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Identification of Nuclear Factor-κB Pathway Genes in Chicken Erythrocytes and their Expression Level in Erythrocytes after Infection with *Mycoplasma synoviae*

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AK drafted the basic manuscript and analyzed the data. WXT and GFG contributed to conception and design of the research and reviewed the manuscript. YHB reviewed the manuscript. RAM and XYH participated in sample collection and laboratory testing. ARJ, DZ and MLQ analysed the data and revised the manuscript. All authors read and approved the final manuscript.

Key words
Chicken, Erythrocytes, *Mycoplasma synoviae*, Transmission electron microscope quantitative real time PCR, NF-κB signaling pathway

ABSTRACT

Mycoplasma synoviae is one of the most important pathogens in the poultry industry and often causes diseases of a chronic and persistent nature. However, there is limited data available on Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway genes expression in chicken erythrocytes infected with *Mycoplasma synoviae* (*M. synoviae*). Therefore, the aim of the current *in-vitro* study was to determine the interaction between chicken erythrocytes and *M. synoviae* using Transmission electron microscope (TEM) and further to investigate the mRNA gene expression of *MyD88* (Myeloid differentiation primary response 88), *CCL5* (C-C Motif Chemokine Ligand 5), *MDA5* (melanoma differentiation-associated protein 5) *IKBKE* (inhibitor of nuclear factor kappa-B kinase subunit epsilon), *NFKBIA* (NF-kappa-B inhibitor alpha), *NFKBIE* (NF-kappa-B inhibitor epsilon), Interferon Alpha (*IFN-α*), *cMGF* (chicken myelomonocytic growth factor), and *TRAF6* (Tumor necrosis factor receptor-associated factor 6) in chicken erythrocytes infected with *M. synoviae* using quantitative real-time PCR (qRT-PCR) at four different time intervals (0, 2, 6 and 10 h) post-infection and compared to uninfected controls. The results indicated that *M.synoviae* interacted efficiently in chicken erythrocytes, which strongly induced the up-regulation of NF-κB pathway and other immune system genes in response to early bacterial infection such as *NFKBIA, NFKBIE, IKBKE, CCL5, MDA5, MyD88, and TRAF6* at different h's interval. Whereas *cMGF* and *IFN-α* expression were significantly downregulated during early time intervals such as 0 h, 2 h and 6 h while later on the expression level significantly increased. These results will lead to increased insights on *M. synoviae* infection resistance mechanisms and the role of NF-κB signaling pathway and other immune system genes in the control of the host immune response.

INTRODUCTION

Erythrocytes in circulation are the most abundant cells which plays critical role in the exchange and transportation of gases. In the blood, erythrocytes are the key bactericidal cells, which perform pathogens clearance in bloodstream. They attract, engulf, kill and then push killed microorganisms back to blood plasma (Minasyan, 2014). Furthermore, many studies stated that in immunology, an important role is played by chicken erythrocytes (Paolucci *et al*., 2013). Indeed, it was shown that candida albicans stimulated erythrocytes released mediators which in turn enhanced phagocytic capabilities after macrophage activation (Passantino *et al*., 2007). Additionally, in erythrocytes several TLRs transcripts were constitutively expressed (Paolucci *et al*., 2013).

Mycoplasma synoviae is a significant pathogen of domestic poultry, leads to huge economic losses in poultry industry (Kleven, 2008; Umar *et al*., 2017). Infection mainly occurs as a subclinical upper respiratory tract infection, but when virulent *M. synoviae* strains are

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involved along with other respiratory pathogens, may leads to respiratory disease with air saculitis (Lockaby *et al*., 1999). Whereas, acute to chronic infectious synovitis can occur as a results of systemic infection in poultry birds (Kleven., 2008). Most importantly, the birds remain carriers for life after being persistently infected with *M. synoviae* (Raviv *et al*., 2007; Umar *et al*., 2017). It has been reported that several immune genes are modulated in response to *M. synoviae* infection in chicken macrophages (Lavrič *et al*., 2007; 2008).

Innate immunity is the first line of defense against invading pathogens. After TLRs detect the microbial components, they recruit adaptor proteins to active the NF-κB pathway (Kawai and Akira, 2007). Nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, which regulates a large array of genes involved in different processes of the immune and inflammatory responses (Oeckinghaus *et al*., 2009) NF-κB induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines, and also participates in inflammasome regulation. In addition, NF-κB plays a critical role in regulating the survival, activation and differentiation of innate immune cells and inflammatory T cells. Consequently, deregulated NF-κB activation contributes to the pathogenic processes of various inflammatory diseases (Liu *et al*., 2017).

The activation of NF-κB involves two major signaling pathways, the canonical and noncanonical (or alternative) pathways, both being important for regulating immune and inflammatory responses despite their differences in signaling mechanism (Sun, 2017; Vallabhapurapu and Karin, 2009). The canonical NF-κB pathway responds to stim- uli from diverse immune receptors and leads to rapid but transient NF-κB activation (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009; Hu and Sun, 2016). The non-canonical NF-κB pathway regulates important aspects of immune functions, including lymphoid organ development, the cross-priming function of dendritic cells, B cell survival and germinal center reactions, generation and maintenance of effector and memory T cells, and antiviral innate immunity (Sun, 2017). A well-recognized function of NF-κB is regulation of inflammatory responses. In addition to mediating induction of various pro-inflammatory genes in innate immune cells, NF-κB regulates the activation, differentiation and effector function of inflammatory T cells (Lawrence, 2009; Tak and Firestein, 2001). Recent evidence suggests that NF-κB also has a role in regulating the activation of inflammasomes (Sutterwala *et al*., 2014).

Our earlier studies have reported that in immunity against thiram induced TD chickens, the main role had been played by chicken erythrocytes, which may also have a role in apoptosis (Jahejo *et al*., 2020b; Tian *et al*., 2013; Wang *et al*., 2018). In addition, chicken erythrocytes express TLRs 2, 3, 4, 5, 7 and possess immune related functions (Paolucci *et al*., 2013). However, till date there was no evidence, whether NF-κB signaling pathway and other immune system genes expressed in *M. synoviae* infected chicken's erythrocytes or not and how *M. synoviae* affects their genes expressions. Therefore, in current study we planned to determine the interaction between *M. synoviae* and erythrocytes and further to determine the effect of *M. synoviae* strain on the mRNA expression of NF-κB signaling pathway and other immune system genes in chicken erythrocytes.

MATERIALS AND METHODS

Erythrocyte collection

Blood was obtained from Specific pathogen free (SPF) chickens purchased from Longkol Company (Taigu, Shanxi). In the same volume of Alsever's solution (Solarbio, Beijing, China), about 4 mL of fresh venous blood from pterygoid vein of adult SPF chicken was drawn and mixed. To the 4 mL Histopaque-1119 solution (Sigma–Aldrich, Oakville, ON), the diluted blood was carefully added following centrifugation at 2000 r/min for 20 min; consequently, the leukocytes and platelets were removed from the supernatant. Later procedures were done as previously described (Kabanova *et al*., 2009). Moreover, via Wright Giemsa staining 99.9% purity of isolated erythrocytes were determined.

Treatment of Mycoplasma

About 8 mL of FM-4 mycoplasma culture medium was taken during log phase (the medium has just turned yellow). The concentration of mycoplasma was about 1×10^{6}-1×10^{7} mL, followed by centrifugation at 12000 r/min 15min, later supernatant was discarded. After washing twice with PBS again centrifuge at 12000 r/min for 10 min. Lastly cells were than cultured in 98% Dulbecco's Modified Eagle Medium (DMEM) (Solarbio, Beijing, China), added with 2% fetal bovine serum (FBS) and 2% chicken serum (Longkol, Shanxi, China).

Experimental infection of chicken erythrocytes (CER)

Total 50 µl of erythrocytes were obtained from specific pathogen free (SPF) chickens purchased from Longkol Company (Taigu, Shanxi) in a sixteen 2 mL centrifuge tube containing a certain amount of cell maintenance solution, and distributed into four groups *i.e.*, 0, 2, 6 and 10 h. The experiment was performed in an Animal Biosafety Level 2 Laboratory. To an experimental group, 100 ul of *M. synoviae* was added into all experimental four groups with the addition of 900 µl of DMEM to make the final volume 1050 µl. Whereas to the another four group *M. synoviae* was not added and designated as control group.

The cells were then cultured at 37 °C in 5% CO_2 incubator, and each of the respective group was taken out at 0 h, 2 h, 6 h, and 10 h, respectively after the centrifugation for 10 min at 2000 r/min. To the Eppendorf tubes supernatants was drawn and stored. Furthermore, 3 times washing of erythrocytes were done with PBS and stored for upcoming experiments.

Identification of interaction between M. synoviae *and erythrocytes using Transmission Electron Microscope (TEM)*

TEM was performed as described previously (Strunov *et al*., 2016). Briefly, erythrocytes were isolated at 2000 r/min for 10 min and washed 3 times for transmission electron microscopy. The samples were then fixed and sent to Shanxi Medical University for testing where, the ultrastructure was observed in JEM-1400 (JEOL Ltd., Tokyo, Japan) TEM.

Total RNA extraction from chicken erythrocytes, and synthesis of cDNA

From blood samples taken at 0 h, 2 h, 6 h and 10 h from both an experimental and control groups the total RNAs was extracted from erythrocytes that were isolated by RNAiso Plus (Takara Bio Inc., Dalian, China) by following the instructions of the manufacturer, and then dissolved in 20 µL RNase-free water. The extracted RNAs purity and quantity were initially examined through a NanoDrop Bioanalyzer ND1000 (Labtech, Uckfield, UK) and via 1.5% agarose gel electrophoresis. Furthermore, from 500 ng total RNA, the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc., Dalian, China) was used to reverse transcribe the total RNAs to cDNA according to the procedures recommended by the manufacturer. The cDNA samples were then stored at − 20°C after diluted at 1:10 in RNase-free water.

Analysis of mRNA expression of NF-κB signaling pathway and other immune system genes by quantitative real-time PCR (qRT-PCR)

The qRT-PCR was used for the NF-κB signaling pathway and other immune system genes expression analysis, using real-time RT-PCR kit (TaKaRa SYBR Premix Ex Taq™II (Takara Bio Inc.). Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) was used to designed primers of TLR's and other immunity-related genes according to the respective chicken genes coding sequences in NCBI and manufactured by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Thorough info of the NF-κB signaling pathway and other immune system genes primers and annealing temperature fixed in this experiment along with GenBank accession numbers of the reference sequences are presented in Table I. For qRT-PCR, the thermal cycling parameters used were described previously (Sheng *et al*., 2018). The expression level of NF-κB

Table I. Primer sequences and accession numbers used in quantitative RT- PCR.

Gene targeted	Primers sequences (5′→3′)	Sizes (bp)	Annealing temp.	Accession number
NFKBIA	F: CACCAACTACAACGGCCATA R: TGAAGGTCTACGGCCAAGTG	100	55˚C	NM_001001472.2
IKBKE	F: GCAGCAGGATGAGGAAAGTCT R: CGTACGATACCGACTTCATCTG	100	55˚C	XM_015299066.2
NFKBIE	F: CGGTGACACGTTGGTTCA R: CTGGGCTGCTCCAGATAGA	100	55˚C	XM_419490.6
IFN α	F: CCACACCTTCCTCCAAGACA R: GCCTGTGAGGTTGTGGATGT	100	55˚C	EU367971
MYD88	F: CAGAAAGACCTTCAGTTTGTCCA R: AATGACGACCACCATCCTCC	165	55˚C	NM_001030962
MDA5	F: GAGTTTGGATCTCAGCCATA R: TCAAGTGTTCTGCACAGACA	100	55˚C	GU570144.1
CCL5	F: TGATACAACCGTGTGCTGCTT R: TGCTGCCTGTGGGCATTT	100	55˚C	NM_001045832.1
TRAF 6	F: ATGGAAGCCAAGCCAGAGTT R: ACAGCGCACCAGAAGGGTAT	144	55˚C	XM015287208
cMGF	F: CAATCACACGACGTTGGTT R: GGATGTTGGAGGAGAGGTT	62	55˚C	M85034
18SrDNA	F: TTCCGATAACGAACGACAC R: GACATCTAAGGGCATCACAG	139	55˚C	FM165414

signaling pathway and other immune system genes relative to the 18S rRNA housekeeping gene was calculated by the QuantStudio™ 6 Flex Real-Time PCR System Software (Applied Biosystems, USA).

Statistical analysis

The real time PCR data was calculated using 2−ΔΔCt method. The data obtained between control and experiment groups for each time point post-infection was employed to Two-way ANOVA to perform statistical analysis. All graphs were accomplished using GraphPad Prism 5. Significant differences were measured once the *p*-value was $*P < 0.05$, $**P < 0.01$ or $P*** \leq 0.001$

RESULTS

Interaction between M. synoviae *and erythrocytes*

For TEM analysis, we have chosen non-infected chicken erythrocytes and infected chicken erythrocytes with *M. synoviae*. Control erythrocyte was distributed uniform cytoplasm as shown in control section of Figure 1A. But erythrocyte infected with *M. synoviae* contain endosome as shown in Figure 1B. it was determined that *M. synoviae* interacted with erythrocyte.

Expression of NF-κB signaling pathway and other immune system genes

The relative mRNA expression levels of NF-κB signaling pathway genes and other immune system genes in chicken erythrocytes following infection with *M. synoviae* were analyzed and presented in Figure 1 to investigate the degree of effect on gene expression in chicken erythrocytes after interaction with *M. synoviae*. We found that the expression levels of NF-κB signaling pathway genes and other immune functioning genes were significantly varied at different time intervals such as 0 h, 2 h, 6 h, 10-h post-infection (Hpi). The qRT-PCR results indicated that relative mRNA expression of *NFKBIA, IKBKE* and *TRAF6* were significantly upregulated, whereas *IFN-α* and *cMGF* gene expression was significantly downregulated at 0-h -post-inoculation (hpi) ($P < 0.05$), compared to the control group erythrocytes. Moreover, at 2 hpi, the expression of *NFKBIA, IKBKE, NFKBIE, MyD88* and *MDA 5* were significantly upregulated ($P < 0.05$) while cMGF was significantly downregulated ($P < 0.05$). Relative mRNA expression of *NFKBIA, CCL5, MDA5* and *cMGF* were significantly up-regulated ($P < 0.05$) compared to a control group at 6hpi. In contrast we observed significant downregulation ($P < 0.05$) of *IKBKE* and *IFN-α* at 6 hpi. Furthermore, the expression of *NFKBIE, CCL5, TRAF6, IFN-α* and *MDA5* in infected erythrocytes at 10 hpi were also significantly upregulated ($P < 0.05$) while *NFKBIA* expression was significantly down-regulated ($P < 0.05$) at 10 hpi compared with the control group.

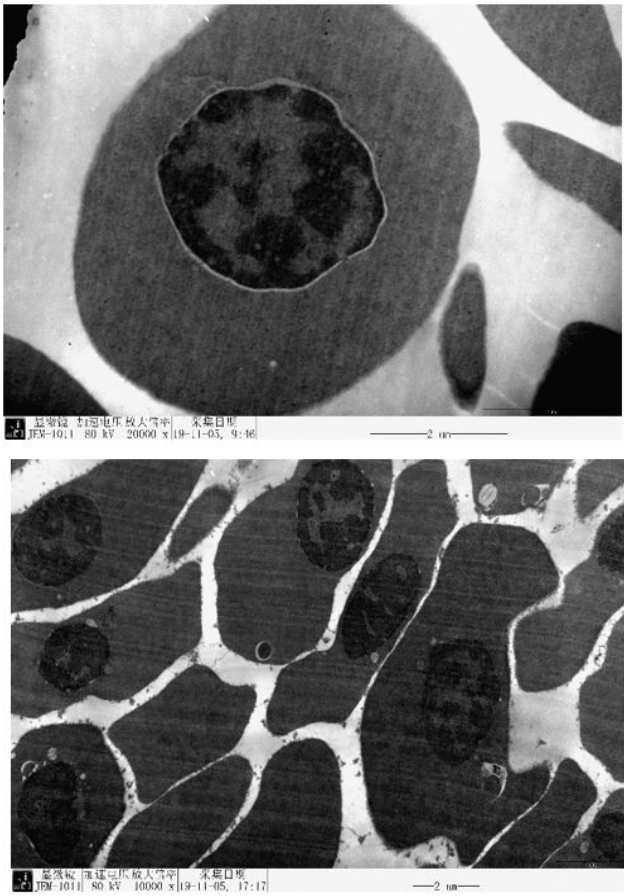

Fig. 1. Transmission electron Microscopy (TEM) of chicken erythrocytes showing interaction with *M. synoviae*. Magnification 20000 × or 10000 ×.

DISCUSSION

M. synoviae infection emerges to cause considerable financial loses to worldwide poultry producers. *M. synoviae* infection controls numerous immune genes in chicken macrophages (Lavrič *et al*., 2007, 2008) and apoptotic genes in chicken chondrocytes (Dušanić *et al*., 2012). Recent studies reports stated that chicken erythrocytes actively participated in important aspects of host immunity (Jahejo *et al*., 2020; Paolucci *et al*.,2013). Optimal NF-κB activation is initiated by bacterial or viral infections through Toll-like receptors, phorbol 12-myristate 13-acetate, or interferon. This process involves the signal-induced phosphorylation and subsequent ubiquitin-mediated degradation of IκB by the classical IκB kinase (IKK)-dependent pathway (Perkins, 2007; Hayden and Ghosh, 2008;

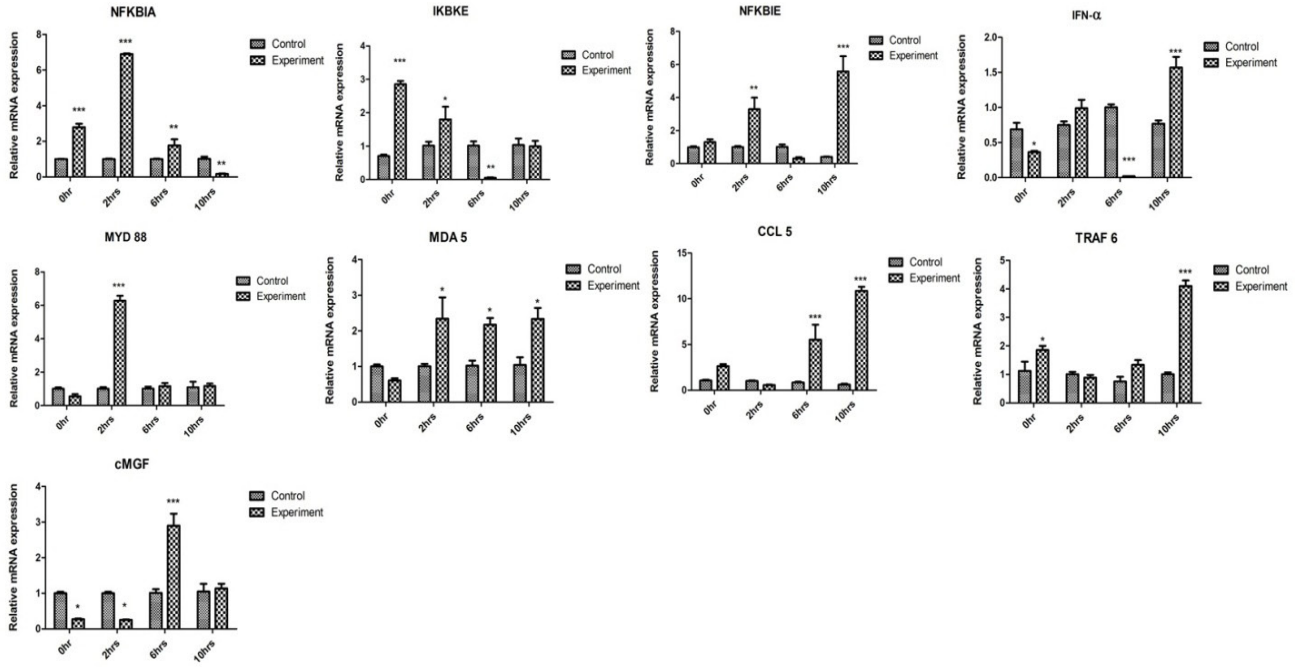

Fig. 2. The expression pattern of NF-κB signaling pathway and other immune system genes in chicken erythrocytes on 0 h, 2 h, 6 h, 10 h post-infection (Hpi).. This experimental study group was as follows: The control group (Con, erythrocytes treated with PBS, n = 3) and the Experiment group (Exp, erythrocytes infected with *M. synoviae*, n = 3). Expression levels of NF-κB signaling pathway and other immune system genes were relatively calculated to that of 18S rRNA the housekeeping genes via quantitative real-time PCR. SEM (standard error of mean) represented by error bars. Bars with asterisks indicate a significant (*$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$) Up or Downregulated relative mRNA expression when compared to uninfected control group.

Karin and Ben-Neriah, 2000). In the current study, for first time it was investigated that chicken erythrocytes constitutively expressed several different NF-kB pathway and other immune system genes (*NFKBIA, NFKBIE, IKBKE, CCL5, MDA5, MyD88, TRAF6, IFN-α* and *cMGF*) at the transcript level and responded to *M. synoviae* by up-regulating different genes expression at certain time interval. Therefore, this result offers a novel perspective for poultry health because, targeting such TLRs for therapeutic purposes can be one of the ways to defend chickens from infection of mycoplasmas, particularly, *M. synoviae*.

There are few reports regarding gene expression of *NFKBIA*, *NFKBIE* and *IKBKE* during bacterial infection. However, genetic variations within *NFKBIA*, *NFKBIE* and *IKBKE* have been shown to influence susceptibility to invasive bacterial infection (Zimmerman *et al*., 2010). The expression of *NFKBIA* was significantly upregulated during early h of infection (0 h, 2 h and 6 h) while there was significant downregulated expression at 10 h post infection on *M. synoviae* infection in chicken erythrocytes. Exposure of cells to various stimulants resulting in release of NF-κB from inhibitor IKB that controls NF-κB activity. Signals activate NF-κB by targeting IKB for proteolysis (Asakrah *et al*., 2013). An NF-κB dependent host response was shown by the significant differential expression of *NFKBIA*. Phosphorylation and the subsequent ubiquitination of IKB, the gene product of the *NFKBIA* gene, are known as key processes required for regulating the innate immune system (Bhoj and Chen, 2009). Furthermore, our results are in agreement with (Tripathi *et al*., 2009) who reported that erythrocytes infected with plasmodium Falciparum upregulates the expression of NF-κB pathway genes including *NFKBIA, NFKBIE* in humans.

The kinase was first identified as an IKK kinase family member of the NF-κB activation by lipopolysaccharides and phorbolesters (Shimada *et al*., 1999; Peters *et al*., 2000). *IKBKE* (*IKKE, IKKi*) is a non-canonical I-kappa-B kinase which can be activated by numerous stimuli (Boehm *et al*., 2007). *IKBKE* mainly mediates NF-κB activation induced by the T cell receptor, phorbol 12-myristate 13-acetate, or interferon. In current study we observed dramatic expression pattern differences of the NF-κB related gene such as *IKBKE* whose expression was significantly upregulated at 0h and 2h while at 10 h post-

infection the expression was significantly downregulated in infected erythrocytes. Previously it was reported that most of the bacterial diseases induces NF-κB related gene expression (Wang *et al*., 2017). Furthermore, (Verhelst *et al*., 2013; Kim *et al*., 2014) reported that *IKBKE* was a major NF-κB signaling mediator. As the significant upregulation of most of NF-κB pathway genes (*NFKBIA, NFKBIE* and *IKBKE*) were detected, it seems that NF-κB pathway was activated in the chicken erythrocytes after *M. synoviae* infection. This study results suggests that NF-κB related genes may play key roles in regulating the immune response to bacterial infection in chickens.

The expression of *NFKBIE* gene was also significantly upregulated at certain time interval (2 h and 10 h) post infection in response to *M. synoviae* infected erythrocytes. This finding could be supported by biological knowledge since NF-κB signaling that is inhibited by *NFKBIE* is transcriptional factor involved in inflammatory immune response (Guthke *et al*., 2005). Moreover, it was also reported that *NFKBIE* itself is upregulated at mRNA level by TNF, whose over expression is feature of inflammatory diseases (Rao *et al*., 2010).

IFNs are cytokines produced in response to viral, bacterial, and fungal pathogens, as well as parasites. The effector mechanisms of IFN-α mainly derive from products of genes which are transcriptionally regulated by type I IFN signaling. Bacteria trigger type I IFN (IFN-α) production mostly following the recognition of bacterial nucleic acids or the Gram-negative cell wall component lipopolysaccharide (LPS) by innate immune receptors (Boxx and Cheng, 2016; Monroe *et al*., 2010). Our results revealed that *IFN-α* can be expressed in chicken erythrocytes in response to *M. synoviae* infection. Interestingly, we observed decreased expression level during early stage of infection (0 h, 2 h and 6 h) surprisingly; expression level increased significantly at later stage (10 h) post infection. It was recently found that chicken erythrocytes constitutively express transcripts for many TLRs as well as for some cytokines such as *IFN-α*, *IFN-β*, and *IL-8* (Paolucci *et al*., 2013).

Our study result also reveals the significant up-regulation of *MyD88* mRNA gene expression in chicken erythrocytes in response to *M. synoviae* strain. Previously it was reported that *MyD88* gene constitutively expressed on almost all tissues (Hardiman *et al*.,1997). Likewise, it was shown previously that in spleen and thymus *MyD88* gene was expressed higher, which is consistent with results of mouse and human (Hardiman *et al*., 1996, 1997). Previous studies also suggested an important role of *MyD88* gene expression in NF-kB activation *in-vitro* in the chicken innate immune response to bacterial infections (Yafeng *et al*., 2008). Recently it was also stated that all *TLR 3* employ the *MyD88* dependent pathway (O'neil, 2006). The significant upregulation of *MyD88* in erythrocyte suggested that this adaptor molecule plays an important role to induce an innate immune response to *M. synoviae* infection.

There is increasing evidence that *MDA5* have additional distinct molecular functionalities in immune signaling (Kasumba and Grandvaux, 2018). It is well-established that the interferon regulatory factor (IRF) and innate immune NF-κB cytokine signaling pathways have many areas of cross-regulation and expression (Czerkies *et al*., 2018). Accordingly, *MDA5* have been shown to activate NF-κB signaling during infection (Yoboua *et al*., 2010; Rückle *et al*., 2012). In current *in-vitro* study for first time it was reported that in *M. synoviae* infected chicken erythrocytes expression of *MDA5* was upregulated in response to bacterial infection. Our results are in agreement with (Ye *et al*., 2018), their results showed up-regulated *MDA5* expression after using a synthetic bacterial analog, Lipopolysaccharide (LPS), representing stimulation by Gram-negative bacteria. Several studies concurrently showed that LPS or bacterial challenge resulted in up-regulation of fish *MDA5*, such as common carp (*Cyprinus carpio*) challenged with Aeromonas hydrophila (Imaizumi *et al*., 2002). Furthermore, chicken *MDA5* is ubiquitously expressed highest in intestine (Su *et al*., 2010; Huang *et al*., 2010). Hence, such results suggesting that *MDA5* might not be involved exclusively in recognizing viral PAMPS, but they are also capable of indirectly distinguishing bacterial PAMPs chickens.

CCL5, a target gene of NF-*κ*B activity, is expressed by T lymphocytes, macrophages, platelets, synovial fibroblasts, tubular epithelium, and certain types of tumor cells (Soria and Ben-Baruch, 2008). NF-*κ*B activation by different stimuli such as CD40 (Aldinucci *et al*., 2012; or IL-15 Chenoweth *et al*., 2012) induces *CCL5* production. In current *in-vitro* study, infection of erythrocytes with *M. synovie* in chicken significantly upregulated the mRNA expression of *CCL5*. Previously upregulated expression of *CCL5* was reported by (Majumder and Silbart, 2015) in chicken macrophages upon direct contact of HD-11 cells with *M. gallisepticum.* In addition, in 4T1 tumour cells *CCL5* was constitutively expressed with upregulated expression (Kurt *et al*., 2001), suggesting that in resistance to immune-surveillance high levels of *CCL5* may play an important role (Adler *et al*., 2003). Moreover, higher expression of *CCL5* gene maybe due to its transcriptional activation that critically dependent on NF- *κ*B signaling pathway (Dandan *et al*., 2013).

TRAF6 has been linked to the function of various immune effector cells. Previous studies have shown that *TRAF6* regulates several signaling cascades involved in

adaptive immunity and innate immunity (Ye *et al*., 2002). *TRAF6* recognizes different binding sites of CD40 and receptor activator of nuclear factor kB, and other members of the TNFR superfamily (Pullen *et al*., 1998). In addition, *MDA5* signaling pathway requires the mitochondrial protein ISP-1 to form a signaling complex with *TRAF6*, which leads to the activation of *TRAF6* and initiation of the innate immune response (West *et al*., 2011). In our study the expression of *TRAF6* was significantly upregulated in response to *M. synoviae* infected erythrocytes, suggesting that *TRAF6* is essential for the induction of effective innate immune responses in chicken erythrocytes. Previously it was observed that abundant *TRAF6* expression was in the spleen, largest lymph organ, in mice, ducks and humans, suggesting that *TRAF6* could be a vital factor for the immune system (Jin *et al*., 2017). In addition, (Stockhammer *et al*., 2010) revealed that in immune-related tissue, such as thymus, spleen and bursa of Fabricius the mRNA expression of *TRAF6* was highest, indicated an innate immune response mediated by *TRAF6* in chickens to pathogenic challenges. Hence, *TRAF6* expression indicated that chicken erythrocytes might have an important role in chicken defense against bacterial infections.

Lastly, in the current study, we examined the mRNA gene expression of *cMGF* in infected chicken erythrocytes. Our results revealed for the first time that M.synoviae infected chicken erythrocytes, there is down regulation of *cMGF* gene in the early phase such as 0h and 2h post infection while with passage of time expression enhanced significantly in later time intervals when compared with an uninfected group. *cMGF* is a 27-kDa glycoprotein that was first described regarding its capability to encourage the growth of macrophage and granulocyte colonies from avian bone marrow progenitor cells *in vitro* (Leutz *et al*., 1984). Previously it was reported the involvement of *cMGF* in the innate immune response through enhancing monocyte or macrophages quantity and activation, resulting in the production of NO with antiviral activity (Djeraba *et al*., 2002). Furthermore, it was also declared that *cMGF* is required for survival and growth for normal and transformed avian myelomonocytic cells (Leutz *et al*., 1984, 1988). Together, all these results show that chicken erythrocytes may have the potential to respond to bacterial infectious agents.

CONCLUSION

This *in vitro* study provided the first evidence that *M. synoviae* interacts with chicken erythrocytes which can also constitutively express NF-kB signaling and other immune system genes. Expression of most of NF-κB signaling pathway and other immune system genes were significantly regulated in chicken erythrocytes after *M. synoviae* infection, indicating their involvement in immune response to bacterial infection. Future studies may be planned to understand how NF-κB signaling and other immune system genes respond to bacterial infections in chicken erythrocytes and their roles in the host's immunity.

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Statement of conflict of interest

The authors have declared no conflict of interest .

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