Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner

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Necroptosis is a physiological cell suicide mechanism initiated by receptor-interacting protein kinase-3 (RIPK3) phosphorylation of mixed-lineage kinase domain-like protein (MLKL), which results in disruption of the plasma membrane. Necroptotic cell lysis, and resultant release of proinflammatory mediators, is thought to cause inflammation in necroptotic disease models. However, we previously showed that MLKL signaling can also promote inflammation by activating the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome to recruit the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and trigger caspase-1 processing of the proinflammatory cytokine IL-1β. Here, we provide evidence that MLKL-induced activation of NLRP3 requires (*i*) the death effector four-helical bundle of MLKL, (*ii*) oligomerization and association of MLKL with cellular membranes, and (*iii*) a reduction in intracellular potassium concentration. Although genetic or pharmacological targeting of NLRP3 or caspase-1 prevented MLKL-induced IL-1β secretion, they did not prevent necroptotic cell death. Gasdermin D (GSDMD), the pore-forming caspase-1 substrate required for efficient NLRP3-triggered pyroptosis and IL-1β release, was not essential for MLKL-dependent death or IL-1β secretion. Imaging of MLKL-dependent ASC speck formation demonstrated that necroptotic stimuli activate NLRP3 cell-intrinsically, indicating that MLKL-induced NLRP3 inflammasome formation and IL-1β cleavage occur before cell lysis. Furthermore, we show that necroptotic activation of NLRP3, but not necroptotic cell death alone, is necessary for the activation of NF-κB in healthy bystander cells. Collectively, these results demonstrate the potential importance of NLRP3 inflammasome activity as a driving force for inflammation in MLKL-dependent diseases.

MLKL | NLRP3 | necroptosis | interleukin-1β | Gasdermin D

Caspase-dependent apoptotic cell death is required for mammalian development and the prevention of autoimmune and neoplastic diseases. Programmed cell death can also act to eliminate pathogen-infected cells, with recent studies highlighting how targeted apoptosis-inducing anticancer compounds can treat viral and intracellular bacterial infections (1, 2). On the other hand, the recently characterized caspase-independent necroptotic cell death pathway is dispensable for organism development but, like apoptosis, can be triggered to kill cells harboring pathogenic microbes (3). A number of studies have also reported how pathological activation of necroptotic signaling may contribute to diverse disease states, such as ischemia–reperfusion injury, atherosclerosis, and liver disease, presumably through cell death and the release of proinflammatory mediators (4).

The execution of necroptosis is dependent on receptor interacting serine–threonine protein kinase 3 (RIPK3) phosphorylation of mixed-lineage kinase domain-like protein (MLKL), and MLKL's association with, and disruption of, plasma membrane integrity (5). In the absence of caspase activity, death receptors, such as TNF receptor 1 (TNFR1), as well as the innate immune Toll-like receptors, TLR3 and TLR4, can activate RIPK3 and MLKL to cause necroptotic cell death. Following TLR or TNFR1 signaling RIPK3 oligomerizes and becomes activated through RIP homotypic interaction motif (RHIM)–RHIM interactions with adaptor proteins, such as RIPK1 and TRIF. The resulting filamentous structure (6), termed the necrosome, triggers RIPK3 phosphorylation of the activation loop in MLKL's pseudokinase domain (7–9), which causes a redistribution of MLKL to cellular membranes, such as the plasma membrane, where it can be detected as high-molecular weight oligomers (10–13). Although MLKL lacks enzymatic activity, phosphorylation of the pseudokinase domain of MLKL is thought to cause a conformational change that exposes the killer N-terminal four-helix bundle (4HB) domain (5, 14). Recombinant MLKL (and the 4HB domain) have been shown to permeabilize artificial liposomes in vitro (12, 13, 15). However, if MLKL alone suffices to induce cell death through membrane disruption, or whether MLKL-induced killing requires other cellular factors, such as ion channel opening (10, 11), remains to be clearly defined.

The lytic nature of necroptotic killing results in the release of intracellular contents, which presumably includes damage-associated

Significance

Necroptotic cell death is mediated by activation of the mixed-lineage kinase domain-like protein (MLKL). The inflammation associated with this form of cell death is thought to be due to the release of proinflammatory cellular contents after plasma membrane rupture. In contrast to this prevailing view, we show that MLKL activates the innate immune receptor nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) in a cell-intrinsic manner. Importantly, we show that MLKL-mediated NLRP3 and caspase-1 activation and the secretion of the proinflammatory cytokine IL-1β is a major determinant of necroptotic-derived inflammatory signals. These findings suggest that NLRP3 and IL-1β may be relevant therapeutic targets in MLKL-driven diseases.

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molecular patterns (DAMPs) that can engage pattern-recognition receptors (PRRs) on bystander cells, thereby generating an inflammatory response (16). This model is widely accepted given that genetic knockout of necroptotic repressors, such as caspase-8 or RIPK1, causes inflammation in vivo through RIPK3 and MLKL activity (4). However, the specific DAMPs, or other potential processes that contribute to the inflammatory response following MLKL signaling need to be clarified.

We and others recently documented how in addition to necroptotic cell death, MLKL signaling also triggers activation of the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome complex (17–19). Inflammasome proteins, such as NLRP3, are cytosolic PRRs that can sense pathogen and host molecules, or ill-defined cellular stresses, to engage the inflammatory caspase, caspase-1. Caspase-1 subsequently cleaves and activates the potent proinflammatory cytokines IL-1β and IL-18 and induces their secretion. Although it has been suggested that cell lysis is required for IL-1β release, it has been reported by a number of groups that mature IL-1β secretion is an active cellular process and can occur in the absence of cell death (20–23).

How MLKL signaling activates NLRP3, and whether this event is separable from cell death, remains of outstanding interest. Similarly, the contribution of MLKL-induced NLRP3 inflammasome activity, as opposed to necroptotic DAMP release, on the activation of inflammatory signaling remains unknown. Here, we demonstrate that MLKL activation of NLRP3 requires a decrease in intracellular potassium levels and correlates with the translocation of MLKL to cellular membranes, but can be separated from MLKL-induced cell lysis. Using a series of inducible activated or necroptosis-defective MLKL constructs, we also show that the necroptotic activity of MLKL is required for NLRP3 inflammasome formation and IL-1β secretion. Notably, our data suggest that MLKL-induced activation of the NLRP3 inflammasome, but not death-induced release of DAMPs, is the dominant means by which necroptotic signaling activates NF-κB in healthy bystander cells. These findings suggest that in MLKL-dependent inflammatory diseases the NLRP3 inflammasome could drive pathology, and thus targeting of NLRP3 and IL-1β with available preclinical and clinical therapies may be of benefit.

Results

RIPK3–MLKL Necroptotic Signaling Activates the NLRP3 Inflammasome. Recent reports suggest that TLR and tumor necrosis factor receptor 1 (TNFR1)-induced RIPK3–MLKL signaling can trigger activation of the NLRP3 inflammasome, resulting in caspase-1 dependent IL-1β maturation and secretion (17–19). To genetically confirm that RIPK3 and MLKL can specifically activate IL-1β, we stimulated bone marrow-derived macrophages (BMDMs) from relevant gene targeted mice with the necroptosis-inducing combination of LPS, Smac-mimetic Compound A (Cp.A) [antagonizes inhibitor of apoptosis (IAP) proteins] and low-dose pan-caspase inhibitor Q-VD-OPh (QVD) [10 μM; does not inhibit caspase-1 activity (24)]. Consistent with our previous studies (17), activation of necroptosis resulted in robust cleavage (activation) and secretion of both caspase-1 and IL-1β from WT BMDMs, which was abrogated in cells lacking either RIPK3 or MLKL (Fig. 1 *A* and *B*). Importantly, RIPK3–MLKL-mediated activation of IL-1β was also dependent on NLRP3 and caspase-1, because their genetic deletion also prevented IL-1β and caspase-1 processing and secretion following LPS/QVD/Cp.A treatment (Fig. 1 *C* and *D*). Of note, inflammasome priming following LPS treatment was similar in WT and all relevant gene knockout BMDMs, as measured by TNF release (Fig. 1 *E* and *F*) and induction of pro–IL-1β expression (Fig. 1 *B* and *D*).

Potassium Efflux Links MLKL-Induced Necroptosis with NLRP3 Activation. Activated MLKL compromises plasma membrane integrity and results in ion fluxes across the plasma membrane (5).

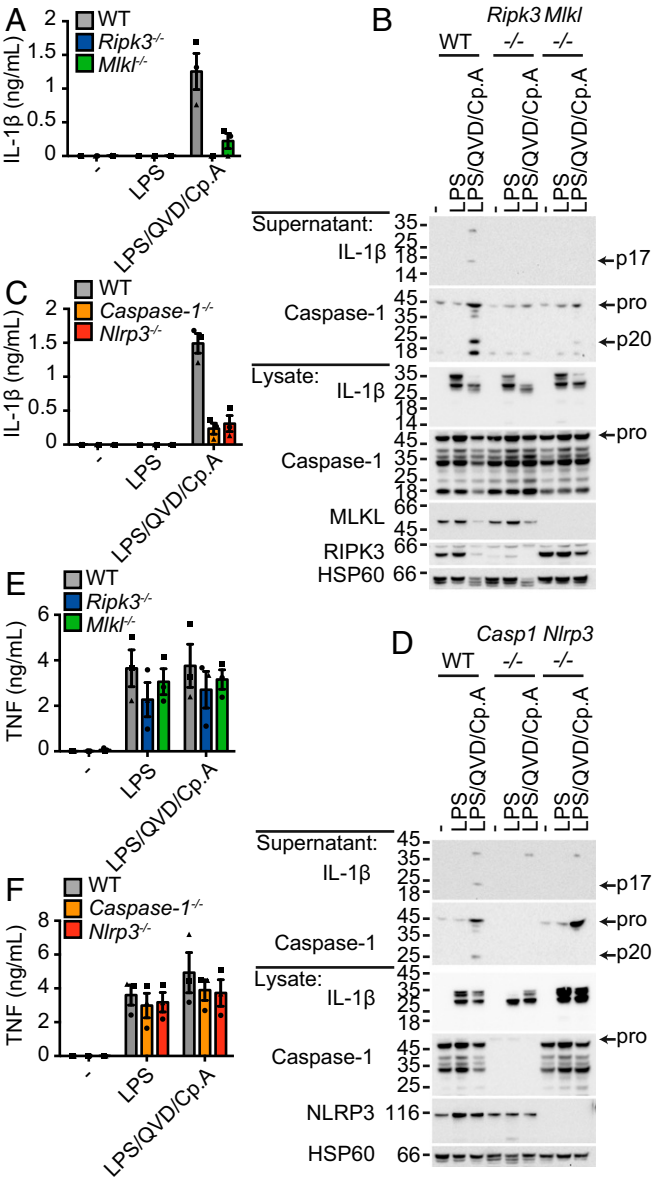

Fig. 1. MLKL activates the NLRP3 inflammasome. WT, *Ripk3*$^{-/-}$, and *Mlkl*$^{-/-}$ BMDMs (*A*, *B*, and *E*) or WT, *Caspase-1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs (*C*, *D*, and *F*) were preincubated with or without LPS (100 ng/mL) for 2–3 h and were then pretreated with Q-VD-OPh (10 μM) for 30 min before stimulation with Cp.A (1 μM) for 5 h. Supernatants were assayed for IL-1β (*A* and *C*) or TNF (*E* and *F*) levels by ELISA. Data are represented as means ± SEM from three independent experiments. (*B* and *D*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. Triangles, circles, and squares in each graph represent independent experiments.

Given that decreased intracellular potassium levels suffice to trigger NLRP3 activity and that high levels of extracellular potassium can inhibit this process (25), we hypothesized that activated MLKL may cause potassium efflux to induce NLRP3 signaling. To test this hypothesis, we increased extracellular potassium levels to 50–150 mM before the activation of MLKL by LPS/QVD/Cp.A treatment. Consistent with our hypothesis, elevated extracellular potassium chloride blocked RIPK3–MLKL-dependent caspase-1 and IL-1β processing and secretion (Fig. 2 *A* and *B*), but did not alter TNF release (Fig. 2*C*). Importantly, necroptotic death was not affected by 50 mM potassium chloride, which maximally

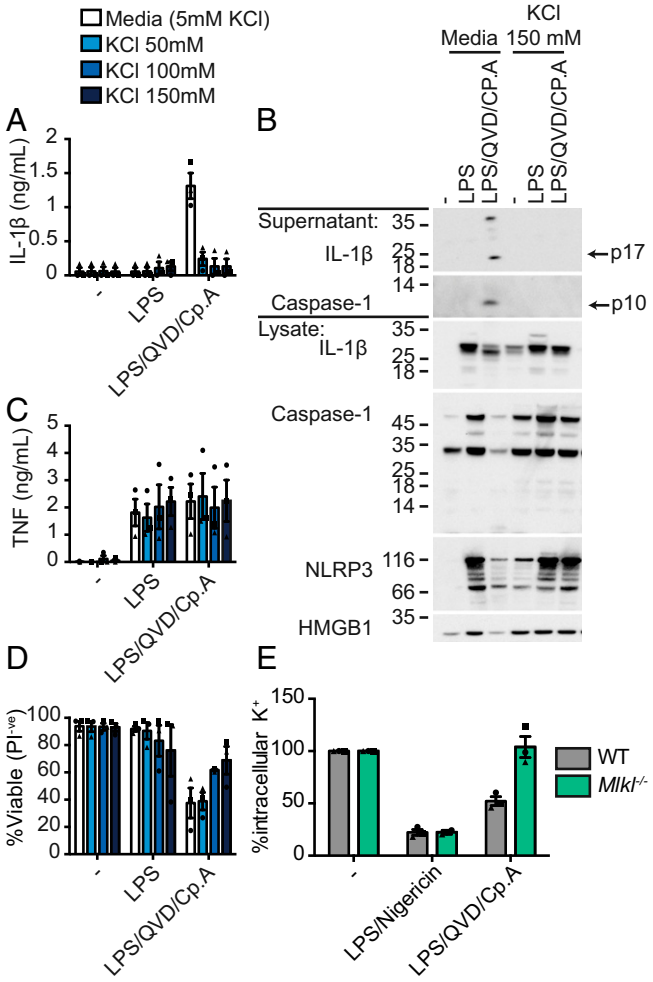

Fig. 2. MLKL induces potassium efflux to activate NLRP3. (*A*–*D*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and increasing concentrations of potassium chloride (50 mM, 100 mM, or 150 mM) 30 min before stimulation with Cp.A (1 μM) for 5 h. (*A* and *C*) Supernatants were assayed for IL-1β (*A*) or TNF (*C*) levels by ELISA. (*B*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. (*D*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*E*) WT and *Mlkl*$^{-/-}$ BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h or nigericin (10 μM) for 2 h, as indicated. The intracellular levels of K^+ were quantified by inductively coupled plasma mass spectrometry. Data are represented as means ± SD from two to three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

inhibits potassium efflux and NLRP3 signaling (25), whereas higher concentrations of potassium (100–150 mM) partially rescued viability following LPS/QVD/Cp.A treatment (Fig. 2*D*). Consistent with potassium efflux being the NLRP3 trigger, the necroptotic stimuli LPS/QVD/Cp.A, like nigericin, decreased intracellular potassium levels in BMDMs in an MLKL-dependent manner, as measured by inductively coupled plasma optical emission spectrometry (Fig. 2*E*).

To investigate if NLRP3 inhibition is specific to increased extracellular potassium chloride, we examined if other alkali halides impacted NLRP3 activity. Strikingly, similar to potassium chloride, 50 mM sodium chloride, rubidium chloride and cesium chloride all markedly reduced NLRP3 activation, as measured by IL-1β secretion resulting from both apoptotic LPS/Cp.A [RIPK3–caspase-8–dependent (17)] or necroptotic LPS/QVD/Cp.A (RIPK3–MLKL-dependent) stimuli (Fig. S1*A*). Importantly, sodium chloride, rubidium chloride and cesium chloride did not impact cellular viability under apoptotic or necroptotic conditions (Fig. S1*B*). In contrast, not only did potassium chloride, rubidium chloride and cesium chloride efficiently prevent nigericin-induced canonical NLRP3 inflammasome activation and IL-1β release, they also inhibited nigericin-mediated pyroptosis (Fig. S1 *A* and *B*). Of note, although cesium chloride did reduce pro-IL-1β levels, the other alkali halides did not consistently impact NLRP3 priming by LPS (Fig. S1*C*), thus highlighting that diminished NLRP3 activation is not simply due to priming defects. These data demonstrate that a range of similarly charged higher, but not lower, atomic weight alkali metal ions can also limit canonical NLRP3 activation and pyroptotic killing.

As expected, extracellular potassium chloride, which blocks MLKL-induced NLRP3 activation, prevented LPS/QVD/Cp.A intracellular potassium levels falling below those observed in untreated macrophages (Fig. S1*D*). Unexpectedly, however, rubidium chloride and cesium chloride, which also inhibit NLRP3, resulted in a dramatic decrease in intracellular potassium even in untreated BMDMs (Fig. S1*D*). How these alkali halides prevent NLRP3 activation therefore remains unclear. Nevertheless, because these larger alkali halides only prevented NLRP3 activation of IL-1β and did not block cell death upon caspase-8 or MLKL signaling, IL-1β activation and cell death are separable events.

MLKL Oligomerization, Membrane Translocation, and Cell Death Occur Simultaneously with NLRP3 Activation. To examine if MLKL-induced IL-1β secretion correlated with MLKL-induced necroptosis, or caspase-1-induced pyroptosis, we examined cell death by propidium iodide (PI) uptake via flow cytometry. Notably, LPS/QVD/Cp.A treatment killed more than 60% of WT BMDMs within 6 h, which was prevented by RIPK3 or MLKL deficiency (Fig. 3*A*). In contrast, LPS/QVD/Cp.A killing was not affected by the absence of NLRP3 or caspase-1 (Fig. 3*B*), which suggests that despite the ability of LPS/QVD/Cp.A to activate caspase-1 in WT cells, necroptosis is the dominant mode of cell death. Consistent with this finding, LPS/QVD/Cp.A resulted in the oligomerization of MLKL and its redistribution from the cytoplasm into a cell membrane fraction within 2 h (Fig. 3*C*), which we have previously documented to be a hallmark for MLKL activation in fibroblasts treated with necroptotic stimuli TNF/QVD/Cp.A (Fig. 3*C*) (15). Indeed, accumulation of activated MLKL at membranes correlated with both macrophage cell death and the secretion of IL-1β (Fig. 3 *D* and *E*).

Activated MLKL has been postulated to directly perturb the plasma membrane, possibly via formation of pores that drive lytic cell death. Although structurally distinct, analogy can be made to the plasma membrane pores formed by the amino-terminal fragment of Gasdermin D (GSDMD). The NLRP3 inflammasome activates caspase-1, which can cleave off the suppressor C-terminal domain (CTD) of GSDMD and liberate the pore-forming N-terminal domain (NTD) to enable efficient pyroptosis and the release of mature IL-1β (26–30). We therefore tested whether GSDMD was also necessary for MLKL-induced IL-1β secretion. However, GSDMD was not essential for IL-1β activation (Fig. 3*F*) despite MLKL-induced IL-1β release requiring both NLRP3 and Caspase-1 (Figs. 1 *C* and *D* and 3*F*). Consistent with this result, GSDMD-deficient BMDMs expressed normal levels of the necroptotic machinery (RIPK3 and MLKL; Fig. S2*A*) and, similar to caspase-1, GSDMD was ultimately dispensable for MLKL killing (Fig. 3*G* and Fig. S2*B*). Hence, in BMDMs, the oligomerization and membrane association of MLKL is likely to trigger membrane damage, potassium efflux and NLRP3 activation to drive IL-1β secretion that coincides with necroptotic death.

Necroptotic Cell Death Activates NLRP3 in a Cell-Intrinsic Manner. Given that necroptotic cell death and NLRP3 activation were detected at the same time, we examined if NLRP3 activation was

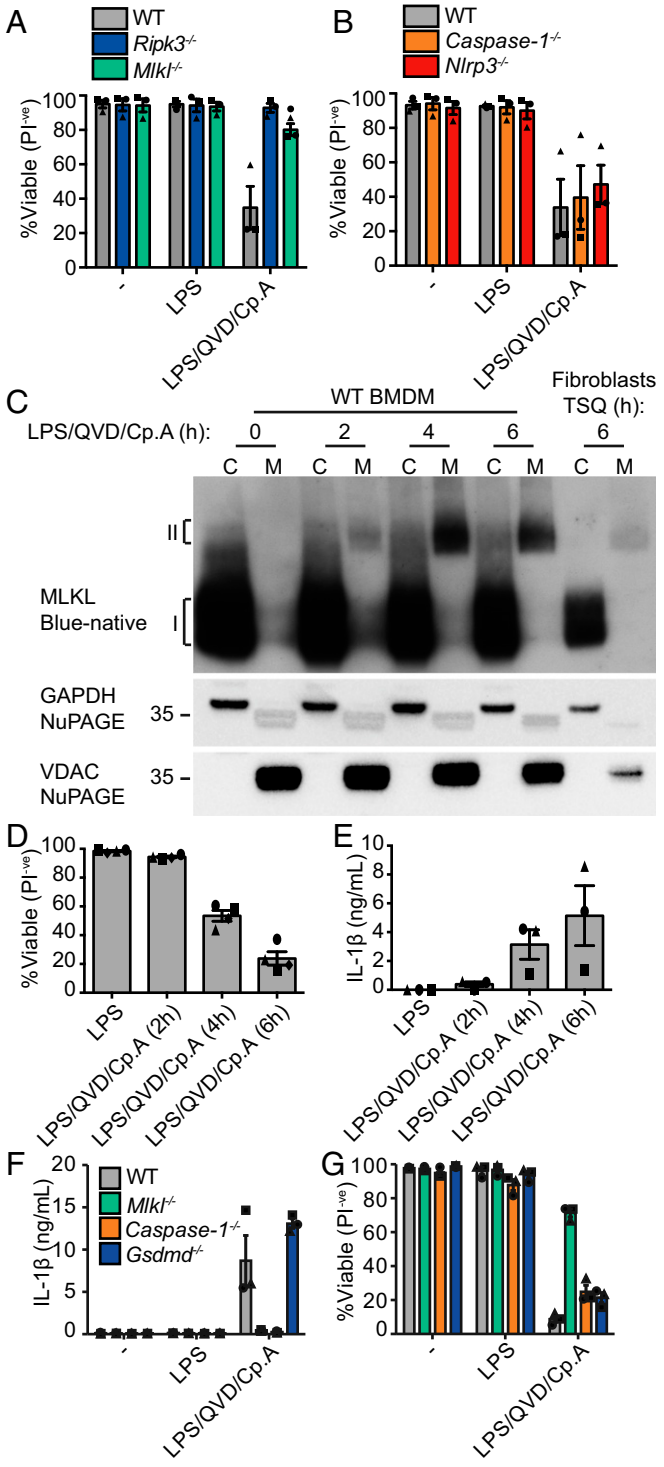

Fig. 3. MLKL oligomerization, membrane translocation and cell death correlates with MLKL-induced IL-1β secretion. (*A* and *B*) WT, *Ripk3*$^{-/-}$, and *Mlkl*$^{-/-}$ BMDMs (*A*) or WT, *Caspase-1*$^{-/-}$, and *Nlrp3*$^{-/-}$ BMDMs (*B*) were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h. Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*C–E*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for the times indicated. MDF cells were stimulated with TNF (100 ng/mL), Q-VD-OPh (10 μM), and Cp.A (1 μM) for 6 h. (*C*) MLKL oligomerization (complex II) formation, and translocation from the cytoplasmic (C) to the membrane (M) fraction, was monitored by Blue Native PAGE. MLKL membrane complex formation was monitored by Blue Native PAGE. Membrane fractionation purity and protein abundance were assessed by immunoblotting for GAPDH and VDAC by NuPAGE gel. Data are representative of three independent repeats. (*D*) Cell death was assessed by flow cytometric analysis of PI uptake. (*E*) Supernatants were assayed for IL-1β levels by ELISA. Data are represented as means ± SEM from three independent experiments. (*F* and *G*) WT, *Mlkl*$^{-/-}$, *Gsdmd*$^{-/-}$, and *Caspase-1*$^{-/-}$ BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 24 h. (*F*) Supernatants were assayed for IL-1β levels by ELISA. (*G*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. Triangles, circles, diamonds, and squares in each graph represent independent experiments.

cell-intrinsic, or was a consequence of released cellular constituents acting on neighboring cells. The osmoprotectant glycine delays the swelling and rupture of cells during lytic cell death, thereby slowing the release of intracellular proteins, such as lactate dehydrogenase (LDH) (31). Incubating BMDMs with increasing amounts of glycine inhibited the release of LDH following either LPS/QVD/Cp.A-induced necroptotic cell death, or ATP-induced pyroptotic cell death (Fig. 4*A*), but importantly did not interfere with LDH assay readout (Fig. S3*A*). Glycine, however, did not block cell death as measured by the staining of cells with PI (Fig. 4*B*), which is consistent with glycine's ability to delay cell swelling and complete rupture, but not plasma membrane disruption (32). Notably, despite the reduction in precursor IL-1β, LDH, caspase-1 p10, and β-actin release, glycine did not block RIPK3–MLKL-induced NLRP3 activation or cytokine release, as reflected by processing and secretion of IL-1β, as well as IL-1α and TNF (Fig. 4 *C–E* and Fig. S3*B*). These data suggest that MLKL-induced NLRP3 inflammasome activation may be cell-intrinsic and not triggered by DAMPs released following plasma membrane rupture.

Glycine treatment only reduced, or in some cases had no effect (e.g., Bid and cytochrome *c*), on the release of cellular proteins following LPS/QVD/Cp.A-induced necroptosis (Fig. S3*B*). Therefore, to better examine if MLKL activates NLRP3 signaling to generate bioactive IL-1β in a cell-intrinsic manner, we examined the ability of necroptotic cells to induce NLRP3 inflammasome formation in neighboring cells. To do so, the formation of NLRP3-induced apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) specks was visualized following LPS/QVD/Cp.A treatment of WT and MLKL-deficient BMDMs, expressing allelic markers Ly5.1 and Ly5.2, respectively. Both genotypes responded to the canonical NLRP3 activating stimuli of LPS/nigericin by forming ASC specks (Fig. S4 *A* and *B*). In contrast, WT but not MLKL-deficient BMDMs, formed ASC specks following necroptotic LPS/QVD/Cp.A treatment (Fig. S4 *A* and *B*), and the formation of ASC specks was prevented by treatment with the NLRP3 inhibitor MCC950 (Fig. S5 *A* and *B*). When WT and MLKL-deficient cells were combined to create a mixed population, the vast majority of necroptotic LPS/QVD/Cp.A induced ASC specks were formed in WT cells and were absent from the cocultured *Mlkl*$^{-/-}$ BMDMs (Fig. 4 *F* and *G* and Fig. S5 *A* and *B*). A small number of ASC specks were observed in the Ly5.2 *Mlkl*$^{-/-}$ cells (Fig. S5*A*), however, these appeared to be due to phagocytosis of dead or dying WT cells. Therefore, necroptotic MLKL activates NLRP3-dependent ASC speck formation in a cell-intrinsic manner, and any released DAMPs have limited, if any, capacity to cause inflammasome formation in bystander cells.

Inducible Expression of Activated MLKL Is Sufficient to Activate NLRP3, Which Can Be Blocked by Increased Extracellular Potassium. We next determined whether MLKL is sufficient to activate IL-1β in the absence of other stimuli. We therefore infected the

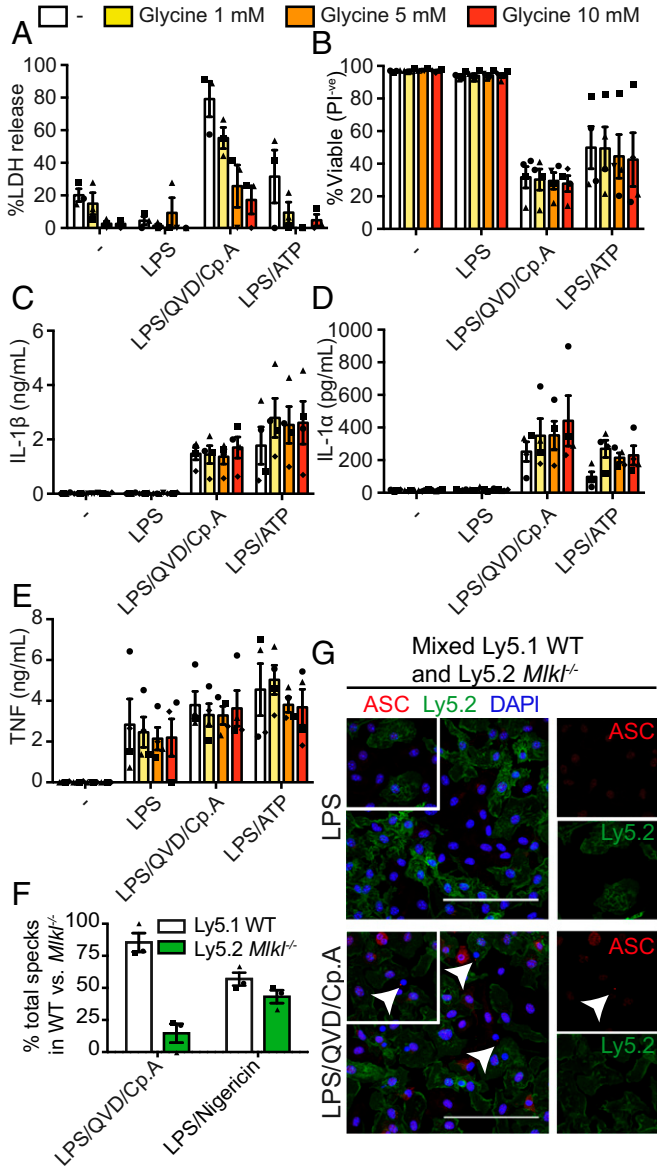

Fig. 4. MLKL activates NLRP3 in a cell-intrinsic manner. (*A*–*E*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and increasing concentrations of glycine (1 mM, 5 mM, or 10 mM) 30 min before stimulation with Cp.A (1 μM) for 5 h or ATP (5 mM) for 2 h. (*A*) Cytoplasmic LDH release into the supernatant was quantified compared with total intracellular LDH of untreated cells (Triton X-lysed). Data are represented as means ± SEM from three independent experiments. (*B*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from four independent experiments. (*C*–*E*) Supernatants were assayed for IL-1β (*C*), IL-1α (*D*), or TNF (*E*) levels by ELISA. Data are represented as means ± SEM from four independent experiments. (*F* and *G*) WT Ly5.1 BMDMs and *Mlkl*$^{-/-}$ Ly5.2 BMDMs were seeded in mixed populations and stimulated with LPS (100 ng/mL) for 2–3 h and then treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 4 h. ASC speck formation (Alexa647, red), Ly5.2 expression (Alexa488, green), and nuclei (DAPI, blue) were assessed by confocal microscopy. (*F*) Specks were assigned to either WT or *Mlkl*$^{-/-}$ cells by automated analysis in FIJI (Fiji Is Just ImageJ) and represented as a percentage of total specks measured. Data are represented as means ± SEM from three independent experiments. (*G*) Representative images from mixed populations treated with LPS or LPS/QVD/Cp.A (necroptotic stimuli) showing ASC speck formation and are representative of five experiments. White arrows indicate ASC specks. (Scale bars: 100 μm.) A second experimental repeat is included in Fig. S5. Triangles, circles, diamonds, and squares in each graph represent independent experiments.

human monocytic THP1 cell line with a range of doxycycline-inducible murine MLKL constructs (7, 14), including (*i*) a full-length WT MLKL, (*ii*) a full-length inactive MLKL mutant that cannot localize with cellular membranes (inactive; E109A, E110A), (*iii*) a CTD and brace region truncation that lacks the membrane damaging 4HB necessary to kill (CTD; residues 124–464), (*iv*) a full-length, constitutively active (CA) MLKL that mimics the activating phosphorylation of MLKL by RIPK3 (phosphomimetic mutant S345D), and (*v*) the NTD of MLKL that harbors the 4HB killing region (residues 1–180) (Fig. S6). Consistent with our previous work in fibroblasts (7, 14), upon doxycycline-induced MLKL expression in THP1 monocytic cells (Fig. 5*A*) neither the inactive or CTD MLKL proteins induced necroptosis (Fig. 5*B*). In contrast, doxycycline-induced expression of CA MLKL or the NTD MLKL efficiently killed cells (Fig. 5*B*). Remarkably, only doxycycline-induced expression of the active MLKL constructs capable of interacting with cellular

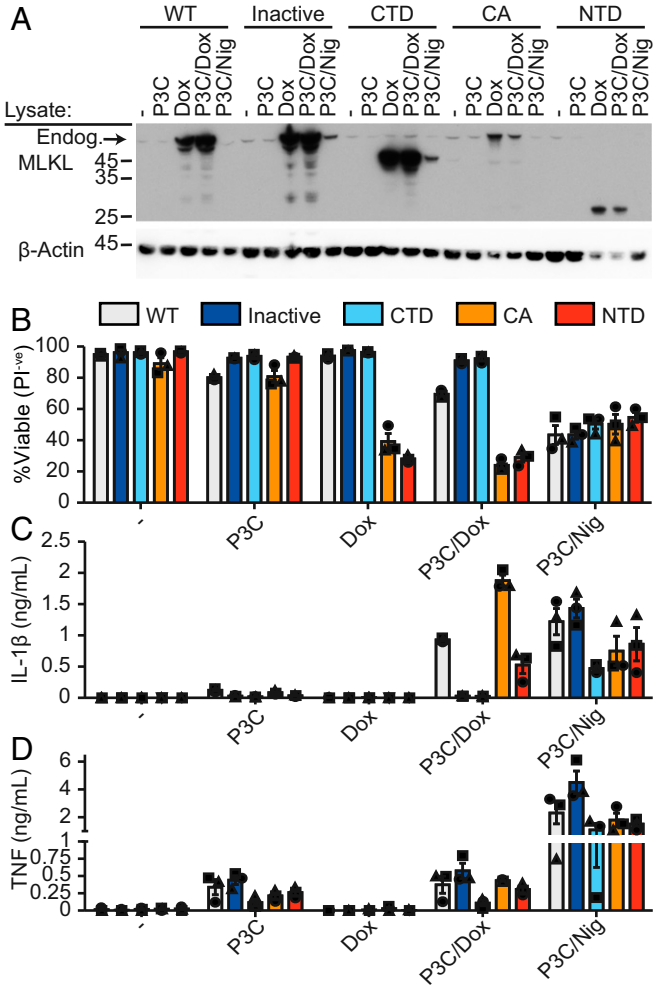

Fig. 5. Necroptosis-inducing MLKL mutants activate NLRP3 to cause IL-1β secretion. (*A*–*D*) THP1 cells infected with doxycycline inducible MLKL constructs (full-length WT, full-length inactive, CTD and brace region truncation, full-length CA, or NTD truncation) were pretreated with P3C (1 μg/mL) for 2 h and then treated with doxycycline (1 μg/mL) for 24 h or nigericin (Nig) (10 μM) for 2 h, as indicated. (*A*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. (*B*) Cell death was assessed by flow cytometric analysis of PI uptake. Supernatants were assayed for IL-1β (*C*) or TNF (*D*) levels by ELISA. Data are represented as means ± SEM from three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

membranes, WT MLKL, CA MLKL or the MLKL NTD, caused robust IL-1β secretion when (TLR1/2)-induced inflammasome priming was induced by Pam_3Cys (P3C) (Fig. 5*C*). In contrast, all THP1 cell lines remained responsive to nigericin-induced canonical NLRP3 inflammasome activation of IL-1β and pyroptosis (Fig. 5 *B* and *C*), as well as P3C-induced TNF secretion (Fig. 5*D*). Collectively, these data show that only necroptotic active MLKL, or the 4HB that causes membrane destabilization, are able to induce IL-1β secretion.

High levels of extracellular potassium (50 mM) efficiently prevented necroptotic (LPS/QVD/Cp.A) activation of NLRP3, but not cell death, in murine BMDMs (Fig. 2). We therefore tested whether increased levels of extracellular potassium could also block NLRP3-caspase-1 and IL-1β processing and secretion in THP1 cells following expression of the CA form of MLKL (S345D). Similar to BMDMs, high extracellular potassium blocked CA MLKL-induced processing, activation and secretion of caspase-1 and IL-1β in THP1 cells but did not prevent cell death (Fig. 6 *A–C*). Similar to potassium chloride, treatment of cells with the specific NLRP3 inhibitor MCC950 (33) abrogated processing and secretion of caspase-1 and IL-1β following CA MLKL induction, but failed to prevent MLKL-induced necroptotic killing (Fig. 6 *D–F*). Importantly, this result was not due to an inhibition of TLR-induced inflammasome priming by MCC950. Consistent with other groups (33), we observed that MCC950 treatment did not alter TLR-induced pro-IL-1β expression or TNF release from THP1 cells, or BMDMs treated with TLR-ligands and NLRP3, apoptotic or necroptotic stimuli (Fig. 6*F* and Fig. S7 *A–E*). These data demonstrate that cell death directed by MLKL, independent of caspase-1 activity, is insufficient to cause IL-1β release but that MLKL must activate the NLRP3 inflammasome to release mature IL-1β.

MLKL Activation of the NLRP3 Inflammasome, but Not Necroptotic Cell Lysis, Is Required for NF-κB Activation in Healthy Bystander Cells. We next tested whether necroptotic DAMP release, or NLRP3 activation, drives the inflammatory potential of MLKL. We examined the ability of supernatants from CA MLKL-killed cells to activate NF-κB signaling in THP1 reporter cells, in which GFP expression is driven by NF-κB signaling (Fig. 7*A*). Notably, most PRRs, including those recognizing host-derived DAMPs, are strong activators of NF-κB, making NF-κB a good readout of both DAMP signaling and NLRP3 induced IL-1β activation.

THP1 cells were differentiated into macrophages by phorbol 12-myristate 13-acetate (PMA) pulse treatment, which induces inflammasome priming and importantly bypasses the requirement for a TLR ligand priming stimulus that would otherwise contaminate the supernatant of MLKL killed cells. Similar to undifferentiated THP1 cells, doxycycline-induced expression of CA MLKL in PMA-differentiated THP1 cells resulted in the secretion of IL-1β (Fig. 7*B*) and significant cell death as measured by both LDH release and PI staining (Fig. 7 *C* and *D*). Importantly, supernatants from differentiated THP1 cells killed by CA MLKL expression activated NF-κB when added to naïve THP1 reporter cells (Fig. 7 *E* and *F*). Pretreatment of differentiated THP1 cells with the NLRP3 inhibitor MCC950 before CA MLKL killing blocked IL-1β secretion but did not prevent necroptotic killing (Fig. 7 *B–D*). Remarkably, despite not blocking necroptosis, MCC950 abrogated the ability of necroptotic supernatants to induce NF-κB (Fig. 7 *E* and *F*).

To confirm that NLRP3 activation and not cellular rupture was required to activate NF-κB signaling in bystander cells, we subjected differentiated THP1 cells to three rounds of freeze-thawing, which resulted in precursor IL-1β release detected by ELISA (Fig. 7*B*) and complete cellular rupture (Fig. 7*C*). However, like necroptotic supernatants, freeze-thawed supernatants failed to activate NF-κB when incubated with THP1 NF-κB reporter cells, unless NLRP3 was activated by CA MLKL signaling (Fig. 7*F*). Collectively, these data show that MLKL-induced activation of the NLRP3 inflammasome, but not necrotic-induced release of potential DAMPs, is the dominant means by which MLKL-mediated necroptosis signals NF-κB activity in healthy bystander cells.

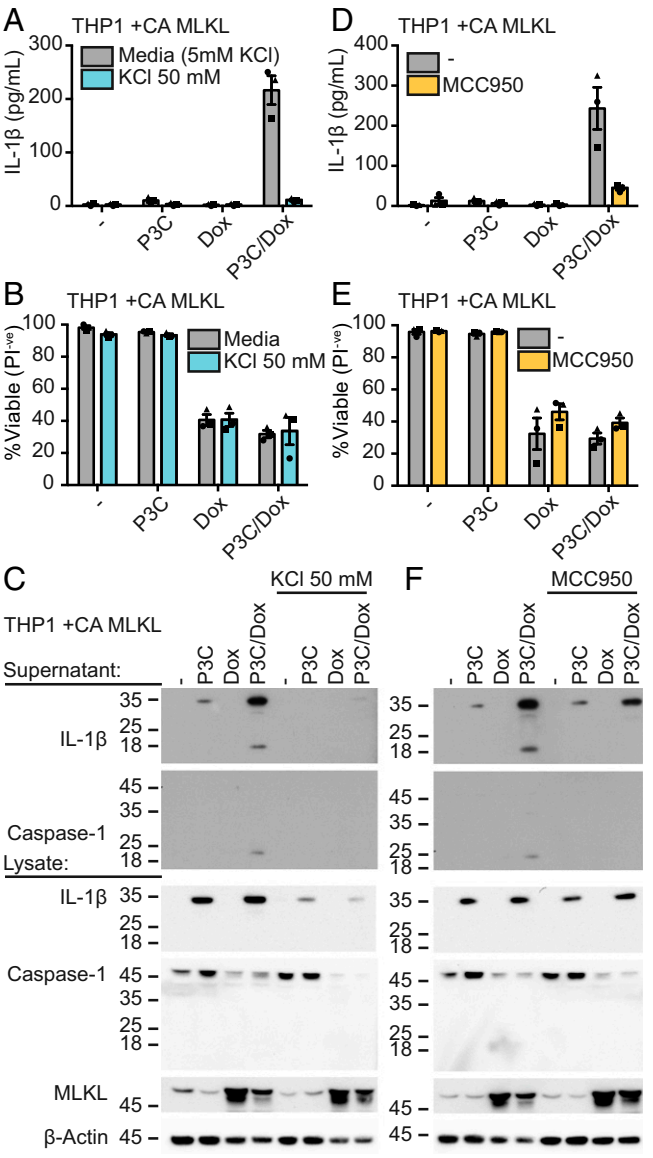

Fig. 6. MLKL-induced IL-1β secretion, but not MLKL killing, is blocked by the NLRP3 inhibitor MCC950 or high extracellular potassium. THP1 cells infected with a doxycycline-inducible CA MLKL construct were treated with P3C (1 μg/mL) and KCl (50 mM) (*A–C*) or the NLRP3 inhibitor MCC950 (1 μM) (*D–F*) before treatment with doxycycline (1 μg/mL) for 24 h. (*A* and *D*) Supernatants were assayed for IL-1β by ELISA. (*B* and *E*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*C* and *F*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. Triangles, circles, and squares in each graph represent independent experiments.

Discussion

Genetic studies have documented how excess necroptotic signaling can cause severe inflammatory disease. Necroptotic cell death is inflammatory in part due to cell lysis and the consequent release of DAMPs, which can induce or amplify inflammatory cytokine levels. We show that MLKL can also drive inflammation through activation of the NLRP3 inflammasome, which induces IL-1β activation and secretion. Our results also reveal that this pathway is responsible for the inflammatory potential of MLKL-killed cells, as measured by their ability to

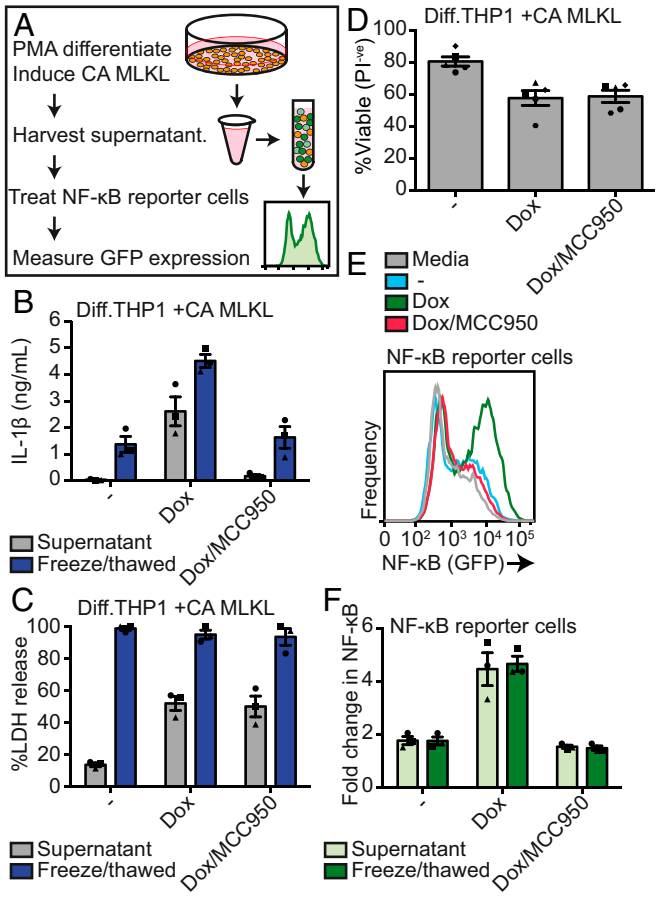

Fig. 7. MLKL-induced NLRP3 inflammasome activation, but not necroptotic cell death, induces NF-κB signaling in healthy bystander cells. (*A*) Schematic of experimental system. (*B–D*) THP1 cells infected with a doxycycline-inducible CA MLKL construct were differentiated by PMA treatment. Cells were treated with the NLRP3 inhibitor MCC950 (1 μM) and then treated with doxycycline (1 μg/mL) as indicated for 24 h. Duplicate plates were subjected to three rounds of freeze thawing to release cytosolic proteins. (*B*) Supernatants from PMA-differentiated cells were assayed for IL-1β by ELISA. (*C*) Cytoplasmic LDH release into the supernatant was quantified compared with total intracellular LDH of untreated cells. Data are represented as means ± SEM from three independent experiments. (*D*) Cell death of PMA-differentiated cells was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from five independent experiments. (*E* and *F*) Cell supernatants were transferred onto THP1 cells bearing an NF-κB GFP-reporter construct. After 8 h of incubation, GFP expression was quantified by flow cytometry. (*E*) Representative histograms of GFP expression representing activation of NF-κB signaling, quantified in *F* as the fold change in mean fluorescence intensity (MFI) relative to THP1 cells in media alone. Data are represented as means ± SEM from three independent experiments. Triangles, diamonds, circles, and squares in each graph represent independent experiments.

activate NF-κB signaling in naïve bystander cells. These findings suggest that NLRP3 may represent an important inflammatory driver in MLKL-dependent diseases and hence is a prospective therapeutic target.

The NLRP3 inflammasome has been reported to be activated in healthy cells following the detection of necrotic cell debris (34). We therefore tested whether necroptotic cell death and DAMP release was the mechanism by which MLKL activated NLRP3 or whether MLKL activation of NLRP3 is a cell-intrinsic process. Glycine has previously been reported to inhibit necrotic cellular lysis, as measured by its ability to prevent LDH release (32). In our hands, the osmoprotectant glycine also inhibited the release of LDH and other, but not all, cellular proteins, and cells still became permeable to propidium iodide. Therefore, a caveat of glycine is that it delays, but does not completely prevent, plasma membrane rupture. As such, we could not conclusively determine if IL-1β secretion was an active or passive process during necroptosis. This question of whether IL-1β can be actively secreted from cells after caspase-1 activation remains controversial (20, 23, 35–37). These experiments nonetheless suggested that NLRP3 activation could be cell-intrinsic, because glycine treatment had no effect on mature IL-1β secretion following MLKL activation. In line with this idea, the assessment of intracellular ASC specks by confocal microscopy demonstrated that necroptotic cells did not activate NLRP3 and ASC speck formation in neighboring cells. Together, these experiments provide strong evidence that MLKL activation of NLRP3 is specific and cell-intrinsic and occurs as a consequence of necroptosis signaling, but before cellular disintegration.

Mechanistically, MLKL appeared to trigger NLRP3 through its necroptotic activity, where oligomerization and translocation of endogenous MLKL to membrane fractions correlated with IL-1β activation. However, external necroptotic activating stimuli can also signal through RIPK1 and RIPK3 and drive multiple inflammatory signaling pathways (38). Therefore, to confirm that NLRP3 activity was dependent on MLKL, we directly induced the expression of necroptotic-competent MLKL, which caused significant NLRP3 signaling and subsequent IL-1β secretion.

Apart from two notable exceptions (39, 40), NLRP3 activators appear to induce a loss of intracellular potassium to specifically cause inflammasome assembly (25, 41). Our data are consistent with a mechanism in which MLKL translocates to the membrane causing potassium efflux. Subsequently, the reduced intracellular potassium concentration leads to activation of NLRP3 in a cell-intrinsic manner (Fig. 8). High levels of extracellular potassium blocked potassium efflux following necroptotic stimuli, thereby preventing NLRP3 activation following both endogenous MLKL activation, or through expression of inducible necroptotically

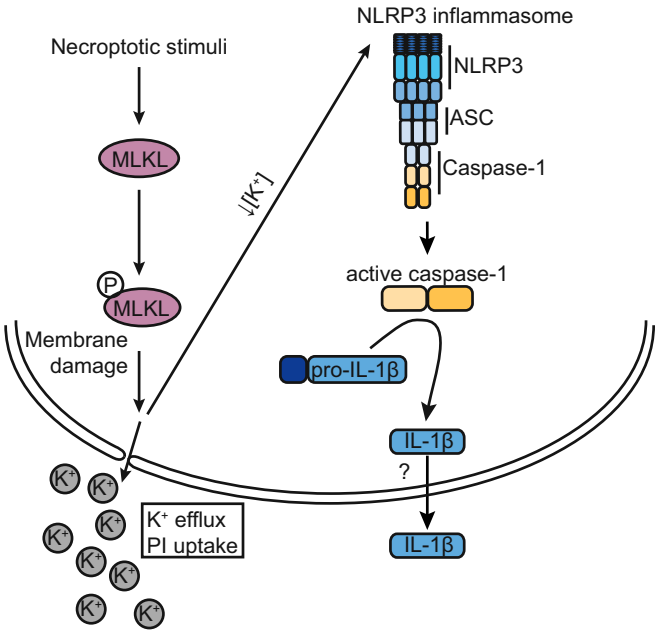

Fig. 8. Model of how MLKL activates the NLRP3 inflammasome. Necroptotic stimuli activate MLKL causing potassium efflux. Potassium efflux nucleates NLRP3 inflammasome formation, resulting in caspase-1 cleavage and activation of IL-1β. Whether IL-1β secretion subsequently occurs by an active or passive mechanism, or a combination of both, is unknown.

active MLKL. It remains unclear how rubidium chloride and cesium chloride inhibit the activation of NLRP3 given that treatment with these cations alone reduced intracellular potassium levels.

Inflammation driven by necroptotic signaling is not necessarily dependent on MLKL killing, but can instead be caused by activation of RIP kinases (42) or through MLKL-activation of NLRP3, driving cytokine secretion (17–19). Our results indicate that MLKL-induced NLRP3 activity and IL-1β secretion, not necroptotic DAMP release, is the dominant activator of NF-κB signaling in healthy bystander cells. Indeed, MLKL-mediated activation of IL-1β has been reported to cause inflammation mediated tissue damage in a model of *Staphylococcus aureus* infection (43). In addition, there are a growing number of studies that have separately implicated necroptosis or NLRP3 in driving pathology of atherosclerosis (44–46), multiple sclerosis (47–49), and ischemia–reperfusion injury of the heart (50, 51) and brain (52–54). Indeed, in models of kidney ischemia–reperfusion injury, both MLKL (42) and NLRP3 (55) deficiency are protective. Based on our findings, it is possible that MLKL-induced NLRP3 signaling is a pathological driver in these inflammatory diseases. Defining the role of MLKL-induced IL-1β signaling in these and other potential necroptotic models (56) will therefore critically inform the development and testing of new disease-specific, antiinflammatory, therapeutic strategies.

Materials and Methods

Cell Culture. BMDMs were generated from bone marrow cells harvested from femoral and tibia bones as described previously (17), except in Fig. 3 *F* and *G* and Fig. S2 which were generated as in ref. 21. BMDMs were seeded at 4×10^5 per well (24-well plate) overnight. *Ripk3*$^{-/-}$, *Nlrp3*$^{-/-}$, *Mlkl*$^{-/-}$, *Gsdmd*$^{-/-}$, and *Caspase-1*$^{-/-}$ (*ICE*$^{-/-}$, with a naturally occurring inactivating caspase-11 deletion) have been described previously (7, 27, 57–60). The Walter and Eliza Hall Institute (WEHI) and University of Queensland Animal Ethics Committee approved all animal experiments. Further experimental details are provided in *SI Materials and Methods*.

Constructs. Inducible lentiviral MLKL constructs were published previously (7, 14, 15) and used to stably infect THP1 cells. NF-κB reporter THP1 monocytic cells were made by infection with the lentiviral reporter vector (pTRH1-NF-κB–dscGFP, TR503PA; System Bioscience) and sorted by flow cytometry for GFP expression.

Cytokine ELISA. Mouse IL-1β [DY401 (R&D Systems) or 88-7013-77 (eBioscience)], human IL-1β (DY201; R&D Systems), mouse TNF (88-7324; eBioscience), human TNF (88-7346; eBioscience), and mouse IL-1α (433401; BioLegend) ELISA kits were used on supernatants according to the manufacturer's instructions.

Cell Viability. BMDMs seeded on non-tissue culture-treated 24-well plates were harvested using 5 mM EDTA/PBS. Viability of both THP1 cells and BMDMs was analyzed by propidium iodide (PI) uptake by flow cytometry on a FACSCalibur or FACSCanto (BD Immunocytometry Systems). Data were analyzed using FlowJo software version 7.6.5. LDH activity was analyzed by Cytotoxicity Detection Kit (LDH) [11644793001 (Roche) or G1780 (Promega)] as per the manufacturer's instructions.

Immunoblotting. Immunoblotting and fractionation was carried out as previously published (14, 17). Blots were probed with antibodies against pro- and cleaved mouse caspase-1 [sc-514 (Santa Cruz Biotechnology) and AG-20B-0042-C100 (Adipogen)], human caspase-1 (3866; Cell Signaling Technology), IL-1β (AF-401-NA; R&D), NLRP3 (AG-20B-0014-C100; Adipogen), ASC (sc-22514-R; Santa Cruz Biotechnology), β-actin (A-1978; Sigma), RIPK3 (PSC-2283-c100; Axxora), MLKL (3H1; in-house), VDAC1 (AB10527; EMD Millipore), GAPDH (2118; Cell Signaling Technology), HMGB1 (ab18256; Abcam), HSP60 (sc-1052; Santa Cruz Biotechnology).

Immunofluorescence of Endogenous ASC Specks by Confocal Microscopy. Immunofluorescence of ASC specks was performed similarly as described (61). Further experimental details are provided in *SI Materials and Methods*.

NF-κB Signaling. Supernatants were removed from cultured THP1 cells and pelleted to remove any debris. Duplicate seedings were subjected to three rounds of freeze thawing, supernatants were then cleared by pelleting at 13,000 rpm. THP1 cells bearing the NF-κB reporter construct were cultured in the conditioned supernatant for 8 h before measuring GFP expression of THP1 cells relative to those in media alone using a FACSCalibur (BD Immunocytometry Systems).

Intracellular Potassium Levels. Intracellular K^+ measurements were performed by inductively coupled plasma optical emission spectrometry as described previously (25).

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