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Salmonella serovar Typhimurium infection modulates the expression of immune-related genes in avian-enriched monocytes

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A B S T R A C T

Salmonella enterica subsp. *enterica* serovar Typhimurium is a gram-negative bacterium that can infect various hosts. *Salmonella* serovar Typhimurium (ST) infection in adult poultry usually results in an asymptomatic intestinal carriage, while the infection in newly hatched chicks may lead to a severe clinical disease. Macrophages play an essential role by limiting bacterial replication in submucosal tissues using several defense mechanisms. Subsequently, *Salmonella* strains have developed countermeasures to evade or subvert the host immune responses to their benefit. We previously showed that the ST challenge can significantly reduce the phagocytic capacity of chicken-enriched peripheral blood monocytes. In the present study, we sought to provide a snapshot of the immune responses against ST challenge in chicken-enriched peripheral blood monocytes by evaluating the transcriptional changes in inflammatory and anti-inflammatory cytokines, pattern recognition receptors, and other immune-related molecules at the mRNA level. Our results indicate that wildtype ST challenge in avian blood monocytes favors the differentiation of macrophages toward the alternatively activated M2-like cells through downregulation of inflammatory IL-1β and upregulation of anti-inflammatory IL-10 cytokines. Our result may partially explain how the bacterium modulates the immune response in professional phagocytes to survive in the hostile environment of host immune cells and further disseminate within the host.

Keywords: *Salmonella serovar Typhimurium, Immune response, Enriched peripheral blood monocyte (EPBM), Inflammatory cytokines, Anti-inflammatory cytokines, Toll-like receptors.*

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1 Introduction

Salmonella serovar Typhimurium (ST) is a gram-negative facultative intracellular bacterium that can infect many hosts (1). In chickens, systemic infection with ST is more transient and, except for newly hatched chicks, causes little clinical disease. However, the gastrointestinal infection may continue for several months and cause horizontal transmission throughout the infected flocks (2). Poultry products contaminated with ST are one of the major causes of zoonotic foodborne illness in humans. Epidemiological surveys focusing on the prevalence of *Salmonella* in poultry have been broadly studied and well-documented (3). However, our understanding of immunological mechanisms involved with invasion, colonization, and intracellular persistence of *Salmonella* in chickens is still limited (4-6).

Pathogenesis of ST is facilitated by two distinct forms of type III secretion system (T3SS) encoded by genes of *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and -2) (7). T3SS produces about 40 distinct effector proteins, enabling *Salmonella* invasion, survival, and replication within the host cells (8). SPI-1 T3SS is mainly expressed in the infected gastrointestinal tract and facilitates the epithelial invasion of the bacterium; SPI-2 T3SS is predominantly expressed inside the infected cells and supports the replication of the bacterium within infected macrophages.

After intestinal colonization, macrophages are the primary immune defender cells that detect the existence of microorganisms and secrete cytokines and chemokines responsible for the recruitment of other immune cells to the site of the infection and thus regulate the inflammatory response (1, 5, 7, 9, 10). Intracellular survival of the bacteria depends on the expression of cytokines and their subsequent inflammation, which define macrophage fates. Excessive inflammation leads to tissue damage, whereas insufficient inflammation will fail to control the infection (8). In addition, different strains of *Salmonella* have developed various mechanisms to avoid or subvert immunity to their benefit, and there is an interplay between the detection and evasion of *Salmonella* in the host (11).

A better understanding of the underlying immunological mechanisms involved in *Salmonella* pathogenicity, at cellular and molecular levels, is crucial to improve the existing control measures, such as vaccination and molecular-based immunotherapeutic strategies against avian salmonellosis. We have previously shown that ST challenge can weaken the phagocytic capacity of chicken-enriched peripheral blood monocytes (EPBMs) (12). The study herein sought to provide a snapshot of the immune responses against ST challenge in chicken EPBMs by evaluating the transcriptional changes in inflammatory (interleukin (IL)-1β, IL-6) and anti-inflammatory (IL-10, transforming growth factor (TGF)-β) cytokines, pattern recognition receptors (PRRs) (Toll-like receptor (TLR)4, TLR5, TLR9), and some other immune-related genes (inducible nitric oxide synthase (iNOS), major histocompatibility complex (MHC)-I, MHC-II, and myeloid differentiation primary response 88 (MyD88)). Our results reveal possible immunomodulatory effects of the ST in chicken EPBMs, which may partially explain how the bacterium survives in the hostile environment of host immune cells and uses such mechanisms to further disseminate within the host body.

2 Materials and Methods

2.1 Bacterial strains

Overnight Luria-Bertani broth (Merck, Darmstadt, Germany) culture of *Salmonella* serovar Typhimurium (ST) (ATCC® 14028) was diluted and grown in Mueller-Hinton agar (Merck, Darmstadt, Germany) at 37°C for 12 h. The corresponding dilution with 4×10^3 colony-forming units (CFUs)/ml of ST was used for the challenge described later in this paper.

2.2 Chicken-enriched peripheral blood monocytes

In previous publications, EPBMs, also called monocyte-derived macrophages, were prepared as described elsewhere in human and porcine model systems (13, 14). Briefly, peripheral blood mononuclear cells were isolated from the blood obtained from 3-week-old broiler chickens (Ross 308) using the Ficoll method. To obtain monocytes, mononuclear-containing cells isolated from chicken were cultured in 24-well tissue culture plates in RPMI (Roswell Park Memorial Institute) culture medium and incubated for two h at 37°C under 5% CO_2 and 95% humidity. The purity of the monocyte cultures was confirmed by the Giemsa staining and under a light microscope (~95% monocytes). The number of viable cells was counted using Trypan Blue vital staining. Eventually, enriched monocytes were divided into 24-well tissue culture plates for further cellular and molecular assays.

2.3 Salmonella challenge

Twenty-four-well plates were seeded with 2×10^6 EPBMs per well and incubated for 24 h at 37°C in a CO_2 incubator. The EPBMs were challenged with ST (50 CFU/cell) or RPMI

media (mock challenge) for two h at 40°C under 5% CO_2 and 95% humidity. The EPBM suspensions were then transferred to 1.5 ml microcentrifuge tubes, centrifuged (1000 × *g*, four °C, 5 minutes) to remove the cell debris, and stored at -80°C before RNA extraction.

2.4 Flow cytometry-based assay to determine phagocytic capacities

At 2 h post-challenge, EPBMs were incubated with fluorescein isothiocyanate (FITC)-loaded polystyrene microparticles (1.0 µm, Sigma-Aldrich; St. Louis, MO) at a ratio of 10 beads/cells for three h at 40 °C under 5% CO_2 and 95% humidity, as previously described (12). The EPBMs were then washed once with ice-cold PBS, harvested on ice, and used to measure the internalization of FITC-loaded beads through flow cytometry (≥10,000 events/sample). Relative phagocytic capacities were presented as the ratio of $FITC^+$ EPBMs relative to the mean values for the mock-challenged group.

2.5 Transcriptional analyses

Transcriptional analyses of proinflammatory (IL-1β, IL-6) and anti-inflammatory (IL-10, TGF-β) cytokines, PRRs (TLR4, TLR5, TLR9), and some other immune-related molecules (iNOS, MHC-I, MHC-II, and MyD88) were performed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using SYBR Green chemistry. RNA was extracted using the FAVOR GENE Kit (Ambion, Thermo Fisher Scientific Inc. Waltham, MA, USA) according to the manufacturer's instructions. A two-step RT-qPCR was initiated by cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Finland). Relative quantification of the mRNA copies of the respective genes was performed by using Rotor-Gene Q (Qiagen, Valencia, CA) real-time PCR machine using YTA SYBR Green qPCR Kit (Yekta Tajhiz Azuma, Tehran, Iran) with the cDNAs synthesized in the previous step. To obviate concerns about genomic DNA contamination, exon junction or intron-spanning proper primers were designed for seven pairs of primers along with five other pairs from references (Table 1).

Table 1. Detailed description of primers used for transcriptional analysis.

Group	Gene	Primer sequence (5′ to 3′)	Amplicon size (bp)	Annealing temperature (°C)	References
TLRs-related	TLR4	F: GAGTGGCAACAGCTCGAAAA R: CAGCCCGTTCATCCTCATAT	129	59	(15)
	TLR5	F: CACACGGCAATAGTAGCA R: CACACCTGGAACTTGGAA	94	56	Designed
	TLR9	F: TCATTGACCTCCACAGAC R: GGAAGACACCAGAGAAGA	77	56.8	Designed
	MyD88	F: AGAAAGAAGGTGTCGGAGGAT R: TGGGGAAAGACTAAGAGCAAAT	100	59	(15)
Cytokines	IL-1β	F: TCATCCAGCCAGAAAGTGAGG R: GTGCCGCTCATCACACAC	140	61.5	Designed
	IL-6	F: CCTGACGAAGCTCTCCAG R: TCGGGATTTATCACCATCTGC	153	60	(16)
	IL-10	F: GACGTTCGAGAAGATGGATGAG R: CTCCTCCTCATCAGCAGGTA	99	61.5	Designed
	TGF-β	F: TGAGTATTGGGCCAAAGAGCTG R: ACACGAAGAAGATGCTGTGG	101	60	Designed
EPBMs-related	iNOS	F: CACTACCTGCCTGGAGAACAT R: TGCCCAATAGCCACCTTCAG	144	60	(16)
	MHC I	F: GCAGTTCCAGAGGCAGTTC R: CCACTCCACGCAGGTTTC	96	61	(16)
	MHC II	F: AGGTATCTGGTCAGGTATGTCTA R: CCACTTCATTCATTCGGTTCTC		61.5	Designed
Housekeeping	GAPDH	F: ATACACAGAGGACCAGGTTG R: AAACTCATTGTCATACCAGG	130	61.5	(17)
	GAPDH[A]	F: AGGGTCTTATGACCACTG R: AGCTCAGGGATGACTTTC	157	58.5	Designed

[A]A second pair of primers was used for GAPDH when the optimal annealing temperature of respective genes was below 59°C.

Table 1 also describes the primer sequences, amplicon sizes, and annealing temperatures used to quantify avian-specific immune-related genes in this study. Primer specificities were verified by NCBI BLAST analysis against the chicken genome and confirmed by observation of specific amplified PCR products on 1.5% agarose gel and melting curve analyses after RT-qPCR. The most optimal annealing temperatures were determined by performing gradient PCR, as appeared in Table 1. Each primer pair was then tested by drawing a standard curve based on the cycle threshold (Ct) values obtained from serially diluted template RNAs to ensure optimal PCR amplification efficiencies for the primer sets. RT-qPCR samples were run in triplicate, where each 20 µl reaction contained one µl (500 ng) of the template cDNAs. The following thermal program was used for RT-qPCR: 10 minutes for pre-denaturation at 95°C followed by 40 cycles of PCR, including 15 seconds of denaturation at 95°C, 20 seconds of annealing at the temperature specific for each primer set (Table 1) and 20 seconds of extension at 72°C prior to melt curve analysis. Target genes were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous standard for gene expression in chicken cells. The relative PCR amplicon concentration was determined by fluorescence signals detected at the end of each qPCR cycle, and their logarithmic values were plotted against the cycle number as Ct values. Relative mRNA expression levels were calculated as fold changes over the Mock group using the $2^{-\Delta\Delta CT}$ method (18).

2.6 *Statistical analysis*

Statistical analysis and data visualization were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA). Statistical differences between the infected and mock groups were determined by performing the independent-sample *t-test*. *P* values equal to or less than 0.05 ($p \leq 0.05$) were considered significant.

3 Results

3.1 *Salmonella serovar Typhimurium reduces the phagocytic capacity of chicken EPBMs*

In great accordance with our previous observation (12), a significant decrease in phagocytic capacities of ST-challenged EPBMs was observed in our flow cytometry-based phagocytosis assay when compared to the mock-challenged group (0.51 ± 0.02) ($p \leq 0.001$) (Figure 1).

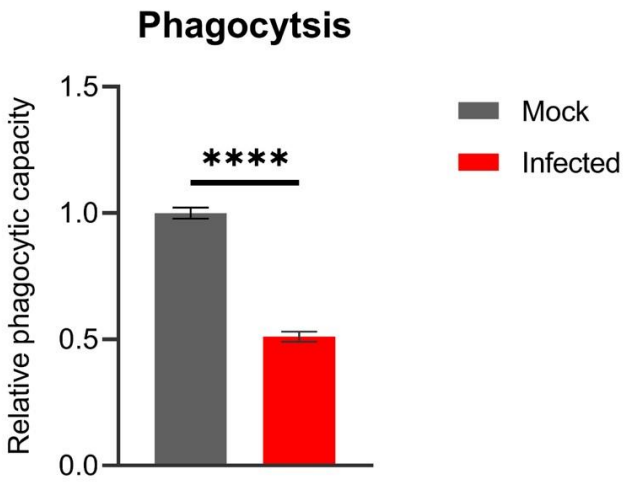

Figure 1. Flow cytometry-based analysis of ST-challenged chicken EPBMs' phagocytic capacities compared to the mock-challenged group. Relative phagocytic capacities were presented as the ratio of $FITC^+$ EPBMs relative to the mean values for the mock-challenged group. Bars indicate the Mean ± SEM of 8 biological replicates per group. Asterisks denote a statistically significant difference between the groups ($^{****}p \leq 0.001$).

3.2 *Salmonella serovar Typhimurium failed to induce a proinflammatory profile in chicken EPBMs*

Salmonella serovar Typhimurium challenge did not increase the expression of proinflammatory cytokines but rather upregulated the expression of anti-inflammatory cytokines in chicken EPBMs at the mRNA level. Figure 2 presents the relative fold changes in the transcription level of the proinflammatory and anti-inflammatory genes investigated in this study. *Salmonella* serovar Typhimurium challenge in chicken EPBMs resulted in a significant downregulation of proinflammatory interleukin (IL)-1β mRNA expression (0.30 ± 0.04), but not IL-6 (0.93 ± 0.23),

compared to non-infected controls ($p \leq 0.05$) (Figure 2A and B). Expression of anti-inflammatory IL-10 was significantly upregulated (1.89 ± 0.28) compared to non-infected controls ($p \leq 0.05$). Transforming growth factor (TGF)-β expression showed a similar pattern of mRNA upregulation (1.60 ± 0.25), though its upregulation compared to non-infected controls was not statistically significant ($p \geq 0.05$) (Figure 2C and D).

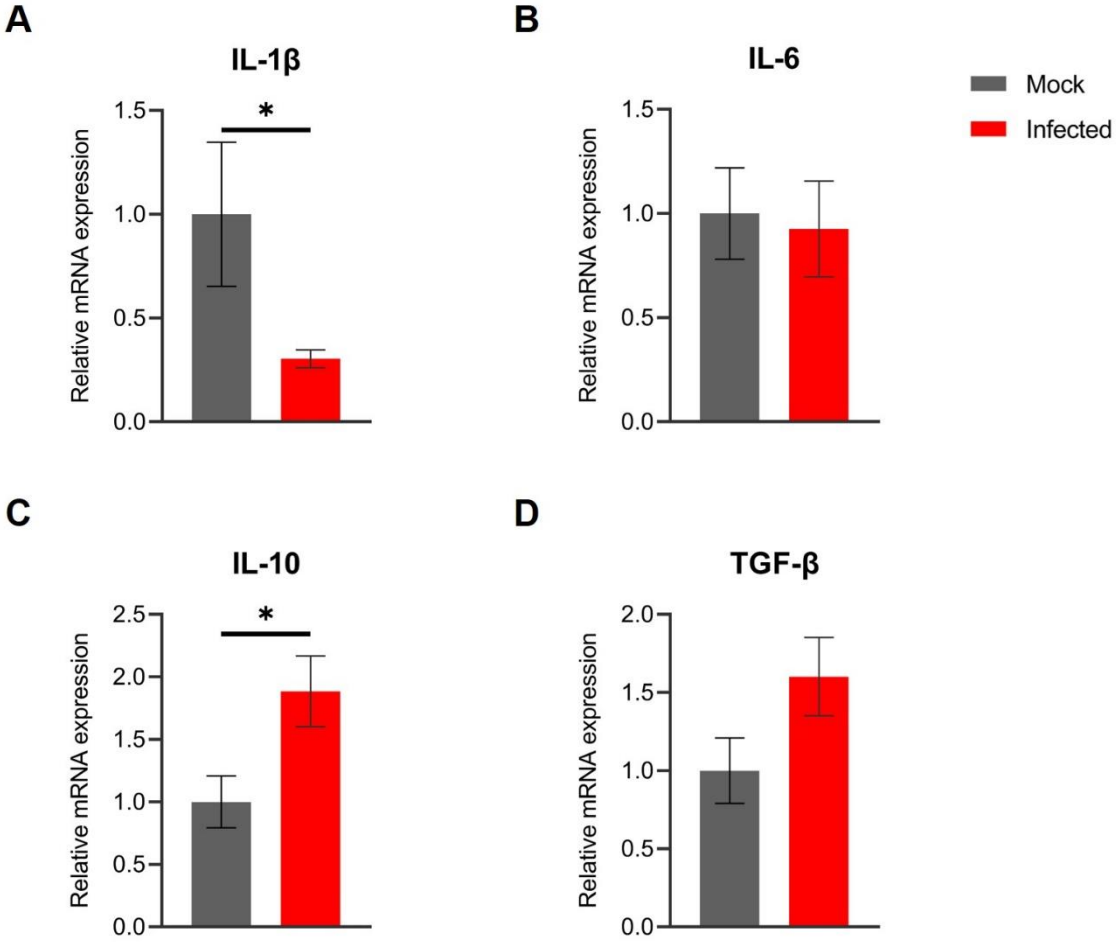

Figure 2. Differential expression of proinflammatory and anti-inflammatory cytokines in ST-challenged chicken EPBMs compared to the mock-challenged group. Relative mRNA expressions of the IL-1β (A), IL-6 (B), IL-10 (C), and TGF-β (D) genes were determined using RT-qPCR. Bars indicate the Mean ± SEM of 12 biological replicates per group. Asterisks denote a statistically significant difference between the groups ($^{*}p \leq 0.05$).

3.3 *Salmonella serovar Typhimurium down-regulated MHC-II but not MHC-I molecules in chicken EPBMs*

The expression of MHC-I mRNA in chicken EPBMs appeared unaffected (1.30 ± 0.33) by ST challenge in this study (Figure 3A). MHC-II mRNA in ST-infected chicken EPBMs was significantly downregulated (0.50 ± 0.11) when compared to the mock group (Figure 2) ($p \leq 0.05$) (Figure 3B). MHC-I expression Although statistically non-significant, inducible nitric oxide synthase (iNOS) seems to be slightly downregulated (0.54 ± 0.23) in response to the ST challenge in this study ($p \geq 0.05$) (Figure 3C).

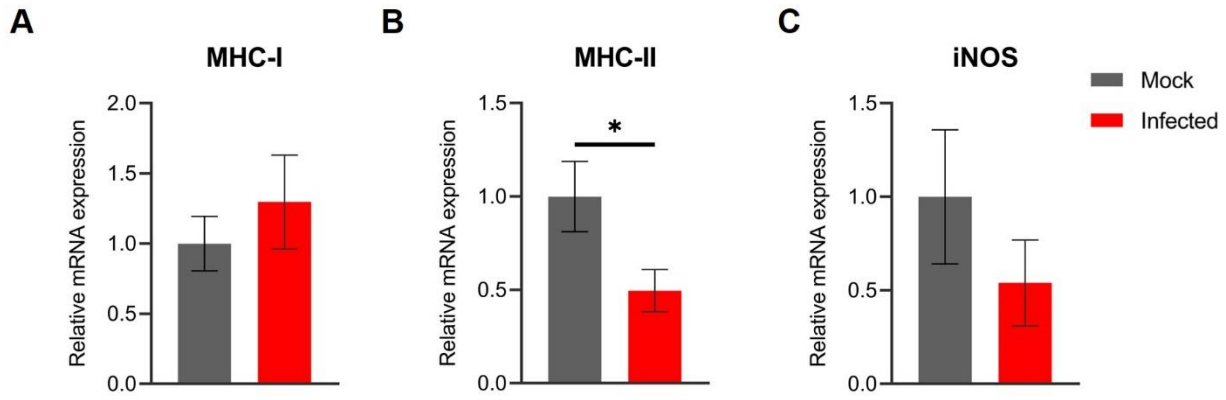

Figure 3. Differential expression of MHC-related genes in ST-challenged chicken EPBMs, compared to the mock-challenged group. Relative mRNA expressions of the MHC-I (A), MHC-II (B), and iNOS (C) genes were determined using RT-qPCR. Bars indicate the Mean ± SEM of 12 biological replicates per group. Asterisks denote a statistically significant difference between the groups ($^{*}p \leq 0.05$).

3.4 *Salmonella serovar Typhimurium upregulated the expression of TLR4 mRNA independent of MyD88 but failed to activate the expression of TLR5 and TLR9 in chicken EPBMs*

Salmonella serovar Typhimurium challenge in this study upregulated the expression of TLR4 mRNA (1.56 ± 0.15, ($p \leq 0.05$)), while the expression of MyD88 was statistically unaffected (0.50 ± 0.12) in comparison to the unchallenged controls ($p \geq 0.05$) (Figure 4A and D). No statistically significant upregulation in the mRNA expression of TLR5 and TLR9 (0.65 ± 0.13 and 0.62 ± 0.24, respectively) in response to the ST challenge was observed in the present study ($p \geq 0.05$) (Figure 4B and C).

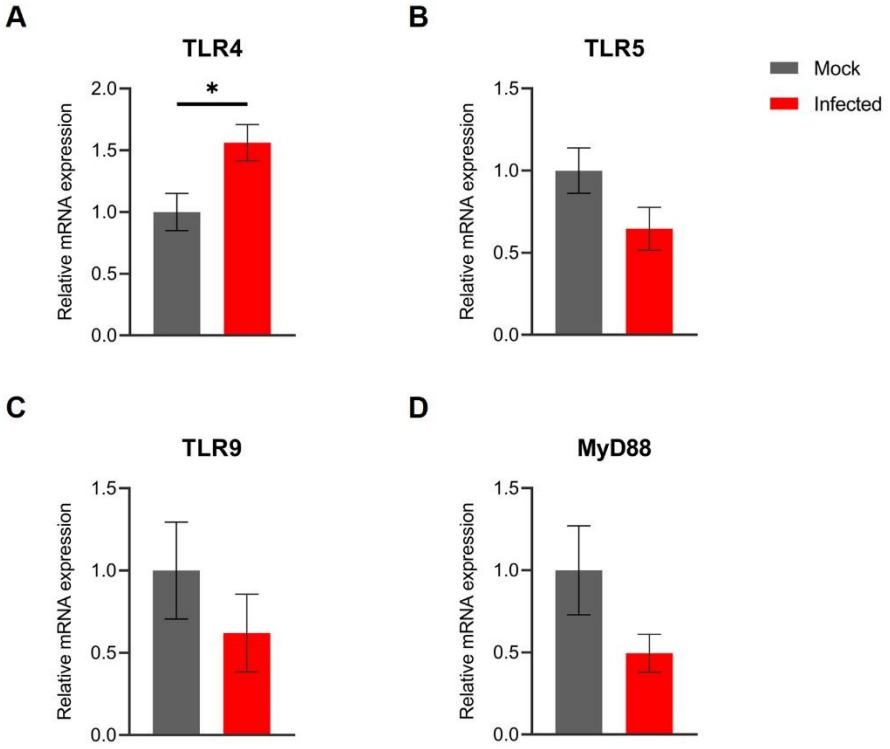

Figure 4. Differential expression of PRR-related genes in ST-challenged chicken EPBMs compared to the mock-challenged group. Relative mRNA expressions of the TLR4 (A), TLR5 (B), TLR9 (C), and MyD88 (D) genes were determined using RT-qPCR. Bars indicate the Mean ± SEM of 12 biological replicates per group. Asterisks denote a statistically significant difference between the groups ($^{*}p \leq 0.05$).

4 Discussion

The ability of *Salmonella* serovar Typhimurium to survive and replicate within macrophages is essential for the bacterial pathogenesis in avian and mammalian hosts, which is tightly regulated by the adaptive expression of bacterial virulence factors required to adapt to the changing microenvironment of the We adequately designed and used exon junction or intron-spanning primers in designing the qPCR to obviate concerns about genomic DNA contamination and thus broader use of these molecules in avian medicine/diseases. Even though the in vivo phenotypic complexity of macrophage cell populations cannot be reproduced in vitro, EPBM culture provides a reliable experimental model to study the underlying mechanisms involved in the interaction between *Salmonella* and the innate immune responses in the host cells (11). Herein, we first confirm that the ST challenge significantly reduces the phagocytic capacity of chicken EPBMs, which is in line with our previous observation (12) (Figure 1). Further, the results of our transcriptional analyses indicate that ST challenge in avian EPBMs favors the differentiation of macrophages toward the alternatively activated M2-like cells through downregulation of inflammatory IL-1β and upregulation of anti-inflammatory IL-10 cytokines (Figure 2A and C), which appears to be necessary for the bacterial survival in the host cells. Similarly, the diminished phagocytic capacities were observed in infected neutrophils and dendritic cells from other animal hosts, arguing that the differentiation of the monocytes to alternatively activated M2 macrophages through redox imbalance and regulation of MAPK/ERK signal transduction pathway post-challenge (13, 19). Further validation of these findings requires additional experiments on stable macrophage cell lines (e.g., HD11) and mature or M1/M2-polarized macrophages.

Proinflammatory cytokines, especially IL-1β, are critical components of the immune response against intracellular pathogens such as ST. Accordingly, several studies reported the upregulation of proinflammatory cytokines, especially IL-1β, following challenge with either bacterial LPS or live or inactivated *Salmonella* in avian and mammalian macrophages (20). In contrast, in our study, proinflammatory cytokines, IL-1β and IL-6, were either downregulated or unaffected, respectively (Figure 2A and B). Unlike the previous studies performed in matured macrophages, we used enriched blood monocytes, which have not yet been differentiated into mature macrophages. Therefore, these contrasting results might be due to the differential immune responses to ST in different cell types. Indeed, ST was previously shown to prevent the maturation of IL-1β in mouse B lymphocytes through the downregulation of the Nod-like receptor family CARD domain-containing protein 4 (NLRC4) induced by bacterial type III secretion system (T3SS) (21).

Similarly, ST has been shown to evade NLRC4 signaling in mouse bone marrow-derived macrophages by repressing flagellin and expressing a mutant SPI2 T3SS rod protein (SsaI) that cannot be recognized by NLRC4 (10, 22). In addition, a recent study suggested the inhibitory role of caspase recruitment domain-containing protein 9 (CARD9) on IL-1β production following *Salmonella* infection (23), notifying that infection with ST may lead to reduced expression of IL-1β due to the overstimulation of CARD9 and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (23). Reduced levels of proinflammatory cytokines may help the intracellular survival of bacterium within the infected cells by inhibiting cell death or production of reactive nitrogen species such as NO, as evident by the even slightly reduced expression of iNOS in this study (Figure 3C), and thus, favoring the *in vivo* persistence and dissemination of bacteria in the host. Herein, we did not test the post-challenge titers to assess the intracellular survival and/or replication of the bacterium in the challenged EPBMs, which calls for additional studies in the future. Furthermore, additional investigations on the role of ST-associated evasion molecules/genes, such as effector proteins, detox enzymes, and virulence genes (e.g., *sopD*, *fliC*, *hilA*, *sipC*, etc.) are necessary to clarify how *Salmonella* is responding to the hostile environment in the macrophages (24).

Anti-inflammatory cytokines, including IL-10 and TGF-β, were generally transcriptionally upregulated in our experiment (Figure 2 and D). Even though the time sequence of the cytokine activation events in our study is not precise, the activated anti-inflammatory pathway in the cells will likely provide an additional description for the observed transcriptional downregulation of proinflammatory cytokines in this study. Interestingly, recent studies suggest that the bacterial-mediated downregulation of the host microRNAs may be responsible for the upregulation of anti-inflammatory cytokines following *Salmonella* infection. *Salmonella* serovar Typhimurium was shown to downregulate the let-7 family of the host microRNAs, responsible for blocking the IL-10 production, thus upregulating the expression of anti-inflammatory IL-10 cytokine (25). Upregulation of TGF-β mRNA expression was previously shown following the receptor-mediated phagocytosis of *Salmonella* Enteritidis by

primary chicken heterophils (26). The same study showed the consequential downregulation of proinflammatory cytokines, including IFN-γ and IL-18 but not IL-1β (26). This may emphasize the phenotypic complexity of macrophage populations where a diverse population of cells adapts dynamically.

It is noteworthy that while the upregulation of proinflammatory cytokines in the presence (27) or absence (2) of anti-inflammatory cytokines was widely recorded following *the Salmonella* challenge in avian and mammalian EPBMs, lack of proinflammatory cytokine activation in our study could be due to an inactive SPI-1, yet an active SPI-2, T3SS in the stationary-phase grown bacteria used for the challenge by previous researchers (28). Future studies require additional experimental time points to assay the time sequence of transcriptional changes at the mRNA level or use protein-based detection methods (e.g., ELISA) to validate their experimental data.

Our study's expression profile of the TLR signaling molecules provided indirect evidence for an active SPI-2 T3SS in the intracellular bacteria (Figure 4). The absence of TLR5 mRNA upregulation following challenge with flagellated ST provides evidence for the SPI-2-mediated repression of bacterial flagellum (29). Similarly, significant downregulation of mRNA expression of TLR5 was observed *in vivo* in the spleen and cecum of chickens after a challenge with *Salmonella* serovar Enteritidis (30). In addition, no differences in TLR5 expression were detected in other studies following *Salmonella* serovars Typhimurium and Enteritidis challenges in the HD11 chicken macrophage cell line (7, 31). The absence of TLR5 activation is possibly due to the reduced expression of flagella in the intracellular environment of chicken cells (32), possibly due to activation of SPI-2 T3SS (29), as previously shown to be important to avoid inflammatory responses to *Salmonella* serovar Enteritidis in chicken oviduct cells (32).

MyD88-independent upregulation of TLR4 in this study has been previously shown in response to LPS in peripheral blood mononuclear cells obtained from different chicken breeds (33) (Figure 4). However, different chicken breeds have shown contrasting patterns of TLR4 expression in response to LPS (33). Although activation of innate immune responses through TLRs may adversely affect the intracellular survival of the bacterium in macrophages (34), TLR-mediated acidification of *Salmonella*-containing vesicles (SCVs) appears to be necessary for bacterial virulence through activation of SPI-2 T3SS (35, 36). The required TLR-mediated activation of *Salmonella* SPI-2 T3SS may describe why only TLR4, but not TLR5 and 9, was upregulated in *Salmonella*-infected chicken EPBMs in this study (Figure 4).

Upon activation of SPI-2 T3SS, *Salmonella* impairs the intracellular trafficking of the endocytic compartments required to mature late endosomes by disrupting the regulation of microtubule motors. It inhibits the recruitment of NADPH oxidase via injection of effector molecules into the host cell (37, 38). Since the proteolytic cleavage of TLR9 appears to be a prerequisite for the formation of mature TLR9 in the endosome (39, 40), the disrupted intracellular trafficking in *Salmonella*-infected cells may inhibit the endosomal maturation required to form an active TLR9 signaling. Reduced activation of TLR9 in *Salmonella*-infected cells may describe ST's inability to activate TLR9 in chicken EPBMs in our study (Figure 4C).

Activation of MyD88-dependent downstream signaling is important for clearing intracellular bacteria such as *Salmonella* (15) and *Brucella abortus* (41). In contrast, alternative up-regulation of TLR4 signaling through the TRIF-dependent pathway was suggested to play a role in endotoxin tolerance in murine cells (42), possibly due to negative regulation of MyD88-dependent signaling by IL-10 (43). Interestingly, endotoxin tolerance in several types of mammalian cells, including mouse embryonic fibroblasts, human monocytes, and murine macrophages, has been shown to upregulate the mRNA expression of IL-10 but downregulate the expression of IL-1β and IL-6 proinflammatory cytokines (42, 44-47). The current study observed a similar gene expression profile for proinflammatory versus anti-inflammatory cytokines following the wildtype ST challenge (Figure 2).

Initiation of adaptive immune responses depends on the ability of professional antigen-presenting cells, such as macrophages, to present the foreign microbial peptides to the CD4+ T-lymphocytes via their MHC molecules. Accordingly, CD4+ T cells appeared crucial for the clearance of *Salmonella* from the murine host (48). Many pathogens have evolved countermeasures to interfere with antigen presentation through MHC molecules to evade cell-mediated immunity and persist in the host. Our results indicate that ST challenge in chicken EPBMs can downregulate MHC-II expression but not MHC-I molecules (Figure 3A and B). Similar expression profiles for the MHC molecules were observed following *the Salmonella* challenge in the THP-1 human monocytic cell line (49) and porcine alveolar macrophages (50). The employment of such evasion mechanisms by *Salmonella* is not surprising. Pathogen survival and dissemination in the host rely on the

ability of the host to survive within the MHC II-expressing antigen-presenting cells.

5 Conclusion

Our results prove insufficient inflammatory responses and preferential differentiation of chicken macrophages toward alternatively activated M2-like cells upon ST infection. Even though our data point to possible immunomodulatory properties of ST in infected macrophages, it cannot describe why such an evasion mechanism might have evolved to enhance bacterial survival in the host cells. Further improvement of the existing control measures, such as vaccination and immunotherapies against avian salmonellosis in future studies, requires a better understanding of mechanisms underlying the immunomodulatory actions of *Salmonella* in host immune cells.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

EA, JM, and SMP participated in the research design. EA and PZD carried out the laboratory work. EA, JM, and SMP analyzed the results and helped draft the manuscript. JM and SMP finalized the manuscript. All authors read and approved the final manuscript for submission to JPSAD.

Data Availability Statement

The data are available upon reasonable request.

Ethical Considerations

All study designs, blood sampling for the *in vitro* cell culture assays, and experimental procedures were conducted according to animal welfare/care regulations/guidelines and approved by the ethical committee of the Faculty of Veterinary Medicine of the University of Tehran.

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