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Research paper

Functional analyses of the interaction of chicken interleukin 23 subunit p19 with IL-12 subunit p40 to form the IL-23 complex

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ABSTRACT

This study represents the first description of the cloning of chicken IL-23p19 (ChIL-23α) and the function of the IL-23 complex in birds. Multiple alignment of ChIL-23α with other known IL-23α amino acid sequences revealed regions of amino acid conservation. The homologies of ChIL-23α, IL-12p35, and similar mammalian subunits ranged between 26% and 42%. ChIL-23α consisted of four exons and three introns; similar to those in humans and mice, and limited conservation of synteny between the human and chicken genomes was observed. Using bioinformatics tools, we identified the NF-κB, C/EBPα-β, c-Jun, c-Rel, AP-1, GATA-1, and ER promoter sites in ChIL-23α. Moreover, IL-23α mRNA was more highly expressed than IL-12p40 and IL-12p35 mRNA in several organs of chickens infected with *Salmonella*. In addition, ChIL-23 complex are associated with IL-23R, IL-12Rβ1 receptors; activate the JAK2/TYK2, STAT1/3, SOCS1 genes, and induced proinflammatory cytokines in immune cells. Collectively, these results indicate that ChIL-23 is a member of the IL-12 family, has proinflammatory properties related to IL-23R and IL-12Rβ1 receptor expression, and activates the JAK/STAT signaling pathway that results in the interaction of ChIL-23α with ChIL-12p40 to form the novel ChIL-23 complex. Our results provide novel insights into the regulation of immunity, inflammation, and immunopathology.

1. Introduction

Type I cytokines, which include IFN-γ, IL-12, IL-23, IL-27, and IL-35, play an important role in protective immunity against intracellular pathogens, as well as inflammatory and organ-specific autoimmune diseases (Trinchieri, 2003). IL-12 consists of p35 and p40 subunits and the biological activities of this cytokine are mediated via binding with the IL-12Rβ1/IL-12Rβ2 complex, in which IL-12Rβ1 binds with IL-12p40 and is associated with TYK2, whereas IL-12Rβ2 recognizes IL-12p35 and is associated with JAK2 (Trinchieri, 2003). Signaling through the IL-12 receptor complex induces several STAT transcription factors (1, 3, 4, 5); however, most of the biological responses to IL-12 are mediated by STAT4 (Gee et al., 2009; Trinchieri, 2003). Recently, IL-23 was identified as a new member of the IL-12 family of cytokines in mammalian tissue (Oppmann et al., 2000). The IL-23 complex is a heterodimeric cytokine that comprises the p40 subunit of IL-12 and a specific p19 subunit of IL-23. The p40-p19 complex is secreted by activated dendritic cells (DCs), T cells, and macrophages (Bettelli and Kuchroo, 2005).

In humans, the IL-23 complex receptor comprises IL-23R and IL-12Rβ1. IL-23 binds to its receptor and is essential for T helper (Th17) (Bettelli and Kuchroo, 2005) and Th1 subset cells (Kobayashi et al., 2008) differentiation, expansion, and survival, and thereby activates transduction pathways, such as the JAK/STAT, MAPK, Erk1/2, PI3K/Akt and TLRs pathways (Cho et al., 2006; Langrish et al., 2005; Toussirot, 2012). Recently, several groups have demonstrated that expression of the p19 gene is regulated by the NF-κB transcription factor, and MAPK, JAK/STAT, and TLRs pathways (Cho et al., 2006; Rodriguez et al., 2014; Zhu et al., 2012). Moreover, human studies have revealed that the IL-23/Th17/Th1 pathway is implicated in the pathophysiology of various autoimmune diseases such as psoriasis (Vaknin-Dembinsky et al., 2006), multiple sclerosis and rheumatoid arthritis (Brentano et al., 2009), autoimmune arthritis and primary biliary cirrhosis (Qian et al., 2013), and inflammatory bowel disease (Fransen et al., 2014). IL-23 induces Th17 and Th1 subset cells population with a distinctive inflammatory gene signature that includes IL-17A, IL-17F, IL-6, and

Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling; TYK2, tyrosine kinase 2; NF-κB, nuclear factor kappa B; C/EBP, CCAAT-enhancer-binding proteins; GATA-1, GATA-binding factor 1; AP-1, Activator protein 1

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particularly IFN-γ (Langrish et al., 2005). Moreover, it is expressed early in pathogen infections and then promote IFN-γ production, so IL-23 may act as a factor important in recruiting early Th1 differentiation by inducing IFN-γ to produce cytokines in response to pathogens (Klasing and Peng, 1987; van de Wetering et al., 2009). In addition, IL-23 production is induced by various stimuli such as Gram-negative bacteria and their component lipopolysaccharide (LPS), such as *Staphylococcus aureus* peptidoglycan (Smits et al., 2004) and the *Sendai* virus (Pirhonen et al., 2002). However, pertinent information is lacking; for example, no data are available on the primary structure of IL-23p19 (IL-23α), and the function of IL-23 in the chicken has not yet been investigated.

In the present study, to clarify the role of the IL-23α subunit, we cloned the entire open reading frame of chicken IL-23α. We adopted a genomics approach based on conservation of synteny, and undertook a characterization and functional analysis of chicken IL-23 as it relates to the chicken HD11 macrophage and CU91 T cell lines. Our findings suggest that chicken IL-23 activates the JAK/STAT signaling pathway and induces cytokine production. Because IL-23 can act as a positive regulator Th1/Th17 immune responses, we also evaluated IL-23 mRNA expression in various tissues of chickens infected with *Salmonella* serovar Enteritidis.

2. Materials and methods

2.1. Reagents

Rabbit anti-chicken SOCS1, anti-chicken STAT1, anti-chicken STAT3 antibodies, and horseradish peroxidase (HRP)-linked anti-rabbit secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following reagents were either purchased from or provided by each respective company: rabbit anti-chicken against JAK2 and TYK2 (Biorbyt, San Francisco, CA, USA); anti-phospho-STAT1 (Ser^{727}), anti-phospho-STAT3 (Ser^{727}), and anti-phospho-JAK2 (Tyr^{1007}/Tyr^{1008}) antibodies (Santa Cruz Biotech, Dallas, Texas, USA); anti-GAPDH antibody (Abcam, Cambridge, MA, USA); anti-His (C-Term)-HRP, Alexa Fluor® 488 Goat Anti-Rabbit IgG (H + L) secondary antibody (Invitrogen, Carlsbad, CA, USA); anti-chicken-IL-12p40 antibody (AbIL-12p40) (Kingfisher Biotech, St. Paul, MN, USA); and mouse monoclonal anti-chicken IFN-γ antibody and recombinant protein (kindly provided from Dr. Hyun Lillehoj, USDA). EZ-Link™ Sulfo-NHS-LC-Biotin, goat anti-mouse IgG-HRP conjugate and HRP-Conjugated Streptavidin were purchased from Thermo Scientific (Waltham, MA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and cell extraction buffer were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Experimental animals and infection

Twenty-four to twenty-six-week-old specified pathogen-free White Leghorn chickens, *S. enterica* serovar Enteritidis (S.E), and tissue samples were provided by Dr. Uma S. Babu of the Center for Food Safety and Applied Nutrition, FDA, USA. Experimental and control groups were kept in separate level 2 biosafety rooms, and commercial feed (without antibiotics or other additives) and drinking water were provided *ad libitum*. Chickens were divided into two groups. One group was infected with 1.0×10^9 CFU of S.E in 1 mL of phosphate-buffered saline (PBS). The second (control) group was mock infected with 1 mL of PBS. Five chickens per group were randomly selected for sample collection at 7 days post infection (dpi). Tissue samples from the small intestine, lung, ovary, liver, spleen, oviduct, and thymus were collected aseptically and placed in liquid nitrogen for total RNA extraction. All procedures were performed according to guidelines for the ethical treatment of animals and were approved by the Institutional Animal Care and Use Committee of the Center for Food Safety and Applied Nutrition, FDA, USA.

2.3. Construction of the chicken IL23α-IL12p40 (ChIL-23) fusion vector

Total RNA was isolated from tissue samples of chickens using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Approximately 2.0 μg of RNA was subjected to reverse transcription, using oligo$(dT)_{18}$ as the primer and SuperScript II Reverse Transcriptase according to the manufacturer's protocol (Thermo Scientific). For amplification of the full-length DNA coding sequences of ChIL-23α (XM_003643958) and ChIL-12p40 (NM_213571), a candidate sequence was identified from the chicken genome database. The PCR products were purified using the QIAQuick gel Extraction Kit (QIAgen, Hilden, Germany) and cloned into the pCR2.1-TOPO vector (Invitrogen), following which, they were transformed using *Escherichia coli* TOP10 high-efficiency competent cells (Invitrogen). The positive clones were sequenced through light blue-white screening at Genotech in Daejeon, Republic of Korea.

ChIL-23α and ChIL-12p40: Full-length ChIL-23α and ChIL-12p40, originally cloned in pCR2.1 (Invitrogen), were excised using *Eco*RI/*Hin*dIII and *Nco*I/*Eco*RI restriction enzymes, respectively, and ligated into the multiple expression vector pTriex™-4 (Novagen, Cambridge, MA, USA). The products were then transformed into *E. coli* BL21 (Invitrogen) and sequenced for confirmation.

Chicken IL12p40-IL23α-(ChIL-23 complex): ChIL-23α and ChIL-12p40 fragments were ligated into pTriex™-4 to obtain an antisense control construct. The entire ChIL-12p40 fragment was amplified with an *Nco*I site at the N terminus for subsequent cloning in the pTriex™-4 expression vector, an *Eco*RI site at the C terminus for ligation to the N-terminal sequence of ChIL-23α, and insertion of a sequence encoding flexible glycine/serine-rich peptide linker sequences (GGGGSGGSGSGGG, $G_{10}S_3$-linker). The primers used for PCR were 5′-CGC CAT GGA TGT CTC ACC TGC TAT TTG C-3′ and 5′-CGG AAT TCT CCA CCT CCA GAA CCG GAT CCA CCT GAT CCA CCT CCA CCT CTG CAA AGC GTG GAC CA-3′ (restriction sites are underlined). The DNA fragment for ChIL-23α was joined with *Hin*dIII at the C terminus, which was directly joined to a 6 × His tag. The PCR primers were 5′-CGG AAT TCA TGG CCC CGC TCC GCC G-3′ (sense) and 5′-CCA AGC TTG CGG TGC TGG CGC CGT-3′ (antisense). This yielded a single-chain ChIL-12p40-IL23α heterodimeric construct in which the IL-12p40 chain was linked to the IL-23α chain by an in-frame $G_{10}S_3$-linker (Stirnemann et al., 2008). The ChIL23α-IL12p40-pTriex™-4 (ChIL-23) structure was transformed into *E. coli* BL21 (Invitrogen) and confirmed by sequencing.

2.4. Recombinant protein production and purification

The expression of each recombinant protein was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; USB Corporation, Cleveland, OH, USA), and bacteria were cultured in Luria-Bertani medium (Difco™ & BBL™, Sparks, MD, USA) containing ampicillin (50 mg/mL; USB Corporation) at 37 °C overnight and 250 rpm. The bacteria were pelleted by centrifugation at 3500 × *g* for 30 min at 4 °C. The supernatant was removed and the pellet was treated with B-PER Bacterial Protein Extraction reagent (Thermo Scientific). Recombinant protein was purified using HisPur Ni-NTA Resin (Thermo Scientific) according to the manufacturer's instructions. To remove bacterial endotoxin contaminants, we combined affinity chromatography with non-ionic detergent washing and dialysis as previously described (Mack et al., 2014; Reichelt et al., 2006). This two-step purification of recombinant protein procedure allows a drastic depletion of endotoxin contaminants. The purified protein was concentrated and the buffer was changed via ultrafiltration using a 3000 molecular weight cut-off membrane (EMD Millipore, Temecula, CA, USA). Dialysis was performed on the samples at 4 °C using Snake Skin™ Dialysis Tubing (Thermo Scientific) overnight with constant stirring, and checked by SDS-PAGE.