



Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis

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Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) constitutes life-threatening autoimmune diseases affecting every organ, including the kidneys, where they cause necrotizing crescentic glomerulonephritis. ANCA activates neutrophils and activated neutrophils damage the endothelium, leading to vascular inflammation and necrosis. Better understanding of neutrophil-mediated AAV disease mechanisms may reveal novel treatment strategies. Here we report that ANCA induces neutrophil extracellular traps (NETs) via receptor-interacting protein kinase (RIPK) 1/3- and mixed-lineage kinase domain-like (MLKL)-dependent necroptosis. NETs from ANCA-stimulated neutrophils caused endothelial cell (EC) damage in vitro. This effect was prevented by (i) pharmacologic inhibition of RIPK1 or (ii) enzymatic NET degradation. The alternative complement pathway (AP) was recently implicated in AAV, and C5a inhibition is currently being tested in clinical studies. We observed that NETs provided a scaffold for AP activation that in turn contributed to EC damage. We further established the in vivo relevance of NETs and the requirement of RIPK1/3/MLKL-dependent necroptosis, specifically in the bone marrow-derived compartment, for disease induction using murine AAV models and in human kidney biopsies. In summary, we identified a mechanistic link between ANCA-induced neutrophil activation, necroptosis, NETs, the AP, and endothelial damage. RIPK1 inhibitors are currently being evaluated in clinical trials and exhibit a novel therapeutic strategy in AAV.

tolerance breakdown toward the ANCA autoantigens (12, 22, 23). In addition, it is conceivable that ANCA-induced NETs participate in the vascular injury process that culminates in necrotizing vasculitis. However, neither the signaling pathways by which ANCA induces NETs nor the potential mechanism(s) by which NETs affect the endothelium are fully understood. We hypothesized that receptor-interacting protein kinases (RIPKs) 1/3, involved in the necroptosis signaling pathway, control ANCA-induced NET generation, and studied NET-mediated endothelial cell (EC) damage mechanisms.

Results

ANCA Stimulation Causes NET Formation, and the RIPK1/3- and Mixed-Lineage Kinase Domain-Like Protein-Dependent Necroptosis Pathway Mediates This Effect. Both monoclonal antibodies (mAbs) to the ANCA antigens PR3 and MPO and ANCA-IgG from AAV patients induced NET formation in TNF α -primed human neutrophils (Fig. 1 *A–F*). Using the cell-impermeable DNA dye propidium iodide, we detected extracellular DNA with the typical filiform NET structures when samples were paraformaldehyde (PFA)-fixed (Fig. 1*A*), and observed typical extracellular DNA circles that surround neutrophils when unfixed samples were stained with Sytox green (Fig. 1*B*). Recently, critical NET-related issues

ANCA | NETs | necroptosis | complement | glomerulonephritis

Systemic necrotizing autoimmune vasculitis is characterized by circulating antineutrophil cytoplasmic antibody (ANCA) that recognizes either proteinase 3 (PR3) or myeloperoxidase (MPO). Both antigens are exclusively expressed by neutrophils and monocytes (1–5). In vitro studies characterized various ANCA-induced neutrophil effector functions that participate in endothelial damage, such as adhesion, granule protein release, respiratory burst, and cytokine generation (reviewed in refs. 6 and 7). Subsequently, animal studies provided mechanistic insights under complex in vivo conditions. Activation of the alternative complement pathway (AP) together with C5a receptor blockade is one such example that was primarily derived from animal studies (8–11). A more recently described neutrophil effector function is the release of neutrophil extracellular traps (NETs) (12–16). Mechanisms controlling NET formation and the clinical relevance of NETs are intensively studied. NETs are lattice-like structures containing DNA, histones, and neutrophil granule proteins, including the ANCA antigens MPO and PR3 (12, 17, 18). ANCA-stimulated neutrophils were shown to release NETs in vitro, and circulating as well as glomerular deposited NETs were detected in AAV patients (12, 19–21). It was suggested that NETs are immunogenic and participate in the

Significance

In this report, we provide evidence of a mechanistic link between antineutrophil cytoplasmic antibody (ANCA)-induced neutrophil activation, regulated necrosis (necroptosis), generation of neutrophil extracellular traps, complement activation, and endothelial cell damage with consecutive vasculitis and glomerulonephritis in autoimmune ANCA-induced vasculitis (AAV). We now show that inhibition of necroptosis-inducing kinases completely prevents ANCA vasculitis and establish a link to activation of the complement system. We suggest that these findings significantly extend our understanding of the pathogenesis of AAV and especially the tight regulation of neutrophil cell death therein. In addition, specific necroptosis inhibitors are currently being evaluated in clinical studies and can possibly complement existing therapeutic strategies in AAV.

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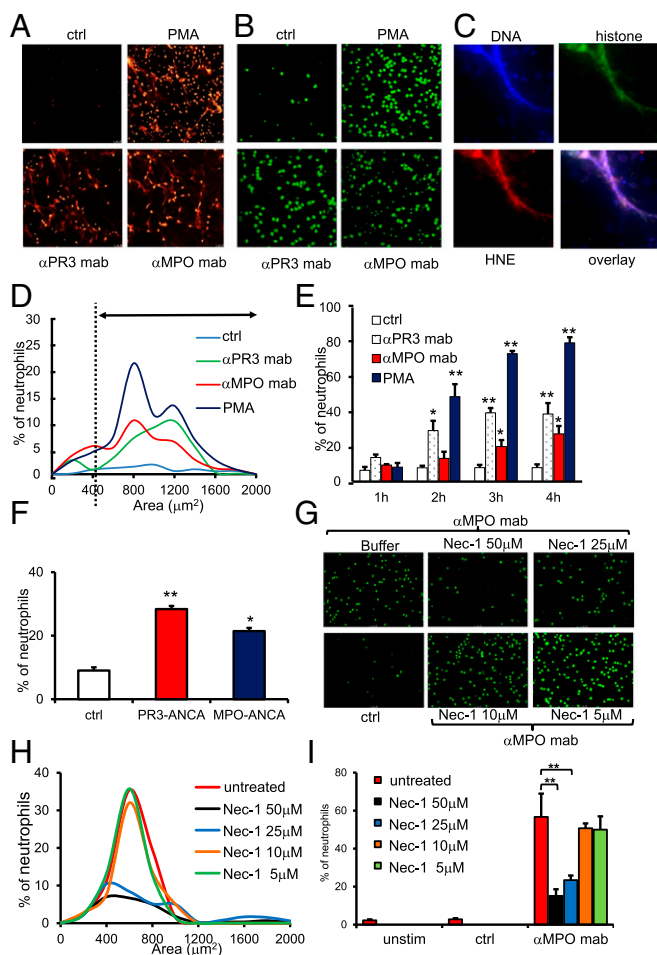


Fig. 1. ANCA induces NET generation in human neutrophils by RIPK1-dependent necroptosis signaling. Isolated human neutrophils were primed with 2 ng/mL TNF α and subsequently stimulated with different monoclonal antibodies (ctrl, isotype control; α PR3 mAb, monoclonal antibody to PR3; α MPO mAb, monoclonal antibody to MPO). (A) PFA-fixed cells were stained with propidium iodide (PI) to detect NETs. (B) Viable cells were stained with Sytox green to detect NETs. (C) A typical example of anti-MPO mAb-induced NETs with strong staining for DNA (blue; DAPI), histone H3 (green), and HNE (red) is depicted. (D) Automated quantification of NET generation in response to mAbs to MPO and PR3, PMA, and isotype control mAb (ctrl) (one representative experiment is shown). The dashed line marks the threshold for definition of NETs generation. (E) Time course of NET formation in response to mAbs to MPO and PR3, PMA, and isotype control mAb; $n = 4$ experiments with different neutrophil donors. (F) Stimulation of TNF α -primed neutrophils with two different human control IgG preparations for 4 h (ctrl), two different human PR3-ANCA IgG preparations, and two different human MPO-ANCA IgG preparations (125 μ g/mL); $n = 4$ experiments with different neutrophil donors. (G) α MPO mAb-induced NET generation by microscopy was dose dependently reduced by necrostatin-1 preincubation. (H) Depiction of the percentage of NET-producing neutrophils and NET area. (I) Statistical analysis of the percentage of NET-producing neutrophils from four independent experiments with different neutrophil donors as in H. Error bars indicate means \pm SEM. Comparisons were made using t test or ANOVA; * $P < 0.05$, ** $P < 0.01$.

were raised, including the need to define NETs by their characteristic composition, use observer-independent quantification methods, identify the upstream pathways that control NET formation in response to a given stimulus, determine the ways by which NETs cause tissue damage, and use physiologic stimuli with clinical relevance rather than artificial phorbol ester (24). ANCA provides such a disease-relevant NET inducer, and ANCA-induced NETs show the characteristic composition of

DNA, histones, and human neutrophil elastase (HNE) (Fig. 1C). Quantitative assessment of NETs using an automated and observer-independent method described by Papayannopoulos et al. (25) indicated that ANCA increased both the percentage of NET-producing cells and the DNA areas surrounding the neutrophils (Fig. 1D). While analyzing NET formation at 1-h intervals up to 4 h, we observed that up to 40% of neutrophils produced NETs in response to anti-PR3 and anti-MPO mAbs by the end of the observation period (Fig. 1E). Incubation of polymorphonuclear neutrophil with IgG purified from patients with active AAV resulted in NET formation, whereas control IgG isolated from healthy controls did not (Fig. 1F).

The pathway(s) that controls ANCA-induced NET formation is not known. NET formation may occur in vital neutrophils (26) or as a consequence of regulated necrosis (27, 28). Necroptosis, the best-studied form of regulated necrosis, depends on RIPK1/3 activation and subsequent phosphorylation of the pseudokinase mixed-lineage kinase domain-like (MLKL) (reviewed in ref. 29). Activation of RIPK3 is prevented by an inactivating conformation of RIPK1 that is maintained by the small molecule necrostatin-1 (Nec-1) or its derivatives (necrostatins) (30). We tested the hypothesis that necroptosis represents the upstream stimulus that drives ANCA-mediated NET formation. Increasing Nec-1 concentrations decreased NET production, as assessed by microscopy (Fig. 1G) and automated quantification (Fig. 1H and I). Significant reduction of NET generation was also observed with the highly specific pharmacologic RIPK1 inhibitor Nec-1s as well as with the downstream MLKL inhibitor necrosulfonamide (Fig. 2A and B). Using a genetic approach, neutrophils from RIPK3-deficient mice exhibited reduced NET generation in response to polyclonal anti-MPO IgG (Fig. 2C and D). Finally, we observed a clearly positive p-MLKL signal in anti-MPO antibody-stimulated human neutrophils that was absent in control antibody-stimulated cells (Fig. 2E–H). Together, these data indicate that RIPK3-dependent necroptosis functions as an upstream activator of ANCA-induced NET generation.

NETs Cause Endothelial Cell Damage, and NET-Dependent Alternative Complement Pathway Activation Contributes to This Effect. EC damage by ANCA-activated neutrophils is a hallmark of AAV. To test whether or not NETs damage the endothelium directly, we incubated NETs isolated from anti-MPO mAb-treated neutrophils with EC monolayers in the presence of serum. Using albumin flux across the EC monolayer as an indicator of EC damage, we observed increased albumin permeability with NETs from anti-MPO mAb-treated neutrophils (Fig. 3A and B). We evaluated the capability of NETs to damage EC by two different approaches: either by NETs degradation with DNase I (Fig. 3A) or by specific necroptosis inhibition with Nec-1. Both of these approaches strongly protected EC from NETs-induced damage (Fig. 3B).

We and others showed recently that AP activation caused necrotizing crescentic glomerulonephritis (NCGN) in murine AAV disease models (8–11). C5a was characterized as an important mediator, and an oral C5a receptor blocker was subsequently established and is currently being evaluated as a treatment target in clinical studies (31). We argued that NETs provide a scaffold for complement activation. We therefore activated TNF α -primed neutrophils with mAbs to MPO or an isotype control for 3 h in HBSS before addition of human serum as a complement source for the last hour of the experiment. After 4 h, NETs were isolated, and the NET components were released by DNase I and subjected to C5a ELISA. We detected C5a generation in anti-MPO mAb-induced NETs that was abrogated by DNase I (Fig. 3E). As an alternative approach, we visualized complement activation by C3d staining and observed that anti-MPO mAb-induced NETs showed strong C3d staining

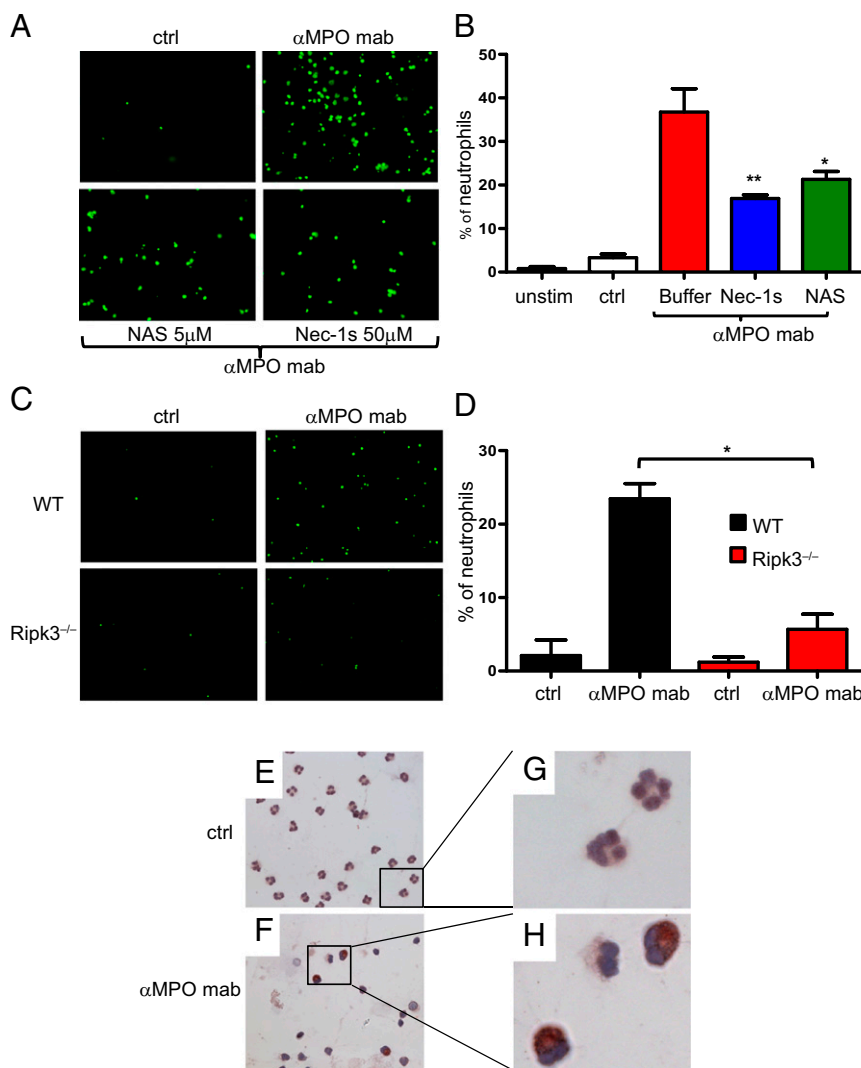


Fig. 2. ANCA induces NET generation in human and murine neutrophils by RIPK1- and MLKL-dependent necroptosis signaling. (A) Isolated TNF α -primed human neutrophils were stimulated with a monoclonal antibody to MPO (α MPO mab) or an isotype control (ctrl). NETs were detected by Sytox green staining. NET generation was reduced by both necrostatin-1s and necrosulfonamide (NAS) preincubation. (B) Corresponding statistical analysis of the percentage of NET-producing neutrophils from three independent experiments with different neutrophil donors. (C) Isolated TNF α -primed murine neutrophils from WT or *Ripk3*^{-/-} mice were stimulated with murine polyclonal anti-MPO IgG, and NET generation was quantified by Sytox green staining. Neutrophils from *Ripk3*^{-/-} mice displayed reduced NET generation. (D) Corresponding statistical analysis of the percentage of NET-producing neutrophils from three independent experiments. (E–H) p-MLKL immunohistochemistry on isolated human neutrophils. α MPO mAb-stimulated TNF α -primed neutrophils displayed a strong p-MLKL signal that was absent in isotype control-stimulated cells; E and F show 63 \times magnification. G and H depict higher digital magnification of the marked areas in E and F. Error bars indicate means \pm SEM. Comparisons were made using *t* test or ANOVA; **P* < 0.05, ****P* < 0.01.

with DNA colocalization in the presence of normal serum. This effect was reversed upon addition of serum and upon DNA degradation by DNase I (Fig. 3C). We then characterized the serum complement components that were required for NET-mediated complement activation and EC damage using sera with defined complement deficiencies. We observed again strong C3d staining in anti-MPO mAb-induced NETs that colocalized with DNA under normal human serum conditions but strongly diminished C3d staining when C3-deficient or factor B-deficient sera were used (Fig. 3D). In contrast, C1q deficiency had no effect on C3d staining. Thus, NETs provide a scaffold for AP activation but not for the classical complement pathway. Importantly, anti-MPO mAb-induced NETs that were generated in C3- or factor B-deficient sera provoked significantly less EC damage compared with NETs produced in normal or C1q-deficient sera (Fig. 3F). In summary, these data identify the in-

terconnection between ANCA-induced neutrophil activation, NETs, the AP, and EC damage.

ANCA-Induced NETs Mediate Anti-MPO-Induced Glomerular Damage.

Finally, we tested the key events from our *in vitro* observations in two murine AAV disease models. First, we used a passive anti-MPO Ab transfer model to induce NCGN and evaluate the effect of DNase I treatment. IgG was prepared from MPO-deficient mice that were immunized with murine MPO and transferred into wild-type (WT) mice. After 7 d, all vehicle-treated mice showed proteinuria, erythrocyturia, and leukocyturia by dipstick (Fig. 4A) as well as glomerular crescents and necrosis by histology (Fig. 4B, C, and E). Importantly, *in vivo* DNase I treatment, started 24 h after anti-MPO Ab transfer, protected mice from the disease (Fig. 4A, B, D, and F), supporting the notion that NETs contribute to glomerular endothelial injury *in vivo*. However, we cannot rule out that DNase had additional effects independent of NET degrada-

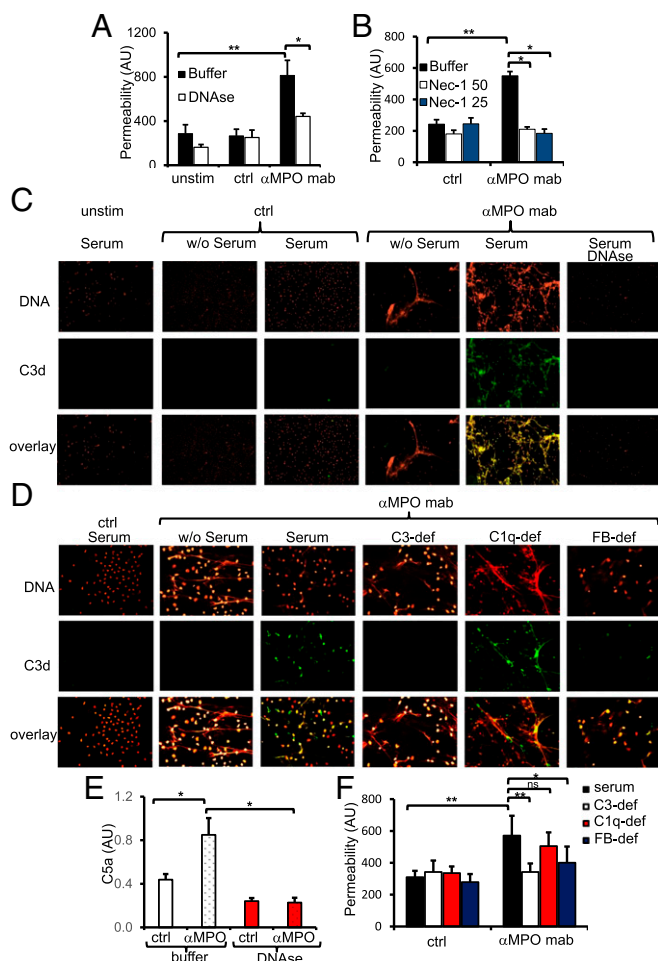


Fig. 3. Endothelial damage and complement activation by ANCA-induced NET generation. (A) HUVECs were cocultured with NETs isolated from either TNF α -primed human neutrophils without further stimulation (unstim), isotype mAb-stimulated (ctrl), or α MPO mAb-stimulated (α MPO) neutrophils. Permeability of the EC monolayer was measured after 1 h using albumin-FITC. NET degradation by DNase protected from endothelial damage. (B) Human neutrophil preincubation with Nec-1 protected from NET-induced endothelial damage. (C) NETs were cocultured in the absence (w/o) or presence of human serum and stained after PFA fixation with PI (DNA; red) and α C3d antibody (FITC; green). Unstimulated cells and isotype-stimulated neutrophils displayed no NETs and no C3d deposition, whereas α MPO mAb-stimulated cells cocultured with serum showed NET formation with strong C3d containing. DNase pretreatment prevented C3d staining. A typical example is depicted. (D) NETs were cocultured with different human sera as indicated and stained after fixation with PI (DNA; red) and α C3d antibody (FITC; green). Isotype-stimulated neutrophils displayed no NETs and no C3d deposition; α MPO mAb-stimulated neutrophils displayed NET formation and—when cocultured with serum—C3d deposition. C3d deposition was not affected by C1q deficiency but strongly reduced in both C3-deficient (C3-def) and complement factor B-deficient (FB-def) sera. (E) α MPO mAb-induced NETs that were cocultured with human serum caused complement activation with C5a generation. The effect was prevented by NET degradation using DNase I. (F) Increased EC permeability by α MPO mAb-induced NETs is mediated by the alternative complement pathway, as both C3-deficient and complement factor B-deficient sera provided protection whereas C1q deficiency did not. Error bars indicate means \pm SEM. Comparisons were made using *t* test or ANOVA; **P* < 0.05, ***P* < 0.01. ns, not significant.

tion. Thus, we used a different intervention by genetically interfering with the necroptosis pathway. We induced disease in WT and RIPK3-deficient mice by passive anti-MPO Ab transfer and found that RIPK3 deficiency prevented NCGN by renal pathology (Fig. 4 *H–L*). To exclude nonnecroptotic functions of RIPK3 (32,

33), we added another approach and induced disease in WT and MLKL-deficient mice. We observed that MLKL deficiency protected from NCGN, thereby definitively implicating necroptosis as an important disease mechanism (Fig. 4 *M–R*). Urine analysis showed less protection with RIPK3 compared with MLKL deficiency, suggesting RIPK3-independent effects on MLKL that may contribute to the pathogenesis. However, to the best of our knowledge, no other kinase has been demonstrated to be capable of phosphorylating MLKL. From these data, we conclude that RIPK3-mediated MLKL phosphorylation contributes to necroptosis as a central pathophysiological contributor of AAV, but cannot rule out RIPK3-independent effects.

The passive transfer model did not allow us to specifically study the effect of RIPK3 deficiency in bone marrow (BM)-derived cells that harbor the NET-producing neutrophils. Thus, we generated chimeric mice that lacked RIPK3 only in BM-derived cells. MPO-deficient mice were immunized with murine MPO, irradiated, and transplanted with BM from either WT or RIPK3-deficient mice. After 8 wk, mice that had received WT BM showed urine abnormalities (Fig. 5*A*) and NCGN by histology (Fig. 5*B, C, and E*), whereas mice transplanted with RIPK3-deficient BM were protected (Fig. 5*A, B, D, and F*). RIPK3 deficiency did not interfere with BM engraftment (Fig. S1). Thus, our *in vitro* data together with the animal data obtained in two models using different gene-deficient mice are in line with an important role of myeloid RIPK1/3, MLKL, and NETs in anti-MPO antibody-mediated NCGN.

In keeping with these data, human kidney biopsy stainings obtained from anti-MPO AAV patients with highly active NCGN exhibited a remarkably specific p-MLKL staining in glomerular neutrophils. Neutrophils were the only glomerular cells found positive for p-MLKL, and there was a notable absence of positivity in parietal epithelial cells, crescents, glomerular endothelial cells, mesangial cells, and mononuclear cells in the ANCA-associated NCGN biopsies and the controls. Out of 12 anti-MPO antibody-associated NCGN biopsies with a total of 174 glomeruli (63 with active crescents, 57 with necrosis), we found 2 p-MLKL-positive glomerular neutrophils (Fig. 5*G–K*). Five biopsies with anti-PR3 antibody-associated NCGN did not show glomerular neutrophils with p-MLKL positivity. Biopsies from disease control NCGN did not show any p-MLKL-positive neutrophils. However, this does not exclude the possibility of a role for necroptosis in cells other than neutrophils, because necroptosis is a rapid form of cell death which is inevitably associated with plasma membrane rupture. Therefore, we expect to lose the p-MLKL signal in tissue sections. Together with the *in vivo* findings of RIPK3- and MLKL-deficient mice, these data suggest that RIPK3-mediated phosphorylation of MLKL and necroptosis in neutrophils play a central role in inducing disease activity in AAV in mice and humans.

Discussion

In summary, we used complementary methods, including quantitative operator-independent analyses, to show that NETs with their characteristic constituents were formed in response to a disease-relevant stimulus, namely ANCA. We provide data supporting the notion that ANCA-induced NET formation is controlled by RIPK1/3 and MLKL, key mediators of the necroptosis pathway. Moreover, we observed that NETs damage the endothelium *in vitro* and that this effect is, at least in part, mediated by NET-associated AP activation. Finally, we used pharmacologic and genetic approaches in murine disease models and provide AAV patient data supporting the concept that necroptosis and NET formation are relevant for *in vivo* contribution to anti-MPO antibody-mediated NCGN.

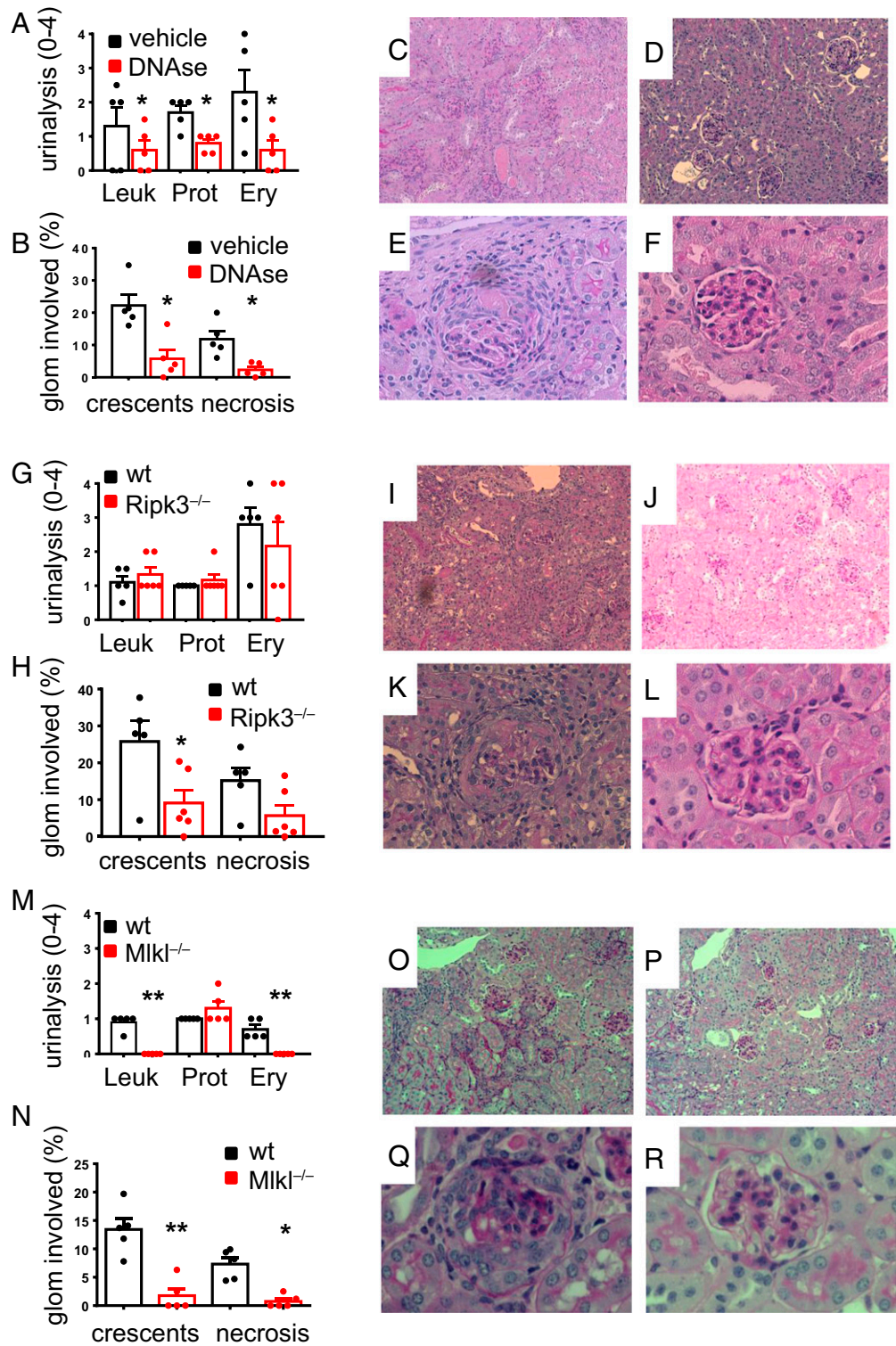


Fig. 4. ANCA-stimulated necroptosis-dependent NET generation induces NCGN. (A–F) NCGN was induced by passive α MPO IgG transfer; NETs were degraded *in vivo* by twice daily *i.p.* DNase injections, which resulted in protection from (A) urine pathology and less crescent and necrosis formation by (B) histology. Depicted are typical renal sections with (C) low (10 \times) and (E) high magnification (40 \times) from vehicle-treated mice as well as (D) low and (F) high magnifications from DNase-treated mice. (G–L) NCGN was induced by passive α MPO IgG transfer into WT or RIPK3-deficient (*Ripk3*^{-/-}) mice. Urine pathology was similar in both groups (G), whereas RIPK3 deficiency resulted in less crescent and necrosis formation (H). (I–L) Representative glomeruli with (I) low and (K) high magnifications from WT mice, whereas J shows low and L shows high magnifications from RIPK3-deficient mice. (M–R) NCGN was induced by passive α MPO IgG transfer into WT or MLKL-deficient (*Mlkl*^{-/-}) mice. Urine pathology showed less leukocyturia and erythrocyturia (M) and less crescent and necrosis formation in MLKL-deficient mice (N). (O–R) Representative glomeruli with low (O) and high (Q) magnifications from WT mice, whereas P shows low and R shows high magnifications from MLKL-deficient mice. Error bars indicate means \pm SEM. Comparisons were made using *t* test or ANOVA; **P* < 0.05, ***P* < 0.01.

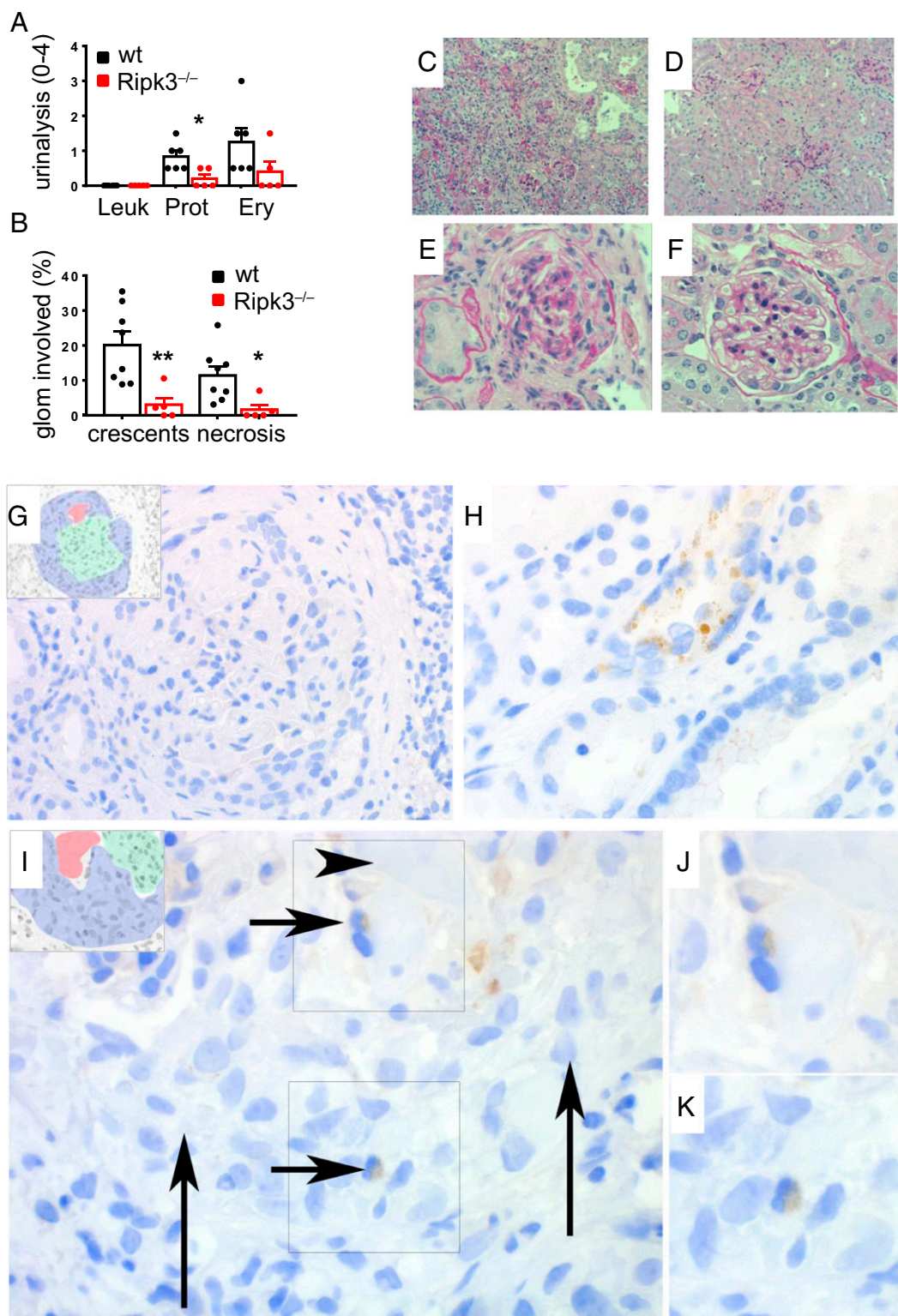


Fig. 5. ANCA-stimulated necroptosis in circulating BM-derived cells induces NCGN. (A–F) NCGN was induced in MPO-deficient mice immunized with murine MPO that were subsequently irradiated and transplanted with either WT or RIPK3-deficient (*Ripk3*^{-/-}) BM. (A–F) RIPK3 deficiency significantly reduced urine pathology (A) and resulted in strong protection from crescent and necrosis induction (B). Depicted are typical renal sections with (C) low and (E) high magnifications from WT mice, whereas (D) shows low and (F) shows high magnifications from RIPK3-deficient mice. (G–K) Exemplary micrograph from a biopsy with active anti-MPO-ANCA-associated NCGN shows fibrinoid necrosis and a cellular crescent; the *Inset* (G) shows the extent of the crescent in blue, necrosis in red, and tuft in green color. No resident mesangial cells, endothelial cells, podocytes, or intracapillary or infiltrating leukocytes are positive (brown) for p-MLKL. (H) Some of the few tubular segments with epithelial positivity for p-MLKL. (I) Biopsy with active anti-MPO-ANCA-associated NCGN and cytoplasmic positivity for p-MLKL in two neutrophils, recognizable by their typical segmented nuclei (short arrows). The colors in the *Left Inset* are as above. The neutrophil in the *Top Right Inset* (arrow) is adjacent to a fibrinoid necrosis area (arrowhead) (I and J). The neutrophil in the *Bottom Right Inset* is adjacent to a fresh, cellular crescent (long arrows). Both neutrophils in the *Right Insets* are magnified in the corresponding two frames (J and K) to better appreciate nuclear morphology. Error bars indicate means \pm SEM. Comparisons were made using *t* test or ANOVA; **P* < 0.05, ***P* < 0.01.

Various infectious and noninfectious mediators were shown to induce NETs. Kessenbrock et al. (12) were the first to demonstrate that ANCA triggers NET formation and deposition in affected glomeruli in AAV patients. NET production by ANCA-activated neutrophils (19, 20, 34, 35) and kidney biopsies from AAV patients with NETs containing citrullinated histones, MPO, and peptidyl arginine deiminase 4 at sites of glomerular fibrinoid necrosis (36) were subsequently reported by others as well. Recently, critical NET-related issues were raised, including the need to define NETs by their characteristic composition, use observer-independent quantification methods, identify the upstream pathways that control NET formation in response to a given stimulus, determine the ways by which NETs cause tissue damage, and use physiologic stimuli with clinical relevance rather than artificial phorbol ester, to name a few (24). In an attempt to address some of these issues, we studied ANCA-induced NETs in vitro and in murine AAV disease models. We found that ANCA triggers NET formation in vitro and detected the typical NET components with extracellular DNA, histones, and human neutrophil elastase in close proximity. This composition characterized the formation of true NETs and distinguished them from any cell-free DNA that could be released after cell lysis. Furthermore, an observer-independent method was introduced by Papayannopoulos et al. (25) that allowed us to quantify NET formation with respect to the percentage of NET-producing cells and NET area.

The pathway(s) that controls NET formation in response to ANCA is not known. In general, NET formation may occur in vital neutrophils (26) or as a consequence of regulated necrosis (27, 28). The intracellular signal transduction events that control the regulated necrotic cell death leading to NET formation are not yet clear and may differ between different NET inducers. Necroptosis is a death pathway of regulated necrosis and is currently defined by its caspase independence and its dependence on receptor-interacting protein kinase 1, RIPK3, and the mixed-lineage kinase domain-like protein (29, 37). Necroptosis was shown to control crystal-induced NET formation (38), whereas the role of necroptosis in phorbol-12-myristat-13-acetate (PMA)-induced NET formation is controversial (38–40). We observed that pharmacologic RIPK1 and MLKL inhibitors as well as RIPK3 deficiency reduced NET formation in response to ANCA, suggesting that necroptosis is upstream of ANCA-induced NET production. Automated quantification indicated that both the percentage of NET-forming cells and the NET area decreased with RIPK1 and MLKL inhibition. The cellular mechanism as to how ANCA induces NETs is currently unclear. It is conceivable that MPO-ANCA interferes with MPO activity. We observed abrogated NET generation in MPO-deficient neutrophils in response to PMA, indicating the importance of MPO for NET generation (Fig. S2). In addition, downstream of p-MLKL, necroptosis may be regulated by the endosomal sorting complex required for transport (ESCRT)-III complex. Thus, we cannot rule out the possibility that additional regulation of the necrotic cell death pathway is involved (41).

Multiple implications of NETs in AAV are conceivable. NETs contain the ANCA antigens MPO and PR3 and present these antigens in an immunogenic way that promotes autoimmunity (22, 42). It is also conceivable that ANCA-induced NETs participate in endothelial injury. In fact, NET-associated histones were recently shown to damage glomerular ECs in vitro and in an antiglomerular basement membrane glomerulonephritis model (43). Moreover, NET-associated matrix metalloproteinases had toxic EC effects in vitro, possibly contributing to vascular injury in systemic lupus erythematosus (44). We observed that isolated NETs induced by ANCA damaged ECs in vitro. DNase and necrostatin-1 reduced EC damage, underscoring the biological importance of necroptosis-induced NET formation in the context of endothelial injury. We made an additional observation, namely that

NETs formed not only a scaffold for the activation of the alternative complement pathway but, in turn, contributed to endothelial damage. Thus, NETs may provide a mechanistic link between ANCA-induced neutrophil activation, complement, and endothelial injury. We believe that these findings provide an additional rationale for complement blockade as a therapeutic principle in AAV.

To test key events of our in vitro findings in complex in vivo conditions, we studied AAV disease models and selected two intervention strategies for proof of principle. First, we used a short-term anti-MPO antibody model for NCGN and administered DNase I to degrade NETs. This intervention reduced glomerular necrosis and crescent formation, supporting the notion that NETs contribute to glomerular endothelial injury in vivo. However, we cannot rule out that DNase had additional effects, independent of NET degradation. Second, we used RIPK3- and MLKL-deficient mice to evaluate the need of the necroptosis machinery for NCGN induction by anti-MPO antibodies. We observed protection in RIPK3- and MLKL-deficient mice, but were not able to differentiate the importance of necroptosis in NET-producing BM-derived cells from necroptosis in other cells, including ECs. Therefore, we used a BM transplantation model to generate mice that were RIPK3-deficient in BM-derived cells only. Anti-MPO antibody-mediated NCGN was strongly attenuated in mice that had received RIPK3-deficient BM compared with WT BM. Thus, our in vivo data are in agreement with the importance of necroptosis-controlled NET formation for anti-MPO antibody-mediated NCGN. However, prospective clinical trials are now possible, and should allow the prognostic potential of p-MLKL staining in AAV patients. In summary, in addition to complement, necroptosis pathway elements may represent molecular targets for therapeutic interventions in AAV. Pharmacologic RIPK1 inhibitors are currently in phase II clinical trials for the treatment of inflammatory bowel diseases, ulcerative colitis, psoriasis, rheumatoid arthritis (GSK2982772), and other indications (45). Repurposing these drugs for the treatment of AAV seems reasonable.

Materials and Methods

Preparation of Neutrophils, IgG, and Human Umbilical Vein Endothelial Cells, NET Production, and Coincubation of Human Umbilical Vein Endothelial Cell Monolayers with NETs. Neutrophils, human umbilical vein endothelial cells (HUVECs), and IgG were prepared as described previously (46). NETs were isolated by detachment using low DNase I concentrations and coincubated with HUVECs. BSA-FITC permeability was assessed as described (47).

Visualization and Quantification of NET Formation. To visualize NETs, neutrophils were stained with either Sytox or propidium iodide. For NET quantification, Sytox images of unfixed neutrophils were analyzed using ImageJ processing software (NIH) as described (25).

Mice, Immunization, NCGN Models, and DNase I Treatment Protocol. *Ripk3*^{-/-} mice were kindly provided by V. Dixit and K. Newton, Genentech, South San Francisco, CA (48), and *Mkl1*^{-/-} mice were kindly provided by James Murphy, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia (45). Male and female mice were used both as BM donors and recipients with equal gender distribution. BM engraftment was assessed 5 wk after bone-marrow transplantation. Passive NCGN was induced in female WT, *Ripk3*^{-/-}, or *Mkl1*^{-/-} mice. Local authorities approved all animal experiments, which followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

p-MLKL Staining of Human Renal Tissue. Sections from 12 renal biopsies with anti-MPO NCGN, 5 anti-PR3 NCGN, 2 anti-glomerular basement membrane-associated GN, and 3 biopsies with focal necrotizing IgA-GN were stained for p-MLKL. Neutrophils from healthy human donors and HUVECs were obtained after approval by Charité and governmental authorities and after written informed consent.

Statistics. Results are given as means \pm SEM. Comparisons were made using *t* test or ANOVA; **P* < 0.05, ***P* < 0.01. See *SI Materials and Methods* for a detailed description.

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