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ORIGINAL RESEARCH PAPER

Pro-inflammatory and anti-inflammatory responses in B cells during *Salmonella* infection

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ABSTRACT

B-cells serve as a niche for *Salmonella* to establish a chronic infection, enabling bacteria to evade immune responses. One mechanism *Salmonella* uses to survive inside B-cells involves inhibiting the NLRC4 inflammasome activation, thereby preventing pyroptotic cell death. This study investigates whether *Salmonella*-infected B-cells can mount bactericidal responses to control intracellular bacteria. Our results show that *Salmonella*-infected B-cells can produce and release TNFα, IL-6, and IL-10, but not RANTES. Furthermore, priming B-cells with TNFα, IL-1β, or IFNγ enhances their bactericidal activity by promoting the production of reactive oxygen and nitrogen production species, reducing intracellular load. These results suggest that B-cells can clear *Salmonella* infection within a pro-inflammatory environment. However, the concurrent production of IL-10 may counteract the effects of pro-inflammatory cytokines, potentially modulating the immune response in the microenvironment.

KEYWORDS

B-cells, *Salmonella*, pro-inflammatory response, anti-inflammatory response, infection

INTRODUCTION

Salmonella is a Gram-negative bacterium that can infect many hosts [1]. In humans, *Salmonella* infection produces symptoms ranging from self-limiting gastroenteritis to the systemic infection called typhoid fever. Typhoid fever is produced exclusively by *Salmonella enterica* serovar Typhi and *S. enterica* serovar Paratyphi. In contrast, non-Typhi serovars produce self-limiting gastroenteritis [2]. Typhoid fever is a global health problem in developing countries. Indeed, every year, 9.3 to 12.6 million new cases are reported, with almost 65,000 to 188,000 deaths worldwide [3]. Moreover, it has been reported that 2–5% of recovered patients remain as chronic carriers [1]. *Salmonella* infects epithelial cells, macrophages, dendritic cells, and B-cells [2, 4–7]. The host response against this pathogen involves the innate and adaptive immune systems. The host innate immune system recognizes *Salmonella* in the intestinal epithelium and releases pro-inflammatory cytokines and chemokines, such as IL-6 and IL-8 [9]. Bacteria recognition by the immune system results in the recruitment and activation of other immune cells, such as macrophages and neutrophils.

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Indeed, these cells are an essential source of TNFα and IL-1β. Thus, TNFα and IL-1β, together with IFN-γ, are of utmost importance for controlling *Salmonella* infection in the early stages [9, 10]. For instance, mice lacking the TNF receptor showed higher bacterial proliferation and prompt death than the wild-type (WT) mice, which is also associated with the impairment in the recruitment and assembly of the NADPH oxidase complex to the *Salmonella*-containing vacuole [10]. IFN-γ, is a pro-inflammatory cytokine essential to macrophage activation during *Salmonella* infection; in the absence of IFN-γ, the production of iNOS is reduced, resulting in increased intracellular multiplication and subsequent mice death [11].

A protective adaptive immune response against *Salmonella* requires the coordinated action of T and B cells. For instance, immunized mice become vulnerable to a secondary challenge when T cells are depleted [9]. Furthermore, transferring antigen-specific T-cells to naïve mice confers protection to the host [10]. Conversely, B-cells play multiple roles during *Salmonella* infection, including production of specific antibodies against the pathogen, prime TH1 T-cells, and present antigens to T-cells [10–12]. In addition to these activities, B cells can serve as a niche for *Salmonella* to establish a chronic infection. To do so, *Salmonella* uses two mechanisms to infect B-cells; the first involves the induction of localized ruffling in the cell membrane to promote its entry, and the bacterium can use antigen-specific B-cell receptors to invade these cells [5, 7]. Once inside, bacterium manipulates the activation of the inflammasome and the Akt-YAP pathway in B-cells while inhibiting autophagy to create a niche that supports its persistence within the cells [13, 14].

Although some of the mechanisms used by *Salmonella* to establish chronic infection in B cells have been described, it is not fully understood how this pathogen can survive inside B cells for a long time. The characterization of *Salmonella*-infected B-cell's role in producing pro- and anti-inflammatory mediators has yet to be described. This study demonstrates that *Salmonella*-infected B-cells release TNFα and IL-6 but not RANTES. Moreover, primed B-cells with pro-inflammatory cytokines increase their bactericidal activity, which mediates ROS and RNS production. Suppressively, *Salmonella*-infected B-cells also release IL-10. These findings suggest that B-cells can control intracellular *Salmonella* in a pro-inflammatory environment. However, *Salmonella* can also subvert B-cell microbicidal activity by producing IL-10. More studies are needed to understand how B-cells respond to *Salmonella* infection.

MATERIAL AND METHODS

Bacterial strains and growth conditions

In this study, we use *S. enterica* Serovar Typhimurium (*Salmonella* Typhimurium) 14028 (ATCC® 14028), *Salmonella* Typhimurium harboring a plasmid that encodes the Green Fluorescent Protein (*Salmonella* Typhimurium GFP). Both strains were grown on 5 mL of LB broth at 180 RPM overnight at 37 °C. The LB broth was supplemented with 100 μg mL^{-1} ampicillin for *Salmonella* Typhimurium GFP. The following day, a 1:30 dilution was made in fresh LB broth, and the culture was continued shaking at 37 °C until reaching the logarithmic phase.

B-cell purification and *in vitro* infection

Spleens were obtained from 6- to 8-week-old male or female Balb/c mice. Cell suspension was prepared, and B-cells were obtained by negative selection (Miltenyi Biotec). Briefly, cells were obtained by mechanical disaggregation of the spleen and incubated with red cells lysis buffer for 10 min at room temperature. Following lysis, the cells were washed with PBS and resuspended in cold PBS supplemented with 0.5% fetal bovine serum and 2 mM EDTA. The cell suspension was incubated with the Biotin-Antibody Cocktail for 5 min at 4 °C, followed by the addition of Anti-Biotin MicroBeads. Cell suspension was incubated for 10 min at 4 °C. The cell suspension was then passed through the magnetic column, and the flow-through was collected. This yielded pure resting B-cells with approximately 95% purity. The purified B-cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (RP10) (Gibco BRL). For infection, 1×10^6 B-cells were incubated with *Salmonella* Typhimurium 14028 at the indicated multiplicities of infection (MOI) for 30 min. Cells were washed twice with PBS, and the culture was continued in RP10 medium supplemented with gentamicin (80 μg mL^{-1}) for 24 h [6].

Bone marrow-derived macrophage obtention (BMDM) and *in vitro* infection

Bone marrow cells were isolated from the femurs and tibias of 6- to 8-week-old male or female Balb/c mice and cultured at 37 °C and 5% CO_2 for seven days in RP10 medium supplemented with 30% L-929 cell supernatant and antibiotics [15]. After differentiation, cells were washed with cold PBS, and BMDM were collected and seeded in 24-well plates at a density of 1×10^6 cells/well in RP10 medium. For infection, *Salmonella* Typhimurium was added at the indicated MOI in 300 μl of medium. To synchronize the infection, plates were centrifuged at 1800 RPM for 5 min at room temperature, followed by incubation at 37 °C and 5% CO_2 for 30 min. After incubation, cells were washed twice with PBS, and the culture was continued in an RP10 medium supplemented with 80 μg mL^{-1} gentamicin for 24 h.

Salmonella survival in stimulated B-cells and BMDM

B-cells were purified and stimulated with recombinant TNFα, IL-1β, or IFN-γ (R&D Systems) for 3 h, followed by infection with *Salmonella* Typhimurium at an MOI of 50 for 30 min. Cells were washed twice with PBS, and at 1, 3, and 24 h post-infection, cells were recovered by centrifugation and lysed with 1 mL of PBS containing 1% Triton-X 100. Serial dilutions of the cell lysates were plated on LB agar to determine the bacterial counts (Colony Forming Units,

CFUs). The percentage of Salmonella survival was calculated using the following formula: (CFUs at 24 h post-infection/ CFUs at 1-h post-infection) x 100%.

Reactive nitrogen and oxygen species detection

To determine reactive nitrogen species (RNS) production, 1×10^6 B-cells or BMDM were stimulated and infected as described above. Cell culture supernatants were collected 24 h post-infection, and RNS production was quantified using the Griess method according to the manufacturer's instructions (Molecular Probes) [16]. For reactive oxygen species (ROS) production, stimulated B-cells or BMDM were incubated with the fluorescent probe 5-(-6)-Chloromethyl-2′7′-Dichlorodihydrofluorescein Diacetate Acetyl Ester (CM-H_2DCFDA) (Molecular Probes) for 30 min at 37 °C [17]. Cells were then infected with *Salmonella* Typhimurium at an MOI of 50 for 30 min post-infection and analyzed by flow cytometry in a Cyan ADP flow cytometer (*Beckman Coulter*).

Expression of IL-10 and IL10Rα in splenic B-cells

B-cells from Balb/c male or female mice were infected with *Salmonella* GFP at an MOI of 50. At 20 h post-infection, brefeldin A was added, and cells were cultured for an additional 4 h to reach 24 h post-infection. Cells were stained with the following antibodies: PB anti-mouse CD19 (clone 6D5, Bio-Legend), PE anti-mouse CD210 (IL-10Rα) (clone 1B1.3a, Bio-Legend) or PerCP-Cy5.5 anti-mouse IL-10 (clone JES5-16E3, BD Pharmigen). Stained cells were analyzed by flow cytometry in a *LSR Fortessa* flow cytometer.

In vivo infection

Balb/c male or female mice (6- to 8-week-old) were intraperitoneally injected with 100 CFUs of *Salmonella* Typhimurium in 100 µL of PBS. At 3- and 5 days post-infection, mice were euthanized, and the spleens were harvested. A cell suspension was prepared, and bacterial load was determined by lysing 1×10^6 cells and plating serial dilutions on LB agar. B-cells were purified by negative selection and used to asses IL-10 production and expression of IL-10Rα *ex-vivo* by flow cytometry.

ELISA assays

B-cells were infected *in vitro* as described above. Twenty-four hours after infection, cell culture supernatants were collected, and the concentrations of TNFα, IL-6, IL-10, and RANTES were quantified by ELISA (R&D Systems) according to the manufacturer's instructions.

RESULTS

B-cells infected with *Salmonella* produce pro-inflammatory cytokines

During *Salmonella* infection, pro-inflammatory cytokines such as TNFα and IL-6 are produced by different types of cells, including epithelial cells, macrophages, dendritic cells, and neutrophils [8, 18]. These cytokines are key for promoting bacterial clearance. For instance, RANTES (CCL5) is produced by macrophages and dendritic cells in response to *Salmonella* infection; this chemokine recruits T-cells, natural killer cells, dendritic cells, and monocytes [19]. Previous studies have shown that B-cells can produce TNFα and IL-6 when stimulated with CD40L, BCR, and inflammatory stimuli, including viral infections and LPS [18, 20, 21]. On the other hand, B-cells can produce RANTES when they are stimulated with LPS [22]. We evaluated whether *Salmonella*-infected B-cells can produce TNFα, IL-6, and RANTES. Splenic B-cells were infected with *Salmonella*, and 24 h post-infection, supernatant of infected cells was collected to determine the production of the cytokines by ELISA. The results show that B-cells infected with *Salmonella* release significant TNFα and IL-6 when we used an MOI of 25 and 50 (Fig. 1A and 1B).

In contrast, B-cells infected with *Salmonella* do not release RANTES (Fig. 1C). Thus, B-cells might initiate or amplify an inflammatory response during *Salmonella* infection. However, the absence of RANTES production in infected B-cells is notable. RANTES is a chemokine that recruits

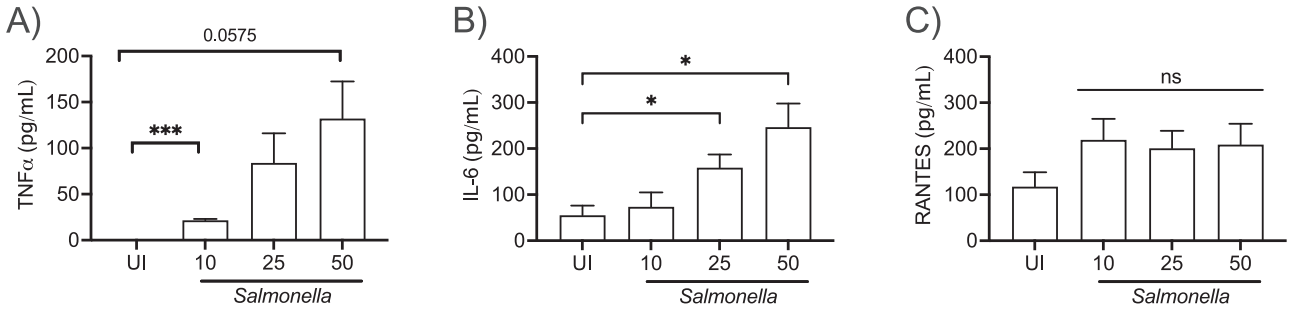

Fig. 1. **Infected B-cells with *Salmonella* release pro-inflammatory cytokines**. A million splenic B-cells were infected with *Salmonella* Typhimurium at the indicated MOI and incubated for 24 h. After incubation, cell culture supernatant was recovered to quantify the (**A**) TNFα, (**B**) IL-6, and (**C**) RANTES released by ELISA. The data shown comprises three independent experiments that were duplicated ($n = 6$). Bars present the mean ± SEM, one-way ANOVA analyzed data, and Welch's multiple comparison tests were performed to compare the treated groups with the control group. Significant changes are indicated by $^{*}P \leq 0.05$, $^{***}P \leq 0.001$. ns = not significant

T cells, monocytes, and other immune cells to sites of infection [23]. This result suggests that, while B-cells respond to *Salmonella* by producing inflammatory cytokines, they may not directly contribute to the chemokine-mediated recruitment of immune cells.

TNFα, IL-1β, and IFN-γ induce bactericidal activity on B-cells

Pro-inflammatory cytokines, including TNFα, IL-1β, and IFN-γ are critical for controlling *Salmonella* infection [24–28]. For example, TNFα stimulation prevents M2 macrophage polarization, disrupting the establishment of persistent infections [18]. The cytokine IL-1β enhances antimicrobial immunity against intracellular bacteria [29], while IFN-γ boosts macrophage's capacity to clear intracellular *Salmonella* [30]. Thus, pro-inflammatory cytokines are pivotal in activating macrophages to kill intracellular bacteria. In contrast, we have previously shown that *Salmonella* can survive and establish a chronic infection in B-cells [4, 31]. Therefore, we assessed whether the pre-activation of B-cells with pro-inflammatory cytokines can induce anti-bactericidal activity in these cells. After 24 h of infection, about 70% of *Salmonella* persists within B-cells, while only 1% survives in macrophages when both types of cells are infected under resting conditions (Fig. 2). Pre-activation of B-cells with TNFα or IFN-γ resulted in a significative reduction in viable bacteria at 24 h post-infection (Fig. 2A and 2C). B-cells stimulated with IL-1β internalized twice as many bacteria as mock-stimulated cells. They were also able to control bacterial growth (Fig. 2B). In macrophages, only high concentrations of cytokines led to a slight reduction in bacterial load after 24 h of infection (Fig. 2D–F). To assess bacterial control, we calculated the percentage of *Salmonella* survival under different conditions in B-cell and macrophages. We found that TNFα, IL-1β, or IFN-γ stimulation significantly reduced bacterial survival inside B-cells from 70% in non-primed cells to 9–20% in cytokine-primed cells (Fig. 2G). All these data showed that B-cells can significantly reduce *Salmonella* survival when primed with pro-inflammatory cytokines. In contrast, in macrophages, only the high concentration of IFN-γ (1,250 pg mL^{-1}) had a significant effect on *Salmonella* survival (Fig. 2H). These findings highlight the complexity of the interaction of *Salmonella* with the different immune cell types and the microenvironment, which ultimately results in the elimination or persistence of the bacteria in the host.

B-cells stimulated with TNFα, IL-1β, and IFNγ induce ROS and RNS activity

The production of Reactive Oxygen and Nitrogen Species (ROS and RNS, respectively) is a crucial mechanism for controlling intracellular pathogens, including *Salmonella* [25]. Cytokines such as TNFα, IL-1β, and IFNγ are known to enhance the production of these microbicidal mediators [32–34]. As previously described, B-cells primed with TNFα, IL-1β, or IFN-γ efficiently control intracellular *Salmonella*. Therefore, we assessed whether B-cells primed with these cytokines could produce ROS and RNS during infection with *Salmonella*. Our results showed that stimulation with TNFα, IL-1β, or IFN-γ alone did not induce ROS or RNS production (data not shown). However, ROS and RNS production was triggered after *Salmonella* infection in cytokine-primed B-cells (Fig. 3). *Salmonella* infection induces ROS production in 2.7% of unprimed B-cells (Fig. 3A). In cytokine-primed B-cells, the percentage of cells-producing ROS increased, with stimulation by IFN-γ (1,250 ng mL^{-1}) raised the proportion to 8% (Fig. 3A). In macrophages, the lower concentration of pro-inflammatory cytokines and *Salmonella* infection did not induce a robust production of ROS. Nevertheless, the *Salmonella* infection of B-cells primed with higher concentrations of pro-inflammatory cytokines induces a strong production of ROS (greater than 26.6% of B-cells) (Fig. 3B).

Next, we assessed the production of RNS in B-cells and macrophages following *Salmonella* infection. The results showed that B-cells primed with TNFα, IL-1β, or IFN-γ exhibited a 3 to 6-fold increase in RNS production compared to untreated B-cells upon infection (Fig. 3C). Notably, stimulation of B-cells with IL-1β resulted in a significantly increased production of RNS. In contrast, in macrophages, only those primed with 1,250 pg mL^{-1} IFN-γ showed a marked increase in the RNS production relative to unprimed macrophages (Fig. 3D). These results indicate that priming B-cells with TNFα, IL-1β, or IFN-γ enhances the production of both ROS and RNS during *Salmonella* infection. This suggests that the production of these oxidative molecules in B-cells plays a role in facilitating bacterial clearance.

Salmonella-infected B-cells produce IL-10

The host's inflammatory responses often play a crucial role in controlling bacterial infections. However, certain bacterial pathogens, such as *Salmonella*, have developed strategies to evade immune detection, enabling them to establish a chronic infection [35]. One key mechanism for evading innate immunity involves the production of IL-10, a potent anti-inflammatory cytokine [36, 37]. IL-10 is secreted by various immune cells, including neutrophils, macrophages, dendritic cells, T-cells, and B-cells [38]. During systemic *Salmonella* infections, IL-10 is primarily produced by B- and T-cells [36, 39]. Previous research showed that Epstein-Bar virus-transformed lymphoblastoid B *cell* lines infected with *Salmonella* produced IL-10 [40]. However, whether infected B-cells produce IL-10 remains unclear. To investigate this, we isolated B-cells from the spleen and infected them with *Salmonella* expressing the Green Fluorescent Protein (*Salmonella* GFP) to identify B-cells harboring the bacteria. At 24 h post-infection, we assessed the presence of intracellular IL-10 and the expression of IL-10R on the cell surface. In line with our previous findings [15, 41], a small percentage of B-cells are infected with *Salmonella* (GFP^+ cells) (Fig. 4A). After incubation, only about 3.5% of uninfected B-cells produce IL-10, and the percentage of IL-10-producing B-cells increased ~ 3-fold in cells that were

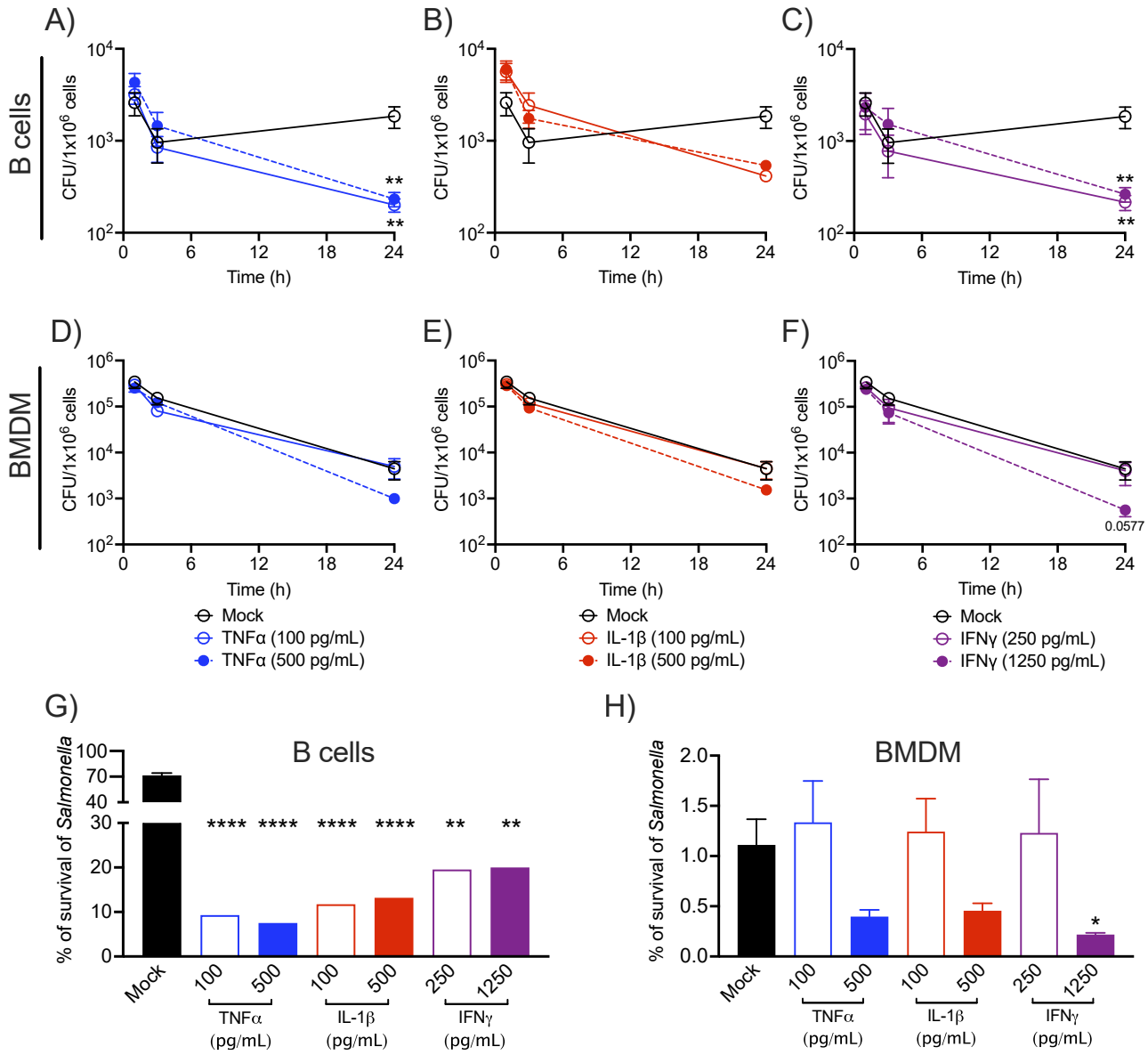

Fig. 2. **Primed B-cells control intracellular *Salmonella*.** A million of splenic purified B-cells (**A–C**) or bone marrow-derived macrophages (BMDM) (**D–F**) were mock stimulated or stimulated with (**A, D**) TNFα, (**B, C**) IL-1β, or (**C, F**) IFNγ for 3 h with the indicated concentrations, and then infected with *Salmonella* Typhimurium at MOI = 50. After 1-, 3-, and 24 h post-infection, cells were lysed with Triton-X 1% (v/v), and Colony Forming Units (CFUs) were determined by plating serial dilutions on LB agar. The percentage of *Salmonella* survival in B-cells (**G**) and BMDM (**H**) at 24 h post-infection was quantified. The data shown comprises two independent experiments by triplicates ($n = 6$) and is presented as the mean ± SEM. Data were analyzed by one-way ANOVA. Welch's multiple comparison tests compared the treated groups with the control group (mock primed cells), for CFUs data were analyzed within treatments at each time point. Significant changes are indicated by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$

in contact with the bacterium. Less than 1% of uninfected cells produced IL-10 or expressed IL-10. Similar results were observed in B-cells that were in contact with *Salmonella* but did not internalize bacteria (GFP^{-} cells). However, the percentage of B-cells producing IL-10 increased significantly in cells harboring *Salmonella* (GFP^{+} cells) (Fig. 4B and 4C). Similar results were observed for the expression of IL-10R (Fig. 4B and 4D).

To further investigate whether *Salmonella* can induce IL-10 production in B-cells during *in vivo* infection, we infected mice intraperitoneally with *Salmonella*, and 3- and 5-days post-infection, the spleen was harvested, and B-cells were isolated. Subsequently, 1×10^7 B-cells were either cultured alone or restimulated with *Salmonella* for 24 h *ex vivo*; the concentration of IL-10 in the cell culture supernatant was determined by ELISA. *Salmonella* seemed to induce secretion of IL-10 even in cells from uninfected mice, and IL-10 production was significantly increased in B-cells from mice that were infected for five days (Fig. 4E). Moreover, the bacterial burden was higher at five than at three days post-infection (Fig. 4F), suggesting a positive correlation between the bacterial load and the capacity of B-cells to produce IL-10. These findings demonstrate that *Salmonella* infection induces IL-10 production in B-cells and upregulates IL-10R

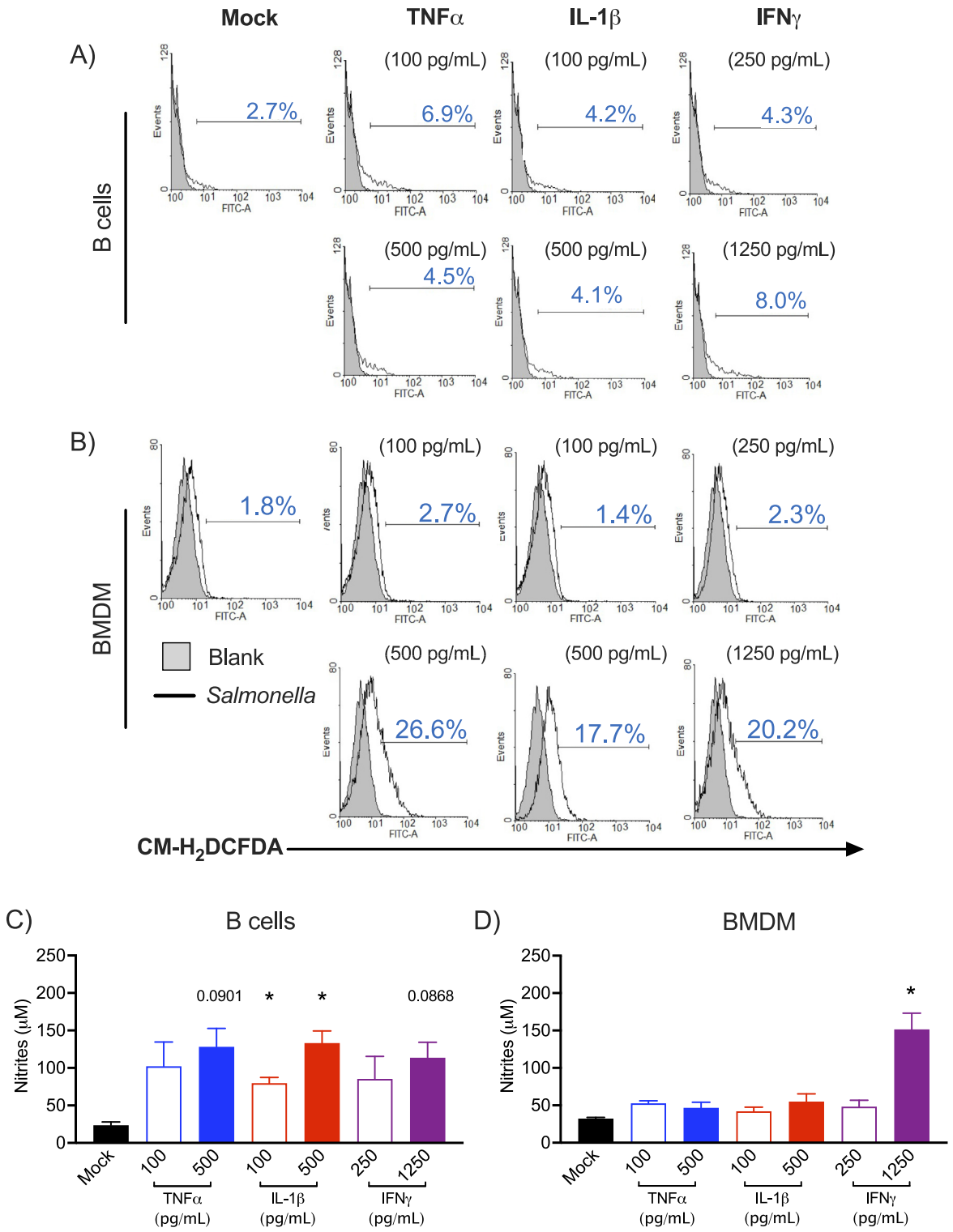

Fig. 3. **B-cells infected with *Salmonella* produce ROS and RNS.** A million splenic B-cells (**A**) or BMDM (**B**) were mock-stimulated or stimulated with the indicated pro-inflammatory cytokines for 3 h. Cells were incubated in the presence of CM-H_2DCFDA for 30 min and then infected with *Salmonella* Typhimurium at an MOI = 50 for 30 min. At 30 min post-infection, cells were analyzed by flow cytometry to determine the percentage of cells producing reactive oxygen species. A representative histogram for each condition is presented. One million splenic B-cells (**C**) or BMDM (**D**) were mock stimulated or stimulated with pro-inflammatory cytokines for 3 h. Then, cells were infected with *Salmonella* Typhimurium at an MOI = 50. At 24 h post-infection, cell culture supernatants were harvested to quantify the nitrite production using the Griess method. The data shown comprise two independent experiments by triplicates ($n = 6$). Bars present the mean ± SEM; one-way ANOVA analyzed data. Welch's multiple comparison tests were performed to compare the treated groups with the control group (mock primed cells). Significant changes are indicated by $^{*}P \leq 0.05$

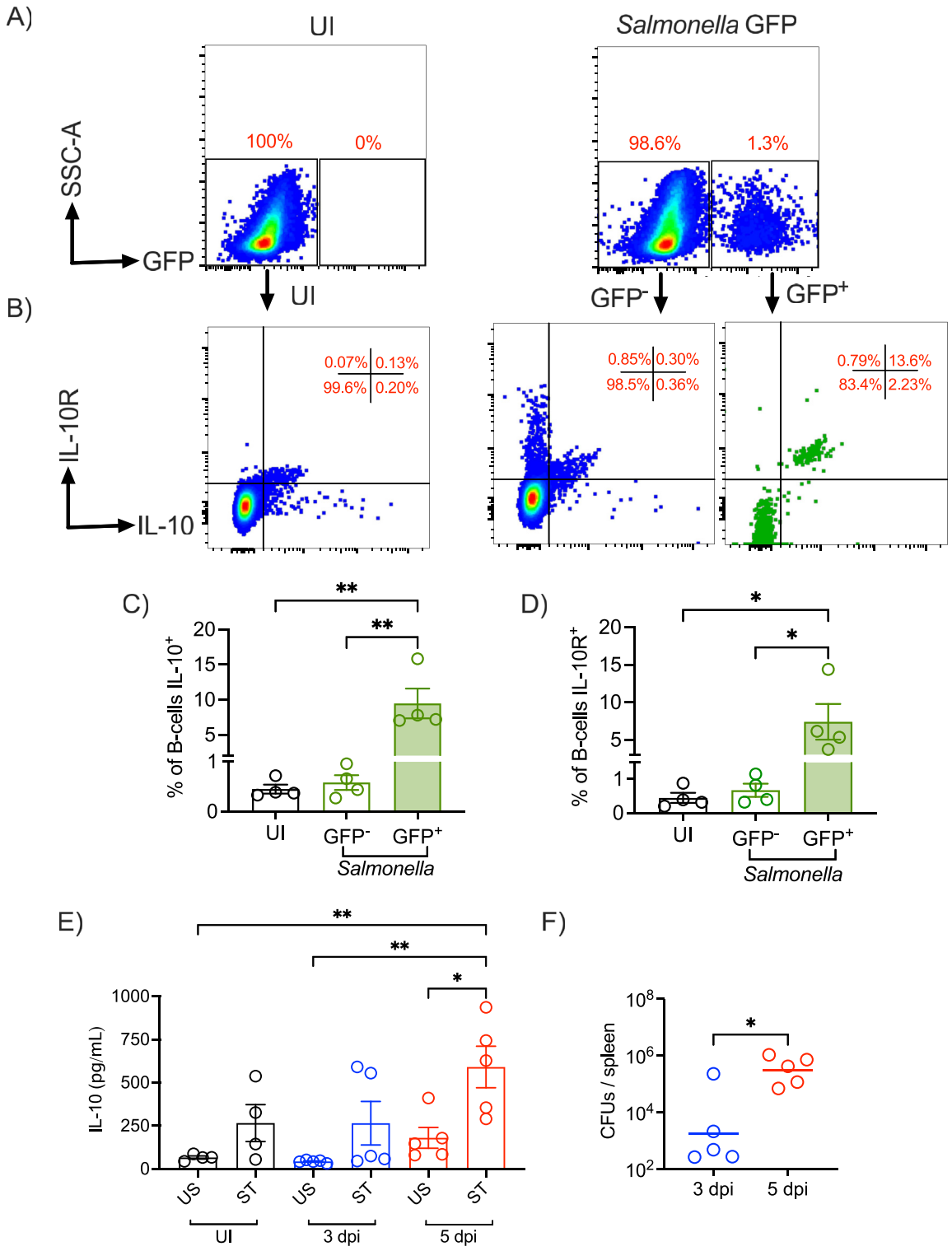

Fig. 4. **IL-10 production in B-cells upon infection with *Salmonella*.** (**A–D**) B-cells were infected with *Salmonella* GFP at MOI = 50; at 20 h post-infection, brefeldin A was added for 4 h, and cells were analyzed by flow cytometry to determine the percentage of IL-10-producing B-cells. Representative dot plots of uninfected cells and infected cells (GFP^- and GFP^+ cells) (**A**) and IL-10^+ B-cells or IL10R^+ B-cells are shown (**B**). The percentage of IL-10^+ B (**C**) cells or IL10R^+ B-cells (**D**) was determined. (**E, F**) Mice were intraperitoneally infected with a dose of 100 CFUs of *Salmonella* Typhimurium, 3 or 5 days post-infection, mice were euthanized, and the spleen was harvested. (**E**) B-cells were enriched, and 1 × 107 cells were cultured alone or stimulated with Salmonella Typhimurium for 24 h. Then, the cell culture supernatant was recovered to determine IL-10 concentration using ELISA. (**F**) Serial dilutions from spleen homogenates determined bacterial load and plated them on LB agar. The data shown comprises four (**C, D**) or two (**E, F**) independent experiments. Bars present the mean ± SEM (**C–E**) or geometric mean (**F**). Each circle represents a mouse (**E, F**). The data were analyzed using a one-way ANOVA test. Tukey's multiple comparisons test was performed to compare groups with each other (**C–E**) or Mann-Whitney's test (**F**). Significant changes are indicated by $^*P \leq 0.05$, $^{**}P \leq 0.01$

expression in infected cells. This suggests that IL-10 may trigger autocrine signaling, potentially facilitating the establishment of chronic infection within B-cells.

DISCUSSION AND CONCLUSIONS

B-cells are essential for controlling secondary infections with *Salmonella* serovars, primarily by producing pathogen-specific antibodies. Studies have shown that B-cell deficient mice immunized with a *Salmonella* attenuated strain and subsequently challenged with a virulent strain fail to clear the infection efficiently [42]. However, B-cells also play a critical role during *Salmonella* primary infection. Research from our group and others has demonstrated that B-cells are a target of *Salmonella* infection [4, 7, 43]. The invasion of B-cell by *Salmonella* depends on virulence genes encoded in the pathogenicity island 1 (IP1) [7]. Once inside B-cells, the pathogen can persist up to 60 days in B-cells. One key mechanism *Salmonella* employs to ensure its long-term survival in B-cells is inhibiting inflammasome activation. This suppression prevents IL-1β and blocks pyroptotic cell death, allowing the bacteria to evade immune detection and elimination [13], potentially contributing to chronic infection and bacterial carriage.

On the other hand, we observed that B-cells secrete pro-inflammatory cytokines such as TNFα and IL-6, whose secretion does not relay on inflammasome activation (Fig. 1A and 1B). These findings suggest that B-cells can initiate a pro-inflammatory response during *Salmonella* infection. Previous studies have shown that B-cell receptor (BCR) and CD40L stimulation can induce the production of TNFα and IL-6 in B-cells [44]. Additionally, B-cells produce these cytokines in the context of infection. For example, human B-cells transformed with Epstein Barr Virus [45, 46] and B-cells from tonsils or peripheral blood stimulated with *Staphylococcus aureus* in combination with IL-2 are capable of producing TNFα and IL-6 [47].

On the other hand, we observed that the secretion of IL-6 was increased only when B-cells were infected at higher MOIs (25 and 50). This suggests that a threshold of bacterial load is required to increase the production of IL-6. *In vivo*, *Salmonella* can accumulate in specific tissues, such as the spleen, where localized bacterial concentrations may reach levels high enough to infect B-cells. These conditions may reflect the conditions used during *in vitro* experiments, supporting the physiological relevance of our findings. While B-cells can produce chemokines such as RANTES (CCL5) during infections like *Trypanosoma cruzi* [22], we found that *Salmonella* infection does not significantly alter RANTES production in B-cells. RANTES is a chemokine that recruits T-cells, monocytes, and other immune cells to sites of infection [23], and its absence in the context of *Salmonella* infection suggests that B-cells may not play a direct role in chemokine-mediated immune cell recruitment during this particular infection. This finding underscores the context-dependent nature of B-cell responses and highlights the complexity of their role in different infections.

Cytokines like TNFα, IL-1β, and IFN-γ play a central role in the host defense against *Salmonella*. Blocking TNFα with specific antibodies increases susceptibility to infection in mice [27], while administration of recombinant TNFα enhances resistance to the infection [48]. IL-1β promotes antimicrobial immunity against intracellular bacteria [29], and IFN-γ is crucial to control systemic infections. Neutralizing IFNγ during infection increases bacterial burden and host mortality, as this cytokine is a key enhancer of antibacterial activity in macrophages [27, 49]. Consistent with these findings, we observed a significant reduction in *Salmonella* survival in macrophages treated with recombinant IFN-γ (Fig. 2H). Other studies have shown that stimulating macrophages with TNFα or IL-1β enhances their antimicrobial functions [29, 50]. In the case of B-cells, we found that stimulation of the cells with TNFα, IL-1β, or IFN-γ promotes infection control, significantly reducing bacterial survival after 24 h post-infection. This suggests that a pro-inflammatory environment may help B-cells clear the infection (Fig. 2A–C and 2G). ROS and NOS production are among the mechanisms that control intracellular bacteria [51]. When B-cells were stimulated with TNFα, IL-1β, or IFN-γ we observed an increase in the production of ROS and NOS, indicating that these microbicidal mechanisms contribute to controlling *Salmonella* infection in cytokine-stimulated B-cells (Fig. 3). Thus, *in vitro*, B-cells can produce pro-inflammatory cytokines and effectively control *Salmonella* infection in the presence of a pro-inflammatory environment. The ability of B-cells to respond to cytokine stimulation and produce ROS and NOS suggests that they can act as auxiliary effector cells in the immune response to intracellular pathogens. However, the extent to which B-cells contribute to bacterial clearance *in vivo*, particularly in chronic or systemic infections, remains an area for further investigation.

Conversely, B-cells also produce anti-inflammatory cytokines such as IL-10, IL-35, and TGFβ [52]. IL-10-producing B-cells have been shown to suppress the progression of colitis and experimental arthritis in mice [53, 54]. In the context of infection, *Brucella abortus* induces TGF-β and IL-10 production in infected B-cells, and B-cell-deficient mice are more resistant to the infection [55]. We found that *Salmonella*-infected B-cells produce IL-10 and express the IL-10 receptor. B-cells isolated from the spleen of infected mice produce IL-10 upon restimulation with *Salmonella*, and the levels of production of IL-10 by B-cells correlate with the bacterial burden in the spleen (Fig. 4). The production of IL-10 B-cells by *Salmonella*-infected B-cells is interesting, as this cytokine can inhibit the activation of macrophages, dendritic cells and T cells [56], thereby limiting the host's ability to mount an effective immune response. By inducing IL-10 production, *Salmonella* may exploit B-cells to create an immunosuppressive niche that allows the bacteria to evade immune detection and persist within the host. This may be another mechanism by which *Salmonella* is able to survive within B cells for extended periods.

Overall, these findings highlight the complex and multifaceted role of B-cells in the immune response to *Salmonella* infection. On one hand, B-cells can contribute to bacterial clearance by producing pro-inflammatory cytokines, ROS,

and RNS, particularly when primed with pro-inflammatory cytokines. On the other hand, the production of IL-10 by infected B-cells may help *Salmonella* evade immune detection and establish chronic infection. It also raises important questions about how pathogens like *Salmonella* manipulate B-cell responses to their advantage. Further research is needed to elucidate the mechanisms by which *Salmonella* induces IL-10 production in B-cells and to explore whether targeting this pathway could enhance host immunity and reduce bacterial persistence.

Ethics statement: All animal experimental protocols were implemented by the instructions of the ethics committee.

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