ORIGINAL ARTICLE

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

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Abstract

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

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

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

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

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

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

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At a Glance Commentary

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

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

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

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

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

Methods

Human Lung Tissue and Cells

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

Animals

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

UTY-LacZ Assay

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

Statistics and Software

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

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

Results

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

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

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

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

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

Table 1. Patient Characteristics

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

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

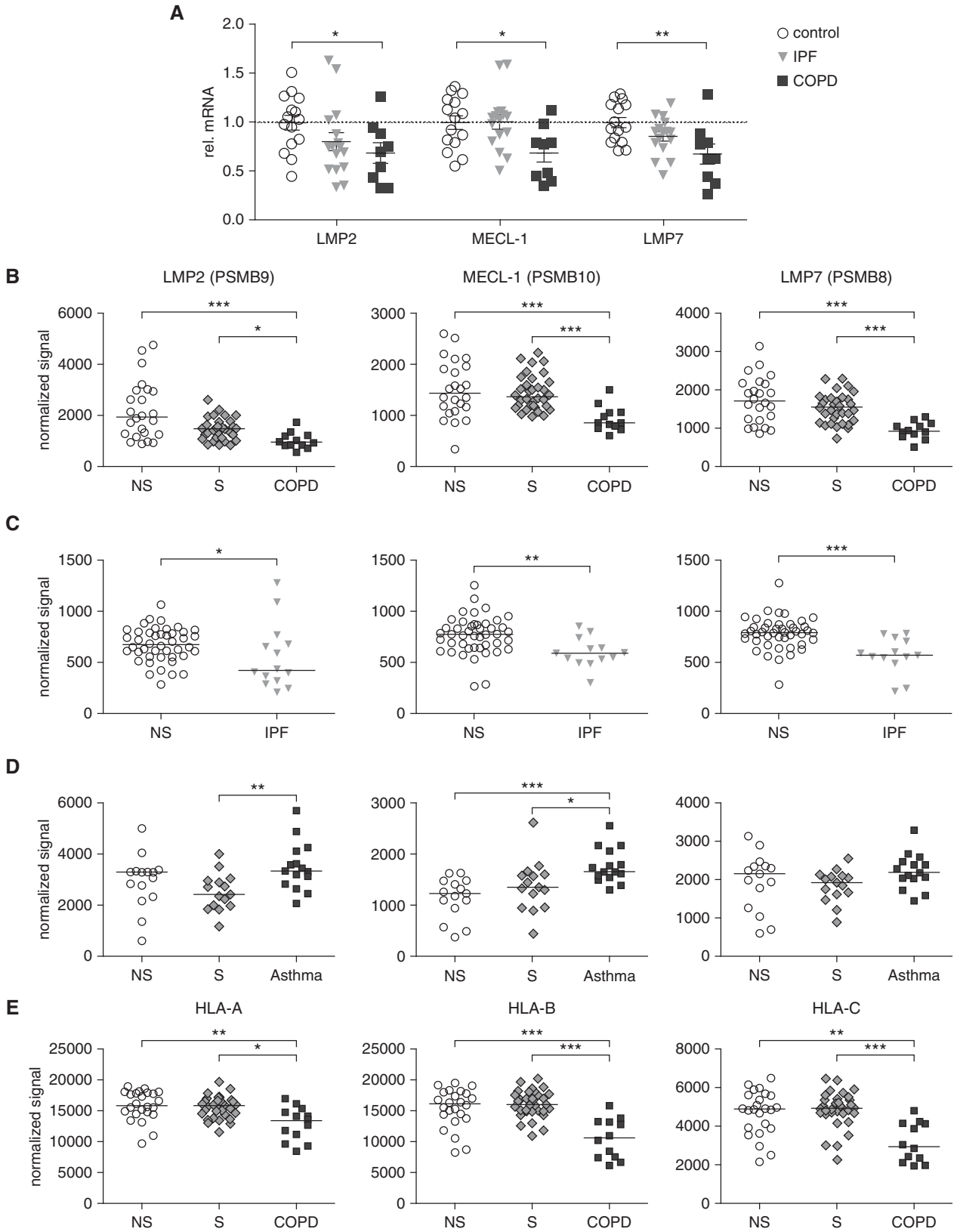

Figure 1. Reduced immunoproteasome transcripts in bronchoalveolar lavage of patients with chronic obstructive pulmonary disease (COPD). (*A*) Quantitative reverse transcriptase polymerase chain reaction mRNA analysis of immunoproteasome subunits low molecular mass protein (LMP) 2, multicatalytic endopeptidase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

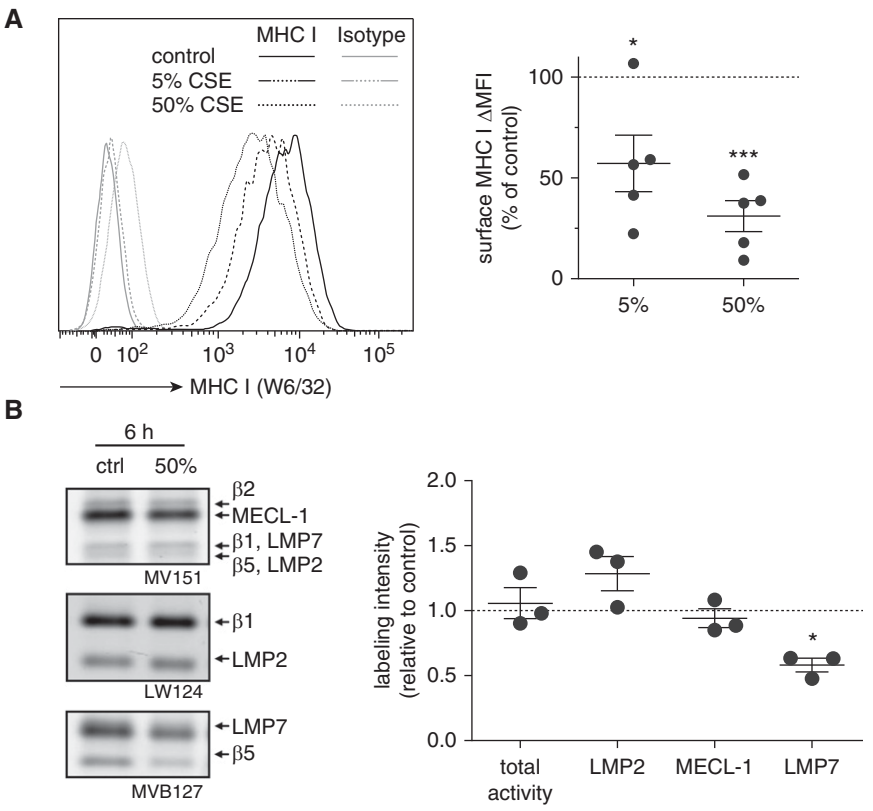

Figure 2. Cigarette smoke extract (CSE) impairs major histocompatibility class I (MHC I) antigen presentation in human blood monocyte-derived macrophages. (*A*) Human monocyte-derived macrophages (n = 5 different donors) were treated with 5 or 50% CSE for 6 hours and stained with anti-MHC I antibody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

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

CSE Impairs Immunoproteasome-mediated Antigen Presentation in Splenic Immune Cells

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

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

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

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

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

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

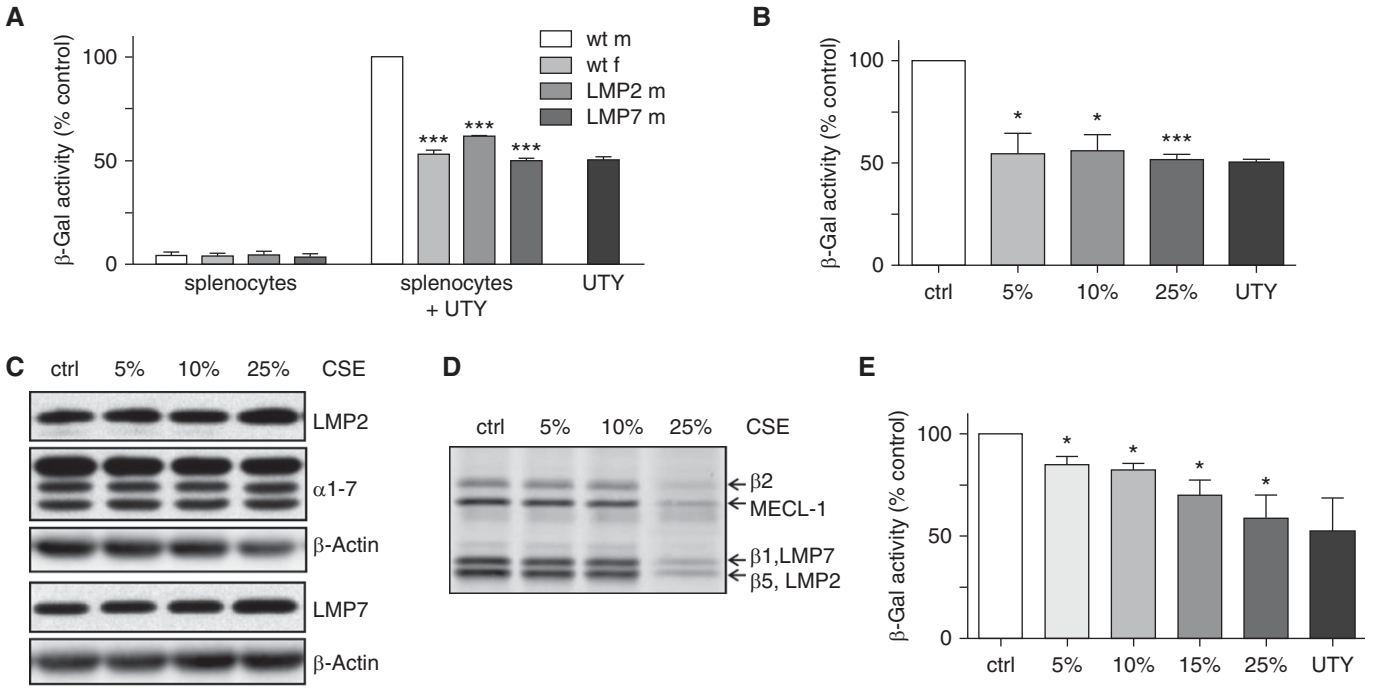

Figure 3. Cigarette smoke extract (CSE) impairs immunoproteasome-mediated antigen presentation of UTY peptide in antigen-presenting cells of spleen and lung. (*A*) Validation of the $UTY_{246–254}$ peptide presentation assay. UTY cells are activated by immunoproteasome-dependent presentation of the male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

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

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

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

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

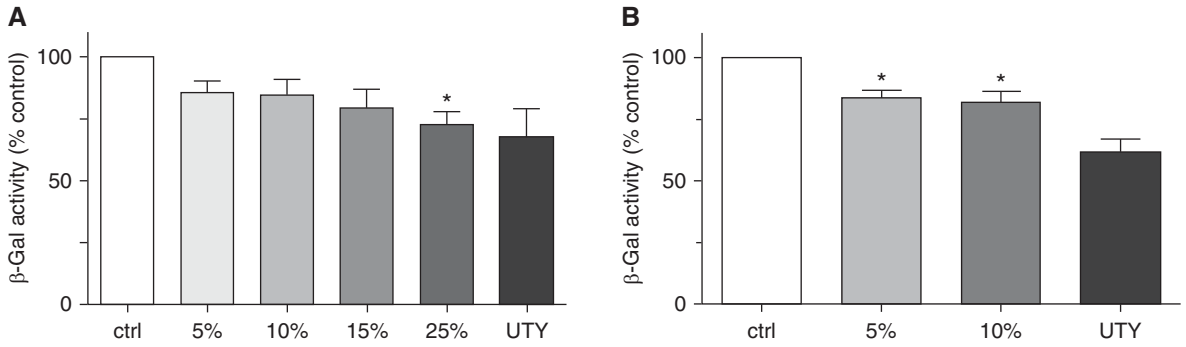

Figure 4. Cigarette smoke extract decreases UTY peptide presentation in antigen-presenting cells of the lung. β-Galactosidase activity of UTY reporter cell line coincubated with (*A*) magnetic-activated cell-sorted CD11c$^+$ lung cells or (*B*) bronchoalveolar lavage cells (>95% alveolar macrophages)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

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

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

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

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

Discussion

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

Regulation of the Immunoproteasome by Cigarette Smoke and in COPD

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

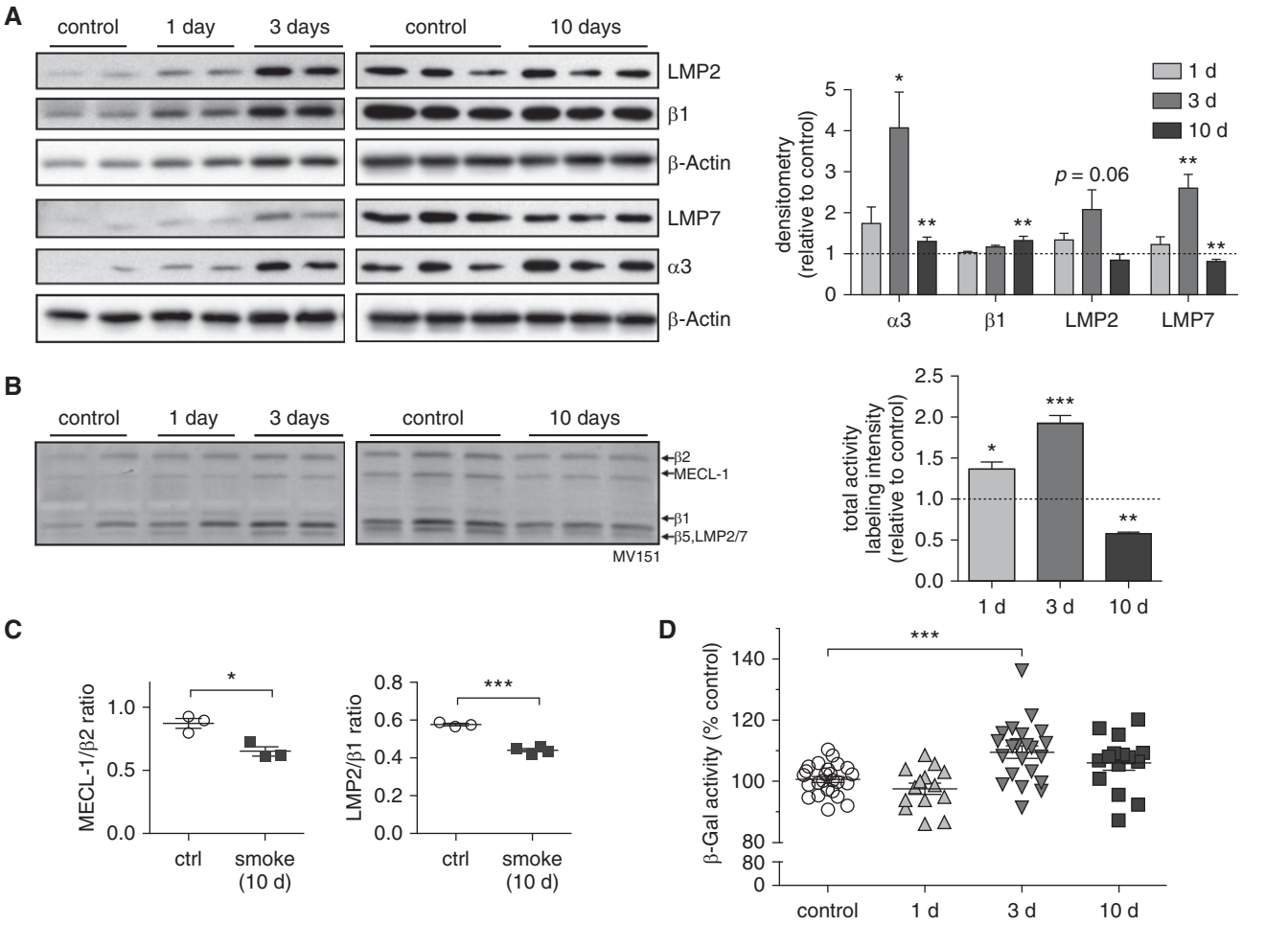

Figure 5. Cigarette smoke dynamically regulates proteasome function and UTY presentation in bronchoalveolar lavage cells of cigarette smoke–exposed mice. (*A*) Proteasome protein expression in isolated alveolar macrophages from air-exposed control animals or mice that had been exposed to cigarette smoke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

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

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

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

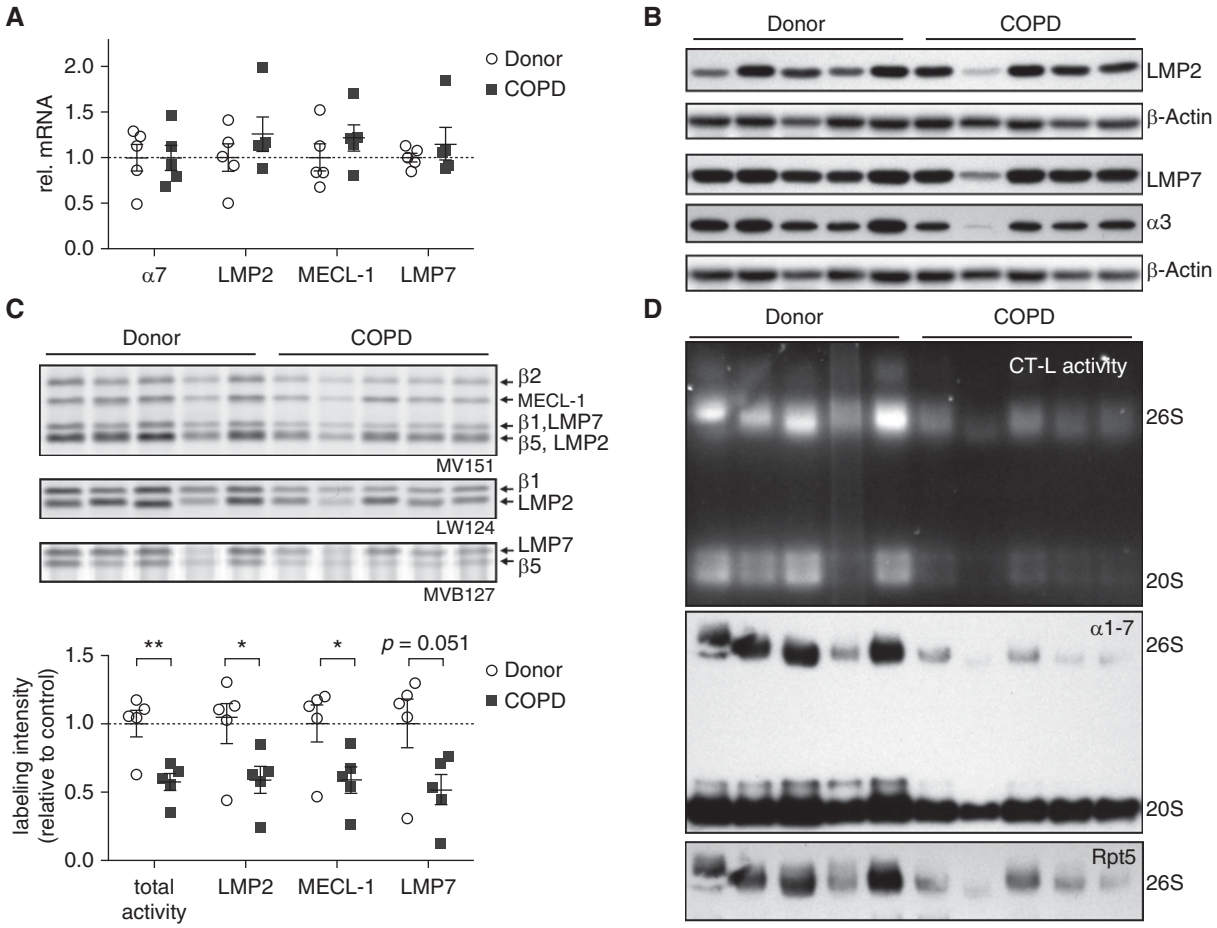

Figure 6. Impaired immunoproteasome activity in patients with chronic obstructive pulmonary disease (COPD). (*A*) Quantitative reverse transcriptase polymerase chain reaction mRNA analysis of 20S proteasome subunit α7 and immunoproteasome subunits low molecular mass protein (LMP) 2, multicatalytic endopeptidase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

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

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

Cigarette Smoke Alters MHC I Antigen Presentation: Implications for COPD

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

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

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

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

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

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

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

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