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Molecular cloning of chicken interleukin-17B, which induces proinflammatory cytokines through activation of the NF-κB signaling pathway

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ABSTRACT

Interleukin (IL)-17B is a little known member of the IL-17 cytokine family, which plays an important role in immunity by regulating the expression of proinflammatory cytokines. In this study, we determined the coding sequence and biological functions of a novel chicken IL-17B (chIL-17B). The full-length chIL-17B coding sequence includes 567 nucleotides encoding 188 amino acids, which was identified in small intestinal epithelial cells. The chIL-17B protein shares 96.48% amino acid sequence identity with turkey, 92.57% with duck, and 44.92–64.06% with mammalian IL-17B proteins. ChIL-17B shares three exons and two introns with mammals, turkey, and duck. Moreover, IL-17B mRNA was more highly expressed than IL-17A mRNA in several organs of chickens infected with *Salmonella* and was upregulated in chicken cell lines following LPS stimulation. In addition, in chicken cell lines, chIL-17B induced the mRNA expression of several proinflammatory cytokines, including IL-1β, IL-6, LITAF, and INF-γ, but not IL-17A, and activated MyD88, TAK1, NF-κB1, and SOCS1, which are associated with the NF-κB signaling pathway. Taken together, chicken interleukin-17B plays a critical role in host defense against the bacterial pathogens, and regulates proinflammatory cytokines by activating the NF-κB signaling pathway.

1. Introduction

Interleukin-17A (IL-17A or IL-17) was the first IL-17 discovered (Rouvier et al., 1993) and determined in humans (Yao et al., 1995). The interleukin 17 (IL-17) family includes six members (IL-17A to IL-17F), which were identified by amino acid sequence homology and were shown to have various biological roles in mammals (Iwakura et al., 2011). They bind to IL-17 receptors (Gaffen, 2009; Gu et al., 2013) and activate specific biological functions by triggering downstream signaling pathways, such as the NF-κB, MAPKs, and C/EBP pathways (Maezawa et al., 2006; Song and Qian, 2013; Song et al., 2011; Yang et al., 2008). This cytokine family plays important roles in immunology by inducing proinflammatory cytokines, chemokines, and antimicrobial peptides (Iwakura et al., 2011; Kawaguchi et al., 2004). In addition, IL-17 family members have been implicated in the development of autoimmune diseases (Gu et al., 2013). In mammals, IL-17A and IL-17F have been well characterized. They share the highest amino acid sequence identity, are produced by T helper (T_h) 17 cells, and signal through the same receptors, IL-17RA and IL-17RC (Gaffen, 2009; Iwakura et al., 2011). IL-17A and IL-17F cooperate with other cytokines, such as TNF-α and IL-1β, to amplify and maintain proinflammatory responses (Miossec, 2003). A recent study showed that IL-17C also binds to IL-17RA and may have similar functions with IL-17A and IL-17F such as the promotion of antimicrobial peptide production. However, IL-17C is produced in epithelial cells and is an important factor in the mucosal immune system (Ramirez-Carrozzi et al., 2011). Although IL-17E is a member of the IL-17 family, its function differs from that of IL-17A, which promotes cytokine and chemokine production for neutrophil recruitment. In contrast, IL-17E (IL-25) promotes the production of type 2 cytokines, such as IL-4, IL-5, and IL-13, which function against infectious diseases (Gu et al., 2013). IL-17D shows the highest sequence homology with IL-17B, suggesting similar functions (Starnes et al., 2002). A previous study showed that IL-6 and IL-8 mRNA expression were upregulated by chIL-17D stimulation in fibroblast cell line (Hong et al., 2008).

In mammals, IL-17B is expressed in several tissues, including

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the pancreas, small intestine, and stomach of adult humans (Li et al., 2000); the spinal cord in Charcot-Marie-Tooth disease (Moore et al., 2002); and the paws of arthritic mice (Yamaguchi et al., 2007). Previous studies suggested that IL-17B plays a critical role in the pathogenesis of inflammatory arthritis in CIA mice (Yamaguchi et al., 2007) and in chondrogenesis and osteogenesis in mouse embryonic limb buds (Reynolds et al., 2015). Moreover, IL-17B stimulates the expression of TNF-α, IL-1β, and IL-6 in mouse and human macrophage cells (Li et al., 2000; Yamaguchi et al., 2007) as well as the expression of G-CSF and IL-6 along with TNF-α in fibroblast cell line (Kouri et al., 2014). IL-17B and IL-17E (IL-25) both bind to the IL-17RB receptor, and IL-17B is inhibited by IL-25-driven inflammation (Reynolds et al., 2015). The IL-17B/IL-17RB complex has been reported to activate nuclear factor-kappaB (NF-κB) and upregulate the antiapoptotic pathway (Huang et al., 2014). However, there is a lack of information regarding the function of IL-17B in chicken, as it has not yet been investigated.

Here, we characterized the biological function of IL-17B in chicken macrophage, fibroblast and T cell lines. We showed that chIL-17B activated the NF-κB signaling pathway and induced cytokine production in chicken cell lines.

2. Materials and methods

2.1. Reagents

Phospho-NFκB1 (Ser^{932}) antibody and phospho-TAK1 (Ser^{192}) antibody were purchased from Biorbyt (San Francisco, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-MyD88 antibody was obtained from Novus Biologicals (Littleton, CO, USA). Anti-GAPDH antibody, anti-6 × His-tagged antibody (HRP), and Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG (H + L) secondary antibodies were purchased from Abcam (Cambridge, MA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and Cell Extraction Buffer were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Experimental animals and challenge

Twenty-four to twenty-six-week-old specific pathogen-free White Leghorn chickens were infected with *S. enterica serovar* Enteritidis (S.E.), and tissue samples were kindly provided by Dr. Uma S. Babu of the Center for Food Safety and Applied Nutrition, USFDA. The treatment and control groups of chickens were housed in separate biosafety level 2 rooms, and commercial feed (without antibiotics or other additives) and drinking water were provided *ad libitum*. For the experiment, chickens were divided into two groups. One group was infected with 1.0×10^{10} CFU of S.E. in 1 mL of phosphate-buffered saline (PBS), and the second control group was mock infected with 1 mL of PBS. Seven days post-infection, tissues sample from seven organs (the small intestine, lung, ovary, liver, spleen, oviduct, and thymus) were collected from five randomly selected chickens per group. Under sterile conditions, the samples were placed in liquid nitrogen and ground with a mortar and pestle for total RNA extraction. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Center for Food Safety and Applied Nutrition, USFDA.

2.3. IL-17B gene cloning

Total RNA was isolated from each chicken tissue sample using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (5 μg) was treated with RNase-free DNase I (Thermo Scientific, Waltham, MA, USA) and incubated at 37 °C for 30 min. Single-stranded cDNA was synthesized from DNase I-treated total RNA using oligo $(dT)_{18}$ primers and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. The chicken IL-17B coding sequence (GenBank: XM_425192) was predicted based on nucleotide sequence homology with mammalian sequences and was amplified from pooled cDNA using the following specific primers (with *Hin*dIII and *Eco*RI restriction enzyme sites, which are underlined): forward 5′-CGGAATTCATGGAGCGGGCTCCCAACCT-3′ and reverse 5′-CCAAGCTTGAAGATGCAAGTGCAGCCCACG-3′. The amplified PCR product was purified using the QIAQuick gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCR2.1-TOPO vector (Invitrogen). Positive chicken IL-17B plasmid clones were sequenced by Genotech (Daejeon, Republic of Korea), ligated into the *Escherichia coli* expression vector pET32a(+) (Novagen, Madison, WI, USA), and transformed into *E. coli* BL21 (DE3) competent cells (Invitrogen).

2.4. Recombinant protein production

E. coli BL21 cells containing a chIL-17B/pET32a (+) clone were cultured in LB medium (Difco™ & BBL™, Sparks, MD, USA) with 50 mg/mL ampicillin (USB Corporation, Cleveland, OH, USA) overnight at 37 °C with shaking, and chIL-17B expression was induced by the addition of 1 mM IPTG (USB Corporation) for 8 h at 37 °C. The cultured bacteria were collected by centrifugation, and the recombinant protein was extracted using the B-PER Bacterial Protein Extraction kit (Thermo Scientific) with HisPur Cobalt Resin (Thermo Scientific). Bacterial endotoxin was removed by a combination of affinity chromatography and a non-ionic detergent washing step as described by Reichelt (Reichelt et al., 2006). The buffer for the eluted protein was changed to PBS by dialysis for 24 h at 4 °C using Snakeskin™ Dialysis Tubing (Thermo Scientific) with stirring. The identity of the recombinant protein was confirmed by SDS-PAGE as a band with the correct molecular weight.

2.5. Cell culture and LPS stimulation

A chicken macrophage cell line (HD11) (Klasing and Peng, 1987), fibroblast cell line (OU2) (Ogura and Fujiwara, 1987), and T cell line (CU91) (Schat et al., 1992) were cultured in complete RPMI 1640 medium (Thermo Scientific) containing 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Thermo Scientific) in a humidified 5% CO_2 atmosphere at 41 °C. Approximately 1.0×10^6 cells were incubated in a 12-well plate (Thermo Scientific) containing 2 mL of culture medium and treated with 1 μg of LPS from S.E. (Sigma-Aldrich) for different times (0.5, 1, 2, 4, 8, 12, and 24 h). Cells were collected, and total RNA was extracted as described above.

2.6. Biological activity of recombinant chIL-17B and Western blotting

HD11, OU2, and CU91 cells seeded in a 12-well plate (1.0×10^6/well) were stimulated with recombinant chIL-17B protein (100 ng/mL) for 0.5, 1, 2, and 4 h. Pilot experiments demonstrated significantly greater activation of certain kinases in the presence of 100 ng/mL than other concentrations. (data not shown). After 0.5, 1, 2, and 4 h of stimulation, total RNA was isolated with TRIzol.

Intracellular protein was extracted with Cell Extraction Buffer (Invitrogen) according to the manufacturer's instruction. The protein concentration was determined by the Bradford assay (Bradford, 1976).

Protein (100 ng/mL) was loaded on an SDS-PAGE gel and then transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) using a Western Blot Gel Transfer Device (Bio-Rad). The membrane was blocked using 5% skim milk in PBS containing 0.05% Tween 20 (PBST) for 2 h, and then incubated with the primary antibody overnight at 4 °C. After washing three times in PBST, the membrane was incubated with an HRP-conjugated secondary antibody for 1 h at RT. Antibody-labeled protein bands were detected with Western Lightning Plus-ECL substrate (Thermo Scientific) and exposure to Hyperfilm (GE Healthcare, LC, UK).

2.7. Quantitative real-time PCR for gene expression analysis

To analyze cytokine gene expression, primers were designed with DNASTAR (Madison, WI, USA) (Table 1), and qRT-PCR was performed using 2 × Power SYBR Master Mix (Roche, Indianapolis, IN, USA) with a LightCycler 96 system (Roche) according to the manufacturer's instructions. Chicken *GAPDH* was used for normalization. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method after normalization to *GAPDH* (Livak and Schmittgen, 2001). All quantitative RT-PCR assays were performed in triplicate.

2.8. Immunocytochemistry staining

Approximately 1.0×10^2 cells/well were cultured in a chamber slide (Thermo Scientific). Cells were stimulated with or without recombinant chIL-17B (100 ng/mL) for various times in a humidified 5% CO_2 atmosphere at 41 °C. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min and then incubated with PBS containing 0.25% Triton X-100 for 10 min at RT. Next, cells were blocked with 1% BSA in PBS for 30 min at RT and treated with the primary antibody for 1 h at RT. Then, cells were incubated with an Alexa Fluor® 488-conjugated secondary antibody for 1 h and stained with DAPI for 5 min at RT. Finally, the images were captured on an EVOS® FLoid® Cell Imaging Station (Invitrogen).

Table 1
Sequences of the primers for real-time PCR analyses of cytokine expression.

Primer	F/R	Nucleotide sequence (5′-3′)	GenBank accession no.
GAPDH	F	TGCTGCCCAGAACATCATCC	NM_204305
	R	ACGGCAGGTCAGGTCAACAA	
IFN-γ	F	AGCTGACGACGGTGGACCTATTATT	HQ739082
	R	GGCTTTGCGCTGGATTC	
IL-6	F	CAAGGTGACGGAGGAGGAC	JQ897539
	R	TGGCGAGGAGGGATTTCT	
IL-17A	F	TGTCTCCGATCCCTTGTTCT	AM773756
	R	GTCCTGGCCGTATCACCTT	
LITAF	F	TGTGTATGTGCAGCAACCCGTAGT	AY765397
	R	GGCATTGCAATTTGGACAGAAGT	
IL-1β	F	TCGGGTTGGTTGGTGATG	NM_204524
	R	TGGGCATCAAGGGCTACA	
SOCS1	F	CTACTGGGGACCGCTGACC	NM_001137648
	R	TTAACACTGATGGCAAAGAAACAA	
IL-17B	F	AAGCCAAGGATGAAAGCAGA	XM_425192
	R	CATTGGAGTGTAGGGGTCATC	
MyD88	F	GGTTCTGGACAAGACTGGCA	NM_001030962
	R	ATGCTGTAGGAACACCGTGG	
NFκB1	F	AGAAAAGCTGGGTCTTGGCA	NM_205134
	R	CCATCTGTGTCAAAGCAGCG	
IKKβ	F	GGGTCAGATCTGGCACACAA	NM_001031397
	R	TGGGAAAGGGAGGCCATAGA	
IKKα	F	TACTGCTTCCTGCCTGTGTG	NM_001012904
	R	GGAGGCCACAGTCCGTAAAA	
TAK1	F	CCAGGAAACGGACAGCAGAG	XM_015284677
	R	GGTTGGTCCCGAGGTAGTGA	

F: forward; R: reverse.

2.9. Bioinformatics analyses

Multiple sequence alignments were generated with CLUSTALX2 (http://www.clustal.org) and BioEdit version 7 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA7 (http://www.megasoftware.net/mega7). A signal peptide and N-like glycosylation site were predicted using SignalP v. 4.1 (http://www.cbs.dtu.dk/services/SignalP) and NetNGlyc software (http://www.cbs.dtu.dk/services/NetNGlyc), respectively.

2.10. Statistical analysis

Data are shown as the mean ± standard error of mean (SEM) of three independent experiments (n = 3) and were analyzed using Student's t-test and one-way analysis of variance (ANOVA) using the SAS® 9.4 statistical program (SAS Institute, Inc., Cary, NC, USA) and were compared between groups using Duncan's multiple comparison. Differences were considered significant at p values less than 0.05.

3. Results

3.1. Cloning and sequence analysis of chIL-17B

The full-length chIL-17B coding sequence contained 567 nucleotides and was predicted to encode a protein of 188 amino acids (Fig. S1). The nucleotide sequence comparison showed that chicken IL-17B shares high homology with IL-17B from duck and turkey (93.28 and 96.96%, respectively), moderate homology with mouse, whale, and human (71.4–74.3%), and low homology with pig (54.80%; Table 2). Moreover, a protein homology analysis revealed that the chIL-17B protein shared 92.57–96.48% amino acid identity and 94.92–97.26% amino acid similarity to duck and turkey IL-17, respectively (Table 2).

Multiple sequence alignment showed that the chIL-17B protein sequence contains eight cysteines that are conserved among chicken, human, and mouse (Fig. 1A). In addition, chIL-17B also includes a signal sequence (amino acids 1–21) and one potential N-linked glycosylation site in the extracellular region, located at position 74 (Fig. 1A).

Next, a phylogenetic analysis including the chicken IL-17B coding sequence and representative avian and mammal IL-17B

Table 2
Percent similarity and identity between chicken IL-17B and IL-17B of other species.

Species	Chicken IL-17B				
	Nucleotide		Protein		
	Identity	Acc. no.	Identity	Similarly	Acc. no.
Turkey	96.96	XM_003210300	96.48	97.26	XP_003210348
Duck	93.28	XM_005009333	92.57	94.92	XP_005009390
Human	74.30	NM_014443	64.06	72.26	NP_055258
Mouse	71.40	NM_019508	64.06	71.48	NP_062381
Whale	74.04	XM_004280369	64.06	72.26	XP_004280417
Pig	54.80	XM_003124086	44.92	53.12	XP_003124134

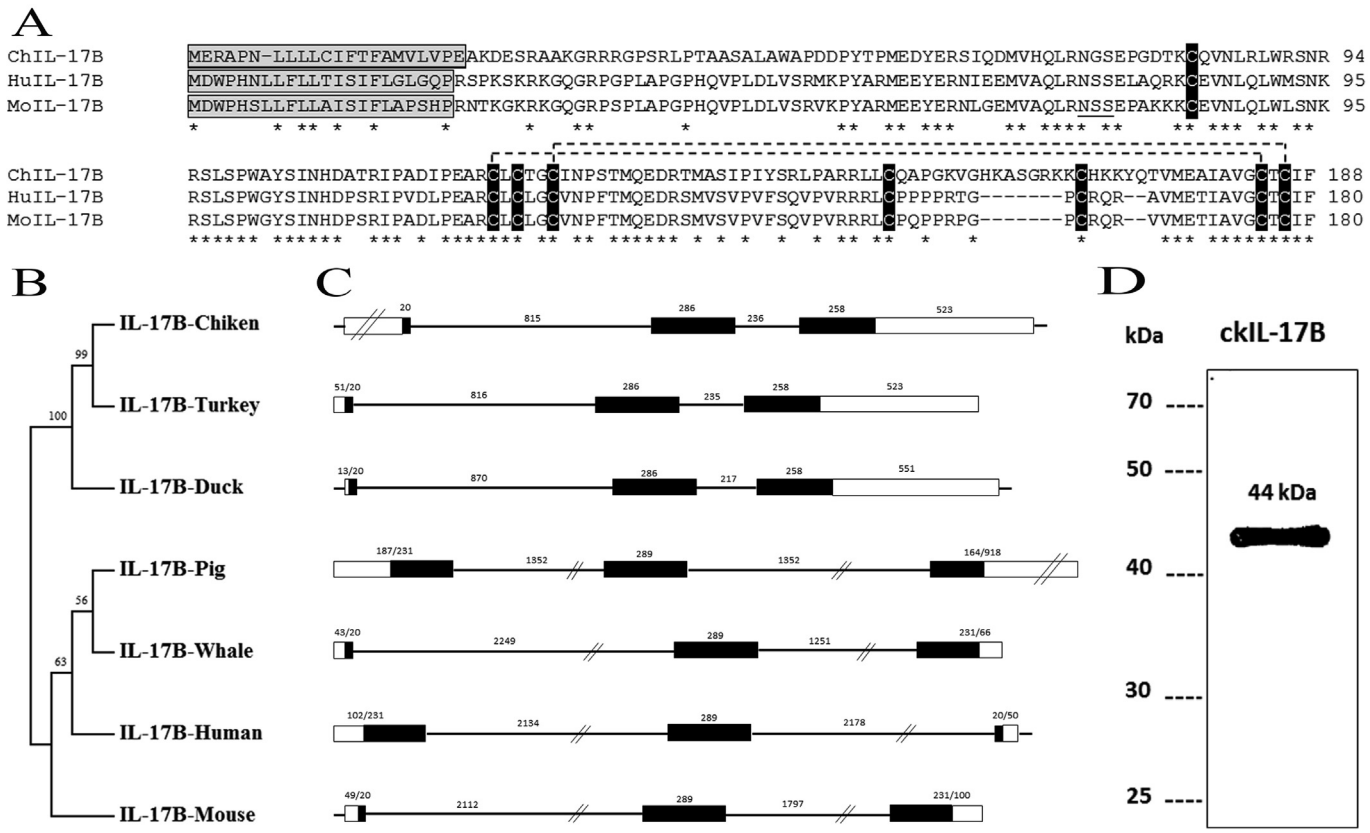

Fig. 1. Molecular characterization of chicken IL-17B. (A) Alignment of the protein sequences of chicken, human, and mouse IL-17B. The signal peptide sequences are shown in grey boxes, the eight conserved cysteine residues are marked with black boxes, and the predicted N-linked glycosylation site is underlined. Two important disulfide bonds in IL-17A that are highly conserved in IL-17 family members are indicated by dashed lines. (B) Phylogenetic tree showing the relationship between the chicken IL-17B amino acid sequence and The IL-17B sequences of other vertebrates. (C) Comparison of the genomic structure of the chicken IL-17B gene to the genes of other species. The lines represent the introns, the black boxes represent the exons, and the white boxes indicate the UTRs. (D) Recombinant protein confirmed by western blotting using an anti-6 × His tag antibody (HRP).

sequences demonstrated that chicken IL-17B is more closely related to turkey and duck than to human and mouse (Fig. 1B). The structure of the chicken IL-17B gene was quite similar to that of turkey, duck, and mammalian IL-17B. ChIL-17B consists of three exons and two introns (Fig. 1C).

3.2. Pathogenic stimulation induced IL-17B expression in vivo and in vitro

To study the role of IL-17B in host defense against bacteria pathogens, we challenged chickens with 1.0×10^{10} CFU/bird of *Salmonella* Enteritis. Seven days after the challenge, tissues were collected from seven organs (small intestine, liver, lung, ovary, oviduct, spleen, and thymus), and IL-17B mRNA expression levels were quantified by qRT-PCR. IL-17B transcript expression was significantly upregulated in the liver, lung, and ovary and was downregulated in the oviduct, spleen, and thymus, compared to the levels in the uninfected controls (Fig. 2B). In contrast, IL-17A expression was upregulated in the lung, although just slightly, and downregulated in the liver and oviduct compared to the levels in the control group (Fig. 2A).

In addition, we evaluated IL-17B expression levels in chicken macrophage cell line (HD11) and fibroblast cell line (OU2) following LPS (S.E.) stimulation. The results showed that chIL-17B mRNA expression was markedly increased for up to 2 h and 0.5 h in the HD11 and OU2 cell lines, respectively (Fig. S2A, B), compared to the levels in the uninfected control. Taken together, these results demonstrate the distinct regulation of IL-17B gene expression by pathogenic stimulation in the chicken as well as in different chicken cell lines.

3.3. Chicken IL-17B activates the NF-κB pathway

Chicken IL-17B recombinant protein was observed as a single band with a molecular weight of approximately 43.8 kDa (Fig. 1D), which is the sum of the predicted molecular weight of ChIL-17B (21.8 kDa) and the TrX protein tag (22 kDa) that were encoded by the expression vector.

To determine the role of IL-17B in intracellular signaling, we investigated the effect of chIL-17B on NF-κB signaling pathway-related gene expression in three chicken cell lines by quantitative RT-PCR and Western blotting. The expression of the signal genes, *MyD88*, *TAK1*, and *NFkB1*, reached maximum levels at 1 h after treatment in the HD11 cell line and at 2 h after treatment in the OU2 and CU91 cell lines (data not shown). All tested cell lines showed significantly higher *MyD88*, *TAK1*, and *NFκB1* expression compared to the levels in the control, and highest levels were observed in the T cell line (Fig. 3A, B, C).

Phosphorylated (Ser^{192}) TAK1 and NFkB1 (Ser^{932}) proteins were detected 30 min after chIL-17B treatment in all cell lines (Fig. 3D, E, F), and the levels peaked at 1 h in HD11 cells (Fig. 3D) and at 2 h in both the OU2 and CU9 cell lines (Fig. 3E and F). Overall, the

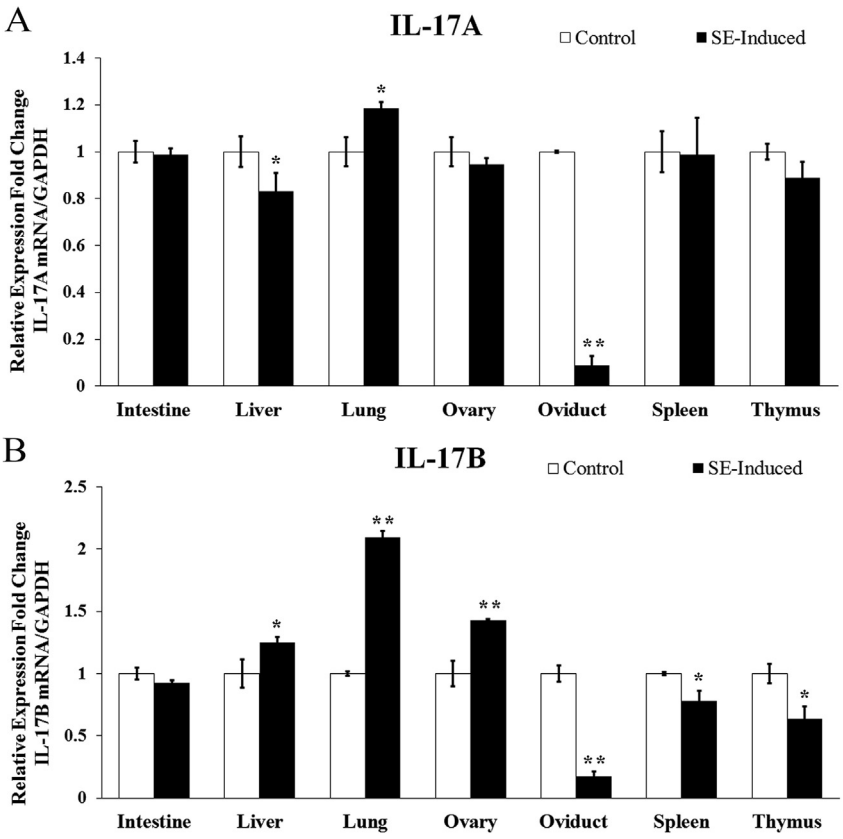

Fig. 2. Expression of IL-17A mRNA (A) and IL-17B mRNA (B) by quantitative real-time PCR. mRNA expression in the tissues of chickens infected with 1.0×10^{10} CFU/mL *Salmonella* serovar Enteritidis (S.E.), compared to the expression levels in non-infected chickens. The tissue samples were collected at 7 days post infection (dpi). Error bars indicate the SE (n = 5) of technical replicates that were performed in triplicate. Significant differences in mRNA expression levels between infected and non-infected chickens are indicated as follows: $^{*}p < 0.05$ and $^{**}p < 0.01$.

expression levels of MyD88, TAK1, and NFκB1 mRNA and protein were higher in the CU91 T cell line than in the HD11 and OU2 cell lines after stimulation with recombinant chicken IL-17B protein.

To determine the mode of NF-κB activation, we evaluated the expression of IKKα and IKKβ, since their catalytic activities are important for NF-κB activation (Zandi et al., 1997). The results showed that the expression of both genes is higher in HD11 and OU2 cells than in the CU91 T cell line (Fig. 3G and H). SOCS1 gene expression was also higher in all chIL-17B-treated chicken cell lines (Fig. 3I).

To visualize the activated NFκB1/TAK1/MyD88 proteins within the chicken cells, immunocytochemical staining was carried out using fluorescent-conjugated antibodies. Strong cellular localization of MyD88, phospho-TAK1, and phospho-NFκB1 protein was observed in all cell lines following treatment with chIL-17B (HD11 [Fig. 4A], OU2 [Fig. 4B], and CU91 [Fig. S3]). Distinct MyD88, TAK1, and NF-κB1 protein expression was observed in the cytoplasm (green color) after treatment with chIL-17B. Taken together, these results suggest that chIL-17B is a mediator of immune responses through the NF-κB intracellular signaling pathway based on the induction of cytokine expression in chicken cells.

3.4. Cytokine production induced by recombinant chicken IL-17B protein

To clarify the role of chIL-17B, we investigated cytokine expression in HD11, OU2, and CU91 cell lines following treatment with chIL-17B. IL-1β and IL-6 mRNA transcripts were significantly upregulated by chIL-17B in HD11 and OU2 cells, but not in CU91 cells (Fig. 5). The effects of recombinant chIL-17B protein on LITAF and IFN-γ were also examined. Both genes were abundantly expressed in all tested cell lines. In contrast, IL-17A transcript levels decreased after chIL-17B treatment in all cell lines except the CU91 cell line (Fig. 5).

4. Discussion

In this study, we cloned and characterized chicken IL-17B, which is a novel member of the interleukin-17 cytokine family. The chIL-17B coding sequence and its protein share high homology with human IL-17B and mouse IL-17B. They also share eight conserved cysteines, four of which are conserved among the mammalian IL-17 cytokine family, and were demonstrated to be important for the formation of disulfide bonds in IL-17A and IL-17F (Gerhardt et al., 2009; Hymowitz et al., 2001; Zhang et al., 2011).

The expression patterns of IL-17 family members in various diseases differ (Johansen et al., 2009; Pappu et al., 2011; Yamaguchi et al., 2007), and in some cases, they can enhance or even inhibit one other (Reynolds et al., 2015); for example, chicken IL-17A gene expression was decreased in chIL-17B-treated OU2 and HD11 cells (Fig. 5). ChIL-17A mRNA was downregulated in some S.E.-challenged organs (liver and ovary) and cell lines following

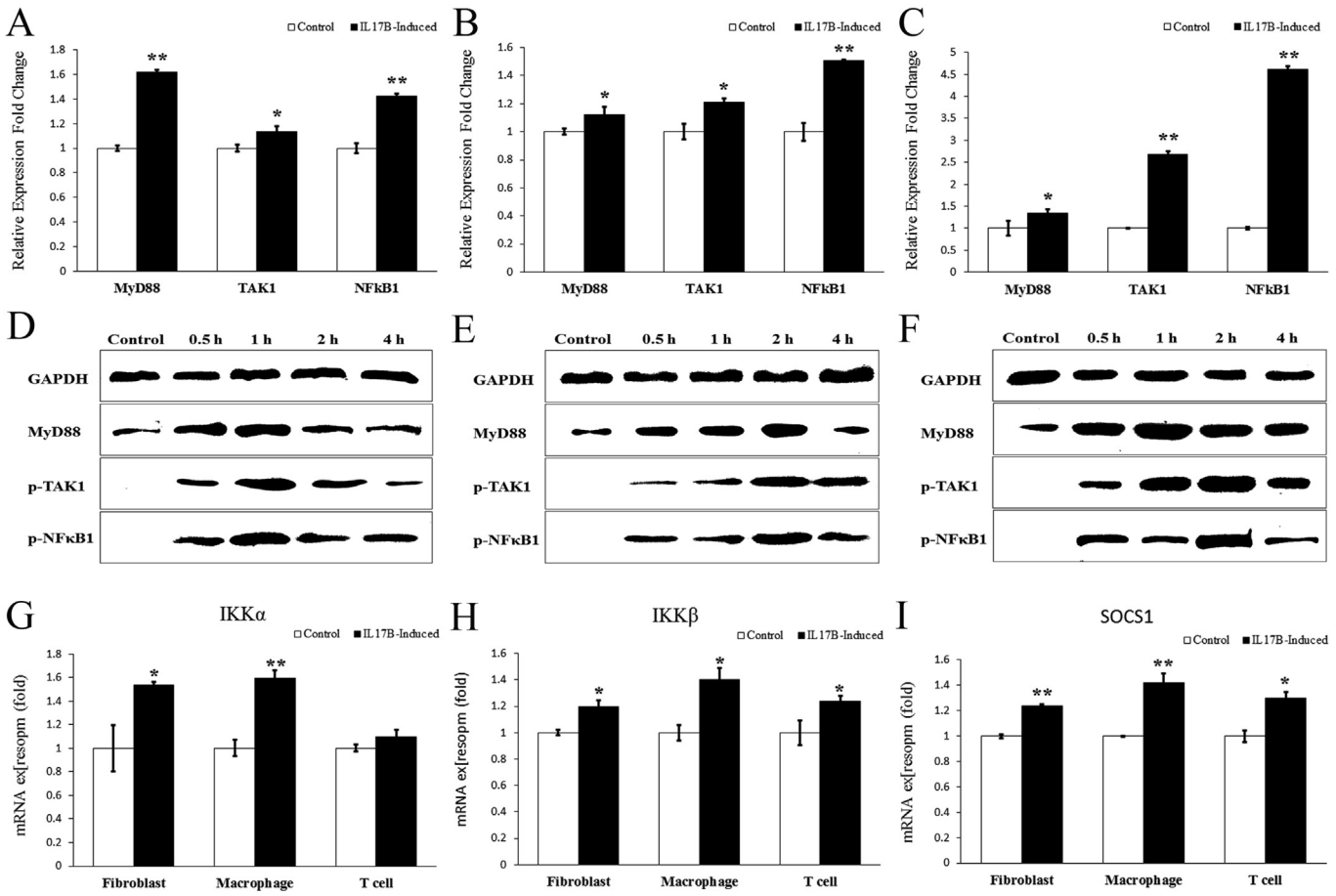

Fig. 3. The NF-κB pathway is activated by chIL-17B in chicken cell lines. The expression of MyD88, TAK1, and NFκB1 mRNA was measured in HD11 (A), OU2 (B), and CU91 cells (C). Western blotting of recombinant chicken IL-17B protein (100 ng/mL)-stimulated HD11 (D), OU2 (E), and CU91 cells (F) was performed using GAPDH, MyD88, p-TAK1, and p-NFκB1 antibodies at 0.5, 1, 2, and 4 h. The expression of two kinase subunits, IKKα (G) and IKKβ (H), and SOCS1 (I) expression was observed in chicken cell lines. Data are the mean (±SEM) of three replicate experiments. Significant differences between treated and untreated (control) cells are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$.

recombinant chIL-17B protein treatment, which might be a competitive effect due to competition for the same receptor. This is similar to the previous report that IL-17B is an antagonist of IL-17E-mediated functions (Reynolds et al., 2015). Although there are numerous studies on other IL-17 family members (e.g., IL-17A and IL-17F) in disease models and other species, only a few mammalian studies have included IL-17B, and there have been no studies in chicken. In the present study, we found that IL-17B was significantly expressed in numerous organs of S.E.-challenged chickens, including the lung, liver, and ovary, with the highest expression in the lung. These data are similar to the expression of IL-17B reported in a wide range of mammalian organs (Li et al., 2000; Moore et al., 2002; Yamaguchi et al., 2007).

NF-κB plays a critical regulatory role in the transcription of mammalian immune system components in response to different stimuli, such as, cytokines, chemokines, and growth factors (Liu et al., 2002). In mammals, the NFκB family has five members, p65 (RelA), RelB, c-Rel, p50/105 (NFκB1), and p52/p100 (NFκB2) (Hayden and Ghosh, 2011). The results of our study clearly indicate that the major pathway utilized by chIL-17B is the NF-κB pathway, as evidenced by the rapid upregulation of phospho-TAK1 and phospho-NFkB1 (30 min post-stimulation) in all tested cell lines. The NF-κB signaling pathway is activated two ways, the canonical pathway, which is activated by a variety of inflammatory signals, including microbial products and proinflammatory cytokines such as TNFα, IL-1β, and LPS, leading to activation of the RelA/p50 (NFκB1) complex; and the alternative pathway with non-canonical NF-κB, which activates RelB/p52 complexes through LTβR, BAFF, and CD40L (Bonizzi and Karin, 2004; Hayden and Ghosh, 2014; Sun, 2012). The NF-κB pathway is characterized by the IKK complex, which consists of two kinase subunits, IKKα and IKKβ (Senftleben et al., 2001; Sun, 2012; Zandi et al., 1997), and both IKKα and IKKβ were upregulated following stimulation with recombinant chIL-17B protein in the tested macrophage and fibroblast cell lines, whereas IKKα was not upregulated in the T cell line (Fig. 3G). IKKβ expression is associated with activation of the canonical pathway in chicken. TGFβ activated kinase 1 (TAK1) mediates signal transduction and controls a variety of cell functions, including transcriptional regulation and apoptosis. Its biological function is the phosphorylation of IKKβ, and its enzymatic activity is increased through the signal transducer TRAF6, leading to NF-κB activation (Wang et al., 2001), which was confirmed in our study (Fig. 3). Furthermore, MyD88 can regulate the NF-κB signaling pathway through activation of the IRAKs-TRAF6-TAK1-IKKα/β pathway (Kawai and Akira, 2007).

The SOCS family plays an important role as a negative-feedback regulator of many cytokine signaling systems, and SOCS1 was shown to be expressed following exposure to pathogens and

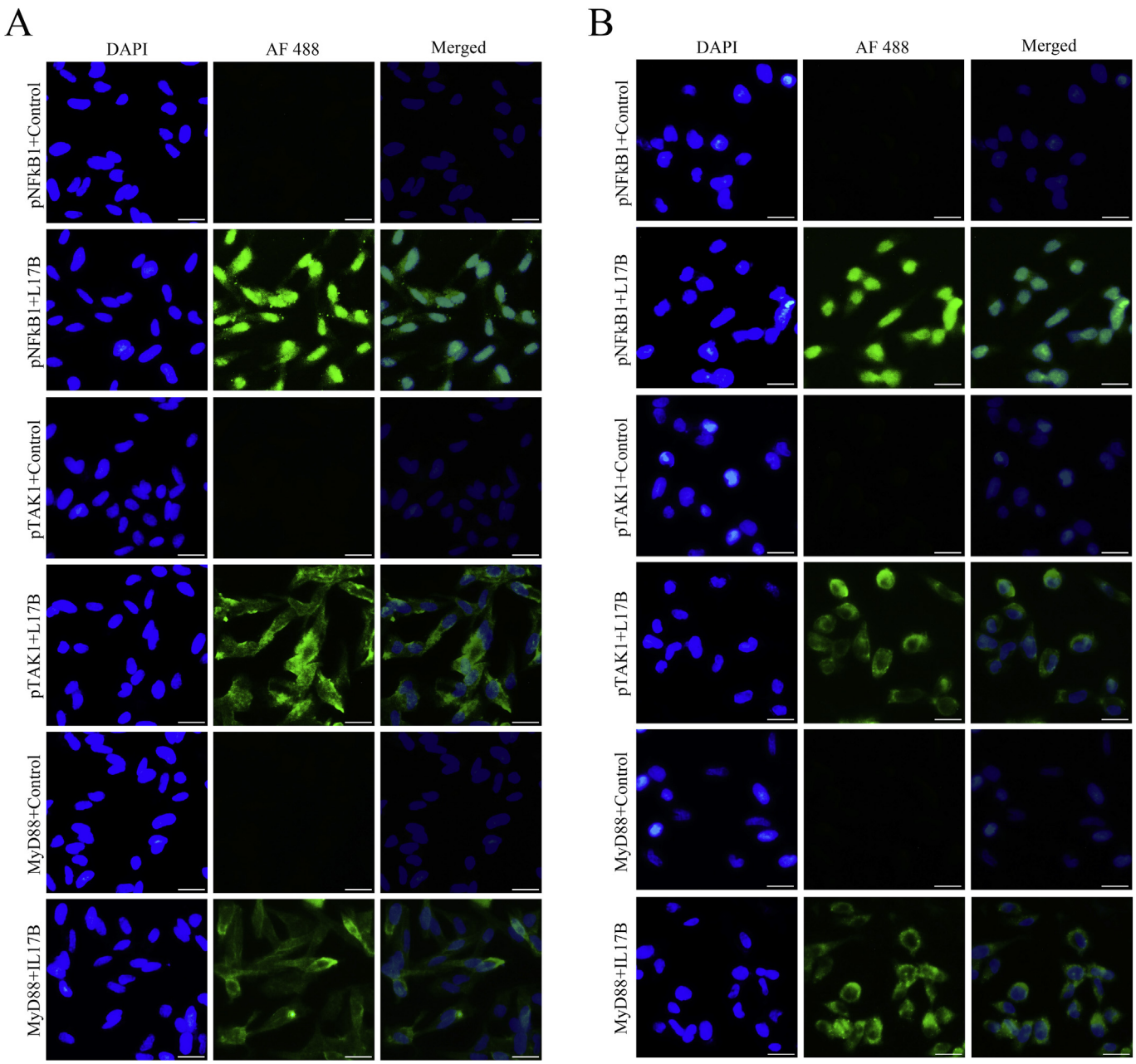

Fig. 4. Immunocytochemical staining of MyD88, p-TAK1, and p-NFκB1 signaling proteins in the HD11 (A) and OU2 (B) cell lines. Both untreated control and recombinant chIL-17B-treated cells were incubated with specific primary antibodies and stained with Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG (H + L) secondary antibody and DAPI (blue). Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proinflammatory cytokines (Greenhalgh and Hilton, 2001). In our study, SOCS1 mRNA was significantly upregulated in all tested cell lines (HD11, OU2, and CU91) following treatment with recombinant chicken IL-17B protein. These data indicated that SOCS1 mRNA expression is regulated by chIL-17B and following NF-κB signaling pathway activation.

Previously, several groups reported that IL-17B stimulates cells to produce inflammatory cytokines, such as TNFα and IL-1β in macrophages, fibroblasts, and THP-1 cell line (Senftleben et al., 2001; Yamaguchi et al., 2007), and IL-6 in macrophages (Yamaguchi et al., 2007). Here, we also showed that the mRNA expression of IL-1β, IL6, IFN-γ, and LITAF was significantly upregulated in HD11, OU2, and CU91 cell lines after treatment with chIL-17B protein.

In conclusion, we cloned the chicken IL-17B gene, analyzed its structure, examined its expression patterns, and performed a functional analysis in chicken macrophages, fibroblasts, and T cells. We demonstrated that chIL-17B is an important cytokine in the host defense against the bacterial pathogens, functioning as a regulator of proinflammatory cytokine production and the NF-κB pathway in chicken macrophage, fibroblast, and T cell lines.

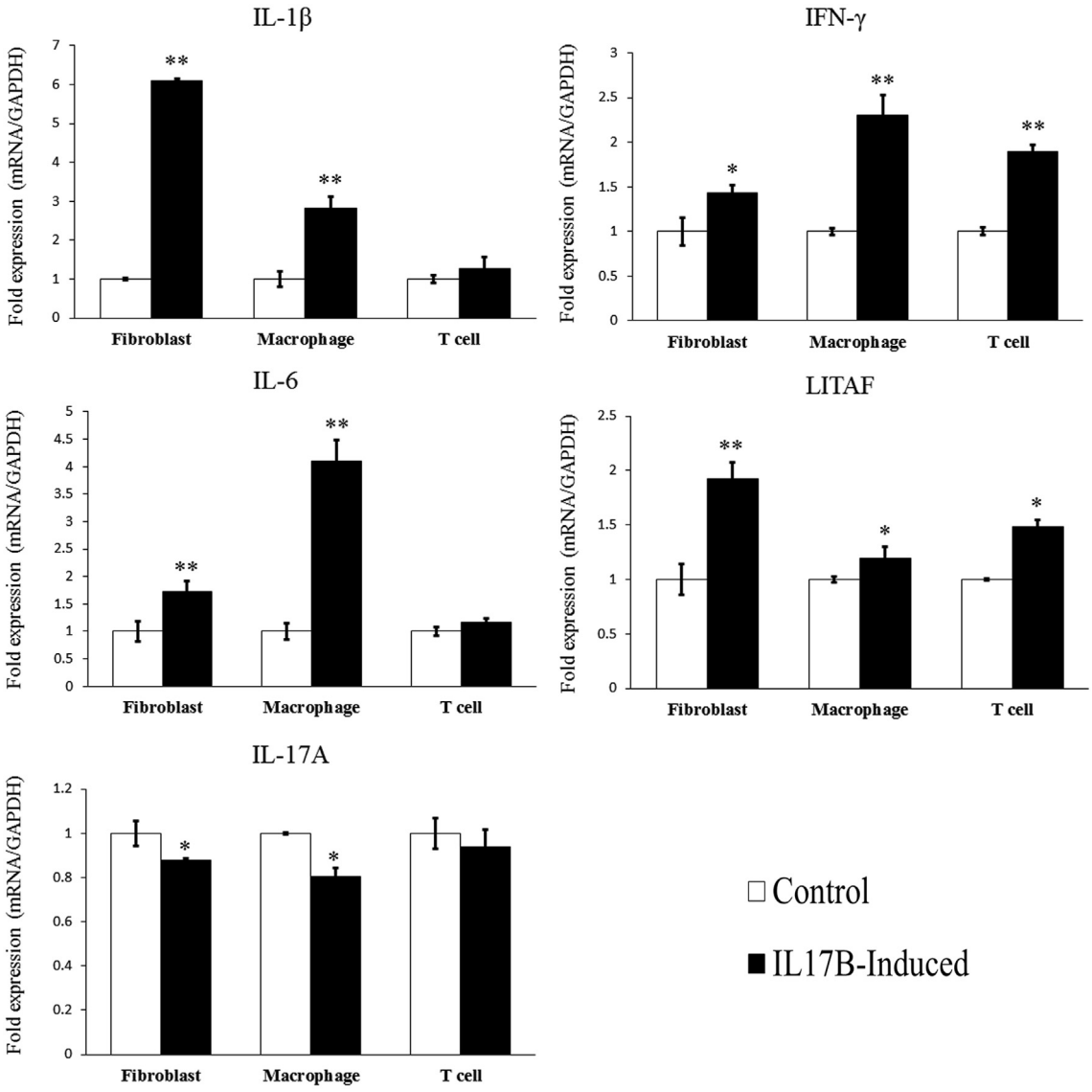

Fig. 5. Cytokine expression induced by recombinant chIL-17B protein in HD11 cells at 1 h and in OU2 and CU91 cells at 2 h. The significance of differences in mRNA expression levels between control (untreated) and IL-17B-treated cells is indicated. Data are the mean (±SEM) of three replicate experiments: $^{*}p < 0.05$, $^{**}p < 0.01$.

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Abbreviations

NFκB	Nuclear factor-kappaB
SOCS	suppressor of cytokine signaling
IL	interleukin
TNF	tumor necrosis factor
LITAF	lipopolysaccharide-induced TNFα factor
S.E.	*Salmonella enterica* serovar Enteritidis
LPS	lipopolysaccharide
IPTG	isopropyl β-D-1-thiogalactopyranoside
IKK	inhibitor of nuclear factor kappa-B kinase
TRAF	TNF receptor associated factor
TAK1	transforming growth factor-beta activated kinase 1
BAFF	B-cell-activating factor belonging to the TNF family
LTβR	Lymphotoxin beta receptor
CD40L	CD40 ligand.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2017.04.010.

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