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Anthrax toxin induces macrophage death by p38 MAPK inhibition but leads to inflammasome activation via ATP leakage

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Abstract

Detection of microbial constituents by membrane associated and cytoplasmic pattern recognition receptors is the essence of innate immunity, leading to activation of protective host responses. However, it is still unclear how immune cells specifically respond to pathogenic bacteria. Using virulent and non-virulent strains of *Bacillus anthracis*, we have shown that secretion of ATP by infected macrophages and the sequential activation of the P2X7 purinergic receptor and nucleotide binding oligomerization domain (NOD)- like receptors are critical for IL-1-dependent host protection from virulent *B. anthracis*. Importantly, lethal toxin produced by virulent *B. anthracis* blocked activation of protein kinases, p38 MAPK and AKT, resulting in opening of a connexin ATP release channel and induction of macrophage death. Prevention of cell death or ATP release through constitutive p38 or AKT activation interfered with inflammasome activation and IL-1 β production, thereby compromising anti-microbial immunity.

INTRODUCTION

Two major types of pattern recognition receptors (PRRs)- *Toll like receptors* (TLRs) and *NOD like receptors* (NLRs) recognize microbial components and activate mammalian innate immunity (Meylan et al., 2006). TLRs are directly activated by microbial components collectively known as pathogen associated molecular patterns (PAMPs), but the mechanism of NLR activation is more obscure and complex (Martinon et al., 2009). Despite their name, most PAMPs, including lipopolysaccharides, peptidoglycans, lipopeptides, and flagellin are expressed by virulent and non-virulent bacteria alike. Thus, it is not entirely clear how immune cells discriminate between non-virulent commensals and virulent pathogens or non-virulent and virulent variants of the same bacterial species. One signaling system that seems

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capable of such discrimination is the NLR-activated inflammasome system (Martinon et al., 2009). Inflammasomes are NLR-containing multiprotein complexes that control activation of caspase-1 and the processing and secretion of pro-inflammatory cytokines interleukin (IL)-1 β and IL-18 (Martinon et al., 2009). Inflammasome activators include certain PAMPs and danger associated molecular patterns (DAMPs), such as proteins released by dying cells, extracellular ATP, reactive oxygen species (ROS), urea crystals and bacterial pore-forming toxins such as listeriolysin O of *Listeria monocytogenes* and alpha-hemolysin of *Staphylococcus aureus* (Franchi et al., 2009; Hruz et al., 2009; Mariathasan et al., 2004). The pathways connecting these elicitors of inflammasome activation remain largely unknown.

We have investigated the mechanisms that allow a toxin-producing strain of the Gram-positive bacterial pathogen *Bacillus anthracis*, the causative agent of anthrax (Tournier et al., 2009), to activate the inflammasome, whereas non-toxin producing derivatives of the same strain do not. Anthrax pathogenesis depends on production of lethal toxin (LT) and edema toxin (ET) (Moayeri and Leppla, 2009). LT is composed of two subunits, protective antigen (PA), which also serves as a subunit of ET, and lethal factor (LF), a zinc metalloprotease that specifically cleaves the N-termini of mitogen-activated protein kinase (MAPK)-kinases (MEK or MKK) within the host cell (Moayeri and Leppla, 2009). Importantly, LF-dependent inhibition of p38 MAPK results in macrophage apoptosis (Park et al., 2002). LT can also induce assembly of the NALP1 inflammasome (Boyden and Dietrich, 2006; Bruey et al., 2007; Faustin et al., 2007; Hsu et al., 2008). However, it is not clear whether LT is directly recognized as a PAMP by NALP1 or whether its catalytic activity promotes inflammasome activation indirectly. Another enigma is the host-based variation in NALP1 inflammasome activation by LT, caused by genetic polymorphisms in the *Nalp1b* locus (Boyden and Dietrich, 2006). Notably, live *B. anthracis* can induce IL-1 β production also in mouse strains, such as C57BL/6 (Hsu et al., 2008), whose macrophages are refractory to LT-induced NALP1 activation (Boyden and Dietrich, 2006). Given these differences and complications, we pursued studies with isogenic live bacterial strains that differ in LT production rather than use purified LT in the absence of other bacterial components.

Using isogenic *B. anthracis* strains that differ only in LF expression, we found that LT affects the host response in a manner dependent on activation of NALP1 and caspase-1, and that a LF-deficient strain cannot induce inflammasome activation. Specifically, LT-dependent inhibition of p38 and AKT signaling resulted in release of ATP from infected macrophages through connexin channels leading to subsequent inflammasome activation mediated by the ATP-responsive P2X7 purinergic receptor. This pathway plays a critical role in protecting the host from LT-producing *B. anthracis*.

RESULTS

The NLR associated molecules, RIP2 and IPAF, are required for *B. anthracis*-mediated NALP1 inflammasome activation

B. anthracis infection activates a NALP1 inflammasome complex leading to IL-1 β processing and secretion (Hsu et al., 2008). To elucidate the composition of the *B. anthracis*-activated NALP1 inflammasome complex, murine peritoneal macrophages were infected with *B. anthracis* (Sterne strain), at a multiplicity of infection (MOI) of 2 bacteria/cell. Cell lysates were immunoprecipitated with a caspase-1 antibody followed by immunoblotting for candidate NLRs and adaptor proteins. *B. anthracis* infection induced association of NALP1, NOD2, RIP2 and IPAF (NLRC4) with caspase-1 (Figure 1A). By contrast, NALP3 and ASC were not present in caspase-1 immunoprecipitates following *B. anthracis* infection.

To determine if IPAF and RIP2 are required for *B. anthracis*-induced IL-1 β secretion, wild type (WT), *Nlr4*^{-/-} or *Ripk2*^{-/-} macrophages were infected as above and IL-1 β secretion was analyzed by ELISA. *Ripk2*^{-/-} and *Nlr4*^{-/-} macrophages released significantly less IL-1 β upon *B. anthracis* infection than WT macrophages (Figure 1B). By contrast, RIP2 or IPAF made little contribution to induction of pro-IL-1 β mRNA in *B. anthracis* infected macrophages (Figure 1C). *Ripk2* ablation also did not decrease the expression of caspase-1, Nod2, Nalp1 and Nalp3 mRNAs (Figure S1). Notably, RIP2 was important for *B. anthracis*-induced caspase-1 activation and processing as detected by release of the caspase-1 p10 fragment along with mature (p17) IL-1 β (Figure 1D).

***B. anthracis* activates the NALP1 inflammasome in a p38-dependent manner**

LF, the active component of LT, is a protease that cleaves the N-termini of all MEKs (or MKKs) with the exception of MEK5 (Vitale et al., 2000). As expected, infection of macrophages with WT *B. anthracis* Sterne strain led to disappearance of full length MEK1, MEK4 and MEK6 (Figures 2A and S2A). All 3 proteins remained intact in macrophages infected with an isogenic LF-deficient mutant of the *B. anthracis* Sterne strain (Δ LF). Correspondingly, infection of macrophages with the Δ LF mutant triggered much stronger activation of ERK, JNK and p38 MAPKs than did infection with the parental *B. anthracis* strain (WT; Figure 2B). Whereas WT *B. anthracis* induced IL-1 β release, infection with the Δ LF strain did not (Figure S2B). Accordingly, WT but not Δ LF *B. anthracis* induced association of caspase-1 with NOD2 (Figure S2C). To determine whether LF-mediated inhibition of MAPK activation contributes to inflammasome activation and IL-1 β secretion, we treated Δ LF-infected macrophages with different MAPK and MEK inhibitors [PD98059, an inhibitor of MEK1 and MEK2 which activate ERK, SP600125 to inhibit JNK and SB202190 for p38 α and β isoforms]. However, inhibition of p38, JNK or ERK signaling in Δ LF-infected macrophages failed to restore IL-1 β secretion (Figure S2B). However, as previously reported (Park et al., 2002), the p38 inhibitor reduced IL-1 β mRNA in Δ LF-infected macrophages to amounts similar to those found in WT *B. anthracis*-infected cells (Figure S2B). Activation of p38 regulates CREB (c-AMP response element binding transcription factor) activity that is required for transcriptional regulation of several molecules including plasminogen activator inhibitor-2 (PAI-2), cyclooxygenase-2 (COX-2) and IL-1 β (Park et al., 2005). Notably, the amounts of IL-1 β mRNA induced by the Δ LF mutant were considerably higher than the amounts of IL-1 β mRNA induced by WT *B. anthracis*.

Due to the interference of p38 inhibition with optimal IL-1 β mRNA induction, we undertook a different approach to understand whether MEK6 cleavage and inhibition of p38 activation contribute to inflammasome activation. We thus transfected RAW264.7 macrophages with a construct encoding MEK6 Δ CR, a variant form of MEK6 that is resistant to the inhibitory effect of LF (Park et al., 2002). RT-PCR with mutant gene-specific primers confirmed MEK6 Δ CR mRNA expression in transfected RAW 264.7 macrophages (Figure S2D); amounts were comparable to those of WT MEK6 mRNA in these cells (data not shown). Over-expression of MEK6 Δ CR in *B. anthracis*-infected macrophages restored p38 MAPK activation (Figure 2C). As found previously using purified LT (Park et al., 2002), over-expression of MEK6 Δ CR prevented *B. anthracis*-induced cell death (Figure 2D). MEK6 Δ CR expression also completely blocked IL-1 β release in response to WT *B. anthracis* infection (Figure 2E). In contrast, IL-6 release was increased by MEK6 Δ CR expression (Figure 2F) and induction of IL-1 β mRNA was also slightly enhanced (Figure S2E). In conclusion, MEK6 cleavage by LF is required for IL-1 β release by *B. anthracis* infected macrophages.

LF-mediated inhibition of AKT contributes to IL-1 β release

AKT is a serine-threonine protein kinase with important roles in multiple cellular processes. LF inhibits T-cell receptor-induced AKT activation (Comer et al., 2005). We observed LF-dependent inhibition of AKT activation in macrophages, as evidenced by elevated AKT S473 phosphorylation in Δ LF-infected cells relative to cells infected with WT *B. anthracis* (Figure 3A). We postulated that reduced AKT activity could also contribute to *B. anthracis*-induced cell death and IL-1 β release along with inhibition of p38 activation. To test this hypothesis, macrophages were transduced with retroviral vectors containing a constitutively active variant of AKT (myristoylated-AKT or myr-AKT) or no insert and were then infected with *B. anthracis*. Myr-AKT expressing macrophages were significantly protected from *B. anthracis*-induced cell death (Figure 3B), suggesting an important contribution of AKT to host cell survival during infection. Given the protective functions of both p38 and AKT, we explored the possibility of cross-talk between the two kinases. Using MEK6 Δ CR-expressing RAW264.7 cells, we found that expression of cleavage-resistant MEK6 Δ CR enhanced AKT activation in *B. anthracis* infected cells (Figure 3C). However, expression of myr-AKT did not prevent inhibition of p38 activation in *B. anthracis* infected macrophages (Figure 3D). Thus, *B. anthracis*-mediated inhibition of p38 activation prevents AKT phosphorylation in infected macrophages, but AKT activation does not affect p38 activity. Nonetheless, expression of myr-AKT blocked IL-1 β release without affecting IL-6 release by infected macrophages (Figure 3E and 3F). Thus, inhibition of both p38 and AKT is required for inflammasome activation and IL-1 β release in *B. anthracis* infected macrophages.

ATP secretion is required for inflammasome activation upon *B. anthracis* infection

ATP is released from mononuclear phagocytes stimulated with various PAMPs (Piccini et al., 2008) and extracellular ATP is one of the most pleiotropic inflammasome activators (Ogura et al., 2006). To examine a potential role for ATP in *B. anthracis*-induced inflammasome activation, we measured extracellular ATP released from macrophages infected with either WT or the Δ LF mutant *B. anthracis*. Infection with WT *B. anthracis* resulted in time-dependent ATP release, but this was not seen in macrophages infected with the Δ LF mutant (Figure 4A). Cell death was minimal (around 3-5%) at 3 hr post-infection suggesting that macrophage death may not be the direct cause of early ATP release. To determine if the secreted ATP contributed to inflammasome activation, we blocked ATP signaling via the P2X7 purinergic receptor with the antagonist KN-62 (Di Virgilio, 2007b; Piccini et al., 2008). P2X7 blockade abolished *B. anthracis*-induced IL-1 β secretion (Figure 4B), without affecting pro-IL-1 β mRNA synthesis (data not shown). Secretion of IL-6 by *B. anthracis*-infected macrophages was unaffected by P2X7 blockade (Figure 4C), suggesting an inflammasome-specific phenomenon rather than a general requirement for P2X7 in cell activation or cytokine secretion. If lack of ATP accounts for inability of Δ LF mutant-infected macrophages to activate caspase-1, we hypothesized that co-treatment with exogenous ATP should compensate for this difference. Indeed, IL-1 β release was detected in the culture medium of Δ LF mutant-infected macrophages upon concomitant ATP treatment (Figure S3A). In fact, in the presence of exogenous ATP, the Δ LF mutant induced more IL-1 β secretion than WT *B. anthracis*.

Furthermore, release of ATP from *B. anthracis*-infected macrophages was blocked by expression of constitutively active myr-AKT or MEK6 Δ CR (Figure 4D), suggesting a role for LF-mediated inhibition of p38 and AKT signaling in triggering ATP release. Overexpression of either p38 or AKT by itself had no inhibitory effect on inflammasome activation, as induction of IL-1 β release by co-treatment of Δ LF + ATP was unaffected, by MEK6 Δ CR or myr-AKT expression in RAW264.7 cells (Figure S3B and data not shown). In contrast to p38 and AKT- signaling, caspase-1 was dispensable for ATP secretion as

similar amounts of ATP were released from *B. anthracis*-infected WT and *Casp1*^{-/-} macrophages (Figure S3C).

Activation of P2X7 by ATP causes rapid K⁺ efflux from the cytosol and release of intracellular Ca⁺⁺ (Di Virgilio, 2007b). To study the role of these events in *B. anthracis*-induced IL-1 β release, we blocked K⁺ efflux by increasing extracellular KCl to 130 mM. Blockade of K⁺ efflux inhibited *B. anthracis*-induced IL-1 β secretion without affecting pro-IL-1 β mRNA expression (Figure 4E and data not shown). Neither extracellular KCl nor KN-62-mediated blockade of P2X7 affected MEK proteolysis in *B. anthracis*-infected macrophages (Figures 4F and S3D), indicating that the observed inhibition of IL-1 β release is not due to alterations in the infection process or LF catalytic activity.

AKT prevents ATP release by controlling a connexin-43 channel

The above experiments suggested that ATP released by *B. anthracis*-infected macrophages is a key signal for activation of inflammasome-dependent host immunity. Live cells release ATP through a number of channels, including volume-sensitive channels, pannexin and connexin channels (Chekeni et al., 2010; Faigle et al., 2008; Pangrsic et al., 2007; Sabirov et al., 2001), as well as through vesicular exocytosis (Pangrsic et al., 2007). To examine the potential involvement of the connexin-43 channel (Eltzschig et al., 2006; Faigle et al., 2008) in *B. anthracis* induced ATP release, mouse macrophages were infected with *B. anthracis* in the presence or absence of the connexin-43 inhibitor, carbenoxolone (CBX) (Kang et al., 2008). Notably, release of ATP by *B. anthracis*-infected macrophages was attenuated following CBX exposure (Figure 5A). In contrast, CBX treatment did not inhibit lactate dehydrogenase (LDH) release; on the contrary, CBX treated macrophages exhibited enhanced LDH release (Figure 5B). To analyze the role of connexin-43 in inflammasome activation, mouse macrophages were infected with *B. anthracis* in the presence or absence of CBX, and IL-1 β and caspase-1 secretion were examined. Incubation with CBX inhibited secretion of activated caspase-1 and IL-1 β by *B. anthracis*-infected macrophages (Figures 5C and S4A). Since, CBX may affect other pathways (Chekeni et al., 2010; Faigle et al., 2008; Pangrsic et al., 2007; Sabirov et al., 2001), we validated our results using shRNA-mediated silencing of connexin-43. shRNA-mediated silencing of connexin-43 (Figure 5D), reduced *B. anthracis*-induced ATP (Figure 5E), caspase-1 and IL-1 β release (Figure 5F) compared to control treated cells. Neither *B. anthracis*-induced IL-1 β mRNA expression nor cell death were affected by connexin-43 silencing (data not shown). Collectively, the connexin-43 silencing studies and pharmacological inhibition by CBX support a role for connexin-43 in ATP release and inflammasome activation during *B. anthracis* infection.

Connexin-43 has multiple serine residues that can be phosphorylated by PKC, MAPK, or AKT (Kang et al., 2008) and the phosphorylation of connexins negatively regulates their opening (Faigle et al., 2008). Since *B. anthracis* inhibits activation of MAPKs and AKT, we asked whether inhibition of these protein kinases impacts the phosphorylation of connexin-43. In non-infected macrophages, connexin-43 was phosphorylated at Ser279 and Ser282 based on reactivity with a phospho-specific connexin-43 antibody (Leykauf et al., 2003). Initially, *B. anthracis* infection enhanced connexin-43 phosphorylation, but after 2 hr connexin-43 phosphorylation was almost completely abolished (Figure 5G). In contrast, infection of macrophages with Δ LF *B. anthracis* had no inhibitory effect on connexin-43 phosphorylation (Figure 5H). The kinetics of connexin-43 dephosphorylation correlated with the kinetics of ATP release (Figure S4B). To gain further insight into the regulation of connexin-43 phosphorylation, we examined it in RAW264.7 cells expressing myr-AKT. Notably, expression of constitutively active myr-AKT, which inhibits ATP release and inflammasome activation in infected macrophages (Figures 4D and 3E), prevented the *B. anthracis*-induced decrease in connexin-43 phosphorylation (Figure 5H). Together, our

results suggest that LT-dependent inhibition of p38 and AKT signaling results in opening of connexin-43 channel and ATP release for inflammasome activation (Table S1).

IL-1 β production protects mice from *B. anthracis*

IL-1 β signaling plays a prominent role in immune and inflammatory responses (Meylan et al., 2006; Miller et al., 2006). To determine the immunological role of *B. anthracis*-induced inflammasome activation and IL-1 β production, we infected WT *C57BL/6* mice and different mutants in the same genetic background with WT or Δ LF *B. anthracis*. Although *C57BL/6* mice are resistant to pharmacological challenge with purified LF (Boyden et al., 2006), they died when infected with WT *B. anthracis* but not upon infection with the Δ LF mutant (Figure S5A). Interestingly, the *C57BL/6* strain exhibited enhanced *B. anthracis*-induced mortality relative to the *Balb/c* strain (Figure S5B), in contrast to the response elicited by purified LT in the two strains (Moayeri et al., 2003). Although purified edema toxin causes lethality of *Balb/c* mice (Moayeri and Leppla, 2009), Δ LF that expresses intact ET was unable to induce mortality in *C57BL/6* (Figure S5A) as well as in *Swiss* mice (Pezard et al., 1991). Infected mice cleared Δ LF mutant bacteria from their spleen more efficiently than WT *B. anthracis* (Figures S5C). Furthermore, WT *B. anthracis*, but not the Δ LF mutant, induced cell death in spleens of infected mice (data not shown). These results demonstrate that lethal *B. anthracis* infection of *C57BL/6* mice is indeed LF-dependent.

Both caspase-1 (*Casp1*^{-/-}) and IL-1 receptor (*Il1r*^{-/-}) deficient mice exhibited enhanced mortality upon infection with WT *B. anthracis* relative to WT mice (Figure 6A). Furthermore, *B. anthracis*-infected mice displayed enhanced mortality when treated with Anakinra (Kineret), an FDA-approved IL-1 receptor (IL-1R) antagonist (Figure 6A). No mortality of either WT or *Casp1*^{-/-} mice was seen upon infection with the Δ LF mutant (data not shown). Furthermore, infection with a low inoculum (0.5×10^6 cfu/mouse) that had no effect on survival of WT mice was sufficient to induce mortality of *Casp1*^{-/-} mice (Figure S5D). Together, the results suggest that inflammasome activation and IL-1 β signaling are critical for innate immunity to LT-producing *B. anthracis*. Furthermore, counter to previous expectations (Boyden and Dietrich, 2006), assembly of inflammasome components and caspase-1 activation was not the cause of *B. anthracis*-induced mortality. Consistent with these observations, mice deficient in critical LF-responsive inflammasome components, including *Nlrp4*^{-/-}, *Ripk2*^{-/-} and *Nod2*^{-/-} mice, also exhibited enhanced mortality upon *B. anthracis* infection (Figure 6B).

To determine the effect of reduced inflammasome responses in *Casp1*^{-/-} mice on host leukocytes, we harvested spleens from *B. anthracis*-infected WT and *Casp1*^{-/-} mice 3 days after infection and evaluated cell death by TUNEL staining. In contrast to previous reports that LT-induced cell death in vitro is caspase-1-dependent (Boyden and Dietrich, 2006), we observed a dramatic increase in splenocyte death in *Casp1*^{-/-} mice relative to WT mice (Figure 6C). This effect correlated with decreased bacterial clearance from spleens of *Casp1*^{-/-} mice (data not shown).

Although neutrophils dominate IL-1 β -dependent responses to bacterial challenge (Miller et al., 2007), downstream effects of IL-1 β signaling during bacterial infection have not been well studied. To determine how inflammasome activation benefits the infected host, peritoneal macrophages from WT and *Casp1*^{-/-} mice were challenged with *B. anthracis* and cell death was assessed by LDH release. As found in vivo, *Casp1*^{-/-} macrophages exhibited enhanced death (Figure 6D) and impaired bacterial clearance (Figure 6E). Corroborating that caspase-1 exerts its effect through IL-1 β , Anakinra treatment also increased the extent of macrophage death in response to infection with WT, but not Δ LF, *B. anthracis* (Figure S5E). Since survival of pathogen-activated macrophages depends on p38 and NF- κ B activation (Park et al., 2002), we examined whether caspase-1 was required for induction of

NF- κ B target genes in *B. anthracis*-infected macrophages. Indeed, expression of several known NF- κ B target genes in mouse macrophages, including *Ptgs2* and *Nos2* was higher in WT macrophages relative to *Casp1*^{-/-} macrophages 4 hr after infection with WT *B. anthracis* (Figure S5F and data not shown). Nonetheless and despite existence of this amplification loop, at very early stages of macrophage infection, both WT and Δ LF *B. anthracis* led to similar NF- κ B activation responses as indicated by the kinetics and extent of I κ B α degradation (Figure 2B). Collectively, these findings indicate a critical role of IL-1 β in mounting immunity against virulent *B. anthracis*.

DISCUSSION

We have used two *B. anthracis* strains that differ in expression of LF, an essential component of LT, a critical virulence factor, to interrogate how the host innate immune system discriminates between virulent and non-virulent bacteria. Our results indicate that the major and most critical difference in the host responses elicited by the two bacteria lies in their ability to activate the inflammasome system and induce IL-1 β secretion. Yet, both *B. anthracis* strains were equally effective in their ability to induce I κ B degradation, a response that is most likely dependent on TLR activation. In fact, at early time points after infection, the non-virulent bacterium led to more effective induction of IL-1 β mRNA and I κ B α re-synthesis, responses that depend on activation of both NF- κ B and MAPKs (Park et al., 2005). IL-1 β secretion was essential for host protection against WT *B. anthracis*, attenuation of macrophage death and bacterial clearance. Dissection of the molecular events involved in *B. anthracis*-mediated inflammasome activation revealed that the key difference between the two strains lies in their ability to induce ATP release from infected macrophages. Our results strongly suggest that the major source of ATP release from *B. anthracis* infected macrophages is the connexin-43 ATP-release channel. Whereas WT *B. anthracis* induces dephosphorylation and presumably opening of connexin-43, the non-virulent Δ LF mutant fails to do so. Most likely, connexin-43 is dephosphorylated in WT *B. anthracis* infected cells due to inhibition of AKT, one of several protein kinases known to keep connexin-43 in a closed state (Park et al., 2007; Solan and Lampe, 2007; Spinella et al., 2003; Warn-Cramer et al., 1996) In addition to elucidating the mechanism of *B. anthracis*-induced inflammasome activation, our results demonstrate that inflammasomes, containing NALP1, NOD2, RIP2 and IPAF, were responsible for discrimination between virulent and non-virulent *B. anthracis*. Interestingly, however, inflammasomes are not directly responsible for recognition of LF, the one molecule that differs between the two bacterial strains used in this study. Rather, the inflammasomes that are activated upon *B. anthracis* infection are responsive to K⁺ ions, whose efflux is stimulated by the ATP that is released from infected cells. Thus, it is the inflammasome, and not the TLR system, that is responsible for recognition of “danger” (Meylan et al., 2006), which in this case is caused by infection with virulent, LT-producing, *B. anthracis*. The initial induction of IL-1 β mRNA, which depends on activation of NF- κ B and other transcription factors, is most likely due to recognition of PAMPs, which are present on both virulent and non-virulent bacteria, by different TLRs expressed by the infected macrophage.

Our results suggest that upon virulent *B. anthracis* infection, caspase-1 interacts with NALP1, NOD2, RIP2 and IPAF, but it is not clear whether all of these molecules form a single inflammasome complex. In vitro studies using purified LT in the absence of other *B. anthracis* components has identified NALP1 as a key component of the LT-activated inflammasome (Boyden and Dietrich, 2006). In these studies, however, LT activated the NALP1 inflammasome only in *Balb/c*, but not in *C57BL/6*, macrophages and the differential response was attributed to polymorphisms at the *Nalp1* locus (Boyden and Dietrich, 2006). The molecular mechanism by which these polymorphisms affect NALP1 activation was never identified, neither was it shown that LT or LF is directly recognized by NALP1. Our

results indicate that although less responsive to purified LT, *C57BL/6* macrophages are perfectly capable of responding to live *B. anthracis*, which is the only physiologically relevant route of LT delivery into these cells. Furthermore, *C57BL/6* mice succumbed to infection with WT *B. anthracis* and the mortality was fully dependent on LT production. As NALP1 is unlikely to be directly involved in LT (or LF) recognition, it is plausible that when encountering recombinant LT, which may not be as active as the bacterially-produced toxin within the host macrophage, *Balb/c* macrophages may be more sensitive to ATP or ATP-induced K⁺ efflux than *C57BL/6* macrophages, a difference that is due to expression of different NALP1 variants.. It should also be noted that in vitro, macrophage killing by LT is caspase-1-dependent, whereas in vivo, at least in *C57BL/6* mice, caspase-1 activation is important for host protection. Furthermore, the *C57BL/6* strain is more susceptible to *B. anthracis*-induced mortality than the *Balb/c* strain, in contrast to the in vitro response elicited by purified LT in macrophages of the two strains (Boyden and Dietrich, 2006), but in agreement with another in vivo infection study using the Sterne strain of *B. anthracis* (Welkos et al., 1986). Furthermore, purified LT induced enhanced mortality in *Balb/c* mice relative to *C57BL/6* mice (Moayeri et al., 2003). Clearly, the response to the intact bacterium is entirely different from the response to the purified toxin.

Previously, we found that *B. anthracis* infection of macrophages induces NALP1- and NOD2-dependent caspase-1 activation and IL-1 β release (Hsu et al., 2008). These NLRs could be involved in sensing of peptidoglycans or other PAMPs produced by this Gram positive bacterium (Martinon, 2007; Schroder and Tschopp, 2010). However, both toxigenic and non-toxigenic *B. anthracis* are probably identical in the PAMPs they express and the key to their differential ability to activate caspase-1 lies in their differential ability to induce ATP release from infected macrophages. Addition of exogenous ATP to macrophages infected with the non-virulent Δ LF mutant restored inflammasome activation and IL-1 β secretion. Consistent with previous publications (Di Virgilio, 2007a, b; Piccini et al., 2008), we find that ATP is likely to cause inflammasome activation through engagement of the P2X7 purinergic receptor and induction of K⁺ efflux.

Multiple mechanisms can mediate release of intracellular ATP in response to mechanical stimulation, stress, osmotic swelling or shrinking of cells, physical perturbation, or host-pathogen interactions (Corriden and Insel, 2010; Piccini et al., 2008). Various membrane channels can release ATP, including connexin and pannexin hemichannels, maxi-anion channels, and volume-regulated anion channels (Corriden and Insel, 2010). In addition to channel-mediated release, ATP can be released by exocytotic mechanisms in neurons, or by membrane permeabilization in dying cells (Chekeni et al., 2010; Faigle et al., 2008; Pangrsic et al., 2007; Sabirov et al., 2001). ATP release was previously shown to be associated with induction of macrophage death through a process that has been named pyroptosis (Iyer et al., 2009), which was implicated in IL-1 β release nearly 20 years ago (Hogquist et al., 1991). Indeed, LT by virtue of its ability to inhibit p38 activation can promote macrophage death (Park et al., 2002). Activation of p38 is required for synthesis of several anti-apoptotic molecules including Bfl1 and PAI-2 (Park et al., 2005) and as we show here, it is also needed for maintenance of AKT activity. Furthermore, the release of ATP from *B. anthracis*-infected macrophages is strongly inhibited by activation of either p38 or AKT but is independent of caspase-1 activation. Rather than be a direct and non-specific consequence of cell death, a major part of the early ATP release by *B. anthracis* infected macrophages depends on the opening of connexin-43 channels as indicated by its inhibition by carbenoxolone, a connexin-43 gap junction inhibitor (De Vuyst et al., 2007) and by connexin-43 silencing.

Previous work has implicated connexin-43 channels in ATP release from various cells including astrocytes, neuronal precursors, neutrophils and myocytes, in response to different

stimuli such as hypoxia, ischemia as well as PAMPs (Clarke et al., 2009; Dobrowolski et al., 2008; Eltzschig et al., 2006; Faigle et al., 2008; Goldberg et al., 2002; Kang et al., 2008; Lampe and Lau, 2004; Liu et al., 2010; Solan and Lampe, 2007). Connexin-43 is maintained in a closed state through phosphorylation by several kinases including PKC, MAPK and AKT (Faigle et al., 2008; Lampe and Lau, 2004) and is opened upon its dephosphorylation (Faigle et al., 2008). We found that macrophage infection with *B. anthracis* resulted in dephosphorylation of connexin-43, which could be prevented by constitutive AKT activation. Although AKT is not a direct target for LF-mediated proteolysis, it is phosphorylated by the p38-responsive protein kinase MK2 at Ser473 (Seimon et al., 2009; Taniyama et al., 2004). In fact, MK2 in a complex with p38, hsp27, and AKT, is activated by p38 to subsequently phosphorylate AKT at Ser473 (Seimon et al., 2009; Taniyama et al., 2004). Thus, cleavage of MEK6 by LF is directly responsible for loss of p38 MAPK activity in *B. anthracis*-infected macrophages and this leads to diminished expression of survival genes and loss of AKT activity. The latter is likely to be responsible for connexin-43 opening and initial ATP release by infected macrophages. The amount of ATP released prior to the onset of massive macrophage death is sufficient for inflammasome activation and IL-1 β secretion (Figure S13). Interestingly, IL-1 β maintains macrophage survival and this may limit further ATP release. However, if the initial release of IL-1 β is not sufficient for prevention of macrophage death, more ATP is likely to be released at later stages when membrane integrity is lost, leading to inflammasome activation in neighboring macrophages that are yet to die. Thus, inflammasome-activation may be controlled by a competition between *B. anthracis*-induced macrophage death and IL-1 β -induced macrophage survival. Importantly, by directly responding to a “danger” signal, the inflammasome is only activated upon infection with a virulent bacterium that cannot evade this critical component of innate immunity whose activation depends on an abundant molecule that is important to all life forms-ATP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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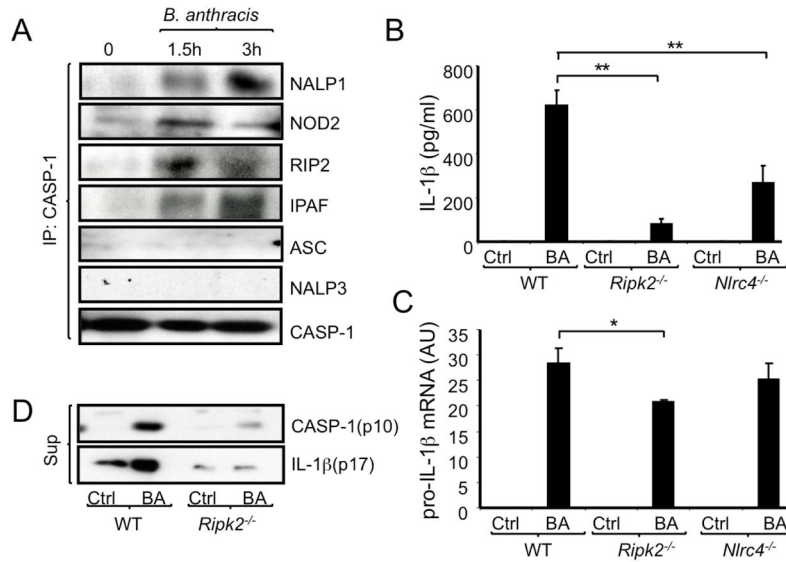


Figure 1. Molecular constituents of *B. anthracis*-activated inflammasomes

(A) Peritoneal macrophages from *C57BL/6* mice were infected with *B. anthracis* at a multiplicity of infection (MOI) = 2. When indicated, macrophage lysates were prepared and immunoprecipitated (IP) with a caspase-1 antibody. Composition of the immunoprecipitates was examined by immunoblotting of three different blots of the same experiment, which was repeated twice with similar results.

(B) Macrophages from the indicated mouse strains were infected with *B. anthracis* as above. After 8 hr, IL-1β in the culture medium was measured by ELISA. This and all similar experiments were repeated at least 3 times and the results of one representative experiment done in triplicates are shown. Values represent means ± SD. **p < 0.01 denote significant differences between the groups.

(C) Macrophages were infected as above and 3 hr later amounts of pro-IL-1β mRNA were measured by quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR). Results were normalized to the amount of GAPDH mRNA. Values represent means ± SD. *p < 0.05 denotes significant differences between the groups. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown.

(D) WT and *Ripk2*^{-/-} macrophages were infected with *B. anthracis* as above and release of active caspase-1 (p10) and IL-1β (p17) to the culture medium was examined after 12 hr by immunoblotting. This experiment was repeated twice with similar results.

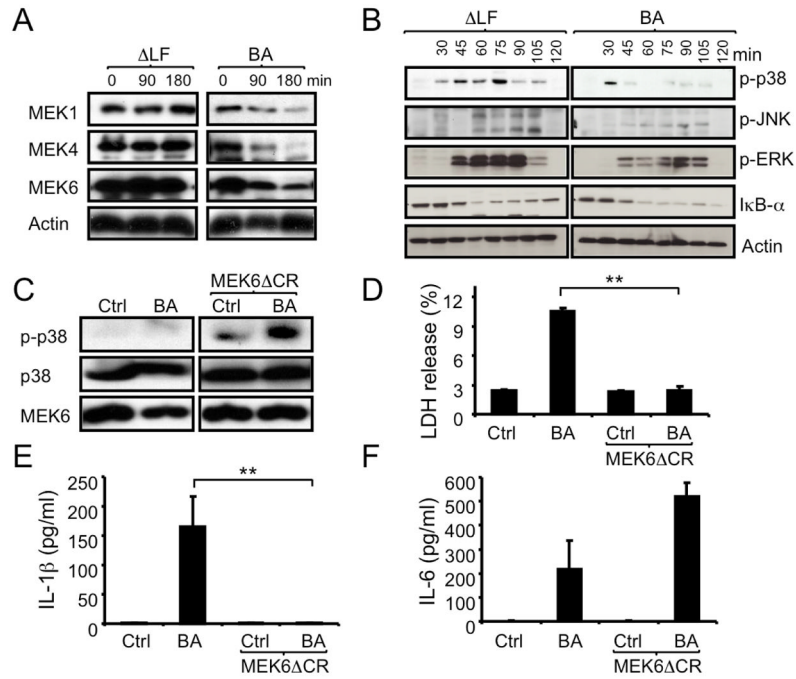


Figure 2. *B. anthracis*-induced inflammasome activation depends on LF and inhibition of p38 (A,B) C57BL/6 macrophages were infected with WT (BA) or Δ LF *B. anthracis*. At the indicated times, cell lysates were analyzed by immunoblotting of 2 separate gels of the same experiment for MEK proteolysis (A) and MAPK phosphorylation (B). (C,D) RAW264.7 macrophages were transfected with MEK6 Δ CR or an empty vector. Transfected cells were left uninfected or infected with *B. anthracis* and 1 hr later cell lysates were prepared and analyzed for p38 phosphorylation by immunoblotting (C). Lactate dehydrogenase (LDH) release to the culture supernatant was measured after 4 hr of infection (D). Results are means \pm SD, **p < 0.01 denote significant differences between the groups. (E,F) RAW264.7 cells were transfected and infected as above. After 8 hr, IL-1 β (E) and IL-6 (F) in culture supernatants were measured by ELISA. Results are means \pm SD, **p < 0.01 denote significant differences between the groups. All experiments were repeated at least 3 times and the results of one representative experiment are shown.

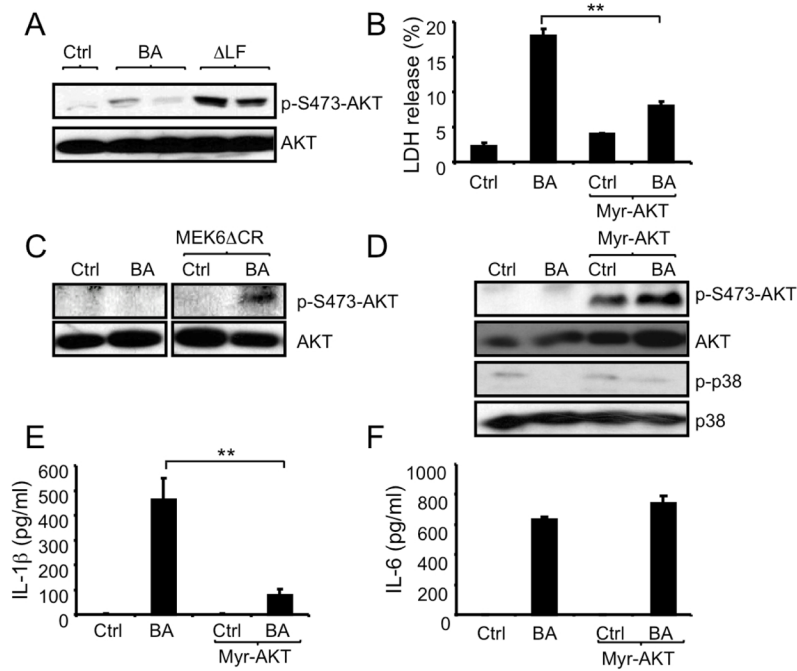


Figure 3. *B. anthracis*-induced cell death and inflammasome activation depend on AKT inhibition

(A) RAW264.7 cells were infected with WT (BA) or Δ LF *B. anthracis* and 2 hr later, cell lysates were prepared and analyzed by immunoblotting for AKT Ser473 phosphorylation.

(B) RAW264.7 cells were retrovirally transduced with myr-AKT or an empty vector. Transduced cells were left uninfected or infected with *B. anthracis*. LDH in culture supernatants was measured 4 hr later. Results are means \pm SD, ** $p < 0.01$ denote significant differences between the groups.

(C) RAW264.7 cells were transfected with MEK Δ 6CR or an empty vector. Transfected cells were left uninfected or infected with *B. anthracis* and 2 hr later, cell lysates were prepared and analyzed by immunoblotting for AKT phosphorylation.

(D) Myr-AKT-transduced or control RAW264.7 cells were infected with *B. anthracis* as above. After 1 hr, cell lysates were prepared and analyzed by immunoblotting of 2 separate gels for AKT and p38 phosphorylation.

(E,F) RAW264.7 cells transfected with myr-AKT or an empty vector were infected as above. IL-1 β (E) and IL-6 (F) in culture supernatants were measured 8 hr after infection by ELISA. Results are means \pm SD, ** $p < 0.01$. All experiments were repeated at least 3 times and the results of one representative experiment are shown.

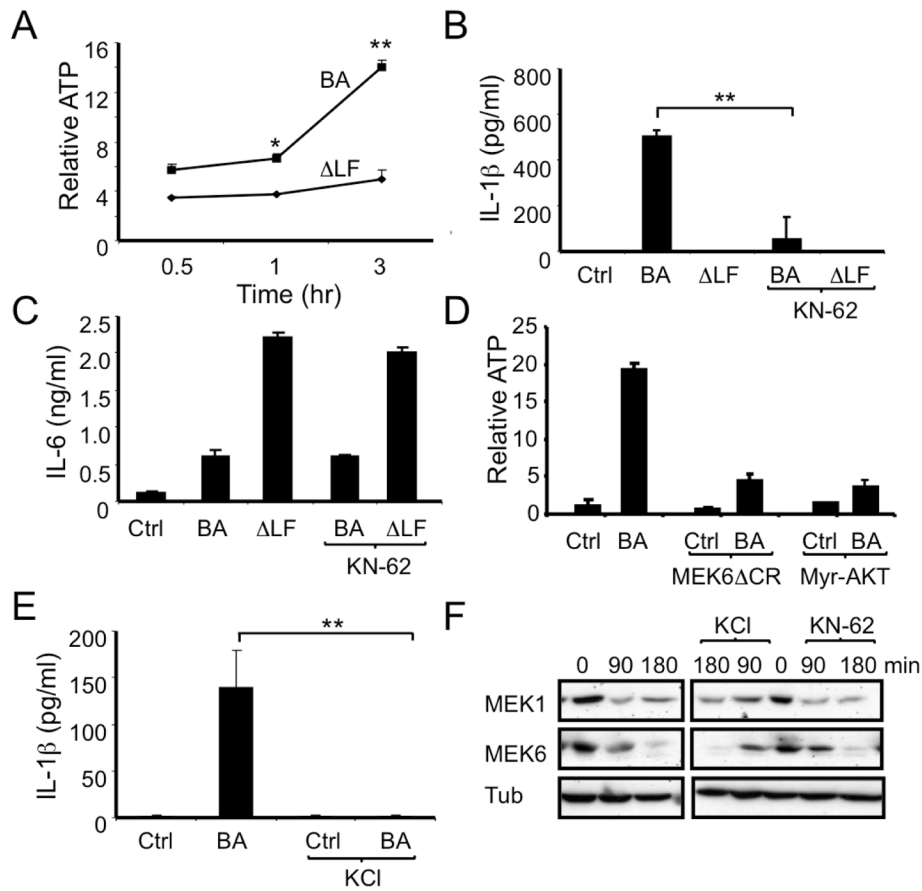


Figure 4. *B. anthracis* induces ATP release from infected macrophages in LF-, p38- and AKT-dependent manner

(A) *C57BL/6* macrophages were infected with WT (BA) or Δ LF *B. anthracis*. At the indicated times points, ATP in the culture supernatants was measured. This experiment was repeated at least 4 times and the results of one representative experiment done in triplicates are shown. Results are means \pm SD, * $p < 0.05$, ** $p < 0.01$ denote significant differences between the groups.

(B,C) Mouse macrophages were infected with WT (BA) or Δ LF *B. anthracis* in the absence or presence of KN-62 (10 μ M). After 8 hr, IL-1 β (B) and IL-6 (C) in culture supernatants were measured by ELISA. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown. Results are means \pm SD, ** $p < 0.01$ denote significant differences between the groups.

(D) RAW264.7 cells transfected with MEK6 Δ CR, myr-AKT or empty vector were left uninfected or infected with *B. anthracis* and 2 hr later ATP release to culture supernatants was measured. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown.

(E) *C57BL/6* macrophages were infected with WT *B. anthracis* in the absence or presence of 130 mM KCl and 8 hr later the amount of IL-1 β in culture supernatants was determined by ELISA. This experiment was repeated 3 times and the results of one representative experiment done in duplicates are shown. Results are means \pm SD, ** $p < 0.01$ denote significant differences between the groups.

(F) Mouse macrophages were infected with WT *B. anthracis* in the absence or presence of KN-62 (10 μ M) or KCl (130 mM) and at the indicated times cell lysates were prepared and analyzed by immunoblotting of 2 separate gels from the same experiment for MEK

proteolysis. This experiment was repeated 3 times and the results of one representative experiment are shown.

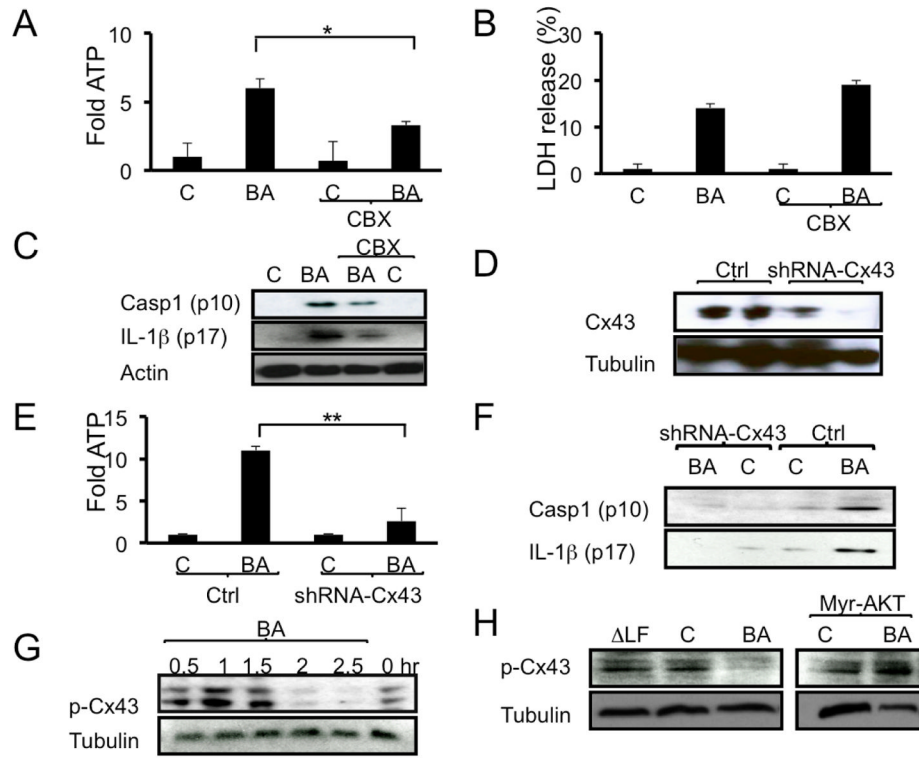


Figure 5. *B. anthracis*-induced ATP release and inflammasome activation depend on connexin-43 (A-C) *C57BL/6* macrophages were infected with *B. anthracis* as above and 45 min later the connexin-43 inhibitor carbenoxolone (CBX, 2.5 μM) was added to the culture medium. ATP release was measured 1 hr later (A) and LDH release was measured after 2 hr (B). Secretion of active caspase-1 (p10) and IL-1β (p17) to the culture medium was examined 12 hr after CBX addition. Cellular actin was used as a control. This experiment was repeated 3 times and the results of one representative experiment are shown.

(D) RAW264.7 cells were transduced with lentiviruses expressing connexin-43 shRNA or control shRNA (Ctrl.). Connexin-43 expression in the transduced cells was analyzed by immunoblotting. This experiment was repeated 2 times and the results of one representative experiment are shown.

(E,F) RAW264.7 cells were transduced with lentiviruses expressing connexin-43 shRNA or control shRNA. Transduced cells were left uninfected or infected with *B. anthracis*. ATP release was measured 2 hr later (E). Caspase-1 (p10) and IL-1β (p17) secretion was examined after 12 hr (F). This experiment was repeated twice and the results of one representative experiment are shown. Results are means ± SD, **p < 0.01 denote significant differences between the groups.

(G) *C57BL/6* macrophages were treated as above and at the indicated times cell lysates were prepared and analyzed by immunoblotting for phosphorylation of connexin-43 at Ser279 and Ser282. This experiment was repeated 3 times and the results of one representative experiment are shown.

(H) Myr-AKT-expressing or parental RAW264.7 cells were infected as above. After 2 hr, cell lysates were prepared and analyzed by immunoblotting for connexin-43 phosphorylation and tubulin content. This experiment was repeated 3 times and the results of one representative experiment are shown.

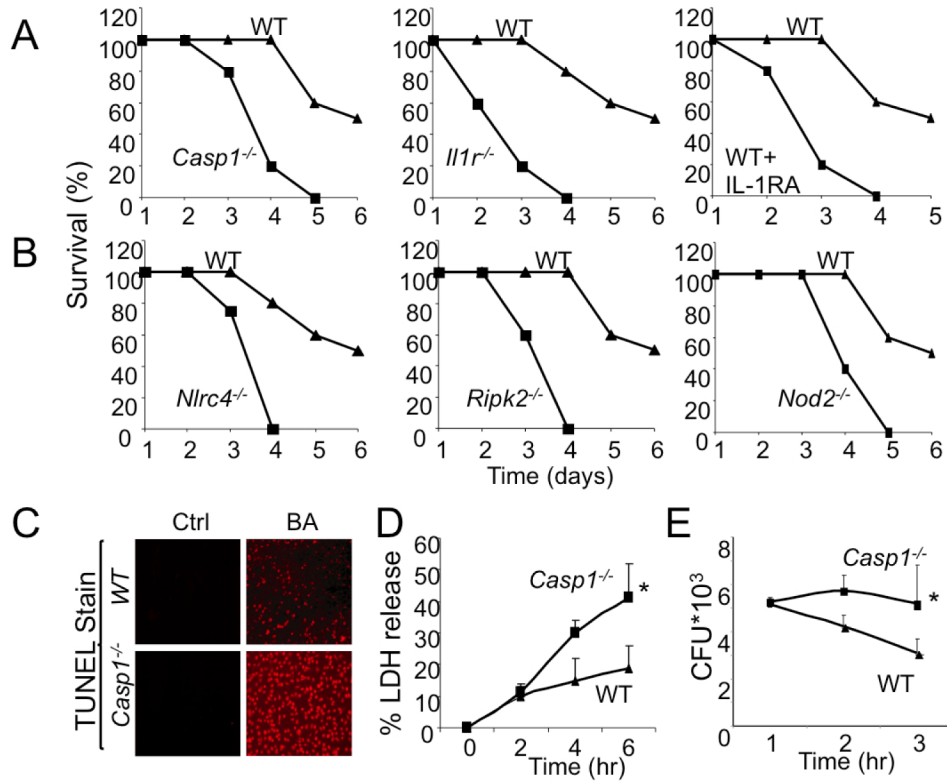


Figure 6. Inflammation protects mice from *B. anthracis* infection

(A,B) Mice of the indicated genotypes were infected with WT *B. anthracis* (2×10^6 cfu/mouse given i.p., n=5–8 mice per group). Where indicated, Anakinra (Kineret, IL-1RA, 100 mg/kg) was s.c. injected every 6 hr over a period of 24 hr post-infection. Mouse survival was analyzed over the course of 6 days post-infection. This experiment was repeated 3 times and the results of one representative experiment are shown.

(C) WT and *Casp1*^{-/-} mice were infected as above and 48 hr later their spleens were collected and examined for cell death by a TUNEL assay. The experiment was repeated twice with similar results.

(D) Macrophages from WT and *Casp1*^{-/-} mice were infected with WT *B. anthracis* and at the indicated times, LDH release was measured. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown. Results are means \pm SD, *p < 0.05 denote significant differences between the groups.

(E) Macrophages from WT and *Casp1*^{-/-} mice were infected with WT *B. anthracis* and at the indicated times intracellular bacterial counts were determined. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown. Results are means \pm SD, *p < 0.05 denote significant differences between the groups.