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## Short Communication

### Comparative aspects of murine proteinase 3

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## **Abstract**

The neutrophilic granule protein proteinase 3 (PR3) is the preferred target antigen of antineutrophil cytoplasmic antibodies (ANCA) found in the serum of patients with Wegener's granulomatosis, a systemic small-vessel vasculitis.

Due to the lack of data concerning the murine homologue of human PR3, we assessed the neutrophil marker polypeptide PR3 in mice by generating a murine-specific PR3 antibody.

In contrast to humans, peripheral blood leukocytes are not the main resource of murine PR3. Interestingly, we could show that the mouse bone marrow is the main PR3 source, indicating that it is a large reservoir for functional neutrophils. This pool of neutrophils could be rapidly mobilized after injection of IL-8.

The development of the new PR3 antibody provides a new tool for studying the maturation processes of the murine hematopoietic system and will also support the generation of infectious disease or vasculitis mouse models.

**Keywords:** proteinase 3, vasculitis, neutrophils, hematopoiesis

## Introduction

Human neutrophils, which constitute 50-70 % of blood leukocytes, are the first cell type at sites of inflammation. They form the first line of defence against harmful microorganisms and are equipped with bactericidal proteins in different subsets of granules and vesicles [reviewed in [1]]. For inbred mice, it has been shown, that the average neutrophil count is extremely variable from strain to strain [2] and that the lymphocytes are the dominant leukocytes in almost all strains [2, 3].

Anti-neutrophil cytoplasmic antibodies (ANCA) constitute a group of antibodies that are directed against myeloid proteins and could be detected in autoimmune vasculitis including Churg-Strauss syndrome, microscopic polyangiitis and Wegener granulomatosis [4]. The neutrophilic granule proteins proteinase 3 (PR3) and myeloperoxidase (MPO) have been identified as the primary target antigens in these disorders [5, 6].

Several attempts have been made to clarify whether these auto-Abs are directly involved in causing ANCA-associated glomerulonephritis and (pulmonary) vasculitis. In almost all of these studies, rodent models have been used to explore the role of the two most common autoantigens, proteinase 3 (PR3) and myeloperoxidase (MPO), in these diseases.

Whereas numerous animal models of MPO ANCA-mediated vasculitis have demonstrated the direct potential of anti-MPO Abs to induce glomerulonephritis and vasculitis in mice and rats [7-13], the development of PR3-ANCA vasculitis models has proven much more difficult. In one model, it was shown that circulating anti-PR3 antibodies lead to a local inflammation of the skin but not to systemic vasculitic lesions [14]. Although different mouse strains have been used to create PR3-associated vasculitis models, none of them could sufficiently reproduce the clinical manifestations that are noticed in humans.

In order to assemble more data concerning the expression of PR3 in different mouse strains, we generated a new antibody, which recognizes a defined epitope of murine PR3. This antibody was used to detect the neutrophil marker PR3 in the bone marrow, spleen and in the peripheral blood.

Our work presents previously unknown features of murine PR3, which may contribute to a more comprehensive understanding of the mouse hematopoietic system and will facilitate the generation of infectious disease or vasculitis models.

## **Materials and methods**

### **Animals**

All mice were bred and housed in a specific-pathogen-free animal facility at the Johannes-Gutenberg University Mainz. Research involving mice has been conducted according to the guidelines of the German Animal Protection Law. The application for the experiments was approved by the responsible local authorities (Landesuntersuchungsamt Rheinland-Pfalz, reference number (1.5 177-07/021-13)).

### **Detection of human PR3**

For Western blot analysis of human PR3, PBL were isolated by dextran sedimentation (2 % dextran 500) from buffy coat. Mouse anti-huPR3 monoclonal antibody (1:1000; Peliclust, 200 ng/ $\mu$ l) was used to detect huPR3 in 20  $\mu$ g PBL or bone marrow lysate (Biocat, Troy, VA, USA).

## Differential cell counts

Blood samples from cardiac puncture (of three mice per strain) were taken into heparin to determine the relative number of neutrophil granulocytes in the peripheral blood of FVB-, 129-, BALB/C, MRL+/+- and MRL/Mp-Tnfrsf6<sup>lpr/lpr</sup> (MRL/lpr-mice). For this purpose, a drop of blood was placed onto a clean glass slide, fixed and stained with May-Gruenwald/Giemsa according to Pappenheim. Cell types were expressed as a percentage of white blood cells after manual counting 200 to 250 cells under light microscopy.

## Antibody generation and western blot analyses of mouse PR3

A rabbit anti-mouse PR3 polyclonal antibody was generated and affinity-purified using a muPR3-specific peptide N-RLGTQAPTPR-C (Eurogentec, Liège, Belgium) cross-linked to the carrier protein keyhole limpet hemocyanin (KLH). This peptide antibody was called EU90. A homology search for this sequence using a NCBI BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; blastp) found no significant homology except with murine proteinase 3.

Protein was isolated from cells or tissue in RIPA buffer with freshly added protease inhibitors (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml PMSF, 30 µg/ml aprotinin), and the protein concentration was quantified using the Bradford method (Protein Microassay; Bio-Rad) as described by the manufacturer. Homogenates were separated by SDS-PAGE in 12 % polyacrylamide gels, transferred to nitrocellulose (Schleicher&Schuell) and immunoprobed overnight with our own rabbit anti-mouse PR3 peptide antibody EU90 (diluted 1:500) or with the goat anti-mouse PR3 antibody P-20 (Santa Cruz, sc-19748, diluted 1:500). The latter antibody is raised against an epitope within the amino acids 91-150 of mouse PR3 (Santa Cruz, personal communication). The filters were probed with goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004

diluted 1:5,000) or with rabbit anti-goat IgG-HRP (Santa Cruz, sc-2922 diluted 1:5,000) for 1 h. The HRP signal was visualized using an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, England).

## Isolation of leukocytes and bone marrow cells

Leukocytes were isolated from FVB, BALB/c, 129S6, C57BL6, MRL<sup>+/+</sup> and MRL/lpr mice according to Giuliano and Warner [15]. Briefly, a mouse anesthetized and then blood was collected by cardiac puncture (~1 ml per mouse) with a heparinized syringe. Peripheral leukocytes were isolated from whole blood by dextran sedimentation of erythrocytes. Following sedimentation of the red cells, the upper leukocyte-containing layer was removed and deprived of contaminating erythrocytes by hypotonic lysis. We isolated bone marrow from mice as previously described [16]. The erythrocytes were lysed with NH<sub>4</sub>Cl and the remaining cells were passed through a nylon mesh (40 µm; Falcon) to remove debris. Subsequently, bone marrow cells and leukocytes were lysed with RIPA buffer containing protease inhibitors and the protein concentration was determined by a protein microassay (Bio-Rad) according to Bradford [17]. 20 µg of each leukocyte extract and 3-5 µg of each bone marrow lysate was used for western blot analyses.

## Immunocytochemistry of leukocytes and bone marrow cells

Leukocytes and bone marrow were isolated as described above. After the last centrifugation step, cells were resuspended in PBS and adjusted at a concentration of  $5 \times 10^5$  cells per 20 µl. A drop of these cell preparations was dropped on poly-L-lysine-treated glass slides. For immunocytochemistry, the slides were air dried, fixed in ice-cold methanol for 5 min, and stored at -20° C before use. Detection of PR3 was performed using the affinity purified rabbit anti mouse PR3 antiserum described above (primary antibody) and biotinylated goat anti-rabbit IgG

(secondary antibody, Linaris). applied at 1:400 dilution and incubated for 1 h at room temperature. After a 1 h incubation with avidin–biotin complex at room temperature (Vector Laboratories) the color development was achieved by application of the chromogen Diamino Benzidine (DAB, Vector Laboratories). Brown stain was visualized after 2–3 minutes by light microscopy. Slides were rinsed in tap water, counterstained in Mayer's hematoxylin, dehydrated, cleared, and coverslipped. Negative controls were prepared on by following all immunostaining procedures except that the primary antibody was replaced with purified rabbit serum IgG, diluted in the same way as the primary correlate.

## Isolation of kidney cells and splenocytes

Both kidneys and the spleen of FVB or MRL/lpr mice were removed and kept frozen at -70°C until used. 0.1 g of kidney and spleen tissue was homogenized in ice cold RIPA buffer [PBS (PH 7.4), 0.5 % (wt/vol) sodium deoxycholate, 1 % (vol/vol) Nonidet P-40, 0.1 % (wt/vol) SDS, 3 % (vol/vol) aprotinin, 1 mmol/l  $\text{Na}_3\text{VO}_4$ , and 100  $\mu\text{g}/\text{ml}$  phenylmethylsulfonyl fluoride (PMSF)] for 30 min. Homogenates were centrifuged at 10,000 g for 10 min at 4°C, and protein concentrations were determined on supernatants using the Bradford method (Protein Microassay; Bio-Rad).

## IL-8 mobilization of neutrophils

Neutrophils were mobilized as described by Laterveer et al. [18]. Per FVB or BALB/C mouse, 5  $\mu\text{g}$  purified, recombinant human IL-8 (more active 72 aa-variant [19], Hölzel Diagnostika) was diluted in 100  $\mu\text{l}$  PBS and administered as a single intraperitoneal injection. 20-30 minutes after the injection, peripheral blood was drawn by cardiac puncture. The release of neutrophils from the bone marrow into the blood stream was controlled with standard H&E-stain of blood smear. Whole-cell extracts of peripheral blood leukocytes and bone marrow lysates were fractionated by

polyacrylamide gel electrophoresis, blotted to a nitrocellulose membrane and then PR3 was visualized as described above.

## Results

### The proportion of neutrophils varies greatly between inbred strains

Evaluating the relative count of blood neutrophils there were significant differences between different mouse strains. The number of neutrophils varied from 7.2 to 28.0 % in proportion to other blood cells (Fig. 1) but never reached the percentage observed for human blood neutrophils (40-75 %). The strains with the lowest relative count were FVB (7.2 %) and 129S6 (10.7 %), the mice with the highest relative count MRL<sup>+/+</sup> mice (28.0 %).

### In the bone marrow, proteinase 3 is considerably more expressed than in the peripheral blood

Due to the lack of data concerning the murine homologue of human PR3, we generated a anti-PR3 rabbit antiserum and tested for PR3 specificity by immunostaining of bone marrow cells and leukocytes of BALB/c mice. To enhance the specificity of the antibody for murine PR3, a defined PR3-epitope of ten amino acid residues and not the whole protein was used for immunization (Fig. 2A). Whereas the bone marrow cells showed variable staining results (from poor to intensive), the polymorphonuclear leukocytes of the peripheral blood were intensively stained. As the lymphocytes and monocytes were not stained for PR3, the antibody was proved to be specific for polymorphonuclear cells, i.e. neutrophils.

Western blot analysis of six different mouse strains revealed that the “lupus-prone” strains MRL+/+ and MRL/lpr had the highest concentration of PR3 in PBL, followed by BALB/C-, 129S6- and C57BL/6-mice, whereas the PR3 antigen concentration was low in PBL of FVB mice (Fig. 2B). The PR3 content corresponded well with the neutrophil count in the peripheral blood (Fig. 1) of these mouse strains. In every case, the PR3 content was always much higher in the bone marrow than in the peripheral blood.

Mouse PR3 was identified as a triplet band at about 29-kDa in six different mouse strains by SDS-PAGE followed by western blotting. Two potential N-glycosylation sites could be identified by the presence of the consensus sequence Asn-Xaa-Thr and the presence of nonpolar amino acids around the glycosylation sites[20] (Fig. 2A). An additional double band at 22 kDa could also be detected (2B). This peptide is always coexpressed with full-length PR3, independently of the protease inhibitor cocktail used during the isolation procedure. This result could be confirmed by the use of the commercial PR3-antibody P-20, which recognizes a different epitope upstream of our antibody EU90.

Strong expression of mouse PR3 was observed in bone marrow cell lysates, but expression in blood leukocytes was either very low (FVB mice) or much lower than that seen in bone marrow. This observation was in contrast to the human system, where most of the PR3 antigen could be found in the PBL but not in the bone marrow (Fig. 3).

## FVB and BALB/C inbred mice respond to IL-8 injection with neutrophils' release from bone marrow

Both, FVB and BALB/C mice were treated with IL-8 or PBS buffer as a negative control. In the case of FVB mice PR3 antigen wasn't almost detectable in the blood of PBS-treated mice, but became visible by immunoblot analysis 20-30 min after the IL-8 injection (Fig. 4A). PR3 antigen

could neither be detected in the blood serum, nor in the supernatant of bone marrow cells, indicating that IL-8 didn't function as a degranulating agent for mouse neutrophils, as it had been described for human neutrophils [21]. The detection of PR3 in the blood coincided with the release of neutrophils from the bone marrow of FVB mice (data not shown). In general, the bone marrow, as well as the blood of BALB/C mice contained more PR3 antigen than that of FVB mice (Fig. 2A). The higher PR3 content of BALB/C-blood was correlated with a higher relative neutrophil count in the BALB/C strain in comparison to FVB mice. As could be demonstrated by H&E stain and western blot analysis, the IL-8 injection in BALB/C mice also resulted in a relative higher neutrophil count (data not shown) and in consequence, to a higher PR3 content of peripheral blood.

## **Discussion**

In the past years there has been many attempts aiming at clarifying the pathogenesis of ANCA-associated vasculitis *in vivo*. In almost all of these studies, rodent models have been used to explore the role of the two most common autoantigens, proteinase 3 (PR3) and myeloperoxidase (MPO), in these diseases. Whereas numerous animal models of MPO ANCA-mediated vasculitis have demonstrated the direct potential of anti-MPO Abs to induce glomerulonephritis and vasculitis in mice and rats [7-13], the development of PR3-ANCA vasculitis models has been proven much more difficult.

Since cloning and genomic localization of the murine PR3 [22-24], only a few further reports were published concerning mouse PR3. In one study, it was shown that circulating anti-PR3 antibodies only lead to a local inflammation of the skin but not to systemic vasculitic lesions [14]. In a second PR3-ANCA model, no pathological abnormalities could be detected neither in the kidney nor in lungs of rodents immunized with chimeric human/mouse PR3 [25].

One more reason for the inability to create a functional PR3-vasculitis model is the fact that human PR3 is more basic than murine PR3, which accounts for a stronger interaction of the antigen with the negatively charged cell surface and thus for an increased pathogenicity of the antigen-ANCA complex in humans [26]. Furthermore, Jenne and colleagues have pointed out that there is a 69 % homology between human and mouse PR3 indicating that the epitopes of human PR3 recognized by human autoantibodies are not preserved on mice PR3 [27].

As neutrophils are the main source of human PR3, we first studied the relative count of neutrophils in the peripheral blood of six different inbred mouse strains. We were able to show that the relative neutrophil count in the blood of mice is strongly reduced compared to human blood. The number of granulocytes varied greatly between strains (7.2-28.0 %) but never reached the quantities observed for human blood neutrophils (40-75 %). Thus, our data is consistent with formerly published quantitative studies [2, 3], with the additional observation that the neutrophil count is highest in autoimmune strains with MRL-background. Moreover, the neutrophil count in different mouse strains seems to be genetically determined because it hasn't changed over some decades. Similarly to the high relative count of neutrophils in the blood of MRL/lpr mice, the PR3 expression proved to be respectively high too. Although MRL mice are prone to a lupus-like symptoms (and lupus is not primarily a neutrophil disease), the inflammatory processes which are associated with this disease obviously lead to a release of neutrophils from the bone marrow to the peripheral blood.

For further investigations an anti-muPR3 rabbit antiserum was generated and tested for PR3 specificity. The immune serum recognized a major band of 29 kDa and two minor bands with a slightly higher molecular mass in the bone marrow, blood and spleen of six different mouse strains. This triplet of PR3 isoforms could also be detected for the human homologue and has first been described for human PR3 independently from each other by three groups [28-30]. It has also been

shown for the human enzyme, that its isoforms are generated by a differential “trimming” of Asn-linked oligosaccharides, rather than proteolytic processing of the protein core [31, 32]. Our results demonstrate that mouse PR3 does not only have the same molecular mass as its human counterpart, but also seems to be processed in the same manner.

However, immunoblots of mouse bone marrow and PBL showed an additional double band of about 22 kDa (Fig. 2B), which we had never detected in human PR3-preparations (data not shown). The doublet could also be detected by the alternative antibody P-20 reacting with a different epitope than EU90 (Fig. 2C). Although, there is a second in frame methionine codon within the PR3 mRNA (GenBank accession: NM\_011178), with exactly the same sequence context as the first one (GCCUGGCCAUGG), the antibody P-20 would not be able to bind to the related truncated peptide (see Figure 2A). For this reason, it is likely that these additional bands are breakdown peptides and not artefact bands. As addition of a cocktail of protease inhibitors before cell lysis did not efficiently stabilize murine PR3, our study indicate that this enzyme is more prone to degradation than its human homologue.

In humans PR3 is one of the most abundant antigens in circulating neutrophils and only traces of this enzyme could be detected in the bone marrow (Fig. 3B). However, strong expression of mouse PR3 was observed in bone marrow cell lysates, but the expression in blood leukocytes wasn't almost detectable (FVB mice) or much lower than that seen in bone marrow (Fig. 3A). This comes in accordance with the low relative neutrophil count of PVB in peripheral blood. Moreover, it was interesting to examine whether this peripheral “neutropenia” is responsive to stimulating factors as IL-8 [18, 33]. Indeed, 20-30 min after IL-8 injection an abrupt rise of the neutrophil marker protein PR3 could be detected in PBL lysates. Boxio et al. have pointed out that mouse bone marrow contains large numbers of functionally competent neutrophils [34]. It is evident that this reservoir is capacious enough to protect the “neutropenic” FVB mice from severe infections, as

it can be released within a few minutes by small chemotactic cytokines [35]. Furthermore, we could show that IL-8 didn't function as a degranulating agent for mouse neutrophils, as it had been described for human neutrophils [21].

In conclusion, our work shows that the expression profile of PR3 differs from mouse to human and presents previously unknown features of murine PR3, which may contribute to a more comprehensive understanding of the mouse hematopoietic system. In any case further studies for a thorough examination of murine PR3 and its characteristics in comparison to humans are necessary.

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## Figure legends

**Fig. 1** Proportion of lymphocytes and neutrophils in mouse blood. PBL from FVB, 129S6, BALB/c, MRL/lpr and MRL+/+ mice (three male mice per strain) were counted. The mean and standard deviation (error bars) of triplicate lymphocyte and neutrophil counts is given as a percentage of whole leukocyte content. The neutrophil columns represent 7.2 % (FVB), 10.7 % (129S6), 18.7 % (BALB/c), 19.0 % (MRL/lpr) and 28.0 % (MRL+/+) of total leukocytes

**Fig. 2** Expression and immunostaining of PR3 in blood and bone marrow of different mouse strains.

**a** Immunostaining of proteinase 3 in bone marrow and blood of BALB/c mice with a sequence-specific peptide antibody. BALB/c mice were used for staining due to the high neutrophil count in the peripheral blood compared to other mouse strains. Detection of PR3 was performed using affinity purified rabbit anti-mouse PR3 antiserum directed against the residues 161-170 (blue letters) of the preproPR3. The serum only recognized polymorphonuclear granulocytes and their precursors (brown) and not lymphocytes (grey). Two potential N-glycosylation sites (N) of the muPR3 are highlighted, as well as the three methionine residues (M) and the epitope recognized by the peptide antibody P-20 (red). Note: Slides were counterstained in Mayer's hematoxylin

**b** Expression of PR3 in blood and bone marrow of FVB-, C57BL/6-, 129S6-, BALB/c-, MRL/+/- and MRL/lpr-mice. Note: 20 µg PBL lysate (PBL) but only 3 µg bone marrow lysate (BM) were loaded per lane. Mouse PR3 was identified as a triple band at 29 kDa in all different mouse strains. An additional double band at 22 kDa could also be detected.

**c** Immunoblot analysis of the specificity of anti PR3 peptide antibody Eu90 and the commercial peptide antibody P-20 (Santa Cruz). Although these two antibodies detect different PR3-epitopes, both antibodies did not only

detect the 29 kDa PR3 polypeptide in the bone marrow lysate but also a smaller peptide of about 22 kDa. Note that an extra band of about 16.5 kDa is solely recognized by the antibody P-20. Kidney lysate (ki) was used as a negative control.

**Fig. 3** Expression of PR3 in human and mouse PBL and bone marrow cells. PBL and bone marrow protein lysates were separated by standard 12 % SDS-polyacrylamide gel electrophoresis and immunoblotted with an anti-mouse PR3-peptide antibody (**a**) or a monoclonal anti-human PR3-antibody (**b**). **a** Expression of PR3 in blood, bone marrow, kidney and spleen of FVB mice. Note, that 40 µg lysate of PBL, kidney and spleen, but only 5 µg bone marrow lysate (BM) were loaded per lane. **b** Detection of PR3 in human PBL and BM lysates (10 µg/lane). Purified huPR3 antigen was used as a positive control. Full-length PR3 bands are marked by arrows

**Fig. 4** Detection of muPR3 in FVB- and BALB/C mice after IL-8 injection. Protein lysates from different mouse strains were separated by polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with a muPR3-specific peptide antibody. **a** In the case of FVB mice the neutrophil marker protein PR3 wasn't almost detectable in the blood of PBS-treated mice (**a**, left side), but become visible by immunoblot analysis 20 min after the huIL-8 injection (**a**, right side). **b** A significant amount of PR3 can be constitutively detected in the blood of untreated BALB/c mice, which is considerably increased after IL-8 injection.



