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Impairment of immunoproteasome function by cigarette smoke and in COPD

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At a Glance Commentary:

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

What This Study Adds to the Field: For the first time, we show that immunoproteasome expression and activity is directly altered by cigarette smoke *in vitro* and *in vivo* resulting in disturbed MHC I antigen presentation. As immunoproteasome expression is downregulated and immunoproteasome activity is impaired in BAL and total lungs of COPD patients, respectively, this may thus contribute to a distorted adaptive immune response in COPD patients.

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ABSTRACT

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

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

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

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

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

Word count: 250

Key words: cigarette smoke, alveolar macrophages, MHC class I antigen presentation, immunoproteasome

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INTRODUCTION

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

The ubiquitin-proteasome system degrades more than 90 % of all cellular proteins - including old and damaged ones - into small peptides. These are used for recycling of amino acids but also for presentation of MHC I epitopes to define the “cellular self” towards the immune system (6–8). The proteasome consists of a barrel-shaped 20S proteolytic core particle which is activated by different proteasome regulators such as the 19S activator to form the 26S, the main proteasome complex for ubiquitin-mediated protein degradation (9). The 20S core is composed of four heptameric rings comprising of α- and β-subunits. In standard proteasomes, three of the seven β-subunits - namely β1, β2, and β5 - exhibit proteolytic activities. They can be exchanged by their inducible counterparts, i.e. low molecular mass protein (LMP) 2, multicatalytic endopeptidase complex-like 1 (MECL-1), and LMP7, to form the immunoproteasome. Expression of immunoproteasomal subunits is induced in response to interferon (IFN) γ or tumor necrosis factor (TNF) α as part of the early innate immune response to virus infections (10–12). Immunoproteasomes are constitutively expressed in immune cells compared to very low basal expression in most parenchymal cells (13). The

newly assembled immunoproteasomes have altered cleavage kinetics compared to their 20S standard counterparts (14), and generate antigenic peptides that are preferentially presented by MHC I molecules (13) contributing to the efficient elimination of infected cells via the adaptive immune system (12, 13). While several studies - including ours - suggest impairment of proteasome function by smoke exposure and in COPD (15–19), the effect of cigarette smoke on immunoproteasome function and its role in COPD pathogenesis have not been investigated so far.

In the current study, we analysed the effect of acute cigarette smoke exposure on immunoproteasome expression *in vitro* and *in vivo*, as well as in bronchoalveolar lavage (BAL) cells from early stage and from lungs of end-stage COPD patients. Furthermore, we investigated the functional effects of cigarette smoke on immunoproteasome-mediated antigen presentation.

Some of the results of these studies have been previously reported in the form of abstracts (20–22).

METHODS

Human lung tissue and cells: BAL cells were obtained as previously described (23) with approval by the local ethics committee of Albert-Ludwig University Freiburg (No. 231/03). The use of explanted human lung tissues and blood from healthy donors was approved by the University hospital of the Ludwig-Maximilians-University in Munich (No. 333-10 and 071-06 – 075-06). Human macrophages were differentiated from peripheral blood monocytes according to Martinez et al. (24).

Animals: Tissues or cells were isolated from C57BL/6J wildtype, LMP2$^{-/-}$ (Psmb9^{tm1Stl} (25)) or LMP7$^{-/-}$ (Psmb8^{tm1Hjf} (26)) mice with C57BL/6J background. For smoke exposure

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experiments, C57BL/6J wildtype males or BALB/cAnCrl females were used (Charles Rivers Laboratories, Germany). All animal procedures were conducted according to international guidelines and with approval of the Bavarian Animal Research Authority in Germany.

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

Statistics & Software: Data were analyzed with ImageLab (Biorad), ImageJ (http://imagej.nih.gov/ij/), or Prism5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistics were performed using Prism5 with initial Grubbs' test for outliers and D'Agostino and Pearson omnibus normality test. Normally distributed data were analyzed using parametric tests with appropriate *post hoc*-analysis, otherwise non-parametric tests were chosen. *P*-values < 0.05 were considered statistically significant. Details on the statistics are given in the figure legends.

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

RESULTS

Immunoproteasome and MHC I expression is reduced in BAL cells of COPD patients.

Our previous study on immunoproteasome expression in the lung identified alveolar macrophages as the main cell type expressing active immunoproteasomes (27). We thus first analyzed immunoproteasome expression in BAL cells of COPD patients (see Table 1 for the clinical characteristics of these patients). Of note, mRNA expression of all three immunoproteasome subunits LMP2, MECL-1, and LMP7 was significantly decreased in total BAL cells from COPD patients compared to controls. There was also a trend towards downregulation of immunoproteasomes in BAL cells of another smoke-related chronic lung disease, namely idiopathic pulmonary fibrosis (IPF), which was, however, not significant (Figure 1A). Cellular composition of BAL cells was not significantly different between control and COPD groups, but clearly altered in IPF patients (Supplemental Figure E1). Independent evidence for reduced immunoproteasome expression in alveolar macrophages of COPD patients was obtained from published microarray data confirming downregulation of immunoproteasome expression in COPD patients compared to non-smokers and healthy smokers ((28), Figure 1B). Of note, we observed downregulation of all three immunosubunits also in isolated alveolar macrophages of IPF patients as determined by analysis of a publicly available but unpublished microarray data set (Figure 1C). In contrast, alveolar macrophages from non-smoking asthma patients had rather increased levels of LMP2 and MECL-1 compared to the smoking controls as revealed by bioinformatical analysis of publicly available array data ((29), Figure 1D). These results suggest specific downregulation of the immunoproteasome in isolated alveolar macrophages of patients with smoke-related chronic lung diseases such as COPD and IPF. As immunoproteasomes play a pivotal role in MHC I antigen presentation, we also analyzed other components involved in the MHC I antigen presentation machinery. We observed uniform downregulation of several genes encoding the

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MHC I heavy chain molecules, i.e. human leukocyte antigen (HLA)-A, -B, -C, as well as components of the peptide loading complex such as transporter associated with antigen presentation (TAP) 1 in COPD patients group compared to non-smokers or healthy smokers, respectively (Figure 1E, Supplemental Figure E2). Several genes of the MHC I antigen presentation machinery were also found to be downregulated in alveolar macrophages from IPF patients very similar to the COPD samples, while these genes were unchanged or even upregulated in alveolar macrophages from asthmatics (an overview is provided in Supplemental Figure E2).

Cigarette smoke extract impairs immunoproteasome activity and MHC I surface expression of human macrophages.

To investigate whether immunoproteasome function and MHC I antigen presentation are concertedly regulated in response to cigarette smoke, we exposed primary human blood monocyte-derived macrophages to cigarette smoke extract (CSE) and quantified cell surface MHC I expression by flow cytometry. Of note, non-toxic doses of CSE (Supplemental Figures E3A and B) decreased surface MHC I expression in cells from four out of five individual blood donors after 6 hours (Figure 2A). We next correlated MHC I surface expression with immunoproteasome activity, and, labeled therefore live macrophages with a set of activity-based probes (ABPs) that specifically attach to the active catalytic β-subunits allowing quantification of individual activities depending on the specificity of the probe. Of note, while total proteasome activity was not grossly altered, the activity of LMP7, the rate-limiting subunit for MHC I peptide supply (26), was significantly reduced after 6 hours of CSE exposure (Figure 2B and Supplemental Figure E3C). Protein expression of proteasome subunits and HLA-A was not significantly affected by CSE exposure except for LMP2 (Supplemental Figure E3D). These data demonstrate that diminished immunoproteasome

activity goes along with reduced MHC I surface expression on human blood-derived macrophages confirming previous data from immunoproteasome knockout mice (26). Moreover, we here provide first evidence that immunoproteasome function is impaired by an environmental insult – here cigarette smoke – contributing to diminished MHC I expression on the cell surface.

Cigarette smoke extract impairs immunoproteasome-mediated antigen presentation in splenic immune cells.

To establish a causal link between cigarette smoke-mediated regulation of immunoproteasome activity and MHC I-mediated antigen presentation, we made use of a functional antigen presentation assay that allows assessment of the specific T-cell response to the presentation of an immunoproteasome-dependent MHC I epitope in C57BL/6-derived immune cells: The male HY-antigen $UTY_{246-254}$ is generated by immunoproteasome subunits LMP2 and LMP7, and presented to the T-cell hybridoma reporter cell line UTY (30). Antigen-mediated activation of UTY cells can be quantified by lacZ assays due to the IL-2 promotor-driven β-galactosidase expression.

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

Of note, treatment of male wildtype splenocytes with non-toxic concentrations of CSE for 24 h impaired UTY activation already at the lowest dose of 5 % of CSE, and full suppression of UTY activation was achieved with 25 % CSE (Figure 3B, and Suppl. Figure E4). While

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expression of the immunoproteasome subunits LMP2 and LMP7 was not altered, overall proteasome and immunoproteasome activities were clearly reduced, as assessed by specific ABP labeling (Figures 3C and D). Impaired presentation of $UTY_{246-254}$ in response to increasing doses of CSE was also confirmed for $CD11c^+$ splenic dendritic cells (Figure 3E).

Cigarette smoke extract impairs immunoproteasome-mediated antigen presentation in immune cells of the lung.

We next exposed immune cells of the lung, i.e. $CD11c^+$ lung cells (mainly composed of alveolar macrophages and dendritic cells) as well as BAL cells of mouse lungs (mainly alveolar macrophages (31)), to non-toxic doses of CSE and performed UTY assays. Very similar to our results obtained with splenic cells, CSE exposure significantly reduced antigen presentation of the $UTY_{246-254}$ peptide both in BAL cells and $CD11c^+$ immune cells of the lung (Figures 4, Supplemental Figure E4). These *in vitro* data thus reveal that a) immunoproteasome-mediated MHC I presentation of $UTY_{246-254}$ antigen follows immunoproteasome activity and b) immunoproteasome-dependent antigen presentation is impaired by CSE.

Cigarette smoke dynamically regulates immunoproteasome function in BAL cells *in vivo*.

Immunoproteasome-mediated antigen presentation was next analyzed *in vivo* using male C57BL/6 mice that were acutely exposed to cigarette smoke for 1, 3, and 10 days (for total and differential cell count, see Supplemental Figures E5A and B). Isolated alveolar macrophages of smoke-exposed mice showed transient upregulation of immunoproteasome expression with highest protein levels after 3 days of smoke exposure and subsequent reduction after 10 days of exposure to levels below those of air-exposed controls (Figure 5A).

This dynamics of proteasome expression was closely followed by transient activation of standard and immunoproteasomes as determined by ABP labeling of catalytic subunits (Figure 5B). Notably, after 10 days of smoke-exposure, the activity of the immunoproteasome subunits was reduced resulting in a shift in the activity ratio from immunoproteasome to their standard proteasome counterparts (Figure 5C and Supplemental Figure E5C). Analysis of $UTY_{246-254}$ antigen presentation in the C57BL/6 mice revealed significant activation of the UTY T-cell response in BAL cells of 3 days smoke-exposed mice which was lost after 10 days of smoke exposure (Figure 5D). Antigen presentation thus again closely followed the course of immunoproteasome activity *in vivo.*

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

Immunoproteasome activity is impaired in end-stage COPD lungs but not in cigarette smoke-exposed mice.

We further investigated immunoproteasome activity in explanted lung tissues from end-stage COPD patients versus control organ donors. Additionally, we analyzed immunoproteasome function in lungs of mice that were chronically exposed to smoke for 4 months and had

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developed smoke-induced emphysema (32). Of note, we did not observe any change in RNA expression of standard (α7) and immunoproteasome subunits in COPD and donor lungs (Figure 6A). Protein expression analysis of the human samples revealed heterogeneous expression levels but no significant alterations in the immunoproteasome subunits or total MHC I (Figure 6B, densitometric analysis in Supplement Figure E8). Similarly, RNA and protein levels of immunoproteasome subunits were not grossly altered in lungs of smoke-exposed mice (Supplemental Figures E7A and B). On the contrary, we observed a uniform decrease in total proteasome activity in native lysates of end-stage COPD lungs as determined by ABP analysis, allowing us to attribute the loss of activity to the standard as well as the immunoproteasome proteolytic activities (Figure 6C). We confirmed this striking impairment of proteasome function using native gels with substrate overlay assay and observed a drastic and uniform impairment of both 20S and 26S proteasome activities in COPD lung tissue compared to controls. Reduced activity of the proteasome complexes was assigned to diminished 20S and 26S proteasome formation, as determined by blotting of the native gels for 20S and 26S proteasome subunits, respectively. Of note, both standard and immunoproteasome activities were rather elevated in lungs of chronically smoke-exposed mice as determined by ABP labeling and native gel analysis (Supplemental Figures E7C and D). These results indicate that in contrast to end-stage COPD lungs, emphysematous lungs of smoke-exposed mice are still able to maintain proteasome and immunoproteasome activities, an observation that is well in agreement with the different lung pathologies, showing only minor changes in smoke-exposed mice but detrimental lung damage in end-stage COPD.

DISCUSSION

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

Regulation of the immunoproteasome by cigarette smoke and in COPD.

Immunoproteasomes are constitutively expressed in immune cells of the lung as shown previously by us and others (27, 33). Parenchymal expression of immunoproteasomes is low but can be rapidly induced upon virus infection (27). We did not observe any upregulation of immunoproteasome expression in lungs of smoke-exposed mice and end-stage COPD lungs. This is in accordance with the study by Baker et al. who analysed immunoproteasome expression in COPD lungs (34). These data thus refute the notion that immunoproteasomes are induced as part of a protective oxidative stress response (35), and rather support contrary reports (36). In contrast to our observation, Fujino et al. reported increased LMP2 and LMP7 RNA expression in primary alveolar type II cells of patients with early COPD stages (37). Despite the absence of expressional alterations, standard and immunoproteasome activities were markedly impaired in COPD lungs as determined by two different activity assays, i.e. ABP- and native gel-based analysis. An overall impairment of proteasome activity in COPD

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lungs has previously been noted by Malhotra et al. (17). Intriguingly, in lungs of chronically smoke-exposed mice, we and others observed the rather opposite effect on proteasome activity, i.e. an overall activation of both standard and immunoproteasome activities (17). The data suggest that the murine model of chronic smoke exposure does not fully reflect the complex features of chronic lung disease in COPD patients.

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

Our *in vivo* data from alveolar macrophages of acutely smoke-exposed mice indicate that there is dynamic regulation of immunoproteasome expression and activity in response to cigarette smoke: both, standard and immunoproteasome, expression and activity were strongly activated after 3 days of smoke exposure while expression of the immunoproteasome was significantly reduced after 10 days. In addition, we observed a shift of standard versus immunoproteasome activities in smoke-activated macrophages. Although the smoke-induced changes in the ratio of standard versus immunoproteasome activities were minor and based on a rather small sample size, they were confirmed for two different mouse strains and in both sexes, i.e. female BALB/c and male C57BL/6 mice. Furthermore, *in vitro* exposure of splenic

immune cells and human blood-derived macrophages to cigarette smoke extract confirmed inactivation of the immunoproteasome by smoke in different cell types and human immune cells. The divergent results obtained *in vitro* and *in vivo* may relate to the well-known differences between cigarette smoke extract and full smoke, which also makes comparative dosing difficult (38). Furthermore, *in vivo* smoking activates alveolar macrophages, e.g. by acute neutrophil-mediated release of IFNγ and TNFα (31, 39). These cytokines induce immunoproteasome expression (13) but also activate standard proteasomes (Chen, Kammerl et al., manuscript in revision). Indeed, neutrophil numbers peaked in BAL after 3 days of smoke exposure, which may contribute to activation of isolated alveolar macrophages. Taken together, our data reveal a previously unrecognized alteration of immunoproteasome expression and activity in immune cells in response to cigarette smoke and in COPD pathogenesis.

Cigarette smoke alters MHC I antigen presentation - implications for COPD.

We are the first to show that smoke-mediated changes in immunoproteasome activity directly affect MHC I antigen presentation and T-cell mediated immune responses. In human blood-derived macrophages, CSE acutely inhibited immunoproteasome activity which was associated with diminished cell surface expression of MHC I molecules. Treatment of splenic and lung immune cells with CSE not only reduced the activity of the immunoproteasome but also impaired MHC I-mediated antigen presentation of the immunoproteasome-specific $UTY_{246-254}$ epitope to a T-cell hybridoma. This functional assay directly monitors the immunological consequences of impaired immunoproteasome function (30). CSE-mediated impairment of immunoproteasome activity thus most likely prevents efficient generation of the $UTY_{246-254}$ epitope and surface expression of these peptide/MHC I complexes resulting in reduced T-cell activation. Proteasome-mediated generation of antigenic peptides is a rate-

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

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

Data on MHC I antigen presentation in COPD are limited while innate and MHC II-mediated immune responses in COPD are well studied (39). Several lines of evidence support a role of MHC I mediated antigen presentation for the pathogenesis of COPD and in viral and bacterial

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

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

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FIGURE LEGENDS

FIGURE 1: Reduced immunoproteasome transcripts in BAL of COPD patients. (A) qRT-PCR mRNA analysis of immunoproteasome subunits LMP2, MECL-1, and LMP7 in BAL cells of control subjects (n=15), IPF (n=16) and COPD patients (n=9). Rpl19 was used as housekeeping gene (mean +/- SEM, One-Way-ANOVA with Dunnett's Post test, * = $p<0.05$, ** = $p<0.01$). (B-D) Microarray results from isolated alveolar macrophages of non-smokers and (B) healthy smokers and COPD patients (28), (C) IPF patients (GSE13896) and (D) healthy smokers and non-smoking asthmatic patients (29) for immunoproteasome subunits LMP2, MECL-1, and LMP7. (E) Analysis of the same samples as in (B) for genes encoding human MHC I genes human leukocyte antigen (HLA)-A, HLA-B, HLA-C, (B-E: median, Mann-Whitney-U or Kruskal-Wallis test with Dunn's Post test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). NS, non-smokers; rel., relative; S, smokers.

FIGURE 2: Cigarette smoke extract impairs MHC I antigen presentation in human blood monocyte-derived macrophages. (A) Human monocyte-derived macrophages (n=5 different donors) were treated with 5 or 50 % CSE for 6 h and stained with anti-MHC I antibody W6/32 or isotype control, and propidium iodide. Median fluorescence intensities were determined on gated live single cells and the isotype-corrected median fluorescence intensity (ΔMFI) was normalized to untreated cells in 5 independent experiments (100 %; mean +/- SEM, one-sample t-test, * = $p<0.05$, *** = $p<0.001$). (B) The same samples as in (A) were labeled with activity-based probes MV151 (labeling all active subunits), LW124 (specific for β1 and LMP2) or MVB127 (specific for β5 and LMP7). Densitometric analysis combines data from three different donors (replicates are shown in Supplemental Figure E3C), values were normalized to untreated cells (mean +/- SEM, one-sample t-test (compared to 1), * = $p<0.05$). CSE, cigarette smoke extract; ctrl, control.

FIGURE 3: Cigarette smoke extract impairs immunoproteasome-mediated antigen presentation of UTY peptide in antigen presenting cells of spleen and lung. (A) Validation of the $UTY_{246-254}$-peptide presentation assay. UTY cells are activated by immunoproteasome-dependent presentation of the male $UTY_{246-254}$ peptide on splenocyte MHC I ($H\text{-}2D^b$) as quantified by measurement of reporter gene β-galactosidase activity. β-galactosidase activity of UTY CTLs alone (UTY), co-incubated with splenocytes (female or male wildtype, male LMP2 or LMP7 k.o.) or splenocytes alone was measured by colorimetric assay of substrate turnover. Data are combined results from three independently performed experiments (splenocytes from several individual male mice were isolated, pooled and divided for the different treatment groups. Cell preparations and stimulations were repeated on different days. Results are normalized to the signal of maximum induction of UTY cells by male splenocytes (mean + SEM)). (B) β-galactosidase-activity of UTY hybridoma cells co-incubated with male wildtype splenocytes that had been treated with increasing concentrations of CSE for 24 h, displayed as percentage of maximum induction of control untreated splenocytes (n=3, mean + SEM). (C) Splenocytes were treated for 24 h with the indicated CSE concentrations. Living cells were first incubated with activity-based probe MV151 and then lysed with RIPA buffer. Proteasome expression (immunosubunits LMP2 and LMP7, total 20S α-subunits (α1-7)) and (D) activity were assessed by immunoblotting and SDS-PAGE, respectively. Results are representative for three independent experiments. (E) β-galactosidase-activity of UTY cells co-incubated with isolated male $CD11c^+$ splenic dendritic cells that had been treated with increasing concentrations of CSE for 24 h, displayed as percentage of maximum induction of control untreated cells (n=4, mean + SEM). Statistical analysis: one-sample t-test (compared to 100 %) * = $p<0.05$, *** = $p<0.001$. β-gal,

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FIGURE 4: Cigarette smoke extract decreases UTY-peptide presentation in antigen presenting cells of the lung. β-galactosidase-activity of UTY reporter cell line co-incubated with (A) Magnetic-activated cell (MAC)-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 %), * = $p<0.05$,). BAL, bronchoalveolar lavage; CSE, cigarette smoke extract; ctrl, control; UTY, $UTY_{246-254}$ hybridoma cell line.

FIGURE 5: Cigarette smoke dynamically regulates proteasome function and UTY presentation in BAL cells of cigarette smoke-exposed mice. (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, 1 day), 3 or 10 days. Western blots display immunosubunits LMP2 and LMP7 as well as standard subunit β1 and α3. β-Actin served as loading control. Combined densitometric analysis of Western blots for 20S standard subunits α3 and β1, as well as immunosubunits LMP2 and LMP7. Results are combined data from two to three independent experiments and are displayed as fold over air-exposed controls (mean + SEM, one-sample t-test (compared to 1), * = $p<0.05$, ** = $p<0.01$). (B) Proteasome activity (activity-based probe MV151) of alveolar macrophages and densitometric analysis thereof (mean + SEM, one-sample t-test (compared to 1), * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). (C) MECL-1/β2 and LMP2/β1 (corresponding gel see

Supplemental Figure E5C) activity ratios in alveolar macrophages from mice exposed to cigarette smoke for 10 days compared to controls (ctrl, 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 1 day (n=14), 3 days (n= 22), or 10 days (n=15) compared to air controls (n=25, set to 100 %) (mean +/- SEM, One-Way-ANOVA with Dunnett's Post test, *** = $p<0.001$).

FIGURE 6: Impaired immunoproteasome activity in COPD patients. (A) qRT-PCR mRNA analysis of 20S proteasome subunit α7 and immunoproteasome subunits LMP2, MECL-1, and LMP7 in total lungs of donors (n=5) and end-stage COPD patients (n=5). (B) Western blot of the same donor and end-stage COPD lungs for immunosubunits LMP2 and LMP7 as well as standard subunit α3. β-Actin served as a loading control. Densitometric analysis can be found in Supplement Figure E8B. (C) Activity-based probe profiling of the same patients as in (B): native lung lysates were labeled with pan-reactive ABP MV151 or LW124 (labeling LMP2 and β1) and MVB127 (labeling MECL-1 and β2) and separated on denaturing SDS gels. Densitometric analysis revealed total activity (MV151 signal) and single subunit activity and is shown normalized to the mean of donor activities (mean +/- SEM, Student's t-test). (D) Native gel analysis with chymotrypsin-like (CT-L) substrate overlay analysis and immunoblotting of native lung lysates to detect 20S and 26S (20S + 19S) proteasome complexes with antibodies detecting α1-7 subunits (20S) or Rpt5 (19S subunit).

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Table 1: Patients' characteristics

Group	**BAL[*] (Figures 1A, E1)**					**Lung Tissue[†] (Figure 6)**
	Controls	**IPF**	**p-value**	**COPD**	**p-value**	**COPD**
N	15	16		9		5
Gender (m/f)	8/7	15/1	<0.05[‡]	9/0	<0.05[‡]	1/4
Age (y; median (range))	59 (45-71)	68 (55-86)	<0.05[§]	67 (48-72)	n.s.[§]	53 (44-63)
Smoking status (NS/Ex-smoker)	4/11	6/10	n.s.[‡]	0/9	n.s.[‡]	n.a.
Pack years (median (range))	12.5 (0-30)	15 (0-30)	n.s.[§]	40 (20-60)	<0.001[§]	n.a.
GOLD stage (I/II/III/IV)	n.a.	n.a.		1/6/2/0		0/0/0/5

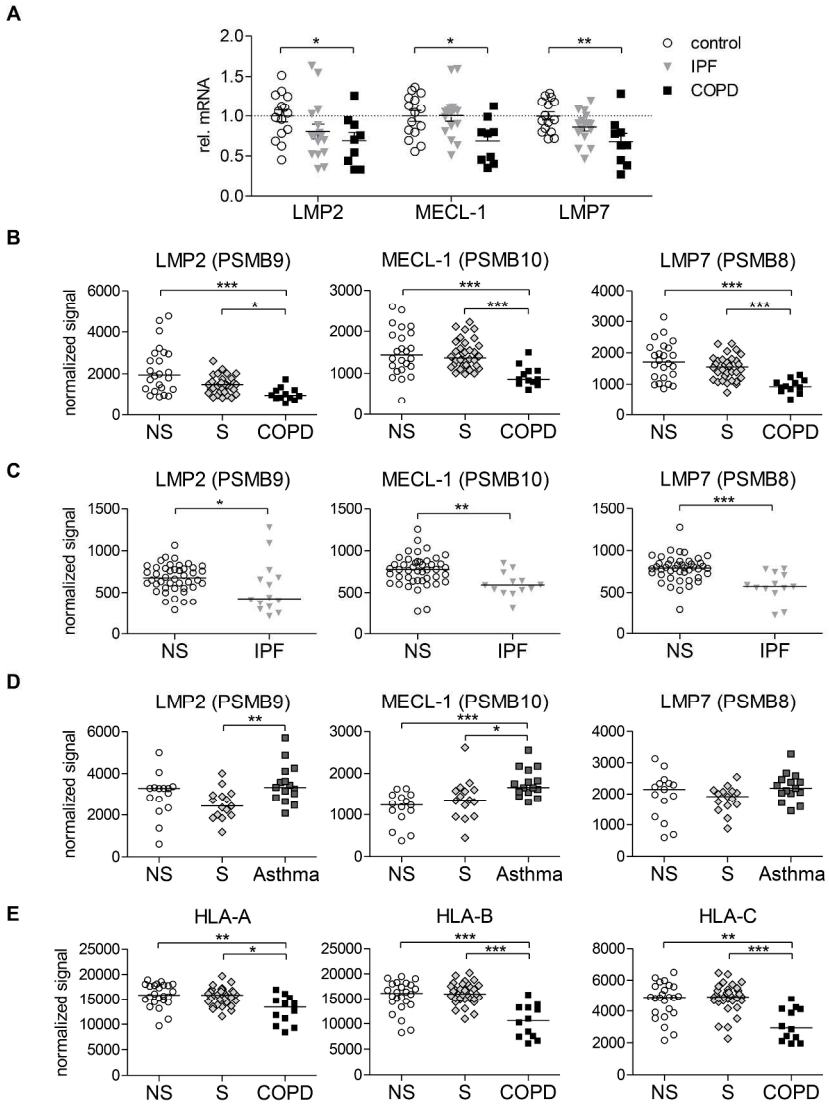
[*] BAL cells were obtained as previously described (23).

[†] According to European organ transplant guidelines, donors are anonymous.

[‡] Statistical analysis was performed using Fisher's exact test compared to controls

[§] Statistical analysis was performed using Kruskal-Wallis test compared to controls

[ll] Abbreviations: BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; f, female; GOLD, global initiative for obstructive lung disease; IPF, idiopathic pulmonary fibrosis; m, male; n, number; n.a., not available; NS, non-smoker; y, year.



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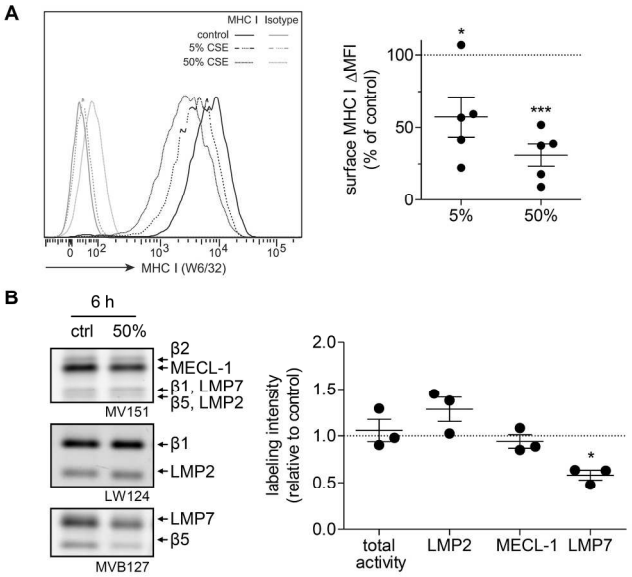

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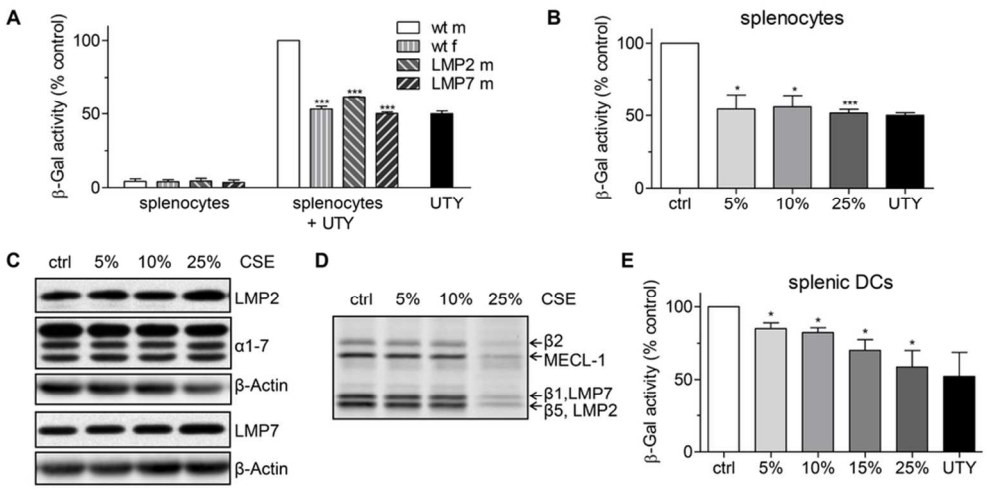

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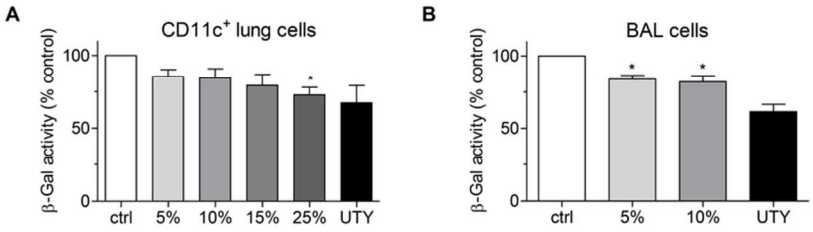

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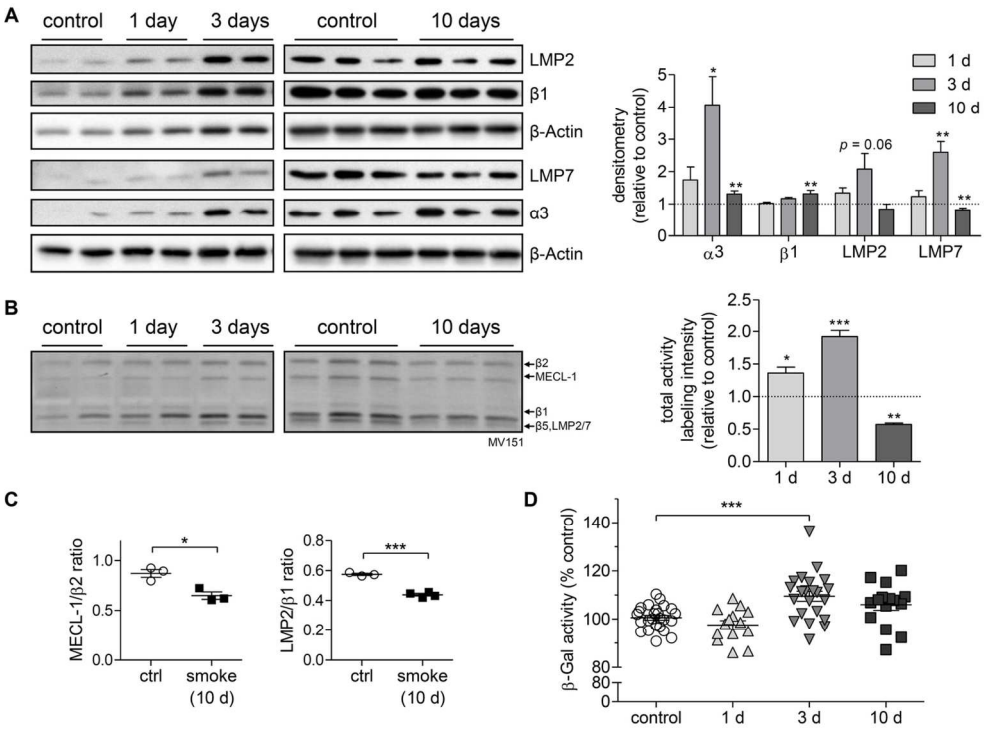

FIGURE 5: Cigarette smoke dynamically regulates proteasome function and UTY presentation in BAL cells of cigarette smoke-exposed mice. (A) Proteasome protein expression in isolated alveolar macrophages from air-exposed controls or mice that had been exposed to cigarette smoke for one exposure cycle (50 min, 1 day), 3 or 10 days. Western blots display immunosubunits LMP2 and LMP7 as well as standard subunit β1 and α3. β-Actin served as loading control. Combined densitometric analysis of Western blots for 20S standard subunits α3 and β1, as well as immunosubunits LMP2 and LMP7. Results are combined data from two to three independent experiments and are displayed as fold over air-exposed controls (mean + SEM, one-sample t-test (compared to 1), * = $p<0.05$, ** = $p<0.01$). (B) Proteasome activity (activity-based probe MV151) of alveolar macrophages and densitometric analysis thereof (mean + SEM, one-sample t-test (compared to 1), * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). (C) MECL 1/β2 and LMP2/β1 (corresponding gel see Supplemental Figure E5C) activity ratios in alveolar macrophages from mice exposed to cigarette smoke for 10 days compared to controls (ctrl, 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 1 day (n=14), 3 days (n= 22), or 10 days (n=15) compared to air controls (n=25, set to 100 %) (mean +/- SEM, One-Way-ANOVA with Dunnett's Post test, *** = $p<0.001$).
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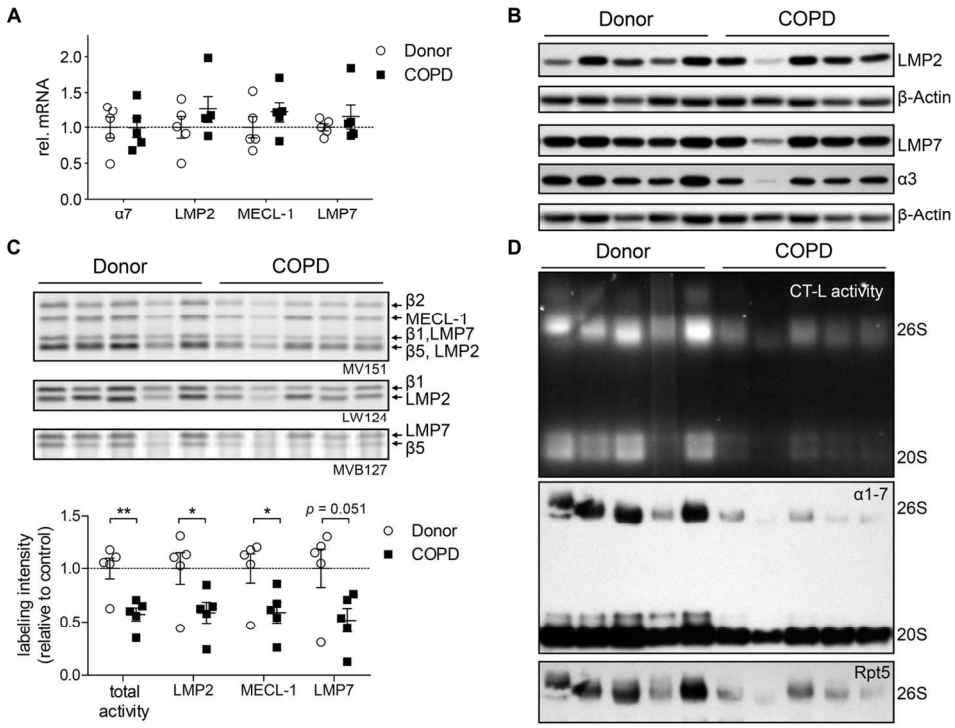

FIGURE 6: Impaired immunoproteasome activity in COPD patients. (A) qRT-PCR mRNA analysis of 20S proteasome subunit α7 and immunoproteasome subunits LMP2, MECL-1, and LMP7 in total lungs of donors (n=5) and end-stage COPD patients (n=5). (B) Western blot of the same donor and end-stage COPD lungs for immunosubunits LMP2 and LMP7 as well as standard subunit α3. β-Actin served as a loading control. Densitometric analysis can be found in Supplement Figure E8B. (C) Activity-based probe profiling of the same patients as in (B): native lung lysates were labeled with pan reactive ABP MV151 or LW124 (labeling LMP2 and β1) and MVB127 (labeling MECL-1 and β2) and separated on denaturing SDS gels. Densitometric analysis revealed total activity (MV151 signal) and single subunit activity and is shown normalized to the mean of donor activities (mean +/- SEM, Student's t-test). (D) Native gel analysis with chymotrypsin-like (CT-L) substrate overlay analysis and immunoblotting of native lung lysates to detect 20S and 26S (20S + 19S) proteasome complexes with antibodies detecting α1-7 subunits (20S) or Rpt5 (19S subunit).
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Online Data Supplement

Impairment of immunoproteasome function by cigarette smoke and in COPD

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SUPPLEMENT METHODS

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

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

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

Smoke exposure of mice: Eight week old mice were exposed to cigarette smoke for 50 min once ("1 day" group), two days for two times 50 min and the third day once for the "3 days" group or once daily for 10 consecutive days ("10 days" group). The smoke of 10 3R4F

research-grade cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) without filter was drawn into the exposure chamber using a peristaltic pump for each 50 min exposure cycle. The mean particle concentration was ~340 mg/m^3. Directly after the last smoke exposure, mice were euthanized and samples were prepared. For immunohistochemistry, non-lavaged lungs were fixed with 4 % paraformaldehyde and embedded in paraffin. Chronic exposure of mice was performed as described previously (E3).

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

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

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

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

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

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

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

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

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

Cell viability:

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

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

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

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

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

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

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

Native gel analysis and substrate overlay:

Chymotrypsin-like proteasome activity in native cell lysates was assessed using the synthetic peptide substrate Suc-LLVY-AMC (Enzo Life Sciences, Farmingdale, NY, USA) and was performed as previously described (E4). Equal amounts of protein (15 µg) of hyposmotic lysates were diluted with 5x native loading buffer (50 % v/v glycerol, 250 mM Tris, 0.1 % w/v bromophenol blue, pH 7.5) and subjected to electrophoresis (4 h, 150 V, 4 °C) on 3-8 % non-denaturing Tris-Acetate gels (Life Technologies). Proteasome activity was detected after incubating the gels for 30 min at 37 °C in substrate buffer (50 µM Suc-LLVY-AMC, 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT). Gels were analyzed using the ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA) with an excitation wavelength of 380 nm and emission wavelength of 460 nm. Band intensity was quantified with the Image Lab software package (Bio-Rad). Afterwards, proteins were denatured by incubation of the gel in solubilization buffer (2 % w/v SDS, 66 mM Na_2CO_3, 1.5 % v/v 2-ME) for 15 min and proteins were blotted onto PVDF membranes. 20S and 26S bands were identified by using antibodies detecting 20S α1-7 subunits and the 19S subunit Rpt5.

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SUPPLEMENT TABLE

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

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

* Statistical analysis was performed using Fisher's exact test

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

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

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TABLE E2: Primer sequences

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

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

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

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

TABLE E3: Antibodies for Western blotting

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

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SUPPLEMENT FIGURES

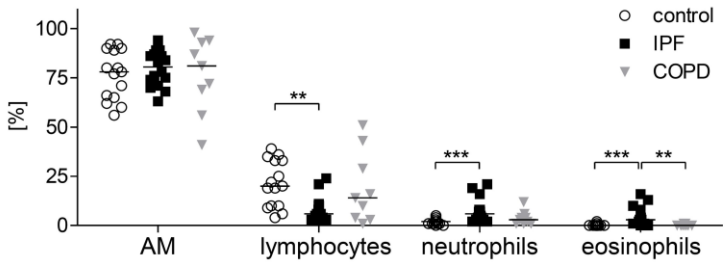

FIGURE E1: BAL characterization of control subjects, COPD, or IPF patients. Cellular composition of human BAL obtained from control subjects, IPF, or COPD patients. (median, Kruskal-Wallis test with Dunn's Post test, ** = $p<0.01$, *** = $p<0.001$). AM, alveolar macrophages; COPD, chronic obstructive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

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

\+ significantly increased
\- significantly decreased
= no change

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

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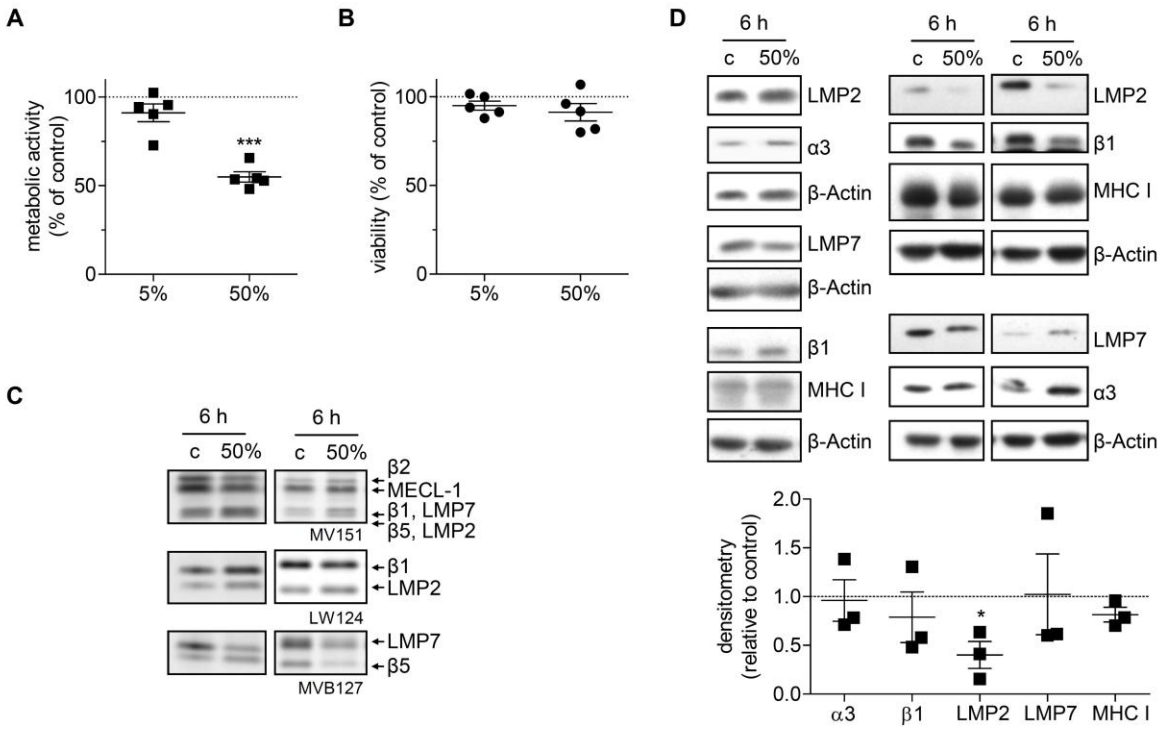

FIGURE E3: Cigarette smoke extract effect on human macrophages. Metabolic activity and viability of human macrophages (n=5) 6 h after treatment with 5 or 50 % cigarette smoke extract compared to untreated cells were tested with (A) MTT or (B) propidium iodide-exclusion assay measured by FACS analysis.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

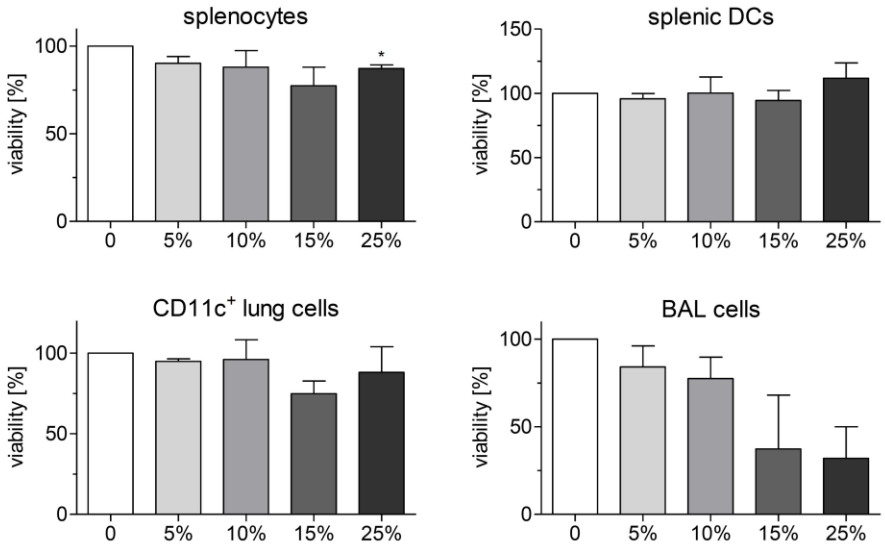

FIGURE E4: Viability of primary cells exposed to cigarette smoke *in vitro*. Viability, measured by trypan blue exclusion assay or MTT, of splenocytes, $CD11c^+$ splenic dendritic cells (DCs), $CD11c^+$ lung cells, or BAL cells treated with increasing percentages of cigarette smoke extract for 24 h (mean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

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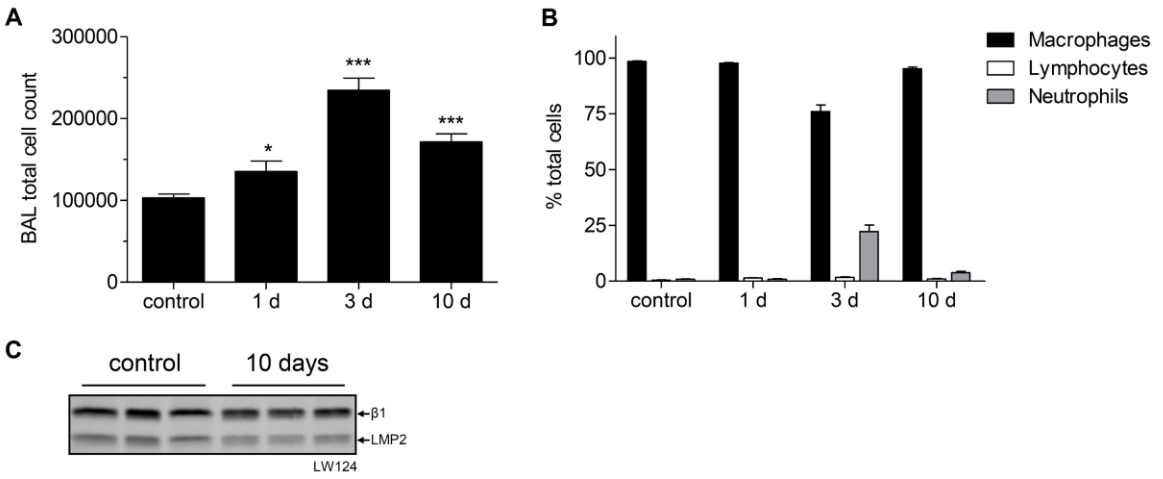

FIGURE E5: BAL characteristics of cigarette smoke-exposed C57BL/6 mice. (A) BAL total and (B) differential cell analysis for male C57BL/6 wt mice exposed to cigarette smoke for 1, 3, or 10 days and controls (mean + SEM, One-Way-ANOVA with Dunnett's Post test, * = $p<0.05$, *** = $p<0.001$, compared to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

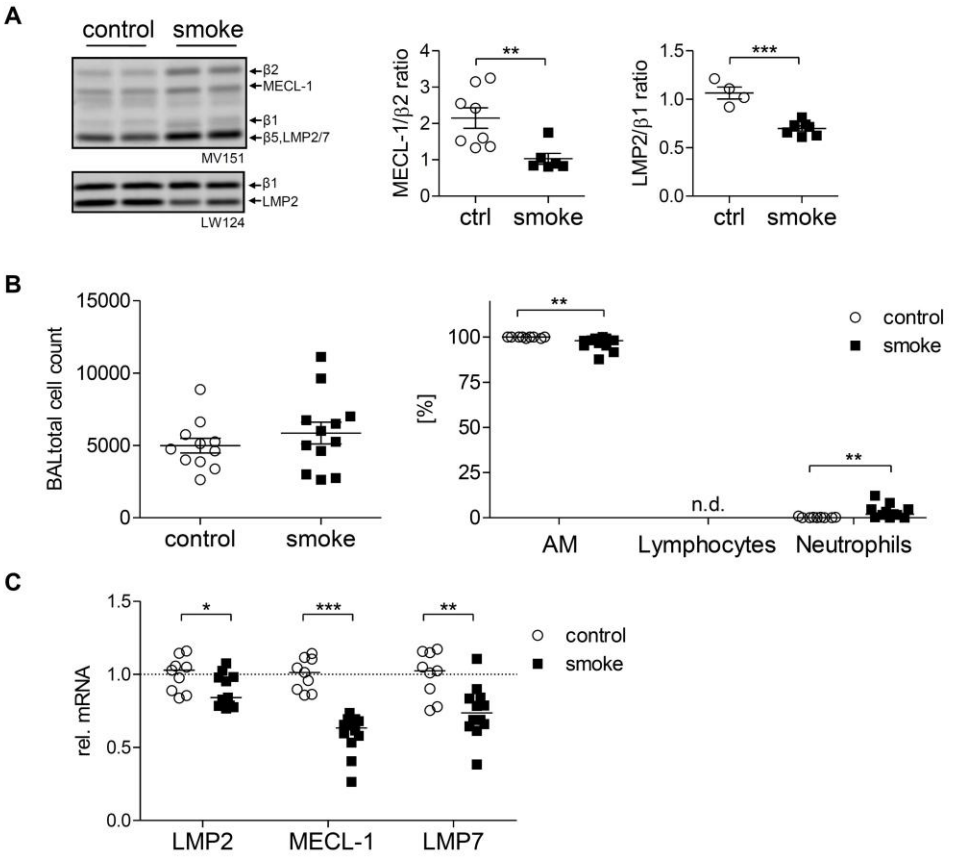

FIGURE E6: Immunoproteasome expression and activity in alveolar macrophages of cigarette smoke-exposed BALB/c mice. (A) Proteasome activity in isolated alveolar macrophages from female BALB/c mice exposed to cigarette smoke for 10 days compared to controls labeled with pan-reactive activity-based probe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

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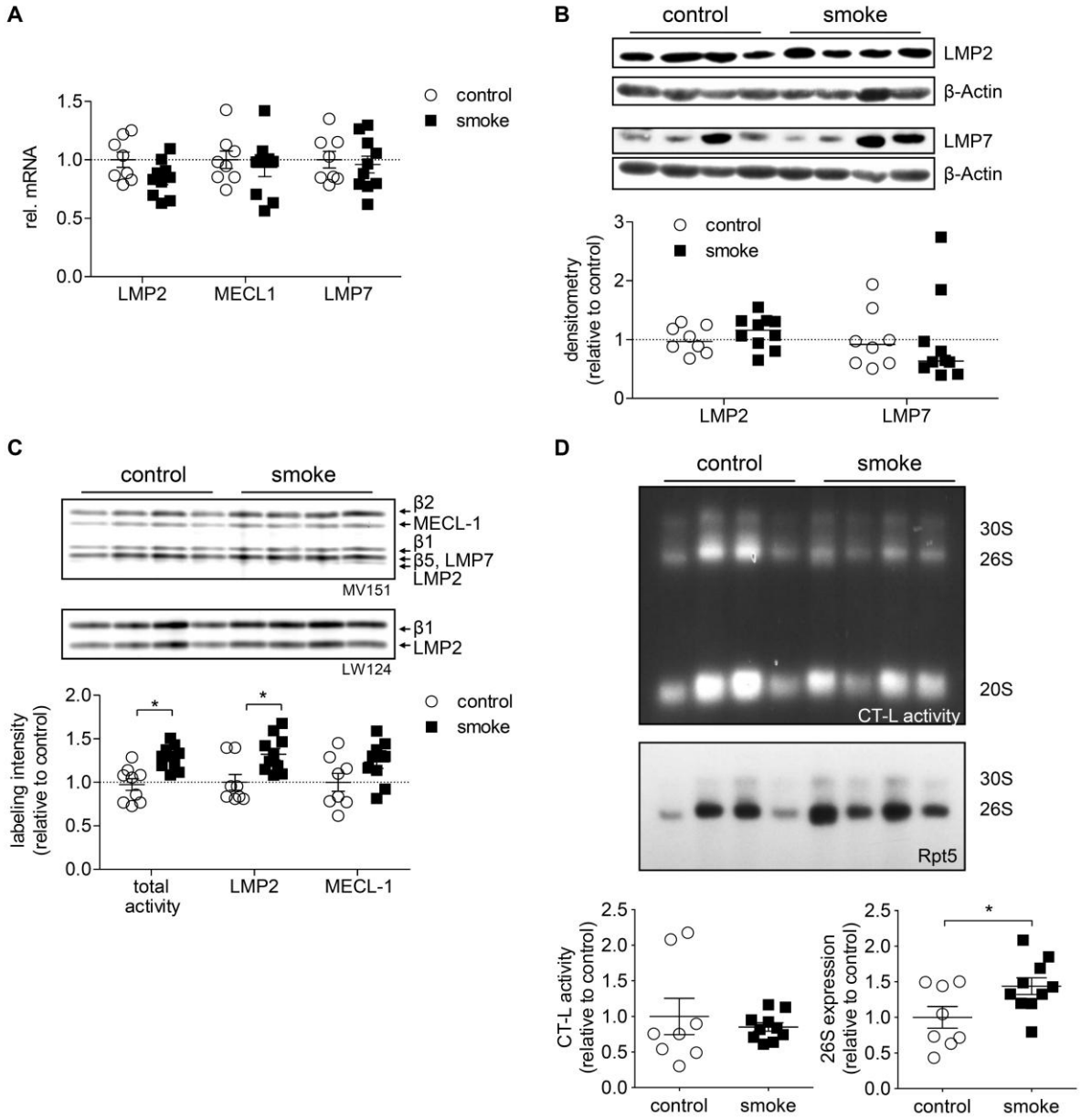

FIGURE E7: Cigarette smoke affects activity of proteasomes in the mouse lung. (A) qRT-PCR mRNA analysis of immunoproteasome subunits LMP2, MECL-1, and LMP7 in total lungs of control mice (n=8) and mice that have been exposed to cigarette smoke for 4 months (n=10), (mean +/- SEM, Student's t-test). (B)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

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

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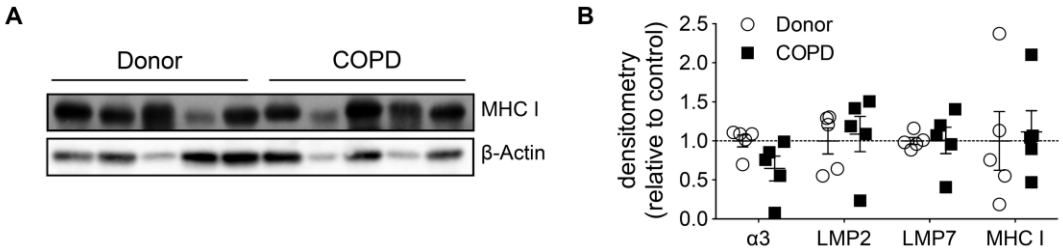

FIGURE E8: Western blot analysis of human COPD tissue. (A) Western blot detecting total MHC I (HLA-A) in total lung lysate of donor or COPD tissue. (B) Densitometric analysis of Western blots shown in Figures 6B and E8A. COPD, chronic obstructive pulmonary disease; HLA, human leukocyte antigen; MHC I,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