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Critical role for the AIM2 inflammasome during acute central nervous system bacterial infection

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Abstract

Interleukin-1 β (IL-1 β) is essential for eliciting protective immunity during the acute phase of *Staphylococcus aureus* (*S. aureus*) infection in the central nervous system (CNS). We previously demonstrated that microglial IL-1 β production in response to live *S. aureus* is mediated through the Nod-like receptor protein 3 (NLRP3) inflammasome, including the adapter protein ASC (apoptosis-associated, speck-like, caspase-1 recruiting domain-containing protein), and pro-caspase-1. Here we utilized NLRP3, ASC, and caspase-1/11 knockout (KO) mice to demonstrate the functional significance of inflammasome activity during CNS *S. aureus* infection. ASC and caspase-1/11 KO animals were exquisitely sensitive, with approximately 50% of mice succumbing to infection within 24 h. Unexpectedly, the survival of NLRP3 KO mice was similar to WT animals, suggesting the involvement of an alternative upstream sensor, which was later identified as absent in melanoma 2 (AIM2) based on the similar disease patterns between AIM2 and ASC KO mice. Besides IL-1 β , other key inflammatory mediators, including IL-6, CXCL1, CXCL10, and CCL2 were significantly reduced in the CNS of AIM2 and ASC KO mice, implicating autocrine/paracrine actions of IL-1 β , since these mediators do not require inflammasome processing for secretion. These studies demonstrate a novel role for the AIM2 inflammasome as a critical molecular platform for regulating IL-1 β release and survival during acute CNS *S. aureus* infection.

Keywords

Inflammasome; interleukin-1; AIM2; ASC; caspase-1; *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a serious human health threat because of its ability to cause a wide array of diseases, ranging from skin and soft tissue infections to more life-

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threatening conditions such as sepsis, endocarditis, and brain abscess (Lowy 1998). Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a frequent cause of hospital- and community-associated infections, which complicates treatment (Drago *et al.* 2007) and MRSA isolates have been recovered from brain abscesses (David *et al.* 2012, Naesens *et al.* 2009, Sifri *et al.* 2007, Arora *et al.* 2012). Brain abscesses can originate from many sources, including: 1) sub-acute or chronic otitis media, mastoiditis, frontal or ethmoid sinusitis, or dental infection; 2) hematogenous dissemination from peripheral sites of infection, including bacterial endocarditis or lung abscesses (Wadhwa *et al.* 2012, Kassis *et al.* 2010, Madsen 1983); 3) immunocompromised patients with HIV infection, organ transplantation, chemotherapy, or steroid usage (Young & McGwire 2005, Muzumdar *et al.* 2011); 4) penetrating trauma to the calvarium; or 5) infectious complications following neurosurgical procedures. Without timely treatment intervention, a brain abscess can rupture, which is associated with a high mortality rate. Therefore, elucidating mechanisms of protection as well as pathophysiology elicited by *S. aureus* after CNS colonization may unveil novel therapeutic targets.

Numerous proinflammatory cytokines are elicited during acute CNS *S. aureus* infection, including IL-1 β , IL-6, and TNF- α (Xiong *et al.* 2012, Garg *et al.* 2009, Kielian *et al.* 2007b, Kielian *et al.* 2004, Stenzel *et al.* 2005). Our prior studies utilizing IL-1 β and IL-1RI KO mice demonstrated a critical role for IL-1 signaling during early brain abscess pathogenesis, as both mouse strains displayed marked susceptibility to infection and elevated bacterial burdens compared to WT animals (Kielian *et al.* 2004, Xiong *et al.* 2012). Our recent report showed that microglial IL-1 β production in response to live *S. aureus* was mediated by the NLRP3 inflammasome (Hanamsagar *et al.* 2011), which has been studied extensively for its role in IL-1 β secretion in response to diverse pathogenic signals, including *S. aureus* α -hemolysin (Craven *et al.* 2009). However, little is known about the functional importance of inflammasomes during CNS bacterial infection, with only one report documenting that IL-1 β release during *S. pneumoniae* meningitis is controlled via the NLRP3 inflammasome (Hoegen *et al.* 2011). To further dissect the underlying mechanisms mediating IL-1 β release during early CNS infection, we focused on the NLRP3 inflammasome utilizing a well-established mouse model of *S. aureus*-induced brain abscess that recapitulates various aspects of human disease. We found that ASC and caspase-1/11 were critical for survival during the first 24 h after infection, whereas NLRP3 was dispensable, revealing the involvement of a NLRP3-independent, ASC-dependent inflammasome during acute CNS *S. aureus* infection. In addition to NLRP3, other ASC-dependent inflammasomes have been described including AIM2, NLRC4, and NLRP6 (Broz *et al.* 2010, Elinav *et al.* 2011, Rathinam *et al.* 2010, Hornung *et al.* 2009, Fernandes-Alnemri *et al.* 2009). When considering alternative inflammasomes that could play a role during CNS *S. aureus* infection, AIM2 seemed the most plausible since intracellular survival of *S. aureus* has been reported in phagocytes enabling the detection of cytoplasmic dsDNA, whereas NLRC4 and NLRP6 recognize motifs that are unrelated to *S. aureus* (Watanabe *et al.* 2007, Gresham *et al.* 2000). Subsequent experiments identified this upstream adaptor as AIM2, which was unexpected, since prior to our study, AIM2 had been implicated in sensing dsDNA in macrophages following viral or *L. monocytogenes* infection (Rathinam *et al.* 2010, Fernandes-Alnemri *et al.* 2010, Warren *et al.* 2010). To our knowledge, this is the first study

to examine the functional role of inflammasome components during CNS *S. aureus* infection and reveals a unique role for AIM2 in *S. aureus* detection in the brain.

MATERIALS AND METHODS

Animals

NLRP3 and ASC KO mice were generously provided by Dr. Vishva Dixit (Genentech, San Francisco, CA) and AIM2 and caspase-1/11 KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All KO mice were on a C57BL/6 background. Age- and sex-matched C57BL/6 mice were used as WT (wild type) controls. Studies were performed with mice between 8–12 weeks of age.

Experimental brain abscess model

Brain abscesses were induced in mice as previously described by the intracerebral injection of a MRSA USA300 isolate (CAV1002) recovered from an otherwise healthy individual who succumbed to a brain abscess (Kielian *et al.* 2007a, Sifri *et al.* 2007, Kielian *et al.* 2001). The animal use protocol, approved by the University of Nebraska Medical Center Animal Care and Use Committee, is in accord with the National Institutes of Health guidelines for the use of rodents. This study was performed in accordance with the ARRIVE guidelines.

Quantitation of bacterial titers and proinflammatory mediator expression

Mice were euthanized at the indicated intervals after infection and perfused transcardially with ice-cold PBS. Brain abscesses were dissected within 1–2 mm of the lesion margins and immediately disrupted in 500 μ l of homogenization buffer [1X PBS supplemented with a protease inhibitor cocktail tablet (Roche, Mannheim, Germany) and RNase inhibitor (Promega, Madison, WI)]. Serial 10-fold dilutions of brain abscess homogenates were plated on trypticase soy-agar (TSA) plates supplemented with 5% sheep blood (Hemostat Laboratories, Dixon CA) to determine bacterial titers (expressed as $\log_{10}$ CFU/g wet tissue weight). To compare proinflammatory mediator expression profiles between KO versus WT mice, a multi-analyte microbead array was utilized according to the manufacturer's instructions (MILLIPLEX; Millipore, Billerica, MA) that detects IL-1 α , IL-1 β , TNF- α , IFN- γ , IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, CXCL1, CXCL2, CXCL9, CXCL10, CCL2, CCL3, CCL4 and CCL5. Results were analyzed using a Bio-Plex workstation (Bio-Rad, Hercules, CA) and adjusted based on the amount of total protein extracted from brain abscess homogenates for normalization using a colorimetric Bio-Rad DC Protein Assay kit (Bio-Rad). IL-1 β was also measured by sandwich ELISA (BD OptiEIA, San Diego, CA).

Statistical analyses

Significant differences between experimental groups were determined by one-way ANOVA followed by Pairwise Multiple Comparison (Tukey Test) or a Student's *t*-test to compare between two groups. For both analyses, $p < 0.05$ was considered statistically significant.

RESULTS

ASC but not NLRP3 KO mice show enhanced susceptibility to CNS *S. aureus* infection

Our recent study revealed that the NLRP3 inflammasome was critical for microglial IL-1 β secretion in response to live *S. aureus* (Hanamsagar et al. 2011). To determine whether these findings extended to *in vivo* infection, NLRP3 and ASC KO mice were challenged intracerebrally with *S. aureus* and disease progression was monitored. Survival rates between NLRP3 KO and WT mice were not significantly different (Fig 1A), whereas ASC KO animals displayed increased susceptibility to CNS *S. aureus* infection, evidenced by a 50% reduction in survival within the first 24 h compared with WT mice (Fig 1B). This was unexpected, as numerous studies have reported that NLRP3 and ASC KO mice display comparable phenotypes in several disease models (Gris *et al.* 2010, Jha *et al.* 2010, Zaki *et al.* 2010, Hoegen et al. 2011). To eliminate potential confounds from survival bias, ASC KO animals were evaluated at 12 and 18 h post-infection in all subsequent experiments, whereas NLRP3 KO animals were assessed at 3 and 7 days. NLRP3 KO mice were examined at later intervals, since early experiments did not reveal any significant differences in survival or inflammation at earlier time points (data not shown). Interestingly, bacterial burdens were similar between ASC KO and WT mice at both 12 and 18 h after infection (Fig 1E), demonstrating that the enhanced susceptibility of ASC KO mice was not attributable to unchecked bacterial growth. Likewise, *S. aureus* burdens were similar between NLRP3 KO and WT animals at day 3 after infection; however, bacterial clearance was impaired in NLRP3 KO mice at later intervals (i.e. day 7; Fig 1D). The dichotomy between NLRP3 and ASC KO phenotypes suggested either the involvement of an alternative NLR sensor that is ASC-dependent or that ASC assumes a non-canonical role during acute CNS *S. aureus* infection. The former possibility appeared more plausible, since caspase-1/11 KO animals displayed a similar sensitivity to CNS *S. aureus* infection as ASC KO mice, with no changes observed in bacterial burdens (Fig 1C & F).

ASC loss has a broad impact on proinflammatory mediator production in the infected CNS

Based on the profound sensitivity of ASC KO mice to CNS *S. aureus* infection, we next evaluated whether this translated into altered proinflammatory mediator expression profiles. As expected, IL-1 β production was significantly attenuated in ASC KO animals (Fig 2A). In addition, several other cytokines and chemokines, including IL-6, CCL2 and CXCL10 were also significantly reduced in ASC KO mice (Fig 2B–D). This was intriguing, since these mediators do not rely on inflammasome processing for their secretion, suggesting that IL-1 β influences cytokine/chemokine production in an autocrine/paracrine manner during acute CNS *S. aureus* infection. Interestingly, IL-18 expression was equivalent between ASC KO and WT mice at all intervals examined (data not shown), corroborating our prior microglial data where IL-18 was processed in an inflammasome-independent manner (Hanamsagar et al. 2011). Other cytokines, such as IL-10, TNF- α , and IFN- γ , and innate immune cell infiltrates (i.e. neutrophils, monocytes, and macrophages) were not significantly different between WT and ASC KO animals at either time point examined (data not shown).

AIM2 is a critical inflammasome sensor during acute *S. aureus* CNS infection

Based on the observed dichotomy in survival patterns between NLRP3 KO versus ASC and caspase-1/11 KO mice following CNS *S. aureus* infection, the involvement of an alternative ASC-dependent inflammasome sensor was investigated. Several inflammasomes including AIM2, NLRC4, and NLRP6 are known to recruit ASC, leading to downstream caspase-1 activation (Hornung et al. 2009, Broz et al. 2010, Elinav et al. 2011). Based on its ability to recognize bacterial dsDNA in the cytoplasm of infected cells and the fact that intracellular survival of *S. aureus* has been reported (Hornung et al. 2009, Fernandes-Alnemri et al. 2010, Kim et al. 2010, Watanabe et al. 2007, Gresham et al. 2000), we examined disease pathogenesis in AIM2 KO mice. Interestingly, AIM2 KO animals showed enhanced susceptibility to *S. aureus* infection in a manner that was similar to ASC and caspase-1/11 KO animals (Fig 3A), suggesting that AIM2 is a critical molecule during acute *S. aureus* infection in the brain. In addition, the expression of the same proinflammatory mediators that were decreased in ASC KO mice (Fig 2) were also significantly inhibited in AIM2 KO animals (Fig 3C–F), providing further support for an AIM2/ASC pathway in sensing CNS *S. aureus* infection. To our knowledge, this is the first report implicating AIM2 in protective immunity to *S. aureus* as well as its action within the CNS in any disease model.

DISCUSSION

We previously established a pivotal role for the NLRP3 inflammasome in mediating IL-1 β release from microglia in response to live *S. aureus* (Hanamsagar et al. 2011). In the current report, we have extended these findings to identify the molecular composition of the inflammasome that is critical for protection during acute CNS bacterial infection *in vivo*. Prior work from our laboratory demonstrated that IL-1RI KO mice succumb to CNS *S. aureus* infection within the first 24 h following bacterial exposure (Xiong et al. 2012); therefore, we predicted that similar results would be observed for both NLRP3 and ASC KO animals, since this inflammasome platform has the largest range of molecular triggers leading to IL-1 β processing and release. Unexpectedly, ASC, but not NLRP3 KO mice were exquisitely sensitive to CNS *S. aureus* infection. The observed disconnect between ASC and NLRP3 phenotypes differs from most of the current inflammasome literature (Gris et al. 2010, Jha et al. 2010, Zaki et al. 2010, Hoegen et al. 2011), although divergent involvement of NLRP3 versus ASC has been reported in models of experimental autoimmune encephalomyelitis and bacterial pneumonia (Shaw et al. 2010, van Lieshout et al. 2013). Differential requirements for NLRP3 versus ASC in eliciting IL-1 β production following *S. aureus* infection could be one explanation, since cytokine levels were significantly reduced in ASC KO mice but not NLRP3 KO animals (data not shown). The inability to elicit wild type IL-1 β production in ASC KO mice functionally equates with the inability of the cytokine to signal via the IL-1RI, which agrees with the finding that both ASC KO and IL-1RI KO animals in our previous study (Xiong et al. 2012) displayed a similar heightened sensitivity to CNS *S. aureus* infection, with approximately 33–50% of mice succumbing within the first 24 h. Collectively, these findings highlight the essential role of IL-1 signaling for protection after acute CNS bacterial exposure. IL-1 β exerts numerous functions within the CNS, including modulation of blood-brain barrier (BBB) permeability, activation of resident glia, and indirectly affecting leukocyte recruitment by augmenting

chemokine and adhesion molecule expression at the BBB (Gosselin & Rivest 2007). In the context of CNS *S. aureus* infection, IL-1 β is critical for survival and bacterial containment, and plays an indirect role in dictating leukocyte influx by augmenting chemokine expression (Xiong et al. 2012, Kielian et al. 2004). However, we did not detect any significant differences in peripheral leukocyte infiltrates or bacterial burdens between WT and ASC or AIM2 KO mice in this study, which may result from the combined actions of IL-1 β and IL-1 α loss in IL-1RI KO animals in our previous report (Xiong et al. 2012), since both cytokines have overlapping biological activities. In addition, it is likely that IL-1 β exerts beneficial effects during acute CNS infection that remain to be defined. A similar protective role for IL-1 β has been reported in West Nile encephalitis (Ramos *et al.* 2012). Despite the marked reduction in IL-1 β in ASC KO mice, residual cytokine remained, suggesting that pro-IL-1 β may also be processed by currently unknown alternative mechanisms, albeit relatively minor. For example, caspase-1 may be activated via other inflammasomes responsive to *S. aureus*-related triggers or DAMPs released from necrotic cells (Davis *et al.* 2011, Willingham *et al.* 2009, Lamkanfi *et al.* 2010). Alternatively, other caspases or enzymes (i.e. cathepsins) may be involved in pro-IL-1 β processing (and potentially IL-18 cleavage) in this model (Terada *et al.* 2010).

One unexpected finding during our *in vivo* studies was the distinct outcomes in NLRP3 and ASC KO mice following CNS *S. aureus* infection, which differs from other inflammatory models that generally reported similar phenotypes in both strains (Gris et al. 2010, Jha et al. 2010, Zaki et al. 2010, Hoegen et al. 2011). This raised the possibility that alternative ASC-dependent inflammasome sensor(s) were likely upstream effectors for triggering IL-1 β release and host survival during CNS *S. aureus* infection. Other inflammasomes known to recruit ASC for caspase-1 activation include AIM2, NLRC4, and NLRP6 (Hornung et al. 2009, Broz et al. 2010, Elinav et al. 2011). The AIM2 inflammasome senses cytosolic dsDNA following bacterial or viral infections (Hornung et al. 2009). Phagocytosis and intracellular killing of *S. aureus* by host cells may lead to the release of bacterial DNA into the cytoplasm and subsequent AIM2 inflammasome activation. Alternatively, numerous reports have demonstrated intracellular survival of *S. aureus* (Watanabe et al. 2007, Gresham et al. 2000), where the organism may be capable of triggering cytosolic AIM2. Indeed, here we show that AIM2 KO mice displayed a phenotype that closely paralleled that of ASC KO animals in terms of survival after infection and impaired proinflammatory mediator production. Collectively, these findings reveal a novel role for AIM2, and not the NLRP3 inflammasome, for host survival following *S. aureus* infection in the CNS. However, another layer of complexity is that AIM2 can also act independent of caspase-1, by recruiting caspase-8 and inducing caspase-8-dependent apoptosis, as shown in a study of *Francisella* infection (Pierini *et al.* 2012). In addition, several inflammasomes, including NLRP3, AIM2, and NLRC4 are known to act cooperatively, likely as an evolutionary mechanism to ensure maximal pathogen eradication or as a means to add multiple layers of regulatory control over cytokine secretion (Kim et al. 2010, Wu *et al.* 2010, Coll & O'Neill 2011, McCoy *et al.* 2010). Therefore, it is possible that the concerted action of several ASC-dependent inflammasomes is elicited following CNS *S. aureus* infection, though this remains to be determined. Assessing the cellular localization and spatial distribution patterns

of these inflammasome molecules may provide insights into their role in disease pathogenesis.

Although we predict that AIM2 and ASC are primarily active in microglia during acute CNS infection, it remains possible that astrocytes and/or infiltrating monocytes/macrophages may also play a role. However, unpublished data from our laboratory show that highly purified astrocytes do not secrete IL-1 β in response to live *S. aureus* challenge *in vitro*. It is possible that in an *in vivo* setting, astrocytes may interact with microglia to secrete IL-1 β , which is supported by a recent report (Holm *et al.* 2012); however, any such release might be small and insignificant in comparison with microglia. Macrophages and monocytes express both AIM2 and ASC (Fernandes-Alnemri *et al.* 2009, Rathinam *et al.* 2010, Mariathasan *et al.* 2005) and are activated during bacterial infection. However, we previously showed that CNS intrinsic cells were critical for the initial sensing of CNS *S. aureus* infection (Garg *et al.* 2009) and the rapid kinetics of susceptibility of AIM2 and ASC KO mice to intracerebral *S. aureus* challenge suggests the involvement of CNS intrinsic sensors of infection. Therefore, although it is possible that the lack of AIM2 or ASC in infiltrating monocytes/macrophages is involved in regulating IL-1 β release and infection outcome at later stages in the disease process, the sum of currently available evidence points to a key role for CNS intrinsic cells during the early phase of infection. However, this prediction remains to be tested and would require the use of Cre-specific mice to ablate AIM2 or ASC expression in astrocytes or microglia/macrophages combined with the use of radiation bone marrow chimeras to discriminate between the involvement of the latter two populations.

Reactive oxygen species (ROS) have been shown to be an important upstream activator of the NLRP3 inflammasome in response to numerous stimuli (Zhou *et al.* 2011, Martinon 2010). However, we have previously demonstrated that ROS does not induce IL-1 β secretion in the context of live *S. aureus* challenge in microglia (Hanamsagar *et al.* 2011). Similarly, a recent study has shown that mycobacterium-induced AIM2 inflammasome activation is not ROS-dependent (Yang *et al.* 2013). Therefore, we do not anticipate a role of ROS in the observed pathology of AIM2 or ASC KO mice reported here; however, this remains to be examined.

In conclusion, our studies reveal the existence of a complex regulatory network for inflammasome activation and cytokine secretion during CNS *S. aureus* infection that is primarily AIM2/ASC-dependent and NLRP3-independent. To our knowledge, a role for the AIM2 inflammasome during *S. aureus* infection either in the CNS or the periphery has not yet been demonstrated. Although sustained inflammation following CNS infection can be detrimental, it is clear that the early release of proinflammatory cytokines, such as IL-1 β , is essential for host survival. Therefore, it will be critical to design future studies to understand the temporal aspects of inflammasome function to develop therapeutic interventions that are safe and effective based on the nature of the insult.

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Figure 1. The inflammasome molecules ASC and caspase-1/11, but not NLRP3, are critical for survival during acute CNS *S. aureus* infection

C57BL/6 WT and NLRP3 KO (A and D), ASC KO (B and E), or caspase-1/11 KO (C and F) mice were injected intracranially with 10^4 cfu of *S. aureus* USA300 and assessed for survival (A–C; $n=7$ –16/group from 2–6 independent experiments). Bacterial burdens from NLRP3 KO mice (D) were assessed at days 3 and 7, whereas ASC and caspase-1/11 KO mice (E and F, respectively) were determined at 12 and 18 h after infection, to avoid potential confounds from survival bias ($n=7$ –12/group). Significant differences in survival and bacterial burdens between groups were determined by a log-rank test and Student's *t*-test, respectively (*, $p < 0.05$; **, $p < 0.01$).

Figure 2. ASC loss affects several inflammatory mediators besides IL-1 β

C57BL/6 WT and ASC KO mice (7–8/group) were injected intracranially with 10^4 cfu of *S. aureus* USA300, whereupon abscess homogenates were collected at the indicated time points after infection for quantitation of IL-1 β (A), IL-6 (B), CCL2 (C), and CXCL10 (D) expression by MILLIPLEX multi-analyte bead arrays. Results were normalized to total protein content for each sample and significant differences in mediator expression were determined by Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Figure 3. AIM2 is critical for survival and inflammatory mediator induction during acute CNS infection

C57BL/6 WT and AIM2 KO mice (7–8/group) were injected intracranially with 10^4 cfu of *S. aureus* USA300, whereupon survival (A), bacterial burdens (B), and IL-1 β (C), IL-6 (D), CCL2 (E), and CXCL10 (F) levels in abscess homogenates were quantified by MILLIPLEX multi-analyte bead arrays. Results were normalized to total protein content for each sample and significant differences in mediator expression were determined by Student's *t*-test (*, $p < 0.05$).

Figure 4. The AIM2 inflammasome is protective during acute CNS *S. aureus* infection

A disconnect in phenotypes between the inflammasome sensor NLRP3 and its adaptor ASC during acute CNS *S. aureus* infection led to the discovery of AIM2 as a critical inflammasome sensor. The AIM2 inflammasome is potentially triggered by dsDNA in cells harboring intracellular *S. aureus*, leading to ASC and caspase-1 recruitment, resulting in pro-IL-1 β processing and cytokine secretion. This cascade, in turn, is protective to the host during acute infection that is independent of controlling bacterial burdens. The NLRP3 inflammasome is also activated in response to *S. aureus* challenge by α -hemolysin (*hla*); however, it is not critical for host survival. Furthermore, the cross-talk between AIM2 and NLRP3 inflammasomes is also a possibility as described in recent studies (Wu et al. 2010, Kim et al. 2010). ASC also regulates the production of other inflammatory mediators, presumably via indirect effects mediated by IL-1 β action.