Short communication

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

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ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 4 April 2016
Accepted 4 April 2016
Available online 6 April 2016

Keywords:
Ducks
Interleukin-17D
Riemerella infection
Avian IL-17D

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 fibroblasts 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.

http://dx.doi.org/10.1016/j.dci.2015.06.002
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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 salmonicida (Kumari et al., 2009). This effect was also observed in the head kidney of grass carp intraaperitoneally 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 anatipes-tifer, 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 \times 10^6 CFU of R. anatipes-tifer serotype 7 in 200 μl phosphate buffer saline (PBS). The bacterial used in this study (Jeong et al., 2014) was isolated from a commercial duck farm in Changwon, Gyeongnam, South Korea and serotyped in Chonbuk 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% CO2. Splenic lymphocytes were resuspended to 5 \times 10^6 cells/ml and stimulated with 25 μg/ml polynosinic-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% CO2 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’-GATCGAATTCCTCACTCTCAATCCGAC-3’ and reverse 5’-GATCTCGAGCTTACATTTCCGTCGAGTTACGCGATTAGTCTCTGAAGATGCTGGGTGTT3’. The primers contained EcoRI and XhoI restriction enzyme sites (underlined with solid lines above) and a FLAG-expressing sequence (underlined with a dashed line). PCR products were digested with EcoRI and XhoI 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% CO2. To assess the biological activity of recombinant duck IL-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 6 h at 41 °C. The cytokine expression was analyzed by qRT-PCR.

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 1 (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/).
levels of individual transcripts were normalized to those of ampli obtained at the end of each run to verify the presence of a single 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 1–10) that is conserved in the IL-17 family (Starnes et al., 2011). The cloned chicken IL-17D (chIL-17D) 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 shorter 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 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  | (Fwd): GATGACCTGCTGCAGTCTACA | Present study |
|             | 3’RACE  | (Rev): CCGCAAGCTTACTCGCTTG  | Present study |
|             | qRT-PCR | (Fwd): CGCGAGATCCTAAGTACATTCC | Present study |
|             |         | (Rev): CTGGGAAGTGGAAAATTCT   | Present study |
|             |         | (Rev): CCCTATACCTCCCTGTA      | Present study |
|             |         | (Rev): CGCGCTTCTCCACTCCCTCC  | Present study |
|             |         | (Rev): ATACGGAACAGCCTTACGCCAGC | Present study |
|             |         | (Rev): GCACACCTCTCTGTCCCTTCCT | Present study |
|             |         | (Rev): CTTCTCTTACCGCTTGGAC   | Present study |
|             | qRT-PCR | (Rev): GTAGTTGCCGATGGACTCT   | Present study |
|             |         | (Rev): CGGCAAGGATGAGAAGAAC  | Present study |
|             |         | (Rev): CGGCCAAGCTGATAGAATGAT | Present study |
|             |         | (Rev): GCTATGTCGCCCTGGATTTC | Present study |
|             |         | (Rev): CAAGGAACCTGATACCAAGAA | Present study |

(Pwd): Forward primer; (Rev): Reverse primer; qRT-PCR: quantitative real-time RT-PCR.
Fig. S1B]. Amino acid sequence comparison using Clustal Omega indicated that duLL-17D shares approximately 94.7% identity with its chicken counterpart identified in this work. The duLL-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 duLL-17D mRNA in healthy tissues and mitogen-activated splenic lymphocytes

We performed qRT-PCR analysis to examine the expression of duLL-17D transcript in various healthy tissues and in mitogen-stimulated splenic lymphocytes (Fig. 1D and E). The expression of duLL-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 duLL-17D transcript (Fig. 1D). This result was somewhat consistent with that of chLL-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, duLL-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 duLL-17D

A construct expressing FLAG-tagged duLL-17D or empty vector was transiently transfected into COS-7 cells. Molecular weights of duLL-17D were identified in PNGase F-treated COS-7 cells transfected with a FLAG-tagged duLL-17D construct. A 23 kDa protein (lower arrow; calculated molecular weight of 23 kDa) is likely the backbone of duLL-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 duLL-17D in primary DEF. When we compared the effect of adding tissue culture supernatant from COS-7 cells transfected with FLAG-tagged duLL-17D to supernatant from cells transfected with the empty vector, we found that supernatant containing duLL-17D induced significantly higher expression of IL-1β, IL-6, and IL-8 transcripts, but not TGF-β (Fig. 1C).

3.4. Downregulation of duLL-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 duLL-17D mRNA, ducks were infected intramuscularly with R. anatipestifer. Compared to uninfected ducks, the level of duLL-17D mRNA remained unchanged or was significantly downregulated in the liver and spleen (Fig. 2A). However, duLL-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 duLL-17D exhibits higher conservation than duLL-17A and duLL-17F between mammals and birds. The putative protein of duLL-17D showed 62.6–68.4% and 94.7% identities with the corresponding mammalian homologues and chLL-17D identified in this study, respectively. Our previous studies indicated that duLL-17A and duLL-17F shared 46–47% and 37–46% identities with mammalian homologues, respectively. Furthermore, duLL-17A and duLL-17F exhibited 84% and 77.5% identities with chLL-17A and chLL-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, duLL-17A and duLL-17F possess six of the conserved cysteine residues (Yoo et al., 2009; Kim et al., 2015), indicating that the avian IL-17D sequence contained two additional cysteines compared to the duLL-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 other IL-17 family members that we previously cloned, namely duLL-17A and duLL-17F (Yoo et al., 2009; Kim et al., 2005), duLL-17D treatment led to increased expression of IL-1β, IL-6, and IL-8 in primary DEF. However, duLL-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 duLL-17D (Fig. 1D) and chLL-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 duLL-17D (Fig. 1D) and chLL-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 duLL-17D could be modulated, splenic lymphocytes were stimulated in vitro and duLL-17D mRNA was assessed (Fig. 1E). The level of duLL-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.
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 (AF493595), chicken IL-17C (Q776598), duck IL-17A (P751329), duck IL-17F (K003421), salmon IL-17D (EU689087), trout IL-17D (NM_001243399), and frog IL-17D (NM_00114247). (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-old chickens. (E) Activation of splenic lymphocytes with ConA, LPS, or Poly I:C. (F) Western blot analysis of supernatant and cell lysate from chickens treated with recombinant duIL-17D. (G) Flow cytometry analysis of IL-6, IL-8, IL-1β, and TGFβ1 expression in chickens treated with duIL-17D and TNFα.
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.


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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.jdi.2016.04.002.

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2015R1D1A1A02059953).

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**R. anatipestifer** is a Gram-negative, non-motile, non-sporulating, 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, septicaemia, and meningitis, and is associated with a 5%-75% mortality rate depending on the virulence of the strain (Pathanasonphon 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 levels in the spleen of ducks and during pathogenic bacterial infections. It is interesting to note that changed or downregulated to facilitate effective host immunity in Atlantic salmon was significant interacting, whereas Hong et al. (2008) used an intracellular parasite E. maxima, whereas Kim et al. (2013) and 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.

**Acknowledgements**

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**dx.doi.org/10.1016/j.jdi.2016.04.002.**
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