Phagocyte NADPH Oxidase Restrains the Inflammasome in ANCA-Induced GN

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ABSTRACT

ANCA-activated phagocytes cause vasculitis and necrotizing crescentic GN (NCGN). ANCA-induced phagocyte NADPH oxidase (Phox) may contribute by generating tissue-damaging reactive oxygen species. We tested an alternative hypothesis, in which Phox restrains inflammation by downregulating caspase-1, thereby reducing IL-1β generation and limiting NCGN. In an antimyeloperoxidase (anti-MPO) antibody-mediated disease model, mice transplanted with either gp91phox-deficient or p47phox-deficient bone marrow showed accelerated disease with increased crescents, necrosis, glomerular monocytes, and renal IL-1β levels compared with mice transplanted with wild-type bone marrow. IL-1β receptor blockade abrogated aggravated NCGN in gp91phox-deficient mice. In vitro, challenge with anti-MPO antibody strongly enhanced caspase-1 activity and IL-1β generation in gp91phox-deficient and p47phox-deficient monocytes compared with wild-type monocytes. This enhanced IL-1β generation was abrogated when caspase-1 was blocked. ANCA-induced superoxide and IL-1β generation were inversely related in human monocytes. Furthermore, transplantation of gp91phox/caspase-1 double-deficient bone marrow rescued the accelerated NCGN phenotype in gp91phox bone marrow-deficient mice. These results suggest that Phox-generated reactive oxygen species downregulate caspase-1, thereby keeping the inflammasome in check and limiting ANCA-induced inflammation. IL-1 receptor blockade may provide a promising strategy in NCGN, whereas our data question the benefit of antioxidants.


ANCA with specificity to proteinase 3 (PR3), myeloperoxidase (MPO), or lysosomal-associated membrane protein 2 is found in patients with systemic small vessel vasculitis and necrotizing crescentic GN (NCGN).1–4 Animal models firmly established ANCA pathogenicity for NCGN.5–10 Numerous in vitro experiments suggest that phagocyte NADPH oxidase (Nox) and granule proteins, including neutrophil serine proteases (NSPs), participate in ANCA-mediated endothelial damage.2,11–13 We used a murine ANCA NCGN model and recently showed that IL-1β provides an important disease mediator and that proteolytically active NSPs are essential for processing pro–IL-1β to IL-1β.14 The in vivo role of phagocyte Nox (Phox) in NCGN is, however, not yet established. Activated Phox consists of a heterodimeric heme protein from gp91phox (Nox2) and p22phox, complemented by p47phox, p67phox, p40phox, and Rac2.15 Catalytic oxidase activity is tightly regulated to precisely control reactive oxygen species (ROS) effects. Phox-induced ROS causes oxidative tissue damage through lipid and protein oxidation in several disease models.16–22 However, data from mice and humans indicate that ROS may also downregulate inflammation by not yet fully understood processes.23–26 In fact, failure to terminate the inflammation may...
provide a central mechanism for nonresolving inflammatory disease, including ANCA vasculitis.

We used a mouse model of anti-MPO Ab-induced NCGN and bone marrow (BM) from wild-type (WT), gp91phox-deficient, p47phox-deficient, and gp91phox/caspase-1 double-deficient mice, treatment with the IL-1 receptor antagonist, Anakinra, in vivo, and ROS-modifying maneuvers in ANCA-stimulated monocytes in vitro to establish that Phox activity is pivotal for restraining caspase-1 activity, thereby reducing IL-1β generation and abrogating NCGN. Recognition of this mechanism is important when considering ROS as a treatment target in ANCA vasculitis and probably, beyond.

RESULTS

BM from gp91phox−/− Mice Aggravated Disease in Anti-MPO Ab-Induced NCGN

We determined whether ROS generation by Phox exerts protective or detrimental effects in anti-MPO Ab-induced NCGN in vivo using our previously described mouse model.9 MPO-deficient animals were immunized with murine MPO, irradiated, and transplanted with BM from either WT or Phox-deficient (gp91phox−/−) animals. After 8 weeks, we found marked erythrocyturia, leukocyturia, and proteinuria in WT BM-transplanted mice. Hematuria and proteinuria were worse in mice transplanted with gp91phox−/− BM, whereas anti-MPO Ab titer did not differ between the groups (Figure 1, A and B). All mice in both groups developed glomerular necrosis and crescents. However, the percentage of these findings was markedly increased in mice that received gp91phox−/− BM instead of WT BM (60.7±8.9% versus 13.0±2.5% for crescents) (Figure 1, C and D). Thus, mice lacking a functional Phox were prone to severely accelerated anti-MPO Ab-induced NCGN. Moreover, neutrophil and macrophage influx in WT BM-transplanted mice was significantly increased when gp91phox−/− BM was transplanted (Figure 1, E and F). We did not observe granulomatous lesions in lungs or kidneys in any of the mice.

Accelerated NCGN Is Recapitulated in Mice Transplanted with p47phox−/− BM

Next, we used a different Phox-deficient mouse strain and tested whether the aggravated phenotype of anti-MPO Ab-induced NCGN occurred when BM from p47phox−/− mice was used. As before, mice were housed in specific pathogen-free facilities, but we extended the antibiotic prophylaxis (cotrimoxazole and neomycin) to exclude the possibility that disease acceleration was merely a consequence of infection. Moreover, an additional control group of MPO−/− mice that were not MPO-immunized but irradiated and transplanted with either WT or p47phox−/− BM was studied (n=5 in each group). None of these control mice developed infections (wasting, urine abnormalities, or pulmonary or renal signs of bacterial or fungal infection by histology) or any renal abnormalities within 8 weeks (data not shown). Compared with WT BM-transplanted mice, mice transplanted with p47phox−/− BM showed a trend to more urine pathology, had similar anti-MPO antibody titer, and had markedly accelerated renal disease (16.7±5.0% versus 45.5±10.1% for crescents) (Figure 2, A–D). Again, WT BM-transplanted mice showed a strong glomerular neutrophil and macrophage influx, with the latter being significantly increased in p47phox−/− BM-transplanted animals (Figure 2, E and F). Thus, we confirm accelerated anti-MPO Ab-induced NCGN with increased macrophage influx in two different Phox-deficient mice strains and exclude infection as the reason for accelerated NCGN.

Renal IL-1β Was Increased with Phox-Deficient BM, Whereas Anakinra Was Protective

Exploring potential mechanisms by which Phox-deficient myeloid cells accelerated anti-MPO Ab-induced NCGN, we asked whether Phox-deficient monocytes display a migration advantage to inflammatory sites. Phox-deficient and WT murine monocytes showed similar migration toward CCL2, ruling out an intrinsic migration advantage (Figure 3A). We next explored whether IL-1β could serve as a potent chemoattractant accelerating monocyte influx. However, human monocytes displayed no migration toward increasing IL-1β concentrations (Figure 3B). We asked whether metalloproteinase 9 (MMP-9), a metalloproteinase known to promote recruitment of proinflammatory macrophages into the kidney, was involved.27 MMP-9 activity in murine monocytes was upregulated in response to murine anti-MPO IgG using zymography (Figure 3, C and D). However, this induced MMP-9 activity did not differ between WT, p47phox−/−-deficient, and gp91phox−/−-deficient cells.

We focused on the inflammasome, because we recently found that IL-1β generation by monocytes was instrumental in inducing necrosis and crescents in this ANCA disease model. In fact, active NSPs were more important than caspase-1 in ANCA-induced IL-1β generation by monocytes.14 In our experiments, the accelerated disease phenotype of Phox-deficient BM showed strongly increased macrophage influx and IL-1β in kidneys from mice transplanted with gp91phox−/− and p47phox−/− BM compared with WT BM. The IL-1β values were 757.0±120.3 pg/mg with WT BM versus 1708.7±560.3 pg/mg in the two combined Phox-deficient BM-transplanted animals (P<0.01). To determine whether IL-1β is mechanistically involved in the aggravated NCGN phenotype, p47phox−/− BM-transplanted mice were either untreated (vehicle-treated) or received Anakinra. Anakinra significantly reduced but did not completely prevent urine pathology, crescents, necrosis, and glomerular inflammatory cell influx, whereas anti-MPO titers were not affected (Figure 4). These data implicate IL-1β as a mechanistic link for accelerated ANCA-induced NCGN in Phox-deficient mice. We reasoned that increased renal IL-1β could be merely a consequence of more infiltrating monocytes but also, could reflect increased anti-MPO IgG-induced IL-1β generation by the individual monocyte.
Figure 1. Aggravated NCGN in mice transplanted with gp91phox−/− bone marrow. Urine analysis and renal histology in MPO-immunized mice transplanted with WT or gp91phox−/− BM (n=6 per genotype), respectively: (A) dipstick analysis, (B) anti-MPO Ab by ELISA (AU is arbitrary units), (C) glomerular crescents and necrosis expressed as mean percentage of affected glomeruli, (D) typical examples for each transplanted group depicted with ×10 magnification in the upper panel and ×40 magnification in the lower panel, (E) glomerular monocyte/macrophage (MoMa) and neutrophil (PMN) influx expressed as the number of infiltrating cells per glomerulus, and (F) typical examples for each transplanted group depicted with ×40 magnification. *P<0.05; **P<0.01.
Phox-Deficient Monocytes Showed Increased Caspase-1 but Similar NSP Activity

We next investigated whether Phox deficiency resulted in more monocyte caspase-1 and/or NSP activity, thereby increasing IL-1β generation. We speculated that ROS may inhibit the catalytic activity of these enzymes and that Phox deficiency abolishes this inhibitory effect. Monocytes from either WT or Phox-deficient mice were stimulated in vitro with murine

Figure 2. Aggravated NCGN in mice transplanted with p47phox<sup>−/−</sup> bone marrow. Urine analysis and renal histology in MPO-immunized mice transplanted with WT or p47phox<sup>−/−</sup> BM (n=5 per genotype), respectively: (A) dipstick analysis, (B) anti-MPO Ab by ELISA, (C) glomerular crescents and necrosis expressed as mean percentage of affected glomeruli, (D) typical examples for each transplanted group depicted with ×10 magnification in the upper panel and ×40 magnification in the lower panel, (E) glomerular monocyte/macrophage (MoMa) and neutrophil (PMN) influx expressed as the number of infiltrating cells per glomerulus, and (F) typical examples for each transplanted group depicted with ×40 magnification. *P<0.05.
anti-MPO IgG or control IgG from mice immunized with BSA. Compared with WT monocytes, we observed significantly more caspase-1 activity in both gp91phox−/− and p47phox−/− monocytes challenged with anti-MPO IgG (Figure 5, A and B). Moreover, anti-MPO IgG-induced IL-1β generation was also significantly higher in gp91phox−/− and p47phox−/− monocytes compared with WT cells (Figure 5, C and D). Interestingly, anti-MPO IgG-induced IL-1β generation was similar in WT and Phox-deficient cells when a caspase-1 inhibitor was present, suggesting that increased IL-1β in Phox-deficient monocytes is mainly caspase-1–mediated. Given the fact that NSPs can also generate mature IL-1β, we then tested whether ROS inactivates their enzymatic activity. Cell lysates and supernatants were assayed under either basal or to induce ROS, PMA-stimulated conditions. We observed no difference in NSP activity between WT and Phox-deficient cells under either of these conditions (Figure 5, E and F). These findings support the contention that accelerated IL-1β generation in

Figure 3. Migration and MMP-9 activity do not differ between WT, gp91phox−/−, p47phox−/− monocytes and human monocytes do not migrate towards IL-1β. (A) Murine monocytes from WT, gp91phox−/−, or p47phox−/− mice were loaded to the upper well, whereas the lower well received either buffer control (unstim) or CCL2 (20 ng/ml). Migration was assessed after 4 hours. (B) Migration toward increasing IL-1β concentrations was estimated in human monocytes. (C and D) Murine monocytes were treated with buffer (unstim), TNF-α/anti-MPO IgG (T αMPO), or TNF-α/anti-BSA IgG (T αBSA). Active MMP-9 release in WT, gp91phox−/−, and p47phox−/− monocytes was assessed by zymography. (C and D) Typical SDS-PAGE gels are shown (upper panel) together with the corresponding statistical analysis (lower panel). AU, arbitrary units. *P<0.05; **P<0.01.
Phox-deficient monocytes was a consequence of accelerated caspase-1 activity. When we studied neutrophils, we observed much lower IL-1β amounts in response to anti-MPO IgG compared with monocytes. However, IL-1β was significantly higher in neutrophils from both gp91phox−/− and p47phox−/− mice compared with cells from WT mice (Figure 5, G and H). With these rather small IL-1β levels, caspase-1 activity was hardly detectable in either WT or Phox-deficient neutrophils (not shown).

**Phox-Dependent ROS in Anti-MPO Ab-Stimulated Human Monocytes Suppressed IL-1β**

We used human monocytes to study the relationship between Phox-dependent ROS generation and IL-1β production. We used SOD to shift superoxide to hydrogen peroxide and catalase to further shift the reaction toward oxygen and water. Primed human monocytes stimulated with anti-MPO Ab generated significant superoxide amounts, and superoxide was reduced to basal levels by a combination of SOD and catalase (Figure 6A). Parallel assessment of anti-MPO Ab-induced IL-1β generation by Western blot analysis (Figure 6B and C) and ELISA (Figure 6D) revealed an inverse relationship between ROS and IL-1β in that anti-MPO Ab increased IL-1β, and this increase was significantly higher when superoxide levels were decreased with SOD/catalase. This increased IL-1β generation under abolished superoxide conditions (SOD/catalase) was significantly reduced with caspase-1 inhibition, whereas caspase-1 inhibition had little effect on IL-1β generation when anti-MPO Ab-triggered superoxide was not abolished. These data from human monocytes are in agreement with the data obtained in Phox-deficient mouse monocytes in the absence or presence of a caspase-1 inhibitor (Figure 5) and compatible with a superoxide-mediated suppression of caspase-1 activity-dependent IL-1β generation.

**Caspase-1 Deficiency Protected from Accelerated NCGN in gp91phox−/− BM-Transplanted Mice**

To further substantiate the pivotal role of caspase-1 in accelerated ANCA-mediated NCGN, we generated gp91phox−/−/caspase-1 double-deficient mice by breeding caspase-1−/− and gp91phox−/− mice. In this set of experiments, MPO-deficient animals were immunized with recombinant instead of native murine MPO, which resulted in lower antibody titer (data not shown) and somewhat weaker renal disease after WT BM was transplanted (Figure 7). Assessing urine and renal histology in animals transplanted with BM from WT, gp91phox−/−, caspase-1−/−, or gp91phox−/−/caspase-1−/− double-deficient mice confirmed accelerated disease with gp91phox−/− BM (Figure 7); gp91phox−/− BM caused more hematuria, accelerated NCGN, and showed more leukocyte influx. In contrast, gp91phox−/−/caspase-1−/− double-deficient BM rescued this severely aggrivated disease phenotype in gp91phox−/− BM-transplanted mice and reduced crescents and necrosis to a similar extent as observed in WT BM-transplanted mice. These genetic data unequivocally establish the fact that increased caspase-1 activity, as a consequence of Phox deficiency, caused accelerated ANCA-induced NCGN. Our data also show that mice reconstituted with caspase-1−/− BM from WT showed similar disease. The data from these experiments suggested that caspase-1 activity caused accelerated NCGN when Phox was deficient but did not significantly contribute to the disease when Phox was functioning. Anti-MPO antibody titer did not differ between all four groups (data not shown).

**DISCUSSION**

Failure to terminate inflammatory responses may lead to nonresolving inflammation.28 This study provides genetic evidence that ANCA-induced Phox activity presents an important inflammation-limiting mechanism by downregulating the inflammasome and thereby, protecting from NCGN. Phox-deficient mice developed accelerated NCGN in an ANCA disease model with more monocyte influx and more renal IL-1β. Phox-deficient murine monocytes were unable to generate ROS and showed more caspase-1 (but similar NSP...
Figure 5. Increased caspase-1 activity and IL-1β generation in phox-deficient ANCA-treated monocytes and increased IL-1β generation in phox-deficient ANCA-treated neutrophils. Caspase-1 activity (AU is arbitrary units), IL-1β generation, and NSP activity in monocytes and neutrophils from WT, gp91phox<sup>−/−</sup>, and p47phox<sup>−/−</sup> mice. (A–D) Monocytes were either left untreated (unstim) or primed with TNF-α and subsequently stimulated with anti-BSA IgG (TαBSA) or anti-MPO IgG (TαMPO). Supernatants were collected.
activity) and higher IL-1β generation after stimulation with anti-MPO Ab compared with WT cells. This increased IL-1β generation in Phox-deficient cells was caspase-1-dependent and inversely correlated with superoxide amounts when cells were activated by anti-MPO Ab. Accelerated ANCA-induced NCGN in Phox-deficient mice was rescued genetically with concomitant caspase-1 deficiency, firmly establishing that increased caspase-1 activity as a consequence of Phox deficiency caused accelerated ANCA-induced NCGN. Our data question the use of medical interventions blocking Phox activity in ANCA vasculitis but implicate IL-1 receptor blockade as a promising therapeutic strategy.

Phagocytes are equipped with a strong Phox that is activated by several inflammatory conditions, including ANCA. After the Phox complex is assembled, the flavocytochrome transports electrons from NADPH to oxygen, resulting in superoxide and its subsequent derivatives. ROS are not only important for killing bacteria and fungi but also modify proteins and lipids and thereby, control cellular functions. However, if Phox activation is too strong, continues for too long, or occurs in the wrong localization, cytotoxic effects may occur. In addition to these well appreciated proinflammatory effects, Phox activity may be important for downregulating and terminating the inflammatory response. Patients with chronic granulomatous disease who lack functional Phox are not only susceptible to certain infections but also, present with uncontrolled noninfectious inflammation, including sterile tissue granulomas, bowel inflammation, and autoimmune disorders. The molecular basis for increased inflammation in chronic granulomatous disease is not fully understood. Gene expression profiling suggested increased proinflammatory transcriptional activity in Phox-deficient leukocytes. More recently, it was shown in patients that Phox deficiency caused increased caspase-1 activity and IL-1β generation. However, this issue remains a matter of debate, because several groups showed that ROS had either no effect or an accelerating effect on inflammasome-mediated IL-1β. Furthermore, the ROS source and the specific ROS molecule that controls inflammasome activity are not yet characterized. Some investigators favor Phox and other sources such as mitochondria. Complexity is further increased in that different NOD, LRR, and pyrin domain-containing 3 activators (e.g., asbestos versus noncystalline compounds) may use different activation pathways. Thus, it is mandatory to determine the interaction of Phox, ROS, and caspase-1 activity for each and every disease entity. Understanding the effects of the Phox–ROS–inflammasome pathway in a specific disease setting is particularly important when antioxidant treatment is considered. Numerous in vitro studies established an ANCA-mediated robust Phox-dependent respiratory burst in neutrophils and although less well studied, monocytes. Together with the fact that ROS modify and damage proteins and lipids, ANCA-induced ROS release is considered a key pathogenic component for necrotizing small vessel vasculitis. However, our data underscore the need to study candidate mechanisms in complex in vivo conditions. Using a murine ANCA disease model and two different Phox-deficient mouse strains, we show that Phox activation also provides an important protective element limiting NCGN in the presence of ANCA. These data seem a priori unexpected given the aforementioned assumptions derived from in vitro experiments using ANCA and phagocytes. It is reasonable to assume that ROS-mediated tissue damage also occurs in vivo but that Phox-induced ROS have additional consequences, including important inflammation-limiting effects that need to be considered. Our study was carried out in a disease model that strongly depends on an autoantibody that activates phagocytes. The role of Phox may differ in other disease models with different pathogenic components. In fact, Devi et al. showed in an antiglomerular basement membrane GN model that gp91phox can promote glomerular injury.

In our study, Phox deficiency was associated with higher monocyte influx. In vitro experiments suggested that this increased monocyte influx was not directly mediated by IL-1β as a chemoattractant, an intrinsic migration advantage in Phox-deficient macrophages, or differences in MMP-9 activity. It is conceivable that increased macrophage accumulation may result from increased chemoattractants released from either ROS-deficient macrophages or neutrophils or from resident glomerular cells interacting with these phagocytes. For example, IL-1β was reported to be a potent inducer of other cytokines, including IL-8 or CCL2. We found glomerular monocyte accumulation, significantly elevated renal IL-1β levels, and pharmacological IL-1 receptor blockade that protected Phox-deficient mice from NCGN, making Phox-regulated IL-1β generation an attractive candidate mechanism for disease acceleration. In the presence of ANCA, Phox deficiency caused accelerated monocyte caspase-1 activity and increased IL-1β generation in vitro. These effects were much more prominent in monocytes compared with neutrophils.

after 4 hours and analyzed. (A and B) Four or (C and D) six independent experiments, each with monocytes from three different mice, were performed. (E and F) NSP activity was determined in cell lysates (LYs) and supernatants (SNs) from unstimulated or PMA-stimulated monocytes. Three independent experiments, each with monocytes or neutrophils from two different mice, were performed. (A) Caspase-1 activity after 30 minutes of stimulation in WT and gp91phox−/− monocytes. (B) Caspase-1 activity in WT and p47phox−/− monocytes after 30 minutes of stimulation (n=4). (C) IL-1β generation in WT and gp91phox−/− monocytes. (D) IL-1β generation in WT and p47phox−/− monocytes. (E) NSP activity in WT, gp91phox−/−, and p47phox−/− monocyte lysates. (F) NSP activity in WT, gp91phox−/−, and p47phox−/− monocyte supernatants. (G) IL-1β generation in WT and gp91phox−/− neutrophils. (H) IL-1β generation in WT and p47phox−/− neutrophils. CI, preincubation with caspase-1 inhibitor. *P<0.05.
Caspase-1 inhibition significantly blocked ANCA-induced IL-1β generation in monocytes from both Phox-deficient strains in vitro but had little effect on ANCA-stimulated WT cells. These data implicate caspase-1 as an important mechanistic link between Phox deficiency and IL-1β generation and suggest that unrestrained caspase-1-mediated IL-1β generation is responsible for accelerated disease in the absence of Phox-generated ROS. Moreover, our in vitro data suggest that superoxide is instrumental in reducing caspase-1 activity and IL-1β generation. In agreement with our findings, Meissner et al. showed previously reduced caspase-1 activity in SOD1-deficient mice that were unable to further process superoxide. ROS decreased caspase-1 activity through oxidation of two redox cysteine residues, thereby preventing IL-1β maturation. As an alternative explanation for increased IL-1β under Phox-deficient conditions, we assayed monocyte NSP activity that can be inhibited by ROS. We showed previously that active NSPs, most likely PR3, process pro-IL-1β to mature IL-1β and that mice deficient in dipeptidylpeptidase I as well as PR3/elastase double-deficient mice produced less IL-1β and were protected from ANCA-induced NCGN. However, Phox deficiency was not associated with increased NSP activity in myeloid cells.

Our data strongly implicate Phox-regulated caspase-1 activity as a control mechanism for ANCA-induced NCGN. However, conceivably, ROS-dependent but inflammasome-independent mechanisms could have caused the accelerated ANCA disease phenotype in Phox-deficient mice. ROS-regulated candidates promoting resolution of inflammation include indoleamine 2,3-dioxygenase, which requires superoxide for its proper function, and the redox-sensitive transcription factor NF erythroid 2-related factor 2, which is not ubiquitinated after oxidation of the adaptor protein Kelch-like ECH-associated protein 1. Furthermore, Phox-generated ROS could play a role in lymphocyte survival and function. To determine whether the inflammasome is, indeed, causatively involved in the accelerated disease phenotype when Phox is missing, we generated Phox−/−/caspase-1−/−

Figure 6. SOD/catalase reduce superoxide and increase IL-1β generation in a caspase-1 dependent fashion in human monocytes. (A) Three or (B and C) four independent experiments, were performed. (A) Superoxide generation of unstimulated (unstim) or TNF-α–primed monocytes treated with control IgG (T ctrl or iso), anti-MPO mAb (T αMPO), and anti-MPO mAb after preincubation with a combination of SOD and catalase (T αMPO S/C). (B) Typical Western blot analysis for active IL-1β (17-kD fragment) in monocytes with or without preincubation with S/C or a caspase-1 inhibitor (inh). (C) Quantitative analysis of IL-1β Western blot analysis from monocytes. AU OD, arbitrary units of optical density. (D) IL-1β from monocyte supernatants by ELISA (n=4). *P<0.05; **P<0.01.
Figure 7. gp91phox/Caspase-1 double-deficiency rescued from the accelerated NCGN observed in gp91phox−/− BM-transplanted animals. Urine analysis and renal histology in MPO-immunized mice transplanted with WT, gp91 phox−/−, caspase-1−/−, and gp91phox−/−/caspase−/− double-deficient BM (n=8 per genotype): (A) dipstick analysis, (B) glomerular crescents and necrosis expressed as mean percentage of affected glomeruli, (C) typical examples for each transplanted group depicted with ×40.
double-deficient mice. BM from these double-deficient animals reduced ANCA-induced NCGN from gp91phox−/− BM-transplanted mice to a disease level observed in WT BM-transplanted animals. Interestingly, caspase-1−/− BM did not provide protection compared with WT BM, suggesting that caspase-1 activity might be very much downregulated by Phox-dependent ROS generation in the WT situation. However, when ROS activity is not properly maintained, this negative loop is lacking, and unrestrained inflammasome activation may proceed.

Bringing our data and the previous NSP study together, we postulate that two independently acting ANCA-mediated pathways induce IL-1β generation in monocytes and thereby, cause NCGN, namely an NSP-dependent, inflammasome/caspase-1−/− independent pathway and a parallel inflammasome/caspase-1−/− dependent pathway (Supplemental Figure 1). The latter seems to be restrained by ANCA-induced Phox-generated ROS providing a pivotal control loop limiting and terminating ANCA-induced inflammation. Assessing patients with ANCA for mutations in Phox genes could be an interesting test of our hypothesis. Loss-of-function mutations could be associated with worse outcome, nonresolving grumbling disease, or relapses. Examples of such disease-modifying mutations are neutrophil cytosolic factor 1 (Ncf1; also known as p47phox) and Ncf4 in patients with rheumatoid arthritis and NCF2 in patients with systemic lupus erythematosus and inflammatory bowel disease.49–52 Our data also add a caveat for ROS-targeted interventions, whereas IL-1 receptor blockade could be a promising strategy in patients with active ANCA vasculitis.

**CONCISE METHODS**

**Materials**

Anakinra (Kineret) was obtained from Amgen, Inc. (Munich, Germany), and urine dipsticks were from Roche Diagnostics (Indianapolis, IN). Human cells were stimulated with an mAb to MPO (clone 2C7) from Acris Antibodies Germany (Herford, Germany) or an isotype control as the treatment group. The treatment arm received Anakinra at a dose of 100 mg/kg body wt intraperitoneally two times per day up to 4 weeks. Mice were euthanized thereafter or earlier if we had to assume that the animal would not survive until the next day.

**Treatment Protocol**

Treatment started 4 weeks after BM transplantation. The control group received food and water ad libitum and was injected with buffer control as the treatment group. The treatment arm received Anakinra for 10 days intraperitoneally two times per day. Mice were euthanized thereafter or earlier if we had to assume that the animal would not survive until the next day.

**Analysis of Kidney Cytokines by ELISA**

Frozen mouse kidneys were homogenized in 0.5 ml cold PBS and a cooled Precellys 24 tissue homogenizer using Precellys Ceramic Kit (Peqlab, Erlangen, Germany) and centrifuged two times; clear supernatants were collected and stored at −80°C. Cytokine concentrations were determined by ELISA (R&D Systems).

**Measurement of Respiratory Burst by Reduction of Ferricytochrome C**

Superoxide was measured using the ferricytochrome C reduction assay as described before.53 Monocytes were pretreated with 5 μg/ml cytochalasin B for 15 minutes. Cells (0.75×10⁶) were pretreated with SOD (300 units/ml) and catalase (300 units/ml) for 15 minutes and primed with 2 ng/ml TNF-α for 15 minutes before 10 μg/ml mAbs (either isotype or MPO-specific). Experiments were done in duplicate. Samples were incubated in 96-well plates at 37°C for up to 60 minutes, and the absorption of samples was scanned repetitively at 550 nm using a Microplate Autoreader (Molecular Devices, Munich, Germany).

**Functional Evaluation of Renal Injury**

Mice were placed in metabolic cages 1 day before euthanasia, and urine was collected for 18 hours overnight. Urine was tested by dipstick for hematuria, leukocyturia, and proteinuria, and the extent is expressed as the mean on a scale of 0 (none) to 4 (severe) for leukocyturia and hematuria and 0–3 for proteinuria.

**Histologic Evaluation of Renal Injury**

Kidney tissue was collected at the time of euthanasia, fixed in 10% formalin, and embedded in paraffin using routine protocols; 4-μm coronal sections of specimens were stained with hematoxylin and eosin or periodic acid–Schiff and evaluated by light microscopy.

Magnification, and (D) glomerular monocyte/macrophage (MoMa) and neutrophil (PMN) influx expressed as the number of infiltrating cells per glomerulus. Typical examples for monocyte (E) and neutrophil (F) influx are shown for each transplanted group. *P<0.05.
Glomerular crescents and necrosis were expressed as the mean percentage of glomeruli with crescents and necrosis in each animal. For detection of leukocytes, sections of snap-frozen tissue were stained with rat antibodies to neutrophils (anti-Gr-1) and monocytes/macrophages (anti-CD68) and a peroxidase detection system.

**Murine and Human Monocyte Preparations and Stimulation**

For the isolation of BM neutrophils and monocytes, mice were euthanized, femurs and tibias were dissected, and BM was flushed with ice-cold sterile PBS without calcium and magnesium. Neutrophils and monocytes were further isolated by Ficoll–Hypaque density gradient centrifugation. Contaminating red blood cells in the neutrophil fraction were lysed by incubation with hypotonic saline for 15 seconds. The PBMC fraction was cultured for 1 hour at 37°C in DMEM in cell culture dishes. Attached cells were washed two times in PBS and resuspended in RPMI/10% FCS. Cell viability by trypan blue exclusion was found to be >99% in every experiment. For activation, cells were primed with 2 ng/ml TNF-α for 15 minutes before adding 150 μg/ml murine anti-MPO IgG, 150 μg/ml murine anti-BSA IgG, or 10 μg/ml mAbs (either isotype or MPO-specific) for 4 hours as indicated. In some experiments, cells were preincubated with 10 μM caspase-1 inhibitor or a combination of 300 units/ml SOD and 300 units/ml catalase for 15 minutes as indicated.

**Detection of NSP Activity**

NSP activity was measured in cell lysates and supernatants. Monocytes were left untreated or stimulated with PMA (25 ng/ml) for 60 minutes at 37°C, supernatants were kept on ice, and cell pellets were lysed without protease inhibitors. Enzymatic NSP activity was evaluated by measuring the hydrolysis of Boc-Ala-Pro-Nva p-chlorothiobenzyl ester (Sigma-Aldrich) in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) at A405 nm and is given as Vmax values.

**Detection of MMP-9 Activity**

MMP-9 activity was measured in supernatants of unstimulated and stimulated monocytes by gelatin zymography; 5 μl/lane for each sample was mixed with sample buffer and loaded onto a 8% polyacrylamide gel with 0.1% gelatin for electrophoresis. Gels were washed in 2.5% Triton X-100, incubated for 18 hours at 37°C in collagenase buffer, and stained for 20 minutes with Coomassie dye. MMP-9 activity was quantified by OD measurement of the 92-kD band using a digital imaging analysis system.

**Transmigration Assay**

Migration was tested in fibronectin-coated transwells (3.0 μm, 6.5 mm; Corning). Murine or human monocytes (1.5×10⁶ in HBSS+*) were stimulated at 37°C with 20 ng/ml murine CCL2, human IL-1β, or TNF-α applied to the lower well. After 4 hours, transmigrated cells were quantified by fluorescence labeling using calcein and a standard curve.

**Statistical Analyses**

Results are given as means±SEMs. Comparisons were made using ANOVA with post hoc analysis by GraphPad Prism5 software. Differences were considered significant at P<0.05.

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**DISCLOSURES**

None.

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