smoke extract (fig. E3).
Claudia Staab-Weijnitz	supervision of A. Schamberger.
Jie Jia	cigarette smoke exposure of mice (fig. E4).
Ali O. Yildirim	supervision of J. Jia.
Melanie Königshoff	supervision of F.E. Uhl and K. Mutze.
Stefanie M.Hauck	supervision of J. Merl-Pham; editing of manuscript.
Oliver Eickelberg	supervision of A. Schamberger and C. Staab-Weijnitz; editing of manuscript.
Silke Meiners	supervision of A. Mossina and C. Lukas, study design and editing of figures and manuscript.

2.2 Impairment of immunoproteasome function by cigarette smoke and in chronic obstructive pulmonary disease

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ORIGINAL ARTICLE

Impairment of Immunoproteasome Function by Cigarette Smoke and in Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: Patients with chronic obstructive pulmonary disease (COPD) and in particular smokers are more susceptible to respiratory infections contributing to acute exacerbations of disease. The immunoproteasome is a specialized type of proteasome destined to improve major histocompatibility complex (MHC) class I mediated antigen presentation for the resolution of intracellular infections.

Objectives: To characterize immunoproteasome function in COPD and its regulation by cigarette smoke.

Methods: Immunoproteasome expression and activity were determined in bronchoalveolar lavage (BAL) and lungs of human donors and patients with COPD or idiopathic pulmonary fibrosis (IPF), as well as in cigarette smoke exposed mice. Smoke mediated alterations of immunoproteasome activity and MHC I surface expression were analyzed in human blood derived macrophages. Immunoproteasome specific MHC I antigen presentation was evaluated in spleen and lung immune cells that had been smoke exposed *in vitro* or *in vivo*.

Measurements and Main Results: Immunoproteasome and MHC I mRNA expression was reduced in BAL cells of patients with COPD and in isolated alveolar macrophages of patients with COPD or IPF. Exposure of immune cells to cigarette smoke extract *in vitro* reduced immunoproteasome activity and impaired immunoproteasome specific MHC I antigen presentation. *In vivo*, acute cigarette smoke exposure dynamically regulated immunoproteasome function and MHC I antigen presentation in mouse BAL cells. End stage COPD lungs showed markedly impaired immunoproteasome activities.

Conclusions: We here show that the activity of the immunoproteasome is impaired by cigarette smoke resulting in reduced MHC I antigen presentation. Regulation of immunoproteasome function by cigarette smoke may thus alter adaptive immune responses and add to prolonged infections and exacerbations in COPD and IPF.

Keywords: cigarette smoke; alveolar macrophages; MHC class I antigen presentation; immunoproteasome

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Author Contributions: I.E.K., A.D., and S.M. designed the research. P.N., H.S.O., A.P., I.O.R., S.K. E., M.K., G.P., H.W., M.L., R.H., J.B., K.H., A.O.Y., and O.E. provided (clinical) samples and reagents. I.E.K., A.D., A.M., D.B., C.L., O.V., P.N., and T.M.C. performed experiments. I.E.K., A.D., A.M., D.B., O.V., P.N., T.M.C., D.E.W., T.S., A.O.Y., and E.N. analyzed data. I.E.K., A.D., C.L., O.V., P.N., T.M.C., and S.M. interpreted results. I.E.K. and A.D. prepared figures. I.E.K., A.D., and S.M. drafted manuscript. I.E.K., A.D., A.M., S.K.E., E.N., O.E., and S.M. edited and revised the manuscript. All authors approved the final version.

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At a Glance Commentary

Scientific Knowledge on the Subject: Immunoproteasomes are specialized types of proteasomes involved in major histocompatibility class I mediated adaptive immune reactions. Although it has been shown that cigarette smoke decreases proteasome function in chronic obstructive pulmonary disease (COPD), the effect of smoke on immunoproteasome function in COPD has not been investigated.

What This Study Adds to the Field: We show that immunoproteasome expression and activity is directly altered by cigarette smoke *in vitro* and *in vivo*, resulting in disturbed major histocompatibility class I antigen presentation. Because immunoproteasome expression is down regulated and immunoproteasome activity is impaired in bronchoalveolar lavage and total lungs of patients with COPD, respectively, this may contribute to a distorted adaptive immune response in patients with COPD.

Chronic obstructive pulmonary disease (COPD) affects more than 200 million people worldwide and is estimated to become the third leading cause of death in 2030 (1). Tobacco smoking is considered to be the main risk factor for COPD (1). Bacterial and viral infections drive exacerbations contributing to high morbidity and mortality of patients with COPD (2 4). The major adaptive immune response against virus infected cells involves major histocompatibility class I (MHC I) mediated antigen presentation of viral antigens to $CD8^+$ T cells. Virus derived antigens are generated by the ubiquitin proteasome system, mounted onto MHC I molecules and exposed at the cell surface to patrolling $CD8^+$ T cells. These cytotoxic T cells then efficiently eliminate virus infected cells as part of the adaptive immune response (5).

The ubiquitin proteasome system degrades more than 80% of all cellular proteins (including old and damaged ones) into small peptides. These are used for recycling of amino acids but also for presentation of MHC I epitopes to define the "cellular self" toward the immune system (6 8). The proteasome consists of a barrel shaped 20S proteolytic core particle that is activated by different proteasome regulators, such as the 19S activator to form the 26S, the main proteasome complex for ubiquitin mediated protein degradation (9). The 20S core is composed of four heptameric rings comprised of α and β subunits. In standard proteasomes, three of the seven β subunits (β1, β2, and β5) exhibit proteolytic activities. They can be exchanged by their inducible counterparts (i.e., low molecular mass protein [LMP] 2, multicatalytic endopeptidase complex like 1 [MECL 1], and LMP7) to form the immunoproteasome. Expression of immunoproteasomal subunits is induced in response to IFN γ or tumor necrosis factor α as part of the early innate immune response to virus infections (10 12). Immunoproteasomes are constitutively expressed in immune cells compared with very low basal expression in most parenchymal cells (13). The newly assembled immunoproteasomes have altered cleavage kinetics compared with their 20S standard counterparts (14), and generate antigenic peptides that are preferentially presented by MHC I molecules (13) contributing to the efficient elimination of infected cells via the adaptive immune system (12, 13). Although several studies, including ours, suggest impairment of proteasome function by smoke exposure and in COPD (15 18), the effect of cigarette smoke on immunoproteasome function and its role in COPD pathogenesis have not been investigated so far.

In the current study, we analyzed the effect of acute cigarette smoke exposure on immunoproteasome expression *in vitro* and *in vivo*, and in bronchoalveolar lavage (BAL) cells from early stage and from lungs of patients with end stage COPD. Furthermore, we investigated the functional effects of cigarette smoke on immunoproteasome mediated antigen presentation. Some of the results of these studies have been previously reported in the form of abstracts (19 21).

Methods

Human Lung Tissue and Cells

BAL cells were obtained as previously described (22) with approval by the local ethics committee of the Albert Ludwig University Freiburg (No. 231/03). The use of explanted human lung tissues and blood from healthy donors was approved by the University Hospital of the Ludwig Maximilians University in Munich (Nos. 333 10 and 071 06 075 06). Human macrophages were differentiated from peripheral blood monocytes according to Martinez and colleagues (23).

Animals

Tissues or cells were isolated from C57BL/6J wild type, LMP2 $^{/}$ ($Psmb9^{tm1Stl}$ [24]), or LMP7 $^{/}$ ($Psmb8^{tm1Hjf}$ [25]) mice with C57BL/6J background. For smoke exposure experiments, C57BL/6J wild type males or BALB/cAnCrl females were used (Charles River Laboratories, Sulzfeld, Germany). All animal procedures were conducted according to international guidelines and with approval of the Bavarian Animal Research Authority in Germany.

UTY-LacZ Assay

To measure T cell responses specific for the male antigen $UTY_{246–254}$ presented on H $2D^b$, 0.5 1×10^5 cigarette smoke extract (CSE) treated or freshly isolated cells from male smoke exposed mice or control animals were cocultured with the same number of cells of the $UTY_{246–254}$ specific T cell hybridoma in 96 well plates. After overnight incubation, cells were centrifuged, medium was aspirated, and cells were incubated in 150 μl LacZ buffer (9 mM $MgCl_2$, 0.15 mM chlorophenol red β galactoside, 100 mM 2 ME, 0.125% Nonidet P 40 in phosphate buffered saline) at 37°C until a color change was observed (approximately 4 h). Colorimetric measurement of LacZ activity was done at 570 nm (reference wavelength at 620 nm) using a Sunrise plate reader (Tecan, Männedorf, Switzerland). The background signal of the cells was subtracted and maximum induction was set to 100%. Cells from female mice and LMP2 or LMP7 deficient mice served as controls.

Statistics and Software

Data were analyzed with ImageLab (Biorad, Hercules, CA), ImageJ (http://imagej.nih.gov/ij/), or Prism5 (GraphPad Software, Inc., La Jolla, CA). Statistics were performed using Prism5 with initial Grubbs' test for outliers and D'Agostino and Pearson omnibus normality test.

Normally distributed data were analyzed using parametric tests with appropriate *post hoc* analysis, otherwise nonparametric tests were chosen. *P* values less than 0.05 were considered statistically significant. Details on the statistics are given in the figure legends. Additional details on the methods are provided in the online supplement.

Results

Immunoproteasome and MHC I Expression Is Reduced in BAL Cells of Patients with COPD

Our previous study on immunoproteasome expression in the lung identified alveolar macrophages as the main cell type expressing active immunoproteasomes (26). We thus first analyzed immunoproteasome expression in BAL cells of patients with COPD (*see* Table 1 for the clinical characteristics of these patients). Of note, mRNA expression of all three immunoproteasome subunits LMP2, MECL 1, and LMP7 was significantly decreased in total BAL cells from patients with COPD compared with control subjects. There was also a trend toward down regulation of immunoproteasomes in BAL cells of another smoke related chronic lung disease, namely idiopathic pulmonary fibrosis (IPF), which was, however, not significant (Figure 1A). Cellular composition of BAL cells was not significantly different between control and COPD groups, but clearly altered in patients with IPF (*see* Figure E1 in the online supplement). Independent evidence for reduced immunoproteasome expression in alveolar macrophages of patients with COPD was obtained from published microarray data confirming down regulation of immunoproteasome expression in patients with COPD compared with nonsmokers and healthy smokers (Figure 1B) (27). Of note, we observed down regulation of all three immunosubunits also in isolated alveolar macrophages of patients with IPF as determined by analysis of a publicly available but unpublished microarray data set (Figure 1C). In contrast, alveolar macrophages from nonsmoking patients with asthma had rather increased levels of LMP2 and MECL 1 compared with the smoking control subjects as revealed by bioinformatical analysis of publicly available array data (Figure 1D) (28).

These results suggest specific down regulation of the immunoproteasome in isolated alveolar macrophages of patients with smoke related chronic lung diseases, such as COPD and IPF. Because immunoproteasomes play a pivotal role in MHC I antigen presentation, we also analyzed other components involved in the MHC I antigen presentation machinery. We observed uniform down regulation of several genes encoding the MHC I heavy chain molecules (i.e., *HLA A*, *B*, and *C*), and components of the peptide loading complex, such as transporter associated with antigen presentation 1, in patients with COPD compared with nonsmokers or healthy smokers, respectively (Figure 1E; *see* Figure E2). Several genes of the MHC I antigen presentation machinery were also found to be down regulated in alveolar macrophages from patients with IPF very similar to the COPD samples, whereas these genes were unchanged or even up regulated in alveolar macrophages from subjects with asthma (*see* Figure E2 for an overview).

CSE Impairs Immunoproteasome Activity and MHC I Surface Expression of Human Macrophages

To investigate whether immunoproteasome function and MHC I antigen presentation are concertedly regulated in response to cigarette smoke, we exposed primary human blood monocyte derived macrophages to CSE and quantified cell surface MHC I expression by flow cytometry. Of note, nontoxic doses of CSE (*see* Figures E3A and E3B) decreased surface MHC I expression in cells from four out of five individual blood donors after 6 hours (Figure 2A). We next correlated MHC I surface expression with immunoproteasome activity, and labeled therefore live macrophages with a set of activity based probes (ABPs) that specifically attach to the active catalytic β subunits allowing quantification of individual activities depending on the specificity of the probe. Of note, although total proteasome activity was not grossly altered, the activity of LMP7, the rate limiting subunit for MHC I peptide supply (25), was significantly reduced after 6 hours of CSE exposure (Figure 2B; *see* Figure E3C). Protein expression of proteasome subunits and HLA A was not significantly affected by CSE exposure except for LMP2 (*see* Figure E3D). These data demonstrate that diminished immunoproteasome activity goes along with reduced MHC I surface expression on human blood derived macrophages confirming previous data from immunoproteasome knockout mice (25). Moreover, we here provide first evidence

Table 1. Patient Characteristics

Group	BAL* (Figure 1A; *see* Figure E1)					Lung Tissue† (Figure 6)
	Control Subjects	IPF	*P* Value	COPD	*P* Value	COPD
N	15	16		9		5
Sex, M/F	8/7	15/1	<0.05‡	9/0	<0.05‡	1/4
Age, yr, median (range)	59 (45–71)	68 (55–86)	<0.05§	67 (48–72)	n.s.§	53 (44–63)
Smoking status, NS/ex-smoker	4/11	6/10	n.s.‡	0/9	n.s.‡	n.a.
Pack-years, median (range)	12.5 (0–30)	15 (0–30)	n.s.§	40 (20–60)	<0.001§	n.a.
GOLD stage, I/II/III/IV	n.a.	n.a.		1/6/2/0		0/0/0/5

Definition of abbreviations: BAL bronchoalveolar lavage; COPD chronic obstructive pulmonary disease; GOLD Global Initiative for Chronic Obstructive Lung Disease; IPF idiopathic pulmonary fibrosis; M/F male/female; n.a. not available; n.s. not significant; NS nonsmoker.
*BAL cells were obtained as previously described (23).
†According to European organ transplant guidelines, donors are anonymous.
‡Statistical analysis was performed using Fisher exact test compared with control subjects.
§Statistical analysis was performed using Kruskal Wallis test compared with control subjects.

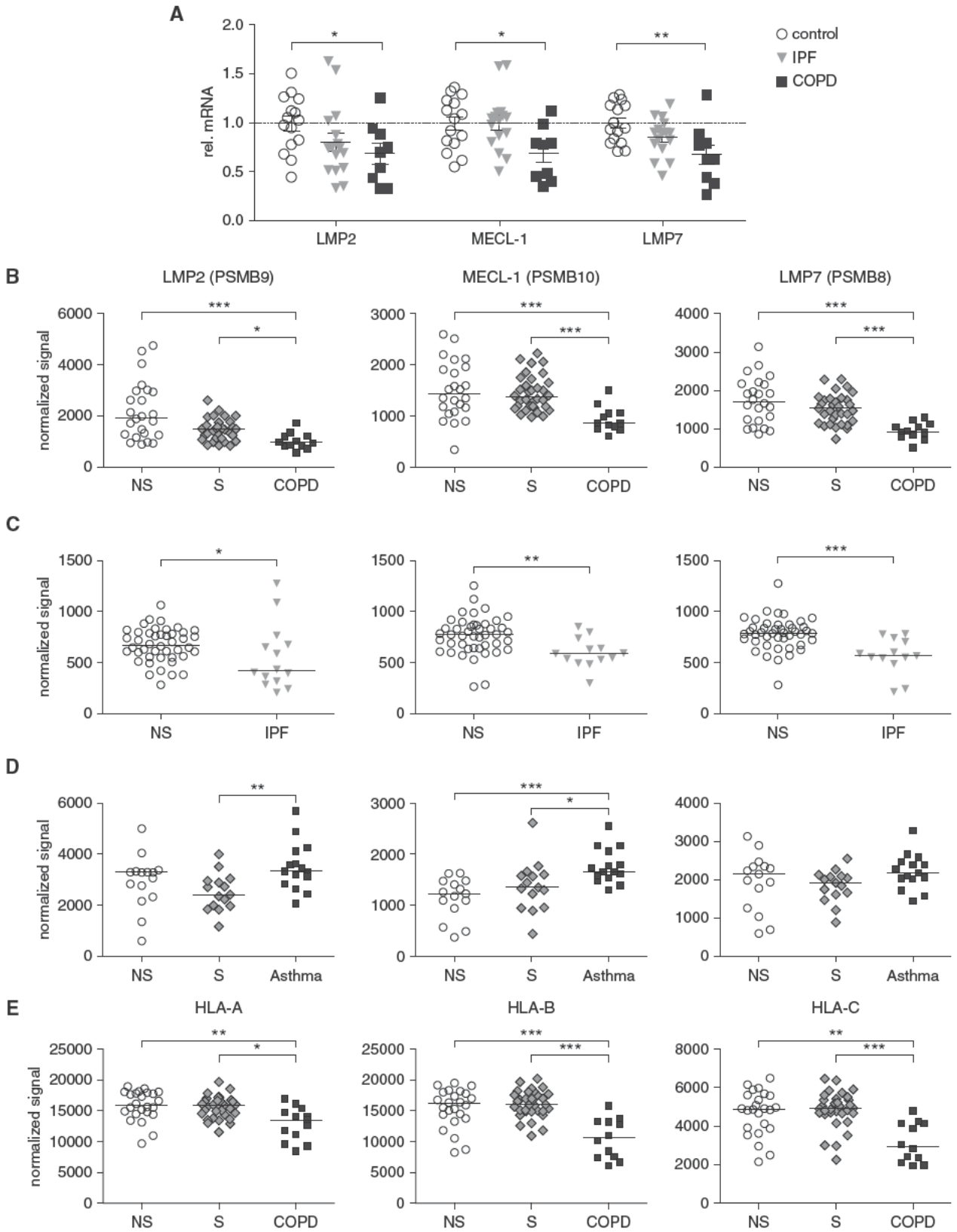

Figure 1. Reduced immunoproteasome transcripts in bronchoalveolar lavage of patients with chronic obstructive pulmonary disease (COPD). (*A*) Quantitative reverse transcriptase polymerase chain reaction mRNA analysis of immunoproteasome subunits low molecular mass protein (LMP) 2, multicatalytic endopeptidase complex like 1 (MECL 1), and LMP7 in bronchoalveolar lavage cells of control subjects (n 15), idiopathic pulmonary fibrosis

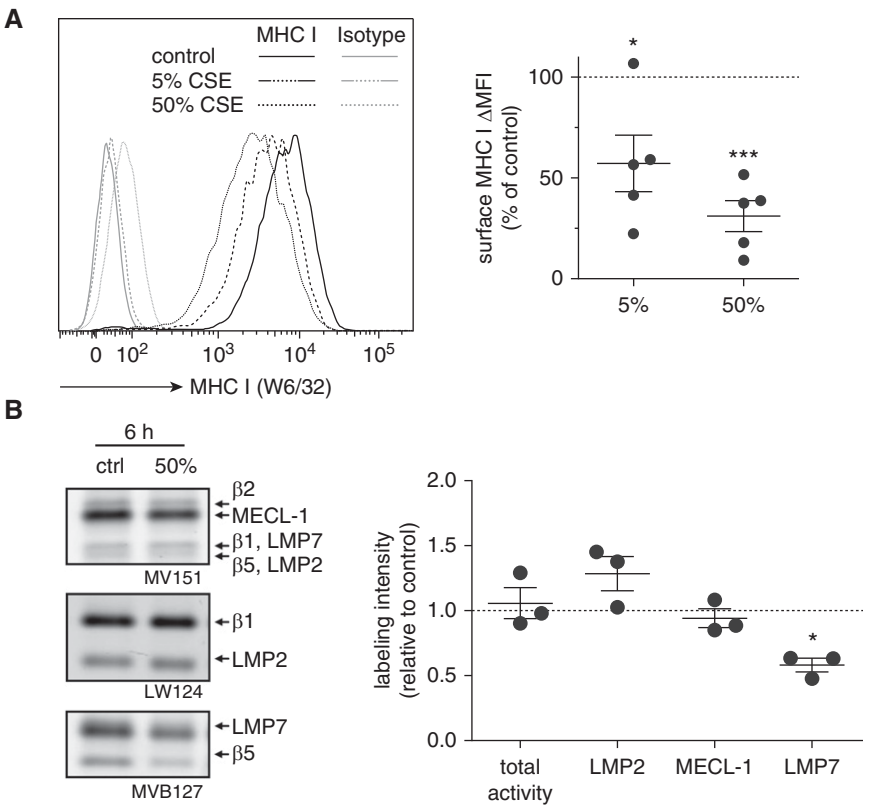

Figure 2. Cigarette smoke extract (CSE) impairs major histocompatibility class I (MHC I) antigen presentation in human blood monocyte derived macrophages. (*A*) Human monocyte derived macrophages (n 5 different donors) were treated with 5 or 50% CSE for 6 hours and stained with anti MHC I antibody W6/32 or isotype control, and propidium iodide. Median fluorescence intensities were determined on gated live single cells, and the isotype corrected median fluorescence intensity (ΔMFI) was normalized to untreated cells in five independent experiments (100%; mean ± SEM, one sample *t* test, $^*P < 0.05$, $^{***}P < 0.001$). (*B*) The same samples as in *A* were labeled with activity based probes MV151 (labeling all active subunits), LW124 (specific for β1 and low molecular mass protein [LMP] 2), or MVB127 (specific for β5 and LMP7). Densitometric analysis combines data from three different donors (replicates are shown in Figure E3C); values were normalized to untreated cells (mean ± SEM; one sample *t* test [compared with 1]; $^*P < 0.05$). ctrl control; MECL 1 multicatalytic endopeptidase complex like 1.

that immunoproteasome function is impaired by an environmental insult (here cigarette smoke) contributing to diminished MHC I expression on the cell surface.

CSE Impairs Immunoproteasome-mediated Antigen Presentation in Splenic Immune Cells

To establish a causal link between cigarette smoke mediated regulation of immunoproteasome activity and MHC I mediated antigen presentation, we made use of a functional antigen presentation assay that allows assessment of the specific T cell response to the presentation of an immunoproteasome dependent MHC I epitope in C57BL/6 derived immune cells. The male HY antigen $UTY_{246–254}$ is generated by immunoproteasome subunits LMP2 and LMP7, and presented to the T cell hybridoma reporter cell line UTY (29). Antigen mediated activation of UTY cells can be quantified by lacZ assays because of the IL 2 promotor driven β galactosidase expression.

To first validate the $UTY_{246–254}$ antigen presentation assay, splenocytes from female or male wild type, male LMP2, or LMP7 knockout mice were isolated, then coincubated with the UTY hybridoma cell line and β galactosidase activity was measured (Figure 3A). Only splenocytes from male wild type mice specifically activated the UTY T cells with a doubling of the β galactosidase reporter signal. The results from these experiments validate the assay as an appropriate readout for immunoproteasome dependent antigen presentation.

Of note, treatment of male wild type splenocytes with nontoxic concentrations of CSE for 24 hours impaired UTY activation already at the lowest dose of 5% CSE, and full suppression of UTY activation was achieved with 25% CSE (Figure 3B; *see* Figure E4). Although expression of the immunoproteasome subunits LMP2 and LMP7 was not altered, overall proteasome and immunoproteasome activities were clearly reduced, as assessed by specific ABP labeling (Figures 3C and 3D). Impaired presentation of $UTY_{246–254}$ in response to increasing doses of CSE was also confirmed for $CD11c^+$ splenic dendritic cells (Figure 3E).

CSE Impairs Immunoproteasome-mediated Antigen Presentation in Immune Cells of the Lung

We next exposed immune cells of the lung (i.e., $CD11c^+$ lung cells [mainly composed of alveolar macrophages and dendritic cells] and BAL cells of mouse lungs [mainly alveolar macrophages] [30]), to nontoxic doses of CSE and performed UTY assays. Very similar to our results obtained with splenic cells, CSE exposure significantly reduced antigen presentation of the $UTY_{246–254}$ peptide both in BAL cells and $CD11c^+$ immune cells of the lung (Figure 4; *see* Figure E4). These *in vitro* data thus reveal that immunoproteasome mediated

Figure 1. (Continued). (IPF) (n 13), and patients with COPD (n 9). Rpl19 was used as a housekeeping gene (mean ± SEM, one way analysis of variance with Dunnett *post hoc* test, $^*P < 0.05$, $^{**}P < 0.01$). (*B D*) Microarray results from isolated alveolar macrophages of nonsmokers and (*B*) healthy smokers and patients with COPD (27), (*C*) patients with IPF (GSE13896), and (*D*) healthy smokers and nonsmoking patients with asthma (28) for immunoproteasome subunits LMP2 (*PSMB9*), MECL 1 (*PSMB10*), and LMP7 (*PSMB8*). (*E*) Analysis of the same samples as in *B* for genes encoding human major histocompatibility class I genes *HLA A*, *HLA B*, and *HLA C* (*B E*: median, Mann Whitney *U* or Kruskal Wallis test with Dunn *post hoc* test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). NS nonsmokers; rel. relative; S smokers.

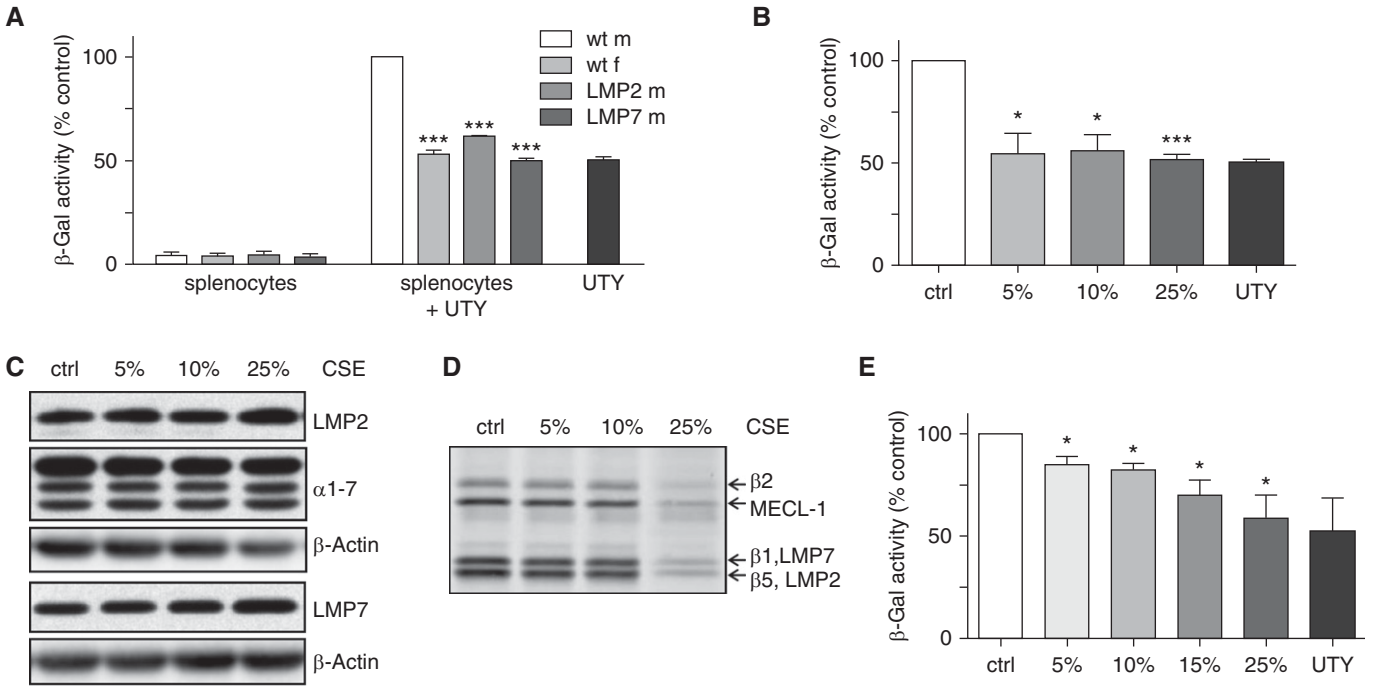

Figure 3. Cigarette smoke extract (CSE) impairs immunoproteasome mediated antigen presentation of UTY peptide in antigen presenting cells of spleen and lung. (*A*) Validation of the $UTY_{246–254}$ peptide presentation assay. UTY cells are activated by immunoproteasome dependent presentation of the male $UTY_{246–254}$ peptide on splenocyte major histocompatibility class I (H $2D^b$) as quantified by measurement of reporter gene β galactosidase activity. β Galactosidase activity of UTY cytotoxic T lymphocytes alone (UTY), coincubated with splenocytes (female or male wild type, male low molecular mass protein [LMP] 2 or LMP7 knockout) or splenocytes alone was measured by colorimetric assay of substrate turnover. Data are combined results from three independently performed experiments (splenocytes from several individual male mice were isolated, pooled, and divided for the different treatment groups). Cell preparations and stimulations were repeated on different days. Results are normalized to the signal of maximum induction of UTY cells by male splenocytes (mean + SEM). (*B*) β Galactosidase activity of UTY hybridoma cells coincubated with male wild type splenocytes that had been treated with increasing concentrations of CSE for 24 hours, displayed as percentage of maximum induction of control untreated splenocytes (n 3; mean + SEM). (*C*) Splenocytes were treated for 24 hours with the indicated CSE concentrations. Living cells were first incubated with activity based probe MV151 and then lysed with RIPA buffer. Proteasome expression (immunosubunits LMP2 and LMP7; total 20S α subunits [α1 7]) and (*D*) activity were assessed by immunoblotting and sodium dodecyl sulfate polyacrylamide gel electrophoresis, respectively. Results are representative for three independent experiments. (*E*) β Galactosidase activity of UTY cells coincubated with isolated male $CD11c^+$ splenic dendritic cells that had been treated with increasing concentrations of CSE for 24 hours, displayed as percentage of maximum induction of control untreated cells (n 4; mean + SEM). Statistical analysis: one sample *t* test (compared with 100%) $^*P < 0.05$, $^{***}P < 0.001$. β gal β galactosidase; ctrl control; f female; m male; MECL 1 multicatalytic endopeptidase complex like 1; UTY $UTY_{246–254}$ hybridoma cell line; wt wild type.

MHC I presentation of $UTY_{246–254}$ antigen follows immunoproteasome activity, and immunoproteasome dependent antigen presentation is impaired by CSE.

Cigarette Smoke Dynamically Regulates Immunoproteasome Function in BAL Cells *In Vivo*

Immunoproteasome mediated antigen presentation was next analyzed *in vivo* using male C57BL/6 mice that were acutely exposed to cigarette smoke for 1, 3, and 10 days (for total and differential cell count, *see* Figures E5A and E5B). Isolated alveolar macrophages of smoke exposed mice showed transient up regulation of immunoproteasome expression with highest protein levels after 3 days of smoke exposure and subsequent reduction after 10 days of exposure to levels below those of air exposed control animals (Figure 5A). These dynamics of proteasome expression were closely followed by transient activation of standard and immunoproteasomes as determined by ABP labeling of catalytic subunits (Figure 5B). Notably, after 10 days of smoke exposure, the activity of the immunoproteasome subunits was reduced resulting in a shift in the activity ratio from immunoproteasome to their standard proteasome counterparts (Figure 5C; *see* Figure E5C). Analysis of $UTY_{246–254}$ antigen presentation in the C57BL/6 mice revealed significant activation of the UTY T cell response in BAL cells of 3 days smoke exposed mice, which was lost after 10 days of smoke exposure (Figure 5D). Antigen presentation thus again closely followed the course of immunoproteasome activity *in vivo*.

We confirmed the relative impairment of immunoproteasome activity in isolated alveolar macrophages of a second mouse strain (i.e., BALB/c mice that had been exposed to cigarette smoke for 10 days) (*see* Figure E6A). Differential BAL count revealed that BAL cells were mainly composed of alveolar macrophages (*see* Figure E6B). Of note, RNA expression of all three immunoproteasome subunits was significantly reduced in alveolar macrophages (*see* Figure E6C), thus resembling our data from human BAL of patients with COPD (Figure 1A). In summary, our *in vivo* data demonstrate a direct effect of cigarette smoke on immunoproteasome expression and activity

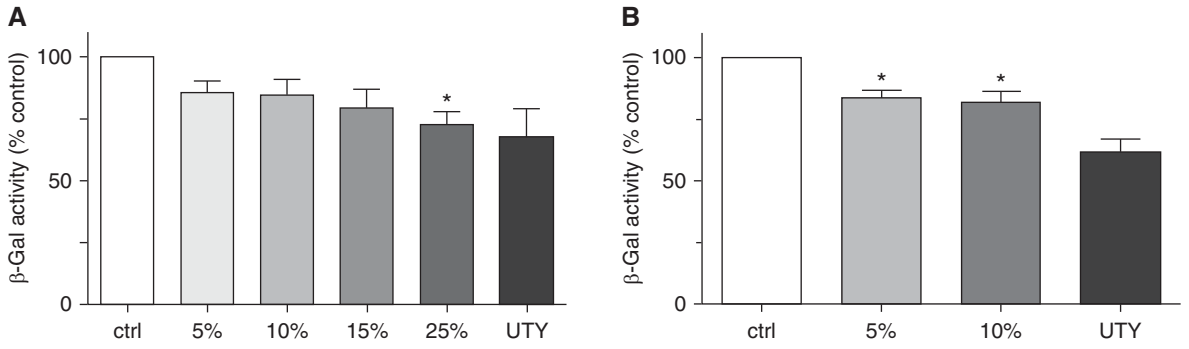

Figure 4. Cigarette smoke extract decreases UTY peptide presentation in antigen presenting cells of the lung. β Galactosidase activity of UTY reporter cell line coincubated with (*A*) magnetic activated cell sorted $CD11c^+$ lung cells or (*B*) bronchoalveolar lavage cells (>95% alveolar macrophages) from male mice that had been treated with increasing cigarette smoke extract concentrations for 24 hours. Data are combined results of three to four independent experiments normalized to the signal of maximum induction of untreated cells coincubated with UTY cells (100%) (mean + SEM; one sample *t* test [compared with 100%]; $*P < 0.05$). β Gal β galactosidase; ctrl control; UTY $UTY_{246–254}$ hybridoma cell line.

in BAL cells. These changes depend on the extent of smoke exposure and result in altered MHC I antigen presentation.

Immunoproteasome Activity Is Impaired in End-Stage COPD Lungs but Not in Cigarette Smoke–exposed Mice

We further investigated immunoproteasome activity in explanted lung tissues from patients with end stage COPD versus control organ donors. Additionally, we analyzed immunoproteasome function in lungs of mice that were chronically exposed to smoke for 4 months and had developed smoke induced emphysema (31). Of note, we did not observe any change in RNA expression of standard (α7) and immunoproteasome subunits in COPD and donor lungs (Figure 6A). Protein expression analysis of the human samples revealed heterogeneous expression levels but no significant alterations in the immunoproteasome subunits or total MHC I (Figure 6B; *see* densitometric analysis in Figure E8). Similarly, RNA and protein levels of immunoproteasome subunits were not grossly altered in lungs of smoke exposed mice (*see* Figures E7A and E7B). On the contrary, we observed a uniform decrease in total proteasome activity in native lysates of end stage COPD lungs as determined by ABP analysis, allowing us to attribute the loss of activity to the standard and the immunoproteasome proteolytic activities (Figure 6C).

We confirmed this striking impairment of proteasome function using native gels with substrate overlay assays and observed a drastic and uniform impairment of both 20S and 26S proteasome activities in COPD lung tissue compared with control subjects. Reduced activity of the proteasome complexes was assigned to diminished 20S and 26S proteasome formation, as determined by blotting of the native gels for 20S and 26S proteasome subunits, respectively. Of note, both standard and immunoproteasome activities were rather elevated in lungs of chronically smoke exposed mice as determined by ABP labeling and native gel analysis (*see* Figures E7C and E7D). These results indicate that in contrast to end stage COPD lungs, emphysematous lungs of smoke exposed mice are still able to maintain standard and immunoproteasome activities, an observation that is well in agreement with the different lung pathologies, showing only minor changes in smoke exposed mice but detrimental lung damage in end stage COPD.

Discussion

We show for the first time that cigarette smoke alters expression and activity of immunoproteasomes in immune cells *in vitro* and *in vivo*. Immunoproteasome expression was specifically down regulated in total BAL of patients with COPD and in isolated alveolar macrophages of patients with COPD and IPF. Both standard and immunoproteasome activities were strongly impaired in end stage COPD lung tissues. Importantly, smoke mediated alteration in immunoproteasome content resulted in altered MHC I surface expression and MHC I mediated presentation of an immunoproteasome specific antigen. The effect of cigarette smoke on immunoproteasome mediated MHC I antigen presentation may thus contribute to a distorted adaptive immune response in viral and bacterial exacerbations of patients with COPD.

Regulation of the Immunoproteasome by Cigarette Smoke and in COPD

Immunoproteasomes are constitutively expressed in immune cells of the lung as shown previously by us and others (26, 32). Parenchymal expression of immunoproteasomes is low but can be rapidly induced upon virus infection (26). We did not observe any up regulation of immunoproteasome expression in lungs of smoke exposed mice and end stage COPD lungs. This is in accordance with the study by Baker and colleagues (33) who analyzed immunoproteasome expression in COPD lungs. These data thus refute the notion that immunoproteasomes are induced as part of a protective oxidative stress response (34), and rather support contrary reports (35). In contrast to our observation, Fujino and colleagues (36) reported increased LMP2 and LMP7 RNA expression in primary alveolar type II cells of patients with early COPD stages. Despite the absence of expressional alterations, standard and immunoproteasome activities were markedly impaired in COPD lungs as determined by two different activity assays (i.e., ABP and

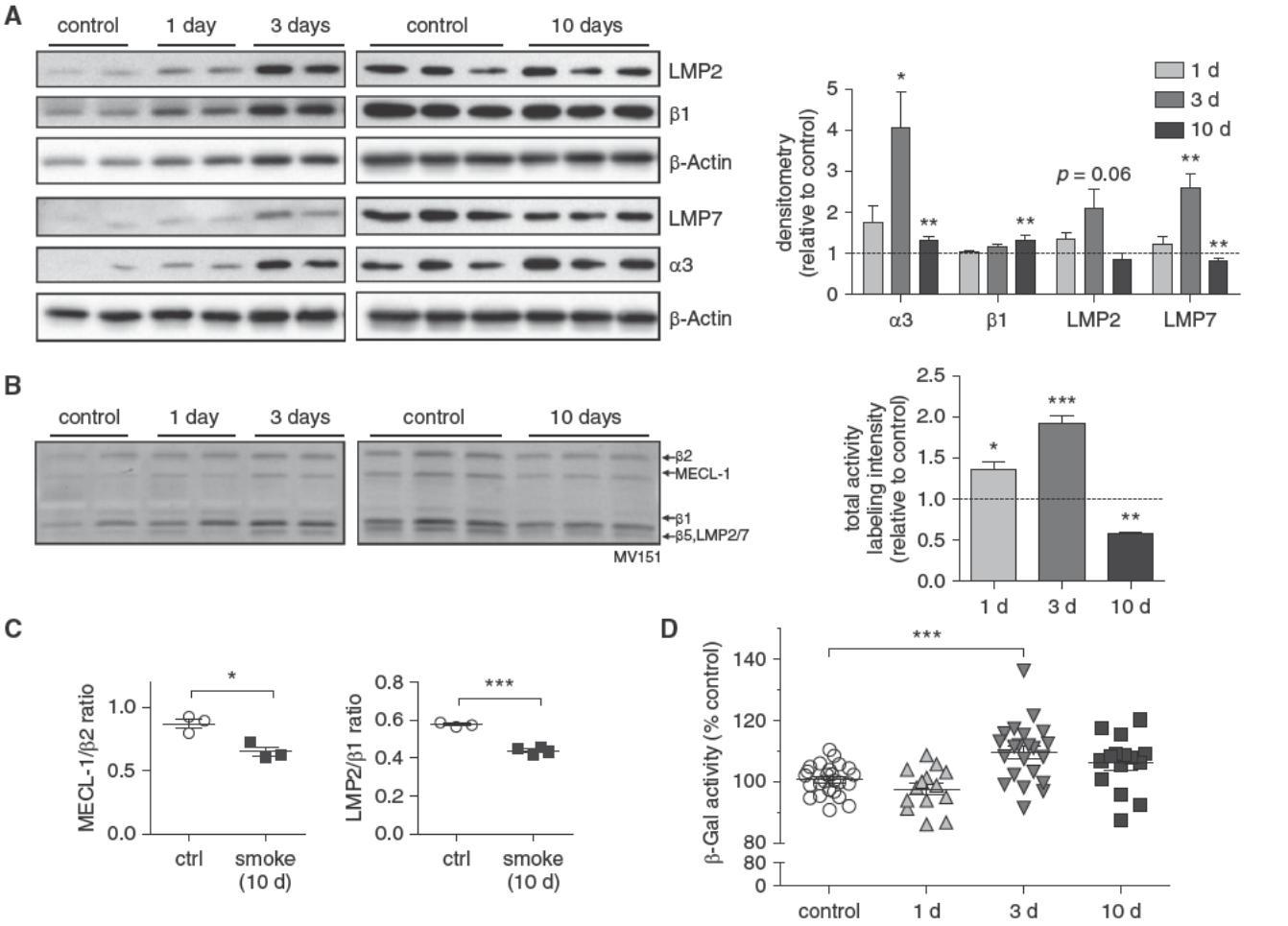

Figure 5. Cigarette smoke dynamically regulates proteasome function and UTY presentation in bronchoalveolar lavage cells of cigarette smoke exposed mice. (*A*) Proteasome protein expression in isolated alveolar macrophages from air exposed control animals or mice that had been exposed to cigarette smoke for one exposure cycle (50 min; 1 d), 3 or 10 days. Western blots display immunosubunits low molecular mass protein (LMP) 2 and LMP7 and standard subunit β1 and α3. β Actin served as loading control. Combined densitometric analysis of Western blots for 20S standard subunits α3 and β1, and immunosubunits LMP2 and LMP7. Results are combined data from two to three independent experiments and are displayed as fold over air exposed controls (mean + SEM; one sample *t* test [compared with 1]; $^{*}P < 0.05$; $^{**}P < 0.01$). (*B*) Proteasome activity (activity based probe MV151) of alveolar macrophages and densitometric analysis thereof (mean + SEM; one sample *t* test [compared with 1]; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (*C*) Multicatalytic endopeptidase complex like 1 (MECL 1)/β2 and LMP2/β1 (for corresponding gel, *see* Figure E5C) activity ratios in alveolar macrophages from mice exposed to cigarette smoke for 10 days compared with control animals (ctrl, mean ± SEM; Student's *t* test; $^{*}P < 0.05$; $^{***}P < 0.001$). (*D*) β Galactosidase activity of UTY reporter cell line coincubated with *ex vivo* bronchoalveolar lavage cells from male mice that had been exposed to cigarette smoke for 1 day (n 14), 3 days (n 22), or 10 days (n 15) compared with air control animals (n 25, set to 100%) (mean ± SEM; one way analysis of variance with Dunnett *post hoc* test; $^{***}P < 0.001$). β Gal β galactosidase; UTY $UTY_{246–254}$ hybridoma cell line.

native gel based analysis). Intriguingly, in lungs of chronically smoke exposed mice, we observed the rather opposite effect on proteasome activity (i.e., an overall activation of both standard and immunoproteasome activities). The data suggest that the murine model of chronic smoke exposure does not fully reflect the complex features of chronic lung disease in patients with COPD.

Quite contrary to total lung tissue, BAL cells of patients with COPD showed significantly reduced RNA expression of immunoproteasome subunits compared with control subjects. This finding was confirmed by microarray analysis of isolated alveolar macrophages using publicly available data sets. Moreover, significantly reduced immunoproteasome expression was also observed in isolated alveolar macrophages of patients with IPF. The correlation of immunoproteasome down regulation and smoking history (i.e., number of pack years) was close to significance ($P = 0.057$ for LMP2/*PSMB9* and $P = 0.067$ for MECL 1/*PSMB10*, respectively) supporting the link between smoke exposure and immunoproteasome down regulation. We did not, however, observe significant down regulation of immunoproteasome expression in healthy smokers suggesting that smoke exposure alone is probably not sufficient to mediate sustained reduction in immunoproteasome expression.

Our *in vivo* data from alveolar macrophages of acutely smoke exposed mice indicate that there is dynamic regulation of immunoproteasome

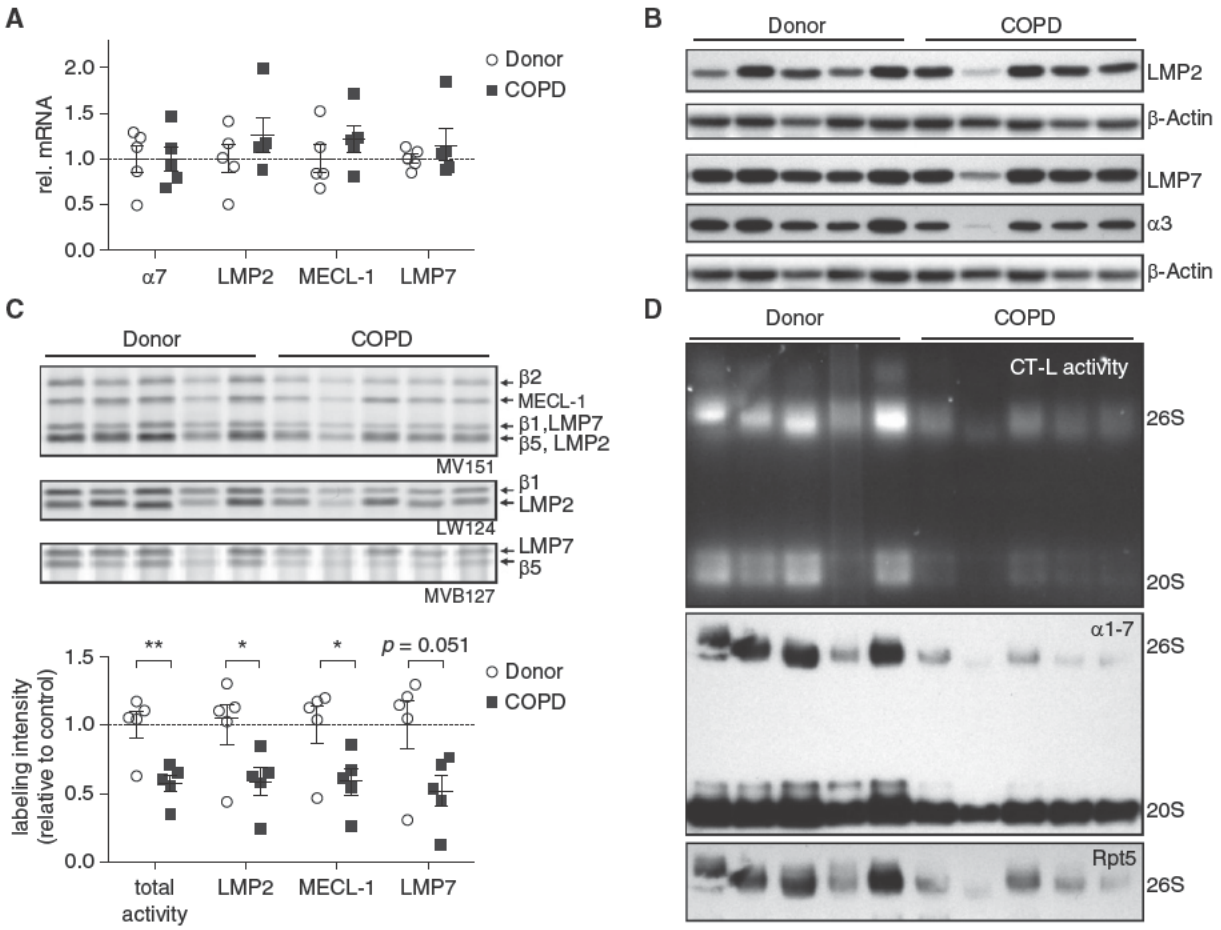

Figure 6. Impaired immunoproteasome activity in patients with chronic obstructive pulmonary disease (COPD). (*A*) Quantitative reverse transcriptase polymerase chain reaction mRNA analysis of 20S proteasome subunit α7 and immunoproteasome subunits low molecular mass protein (LMP) 2, multicatalytic endopeptidase complex like 1 (MECL 1), and LMP7 in total lungs of donors (n 5) and patients with end stage COPD (n 5). (*B*) Western blot of the same donor and end stage COPD lungs for immunosubunits LMP2 and LMP7 and standard subunit α3. β Actin served as a loading control. Densitometric analysis can be found in Figure E8B. (*C*) Activity based probe profiling of the same patients as in *B*: native lung lysates were labeled with pan reactive activity based probe MV151 or LW124 (labeling LMP2 and β1) and MVB127 (labeling MECL 1 and β2) and separated on denaturing SDS gels. Densitometric analysis revealed total activity (MV151 signal) and single subunit activity and is shown normalized to the mean of donor activities (mean ± SEM; Student's *t* test; $^*P < 0.05$; $^{**}P < 0.01$). (*D*) Native gel analysis with chymotrypsin like (CT L) substrate overlay analysis and immunoblotting of native lung lysates to detect 20S and 26S (20S + 19S) proteasome complexes with antibodies detecting α1 7 subunits (20S) or Rpt5 (19S subunit). rel. relative.

expression and activity in response to cigarette smoke: standard and immunoproteasome expression and activity were strongly activated after 3 days of smoke exposure, whereas expression of the immunoproteasome was significantly reduced after 10 days. In addition, we observed a shift of standard versus immunoproteasome activities in smoke activated macrophages. Although the smoke induced changes in the ratio of standard versus immunoproteasome activities were minor and based on a rather small sample size, they were confirmed for two different mouse strains and in both sexes (i.e., female BALB/c and male C57BL/6 mice). Furthermore, *in vitro* exposure of splenic immune cells and human blood derived macrophages to CSE confirmed inactivation of the immunoproteasome by smoke in different cell types and human immune cells.

The divergent results obtained *in vitro* and *in vivo* may relate to the well known differences between CSE and full smoke, which also makes comparative dosing difficult (37). Furthermore, *in vivo* smoking activates alveolar macrophages (e.g., by acute neutrophil mediated release of IFN γ and tumor necrosis factor α) (30, 38). These cytokines induce immunoproteasome expression (13) but also activate standard proteasomes (39). Indeed, neutrophil numbers peaked in BAL after 3 days of smoke exposure, which may contribute to activation of isolated alveolar macrophages. Taken together, our data reveal a previously unrecognized alteration of immunoproteasome expression and activity in immune cells in response to cigarette smoke and in COPD pathogenesis.

Cigarette Smoke Alters MHC I Antigen Presentation: Implications for COPD

We are the first to show that smoke mediated changes in immunoproteasome activity directly affect MHC I antigen presentation and T cell mediated immune responses. In human blood derived macrophages, CSE acutely inhibited

immunoproteasome activity that was associated with diminished cell surface expression of MHC I molecules. Treatment of splenic and lung immune cells with CSE not only reduced the activity of the immunoproteasome but also impaired MHC I mediated antigen presentation of the immunoproteasome specific $UTY_{246–254}$ epitope to a T cell hybridoma. This functional assay directly monitors the immunologic consequences of impaired immunoproteasome function (29). CSE mediated impairment of immunoproteasome activity thus most likely prevents efficient generation of the $UTY_{246–254}$ epitope and surface expression of these peptide/MHC I complexes resulting in reduced T cell activation.

Proteasome mediated generation of antigenic peptides is a rate limiting step for MHC I antigen presentation, because loading of antigenic peptide to the MHC I binding groove stabilizes MHC I complexes in the endoplasmic reticulum and enhances their transport to the cell surface (13). Accordingly, inhibition of the proteasome impairs MHC I driven immune responses toward lymphocytic choriomeningitis virus infections (40) and mice lacking immunoproteasome subunits have severely impaired MHC I antigen presentation (41). Our MHC I flow cytometry analysis of human macrophages revealed a significant acute reduction of MHC I surface expression, whereas total MHC I expression was not affected. These data may be indicative for reduced MHC I complex loading caused by impaired proteasome activity. We cannot, however, rule out that CSE alters MHC I surface expression by other mechanisms related to oxidative or endoplasmic reticulum stress (42). In addition, CSE may also directly affect peptide/MHC I interactions thereby contributing to reduced T cell activation. Indeed, Fine and colleagues (43) showed that tobacco extract reduces membrane HLA class I levels and concomitant immune responses. This is in line with the observation of significantly diminished MHC I levels on alveolar macrophages of smokers with COPD (44). In addition, cigarette smoke may oxidatively modify MHC I epitopes thereby reducing their affinity to T cell receptors and impacting T cell activation and proliferation (45).

Our *in vivo* data revealed that BAL cells from cigarette smoke exposed mice had significantly increased immunoproteasome activity and MHC I antigen presentation after 3 days of smoke exposure. After 10 days, antigen presentation was still elevated, although to a lesser extent and immunoproteasome activity was reduced compared with standard proteasome function. These data suggest that longer smoke exposure impairs immunoproteasome activity and concomitant MHC I antigen presentation, which still needs to be tested.

Data on MHC I antigen presentation in COPD are limited, whereas innate and MHC II mediated immune responses in COPD are well studied (38). Several lines of evidence support a role of MHC I mediated antigen presentation for the pathogenesis of COPD and in viral and bacterial exacerbations (46): $CD8^+$ T cells are abundantly present in COPD tissue and chronic smoke exposure induces proliferation of $CD8^+$ T cells in the lung (47 49). Moreover, $CD8^+$ T cell depletion or genetic ablation protects mice from emphysema formation suggesting an essential role of MHC I mediated immune responses for smoke induced emphysema development (50, 51). Cigarette smoke generally dampens the host's immune system in its response to infections (52, 53) because alveolar macrophages become less responsive to IFN γ and are less protective against bacterial and viral infections (54). Although the role of viral exacerbations in IPF is not clear (55, 56), our analysis of microarray data from isolated alveolar macrophages of patients with IPF suggests down regulation of not only the immunoproteasome but also of some MHC I molecules. Moreover, in an unbiased bioinformatics approach that compared the gene expression signatures in isolated alveolar macrophages of healthy smokers, smokers with COPD, patients with IPF, and nonsmoking patients with asthma, we observed uniform down regulation of several genes involved in antiviral immune responses that was specific for macrophages from patients with COPD and patients with IPF and not evident in healthy smokers or patients with asthma (data not shown). We thus envision sustained dampening of antiviral immune responses as a characteristic feature of chronic smoke related lung diseases that may add to an increased susceptibility of patients with COPD and patients with IPF to viral exacerbations.

In conclusion, we are the first to provide evidence for a novel pathomechanism involving dysfunction of the immunoproteasome and MHC I antigen presentation by cigarette smoke in lung immune cells that may contribute to impaired clearance of pathogens and to sustained infections in smokers and exacerbations in COPD and possibly also in patients with IPF. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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2.2.1 Supplementary information

Supplementary information for the manuscript

Impairment of immunoproteasome function by cigarette smoke and in COPD

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SUPPLEMENT METHODS

Microarray analysis: Microarray expression values were extracted with the robust multi-array average (RMA) procedure. Zero variance probe sets were removed and many-to-one probe sets-to-gene relationships were resolved by retaining only the probe sets with the highest variance across all experimental conditions. The microarrays used in this study can be found under GEO accession GSE13896 (non-smokers, smokers, and COPD patients (E1)), GSE49072 (non-smokers and IPF patients, for patient characteristics, see Supplementary Table E1), and GSE2125 (non-smokers, smokers, and non-smoking asthma patients (E2)).

Human macrophages: Briefly, peripheral blood of healthy donors (n=5, non-smokers) was separated by ficoll density gradient centrifugation and monocytes were isolated using $CD14^+$ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were then cultivated at 5 x 10^6 cells/4 ml in 6-well plates (AIM-V/1 % human serum). Recombinant human M-CSF (50 ng/ml, R&D Systems, Minneapolis, MN, USA) was supplemented on days 0, 2 and 5. On day 7, M-CSF macrophages were harvested and reseeded at 0.4 x 10^6 cells/ml in CSE medium (AIM-V/10 % FBS with indicated concentrations of CSE) for 6 h. Afterwards, cells were harvested for FACS and proteasome analysis. Cell viability after CSE incubation was determined by MTT assay or propidium iodide staining followed by FACS analysis.

Flow cytometry: Cells were harvested and stained for MHC class I using hybridoma supernatant W6/32 (ATCC HB-95) and PE-labeled goat-anti-mouse IgG. Dead cells were determined using propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). Analysis employed the LSRII cytometer (Becton-Dickenson, Franklin Lakes, NJ, USA) and FlowJo Software (TreeStar, Ashland, OR, USA).

Smoke exposure of mice: Eight week old mice were exposed to cigarette smoke for 50 min once ("1 day" group), two days for two times 50 min and the third day once for the "3 days" group or once daily for 10 consecutive days ("10 days" group). The smoke of 10 3R4F research-grade cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) without filter was drawn into the exposure chamber using a peristaltic pump for each 50 min exposure cycle. The mean particle concentration was ~340 mg/m^3. Directly after the last smoke exposure, mice were euthanized and samples were prepared. For immunohistochemistry, non-lavaged lungs were
fixed with 4 % paraformaldehyde and embedded in paraffin. Chronic exposure of mice was performed as described previously (E3).

Mouse cell isolation and culture: All different primary mouse cell-types were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 100 U/ml of penicillin/streptomycin (Life Technologies). Cells were grown at 37 °C in a humidified atmosphere containing 5 % CO_2.

BAL: Mouse lungs were lavaged by inserting a cannula into the trachea and instilling 10 times 500-800 µl of ice-cold sterile PBS into the lungs. Cells were collected and washed with ice-cold PBS before they were taken in culture.

Lung cells: Following euthanasia, the lungs were removed and lung tissue was cut into small pieces with scissors and digested for 45 min at 37 °C in 1 mg/ml collagenase A (Roche, Basel, Switzerland) in RPMI medium. Samples were passed through a 40 µm nylon mesh (Corning, NY, USA) to obtain a single cell suspension. Remaining red blood cells were lysed using RBC Lysis Buffer (eBioscience).

Splenocytes: Splenocytes were isolated by passing whole spleens through a 40 µm nylon mesh. Cells were washed with ice-cold PBS, centrifuged at 1000 rpm at 4 °C and remaining red blood cells were lysed using RBC Lysis Buffer (eBioscience). 1 x 10^5 cells per well were seeded in 96-well plates in FBS-containing medium and CSE was added.

Splenic DCs and Lung CD11c$^+$ cells: CD11c positive cells were isolated using magnetic bead purification (CD11c$^+$ Cell Isolation Kit, Miltenyi Biotec), according to the manufacturer`s instructions.

UTY cell line: The T cell hybridoma cell line UTY, specific for the $UTY_{246-254}$ peptide presented on H-2D^b, was a kind gift from N. Shastri (University of California, Berkeley, CA, USA).

Mouse bronchoalveolar lavage (BAL) cell analysis: For BALB/c total BAL cell analysis, cytospins from 3 x 500 µl lavages were performed, for C57BL/6J, lavages from 10 x 500-800 µl were counted. A maximum of 3 x 10^4 cells were used for cytospins. These were stained according to May-Grünwald (Merck, Whitehouse Station, NJ, USA) and cellular composition was assessed by counting 300 cells per slide.

Mouse alveolar macrophage analysis: BAL cells were prepared as described above, counted, and seeded into 24-well plates with RPMI medium supplemented with 10 % FBS. Cells were allowed to adhere for 30 min. Non-adherent cells were removed by washing twice with PBS. Adherent cells were directly lysed for mRNA or protein analysis or incubated with activity-based probes for 1 hour at 37 °C and were then lysed in RIPA buffer.

Cigarette smoke extract (CSE) preparation: CSE was prepared as previously described (E4). Briefly, a CSE stock was prepared by drawing the smoke of two research-grade cigarettes (3R4F, Tobacco and Health Research Institute) through 50 ml of serum-free medium at RT. Eight of these preparations were pooled, sterile filtered through a 0.20 µm filter (Minisart, Sartorius Stedim Biotech, Göttingen, Germany), aliquoted, and stored at -20 °C until use. For each experiment, 10 % FBS was added freshly. This solution was considered as 100 % CSE and diluted accordingly with serum-containing medium.

Cell viability:

MTT assay: Metabolic activity was evaluated by a colorimetric MTT assay (Tetrazolium Blue, Sigma-Aldrich) as already described (E4, E5). 96-well plates were read on a Sunrise™ plate reader (Tecan, Männedorf, Switzerland) using a wavelength of 570 nm.

Trypan blue exclusion assay: Cell viability was assessed by the trypan blue exclusion method. The number of total and dead cells was counted in duplicates. Treatment of cells with a cell viability < 75 % was excluded.

Quantitative real-time RT-PCR: Total RNA from cells was isolated using Roti®-Quick-Kit (Carl Roth, Karlsruhe, Germany). 100-1000 ng per sample of total RNA was reverse-transcribed using random hexamers (Life Technologies) and M-MLV reverse transcriptase (Sigma-Aldrich). Quantitative PCR was performed using the SYBR Green LC480 System (Roche Diagnostics, Mannheim, Germany) or fluorescent labeled probes were used as previously described (E6), gene-specific primer and probe sequences are listed in Table E2.

Western blotting: Cell and tissue lysis with RIPA buffer as well as Western blot analysis was performed as previously described (E7), antibodies and dilutions are listed in Supplementary Table E3.

Activity-based probe labeling: Activity of standard and immunoproteasome subunits was monitored by using a set of activity-based probes (ABP) (E8). The pan-reactive proteasome ABP MV151 (E9) was used for assessing of total and β2/MECL-1 activities, LW124 for β1/LMP2 activity, and MVB127 was used to label β5/LMP7 (E10).

Hyposmotic native lysates of lungs were labeled with ABPs as described previously (E7), except that instead of ddH_20 for lysis of cells, we used TSDG buffer (10 mM Tris/HCl, 1.1 mM $MgCl_2$, 10 mM NaCl, 0.1 mM EDTA, 1 mM NaN_3, 1 mM DTT, 2 mM ATP, 10 % v/v glycerol, pH 7.0) containing cOmplete protease inhibitors (Roche).

Primary macrophages were directly labeled in full medium containing 0.5 µM MV151 or a combination of 0.25 µM LW124 and 1 µM MVB127 for 1-2 h at 37 °C, washed with PBS and lysed in RIPA buffer. 2 µg of protein were denatured with 6x Laemmli Buffer to a final 1x concentration for gel analysis or Western blotting.

Native gel analysis and substrate overlay:

Chymotrypsin-like proteasome activity in native cell lysates was assessed using the synthetic peptide substrate Suc-LLVY-AMC (Enzo Life Sciences, Farmingdale, NY, USA) and was performed as previously described (E4). Equal amounts of protein (15 µg) of hyposmotic lysates were diluted with 5x native loading buffer (50 % v/v glycerol, 250 mM Tris, 0.1 % w/v bromophenol blue, pH 7.5) and subjected to electrophoresis (4 h, 150 V, 4 °C) on 3-8 % non-denaturing Tris-Acetate gels (Life Technologies). Proteasome activity was detected after incubating the gels for 30 min at 37 °C in substrate buffer (50 µM Suc-LLVY-AMC, 50 mM Tris,

pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT). Gels were analyzed using the ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA) with an excitation wavelength of 380 nm and emission wavelength of 460 nm. Band intensity was quantified with the Image Lab software package (Bio-Rad). Afterwards, proteins were denatured by incubation of the gel in solubilization buffer (2 % w/v SDS, 66 mM Na_2CO_3, 1.5 % v/v 2-ME) for 15 min and proteins were blotted onto PVDF membranes. 20S and 26S bands were identified by using antibodies detecting 20S α1-7 subunits and the 19S subunit Rpt5.

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SUPPLEMENT TABLE

TABLE E1: Controls and IPF patients' data from microarray analysis GSE49072 (personal communication by Ivan Rosas)

	Controls (n=45)	IPF (n=14)	p-value
Gender (m/f)	30/15	13/1	n.s.*
Age (years; mean (SEM))	48 (± 2)	62 (± 2)	<0.001†
Smoking status (NS/smoker)	45/0	5/9	n.s.*
Pack years (median (range))	0	7 (0-40)	<0.01‡

* Statistical analysis was performed using Fisher's exact test

† Statistical analysis was performed using Student's t-test

‡ Statistical analysis was performed using Wilcoxon Signed Rank test (compared to 0)

TABLE E2: Primer sequences

Name	Acc. No.	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)
Mouse (SYBR Green)			
Psmb8	NM_010724.2	TGCTTATGCTACCCACAGAGACAA	TTCACTTTCACCCAACCGTC
Psmb9	NM_013585.2	GTACCGTGAGGACTTGTTAGCGC	GGCTGTCGAATTAGCATCCCT
Psmb10	NM_013640.3	GAAGACCGGTTCCAGCCAA	CACTCAGGATCCCTGCTGTGAT
Rpl19	NM_001159483.1	CGGGAATCCAAGAAGATTGA	TTCAGCTTGTGGATGTGCTC
Human (SYBR Green, Figure 6A)			
PSMA3	NM_002788.3	ACAGTGTGAATGACGGTGCG	GCAGCTTGCCTGGCTTTG
PSMB8	NM_148919.3	AGTACTGGGAGCGCCTGCT	CCGACACTGAAATACGTTCTCCA
PSMB9	NM_002800.4	ATGCTGACTCGACAGCCTTT	GCAATAGCGTCTGTGGTGAA
PSMB10	NM_002801.3	TGCTGCGGACACTGAGCTC	GCTGTGGTTCCAGGCACAAA
RPL19	NM_000981.3	GAGACCAATGAAATCGCCAATG	GCGGATGATCAGCCCATCTT

Human (Fluorescent Reporter Probe, Figure 1A, E1A)

Primer	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)	Probe (5‘-3‘)*
PSMB8	AGTACTGGGAGCGCCTGCT	CCGACACTGAAATACGTTCTCCA	TCGCAGATAGTACAGCCTGCATTCCTTGG
PSMB9	CGTTGTGATGGGTTCTGATTCC	GACAGCTTGTCAAACACTCGGTT	CACCGCCTCGCCTGCAGACACT
PSMB10	TGCTGCGGACACTGAGCTC	GCTGTGGTTCCAGGCACAAA	CCCGTGAAGAGGTCTGGCCGCTAC
RPL19	GAGACCAATGAAATCGCCAATG	GCGGATGATCAGCCCATCTT	CAACTCCCGTCAGCAGATCCGGAA

*Probes labeled with BHQ-1 (PSMA3-PSMB10) or TAMRA (RPL19)

TABLE E3: Antibodies for Western blotting

Antibody	Order number	Manufacturer	Dilution
α1+2+3+5+6+7	ab22674	Abcam (Cambridge, UK)	1:1000
β-Actin	A3854	Sigma-Aldrich (St. Louis, MO, USA)	1:40 000
β1	sc-67345	Santa Cruz (Dallas, TX, USA)	1:200
HLA-A	ab52922	Abcam	1:8000
LMP2	ab3328	Abcam	1:1500
LMP7	ab3329	Abcam	1:1500
PSMA4 (α3)	ab119419	Abcam	1:1000
Tbp1 (Rpt5)	A303-538A	Bethyl Laboratories (Montgomery, TX, USA)	1:3000

SUPPLEMENT FIGURES

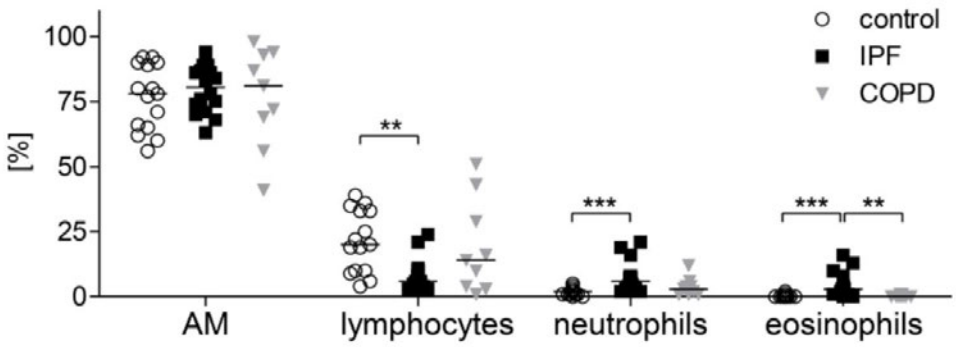

FIGURE E1: BAL characterization of control subjects, COPD, or IPF patients. Cellular composition of human BAL obtained from control subjects, IPF, or COPD patients. (median, Kruskal-Wallis test with Dunn's Post test, ** = $p<0.01$, *** = $p<0.001$). AM, alveolar macrophages; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis.

Group	Smoker	Smoker	COPD	IPF	Asthma
GEO accession	GSE13896	GSE2125	GSE13896	GSE49072	GSE2125
PSMB9 (LMP2)	=	=	-	-	=
PSMB10 (MECL-1)	=	=	-	-	+
PSMB8 (LMP7)	=	=	-	-	=
HLA-A	=	=	-	=	=
HLA-B	=	=	-	-	+
HLA-C	=	=	-	=	+
HLA-E	=	=	-	-	=
HLA-F	-	=	-	-	=
HLA-G	=	=	-	-	+
HLA-J	=	=	-	-	=
B2M	=	=	=	=	=
TAP1	=	=	-	=	=
TAP2	=	=	=	-	+
TAPBP	=	=	-	=	=
CALR	=	=	=	=	=

\+ significantly increased

\- significantly decreased

= no change

FIGURE E2: Microarray expression data from alveolar macrophages of smokers, COPD, IPF, and asthma patients. Summary of microarray results for genes related to MHC I antigen presentation in isolated alveolar macrophages of smokers, COPD, IPF, and asthma patients compared to non-smokers. Mann-Whitney-U or Kruskal-Wallis Test with Dunn's Post Test.

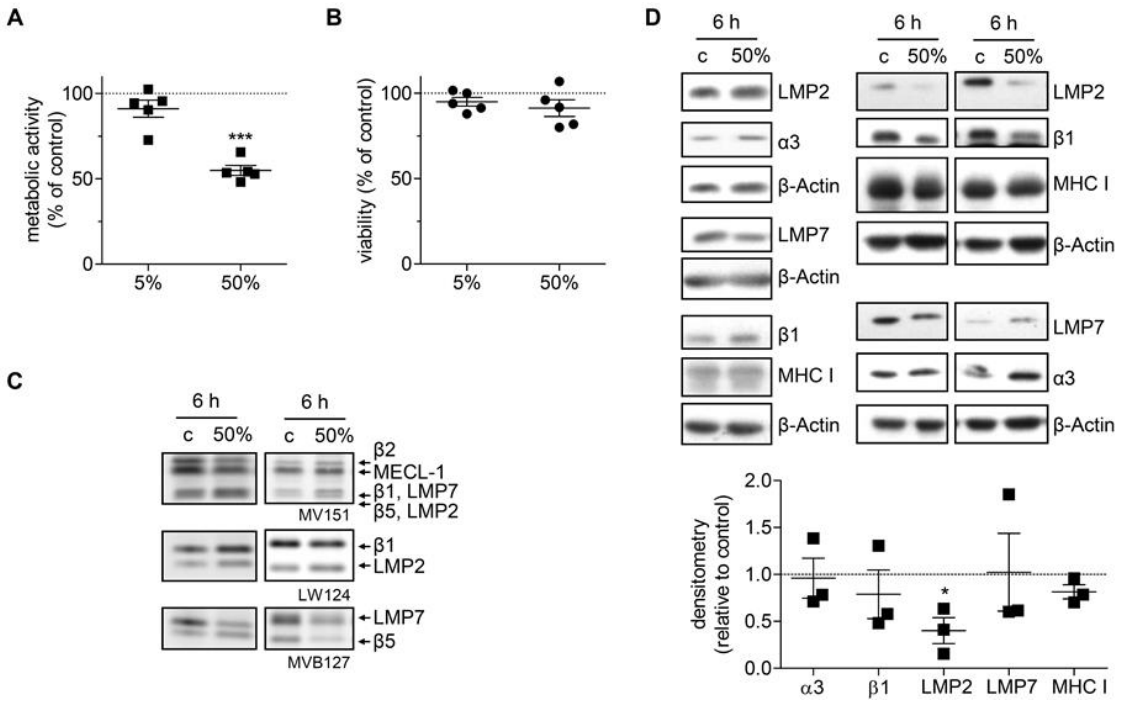

FIGURE E3: Cigarette smoke extract effect on human macrophages. Metabolic activity and viability of human macrophages (n=5) 6 h after treatment with 5 or 50 % cigarette smoke extract compared to untreated cells were tested with (A) MTT or (B) propidium iodide-exclusion assay measured by FACS analysis. (C) Replicates of ABP-gels shown in Figure 2B. (D) Western blot and densitometric analysis of human macrophages after 6 h of 50 % CSE treatment (n=3) of standard proteasome subunits α3 and β1, as well as immunosubunits LMP2 and LMP7, and MHC I (HLA-A) normalized to β-Actin. Statistical analysis: mean +/- SEM, one-sample t-test (compared to 100 % (A,B) or 1 (D)), * = p<0.05, ** = p<0.01, *** = p<0.001). c, control.

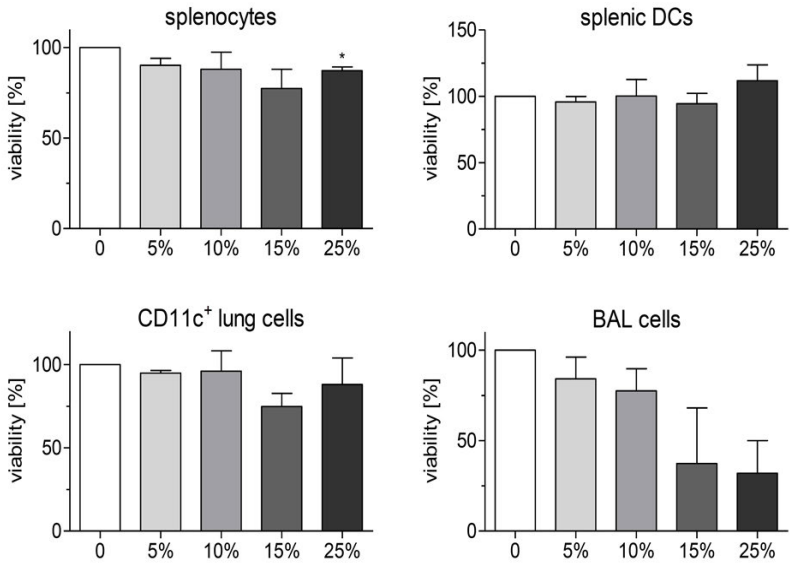

FIGURE E4: Viability of primary cells exposed to cigarette smoke *in vitro*. Viability, measured by trypan blue exclusion assay or MTT, of splenocytes, CD11c$^+$ splenic dendritic cells (DCs), CD11c$^+$ lung cells, or BAL cells treated with increasing percentages of cigarette smoke extract for 24 h (mean + SEM, one-sample t-test, * = p<0.05). Shown are the combined data from two to three experiments, controls were set to 100 %. BAL, bronchoalveolar lavage; DC, dendritic cell

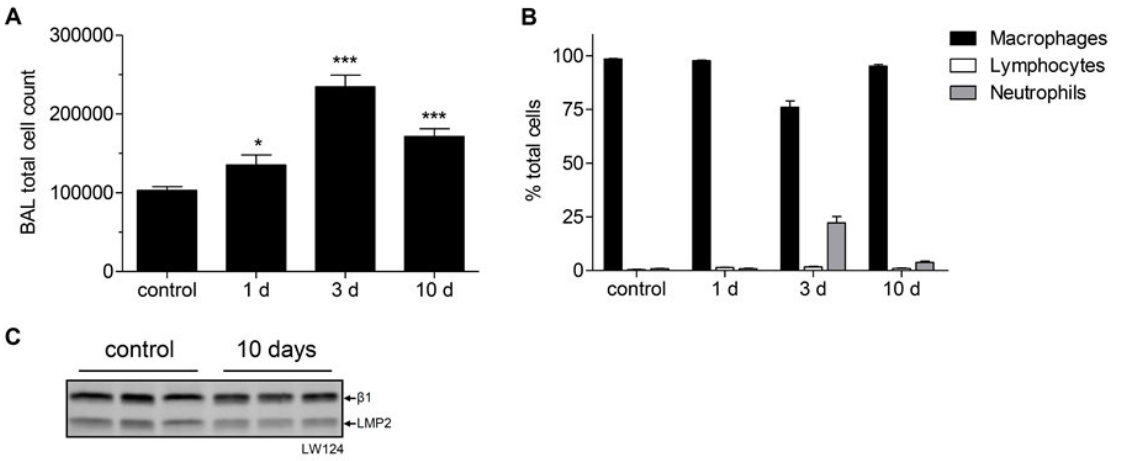

FIGURE E5: BAL characteristics of cigarette smoke-exposed C57BL/6 mice. (A) BAL total and (B) differential cell analysis for male C57BL/6 wt mice exposed to cigarette smoke for 1, 3, or 10 days and controls (mean + SEM, One-Way-ANOVA with Dunnett's Post test, * = $p<0.05$, *** = $p<0.001$, compared to control). (C) ABP-labeling of isolated macrophages from mice exposed to cigarette smoke for 10 days with probe LW124 detecting β1 and LMP2. BAL, bronchoalveolar lavage; d, day.

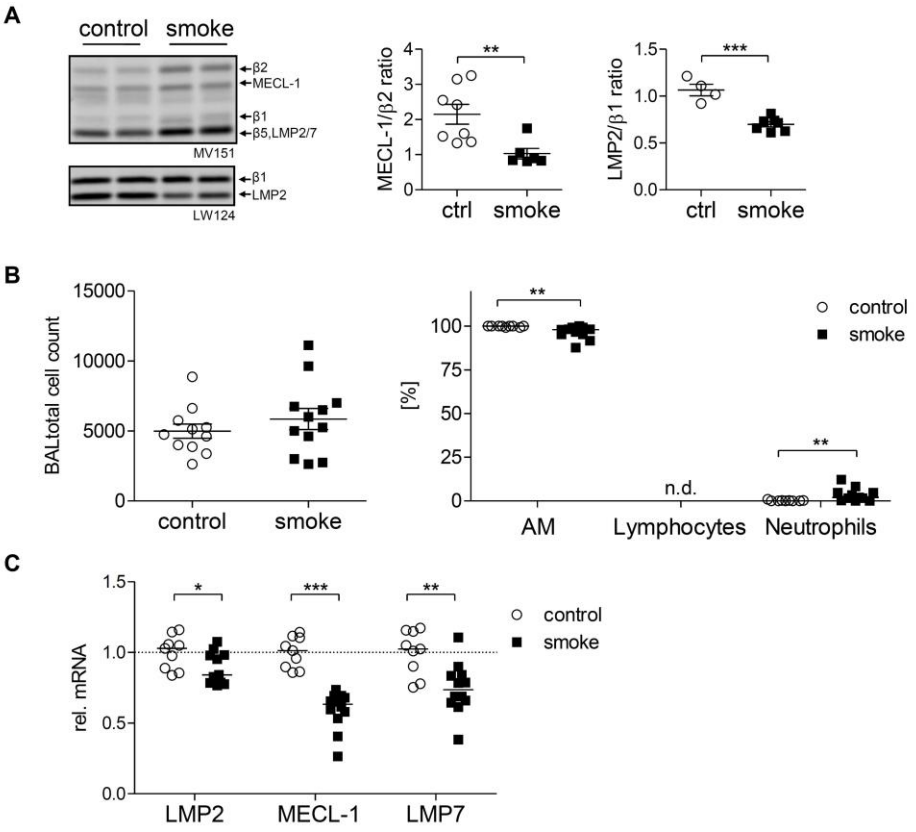

FIGURE E6: Immunoproteasome expression and activity in alveolar macrophages of cigarette smoke-exposed BALB/c mice. (A) Proteasome activity in isolated alveolar macrophages from female BALB/c mice exposed to cigarette smoke for 10 days compared to controls labeled with pan-reactive activity-based probe (ABP) MV151 or LMP2/β1-specific ABP LW124. Ratios of MECL-1/β2 or LMP2/β1 activities were analyzed by densitometry. Results are combined data from three (MV151) or two (LW124) independent experiments with several mice per group (mean +/- SEM, Student's t-test ** = p<0.01, *** = p<0.001). (B) BAL total and differential cell analysis for BALB/c mice smoke-exposed for 10 days and air-exposed controls (mean +/- SEM, Student's t-test (total cell count), median, Mann-Whitney-U test (differential cell count) ** = p<0.01). (C) mRNA analysis of immunoproteasome subunits LMP2, MECL-1, and LMP7 in alveolar macrophages isolated from control or smoke-exposed mice. Rpl19 was used as a housekeeping gene (median, Mann-Whitney-U test, * = p<0.05, ** = p<0.01, *** = p<0.001). AM, alveolar macrophage; BAL, broncholaveolar lavage; ctrl, control; n.d., not detected.

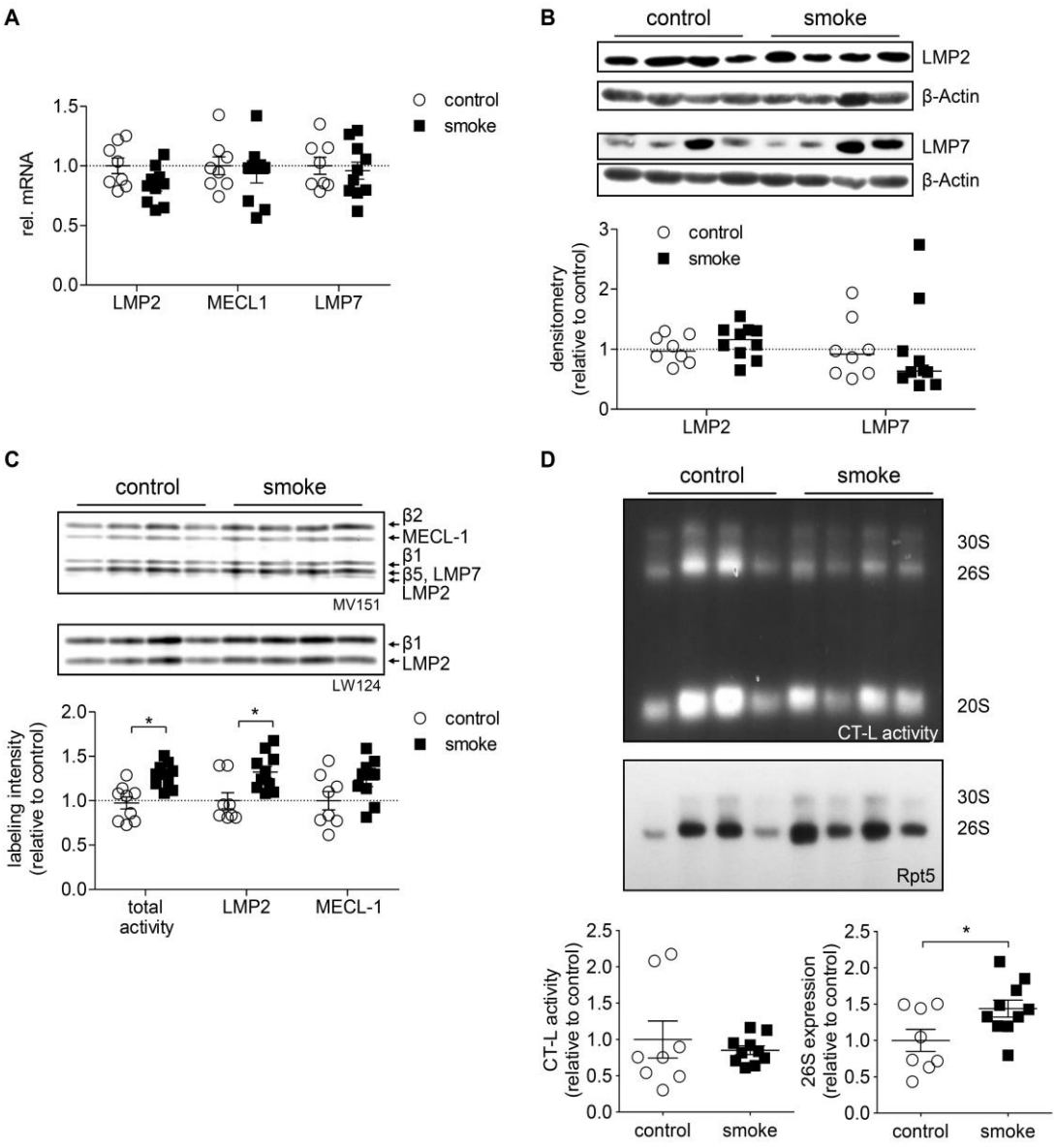

FIGURE E7: Cigarette smoke affects activity of proteasomes in the mouse lung. (A) qRT-PCR mRNA analysis of immunoproteasome subunits LMP2, MECL-1, and LMP7 in total lungs of control mice (n=8) and mice that have been exposed to cigarette smoke for 4 months (n=10), (mean +/- SEM, Student's t-test). (B) Representative Western blot of total lung lysates of controls or cigarette smoke-exposed mice for immunosubunits LMP2 and LMP7. β-Actin served as loading control. Densitometric analysis from normalized data of two independent mouse experiments is shown (median, Mann-Whitney-U test). (C) Activity-based probe profiling of the same lungs as in (B): native lung lysates were labeled with pan-reactive ABP MV151 (labeling all six active proteasome sites) or LW124 (labeling LMP2 and β1), and separated on denaturing SDS

gels. Densitometric analysis of total activity (MV151 signal) and single subunit activity is normalized to the mean of controls activities, densitometric results are combined data from two independent experiments (mean +/- SEM, Student's t-test, * = $p<0.05$). (D) Native gel analysis with chymotrypsin-like (CT-L) substrate overlay analysis and immunoblotting of native lung lysates to detect 26S (20S + 19S) proteasome complexes with an Rpt5 (19S subunit)-specific antibody. Densitometric analysis of chymotrypsin-like activity and 26S expression (Rpt5 signal) are shown, results are combined data from two independent experiments (mean +/- SEM, Student's t-test, * = $p<0.05$).

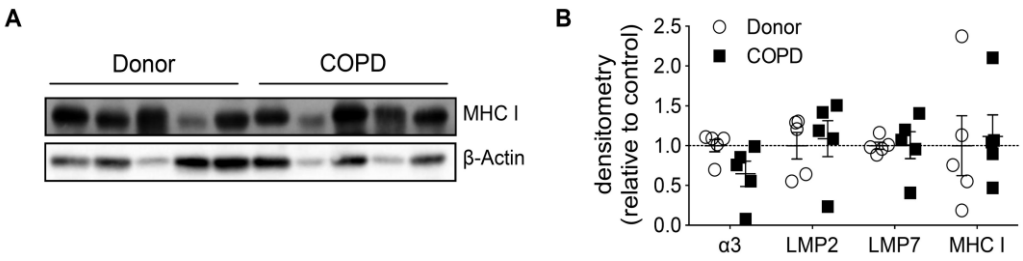

FIGURE E8: Western blot analysis of human COPD tissue. (A) Western blot detecting total MHC I (HLA-A) in total lung lysate of donor or COPD tissue. (B) Densitometric analysis of Western blots shown in Figures 6B and E8A. COPD, chronic obstructive pulmonary disease; HLA, human leukocyte antigen; MHC I, major histocompatibility complex class I.

2.2.2 Author's contribution

Ilona E. Kammerl	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. 3B/C/D/E, 4, 5D, E4); cigarette smoke exposure of mice (fig. 5A/B/C, E5); quantitative real-time RT-PCR (fig. 1A, E6C, E7A); analysis of published microarray data (fig. 1B/C/D/E, E2); $UTY_{246-254}$ assay (fig. 3A/B/E, 5D); Western Blot (fig. 3C, 5A, 6B/D, E7D); Western Blot analysis (fig. 3C, 5A, E3D, E7D, E8); proteasome activity assay with activity-based probes (fig. 2B, 3D, 5B/C, 6C, E3C, E5C, E6A, E7C); proteasome activity assay with native gel and substrate overlay (fig. 6D, E7D); study design; preparation and editing of figures and manuscript.
Angela Dann	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. 3D, 4, 5D, E4); cigarette smoke exposure of mice (fig. 5A/B/C, E5); $UTY_{246-254}$ assay (fig. 3A/B/E, 5D); study design; preparation and editing of figures and manuscript.
Alessandra Mossina	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. 2B, E3); Western Blot (fig. E3D, E8); supervision of C. Lukas; study design; preparation and editing of figures and manuscript.
Dorothee Brech	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. 2B, E3); FACS experiments and analysis (fig 1A, E3B); preparation and editing of figures; editing of manuscript.
Christina Lukas	in vitro cell culture experiments of exposure to cigarette smoke extract (fig. 3D, 4, 5D, E4); quantitative real-time RT-PCR (fig. 6A); Western Blot (E3D, E7B).
Oliver Vosyka	proteasome activity assay with activity-based probes analysis (fig. 6C); proteasome activity assay with native gel analysis (fig. 6B).
Petra Nathan	cigarette smoke exposure of mice (fig. 5); BAL analysis (fig. E6B)
Thomas M. Conlon	cigarette smoke exposure of mice (fig. E7)
Darcy E. Wagner	microarray analysis (fig. 1)
Hermen S. Overkleeft	provision of activity based probes (fig. 2B, 3D, 5B/C, 6C, E3C, E5C, E6A, E7C).
Antje Prasse	provision of human BAL samples (fig. 1A, E1)

Ivan O. Rosas	provision of clinical data and microarray results of IPF patients (fig. 1C, E2)
Tobias Straub	microarray analysis (fig. 1, E2)
Susanne Krauss-Etschmann	supervision of P. Nathan; editing of manuscript.
Melanie Königshoff	supervision of D.E. Wagner.
Gerhard Preissler	provision of human lung samples (fig. 6).
Hauke Winter	provision of human lung samples (fig. 6).
Michael Lindner	provision of human lung samples (fig. 6).
Rudolf Hatz	provision of human lung samples (fig. 6).
Jürgen Behr	provision of human lung samples (fig. 6).
Katharina Heinzelmann	provision and preparation of human lung samples (fig. 6).
Ali Ö Yildirim	supervision of T.M. Conlon.
Elfride Nößner	provision of normalized microarray data (fig. 1B/D/E, E2); supervision of D. Brech; editing of manuscript.
Oliver Eickelberg	provision of human lung samples (fig. 6); supervision of K. Heinzelmann; editing of manuscript.
Silke Meiners	supervision of I.E. Kammerl, A. Dann, A. Mossina, C. Lukas and O. Vosyka, study design and editing of figures and manuscript.

3 DISCUSSION

Within the two presented publications, the biological consequence of exposure to cigarette smoke has been investigated *in vitro* in lung alveolar cells and in lung immune cells, and *in vivo* in mouse models and in lung tissue of COPD patients. More specifically, in the first study, the effects of cigarette smoke exposure were studied in the context of overall proteome alterations and protein homeostasis, while the second study focused on cigarette smoke-mediated effects on proteasome-dependent MHC class I antigen presentation.

3.1 Tobacco smoke and protein homeostasis: implications for COPD

3.1.1 Cigarette smoke, proteostatic stress and alterations of the cellular proteome

The cellular proteome is a complex mixture of structural and regulatory proteins that requires to be kept in proper balance according to the functions and dynamic needs of the cell. Protein biogenesis and degradation machineries work to maintain proteome fidelity and a balanced protein flux within the cell (Fig. 5)[81]. Cigarette smoke, as previously mentioned, has inhibitory effects on the degradation capacity of the cell and thus potentially alters cellular localization and abundance of cellular proteins.

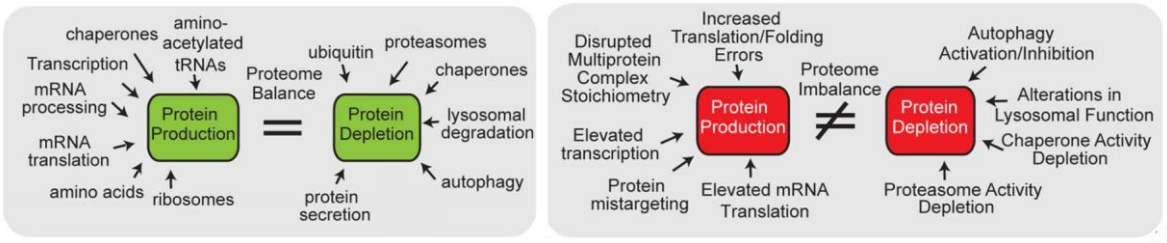

Figure 5 – Dynamic control of proteome complexity. In a balanced proteome, cellular mechanisms that contribute to protein anabolism and protein catabolism work together to execute the dynamic control of proteome complexity (left panel). Changes in protein degradation due to alterations in proteasome and autophagy functions, if not properly counteracted by protein production mechanisms, will lead to an imbalance in the proteome promoting proteotoxic stress (right panel)[81].

To investigate protein homeostasis functionality and proteome fidelity upon exposure to cigarette smoke the most common methodology used is quantitative-MS (mass spectrometry) that allows identifying proteomic changes due to transient or chronic stress. In the past years, several proteomic studies have been applied to lung tissue and cells with the aim of identifying possible alteration in the abundance and in the posttranslational modification of proteins affected by the exposure to cigarette smoke[82]. Most of these studies involved 2D gel electrophoresis-MS analysis of *in vivo* samples, including sputum, lung tissue, bronchoalveolar lavage and epithelial lining fluids from non-smokers, healthy smokers and COPD subjects[83–87] or from animal models[88]. Some recent studies investigated the effect of chronic cigarette smoke exposure on C57BL/6 mice and lung cancer cells by complementing 2D gel electrophoresis-MS analysis with a liquid chromatography-tandem mass spectrometry workflow using isobaric mass tags[89–91] or stable isotope labeling with amino acids in cell cultures (also known as SILAC)[92,93]. Other *in vitro* proteomic studies have been conducted on pulmonary human fibroblasts and bronchial airway epithelium cells exposed to cigarette smoke[84,94–96]. However, no study has investigated the effect of cigarette smoke in pulmonary epithelial cells forming the critical barrier between the internal and external environment and where reactive compounds of cigarette smoke will interact with and consequently modify lipids, nucleic acids and proteins[82,97]. In addition, in none of the previous studies cell compartment-specific-changes have been investigated. For this reason we used alveolar epithelial cells and subcellular fractionation coupled with label-free quantitative mass spectrometry to identify possible alteration in the cellular compartment-specific proteome. While we did not notice major alterations in the subcellular distribution of proteins upon exposure of alveolar epithelial cells to non-toxic doses of cigarette smoke, we observed the most prominent changes in the cellular secretome[76]. As we did not observe any obvious sign of proteostatic stress upon cigarette smoke exposure, we cannot establish a direct connection between the altered abundance of secreted proteins and proteostatic stress responses. Proteomic changes might be due to a slight impairment in the secretion process as well as in the degradation machinery, as shown to occur in alveolar epithelial cells exposed to cigarette smoke[50]. By studying more deeply some altered proteins, we noticed that downregulation of proteins was reflected also on transcript levels, possibly indicating indirect effect of an imbalanced proteostasis. In fact, cumulative evidence indicates that the proteasome not only helps maintaining a stable proteome through posttranslational degradation, but also by affecting transcription and RNA stability and therefore regulating gene expression. For example, the proteolytic activity of the proteasome might influence location and lifetime of transcriptional activators, co-activators and repressors, leading to the hypothesis that changes in proteome stability might be indirectly responsible for altered transcriptional responses[98,99]. Previous studies have observed different gene expression profiles due to inhibition of proteasome function[100,101]. Bieler and colleagues identified more than 50 nuclear proteins altered upon chemical inhibition of the proteasome, with

particular enrichment of proteins involved in RNA splicing, binding and transport, emphasizing the role of the proteasome also in protein biogenesis[102]. In our study, about a fourth of the proteins significantly regulated by CSE were nuclear proteins with functions in splicing and translation cellular activities[76]. This observation further suggests that inhibition of the proteasome activity observed upon CSE exposure might alter the proteome balance by affecting the abundance of proteins involved in transcriptional process. The interplay between proteasome activity inhibition and reduced mRNA abundance should be further investigated.

Tobacco smoke not only destabilizes the cellular proteome but it may also directly modify and impair protein complexes involved in protein control pathways. Indeed, *in vitro* exposure of purified 20S proteasomes to cigarette smoke extracts provoked a dose-dependent inhibition of proteasomal activity, leading to the conclusion that cigarette smoke can have a direct effect on the proteasome[50]. It is not fully understood how cigarette smoke alters proteasome activity. One possibility is that cigarette smoke affects the interaction of proteasome with proteasome alternative regulators (i.e.: PA28αβ, PA28γ, PA200 and PI31), directly through oxidative modifications or indirectly as a secondary effect of proteasome activity inhibition upon CS exposure, as suggested by our group[103]. Previous studies have shown that stability of the 26S complex is compromised upon oxidative stress induction[104–106]. Giving the fact that cigarette smoke influences the redox state of the cell, we hypothesized that the composition and the integrity of the proteasome are altered when exposed to tobacco smoke. Recently published data from our group[107] show that cigarette smoke induces changes in the stability of proteasome complexes. We observed that 26S proteasome complexes become slightly instable in response to acute cigarette smoke exposure both *in vitro* (A549 cells) and *in vivo* (mice). More specifically, using proteomic approaches, we found diminished interactions of 19S subunits with 20S proteasome complexes, indicating a partial disassembly of 26S proteasomes upon CSE exposure. In addition, the composition of 26S proteasome isolated from A549 lung epithelial cells or from lung tissue of mice exposed to cigarettes smoke was analyzed by mass spectrometry. In both cases, the proteasome alternative regulators PA200 and PA28αβ were found to be less stably bound, suggesting that cigarette smoke affects the interaction of 26S proteasomes with alternative regulators. The 20S CP alone is ineffective in protein degradation, as the entry to the catalytic core is obstructed by the N-termini of the outer α-subunits[35,108]. The association with proteasome regulators therefore is particularly relevant because it determines the opening of the CP gate and subsequently proteasome activity and function. Meiners and colleagues proposed that proteasome complexes can be considered as dynamic building blocks that assemble or disassemble according to cellular needs and status[35]. The association of the 20S proteasome with the regulatory particles might represent a rapid mechanism by which the cell responds to some specific stimuli and signaling. According to our unpublished data, tobacco smoke appears to weaken the binding of regulators to the 20S CP which may contribute to the reduction in

proteasome activity observed in alveolar epithelial cells and mice upon acute tobacco smoke exposure[50,51]. Interestingly, the reduced interaction of the 20S proteasome with its regulators is not due to cigarette smoke-induced post-translational modifications (PTMs) of proteasome subunits, as in our study we didn't identify any stable modification of proteasomal proteins[107].
A balanced proteome is achieved by regulated protein biogenesis involving transcriptional, splicing, mRNA stabilizing and folding processes and by controlled protein depletion mechanisms (UPS and ALP) and secretion processes (Fig. 5). The observation in our study that both proteins and mRNAs levels are altered suggests that cells exposed to cigarette smoke have lost their capacity of maintaining a balanced proteome. Cellular events observed upon exposure to cigarette smoke such as increased folding errors, impaired transcription rates, proteasome activity inhibition, accumulation of autophagosomes and decreased lysosomal function (as described in section 1.2.1) may contribute to proteome imbalance promoting proteotoxic stress. Further proteomic studies might help in identifying which proteins levels are altered upon tobacco smoke exposure and establish a causal relationship between those proteins and their implications for the development of the pathological characteristics typical of COPD.

3.1.2 Implications of secretome alterations for COPD

In our study, we observed the most prominent changes in the cellular secretome of human and murine alveolar epithelial lung cells, identifying in particular alterations in proteins involved in wound-healing response and ECM reorganization. More specifically, progranulin was found downregulated on protein and transcript levels in human and murine lung epithelial cell culture as well as in primary murine alveolar cells[76]. Progranulin is a wound-healing mediator needed for the formation of a fibronectin scaffold necessary for subsequent collagen deposition and proper tissue regeneration[109,110]. The reduce levels of progranulin indicates a possible impairment in the healing process of epithelial cells after acute exposure to cigarette smoke. Similarly, SPARC (secreted protein acidic and rich in cysteine) has been identified downregulated in our proteomic profiling and subsequently confirmed on protein and mRNA levels in both human and murine alveolar epithelial lung cells and in the more physiological relevant setting of the three-dimensional *ex-vivo* lung tissue cultures[76]. SPARC is a matricellular protein that does not contribute structurally to the ECM, but modulates interactions between cells and the extracellular environment. SPARC is highly expressed in tissues undergoing injury repair and/or development process[111,112] and acts by binding several proteins resident of the extracellular matrix and concerting the activity of extracellular proteases and growth factors[113]. SPARC-null mice have several altered phenotypes, related to ECM dysregulation, i.e. skin and connective tissue of heart with less fibrillar collagen and osteopenia[114,115]. Giving its role as communicator at the cell-ECM interface, SPARC regulates

ECM assembly and deposition as well as growth factor signaling thereby contributing to pathological remodeling of the extracellular matrix. Moreover, several studies indicate a reciprocal regulation of SPARC and TGF-β (transforming growth factor-β), one of the main proteins that orchestrates ECM remodeling and whose superfamily members were also found downregulated in our proteomic profiling[116,117]. Although SPARC is quite well studied in the contest of lung cancer and pulmonary fibrosis, little is known about its role in other lung diseases characterized by ECM remodeling, such as COPD[118]. Our finding that SPARC expression is downregulated upon cigarette smoke exposure in alveolar epithelial lung cells suggests that its dysregulation might be connected to the aberrant extracellular matrix remodeling that is considered an hallmark of COPD[119–121].

Besides its role in extracellular matrix remodeling and wound repair, it has been proposed that SPARC might have a pleiotropic role in the immune and inflammatory response[118]. For example, SPARC null-mice show impaired leukocyte recruitment and in different disease models SPARC is associated to decreased or oppositely to enhanced immune and inflammatory responses[122–126]. These studies pose the question of the possible role of SPARC in the aberrant and persistent activation of the immune system in chronic diseases like COPD.

3.2 Tobacco smoke and the immune system: implications for COPD

MHC class I antigen presentation is the main mechanisms by which cells communicate to the immune system the presence of a foreign antigen. By producing peptides that efficiently bind MHC class I molecules, immunoproteasome are specialized types of proteasomes and central players in the process of antigen presentation. As for proteasomes, immunoproteasomes might as well be affected by cigarette smoke. The fact that immunoproteasomes have different cleavage capacities compared to standard proteasome, has posed the question on the possible different ability of immunoproteasomes in degrading oxidatively modified proteins. This question is still a matter of debate, as contrasting results have been reported[127,128]. In our study we investigated the expression and activity of the immunoproteasome upon cigarette smoke exposure. Confirming previous data on expression of the immunoproteasome[129,130], our own results identified that both standard and immunoproteasome subunits are not altered in their expression level in end-stage COPD lung tissue[131]. Most importantly, while the expression was unaltered, we observed a pronounced decrease in both standard and immunoproteasome activity as determined by two different activity assays, namely the activity-based probe (ABP) labeling and the native gel analysis with chymotrypsin substrate overlay[131]. Similar results were obtained also *in vitro* when

murine splenocytes where exposed to non-toxic concentration of cigarette smoke extract. In fact, while the expression of the immunoproteasome subunits LMP2 and LMP7 was not altered, we observed proteasome and immunoproteasome decreased activities[131]. On the contrary, we noticed an overall increase of both standard and immunoproteasome activities in total lung tissue of mice chronically exposed to cigarette smoke [132]. This result might be due to the fact that perhaps our murine model of chronic exposure does not fully reflect the complex features typical of end-stage COPD lungs. In alveolar macrophages of mice acutely exposed to cigarette smoke, however, both standard and immunoproteasome are dynamically regulated with increased expression and activity after 3 days of smoke exposure, followed by a decrease at day 10[132]. The dissimilarity of results obtained *in vivo* and *in vitro*, might be partially explained by the differences between CSE and full smoke.

The altered regulation of immunoproteasome upon exposure to cigarette smoke is of particular relevance if we take in consideration that this protease is also one important player of the antigen presentation pathway. Indeed, immunoproteasomes not only are the main form of proteasome expressed in immune cells, but they also have altered cleavage kinetics that favor the generation of antigenic peptides preferentially presented by MHC class I molecules. As such, immunoproteasomes contribute in mounting a proper adaptive immune response against virus-infected cells. Failure in generating this response could lead to impaired clearance of pathogens, sustained viral infections and susceptibility to bacterial diseases that are associated with COPD exacerbations (Fig. 6)[12,62,133]. This hypothesis is further corroborated by our observation that in human blood-derived macrophages exposed to cigarette smoke extract not only the activity of the immunosubunits LMP7 was decreased, but also the expression of MHC class I molecules on the cell surface[131]. In addition, our results showed that tobacco smoke impaired MHC-I mediated antigen presentation of a specific immunoproteasome epitope in splenic and lung antigen presenting cells[131]. This observation implies that cigarette smoke impairs not only immunoproteasome activity but also affects MHC class I antigen presentation and $CD8^+$ T cell-mediates immune response (Fig. 6). In accordance to our results, Kincaid E.Z. et al. showed that an immunoproteasome triple knock-out mouse, lacking all three immunoproteasome subunits, has severely impaired MHC class I antigen presentation[134]. Protecting specifically the immunoproteasome from the detrimental effects of tobacco smoke or compensating for the loss of its activity could be a potential mechanism to improve immune responses against infection and reduce the likelihood of exacerbation in COPD.

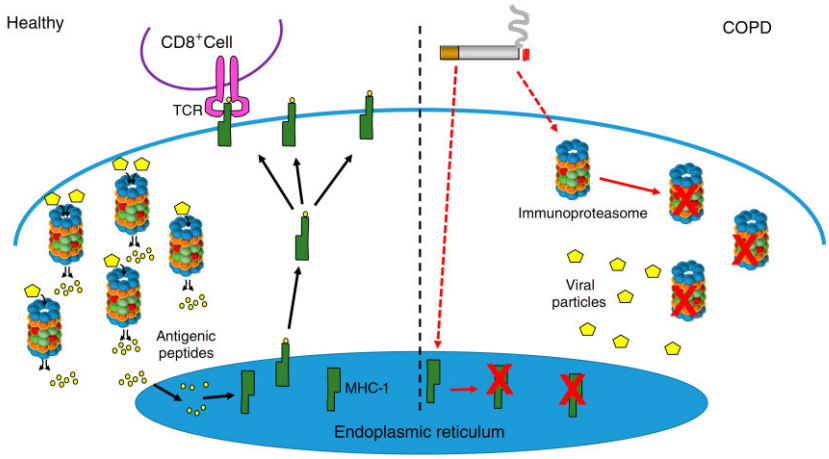

Figure 6 – Implications of immunoproteasome impairment in COPD

In a healthy cell, viral particles are processed by the immunoproteasome that generates antigenic peptides preferentially loaded onto MHC class I molecules pockets. The antigenic peptides are therefore presented on the cell surface where they can interact with the immune system via $CD8^+$ T cells and induce an effective adaptive immune response. In a COPD cell, cigarette smoke affects immunoproteasome activity and consequently reduces the generation of antigenic peptides that can be presented at the immune system via MHC class I molecules[135].

3.2.1 Potential consequences of altered immunoproteasome activity in autoimmune mechanisms of COPD

CD8+ T cells infiltration of the lungs in response to cigarette smoke exposure is a feature of the chronic inflammation in COPD[60,63]. The fact that such inflammation persists even after smoking cessation suggests the possibility that there has been a break of tolerance of the immune system to self-proteins rising from the initial noxious stimuli[60]. The alteration in the immunoproteasome activity that we observed in our study[131] might play a pivotal role in the induction of autoimmunity typical of COPD patients.

As previously mentioned, the immunoproteasome operates in the negative selection of T cells that takes place in the thymus. If the activity of the immunoproteasome (and possibly of the thymoproteasome) is decreased upon exposure to tobacco smoke, the generation of the peptides required for the positive selection would be altered as well. The survival of T cell therefore could be diminished with a direct impact on the proper production of the $CD4^+$ and $CD8^+$ lineages. On the other hand, if the activity of the immunoproteasome fails during the process of negative selection, the MHC class I antigen repertoire, generated for the elimination of those T cells that

bind too strongly the complex MHC class I-peptide, would be altered and/or incomplete. This possible scenario is important in the process of autoimmunity, as some T cells that recognize autoantigens could escape the negative selection, migrate to the lymphonodes and be subsequently activated against “self” antigens.

Immunoproteasome are highly expressed in APCs where they process cellular proteins, but also viral or bacterial proteins, presenting the resulting epitopes to naïve T cells for priming of active T cells and induction of an adaptive immune response. On the contrary, in most non-immune tissues the basal expression of immunoproteasome is very low[43]. This means that in normal conditions the priming of T cells occurs against peptides mostly produced by the immunoproteasome. $CD8^+$ T cells would be therefore not activated against those epitopes that are instead produced by constitutive proteasomes, expressed by non APCs, in non-lymphoid organs and not in the presence of co-stimulatory cytokines[43]. The data presented in our study indicate a dysfunction in immune cells of the lung as a consequence of exposure to cigarette smoke extract[131]. Under this condition, murine lung $CD11c^+$ cells (which include macrophages and dendritic cells) failed in presenting an immunoproteasome-dependent MHC class I peptide. As a direct effect of the reduced immunoproteasome activity, professional antigen presenting cells of the lung would present on their surfaces more epitopes produced through standard proteasomes and the priming of T cells would occur against standard proteasome-derived self-peptides. This would generate and perpetuate the production of autoreactive $CD8^+$ T cells that cause an autoimmune attack against epitopes normally presented in uninflamed tissues[43].

There is finally a third possibility for an immunoproteasome-dependent induction of autoimmunity in COPD patients. Some PTMs, generated on proteins through the oxidative stress caused by cigarette smoke, might create new antigenic peptides for which immune tolerance does not exist (Fig. 7B, possibility a). The mechanisms of the breakdown of tolerance by post-translationally modified proteins are still not clear. One hypothesis is that some specific PTMs are not present during the selection process that occurs in the thymus. Autoreactive T-cells would then be allowed to escape the negative selection and migrate to the periphery[136]. There is the possibility that the post-translational modification alters the binding of the peptide to the MHC class I groove, but in some cases the presence of a PTM does not affect at all the binding peptide-MHC I[137]. Some modification might occur even after the association of the peptide with MHC class I molecules[136] (Fig. 7B, possibility c). New antigenic peptides might arise not only by the addition of a PTM to a non-immunogenic peptide, but also by the generation of a new set of peptides. Amino acid modifications in fact could directly affect how proteins are processed by the immunoproteasome. PTMs might hide or expose cleavage sites normally recognized by the immunoproteasome, resulting in the creation of new autoantigens[136,138] (Fig. 7b, possibility b). The decreased presentation of an immunoproteasome-dependent MHC I peptide observed in our

study[131] might be due to the fact that a possible PTM impedes the cleavage of the proteins or alters the cleavage site for the production of the specific peptide.

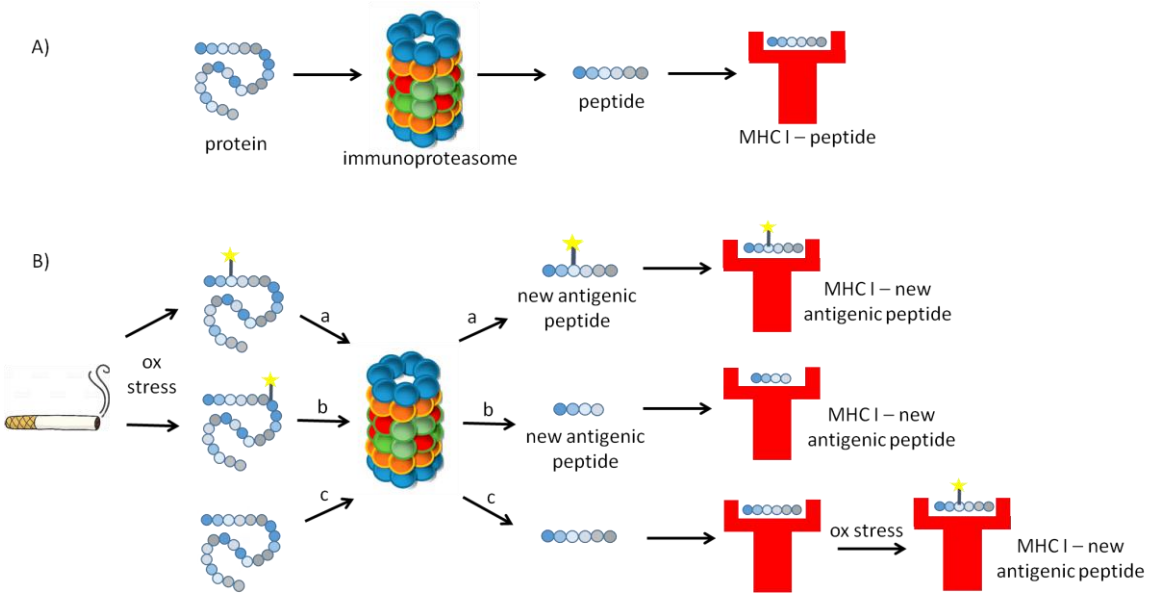

Figure 7 – Generation of new antigenic peptides – a hypothesis. A) In the absence of cigarette smoke, a protein is cleaved by the immunoproteasome into peptides subsequently loaded onto the MHC class I for presentation to the immune system. B) In the presence of cigarette smoke the proteins might be modified via oxidative stress (PTM represented as a star) and new antigenic peptides are generated. Peptides could have the same amino acid sequence and carry a PTM (a) or have a different amino acid sequence if the immunoproteasome recognizes a different cleavage site (b). In some cases, PTMs might even occur after the binding of the peptide into the MHC class I groove (c).

The post-translational modification of antigens is one way by which immune tolerance might be bypassed. The identification of several autoimmune autoantibodies in COPD patients, including antibodies directed against carbonylated proteins, suggest not only that COPD can indeed be considered an autoimmune disease, but also that post-translationally modified proteins can be a relevant factor for the development of such disease[73,74]. The pathophysiological mechanisms of the autoimmune component in COPD have not been well studied. Whether it is the PTMs of proteins or a failure in the negative T cell selection process in the thymus, or a combination of both will have to be further investigated.

3.3 Conclusion and Outlook

Future work should highlight the role of proteasome and immunoproteasome in the development and progression of COPD. Proteostasis and immune system are both affected in COPD patients. The decrease in proteasome activity observed upon exposure to cigarette smoke extract[50] might have detrimental effects on cellular proteostasis and affects the abundance of protein expressed[76] resulting in a severe proteome imbalance and alteration in proteome composition. The progressive loss of proteome balance will in return overboard the proteostasis system further driving proteostatic stress typical of COPD.

Similarly, reduced expression and activity of the immunoproteasome might affect the generation of the antigenic peptides that are presented to $CD8^+$ T cells via MHC class I molecules. This would have consequences on the proper function of the immune system, contributing to impaired clearance of pathogens and susceptibility to exacerbations in COPD patients.

In the light of our studies and previous observations, the evaluation of proteasome and immunoproteasome activities in blood immune cells of COPD patients should be further studied and might be considered as possible biomarker for the development of the disease. At the moment there are not predictive biomarkers for identification of individual at risk and their possible lung function deterioration. In particular, the profiling of immunoproteasome activity might correlate directly with the abnormal immune response of the COPD lung and could serve as biomarker for patients susceptible to sustained infections and exacerbation.

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