Salmonella Downregulates Nod-like Receptor Family CARD Domain Containing Protein 4 Expression To Promote Its Survival in B Cells by Preventing Inflammasome Activation and Cell Death

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Salmonella Downregulates Nod-like Receptor Family CARD Domain Containing Protein 4 Expression To Promote Its Survival in B Cells by Preventing Inflammasome Activation and Cell Death

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Salmonella infects and survives within B cells, but the mechanism used by the bacterium to promote its survival in these cells is unknown. In macrophages, flagellin secreted by Salmonella activates the Nod-like receptor (NLR) family CARD domain containing protein 4 (NLRC4) inflammasome, leading to the production of IL-1β and pyroptosis of infected cells. In this study, we demonstrated that the NLRC4 inflammasome is functional in B cells; however, in Salmonella-infected B cells, IL-1β secretion is prevented through the downregulation of NLRC4 expression. A functional Salmonella pathogenicity island 1 type III secretion system appears to be required for this process. Furthermore, infection induces Yap phosphorylation and promotes the interaction of Yap with Hck, thus preventing the transcriptional activation of NLRC4. The ability of Salmonella to inhibit IL-1β production also prevents B cell death; thus, B cells represent an ideal niche in which Salmonella resides, thereby promoting its persistence and dissemination. The Journal of Immunology, 2013, 190: 000–000.

During Salmonella infection, B cells produce a robust response against protein and nonprotein Ags (1). B cells are essential for protective immunity to oral challenge; Salmonella-specific Abs protect against secondary infection (2). Furthermore, it has been demonstrated that B cells are required for priming the CD4+ and CD8+ T cell responses during infection (3). However, B cells have additional roles during Salmonella infection, and there is evidence that Salmonella infects B cells in vivo and in vitro (3–9). Within B cells, Salmonella resides in a late endosomal compartment that is distinct from the Salmonella-containing vacuoles observed in macrophages (8). In addition, the bacteria infect B cell precursors in the bone marrow, and these cells may serve as reservoirs of Salmonella infection (10).

During infection, pathogen recognition by the innate immune system occurs through a number of pattern-recognition receptors, such as TLRs, Nod-like receptors (NLRs), and others. Activation of pattern-recognition receptors is fundamental for the initiation of signaling networks that culminate in a proinflammatory immune response. TLRs are located in the plasma membrane and vacuolar compartments, whereas NLR receptors are located in the cytoplasm (11). During Salmonella infection, LPS and other Ags activate TLR pathways to produce TNF-α, IL-6, IL-12, and various chemokines (12), whereas the NLR family CARD domain containing protein 4 (NLRC4) inflammasome recognizes flagellin monomers and triggers caspase-1 activation and the subsequent processing of pro–IL-1β and secretion of the mature form of IL-1β (13, 14). IL-1β induces pyroptosis in infected cells, promoting bacterial dissemination. In fact, caspase-1–deficient mice survive Salmonella infection because the spread of bacteria is abolished because of the absence of cell death (15).

NLRC4 was initially described as a proapoptotic molecule (16), and its transcription is activated by the p53 transcription factor family (17). The most efficient transcription of NLRC4 occurs when the p73/Yap heterodimer binds to its promoter region (18, 19). Negative regulation of the expression p73/Yap target genes requires cytosolic sequestering by two mechanisms: Yap phosphorylation at S127 (20) or a direct interaction of Yap with Hck (18). Both mechanisms prevent the nuclear translocation of Yap and the subsequent activation of gene expression, including NLRC4.

In this work, we demonstrate for the first time, to our knowledge, that Salmonella infection inhibits IL-1β secretion through the downregulation of NLRC4 in B cells. This event could explain why B cells are resistant to Salmonella-induced cell death. Yap phosphorylation and Yap–Hck interaction are involved in the downregulation of NLRC4 during Salmonella infection. We suggest that NLRC4 downregulation is a virulence mechanism of Salmonella that promotes its survival in B cells. These findings indicate that B cells may facilitate the dissemination and persistence of Salmonella during infection.
Materials and Methods

Mouse primary cells

Primary cells were obtained from 8-wk-old female BALB/c mice (Experimental Medicine Department, School of Medicine, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico). Splenic B cells were obtained by CD43 negative selection (Milenyi Biotec, Cambridge, MA) and resuspended in RPMI 1640 medium (Milenyi Biotec) supplemented with 10% FBS (Life Technologies, Carlsbad, CA). Bone marrow cells were obtained from the femurs of mice and cultured in RPMI 1640 medium supplemented with 10% FBS, antibiotics (Life Technologies BRL), and 30% L-929 cell culture supernatant for 5 d. After washing with PBS, bone marrow–derived macrophages (BMDMs) were collected (21). For IL-1β secretion assays, B cells and BMDMs were preactivated with 100 ng/ml LPS for 16 h before infection (14).

Bacterial strains, growth conditions, and cell culture

Wild-type Salmonella enterica serovar Typhimurium 14028 (American Type Culture Collection) was used in this study. The Salmonella mutant prgH (ΔpbgH) strain was kindly donated by Dr. Samuel I. Miller (Washington University, Seattle, WA). Salmonella 14028 carrying the plasmid pEM180, which expresses GFP under the ampicillin promoter, was grown in the presence of 100 μg/ml ampicillin (22), and it was used as GFP-expressing S. typhimurium (S. typhimurium-GFP). The mutant ΔpbgH was grown in medium supplemented with 35 μg/ml kanamycin. All strains were grown overnight in Luria Bertani (LB) medium at 37°C with shaking, diluted 1:30 with fresh LB medium, and cultured until reaching the logarithmic phase. An OD of 0.6 at 540 nm was used to adjust the bacterial concentration and obtain the desired multiplicity of infection (MOI). Bacteria grown in LB broth containing 300 mM NaCl was used to express the Salmonella pathogenicity island 1 (SPI-1) genes (23). LB-cultured bacteria supplemented with 100 μg/ml chloramphenicol and incubated for an additional 1.5 h at 37°C without shaking were used to inhibit protein synthesis (24). Cells were obtained from a logarithmic phase culture, washed with PBS, and fixed with 0.5% paraformaldehyde (PFA) for 1 h at room temperature to fix bacteria. The bacteria were centrifuged at 10,000 rpm for 10 min and washed with PBS to remove the PFA.

Cell infection and intracellular survival assay

B cell infection was performed in suspension (6), whereas BMDM infection was conducted using a monolayer cell model (25). For both of these assays, 1 × 10^6 cells were infected for 30 min at the indicated MOI and cultured in RPMI 1640 supplemented with 30 mg/ml gentamicin. Bacterial CFUs were recovered at 1, 3, and 24 h postinfection (25).

Cytokine assays

The levels of TNF-α, IL-1β, and IL-6 in culture supernatants were determined by a quantitative sandwich enzyme immunoassay (R&D Systems, Minneapolis, MN) (14). B cells and BMDMs were infected at the indicated MOI as described previously, and culture supernatants were collected at 24 h postinfection for the ELISAs.

FACS sorting

B cells were placed in contact with Salmonella-GFP for 30 min, washed with PBS supplemented with 100 μg/ml gentamicin three times, and then immediately separated into GFP+ (cells with internalized Salmonella) and GFP− (cells that were only in contact with Salmonella) pools using a MoFlo cell sortor. GFP+ and GFP− cells were cultured in RPMI supplemented with FBS (10%) and gentamicin (100 μg/ml).

RT-PCR

Total RNA was isolated from 3 × 10^6 B cells or BMDMs using TRizol reagent (Invitrogen, Carlsbad, CA). Semiquantitative RT-PCR was performed using a one-step RT-PCR kit (Invitrogen). The primers used for amplification are NLRC4 sense 5'-TTGCACTTGAACCCGGAACGAC-3', antisense 5'-ACTTCTTCTCTCGTGTTGAGTA-3'; caspase-1 sense 5'-TTGCTCTTGCTGCTGTGAAGA-3', antisense 5'-AGCAGATGTTTCGGCTGTCG-3'; IL-1β sense 5'-ATGCGCA CGTTCCTGAACTCAACT-3', antisense 5'-CAGACAGAAGTGATATGACCTTCTC-3'; and Hprt sense 5'-TCTCACCTGGAGAGCC-3', antisense 5'-CCTACTTGAGTGTCAGCA-3'. For NLRC4 quantitative RT-PCR (qRT-PCR), cDNA was synthesized using a high-capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA), and qRT-PCR reactions were performed using following primers: sense 5'-ATGCTTATTCTGGACCTCTG-3' and SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. A standard curve was used to quantify NLRC4 expression. The data were analyzed using ABI StepOne software.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChiP) assay that we devised for our work was an extension of previous protocols with some modifications (26). In brief, 5 × 10^6 B cells were cross-linked with formaldehyde (1% final concentration) 5 min at room temperature after glycine was added at a final concentration of 0.125 M. After washing twice with ice-cold PBS, cells were resuspended in cell lysis buffer (Beyotime) and incubated on ice for 10 min. Nuclei were recovered by centrifugation, resuspended in lysis buffer 2, and incubated 10 min on ice. Samples were centrifuged and resuspended in 2 ml sonication buffer. Chromatin samples were precleared for 1 h at 4°C by adding 50 μl protein A-agarose beads (Invitrogen) and 1 μg DNA from salmon sperm (Sigma-Aldrich), followed by incubation with 1 μg Yap Ab (Santa Cruz Biotechnology) in nondenaturing lysis buffer at 4°C overnight. Immunocomplexes were recovered at 4°C for 2 h by using 60 μl protein A-agarose beads and 1 μg DNA from salmon sperm. Complexes were washed five times with IP1, once with IP2, and twice with Tris-EDTA pH 8.0. Immunoprecipitation reactions and input chromatin were digested with 200 μg/ml RNase A for 1 h and 200 μg/ml proteinase K in Tris-EDTA buffer with 0.5% SDS for 2 h at 55°C. Cross-links were reversed overnight at 65°C. Samples were obtained using a DNA extraction kit (Zymo Research, Irvine, CA) and analyzed by real-time PCR using following primers: sense 5'-TGGCCTTCTACTAATGCGTAC-3', antisense 5'-CCCTTCTTGGCAGACCTCTC-3' and SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions.

Stimulation of cells with soluble flagellin and caspase-1 activity assay

To stimulate the cytosolic NLRC4 with soluble flagellin, we used Dharmafect-1 (DF) (Thermo Scientific, Suwanee, GA) according to the manufacturer’s instructions. DF (2 μl) was diluted in 200 μl serum-free medium and incubated for 30 min with 2 μg purified flagellin (Invitrogen, San Diego, CA). After incubation, 200 μl medium was added, and a 100-μl aliquot was used to stimulate B cells or BMDMs (1 × 10^5), seeded in 96-well plates for 3 h for the caspase-1 activity assay or for 16 h for the IL-1β secretion assays. For caspase-1 activity, cells were then stained with an active caspase-1–specific fluorescent probe (FAM-TYD-FLK) (Immunochemistry Technologies, Bloomington, MN) according to the manufacturer’s instructions. For infected cells, bacteria were stained with a polyclonal anti-Salmonella Ab and an anti-rabbit tetramethylrhodamine isothiocyanate Ab (Sigma). Cells were analyzed by confocal microscopy (LSM 510 Meta; Zeiss).

Cytotoxicity assay

B cells or BMDMs were infected or stimulated as described previously (13). Twenty-four hours later, cytotoxicity was determined by lactate dehydrogenase (LDH; Promega, Madison, WI) release assay. The percentage of LDH release was calculated using the following formula: percentage of release = (experimental LDH release − spontaneous LDH release)/(maximal LDH release − spontaneous LDH release) × 100% (13).

Western blot and immunoprecipitation

Cytosolic and nuclear proteins were obtained from noninfected and infected B cells using a ProteoJet kit (Fermentas, Waltham, MA) according to the manufacturer’s instructions and solubilized in Laemmli sample buffer. Samples were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in BSA (5%) in PBS containing 0.1% Tween and incubated with anti-p53 S127 Ab (Cell Signaling), anti-Yap (Cell Signaling, Beverly, MA), anti–laminin B (Santa Cruz Biotechnology), or anti–β-tubulin Ab (Zymed, San Francisco, CA). Proteins were detected by chemiluminescence (Thermo Scientific). For immunoprecipitation, lysates from noninfected and infected B cells were precleared with protein A-Sepharose beads (Invitrogen, Carlsbad, CA) for 1 h, followed by immunoprecipitation with anti-p53 Ab (Santa Cruz Biotechnology) or anti–β-tubulin Ab (Santa Cruz Biotechnology). NLRC4 was immunoprecipitated using a polyclonal anti-NLRC4 Ab and by immunoblotting with the same Ab (Cell Signaling).

Apoptosis assay

B cells were treated with doxorubicin (500 ng/ml; Sigma), infected with Salmonella, and treated with doxorubicin alone or LPS (100 ng/ml) in...
addition to doxorubicin for 4 h. Cells were fixed with 2% PFA and labeled by TUNEL using an In Situ Cell Death Detection Kit (Roche, Basel, Sweden). Cells were analyzed by flow cytometry or fluorescence microscopy (23).

Statistical analysis

The data represent the mean ± SD and were analyzed by one-way ANOVA followed by a post hoc Tukey’s comparison. In some cases, data with normal distribution were analyzed using Student t test. The p values <0.05 was considered significant. The data were analyzed using GraphPad Prism 3 software.

Results

Salmonella prevents IL-1β secretion in B cells

In response to Salmonella infection, macrophages produce proinflammatory cytokines such as TNF-α and IL-6 through TLR activation (27) and IL-1β and IL-18 through NLR activation (13, 14). These cytokines promote the control of bacterial infection (22, 28). We investigated whether B cells produced proinflammatory cytokines in response to Salmonella infection. For this purpose, we purified B cells from spleens (purity > 99%) (Supplemental Fig. 1A). B cells were infected with Salmonella at different MOIs, and we found that IL-1β secretion decreased with MOI of 10, 56.4 ± 11.0 pg/ml IL-1β, and 11.2 ± 2.2 pg/ml IL-1β at MOI of 50. In contrast, as has been reported previously (13, 14), macrophages increased IL-1β secretion in a dose-dependent manner during infection (MOI 10: 292.0 ± 121.6 pg/ml; MOI 50: 1445.0 ± 176.8 pg/ml; Fig. 1A). We also found that IL-18 secretion is inhibited in B cells, but not in macrophages (Supplemental Fig. 2). TNF-α and IL-6, which are secreted in response to TLR stimulation, were secreted in a dose-dependent manner in both B cells and macrophages (Fig. 1B, 1C). These results suggest that activation of the inflammasome is different in B cells than in macrophages because we observed that Salmonella could modulate the activation of this complex and inhibit IL-1β and IL-18 secretion in B cells, but not in macrophages.

The NLRC4 inflammasome is active in B cells

Because we observed that the ability of B cells to produce IL-1β is dampened in the presence of Salmonella, we evaluated the expression of the NLRC4 inflammasome components caspase-1 and IL-1β using semiquantitative RT-PCR. We found that in infected B cells, the expression of NLRC4 was decreased, whereas that of caspase-1 and IL-1β was not affected (Supplemental Fig. 3A, 3B). In addition, we analyzed the functionality of the NLRC4 inflammasome in B cells. The cytosol of B cells or macrophages was loaded with flagellin, and the secretion of IL-1β and the activity of caspase-1 were evaluated. Whereas B cells were able to secrete IL-1β (145 ± 26.7 pg/ml) when they were stimulated with flagellin (Fig. 2A, gray bars), they secreted only 19.1 ± 9.0 pg/ml IL-1β when they were infected with Salmonella at an MOI of 50. By contrast, macrophages secreted IL-1β in response to both cytosolic flagellin and bacteria (Fig. 2A, black bars). The enzymatic activity of caspase-1 is required for IL-1β secretion (29); thus, we evaluated the activity of caspase-1 in Salmonella-infected flagellin-stimulated B cells using an fluorescent labeled inhibitor of caspases (FLICA). Thirty-five percent of B cells exhibited caspase-1 activity when they were stimulated with flagellin, and 12.9% of Salmonella-infected B cells displayed caspase-1 activity, similar to baseline (9%). Moreover, the FLICA signal was stronger in flagellin-stimulated cells than in Salmonella-infected cells (Fig. 2B). Macrophages exhibited caspase-1 activity when they were stimulated with flagellin and during infection (Fig. 2C). These data demonstrate that the NLRC4 inflammasome is thoroughly active in B cells, but Salmonella can impair its activity, thus preventing the secretion of IL-1β.

To further investigate this, we evaluated the effect of cytosolic flagellin when B cells were infected with Salmonella. B cells were infected with Salmonella and stimulated with flagellin at 5 h postinfection, and the secretion of IL-1β and activation of caspase-1 were evaluated. Cells stimulated with cytosolic flagellin could secrete IL-1β, and 67% of these cells had active caspase-1; however, when cells were infected and then stimulated with flagellin, they secreted very low amounts of IL-1β, and only 39% had active caspase-1. These results confirm that when B cells are infected with Salmonella, there is not enough NLRC4 protein to be activated by cytosolic flagellin (Fig. 2D, 2E).

Synthesis of Salmonella proteins associated with the IP-1 secretion system during contact with B cells is needed for the downregulation of NLRC4

Using qRT-PCR, we demonstrated that Salmonella induced the downregulation of NLRC4 expression in B cells. NLRC4 expression in B cells was decreased 3-fold at 24 h postinfection (Fig. 3A). Because the activation of the NLRC4 inflammasome is important for the control of Salmonella and other pathogens (13, 14, 30), we tested whether this downregulation of NLRC4 also occurred in macrophages. In correlation with the IL-1β secretion presented previously, NLRC4 expression in macrophages was not decreased (Fig. 3B). In addition, we analyzed NLRC3 expression and observed no changes in its expression in infected B cells (Supplemental Fig. 4); therefore, we concluded that Salmonella specifically downregulates NLRC4 in B cells.
FIGURE 2. The NLRC4 inflammasome is functional in B cells. (A) B cells (gray bars) or BMDMs (black bars) were stimulated for 24 h with LPS (100 ng/ml) and then infected with *Salmonella* at the indicated MOI or loaded with flagellin (500 ng) using DF. Twenty-four hours later, IL-1β levels were measured in the cell culture supernatant by ELISA. (B and C) B cells (B) or BMDMs (C) were infected with *Salmonella* or loaded with flagellin as previously described. Three hours later, cells were exposed to the fluorescent caspase-1 substrate FLICA for 1 h. Cells were analyzed by confocal microscopy. The percentage of B cells (gray bars) or BMDMs (black bars) with active caspase-1 is shown (right panel). (D) B cells were stimulated for 24 h with LPS (100 ng/ml) and then infected with *Salmonella* at MOI of 50, loaded with flagellin (500 ng), or infected with *Salmonella*. At 5 h postinfection, the cells were loaded with flagellin, and after 24 h, IL-1β levels were measured in the cell culture supernatant by ELISA. (E) B cells were stimulated for 24 h with LPS (100 ng/ml) and then infected with *Salmonella* at MOI of 50, loaded with flagellin (500 ng), or infected with *Salmonella*. At 5 h postinfection, the cells were loaded with flagellin, after 3 h, they were exposed to the fluorescent caspase-1 substrate FLICA for 1 h (left panel). Cells were analyzed by microscopy. The percentage of B cells with active caspase-1 is shown (right panel). Data are representative of two and three independent experiments. Mean ± SD is shown. Scale bar represents 5 and 10 μm in the B cell and BMDM images, respectively. *p < 0.05, **p < 0.01, ***p < 0.001.
In addition, we analyzed the expression of NLRC4 in B cells according to their internalization of Salmonella. We infected splenic B cells with Salmonella expressing GFP, and postinfection, cells were separated by FACS into GFP+ (cells with internalized bacteria) and GFP− (cells with no internalized bacteria) pools. Then the sorted cells were cultured for 1, 3, or 24 h, and the expression of NLRC4 was analyzed. The expression of NLRC4 was decreased 3-fold in both GFP+ and GFP− cells (Fig. 3C). We reasoned that Salmonella interferes with the expression of NLRC4 through an extracellular signal in B cells.

We then clarified which components of the bacteria are involved in the downregulation of NLRC4. In B cells stimulated with different concentrations of LPS, the expression of NLRC4 did not fluctuate (Fig. 3D). However, when bacteria were fixed or treated with chloramphenicol to inhibit protein synthesis, Salmonella was unable to downregulate NLRC4 (Fig. 3E). Salmonella possesses several virulence factors, including SPI-1, which contains genes important for bacterial entry into B cells (31). We evaluated whether the type III secretion system (TTSS) of SPI-1 is required for NLRC4 downregulation in B cells. A Salmonella ΔpgrH mutant, which lacks a TTSS, did not alter the expression of NLRC4. These data may indicate that an effector of SPI-1 is responsible for the downregulation of NLRC4; therefore, if we induced the expression of SPI-1 effectors in Salmonella, then we would expect depletion of NLRC4 expression in B cells. Surprisingly, when Salmonella was grown in the presence of NaCl (300 mM), a condition reported to induce the expression of SPI-1 effectors (23), we observed a 2-fold increase in the expression of NLRC4 (Fig. 3F). This result could imply that the protein involved in NLRC4 downregulation is secreted by the TTSS encoded in SPI-1 but is not under the transcriptional control of SPI-1. Altogether, these results establish that in B cells, but not in macrophages, Salmonella induces a decrease in NLRC4 expression, and that this occurs in an extracellular manner and requires the TTSS encoded in SPI-1.

Salmonella impairs Yap coactivator activity in B cells

NLRC4 transcription is activated by p53 (17) and p73/Yap (18). It has been previously observed that the transcription of p53 target genes is more efficient when they are activated by p73/Yap (19). The mechanisms related to the negative control of the expression of p73/Yap target genes involve the phosphorylation of Yap at S127 (20) or its interaction with Hck (18). We analyzed the phosphorylation status of Yap at S127 and its interaction with Hck in infected B cells. We observed that Yap was phosphorylated at 1 h postinfection and remained phosphorylated until 24 h postinfection (Fig. 4A); likewise, Yap interacted with Hck at 2 h postinfection, and this interaction continued until 24 h postinfection (Fig. 4B). These results reveal that Salmonella induces Yap phosphorylation and its interaction with Hck. These events prevent Yap translocation to the nucleus; consequently, this coactivator is not present in the NLRC4 promoter region, resulting in its downregulation. Both of these events could result in Yap retention in the cytosol and prevent its translocation to the nucleus, resulting in inefficient NLRC4 transcription. To test this hypothesis, we evaluated Yap in the nuclei of infected B cells and found that the presence of Yap in the nuclei of infected cells is significantly diminished (Fig. 4C). If the presence of Yap is diminished in the nucleus during infection, it might have consequences on Yap binding to the NLRC4 promoter region; we thus performed a ChIP assay to test this, and we observed that Yap is not present on the NLRC4 promoter region in infected B cells (Fig. 4D). Moreover, the absence of Yap in the NLRC4 promoter region correlated with the downregulation of NLRC4 mRNA. Finally, we evaluated whether the downregulation of NLRC4 mRNA correlated with the protein level of this molecule by immunoprecipitation and Western blotting with a polyclonal NLRC4 Ab. NLRC4 protein expression was diminished in infected B cells at 24 h postinfection (Fig. 4E), which correlated with the downregulation of NLRC4 mRNA. These results together suggest a mechanism by which Salmonella downregulates NLRC4 expression, avoiding inflammasome activation and subsequently preventing the secretion of IL-1β, which is required for the control of infection.

B cells are resistant to the cytotoxic effect of Salmonella

The production of proinflammatory cytokines such as IL-1β promotes the control of bacterial infection by macrophages. Given that B cell IL-1β secretion is impaired during Salmonella infection, we evaluated the survival of the bacterium in B cells. Macrophages control the Salmonella infection better than B cells because the numbers of CFUs recovered at 24 h postinfection remain constant (Fig. 5A), and we found that 68.5 ± 4.5% of bacteria survived in B cells, whereas only 1.6 ± 0.1% survived in macrophages (Fig. 5B).
Salmonella prevents NLRC4 activity in B cells

The expression of NLRC4 is induced by doxorubicin (32), a drug that also induces cell death (17, 18). We analyzed whether Salmonella infection would be able to prevent B cell expression of NLRC4 B and cell death induced by doxorubicin. Indeed, we found that doxorubicin induced cell death in B cells, as 68.0 ± 6.5% of the cells were TUNEL+, whereas only 41.1 ± 1.5% of Salmonella-infected cells treated with doxorubicin were TUNEL+. These data correlate with our previous finding of a function of the NLRC4 inflammasome in macrophages during Salmonella infection results in cell death because macrophages deficient in NLRC4 do not die when they become infected with Salmonella (13). Therefore, we analyzed the cytotoxic effect on B cells with increased doses of bacterial infection. In comparison with macrophages, B cell death did not increase independent of the MOI (Fig. 5C, 5D). When we sorted Salmonella-infected (GFP+) and -uninfected (GFP−) B cells, the level of cytotoxicity remained similar (Fig. 5E). However, when B cells were stimulated with cytosolic flagellin, cell death rate increased to 50% (Fig. 5F). These data correlate with our previous finding of a function of the NLRC4 inflammasome in B cells, because of adequate IL-1β secretion in addition of cell death induction in the presence of flagellin in the cytosol. These results demonstrate that unlike macrophages, B cells are resistant to the cytotoxic effect induced by Salmonella.

Discussion

We have previously demonstrated that Salmonella infects B cells. In fact, Salmonella survives more efficiently within B cells than in macrophages, and it can be recovered from splenic B cells, bone marrow B cell precursors, and plasma cells after a single-dose inoculation (8). However, the mechanisms used by Salmonella to survive within B cells are not established.

During Salmonella infection, macrophages and nonphagocytic cells produce proinflammatory cytokines that promote control of the infection (33). Among the mechanisms involved in the production of proinflammatory cytokines are the TLR and NLR pathways (34, 35). NLRs recognize their ligands to promote the formation of a protein complex called the inflammasome. This complex is responsible for activating caspase-1, to generate the mature form of IL-1β (29). It has previously been shown that IL-1β is secreted by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from
by macrophages infected by *Salmonella*, and this response is based on the recognition of flagellin by NLRC4 (13). By contrast, in this article, we revealed that in a dose-dependent manner, *Salmonella* can inhibit IL-1β secretion when it infects B cells. This result clearly indicates that macrophages and B cells respond differently to *Salmonella* infection. In fact, *Salmonella* downregulated NLRC4 transcription in B cells, but not in macrophages (Fig. 3B).

Previous findings demonstrated that other bacterial and viral pathogens have different mechanisms to inhibit the activity of the inflammasome complex, including the expression of proteins homologous to components of the inflammasome or proteases that inhibit the activation of caspase-1 (36, 37). To our knowledge, NLRC4 downregulation had not been previously observed in any type of viral or bacterial infection. Thus, downregulation of the inflammasome might be a strategy for evading the innate immune response to permit *Salmonella* survival within B cells. Interestingly, internalization of the bacterium is not necessary for this downregulation to occur, suggesting that some structure on the surface of the bacteria is involved in this phenomenon. *Salmonella* requires the TTSS and the effectors of SPI-1 to infect B cells (26). The requirement of SPI-1 for the invasion of epithelial cells has been extensively studied, and we know that the TTSS is engaged when the bacteria come into contact with the cell and is used to translocate effector proteins to induce *Salmonella* entry (38, 39). We found that the TTSS is required to decrease NLRC4 expression; we then concluded that the TTSS is assembled when the bacteria contact the B cell, as observed in epithelial cells, and *Salmonella* might secrete a protein responsible for the downregulation of NLRC4. Moreover, the requirement of the TTSS for the downregulation of NLRC4 might explain why this phenomenon is observed only in B cells, because the TTSS is involved in bacterial entry into B cells, but it is not required for entry into macrophages (40). However, when bacteria are grown under SPI-1–inducing conditions, we observed an increase in NLRC4 expression. This suggests that although the protein is secreted by the TTSS, the effectors encoded by the SPI-1 are not responsible for the downregulation of NLRC4; those effectors might function in a similar manner as they do in epithelial cells. In fact, it has been reported that in epithelial cells, the translocation of AvrA, an SPI-1 effector protein, increases p53 acetylation and, therefore, promotes the transcripational activity of this transcription factor (41). It has also been demonstrated that p53 is a positive transcriptional regulator of NLRC4 (17).

In addition to p53, p73 is also involved in the expression of NLRC4 (17, 18). Whereas p53 binds to the promoter region of its target genes as a homodimer (42), p73 forms a heterodimer with Yap. The p73-Yap heterodimer is more efficient than the p53 homodimers, because Yap increases stability and accumulation of p73 on the promoter region (19). Moreover, the mechanisms responsible for the negative regulation of NLRC4 expression are the phosphorylation of Yap by AKT kinase and the interaction of Hck with Yap, and both mechanisms retain Yap in the cytosol and consequently prevent the transcription of NLRC4 (18, 20). In *Salmonella*-infected B cells, we observed an increase in the amount of phosphorylated Yap, and we also observed heterodimers formed by Yap and Hck; consequently, this prevents Yap translocation to the nucleus, resulting in downregulation of NLRC4 mRNA and thus NLRC4 protein.

Activation of NLRC4 in macrophages during *Salmonella* infection, in addition to the production of the mature form of IL-1β, also induces cell death. In fact, NLRC4 was initially described as a proapoptotic molecule (17). In contrast with macrophages (13), B cells were resistant to cell death induced by *Salmonella*. However, if B cells are stimulated with cytosolic flagellin, cell death is observed as it has been described in macrophages (13). This result demonstrated that NLRC4 is fully functional in B cells and presents the possibility that flagellin is not efficiently translocated to the cytosol during infection. Additional evidence that the downregulation of NLRC4 by *Salmonella* affects B cell survival is demonstrated by the fact that infected B cells are more resistant to cell death induced by doxorubicin, a chemical agent

![FIGURE 7](http://www.jimmunol.org/)
Mechanism used by *Salmonella* to downregulate NLRC4 in B cells. A *Salmonella*-secreted protein injected by the TTSS into the B cell cytosol induces the phosphorylation of Yap and its interaction with Hck. This protein retains Yap in the cytosol and prevents its translocation to the nucleus. This results in a decrease in the efficiency of NLRC4 transcription with a consequent decrease in the expression of this receptor in B cells, preventing both the secretion of IL-1β and the induction of cell death.
that promotes the expression of NLRC4. Moreover, delivering flagellin to the cytosol of infected B cells does not result in caspase-1 activation and IL-1β secretion.

In addition to the participation of NLRC4 in IL-1β secretion and cytotoxicity, this molecule also has a role in controlling Legionella replication (43–45) and phagosome maturation during Legionella infection (30). Hence, the downregulation of NLRC4 in B cells may also have an impact on Salmonella replication and phagosomal maturation. Indeed, we have observed that Salmonella resides in a lysosomal compartment, and large numbers of bacteria are recovered from this compartment (8).

Via the aforementioned mechanism, B cells are an ideal niche for Salmonella. B cells permit the bacteria to remain for long periods in the host and spread to different organs. In this context, we have revealed that bone marrow cells are infected after a single dose of bacteria (10).

In summary, this study demonstrates that Salmonella induces downregulation of NLRC4 in B cells by a protein secreted through bacteria (10). This may help the survival of Salmonella in B cells (Fig. 7). These events provide further support that B cells are an amenable intracellular environment for Salmonella, enabling its survival and favoring its persistence and dissemination.

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Disclosures

The authors have no financial conflicts of interest.

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