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Targeting Cathepsin C in PR3-ANCA Vasculitis

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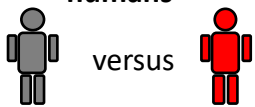
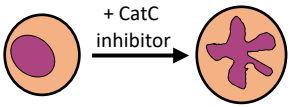
CatC gene-deficiency in humans



Healthy versus Papillon-Lefèvre syndrome

Pharmacological CatC inhibition in human neutrophils



$CD34^{pos}$ stem cell-derived neutrophils

Neutrophil Serine Protease (NSP) zymogens

degradation

CatC

mature, proteolytically active Neutrophil Serine Proteases

Reduction of

- **NSP proteins**
- **NSP proteolytical acticity**
- **PR3-ANCA induced neutrophil activation**
- **glomerular endothelial injury**
- **PR3 autoantigen pool**

Conclusion: Gene-deficiency and pharmacological inhibition of CatC diminishes NSPs, including the ANCA autoantigen PR3. Reducing NSPs together with PR3 autoantigen-dependent effects by CatC inhibition could be explored as adjunctive treatment in PR3-AAV patients.

Significance Statement:

Steroids and immunosuppressive drugs are standard-of-care for treating ANCA vasculitis patients. Cathepsin C (CatC) provides a potential novel treatment target by controlling neutrophil serine proteases (NSPs) in both human neutrophils and monocytes. CatC gene-deficiency in humans and pharmacological CatC inhibition in a human stem-cell model effectively downregulated neutrophil serine proteases (NSPs), including the PR3 autoantigen. Consequently, these neutrophils showed a diminished activation response to PR3- but not MPO-ANCA. Moreover, both genetic and pharmacological NSP-depletion resulted in less neutrophil-induced injury of glomerular microvascular endothelial cells. Pharmacological CatC inhibition is currently tested in patients with inflammatory lung diseases. Our findings may encourage clinical studies with adjunctive CatC inhibitor administration in PR3-AAV patients.

ABSTRACT

Background The ANCA autoantigens proteinase 3 (PR3) and myeloperoxidase (MPO) are exclusively expressed by neutrophils and monocytes. ANCA-mediated activation of these myeloid cells is the key-driver to the vascular injury process and neutrophil serine proteases (NSPs) are disease mediators. NSPs, including the PR3 autoantigen, are activated in its proteolytic function by cathepsin C (CatC) from zymogens. Lack of NSP zymogen activation results in neutrophils with strongly reduced NSP proteins. We explored AAV-relevant consequences of blocking NSP zymogen activation by CatC.

Methods We assessed NSPs and NSP-mediated endothelial cell injury using myeloid cells from CatC-deficient Papillon-Lefèvre syndrome (PLS) patients, pharmacological CatC inhibition in neutrophil-differentiated human hematopoietic stem-cells (HSC), primary human umbilical

vein (HUVEC), and primary glomerular microvascular endothelial cells (glomerular microvascular EC).

Results PLS patients showed strongly reduced NSPs in both neutrophils and monocytes. PLS neutrophils produced a negative PR3-ANCA test, presented less PR3 on the surface of viable and apoptotic cells, and caused significantly less damage in HUVECs. These findings were recapitulated in human stem-cells, where a highly specific CatC inhibitor, but not prednisolone, effectively reduced NSPs without affecting neutrophil differentiation, reduced membrane-PR3, and diminished neutrophil activation upon PR3-ANCA but not MPO-ANCA stimulation. Compared to HC, PLS neutrophils transferred less proteolytically active NSPs to glomerular microvascular EC, the targeted cell type in ANCA-induced necrotizing crescentic glomerulonephritis. Finally, both genetic CatC-deficiency and pharmacological CatC inhibition, but not prednisolone, reduced the neutrophil-induced glomerular microvascular EC damage.

Conclusions Our findings may encourage clinical studies with adjunctive CatC inhibitor administration in PR3-AAV patients.

INTRODUCTION

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is a systemic autoimmune disease that frequently affects the kidneys, manifesting as necrotizing crescentic glomerulonephritis (NCGN).[1, 2] The major ANCA autoantigens proteinase 3 (PR3) and myeloperoxidase (MPO) are exclusively expressed by neutrophils and monocytes.[2-5] ANCA binding to their target antigens on the cell surface initiates cell activation subsequently resulting in endothelial injury and necrotizing vasculitis.[6] Current immunosuppressive treatments improved clinical outcomes but are associated with significant side effects.[7, 8] Characterization and pharmacological targeting of AAV disease mediators may lead to more disease-specific treatments. The neutrophil serine protease (NSP) family, consisting of PR3, neutrophil elastase (NE), cathepsin G (CatG), and low-abundant neutrophil serine protease 4 (NSP4), provides a potential candidate for a druggable disease mediator.[9] Several AAV-relevant NSP actions have been identified, including NSP transfer to and injury of endothelial cells (EC)[10], promotion of receptor-interacting protein kinase (RIPK1/3)-dependent neutrophil extracellular trap (NET) formation[11-13] and monocyte and neutrophil IL-1β generation[14]. PR3 is a unique NSP because it acts not only as a serine protease, but also as a disease-driving autoantigen in PR3-AAV. PR3 on the neutrophil and monocyte membrane (mPR3) binds PR3-ANCA thereby initiating cell activation and PR3 on the surface of apoptotic neutrophils promotes proinflammatory efferocytosis and PR3 autoimmunity with Th17 polarization in the presence of PR3-ANCA.[15] Thus, some NSP-mediated disease mechanisms pertain to both PR3- and MPO-AAV, whereas others have additional implications specifically in PR3-AAV.

Cathepsin C (CatC) is a highly conserved lysosomal cysteine dipeptidyl aminopeptidase that proteolytically activates NSPs zymogens during hematopoietic stem cell (HSC) differentiation in the bone marrow (BM).[16] We observed previously that CatC gene-deficient

mice were protected from AAV.[14] CatC loss-of-function mutations in humans cause rare autosomal-recessive Papillon-Lefèvre syndrome (PLS, OMIM:245000) affecting 1-to-4 people per million.[17, 18] PLS patients feature periodontitis, plantar, and palmar hyperkeratosis but do not show marked immunodeficiency despite NSP depletion. Moreover, blood neutrophils from PLS patients were found to be fully capable of bactericidal activity against *S. aureus* and *K. pneumoniae* in vitro indicating that NSPs are not the only tool to clear common bacterial pathogens.[12] Pharmacological CatC inhibition is currently tested in patients with NSP-mediated diseases, such as bronchiectasis.[19] We explored AAV-relevant consequences of CatC deficiency in humans using cells from PLS patients and developed and tested a highly specific pharmacological CatC inhibitor in a human stem cell model.

METHODS

Preparation of human neutrophils and monocytes

Blood from healthy human controls (HC), PLS patients, and active PR3-AAV patients were obtained after approval by the Charité University and after written informed consent (Ethics vote EA4/025/18). The two PLS patients were treated by P.E. and K.N. and showed the typical palmoplantar hyperkeratosis and aggressive periodontitis resulting in premature tooth loss. Clinical characteristics of PLS patient 1 (PLS1) were described[20] as well as the compound heterozygous *CatC* gene mutations in exon 7 (c.947T>G and c.1268G>C, family 2).[21] Clinical symptoms of PLS2 were reported[22] and a frame shift mutation was found in exon 4 of the *CatC* gene (c.566-572Del, family 9).[21]

Neutrophils were isolated from heparinized whole blood using dextran sedimentation and density gradient centrifugation as described previously.[23] Cells were >95% neutrophils as determined by morphological analysis and >99% viable by Trypan blue dye exclusion. For the

preparation of a monocyte-enriched cell population, monocytes from the interphase were positively sorted with anti-CD14 magnetic beads on LD columns according to the manufacturer's instructions (Miltenyi, Bergisch-Gladbach, Germany).

Sorting algorithm for neutrophils and monocytes from healthy controls

After density gradient centrifugation and red blood cell lysis, neutrophils were negatively MACS sorted using a cocktail of biotinylated antibodies against CD3, CD19, CD36, CD49d, CD56, CD235a, followed by incubation with anti-biotin magnetic beads (all from Miltenyi). The resulting population contained >99% pure $CD16^{pos}$ neutrophils. After density gradient centrifugation, monocytes from the interphase were FACS sorted (FACSAria II cell sorter, BD Biosciences, San Diego, CA) using light scatter characteristics and cell staining with antibodies to CD11b (Biolegend, San Jose, CA), CD15 and CD14 (both BD Biosciences), respectively. The resulting population contained approximately 99% pure $CD14^{hi}$ monocytes.

Preparation of human IgG

Normal- and ANCA-IgG were prepared from HC and patients with active MPO- and PR3-ANCA disease using a High-Trap-protein-G column in an Äkta-FPLC system (Cytiva Europe GmbH, Freiburg, Germany).

Cathepsin C inhibitor

The small molecule cathepsin C inhibitor BI-9740 was obtained via Boehringer Ingelheim´s open innovation platform OpnMe (https://opnme.com). BI-9740 inhibits human CatC with an IC50 of 2 nM and mouse CatC with an IC50 of 0.8 nM. It is highly selective (> 1000 fold) for CatC versus the other Cathepsins (B, F, H, K, L, S) and highly potent in reducing NSP

activity *in vivo*. Detailed data on structure potency, selectivity as well as PK profile and *in vivo* activity data can be found under https://opnme.com/sites/default/files/opnMe_M2O_profile_BI-9740.pdf.

Differentiation of human neutrophils from hematopoietic stem cells

Umbilical cord blood was collected (Ethics vote EA4/025/18). Cells were washed and stained using $CD34^{pos}$ progenitor isolation kit (Miltenyi) and sorted on LS column according to the manufacturer's instructions. Cells were expanded in stem span serum-free medium (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin/streptomycin, 100 ng/mL stem cell factor, 20 ng/mL TPO, and 50 ng/mL FLT3-L (PeproTech, London, United Kingdom). Neutrophil differentiation was in RPMI with 10% FCS and 10 ng/mL G-CSF (PeproTech), and either buffer control, 1 µM CatC inhibitor BI-9740 (BI-I), or 100 µM prednisolone for 10 days.

Assessment of EC damage by phalloidin staining and microscopy

Confluent human umbilical vein endothelial cells (HUVECs) or primary glomerular microvascular endothelial cells (glomerular microvascular EC) (Cell Systems, Kirkland, WA) were grown on glass coverslips. Cells were washed and incubated with cell-free supernatants (cf-SN) from resting or 2.5 µM ionophore A23187 (Merck Milipore, Calbiochem, Darmstadt, Germany) stimulated neutrophils, and 10 µM α1-AT (Sigma-Aldrich) for 8 h as indicated. The cf-SN from blood neutrophils was used at a 1:4, and from stem cell-differentiated neutrophils at a 1:2 dilution in basal medium. EC cells were fixed in 4% paraformaldehyde (15 min, RT) and permeabilized in 0.5% Tx-100 (2 min, RT) and actin filaments were stained with phalloidin-Alexa488 and nuclei with DAPI (both Invitrogen). Fluorescence images were acquired using a

Leica DMI6000 B Microscope with 40x objective. EC damage was assessed by quantification of at least five black pixel areas with ImageJ 1.48v software (https://imagej.nih.gov/ij).

Apoptosis assessment in neutrophils and glomerular microvascular EC-by annexin V and 7-AAD staining

2 x 10^6 human neutrophils were kept in RPMI medium supplemented with 10% FCS for 20 h for constitutive apoptosis. For induced apoptosis, neutrophils from differentiated HSC were treated with 25 µM MG-132 (Merck, Darmstadt, Germany) for 20 h. Phosphatidylserine exposure on neutrophils or glomerular microvascular EC that were treated for 16 h with buffer or cf-SN from neutrophils was determined by Annexin V-FITC (BD Biosciences, San Jose, CA) and necrosis by 7-AAD (Merck, Darmstadt, Germany) staining for 15 min at RT.

SDS-PAGE and immunoblotting analysis

Cell lysis, SDS-PAGE, and immunoblot were performed as described previously.[14] In brief, neutrophils or glomerular microvascular EC treated with buffer or cf-SN from neutrophils for 1h were lysed, incubated in loading buffer, separated on a 12% SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membrane, developed with the indicated antibodies, and visualized by an ECL detection system (Super Signal West Dura Extended Duration substrate, Fisher Scientific, Schwerte, Germany) on a Chemi Only Imager (VWR International, Darmstadt, Germany). Antibodies used for immunoblotting were: monoclonal (mab) rabbit anti-human PR3 (1:4.000, Abcam, Cambridge, UK), polyclonal rabbit anti-NE (1:1,000, Abcam), polyclonal goat anti-CatG (1:500) and monoclonal mouse anti-CatC (1:1,000, both Santa Cruz Biotechnology, Dallas, TX, USA), polyclonal rabbit anti-MPO (1:1,000, Merck, Darmstadt, Germany), polyclonal rabbit anti-actin (1:2,000, Cell Signaling

Europe, Frankfurt/Main, Germany). Corresponding secondary horseradish peroxidase conjugated antibodies (1:1,000) were used.

Fluorescence resonance energy transfer (FRET) and NSP-specific proteolytic activity

NSP-specific proteolytic activity was measured as described previously.[24] Briefly, neutrophils or glomerular microvascular EC treated with buffer or cf-SN from neutrophils for 1 h were lysed on ice, soluble fractions were separated from cell debris by centrifugation, and 5 to 30 µg cell lysates in 150 µl HEPES buffer containing 0.02% lauryl maltoside (LM) were incubated with FRET substrate (20 µM final). Fluorescence was measured by plate reader (excitation 320 nm, emission 420 nm, SpectraMax M5, Molecular Devices, CA) and the corresponding Vmax is reported. For the calculation of the assay baseline (auto- and unspecific hydrolysis of FRET substrate) 20 µM α1-antitrypsin (Merck) was added and the corresponding Vmax was subtracted from sample values. The selective FRET substrates for human NSPs were: PR3: 2-Abz-VAD-(nor)V-ADYQ-EDA-Dnp; NE: 2-Abz-APEEIMRRQ-EDA-Dnp; CatG: 2-Abz-EPFWEDQ-EDA-Dnp.

Flow cytometry to assess neutrophil mPR3 and mCD177

For double staining of mPR3 and mCD177, cells were incubated first with 5 µg/ml Alexa488 conjugated anti-PR3 (clone 43-8-3) or corresponding Alexa488-isotype for 15 min on ice. After washing CD177 was assessed with anti-CD177^{PE} (15 min, ice), mabs mouse IgG^{PE} were used as isotype control (both Beckman Coulter, Munich, Germany). For MPO, cells were stained with 5 µg/ml polyclonal rabbit anti-MPO (Merck) or normal rabbit IgG (Santa Cruz Biotechnology) followed by Alexa488-conjugated secondary abs. 10,000 events per sample

were collected using a BD FACS Calibur or a BD FACS CANTO II and analyzed with FlowJo software (TreeStar, Ashland, OR).

ANCA staining by indirect immunofluorescence

Neutrophils were centrifuged on glass slides using a Cytospin Hettich Universal device (Hettich GmbH, Tuttlingen, Germany), and permeabilized in ice-cold 99% ethanol. Slides were stored at -20 °C until use. Slides were incubated with sera from PR3- or MPO-ANCA patients (1:10 diluted in PBS) for 60 min at RT. After washing, cells were stained with Alexa488-conjugated anti-human IgG (1:250, Molecular Probes, Eugene, Oregon, USA) for 60 min at RT in the dark, washed with PBS, and covered with fluoromount (Southern Biotech, Birmingham, AL, USA). Fluorescence images were acquired using a Leica Microscope (Leica DMI6000 B, Wetzlar, Germany) with a 40x objective.

Measurement of superoxide release

Superoxide was measured using superoxide dismutase (SOD)-inhibitable ferricytochrome c (final concentration 50 µM) reduction as described previously.[25] Briefly, 2.5x 10^5 cells/ml were pretreated with cytochalasin B (5 µg/ml, 15 min), primed with TNFα (2 ng/ml, 15 min) before stimulating antibodies (mabs 5 µg/ml, or 75 µg/ml purified IgGs) were added. Assay was performed in 96-well plates at 37 °C, and the absorption of samples with and without SOD (300 U/ml) was scanned repetitively at 550 nm using a Microplate Reader (Molecular Devices). 45 min results are reported.

Statistics

Results are given as means ± SEM. Comparisons were made using ANOVA with post-hoc analysis, comparisons between two groups were done by unpaired t-test using GraphPad Prism8 software. Differences were considered significant at $p<0.05$.

RESULTS

Reduced NSPs in neutrophils and monocytes, and less cell-surface PR3 in PLS patients

We first characterized NSPs in neutrophils and monocytes from two PLS patients. Compared to healthy control (HC) neutrophils, PLS neutrophils lacked CatC protein and showed strongly reduced PR3, NE, and CatG protein and proteolytical activity (Figure 1A). MPO was not affected by CatC gene-deficiency. We extended our observations to monocytes because these cells also contribute to ANCA-induced inflammation and injury[26, 27] and have not been studied in PLS. We first assessed NSPs in highly pure sorted human HC monocytes and neutrophils (Figure 1B). Both cell types contained PR3, NE, and CatG protein, although monocytes harbored less NSPs than neutrophils. Because this sorting approach was not feasible with the limited PLS patient material, we prepared a monocyte-enriched cell population from HC and PLS patient with 80%±5 purity using CD14 magnetic beads. The preparation from the PLS patient showed strongly reduced NSP proteins and proteolytical activity compared to HC cells (Figure 1C).

Because PR3-ANCA binding to mPR3 on the neutrophil and monocyte surface initiates cell activation, we assessed mPR3 on both cell types from HC and PLS patients (Figure 1D). HC neutrophils showed the typical bimodal mPR3 pattern with distinct $CD177^{neg}/mPR3^{lo}$ and $CD177^{pos}/mPR3^{hi}$ populations that is caused by the subset-restricted expression of the PR3-presenting CD177 receptor[28], whereas PLS neutrophils revealed strongly reduced mPR3 on

both CD177 subsets. In contrast to neutrophils, mPR3 staining in HC monocytes was monomodal because these cells lack the neutrophil-specific CD177 receptor. Compared to HC, PLS monocytes showed strongly reduced mPR3.

PLS neutrophils expose less PR3, cause less endothelial injury, and produce a negative ANCA test

We next studied additional AAV-relevant findings in HC and PLS neutrophils. We performed an immunofluorescence (IF) ANCA test as done in clinical medicine using serum from a PR3-ANCA positive AAV patient. The test produced the typical cytoplasmic (c-ANCA) staining pattern with HC neutrophils but was negative on PLS neutrophils (Figure 2A).

We then investigated mPR3 on the surface of apoptotic neutrophils suggested to provide a do-not-eat-me signal thereby promoting inflammatory efferocytosis and PR3 autoimmunity.[15, 29] Culturing HC and PLS neutrophils overnight resulted in constitutive apoptosis indicated by AnnexinV^{pos} neutrophils. The apoptotic PLS neutrophils showed strongly reduced mPR3 compared to HC cells (Figure 2B).

We next produced cell-free supernatants (cf-SN) from resting and activated HC and PLS neutrophils and incubated primary human umbilical vein endothelial cell (HUVEC) monolayers with this material. cf-SN from activated PLS neutrophils contained significantly less proteolytically active NSPs compared to cf-SN from HC neutrophils as exemplified by PR3 (Figure 2C). Proteolytical PR3 activity in cf-SN from HC neutrophils was strongly reduced by α1-antitrypsin (AAT), the major natural NSP inhibitor. HUVEC treatment with cf-SN from activated HC neutrophils profoundly disturbed the actin structure of the EC monolayer and this effect was strongly reduced when cf-SN from PLS neutrophils was used or when cf-SN from HC neutrophils were treated with AAT (Figure 2D). Together, these data indicate that

human CatC gene-deficiency strongly reduced NSPs in neutrophils and monocytes. This effect included less PR3 autoantigen in cells and on their surface. Moreover, CatC gene-deficiency reduced HUVEC injury by activated neutrophils.

Pharmacological CatC inhibition reduces NSPs, neutrophil mPR3, PR3-ANCA induced neutrophil activation, and NSP-dependent EC damage.

Proteolytic NSP maturation by CatC occurs in early neutrophil progenitors in the BM. We tested the potent and highly-selective CatC inhibitor BI-9740 (BI-I) in a human $CD34^{pos}$ HSC model. BI-I treatment did not interfere with neutrophil differentiation over the 10-day differentiation period (Figure 3A) but strongly reduced NSP proteins and their proteolytical activity whereas MPO was not affected (Figure 3B). In contrast, prednisolone that is part of the standard AAV treatment did not reduce NSPs. mPR3 on HSC-derived neutrophils was, similar to human blood neutrophils, largely presented on the $CD177^{pos}$ subset. BI-I treatment reduced mPR3 by 83%±5 (Figure 3C). Consequently, neutrophils differentiated in the presence of the BI-I released significantly less superoxide in response to PR3- but not to MPO-ANCA (Figure 3D). Superoxide reduction by BI-I was approximately 50% but did not achieve levels seen with isotype and normal IgG controls. Conceivably, incomplete reduction was caused by PR3-ANCA binding to residual mPR3. An 18-hour incubation with the proteasome inhibitor MG-132 on differentiation day 10 induced apoptosis in 16%±3 of the cells. BI-I treatment reduced mPR3 on both viable and apoptotic cells (Figure 3E). Finally, cf-SN from activated neutrophils differentiated in the presence of BI-I contained less NSP activity and caused significantly less HUVEC injury than cf-SN from control neutrophils (Figure 3F).

Thus, pharmacological CatC inhibition in a human neutrophil differentiation model recapitulated key findings from CatC gene-deficient PLS patients without affecting the

differentiation process. NSPs were strongly reduced resulting in less neutrophil-mediated endothelial damage. Diminished mPR3 had a functional consequence, namely less neutrophil activation by PR3- but not MPO-ANCA.

Healthy control but not PLS neutrophils transfer NSPs to glomerular microvascular EC and both CatC gene-deficiency and pharmacological CatC inhibition lead to less neutrophil-induced glomerular microvascular EC injury

HUVECs provide a robust primary cell type to study NSP effects, but glomerular microvascular EC are more relevant for ANCA-induced NCGN. We assessed the NSP transfer from neutrophils to glomerular microvascular EC and the subsequent endothelial injury. Incubation of glomerular microvascular EC monolayers with cf-SN from activated HC but not PLS neutrophils led to NSP acquisition by glomerular microvascular EC, exemplified by immunoblotting using a PR3-specific mab (Figure 4A). Importantly, acquired PR3 preserved its proteolytic activity by FRET assay (Figure 4B). Finally, glomerular microvascular EC-treatment with cf-SN from activated HC or HSC-derived neutrophils caused glomerular microvascular EC injury by phalloidin (Figure 4C) and annexinV/7AAD (Figure 4D) staining. In contrast, cell injury was strongly reduced with cf-SN from activated PLS neutrophils or from HSC-derived neutrophils differentiated in the presence of the BI-I. Note that glomerular microvascular EC injury was not diminished when cf-SN from neutrophils differentiated in the presence of prednisolone was used.

DISCUSSION

We explored CatC as a potential AAV treatment target to down-regulate NSPs, including the PR3 autoantigen that drives PR3-AAV. Our study revealed several new findings.

First, human CatC gene-deficiency in PLS patients strongly reduced NSPs in both neutrophils and monocytes resulting in less neutrophil-mediated EC damage. Notably, the PR3 autoantigen on the cell surface of viable and apoptotic neutrophils was diminished. Second, pharmacological CatC inhibition in a human HSC model, but not prednisolone, effectively reduced NSPs without affecting neutrophil differentiation. CatC inhibition recapitulated findings from PLS neutrophils and established diminished neutrophil activation by PR3- but not MPO-ANCA as a functional consequence of mPR3 reduction. Third, CatC gene-deficiency and pharmacological CatC inhibition strongly diminished neutrophil-induced injury of glomerular microvascular EC that are highly relevant to ANCA-induced NCGN.

ANCA-activated neutrophils and monocytes are central to the vascular inflammation and injury process. Activation starts with ANCA binding to its corresponding autoantigen on the cell surface resulting in cell activation and release of mediators that contribute to the vascular damage. Characterization of druggable disease mediators will possibly extend treatment options for AAV patients as recently shown for complement C5a.[30-33] We explored NSPs downregulation by targeting CatC as an additional approach. The rationale was that all NSP family members have mechanistic implications in AAV as proteolytically active serine proteases. Because PR3 is a protease but also provides the autoantigen in PR3-AAV, reducing PR3 may have particular beneficial effects for PR3-AAV patients. Moreover, we showed previously that PR3/NE double gene-deficient mice and CatC gene-deficient mice were protected from NCGN in an MPO-ANCA disease model.[14]

PLS is a rare autosomal-recessive disease characterized by CatC loss-of-function mutations (PLS, OMIM:245000). PLS affects 1-4 people per million featuring periodontitis, palmar and plantar hyperkeratosis but no marked immunodeficiency.[17, 18] We show for the first time that, in addition to PLS neutrophils, PLS monocytes also harbor marginal NSP activity

at best. NSP reduction in PLS resulted in less endothelial damage by activated neutrophils, a hallmark of AAV. CatC deficiency diminished PR3 on viable and apoptotic PLS neutrophils and resulted in a negative PR3-ANCA IF test indicating less PR3-ANCA binding to these neutrophils - an effect that reduces neutrophil activation by PR3- but not MPO-ANCA as we showed in a neutrophil HSC model using the CatC inhibitor that we recently developed.

Potent chemical compounds inhibiting CatC in BM cells are currently explored for NSP downregulation in preclinical models and patients.[16] We developed the highly-selective CatC inhibitor BI-9740 (BI-I) that blocks human CatC activity *in vitro* with an IC_{50} of 1.8 nM, shows more than 1500x selectivity versus the related proteases CatB, F, H, K, L and S, and has no activity against 34 unrelated proteases from different classes up to a concentration of 10 µM (https://opnme.com/molecules/cathepsin-c-inhibitor-bi-9740). We found that BI-I treatment of human $CD34^{pos}$ HSC did not interfere with neutrophil differentiation. This is an important finding because PR3 was initially named myeloblastin and shown to control neutrophil differentiation.[34] Most importantly, BI-I strongly reduced NSP activity in *in vitro* differentiated neutrophils by more than 95%. The fact that CatC controls NSPs but not neutrophil differentiation is supported by genetic and pharmacological data from the literature. Sorensen et al. performed a pulse-chase biosynthesis study using PLS patient's bone-marrow cells.[12] NSP production and sorting in promyelocytes was normal, whereas NSPs were degraded later, before mature neutrophil stages were reached and the cells entered blood circulation. Red blood cells, platelets, leukocytes number and differential counts were normal despite CatC deficiency. In addition, pharmacological CatC inhibition in *ex vivo* cultured human bone-marrow cells reduced NSPs, but again, did not affect neutrophil differentiation.[35] Importantly, NSP reduction was not seen with prednisolone that is part of AAV standard treatment protocols suggesting that CatC inhibition provides an additional therapeutic angle not covered

by current strategies. Pharmacological CatC inhibition recapitulated reduced endothelial injury by activated neutrophils seen with PLS neutrophils. mPR3 was reduced resulting in significantly less superoxide release in response to PR3 but not to MPO-ANCA. Thus, the HSC model extends the PLS data by showing reduced neutrophil activation by PR3-ANCA as a functional consequence of mPR3 reduction. However, the reduction was incomplete, presumably limited by residual mPR3 interacting with PR3-ANCA.

Thus, our studies with human cells demonstrated that CatC controls NSPs in neutrophils and monocytes. This effect includes the PR3 autoantigen in and on the surface of viable and apoptotic neutrophils. Pharmacological CatC inhibition effectively reduced NSPs with implications for both PR3- and MPO-AAV such as endothelial damage. Reduced PR3 autoantigen has additional implications for PR3-AAV such as neutrophil activation by PR3-ANCA. Moreover, less PR3 on apoptotic neutrophils would possibly reduce inflammatory efferocytosis and PR3 autoimmunity.[15]

We reported recently that effective pharmacological NSP abrogation needs sustained CatC inhibition in differentiating neutrophil progenitors in human bone marrow (BM) *in vitro* and in a non-human primate model.[35] Moreover, we found that NSP reduction by pharmacologic CatC inhibition was protective in murine arthritis[36] and lung transplantation[37] models. Unfortunately, no suitable PR3-ANCA vasculitis model is currently available preventing testing CatC inhibition in an appropriate preclinical AAV model. Several attempts failed, including our own approach to generate a human transgenic PR3-AAV model.[38] However, the therapeutic principle of CatC inhibition enters clinical medicine and was recently tested in patients with bronchiectasis. CatC inhibitor administration prolonged time to exacerbation without increasing the risk for severe infections.[19] Additional clinical trials exploring CatC inhibition in bronchiectasis (phase 3, NCT04594369) and in SARS-CoV-2

(NCT04817332) infected patients are underway. These trials will generate more data on both efficacy and potential side effects of pharmacological CatC inhibition in inflammatory diseases.

Together, our study shows that genetic deficiency and pharmacological inhibition of CatC significantly diminished NSPs, including the PR3-ANCA target autoantigen. Conceivably, reducing NSP activity together with PR3 autoantigen-dependent disease mechanisms has additive protective effects in PR3-AAV patients. This state-of-affairs can currently not be directly studied in animal models. However, our findings may encourage clinical studies with adjunctive CatC inhibitor administration in PR3-AAV patients.

Author contributions

R.K. and B.K designed the study; and U.J., C.E., A.R., S.K, M.G. carried out experiments; K.N. and P.E. treated the PLS patients, R.K., B.K., U.J., A.S., C.E., M.G., S.K., P.N. analyzed the data; U.J., S.K., and R.K. generated the figures; R.K. and U.J. drafted the manuscript; all authors revised the paper and approved the final version of the manuscript.

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DICLOSURE STATEMENT

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References:

1. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, et al.: Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker for disease activity in Wegener's granulomatosis. *Lancet,* i**:** 425-429, 1985

2. Falk RJ, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med,* 318**:** 1651-1657, 1988

3. Ludemann J, Utecht B, Gross WL: Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. *J Exp Med,* 171**:** 357-362, 1990

4. Goldschmeding R, van der Schoot CE, ten Bokkel Huinink D, Hack CE, van den Ende ME, Kallenberg CG, et al.: Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. *J Clin Invest,* 84**:** 1577-1587, 1989

5. Gupta SK, Niles JL, McCluskey RT, Arnaout MA: Identity of Wegener's autoantigen (p29) with proteinase 3 and myeloblastin. *Blood,* 76**:** 2162, 1990

6. Kettritz R: How anti-neutrophil cytoplasmic autoantibodies activate neutrophils. *Clin Exp Immunol,* 169**:** 220-228, 2012 10.1111/j.1365-2249.2012.04615.x

7. Little MA, Nightingale P, Verburgh CA, Hauser T, De Groot K, Savage C, et al.: Early mortality in systemic vasculitis: relative contribution of adverse events and active vasculitis. *Ann Rheum Dis*, 2009

8. Flossmann O, Berden A, de Groot K, Hagen C, Harper L, Heijl C, et al.: Long-term patient survival in ANCA-associated vasculitis. *Ann Rheum Dis,* 70**:** 488-494, 2011 10.1136/ard.2010.137778

9. Kettritz R: Neutral serine proteases of neutrophils. *Immunol Rev,* 273**:** 232-248, 2016 10.1111/imr.12441

10. Jerke U, Hernandez DP, Beaudette P, Korkmaz B, Dittmar G, Kettritz R: Neutrophil serine proteases exert proteolytic activity on endothelial cells. *Kidney Int,* 88**:** 764-775, 2015 10.1038/ki.2015.159

11. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A: Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol,* 191**:** 677-691, 2010 10.1083/jcb.201006052

12. Sorensen OE, Clemmensen SN, Dahl SL, Ostergaard O, Heegaard NH, Glenthoj A, et al.: Papillon-Lefevre syndrome patient reveals species-dependent requirements for neutrophil defenses. *J Clin Invest,* 124**:** 4539-4548, 2014 10.1172/JCI76009

13. Schreiber A, Rousselle A, Becker JU, von Massenhausen A, Linkermann A, Kettritz R: Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. *Proc Natl Acad Sci U S A,* 114**:** E9618-E9625, 2017 10.1073/pnas.1708247114

14. Schreiber A, Pham CT, Hu Y, Schneider W, Luft FC, Kettritz R: Neutrophil Serine Proteases Promote IL-1beta Generation and Injury in Necrotizing Crescentic Glomerulonephritis. *Journal of the American Society of Nephrology : JASN,* 23**:** 470-482, 2012 10.1681/ASN.2010080892

15. Millet A, Martin KR, Bonnefoy F, Saas P, Mocek J, Alkan M, et al.: Proteinase 3 on apoptotic cells disrupts immune silencing in autoimmune vasculitis. *J Clin Invest,* 125**:** 4107-4121, 2015 10.1172/JCI78182

16. Korkmaz B, Caughey GH, Chapple I, Gauthier F, Hirschfeld J, Jenne DE, et al.: Therapeutic targeting of cathepsin C: from pathophysiology to treatment. *Pharmacol Ther,* 190**:** 202-236, 2018 10.1016/j.pharmthera.2018.05.011

17. Hart TC, Hart PS, Bowden DW, Michalec MD, Callison SA, Walker SJ, et al.: Mutations of the cathepsin C gene are responsible for Papillon-Lefevre syndrome. *J Med Genet,* 36**:** 881-887, 1999

18. Toomes C, James J, Wood AJ, Wu CL, McCormick D, Lench N, et al.: Loss-of-function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis. *Nat Genet,* 23**:** 421-424, 1999 10.1038/70525

19. Chalmers JD, Haworth CS, Metersky ML, Loebinger MR, Blasi F, Sibila O, et al.: Phase 2 Trial of the DPP-1 Inhibitor Brensocatib in Bronchiectasis. *N Engl J Med,* 383**:** 2127-2137, 2020 10.1056/NEJMoa2021713

20. Eickholz P, Kugel B, Pohl S, Naher H, Staehle HJ: Combined mechanical and antibiotic periodontal therapy in a case of Papillon-Lefevre syndrome. *J Periodontol,* 72**:** 542-549, 2001 10.1902/jop.2001.72.4.542

21. Noack B, Gorgens H, Schacher B, Puklo M, Eickholz P, Hoffmann T, et al.: Functional Cathepsin C mutations cause different Papillon-Lefevre syndrome phenotypes. *J Clin Periodontol,* 35**:** 311-316, 2008 10.1111/j.1600-051X.2008.01201.x

22. Rudiger S, Petersilka G, Flemmig TF: Combined systemic and local antimicrobial therapy of periodontal disease in Papillon-Lefevre syndrome. A report of 4 cases. *J Clin Periodontol,* 26**:** 847-854, 1999

23. von Vietinghoff S, Tunnemann G, Eulenberg C, Wellner M, Cristina Cardoso M, Luft FC, et al.: NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. *Blood,* 109**:** 4487-4493, 2007

24. Korkmaz B, Attucci S, Juliano MA, Kalupov T, Jourdan ML, Juliano L, et al.: Measuring elastase, proteinase 3 and cathepsin G activities at the surface of human neutrophils with fluorescence resonance energy transfer substrates. *Nat Protoc,* 3**:** 991-1000, 2008 10.1038/nprot.2008.63

25. Schreiber A, Luft FC, Kettritz R: Membrane proteinase 3 expression and ANCA-induced neutrophil activation. *Kidney Int,* 65**:** 2172-2183, 2004

26. Popat RJ, Hakki S, Thakker A, Coughlan AM, Watson J, Little MA, et al.: Anti-myeloperoxidase antibodies attenuate the monocyte response to LPS and shape macrophage development. *JCI Insight,* 2**:** e87379, 2017 10.1172/jci.insight.87379

27. Rousselle A, Kettritz R, Schreiber A: Monocytes Promote Crescent Formation in Anti-Myeloperoxidase Antibody-Induced Glomerulonephritis. *Am J Pathol,* 187**:** 1908-1915, 2017 10.1016/j.ajpath.2017.05.003

28. Eulenberg-Gustavus C, Bahring S, Maass PG, Luft FC, Kettritz R: Gene silencing and a novel monoallelic expression pattern in distinct CD177 neutrophil subsets. *J Exp Med,* 214**:** 2089-2101, 2017 10.1084/jem.20161093

29. Kantari C, Pederzoli-Ribeil M, Amir-Moazami O, Gausson-Dorey V, Moura IC, Lecomte MC, et al.: Proteinase 3, the Wegener autoantigen, is externalized during neutrophil apoptosis: evidence for a functional association with phospholipid scramblase 1 and interference with macrophage phagocytosis. *Blood,* 110**:** 4086-4095, 2007 10.1182/blood-2007-03-080457

30. Huugen D, van Esch A, Xiao H, Peutz-Kootstra CJ, Buurman WA, Tervaert JW, et al.: Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int,* 71**:** 646-654, 2007

31. Schreiber A, Xiao H, Jennette JC, Schneider W, Luft FC, Kettritz R: C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis. *J Am Soc Nephrol,* 20**:** 289-298, 2009

32. Xiao H, Dairaghi DJ, Powers JP, Ertl LS, Baumgart T, Wang Y, et al.: C5a Receptor (CD88) Blockade Protects against MPO-ANCA GN. *J Am Soc Nephrol*, 2013 10.1681/ASN.2013020143

33. Jayne DRW, Merkel PA, Schall TJ, Bekker P, Group AS: Avacopan for the Treatment of ANCA-Associated Vasculitis. *N Engl J Med,* 384**:** 599-609, 2021 10.1056/NEJMoa2023386

34. Bories D, Raynal MC, Solomon DH, Darzynkiewicz Z, Cayre YE: Down-regulation of a serine protease, myeloblastin, causes growth arrest and differentiation of promyelocytic leukemia cells. *Cell,* 59**:** 959-968, 1989 10.1016/0092-8674(89)90752-6

35. Guarino C, Hamon Y, Croix C, Lamort AS, Dallet-Choisy S, Marchand-Adam S, et al.: Prolonged pharmacological inhibition of cathepsin C results in elimination of neutrophil serine proteases. *Biochem Pharmacol,* 131**:** 52-67, 2017 10.1016/j.bcp.2017.02.009

36. Korkmaz B, Lesner A, Wysocka M, Gieldon A, Hakansson M, Gauthier F, et al.: Structure-based design and in vivo anti-arthritic activity evaluation of a potent dipeptidyl cyclopropyl nitrile inhibitor of cathepsin C. *Biochem Pharmacol,* 164**:** 349-367, 2019 10.1016/j.bcp.2019.04.006

37. Rehm SRT, Smirnova NF, Morrone C, Gotzfried J, Feuchtinger A, Pedersen J, et al.: Premedication with a cathepsin C inhibitor alleviates early primary graft dysfunction in mouse recipients after lung transplantation. *Sci Rep,* 9**:** 9925, 2019 10.1038/s41598-019-46206-8

38. Schreiber A, Eulenberg-Gustavus C, Bergmann A, Jerke U, Kettritz R: Lessons from a double-transgenic neutrophil approach to induce antiproteinase 3 antibody-mediated vasculitis in mice. *J Leukoc Biol,* 100**:** 1443-1452, 2016 10.1189/jlb.5A0116-037R

FIGURE LEGENDS

Figure 1. Neutrophil serine proteases are strongly decreased in and on the surface of neutrophils and monocytes from CatC gene-deficient Papillon-Lefèvre syndrome (PLS) patients. (A) CatC, NE, PR3, and CatG in isolated blood neutrophils from a healthy individual (HC) and a PLS patient (PLS) by immunoblotting and by protease-specific FRET assays. MPO served as control and 42 kDa actin indicates equal sample loading. **(B)** NSP proteins in highly pure sorted HC monocytes (Mo) and neutrophils (Neu) by immunoblotting. Flow cytometry documents the purity of the sorted neutrophils and monocytes. **(C)** NSP protein expression and proteolytic activity in $CD14^{hi}$ sorted HC and PLS monocytes. **(D)** Flow cytometry of neutrophils stained for membrane-PR3 (mPR3) and CD177. mPR3 expression on $CD11b^{pos}/CD14^{hi}/CD15^{neg}$ blood monocytes from a HC and a PLS patient by flow cytometry.

Figure 2. PLS neutrophils produce a negative clinical PR3-ANCA immunofluorescence test, expose less PR3 on apoptotic neutrophils, and cause less NSP-mediated endothelial injury. (A) Indirect immunofluorescence using HC and PLS neutrophils and sera from PR3- and MPO-patients, respectively. **(B)** A percentage of overnight cultured neutrophils showed constitutive apoptosis by Annexin V-staining. mPR3 was analyzed after gating on apoptotic (Ann^{pos}) cells, respectively. **(C)** Cell-free supernatants (cf-SN) from resting and activated (2.5 µM ionophore A23187) HC and PLS neutrophils were assessed for PR3 protein by immunoblotting and

proteolytic activity by FRET assay. **(D)** Confluent HUVEC monolayers were incubated with cf-SN from resting and activated HC and PLS neutrophils, respectively. When indicated cf-SN from activated neutrophils were treated with α1-antitrypsin (AAT) prior to EC incubation. EC damage was visualized by actin staining with phalloidin-FITC (green) and nuclear DAPI staining (blue), and quantified by determining the black pixel areas using a Leica microscope (x40) and ImageJ 1.48v software. ** $p<0.01$.

Figure 3. Pharmacological CatC inhibition strongly reduces NSPs in neutrophils differentiated from hematopoietic stem cells (HSC) and subsequently reduces neutrophil activation by PR3-ANCA and EC injury. Neutrophils were differentiated from human $CD34^{pos}$ HSC for 10 days in the presence of buffer control (bu), CatC inhibitor (BI-I), and prednisolone (pred) as indicated. **(A)** Neutrophil differentiation was assessed using the indicated surface markers. **(B)** NSP proteins were assessed by immunoblotting and the optical densities (OD) of the NSP bands were quantified. Proteolytic activity was measured by FRET assay. MPO served as a control. **(C)** Neutrophils differentiated for 10 days were double-stained for mPR3 and CD177 and analyzed by flow cytometry. A typical experiment together with the mPR3 and CD177 MFIs are depicted. **(D)** Superoxide release by neutrophils differentiated for 10 days was assessed using mabs to PR3 and MPO, human PR3- and MPO-ANCA IgG, and appropriate controls as indicated. **(E)** mPR3 on viable ($AnnV^{neg}$) and apoptotic (Ann^{pos}) neutrophils differentiated for 10 days was assessed by flow cytometry. **(F)** The effect of cf-SN from resting and activated neutrophils differentiated for 10 days on EC injury was assessed by phalloidin-FITC (green) and nuclear DAPI staining (blue) and microscopy. * indicates $p<0.05$ and ** $p<0.01$.

Figure 4. Healthy control but not PLS neutrophils transfer proteolytically active PR3 to glomerular microvascular EC and both CatC gene-deficiency and pharmacological CatC inhibition result in less neutrophil-induced glomerular microvascular EC injury. Confluent glomerular microvascular EC (gMVEC) monolayers were incubated with cf-SN from resting and activated HC or activated PLS neutrophils as indicated. After 1 h, PR3 acquisition by glomerular microvascular EC was assessed by **(A)** immunoblotting using a PR3-specific mab, and **(B)** FRET assay measuring proteolytical PR3 activity. Two independent experiments each with two HC and PLS2 neutrophils were performed. For assessing cell injury, glomerular microvascular EC monolayers were incubated with cf-SN from HC or PLS neutrophils or from neutrophils that were differentiated from HSC in the presence of buffer, BI-I, or prednisolone as indicated. Each independent experiment includes neutrophils from two HC and PLS2, or two different HSC donors. Glomerular microvascular EC injury was determined **(C)** by phalloidin staining with the analysis of black pixel areas as in 2D. The corresponding statistics is given (n=2), and **(D)** by AnnexinV/7-AAD staining and flow cytometry (n=3). Contour plots of a typical experiment with cf-SN from resting and activated HC and PLS neutrophils are depicted together with the corresponding statistics of all experiments. * indicates $p<0.05$ and ** $p<0.01$.

Figure 1

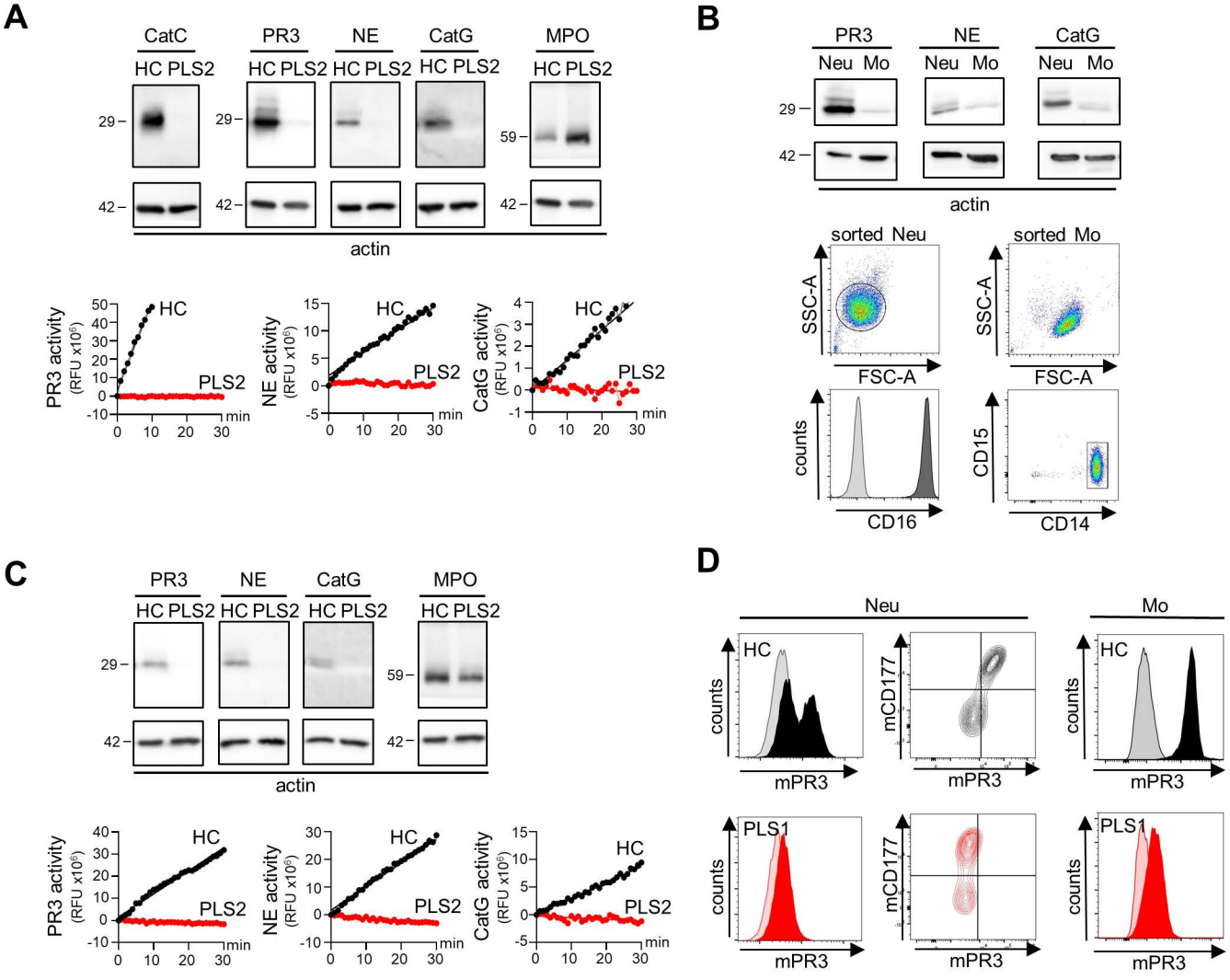

A
CatC
PR3
NE
CatG
MPO
HC PLS2
29
42
59
actin
PR3 activity (RFU x10⁶)
NE activity (RFU x10⁶)
CatG activity (RFU x10⁶)
HC
PLS2
min
B
PR3
NE
CatG
Neu Mo
actin
sorted Neu
sorted Mo
SSC-A
FSC-A
counts
CD16
CD15
CD14
C
PR3
NE
CatG
MPO
HC PLS2
actin
PR3 activity (RFU x10⁶)
NE activity (RFU x10⁶)
CatG activity (RFU x10⁶)
HC
PLS2
min
D
Neu
Mo
HC
PLS1
counts
mCD177
mPR3

Figure 2

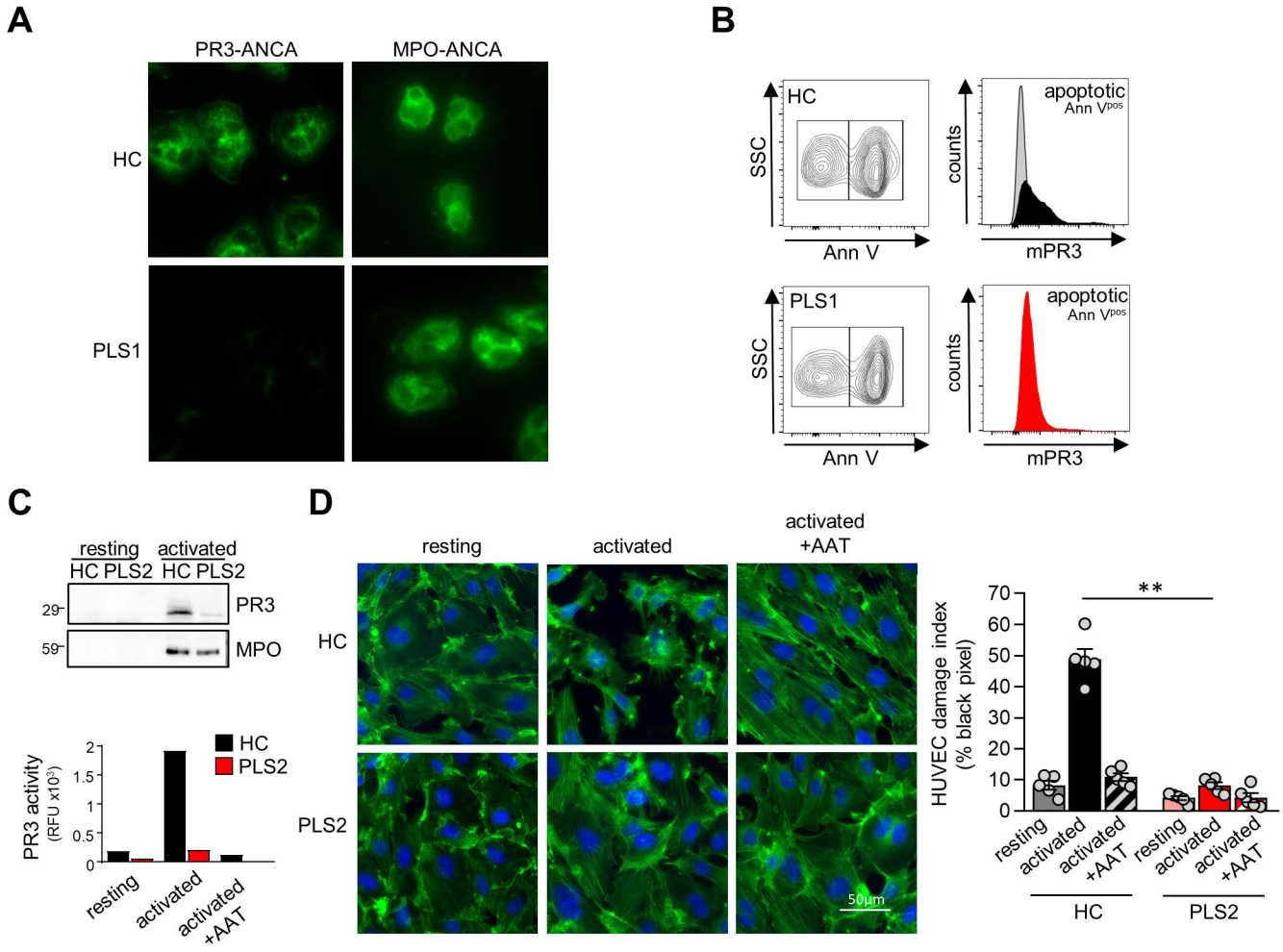

A
PR3-ANCA
MPO-ANCA
HC
PLS1
B
HC
SSC
Ann V
apoptotic
Ann V^pos
counts
mPR3
PLS1
C
resting
activated
HC PLS2 HC PLS2
29
59
PR3
MPO
PR3 activity
(RFU x10^3)
HC
PLS2
resting
activated
activated
+AAT
D
resting
activated
activated
+AAT
HC
PLS2
50µm
HUVEC damage index
(% black pixel)
**
HC
PLS2

Figure 3

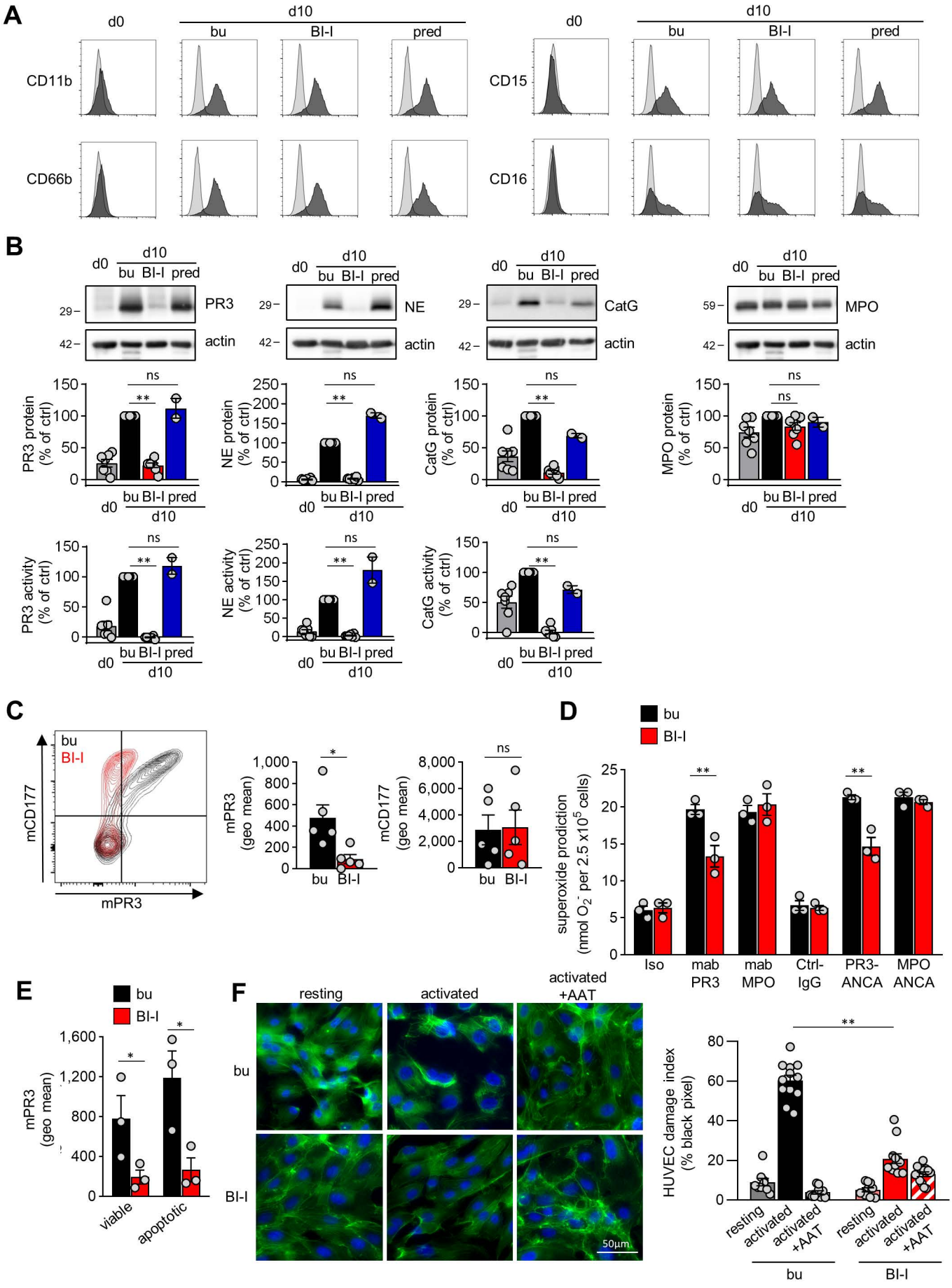
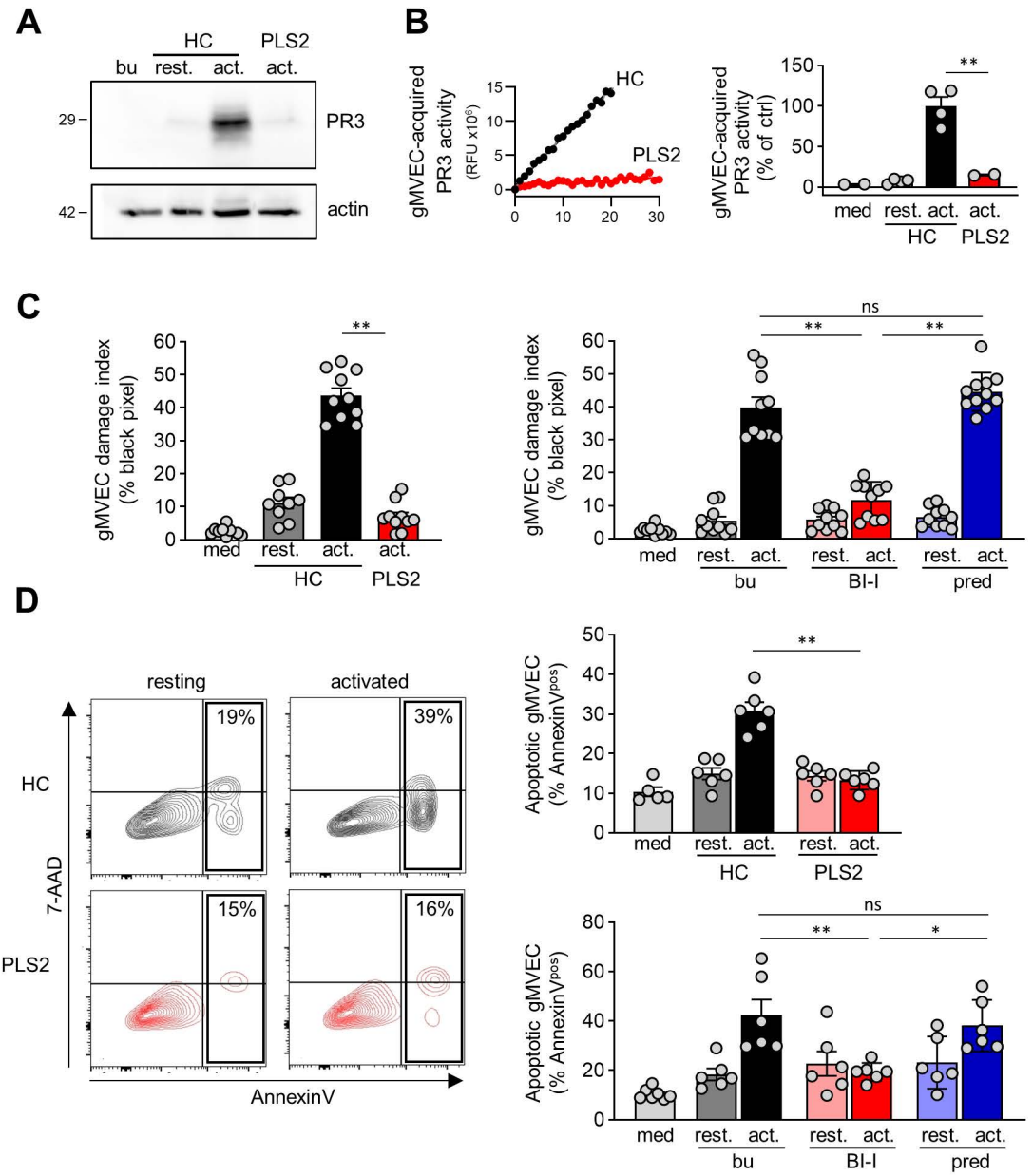

A
d0
d10
bu
BI-I
pred
CD11b
CD66b
CD15
CD16
B
PR3
NE
CatG
MPO
actin
PR3 protein (% of ctrl)
NE protein (% of ctrl)
CatG protein (% of ctrl)
MPO protein (% of ctrl)
PR3 activity (% of ctrl)
NE activity (% of ctrl)
CatG activity (% of ctrl)
ns
C
mCD177
mPR3
mPR3 (geo mean)
mCD177 (geo mean)
D
superoxide prodiction (nmol O2- per 2.5 x10^5 cells)
Iso
mab PR3
mab MPO
Ctrl-IgG
PR3-ANCA
MPO ANCA
E
viable
apoptotic
F
resting
activated
activated +AAT
50µm
HUVEC damage index (% black pixel)

Figure 4


A
HC
PLS2
bu
rest.
act.
act.
29
PR3
42
actin
B
gMVEC-acquired PR3 activity
(RFU x10⁶)
HC
PLS2
gMVEC-acquired PR3 activity (% of ctrl)
**
med
rest.
act.
act.
HC
PLS2
C
gMVEC damage index (% black pixel)
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bu
BI-I
pred
D
resting
activated
HC
PLS2
7-AAD
19%
39%
15%
16%
AnnexinV
Apoptotic gMVEC (% AnnexinV^pos)
**
ns
*