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Anti-PR3 immune responses induce segmental and necrotizing glomerulonephritis

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Summary

Wegener's granulomatosis (WG) is a life-threatening autoimmune vasculitis that affects lungs, kidneys and other organs. A hallmark of WG is the presence of classic anti-neutrophil cytoplasmic antibodies (c-ANCA) against self-proteinase 3 (PR3). Little is known about the role of these antibodies and PR3-specific immune responses in disease development. In this study, we demonstrate that PR3-specific autoimmune responses are pathogenic in non-obese diabetic (NOD) mice with an impaired regulatory arm of the immune response. Immunization of autoimmunity prone NOD mice with rmPR3 (recombinant mouse PR3) in complete Freund's adjuvant (CFA) resulted in high levels of c-ANCA, without detectable disease development. However, when splenocytes from these immunized mice were transferred into immunodeficient NOD–severe combined immunodeficiency (SCID) mice, the recipient mice developed vasculitis and severe segmental and necrotizing glomerulonephritis. No disease developed in NOD–SCID mice that received splenocytes from the CFA-alone-immunized donors (controls), indicating that disease development depends upon PR3-specific immune responses. In contrast to the pathology observed in NOD–SCID mice, no disease was observed when splenocytes from rmPR3-immunized C57BL/6 mice were transferred into immunodeficient C57BL/6-RAG-1$^{-/-}$ mice, suggesting that complex and probably multi-genetic factors play a role in the regulation of disease development.

Keywords: animal models/mice/rats, anti-neutrophil cytoplasmic antibodies/anti-PR3/myeloperoxidase, autoimmunity, glomerulonephritis, Wegener's granulomatosis

Introduction

Wegener's granulomatosis (WG) is a debilitating and life-threatening autoimmune disease of unknown aetiology characterized by necrotizing granulomas of the upper and lower respiratory tract, necrotizing vasculitis and glomerulonephritis [1]. WG is one of several vasculitic syndromes characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA).

There are two classes of ANCA: classic ANCA (c-ANCA) and perinuclear ANCA (p-ANCA), which show different staining patterns of neutrophilic cytoplasm. c-ANCA (also called PR3-ANCA) shows a diffusely granular cytoplasmic staining and corresponds in most cases with antibodies to proteinase 3 (PR3). p-ANCA (also called MPO-ANCA) shows a perinuclear pattern of cytoplasmic staining and corresponds in most cases with antibodies to myeloperoxidase (MPO) [2].

The pathogenicity of these ANCAs, however, remains unproven. The aetiological agents that provoke the autoimmune response with the loss of tolerance to neutrophil enzymes and concomitant development of ANCAs are unknown, although certain drugs, infections and genetic factors have been implicated. The most accepted model of pathogenesis suggests that there is a breaking of self-tolerance towards PR3 resulting in the production of ANCAs. ANCAs activate cytokine-primed neutrophils within the microvasculature, leading to bystander damage to endothelial cells themselves and a rapid escalation of inflammation with recruitment of mononuclear cells [2,3].

Although c-ANCA occurs particularly in WG and p-ANCA in microscopic polyangiitis (MPA), some overlap does occur [3–6]. Nevertheless, c-ANCA is extremely specific for Wegener's pauci-immune necrotizing crescentic glomerulonephritis and its presence indicates the disease with >90% certainty [3].

The target antigen of c-ANCA is PR3, a neutral serine protease found in neutrophils and monocytes [7]. It is a highly folded protein with four disulphide bridges stabilizing its three-dimensional structure [8]. PR3 has an elastase-like enzymatic activity and can degrade extracellular matrix proteins, promoting the migration of neutrophils through the basement membrane [9–11]. PR3 is present in azurophilic granules and secretory vesicles as well as on the plasma membrane of resting neutrophils [12,13]. Following neutrophil and monocyte activation, an increased amount of PR3 is expressed on the cell surface [14,15]. *In vitro* studies show that the binding of PR3-ANCA to PR3 on the surface of neutrophils results in neutrophil activation, degranulation, release of superoxide and lipid mediators, stimulation of neutrophil-related endothelial cytotoxicity and secretion of cytokines *in vitro* [16,17].

While these findings support a pathogenic role for PR3-ANCA in the development of WG, there is no clear *in vivo* evidence that PR3-specific autoimmune responses can cause disease development. Recent findings that PR3-ANCA enhances cutaneous inflammation induced by local tumour necrosis factor (TNF) injection suggest that these antibodies may contribute to inflammation, but only in conjunction with other proinflammatory mediators [18]. However, it is also possible that PR3-ANCA may not be the cause of disease but simply a co-factor, as it happens in many autoimmune diseases. It is unclear whether PR3-ANCA titres in patients correlate with disease activity and relapse [5,19,20].

Various rodent models have implicated ANCA in the pathogenesis of vasculitis [18,21–31]. In several studies, antibodies specific for human PR3 were generated and reported to induce kidney pathology [27,31]. However, human PR3-ANCA does not cross-react with murine PR3, and it is therefore unlikely that the pathology observed under such circumstances is mediated by binding of the antibodies to murine PR3 [31].

In a recent study [32], chimeric human/mouse PR3 proteins were used as tools to induce an autoimmune response to PR3 in rats and mice. While autoimmune PR3-specific antibodies were detected in both mice and rats, no gross pathological abnormalities were detected in kidneys or lungs of these animals, suggesting that anti-PR3 immune responses may not be pathogenic. However, another recent study [28] showed that PR3/neutrophil elastase (NE) double-deficient mice immunized with rmPR3 developed PR3-ANCA and that a passive transfer of these antibodies to the wild-type recipients aggravated the inflammatory responses elicited by local TNF injection. This study suggested that PR3-ANCA is pathogenic, but only in conjunction with other immunological insults [28]. To test if PR3-specific immune responses may be pathogenic in an environment that facilitates development of autoimmunity we used NOD mice, which develop spontaneous autoimmune diabetes.

In the present study, we demonstrate that breaking tolerance towards self-PR3 in wild-type NOD mice is not sufficient to induce disease, despite the presence of high levels of PR3-ANCA. However, adoptive transfer of splenocytes from rmPR3-immunized NOD mice into immunodeficient NOD–SCID mice resulted in the appearance of PR3-ANCA in blood, rapid loss of renal function and the development of vasculitis and glomerulonephritis. No disease developed in mice that received splenocytes from the complete Freund's adjuvant (CFA)-alone-immunized donors (controls), indicating that the disease development depended upon PR3-specific immune responses.

Interestingly, the disease was not observed when splenocytes from rmPR3-immunized C57BL/6 mice were transferred into immunodeficient C57BL/6-RAG-$1^{-/-}$ mice, suggesting that multi-genetic factors play a role in the regulation of disease development. Our findings strongly support a pathogenic role for anti-PR3 immune responses in c-ANCA-associated renal diseases.

Materials and methods

Cloning of mPR3 gene

The FLAG-tagged, full-length cDNA for mouse PR3 (GenBank Accession no. NM_011178) was generated by polymerase chain reaction (PCR) from IMAGE clone 437587, using the following primers: mPR3 forward primer (5′-GATAGATCTATGTCTGGAGCTACCCATC-3′) and mPR3 reverse primer (5′-GGACTCGAGTCACTTATC ATCATCATCCTTATAAT CGGGCTCTGCGCCCCGCA-3′). The forward primer contained a *Bgl*I site and reverse primer a *Xho*I site, a stop codon and a FLAG tag for affinity purification (underlined).

The mPR3 cDNA was amplified in 100 μl reaction, using 1 U Pfu-DNA polymerase (Stratagene, La Jolla, CA, USA), 100 nM 2′-deoxynucleosides 5′triphosphate (dNTPs), and 20 pM of each primer for 25 cycles (94°C 45″; 55°C 45″; 72°C 1″). The DNA from the PCR reaction was separated on a 1·5% agarose gel, and a single product of 0·75 kb was eluted using the Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA) followed by digestion with *Bgl*I and *Xho*I. After ligation of the PCR product with T4 ligase (Roche, Mannheim, Germany) into the *Bam*H1/*Sna*BI sites of pFastBac1 vector (Invitrogen, Carlsbad, CA, USA), the *Escherichia coli* strain TOP10F was transformed and ampicillin-resistant colonies were obtained. Upon sequence confirmation, the corresponding miniprep DNA was used

to transform *E. coli* DH10Bac (Invitrogen) to obtain FLAG-mouse PR3 bacmid.

Transfection and infection of SF9 cells with baculovirus encoding rmPR3

We followed the protocol described in the Bac-to-Bac baculovirus expression system (Invitrogen) using Cellfectin reagent (Invitrogen). Sf9 cells were cultured in suspension using serum-free conditions with SF-900 II serum-free medium (SFM) without antibiotics (Invitrogen). After 4 days, the supernatant was collected and the baculoviral stock was amplified later to obtain the highest viral titre. When SF9 cells reached the mid-logarithmic phase of growth at a density of 2×10^6 cell/ml, they were infected with the baculovirus encoding for mPR3 at a multiplicity of infection (MOI) of 10. Four days after infection, the supernatant and the cells were collected for the purification of rmPR3-FLAG.

Affinity chromatography of FLAG rmPR3

Purification of the FLAG tagged mPR3 was performed using anti-FLAG M2 affinity gel (Sigma, St Louis, MO, USA). Recombinant mPR3-FLAG from the lysed cells and supernatant was purified using a column format and was eluted by competition with 100 μg/ml FLAG peptide (Sigma). Purity of the different eluates was checked by Western blot, and the aliquots were pooled and concentrated using centrifugal filter devices (Centriplus; Millipore, Billerica, USA). The concentrated rmPR3 was stored at 4°C in 10 mM Tris HCl, 150 mM NaCl at pH 7·4.

Western blotting

Cell extracts and supernatant of infected Sf9 cells were resolved on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Harvested cells were lysed with Triton lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100, pH 7·4]. The eluates from the anti-FLAG affinity column were resolved by SDS-PAGE electrophoresis followed by silver stain. Purified rmPR3 and commercially available human PR3 were used for protein electroblotting on Immuno-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Richmond, CA, USA). We detected rmPR3 by staining with anti-FLAG M2 antibody (Sigma), as well as with anti-mouse PR3 D-20 (sc19747) and anti-mouse PR3 P-20 (sc-19748) followed by staining with the Immuno-Star horseradish peroxidase (HRP) chemiluminescence kit (Bio-Rad). Anti-FLAG M2 monoclonal antibody (Sigma) binds to FLAG fusion proteins. PR3 (D-20): *sc-19747* and PR3 (P-20): *sc-19748* are affinity purified goat antibodies raised against peptides from two different internal regions of mouse PR3 (Santa Cruz Biotechnology, CA, USA).

Proteolytic assay and kinetic activity of rmPR3

Protease activity was assayed by an electrophoretic zymogram gel. Twenty μl of rmPR3 immunoprecipitated with FLAG antibody was loaded on a 12% zymogram gel without boiling or adding reducing reagents. Human elastase (Sigma) was used as a positive control. Following electrophoresis, the gel was incubated with zymogram renaturation buffer for 30 min. The gel was then incubated in zymogram development buffer for 24 h at 37°C before staining with 0·5% Coomassie Brilliant Blue.

Protease activity of rmPR3 was also assayed using the synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MeSuc-AAPV-pNA) at 4 mM from Sigma. The reaction was carried out in 100 μl volumes of 0·1 M Tris, pH 8, at 37°C using different concentrations of rmPR3. The proteolysis of MeSuc-AAPV-pNA was determined by measuring the change in absorbance at 410 nm. The specificity was confirmed by an inhibition experiment using α-antitrypsin at 0·5 mg/ml (Sigma).

To characterize further the functional activity of rmPR3, an enzymatic reaction was started by adding 10 μl of the recombinant enzyme to the reaction buffer (0·1 M Tris, pH 8) containing between 1·25 and 6·25 mM MeSuc-AAPV-pNA. Changes in optical density ($OD_{410\,nm}$) were recorded continuously using a Hitachi U-2000 photometer. Readings from control experiments (substrate and buffer) were used to determine the baseline activity.

Immunization of mice and adoptive transfer of splenocytes

NOD-LtJ, NOD–SCID, C57BL/6 and C57BL/6-RAG1$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed under specific pathogen-free conditions at Massachusetts General Hospital.

Eight to 12-week-old C57BL/6 or NOD mice were primed with 100 μg of purified rmPR3 [diluted in phosphate-bufferd saline (PBS)] and emulsified in complete Freund's adjuvant (CFA) on day 0. The mice were boosted intraperitoneally with 40 μg of rmPR3 emulsified in incomplete Freund's adjuvant (IFA) on days 20, 40 and 50. The control mice were immunized with emulsified CFA on day 0 and boosted with emulsified IFA on days 20, 40 and 50.

We isolated splenocytes from immunized and control C57BL/6 or NOD mice by disrupting the spleens using cold RPMI-1640 medium. Red blood cells were removed with lysis buffer (ACK Lysis Buffer; BioWhittaker, Walkersville, MD, USA) followed by washing with media. Suspensions of $6–8 \times 10^7$ NOD splenocytes resuspended in PBS were administrated via the tail vein (1 ml/mouse) into NOD–SCID mice, while $6–8 \times 10^7$ C57BL/6 splenocytes were

administrated via the tail vein into C57BL/6-RAG-1$^{-/-}$ mice. The mice were bled at different time-points and tissue samples were collected for histological examination.

Detection of anti-mPR3 antibodies by enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FCM)

ELISA assay was used to monitor anti-mPR3 in serum. Microtitre plates were coated with rmPR3 at 0·5 μg/well and mouse sera were applied at a dilution of 1/100. Antibody binding was detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) at a concentration of 1/20 000. Both incubations were performed in PBS containing 3% bovine serum albumin (BSA) at room temperature for 60 min. The substrate was *p*-nitro phenyl phosphate (Sigma) at a concentration of 1 mg/ml in 0·2 M Tris buffer. The $OD_{405\,nm}$ was determined at 60 min. A competitive inhibition ELISA assay was performed in the presence of different doses of FLAG peptide (0, 0·01, 0·1 and 1 mg/ml).

For flow cytometric analysis, the white cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD19, T cell receptor (TCR), Mac-1 and rat anti-mouse immunoglobulin G-phycoerythrin (Ig-PE)-conjugated antibodies, and analysed by three-colour FCM (Becton Dickinson, Mountain View, CA, USA). To detect binding of PR3-specific antibodies to activated neutrophils from peritoneal lavage, the cells were first incubated with the sera from PR3-immunized, CFA-alone-immunized controls or with pre-immunization sera. All antibodies used were from Becton Dickinson.

Detection of anti-mPR3 antibodies by indirect immunofluorescence

Peritoneal lavage cells, which contained ~80% activated neutrophils, were obtained after intraperitoneal injection of 1·5 ml of a sterile 10% solution of protease peptone into NOD–SCID mice. The isolated cells (1×10^6 cells in 100 μl of PBS/BSA/azide) were incubated for 30 min at 4°C with 10 μl of sera from rmPR3-immunized mice. Pre-immunization sera from the same mice were used as controls. Non-specific FcγR binding was blocked with 10 μl of undiluted culture supernatant containing rat anti-mouse FcγR monoclonal antibody (mAb) 2·4G2. Cells were washed and incubated with FITC-labelled rat anti-mouse Ig (BD Biosciences, San Diego, CA, USA) for 30 min at 4°C. The cytospin slides were prepared by centrifugation of 2×10^4 cells on glass slides at 500 rpm (27 ***g*** Cytospin 4; Shandon, Cheshire, UK) for 5 min and fixed with 100% ethanol for 10 min. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with 4,6-diamidino-2-phenylindole (DAPI) and analysed by indirect immunofluorescence (IIF).

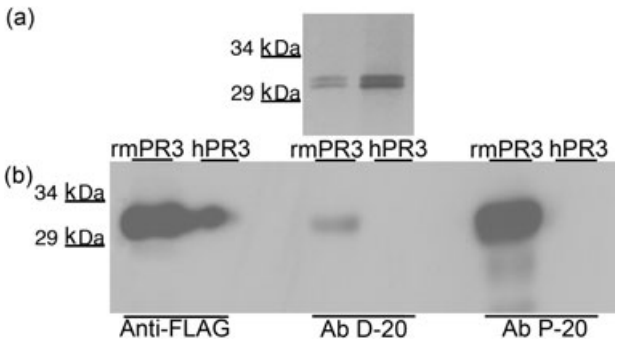

Fig. 1. Recombinant mouse proteinase 3 (rmPR3) expression using the baculovirus system. Insect cells were infected with PR3-baculovirus for 4 days and rmPR3 was purified by affinity chromatography from the media and cell lysate. (a) Silver staining of a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following electrophoresis of eluates. (b) Immunoblot staining with anti-FLAG antibodies (left), anti-D-20 antibodies (middle) and anti-P-20 antibodies (right). P-20 and D-20 antibodies did not cross-react with human PR3.

Evaluation of kidney injury

Mouse sera were obtained by tail incision. After centrifugation, the samples were stored at –20°C for subsequent analysis. Serum creatinine and blood urea nitrogen (BUN) were measured using manual kits (Stanbio Laboratory, Borne, TX, USA). The mice were euthanized with methoxyflurane and kidney samples were fixed in 10% formalin and processed for light microscopy. All tissue sections were stained with haematoxylin and eosin (H&E).

Statistical analysis

Statistical significance was determined using Student's *t*-test. A *P*-value of less than 0·05 was considered to be statistically significant.

Results

Generation of rmPR3

rmPR3 expressed in *E. coli* or *Pichia pastoris* was not processed and folded like the naturally occurring mammalian protein [31,33,34]. Therefore, we expressed FLAG-tagged rmPR3 in insect cells utilizing a baculovirus expression system.

We obtained a protein which, when affinity purified, migrated as a 30 kDa species in SDS-polyacrylamide gel (Fig. 1a). Its identity was confirmed as mouse PR3 using two different commercially available goat anti-mouse PR3 peptide antibodies, D-20 and P-20, in Western blot analysis. These antibodies were mPR3-specific and did not recognize human PR3 in Western blot analysis (Fig. 1b). There were two additional bands detected in Western blot analysis when P-20 antibodies were used. However, these bands were not

detected by either anti-FLAG or D-20 antibodies, and there are no known isotypes of PR3.

Functional status of rmPR3

To assess the functional status of the expressed rmPR3, we first assessed its protease activity using an electrophoretic zymogram assay (Fig. 2a). The enzymatic activity of the rmPR3, immunoprecipitated with anti-FLAG antibodies, was observed at the expected molecular weight (MW) of approximately 30 kDa. We next measured rmPR3 enzymatic activity in a standard assay for PR3 [9], using the tetrapeptide MeSuc-AAPV-pNA as a substrate in the absence or presence of the inhibitor, α-1-anti-trypsin (Fig. 2b). Our rmPR3 hydrolyzed MeSuc-AAPV-pNA, and its proteolytic activity was inhibited completely by α-anti-trypsin (Fig. 2b). The enzymatic activity of rmPR3 was also measured using different concentrations of substrate MeSuc-AAPV-pNA. Representative results of three independent experiments are shown in Fig. 2c. The proteolytic activity of rmPR3 showed a linear dependence on substrate concentration. With increasing concentrations of the substrate, from 1·25 mM to 6·25 mM, the rates of product formation increased almost proportionally with the concentrations of substrate from 4×10^{-4} (s^{-1}) to $1{\cdot}1 \times 10^{-3}$ (s^{-1}), demonstrating a non-competitive mechanism (Fig. 2c). After plotting these data in a Lineweaver–Burk graph, values for K_M of $2{\cdot}5 \times 10^{-3}$ (M) and k_{cat} of 3×10^{-3} (1/s) with a k_{cat}/K_M of 1·2 (1/M × s) were obtained. These findings demonstrate that rmPR3 expressed in insect cells was a functional enzyme.

Immunization with rmPR3 resulted in the breaking of tolerance toward self-PR3

NOD mice were selected for immunization with rmPR3 because of their defects in both central and peripheral tolerance, which may predispose them to autoimmunity [35]. NOD mice were primed with rmPR3 in CFA on day 0, and then boosted intraperitoneally with rmPR3 in IFA on days 20, 40 and 50. Control NOD mice were immunized with CFA and IFA alone. Mouse serum was collected at different time-points during the immunization, and rmPR3 antibody concentrations were determined. The concentration of antibodies against mPR3 in serum was monitored by anti-PR3 ELISA (Fig. 3a,b), FCM analysis of circulating white blood cells (Fig. 3c) and indirect immunofluorescence microscopy of peritoneal neutrophils/monocytes (Fig. 3d,e).

Using anti-PR3 ELISA, we detected that NOD mice immunized with rmPR3 developed high levels of PR3-specific antibodies on days 21, 42 and 63 post-immunization (dark columns, Fig. 3a). No PR3-specific antibodies were detected in control mice (light columns, Fig. 3a).

Because FLAG-tagged rmPR3 was used for the immunization, the possibility that the induced immune response was directed against the FLAG peptide and not against rmPR3 was analysed. We performed a competitive inhibition ELISA assay. Sera from rmPR3-immunized mice collected at different time-points were pooled and anti-PR3 ELISA performed in the presence of increasing doses of FLAG peptide (between 0 and 1 mg/ml). The presence of FLAG peptide did not influence ELISA binding, demonstrating that the antibodies were directed against rmPR3 and not FLAG peptide (Fig. 3b).

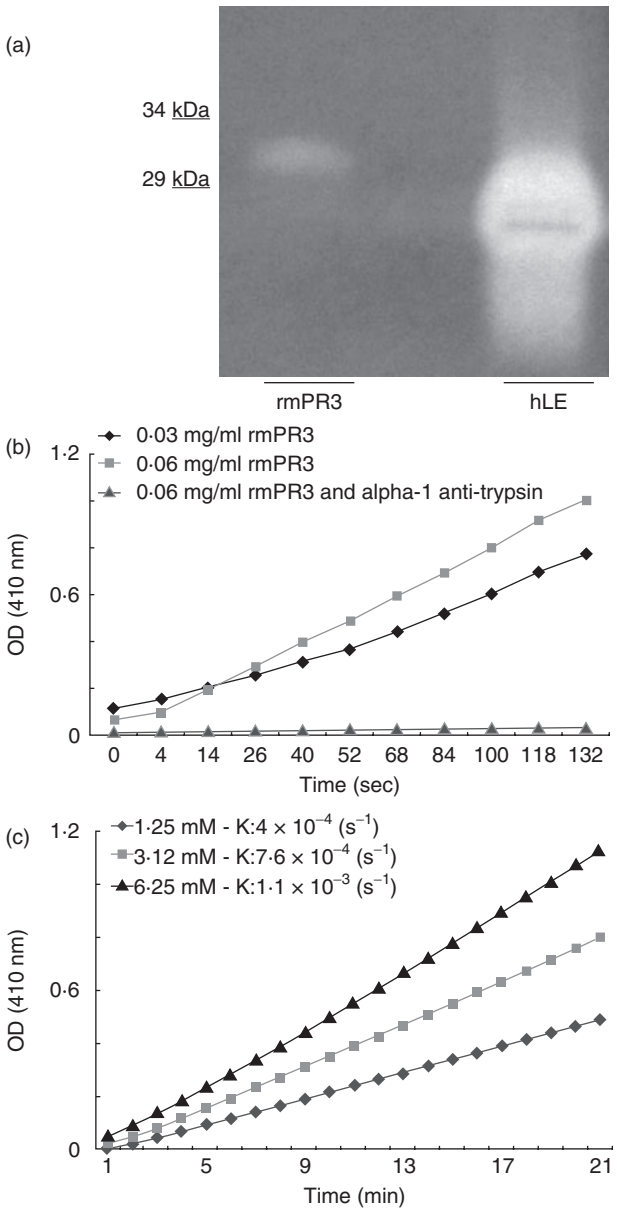

Fig. 2. Recombinant mouse proteinase 3 (rmPR3) has enzymatic activity. (a) Zymogram gel of purified rmPR3. Purified human neutrophil elastase (hLE) was used as a positive control. (b) Proteolytic activity expressed as optical density (OD) measured at 410 nm using a specific nitroanilide substrate at two concentrations of rmPR3. The proteolytic activity was inhibited by the addition of α-1-anti-trypsin at 0·06 mg/ml of rmPR3. (c) PR3 enzymatic activity, expressed as optical density (OD) in the presence of different concentrations of nitroanilide substrate. The results shown are representative of three independent experiments. Note that for kinetic purposes, the rates are calculated in seconds.

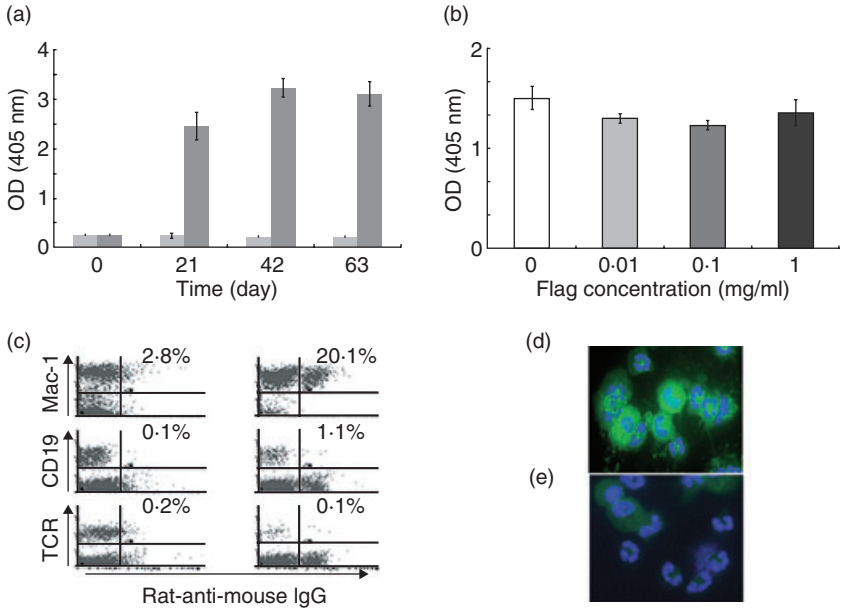

Fig. 3. Non-obese diabetic (NOD) mice immunized with recombinant mouse proteinase 3 (rmPR3) develop classic anti-neutrophil cytoplasmic antibodies (c-ANCA). (a) NOD mice were primed by immunization with rmPR3 in complete Freund's adjuvant (CFA) on day 0, followed by boosts on days 20 and 40 with rmPR3 in incomplete Freund's adjuvant (IFA) (■). The titre of anti-mPR3 antibodies in sera was monitored using enzyme-linked immunosorbent assay (ELISA) at different time-points. Control mice received CFA and IFA without antigen (■). Values shown are mean ± standard deviation ($n = 4$), $P < 0{\cdot}005$. (b) Anti-PR3 ELISA was performed in the presence of increasing concentrations of FLAG-peptide (0–1 mg/ml). (c) Autoantibodies are present on peripheral neutrophils/monocytes in NOD mice immunized with rmPR3 (right panel) but not CFA-alone-immunized mice (left panel). Indirect immunofluorescence staining of neutrophils with serum from NOD mice immunized with rmPR3. (d) This staining resulted in the characteristic granular cytoplasmic staining pattern. (e) Indirect immunofluorescence staining of neutrophils with serum from CFA-alone-immunized NOD mice. Original magnification of both images (d,e) ×600.

To test if the anti-PR3 antibodies have the binding pattern expected for PR3-ANCA, we used FCM to evaluate whether the induced autoantibodies against rmPR3 were bound to the cell surface of peripheral circulating neutrophils/monocytes. FCM analysis of peripheral blood cells of rmPR3-immunized NOD mice demonstrated that approximately 20% of white blood cells were coated with mouse antibodies, while only 2·8% of cells from the control mice were coated with mouse antibodies. All of the coated cells were Mac-1 positive. (Fig. 3c, upper panel). We did not detect any coating of peripheral $CD19^+$ cells (B cells) (Fig. 3c, middle panel), nor of peripheral TCR^+ cells (T cells) with mouse antibodies in either rmPR3-immunized or control mice (Fig. 3c, lower panel). This pattern of binding is consistent with the expected binding of PR3-specific c-ANCA [13].

The presence of PR3-ANCA antibodies in the serum of rmPR3-immunized NOD mice was confirmed by *in situ* staining of fixed neutrophils. The incubation of activated neutrophils with c-ANCA-containing serum from rmPR3-immunized mice followed by FITC-conjugated secondary rat anti-mouse IgG antibodies resulted in a typical c-ANCA granular cytoplasmic staining profile (Fig. 3d). Pre-immunization serum and serum from CFA-alone-immunized mice showed no cytoplasmic staining, confirming that c-ANCA granular cytoplasmic staining profile was due to anti-rmPR3 antibodies (Fig. 3e).

NOD mice immunized with PR3/CFA and boosted with PR3/IFA and NOD mice immunized with CFA/IFA (controls) were observed for 12 months after immunization. Despite the long-term presence of PR3-ANCA, rmPR3-immunized mice did not develop vasculitis, glomerulonephritis, interstitial nephritis or tubular injury, as determined by pathohistological examination of kidney tissues harvested from these mice up to 1 year post-immunization (data not shown). In addition, these mice appeared clinically healthy for the duration of the observation. We also followed the level of blood glucose in these mice because untreated NOD mice develop type I diabetes spontaneously. As reported previously [36,35], the immunization of NOD mice with CFA prevented the development of diabetes and no mice developed hyperglycaemia during the experiment.

Development of kidney failure after the secondary transfer of splenocytes from immunized NOD mice to immunodeficient NOD–SCID recipients

To test if lymphocytes from rmPR3-immunized NOD mice had the capacity to induce autoimmune processes in the absence of regulatory T cells, we transferred splenocytes

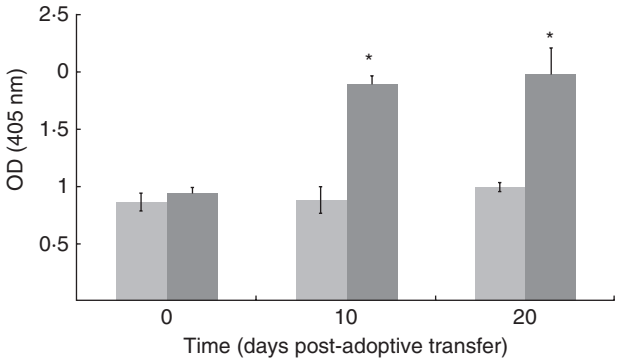

Fig. 4. Non-obese diabetic–severe combined immunodeficiency (NOD–SCID) mice transferred adoptively with splenocytes from recombinant mouse proteinase 3 (rmPR3)-immunized mice develop anti-PR3 antibodies. The NOD–SCID mice transferred adoptively with splenocytes from rmPR3-immunized mice ($n = 3$) (■) and adjuvant-alone-immunized mice ($n = 2$) (■) were bled at different time-points and the anti-PR3 antibody titre was measured using enzyme-linked immunosorbent assay.

from rmPR3-immunized NOD mice into immunodeficient NOD–SCID mice. Splenocytes from rmPR3-immunized and control adjuvant-alone-immunized mice were transferred into immunodeficient NOD–SCID mice, and the recipients were evaluated for immune reconstitution, presence of PR3-ANCA, clinical status, survival, kidney function and development of glomerulonephritis.

Splenocytes ($6–8 \times 10^7$) from rmPR3-immunized or control adjuvant-alone-immunized NOD mice, were injected via the tail vein into NOD–SCID mice. Flow cytometry analysis of recipient mice peripheral blood confirmed the reconstitution of CD4 and CD8 T cells and B cells 1 week after the adoptive transfer (data not shown).

All recipient mice were bled, and the level of PR3-specific antibodies in serum was determined using ELISA. Ten and 20 days after the adoptive transfer, a significant rise in PR3-ANCA was detected in serum of all NOD–SCID mice which had received splenocytes from rmPR3-immunized donors (dark columns), but not of CFA-alone-immunized donors (light columns) (Fig. 4).

NOD–SCID recipient mice were observed for early clinical evidence of renal failure by assessment of changes in fur texture and posture (hunching). Twenty to 40 days after the adoptive transfer of splenocytes from rmPR3-immunized mice, NOD–SCID recipients developed generalized sickness and acute kidney failure followed by death (Fig. 5a). There were no deaths observed in the recipients of splenocytes from mice immunized with CFA alone.

BUN and creatinine were measured in the blood of NOD–SCID recipients at various time-points after the adoptive transfer. While NOD–SCID mice that received splenocytes from NOD mice immunized with CFA alone had normal values of BUN (<40 mg/dl) and creatinine (<0·5 mg/dl), NOD–SCID mice that received splenocytes from rmPR3-immunized mice had high values of BUN (120–300 mg/dl) and creatinine (2–3 mg/dl), as measured 1–3 days prior to death (days 20–40 post-adoptive transfer), consistent with rapidly progressive glomerulonephritis (Fig. 5b,c).

Histopathological evaluation was performed on the kidneys harvested from NOD–SCID mice that received splenocytes from adjuvant-alone-immunized ($n = 3$) or rmPR3-immunized NOD mice ($n = 5$). All morphological evaluations were performed in a blinded fashion by the same pathologist (R. N. S.). All NOD–SCID mice that received splenocytes from PR3-immunized mice developed kidney pathology (Fig. 6a–c). In these mice, we found small artery fibrinoid necrosis and protein casts (Fig. 6a), glomerular fibrinoid necrosis (Fig. 6b) and crescentic glomerulonephritis (Fig. 6c). Fifty glomeruli were scored in each animal for necrosis, crescents and hypercellularity/apoptotic debris. The average percentage of glomeruli with segmental necrosis was 56·4% (standard deviation 24·9%; range 33·4–78·2%). The average percentage of glomeruli with crescents

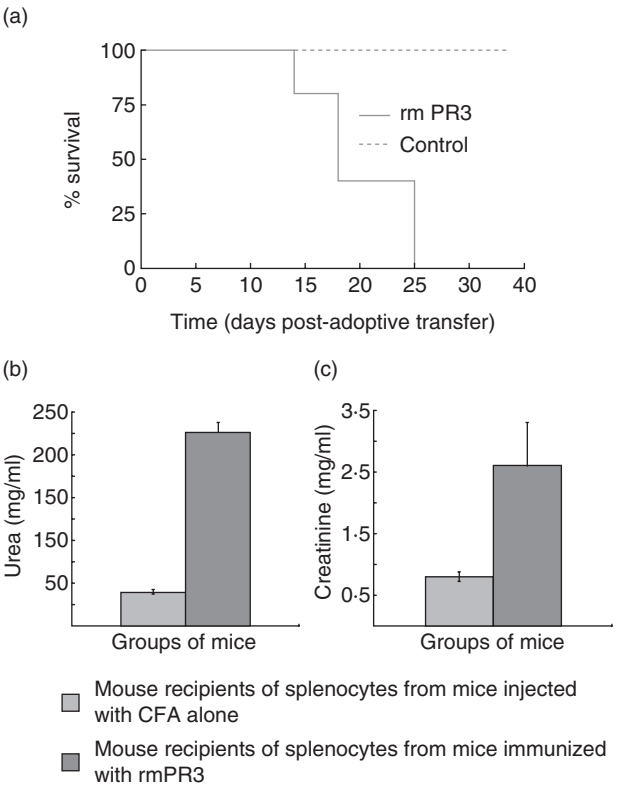

Fig. 5. Non-obese diabetic–severe combined immunodeficiency (NOD–SCID) mice transferred adoptively with splenocytes from recombinant mouse proteinase 3 (rmPR3)-immunized mice develop lethal kidney failure. (a) Survival curve of NOD–SCID mice after adoptive transfer of splenocytes from rmPR3-immunized or adjuvant-alone-immunized, control mice. (b) Blood urea nitrogen (BUN) and (c) creatinine serum levels in NOD–SCID mice 20 days after they received 8×10^7 splenocytes from mice immunized with rmPR3 (■) or with adjuvant alone (■).The normal range for BUN is 18–29 mg/dl and 0·2–0·8 mg/dl for creatinine in mouse serum.

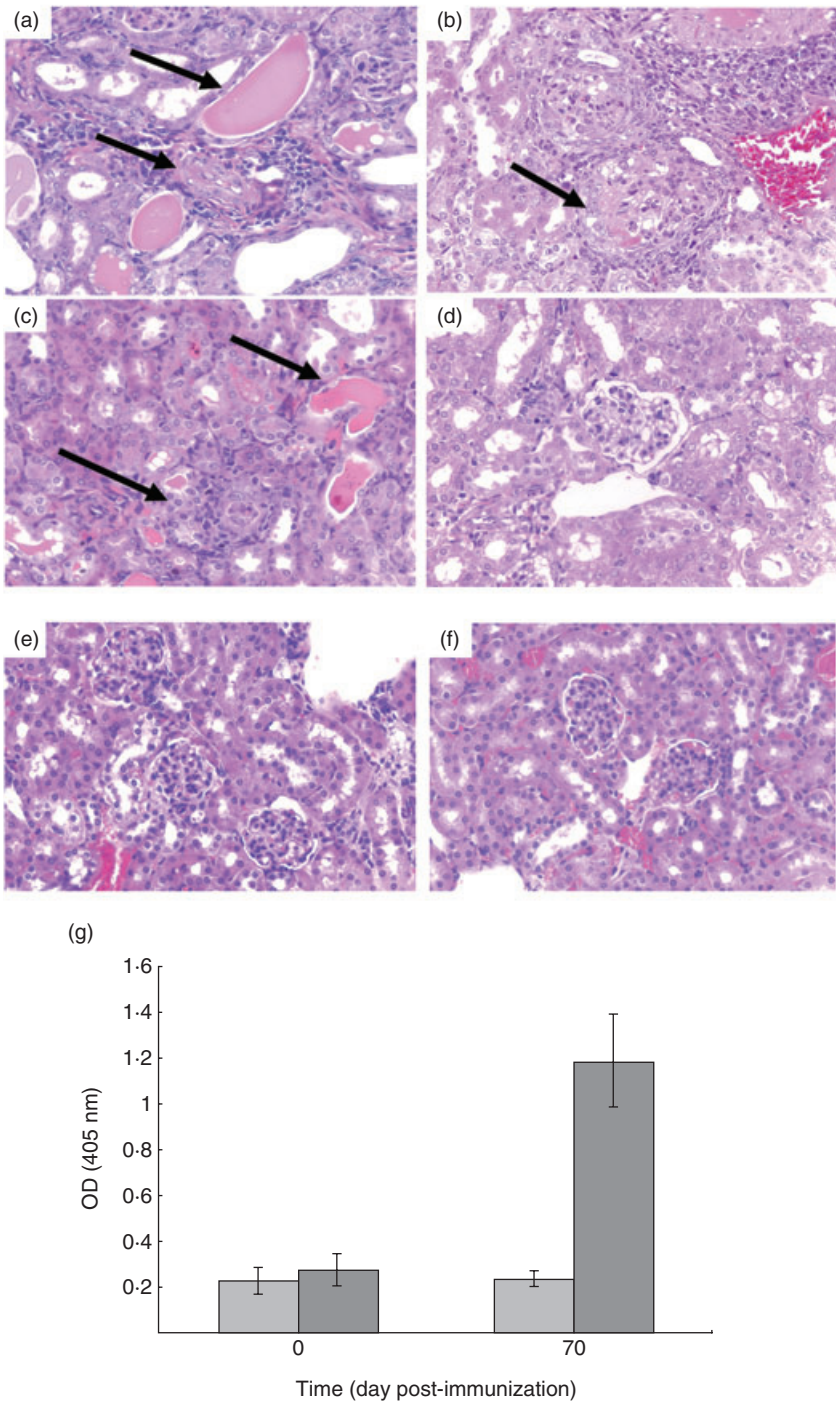

Fig. 6. Kidney sections from adoptively transferred mice. All NOD–SCID mice that received splenocytes from recombinant mouse proteinase 3 (rmPR3)-immunized NOD mice developed kidney pathology: (a) small artery fibrinoid necrosis (arrow) and protein cast (arrow); (b) glomerular fibrinoid necrosis (arrow); (c) crescentic glomerulonephritis (arrow). (d) No pathology was observed in any NOD–SCID mice that received splenocytes from mice immunized with complete Freund's adjuvant (CFA) alone. Kidney sections from C57BL/6-RAG-1$^{-/-}$ mice that received splenocytes from either (e) rmPR3-immunized mice or (f) adjuvant-alone-immunized mice reveal no pathology. Original magnification is ×400. (g) The anti-PR3 antibody titre was measured using enzyme-linked immunosorbent assay. The C57BL/6RAG1$^{-/-}$ mice adoptively transferred with splenocytes from rmPR3-immunized mice (■) ($n = 3$) and adjuvant-alone-immunized mice (■) ($n = 2$) were bled at different time-points.

was 19·0% (standard deviation 7·6%; range 15·6–31·0%). The average percentage of glomeruli with either hypercellularity or apoptotic debris was 18·4% (s.d. 5·5%; range 11·2–29·6%). There was no evidence of interstitial nephritis. Necrosis and crescents were not observed in any NOD–SCID mice that received splenocytes from CFA-alone-immunized mice (Fig. 6d).

No gross pathological changes were observed in mice that developed nephritis. We conducted histological examination of heart, lungs and large vessels and found no signs of pathological changes.

Kidney pathology does not develop after transfer of splenocytes from immunized C57BL/6 mice to immunodeficient C57BL/6-RAG-1$^{-/-}$ recipients

To assess if the anti-PR3 immune response would result in kidney pathology in another mouse strain which does not

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develop autoimmune disease spontaneously, we isolated splenocytes from rmPR3-immunized and adjuvant-alone-immunized C57BL/6 mice. Splenocytes ($6–8 \times 10^7$) from immunized mice were administrated via the tail vein into immunodeficient C57BL/6-RAG-$1^{-/-}$ mice.

We used C57BL/6-RAG-$1^{-/-}$ mice to achieve similar T and B cell immunodeficiency on a C57BL/6 background. RAG-$1^{-/-}$ mice, rather than SCID mice, were used to avoid 'leakiness'. Note that although the SCID mutation is not significantly 'leaky' on a NOD background, it is 'leaky' on a C57BL/6 background (Jackson Laboratories; strain description, http://jaxmice.jax.org/strain/001303.html).

In contrast to NOD–SCID recipients, which showed clinical signs of renal failure, C57BL/6-RAG-$1^{-/-}$ recipients appeared healthy and had no clinical signs of renal failure. Histopathological analysis of kidney tissues from C57BL/6-RAG$1^{-/-}$ recipients did not reveal pathology in any of the recipients up to 70 days after the transfer of splenocytes from either rmPR3-immunized or adjuvant-only-immunized donors (Fig. 6e,f). While the recipient mice did not develop kidney pathology, the titres of PR3-specific antibodies in the serum of the recipients, which received cells from rmPR3-immunized C57BL/6 donor mice, were increased significantly compared to the antibody titres in the control recipient mice, as determined by rmPR3-specific ELISA (Fig. 6g).

Discussion

Vasculitis and severe segmental and necrotizing glomerulonephritis consistent with WG developed in immunodeficient NOD–SCID mice upon transfer of splenocytes from the rmPR3-immunized NOD mice. Our findings strongly support a pathogenic role for anti-PR3 immune responses in PR3-ANCA-associated renal diseases. However, our data also indicate that PR3-specific autoimmune responses need other factors, such as genetic background and autoimmune predisposition of the strain for the development of kidney pathology. Similar to findings in humans, anti-PR3 responses were not always pathogenic. Kidney pathology developed only when the anti-PR3 response was transferred adoptively into NOD–SCID mice, but not when anti-PR3 responses were induced in wild-type NOD mice. Interestingly, the disease did not develop in immunodeficient C57BL/6 mice after the adoptive transfer, suggesting that complex and probably multi-genetic factors play a role in the regulation of disease development.

Previous attempts to determine if anti-PR3 immune responses were pathogenic were complicated by the low homology between human and murine PR3. Immunization of mice or rats with human PR3 resulted in anti-human PR3 antibodies, which did not cross-react with the murine homologue [32]. It was therefore difficult to determine if the lack of observed pathology in the immunized animals was the result of anti-human PR3 antibodies not recognizing the appropriate antigenic epitopes on the murine homologue, or if this was an indication that the anti-PR3 immune response is not pathogenic.

In the present study, we generated an enzymatically active mouse recombinant PR3 protein using a baculovirus/insect cell system. The rmPR3 was recognized by two different monoclonal antibodies specific for mouse PR3. Functional status of the rmPR3 was demonstrated by proteolytic activity towards a specific substrate. We have shown previously that this rmPR3 induces proinflammatory cytokine interleukin (IL)-18 and IL-18-dependent liver injury when injected in mice, further confirming its functional activity [37].

To elicit PR3-specific immune responses in mice, we immunized autoimmunity-prone NOD mice with enzymatically functional rmPR-3. This resulted in a high level of PR3-specific autoantibodies in the serum of the immunized mice.

The serum of rmPR3-immunized-, but not CFA-alone-immunized mice showed a staining pattern consistent with c-ANCA found in humans. The sera from rmPR3-immunized mice produced an intense granular cytoplasmic staining of neutrophils, resembling strongly c-ANCA-mediated staining of human neutrophils from patients with WG.

FCM analysis of peripheral blood of rmPR3-immunized mice revealed that approximately 20% of circulating white blood cells were coated with mouse IgG and all the coated cells were Mac-1 positive. This coating was not found in CFA-only-immunized mice, suggesting that the coating antibodies are PR3-specific.

Collectively, our data indicate that anti-rmPR3 antibodies generated in NOD mice after immunization with rmPR3 in CFA had binding profiles indistinguishable from c-ANCA detected in patients with WG, making our mouse model of autoimmune response to PR3 a suitable system to study the potential pathogenic role of anti-PR3 immune responses *in vivo*.

Despite the high titre of c-ANCA, rmPR3-immunized NOD mice showed no clinical signs of histopathological disease even 12 months after immunization, consistent with an interpretation that c-ANCA may not be always pathogenic. This interpretation is also consistent with reports of persistently high levels of circulating c-ANCA in some patients without any form of active vasculitis [2–4].

However, in addition to the intrinsic lack of pathogenicity of anti-PR3 immune responses, the absence of pathology may have resulted alternatively from rmPR3-specific antibodies not binding the mouse PR3 epitope related to the pathogenicity of c-ANCA. It is also possible that PR3-specific antibodies are a necessity, but not sufficient factor in disease induction. Finally, the regulatory arm of the immune responses may be able to control the pathology under some circumstances. Such control of autoimmune responses by the regulatory T cells has been demonstrated in multiple experimental systems [38–43].

Xiao *et al.* [28] have also demonstrated development of autoimmune responses when splenocytes or antibodies from $MPO^{-/-}$ mice immunized with murine MPO were transferred into $RAG^{-/-}$ mice, but not if the autoimmune cells were transferred into normal recipients.

To determine if anti-PR3 immune responses were pathogenic when the function of the regulatory arm of the immune responses was impaired, we transferred splenocytes from the rmPR3-immunized NOD mice into immunodeficient NOD–SCID mice. The immunodeficient recipient mice developed vasculitis and severe segmental and necrotizing glomerulonephritis, consistent with WG. No disease developed in NOD–SCID mice that received splenocytes from the control (CFA-alone-immunized donors), indicating that the development of autoimmunity was linked specifically to the presence of anti-PR3 immune responses, rather than immune responses against a component of CFA.

The vasculitis and necrotizing glomerulonephritis resulted in the death of 100% of the recipient mice, while no deaths were observed in mice that received splenocytes from CFA-only-immunized NOD mice. Histological analysis of the affected kidneys showed protein casts, crescentic glomerulonephritis, glomerular and small artery fibrinoid necrosis. Many of these features are found in patients with WG. It is possible that, when the function of regulatory arm of the immune responses is impaired, anti-PR3 immune responses result in a lethal vasculitis and glomerulonephritis.

Interestingly, immunization of C57BL/6 mice with rmPR3 also resulted in high levels of anti-PR3 antibodies, but the transfer of splenocytes from these mice into $RAG^{-/-}$ recipients did not result in kidney pathology. Multiple mechanisms could account for this observation. It is possible that the immunization of C57BL/6 mice, which are not prone to autoimmunity, did not result in the immune responses against antigenic epitopes of PR3 responsible for c-ANCA-mediated pathologies. It is also possible that multiple mechanisms contribute to the control of development of autoimmune vasculitis.

In summary, our results demonstrate that anti-PR3 immune responses have a potential to induce lethal vasculitis and glomerulonephritis with pathological changes in kidneys similar to those observed in patients with WG. PR3-specific autoimmune responses were pathological in NOD–SCID mice, but not wild-type NOD mice, suggesting that under some circumstances the functional immune response may be able to control the development of autoimmune processes. Further studies will be needed to elucidate the separate roles of CD4 and CD8 T cells, B cells and antibodies as well as the role of genetic background in this new mouse model of WG.

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Disclosure

The authors have nothing to disclose.

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