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Cirtical role for *Salmonella* effector SopB in regulating inflammasome activation

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ABSTRACT

Objective: *Salmonella* is known to evolve many mechanisms to avoid or delay inflammasome activation which remain largely unknown. In this study, we investigated whether the SopB protein critical to bacteria virulence capacity was an effector that involved in the regulation of inflammasome activation.

Methods: BMDMs from NLRC4-, NLRP3-, caspase-1/-11-, IFI16- and AIM2-deficient mice were pretreated with LPS, and subsequently stimulated with a series of SopB-related strains of *Salmonella*, inflammasome induced cell death, IL-1β secretion, cleaved caspase-1 production and ASC speckle formation were detected.

Results: We found that SopB could inhibit host IL-1β secretion, caspase-1 activation and inflammasome induced cell death using a series of SopB-related strains of *Salmonella*; however the reduction of IL-1β secretion was not dependent on sensor that contain PYD domain, such as NLRP3, AIM2 or IFI16, but dependent on NLRC4. Notably, SopB specifically prevented ASC oligomerization and the enzymatic activity of SopB was responsible for the inflammasome inhibition. Furthermore, inhibition of Akt signaling induced enhanced inflammasome activation.

Conclusions: These results revealed a novel role in inhibition of NLRC4 inflammasome for *Salmonella* effector SopB.

1. Introduction

Inflammasomes are cytosolic multiprotein complexes that promote the maturation and release of pro-inflammatory cytokines and cell death known as pyroptosis in response to infections and cellular stress (Kapetanovic et al., 2015; Santoni et al., 2015). Assembly of the inflammasome is initiated by the intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) or HIN200 protein families. Activated NLRs or HIN200 protein families, in most cases, recruit a bipartite protein known as apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) to recruitment of caspase-1 into the ASC speckle, where caspase-1 induces proteolysis processing of pro-IL-1β and pro-IL-18 (Plantinga et al., 2013; Arbore and Kemper, 2016). While inflammasome activation has been increasingly recognized as an important cytoplasmic inflammatory respond against microbial infection, aberrant activation of inflammasome has been implicated in many human diseases (Chen et al., 2015; Dinarello et al., 2010). Inhibition of the inflammasome by host or pathogens is a common mechanism to limit or facilitate infection. Autoinhibition and modification of receptors from the host are critical in limiting aberrant inflammatory response (Jin et al., 2013; Han et al., 2015). To avoid inflammasome activation, many pathogens encode inhibitors of inflammasome functions, so as to reach the purpose of immune escape (Cunha et al., 2015; LaRock and Cookson, 2012; Zheng et al., 2011).

Salmonella is a Gram-negative facultative intracellular bacterial pathogen capable of infecting a number of hosts and causing significant morbidity and mortality globally (Coburn et al., 2007; Scallan et al., 2011). *Salmonella* pathogenicity depends on the ability to invade and survive within host cells. Type III secretion systems (T3SS) encoded pathogenicity islands (SPI) -1 and -2 which is responsible for the secretion and translocation of a set of bacterial effectors into host cells. Flagellin injected into host cells by invading *Salmonella* is sensed by NLRC4 and NLRP3 inflammsome also activated by unknown signals in this process (Mariathasan et al., 2004; Broz et al., 2010). To survive and replicated in host cells, *Salmonella* has evolved mechanisms to avoid or delay inflammasome activation (Wynosky-Dolfi et al., 2014; Rosales-Reyes et al., 2012) and many of which remain largely unknown.

Salmonella outer protein B (SopB) is an important effector protein

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encoded in SPI-1 with inositol phosphatase activaty, an enzyme that hydrolyzes a variety of phosphoinositides and inositol phosphates at the host cell membrane during *Salmonella* invasion (Roppenser et al., 2012; Khoo et al., 2015; Rogers et al., 2011). It has been reported that SopB affects several cellular pathways during infection, including membrane ruffling, inhibition of SCV fusion with lysosomes (Bakowski et al., 2010; Mallo et al., 2008). SopB can be detected in cell up to 12 h post *Salmonella* infection indicating that it performs more than one role in *Salmonella* infection (Drecktrah et al., 2005). Therefore, we have investigated the role of SopB in inflammasome activation. We demonstrated that SopB is an inhibitor of NLRC4 inflammasome during *Salmonella* infection. SopB mediated inhibition might results from its ability to activate Akt signal, in turn inhibiting ASC pyroptosome formation, caspase-1 cleavage and IL-1β secretion.

2. Materials and methods

2.1. Mice

NLRC4- and caspase-1-deficient mice were on the C57BL/6J background; NLRP3-, AIM2- and IFI16-deficient mice were subsequently backcrossed onto the C57BL/6J background for another eight generations. All animal studies were conducted according to experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University.

2.2. Bacteria and inflammasome activation

Salmonella SL1344 was a gift from Dr. Xiang-Chao Cheng (Henan University of Science and Technology, Luoyang, China). *sopB* deleted *Salmonella* and other mutant strains are constructed in this work. For inflammasome activation, BMDMs were pre-treated with 500 ng/mL LPS (sigma) for 4 h, and subsequently washed twice with PBS, then *Salmonella* and the mutant strains were added at MOI = 20 and infected for 1 h, then cells were washed twice, incubated for an additional 3 h with culture medium containing genttamicin (50 μg/mL). Cytokines were measured in the supernatants generated during the final incubation.

2.3. ASC pyrotosomes detection

ASC pyrotosomes were detected as previously reported (Fernandes-Alnemri et al., 2007). Macrophages were seeded in 12-well plates (1.5×10^6 cells per well) and treated with different stimuli. The cells were collected by centrifugation and resuspended in 0.5 mL ice-cold buffer containing 20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1% Nonidet P-40, 0.1 mM PMSF and a protease inhibitor mixture, and lysed by shearing 10 times through a 21-gauge needle. The cell lysates were then centrifuged at 5000 × *g* for 10 min at 4 °C, and the resultant pellets were washed twice with PBS and resuspended in 0.5 mL PBS. Next, the resuspended pellets were crosslinked with fresh DSS (sigma; 4 mM) for 30 min and pelleted by centrifugation at 5000 × *g* for 10 min. The crosslinked pellets were resuspended in 30 μL SDS sample buffer, separated by 12% SDS-PAGE and immunoblotted with anti-mouse ASC antibodies.

2.4. ASC speckle detection

ASC speckle was detected via indirect immunofluorescence. After stimulation, BMDMs were plated on 24-well chamber slides, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (sigma). After blocking with 5% goat serum for 1 h at room temperature, the cells were incubated with anti-ASC (santa curz; 1:100) and Alexa Flour 488-conjugated anti-rabbit IgG (Invitrogen; 1:1000). DAPI was used to stain nuclei. After sequential excitation, images of the same cell were saved with cellSens dimension software and analyzed using Image J software.

2.5. Western blotting

Cell culture supernatants were precipitated by the addition of an equal volume of methanol and 0.25 vols of chloroform and then vortex for 10 s. After standing for 5 min at room temperature, the mixtures were centrifuged at 13,000 × *g* for 10 min. The upper phase was discarded, and 200 μL of methanol was added to the interphase. The mixture was centrifuged at 13,000 × *g* for 10 min, and the protein pellets were dried at 55 °C, resuspended and boiled for 5 min at 95 °C. The samples were separated by SDS-PAGE and immunoblotted.

2.6. Cytokine production

The secretion of IL-1β and TNF-α in cell-free supernatants were measured by ELISA following R & D Systems according to the manufacturer's manual. The assays were independently performed three times in triplicate.

2.7. Statistics

GraphPad Prism 5.0 software was used for data analysis. Data are showed as mean with SD. Data were compared by an unpaired two-tailed Student's *t*-test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. SopB inhibits IL-1β secretion and caspase-1 activation

To investigate the effect of SopB on inlfammasome activation, murine bone marrow-derived macrophages (BMDMs) were pre-treated with lipopolysaccharide (LPS) and subsequently infected with wild type strain SL1344 or a *sopB*-defective strain *ΔsopB* to prime the inflammasome. The inflammasome activation causes a manner of cell death termed as "pyroptosis". Therefore, cell death was measured firstly. Compared to SL1344, the *ΔsopB* resulted in a significant higher cell death rate (Fig. 1A) which indicated that *ΔsopB* might enhance the inflammasome activation. Subsequently, we tested whether the enhanced cell death was induced by elevated inflammasome activation. The amount of bioactive IL-1β secretion and caspase-1 cleavage were measured. Results showed that *ΔsopB* induced an enhancement of bioactive IL-1β and caspase-1 secretion in supernatants compared to SL1344; however, the expression of pro-IL-1β and pro-caspase-1 in cell lysates have no difference between SL1344 and *ΔsopB* infection (Fig. 1B), suggesting that *ΔsopB* has no role on the expression of pro-caspase-1 and pro-IL-1β, but promotes pro-caspase-1 and pro-IL-1β processing. Consistent with immunoblotting results, *ΔsopB* induced an increased production of mature IL-1β in cell supernatants (Fig. 1C). IL-1β secretion can be stimulated with TNF-α, therefore the production of TNF-α in supernatants was examined. As the result showed, no significant difference was found between SL1344 and *ΔsopB* infection (Fig. 1D), indicating that the enhancement of IL-1β production was caused by elevated inflammasome activation.

Inflammasome activation is important for antibacterial defense. Recently a non-canonical infammasome response to Gram-negative bacteria that actives caspase-11 and mediates pyroptosis leading to release of IL-1a and IL-1β was reported (Broz et al., 2012). In this study, we found that *sopB* deletion did not influence the activation of caspase-11 (Fig. 1E) indicating that SopB involved in the inhibition of canonical inflammasome activation. Collectively, the above results indicate that SopB inhibits certain inflammasome activation; however, it does not affect the expression of pro-IL-1β and pro-caspase-1 in cell lysate.