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Essential role of *Salmonella* Enteritidis DNA adenine methylase in modulating inflammasome activation



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Abstract

Background: *Salmonella* Enteritidis (SE) is one of the major foodborne zoonotic pathogens of worldwide importance which can induce activation of NLRC4 and NLRP3 inflammasomes during infection. Given that the inflammasomes play an essential role in resisting bacterial infection, *Salmonella* has evolved various strategies to regulate activation of the inflammasome, most of which largely remain unclear.

Results: A transposon mutant library in SE strain C50336 was screened for the identification of the potential factors that regulate inflammasome activation. We found that T3SS-associated genes *invC*, *prgH*, and *spaN* were required for inflammasome activation in vitro. Interestingly, C50336 strains with deletion or overexpression of Dam were both defective in activation of caspase-1, secretion of IL-1 β and phosphorylation of c-Jun N-terminal kinase (Jnk). Transcriptome sequencing (RNA-seq) results showed that most of the differentially expressed genes and enriched KEGG pathways between the C50336-VS-C50336 Δ *dam* and C50336-VS-C50336::*dam* groups overlapped, which includes multiple signaling pathways related to the inflammasome. C50336 Δ *dam* and C50336::*dam* were both found to be defective in suppressing the expression of several anti-inflammasome factors. Moreover, overexpression of Dam in macrophages by lentiviral infection could specifically enhance the activation of NLRP3 inflammasome independently via promoting the Jnk pathway.

Conclusions: These data indicated that Dam was essential for modulating inflammasome activation during SE infection, there were complex and dynamic interplays between Dam and the inflammasome under different conditions. New insights were provided about the battle between SE and host innate immunological mechanisms.

Keywords: *Salmonella* Enteritidis, DNA adenine methylase, Inflammasome, Cytotoxicity, Caspase-1, Interleukin-1 β

Background

Salmonella enterica subsp. enterica is a Gram-negative facultative intracellular bacterial pathogen that causes over 1.3 billion illnesses and 200,000 deaths in humans annually worldwide [1]. Salmonellosis ranges from self-limiting gastroenteritis to lethal bacteremia in immunocompromised

individuals [2]. Although more than 2500 different serotypes have been discovered, *Salmonella enterica subsp. enterica* serotype Enteritidis (SE) is one of the most important serovars transmitted from animals to humans and has replaced *Salmonella enterica subsp. enterica* serotype Typhimurium (ST) as the primary cause of salmonellosis globally since 1980 [3]. SE has been one of the major causes of outbreaks of food poisoning, poultry meat, eggs, and products are considered to be the major source of infection [4]. SE infection is a major public health problem, which could also cause

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severe economic losses in the poultry industry worldwide [5]. The elucidation of the host anti-infection immunity mechanism and development of new therapeutic strategies against SE infection would be of great importance.

After invading the host through the gastrointestinal tract, *Salmonella* traverses the epithelial barrier either through invasion of intestinal epithelial and microfold cells overlying Peyer's Patches or capture by CD18⁺ immune cells directly from the intestinal lumen [6]. *Salmonella* cells that survive and replicate within macrophages are critical for the infection to extend beyond the intestinal mucosa [7]. The ability of *Salmonella* to invade host cells is dependent upon the *Salmonella* pathogenicity island-1 (SPI-1)-encoded type III secretion system (T3SS) which injects effector proteins into the host cell cytosol to modulate cellular responses, while the SPI-2 T3SS subsequently translocates virulence proteins to subvert the bactericidal properties of macrophages and create a specialized *Salmonella*-containing vacuole (SCV) for replication [8]. This special intracellular lifestyle exposes *Salmonella* to detection by inflammasomes, which have been identified as playing an essential role in the innate immune response against *Salmonella* infection [9].

Inflammasomes are cytoplasmic multi-protein sensors consisting of varying protein components, generally including pattern recognition receptors (PRRs, such as Nod-like receptors (NLRs) and melanoma 2 (AIM2)-like receptor), an adaptor protein ASC (apoptosis-associated speck-like proteins containing caspase recruitment domains), and an effector subunit caspase (primarily caspase-1) [10]. It is widely reported that NLRP3 and NLRC4 inflammasomes are activated during *Salmonella* infection [11]. After *Salmonella* invades host cells, the T3SS apparatus and pore-forming toxins are detected by NLRP3, while NLRC4 recognizes bacterial flagellin, T3SS needle, and basal rod proteins [12]. Inflammasome assembly is triggered to activate pro-caspase-1 cleavage, leading to cleavage and secretion of caspase-1-dependent pro-inflammatory cytokines interleukin-1 β (IL-1 β) and IL-18 and rapid lytic cell death known as pyroptosis [13]. *Salmonella* cells are subsequently released and exposed to neutrophil-mediated killing, which are recruited by IL-1 β [14].

A previous study demonstrated that mice deficient in caspase-1, IL-1 β , or IL-18 succumbed to infection earlier than wild type (WT) mice and had significantly higher *Salmonella* burdens in the Peyer's patches, mesenteric lymph nodes, and spleens [15]. In addition, genetic ablation of both NLRC4 and NLRP3 in mice leads to increased susceptibility to *Salmonella* infection [16]. Given the emphasis on inflammasomes in resisting bacterial infection, *Salmonella* has evolved multiple mechanisms to avoid or delay inflammasome activation [17]. To evade NLRC4 detection, flagellin and SPI-1 are strongly

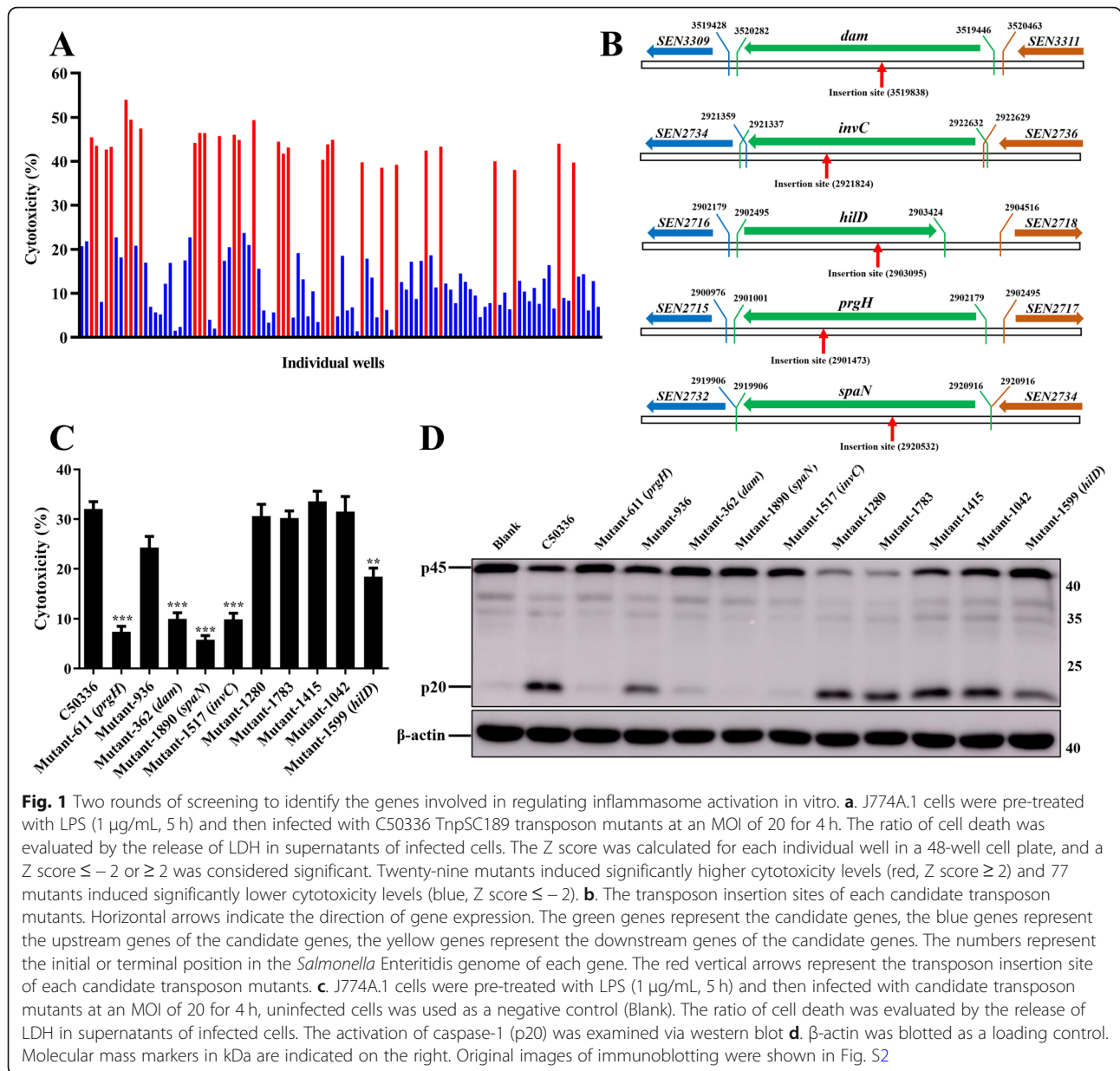
repressed in the intracellular environment through the PhoP-PhoQ regulatory system of *Salmonella* [18]. A recent study reported that *Salmonella* uses the SPI-1 T3SS effector SopB to suppress NLRC4 inflammasome activation by preventing ASC oligomerization [19]. *Salmonella* can also down-regulate NLRC4 and interfere with other host pathways to regulate inflammasome activation, such as autophagy [20]. In addition, *Salmonella* tricarboxylic acid cycle enzymes have been reported to limit NLRP3 inflammasome-dependent antibacterial immune responses through mitochondrial reactive oxygen species [21]. However, the many strategies of *Salmonella* regulation of the activation of the inflammasome remain largely unknown.

Until now, research on the interplay between *Salmonella* and inflammasomes has focused on ST, with SE inflammasomes still poorly understood. To identify potential factors involved in regulating the activation of the inflammasome, we constructed a transposon mutant library in SE strain C50336 and screened for increased or decreased inflammasome activation in the mouse macrophage cell line J774A.1. Sequencing of candidate mutants identified *dam*, the gene encoding DNA adenine methylase, which is an essential factor for *Salmonella* virulence [22]. Intriguingly, both deletion and overexpression of *Dam* failed to induce inflammasome activation, and RNA-seq assays suggested that the absence and overexpression of *Dam* had similar effects on infected macrophages. Notably, overexpression of *Dam* in J774A.1 cells by lentiviral infection promoted activation of the NLRP3 inflammasome. Collectively, these results indicated that SE *Dam* plays a critical role in inflammasome activation during infection.

Results

Screening candidate mutants for alterations in inflammasome activation

To screen potential genes involved in modulating the activation of the inflammasome, a *mariner*-based transposon (TnpSC189) mutant library in SE strain C50336 was generated. Thus far, 2062 transposon mutants have been tested individually, and candidate mutants were selected by increased or decreased Lactate dehydrogenase (LDH) release relative to the C50336 parental strain 4 h after infection of the mouse macrophage cell line J774A.1. Following the first round of screening, 106 mutants were considered candidates for the second round of validation (Fig. 1a). The cytotoxicity induced by 29 mutants (Z score ≥ 2) was significantly higher than that induced by C50336, while 77 mutants (Z score ≤ -2) induced significant lower cytotoxicity. To verify the selected candidates, each was tested individually in another screening round. The candidate mutants were rescreened in J774A.1 cells, LDH release was quantified,



and caspase-1 activation was determined by western blot. The candidate transposon mutants 611, 362, 1890, 1517, and 1599 induced significant lower levels of cytotoxicity and activation of caspase-1 compared to WT (Fig. 1c and d). The transposon insertion sequencing of the candidate mutants identified these five genes were *dam*, *invC*, *hilD*, *prgH* and *spaN*, the transposon insertion sites were shown in Fig. 1b. The *dam* gene encodes a DNA adenine methylase, *invC* encodes a FliI/YscN family ATPase, *hilD* encodes a transcriptional regulator, *prgH* encodes a T3SS inner membrane ring protein, and *spaN* encodes an SPI-1 T3SS protein.

Identification of candidate genes regulate the inflammasome activation

To further investigate the function of these five genes in regulating inflammasome activation, the in-frame deletion mutants of *dam*, *invC*, *hilD*, *prgH*, and *spaN* genes were constructed. There was no significant difference in growth characteristics among WT strain, each mutant, complementation, and overexpression strains (Fig. S1). As shown in Fig. 2a, infection with *dam*, *invC*, *prgH*, and *spaN* mutant strains resulted in greatly decreased LDH release in comparison with J774A.1 cells infected by WT. We subsequently examined whether reduced cell

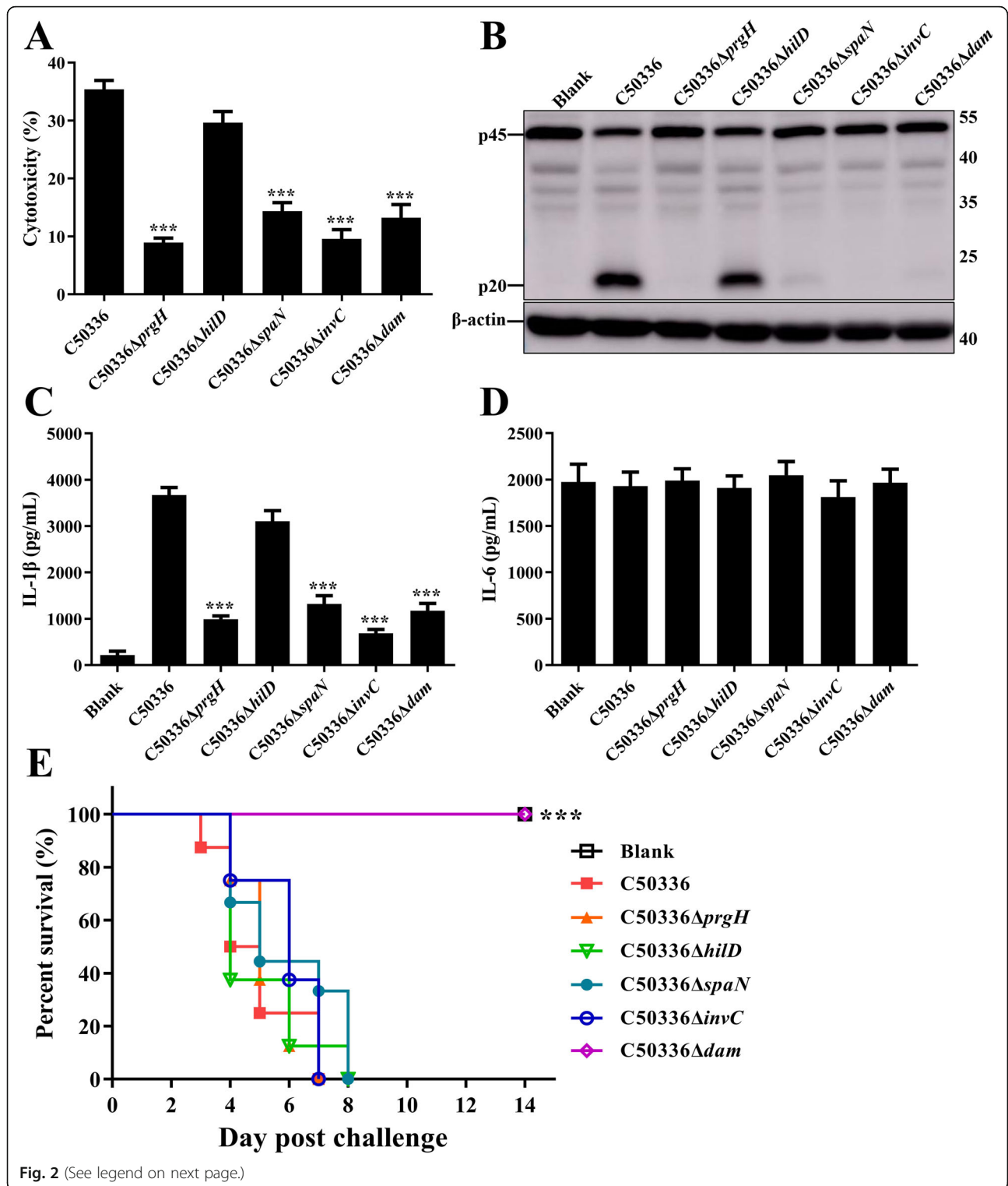


Fig. 2 (See legend on next page.)

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Fig. 2 Deletion mutants of *dam*, *invC*, *prgH*, and *spaN* failed to induce inflammasome activation. J774A.1 cells were pre-treated with LPS (1 µg/mL, 5 h) and then infected with WT strain C50336 and *dam*, *invC*, *hilD*, *prgH*, and *spaN* gene deletion mutants at an MOI of 20 for 4 h, uninfected cells was used as a negative control (Blank). **a.** The ratio of cell death was evaluated by the release of LDH in supernatants of infected cells. **b.** The activation of caspase-1 (p20) was examined via western blot. β-actin was blotted as a loading control. Molecular mass markers in kDa are indicated on the right. Original images of immunoblotting were shown in Fig. S3. The production of IL-1β **c** and IL-6 **d** in supernatants were examined via ELISA. ****p* < 0.001 for one-way ANOVA followed by Bonferroni's multiple comparison test indicate significant findings in comparison with cells infected with WT strain C50336. Data are presented as mean ± SEM of triplicate samples per experimental condition from three independent experiments. **e.** C57BL/6 mice were intraperitoneally injected with PBS (negative control, Blank), C50336, and gene deletion mutants at a dose of 1×10^5 CFU per mouse. The mortality was recorded over 14 dpc. ***, *p* < 0.001 compared with the C50336-infected group by log-rank (Mantel-Cox) test for the survival curve

death was caused by decreased inflammasome activation. As expected, the WT triggered strong caspase-1 activation and IL-1β expression in infected J774A.1 cells, while the production of cleaved caspase-1 p20 subunits and caspase-1-dependent cytokine IL-1β was significantly suppressed in cells infected with *dam*, *invC*, *prgH*, or *spaN* deletion mutant strains (Fig. 2b and c). Simultaneously, to further verify whether these genes can specifically influence the inflammasome pathway, we also measured the levels of non-inflammasome inflammatory cytokine IL-6 in supernatants. There was no significant difference in IL-6 secretion between WT- and deletion mutant strains-infected cells (Fig. 2d). These results suggested that *dam*, *invC*, *prgH*, and *spaN* specifically influenced inflammasome activation during SE infection in J774A.1 cells. In addition, the mouse was used as the animal model to determine the virulence of gene-deficient strains. None of the mice died in the C50336Δ*dam*-infected group, whereas all mice infected with other gene deletion mutants or WT C50336 (50) died within 8 days post-challenge (dpc) (Fig. 2e). No clinical symptoms were detected in the mice infected with C50336Δ*dam*, suggesting that Dam played an important role in the virulence of SE.

Identification of proteins transferred into host cells

Salmonella injects various effector proteins into host cell cytosol to stimulate or interfere with cellular processes. To identify whether Dam, InvC, PrgH, and SpaN can be translocated into the cytosol of infected cells, β-lactamase TEM-1 was fused with each of these four proteins. The intracellular translocation was assayed via fluorescence resonance energy transfer (FRET) in infected HeLa cells (Fig. 3a). Uninfected HeLa cells and cells infected with C50336 contain an empty plasmid pCX340 appeared green, indicating the absence of TEM-1 activity in these cells. While, no blue fluorescence was detected in cells infected with C50336-pCX340-*invC* and C50336-pCX340-*spaN*, suggesting that the fusion proteins InvC-TEM and SpaN-TEM encoded by C50336 were not secreted and/or able to cleave the β-lactam ring of CCF2-AM. In contrast, infection with C50336 expressing Dam-TEM and PrgH-TEM resulted in a significant population of blue fluorescent cells. The transfer efficiency of PrgH-TEM was about

10.3% (Fig. 3b), while the expressed Dam-TEM was translocated at a higher efficiency (~ 21.1%). These data indicate that Dam and PrgH could be secreted and then translocated into the host cells during SE infection.

Deletion of *dam* or *prgH* does not affect synthesis of inflammasome components

Previous studies have shown that the expression of PrgH is regulated by Dam [23]. To further confirm the role of *dam* and *prgH* in regulating inflammasome activation, complementation strains and overexpression strains were constructed by employing the plasmid pMMB207. Primary bone marrow-derived macrophages (BMDMs) obtained from WT C57BL/6 mice were infected with indicated SE strains. The expression of NLRP3, NLRC4, ASC, and pro-IL-1β were analyzed by immunoblotting (Figs. 4 and 5). The results demonstrated that neither *dam* or *prgH* gene deletion nor overexpression could significantly suppress the expression of inflammasome components, indicating that the effect of Dam and PrgH on inflammasome activation was not achieved by influencing the synthesis of inflammasome components.

Similarly, LDH release was significantly decreased in BMDMs infected with C50336Δ*prgH* or C50336Δ*dam* as compared to WT strains, as was caspase-1 activation and IL-1β secretion (Figs. 4 and 5). The decreased cytotoxicity and expression of caspase-1 and IL-1β induced by C50336Δ*prgH* was recovered to a normal level when infected with C50336Δ*prgH*::*prgH*, whereas overexpression of *prgH* could not induce stronger inflammasome activation. However, the most interesting observation was that caspase-1 activation, IL-1β secretion, and LDH release were all obviously suppressed in BMDMs infected with complementation strain C50336Δ*dam*::*dam* and overexpression strain C50336::*dam* (Fig. 5), suggesting Dam may play a variety of roles in modulating inflammasome activation during SE infection. The addition of IPTG during the culture of bacteria containing plasmid pMMB207 had a great effect on promoting the expression of target proteins [24]. Moreover, we found that SE bearing plasmid pMMB207-*dam* failed to activate the inflammasome, and seemed reasonable because the expressions of Dam in both the complementation strain and overexpression strain were

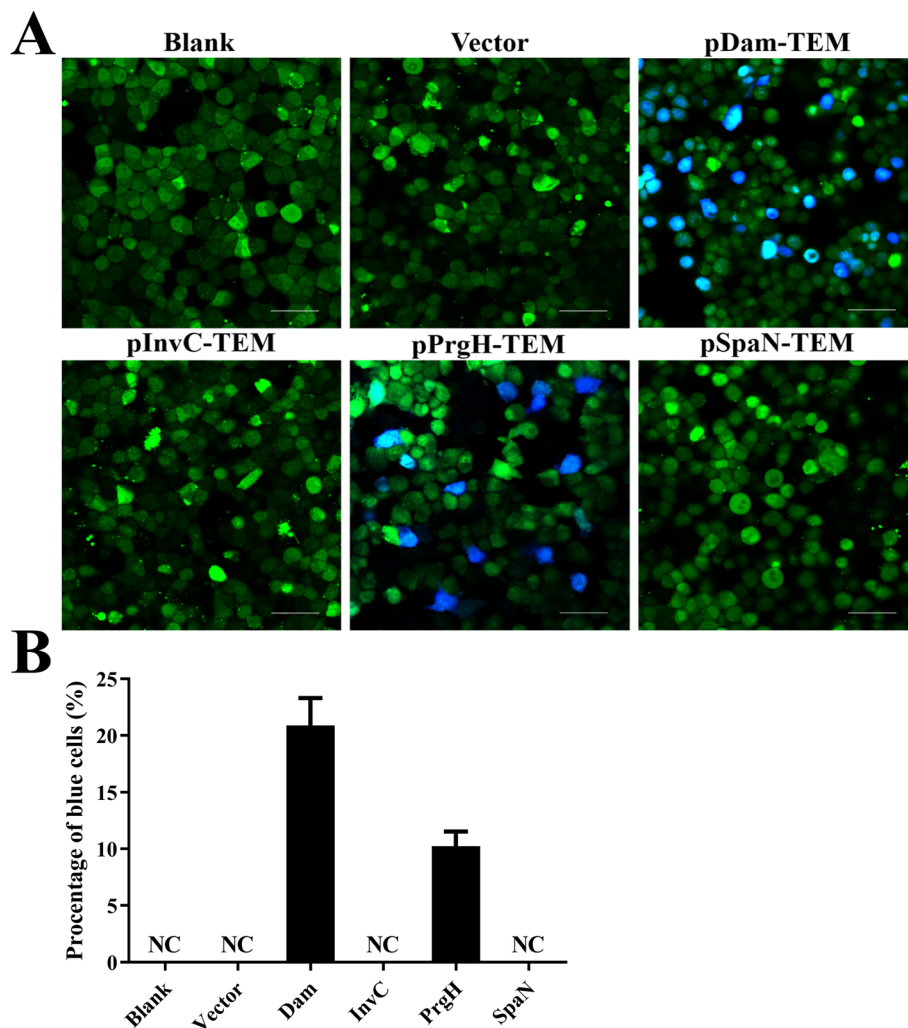


Fig. 3 Target proteins Dam and PrgH were able to be translocated into the infected cells. HeLa cells were infected with WT C50336 bearing empty plasmid pCX340 or expressing different TEM-1 fusion proteins at an MOI of 100, uninfected HeLa cells was used as a negative control (Blank). Cells were washed and loaded with CCF2-AM after infection. **a**. Translocation of TEM-1 fusion proteins into the cell cytosol results in cleavage of CCF2-AM, emission of blue fluorescence revealed the activity of TEM β -lactamase, whereas uncleaved CCF2-AM emitted green fluorescence. Scale bar, 50 μ m. **b**. The percentages of cells emitting blue fluorescence. For a particular cell well, six pictures were taken and approximately 1200–2000 cells were counted. Each picture was considered an independent observation and used to calculate the percentage of blue fluorescent cells. Data are presented as mean \pm SEM of triplicate samples per experimental condition from three independent experiments

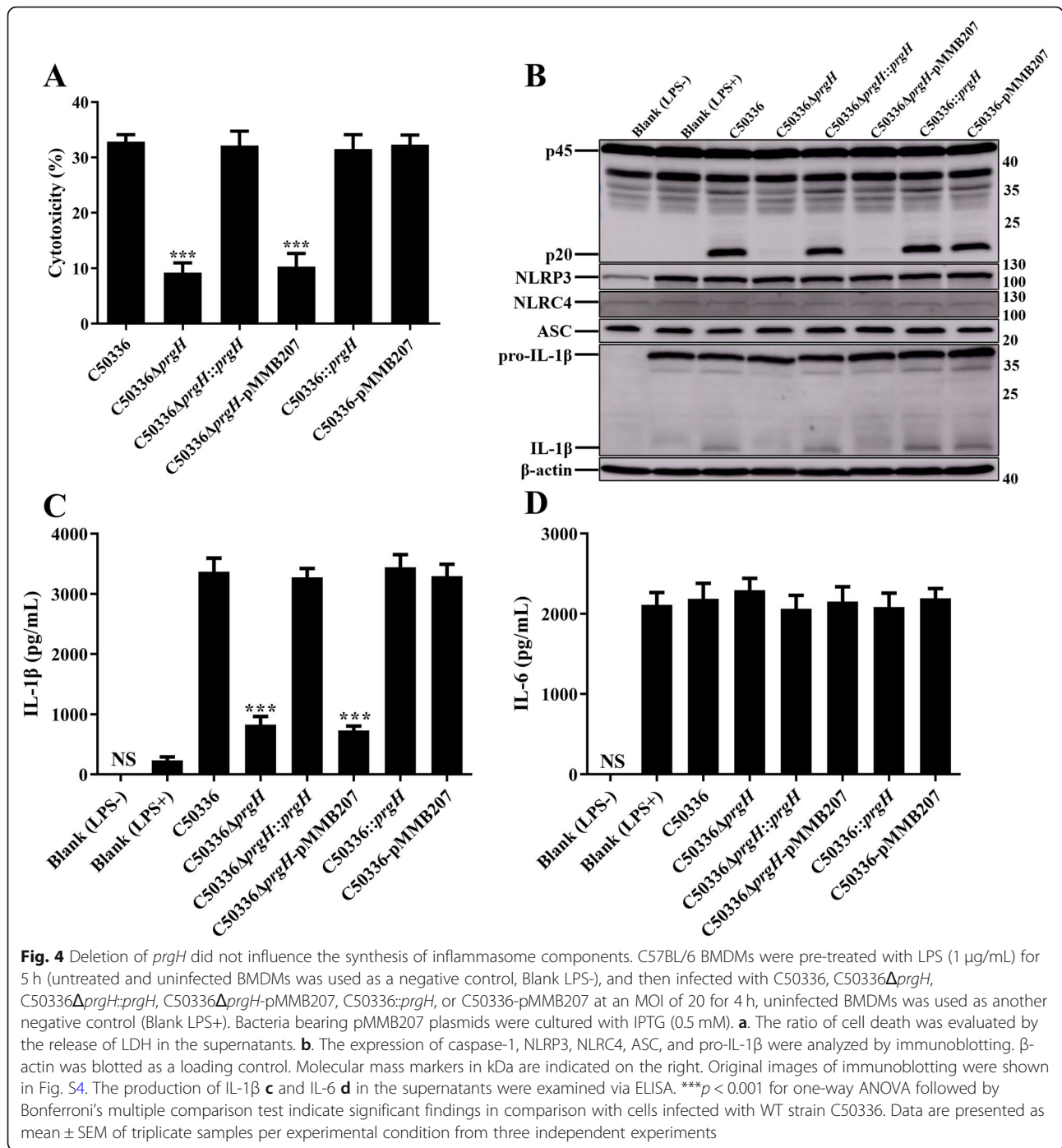
maintained at a high level in the presence of IPTG. The expression level of Dam may be a key factor in regulating inflammasome activation.

To verify this hypothesis, the complementation strains and overexpression strains cultured without IPTG were used to infect WT BMDMs. The results showed that the complementation strain induced a significant enhancement of cytotoxicity, bioactive IL-1 β and caspase-1 secretion compared to C50336 Δ dam (Fig. 6), although the ability of C50336 Δ dam::dam to activate the inflammasome did not return to a normal level like C50336 Δ prgH::prgH induction. Furthermore, the C50336::dam cultured without IPTG still induced relatively lower levels of cytotoxicity, IL-1 β and caspase-1 compared with C50336-

pMMB207. However, inflammasome activation induced by C50336::dam was significantly stronger than that induced by C50336 Δ dam. Taken together, our results demonstrate that different expression levels of Dam could lead to different degrees of inflammasome activation during SE infection in vivo, suggesting that there may be a complex interaction between Dam and the inflammasome.

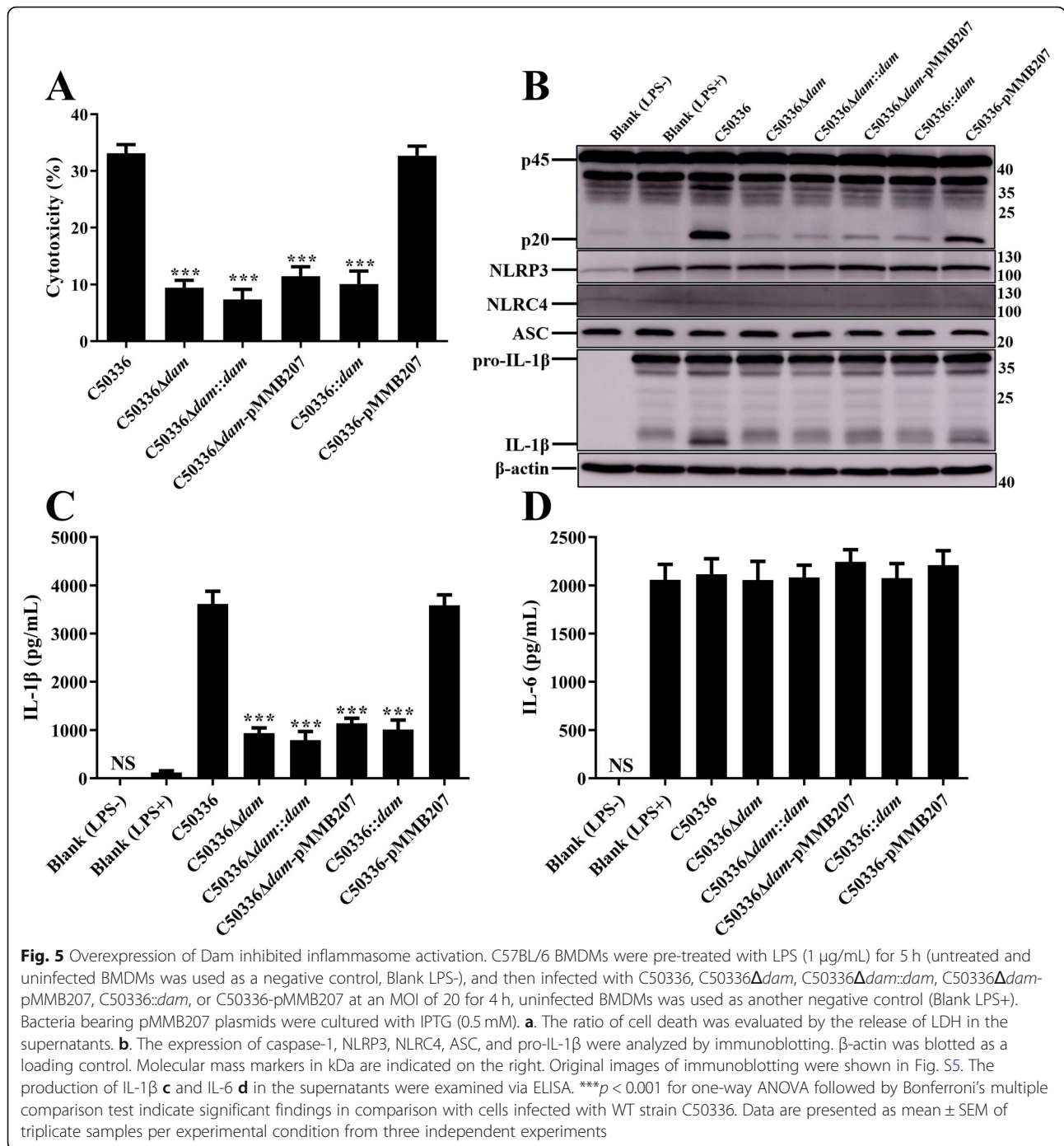
Global transcriptome of J774A.1 cells infected with SE

Since the expression level of Dam was found to be important in regulating inflammasome activation, transcriptome analysis of J774A.1 cells infected with C50336, C50336 Δ dam, or C50336::dam (cultured with IPTG) strains were carried out. The criteria for the selection of



differentially expressed genes (DEGs) were false discovery rate (*p*-adjust) < 0.05 and a fold change value |log₂-Ratio| ≥ 1. In total, 2237 DEGs were identified between the C50336-infected and C50336Δ*dam*-infected groups, including 2039 up-regulated genes and 198 down-regulated genes (Fig. 7a). Similarly, 2526 DEGs were found in the C50336-infected group compared with the C50336::*dam*-infected group, with 2151 genes up-regulated and

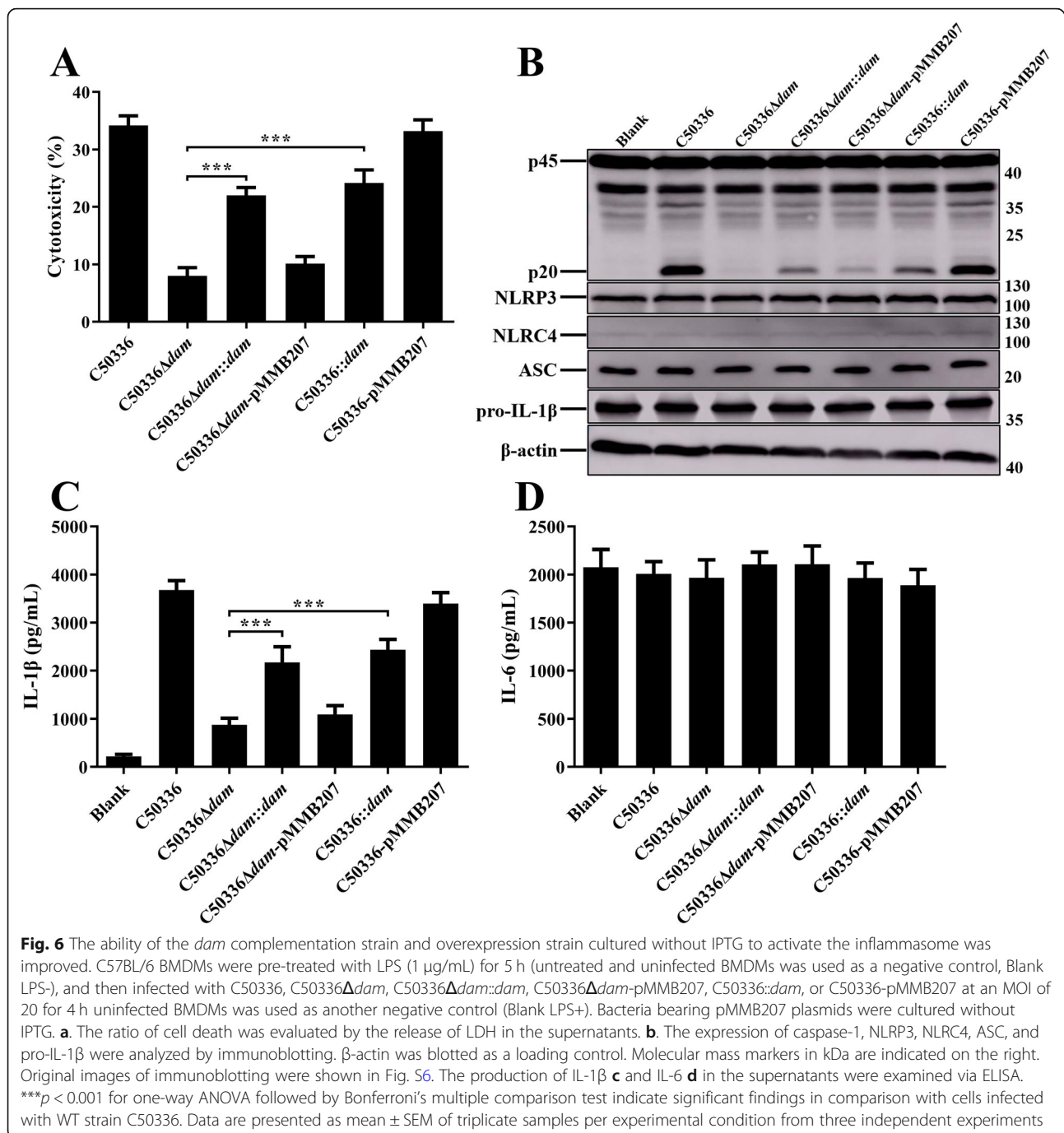
375 genes downregulated (Fig. 7b). Only 56 DEGs were screened between the C50336Δ*dam*-infected and the C50336::*dam*-infected groups (Fig. 7c), indicating that the gene expression pattern was similar between these two groups, which was further confirmed by Venn diagram analysis. As shown in Fig. 7d, 1738 overlapping genes were identified between the C50336-VS-C50336Δ*dam* and C50336-VS-C50336::*dam* groups,



suggesting that the deletion and overexpression of Dam may had a similar effect in modulating cell responses during SE infection.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed with the DEGs between these three groups to identify the potential pathways and genes affected by Dam. The 30 pathways that were most significantly enriched were shown in Fig. 8. The most significantly enriched pathways were

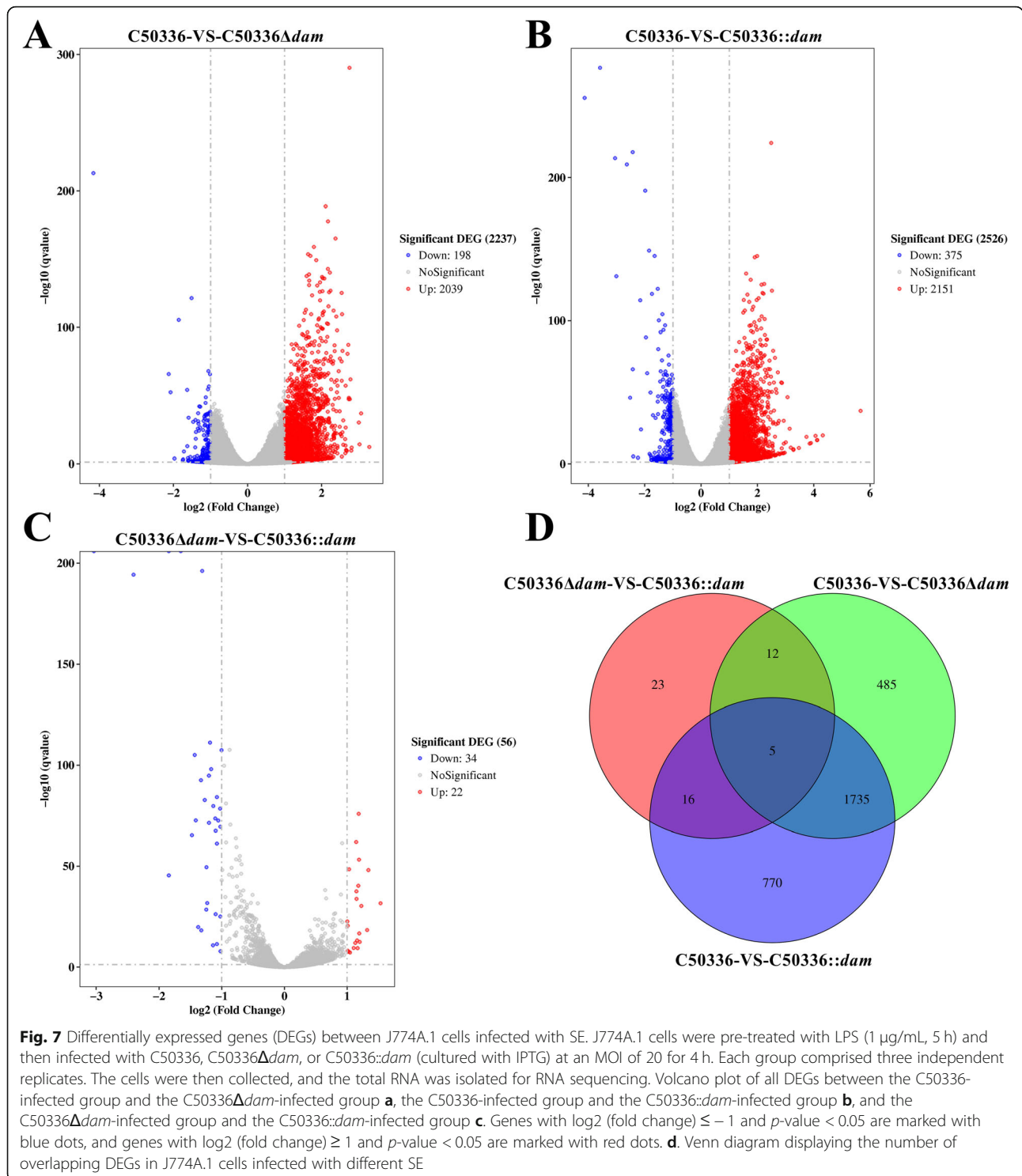
related to cancer, mitogen-activated protein kinase (MAPK) signaling, and human papillomavirus infection between the C50336-infected group and the C50336 Δdam -infected group (Fig. 8a). The significantly enriched pathways were mainly involved in viral carcinogenesis, MAPK signaling, and tumor necrosis factor (TNF) signaling between the C50336-infected group and the C50336: dam -infected group (Fig. 8b). As expected, 18 overlapping pathways were found in the top 30 significantly enriched pathways between



the C50336-VS-C50336 Δ *dam* group and the C50336-VS-C50336::*dam* group, which further suggested that the deletion and overexpression of Dam may play a similar role in regulating cellular processes during SE infection. In contrast, the most significantly enriched pathways within a maximum of 10 DEGs were cytokine-cytokine receptor interaction, TNF signaling, IL-17 signaling, and AGE-RAGE signaling in diabetic complications between the C50336 Δ *dam*-infected group and C50336::*dam*-infected group (Fig. 8c).

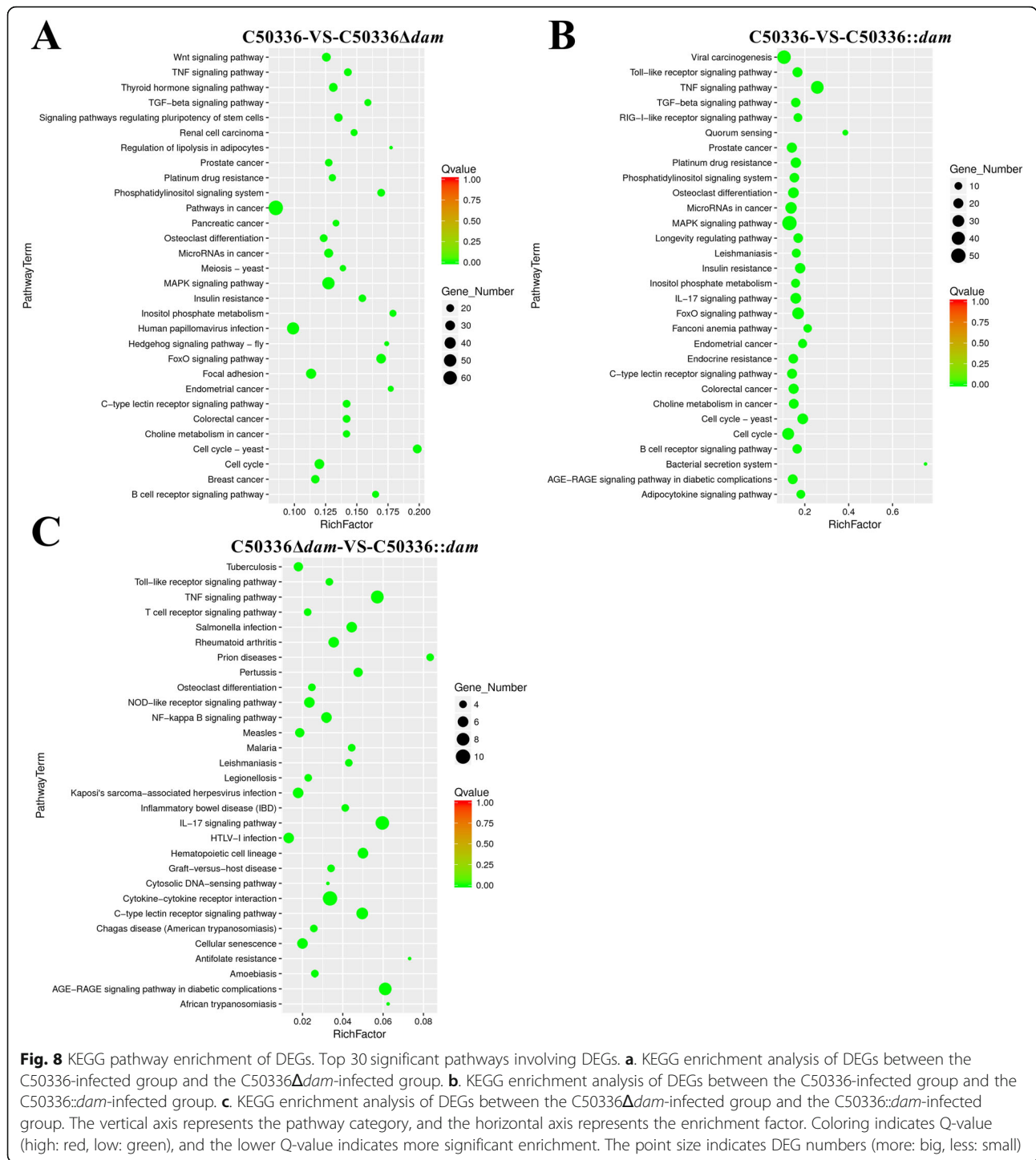
Identification of the expression patterns of DEGs based on quantitative real-time PCR

We verified RNA-seq results by selecting the differentially expressed genes in infected J774A.1 cells including 7 overlapping DEGs from MAPK signaling pathway, 7 overlapping DEGs from transforming growth factor- β (TGF- β) signaling pathway, 8 overlapping DEGs from TNF signaling pathway, and 8 overlapping DEGs from forkhead box, sub-group O (FoxO) signaling pathway between the C50336-VS-C50336 Δ *dam* and C50336-VS-



C50336::dam groups. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the gene expression. The expression of the selected overlapping DEGs was consistent with the results of RNA-seq in both C50336-VS-C50336 Δ dam (Fig. 9) and C50336-VS-C50336::

dam groups (Fig. 10). In addition, the fold changes of overlapping DEGs were similar between the C50336-VS-C50336 Δ dam and C50336-VS-C50336::dam groups indicating that both deletion and overexpression of Dam played a similar role of regulating cellular processes during SE

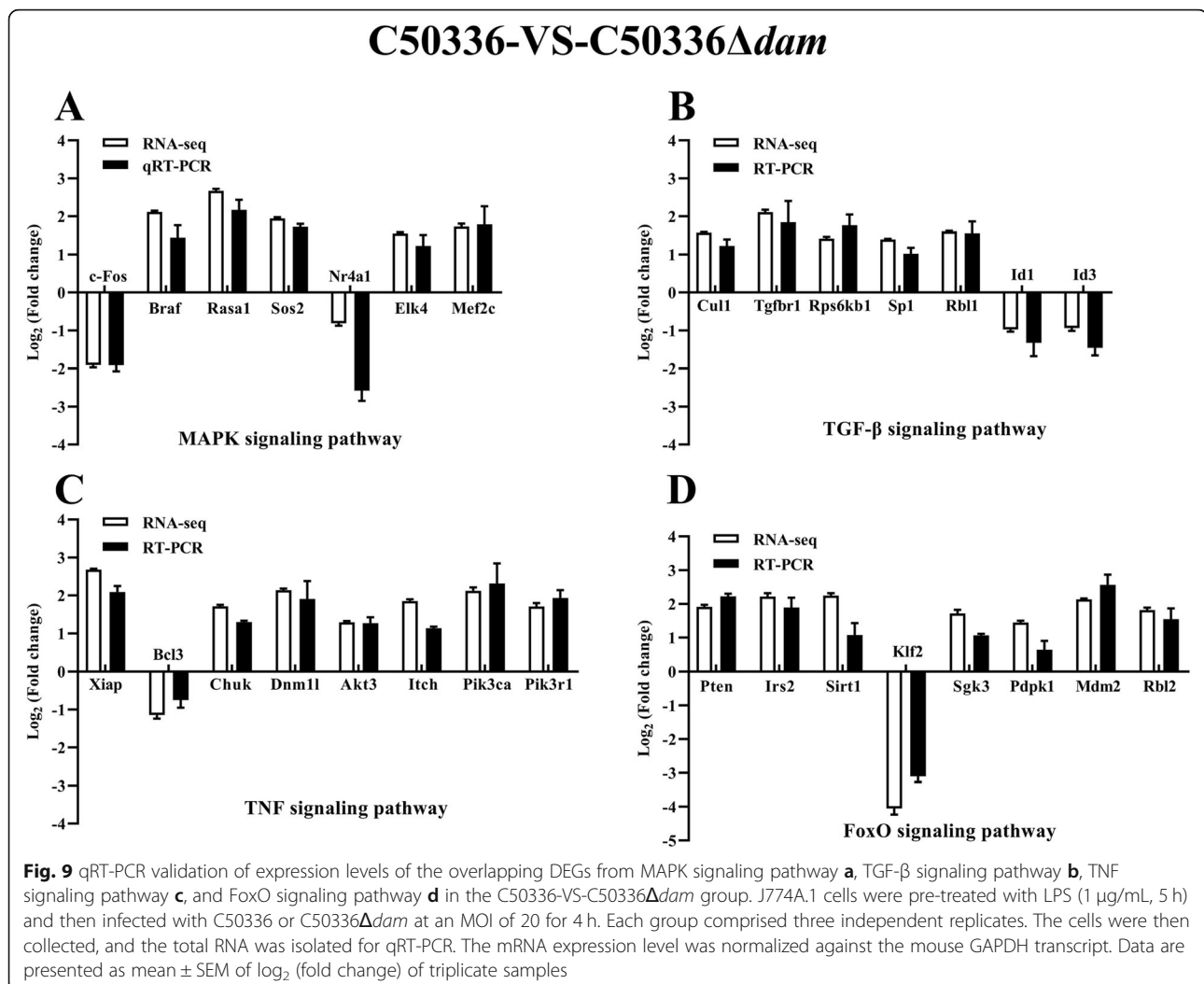


infection. These results demonstrated that the expression levels of Dam was essential for the regulation of these signaling pathways.

Dam was required for Jnk pathway activation in vitro

To further explore the mechanism of Dam regulates the activation of inflammasome, western blot was used to

determined the activation of MAPK pathway based on the data of RNA-seq. The phosphorylation level of c-Jun N-terminal kinase (Jnk) induced by dam mutant strain was significantly lower than that induced by C50336 in J774A.1 cells, which was accompanied with the decreased activation of caspase-1 (Fig. 11). The Jnk phosphorylation was more significantly induced by dam

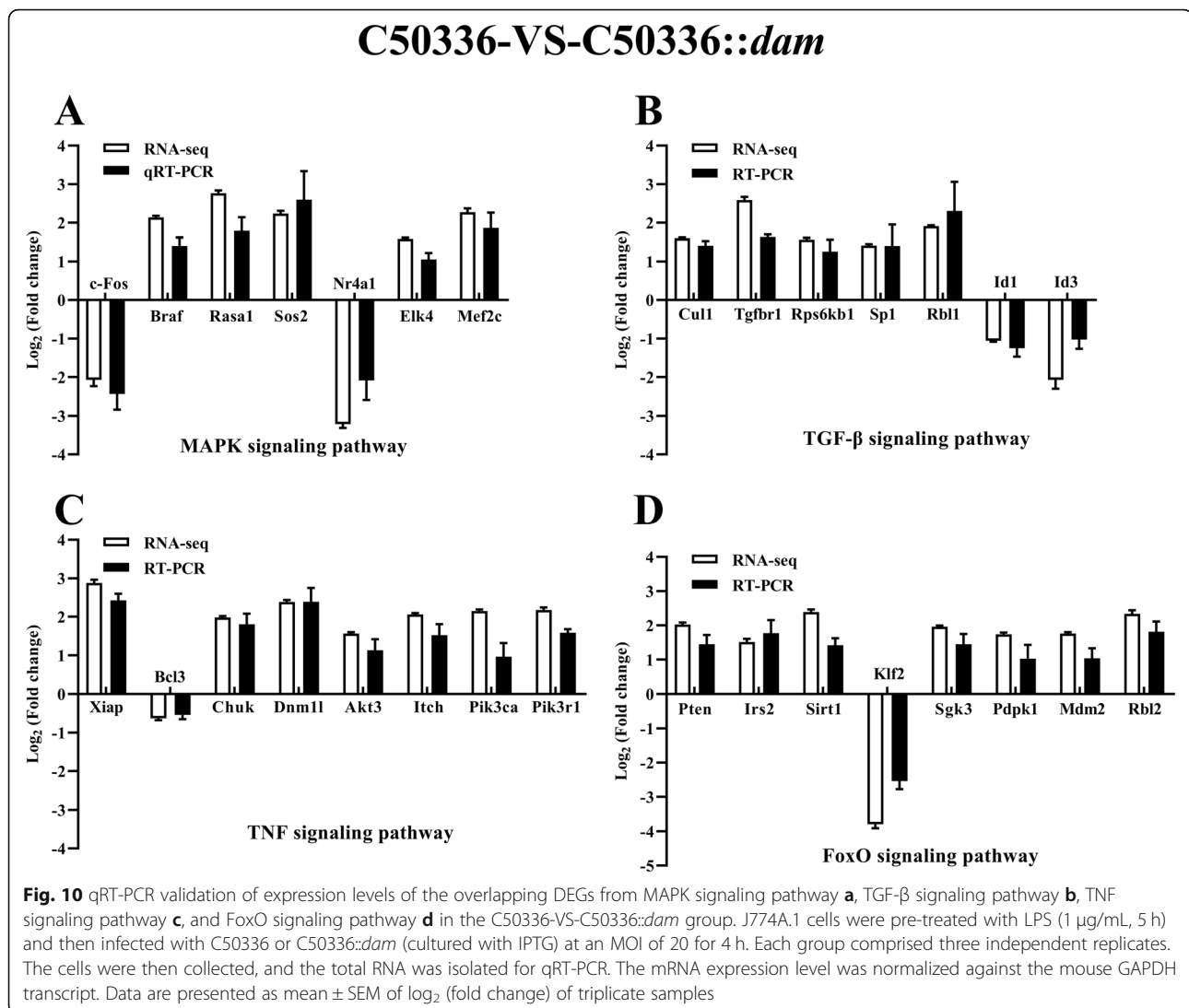


complementation strain than by C50336 Δ dam. In addition, the overexpression of Dam also induced a lower phosphorylation level of Jnk compared to WT. Furthermore, the phosphorylation level of p38 and extracellular regulated protein kinases 1/2 (ERK1/2) induced by C50336 Δ dam was slightly lower than that induced by C50336, which was not significant. These results suggested that deletion of *dam* blocked the Jnk pathway in infected macrophages, which led to the inhibition of inflammasome activation.

Dam promoted NLRP3 inflammasome activation independently via Jnk pathway in lentiviral infection assays

To further explore the effect of Dam on inflammasome activation, lentiviral vectors pGLV5-*dam* (EF-1aF/GFP&Puro) were constructed, and the lentivirus LV5-Dam particles were packaged and transfected into J774A.1 cells. To test the efficacy of lentivirus delivery

into the macrophages, green fluorescent protein (GFP) expression was examined. The cells with LV5-Dam or LV5-negative lentivirus treatment exhibited robust GFP expression, indicating the effective infection of lentivirus in mice macrophages. After LPS priming, J774A.1 cells were stimulated with a classical NLRP3 inflammasome activator adenosine triphosphate (ATP, 1.25 mM). Significantly up-regulated inflammasome signals were detected in Dam-expressing J774A.1 cells, compared with those in negative lentivirus transduced cells and control cells (Fig. 12a and c). Furthermore, the expression of IL-6 and the synthesis of NLRP3 inflammasome components, including NLRP3, ASC, and pro-IL-1 β were not affected by lentiviral infection (Fig. 12b and c). In addition, the deletion of *dam* failed to induce Jnk phosphorylation in infected J774A.1 cells, the phosphorylation of Jnk was also determined by immunoblotting with the infection of lentivirus LV5-Dam. As expected, there was a significant

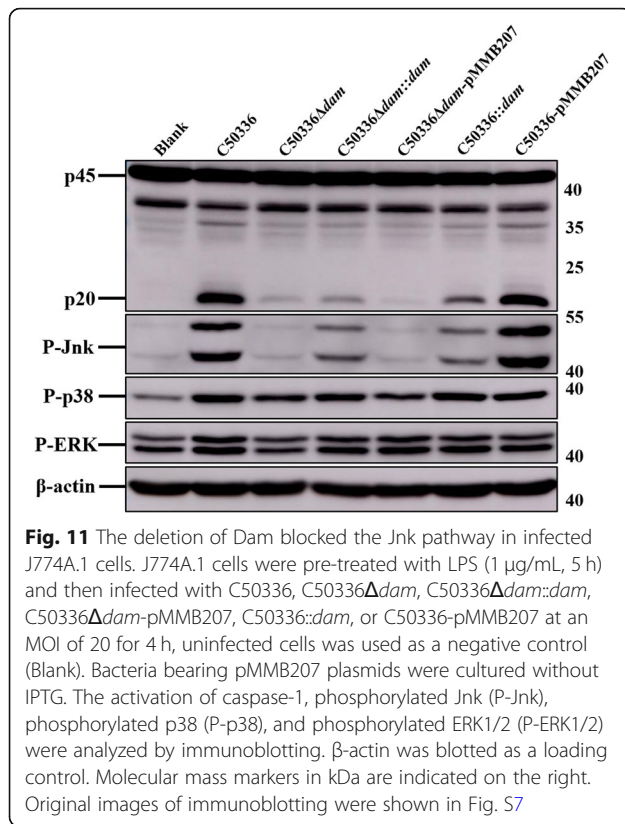


increased phosphorylation level of Jnk in Dam-expressing macrophages compared to those in negative lentivirus transduced cells and control cells. These results demonstrated that Dam could specifically enhance the NLRP3 inflammasome activation independently via promoting Jnk activation in macrophages.

Discussion

Inflammatory caspases are multi-functional proteins, and activation of caspases not only play an essential role in mediating host defense against infection by pathogens but are also indispensable for the regulation of tumor development, metabolic syndromes, autoinflammatory disease, tissue repair, and cell survival [25]. NLRP3 and NLRP3 inflammasomes can detect exogenous and endogenous molecules that serve as indicators of *Salmonella* infection. Inflammasome

activation triggers the secretion of the pro-inflammatory cytokines IL-1 β and IL-18, as well as rapid cell lysis. Intracellular *Salmonella* is then released into the extracellular space, which can be a double-edged sword for both the host and bacteria. *Salmonella* exposed to neutrophil-mediated killing is extremely fragile [26], but the interaction also provides an opportunity for *Salmonella* to invade the intestinal epithelium or access the liver and spleen, which may promote systemic infection [27]. Inflammasomes play a key and multilayered role at distinct stages of immune defense against *Salmonella* infection, although unexpected redundancy was found among NLRP3 and NLRC4 in vivo [16]. To further understand the complex interplay of detection and evasion between SE and the inflammasome, a transposon library based on SE strain C50336 was

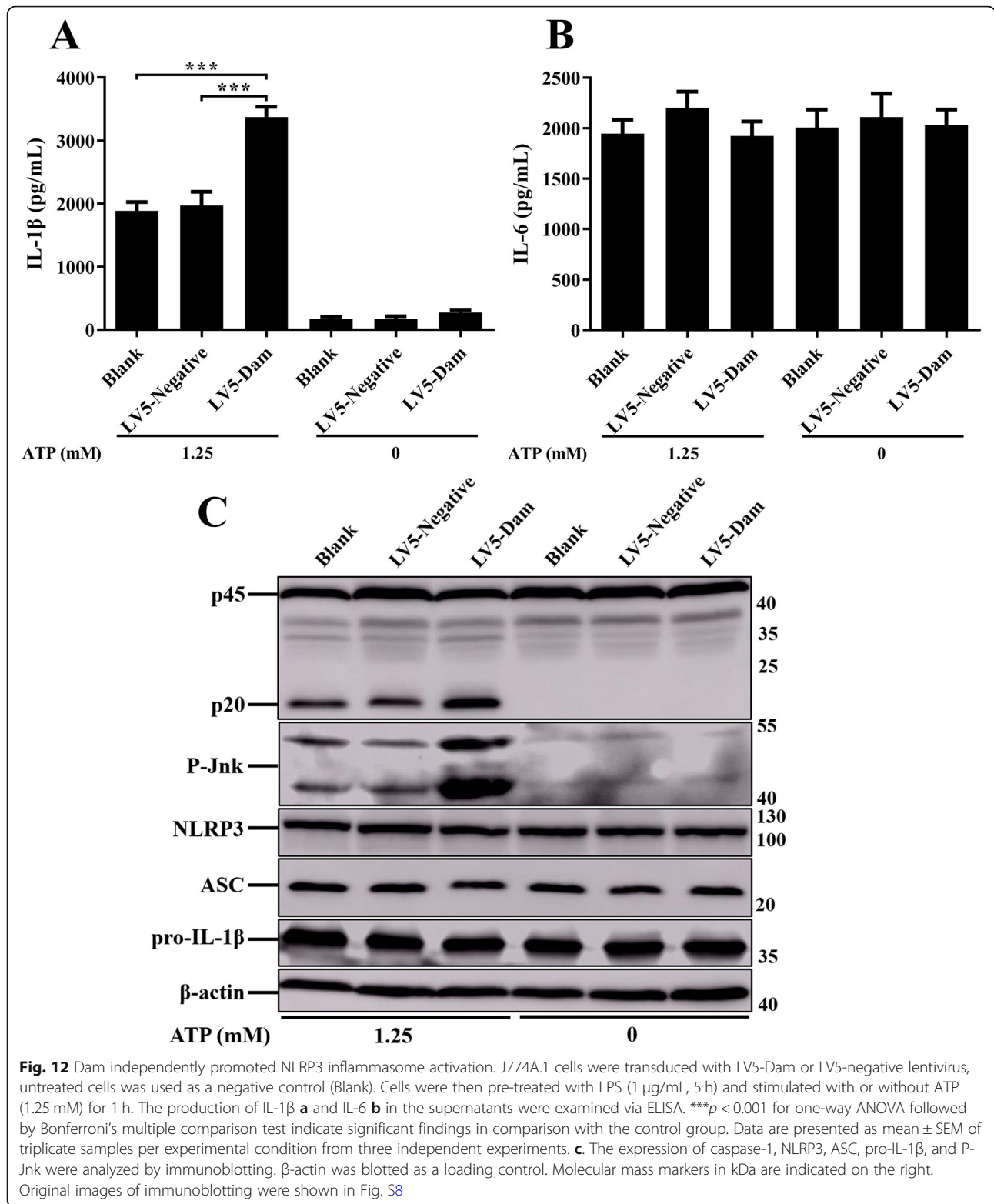


generated and screened in this study. The mutant strains were identified by increased or decreased LDH release compared to the WT C50336 parental strain after infection of J774A.1 cells in the first round of screening. Then, the candidate mutant strains were further rescreened for LDH release and activation of caspase-1 in the second round screening. Four T3SS related genes (*dam*, *invC*, *prgH*, and *spaN*) were identified after two rounds of screening, which the mutants of these four genes failed to activate the inflammasome indicating the importance of T3SS during modulating inflammasome activation. Our study offers new insights into understanding the interactions between SE and the inflammasome.

T3SS, as an important apparatus for inserting virulence factors into host cells, is often utilized by *Salmonella* to regulate the inflammasome responses. T3SS1 effector SopB was found to inhibit the activation of NLR4 inflammasome during ST infection [19]. The SPI-2 T3SS is employed by ST to subvert human NLRP3 and NLR4 inflammasome activation [28]. An essential component of T3SS is a highly conserved SPI-1 associated ATPase InvC in ST, which could couple with proton motive force to provide energy for the secretion process [29]. The deletion of *invC* gene resulted in a significant decrease in

secretion and translocation of effectors, which is required for *Salmonella* entry into epithelial cells [30]. Additionally, we also identified the *spaN* gene, which was known to be a target of the invasion secretion system [31]. The invasion-defective *invC* mutant of ST failed to secrete SpaN upon contact with live epithelial cells [32], which could explain why InvC and SpaN mutant strains are defective in activation of the inflammasome. In addition, InvC and SpaN were both reported to be essential for *Salmonella* virulence in a murine model [33]. The InvC deficient mutant of ST significantly reduced virulence in swine [34].

The needle complex (NC) is a structure of connected oligomeric rings assembled by T3SS that spans both membranes of bacteria and host cells for the delivery of virulence effectors [35]. The earliest step in NC morphogenesis is the association of PrgH and PrgK to form a base substructure and inner membrane rings. PrgH and PrgK are demonstrated to be the only T3SS components that are absolutely required for the formation of NC [36]. The previous studies showed that the NC rod proteins PrgI and PrgJ can be detected by NLR4, and resulting in rapid activation of the inflammasome [14]. However, the rod proteins could not be detected in a correct location within the T3SS apparatus [11]. If T3SS exports excess rod monomers from the bacterial cytosol or if rod monomers slough into the interior channel of the needle apparatus, NLR4 can respond to such inadvertent translocation of PrgI and PrgJ monomer [37]. In this study, the β-lactamase-based translocation assay indicated that the PrgH of SE could be transferred into host cells (Fig. 3). The transfer efficient of PrgH-TEM fusion protein was lower, and this is understandable considering that PrgH is a structural protein of NC and may also depend on inadvertent translocation, like PrgJ [11]. The persistent overexpression of PrgJ resulted in strong detection via NLR4 and the complete clearance of ST in mice [38]. However, overexpression of PrgH induced by exogenous plasmid pMMB207 failed to trigger stronger expression of bioactive caspase-1 and IL-1β in this work, indicating that NLR4 could not directly detect PrgH to activate the inflammasome. None of the mutant strains C50336Δ*invC*, C50336Δ*spaN*, and C50336Δ*prgH* presented here decreased the mortality rate in the C57BL/6 mice model, in comparison with WT C50336. Whereas all of three gene deletion mutant strains were defective in inducing macrophage death in vitro, suggesting that the role of *Salmonella* SPI-1 T3SS in pathogenesis was different in vivo and in vitro. Besides, there may be other potential mechanisms for T3SS-defective SE mutants to induce activation of inflammasome in vivo.



Crucial for DNA methylation, Dam modulates bacterial gene expression through N-6 methylation of adenosine residues in GATC sites [39]. While a broad range of

processes are modulated by Dam, such as DNA replication and mismatch repair, chromosome segregation, surface protein expression, and transcription of insertion

elements, lack of Dam does not impair bacterial viability [22, 40]. In this study, a *dam* deletion mutant strain of SE was found to be defective in inducing activation of the inflammasome and pyroptosis, which was consistent with the findings of another study that found that IL-1 β secretion is reduced in the absence of Dam compared with a WT ST strain [41]. Unlike the T3SS component deletion strains, *dam* mutations had an LD₅₀ that was > 10⁴ higher than the WT parent strain in mice [22]. Similar results were found in this study. *Salmonella* Dam mutants were also attenuated for virulence in avian [42, 43], sheep [44], and bovine models [45, 46], which has been shown to confer cross-protective immunity to multiple *Salmonella* to prevent salmonellosis in animals and have been demonstrated as safe. Previous studies indicated that Dam is involved in the regulation of LPS synthesis, flagellar genes, fimbrial genes, and SPI-1 effector genes, and *prgH* also showed Dam-dependent regulation in both stationary-phase and static cultures [23], which seemed to be correlated with certain defects in the induction of pyroptosis and caspase-1 activation. Even then, *dam* mutants were able to persist in the liver, spleen, and lymph nodes of infected mice [47]. One likely explanation was that the reduced activation of the inflammasome induced by *dam* mutants resulted in *Salmonella* colonization in organs that could not be subjected to rapid and complete clearance, given that pyroptosis and secretion of IL-1 β and IL-18 were an effective mechanism by which caspase-1 clears *Salmonella* [15]. A previous study showed that an ST strain that persistently expresses the flagellin protein FlhC failed to evade NLRC4 detection and had been cleared efficiently [14].

Interestingly, the overexpression of Dam still prevented caspase-1 activation and cell death, which was consistent with the findings that a Dam-overproducing ST strain was highly attenuated in mice [22]. Therefore, we speculated that the deletion and overexpression of Dam may have a similar effect on infected macrophages, and confirmed by RNA-seq and quantitative real-time PCR (qRT-PCR). In infected macrophages, the number of overlapping DEGs and enriched pathways were very high between the C50336-VS-C50336 Δ *dam* and C50336-VS-C50336::*dam* groups, suggesting that homeostasis of *dam* expression was essential for *Salmonella* virulence and the ability to induce inflammasome activation. The MAPK signaling pathway [48], TGF- β signaling pathway [49], TNF signaling pathway [50, 51], and FoxO signaling pathway [52] from overlapping enriched pathways were all reported to be related to the activation of inflammasome. The transcription regulator c-Fos plays an important role in regulating multiple signal pathways including MAPK and TNF [53]. A recent study reported that the c-Fos could bind to the promoter region of NLRP3

gene and positively regulate the inflammasome activation [54]. In this study, the expression levels of c-Fos induced by C50336 Δ *dam* and C50336::*dam* were both significantly lower than that induced by WT in J774A.1 cells (Figs. 9 and 10), which could explain the defective activation of caspase-1 in both strains. Braf is an adaptor kinase in the MAPK pathway that transmits stimulatory signals of receptor binding growth factors [55]. Braf inhibitors were reported to upregulate the inflammasome activation and the production of IL-1 β in dendritic cells [56]. Cullin1 (Cul1) was a key component of the Skp1-Cul1-F-box E3 ligase, which could bind and promote NLRP3 ubiquitination to repress systematic inflammasome activation [57]. Tgfb1 and Pten were able to inhibit the expression of NLRP3, ASC, caspase-1, IL-1 β , and IL-18 in a mouse squamous cell carcinoma of the head and neck model [58]. X-linked inhibitor of apoptosis protein (Xiap) was reported to restrict TNF- and RIP3-dependent cell death and inflammasome activation [50]. Besides, the IKK α (Chuk) was reported as a critical negative regulator of ASC-dependent inflammasomes, while the loss of IKK α kinase activity result in inflammasome hyperactivation [59]. In the FoxO pathway, the overexpression of Irs2 was able to inhibit the IL-1 β promoter activity [60]. The anti-inflammatory factor Sirtuin 1 (Sirt1) is a conserved NAD-dependent protein deacetylase, which has been demonstrated to attenuate the activation of NLRP3 inflammasome [61]. In this study, we observed that both C50336 Δ *dam* and C50336::*dam* could induce the high expression level of anti-inflammasome factors including Braf, Cul1, Tgfb1, Pten, XIAP, Chuk, Irs2, and Sirt1 (Figs. 9 and 10), which could further explain why inflammasome could not be activated in the Dam deletion and overexpression strains.

A previous study demonstrated that apoptotic cell rates and NOS-2 and COX-2 expression were diminished in *dam* mutant strain-infected cells, coinciding with impaired activation of p38 and ERK MAPKs [62]. However, our result showed that the absence of Dam could not sufficiently inhibit the phosphorylation level of p38 and ERK1/2 for blocking the inflammasome activation. In addition, the Jnk pathway has been shown to regulate both at the priming stage and activation stage of NLRP3 inflammasome activation [63]. The phosphorylated Jnk is essentially required for ASC oligomerization, which is a critical step for NLRP3 inflammasome activation [64]. Recent studies reported that Jnk inhibitor could mediate Jnk inactivation, and further decrease the secretion of IL-1 β and NLRP3 inflammasome activation [65]. In this study, both the deletion and overexpression of Dam decreased the phosphorylation level of Jnk, which could be related to the failure of activating inflammasome in SE infected

macrophages, indicating that Jnk might become a target for SE in manipulating inflammasomes.

Moreover, the most surprising discovery in this study was that SE Dam specifically enhanced the activation of NLRP3 inflammasome independently via promoting Jnk phosphorylation in the absence of bacteria. Unlike the T6SS effector EvpP of *Edwardsiella tarda*, which could suppress activation of the NLRP3 inflammasome independently by preventing Jnk activation in macrophages [66]. Dam was not a direct activator and was unable to activate the NLRP3 inflammasome without the help of ATP, indicating that the function of Dam expressed in host cells independently may not be the same as that in bacteria. It is accepted that the extracellular stimuli could promote NLRP3 inflammasome activation by inducing various molecular mechanisms including lysosomal permeability, potassium efflux, and mitochondrial reactive oxygen species (ROS) production [67, 68]. The activation of Jnk pathway could enhance the activation of NLRP3 inflammasome through the production of MAPKs upstream signal ROS [65]. In this study, the expression level of Nr4a1 induced by C50336 was significantly higher than that induced by C50336 Δ dam, while the overexpression of Nr4a1 could enhance the production of ROS [69]. Jnk-mediated caspase activation has been known to enhance the cleavage of Xiap and disrupt its function [70]. Pten inactivation was associated with increased activity of the Jnk pathway in human prostate cancer [71]. The expression level of Sirt1 protein was reported to be downregulated via Jnk activation-mediated protein degradation [72]. In this study, the expression level of Xiap, Pten, and Sirt1 induced by WT C50336 was significantly lower than that induced by dam deletion mutant strain, which is consistent with studies mentioned above. The increasing of intracellular Ca²⁺ was essential for activation of NLRP3 inflammasome [73]. The ASC oligomerization requires the release of Ca²⁺, which could activate the TAK1-Jnk pathway by an upstream kinase TAK1, and trigger the activation of NLRP3 inflammasome [63]. Base on the findings above, we speculated that Dam might have a potential function in regulating Ca²⁺ release via Jnk pathway, which could subsequently influence the inflammasome activation.

Conclusions

In summary, we identified that Dam is a regulator of T3SS, which play an essential role in inducing inflammasome activation and pyroptosis during SE infection. We first described that the deletion and overexpression of Dam had a similar effect on inducing the macrophage death and secretion of inflammatory cytokines. The results of RNA-seq and qRT-PCR

suggested that both C50336 Δ dam and C50336::dam were defective in inhibiting the expression of several anti-inflammasome factors (such as Braf, Cul1, Xiap, Pten, and Sirt1) compared to WT. In addition, both the absence and overexpression of Dam blocked the activation of Jnk pathway during SE infection. To our knowledge, it was the first demonstration that Dam could enhance the activation of the NLRP3 inflammasome independently via promoting Jnk phosphorylation in the absence of bacteria. Further research will focus on exploring more specific molecular mechanism of Dam regulates the activation of inflammasome and the effect of different expression levels of Dam on inflammasome activation. Collectively, our results provides a novel role of Dam in modulating inflammasome response and offers a new insights of the complex interplay between Dam and the inflammasome.

Methods

Bacterial strains

The wild-type SE strain C50336 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Escherichia coli* X7213 λ pir is stored in our laboratory. The bacterial strains and plasmids used in this study are listed in Table 1. The gene deletion mutant strains were constructed by double exchange of homologous recombination as described previously [77]. The upstream and downstream fragments of target genes were amplified using PCR by employing the primers listed in Table 2. The PCR products were purified using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver 4.0 (TaKaRa Biotechnology Co. Ltd., Dalian, China). The pDM4 plasmid was digested by restriction endonucleases *Sal* I and *Sac* I (TaKaRa). The purified plasmid and upstream and downstream fragments were fused using the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotechnology Co. Ltd., Nanjing, China). The recombinant plasmids were transferred into X7213 λ pir cells and sequenced. Single-crossover mutants were obtained by conjugal transfer of the recombinant suicide plasmids into C50336. Deletion mutants were screened on 15% sucrose Luria-Bertani (LB) plates. Construction of gene complementation strains and overexpression strains were performed as described previously [24]. Target genes were cloned into the low copy number plasmid pMMB207 by employing the ClonExpress II One Step Cloning Kit (Vazyme). The recombinant plasmids were mated from X7213 λ pir into mutant or WT C50336 by conjugation. Chloramphenicol resistant transconjugants were selected, and the presence of the plasmids was confirmed by PCR analysis with flanking primers and sequencing.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Escherichia coli		
X7213 λ pir	Host for π requiring plasmids, conjugal donor	Laboratory collection
X7213 λ pir-pSC189	X7213 λ pir with pSC189, Km ^r , Cm ^r	This study
Salmonella Enteritidis		
C50336	Wild type	Obtained from Chinese National Institute for the Control of Pharmaceutical and Biological
C50336 Δ dam	C50336, In-frame deletion in <i>dam</i>	This study
C50336 Δ invC	C50336, In-frame deletion in <i>invC</i>	This study
C50336 Δ hilD	C50336, In-frame deletion in <i>hilD</i>	This study
C50336 Δ prgH	C50336, In-frame deletion in <i>prgH</i>	This study
C50336 Δ spaN	C50336, In-frame deletion in <i>spaN</i>	This study
C50336 Δ dam::dam	C50336 Δ dam with pMMB207 expressing the <i>dam</i> gene, Cm ^r	This study
C50336 Δ invC::invC	C50336 Δ invC with pMMB207 expressing the <i>invC</i> gene, Cm ^r	This study
C50336 Δ hilD::hilD	C50336 Δ invC with pMMB207 expressing the <i>hilD</i> gene, Cm ^r	This study
C50336 Δ prgH::prgH	C50336 Δ prgH with pMMB207 expressing the <i>prgH</i> gene, Cm ^r	This study
C50336 Δ spaN::spaN	C50336 Δ dam with pMMB207 expressing the <i>dam</i> gene, Cm ^r	This study
C50336-pMMB207	C50336 with pMMB207, Cm ^r	This study
C50336::dam	C50336 with pMMB207 expressing the <i>dam</i> gene, Cm ^r	This study
C50336::prgH	C50336 with pMMB207 expressing the <i>prgH</i> gene, Cm ^r	This study
C50336 Δ dam-pMMB207	C50336 Δ dam with pMMB207, Cm ^r	This study
C50336 Δ prgH-pMMB207	C50336 Δ prgH with pMMB207, Cm ^r	This study
C50336-pCX340	C50336 with pCX340, Tet ^r	This study
C50336-pCX340-dam	C50336 with pCX340 expressing the <i>dam</i> gene, Tet ^r	This study
C50336-pCX340-invC	C50336 with pCX340 expressing the <i>invC</i> gene, Tet ^r	This study
C50336-pCX340-prgH	C50336 with pCX340 expressing the <i>prgH</i> gene, Tet ^r	This study
C50336-pCX340-spaN	C50336 with pCX340 expressing the <i>dam</i> gene, Tet ^r	This study
Plasmids		
pSC189	Transposon delivery vector, R6K, Km ^r , Cm ^r	[74]
pDM4	Suicide vector, <i>pir</i> dependent, R6K, <i>SacBR</i> , Cm ^r	[75]
pMMB207	IncQ <i>lacI^q</i> Δ <i>bla</i> P _{tac-lac} <i>lacZ</i> α , Cm ^r	[24]
pMMB207-dam	pMMB207 derivative containing <i>dam</i> , Cm ^r	This study
pMMB207-invC	pMMB207 derivative containing <i>invC</i> , Cm ^r	This study
pMMB207-hilD	pMMB207 derivative containing <i>hilD</i> , Cm ^r	This study
pMMB207-prgH	pMMB207 derivative containing <i>prgH</i> , Cm ^r	This study
pMMB207-spaN	pMMB207 derivative containing <i>spaN</i> , Cm ^r	This study
pCX340	pBR322 derivative, cloning vector used to fuse effectors to TEM-1- β -lactamase, Tet ^r	[76]
pCX340-dam	pCX340 derivative containing <i>dam</i>	This study
pCX340-invC	pCX340 derivative containing <i>invC</i>	This study
pCX340-prgH	pCX340 derivative containing <i>prgH</i>	This study
pCX340-spaN	pCX340 derivative containing <i>spaN</i>	This study

The antibiotics as follows: kanamycin (Km^r), 100 μ g/mL; chloramphenicol (Cm^r), 25 μ g/mL; tetracycline (Tet^r), 12.5 μ g/mL

Table 2 Primers used in this study

Primer name	Primer sequence (5' to 3')	Target
AB1	GGCCACGCGTCTGACTAGTACNNNNNNNNNACGCC	For transposon insertion sequencing
AB2	GGCCACGCGTCTGACTAGTACNNNNNNNNNCCTGG	For transposon insertion sequencing
AB3	GGCCACGCGTCTGACTAGTACNNNNNNNNNCCTCG	For transposon insertion sequencing
ABS	GGCCACGCGTCTGACTAGTAC	For transposon insertion sequencing
SP1	GCTGACCGCTTCCTCGTCTTTACG	For transposon insertion sequencing
SP2	CATCGCCTTCTATCGCCTTCTTGAC	For transposon insertion sequencing
pSC189-seq	CGCGAAGTTCCTATCCGAAGTTC	For transposon insertion sequencing
pDM4-F	GGTGCTCCAGTGGCTTCTGTTTCTA	For deletion mutants
pDM4-R	CAGCAACTTAAATAGCCTCTAAT	For deletion mutants
<i>dam</i> -up-F	GAGCGGATAACAATTTGTGGAATCCCGGAGACGCC GAAAGCGACCACCACGACG	For <i>dam</i> deletion mutant
<i>dam</i> -up-R	TTGAGAATTACATGCTGACTAACTAATTACACCTT	For <i>dam</i> deletion mutant
<i>dam</i> -down-F	AGTCAGCATGTAATTCTCAAGGAGAAGCGGATGAA	For <i>dam</i> deletion mutant
<i>dam</i> -down-R	AGCGGAGTGTATATCAAGCTTATCGATACCCGATCTC GCCGATATTGTTACCTT	For <i>dam</i> deletion mutant
<i>dam</i> -in-F	CGAGTGCCTTGTGCAACCTTTTGTG	For <i>dam</i> deletion mutant
<i>dam</i> -in-R	ACAGAGCCAGCAGTTCGTCCACCTT	For <i>dam</i> deletion mutant
<i>dam</i> -out-F	AACCACGACTGCGGAACCGAAGAAA	For <i>dam</i> deletion mutant
<i>dam</i> -out-R	TCGGGTTTATCGAAAATTGCCGACC	For <i>dam</i> deletion mutant
<i>invC</i> -up-F	GAGCGGATAACAATTTGTGGAATCCCGGAGTCCTC CTTACGTCTGTGATGTCC	For <i>invC</i> deletion mutant
<i>invC</i> -up-R	GCGAATGCATTATCTCATTAGCGACCGACTAAAA	For <i>invC</i> deletion mutant
<i>invC</i> -down-F	AATGAGATGAATGCATTCTGCTGACCAGAATTAAG	For <i>invC</i> deletion mutant
<i>invC</i> -down-R	AGCGGAGTGTATATCAAGCTTATCGATACCAATGCTT CTGATAAACCGCCAACCT	For <i>invC</i> deletion mutant
<i>invC</i> -in-F	GGGAACGCACCGTGTGAGCCTTAT	For <i>invC</i> deletion mutant
<i>invC</i> -in-R	CCAGGACGATATTCTCCCAAGTCAA	For <i>invC</i> deletion mutant
<i>invC</i> -out-F	CAGTACCTTCTCAGCCTTGACCCG	For <i>invC</i> deletion mutant
<i>invC</i> -out-R	GCATTACGAAAGCATCGCCATAGTC	For <i>invC</i> deletion mutant
<i>hilD</i> -up-F	GAGCGGATAACAATTTGTGGAATCCCGGAGTCAT GGAGTATAATTTGCGTCTG	For <i>hilD</i> deletion mutant
<i>hilD</i> -up-R	AAAAATGTTACATATTATCCCTTTGTTGATGTTAT	For <i>hilD</i> deletion mutant
<i>hilD</i> -down-F	GGATAATATGTAACATTTTTGTATCTGTCACTTA	For <i>hilD</i> deletion mutant
<i>hilD</i> -down-R	AGCGGAGTGTATATCAAGCTTATCGATACCTGAAAGA CTGTTTTTAATGGTGCGC	For <i>hilD</i> deletion mutant
<i>hilD</i> -in-F	GTCAGACTCAGCAGTTACCATCAA	For <i>hilD</i> deletion mutant
<i>hilD</i> -in-R	CATTATGGTTGCCTATGCGTAAAAG	For <i>hilD</i> deletion mutant
<i>hilD</i> -out-F	TTCACCGACCTGTATTGGCGTATT	For <i>hilD</i> deletion mutant
<i>hilD</i> -out-R	TTTTGGGGTGAAATGCTGCTTATT	For <i>hilD</i> deletion mutant
<i>prgH</i> -up-F	GAGCGGATAACAATTTGTGGAATCCCGGACACCAA CATCCCAAGTTTCGTCACAG	For <i>prgH</i> deletion mutant
<i>prgH</i> -up-R	CGTTAAATTACATATATACTGTTAGCGATGTCTGT	For <i>prgH</i> deletion mutant
<i>prgH</i> -down-F	AGTATATATGTAATTTAACGTAATAAGGAAGTCA	For <i>prgH</i> deletion mutant
<i>prgH</i> -down-R	AGCGGAGTGTATATCAAGCTTATCGATACCTTCAACA GCCCCGACTCCTTACGA	For <i>prgH</i> deletion mutant
<i>prgH</i> -in-F	GTTTGCTGCTCGTTTGGGATAAGTG	For <i>prgH</i> deletion mutant
<i>prgH</i> -in-R	GGCAAGGGTCATTACCAGCAGAAAG	For <i>prgH</i> deletion mutant

Table 2 Primers used in this study (Continued)

Primer name	Primer sequence (5' to 3')	Target
<i>prgH</i> -out-F	GAACGGCTGTGAGTTCCATTGCTG	For <i>prgH</i> deletion mutant
<i>prgH</i> -out-R	GACGGGCTCTGAGTATTCTACATC	For <i>prgH</i> deletion mutant
<i>spaN</i> -up-F	GAGCGGATAACAATTTGTGGAATCCCGGGAATTGAC TTGGGAGAATATCGTCCTG	For <i>spaN</i> deletion mutant
<i>spaN</i> -up-R	AATGACATCACATTAATATCTCCTCTGACTCGG	For <i>spaN</i> deletion mutant
<i>spaN</i> -down-F	TAATTTAATGTGATGTCATTGCGTGTGAGACAGAT	For <i>spaN</i> deletion mutant
<i>spaN</i> -down-R	AGCGGAGTGTATATCAAGCTTATCGATACCCGCACGG GAAGTACGAATCAGGAGT	For <i>spaN</i> deletion mutant
<i>spaN</i> -in-F	CRACTATGGCGATGCTTTCGTAATG	For <i>spaN</i> deletion mutant
<i>spaN</i> -in-R	CAAACGATGTTCAACCTGCGTATTT	For <i>spaN</i> deletion mutant
<i>spaN</i> -out-F	AAAGCGTAAGCCGCGTTTTTGGACA	For <i>spaN</i> deletion mutant
<i>spaN</i> -out-R	CAATTGATTCAAGCCAGGCAGAGTT	For <i>spaN</i> deletion mutant
pMMB207-F	CTCCCGTTCTGGATAATGTT	For complemented mutants
pMMB207-R	GGCGTTTCACTTCTGAGTTCC	For complemented mutants
pMMB207- <i>dam</i> -F	AGCTCGGTACCCGGGATCCTCTAGCTAAAGGAAGA CGTTATGAAAAAATCGCGCTTTTTTGA	For complemented mutant of Δdam
pMMB207- <i>dam</i> -R	TCTCATCCGCCAAAACAGCCAAGCTTTATTTTCTTGA GGCGTTGCGACT	For complemented mutant of Δdam
pMMB207- <i>invC</i> -F	AGCTCGGTACCCGGGATCCTCTAGCTAAAGGAAGA CGTTATGAAAACACCTCGTTTACTGCAAT	For complemented mutant of $\Delta invC$
pMMB207- <i>invC</i> -R	TCTCATCCGCCAAAACAGCCAAGCTTTAATTCTGGTC AGCGAATGCATTC	For complemented mutant of $\Delta invC$
pMMB207- <i>prgH</i> -F	AGCTCGGTACCCGGGATCCTCTAGCTAAAGGAAGA CGTTATGGAACATCAAAAGAGAAGACGA	For complemented mutant of $\Delta prgH$
pMMB207- <i>prgH</i> -R	TCTCATCCGCCAAAACAGCCAAGCTTTAAAGTGGGCT TGGGAAATACCAA	For complemented mutant of $\Delta prgH$
pMMB207- <i>spaN</i> -F	AGCTCGGTACCCGGGATCCTCTAGCTAAAGGAAGA CGTTATGGGCGATGTGTGAGCTGTCAGTT	For complemented mutant of $\Delta spaN$
pMMB207- <i>spaN</i> -R	TCTCATCCGCCAAAACAGCCAAGCTTCAGGCGTCATC CTCCTCGCCAGAT	For complemented mutant of $\Delta spaN$
pCX340-F	AGACAATCTGTGTGGGCACTCGACC	For β -lactamase TEM-1 fusion plasmid
pCX340-R	TTCTGAGAATAGTGTATGCGGGCAG	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>dam</i> -F	AAGGAGGAATAACATATGATGAAAAAATCGCGCT TTTTTGA	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>dam</i> -R	GTGCGAATTCTCCGCGGAGGTACCTTTTCTTGACGGC GTTGCGACT	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>invC</i> -F	AAGGAGGAATAACATATGATGAAAACACCTCGTTTACTGCAAT	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>invC</i> -R	GTGCGAATTCTCCGCGGAGGTACCATTTCTGGTCAGCGAATGCATTC	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>prgH</i> -F	AAGGAGGAATAACATATGATGAAAACATCAAAAGAGAAGACGA	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>prgH</i> -R	GTGCGAATTCTCCGCGGAGGTACCAAGTGGGCTTGGGAAATACCAA	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>spaN</i> -F	AAGGAGGAATAACATATGATGGGCGATGTGTGAGCTGTCAGTT	For β -lactamase TEM-1 fusion plasmid
pCX340- <i>spaN</i> -R	GTGCGAATTCTCCGCGGAGGTACCGGCGTCATCCTCCTCGCCAGAT	For β -lactamase TEM-1 fusion plasmid

Construction of transposon mutant library and transposon insertion sequencing

Random transposition of TnpSC189 into the C50336 chromosome was achieved by using the delivery plasmid pSC189 [74]. For conjugation, the X7213

λpir donor strain bearing the plasmid pSC189 was cultivated in LB broth containing 50 μg/mL DAP (diaminopimelic acid) and 100 μg/mL kanamycin, while recipient strain C50336 was cultured in ordinary LB broth and grown until mid-logarithmic phase

($OD_{600} = 0.6$). Equal volumes (100 μ L) of bacterial suspensions were mixed after two washes with fresh LB medium. The mixed suspension was plated on nitrocellulose filters placed on the surface of LB agar plates supplemented with DAP (50 μ g/mL) and incubated for 12 h at 37 °C. The conjugation mixtures were then diluted with sterile phosphate-buffered saline (PBS) and plated on LB agar supplemented with kanamycin (100 μ g/mL).

Two-round semi-arbitrary PCR was used to determine the location of the transposon insertion [78]. PrimeSTAR® Max DNA Polymerase (TaKaRa) was employed in a 10- μ L reaction volume consisting of 2 \times PrimeSTAR Max Premix (5 μ L), 0.25 μ M of SP1 primer, 0.1 μ M of AB1, AB2, and AB3 primers (Table 2), and 1 μ L of an overnight LB-grown culture as the source of DNA. The first-round reaction conditions were as follows: 1 cycle of 95 °C for 5 min, 6 cycles of 95 °C for 30 s, 42–36 °C for 30 s (1 °C reduction per cycle), and 72 °C for 3 min, then 26 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 3 min, followed by 1 cycle of 72 °C for 10 min. The second-round reaction was performed in a total volume of 30 μ L containing 15 μ L of 2 \times PrimeSTAR Max Premix, 0.25 μ M of SP2 and ABS primers (Table 2), with 2 μ L of the first-round reaction product as the source of DNA. The second-round PCR amplifications were performed as follows: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 3 min, followed by 1 cycle of 72 °C for 10 min. The second-round PCR products were sequenced using the pSC189-seq primer (Table 2) and the sequences were compared with the GenBank DNA sequence database using the BLASTX program.

Mice and cell culture

Specific pathogen-free (SPF) female C57BL/6 mice (age, 6–8 weeks; body weight, 20 \pm 2 g) were obtained from the Comparative Medical Center of Yangzhou University (Yangzhou, China). All mice were bred under specific pathogen-free conditions, in the mouse isolators (Suzhou monkey animal experiment equipment Technology Co. Ltd., Suzhou, China). Five mice were raised in a cage, fed with pathogen-free diet and water. All animal experiments were approved by the Animal Welfare and Ethics Committees of Yangzhou University and complied with the guidelines of the Institutional Administrative Committee and Ethics Committee of Laboratory Animals (IACUC license number: YZUDWLL-201811-001). All animals were subjected to a clinical examination to assess their physical appearance and the normality of their behavior, and those presenting signs of disease were removed. All animals were humanely handled. For bone

marrow preparations animals were sacrificed by cervical dislocation after anesthesia by intraperitoneal injection of 20% urethane (ethyl carbamate) solution. Animals used for the evaluation of bacterial virulence in this study were euthanized by CO₂ delivery from a compressed CO₂ gas cylinder directly in the mouse cages to reduce stress. Euthanasia was accomplished by slow exposure to increasing levels of CO₂, replacing approximately 15–30% of the cage volume per minute, outside the mouse cages. Flow was kept for 2 min and the animals were maintained in the same cage for an additional 5 min.

BMDMs are primary macrophages obtained from C57BL/6 mouse bone marrow of the tibia and femur as described previously [79]. Three mice were used for BMDM preparation in each individual experiment, three independent experiments were performed. Bone marrow cells were cultured at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, 100 μ g/mL streptomycin (Gibco), and 25 ng/mL macrophage colony-stimulating factor (M-CSF, PeproTech, Rocky Hill, NJ, USA). Fresh medium was added on day 3. Differentiated BMDMs were collected for experiments on day 6. J774A.1 and HeLa cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA) and cultured in complete DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μ g/mL). Cell viability and number were determined by trypan blue exclusion assays.

Salmonella infection

Each single SE colony was cultured in LB medium, followed by overnight incubation at 37 °C with shaking at 180 rpm. Overnight cultures were diluted 1:100 and cultured for another 3 h to an OD_{600} of 0.7 to induce SPI-1 expression [80]. J774A.1 cells or BMDMs were seeded into cell culture plates the day before infection. For inflammasome activation, the cells were washed with Dulbecco's PBS (DPBS, Gibco) and pre-treated with 1 μ g/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) diluted in Opti-MEM (Gibco) for 5 h. Bacteria strains were washed twice with sterile PBS, added to the cells at an MOI of 20:1, and spun onto the cells at 1000 rpm for 10 min. Cells were incubated at 37 °C for 1 h, subsequently washed twice, and incubated for an additional 3 h with Opti-MEM containing 50 μ g/mL gentamicin.

In total, 70 female C57BL/6 mice (6–8 weeks old) were randomly assigned to seven groups ($n = 10$). Each group was intraperitoneally infected with 100 μ L of WT C50336 or gene deletion mutant strains diluted in PBS at a dose of 1×10^5 CFU per mouse. The control mice

received 100 μ L of PBS via the same route. All mice deaths were recorded over 14 dpc.

Cytotoxicity assays and cytokine measurements

J774A.1 cells or BMDMs were seeded at a concentration of 1×10^5 cells per well in 48-well plates. The cells were infected with SE strains as described above, and supernatants were harvested at 4 h after infection and centrifuged at 2000 rpm for 5 min to remove cell debris. LDH release was quantified using the LDH Cytotoxicity Assay Kit (Beyotime Biotechnology Co. Ltd., Haimen, China) according to the manufacturer's instructions. Quantitative determination of pro-inflammatory cytokines IL-1 β and IL-6 in supernatants were performed through an enzyme-linked immunosorbent assay (ELISA) by employing Mouse IL-1 beta/IL-1F2 DuoSet ELISA and Mouse IL-6 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's manual.

Immunoblotting and antibodies

J774A.1 cells or BMDMs were seeded into 12-well plates at a density of 5×10^5 cells per well and infected with bacteria as described above. After harvesting the supernatants, the remaining cells were directly lysed with 300 μ L cell lysis buffer per well for western and IP (Beyotime). For each single well, the supernatant and lysate were pooled separately. The mixtures were centrifuged at 2000 rpm for 5 min to remove cell debris. An equal volume of methanol and a 0.25 volume of chloroform were added, and the solution was vortexed vigorously for 30 s. The samples were centrifuged at 12000 rpm for 5 min. The upper aqueous phase was removed, and an equal volume of methanol was added to each sample. The mixtures were centrifuged at 12000 rpm for 5 min after vortexing for 10 s. The protein pellets were dried at 55 $^{\circ}$ C for 5–10 min, resuspended with 40 μ L of 1 \times SDS-PAGE Sample Loading Buffer (Beyotime), and boiled for 10 min at 95 $^{\circ}$ C. The samples were loaded onto 15% Tris-glycine gels. The proteins were then transferred from the gels onto nitrocellulose membranes, which were blocked with blocking buffer (3% nonfat dry milk in PBS) for 2 h at room temperature. The membranes were subsequently incubated on a rotator overnight at 4 $^{\circ}$ C with a primary antibody (diluted 1:1000 in blocking buffer). After 5 washes with PBST (0.05% Tween 20 in PBS), the membranes were incubated at room temperature for 1.5 h with a secondary antibody diluted 1:5000 in blocking buffer. Images of antibody reactions with an ECL chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA) were acquired using an Amersham Imager 600 Imaging System (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

The primary antibodies used in this study were as follows: anti-caspase-1 p20 antibody (AG-20B-0042, AdipoGen, San Diego, CA, USA), anti-NLRP3/NALP3 antibody (AG-20B-0014, AdipoGen), anti-NLRC4 antibody (ab201792, Abcam, Cambridge, UK), anti-ASC/TMS1 antibody (67824S, Cell Signaling Technology, Danvers, MA, USA), anti-IL-1 β antibody (12507S, Cell Signaling Technology), anti- β -actin antibody (A5441, Sigma-Aldrich), anti-Phospho-SAPK/Jnk antibody (4671S, Cell Signaling Technology), anti-Phospho-p44/42 MAPK (ERK1/2) antibody (4377S, Cell Signaling Technology), and anti-Phospho-p38 MAPK antibody (9215S, Cell Signaling Technology). The secondary antibodies were goat anti-mouse IgG-HRP (401,215, Sigma-Aldrich) and goat anti-rabbit IgG-HRP (BS13278, Bioworld Technology, Bloomington, MN, USA).

Protein translocation assays

Plasmid pCX340 was used for a TEM-1 β -lactamase fusion protein FRET assay as described previously [76]. The target genes were amplified from the genome of wild-type C50336 and then ligated into the *Nde* I and *Kpn* I restriction sites in the multiple cloning region of pCX340 using the ClonExpress II One Step Cloning Kit (Vazyme). All recombinant plasmids were transferred into C50336 by electroporation. Overnight bacterial cultures were diluted to 1:100 in LB broth supplemented with 12.5 μ g/mL tetracycline for 3 h and induced with 1 mM IPTG for another 3 h.

HeLa cells were trypsinized and seeded in black, clear-bottomed 24-well plates (Cellvis, Mountain View, CA, USA) at 2×10^5 cells per well. Bacterial strains bearing plasmid pCX340 were washed twice with DMEM, added to the cells at an MOI of 100:1, and spun onto the cells at 1000 rpm for 10 min. The cells were incubated for 3 h, subsequently washed 4 times, and incubated for an additional 4 h with DMEM. The cells were washed with DMEM and covered with 300 μ L DMEM plus 60 μ L of 6 \times CCF2/AM solution freshly prepared with the CCF2/AM loading kit (CCF2/AM final concentration, 1 μ M; Invitrogen, Carlsbad, CA, USA). The plates were incubated for 2 h at room temperature, and images were acquired using a Leica confocal microscope (Leica Microsystems, Wetzlar, Germany). The appropriate filters were selected to allow for excitation of coumarin (~ 410 nm) and detection of blue coumarin (~ 450 nm) and green fluorescein (~ 520 nm) emissions, enabling the simultaneous observation of green fluorescence emitted by the CCF2 substrate and blue fluorescence emitted by the cleaved CCF2 product. Translocation of β -lactamase fusions into CCF2-loaded cells was determined by counting the number of blue fluorescent cells in the images. Cells that did not exhibit secretion of TEM fusions appeared green. For a particular cell well from three

separate experiments, six pictures were taken, and an average of 1200–2000 cells were counted. Each picture was considered an independent observation and used to calculate the percentage of blue fluorescent cells.

Lentivirus infection assays

Lentiviral vectors pGLV5-*dam* (EF-1aF/GFP&Puro) and pGLV5-*dam*-His (EF-1aF/GFP&Puro) for the Dam gene encoding a GFP sequence were designed, constructed, amplified, and purified by GenePharma (Shanghai, China). The packaging, collection, and titer determination of the lentivirus were also conducted by GenePharma. In general, 293 T producer cells were transfected with optimized packaging plasmids (pGag/Pol, pRev, and pVSV-G) along with lentiviral vectors expressing Dam or Dam-His constructs by lipofectamine. Six hours post-transfection, the transfection mix was replaced with fresh DMEM containing 10% FBS. The virus-containing supernatant was harvested 72 h post-transfection, centrifuged at 4 °C and 4000 rpm for 4 min, filtered through a 0.45 µm filter (Millipore, Burlington, MA, USA), and centrifuged at 4 °C and 20,000 rpm for 2 h. The lentivirus particles were titrated by adding serial dilutions to fresh 293 T cells and assessed using GFP expression after 72 h. The viral titers of approximately 1×10^9 infectious units/mL were obtained and stored at -80 °C before infection. The negative control lentivirus Lv-NC (10^9 TU/mL) was also provided by GenePharma.

J774A.1 cells were seeded into 12-well plates at a density of 1×10^5 cells per well. A 100 µL volume of lentivirus was mixed with 400 µL of complete DMEM, and polybrene (GenePharma) was added to a final concentration of 5 µg/mL. The cells were incubated at 37 °C for 24 h, the medium was removed, and the cells were cultured in 500 µL of complete DMEM for another 48 h. The efficiency of lentivirus infection was evaluated by detection of GFP fluorescence. The cells were pre-treated with LPS as described above and stimulated with 1.25 mM ATP (Sigma) diluted in Opti-MEM (Gibco) for 1 h. Then supernatants and cell lysates were collected for immunoblotting or ELISA as described above.

RNA sequencing

Overnight cultures of the wild-type strain C50336, gene deletion mutant strain C50336Δ*dam*, and overexpression strain C50336::*dam* were diluted to 1:100 in LB broth and cultured to logarithmic phase ($OD_{600} = 0.7$). J774A.1 cells were seeded at a density of 5×10^6 cells per 10-cm dish. The cells were pre-treated with LPS and infected with SE strains as described above. Each group comprised three independent replicates. The cells were then collected with 1 mL Trizol (Ambion, Carlsbad, CA, USA) and sent to GENEWIZ (Suzhou,

China) for analysis. The total RNA was isolated, purified, and quantified by GENEWIZ. The eukaryotic transcriptome libraries were pooled and sequenced on an Illumina platform (Illumina, San Diego, CA, USA). The subsequent procedures and statistical analyses were conducted by GENEWIZ as previously described [81]. All sequences were deposited at NCBI Sequence Read Archive (SRA) with accession number SRP253144.

qRT-PCR analysis

J774A.1 cells were seeded into 12-well plates at a density of 5×10^5 cells per well and infected with WT C50336, *dam* deletion mutant, complementation, and overexpression strains as described above. After infection for 4 h, cells were harvested and total RNA was extracted using E.Z.N.A.[™] Total RNA Kit I (Omega Bio-tek, Norcross, GA, USA) by following the manufacturer's instructions. RNase-free DNase I (TaKaRa) was used to remove contaminating DNA in purified total RNA, in accordance with the manufacturer's instructions. RNA of the infected cells were quantified using One drop[™] spectrophotometer (Wins Technology Co. Ltd., Nanjing, China). PrimeScript RT Reagent Kit (TaKaRa) was used to reverse transcribe total RNA into cDNA. Reaction was performed in a total volume of 20 µL, containing 1 µg of total RNA, 1 µL of PrimeScript RT Enzyme Mix I, 4 µL of RT Primer Mix, 4 µL of $5 \times$ PrimeScript Buffer 2. The mixture was incubated at 37 °C for 15 min, followed by incubation at 85 °C for 5 s, subsequently stored at -20 °C until further use.

The Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to measure the mRNA expression levels of *c-Fos*, *Braf*, *Rasa1*, *Sos2*, *Nr4a1*, *Elk4*, *Mef2c*, *Cul1*, *Tgfb1*, *Rps6kb1*, *Rbl1*, *Id1*, *Id3*, *Xiap*, *Bcl3*, *Chuk*, *Dnm1*, *Akt3*, *Itch*, *Pik3ca*, *Pik3r1*, *Pten*, *Irs2*, *Sirt1*, *Klf2*, *Sgk3*, *Pdpk1*, *Mdm2*, and *Rbl2* as well as of the internal control gene, mouse GAPDH. Primers were designed using Primer Express software v3.0 (Applied Biosystems, Carlsbad, CA, USA) and were listed in Table 3. The qRT-PCR reaction was performed in a total volume of 20 µL, containing 200 ng of cDNA, 10 µL of $2 \times$ SYBR Green Master Mix (TaKaRa), 0.6 µL each of 10 µM forward and reverse primers, and 6.8 µL RNase-free water. The comparative threshold cycle ($2^{-\Delta\Delta C(T)}$) method) was used to calculate relative concentrations. All qRT-PCR reactions were performed in triplicates.

Statistical analysis

The Z score was calculated for each well in a 48-well plate as described previously [82]. For a given well, the Z score was calculated by subtracting the mean value of the wells on that plate from the value of the given well and dividing by the standard deviation value for all of

Table 3 Primers used in qRT-PCR

Primer name	Primer sequence (5' to 3')	Target
c-Fos-F	CTCCCGTGGTCACCTGACT	Detection of mRNA
c-Fos-R	TTGCCTTCTGACTGCTCA	Detection of mRNA
Braf-F	GTAGCGCCTGTTAGTCCCTC	Detection of mRNA
Braf-R	GAGCAGCCTGAGTGGTTAGG	Detection of mRNA
Rasa1-F	CGGGGTCTTTGTACTTTCA	Detection of mRNA
Rasa1-R	TGGTGGTCAACTGGATAGA	Detection of mRNA
Sos2-F	CAGTCTCTTGCCCACTCA	Detection of mRNA
Sos2-R	GTGGAATAGCAGGAGGGTCA	Detection of mRNA
Nr4a1-F	CTTGAGTTCGGCAAGCCTAC	Detection of mRNA
Nr4a1-R	CGAGGATGAGGAAGAAGACG	Detection of mRNA
Elk4-F	AGCTTTGCCAGAAAAGGACA	Detection of mRNA
Elk4-R	TGGTGAAGAGACGCTGTGCG	Detection of mRNA
Mef2c-F	ACGCCTGTCACCTAACATCC	Detection of mRNA
Mef2c-R	AGCTCTCAAACGCCACACTT	Detection of mRNA
Cul1-F	GAATTGGGGCTGAATGAAGA	Detection of mRNA
Cul1-R	AACTCTCCGCTGTTCTCAA	Detection of mRNA
Tgfb1-F	GGCGAAGGCATTACAGTGTT	Detection of mRNA
Tgfb1-R	TGCACATACAAATGGCCTGT	Detection of mRNA
Rps6kb1-F	GCTGTGATTGGTGGAGTTT	Detection of mRNA
Rps6kb1-R	GCTTGGACTTCTCCAGCATC	Detection of mRNA
Sp1-F	TGCAGCAGAATTGAGTACC	Detection of mRNA
Sp1-R	CACAACATACTGCCACCAG	Detection of mRNA
Rbl1-F	AAACCTTGACCACAAGTACG	Detection of mRNA
Rbl1-R	GCACAGGAGACATTTGATCAT	Detection of mRNA
Id1-F	CCAGTGGTAGAGGGTTTGA	Detection of mRNA
Id1-R	AGAAATCCGAGAAGCACGAA	Detection of mRNA
Id3-F	ACTCAGCTTAGCCAGGTGGA	Detection of mRNA
Id3-R	GTCAGTGGCAAAGCTCCTC	Detection of mRNA
Xiap-F	TTGGAACATGGACATCTCA	Detection of mRNA
Xiap-R	TGCCCTTCTCATCCAATAG	Detection of mRNA
Bcl3-F	TACTCTACCCGACGATGG	Detection of mRNA
Bcl3-R	CCAAGCTTGAAGGCTGAG	Detection of mRNA
Chuk-F	TGGAGCCTACGAAGCTGTT	Detection of mRNA
Chuk-R	CCCTCATTAGTTGCGGTGTT	Detection of mRNA
Dnm11-F	ACCCGGAGACTCTCATTCT	Detection of mRNA
Dnm11-R	GGCGAGAAAACCTTGAGATG	Detection of mRNA
Akt3-F	GAACTGGCCACTTCTGCTC	Detection of mRNA
Akt3-R	ACTGAGGTGTGGTGGAGACC	Detection of mRNA
Itch-F	CATGTGGTTTTGGCAGTTTG	Detection of mRNA
Itch-R	TTGTAAGGTGGGAGGTCAG	Detection of mRNA
Pik3ca-F	ACTGTTCCAGAGAGGCCAGGA	Detection of mRNA
Pik3ca-R	CGGTTGCCTACTGGTTCAAT	Detection of mRNA
Pik3r1-F	GCGTGACATGTAGGCTCTCA	Detection of mRNA
Pik3r1-R	CAGTTTCCTTGGCTTTGCTC	Detection of mRNA

Table 3 Primers used in qRT-PCR (Continued)

Primer name	Primer sequence (5' to 3')	Target
Pten-F	CATAACGATGGCTGTGGTTG	Detection of mRNA
Pten-R	CGGGGTAAGGCTGTTTTACA	Detection of mRNA
Irs2-F	GTAGTTCAGGTCGCCTCTGC	Detection of mRNA
Irs2-R	CAGCTATTGGGACCACCACT	Detection of mRNA
Sirt1-F	AGTTCAGCCGTCTCTGTGT	Detection of mRNA
Sirt1-R	CTCCACGAACAGTTCACAA	Detection of mRNA
Klf2-F	GCCTGTGGGTTGCTATAAA	Detection of mRNA
Klf2-R	AAGGAATGGTCAGCCACATC	Detection of mRNA
Sgk3-F	ATCCAGATGTCCGAGCATTTC	Detection of mRNA
Sgk3-R	GAAGAACCTTGCCAAAGCTG	Detection of mRNA
Pdpk1-F	GGTCCAGTGGATAAGCGAAA	Detection of mRNA
Pdpk1-R	TTTCTGCACCACTTGTGAGC	Detection of mRNA
Mdm2-F	TGCAAGCACCTCACAGATTC	Detection of mRNA
Mdm2-R	ACACAATGTGCTGCTGCTTC	Detection of mRNA

the plate wells. A Z score ≤ -2 or ≥ 2 was considered significant. All data are presented as mean \pm standard error (SEM) of triplicate samples per experimental condition from three independent experiments using GraphPad Prism 5 software (La Jolla, USA). To detect significant differences between experimental groups, an one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test were conducted. Statistical significance was determined at p values of < 0.05 (*), < 0.01 (**), or < 0.001 (***)

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12866-020-01919-z>.

Additional file 1: Figure S1. Growth curves of *dam*, *invC*, *hilD*, *prgH*, and *spaN* gene deletion mutants, complementation, and overexpression strains. **Figure S2.** Two rounds of screening to identify the genes involved in regulating inflammasome activation in vitro (original images of immunoblotting). **Figure S3.** Deletion mutants of *dam*, *invC*, *prgH*, and *spaN* failed to induce inflammasome activation (original images of immunoblotting). **Figure S4.** Deletion of *prgH* did not influence the synthesis of inflammasome components (original images of immunoblotting). **Figure S5.** Overexpression of Dam inhibited inflammasome activation (original images of immunoblotting). **Figure S6.** The ability of the *dam* complementation strain and overexpression strain cultured without IPTG to activate the inflammasome was improved (original images of immunoblotting). **Figure S7.** The deletion of Dam blocked the Jnk pathway in infected J774A.1 cells (original images of immunoblotting). **Figure S8.** Dam independently promoted NLRP3 inflammasome activation (original images of immunoblotting).

Abbreviations

SE: *Salmonella* Enteritidis; SPI: *Salmonella* pathogenicity island; T3SS: type III secretion system; SCV: *Salmonella*-containing vacuole; PRRs: pattern recognition receptors; NLRs: Nod-like receptors; AIM2: Melanoma 2; ASC: Apoptosis-associated speck-like proteins containing caspase recruitment domains; IL-1 β : Interleukin-1 β ; WT: Wild type; Dam: DNA adenine methylase; LDH: Lactate dehydrogenase; DEGs: Differentially expressed genes; LB: Luria-Bertani; DAP: Diaminopimelic acid; PBS: Phosphate-buffered saline;

DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; M-CSF: Macrophage colony-stimulating factor; DPBS: Dulbecco's phosphate-buffered saline; dpc: Days post-challenge; ELISA: Enzyme-linked immunosorbent assay; FRET: Fluorescence resonance energy transfer; Km^r: Kanamycin; Cm^r: Chloramphenicol; Tet^r: Tetracycline; MAPK: Mitogen-activated protein kinase; KEGG: Kyoto Encyclopedia of Genes and Genomes; TNF: Tumor necrosis factor; qRT-PCR: Quantitative real-time PCR; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Jnk: C-Jun N-terminal kinase; ERK1/2: Extracellular regulated protein kinases 1/2; GFP: Green fluorescent protein; ATP: Adenosine triphosphate; FoxO: Forkhead box, sub-group O; Xiap: X-linked inhibitor of apoptosis protein; ROS: Reactive oxygen species

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Authors' contributions

YG, DG, and ZP conceived of and designed the experiments. YG, DG, and TH performed the experiments and analyzed the experimental results. LC, XZ, YZ, and KW analyzed experiment performance. YG, XK, and CM analyzed and interpreted all of the data. YG performed the statistical analysis and wrote manuscript. XJ and ZP did the supervised study. All authors read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Any additional information will be made available from the corresponding author on reasonable request. RNA sequences were deposited at NCBI Sequence Read Archive (SRA) with accession number SRP253144.

Ethics approval and consent to participate

This manuscript reports data obtained in the mouse. All mice were bred under specific pathogen-free conditions, in the mouse isolators, fed with pathogen-free diet and water. All animal experiments were approved by the Animal Welfare and Ethics Committees of Yangzhou University and complied with the guidelines of the Institutional Administrative Committee and Ethics Committee of Laboratory Animals (IACUC license number: YZUDWLL-201811-001). All animals were humanely handled.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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