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Short communication

Identification and expression analysis of duck interleukin-17D in *Riemerella anatipestifer* infection

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ABSTRACT

Interleukin (IL)-17D is a proinflammatory cytokine with currently largely unknown biological functions. Here we provide the description of the sequence, bioactivity, and mRNA expression profile of duck IL-17D homologue. A full-length duck IL-17D (duIL-17D) cDNA with a 624-bp coding region was identified from the large intestine. duIL-17D shares approximately 94.7% identity with its chicken counterpart, which is also identified in this work. duIL-17D exhibits 62.6–68.4% and 52.1–53.1% identity with mammalian and piscine homologues. Recombinant duIL-17D promoted the expression of proinflammatory cytokines such as IL-6, IL-8, and IL-1β in duck embryo fibroblast cells. Very low levels of duIL-17D transcript were observed in healthy lymphoid tissues, including bursa, thymus, and spleen, while duIL-17D expression was relatively high in the heart. The duIL-17D expression profiles were examined in mitogen-stimulated splenic lymphocytes, as well as tissues affected by *Riemerella anatipestifer* infection. The levels of duIL-17D were mostly upregulated in mitogen-activated splenic lymphocytes but downregulated in the liver and spleen of *R. anatipestifer*-infected ducks. These results provide new insights into the roles of IL-17D in host protective immune responses to *Riemerella* infection, which can therefore lead to further studies of its biological functions in different disease models of ducks and other avian species.

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1. Introduction

The interleukin (IL)-17 cytokine family plays a broad role in the host immune response and the pathogenesis of multiple inflammatory disorders (Iwakura et al., 2011; Pappu et al., 2012). Six IL-17-related cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) have been identified in mammals, each with a varying degree of intermolecular amino acid sequence homology and functional activities. These cytokines generally function through engagement of the IL-17 receptor (IL-17R) family, which includes five members (IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE), by binding as homodimers or heterodimers (Onishi and Gaffen, 2010; Mensikova et al., 2013). IL-17A and IL-17F are the most intensively studied members of the family and are produced by activated T cells and a variety of innate cells to mediate protective immunity against microbial pathogens, as well as the pathogenesis of autoimmune diseases (Iwakura et al., 2011; Gu et al., 2013; Min et al., 2013). Recently, IL-17C was shown to have similar activities as IL-17A and IL-17F even though IL-17C is expressed mainly by epithelial cells and was found to be a critical factor in mucosal immunity (Ramirez-Carozzi et al., 2011; Song and Qian, 2013). IL-17E enhances type 2 immune responses by promoting the production of Th2 cytokines, such as IL-4, IL-5, and IL-13, and contributes to host defense against parasitic infections (Gu et al., 2013; Song and Qian, 2013). Although IL-17B and IL-17D play a similar role in inducing inflammatory mediators such as IL-17A and IL-17F, their biological functions remain largely unknown.

IL-17D shares the highest sequence homology with IL-17B and is known to be preferentially expressed outside the immune system.

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IL-17D reportedly stimulates the expression of IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human vascular endothelial cells, and exerts an inhibitory effect on the hematopoiesis of myeloid progenitor cells (Starnes et al., 2002). In contrast, IL-17D expression is decreased in psoriatic skin (Johansen et al., 2009) and undetectable in human synovial fluid mononuclear cells (Hwang and Kim, 2005). Additionally, a significant increase in IL-17D mRNA was detected in certain unedited tumors, but not in edited mouse tumor cell lines (O'Sullivan et al., 2014). In infection experiments, IL-17D mRNA expression was significantly enhanced in the head kidney but not in the spleen of Atlantic salmon that were injected intraperitoneally with *Aeromonas salmoncida* (Kumari et al., 2009). This effect was also observed in the head kidney of grass carp intraperitoneally infected with *Aeromonas hydrophila* (Du et al., 2014).

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 (Gibson et al., 2012; Jeong et al., 2015; Stewart et al., 2013), our understanding of avian immunobiology increased extensively over the past decade. Recently, the growing literature on the critical roles of the IL-17/IL-17R axis in host immune responses includes many reports that describe the importance of the IL-17 family of cytokines in avian immunity (Hong et al., 2008; Kim et al., 2012, 2014; Min et al., 2013; Rychlik et al., 2014; Zhao et al., 2014). Given the role that IL-17D plays in various diseases, understanding its potential contribution in avian health is of considerable interest. In this study, we report the discovery and characterization of a full-length cDNA encoding a duck homologue of mammalian IL-17D. By also examining the expression and bioactivity of this cytokine, we sought to gain better insight into its function in ducks. Finally, using quantitative real-time RT-PCR (qRT-PCR), we evaluated the expression profile of duck IL-17D (duIL-17D) in birds infected with *Riemerella anatipestifer*, one of the major pathogens afflicting the duck industry.

2. Materials and methods

2.1. Animals and infections

Pekin ducklings (Joowon ASTA Ducks, South Korea) were given unlimited access to feed and water. Constant light was provided throughout the experiment. Fifty two-week-old ducks were infected intramuscularly with 5×10^7 CFU of *R. anatipestifer* serotype 7 in 200 μl phosphate buffer saline (PBS). The bacterium used in this study (Jeong et al., 2014) was isolated from a commercial duck farm in Changwon, Gyeongnam, South Korea and serotyped in Chonbuk National University. The isolate was grown in sheep blood agar plates (Asan Pharmaceutical, Korea) at 37 °C in 5% CO_2. A single isolated colony was picked and inoculated into Tryptic Soy Broth (Difco, USA) and incubated at 37 °C in a shaking incubator until the logarithmic growth phase was achieved. The final inoculum concentration was determined by plating 10-fold serial dilutions onto sheep blood agar plates. Control birds were given the same volume of PBS as the volume of bacteria used for infection. Liver and spleen were collected from five birds per group at 1, 4, 7, and 10 days post-infection. All animal experiments were performed in accordance with the Gyeongsang National University Guidelines for the Care and Use of Experimental animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Gyeongsang National University, Jinju, Republic of Korea (GNU-150504-C0026).

2.2. Cloning of duIL-17D and sequence analysis

Total RNA was extracted from the large intestine of a healthy duck using RiboEx (GeneAll, Korea) and RNeasy Mini Kit (Qiagen, Germany). The RNA was then treated with RNase-free DNAse I (Fermentas, Canada) and cDNA was synthesized from the treated total RNA using the first strand synthesis kit, HyperScript (GeneAll). Based on a predicted cDNA sequence (GenBank accession number XM_005028731.2), 5′/3′-Rapid Amplification of cDNA Ends (RACE) was performed using duIL-17D-specific primers (Table 1) with the large intestine cDNA, a high-fidelity DNA polymerase (Bioneer, Korea), and a 5′/3′ RACE kit (5′/3′ RACE kit, 2nd Generation; Roche Applied Science, Germany) according to the manufacturer's instructions. PCR products were cloned into a TA vector (RBC, Taiwan) and sequenced (Macrogen, Korea). PCR was performed on a DNA Engine thermocycler (Bio-Rad, USA) with the following program: 5 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C; and a final 5 min extension at 72 °C. duIL-17D cDNA sequence was submitted to GenBank (accession number KT932633). Protein identification was conducted using the Expert Protein Analysis System (ExPASy; www.expasy.org/tools/) and multiple amino acid alignments were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). A signal peptide was predicted using the Signal P 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and the potential *N*-linked glycosylation site was determined by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.3. Cell culture

Duck splenic lymphocytes isolated from two-week-old ducks and duck embryonic fibroblast cells (DEF) obtained from ten-day-old embryos were cultured as previously described (Jeong et al., 2015; Kim et al., 2015). Cells were collected, washed twice with PBS, and cultured in DMEM (Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies) and penicillin-streptomycin (10,000 unit/ml) in a 41 °C incubator in 5% CO_2. Splenic lymphocytes were resuspended to 5×10^6 cells/ml and stimulated with 25 μg/ml polyinosinic:polycytidylic acid (poly I:C), 10 μg/ml lipopolysaccharide (LPS from *Escherichia coli*, O111:B4) (all purchased from Sigma-Aldrich, Germany), or 10 μg/ml concanavalin (ConA) (Amersham Bioscience, Sweden) for 0, 4, 8, and 24 h. DEF was stimulated with the conditioned medium from COS-7 cells transfected with duIL-17D-FLAG or empty vector (pcDNA 3.1) for 6 h at 41 °C in 5% CO_2, and then proinflammatory cytokine expression was analyzed by qRT-PCR.

2.4. Production and biological activity of recombinant duIL-17D

duIL-17D cDNA harboring a FLAG-tag expression sequence (duIL-17D-FLAG) was amplified by PCR from single-stranded cDNA isolated from duck large intestine using the following specific primers: forward 5′-GATCGAATTCATGCAGCGAGGCAGGGTGCT-3′ and reverse 5′-GATCCTCGAGTCACTTGTCGTCATCGTCTTTGTAGTCTTCTGAAGATGGCTTGTT-3′. The primers contained *Eco*RI and *Xho*I restriction enzyme sites (underlined with solid lines above) and a FLAG-expressing sequence (underlined with a dashed line). PCR products were digested with *Eco*RI and *Xho*I and cloned into the corresponding restriction sites of the pcDNA 3.1 + vector (Invitrogen, USA). COS-7 cells were transiently transfected with 10 μg of the construct or empty vector (negative control) using FuGENE 6 transfection reagent (Promega, USA) according to the manufacturer's instructions. Transfected cells were cultured in Opti media (Gibco Life Technologies, USA), a serum-free medium, for 48 h at 37 °C in 5% CO_2. To assess the biological activity of recombinant duIL-17D, primary DEF was obtained from ten-day-old embryos and cultured as described above. Supernatants from transfected COS-7 cells were added to DEF and then incubated for

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

Gene target	Purpose	Primer and sequence (5′-3′)	References
duIL-17D	5′RACE 3′RACE	(Fwd) GATGACGGTGGGCATGTACA (Rev) CCCGAAGCGTACTGCCTGTG	Present study
duIL-17D	qRT-PCR	(Fwd) CACGAGATACCCTAAGTACATTC (Rev) CTGCGGAAGTGGAAATTCT	Present study
duIL-17A	qRT-PCR	(Fwd) ATGTCTCCAACCCTTCGT (Rev) CCGTATCACCTTCCCGTA	Kim et al. (2015)
IL-6	qRT-PCR	(Fwd) GCGGTCTCCGACTCCTCC (Rev) ATAGCGAACAGCCCTCACG	Wei et al. (2014b)
IL-8	qRT-PCR	(Fwd) GCTGTCCTGGCTCTTCTCCT (Rev) GCACACCTCTCTGTTGTCCTTC	Wei et al. (2014b)
IL-1β	qRT-PCR	(Fwd) TCATCTTCTACCGCCTGGAC (Rev) GTAGGTGGCGATGTTGACCT	Wei et al. (2014a)
TGF-β	qRT-PCR	(Fwd) CGGGACGGATGAGAAGAAC (Rev) CGGCCCACGTAGTAAATGAT	Shanmugasundaram and Selvaraj (2012)
β-actin	qRT-PCR	(Fwd) GCTATGTCGCCCTGGATTTC (Rev) CACAGGACTCCATACCCAAGAA	Liu et al. (2012)

(Fwd): Forward primer; (Rev): Reverse primer; qRT-PCR: quantitative real-time RT-PCR.

6 h at 41 °C in 5% CO_2. Total RNA was extracted from the stimulated cells and cDNA synthesis was performed using oligo (dT) primer and the first strand synthesis kit, HyperScript (GeneAll). Analysis of duIL-17D-induced gene expression was performed by qRT-PCR using the primers listed in Table 1.

2.5. Western blot analysis

Cell lysates and supernatants of transfected COS-7 cells 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 12% SDS-polyacrylamide gels, and electroblotted onto polyvinyl difluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked with a PBS and 0.05% Tween-20 (PBS-T) solution containing 4% nonfat dry milk for 2 h at room temperature and then incubated with monoclonal anti-FLAG mouse antibody (Cell Signaling Technology, USA) at 4 °C overnight. The membrane was then washed with PBS-T five times and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Promega) in 4% nonfat dry milk in PBS-T for 1 h at room temperature. The membrane was then washed five times with PBS-T. It was then incubated with chemiluminescent reagent EZ West Lumi plus (Atto, Japan) and visualized using the ChemiDoc Imaging System (Bio-Rad).

2.6. Cytokine induction assay and quantitative real-time RT-PCR (qRT-PCR)

Healthy tissues, activated splenic lymphocytes, and tissue samples from ducks infected with *R. anatipestifer* serotype 7 were subjected to qRT-PCR analysis in duplicate. RiboEx (GeneAll) was used to extract total RNA from samples homogenized with a tissue grinder (Daihan Sci. Korea) or by vortexing cells according to manufacturer's instructions. It was then purified with the RNeasy Mini kit (Qiagen) and stored at −70 °C. To remove any contaminating genomic DNA, samples were treated with DNase I (Fermentas). Single-stranded cDNA was synthesized with the first strand synthesis kit, HyperScript (GeneAll), from the total RNA using oligo (dT) primer. The qRT-PCR was performed using the CFX 96 real-time PCR system (Bio-Rad, USA) with SYBR Green (Bioneer, South Korea) 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. Relative expression levels of individual transcripts were normalized to those of β-actin using Bio-Rad CFX software as previously described (Kim et al., 2015).

2.7. Statistical analysis

Data were analyzed by the Student's *t*-test using InStat® software (GraphPad, USA). Differences with a *p*-value less than 0.05 were considered statistically significant. Data are expressed as the mean ± standard error (SE).

3. Results

3.1. Cloning and characterization of duIL-17D

The full-length cDNA encoding a duck homologue of mammalian IL-17D was first cloned from duck large intestine cDNA. The cloned duIL-17D sequence contained a 624-bp open reading frame (ORF) predicted to encode a putative 207 amino acid protein with a calculated molecular weight of 23 kDa (non-glycosylated) and an isoelectric point of 8.97. The duIL-17D amino acid sequence contained a signal sequence (amino acids 1–22), one potential *N*-linked glycosylation site (Asn-X-Ser/Thr) in the extracellular region, and eight cysteine residues involved in the interchain disulfide bond formation that is conserved in the IL-17 family (Starnes et al., 2002; Moseley et al., 2003) (Fig. 1A and B). Interestingly, the duIL-17D protein identified in this study is 91 amino acids longer than the previously cloned chicken IL-17D (GenBank accession number ABU51881) (Hong et al., 2008), which is predicted to encode a putative 116 amino acid protein with a calculated molecular weight of 13.3 kDa, suggesting the possibility that the ABU51881 clone may be a short isoform or a truncated form of IL-17D.

To confirm this possibility, chicken IL-17D (chIL-17D) was screened from chicken skeletal muscle cDNA based on the duIL-17D cDNA sequence, and a full-length chIL-17D cDNA was also identified in this study (GenBank accession number ALM23739). The chIL-17D sequence that was similar to duIL-17D contained a 621-bp ORF predicted to encode a putative 206 amino acid protein with a calculated molecular weight of 23 kDa (non-glycosylated) and an isoelectric point of 8.97. The chIL-17D amino acid sequence contained a signal sequence (amino acids 1–22), one potential *N*-linked glycosylation site in the extracellular region, and eight cysteine residues (Supplementary Fig. S1A). Multiple amino acid alignment revealed that, compared to the chIL-17D cDNA cloned in this study, the previously reported cDNA (GenBank accession no. ABU51881; Hong et al., 2008) was completely identical only to amino acids 102–206 of full-length chIL-17D (Supplementary

Fig. S1B). Amino acid sequence comparison using Clustal Omega indicated that duIL-17D shares approximately 94.7% identity with its chicken counterpart identified in this work. The duIL-17D shares 62.6–68.4% identity with mammalian homologues and 52.1–53.1% identity with its piscine counterparts (Fig. 1C).

3.2. Distribution of duIL-17D mRNA in healthy tissues and mitogen-activated splenic lymphocytes

We performed qRT-PCR analysis to examine the expression of duIL-17D transcript in various healthy tissues and in mitogen-stimulated splenic lymphocytes (Fig. 1D and E). The expression of duIL-17D transcripts was detected in all tissues, especially the heart, which exhibited a relatively high expression level. Healthy lymphoid tissues such as bursa, thymus, and spleen generally expressed very low levels of duIL-17D transcript (Fig. 1D). This result was somewhat consistent with that of chIL-17D, which showed very low expression levels in chicken lymphoid tissues such as bursa, thymus, cecal tonsil, and spleen (Supplementary Fig. S1C). As shown in Fig. 1E, duIL-17D mRNA transcript was generally upregulated at all time points of ConA, LPS and poly I:C stimulation examined in splenic lymphocytes compared with unstimulated and cultured lymphocytes.

3.3. Molecular weight and bioactivity of recombinant duIL-17D

A construct expressing FLAG-tagged duIL-17D or empty vector was transiently transfected into COS-7 cells. Molecular weights of duIL-17D were identified in PNGase F-treated COS-7 cells transfected with a FLAG-tagged duIL-17D construct. A 23 kDa protein (lower arrow; calculated molecular weight of 23 kDa) is likely the backbone of duIL-17D, and the 27 kDa protein (upper arrow) represents an *N*-linked glycosylated form of the protein. Induction of proinflammatory cytokines is a characteristic of IL-17 family members (Iwakura et al., 2011; Pappu et al., 2011). Thus, the supernatant was used for investigating the biological activity of recombinant duIL-17D in primary DEF. When we compared the effect of adding tissue culture supernatant from COS-7 cells that were transiently transfected with FLAG-tagged duIL-17D to supernatant from cells transfected with the empty vector, we found that supernatant containing duIL-17D induced significantly higher expression of IL-1β, IL-6, and IL-8 transcripts, but not TGF-β (Fig. 1G).

3.4. Downregulation of duIL-17D in R. anatipestifer-infected ducks

Although many studies have demonstrated the biological function of IL-17 family cytokines such as IL-17A in different animal and disease models, only a few have investigated the role of IL-17D in host defense against major pathogens. *R. anatipestifer*, one of most harmful bacterial pathogens of ducks, has been implicated in a range of diseases, including fibrinous pericarditis, polyserositis, and septicemia (Ruiz and Sandhu, 2013). In an initial effort to determine the expression pattern of duIL-17D mRNA, ducks were infected intramuscularly with *R. anatipestifer*. Compared to uninfected ducks, the level of duIL-17D mRNA remained unchanged or was significantly downregulated in the liver and spleen (Fig. 2A). However, duIL-17A mRNA was upregulated in both liver and spleen compared to that of healthy controls (Fig. 2B). On days 7 and 10, IL-17A expression was dramatically reduced in the spleen of *R. anatipestifer*–infected ducks (Fig. 2B). To confirm infection, bacterial burden was monitored in the spleen and liver post-infection, and was found in both organs of infected ducks (Fig. 2C).

4. Discussion

IL-17D is a proinflammatory cytokine of the IL-17 cytokine family. To date, IL-17D is the least studied member of this family. Here, a full-length cDNA encoding the duck homologue of mammalian IL-17D was cloned and characterized. We also found that duIL-17D exhibits higher conservation than duIL-17A and duIL-17F between mammals and birds. The putative protein of duIL-17D showed 62.6–68.4% and 94.7% identities with the corresponding mammalian homologues and chIL-17D identified in this study, respectively. Our previous studies indicated that duIL-17A and duIL-17F shared 46–47% and 37–46% identities with mammalian homologues, respectively. Furthermore, duIL-17A and duIL-17F exhibited 84% and 77.5% identities with chIL-17A and chIL-17F, respectively (Min and Lillehoj, 2002; Yoo et al., 2009; Kim et al., 2012, 2015). Multiple sequence alignment revealed the presence of eight cysteine residues potentially involved in the formation of disulfide bonds are conserved in both avian and mammalian IL-17D. Eight of the conserved cysteine residues were also found in IL-17D of Atlantic salmon (Kumari et al., 2009). However, duIL-17A and duIL-17F possess six of the conserved cysteine residues (Yoo et al., 2009; Kim et al., 2015), indicating that the avian IL-17D sequences contained two additional cysteines compared to the duIL-17A and IL-17F sequences. Thus, our data suggest that the newly cloned IL-17D cDNA is an avian ortholog of mammalian IL-17D.

The IL-17 family cytokines induce the production of proinflammatory cytokines, chemokines, and matrix metalloproteinases (Weaver et al., 2007; Iwakura et al., 2011). Similar to the other IL-17 family members that we previously cloned, namely duIL-17A and duIL-17F (Yoo et al., 2009; Kim et al., 2015), duIL-17D treatment led to increased expression of IL-1β, IL-6, and IL-8 in primary DEF. However, duIL-17D did not induce the expression of duck TGF-β. IL-17D induced IL-6, IL-8, and GM-CSF production in human endothelial cells (Starnes et al., 2002), as well as IL-1β, CXCL-8, and TNF-α mRNA expression in grass carp primary head kidney cells (Du et al., 2014).

Like in human and fish species (Starnes et al., 2002; Kumari et al., 2009; Korenaga et al., 2010; Du et al., 2014), constitutive expression of duIL-17D (Fig. 1D) and chIL-17D (Supplementary Fig. S1C) transcripts was ubiquitous in both bird organs examined. Duck and chicken IL-17D transcripts were expressed at a relatively low level in healthy lymphoid tissues such as spleen, thymus, and bursa. These observations are in accordance with the low abundance of IL-17D mRNA in the spleen of grass carp (Du et al., 2014), Atlantic salmon (Kumari et al., 2009), broiler chickens (Hong et al., 2008), and humans (Starnes et al., 2002). However, IL-17D was expressed at different levels across these species in the thymus; IL-17D was expressed at low levels in grass carp and human while Atlantic salmon and broiler chickens showed moderate or high expression levels. Relatively high expression of duIL-17D (Fig. 1D) and chIL-17D (Supplementary Fig. S1C) transcripts has been noted in the heart, which is consistent with findings in humans (Starnes et al., 2002) and teleost fish tongue sole (Chi and Sun, 2015). To investigate whether the mRNA expression profiles of duIL-17D could be modulated, splenic lymphocytes were stimulated *in vitro* and duIL-17D mRNA was assessed (Fig. 1E). The level of duIL-17D expression remained unchanged or increased significantly in splenic lymphocytes stimulated with ConA, LPS, or poly I:C. Similarly, reports have demonstrated that, after LPS stimulation, IL-17D expression was unchanged or increased significantly in head kidney cells of pufferfish (Korenaga et al., 2010) and significantly induced at all time points examined in head kidney leukocytes of grass carp (Du et al., 2014). These findings suggest that *in vitro* expression profiles of IL-17D may be similar between ducks and fish.

A

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ATGCAGCGAGGCAGGGTGCTGGCGGCGCTGCTGTGCGCGGCGCTGCTGCCGCTGCCCTCGGACGCCGCCAAAGCTCCCAAGCGGCCGGCTCGGCCCCGGGGCTGCGGGGAGAGGCCCGAG 120
 M  Q  R  G  R  V  L  A  A  L  L  C  A  A  L  L  P  L  P  S  D  A  A  K  A  P  K  R  P  A  R  P  R  G  C  G  E  R  P  E  40
GAGCTGCTGGAGCAGCTGTACGGGCGGCTGGCCGCCGGCATGCTCAGCGCCTTCCACCACACGCTGCAGCCCGAGCCGCCCGGCCGCCAGCAGCACAACGCCAGCTGCCCCGCGGGGGCA 240
 E  L  L  E  Q  L  Y  G  R  L  A  A  G  M  L  S  A  F  H  H  T  L  Q  P  E  P  P  G  R  Q  Q  H  N  A  S  C  P  A  G  A  80
CGGCCGGCCGGCGACAAGAGGCTGCGGCTGCCCGTCAACATGCGCAGCGCCTCGCCCTGGGCGTACAGAATTTCATACGATCCCACGAGATACCCTAAGTACATTCCCGAAGCGTACTGC 360
 R  P  A  G  D  K  R  L  R  L  P  V  N  M  R  S  A  S  P  W  A  Y  R  I  S  Y  D  P  T  R  Y  P  K  Y  I  P  E  A  Y  C  120
CTGTGCAAGGGCTGCCTCATGGGGATCTTCGGCGAGGAGAATTTCCACTTCCGCAGCACCCCCGTGTACATGCCCACCGTCATCCTCCGCCGCACCTCGTCCTGTGCCGGGGGCCGCTAC 480
 L  C  K  G  C  L  M  G  I  F  G  E  E  N  F  H  F  R  S  T  P  V  Y  M  P  T  V  I  L  R  R  T  S  S  C  A  G  G  R  Y  160
GTGTACACGGAAGACTACGTCACTATCCCCGTGGGCTGCACTTGTGTCCCCGAGCCAGAGAAAGAGGCCGAAAGCGTAAATTCCAGCATAGATAAGCAAGAAATGAAGTTGCTCGTAAAC 600
 V  Y  T  E  D  Y  V  T  I  P  V  G  C  T  C  V  P  E  P  E  K  E  A  E  S  V  N  S  S  I  D  K  Q  E  M  K  L  L  V  N  200
CAGAACAAGCCATCTTCAGAATGA                                                                                                624
 Q  N  K  P  S  S  E  -                                                                                                 207
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B

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Chicken   --MQRGRVLTALL-CAALLPLRSDAAKAPKRPARPRSCGERPEELLEQLYGRLAAGMLGAFHHTLQPEPPG-RHHNASCPAGARPGGDKRFRLPVNLRSASPWAYRISYDPTRYPKYIPE 116
Duck      --MQRGRVLAALL-CAALLPLPSDAAKAPKRPARPRGCGERPEELLEQLYGRLAAGMLSAFHHTLQPEPPGRQQHNASCPAGARPAGDKRLRLPVNMRSASPWAYRISYDPTRYPKYIPE 117
Human     -------MLVAGFLLALPPSWAAGAPRAGRRPARPRGCADRPEELLEQLYGRLAAGVLSAFHHTLQLGPRE-QARNASCPAGGRPGDRRFRPPTNL-RSVSPWAYRISYDPARYPRYLPE 111
Mouse     MLGTLVWMLAVGFLLALAPGRAAGALRTGRRPARPRDCADRPEELLEQLYGRLAAGVLSAFHHTLQLGPRE-QARNASCPAGGRAADRRFRPPTNL-RSVSPWAYRISYDPARFPRYLPE 118
                 *      *        *      ****** *  ***************** *******  *      ******* *             ** *********** * * * **

Chicken   AYCLCKGCLMGIFGEENFHFRSTPVYMPTVILRRTSSCAGGRYVYTEDYVTIPVGCTCVPEQEKEAEDVNSSIDKQEMKLLVNQNKPSSE- 206
Duck      AYCLCKGCLMGIFGEENFHFRSTPVYMPTVILRRTSSCAGGRYVYTEDYVTIPVGCTCVPEPEKEAESVNSSIDKQEMKLLVNQNKPSSE- 207
Human     AYCLCRGCLTGLFGEEDVRFRSAPVYMPTVVLRRTPACAGGRSVYTEAYVTIPVGCTCVPEPEKDADSINSSIDKQGAKLLLGPNDAPAGP 202
Mouse     AYCLCRGCLTGLYGEEDFRFRSTPVFSPAVVLRRTAACAGGRSVYAEHYITIPVGCTCVPEPDKSADSANSSMDKL----LLGPADRPAGR 205
          ***** *** *  ***    *** **  * * ****  ***** ** * * ********** * *    *** **     *
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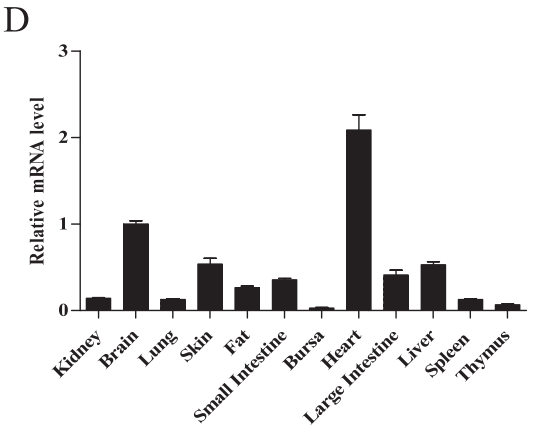
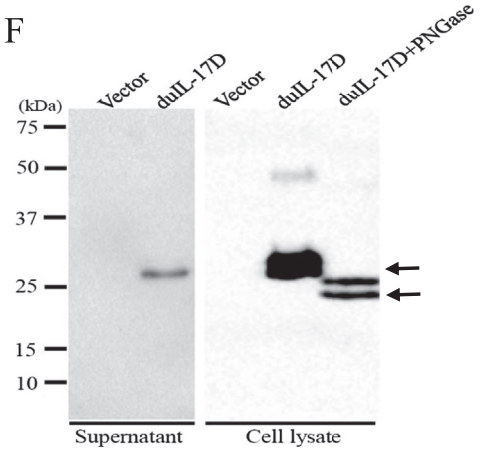
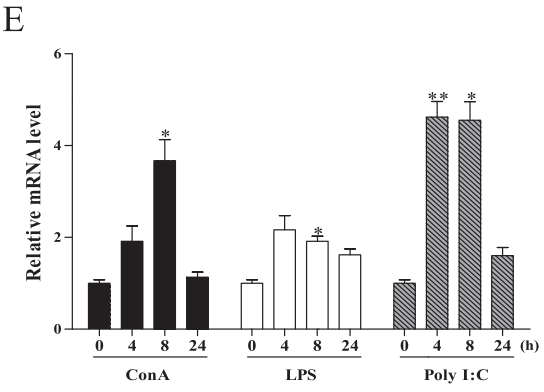
C

	human/mouse						chicken/duck			salmon/trout	frog
duIL-17D	IL-17A	IL-17B	IL-17C	IL-17D	IL-17E	IL-17F	IL-17A	IL-17F	IL-17D	IL-17D	IL-17D
	21.2-21.4	21.6-25.8	23.2-26	62.6-68.4	16.5-22.4	17.5-18.1	22.0-25.2	20.6-22.6	94.7	52.1-53.1	61.7







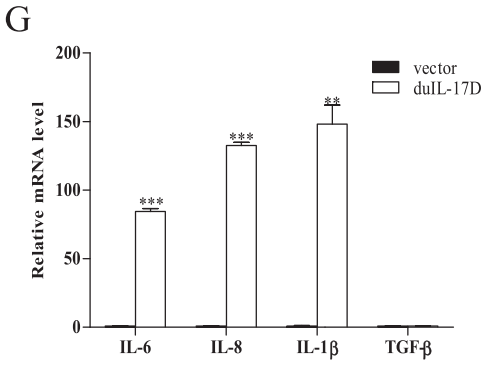


Fig. 1. Molecular characterization of duIL-17D. (A) Nucleotide and deduced amino acid sequences of duIL-17D. The predicted signal sequence is underlined. The eight conserved cysteine residues are highlighted with black boxes. The potential *N*-linked glycosylation site is highlighted with a white box. (B) Multiple alignment of avian and mammalian IL-17D deduced amino acid sequences. Sequences were aligned using Clustal Omega software (www.ebi.ac.uk/Tools/msa/clustalo/). Asterisks (*) indicate identical residues. The predicted signal sequence is underlined. The potential *N*-linked glycosylation site is denoted in bold and underlined. The eight conserved cysteine residues are identified with white boxes. (C) Percentage of amino acid identities between duIL-17D and IL-17 family members from other species. The GenBank accession numbers used in this comparison were: human IL-17A (U32659), human IL-17B (NM_014443), human IL-17C (NM_013278), human IL-17D (AF458062), human IL-17E (AF305200), human IL-17F (NM_052872), mouse IL-17A (NM_010552), mouse IL-17B (NM_019508), mouse IL-17C (AF458061), mouse IL-17D (AF458063), mouse IL-17E (NM_080729), mouse IL-17F (AF458064), chicken IL-17A (AJ493595), chicken IL-17F (JQ776598), duck IL-17A (FJ755182), duck IL-17F (KM035421), salmon IL-17D (EU689087), trout IL-17D (NM_001124399), and frog IL-17D (NM_001114247). (D, E) Expression of duIL-17D mRNA in healthy tissues and activated-splenic lymphocytes. (D) Total RNA was extracted from various tissues from two-week-

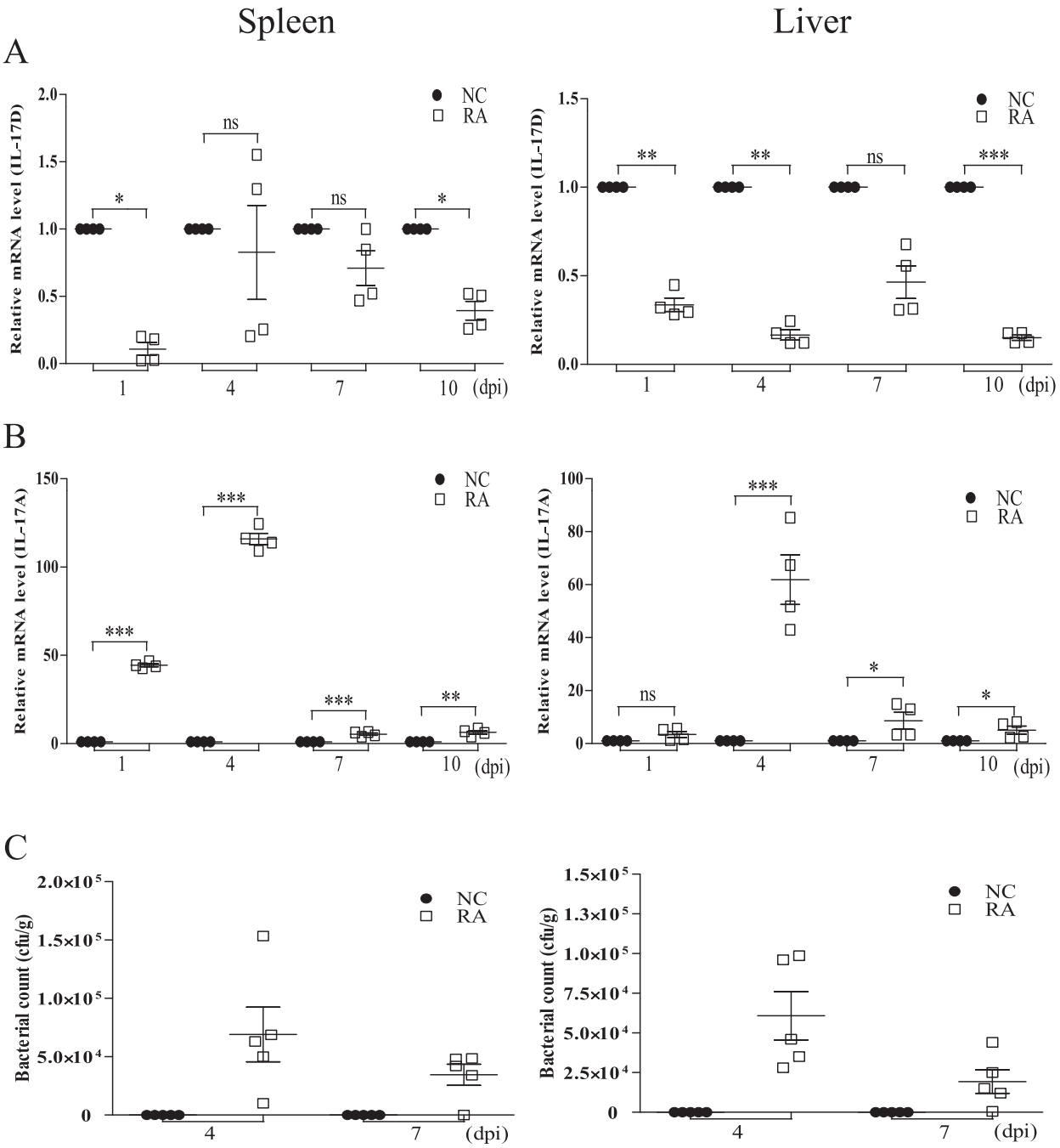


Fig. 2. Expression profiles of duIL-17D and duIL-17A mRNA in *R. anatipestifer*-infected ducks. Fourteen-day-old ducks were infected via intramuscular injection with 5×10^7 colony-forming units (cfu) of *R. anatipestifer*. The liver and spleen of *R. anatipestifer*-infected ducks were collected on days 1, 4, 7, and 10 post-infection. Expression levels of duIL-17D (A) and duIL-17A (B) transcript were quantified by qRT-PCR. Gene expression levels were normalized with β-actin and calibrated with expression found in the uninfected group. Error bars represent the mean ± SE (n = 4). $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$ was considered significant compared to the uninfected group, which was used as a negative control (NC). (C) Bacterial load of *R. anatipestifer* in the liver and spleen. Five ducks were sacrificed at each time point and then the liver and spleen were aseptically collected for bacterial recovery. One representative experiment of two independent experiments performed with similar results is shown. RA, *R. anatipestifer*; NC, negative control; dpi, days post-infection; ns, no statistical significance.

old healthy ducks. Tissue samples were pooled from five ducks and were subjected to qRT-PCR analysis. Gene expression levels were normalized with β-actin and calibrated with brain tissue expression. Data represent the mean ± SE from two independent experiments. (E) Splenic lymphocytes were isolated from two-week-old ducks, and activated with 10 μg/ml ConA, 10 μg/ml LPS, or 25 μg/ml poly I:C for the indicated times. Gene expression levels were normalized to β-actin and calibrated with unstimulated and cultured splenic lymphocytes. The y-axis represents the fold change in expression of duIL-17D in activated lymphocytes compared to unstimulated and cultured splenic lymphocytes. All data represent the mean ± SE from two independent experiments. $^*P < 0.05$ or $^{**}P < 0.01$ was considered to be statistically significant. (F, G) Molecular weight and bioactivity of duIL-17D. (F) Detection of duIL-17D protein by western blot analysis. COS-7 cells were transiently transfected with duIL-17D-FLAG or vector pcDNA 3.1. Supernatants and cell lysates were collected after 48 h and separated by SDS-PAGE under reducing conditions. Cell lysates were deglycosylated by 100 U peptide-*N*-glycosidase F (PNGase-F) at 37 °C for 2 h. The arrows indicate specific bands. One representative experiment of two independent experiments performed with similar results is shown. (G) Induction of proinflammatory cytokines in primary DEF. Primary DEF was obtained from ten-day-old embryos as previously described (Kim et al., 2015) and then stimulated with the conditioned medium from COS-7 cells transfected with duIL-17D or empty vector (pcDNA 3.1+) for 6 h at 41 °C in 5% CO_2. Total RNA was extracted and then cytokine gene expression was analyzed by qRT-PCR. Expression levels were normalized to those of β-actin. Data represent the mean ± SE from two independent experiments. $^{**}P < 0.01$ or $^{***}P < 0.001$ was considered to be statistically significant.

R. anatipestifer is a Gram-negative, non-motile, non-spore-forming, rod-shaped, and extracellular bacterium that causes one of the most economically harmful and contagious diseases in duck farms worldwide (Hu et al., 2012; Zhou et al., 2013). *R. anatipestifer* infection causes polyserositis, septicemia, and meningitis, and is associated with a 5%–75% mortality rate depending on the virulence of the strain (Pathanasophon et al., 2002). This pathogen enters through skin wounds or the respiratory tract and is carried to various organs, including the spleen and liver (Ruiz and Sandhu, 2013). In this study, duIL-17D, unlike duIL-17A, was largely downregulated in the liver and spleen during *R. anatipestifer* infection compared to uninfected ducks. Previous studies with pathogenic bacteria and parasites showed a variation in IL-17D expression pattern (unresponsive, decrease or increase) depending on the tissues examined after infection (Hong et al., 2008; Kumari et al., 2009; Du et al., 2014). In grass carp, infection with *Aeromonas hydrophila* for 1 and 3 days led to increased IL-17D expression in the head kidney; expression of this transcript returned to basal levels at day 7 (Du et al., 2014). IL-17D expression in Atlantic salmon was significantly increased in the head kidney after *Aeromonas salmonicida* infection and returned to basal levels at day 4. In contrast, IL-17D was unchanged in the spleen during the infection (Kumari et al., 2009). The expression level of IL-17D was unchanged in the spleen of teleost fish tongue sole during *Vibrio harveyi* infection (Chi and Sun, 2015). In broiler chickens infected with the intracellular parasite *E. maxima*, the level of IL-17D mRNA was increased in the jejunum, bursa, lung, and spleen, but decreased in thymus (Hong et al., 2008). Taken together, these observations suggest the possibility that expression levels of IL-17D in the spleen of ducks and fish may be unchanged or downregulated to facilitate effective host immunity during pathogenic bacterial infections. It is interesting to note that IL-17D treatment showed a suppressive effect on myeloid progenitor cell proliferation (Starnes et al., 2002). However, discrepancies in IL-17D expression levels occur in the spleen of *Eimeria*-infected chickens where its expression was increased. This discrepancy is likely attributable to assessing different pathogens with different pathogenic mechanisms. We employed the bacterial pathogen *R. anatipestifer*, whereas Hong et al. (2008) used an intracellular parasite *E. maxima*. While *R. anatipestifer* is translocated to various organs, including the spleen and liver (Ruiz and Sandhu, 2013), *E. maxima* targets the enterocytes and causes significant damage to the intestinal epithelial layer (Min et al., 2013; Chapman, 2014). Further investigation is needed to assess the differential expression of duIL-17D in response to various pathogens and its differential functions in host immunity and defense mechanisms against those pathogens.

In summary, we cloned duIL-17D and examined its expression patterns and functions. Molecular analysis indicated that duIL-17D possesses several conserved features characteristic of the Th17 family of cytokines. When compared to duIL-17A and duIL-17F, duIL-17D possesses distinct characteristics in tissue distribution and expression patterns in mitogen-activated splenic lymphocytes and in *R. anatipestifer*-infected ducks, indicating that duIL-17D is a unique member of the IL-17 family of cytokines.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.04.002.

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