Interleukin (IL)-1β Gene Expression Analysis after *Salmonella enterica* serovar Typhimurium Challenge in Chicken Monocyte-Derived Macrophages

Elya Abbaszadeh[1], Jalil Mehrzad[2], Seyed Mostafa Peighambari[1*], Paniz Zarghami[1], Vahid Karimi[1], Ramak Yahyaraeyat[2]

[1.]Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[2.]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Abstract

BACKGROUND: *Salmonella enterica* serovar Typhimurium (ST) is a gram-negative facultative intracellular bacterium with the ability to infect a wide range of hosts.

OBJECTIVES: This study aimed to provide a snapshot of the immune responses against ST challenge in primary chicken monocyte-derived macrophages (MDMs) by evaluating the transcriptional changes in inflammatory cytokine interleukin (IL)-1β.

METHODS: After preparing blood MDMs, cell monolayers were challenged with ST at a multiplicity of infection of 50. Transcriptional analyses of inflammatory cytokine IL-1β were performed by reverse transcription-quantitative polymerase chain reaction using SYBR Green dye.

RESULTS: The results indicated that wildtype ST challenge in avian MDMs favors the differentiation of macrophages toward the alternatively activated M2-like cells through downregulating inflammatory IL-1β.

CONCLUSIONS: The findings demonstrated the preferential differentiation of chicken macrophages toward the alternatively activated M2-like cells upon ST infection. Further improvement of the existing control measures, such as vaccination and molecular-based immunotherapeutic strategies against poultry salmonellosis requires a better understanding of mechanisms involved in the immunomodulatory actions of *Salmonella* in immune cells in future studies.

KEYWORDS: Immune response, Inflammatory cytokine, Interleukin-1β, Monocyte-derived macrophage, *Salmonella enterica* serovar Typhimurium

Introduction

Salmonella enterica serovar Typhimurium (*ST*) is a gram-negative facultative intracellular bacterium that is capable of causing disease in a range of hosts (He *et al.*, 2018). Except for very young chickens, ST rarely causes clinical disease. However, the bacterium can colonize the poultry intestinal tract (Barrow *et al.*, 1988; Barrow *et al.*, 1990). Poultry products contaminated with ST are one of the major causes of zoonotic foodborne diseases in humans (Scallan *et al.,* 2011). Extensive epidemiological surveys focused on the prevalence of *Salmonella* in poultry have been performed and well documented (Foley *et al.,* 2011). However, our understanding of immunological mechanisms contributing to the invasion, colonization, and intracellular persistence of *Salmonella* in chickens is still limited (Boyd *et al.*, 2007; Lillehoj *et al.*, 2007; Chappell *et al.*, 2009; Wisner *et al.*, 2010; Wisner *et al.*, 2011).

Pathogenesis of ST is facilitated by two distinct forms of type III secretion system (T3SS) encoded by the genes of *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and -2) (Balan and Babu, 2017). The T3SS plays a key role in targeting host cell and triggers the production of pro-inflammatory cytokines during the invasion of mammalian hosts. The T3SS produces about 40 distinct effector proteins to enable *Salmonella* invasion, survival, and replication within the host cells (Haraga *et al.*, 2008; Ibarra and Steele-Mortimer, 2009; Malik Kale *et al.*, 2011). The SPI-1 T3SS is primarily expressed in the gut lumen and promotes *Salmonella* invasion to the gut epithelial cells. Alternatively, SPI-2 T3SS is mainly expressed when the bacterium lives within the vacuolar compartment of macrophages and epithelial cells. Therefore, SPI-2 T3SS is important for intracellular replication and survival of the bacterium and causes a systemic disease (Miao and Rajan, 2011; Braukmann *et al.*, 2015; Balan and Babu, 2017).

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 infection. Therefore, it regulates the inflammatory response (Babu *et al.*, 2006; Chappell *et al.*, 2009; Miao *et al.,* 2010; Setta *et al.*, 2012). Intracellular survival of the bacteria depends on the expression of cytokines and their subsequent inflammation, which defines macrophage cell fates (Ibarra and Steele-Mortimer, 2009). Consequently, different strains of *Salmonella* have developed various mechanisms to avoid immune reactions or subvert immunity to their benefit and there is an interplay between the detection and evasion of *Salmonella* in the host (Miao and Rajan, 2011).

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 against poultry salmonellosis, including vaccination and molecular-based immunotherapeutic strategies. This study sought to provide a snapshot of the immune responses against ST challenge in primary chicken monocyte-derived macrophages (MDMs) by evaluating the transcriptional changes in inflammatory cytokine interleukin (IL)-1 β. Possible immunomodulatory impacts of the bacterium on chicken MDMs were revealed, which may partially explain how the bacterium survives in the environment of host immune cells and uses such mechanisms to further disseminate within the host body.

Materials and Methods

Bacterial Strains

Overnight Luria-Bertani broth (Merck, Darmstadt, Germany) culture of *Salmonella enterica subsp. enterica (Salmonella enterica* serovar Typhimurium*)* 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 (CFU)/ml of ST ATCC® 14028 was used to induce infection as described later in this paper.

Chicken MDMs

Blood MDMs were prepared as described in human and porcine model systems (Bahari *et al.*, 2014). 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 chickens were cultured in 24-well tissue culture plates in RPMI medium and incubated for 2 h at 37°C, 5% CO_2, and 95% humidity. The purity of the monocyte cultures was confirmed by Giemsa staining under a light microscope. The number of viable cells was counted using Trypan Blue vital staining.

Salmonella Challenge

Twenty-four-well plates were seeded with 2×10^6 million cells per well and incubated for 16-18 h. Cell monolayers were challenged with ST at a multiplicity of infection of 50 for 2 h at 40°C, 5% CO_2, and 95% humidity. Cell suspensions were obtained by centrifugation at 1000 g for 5 min at 4°C and were stored at -70°C prior to RNA extraction.

Reverse Transcription-Quantitative Polymerase Chain Reaction

Transcriptional analysis of inflammatory IL-1β was performed by reverse transcription-quantitative polymerase chain reaction (RT-PCR) using SYBR Green dye. Briefly, RNA was extracted using the Favorgen 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 utilizing the RevertAid First Strand cDNA Synthesis Kit and oligo(dT) primer (Fermentas, Finland). Relative quan-

tification of the mRNA copies of the respective genes was performed by Rotor-Gene Q (Qiagen, Valencia, CA) real-time PCR machine using YTA SYBR Green qPCR Kit (Yekta Tajhiz Azma, Tehran, Iran) with the cDNAs synthesized in the previous step. Table 1 provides a detailed description of the primer sequences, amplicon sizes, and annealing temperatures used for the quantification of IL-1β and glyceraldehyde-3-phosphate dehyd-rogenase (GAPDH) genes in this study. Primer specificities were verified by NCBI BLAST analysis against the chicken genome and were confirmed by observing specific amplified PCR products on 1.5% agarose gel and melting curve analysis after RT-qPCR. The most optimal annealing temperatures were determined by performing gradient PCR as shown in Table 1. Afterwards, each primer pair was 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. The RT-qPCR samples were run in triplicate where each 20 µL reaction solution contained 1 µL (500 ng) of the template cDNAs. The thermal program used for RT-qPCR included 10 min pre-denaturation at 95°C followed by 40 cycles of PCR, including 15 sec of denaturation at 95°C, 20 sec of annealing at the temperature specific for each primer set, and 20 sec of extension at 72°C prior to melting curve analysis. Normalization of target genes was performed using 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 PCR cycle, and their logarithmic values were plotted against the cycle number as Ct values. The corresponding Ct values for each sample were used to calculate the fold-changes in gene expression using the ΔΔCt method.

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

Gene	Primer sequence (5′ to 3′)		Amplicon size (bp)	Annealing temperature (°C)	References
IL-1β	F: TCATCCAGCCAGAAAGTGAGG		140	61.5	Designed
	R: GTGCCGCTCATCACACAC				
GAPDH	F: ATACACAGAGGACCAGGTTG		130	61.5	(Zheng *et al.*, 2014)
	R: AAACTCATTGTCATACCAGG				

Statistical differences between transcriptional fold-change values were determined by the independent-samples t-test using the SPSS software version 25 (IBM, Chicago, Ill., USA). P-values equal to or less than 0.05 ($P \leq 0.05$) were considered statistically significant.

Results

The specificity of the primers for their target genes was confirmed by observing a single peak in melting curve analysis (Figure 1), as well as visualizing a single band within its expected length on the agarose gel (Figure 2). The accuracy of RT-qPCR for determining gene copy numbers was confirmed based on the presence of a linear relationship between the extrapolated Ct values and the concentration of cDNA used in the reaction (Figure 3). A gradual elevation in the sample Ct values was also evident in the replication curve using the decreasing amounts of the input templates (Figure 4).

The Ct values acquired from the RT-qPCR were used to assess the fold changes in the transcription level of the IL-1β gene in chicken MDMs in the presence or absence of *Salmonella* infection. Interestingly, the ST challenge in chicken MDMs resulted in a significant downregulation of IL-1β mRNA expression (0.4 or -1.3 Log_2 mean fold change), compared to the non-infected controls ($P \leq 0.05$) (Figure 5).
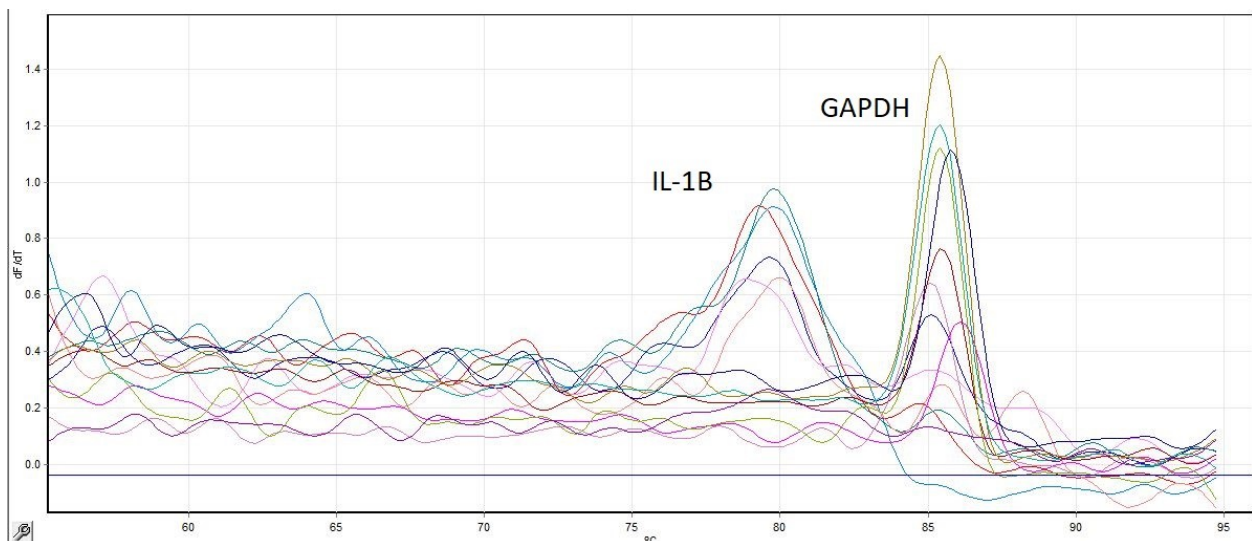

Figure 1. Melting curve was drawn for proinflammatory IL-1β and GAPDH genes to evaluate the specificity of the qRT-PCR in replication of the target genes.
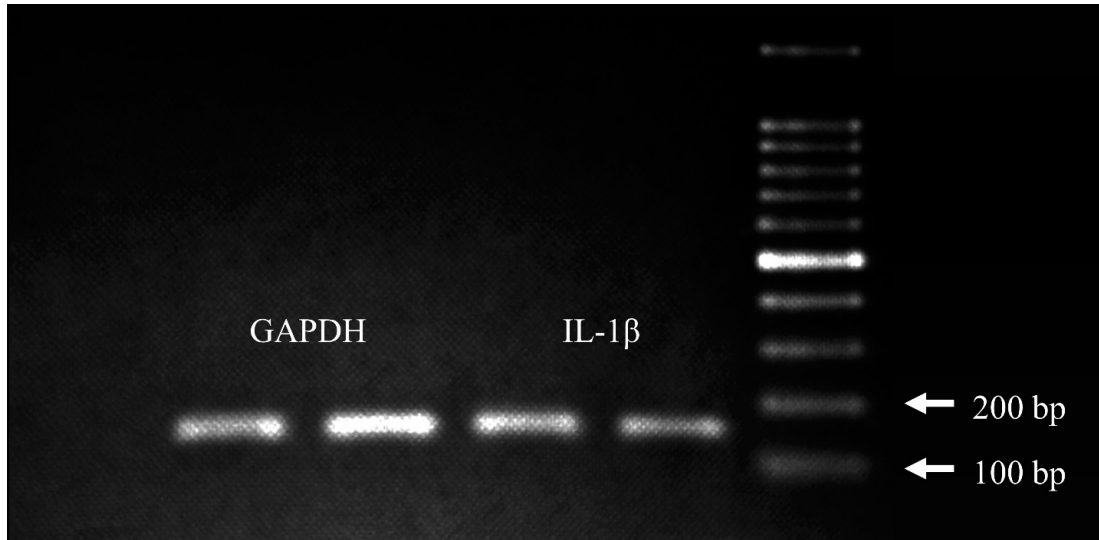
Figure 2. Gene products visualized on 1.5% agarose gel to confirm the specificity of replicated amplicons in qPCR.
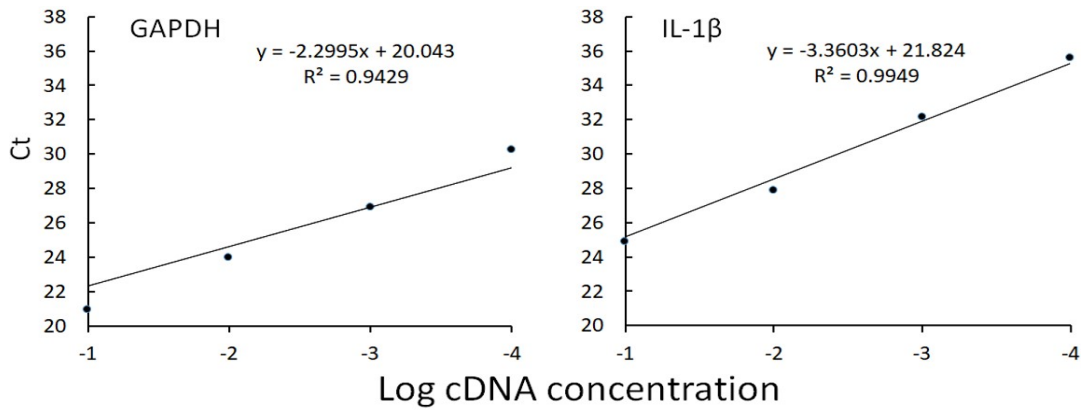

Figure 3. Presence of a linear relationship between qRT-PCR Ct values and concentration of input cDNA confirms the accuracy of qRT-PCR reaction in determination of gene copy numbers.
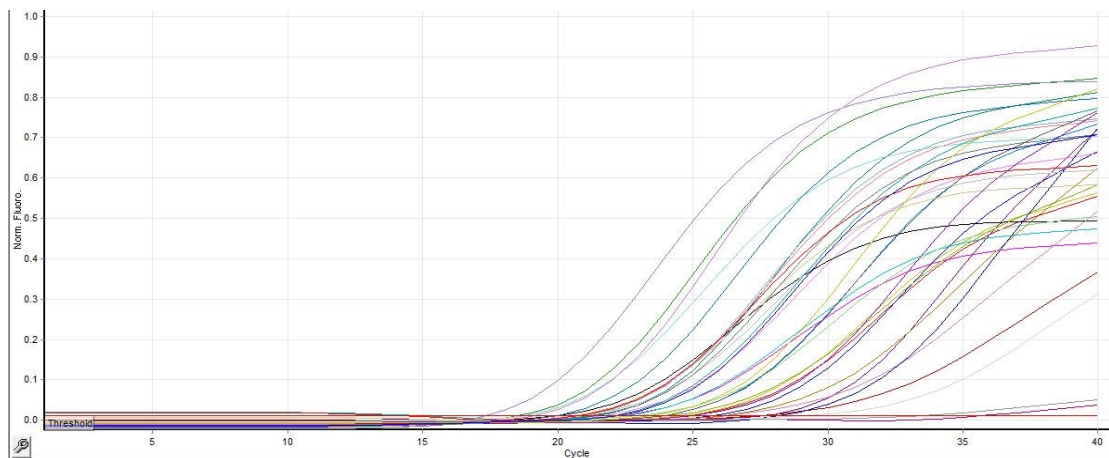

Figure 4. Replication curves related to decreasing concentrations of IL-1β and GAPDH genes.
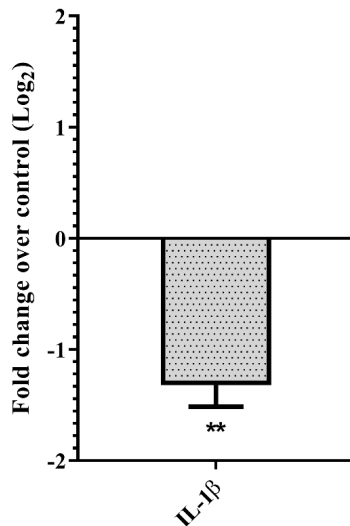
Figure 5. Fold changes in transcription level of IL-1β gene in RNA extracted from chicken monocyte-derived macrophages (MDMs) challenged with *Salmonella* Typhimurium, when compared to uninfected controls. The bars represent the Log2 based fold changes in transcription level of the gene. Error bars represent mean ± SEM fold changes for twelve treatment replicates when compared to the twelve uninfected control samples. Asterisks denote statistically significant differences compared with uninfected controls (**$P{\leq}0.01$).

Discussion

The ability of ST for surviving and replicating within macrophages appears to be essential for 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 host cells (Miao and Rajan, 2011). Although the in vivo phenotypic complexity of macrophage cell populations cannot be simply reproduced in vitro, MDM culture provides a reliable experimental model to study the underlying mechanisms involved in the interaction between *Salmonella* and the innate immune responses of the host cells (Miao and Rajan, 2011). Our results indicated that wildtype ST infection in avian MDMs favors the differentiation of macrophages toward the alternatively activated M2-like cells through downregulating IL-1β ($P{\leq}0.05$), which seems to be necessary for bacterial survival in the host cells (Figure 1).

Pro-inflammatory cytokines, especially IL-1β, are the critical components of the immune response against intracellular pathogens, such as *ST*. Accordingly, several studies reported the upregulation of pro-inflammatory cytokines, especially IL-1β, following infection with either bacterial lipopolysaccharide, live, or inactivated *Salmonella* in avian and mammalian macrophages (Monack *et al.*, 2001; Raupach *et al.*, 2006). However, the pro-inflammatory cytokine IL-1β showed to be downregulated in our study. This is in line with the findings of a recent study that suggested the inhibitory role of caspase recruitment domain-containing protein 9 (CARD9) on IL-1β production following *Salmonella* infection (Pereira *et al.*, 2016). This shows 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 (Pereira *et al.*, 2016).

Similarly, the activation of pattern-recognition receptors (PRRs), such as toll-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), appears to be crucial for the initiation of a cascade of events that result in a robust pro-inflammatory immune response (Kumar *et al.*, 2011). For example, a family of PRRs known as NLR family CARD domain-containing protein 4 (NLRC4) could potentially recognize ST infection by sensing the flagellin monomers and trigger the maturation of IL-1β (Miao *et al.*, 2006). The maturation of IL-1β to its active form requires several enzymatic complexes, the most important of which happens in the inflammasomes (Lamkanfi and Dixit, 2014). Inflammasomes are assembled in the cell cytoplasm and utilize caspases, namely caspase-1 and caspase-8, for cytokine processing (Lamkanfi and Dixit, 2014). However, ST was also previously shown to prevent the maturation of IL-1β in murine B cells through the downregulation of NLRC4 induced by the bacterial SPI-1 T3SS (Perez-Lopez *et al.*, 2013).

Similarly, ST has been shown to evade NLRC4 signaling in mouse bone marrow-derived macrophages by repressing flagellin and expressing a mutant SPI-2 T3SS rod protein (SsaI) that cannot be detected by NLRC4 (Miao, *et al.*, 2010; Miao and Warren, 2010). As a result, flagellated bacterial pathogens can evade flagellin-based NLRC4 detection by mutating or transcriptionally repressing the flagellin gene (Lightfield *et al.*, 2008).

Reduced levels of pro-inflammatory cytokines may help the intracellular survival of bacterium within the infected cells by inhibiting cell death or preventing the production of reactive oxygen species, such as nitric oxide. Therefore, the whole procedure favors the in vivo persistence and dissemination of bacteria in the host.

Conclusion

In conclusion, our results provide evidence for the preferential differentiation of chicken macrophages toward the alternatively activated M2-like cells upon ST infection. Although our data point out possible immunomodulatory roles of ST in infected macrophages, the reason for the evolving of such evasion mechanism to enhance bacterial survival in the host cells cannot be described. Further improvement of the existing control measures, including vaccination and molecular-based immunotherapeutic strategies against poultry salmonellosis requires further investigation on the molecular mechanisms involved in the immunomodulatory actions of *Salmonella* in immune cells.

Acknowledgments

Conflict of Interest

The authors declared no conflict of interest.

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مطالعه بیان ژن اینترلوکین 1β (IL-1β) بعد از چالش ماکروفاژهای مشتق شده از مونوسیت‌های ماکیان با سالمونلا تیفی موریوم

الیا عباس زاده[۱]، جلیل مهرزاد[۲]، سید مصطفی پیغمبری[۱]*، پانیذ ضرغامی[۱]*، وحید کریمی[۱]، رامک یحیی رعیت[۲]

[۱]گروه بیماری‌های طیور، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران

[۲]گروه میکروب شناسی و ایمنی‌شناسی، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران

زمینه مطالعه: باکتری گرم منفی *سالمونلا* تیفی موریوم توانایی زنده‌مانی و تکثیر درون سلول‌های ایمنی مانند ماکروفاژها را دارد. لذا بررسی زوایای مبهم پاسخ ایمنی ذاتی ماکیان به *سالمونلا* از اهمیت بسزایی در طراحی واکسن‌های موثرتر و همچنین بهبود واکسن‌های موجود در ماکیان برخوردار است که در نهایت می‌تواند سبب کنترل بیماری گردد.

هدف: بررسی زوایای پاسخ ایمنی ذاتی توسط مونوسیت‌های شبه ماکروفاژ خون ماکیان در پاسخ به چالش با *سالمونلا انتریکا* تحت گونه انتریکا سرووار تیفی موریوم در شرایط برون‌تن.

روش کار: پس از آماده کردن مونوسیت‌های شبه ماکروفاژ خونی، سلول‌ها توسط باکتری *سالمونلا* سرووار تیفی موریوم ۱۴۰۲۸ATCC به تعداد ۵۰ عدد باکتری به ازای هر سلول چالش داده شدند. آنالیزهای رونویسی سایتوکاین التهابی اینترلوکین 1β توسط qRT-PCR با استفاده از سایبرگرین انجام شد. داده‌های بهدست آمده با سطح بیان ژن housekeeping که در مطالعه صورت گرفته ژن GAPDH است، نرمال شده و اختلاف سطوح بیان ژن‌های مربوطه در گروه چالش نسبت به گروه شاهد مورد تجزیه و تحلیل آماری قرار گرفتند.

نتیجه‌گیری نهایی: در چالش سلول‌های مونوسیت شبه ماکروفاژ پرندگان با *سالمونلا* تیفی موریوم سوبه وحشی، سلول‌های ماکروفاژ از طریق کاهش معنادار بیان سایتوکاین التهابی اینترلوکین 1β به سمت تمایز به سلول‌های شبه M2 ماکروفاژ سوق پیدا می‌کنند.

واژه‌های کلیدی: *سالمونلا* تیفی موریوم، پاسخ ایمنی، مونوسیت شبه ماکروفاژ، سایتوکاین التهابی، اینترلوکین1β

نویسندهٔ مسئول: سید مصطفی پیغمبری، گروه بیماری‌های طیور، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران. ایمیل: mpeigham@ut.ac.ir