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Downregulation of common cytokine receptor γ chain inhibits inflammatory responses in macrophages stimulated with *Riemerella anatipestifer*

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ABSTRACT

Th17-cell-mediated inflammation is affected by the soluble form of common cytokine receptor γ chain (γ_c). We previously suggested that inflammatory cytokines including interleukin (IL)-17A are associated with *Riemerella anatipestifer* infection, which a harmful bacterial pathogen in ducks. Here, the expression profiles of membrane-associated γ_c (duγ_c-a) and soluble γ_c (duγ_c-b) in *R. anatipestifer*-stimulated splenic lymphocytes and macrophages, and in the spleens and livers of *R. anatipestifer*-infected ducks, were investigated. *In vitro* and *in vivo* results indicated that the expression levels of both forms of γ_c were increased, showing that marked increases were detected in the expression of the duγ_c-b form rather than the duγ_c-a form. Treatment with γ_c-specific siRNA downregulated mRNA expression of Th17-related cytokines, including IL-17A and IL-17F, in duck splenic macrophages stimulated with *R. anatipestifer*, whereas the expressions of interferon (IFN)-γ and IL-2 were enhanced. The results showed that the upregulation of γ_c, especially the duγ_c-b form, was associated with expression of Th17-related cytokines during *R. anatipestifer* infection.

1. Introduction

Riemerella anatipestifer infection is a significant disease confronting the duck industry worldwide. *R. anatipestifer* is a gram-negative, non-motile, and non-sporulating bacterium (Ruiz and Sandhu, 2013). To date, at least 21 serotypes of *R. anatipestifer* that vary in virulence both between and sometimes within a given serotype have been identified, with no significant cross-protection between these serotypes (Sandhu and Leister, 1991; Pathanasophon et al., 2002). *R. anatipestifer* infection causes an acute or chronic septicemia, polyserositis, fibrinous pericarditis, perihepatitis, and airsacculitis, which leads to high mortality and weight loss, and consequently to major economic losses in duck farmers (Ruiz and Sandhu, 2013). Thus, several studies have been performed to understand the host immune responses to *R. anatipestifer*. Using immunoproteomics, a variety of immunogenic proteins were identified using duck or rabbit antisera to *R. anatipestifer* (Hu et al., 2012; Zhai et al., 2012). Host genes related to inflammatory responses were identified in the liver of *R. anatipestifer*-infected ducks (Zhou et al., 2013). Recently, we suggested that upregulation of interleukin (IL)-17A in ducks was strongly associated with *Riemerella* infection, by comparative expression analyses of immune-related genes in *R. anatipestifer*-infected tissues obtained from chickens and ducks (Fernandez et al., 2016, 2017; Diaz et al., 2016).

The IL-17 family of cytokines consists of six members (IL-17A to IL-17F) with varying degrees of intermolecular amino acid sequence homology and related biological activities in mammals (Korn et al., 2009; Min et al., 2013). IL-17A is the best characterized family member and is produced mainly by IL-17A-producing $CD4^+$ T helper cells, also called Th17 cells. IL-17A is produced by multiple cell types including Th17 cells, lymphoid tissue inducer-like cells, γδ T cells, invariant natural killer T (iNKT) cells, NKT cells, mast cells,

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and neutrophils (Pappu et al., 2011; Reynolds et al., 2010). Th17 cell development and differentiation is influenced by various cytokines, with distinct roles for IL-6, transforming growth factor beta (TGFβ), IL-1β, IL-21, and IL-23 (Bhaumik and Basu, 2017; Reynolds et al., 2010). Thus, IL-17A plays a critical role in host protective immunity against various microbial pathogens and tissue inflammation, and aberrant IL-17A expression is involved in the pathogenesis of several inflammatory disorders (Pappu et al., 2011; Chen and Kolls, 2017).

Recent studies suggested that exacerbation of Th17 cell-mediated inflammation can be affected by the soluble form of the common cytokine receptor γ chain (γ_c) (Hong et al., 2014; Lee and Hong, 2015). The γ_c, which is also known as interleukin-2 receptor (IL-2Rγ) or CD132, is a subunit shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and it is expressed on T cells, B cells, NK cells, monocytes, macrophages, granulocytes, and dendritic cells, where it mediates lymphoid development, proliferation, and homeostasis in both innate and acquired immunity (Walsh, 2012; Overwijk and Schluns, 2009). Our previous studies identified conventional and soluble forms of γ_c ($s\gamma_c$) in birds that can be generated by alternative splicing of mRNA transcripts lacking a transmembrane region (in ducks) or by proteolytic cleavage (in chickens) (Min et al., 2002; Jeong et al., 2011, 2015). Thus, we were interested in elucidating the role of the γ_c gene in the upregulation of IL-17A expression during *R. anatipestifer* infection in ducks, and we have demonstrated here that reduced expression of the γ_c gene by γ_c-specific small interfering RNA ($si\gamma_c$) downregulated the expression of inflammatory cytokines, including IL-17A.

2. Materials and methods

2.1. Animals and infections

Peking ducklings (*Anas platyrhynchos*) were obtained from Joowon ASTA Ducks (Gyeongnam, Korea) and given unlimited access to antibacterial/anticoccidial-free feed and water. Constant light was provided for the duration of the experiments. Two-week-old ducks were randomly divided into two groups (infected and non-infected) with separate housing for each group. *R. anatipestifer* serotype 7 was grown on 5% sheep blood agar plates (Asan Pharmaceutical, Seoul, Korea) at 37 °C in 5% CO_2. A single colony was inoculated into tryptic soy broth (Difco, Livonia, MI, USA) and incubated in a shaking incubator at 37 °C until growth phase was achieved. The final inoculum concentration was determined by plating 0.1 mL of 10-fold serial dilutions onto 5% sheep blood agar plates. Ducks were infected intramuscularly with 0.2 mL suspensions containing 5×10^7 colony-forming unit (CFU) *R. anatipestifer*. The same volume of phosphate-buffered saline (PBS) was provided to the control ducks. Spleens and livers were collected from each group of ducks at 4 and 7 days post-infection. Animal sustenance and experiments were accomplished 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, Korea (GNU-150504-C0026).

2.2. Bacterial recovery

Bacteria were recovered from 0.1 g liver and 0.05 g duck spleen, and were aseptically collected and homogenized separately in 500 μL tryptic soy broth using tissue homogenizers. Homogenized samples were diluted serially 10- or 100-fold, plated on 5% sheep blood agar plates, and incubated at 37 °C under 5% CO_2. The bacterial colonies were calculated as CFU/g tissue.

2.3. In vitro stimulation of splenic lymphocytes and macrophages

From two- or three-week-old healthy ducks, spleens were collected, minced, and sieved with a 40-μm nylon strainer. Splenic lymphocytes were isolated using Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, Hertfordshire, UK) following the manufacturer's instructions, washed with PBS, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (10,000 unit/mL) (Hyclone), then incubated at 41 °C in 5% CO_2. Splenic lymphocytes were resuspended to 5×10^6 cells/mL in 6-well plates, and stimulated with heat-killed *R. anatipestifer* (1×10^6 CFU/mL) for 12 h or 24 h. Spleen cells for macrophages were cultured at 10^6 cells/mL for approximately 3 h at 41 °C in 5% CO_2, as previously described (Chung et al., 2000; Kim et al., 2003). Briefly, nonadherent cells were removed by washing with DMEM until visual inspection revealed a lack of lymphocytes. The adherent cells were removed from plates by incubating for 3–5 min with ice-cold Accutase® Cell Detachment Solution (Innovative Cell Technologies, San Diego, CA, USA) and rinsing repeatedly. The adherent cells were incubated with heat-killed *R. anatipestifer* (1×10^6 CFU/mL) for 12 h or 24 h. The heat-killed *R. anatipestifer* was prepared by heating in a water bath at 100 °C for 5 min.

2.4. RNA interference

Macrophages were transfected with 10 nM γ_c-specific small interfering RNA ($si\gamma_c$) using Lipofectamine® RNAiMAX (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, macrophages were seeded at a density of 5×10^5 cells per well in 6-well plates and transfected with $si\gamma_c$-Lipofectamine® complexes. After 24 h post-transfection, cells were stimulated with heat-killed *R. anatipestifer* (1×10^6 CFU/mL) for 24 h. Total RNA was extracted from the cultured cells using RiboEx reagent (Geneall Biotechnology, Seoul, Korea), treated with RNase-free DNase I (Fermentas, Burlington, Ontario, Canada), and used for cytokine expression analyses by quantitative reverse-transcription RT-PCR (qRT-PCR) with gene-specific primers (Table 1). A nonsense siRNA (siNC) was used as a negative control for non-sequence specific effects (Bioneer, Daejeon, Korea).

2.5. Cotransfection of $si\gamma_c$ and γ_c constructs

COS-7 cells were maintained in DMEM supplemented by 10% FBS and penicillin/streptomycin (10,000 unit/mL). One day before transfection, these cells were trypsinized and resuspended in DMEM, and plated onto a 6-well plate. COS-7 cells were cotransfected with $si\gamma_c$ or siNC (Table 1) and γ_c constructs ($du\gamma_c$-a flag or $du\gamma_c$-b flag) (Jeong et al., 2015) using Lipofectamine® reagent (Invitrogen) according to the manufacturer's instructions. The cells were harvested for Western blot analyses 48 h of cotransfection. We used 10 nM $si\gamma_c$ and 10 μg constructs for transfections.

2.6. Western blot analysis

Cell supernatants and lysates 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 transferred to polyvinyl difluoride membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with PBS containing 5% nonfat dry milk for 2 h at room temperature and incubated with anti-flag M2 antibody (Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. After washing three times with PBS containing 0.1% Tween 20 (PBS-T), membranes were incubated with horseradish