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Cytosolic LPS-induced caspase-11 oligomerization and activation is regulated by extended synaptotagmin 1

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SUMMARY

Caspase-11 (Casp-11) is known to induce pyroptosis and defends against cytosol-invading bacterial pathogens, but its regulation remains poorly defined. Here, we identified extended synaptotagmin 1 (E-Syt1), an endoplasmic reticulum protein, as a key regulator of Casp-11 oligomerization and activation. Macrophages lacking E-Syt1 exhibited reduced production of interleukin-1β (IL-1β) and impaired pyroptosis upon cytosolic lipopolysaccharide (LPS) delivery and cytosol-invasive bacterial infection. Moreover, cleavage of Casp-11 and its downstream substrate gasdermin D were significantly diminished in *ESyt1*$^{-/-}$ macrophages. Upon LPS stimulation, E-Syt1 underwent oligomerization and bound to the p30 domain of Casp-11 via its synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain. E-Syt1 oligomerization and its interaction with Casp-11 facilitated Casp-11 oligomerization and activation. Notably, *ESyt1*$^{-/-}$ mice were susceptible to infection by cytosol-invading bacteria *Burkholderia thailandensis* while being resistant to LPS-induced endotoxemia. These findings collectively suggest that E-Syt1 may serve as a platform for Casp-11 oligomerization and activation upon cytosolic LPS sensing.

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AUTHOR CONTRIBUTIONS

Y.M. designed and performed most experiments and analyzed the data; R.Z. and Q.T. prepared BMDMs and performed some of the immunoblot assays; H.G. helped Y.M. with BMDM electroporation and data analysis; W.Y.L. and W.L. edited the manuscript; Jun Zhang and Jian Zhang conceived and supervised the research and analyzed data; Y.M. and Jian Zhang wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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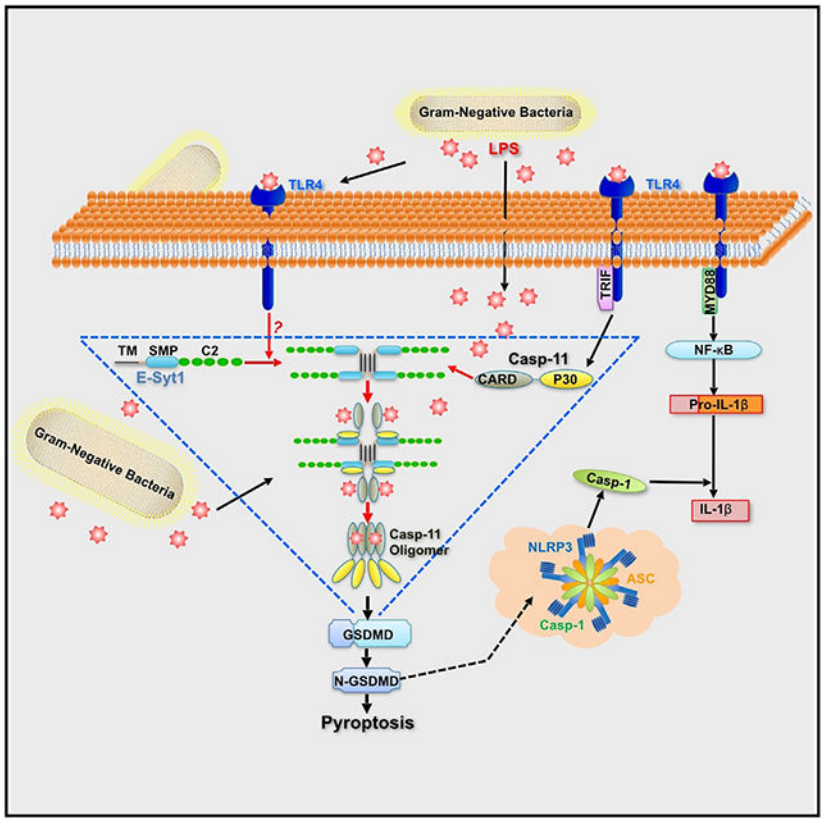
Graphical Abstract



In brief

Ma et al. discovered that E-Syt1 oligomerizes upon LPS stimulation and interacts with Casp-11's p30 domain through its SMP domain. E-Syt1 oligomerization and its interaction with Casp-11 promotes Casp-11 oligomerization and cleavage, suggesting E-Syt1 as a platform for Casp-11 activation upon cytosolic LPS sensing.

INTRODUCTION

Surveillance of lipopolysaccharide (LPS), a key component of the outer membrane of gram-negative bacteria, plays crucial roles both in host defense against infection and immunopathology.[1] Caspase-11 (Casp-11; Casp-4 and −5 in humans) has recently been recognized as an intracellular receptor for cytosolic LPS.[1] Upon sensing of LPS in innate immune cells by its cytosolic receptor, Casp-11/Casp-4, a higher-order structure is assembled called the non-canonical inflammasome.[1,2] This results in Casp-11/Casp-4 clustering, dimerization/oligomerization of the catalytic subunits, and acquisition of protease function, leading to cleavage-mediated activation of GSDMD (gasdermin D).[2-4] The resultant N-terminal GSDMD fragment, GSDMD-N, binds to membrane phospholipids and stimulates oligomerization-mediated pore formation in the plasma membrane, resulting in its permeabilization and lytic cell death (pyroptosis), as well as release of lactate dehydrogenase (LDH) and the mature forms of interleukin-1β (IL-1β) and IL-18.[5,6] While

Casp-11 has been shown to play a vital role in host defense against lethal infections of gram-negative bacteria that are able to invade the cytosol,[7,8] dysregulated Casp-11 activation can contribute to septic shock and mortality. Indeed, it was reported that Casp-11 promotes disease severity and reduces survival time during severe endotoxemia.[9,10] Thus, although Casp-11-dependent non-canonical inflammasome activation appears to be a prerequisite for a competent immune response, it can also result in severe side effects. However, the mechanisms for regulation of Casp-11-dependent non-canonical inflammasome activation are largely unknown.

E-Syt1 (extended synaptotagmin 1), one member of the E-Syt family (E-Syt1, E-Syt2, and E-Syt3), is an endoplasmic reticulum (ER) protein composed of an N-terminal transmembrane (TM) region, a central SMP (synaptotagmin-like mitochondrial lipid-binding protein) domain, and five C2 domains.[11,12] E-Syt1 binds to lycerophospholipids in a barrel-like domain and is involved in cellular lipid transport.[13,14] It also binds to calcium (via the C2 domains) and translocates to ER-cell membrane contact sites in response to elevated cytosolic calcium levels.[13] It has been documented that E-Syt1 facilitates the tethering of ER to the cell membrane and the formation of appositions.[15] Although the membrane-tethering-independent role of E-Syts in activation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in T cells has been reported before,[16] their role in innate immunity is completely unknown.

In this study, we investigated the role of E-Syt1 in inflammasome activation. Strikingly, macrophages lacking E-Syt1 produced less IL-1β and underwent less pyroptosis upon cytosolic LPS sensing and cytosol-invading gram-negative bacterial infection. These findings suggest that E-Syt1 regulates Casp-11-mediated non-canonical inflammasome activation. Mechanistically, we observed that E-Syt1 undergoes self-oligomerization and binds to Casp-11, promoting Casp-11 oligomerization and activation. Our data collectively indicate that E-Syt1 may serve as a platform for facilitating Casp-11 oligomerization and activation via recruiting and assembling Casp-11 monomers. This event is crucial for Casp-11-mediated innate immune responses against intracellular gram-negative bacterial infections and endotoxemia.

RESULTS

E-Syt1 is required for Casp-11-mediated non-canonical inflammasome activation

As a member of the ER-localized protein family, the role of E-Syt-1 in ER-plasma membrane (PM) tethering and lipid transfer has been clearly dissected in the past decades.[13,14] To determine the role of E-Syt1 in regulating canonical and non-canonical inflammasome activation, we stimulated LPS-primed bone marrow-derived macrophages (BMDMs) from wild-type (WT) and *ESyt1*$^{-/-}$ mice with (1) ATP and nigericin, which activate the NLRP3 inflammasome[17-20]; (2) cholera toxin B (CTB) and *EHEC,* which activate the non-canonical inflammasome[21]; (3) poly (dA:dT), which activates the AIM2 inflammasome[19,22]; or (4) flagellin, which activates the NLRC4 inflammasome.[19,23] While there was no difference in IL-1β production between WT and *ESyt1*$^{-/-}$ BMDMs upon stimulation with flagellin, poly (dA:dT), ATP, and nigericin, *ESyt1*$^{-/-}$ BMDMs showed significantly reduced IL-1β production in response to CTB/LPS stimulation and enterohemorrhagic *Escherichia coli* (*EHEC*) infection (Figures 1A and S1A), despite

comparable levels of tumor necrosis factor α (TNF-α) and IL-6 production under the same stimulation conditions (Figures S1C and S1D).

Sensing of cytosolic LPS by Casp-11 results in oligomerization and activation of Casp-11, which cleaves GSDMD to generate an N-terminal truncated GSDMD (N-GSDMD) fragment, eliciting pyroptosis.[4] We found that LPS-primed *ESyt1*$^{-/-}$ BMDMs displayed significant reduced pyroptosis, as revealed by decreased release of LDH in response to *EHEC* infection or CTB/LPS stimulation (Figure 1B), and showed no difference in pyroptosis caused by flagellin, poly (dA:dT), ATP, or nigericin stimulation (Figure S1B). Moreover, kinetic analysis of LDH and IL-1β release and GSDMD cleavage in BMDMs all showed that *ESyt1* deficiency significantly inhibited pyroptosis caused by *EHEC* infection or cytosolic LPS stimulation (Figures S1E-S1I). Meanwhile, we measured Casp-11, GSDMD, and Casp-1 cleavages in BMDMs induced by cytosolic LPS. Compared with LPS priming only, LPS priming plus CTB stimulation led to increased expression of Casp-11 p26 and N-GSDMD in cell lysates and Casp-1 p10 in the supernatant (Figure 1C). However, all of these were markedly diminished in *ESyt1*$^{-/-}$ BMDMs stimulated with LPS plus CTB (Figure 1C). All this suggests that E-Syt-1 regulates Casp-11-dependent non-canonical inflammasome activation. To confirm this, we directly delivered LPS into the cytosol of BMDMs by means of electroporation or lipofectamine transfection as described previously.[2] In response to cytosolic LPS delivered by electroporation or lipofectamine transfection, *ESyt1*$^{-/-}$ BMDMs underwent less pyroptosis and released less IL-1β than WT BMDMs (Figures 1A and 1B). Immunoblots showed that the expression of Casp-11 p26 and N-GSDMD in cell lysates and Casp-1 p10 in the supernatant was significantly lower in BMDMs from *ESyt1*$^{-/-}$ mice than that of WT mice at 2 h post-LPS electroporation (Figure 1D). The attenuated Casp-11 inflammasome activation observed in *ESyt1*$^{-/-}$ BMDMs was not due to reduced protein levels of Casp-11, given that there was no change in the expression of the pro-form of Casp-11 by BMDMs derived from *ESyt1*$^{-/-}$ mice in the presence or absence of LPS or CTB stimulation (Figure 1E). We then extended the study to verify whether E-Syt-1 is also required for human Casp-4 (homologous to mouse Casp-11)-mediated non-canonical inflammasome activation. To this end, we knocked down *ESyt1* expression in THP-1-derived macrophages by transfecting *ESyt1* small interfering RNA (siRNA). Upon delivering LPS into the cytosol by CTB, scramble siRNA-treated macrophages underwent pyroptosis, displayed apparent Casp-4 and GSDMD cleavage in cell lysates, and released IL-1β p17 and Casp-1 p20 in the supernatants (Figures 1F-1H). However, all of these responses were greatly inhibited in macrophages deficient in E-Syt-1 (Figures 1F-1H). Therefore, our data collectively indicated that E-Syt1 is required for Casp-4/Casp-11-mediated non-canonical inflammasome activation.

E-Syt1 potentiates Casp-4/Casp-11 oligomerization induced by cytosolic LPS sensing

Since the absence of E-Syt1 affects Casp-11 activation and E-Syt1 is a TM protein that binds to lipids, we hypothesized that E-Syt1 might be involved in the delivery of LPS into the cytosol. To investigate this, we primed WT and *ESyt1*$^{-/-}$ BMDMs with fluorescein isothiocyanate (FITC)-conjugated LPS, stimulated them with CTB, and analyzed the cytosolic localization of LPS by flow cytometry. Surprisingly, we found that the cytosolic localization of LPS was comparable between BMDMs with or without E-Syt1

(Figure 2A), suggesting that E-Syt1 is dispensable for LPS cytosolic delivery. To further confirm this, we extracted cytosol from BMDMs treated with LPS or CTB plus LPS. By subjecting the cytosol fraction (GAPDH) and the residual non-cytosolic fraction (EEA1 and E-cadherin) to LAL (limulus amebocyte lysate) assay, we observed similar amounts of LPS in the cytosol of both WT and *ESyt1*$^{-/-}$ BMDMs (Figure 2B). Subsequently, we aimed to determine whether LPS binding capacity to Casp-11, and the accessibility to Casp-11 by cytosolic LPS, is affected in the absence of E-Syt1. To address this, we incubated biotinylated LPS with lysates of WT and *ESyt1*$^{-/-}$ BMDMs primed with LPS or directly electroporated biotinylated LPS into LPS-primed BMDMs. The LPS-binding proteins were then affinity precipitated by avidin beads as described previously,[2] and the resulting precipitates were blotted using an antibody against Casp-11. We found that comparable amounts of pro-Casp-11 were detected in LPS pull-down products generated from WT and *ESyt1*$^{-/-}$ BMDMs (Figure 2C). Thus, our data indicated that E-Syt1 does not affect the binding of LPS to Casp-11.

Casp-11 oligomerization is essential for optimal Casp-11 activation.[2,24,25] To assess whether E-Syt1 affects Casp-11 oligomerization, we utilized disuccinimidyl suberate (DSS), a membrane-permeable protein cross-linking reagent, to stabilize protein oligomers.[26-28] LPS-primed BMDMs, LPS-primed BMDMs stimulated with LPS plus CTB or LPS directly delivered to the cytosol by electroporation, were treated with DSS and cell lysates were subjected to western-blot analysis. In WT BMDMs, delivery of LPS into the cytosol resulted in the appearance of distinct Casp-11 oligomer bands (representing dimers, tetramers, and octamers) with significantly higher molecular weights compared with the Casp-11 monomer (Figure 2D). Strikingly, E-Syt1 deficiency significantly reduced the Casp-11 oligomer formation in BMDMs following cytosolic LPS delivery, regardless of CTB stimulation or electroporation (Figure 2D). Consistently, N-GSDMD oligomer formation, which occurs subsequent to Casp-11 activation, was also attenuated in *ESyt1*$^{-/-}$ BMDMs treated with CTB plus LPS (Figure 2E). To confirm these findings, we transfected HEK293T cells with FLAG-tagged Casp-11 alone or in combination with Myc-tagged E-Syt1, followed by LPS electroporation and DSS treatment. Similar to the results observed in BMDMs, overexpression of E-Syt1 in HEK293T cells indeed potentiated Casp-11 oligomerization (Figure S2A). Co-expression of E-Syt1 with Casp-11 in HEK293T cells also enhanced its catalytic activity, as indicated by an increased level of Casp-11 p26 (Figure S2B). Furthermore, interfering with E-Syt-1 expression in human macrophages significantly inhibited cytosol LPS-induced Casp-4 oligomer formation (Figure 2F), similar to the observations made in mouse BMDMs. Therefore, the data described above demonstrated that E-Syt1 is required for cytosolic LPS-induced Casp-4/Casp-11 oligomerization, which is a prerequisite for their catalytic activity.

E-Syt1 SMP domain interacts with Casp-11 p30 domain and is required for Casp-11 cleavage

To determine the molecular interaction between E-Syt1 and Casp-11, we performed a biotinylated-LPS pull-down assay using WT and *Casp11*$^{-/-}$ BMDMs, as described in Figure 2B. The precipitated biotinylated LPS complexes were probed with an antibody against E-Syt1. Our findings revealed that biotinylated LPS pulled down not only Casp-11 but also

E-Syt1 in WT BMDMs when it was incubated with lysates of LPS-primed WT BMDMs receiving cytosolic LPS (Figure 3A). However, in BMDMs lacking Casp-11, E-Syt1 was not detected in the biotinylated LPS precipitates (Figure 3B), suggesting that E-Syt1 does not directly bind to LPS but instead interacts directly with Casp-11. To verify this interaction, we performed a co-immunoprecipitation assay. Lysates of LPS-primed BMDMs from WT and *ESyt1*$^{-/-}$ mice were immunoprecipitated with anti-Casp-11 and subsequently probed with anti-E-Syt1. As expected, E-Syt1 was observed to bind with Casp-11 (Figure 3C). Similarly, in THP-1-derived macrophages, a direct interaction of E-Syt-1 with inflammatory Casp-11/Casp-4 was evident (Figure 3D). To corroborate these findings, we transfected HEK293T cells with plasmids encoding Myc-tagged E-Syt1 and FLAG-tagged Casp-11. The cell lysates were immunoprecipitated with anti-Myc or anti-FLAG and subsequently blotted with anti-FLAG or anti-Myc antibodies. As depicted in Figure 3E, E-Syt1 indeed bound to Casp-11. Therefore, our data strongly support the conclusion that E-Syt1 directly interacts with Casp-11, establishing E-Syt1 as a bona fide binding partner of Casp-11.

It is well known that E-Syt1 is an ER-localized multidomain-containing protein consisting of a TM domain, an SMP domain, and five C2 domains (C2A–C2E)[11] (Figure 3F). The SMP domain is primarily responsible for lipid binding and transport and has the potential to interact with other proteins.[13,14] Among the five C2 domains, only the C2A and C2C domains possess calcium-binding capacity, while the C2E domain directly binds with membrane PI(4,5)P2.[11,13] To determine which domain of E-Syt1 is responsible for its binding to Casp-11, we generated a series of deletion mutants from the N terminus to the C terminus, tagged with Myc. These mutants were designated as Myc-E-Syt1-ΔTM, Myc-E-Syt1-C2(A–E), and Myc-E-Syt1-C2(B–E). When these full-length (FL) E-Syt1 constructs and the deletion mutants were individually transfected into HEK293T cells along with FLAG-tagged Casp-11, we observed that only FL E-Syt1 and E-Syt1-ΔTM, but not the E-Syt1 C2 domains, were able to bind to FLAG-tagged Casp-11. These results indicate that the SMP domain of E-Syt1 is required for its interaction with Casp-11 (Figure 3F).

Casp-11 belongs to a family of aspartate-specific cysteine proteases,[2,21,25] characterized by an N-terminal caspase recruitment domain (CARD) and a C-terminal catalytic domain (p30).[24,29] We then mapped the region(s) of Casp-11 that may interact with E-Syt1. To this end, we transfected HEK293T cells with FLAG-tagged Casp-11, the Casp-11 CARD domain, or Casp-11 p30, along with Myc-tagged E-Syt1. We found that FL Casp-11 and the Casp-11 p30 domain were capable of binding to E-Syt1, whereas the Casp-11 CARD domain did not show any interaction with E-Syt1 (Figure 3G). These results indicate that the interaction between E-Syt1 and Casp-11 occurs through the direct binding of the SMP domain of the E-Syt1 to the p30 domain of Casp-11. To confirm these findings, we transfected HEK293T cells with Myc-tagged E-Syt1-SMP and FLAG-tagged Casp-11 p30. Remarkably, we observed that the E-Syt1-SMP domain bound to FLAG-tagged Casp-11 p30 (Figure 3H), providing additional evidence that E-Syt1 interacts with Casp-11 through its SMP domain and the Casp-11 p30 domain.

Although we have established the interaction between E-Syt1 and Casp-11, it remains unknown whether this binding event potentiates Casp-11 activation. To this end, we transfected HEK293T cells with Myc-tagged E-Syt1 TM-SMP, E-Syt1-C2, or FL E-Syt1,

along with plasmids harboring FLAG-tagged Casp-11. Following LPS electroporation, we observed that both Myc-tagged FL E-Syt1 and the E-Syt1 TM-SMP domain construct facilitated the cleavage of pro-Casp-11 into Casp-11 p26, while the C2 domain had no impact on Casp-11 cleavage, unlike Casp-11 alone (Figure 3I). Furthermore, we revealed that the promotion of Casp-11 cleavage by E-Syt1 relied on the presence of the Casp-11 CARD domain, which is responsible for LPS sensing (Figure 3J). Collectively, these data strongly suggest that E-Syt1 promotes Casp-11 activation upon cytosolic LPS delivery through its interaction with Casp-11.

E-Syt1 oligomers are required for Casp-11 oligomerization and activation

Several previous studies have demonstrated that within the E-Syt family, E-Syt1, E-Syt2, and E-Syt3 could form heterodimers or homodimers,[11-14] but the physiological roles of E-Syt1 dimers, especially in immune responses, have not been investigated. To determine whether E-Syt1 undergoes oligomerization upon LPS stimulation, we stimulated WT BMDMs with LPS for varying durations and then treated them with DSS. Western blot analysis of cell lysates revealed higher molecular weight E-Syt1 proteins, potentially corresponding to tetramers (Figure 4A). Moreover, as the stimulation time with LPS increased and cross-linking by DSS occurred, the high molecular weights of E-Syt1 oligomers gradually increased in WT BMDMs, while the levels of E-Syt1 monomers decreased (Figure 4B). Based on the above results that E-Syt1 promoted Casp-11 activation via protein-to-protein interactions, we aimed to determine which form of E-Syt1 binds to Casp-11 and whether this interaction is crucial for Casp-11 oligomerization and activation. To address this, we primed WT BMDMs with LPS, stimulated them with CTB, treated them with DSS, and lysed the cells. The resulting cell lysates were immunoprecipitated with anti-Casp-11 and then probed with anti-E-Syt1. We observed that not only E-Syt1 monomers but also its oligomers were present in Casp-11 immunoprecipitates (Figure 4C). This indicates that the binding of E-Syt1 to Casp-11 can lead to oligomerization and that these E-Syt1 oligomers may serve as a means to recruit Casp-11 in the cytosol.

To determine the biological significance of E-Syt1 oligomers, we used FLAG-tagged FL E-Syt1 and three E-Syt1 deletion mutants (E-Syt1-ΔTM, E-Syt1-C2(A–E) and E-Syt1-C2(B–E)). When these FLAG-tagged deletion mutants were transfected into HEK293T cells along with Myc-tagged FL E-Syt1, we found that only FLAG-tagged FL E-Syt1 could bind to Myc-tagged FL E-Syt1 (Figure 4D), suggesting that the E-Syt1 TM domain is required for E-Syt1 oligomerization. As E-Syt1 markedly enhances Casp-11 cleavage when they were co-expressed in HEK293T cells compared with Casp-11 alone, we then tested whether E-Syt1 oligomerization is essential for Casp-11 activation. As shown in Figure 4E, the Casp-11 p26 fragment was completely abolished in HEK293T cells expressing the E-Syt1-ΔTM, despite its ability to bind to Casp-11. To further verify the crucial role of the E-Syt1 TM domain in non-canonical inflammasome activation, we carried out a rescue experiment by transfecting Myc-tagged E-Syt1, E-Syt1-ΔTM, E-Syt1-C2(A–E), and E-Syt1-TM-SMP into *ESyt1*$^{-/-}$ BMDMs using electroporation. Empty vector-transfected WT BMDM and *ESyt1*$^{-/-}$ BMDMs served as controls. Upon LPS plus CTB stimulation, only E-Syt1 and the E-Syt1 TM-SMP domain, but not E-Syt1ΔTM or E-Syt1-C2(A–E), efficiently restored Casp-11 cleavage, oligomerization, and GSDMD cleavage, comparable to that observed in

WT BMDMs transfected with empty vector (Figures 4F and 4G). Consistent with these data, the FLAG-tagged E-Syt1 TM-SMP domain bound to Myc-tagged E-Syt1 and the E-Syt1-TM-SMP domain when overexpressed in HEK293T cells, indicating that the E-Syt1 TM-SMP domain alone is sufficient for its oligomerization (Figure 4H). These results also ruled out the possibility that the Casp-11 activation facilitated by E-Syt1 is dependent on calcium regulation, even though cytosolic calcium flux increases Casp-11 activation and E-Syt1 C2A and C2C domains have high calcium-binding affinity.[11,13] Hence, the E-Syt1 TM domain, which mediates E-Syt1 oligomerization, is crucial for Casp-11 oligomerization and activation when LPS is present in the cytosol.

E-Syt1 deficiency protects mice from endotoxemia

After establishing the essential role of E-Syt1 in Casp-11 oligomerization and activation, we sought to verify its relevance in an *in vivo* setting. To this end, we performed experiments to assess the role of E-Syt1 in an endotoxemia model that has been developed to assess the non-canonical inflammasome *in vivo.*[9,10,30] Mice were initially primed by intraperitoneal injection of poly (I:C) and subsequently rechallenged with LPS to induce endotoxemia. Consistent with previous reports,[30] poly (I:C)-primed WT mice rapidly succumbed within 20 h after LPS challenge, while 75% of *Casp-11*$^{-/-}$ mice were resistant to LPS challenge, confirming the idea that Casp-11 activation is essential for cytosolic LPS-induced endotoxic shock. Consistent with the observed critical role of E-Syt-1 in Casp-11 activation, 37% of *ESyt1*$^{-/-}$ mice survived lethal endotoxemia (Figure 5A). To investigate the underlining inflammatory response in these mice, we measured serum levels of IL-1α, IL-1β, TNF-α, and IL-6 at 4 h post-LPS challenge, as previously reported.[30] Similar to *Casp11*$^{-/-}$ mice, *ESyt1*$^{-/-}$ mice produced significantly lower levels of all four cytokines analyzed (Figures 5B-5E). Therefore, the absence of E-Syt1 protects mice from lethal endotoxemia, likely attributed to the inhibition of inflammatory cytokines subsequent to non-canonical Casp-11 inflammasome activation.

E-Syt1 protects mice against *B. thailandensis* infection

The inflammasome could discriminate pathogens from non-pathogens by detecting cytosolic contamination or perturbation of the cytosolic compartment.[1] *Burkholderia pseudomallei,* which is closely related to the far less virulent *B. thailandensis,* is a gram-negative bacterium endemic to South Asia that causes melioidosis and is a potential biologic weapon. *B. pseudomallei* uses a type 3 secretion system (T3SS) to escape from the phagosome and to replicate in the cytosol, thereby activating Casp-11 and enhancing clearance of the bacterium *in vivo.*[7] To further determine whether E-Syt-1 is essential for Casp-11-mediated host responses *in vivo,* we intraperitoneally challenged WT mice, *ESyt1*$^{-/-}$ mice, and *Casp-11*$^{-/-}$ mice with *B. thailandensis,* as previously reported.[7] While all WT mice survived the challenge, all *Casp11*$^{-/-}$ mice died of infection within 2 days, and 50% of *ESyt1*$^{-/-}$ mice succumbed to infection during the first 3 days (Figure 6A). This suggests that E-Syt1 may protect mice against *B. thailandensis* infection via facilitating Casp-11 activation. When infected with *B. thailandensis,* WT BMDMs displayed markedly increased IL-1β production and LDH release, both of which were inhibited by E-Syt1 and Casp-11 deficiency (Figures 6B and 6C). Consistent with these findings, although infection of WT BMDMs with *B. thailandensis* induced robust Casp-11 inflammasome activation (as evidenced by Casp-11

and GSDMD cleavage in cell lysates and Casp-1 p10 release in the supernatant), GSDMD cleavage subsequent to Casp-11 activation was almost abolished in Casp-11-deficient cells and markedly inhibited in E-Syt1-deficient cells (Figure 6D). To exclude the possibility that other functions of E-Syt1 my complicate the observed effects of E-Syt1 in our *in vivo* study, we used *P. aeruginosa* (*Pseudomonas aeruginosa*) infection, a well-known pathogen that specifically activate NLRC4 inflammasome, as a negative control. As expected, we found that knockout of either E-Syt1 or Casp-11 did not show any impact on the survival rate of *P. aeruginosa*-infected mice compared with that of WT mice (Figure S3A). Accordingly, *in vitro* studies showed that BMDMs from WT and *ESyt1*$^{-/-}$ mice release comparable amounts of LDH and IL-1β when infected with *P. aeruginosa* (Figures S3B and S3C). Therefore, E-Syt1 protects mice against *B. thailandensis* infection by facilitating Casp-11 activation, which is essential for the clearance of *B. thailandensis*.

DISCUSSION

The sensing of LPS by the CARD domain of Casp-11 (human Casp-4 and Casp-5) induces its oligomerization and activation, which cleaves GSDMD to generate N-GSDMD, thus eliciting pyroptosis.[3,4,25] It has been shown that Casp-4/Casp-11 undergoes oligomerization upon binding to the LPS LPA domain, resulting in its activation in a cell-free system.[2] However, in view of the complex environment inside cells, how Casp-11 oligomerization is organized, or whether Casp-11 monomers are assembled onto a complex prior to or after accessing LPS, similar to other members of Casp family, remains unknown. In this study, we demonstrated that E-Syt1 binds to Casp-11 via the interaction between its SMP and Casp-11 p30 domains and promoted Casp-11 activation in a manner dependent on this interaction. Mechanistically, loss of E-Syt1 in BMDMs or knockdown of *ESyt1* in human macrophages greatly inhibited Casp-11/Casp-4 oligomer formation, respectively, even in the presence of cytosolic LPS. The enhancing effect of E-Syt1 on Casp-11 oligomerization and proteolysis is also reproduced by co-transfecting 293T cells with E-Syt1 and Casp-11 and subjecting the cells to LPS electroporation. It should be noted that basal levels of Casp-11 oligomers and cleavage are still observed in *ESyt1*$^{-/-}$ BMDMs. This may be attributed to the increased chances of interaction among Casp-11 monomers facilitated by the abundant expression of Casp-11 following LPS priming, which shortens the space gap between the monomers. This observation was also reflected in the fact that insect-cell-purified Casp-11 or overexpressed Casp-11 only in 293T cells underwent oligomerization and proteolysis without accessing LPS.[2,4]

It is well demonstrated that all three members of the E-Syt family are capable of forming homodimers, heterodimers, or heterotrimers. However, the physiologic roles of these E-Syt dimers and trimers have never been investigated. Here, we found that, E-Syt1 oligomers are readily detected in cell lysates of BMDMs treated with DSS and that their levels increased with continuous LPS stimulation, indicating their potential immunologic roles. A series of biochemical and rescue studies proved that the TM domain of E-Syt1 is not only essential for E-Syt1 oligomerization itself but also critical for Casp-11 oligomerization and activation. This finding indicates that E-Syt1 oligomers are responsible for its role in facilitating Casp-11 oligomerization and activation. Consistent with this finding, reconstituting E-Syt1-deficient BMDMs with the E-Syt1 TM-SMP domain alone was as effective as using FL

E-Syt1 in restoring Casp-11 oligomerization and activation. Taken together, we propose that LPS priming, as the prerequisite for Casp-11 activation, not only up-regulates Casp-11 expression but also increases E-Syt1 oligomer formation, which may facilitate Casp-11 oligomer formation via their direct interaction. Once LPS is internalized into the cytosol, or endocytosed bacteria shedding LPS into the cytosol, the aggregated Casp-11 by E-Syt1 oligomers may have a higher chance to oligomerize upon binding to LPS through its CARD domain. Importantly, the increased E-Syt1 oligomer formation upon LPS priming is not due to an increase in E-Syt1 proteins levels since we did not observe any change in E-Syt1 expression upon LPS stimulation (Figure 1E). Future studies will focus on the critical signaling pathway engaged by LPS priming that regulates E-Syt1 oligomer formation or whether the engagement of other pattern recognition receptors (PRRs) could also regulate E-Syt1 oligomerization. Understanding the underlying mechanisms may provide valuable insights for the development of strategies to modulate Casp-11 activation induced by cytosolic LPS.

E-Syt1 contains 5 C2 domains at its C terminal, and the binding of calcium by its C2A and/or C2C domain is known to be responsible for its structural transformation and lipid transfer function.[11,13,14] Recently, calcium influx caused by bacteria infection was reported to be an upstream regulator of Casp-11 activation in a mouse sepsis model.[31] However, in our study, we found that E-Syt1 oligomer formation and its role in facilitating Casp-11 activation is independent of calcium, as demonstrated by the ability of the E-Syt1 TM-SMP domain alone to form oligomers and restore Casp-11 oligomerization in BMDMs lacking E-Syt1.

E-Syt1 is an ER-anchored protein that shares high homology with E-Syt2 and E-Syt3.[11] However, we did not observe E-Syt2 and E-Syt3 in immunoprecipitates directed by antibody against Casp-11. In addition, double knockout of *ESyt1* and *ESyt2* or triple knockout of all three *ESyt* family members did not provide additional improvements in the survival rate of mice with endotoxic shock compared with mice with *ESyt1* single knockout (Figure S4). These findings suggest that E-Syt2 and E-Syt3 are unlikely to be involved in regulating Casp-11-mediated non-canonical inflammasome activation. One previous report showed that Casp-4 (human homolog of mouse Casp-11) localized to the ER and underwent cleavage in cells treated with ER stress-inducing reagent.[32] It is highly possible that Casp-11 may also localize to the ER once it binds with E-Syt1, an ER-anchored protein. But the exact subcellular localization of Casp-11 and whether it assembles on the ER require further investigation in future studies.

As with other ER-localized proteins, the role and mechanism of E-Syt family members in regulating ER-PM tethering have been extensively studied in the past decades.[11-14] Although knockdown of the three E-Syt family members impaired the Ca^{2+}-induced increase of ER-PM appositions in cell lines, mice deficient in these three proteins do not show any defects under normal conditions.[11] Here, for the first time, we investigated the *in vivo* roles of E-Syt1 in inflammatory disease using *ESyt1*$^{-/-}$ mice. We identified its contribution to two Casp-11-dependent disease models, namely LPS endotoxemia and *B. thailandensis* infection. Our mechanistic study further verified the critical role of E-Syt1 in regulating Casp-11 inflammasome activation in BMDMs in response to cytosolic LPS

and *B. thailandensis* infection. However, knockout of E-Syt1 in BMDMs was incapable of completely abolishing Casp-11 oligomerization and GSDMD cleavage. This observation aligns with the apparent difference in survival between *ESyt1*$^{-/-}$ mice and *Casp-11*$^{-/-}$ mice, not only in the endotoxemia model but also in the *B. thailandensis* infection model. These findings imply that other, unknown factors may independently or cooperatively regulate Casp-11 oligomerization and activation in response to its stimulants, in addition to E-Syt1.

In conclusion, we have identified E-Syt1 as a key molecule controlling Casp-4/Casp-11 oligomer formation and activation, which is essential for Casp-4/Casp-11-mediated innate immune responses. To our knowledge, this is a novel mechanism by which Casp-4/Casp-11 oligomerization and activation is regulated. Therefore, targeting E-Syt1 may offer a therapeutic approach for addressing aberrant Casp-4/Casp-11-mediated responses in host defense against cytosolic gram-negative bacterial infections and sepsis.

Limitations of the study

Our current study has revealed that E-Syt1 oligomer formation plays a critical role in regulating Casp-11 oligomerization and activation in response to cytosolic LPS. However, there are still a few limitations in this work that should be acknowledged. First, we found that the SMP and TM domains of E-Syt1 are both required for its activity, but the mechanistic coordination between these two domains in promoting Casp-11 activation remains unclear. Secondly, while we have proved the interaction between the E-Syt1 SMP domain and the Casp-11 p30 domain, the specific binding site between these two proteins needs to be identified. Additionally, it should be noted that although we demonstrated the positive regulatory role of E-Syt1 on Casp-4 activation, THP-1-derived macrophages may not be the optimal cell model for studying human-specific processes.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests regarding resources and reagents should be directed to lead contact, Yilei Ma (3316045@zju.edu.cn).

Materials availability—The recombinant DNA generated in this study are available from the lead contact upon request (subject to their availability at the time of request).

Data and code availability—All data reported in this study are available from the lead contact upon request.

This paper dose not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice—*C57BL/6J* and *ESyt1*$^{-/-}$*ESyt2*$^{-/-}$*ESyt3*$^{-/-}$ (stock number:030196) mice were purchased from the Jackson Laboratory. We then crossed *ESyt1*$^{-/-}$*ESyt2*$^{-/-}$*ESyt3*$^{-/-}$

mice with *C57BL/6J to generate ESyt1*$^{+/-}$*ESyt2*$^{+/-}$*ESyt3*$^{+/-}$ mice. *ESyt1*$^{-/-}$ and *ESyt1*$^{-/-}$ *ESyt2*$^{-/-}$ mice were generated from the off-springs of *ESyt1*$^{+/-}$*ESyt2*$^{+/-}$*ESyt3*$^{+/-}$ mice. *Casp-11*$^{-/-}$ mice[25] were purchased from the Jackson Laboratory.

All mice were 8–12 weeks of age when used, and both male and female mice were used in this study. All animal experimentation involving LPS injection was approved by the Institutional Animal Care and Use Committees of the University of Iowa.

Cell culture—Human THP-1 cells and 293T cells were cultured for in RPMI-1640 medium containing 10% FBS. Primary BMDMs were cultured in DMEM containing 10% FBS.

Bacteria—The growth conditions for *EHEC* (700927 strain) and *P.aeruginosa* (PAO1) were described previously.[31] *Burkholderia thailandensis* (700388 strain) was purchased from ATCC and cultured in LB broth at 37°C.

METHOD DETAILS

Plasmids and transfection—PCMV plasmids carrying Myc-tagged E-Syt1, E-Syt2 and E-Syt3 were kindly provided by Dr. Pietro De Camilli at Yale University via MTA. PCMV plasmid carrying flag-tagged Casp-11(pCMV-flag-Caspase-11) was purchased from Addgene. E-Syt1 truncated fragments and Casp-11 truncated fragments were PCR-amplified using PrimeSTAR HS DNA Polymerase (R010A, Takara), and then ligated into PCMV6 plasmids carrying a flag or Myc tag using Gibson Assembly Master Mix (E2611s, NEB). HEK293T cells were transfected with various plasmids by calcium precipitation.

***ESyt1*$^{-/-}$ BMDM reconstitution—**To assay the sufficiency of FL-E-Syt1 and its series of deletions in restoring Casp-11 activation in *ESyt1*$^{-/-}$ BMDMs, we electroporated 5 μg of empty PCMV vectors, Myc-FL-E-Syt1 and its deletions (Esyt1-Δ-TM, E-Syt1-C2(A-E) and E-Syt1-TM-SMP into *ESyt1*$^{-/-}$ BMDMs ($2.5x10^6$) using P2 Primary Cell 4D-Nucleofector X Kit (Lonza) according to the manufacturer's instruction, while electroporating 5ug of empty PCMV vectors into WT BMDMs as a control.

Generation of BMDMs and activation of inflammasomes—Bone marrow (BM) cells from WT mice and various mouse strains were harvested from the femurs and tibias of mice. Cells were cultured in DMEM containing 10% FBS and 30% conditioned medium from L929 cells expressing M-CSF. After 1 week of culture, non-adherent cells were removed, and adherent cells were 80%–90% $F4/80^+CD11b^+$as determined by flow cytometric analysis. LPS-primed BMDMs were infected with *EHEC* (MOI = 25:1), or *P. aeruginosa* (MOI = 30:1) for 1.5 h and then cultured in 100 μg/mL gentamycin. LPS-primed BMDMs were infected with *Burkholderia thailandensis* (MOI = 50:1) for 1 h and then cultured in 300 μg/mL kanamycin for another 3 h. Other stimulations included ATP (2.5 mM, 30 min), CTB (20 μg/mL, 16 h), nigericin (20 μM, 3 h). Poly (dA:dT) (1 μg/10^6 cells, 6 h) and LPS (2.5ug/10^6 cells) were transfected using Lipofectamine (Invitrogen). Flagellin (2 μg/10^6 cells, 4 h) were transfected using DOTAP liposomal transfection reagent (144189731, Sigma-Aldrich). In some experiments, 1 μg of LPS was directly delivered into the cytosol of HEK293T cells or LPS-primed BMDMs by electroporation to activate

Casp-11 non-canonical inflammasome, using P2 Primary Cell 4D-Nucleofector X Kit (Lonza) according to manufacturer's instruction. Two hours post LPS electroporation, the cells were collected and lysed for Western-blot studies.

Generation of human macrophages, silencing of the *ESyt1* gene, and activation of Casp-4 inflammasome—Human THP-1 cell-derived macrophages were generated as following: THP-1 (2×10^6 cells/ml) cell were cultured for 5 days in RPMI-1640 medium containing 200 ng/mL phorbol 12-myristate-13-acetate (PMA) at 37°C. On day 5, the differentiated macrophages were transfected with control siRNA or human *ESyt1* siRNA (80 nM) (Assay ID:523606; ThermoFisher) by using Lipofectamine RNAiMAX Transfection Reagent (13778030, ThermomFisher). After 36 h, the macrophages were washed, primed with LPS (500 ng/mL), and then stimulated CTB (20μg/ml) plus LPS (1 μg/ml) for 16 h.

Isolation of cytosol fraction from BMDMs—BMDMs cytosol extraction was conducted by a digitonin-based fractionation method. Briefly, 4×10^6 BMDMs primed with LPS or stimulated with CTB (20μg/ml) plus LPS (1ug/ml) were washed with sterile cold PBS 4 times. Cells were subsequently treated with 300 μL of 0.005% digitonin extraction buffer for 8 min and the supernatant containing cytosol was collected. Cytosol and residual fractions were subjected to LAL assay for LPS quantification. Additionally the fractions were immunoblotted for EEA1, E-Cadherin, E-Syt1 and GAPDH to confirm the purity of cytosol fraction.

ELISA and cell death assay—Cell culture supernatants and serum were assayed by ELISA for IL-1β, IL-1α, IL-6, and TNF-α. Pyroptosis was measured by testing LDH released into supernatant using Cytotoxicity Detection KitPLUS (LDH) kit (04744926001, Sigma-Aldrich).

LPS-induced endotoxemia—Various groups of mice at 8–12 weeks of age were injected i.p. with low dose of Poly I:C (10 mg/kg) (P1530, Sigma). Six hours later, the primed mice were challenged with LPS (5 mg/kg; E. coli 0111:B4) and monitored every 4 h. To determine the serum IL-1β, IL-1α, IL-6, and TNF-α levels, serum was collected at 4h after LPS injection.

***B. thailandensis* and *P.aeruginosa* infection—**For *B. thailandensis* infection, various groups of mice at 8–12 weeks of age were injected i.p. with 2×10^7 cfu of *B. thailandensis* (overnight cultured) as previously reported,[7] and the survival of the mice was monitored two times a day for consecutive 10 days. For *P.aeruginosa,* various groups of mice at 8–12 weeks of age were infected by intrachracheal injected with 5×10^6 cfu of *P.aeruginosa* (PAO1) (overnight cultured), and survival of infected mice was followed every 12 h over a period of 4 days.

Immunoprecipitation—For Co-IP assays of the interaction between E-Syt1 and Casp-11, WT BMDMs and *ESyt1*$^{-/-}$ BMDMs were primed with LPS (500 ng/mL) for 5 h. Cells were lysed in 0.5% NP-40 lysis buffer. The cell lysates were immunoprecipitated with anti-Casp-11 (1:100) and blotted with anti-E-Syt1 (1:1000). For Co-IP assays of the interaction

between E-Syt1 and Casp-4, THP-1 derived macrophages were stimulated with LPS (500 ng/mL, 5 h) or not. Cells were lysed in 0.5% NP-40 lysis buffer. The cell lysates were immunoprecipitated with anti-Casp-4 (1:100) and blotted with anti-E-Syt1 (1:1000). To map the interaction domains between E-Syt1 and Casp-11, HEK293T cells were transfected with Myc-tagged full-length E-Syt1 or its various deletions, and Flag-tagged full-length Casp-11 or its deletions. The cell lysates were immunoprecipitated with anti-Myc (1:100) and blotted with anti-Flag (1:1000) or, alternatively, immunoprecipitated with anti-flag (1:100) and blotted with anti-Myc (1:1000).

Oligomer detection—To detect caspase-11/caspase-4 oligomer or E-syt1 oligomer using SDS-PAGE and Western-blot, DSS (disuccinimidyl suberate) (A39267; Thermo Fisher), which is non-cleavable and membrane permeable crosslinker, was used to stabilize the oligomers preformed inside cells. After the indicated stimulation above, BMDMs ($4x10^6$) or THP-1 derived macrophages were collected and washed with ice-cold 1xPBS for at least three times and then the cross-linking was performed by treating the cells with 500 μM DSS for 30 min at room temperature. The reaction was quenched by addition of Tris-HCI (pH 7.5) to 100 mM final concentration. Before lysing by 0.5% SDS lysis buffer, the cells were washed three times using ice-cold 1xPBS. The lysates were analyzed by 4–15% protein gels (4561084, Bio-Rad) and Western-blot.

Cell supernatant protein concentration—For Western-Blot assay of extracellular caspase-1, caspase-11 or IL-1β, FBS-free cell supernatants were collected and the proteins released by cells were concentrated as following: 500 μL methanol and 125μL chloroform were added into 500μL of cell supernatants, and then vigorously mixed. After centrifugation (13000 rpm, 5mins), the upper clear liquid was removed, leaving the white pellet untouched. At this time, 500 μL of methanol was added and the white pellet was blown and dispersed into small pieces. With another round of centrifugation (13000 rpm, 5 min), the white pellet was left for air drying at room temperature until no visible liquid was evident. Finally, 1xSDS loading buffer was added to the dry proteins and heated for 10 min at 100°C, followed by immunoblotting analyses.

Biotin-LPS pull-down—To assay LPS binding to Casp-11 in cell lysates, BMDMs ($1x10^7$) from WT and E-Syt1 KO mice were primed with LPS. Cells were collected and lysed in a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA and 1% Triton X-100 supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals) for 30 min. After centrifugation (13000rpm, 30mins), the supernatants were collected and incubated with 4 μg of biotin-conjugated LPS (0111:B4; InvivoGen) and 40 μL of streptavidin-sepharose beads (#20347; Thermo Fisher) at 4°C with constant rotation overnight. The precipitates were washed three times with lysis buffer before they were eluted in 1xSDS loading buffer. To assay the role of E-Syt1 on LPS binding to endogenous Casp-11, biotin-conjugated LPS was directly electroporated into LPS-primed BMDMs ($2x10^7$) from WT and E-Syt1 KO mice. The cells were collected and washed three times with ice-cold 1xPBS. The pre-cleared cell lysates were then incubated with streptavidin Sepharose beads at 4°C with constant rotation overnight. The precipitates were then washed and eluted in 1xSDS sample buffer followed by immunoblotting analyses.

QUANTIFICATION AND STATISTICAL ANALYSIS

ELISA data of cytokines and LDH level were analyzed by using the Student's t test. Survival data of mouse the endotoxemia model and *B. thailandensis* infection model were analyzed by using the Kaplan-Meier log-rank test. Differences were considered significant at $p < 0.05$. No animals were excluded from the analysis. Mice were allocated to experimental groups based on their genotypes and were randomized within their sex- and age-matched groups. No statistical method was used to predetermine sample size. It was assumed that normal variance occurred between the experimental groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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Highlights

- E-Syt1 is required for Casp-11 oligomerization and cleavage
- E-Syt1 SMP domain interacts with Casp-11's p30 domain
- E-Syt1's TM domain mediates its oligomerization upon LPS stimulation
- Both E-Syt1 TM domain and SMP domain are essential for promoting Casp-11 cleavage

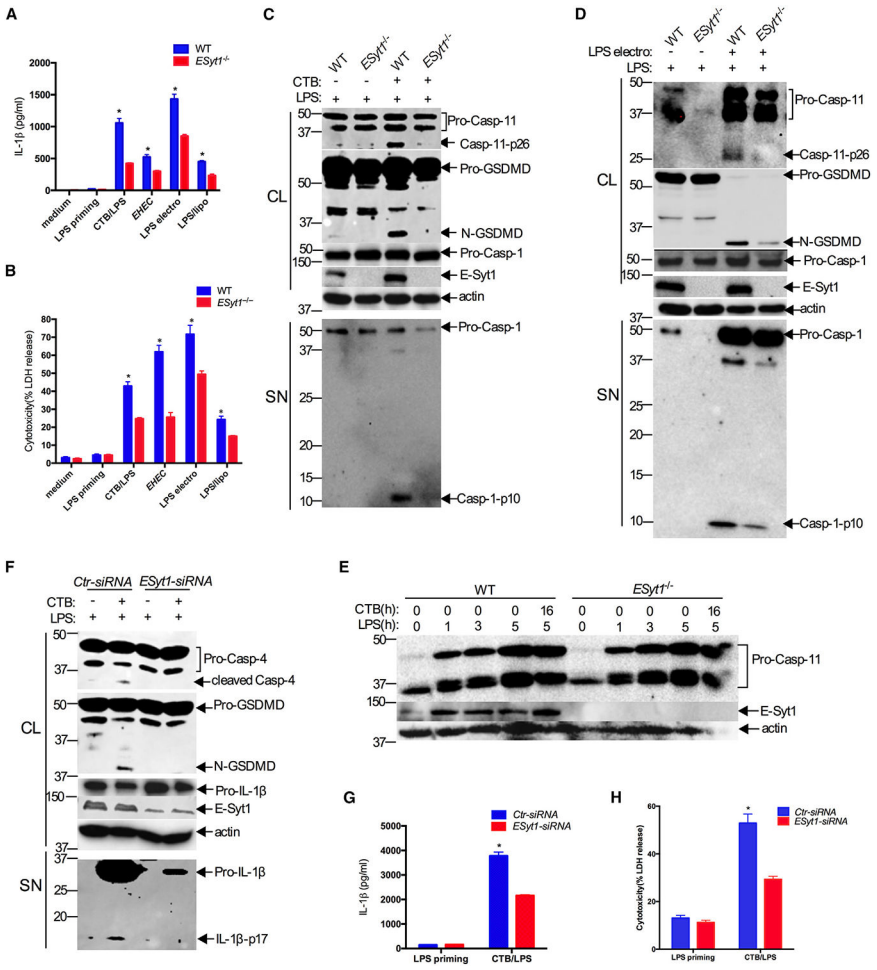

Figure 1. E-Syt1 is required for Casp-11 mediated non-canonical inflammasome activation

(A and B) IL-1β and lactate dehydrogenase (LDH) release by LPS-primed BMDMs from WT and *ESyt1*$^{-/-}$ mice stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) for 16 h, infected with *EHEC* (MOI = 25:1) for 12 h, or transfected with LPS by electroporation or lipofectamine.

(C) Immunoblots of pro-Casp-11, Casp-11-p26, E-Syt1, pro-Casp-1, pro-GSDMD, and N-GSDMD expression in cell extracts and pro-Casp-1 and Casp-1-p10 in the supernatants of LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) for 16 h. CL, cell lysate; SN, supernatants.

(D) Immunoblots of pro-Casp-11, Casp-11-p26, E-Syt1, pro-Casp-1, pro-GSDMD, and N-GSDMD expression in cell extracts and pro-Casp-1 and Casp-1-p10 in the supernatants of LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs transfected with LPS by electroporation.

(E) Immunoblots of pro-Casp-11 and E-Syt1 expression in cell extracts of WT and *ESyt1*$^{-/-}$ BMDMs primed with LPS (500 ng/mL) for the indicated times (1, 3, and 5h) or stimulated with CTB (20 μg/mL) for 16 h. Macrophages differentiated from THP-1 (2×10^6 cells/mL) cells by PMA (200 ng/mL) stimulation were transfected with control siRNA or human *ESyt1* siRNA (80 nM).

(F) Immunoblots of Casp-4, E-Syt1, pro-IL-1β, pro-GSDMD, and N-GSDMD expression in cell extracts and pro-IL-1β and IL-1β-p17.

(G and H) IL-1β and LDH release in the supernatants of macrophages primed with LPS (500 ng/mL) and then stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) for 16 h. CL, cell lysate; SN, supernatants.

Data are shown as mean ± SD. Data are representative of three independent experiments. *$p < 0.05$; Student's t test.

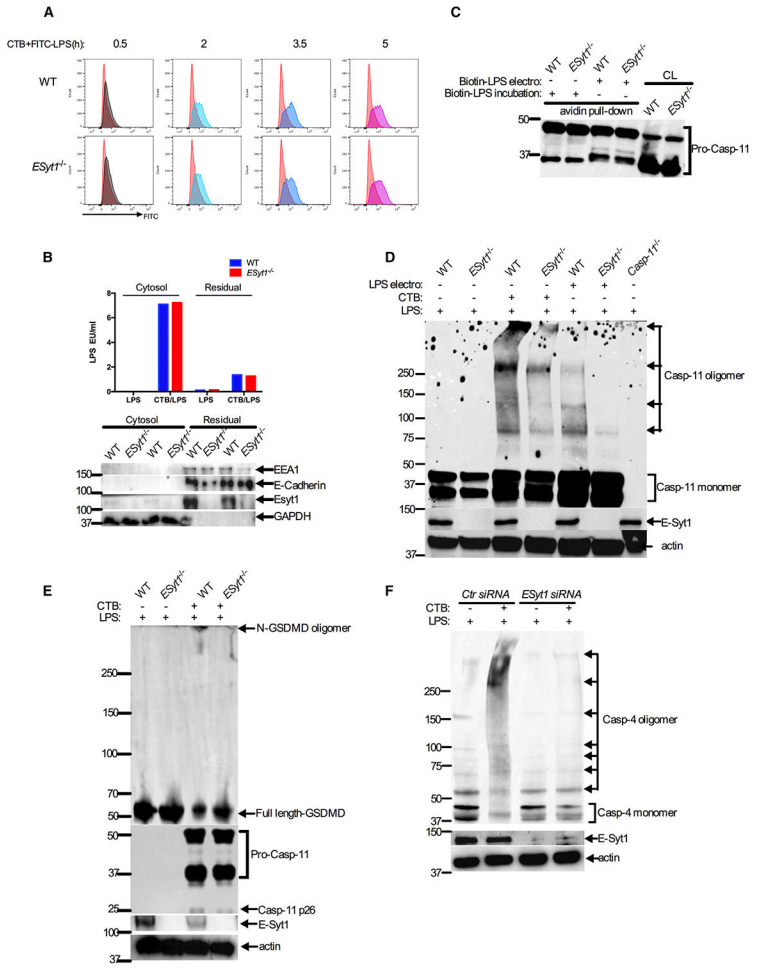

Figure 2. Absence of E-Syt1 inhibits cytoplasmic LPS-induced Casp-11 oligomerization

(A) Flow cytometry analysis of FITC-LPS internalization by WT and *ESyt1*$^{-/-}$ BMDMs stimulated with CTB (20 μg/mL) plus FITC-LPS (1 μg/mL) for the indicated times (0.5, 2, 3.5, and 5 h).

(B) Immunoblots for EEA1, E-cadherin, E-Syt1, and GAPDH, LAL (limulus amebocyte lysate) assay for LPS (EUs [endotoxin units]) in the cytosol, and residual fraction of BMDMs stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) or LPS (500 ng/mL) only.

(C) The cell lysates of LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs were incubated with biotin-LPS or LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs were transfected with biotin-LPS using electroporation. LPS-binding proteins were pulled down by avidin beads and blotted with anti-Casp-11 antibody.

(D) LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs were stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) for 8 h or transfected with LPS (1 μg/10^6 cells) for 2 h, and then the cells were treated with DSS (500 μM, 30 min). The cell lysates were immunoblotted with anti-Casp-11 and E-Syt1.

(E) LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs were stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) for 8 h, and then the cells were treated with DSS (500 μM, 30 min). The cell lysates were immunoblotted with anti-Casp-11, anti-GSDMD, and anti-E-Syt1.

(F) THP-1-derived macrophages were first transfected with control siRNA or human *ESyt1* siRNA (80 nM). 36 h later, these transfected macrophages were primed with LPS and stimulated with CTB (20 μg/mL) plus LPS (1 μg/mL) for 8 h, and then the cells were treated with DSS (500 μM, 30 min). The cell lysates were immunoblotted with anti-Casp-4 and E-Syt1.

Data are representative of three independent experiments.

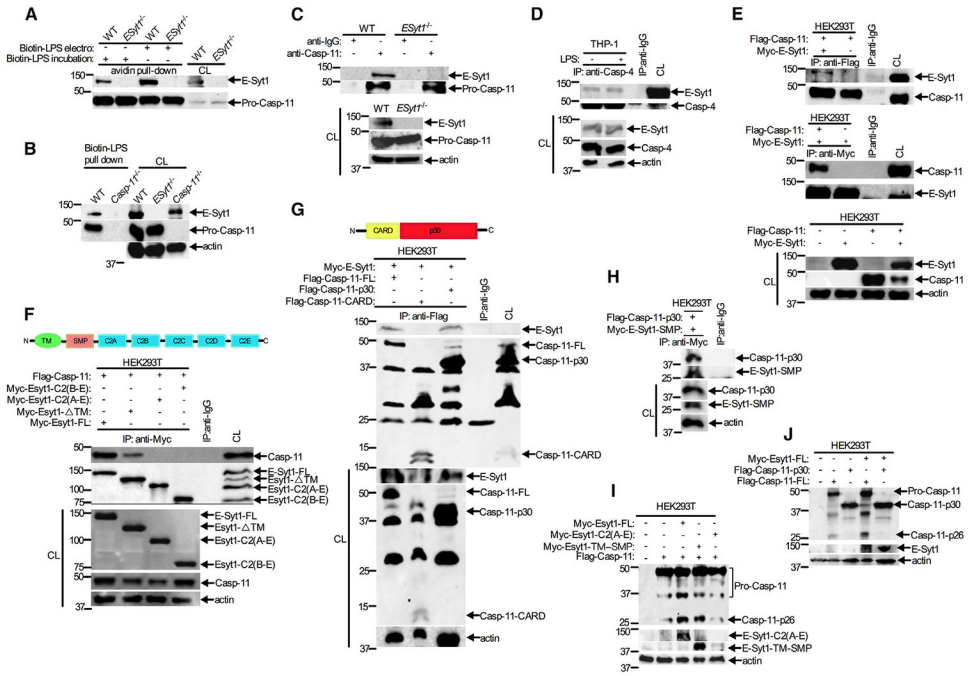

Figure 3. The Esyt1 SMP domain interacts with the Casp-11 p30 domain and is required for boosting Casp-11 cleavage

(A) The cell lysates of LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs were incubated with biotin-LPS or LPS-primed WT and *ESyt1*$^{-/-}$ BMDMs were transfected with Biotin-LPS using electroporation. LPS-binding proteins were pulled down by avidin beads and blotted with anti-Casp-11 and anti-E-Syt1 antibodies.

(B) The cell lysates of LPS-primed WT and Casp-11$^{-/-}$ BMDMs were incubated with biotin-LPS, and then LPS-binding proteins were pulled down by avidin beads and blotted with anti-Casp-11 and anti-E-Syt1 antibodies.

(C) Immunoblot of E-Syt1 in anti-Casp-11 immunoprecipitates of lysates of WT and *ESyt1*$^{-/-}$ BMDMs primed with LPS.

(D) THP-1-derived macrophages stimulated with LPS (500 ng/mL, 5 h) or not. Then, immunoblots of E-Syt1 in anti-Casp-4 immunoprecipitates were performed.

(E) Immunoblot analysis of FLAG or Myc immunoprecipitates of lysates of HEK293T cells transfected with FLAG-tagged Casp-11 and Myc-tagged E-Syt1.

(F) Immunoblot analysis of Myc immunoprecipitates of lysates of HEK293T cells transfected with FLAG-tagged Casp-11 and Myc-tagged full-length E-Syt1 (Myc-E-Syt1-FL) or E-Syt1 mutants (E-Syt1-C2(A–E), E-Syt1-C2(B–E), E-Syt1-ΔTM).

(G) Immunoblot analysis of FLAG immunoprecipitates of lysates of HEK293T cells transfected with Myc-tagged E-Syt1 and FLAG-tagged Casp-11 or Casp-11 mutants (Casp-11-CARD, Casp-11-p30).

(H) Immunoblot analysis of Myc immunoprecipitates of lysates of HEK293T cells transfected with Myc-tagged E-Syt1-SMP and FLAG-tagged Casp-11 p30.

(I) HEK293T cells transfected with FLAG-tagged Casp-11 and Myc-tagged E-Syt1-FL or its mutants (E-Syt1-TM-SMP, E-Syt1-C2(A–E)) were electroporated with LPS, and cell lysates were bolted with anti-Casp-11 and anti-Myc.

(J) HEK293T cells transfected with Myc-tagged E-Syt1-FL and FLAG-tagged Casp-11, or FLAG-tagged Casp-11-p30, were electroporated with LPS, and cell lysates were bolted with anti-Casp-11 and anti-Myc.

Data are representative of three independent experiments.

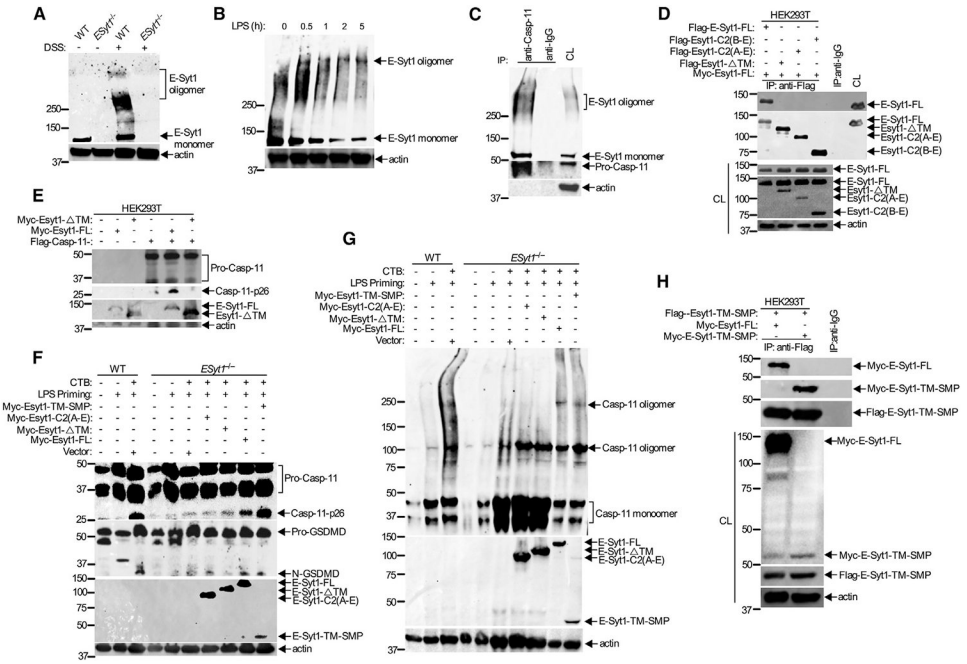

Figure 4. TM domain-mediated E-Syt1 oligomerization is required for Casp-11 oligomerization and cleavage

(A) Immunoblot analysis of E-Syt1 in cell lysates of WT and *ESyt1*$^{-/-}$ BMDMs primed with LPS (500 ng/mL, 5 h) and treated with DSS.

(B) Immunoblot analysis of E-Syt1 in cell lysates of WT and *ESyt1*$^{-/-}$ BMDMs stimulated with LPS (500 ng/mL) for various times (0.5, 1, 2, and 5 h) and treated with DSS.

(C) Immunoblot analysis of Casp-11 immunoprecipitates of lysates of WT BMDMs primed with LPS (500 ng/mL, 5 h) and treated with DSS.

(D) Immunoblot analysis of FLAG immunoprecipitates of lysates of HEK293T cells transfected with Myc-tagged E-Syt1 and FLAG-tagged E-Syt1 or its FLAG-tagged mutants (E-Syt1-C2(A–E), E-Syt1-C2(B–E), and E-Syt1-ΔTM).

(E) HEK293T cells transfected with FLAG-tagged Casp-11 and Myc-tagged E-Syt1-FL or Myc-tagged E-Syt1-ΔTM were electroporated with LPS, and cell lysates were bolted with anti-Casp-11 and anti-Myc.

(F) *ESyt1*$^{-/-}$ BMDMs were transfected with empty vector, Myc-tagged E-Syt1, or its mutants (E-Syt1-C2(A–E), E-Syt1-C2(B–E), E-Syt1-ΔTM, and E-Syt1-TM-SMP) by electroporation, using non-transfected *ESyt1*$^{-/-}$ BMDMs or WT BMDMs transfected with empty vector as controls. The transfected BMDMs were then primed with LPS and stimulated with CTB (20 μg/mL) for 16 h, and cell lysates were blotted with anti-Casp-11, anti-GSDMD, and anti-Myc.

(G) WT and *ESyt1*$^{-/-}$ BMDMs transfected and stimulated as described in (F) were treated with DSS (500 μM, 30min), and cell lysates were blotted with anti-Casp-11 and anti-Myc.

(H) Immunoblot analysis of FLAG immunoprecipitates of lysates of HEK293T cells transfected with FLAG-tagged E-Syt1-TM-SMP and Myc-tagged E-Syt1-TM-SMP or Myc-tagged E-Syt1-FL.

Data are representative of three independent experiments.

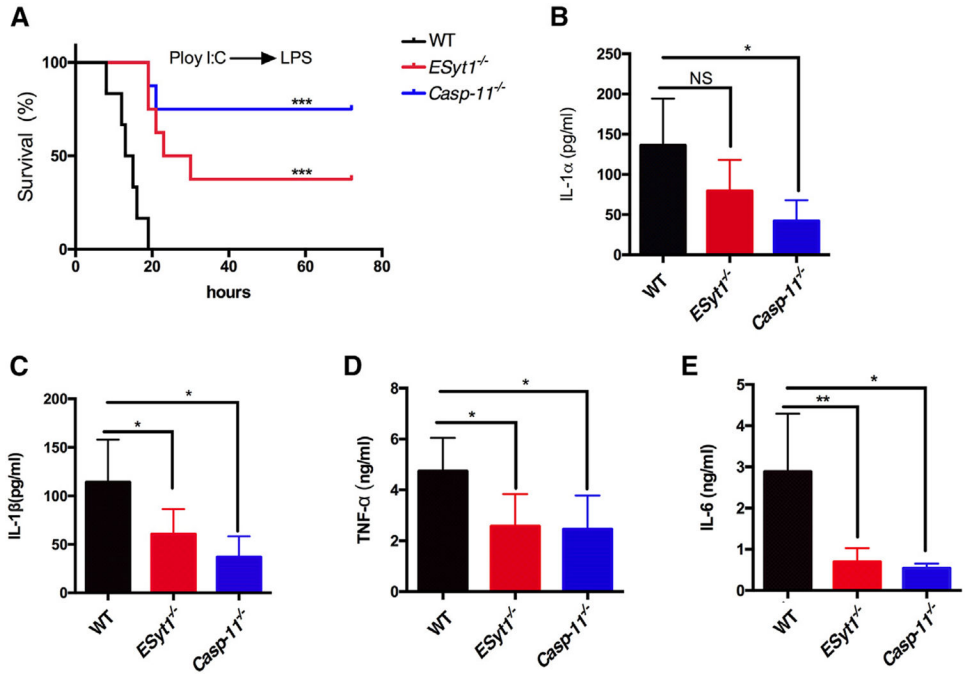

Figure 5. E-Syt1 deficiency protects mice from endotoxemia death

(A) Kaplan-Meier survival curve of WT, *ESyt1*$^{-/-}$, and *Casp-11*$^{-/-}$ mice primed with poly (I:C) (10 mg/kg) and then challenged 6 h later with LPS (4 mg/kg). WT mice (n = 6), *ESyt1*$^{-/-}$ mice (n = 8), *Casp-11*$^{-/-}$ mice (n = 8). ***p < 0.001; log rank test.

(B–E) WT, *ESyt1*$^{-/-}$, and *Casp-11*$^{-/-}$ mice (five mice per group) were primed with poly (I:C) (10 mg/kg) and then challenged 6 h later with LPS (4 mg/kg). 4 h post-LPS challenge, serum levels of IL-1β, IL-1α, TNF-α, and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA).

Data are shown as mean ± SD. *p < 0.05; **p < 0.01; Student's t test. Data are representative of three independent experiments.

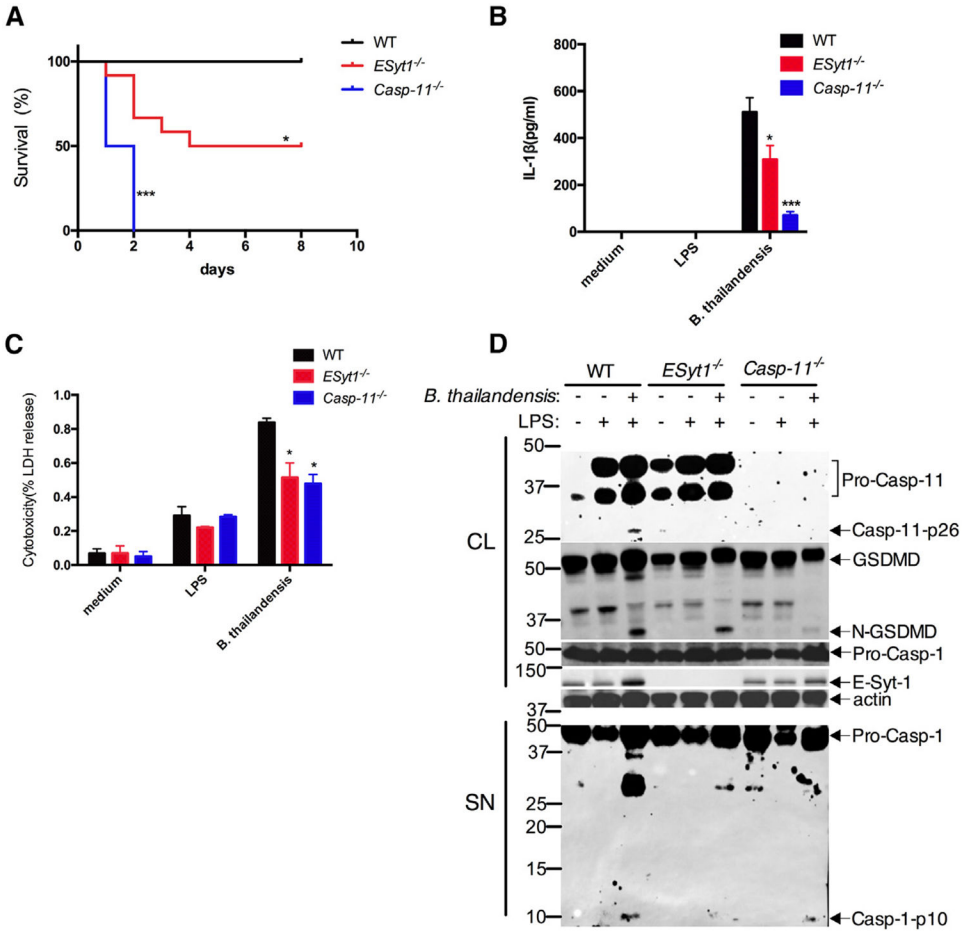

Figure 6. E-Syt1 protects mice against *B. thailandensis* infection

(A) Kaplan-Meier survival curve of WT, *ESyt1*$^{-/-}$ and *Casp-11*$^{-/-}$ mice infected with 2 × 10^7 colonyforming units (CFUs) intraperitoneally (i.p.) with *B. thailandensis*. WT mice (n = 10), *ESyt1*$^{-/-}$ mice (n = 12), *Casp-11*$^{-/-}$ mice (n = 8). ***p < 0.001; *p < 0.05; 1og rank test.

(B–D) LPS-primed BMDMs from WT, *ESyt1*$^{-/-}$, and *Casp-11*$^{-/-}$ mice were infected with *B. thailandensis* for 4 h. IL-1β release (B) was determined by ELISA. Cytotoxicity (C) was determined by LDH release assay. (D) Immunoblots of pro-Casp-11, Casp-11-p26, E-Syt1, pro-GSDMD, pro-Casp-1, and N-GSDMD expression in cell extracts and pro-Casp-1 and Casp-1-p10 in the supernatants. CL, cell lysate; SN, supernatants.

Data are shown as mean ± SD. Data are representative of three independent experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Caspse-1 p10	Santa Cruz	Cat# sc-514; RRID:AB_2068895
Mouse monoclonal anti-Caspse-4	Santa Cruz	Cat# sc-56056; RRID:AB_781828
Mouse monoclonal anti-IL-1β	Santa Cruz	Cat# sc-32294; RRID:AB_627790
Rat monoclonal anti-Caspase-11	Cell Signaling Technology	Cat# 14340; RRID:AB_781818
Rabbit polyclonal anti-GSDMD	Cell Signaling Technology	Cat# 93709; RRID:AB_2800210
Rabbit monoclonal anti-flag	Cell Signaling Technology	Cat# 14793; RRID:AB_2572291
Mouse monoclonal anti-Myc	Cell Signaling Technology	Cat# 2276; RRID:AB_2314825
Rabbit monoclonal anti-EEA1	Cell Signaling Technology	Cat# 3288; RRID:AB_2096811
Mouse monoclonal anti-E-Cadherin	Cell Signaling Technology	Cat# 14472; RRID:AB_2728770
Rabbit monoclonal anti-Caspase-1	Cell Signaling Technology	Cat# 3866; RRID:AB_2069051
Rabbit monoclonal anti-GAPDH	Cell Signaling Technology	Cat# 5174; RRID:AB_10622025
Rabbit monoclonal anti-β-Actin	Cell Signaling Technology	Cat# 4970; RRID:AB_2223172
Rabbit polyclonal anti-E-Syt1	Invitrogen	Cat# PA5-90448; RRID:AB_2857152
Rabbit polyclonal anti-E-Syt2	Invitrogen	Cat# PA5-69613; RRID:AB_2641435
Rabbit polyclonal anti-E-Syt3	Invitrogen	Cat# PA5-106977; RRID:AB_1860382
HRP-Rabbit anti-mouse IgG (H + L)	Invitrogen	Cat# 61-6520; RRID:AB_2533933
HRP-Goat anti-rabbit IgG (H + L)	Kirkegaard & Perry Laboratories	Cat# 816120; RRID:AB_87750
HRP-Goat anti-rat IgG (H + L)	R&D	Cat# HAF005; RRID:AB_1512258
Bacterial and virus strains		
EHEC	ATCC	700927
P.aeruginosa	ATCC	BAA-47
Burkholderia thailandensis	ATCC	700388
Chemicals, peptides, and recombinant proteins		
Gentamycin	Sigma-Aldrich	Cat# 1405-41-0
Kanamycin	Sigma-Aldrich	Cat# 25389-94-0
LPS	Sigma-Aldrich	Cat# LPS25
ATP	MedChemExpress (MCE)	Cat# HY-B2176
CTB (Cholera Toxin B subunit)	Sigma-Aldrich	Cat# c9903
Flagellin	InvivoGen	Cat# tlrl-pafla
Poly I:C	InvivoGen	Cat# tlrl-picw-250
Poly (dA:dT)	InvivoGen	Cat# tlrl-patn
Nigericin	Sigma-Aldrich	Cat# N7143
Lipofectamine	Thermo Fisher	Cat# L3000150
DOTAP liposomal Transfection Reagent	Sigma-Aldrich	Cat# 144189-73-1
phorbol 12-myristate-13-acetate (PMA)	Sigma-Aldrich	Cat# 16561-29-8
Digitonin	MedChemExpress (MCE)	Cat# HY-N4000
Biotin-conjugated LPS	InvivoGen	Cat# tlrl-lpsbiot
FITC-conjugated LPS	Sigma-Aldrich	Cat# F8666
Streptavidin-sepharose beads	Thermo Fisher	Cat# 20347

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protein G-sepharose	GE Healthcare	N/A
DSS (disuccinimidyl suberate)	Thermo Fisher	Cat# A39267
Critical commercial assays		
Mouse TNF-α ELISA kit	Biolegend	Cat# 430904
Mouse IL-6 ELISA kit	Biolegend	Cat# 31301
Mouse IL-1β ELISA kit	Biolegend	Cat# 432604
Mouse IL-α ELISA kit	Biolegend	Cat# 433404
PrimeSTAR HS DNA Polymerase	Takara	Cat# R010A
Gibson Assembly® Master Mix	NEB (New England Biolabs)	Cat# E2611s
P2 Primary Cell 4D-NucleofectorTM X Kit	Lonza	Cat# V4XP-2024
Lipofectamine™ RNAiMAX	ThermomFisher	Cat# 13778030
LAL (limulus amebocyte lysate) kit	Xiamen Bioendo Technology Co.,Ltd.	Cat# KTE20
Cytotoxicity Detection KitPLUS (LDH) kit	Sigma-Aldrich	Cat# 04744926001
Experimental models: Cell lines		
THP-1	ATCC	TIB-202
293T	ATCC	CRL-3216
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Jackson Laboratory	RRID:IMSR_JAX:000664
Mouse: *ESyt1*$^{-/-}$ *ESyt2*$^{-/-}$ *ESyt3*$^{-/-}$	Jackson Laboratory	RRID:MMRRC_JAX:042272
Mouse: *Casp-11*$^{-/-}$	Jackson Laboratory	RRID:IMSR_JAX:024698
Mouse: *ESyt1*$^{-/-}$	This manuscript	N/A
Mouse: *ESyt1*$^{-/-}$ *ESyt2*$^{-/-}$	This manuscript	N/A
Oligonucleotides		
human *ESyt1* siRNA	ThermoFisher	Cat# 4392420; Assay ID:s23606
control *siRNA*	ThermoFisher	Cat# AM4611
Recombinant DNA		
Myc-tagged E-Syt1	Yale University	N/A
flag-tagged Casp-11	Addgene	Cat# 21145
Myc-Esyt1-Δ-TM	This manuscript	N/A
Myc-E-Syt1-C2 (A-E)	This manuscript	N/A
Myc-E-Syt1-TM-SMP	This manuscript	N/A
Flag-tagged E-Syt1	This manuscript	N/A
Flag-Casp-11 p30	This manuscript	N/A
Flag-Casp-11 CARD	This manuscript	N/A
Flag-Esyt1-Δ-TM	This manuscript	N/A
Flag-E-Syt1-C2(A-E)	This manuscript	N/A
Flag-E-Syt1-C2(B-E)	This manuscript	N/A
Software and algorithms		
GraphPad Prism 6.0	GraphPad Software	N/A
Flow Jo™ 10	Flow Jo	N/A