Molecular Immunology 95 (2018) 20–29

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Identification of duck IL-4 and its inhibitory effect on IL-17A expression in *R. anatipestifer*-stimulated splenic lymphocytes

Cherry P. Fernandez[a], Fahmida Afrin[a], Rochelle A. Flores[a], Woo H. Kim[c], Jipseol Jeong[b], Suk Kim[a], Hyun S. Lillehoj[c], Wongi Min[a,*]

[a] College of Veterinary Medicine & Institute of Animal Medicine, Gyeongsang National University, Jinju, 52828, Republic of Korea
[b] Environmental Health Research Division, National Institute of Environmental Research, Incheon, 22689, Republic of Korea
[c] Animal Biosciences and Biotechnology Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD, 20705, USA

ARTICLE INFO

Keywords:
Duck
Splenic lymphocyte
Interleukin-4
Inhibition of Th17 cytokine expression
Riemerella anatipestifer

ABSTRACT

As the dysregulation of IL-17 is implicated in the pathogenesis of various autoimmune and inflammatory diseases, the suppression of IL-17 production by Th2 cytokines could alleviate the development of these diseases. Previously, we confirmed that inflammatory cytokines including IL-17A are strongly associated with *R. anatipestifer* infection, which is one of the most important bacterial pathogens in the duck industry. Here, we found that IL-4 treatment downregulated the expression of IL-17A and IL-17F transcripts in splenic lymphocytes stimulated with *R. anatipestifer*. Moreover, duck IL-4 (duIL-4) treatment in *R. anatipestifer*-stimulated lymphocytes suppressed the expression of IL-23p19 and IL-12p40 transcripts compared to untreated and stimulated lymphocytes. Conversely, duIL-4 increased levels of IFN-γ and IL-10. We identified a full-length duIL-4 cDNA encoding 136 amino acids from ConA-activated splenic lymphocytes that shares 49.3–50% amino acid sequence identity with chicken and quail IL-4 and 21–29.7% with mammalian and piscine homologues. Low or moderate levels of duIL-4 transcript were observed in healthy tissues, including the spleen, bursa, and thymus, whereas duIL-4 expression was higher in the kidney and lung. Levels of duIL-4 were generally upregulated in mitogen-activated splenic lymphocytes but lower in the liver and spleen of *R. anatipestifer*-infected ducks compared to those of infected chickens. Recombinant duIL-4 promoted nitric oxide synthesis in duck macrophages stimulated by *R. anatipestifer* compared to untreated and stimulated control macrophages. These results demonstrate that IL-4 is an important Th2 cytokine that inhibits inflammatory responses in splenic lymphocytes stimulated with *R. anatipestifer*.

1. Introduction

Interleukin-4 (IL-4), a hallmark Th2 cytokine, was initially identified in mice as a B-cell growth factor (Howard et al., 1982) and is produced by a wide range of innate and adaptive immune cells including CD4+ T cells, CD8+ T cells, NK cells, and γδT cells (Seder et al., 1992; Leite-de-Moraes and Dy, 1997; Gerber et al., 1999). IL-4 is an important effector cytokine with diverse biological functions; it not only promotes immune response and homeostasis but is also involved in immune regulation, particularly the balance among Th1, Th2, and Th17 cytokines (Bettelli et al., 2008; Min et al., 2013). Recent studies show that dysregulated expression of IL-4 is implicated in allergic inflammation and asthma (Steinki and Borish, 2001; Liang et al., 2012) and leukemic cutaneous T-cell lymphoma (Guenova et al., 2013). Several groups demonstrated that IL-4 therapy abrogates inflammatory autoimmune diseases such as experimental autoimmune encephalomyelitis (Racke et al., 1994), insulitis and diabetes (Mueller et al., 1996), psoriasis (Ghoreschi et al., 2003), arthritis (Sarkar et al., 2009), and colitis (Sheikh et al., 2011). Recent studies established the involvement of Th1 and Th17 in disease pathogenesis and show that multiple sclerosis, non-obese diabetes and autoimmune encephalomyelitis can be attenuated by neutralization or suppression of Th1 and Th17 (Solt et al., 2011; Ikeda et al., 2014).

R. anatipestifer, a gram-negative extracellular bacterium, is the most economically important major infectious pathogen affecting the duck industry (Zhou et al., 2013). Although other avian species are also susceptible to *R. anatipestifer* infection, ducks are the most susceptible (Ruiz and Sandhu, 2013, Cha et al., 2015, Fernandez et al., 2016). At least 21 serotypes of *R. anatipestifer* have been identified and are associated with a 5–75% mortality rate depending on the virulence of the strain (Pathanasophon et al., 2002; Ruiz and Sandhu, 2013). Several efforts to study *R. anatipestifer* have primarily focused on identification

* Corresponding author at: College of Veterinary Medicine, Gyeongsang National University, 501 Jinju-daero, Jinju, Gyeongnam 52828, Republic of Korea.
E-mail address: wongimin@gnu.ac.kr (W. Min).

https://doi.org/10.1016/j.molimm.2018.01.009
Received 14 August 2017; Received in revised form 29 December 2017; Accepted 19 January 2018

of various immunoreactive proteins (Hu et al., 2012; Zhai et al., 2012) and several virulence factors, including outer membrane protein A, CAMP cohemolysin, virulence-associated protein D, and lipopolysaccharide (LPS)-associated genes (Hu et al., 2011; Crasta et al., 2002; Chang et al., 1998; Wang et al., 2014). Recent report suggests that Th17 cytokines, including IL-17A and IL-17F, are significantly upregulated in *R. anatipestifer*-infected ducks compared to *R. anatipestifer*-infected chickens, whereas IL-4 is generally unchanged or downregulated in infected ducks but upregulated in infected chickens (Fernandez et al., 2016).

The role of cytokines in avian species is poorly understood. IL-4 transcript levels have been monitored in intestinal lymphocytes of *Eimeria*-infected chickens (Hong et al., 2006), splenic tissues of chickens infected with a highly oncogenic strain of Marek's disease virus (Heidari et al., 2008), and macrophages and lungs of H9N2-infected chickens (Xing et al., 2008). However, little is known about whether avian IL-4 serves a protective and pathogenic role in certain diseases. This prompted us to investigate whether duck IL-4 (duIL-4) could suppress the production of Th17 cytokines induced by *R. anatipestifer*. Here, we provide the first description of full-length duIL-4 cDNA, the bioactivity of recombinant duIL-4 protein, and the expression profiles of duIL-4 transcript in various healthy tissues and mitogen-stimulated splenic lymphocytes using quantitative reverse-transcription polymerase chain reaction (qRT-PCR), Western blot analysis, and nitrite assay. We also examined comparative expression profiles of inflammatory and related cytokines in duck splenic lymphocytes treated with recombinant duIL-4 protein and then stimulated with *R. anatipestifer*. Our results indicate that IL-4 may inhibit the production of Th17 cytokines induced by *R. anatipestifer*.

2. Materials and methods

2.1. Animals and infections

Pekin ducklings (*Anas platyrhynchos*) and Ross chickens were obtained from Joowon ASTA Ducks (Gyeongnam, Korea) and Samhwa (Chungnam, Korea), respectively. Animals were raised in wire cages in a temperature-controlled environment with unlimited access to anticoccidial/antibiotic-free feed, water, and constant light for the duration of the experiments. Birds were randomly divided into infected and non-infected control groups (n = 75/group), which were housed in separate buildings. *R. anatipestifer* serotype 7 was grown in blood agar plates with 5% sheep blood (Asan Pharmaceutical, Korea), as previously described (Fernandez et al., 2017). Two-week-old ducks and chickens were infected intramuscularly with 5×10^7 CFU of *R. anatipestifer* serotype 7 in 200 µl phosphate-buffered saline (PBS) in the thigh muscle using a standard needle (26 gauge). The same volume of PBS was given to control birds via intramuscular injection in the thigh muscle. Five birds from each group of ducks and chickens were euthanized by atlanto-occipital dislocation, and spleen and liver tissues were aseptically collected 0, 1, and 4 days post-infection. All animal maintenance and experimental procedures were performed in accordance with 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.

2.2. Molecular cloning of duIL-4 cDNA and sequence analysis

Total RNA was extracted from ConA-activated duck splenic lymphocytes using RiboEx reagent (GeneAll, Korea) and RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted total RNA was treated with RNase-free DNase I (Fermentas, Canada) to remove any contaminating genomic DNA, and single-stranded cDNA was synthesized from the treated total RNA using the QuantiTect reverse-transcription kit (Qiagen). Based on a predicted cDNA sequence (GenBank accession no. XM_005024359.3), full-length duIL-4 cDNA was obtained from splenic lymphocyte cDNA by performing 5′/3′-Rapid Amplification of cDNA Ends (RACE) using duIL-4 specific primers (for 5′ RACE 5′-GTGCTGGAGTGCTCGATCTT-3′ and for 3′ RACE 5′-GTGACAAAGAGCACCAGCCT-3′), high-fidelity DNA polymerase (Bioneer, Korea), and a 5′/3′ RACE kit (2nd generation; Roche Applied Science, Germany) according to the manufacturer's instructions. A DNA Engine thermocycler (Bio-Rad, USA) was used for PCR with the following conditions: 1 cycle for 5 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C, and 1 min extension at 72°C, with a final 5 min extension at 72°C. The cloned cDNA in TA vector (RBC, Taiwan) was confirmed by sequencing (Macrogen, Korea), and the nucleotide sequence was submitted to GenBank (accession no. MF346730).

Protein identification was performed by computer-assisted sequence analysis using the Expert Protein Analysis System (ExPASy; www.expasy.org/tools/). Amino acid multiple alignments and percent identity and similarity were generated using Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) and EMBOSS Needle pairwise sequence alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), respectively. 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 using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.3. Cell culture

Duck splenic lymphocytes isolated from 2-week-old ducks were cultured as previously described (Fernandez et al., 2017). Briefly, duck spleens were gently passed through a cell strainer (SPL Life Science, Korea) with a syringe plunger to obtain single cell suspensions in Hank's balanced salt solution (HBSS) (Sigma-Aldrich, USA). Splenic lymphocytes were collected by Ficoll-Paque PLUS (GE Healthcare, USA) according to the manufacturer's protocol. Cells were collected, washed twice with PBS, and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies) and penicillin-streptomycin (10,000 unit/ml) in a 41°C incubator with 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; Sigma-Aldrich, Germany), 10 µg/ml LPS (from *E. coli*, O111:B4; Sigma-Aldrich), or 10 µg/ml concanavalin (ConA; Amersham Bioscience, Sweden) for 4, 8, or 24 h. COS-7 cells were cultured as described above in a 37°C incubator with 5% CO_2. Total RNA was extracted from stimulated cells, and cDNA synthesis was performed using the QuantiTect reverse-transcription kit (Qiagen) and random hexamer primers. Analysis of duIL-4-induced gene expression was performed by qRT-PCR using the primers listed in Table 1.

2.4. Production of recombinant duIL-4 protein

The duIL-4 nucleotide coding sequence was amplified by PCR using the following specific primers: for pCDNA 3.1(+) vector, forward 5′-GATCAAGCTTATGAGCACCTCCTTCTCCGT-3′ and reverse 5′-GATCGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCCAAATTCTCTTTTGCTA-3′; for pET28b(+), forward 5′-GATCGAATTCGCAGCACCTCCTTCTCCGTCCT-3′ and reverse 5′-GATCAAGCTTTCACAAATTCTCTTTTGCTACTC-3′. The primers contained Hind*III* and EcoR*I* restriction enzyme sites (solid lines) and a MYC tag sequence (dashed line). PCR products were digested with Hind*III* and EcoR*I* and cloned into the corresponding restriction enzyme sites of the pCDNA 3.1(+) (Invitrogen, USA) and pET28b(+) (Novagen, USA) vectors. duIL-4 cloned in pET28b(+) was transformed into *E. coli* BL21 (DE3) competent cells (RBC). The duIL-4 harboring a MYC-tag expression sequence (duIL-4-MYC) and empty vector were transiently transfected into COS-7 cells using FuGene 6 transfection reagent (Promega, USA)