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Chicken IL-17F: Identification and comparative expression analysis in *Eimeria*-infected chickens

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ABSTRACT

Interleukin-17F (IL-17F) is a proinflammatory cytokine, which plays an important role in gut homeostasis. A full-length chicken IL-17F (chIL-17F) cDNA with a 510-bp coding region was identified from ConA-activated chicken splenic lymphocytes. ChIL-17F shares 53% amino acid sequence identity with the previously described chicken IL-17 (chIL-17A) and 38–43% with mammalian homologues. The locus harboring chIL-17 and chIL-17F displayed inverted order compared to those of mammals. ChIL-17F transcript expression was high in lymphoblast cell line CU205 and at moderate levels in small and large intestines and liver. ChIL-17F and chIL-17 expression profiles were examined by quantitative real-time RT-PCR in mitogen-stimulated splenic lymphocytes and intestinal areas affected by *Eimeria maxima* and *Eimeria tenella* infections. Expression levels of chIL-17F, like chIL-17, were elevated in mitogen-activated splenic lymphocytes. ChIL-17F, but not chIL-17, expression was upregulated in intestinal tissues affected by *E. maxima* and *E. tenella* infections. Recombinant chIL-17F biological activities were similar to that of chIL-17 in primary chicken embryonic fibroblasts. These results suggest that chIL-17F is a unique member of the IL-17 family of cytokines.

1. Introduction

The IL-17 family consists of six similar cytokine members designated IL-17A (or IL-17), IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. The IL-17A and IL-17F genes are located in opposite directions at the same chromosomal locus and share high amino acid sequence identity (approximately 50%) (Moseley et al., 2003; Weaver et al., 2007). Both IL-17A and IL-17F can be produced as disulfide-linked homodimers or heterodimers and may bind the same receptor. Thus, these two cytokines are involved in a broad range of cellular activities against infection/inflammation by inducing the production of proinflammatory cytokines (IL-1, IL-6, GM-CSF, G-CSF), chemokines (CXCL1, CXCL2, CXCL5, IL-8), antimicrobial peptides (mucins, β-defensins), and matrix metalloproteinases (MMP1, MMP3, MMP13) by a large variety of cells. IL-17A and IL-17F also recruit neutrophils to inflammatory sites (Weaver et al., 2007; Iwakura et al., 2011; Hot and Miossec, 2011; Reynolds et al., 2010). Although IL-17A and IL-17F likely have similar biological activities (Moseley et al., 2003; Hirahara et al., 2010; Iwakura et al., 2011), different functions have been suggested for these closely related cytokines in host immune responses and defense mechanisms (Yang et al., 2008; Ishigame et al., 2009; Reynolds et al., 2010).

IL-17F was initially identified in the human genomic sequence using IL-17A sequence, which is composed of three exons and two introns, resulting in a protein containing 163 amino acids (Hymowitz et al., 2001; Korenaga et al., 2010). IL-17F, like IL-17A, is produced by $CD4^+$ T cells, $CD8^+$ T cells, NK cells, γδ T cells, and neutrophils (Weaver et al., 2007; Iwakura et al., 2011). However, IL-17F expression in activated basophils, activated mast cells, and ragweed allergen-specific T cell clones with different cytokine profiles (Th1 and Th2) was unique when compared with IL-17A expression. Additionally, IL-17F expression differed between various tissues, such as liver, lung, ovary, and fetal liver (Kawaguchi et al., 2001, 2003), suggesting that IL-17F is produced by a wider range of cell types and tissues and is involved in more biological activities than is IL-17A. IL-17F inhibits the angiogenesis of endothelial cells and hepatocellular carcinoma (Starnes et al., 2001; Xie et al., 2010). Defective expression of IL-17F has been implicated

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in the pathogenesis of asthma and chronic obstructive pulmonary diseases (Hizawa et al., 2006; Kawaguchi et al., 2006), chronic intestinal inflammation (Leppkes et al., 2009), and psoriasis (Fujishima et al., 2010).

The avian immune system provides an important model for the study of basic and applied immunology. Despite the general lack of cross-reactivity and the low level of sequence conservation between avian and mammalian cytokines, many chicken genes are homologous to their mammalian counterparts (Staeheli et al., 2001; Min and Lillehoj, 2002, 2004). Within the IL-17 family in chickens, only IL-17 and IL-17D have been identified in an expressed sequence tag (EST) cDNA library prepared from intestinal intraepithelial lymphocytes (IELs) of chickens infected with *Eimeria* (Min and Lillehoj, 2002) and from a testis cDNA library prepared from Korean native chickens (Hong et al., 2008), respectively. Thus, it is difficult to study any relationship and comparative analysis between chIL-17 and chIL-17F which is remained to be identified. In this study, we identified a full-length cDNA encoding a chicken homologue of mammalian IL-17F. Through real-time RT-PCR and Western blot analysis, the tissue distribution of chIL-17F transcripts and the chIL-17F molecular weight were analyzed. Furthermore, real-time RT-PCR was used to evaluate expression profiles of chIL-17F and chIL-17 in mitogen-stimulated splenic lymphocytes and intestinal tissues from chickens infected with two different *Eimeria* species, *Eimeria tenella* and *Eimeria maxima*, that preferentially infect the cecum and jejunum, respectively.

2. Materials and methods

2.1. Animals and infections

Eggs from ROSS chickens were obtained from Samhwa (Chungnam, Korea) and hatched at the Gyeongsang National University. Chickens were given unlimited access to feed and water. Constant light was provided for the duration of the experiments. Ten-day-old chickens were orally infected with 1×10^4 sporulated *E. tenella* (Korean isolate 291-7) (Jeong et al., 2012) or *E. maxima* (Korean isolate 291-3) (Yim et al., 2011) oocysts and transferred to disposable cages (Yoo et al., 2011). *Eimeria* spp. were cleaned by flotation on 5.25% sodium hypochlorite and washed three times with PBS. Control chickens were inoculated with the same volume of PBS. Intestinal samples were collected on days 0, 1, 2, 4, 7, and 10 post-infection. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Gyeongsang National University, Jinju, Republic of Korea.

2.2. Cloning of chIL-17F

Chicken spleens were gently passed through a cell strainer (SPL Life Sciences, Korea) with a syringe plunger to obtain single-cell suspensions in Hank's balanced salt solution (HBSS) (Sigma–Aldrich, USA). Total RNA was extracted from ConA-activated splenic lymphocytes using RiboEx reagent (Geneall, Korea) and treated with RNase-free DNase I (Fermentas, Canada). Single-stranded cDNA was synthesized from total RNA using oligo dT primers and a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Germany).

Based on a partial cDNA sequence (http://www.ensembl.org/; accession number: ENSGALT00000026912), 5′/3′-Rapid Amplification of cDNA Ends (RACE) was performed using chIL-17F-specific primers (for 5′ RACE, 5′-GCCGAGGACGAACTACCTTC-3′; for 3′ RACE, 5′-AGACTGCCTGAACCAAAAGGAT-3′) with splenic lymphocyte cDNA, a high-fidelity DNA polymerase (Bioneer, Korea) and a 5′/3′ RACE kit (5′/3′ RACE 2nd Generation; Roche Applied Science) according to the manufacturers' instructions. PCR products were cloned into TA vectors (RBC, Taiwan) and sequenced (Macrogen, Korea). PCR was performed on a DNA Engine thermocycler (Bio-Rad, USA) as follows: 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final 5 min extension at 72 °C. The cDNA sequence was submitted to GenBank with accession number JQ776598.

2.3. Sequence analysis

Protein identification was conducted using the Expert Protein Analysis System (ExPASy; http://www.expasy.org/tools/). The signal peptide sequence was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP). Amino-acid multiple alignments were generated using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/). Homology analysis was performed using MatGat software (Campanella et al., 2003). Phylogenetic trees were created using the neighbor-joining method with the MEGA program version 5 (Tamura et al., 2011) and were bootstrapped 10,000 times.

2.4. Cell culture

The chicken macrophage cell line HD11 (Beug et al., 1979), the reticuloendotheliosis virus (REV)-transformed chicken lymphoblast cell lines CU205 and CU91 (Schat et al., 1992), and splenic lymphocytes obtained from 3-week-old chickens were cultured in Dulbecco's modified eagle's medium (DMEM) (Hyclone, USA) supplemented with 10% FBS and penicillin/streptomycin (10,000 unit/ml) (Hyclone) at 41 °C in 5% CO_2. Splenic lymphocytes and HD11 cells were resuspended to 5×10^6 cells/ml and stimulated with 25 μg/ml poly I:C, 10 μg/ml lipopolysaccharide (LPS from *Escherichia coli*, 0111:B4), 10 μg/ml lipoteichoic acid (LTA) (all Sigma–Aldrich) or 10 μg/ml concanavalin (ConA) (Amersham Bioscience, Sweden).

2.5. Production and biological activity of recombinant chIL-17F and chIL-17

Full-length chIL-17F and chIL-17 cDNAs harboring influenza virus hemagglutinin (HA) were amplified by PCR from single-stranded cDNA from splenic lymphocytes using the following primers: chIL-17-HA: 5′-GATCGAATTCGCAATGTCTCCGATCCCTTATTCTCC-3′ and 5′-GATCTCTAGATTAAGCGTAATCTGGAACATCGTATGGGTA AGCCTGGTGCTGGATCAGT-3′ and chIL-17F-HA: 5′-GATCGAATTC GCAATGGCTTTTGCTAGCTGTGCTG-3′ and 5′-GATCTCTAGACTA AGCGTAATCTGGAACATCGTATGGGTAGGACTGGTGCTGGATGACC-3′. Primers contained *Eco* RI and *Xba* I restriction enzyme sites (single underline) and HA sequences (dashed underline). PCR products were digested with *Eco* RI and *Xba* I and cloned into the corresponding restriction sites of pcDNA 3.1 (Invitrogen, USA). Cells were transiently transfected into COS-7 cells with 10 μg of constructs or empty vector (negative control) using Lipofectamine Reagent (Invitrogen) following the manufacturer's instructions. Growth media were replaced with serum-free media 5 h post-transfection. Cells were incubated for an additional 48 h in serum-free DMEM at 37 °C in 5% CO_2.

To assay for biological activities, primary chicken embryonic fibroblasts (CEFs) were obtained from 11-day-old embryos and cultured as described above. Serial dilutions of the supernatants from transfected COS-7 cells were added to CEFs followed by incubation for 6 h at 41 °C. Total RNA was extracted from the stimulated cells and cDNA synthesis was performed using random hexamer primers. Analysis of chIL-17- and chIL-17F-induced gene expression was performed using real-time RT-PCR using the primers listed in Table 1.

Table 1
List of primers used in quantitative real-time RT-PCR.

Gene target	Primer and sequence	Efficiency	References
IL-17F	(For) 5′-TGAAGACTGCCTGAACCA-3′ (Rev) 5′-AGAGACCGATTCCTGATGT-3′	1.991	JQ776598
IL-17	(For) 5′-GAGAAGAGTGGTGGGAAAG-3′ (Rev) 5′-TCTACAAACTTGTTTATCAGCAT-3′	2.107	Min and Lillehoj (2002)
IL-6	(For) 5′-CAAGGTGACGGAGGAGGAC-3′ (Rev) 5′-TGGCGAGGAGGGATTTCT-3′	–	Hong et al. (2006a,b)
IL-8	(For) 5′-GGCTTGCTAGGGGAAATGA-3′ (Rev) 5′-AGCTGACTCTGACTAGGAAACTGT-3′	–	Hong et al. (2006a,b)
IL-1β	(For) 5′-TGGGCATCAAGGGCTACA-3′ (Rev) 5′-TCGGGTTGGTTGGTGATG-3′	–	Hong et al. (2006a,b)
TGF-β1	(For) 5′-CGGGACGGATGAGAAGAAC-3′ (Rev) 5′-CGGCCCACGTAGTAAATGAT-3′	–	Hong et al. (2006a,b)
β-Actin	(For) 5′-CACAGATCATGTTTGAGACCTT-3′ (Rev) 5′-CATCACAATACCAGTGGTACG-3′	–	De Boever et al. (2008)

2.6. Western blot analysis

Cell lysates and supernatants were mixed with equal volumes of sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue), heated for 5 min at 95 °C, resolved on 10% SDS–polyacrylamide gels, and electroblotted onto polyvinyl difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with PBS containing 1% nonfat dry milk for 16 h at 4 °C, incubated with monoclonal anti-HA antibody (Sigma–Aldrich) at 4 °C overnight, washed three times with PBS containing 0.05% Tween 20 (PBS-T), and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Promega, USA) in PBS containing 1% nonfat dry milk for 40 min at room temperature. Membranes were washed five times with PBS-T followed by five washes with distilled water, visualized using an Enhanced Chemiluminescence Kit and Western Blotting Detection Reagents (GE Healthcare Life Sciences, USA), and exposed to X-ray film (AGFA, Belgium) as described previously (Jeong et al., 2012).

2.7. Quantitative real-time RT-PCR

Normal tissues, activated splenic lymphocytes, and tissue samples pooled from five chickens infected with *Eimeria* spp. were subjected to real-time RT-PCR analysis in triplicate. cDNA synthesis was performed using random hexamer primers. Real-time RT-PCR was performed on a CFX96 real-time PCR system (Bio-Rad) with SYBR Green (Bioneer) and the primers listed in Table 1. A melting curve was obtained at the end of each run to verify the presence of a single amplification product without primer dimers. Standard curves were generated using serial, 5-fold dilutions of ConA-activated splenic lymphocyte cDNA. The relative expression levels of individual transcripts were normalized to those of β-actin using Bio-Rad CFX software. Gene expression levels were quantified using the comparative ΔCt method with β-actin as a reference for normalization as described previously (Jeong et al., 2012).

2.8. Statistical analysis

Data were analyzed using Student's *t*-tests. Differences were considered significant at $P < 0.05$. Data are expressed as means ± standard error (SE).

3. Results

3.1. Cloning and characterization of chIL-17F cDNA

Full-length chIL-17F cDNA was first cloned from ConA-activated chicken splenic lymphocytes using 5′/3′-RACE based on the sequence of an EST fragment (accession No. ENSGALT00000026912). The chIL-17F cDNA was approximately 0.9 kb and contained a 510-bp open reading frame (ORF) predicted to encode a putative 169-amino acid protein with a 25-amino-acid NH_2-terminal hydrophobic leader sequence and a potential N-linked glycosylation site. The predicted molecular weight was 18.9 kDa (non-glycosylated) and the calculated isoelectric point was 8.75 (accession No. JQ776598). The 3′-UTR contained a single consensus AATAAA polyadenylaytion signal and five AU-rich (ATTTA) sequences shown previously to be involved in regulating mRNA stability in many cytokines, growth factors, and oncogenes (Caput et al., 1986; Shaw and kamen, 1986). In addition, chIL-17F contained the six cysteine residues involved in interchain disulfide bond formation that are conserved among the Th17 family members and the open reading frame 13 of *Herpesvirus saimiri* (HVS 13) (Yao et al., 1995) (Fig. 1A).

Amino acid sequence comparisons using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/) indicated that chIL-17F shared 53% amino acid sequence identity with the previously described chIL-17 and duck IL-17 (Min and Lillehoj, 2002; Yoo et al., 2009). Chicken IL-17F shared 33–43% identity with mammalian homologues and HSV 13, and 37–40% identity with human, mouse, rat, swine and bovine IL-17A (Table 2). Phylogenetic and molecular evolutionary analysis was performed using IL-17A and IL-17F amino acid sequences (Tamura et al., 2011). Interestingly, the phylogenetic tree showed that avian IL-17 and IL-17F form a distinct cluster (Fig. 1B), indicating that IL-17 and IL-17F likely evolved independently in avians and mammals.

A high degree of synteny was found between the chicken and mammalian genomes. However, the analysis of synteny indicated that PKHD1, IL-17A, IL-17F, MCM3, PAQR8, and EFHC1 were present in the same order on human chromosome 6, rat chromosome 9, and mouse chromosome 1 (Moseley et al., 2003; Weaver et al., 2007). Analysis of the chicken genome from the GenBank database indicated that the order of genes was EFHC1, PAQR8, MCM3, IL-17F, IL-17, and PKHD1 on chromosome 3, indicating that the genetic locus, including the synteny, was inverted in chickens compared to mammals.

3.2. Distribution of chIL-17F mRNA in normal tissues and cell lines

The expression of chIL-17F mRNA in various tissues and cell lines was monitored using real-time RT-PCR and was compared to that of chIL-17. The chIL-17F transcripts were widely expressed, albeit at low levels, in most normal tissues compared to chIL-17 transcript expression. However, relatively high levels of chIL-17F transcripts were detected in small and large intestinal tissues and liver (Fig. 2A). Thus, chIL-17F expression levels in intestinal tissues were further examined in detail. As shown in Fig. 2B, chIL-17F

A

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Chicken IL-17    --MSPIPYSPLFRPLLLVLLAMLSASISAHGKVIRPGLEPESLFKKADAGCLTQKDGK 56
HVS 13           --MTFRMTSLV---LLLLLSIDCIVKSE----ITSAQ----------TPRCLAANN-S 38
Human IL-17F     MTVKTLHGPAMVKYLLLSILGLAFLSEAAARKIPKVGHT----FFQKPESCPPVPG-- 49
Rat IL-17F       ----------MVKSLLLLMLGFAIISSGAARRNPKVGLS----ALQKAGNCPPLED-- 42
Mouse IL-17F     --MKCTRETAMVKSLLLLMLGLAILREVAARKNPKAGVP----ALQKAGNCPPLED-- 50
Chicken IL-17F   --MAFASCAAVFRSLLLVLVLALTVWSSPHGKVVRPRPRKDGGSEKLSEDCLNQKDPS 56
                               ***                                 *

Chicken IL-17    FPQTVRVNISISN---MNQDTKVTLDISKRSLAPWDYRIDEDHNRFPRLVADAQCRHS 111
HVS 13           FPRSVMVTLSIRN-WNTSS--KRASDYYNRSTSPWTLHRNEDQDRYPSVIWEAKCRYL 93
Human IL-17F     --GSMKLDIGIIN---ENQRVSMSRNIESRSTSPWNYTVTWDPNRYPSEVVQAQCRNL 105
Rat IL-17F       --NSVRVDIRIFN---QNQGISVPRDFQNRSSSPWDYNITRDPDRFPSEIAEAQCRHS 95
Mouse IL-17F     --NTVRVDIRIFN---QNQGISVPREFQNRSSSPWDYNITRDPHRFPSEIAEAQCRHS 103
Chicken IL-17F   FPTMVKVDIRIGS---SDPASRMIHDIRNRSLAPWNYRLDEDPNRFPQVIADAECRLL 111
                                          **  **      *  * *     * **

Chicken IL-17    RCVNSAGQLDHSVNSVPIKQEILVLRREPKGCQHSYRLEKKMITVGCTCVTPLIQHQA 169
HVS 13           GCVNADGNVDYHMNSVPIQQEILVVRKGHQPCPNSFRLEKMLVTVGCTCVTPIVHNVD 151
Human IL-17F     GCINAQGKEDISMNSVPIQQETLVVRRKHQGCSVSFQLEKVLVTVGCTCVTPVIHHVQ 163
Rat IL-17F       GCINAQGQEDGSMNSVPIQQEILVLRREPQGCSNSFRLEKMLIKVGCTCVTPIVHHAA 153
Mouse IL-17F     GCINAQGQEDSTMNSVAIQQEILVLRREPQGCSNSFRLEKMLLKVGCTCVKPIVHQAA 161
Chicken IL-17F   GCLNSLGQEDRSLNSVPITQEILVLRREQRGCQPTYHLEKKLITVGCTCAAPVIQHQS 169
                  * *     *   *** * ** **       *       **    *****
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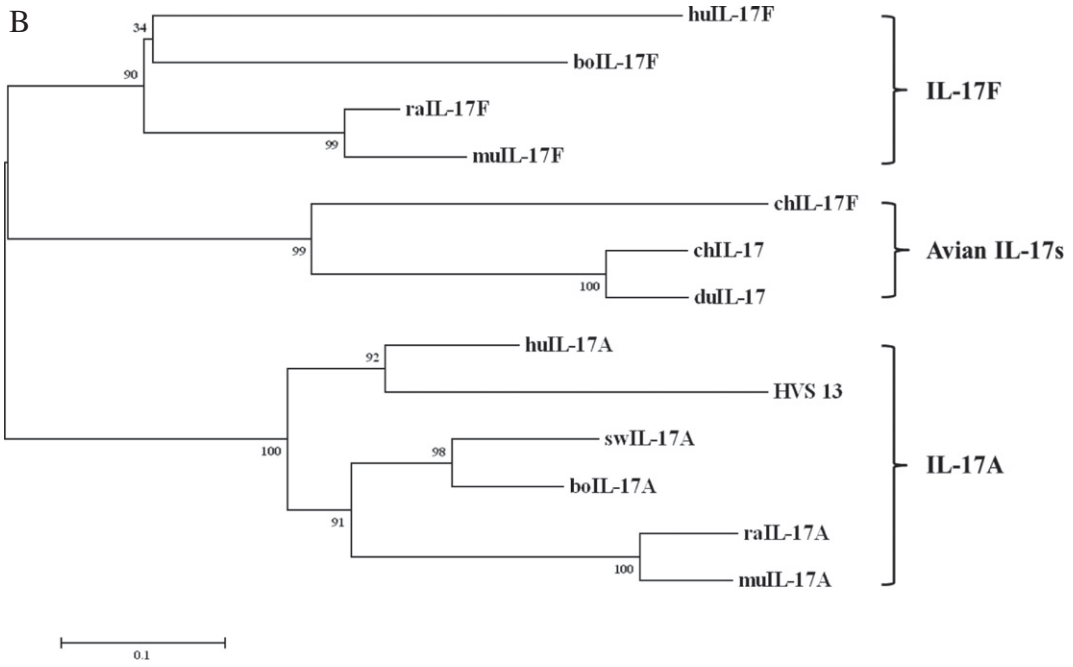

Fig. 1. Molecular features of chicken IL-17F. (A) Multiple alignment of HVS 13 and chicken IL-17 and IL-17F deduced amino acid sequences. Sequences were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Asterisks (*) indicate identical residues among sequences. The six conserved cysteine residues are highlighted by black boxes. HVS 13 indicates the open reading frame 13 of *Herpesvirus saimiri*. (B) Phylogenetic tree of avian and mammalian IL-17A and IL-17F. The tree was constructed by the neighbor-joining method using amino acid sequences aligned with ClustalW2 and was bootstrapped 10,000 times. Branch lengths represent units of substitutions per site of the sequence alignment. The GenBank accession numbers used in the comparison are AAC50341 (human IL-17A), CAA45636 (HVS 13), NP_001100367 (rat IL-17A), AAB05222 (mouse IL-17A), CAD38489 (chicken IL-17), AAQ03220 (bovine IL-17A), NP_443104 (human IL-17F), NP_001015011 (rat IL-17F), NP_665855 (mouse IL-17F), NP_001179011 (bovine IL-17F), BAD52431 (swine IL-17), ABY68458 (duck IL-17), and JQ776598 (chicken IL-17F).

transcripts were highly expressed in all intestinal tissues, including duodenum, jejunum, ileum, cecum, and colorectum, indicating that intestinal tissues preferentially express chIL-17F compared to other tissues. Of the three cell lines, only CU205 expressed high levels of chIL-17F transcripts at levels similar to chIL-17 (Fig. 2C).

Table 2
Percentage of amino acid identities of HVS 13 and avian and mammalian IL-17A and IL-17F.

IL-17 (IL-17A)							IL-17F					HVS13
Human	Mouse	Rat	Bovine	Swine	Chicken	Duck	Human	Mouse	Rat	Bovine	Chicken	
40.9	37.8	37.8	38.5	38.5	53.8	53.8	38.2	39.6	43.2	38.7	100	36.1
42.1	38.4	38.4	42.0	44.4	100	84.0	39.2	43.8	47.9	37.4	53.8	38.5

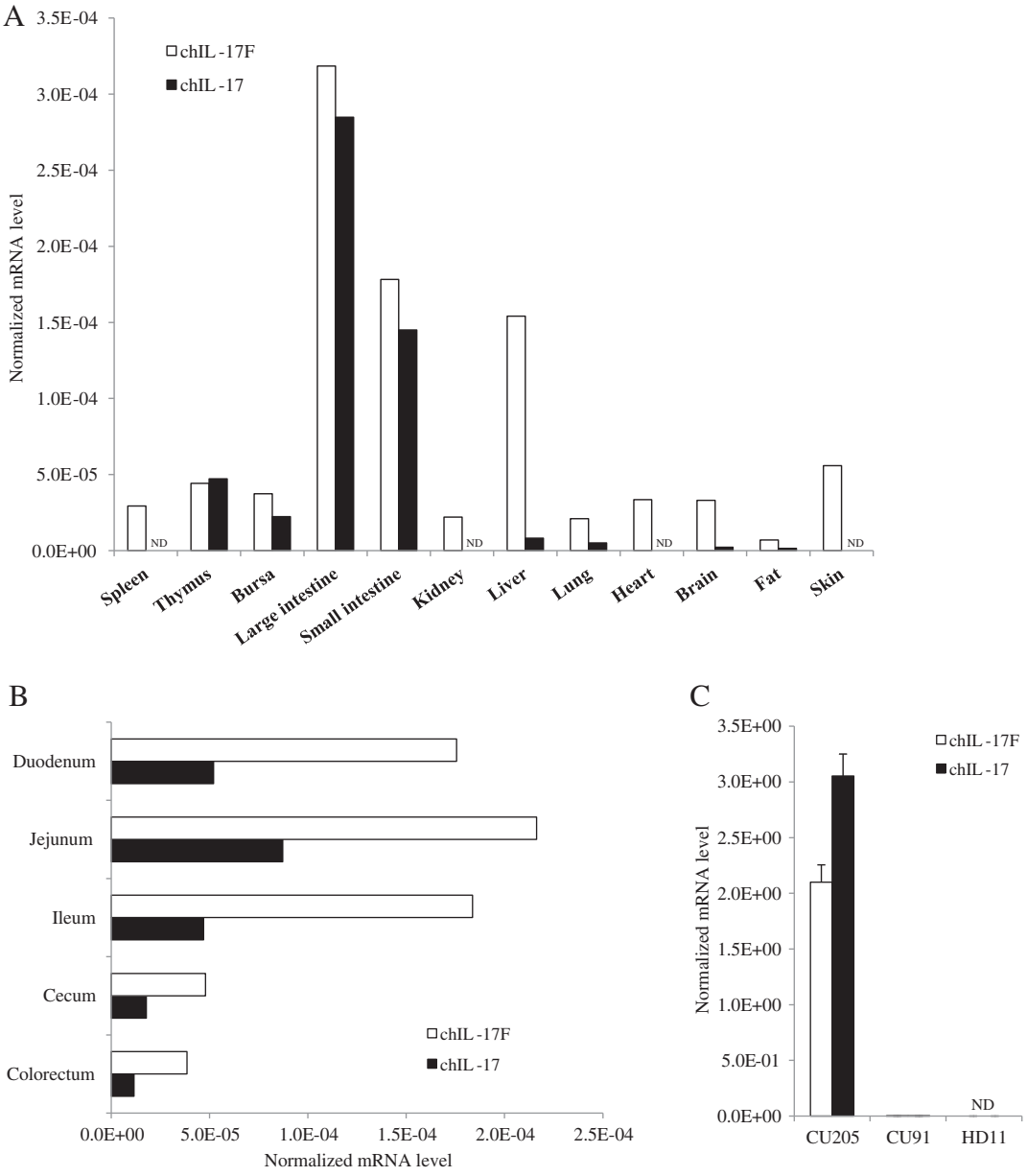

Fig. 2. Distribution of chIL-17F mRNA in normal tissues and cell lines. Total RNA was isolated from various tissues from 2 or 3-week-old chickens and analyzed using real-time RT-PCR. Tissue samples (A, B) were pooled from five chickens and subjected to real-time RT-PCR analysis in triplicate. (C) Data from cell lines represent means ± standard error of triplicate samples. Expression levels were normalized to those of β-actin. Data are representative of two independent experiments with similar pattern results. CU205 and CU91, REV-transformed lymphoblast cell lines; HD11, macrophage cell line; ND, not detected.

3.3. Molecular weight and biological activity of chIL-17F

Recombinant chIL-17 and chIL-17F were expressed with a HA tag in COS-7 cells to allow detection by Western blot using an anti-HA mouse antibody. Molecular weight bands at 20.5 kDa were detected for chIL-17F from both culture supernatants and cell lysates. The molecular weight of chIL-17F was very similar to that of chIL-17 (Fig. 3A). To assay the biological activity of recombinant chIL-17F on the production of cytokines in primary CEFs, CEFs were incubated with serial dilutions of supernatants from COS-7 cells transfected with chIL-17F for 48 h. ChIL-17F and chIL-17 treatments led to increased expression of IL-1β, IL-6, and IL-8, but not

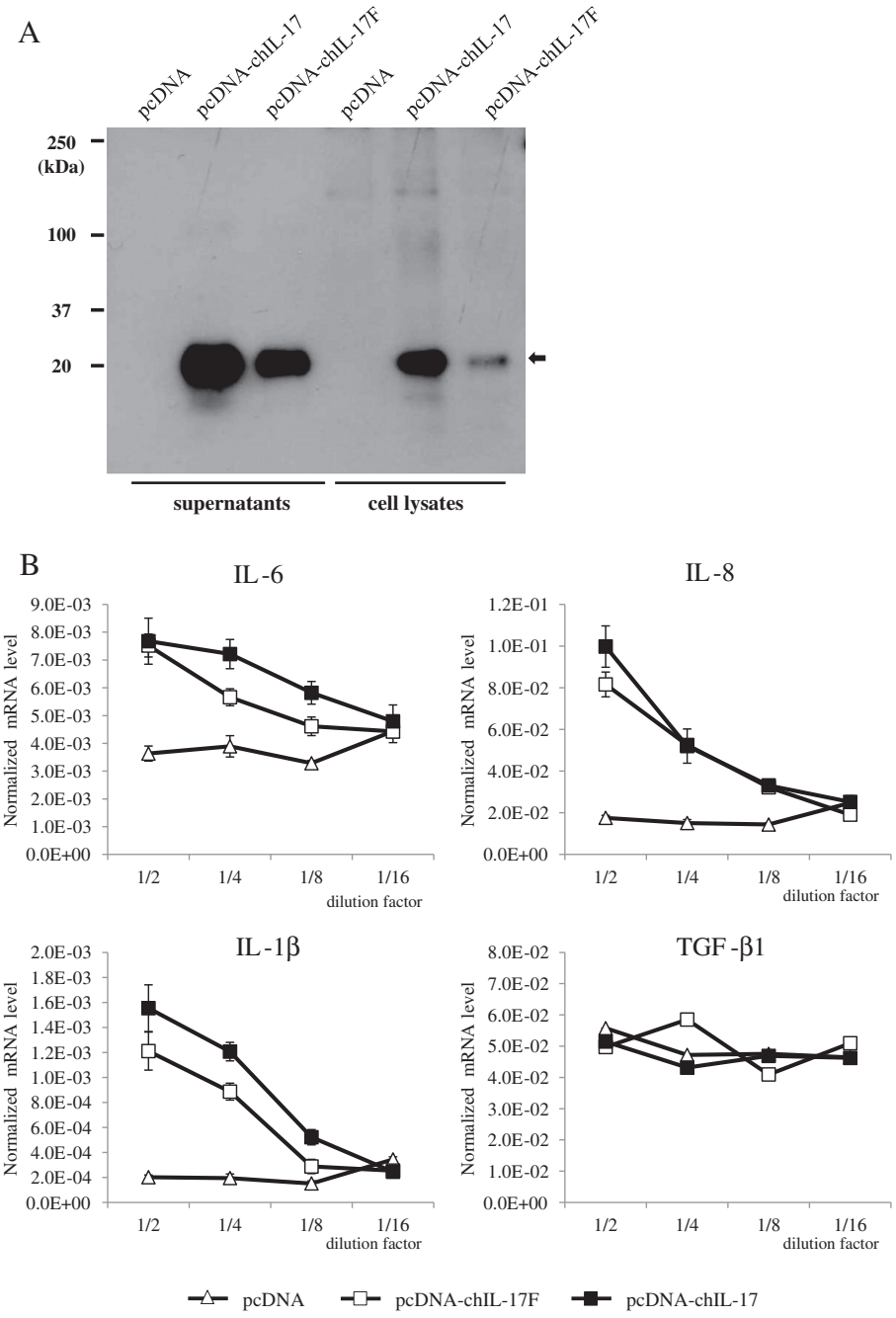

Fig. 3. Biological activity and molecular weight of chIL-17F. (A) Detection of chIL-17F protein by Western blot analysis. Supernatants and whole-cell lysates from COS-7 cells were collected 48 h after transient transfection with the chIL-17-HA or chIL-17F-HA constructs or empty pcDNA 3.1. Samples were separated by SDS–PAGE under reducing conditions. Arrow indicates specific bands. Data are representative of three independent experiments with similar pattern results. (B) Biological activity of chIL-17F. Primary chicken embryonic fibroblasts (CEFs) were obtained from 11-day-old embryos, stimulated with serial dilutions of the conditioned medium from COS-7 cells transfected with the chIL-17 construct (■), the chIL-17F construct (□), or empty vector (Δ) for 6 h, and analyzed by real-time RT-PCR. Expression levels of the indicated cytokines were normalized to those of β-actin. Data represent means ± standard error of triplicate samples and are representative of two independent experiments with similar pattern results.

TGF-β1 (also designated TGF-β4) (Pan and Halper, 2003; Halper et al., 2004) (Fig. 3B).

3.4. Quantitative analysis of chIL-17F mRNA expression in mitogen-activated splenic lymphocytes and intestinal tissues of Eimeria-infected chickens

Using real-time RT-PCR, expression profiles of chIL-17F and chIL-17 were determined in ConA-, LPS-, LTA-, and poly I:C-stimulated splenic lymphocytes (Fig. 4A) and in intestinal tissues of chickens infected with *E. maxima* (Fig. 4B) or *E. tenella* (Fig. 4C). As shown in Fig. 4A, chIL-17F mRNA levels generally increased in a time-dependent manner and peaked 24 h after mitogen stimulation. Compared with the expression levels of chIL-17, chIL-17F mRNA transcripts were relatively high after 24 h in LPS-, LTA-, and poly I:C-stimulated splenic lymphocytes, but not in ConA-stimulated samples. Expression levels of chIL-17 were significantly elevated in all mitogen-activated splenic lymphocytes compared to normal splenic lymphocytes, except 8 h after poly I:C stimulation. Compared with 4 and 8 h after poly I:C stimulation, chIL-17F tran-

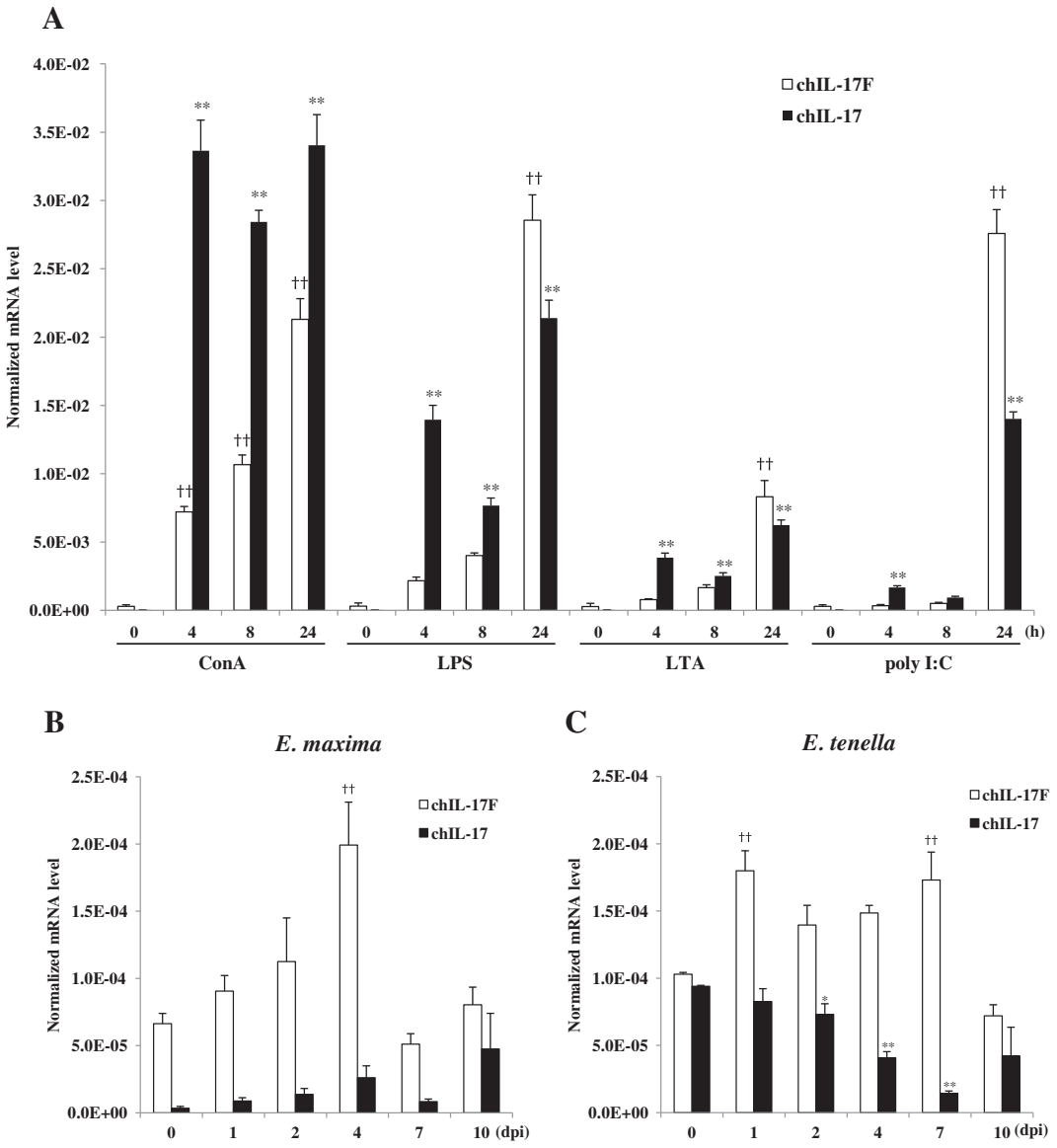

Fig. 4. mRNA expression profiles of chIL-17F in mitogen-stimulated splenic lymphocytes and *Eimeria*-infected chickens. (A) Splenic lymphocytes were isolated from 3-week-old chickens, activated with 10 μg/ml ConA, 10 μg/ml LPS, 10 μg/ml LTA, or 25 μg/ml Poly I:C for the indicated times and analyzed by real-time RT-PCR. Expression levels were normalized to those of β-actin. Data represent means ± standard error of triplicate samples and are representative of two independent experiments with similar pattern results. ** and †† indicate significant differences ($P < 0.01$) as compared to chIL-17 and chIL-17F levels, respectively, from untreated lymphocytes. (B, C) Ten-day-old chickens were orally infected with 1×10^4 sporulated *E. tenella* or *E. maxima* oocysts. Intestinal tissues (duodenum for *E. maxima* and cecum for *E. tenella*) were collected on days 0, 1, 2, 4, 7, and 10 after infection. Tissue samples were pooled from five chickens and subjected to real-time RT-PCR analysis in triplicate. Expression levels were normalized to those of β-actin. Data are representative of two independent experiments with similar pattern results. * and † indicate significant differences ($P < 0.05$) as compared to chIL-17 and chIL-17F levels, respectively, from uninfected chickens. *$P < 0.05$, **, ††$P < 0.01$. dpi = Days post-infection.

script levels 24 h after poly I:C stimulation were approximately 82 and 53 times higher, respectively. Thus, chIL-17F expression may be preferentially regulated indirectly by other factors rather than by the specific stimulator.

ChIL-17F transcripts were highly expressed in normal intestinal tissues (Fig. 2A and B), and IL-17F has been implicated in intestinal inflammation (Seiderer et al., 2008; Leppkes et al., 2009). Thus, chIL-17F transcript levels were monitored in intestinal tissues of *Eimeria*-infected chickens. In *E. maxima*- and *E. tenella*-infected chickens (Fig. 4B and C), chIL-17F transcript levels were up-regulated or unchanged in both jejunum and cecum compared to those from uninfected chickens. However, chIL-17 mRNA expression was unchanged or down-regulated compared to that of uninfected chickens.

4. Discussion

IL-17F, like IL-17A, is a proinflammatory cytokine and is categorized in the Th17 lineage. Here, a full-length cDNA encoding the chicken homologue of mammalian IL-17F was cloned. Like chIL-17 that we previously cloned (Min and Lillehoj, 2002), chIL-17F induced production of other cytokines and chemokines such as IL-1β, IL-6, and IL-8. However, chIL-17F transcripts were widely detected in various normal tissues compared to chIL-17 transcripts. Additionally, chIL-17F transcripts were highly expressed in intestinal tissues of chickens infected with two different *Eimeria* species, *E. tenella* and *E. maxima*. ChIL-17 transcript expression was unchanged or down-regulated under these same conditions. Interestingly, synteny analysis indicated that the genetic locus harboring

IL-17 and IL-17F was inverted on the chicken chromosome compared to mammalian chromosomes.

Avian cytokines show low levels of sequence conservation (approximately 30%) compared to their mammalian counterparts (Staeheli et al., 2001) and form distinct branches in phylogenetic analyses (Sundick and Gill-Dixon, 1997; Lillehoj et al., 2001; Balu and Kaiser, 2003). When compared to mammalian IL-17A and IL-17F sequences, chIL-17F showed approximately 37–40% and 38–43% identities, respectively. Additionally, IL-17A and IL-17F formed new branches with chIL-17 and duck IL-17 that share 53.8% amino acid identity with chIL-17F in a phylogenetic tree (Min and Lillehoj, 2002; Yoo et al., 2009). Furthermore, unlike mammalian IL-17F and IL-17A, including human, rat, and mouse IL-17F and IL-17A, the length of chIL-17 and chIL-17F amino acid sequences is identical (Fig. 1B). In contrast to similarities between chIL-17 and chIL-17F, several lines of evidence suggest that chIL-17 and chIL-17F are unique cytokines. First, unlike chIL-17 expression, chIL-17F transcript expression was widely detected in various normal tissues. Second, the genetic locus, including the synteny, was inverted in chicken chromosome 3. Third, a high degree of synteny was found between the chicken and turkey genomes, in which EFHC1, PAQR8-like, MCM3-like, IL-17F-like (LOC100547361), IL-17F-like (LOC100546746), and PKHD1 are on turkey chromosome 2 (data not shown). In addition, chIL-17F showed 93.5% and 53.8% amino acid identities to IL-17F-like (LOC100547361) and IL-17F-like (LOC100546746), respectively. ChIL-17 showed 52.7% and 95.9% amino acid identities to IL-17F-like (LOC100547361) and IL-17F-like (LOC100546746), respectively (data not shown). Thus, our data suggest that chIL-17F is homologous to mammalian IL-17F and is unique from chIL-17.

The IL-17 family induces production of pro-inflammatory cytokines, chemokines, and matrix metalloproteinases (Weaver et al., 2007; Iwakura et al., 2011). ChIL-17F and chIL-17, like their mammalian counterparts, appear to affect the production of cytokines (IL-1β, IL-6, IL-8) in primary CEFs. However, chIL-17F and chIL-17 did not induce the expression of chicken TGF-β1 (or TGF-β4 in chickens) (Pan and Halper, 2003), even though recombinant human IL-17F stimulated TGF-β1 expression by endothelial cells (Starnes et al., 2001), suggesting that immune functions involving IL-17F may differ between mammals and avians.

In the present study, chIL-17F transcript levels were, in general, up-regulated or unchanged in chickens infected with *E. maxima* or *E. tenella* compared to uninfected chickens. As shown in Fig. 4B and C, chIL-17 mRNA expression in chickens infected with *E. maxima* or *E. tenella* was unchanged or down-regulated compared to uninfected chickens. This indicates that chIL-17F and chIL-17 expression levels were differentially regulated in intestinal tissues of chickens infected with *E. maxima* or *E. tenella*. In our previously work (Min and Lillehoj, 2002), ChIL-17 transcripts were not detected by Northern blot analysis using 15 μg of total RNA from intestinal intraepithelial lymphocytes from *E. acervulina* -infected chickens. However, chIL-17 transcript expression in intestinal intraepithelial lymphocytes is highly up-regulated following primary infection with *E. acervulina* or *E. maxima*, but not *E. tenella* (Hong et al., 2006a,b). chIL-17 transcript expression increases approximately 1650-fold following *E. maxima* infection, 2020-fold following *E. acervulina* infection, and only 3-fold following *E. tenella* infection (Hong et al., 2006a,b). The discrepancy between these reports and our data is likely attributable to the use of different parasite and chicken strains and different tissue samples. Hong et al. (2006a,b) used intestinal intraepithelial lymphocytes, whereas we used intestinal tissues. *Leishmania major*-infected BALB/c mice produce more IL-17 than do infected C57BL/6 mice (Kostka et al., 2009). Thus, further investigations are needed to assess differences in chIL-17F expression levels between different chicken strains and also the differential functions of these closely related cytokines in host immune responses and defense mechanisms against pathogens.

In summary, we cloned chIL-17F and examined its expression patterns and functions. Molecular analysis indicated that chIL-17F possesses a number of conserved features of the Th17 family of cytokines. In addition, when compared to chIL-17, chIL-17F possesses distinct characteristics in tissue distribution and expression patterns in mitogen-activated splenic lymphocytes and in *Eimeria*-infected chickens, indicating that chIL-17F is a unique member of the IL-17 family of cytokines.

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