



Activation of immune cell proteasomes in peripheral blood of smokers and COPD patients: implications for therapy

Ilona E. Kammerl¹, Sophie Hardy^{1,2}, Claudia Flexeder³, Andrea Urmann¹, Julia Peierl¹, Yuqin Wang¹, Oliver Vosyka¹, Marion Frankenberger^{1,4}, Katrin Milger^{1,5}, Jürgen Behr^{1,5}, Andrea Koch^{1,6}, Juliane Merl-Pham⁷, Stefanie M. Hauck⁷, Charles Pilette², Holger Schulz³ and Silke Meiners^{1,8,9}

¹Comprehensive Pneumology Center (CPC), University Hospital, Ludwig Maximilians University, Helmholtz Zentrum München, Member of the German Center for Lung Research (DZL), Munich, Germany. ²Cliniques Universitaires Saint-Luc, Dept of Pulmonology and Institute of Experimental and Clinical Research (IREC), Pole of Pulmonology, ENT and Dermatology, Université Catholique de Louvain, Brussels, Belgium. ³Institute of Epidemiology, Helmholtz Zentrum München, Member of the German Center for Lung Research (DZL), Neuherberg, Germany. ⁴Institute of Lung Biology and Disease and Comprehensive Pneumology Center with the CPC-M bioArchive, Helmholtz Zentrum München, Member of the German Center for Lung Research (DZL), Munich, Germany. ⁵Dept of Medicine V, University Hospital, Ludwig Maximilians University, Member of the German Center for Lung Research (DZL), Munich, Germany. ⁶Dept of Pneumology, Teaching Hospital Pyhrn-Eisenwurzen Klinikum Steyr, Steyr, Austria. ⁷Research Unit Protein Science, Metabolomics and Proteomics Core, Helmholtz Zentrum München, Munich, Germany. ⁸Research Center Borstel, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Borstel, Germany. ⁹Institute of Experimental Medicine, Christian-Albrechts University, Kiel, Germany.

Corresponding author: Silke Meiners (silke.meiners@helmholtz-muenchen.de)



Shareable abstract (@ERSpublications)

This study demonstrates distinct activation of immunoproteasomes in peripheral blood cells of young smokers and COPD patients. Specific inhibition of the immunoproteasome might represent a novel therapeutic concept for COPD treatment. <https://bit.ly/3rEwaU7>

Cite this article as: Kammerl IE, Hardy S, Flexeder C, *et al.* Activation of immune cell proteasomes in peripheral blood of smokers and COPD patients: implications for therapy. *Eur Respir J* 2022; 59: 2101798 [DOI: 10.1183/13993003.01798-2021].

Copyright ©The authors 2022.

This version is distributed under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0. For commercial reproduction rights and permissions contact permissions@ersnet.org

This article has an editorial commentary: <https://doi.org/10.1183/13993003.02557-2021>

Received: 22 March 2021
Accepted: 19 July 2021

Abstract

Background Immune cells contain a specialised type of proteasome, *i.e.* the immunoproteasome, which is required for intracellular protein degradation. Immunoproteasomes are key regulators of immune cell differentiation, inflammatory activation and autoimmunity. Immunoproteasome function in peripheral immune cells might be altered by smoking and in chronic obstructive pulmonary disease (COPD), thereby affecting immune cell responses.

Methods We analysed the expression and activity of proteasome complexes in peripheral blood mononuclear cells (PBMCs) isolated from healthy male young smokers as well as from patients with severe COPD and compared them with matching controls.

Results Proteasome expression was upregulated in COPD patients as assessed by quantitative reverse transcriptase-PCR and mass spectrometry-based proteomic analysis. Proteasome activity was quantified using activity-based probes and native gel analysis. We observed distinct activation of immunoproteasomes in the peripheral blood cells of young male smokers and severely ill COPD patients. Native gel analysis and linear regression modelling confirmed robust activation and elevated assembly of 20S proteasomes, which correlated significantly with reduced lung function parameters in COPD patients. The immunoproteasome was distinctly activated in COPD patients upon inflammatory cytokine stimulation of PBMCs *in vitro*. Inhibition of the immunoproteasome reduced pro-inflammatory cytokine expression in COPD-derived blood immune cells.

Conclusions Given the crucial role of chronic inflammatory signalling and the emerging involvement of autoimmune responses in COPD, therapeutic targeting of the immunoproteasome might represent a novel therapeutic concept for COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major chronic lung disease estimated to become the third leading cause of death worldwide in 2030 [1]. Notably, there is a lack of innovative therapies for this



disease. Cigarette smoke is the main risk factor for the development of COPD [2]. It causes oxidative stress that damages DNA and proteins [3], results in degradation and remodelling of lung tissue, and initiates innate and adaptive immune dysfunction driving COPD disease development [4, 5].

The ubiquitin–proteasome system is the main protein degradation pathway in the cell. The proteasome hydrolyses most cellular proteins, including short-lived cellular regulators such as transcription factors, cell cycle and signalling molecules, into small peptides [6, 7]. Degradation products are used for amino acid recycling and as major histocompatibility complex (MHC) class I antigens enabling immune surveillance by CD8⁺ T-cells [8, 9]. The most prominent proteasome complexes are the 26S and 20S proteasomes, with 26S consisting of the 20S catalytic core and one or two 19S regulators (figure 1a) [7]. Immune cells contain a specialised type of proteasome, *i.e.* the immunoproteasome, harbouring three distinct catalytic subunits: LMP2, MECL-1 and LMP7 [8, 9]. Immunoproteasomes are key regulators of immune cell activation and differentiation [9, 10]. In particular, they play a major role in inflammatory signalling by regulating activation of inflammatory transcription factors such as NF- κ B [11]. Specific inhibition of the immunoproteasome counteracts autoimmunity and inflammatory immune responses [12, 13].

We and others have previously demonstrated that lung tissue proteasomes are inhibited by cigarette smoke, resulting in accumulation of oxidatively damaged proteins and altered MHC class I antigen presentation [14–17]. Proteasome activity in lung tissue of end-stage COPD patients is severely impaired [16, 18] and protein aggregates accumulate in COPD lungs [19]. These data indicate that proteasome function and proteostasis in lungs of COPD patients is severely disturbed, possibly contributing to exacerbation of disease, altered MHC class I antigen presentation and susceptibility to virus infections [16, 20].

In this study, we extend our understanding of proteasome function in COPD by focusing on the analysis of the proteasome in peripheral blood immune cells of young male smokers and COPD patients. We demonstrate distinct activation of immune cell proteasomes in smokers and severely ill COPD patients.

Methods

Further details on the methods, primers and antibodies used in this study can be found in the supplementary material.

Human samples

For the first study arm, EDTA-blood samples of 20 young, self-reported healthy never-smokers and 20 current smoking subjects were obtained (table 1). We chose male participants to exclude any potential hormonal variations. Inclusion criteria were male gender, age 18–30 years, body mass index (BMI) 18–30 kg·m⁻², at least 10 cigarettes per day within the last year or never-smoking; exclusion criteria were chronic diseases, long-term medication or infectious disease within the last 3 weeks. Cotinine was assayed

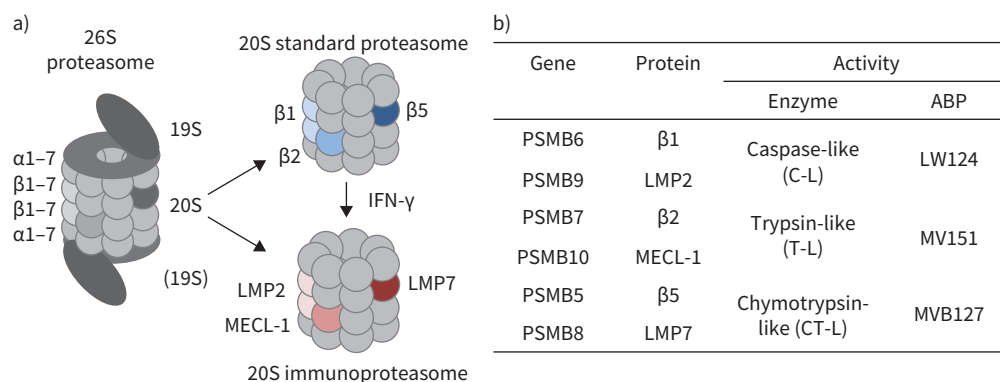


FIGURE 1 Overview on the analysis of proteasome complexes in this study. **a)** The 26S proteasome complex consists of a central 20S core particle capped with one or two 19S regulatory particles. The 20S particle consists of four rings with seven subunits each, two outer α -rings and two inner β -rings, where the three proteolytically active subunits reside: β 1, β 2 and β 5 constitute the standard proteasome; the three immunosubunits LMP2, MECL-1 and LMP7 can be induced, *e.g.* by interferon (IFN)- γ , but are constitutively expressed at high levels in immune cells. **b)** Nomenclature of gene/protein names, enzymatic activity and activity-based probes (ABPs) used to differentiate immune and standard proteasomes.

TABLE 1 Study population of young healthy male smokers and nonsmokers

	Never-smoker		Smoker		p-value [#]
	% or median (range)	n/N or N	% or median (range)	n/N or N	
Allergies					
No	90	18/20	95	19/20	
Yes	10	2/20	5	1/20	1.000
Pack-years	NA	20	3.5 (1.5–10.0)	20	NA
Age, years	24 (18–30)	20	24.5 (19–29)	20	0.978
BMI, kg·m⁻²	22.70 (18.59–27.45)	20	23.27 (19.60–28.60)	20	0.387
Cotinine, ng·mL⁻¹	0.88 (0.62–1.28)	20	314.2 (142.6–693.6)	20	<0.001*

BMI: body mass index; NA: not applicable. [#]: differences between groups were tested using Fisher's exact test for categorical variables and the Wilcoxon rank sum test for continuous variables. *: p<0.05.

in blood plasma via ELISA according to the assay manufacturer's recommendations (Cotinine ELISA CO096D; Calbiotech, El Cajon, CA, USA) to confirm current smoking status.

For the second arm, analysis was performed in EDTA-blood samples from 30 stable COPD patients (no exacerbation of the disease since at least 6 weeks) and 24 healthy age-matched control subjects collected from the clinics of the Ludwig Maximilians University (LMU) and the outpatient unit of the Comprehensive Pneumology Center (CPC) in Munich, Germany (table 2). We also obtained blood samples for our *in vitro* stimulation experiments from the same locations (supplementary table S1).

All donors gave written consent. The study was approved by the Ethics Committee of the Medical Faculty of the LMU (study number 382-10).

TABLE 2 Study population of lung healthy controls and chronic obstructive pulmonary disease (COPD) patients

	Never-smoking control		Ever-smoking control		COPD		p-value [#]
	% or median (range)	n/N or N	% or median (range)	n/N or N	% or median (range)	n/N or N	
Sex							
Female	62.50	10/16	75.00	6/8	53.33	16/30	
Male	37.50	6/16	25.00	2/8	46.67	14/30	0.550
Age, years	56 (47–64)	16	54.5 (48–58)	8	60 (47–84)	29	0.006*
BMI, kg·m⁻²	23.53 (20.07–33.43)	15	25.82 (20.76–32.00)	8	22.45 (17.18–32.00)	28	0.258
Comorbidities[¶]							
No	81.25	13/16	62.50	5/8	73.33	22/30	
Yes	18.75	3/16	37.50	3/8	26.67	8/30	0.571
Immunosuppressive medication[†]							
No	100.00	15/15	100.00	8/8	57.14	16/28	
Yes	0.00	0/15	0.00	0/8	42.86	12/28	0.001*
Smoking status							
Current	0.00	0/16	75.00	6/8	0.00	0/30	
Former	0.00	0/16	25.00	2/8	96.67	29/30	
Never	100.00	16/16	0.00	0/8	3.33	1/30	<0.001*
Pack-years	0 (0–0)	16	15 (0–35)	7	40 (0–80)	28	<0.001*
GOLD stage							
II/III/IV						2/3/23	
B/C/D						2/3/16	
FEV₁/FVC, %	78.00 (69.00–85.00)	15	81.00 (68.00–85.00)	6	44.50 (29.00–70.00)	24	<0.001*
FEV₁/FVC % pred GLI[§], %	96.40 (86.55–106.86)	15	101.25 (85.12–105.85)	6	55.29 (37.41–88.99)	23	<0.001*

BMI: body mass index; GOLD: Global Initiative for Chronic Obstructive Lung Disease; FEV₁: forced expiratory volume in 1 s; FVC: forced vital capacity; GLI: Global Lung Function Initiative. [#]: differences between groups were tested using Fisher's exact test for categorical variables and the Wilcoxon rank sum test for continuous variables; [¶]: hypertension, myocardial infarction, stroke and/or diabetes; [†]: oral immunosuppressive drugs (e.g. cortisone); [§]: percent predicted values according to GLI [26]. *: p<0.05.

Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep and SepMate tubes according to the manufacturer's instructions (STEMCELL Technologies, Cologne, Germany) and stored in aliquots at -80°C until analysis. Flow cytometry of full EDTA-blood was performed as detailed in the supplementary material.

Activity-based probe labelling

Native protein lysates were extracted from PBMCs with 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 5 mM MgCl_2 , 10% glycerol, 2 mM ATP, 0.05% digitonin and complete protease inhibitor (Roche Diagnostics, Mannheim, Germany). Activity of catalytic subunits was monitored by using activity-based probes (ABPs) as described previously [21].

Native gel analysis and substrate overlay

Native gel analysis and subsequent immunoblotting with an antibody detecting the 20S α 1–7 subunits (ab22674; Abcam, Cambridge, UK) was performed as described previously [22].

PBMC in vitro stimulation

Isolated PBMCs were plated in 24-well plates (2×10^6 cells per well), cultivated in RPMI medium (containing 10% FBS (Biochrom, Berlin, Germany) and $100 \text{ U} \cdot \text{mL}^{-1}$ penicillin/streptomycin) and treated with or without $75 \text{ U} \cdot \text{mL}^{-1}$ interferon (IFN)- γ (Roche) or $1 \mu\text{g} \cdot \text{mL}^{-1}$ lipopolysaccharide (LPS) (Sigma, Darmstadt, Germany) for 24 h. PBMCs were harvested and RNA or proteins were extracted. Immunoproteasome inhibitor LU-005i was kindly provided by Hermen Overkleeft (Leiden University, Leiden, The Netherlands) [23]. At 2 h before LPS stimulation, cells were treated with $0.5 \mu\text{M}$ LU-005i.

Luminescent activity assay

Chymotrypsin-, caspase- and trypsin-like activities were measured with the Proteasome-Glo Assay kit according to the manufacturer's protocol (Promega, Walldorf, Germany) and as described previously [24].

Statistics

All analyses were performed using the statistical software package R version 4.0.3 [25]. Details of the data transformation and regression models are given in the supplementary material. Outliers exceeding $\text{mean} \pm 4\text{SD}$ were excluded from the analyses. Differences between groups (nonsmoker *versus* smoker and control *versus* COPD) were tested using Fisher's exact test for categorical variables and the Mann-Whitney-Wilcoxon rank sum test or Kruskal-Wallis rank sum test for continuous variables. Reference equations for spirometry according to the Global Lung Function Initiative were applied to calculate percentage predicted values of forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) [26]. A p-value < 0.05 was used to indicate statistical significance.

Results

To study immunoproteasome function in peripheral blood in detail, we used a two-armed study design reflecting the extremes of the control and disease groups: the first arm included analysis of healthy male current smokers and never-smokers aged 20–30 years (table 1), and the second arm contained mainly end-stage COPD patients and lung healthy controls aged 47–84 years, including 16 never-smokers, two former smokers and six current smokers (table 2). The control groups were not overlapping and were age-matched to the respective study arms.

Activation of immunoproteasome in peripheral blood cells of young smokers

The first study arm evaluated the effect of cigarette smoke exposure on proteasome function in PBMCs of young male smokers (table 1). Current tobacco smoking was confirmed by elevated levels of the metabolic byproduct of nicotine, cotinine, in the blood plasma of smokers (table 1). Flow cytometry analysis of blood cells revealed a significant increase in the absolute number of all analysed immune cell types in smokers compared with the never-smoking control group (supplementary figure S1a). However, the relative cellular composition of monocytes and leukocytes was not altered between smokers and nonsmokers (supplementary figure S1b and supplementary table S2). Proteasome activity of blood mononuclear cells was analysed using two distinct methods. First, we assessed the number of active standard and immunoproteasome complexes using specific ABPs [27]. These ABPs covalently bind to and label catalytically active proteasome subunits which are then identified according to their molecular weight in denaturing sodium dodecyl sulfate gels [27]. A set of three ABPs was used to differentiate the three standard catalytic subunits of the proteasome β 1, β 2 and β 5 and the immunoproteasome sites LMP2, MECL-1 and LMP7 (figure 1b). ABP labelling confirmed that the immunoproteasome is the predominant type of proteasome in PBMCs (figure 2a) [28]. The catalytic activity in PBMCs was largely preserved upon tobacco smoke consumption (figure 2a). β 5 activity was almost below the level of detection in

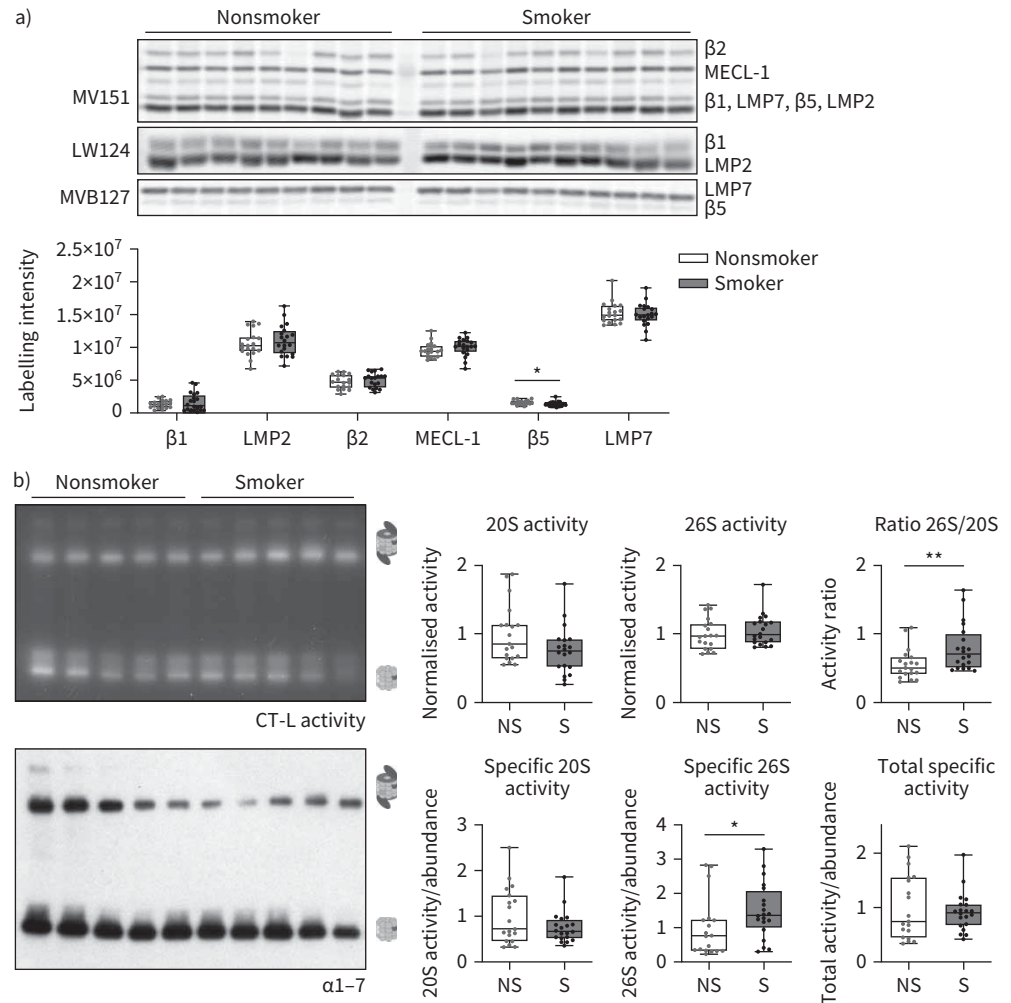


FIGURE 2 Proteasome activity profiling in peripheral blood mononuclear cells (PBMCs) of young healthy smokers and nonsmokers. **a)** Activity-based probe (ABP) analysis of proteasome activity in PBMCs of smokers (n=20) and nonsmokers (n=18-19) with signal quantification (labelling intensity) using the pan-reactive MV151, the β1- and LMP2-specific LW124, and the β5- and LMP7-specific MVB127 ABPs. All samples were run on one large gel to allow direct comparison of signals. **b)** Native gel analysis of native protein lysates of PBMCs of smokers (S) (n=20) and nonsmokers (NS) (n=19) with fluorescent activity assay for the chymotrypsin-like (CT-L) activity of the proteasome. Subsequent immunoblotting of the native gels using an antibody against the α1-7 subunits of the 20S catalytic core was applied to quantify proteasome complex abundance. Densitometry analysis of the gels is shown for the activities as relative signal intensity. Samples were run on four different gels and each sample was normalised to the mean intensity of the controls. The specific activity is defined as activity/abundance; specifically, the activity signal of the 20S, 26S or sum of both (total activity) divided by the densitometric signal for immunostaining, *i.e.* abundance of the respective complex. Data are presented as median±quartile; whiskers indicate the range. Mann-Whitney U-test: *: p<0.05; **: p<0.01.

isolated PBMCs, as described previously (figure 2a) [29]. In a second approach, we dissected the different proteasome complexes, *i.e.* the 26S and the free 20S proteasomes, using native gel analysis. With this method, the proteasome complexes maintain their activity and can be resolved according to their size [22]. The enzymatic activity of the proteasome complexes was quantified by in-gel degradation of a fluorescently quenched substrate for the chymotrypsin-like activity of the proteasome. Of note, 20S proteasome activity was slightly reduced in smokers compared with never-smokers, while 26S and total proteasome activities were not altered (figure 2b and supplementary figure S2a). This shift in activities between 20S and 26S proteasome complexes increased the ratio of 26S/20S activity in smokers (figure 2b). Blotting of the native gels and immunodetection of the 20S catalytic core allowed us to quantify the amount of proteasome complexes in the PBMC samples [22]. The abundance of 20S and 26S proteasome

complexes was not different between the two groups (supplementary figure S2a). By calculating the activity/abundance ratio, we determined the specific activity of distinct proteasome complexes. The specific activity of the 26S proteasome was significantly elevated in smokers compared with nonsmokers (figure 2b), suggesting that the 26S proteasomes are more active in peripheral blood cells of young healthy smokers. As the overall number of active sites of the proteasome was not grossly altered as determined by our ABP analysis (figure 2a), these data suggest that tobacco smoke exposure in healthy individuals does not increase the expression and amount of proteasome complexes but rather activates the enzymatic activity of the 26S proteasome in the peripheral blood cells. This notion is supported by the comparable RNA expression of multiple proteasomal subunits of the 26S proteasome in PBMCs of smokers and never-smoking males (supplementary figure S2b and supplementary table S2).

Immunoproteasome activation in peripheral immune cells of COPD patients

For our second study arm, we applied native gel proteasome activity profiling to analyse proteasome activity in PBMCs isolated from patients with severe COPD (mainly Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV/D) and compared the results with lung healthy age-matched controls (table 2). Of note, we observed substantial activation of 20S and total proteasome activity in COPD patients (figure 3 and supplementary figure S3a). In addition, the abundance of all proteasome complexes was increased, indicating elevated assembly of both 20S and 26S proteasome complexes in blood immune cells of COPD patients (figure 3). The specific activity (activity/abundance) of the 26S proteasome complex and of total proteasomes, however, was significantly reduced in COPD patients (figure 3). The ratio of the two complexes was not altered (supplementary figure S3b). Increased abundance of immune cell proteasome complexes in COPD patients was confirmed by RNA and protein expression analysis: mRNA expression of several proteasome subunits such as the immunoproteasomal genes PSMB9 (encoding LMP7) and PSMB10 (encoding MECL-1), and the 19S regulatory subunit genes PSMC3 and PSMD11, was significantly elevated in COPD PBMCs (figure 4a). Moreover, mass spectrometry-based protein analysis of PBMCs revealed concerted upregulation of multiple proteasome subunits in COPD patients compared with controls (figure 4b). These expression data thus support the observation that COPD patients assemble more proteasome complexes in their peripheral immune cells, which might be part of an adaptive response to compensate for diminished specific 26S proteasome activity.

Robust activation of 20S immunoproteasomes correlates with reduced lung function in COPD

To determine whether the changes in proteasome complexes correlate with altered lung function of COPD patients, we performed correlation analyses for proteasome function and FEV₁/FVC impairment (figure 5a and supplementary figure S3c). Of note, we observed a statistically significant negative correlation of 20S activity as well as 20S, 26S and total abundance with FEV₁/FVC % pred, while the specific 26S and total activities correlated positively with this lung function parameter (figure 5a). These data indicate that patients with severe lung function alterations have higher levels of both 20S and 26S proteasome complexes in their PBMCs. This is associated with a higher activity of 20S immunoproteasomes but not of 26S proteasome complexes. Rather contrary, the 26S and total specific activities, *i.e.* the activity per complex, are higher in blood immune cells of patients with better lung function. These data demonstrate a complex change in immunoproteasome function in peripheral immune cells of COPD patients, which correlates with the degree of lung function impairment.

Altered immunoproteasome activity might be caused by the skewed immune cell composition in the blood of COPD patients. Our flow cytometry analysis revealed an elevated percentage of granulocytes and monocytes but not lymphocytes in our COPD study cohort (supplementary figure S4a and supplementary table S3). These latter two cell types represent the main immune cells present in our PBMC isolates (supplementary figure S4b). Extracted data from the ImmProt database [28] and our own preliminary RNA analysis of sorted blood immune cells (data not shown) indicated that proteasomal protein abundance (copy number) is quite similar in lymphocytes, natural killer cells, plasmacytoid dendritic cells and monocytes at baseline, and does not grossly diverge upon immune cell activation (supplementary figure S5). These data suggest that the amount of proteasomes is rather similar and stable in different immune cell subsets, and might thus not be the underlying cause for the observed changes in proteasome activity in COPD patients. We further validated our data by linear regression modelling where we adjusted for multiple parameters of our study cohort such as age, sex, BMI, comorbidities as well as differential blood composition and immunosuppressive medication (figure 5b–c and supplementary table S4). Of note, these various parameters did not affect the significant activation of 20S activity and 20S abundance as well as activation of total proteasome activity in blood leukocytes of COPD patients (figure 5c). Moreover, we performed sensitivity analysis on the effect of the eight ever-smokers in our control groups. As evident from supplementary table S5, there was no major change in the β estimator and the overall alterations in immunoproteasome function were similar. Activation of the 20S proteasome in peripheral immune cells

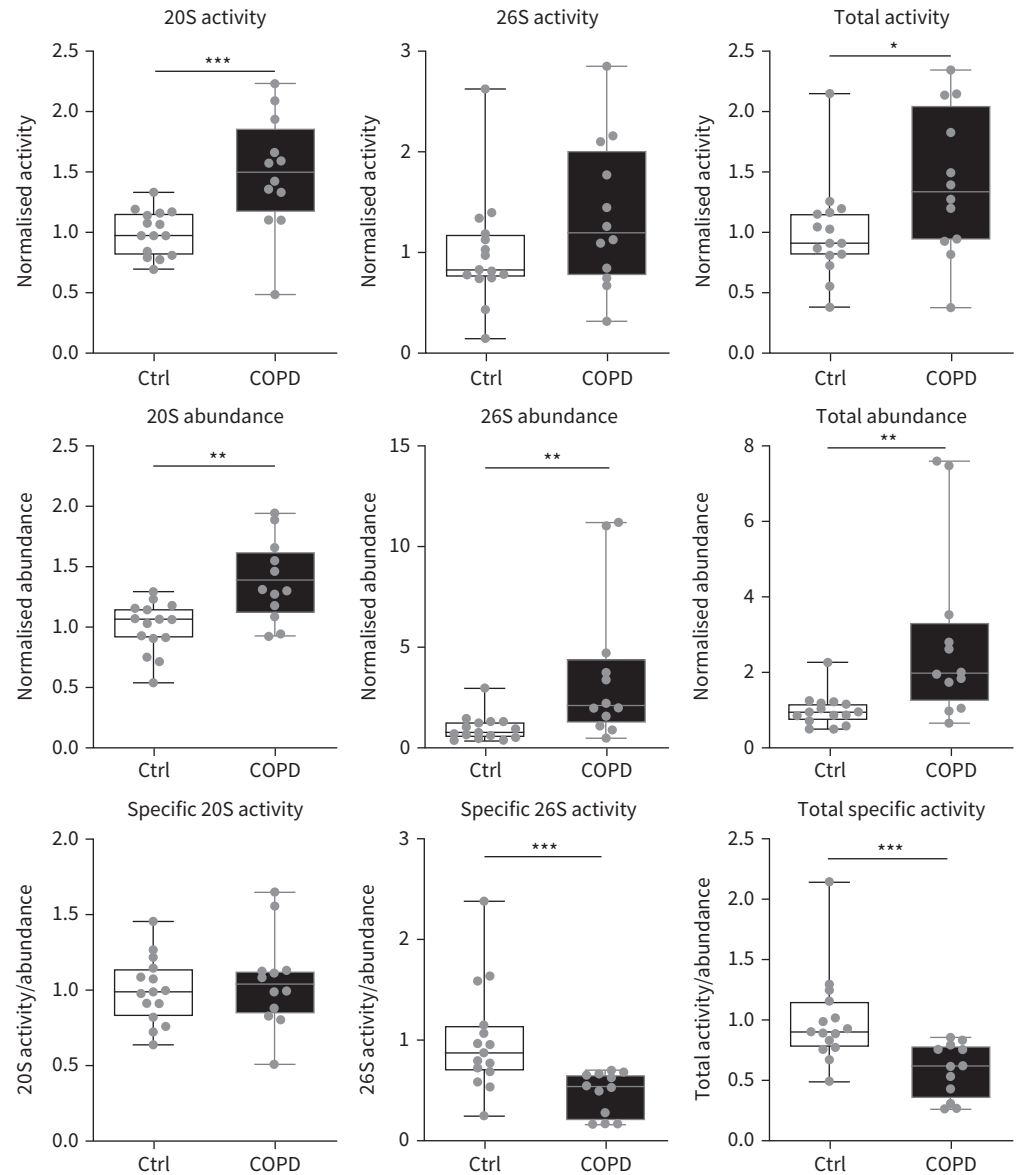


FIGURE 3 Proteasome activity in peripheral blood mononuclear cells (PBMCs) of healthy control subjects and chronic obstructive pulmonary disease (COPD) patients. Native gel analysis of PBMC native protein lysates of healthy control (Ctrl) subjects (n=15: n=9 never-smokers, n=2 former smokers and n=4 current smokers) and COPD patients (n=12) with fluorescent activity assay for the chymotrypsin-like activity of the proteasome and subsequent immunoblotting of the native gel using an antibody against the α 1-7 subunits for detection of proteasome complex abundance (supplementary figure S3a). Samples were run on four different gels and each sample was normalised to the mean intensity of the controls. Densitometry analysis of the gels is shown as normalised signal to the mean of the controls. The specific activity is given as the ratio of activity per abundance. Statistical analysis: median±quartile; whiskers indicate the range. Mann-Whitney U-test: *: p<0.05; **: p<0.01; ***: p<0.001.

can thus be regarded as a robust feature of patients with severe COPD and unrelated to the smoking status. In contrast, 26S proteasome function appears to be less robust and regulated by additional factors (Model 6). This finding requires further analysis.

Inflammatory immunoproteasome regulation in COPD patients

To further investigate whether the activation of the immune cell proteasomes in COPD patients extends to activated immune cells, we challenged freshly isolated PBMC samples from healthy donors and severe

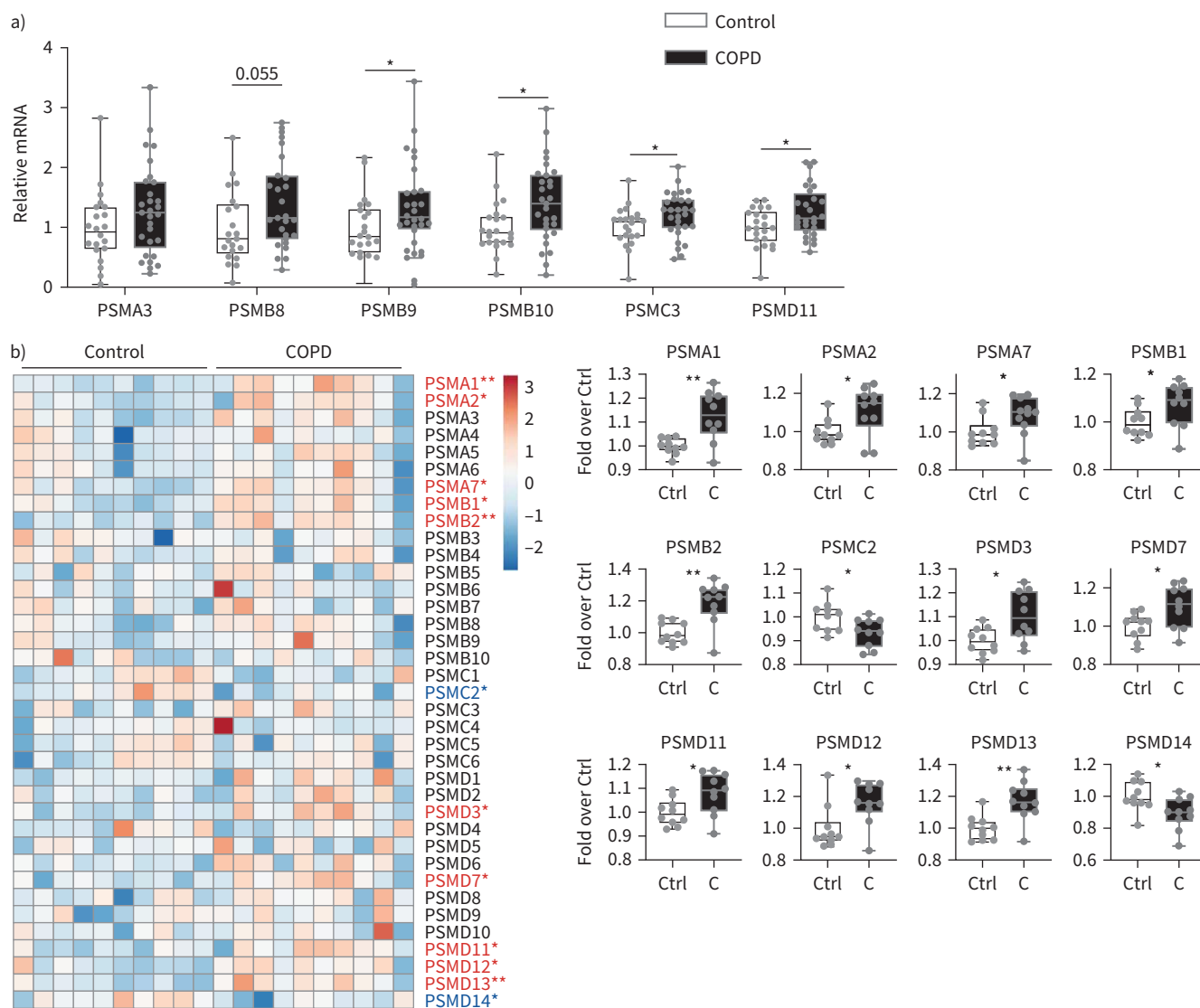


FIGURE 4 Profiling of proteasome expression in peripheral blood mononuclear cells (PBMCs) of healthy control subjects and chronic obstructive pulmonary disease (COPD) patients. **a)** Quantitative reverse transcriptase-PCR analysis of the α -subunit PSMA3, immunoproteasome subunits PSMB8–10, and 19S subunits PSMC3 and PSMD11 in PBMCs of healthy controls ($n=21$ – 22) and COPD patients ($n=27$ – 29). RPL19 and HPRT were used as housekeeping genes. Fold change over control is shown. **b)** Mass spectrometry proteomic analysis of total proteins in PBMCs of COPD (C) patients ($n=10$) and control (Ctrl) subjects ($n=10$) with a heatmap of all 20S (PSMA and PSMB) and 19S subunits (PSMC and PSMD). Significantly upregulated subunits are highlighted in red, significantly downregulated subunits are highlighted in blue. Data are presented as median \pm quartile; whiskers indicate the range. Mann–Whitney U-test: *; $p<0.05$; **; $p<0.01$.

COPD patients (supplementary table S1) with the inflammatory stimuli IFN- γ or LPS for 24 h and analysed proteasome activity. We tested for the three main activities of the proteasome, *i.e.* chymotrypsin-, caspase- and trypsin-like proteasome activity, using a luminogenic substrate assay. Of note, all three activities significantly increased in COPD patients upon stimulation of PBMCs with IFN- γ (figure 6a) but were less strongly activated by LPS (figure 6b) compared with healthy controls. These data demonstrate inflammatory immunoproteasome activation in COPD patients. In an exploratory analysis, we next investigated whether the inhibition of the immunoproteasome affects LPS-induced inflammatory cytokine expression. For that, we pre-treated PBMCs isolated from controls or COPD patients with the specific immunoproteasome inhibitor LU-005i [23] for 2 h before LPS stimulation for 24 h and then assessed inflammatory cytokine expression on the RNA level. After 24 h of LPS stimulation, the immunoproteasome was still effectively inhibited as evidenced both by Western blot-based detection of mass-shifted LMP2 and LMP7 subunits upon covalent binding of the inhibitor (supplementary figure S6a)

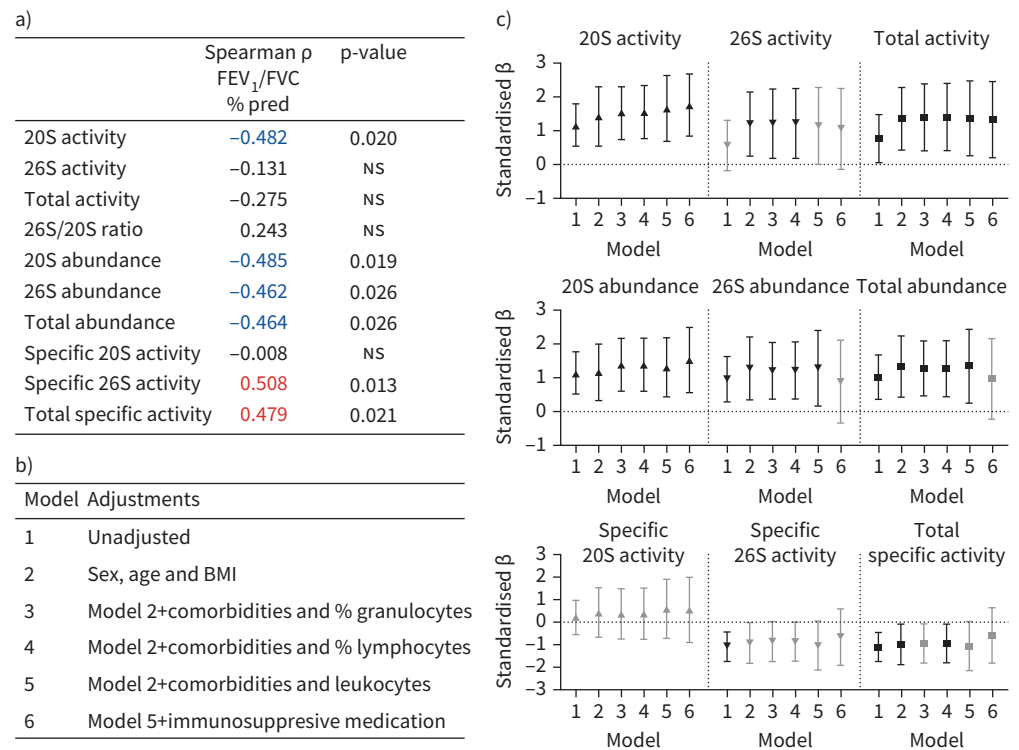


FIGURE 5 Correlation and linear regression analysis of the proteasome complex with selected clinical parameters. **a)** Spearman correlations of lung function (forced expiratory volume in 1 s (FEV₁)/forced vital capacity (FVC) (FEV₁/FVC) % pred [26]) with the proteasome complex parameters activity, abundance and specific activity (activity/abundance). Significant correlations are highlighted in blue (negative correlation) and red (positive correlation). **b, c)** Parameters of the proteasome complex analysis were adjusted for sex, age and body mass index (BMI) (Model 2), additionally comorbidities and percentage granulocytes (Model 3), percentage lymphocytes (Model 4) or the number of leukocytes per μ L blood (Model 5) and also for immunosuppressive medication (Model 6). Model 1 shows the unadjusted values. Data were standardised prior to calculation of the regression coefficient β (with 95% confidence intervals). Significant regression estimates are depicted in black, nonsignificant regression estimates are depicted in grey. See supplementary table S4 for the corresponding values.

and chemiluminescent activity assays (supplementary figure S6b). LPS-induced transcriptional activation of interleukin (IL)-1B, IL-6 and IL-8 was clearly attenuated by immunoproteasome inhibition, while IL-10 was upregulated by immunoproteasome inhibition (figure 6c). This was most prominent in COPD patients where LPS stimulation strongly activated the expression of these inflammatory cytokines. These data thus provide first proof-of-concept evidence for a potential beneficial effect of therapeutic immunoproteasome inhibition on inflammatory cytokine expression in COPD.

Discussion

In this study, we show that the proteasome is distinctly activated in peripheral blood cells of young smokers and in patients with severe COPD. Activation of the 20S immunoproteasome correlates with lung function impairment. Moreover, inflammatory stimuli alter immunoproteasome activation in COPD patients and inflammatory cytokine expression is attenuated by immunoproteasome inhibition *in vitro*. This study thus presents the first evidence for systemic activation of the immunoproteasome in peripheral blood cells of severely ill COPD patients. Given the key role of the immunoproteasome for immune cell activation and autoimmune responses [9, 12], our data suggest that specific inhibition of the immunoproteasome might represent a novel therapeutic concept for COPD treatment.

Regulation of the immunoproteasome by cigarette smoke and in COPD

We and others previously demonstrated inhibition of the proteasome by cigarette smoke *in vitro*, *in vivo* and in explanted lungs of severely ill COPD patients [14–18]. Impaired protein degradation by the

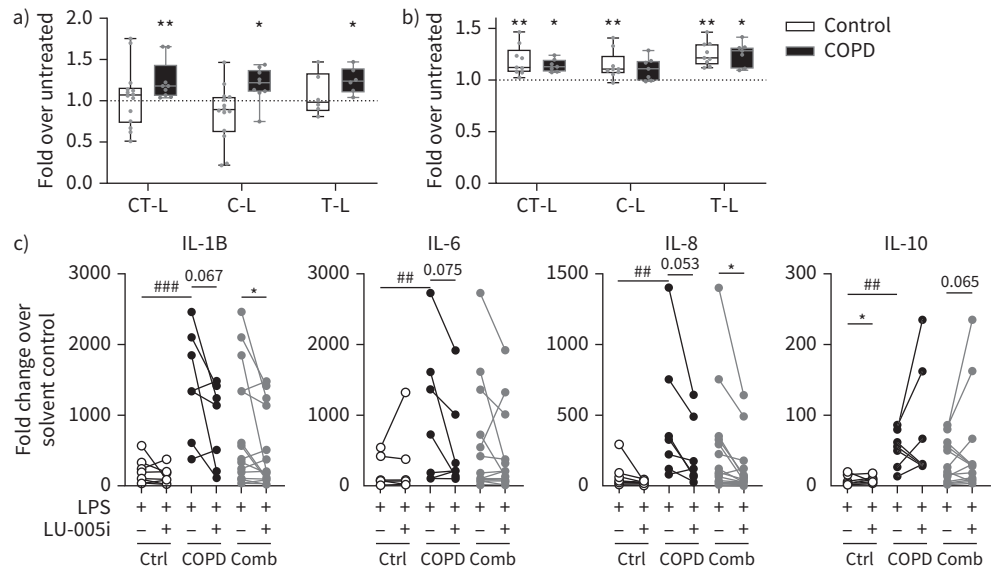


FIGURE 6 Alterations on proteasome activities upon interferon (IFN)- γ or lipopolysaccharide (LPS) treatment of peripheral blood mononuclear cells (PBMCs) isolated from healthy controls and chronic obstructive pulmonary disease (COPD) patients. PBMCs of healthy controls ($n=6-14$) and COPD patients ($n=6-10$) were treated with **a**) $75 \text{ U}\cdot\text{mL}^{-1}$ IFN- γ or **b**) $1 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ LPS for 24 h. Proteasome chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) activities were analysed using chemiluminescent substrates specific for the respective activities and are shown as fold over the respective untreated control. **c**) mRNA expression of the cytokines interleukin (IL)-1B, IL-6, IL-8 and IL-10 was measured in PBMCs of healthy controls ($n=9$) or COPD patients ($n=7$) treated with/without the immunoproteasome inhibitor LU-005i ($0.5 \text{ }\mu\text{M}$, 2 h pre-treatment) and LPS ($1 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$, 24 h). RPL19 and HPRT were used as housekeeping genes. Fold change over solvent control in the control (Ctrl) and COPD groups and with all samples combined (Comb) is shown. **a**, **b**) Data are presented as median \pm quartile; whiskers indicate the range. One sample Wilcoxon test: *: $p<0.05$; **: $p<0.01$. **c**) Paired t-test within groups: *: $p<0.05$. Mann-Whitney U-test for difference between LPS-treated control and COPD groups: ##: $p<0.01$; ###: $p<0.001$.

proteasome contributes to the accumulation of damaged proteins and augmented protein stress in lung cells as also demonstrated for neurodegenerative and cardiovascular diseases [30–33].

Here, we show activation of the immunoproteasome, a specialised type of immune cell proteasome, in peripheral blood cells of healthy young smokers and severely ill COPD patients. Our sophisticated native gel analysis allowed us to dissect proteasome activities of distinct 20S and 26S complexes, which are well known to be differentially regulated [34, 35]. In young smokers, the specific activity of the 26S proteasome was significantly increased, while overall proteasome expression and activity were not grossly altered. Activation of the 26S proteasome, which degrades ubiquitinated proteins, might be part of an adaptive response to adjust proteasome function to an increased protein turnover [34, 36]. This finding is supported by experimental data from chronically smoke-exposed mice, where proteasome activity and expression were increased in the mouse lung [16, 37].

In peripheral blood cells of COPD patients, however, assembly and activity of the 20S proteasome complexes were activated. Despite the small size of the study population, the effect was robust even when adjusting for various parameters such as sex, age, BMI, comorbidities, differential blood cell count and immunosuppressive medication. Importantly, elevated 20S immunoproteasome activity in the peripheral blood cells of COPD patients correlated significantly with the extent of lung function impairment. These data are well in line with the established concept that the 20S proteasome is activated upon severe oxidative stress to enable ubiquitin-independent degradation of oxidatively modified and damaged proteins [36, 38]. Induction of the immunoproteasome is also part of a conserved protective response to oxidative stress [39, 40]. Increased assembly and activity of the proteasome is most likely due to transcriptional activation as we observed elevated mRNA and protein levels in peripheral immune cells of COPD patients. We cannot rule out, however, that the increased levels of 20S proteasomes also involve disassembly of the 26S proteasome in COPD patients. Dissociation of the 26S into its 19S and 20S subcomplexes takes place

in response to oxidative stress [41, 42]. Accordingly, we have previously shown that the 26S proteasome becomes unstable in cells and lungs exposed to cigarette smoke [17]. The abundance of 26S proteasome complexes was elevated in COPD patients together with increased protein levels of 19S and 20S subunits. 26S proteasome activity, however, was not equally elevated, but the specific 26S activity was reduced instead. We speculate that 26S assembly is activated as a frustrated attempt of the immune cells to compensate for the loss of 26S proteasome activity in immune cells of COPD patients. The activation of immunoproteasome function in peripheral blood immune cells observed here is in contrast to the previously described inhibition of proteasome activity in lung tissue of COPD patients. This discrepancy might be resolved by the short-lived nature of peripheral immune cells, which prevents sustained accumulation of oxidative damage but favours acute adaptation to oxidative and inflammatory stress in COPD. Moreover, we speculate that there is an exposure dose or damage-related threshold for proteasome function, which will either allow adaptive activation or detrimental inhibition depending on the duration and extent of damage. This concept accords with homeostatic regulation circuits that are common for cellular stress responses [43] and reflects the complexity of protein quality control in the cell.

Our present study is limited to the analysis of severe cases of COPD but provides first proof-of-concept evidence for disease-related regulation of the immunoproteasome in peripheral blood cells. Of note, altered immunoproteasome activity in blood immune cells of severe COPD patients might represent a potential circulating biomarker for COPD severity, disease progression and exacerbation frequency. The analysis of large cohorts with longitudinal data from COPD patients of different GOLD stages and lung healthy control samples, which we are currently pursuing, will deliver the required statistical power to test our biomarker hypothesis. This approach may also allow us to delineate whether the activation of the immunoproteasome is an epiphenomenon of severe COPD or an early event and related to COPD disease severity.

Therapeutic targeting of the immunoproteasome in COPD

Here, we describe a novel role for the immunoproteasome in COPD patients. The immunoproteasome was activated in PBMCs isolated from severely ill COPD patients compared with healthy controls. Moreover, it was further activated upon *ex vivo* stimulation of PBMCs with the inflammatory cytokine IFN- γ or LPS. Importantly, inhibition of the immunoproteasome with the specific inhibitor LU-005i attenuated LPS-induced expression of pro-inflammatory cytokines such as IL-1B, IL-6 and IL-8 from isolated PBMCs *in vitro*. In contrast, expression of IL-10 was activated by immunoproteasome inhibition, which may contribute to protective immune regulation by this key anti-inflammatory cytokine [44]. One may speculate that therapeutic application of immunoproteasome inhibitors may thus contribute to the restoration of the dysfunctional immune system in COPD. Our data are well in line with studies showing regulation of pro-inflammatory cytokine secretion such as IL-6, IFN- γ and tumour necrosis factor- α upon immunoproteasome inhibition [45–48]. Immunoproteasome function also shapes dendritic cell programmes and controls T- and B-cell differentiation [10]. In particular, immunoproteasome activity is crucial for the differentiation and function of T-helper (Th) cell lineages, namely Th1 and Th17 differentiation [49]. Specific inhibition of the immunoproteasome revealed an extended function for immunoproteasomes in autoimmunity with suppression of pro-inflammatory cytokine secretion, plasma cell-mediated antibody production and Th17 differentiation [10, 45, 46, 50]. Accordingly, immunoproteasome inhibitors are currently being tested in clinical trials for the treatment of autoimmune polymyositis and lupus nephritis [12, 13]. Given the crucial role of chronic inflammatory signalling in the propagation of COPD as a systemic disease [4, 51], the prominent role of T-regulatory cell Th1/Th17 function [52, 53] and the potential involvement of autoimmune responses in COPD [54], therapeutic targeting of the immunoproteasome might represent a novel therapeutic concept for COPD. Our *in vitro* data on the reduced activation of inflammatory cytokines from circulating blood mononuclear cells suggest a beneficial systemic effect of immunoproteasome inhibition that may diminish pro-inflammatory signalling in COPD lungs and attenuate disease progression.

Acknowledgements: We gratefully acknowledge the provision of human biomaterial and clinical data from the CPC-M bioArchive and its partners at the Asklepios Biobank Gauting, the Klinikum der Universität München and the Ludwig-Maximilians-Universität München. We are thankful for the excellent support by Patricia Sklarek from the CPC (Munich, Germany) outpatient unit.

Author contributions: I.E. Kammerl, S. Hardy and S. Meiners conception and design of research. M. Frankenberger, K. Milger, A. Koch, C. Pilette and J. Behr provided (clinical) samples and reagents. I.E. Kammerl, S. Hardy, Y. Wang, A. Urmann and J. Peierl performed experiments. I.E. Kammerl, S. Hardy, Y. Wang, O. Vosyka, S.M. Hauck, J. Merl-Pham, C. Flexeder and S. Meiners analysed data. I.E. Kammerl, S. Hardy, H. Schulz and S. Meiners interpreted results. I.E. Kammerl and C. Flexeder prepared the tables and figures. I.E. Kammerl, S. Hardy,

C. Flexeder, H. Schulz and S. Meiners drafted the manuscript. I.E. Kammerl and S. Meiners edited and revised the manuscript. All authors approved the final version.

Conflict of interest: I.E. Kammerl has nothing to disclose. S. Hardy has nothing to disclose. C. Flexeder has nothing to disclose. A. Urmann has nothing to disclose. J. Peierl has nothing to disclose. Y. Wang has nothing to disclose. O. Vosyka has nothing to disclose. M. Frankenberger has nothing to disclose. K. Milger reports personal fees for lectures from AstraZeneca, BerlinChemie, GSK, Novartis and Sanofi, outside the submitted work. J. Behr has nothing to disclose. A. Koch has nothing to disclose. J. Merl-Pham has nothing to disclose. S.M. Hauck has nothing to disclose. C. Pilette has nothing to disclose. H. Schulz has nothing to disclose. S. Meiners has nothing to disclose.

Support statement: The research described in this manuscript was funded by the Helmholtz Zentrum München and the Else-Kröner Fresenius Stiftung (2016_A22). S. Hardy was funded by the Belgian Respiratory Society, the German Academic Exchange Service and the Université Catholique de Louvain (ERASMUS grant). Y. Wang was funded by the Chinese Guangzhou Elite Scholarship Council. Funding information for this article has been deposited with the Crossref Funder Registry.

References

- 1 Decramer M, Janssens W, Miravittles M. Chronic obstructive pulmonary disease. *Lancet* 2012; 379: 1341–1351.
- 2 Postma DS, Bush A, van den Berge M. Risk factors and early origins of chronic obstructive pulmonary disease. *Lancet* 2015; 385: 899–909.
- 3 Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 1985; 64: 111–126.
- 4 Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 2011; 378: 1015–1026.
- 5 Caramori G, Casolari P, Barczyk A, et al. COPD immunopathology. *Semin Immunopathol* 2016; 38: 497–515.
- 6 Schmidt M, Finley D. Regulation of proteasome activity in health and disease. *Biochim Biophys Acta* 2014; 1843: 13–25.
- 7 Coux O, Zieba BA, Meiners S. The proteasome system in health and disease. *Adv Exp Med Biol* 2020; 1233: 55–100.
- 8 Basler M, Kirk CJ, Groettrup M. The immunoproteasome in antigen processing and other immunological functions. *Curr Opin Immunol* 2013; 25: 74–80.
- 9 Kammerl IE, Meiners S. Proteasome function shapes innate and adaptive immune responses. *Am J Physiol Lung Cell Mol Physiol* 2016; 311: L328–L336.
- 10 Groettrup M, Kirk CJ, Basler M. Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol* 2010; 10: 73–78.
- 11 Beling A, Kespohl M. Proteasomal protein degradation: adaptation of cellular proteolysis with impact on virus- and cytokine-mediated damage of heart tissue during myocarditis. *Front Immunol* 2018; 9: 2620.
- 12 Basler M, Mundt S, Bitzer A, et al. The immunoproteasome: a novel drug target for autoimmune diseases. *Clin Exp Rheumatol* 2015; 33: Suppl. 92, S74–S79.
- 13 Ettari R, Zappalà M, Grasso S, et al. Immunoproteasome-selective and non-selective inhibitors: a promising approach for the treatment of multiple myeloma. *Pharmacol Ther* 2018; 182: 176–192.
- 14 van Rijt SH, Keller IE, John G, et al. Acute cigarette smoke exposure impairs proteasome function in the lung. *Am J Physiol Lung Cell Mol Physiol* 2012; 303: L814–L823.
- 15 Somborac-Bacura A, van der Toorn M, Franciosi L, et al. Cigarette smoke induces endoplasmic reticulum stress response and proteasomal dysfunction in human alveolar epithelial cells. *Exp Physiol* 2013; 98: 316–325.
- 16 Kammerl IE, Dann A, Mossina A, et al. Impairment of immunoproteasome function by cigarette smoke and in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2016; 193: 1230–1241.
- 17 Kammerl IE, Caniard A, Merl-Pham J, et al. Dissecting the molecular effects of cigarette smoke on proteasome function. *J Proteomics* 2019; 193: 1–9.
- 18 Baker TA, Bach HH, Gamelli RL, et al. Proteasomes in lungs from organ donors and patients with end-stage pulmonary diseases. *Physiol Res* 2014; 63: 311–319.
- 19 Min T, Bodas M, Mazur S, et al. Critical role of proteostasis-imbalance in pathogenesis of COPD and severe emphysema. *J Mol Med* 2011; 89: 577–593.
- 20 Donnelly LE. Could protecting the immunoproteasome reduce exacerbations in chronic obstructive pulmonary disease? *Am J Respir Crit Care Med* 2016; 193: 1188–1190.
- 21 Keller IE, Vosyka O, Takenaka S, et al. Regulation of immunoproteasome function in the lung. *Sci Rep* 2015; 5: 10230.
- 22 Yazgılı AS, Meul T, Welk V, et al. In-gel proteasome assay to determine the activity, amount, and composition of proteasome complexes from mammalian cells or tissues. *STAR Protoc* 2021; 2: 100526.

- 23 de Bruin G, Huber EM, Xin B-T, *et al.* Structure-based design of either β 1i or β 5i specific inhibitors of human immunoproteasomes. *J Med Chem* 2014; 57: 6197–6209.
- 24 Welk V, Coux O, Kleene V, *et al.* Inhibition of proteasome activity induces formation of alternative proteasome complexes. *J Biol Chem* 2016; 291: 13147–13159.
- 25 R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria, 2020.
- 26 Quanjer PH, Stanojevic S, Cole TJ, *et al.* Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur Respir J* 2012; 40: 1324–1343.
- 27 de Bruin G, Xin BT, Kraus M, *et al.* A set of activity-based probes to visualize human (immuno)proteasome activities. *Angew Chem Int Ed Engl* 2016; 55: 4199–4203.
- 28 Rieckmann JC, Geiger R, Hornburg D, *et al.* Social network architecture of human immune cells unveiled by quantitative proteomics. *Nat Immunol* 2017; 18: 583–593.
- 29 Schmidt C, Berger T, Groettrup M, *et al.* Immunoproteasome inhibition impairs T and B cell activation by restraining ERK signaling and proteostasis. *Front Immunol* 2018; 9: 2386.
- 30 Powers ET, Morimoto RI, Dillin A, *et al.* Biological and chemical approaches to diseases of proteostasis deficiency. *Annu Rev Biochem* 2009; 78: 959–991.
- 31 Bouchecareilh M, Balch WE. Proteostasis: a new therapeutic paradigm for pulmonary disease. *Proc Am Thorac Soc* 2011; 8: 189–195.
- 32 Meiners S, Eickelberg O. What shall we do with the damaged proteins in lung disease? Ask the proteasome! *Eur Respir J* 2012; 40: 1260–1268.
- 33 Wei J, Rahman S, Ayaub EA, *et al.* Protein misfolding and endoplasmic reticulum stress in chronic lung disease. *Chest* 2013; 143: 1098–1105.
- 34 Rousseau A, Bertolotti A. Regulation of proteasome assembly and activity in health and disease. *Nat Rev Mol Cell Biol* 2018; 19: 697–712.
- 35 VerPlank JJS, Lokireddy S, Zhao J, *et al.* 26S Proteasomes are rapidly activated by diverse hormones and physiological states that raise cAMP and cause Rpn6 phosphorylation. *Proc Natl Acad Sci USA* 2019; 116: 4228–4237.
- 36 Wang X, Meul T, Meiners S. Exploring the proteasome system: a novel concept of proteasome inhibition and regulation. *Pharmacol Ther* 2020; 211: 107526.
- 37 Conlon TM, John-Schuster G, Heide D, *et al.* Inhibition of LT β R signalling activates WNT-induced regeneration in lung. *Nature* 2020; 588: 151–156.
- 38 Kumar Deshmukh F, Yaffe D, Olshina MA, *et al.* The contribution of the 20S proteasome to proteostasis. *Biomolecules* 2019; 9: 190.
- 39 Pickering AM, Koop AL, Teoh CY, *et al.* The immunoproteasome, the 20S proteasome and the PA28 $\alpha\beta$ proteasome regulator are oxidative-stress-adaptive proteolytic complexes. *Biochem J* 2010; 432: 585–594.
- 40 Seifert U, Bialy LP, Ebstein F, *et al.* Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. *Cell* 2010; 142: 613–624.
- 41 Wang X, Yen J, Kaiser P, *et al.* Regulation of the 26S proteasome complex during oxidative stress. *Sci Signal* 2010; 3: ra88.
- 42 Livnat-Levanon N, Kevei E, Kleifeld O, *et al.* Reversible 26S proteasome disassembly upon mitochondrial stress. *Cell Rep* 2014; 7: 1371–1380.
- 43 Martins I, Galluzzi L, Kroemer G. Hormesis, cell death and aging. *Aging* 2011; 3: 821–828.
- 44 Ouyang W, Rutz S, Crellin NK, *et al.* Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2011; 29: 71–109.
- 45 Muchamuel T, Basler M, Aujay MA, *et al.* A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 2009; 15: 781–787.
- 46 Basler M, Mundt S, Muchamuel T, *et al.* Inhibition of the immunoproteasome ameliorates experimental autoimmune encephalomyelitis. *EMBO Mol Med* 2014; 6: 226–238.
- 47 Basler M, Claus M, Klawitter M, *et al.* Immunoproteasome inhibition selectively kills human CD14⁺ monocytes and as a result dampens IL-23 secretion. *J Immunol* 2019; 203: 1776–1785.
- 48 Santos RDLA, Bai L, Singh PK, *et al.* Structure of human immunoproteasome with a reversible and noncompetitive inhibitor that selectively inhibits activated lymphocytes. *Nat Commun* 2017; 8: 1692.
- 49 Kalim KW, Basler M, Kirk CJ, *et al.* Immunoproteasome subunit LMP7 deficiency and inhibition suppresses Th1 and Th17 but enhances regulatory T cell differentiation. *J Immunol* 2012; 189: 4182–4193.
- 50 Huber EM, Basler M, Schwab R, *et al.* Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* 2012; 148: 727–738.
- 51 Brightling C, Greening N. Airway inflammation in COPD: progress to precision medicine. *Eur Respir J* 2019; 54: 1900651.
- 52 Le Rouzic O, Pichavant M, Frealle E, *et al.* Th17 cytokines: novel potential therapeutic targets for COPD pathogenesis and exacerbations. *Eur Respir J* 2017; 50: 1602434.
- 53 Xu W, Li R, Sun Y. Increased IFN- γ -producing Th17/Th1 cells and their association with lung function and current smoking status in patients with chronic obstructive pulmonary disease. *BMC Pulm Med* 2019; 19: 137.
- 54 Scambler T, Holbrook J, Savic S, *et al.* Autoinflammatory disease in the lung. *Immunology* 2018; 154: 563–573.