**Staphylococcus aureus α-Hemolysin Activates the NLRP3-Inflammasome in Human and Mouse Monocytic Cells**

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**Abstract**

Community Acquired Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) causes severe necrotizing infections of the skin, soft tissues, and lungs. Staphylococcal α-hemolysin is an essential virulence factor in mouse models of CA-MRSA necrotizing pneumonia. *S. aureus* α-hemolysin has long been known to induce inflammatory signaling and cell death in host organisms, however the mechanism underlying these signaling events were not well understood. Using highly purified recombinant α-hemolysin, we now demonstrate that α-hemolysin activates the Nucleotide-binding domain and leucine-rich repeat containing gene family, pyrin domain containing 3 protein (NLRP3)-inflammasome, a host inflammatory signaling complex involved in responses to pathogens and endogenous danger signals. Non-cytolytic mutant α-hemolysin molecules fail to elicit NLRP3-inflammasome signaling, demonstrating that the responses are not due to non-specific activation of this innate immune signaling system by bacterially derived proteins. In monocyte-derived cells from humans and mice, inflammasome assembly in response to α-hemolysin results in activation of the cysteine protease, caspase-1. We also show that inflammasome activation by α-hemolysin works in conjunction with signaling by other CA-MRSA-derived Pathogen Associated Molecular Patterns (PAMPs) to induce secretion of pro-inflammatory cytokines IL-1β and IL-18. Additionally, α-hemolysin induces cell death in these cells through an NLRP3-dependent program of cellular necrosis, resulting in the release of endogenous pro-inflammatory molecules, like the chromatin-associated protein, High-mobility group box 1 (HMGB1). These studies link the activity of a major *S. aureus* virulence factor to a specific host signaling pathway. The cellular events linked to inflammasome activity have clear relevance to the disease processes associated with CA-MRSA including tissue necrosis and inflammation.

**Introduction**

CA-MRSA is the most commonly identified cause of skin infections seen in emergency rooms in the United States [1]. CA-MRSA can also cause severe, life threatening infections including necrotizing pneumonias and fasciitis, which are associated with very high mortality rates even in previously healthy patients [2,3]. These necrotizing infections are characterized by localized necrosis and severe inflammation. People colonized with CA-MRSA suffer from some form of *S. aureus* infection at rates about seven times higher than people colonized with other *S. aureus* strains [4]. In addition to resistance to our most commonly used anti-staphylococcal antibiotics, CA-MRSA has increased virulence that is associated with the production of specific factors that assist this pathogen in causing disease [5].

All *S. aureus* produce secreted exotoxin virulence factors including several cytolytic, pore-forming toxins [6]. Both α-hemolysin (Hla) and Panton Valentine leukocidin (PVL) have been implicated in the pathogenesis of *S. aureus* necrotizing pneumonia [7,8]. Pulmonary delivery of purified α-hemolysin has been shown to induce pulmonary hypertension and inflammation in rat and rabbit [9,10]. Similar studies in mice with PVL, have show that PVL can induce pulmonary inflammation and pneumonitis in mice, in the absence of the bacteria that produce it [8]. More recently, α-hemolysin has been shown to be required for *S. aureus* strains, including CA-MRSA, to promote pneumonia in a mouse.
model of this disease [7,11]. Immunization with inactive α-hemolysin protects mice from S. aureus pneumonia and prevents production of IL-1β and other cytokines associated with pulmonary exposure to S. aureus [12]. Most recently, passive immunization with α-hemolysin-neutralizing antibodies has been shown to protect mice from fatal S. aureus pneumonia [12,13].

In addition to inducing pulmonary inflammation, S. aureus α-hemolysin has been shown to induce inflammatory reactions in the eye, skin, and abdomen when injected into rodents [14,15,16]. The mechanisms by which α-hemolysin induces inflammation in these intact animals have not been fully evaluated. In endothelial cells, α-hemolysin induces platelet activating factor production [17,18,19]. In pulmonary epithelial derived cell lines, exposure to α-hemolysin causes release of nitric oxide and other inflammatory mediators [20]. α-hemolysin has also been shown to act synergistically with S. aureus particles to induce some cytokine secretion, including IL-6 and IL-1α in mouse peritoneal macrophages [21]. Finally, α-hemolysin has been shown to induce cell death and IL-1β secretion from human monocytes [22]. The intracellular signaling mechanisms that link α-hemolysin to secretion of cytokines and other inflammatory mediators are unknown.

Physiologic inflammatory signaling can be triggered by pathogen and host derived molecules. The family of nucleotide binding domain and leucine rich repeat containing proteins known as NLR (formerly NALP, NOD, PYPAF, and CATERPILLER) have recently been implicated in several different inflammatory signaling pathways [23,24]. Mutations in the gene encoding the NLR family member, NLRP3, are associated with hyperactive inflammatory signaling characterized by excessive IL-1β [25,26,27]. Many NLR proteins, including NLRP3, have been found to oligomerize into a macromolecular complex known as the inflammasome [28,29]. The NLRP3-inflammasome is a signaling complex that activates procaspase-1 and induces processing of caspase-1 dependent inflammatory cytokines (particularly IL-1β and IL-18). The NLRP3-inflammasome is activated in response to a multitude of pro-inflammatory stimuli, including many pathogen-derived molecules, endogenous inducers of sterile inflammation, and non-pathogen microbial pore-forming toxins, including nigericin and maitotoxin [30,31,32,33,34,35]. Recently, activation of NLRP3 has been found to initiate both caspase-1 dependent cytokine production and caspase-independent pro-inflammatory program of necrotic cell death [36,37].

Living S. aureus activates NLRP3-mediated signaling in cell culture, but the S. aureus-derived factors involved in initiating this signaling remained to be identified [32]. We now show that S. aureus α-hemolysin induces NLRP3-mediated signaling triggering caspase-1 activation and programmed necrosis.

Results

Prior studies have demonstrated that S. aureus α-hemolysin could induce IL-1β secretion in human monocytes [22]. We sought to determine whether we could see similar induction of IL-1β secretion by α-hemolysin in THP-1 cells, a human monocyte-derived cell line. Commercial preparations of α-hemolysin are relatively crude, with manufacturers reporting ~50% of preparations being protein and the homogeneity of the protein present not reported. In order to study α-hemolysin-induced inflammatory signaling we sought to prepare highly purified S. aureus α-hemolysin. Recombinant α-hemolysin bearing a hexa-histidine tag was prepared after expression in Escherichia coli. The recombinant α-hemolysin was isolated from crude E. coli homogenates using immobilized metal affinity chromatography. In order to eliminate the possibility of E. coli LPS contamination of our preparation, the protein was washed extensively in pyrogen free buffers. After elution, the recombinant protein was exchanged into pyrogen free phosphate buffered saline using size exclusion chromatography. The resulting recombinant α-hemolysin runs as a single homogenous protein band on Coomassie Blue stained SDS-PAGE (Fig. 1A). This protein is immunoreactive with multiple anti-α-hemolysin antibodies (Fig. 1B & Fig. 2C). This preparation of α-hemolysin induced IL-1β secretion from THP-1 cells (Fig. 1C). In order to confirm that the ability to activate IL-1β secretion depended on functional α-hemolysin, and not a contaminating substance in the preparation, we prepared an inactive mutant of α-hemolysin (H35L) that lacks hemolytic activity [12]. The mutant α-hemolysin was highly purified, just as the wild-type preparation, yet failed to elicit IL-1β secretion (Fig. 1A, B, & C). A monoclonal antibody that neutralizes α-hemolysin activity and has been shown to protect mice from S. aureus pneumonia also reduced IL-1β secretion induced by α-hemolysin when preincubated with the toxin prior to treatment of the cells (Fig. 1D) [13]. In order to determine whether α-hemolysin was inducing IL-1β secretion through activation of caspase-1, caspase-1 activation was assessed by immunoblot analysis for the p10 subunit of active caspase-1 after THP-1 cells were treated with wild type or H35L mutant α-hemolysin. Caspase-1 p10 was observed only in cells treated with active α-hemolysin (Fig. 1E). These data all support the hypothesis that α-hemolysin activates IL-1β processing and secretion in a manner similar to other non-pathogen derived pore forming toxins, nigericin and maitotoxin [32].

Unlike preparations of highly purified α-hemolysin, treatment of resting THP-1 cells with commercial preparations of α-hemolysin did not induce detectable levels of IL-1β secretion (Fig. 2A). We sought to further characterize these commercial preparations of α-hemolysin. Using SDS-PAGE and Coomassie Blue protein staining, we found that these preparations contained at least 3 predominant protein bands (Fig. 2B). Immunoblot analysis of these proteins using antibodies directed to α-hemolysin revealed only a single band of immunoreactivity, suggesting the additional proteins within the preparation were derived from other S. aureus proteins rather than proteolysis of α-hemolysin (Fig. 2C). These data indicate that significantly less α-hemolysin was added to the cells, when compared to highly purified recombinant α-hemolysin.

Production of mature IL-1β is frequently studied in cells pretreated with lipopolysaccharide (LPS) or other Toll-like receptor (TLR) agonists, which are thought to induce expression of pro-IL-1β. Indeed studies demonstrating that secretion of IL-1β could be induced by α-hemolysin were carried out in LPS-treated monocytes [22]. Because S. aureus does not produce LPS, we sought to determine whether priming of THP-1 cells with heat killed S. aureus (HKSA) could support IL-1β secretion in a manner analogous to LPS treatment. Treatment of THP-1 cells with heat killed S. aureus also resulted in minimal IL-1β secretion (Fig. 2A). However, exposure of HKSA-primed THP-1 cells to either commercial or recombinant α-hemolysin induced much more robust IL-1β secretion (Fig. 2A). Consistent with its lower α-hemolysin content, the commercial α-hemolysin preparation induced less IL-1β secretion than an equivalent dose of recombinant α-hemolysin. Two S. aureus derived TLR ligands [38,39,40], peptidoglycan, PGN, (TLR2) and lipotechoic acid, LTA, (TLR4) also caused a synergistic induction of IL-1β secretion in conjunction with α-hemolysin treatment, while causing little or no IL-1β secretion by themselves (Fig. 2D). Immunoblot analysis of cell lysates treated with HKSA, α-hemolysin, or both demonstrated that HKSA did indeed induce production of pro-IL-1β while α-hemolysin alone did not (Fig. 2E). Processing of IL-1β, reflected by reduced pro-IL-
levels and detectable secreted processed IL-1β, could only be detected by immunoblot in the HKSA-primed, α-hemolysin-treated cells (Fig. 2E). These data suggest that α-hemolysin acts downstream of pro-IL-1β synthesis, at the level of processing or secretion, in order to induce IL-1β secretion. Overall, these data demonstrate that multiple S. aureus-derived TLR ligands enhance α-hemolysin-induced IL-1β secretion, though they are not required for caspase-1 activation.

The NLRP3 inflammasome has been identified as a critical signaling complex involved in activation of caspase-1 and subsequent IL-1β secretion in response to many inflammatory stimuli, including nigericin and maitotoxin [32,41]. We sought evidence that the NLRP3 inflammasome was required for α-hemolysin-mediated IL-1β secretion. For these studies, we utilized THP-1 derived cell lines that have been transduced with recombinant retrovirus expressing shRNA that “knock down” expression of the inflammasome components, ASC or NLRP3 [36,42]. When compared to control cell lines expressing no shRNA or a scrambled shRNA, cells lacking expression of either NLRP3 or ASC failed to mount robust IL-1β secretion responses to α-hemolysin treatment (Fig. 3A). To confirm these findings, we carried out a similar set of experiments in peritoneal macrophages from C57Bl/6 mice with genetic knock out of several inflammasome components: Nlrp3<sup>−/−</sup>, Asc<sup>−/−</sup>, and Casp1<sup>−/−</sup>. IL-1β secretion was reduced in HKSA-primed, α-hemolysin-treated peritoneal macrophages derived from Nlrp3<sup>−/−</sup>, Asc<sup>−/−</sup>, and Casp1<sup>−/−</sup> mice when compared to wild-type controls (Fig. 3B). We also sought further evidence that α-hemolysin was inducing IL-1β secretion through NLRP3-inflammasome dependent activation of caspase-1. We tested whether α-hemolysin treatment also led to the secretion of IL-18, another caspase-1 processed cytokine. As expected, control THP-1 cells secreted IL-18 in response to α-hemolysin. Like IL-1β secretion, IL-18 secretion was also enhanced by pretreatment with HKSA. THP-1 cells lacking

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Figure 1. S. aureus α-hemolysin induces IL-1β secretion and caspase-1 activation in THP-1 cells. (A) recombinant hexahistidine-tagged α-hemolysin preparations were analyzed by SDS-PAGE and Coomassie Blue staining. Both Wild Type (W.T.) and inactive mutant (H35L) Hla were prepared as described in the materials and methods. (B) The Hla preparations described in (A) were analyzed by immunoblot analysis using polyclonal antisera directed against S. aureus α-hemolysin, Abcam product # ab15948. (C) THP-1 cells were untreated (N.T.) or treated with each of the α-hemolysin preparations described in (A) for 1 hour. The cell culture supernatants were analyzed for secreted IL-1β using ELISA. Asterisk (*) denotes cytokine levels below detectable limits (4 pg/ml). (D) Wild type α-hemolysin was prepared with the addition of saline (Hla), control IgG (Hla + IgG), or anti-α-hemolysin IgG (Hla + anti-Hla). THP-1 cells were untreated (N.T.) or treated with the aforementioned α-hemolysin preparations for 1 hour. The cell culture supernatants were analyzed for secreted IL-1β using ELISA. (E) THP-1 cells were either untreated (N.T.) or treated with highly purified recombinant α-hemolysin (W.T. or H35L) for 1 hour, cell lysates were prepared and analyzed by immunoprecipitation and immunoblot analysis for the p10 subunit of activated caspase-1 and actin (loading control) as described in the materials and methods. The double arrow («) indicates the position of protein G used in immunoprecipitation, which binds immunoblot antibodies nonspecifically. Representative data from at least 3 independent experiments are shown. doi:10.1371/journal.pone.0007446.g001
Figure 2. *S. aureus* derived PAMPS cooperate with α-hemolysin in the induction of IL-1β secretion. (A) THP-1 cells were treated with and without Heat Killed *S. aureus* (HKSA, 10⁶ particle/ml) for 3 hours then subsequently treated with 1.0 μg/ml α-hemolysin preparations, commercial (Com.) or recombinant α-hemolysin (rHla) described in Fig. 1, for 1 hour. The cell culture supernatants were subsequently analyzed for secreted IL-1β using ELISA. (B) Commercial (Com.) α-hemolysin from Sigma Aldrich and recombinant (Rec.), hexahistidine-tagged α-hemolysin preparations were analyzed by SDS-PAGE and Coomassie Blue staining. Both Wild Type (W.T.) and inactive mutant (H35L) Hla were prepared as described in the materials and methods. (C) The Hla preparations described in (B) were analyzed by immunoblot analysis using polyclonal antibodies directed against *S. aureus* α-hemolysin (Toxin Technologies product # SLH101). (D) THP-1 cells were untreated (N.T.) or treated with Heat Killed *S. aureus* (HKSA, 10⁶ particle/ml), *S. aureus* lipotechoic acid (LTA, 100 ng/ml), or *S. aureus* peptidoglycan (PGN, 100 ng/ml) for 3 hours. The cells were subsequently treated either with or without *S. aureus* hemolysin (Hla, 1.0 μg/ml, Sigma Aldrich) for an additional 1 hour. IL-1β secretion into the culture media was measured using ELISA. Asterisk (*) denotes cytokine levels below detectable limits (4 pg/ml). Representative data from at least 3 independent experiments are shown. (E) THP-1 cells were treated as described in (A) with Heat Killed *S. aureus* (HKSA) followed by commercial (Hla) or recombinant α-hemolysin (rHla). Lysates were prepared from the cells and analyzed by immunoblot using antibodies directed against actin (loading control, right/top panel). Lysates were analyzed by immunoblot with antibodies directed against IL-1β (left panel). Processed IL-1β was detected in the cell culture supernatants (right/bottom panel). A mature IL-1β (p18) standard is run in the far right lane.

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either NLRP3 or ASC expression failed to secrete significant levels of IL-1β (Fig. 3C). The activation of caspase-1 could be also be detected in THP-1 cells treated with α-hemolysin using immunoblot analysis for the active p10 subunit of caspase-1 in the absence of HKSA-priming. Intact NLRP3 and ASC expression was required for α-hemolysin induced caspase-1 activation as well (Fig. 3D). Immunoblot analysis also confirmed that the THP-1 cell lines lacking NLRP3 or ASC do induce substantial pro-IL-1β production in response to priming with HKSA (Fig. 3E). Together these data suggest that the primary defect in α-hemolysin responsiveness in NLRP3 inflammasome deficient cells is in activation of caspase-1.

Mariathasan et al. have demonstrated that bone marrow derived macrophages secrete IL-1β in response to live S. aureus. This IL-1β secretion in bone marrow derived macrophages was not significantly reduced when the cells were treated with S. aureus lacking α-hemolysin [32]. These reports suggest that either S. aureus expresses multiple toxins that activate the NLRP3 inflammasome or that α-hemolysin activates NLRP3 in some but not all monocytic-derived cell lineages. We treated bone marrow derived macrophages with HKSA, α-hemolysin, or the combination of agents and measured IL-1β secretion. Surprisingly, we found wild type, Nlrp3−/−, and Asc−/− derived BMDM failed to secrete significant levels of IL-1β to any of these stimuli (Fig. 4A). This suggests that BMDM do not respond to α-hemolysin at the doses used in these studies, unlike the other monococyte-derived cells studied. These BMDM produced pro-IL-1β in response to LPS but not to HKSA pre-conditioning at the doses used (Fig. 4B). As observed by others, treatment of LPS-primed BMDM with ATP-induced processing of pro-IL-1β and secretion of processed IL-1β [30,31,32,33]. At the same time, α-hemolysin did not activate processing of LPS-induced pro-IL-1β (Fig. 4B). Our data support the fact that α-hemolysin can indeed activate the NLRP3 inflammasome, but suggests that this host response is likely to vary depending on the site of infection and types of host inflammatory cells present to combat it.

In addition to causing IL-1β secretion, α-hemolysin is known to induce cell death in numerous cell types [22]. We found that α-hemolysin induced death in THP-1 cells in a robust fashion, as demonstrated by release of cytoplasmic LDH into the culture media from THP-1 cells treated with the toxin (Fig. 5A & B.). This activation of cell death was rapid, reaching maximal effect within 60 min. Paralleling our findings in studies of α-hemolysin-induced IL-1β secretion, the mutant α-hemolysin failed to elicit cell death at 1 hour (Fig. 5A), though at longer time points LDH release was observed with the mutant. Also like IL-1β secretion, the induction of cell death in THP-derived cells by α-hemolysin required intact NLRP3 and ASC expression (Fig. 5B).

S. aureus α-hemolysin has been shown to induce both apoptosis and necrotic cell death in peripheral blood mononuclear cells [43,44]. Our lab and others have recently demonstrated that NLRP3 activation leads to programmed necrosis, termed...
Pyroptosis [36,37,45]. Pyroptosis is a form of inflammatory cell death and is distinct and independent of the process of apoptosis, which normally results in an orderly disassembly of the dying cells that limits inflammatory responses to the death. In order to confirm that α-hemolysin causes NLRP3-dependent pyroptosis, we first sought to demonstrate that α-hemolysin-induced death lacked hallmarks characteristic of apoptosis including: activation of caspase-3, cleavage of PARP, and intranucleosomal cleavage of DNA. Immunoblot analysis of lysates from cells treated with α-hemolysin showed no evidence of caspase-3 activation or cleavage of PARP, both of which could be induced in the THP-1 cells by treatment with Staurosporine (which is known to activate apoptosis) (Fig. 6A). Intranucleosomal cleavage of DNA occurs in the setting of apoptosis and is reflected by reduced propidium iodide staining (sub-G1 DNA content) of fixed cells detected by flow cytometry. While staurosporine induced accumulation of sub-G1 DNA content, α-hemolysin treated cells maintained DNA content profiles similar to untreated controls (Fig. 6B). Overall, these studies demonstrate that α-hemolysin-induced NLRP3-mediated cell death lacks traditional apoptotic features.

To demonstrate that α-hemolysin induces programmed necrosis in THP-1 cells, we used Transmission Electron Microscopy to examine the morphology of α-hemolysin treated cells. The α-hemolysin treated cells lacked morphologic features of apoptotic cells seen in staurosporine treated cells, including chromatin condensation and blebbing of apoptotic bodies from the plasma membrane (Fig. 6C). Instead they have necrotic morphology with

Figure 4. Murine Bone Marrow Derived macrophages are resistant to α-hemolysin induced NLRP3-inflammasome activation. (A) Bone marrow-derived macrophages were cultured from the femurs of C57BL/6 mice, which were either wild type (WT), Nlrp−/−, Asc−/−, or Casp1−/−. The macrophages were subsequently pretreated with HKSA or without HKSA for 3 hours then treated for 1 hour with Hla (1 μg/ml) as described in Fig. 2A. Culture supernatant was removed and assayed for the presence of IL-1β using ELISA. Representative data from 3 independent experiments are shown. (B) Bone marrow-derived macrophages were cultured from the femurs of wild type C57BL/6 mice, as described in (A). The cells were then pretreated with nothing (N), LPS (100 ng/ml, L), or HKSA (1×10^6 particles, H) for 3 hours. The cells were then treated for 1 hour with nothing (N.T.), 1 mM ATP, commercial α-hemolysin (1 μg/ml, Hla), or recombinant α-hemolysin (1 μg/ml, rHla). Cellular lysates and culture supernatants were prepared and subjected to immunoblot analysis with anti-IL-1β antibody. doi:10.1371/journal.pone.0007446.g004

Figure 5. *S. aureus* induces NLRP3-inflammasome-dependent cell death in THP-1 cells. (A) THP-1 cells were treated with 1.0 μg/ml recombinant wild type (W.T.) α-hemolysin or recombinant mutant (H35L) α-hemolysin for 4 hours. Treatment induced cell death was assayed at the indicated time points by measuring LDH activity released into culture supernatant. Levels of LDH are reported as a percent of the maximal LDH activity detected after detergent lysis. (B) THP-1 derived cell lines stably transduced with shRNA expressing retrovirus were treated with or without Hla (1.0 μg/ml, Sigma Aldrich) for 1 hour and cell death was assayed by measuring LDH activity released into culture supernatant. Levels of LDH are reported as a percent of the maximal LDH activity detected after detergent lysis. Representative data from at least 3 independent experiments are shown. doi:10.1371/journal.pone.0007446.g005
loss of plasma membrane integrity and cytoplasmic contents as well as intact nuclei with loose chromatin. Necrotic cell death results in the release of the chromosomal protein HMGB1. Release of HMGB1 is also observed from apoptotic cells in tissue culture as a result of secondary necrosis [36]. Immunoblot analysis of cell culture supernatants from a-hemolysin treated THP-1 cells demonstrates that a-hemolysin induced death is accompanied by early release of HMGB1 protein, which is not seen in staurosporine treated cells (Fig. 6D). Staurosporine-induced apoptotic death was confirmed in these cultures by immunoblot analysis for PARP cleavage (Fig. 6D). Prior studies have demonstrated that induction of IL-1β processing through activation of the NLRP3-inflammasome in response to the pore-forming toxin Nigericin requires intracellular potassium depletion. Both NLRP3-inflammasome dependent IL-1β secretion and necrotic cell death were inhibited when cells were treated with S. aureus a-hemolysin in the presence of 130 mM KCl (Fig. 7A & B). This data, combined with the inability of mutant a-hemolysin to induce IL-1β secretion or rapid necrosis, suggests that S. aureus a-hemolysin activates the inflammasome through potassium depletion and not through direct interaction with other host signaling proteins. In total, this work demonstrates that a-hemolysin actually requires host NLRP3 signaling in order to induce necrotic cell death.

Though the NLRP3 inflammasome activates the cysteine proteinase, caspase-1, NLRP3-dependent programmed necrosis has been shown to be independent of caspase activation. Pretreatment of THP-1 cells with a permeable pancaspase inhibitor did not block a-hemolysin induced cell death (Fig. 8A). We confirmed the inhibitor was functioning by examining a-hemolysin-mediated IL-1β secretion in the treated cells, which was completely abrogated by treatment with caspase inhibitor (Fig. 8B). We also studied induction of pyronecrosis in peritoneal derived macrophages treated with a-hemolysin. While a-hemolysin induced release of HMGB1 into the culture supernatant at levels equivalent to detergent induced lysis from wild type peritoneal macrophages, Nlrp32/2 macrophages were resistant to a-hemolysin induced HMGB1 release (Fig. 8C). Consistent with our findings using caspase inhibitors, release of...
Discussion

We have demonstrated that highly purified recombinant S. aureus α-hemolysin is capable of inducing activation of caspase-1 through the NLRP3 inflammasome. Activation of NLRP3 by α-hemolysin induces cytokine processing and programmed necrosis, both of which can explain many of the pro-inflammatory properties of α-hemolysin that have been observed in the past. In fact, these studies identify the mechanism by which α-hemolysin induces IL-1β secretion, an observation that was published almost twenty years ago [22].

In the initial report of S. aureus activating NLRP3-mediated IL-1β secretion, Mariathasan et al. found that S. aureus lacking α-hemolysin, β-hemolysin, or γ-hemolysin still activated IL-1β secretion [32]. Our findings using purified toxin clearly demonstrate that NLRP3 is activated by α-hemolysin. Because bone marrow derived macrophages do not respond robustly to α-hemolysin in our studies, the findings of Mariathasan et al. are not surprising. Additionally, given the large number of pore-forming and membrane disrupting toxins produced by S. aureus, it seems likely S. aureus may have redundant factors that activate NLRP3-signaling. Supporting this possibility, we have found that other pore-forming S. aureus toxins (particularly Panton Valentine Leukocidin) also induce NLRP3-dependent cytolysis (data not shown). Since some of these toxins exert differential cytolytic activity based on cell type, the ability to activate NLRP3-mediated signaling may be important in establishing infection in a variety of infection sites [22].

The ability of α-hemolysin to activate programmed cell death in host cells has been the subject of numerous studies. In early studies, α-hemolysin was shown to activate apoptotic machinery in studies carried out primarily in lymphocyte derived cell lines [46]. This report demonstrated that apoptotic caspases were activated and down stream apoptotic effects were observed, including cleavage of α-fodrin and intranucleosomal DNA cleavage. However, these effects were studied at times between 4 and 24 hours. A second report by this group later demonstrated that α-hemolysin induced cell death, as measured by loss of membrane integrity, occurred even if apoptotic machinery was inhibited [44]. Additionally, they demonstrated that α-hemolysin induced death...
was accompanied by morphologic features of necrosis and release of HMGB1 into the culture supernatant. We now show that pyroptosis is activated very rapidly by α-hemolysin, in a timeframe earlier than has previously been characterized for α-hemolysin-induced death and with features consistent with these other reports. Namely, we report α-hemolysin-induced death is independent of caspase-activation, has morphologic features of necrosis, and is accompanied by early release of HMGB1. Interestingly, when taken out to 16 hours, we did observe some cells with sub-G1 DNA content, which may represent activation of apoptotic machinery in cells from the population that resisted activation of pyroptosis. Overall, we have demonstrated that, in monocytic cells, activation NLRP3-dependent pyroptosis by α-hemolysin is the primary mechanism underlying the cytolytic activity of the toxin.

The activation of host NLRP3 by α-hemolysin requires the hemolytic activity of the toxin. Our data demonstrates that mutations in the toxin that eliminate hemolytic activity also eliminate the toxin’s cytolytic activity in monocyte derived cells and ability to induce NLRP3-inflammasome signaling. A recent study of a similar α-hemolysin mutant demonstrates that this mutant toxin is capable of inducing an apoptotic host response [47]. Our experiments also demonstrate that although mutant α-hemolysin does not induce NLRP3-dependent IL-1β secretion, it does induce cell death with a time delay (Fig. 5). We suspect that this death may represent apoptotic death observed by others using mutant α-hemolysin, though formal demonstration of this requires further investigation. Interestingly, this mutation also renders the toxbin incapable of causing death in animal models of α-hemolysin induced peritonitis, presumably due to reduced activation of inflammatory signaling [48]. The α-hemolysin gene, Hla, is a critical virulence factor in cellulitis, mastitis, and pneumonia models of S. aureus infection [49,50]. We speculate that activation of inflammatory signaling through the inflammasome may be essential for α-hemolysin to mediate virulence. In support of this hypothesis, α-hemolysin deficient S. aureus fail to establish lethal infection or induce significant pulmonary infection [7,11]. Additionally, immunization with mutant α-hemolysin protects mice from S. aureus pneumonia and reduces IL-1β production in the lungs of the challenged mice [12]. Furthermore, we have now shown a monoclonal antibody, which is protective against S. aureus pneumonia in passive immunization studies of mice, inhibits NLRP3-mediated signaling in cultured cells (Fig. 1D) [13]. It is certainly possible that host inflammatory responses and programmed necrosis could damage host tissue architecture and compromise the innate capacity of these tissues to resist infection. Further experimentation is clearly required to determine the role of NLRP3 inflammasome signaling in the pathogenesis of S. aureus infection. If this hypothesis is born out, inhibition of inflammasome signaling may turn out to be a useful adjuvant therapy to appropriate antibiotic use in severe S. aureus infections.

Materials and Methods

Ethics Statement

All protocols used in this study were approved by the Institutional Animal Care and Use Committees at the University of North Carolina.

Preparation of recombinant α-hemolysin

E. coli (BL21/DE3) harboring a hexahistidine-tagged S. aureus α-hemolysin (wild type and H35L mutant)-expressing plasmid, construction described by Ragle and Bubeck Wardenburg [13], were grown overnight in LB supplemented with kanamycin (50 μg/ml). Overnight cultures were diluted 1:10 into 100 ml LB with kanamycin (50 μg/ml) and grown for 3 hours. α-hemolysin expression was induced by addition of IPTG to 1 mM. After 2 hours at 37°C, the E. coli were harvested by centrifugation and lysed in Dulbecco’s Phosphate Buffered Saline (D-PBS) supplemented with complete protease™ inhibitor cocktail (Roche, product # 04 693 116 001) by sonication. The lysate was clarified by centrifugation at 13,000 x g for 30 min and applied to immobilized nickel resin (PrepEase Histidine-tagged Protein Purification Sample Kit, USB, product # 78810). The bound protein was washed extensively (50 column volumes) with pyrogen free tissue culture grade D-PBS (Invitrogen/Gibco, product # 14040-133) and eluted in D-PBS supplemented with 200 mM imidazole. Imidazole was removed from the eluted proteins by size exclusion chromatography; 2 sequential passages through a sephadex G-25 (Disposable PD-10 Desalting Columns, G.E. Healthcare, product # 17-0851-01) equilibrated with pyrogen free PBS. Final protein preparations concentration was determined using Bradford protein assay reagent (Bio-Rad Protein Assay, Bio-Rad, product # 500-0006) with albumin protein standards, purity of the proteins was assessed using SDS-PAGE analysis.

Mouse strain maintenance and care

The generation of Nlpr3<sup>−/−</sup>, Asc<sup>−/−</sup>, and Casp1<sup>−/−</sup> mice has been described previously [30,51]. The mice were kindly provided by Millenium Pharmaceuticals and Dr. Vishva Dixit at Genentech, and Dr. Richard Flavell at Yale University, respectively. The mice have been backcrossed onto the C57BL/6J genetic background for at least nine generations. Age- and sex-matched C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were used as wild-type controls. The mice were maintained according to institutional policies.

Culture of mammalian cells

The construction of THP-1-derived cell lines expressing shRNA targeting NLRP3 and ASC as well as control cell lines using recombinant retrovirus has been previously described [36,42]. All cell lines were maintained in RPMI Media 1640 (Invitrogen/ Gibco, product # 12633-012) supplemented with 10% fetal calf serum and penicillin and streptomycin. Resting peritoneal macrophages were obtained by peritoneal lavage of euthanized mice with Hanks Buffered Saline supplemented with 10% fetal calf serum and penicillin/streptomycin. Lavaged cells were counted and plated at a density of 2x10<sup>5</sup>/ml in 96 well tissue culture plates. Bone marrow-derived macrophages were prepared as previously described [36]; briefly, bone marrow was harvested by necrotic dissection of euthanized mice. The cells were resuspended in 25 ml/fe/mur DMEM supplemented with 10% FBS (Hyclone Characterized Fetal Bovine Serum, Thermo Scientific, product # SH30071), L-glutamine (Invitrogen/GIBCO, product # 21051-024), 100 μM Non-Essential Amino Acids (Invitrogen/ GIBCO, product # 11140-050), 20% L929 conditioned media, and plated at 12.5 ml/100×20 mm non-tissue culture treated plates. The adherent bone marrow-derived macrophages were cultured in DMEM with 20% L929 cell supernatant (as a source of M-CSF) for 7 days. The cells were then treated as indicated (treatments detailed below).

Treatment of cultured cells with α-hemolysin

When indicated cells were preincubated with Heat Killed S. aureus (HKSA, Invivogen, product # hkl-hksa) at 1 particle/cell (typically 10<sup>4</sup>/ml) for 3 hours. S. aureus α-hemolysin (Sigma-Aldrich, product # H9395) or recombinant α-hemolysin (prepa-
A similar volume of DMSO (1 from a stock of inhibitor in DMSO at a concentration of 20 mM. gluteraldehyde, 0.15 M sodium phosphate, pH 7.4 solution for used, Z-VAD(OMe)-FMK, (Alexis Biochemicals, product # ALX-260-039) was added to the cells to a final concentration of 20 mM from a stock of inhibitor in DMSO at a concentration of 20 mM. A similar volume of DMSO (1 µg/ml) was obtained from Sigma-Aldrich (product # S6942) and added to the culture to final concentration of 1 µM. When caspase inhibitors were used, Z-VAD(OMe)-FMK, (Alexis Biochemicals, product # ALX-260-039) was added to the cells to a final concentration of 20 mM from a stock of inhibitor in DMSO at a concentration of 20 mM. When indicated, culture supernatants are run on SDS PAGE for analysis. In Vitro caspase gels (NuPAGE) were used for all gel-based analyses. Proteins were transferred to nitrocellulose using the Invitrogen iBlot system and immunoblot analysis carried out using antibodies directed against: human and mouse IL-1β (R & D Systems, product # AF-201-NA & AF-401-NA, respectively), anti–active caspase-3 (Cell Signaling, product # 9662), anti-PARP (Cell Signaling, product # 9542), anti-HMG1 (Abcam, product # ab18256), anti (Santa Cruz Biotechnology, product # sc-8432). 

Immunoblot analysis

Cell pellets were lyzed using PBS supplemented with 1% Igepal, protease inhibitor, 2 mM DTTO and soluble lysates for immunoblot analysis were generated by centrifugation at 13,000 xg for 15 min. When indicated, culture supernatants are run on SDS PAGE for analysis. In Vitro caspase gels (NuPAGE) were used for all gel-based analyses. Proteins were transferred to nitrocellulose using the Invitrogen iBlot system and immunoblot analysis carried out using antibodies directed against: human and mouse IL-1β (R & D Systems, product # AF-201-NA & AF-401-NA, respectively), anti–active caspase-3 (Cell Signaling, product # 9662), anti-PARP (Cell Signaling, product # 9542), anti-HMG1 (Abcam, product # ab18256), anti (Santa Cruz Biotechnology, product # sc-8432). 

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