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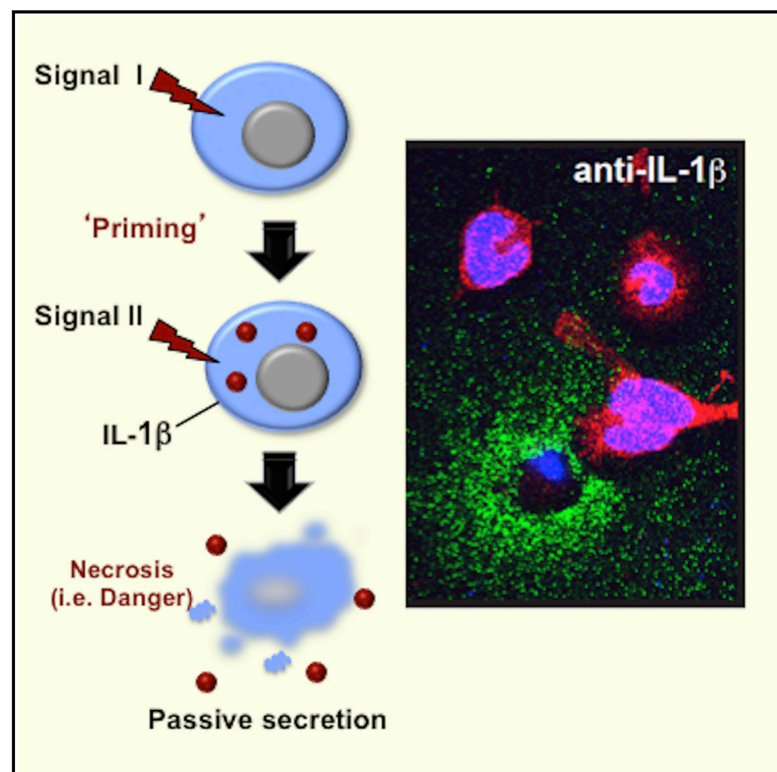
Diverse Activators of the NLRP3 Inflammasome Promote IL-1β Secretion by Triggering Necrosis

Graphical Abstract



Authors

Sean P. Cullen, Conor J. Kearney, Danielle M. Clancy, Seamus J. Martin

Correspondence

martinsj@tcd.ie

In Brief

A two-signal model has emerged for NLRP3 inflammasome-dependent IL-1β maturation, but the mechanism of IL-1β release remains unclear. Here, Cullen et al. show that all inflammasome "signal II" agents examined triggered necrosis, with IL-1β secretion confined to necrotic cells, suggesting that IL-1β represents an inducible danger signal.

Highlights

- Diverse NLRP3 activators promote necrosis
- IL-1β release is confined to necrotic cells
- "Signal II" represents a necrotic trigger
- IL-1β functions as a danger signal

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Diverse Activators of the NLRP3 Inflammasome Promote IL-1β Secretion by Triggering Necrosis

Sean P. Cullen,[1,2] Conor J. Kearney,[1] Danielle M. Clancy,[1] and Seamus J. Martin[1,2,*]
[1]Molecular Cell Biology Laboratory, Department of Genetics, The Smurfit Institute
[2]Immunology Research Centre
Trinity College, Dublin 2, Ireland
*Correspondence: martinsj@tcd.ie
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SUMMARY

The NLRP3 inflammasome is involved in caspase-1-dependent maturation of IL-1β in many contexts. A two-signal model has emerged for IL-1β maturation, with LPS providing "signal I" and diverse agents such as ATP, Nigericin, streptolysin O, uric acid crystals, and alum salts capable of acting as "signal II." In the absence of signal II, pro-IL-1β is upregulated but typically fails to be processed or released. What unites signal II stimuli has been debated, with the ability to promote K+ efflux suggested as a common factor, but the mechanism of IL-1β release remains unclear. Here, we show that all examined inflammasome signal II agents triggered necrosis, which was highly correlated with their ability to promote IL-1β release. IL-1β secretion occurred in tandem with the release of many additional proteins and was confined to necrotic cells. Thus, signal II agents initiate inflammation by promoting necrosis-driven IL-1β release, suggesting that IL-1β represents an inducible danger signal.

INTRODUCTION

The NLRP3 inflammasome is a caspase-1-activating complex that is involved in IL-1β processing and activation in response to pathogens or sterile injury (Agostini et al., 2004; Mariathasan et al., 2006; Franchi et al., 2012). This complex, which is comprised of NLRP3, ASC, and caspase-1, is assembled in response to bacterial lipopolysaccharide (LPS) and is activated by diverse stimuli (Agostini et al., 2004; Mariathasan et al., 2006). Most cell types are devoid of IL-1β and only synthesize this in response to detection of LPS via TLR4. However, LPS alone fails to induce robust processing and activation of IL-1β in most cell types (Franchi et al., 2012), which has led to the emergence of a two-signal model for IL-1β processing and release.

In the two-signal model of inflammasome activation, "signal I" (LPS or another pathogen-associated molecular pattern) induces the expression of pro-IL-1β as an inactive 32-kDa precursor but fails to trigger maturation or release of this cytokine. For productive IL-1β processing and secretion, a second signal ("signal II") induces processing of pro- IL-1β to the p17 active form, which is then released into the extracellular space. The mechanism of IL-1β release has been much debated, with some suggesting that caspase-1 is involved in the selective transport of IL-1β across the plasma membrane (Brough and Rothwell, 2007), whereas others suggest that membrane microvesiculation is involved (MacKenzie et al., 2001). Yet another putative mechanism involves ATP-mediated triggering of membrane P2X7R channels, resulting in P2X7R-dependent formation of multi-vesicular bodies containing inflammasome components, with subsequent membrane fusion and release of IL-1β-containing exosomes (Qu et al., 2007). However, a mechanism for IL-1β release that unites all signal II stimuli has yet to emerge.

Somewhat perplexingly, a plethora of diverse agents have been shown to be capable of acting as signal II for IL-1β processing and secretion. These include the pore-forming toxins streptolysin O (Harder et al., 2009), Nigericin (Franchi et al., 2012), maitotoxin (Mariathasan et al., 2006), and valinomycin (Gurcel et al., 2006), as well as the P2X7 channel activator ATP (Perregaux et al., 1996), uric acid, and silica crystals (Martinon et al., 2006; Dostert et al., 2008) and the widely used adjuvant aluminum hydroxide (alum; Eisenbarth et al., 2008). Many of these agents are capable of triggering K+ efflux from the cell, and indeed, addition of excess K+ to the extracellular space suppresses activation of the NLRP3 inflammasome and IL-1β release (Pétrilli et al., 2007; Muñoz-Planillo et al., 2013). However, numerous additional agents have also been found to induce processing and release of IL-1β, ranging from the cytotoxic drug daunorubicin, UV treatment, and histones (Feldmeyer et al., 2007; Sauter et al., 2011; Allam et al., 2013). What all of these agents share in common is unclear, and no unified mechanism of NLRP3-dependent IL-1β processing and release has emerged.

One hypothesis is that extracellular ATP, released during inflammation, might act as a common signal for the opening of P2X7 membrane channels, thereby facilitating K+ release (Sanz and Di Virgilio, 2000; Labasi et al., 2002). However, P2X7-null cells are capable of activating the NLRP3 inflammasome and degradation of extracellular nucleotides did not inhibit LPS-driven inflammation in vivo (Franchi et al., 2007; He et al., 2013). In addition, concentrations of ATP that are required to promote P2X7-dependent inflammasome activation in

experimental settings are typically in the non-physiological, micromolar range and are unlikely to be achieved in vivo. Furthermore, inflammasome activation by flagellin or intracellular bacteria, as well as in vivo administration of LPS, proceeded independently of the P2X7R (Franchi et al., 2007; He et al., 2013).

A caspase-activating complex similar to the NLRP3 inflammasome, the Apaf-1/caspase-9 apoptosome, is activated by numerous divergent stimuli and promotes apoptosis-associated caspase activation (Taylor et al., 2008; Adrain and Martin, 2009). Whereas numerous cytotoxic stimuli activate the inflammasome, all of these ultimately converge on mitochondria to trigger assembly of the Bax/Bak channel and efflux of cytochrome c from mitochondria. Within the cytosol, cytochrome c triggers the assembly of the Apaf-1 apoptosome and is the only known activator of this complex. Thus, whereas activators of the apoptosome are chemically and physically highly diverse, they all ultimately utilize the same mechanism of activating this complex: mitochondrial permeabilization. Thus, the Apaf-1 apoptosome acts as a sensor for mitochondrial permeabilization by binding to a molecule (cytochrome c) that is normally sequestered within healthy mitochondria.

Given the precedent established by activation of the Apaf-1/caspase-9 apoptosome, it is highly probable that the highly diverse triggers of the NLRP3 inflammasome also converge on some common event that is sensed by this complex. Here, we demonstrate that all signal II stimuli examined led to non-selective permeabilization of plasma membranes to promote necrosis and IL-1β release. We also show that signal-II-initiated necrosis and associated IL-1β maturation and release were all blocked through raising extracellular K+. Taken together, these data argue that agents capable of triggering plasma membrane damage or permeabilization, leading to a decline in intracellular K+ levels and concurrent necrosis, can serve as signal II for activation of the NLRP3 inflammasome. Thus, similar to the apoptosome, which detects permeabilization of mitochondrial membranes, the NLRP3 inflammasome detects permeabilization of the plasma membrane and acts as a sensor for "danger" or "necrotic cell death".

RESULTS

LPS Treatment Upregulates Pro-IL-1β but Leads to Little Processing or Release

As previously described in many studies, LPS stimulation of THP-1 monocytic cells led to the production of multiple cytokines and chemokines and robust upregulation of pro-IL-1β but little processing or secretion of IL-1β into the extracellular space (Figures 1A and 1B). However, addition of diverse agents, such as Nigericin, ouabain, valinomycin, streptolysin O, ATP, or LLOMe, which are all known to be capable of acting as signal II stimuli for the NLRP3 inflammasome, led to robust IL-1β processing and release of mature p17 IL-1β from the same cells (Figures 1C and 1D). To demonstrate that the processed and secreted p17 IL-1β was indeed active, we transferred THP-1 cell supernatants onto HeLa cells, which are IL-1 responsive (but LPS insensitive) and generate robust amounts of IL-6 and IL-8 in response to IL-1β (Afonina et al., 2011). All signal II agents tested induced robust IL-1β activation and secretion from THP-1 cells, as assessed by IL-6 and IL-8 secretion in the HeLa cell bioassay (Figure 1E). We confirmed that the latter cytokines were generated by HeLa cells in an IL-1β-dependent manner through use of specific neutralizing antibodies to the latter, whereas neutralizing antibodies directed against IL-1α, IL-33, or TNF failed to inhibit IL-6/IL-8 production in this context (Figure 1F).

Signal II Stimuli Share in Common the Ability to Promote Necrosis

What signal II agents have in common has been debated, but it is clear that a drop in intracellular K+ is one of the common effects of many of these agents (Muñoz-Planillo et al., 2013). The mechanism of IL-1β release also remains unresolved; however, we considered the simple hypothesis that agents capable of acting as signal II simply trigger necrosis, which is invariably preceded by a drop in intracellular K+ as plasma membrane permeability is lost, which would lead to inflammasome activation followed rapidly by IL-1β release as a consequence of the complete loss of membrane integrity.

This hypothesis makes three predictions: first, that the ability of signal II agents to promote IL-1β processing and release is closely correlated with their ability to promote necrosis; second, that IL-1β release is non-selective and accompanied by numerous additional cytosolic proteins; and third, that IL-1β release only occurs from necrotic, but not viable, cells. To explore whether necrosis is the common mechanism of inflammasome-dependent IL-1β secretion, we initially asked whether signal II stimuli induced necrosis within the ranges that they exhibit activity as initiators of IL-1β maturation and release. As Figures 2A and 2B demonstrate, all signal II stimuli tested induced cell death in a dose-dependent manner, which was highly correlated with their ability to trigger mature IL-1β release, as measured by the HeLa bioassay. Of note, signal II agents promoted cell death irrespective of whether cells were treated with LPS or not (Figure S1), suggesting that the cell death seen in this context was independent of inflammasome activation. In further support of this, the poly-caspase inhibitor zVAD-fmk failed to block LPS+signal II-driven cell death but completely inhibited LPS+signal II-initiated IL-1β production, as indicated by the HeLa bioassay (Figure S1). Furthermore, knockdown of the inflammasome constituents NLRP3, ASC, or caspase-1 (Figure S2A) failed to attenuate signal II-associated cell death (Figures S2B and S2D–S2F) but profoundly attenuated IL-1β processing (Figure S2C) and the release of bioactive IL-1β (Figures S2B and S2D–S2F). Collectively, these data suggest that signal II agents are intrinsically cytotoxic and induce cell death independent of LPS or inflammasome activation.

Signal-II-Induced Cell Death Occurs via Conventional Necrosis

The morphology of cell death observed in response to signal II agents appeared to be necrotic, rather than apoptotic, as cells underwent high-amplitude swelling and ballooning, followed by rapid membrane permeabilization in response to signal II stimuli (Figure 2C). Moreover, cells treated with the latter agents failed to display nuclear hypercondensation or fragmentation typical of apoptosis and underwent simultaneous uptake of the vital

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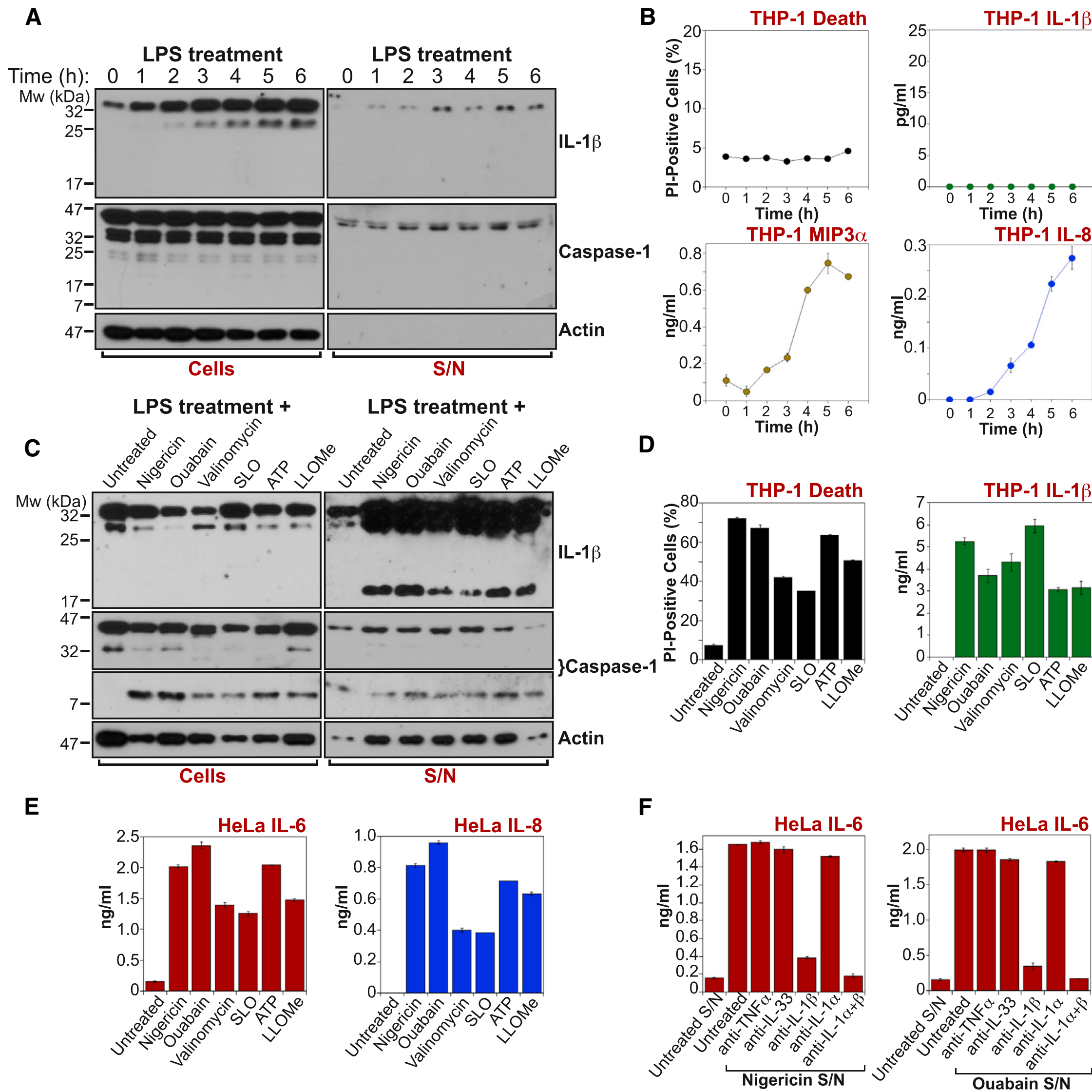

Figure 1. Diverse Signal II Agents Promote IL-1β Processing and Release

(A) THP-1 cells were primed with LPS (1 μg/ml) for the indicated durations. Cell lysates and clarified culture supernatants were analyzed by western blotting for the indicated proteins.

(B) THP-1 cells from (A) were scored for necrosis by PI staining by flow cytometry, and cytokine concentrations in the culture supernatants were measured by ELISA.

(C) THP-1 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with Nigericin (2.5 μM), ouabain (5 μM), valinomycin (400 μM), SLO (1.0 μg/ml), ATP (30 mM), or LLOMe (0.5 mM). After 6 hr, cell lysates and culture supernatants were analyzed by western blotting for the indicated proteins.

(D) Cells from (C) were scored for necrosis by PI staining by flow cytometry, and IL-1β concentrations in the culture supernatants were measured by ELISA.

(E) THP-1 culture supernatants from (C) were diluted 1:100 in culture medium and added to HeLa cells, which express IL-1R, to measure active IL-1. After 24 hr, IL-6 and IL-8 concentrations in the HeLa culture supernatants were measured by ELISA.

(F) To determine the contribution of IL-1β to HeLa bioactivity, Nigericin and ouabain THP-1 culture supernatants from (E) were immunodepleted for the indicated cytokines and added to HeLa cells. After 24 hr, IL-6 concentrations in the culture supernatants were measured by ELISA. Results shown are representative of least two independent experiments.

Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S1.

dye propidium iodide in tandem with annexin V binding, characteristic of necrosis (Figure 2C). Furthermore, signal II agents failed to activate apoptotic caspases (caspases-3/-7) or process caspase substrates that are typically cleaved during apoptosis (Figure 2D). In addition, knockdown of the "apoptotic" caspases, caspase-3 and caspase-7, failed to attenuate cell death in response to signal II but did so in response to actinomycin D, a conventional pro-apoptotic stimulus (Figure 2E). Collectively, these data suggest that necrosis could be a common mechanism for IL-1β release induced by signal II stimuli.

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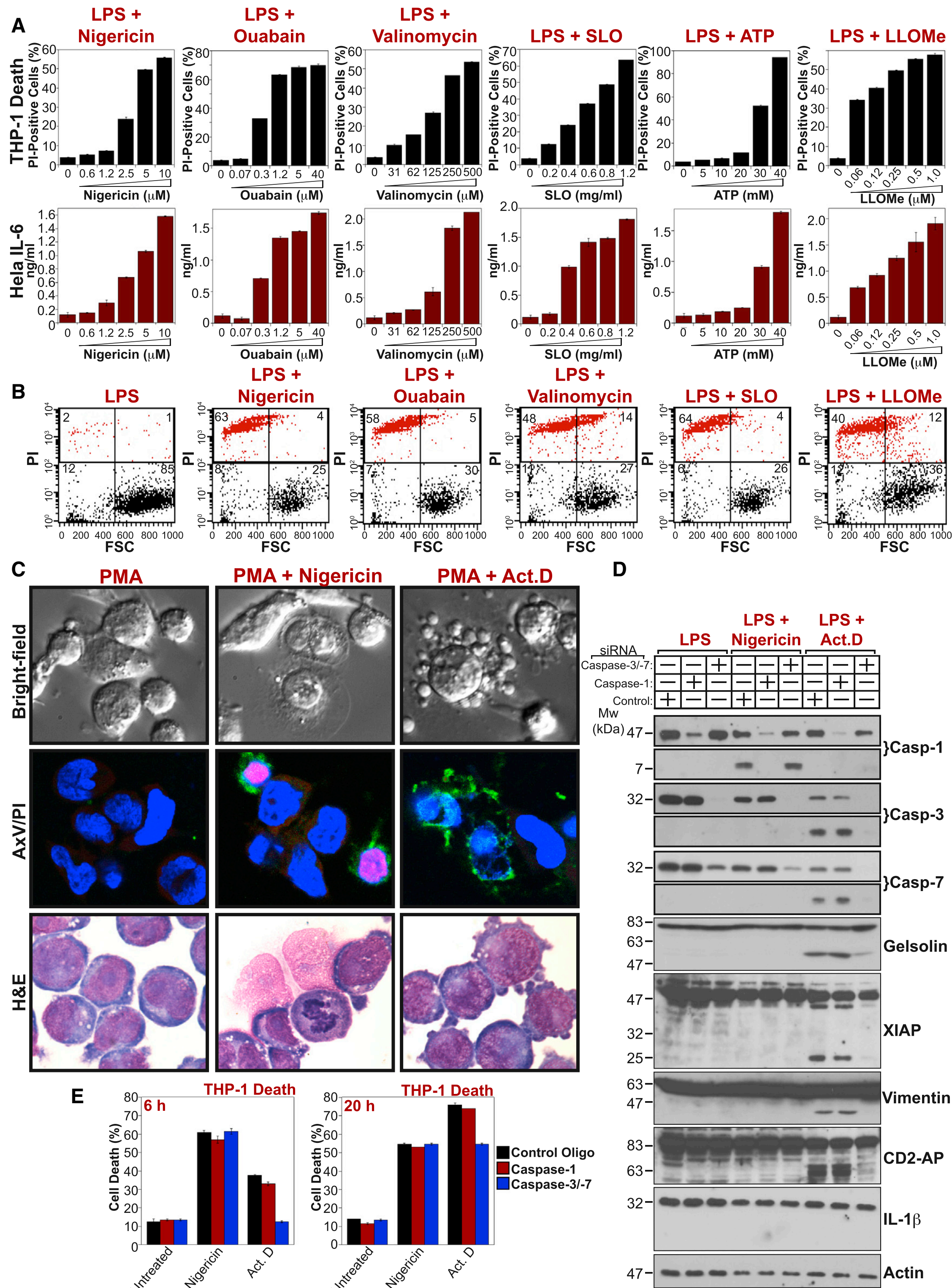

Figure 2. Signal II Stimuli Are Cytotoxic and Promote Necrosis

(A) THP-1 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with the indicated signal II agents. Six hours later, necrosis was scored by PI staining by flow cytometry and clarified supernatants were added to HeLa cells at a 1:100 dilution. Twenty-four hours later, IL-6 concentrations in the HeLa culture supernatants were measured by ELISA.

(B) Flow cytometry analysis from (A).

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Because certain forms of necrosis occur in a programmed manner, called necroptosis, we also asked whether the necroptosis inhibitor necrostatin-1 had any inhibitory effect on LPS+signal II-induced necrosis. However, as shown in Figures S3A and S3B, necrostatin-1 failed to attenuate LPS+signal II-induced necroptosis but did block TNF/zVAD-induced necroptosis in HeLa cells stably overexpressing RIPK3, as expected. Similarly, knockdown of the necroptosis regulatory molecules RIPK1, RIPK3, or MLKL also failed to block LPS+signal II-induced necrosis (Figures S3C–S3F), but this was effective in arresting TNF/zVAD-induced necroptosis, as expected. Thus, signal II agents appear to induce conventional necrosis rather than necroptosis.

The Degree of Necrosis Induced by Signal II Stimuli Is Highly Correlated with Their Ability to Promote IL-1β Maturation and Release

We next explored whether the ability of signal II agents to promote necrosis correlated with their ability to promote IL-1β processing, IL-1β release into the cell supernatant, as well as the generation of IL-1β bioactivity. As Figure 3A demonstrates, we observed a very close correlation between the degree of necrosis observed at a given dose of Nigericin and the extent of IL-1β processing, IL-1β secretion, and the appearance of IL-1β bioactivity in the culture supernatants. Of particular note, we also observed release of multiple additional irrelevant proteins (XIAP, p23, Bax, vimentin, β-catenin, and actin) into the cell supernatants in concert with mature p17 IL-1β, which strongly suggests that release of this cytokine is unselective (Figure 3A). Indeed, where p17 IL-1β release was detected, we also observed release of pro-IL-1β in tandem (Figure 3A). Essentially identical results were observed with three other signal II agents (ouabain, valinomycin, and SLO) tested (Figures 3B–3D). Thus, IL-1β release mirrors the ability of signal II agents to promote cell death.

The Onset of IL-1β Release Correlates Precisely with the Onset of Necrosis

To explore further whether inflammasome-dependent IL-1β release was due to necrosis, we conducted time course analyses at closely spaced time intervals to determine whether the onset of IL-1β processing and release was coincident with the appearance of necrotic cells in these cultures. As shown in Figures 4A and 4B, the appearance of IL-1β bioactivity, as well as the release of processed p17 IL-1β, in THP-1 cell supernatants was highly correlated with the onset of necrosis induced by Nigericin and ouabain. We also made very similar observations with valinomycin (Figure S4A). Furthermore, similar observations were also made using the murine macrophage cell line J774 (Figures S4B and S4C). Once again, we also observed the release of multiple additional cytosolic proteins into the cell supernatants in tandem with IL-1β (Figures 4A, 4B, and S4A–S4C), consistent with a non-selective release mechanism. These data argue that signal II agents promote necrosis, which acts as a driver of IL-1β secretion simply as a consequence of plasma membrane rupture.

IL-1β Processing Occurs Intracellularly

The observation that both pro- and mature IL-1β could be detected in the cell supernatants (Figures 3 and 4) presented the possibility that IL-1β maturation could occur extracellularly. To explore this possibility, we used two different strategies. First, we added recombinant full-length pro-IL-1β to the cell culture medium of cells treated with LPS/Nigericin or LPS/SLO to ask whether processing of extracellular His-tagged IL-1β occurred under conditions where endogenous IL-1β was processed and released. However, we failed to detect processing of extracellular His-tagged IL-1β, above background levels, under conditions where cell-associated IL-1β underwent processing and release (Figures S5A and S5B). In contrast, recombinant caspase-1 readily processed recombinant His-tagged IL-1β, demonstrating that it was not incapable of undergoing proteolytic maturation (Figures S5A and S5B). Second, we also added the caspase-1-inhibitory serpin PI-9 to the cell culture medium to determine whether this could inhibit LPS+signal II-induced IL-1β processing (Figures S5C–S5E). As Figure S5E shows, PI-9 failed to inhibit LPS+Nigericin or LPS+SLO-mediated IL-1β processing when added to cell culture, whereas PI-9 readily inhibited recombinant caspase-1-mediated IL-1β processing in control reactions (Figure S5D).

Collectively, these data suggest that, although pro- and mature IL-1β are released in tandem, IL-1β processing does not occur in the extracellular space to any significant degree.

Viable Cells Retain IL-1β after Exposure to Signal II Agents

Although the onset as well as the magnitude of necrosis observed in the preceding experiments correlated very well with the onset and magnitude of IL-1β release, it was formally possible that viable, non-necrotic, cells could contribute to the IL-1β release seen in mixed (i.e., viable and necrotic) cell populations. To rule out this possibility, we also explored inflammasome-dependent IL-1β release using several additional approaches. In the first, we used flow cytometry to stain for intracellular IL-1β versus forward scatter, which correlates very well with loss of plasma membrane permeability (Figures 2B and 5A, left hand column). As Figure 5A demonstrates, LPS treatment alone dramatically increased intracellular IL-1β staining, as expected. However, when THP-1 cells were stimulated with LPS in combination with ouabain to promote IL-1β maturation

(C) THP-1 cells treated with PMA (250 nM) and then plated on glass slides. Sixteen hours later, cells were treated for 8 hr with Nigericin (2.5 μM) or actinomycin D (2.5 μM). Cells were analyzed by phase contrast microscopy and then stained with either annexin V-FITC, PI, or Hoescht and analyzed by UV microscopy. Alternatively, cytospins were generated and stained for hematoxylin and eosin, followed by analysis by microscopy.

(D and E) THP-1 cells were transfected with either nonsilencing siRNA or siRNAs directed against caspase-1 or caspase-3/-7 as indicated. Forty-eight hours later, cells were primed with LPS (1 μg/ml) for 4 hr and then treated with Nigericin (2.5 μM) or actinomycin D (2.5 μM). At the indicated time points, cells were scored for necrosis by PI staining by flow cytometry, whereas cell lysates were analyzed by western blotting for the indicated proteins. Results shown are representative of least two independent experiments.

Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S2.

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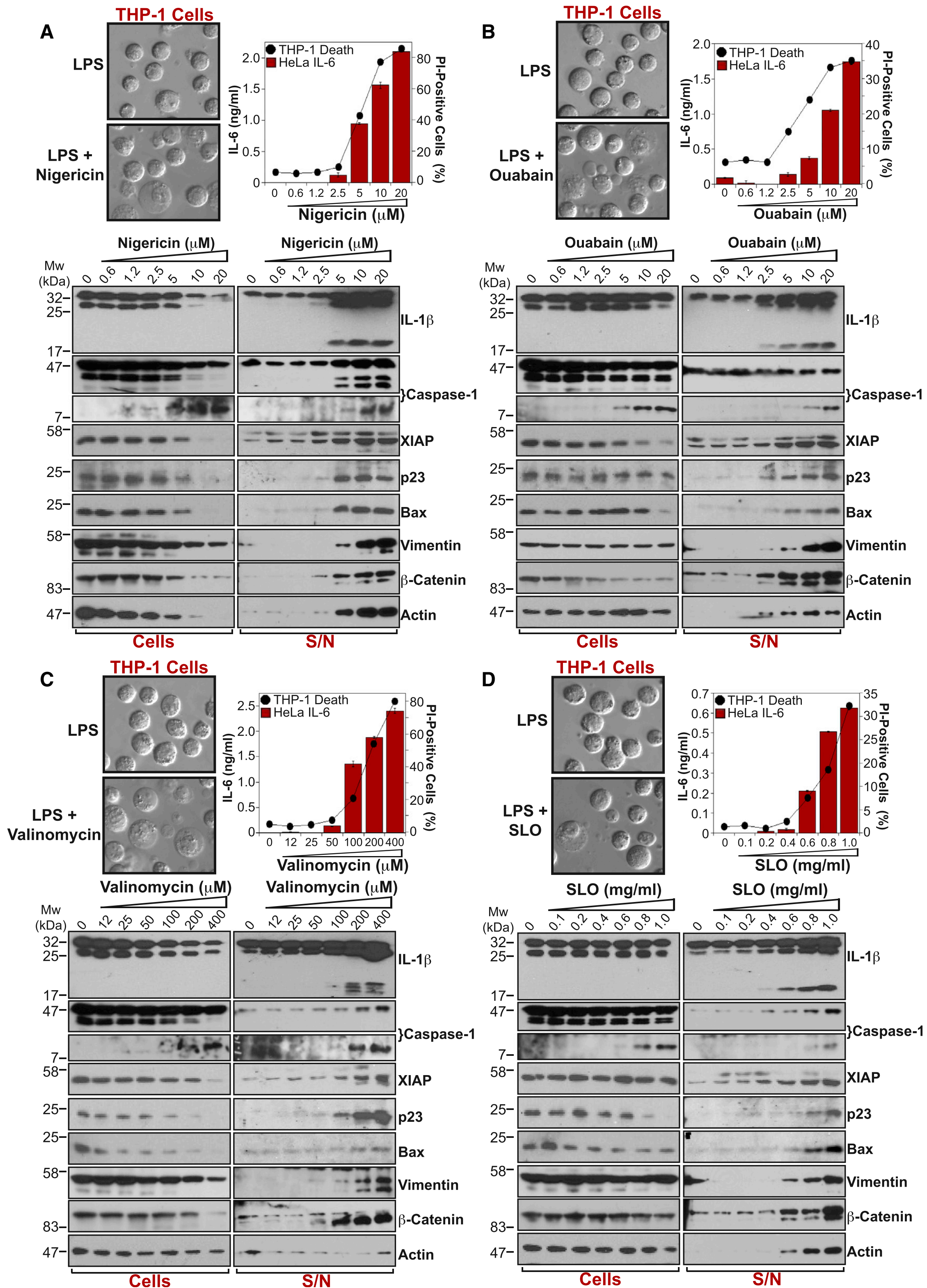

Figure 3. Signal-II-Induced IL-1β Release Is Unselective and Closely Correlated with Necrosis

(A–D) THP-1 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with the indicated doses of Nigericin (A), ouabain (B), valinomycin (C), and SLO (D). Six hours later, cells were analyzed by phase contrast microscopy and scored for necrosis by PI staining by flow cytometry, whereas cell

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and release, we observed a dramatic reduction in forward scatter (which occurs as a consequence of membrane permeabilization) and these cells stained markedly less intensely for IL-1β than their viable counterparts (Figure 5A). Furthermore, we also stained for a number of additional intracellular proteins under the same conditions and observed similar non-selective release of p62, Bax, cytochrome c, and actin under conditions where IL-1β was released (Figure 5A). In all cases, cells that stained negative for the latter proteins exhibited low forward scatter characteristics, indicative of necrosis.

In the second approach, we plated cells on coverslips followed by treatment with PMA to promote adherence as well as pro-IL-1β production. Cells were treated with PMA alone or in combination with Nigericin followed by staining with Mitotracker, which is only taken up by viable cells that contain polarized mitochondria. Cells were then fixed and permeabilized to stain for intracellular IL-1β. As Figure 5B demonstrates, only Mitotracker-positive cells were positive for intracellular IL-1β, whereas neighboring Mitotracker-negative cells were essentially devoid of IL-1β. Once again, these data argue that necrotic cells are responsible for the majority of IL-1β secretion in response to signal II agents.

In the third approach, we used quantitative ELISA assays to measure the precise amount of intracellular IL-1β available for release (i.e., cell-associated) versus the amount actually released (i.e., supernatant-associated) in response to LPS+Nigericin or LPS+ouabain and asked whether this correlated with the percentage of cell death observed in response to these treatments. As Figures 5C and 5D demonstrate, cell viability and IL-1β retention, or conversely cell death and IL-1β release into the medium, were very highly correlated.

Single Cell Analysis Reveals that Only Necrotic Cells Release IL-1β

As an additional approach to explore the mechanism of IL-1β release in response to signal II agents, we used confocal microscopy in tandem with a novel strategy to detect IL-1β secretion from cells in situ. Here, we coated coverslips with anti-IL-1β capture antibodies, followed by plating of live cells onto the capture antibody-coated coverslips. Adherent cells were then treated with LPS in the presence or absence of signal II agents. Prior to termination of the experiments, we added a FITC-tagged secondary antibody to the cultures to detect secreted IL-1β that was captured by the slide-immobilized antibodies. Furthermore, cultures were also incubated with Mitotracker, which is only incorporated into live cells. Following this, cells were then washed to remove excess unbound anti-IL-1β antibodies and were fixed. Using this approach, only extracellular IL-1β is detected as neither the capture or detection anti-IL-1β antibodies can penetrate viable cells.

In this scenario, if viable cells released IL-1β, this would be expected to lead to a halo of IL-1β reactivity (i.e., green/FITC) surrounding Mitotracker-positive (i.e., red) cells. However, if necrotic (dead) cells were the only cells to release IL-1β, then all cells with IL-1β "halos" would be expected to be Mitotracker negative. As illustrated in Figure 6, all of the cells where IL-1β halos were detected were essentially Mitotracker negative. Indeed, we failed to detect any extracellular IL-1β halos surrounding Mitotracker-positive cells. Once again, these data argue that necrosis is the primary driver of IL-1β release in response to signal II agents.

Known Inhibitors of IL-1β Release Block Cell Death

Collectively, the preceding results suggested that cell death was necessary for IL-1β release in response to signal II agents. We therefore wondered whether agents known to block IL-1β release did so by blocking cell death. We addressed this question using two different approaches. Elevated extracellular K+ is well known to block inflammasome activation and IL-1β release (Perregaux and Gabel, 1994; Pétrilli et al., 2007). If cell death is the primary route of IL-1β release, our model would predict that elevations in extracellular K+ would block IL-1β release by suppressing cell death induced by signal II agents. To test whether this was indeed the case, we treated LPS-primed THP-1 cells with Nigericin in the presence of range of KCl concentrations. Elevated extracellular K+ efficiently blocked maturation and release of mature IL-1β, as expected (Figures 7A and 7C). However, in agreement with the idea that IL-1β release occurs via necrosis, K+ also blocked cell death associated with Nigericin treatment (Figure 7A). This effect was observed at K+ concentrations ranging from 10 to 40 mM, with the degree of suppression of IL-1β release correlating very well with suppression of cell death (Figures 7A and 7C). We also made similar observations with SLO (Figures 7B and 7C) as well as ouabain (data not shown), where the suppression of IL-1β release also occurred in tandem with suppression of cell death.

The type 2 diabetes drug glyburide has also been shown to block IL-1β release, through an unknown mechanism (Lamkanfi et al., 2009). As shown in Figures 7D–7F, glyburide-mediated inhibition of IL-1β release was also associated with inhibition of cell death associated with signal II treatment. Thus, two well-known inhibitors of IL-1β secretion also blocked cell death associated with signal II agents, which strongly supports the idea that necrosis is the major route of IL-1β release.

Where Signal II Is Not Required, Signal I Is Sufficient to Promote Necrosis

Certain cell types, such as bone-marrow-derived macrophages (BMDMs), do not require signal II agents to promote IL-1β release, and in these cells, LPS treatment alone is sufficient (Figure 7G). The necrosis-release model would predict that, in such cells, LPS treatment alone should induce cell death, which is why signal II is not required. Therefore, we asked whether this was indeed the case. As Figures 7H and 7I demonstrate, LPS was sufficient to promote necrosis of primary BMDMs, with the

lysates and culture supernatants were analyzed by western blotting for the indicated proteins. In addition, clarified supernatants were added to HeLa cells at a 1:100 dilution. Twenty-four hours later, IL-6 concentrations in the HeLa culture supernatants were measured by ELISA. Results shown are representative of least two independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S3.

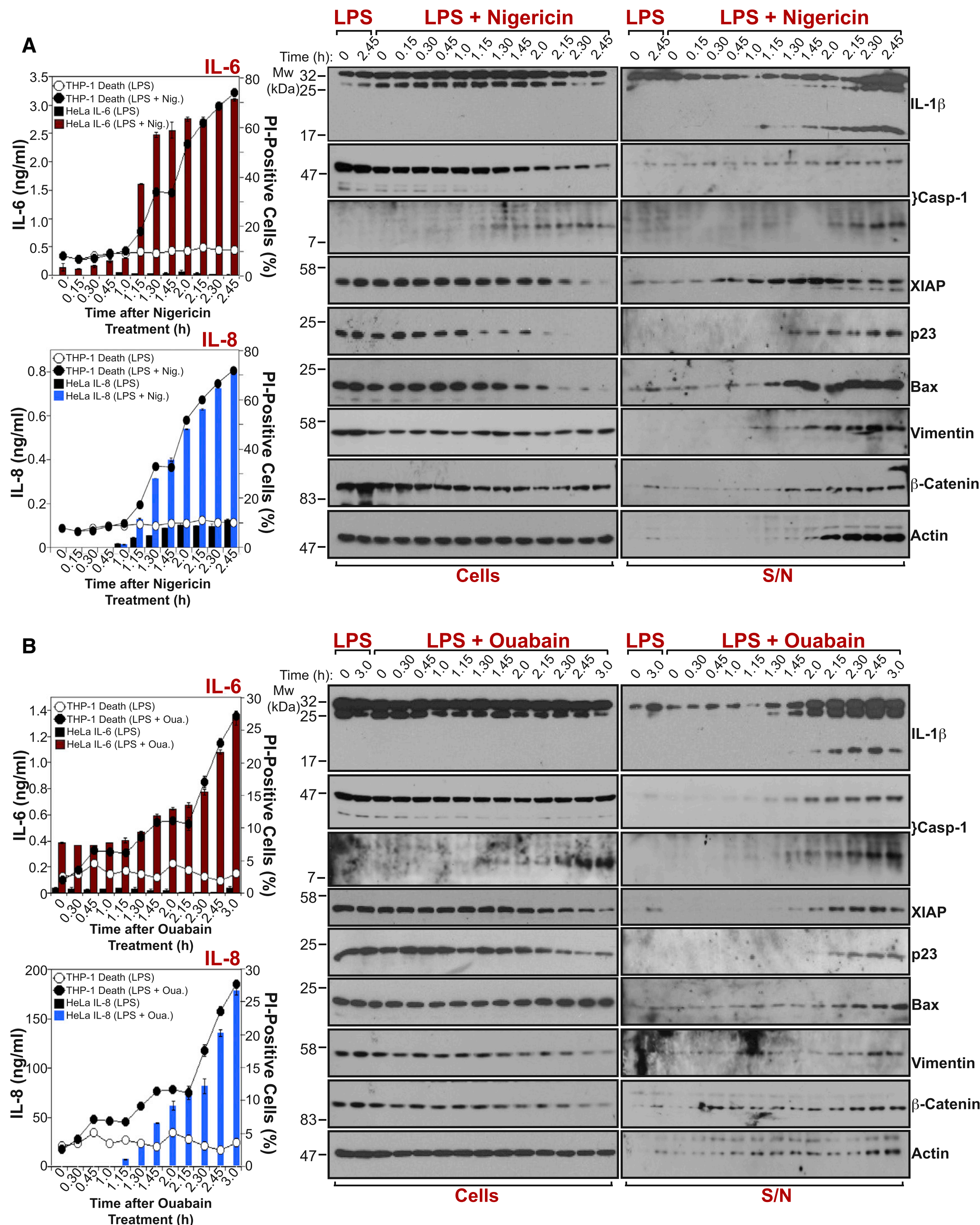

Figure 4. IL-1β Release Is Coincident with the Onset of Necrosis

(A and B) THP-1 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with Nigericin (5 μM) (A) or ouabain (20 μM) (B) and samples taken at the indicated time points. Cells were scored for necrosis by PI staining by flow cytometry, whereas cell lysates and culture supernatants were analyzed by western blotting for the indicated proteins. In addition, clarified supernatants were added to HeLa cells at a 1:100 dilution. Twenty-four hours later, IL-6 and IL-8 concentrations in the HeLa culture supernatants were measured by ELISA. Results shown are representative of least two independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S4.

degree of necrosis observed once again correlating very well with the release of mature IL-1β seen.

Collectively, these results demonstrate that diverse signal II agents promote LPS-initiated IL-1β maturation and release as a consequence of their ability to trigger membrane permeabilization and necrosis.

DISCUSSION

Here, we have shown that diverse signal II agents, including Nigericin, ouabain, valinomycin, streptolysin O, ATP, and LLOMe, share in common the ability to promote secretion of bioactive mature IL-1β by inducing necrosis. Signal-II-induced IL-1β release was non-selective, as this was accompanied by the release of numerous intracellular proteins, including XIAP, p23, Bax, and vimentin. Single cell analysis also revealed that the only cells to release IL-1β in response to signal II agents were those that underwent necrosis. These data suggest that IL-1β behaves as an inducible "danger-associated molecular pattern" (DAMP), with release of the latter cytokine contingent upon the presence of a cytotoxic stimulus such as a bacterial virulence factor.

The mechanism of IL-1β release has been debated, and although cell lysis has commonly been reported in studies that have sought to define the mode of IL-1β release, it is typically argued that cytokine release precedes death. However, such claims have been based primarily on insensitive cell death assays such as GAPDH release. Here, using more-sensitive assays, such as single cell analysis, we have shown that signal-II-induced cell death and IL-1β release are highly correlated. Moreover, agents that block IL-1β release, such as elevated extracellular K+ as well as glyburide, also blocked cell death.

Whereas in this study we have focused on the NLRP3 inflammasome that is activated by LPS, it is interesting to note that caspase-1 activation and IL-1β release in the context of other inflammasomes are well known to be associated with cell death. Cytosolic bacterial flagellin activates the NAIP5/NLRC4 inflammasome, promoting caspase-1 activation and IL-1β processing (Zhao et al., 2011). Interestingly, flagellin also promotes lysis of infected cells, through caspase-dependent and independent mechanisms (Miao et al., 2010; Lage et al., 2013). Similarly, the AIM2 inflammasome responds to intracellular DNA detection by instigating caspase-dependent IL-1β processing and pyroptosis (Rathinam et al., 2010; Wu et al., 2010) as does the NLRP1 inflammasome in response to anthrax lethal toxin (Masters et al., 2012). In light of our findings, it will be interesting to determine the role of cell lysis as a facilitator of mature IL-1β release in response to activation of other inflammasomes.

Whereas a unifying trigger for inflammasome activation has yet to be firmly established, much evidence now strongly suggests that a decline in intracellular K+ ions is a pre-requisite (Perregaux and Gabel, 1994; Walev et al., 1995; Pétrilli et al., 2007, Muñoz-Planillo et al., 2013). In this regard, it is possible that initial signal II-mediated membrane permeabilization, without overt cell lysis, may facilitate sufficient potassium efflux to promote inflammasome activation and IL-1β maturation. Signal-II-mediated membrane permeabilization may then overwhelm the cell, leading to necrosis and mature IL-1β release. In this scenario, signal-II-mediated membrane permeabilization is responsible for the intracellular K+ drop, which leads to activation of the inflammasome and also for the downstream release of IL-1β through eventual cell lysis. Importantly, this scenario does not preclude the possibility that activation of the inflammatory caspases contribute, at least in part, to the membrane rupture that eventually leads to IL-1β release. Thus, the K+ efflux that occurs in response to diverse signal II agents may trigger two closely related events: activation of the inflammasome followed by plasma membrane rupture, which may well be facilitated by the actions of active inflammatory caspases. However, we have failed to find evidence for involvement of the inflammasome in signal-II-induced cell death, either through blocking caspase activity or knockdown of inflammasome constituents. This suggests that signal II agents are intrinsically cytotoxic, without recourse to the participation of the inflammasome in this process.

The observation that necrosis is the common event that promotes IL-1β release fits well with Matzinger's "danger model," which argues that cell death (danger) rather than detection of "non-self" is a key factor in the decision to initiate an immune response (Matzinger, 1994). Non-self entities (such as LPS) may serve only to prime responding cells, whereas the defining event in the initiation of inflammation may be the detection of a second, membrane-damaging signal that drives the K+ drop necessary for activation of the inflammasome and release of IL-1β and other DAMPs (also called alarmins). In this light, IL-1β serves as an inducible DAMP that is generated as a consequence of PAMP detection. Because a common feature of pathogenic organisms is to cause cell death within the host, this two-step model incorporating tissue damage explains many of the ambiguities in immunity, such as why PAMPs only typically induce robust immunity when co-administered with noxious, tissue-damaging adjuvants (Matzinger 1994), unless the PAMP itself is directly cytotoxic.

In conclusion, here we have shown that signal II agents share in common the ability to promote cell lysis, which closely correlated with their ability to facilitate inflammasome activation and mature IL-1β release. Importantly, signal-II-induced IL-1β release was unselective and was confined to necrotic cells. Thus, cell-rupture-associated IL-1β release, downstream of PAMP detection, may permit the immune system to discriminate between pathogenic (i.e., cell-death-inducing) and non-pathogenic micro-organisms, thereby qualifying the decision to respond on the basis of whether a PAMP is detected in the context of danger (i.e., necrotic cell death) or not.

EXPERIMENTAL PROCEDURES

Reagents

Antibodies specific to caspase-3, caspase-7, RhoGDI, XIAP, gelsolin, RIPK1, and p65 were obtained from BD Biosciences. Anti-CD2-AP, anti-human caspase-1, and mouse caspase-1 (p20) antibodies were from Santa Cruz Biotechnology, whereas mouse anti-caspase-1 was from Adipogen. Anti-IL-1β antibody was from R&D Systems, whereas anti-co-chaperone p23 antibody was purchased from Affinity Bioreagents. Anti-human ASC, caspase-4, and caspase-5 (M060-3, which also detects caspase-4) were from MBL International. Anti-ERK antibody was from Cell Signaling Technology. Mitotracker CMXROS was from Invitrogen. Nigericin was from Enzo Life

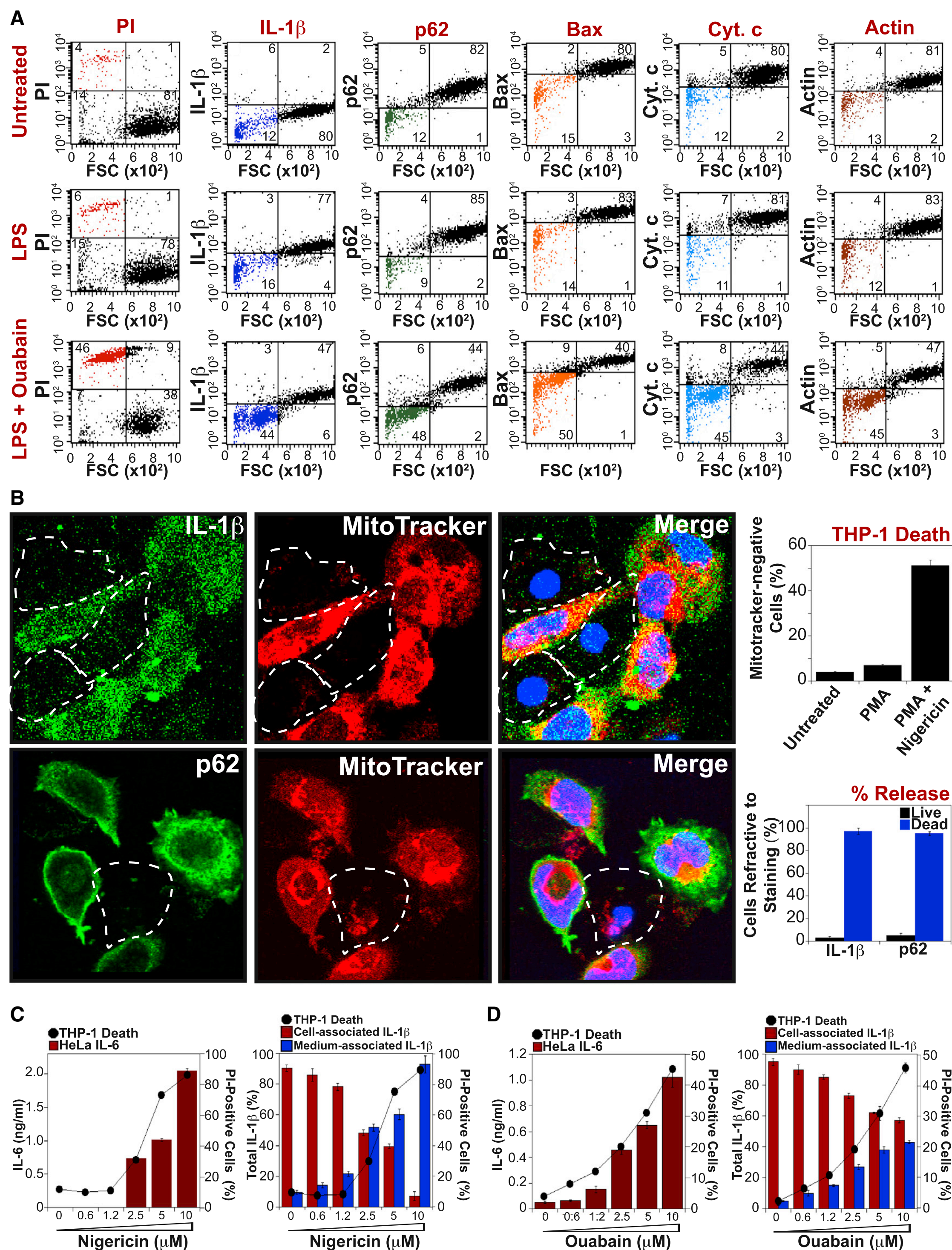

Figure 5. Viable Cells Retain IL-1β after Exposure to Signal II Agents

(A) THP-1 cells were left untreated or primed with LPS (1 μg/ml) for 20 hr and then treated with ouabain (1.25 μM). Four hours later, necrosis was measured by PI staining by flow cytometry (top panel) and the cells were fixed with paraformaldehyde and permeabilized with Triton X-100 and then immunostained for intracellular IL-1β, p62, Bax, cytochrome c, or actin and analyzed by flow cytometry. The percentage of live (high-FSC) or dead (low-FSC) THP-1 cells that released IL-1β is shown (bottom panel).

(B) THP-1 cells were plated on glass slides in medium containing PMA (250 nM) to encourage cells to stick to the slides and to prime pro-IL-1β production. Twenty hours later, the monolayer was washed in RPMI and RPMI containing Nigericin (1.25 μM) was added. Three hours later, Mitotracker CMX-ROS (25 nM)

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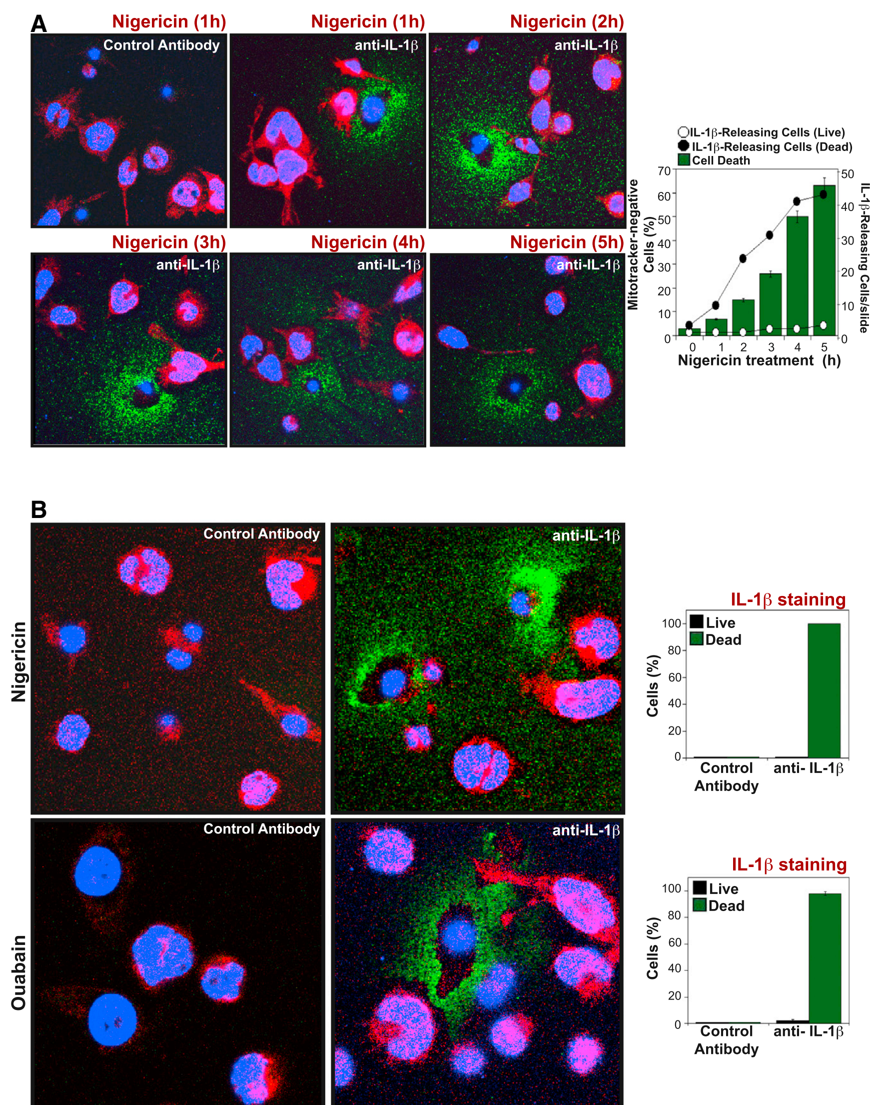


Figure 6. Single Cell Analysis Reveals that Only Necrotic Cells Release IL-1β

(A) Glass slides were coated with IL-1β capture antibody. Twenty-four hours later, slides were washed and THP-1 cells were plated in medium containing PMA (250 nM) to encourage cells to stick to the slides and to prime pro-IL-1β production. Twenty hours later, the monolayer was washed in RPMI and RPMI containing Nigericin (1.25 μM) was added to promote the release of IL-1β, which would subsequently be captured on the slide-bound antibody surrounding the cells. At the indicated time points, cells were washed gently in PBS, 1% BSA, and slide-bound IL-1β was detected by addition of streptavidin-FITC-conjugated IL-1β secondary antibody, which was added in PBS, 1% BSA. Twenty minutes later, cells were washed again and Mitotracker CMX-ROS (25 nM) was added for 15 min to stain exclusively for viable cells. Finally, cells were washed gently in PBS and then fixed and the nuclei stained with Hoescht, followed by visualization of IL-1β release by UV-confocal microscopy. The percentage of live (Mitotracker-positive) or dead (Mitotracker-negative) THP-1 cells that released IL-1β is shown.

(B) Cells were treated as in (A), except that Nigericin (1.25 μM) or ouabain (10 μM) were added for 3 hr. Results shown are representative of least two independent experiments.

Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

Sciences, and streptolysin O was from Aalto Bioreagents. Unless otherwise indicated, all other reagents were purchased from Sigma.

Measurement of Cytokines and Chemokines

Cytokines and chemokines were measured from cell culture supernatants using specific ELISA kits obtained from R&D Systems (human and mouse IL-1β, human and mouse IL-6, and human IL-8). Each assay was repeated a minimum of three times, and all cytokine assays were carried out using triplicate samples from each culture.

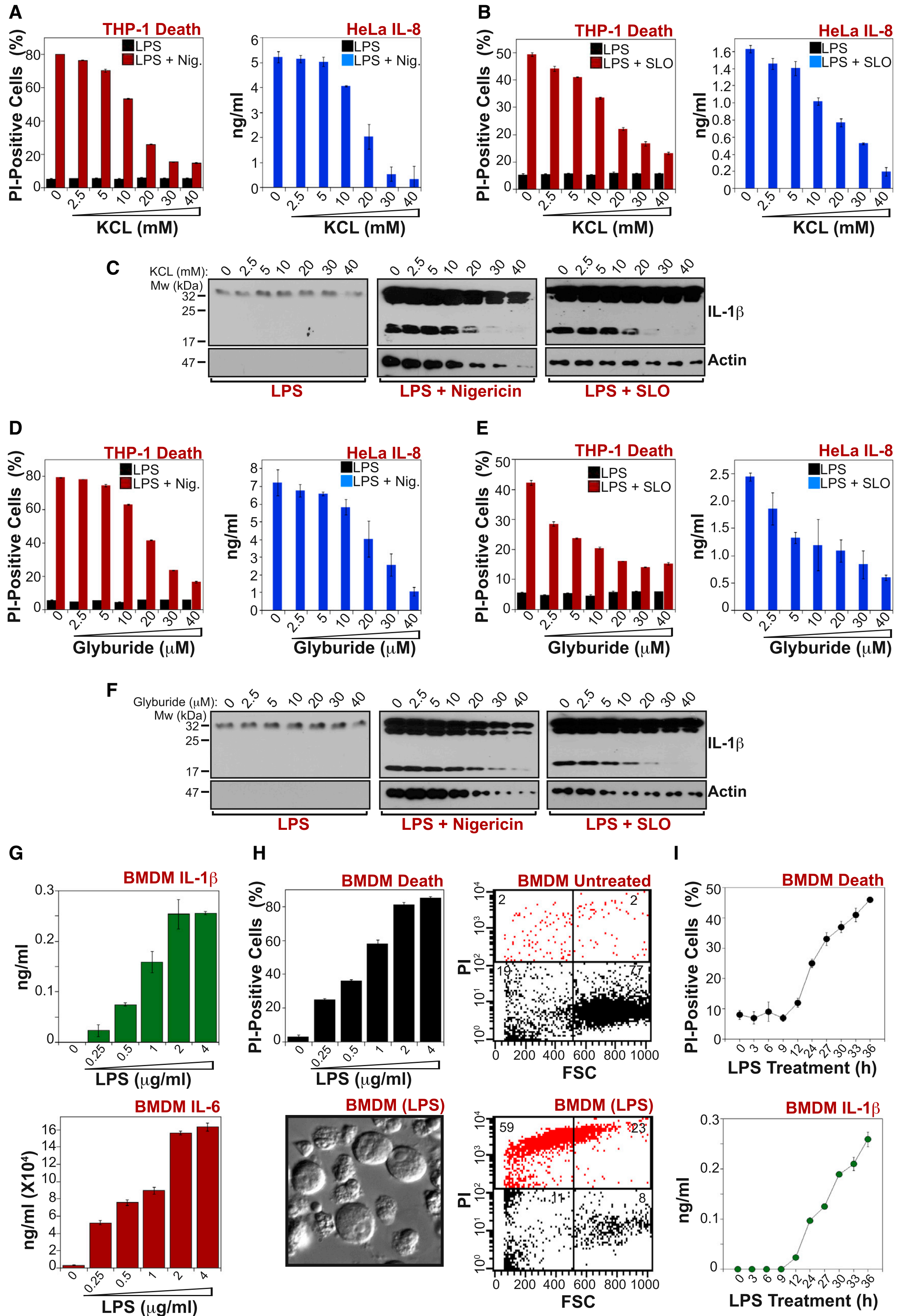
Treatment of THP-1 and J774 Cells with Signal II Agents and HeLa Bioassay

THP-1 or J774 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with signal II agents for 2–6 hr. A sample of cells was then taken for necrosis measurement by PI staining by flow cytometry and the remaining cells separated from the medium by centrifugation at 600 *g* for 10 min, followed by lysing in SDS-PAGE sample buffer. The medium was further clarified at 7,000 *g* for 5 min, which was important to make sure all cells were removed, which would otherwise lead to false positives. 1:100 dilutions of THP-1 supernatant were then added to HeLa cells and incubated overnight at 37°C. After 24 hr, IL-6 and IL-8 concentrations in the HeLa culture supernatants were measured by ELISA. To determine the contribution of IL-1β to HeLa bioactivity, Nigericin and ouabain THP-1 culture supernatants were immunodepleted using antibodies directed against IL-1β, IL-1α, TNFα, or IL-33 (1 μg/ml; 2 hr), which had been pre-coupled to A/G agarose (20 μl). Two hours later, the agarose/antibody/protein complexes were removed by centrifugation and the depleted supernatants added to the HeLa bioassay. Alternatively, bioactive mouse IL-1β was measured directly

was added for 15 min to stain exclusively for viable cells. Cells were then fixed and permeabilized as in (A) and immunostained for intracellular IL-1β or p62, followed by staining of nuclei with Hoescht and analysis by UV confocal microscopy. The percentage of live (Mitotracker-positive) or dead (Mitotracker-negative, indicated by circles) THP-1 cells that released IL-1β is shown (side panel).

(C and D) THP-1 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with the indicated doses of Nigericin (C) or ouabain (D). Four hours later, necrosis was measured by PI staining by flow cytometry, and the clarified supernatants were added to HeLa cells at a 1:100 dilution followed by IL-6 measurement in the HeLa supernatants by ELISA after a further 24 hr. At the 4-hr time point, THP-1 cells were also permeabilized with Trition-X100, and IL-1β concentration in cells versus supernatants was determined by ELISA.

Results shown are representative of least two independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S5.



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by ELISA. For experiments utilizing caspase or necroptosome inhibition, zVAD-fmk (10 μM) or Necrostatin-1 (5 μM) was added to LPS-primed THP-1 for 1 hr before addition of signal II agents as described above.

Quantitative ELISA for Comparison of Cell- and Medium-Associated IL-1β

THP-1 cells were primed with LPS (1 μg/ml) for 4 hr and then treated with signal II agents for 3–6 hr. A cell sample was then taken for necrosis measurement by PI staining by flow cytometry and the remaining cells separated from the medium by centrifugation at 600 *g* for 10 min, whereas the medium was further clarified at 5,000 *g* for 5 min. Cell pellets were lysed in buffer containing 1% TX-100 for 15 min. Lysates were then clarified by centrifugation at 20,000 *g* for 15 min. IL-1β levels in cell lysates and supernatants were determined by ELISA. Additionally, a HeLa bioassay was performed with the clarified supernatants.

RNAi

To ablate protein expression in THP-1 or HeLa cells, 5×10^5 cells were transfected with 200–400 nM siRNA using nucleofection (Amaxa; Program T-001 for THP-1; I-19 for HeLa) as per manufacturer's instructions. siRNA were as follows: NLRP3 no. 1: sense: 5′-gguguuggaauuagacaac-3′; NLRP3 no. 2: sense: 5′-aagcuucagguguuggaauua-3′; ASC no. 1: sense: 5′-gaugcggaagcucuuca gu-3′; ASC no. 2: sense: 5′-aagagcuuccgcaucuugcuugggu-3′; caspase-1 no. 1: sense: 5′-gguucgauuuucauuugag-3′; caspase-1 no. 2: sense: 5′-gguucg auuuucauuugag-3′; caspase-4 no. 1: sense: 5′-guguagauguagaaga-3′; caspase-4 no. 2: sense: 5′-aaguggccucuucacagucau-3′; caspase-5 no. 1: sense: 5′-gcuccaucuucauuacgga-3′; caspase-5 no. 2: sense: 5′-gcacucaucucuucac agu-3′; MLKL no. 1: sense: 5′-gagauccaguucaacgaua-3′; MLKL no. 2: sense: 5′-ggaauaccguuucagaugu-3′; RIPK3 no. 1: sense: 5′-cccgacgaugucuucuguc aa-3′; RIPK3 no. 2: sense: 5′-aagauuaaccauagccuucaccuccca-3′; caspase-3: sense: 5′-ggaauaucccuggacaaca-3′; and caspase-7: sense: 5′-ccacgguuc caggcuauua-3′.

Intracellular Cytokine Staining

THP-1 cells were treated with PMA (250 nM) and plated overnight on coverslips. Cells were then incubated in RPMI for 2 hr to encourage attachment to the slides and then treated with PMA (250 nM) and ouabain (1.25 μM) or Nigericin (2 μM) for 1–5 hr. Mitotracker CMX-ROS (200 μM) was then used to stain live cells, and the monolayer was washed and cells were fixed in 2.7% paraformaldehyde and permeabilized in 0.15% Triton X-100 in PBS (pH 7.2). Cells were incubated in 2% BSA (Roche) for 30 min and antibodies against IL-1β or p62 were added at 2.5–5 μg/ml for 1 hr followed by PBS washing and addition of fluorescently conjugated secondary antibodies. Final washings included incubation with 20 μM Hoechst (Sigma) for 10 min. Cells were mounted with Slow Fade (Molecular Probes) and observed on a laser scanning microscope (Olympus FV1000) using a 488-nm argon laser (green fluorescence), a 543-nm HeNe laser (Mitotracker), and a 405-nm LD laser (Hoechst). Confocal images were acquired with Fluoview 1000 V.1 application software, and images were processed with Indesign (Adobe). For analysis by FACS, THP-1 cells were treated overnight with LPS (1 μg/ml) and then treated with ouabain (5 μM) for 3 hr. Mitotracker CMX-ROS (200 μM) was then used to stain live cells, and cells were washed once in PBS and then fixed, permeabilized, and stained with antibodies directed toward IL-1β, p62, Bax, cytochrome c, or Actin, followed by PBS washing and addition of fluorescently conjugated secondary antibodies. Cells were then analyzed by flow cytometry.

Assessment of Extracellular IL-1β Proteolysis

THP-1 cells (2×10^6/ml) were primed with LPS (1 μg/ml) for 4 hr and then cells were aliquoted to two equal fractions, one of which was doped with recombinant His-tagged pro-IL-1β (1 ng/ml). Cells were treated with signal II agents and then 8 hr later cell death was determined by PI staining by flow cytometry and the supernatants were clarified and immunoblotted with anti-IL-1β or anti-His to determine the fraction of cleaved extracellular IL-1β. Alternatively, the known caspase-1 inhibitor PI-9 was added into the medium of a standard signal II assay, before signal-II addition, at concentrations that were sufficient to block recombinant caspase-1-mediated proteolysis of recombinant pro-IL-1β. IL-1β proteolysis was then determined by immunoblotting.

Staining Extracellular IL-1β

Coverslips were coated overnight with IL-1β capture antibody (5 μg/ml) and then THP-1 cells, treated with PMA (250 nM), were plated overnight. Cells were then incubated in RPMI for 2 hr to encourage attachment to the slides and then treated with PMA (250 nM) and Nigericin (2 μM) or ouabain (1.25 μM) for 1–5 hr. FITC-conjugated IL-1β secondary antibody was then added and incubated for 15 min, followed by a gentle PBS wash. Mitotracker CMX-ROS (200 μM) was used to stain live cells, and cells were washed with PBS and then fixed and washed again with PBS, including 20 μM Hoechst for 10 min. Cells were mounted with Slow Fade (Molecular Probes) and observed on a laser scanning microscope (Olympus FV1000) using a 488-nm argon laser (green fluorescence) and a 405-nm LD laser (Hoechst). Confocal images were acquired with Fluoview 1000 V.1 application software, and images were processed with Indesign (Adobe).

Inhibition of IL-1β Release by KCL or Glyburide

THP-1 cells were primed with LPS (1 μg/ml). Four hours later, cells were either left untreated or treated with KCL or glyburide (2.5–40 mM) for 1 hr, followed by treatment with signal II agents. Cells and supernatants were then processed as described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.003.

AUTHOR CONTRIBUTIONS

S.P.C. designed and performed experiments, analyzed data, generated most of the figure panels, and wrote the figure legends. C.J.K. and D.M.C. performed experiments and generated some of the figure panels. S.J.M. conceived the study, designed and analyzed experiments, supervised the study, and wrote the paper with contributions from S.P.C. S.J.M. obtained the funding to support the study.

Figure 7. Elevated Extracellular K+ and Glyburide Inhibit IL-1β Release and Necrosis

(A–C) THP-1 cells were primed with LPS (1 μg/ml). Four hours later, cells were either left untreated or treated with then indicated concentrations of KCL for 1 hr. Nigericin (5 μM; A) or SLO (1 μg/ml; B) was then added and the cells incubated for a further 4 hr. Necrosis was measured by PI staining by flow cytometry, and clarified THP-1 supernatants were added to HeLa cells at a 1:100 dilution followed by IL-8 measurement in the HeLa supernatants by ELISA after a further 24 hr (A and B). In addition, culture supernatants were analyzed by western blotting for the indicated proteins (C).

(D–F) THP-1 cells were primed with LPS (1 μg/ml). Four hours later, cells were either left untreated or treated with the indicated concentrations of glyburide for 1 hr. Nigericin (5 μM; D) or SLO (1 μg/ml; E), was then added and the cells incubated for a further 4 hr. The cells were then processed and analyzed as in (A–C).

(G) Mouse bone-marrow-derived macrophages (BMDM) were treated with the indicated doses of LPS. Forty-eight hours later, cytokine concentrations in the supernatants were measured by ELISA.

(H) Cells from (G) were scored for necrosis by PI staining by flow cytometry and viewed by phase contrast microscopy.

(I) BMDMs were treated with LPS (2 μg/ml) and then necrosis was measured by PI staining by flow cytometry, and IL-1β concentrations in the supernatants were measured by ELISA. Results shown are representative of least two independent experiments.

Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

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