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c-ANCA induce neutrophil-mediated lung injury –a model of acute Wegener´s Granulomatosis

Short title: Anti-PR3-antibodies mediate acute lung injury

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Abstract

Anti-neutrophil-cytoplasmic antibodies (c-ANCA) targeting proteinase 3 (PR3) are implicated in the pathogenesis of Wegener´s Granulomatosis (WG). Fulminant disease can present as acute lung injury (ALI).

In this study, a model of ALI in WG was developed using isolated rat lungs. Isolated human polymorphonuclear leukocytes (PMN) were primed with tumor necrosis factor (TNF) to induce surface expression of PR3. Co-perfusion of TNF-primed neutrophils and monoclonal anti-PR3-antibodies (anti-PR3) induced a massive weight gain in isolated lungs. This effect was not observed when control-IgG (IgGc) was co-perfused with TNF-primed PMN. The ANCA-induced edema formation was paralleled by an increase in the capillary filtration coefficient as a marker of increased pulmonary endothelial permeability. In contrast, pulmonary artery pressure was not affected. In the presence of the oxygen radical scavenger superoxide dismutase and a NADPH-oxidase inhibitor, ANCA-induced lung edema could be prevented. Inhibition of neutrophil elastase was equally effective in preventing ANCA-induced lung injury.

In conclusion, anti-PR3-antibodies induce neutrophil mediated, elastase- and oxygen radical-dependent ALI in the isolated lung. This experimental model supports the hypothesis of a pathogenic role for c-ANCA in WG and offers the possibility to develop therapeutic strategies for the treatment of lung injury in fulminant WG.

Keywords

acute lung injury, anti-neutrophil-cytoplasmic antibodies, neutrophil activation, Wegener´s Granulomatosis

Introduction

Acute lung injury (ALI) is a rare but life-threatening complication of Wegener´s Granulomatosis (WG) [1]. In WG, ALI usually occurs together with acute glomerulonephritis as pulmonary-renal syndrome, and is characterized by diffuse alveolar hemorrhage with edema formation and extravasation of blood components, mainly erythrocytes and neutrophils, into the alveolar space [2]. Histologically, ALI in WG is defined by pulmonary capillaritis with disruption of pulmonary endothelial integrity as a putative underlying pathophysiological mechanism [3].

Activation and transmigration of neutrophils are thought to play a major role in the development of ALI [4] and in some experimental models, the severity of ALI is diminished upon neutrophil depletion [5]. Neutrophil-derived mediators implicated in the pathogenesis of ALI are reactive oxygen species (ROS), proteases, cationic proteins as well as proinflammatory cytokines such as TNF [6, 7]. These mediators were also found to be elevated in the circulation and, in case of MPO and peroxidase, in the broncho-alveolar fluid (BALF) of patients with active WG. The levels of these neutrophil-derived products were shown to correlate with clinical disease activity [8-10]. Moreover, circulating neutrophils display an activated phenotype in active WG [11] and neutrophil depletion substantially diminished the severity of experimentally induced ANCA-associated vasculitis [12]. Thus, activated neutrophils seem to be major effector cells in the development of inflammatory tissue injury associated with WG.

The mechanisms of neutrophil activation in WG have been extensively studied over the past decades, and, apart from their outstanding role as diagnostic marker, circulating “classic” anti-neutrophil cytoplasmic autoantibodies (c-ANCA) targeting the neutrophil granule protein proteinase-3 (PR3) have been attributed with pathogenic potential in the development of inflammatory vascular lesions in WG, as they can activate neutrophils *in vitro* [13]. In

neutrophils, the autoantigen PR3 is expressed on the cell surface in response to priming with proinflammatory cytokines such as TNF [14]. Binding of anti-PR3-antibodies to neutrophils activates a wide variety of inflammatory cellular functions such as respiratory burst, degranulation and the release of lipid mediators and cytokines [15-17]. In leukocyte-endothelial co-cultures, ANCA-activated PMN adhere to and lyse endothelial cells [18, 19]. While ANCA targeting myeloperoxidase (MPO) have recently been shown to cause vasculitis and glomerulonephritis in an animal model [20], little is known about the pathogenic role of anti-PR3-antibodies in the development of pulmonary lesions in active WG. Against this background, the aim of the present study was to clarify whether PR3-ANCA are involved in the pathogenesis of pulmonary dysfunction in WG. In essence, it was found that PR3-ANCA potently activate neutrophils in the pulmonary circulation of isolated rat lungs resulting in the development of pulmonary edema. Edema formation was attributable to neutrophil release of elastase and oxygen radicals in response to anti-PR3-antibodies. Thus, a pathogenic role of PR3-ANCA in lung injury in WG is suggested.

Materials and Methods

Materials

Sterile Krebs-Henseleit-hydroxyethylamylopectin buffer was obtained from Serag-Wiessner (Naila, Germany). The buffer contained 120 mM NaCl, 4.3 mM KCL, 1.1 mM KH_2PO_4, 24 mM $NaHCO_3$, 2.4 mM $CaCL_2$, 1.3 mM $MgCL_2$ and 2,4 g/l of glucose as well as 5% (wt/vol) hydroxyethylamylopectin (mol wt 2000.000) as an oncotic agent. O_2, CO_2, and N_2 were obtained from Messer Griesheim (Herborn, Germany).

Ficoll-Paque was purchased from Pharmacia (Upsalla, Sweden). Superoxide dismutase (SOD), AAPVKC, and isotype control mouse IgG_1 (MOPC-21) were obtained from Sigma (Deisenhofen, Germany), while recombinant human TNF-α was from R&D Systems (Wiesbaden, Germany). The NADPH oxidase inhibitor diphenylene iodonium (DPI) was obtained from Merck (Schwalbach, Germany), while the 5-lipoxygenase-(5-LO) activation inhibitor MK-886 was from Biomol (Hamburg, Germany).

Phosphate-buffered saline was obtained from Gibco Laboratories (Grand Island, NY, USA). All other biochemicals were obtained from Merck (Darmstadt, FRG).

The murine monoclonal anti-PR3-antibody 4A5 (mouse IgG) was purchased from Wieslab AG (Lund, Sweden), while the rabbit polyclonal anti-human MPO-antibody (DAKO A398) used for histologic staining was from Dako (DAKO, Hamburg, Germany). Phycoerythrin-(PE)-conjugated goat-anti-mouse IgG was from Dako (Hamburg, Germany), and pooled human IgG (Octagam) was from Octapharma (Langenfeld, Germany).

Isolation of human neutrophils

Neutrophils were isolated from venous blood of healthy donors by centrifugation over a Ficoll-Paque gradient as previously described [17]. In brief, EDTA-anticoagulated blood was layered over Ficoll-Paque and centrifuged at 400 × *g* for 35 min. After removal of mononuclear cells, erythrocytes were sedimented in 1% polyvinyl alcohol. Residual

erythrocytes were removed by hypotonic lysis, cells were washed twice in Ca^{2+}/Mg^{2+}-free PBS, and resuspended in PBS containing $CaCl_2$ (1 mM) and $MgSO_4$ (1 mM) at 10^7 PMN/ml. Cell purity was >97%, as quantified by flow cytometry, and cell viability was >96%, as assessed by trypan blue dye exclusion. Prior to addition to the lung perfusate, neutrophils were treated with TNF (2 ng/ml) for 30 min to induce surface expression of PR3.

Flow cytometry

For the determination of surface expression of PR3, flow cytometry was performed. In brief, isolated neutrophils (5×10^6/ml) were incubated with TNF (2 ng/ml) or sham-incubated for 30 min. Cells were pelleted at 4 °C, and resuspended in PBS containing 0.1% BSA and 0.02% sodium azide. Then, 2×10^5 cells were distributed to each well of flexible round bottom microtiter plates and washed. Prior to the addition of monoclonal antibodies, 20 µl of pooled human immunoglobulin (100 µg/ml) were added to block Fcgamma receptors. Murine monoclonal anti-PR3-antibodies (10µg/ml) or mouse control IgG (10µg/ml) were then added, and incubated for 30 min at 4 °C. After three washes, the secondary antibody, a PE-conjugated goat-anti-mouse IgG (50 µg/ml) was added and again incubated for 30 min at 4 °C. After three final washes, cells were resuspended in PBS and kept on ice until flow cytometric analysis. Flow cytometry was performed in a FACScan (Becton-Dickinson, Mountain View, CA, USA) using forward and orthogonal light scatter to select viable cells. Software CellQuest® research software (Becton-Dickinson, Mountain View, CA, USA) was used to analyze the generated data.

Lung isolation and perfusion

The model of the isolated rat lung has already been described in detail [21, 22]. In brief, male CD rats (body weight 350-450 g) were deeply anaesthetized with sodium pentobarbital (100 mg/kg body weight i.p.). After local anesthesia with 2% xylocaine and median incision,

the trachea was dissected and a tracheal cannula was immediately inserted. A median laparotomy was performed and subsequently the rats were anticoagulated with 1000 units of heparin. Mechanical ventilation was started with 5.3 % CO_2, 21% O_2 and 73.7% N_2 (tidal volume 4 ml, frequency 65/min, end exspiratory pressure 2 cmH_2O) using a small animal respirator KTR-4 (Hugo Sachs Electronic, Germany). After midsternal thoracotomy the right ventricle was incised, a cannula was fixed in the pulmonary artery, and the apex of the heart was cut off to allow pulmonary venous outflow. Simultaneously pulsatile perfusion with KHB containing 5% hydroxyethyl starch was performed and the lungs were carefully excised and placed in a supine position. A cannula was fixed through the left ventricle in the left atrium to obtain a closed perfusion circuit without leakage. After extensive rinsing of the vascular bed, the lungs were perfused with a pulsatile flow of 13 ml/min (total volume 150 ml) in a recirculating model. The left atrial pressure was set at 2 mm Hg under baseline conditions (0 referenced at the hilum) and the whole perfusion system was equilibrated at 37.5 °C. Lungs selected for the study were those that 1) had a homogenous white appearance without signs of hemostasis or edema formation, 2) had pulmonary artery and ventilation pressures in the normal range and 3) were isogravimetric during a steady state period of 30 min.

The pulmonary artery pressure (P_{pa}) , pulmonary venous pressure (P_{pv}) and the weight of the isolated organ were registered continuously, with P_{pa} being a direct measure of pulmonary vascular resistance.

The venous pressure was repeatedly elevated by sequential hydrostatic challenge of 10 cm H_2O for 8 min. After the steady state period lung weight was set at zero and lung weight gain was calculated as the weight difference before and after each hydrostatic challenge maneuver.

The capillary filtration coefficient was calculated from the slope of weight gain as described previously [23].

Experimental protocols

Lungs without any drug application were perfused under standard conditions for 180 min. After a control hydrostatic challenge (30 min after the initiation of perfusion) TNF-primed neutrophils were added to the perfusate at a final concentration of 10^6/ml. When indicated, anti-PR3-antibodies (4A5, 10 µg/ml) or equal amounts of isotype-matched control-IgG (MOPC-21) were co-perfused with human neutrophils. All pharmacological agents (DPI, SOD, MK-886 and AAPVKC) were admixed to the perfusate before the first hydrostatic challenge, and were re-administered prior to the third hydrostatic challenge.

Histologic Examination.

At the end of the perfusion period, one of each rat lungs were fixed by instillation with formaldehyde solution (4.5%, pH 7.0) via the main bronchi. Fixation was allowed to proceed overnight at RT. Subsequently, tissues were dissected and routinely embedded in paraffin and sections of 5 µm were stained with hematoxylin-eosin. The other lungs were snap-frozen in liquid nitrogen after instillation with OCT (Tissue Tek, Sakura, Japan) via the main bronchi. Frozen tissues were stored at -80 °C. A few minutes before the preparation of cryosections using a cryomicrotome (Leica Jung CM 3000 Bensheim, Germany) the lung tissues were dissected into smaller tissue blocks and 5 µm cryosections were prepared. After drying at RT overnight, these sections were stored at -20 °C for a few days and stained with anti-myeloperoxidase (1:6000) using the APAAP ChemMate kit (K5000, DAKO, Hamburg). Hematoxylin was used for counterstaining. A Zeiss Axioskop 40 (Carl Zeiss, Jena, Germany) with Plan-NEO Fluar 20×/0.5 NA objective lenses, in connection with a JVC KY-S75U digital camera (JVC, Friedberg, Germany) was used to acquire the micrographs. Images were

processed with Diskus acquisition software, version 4.5 (Hilgers, Königswinter, Germany). A neutrophil aggregate was defined as intravascular neutrophil accumulation reaching an extent of more than tree diameters of alveolar septa in at least one dimension. Quantitation was done in duplicate by two blinded reviewers (R.M.B., R.M.).

Statistics

For statistical comparison, one-way analysis of variance (ANOVA) was performed, followed by Tukey`s honestly significant difference test when appropriate. A level of $P<0.05$ was considered to be significant.

Results

Anti-PR3 antibodies induce neutrophil-dependent edema formation in isolated rat lungs

In order to determine neutrophil surface expression of PR3, a prerequisite for specific ANCA-targeting, neutrophils were subjected to flow-cytometry after the isolation procedure. Freshly isolated neutrophils exhibited minor surface expression of the ANCA target antigen, while neutrophil priming with TNF (2 ng/ml) induced marked surface expression of PR3, as assessed by flow cytometry (Figure 1).

Under baseline conditions, isolated rat lungs were perfused for 180 min and baseline lung weight remained stable over the entire perfusion period (control). Perfusion of TNF-primed human neutrophils (10^6/ml) did not induce any weight gain, whereas after simultaneous perfusion of TNF-primed human neutrophils (10^6/ml) and monoclonal anti-PR3-antibodies (10 µg/ml) a massive pulmonary edema formation, quantified as weight increase, developed (Figure 2). Significant weight gain was noted after 140 min of perfusion. Occasionally the experiment was prematurely terminated due to massive fluid accumulation after 180 min. In contrast, co-perfusion of isotype-matched control-IgG and TNF-primed human neutrophils did not exert any effect on pulmonary physiology. Likewise, perfusion of anti-PR3-antibodies in the absence of human neutrophils or in the presence of unprimed neutrophils (data not given) was ineffective.

Histological and immunohistochemical examination of isolated lungs revealed the retention of human neutrophils in the isolated perfused rat lung. Neutrophil transmigration into the alveolar lumen was sometimes observed in lungs perfused with human neutrophils (Figure 3 a-c). However, there was no differences in the degree of neutrophil infiltration between anti-PR3 and control-IgG perfusion (table 1).

Edema formation in anti-PR3-treated lungs is accompanied by microvascular leakage response

The capillary filtration coefficient ($K_{f,c}$), calculated from the slope of weight gain after stepwise elevation of the venous pressure, remained stable during the entire perfusion period in untreated lungs as well as upon perfusion with TNF-primed neutrophils (10^6/ml). However, when anti-PR3-antibodies were co-perfused with human neutrophils, a dramatic increase in $K_{f,c}$ values paralleled edema formation (Figure 4). Significant elevations of $K_{f,c}$ values were noted after perfusion of TNF-primed neutrophils and anti-PR3 antibodies. As anticipated from the lung weight data, perfusion of anti-PR3-antibodies alone, as well as co-perfusion of TNF-primed neutrophils and mouse control IgG did not induce any alterations to the microvascular barrier function in isolated lungs.

The pulmonary artery pressure is not affected by anti-PR3-antibodies

In control experiments, P_{pa} values were constant (6-7 mmHg) throughout the entire experimental period. While the P_{pa} did rise slightly immediately after addition of human neutrophils, the P_{pa} remained stable over the three-hour perfusion period and, notably, co-perfusion of anti-PR3-antibodies or control-IgG did not induce any significant changes in pulmonary artery pressure (Figure 5).

Effect of oxygen radical scavengers on anti-PR3-induced pulmonary edema formation

In the presence of SOD (300 U/ml), catalyzing the dismutation from superoxide (O_2^-) to hydrogen peroxide (H_2O_2), the autoantibody-induced weight increase was strongly attenuated (Figure 6a). The NADPH oxidase inhibitor DPI (5 µg/ml) was equally effective as SOD, thus suggesting that neutrophil NADPH oxidase was the source of superoxide in our model. Similarly impressive was the effect of these oxygen radical suppressors on the disturbed microvascular barrier function: in the presence of SOD, the elevation of $K_{f,c}$ values upon

treatment of isolated lungs with anti-PR3-antibodies and TNF-primed neutrophils was nearly normalized, and the NADPH oxidase inhibitor was equally protective (Figure 6b).

Role of neutrophil elastase, but not 5-lipoxygenase products in anti-PR3-induced lung edema formation

As anti-PR3-induced pulmonary edema formation was clearly dependent on the presence of neutrophils, the contribution of other neutrophil mediators to ALI was investigated. Inhibition of 5-lipoxygenase activation with MK-886 (7.5 μM) did not prevent pulmonary edema formation, as measured by changes in the weight of isolated lungs (Figure 7a) or increase in $K_{f,c}$ values (Figure 7 b). Interestingly, both responses were strongly attenuated, and in case of $K_{f,c}$, normalized to control values, when the specific neutrophil elastase inhibitor AAPVKC (5 μM) was co-perfused with neutrophils and anti-PR3-antibodies (Figure 6a and 6b). Combined inhibition of ROS by SOD and neutrophil elastase by AAPVKC had no additive or synergistic effect (data not given).

Discussion

Severe clinical presentations of WG, such as pulmonary-renal syndrome or isolated alveolar hemorrhage are characterized by the occurrence of ALI and require immediate treatment. Since anti-PR3-antibodies have been suggested to posses pathogenic potential in WG, the question was posed weather these autoantibodies could induce lung injury.

Compelling evidence is provided in the present study documenting that anti-neutrophil-cytoplasmic antibodies, targeting the neutrophil serine protease PR3, are pathogenic in an isolated rat lung model causing ALI. Upon perfusion of monoclonal anti-PR3-antibodies and TNF-primed human neutrophils in isolated rat lungs, an increase in lung vascular permeability occurred which proceeded to severe vascular leakage and edema formation. This effect was not attributable to enhanced capillary filtration pressure, as the pulmonary artery pressure remained stable despite anti-PR3-challenge. Anti-PR3-induced lung injury was strictly dependent on the presence of target-antigen expressing, pre-activated human neutrophils, and was caused by neutrophil oxygen radical formation and elastase degranulation, whereas 5-LO-metabolites were not involved.

The isolated lung model was selected to investigate the pathogenic role of anti-PR3-antibodies for several reasons: *First*, although, an elegant model for anti-MPO-associated renal vasculitis has been established [20], no valid animal model for pulmonary WG exists. *Second,* the lungs are, apart from the kidneys, the organ system most frequently affected in fulminant presentations of WG, and *third*, the isolated lung model allows the registration of acute pathophysiological changes typical for ALI, such as edema formation, and increases in vascular permeability or pulmonary artery pressure.

The experimental model facilitated the perfusion of isolated rat lungs with TNF-primed human neutrophils and the well defined and widely used mouse anti-human monoclonal anti-

PR3-antibody 4A5. The co-perfusion of human neutrophils as target cells for the murine monoclonal anti-PR3-antibody 4A5 was necessary, as mouse-anti-human PR3-abs did not cross-react with rat neutrophils in previous studies [24, 25] and antibodies to rat PR3 are, as the state of our knowledge, not available. Clearly, the isolated lung model only reflects acute pathogenic effects of anti-PR3-antibodies and does not replace an animal model of anti-PR3 associated vasculitis. Recently, successful immunization of rodents with chimeric human/mouse PR3 and subsequent induction of an autoimmune response to mouse and rat PR3 has been performed; thus providing new perspectives to study the pathogenesis of anti-PR3 associated vasculitis [25].

Human neutrophils have been used in various neutrophil-dependent models of organ injury in the rat [22, 26], and were currently shown to be effectively "trapped" in the alveolar capillaries of these animals. However, although only anti-PR3-antibodies, and not control-IgG induced neutrophil mediated lung injury in the current model, we could not observe any morphological differences between anti-PR3 and control-IgG perfused lungs. This discrepancy might be due to the fact that the documentation of PMN infiltration, which did not differ in terms of quantity for both anti-PR3 and IgG perfused lungs, does not necessarily reflect any changes in the activation status of neutrophils, as also described for other models of lung injury (27). The quantitation of *in situ* neutrophil activation, for example, by *in situ* detection of ROS or elastase degranulation, would be helpful in this context, but is beyond the scope of the current study. Moreover, severe damage to the structural integrity of the lungs cannot be expected to occur in the current short-time experiments and due to methodological limitations (for example, the use of a protein-free perfusate), edema formation could only be assessed by physiologic parameters, such as weight gain and increase in the capillary filtration coefficient, which was indeed specific for anti-PR3 antibodies.

The capacity of the anti-PR3-antibodies to induce lung injury was strictly dependent upon the presence of PR3-expressing, TNF-primed human neutrophils, as the antibody *per se* did not induce any changes in the isolated rat lung physiology over the entire perfusion period. Thus, any unspecific disruption of pulmonary microvascular integrity by the currently used anti-PR3-antibody may be excluded. Moreover, neither perfusion of isotype-matched control-IgG and TNF-primed neutrophils did increase lung vascular permeability, nor did co-perfusion of anti-PR3 and unprimed neutrophils, which do not express surface PR3, as documented by the currently performed flow cytometric studies. Therefore, specific targeting of PR3, which was shown to be expressed on the surface of isolated leukocytes after TNF-priming, in line with previous studies [14], and the subsequent initiation of multiple, now well characterized signaling events [28, 29], seem to underlie the ANCA-mediated lung injury. As the experiments were performed in the absence of complement sources, complement-dependent mechanisms, which have been implicated in many forms of antibody-dependent leukocyte activation, including models of transfusion-related ALI [30], were not operative in the anti-PR3-associated lung injury.

Although the exact molecular mechanisms of anti-PR3-induced leukocyte activation are not completely understood, anti-PR3-related lung injury was clearly caused by reactive oxygen species formation as well as elastase liberation from anti-PR3-activated neutrophils: in the presence of the oxygen radical scavenger SOD, catalyzing the reduction from superoxide (O_2^-) to hydrogen peroxide (H_2O_2), the ANCA-induced edema formation was completely reversed. Obviously, superoxide and not hydrogen peroxide is the primary oxygen metabolite responsible for the ANCA-mediated increase in vascular permeability. The fact that edema formation could be prevented by the NADPH oxidase inhibitor DPI suggests that NADPH oxidases are the major source of superoxide release in ANCA-induced lung injury. Although DPI interferes with the mitochondrial superoxide production [31], the clear dependence of microvascular leakage on the presence of human neutrophils gives strong arguments for

neutrophil NADPH-Oxidase as the source of superoxide in anti-PR3-induced lung injury. Well in line with this reasoning, the capacity of anti-PR3-antibodies to induce NADPH oxidase-dependent neutrophil superoxide formation has been repeatedly proven *in vitro* [15, 16], and *in vivo*, the degree of tissue injury in WG correlates with the presence of toxic oxygen metabolites released by activated neutrophils [32]. Moreover, increased levels of markers of oxidative stress, such as exhalative H_2O_2 and blood lipid peroxidation levels were found in patients with ALI [7].

Apart from oxygen radicals, elastase was apparently centrally involved in mediating ANCA-induced lung injury, as demonstrated by the protective effect of the highly specific elastase inhibitor AAPVCK [33]. A key role for neutrophil elastase has been postulated in models of lung injury due to its capacity to degrade extracellular matrix proteins and protease inhibitors [34]. A role for elastase in the pathogenesis of pulmonary injury in WG is well established: the protease has been found to be elevated in the BALF and in the circulation of patients with active disease [8, 10], is liberated from neutrophils upon stimulation with anti-PR3-antibodies [15, 17] and was recently defined to mediate endothelial cell damage by ANCA-activated neutrophils in vitro [34], thus well in line with the present results.

Interestingly, inhibition of elastase *or* superoxide formation completely reversed the anti-PR3-induced lung injury and combined application of both inhibitors could not amplify this effect. These findings might be related to the previously postulated synergy between oxidants and neutrophil elastase in lung injury: while elastase was shown to increase oxygen radical formation in macrophages [35] and pulmonary epithelial cells [36], oxygen radicals were found to inactivate endogenous elastase inhibitors and to increase elastase toxicity in the isolated rat lung [37]. Thus, oxidant- and elastase-mediated tissue destruction are obviously not independent events in this model of ANCA-induced lung injury either, and the simultaneous release of both mediators is mandatory for the effects observed.

It has previously demonstrated that neutrophils release leukotrienes in response to stimulation with anti-PR3-antibodies [17], and 5-LO-products have been shown to be involved in leukocyte-mediated pulmonary injury [30]. However, a highly specific inhibitor of 5-lipoxygenase activation did not exert any protective effects in anti-PR3-induced lung injury here. Thus, leukotrienes do not contribute to tissue injury in this model. This might be related to the fact that leukotriene generation is critically dependent on the presence of the substrate arachidonic acid which was not provided in the present studies. Although a role for leukotrienes in mediating ANCA-induced tissue injury can not be ruled out *in vivo*, these mediators do not contribute to ALI under the experimental conditions employed.

In conclusion, autoantibodies targeting neutrophil PR3 may be crucially involved in the development of lung injury in WG. Synergistic effects of anti-PR3- induced neutrophil oxygen radical formation and elastase degranulation were identified as central mechanisms mediating microvascular damage. Pharmacological interference with these mediators might be protective in acute lung injury in Wegener´s Granulomatosis.

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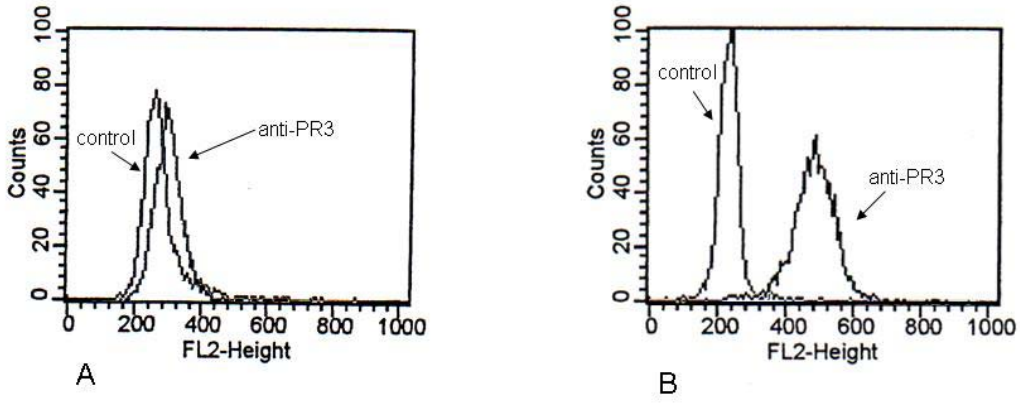
Figure legends

Figure 1

Surface expression of PR3 in isolated neutrophils in response to TNF

Isolated neutrophils (5×10^6/ml) were either sham-incubated (A) or primed with TNF (2 ng/ml) (B) for 30 min. Following incubation with isotype control IgG (control, 10 µg/ml) or anti-PR3-antibodies (anti-PR3, 10 µg/ml), a PE-labeled goat-anti-mouse IgG (50 µg/ml) was added, and flow cytometry was performed. Note the strong staining for PR3 in TNF-primed versus unprimed PMN, as depicted in one out of four representative flow cytometric studies.

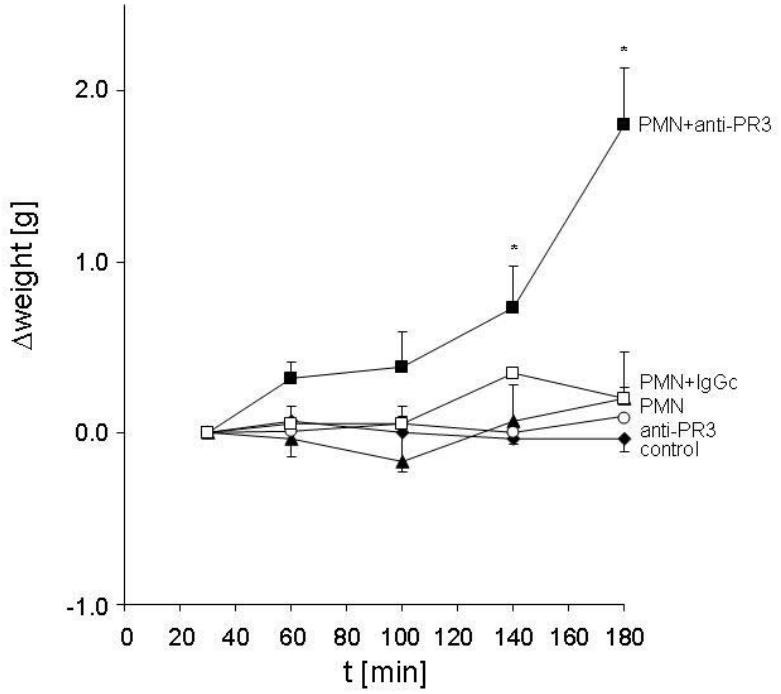


Hattar et al., Figure 1

Figure 2

Effect of anti-PR3-antibodies on lung weight in isolated rat lungs.

After an isogravimetric steady-state period of 30 min, a control hydrostatic challenge was performed, and lung weight was set to zero. Isolated rat lungs were either perfused with TNF-primed neutrophils (10^6/ml) alone (PMN) or PMN were given together with murine monoclonal anti-PR3 antibodies (10 µg/ml) (PMN+anti-PR3) or equal concentrations of an isotype-matched control-IgG (PMN+IgG_c). As a control group, lungs were buffer-perfused without the administration of human neutrophils (control) over the entire perfusion period or anti-PR3-antibodies were perfused in the absence of neutrophils (anti-PR3). Lung venous pressure elevations were performed at indicated time points. Changes in lung weight (Δ weight) were quantified after each hydrostatic maneuver and are expressed as change from baseline. Data reflect mean ± SEM of at least five independent experiments. Values indicated (lungs perfused with anti-PR3-antibodies and neutrophils) differ significantly from controls, PMN, PMN+IgG, and mono-anti-PR3-perfused lungs ($P< 0.05$).

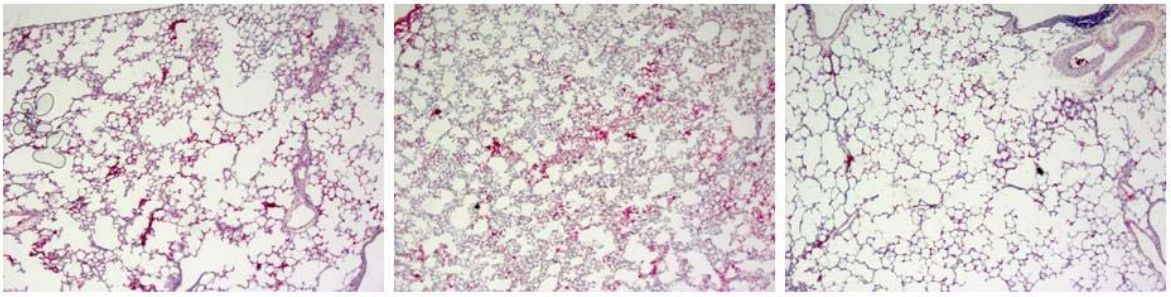


Hattar et al., Figure 2

Figure 3

Accumulation of human neutrophils in isolated rat lungs

Isolated rat lungs were buffer-perfused (A) or perfusion with TNF-treated human neutrophils (10^6/ml) (B + C) was performed. Anti-PR3-antibodies (10 µg/ml) (B) or equal amounts of isotype-matched control-IgG (C) were co-perfused with human neutrophils. Repeatedly, hydrostatic challenges were performed by elevation of the venous pressure**.** At the end of the perfusion period of 180 min, rat lungs were harvested. Paraffin-embedded tissues and OCT-embedded frozen tissues of superior lobe were stained with hematoxylin-eosin, additionally paraffin-embedded tissue sections were stained with anti-myeloperoxidase/hematoxylin. Note the accumulation of human neutrophils in the alveolar capillaries of rat lungs (B+C), as depicted for representative (n=3) immunostained slides of each group (2.5 × magnification).



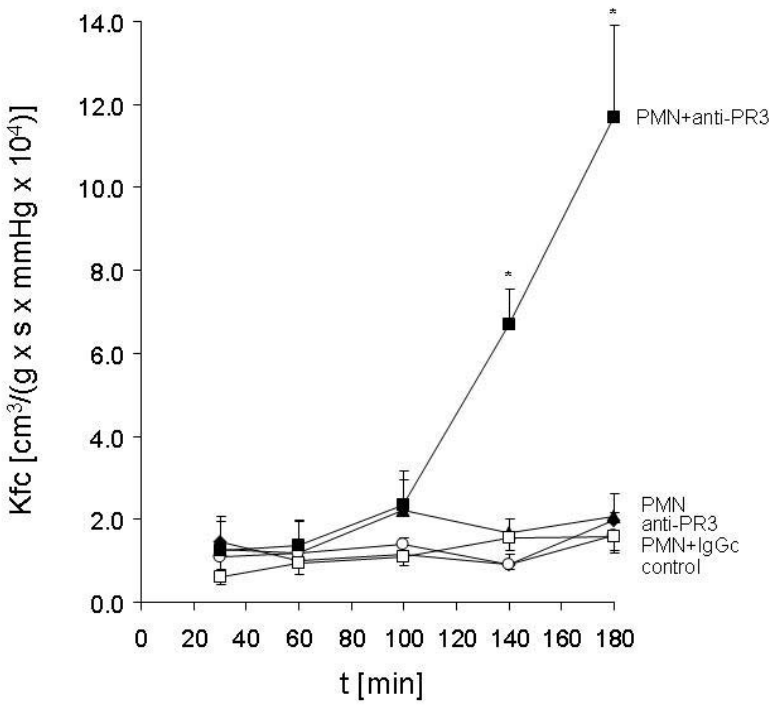
A B C

Hattar et al., Figure 3

Figure 4

Effect of anti-PR3-antibodies on microvascular leakage in isolated rat lungs.

Following an isogravimetric steady state period of 30 min, isolated rat lungs were either perfused with TNF-primed neutrophils (10^6/ml) alone (PMN) or PMN were given together with murine monoclonal anti-PR3 antibodies (10 µg/ml) (PMN+anti-PR3) or equal concentrations of an isotype-matched control-IgG (PMN+IgG_c); alternatively, anti-PR3-antibodies were perfused in the absence of neutrophils (anti-PR3). Controls represent buffer-perfused lungs. Lung venous pressure elevations for determination of the capillary filtration coefficient ($K_{f,c}$) were performed at indicated time points. Data reflect mean ± SEM of at least five independent experiments. Values marked (lungs perfused with anti-PR3-antibodies and neutrophils) differ significantly from controls, PMN, PMN+IgG, and mono-anti-PR3-perfused lungs ($P < 0.05$).

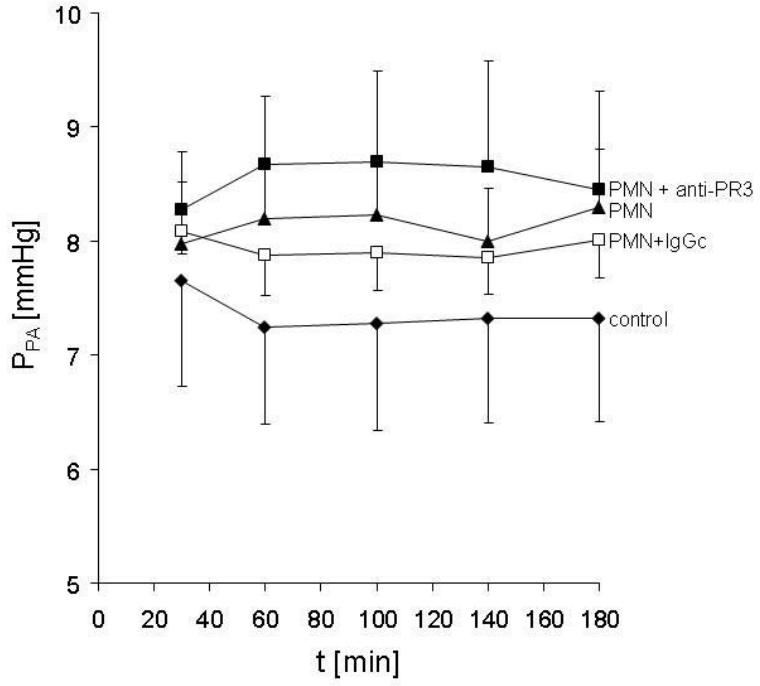


Hattar et al., Figure 4

Figure 5

Effect of anti-PR3-antibodies on the pulmonary artery pressure in isolated rat lungs.

Isolated rat lungs were either buffer-perfused for 180 min (control) after a steady state period, or perfusion with TNF-primed human neutrophils (10^6/ml) alone (PMN) or in combination with murine monoclonal anti-PR3 antibodies (10 µg/ml) (PMN+anti-PR3) was performed. Alternatively, isotype-matched control IgG (10µg/ml) was perfused with PMN (PMN+IgG_c). The pulmonary artery pressure (P_{pa}) as a direct marker of vascular resistance was registered continuously. Data reflect mean ± SEM of at least five independent experiments. No significant changes in P_{pa} were noted between the different experimental groups.



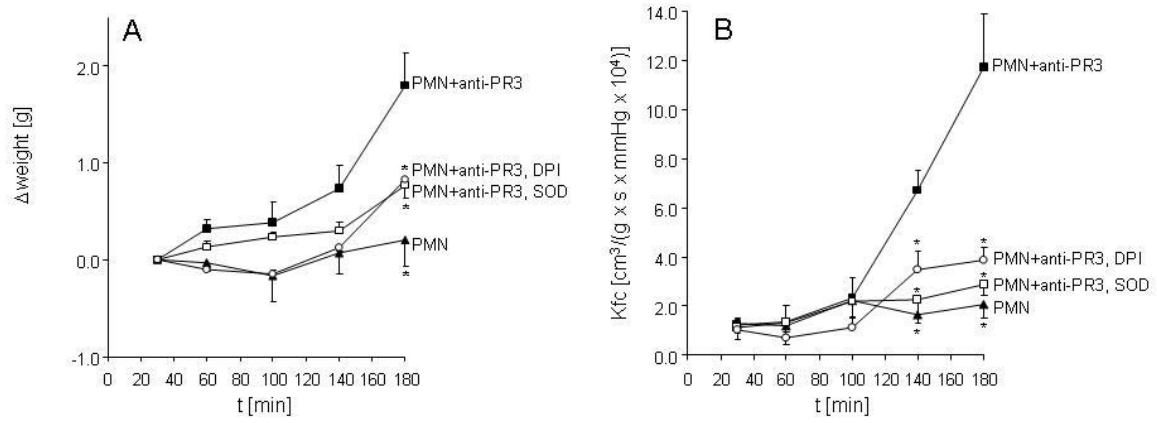
Hattar et al., Figure 5

Figure 6

Effect of oxygen radical suppressors in anti-PR3-induced edema formation (Figure 6a) and leakage response (Figure 6b) in isolated rat lungs.

After an isogravimetric steady state period of 30 min, isolated rat lungs were either perfused with TNF-primed neutrophils (10^6/ml) alone (PMN) or PMN were administered together with murine monoclonal anti-PR3 antibodies (10 µg/ml) in the absence of any pharmacological agents (PMN+anti-PR3) or in the presence of SOD (300 U/ml) (PMN+anti-PR3, SOD) or DPI (5 µg/ml) (PMN+anti-PR3, DPI). SOD and DPI were admixed before the first hydrostatic challenge (30 min) and again given before the third hydrostatic challenge (90 min). Lung venous pressure elevations were performed at indicated time points, and lung weight gain was quantified after each hydrostatic

maneuver and is expressed as change from baseline (Figure 6a), while the capillary filtration coefficient was determined from the slope of lung weight gain (Figure 6b). Data reflect mean ± SEM of at least three independent experiments. Values marked differ significantly from lungs treated with human neutrophils and anti-PR3-antibodies (PMN+anti-PR3) in the absence of any pharmacological agent ($P < 0.05$).



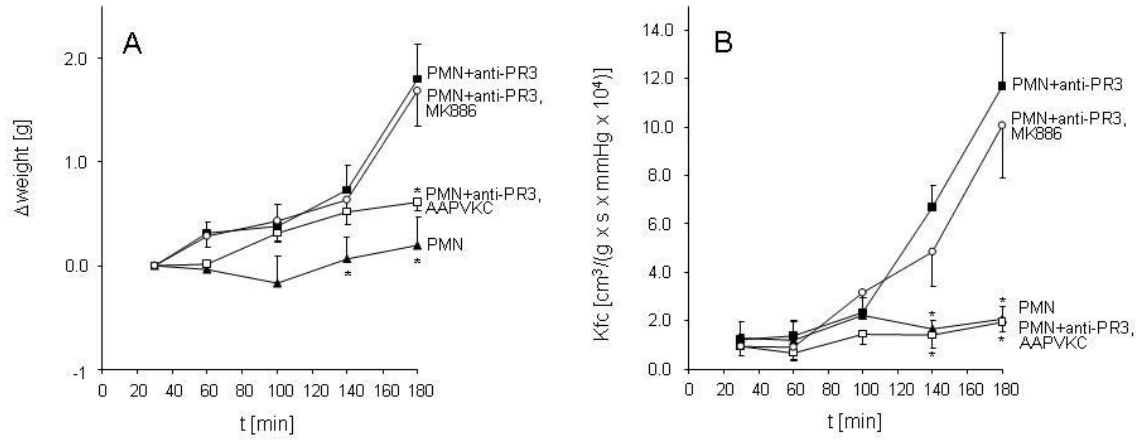
Hattar et al., Figure 6

Figure 7

Effect of elastase and leukotriene inhibition in anti-PR3-induced edema formation (Figure7a) and leakage response (Figure 7b) in isolated rat lungs.

Isolated rat lungs were either perfused with TNF-primed neutrophils (10^6/ml) alone (PMN) or PMN and murine monoclonal anti-PR3 antibodies (10µg/ml) (PMN+anti-PR3)

in the absence of any pharmacological agents (PMN+anti-PR3), or the elastase inhibitor AAPVKC (5 µM) (PMN+anti-PR3, AAPVKC) or the 5-LO-antagonist MK-886 (7.5 µM) (PMN+anti-PR3, MK-886) were co-perfused with PMN and anti-PR3-antibodies. Both inhibitors were administered before the first and again before the third hydrostatic challenge. Hydrostatic challenges were repeatedly performed at indicated time points and lung weight gain was quantified after each hydrostatic maneuver and is expressed as change from baseline (Figure 7a). In Figure 7b, the capillary filtration coefficient is given and expressed in $cm^3/(g \times s \times mmHg \times 10^4)$. Data reflect mean ± SEM of at least three independent experiments. Values marked differ significantly from lungs treated with human neutrophils and anti-PR3-antibodies (PMN+anti-PR3) in the absence of elastase or 5-LO inhibition ($P < 0.05$).



Hattar et al., Figure 7

Table 1

Semiquantitative analysis of human neutrophil retention in isolated rat lungs

Isolated rat lungs were buffer-perfused (control) or perfusion with TNF-treated human neutrophils (10^6/ml) and anti-PR3-antibodies (PMN+anti-PR3) or istotype-matched control-IgG (PMN + IgGc) was performed. Repeatedly, hydrostatic challenges were performed by elevation of the venous pressure**.** At the end of the 180-min perfusion period, rat lungs were harvested. Paraffin-embedded tissues and OCT-embedded frozen tissues of superior lobe origin were stained with hematoxylin-eosin, additionally paraffin-embedded tissues were stained with anti-myeloperoxidase/hematoxylin. Neutrophil retention was expressed as number of neutrophil aggregates, which were defined as intravascular neutrophil accumulation reaching an extent of more than tree diameters of alveolar septa in at least one dimension. Quantitation was done in duplicate from the upper lobe in an tissue area of 21,36 mm² (rectangular area selected at low power view) by two blinded reviewers (R.M.B., R.M.) The number of neutrophil aggregates is given/mm^2 and values are expressed as means +/- SEM, n=3. Values marked (*) differ significantly from controls. No differences were obtained between the reviewers, nor between the anti-PR3 and IgGc group.

	PMN aggregates/mm^2 (Reviewer 1)	PMN aggregates/mm^2 (Reviewer 2)
control	0 +/- 0	0 +/- 0
PMN+anti-PR3	*10.33 +/- 3.95	*15.00 +/- 6.6
PMN+IgGc	*9.33 +/- 1.66	*12.33 +/- 2.68