Salmonella Serovar Typhimurium Infection Modulates Expression of Immune-Related Genes in Avian Enriched Monocytes

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Research Article

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Abstract

Salmonella enterica subsp. enterica serovar Typhimurium is a gram-negative bacterium with the ability to infect a wide variety of 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 important 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 own benefit. We previously showed that ST challenge can greatly 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 some other immune-related molecules at 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 modulate the immune response in professional phagocytes in order to survive in the hostile environment of host immune cells and further disseminate within the host.

Introduction

Salmonella serovar Typhimurium (ST) is a gram-negative facultative intracellular bacterium with the ability to infect a wide range of 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 human. Epidemiological surveys focusing on prevalence of *Salmonella* in poultry has been broadly studied and well documented [3]. However, our understanding about 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 to enable *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 bacterium within infected macrophages.

After intestinal colonization, macrophages are the primary immune defender cells which 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 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 own benefit and there is an interplay between 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 showed that ST challenge can weakens 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 survive in the hostile environment of host immune cells and use such mechanisms to further disseminate within the host body.

Material And Methods

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 challenge as described later in this paper.

Chicken enriched peripheral blood monocytes

EPBMs, also referred to as monocyte-derived macrophages in previous publications, were prepared as described elsewhere in human and porcine model system [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 2 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 were counted using Trypan Blue vital staining. Eventually, enriched monocytes were divided into 24-well tissue culture plates for further cellular and molecular assays.

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 2 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*, 4°C, 5 minutes) to remove the cell debris, and stored at -80°C prior to RNA extraction.

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 3 h at 40 °C under 5% CO_2 and 95% humidity, as previously described [12]. The EPBMs were then washed once with an 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.

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. Briefly, 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 Azma, Tehran, Iran) with the cDNAs synthetized in the previous step. To obviate concerns about genomic DNA contamination, exon junction or intron-spanning proper primers were designed for 7 pairs of primers along with other 5 pairs from references (Table 1). Table 1 also provides a detailed description of the primer sequences, amplicon sizes, and annealing temperatures used for quantification of 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 subsequent to RT-qPCR as well. The most optimal annealing temperatures were determined by performing gradient PCR, as appeared in Table 1. Each primer pair were 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 reactions contained 1 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. Normalization of target genes was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous standard for the 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 [15].

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-samples *t* test. *P* values equal or less than 0.05 ($P \leq 0.05$) were considered significant.

Results

Salmonella serovar Typhimurium reduce the phagocytic capacity of chicken EPBMs

In great accordance with our previous observation [12], a significant decrease in phagocytic capacities of ST-challenged EPBMs were observed in our flow cytometry-based phagocytosis assay, when compared to the mock-challenged group (0.51 ± 0.02) ($P \leq 0.0001$) (Figure 1).

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 mRNA level. Figure 2 presents the relative fold changes in 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).

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 to be 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).

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).

Discussion

The ability of *Salmonella* serovar Typhimurium to survive and replicate within macrophages appears to be essential for the bacterial pathogenesis in avian and mammalian hosts, which is tightly regulated by adaptive expression of bacterial virulence factors required to adapt to the changing microenvironment of the host cells . We properly designed and used exon junction or intron-spanning primers in designing the qPCR so as 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 simply reproduced in vitro, EPBMs culture provides a reliable experimental model to study the underlying mechanisms involve in the interaction between *Salmonella* and the innate immune responses in the host cells [11]. Herein, we first confirm that ST challenge results in a significant reduction in 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 the differentiation of the monocytes to alternatively activated M2 macrophages through redox imbalance and/or regulation of MAPK/ERK signal transduction pathway post-challenge [13, 16]. Further validation of these finding requires additional experiments on stable macrophage cell lines (e.g. HD11), mature macrophages, or M1/M2-plarized 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 [17]. In contrast, proinflammatory cytokines, IL-1β and IL-6, in our study were either downregulated or unaffected, respectively (Figure 2A and B). Unlike the previous studies performed in matured macrophages, we used enriched blood monocytes which are not differentiated into mature macrophages yet. 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 Nod-like receptor family CARD domain containing protein 4 (NLRC4) induced by bacterial type III secretion system (T3SS) [18]. 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, 19]. In addition, a recent study suggested the inhibitory role of caspase recruitment domain-containing protein 9 (CARD9) on IL-1β production following *Salmonella* infection [20], 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) [20]. 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 [21].

Anti-inflammatory cytokines, including IL-10 and TGF-β, were generally transcriptionally upregulated in our experiment (Figure 2C and D). Even though the time-sequence of the cytokine activation events in our study is not clear, the activated anti-inflammatory pathway in the cells is likely to provide an additional description for the observed transcriptional downregulation of proinflammatory cytokines in this study. Interestingly, recent studies suggest 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, and thus upregulate the expression of anti-inflammatory IL-10 cytokine [22]. Upregulation of TGF-β mRNA expression was previously shown following the receptor-mediated phagocytosis of *Salmonella* Enteritidis by primary chicken heterophils [23]. The same study showed the consequential downregulation of proinflammatory cytokines including IFN-γ and IL-18 but not IL-1β [23]. This may emphasize on the phenotypic complexity of macrophage populations where a diverse population of cells are dynamically adapting themselves to the situation.

It is noteworthy that while the upregulation of proinflammatory cytokines in the presence [24] or absence [2] of anti-inflammatory cytokines was widely recorded following *Salmonella* challenge in avian and mammalian EPBMs, lack of pro-inflammatory cytokine activation in our study could be possibly 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 [25]. Future studies require additional experimental time-points to assay the time-sequence of transcriptional changes at mRNA level or use of protein-based detection methods (e.g. ELISA) to validate their experimental data.

Looking at the expression profile of the TLR signaling molecules in our study provided an 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 [26]. Similarly, significant downregulation of mRNA expression of TLR5 was observed *in vivo* in spleen and cecum of chickens after challenge with *Salmonella* serovar Enteritidis [27]. In addition, no differences in TLR5 expression were

detected in other studies following *Salmonella* serovars Typhimurium and Enteritidis challenges in HD11 chicken macrophage cell line [7, 28]. Absence of TLR5 activation is possibly due to the reduced expression of flagella in the intracellular environment of chicken cells [29], possibly due to activation of SPI-2 T3SS [26], as previously shown to be important to avoid inflammatory responses to *Salmonella* serovar Enteritidis in chicken oviduct cells [29].

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 [30] (Figure 4). However, contrasting patterns of TLR4 expression in response to LPS has been shown in different chicken breeds [30]. Although activation of innate immune responses through TLRs may adversely affect the intracellular survival of bacterium in macrophages [31], TLR-mediated acidification of *Salmonella*-containing vesicles (SCVs) appears to be necessary for the bacterial virulence through activation of SPI-2 T3SS [32, 33]. 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 for the maturation of late endosomes by disrupting the regulation of microtubule motors and inhibits the recruitment of NADPH oxidase via injection of effector molecules into the host cell [34, 35]. Since the proteolytic cleavage of TLR9 appears to be a prerequisite for the formation of mature TLR9 in the endosome [36, 37], 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 the inability of ST in activation of TLR9 in chicken EPBMs in our study (Figure 4C).

Activation of MyD88-dependent downstream signaling appears to be important for the clearance of intracellular bacteria such as *Salmonella* [38] and *Brucella abortus* [39]. 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 [40], possibly due to negative regulation of MyD88-dependent signaling by IL-10 [41]. Interestingly, endotoxin tolerance in several types of mammalian cells including mouse embryonic fibroblasts, human monocytes, and murine macrophages have been shown to upregulate the mRNA expression of IL-10 but downregulate the expression of IL-1β and IL-6 proinflammatory cytokines [40, 42-45]. A similar gene expression profile for proinflammatory versus anti-inflammatory cytokines was observed following the wildtype ST challenge in the current study (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 to be crucial for the clearance of *Salmonella* from the murine host [46]. Many pathogens have evolved countermeasures to interfere with antigen presentation through MHC molecules in order to evade the cell-mediated immunity and persist in the host. Our results indicate that ST challenge in chicken EPBMs can downregulate the expression of MHC-II but not MHC-I molecules (Figure 3A and B). Similar expression profiles for the MHC molecules were observed following *Salmonella* challenge in THP-1 human monocytic cell line [47] and porcine alveolar macrophages [48]. The employment of such evasion mechanisms by *Salmonella* is not surprising where the pathogen survival and dissemination in the host relies on its ability to survive within the MHC II-expressing antigen-presenting cells.

Conclusion

Our results provide an evidence for insufficient inflammatory responses along with 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 evasion mechanism might have been evolved to enhance the bacterial survival in the host cells. Further improvement of the existing control measures such as vaccination and immunotherapies against avian salmonellosis in the future studies requires a better understanding of mechanisms underlying the immunomodulatory actions of *Salmonella* in host immune cells.

Declarations

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Authors' Contributions EA, JM and SMP participated in the design of the research work. EA and PZ carried out the laboratory works. EA, JM and SMP participated in the analysis of the results and helped to draft the manuscript. JM and SMP finalized the manuscript. All authors read and approved the final manuscript for submission to "*Molecular Biology Reports*".

Compliance with ethical standards

Conflict of Interest The authors declare no conflict of interest.

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

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Table

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	129	59	[38]
		R: CAGCCCGTTCATCCTCATAT			
	TLR5	F: CACACGGCAATAGTAGCA	94	56	Designed
		R: CACACCTGGAACTTGGAA			
	TLR9	F: TCATTGACCTCCACAGAC	77	56.8	Designed
		R: GGAAGACACCAGAGAAGA			
	MyD88	F: AGAAAGAAGGTGTCGGAGGAT	100	59	[38]
		R: TGGGGAAAGACTAAGAGCAAAT			
Cytokines	IL-1β	F: TCATCCAGCCAGAAAGTGAGG	140	61.5	Designed
		R: GTGCCGCTCATCACACAC			
	IL-6	F: CCTGACGAAGCTCTCCAG	153	60	[49]
		R: TCGGGATTTATCACCATCTGC			
	IL-10	F: GACGTTCGAGAAGATGGATGAG	99	61.5	Designed
		R: CTCCTCCTCATCAGCAGGTA			
	TGF-β	F: TGAGTATTGGGCCAAAGAGCTG	101	60	Designed
		R: ACACGAAGAAGATGCTGTGG			
	iNOS	F: CACTACCTGCCTGGAGAACAT	144	60	[49]
		R: TGCCCAATAGCCACCTTCAG			
EPBMs-related	MHC I	F: GCAGTTCCAGAGGCAGTTC	96	61	[49]
		R: CCACTCCACGCAGGTTTC			
	MHC II	F: AGGTATCTGGTCAGGTATGTCTA		61.5	Designed
		R: CCACTTCATTCATTCGGTTCTC			
Housekeeping	GAPDH	F: ATACACAGAGGACCAGGTTG	130	61.5	[50]
		R: AAACTCATTGTCATACCAGG			
	GAPDH[A]	F: AGGGTCTTATGACCACTG	157	58.5	Designed
		R: AGCTCAGGGATGACTTTC			

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

Figures

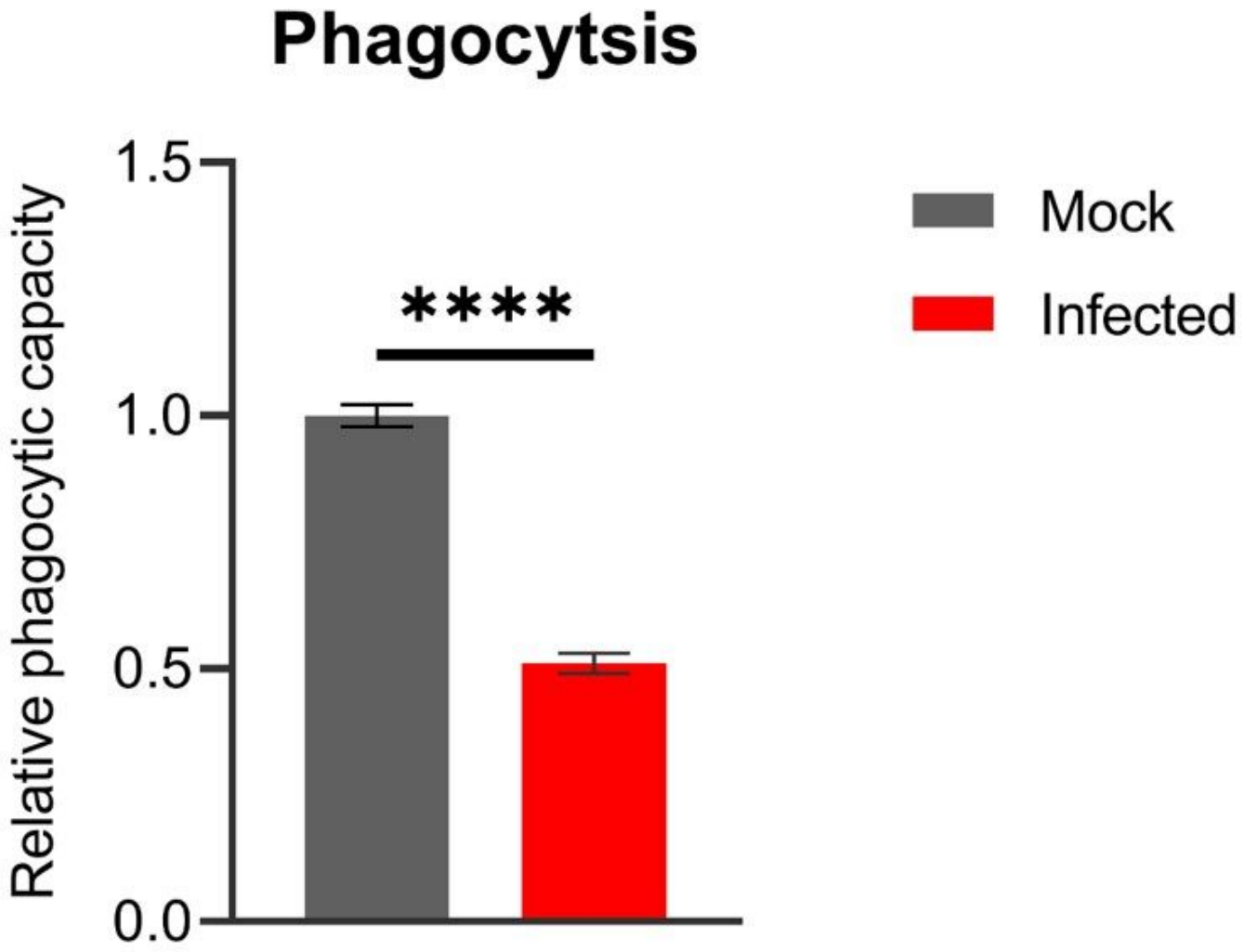

Figure 1

Flow cytometry-based analysis of phagocytic capacities of ST-challenged chicken EPBMs, when 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 mean ± SEM of 8 biological replicates per groups. Asterisks denote a statistically significant difference between the groups (****P ≤ 0.0001).

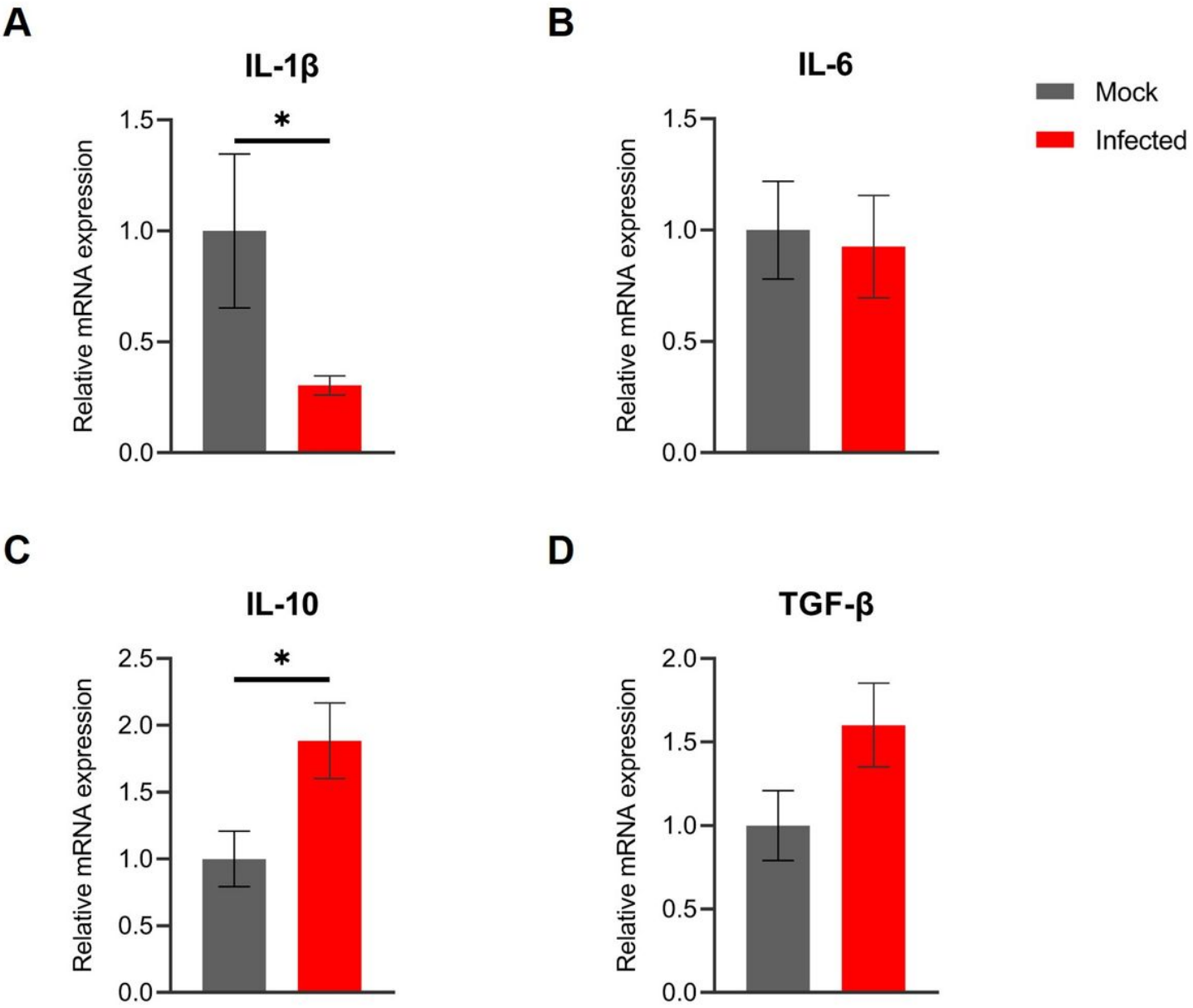

Figure 2

Differential expression of proinflammatory and anti-inflammatory cytokines in ST-challenged chicken EPBMs, when 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 mean ± SEM of 12 biological replicates per groups. Asterisks denote a statistically significant difference between the groups (*$P \leq 0.05$).

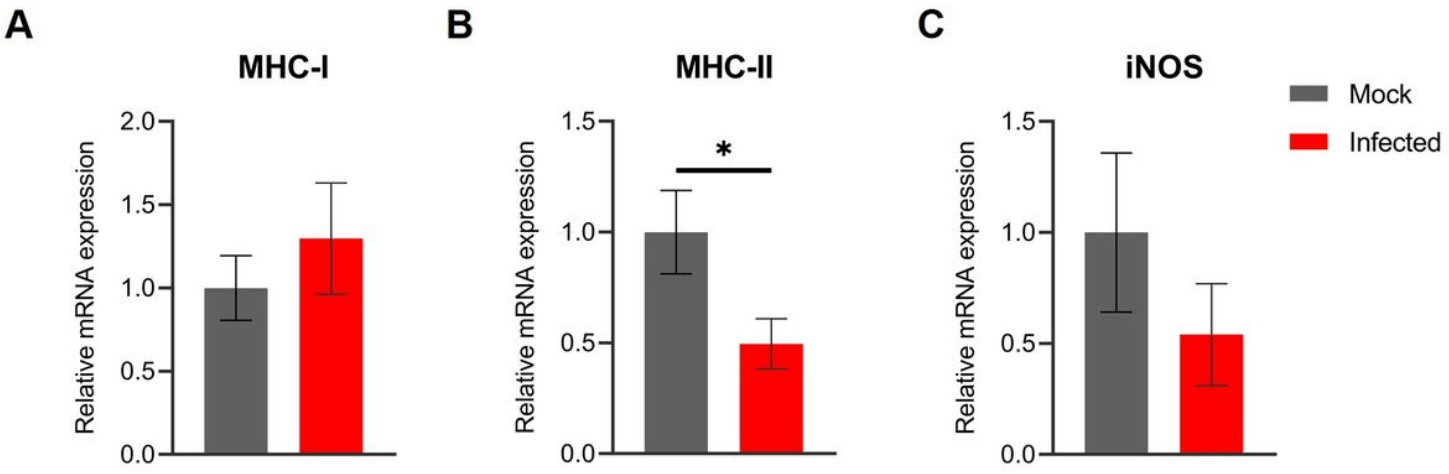

Figure 3

Differential expression of MHC-related genes in ST-challenged chicken EPBMs, when 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 mean ± SEM of 12 biological replicates per groups. Asterisks denote a statistically significant difference between the groups (*$P \leq 0.05$).

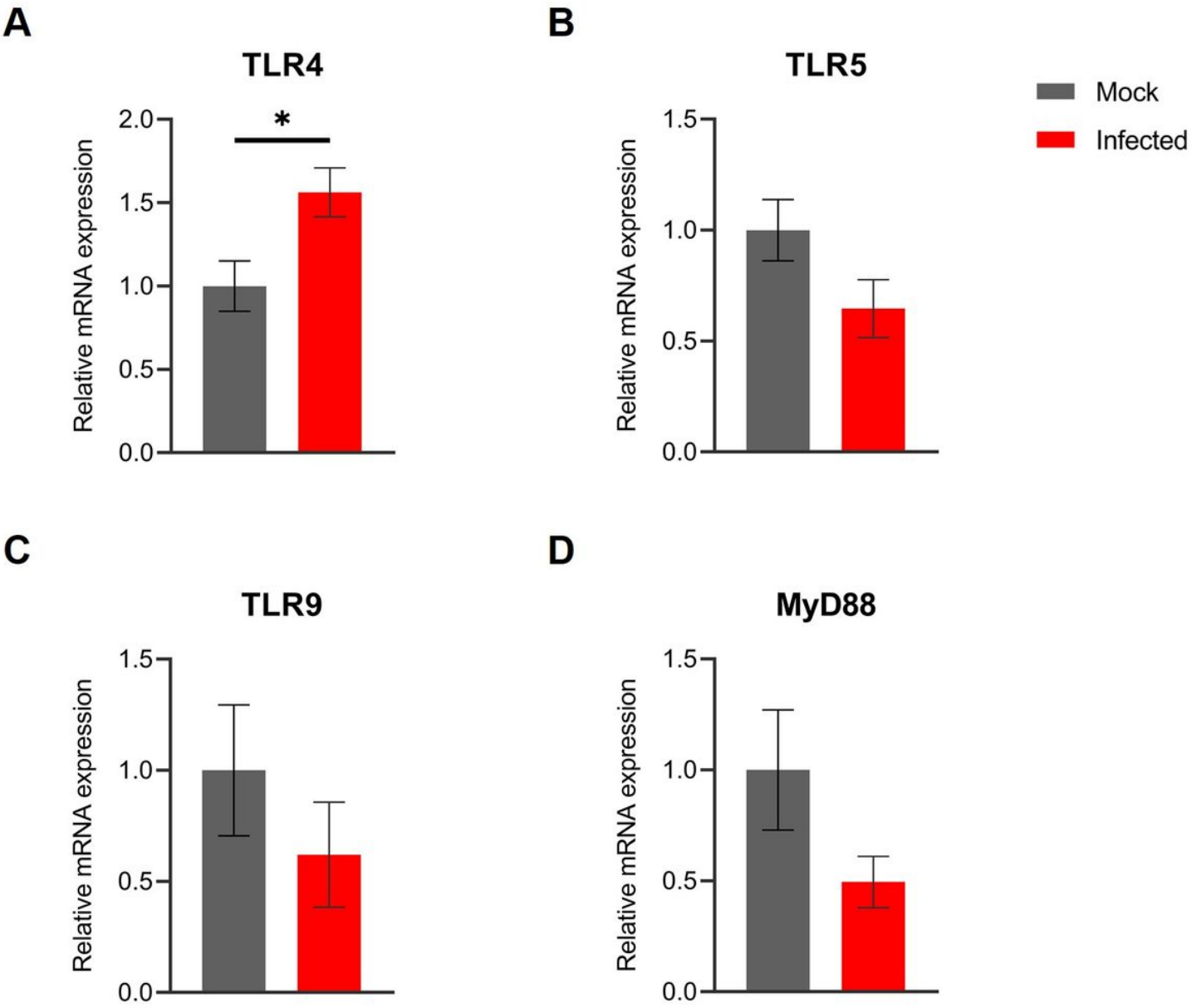

Figure 4

Differential expression of PRR-related genes in ST-challenged chicken EPBMs, when 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 mean ± SEM of 12 biological replicates per groups. Asterisks denote a statistically significant difference between the groups (*$P \leq 0.05$).