CRISPR-Cas9 mediated proteinase 3 autoantigen deletion as a treatment strategy for anti-neutrophil cytoplasmic autoantibody-associated vasculitis

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Abstract

Introduction: Proteinase 3 (PR3) is a major autoantigen in patients with anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV). Here, we performed a proof-of-principle study using *ex vivo* CRISPR-Cas9 guided gene editing to eliminate the PR3 autoantigen as an alternative to suppressing the autoimmune response to PR3.
Methods: A ribonucleoprotein (RNP) complex of Cas9 protein and a PR3-specific single guide-RNA was transfected into human $CD34^+$ hematopoietic stem and progenitor cells (HSPC) by electroporation. Effects on PR3 protein abundance, neutrophil differentiation, and ANCA-dependent and -independent neutrophil responses were assessed.
Results: Gene editing introduced a frame shift in exon 2 of *PRTN3*. Consequently, PR3 protein was efficiently reduced in neutrophil-differentiated HSPCs as demonstrated by immunoblotting, ELISA, microscopy, and the complete absence of PR3-specific proteolytic activity. Human neutrophil elastase served as control and was not affected. PR3-deleted ($PR3^{KO}$)- and PR3 wild-type ($PR3^{WT}$)-HSPCs showed similar neutrophil differentiation. Importantly, general neutrophil defense functions to non-ANCA receptor-independent and -dependent stimuli were similar in $PR3^{KO}$- and $PR3^{WT}$-neutrophils as was constitutive apoptosis. Flow cytometry showed that cell membrane-PR3 was significantly reduced on $PR3^{KO}$-neutrophils and consequent neutrophil activation to either monoclonal antibodies to PR3 or human PR3-ANCA was attenuated. In contrast, myeloperoxidase-ANCA stimulation was not affected.
Conclusions: We show the feasibility and efficacy of depleting the PR3 autoantigen in human $CD34^+$ HSPCs using CRISPR-Cas9. Depleting the PR3 autoantigen instead of suppressing the autoimmune response to PR3 could potentially lead to drug-free remission, particularly in patients with refractory or relapsing disease.

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Translational Statement

Patients with treatment-resistant and relapsing anti-neutrophil cytoplasmic autoantibody (ANCA) vasculitis require prolonged and repetitive immunosuppression that contributes to increased morbidity and mortality. We envision that these patients benefit from an alternative treatment strategy that deletes the proteinase 3 (PR3) autoantigen instead of suppressing the autoimmune response. We achieved effective and specific PR3 autoantigen depletion in neutrophils derived from clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 PR3 gene-edited human hematopoietic stem and progenitor cells (HSPCs). We propose that autologous HSPC transplantation could be combined with *ex vivo* PR3 gene editing to possibly achieve immunosuppression-free sustained remission and cure PR3-ANCA vasculitis. Future technology advances are expected to improve tolerability of gene and cell therapies.

Anti-neutrophil cytoplasmic autoantibody (ANCA)–associated vasculitides (AAVs) are life-threatening systemic autoimmune diseases. Patients lose tolerance to either proteinase 3 (PR3) or myeloperoxidase (MPO) and consequently develop PR3-ANCA or MPO-ANCA. Autoantigen-driven adaptive and innate immune responses cooperate in the disease process, leading to highly inflammatory,

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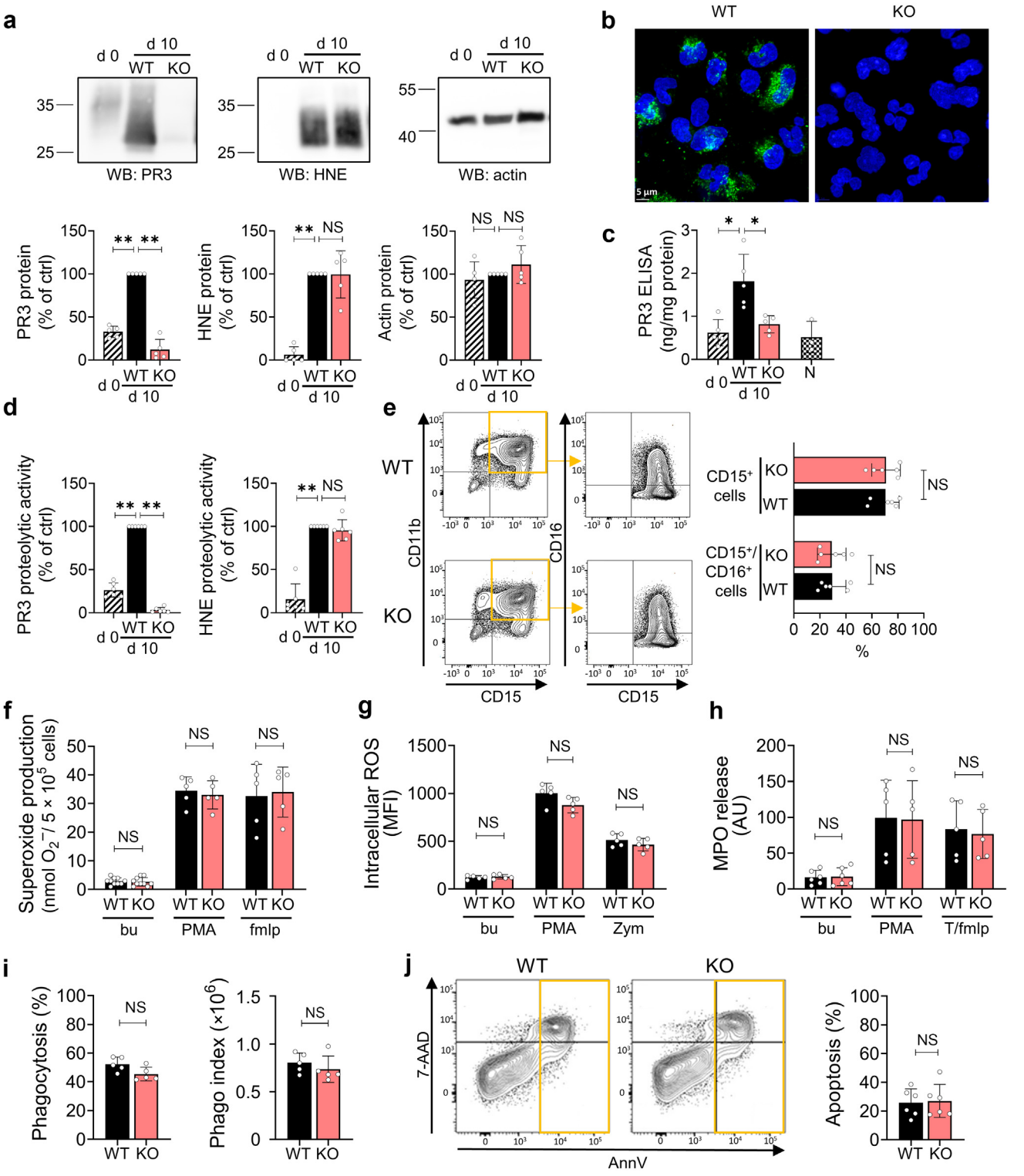

Figure 1 | Proteinase 3 (PR3) gene editing in CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) depletes PR3 protein in differentiated neutrophils without affecting their differentiation or physiological inflammatory responses. (**a**) Protein expression was analyzed in PR3^{KO} (knockout [KO]; red bars) and PR3^{WT} (wild type [WT]; black bars) neutrophils by immunoblotting. Compared with PR3^{WT}, PR3 protein was efficiently reduced in PR3^{KO} neutrophils at day 10 (d 10) of differentiation (left panel). Human neutrophil elastase (HNE; middle panel) served as control (ctrl), and 42-kDa actin indicates equal sample loading (right panel). Additionally, corresponding protein expression at day 0 (d 0) before differentiation is shown (shaded bars). Optical densities of the protein bands were quantified (n = 5). (**b**) Superimposed confocal laser-scanning microscopy images of PR3^{KO} and PR3^{WT} neutrophils at d 10 of differentiation. PR3 staining is shown in green, and 4′,6-diamidino-2-phenylindole nuclear staining in blue. Zeiss LSM, 63× oil objective, image acquisition with Zeiss ZEN software. (**c**) PR3 was assessed in PR3^{KO} and PR3^{WT} neutrophil lysates by enzyme-linked immunosorbent assay (ELISA; n = 5). PR3-nonexpressing control cells (continued)

necrotizing, small-vessel vasculitis.[1] ANCA-activated neutrophils and monocytes are central to this process, because these cells exclusively express the ANCA autoantigens, are bound and activated by ANCA, and function as important effector cells for vascular inflammation and injury.

Current AAV treatments include glucocorticoids, cytotoxic drugs, antibody-induced B-cell depletion, and C5a-receptor blocker to suppress the autoimmune responses toward the ANCA autoantigens. Treatment-refractory AAV, relapses, and treatment-related adverse events prevent better patient outcomes, demonstrating the need for alternative treatments.

We explored the feasibility of gene editing to eliminate the PR3 autoantigen as a treatment strategy for PR3-AAV. Gene editing uses programmable nucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9), to specifically induce DNA changes at specified sites in the genome of the transfected cells. Induction of DNA breaks in protein-coding genes can force insertion and deletions through error-prone DNA-repair pathways, compromising protein expression and function. Conceivably, AAV is suited for a gene editing approach because the disease features autoimmunity to a single autoantigen (namely, PR3 or MPO).

We performed a proof-of-principle study and tested the hypothesis that *ex vivo* CRISPR-Cas9 gene editing of human hematopoietic stem and progenitor cells (HSPCs) effectively eliminates the PR3 protein, thereby reducing PR3-ANCA binding to and activation of neutrophil-differentiated HSPCs without compromising general neutrophil defense responses.

METHODS

Single-guide RNA#-1 targeting exon 2 of human *PRTN3* gene was designed, chemically modified, and nucleofected into human $CD34^+$ HSPCs. PR3 protein abundance, neutrophil differentiation, general defense functions, and ANCA-induced neutrophil activation in PR3 protein-disrupted ($PR3^{KO}$) and wild-type control ($PR3^{WT}$) neutrophils were assessed. Full methods are provided in the Supplementary Methods.

RESULTS

We first designed, modified, and synthesized a PR3-specific single-guide RNA-#1 (Supplementary Table S1) that induced an insertion and deletion rate of 93.9% and a frame shift in the *PRTN3* gene by Sanger sequencing (Supplementary Figure S1) using primers listed in Supplementary Table S2. A ribonucleoprotein complex of Cas9 protein and single-guide RNA-#1 was then electroporated into cytokine-activated, proliferating human $CD34^+$ HSPCs. Transfected and control HSPCs were differentiated into neutrophils (Supplementary Figure S2) and assessed for PR3 protein and proteolytic PR3 activity, respectively. PR3 protein abundance was strongly reduced in gene-edited $PR3^{KO}$ compared with $PR3^{WT}$ neutrophils by immunoblotting (Figure 1a). Effective PR3 protein reduction in $PR3^{KO}$ neutrophils was confirmed using a PR3-specific enzyme-linked immunosorbent assay, confocal microscopy, and a PR3-specific fluorescence resonance energy transfer assay measuring the enzymatic activity (Figure 1b–d). Human neutrophil elastase served as a control because it is encoded within 4.1 kb downstream of the *PRTN3* gene on chromosome 19.

To assess whether PR3 disruption, also named myeloblastin, affects neutrophil differentiation, CD34+ HSPCs were expanded, gene edited as indicated, and subsequently differentiated into neutrophils. PR3 gene editing did not affect the percentage of differentiated neutrophils using CD15 and CD16 differentiation markers (Figure 1e). To investigate whether disrupting PR3 protein and enzymatic activity compromised important host defense functions, PR3KO and PR3WT neutrophils were treated with receptor-independent and receptor-dependent stimuli. We found no differences in the activation responses between PR3wt and PR3KO neutrophils for extracellular superoxide release, intracellular reactive oxygen species production, degranulation, phagocytosis of Escherichia coli particles, and constitutive apoptosis (Figure 1f–j). These results support the notion that PR3 is dispensable for a variety of neutrophil defense responses and for constitutive apoptosis that is pivotal to inflammation resolution.

Performing indirect immunofluorescence on ethanol-permeabilized neutrophils using sera from patients with PR3-AAV, we observed typical cytoplasmic ANCA staining with $PR3^{wt}$ but not $PR3^{KO}$ neutrophils (Figure 2a). This finding underscores the efficacy of PR3 disruption and supports the notion that PR3 is the single autoantigen in PR3-AAV.

To activate neutrophils, PR3-ANCA binds to PR3 on the neutrophil cell membrane (mPR3). Subset-restricted CD177 surface receptor expression results in distinct

Figure 1 | (continued) (N; checkered bar) served as an additional control (n = 3). (**d**) PR3 gene editing abolished PR3 but not HNE proteolytic activity, as detected by specific fluorescence resonance energy transfer substrates (n = 6). (**e**) HSPC differentiation into neutrophils was assessed by flow cytometry using the indicated surface markers and the depicted gating strategy (left panel). Quantification of the $CD15^+$ and $CD15^+/CD16^+$ cells shows similar percentages of differentiated cells in $PR3^{WT}$ (WT; black bars) and $PR3^{KO}$ samples (KO; red bars; n = 6; right panel). (**f–i**) $PR3^{WT}$ and $PR3^{KO}$ neutrophils were treated with buffer control (bu), the receptor-independent stimulus phorbol-12-myristate-12-acetate (PMA), or receptor-dependent stimuli (N-formyl-methionyl-leucyl-phenylalanine [fmlp], zymosan [Zym], or tumor necrosis factor [T] + fmlp), respectively. Similar responses for $PR3^{WT}$ and $PR3^{KO}$ neutrophils were observed for (**f**) extracellular superoxide (O_2^-) release (bu; n = 10; PMA and fmlp, n = 5), (**g**) intracellular reactive oxygen species (ROS) generation (n = 5), (**h**) degranulation of myeloperoxidase (MPO; n = 5), and (**i**) phagocytosis of pH-sensitive pHrodo BioParticles. Phagocytic activity is depicted as percentage fluorescent positive cells (left panel) and as phagocytic index (phagocytic index = percentage fluorescent positive cells × mean fluorescence intensity [MFI]; right panel; n = 5). (**j**) Constitutive apoptosis of $PR3^{WT}$ and $PR3^{KO}$ neutrophils were after 24 hours assessed by annexin V (AnnV) staining and flow cytometry (n = 5). Individual data points, mean, and SD are given. For statistical analysis, paired *t* test (2 tailed) or repeated-measures 1-way analysis of variance with Sidak *post hoc* test was performed. $^*P < 0.05$, $^{**}P < 0.01$. 7-AAD, 7-aminoactinomycin D; AU, arbitrary unit; NS, nonsignificant; WB, Western blot. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

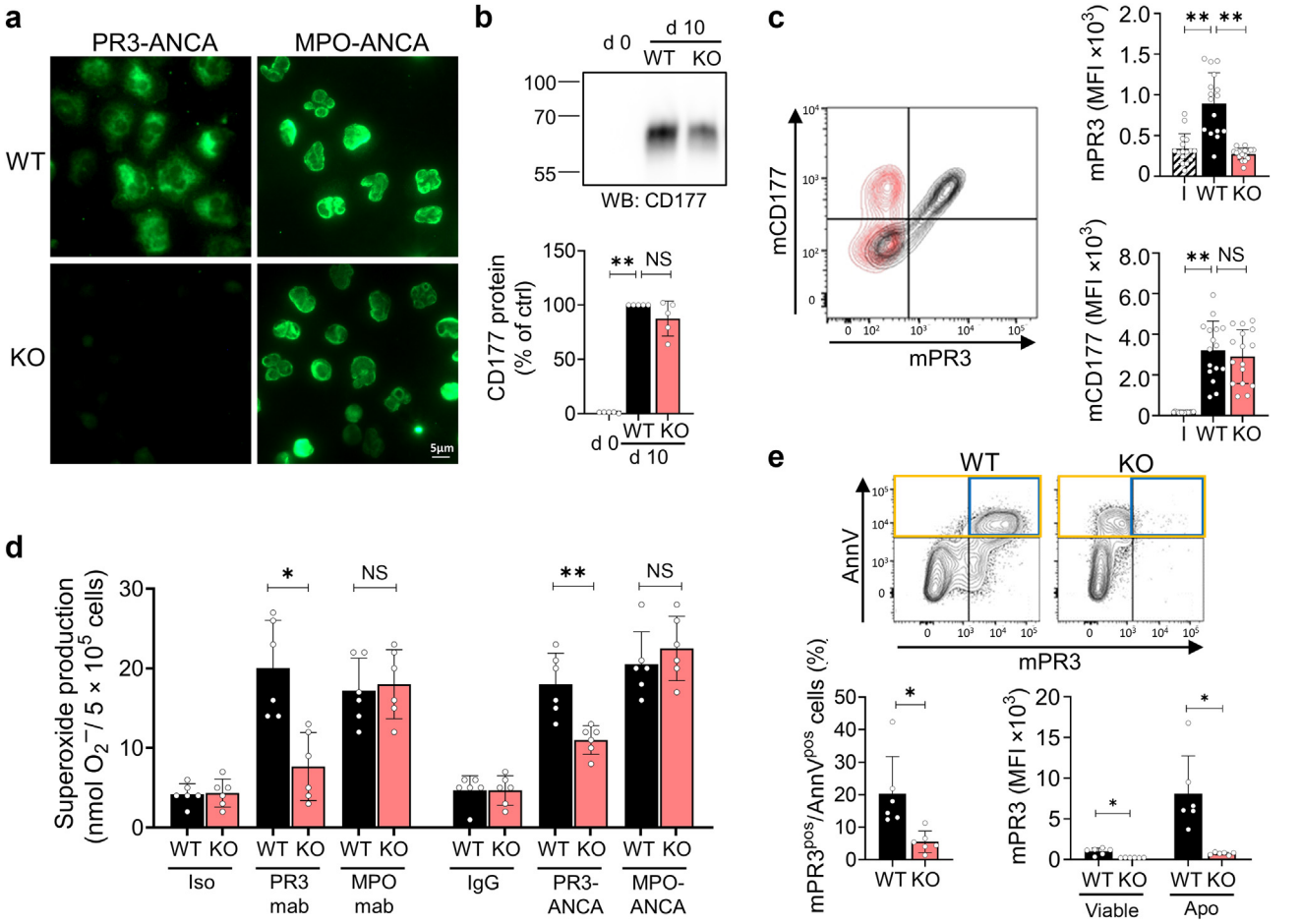

Figure 2 | Disruption of the proteinase 3 (PR3) autoantigen reduces PR3 on the neutrophil cell membrane (mPR3), binding of PR3–anti-neutrophil cytoplasmic autoantibody (ANCA) from patients with ANCA-associated vasculitis (AAV) to neutrophils, and PR3-ANCA induced neutrophil activation. (**a**) Indirect immunofluorescence using $PR3^{WT}$ and $PR3^{KO}$ neutrophils and sera from PR3-ANCA or myeloperoxidase (MPO)–ANCA positive patients with AAV. PR3-ANCA or MPO-ANCA staining is shown in green. Leica DMI6000 B microscope, 63× glycerol objective. Note the typical cytoplasmic staining pattern for PR3-ANCA in $PR3^{WT}$ but not $PR3^{KO}$ neutrophils, and the perinuclear staining for MPO-ANCA in both cell types. (**b**) CD177 protein levels were assessed in hematopoietic stem and progenitor cells prior (day 0 [d 0]) and after (day 10 [d 10]) of differentiation in $PR3^{WT}$ (wild type [WT]; black bars) and $PR3^{KO}$ neutrophils (knockout [KO]; red bars) by immunoblotting. A typical experiment together with the corresponding statistics shows similar CD177 protein in $PR3^{WT}$ and $PR3^{KO}$ neutrophils (n = 5). (**c**) mPR3 (upper panel) and mCD177 (lower panel) on the cell membrane of viable $PR3^{WT}$ and $PR3^{KO}$ neutrophils was measured by flow cytometry. Isotype stainings (I; shaded bars) are shown as controls (ctrls). A typical experiment together with the corresponding statistics shows strong mPR3 reduction in $PR3^{KO}$ neutrophils (n = 16). (**d**) $PR3^{WT}$ and $PR3^{KO}$ neutrophils were stimulated with monoclonal antibodies (mabs) to PR3 and MPO, human PR3-ANCA, and MPO-ANCA, respectively. Superoxide generation was measured using the ferricytochrome C reduction assay (n = 6 independent experiments, each using 3 different ANCA preparations). (**e**) Constitutive overnight apoptosis and mPR3 were assessed in $PR3^{WT}$ and $PR3^{KO}$ neutrophils by annexin V (AnnV) and PR3 staining, followed by flow cytometry. Samples were analyzed for the percentage of AnnV/PR3 double-positive cells (left panel; blue gate) and for the mPR3 amount (mean fluorescence intensity [MFI]) on viable and apoptotic (Apo) cells (right panel; yellow gate; n = 6). The percentage of mPR3/AnnV double-positive cells, the mPR3 MFI on Apo cells, and the MFI on viable cells all decreased significantly in $PR3^{KO}$ neutrophils. Individual results and mean ± SD are given. Paired *t* test (2 tailed) or repeated-measures 1-way analysis of variance with Sidak *post hoc* test was performed. $*P < 0.05$, $**P < 0.01$. Iso, isotype control; NS, nonsignificant; WB, Western blot. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

$CD177^{neg}/mPR3^{low}$ and $CD177^{pos}/mPR3^{high}$ neutrophils.[2] We evaluated the possibility that residual PR3 amounts were present on the surface of viable neutrophils despite PR3 gene disruption. CD177 protein amounts were similar in $PR3^{wt}$ and $PR3^{KO}$ neutrophils (Figure 2b). Importantly, double staining and flow cytometry indicated that mPR3 was strongly reduced on the surface of $PR3^{KO}$ compared with $PR3^{wt}$ neutrophils (Figure 2c). Next, we evaluated neutrophil activation in response to monoclonal antibodies to PR3 and to PR3-ANCA IgG from different patients with AAV. Using a robust respiratory burst assay and appropriate controls, we observed strong superoxide generation in $PR3^{wt}$ neutrophils treated with monoclonal antibodies to PR3 and PR3-ANCA IgG, respectively (Figure 2d). Activation was significantly reduced when $PR3^{KO}$ neutrophils were treated with these antibody preparations. Monoclonal antibody to MPO and MPO-ANCA IgG served as controls causing similar activation of $PR3^{wt}$ and $PR3^{KO}$ neutrophils.

Finally, PR3 on the surface of apoptotic cells was suggested as a do-not-eat-me signal and trigger for inflammatory cytokine release during efferocytosis by murine macrophages.[3] We observed high PR3 amounts on annexinV^{+} apoptotic PR3^{wt}-neutrophils, and both the percentage of PR3^{pos} cells and the mean PR3 fluorescence intensity were strongly reduced on apoptotic PR3^{KO} neutrophils (Figure 2e).

DISCUSSION

Our study revealed several new findings. First, gene editing of human HSPCs using CRISPR-Cas9 and an appropriate single-guide RNA effectively disrupted the PR3 protein. Second, PR3 disruption did not affect neutrophil differentiation and was dispensable for a variety of neutrophil defense functions. Third, PR3 gene disruption strongly reduced the PR3 autoantigen, PR3-ANCA binding to neutrophils, and consequently neutrophil activation.

PR3 is a member of the neutrophil serine protease family that also includes human neutrophil elastase. Neutrophil serine proteases are proteolytically activated by cathepsin C. *In vitro* studies demonstrated effective neutrophil serine protease downregulation by pharmacologic cathepsin C inhibitors, including PR3.[4] Clinical studies revealed that cathepsin C inhibitors reduced exacerbations in patients with bronchiectasis with an acceptable adverse event spectrum[5] but also that neutrophil serine protease activity was (only) halved.[6] Our CRISPR-Cas9 *PRTN3* gene editing was highly efficient in disrupting the PR3 gene and protein. Conceivably, this PR3-specific intervention will have even fewer adverse effects than pharmacologic cathepsin C inhibition. Along the same lines, the lack of PR3 protein (i.e., myeloblastin) did not compromise HSPC differentiation into neutrophils, neutrophil defense responses to receptor-dependent and receptor-independent stimuli, and constitutive apoptosis. These findings support feasibility and safety of a PR3 gene editing approach.

Importantly, PR3 gene editing strongly reduced PR3-ANCA binding to and activation of neutrophils—a central vascular injury mechanism in AAV. Moreover, we reported recently that increased PR3 protein pools in plasma and neutrophils of patients with PR3-AAV correlated with systemic inflammation, kidney injury, and PR3-ANCA titer, underscoring the PR3 antigen-driven disease process.[7] Conceivably, deleting the PR3 autoantigen will diminish the autoimmune response.

Current AAV treatments achieve remission in ∼75% of patients, and more than half experience relapses, particularly in PR3-AAV. Patients with treatment-resistant AAV and those with repeated relapses require prolonged and repetitive immunosuppression cycles that contribute to increased morbidity and excess mortality.[8] Conceivably, these patients would benefit from an alternative treatment strategy that deletes the PR3 autoantigen. Gene and cell therapies are gaining attraction in autoimmune diseases, such as lupus erythematosus, systemic sclerosis, polymyositis, and autoimmune neurologic disorders. HSPC treatments, at least currently, use preconditioning preceding cell infusion. We propose that autologous HSPC transplantation, already used to reset autoimmunity in a variety of autoimmune diseases,[9] could be combined with *ex vivo* PR3 gene editing. This approach has the potential to induce immunosuppression-free remission in carefully selected patients with PR3-AAV.

Together, we provide a proof-of-principle study showing efficacy and specificity of PR3 gene editing in human HSPCs that could be further developed for clinical application in patients with PR3-AAV in whom current standard treatments fail.

DISCLOSURE

UJ, CE-G, DLW, and RK declare that there is a pending patent application related to the work described in this article, with the authors being coinventors on this application (Europäische Patentanmeldung 24 202 670.6 "Genomeditierung zur Ausschaltung des Proteinase 3 Autoantigens als Therapiestrategie der ANCA-assoziierten Vaskulitis"). DLW is a cofounder of TCBalance Biopharmaceuticals GmbH, which is focused on regulatory T-cell therapy. DLW's laboratory at Charité–Universitätsmedizin Berlin has received gene editing reagents from Integrated DNA Technologies and GenScript Inc. The other author declared no competing interests.

DATA STATEMENT

The authors confirm that the data supporting the findings of this study are available in the article and its supplementary materials. The custom resources related to the article are available from the corresponding author on request. This article does not report large data sets, original code, or reanalyzed data.

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Supplementary material is available online at www.kidney-international.org.

REFERENCES

1. Falde SD, Fussner LA, Tazelaar HD, et al. Proteinase 3-specific antineutrophil cytoplasmic antibody-associated vasculitis. *Lancet Rheumatol*. 2024;6:e314–e327.
2. von Vietinghoff S, Tunnemann G, Eulenberg C, et al. NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. *Blood*. 2007;109:4487–4493.
3. Millet A, Martin KR, Bonnefoy F, et al. Proteinase 3 on apoptotic cells disrupts immune silencing in autoimmune vasculitis. *J Clin Invest*. 2015;125:4107–4121.
4. Jerke U, Eulenberg-Gustavus C, Rousselle A, et al. Targeting cathepsin C in PR3-ANCA vasculitis. *J Am Soc Nephrol*. 2022;33:936–947.
5. Chalmers JD, Haworth CS, Metersky ML, et al. Phase 2 trial of the DPP-1 inhibitor brensocatib in bronchiectasis. *N Engl J Med*. 2020;383:2127–2137.
6. Palmer R, Maenpaa J, Jauhiainen A, et al. Dipeptidyl peptidase 1 inhibitor AZD7986 induces a sustained, exposure-dependent reduction in neutrophil elastase activity in healthy subjects. *Clin Pharmacol Ther*. 2018;104:1155–1164.
7. Ebert M, Jerke U, Eulenberg-Gustavus C, et al. Protective alpha1-antitrypsin effects in autoimmune vasculitis are compromised by methionine oxidation. *J Clin Invest*. 2022;132:e160089.
8. Sanchez Alamo B, Moi L, Bajema I, et al. Long-term outcomes and prognostic factors for survival of patients with ANCA-associated vasculitis. *Nephrol Dial Transplant*. 2023;38:1655–1665.
9. Alexander T, Greco R, Snowden JA. Hematopoietic stem cell transplantation for autoimmune disease. *Annu Rev Med*. 2021;72:215–228.