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The pseudokinase MLKL activates PAD4-dependent NET formation in necroptotic neutrophils

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Abstract

Neutrophil extracellular trap (NET) formation can generate short-term, functional anucleate cytoplasts and trigger loss of cell viability. We demonstrated that the necroptotic cell death effector mixed lineage kinase domain-like (MLKL) translocated from the cytoplasm to the plasma membrane and stimulated downstream NADPH oxidase-independent ROS production, loss of cytoplasmic granules, breakdown of the nuclear membrane, chromatin decondensation, histone hypercitrullination, and extrusion of bacteriostatic NETs. This process was coordinated by receptor-interacting protein kinase-1 (RIPK1), which activated the caspase-8-dependent apoptotic or RIPK3/MLKL-dependent necroptotic death of mouse and human neutrophils. Genetic deficiency of RIPK3 and MLKL prevented NET formation but did not prevent cell death, which was because of residual caspase-8-dependent activity. Peptidylarginine deiminase 4 (PAD4) was activated downstream of RIPK1/RIPK3/MLKL and was required for maximal histone

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hypercitrullination and NET extrusion. This work defines a distinct signaling network that activates PAD4-dependent NET release for the control of methicillin-resistant *Staphylococcus aureus* (MRSA) infection.

INTRODUCTION

Programmed apoptotic cell death is a fundamental response to infection that can reduce pathogen burden (1, 2). However, some pathogens can inhibit host apoptotic pathways, and these pathogens may, in turn, trigger alternative forms of regulated host cell death, such as necroptosis (3). Necroptosis is an inflammatory form of cell suicide characterized by caspase-independent cell rupture. Stimulated by receptor-interacting protein kinase-3 (RIPK3) and mixed lineage kinase domain-like (MLKL) activity, necroptosis can spur inflammasome activation and the production of inflammatory cytokines (4–6). However, the cell-intrinsic effector functions associated with necroptosis remain poorly defined.

Necroptotic signaling is activated by loss of cellular inhibitor of apoptosis proteins cIAP1, cIAP2, and, in innate immune cells, X-linked IAP (XIAP), as well as inhibition of caspase-8 (7–10). Pharmacological targeting of the IAPs and caspase activity can therefore be exploited to induce necroptosis. Loss of cIAP1/2 promotes tumor necrosis factor (TNF)– and RIPK1-dependent apoptosis through caspase-8. However, simultaneous inhibition of caspase-8 allows for necroptotic cell death (7–9, 11, 12). The pseudokinase MLKL is central to the execution of necroptotic cell death, but its precise cellular effects are yet to be fully established (12). Recruitment of multimeric complexes of phosphorylated MLKL (pMLKL) to the plasma membrane is associated with membrane permeabilization and cell death (13–15). However, whether this membrane localization represents the end of this pathway is controversial; additional factors may exist downstream to induce mitochondrial fragmentation and/or a calcium current across the plasma membrane to kill the cell (14, 16–20).

Neutrophil extracellular traps (NETs) are networks of extracellular DNA and microbicidal proteins released from neutrophils (21, 22). NETs can restrict the replication of *Staphylococcus aureus*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, and *Shigella flexneri* (22–25), and their release and/or prolonged presence has been implicated in the pathogenesis of systemic lupus erythematosus (26), rheumatoid arthritis (27), diabetes (28), acute lung injury (29), and thrombosis (30–32). Despite this, the biochemical regulators of NET formation in these disease settings as well as the possible contribution of regulated forms of cell death to the phenomenon of NET formation remain unclear.

Here, we examine the cellular and biochemical consequences of activating RIPK1-, RIPK3-, and MLKL-dependent necroptosis in mouse and human neutrophils. We describe how activation of RIPK3 and MLKL stimulates a downstream signaling network that includes protein arginine deiminase 4 (PAD4), an important factor in NET formation (33). Our data also reveal how RIPK1 orchestrates necroptotic or apoptotic cell death according to substrate availability to control the release of networks of extracellular DNA, hypercitrullinated histones, and microbicidal proteins resembling NETs.

RESULTS

RIPK1 can engage multiple cell death pathways depending on substrate availability

To examine the role of RIPK1 signaling in neutrophil apoptotic and necroptotic cell death, we used mice expressing a kinase-dead form of RIPK1 (*Ripk1^{D138N/D138N}*), which specifically abrogates RIPK1 kinase activity and prevents necroptosis (34). To induce apoptosis, we treated neutrophils with birinapant, a second SMAC-mimetic that degrades cIAP1/2 and activates caspase-8 and caspase-3. Necroptosis was triggered with birinapant treatment in combination with the widely used irreversible pan-caspase inhibitor z-VAD-fmk, which resulted in phosphorylation and activation of MLKL (pMLKL; Fig. 1A and fig. S1). The kinase activity of RIPK1 was required for both birinapant-induced caspase-8-dependent apoptosis (35) and RIPK3/MLKL-dependent necroptosis, as the D138N mutation in RIPK1 protected neutrophils from cell death (Fig. 1B). Likewise, chemical inhibition of the kinase activity of RIPK1 using necrostatin-1s (Nec-1s), which has become synonymous with blocking RIPK3/MLKL-dependent necroptosis (7, 8, 11, 12), blocked cell death (Fig. 1B), consistent with previous reports (36, 37).

Despite the fundamental requirement for RIPK1 kinase activity in necroptotic cell death, we surprisingly found that *Ripk3^{-/-}*, *Mkl1^{-/-}*, and *Ripk3^{-/-}Mkl1^{-/-}* neutrophils were still highly sensitive to birinapant/z-VAD-fmk-induced cell death (Fig. 1C). Because z-VAD-fmk does not completely inhibit apoptotic caspases (38) and can stabilize cleaved caspases (39), we postulated that residual cleaved (active) caspase-8 may be sufficient to drive apoptotic signaling. In support of this notion, concomitant deletion of caspase-8 and RIPK3 rendered neutrophils resistant to the birinapant/z-VAD-fmk-induced cell death seen in RIPK3-deficient neutrophils (Fig. 1D). To examine whether residual caspase-8 activity limited necroptotic signaling in neutrophils, we examined key signaling events after birinapant/z-VAD-fmk treatment in wild-type, *Ripk3^{-/-}*, and *Mkl1^{-/-}* neutrophils. As anticipated, cleaved (active) caspase-8 was detectable in the lysates of birinapant/z-VAD-fmk-treated *Ripk3^{-/-}* and *Mkl1^{-/-}* neutrophils, despite no overall increase in pro-caspase-8 expression (Fig. 1E). Cleaved caspase-8 was not observed in wild-type neutrophils, but active pMLKL was present, suggesting that necroptosis occurs in a wild-type setting (Fig. 1E). These results indicate that RIPK1 determines cell fate according to downstream substrate availability in neutrophils, whereby caspase-8 inhibition drives RIPK3/MLKL-dependent necroptosis, whereas the loss of RIPK3 or MLKL increases the potential for RIPK1 to engage residual caspase-8 and drive apoptotic cell death (Fig. 1F).

RIPK1 kinase activity regulates NET formation

To further investigate the cellular and biochemical outcomes of engaging RIPK1, RIPK3, and MLKL in mouse neutrophils, we visualized birinapant/z-VAD-fmk-treated cells using electron microscopy. In neutrophils treated with birinapant/z-VAD-fmk but not in control samples, we observed decondensed chromatin emerging from discrete membrane-disrupting structures on the plasma membrane (Fig. 2, A to D, and fig. S2, A to D). Immunogold electron microscopy revealed extracellular structures containing double-stranded DNA (dsDNA; Fig. 2B and fig. S2C), hypercitrullinated histone H3 (H3Cit) (R2/R8/R17; Fig. 2C and fig. S2D), and neutrophil elastase extruding from necroptotic cells (Fig. 2D), all

hallmarks of NET formation. In untreated controls, intracellular azurophilic granules were positive for neutrophil elastase (fig. S2, E and F), and dsDNA staining was evident in the nucleus (Fig. 2, E and F), confirming the specificity of the markers. In contrast, when neutrophils were cotreated with birinapant/z-VAD-fmk and Nec-1s, we observed no loss of cytoplasmic granules and chromatin decondensation, suggestive of preserved nuclear membrane integrity and abrogation of NET release (Fig. 2F and fig. S2F). Notably, the extrusion of dsDNA appeared to be an early event in necroptosis because small portions of dsDNA were extruded from viable neutrophils, leaving most of the nuclear DNA and cytoplasmic granules intact (Fig. 2, A and C, and fig. S2, C and D). In some instances, anucleate neutrophil cytoplasts could be found after DNA release (Fig. 2, B and D), albeit with a loss of cytoplasmic granules. These data suggest that necroptotic cell death was associated with the induction of NETosis.

To further confirm NET formation in response to necroptotic stimuli, we visualized necroptotic neutrophils by immunofluorescence microscopy (Fig. 2G). Similar to neutrophils treated with PMA, a known agonist of NET formation (21), SMAC-mimetic (birinapant or compound B)/z-VAD-fmk-treated neutrophils displayed elongated DNA that colocalized with H3Cit. In line with our electron microscopy results, the addition of Nec-1s abrogated birinapant/z-VAD-fmk-dependent NET formation, as shown by the identical staining pattern when compared to negative controls (Fig. 2G). Together, these data suggest that necroptotic neutrophils generate NETs that are dependent on the kinase activity of RIPK1.

Neutrophil necroptosis, not apoptosis, induces NET formation

Because of the requirement for RIPK1 kinase activity in both RIPK3/MLKL- dependent necroptosis and caspase-8- dependent apoptosis, we wanted to compare the effects of apoptotic and necroptotic signaling on NET formation. To do this, we examined the morphology of necroptotic (birinapant/z-VAD-fmk; Fig. 3A) versus apoptotic (FasL; Fig. 3B) neutrophils by imaging flow cytometry using a gating strategy that identified neutrophils extruding DNA (fig. S2, G and H). H3Cit and dsDNA colocalized on external structures attached to necroptotic (Fig. 3A and fig. S2H) but not apoptotic (Fig. 3B and fig. S2H) neutrophils, indicating that histone hypercitrullination is a feature of neutrophil necroptosis. Decondensed nuclei were also identified in some necroptotic neutrophils, consistent with the nuclear decondensation seen by electron microscopy (Fig. 3A).

The PS marker annexin V is commonly used to quantitate apoptosis, but necroptotic neutrophils also stained positively (Fig. 3A). Annexin V colocalized with extracellular structures containing H3Cit and dsDNA, thereby suggesting that membrane components can be associated with NET structures as they emerge from necroptotic neutrophils (Fig. 3A). Regardless of the stimuli, flow cytometric analysis of apoptotic and necroptotic neutrophils exhibited nearly equivalent maximal annexin V staining. However, necroptotic neutrophils after birinapant/z-VAD-fmk treatment displayed a wide distribution of annexin V staining, reflecting a continuum of PS exposure (Fig. 3C). These data suggest a model of progressive increases in membrane damage caused by MLKL resulting in PS exposure. These data are consistent with other recent studies demonstrating that activation of MLKL triggers

membrane PS externalization, which precedes cellular lysis (40, 41). Furthermore, these data may suggest that induction of neutrophil necroptosis and the consequential release of NET-like structures are highly regulated processes.

Mouse and human neutrophils respond differentially to SMAC mimetics

To study how NET formation is regulated in response to necroptotic stimuli, we modified an established flow cytometry assay to quantify H3Cit and dsDNA release (42). Viability was measured as a percentage of dsDNA-negative events, whereas NET formation was quantified by calculating H3Cit⁺dsDNA⁺ events as a percentage of all neutrophils in the population (Fig. 3, D to F). To confirm the specificity of the flow cytometry assay for NET structures, we used DNase I to degrade extracellular dsDNA before staining, resulting in a reduction of H3Cit⁺dsDNA⁺ events in necroptotic neutrophils (Fig. 3E).

To understand the requirements for cell death signaling in mouse and human neutrophils, we next compared the sensitivity of these cells to a panel of SMAC mimetics. Human neutrophils were sensitive to compound B–induced NETosis of a comparable magnitude to the conventional NET inducer PMA (fig. S3A). In contrast to mouse neutrophils, human peripheral blood neutrophils were not sensitive to equivalent concentrations of birinapant but were sensitive to SMAC mimetics that target XIAP, in addition to cIAP1 and cIAP2 (compounds A to D; Fig. 3G, 3H). Birinapant had an effect at supraphysiological doses that may inhibit XIAP or have other off-target effects (fig. S3, B to E) (10, 43). These data suggest that XIAP, cIAP1, and cIAP2 may be differentially expressed between mouse and human neutrophils. Combination with Nec-1s inhibited the production of NETs (Fig. 3G) and necroptosis (Fig. 3H), indicating that NET formation and death in human neutrophils are dependent on RIPK1 kinase activity.

RIPK1 induces PAD4-dependent NET formation

PAD4 is required for H3Cit, which, along with chromatin decondensation, are hallmarks of NET formation triggered by PMA and microbes (33). Examination of *Pad4*^{−/−} neutrophils confirmed PAD4 involvement in birinapant/z-VAD-fmk–induced NETosis (Fig. 4A). As expected, quantification of NET formation upon loss or inhibition of RIPK1 kinase activity revealed reduced PAD4-dependent H3Cit staining, which was associated with less NET formation (Fig. 4, A and B).

To further tease apart how RIPK1 affects NETosis, we examined events at a molecular level. We observed a time- dependent increase in H3Cit in the supernatant of wild-type neutrophils treated with birinapant/z-VAD-fmk that corresponded with the appearance of pMLKL in neutrophil lysates (Fig. 4C and fig. S4A). In contrast, in *Ripk1*^{D138N/D138N} neutrophils treated with birinapant/z- VAD-fmk, pMLKL was absent, and H3Cit was substantially reduced, suggesting that necroptosis and optimal PAD4 activity are dependent on RIPK1 kinase. The appearance of pMLKL but not H3Cit in PAD4- deficient neutrophils also suggests that PAD4-mediated H3Cit occurs downstream of MLKL phosphorylation (Fig. 4C and fig. S4A). Elastase release into the supernatant corresponded with a reduction in neutrophil lysates and appeared to be partly dependent on RIPK1 kinase activity and phosphorylation of MLKL but independent of PAD4 activity (Fig. 4C and fig. S4A).

To examine the role of PAD4 in necroptosis, we studied *Pad4*^{-/-} neutrophils in response to birinapant/z-VAD-fmk. *Pad4*^{-/-} neutrophils exhibited reduced NET formation as expected but increased necroptotic cell death (Fig. 4, D and E), implying a role for PAD4 in the negative regulation of necroptosis. How PAD4 affects necroptosis is unknown, but it may act through epigenetic and/or transcriptional changes in neutrophils at steady state, or after depletion of IAPs. As previously described, loss of IAPs activates RIPK1-driven neutrophil death and also induces TNF, which acts in a paracrine, or autocrine, manner to trigger cell death (fig. S5, I and J) (44–48). TNF deficiency (*Tnfa*^{-/-}) protected neutrophils from birinapant/z-VAD-fmk-induced death and prevented NET formation (Fig. 4F). Together, these data indicate that RIPK1 kinase activity induces PAD4-dependent NET formation downstream of MLKL and that this is also dependent on a paracrine, or autocrine, loop of TNF driving necroptotic cell death.

We also did not detect NET formation in either *Ripk3*^{-/-} or *Mlkl*^{-/-} neutrophils treated with birinapant/z-VAD-fmk (Fig. 4F). As observed in *Ripk1*^{D138N/D138N} neutrophils, H3Cit induction and secretion were reduced in the absence of MLKL (Fig. 4G and fig. S4B) and RIPK3 (Fig. 4H and fig. S4C), confirming that triggering necroptosis generates H3Cit and NET formation. The residual H3Cit in *Ripk1*^{D138N/D138N}, *Ripk3*^{-/-}, and *Mlkl*^{-/-} neutrophils may be attributable to necroptosis-independent activation of PAD4 (Fig. 4, A, C, G, and H), whereas the low-intensity H3Cit seen in *Pad4*^{-/-} neutrophils (Fig. 4A) may be due to PAD4-independent factors, such as PAD2 (49).

We sought to further confirm the role of RIPK1 kinase activity, RIPK3, MLKL, and PAD4 in necroptotic NET formation using transmission electron microscopy. Immunogold electron microscopy revealed dsDNA extruding from the surface of wild-type neutrophils after treatment with birinapant/z-VAD-fmk (Fig. 5A). H3Cit was also associated with these extracellular structures (Fig. 5A). As expected, normal polymorphonuclear morphology was seen in *Ripk1*^{D138N/D138N}, *Ripk3*^{-/-}, and *Mlkl*^{-/-} neutrophils despite birinapant/z-VAD-fmk treatment, shown by nuclear dsDNA labeling (Fig. 5A). These data suggest that RIPK1 kinase activity and the actions of RIPK3 and MLKL contribute to PAD4-dependent H3Cit generation and dsDNA extrusion. Unexpectedly, despite reduced NET formation after birinapant/z-VAD-fmk treatment (Fig. 4D), *Pad4*^{-/-} neutrophils also exhibited decondensed chromatin and a loss of polymorphonuclear architecture (Fig. 5, A and B). These data suggest that RIPK1, RIPK3, and MLKL activation leads to loss of intracellular architecture and chromatin decondensation, but PAD4 is only required for the externalization of NETs.

MLKL accumulates in the membrane at the site of chromatin release

Because MLKL phosphorylation coincides with the release of H3Cit into the supernatant (Fig. 4, C and G), we examined the localization of MLKL protein and pMLKL by immunogold electron microscopy. We found that MLKL was also detected at the nuclear membrane (Fig. 6A). In addition, MLKL was found on the plasma membrane surrounding regions of DNA release in necroptotic neutrophils (Fig. 6A and fig. S5K) but not in DMSO-treated controls (Fig. 6B and fig. S5K). Nec-1s inhibited MLKL plasma membrane localization (Fig. 6C and fig. S5K), indicating that RIPK1 kinase activity controls the localization of MLKL to the membrane. Active pMLKL (S345) was also detected at the

plasma membrane in necroptotic neutrophils (Fig. 6D and fig. S5K) but not DMSO-treated (Fig. 6E and fig. S5K) or Nec-1s-treated (Fig. 6F and fig. S5K) neutrophils. These data suggest that RIPK1 kinase-dependent necroptosis controls the localization of pMLKL, which may help to recruit additional MLKL to nucleate membrane-disrupting complexes and trigger DNA release (13, 20). MLKL was also found associated with NETs in the extracellular space (Fig. 6A), likely through its association with NET-associated membrane components (Fig. 3A). Together, these data support a model of MLKL translocation to the plasma membrane during necroptosis to contribute to membrane disruption and the release of DNA. MLKL-driven membrane disruption may also be required for the release of granular proteins, such as neutrophil elastase, from azurophilic vesicles, which may, in turn, contribute to NET formation.

Necroptotic NET formation is driven by NADPH oxidase-independent reactive oxygen species

Necroptosis and NETosis are associated with reactive oxygen species (ROS) production and do not require apoptotic caspases (21, 50). Inhibition of ROS using *N*-acetyl-cysteine (NAC) impaired necroptotic death of mouse neutrophils, whereas *Ncf1*-mutant (p47^{phox}-deficient) neutrophils and diphenyleneiodonium- treated neutrophils were unaffected, suggesting NADPH oxidase-independent sources of ROS contribute to neutrophil necroptosis (fig. S5, A to C). NAC treatment also inhibited necroptosis-induced NET formation (fig. S5D). In contrast, PMA- induced NETs are dependent on NADPH oxidase and were sensitive to NAC inhibition (fig. S5E) but were not sensitive to Nec-1s (fig. S5, E and F), RIPK3 deficiency, or MLKL deficiency (fig. S5G). Electron microscopy of PMA-stimulated wild-type, *Ripk3*^{-/-}, and *Mkl1*^{-/-} neutrophils also revealed extensive membrane ruffling, lamellipodia, and filopodia (fig. S5H). This morphology is fundamentally different to the morphology of birinapant/z-VAD-fmk-treated neutrophils (Figs. 2, 5, and 6, and fig. S2), suggesting that necroptotic NET formation may be distinct from PMA-driven NET formation.

NETs released from necroptotic neutrophils restrict the growth of methicillin-resistant *S. aureus*

To determine whether necroptosis-stimulated NET formation was required for the neutrophil pathogen response, we cultured human neutrophils with methicillin-resistant *S. aureus* (MRSA) in vitro in the presence of compound B or z-VAD-fmk/compound B. MRSA growth was restricted after coculture with necroptotic human neutrophils treated with compound B or z-VAD-fmk/compound B but not when neutrophils were coincubated in the presence of DNase I or Nec-1s (Fig. 7A). We obtained similar results with birinapant/ z-VAD-fmk-stimulated neutrophils (Fig. 7B). DNase I-treated cells failed to block MRSA replication (Fig. 7, A and B), indicating that it is the NET structure conferring antimicrobial activity in necroptotic neutrophils, and not the individual components—histones, proteases, or DNA-free cytoplasts. Restriction of MRSA replication by birinapant/z-VAD-fmk-stimulated, IFN- γ -primed neutrophils was dependent on MLKL, confirming that necroptosis-induced NET formation can control MRSA in vitro (Fig. 7C). Incubation of mouse neutrophils with irradiated MRSA reduced neutrophil viability but did so independently of MLKL, possibly by engaging caspase-8-dependent apoptosis in the absence of MLKL (Figs. 1, C, D, and F, and 7D). These data demonstrate that in addition to

the canonical antibacterial pathways used by neutrophils, such as phagocytosis, degranulation, and superoxide production, necroptosis can also function as an antibacterial cell-intrinsic effector pathway through the generation of bacteriostatic NETs.

To examine the role of MLKL in responses to bacterial infection in vivo, mice were challenged with a high dose (10^7) of MRSA by bloodstream inoculation. Infection resulted in accelerated weight loss and thus increased morbidity in *Mkl1*^{-/-} mice (Fig. 7E). When mice were challenged retro-orbitally with a low dose (10^6 CFU) of MRSA to establish a chronic infection, an increase in MRSA burden was observed in the blood and kidney of *Mkl1*^{-/-} mice at specific time points (Fig. 7, F and G, and fig. S6, A and B). Neutrophil numbers in the peripheral blood of *Mkl1*^{-/-} mice were elevated 24 hours after retro-orbital challenge, particularly with high dose of MRSA (10^7 CFU) compared to wild type (Fig. 7H). Furthermore, we confirmed a role for MLKL in blocking MRSA dissemination using an intraperitoneal infection model (51) and demonstrated that *Mkl1*^{-/-} mice failed to control MRSA infection with increased CFU in the blood at one time point (Fig. 7I and fig. S6, C and D). Together, these data suggest that necroptosis serves to control the bacterial load in the organs and prevents dissemination of MRSA in the bloodstream. We obtained similar results when we infected mice with a neutrophil-specific deletion of caspase-8 (*Casp8*^{PMN}), a key negative regulator of RIPK3/MLKL (fig. S6, E to G). Elevated numbers of MRSA were detected in the bone marrow and spleen of *Casp8*^{PMN} mice 22 hours after retro-orbital infection (Fig. 7J). These data indicated a key regulatory role of caspase-8 in neutrophils during MRSA infection and suggest that loss of positive or negative regulators of the necroptosis cascade can interfere with innate immune responses to MRSA.

DISCUSSION

The activation of RIPK3/MLKL-dependent cell death is inflammatory in vivo and thereby acts as an immunomodulatory event. Why this pathway has evolved to counter infection, how it is used in different innate immune cells, and whether it functions beyond the elicitation of high amounts of inflammatory cytokines have remained largely obscure. This study demonstrated that neutrophils can co-opt the MLKL-dependent pathway, in combination with PAD4, to generate NETs in response to pharmacologic activation of cell death. RIPK1 acted as a master regulator of caspase-8-dependent cell death, and RIPK3/MLKL-dependent NET formation and necroptotic death. In doing so, RIPK1 may respond to the availability of its substrates to orchestrate the activation of caspase-8- or RIPK3/MLKL-dependent cell death programs. Similar plasticity in cell death signaling has been described for caspase-8- and MLKL-deficient cell lines engineered for inducible RIPK3 dimerization (52). Likewise, specific RIPK3 kinase mutations (D161N) and RIPK3 kinase inhibitors (for example, GSK'872) can limit necroptosis signaling but trigger caspase-8-mediated apoptosis (53, 54).

Necroptotic neutrophils release NETs at membrane locations that are surrounded by MLKL. This process is positively regulated by TNF α , dependent on PAD4, and can be triggered by the kinase activity of RIPK1. The role of MLKL now appears more complex than previously appreciated, acting not only in disrupting the plasma membrane in necroptotic cells but also as an intermediary in other biochemical pathways. Furthermore, its localization to nuclear

and vesicular membranes suggests alternate roles that require further investigation. This is supported by infection studies showing that human cytomegalovirus can inhibit necroptosis downstream of pMLKL (55). In MLKL-deficient neutrophils, the ultrastructure of cells was unaffected by birinapant treatment and caspase inhibition, indicating that MLKL also contributed to nuclear membrane breakdown and chromatin decondensation leading to NET formation. PAD4 catalyzes the conversion of positively charged arginine residues to neutral citrulline residues, thereby disrupting histone-DNA electrostatic interactions within the nucleosome (33). However, our data indicate that PAD4-independent processes, such as disruption of granule membrane integrity and neutrophil protease activity may also contribute to chromatin decondensation for necroptotic NET formation. Furthermore, in the absence of PAD4, low amounts of H3Cit persisted, which may suggest the involvement of other PAD family members, such as PAD2. PAD4 also appeared to negatively regulate necroptotic signaling but did so without changing pMLKL abundance. Electron microscopy demonstrated that MLKL was associated with necroptotic NET structures, raising the possibility that PAD4 may facilitate the removal of MLKL from the membrane to the extracellular space, in a manner analogous to the removal of MLKL from the membrane by ESCRT- III complexes (40, 41, 56).

The adsorption of antimicrobial proteins onto the burgeoning NET appears to precede plasma membrane rupture and extrusion of the DNA-protein mesh into the extracellular space (57). How MLKL triggers these events before NET release is not clear, but this pseudokinase may act directly by disrupting the membrane of the nucleus (58), azurophilic granules, and/or the plasma membrane. Alternatively, MLKL activity may indirectly drive NADPH oxidase-independent ROS generation, leading to disruption of the aforementioned structures and NET formation.

Several biochemical pathways lead to the generation of NETs. Our data are consistent with previous studies describing RIPK3-independent NET generation in response to PMA (59). Our findings also described stimuli that engage the RIPK3/MLKL-dependent pathway in mouse (birinapant/z-VAD-fmk) and human (compound B/z-VAD-fmk) neutrophils by inhibiting cIAP1/2 and cIAP1/2/XIAP, respectively. The role of necroptosis in NET formation does not exclude alternative cell death pathways in NET formation; rather, the current description of necroptotic NET formation supports the presence of alternative biochemical pathways that may generate subtly different outcomes or types of chromatin structures in the extracellular space upon regulated lytic death.

Necroptosis has been implicated in the pathogenesis of pulmonary and cutaneous *S. aureus* infections in mouse models (60, 61). In our acute and chronic models of MRSA infection, MLKL prevented replication of MRSA in the spleen, kidney, bone marrow, and blood. In MRSA-infected *Casp8^{PMN}* mice, premature infection-triggered neutrophil necroptosis appeared to interfere with efficient clearance of MRSA. In this setting, severe systemic infection leading to widespread necroptosis and NET formation may drive tissue damage, pathological inflammation, and impaired immune responses. Further study with other neutrophil-specific conditionally targeted regulators of necroptosis will be required to establish the role of the RIPK3/ MLKL cell death pathway in response to bacterial pathogens.

Necroptosis is associated with the release of interleukin-1 α (IL-1 α) and IL-1 β (4, 62), and it is possible that NETs from necroptotic neutrophils synergize with IL-1 to bolster antimicrobial responses. NET-associated neutrophil proteases may additionally contribute to the processing and regulation of IL-1 family members in this setting. The orchestrated release of antimicrobial and inflammatory cellular contents including NETs by MLKL membrane-disrupting complexes may be a general feature of necroptotic cells, but this is likely to be limited by PAD4 expression. Future work will be required to determine the contribution of RIPK3/MLKL- and PAD4-dependent signaling in necroptotic NET formation during infection, inflammation, and autoimmunity.

MATERIALS AND METHODS

Mice

The *Ripk1*^{D138N/D138N} (34), *Ripk3*^{-/-} (63), *Tnfa*^{-/-} (64), *Casp-8*^{S100a8Cre/ S100a8Cre} (*Casp8*^{PMN}) (65, 66), *Casp-8*^{-/-}*Ripk3*^{-/-} (10), *S100a8-Cre/GtROSA26-eYFP* (66, 67), *Ncf1* (68), *Pad4*^{-/-} (33), and *Mkl1*^{-/-} (20) mouse strains were generated on or had been backcrossed at least 10 generations with the C57BL/6J background. All animal experiments complied with the regulatory standards of, and were approved by, the Institutional Animal Ethics Committee at Boston Children's Hospital and The Walter and Eliza Hall Institute of Medical Research.

Human peripheral blood neutrophils

Peripheral blood was obtained from normal healthy donors after obtaining informed consent under an approved institutional review board protocol. Donors were 18 years of age or older and had not taken anti-inflammatory medications during the 2 weeks before donating. Red blood cells were removed by two rounds of hypotonic lysis in 0.168 M NH₄Cl, 11.9 mM NaHCO₃, and 10 μ M EDTA (pH 7.3) on ice. Human neutrophils were then isolated on a three-layer Percoll gradient, as described for mouse bone marrow neutrophils below. The purity of neutrophil preparations was 93 $\pm$ 1% (mean $\pm$ SD, n = 3) as assessed by May-Grünwald-Giemsa staining. Contaminating cells were composed almost exclusively of eosinophils (7 $\pm$ 1%).

Mouse bone marrow neutrophil purification

Mouse bone marrow cells from the femur and tibia were isolated in Hanks' balanced salt solution (HBSS) supplemented with 0.075% bovine serum albumin (BSA) and 15 mM EDTA. Cells were overlaid on 78, 68, and 52% Percoll layers and centrifuged at 400g for 30 min at 4°C. Neutrophils were removed from the 68/78% interface and resuspended in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS) for survival assays, or DMEM without FBS for NET assays. The purity of neutrophil preparations was 97 $\pm$ 1% (mean $\pm$ SD, n = 8) as assessed by May-Grünwald-Giemsa staining. Contaminating cells were composed of lymphocytes (2 $\pm$ 1%) and nucleated red blood cells (1 $\pm$ 1%). For assays, neutrophils were primed for 1 hour in recombinant human G-CSF (100 ng/ml; Amgen) or recombinant mouse IFN- γ (100 ng/ml; PeproTech) and then treated with combinations of recombinant mouse TNF α (10 ng/ml; PeproTech), 2 μ M birinapant (Tetralogic Pharmaceuticals), 10 μ M z-VAD-fmk (Sigma-Aldrich), 10 μ M Nec-1s (Enzo

Life Sciences), DNase I (100 U/ml; Worthington Bioscience), or 10 mM NAC (Enzo Life Sciences). Compound B (GT13030–002A) and birinapant were provided by Tetralogic Pharmaceuticals. Compound B functionally inhibits cIAP1, cIAP2, and XIAP. Birinapant inhibits cIAP1 and cIAP2 activity.

Flow cytometry and imaging flow cytometry

Flow cytometric analysis of neutrophils was performed using a BD Biosciences LSRFortessa cell analyzer or a BD Biosciences LSR II cell analyzer. Imaging flow cytometry was performed on the Amnis ImageStream X Mark II. Neutrophils were stained for 30 min with antibodies specific to dsDNA (ab27156, Abcam) or H3Cit (ab5103, Abcam) and then washed twice in 1% BSA/HBSS before primary antibodies were detected using goat anti-rabbit Alexa Fluor 647 (2 µg/ml; A-21245, Life Technologies) for 15 min. For some experiments, dsDNA was detected using 1/2000 of Quant-iT PicoGreen dsDNA Reagent (P7581, Life Technologies), and PS was detected with annexin V (BioLegend).

Immunofluorescence

Treated cells were washed with 1% BSA/HBSS and fixed in 4% paraformaldehyde for 30 min on ice and blocked in 3% (w/v) BSA/HBSS overnight. Antibody specific to H3Cit (R2/R8/R17) (ab5103, Abcam) was applied for 2 hours and detected using goat anti-rabbit Alexa Fluor 647 (A-21245, Life Technologies) for 1 hour. dsDNA was detected using 1/2000 of Quant-iT PicoGreen dsDNA Reagent (P7581, Life Technologies). Fluorescent images were acquired at ×400 magnification using an Axiovert 200 widefield fluorescence microscope (Zeiss) in conjunction with an AxioCam MRm monochromatic charge-coupled device (CCD) camera (Zeiss) and analyzed with Zeiss AxioVision software and ImageJ (National Institutes of Health). Exposure times are identical between images and samples. Quantification was performed using inverted images of five fields of view from triplicate wells per sample.

Immunoblotting

Treated cells were incubated in serum-free DMEM, and the supernatant was collected before lysis of the cell pellet in 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM NaVO₄, and 1× cOmplete Protease Inhibitor Cocktail Tablets (Roche). Proteins from the supernatant were precipitated using 20% (v/v) trichloroacetic acid. Antibodies specific to H3Cit (R2/R8/R17) (ab5103, Abcam), mouse MLKL (ab194699, Abcam), mouse pMLKL (S345) (ab196436, Abcam), mouse caspase-8 (1C12) (#9746, Cell Signaling Technology), mouse cleaved caspase-8 (Asp387) (#9429, Cell Signaling Technology), caspase-3 (#9662, Cell Signaling Technology), elastase (ab68672, Abcam), and ERK (p44/42 MAPK) (#9102, Cell Signaling Technology) were used to analyze cell lysates and supernatants by luminescence-based quantitative Western blots using a Bio-Rad ChemiDoc and Bio-Rad Image Lab Software.

Bacterial infections

Purified neutrophils were primed with IFN- γ for 1 hour, followed by treatment with combinations of birinapant, compound B, z-VAD-fmk, and Nec-1s for 4 hours. MRSA

[multiplicity of infection (MOI), 1] was added to cells for a further 30 min in the presence or absence of DNase I (10 U/ml). For neutrophil cell death assays, cultures of MRSA were irradiated with 650 gray before exposure to neutrophils at MOI of 10 for 18 hours. For in vivo experiments, mice were injected with 10^6 to 10^7 CFU of MRSA retro-orbitally or intraperitoneally. Bacterial load was assessed at days 1, 7, and 14 by culturing blood, spleen, bone marrow, or kidney lysates on tryptic soy agar plates supplemented with methicillin (5 mg/ml). Zero CFU values were marked as below the detection limit.

Electron microscopy

Neutrophils were fixed for at least 2 hours at room temperature in 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4). Cell pellets were washed in 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide (OsO_4)/1.5% potassium ferrocyanide (KFeCN_6) for 1 hour, washed in water three times, and incubated with 1% aqueous uranyl acetate for 1 hour, followed by two washes in water and subsequent dehydration in grades of alcohol (10 min each; 50, 70, and 90%; 2×10 min, 100%). The samples were treated with propylene oxide for 1 hour and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc.). The following day, the samples were embedded in TAAB Epon and polymerized at 60°C for 48 hours. Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut S microtome and then placed on copper grids stained with lead citrate and examined in a JEOL 1200EX transmission electron microscope. Images were recorded with an Advanced Microscopy Techniques (AMT) 2k CCD camera.

Immunogold electron microscopy (Tokuyasu method)

For preparation of cryosections, the cells were fixed in 4% paraformaldehyde for 2 hours at room temperature before fixative was replaced with phosphate-buffered saline (PBS). Before freezing in liquid nitrogen, the cell pellets were infiltrated with 2.3 M sucrose in PBS (containing 0.2 M glycine to quench free aldehyde groups) for 15 min. Frozen samples were sectioned at -120°C , and the sections were transferred to formvar/carbon-coated copper grids. Grids were floated on PBS until the immunogold labeling was carried out. The gold labeling was carried out at room temperature on a piece of Parafilm. All antibodies and protein A-gold were diluted in 1% BSA. The diluted antibody solution was centrifuged at 14,000 rpm for 1 min before labeling to remove possible aggregates. Grids were floated on drops of 1% BSA to block nonspecific labeling, transferred to 5 μl of drops of primary antibody, and incubated for 30 min. The grids were then washed in four drops of PBS for a total of 15 min, transferred to 5 μl of drops of protein A-gold for 20 min, and washed in four drops of PBS for 15 min and six drops of double-distilled water. Contrasting and embedding of the labeled grids were carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 min. Grids were picked up, and the excess liquid was removed by streaking on filter paper (Whatman #1), leaving a thin coat of methyl cellulose. The grids were examined in a JEOL 1200EX, and images were recorded with an AMT 2k CCD camera.

Statistics

Comparisons were performed using Mann-Whitney tests, *t* tests, or ANOVA, followed by Tukey's multiple comparisons test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Substrate availability determines RIPK1 signaling by caspase-8–driven apoptosis or RIPK3/MLKL-dependent necroptosis.

(A) Western blot analysis for the indicated proteins in lysates from wild-type (WT) and *Ripk1*^{D138N/D138N} bone marrow neutrophils primed with interferon- γ (IFN- γ) for 1 hour and treated with combinations of birinapant, z-VAD-fmk, and Nec-1s for 6 hours. Extracellular signal-regulated kinase (ERK) [p44/42 mitogen-activated protein kinase (MAPK)] was used as a loading control. Blots are representative of two to four independent experiments using bone marrow from two to three mice per sample. For quantification of pooled experiments, see fig. S1. MW, molecular weight; WCL, whole-cell lysate. (B) Flow cytometric analysis of mouse bone marrow neutrophil viability assessed by PicoGreen DNA dye exclusion on cells from WT and *Ripk1*^{D138N/D138N} mice treated for 6 hours as indicated after IFN- γ priming. Data are means $\pm$ SEM of six independent experiments. DMSO, dimethyl sulfoxide. (C) Flow cytometric analysis of mouse bone marrow neutrophil viability assessed by PicoGreen DNA dye exclusion on cells from WT, *Ripk3*^{-/-}, *Mkl1*^{-/-}, and *Ripk3*^{-/-}*Mkl1*^{-/-} mice treated with IFN- γ for 12 hours as indicated. Data are means $\pm$ SEM of six independent experiments. (D) Flow cytometric analysis of mouse bone marrow neutrophil viability assessed by PicoGreen DNA dye exclusion on cells from WT and *Casp8*^{-/-}*Ripk3*^{-/-} mice treated with IFN- γ for 14 hours as indicated. Data are means $\pm$ SEM of six independent experiments. (E) Western blot analysis for the indicated proteins in

lysates from WT, *Ripk3*^{-/-}, and *Mkl1*^{-/-} bone marrow neutrophils primed for 1 hour with IFN- γ and treated for 4 hours as indicated. ERK (p44/42 MAPK) was used as a loading control. Blots are representative of four independent experiments. (F) In the presence of z-VAD-fmk, birinapant preferentially promotes RIPK3/MLKL-dependent necroptosis. In the absence of the necroptotic effectors RIPK3 and MLKL, RIPK1 promotes caspase-8-dependent apoptosis. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ by analysis of variance (ANOVA)/Tukey's tests.

Fig. 2. Neutrophil necroptosis is morphologically similar to NETosis.

(A) Transmission electron microscopy analysis of IFN- γ -primed mouse bone marrow neutrophils treated as indicated. Black-filled arrowheads highlight NET formation, and open arrowheads indicate NET absence. (B to F) Immunogold transmission electron microscopy analysis of IFN- γ -primed mouse bone marrow neutrophils treated as indicated. Sections were stained for dsDNA (B, E, and F), H3Cit (C), or neutrophil elastase (Neut elastase) (D). Black-filled arrowheads highlight immunogold staining of NETs, whereas open arrowheads highlight cytoplasmic or nuclear staining. Images are representative of 25 images taken over the course of three independent experiments. Scale bars, 1 μ m. (G and H)

Immunofluorescence microscopy analysis of dsDNA (green channel) and H3Cit (red channel) in IFN- γ -primed neutrophils treated as indicated. Images (G) are representative of three independent experiments performed in triplicate. Quantified data (H) are means $\pm$ SEM of all experiments. * P < 0.05 by ANOVA/Tukey's tests. PMA, phorbol 12-myristate 13-acetate; CpB, compound B.

Fig. 3. Human and mouse necroptotic neutrophils release NETs that are sensitive to DNase I and inhibited by Nec-1s.

(**A** and **B**) Imaging flow cytometric analysis of membrane permeability, phosphatidylserine (PS) exposure, and NET formation assessed by annexin V (AnV), Sytox DNA dye, and H3Cit staining of murine WT bone marrow neutrophils treated as indicated. Black-filled arrowheads highlight NET formation, whereas open arrowheads highlight decondensed nuclei. Data are representative of four independent experiments. For quantification of pooled experiments, see fig. S2G. FasL, Fas ligand; BF, bright field. (**C**) Flow cytometric analysis of membrane permeability and PS exposure assessed by propidium iodide and annexin V staining of neutrophils stimulated for 12 hours as indicated. Data are representative of five independent experiments. G-CSF, granulocyte colony-stimulating factor. (**D** to **H**) Flow cytometric analysis of cellular viability and NET formation assessed by extracellular DNA and H3Cit staining of IFN- γ -primed mouse bone marrow neutrophils treated as indicated. Deoxyribonuclease I (DNase I) pretreatment eliminated the appearance of H3Cit⁺PicoGreen⁺ NET-producing cells (**E**), without effecting PicoGreen⁻ viable cells (**F**). Representative dot plots (**D**) were quantified (**E** and **F**), and data are means $\pm$ SEM of four independent experiments. (**G** and **H**) Flow cytometric analysis of cellular viability and NET formation of IFN- γ -primed mouse bone marrow neutrophils treated as indicated. NET formation (**G**) and viability (**H**) are means $\pm$ SEM of three independent experiments. * P < 0.05, ** P < 0.01, and *** P < 0.005 by Student's t test or ANOVA/Tukey's tests.

Fig. 4. RIPK1 activates PAD4-dependent NET formation.

(A) Flow pMLKL cytometric analysis of cellular viability and NET formation assessed by 50 - WCL extracellular DNA and H3Cit staining of birinapant/z-VAD-fmk-treated 50 WT $\pm$ Nec-1s, *Ripk1^{D138N/D138N}*, and *Pad4^{-/-}* neutrophils. Data are representative of six independent biological replicates from two independent experiments. (B) Flow cytometric analysis of NET formation by IFN- γ -primed WT and *Ripk1^{D138N/D138N}* bone marrow neutrophils treated H for 6 hours as indicated. Data are means $\pm$ SEM of three biological replicates representative from two independent experiments. (C) Western blot analysis of the indicated proteins in supernatants and WCLs from IFN- γ -primed WT, *Ripk1^{D138N/D138N}*, and *Pad4^{-/-}* bone marrow neutrophils treated with birinapant/z-VAD-fmk for the indicated times. Blots are representative of three independent experiments. For quantification of pooled experiments, see fig. S4A. (D and E) Flow cytometric analysis of NET formation (E) and cellular viability (F) from WT and *Pad4^{-/-}* neutrophils treated for 6 hours as indicated. Data are means $\pm$ SEM of six independent biological replicates from two independent experiments. (F) Flow cytometric analysis of NET formation from IFN- γ -primed WT, *Ripk3^{-/-}*, *Mlkl^{-/-}*, and *Tnfa^{-/-}* mouse bone marrow neutrophils treated for 6 hours as indicated. Data are means $\pm$ SEM of three independent biological replicates from three

independent experiments. **(G)** Western blot analysis of the indicated proteins in supernatants and WCLs from IFN- γ -primed WT and *Mkl1*^{-/-} bone marrow neutrophils treated with birinapant/z-VAD-fmk for the indicated times. Blots are representative of three independent experiments. For quantification of pooled experiments, see fig. S4B. **(H)** Western blot analysis of H3Cit in supernatants and WCLs of IFN- γ -primed WT and *Ripk3*^{-/-} bone marrow neutrophils and treated with birinapant/z-VAD-fmk for the indicated times. Blots are representative of three independent experiments. For quantification of pooled experiments, see fig. S4C. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ by ANOVA/Tukey's tests.

Fig. 5. Chromatin decondensation in *Pad4*^{-/-} neutrophils.

(**A** and **B**) Transmission electron microscopy analysis of immunogold staining for dsDNA or H3Cit (black spots) in bone marrow neutrophils from WT, *Ripk1^{D138N/D138N}*, *Ripk3^{-/-}*, *Mkl^{-/-}*, and *Pad4^{-/-}* mice treated with IFN- γ /birinapant/z-VAD-fmk for 12 hours. Arrowheads highlight immunogold particles, black-filled arrowheads highlight NETs, and open arrowheads highlight cytoplasmic or nuclear staining. Images (**A**) are representative of two independent experiments. Quantified data (**B**) on the percentage of neutrophils with dsDNA-stained polymorphonuclear architecture versus decondensed nuclear morphology

are means $\pm$ SEM of 45 to 64 neutrophils for each genotype from two independent experiments. (C) Necrotic stimuli may activate MLKL to trigger numerous cellular and biochemical changes, leading to PAD4-dependent H3Cit and NET formation. Scale bars, 500 nm. $P < 0.0001$ by χ^2 test (B).

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Fig. 6. Activated MLKL colocalizes to sites of DNA release in the plasma membrane. (A to F) Transmission electron microscopy analysis of immunogold staining for MLKL (A to C) and pMLKL (D to F) in mouse bone marrow neutrophils treated with IFN- γ /birinapant/z-VAD-fmk (A and D), IFN- γ , (B and E), or IFN- γ /birinapant/z-VAD-fmk/Nec-1s (C and F). pMLKL was visualized in neutrophils treated with IFN- γ /birinapant/z-VAD-fmk (D), IFN- γ (E), or IFN- γ /birinapant/z-VAD-fmk/Nec-1s (F). Arrows highlight immunogold particles, black-filled arrowheads highlight NETs, and open arrowheads highlight cytoplasmic or nuclear staining. Images are representative of two independent experiments. For quantification of pooled experiments, see fig. S5K. Scale bars, 500 nm.

Fig. 7. Necroptosis leads to bacteriostatic NET formation in human and mouse neutrophils. (A and B) Growth of MRSA bacteria measured by colony formation assay after coculture with IFN- γ -primed human (A) or mouse (B) neutrophils treated as indicated. Data are means $\pm$ SEM pooled from three to six independent experiments performed in triplicate. (C) Growth of MRSA bacteria measured by colony formation assay after coculture with WT or *Mikl*^{-/-} mouse bone marrow neutrophils treated as indicated. Data are means $\pm$ SEM representative of three independent experiments performed in triplicate. (D) Flow cytometric analysis of cellular viability in WT or *Mikl*^{-/-} mouse bone marrow neutrophils stimulated with irradiated MRSA for 18 hours in the presence of IFN- γ (100 ng/ml) or G-CSF (100 ng/ml). Data are means $\pm$ SEM pooled from three independent experiments. (E) Body weight of WT and *Mikl*^{-/-} mice infected retro-orbitally with 10^7 colony-forming unit (CFU) of MRSA at the indicated times after infection. (F and G) Bacterial growth in WT and *Mikl*^{-/-} mice infected retro-orbitally with 10^6 CFU of MRSA as assessed by colony formation assay on the blood at day 7 (F) and kidney at day 14 (G) after infection. Data from at least 11 mice per group are pooled from two independent experiments. (H) Flow cytometric analysis of peripheral blood neutrophil number in WT and *Mikl*^{-/-} mice at 24 hours after retro-orbital infection with the indicated dose of MRSA. Data are means $\pm$ SEM of two independent experiments. (I) Bacterial growth in the blood of WT and *Mikl*^{-/-} mice at 24 hours after peritoneal infection with 10^7 CFU of MRSA. Data from at least 17 mice per group are pooled from two independent experiments. (J) Bacterial growth in the spleen, bone marrow, and blood of WT and neutrophil-specific *Casp8*^{-/-} (*Casp8*^{fl/fl} $\times$ *S100a8-Cre*) mice at 24 hours after retro-orbital infection with 10^7 CFU of MRSA. Data from at least 10 mice per group are pooled from two independent experiments. Dashed lines indicate the limit of detection (LOD) of colony formation assays. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ by Mann-Whitney test or Student's *t* test.