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Proteasome and immunoproteasome function in cigarette smoke-mediated chronic lung disease

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Table of contents

List of abbreviations

19S	19S proteasome activator
20S	20S proteasome core particle
26S	20S proteasome in complex with one 19S activator
30S	20S proteasome in complex with two 19S activators
ABP	activity-based probe
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
BMDC	bone marrow-derived dendritic cell
CD	cluster of differentiation
CHIP	carboxyl terminus of the Hsc70-interacting protein
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
CSE	cigarette smoke extract
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
EFRAIM	Mechanisms of early protective exposures on allergy development
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FDA	Food and Drug Administration
GOLD	Global initiative for obstructive lung disease
HLA	human leukocyte antigen
Hsp	heat shock protein
ICAM	intercellular adhesion molecule
IFNγ	interferon-γ
LCMV	lymphocytic choriomeningitis virus
LMP	low molecular mass protein
MDa	megadalton
MECL-1	multicatalytic endopeptidase complex-like 1
MHC	major histocompatibility complex

MHV	murid herpesvirus
mTEC	medullary thymic epithelial cells
PA	proteasome activator
PASTURE	Protection against Allergy: Study in Rural Environments
PBMC	peripheral blood mononuclear cell
PI	proteasome inhibitor
polyI:C	polyinosinic:polycytidylic acid
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
TCR	T cell receptor
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
UPR	unfolded protein response
wt	wildtype

Publications included in this thesis

Peer-reviewed publications

Acute cigarette smoke exposure impairs proteasome function in the lung

Sabine H. van Rijt, **Ilona E. Keller**, Gerrit John, Kathrin Kohse, Ali Ö. Yildirim, Oliver Eickelberg, and Silke Meiners. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L814–823.

Regulation of Immunoproteasome Function in the Lung

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Publications not included in this thesis

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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, and Silke Meiners. *Am J Respir Crit Care Med*. First published online 12 Jan 2016 as doi: 10.1164/rccm.201506-1122OC

Regulation of the Proteasome: Evaluating the Lung Proteasome as a New Therapeutic Target

Silke Meiners, **Ilona E. Keller**, Nora Semren, and Anne Caniard. *Antioxid Redox Signal* 2014 Dec 10;21(17):2364-82.

Summary

Chronic obstructive pulmonary disease (COPD) is projected to be the third leading cause of death by 2020 with cigarette smoke exposure being the main risk factor. Cigarette smoke leads to oxidative stress in the lung, resulting in protein damage and adaptive immune responses. Also, smokers and COPD patients are more susceptible to viral infections often followed by acute exacerbations of COPD pathogenesis. Lungs of COPD patients exhibit increased numbers of innate and adaptive immune cells, among these $CD8^+$ T cells, whose abundance correlates with disease severity. The proteasome degrades more than 90 % of intracellular proteins - including damaged ones - into small peptides and is important to protect the cell from proteotoxic stress. Furthermore, the immunoproteasome, a specialized proteasome subtype which is expressed by default in antigen presenting cells and induced during infection, is involved in shaping adaptive immune responses by enhancing antigen presentation via major histocompatibility complex (MHC) I to cytotoxic $CD8^+$ T cells. The effects of cigarette smoke on (immuno-)proteasome function have not been investigated so far.

The first publication included in this thesis (van Rijt et al. 2012) explored the effects of acute cigarette smoke exposure on proteasome expression and activity. We observed that short-term exposure of cells to extracts of cigarette smoke directly impaired proteasome activity, while proteasomal protein expression was not altered. Oxidatively modified and polyubiquitinated proteins accumulated, suggesting augmentation of oxidative stress in cigarette smoke-treated cells. In lungs of mice acutely exposed to cigarette smoke, a similar effect could be observed: one of the three proteasome activities was significantly reduced, and ubiquitinated substrates for the proteasome were found to be accumulated, while proteasome expression levels were not changed.

The second publication in this thesis (Keller et al. 2015) shows for the first time the cell-specific expression of immunoproteasomes in the lung and their induction by interferon-γ *in vitro* and by murid herpesvirus 68 (MHV-68) infection *in vivo*. Within these experiments, activity-based probes were used to clearly define the kinetics of standard and immunoproteasome subunit incorporation. In human lungs from controls or early-stage COPD patients, immunoproteasome expression was not changed. Immunoproteasomes localized mainly to alveolar macrophages, but not to parenchymal cells in both donors and end-stage COPD.

Results from recent experiments were accepted for publication in the meantime (Kammerl et al. 2016): we investigated MHC I antigen presentation in cigarette smoke extract-treated primary immune cells and bronchoalveolar lavage (BAL) cells from mice exposed to cigarette smoke for ten days. *In vitro*

treatment of primary immune cells with cigarette smoke extract led to a decrease in the presentation of an immunoproteasome-dependent "self"-epitope. With the help of activity-based probes, we observed a shift from immuno- to standard proteasome activity in isolated alveolar macrophages from smoke-exposed mice. This shift, however, was not sufficient to impact antigen presentation of an immunoproteasome-dependent epitope. The altered ratio of standard and immunoproteasome might be explained by transcriptional downregulation of immuno-, but not standard proteasomes by cigarette smoke in isolated alveolar macrophages of smoke-exposed mice, which was also observed in total BAL cells of early-stage COPD patients. In the lungs of end-stage COPD patients, activities of both standard and immunoproteasome subunits were significantly decreased, while total proteasome protein levels were not changed.

Taken together, we show that cigarette smoke directly impairs proteasome function *in vitro* and *in vivo*, which may exacerbate oxidative stress resolution in response to cigarette smoke, since the degradation of oxidatively modified and misfolded proteins is impaired. In addition, we observed alterations in immunoproteasome-dependent MHC I antigen presentation, which may contribute to increased susceptibility to virus-induced exacerbations, prolonged infection and possibly result in autoimmune responses.

Zusammenfassung

Die chronisch obstruktive Lungenerkrankung (chronic obstructive pulmonary disease, COPD) wird laut Hochrechnungen die weltweit dritthäufigste Todesursache im Jahr 2020 darstellen. Zigarettenkonsum gilt als Hauptrisikofaktor für die Entstehung der COPD. Das Rauchen von Zigaretten führt in der Lunge zu oxidativem Stress, welcher zu Beschädigung von Proteinen und Induktion einer adaptiven Immunantwort führt. Raucher und COPD-Patienten sind außerdem anfälliger für Virusinfektionen, die oft in einer akuten Exazerbation der COPD-Pathogenese resultieren. Die Lungen von COPD-Patienten weisen hierbei eine erhöhte Anzahl an Zellen des angeborenen und adaptiven Immunsystems auf, darunter befinden sich auch $CD8^+$ T-Zellen, deren Häufigkeit mit dem Krankheitsstadium korreliert. Das Proteasom baut mehr als 90 % aller intrazellulären, einschließlich beschädigter Proteine zu kurzen Peptiden ab und schützt die Zelle so vor proteotoxischem Stress. Das Immunproteasom stellt eine besondere Proteasomform dar und ist in antigenpräsentierenden Zellen ständig exprimiert oder kann durch Infektion induziert werden. Es ist weiterhin maßgeblicher Bestandteil der adaptiven Immunantwort, da es die Antigenpräsentation über den Haupthistokompatibilitätskomplex (major histocompatibility complex, MHC) Klasse I zu $CD8^+$ T-Zellen verbessert. Der Effekt von Zigarettenrauch auf die Funktion des (Immun-)Proteasoms wurde noch nicht untersucht.

Die erste Veröffentlichung in dieser Dissertation (van Rijt et al. 2012) untersuchte die akuten Effekte der Zigarettenrauch-Exposition auf Proteasomexpression und -aktivität. Dabei konnten wir beobachten, dass kurzzeitige Exposition gegenüber Zigarettenrauch die Proteasomaktivität direkt beeinträchtigte, während sich die Proteasomexpression nicht änderte. Die Anreicherung oxidativ-modifizierter und ubiquitinierter Proteine legte eine Verstärkung des oxidativen Stresses in Zellen nahe, die mit Zigarettenrauch behandelt wurden. In Lungen von Mäusen, die akut Zigarettenrauch ausgesetzt waren, wurde ein ähnlicher Effekt beobachtet: Eine der drei Proteasomaktivitäten war signifikant reduziert, während ubiquitinierte Proteasomsubstrate akkumulierten, die Proteasomexpression jedoch unverändert war.

Die zweite Veröffentlichung dieser Dissertation (Keller et al. 2015) zeigt erstmals die zellspezifische Expression von Immunproteasomen in der Lunge sowie ihre Induktion durch Interferon-γ *in vitro* und nach Infektion mit dem murinen Herpesvirus 68 (MHV-68) *in vivo*. Für diese Untersuchungen wurde von Aktivitäts-basierten Sonden Gebrauch gemacht, um die Kinetik der Inkorporation von Standard- und Immunproteasom-Untereinheiten genau zu beschreiben. In humanen Lungen von Kontrollpersonen oder Patienten mit COPD im Frühstadium wurde keine Änderung der

Immunproteasomexpression beobachtet. Außerdem wurden Immunproteasomen hauptsächlich in Alveolarmakrophagen von Organspendern und COPD-Patienten im Endstadium detektiert, jedoch nicht in deren parenchymalen Zellen.

Neu erhobene Daten wurden vor kurzem zur Publikation angenommen (Kammerl et al. 2016): Wir untersuchten MHC I Antigenpräsentation in primären Immunzellen, die mit Zigarettenrauchextrakt behandelt wurden, sowie in Bronchoalveolär-lavagierten (BAL)-Zellen, die von Mäusen stammten, welche zehn Tage lang Zigarettenrauch ausgesetzt waren. Die *in vitro* Behandlung von primären Immunzellen mit Zigarettenrauchextrakt führte zur Verminderung der Präsentation eines Immunproteasom-abhängigen „Selbst"-Epitops. Mit Hilfe von Aktivitäts-basierten Sonden beobachteten wir eine Verschiebung von Immun- zu Standardproteasomaktivität in isolierten Alveolarmakrophagen von berauchten Mäusen. Jedoch war diese Verschiebung nicht ausreichend, um die Antigenpräsentation eines Immunproteasom-abhängigen Epitops zu verändern. Das verschobene Verhältnis von Standard- und Immunproteasom könnte durch die transkriptionelle Herabregulierung der Immunproteasom-Untereinheiten durch Zigarettenrauch in isolierten Alveolarmakrophagen erklärt werden. Die Standardproteasom-Untereinheiten blieben hierbei unverändert. Dieser Effekt wurde auch in BAL-Zellen von COPD-Patienten im Frühstadium beobachtet. Die Lungen von COPD-Patienten im Endstadium wiesen signifikant verminderte Standard- und Immunproteasomaktivitäten auf, während auf Proteineben die Proteasomexpression unverändert war.

Zusammenfassend haben wir gezeigt, dass Zigarettenrauch die Proteasomfunktion *in vitro* und *in vivo* direkt beeinträchtigt, was möglicherweise die Beseitigung des Zigarettenrauch-induzierten oxidativen Stresses erschwert, da der Abbau oxidativ-modifizierter und fehlgefalteter Proteine beeinträchtigt ist. Außerdem konnten wir Veränderungen der Immunproteasom-abhängigen MHC I-Antigenpräsentation beobachten, was eventuell zu einer gesteigerten Anfälligkeit für Virus-induzierte Exazerbationen, verlängerter Infektionsdauer und zu Autoimmunprozessen führt.

1. Introduction[1]

1.1 Pathogenesis of chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) affects more than 300 million people worldwide and is estimated to become the third leading cause of global deaths in 2020 (Decramer et al. 2012; Vos et al. 2012). COPD is defined by progressive airflow limitation and airway inflammation, chronic activation of immune responses and chronic bronchitis, mucus hypersecretion and loss of alveolar septa, i.e. emphysema formation (Decramer et al. 2012). These hallmarks of COPD result in reduced lung function, impaired gas exchange and severely diminished quality of life. COPD is uncurable with available treatments only improving symptoms and slowing down disease progression (Decramer et al. 2012). Risk factors for the development of COPD include, amongst others, genetic susceptibility, exposure to particles (cigarette smoke, air pollution), and age (Postma et al. 2015). COPD diagnosis was initially categorized by the "Global Initiative for Chronic Obstructive Lung Disease" (GOLD) into four stages (I – IV) according to spirometry of the patients, but only recently, a modified assessment for COPD has been released (GOLD Report 2015), which also takes symptoms and comorbidities into account (group A-D). Acute exacerbations of the disease are often associated with viral and bacterial respiratory infections and contribute to reduced quality of life, acceleration of decline in lung function, hospitalization, or even death (Donaldson et al. 2002; Sethi & Murphy 2008; Decramer et al. 2012).

1.1.1 Tobacco smoke induces oxidative stress and is the main risk factor for COPD

Tobacco smoking is the main risk factor for COPD. However, air-pollution and biomass fuel exposure are becoming more evident as risk factors, especially in low-income countries (Mannino & Buist 2007; Salvi & Barnes 2009). While 5 % of the general global population is affected by COPD, the prevalence goes up to 50 % in heavy smokers (Rennard & Vestbo 2006; Vos et al. 2012). Cigarette smoke consists of more than 4700 chemical compounds, including toxins, oxidants and carcinogens, and is a major source of oxidative stress which leads to reduced antioxidant capacities (Smith & Hansch 2000). The highly reactive compounds of cigarette smoke are known to cause DNA adducts, peroxidation of lipids, and protein modifications (Church & Pryor 1985; Cai et al. 2009; F. Liu et al. 2010). Protein modifications can result in misfolding, unfolding, and loss of function. Misfolded proteins have been

[1] partially adapted from Meiners et al. 2014

shown to lead to endoplasmic reticulum (ER) stress and induction of the unfolded protein response (UPR). When production of misfolded proteins exceeds the cell's capacity to degrade them, this can result in protein stress or even proteotoxicity (Wei et al. 2013). Perpetuated exposure to oxidative components of cigarette smoke thus challenges proteostasis in lung cells and can lead to apoptosis. Components of the UPR have been shown to be increased in healthy smokers (Kelsen et al. 2008) as well as COPD patients (Malhotra et al. 2009; Min et al. 2011), suggesting a role for proteostasis imbalance in COPD pathogenesis.

While cigarette smoke might play an important role in initiating COPD pathogenesis, the disease is not reversible when patients quit smoking, although smoking cessation might slow down lung function decline and is highly recommended as a first therapeutic measure (Tashkin & Murray 2009; Decramer et al. 2012). The irreversibility might be partially explained with irreversible epigenetic changes induced by cigarette smoke (Stämpfli & Anderson 2009; Besingi & Johansson 2014; Schamberger et al. 2014), persistence of oxidative stress (Louhelainen et al. 2009), irreversibly altered microbiome colonization (Marsland & Gollwitzer 2014), and sustained activation of (autoreactive) adaptive immune responses, leading to self-propagation of inflammation and tissue destruction (Rutgers et al. 2000; Morissette et al. 2014). Importantly, cigarette smoke is harmful not only to the lung, but also to other organ systems, including the immune system (Kitamura 1987; Stämpfli & Anderson 2009).

1.1.2 Immune responses in smokers and COPD patients

The manifold components of cigarette smoke include both pro-inflammatory (e.g. LPS) and immune-suppressive agents (Hogg 2003; Mehta et al. 2008; Stämpfli & Anderson 2009; Gonçalves et al. 2011; Larsson et al. 2012). Cigarette smoke directly damages the integrity of the airway epithelial cell layer, increases permeability of this physical barrier, and impairs mucociliary clearance of pathogens (Stämpfli & Anderson 2009). Cigarette smoking results in activation of epithelial cells to secrete "danger signals", which act as Toll-like receptor (TLR) agonists contributing to the secretion of proinflammatory cytokines, and recruitment of macrophages and neutrophils (Cosio et al. 2009; Brusselle et al. 2011). Macrophages and neutrophils secrete proteases, leading to degradation of extracellular matrix and tissue injury and propagate inflammation (Kirkham & Barnes 2013).

At the same time, cigarette smoke has been shown to impair monocyte and macrophage function (e.g. phagocytosis of apoptotic cells and bacteria; cytokine production) (Ouyang et al. 2000; McMaster et al. 2008; Kollert et al. 2009; Bozinovski et al. 2011; Karavitis & Kovacs 2011; Minematsu et al. 2011; O'Leary et al. 2014; van Zyl-Smit et al. 2014) and possibly skewing their polarization state towards an anti-inflammatory M2 phenotype (Shaykhiev et al. 2009; Stämpfli & Anderson 2009; Hodge et al. 2011; Kaku et al. 2014).

The sustained presence of TLR agonists also leads to maturation of lung-residing immature dendritic cells and their trafficking to lymph nodes, where they present antigens, e.g. extracellular matrix degradation products, to T cells and an adaptive immune response is shaped (Cosio et al. 2009). Accordingly, the lungs of COPD patients exhibit not only increased levels of innate (neutrophils, macrophages), but also adaptive immune cells (cluster of differentiation (CD)8^+ T cells, $CD4^+$ T cells, B cells), highlighting the role of the immune system in the pathogenesis of COPD (Barnes 2008; Brusselle et al. 2011; Baraldo et al. 2012; Holloway & Donnelly 2013).

It is well established that COPD lungs exhibit increased numbers of $CD8^+$ T cells, which has been shown to correlate with disease severity (Saetta et al. 1998; Majo et al. 2001; Hogg et al. 2004; Tzanakis et al. 2004). Furthermore, it was demonstrated that the functionality of $CD8^+$ T cells is altered in COPD (Freeman et al. 2010; Freeman et al. 2013; Grundy et al. 2013). Novel insight into the role of $CD8^+$ T cells for COPD pathogenesis has been gained by using mouse models of COPD. Like in human COPD, $CD8^+$ T cells accumulate in mice exposed to cigarette smoke for six months and persist upon smoking cessation (Motz et al. 2008). By diminishing $CD8^+$ T cells with a specific depleting antibody, inflammatory and emphysematous responses in mice could be blunted compared to isotype-treated control mice (Podolin et al. 2013). Furthermore, CD8-deficient mice were protected from emphysema development after six months of cigarette smoke exposure and exhibited reduced inflammatory bronchoalveolar lavage (BAL) cells (Maeno et al. 2007). The lab of Michael Borchers showed that pulmonary T cells of mice exposed to cigarette smoke for six months are capable of recapitulating emphysematous changes in cigarette smoke naïve immunodeficient as well as immunocompetent mice (Motz et al. 2010; Eppert et al. 2013). In these studies, transfer of only $CD8^+$ T cells was not sufficient, but required co-transfer of $CD4^+$ T cells as well. However, they showed that the effect was dependent on major histocompatibility complex (MHC) class I antigen presentation, as the mice deficient for β2-microglobulin, a crucial component of the MHC I complex, did not develop emphysema after T cell transfer from cigarette smoke-exposed mice. These data suggest a role for $CD8^+$ T cell-mediated immune response in COPD pathogenesis.

Cigarette smoke increases susceptibility to infection

Cigarette smoking has been associated with increased susceptibility to respiratory infections (Arcavi & Benowitz 2004; Stämpfli & Anderson 2009; Shang et al. 2011; Feldman & Anderson 2013; Sajjan 2013). Moreover, cigarette smoke has been shown to dampen the host's immune system to combat bacteria and viruses. The molecular mechanism behind this effect involves reduction of interferon (IFN) signaling both in immune (Braun et al. 1998; Edwards et al. 1999; Mian et al. 2009) and parenchymal cells (Bauer et al. 2008; Modestou et al. 2010; Eddleston et al. 2011; Proud et al. 2012; Hudy et al. 2014) in response to smoke *in vitro*.

When mice were exposed to cigarette smoke prior to influenza infection, they exhibited increased virus titers in the lung (Gualano et al. 2008). Another study also showed an amplified inflammatory response in smoke-exposed mice that were either given a viral mimic, polyinosinic:polycytidylic acid (polyI:C), or were infected with influenza virus, characterized by increased levels of inflammatory cytokines and interferons in the lung (Kang et al. 2008). However, one study found reduced production of interferons in influenza infected and cigarette smoke-exposed mice, compared to virus-infection alone (Wu et al. 2014). These differences might be explained by the different smoking protocols and durations of cigarette smoke exposure (two vs. six weeks in the latter).

Virus-induced COPD exacerbations

COPD patients, like smokers, are more susceptible to respiratory infections, have a more severe course of disease and need more time to resolve the infection (Stämpfli & Anderson 2009; Mallia et al. 2011; Beasley et al. 2012). Respiratory infections in COPD patients often result in acute exacerbations, but also non-infectious causes for exacerbations are known, such as pneumothorax, pulmonary embolism, or air pollution (Sethi & Murphy 2008; Decramer et al. 2012). Acute exacerbations in COPD are defined by short periods (at least 48 h) of increased cough, dyspnea, and production of sputum that can become purulent (Decramer et al. 2012). The frequency of acute exacerbations is directly linked with the outcome: frequent exacerbators exhibit a faster decline in lung function, and are more likely to be hospitalized than infrequent exacerbators, moreover, their mortality is increased (Wedzicha et al. 2013). The majority of acute exacerbations (60-80 %) is caused by infections and occurs one to two times per year in COPD patients, but frequency increases with severity of disease (Sethi & Murphy 2008). Typical pathogens found during exacerbations include bacterial strains of *Haemophilus influenza* (cause of 20-30 % of exacerbations) and *Streptococcus pneumoniae* (10-15 %), but also rhinovirus (20-25 %), parainfluenza virus (5-10 %) and influenza virus (5-10 %) are frequently found (Sethi & Murphy 2008; Zwaans et al. 2014). Although the prevalence of influenza virus is low, this pathogen is associated with more severe exacerbations requiring hospitalization of COPD patients (Sethi & Murphy 2008).

While it was thought for a long time that the lower airways are sterile in health, improved methodology for sampling, identification and quantification of bacteria has led to the insight that different regions of the lung can harbor diverse bacterial strains, the composition of the microbiome, however, changes with cigarette smoking and COPD (Marsland & Gollwitzer 2014; Sze et al. 2015). Bacterial colonialization affects virus entry and modulates immune responses and, vice versa, viral infections are capable of paving the way for the expansion of pathogenic bacteria (Papi et al. 2006; Wilkinson et al. 2006; Mallia et al. 2012; Sajjan 2013; Marsland & Gollwitzer 2014).

Autoimmune aspects of COPD

Several lines of evidence support the assumption that COPD involves autoimmune processes that are facilitated by cigarette smoke (Agusti 2003; Grumelli et al. 2004; Taraseviciene-Stewart et al. 2005; Feghali-Bostwick et al. 2008; Stefanska & Walsh 2009; Arnson et al. 2010; Duncan 2010; Duncan 2011; Kheradmand et al. 2012; Tzouvelekis et al. 2012; Bieber et al. 2013). These include the presence of tertiary lymphoid follicles in COPD lungs, which consist of B and T cells as well as dendritic cells, indicating ongoing adaptive immune responses against a prevailing antigen. Furthermore, these follicles persist after smoking cessation (Morissette et al. 2014). Whether lymphoid follicles are beneficial or harmful in COPD, however, is still under debate (Brusselle et al. 2009; Brusselle et al. 2011; Hansbro & Knight 2013; Yadava & Marsland 2013; John-Schuster et al. 2014). Within lymphoid follicles, B and T cells are primed by antigen presenting dendritic cells and clonally expand in response to antigens (Brusselle et al. 2011). These antigens include microbial antigens, cigarette smoke-derived antigens or (modified) autoantigens such as extracellular matrix degradation products (Brusselle et al. 2009). Indeed, autoantibodies against multiple self-antigens have been described in COPD patients, possibly correlating with disease severity and/or smoking status (Lee et al. 2007; Leidinger et al. 2009; Packard et al. 2013; Morissette et al. 2014).

1.2 The proteasome system

The proteasome is a 2.5 MDa protease complex and the main protein degradation system within the cell. More than 90 % of all cellular proteins are processed by the proteasome into peptides of 3-22 amino acids in length. These can be used to recycle amino acids or are loaded onto MHC I molecules to communicate the intracellular protein composition to the immune system (Kisselev et al. 1999; Goldberg 2003; Kloetzel 2004; Finley 2009). However, less than 0.1 % of the peptides generated by the proteasome are presented at the cell surface (Yewdell et al. 2003). Due to the broad nature of substrates, the proteasome is involved in many essential cellular functions such as protein quality control, degradation of (oxidatively) damaged proteins, transcription, immune responses, cell signaling, and apoptosis (Finley 2009; Schmidt & Finley 2014). In the normal course of a protein's lifetime, synthesis and degradation rate determine the half-life of both short- and long-lived proteins for cellular maintenance (Yewdell 2001). Controlled protein breakdown by the proteasome involves tagging of protein substrates with ubiquitin chains linked at the lysine at position 48 (K48) via a cascade of E1, E2 and E3 enzymes. However, ubiquitin-independent degradation by the proteasome has also been described (Kish-Trier & Hill 2013; Schmidt & Finley 2014).

The proteasome consists of a central 20S catalytic core particle, which needs to be activated by proteasome regulators (Figure 1). Several regulators are known that bind to and thus mediate opening of the 20S for substrate entry (Meiners et al. 2014). The 19S is the best studied regulator: it consists of

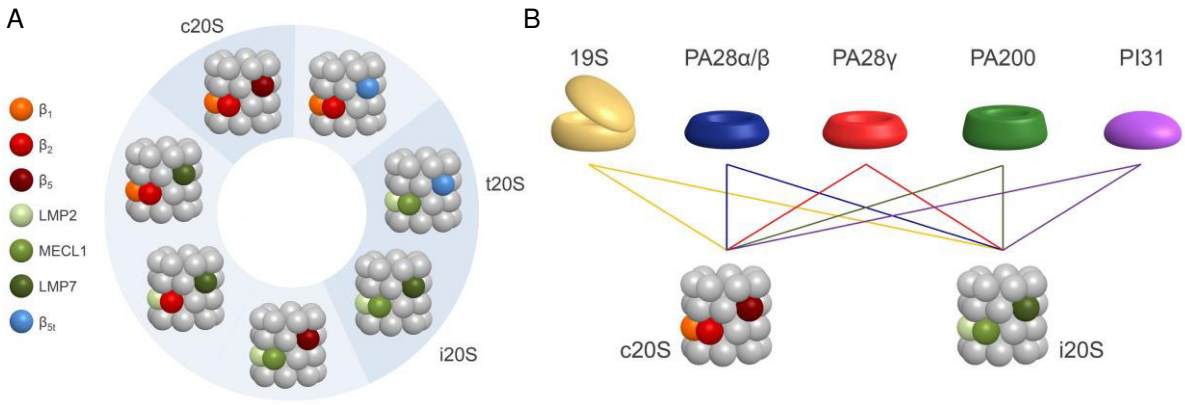

Figure 1: Diversity of 20S proteasome complexes. (A) The 20S core particle is composed of four stacked heptameric rings. The outer α-rings close the pore and protect from uncontrolled substrate entry. The inner two β-rings comprise three catalytically active subunits each: the standard c20S proteasome contains β1, β2, and β5, the immunoproteasome (i20S) incorporates LMP2, MECL-1, and LMP7. In cortical thymic epithelial cells, the thymus-specific catalytic subunit β5t is expressed to form the thymoproteasome (t20S). Intermediate proteasomes containing a mixture of standard and immunosubunits have also been reported. (B) Proteasome substrate specificity and turnover are modulated through binding of regulatory particles to the 20S core particles. Four types of regulatory particles are known: 19S, the two 11S-types PA28α/β and PA28γ, PA200, and PI31. These regulators can bind to one or both sides of c20S and i20S. c20S, standard 20S proteasome; i20S, immunoproteasome; LMP, low molecular mass protein; MECL-1, multicatalytic endopeptidase complex-like 1: PA, proteasome activator; PI, proteasome inhibitor; t20S, thymoproteasome. Figures taken from (Meiners et al. 2014).

at least 18 different subunits, including ubiquitin receptors, and is thus accountable for ubiquitin- and ATP-dependent degradation of substrates (Lander et al. 2012). Together with the 20S, it forms the 26S/30S proteasome by binding to one or both sides, respectively. Two 11S-types of regulators are known: the IFNγ-inducible heteroheptameric PA28α/β and the homoheptameric PA28γ, which can only be found in the nucleus. Furthermore, two monomeric regulators, PA200 as well as PI31, have been described. Proteasome regulators have been shown to determine substrate specificity and turnover rate. However, their function is not well understood. This is especially true for PA200 and PI31, and PI31 might even serve as an inhibitor for 20S activity (Li et al. 2014).

1.2.1 The proteasome 20S catalytic core particle

The 20S proteasome consists of a barrel-shaped core particle composed of four rings comprising seven subunits each (Figure 1A). Seven related, but distinct α-subunits form the two outer α-rings (Finley 2009). Because the N-termini of the α-subunits close the entry pore and inhibit substrate entry, the 20S core particle is rather inert in itself and needs regulators for activation (Finley 2009). Three of the seven β-subunits that constitute each of the two inner β-rings are catalytically active and mediate the proteolytic capacity of the 20S proteasome.

These three β-subunits determine the species of the 20S core particle: depending on the cell-type, cytokine milieu or activation state of the cell, different β-subunits are expressed and incorporated into mature 20S. The standard 20S proteasome is expressed in every cell-type and integrates the β1, β2,

and β5 subunits, which cleave after acidic, basic, or hydrophobic amino acids, respectively (Huber et al. 2012). In lymphoid cells, however, three different β-subunits are constitutively expressed (Sijts & Kloetzel 2011): low molecular mass protein (LMP) 2, multicatalytic endopeptidase complex-like 1 (MECL-1), and LMP7 (also called $\beta1_i$, $\beta2_i$, and $\beta5_i$). In non-immune cells, these three so-called immunosubunits can be induced by IFNγ or tumor necrosis factor (TNF) α signaling (Aki et al. 1994; Hallermalm et al. 2001). When immunosubunits are expressed, they are preferentially incorporated into newly assembled 20S immunoproteasomes (Kingsbury et al. 2000; Joeris et al. 2012). Furthermore, they exhibit altered cleavage preferences compared to standard proteasomes, with a strongly reduced post-acidic cleavage activity based on the β1/LMP2 exchange, leading to generation of peptides that are preferentially loaded onto MHC I molecules compared to peptides derived from standard proteasomes (Groettrup et al. 2001). In addition, mixed proteasomes have been described consisting of both standard and immunoproteasome subunits, increasing the peptide pool even more (Zanker et al. 2013; Dahlmann et al. 2000).

A general overview of the role of immunoproteasomes and their regulation can be found in the recent review by Meiners and colleagues, in which the authors also highlight the known roles of (immuno-)proteasomes in lung diseases (Meiners et al. 2014). The manifold functions of immunoproteasomes that have emerged in the past years go far beyond their initial proposed function of improved generation of antigenic peptides (Figure 2) and include, amongst others, cytokine production, resolution of oxidative stress, NFκB signaling as well as T cell differentiation (summarized in the following reviews: Groettrup et al. 2010; Angeles et al. 2012; Ebstein et al. 2012; Basler et al. 2013).

A third proteasome species is the 20S thymoproteasome that incorporates the $\beta5_t$-subunit and the two immunosubunits LMP2 and MECL-1. The $\beta5_t$-subunit exhibits reduced post-hydrophobic cleavage, is exclusively expressed in cortical thymic epithelial cells and is important for positive selection of $CD8^+$ T cells in the thymus (Murata et al. 2007; Klein et al. 2009; Xing et al. 2013; Klein et al. 2014).

1.2.2 Function of proteasomes in oxidative stress responses

The ubiquitin-proteasome system plays an important role in the degradation of damaged and misfolded proteins, which might aggregate and exhibit cytotoxic potential (Goldberg 2003; Meiners & Eickelberg 2012). Proteins can be damaged by reactive agents, such as present in cigarette smoke, or which are generated at conditions of oxidative stress and oxidatively modify amino acids, resulting in loss of protein function and rendering them prone for selective degradation by the proteasome (Goldberg 2003).

It has been proposed that the 20S proteasome is able to degrade oxidatively-modified proteins in an ATP- and ubiqutitin-independent manner, which was shown by *in vitro* digestion of native or oxidized

proteins by isolated proteasomes (Davies 2001; Pickering et al. 2010; Pickering & Davies 2012; Jung et al. 2013). Within these studies, the role of several proteasome regulators was examined: while 20S proteasomes alone were not very efficient, addition of PA28α/β substantially increased degradation rate, while 19S and PA200 did not enhance or even inhibited degradation of oxidized substrate. The relevance of these results in a cellular context or *in vivo* has yet to be demonstrated.

A special role for immunoproteasomes in the resolution of oxidative stress and turnover of oxidatively damaged proteins has been discussed. Firstly, immunoproteasome levels have been reported to increase in response to oxidative conditions *in vitro* by treatment of cells with hydrogen peroxide (Pickering et al. 2010) and *in vivo* in an experimental model of neurodegeneration induced by oxidative stress, which was reversible by treatment with antioxidants (Launay et al. 2013). Secondly, knock-out mice of immunoproteasome subunit LMP2 showed increased levels of carbonylated proteins compared to wildtype controls at both four and twelve months of age (Ding et al. 2006) and immunoproteasome-deficient cells needed more time to resolve IFNγ-induced oxidatively modified, i.e. carbonylated, proteins (Seifert et al. 2010). In the latter publication, the authors suggested a specific role of immunoproteasomes in the oxidative stress response. However, these results, including an increase of polyubiquitinated proteins after IFNγ induction, could not be reproduced by Nathan and colleagues (Nathan et al. 2013). Thus, the specific function of immunoproteasomes in response to oxidative stress remains controversial.

1.2.3 Function of immunoproteasomes in MHC I antigen presentation

Immunoproteasomes enable rapid resolution of viral infections

Immunoproteasomes play an essential role at three crucial checkpoints of $CD8^+$ T cell-mediated adaptive immune responses against intracellular infections (McCarthy & Weinberg 2015). Firstly, immunoproteasomes are important for negative selection of autoreactive $CD8^+$ T cells in the thymus: immunoproteasomes are expressed in medullary thymic epithelial cells (mTECs), where they present the cellular "self" peptide repertoire to $CD8^+$ T cells (Osterloh et al. 2006) and enable selection of only those T cells that do not bind to "self" peptide/MHC I complexes, as cells with a high affinity are eliminated (Anderton & Wraith 2002; Groettrup et al. 2010). The remaining naïve $CD8^+$ T cells migrate to lymph nodes and persist until they are activated by antigen-presenting cells (APCs) in order to execute their effector function and combat infections.

Secondly, APCs, especially dendritic cells, mainly express immunoproteasomes and are able to engulf apoptotic or necrotic particles of infected cells (Sijts & Kloetzel 2011). Subsequently, APCs mature and traffic to draining lymph nodes, where they present immunoproteasome-derived pathogen-peptides on MHC I together with co-stimulatory molecules to evoke a specific $CD8^+$ T cell responses (so-called cross-presentation). With the help of APCs, intracellular viral or bacterial infections are thus

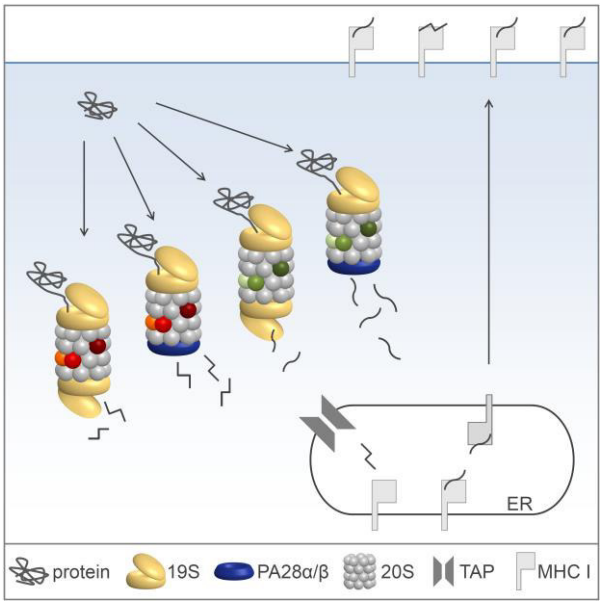

Figure 2: Immunoproteasome function in antigen presentation. Different proteasome populations contribute to antigen processing. Cytosolic and nuclear proteins have been shown to be degraded into peptides by standard (red), mixed, and immunoproteasomes (green) and may contribute to differential protein cleavage and epitope generation. In addition, the different catalytic subcomplexes can associate with several regulatory particles, for example, the 19S and PA28α/β complex. Less than 0.1 % of the generated peptides are translocated into the lumen of the ER by the TAP transporter and are loaded onto MHC I molecules. MHC I/peptide complexes traffic from the ER to the outer cell membrane, where they can be detected by $CD8^+$ T cells. ER, endoplasmic reticulum; MHC, major histocompatibility complex; TAP, transporter associated with antigen presentation. Figure taken from (Meiners et al. 2014).

communicated to naïve $CD8^+$ T cells to induce a pathogen-specific adaptive immune response. After activation, the $CD8^+$ T cells move to the site of infection and patrol the infected organ in search for their specific epitope bound to MHC I to kill the infected cell.

Thirdly, to limit pathogen replication by selectively killing infected cells, cells need to signal their infection status to patrolling activated $CD8^+$ T cells. In order to be recognized by $CD8^+$ T cells, infected cells upregulate immunoproteasome expression to present the exact same immunoproteasome-generated pathogen peptide as during $CD8^+$ T cell activation by the APC (Khan et al. 2001; Shin et al. 2006).

For all these aforementioned processes, immunoproteasomes enhance antigen presentation by increasing the quantity (Deol et al. 2007; Mishto et al. 2014) and/or quality of peptides for MHC I antigen presentation (Fehling et al. 1994; Groettrup et al. 2001; Toes et al. 2001; Van den Eynde 2001; Dalet et al. 2011). Immunoproteasomes have been reported to shape the MHC I peptide repertoire, which was illustrated by the use of proteasome inhibitors or immunoproteasome knock-out mice, either of single or all three immunosubunits (Groettrup et al. 1995; Morel et al. 2000; Chen et al. 2001; de Verteuil et al. 2010; Guillaume et al. 2010; Kincaid et al. 2012). Accordingly, immunoproteasomes dictate generation of the T cell receptor (TCR) repertoire on $CD8^+$ T cells after infection, which has been shown in several mouse models of viral or bacterial infections. In these models, and strongly

depending on the pathogen, immunoproteasome (subunit) deficiency effects ranged from no detectable differences in virulence (Nussbaum et al. 2005; Brosch et al. 2012), altered antigenic peptide presentation and $CD8^+$ T cell response (Van Kaer et al. 1994; Sibille et al. 1995; Schwarz, de Giuli, et al. 2000; Schwarz, van Den Broek, et al. 2000; Pang et al. 2006; Jäkel et al. 2009; Basler et al. 2011; de Graaf et al. 2011; Hutchinson et al. 2011; Zanker et al. 2013) to even increased morbidity and mortality (Tu et al. 2009; Opitz et al. 2011). These publications show the importance of immunoproteasomes during infection to enhance antigen presentation and to increase pathogen-derived peptides. It is, however, equally important to downregulate immunoproteasomes after the infection is resolved to limit autoreactive $CD8^+$ T cells that might have been evoked during infection (Groettrup et al. 2001; Groettrup et al. 2010).

Immunoproteasomes protect from autoimmunity

Intriguingly, the cell type- and tissue-specific distribution of immunoproteasomes is important for protecting the organism from autoimmunity after infection. Immune cells such as APCs express immunoproteasomes constitutively, whereas parenchymal cells only express them in response to inflammatory cytokines such as IFNγ or TNFα. During $CD8^+$ T cell priming in the lymphatic tissues, both immunoproteasome-derived pathogen-, but also "self"-peptides are presented on MHC I by the APC. If a "self"-reactive $CD8^+$ T cell, despite thymic selection, would be activated during infection by an APC, the same immunoproteasome-dependent "self"-peptide might be presented by an infected parenchymal cell, however, the epitope would cease to be presented by parenchymal cells after the infection is resolved, because immunoproteasomes are gradually replaced by standard proteasomes (Heink 2005). Certain immunoproteasome-derived "self"-peptides are thus presented to the immune system only during infection, thereby protecting from autoreactive immune responses (Groettrup et al. 2001; Shin et al. 2006; Eleftheriadis 2012).

Indeed, it has been shown that immunoproteasomes are expressed in human autoimmune disorders (Egerer et al. 2006; Krause et al. 2006; Mishto et al. 2010; Ghannam et al. 2014) and experimental models of autoimmunity (Basler et al. 2010; Belogurov et al. 2015). Furthermore, single nucleotide polymorphisms (SNP) of proteasome subunits have been associated with autoimmune diseases with partially conflicting results (an overview can be found in Supplementary Table S1 in Meiners et al. 2014). Recently, several mutations in the human *PSMB8* gene encoding the LMP7 immunoproteasome subunit have been identified that lead to autoinflammatory disorders (Agarwal et al. 2010; Arima et al. 2011; Kitamura et al. 2011; Liu et al. 2012; McDermott et al. 2015).

Accordingly, the use of immunoproteasome-specific inhibitors has been proposed for treatment of autoimmune disorders (Bird 2009; Bellavista et al. 2014; Kisselev & Groettrup 2014; Kniepert & Groettrup 2014; Verbrugge et al. 2015), as they have been proven to successfully counteract

autoimmune responses in several experimental models of autoimmune diseases (Muchamuel et al. 2009; Basler et al. 2010; Zaiss et al. 2011; Ichikawa et al. 2012; Nagayama et al. 2012; Basler et al. 2014). In these models, immunoproteasomes have been shown to not only alter antigen processing, but also to modulate cytokine production and T cell differentiation as demonstrated previously (Kalim et al. 2012).

1.3 Objectives

The main risk factor for the development of COPD is chronic exposure to cigarette smoke, which induces oxidative stress in the lung and evokes adaptive immune responses, both of which are hallmarks of COPD pathogenesis and are ongoing even after smoking cessation. In the course of COPD, viral infections exacerbate pathogenesis and worsen quality of life and prognosis of the patients.

The proteasome is the main proteolytic system to degrade oxitatively modified proteins and is involved in shaping adaptive immune responses via MHC I antigen processing. While oxidative stress has been shown to impair the proteolytic activity of the proteasome, this has not been studied for cigarette smoke exposure. Moreover, the effect of oxidative stress in general and cigarette smoke in particular on proteasome-mediated MHC I antigen presentation is unknown.

The aims of this thesis were: first, to comprehensively analyze the regulation of proteasome function in response to cigarette smoke *in vitro* and *in vivo* using expression analysis and activity assays; second, to investigate the function of immunoproteasomes, a specialized proteasome form for enhancing MHC I antigen presentation, in the lung; and third, to analyze how proteasome-mediated MHC I antigen presentation is altered in response to cigarette smoke. These results may explain the increased susceptibility to virus infections as observed in smokers and COPD patients.

2. Results: Publications originating from this thesis

2.1 Summary of publications

Acute cigarette smoke exposure impairs proteasome function in the lung: van Rijt et al. 2012

The first publication included in this thesis examined the effects of cigarette smoke on proteasome activity by exposing lung cell lines to extracts of cigarette smoke and by exposing mice to mainstream cigarette smoke. In this study, we investigated the effect of cigarette smoke on general protein oxidative modifications and ubiquitination, but also the specific effects of cigarette smoke on proteasome function *in vitro* in lung cell lines and *in vivo* in the lung. Acute exposure of lung cell lines (2-24 h) to cigarette smoke extract (CSE) led to an increase of reactive oxygen species, oxidative protein modifications, and ubiquitinated proteins. The proteasome itself was not affected in terms of subunit expression, but proteasome activity was significantly reduced in a time- and dose-dependent manner. With native gel analysis and substrate overlay techniques, we could show that both 20S and 26S activity were affected by CSE exposure.

When mice were exposed to mainstream cigarette smoke, the trypsin-like (post-basic) activity was significantly reduced in whole lung tissue. At the same time, levels of ubiquitinated proteins were significantly increased. With the commercially available fluorogenic substrates used in this publication, however, it is not possible to distinguish between standard- and immunoproteasome activities, which leaves the question unanswered to which degree immunoproteasome activity is affected by cigarette smoke in particular. In summary, this study showed for the first time a direct impairment of the proteasome by cigarette smoke *in vitro* and *in vivo*.

Regulation of immunoproteasome function in the lung: Keller et al. 2015

The second publication included in this thesis explored the cell-specific expression of immunoproteasomes in the lung and their induction by IFNγ *in vitro* and after murid herpesvirus 68 (MHV-68) infection *in vivo*. While it was previously demonstrated that virus infection induces immunoproteasome subunits incorporated into 20S in the lung (Kremer et al. 2010), we here show that MHV-68 infection leads to incorporation of immunosubunits into both 20S and 26S proteasome complexes. Furthermore, with the use of fluorescently-labeled activity-based probes we dissected the kinetics of standard vs. immunosubunit activity up to 148 days post-infection. With this unique methodology, we have been able to show that the lung is capable of presenting immunoproteasome-

dependent pathogen-derived antigenic peptides on MHC I, which is essential for rapid resolution of infection.

In lung cancer resectates of non-smokers, smokers, and COPD GOLD stage I or II patients, we did not observe obvious alterations in immunoproteasome expression. When human lung explants from donors or end-stage COPD patients were examined, surprisingly no change in cellular distribution was observed: immunoproteasomes localized mainly to alveolar macrophages, but not to parenchymal cells, even though COPD is described as an inflammatory disorder with increased IFNγ and TNFα levels (Barnes 2008; Brusselle et al. 2011).

With this work, we showed for the first time the cell-specific distribution and regulation of immunoproteasomes in the lung.

2.2 Acute cigarette smoke exposure impairs proteasome function in the lung

Sabine H. van Rijt, **Ilona E. Keller**, Gerrit John, Kathrin Kohse, Ali Ö. Yildirim, Oliver Eickelberg and Silke Meiners

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Acute cigarette smoke exposure impairs proteasome function in the lung

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van Rijt SH, Keller IE, John G, Kohse K, Yildirim AÖ, Eickelberg O, Meiners S. Acute cigarette smoke exposure impairs proteasome function in the lung. *Am J Physiol Lung Cell Mol Physiol* 303: L814–L823, 2012. First published September 7, 2012; doi:10.1152/ajplung.00128.2012.—Cigarette smoke mediates DNA damage, lipid peroxidation, and modification and misfolding of proteins, thereby inducing severe cellular damage. The ubiquitin proteasome system serves as the major disposal system for modified and misfolded proteins and is thus essential for proper cellular function. Its role in cigarette smoke-induced cell damage, however, is largely unknown. We hypothesized that the ubiquitin-proteasome system is involved in the degradation of cigarette smoke-damaged proteins and that cigarette smoke exposure impairs the proteasome itself. Here, we show that treatment of human alveolar epithelial cells with cigarette smoke extract (CSE) induced time- and dose-dependent cell death, a rise in intracellular reactive oxygen species, and increased levels of carbonylated and polyubiquitinated proteins. While high doses of CSE severely impaired all three proteasomal activities, low CSE concentrations significantly inhibited only the trypsin-like activity of the proteasome in alveolar and bronchial epithelial cells. Moreover, acute exposure of mice to cigarette smoke significantly impaired the trypsin-like activity by 25% in the lungs. Reduced proteasome activity was not due to transcriptional regulation of the proteasome. Notably, cigarette smoke exposure induced accumulation of polyubiquitinated proteins in the soluble and insoluble protein fraction of the lung. We show for the first time that acute exposure to cigarette smoke directly impairs proteasome activity in the lungs of mice and in human epithelial cells at low doses without affecting proteasome expression. Our results indicate that defective proteasomal protein quality control may exacerbate the detrimental effects of cigarette smoke in the lung.

ubiquitin proteasome system; cigarette smoke; protein quality control; oxidative stress; chronic obstructive pulmonary disease

CIGARETTE SMOKE IS ONE OF the primary risk factors for the pathogenesis of chronic obstructive pulmonary disease (COPD) (10). COPD is a major and rapidly increasing cause of death and morbidity worldwide (4). The pathology of COPD can be regarded as a persistent inflammatory immune response to oxidative and chemical injury by noxious particles such as cigarette smoke (18). Cigarette smoke contains over 4,700 chemical components, including many reactive compounds such as quinones and aldehydes. The highly reactive compounds of cigarette smoke are known to induce DNA damage, peroxidation of lipids, and protein modifications (7). Repeated and persistent exposure to cigarette smoke thus mediates severe damage in the cell and contributes to cell death, induction of immune responses, and subsequent destruction of lung tissue (36).

The ubiquitin proteasome system is the main protein waste disposal and recycling system of the cell. Proper proteasome function is essential for numerous cellular processes such as proliferation and signaling, transcriptional regulation, and immune responses (2, 21, 23). Before degradation, proteins are tagged with polyubiquitin chains via an enzymatic cascade. The structure and length of the polyubiquitin chains can be highly diverse; however, ubiquitin chains conjugated through the lysine at position 48 of ubiquitin (Lys^{48}) specifically lead to proteasomal degradation, whereas chains that are for instance linked at Lys^{63} are implicated in signaling or trafficking events (31). The 26S proteasome consists of one catalytic (20S) and two regulatory (19S) particles. Polyubiquitinated proteins are recognized by the regulatory particle (19S) of the proteasome where the substrates are deubiquitinated and unfolded for entry into the 20S catalytic core of the proteasome (12). The 20S core proteasome is built of four staggered rings of seven related but different α-subunits and seven distinct β-subunits that form a αββα barrel-like structure (16). Inside the barrel-shaped 20S, the three catalytically active subunits, caspase-like (β_1), trypsin-like (β_2), and chymotrypsin-like (β_5) activities, cleave the proteins into small peptides that can be used for major histocompatibility complex (MHC) class I antigen presentation (33). Proteasome function is not only essential for the normal turnover of most cytoplasmic and nuclear proteins, it also serves as the central quality control system to rapidly destroy misfolded and modified proteins, among them oxidatively modified proteins (13, 19, 27). Importantly, the proteasome itself can be a target for oxidative and chemical modification and inactivation (3, 5, 6, 9, 14, 32). It has been proposed that impaired proteasome function can result in a vicious cycle of detrimental accumulation of modified and ubiquitinated proteins, cellular dysfunction, and cell death (8). Accordingly, impaired proteasome function has been implicated in several protein quality diseases such as neurodegenerative disorders, cardiac and endothelial dysfunction, and cataract formation (8, 9, 17, 34). In the lung, reduced expression of proteasomal subunits and diminished proteasomal activity has been found to inversely correlate with lung function in COPD patients (25).

Surprisingly, not much is known about the role of the proteasome in cigarette smoke-induced cellular damage or whether the proteasome itself is affected by cigarette smoke. We provide evidence that acute cigarette smoke treatment induces accumulation and aggregation of polyubiquitinated proteins in a lung epithelial cell line as well as in the lungs of cigarette smoke-exposed mice. Moreover, cigarette smoke directly impairs proteasome activity, without affecting proteasome expression. Specifically, the trypsin-like activity of the

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proteasome is inhibited in alveolar and bronchial lung epithelial cells at nontoxic doses of cigarette smoke extract (CSE) as well as in cigarette smoke-exposed mice lungs.

EXPERIMENTAL PROCEDURES

Cell culture. The A549 human alveolar epithelial and the 16HBE human bronchial epithelial cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA). A549 cells were maintained in DMEM media (LifeSciences) and 16HBE cells in MEM media (LifeSciences). Media was supplemented with 10% FBS. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of CSE. Research-grade cigarettes (3R4F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). Stocks of CSE for treatment of A549 cells were prepared by bubbling smoke from 29 cigarettes through 400 ml of DMEM cell culture media at puffing speed in a closed environment with limited air flow. CSE was sterile filtered through a 0.20-μm filter (Minisart; Sartorius Stedim Biotech), separated into aliquots, and stored at −20°C for future use. This stock was considered as 100% CSE extract. For cell treatment, CSE stock was supplemented with FBS (to a final concentration of 10%) and serially diluted to the stated concentrations with full media. The CSE could be frozen and thawed again without loss of potency. For the 16HBE cells, 16 cigarettes were smoked by bubbling smoke through 400 ml of MEM media. For serial dilutions, the CSE concentrations used for the 16HBE cells were calculated relative to the 100% CSE stock used for the A549 cells.

Cytotoxicity assay. Cytotoxicity of CSE was assessed using the 2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 6×10^4 cells/well were seeded in 24-well plates. After seeding (24 h), cells were treated with CSE. After treatment, 100 μl of freshly prepared solution of 5 mg thiazolyl blue tetrazolium bromide per milliliter PBS (Sigma) were added to each well and incubated at 37°C for 1 h. The supernatant was then aspirated, and the blue crystals were dissolved in 500 μl isopropanol + 0.1% Triton X-100. Absorbance was measured at 570 nm using a Tristar LB 941 plate reader (Berthold Technologies).

Detection of intracellular oxidative stress. For the detection of reactive oxygen species (ROS), the fluorescent probe carboxy-H_2DCF-DA (Invitrogen) was used. In short, 3×10^5 cells were seeded per well in six-well plates. After seeding (24 h), cells were treated with different concentrations of CSE and trypsinized after 2, 6, or 24 h. After being washed with PBS, cells were incubated for 20 min in a 5 μM carboxy-H_2DCF-DA solution in PBS at 37°C. Cells were then resuspended in ice-cold PBS and analyzed by fluorescence-activated cell sorter (FACS) analysis (Becton-Dickenson LSRII).

Live/dead assay using annexin V. Induction of apoptosis or necrosis was investigated in A549 cells using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining. For that, 3×10^5 cells were seeded per well in six-well plates and treated 24 h after seeding with CSE with the indicated concentrations. CSE-containing media was then removed, and cells were washed, trypsinized, and stained with annexin V-FITC and PI in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) at room temperature for 15 min. Samples were then analyzed by FACS analysis (BD LSRII) using FlowJo software (version 7.6.5).

Proteasome activity assay. The chymotrypsin-like, trypsin-like, and caspase-like proteasome activities in cell and tissue lysates were assessed using luminogenic substrates Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin, and Z-nLPnLD-aminoluciferin, respectively (Proteasome-Glo Assay System; Promega). For each assay, 3×10^5 cells were seeded per well in six-well plates. After seeding (24 h), cells were treated with CSE. Cells were then harvested by scraping and lysed under hypoosmotic conditions by repeated freezing (liquid nitrogen) and thawing (37°C) in distilled water containing protease inhibitor cocktail (Complete; Roche). Whole lung tissue was homogenized using the Mikro-Dismembrator (Sartorius Stedim Biotech) and lysed as described above. After removal of cellular debris by centrifugation, supernatants were used for determination of protein concentration (Pierce BCA kit; Thermo Scientific) and proteasome activity. The chymotrypsin-like, caspase-like, and trypsin-like proteasome activities were determined in cell lysates using the Proteasome-Glo 3 Substrate System (Promega) according to the manufacturer's instructions. The luminescent signal was measured in a Tristar LB 941 plate reader (Berthold Technologies). Enzymatic activity was normalized to protein concentration. Data are expressed relative to the activity of untreated control lysates; the average of all individual control samples within an experiment was taken and set as one; individual control samples and treated samples are shown relative to this average.

Native gel analysis. Chymotrypsin-like proteasome activity in cell lysates was assessed using the synthetic peptide substrate Suc-LVVY linked to the fluorescent molecule AMC (Enzo Life Sciences). For native gel analysis, equal amounts of protein (25 μg) from hypoosmotic lysates were subjected to electrophoresis (16 h, 50 V, 4°C) on 5% nondenaturing gels. Proteasome activity was detected by incubating the gels for 30–60 min at 37°C in an ATP-regenerating buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 1 mM dithiothreitol) containing 50 μM Suc-LVVY-AMC. Gels were analyzed using the ChemiDoc XRS+ (Bio-Rad) with an excitation wavelength of 380 nm and emission wavelength of 460 nm. Band intensity was quantified with the Image Lab software package (version 3.0.1 beta 2) from Bio-Rad. Equal protein loading was assessed by PAGE-Blue (Fermentas) staining of native gels after overlay assays according to the manufacturer's instructions.

In vitro proteasome activity. Purified 20S proteasome was kindly provided by Burkhardt Dahlmann from the Institute of Biochemistry of the Charité Berlin. To confirm the purity of the purified 20S batch, 10 ng of 20S were run on a 10% SDS gel. Silver staining confirmed the typical band pattern of highly purified 20S proteasomes (data not shown). We exposed 50 ng of purified 20S solution in 10 mM HEPES buffer (pH 7.6) to CSE in 10 mM HEPES buffer for 15 min at room temperature. The chymotrypsin-like, caspase-like, and trypsin-like proteasome activities were determined using the Proteasome-Glo 3 Substrate System according to the manufacturer's instructions. The luminescent signal was quantified in a Tristar LB 941 plate reader (Berthold Technologies). Enzymatic activity was expressed relative to the activity of untreated controls.

Antibodies and western blot. Anti-α_5 proteasome subunit antibody was purchased from BostonBiochem, and anti-β_1 and anti-β_2 proteasome subunit antibodies were purchased from Santa Cruz Biotechnology. The anti-polyubiquitin (FK1 clone) antibody was obtained from Enzo Life Sciences, the anti-Lys^{48}-polyubiquitin antibody was obtained from Millipore, the β-actin antibody was obtained from CellSignaling, and the OxyBlot protein oxidation detection kit was purchased from Millipore. For Western blot analysis of cultured cells, 3×10^5 cells were seeded per well in six-well plates. After seeding (24 h), cells were treated with CSE, trypsinized, washed with PBS, and lysed in RIPA buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail (Complete; Roche). Frozen whole lung tissue was homogenized using the Mikro-Dismembrator (Sartorius Stedim Biotech) and lysed in RIPA buffer. For detecting ubiquitinated proteins in the insoluble fraction, cell or tissue lysate pellets were resuspended in 8 M urea in Tris buffer (pH 7.6). Protein content was determined in the supernatants using the Pierce BCA protein assay kit (Thermo Scientific). To detect oxidatively modified proteins, the OxyBlot kit was used according to the manufacturer's instructions.

For Western blot analysis, equal amounts of protein (10–20 μg) were subjected to electrophoresis on 10 or 12% SDS-PAGE gels and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were treated with antibodies using standard Western blot techniques. The ECL Plus Detection Reagent (GE Healthcare) was used for chemiluminescent detection, and membranes were analyzed

using Kodak X-Omal LS films (Sigma-Aldrich) in a Curix 60 developer (Agfa) or with the ChemiDoc XRS+ (Bio-Rad). Amido Black staining (Sigma-Aldrich) of the PVDF membranes was performed for protein loading control according to the manufacturer's instructions. Densitometry analysis was performed including all amido black-stained bands.

Glutathione levels. Glutathione (GSH) levels in whole blood and in tissue extracts were analyzed using the GSH-Glo Glutathione assay (Promega) according to the manufacturer's instructions. The luminescent signal was measured in a Tristar LB 941 plate reader (Berthold Technologies). Enzymatic activity was standardized to protein content and expressed relative to the activity of untreated controls.

Animals and maintenance. Female C57BL/6 mice were obtained from Charles River and housed in rooms maintained at constant temperature and humidity with a 12:12-h light cycle. Animals were allowed food and water ad libitum. All animal experiments were conducted under strict governmental and international guidelines and were approved by the local government for the administrative region of Upper Bavaria.

Cigarette smoke exposure of mice. Mice were exposed to mainstream cigarette smoke of a concentration of 500 mg/m^3 total particulate matter for 50 min two times per day for 3 days. The smoke was generated using 10 3R4F Research Cigarettes (Tobacco Research Institute, University of Kentucky) per exposure cycle and was drawn into the exposure chamber via a peristaltic pump. Control mice were kept in a filtered air environment. Immediately after the last exposure, mice were killed with an overdose of ketamine/xylazine followed by exsanguination. Mice were dissected, and bronchoalveolar lavage (BAL) fluid was obtained to perform BAL differential cell counts. Blood was collected from the femoral artery. Lung tissue was shock-frozen in liquid nitrogen and used for protein extraction or fixed by intratracheal instillation of paraformaldehyde and embedded into paraffin for hematoxylin-eosin staining.

Preparation of BAL. The lungs were lavaged using a cannula inserted in the trachea and instilling the lungs with 4 × 0.5 ml aliquots of sterile PBS (Life Technologies). Total cell counts were determined in a hemocytometer via trypan blue exclusion.

Statistical analysis. The one-way ANOVA and Bonferroni posttest was used for statistical analysis of the in vitro data and the Mann-Whitney test for the animal data using GraphPad Prism software (version 5.00). Outlier tests were performed on all datasets using GraphPad Prism software (version 5.00).

RESULTS

CSE induces cell death and oxidative stress in human alveolar epithelial cells. The effects of CSE on cell death and oxidative stress were investigated in A549 human lung epithelial cells. Exposure of A549 cells to increasing doses of CSE resulted in a concentration-dependent decrease of cell survival after 24 h as determined by MTT assays (Fig. 1*A*). Exposure to 100% CSE induced nearly complete cell death after 24 h, whereas 50% CSE diminished cell survival by one-half. Exposure to 25% CSE, however, did not significantly compromise survival of A549 cells. Of note, even high doses of CSE had only minor acute effects on viability of A549 cells as observed in MTT assays after 2 and 6 h (Fig. 1*A*). To determine the nature of CSE-induced cell death, we performed double staining of cells with PI and annexin V-FITC. Annexin V is a sensitive probe for identifying early apoptotic cells, whereas PI is taken up by necrotic cells after breakdown of plasma membranes. CSE induced necrosis in A549 cells in a dose-dependent manner as observed by annexin V-negative and PI-positive staining after 24 h (Fig. 1*B*). After 48 h, cells exposed to 100% CSE had all died; however, cells exposed to 10 and 25% CSE had completely recovered, showing no positive PI staining (data not shown). Because it is known that cigarette smoke mediates oxidative damage in the cell (7),

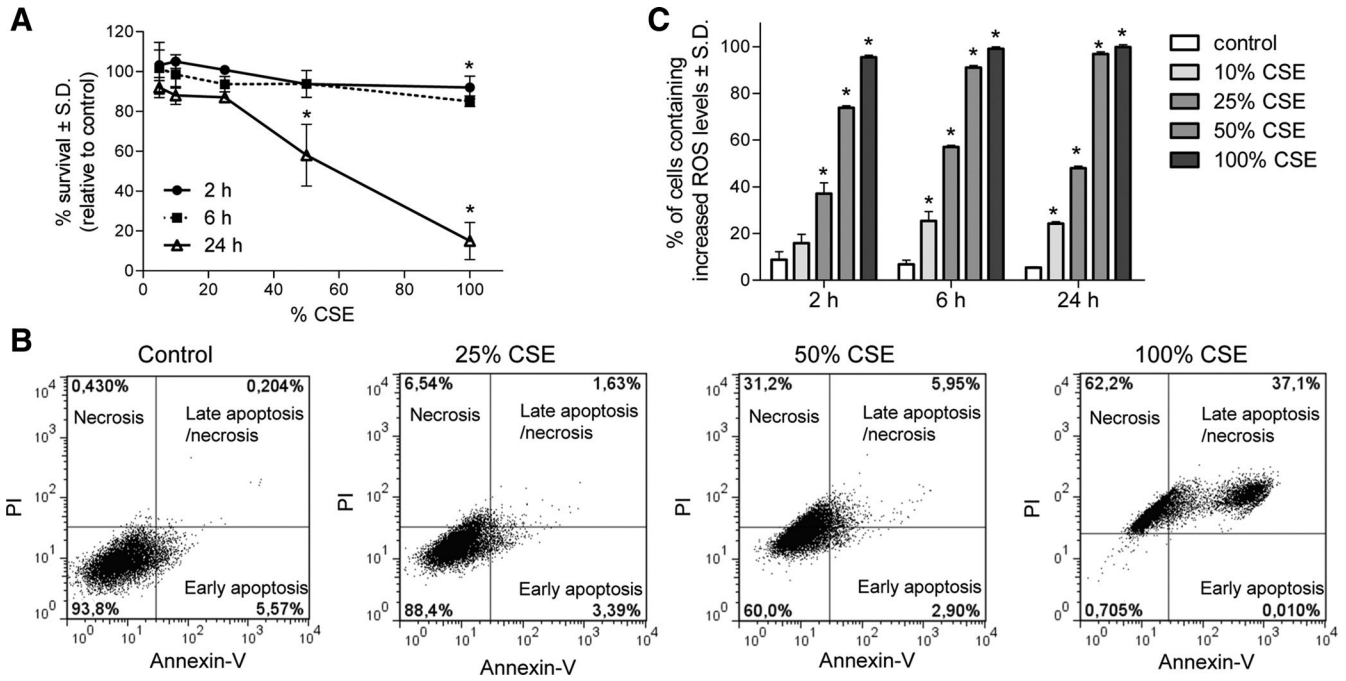

Fig. 1. Cigarette smoke extract (CSE) causes cell death and oxidative stress in lung epithelial cells. *A*: CSE caused a concentration-dependent decrease in cell survival in A549 cells after 24 h but not after 2 or 6 h of exposure as determined by MTT assays. *B*: 24 h of 25, 50, and 100% of CSE exposure induced dose-dependent necrosis in A549 cells as assessed by annexin V-fluorescein isothiocyanate and propidium iodide (PI) double staining and fluorescence-activated cell sorter analysis. *C*: exposure of A549 cells to CSE resulted in a concentration-dependent increase of reactive oxygen species (ROS) after 2, 6, and 24 h (n = 3 ± SD for all experiments, *$P < 0.05$).

intracellular levels of ROS were assessed by FACS analysis using the carboxy-H_2DCF-DA fluorescent probe. Increased levels of ROS were already observed at low CSE concentrations and short exposure times of 2 and 6 h in A549 cells. Furthermore, the increased amount of intracellular ROS was concentration-dependent and persisted even with the low CSE doses for at least 24 h (Fig. 1*C*). These data accord with published data on the effect of CSE on epithelial cells (22, 24).

Accumulation of oxidatively modified and polyubiquitinated proteins by CSE. Next, we determined whether the increased levels of oxidative stress due to CSE exposure also led to the accumulation of oxidatively modified proteins. CSE induced time- and concentration-dependent accumulation of oxidatively modified proteins that was evident after 2, 6, and 24 h of CSE exposure (Fig. 2*A*). Importantly, a transient accumulation of polyubiquitinated proteins was also seen by Western blotting (Fig. 2*B*), suggesting that ubiquitin-mediated degradation contributes to the elimination of cigarette smoke-modified proteins. Moreover, high CSE doses resulted in the accumulation of insoluble ubiquitinated proteins already after 2 h of exposure (Fig. 2*C*). These data indicate that, at high doses of CSE, the ubiquitin proteasome system is unable to cope with severely damaged proteins that accumulate in the cell in the form of insoluble polyubiquitinated aggregates.

Inhibition of the proteasome by CSE. The above data suggest that CSE challenges the proteolytic capacity of the ubiquitin proteasome system. We thus investigated if the proteasome itself is a target for inactivation by cigarette smoke exposure. A549 cells were treated with increasing amounts of CSE for 2, 6, and 24 h, and proteasome activity was analyzed using substrate-specific luminescent probes for the three different activity sites. After only 2 h of CSE exposure, a significant decrease of the chymotrypsin-like and a distinct increase of the caspase-like activity site were observed for high concentrations of CSE (Fig. 3*A*). After 6 h, reduced chymotrypsin-like activity was observed also for lower CSE concentrations, and a reproducible decrease of the trypsin-like activity was observed for 100% CSE (Fig. 3*A*). After 24 h, all three activities were significantly impaired at high CSE concentrations of 50 and 100%. Importantly, the trypsin-like active site was also inhibited at the lower and nontoxic CSE dose of 25%. The inhibition of the chymotrypsin-like activity by CSE was confirmed with a second method, i.e., native gel analysis. This method allows the detection and discrimination of the two main proteasomal complexes in the cell, the 26S and 20S proteasomes, based on an activity assay. Band intensities of 26S and 20S complexes were quantified and clearly showed a dose-dependent decrease of the chymotrypsin-like activity for both subtypes of the

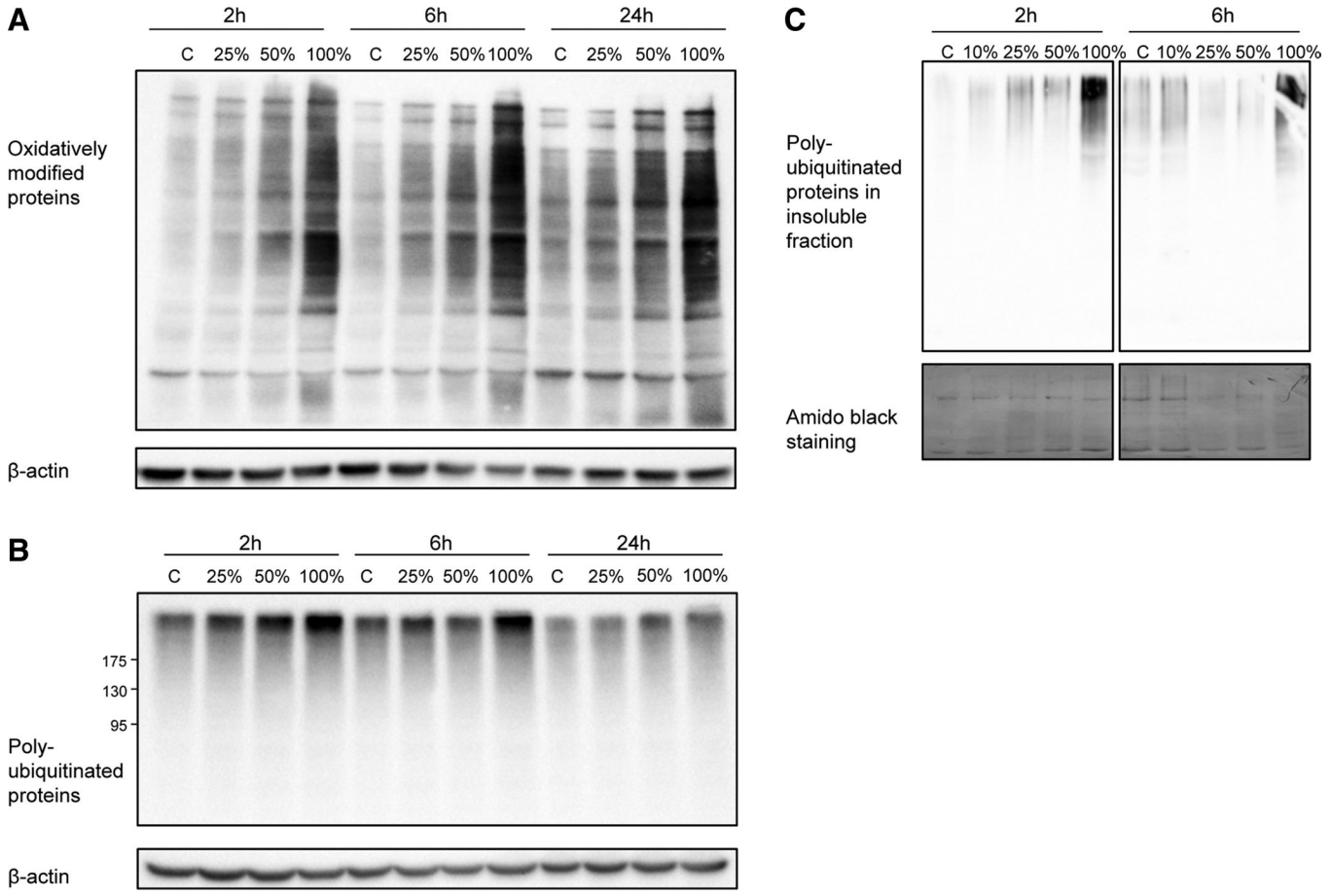

Fig. 2. Exposure to CSE leads to the accumulation of carbonylated and polyubiquitinated proteins. *A*: OxyBlot analysis of A549 cells exposed to increasing doses of CSE showed an accumulation of oxidatively modified proteins after 2, 6, and 24 h. *B*: Western blot analysis of A549 protein extracts revealed accumulation of polyubiquitinated proteins after 2, 6, and 24 h. *C*: Western blot analysis of the insoluble protein fraction (cell pellets) of A549 protein extracts showed CSE-dependent accumulation of polyubiquitinated proteins after 2 and 6 h. Equal protein loading of blots was confirmed by amido black staining of membranes. Representative Western blots of 3 independent experiments are shown.

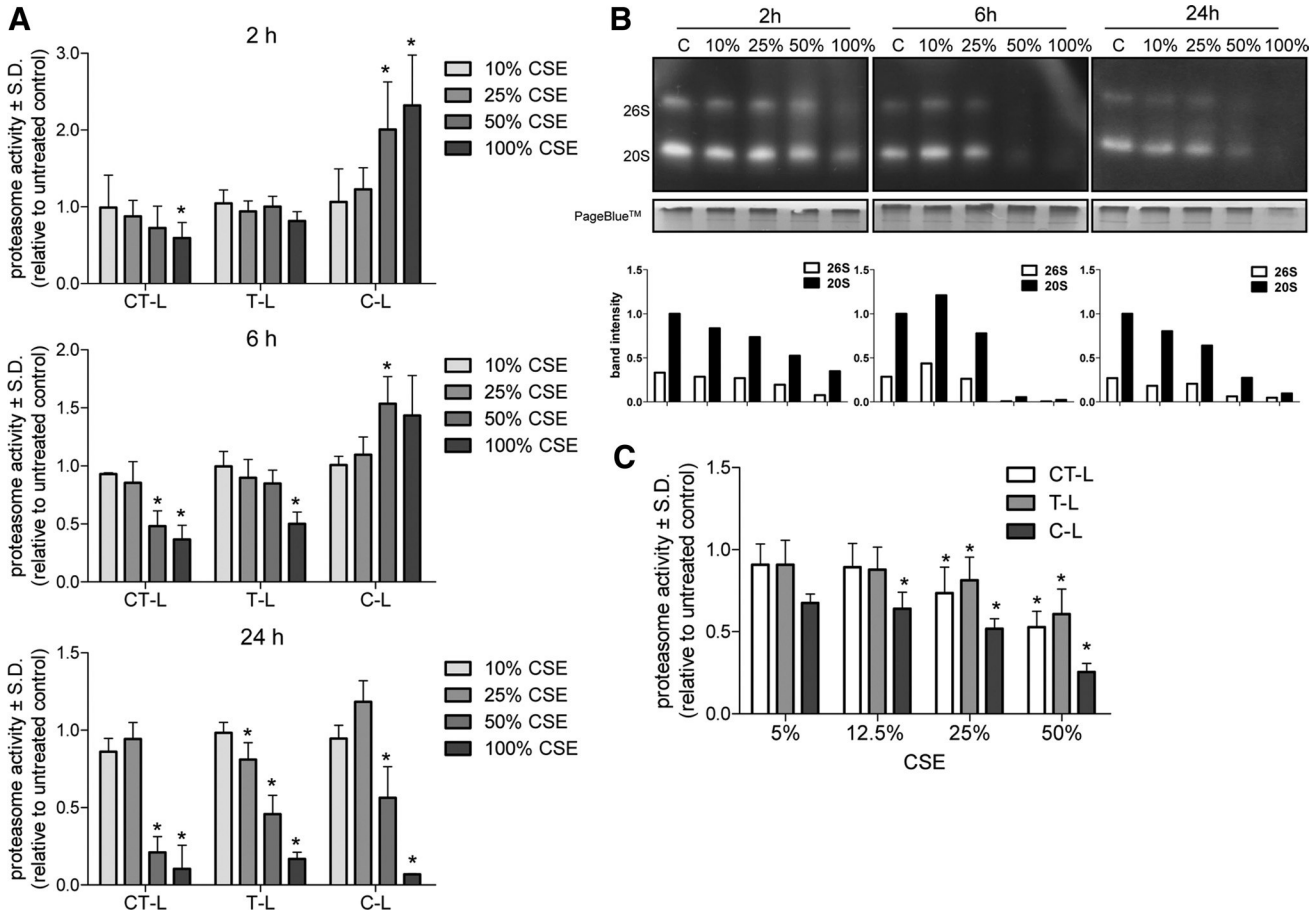

Fig. 3. Exposure to CSE leads to proteasome inhibition in A549 cells. *A*: significant impairment of the proteasome was observed after 24 h for the chymotrypsin-like (CT-L), trypsin-like (T-L), and the caspase-like (C-L) activity sites. The trypsin-like site was inhibited also at nontoxic CSE doses after 24 h. *B*: dose-dependent impairment of the chymotrypsin-like site of the proteasome was confirmed by native gel analysis and overlay activity assay. PageBlue staining confirmed equal protein loading. *C*: incubation of purified 20S proteasome with CSE led to a concentration-dependent inhibition of all three activity sites after 15 min ($n = 3 \pm$ SD for all experiments, $^*P < 0.05$).

proteasome after 2, 6, and 24 h (Fig. 3*B*). The ratio between proteolytically active 26S and 20S was not affected by CSE treatment of A549 cells.

To assess if oxidative stress alone is responsible for the observed impairment of the proteasome, cells were treated with varying concentrations of hydrogen peroxide (H_2O_2) for 24 h and checked for proteasome activity (data not shown). Treatment (24 h) with lethal doses of H_2O_2 did not cause any impairment of proteasome activity but rather resulted in increased activity of the chymotrypsin- and caspase-like activity sites.

To evaluate whether CSE can directly inhibit the proteasome, purified 20S core particle was exposed to increasing amounts of CSE, and proteasome activity was measured. A dose-dependent inhibition of all three active sites was observed after only 15 min of exposure to CSE (Fig. 3*C*). Surprisingly, the caspase-like activity site was inhibited most effectively. These results clearly indicate an acute effect of CSE on proteasome activity.

Expression of the proteasome is not altered by CSE. Next, we assessed whether the impairment of proteasome activity by CSE is the result of expressional changes of the proteasome. A549 cells were treated with low (25%) and high (100%) doses of CSE for up to 24 h, and expression of several proteasomal subunits was analyzed by Western blotting. As depicted in Fig. 4, all three 20S proteasome core subunits analyzed, i.e., the α_5-subunit (outer ring), β_1-subunit (caspase-like activity), and β_2-subunit (trypsin-like activity), were not affected by CSE treatment.

CSE impairs proteasome activity in human bronchial epithelial cells. Because bronchial epithelial cells represent the first line of defense against cigarette smoke, we investigated whether proteasome activity of human bronchial lung epithelial cells is also affected by CSE. The 16HBE bronchial cells showed a concentration-dependent decrease of survival after 24 h of CSE exposure as assessed by MTT assays (Fig. 5*A*). 16HBE cells were more sensitive to CSE treatment than A549 alveolar epithelial cells, since 25% CSE resulted in >50% cell death of the 16HBE cells, whereas it was nontoxic in A549 cells (Fig. 1*A*).

Importantly, proteasome inhibition was also seen in 16HBE bronchial epithelial cells after 24 h of CSE exposure (Fig. 5*B*). Inhibition of the chymotrypsin-like activity site was only observed for high and toxic CSE doses of 25%. However, the trypsin-like activity was significantly inhibited also at lower and nontoxic concentrations of CSE. These data indicate that inhibition of the proteasome due to acute smoke exposure is

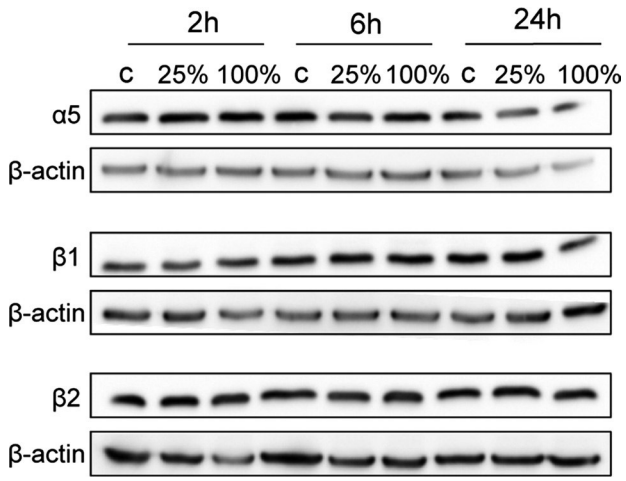

Fig. 4. Exposure of CSE does not lead to expressional changes of proteasome subunits. The α_5 (outer ring)-, β_1 (caspase-like activity)-, and β_2 (trypsin-like activity)-subunits of the 20S core particle showed no altered expression after 2, 6, and 24 h of nontoxic (25%) and toxic (100%) CSE treatment as determined by Western blot analysis. Representative Western blots of 3 independent experiments are shown. C, control.

not cell line specific but can be observed in bronchial and alveolar epithelial cells.

Oxidative stress response upon acute cigarette smoke exposure in mice. We next sought to determine the in vivo effects of acute cigarette smoke exposure on ubiquitin-mediated protein quality control in the lung. For that, mice were exposed to mainstream cigarette smoke for 50 min two times daily for 3 days. Analysis of BAL showed only a slight increase in total cell counts in cigarette smoke-treated mice compared with air-treated controls, indicative of a mild inflammatory response (data not shown). Histological analysis revealed that lung morphology was not affected by acute smoke exposure (data not shown). We were unable to detect infiltration of inflammatory cells into the lung tissue of smoked mice (data not shown). These data indicate that our model of acute cigarette smoke exposure induced only a mild inflammatory response but no detectable lung tissue alterations. Accordingly, mice showed a mild and partial oxidative stress response to cigarette smoke as assessed by quantifying GSH levels in blood and lung tissue and by OxyBlot analysis. Although GSH levels in the blood of smoked mice were significantly decreased compared with control mice (Fig. 6*A*), GSH levels in the lung remained unchanged (Fig. 6*B*). Correspondingly, we were unable to detect any increased levels of oxidatively modified proteins in the lungs of CS-exposed mice (Fig. 6*C*).

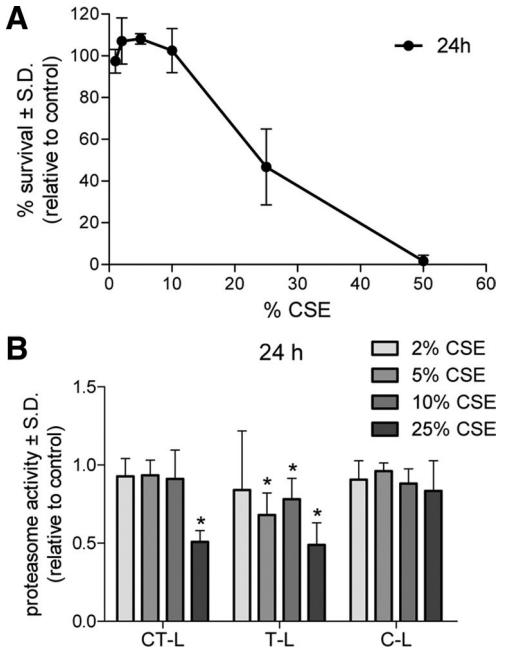

Fig. 5. CSE causes cell death and impairs proteasome activity in human bronchial epithelial cells. *A*: CSE caused a concentration-dependent decrease in cell survival in 16HBE cells as assessed by MTT assay. *B*: significant impairment of the proteasome was observed after 24 h for the CT-L and T-L activity sites at high CSE doses but not for the C-L site. The trypsin-like activity site was also inhibited at nontoxic CSE doses ($n = 3 \pm$ SD, $^*P <$ 0.05).

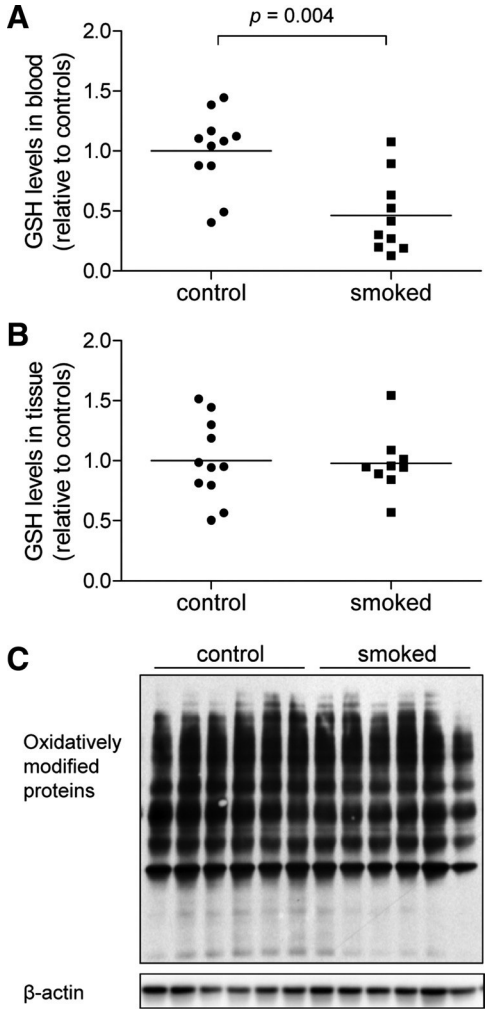

Fig. 6. Oxidative stress response upon acute cigarette smoke exposure in mice. *A*: glutathione (GSH) levels in whole blood were significantly decreased ($p =$ 0.04) in smoked mice compared with air-exposed control mice ($n = 11$ for control group mice and $n = 10$ for smoked mice). *B*: no difference of GSH levels in tissue extracts of smoked mice compared with control mice could be detected ($n = 11$ for control group mice and $n = 10$ for smoked mice). *C*: no accumulation of oxidatively modified proteins was observed in lungs of smoked mice compared with control mice by OxyBlot ($n = 6$/group).

Accumulation of polyubiquitinated proteins upon acute cigarette smoke exposure of mice. Despite low levels of oxidative stress in the lungs of CS-exposed mice, we observed a significant accumulation of Lys^{48}-linked polyubiquitinated proteins in smoked vs. control lungs as determined by Western blot analysis (Fig. 7*A* and for densitometric analysis Fig. 7*B*). Furthermore, accumulation of Lys^{48}-linked polyubiquitinated proteins was also observed in the insoluble protein fraction for low-molecular-weight proteins (Fig. 7*C*). This finding is fully in line with our in vitro results and indicates that ubiquitin-mediated degradation of proteins is part of the protein quality control response to cigarette smoke. Accumulation of insoluble polyubiquitinated protein aggregates suggests that the proteasome is unable to degrade proteins in a timely fashion after acute cigarette smoke exposure.

Impaired proteasome function upon acute cigarette smoke exposure of mice. To assess whether the proteasome itself was also affected by acute cigarette smoke in vivo, whole lung tissue of cigarette smoke- or air-treated control mice was homogenized, and protein extracts were analyzed for proteasome activity. Of note, we observed significant inhibition of 25% of the trypsin-like activity in the lungs of smoked mice (Fig. 8). The chymotrypsin- and caspase-like activities of the lung proteasome were, however, not affected by exposure to cigarette smoke, indicating only partial inhibition of the proteasome. Proteasome activity was also assessed in isolated peripheral blood mononuclear cells from control and cigarette smoke-treated mice. A general trend for decreased proteasome activity was observed for all three activity sites (data not shown), however, not significant.

Proteasome expression is not affected by acute cigarette smoke exposure of mice. To assess whether the decreased proteasome activity in smoke-exposed lungs is the result of transcriptional changes of the proteasome, expression of different proteasome subunits was analyzed by Western blotting. Similar to our in vitro results, all three proteasomal subunits (α_5, β_1, and β_2) analyzed showed no alteration in expression levels in lungs of smoked mice compared with control mice (Fig. 9). RNA expression of these subunits was also not affected, as analyzed by qRT-PCR analysis (data not shown).

DISCUSSION

Cigarette smoke mediates modification and misfolding of proteins, DNA damage, and induces cellular (oxidative) stress.

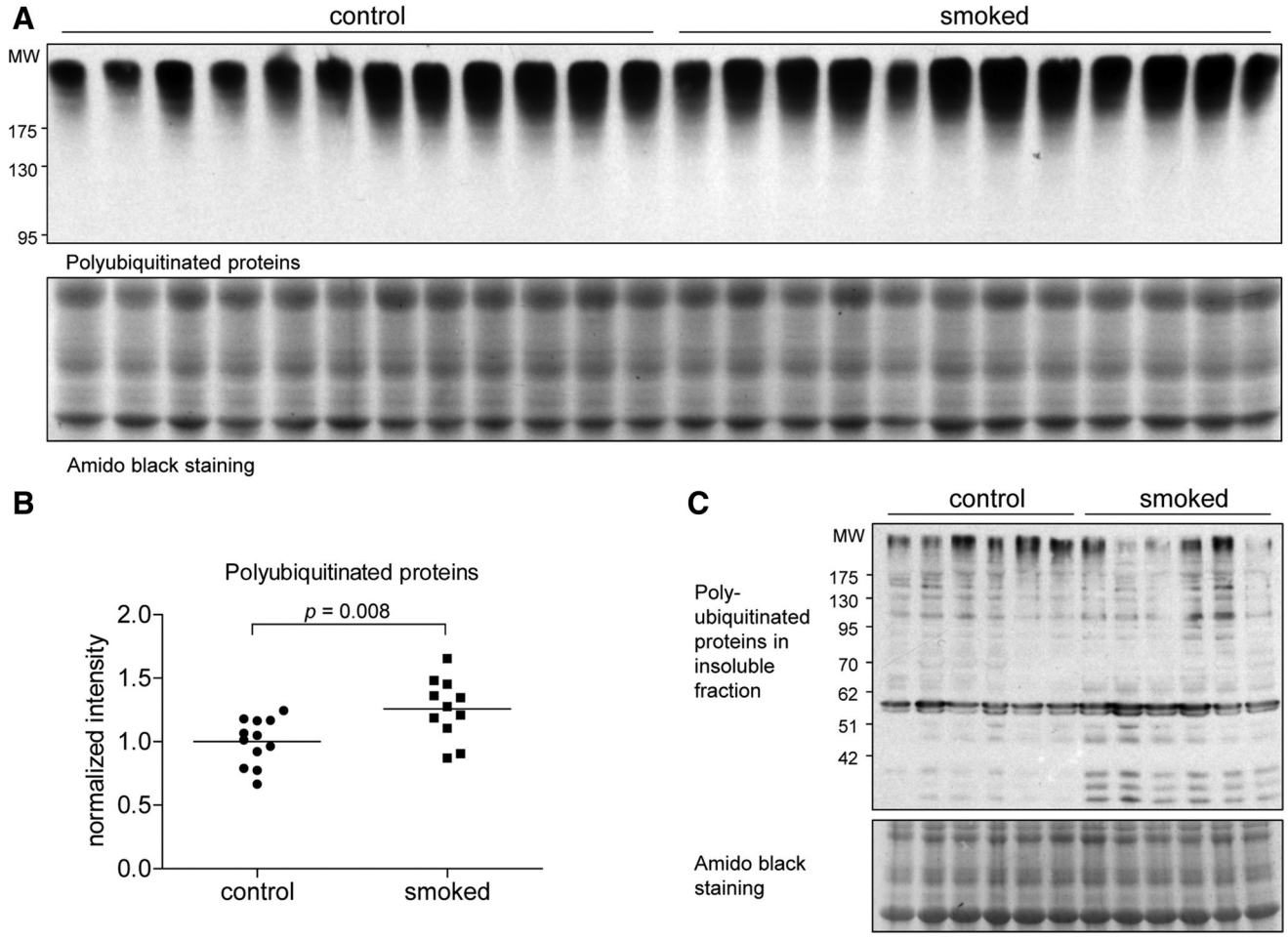

Fig. 7. Accumulation of polyubiquitinated proteins upon acute cigarette smoke exposure of mice. *A*: Western blot analysis of whole lung protein extracts showed accumulation of lysine at position 48 of ubiquitin (Lys^{48})-specific polyubiquitinated proteins in lungs of acutely smoked mice compared with lungs from control mice with $n = 12$/group. MW, mol wt. *B*: band intensity analysis shows a significant increase ($P = 0.008$) of Lys^{48}-specific polyubiquitinated proteins in smoked mice. *C*: accumulation of low molecular polyubiquitinated proteins was also observed in the insoluble protein fraction of smoked mice with $n = 6$/group.

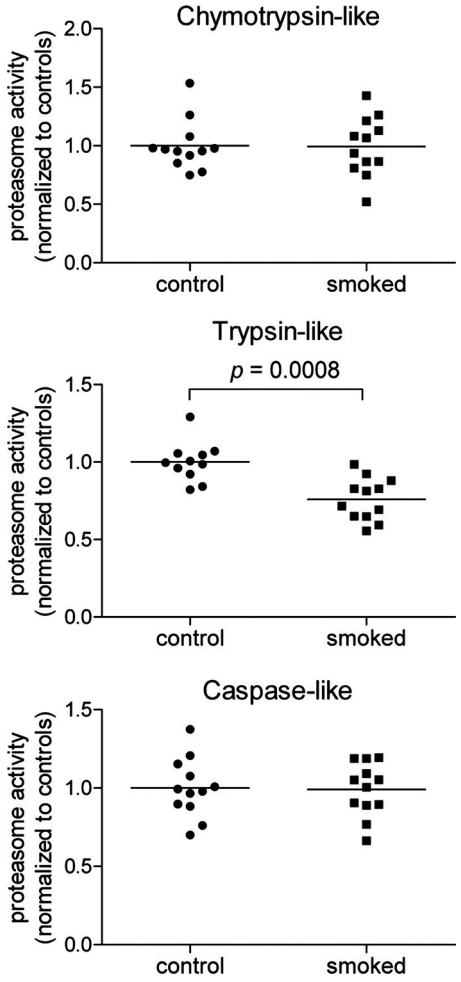

Fig. 8. Impaired proteasome function upon acute cigarette smoke exposure of mice. The trypsin-like activity site of the proteasome was significantly (P = 0.0008) inhibited in protein extracts of smoked mice lungs compared with control lungs. The chymotrypsin-like and caspase-like activity sites remained unaffected (n = 11 for the control group and n = 12 for the smoked mice group).

In the present study, we investigated whether the bulk of smoke-damaged proteins is disposed by the ubiquitin proteasome system, the major protein quality control system in the cell, and also whether the proteasome itself is affected by cigarette smoke.

Cigarette smoke induced oxidative stress. Because cigarette smoke is known to induce oxidative stress, we first analyzed if cigarette smoke is also able to increase markers of oxidative stress and oxidative protein modifications in our model systems, i.e., A549 lung epithelial cells exposed to CSE and mice acutely smoked for 3 days. We are well aware of the limitations of CSE. Because cigarette smoke is bubbled through media and subsequently filtered, some reactive components that are in the smoke gas phase may not be present in the CSE. Our in vivo data, however, clearly indicate that the observed in vitro effects with CSE are reproducible with full mainstream cigarette smoke.

A549 cells exposed to CSE resulted in a rise of intracellular ROS levels that was time- and dose-dependent. In accordance with enhanced oxidative stress levels, a dose-dependent increase in carbonylated (i.e., oxidatively modified) proteins was observed after 2, 6, and 24 h of CSE exposure. Reduction of GSH levels, another marker for oxidative stress, was observed in the blood of mice exposed to cigarette smoke, indicating an acute response to the CS. However, GSH levels in lung tissue of smoked mice remained unaltered compared with the lungs of control mice. Decreased GSH levels upon acute CS exposure (5 h) in mice have been observed previously in lung epithelial lining fluid, plasma, and liver, whereas, similar to our findings, lung GSH levels remained unchanged (15). The lack of detectable oxidative stress levels in the lungs was further supported by our finding that oxidatively modified proteins did not accumulate in protein extracts of smoked lungs. Although a slight increase in total cell counts indicative of a mild inflammatory response was detected in BALs of smoked mice, we saw neither infiltration of inflammatory cells in the lung nor any morphological changes of the lung. Taken together, these data clearly indicate that the acute 3-day exposure of mice to cigarette smoke had no toxic effects.

Cigarette smoke induced accumulation of polyubiquitinated proteins. The proteasome serves, among many other functions, as the central quality control system to rapidly degrade misfolded and modified proteins, among them oxidatively modified proteins. Before degradation, damaged proteins are polyubiquitinated with ubiquitin chains of varying length and structures. In particular, ubiquitin chains conjugated through Lys^{48} target proteins specifically for proteasomal degradation. CSE-exposed human alveolar epithelial cells showed a time- and concentration-dependent accumulation of bulk polyubiquitinated proteins after 2 and 6 h with a persisting accumulation of polyubiquitinated proteins after 24 h. Importantly, a significant increase in Lys^{48}-linked polyubiquitinated proteins was also observed in lung tissue of smoke-exposed mice. To our knowledge, our data are the first evidence for a specific role of ubiquitin-mediated protein degradation in response to cigarette smoke-induced cell damage. Our finding is fully in line with observations made by others that cigarette smoke modifies specific cellular proteins, thereby making them prone for ubiq-

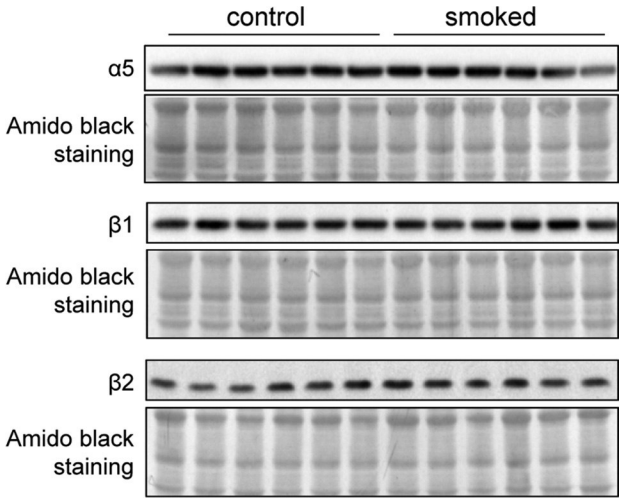

Fig. 9. Proteasome expression is not affected by acute cigarette smoke exposure of mice. Expression of the α_5 (outer ring)-, β_1 (caspase-like activity)-, and β_2 (trypsin-like activity)-subunits of the 20S core particle for smoked and nonsmoked mice lungs remained unaffected as shown by Western blot analysis (n = 6/group).

uitin-mediated proteasomal degradation (28). Accumulation of ubiquitinated proteins in acutely cigarette smoke-exposed mice has recently been shown by fluorescent microscopy (29). However, the antibody used in that study did not discriminate between free, mono-, or polyubiquitin and was therefore not specific to detect ubiquitin-mediated protein degradation.

We also observed that acute cigarette smoke exposure resulted in the accumulation of insoluble polyubiquitinated proteins in human alveolar epithelial cells as well as in the lungs of smoked mice. Similarly, Min et al. (29) detected increased amounts of insoluble ubiquitinated proteins in lungs of COPD patients with smoke history. These data suggest that cigarette smoke has the potential to induce extensive damage of proteins, resulting in misfolding and subsequent formation of insoluble protein aggregates. Formation of such insoluble aggregates is driven by extensive crosslinking of unstable misfolded proteins and has been implicated in several neurodegenerative and cardiovascular diseases as well as in age-related macular degeneration. The formation of these aggregates can be regarded as a hallmark of protein quality disease (8, 9, 17, 34). Here, we show that it can also be detected in lungs of mice exposed to acute cigarette smoke.

Acute cigarette smoke exposure impairs proteasome activity. Protein modification and subsequent loss of function may also apply to the protein quality control system itself, the proteasome. Indeed, the proteasome has been shown to be impaired in response to oxidative and chemical stressors in vitro and in vivo (11, 26). In addition, expression of the proteasome can be deregulated in several diseases, such as in neurodegenerative and cardiovascular diseases, cancer, cachexia, and COPD (9, 25). Here, we report direct impairment of proteasomal activities by cigarette smoke in the absence of transcriptional deregulation. Exposure to high cigarette smoke doses resulted in the pronounced inhibition of all three proteasomal activities in human alveolar epithelial cells and in purified 20S proteasomes. Importantly, only the trypsin-like activity site of the proteasome was inhibited at low and nontoxic doses of CSE in the alveolar epithelial cell line. A similar inhibition of only the trypsin-like activity at nontoxic doses was observed in bronchial epithelial cells, which clearly demonstrates that proteasome inhibition by cigarette smoke is not a cell type-specific effect and is not related to cell toxicity. Bronchial epithelial cells were more susceptible to CSE, with 25% CSE as a high and toxic dose, indicating that different cell lines have different susceptibilities to CSE. We cannot exclude, however, that this may solely be an in vitro phenomenon.

Importantly, mice acutely exposed to low cigarette smoke doses showed a very similar decrease of only trypsin-like activity by about 25% compared with air-exposed controls in the absence of toxicity. Proteasome expression was unaffected by cigarette smoke exposure in both in vitro and in vivo models. Our observation that partial and active site-specific inhibition of the proteasome takes place in the absence of any transcriptional regulation strongly argues in favor of a direct effect of cigarette smoke components on the proteasome. This argument is further supported by the fast and direct CSE-dependent inhibition of purified 20S proteasomes and also by the observation that high doses of H_2O_2 did not impair proteasome activity in A549 cells (data not shown). The finding that polyubiquitinated proteins accumulated in the absence of increased levels of oxidative stress in smoke-exposed lungs of mice also further strengthens this notion. Hence, cigarette smoke either contains some (unknown) proteasome inhibitors or it may induce chemical modifications to the proteasome, inferring a structural change that renders the proteasome less active. Cigarette smoke contains over 4,700 compounds, many of which are still unknown, making the identification of specific compound(s) responsible for the observed proteasome impairment challenging. Reactive α,β-unsaturated aldehydes, such as acrolein and crotonaldehyde, present in cigarette smoke can cause protein crosslinking (1) and may affect proteasome structure and activity. Initial experiments using acrolein did not reveal any inhibition of the proteasome at the relevant concentrations in epithelial cells or in purified 20S proteasomes (data not shown).

Most studies have assumed that the chymotrypsin-like activity of the proteasome is most important for protein breakdown. Consequently, the relative contributions of the two other active sites to protein degradation in mammalian cells are not well studied (20, 30). This is surprising since these different activity sites have evolved specifically in eukaryotes and show different cleavage preferences. It has been reported that the importance of the trypsin-like activity site in protein degradation varies strongly, depending on the substrate content of basic residues (20). Consequently, impairment of one or more of the proteasome activity sites does not only affect the rate of protein degradation but will also result in qualitative changes of protein processing and thus altered peptide products (20, 35). Partial inhibition of the trypsin-like site by cigarette smoke may therefore result in the generation of an altered peptide repertoire presented via MHC class I to the immune system. Moreover, impaired proteasome activity in general may contribute to increased stress and an exaggerated stress response similar to what is seen in COPD.

In conclusion, we provide evidence that cigarette smoke-modified proteins of the lung are disposed by the ubiquitin proteasome system. Here, we show for the first time that acute exposure to cigarette smoke directly impairs proteasome activity in the lungs of mice and in human alveolar and bronchial epithelial cells without affecting proteasome expression. Our results indicate that defective proteasomal protein quality control may exacerbate the detrimental effects of cigarette smoke in the lung. This finding not only adds to the understanding of how the environmental pollutant cigarette smoke may contribute to the detrimental changes as seen in chronic lung disease but also highlights the central pathophysiological role of proteasomal protein degradation in human diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.H.v.R. and S.M. conception and design of research; S.H.v.R., I.E.K., G.J., and K.K. performed experiments; S.H.v.R., I.E.K., G.J.,

and K.K. analyzed data; S.H.v.R., I.E.K., and G.J. interpreted results of experiments; S.H.v.R. and I.E.K. prepared figures; S.H.v.R. drafted manuscript; S.H.v.R., I.E.K., A.O.Y., O.E., and S.M. edited and revised manuscript; S.H.v.R., I.E.K., G.J., K.K., A.O.Y., O.E., and S.M. approved final version of manuscript.

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2.2.1 Supplementary information

Acute cigarette smoke exposure impairs proteasome function in the lung

Sabine H. van Rijt, **Ilona E. Keller**, Gerrit John, Kathrin Kohse, Ali Ö. Yildirim, Oliver Eickelberg and Silke Meiners

SUPPLEMENTAL DATA

Isolation of PBMC's from mouse blood: Whole blood collected from the femoral artery was diluted 5x in PBS. This was carefully transferred into a tube containing Biocoll (Biochrom AG) in a 2:1 (Biocoll:blood/PBS) ratio. This mixture was then centrifuged for 30 min at 800 x g. After centrifugation, the interphase was taken, and centrifuged for 10 min at 800 x g. The cell pellet was washed once with PBS and then analyzed for protein content and proteasome activity as described in the experimental section for cultured cells.

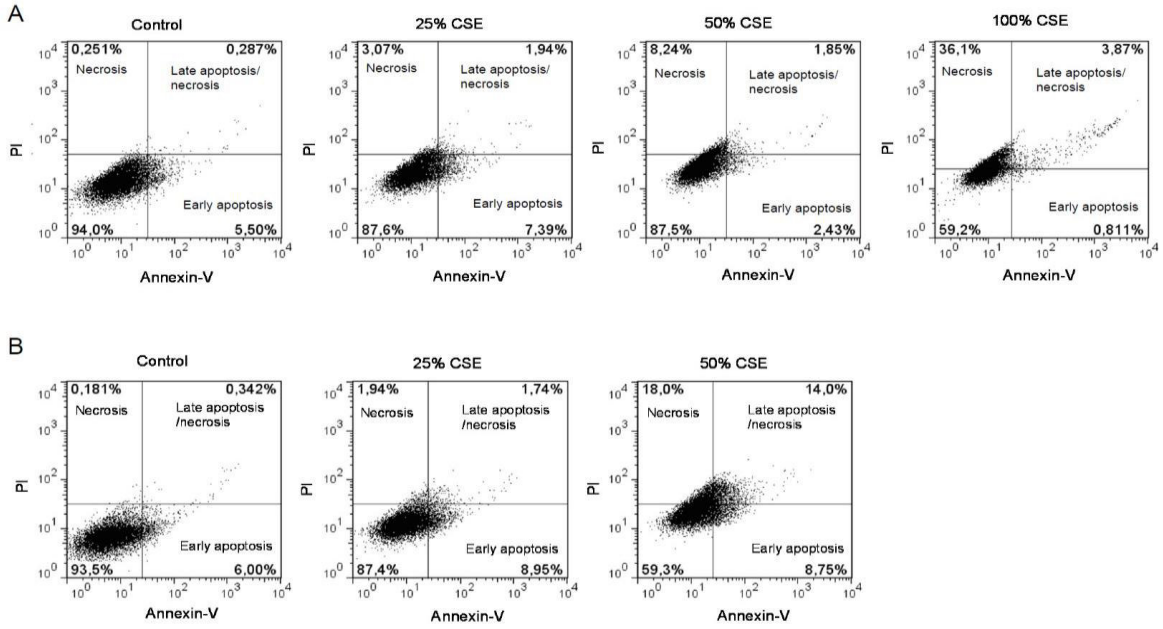

FIGURE S1. CSE induces necrosis in A549 cells. Cells treated with 25%, 50%, and 100% of CSE showed dose-dependent necrosis in A549 cells as assessed by Annexin-V-FITC and propidium iodide (PI) double staining using FACS analysis, in *A:* after 4h of CSE exposure and in *B:* after 48h of CSE exposure. Note, 100% CSE treated cells were dead after 48 h exposure.

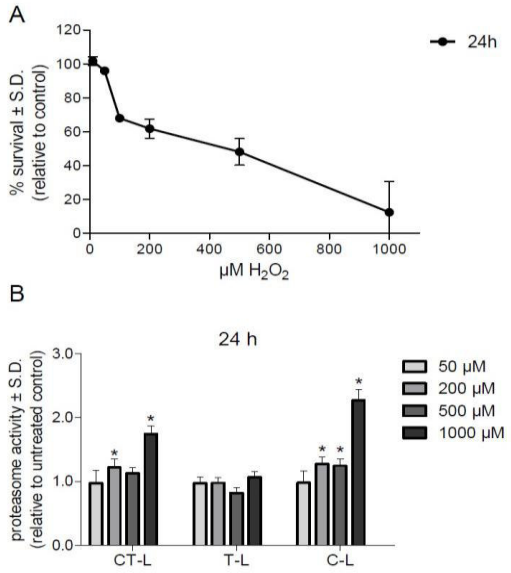

FIGURE S2. Treatment of A549 cells with high doses of hydrogen peroxide (H_2O_2) for 24 h resulted in increased proteasome activity. *A*: Treatment with H_2O_2 caused a concentration dependent decrease of cell survival in A549 cells after 24 h of exposure as determined by MTT assays. *B*: A significant (* = $p < 0.05$) increase of chymotrypsin-like (CT-L) and caspase-like (C-L) activity was observed for cells treated with high (lethal) doses of H_2O_2. The trypsin-like (T-L) activity site remained unaltered. n = 3 ± S.D.

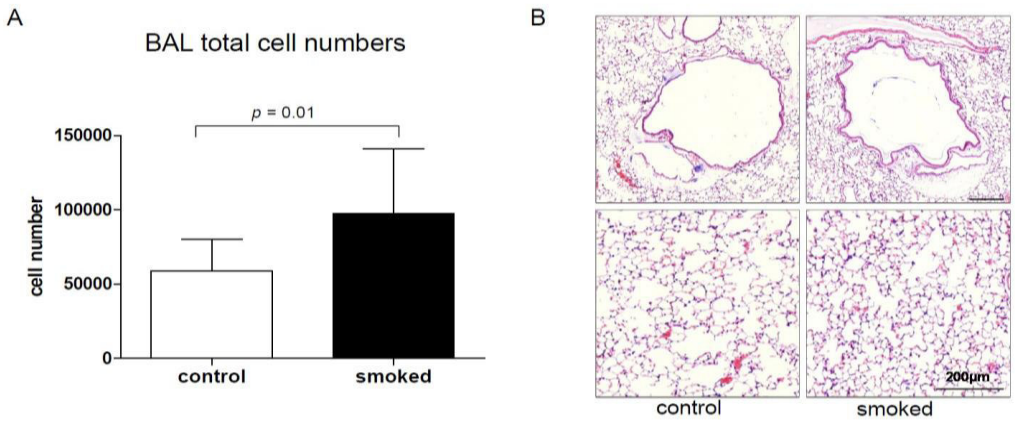

FIGURE S3. Mice were exposed to nontoxic doses of cigarette smoke. *A:* Total cell counts are slightly increased in BAL fluid isolated from lungs of smoked mice compared to air-exposed mice indicating a mild inflammatory response. *B:* Paraffin sections of lung tissue show no differences in lung morphology indicating that the lungs were not severely damaged by acute cigarette smoke exposure.

Figure S4

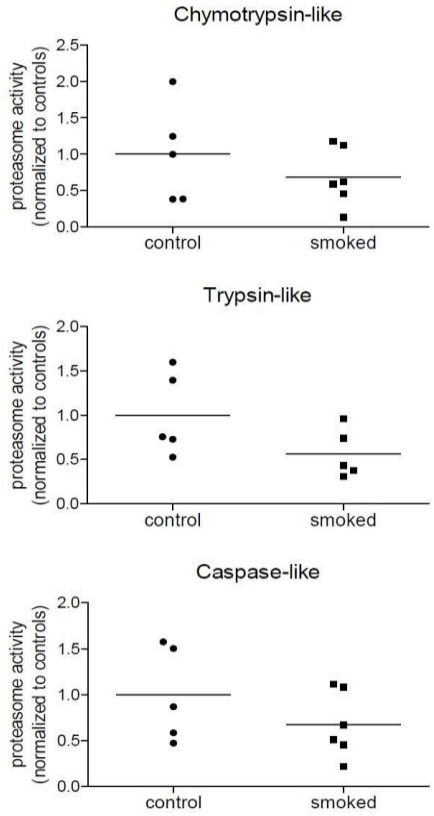

FIGURE S4. Proteasome activity in isolated PBMCs**.** Proteasome activity in isolated PBMCs show a trend to decreased activity of all three activity sites, however not significant.

2.2.2 Authors' contributions

Sabine H. van Rijt	*in vitro* cell culture experiments with cigarette smoke extract: viability testing (Fig. 1A, 5, S2A), FACS analysis of reactive oxygen species and cell death (Fig. 1B/C, S1), Western Blot (Fig. 2C, 4), proteasome activity assays (Fig. 3A/C, 5B, 8, S2B), native gel analysis (Fig. 3B), glutathione measurement (Fig. 6A/B), PBMC isolation (Fig. S4), study design, preparation and editing of figures and manuscript
Ilona E. Keller	*in vitro* cell culture experiments with cigarette smoke extract, Oxyblot (Fig. 2A, 6C), Western Blot analysis (Fig. 2B, 7, 9), preparation of figures, editing of manuscript
Gerrit John	cigarette smoke exposure of mice, BAL analysis (Fig. S3A), histology (Fig. S3B)
Kathrin Kohse	cigarette smoke exposure of mice, BAL analysis (Fig. S3A)
Ali Ö. Yildirim	supervision of G. John and K. Kohse
Oliver Eickelberg	editing of manuscript
Silke Meiners	design of the study, supervision of S.H. van Rijt and I.E. Keller, preparation and editing of manuscript

2.3 Regulation of immunoproteasome function in the lung

Ilona E. Keller, Oliver Vosyka, Shinji Takenaka, Alexander Kloß, Burkhardt Dahlmann, Lianne I. Willems, Martijn Verdoes, Hermen S. Overkleeft, Elisabeth Marcos, Serge Adnot, Stefanie M. Hauck, Clemens Ruppert, Andreas Günther, Susanne Herold, Shinji Ohno, Heiko Adler, Oliver Eickelberg and Silke Meiners

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Regulation of Immunoproteasome Function in the Lung

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Impaired immune function contributes to the development of chronic obstructive pulmonary disease (COPD). Disease progression is further exacerbated by pathogen infections due to impaired immune responses. Elimination of infected cells is achieved by cytotoxic CD8$^+$ T cells that are activated by MHC I-mediated presentation of pathogen-derived antigenic peptides. The immunoproteasome, a specialized form of the proteasome, improves generation of antigenic peptides for MHC I presentation thereby facilitating anti-viral immune responses. However, immunoproteasome function in the lung has not been investigated in detail yet. In this study, we comprehensively characterized the function of immunoproteasomes in the human and murine lung. Parenchymal cells of the lung express low constitutive levels of immunoproteasomes, while they are highly and specifically expressed in alveolar macrophages. Immunoproteasome expression is not altered in whole lung tissue of COPD patients. Novel activity-based probes and native gel analysis revealed that immunoproteasome activities are specifically and rapidly induced by IFNγ treatment in respiratory cells *in vitro* and by virus infection of the lung in mice. Our results suggest that the lung is potentially capable of mounting an immunoproteasome-mediated efficient adaptive immune response to intracellular infections.

The lung is constantly exposed to acute environmental agents such as noxious gases, aerosols, and pathogens[1]. Efficient clearance and defense mechanisms are thus indispensable to protect the lung from injury and maintain lung function. Failure of these defense mechanisms results in sustained inflammation and activation of the immune system, contributing to chronic pulmonary diseases with impaired lung structure and function[2]. This is particularly evident for chronic obstructive pulmonary disease (COPD): lungs of COPD patients show increased levels of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interferon-γ (IFNγ) as well as increased numbers of both innate and adaptive immune cells[2,3]. In addition, bacterial or viral infections in COPD patients often result in acute exacerbations and

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accelerate disease progression, suggesting that, amongst others, the adaptive immune system is unable to efficiently detect and eliminate infected lung cells to terminate pathogen amplification. Intracellular antigens are detected by pathogen-specific activated $CD8^+$ T cells that patrol the lungs for pathogen-derived peptides presented in complex with major histocompatibility complex (MHC) I on the cell surface of infected cells.

The ubiquitin-proteasome system is the major peptide provider for MHC I antigen presentation. It degrades more than 90 % of all cellular proteins - including old and damaged ones - into small peptides[4–6]. The proteasome consists of a barrel-shaped 20S proteolytic core particle which is activated by different proteasome regulators to form for instance the 26S, which degrades poly-ubiquitinated proteins in an ATP-dependent manner, and hybrid proteasomes[7]. The 20S core is composed of four heptameric rings comprising α- and β-subunits with $\alpha_7\beta_7\beta_7\alpha_7$ structure. In standard proteasomes, three of the seven β-subunits - namely $\beta1$, $\beta2$, and $\beta5$ - exhibit proteolytic activity. A replacement of these β-subunits by their immunosubunit counterparts, i.e. low molecular mass protein (LMP) 2, multicatalytic endopeptidase complex-like 1 (MECL-1), and LMP7, also termed $\beta1_i$, $\beta2_i$, and $\beta5_i$, respectively, results in formation of so-called immunoproteasomes. Immunoproteasomes are constitutively present in lymphoid cells but their synthesis can be induced rapidly also in non-immune cells by IFNγ, or TNFα, e.g. upon viral or bacterial infection[8]. The newly assembled immunoproteasomes have altered cleavage kinetics compared to their 20S standard counterparts and generate antigenic peptides that are preferentially presented by MHC I molecules[9]. As such, rapid and specific induction of immunoproteasomes is required for efficient elimination of infected cells via the adaptive immune system. Increasing evidence suggests impairment of proteasome function by smoke exposure and in COPD[10–13], however, until now it is not known whether immunoproteasome function is affected as well. Moreover, cell-specific expression of immunoproteasomes in the lung has not been analyzed so far and it is unclear to which degree immunoproteasome activity can be induced upon virus infection *in vivo*.

In this study, we comprehensively characterized immunoproteasome function, i.e. activity, in the lung by dissecting IFNγ-mediated regulation of specific catalytic activities of the immunoproteasome in different respiratory cell types *in vitro* and upon MHV-68 infection of the lung *in vivo*.

Results

Immunoproteasome expression in the murine lung. As immunoproteasome expression in the lung has not been investigated in detail so far, we first examined total expression levels of immunoproteasome subunits in the murine lung compared to liver and spleen including tissues from LMP2 and LMP7 deficient mice as controls (Fig. 1a). Wildtype lungs contained intermediate amounts of the immunoproteasomal subunits LMP2 and LMP7 compared to liver and spleen. While LMP7 levels were unchanged in LMP2 deficient mice, LMP2 protein levels were evidently decreased in LMP7 deficient mice and the unprocessed pro-form of LMP2 accumulated in spleens of LMP7 deficient mice. To confirm incorporation of immunoproteasome subunits into active 20S proteasomes and their relative distribution compared to standard β-subunits, we next isolated active 20S proteasomes from the lungs of healthy wildtype mice via sucrose-gradient fractionation and separated them on a 2D gel (Fig. 1b and Supplementary Fig. S1). By mass spectrometry of excised spots, we detected all different 14 subunits of the 20S proteasome. All three immunoproteasome subunits were present in addition to the three standard proteasome subunits $\beta1$, $\beta2$, and $\beta5$. Each immunoproteasome subunit was identified from three distinct spots, indicating post-translational modifications of these subunits.

To specify the pulmonary cell types that contain immunoproteasomes, we stained murine lungs with LMP2- and LMP7-detecting antibodies using lungs of the respective knockout animals as controls. Although we tested several commercially available antibodies, immunohistochemical detection of LMP7 proved to be unspecific as controlled by lungs of LMP7 knockout mice while staining for LMP2 was specific (Fig. 1c): Alveolar epithelial and parenchymal cells of the lung expressed only very low amounts of LMP2, whereas alveolar macrophages were highly positive for this immunoproteasome subunit. Individual cells in the vicinity of airways also exhibited prominent LMP2 staining.

Immunoproteasome expression is not altered between donor and COPD lungs. In a next step, we thoroughly investigated immunoproteasome expression in the human lung by using native PAGE and immunoblotting of human donor lung tissue. We unambiguously identified the immunoproteasome subunits LMP2 and LMP7 mainly in active 20S but also to some extent in 26S fractions as confirmed by blotting for respective proteasomal 19S (Rpt5) and 20S subunits (α1-7) (Fig. 2a).

Immunohistochemical analysis of LMP2 in end-stage COPD tissue (GOLD stage III and IV) from explanted lungs revealed no obvious alteration in cell-type specific expression of LMP2 compared to lungs from human donors (Fig. 2b). We observed prominent but variable staining for LMP2 mainly in alveolar macrophages. While bronchial epithelial cells showed some positive staining, alveolar epithelial cells were negative for LMP2. In addition to alveolar macrophages, cells in the vicinity of airways also showed some LMP2 reactivity. However, our immunohistochemical staining was heterogeneous and did not allow a reliable quantification of LMP2 expression levels in lung tissue samples of COPD patients compared to controls.

In human lung tissue homogenates from cancer resections of never-smokers, ex-smokers, and COPD GOLD stage I and II classified patients, we again did not observe any significant difference in the levels

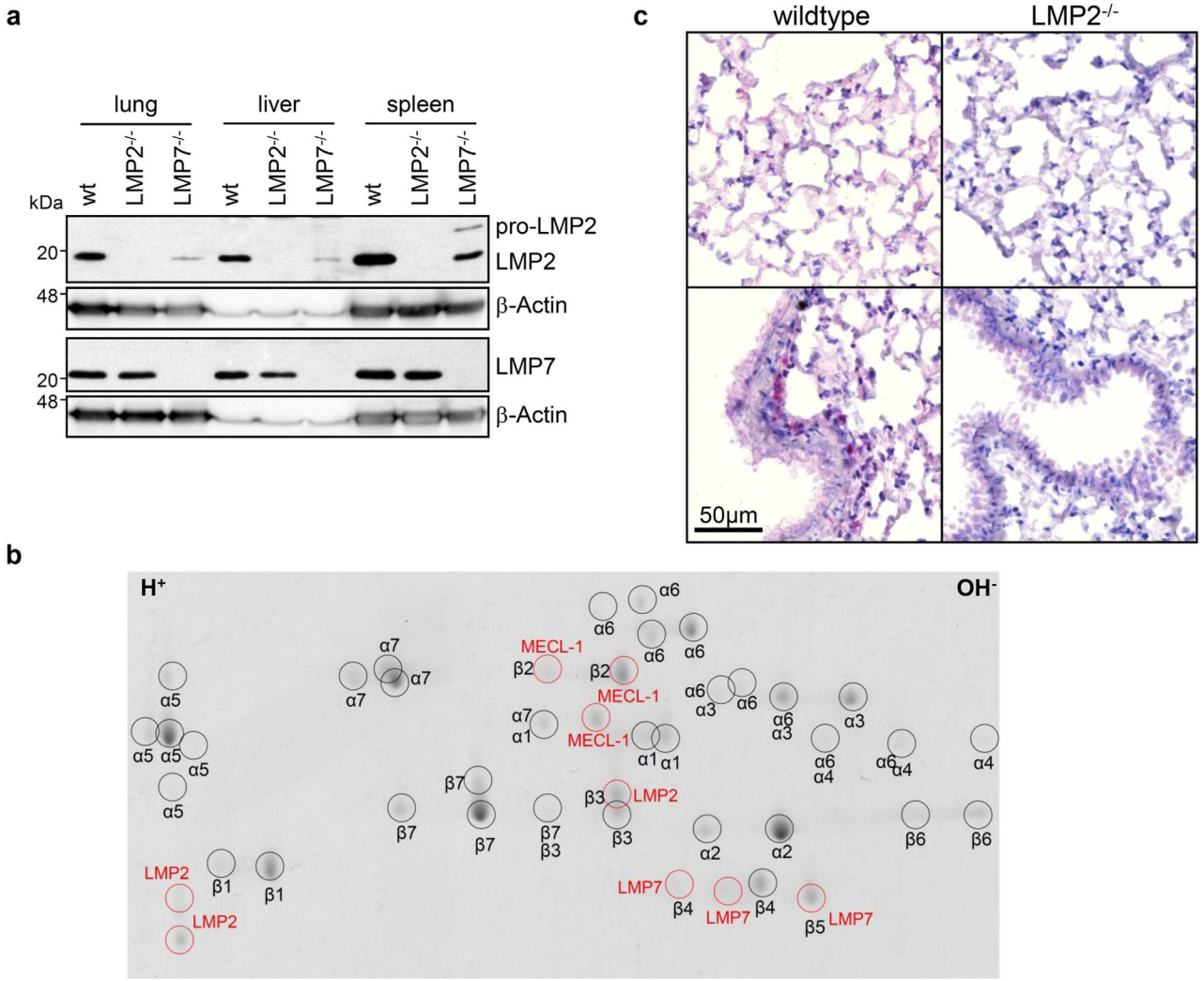

Figure 1. Immunoproteasome expression in mouse lungs. (**a**) Immunoproteasome expression in homogenates of whole lung, liver, and spleen in C57BL/6 wildtype (wt), LMP2$^{-/-}$ or LMP7$^{-/-}$ mice. (**b**) Coomassie stained 2D-gel of purified 20S proteasomes from C57BL/6 mouse lungs. Protein spots were identified by mass spectrometry, immunoproteasome subunits are indicated in red. (**c**) Immunohistochemistry analysis of LMP2 expression in wildtype and LMP2$^{-/-}$ mice. Scale bar represents 50 μm.

of LMP2 and LMP7 between these groups (Fig. 2c). Of note, expression of the 20S proteasome was also not altered in COPD tissue compared to non-COPD controls, indicating that the proteasome is not obviously dysregulated in these samples.

Active immunoproteasomes are induced by IFNγ in parenchymal cells of the lung. With the basal expression levels of immunoproteasomes being low in parenchymal cells, but high in immune cells of the lung, we next investigated to what extent immunoproteasomes can be induced in parenchymal cells by IFNγ, which has been shown as a major cytokine involved in acute virus infection and a major inducer of immunoproteasomes[14]. We confirmed IFNγ-mediated induction of immunoproteasomes in primary parenchymal cells of the murine and human lung: IFNγ strongly induced immunoproteasomal gene expression in mouse primary alveolar type II cells (pmATII) after 24 h of treatment (Fig. 3a). LMP2 and LMP7 protein levels were both strongly induced in primary human (phLF) and mouse lung fibroblasts (pmLF) after IFNγ stimulation for 24 h (Fig. 3b). Similar to tissue homogenates shown in Fig. 1a, LMP7 was induced in fibroblasts from LMP2 deficient mice to the same degree as in wildtype mice, but the unprocessed pro-LMP2 accumulated in LMP7 deficient fibroblasts.

To define the kinetics and activities of newly formed immunoproteasomes after IFNγ stimulation in detail, we treated the human alveolar epithelial cell line A549 from 2 up to 72 h with IFNγ. mRNA levels of all three immunoproteasome subunits were upregulated after 2 h and further increased up to 24 h in

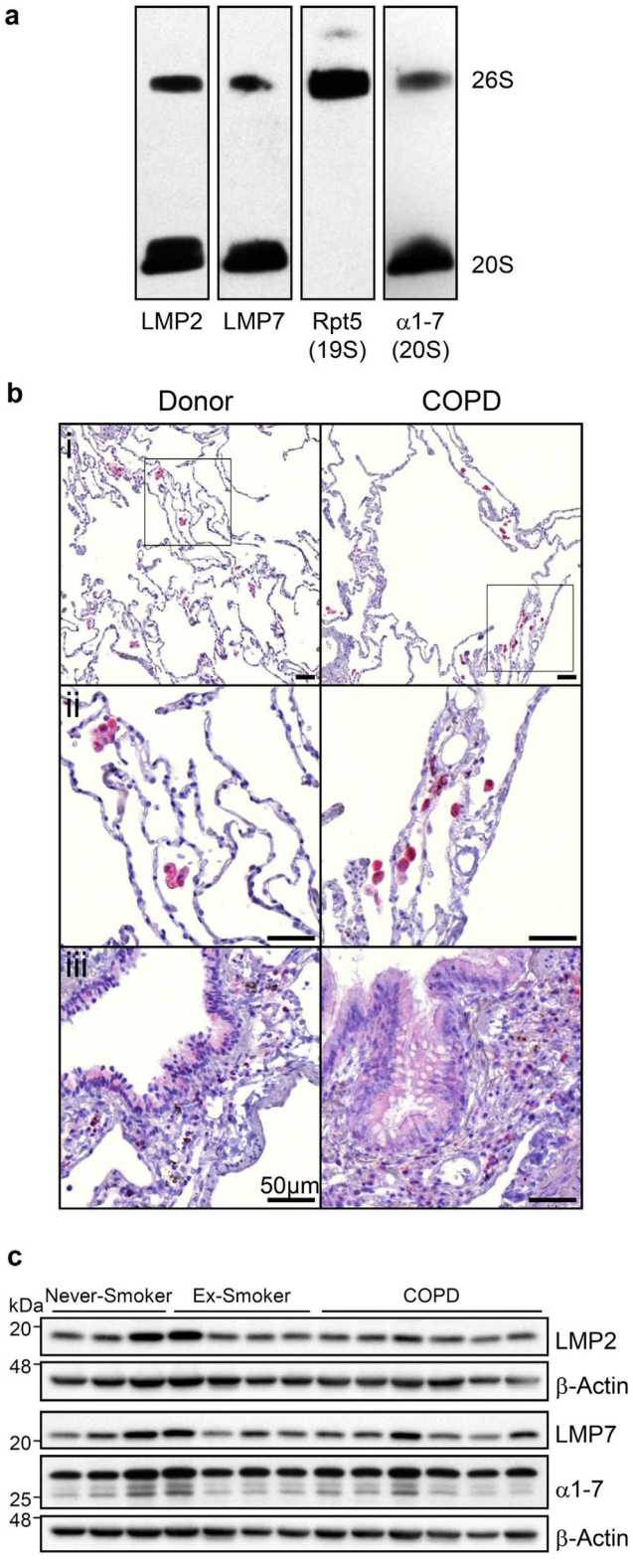

Figure 2. Immunoproteasome expression in human donor and COPD lungs. (**a**) Immunoproteasome expression in human donor lung lysate under native conditions. Native gels were blotted and LMP2, LMP7, α1-7 subunits (20S), Rpt5 (19S) was detected with respective antibodies. (**b**) LMP2 staining of human lung sections from donors (n = 5) and COPD (n = 9) patients: (i) alveolar parenchyma, (ii) alveolar macrophages, (iii) bronchial epithelium with goblet cell hyperplasia in COPD. Scale bar represents 50 μm. (**c**) Protein expression of immunoproteasome subunits LMP2 and LMP7 and total 20S (α1-7) proteasomes in lungs of human organ donors (never-smoker or ex-smoker) and COPD patients.

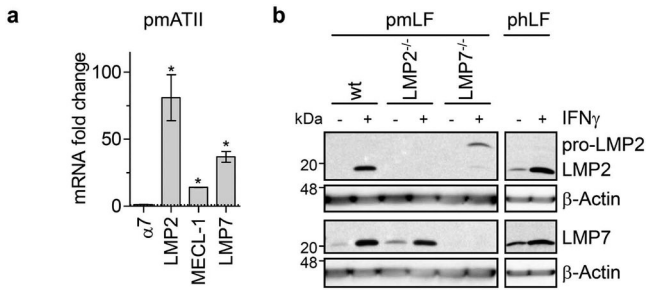

Figure 3. Immunoproteasomes are induced by IFNγ in lung parenchymal cells. (**a**) mRNA fold change of 20S α7-subunit and immunoproteasome subunits in primary mouse alveolar type II cells (pmATII) after 24 h of IFNγ treatment compared to control. Results are combined data from three independent experiments (mean +/− SEM, Mann-Whitney-U test, * = $p < 0.05$). (**b**) Western Blot showing induction of LMP2 or LMP7 in primary mouse (wildtype (wt), LMP2$^{-/-}$, or LMP7$^{-/-}$; pmLF) and human donor lung fibroblasts (phLF) after 24 h of IFNγ treatment. Results are representative for two independent experiments.

A549 cells upon IFNγ treatment (Fig. 4a). Transcript levels of NLRC5, a recently identified transactivator of LMP2 and MHC class I genes[15], transiently peaked at 6 h but declined after 24 h (Fig. 4a).

Protein levels of both LMP2 and LMP7, were upregulated already after 6 h and stayed elevated until 72 h after IFNγ treatment (Fig. 4b). The unprocessed pro-form of LMP2, which indicates that the protein is not yet incorporated into mature 20S proteasome complexes[16], was detected between 6 and 24 h of IFNγ treatment suggesting that LMP2-containing immunoproteasomes are only finally assembled 48 h after IFNγ stimulation. As total 20S proteasome levels, however, were not altered, these results indicate a shift from standard 20S towards immunoproteasome expression in IFNγ-exposed lung alveolar cells (Fig. 4b). *De novo* assembly of active immunoproteasomes was further proven by use of activity-based probes (ABP). ABPs covalently bind to and label only active catalytic β-subunits of the intact 20S catalytic core[17]. Here, we made use of three distinct site-specific ABPs that allowed us to discriminate the active standard and immunoproteasome subunits[18]. Native lysates of IFNγ-treated A549 were incubated with the respective ABPs and then separated under denaturing conditions to quantify the labeled catalytic subunits of the proteasome: The activity of all three immunoproteasome subunits, LMP2, MECL-1, and LMP7, increased up to 24 h and slightly decreased at 72 h after IFNγ treatment while the standard catalytic subunits β1, β2, and β5 were inversely regulated after an initial 24 h activation burst (Fig. 4c). The novel technique of ABP detection in native gels revealed ABP labeling of five different active 26S and hybrid proteasome complexes with a slight shift from active 26S to 20S proteasomes after 72 h of IFNγ treatment (Fig. 4d). LMP2 and LMP7 were incorporated into both, active 20S and 26S complexes, as shown by immunoblotting.

Active immunoproteasomes are induced by MHV-68 infection in the lung. As IFNγ-mediated induction of immunoproteasome is indispensable for efficient antigen presentation of viral proteins during infection, we investigated the kinetics of immunoproteasome expression and activity in the lung after murine gammaherpesvirus-68 (MHV-68) infection *in vivo*. MHV-68 infection strongly induced immunoproteasome expression: mRNA levels of all three immunoproteasome subunits were highest at day 14 post infection and declined to control levels after 148 days, even though IFNγ and TNFα transcript levels were still increased at that time point (Fig. 5a and Supplementary Fig. S3a). NLRC5 mRNA levels showed similar expression kinetics, but were still elevated after 148 days. Expression of standard proteasome subunits was not obviously altered upon infection and even slightly decreased over time (Fig. 5b).

On the protein level, immunoproteasomes were strongly induced after 14 days and were still found to be slightly elevated 148 days after infection (Fig. 5c). The α3 as well as the β1 and β2 constitutive subunits were also increased after 14 days of infection, although to a lesser extent (Fig. 5c). The inducible immunoproteasome subunits LMP2 and LMP7 were found in both, 20S and 26S, complexes, as determined by native gel immunoblotting (Fig. 5d). LMP2 staining of virus-infected mouse lungs revealed that the overall increase of LMP2 protein levels after 47 days was mainly attributable to enhanced LMP2 expression in alveolar epithelial cells and alveolar macrophages (Fig. 5e).

ABP labeling of native lung lysates of infected mice revealed that the specific activity of LMP2 and MECL-1 was transiently increased during the course of infection and normalized to control levels after 148 days (Fig. 6a). In these mouse samples, we were not able to discriminate LMP7 and β5 activities as both mouse subunits have a similar molecular weight (Fig S2), different from the human subunits (Fig. 4). Of note, activity of standard subunits β1 and β2 was also increased, but to a lesser extent than their respective immunoproteasome subunit counterparts LMP2 and MECL-1 (Fig. 6a). The pronounced rise in specific immunoproteasome activity during the course of virus infection closely followed a transient increase in total proteasome activity with similar kinetics and resulted in a considerable shift from standard to immunoproteasome activity in these samples (Fig. 6b and Supplemental Fig. S3b).

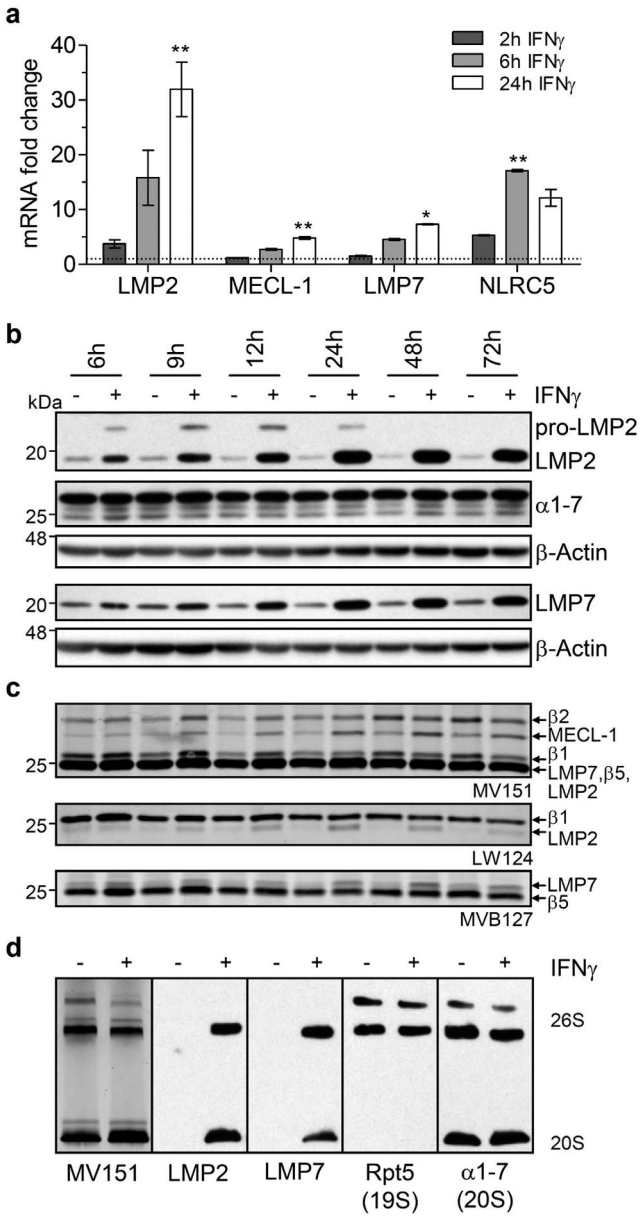

Figure 4. Immunoproteasome induction kinetics in alveolar epithelial cell line. (**a**) mRNA fold change of immunoproteasome subunits and their transcriptional activator NLRC5 in response to IFNγ (75 U/ml) after 2, 6, or 24 h in A549 cells. Results are the combined data of three independent experiments (mean +/− SEM, Kruskal-Wallis Test with Dunn's Post Test, * = $p < 0.05$, ** = $p < 0.01$). (**b**) Time course of expression of immunoproteasome subunits LMP2 and LMP7 and total 20S α-subunits in native lysates of A549 cells from 6 up to 72 h after IFNγ treatment. Results are representative for three independent experiments. (**c**) Fluorescent ABP labeling of the same lysates as in (**b**) with MV151 (labeling all active β-subunits), LW124 (β1 and LMP2 specific) or MVB127 (β5 and LMP7 specific). Results are representative for three independent experiments. (**d**) Native gel analysis of A549 lysates +/− IFNγ treatment for 72 h: MV151-ABP analysis and Western Blot of native lysates with LMP2 and LMP7 antibodies. α1-7 was used to detect 20S complexes, Rpt 5 (19S subunit) was used to detect 26S proteasome complexes. Results are representative for three independent experiments.

The increase in total proteasome activity was attributable to both 20S and 26S complexes by analysis of native PAGE of ABP-labeled lysates (Fig. 6c). Taken together, our data show prominent induction of active immunoproteasomes in the lung by IFNγ in different alveolar cell types and by virus infection *in vivo* indicating that these cells are able to mount efficient immunoproteasome-mediated immune responses to infection. Of note, while the kinetics of specific immunoproteasome activities were similar to the transcript kinetics of immunoproteasome subunits, activation of standard proteasome activity

upon acute virus infection did not involve transcriptional regulation but appears to take place on the post-transcriptional level.

Discussion

Immunoproteasomes play a pivotal role in MHC I antigen presentation. We thus investigated the function and plasticity of immunoproteasomes in human and mouse lungs as well as upon virus-infection.

Protein expression levels of immunoproteasomes in the mouse lung were comparable to those in the liver but lower than in mouse spleen. While we could specifically detect LMP2 and LMP7, immunodetection of the third immunoproteasome subunit MECL-1 was unspecific with several commercially available antibodies. Biochemical purification of lung 20S proteasomes revealed incorporation of both standard and immunoproteasome subunits into active 20S complexes. Of note, each of the three immunoproteasome subunits was found in three distinct protein spots indicating post-translational modifications or isoform expression in the mouse lung. However, our mass spectrometry analysis did not allow us to identify any modifications, which was beyond the scope of this project. Further analysis of native proteasome complexes in the lung by blotting of native PAGE gels revealed that 20S immunoproteasomes can be found both in the 20S and 26S proteasome fraction of mouse and human lungs (Figs. 2a, 4d and 5d), implying that immunoproteasomes contribute to both ubiquitin-dependent (26S) and -independent (20S) degradation of proteins.

While other studies have examined total levels of immunoproteasomes in human tissue, whole rat lungs, and in LCMV-infected mouse lungs and other organs[19–23], cell-specific expression in the lung has not yet been investigated[23,24]. Here, we show that the immunoproteasomal subunit LMP2 is expressed at low basal levels in lung parenchymal cells (alveolar type I and II cells, fibroblasts) and the bronchial epithelium but strongly expressed in alveolar macrophages (Figs. 1c and 2b). Specificity of our staining was confirmed in control lungs of LMP2 deficient mice (Fig. 1c). This is in line with a recent study that demonstrated expression of LMP2 and LMP7 in lung granulomas of sarcoidosis patients[24]. Our *in vitro* data show that primary alveolar type II cells and fibroblasts have the capability to express immunoproteasomes after IFNγ stimulation, thus enabling immunoproteasome-dependent antigen presentation. Interestingly, fibroblasts from LMP7 deficient mice express some unprocessed LMP2 compared to wildtype mice. It was previously shown that LMP7 is necessary for efficient incorporation of LMP2[25], which explains our observation of the presence of unprocessed LMP2 both after stimulation with IFNγ *in vitro* (Fig. 3b) as well as in spleen homogenates of LMP7 deficient mice (Fig. 1a). In A549 cells, the pro-form of LMP2 was detected until 24 h after IFNγ treatment, while pro-LMP7 was not detectable at any time-point (even though the LMP7 antibody we used detects both the unprocessed and mature form of LMP7). This might be due to preferential incorporation of pro-LMP7 into 20S compared to its standard proteasome counterpart pro-β5, as suggested previously[26,27].

Using a novel and specific set of activity-based probes[18], we were able to dissect the six different active sites of the standard and immunoproteasome 20S, which specified immunoproteasome function in the lung. This is not possible with commercially available proteasome substrates. A striking feature of these activity-based probes is that beyond quantification of the three main proteasomal activities, we can specifically discriminate activities for the standard and the respective immunoproteasome subunit counterparts β1/LMP2, β2/MECL-1, and β5/LMP7 as they are labeled within the same lysate (Fig. 4c). The novel combination of activity-based probe labeling of all catalytic active sites of the proteasome with native gel electrophoresis permitted us to assign newly assembled immunoproteasomes to active 20S and 26S proteasome complexes (Fig. 4d). Using this innovative biochemical toolbox, we showed that IFNγ can rapidly induce expression and assembly of active immunoproteasomes in parenchymal cells of the lung. This cannot be achieved with conventional and commercially available proteasome activity assays. With these techniques at hand, we also assessed immunoproteasome activity in the course of virus infection of the lung. For that, we used the model of MHV-68, since intranasal infection of mice leads to productive virus replication in the lung accompanied by virus-induced cell damage and subsequent development of pulmonary fibrosis[28,29]. Viral infections induce immunoproteasomes via IFNγ as part of the adaptive immune response to infections[30] thereby facilitating the specific detection and targeted elimination of infected cells by the immune system: Pathogenic, e.g. viral, proteins are cleaved by immunoproteasomes into antigenic peptides for MHC I presentation[14,31–34]. MHC I epitopes are then recognized by specific cytotoxic $CD8^+$ T cell clones that kill infected cells. To raise a specific clonal T cell response, antigen presenting cells (APC) in the lung take up pathogens and migrate to the lymph nodes to prime $CD8^+$ T cells. Importantly, APCs and infected parenchymal cells need to present the same MHC I epitope to prime an efficient clonal $CD8^+$ T cell response, respectively. As APCs constitutively express immunoproteasomes, IFNγ-mediated upregulation of immunoproteasomes in infected parenchymal cells is thus indispensable for mounting an efficient immune response against the pathogen[14,31–35]. In our MHV-68 infection model (Figs. 5 and 6), we were able to detect increased immunoproteasome transcript and protein levels which were highest 14 days after viral infection. While mRNA transcripts were back at baseline at day 148, protein levels of LMP2 and LMP7 were still increased, suggesting proteasome stabilization and extended half-life of proteasomes after infection. This might also explain the observation of slightly increased standard proteasomes on the protein and activity levels at day 14, which cannot be explained by increased transcript levels (Figs. 5 and 6). Total proteasome activity, as assessed by fluorescent activity-based probes, was transiently increased up to twofold during infection and was

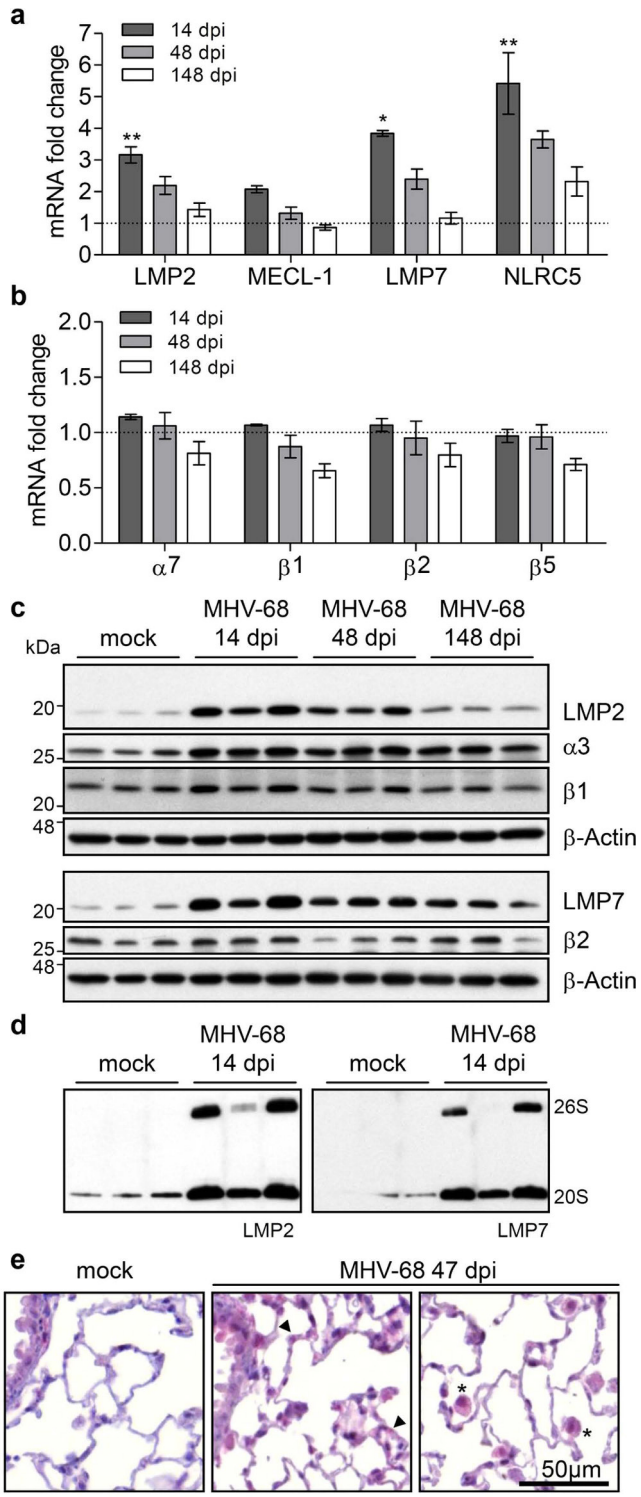

Figure 5. Murine gammaherpesvirus-68 (MHV-68) infection induces immunoproteasomes in the lung. (**a**) and (**b**) relative mRNA levels of standard proteasome subunits α7, β1, β2, β5 and immunoproteasome subunits LMP2, MECL-1, LMP7 and MHC I gene transactivator NLRC5 in the lungs of MHV-68 infected mice (day 14, 48 and 148 post infection) compared to mock-infected controls, Rpl19 served as housekeeping gene, n = 3 per group (mean +/− SEM, Kruskal-Wallis Test with Dunn's Post Test, * = $p < 0.05$, ** = $p < 0.01$). (**c**) Western Blot analysis of LMP2, LMP7, α3, β1 and β2 protein expression of whole lung homogenate of MHV-68 infected mice (day 14, 48 and 148) compared to uninfected controls. (**d**) Native Western Blot of lung lysates from uninfected or MHV-68 infected mice after 14 days. (**e**) Immunohistochemistry analysis of LMP2 expression in wildtype lung slices at 47 dpi. All results are representative for two independent experiments. dpi, days post infection.

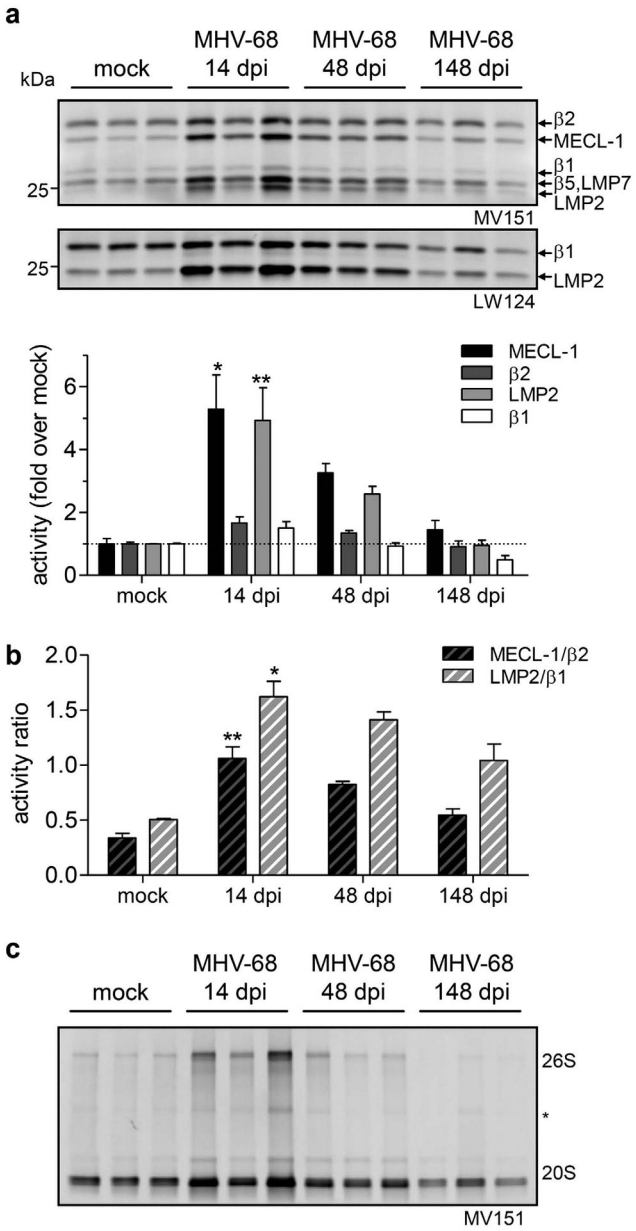

Figure 6. Immunoproteasome and standard proteasome activities in the lung during course of MHV-68 infection. (**a**) Activity-based probe labeling of native whole lung lysates of infected mice (mock, 14, 48, 148 dpi) with MV151 (labeling all active β-subunits), LW124 (β1 and LMP2 specific) and densitometric analysis of MECL-1, β2, LMP2 and β1, depicted as fold increase over uninfected mice. (**b**) Activity ratios of intensities of MECL-1/β2 and LMP2/β1. (**c**) Native gel analysis of lung lysates labeled with activity-based probe MV151. (mean +/− SEM, Kruskal-Wallis Test with Dunn's Post Test, * = $p < 0.05$, ** = $p < 0.01$). All results are representative for two independent experiments. dpi, days post infection.

back at baseline after 148 days. The increase in proteasome activity was attributed to an increase in both 20S and 26S activities which both comprised the virus-induced immunoproteasome subunits LMP2 and LMP7. These data suggest ubiquitin-dependent and -independent degradation of proteins by immunoproteasomes during infection.

Over the course of infection, we observed a shift in standard versus immunoproteasome activity in MHV-68 infection, which was resolved for the catalytic subunit pair MECL-1/β2, but not for LMP2/β1 subunits (Fig. 6b). This indicates that virus infection has a long-term effect on antigen processing by immunoproteasomes. In part, this might be explained by the nature of MHV-68 infection, which can persist latently in lung epithelial cells and macrophages[36,37] and can be spontaneously reactivated. An indicator for such reactivation is the still increased level of IFNγ 148 days post infection.

COPD is characterized by loss of parenchymal tissue, chronic bronchitis, and bacterial colonization of the lower airways[2]. Respiratory infections exacerbate COPD pathology. Smokers and COPD patients suffer longer from respiratory infections and need more time to resolve them[38]. Accordingly, it has been shown that cigarette smoke, the main risk factor for COPD, generally dampens the host's immune system in response to infections as it interferes with STAT-1 and IRF-3 immune signaling[39–43]. Cigarette smoke has also been shown to affect adaptive immune responses such as MHC II antigen presentation[2,44]. The role of the MHC I antigen presentation machinery in COPD in general and in viral exacerbations in particular has not been investigated so far. In this study, we did not detect increased levels of immunoproteasomes in early-staged COPD lungs (Fig. 2c). While Fujino *et al.* observed increased LMP2 and LMP7 transcript levels in primary alveolar type II cells of patients with early COPD stages, a recent study observed no differential expression of immunoproteasomes in lungs of end-stage COPD patients compared to controls[23,45]. This accords with our immunohistochemical analysis of end-stage-diseased COPD tissue, which did not reveal upregulation of the immunoproteasomal LMP2 subunit in alveolar epithelial cells. High immunoproteasome expression in alveolar macrophages, as observed here, may also account for extracellular immunoproteasomes in the BAL fluid of patients with acute respiratory distress syndrome[46]. We also did not observe any consistent change in standard versus immunoproteasome activities in early and late stage COPD lungs (data not shown). Overall proteasome activity has been assessed previously in COPD lungs using conventional proteasome activity assays with conflicting results: While Baker *et al.* did not observe significantly altered levels and activities in COPD lungs, Malhotra *et al.* reported that proteasome expression and activity declined and strongly associated with the severity of lung dysfunction in COPD patients[12,23]. However, as the corresponding author has recently expressed his concern on anomalies in figures in this article, this study has to be considered with caution[47]. It is well established though that proteasome activity can be impaired by cigarette smoke which may then add to development and progression of COPD[10,11].

In this study, we show that lung parenchymal cells express immunoproteasomal subunits at low basal levels, but they can be rapidly induced to form active immunoproteasomes upon IFNγ stimulation *in vitro* or MHV-68 infection *in vivo*. This suggests that the lung is potentially capable of mounting an efficient adaptive immune response to intracellular infections.

Methods

Human lung tissue. For protein extraction, human lung tissue from never-smokers (n = 3), ex-smokers (n = 4), and COPD patients (n = 6) undergoing lung resection surgery for localized lung tumors was collected as previously described[48]. This study was approved by the institutional review board of the Henri Mondor Teaching Hospital (Créteil, France; AFSSAPS reference number B90895-60). All patients and control subjects signed an informed consent document before study inclusion. Paraffin-embedded lung sections of human lung transplant donors (n = 5) or COPD patients (n = 9) with end-stage disease were obtained from the Department of Thoracic Surgery in Vienna, Austria, as described elsewhere[49]. The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (No. 31/93, 84/93, 29/01) and the University of Vienna Hospital ethics committee (EK-Nr 076/2009).

Animals. Tissues or cells were isolated from C57BL/6 wildtype (Charles River Laboratories), LMP2$^{-/-}$ (Psmb9$^{tm1Stl,\ 50}$), or LMP7$^{-/-}$(Psmb8$^{tm1Hjf,\ 51}$) mice. All animal procedures were conducted according to international guidelines and with approval of the Bavarian Animal Research Authority in Germany. All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering.

Virus infection of mice. 8-12 week old female C57BL/6 mice were anesthetized using ketamine/xylazine and infected intranasally with 5×10^4 plaque forming units (PFU) of murine gammaherpesvirus-68 (MHV-68) as described elsewhere[52]. Animals were sacrificed after 14, 48, or 148 days, uninfected mice served as controls and were sacrificed together with the 14 days infected mice, the group size was three per group. Mice were housed in individually ventilated cages during the MHV-68 infection period. All animal experiments were in compliance with the German Animal Welfare Act, and the protocol was approved by the local Animal Care and Use Committee (District Government of Upper Bavaria; permit number 124/08).

Cell culture and reagents. The human A549 alveolar epithelial cell line was obtained from ATCC (ATCC® CCL-185™, American Type Culture Collection, Manassas, VA, USA). Cells were cultured in DMEM (21885025, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, P30-3702, PAN Biotech, Aidenbach, Germany) and 100 U/ml of Pen/Strep (15070063, Life Technologies) and cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Human or mouse recombinant IFNγ (11040596001/11276905001, Roche, Basel, Switzerland) was used at concentrations of 75 U/ml.

Activity-based probe labeling. Activity of the constitutive and immunoproteasome subunits was monitored by using a set of activity-based probes (ABP)[53]. The pan-reactive proteasome ABP MV151[17]

was used for assessing of β2/MECL-1 activities, LW124 for β1/LMP2 activity, and MVB127 was used to label β5/LMP7[18]. Hypoosmotic native lysates of total lung or A549 cells were diluted to a total protein concentration of 0.5 μg/μl with reaction buffer (50 mM HEPES pH 7.4, 100 mM KCl, 10 mM $MgCl_2$). By shaking at 37 °C for 1 h, 30 μl of sample was incubated with 0.5 μM MV151, 0.25 μM LW124 or 1 μM MVB127, respectively, and subsequently quenched by the addition of 6x Laemmli (50 % v/v glycerol, 300 mM Tris·HCl, 6 % w/v SDS, 325 mM DTT, 0.1 % w/v bromophenol blue, pH 6.8) or 5x native loading buffer (50 % v/v glycerol, 250 mM Tris, 0.1 % w/v bromophenol blue, pH 7.5) to a final 1x concentration. Samples were separated on 15 % Tris-glycine SDS polyacrylamide gels or non-denaturing 3–8 % Tris-Acetate gels (Life Technologies) and active proteasome subunits were visualized using a fluorescent scanner (Typhoon TRIO+; Amersham biosciences). Images were taken at 450 PTM and 50 μm pixel resolution with fluorescence Cy3/TAMRA for ABPs MV151 and MVB127 while the Cy2 florescent channel was used for LW124 and analyzed by using ImageJ software. Equal sample loading was verified by staining gels with PageBlue™ (24620, Fisher Scientific, Schwerte, Germany).

Statistics and Software. Data were analyzed with Image Lab™ (Version 3.0.1., Bio-Rad, Hercules, CA, USA), ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), or Prism5 (Version 5.0, GraphPad Software, Inc., La Jolla, CA, USA). Statistics were performed using Prism5 with non-parametric tests and appropriate *post hoc*-analysis. *P*-values < 0.05 were considered statistically significant.

Additional detail on the methods is provided in an online data supplement.

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Author Contributions

I.E.K. and S.M. conception and design of research; L.I.W., M.V., H.S.O., E.M., S.A., C.R., A.G., S.O. and H.A. provided (clinical) samples and reagents; I.E.K., O.V., S.T., A.K. and S.O. performed experiments; I.E.K., O.V., S.T., A.K., B.D., S.M.H. and H.A. analyzed data; I.E.K., O.V., S.T., A.K., B.D., S.M.H., S.H., H.A. and S.M. interpreted results; I.E.K. prepared figures; I.E.K. and S.M. drafted manuscript; I.E.K., O.V., B.D., S.M.H., S.H., H.A., O.E. and S.M. edited and revised manuscript; all authors approved final version.

Additional Information

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2.3.1 Supplemental information

Regulation of immunoproteasome function in the lung

Ilona E. Keller, Oliver Vosyka, Shinji Takenaka, Alexander Kloß, Burkhardt Dahlmann, Lianne I. Willems, Martijn Verdoes, Hermen S. Overkleeft, Elisabeth Marcos, Serge Adnot, Stefanie M. Hauck, Clemens Ruppert, Andreas Günther, Susanne Herold, Shinji Ohno, Heiko Adler, Oliver Eickelberg and Silke Meiners

Supplementary information for the manuscript

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SUPPLEMENT METHODS

Primary lung fibroblast isolation:

Primary mouse or human lung fibroblasts were isolated as described[1]. Mouse fibroblasts were used between passages 2-4, human fibroblasts before passage 6.

Mouse alveolar epithelial cell isolation and culture:

Primary alveolar type II cells (pmATII) were isolated from C57BL/6 mice as described previously[2].

Protein extracts and Western Blotting: Cells or dismembrated frozen tissue was lysed in ice-cold RIPA buffer (50 mM Tris·HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.5), supplemented with protease inhibitor cocktail cOmplete (Roche, Basel, Switzerland). After 20 min incubation on ice, lysates were centrifuged at maximum speed for 20 min at 4°C and supernatants were used for further analysis.

To prepare native lysates, samples were resuspended in distilled water containing cOmplete protease inhibitors and subjected to five cycles of freezing (liquid N_2) and thawing (37°C waterbath). Cell debris was removed by centrifugation as described above. Protein concentrations were assessed using Pierce BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). Western Blot analysis was performed as described[3]. Antibodies directed against LMP2 (1:1,500, ab3328, polyclonal), LMP7 (1:1,500, ab3329, polyclonal), PSMA4 (α3, 1:1,000, ab119419, clone 1H10) or 20S alpha 1+2+3+5+6+7 (α1-7, 1:1,000, ab22674, clone MCP231) were from Abcam (Cambridge, UK). An HRP-coupled antibody detecting β-Actin (1:40,000, A3854, clone AC-15) was purchased from Sigma-Aldrich (St. Louis, MO, USA), the antibody detecting the 19S subunit Tbp1 (Rpt5, 1:3,000, A303-538A, polyclonal) was purchased from Bethyl Laboratories (Montgomery, TX, USA). Antibodies detecting 20S proteasome subunits

β1 and β2 were from Santa Cruz (β1: 1:500, sc-67345, polyclonal; β2: 1:500, sc-58410, clone MCP165; Santa Cruz Biotechnology, Inc., Dallas, TX, USA)

Quantitative real-time RT-PCR: Total RNA from cells was isolated using Roti®-Quick-Kit (Carl Roth, Karlsruhe, Germany). 100-1,000 ng per sample of total RNA were reverse-transcribed using random hexamers (Life Technologies, Carlsbad, CA, USA) and M-MLV reverse transcriptase (Sigma-Aldrich). Quantitative PCR was performed using the SYBR Green LC480 System (Roche Diagnostics, Mannheim, Germany), gene-specific primer sequences are listed in Table S1.

Immunohistochemistry: Human or mouse lung sections (3 µm) were deparaffinized in Xylene and rehydrated. Slides were incubated in solution containing 80% methanol and 1.8% H_2O_2 for 20 min to quench endogenous peroxidase activity. Heat-induced antigen retrieval was performed in 0.05% citraconic anhydride buffer (pH 7.4). Slides were washed with TBST buffer (20 mM Tris, 135 mM NaCl, 0.02% Tween, pH 7.6), blocked with Rodent Block M (Biocare, Concord, CA, USA) for 30 min, washed and incubated for 60 min with an LMP2 specific antibody (1:600, ab3328, Abcam, Cambridge, UK). After another washing step, slides were incubated with rabbit-polymer coupled to alkaline phosphatase (Biocare) for 30 min and washed again. Vulcan Fast Red (Biocare) was used as substrate and incubated for 12 min. Hematoxylin counterstaining was performed, and slides were dehydrated and mounted in Eukitt® (Sigma-Aldrich). Slides were evaluated using a MIRAX scanning system (Zeiss, Oberkochen, Germany).

20S proteasome isolation from mouse lungs: Isolation and purification of proteasomes from lung tissue was performed essentially as described by Dahlmann et al.[4], except for the fact that DEAE-Toyopearl was used for the initial step of anion exchange chromatography and Superose 6 instead of Sepharose for gel chromatography. After chromatography on arginine-Sepharose, the enzyme preparation was concentrated by ultracentrifugation and the precipitate

dissolved in TSDG buffer (10 mM Tris/HCl, 25 mM KCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 10% glycerol, pH 7) containing 2 mM ATP. 20S and 26S proteasomes were then separated by centrifugation in a glycerol gradient (20% - 40% dissolved in TSDG buffer). Centrifugation was performed for 24 h at 25,000 rpm in a Beckman SW28 rotor and afterwards the gradient was fractionated into fractions of 0.5 ml.

Determination of proteolytic activity was performed by use of fluorogenic peptide substrates as described by Dahlmann et al.[5].

For detection of proteasome activity by substrate overlay technique after non-denaturing polyacrylamide gel electrophoresis, the substrate Bz-VGR-MCA was used. This technique as well as non-equilibrium pH gradient and SDS-PAGE were performed as described by Dahlmann et al.[4].

2D gel electrophoresis / In-gel tryptic digest: Purified 20S proteasomes were sepaprated by two dimensional gel electrophoresis (protalys, Berlin, Germany) (1D: Nonequilibrium pH gel electrophoresis-IEF; 2D 2.6-15% SDS-polyacrylamide gel electrophoresis). After visualization of proteins (Colloidal Coomassie-G 250 staining), gel spots were excised for in-gel tryptic digest. Destaining of gel spots was done by washing in 200 µl nanopure water followed by dehydration in 200 µl 60% acetonitrile (each 3 x 10 minutes or until gel spots were completely destained). Acetonitrile was then removed and gel spots were rehydrated in 10 µl digestion-buffer (1 mM Tris-HCl, pH 7.5) containing 0.01 µg/µl trypsin (Sequencing grade Modified Trypsin; Promega). Protein samples were digested over-night at 37°C.

Mass Spectrometry: Proteins were identified using Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or liquid chromatrography electrospray-based (LC-MS/MS) mass spectrometry. Peptide mass fingerprints were obtained on a MALDI-TOF/TOF tandem mass spectrometer (ABI 4700 ProteomicsAnalyzer, Applied Biosystems)[6]. The tryptic digest was diluted with one equivalent of MALDI matrix consisting of

2,5-dihydroxy-benzoic acid (Sigma-Aldrich) (20 mg/ml in 20% acetonitrile, 0.1% TFA) and 2-hydroxy-5-bethoxybenzoic acid (Fluka) (20 mg/ml in 20% acetonitrile, 0.1% TFA) in a 9:1 ratio (v/v), and spotted onto a steel target plate. Peptide mass fingerprint identification of the sample protein was done by comparing peptide masses of the tryptic digest to the virtually trypsinized Ensembl Mouse protein database (database downloaded from www.ensembl.org). The database search was performed using the MASCOT Database search engine v1.9 (Matrix Science Ltd.). Search parameter settings were 150 ppm peptide mass tolerance and one allowed missed cleavage. LC-MS/MS analysis was performed on an Ultimate3000 nano HPLC system (Dionex, Sunnyvale, CA) coupled to a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific) by a nano spray ion source. Samples from in-gel digest were acidified using TFA and automatically loaded to the HPLC system as described by Hauck et al.[7]. The acquired spectra (Thermo raw file) were exported to Mascot Deamon and searched against the Ensembl_Mouse protein database. Search parameters included fixed modification Carbamidomethyl (C) and variable modifications Deaminated (NQ) and Oxidation (M). Peptide tolerance was set to 10 ppm and MS/MS tolerance to 0.6 Da. Only 2, 3 and 4 fold charged peptides were selected for protein identification. Search results were viewed using the Scaffold software (Scaffold 3).

SUPPLEMENT METHODS REFERENCES

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2. Königshoff, M. *et al.* WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. *J. Clin. Invest.* **119,** 772–787 (2009).

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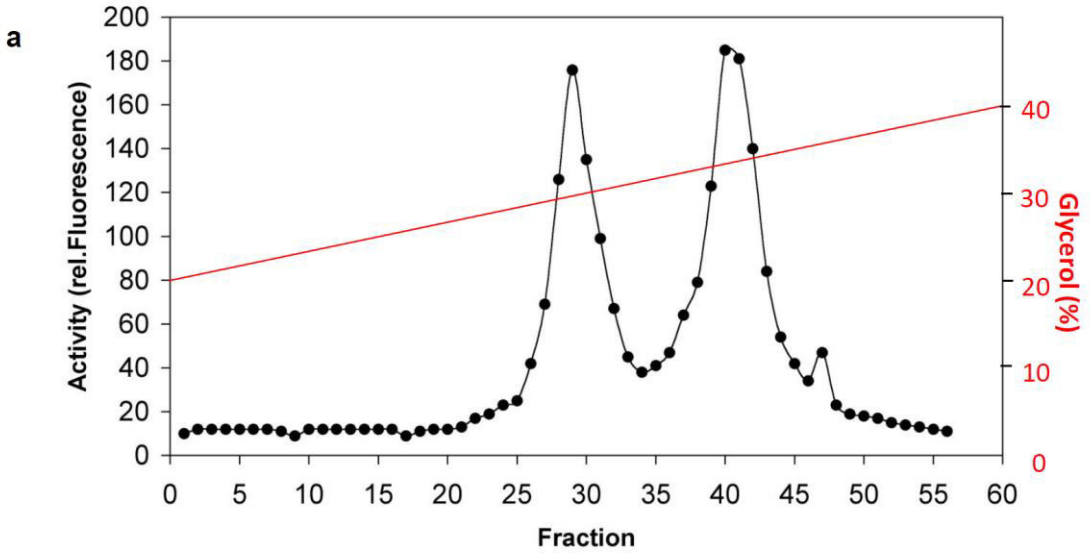
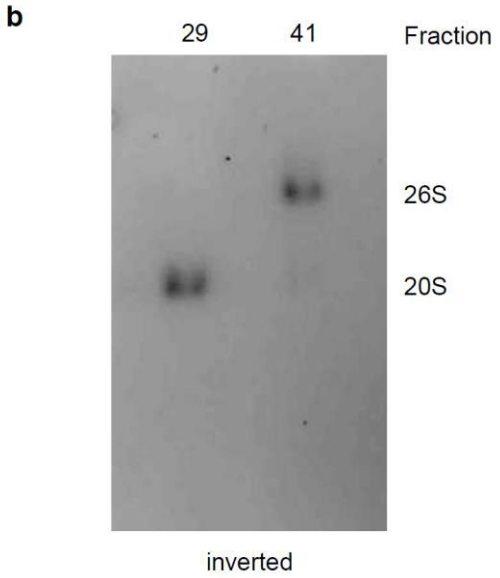
5. Dahlmann, B., Ruppert, T., Kuehn, L., Merforth, S. & Kloetzel, P. M. Different proteasome subtypes in a single tissue exhibit different enzymatic properties. *J. Mol. Biol* **303,** 643–653 (2000).

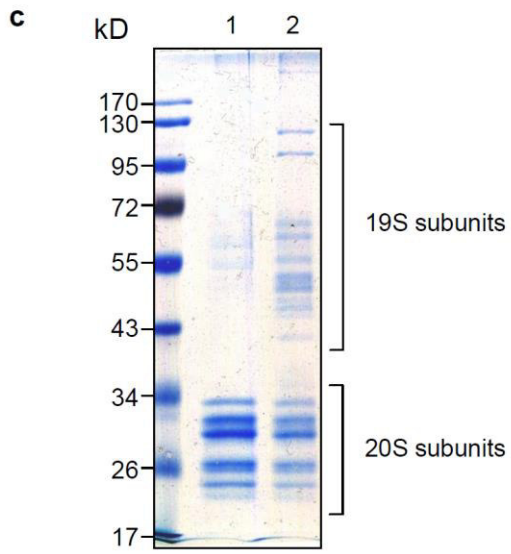
6. Hauck, S. M. *et al.* Retinal Mueller glial cells trigger the hallmark inflammatory process in autoimmune uveitis. *J. Proteome Res.* **6,** 2121–2131 (2007).

7. Hauck, S. M. *et al.* Deciphering membrane-associated molecular processes in target tissue of autoimmune uveitis by label-free quantitative mass spectrometry. *Mol. Cell Proteomics* **9,** 2292–2305 (2010).

SUPPLEMENTARY FIGURES







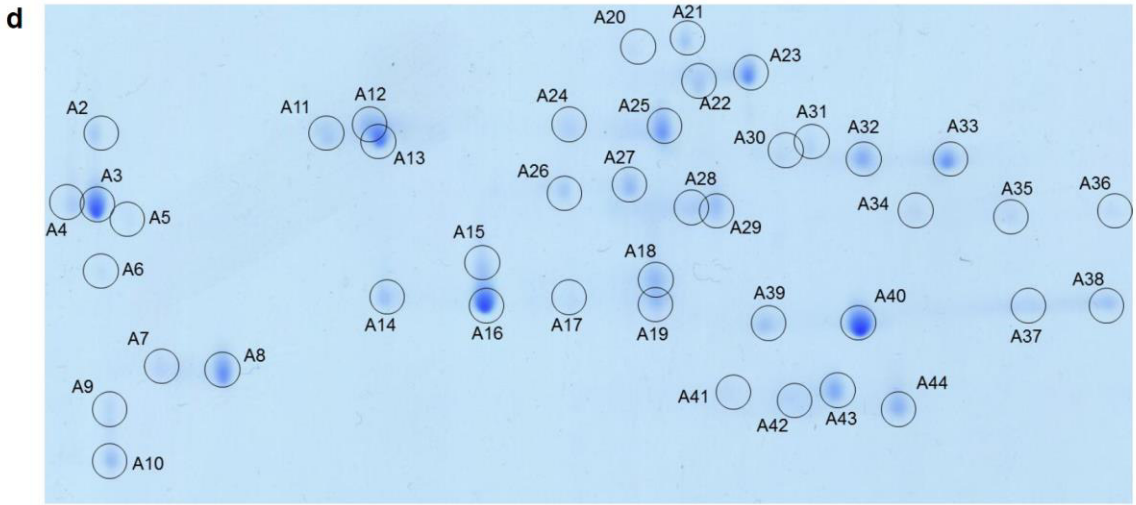


Figure S1. Glycerol gradient analysis to isolate 20S proteasomes from mouse lung for mass spectrometry. (a) Proteasomes were subjected to glycerol gradient centrifugation and afterwards the gradient was fractionated into fractions of 0.5 ml. In each fraction, proteasome activity was measured by use of Suc-LLVY-MCA as substrate to detect the chymotrypsin-like activity of the proteasome. (b) Native PAGE of glycerol gradient fractions 29 and 41: 20 µl of each were subjected to non-denaturing PAGE and after the run proteasome activity was detected by substrate overlay technique (Suc-LLVY-MCA). (c) Coomassie stained SDS-PAGE of 8 µg of each 20S proteasome (lane 1) or 26S proteasome (lane 2) separated by glycerol gradient centrifugation. (d) Coomassie stained 2D gel with annotated spots for mass-spectrometry protein identification (for protein identities, see Supplementary Table S2).

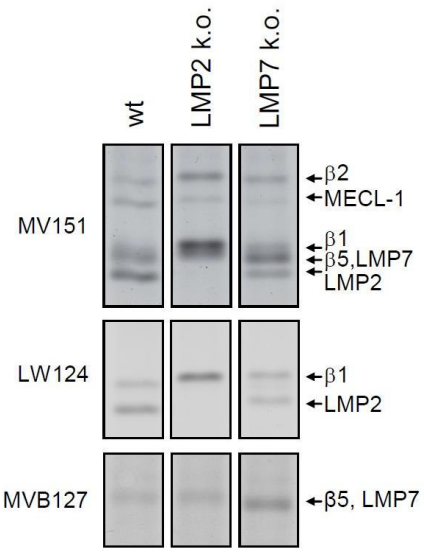


Figure S2. Labeling specificities of activity-based probes. Native splenocyte lysates of wildtype, LMP2 k.o. or LMP7 k.o. mice were labeled with activity-based probes MV151 (labeling all active β-subunits), LW124 (β1 and LMP2 specific) or MVB127 (β5 and LMP7 specific) and separated by SDS-PAGE. Bands originate from the same gel. The molecular weight of mouse β5 and mouse LMP7 is very similar and thus, these subunits separate only marginally on an SDS gel.

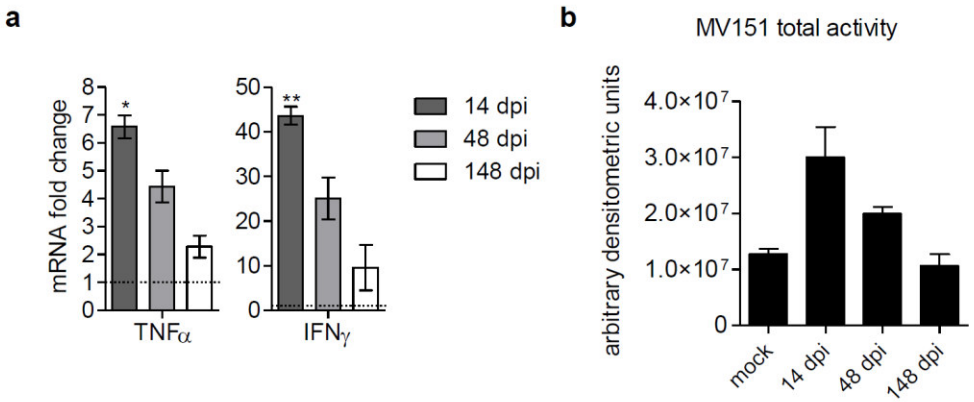


Figure S3. Characterization of MHV68-infected mouse lungs. (a) mRNA levels of inflammatory cytokines TNFα and IFNγ during course of MHV-68 infection displayed as fold over uninfected controls, Rpl19 served as housekeeping gene (mean +/- SEM, Kruskal-Wallis Test with Dunn's Post Test, * = $p<0.05$, ** = $p<0.01$). (b) Densitometric analysis of total proteasome activity of ABP MV151 labeled native lung lysates of uninfected mice or MHV-68 infected mice (day 14, 48 and 148). (mean +/- SEM, Kruskal-Wallis Test with Dunn's Post Test, * = $p<0.05$, ** = $p<0.01$).

SUPPLEMENTARY TABLES

Table S1: Primer sequences

Name	Acc. No.	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)
Mouse			
Nlrc5	NM_001033207.3	AGGCTCCCACTGCTTAGACA	CGGACAGCAAGAGTTTCTCC
Rpl19	NM_001159483.1	CGGGAATCCAAGAAGATTGA	TTCAGCTTGTGGATGTGCTC
Psma3	NM_011184.4	TGAAGAAGGCTCCAATAAACGTCT	AACGAGCATCTGCCAGCAA
Psmb5	NM_011186.1	TGCTCGCTAACATGGTGTATCAGTA	GGCCTCTCTTATCCCAGCCA
Psmb6	NM_008946.4	AGACGCTGTCACTTACCAACTTGG	AAGAGACTGGCGGCTGTGTG
Psmb7	NM_011187.1	TGCCTTATGTCACCATGGGTTC	TTCCTCCTCCATATCTGGCCTAA
Psmb8	NM_010724.2	TGCTTATGCTACCCACAGAGACAA	TTCACTTTCACCCAACCGTC
Psmb9	NM_013585.2	GTACCGTGAGGACTTGTTAGCGC	GGCTGTCGAATTAGCATCCCT
Psmb10	NM_013640.3	GAAGACCGGTTCCAGCCAA	CACTCAGGATCCCTGCTGTGAT
Tnf	NM_013693.3	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Ifng	NM_008337.3	ACGGCACAGTCATTGAAAGCCTA	GTCACCATCCTTTTGCCAGTTCC
Human			
NLRC5	NM_032206.4	CTGCAGCCAAGTTCTTAGGG	TCAGCTGAGGGAGTTGAGGT
RPL19	NM_000981.3	GAGACCAATGAAATCGCCAATG	GCGGATGATCAGCCCATCTT
PSMA3	NM_002788.3	ACAGTGTGAATGACGGTGCG	GCAGCTTGCCTGGCTTTG
PSMB5	NM_002797.4	AGGAATCGAAATGCTTCATGGA	GTAAGCACCCGCTGTAGCCC
PSMB6	NM_002798.2	ACACCTATTCACGACCGCATTT	GTAGGTGACAGCATCAGCTACTGC
PSMB7	NM_002799.3	CTTCAACGACCTGGGCTCC	TCTTGTTGGGCACTGTGTATGG
PSMB8	NM_148919.3	AGTACTGGGAGCGCCTGCT	CCGACACTGAAATACGTTCTCCA
PSMB9	NM_002800.4	CGTTGTGATGGGTTCTGATTCC	GACAGCTTGTCAAACACTCGGTT
PSMB10	NM_002801.3	TGCTGCGGACACTGAGCTC	GCTGTGGTTCCAGGCACAAA

Table S2: Mass spectrometry analysis of 2D gel excised spots

spot ID	Protein Name	Accession Number	Protein MW	Peptide Count	Protein Score	Total Ion Score	Best Ion Score	Total MS Ion Cluster Area	MS Ion Cluster Area Matched %
A2	Psma5|MGI (curated)|Proteasome subunit alpha type-5 (EC 3.4.25.1)(Proteasome zeta chain)(Macropain zeta chain)(Multicatalytic endopeptidase complex zeta chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U1]|3|ENSMUSG00000068749|ENSMUST00000090569	ENSMUSP00000088057	26565	6	95	51	51	363064	26
A3	Psma5|MGI (curated)|Proteasome subunit alpha type-5 (EC 3.4.25.1)(Proteasome zeta chain)(Macropain zeta chain)(Multicatalytic endopeptidase complex zeta chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U1]|3|ENSMUSG00000068749|ENSMUST00000090569	ENSMUSP00000088057	26565	7	345	286	123	1119377	37
A4	Psma5|MGI (curated)|Proteasome subunit alpha type-5 (EC 3.4.25.1)(Proteasome zeta chain)(Macropain zeta chain)(Multicatalytic endopeptidase complex zeta chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U1]|3|ENSMUSG00000068749|ENSMUST00000090569	ENSMUSP00000088057	26565	7	205	150	79	328137	34
A5	Psma5|MGI (curated)|Proteasome subunit alpha type-5 (EC 3.4.25.1)(Proteasome zeta chain)(Macropain zeta chain)(Multicatalytic endopeptidase complex zeta chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U1]|3|ENSMUSG00000068749|ENSMUST00000090569	ENSMUSP00000088057	26565	7	246	186	76	149782	42
A6	Psma5|MGI (curated)|Proteasome subunit alpha type-5 (EC 3.4.25.1)(Proteasome zeta chain)(Macropain zeta chain)(Multicatalytic endopeptidase complex zeta chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U1]|3|ENSMUSG00000068749|ENSMUST00000090569	ENSMUSP00000088057	26565	5	228	196	86	135406	32
A7	Psmb6|MGI (curated)|Proteasome subunit beta type-6 Precursor (EC 3.4.25.1)(Proteasome delta chain)(Macropain delta chain)(Multicatalytic endopeptidase complex delta chain)(Proteasome subunit Y) [Source:UniProtKB/Swiss-Prot;Acc:Q60692]|11|ENSMUSG0000	ENSMUSP00000018430	25591	8	126	55	55	439117	43
A9	Psmb9|MGI (automatic)|Proteasome subunit beta type-9 Precursor (EC 3.4.25.1)(Proteasome subunit beta-1i)(Proteasome chain 7)(Macropain chain 7)(Multicatalytic endopeptidase complex chain 7)(RING12 protein)(Low molecular mass protein 2)(LMP-2d) [Sour	ENSMUSP00000075907	23482	5	130	95	77	225749	27
A11	Psma3|MGI (automatic)|Proteasome subunit alpha type-3 (EC 3.4.25.1)(Proteasome component C8)(Macropain subunit C8)(Multicatalytic endopeptidase complex subunit C8)(Proteasome subunit K) [Source:UniProtKB/Swiss-Prot;Acc:O70435]|12|ENSMUSG000000060073|	ENSMUSP00000071624	28615	12	321	226	61	459544	28
A12	Psma3|MGI (automatic)|Proteasome subunit alpha type-3 (EC 3.4.25.1)(Proteasome component C8)(Macropain subunit C8)(Multicatalytic endopeptidase complex subunit C8)(Proteasome subunit K) [Source:UniProtKB/Swiss-Prot;Acc:O70435]|12|ENSMUSG000000060073|	ENSMUSP00000071624	28615	11	411	327	96	924322	25
A13	Psma3|MGI (automatic)|Proteasome subunit alpha type-3 (EC 3.4.25.1)(Proteasome component C8)(Macropain subunit C8)(Multicatalytic endopeptidase complex subunit C8)(Proteasome subunit K) [Source:UniProtKB/Swiss-Prot;Acc:O70435]|12|ENSMUSG000000060073|	ENSMUSP00000071624	28615	11	399	316	103	1785503	28
A14	Psmb4|MGI (curated)|Proteasome subunit beta type-4 Precursor (Proteasome beta chain)(EC 3.4.25.1)(Macropain beta chain)(Multicatalytic endopeptidase complex beta chain)(Proteasome chain 3) [Source:UniProtKB/Swiss-Prot;Acc:P99026]|3|ENSMUSG00000000577	ENSMUSP00000005923	29211	7	267	222	73	726792	34
A15	Psmb4|MGI (curated)|Proteasome subunit beta type-4 Precursor (Proteasome beta chain)(EC 3.4.25.1)(Macropain beta chain)(Multicatalytic endopeptidase complex beta chain)(Proteasome chain 3) [Source:UniProtKB/Swiss-Prot;Acc:P99026]|3|ENSMUSG00000000577	ENSMUSP00000005923	29211	8	241	184	92	521272	32
A16	Psmb4|MGI (curated)|Proteasome subunit beta type-4 Precursor (Proteasome beta chain)(EC 3.4.25.1)(Macropain beta chain)(Multicatalytic endopeptidase complex beta chain)(Proteasome chain 3) [Source:UniProtKB/Swiss-Prot;Acc:P99026]|3|ENSMUSG00000000577	ENSMUSP00000005923	29211	8	286	228	146	1916248	59
A17	Psmb3|MGI (curated)|Proteasome subunit beta type-3 (EC 3.4.25.1)(Proteasome theta chain)(Proteasome chain 13)(Proteasome component C10-II) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P1]|11|ENSMUSG00000069744|ENSMUST00000103147	ENSMUSP00000099436	23235	7	120	77	52	270844	13
and	Psmb4|MGI (curated)|Proteasome subunit beta type-4 Precursor (Proteasome beta chain)(EC 3.4.25.1)(Macropain beta chain)(Multicatalytic endopeptidase complex beta chain)(Proteasome chain 3) [Source:UniProtKB/Swiss-Prot;Acc:P99026]|3|ENSMUSG00000000577	ENSMUSP00000005923	29211	6	102	65	27	270844	20
A20	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	6	116	76	41	131208	54
A21	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	9	223	161	92	492981	50
A22	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	11	329	247	109	960792	74
A23	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-	ENSMUSP00000033008	29813	11	343	260	126	1488460	69

spot ID	Protein Name	Accession Number	Protein MW	Peptide Count	Protein Score	Total Ion Score	Best Ion Score	Total MS Ion Cluster Area	MS Ion Cluster Area Matched %
	Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM								
A24	Psmb7|MGI (curated)|Proteasome subunit beta type-7 Precursor (EC 3.4.25.1)(Proteasome subunit Z)(Macropain chain Z)(Multicatalytic endopeptidase complex chain Z) [Source:UniProtKB/Swiss-Prot;Acc:P70195]|2|ENSMUSG00000026750|ENSMUST00000028083	ENSMUSP00000028083	30214	7	204	150	58	563024	50
and	Psmb10|MGI (automatic)|Proteasome subunit beta type-10 Precursor (EC 3.4.25.1)(Proteasome subunit beta-2i)(Proteasome MECl-1)(Macropain subunit MECl-1)(Multicatalytic endopeptidase complex subunit MECl-1) [Source:UniProtKB/Swiss-Prot;Acc:O35955]|8|E	ENSMUSP00000034369	29330	1	48	44	44	563024	4
A25	Psmb7|MGI (curated)|Proteasome subunit beta type-7 Precursor (EC 3.4.25.1)(Proteasome subunit Z)(Macropain chain Z)(Multicatalytic endopeptidase complex chain Z) [Source:UniProtKB/Swiss-Prot;Acc:P70195]|2|ENSMUSG00000026750|ENSMUST00000028083	ENSMUSP00000028083	30214	10	293	209	69	1700471	57
and	Psmb10|MGI (automatic)|Proteasome subunit beta type-10 Precursor (EC 3.4.25.1)(Proteasome subunit beta-2i)(Proteasome MECl-1)(Macropain subunit MECl-1)(Multicatalytic endopeptidase complex subunit MECl-1) [Source:UniProtKB/Swiss-Prot;Acc:O35955]|8|E	ENSMUSP00000034369	29330	1	48	45	45	1700471	4
and	Psmb10|MGI (automatic)|Proteasome subunit beta type-10 Precursor (EC 3.4.25.1)(Proteasome subunit beta-2i)(Proteasome MECl-1)(Macropain subunit MECl-1)(Multicatalytic endopeptidase complex subunit MECl-1) [Source:UniProtKB/Swiss-Prot;Acc:O35955]|8|E	ENSMUSP00000034369	29330	9	415	340	185	1365419	59
A28	Psma6|MGI (automatic)|Proteasome subunit alpha type-6 (EC 3.4.25.1)(Proteasome iota chain)(Macropain iota chain)(Multicatalytic endopeptidase complex iota chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9QUM9]|12|ENSMUSG00000021024|ENSMUST00000021412	ENSMUSP00000021412	27811	4	66	47	47	1365419	6
A29	Psma6|MGI (automatic)|Proteasome subunit alpha type-6 (EC 3.4.25.1)(Proteasome iota chain)(Macropain iota chain)(Multicatalytic endopeptidase complex iota chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9QUM9]|12|ENSMUSG00000021024|ENSMUST00000021412	ENSMUSP00000021412	27811	8	250	198	82	496373	52
and	Psma6|MGI (automatic)|Proteasome subunit alpha type-6 (EC 3.4.25.1)(Proteasome iota chain)(Macropain iota chain)(Multicatalytic endopeptidase complex iota chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9QUM9]|12|ENSMUSG00000021024|ENSMUST00000021412	ENSMUSP00000021412	27811	10	327	256	80	654306	61
A30	Mcm2|MGI (automatic)|DNA replication licensing factor MCM2 (Minichromosome maintenance protein 2 homolog)(Nuclear protein BM28) [Source:UniProtKB/Swiss-Prot;Acc:P97310]|6|ENSMUSG00000002870|ENSMUST00000058011	ENSMUSP00000061923	102698	10	66	44	44	654306	37
and	Psma4|MGI (automatic)|Proteasome subunit alpha type-4 (EC 3.4.25.1)(Proteasome component C9)(Macropain subunit C9)(Multicatalytic endopeptidase complex subunit C9)(Proteasome subunit L) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P0]|9|ENSMUSG00000032301|E	ENSMUSP00000034848	29737	6	180	143	66	123819	35
A31	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	6	62	25	25	123819	21
A32	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	10	229	159	71	244083	52
and	Psma4|MGI (automatic)|Proteasome subunit alpha type-4 (EC 3.4.25.1)(Proteasome component C9)(Macropain subunit C9)(Multicatalytic endopeptidase complex subunit C9)(Proteasome subunit L) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P0]|9|ENSMUSG00000032301|E	ENSMUSP00000034848	29737	7	258	217	82	491609	39
A33	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	3	47	34	34	491609	8
A34	Psma4|MGI (automatic)|Proteasome subunit alpha type-4 (EC 3.4.25.1)(Proteasome component C9)(Macropain subunit C9)(Multicatalytic endopeptidase complex subunit C9)(Proteasome subunit L) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P0]|9|ENSMUSG00000032301|E	ENSMUSP00000034848	29737	8	381	331	85	730769	41
and	Psma7|MGI (curated)|Proteasome subunit alpha type-7 (EC 3.4.25.1)(Proteasome subunit RC6-1) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U0]|2|ENSMUSG00000027566|ENSMUST00000029082	ENSMUSP00000029082	28009	9	204	127	61	88216	44
A35	Psma1|MGI (curated)|Proteasome subunit alpha type-1 (EC 3.4.25.1)(Proteasome component C2)(Macropain subunit C2)(Multicatalytic endopeptidase complex subunit C2)(Proteasome nu chain) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P4]|7|ENSMUSG00000030751|ENSM	ENSMUSP00000033008	29813	3	70	54	34	88216	22
A37	Psma7|MGI (curated)|Proteasome subunit alpha type-7 (EC 3.4.25.1)(Proteasome subunit RC6-1) [Source:UniProtKB/Swiss-Prot;Acc:Q9Z2U0]|2|ENSMUSG00000027566|ENSMUST00000029082	ENSMUSP00000029082	28009	9	188	116	77	95074	40
A38	Psmb1|MGI (automatic)|Proteasome subunit beta type-1 Precursor (EC 3.4.25.1)(Proteasome component C5)(Macropain subunit C5)(Multicatalytic endopeptidase complex subunit C5)(Proteasome gamma chain) [Source:UniProtKB/Swiss-Prot;Acc:O09061]|17|ENSMUSG0	ENSMUSP00000014913	26583	11	381	288	106	491567	68
A39	Psmb1|MGI (automatic)|Proteasome subunit beta type-1 Precursor (EC 3.4.25.1)(Proteasome component C5)(Macropain subunit C5)(Multicatalytic	ENSMUSP00000014913	26583	11	377	284	103	698770	69

spot ID	Protein Name	Accession Number	Protein MW	Peptide Count	Protein Score	Total Ion Score	Best Ion Score	Total MS Ion Cluster Area	MS Ion Cluster Area Matched %
	endopeptidase complex subunit C5)(Proteasome gamma chain) [Source:UniProtKB/Swiss-Prot;Acc:O09061]|17|ENSMUSG0								
A40	Psma2|MGI (automatic)|Proteasome subunit alpha type-2 (EC 3.4.25.1)(Proteasome component C3)(Macropain subunit C3)(Multicatalytic endopeptidase complex subunit C3) [Source:UniProtKB/Swiss-Prot;Acc:P49722]|13|ENSMUSG00000015671|ENSMUST00000082305	ENSMUSP00000106140	26024	7	246	186	97	458855	45
A41	Psma2|MGI (automatic)|Proteasome subunit alpha type-2 (EC 3.4.25.1)(Proteasome component C3)(Macropain subunit C3)(Multicatalytic endopeptidase complex subunit C3) [Source:UniProtKB/Swiss-Prot;Acc:P49722]|13|ENSMUSG00000015671|ENSMUST00000082305	ENSMUSP00000106140	26024	9	430	350	146	1490688	66
and	Psmb2|MGI (curated)|Proteasome subunit beta type-2 (EC 3.4.25.1)(Proteasome component C7-I)(Macropain subunit C7-I)(Multicatalytic endopeptidase complex subunit C7-I) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P3]|4|ENSMUSG00000028837|ENSMUST00000030642	ENSMUSP00000030642	23063	6	218	176	79	359746	49
A42	Psmb8|MGI (automatic)|Proteasome subunit beta type-8 Precursor (EC 3.4.25.1)(Proteasome subunit beta-5i)(Proteasome component C13)(Macropain subunit C13)(Multicatalytic endopeptidase complex subunit C13) [Source:UniProtKB/Swiss-Prot;Acc:P28063]|17|E	ENSMUSP00000025196	30526	8	139	87	62	359746	14
and	Psmb8|MGI (automatic)|Proteasome subunit beta type-8 Precursor (EC 3.4.25.1)(Proteasome subunit beta-5i)(Proteasome component C13)(Macropain subunit C13)(Multicatalytic endopeptidase complex subunit C13) [Source:UniProtKB/Swiss-Prot;Acc:P28063]|17|E	ENSMUSP00000025196	30526	10	218	131	86	387945	47
A43	Psmb2|MGI (curated)|Proteasome subunit beta type-2 (EC 3.4.25.1)(Proteasome component C7-I)(Macropain subunit C7-I)(Multicatalytic endopeptidase complex subunit C7-I) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P3]|4|ENSMUSG00000028837|ENSMUST00000030642	ENSMUSP00000030642	23063	5	149	118	73	387945	19
A44	Psmb2|MGI (curated)|Proteasome subunit beta type-2 (EC 3.4.25.1)(Proteasome component C7-I)(Macropain subunit C7-I)(Multicatalytic endopeptidase complex subunit C7-I) [Source:UniProtKB/Swiss-Prot;Acc:Q9R1P3]|4|ENSMUSG00000028837|ENSMUST00000030642	ENSMUSP00000030642	23063	11	612	503	174	1853259	70
and	Psmb8|MGI (automatic)|Proteasome subunit beta type-8 Precursor (EC 3.4.25.1)(Proteasome subunit beta-5i)(Proteasome component C13)(Macropain subunit C13)(Multicatalytic endopeptidase complex subunit C13) [Source:UniProtKB/Swiss-Prot;Acc:P28063]|17|E	ENSMUSP00000025196	30526	13	407	293	96	1260507	60
	Psmb5|MGI (automatic)|Proteasome subunit beta type-5 Precursor (EC 3.4.25.1)(Proteasome epsilon chain)(Macropain epsilon chain)(Multicatalytic endopeptidase complex epsilon chain)(Proteasome subunit X)(Proteasome chain 6) [Source:UniProtKB/Swiss-Pro	ENSMUSP00000107118	22645	3	48	31	31	1260507	12

Spots not significantly identified by MALDI-MS were analyzed by LC-MS/MS:

spot ID	Protein Name	Accession Number	Protein MW	Peptide Count	Protein Score	Best MS/MS Ion Score	Sequence Coverage (%)
A8	Psmb6|MGI Symbol|proteasome (prosome, macropain) subunit, beta type 6 Gene [Source:MGI Symbol;Acc:MGI:104880]|ENSMUSG00000018286|11|ENSMUST00000018430	ENSMUSP00000018430	25591	12	3063	105	38
A10	Psmb9|MGI Symbol|proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2) Gene [Source:MGI Symbol;Acc:MGI:1346526]|ENSMUSG00000024337|17|ENSMUST00000076602	ENSMUSP00000075907	23482	15	2592	118	40
A18	Psmb3|MGI Symbol|proteasome (prosome, macropain) subunit, beta type 3 Gene [Source:MGI Symbol;Acc:MGI:1347014]|ENSMUSG00000069744|11|ENSMUST00000103147	ENSMUSP00000099436 und	23235	27	4586	121	60
A18	Psmb9|MGI Symbol|proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2) Gene [Source:MGI Symbol;Acc:MGI:1346526]|ENSMUSG00000024337|17|ENSMUST00000076602	ENSMUSP00000075907	23482	8	555	107	33
A19	Psmb3|MGI Symbol|proteasome (prosome, macropain) subunit, beta type 3 Gene [Source:MGI Symbol;Acc:MGI:1347014]|ENSMUSG00000069744|11|ENSMUST00000103147	ENSMUSP00000099436 und	23235	39	4577	122	61
A19	Psma6|MGI Symbol|proteasome (prosome, macropain) subunit, alpha type 6 Gene [Source:MGI Symbol;Acc:MGI:1347006]|ENSMUSG00000021024|12|ENSMUST00000021412	ENSMUSP00000021412	27811	5	218	94	21
A26	Psma6|MGI Symbol|proteasome (prosome, macropain) subunit, alpha type 6 Gene [Source:MGI Symbol;Acc:MGI:1347006]|ENSMUSG00000021024|12|ENSMUST00000021412	ENSMUSP00000021412 und	27811	12	899	116	41
A26	Psma3|MGI Symbol|proteasome (prosome, macropain) subunit, alpha type 3 Gene [Source:MGI Symbol;Acc:MGI:104883]|ENSMUSG00000060073|12|ENSMUST00000160027	ENSMUSP00000125548	28615	14	740	114	28
A35	Psma7|MGI Symbol|proteasome (prosome, macropain) subunit, alpha type 7 Gene [Source:MGI Symbol;Acc:MGI:1347070]|ENSMUSG00000027566|2|ENSMUST00000029082	ENSMUSP00000029082 und	28009	21	1892	130	48
A35	Psma1|MGI Symbol|proteasome (prosome, macropain) subunit, alpha type 1 Gene [Source:MGI Symbol;Acc:MGI:1347005]|ENSMUSG00000030751|7|ENSMUST00000033008	ENSMUSP00000033008	29813	19	850	90	34

2.3.2 Authors' contributions

Ilona E. Keller	Western Blots (Fig. 1a, 2c, 3b, 4b, 5c), histology of murine samples (Fig. 1c), native gel analysis (Fig. 2a, 4d, 5d), isolation of murine fibroblasts and cell culture experiments with IFNγ (Fig. 3, 4), mRNA analysis (Fig. 3a, 4a, 5a/b, S3a) activity-based probe analysis (Fig. 6, S3b), design of the study, preparation and editing of figures and manuscript
Oliver Vosyka	ABP native gel and SDS gel analysis (Fig. 4c/d, S2), mass spectrometry analysis of 2D gel excised spots (Table S2), editing of manuscript
Shinji Takenaka	histology of mouse and human samples (Fig. 2b, 5e)
Alexander Kloß	20S isolation from mouse lungs (Fig. 1b, S1a-c)
Burkhardt Dahlmann	supervision of A. Kloß, editing of manuscript
Lianne I. Willems	synthesis of activity-based probe
Martijn Verdoes	synthesis of activity-based probe
Hermen S. Overkleeft	supervision of L.I. Willems, M. Verdoes, provision of activity-based probes
Elisabeth Marcos	provision of human lung samples (tumor resections)
Serge Adnot	supervision of E. Marcos, provision of human lung samples (tumor resections)
Stefanie M. Hauck	mass spectrometry of 2D gel excised spots, analysis, editing of manuscript
Clemens Ruppert	provision of human lung samples (paraffin-embedded lungs)
Andreas Günther	provision of human lung samples (paraffin-embedded lungs)
Susanne Herold	influenza infection of mice for revision of manuscript (data integrated into "response to the reviewers" letter)
Shinji Ohno	MHV-68 infection and section of mice
Heiko Adler	supervision of S. Ohno, provision of MHV-68 infected mouse lungs, editing of manuscript
Oliver Eickelberg	editing of manuscript
Silke Meiners	study design, supervision of I.E. Keller and O. Vosyka, preparation and editing of manuscript

3. Discussion

Within the two presented publications, the role of standard and immunoproteasome activity was investigated in response to cigarette smoke, IFNγ, or virus infection using *in vitro* and *in vivo* mouse models as well as lung tissue of COPD patients. In addition, the results of recent experiments, which were accepted for publication in the meantime (Kammerl et al. 2016), reveal a specific downregulation of immunoproteasome expression and activity in response to cigarette smoke and are partially integrated into the discussion part of this thesis.

3.1 Cigarette smoke induces oxidative stress and reduces proteasome activity

The gaseous and particulate compounds found in cigarette smoke comprise many reactive compounds including oxidants that directly react with and modify lipids, nucleic acids, and proteins (Church & Pryor 1985). Oxidative modifications of proteins might lead to misfolding and loss of function, and make them prone to aggregate, resulting in proteotoxic stress. To prevent accumulation of aggregation-prone proteins, these misfolded and malfunctioning proteins are either refolded with the help of heat shock proteins (Hsp) or tagged with ubiquitin by E3 ligases, which are then recognized by the 19S regulatory particle of the 26S proteasome and subsequently degraded (Goldberg 2003). The major ubiquitin E3 ligase for misfolded proteins is carboxyl terminus of the Hsc70-interacting protein (CHIP), a co-chaperone of Hsp70, thus enabling degradation of misfolded proteins by the proteasome (Rosser et al. 2007). While lung cells might be able to cope with acute and transient impairment of proteasome function in response to cigarette smoke, repetitive injury of the proteasome and continuous imbalance of protein homeostasis might lead to aggregation of proteins which cannot be degraded (Goldberg 2003; Weathington et al. 2013; Balch et al. 2014). Min and colleagues found increased levels of insoluble ubiquitinated proteins in COPD lung tissue and pointed thus indirectly to decreased proteasome activity (Min et al. 2011). Our results also indicated increased levels of ubiquitinated proteins, both in detergent-soluble and insoluble fractions derived from lungs of mice exposed to cigarette smoke (van Rijt et al. 2012).

Increasing evidence suggests impairment of proteasome function by acute smoke exposure *in vitro* (van Rijt et al. 2012; Somborac-Bacura et al. 2013). Not only cigarette smoke has been shown to interfere with proteasome activity, also other combustion products, i.e. diesel exhaust and secondary organic aerosols, have been shown to impair the proteasome system in human white blood cells

(Kipen et al. 2011). It is, however, not known whether biomass smoke exposure, which is also a risk factor for COPD development, or other kinds of air pollution impact the proteasome system in a similar fashion.

Furthermore, the proteasome might be a target for protein modifications itself (Meiners et al. 2014): *in vitro* treatment of purified 20S proteasomes with cigarette smoke extract resulted in a dose-dependent reduction of activity, indicating a direct effect of cigarette smoke on the proteasome (van Rijt et al. 2012). It was shown that upon proteasome inhibition by small molecule inhibitors, a positive feedback-loop leads to concerted upregulation of all 20S and 19S proteasome subunits (Meiners et al. 2003). However, it is currently not fully understood how damaged proteasomes themselves are disassembled and degraded. Due to their long half-lives of approximately two weeks *in vivo* (Tanaka & Ichihara 1989), slightly malfunctioning proteasomes would be present inside the cell for a long time and *de novo* assembly of fully functioning proteasome complexes requires timely effort.

Cigarette smoke might also impact on another layer of proteasome regulation: Complex formation of 20S proteasomes with proteasome regulators, i.e. 19S, PA28α/β, PA28γ, PA200 or PI31, might be affected. The stability of the 26S complex has been shown to be sensitive to the redox state of the cell, which is influenced by cigarette smoke (Wang et al. 2010; van Rijt et al. 2012; Livnat-Levanon et al. 2014; Tsvetkov et al. 2014). While 26S proteasome biology during oxidative stress is well established, another proteasome activator, PA28α/β, was recently described to protect from oxidative stress and might even assemble with 20S proteasomes originating from disassembled 26S proteasomes, but little is known about other activators (Pickering et al. 2010; Li et al. 2011; Pickering et al. 2012; Pickering & Davies 2012; Freudenburg et al. 2013; Hernebring et al. 2013). Cigarette smoke components evoke many more cellular responses than simple oxidative stress inducers such as H_2O_2 alone, which was used in several of the aforementioned studies. We could show that 26S and 20S activity is also reduced in cigarette smoke extract-treated cells already after two hours (van Rijt et al. 2012). The acute reduction in activity was not due to reduced protein levels of proteasome subunits. Whether this effect was due to inhibition of the catalytic active sites or disassembly of the proteasome by cigarette smoke remains to be elucidated. Also, the effect of cigarette smoke on assembly/disassembly of the 20S proteasome with other regulators has not been investigated so far. It would also be interesting to know whether the same effects can be observed *in vivo* after acute smoke exposure of mice.

It has been debated whether 26S proteasomes containing standard or immunoproteasome subunits are better capable of degrading oxidatively modified proteins (Seifert et al. 2010; Nathan et al. 2013) and a protective role of immunoproteasomes in oxidative stress response has been proposed (Pickering et al. 2010; Jung et al. 2013). Our own and unpublished data do not support a protective role of immunoproteasomes in response to cigarette smoke: basal RNA and protein levels of immunoproteasome subunits in lung parenchymal cell lines (A549 and HFL-1) were reduced after three

days of cigarette smoke extract-treatment (data not shown). However, it was not tested whether this is a reversible effect.

A recent study by Yamada and colleagues pointed towards a causal role of decreased proteasomal function (i.e. chymotrypsin-like activity) and the development of emphysema in a murine model of COPD (Yamada et al. 2015). The authors associated this effect with *in vitro* results showing higher susceptibility to cigarette smoke-induced apoptosis of primary cells from transgenic mice that express the thymoproteasome subunit $\beta5_t$.

In COPD patients, one study showed decreased RNA and protein expression of proteasome subunits as well as reduced activity of proteasomes in lungs of patients with moderate and severe COPD (Malhotra et al. 2009). However, the authors have recently raised concern about some of the published figures (2014)[2], so these results should be considered with caution. Fujino and colleagues found increased levels of LMP2 and LMP7 transcripts in isolated alveolar type II cells from early-stage COPD patients, nonetheless, all patients included in this study also suffered from lung cancer (Fujino et al. 2012). Our immunohistochemical analysis did not indicate increased LMP2 staining in alveolar epithelial cells in cancer-free end-stage COPD patients (Keller et al. 2015).

Two recent studies found no difference in total proteasome content in end-stage COPD (Baker et al. 2014; Tomasovic et al. 2015) and no differences in chymotrypsin-like activity of the proteasome (Baker et al. 2014). Our own data on proteasome expression and activity in lungs from end-stage COPD patients exhibited differences: while there was no difference in expression of single subunits (RNA and protein), we observed a clear decrease in active subunits as detected by activity-based probe labeling of native lysates and assembled 20S and 26S complexes as examined by native gel-analysis and chymotrypsin substrate overlay (Kammerl et al. 2016). The decrease in activity was found in both standard and immunoproteasome subunits, implying that cigarette smoke/oxidative stress/other mechanisms decreasing proteasome activity are not selective for one over the other. However, as group size was limited, these experiments should be confirmed in an independent cohort and with larger numbers of patient samples.

3.2 Functional consequences of decreased (immuno-)proteasome activity in COPD pathogenesis

Cigarette smoke is the main risk factor for the development of COPD and has been shown to affect the microbiome in the lung and immune responses to bacterial and viral infection (Stämpfli & Anderson 2009). We have shown that parenchymal primary cells of mouse and human origin respond to IFNγ-treatment with rapid upregulation of immunoproteasomes. Furthermore, lungs from MHV-68

[2] URL: http://www.atsjournals.org/doi/full/10.1164/rccm.190101200#.VYFsAEY3RXU; (Anonymous 2014)

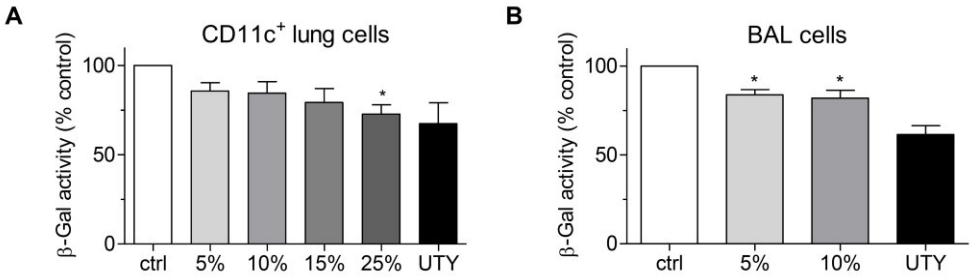


Figure 3: Cigarette smoke extract decreases immunoproteasome-dependent $UTY_{246-254}$-peptide presentation in antigen presenting cells of the lung. β-galactosidase-activity of UTY reporter cell line co-incubated with (A) MACS-sorted $CD11c^+$ lung cells or (B) BAL cells (>95 % alveolar macrophages) from male mice that had been treated with increasing CSE concentrations for 24 h. Data are combined results of three to four independent experiments normalized to the signal of maximum induction of untreated cells co-incubated with UTY cells (= 100 %), (mean + SEM, one-sample t-test (compared to 100 %), * = $p<0.05$,). β-gal, β-galactosidase; BAL, bronchoalveolar lavage; ctrl, control; CSE, cigarette smoke extract; MACS, magnetic-activated cell sorting; UTY, $UTY_{246-254}$ hybridoma cell line.

infected mice exhibited increased levels of immunoproteasomes, suggesting that the lung is fully capable of mounting an immunoproteasome-dependent MHC I-mediated antiviral response (Keller et al. 2015). Our recently published data (Figure 3, (Kammerl et al. 2016)) imply that impaired MHC I antigen presentation, as caused by cigarette smoke, might add on severity and duration of respiratory infections, as observed in smokers and experimentally shown in controlled rhinovirus infection in COPD patients (Stämpfli & Anderson 2009; Mallia et al. 2011). Severe exacerbations often require hospitalization of the patient and lead to accelerated disease progression with loss of lung function and reduced quality of life (Decramer et al. 2012). Clinically relevant viruses in COPD exacerbations include rhinovirus, parainfluenza virus, and influenza virus, with influenza virus infection being less frequent, but more severe in terms of clinical course (Sethi & Murphy 2008). For this reason, annual influenza vaccination is recommended for all COPD patients (Decramer et al. 2012). The GOLD stage of patients is strongly associated with exacerbation frequency, however, the best predictor for frequent exacerbations is a history of frequent exacerbations (Decramer et al. 2012). Importantly, exacerbation frequency has been directly linked with long-term lung function decline (Donaldson et al. 2002). Therefore, it is important to understand the molecular mechanisms of reduced antiviral immune responses in COPD patients to find new therapeutic targets to possibly prevent exacerbations.

It has been shown that rhinovirus-infected primary airway epithelial cells of COPD patients exhibited increased levels of inflammatory cytokines, additionally, downstream targets of IFNs were increased. Viral titers, however, were elevated in COPD epithelial cells compared to normal donors (Schneider et al. 2010). In serum samples of COPD patients hospitalized with an acute exacerbation, levels of IFNγ, the main inducer of immunoproteasomes, were associated with virus infection, but inversely correlated with clinical outcome, implying that the antiviral immune response in COPD exacerbations is strongly dependent on IFNγ-signaling (Almansa et al. 2012). This would also affect immunoproteasome induction for efficient antigen presentation.

3.2.1 Susceptibility to respiratory virus infection

Reduced (immuno-)proteasome activity induced by cigarette smoke might have a substantial effect on MHC I-dependent antiviral immune responses: the proteasome is the main peptide supplier for MHC I antigen presentation, with the immunoproteasome being superior over the standard proteasome to generate MHC I-suitable peptides. The outcome of efficient clearance of a respiratory viral infection via (immuno-)proteasome-dependent MHC I antigen presentation might be strongly dependent on the virus itself. Different viral epitopes can be divided into immunodominant or subdominant epitopes, according to the hierarchy of $CD8^+$ T cell clones (Yewdell 2006; Akram & Inman 2012). The number of epitopes depends on the type of virus as well as on the MHC I genotype of the host. Every nucleated cell in the human body expresses up to six different MHC I molecules, two from each of human leukocyte antigen (HLA)-A, -B- and -C, and for each MHC I locus, several thousand alleles are known. However, one MHC I molecule can bind to multiple ligands sharing biochemical properties of the so-called anchor residues of the peptide that enable binding to the MHC I groove (Klein & Sato 2000).

Several viral epitopes have been shown to be generated or destroyed by the standard or immunoproteasome, respectively. These viruses include influenza virus, a clinically relevant virus in respiratory infection and COPD exacerbation (Van Kaer et al. 1994; Sibille et al. 1995; Pang et al. 2006; de Graaf et al. 2011; Zanker et al. 2013). As most of these studies were conducted in mice, it is not known to which degree these findings can be transferred to the human situation. Our results showed both reduced immuno- and standard proteasome activities in end-stage COPD whole lung tissue, suggesting restrained peptide generation for efficient MHC I antigen presentation (Kammerl et al. 2016). Indeed, it has been demonstrated that mere proteasome inhibition with the Food and Drug Administration (FDA)-approved proteasome inhibitor bortezomib increased susceptibility of mice to lymphocytic choriomeningitis virus (LCMV) infection, accompanied by reduced $CD8^+$ T cell responses and increased viral titers (Basler et al. 2009).

The effect of decreased (immuno-)proteasome activity, however, might differ between diverse cell types and may critically depend on the type of proteasome present in the cell. This demands to distinguish the effects of cigarette smoke on parenchymal and immune cells.

Effect on parenchymal cells

Epithelial cells and fibroblasts in the lung mainly express standard proteasomes, but rapidly upregulate immunoproteasomes in response to IFNγ or during viral infection. In epithelial cells of end-stage COPD patients, immunoproteasomes were not found to be upregulated (Keller et al. 2015). Airway or alveolar epithelial cells are the entry and replication site for several respiratory viruses (Braciale et al. 2012; Yoo et al. 2013). To efficiently eliminate virus-infected cells and thus terminate viral replication, infected respiratory cells need to communicate their infection status to $CD8^+$ T cells via MHC I antigen

presentation. Reduced proteasome activity triggered by cigarette smoke might lead to reduced antigen presentation of virus-derived peptides and thus contribute to impaired clearance of virus-infected cells (Figure 4).

Joeris and colleagues have shown that presence of immunoproteasome subunit LMP7 during infection increases total 20S proteasome abundancy, possibly allowing the cell to cope with changed proteolytic needs during infection and increasing MHC I cell surface expression (Joeris et al. 2012). Actually, LMP7 incorporation to 20S proteasomes has been shown to be the limiting factor for MHC I peptide supply for antigen presentation (Fehling et al. 1994). Reduced levels of LMP7 in response to cigarette smoke might also affect incorporation of the other two immunosubunits: Kingsbury and colleagues have shown that the propeptide of LMP7 is responsible for its favored incorporation into 20S proteasomes over β5, leading to preferred incorporation of LMP2 and MECL-1 as well (Kingsbury et al. 2000), which was confirmed by another group (Joeris et al. 2012). In LMP7-deficient mice, we showed that the pro-form of LMP2 accumulated already at basal conditions and levels of processed and thus incorporated LMP2 were decreased, which was even more evident after IFNγ-treatment of LMP7-deficient cells (Keller et al. 2015).

Several groups reported decreased responsiveness of epithelial cells to interferons/viruses when they were exposed to cigarette smoke extract (Bauer et al. 2008; Modestou et al. 2010; Eddleston et al. 2011; Proud et al. 2012; Hudy et al. 2014). Our own unpublished results expand these findings: the alveolar epithelial cell line A549 exhibited reduced immunoproteasome expression levels after IFNγ-stimulation in the presence of cigarette smoke extract, implying that not only basal levels, but also inducibility of immunoproteasomes are affected by cigarette smoke (data not shown). Reduced immunoproteasome abundancy in infected respiratory epithelial cells might thus add on reduced presentation of viral epitopes or increase presentation of standard proteasome-derived epitopes and lower the possibility of infected epithelial cells to be detected by $CD8^+$ T cells (Figure 4). This may then ultimately limit virus elimination resulting in prolonged infections, as observed in smokers. Indeed, it has been shown that membrane MHC I is reduced in primary keratinocytes exposed to cigarette smoke extract, which could be restored by overexpressing transporter associated with antigen processing (TAP) 1, a crucial integral part of MHC I antigen presentation (Fine et al. 2002).

Effect on immune cells

Immune cells in the lung, such as macrophages and dendritic cells, are the main cell types expressing immunoproteasomes (Keller et al. 2015). In professional APCs of the lung, e.g. dendritic cells, immunoproteasomes are important for evoking adaptive immune responses. At steady state conditions, dendritic cells mainly comprise immunoproteasomes (Macagno et al. 2001). After phagocytosis of viral particles or infected cells, dendritic cells process and cross-present virus-derived

epitopes on MHC I to naïve CD8$^+$ T cells in the lymph node (Figure 4A). Cigarette smoke might have different effects on distinct dendritic cell subsets in the lung: resident dendritic cells might be more affected by cigarette smoke than infiltrating dendritic cells after infection.

It has been shown that cigarette smoke leads to accumulation of immature (Langerin$^+$) dendritic cells

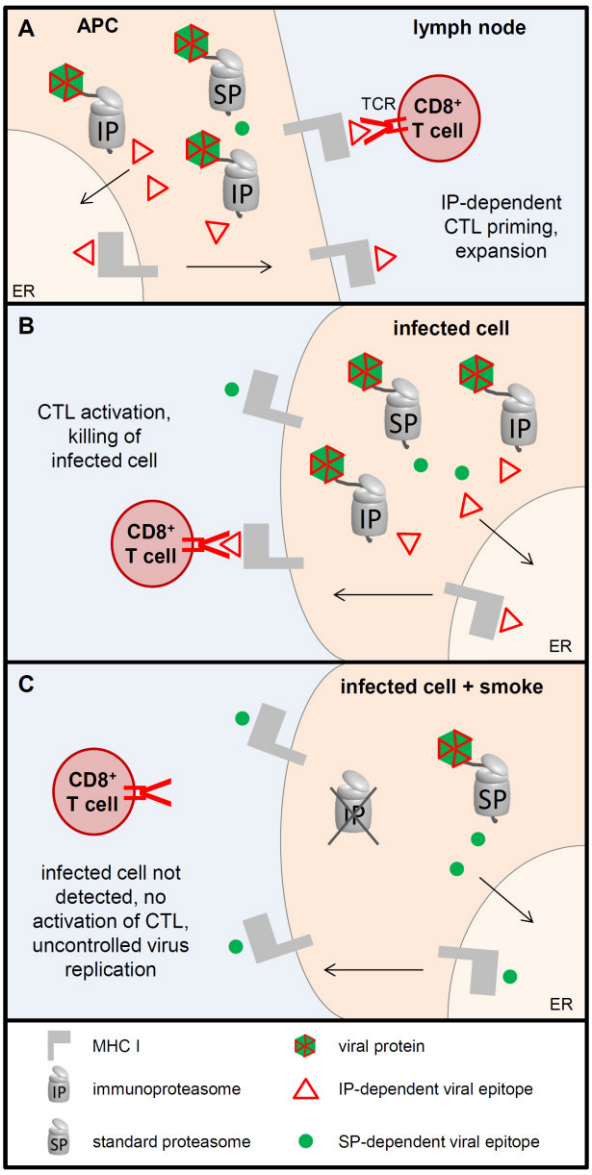


Figure 4: Proposed consequences of decreased immunoproteasome function in infected parenchymal lung cells. (A) Immunoproteasomes in antigen-presenting cells (APCs) degrade viral proteins into peptides, which are translocated into the ER and loaded onto MHC class I molecules. These are transported to the cell surface and presented to naïve CD8$^+$ T cells in the lymph node. Upon activation and clonal expansion, effector CD8$^+$ T cells (also called cytotoxic T lymphocyte (CTL)) infiltrate the lung in search for their specific epitope bound to MHC I to kill infected cells. (B) Infected cells in the lung upregulate immunoproteasomes which degrade viral proteins into the same peptides as the APC did to evoke the CD8$^+$ T cell response, these immunodominant epitopes are presented on MHC I proteins. Infected cells are recognized and killed by immunoproteasome-dependent CD8$^+$ T cells to resolve infection. (C) If immunoproteasome function is impaired in infected cells, the pathogen epitopes presented on MHC class I differ in quantity or quality from the ones that were presented by APCs for CTL activation. Thus, pathogen-specific CD8$^+$ T cells do not recognize infected cells and infection is not resolved. APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; IP, immunoproteasome; MHC I, major histocompatibility complex class I; SP, standard proteasome; TCR, T cell receptor.

in the lung, which was also observed in COPD patients with cell numbers correlating with disease severity (Freeman et al. 2009). Interestingly, these Langerin$^+$ cells have been linked with induction of CD8$^+$ T cell responses through cross-presentation (Heath & Carbone 2009; Brusselle et al. 2011). While it has been shown that dendritic cells do not fully mature in response to cigarette smoke and have lower capacity of priming CD4$^+$ T cells (Robbins et al. 2008), the effect of cigarette smoke on cross-presentation capacity of dendritic cells to evoke CD8$^+$ T cell responses has not been investigated yet. Our unpublished data give first insights on reduced capacity of bone marrow-derived dendritic cells (BMDCs) to cross-present SIINFEKL peptide (derived from full-length ovalbumin) in the presence of cigarette smoke extract (Angela Dann, unpublished). Whether this effect can also be observed *in vivo* needs to be determined in future studies. In this sense, it would be very interesting to evaluate immunoproteasome expression in APCs derived from lung or lymph nodes of cigarette smoke-exposed mice. In addition, cross-presentation *in vivo* and the impact of cigarette smoke exposure on the number, clonality or specificity of CD8$^+$ T cells after respiratory virus infection should be tested. *In vitro* treatment of dendritic cells with cigarette smoke extract has been shown to induce CD8$^+$ T cell proliferation, while CD4$^+$ T cell proliferation was impaired (Mortaz et al. 2009). We evaluated the presentation of an immunoproteasome-dependent epitope as a functional readout for immunoproteasome activity and showed that isolated CD11c$^+$ cells of the lung, mainly consisting of macrophages and dendritic cells, as well as BAL alveolar macrophages exhibited decreased immunoproteasome-dependent antigen presentation when cultured in cigarette smoke-conditioned medium (Figure 3 (Kammerl et al. 2016)). Cigarette smoke extract led to reduced immunoproteasome activity and antigen presentation in primary immune cells of the lung and spleen. However, BAL cells *ex vivo* did not exhibit a reduction in both immunoproteasome activity and antigen presentation after ten days of smoke exposure (Figure 5 (Kammerl et al. 2016)). Within these experiments, immunoproteasome activity and antigen presentation were directly correlated. Transcript levels of immunoproteasomes were reduced in response to smoke after ten days, yet we observed a slight increase of LMP7 in macrophages from smoke-exposed BALB/c mice, while C57BL/6 mice exhibited reduced levels of both LMP2 and LMP7 subunits (Figure 5). In both mouse strains, however, we observed a clear shift towards standard proteasome activity by using activity-based probes. Nevertheless, the effects might be different after longer cigarette smoke exposures, as ten days might not represent a typical chronic exposure.

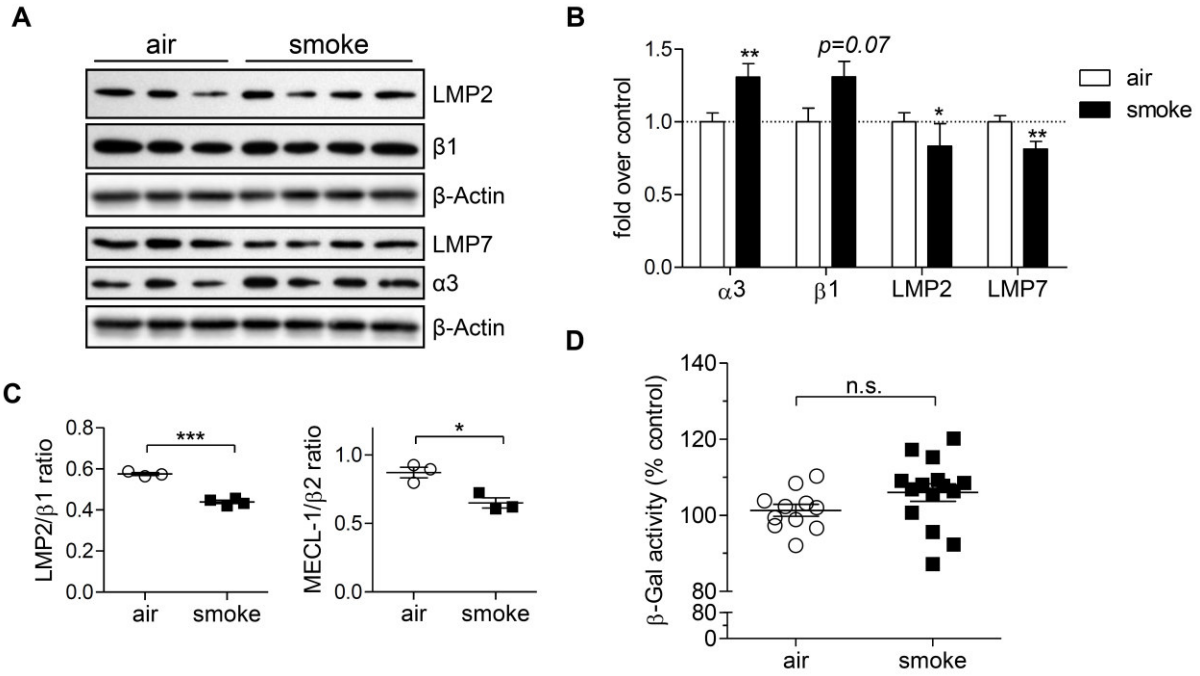


Figure 5: Ten days of cigarette smoke exposure affects immunoproteasome expression in alveolar macrophages without affecting UTY$_{246-254}$ presentation. (A) Proteasome protein expression in isolated alveolar macrophages from air-exposed controls or mice that had been exposed to cigarette smoke for one exposure cycle (50 min/day) for 10 days. Western Blots display immunosubunits LMP2 and LMP7 as well as standard subunit β1 and α3. β-Actin served as loading control. (B) Combined densitometric analysis of Western Blots from three independent experiments as in (A). Results are displayed as fold over air-exposed controls (mean + SEM, Student's t-test, * = $p<0.05$, ** = $p<0.01$). (C) LMP2/β1 and MECL-1/β2 activity ratios in alveolar macrophages derived from densitometry of activity-based probe-labeling of isolated alveolar macrophages (mean ± SEM, Student's t-test, * = $p<0.05$, *** = $p<0.001$). (D) β-galactosidase-activity of UTY reporter cell line co-incubated with *ex vivo* BAL cells from male mice that had been exposed to cigarette smoke for 10 days compared to air controls. Results are combined data from two independent experiments displayed as % of control (mean ± SEM, Student's t-test).

In BAL cells from COPD patients, we found all three immunosubunits to be decreased on the mRNA level compared to controls (Figure 6 (Kammerl et al. 2016)). These results were confirmed in an independent cohort by analysis of a set of publicly available microarray data (GSE13896), which included purified alveolar macrophages of healthy non-smokers, smokers, and early-stage COPD patients (Shaykhiev et al. 2009). In line with this, Hodge et al. found significantly reduced MHC I surface levels on alveolar macrophages of current smokers with COPD (Hodge et al. 2011), indirectly pointing towards reduced peptide supply by the (immuno-)proteasome.

Reduced (immuno-)proteasome activity in dendritic cells due to smoke exposure might reduce the probability of matching peptide/MHC I binding on the APC to the TCR complex on the $CD8^+$ T cell to evoke a specific immune response. Also, reduced abundancy of immunoproteasome subunits in APCs might shift the peptide repertoire towards peptides generated by the standard proteasome subunits and are thus not as suitable for MHC I binding as peptides derived from immunoproteasomes (Figure 7).

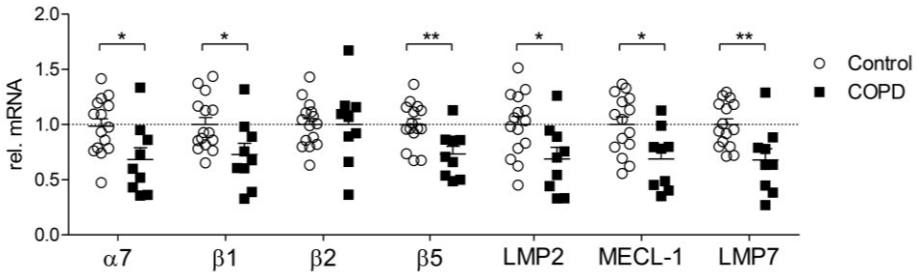


Figure 6: Reduced immunoproteasome transcripts in BAL of COPD patients. qRT-PCR mRNA analysis of 20S proteasome subunits α7, β1, β2, and β5 and immunoproteasome subunits LMP2, MECL-1, and LMP7 in BAL cells of control subjects (n=15) and COPD patients (n=9). Rpl19 was used as housekeeping gene (mean ± SEM, Student's t-test, * = $p<0.05$, ** = $p<0.01$).

Furthermore, a shift in epitope generation due to a shift of standard vs. immunoproteasome activity might lead to altered/dampened $CD8^+$ T cell responses in susceptible smokers, possibly prolonging infection. A counterexample to show a beneficial and clinically relevant shift from immuno- to standard proteasome has been published recently: tumor cells mainly express standard proteasomes, present standard proteasome-derived epitopes on MHC I and thus evade recognition by $CD8^+$ T cells, which are targeted against immunoproteasome-derived tumor antigens. Downregulation of immunoproteasomes and introducing tumor antigens in dendritic cells resulted in profound cytotoxic T lymphocyte (CTL) responses against standard proteasome-derived tumor antigens in melanoma patients (Dannull et al. 2013).

It has been critically discussed whether 26S proteasomes containing standard or immunoproteasome subunits *per se* degrade (oxidatively) modified proteins differently (Seifert et al. 2010; Nathan et al. 2013), or whether immunoproteasomes mainly enhance quantity, but not quality of peptides, as shown by *in vitro* digestions of model substrates (Mishto et al. 2014). Moreover, it has been recently demonstrated that the nature of incorporated β-subunits influences 20S associations with regulators: 20S immunoproteasomes preferentially bound to PA28α/β regulators and less to PI31 compared to 20S proteasomes containing standard subunits (Fabre et al. 2015). As the PA28α/β regulatory complex is inducible by IFNγ as well, it is easily feasible that cigarette smoke interferes with PA28α/β expression in a similar way. In the aforementioned microarray analysis from Shaykhiev et al., both PA28α and PA28β subunits were significantly reduced in alveolar macrophages from COPD patients, and PA28β levels were significantly decreased in healthy smokers (Shaykhiev et al. 2009, data not shown). Reduced levels of PA28α/β in response to cigarette smoke might additionally impact antigen presentation (de Graaf et al. 2011; Raule et al. 2014).

3.2.2 Potential role of decreased (immuno-)proteasome activity in (auto-)immune processes of COPD

Autoimmunity describes the break of tolerance of the immune system to "self"-derived structures and can be triggered by environmental exposures such as virus infection (Olson et al. 2001; Fujinami et al. 2006), which might be modulated by cigarette smoke exposure (Arnson et al. 2010). While research has mainly focused on $CD4^+$ T cell-mediated autoimmunity for a long time, the role of $CD8^+$ effector T cells in several autoimmune diseases has emerged as well (Walter & Santamaria 2005; Gravano & Hoyer 2013).

In COPD, several lines of evidence support an autoimmune component of disease pathogenesis, including humoral responses against self-antigens or "altered self"-structures and increased $CD8^+$ T cells (Agusti 2003; Grumelli et al. 2004; Feghali-Bostwick et al. 2008; Stefanska & Walsh 2009; Arnson et al. 2010; Duncan 2010; Duncan 2011; Kheradmand et al. 2012; Rovina et al. 2013).

Neoantigenesis by cigarette smoke

Cigarette smoke has been shown to modify macromolecules, either directly via adducts of smoke components such as acrolein to DNA and proteins, or indirectly via induction of oxidative stress resulting in oxidatively modified proteins (Cai et al. 2009; X. Liu et al. 2010). Indeed, oxidative stress-induced antibodies directed against carbonyl-modified proteins have been observed in COPD, which correlated with disease severity (Kirkham et al. 2011).

Posttranslational protein modifications might have a strong effect on protein folding and function and may thus ultimately influence the cleavage pattern by the proteasome. Modifications on self-peptides might include direct modifications of peptides that are loaded onto MHC I, or modifications of proteins in the vicinity of the immunogenic peptide that lead to altered processing by the proteasome, resulting in presentation of peptides that were not initially presented in the thymus. It has been shown that an oxidatively modified MHC I epitope could still bind to MHC I, however, recognition of antigen-specific $CD8^+$ T cells was reduced (Weiskopf et al. 2010).

An alternative pathway of protein modifications could be due to the fact that cigarette smoke might affect abundancy of protein-modifying enzymes, as shown for protein citrullination, a modification associated with possible autoantibody production in response to smoke in susceptible rheumatoid arthritis patients (Makrygiannakis et al. 2008; Klareskog & Catrina 2015; Valesini et al. 2015).

Another possibility of "altered self" has been proposed by Tzortzaki & Siafakas: DNA mutations caused by cigarette smoke might result in presentation of "mutated" epitopes and would then contribute to detection of cells as "non-self", and activation of $CD8^+$ T cell response leading to cell death and tissue destruction (Tzortzaki & Siafakas 2009). A prominent example of loss of epitope cleavage site due to mutation includes the tumor suppressor p53 (Theobald et al. 1998): Here, a frequent mutation (R273H)

found in many cancer types resulted in loss of the adjacent proteasomal cleavage site, preventing epitope presentation and lysis of cells bearing the mutation. Several viruses use the same mechanism to evade immune surveillance, and mutational sites have been described within (Ossendorp et al. 1996; Kimura et al. 2005; Cardinaud et al. 2011; Petrovic et al. 2012) or flanking MHC I epitope sequences (Yellen-Shaw et al. 1997; Seifert et al. 2004; Milicic et al. 2005). Similarly, it is conceivable that new proteasome cleavage sites emerge from cigarette smoke-induced mutations.

Interestingly, chronic exposure of mice to an irritant present in cigarette smoke, i.e. acrolein, is able to recapitulate COPD symptoms including emphysema and accumulation of $CD8^+$ T cells in the lungs (Borchers et al. 2007). Intriguingly, mice deficient for CD8 were partially protected from developing emphysema, highlighting the role of $CD8^+$ T cells. In this model, acrolein might cause both DNA damage leading to altered peptide sequences presented on MHC I or directly modify proteins and alter the peptides that are generated by the proteasome. Whether there is a direct effect of acrolein exposure on proteasome activity has not been fully investigated (van Rijt et al. 2012). It would also be interesting to evaluate the effect of other pulmonary irritants, as about one third of COPD patients has never smoked (Salvi & Barnes 2009). These irritants might include environmental pollutants like ozone or industrial air pollutants such as dusts and gases.

The concept of "altered self" peptides and neoantigenesis has been investigated in the context of $CD4^+$/MHC II responses and autoantibody production in several human diseases (Doyle & Mamula 2012; Zavala-Cerna et al. 2014), but not regarding $CD8^+$ T cell auto-cytotoxicity. This is possibly due to the previous difficulties to isolate and characterize $CD8^+$ T cells. However, new technologies allow for deep-sequencing of TCRs (Clemente et al. 2013). It has not been possible to detect modifications in the MHC I peptidome in response to cigarette smoke, but new mass spectrometry approaches might prove useful (Kincaid et al. 2012; Olsen & Mann 2013). Also, it is not feasible to elucidate the effects of smoke on the MHC I peptidome in humans due to the variety of MHC I allele variants between individuals. To test whether cigarette smoke modifies the MHC I peptidome, experimental models with a clearly defined genetic background combining smoke and virus infection are needed, which would permit the purification of MHC I peptides for mass spectrometric analysis.

Priming of autoreactive $CD8^+$ T cells

Autoreactive $CD8^+$ T cells recognizing self-peptides on MHC I are sorted out in the thymus through negative selection. However, in the periphery, $CD8^+$ T cells may encounter modified self-peptides on MHC I, against which they were not selected (Klein et al. 2014). The proteasome, and especially the immunoproteasome, shape the MHC I peptide repertoire by enhancing peptide supply both qualitatively and quantitatively (Groettrup et al. 2001; Zanker et al. 2013; Mishto et al. 2014). It has been recently shown that in healthy individuals, self-reactive, but anergic $CD8^+$ T cells are present,

although low in numbers and kept under control by regulatory T cells (Maeda et al. 2014). In COPD, however, lungs exhibit reduced levels of regulatory T cells (Hou et al. 2013).

As highlighted in the introduction part, immunoproteasomes protect the organism from autoimmunity, because the antigen presentation of both "self" and "non-self", i.e. pathogen-derived peptides, is enhanced only during infection. Due to altered cleavage preferences of the immunoproteasome, certain "self" peptides cease to be presented when infection is resolved and immunoproteasome levels are back to baseline (Groettrup et al. 2001; Heink 2005; Shin et al. 2006; Eleftheriadis 2012). The data presented in this thesis point towards a transcriptional downregulation of immunoproteasomes resulting in a shift from immuno- to standard proteasomes in BAL cells of COPD patients and alveolar macrophages from cigarette smoke-exposed mice (Figures 5 and 6). Murine lung $CD11c^+$ cells, including professional antigen-presenting dendritic cells, exhibited decreased presentation of an immunoproteasome-dependent MHC I peptide when they were exposed to cigarette smoke extract (Figure 3). All together, the data imply a dysfunction of immunoproteasomes in response to cigarette smoke in immune cells of the lung (Kammerl et al. 2016). Reduced immunoproteasome in APCs might lead to augmented presentation of standard proteasome-derived self-peptides during infection, which are also presented by parenchymal cells when infection is resolved and thus might perpetuate autoreactive $CD8^+$ T cell responses (Figure 7).

3.2.3 Hypothesis for the role of immunoproteasomes in COPD pathogenesis

The following model is proposed for the role of immunoproteasomes in COPD pathogenesis: In the course of respiratory viral infection, decreased immunoproteasome function impacts on the cell-types' specific function during viral resolution. In infected parenchymal cells (Figure 4), cigarette smoke-mediated impairment of immunoproteasome function might lead to evasion of virus-infected cells to $CD8^+$ T cell surveillance if antigenic peptides are produced rather by standard proteasomes and not immunoproteasomes. Accordingly, viral peptides on MHC I of infected cells are different in quantity and/or quality from the ones presented on APCs and thus do not match to the specifically activated $CD8^+$ T cell clones. Non-detection of infected cells might enhance infection symptoms or contribute to chronic infections, because the virus has more time to amplify without being detected and to lyse cells, leading to increased tissue damage.

If APCs loose immunoproteasome activity (Figure 7), they might prime an altered set of TCRs on $CD8^+$ T cells, because their MHC I peptides would mainly be generated by standard proteasomes. In that case, $CD8^+$ T cells may still recognize infected cells, but chances are higher that $CD8^+$ T cells were primed against "self"- or "altered self"-derived peptides. Such priming of autoreactive $CD8^+$ T cells against "self"- or "altered self" may then contribute to increased and autoimmune responses during

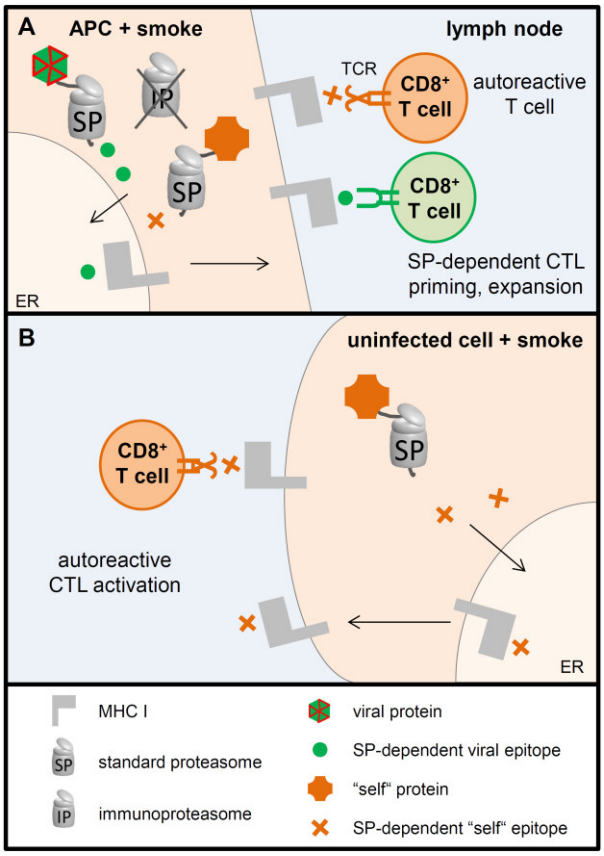


Figure 7: Possible role of immunoproteasome in generation of autoreactive CD8$^+$ T cell responses during infection. (A) When immunoproteasome function is impaired in cross-presenting APCs due to cigarette smoke exposure, viral proteins, but also "self" proteins, are degraded by the standard proteasome and peptides are presented in the lymph node to naïve CD8$^+$ T cells. In this context, "self"-proteins might be modified by posttranslational modifications or directly by cigarette smoke components, possibly resulting in altered cleavage. Presentation of these "self"-derived peptides leads to priming of autoreactive CD8$^+$ T cells, which did not undergo negative thymic selection. (B) If autoreactive CTLs were activated during the course of CD8$^+$ T cell priming, they recognize standard proteasome-dependent and "self"-derived peptides and kill uninfected cells, resulting in tissue damage and inflammation. APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; IP, immunoproteasome; MHC I, major histocompatibility complex class I; SP, standard proteasome; TCR, T cell receptor.

infection, because the same standard proteasome-derived peptides are also presented on MHC I of non-infected cells (Figure 7).

A combination of both scenarios, namely decreased immunoproteasome activity in infected parenchymal cells and APCs, might have additive effects and perpetuate inflammation in the lungs of smokers, leading to secondary (bacterial) infection, immune cell recruitment with activation of proteolytic enzymes and oxidative stress generation, ultimately resulting in loss of tissue and respiratory capacity as observed in COPD.

3.3 Ongoing and future work

Future studies to decipher the role of (immuno-)proteasomes in cigarette smoke-induced lung disease should ideally combine both cigarette smoke exposure and infections with COPD-relevant viruses to

mimic the human situation during COPD exacerbations. Furthermore, mouse models would have the advantage of having a defined MHC I genotype, also, knock-out mice for immunoproteasome subunits are available and the LMP7-specific inhibitor ONX-0914 has proven to be safe and beneficial in several preclinical models. In this way, the dependency of virus epitope generation on immunoproteasomes could be tested.

In addition, the length of smoke exposure might strongly influence the outcome of virus infection. Our data from mice exposed to cigarette smoke for ten days showed that cigarette smoke exposure had an effect on relative immunoproteasome activity, but it was not tested whether longer smoke exposure might lead to further decrease of immunoproteasome activity with impairment of immunoproteasome-dependent antigen presentation, such as the $UTY_{246-254}$-peptide. This would only be possible in mice that were exposed to cigarette smoke for longer durations, e.g. 4-6 months when histologic COPD-like changes are clearly present. These analyses are currently ongoing.

Moreover, it has not been tested so far whether the IFNγ response of cells for upregulation of immunoproteasomes is reversibly changed or whether this process is irrevocably altered even after smoking cessation. This might be easily performed in cell culture experiments with cigarette smoke extract and subsequent IFNγ treatment or in mice that were exposed to cigarette smoke and infected with a respiratory virus or a viral mimic such as polyI:C to evaluate the induction kinetics of the antigen presentation machinery, including the immunoproteasome.

An interesting functional readout for (immuno-)proteasome function in response to virus infection in COPD exacerbations might be to decipher the $CD8^+$ T cell repertoire. Experimental models of acute exacerbations have been established, which include viral infection of smoke-exposed mice (Gaschler et al. 2007; Papi et al. 2007; Foronjy et al. 2014), but also controlled human studies involving rhinovirus infection have been reported (Mallia et al. 2011; Sethi & MacNee 2011; Mallia et al. 2014). Recent advance has been made to study rhinovirus infection in mice transgenic for human intercellular adhesion molecule (ICAM) 1, the cellular receptor for rhinovirus (Bartlett et al. 2008). For viruses such as influenza, the possibilities of detecting presentation of known epitopes is possible due to hybridoma cell lines, and influenza-specific $CD8^+$ T cells can be detected by MHC I-tetramer staining. The MHC I epitopes of several respiratory viruses have been comprehensively identified in the mouse (Gredmark-Russ et al. 2008; Walsh et al. 2013),

Effects on $CD8^+$ T cells might be of both quantitative (reduced numbers of T cell clones) and qualitative (altered TCR repertoire) nature. To translate this model to the human situation, it might be beneficial to use mice that are transgenic for the human MHC I allele HLA-A2 to directly transfer viral peptide identities. With improvements in methodology, mass spectrometry analysis of MHC I-eluted peptides is possible and might help to identify changes in virus antigen presentation in response to smoke. Also, abundancy of presented "self"-derived peptides during infection might be estimated.

Furthermore, it would be interesting to examine the viral peptides and $CD8^+$ T cell responses in the blood of stable COPD patients with a defined MHC I genotype and during exacerbations. A first step could be to sequence the TCRs by using next-generation sequencing techniques in stable vs. virus-induced exacerbated COPD and in virus-infected non-COPD controls to get a first hint on both quantity and quality of $CD8^+$ TCRs.

All these experiments might shed light on the role of (immuno-)proteasome-mediated antigen presentation in response to smoke. Reduced levels of immunoproteasomes may serve as a biomarker for enhanced susceptibility to virus infection, while increased levels might be protective from Th2-associated diseases like asthma. This concept is tested at the moment in the PASTURE/EFRAIM birth cohort, where immunoproteasome transcript levels are evaluated in peripheral blood mononuclear cells (PBMCs) of cord blood of newborns and at the age of one and six years.

Future work might also include evaluations of immunoproteasome activity in PBMCs of stable COPD patients or during exacerbations. Preliminary studies on PBMCs isolated from a well-defined cohort of never-smokers and smokers revealed no obvious differences in general proteasome content or activity between groups (medical thesis of Julia Schimmer). However, recruited study participants were all healthy young males, without any history of chronic diseases and daily medication. Nonetheless, immunoproteasome expression and activity in blood immune cells of COPD patients might be an interesting biomarker for virus susceptibility and outcome of exacerbation.

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