# Functional Characterization of the Avian Inflammatory Mediators Nod1, MIF and IL-22

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## ABSTRACT

Inflammation can be initiated by an innate immune sensor, followed by activation of a signal mediator, resulting in control of immune response by a signal regulator. Mammalian nucleotide-binding oligomerization domain protein 1 (Nod1) and Nod2 initiate host innate immune response by recognition of specific bacterial molecules, resulting in the production of pro-inflammatory cytokines, chemokines, and anti-microbial peptides. A candidate sequence of chicken Nod1 (ChNod1) was identified with no current evidence of ChNod2. Stimulation of transiently overexpressed ChNod1 and its mutants with mammalian Nod-specific ligands was not conclusive of the function of ChNod1 most likely due to self-activation of ChNod1. In vitro studies showed no significant difference in expression of Nod1, its signaling molecules and pro-inflammatory cytokines in stimulated chicken mononuclear cells with synthetic ligands for mammalian Nod1 or Nod2.

A signal mediator, macrophage migration inhibitory factor (MIF) inhibits the random migration of macrophages. Chemotaxis assay using recombinant ChMIF (rChMIF) revealed a substantial decrease in migration of macrophages. qRT-PCR analysis revealed that the presence of rChMIF enhanced levels of IL-1β and iNOS during monocytes stimulation with LPS. Additionally, Con A-stimulated lymphocytes exhibited enhanced IFN-γ and IL-2 transcripts in the presence of rChMIF.

IL-22, which may act as a signal regulator, is an important effector of activated Th1 and Th17 as well as natural killer cells during inflammation. Recombinant ChIL-22 alone did not have an impact on chicken embryo kidney epithelial cells (CKECs); however, co-stimulation of CKECs with LPS and rChIL-22 enhanced the production of pro-inflammatory cytokines and anti-microbial peptides. Furthermore, rChIL-22 alone stimulated acute phase reactants in chicken embryo liver cells. These effects of rChIL-22 were abolished by addition of rChIL22 binding protein. Taken together, these results indicate an important role of ChIL-22 on epithelial cells and hepatocytes during inflammation.

In this project, we identified and characterized the avian inflammatory mediators ChNod1, ChMIF, and ChIL-22. Studying each of their biological function in avian inflammation, especially under pathogenic challenges in epithelial tissues will provide a foundation for understanding the role of these inflammatory mediators in mucosal immunity.

Keywords: Nod1, MIF, IL-22, inflammation, cytokines

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## List of Abbreviations

**AMP:** Antimicrobial peptide

**AP1:** Activator protein 1

**APCs:** Antigen presenting cells

**CARD:** Caspase activation and recruitment domains

**CD:** Cluster of differentiation

**CpG:** Cytosin-phosphate-Guanin

**DAMPs:** Damage-associated molecular patterns

**DCs:** Dendritic cells

**DMEM:** Dulbecco's Modified Eagle Medium

**Duo:** Duodenum

**ERK:** Extracellular signal-regulated kinases

**FCS:** Fetal Calf Serum

**GAPDH:** Glyceraldehyde 3-phophate dehydrogenase

**GM-CSF:** Granulocyte-Macrophage Colony Stimulating Factor

**h:** Hour

**IBD:** Inflammatory bowel disease

**iE-DAP:** γ-$_{D}$-*meso*-diaminopimelic acid

**IFN:** Interferon

**Ig:** Immunoglobulin

**IL:** Interleukin

**Ile:** Ileum

**iNOS:** Inducible nitric oxide synthase

**IRFs:** IFN response fector

**Jej:** Jejunum

**JNKs:** c-Jun N-terminal kinases/stress-activated protein kinase

**LPS:** Lipopolysaccharide

**LRR:** Leucine-rich repeat

**LTA:** Lipoteichoic acid

**MAMPs:** Microbial-associated molecular patterns

**MAPKs:** Mitogen-activated protein kinases

**MDP:** Muramyl dipeptide

**MHC:** Major histocompatability complex

**MIF:** Macrophage migration inhibitory factor

**min:** Minute

**MyD88:** Myeloid differentiation primary response gene 88

**NF-κB:** Nuclear factor kappa-light-chain-enhancer of activated B cells

**NK:** Natural killer

**NLR:** NOD-like receptor

**NO:** Nitric oxide

**NOD:** Nucleotide-binding oligomerization domain

**PBMCs:** Peripheral blood mononuclear cells

**PGN:** Peptidoglycan

**PRRs:** Pattern recognition receptors

**qRT-PCR:** Quantitative real-time polymerase chain reaction

**RIG-I:** Retinoic acid-inducible gene-I

**RIPK2:** Receptor-interacting serine/threonine-protein kinase 2

**RLR:** RIG-I-like receptor

**sec:** Second

**STAT:** Signal transducers and Activators of Transcription

**TAK1:** Transforming growth factor beta-activated kinase 1

**Th:** Helper T cells

**Th1:** Type 1 helper T cell

**Th2:** Type 2 helper T cell

**TLR:** Toll-like receptor

**TNF:** Tumor necrosis factor

# Chapter I

## Introduction

The avian immune system, like that of all vertebrates, is built with innate and adaptive arms. The innate immune system is the first line of defense against invading pathogens in a non-specific manner by recognition of microbial-associated molecular patterns (MAMPs) or endogenous damage-associated molecular patterns via pattern recognition receptors (PRRs) on host epithelial cells, dendritic cells (DCs), heterophils, and macrophages. Engagement of PRRs on host immune cells and consequent activation of signaling cascades including NF-κB, interferon response factors (IRFs), activator protein 1 (AP1), and mitogen-activated protein kinases (MAPK), can promote the production of pro-inflammatory cytokines and antimicrobial peptides, as well as maintenance of epithelial barrier function. Various PRRs have recently been identified in mammals, including Toll-like receptors (TLRs) (Akira et al., 2006; Beutler, 2009), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Inohara et al, 2005; Meylan et al., 2006), and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (Yoneyama et al., 2008; Yoneyama and Fujita, 2009). With the exception of TLRs, there has been little or no identification and characterization of avian PRRs.

Nucleotide-binding oligomerization domain 1 (Nod1) is a member of the NLR family and acts as an intracellular sensor that recognizes bacterial peptidoglycan (PGN) component, γ-$_D$-*meso*-diaminopimelic acid (iE-DAP) (Chamaillard et al., 2003; Girardin et al., 2003a, b). Ubiquitously expressed in adult human tissues, Nod1 is stimulated and activated by recognition of internalized ligands by endocytosis through clathrin-coated pits (Inohara et al., 1999; Lee et al., 2009), resulting in the up-regulation of transcripts of pro-inflammatory genes and defensins via NF-κB activation. Additionally, Nod1, along with Nod2 in mammals, is known to be a

susceptibility gene for inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (Hugot et al., 2001; Inohara et al., 2005; Lu et al., 2010). Several studies showed the association of polymorphism in the Nod1 locus with severity of IBD (Hugot et al., 2001; Inohara et al., 2005; Lu et al., 2010).

Stimulation and activation of Nod1 lead to the release of many cytokines and chemokines; however, more comprehensive characterization of its role in stimulation of adaptive immunity is still required. A recent study of *Helicobacter pylori*, a causative agent of gastric cancer and IBD through production of chronic gastric infection, discussed the characterization of macrophage migration inhibitory factor (MIF) as an innate inflammatory mediator of T-cells following Nod1 activation (Wong et al., 2009). Absence of MIF showed reduced interferon (IFN)-γ and tumor necrosis factor (TNF)-α production during *H. pylori* infection, implying a role of MIF in the activation of adaptive immunity. MIF was originally identified as a soluble factor produced by antigen-activated T lymphocytes. MIF is a pleiotropic cytokine produced by activated T cells (Bloom and Bennett, 1966; David, 1966), macrophages (Calandra et al., 1994), and the pituitary gland (Bernhagen et al, 1994). Mammalian MIF is an immunomodulator that controls macrophage functions, resulting in the promotion of pro-inflammatory cytokine expression (Calandra et al., 1994; Bacher et al., 1996; Donnelly et al., 1997), TLR4 expression (Roger et al., 2001), as well as involvement in T helper 1 (Th1) cells activation and differentiation (Wu et al., 1993; de Jong et al., 2001).

While MIF is a key effector molecule in modulating protective immunity against bacteria (Koebernick et al., 2002; Oddo et al., 2005) and protozoan parasites (Martiney et al., 2000; Satoskar et al., 2001; Reyes et al., 2006), it is also involved in the pathogenesis of several inflammatory diseases, such as chronic colitis (de Jong et al., 2001; Ohkawara et al., 2006),

rheumatoid arthritis (Mikulowska et al., 1997), and atherosclerosis (Kong et al., 2005). In Crohn's disease, as well as in several murine colitis models, inflammation appears to interplay between activated Th1 cells and antigen-presenting cells (APCs) (Parronchi et al., 1997; Papadakis and Targan, 2000; Simpson et al., 2000). Recent research shows an important role of interleukin (IL)-22 produced by newly discovered lineages of $CD4^+$ Th cells, Th17 cells and Th22 cells in colitis (Brand et al., 2006; Feng et al., 2011; Geremia et al., 2011). Additionally, Agnihotri and colleagues (2011) reported an up-regulation of IL-22 transcripts during stimulation of human PBMCs with various synthetic Nod1 ligands. A member of the IL-10 family, IL-22 is produced by Th22 cells (Duhen et al., 2009; Trifari et al., 2009) as well as Th17 cells dependent of IL-23 (Liang et al., 2006; Zheng et al., 2007). Functional studies in murine model systems indicate that IL-22 exerts immunoregulatory properties during infection (Aujla et al., 2008; Munoz et al., 2009; Wilson et al., 2010), inflammation (Brand et al., 2006; Zenewicz et al., 2008; Pickert et al., 2009), autoimmunity (Zheng et al., 2007; Ma et al., 2008), and cancer (Nagakawa et al., 2004; Bard et al, 2008; Zhang et al., 2008). In these models, the functional consequences of IL-22 expression can be either pathologic or protective, depending on the context in which it is expressed. IL-22 confers protective immunity by inducing the production of antimicrobial peptides, neutrophil-recruiting chemokines, and acute phase proteins, as well as promotion of maintenance of epithelial barrier in the intestine and respiratory tract during infection (Aujla et al., 2008; Zheng et al., 2008). On the other hand, IL-22 expression can be pathologic following infection with an intracellular protozoan parasite, by promoting intestinal inflammation and enhanced disease (Munoz et al., 2009; Wilson et al., 2010).

There is clear interplay of these molecules during immune responses to various pathogens and in some cases autoimmune and neoplastic diseases. All of such evidence however has been

demonstrated in mammalian systems, while there is a dearth of similar research described in avian species. The initial goal of this project was to identify and characterize avian Nod1 and determine its role in the avian immune system. However, investigating avian Nod1 led to a more comprehensive characterization of its role in avian immunity, including subsequent studies of other immune molecules, MIF and IL-22. Therefore, the initial goal of the project was expanded and the overall hypothesis was that recognition of MAMPs and subsequent signaling through NLR (specifically Nod1), would lead not only to innate immune responses, but also orchestration of acquired immunity. The emerging role of the innate immune system in setting up the stage for the appropriate adaptive immune responses has attracted much attention in recent years. Unfortunately, none of the above molecules and mechanisms have been identified and characterized in avian species. Thus, the overall goal of this project was to functionally characterize the avian inflammatory mediators Nod1, MIF and IL-22. For this goal, three specific objectives were built: 1) to identify each molecule in the chicken genome; 2) to amplify, clone and express each recombinant form using either eukaryotic or prokaryotic expression system; and 3) to characterize biological function of each molecule using in vitro and ex vivo systems.

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# Chapter II

## Literature Review

### *Avian Immune System: main components and their function*

The avian immune system has provided a useful model for studying basic immunology, with significant contributions to the advances in the knowledge of the mammalian immune system. For example, the bursa of Fabricius is a major primary lymphoid organ for the development of B lymphocytes and humoral immunity (hence the name B cells), whereas the thymus is for the development of T cells involved in cellular immunity. In chickens, the bursa of Fabricius and the thymus are located at anatomically diverse locations; thus it is possible to selectively remove the bursa or the thymus and induce selective humoral or cellular immune deficiency. Experimental removal of the bursa from chickens leads to failure of antibody production, indicating the role of bursa in antibody production. Because of similarities in the lymphoid tissues and immune system of birds and mammals, this research led to the identification of mammalian primary B lymphoid organ, bone marrow (Cooper et al., 1965). Studying the bursa also contributed to the discovery of gene conversion in generating the antibody repertoire. In humans, different B cells produce immunoglobulin (Ig) molecules of different specificities through Ig gene rearrangement among the variable (V) region, a joining (J) region and the constant region. In contrast to mammals, chicken Ig heavy and light chains have limited V(D)J rearrangement due to a single copy of the functional V and J genes (Reynard et al., 1985). To overcome this limited rearrangement and low antibody diversity, chicken antibody repertoire undergos somatic gene conversion using regions of pseudogenes, resulting in greater diversity of V sequences containing portions V pseudogenes incorporated in one functional V segment (Arakawa et al., 2002). Becker and Knight (1990) later reported that gene conversion is

not just limited to birds, but also occurs in rabbits, pigs and other mammalian species. Research of the avian immune system has also contributed to development of vaccines. One such remarkable development was the vaccine against Marek's disease (MD), a naturally occurring neoplastic disease caused by virual infection in chickens. Before the introduction of MD vaccine, morbidity and mortality in laying flocks reached up to 60% or greater, with losses of 30% being common (Powell and Lombardini, 1986). The development of an MD vaccine represents the first example of widespread use of vaccination to protect against a viral induced cancer (Witter, 2001). As described earlier, the bursa and thymus are the primary lymphoid organs where B and T cell precursors differentiate and undergo maturation. After differentiation and maturation, functional immune cells leave the primary organs and populate secondary lymphoid organs, the principal sites of antigen-induced immune responses. Secondary lymphoid organs are characterized by aggregates of lymphocytes and antigen-presenting cells. They are scattered throughout the body, including spleen, bone marrow, gland of Harder (or Harderian gland) and conjunctiva-associated lymphoid tissues (CALT), bronchia-associated lymphoid tissues (BALT), and gut-associated lymphoid tissues (GALT). Also, the bursa may serve as a secondary lymphoid organ. Unlike mammals, which have lymph nodes as main secondary lymphoid organs, chickens have lymphoid nodules along the length of lymph vessels. The round or oval chicken spleen develops primarily after hatching following exposure to antigens. As in mammals, the avian spleen is a major antigen sampling site where immune responses develop to fight infections. Although the spleen is not a primary site for lymphocyte antigen-independent differentiation and proliferation, it plays an important role in embryonic lymphopoiesis. For example, B cell progenitors undergo rearrangement of their Ig genes before colonizing the bursa (Masteller and Thompson, 1994). Chicken bone marrow is distinguished histologically as two

separate compartments; the intravascular compartment is responsible for the erythrocytes and thrombopoiesis, while the extravascular compartment is responsible for lymphopoiesis. Thrombocytes are similar to lymphocytes. The extravascular compartment is filled with granulocytes in different stages of maturation (Jeurissen et al., 1988). In chickens, thrombopoiesis and erythropoiesis occur in the sinuses, while erythropoiesis takes place in the extravascular compartment in mammals (Wu et al., 2010; Heo et al., 2011). The pineal gland is located at the dorsal surface of the brain and functions in immune surveillance of the central nervous system (Okano and Fukada, 2001). Harderian gland is located in the orbit behind the eye and functions in secretion of tear fluid and lymphocytic infiltration (Ohshima and Hiramatsu, 2002; Scott et al., 2005). As part of the GALT, Peyer's patches visibly appear in 10-day old chickens with the major one along the intestine cranial to the ileocecal junction. Most T cells are helper type and mature B cells produce all three Ig isotypes (IgA, IgM and IgY [equivalent to mammalian IgG]) in Peyer's patches (Motyka and Reynold, 1991; Kajiwara et al., 2003). Cecal tonsils are enlarged patches of tissue in the proximal region of each cecum. There are T cells as well as B cells which produce IgM, IgY and IgA antibodies (Kajiwara et al., 2003). Although the primary function of the gastrointestinal tract is to digest and absorb nutrients in order to meet metabolic demands for normal growth, development, and maintenance, it also acts as a vital barrier preventing the entry of several antigens and potentially harmful pathogens from the external environmental (Beal et al., 2006). The GALT make up the largest component of the mucosa-associated lymphoid tissues and are a significant source of immune cells that monitor and protect the mucosal layers of the gut. The GALT are continuously being exposed to food antigens, microflora and ingested pathogens (Yun et al., 2000). Protection of the gut is achieved through use of both the innate and adaptive immune systems. Unlike the mammalian GALT,

chickens do not posses lymph nodes, instead they have scattered lymphoid aggregates as well as organized lymphoid structures such as the cecal tonsils, Meckel's diverticulum and Peyer's patches described above.

Among avian species, the immune system of the domestic chicken has been studied most intensively and extensively, due to its economic importance. Therefore, most information presented is based on studies in chickens. It is likely that the broad principles of immunology identified in chickens will apply to other members of the avian species. Like all other vertebrates, the avian immune system is broadly organized as innate and adaptive immunity.

*Innate Immunity*

The innate immune system is highly conserved evolutionarily and characterized by non-specific defense mechanisms that are present and ready to be active on the day the bird hatches. It is the first line of defense providing immediate protection against microorganisms and other antigens. It includes cellular components, primarily phagocytic and pro-inflammatory cells, including macrophages, heterophils, dendritic cells (DCs), and natural killer (NK) cells. It also includes humoral or secretory components such as bacteriolytic enzymes (e.g. lysozyme), complement, and mannose-binding protein (Hoffmann et al., 1999; Aderem and Ulevitch, 2000; Janeway and Medzhitov, 2002).

Heterophils have been declared the counterpart to the neutrophils in mammals. Polymorphonuclear leukocytes, heterophils are the first line of cellular defense against invading microbial pathogens in birds. Although there are many similarities between heterophils and neutrophils, there are also important differences. Heterophils lack myeloperoxidase, a lysosomal protein abundant in neutrophil granules. In addition, heterophils do not produce significant

amounts of bactericidal activity by oxidative burst (Harmon, 1998). Avian macrophages, the most well studied avian immune cells, are active effector cells that can detect, phagocytize and kill extracellular microorganisms. They are the key regulatory cells of the immune system involved in initiating and directing immune and inflammatory responses. Biological functions of macrophages include tissue homeostasis, pathogen recognition and destruction, modulation of innate immune responses, and activation of the adaptive immune system. During inflammation, monocytes migrate to the appropriate location in response to released chemotactic signals. Although chemokines involved in monocyte migration are still unknown in avian species, histological studies have shown adhesion of avian macrophages to blood vessel walls and their active migration to sites of infection and inflammation (Chu and Dietert, 1988; Golemboski et al., 1990). As early as embryonic day 12, chicken macrophages exhibit phagocytic activity, which is the best known and most conserved function of macrophages, indicating early functional innate immune abilities (Jeurissen and Janse, 1989). Phagocytosis by primary macrophages has been reported with a range of bacterial species, including *Salmonella enterica*, *Escherichia coli*, and *Campylobacter jejuni* (Miller et al., 1990; Myszewski and Stern, 1991; Okamura et al., 2005). In phagocytosis, microorganisms bind to macrophages via receptor-mediated recognition, following uptake of microorganisms, resulting in activation of effector mechanisms for pathogen destruction. This mechanism includes internalization of phagocytosed particles, and fusion with lysosomes to form a phagolysome. Lysosomes contain a variety of anti-microbial proteins as well as enzymes such as acid phosphatase and β-glucuronidase (Fox and Solomon, 1981; Taylor et al., 2005). Avian macrophages not only trigger the uptake and destruction of invading pathogens, but also induce the secretion of inflammatory mediators to boost innate immune response and activate adaptive immunity. The secreted inflammatory

mediators include IL-1, IL-6, IL-18, TNF-α like factor, all known as pro-inflammatory cytokines in mammals (Amrani et al., 1986; Klasing and Peng, 1987; Klasing, 1991). Further studies showed the induction of these pro-inflammatory cytokines from avian macrophages not only against bacterial infections, but also in response to viral and parasitic invasion (Palmquist et al., 2006; Dalloul et al., 2007). Avian DCs have similar characteristics to their mammalian counterparts where bone marrow-derived DCs are capable of phagocytosis and macro-pinocytosis. DCs exist under two forms, immature and mature. By antigen uptaking, processing and presentation, immature DCs become mature, which is the efficient form for capturing and optimal presentation of antigens. Moreover, antigen capture may take place at a site that is anatomically distant from where antigen presentation to lymphocytes occurs, and DCs must therefore have the ability to selectively migrate to such sites (Olah et al., 2003; Reese et al., 2006). Natural killer cells function by releasing small molecules of cytoplasmic granules called perforin and granzyme, which cause apoptosis in of target cells such as virus-infected and tumor cells (Moretta et al., 2008).

The innate immune system is also required for the initiation of efficient adaptive immune responses (Schnare et al., 2001; Janeway and Medzhitov, 2002). Macrophages and DCs activated by foreign antigens are called antigen presenting cells (APCs), and can induce phagocytosis, chemotaxis, or secretion of pro-inflammatory mediators (Hoffmann et al., 1999; Aderem and Ulevitch, 2000; Janeway and Medzhitov, 2002). Following phagocytosis and activation, APCs will present a processed fragment of the pathogen to members of the adaptive immune system, mainly B and T lymphocytes, resulting in differentiation of naïve lymphocytes into appropriate effector cells in order to defeat specific types of pathogens (Lee and Iwasaki, 2007).

*Adaptive Immunity*

Adaptive immunity is more complex than innate immunity and it provides antigen specific protection to the host. It also carries a "memory" feature, which allows future responses against a specific antigen to be quicker and more robust. Briefly, the key players of the adaptive immune system, B and T lymphocytes, recognize an antigen processed by APCs, resulting in their differentiation into effector and memory cells against that specific antigen.

Adaptive immunity is sub-grouped into either humoral or cell mediated immunity. Depending on the characteristics of the pathogen, adaptive immunity will utilize the humoral system, cell-mediated system, or a combination of the two in order to efficiently clear and protect the host. Blood-borne haematopoietic cells, precursors of lymphocytes, differentiate and maturate in the primary lymphoid organs, which are the thymus and bursa of Fabricius for T and B lymphocytes, respectively. Once developed, lymphocytes become effector cells in the secondary lymphoid organs such as the spleen, bone marrow, gland of Harder, conjunctival-associated (CALT), bronchial-associated (BALT), and gut-asociated lymphoid tissues (GALT), where they come into contact with potential pathogens or other antigens (Dalloul and Lillehoj, 2006).

***Inflammation***

A type of innate immune response, inflammation was first described by Aulus Cornelius Celsus as redness, warmth, swelling, and pain (Xie et al., 2001; Ferrero-Miliani et al., 2007). It is part of the complex physiologic response to harmful stimuli such as pathogens, damaged cells, or irritants. The inflammatory response, both localized and systemic, consists of altered patterns

of blood flow, an influx of phagocytic and other immune cells, removal of foreign antigens, and healing of damaged tissue (Xie et al., 2001; Ferrero-Miliani et al., 2007).

Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes in mammals and heterophils in avians) from blood into injured tissues (Xie et al., 2001; Ferrero-Miliani et al., 2007). A cascade of biochemical events that propagates and matures the inflammatory response, involving the local vascular system, the immune system (IL-1, IL-6 and TNF-α are particularly important), and various cells within the injured tissues results in a systemic acute phase response (Ferrero-Miliani et al., 2007). Chronic inflammation develops because of the persistence of an antigen. A chronic inflammatory response leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissues from the inflammatory process (Ferrero-Miliani et al., 2007).

***Pathogen recognition receptors and microbial-associated molecular patterns***

Innate immune responses can be initiated by a system of structurally related proteins that function as receptors for specific microbial components that are highly conserved in evolution. Identification of microbes involves host receptors, termed pattern recognition receptors (PRRs) that recognize conserved molecular motifs on a wide range of different microorganisms. These motifs have been termed microbial-associated molecular patterns (MAMPs), and include such structural molecules as flagellin, peptidoglycan (PGN), lipopolysaccharide (LPS), and double-stranded RNA (Athman and Philpott, 2004). For example, PGN, which is a unique and essential component of the cell wall of virtually all bacteria, is an excellent target for the innate immune

system, since it is not present in eukaryotes. Indeed, higher eukaryotes, including avians and mammals, have several PGN recognition molecules including CD14, TLR2, nucleotide-binding oligomerization domain (NOD)-containing proteins, a family of peptidoglycan receptor protein (PGRP), and PGN-lytic enzymes (lysozyme and amidase) (Dziarski, 2003; Dziarski and Gupta, 2005). These molecules induce host responses to microorganisms, degrade PGN, or have direct antimicrobial effects. PRRs are expressed both at the cell surface and in the cytosol, and by immune and non-immune cells including epithelial cells, macrophages and DCs (Kawai and Akira, 2009; Lavelle et al., 2010). There are three families known to be as major PRRs: Toll-like receptor (TLR), nucleotide-binding oligomerization domain (NOD)-like domain (NLR), and retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) family (Kawai and Akira, 2009; Lavelle et al., 2010). Activation of these PRR families promotes the production of pro-inflammatory cytokines and antimicrobial peptides, as well as the maintenance of epithelial barrier function and epithelial cell proliferation via activated signaling cascades including NF-κB, interferon response factor (IRFs), activator protein 1 (AP1), and mitogen-activated protein kinases (MAPKs) (Kawai and Akira, 2009; Lavelle et al., 2010).

As type I transmembrane glycoproteins, TLRs are the most well studied PRRs in avian species which recognize microbial pathogens and induce an immediate response by innate immune cells (Brownlie and Allan, 2011). Thirteen TLRs (TLR1 – TLR13) have been identified although expression of TLR10 – 13 appears to be species specific (Chuang and Ulevitch, 2000; Du et al., 2000; Tabeta et al., 2004). In comparison with mammals, there are ten avian TLRs (TLR1 – TLR5, TLR7, TLR15, and TLR21) and five of these (TLR2a, 2b, 4, 5, and 7) have clear orthologs in human and mice (Table 2.1) (Brownlie and Allan, 2010).

**Table 2.1. Mammalian and avian TLRs and their specific MAMPs**

| Toll-like Receptors | | MAMPs | Pathogen |
|---|---|---|---|
| Mammalian | Avian | | |
| TLR1 | TLR1a | LTA<br>PGN | Gram-positive<br>Mycoplasma |
| | TLR1b | | |
| TLR2 | TLR2a | | |
| | TLR2b | | |
| TLR6 | | | |
| TLR4 | TLR4 | LPS, Lipid A | Gram-negative |
| TLR5 | TLR5 | Flagellin | Bacteria, Flagellum |
| TLR3 | TLR3 | dsRNA | Virus |
| TLR7 | TLR7 | ssRNA | Virus |
| TLR8 | | | |
| TLR9 | TLR21 | CpG | Bacteria, Virus |
| TLR10 | | Unknown | Unknown |
| TLR11 | | Unknown | Unknown |
| TLR12 | | Unknown | Unknown |
| TLR13 | | Unknown | Unknown |
| | TLR15 | Unknown | Unknown |

Chicken TLR1 (ChTLR1) and ChTLR2 have two isoforms each, and ChTLR15 appears to be unique to avian species although phylogenetic analysis showed that it is related to the mammalian TLR2 group. A recent study reported that CpG DNA, recognized by mammalian TLR9, can activate ChTLR21, an ortholog to TLR21 in fish and amphibians (Keestra et al., 2010). Biologically, avian TLRs 1 – 5 and TLR7 function similarly to those of mammals. As

for other PRR families, analyses of avian genomes including chicken, turkey and zebra finch, confirmed the existence of avian NLR and RLR family sequences; however, no further research is available for these receptors.

***Nucleotide-binding oligomerization domain-like receptor family***

Members of the NLR family are cytosolic MAMPs and damage-associated molecular patterns (DAMPs) sensors, typically characterized by a C-terminal series of leucine rich repeats (LRRs), a central Nod, and an N-terminal signaling domain (Tschopp et al., 2003; Inohara et al., 2005). The C-terminal LRR domain is thought to function in ligand sensing, and the central NACHT domain, also called NOD, is important for oligomerization and activation. The N-terminal signaling domain (also called effort domain) mediates signal transduction to downstream targets through protein-protein interactions (Inohara and Nunez, 2003; Inohara et al., 2005). Members of the NLR family are categorized into at least five subfamilies distinguished by their N-terminal structures. These include NLRA (which contain an acidic transactivation domain), NLRB (contain a baculovirus inhibitor of apoptosis protein repeat 9BIR), NLRC (contain a CARD), NLRP (contain a Pyrin domain), and NLRX (contain an unknown domain) (Figure 2.1). So far, at least 23 human and 34 murine NLR genes have been identified, although the physiological function of most NLRs is poorly understood (Ting et al., 2008).

Binding of a ligand to the LRR domain leads to self-oligomerization of NLR family members; then this self-oligomerization activates the molecule and recruits the specialized signal adaptor molecule (e.g. RIPK2), resulting in production of pro-inflammatory cytokines and anti-microbial peptide (AMP) via NF-κB or MAPK pathway activation (Strober et al., 2006; Franchi

et al., 2008; Shaw et al., 2008). The NF-κB family consists of five members that can exist as dimers and the heterodimer composed of RelA and p50 is considered to be the most frequently activated during NLR signaling (Park et al., 2007; Hasegawa et al., 2008). MAPKs include extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and c-jun N-terminal kinase (JNKs), which phosphorylate the activator protein 1 (AP1) family of transcription factors to regulate transcription or mRNA stability of inflammatory cytokine genes (Strober et al., 2006).

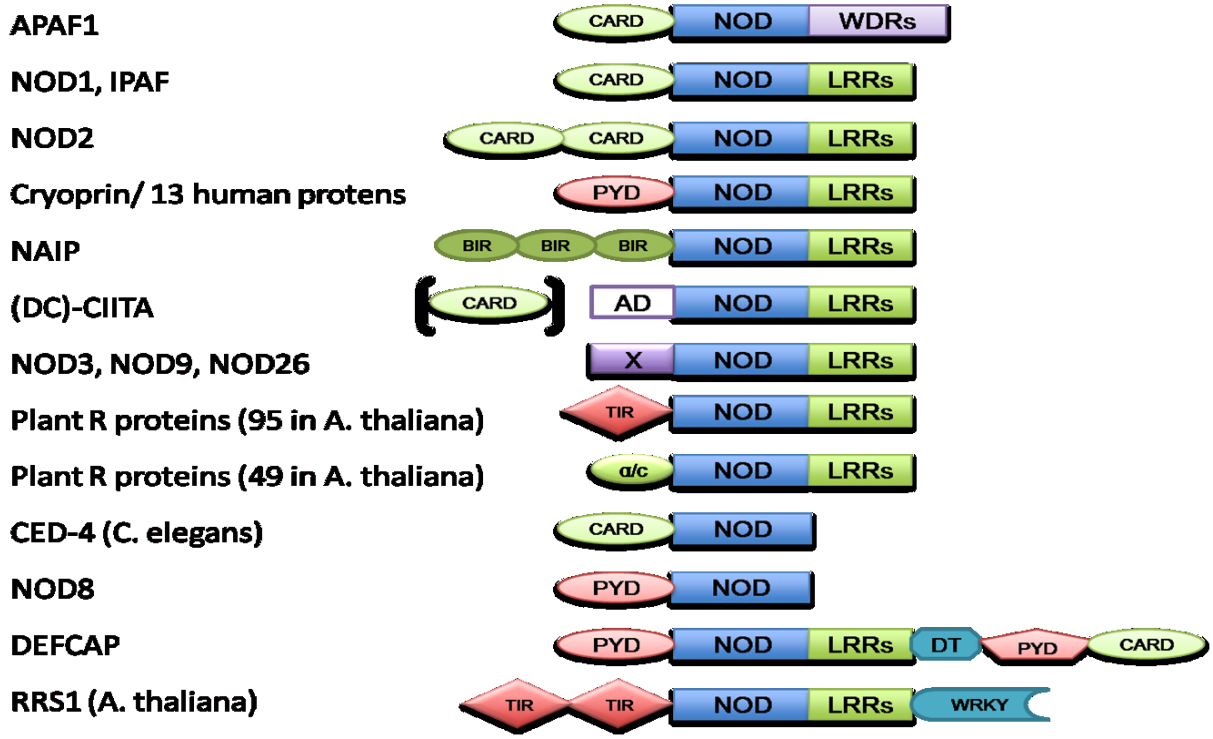


**Figure 2.1. Schematic drawings of domain structure of NLR family.**

As for their role, NLR family members are increasingly implicated in the pro-inflammatory forms of cell death, pyroptosis and pyronecrosis (Kroemer et al., 2009). Thus, during inflammatory conditions where significant mucosal cell death is ongoing, it will be

important to assess the contribution of NLR family members, including NLRP1 and NLRP3 to the inflammatory process.

*Nucleotide-binding oligomerization domain protein 1 (Nod1) and 2 (Nod2)*

Nod1 and Nod2 are well-characterized members of the NLR family, and recognize distinct structural motifs derived from PGN. Nod1 recognizes γ-$_D$-glutamyl-*m*-DAP (iE-DAP), found in the PGN structures of all Gram-negative as well as in several Gram-positive bacteria such as *Bacillus subtilis* and *Listeria monocytogenes* (Chamaillard et al., 2003; Girardin et al., 2003a). In contrast, Nod2 recognizes Muramyl dipeptide (MDP, the largest component of the PGN motif that is also present in all Gram-negative and Gram-positive bacteria (Girardin et al., 2003b; Inohara and Nunez, 2003; Inohara et al., 2003). Nod1 and Nod2 activating ligands are thought to enter cells by endocytosis through clathrin-coated pits (Lee et al., 2009). The internalization of the peptides is pH dependent and the peptides are enzymatically processed in endosomes before transport into the cytosol.

Nod1 is ubiquitously expressed in adult human tissues, whereas Nod2 is found mainly within monocytes, macrophages and intestinal epithelial cells (Inohara et al., 2002; 2003). In addition, expression of Nod2 can be upregulated by inflammatory stimuli such as LPS, TNF-α, or IFN-γ (Gutierrez et al., 2002; Rosenstiel et al., 2003). Nod1 and Nod2 are linked to a specific signaling pathway following activation, associating with a serine-threonine kinase, RIPK2. RIPK2 subsequently activates TAK1 through Lys63-linked ubiquitination, resulting in the activation of NF-κB and MAPK (Inohara et al., 2003; Viala et al., 2004; Park et al., 2007; Hitotsumatsu et al., 2008). The activation of MAPKs via Nod2 requires CARD9 (Hus et al.,

2007). This activation is TLR- and MyD88-independent and thus serves as an alternative pro-inflammatory pathway to aid in host defense.

Intensive studies over the past 8-10 years have focused on the role of Nod1 and Nod2 in chronic inflammatory diseases in the small intestine. Nod1-deficient mice are highly susceptible to infection with *H. pylori* (Viala et al., 2004), whereas Nod2-deficient mice are more susceptible to oral infection with *L. monocytogenes* (Kobayashi et al., 2005). The uptake of *L. monocytogenes* into phagolysosomes generates bacterial peptides that enter the cytosol and interact with Nod2. This interaction is thought to specifically trigger type I IFN responses (Herskovits et al., 2007). Nod2 is expressed in ileal Paneth cells, and production of cryptidin subgroup of antimicrobial peptides is reduced in Nod2-deficient cells (Kobayashi et al., 2005). Furthermore, the expression of α-defensins is reduced in Crohn's disease patients with Nod2 mutations (Wehkamp et al., 2004).

As previously mentioned, recognition of MAMPs by PRRs is an essential element to instruct adaptive immune responses (Medzhitov, 2007). Nod1 stimulation alone has the capacity to drive Th2 responses; however, when given with TLR ligands, Nod1 signaling enhances Th1, Th2 and Th17 responses, suggesting that Nod1 signaling potentiates the adaptive immune responses that are mediated by TLRs (Fritz et al., 2007). Nod2-deficient mice had defects in their ability to mount antibody responses when MDP was given as an adjuvant (Kobayashi et al., 2005). Nod2 mutations are linked to Crohn's disease, and the IL-23-derived and IL-1β-derived Th17 responses induced by Nod2 ligand are abrogated in cells from patients who carry Nod2 mutations (van Beelen et al., 2007).

A putative avian Nod1 nucleotide sequence was uncovered on chromosome 2 during analysis of the chicken genome; however, there was no evidence of avian Nod2.

***Innate inflammatory mediators***

Activation of Nod1 signal leads to the release of many chemokines and cytokines; however, more a comprehensive characterization of their individual roles is still required. There is no clear evidence of a role of MIF during Nod1 activation; however, studies of the enteric pathogen *H. pylori* show potential involvement of MIF during Nod1 activation. Even though *H. pylori* infection is recognized by TLR family members, TLR2, 4, 5, 7, 8 and 9, recent studies showed Nod1-dependent activation of NF-κB, resulting in IL-8 release and β-defensin 2 production during *H. pylori* infection (Viala et al., 2004; Grubman et al., 2010; Kaparakis et al., 2010). Furthermore, gastric gavage of *H. pylori*-derived outer membrane vesicles in mice induced Nod1-dependent chemokine responses in the stomach and outer membrane vesicles-specific serum IgG production (Schroeder and Aebischer, 2009). Activation of Nod1 by *H. pylori* generates signals that ultimately impact the adaptive immune response. Earlier research suggests that infection with *H. pylori* induces production of IL-17 from Th17 cells, which is newly discovered $CD4^+$ T helper cells (Kao et al., 2010; Khamri et al., 2010). However, there is no clear mechanism of how activated Nod1 signal by *H. pylori* activates Th17 cells by IL-23-dependent manner. MIF may be the missing key molecule that bridges activated Nod1 signal and activate Th17 cells. Wong and colleagues (2009) reported that absence of MIF leads to decreased production of TNFα and IFN-γ, as well as reduced number of macrophages and T cells during *H. pylori* infection, implying a role of MIF in the activation of adaptive immunity.

*Macrophage migration inhibitory factor (MIF)*

Macrophage migration inhibitory factor, one of the first lymphocyte-derived cytokines, was originally identified as a soluble factor produced by antigen-activated T lymphocytes and

inhibited the random migration of macrophages (Bloom and Bennett, 1966; David, 1966; Weiser et al., 1989). Further research showed that MIF was produced by macrophages (Calandra et al., 1994), as well as granulocytes and B lymphocytes (Calandra et al., 1994; Lue et al., 2002). Moreover, most epithelial cells seem to express and store MIF, indicating that it plays a role in the early innate host defense since the epithelial cell lining provides a first mechanical barrier against pathogens (Bacher et al., 1996). Interestingly, the MIF protein has also been detected in the pituitary gland, specifically within ACTH-producing cells (Nishino et al., 1995). Given the above descriptions of MIF in different tissues, it appears that it not only plays an important role in host defense within the immune system, but also has other physiological functions that have not yet been well characterized.

Sequence analysis revealed that human MIF is a molecule comprised of 115 amino acids with a molecular weight of 12.5 kDa (Weiser et al., 1989). The secondary structure of MIF consists of two anti-parallel α-helices and six β-pleated sheets that are highly similar to MHC molecules (Suzuki et al., 1996). The crystal structure of MIF revealed the active form to be a 37.5 kDa homotrimer with novel protein folds that defined a new structural superfamily (Sun et al., 1996; Suzuki et al., 1996). In addition to its distinctive structure, MIF possesses a unique enzymatic activity, called tautomerase activity, revealed through its structural homology to several bacterial enzymes. Tautomerase activity mediated by an N-terminal proline residue, allows MIF to catalyze the conversion of the non-physiological substrates $_{D}$-dopachrome or $_{L}$-dopachrome methyl esters to their indole derivatives (Rosengren et al., 1996; Dios et al., 2002). No human physiological substrates from MIF tautomerase have been identified yet.

Although much is known about the effect of MIF on various immune cells, it has taken quite some time for scientists to understand the signal transduction pathway that operates in

many immune cells in response to MIF activation, especially given that this activation requires an extracellular receptor. Experimental work from Leng et al. (2003) extended the knowledge regarding MIF signaling by characterizing CD74 as a potential MIF receptor. In macrophages, CD74 demonstrates high-affinity binding to MIF. This binding of MIF to the extracellular domain of CD74 is required for MIF-mediated MAPK activation and cell proliferation (Leng et al., 2003). Furthermore, CD44, a transmembrane co-receptor, is required for MIF-induced ERK1 and ERK2 kinase phosphorylation (Shi et al., 2006). After receptor binding, the intracellular signaling cascade is activated via the ERK-MAP kinase pathway, resulting in increased cell proliferation via cyclin D1 transcription and subsequent phosphorylation of the Rb gene (Leng et al., 2003). For fast and transient activation of this cascade, there is another pathway that involves Jab-1/CSN5, a protein that serves as an intracellular binding partner of MIF. In addition, a Src tyrosine kinase plays an important role and further enhances cell cycle progression (Liao et al., 2003; Lue et al., 2006).

Recent research shows a more prominent role of MIF as a multi-functional cytokine mediating both innate and adaptive immune responses, though it primarily acts as a pro-inflammatory protein (Donn and Ray, 2004). A role of MIF as a pro-inflammatory protein was determined and evaluated using a mouse model of septic shock (Bernhagen et al., 1993). After applying LPS, a potent activator of the innate immune system, MIF expression and production were increased compared to controls at both mRNA and protein levels. However, when adding recombinant MIF, these effects were counter-regulated. Use of antisense MIF led to decreased MIF concentrations in treated macrophages while higher amounts of MAPK phosphatase and lower concentration of cytokines such as TNFα were observed. This study, therefore, demonstrated the pro-inflammatory potential of MIF (Roger et al., 2005). Following these

observations, knockout mice were generated by different groups in order to examine the possible effect of LPS on these animals with regard to the severity of sepsis. However, the data obtained were subject to controversy as different groups reported different results. Bozza et al. (1999) reported a reduced mortality rate in these knockout mice in response to LPS when compared to the control group. In contrast, Honma et al. (2000) were not able to detect a significant difference in survival between the knockout mice and the controls.

As mentioned above, MIF secretion results in an increased production and release of pro-inflammatory cytokines such as TNF$\alpha$, interleukins and IFN-$\gamma$. In a series of experiments by Roger et al. (2005), MIF was found to regulate the expression of TLR4, which recognizes LPS and induces the activation of monocytes/macrophages. MIF knockout mice were reported to express only low levels of this receptor, suggesting potential involvement of MIF early in innate immune responses (Roger et al., 2001). .

In addition to the previously described effects of MIF on innate immunity, its involvement in adaptive immune response has also been shown. In 1996, investigators demonstrated that antibodies directed against MIF inhibit a delayed-type IV immune response. In vivo, T-cell activation and antibody production is inhibited by MIF (Bacher et al., 1996). MIF-specific antibodies prevent superantigen-induced activation and proliferation of splenocytes, thus supporting the concept that MIF is also a lymphotropic cytokine (Calandra et al., 1998). Moreover, MIF inhibits regulatory effects of cytotoxic $CD8^+$ T cells and regulates lymphocyte trafficking (Abe et al., 2001). Several groups have provided evidence of MIF upregulation in atopic dermatitis, asthma, psoriasis, colitis ulcerosa and rheumatic arthritis (Calandra et al., 1995; Repp et al., 2000; Abe et al., 2001; de Jong et al., 2001; Becker et al., 2006; Bucala and Donnelly, 2007). Becker et al. (2006) demonstrated a correlation between

MIF and disease activity in vasculitis. The potential of MIF inhibition to reduce an immune response was shown in knockout mice afflicted with inflammatory bowel disease and sepsis (Bozza et al., 1999; de Jong et al., 2001).

In birds, the sequence of ChMIF was first identified from early stage embryonic chicken eye lens as a discrete 10-kDa polypeptide (Wistow et al., 1993). Further studies showed elevated MIF mRNA level during *Eimeria* infection, implicating potential involvement of MIF in primary *Eimeria* infection (Hong et al., 2006); however, there has been no further characterization of its function.

***Interleukin-10 family***

IL-10 was initially discovered as an inhibitory factor for the production of Th1 cytokines such as IL-2, TNF-α, and IFN-γ (Fickenscher et al., 2002; Ouyang et al., 2011). Subsequently, pleiotropic inhibitory and stimulatory effects on various types of blood cells were described for IL-10, including its role as a survival and differentiation factor for B cells. Furthermore, IL-10 appears to be a crucial factor for at least some forms of peripheral tolerance and a major suppressor of the immune response and inflammation (Fickenscher et al., 2002; Thomson and Lotze, 2003; Ouyang et al., 2011). With complete human genome sequencing, five novel cytokines that display structural similarity to IL-10 have been identified; IL-19, IL-20, IL-22, IL-24, and IL-26. These "IL-10 homologs" form a group known as the IL-10 family, a major Class II cytokine family along with the interferon family. The human IL-10 gene lies with those of IL-19, IL-20 and IL-24 on chromosome 1, while IL-22 and IL-26 lie on chromosome 12 with the IFN-γ locus (Fickenscher et al., 2002; Conti et al., 2003; Ouyang et al., 2011).

IL-10 family members can be categorized into two subgroups based primarily on their biological functions. The first group contains only IL-10 itself. IL-10 targets various leukocytes and mainly represses excessive inflammatory responses. The second group, namely the IL-20 subfamily cytokines, is composed of IL-19, IL-20, IL-22, IL-24, and IL-26 (Conti et al., 2003; Commins et al., 2008; Ouyang et al., 2011). This group of cytokines primarily acts on various epithelial cells and protects these cells from invasion by extracellular pathogens such as bacteria and yeast. In addition, IL-20 subfamily cytokines enhance tissue remodeling and wound-healing activities, which help maintain tissue integrity and restore homeostasis of epithelial layers during infection and inflammatory responses (Commins et al., 2008; Ouyang et al., 2011).

*The IL-10 family-related receptors and signaling pathway*

The homodimer form of IL-10 is recognized and binds to the IL-10 receptor complex, composed of IL10R1 and IL10R2 (Pestka et al., 2004). IL10R1 is specific for only IL-10, while IL-19, IL-20 and IL-24 bind to a common two-chain receptor complex that consists of IL20R1 and IL20R2. In addition, IL20R2 can also pair with IL22R to form a heterodimeric receptor complex that is preferentially used by IL-20 and IL-24, but not by IL-19. IL-22 and IL-26 share the common β chain receptor IL20R2 with IL-10. However, each of the cytokines binds to its unique α chain receptor, namely IL22R1 and IL20R1 for IL-22 and IL-26, respectively (Pestka et al., 2004; Ouyang et al., 2011). Interestingly, IL-22 has an IL22R1 homolog, the secreted soluble receptor IL22BP. IL22BP shows 33% amino acid sequence identity with the extracellular domain of IL22R1. In addition, IL22BP binds to IL-22 with high affinity and neutralizes its activity in vitro (Dumoutier et al., 2001; Wei et al., 2003).

Binding of IL-10 family members to a specific receptor complex leads to cascade signaling mainly through the Jak-STAT signaling pathway. STAT3 is the key downstream transcription factor used by IL-10 and IL-20 subfamily cytokines (Kotenko et al., 2003). Jak1 and Tyk2 are associated with IL10R1 and IL10R2, respectively (Finbloom and Winestock, 1995; Ho et al., 1995). IL-10 activates STAT1, STAT3 and STAT5, and STAT3 is critical in mediating the function of IL-10 in immune cells (Takeda et al., 1999). The suppressive effects of IL-10 on the production of pro-inflammatory cytokines are completely abolished in absence of STAT3 in mononuclear cells (Takeda et al., 1999). STAT3 is also a major transcription factor used by IL-20 subfamily cytokines, similar with IL-10. Binding of IL-22 to the IL22RA1/IL10R2 heterodimeric receptor complex leads to the activation of the Jak/STAT pathway, including activation of STAT1, STAT3, and STAT5 (Lejeune et al., 2002).

IL-10 family cytokines are produced by various leukocytes and epithelial cells that can secrete some of these cytokines, especially IL-19, IL-20 and IL-22. IL-10 and IL-22 are the best studied among the family members in terms of their expression. IL-10 is expressed by cells of the innate and the adaptive immune system, including DCs, macrophages, mast cells, NK cells, eosinophils, neutrophils, CD4 and CD8 T cells, and B cells (Nagalakshmi et al., 2004). Similarly, IL-22 is produced by various leukocytes, such as CD4, CD8, NK cells (also NK-22 cells) and various lymphoid tissue inducers (LTi)-like subsets. IL-19, IL-20 and IL-24 are primarily generated by myeloid cells (Wolk et al., 2002; Nagalakshmi et al., 2004).

*Biological function of IL-10 family in inflammation*

Stimulated IL-10 family members function mainly in two different fashions during inflammation: 1) IL-20 subfamily cytokines induce innate defense mechanisms from epithelial

cells during infection, and 2) IL-10 and IL-20 subfamily cytokines prevent tissue damage caused by infections and inflammation (Fickenscher et al., 2002; Conti et al., 2003; Pestka et al., 2004; Commins et al., 2008). In the latter case, IL-10 regulates and represses the expression of pro-inflammatory cytokines during the recovery phase of infections and reduces tissue damage caused by these cytokines, while IL-20 subfamily cytokines stimulate epithelial cell proliferation, anti-apoptotic responses and tissue remodeling and healing (Pestka et al., 2004; Ouyang et al., 2011). Unlike the anti-inflammatory cytokine IL-10, which prevents tissue lesions caused by exacerbated adaptive immune response (Wolk et al., 2005; Sanjabi et al., 2009), IL-20 subfamily, especially IL-22, enhances innate host defense mechanism including induction of various anti-microbial peptides, pro-inflammatory cytokines and chemokines in local epithelial tissues (Aujla et al., 2007; Ouyang et al., 2008; Aujla et al., 2009).

In chickens, ChIL-10 is located on chromosome 26 along with ChIL-19, while ChIL-22 is syntenic with ChIL-26 on chromosome 1. Chicken IL-20 and IL-24 are currently absent in the most recent genome assembly. The function of IL-10 appears to be conserved in the chicken, in that it acts as an anti-inflammatory cytokine, downregulating the effect of IFN-γ (Rothwell et al., 2004). There is no published knowledge of the biological role of ChIL-19, ChIL-22 and ChIL-26. Albeit IL-19 research is limited, mammalian IL-19 functions as a Th2 cytokine, and such functions of ChIL-19 and ChIL-22 seem to be conserved in the chicken. ChIL-19 is expressed along with IL-4 and IL-13 following infections with extracellular pathogens such as worms (*A. galli*) or protozoa (*H. meleagridis*). ChIL-22 induces IL-10 expression in chicken kidney epithelial cells and upregulates the expression of β-defensins in heterophils (unpublished observations by Kaiser et al.).

*Interleukin-22*

During the development of vaccine adjuvants, Agnihotri and colleagues (2011) synthesized various Nod1 ligands and tested their effects on human PBMCs using microarrays. In their report, activation of Nod1 led to B and T cell immunity with predominant Th2 polarization and additional Th17 priming. Study of transcriptional profiles revealed a very prominent upregulation of IL-17A (approximately 10-fold) and IL-10 family cytokines, especially IL-22 (approximately 4-fold). Upregulation of IL-17 suggests induction of Th17 cells, implicating increased IL-22 production. Additionally, Kao et al. (2010) reported increased population of Th17 cells in an IL-23 dependent manner during *H. pylori* infection, which in turn stimulates Nod1 signaling pathway, implicating the induction of IL-22 expression by Nod1 signaling. Together, the results implicate an important role of IL-22 in the downstream activation of Nod1.

IL-22 was initially identified as an IL-10-related T cell-derived inducible factor (Dumoutier et al., 2000a) and classified as IL-10 family member based on its similarity with IL-10 (Conti et al., 2003). Human IL-22 consists of 179 amino acids containing a signal peptide at its N-terminus and shows 79% amino acid identity with murine IL-22, and 25% and 22% identity with human and mouse IL-10, respectively (Dumoutier et al., 2000b). As previously mentioned, IL-22 is recognized and binds to heterodimeric receptor complex, IL22RA1 and IL10R2. Binding of IL-22 to its receptor complex induces a cascade of downstream phosphorylation signals, including several MAPK pathway molecules (ERK1/2, MEK1/2, JNK, and p38 kinase), and STAT1, STAT3 and STAT5 via Jak1 and Tyk2 (Lejeune et al., 2002). IL-22 binding protein (IL22BP or IL22RA2) is a soluble receptor for IL-22 (Dumoutier et al., 2001; Kotenko et al., 2001) that prevents its binding to the functional cell surface IL-22 receptor complex. IL-22BP

has a higher affinity for IL-22 than the membrane-bound IL-22 receptor complex (Kotenko et al., 2001), and is capable of neutralizing IL-22 activity (Dumoutier et al., 2001; Kotenko et al., 2001; Xu et al., 2001). IL22BP-Fc fusion protein is not capable of binding to IL-19, IL-20 or IL24 (Dumoutier et al., 2001), and IL22BP fails to neutralize IL-10 activities, indicating specific interaction of IL22BP with IL-22. Thus, IL22BP is a naturally occurring, highly specific IL-22 antagonist.

IL-22 is mainly produced by two newly discovered lineages of $CD4^+$ Th cells; Th17 cells (Liang et al., 2006; Zheng et al., 2007) and Th22 cells (Duhen et al., 2009; Trifari et al., 2009). Th17 cells secrete IL-17A and IL-17F as well as IL-22 (Harrington et al., 2005; Park et al., 2005), whereas Th22 cells only produce IL-22 (Trifari et al., 2009). Both cell types are known to be involved in innate immunity against extracellular pathogens, clearance of microbes that are not completely extirpated by other Th cell subsets, and in the development of autoimmune diseases (Bettelli et al., 2007; Wilson et al., 2007; Duhen et al, 2009). IL-22 can also be produced by γδ T cells (Martin et al., 2009; Simonian et al., 2010), natural killer (NK) cells (Wolk et al., 2002; Cella et al., 2009; Guo and Topham, 2010), lymphoid tissue inducer (LTi) cells (Cupedo et al., 2009), and LTi-like cells (Satoh-Takayama et al., 2008; Takatori et al., 2009). Recent findings revealed that a new subpopulation of mucosal NK-like cells secrete IL-22, particularly in the small intestine (Satoh-Takayama et al., 2008). In addition, a different subset of NK cells, named NK-22, has been shown to be specialized in IL-22 production in mucosal-associated lymphoid tissues in mice and humans, thus affording mucosal protection (Cella et al., 2009).

Functional studies in murine models indicate that IL-22 has immunoregulatory properties during infection (Aujla et al., 2008; Zheng et al., 2008; Munoz et al., 2009; Wilson et al., 2010),

inflammation (Brand et al., 2006; Zenewicz et al., 2008; Pickert et al., 2009), autoimmunity (Zheng et al., 2007; Ma et al., 2008), and cancer (Nagakawa et al., 2004; Bard et al., 2008; Zhang et al., 2008). In these models, the functional consequences of IL-22 expression can be either pathogenic or protective, depending on the context in which it is expressed. IL-22 confers protective immunity by inducing the production of antimicrobial peptides, neutrophil-recruiting chemokines, and acute phase proteins, as well as promotion of maintenance of the epithelial barrier in the intestine and respiratory tract during infection (Aujla et al., 2008; Zheng et al., 2008). On the other hand, IL-22 secretion can be pathogenic following infection by an intracellular protozoan parasite through promoting intestinal inflammation and enhancing disease status (Munoz et al., 2009; Wilson et al., 2010).

A putative avian IL-22 nucleotide sequence was identified during analysis of the chicken genome (Kaiser et al., 2005; Kaiser, 2007). In avians, there are only four members of the IL-10 family; IL-10, IL-19, IL-22, and IL-26. Similar to what is found in humans, ChIL-22 is encoded in the same cluster with IL-26 on chromosome 1 in a region syntenic with human chromosome 12. Although there is no research reporting the biological function of ChIL-22, chicken kidney cells exposed to recombinant ChIL-22 showed induced IL-10 expression. In addition, recombinant ChIL-22 upregulates the expression of β-defensins in heterophils (unpublished observations by Kaiser et al.).

In summary, there are numerous factors and molecules involved in the inflammatory response; however, there is little or no information currently known in avian systems. Though avian TLRs were intensively studied, no other PRRs (especially intracellular PRRs) had been characterized prior to this project. In addition, several studies described the potential roles of ChMIF and ChIL-22 during microbial infections (e.g. *Eimeria*) based only on mRNA expression

level and without further characterization. Additionally, none of the studies showed systemic regulation of avian inflammation through innate immune sensors to immune regulation. Thus, the overall goal of this project was to understand the avian inflammatory response in relation with infectious diseases. To achieve our goal, we performed the first stage of this challenging research, which was the functional characterization of the inflammatory mediators Nod1 as a signal initiator, MIF as an immune modulator, and IL-22 as an immune regulator.

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# CHAPTER III

## The Innate Immune Sensor Nod1 and Its Potential Ligands

**ABSTRACT:** Nucleotide-binding oligomerization domain protein 1 (Nod1) and Nod2 are intracellular pattern recognition receptors (PRRs) of nucleotide-binding oligermization domain (NOD)-like receptor (NLR) family involved in innate immune responses. In mammals, Nod1 and Nod2 are involved in host recognition of specific bacterial molecules and are genetically associated with several inflammatory diseases. Stimulation of Nod1 and Nod2 activates NF-κB through RIPK2, resulting in production of pro-inflammatory cytokines, chemokines and anti-microbial peptides. Upon chicken genome analysis, a candidate sequence of chicken Nod1 (ChNod1) was found; however, there is currently no evidence of ChNod2. To examine the subcellular expression and function of ChNod1, we amplified, cloned into peYFP-N1 vector, and transiently overexpressed WT ChNod1 and its mutant forms, absence of either CARD domain or LRR domain in HEK293T cells, followed by measurement of NF-κB activity. Observation of fused yellow fluorescence protein indicated subcellular localization of ChNod1 in the cytoplasm. Stimulation of transiently overexpressed ChNod1 and its mutants with $\gamma$-$_D$-glutamyl-*meso*-diaminopimelic acid (iE-DAP) or muramyl dipeptide (MDP) could not demonstrate the function of ChNod1 due to self-activation of ChNod1. In vitro stimulation study showed no significant difference of transcripts of Nod1 and its signal molecules as well as pro-inflammatory cytokines during stimulation of chicken PBMCs with synthetic mammalian Nod1 or Nod2 motif. On the other hand, incubation of PBMCs with PGN, which stimulates mammalian Nod1 and Nod2 signal, but not TLR signal, led to induction of pro-inflammatory

cytokines. Although our results could not determine the function of ChNod1 and its specific ligand, they implicate potential involvement of ChNod1 in pathogen recognition.

## Introduction

An essential component of bacterial cell walls, peptidoglycan (PGN) consists of glycan chains with alternating moieties of β-(1, 4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc), where muramic acid acts as a linker between sugars and stem peptides containing four alternating $_{L}$- and $_{D}$-amino acids (Dziarski and Gupta, 2005; Agnihotri et al., 2011) (Figure 3.1). The initial amino acid of these tetrapeptides is an $_{L}$-alanine, and the second and fourth amino acids are $_{D}$-glutamine and $_{D}$-alanine, respectively. In most Gram-positive bacteria, the third amino acid is $_{L}$-lysine, whereas in most Gram-negative and some Gram-positive bacilli (genus *Bacillus* and *Clostridium*), it is *meso*-diaminopimelic acid (*m*-DAP) (Uehara et al., 2006). The components of the innate immune system recognize conserved motifs of microorganisms, called microbial-associated molecular pattern (MAMPs) found only in microorganisms, but not in higher eukaryotes. Since PGN is a unique and essential cell wall component of virtually all bacteria, it is an excellent MAMP for recognition by the pathogen recognition receptors (PRRs) in the eukaryotic innate immune system. Extensive studies revealed that muramyldipeptide (MDP) and γ-$_{D}$-glutamyl-*m*-DAP (iE-DAP) are the minimal structure of PGN to stimulate immunobiological responses (Inohara et al., 2005).

Cellular PRRs are ubiquitous innate immune sensors that recognize specific MAMPs. Unlike Toll-like receptors (TLRs), which are expressed on the cell surface and the endocytoplasmic reticulum, recent research has described cytosolic receptors encompassing the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family (Kufer et al.,

2006). NLR family is characterized by a unique nucleotide-binding oligomerization domain, called NACHT, located at the center of the molecule between an N-terminal caspase-associated recruitment domain (CARD), pyrin domain (PYD), or baculovirus inhibitor of apoptosis repeat (BIR) for protein-protein interaction, and a C-terminal leucine-rich repeat (LRR) domain for pathogen recognition (Inohara et al., 2005; Fritz et al., 2006). The best studied intracellular pathogen detectors, Nod1 and Nod2 are prototype members of NLR family that serve to signal the presence of intracytoplasmic peptidoglycan fragment by sensing iE-DAP (Chamaillard et al., 2003; Girardin et al., 2003a) and MDP (Girardin et al., 2003b; Inohara et al., 2003), respectively. Nod1 has ubiquitous expression in several tissues and cell types while Nod2 is found mainly within monocytes and macrophages (Inohara et al., 2002; Inohara et al., 2003). Nod1 and Nod2 are linked to a specific signaling pathway following activation, associating with a serine-threonine kinase, RIPK2, resulting in the activation of NF-κB (Inohara and Nunez, 2003; Viala et al., 2004; Park et al., 2007). This activation is TLR- and MyD88-independent and thus serves as an alternative pro-inflammatory pathway to aid in host defense.

A putative avian Nod1 nucleotide sequence was located on chromosome 2 during analysis of the chicken genome (Figure 3.2) with no evidence of avian Nod2. In this study, the identified chicken Nod1 (ChNod1) was amplified and cloned with its mutant forms, absence of CARD domain or LRR domain, to characterize its biological function and specific ligands.

## Materials and Methods

*Birds, blood and tissue sources*

Twenty-week old healthy broilers were donated by Dr. Paul Siegel; birds had been housed and reared according to the Institutional Animal Care and Use Committee of Virginia

Tech. Birds were sacrificed by cervical dislocation and blood immediately collected via cardiac puncture. Additionally, tissues were quickly harvested for RNA isolation.

*Cells and stimulants*

Human embryonic kidney cells, HEK293T cells (kindly provided by Dr. Liwu Li, Virginia Tech) were used to measure ChNod1-induced NF-κB activity, since HEK293T cells exhibit no expression of mammalian Nod2 and very low expression of mammalian Nod1. They were maintained in Dulbecco's Modified Eagle Medium and (DMEM; Mediatech, VA) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (FCS) (all supplied by Atlanta Biologicals, GA). Peripheral blood mononuclear cells (PBMCs) were isolated as described in Kim et al. (2010). All ligands used in this study were purchased from InvivoGen (CA), except Lipopolysaccharide (LPS) purchased from Sigma (MO). For stimulation study, ligands were used at different working concentrations as shown in Table 3.1.

*Cloning and expression of recombinant chicken Nod1 (ChNod1) and its mutant forms*

Using total RNA from small intestine, the full-length ChNod1 and its mutant forms were amplified and cloned into yellow fluorescence encoded eukaryotic vector (peYFP-N1) as follows. A candidate sequence was identified from the chicken genome and NCBI databases, and primer sets were designed using the predicted ChNod1 (GenBank Accession # XM_41877) (Table 3.2). Total RNA was isolated from jejunum using RNeasy Mini Kit (Qiagen, CA), followed by synthesis of the first-strand cDNA using iScript (Bio-Rad, CA). The full-length

ChNod1 and its mutant forms were amplified by polymerase chain reaction (PCR) with AccuPrime SuperMix II (Invitrogen, CA) using the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 68°C for 1.5 min, with a final extension at 68°C for 10 min. The freshly synthesized full-length ChNod1 was directly inserted into pCR 2.1-TOPO vector, followed by transformation into *E. coli* TOP10 (Invitrogen, CA). Transformed *E. coli* TOP10 were cultured in Luria-Bertani media (LB; Fisher Scientific, NJ) at 37°C overnight. A transformant was selected by a combination of PCR screening and endonuclease digestion with *Eco*R I (New England Biolabs, MA) and confirmed by sequencing (Virginia Bioinformatics Institute at VT, VA). For sub-cloning into eukaryotic expression vectors (peYFP-N1; Clontech, CA), ChNod1 was digested with endonucleases *Xho* I and *Sac* II (New England Biolabs, MA). The digested ChNod1 fragment was purified from an agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, WI), and ligation was performed with *Xho* I/*Sac* II-digested peYFP-N1 vector at 4°C overnight. *E. coli* TOP10 were transformed with the ligation mixtures, plasmid DNA was prepared from transformants, and subjected to DNA sequencing to confirm identity of recombinant plasmid.

*Co-transfection of HEK293T cells*

One day prior to transfection, HEK293T cells were seeded at 4 x $10^4$ cells/well in a 24-well plate, and incubated 24 h at 37°C and 5% $CO_2$. The cells were gently washed once with DMEM and incubated with serum- and antibiotics-free DMEM for 30 min. DNA cocktail was prepared with Lipofectamine 2000 (Invitrogen, CA) as shown in Table 3.3, according to the

manufacturer's instruction. The prepared DNA mixture was added and cells were incubated 20 h. The media were changed and the cells were observed by the expression of yellow fluorescence by a microscope followed by treatment with various ligands as listed in Table 3.1. For total RNA or protein, cells were directly lysed with either cell lysis buffer (RLT buffer; Qiagen) or RIPA buffer, respectively.

*Dual-Luciferase Assay*

Luciferase assay was performed using Dual-Luciferase Assay Kit (Promega; MI). Briefly, co-transfected HEK293T cells were treated with media alone, iE-DAP, MDP, soluble PGN, and LPS (5 ug/mL of each) for 24 h. After the media were completely removed and the cells gently washed, 1X Passive Lysis Buffer (Promega) was added directly and the plates were incubated on a microplate shaker (700 rpm) for 15 min at room temperature. Twenty microliters of the prepared cell lysis were mixed with 50 μL of Luciferase Assay Buffer II (LAR II), then Firefly luciferase activity was measured using a luminometer. Next, 50 μL of the prepared Stop & Glo reagent were added/mixed, and *Renilla* luciferase activity was measured. The activity of NK-κB is described as the ratio of Firefly luciferase activity verus *Renilla* luciferase activity.

*In vitro stimulation of ChNod1*

The freshly isolated PBMCs were seeded onto 6-well plates and cultured for 24 h. After gentle washing to remove non-adherent cells, PBMCs were treated with the listed ligands (Table 3.1) for 3 and 6 h. Also, PBMCs were also incubated with rChIFN-γ (0.01 μg/mL) for 6 h. Cell

lysis buffer was directly added onto the wells and total RNA was extracted using RNeasy Mini Kit (Qiagen).

*Quantitative real-time PCR (qRT-PCR) analysis of the cytokine transcripts*

To analyze the transcripts of various Nod1 signal molecules and cytokines, primers were designed using Primer Express (Ver 3.0; Applied Biosystems, CA) (Table 3.3), and qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, CA) according to the manufacturer's instructions in an ABI 7500 Fast Real-Time PCR System. Using 1 μg of total RNA, first-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) following manufacturer's instruction. Synthesized cDNA was diluted (1/25) with nuclease-free water, and 2 μL of the solution were used for Fast SYBR Green Master Mix; 100 nM primers and 10 μL of SYBR Green Master Mix in nuclease-free water in a final volume of 20 μL. Samples were heated to 95°C for 20 sec as initial denaturation, followed by 40 cycles of denaturation at 95°C for 3 sec, and annealing/extension at 57°C for 30 sec. Dissociation curves were generated to analyze individual PCR products after 40 cycles. Target gene expression in this study was normalized against the expression of chicken GAPDH mRNA. The results were analyzed using 7500 Software (Ver 2.0; Applied Biosystems, CA).

*Statistical analysis*

All data were analyzed by either Student's t-test or analysis of variance (ANOVA) using Jump software (SAS, JMP Ver 9.0), and significant differences among groups were tested by the Tukey-Kramer Honestly Significant Difference post-hoc procedure.

## Results

*Sequence analysis of ChNod1/2*

Upon genome analysis, ChNod1 was found on chicken chromosome 2, in synteny with human chromosome 7 and mouse chromosome 6 (Figure 3.2A). However, there is no evidence of ChNod2 in chicken genome, even though chicken chromosome 11 showed synteny with human Nod2 locus on chromosome 16 and mouse Nod2 locus on chromosome 8 (Figure 2B). The predicted open reading frame of ChNod1 encodes a 951-amino acid protein (Figure 3.3A). Analysis of the primary structure of ChNod1 using the SMART architecture research program identified no signal peptide, an N-terminal CARD domain (residues 27 – 101), a central NACHTdomain (residues 201 – 340), and multiple C-terminal LRR domains (residues 650 – 947) (Figure 3.3B). Comparison of the deduced amino acid sequences of ChNod1 with those of human and mouse revealed 61% and 59% identity, respectively (Figure 3.4A). In the phylogenetic analysis, avian Nod1 sequences form a monophyletic clade, which is sister to that of Nod1 from mammals (Figure 3.4B). The chicken Nod1 sequence is most similar to those of turkey and zebra finch.

*Tissue distribution of ChNod1*

The expression of ChNod1 was examined in various tissues, including brain, thymus, spleen, bursa, heart, liver, lung, kidney, stomach, and intestinal sections using qRT-PCR (Figure 3.5). ChNod1 expression was normalized with GAPDH, and the relative expression of ChNod1 in various tissues was compared to that of the brain, which showed the lowest expression level. The results showed that ChNod1 is ubiquitously expressed in all tissues tested, with the highest

level found in the jejunum. Expression levels of ChNod1 were similar between thymus and spleen, and the three intestinal sections (duodenum, jejunum and ileum) showed higher expression of ChNod1 than all other examined tissues.

*Cloning, expression and subcellular localization of ChNod1 and its mutants*

To characterize the molecular function of ChNod1, the full-length ChNod1 (termed wild type, WT) and two mutant forms, absence of either CARD domain or LRR domain, were amplified from total RNA isolated from jejunum (Figure 3.6A). The amplified products were cloned into the eukaryotic expression vector peYFP-N1, which encoded yellow fluorescence protein at the C-terminal of target gene. The subcellular localization of the cloned WT ChNod1 and mutants were first determined by observation of fused yellow fluorescence protein via a fluorescence microscope, after transient transfection of ChNod1 into HEK293T cells (Figure 3.6B). All three forms of ChNod1 were present in the cytosol.

*NF-κB activity by various ligands*

To determine whether ChNod1 is activated byligands that stimulate mammalian Nod1, Nod2 or both Nod1 and Nod2, HEK293T cells were transiently transfected with WT or mutant forms of ChNod1. Then, the transfected cells were stimulated with various ligands (iE-DAP, MDP, PGN-Ecndss, LTA and LPS), followed by evaluation of NF-κB activation. Whether stimulated or not, vector only-transfected cells showed little or no NF-κB activity (Figure 3.7). In the presence of WT ChNod1, NF-κB activity was increased even without stimulants in

comparison with that of vector only transfected cells. While the CARD-deletion mutant showed as little NF-κB activity as that of vector only-transfected cells, LRR-deletion mutant showed induced NF-κB activity in non-stimulated sample. Taken together, presence of the CARD in ChNod1 led to self-activation of ChNod1, resulting in induced NF-κB activity.

*In vitro stimulation of Nod1 with various ligands*

Since the ChNod1 activates NF-κB activity by self-activation with a mammalian system, we attempted a different method to find a ligand for ChNod1 by measuring ChNod1 expression. PBMCs were isolated and treated with mammalian Nod1/2 ligands at two different time points, for 3 and 6 h (Figure 3.8). The minimum motif of iE-DAP for Nod1 and MDP for Nod2 did not stimulate Nod1 transcript as well as any expression of signal molecules (RIPK2 and p65) and pro-inflammatory cytokines (IL-1β, IL-8 and IL-18). However, soluble sonicated PGN induced expression of IL-1β, IL-8 and IL-18, though there was no significant difference in expression of Nod1 signaling molecules. In contrast, LPS stimulated cells showed high expression of pro-inflammatory cytokines and signaling molecules, including RIPK2 and MAPK11. Since there was no significant difference in ChNod1 expression, recombinant ChIFN-γ was employed as a stimulant; however, ChNod1 expression was not induced by rChIFN-γ (Figure 3.9), although mRNA levels of TLR2 and IL-1β were induced by the presence of rChIFN-γ. To evaluate ligands of ChNod1, chicken PBMCs were stimulated with various commercially available mammalian Nod1/2 ligands for 6 h (Figure 3.10). However, none of the mammalian Nod1/2 ligands stimulated and induced expression of ChNod1 or its signaling molecules, except M-triDAP and PGN groups, both being Nod1 and Nod2 agonists.

## Discussion

The goal of this study was to characterize the biological function of ChNod1 and its ligand. Upon searching the chicken genome, we found a candidate sequence for ChNod1, but not for ChNod2. Comparative genomic analysis revealed that ChNod1 is syntenic with Zinc and ring finger 2 (ZNRF2), gamma-glutamyl cyclotransferase (GGCT), and glycyl-tRNA synthetase (GARS) on chromosome 2, in similar synteny with human chromosome 7 and mouse chromosome 6. Chicken chromosome 11 is syntenic with human and mouse Nod2 locus; however, there are Naked cuticle 1 homolog (NKD1), sorting nexin 20 (SNX20), and cylindromatosis (CYLD) genes, but not Nod2 gene. Interestingly, even zebrafish has Nod2 gene, but *Xenopus* and *Gallus* are missing this gene. Thus, we hypothesized that ChNod1 may recognize and be stimulated by both mammalian Nod1 and Nod2 ligands.

Like mammalian Nod1, ChNod1 is expressed ubiquitously in most tissues. Especially, ChNod1 is highly expressed in the small intestine, suggesting potential important role of ChNod1 in mucosal immunity. In mammals, absence of Nod1 reduced formation of isolated lymphoid follicles in gut (Bouskra et al., 2008), indicating the role of Nod1 in homeostasis of intestinal lymphoid tissues.

Most mammalian studies used HEK293(T) system to measure NF-κB activity by activation of Nod1 or Nod2 because there is no mammalian Nod2 expression and very low mammalian Nod1 expression in HEK293 cells. In addition, HEK293 cells do not express TLR2. Thus, WT ChNod1and its mutant forms were cloned and transiently transfected into HEK293T cells, following stimulation with various ligands and measurement of NF-κB activity by dual-luciferase assay. However, presence of WT ChNod1 showed high NF-κB activity even in

incubation with medium alone. This induced NF-κB activity was retained in the absence of LRR domain, but abolished in the absence of CARD domain, suggesting potential involvement of CARD domain in self-activation of ChNod1 in the HEK293T system. Chicken cell lines, HTC and DF-1, were also used to determine ChNod1 activity by overexpressing ChNod1; however, those cells did not work due to high expression of internal control, *Renilla* luciferase activity.

To stimulate ChNod1 signal, various ligands for mammalian Nod1, Nod2 or for both were used in this study (Table 3.1). A dipeptide, iE-DAP is a minimum motif recognized by Nod1 and is a component of most Gram-negative and some Gram-positive bacteria, such as *Bacillus*, *Listeria* and *Clostridium* (Chamaillard et al., 2003; Park et al, 2007). C12-iE-DAP is an acylated derivative of the dipeptide iE-DAP (Agnihotri et al., 2011), generated by addition of a lauroyl (C12) group to the glutamic residue of iE-DAP. Similarily to iE-DAP, C12-iE-DAP is recognized by mammalian Nod1 and it stimulates 100- to 1000-fold higher than iE-DAP. The other ligand for mammalian Nod1, Tri-DAP is comprised of the minimum form of iE-DAP plus an $_{L}$-Ala residue, which is naturally present in PGN (Chamaillard et al., 2003; Girardin et al., 2003a; Park et al., 2007). Tri-DAP exhibits around 3-fold higher ability to activate NF-κB than iE-DAP. For mammalian Nod2, MDP is the minimal bioactive PGN motif common to all bacteria, and is the essential structure required for adjuvant activity in vaccines (Girardin et al., 2003b; Inohara et al., 2003; Martinon et al., 2004). L18-MDP, a 6-O-acyl derivative with a stearoyl fatty acid, showed the highest activity, and in an attempt to enhance the protective activity against bacterial infection, numerous derivatives of MDP have been synthesized (Girardin et al., 2003b). L18-MDP was shown to be 10 times more efficient than MDP inducing NF-κB activation. Murabutide is a safe synthetic immunomodulator derived from MDP (Chedid et al., 1982; Vidal et al., 2001). In contrast to MDP, murabutide is devoid of pyrogenic activity

and lacks somnogenic activity. Murabutide has the capacity to synergize with selected therapeutic cytokines to drive the release of Th1 cytokines. MurNAc-L-Ala-γ-D-Glu-mDAP (M-TriDAP), also called DAP-containing muramyl tripeptide is a PGN degradation product found mostly in Gram-negative bacteria (Park et al., 2007). M-TriDAP is recognized by the intracellular sensor Nod1 and to a lesser extent Nod2. The other ligand for mammalian Nod1and Nod2 used in this study were three different forms of PGN; insoluble PGN from *E. coli K12*, soluble PGN from *E. coli K12* and insoluble PGN from *Staphylococus aureus* (only for Nod2). In mammalian trials, these PGN only stimulate mammalian Nod1and Nod2, but not mammalian TLR2 and TLR4 (Dziarski et al., 2004; Travassos et al., 2004).

In this study, incubation of PBMCs with PGN showed induction of pro-inflammatory cytokines including IL-1β and IL-8. In conjunction with mammalian research, our results suggest that ChNod1 is stimulated by PGN – since there is no evidence of ChNod2, and activates pro-inflammatory cytokines via Nod1 signaling pathway. Overall, ChNod1 is stimulated and activated by PGN, although minimal motif of Nod1 and Nod2, iE-DAP and MDP did not activate ChNod1, suggesting ChNod1 may be stimulated by different peptide motif of PGN.

Park and his colleagues (2007) reported that absence of RIPK2 did not affect cytokine responses to TLR agonists, but influenced NF-κB activation in response to iE-DAP and MDP, indicating RIPK2 is critical signal molecule in Nod1/2 signaling pathway. Based on that work, LPS should not stimulate and induce RIPK2 expression. However, stimulation of chicken PBMCs with LPS led to induction of RIPK2 mRNA level. There are two possible explanations: One is that LPS employed here was not ultrapure, so it was perhaps contaminated with PGN fragments that can activate Nod1 signal and induce RIPK2 expression. However, contamination of PGN fragments can explain induction of RIPK2 mRNA level, but cannot justify the "no

change" in ChNod1 expression. The other possibility is that chicken RIPK2 may be an adaptor molecule in chicken TLR signaling pathway or may be involved in a certain member of NLR family signaling pathway.

An earlier publication reported that mammalian Nod1 mRNA level was induced by incubation with IFN-γ, but not other cytokines like IL-1β, TNF-α and TGF-β (Hisamatsu et al., 2003). Additionally, Nod2 mRNA level was induced by IFN-γ and TNF-α (Gutierrez et al., 2002; Rosenstiel et al., 2003). However, incubation of chicken PBMCs with rChIFN-γ did not induce ChNod1 expression. It may be because ChNod1 is regulated not at the mRNA level, but post-translationally at the protein level. Besides, we could not observe any significant change of ChNod1 expression in any *in vitro* stimulation study, even though RIPK2 was induced by LPS. Alternatively, induction of Nod1 by other cytokines like IFN-γ may be a tissue/cell specific, since early publication used epithelial cell lines (Hisamatsu et al., 2003; Rosenstiel et al., 2003).

In summary, WT ChNod1 and its mutant forms were successfully cloned and expressed. However, we failed to measure NF-κB activity using a "conventional" system because of interference of chicken CARD domain. Additionally, we could not clarify whether mammalian Nod2 ligands stimulate ChNod1 because mammalian Nod1 and Nod2 ligands used in this study could not stimulate ChNod1 mRNA as well as its signaling molecules and pro-inflammatory cytokines. However, the mammalian Nod1/2 ligand, PGN, induced pro-inflammatory cytokines, suggesting ChNod1 is activated by certain component(s) of PGN. Further research is clearly warranted to determine the specific ligand of ChNod in order to better characterize its biological function in avian species.

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**Table 3.1. Stimulant and its working concentration**

| PRRs | Product | Working Concentration (μg/mL) | References |
|---|---|---|---|
| Nod1 | C12-iE-DAP | 1 | Chamaillard et al., 2003; Girardin et al., 2003; Park et al., 2007 |
| | iE-DAP | 5 | |
| | Tri-DAP | 0.1 | |
| Nod2 | L18-MDP | 5 | Inohara et al., 2004; Martinon et al., 2004 |
| | MDP | 5 | |
| | Murabutide | 1 | Chedid et al., 1982; Vidal et al., 2001 |
| Nod1/2 | M-TriDAP | 5 | Park et al., 2007 |
| | PGN-Ecndi[1)] | 5 | Travassos et al., 2004; Dziarski, 2004; Girardin et al., 2003 |
| | PGN-Ecndss[2)] | 5 | |
| | PGN-Sandi[3)] | 5 | |
| TLR2 | LTA | 5 | Morath et al., 2001; Schwandner et al., 1999 |
| TLR4 | LPS | 5 | Kim et al., 2010 |

1) Insoluble PGN from *E. coli K12*

2) Soluble PGN from *E. coli K12*

3) Insoluble PGN from *S. aureus*

**Table 3.2. Primer sequences for cloning ChNod1 and its mutant forms, and qRT-PCR analyses of cytokine transcript expressions.**

| Primer Name | Accession No. | Sense sequence | Anti-sense sequence |
|---|---|---|---|
| Cloning | | | |
| WT ChNod1 | XM_418777 | GCACTCGAGATGGAAGGCCACCTTTGTGCTAAC | GATCCGCGGGAAGCAAATGATCCGTTCTTCACT |
| ChNod1ΔCARD | | GCACTCGAGATGCTGGATGAAATAGGTTACCAG | GATCCGCGGGAAGCAAATGATCCGTTCTTCACT |
| ChNod1ΔLRR | | GCACTCGAGATGGAAGGCCACCTTTGTGCTAAC | GATCCGCGGGTAGTCATTGATGTTGTTGTTATCCAA |
| qRT-PCR | | | |
| GAPDH | NM_204305 | AGGGTGGTGCTAAGCGTGTTA | TCTCATGGTTGACACCCATCA |
| IL-1β | NM_204524 | CCCGCCTTCCGCTACA | CACGAAGCACTTCTGGTTGATG |
| IL-8 | NM_205498 | TCCTGGTTTCAGCTGCTCTGT | CGCAGCTCATTCCCCATCT |
| IL-18 | NM_204608 | AGGTGAAATCTGGCAGTGGAAT | TGAAGGCGCGGTGGTTT |
| MAPK11 | NM_001006227 | GCGCTCGCACATCCATACT | AGCTCGGCCTCAGGTTCGT |
| Nod1-LRR | XM_418777 | TGAGGAACCACCCCAGGTT | CTCTGTCGTGATGCCATTGAA |
| Nod1-CARD | XM_418777 | CCAATGAAAGCCTGGGTGAA | TTACCCCAACTGCATCATCAA |
| p65 | NM_205129 | GCGGCGGTGGCTGAA | GCTCCTCCATTGGCACATTGA |
| RIPK2 | NM_001030943 | AAATATACCACTGAATCCCCAAGAG | CCGAAGAGGAAGTGCATCACA |
| TLR2 | NM_204278 | GCGAGCCCCCACGAA | GGAGTCGTTCTCACTGTAGGAGACA |

**Table 3.3. DNA cocktail for co-transfection**

| Tube No. | DNA | | | | | | | Lipofectamine 2000 | |
|---|---|---|---|---|---|---|---|---|---|
| | DNA | Amt (μg/well) | DNA | Amt (μg/well) | DNA | Amt (μg/well) | Medium (μl/well) | Lipofectamine (μl/well) | Medium (μl/well) |
| 1 | pNF-κB-Luc (Clontech, CA) | 0.2 | pRL-TK (Promega, WI) | 0.2 | peYFP-N1 | 0.4 | 50 | 1.0 | 50 |
| 2 | | | | | WT ChNod1 | 0.4 | 50 | 1.0 | 50 |
| 3 | | | | | ChNod1ΔCARD | 0.4 | 50 | 1.0 | 50 |
| 4 | | | | | ChNod1ΔLRR | 0.4 | 50 | 1.0 | 50 |

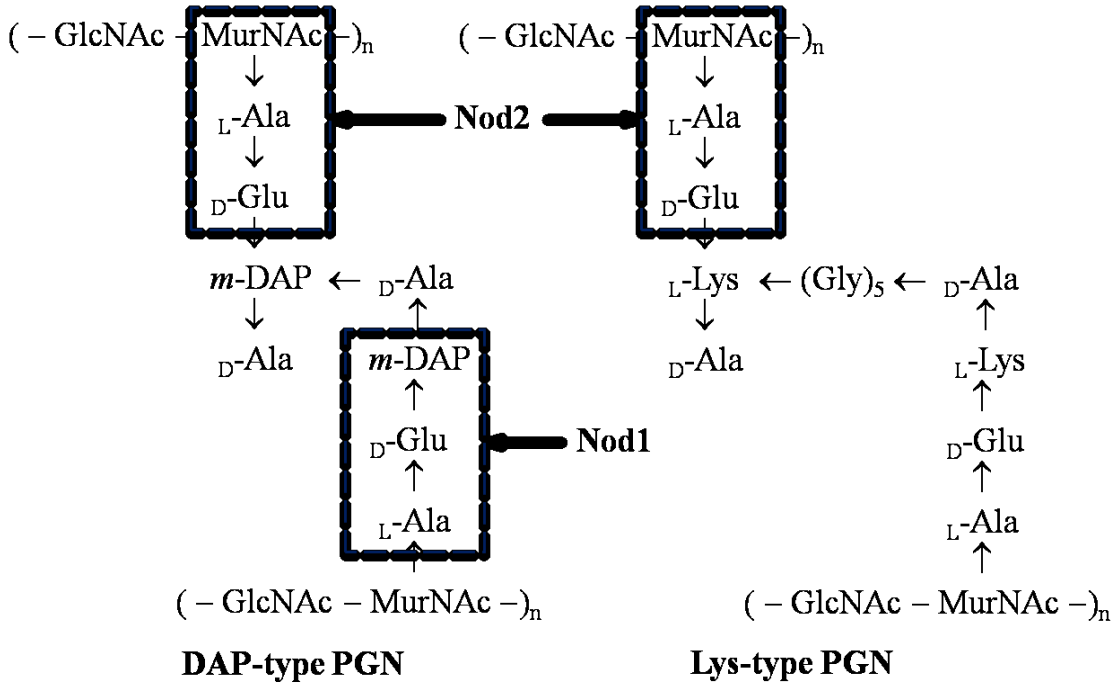



**Figure 3.1. DAP-type and Lys-type PGN and minimal PGN fragments recognized by Nod1 and Nod2**

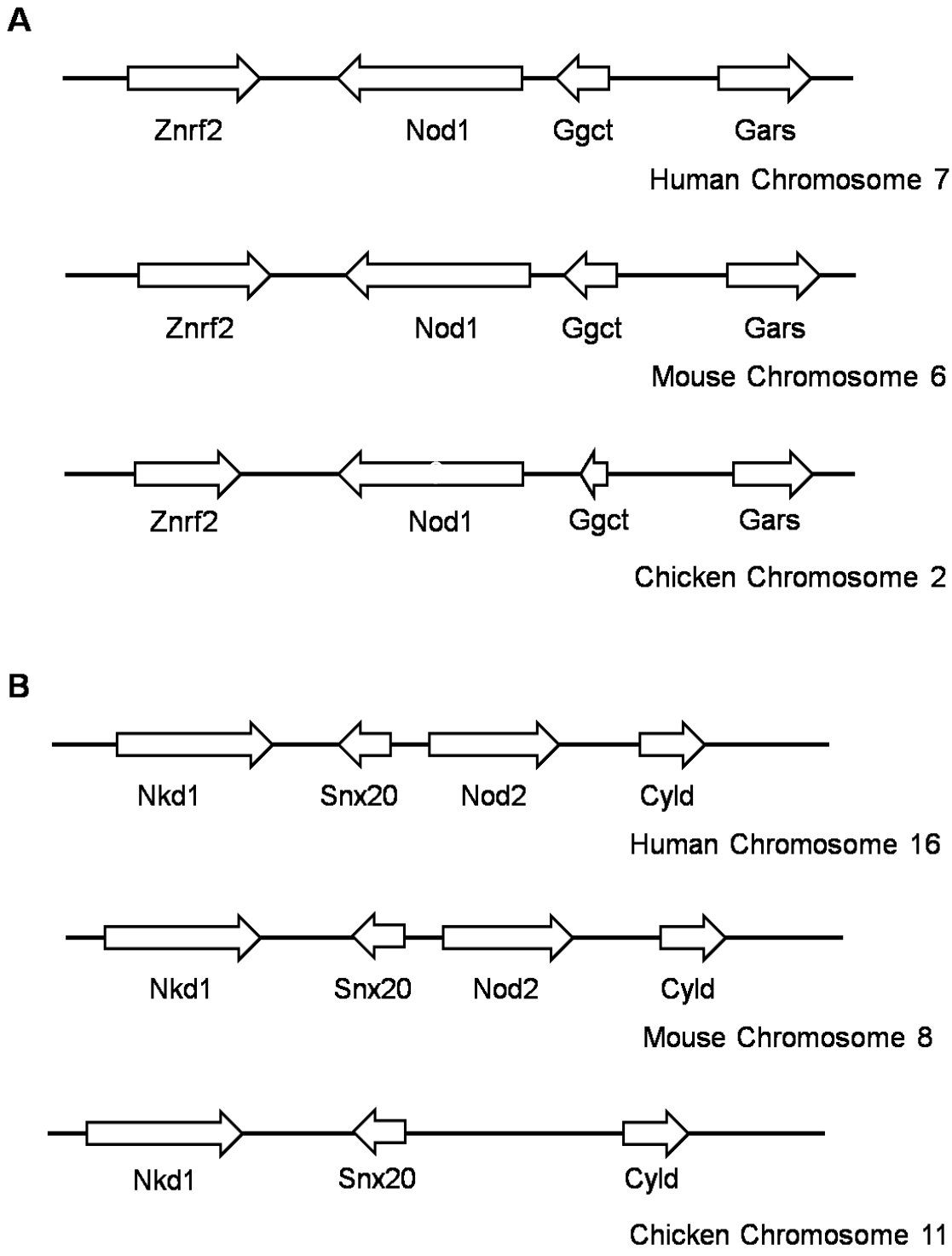



**Figure 3.2. Schematic drawings comparing the human, mouse and chicken Nod1 and Nod2.** **A.** Similar synteny with human and mouse, ChNod1 is located on chromosome 2 along with zinc and ring finger 2 (Znrf2) and gamma-glutamyl cyclotransferase (Ggct), and glycyl-tRNA synthetase (Gars). **B.** There is no evidence of ChNod2 in chromosome 11, which shows similar

synteny of human and mouse Nod2 locus. Naked cuticle 1 homolog (Nkd1), sorting nexin 20 (Snx20), and cylindromatosis (Cyld).

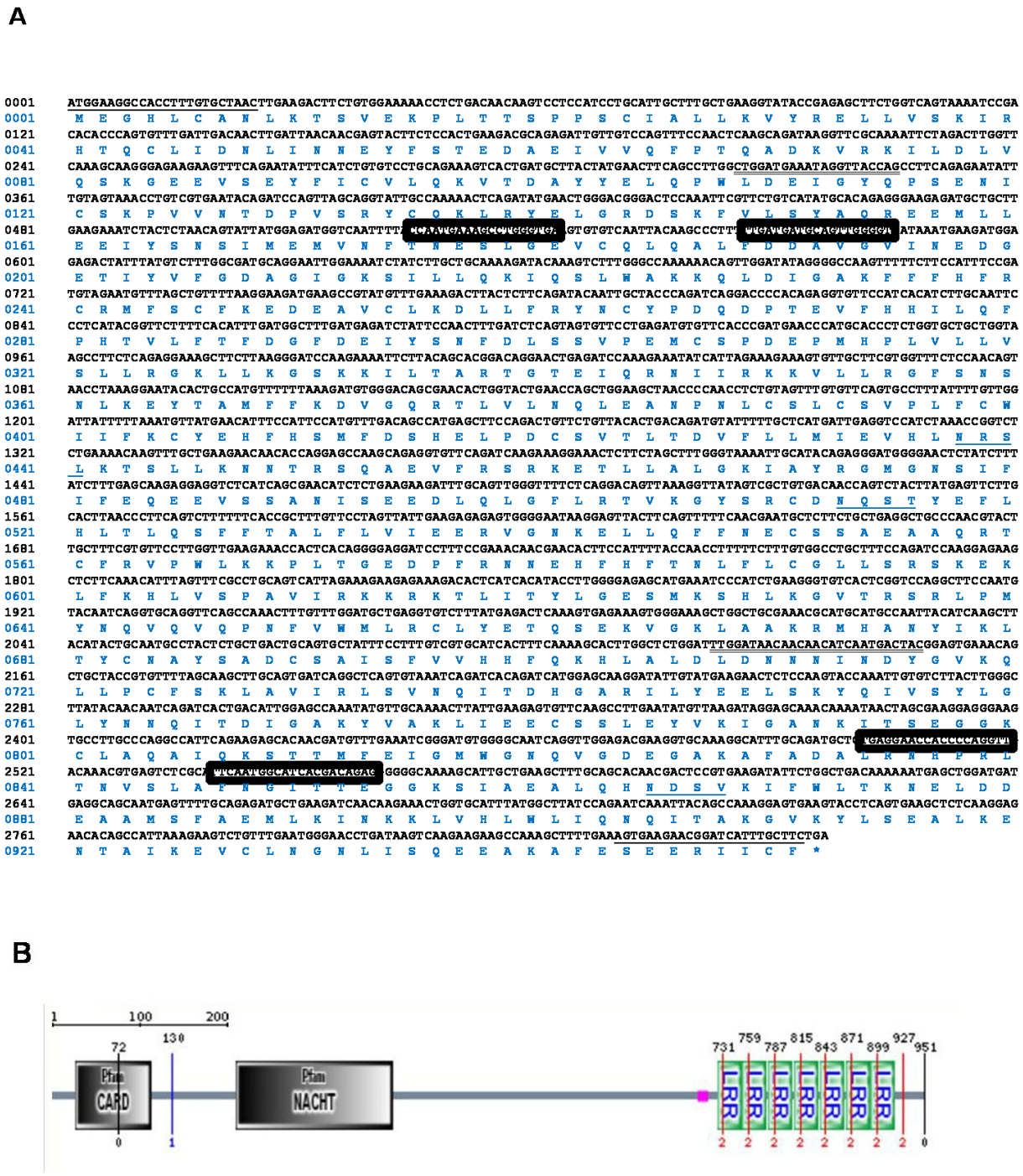



**Figure 3.3. Nucleotide and deduced amino acid sequences of ChNod1 and its architecture.** **A.** Nucleotide and deduced amino acid sequences of ChNod1. The underlined nucleotide sequences indicate the primers used to amplify WT ChNod1, and double underlined nucleotide sequences indicate the primers used to amplify mutant forms of ChNod1. The boxed nucleotide sequences indicate the primers used to detect ChNod1 from various tissues by qRT-PCR. **B.** Architecture of ChNod1. The protein sequence of ChNod1 was analyzed using SMART.

**A**

**CARD domain**

```
Ggal        MEGHLCANLKTSVEKPLTTSPPSCIALLKVYRELLVSKIRHTQCLIDNLINNEYFSTEDA 60
Mgal        MEGHLCANLKTSVEKPLAASPPSCIALLKVYRELLVSKIRHTQCLIDNLINNEYFSTEDA 60
Tgut        MEGQLCANVDISVNKPPGASPQSFIALLKVHRELLVSRIRNTQCLIDNLIKNGYFSTEDA 60
Hsap        MEEQGHSEMEIIP-----SESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDA 55
Mmus        MEEHGHHEMEGTP-----LGCHSHIKLLKINREHLVTNIRNTQCLVDNLLENGYFSAEDA 55
Drer        MGSYKTEN--------------SYLKLLTVHRELLVEQVKNTQCILDNLLMNSFICTEDI 46
            *      :              . : **.  ** ** .:::***::***: * ::.:**

Ggal        EIVVQFPTQADKVRKILDLVQSKGEEVSEYFICVLQKVTDAYYELQPWLDEIGYQPSENI 120
Mgal        EIVVQFPTQADKVRKILDLVQSKGEEVSEFFIHVLQNVTDAYYELQPWLDEIGYQPSENI 120
Tgut        EIVVQFPTQADKVRKILDLVQSKGEEISEYFIYVLQKVTDAYYELQPWLDEIGYKPSENI 120
Hsap        EIVCACPTQPDKVRKILDLVQSKGEEVSEFFLYLLQQLADAYVDLRPWLLEIGFSPSLLT 115
Mmus        EIVCACPTKPDKVRKILDLVQSKGEEVSEFFLYVLQQLEDAYVDLRLWLSEIGFSPSQLI 115
Drer        EIIQRSSTKTDQVRKILELVQSKGEECSAYFTQILHEAYDAYIDLRPWFDEIQYTPLDTI 106
            **:   .*:.*:*****:******** * :*  :*::  *** :*: *: ** : *

Ggal        CSKPVVNTDPVSRYCQKLRYELGRDSKFVLSYAQREEMLLEEIYSNSIMEMVNFTNESLG 180
Mgal        CSKPVINTDPVSRYCQKLRYELGRDSKFVTSYAQREEMLLEEIYSNSIMEMVNFTNESLG 180
Tgut        CSKPVVNTDPVSRYCQKLRHELGRDSKFFMLYAQKEEMLLEEIYSNSIMELVSFTNESLG 180
Hsap        QSKVVVNTDPVSRYTQQLRHHLGRDSKFVLCYAQKEELLLEEIYMDTIMELVGFSNESLG 175
Mmus        RTKTIVNTDPVSRYTQQLRHQLGRDSKFMLCYAQKEDLLLEETYMDTLMELVGFNNENLG 175
Drer        KAIPVVNTDPISKYCEKLRYELGRDTQFITSYSKSEETPLEDLYTDTQMELLNDTGESLG 166
             :  ::****:*:* ::**:.****::*.  *:: *:  **: * :: **::. ..*.**
```

**NACHT domain**

```
Ggal        EVCQLQALFDDAVGVINEDGETIYVFGDAGIGKSILLQKIQSLWAKKQLDIGAKFFFHFR 240
Mgal        EVCQLQALFDDAVGVINEDGETIYVFGDAGIGKSILLQKIQSLWAKKQLDIGAKFFFHFR 240
Tgut        QVGQLEALFDDAVGLINEDGETVYVYGDAGIGKSILLQKIQSLWARKELDIGAKFFFRFR 240
Hsap        SLNSLACLLDHTTGILNEQGETIFILGDAGVGKSMLLQRLQSLWATGRLDAGVKFFFHFR 235
Mmus        SLGGLDCLLDHSTGVLNEHGETVFVFGDAGVGKSMLLQRLQSLWASGRLTSTAKFFFHFR 235
Drer        YLQNLDQLLG-DHGVFNPQAETIFITGDAGVGKSIMLQKLQNLWSRRELKTGAKFFFKFR 225
             :  *  *:.   *::* ..**::: ****:***::**::*.**:  .*   .****:**

Ggal        CRMFSCFKEDEAVCLKDLLFRYNCYPDQDPT-EVFHHILQFPHTVLFTFDGFDEIYSNFD 299
Mgal        CRMFSCFKEDEAVCLKDLLFRYNCYPDQDPT-EVFHHILQFPHTVLFTFDGFDEIYSNFD 299
Tgut        CRMFSCFKEDEAICLKDLLFKYNCYPDQDPT-EVFHHILQFPHTVLFTFDGFDEIYSNFD 299
Hsap        CRMFSCFKESDRLCLQDLLFKHYCYPERDPE-EVFAFLLRFPHVALFTFDGLDELHSDLD 294
Mmus        CRMFSCFKESDMLSLQDLLFKHFCYPEQDPE-EVFSFLLRFPHTALFTFDGLDELHSDFD 294
Drer        CRAFSAFKETDEISLKDLIFKHNCYPDGDPDNEVFAYILRFPETVVFTFDGYDELQMDFD 285
            ** **.*** : :.*:**:*:: ***: **  *** .:*:**...:***** **:  ::*

Ggal        LSSVPEMCSPDEPMHPLVLLVSLLRGKLLKGSKKILTARTGTEIQRNIIRKKVLLRGFSN 359
Mgal        LSSVPEMCSPNEPIHPLVLLVSLLRGKLLKGSKKILTARTGTEIQRNIIRKKVLLRGFSS 359
Tgut        LSSVPEICSPNEPIHPLALLVSLLRGKLLKGSKKILTARTGTEIQKNIIRKKVLLRGFSR 359
Hsap        LSRVPDSSCPWEPAHPLVLLANLLSGKLLKGASKLLTARTGIEVPRQFLRKKVLLRGFSP 354
Mmus        LSRVPDSCCPWEPAHPLVLLANLLSGRLLKGAGKLLTARTGVEVPRQLLRKKVLLRGFSP 354
Drer        LDNVPETVSPEEKTRPLLLLMNLLCGKLLKGSRKILSARSGTEIQSRVIRKKVFLKGFAP 345
            *. **:  .* *  :** ** .** *:****: *:*:**:* *:  ..:****:*:**:
```

```
Ggal        SNLKEYTAMFFKDVGQRTLVLNQLEANPNLCSLCSVPLFCWIIFKCYEHFHSMFDSHE-- 417
Mgal        NNLKEYTAMFFKDVGQRTLILNQLEANPNLHSLCSVPLFCWIIFKCYEHFHSMFDSHE-- 417
Tgut        SNLKEYTAMFFKDEQRRVLVSNQLEANPNLCSLCSVPLFCWIIFKCFEHFHSMFDSHE-- 417
Hsap        SHLRAYARRMFPERALQDRLLSQLEANPNLCSLCSVPLFCWIIFRCFQHFRAAFEGSP-Q 413
Mmus        SHLRAYARRMFPERTAQEHLLQQLDANPNLCSLCGVPLFCWIIFRCFQHFQTVFEGSSSQ 414
Drer        EHLKRYLALHFPEQEHRMLVSDQLDANPHLCGLCSIPLFSWIILKSFKHLQSVYDDFE-- 403
            .:*: *    * :   :  : .**:***:* .**.:***.***::.::*::: ::.

Ggal        LPDCSVTLTDVFLLMIEVHLNRSLKTS-LLKNNTRSQAEVFRSRKETLLALGKIAYRGMG 476
Mgal        LPDCSVTLTDVFLLMIEVHLSRSLKTS-LLKNNVRSQAEVFKSRKETLLALGKIAYKGMR 476
Tgut        LPDSSVTLTDVFLLMIEVHLNRSVKTS-LLKSNIRSQAEMFKSRKESLLALGKMAYKGME 476
Hsap        LPDCTMTLTDVFLLVTEVHLNRMQPSS-LVQRNTRSPVETLHAGRDTLCSLGQVAHRGME 472
Mmus        LPDCAVTLTDVFLLVTEVHLNRPQPSS-LVQRNTRSPAETLRAGWRTLHALGEVAHRGTD 473
Drer        LPGSCITLTNVFLLLSEVFLGHSTARPGLLRRTLRCPTETFKAGEQKLSGFARLALHGIE 463
            **.. :***:****: **.*.:    . *:: . *. .* :::   .* .:..:* :*

Ggal        NSIFIFEQEEVSSANISEEDLQLGFLRTVKGYSRCDNQSTYEFLHLTLQSFFTALFLVIE 536
Mgal        NSIFIFEQEEVSSANISEEDLQLGFLRTVKGYSGCDDQSTYEFLHLTLQSFFTALFLVME 536
Tgut        NSSFIFEQEEVSSANISEEDLQLGFLRTVKGYSGCGSQATYEFLHLTLQSFFTALFLVME 536
Hsap        KSLFVFTQEEVQASGLQERDMQLGFLRALPELGPGGDQQSYEFFHLTLQAFFTAFFLVLD 532
Mmus        KSLFVFGQEEVQASKLQEGDLQLGFLRALPDVGPE-QGQSYEFFHLTLQAFFTAFFLVAD 532
Drer        TSKLVFTLDEAVCCGLNDEDLQFGFLRPASHYDSS-SASSFEFLHETLQAFLAAFSLVLD 522
            .* ::*  :*. .. :.: *:*:****.    .   .  ::**:* ***:*::*: ** :

Ggal        ERVGNKELLQFFNECSSAEAAQRTCFRVPWLKKPLTG------EDPFRNNEHFHFTNLFL 590
Mgal        ERAGNKELLQFFNECSSAEAAQRTCFHVPWLKKPLTG------EDPFRNNEHFHFTNLFL 590
Tgut        EKVGTKELLEFFNECSSLETAQPTCLRIPWLKKQLAG------EDPFQNKEHFNFTNMFL 590
Hsap        DRVGTQELLRFFQEWMPPAGAATTSCYPPFLPFQCLQGSGPAREDLFKNKDHFQFTNLFL 592
Mmus        DKVSTRELLRFFREWTSPGEATSSSCHSSFFSFQCLGGRSRLGPDPFRNKDHFQFTNLFL 592
Drer        AKLNPESILKFFSKCKYKKSSRLSCIPCLKNTKPRES-------DAFQTN--FQFTNLFL 573
             : . ..:*.** :      :  :.                   * *:.:  *:***:**

Ggal        CGLLSRSKEKLFKHLVSPAVIRKKRKTLITYLGESMKSHLKGVTRSRLPMYN--QVQVQP 648
Mgal        CGLLSRSNEKLFKHLVLPAVIRKKRKTLITYLGESMKSHLKGVTRSRLPKYN--QVQVQP 648
Tgut        CGLLSGSRQKLFRHLVSPAVIKRKRKILITYLGESMKSHLKGYTRSRLKSYN--QVQMQP 648
Hsap        CGLLSKAKQKLLRHLVPAAALRRKRKALWAHLFSSLRGYLKSLPRVQVESFN--QVQAMP 650
Mmus        CGLLAKARQKLLRQLVPKAILRRKRKALWAHLFASLRSYLKSLPRVQSGGFN--QVHAMP 650
Drer        CGLLSKSNAALLEHLVPPSALKQKRKILKSYLSNSVKTHLKSLPRSPSTDIEGDKVHAMP 633
            ****: :.  *:.:**  : :::*** * ::*  *:: :**. .*      :  :*:  *

Ggal        NFVWMLRCLYETQSEKVGKLAAKRMHANYIKLTYCNAYSADCSAISFVVHHFQKHLALDL 708
Mgal        NFVWMLRCIYETQNEKVGKLAAKRMHANYIKLTYCNAYSADCSAISFVMHHFQKRLALDL 708
Tgut        NFVWMLRCLYETQSEKVGRMAARRMHANYIKLAYCNACSADCSAISFVLHHFQKRLALDL 708
Hsap        TFIWMLRCIYETQSQKVGQLAARGICANYLKLTYCNACSADCSALSFVLHHFPKRLALDL 710
Mmus        TFLWMLRCIYETQSQKVGRLAARGISADYLKLAFCNACSADCSALSFVLHHFHRQLALDL 710
Drer        NFLWMLRCIFETNSEDVAKMTANGISADYIKIAFCNIYSADCSALNFVLHHRRKHLGVDM 693
            .*:*****::**:.:.*.:::*. : *:*:*:::**  ******:.**:**  ::*.:*:

Ggal        DNNNINDYGVKQLLPCFSKLAVIRLSVNQITDHGARILYEELSKYQIVSYLGLYNNQITD 768
Mgal        DNNNINDYGVKQLLPCFSKLAVIRLSVNQITDHGARILYEELSKYQIVSYLGLYNNQITD 768
Tgut        DNNNINDYGIKQLQPCFSKLAVIRLSVNQITDHGVRILYEELSKYQIVSFLGLYNNQITD 768
```

```
Hsap       DNNNLNDYGVRELQPCFSRLTVLRLSVNQITDGGVKVLSEELTKYKIVTYLGLYNNQITD 770
Mmus       DNNNLNDYGVQELQPCFSRLTVIRLSVNQITDTGVKVLCEELTKYKIVTFLGLYNNQITD 770
Drer       DNNNINDYGVKQLRPSFSKMTVVRFCVNQLTDSGIEVLAEELIRYKIVKVLGLYQNHITD 753
           ****:****:::* *.**:::*:*:.***:** * .:* *** :*:**. ****:*:***

Ggal       IGAKYVAKLIEECSSLEYVKIGANKITSEGGKCLAQAIQKSTTMFEIGMWGNQVGDEGAK 828
Mgal       IGAKYVAKLIEECSSLEYVKIGANKITSEGGKCLAQAIQKSTTMFEIGMWGNQVGDEGAK 828
Tgut       VGAKYVAKLIEECSSLEYVKIGANKITSEGGKCLAQAIQKSKTMFEIGMWGNQVGDEGAK 828
Hsap       VGARYVTKILDECKGLTHLKLGKNKITSEGGKYLALAVKNSKSISEVGMWGNQVGDEGAK 830
Mmus       IGARYVAQILDECRGLKHLKLGKNRITSEGGKCVALAVKNSTSIVDVGMWGNQIGDEGAK 830
Drer       VGAKQVAKIIEECPHLRTVKLGCNNITSVGGKYLASAIHKSKSIFDIGMWGNCIGDEGAE 813
           :**: *::::::**  *  :*:* *.*** *** :* *:::*.:: ::***** :*****:

Ggal       AFADALRNHPRLTNVSLAFNGITTEGGKSIAEALQHNDSVKIFWLTKNELDDEAAMSFAE 888
Mgal       AFAEALRNHPRLTNVSLAFNGITTEGGKSIAEALQHNNSVKIFWLTKNELDDEAAMSFAE 888
Tgut       AFAEALRNHPKLTNVSLAFNGITTEGGKSIAEAMQHNNSVRIFWLTKNELDDEAAMSFAE 888
Hsap       AFAEALRNHPSLTTLSLASNGISTEGGKSLARALQQNTSLEILWLTQNELNDEVAESLAE 890
Mmus       AFAEALKDHPSLTTLSLAFNGISPEGGKSLAQALKQNTTLTVIWLTKNELNDESAECFAE 890
Drer       AFAEALKNHPSLTNLSLSANGITSHGGRSLAQTLKENTSLHIVWLIQNKISDDAASDLAE 873
           ***:**::** **.:**: ***:..**:*:*.::::.* :: :.** :*::.*: *  :**

Ggal       MLKINKKLVHLWLIQNQITAKGVKYLSEALKENTAIKEVCLN-------GNLISQEEAKA 941
Mgal       MLKINKKLVHLWLIQNQITAKGVKYLSEALKENTAIKEVCLN-------GNLISQEEAKA 941
Tgut       MLKVNKKLVHLWLIQNKITAKGVKCLSDALQENTTIKEICLN-------GNLISQEEAKA 941
Hsap       MLKVNQTLKHLWLIQNQITAKGTAQLADALQSNTGITEICLN-------GNLIKPEEAKV 943
Mmus       MLRVNQTLRHLWLIQNRITAKGTAQLARALQKNTAITEICLN-------GNLIKPEEAKV 943
Drer       AFRSNSSLTHLMLIDNEFTIDGARQLSEGLKDNTTLKEVNVRGKHRPWHGAVLNQETHPS 933
            :: *..* ** **:*.:* .*.  *: .*:.** :.*: :.       * ::. *

Ggal       FESE---------ERIICF------------------------------------------ 951
Mgal       FESE---------ERIVCF------------------------------------------ 951
Tgut       FENE---------ERIICF------------------------------------------ 951
Hsap       YEDE---------KRIICF------------------------------------------ 953
Mmus       FENE---------KRIICF------------------------------------------ 953
Drer       YPDVSIQPFCCPKRSFFCPPHLKIKALRDTVPFSRTINPSSNQQRDGSQLTSSPANQRAE 993
           : .          . :.*

Ggal       ----------
Mgal       ----------
Tgut       ----------
Hsap       ----------
Mmus       ----------
Drer       SSQYDDCQFP 1003
```

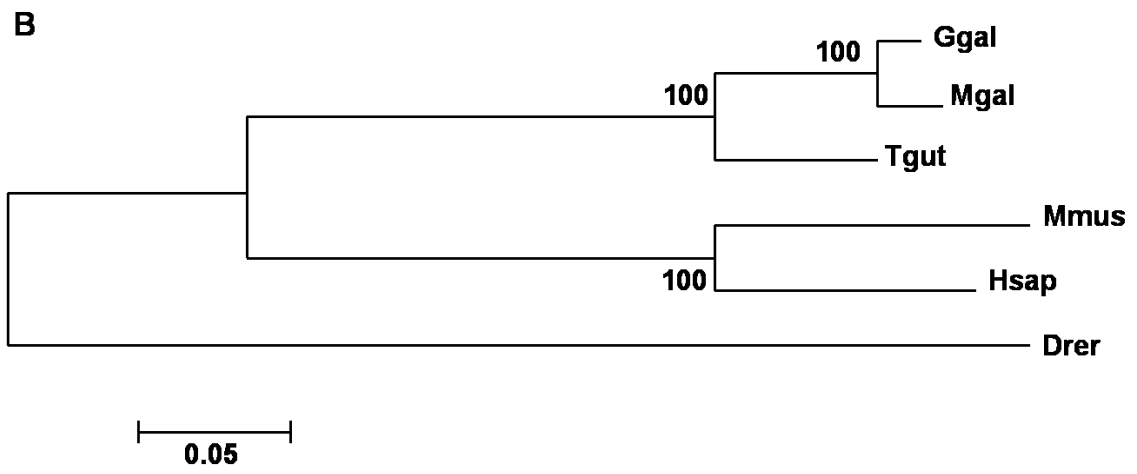



**Figure 3.4. Sequence comparison and phylogenetic tree of amino acids encoded by ChNod1. A.** Multipe alignments of Nod1 protein sequences from human (Hsap), mouse (Mmus), zebrafish (Drer), chicken (Ggal), turkey (Mgal) and zebra finch (Tgut). Identical amino acids are indicated with asterisks, and conxervative substitutions are indicated with "." or ":". CARD domains and NACHT domains are underlined. LRR domains are highlighted with yellow. **B.** Phylogenetic tree of representative vertebrate Nod1 sequences. The tree was constructed using NJ. The numbers at each node represent percentage of bootstrap replicates which resulted in the production of the same branching pattern.

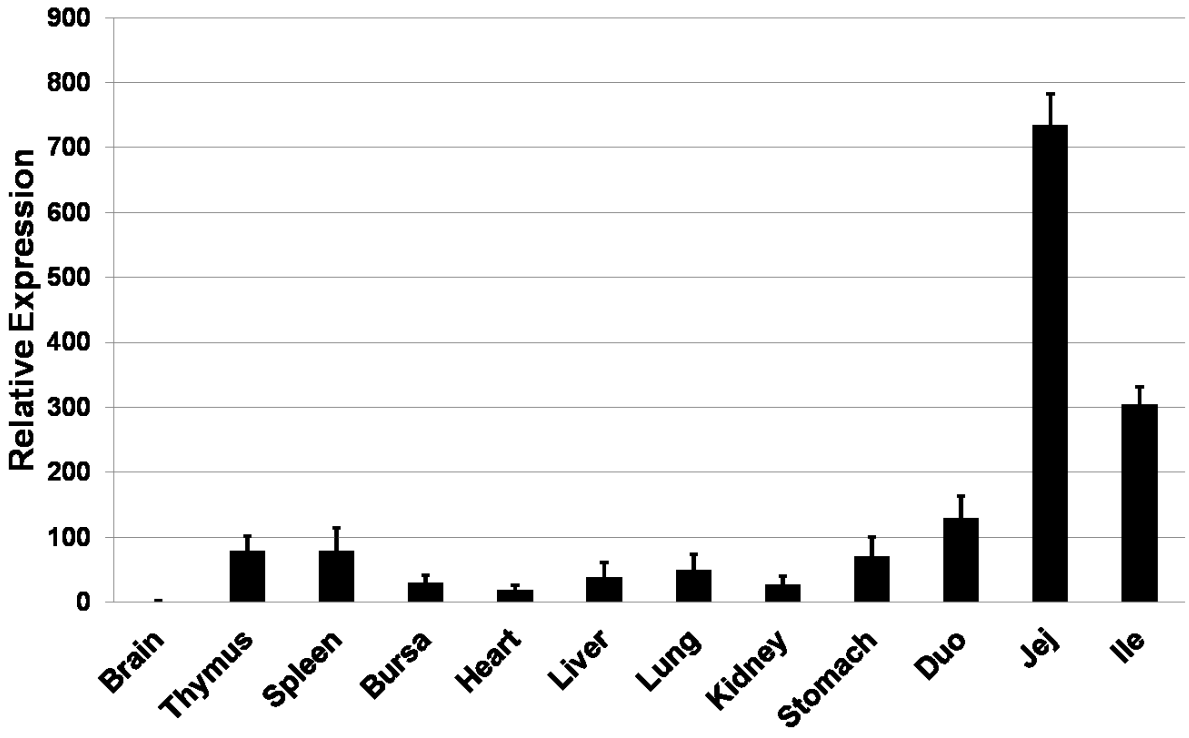



**Figure 3.5. Tissue distribution of ChNod1.** Relative ChNod1 expression was calculated using the $2^{-\Delta\Delta C}{}_{T}$ method with initial normalization of ChNod1 against GAPDH within each sample, and then comparison of tissue expression levels with expression in brain (arbitrarily set at 1.0).

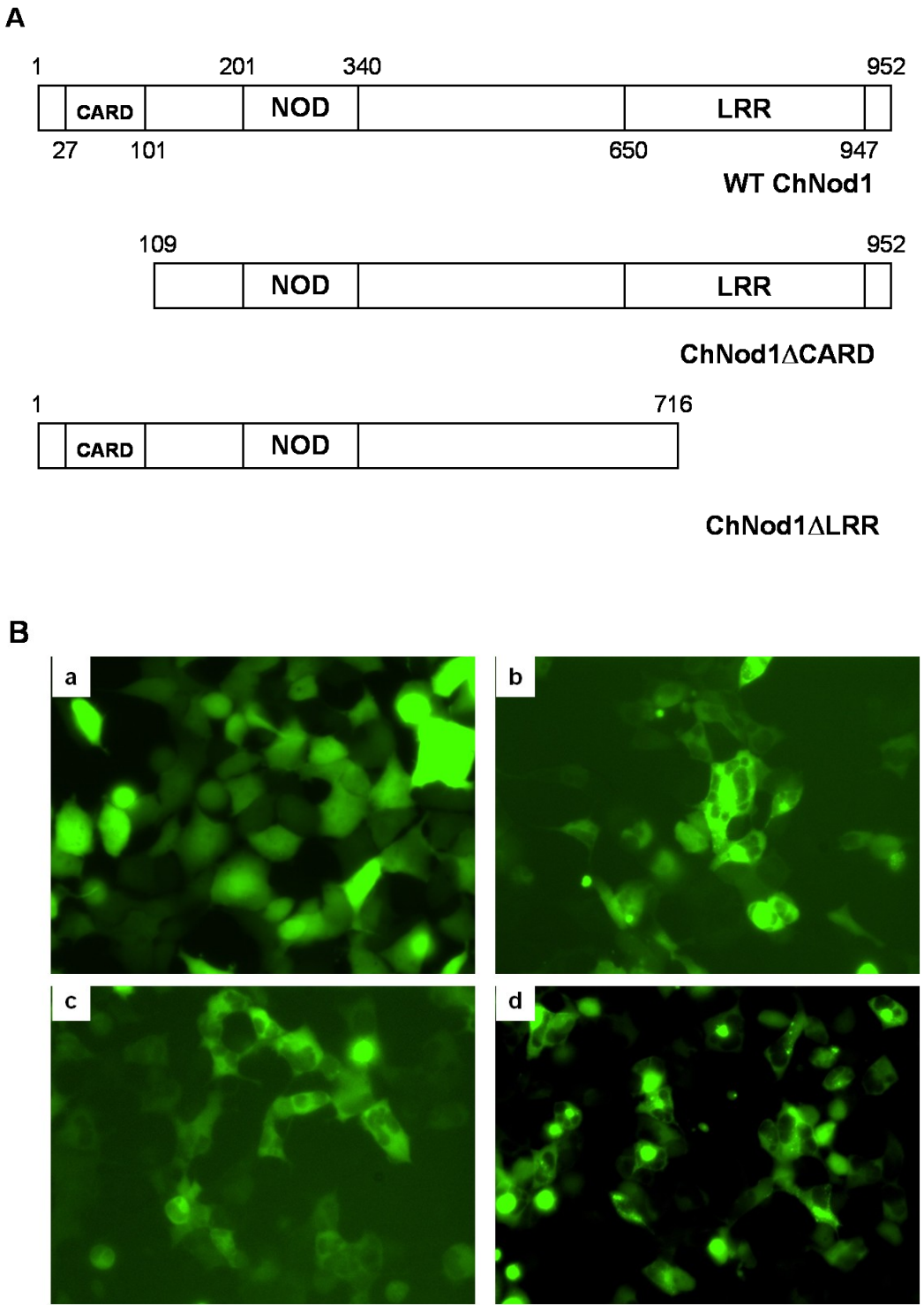



**Figure 3.6. Schematic diagram of cloned ChNod1 forms and their subcellular localization.** **A.** Schematic drawing of WT ChNod1 and its mutant forms. The number indicates amino acid residues. **B.** Subcellular localization of ChNod1. Twenty-four hour post-transfection of HEK293T cells, expression of yellow fluorescence protein was observed through a fluorescence

microscope. a) vector only-transfection; b) WT ChNod1 transfection; c) ChNod1ΔCARD transfection; d) ChNod1ΔLRR transfection.

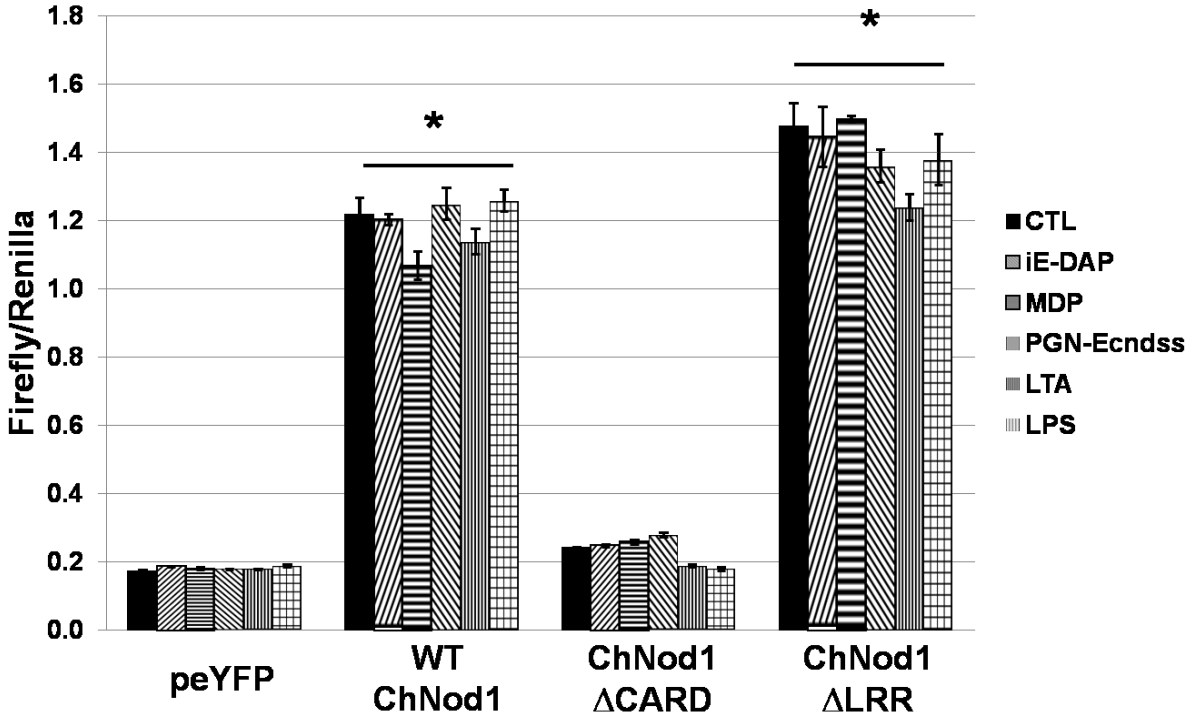
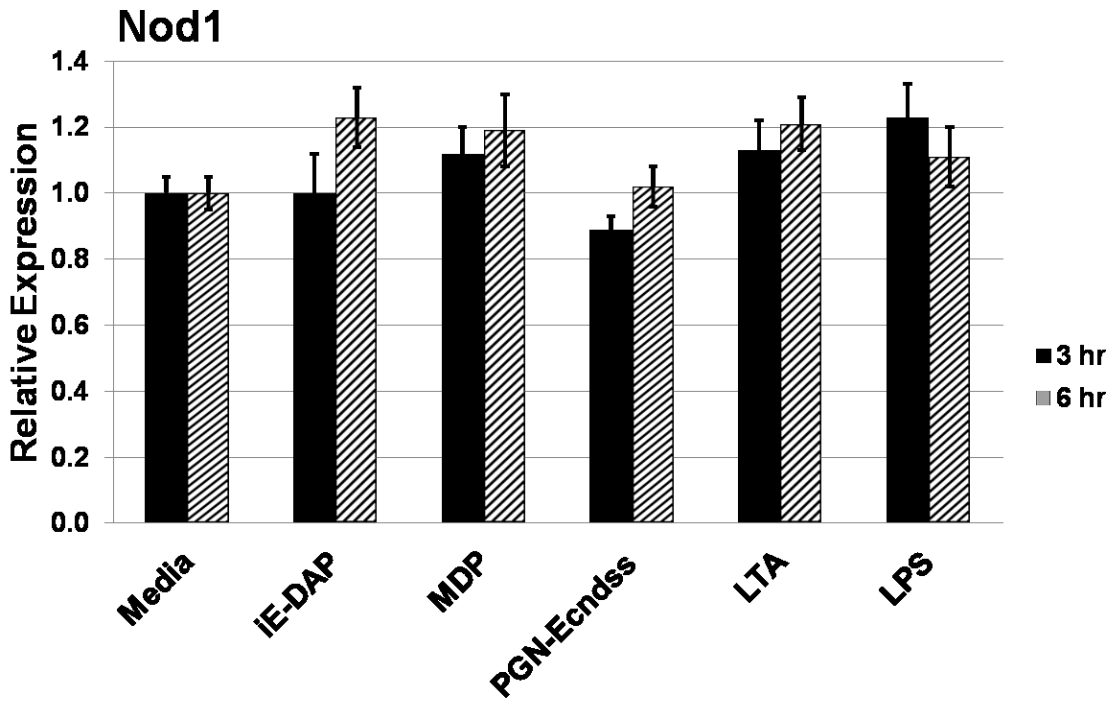
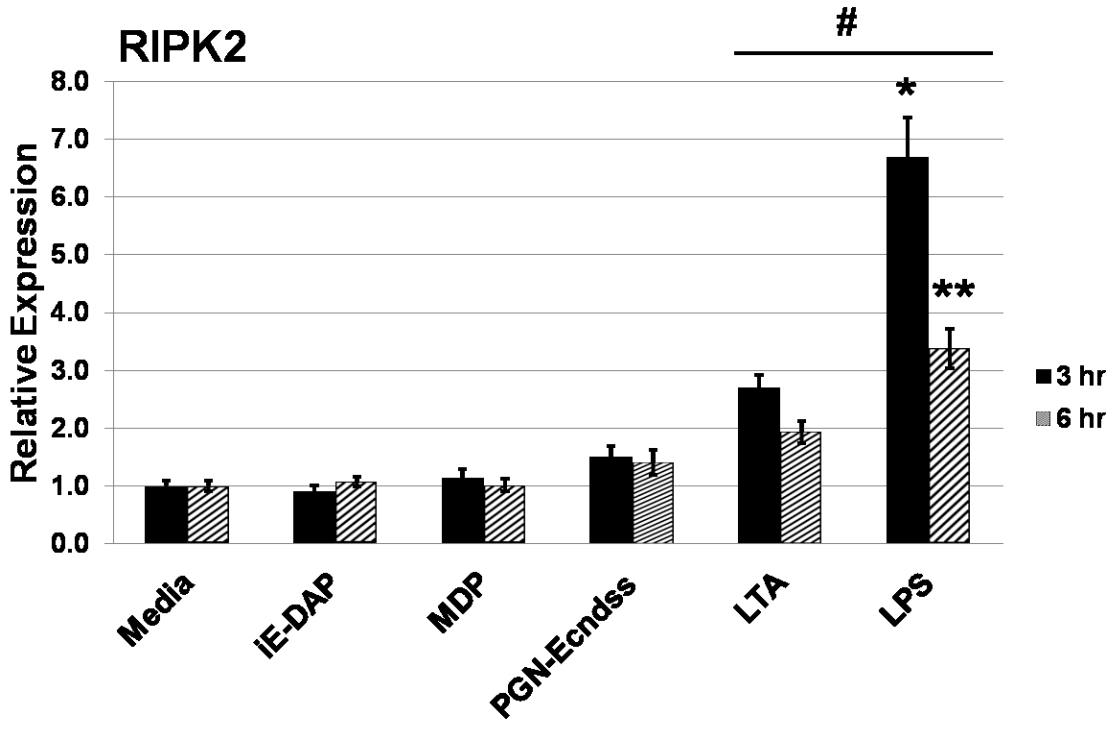
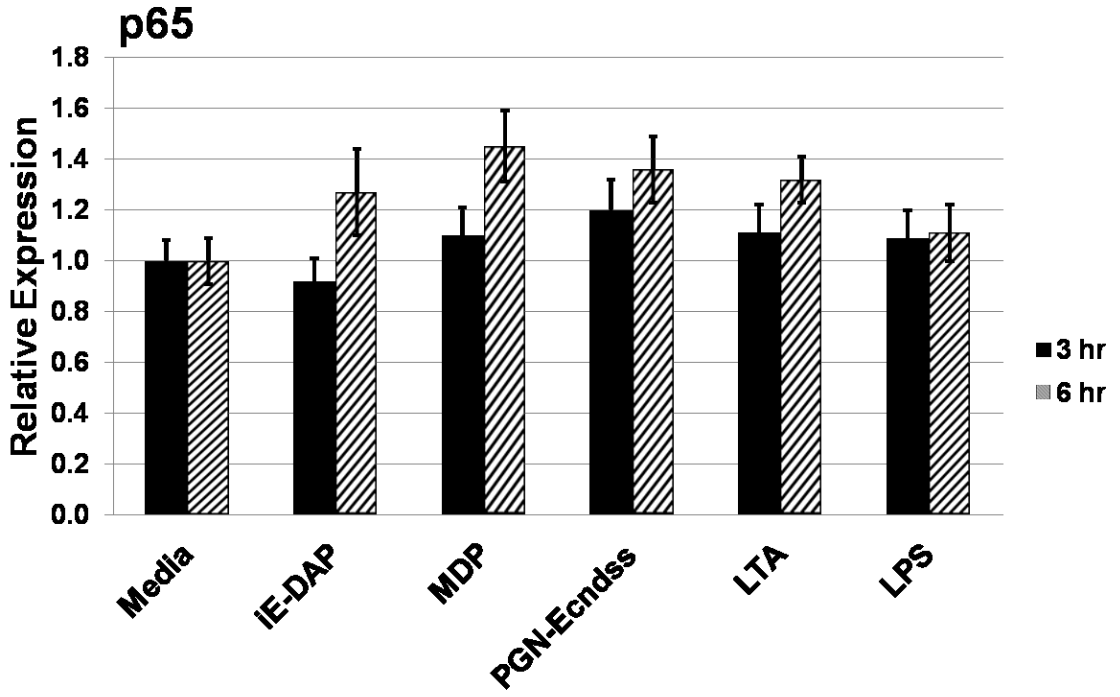
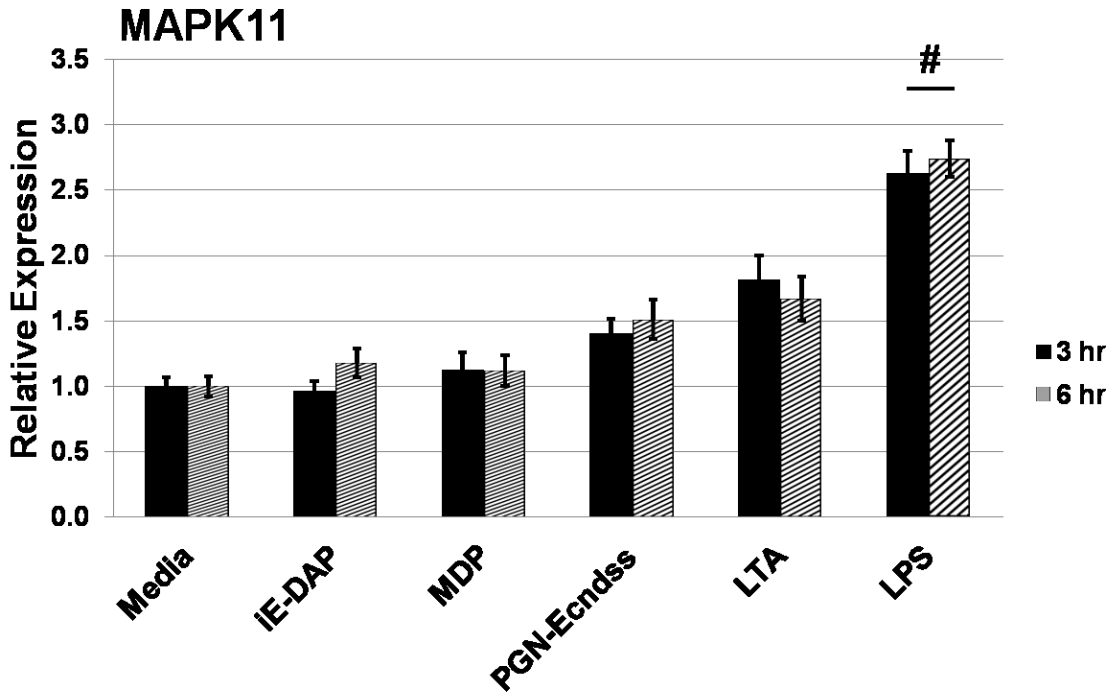


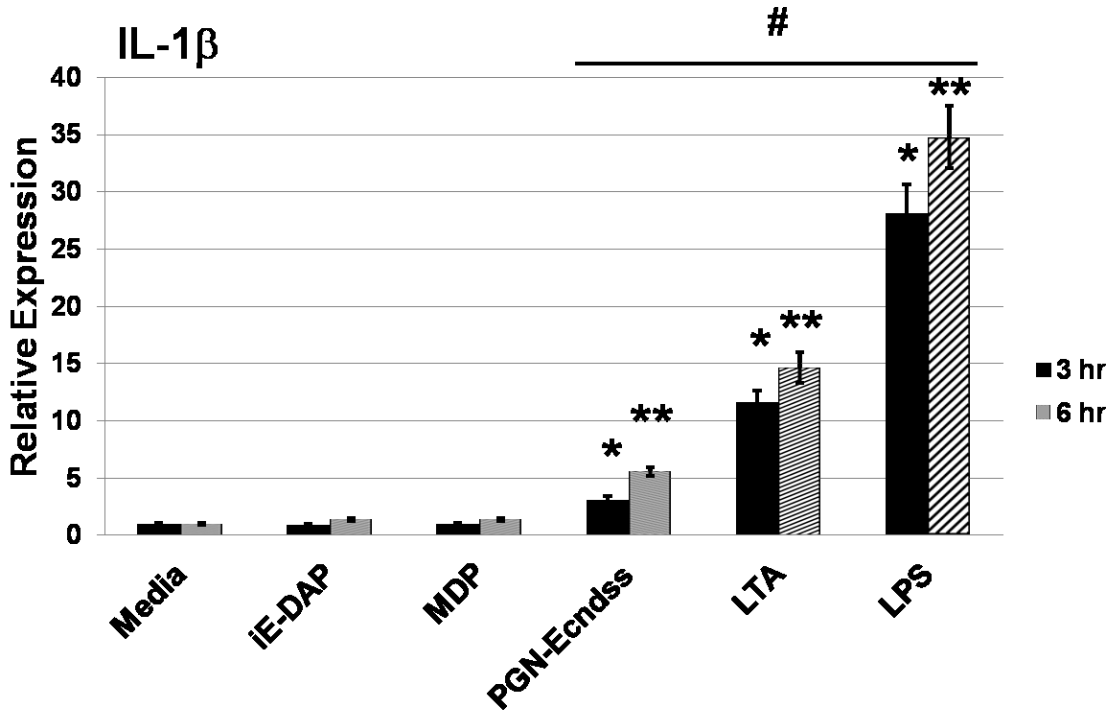

**Figure 3.7. Induction of NF-κB reporter (Luciferase) activity by various ligands.** Transiently transfected HEK293T cells were treated with the working concentration (Table 1) of ligands for 24 h. Values represent means, and error bars indicate the S.D. The results are representative of five independent experiments. The asterisk (*) indicates statistically significant difference ($p < 0.05$)

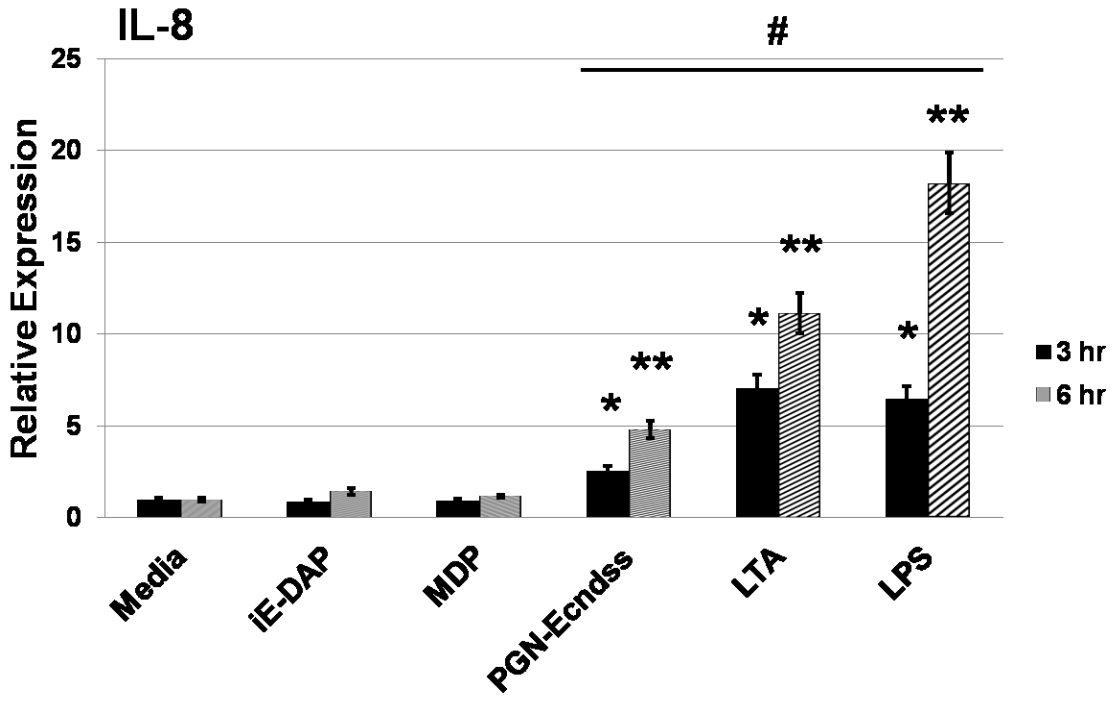

Nod1
Relative Expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Media
iE-DAP
MDP
PGN-Ecndss
LTA
LPS
3 hr
6 hr


RIPK2
#
*
**
Relative Expression
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Media
iE-DAP
MDP
PGN-Ecndss
LTA
LPS
3 hr
6 hr


p65
Relative Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Media
iE-DAP
MDP
PGN-Ecndss
LTA
LPS
3 hr
6 hr


MAPK11
#
Relative Expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Media
iE-DAP
MDP
PGN-Ecndss
LTA
LPS
3 hr
6 hr


IL-1β
#
Relative Expression
40
35
30
25
20
15
10
5
0
*
**
3 hr
6 hr
Media
iE-DAP
MDP
PGN-Ecndss
LTA
LPS


IL-8
#
Relative Expression
25
20
15
10
5
0
*
**
3 hr
6 hr
Media
iE-DAP
MDP
PGN-Ecndss
LTA
LPS

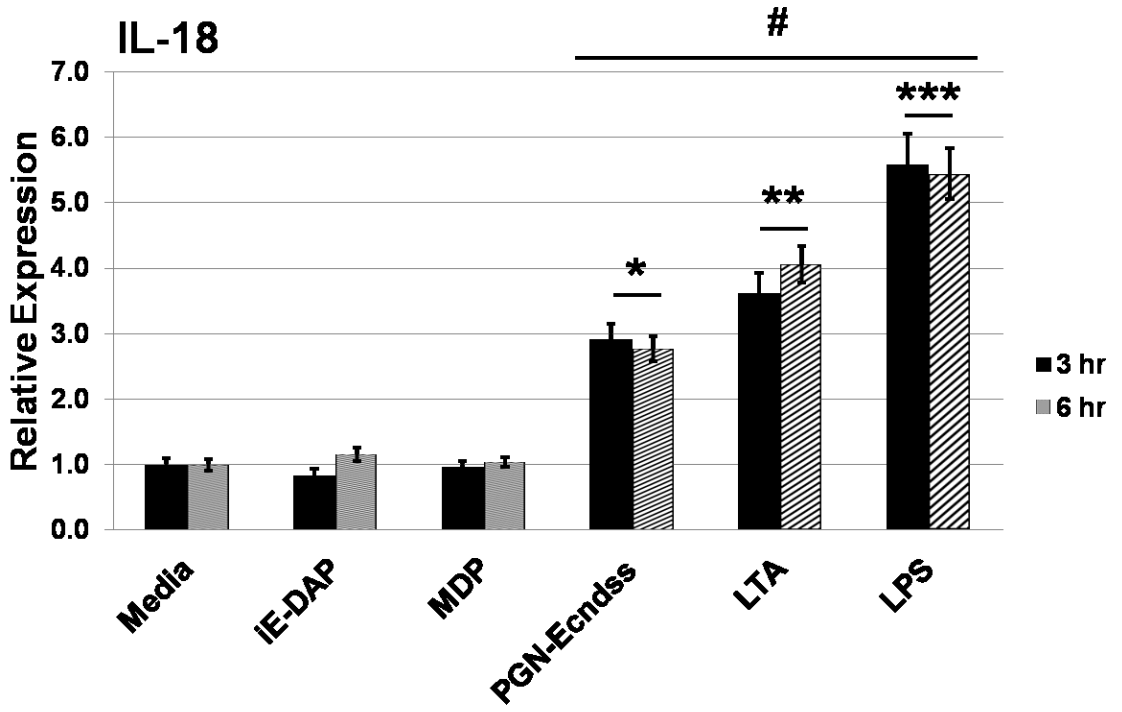



**Figure 3.8. In vitro stimulation of PBMCs with various ligands.** Freshly cultured chicken PBMCs were incubated with iE-DAP, MDP, PGN, LTA and LPS for 3 and 6 h. Expression of Nod1 signal molecules and pro-inflammatory cytokines was measured by qRT-PCR. Asterisks (*) indicate statistically significant difference between 3- and 6-h ($p < 0.05$), and pound sign (#) indicates statistically significant difference from media ($p<0.05$).

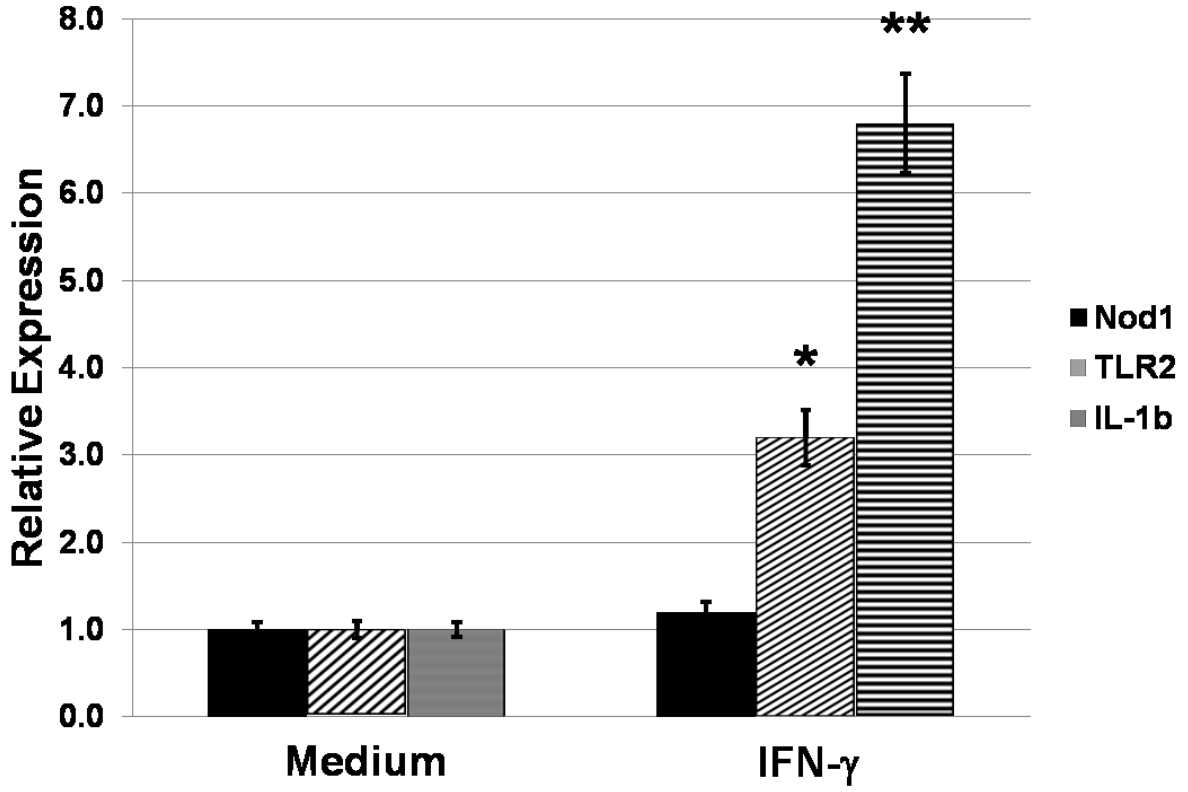
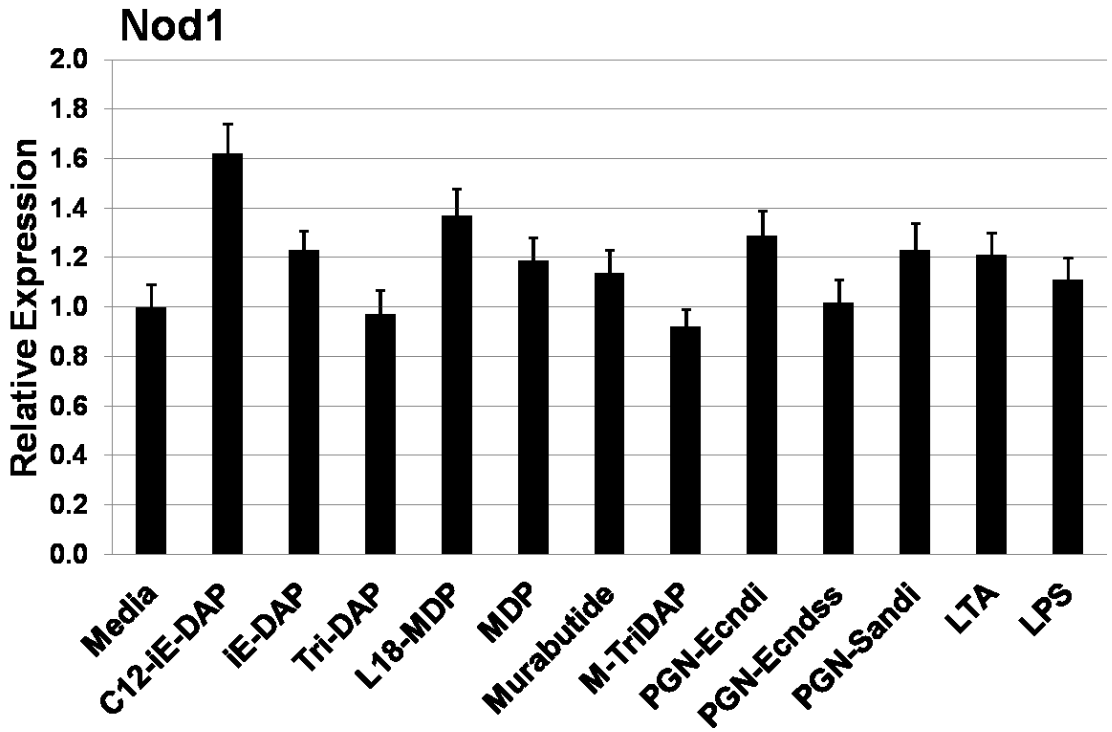
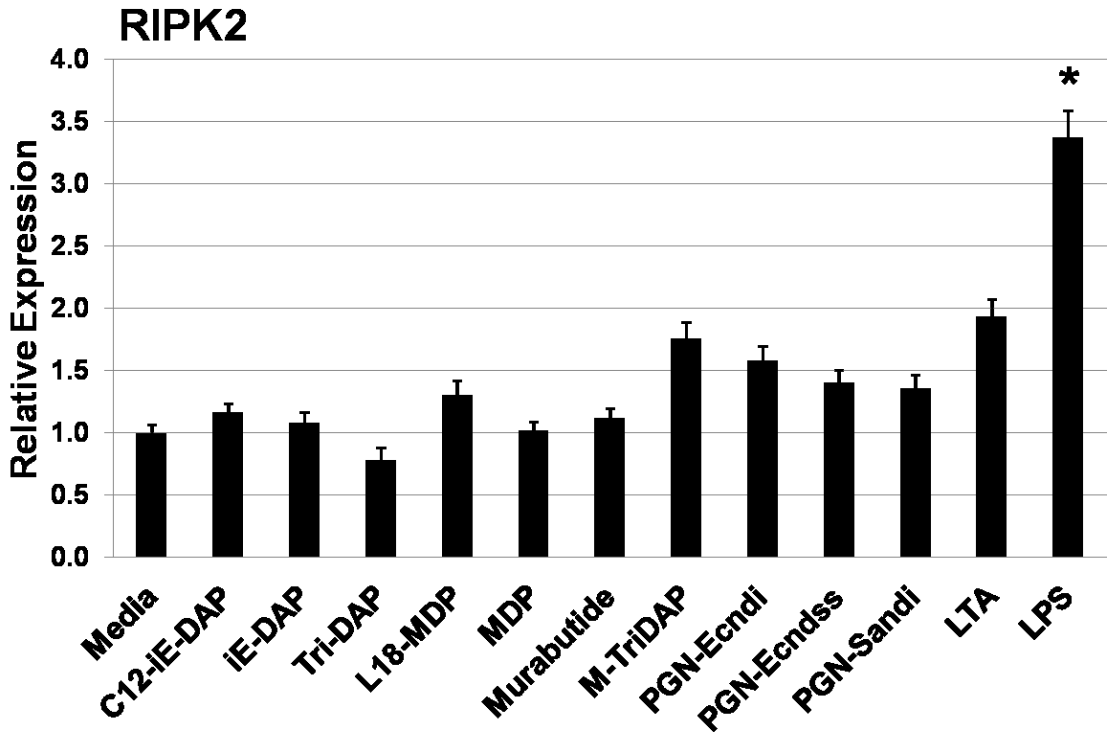
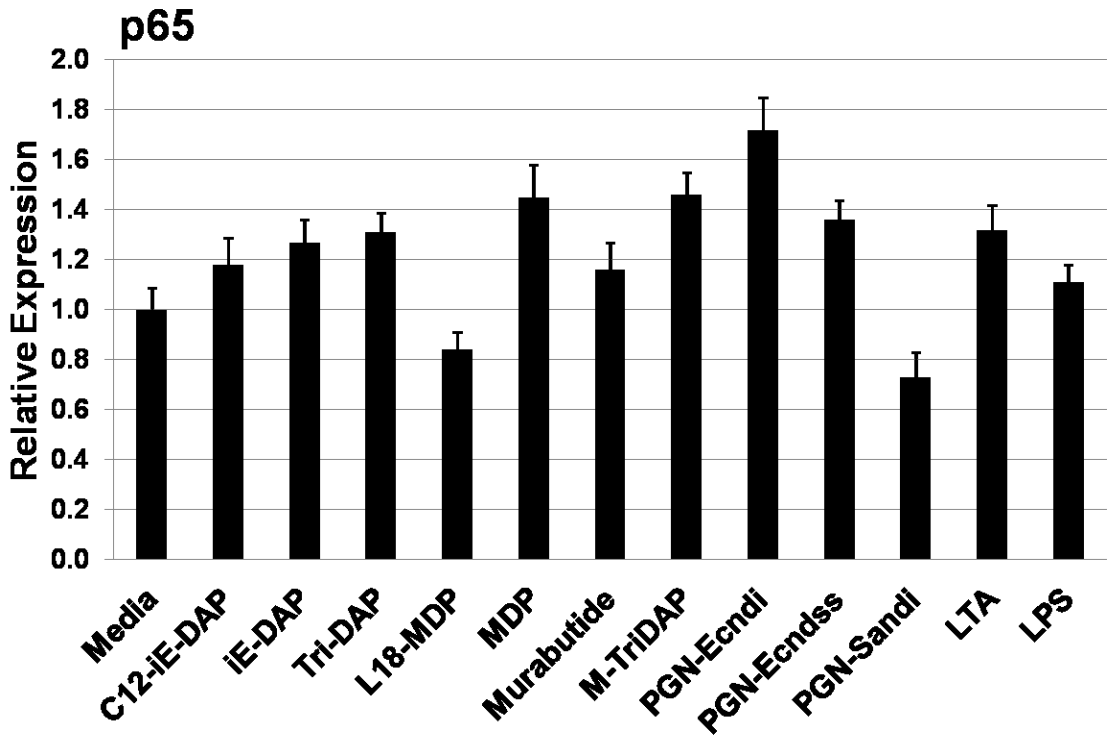
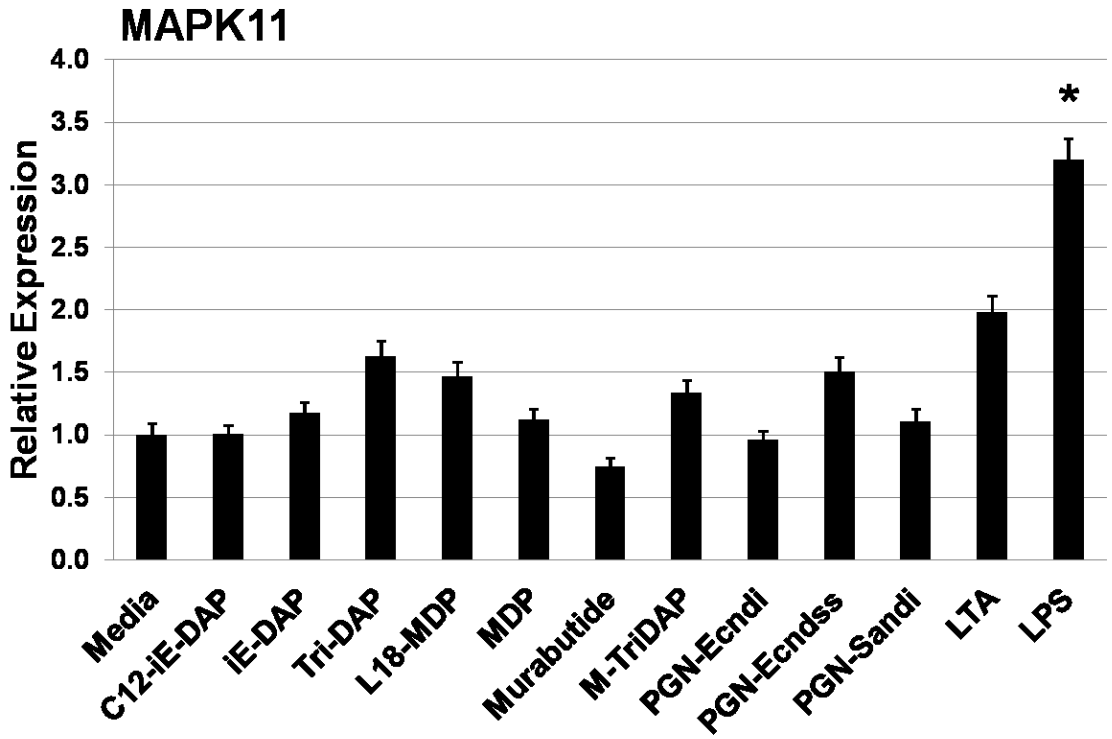


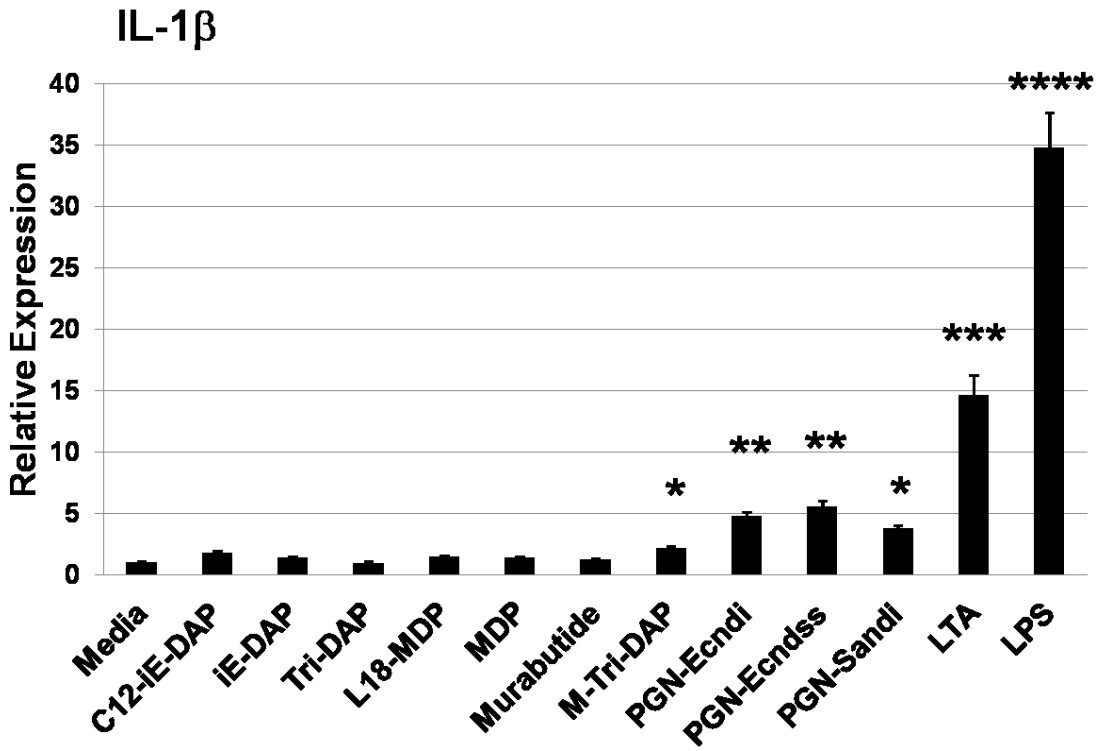

**Figure 3.9. No effect of Nod1 mRNA level by ChIFN-γ.** Isolated PBMCs were treated with rChIFN-γ (0.01 μg/mL) for 6 h, following measurement mRNA level of ChNod1, TLR2 and IL-1β by qRT-PCR. Asterisks (*) indicate statistically significant difference ($p < 0.05$).

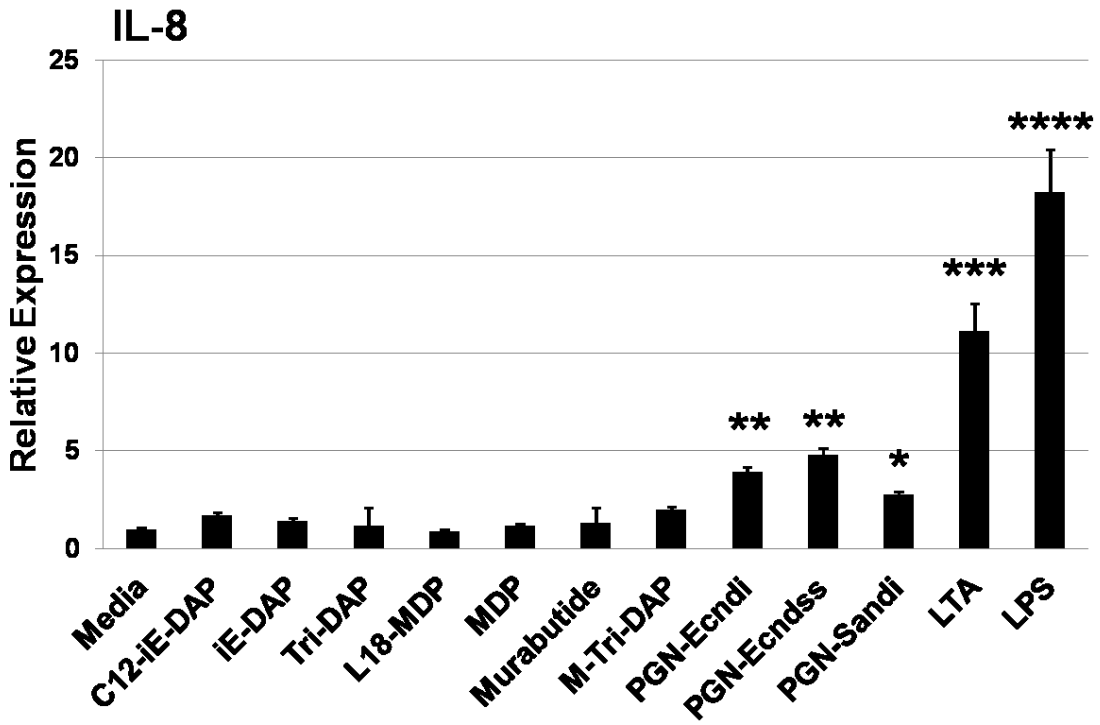

Nod1
Relative Expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Media
C12-iE-DAP
iE-DAP
Tri-DAP
L18-MDP
MDP
Murabutide
M-TriDAP
PGN-Ecndi
PGN-Ecndss
PGN-Sandi
LTA
LPS


RIPK2
Relative Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Media
C12-iE-DAP
iE-DAP
Tri-DAP
L18-MDP
MDP
Murabutide
M-TriDAP
PGN-Ecndi
PGN-Ecndss
PGN-Sandi
LTA
LPS


p65
Relative Expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Media
C12-iE-DAP
iE-DAP
Tri-DAP
L18-MDP
MDP
Murabutide
M-TriDAP
PGN-Ecndi
PGN-Ecndss
PGN-Sandi
LTA
LPS


MAPK11
Relative Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Media
C12-iE-DAP
iE-DAP
Tri-DAP
L18-MDP
MDP
Murabutide
M-TriDAP
PGN-Ecndi
PGN-Ecndss
PGN-Sandi
LTA
LPS


IL-1β
Relative Expression
40
35
30
25
20
15
10
5
0
****
***
**
**
*
*
Media
C12-iE-DAP
iE-DAP
Tri-DAP
L18-MDP
MDP
Murabutide
M-Tri-DAP
PGN-Ecndi
PGN-Ecndss
PGN-Sandi
LTA
LPS


IL-8
Relative Expression
25
20
15
10
5
0
****
***
**
**
*
Media
C12-iE-DAP
iE-DAP
Tri-DAP
L18-MDP
MDP
Murabutide
M-Tri-DAP
PGN-Ecndi
PGN-Ecndss
PGN-Sandi
LTA
LPS

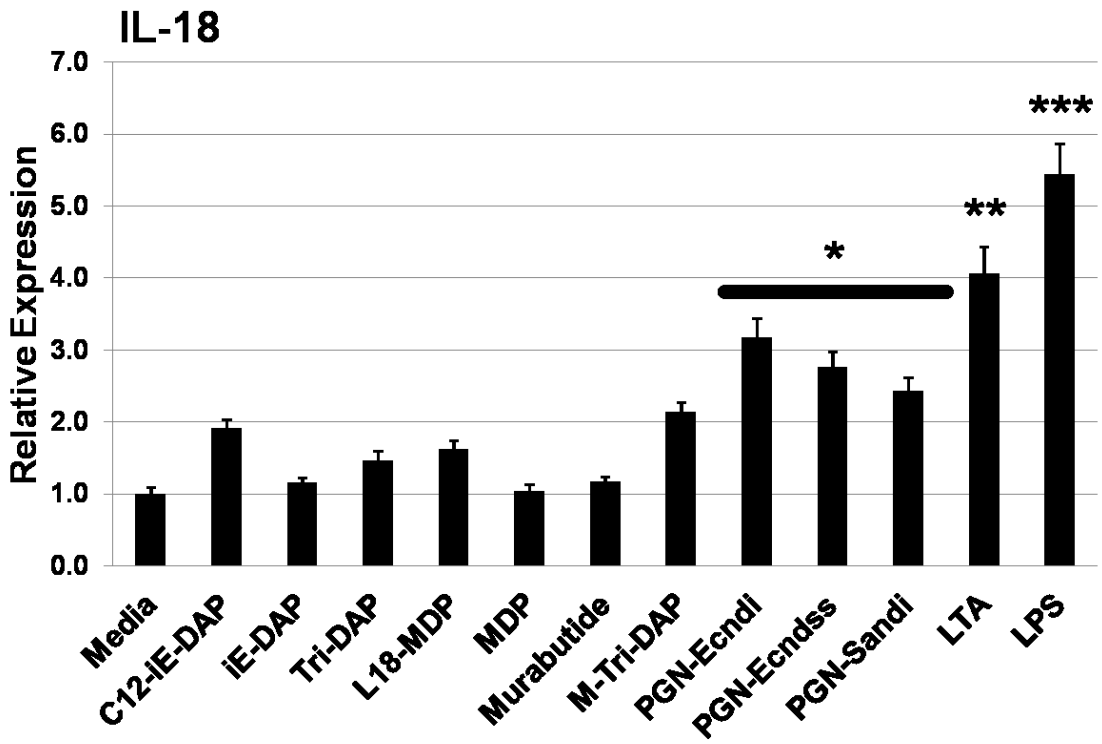



**Figure 3.10. Stimulation of Nod1 signal with commercially available mammalian ligands.** Cultured PBMCs were incubated with the listed ligands (Table 1) for 6 h. Expression of Nod1 signal molecules and pro-inflammatory cytokines was measured by qRT-PCR. Asterisks (*) indicate statistically significant difference ($p < 0.05$).

# CHAPTER IV

## Molecular Cloning and Functional Characterization of the Avian Inflammatory Modulator, Macrophage Migration Inhibitory Factor (MIF)

**ABSTRACT:** Macrophage migration inhibitory factor (MIF) is recognized as a soluble factor produced by sensitized T lymphocytes and inhibits the random migration of macrophages. Recent studies have revealed a more prominent role for MIF as a multi-functional cytokine mediating both innate and adaptive immune responses. This study describes the cloning and functional characterization of avian MIF in an effort to better understand its role in innate and adaptive immunity, and potential use in poultry health applications. The full-length avian MIF gene was amplified from stimulated chicken lymphocytes and cloned into a prokaryotic expression vector. The confirmed 115 amino acid sequence of avian MIF has 71% identity with human and murine MIF. The bacterially expressed avian recombinant MIF (rChMIF) was purified, followed by endotoxin removal, and then tested by chemotactic assay and qRT-PCR. Diff-Quick staining revealed a substantial decrease in migration of macrophages in the presence of 0.01 μg/mL rChMIF. qRT-PCR analysis revealed that the presence of rChMIF enhanced levels of IL-1β and iNOS during PBMCs stimulation with LPS. Additionally, the Con A-stimulated lymphocytes showed enhanced IFN-γ and IL-2 transcripts in the presence of rChMIF. Interestingly, addition of rChMIF to the stimulated PBMCs, in the presence of lymphocytes, showed anti-inflammatory function of rChMIF. To our knowledge, this study represents the first report for the functional characterization of avian MIF, demonstrating the inhibition of macrophage migration, similar to mammalian MIF, and the mediation of inflammatory responses during antigenic stimulation.

## Introduction

Macrophage migration inhibitory factor (MIF), one of the first lymphocyte-derived cytokines, was originally identified as a soluble factor produced by antigen-activated T lymphocytes that inhibited the random migration of macrophages (Bloom and Bennett, 1966; David, 1966; Weiser et al., 1989). Recent research shows a more prominent role of MIF as a multi-functional cytokine mediating both innate and adaptive immune responses. Mammalian MIF is an immunomodulator that controls macrophage functions, resulting in the promotion of pro-inflammatory cytokine expression (TNF-α, IL-1β, IL-2, IL-6, IL-8, IFN-γ) (Bacher et al., 1996; Calandra et al., 1994; Donnelly et al., 1997), NO release (Bernhagen et al., 1994) and cyclooxygenase (COX)-2 activity (Mitchell et al., 2002). In activated macrophages, MIF-induced TNF-α leads to further MIF release, resulting in optimal expression of TNF-α by macrophages (Calandra et al., 1994). MIF also up-regulates the expression of TLR4, which recognizes LPS and induces the activation of monocytes/macrophages, suggesting potential involvement of MIF early in innate immune responses (Roger et al., 2001). Due to its role as a mediator of systemic inflammatory responses, MIF has attracted attention as a therapeutic target (Larson and Horak, 2006; Morand, 2005). Although it has been discovered that MIF is released by activated lymphocytes, little is known about the role of this cytokine in adaptive immunity. MIF is constitutively expressed by T lymphocytes but can also be induced by mitogens, CD3-specific antibody, and glucocorticoids (Bacher et al., 1996; Calandra et al., 1998; Abe et al., 2001). Produced primarily by activated Th2 cells, MIF appears to have a possible autocrine function, resulting in activation and proliferation of T cells and IL-2 production (Bacher et al., 1996). MIF-specific antibodies prevent superantigen-induced activation and proliferation of splenocytes, thus supporting the concept that MIF is also a lymphotropic cytokine (Calandra et

al., 1998). Moreover, MIF inhibits regulatory effects on cytotoxic $CD8^+$ T cells and regulates lymphocyte trafficking (Abe et al., 2001).

In birds, the sequence of chicken MIF (ChMIF) was first identified from early stage embryonic chicken lens as a discrete 10-kDa polypeptide (Wistow et al., 1993). Further studies showed the potential involvement of MIF in primary *Eimeria* infection (Hong et al., 2006); however, there was no further characterization of its molecular function. In this study, the identified ChMIF was cloned, expressed and its biological function characterized. Observed biological effects of rChMIF included inhibitory function of macrophage migration, enhancement of inflammatory response in monocytes and Th1/Th2 cytokines in lymphocytes, and enhancement of proliferation of activated T lymphocytes.

## Materials and Methods

*Chickens, RNA source for cloning*

Nine-week old healthy broiler chickens were housed in accordance with the Institutional Animal Care and Use Committee Guidelines of Virginia Tech. Various tissues were collected including thymus, spleen, bursa, brain, lung, heart, liver, crop, stomach, and intestinal sections. Then, 50 mg of each tissue were mixed with 1 mL of TRI Reagent and homogenized using VWR PowerMax AHS 200 (VWR, PA). By addition of 0.2 mL of chloroform and centrifugation, total RNA was separated from DNA and proteins, and precipitated with 0.5 mL isopropanol, followed by centrifugation at 12,000 x *g* for 5 min at 4°C. Precipitated total RNA was dissolved in RNase-free water and possible genomic DNA was removed by incubation with RNase-free DNase treatment (Promega, WI) at 37° for 1 h.

*Isolation of lymphocytes and peripheral blood mononuclear cells*

PBMCs were isolated using Histopaque-1077 (Sigma, MO). Briefly, 10 mL of blood were collected by wing venipuncture from each bird, diluted with equal volume of Hank's Buffered Salt Solution (HBSS; HyClone, UT), and centrifuged at 50 x *g* for 10 min. The supernatant and buffy coat were collected and carefully overlaid on Histopaqe-1077. Mononuclear cells were separated from plasma and red blood cells by centrifugation at 400 x *g* for 30 min. The collected mononuclear cells were washed twice with Dulbecco's Modified Eagle Medium (DMEM; Mediatech, VA) supplemented with 2 mM $_L$-Glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B, and 10% fetal calf serum (FCS) (all supplied by Atlanta Biologicals, GA), and incubated for 24-hr at 39°C with 5% $CO_2$ humidified air. The non-adherent cells were then removed by washing three times with warm DMEM.

Chicken lymphocytes were prepared from spleens using 0.22 µm cell strainers (BD, CA) followed by serial centrifugation. Lymphocytes were cultured in RPMI-1640 (Mediatech, VA) containing 20% FCS and 1% penicillin/streptomycin and Amphotericin B for 24 hr at 39°C and 5% $CO_2$. Non-adherent cells were collected and seeded onto 24-well plates.

*Sequence and evolutionary analyses*

Nucleotide alignment was constructed using the ClustalX software (Thompson et al., 1997; Saitou and Nei, 1987) with minor manual corrections. Phylogenetic trees were reconstructed from this alignment using the neighbor joining (NJ) method also using ClustalX (Thompson et al., 1997; Saitou and Nei, 1987). The stability of the branching order was

confirmed by performing 1000 bootstrap replicates. The MIF sequence from the sea lamprey was used as an outgroup.

*Construction of recombinant ChMIF (rChMIF) expression plasmid*

For amplification of the full-length ChMIF, a candidate sequence was identified from the chicken genome database. Using the reported ChMIF sequence (GenBank Accession # M95776), primer sets were designed (Table 4.1). Total RNA was isolated from lymphocytes stimulated with Con A (Sigma, MO) using RNeasy Mini Kit (Qiagen, CA), followed by synthesis of the first-strand cDNA using iScript (Bio-Rad, CA). The full-length ChMIF was amplified by PCR using the following conditions; initial denaturation at 92°C for 2 min, 35 cycles of denaturation at 92°C for 15 sec, annealing at 54°C for 15 sec, and extension at 72°C for 30 sec, with a final extension at 72°C for 7 min. The freshly synthesized full-length ChMIF was directly inserted into pCR 2.1-TOPO vector, followed by transformation into *E. coli* TOP10 (Invitrogen, CA). Transformed *E. coli* TOP10 were cultured in Luria-Bertani media (LB; Fisher Scientific, NJ) at 37°C overnight. A transformant was selected by a combination of PCR screening and endonuclease digestion with *Eco*R I (New England Biolabs, MA) and confirmed by sequencing (Virginia Bioinformatics Institute at VT, VA). For sub-cloning into prokaryotic expression vectors, ChMIF was digested with endonucleases *Nde* I and *Nhe* I (New England Biolabs, MA). The digested ChMIF fragment was purified from an agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, WI), and ligation was performed with *Nde* I/*Nhe* I-digested pET11a vector (Novagen, CA) at 4°C overnight. *E. coli* TOP10 were transformed with

the ligation mixtures, plasmid DNA was prepared from transformants, and subjected to DNA sequencing to confirm identity of recombinant plasmid.

*Expression and purification of rChMIF*

*E. coli* (BL21; Novagen, CA) containing recombinant ChMIF-pET11 were cultured in LB containing 100 µg/mL ampicillin in an orbital shaking incubator at 37°C until mid-log phase ($O.D._{600}$ = 0.5). Recombinant ChMIF production was induced by incubating the culture for an additional 4 hr in the presence of 1 µM isopropyl thiogalactopyranoside (IPTG). The cultures were harvested by centrifugation at 25,000 x *g* for 10 min, followed by resuspension of the pellet in 20 mM $NaPO_4$, 500 mM NaCl (pH 7.8) buffer. The cells were lysed by three freeze-thaw cycles between dry ice-ethanol bath and a 37°C water-bath, followed by treatment with 1 U/mL DNase and RNase for 30 min at room temperature. The soluble fraction containing rChMIF was recovered by centrifugation of the extract at 5,000 x *g* for 15 min.

To reduce endotoxin levels, the bacterial lysate containing rChMIF was extracted with octylphonoxypolyethoxyethanol (TX-114; Sigma, MO) prior to purification using a slight modification of a previously described procedure (Liu et al., 1997). Briefly, 10 µl of TX-114 were added to 1 mL of bacterial lysate maintained on ice and the sample was vortexed for about 10 sec. Following incubation at 41°C for 5 min, the sample was centrifuged at 10,000 x *g* for 2 min at 38°C to facilitate phase separation. Following centrifugation, the upper aqueous phase was removed and saved, and the lower detergent phase containing endotoxin was discarded. The procedure was repeated for a total of three extractions. Following TX-114 extraction, rChMIF was purified from the extract by size exclusion high performance liquid chromatography (SEC-

HPLC). The columns consisted of a series of two size exclusion columns (7.7 x 300 mm, Biosuite 5 μm HR; Waters, MA). The mobile phase consisted of 50 mM $K_2HPO_4$, 150 mM NaCl (pH 6.8) and the flow rate was 0.5 mL/min. Absorbance was monitored at 280 and 214 nm using a photo diode array detector (Model 997; Waters, MA). The lysate (5-10 mg/mL) was injected in a 200 μl volume, and fractions (0.5 mL) of eluate were collected and analyzed by electrophoresis. Fractions containing rChMIF were pooled and concentrated by centrifugal filtration (Millipore, MA). Protein concentrations were determined by BCA assay (Thermo Scientific, IL).

To further reduce endotoxin levels, HPLC purified rChMIF samples used for cellular assays were extracted with polymyxin B bound to 45 μm beads (Affi-Prep; Biorad, CA). Beads were washed 3 times with 2 mL 0.1 M NaOH and 5 times with 3 mL of pyrogen-free water. Approximately 200 μl of packed volume of beads were incubated with 800 μl of purified rChMIF. The sample was mixed and then incubated overnight with gentle rocking. Following incubation, the sample was centrifuged and supernatant removed. Samples were reanalyzed for protein concentration and purity was determined by electrophoresis. Protein recovery was estimated to be about 90%. Endotoxin concentrations of samples were determined by the Limulus amebocyte assay (Lonza, MD) performed using the microplate method following the manufacturer's recommendation. Samples were stored frozen (-70°C) until used for assays.

*Production of rabbit anti-rChMIF polyclonal antisera and Western blot analysis*

Antisera production was performed by Pacific Immunology (Romona, CA) by immunizing rabbits with rChMIF purified by SEC-HPLC as described in section 2.5. Briefly,

pre-immune sera were obtained from two rabbits, which were then immunized four times with approximately 200 μg protein each. After immunization, sera were collected from rabbits and frozen (-70°C) in 1 mL aliquots.

The ability of rabbit polyclonal antisera to recognize rChMIF was analyzed by Western blot analysis as previously described (Fetterer and Barfield, 2003). Briefly, samples were separated by polyacrylamide gel electrophoresis using 1 mm thick gradient gels (8 x 9 cm, 4-12% Bis-Tris; Invitrogen, CA). All samples were chemically reduced. For analysis of fractions from SEC-HPLC analysis, gels were stained with Coomassie Blue (Invitrogen, CA). Following electrophoresis, proteins were transferred to a PVDF membrane (Millipore, MA). Rabbit anti-rChMIF polyclonal antisera were used in a dilution of 1:1000. Goat anti-rabbit antibody conjugated with HRP (10-20 ng/mL; Thermo Scientific, IL) served as the secondary antibody. Chemiluminescence of Western blots was visualized with a digital camera (UVP, CA) after exposure to luminol (Super Signal West Dura Extend; Thermo Scientific, IL).

*Chemotaxis assay using modified Boyden Chamber*

The purified rChMIF was prepared by 10-fold serial dilutions (0.001 μg/mL to 1 μg/mL) with DMEM supplemented with 2 mM $_{L}$-Glutamine and 10% FCS. Human MCP-1 and IL-8 (PeproTech, NJ) were also diluted with DMEM to a final concentration of 0.01 and 0.1 μg/mL. The medium supplemented with 10% FCS was used as positive control to induce cell migration, while serum-free medium was used as negative control. Recombinant protein and controls (25 μl) were pipetted into bottom wells of the chamber, separated from the top wells by 5-μm pore of polycarbonate filter membrane. Isolated PBMCs were adjusted to 1 x $10^6$ cells/mL and 50 μl of

suspended cells were loaded into the top wells of the chamber. Migration was allowed to continue for 4 hr at 39°C in humidified air containing 5% $CO_2$. The cells at the lower surface of the membrane were fixed and stained with Diff-Quick Staining (Fisher Scientific, NJ). The stained cells were counted and the percentage of migration inhibition was determined by applying the following formula (Weiser et al., 1989; Jin et al., 2007):

$$\text{Percent migration inhibition} = \left( 1 - \frac{\text{Mean area of migration in experimental group}}{\text{Mean area of migration in control group}} \right) \times 100$$

*In vitro cell stimulation for biological function analysis*

Splenic lymphocytes were cultured at 4 x $10^6$ cells/well in 12-well plates and then treated with medium alone, Con A (10 μg/mL) alone, rChMIF (0.01 or 0.1 μg/mL) or Con A with rChMIF (0.01 or 0.1 μg/mL) for 12 hr. Total RNA was isolated from the treated lymphocytes and analyzed for Th1/Th2 cytokine transcript levels. The PBMCs were cultured at 2 x $10^6$ cells/well in 12-well plates, and then treated with medium alone, LPS (5 μg/mL) alone, rChMIF (0.01 or 0.1 μg/mL), or combination of LPS and rChMIF (0.01 or 0.1 μg/mL) for 6 hr. After incubation, total RNA was extracted using RNeasy Mini Kit.

In order to test the sequential effect of ChMIF on PBMCs and lymphocytes, the isolated PBMCs were seeded and cultured at 2 x $10^6$ cells/well in 12-well plates. Following 2-hr incubation, the cells were gently washed once and the PBMCs were incubated in fresh medium alone for 2 hr, after which cells from the first 3 wells were collected for total RNA isolation. The rest of the cells were stimulated with LPS (5 μg/mL) for 2 hr, followed by harvesting cells

from another set of 3 wells (replicates) and adding isolated lymphocytes (4 x $10^6$ cells/well) to the remaining 6 wells. After 2-hr incubation, cells from 3 wells were collected and the last 3 wells treated with rChMIF (0.01 μg/mL) for 2 hr. Treated cells were collected by adding cell lysis buffer directly. Total RNA was extracted using RNeasy Mini Column to analyze transcription of various cytokines.

*Cell proliferation assay*

Using the treated lymphocytes, a cell proliferation assay was performed using CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega, WI). The isolated lymphocytes were cultured at 1 x $10^5$ cells/mL in a 96-well plate, and then treated with medium alone, Con A, rChMIF (0.01 or 0.1 μg/mL), or combination of Con A and rChMIF (0.01 or 0.1 μg/mL) in the presence or absence of anti-rChMIF polyclonal antisera for 12 hr. The Dye Solution (15 μl) was directly added into each well, followed by 3 hr incubation at 39°C with 5% $CO_2$. Then, 100 μl of the Solubilization Solution/Stop Mix were added and mixed with a multi-channel pipette. After 1 hr incubation at 39°C, the absorbance was measured at 595 nm using a microplate reader. Background value was measured at 630 nm, subtracted, and the results were analyzed in Excel (Microsoft Corp, WA).

*Quantitative real-time PCR (qRT-PCR) analysis of cytokine transcripts*

To analyze the transcripts of various cytokines, primers were designed using Primer Express (Ver 3.0; Applied Biosystems, CA) (Table 4.1), and qRT-PCR was performed using

Fast SYBR Green Master Mix (Applied Biosystems, CA) according to the manufacturer's instructions in an ABI 7500 Fast Real-Time PCR System. Using 1 μg of total RNA, first-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) following manufacturer's instruction. Synthesized cDNA was diluted (1/25) with nuclease-free water, and 2 μl of the solution were used for Fast SYBR Green Master Mix; 100 nM primers and 10 μl of SYBR Green Master Mix in nuclease-free water in a final volume of 20 μl. Samples were heated to 95°C for 20 sec as initial denaturation, followed by 40 cycles of denaturation at 95°C for 3 sec, and annealing/extension at 57°C for 30 sec. Dissociation curves were generated to analyze individual PCR products after 40 cycles. Target gene expression in this study was normalized against the expression of chicken GAPDH mRNA. The results were analyzed using 7500 Software (Ver 2.0; Applied Biosystems, CA).

*Statistical analysis*

All data were analyzed by either Student's *t*-test or ANOVA using JMP software (Ver 7.0), and significant differences among groups were tested by the Tukey-Kramer Honestly Significant Difference post-hoc procedure.

## Results

*Sequence analysis of ChMIF*

The full-length ChMIF was cloned from stimulated T lymphocytes and is 99% identical to the reported nucleotide sequence (Wistow et al., 1993), and encodes 115 amino acids with

71% identity with human and murine MIF (Figure 4.1). Computational analysis showed two possible N-linked glycosylation sites; 73Asn-Lys-Thr and 110Asn-Gly-Ser. Sequence comparison showed three conserved cysteine residues, 57Cys, 60Cys and 81Cys, as well as an amino-terminal proline residue (Figure 4.2A). Secondary structure of ChMIF was predicted to contain two major α-helices between the 1st and 2nd β-sheets and the 4th and 5th β-sheets, respectively, with six-stranded β-sheets. The avian MIF sequences form a monophyletic clade, which is sister to that of MIF from mammals. The chicken MIF sequence is most similar to the turkey MIF, which is not surprising, considering these two species are both galliformes. The MIF sequences from the zebra finch (which is a passerine species) are sister to the MIF of both turkey and chicken. Interestingly, the zebra finch genome contains multiple MIF isoforms, which has not been observed in the chicken or turkey genome.

*Expression of ChMIF*

The expression of ChMIF gene was examined in various tissues, including thymus, spleen, bursa, brain, heart, liver, lung, crop, stomach, and intestinal sections using qRT-PCR (Figure 4.3). ChMIF expression was normalized with GAPDH, and the relative expression of ChMIF in various tissues was compared to that of the brain, which showed the lowest expression level. The results showed that ChMIF is ubiquitously expressed in all tissues tested, with the highest level in stomach and the lowest levels in brain and heart. Expression levels of ChMIF were similar between the 3 small intestinal sections examined.

*Purification and Western blot analysis of rChMIF*

Analysis of bacterial lysates by gel electrophoresis demonstrated a major band of predicted relative molecular weight (MW) for rChMIF (12.2 kDa) and estimates from gel analysis indicate about 30% of soluble protein in lysates was rChMIF. This major band was absent in lysates of non-recombinant bacteria. SEC-HPLC of bacterial lysates from rChMIF recombinant bacteria resulted in a single peak of absorbance with a retention time of approximately 40 min associated with rChMIF (Figure 4.4A). Analysis of this band by gel electrophoresis indicated a primary protein with a MW of 12.2 kDa (Figure 4.4B). Quantification of the bands indicated this protein consisted of more than 90% of the total protein observed. Following extraction with polymyxin beads, the endotoxin concentration of the purified rChMIF was determined to be 0.073 endotoxin units (EU) per μg protein. Western blot analysis with a 1:1000 dilution of rabbit polyclonal antisera made to the purified rChMIF recognized a band with a MW of 12.2 kDa (Figure 4.4C). The pre-immune antisera at the same dilution were non-reactive.

*Determination of chemotactic ability of ChMIF*

The ability of rChMIF to inhibit random migration of macrophages was assessed with a chemotaxis assay. Since commercially available chicken chemoattractant was unavailable, human MCP-1 and IL-8 were tested; however, their chemoattractant ability against chicken cells was not as good as that of medium supplemented with 10% FCS. As shown in Figure 4.5, migration of macrophages was inhibited by rChMIF in a dose-dependent manner. Since 0.01 and 0.1 μg/mL of rChMIF showed noticeable reduction of macrophage migration, these two concentrations were used in further analyses.

*The effect of ChMIF on lymphocyte proliferation*

To determine the effect of ChMIF on cell proliferation, isolated lymphocytes were incubated with Con A with or without rChMIF (0.01 or 0.1 μg/mL) for 24 hr. Although rChMIF alone did not have a significant effect on lymphocyte proliferation, 0.1 μg/mL of rChMIF enhanced proliferation of Con A-stimulated lymphocytes (Figure 4.6). This enhancement was abolished by addition of anti-MIF polyclonal antibody, verifying the specificity of rChMIF on lymphocyte proliferation.

*Modulation of Th1/Th2 cytokines by ChMIF in lymphocytes*

To determine the biological effects of ChMIF on cytokine expression in lymphocytes, chicken splenocytes were isolated and treated with Con A (10 μg/mL) alone or Con A with two different concentrations of rChMIF (0.01 or 0.1 μg/mL) for 12 hr. qRT-PCR was performed to measure transcription of the Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-13) cytokines (Figure 4.7). rChMIF alone had negligible effect on Th1 or Th2 cytokine transcriptions. However, Con A-stimulated lymphocytes demonstrated enhanced transcription of the Th1 cytokines IFN-γ and IL-2 in the presence of rChMIF. The transcription of IFN-γ was enhanced over 1.5-fold by the lower concentration of rChMIF (0.01 μg/mL) relative to that observed with Con A alone. However, with the high concentration of rChMIF (0.1 μg/mL), IFN-γ transcription was not significantly different from that observed in lymphocytes treated with Con A alone. Con A-stimulated lymphocytes showed 1.5- and 1.6-fold enhancement of IL-2 transcription respectively when exposed to the high and low concentrations of rChMIF. Of the Th2 cytokines, IL-4

transcripts were significantly decreased in lymphocytes treated with Con A alone, and Con A with 0.01 μg/mL of rChMIF (0.5- and 0.3-fold, respectively), but not Con A with 0.1 μg/mL of rChMIF. Low concentration of rChMIF (0.01 μg/mL) suppressed transcription of IL-13 (0.1-fold), but high concentration of rChMIF (0.1 μg/mL) induced IL-13 transcription 3-fold. In addition, IL-10 transcription showed a two-fold increase in the incubation with Con A and high concentration of rChMIF compared to controls (Figure 4.7). Taken together, low concentration of rChMIF mainly enhanced Th1 cytokines and high concentration of rChMIF induced Th2 cytokines.

*Modulation of inflammatory cytokines by ChMIF in monocytes*

Transcript levels of cytokines expressed by monocytes treated with rChMIF alone were not different from untreated monocytes. However, monocytes stimulated with LPS showed enhanced pro-inflammatory cytokine production (Figure 4.8). Addition of 0.01 μg/mL of rChMIF enhanced IL-1β and IL-6 transcription by 1.3-fold and 1.4-fold, respectively. LPS-stimulated monocytes exhibited a 1.3-fold increase in IL-8 transcript levels in the presence of 0.01 μg/mL of rChMIF. iNOS and IL-12, which function in differentiation and activation of T lymphocytes, also showed enhanced transcription in the presence of rChMIF; however, IL-4 transcription was not enhanced by rChMIF. Taken together, ChMIF enhanced the transcription of the pro-inflammatory cytokines and chemokines in stimulated chicken monocytes.

Differential expression of cytokines by ChMIF was measured in lymphocytes activated with LPS-stimulated monocytes. LPS-induced IL-1β and IL-8 transcripts (around 1600- and 320-fold, respectively) were remarkably decreased by addition of lymphocytes (8- and 4-fold,

respectively) and rChMIF (2.4- and 1.5-fold, respectively) (Figure 4.9). Unlike IL-1β and IL-8, transcription of IL-12 was not affected by addition of lymphocytes after induction with LPS; however, its transcription was decreased 4-fold by addition of rChMIF. Transcription of iNOS was induced by LPS (80-fold) and the additional enhancement observed upon addition of lymphocytes (6-fold) was maintained in the presence of rChMIF. The Th1 cytokine IFN-γ was highly induced by LPS (60-fold), followed by decrease in the presence of lymphocytes and rChMIF (3- and 2-fold, respectively). Addition of rChMIF showed a 1.3-fold enhancement of IL-10 transcription in comparison with those of LPS and lymphocytes. Transcription of IL-4 was 1.2-fold enhanced by addition of lymphocytes, while addition of ChMIF did not result in any change in IL-4 expression.

## Discussion

The present study is the first report of functional analysis of an avian MIF. The ChMIF gene, located on chicken chromosome 15, consists of three exons, an organization that is conserved in other species (Jin et al., 2007). Sequence analysis showed a conserved amino-terminal proline residue and three cysteine residues as in human and mouse. These residues are not conserved in all vertebrate MIFs such as fish and nematode. The only sequence motif, which is conserved among all MIF sequences, is 57Cys-Als-Leu-Cys60, which is thought to be involved in a single disulfide bond (Jin et al., 2007; Sun et al., 1996). Similarly to mammalian MIF, the secondary structure of chicken MIF indicated the presence of two α-helices and six β-sheets. Therefore, ChMIF may be able to form a trimer through the interaction between α2 and β6. The stabilization of the trimer is formed by a hydrophobic patch between aromatic residues

(Sun et al., 1996). The phylogenetic analysis of vertebrate MIF sequences coincides with the current understanding of vertebrate evolution. The two MIF sequences from galliform birds (turkey and chicken) formed a sister group, which in turn was sister to MIF sequences from the zebra finch. Interestingly, multiple MIF isoforms exist in the zebra finch; however, these are not present in chicken or turkey genomes.

Although tissue expression pattern of ChMIF was very similar to human, mouse, and swine, ChMIF transcripts in stimulated lymphocytes and monocytes differed. An increased expression of MIF was reported in mouse primary T cells stimulated with anti-CD3 antibody or phorbol 12-myristate 12-acetate (PMA) together with ionomycin (PMA/ION), and in mouse macrophage cell line (RAW 264.7) stimulated with toxic shock syndrome toxin 1 (TSST-1) or streptococcal pyrogenic exotoxin A (SPEA)(Bacher et al., 1996; Calandra et al., 1998). Increased MIF expression in LPS-stimulated kidney and spleen was observed in teleost fish (Jin et al., 2007); however, Con A-stimulated lymphocytes or LPS-stimulated monocytes did not show any change in ChMIF expression.

Since preliminary studies suggested that an affinity tag may reduce biological activity of recombinant ChMIF, the rChMIF was expressed without N- or C-terminal modification. The rChMIF was highly expressed in bacterial lysates and could be purified readily by SEC-HPLC. Purified MIF had a MW nearly identical to that of the predicted amino acid sequences. The rabbit polyclonal antisera generated against purified rChMIF recognized the rChMIF in a Western blot demonstrating specificity for ChMIF. In addition, the anti-ChMIF antisera recognized a protein of similar MW (11.6 kDa) in a protein extract made from chicken transformed macrophage cell line (unpublished observation), suggesting that the antibody can recognize both the recombinant and native proteins. Endotoxin was removed from the rChMIF

by first extracting the lysate with TX-114, which is reported to remove about 99% of the endotoxin (Liu et al., 1997) and subsequently treated with polymyxin B to further reduce endotoxin prior to cellular assays. The endotoxin level of .017 EU/μg protein used in the current study is well below the level of 0.5 EU/μg protein deemed acceptable for therapeutic proteins (Chen et al., 2009). In addition, rabbit anti-rChMIF antisera blocked the proliferative activity of rChMIF suggesting the biological activity of rChMIF is not caused by endotoxin or other contaminant.

MIF was initially discovered as an inhibitor of random migration of macrophages (Bernhagen et al., 1994). Inhibition of monocyte migration caused by recombinant mouse MIF had a bell-shaped dose-response curve with peak activity occurring at 0.1 μg/mL, while high MIF concentrations (1–10 μg/mL) had reduced activity (Bernhagen et al., 1994). In the current study, the relationship between rChMIF concentration and inhibition of macrophage migration lacked the bell-shaped dose-response curve. This may be attributed to the limited range of MIF concentrations (0.01 and 0.1 μg/mL) used in this study.

Calandra et al. (1998) reported 40% reduction in proliferation of splenocytes induced with TSST-1 in the presence of anti-MIF antibody, suggesting a clear involvement of MIF in T cell activation. Conversely, addition of rMIF resulted in about a 50% decrease in T-cell proliferation as induced by Con A (Bacher et al., 1996). Interestingly, others reported that T-cell activation was inhibited by anti-MIF antibody, providing further evidence of a role of MIF in T-cell activation (Bacher et al., 1996). In chickens, Con A-stimulated lymphocytes showed enhanced cell proliferation in the presence of 0.1 μg/mL of rChMIF, which was abolished by addition of anti-rChMIF antibody.

Similar to T cell activation, the effect of rChMIF on the expression of Th1/Th2 cytokines in lymphocytes was different from that of mammalian MIF. In the present study, rChMIF alone did not affect transcription of Th1 or Th2 cytokines, whereas addition of murine rMIF led to a 75% reduction of IFN-γ and IL-2 production in Con A-stimulated T cells (Bacher et al., 1996). However, addition of rChMIF to the Con A-stimulated lymphocytes enhanced transcription of the Th1 cytokine IFN-γ and IL-2 at low concentration (0.01 μg/mL) and that of the Th2 cytokine IL-4 and IL-13 at high concentration (0.1 μg/mL). The slight up-regulation of IL-13 observed with the higher concentration of rChMIF (0.1 μg/mL) may represent a switch to transcription of Th2 cytokines and thus downregulation of the Th1 cytokine response. Taken together, the results indicate enhancement of the transcription of both Th1 and Th2 cytokines in stimulated lymphocytes, and suggest that it is dependent on rChMIF levels in culture.

Mammalian MIF is reported to promote pro-inflammatory cytokine expressions (TNF-α, IL-1β, IL-6, IL-8) and NO release (Bernhagen et al., 1994; Calandra et al., 1994; Donnelly et al, 1997). Unlike mammalian MIF, ChMIF alone did not promote the expression of pro-inflammatory cytokines and NO release. However, when monocytes/macrophages were first primed with LPS, rChMIF-induced the expression of mRNA of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and IL-12) as well as NO. These results are consistent with those of Bernhagen et al. (1994), which showed a 10-fold increase in NO production from mouse macrophages treated with a combination of MIF (10 μg/mL) with IFN-γ (100 IU/mL) in comparison with those from mouse macrophage treated with MIF alone.

In summary, following the successful cloning and expression of ChMIF, the molecular function of the first described avian MIF is demonstrated. Our data indicate that avian MIF

functionally enhances pro-inflammatory cytokines and Th1/Th2 cytokines in previously stimulated monocytes and lymphocytes, respectively and that antigen stimulation and cell type affect avian MIF activity. Characterization of chicken MIF provides additional insight into the function of the avian immune system.

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**Table 4.1. Primer sequences for cloning ChMIF and qRT-PCR analyses of cytokine transcripts.**

| Name | Accession No. | Nucleotide sequence (5' → 3') | Application |
|---|---|---|---|
| chMIF_F | XM_425824 | gatcatatgagatctATGCCTATGTTCACCATCCACACC | Cloning of rChMIF |
| chMIF_R | | gatgctagcctaTGCAAAGGTGGAACCGTTCCA | |
| GAPDH | NM_204305 | AGGGTGGTGCTAAGCGTGTTA | qRT-PCR |
| GAPDH | | TCTCATGGTTGACACCCATCA | |
| IFNgamma_F | NM_205149 | GCTCCCGATGAACGACTTGA | |
| IFNgamma_R | | TGTAAGATGCTGAAGAGTTCATTCG | |
| IL-1b_F | NM_204524 | GCTCTACATGTCGTGTGTGATGAG | |
| IL-1b_R | | TGTCGATGTCCCGCATGA | |
| IL-2_F | NM_204153 | CGAGCTCTACACACCAACTGAGA | |
| IL-2_R | | CCAGGTAACACTGCAGAGTTTGC | |
| IL-4_F | NM_001007079 | GCTCTCAGTGCCGCTGATG | |
| IL-4_R | | GAAACCTCTCCCTGGATGTCAT | |
| IL-6_F | NM_204628 | GAACGTCGAGTCTCTGTGCTAC | |
| IL-6_R | | CACCATCTGCCGGATCGT | |
| IL-8_F | NM_205498 | TCCTGGTTTCAGCTGCTCTGT | |
| IL-8_R | | CGCAGCTCATTCCCCATCT | |
| IL-10_F | NM_001004414 | CGCTGTCACCGCTTCTTCA | |
| IL-10_R | | CGTCTCCTTGATCTGCTTGATG | |
| IL-12B_F | NM_213571 | TGCCCAGTGCCAGAAGGA | |
| IL-12B_R | | TCAGTCGGCTGGTGCTCTT | |
| IL-13_F | NM_001007085 | CATGACCGACTGCAAGAAGGA | |
| IL-13_R | | CCGTGCAGGCTCTTCAGACT | |
| iNOS_F | D85422 | CCTGTACTGAAGGTGGCTATTGG | |
| iNOS_R | | AGGCCTGTGAGAGTGTGCAA | |
| MIF_F | XM_425824 | GCCCGCGCAGTACATAGC | |
| MIF_R | | CCCCCGAAGGACATCATCT | |

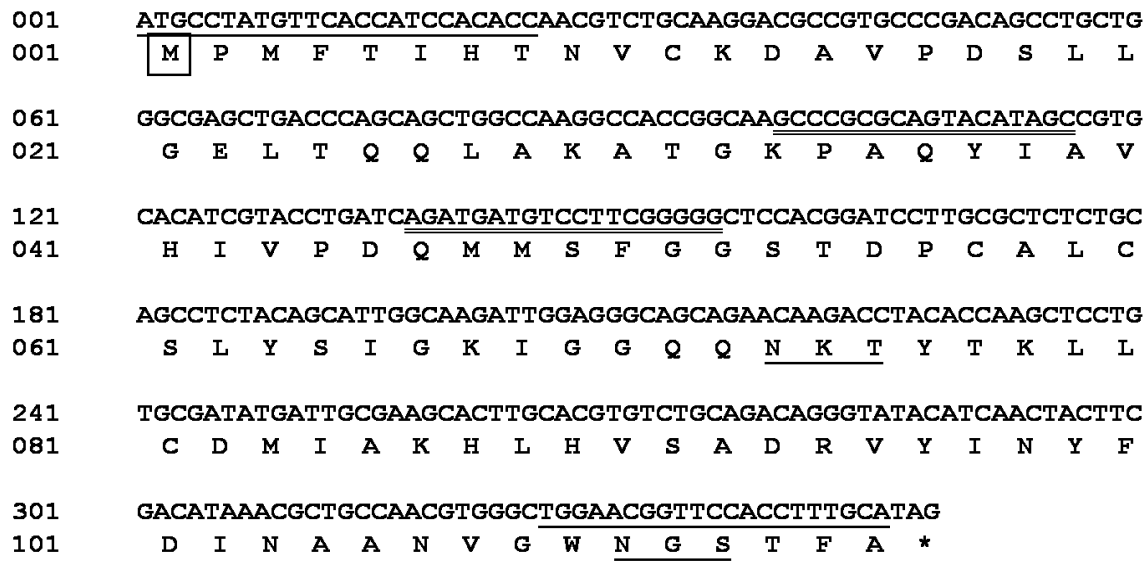
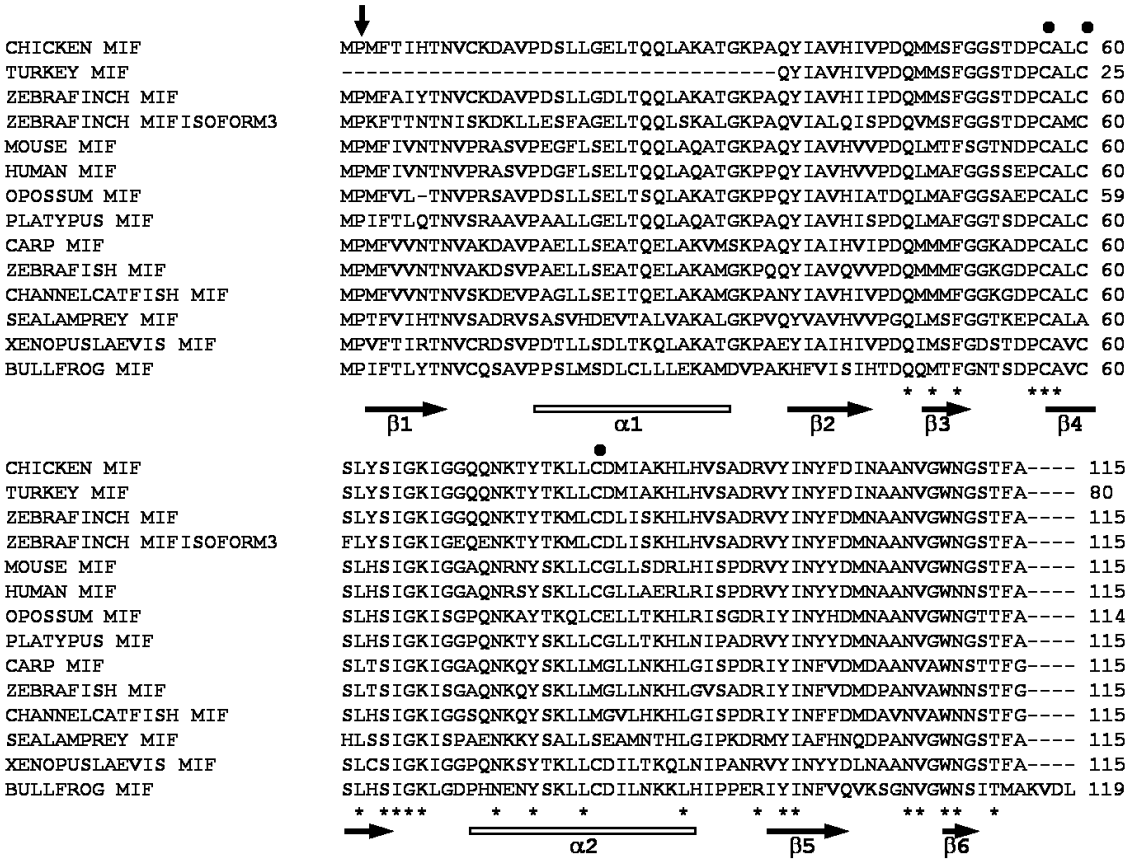



**Figure 4.1. Nucleotide and deduced amino acid sequences of ChMIF.** The methionine at the first position (marked with a box) was used as the translation initiation site for expressing rChMIF. The underlined nucleotide sequences indicate the primers used to amplify the full-length ChMIF, and double underlined nucleotide sequences indicate the primers used to detect ChMIF from various tissues by qRT-PCR. Underlined amino acid sequences show potential N-linked glycosylation sites.

**A**


CHICKEN MIF MPMFTIHTNVCKDAVPDSLLGELTQQLAKATGKPAQYIAVHIVPDQMMSFGGSTDPCALC 60
TURKEY MIF ----------QYIAVHIVPDQMMSFGGSTDPCALC 25
ZEBRAFINCH MIF MPMFAIYTNVCKDAVPDSLLGDLTQQLAKATGKPAQYIAVHIIPDQMMSFGGSTDPCALC 60
ZEBRAFINCH MIFISOFORM3 MPKFTTNTNISKDKLLESFAGELTQQLSKALGKPAQVIALQISPDQVMSFGGSTDPCAMC 60
MOUSE MIF MPMFIVNTNVPRASVPEGFLSELTQQLAQATGKPAQYIAVHVVPDQLMTFSGTNDPCALC 60
HUMAN MIF MPMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCALC 60
OPOSSUM MIF MPMFVL-TNVPRSAVPDSLLSELTSQLAKATGKPPQYIAVHIATDQLMAFGGSAEPCALC 59
PLATYPUS MIF MPIFTLQTNVSRAAVPAALLGELTQQLAQATGKPAQYIAVHISPDQLMAFGGTSDPCALC 60
CARP MIF MPMFVVNTNVAKDAVPAELLSEATQELAKVMSKPAQYIAIHVIPDQMMMFGGKADPCALC 60
ZEBRAFISH MIF MPMFVVNTNVAKDSVPAELLSEATQELAKAMGKPQQYIAVQVVPDQMMMFGGKGDPCALC 60
CHANNELCATFISH MIF MPMFVVNTNVSKDEVPAGLLSEITQELAKAMGKPANYIAVHIVPDQMMMFGGKGDPCALC 60
SEALAMPREY MIF MPTFVIHTNVSADRVSASVHDEVTALVAKALGKPVQYVAVHVVPGQLMSFGGTKEPCALA 60
XENOPUSLAEVIS MIF MPVFTIRTNVCRDSVPDTLLSDLTKQLAKATGKPAEYIAIHIVPDQIMSFGDSTDPCAVC 60
BULLFROG MIF MPIFTLYTNVCQSAVPPSLMSDLCLLLEKAMDVPAKHFVISIHTDQQMTFGNTSDPCAVC 60
β1
α1
β2
β3
β4
CHICKEN MIF SLYSIGKIGGQQNKTYTKLLCDMIAKHLHVSADRVYINYFDINAANVGWNGSTFA---- 115
TURKEY MIF SLYSIGKIGGQQNKTYTKLLCDMIAKHLHVSADRVYINYFDINAANVGWNGSTFA---- 80
ZEBRAFINCH MIF SLYSIGKIGGQQNKTYTKMLCDLISKHLHVSADRVYINYFDMNAANVGWNGSTFA---- 115
ZEBRAFINCH MIFISOFORM3 FLYSIGKIGEQENKTYTKMLCDLISKHLHVSADRVYINYFDMNAANVGWNGSTFA---- 115
MOUSE MIF SLHSIGKIGGAQNRNYSKLLCGLLSDRLHISPDRVYINYYDMNAANVGWNGSTFA---- 115
HUMAN MIF SLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAANVGWNNSTFA---- 115
OPOSSUM MIF SLHSIGKISGPQNKAYTKQLCELLTKHLRISGDRIYINYHDMNAANVGWNGTTFA---- 114
PLATYPUS MIF SLHSIGKIGGPQNKTYSKLLCGLLTKHLNIPADRVYINYYDMNAANVGWNGSTFA---- 115
CARP MIF SLTSIGKIGGAQNKQYSKLLMGLLNKHLGISPDRIYINFVDMDAANVAWNSTTFG---- 115
ZEBRAFISH MIF SLTSIGKISGAQNKQYSKLLMGLLNKHLGVSADRIYINFVDMDPANVAWNNSTFG---- 115
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XENOPUSLAEVIS MIF SLCSIGKIGGPQNKSYTKLLCDILTKQLNIPANRVYINYYDLNAANVGWNGSTFA---- 115
BULLFROG MIF SLHSIGKLGDPHNENYSKLLCDILNKKLHIPPERIYINFVQVKSGNVGWNSITMAKVDL 119
α2
β5
β6

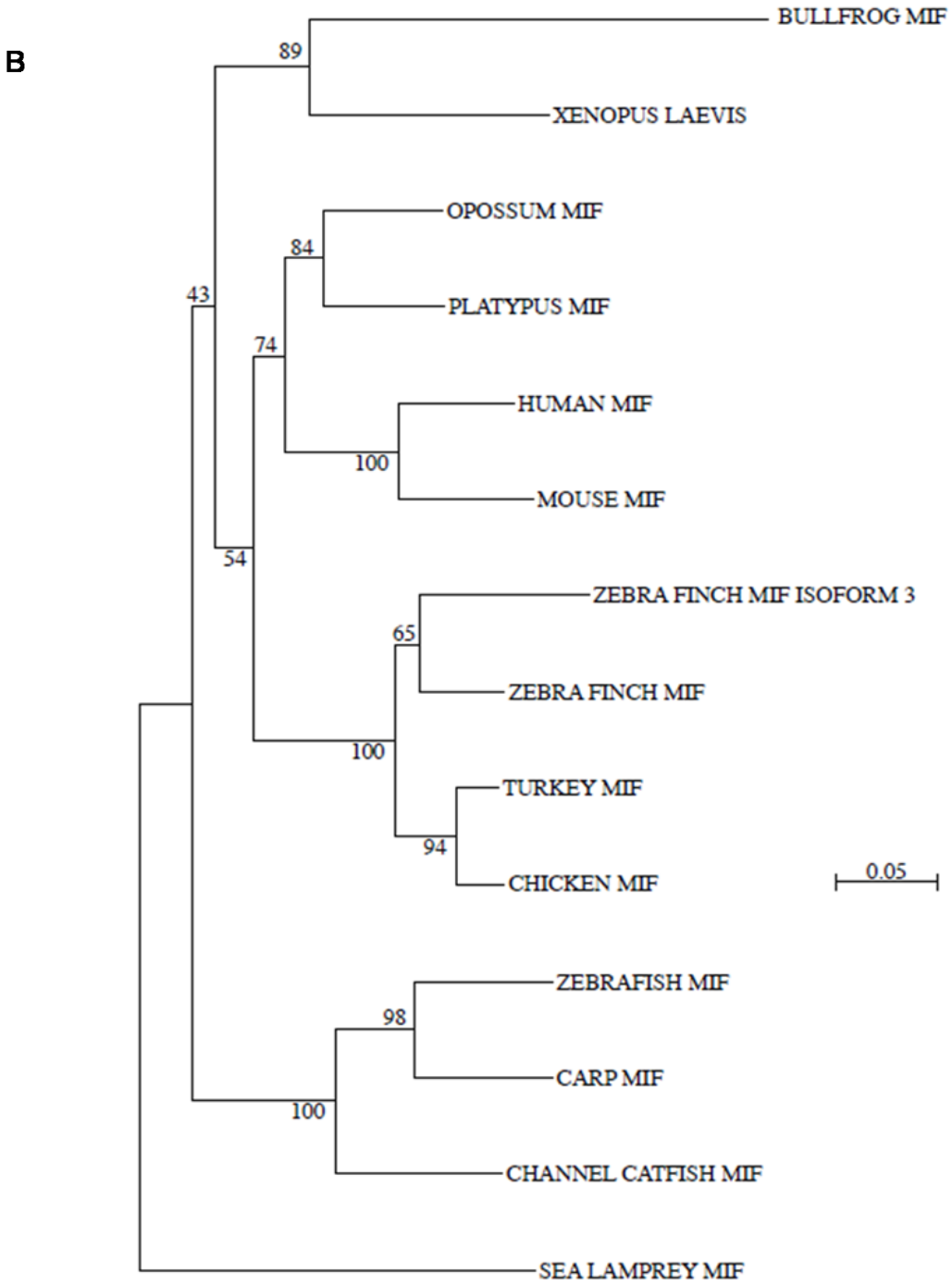



**Figure 4.2. Sequence comparison and phylogenetic tree of amino acids encoded by ChMIF.** (A) Using the known MIF molecules, sequences were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). Asterisks (*) indicate identical residues among sequences. Downward arrow marks the conserved amino-terminal proline residue, and circle

dots (•) indicate the three conserved cysteine residues in vertebrate species. Cylinders show α-helices and dark arrows represent β strands. (B) Phylogenetic tree of representative vertebrate MIF sequences. The tree was constructed using the neighbor joining method. MIF from the sea lamprey was used as outgroup. The numbers at each node represent percentage of bootstrap replicates which resulted in the production of the same branching pattern.

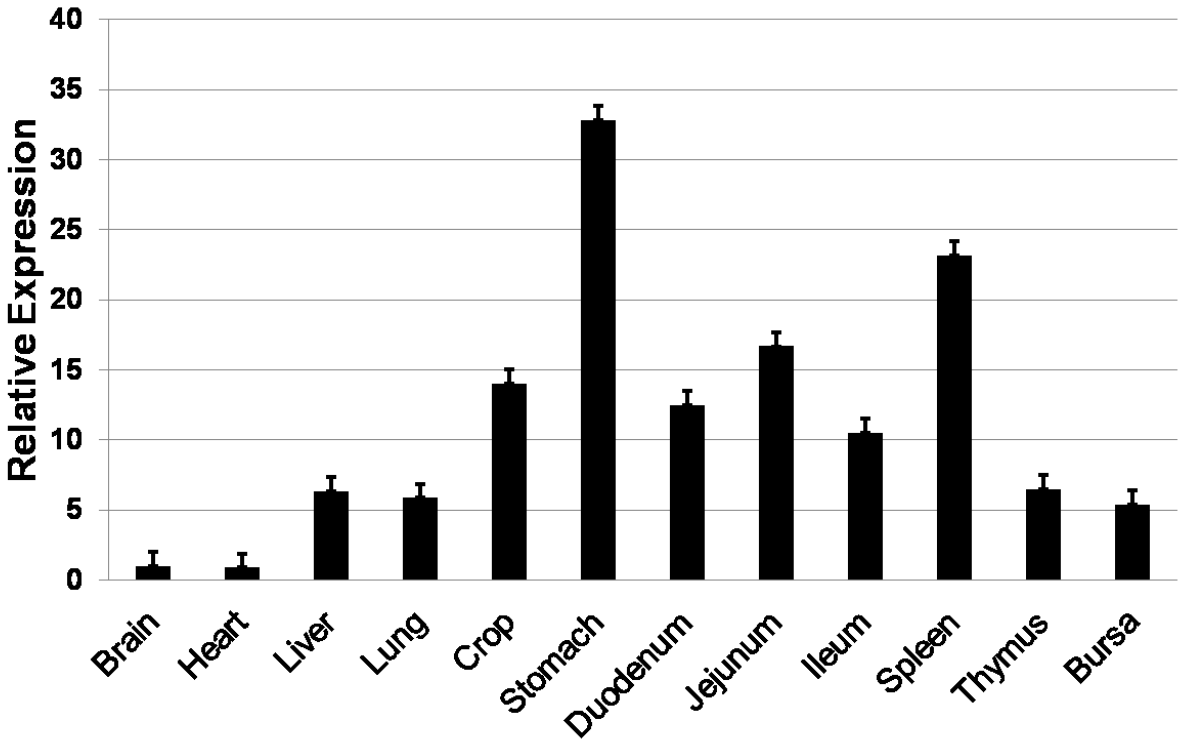



**Figure 4.3. Tissue distribution of ChMIF.** Relative ChMIF expression was calculated using the $2^{-\Delta\Delta CT}$ method with initial normalization of ChMIF against GAPDH within each sample, and then comparison of tissue expression levels with expression in brain (arbitrarily set at 1.0).

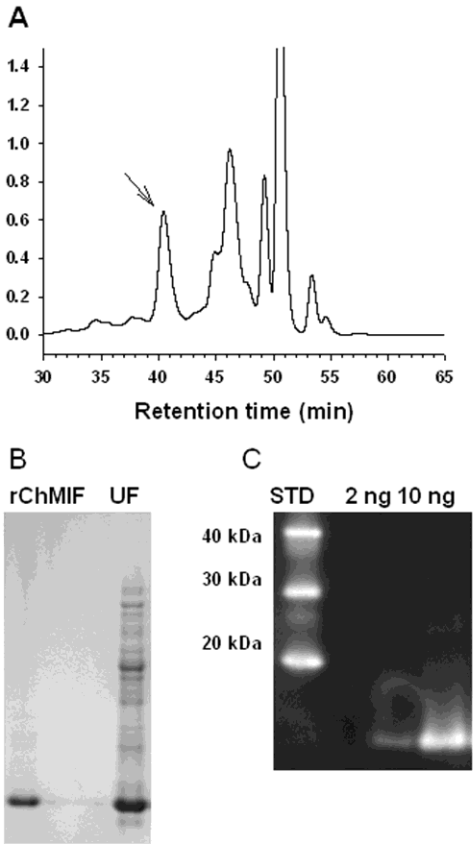



**Figure 4.4. Purification and analysis of rChMIF expressed in bacteria.** (A) Chromatogram of SEC-HPLC purification of a lysate of recombinant bacteria expressing rChMIF. About 1 mg of lysate protein was injected onto the column in a 200 µl volume. The arrow indicates the peak containing purified rChMIF. The vertical axis is absorbance at 280 nm. (B) PAGE analysis of the protein purified by SEC-HPLC (rChMIF) and unfractionated lysate (UF). The gel was stained with Coomassie Blue. (C) Western blot analysis of purified rChMIF (2 or 10 ng per lane). A 1:1000 dilution of rabbit antisera made against purified rChMIF was used as the primary antibody. STD indicates the lane containing molecular weight standards.

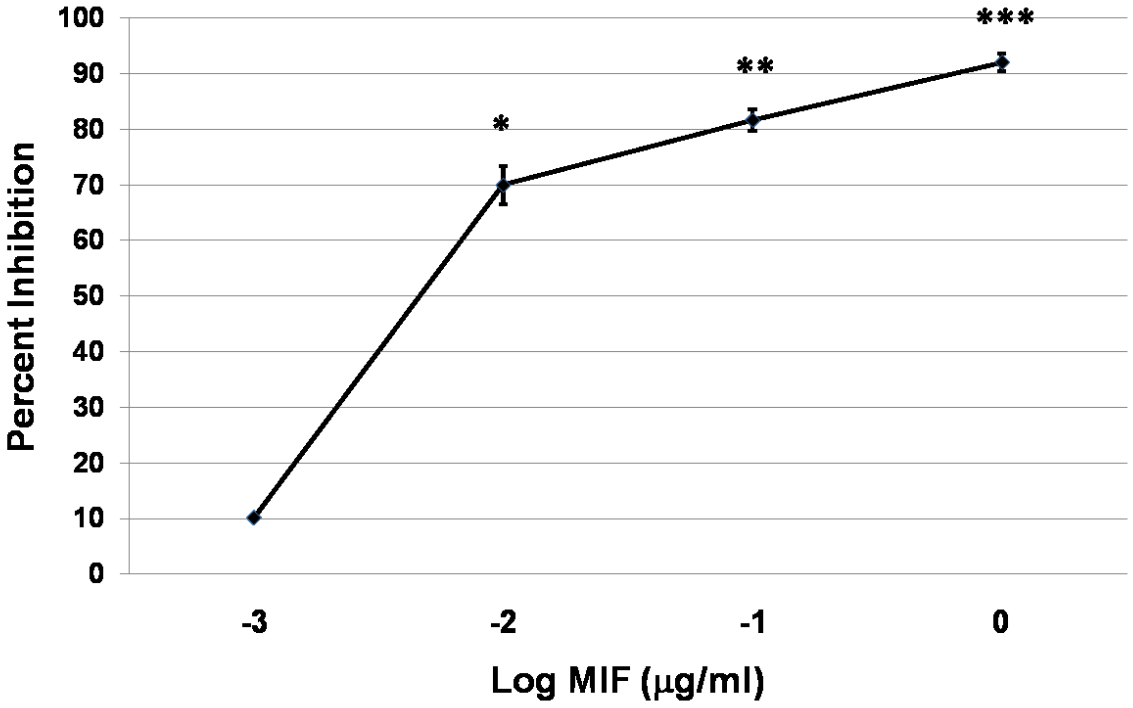



**Figure 4.5. Chemotaxis Assay in modified 48-well Boyden Chambers.** Migration of PBMCs was examined in the presence of different concentration of rChMIF (Log $10^{-3}$ – log $10^{0}$ μg/mL). Isolated PBMCs were placed into the top chamber wells, which were separated by 5-μm pores membrane from rChMIF in the bottom chamber. Chemotaxis chamber was incubated for 4 hr at 37°C in humidified air containing 5% $CO_2$. The cells were stained with DF staining method and the number of cells was counted. Each experiment was set up in triplicate, and the results represent the mean of two individual experiments. The different number of asterisks (*) indicates statistically significant difference ($p < 0.05$).

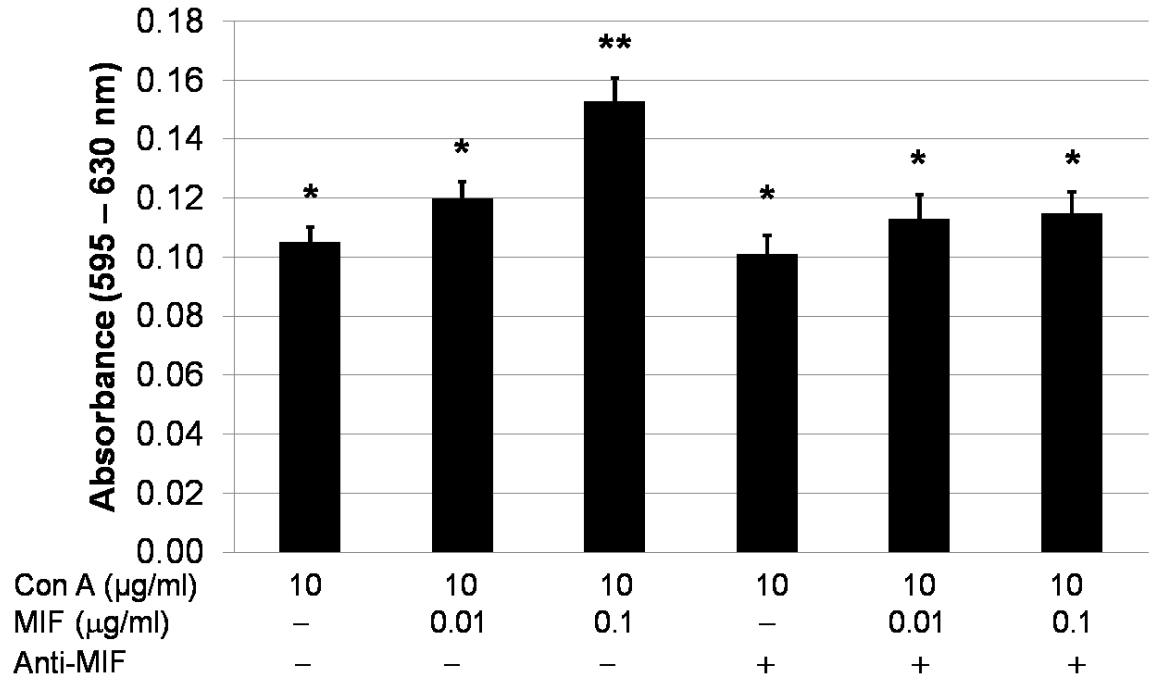
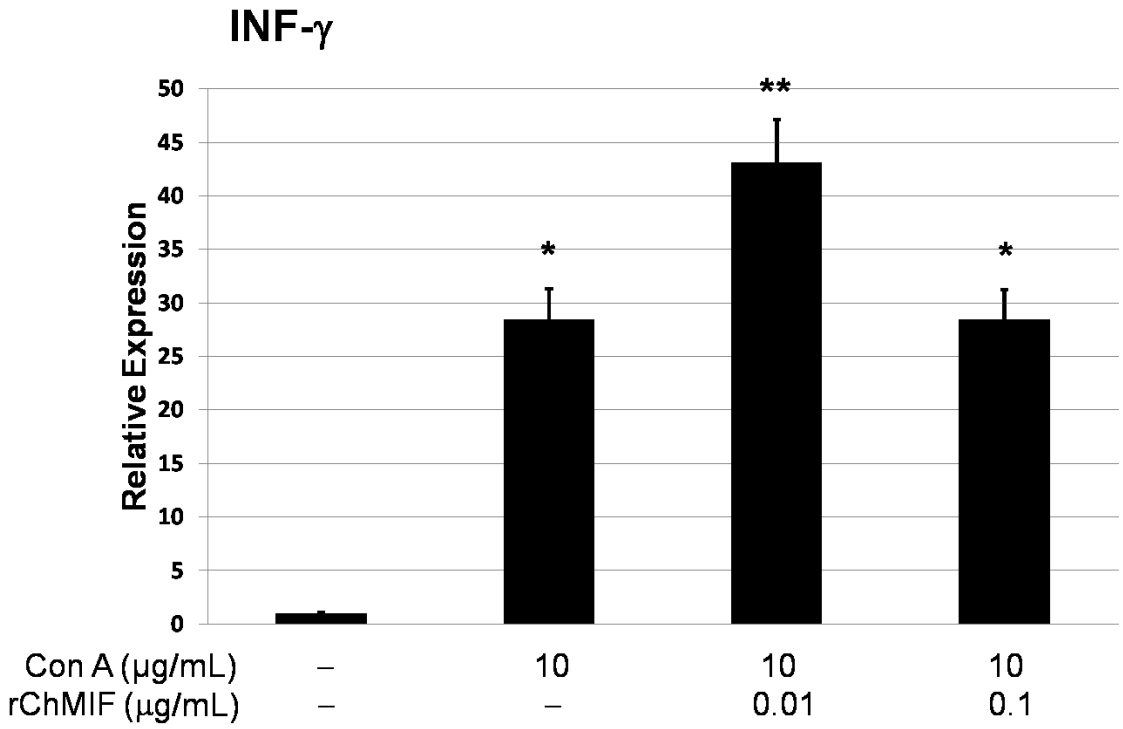



**Figure 4.6. The effect of rChMIF on proliferation of lymphocytes.** Isolated lymphocytes were seeded into a 96-well plate with 1 x $10^5$ cells/well concentration. After 24 hr incubation at 39°C with 5% of $CO_2$, the cells were gently washed once, treated with media alone, Con A (10 μg/mL) alone, Con A with rChMIF (0.01 or 0.1 μg/mL) and Con A/rChMIF with anti-rChMIF polyclonal antibody (1: 1,000 dilution) for 24 hr. Cell proliferation assay was performed using CellTiter 96 Non-radioactive Cell Proliferation Assay kit (Promega) followed by manufacturer's instruction. The different number of asterisks (*) indicates statistically significant difference ($p <$ 0.05).


INF-γ
Relative Expression
50
45
40
35
30
25
20
15
10
5
0
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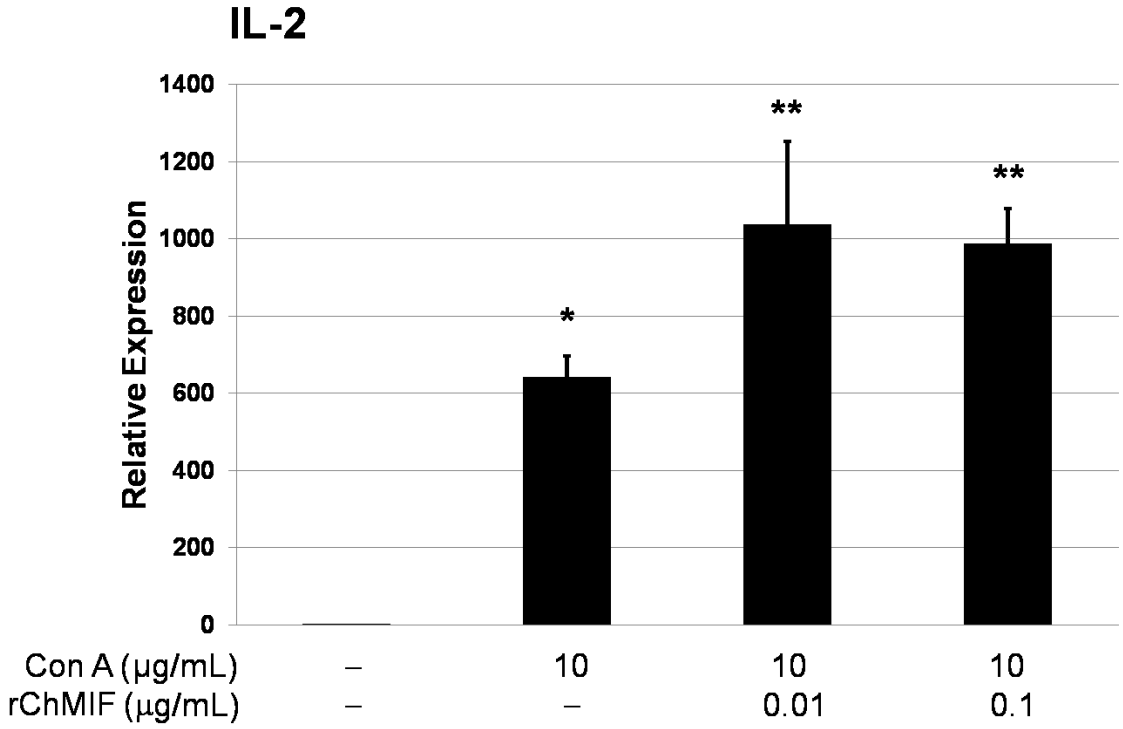
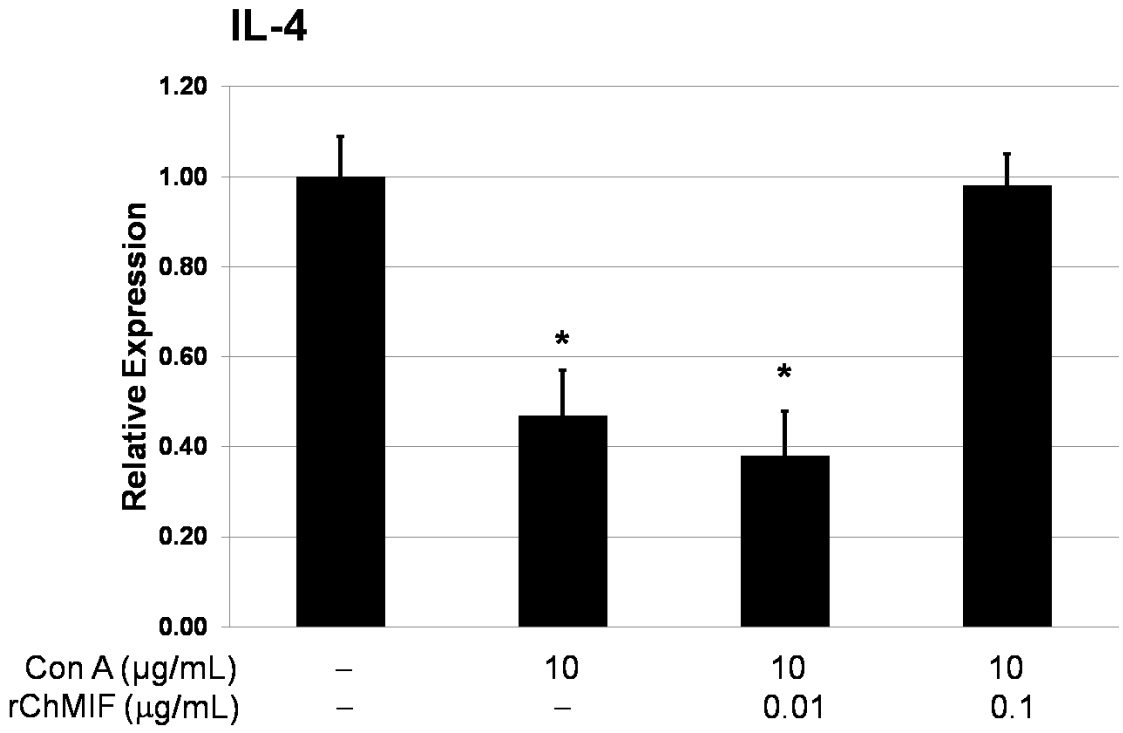
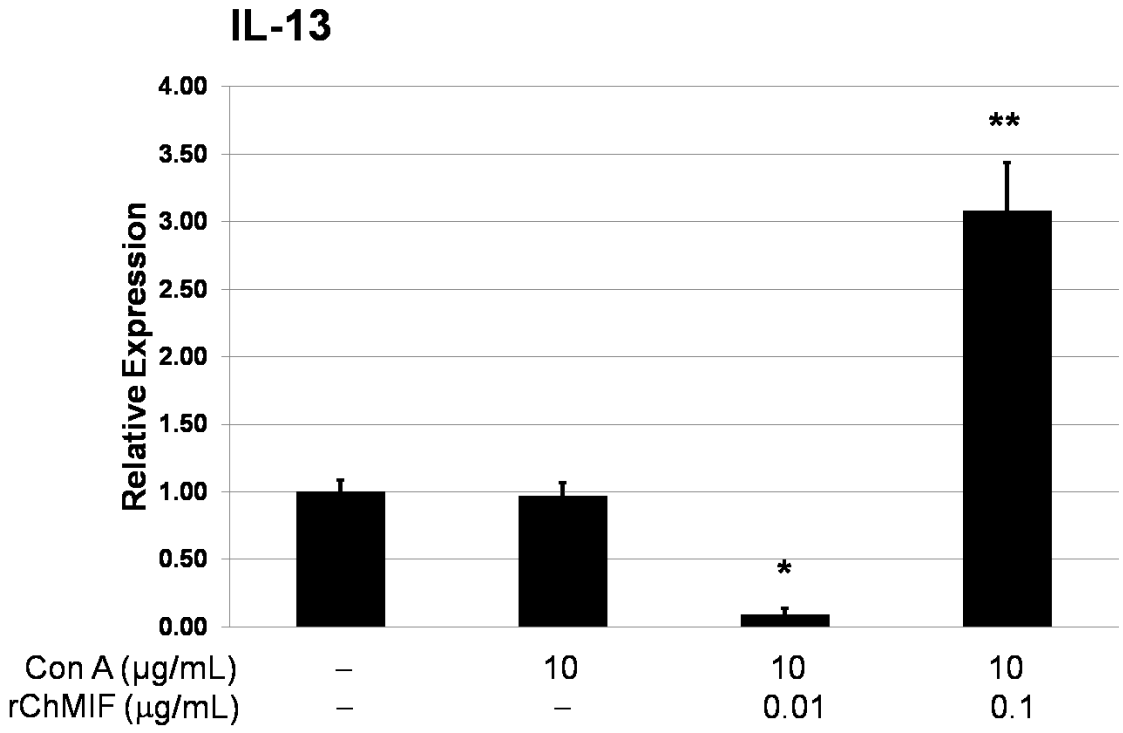
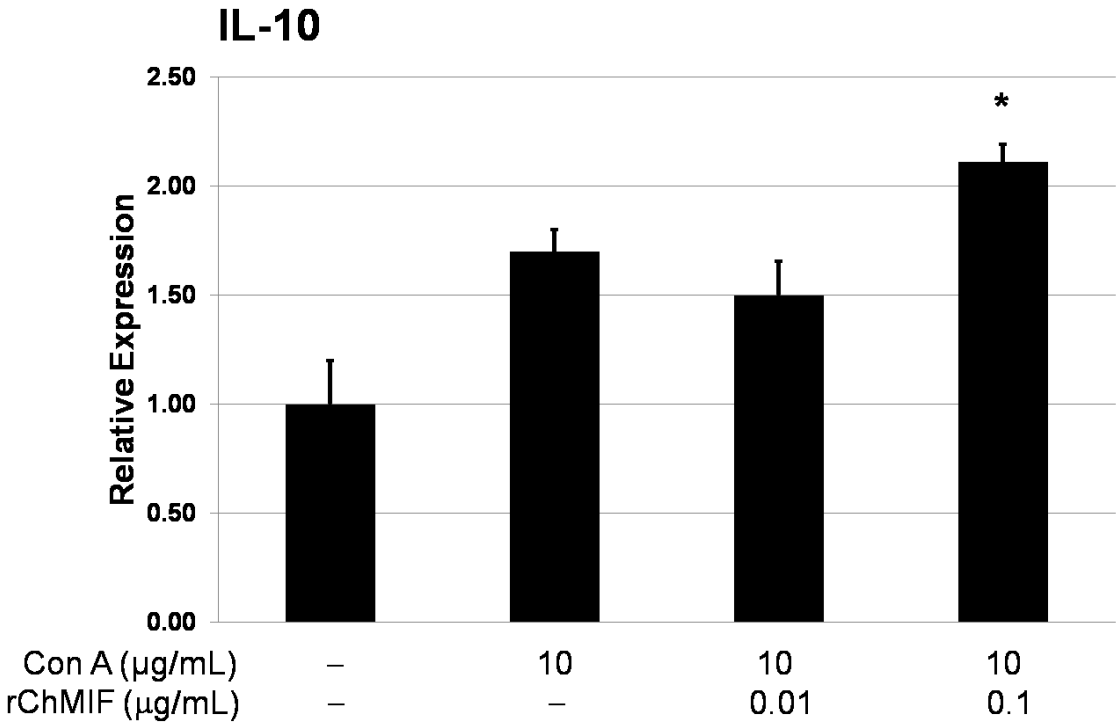
*
*
Con A (μg/mL) – 10 10 10
rChMIF (μg/mL) – – 0.01 0.1


IL-2
Relative Expression
1400
1200
1000
800
600
400
200
0
**
**
*
Con A (μg/mL) – 10 10 10
rChMIF (μg/mL) – – 0.01 0.1


IL-4
Relative Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
*
Con A (µg/mL) – 10 10 10
rChMIF (µg/mL) – – 0.01 0.1


IL-13
Relative Expression
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
*
Con A (µg/mL) – 10 10 10
rChMIF (µg/mL) – – 0.01 0.1


IL-10
Relative Expression
2.50
2.00
1.50
1.00
0.50
0.00
*
Con A (μg/mL) – 10 10 10
rChMIF (μg/mL) – – 0.01 0.1

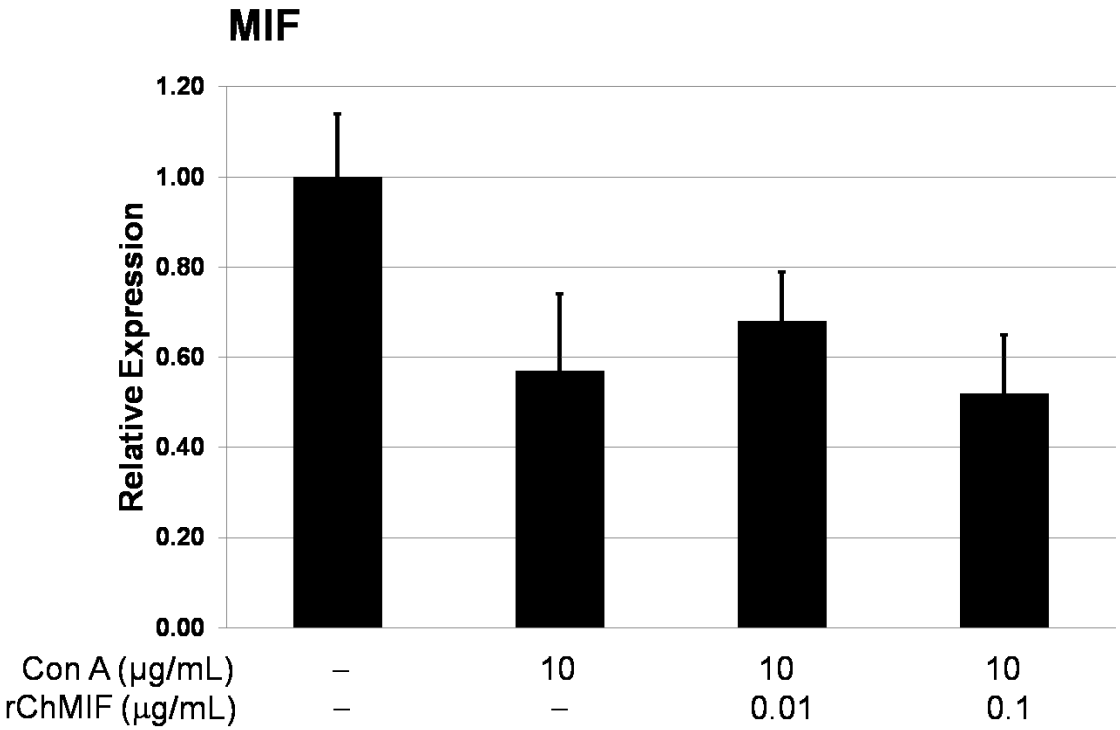
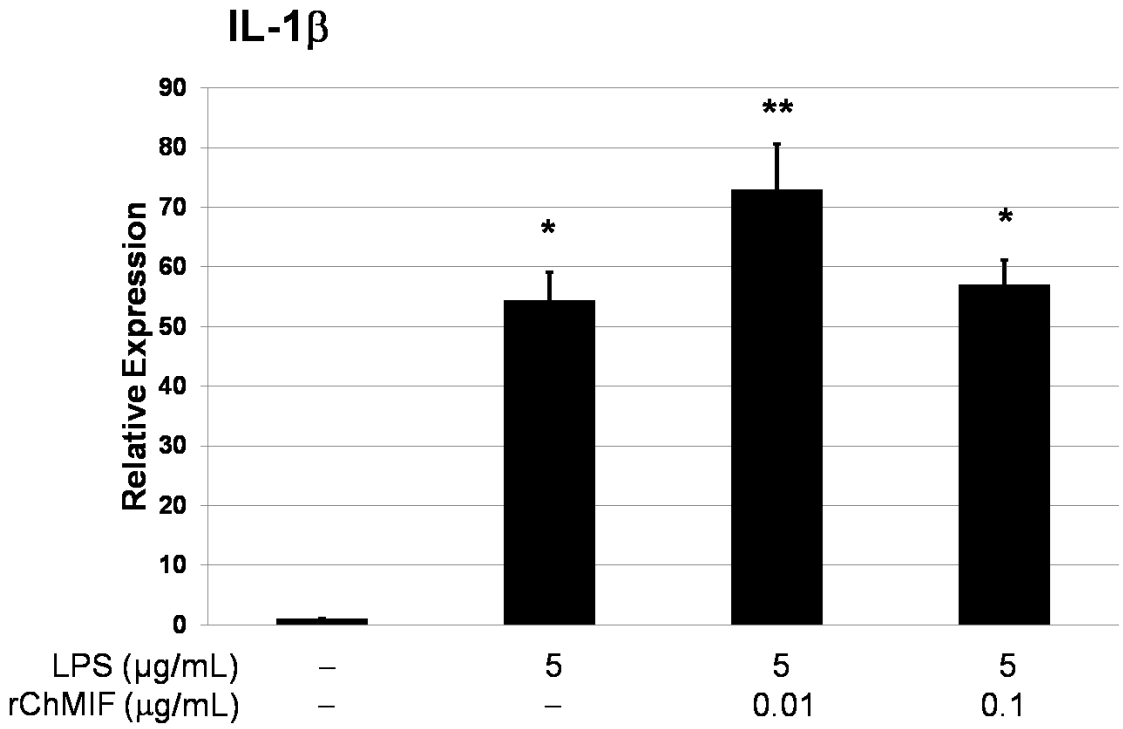
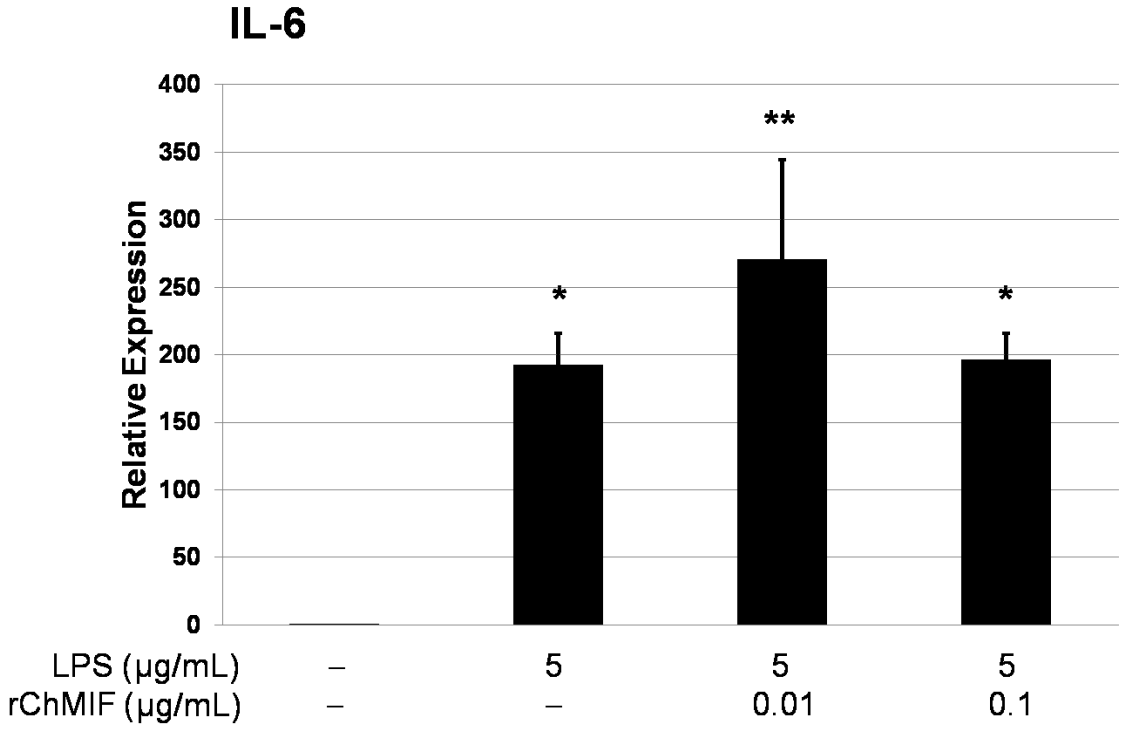
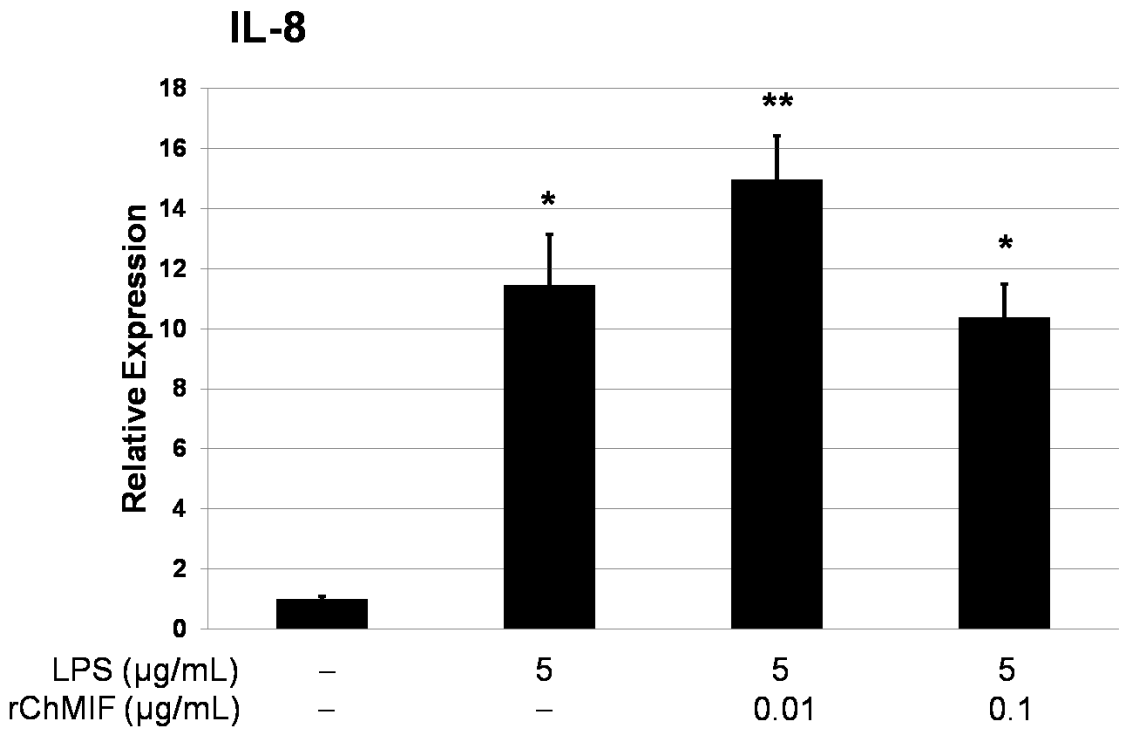
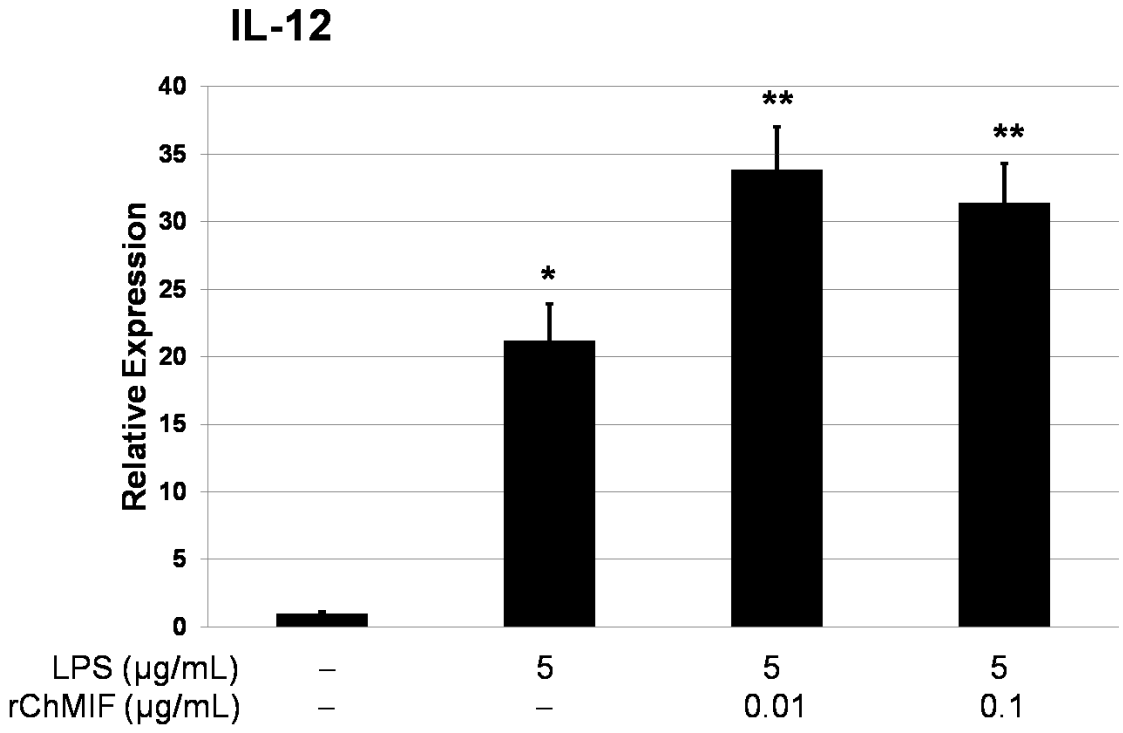

MIF
Relative Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Con A (μg/mL) – 10 10 10
rChMIF (μg/mL) – – 0.01 0.1

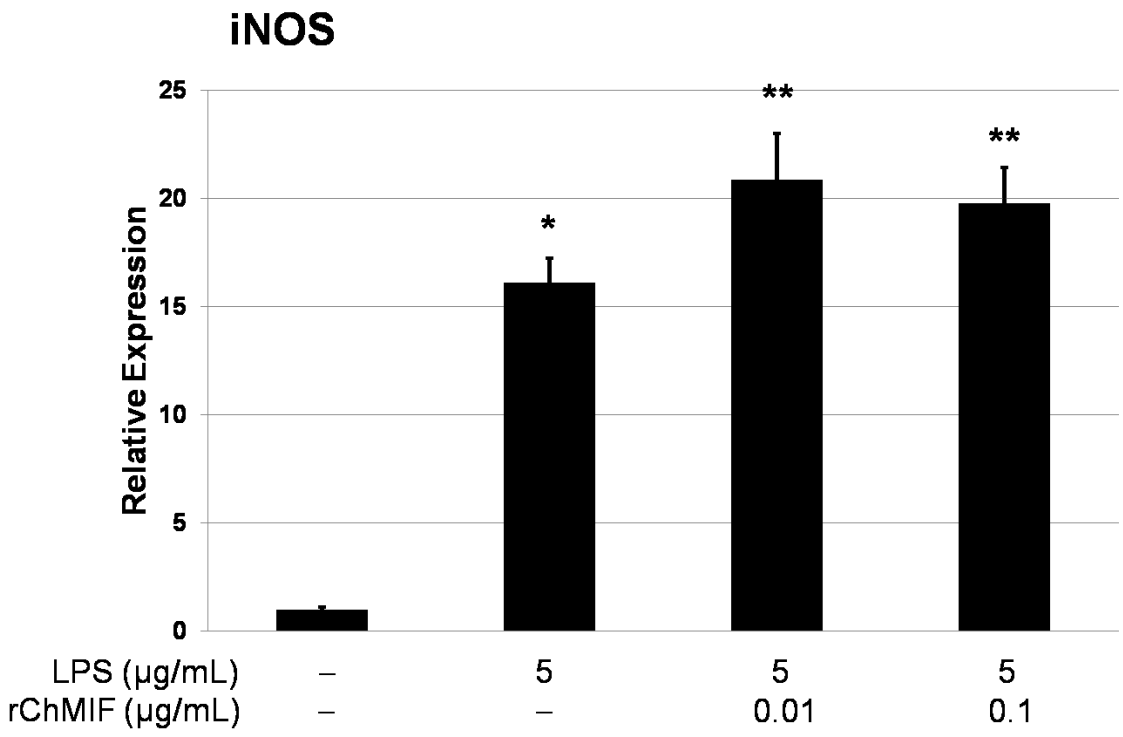
**Figure 4.7. The biological function of rChMIF on lymphocytes.** Splenocytes were treated with media alone, Con A (10 μg/ mL) or Con A with rChMIF (0.01 or 0.1 μg/mL) for 12 hr. The expression of Th1/2 cytokines was measured by qRT-PCR, and their transcripts were compared with control media alone. Each bar represents the mean of 3 different experiments. The different number of asterisks (*) indicates statistically significant difference ($p < 0.05$).


IL-1β
Relative Expression
90
80
70
60
50
40
30
20
10
0
*
**
*
LPS (μg/mL) – 5 5 5
rChMIF (μg/mL) – – 0.01 0.1


IL-6
Relative Expression
400
350
300
250
200
150
100
50
0
*
**
*
LPS (μg/mL) – 5 5 5
rChMIF (μg/mL) – – 0.01 0.1


IL-8
Relative Expression
18
16
14
12
10
8
6
4
2
0
*
**
*
LPS (µg/mL) – 5 5 5
rChMIF (µg/mL) – – 0.01 0.1


IL-12
Relative Expression
40
35
30
25
20
15
10
5
0
*
**
**
LPS (µg/mL) – 5 5 5
rChMIF (µg/mL) – – 0.01 0.1


iNOS
Relative Expression
25
20
15
10
5
0
*
**
**
LPS (μg/mL) – 5 5 5
rChMIF (μg/mL) – – 0.01 0.1

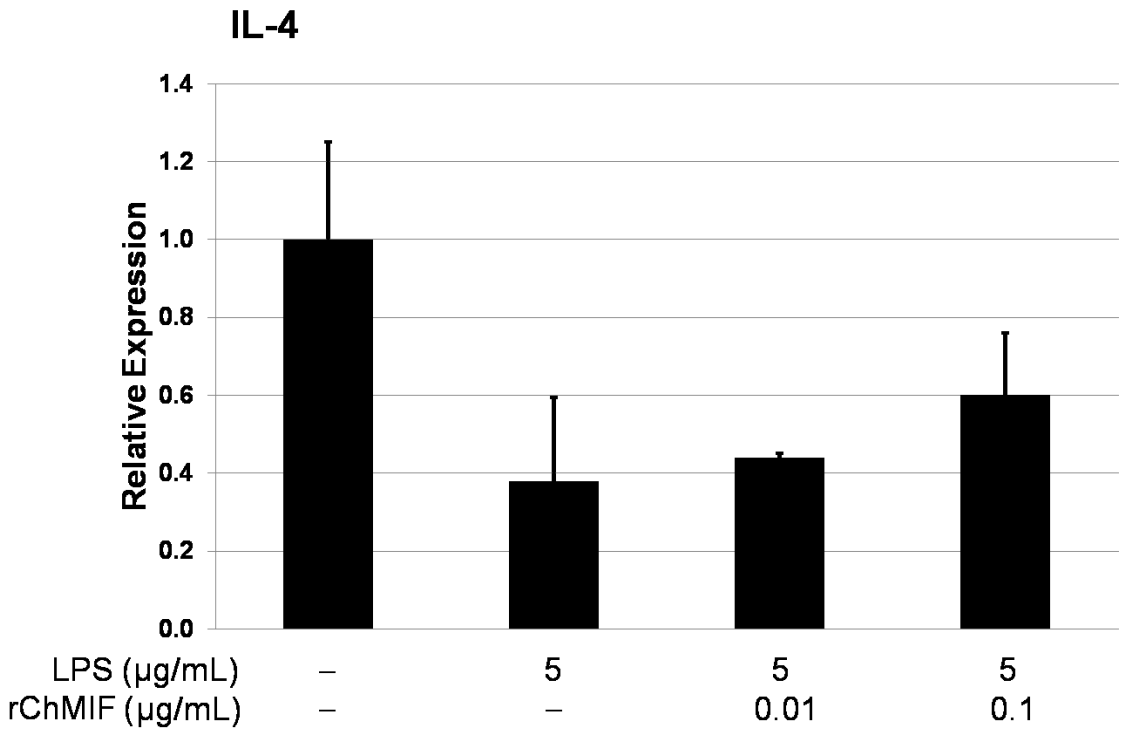
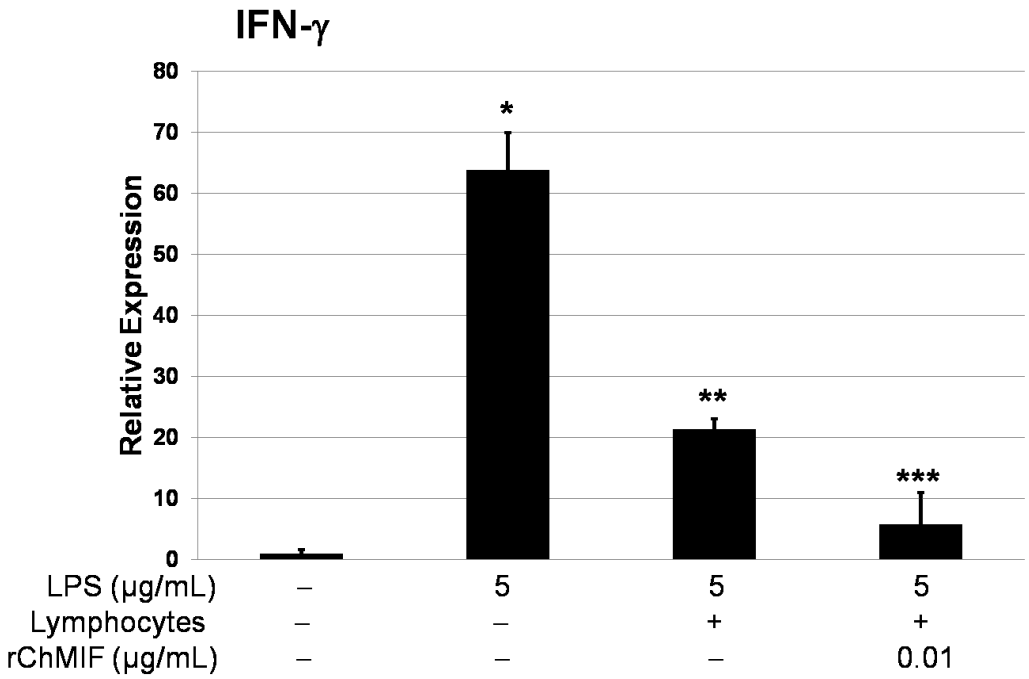
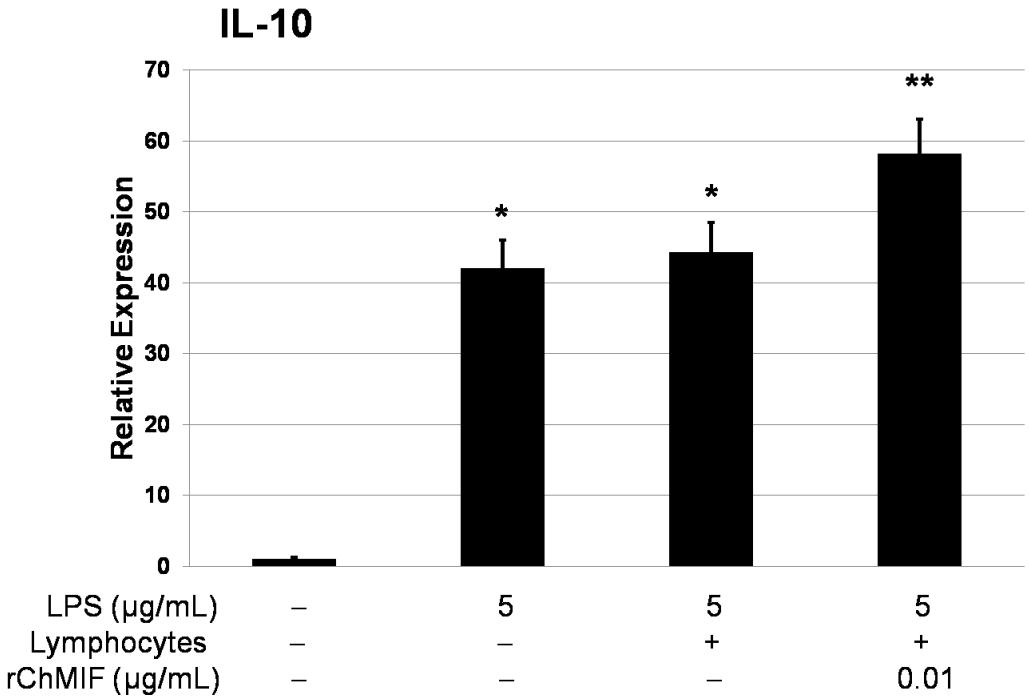
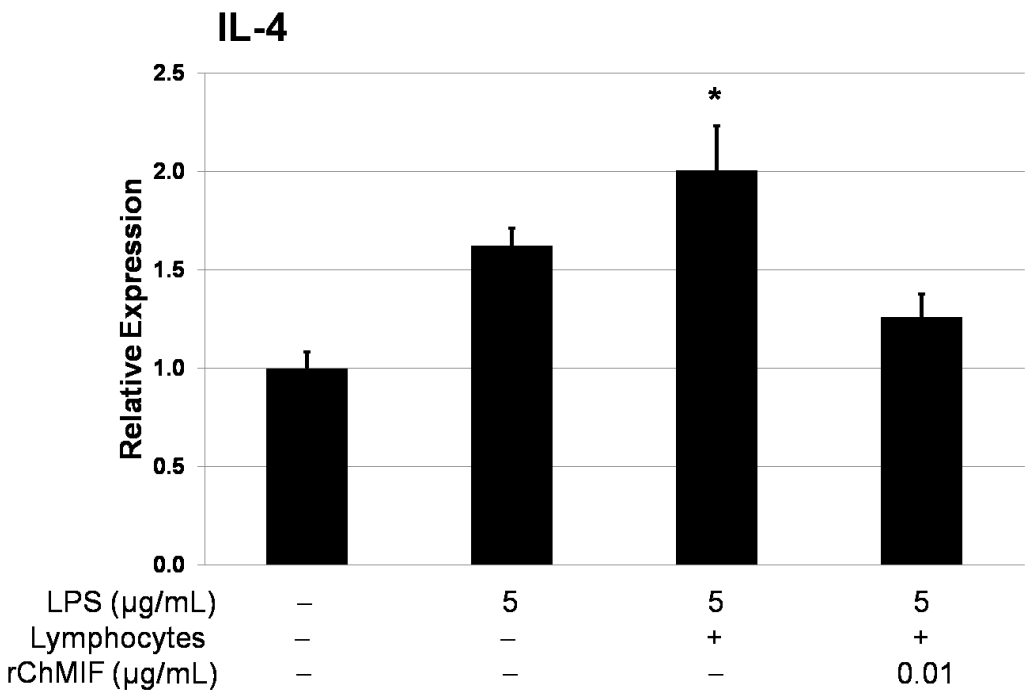
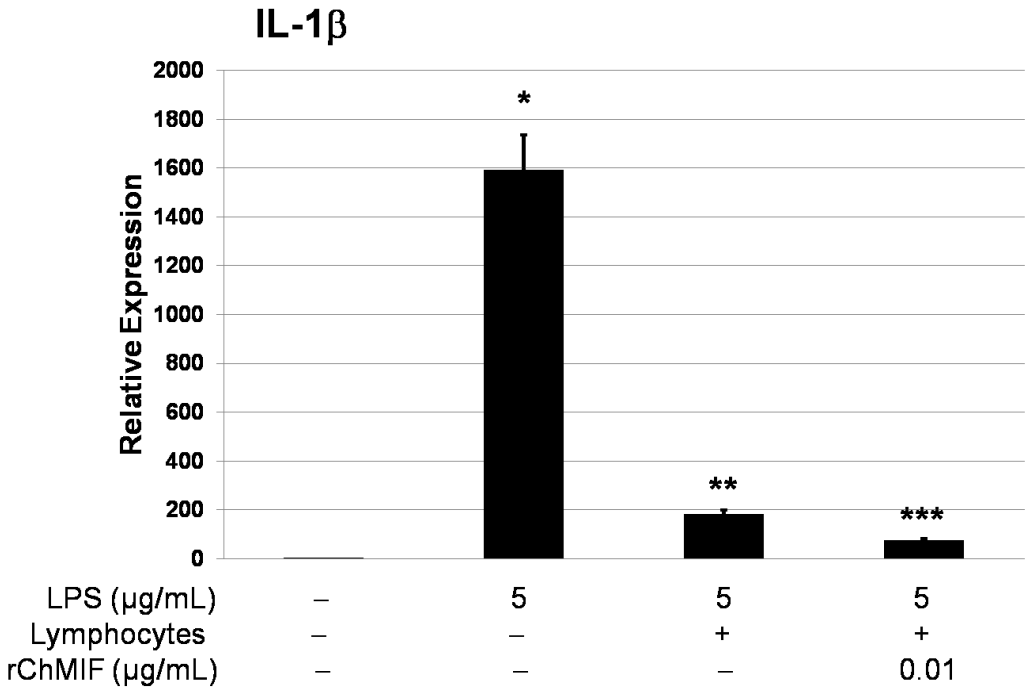

IL-4
Relative Expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LPS (μg/mL) – 5 5 5
rChMIF (μg/mL) – – 0.01 0.1

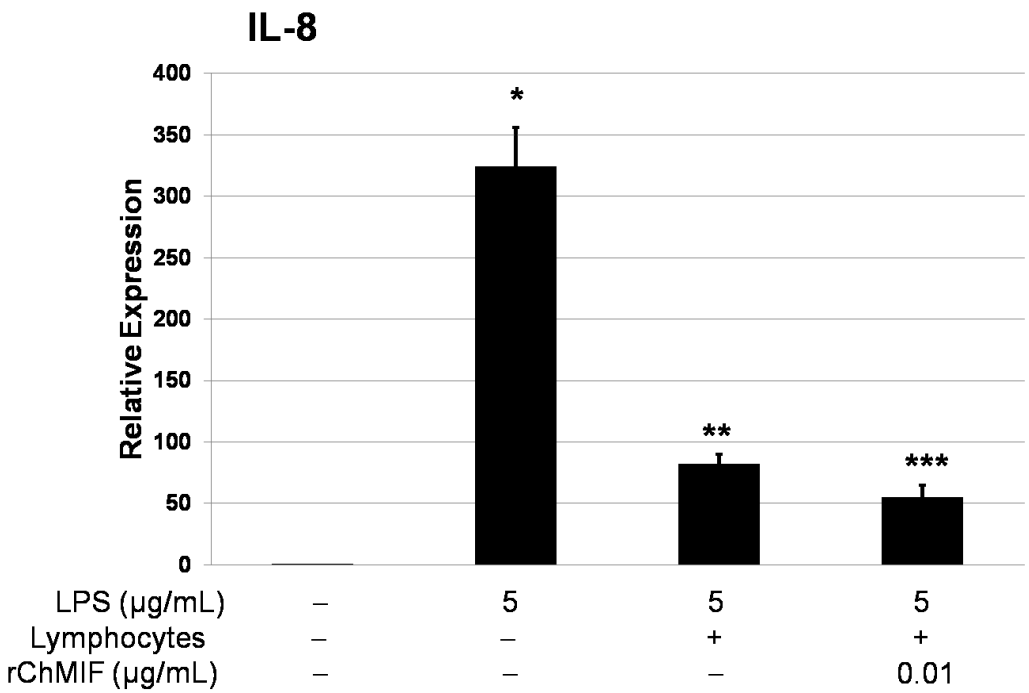
**Figure 4.8. The biological effect of rChMIF on PBMCs.** Isolated PBMCs were treated with media alone, LPS (5 μg/mL) or LPS with rChMIF (0.01 or 0.1 μg/mL) for 6 hr. The expression of pro-inflammatory cytokines were measured by qRT-PCR and compared to control media alone. Each bar represents the mean of 3 different experiments. The different number of asterisks (*) indicates statistically significant difference ($p < 0.05$).

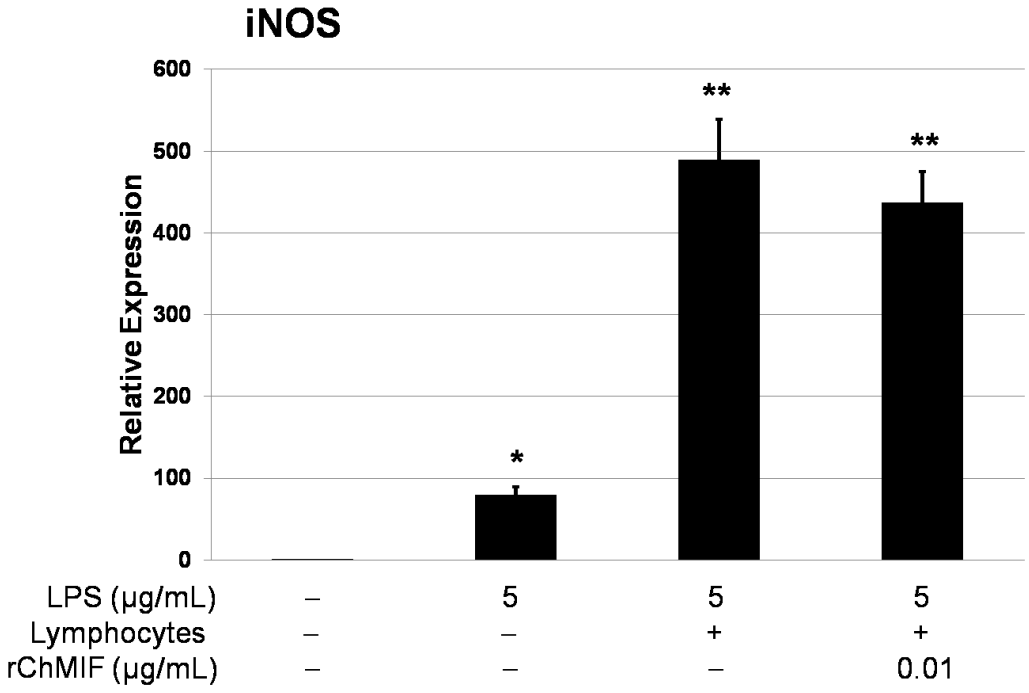

IFN-γ
Relative Expression
80
70
60
50
40
30
20
10
0
*
**
***
LPS (μg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (μg/mL) – – – 0.01

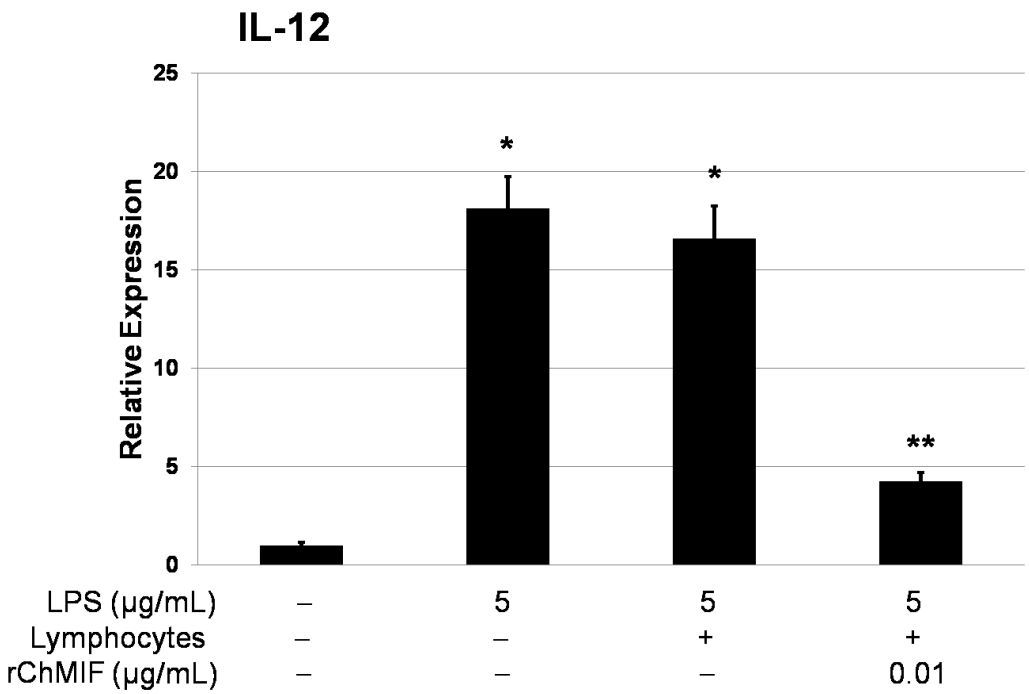

IL-10
Relative Expression
70
60
50
40
30
20
10
0
*
*
**
LPS (μg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (μg/mL) – – – 0.01


IL-4
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
LPS (µg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (µg/mL) – – – 0.01


IL-1β
Relative Expression
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
**
***
LPS (µg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (µg/mL) – – – 0.01


IL-8
Relative Expression
400
350
300
250
200
150
100
50
0
*
**
***
LPS (µg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (µg/mL) – – – 0.01


iNOS
Relative Expression
600
500
400
300
200
100
0
*
**
**
LPS (µg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (µg/mL) – – – 0.01


IL-12
Relative Expression
25
20
15
10
5
0
*
*
**
LPS (µg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (µg/mL) – – – 0.01

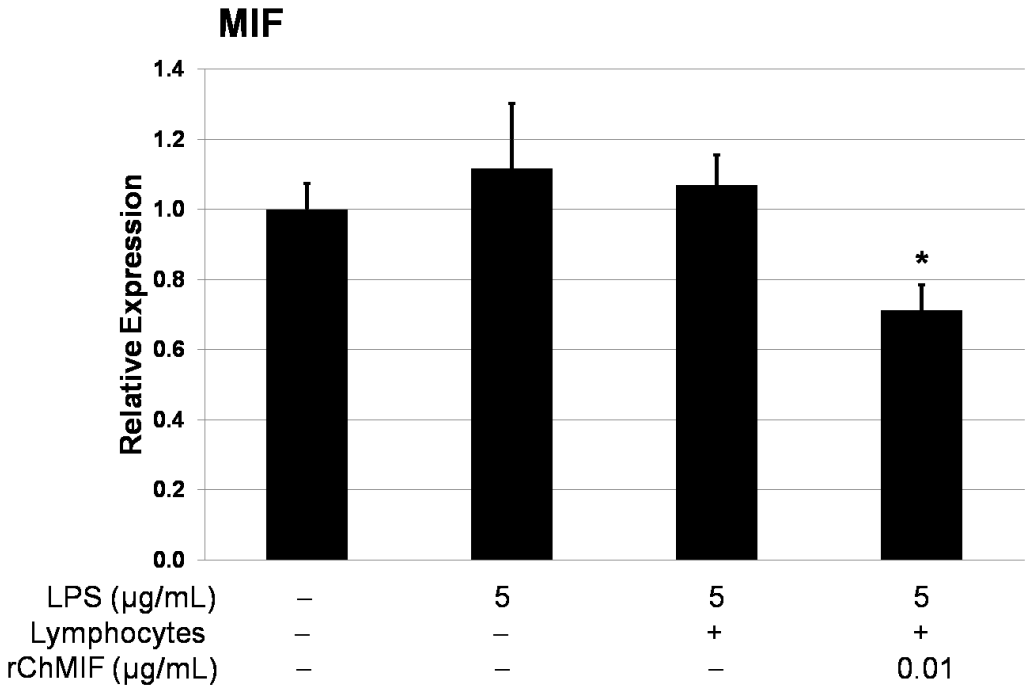

MIF
Relative Expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
LPS (µg/mL) – 5 5 5
Lymphocytes – – + +
rChMIF (µg/mL) – – – 0.01

**Figure 4.9. The effect of rChMIF on the stimulated PBMCs and lymphocytes.** In a 24-well plate, isolated PBMCs (1 x $10^5$ cells/well) were seeded and incubated for 24 hr at 39°C with 5% $CO_2$. After the cells were gently washed, media alone, LPS (5 μg/mL), isolated lymphocytes (4 x $10^6$ cells/well) and rChMIF (0.01 μg/mL) were added into each well at 2-h intervals. The transcripts of various cytokines were measured by qRT-PCR and compared to control media alone. Each bar represents the mean of 2 different experiments. The different number of asterisks (*) indicates statistically significant difference ($p < 0.05$).

# CHAPTER V

## Functional Characterization of the Avian Inflammatory Regulator, Interleukin-22

**ABSTRACT:** A member of the interleukin (IL)-10 family, IL-22 is an important effector of activated Th1 and Th17 as well as natural killer cells during inflammatory responses. Interestingly, recent studies show little involvement of mammalian IL-22 in communication between immune cells. Rather, it mainly acts on epithelial cells, where it functions in antimicrobial defense, regeneration and protection against damage, as well as inducing production of acute phase reactants and chemokines. This study reports the cloning of recombinant chicken IL-22 (rChIL-22) and characterization of its biological effects during stimulation of immune response. Purified rChIL-22 had no effect on either chicken PBMCs or lymphocytes similar to mammals due to the lack of expression of ChIL22RA1. rChIL-22 alone did not affect chicken embryo kidney epithelial cells (CKECs); however, co-stimulation of CKECs with LPS and rChIL-22 enhanced the production of pro-inflammatory cytokines and anti-microbial peptides. Furthermore, rChIL-22 alone stimulated and induced acute phase reactants in chicken embryo liver cells (CKELs). These effects of rChIL-22 were abolished by addition of rChIL22BP. Together, this study indicates an important role of ChIL-22 on epithelial cells and hepatocytes during inflammation.

## Introduction

Interleukin (IL)-22 was initially identified as IL-10-related T cell-derived inducible factor (IL-TIF) (Dumoutier et al., 2000a) and a novel member of the IL-10 family, which is composed of IL-10, IL-19, IL-20, IL-24 and IL-26 (Conti et al., 2003). Human IL-22 consists of 179 amino acids containing a signal peptide at its N-terminus and shows 79% amino acid identity with

murine IL-22, and 25% and 22% identity with human and mouse IL-10, respectively (Dumoutier et al., 2000b).

IL-22 is recognized by a heterodimeric receptor complex consisting of two chains of class II cytokine receptor family, IL22R1 (IL22RA1) and IL10R2 (IL10RB) (Dumoutier et al., 2000b; Xie et al., 2000). While IL10R2 is a common subunit for the IL-10 and IL-22 receptor complexes, IL22R1 is a specific subunit for IL-22 (Xie et al., 2000; Kotenkot et al., 2001a). Binding of IL-22 to its receptor complex induces a cascade of downstream phosphorylation signals, including several MAPK pathway molecules (ERK1/2, MEK1/2, JNK, and p38 kinase), and STAT1, STAT3 and STAT5 via Jak1 and Tyk2 (Lejeune et al., 2002). IL-22 binding protein (IL22BP or IL22RA2) is another soluble receptor for IL-22 (Dumoutier et al., 2001b; Kotenko et al., 2001b), that prevents its binding to the functional cell surface IL-22 receptor complex. IL-22BP has a higher affinity for IL-22 than the membrane-bound IL-22 receptor complex (Kotenko et al., 2001b), and is capable of neutralizing IL-22 activity (Dumoutier et al., 2001b; Kotenko et al., 2001b; Xu et al., 2001). IL22BP-Fc fusion protein is not capable of binding to IL-19, IL-20 or IL24 (Dumoutier et al., 2001a) and IL22BP fails to neutralize IL-10 activities, indicating specific interaction of IL22BP with IL-22. Thus, IL22BP is a naturally occurring, highly specific IL-22 antagonist.

IL-22 is mainly produced by two newly discovered lineages of $CD4^+$ T helper (Th) cells; Th17 cells (Liang et al., 2006; Zheng et al., 2007) and Th22 cells (Duhen et al., 2009; Trifari et al., 2009). Th17 cells secrete IL-17A and IL-17F as well as IL-22 (Harrington et al., 2005; Park et al., 2005), whereas Th22 cells only produce IL-22, but not IL-17 (Trifari et al., 2009). They are known to be involved in innate immunity against extracellular pathogens, clearance of microbes that are not completely extirpated by other Th cells subsets, and in the development of

autoimmune disease (Bettelli et al., 2007; Wilson et al., 2007; Duhen et al, 2009). IL-22 can also be produced by γδ T cells (Martin et al., 2009; Simonian et al., 2010), natural killer (NK) cells (Wolk et al., 2002; Cella et al., 2009; Guo and Topham, 2010), lymphoid tissue inducer (LTi) cells (Cupedo et al., 2009) and LTi-like cells (Satoh-Takayama et al., 2008; Takatori et al., 2009). Recent findings revealed that a new subpopulation of mucosal NK-like cells secrete IL-22, particularly in the small intestine (Satoh-Takayama et al., 2008). In addition, a different subset of NK cells, named NK-22 has been shown to be specialized in IL-22 production in mucosal-associated lymphoid tissues in mice and humans, providing mucosal protection (Cella et al., 2009).

Functional studies in a murine model system indicate that IL-22 has immunoregulatory properties during infection (Aujla et al., 2008; Zheng et al., 2008; Munoz et al., 2009; Wilson et al., 2010), inflammation (Brand et al., 2006; Zenewicz et al., 2008; Pickert et al., 2009), autoimmunity (Zheng et al., 2007; Ma et al., 2008) and cancer (Nagakawa et al., 2004; Bard et al., 2008; Zhang et al., 2008). In these models, the functional consequences of IL-22 expression can be either pathogenic or protective, depending on the context in which it is expressed. While IL-22 confers protective immunity by inducing the production of antimicrobial peptides, neutrophil-recruiting chemokines, and acute phase proteins, as well as promotion of maintenance of epithelial barrier in the intestine and respiratory tract during infection (Aujla et al., 2008; Zhang et al., 2008), IL-22 secretion is pathogenic following infection with an intracellular protozoan parasite by promotion of intestinal inflammation and enhanced disease (Munoz et al., 2009; Wilson et al., 2010).

A putative avian IL-22 nucleotide sequence was found during analysis of the chicken genome (Kaiser et al., 2005; Kaiser, 2007). In avians, there are only four members of the IL-10

family; IL-10, IL-19, IL-22, and IL-26. Similar to what is found in humans, chicken IL-22 (ChIL-22) is encoded in the same cluster with IL-26 on chromosome 1 in a region syntenic with human chromosome 12. Although there is no research reporting the biological function of ChIL-22, chicken kidney cells exposed to recombinant ChIL-22 (rChIL-22) showed induced IL-10 expression. In addition, rChIL-22 upregulates the expression of β-defensins in heterophils (unpublished observations by Kaiser et al.).

In this report, we describe the sequences of the avian IL-22 and its soluble receptor molecule, ChIL22BP. Additionally, in order to gain a better insight into the biological function of avian IL-22, rChIL-22 and rChIL22BP were produced in *E. coli* and their bioactivities were studied.

## Materials and Methods

*Birds*

Twenty-week old healthy broiler chickens were donated by Dr. Paul Siegel (Virginia Tech); birds had been housed and reared according to the Institutional Animal Care and Use Committee of Virginia Tech. Various tissues were collected including thymus, spleen, bursa, brain, lung, heart, liver, crop, stomach and small intestinal sections.

*Cells*

Chicken embryo fibroblast cell line, DF-1, and chicken macrophage transformed cell line, HTC, were maintained in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, VA) supplemented with 2 mM $_{L}$-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL Amphotericin B, and 10% fetal calf serum (FCS) (all supplied by Atlanta Biologicals,

GA). Peripheral blood mononuclear cells (PBMCs) and splenocytes were isolated as described in Kim et al. (2010).

Chicken embryo kidney cells (CEKCs) and chicken embryo liver cells (CELCs) were isolated and cultured as described by Cori (1972). Liver and kidneys were removed from 18-day-old chicken embryos. After collected tissues were washed with calcium/magnesium-free HBSS with antibiotics three times, the cells were dissociated with 0.25% trypsin. The isolated CEKCs and CELCs were resuspended with minimal essential medium (MEM) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL Amphotericin B, and 10% FCS, then seeded into 6-well plates. The cells were gently washed after 24-hour (h) culture and further incubated for 24 h.

*Cloning and expression of rChIL-22 and its binding protein in E. coli*

The full-length ChIL-22 and IL22BP were amplified, cloned and the recombinant proteins expressed from *E. coli* as described previously (Kim et al., 2009). Briefly, a candidate sequence of ChIL-22 was identified from the chicken genome database and NCBI database. Using the submitted ChIL-22 and ChIL22BP sequences (GenBank Accession # NM_001199614 and XM_001233761, respectively), primer sets were designed (Table 5.1). The full-length ChIL-22 and ChIL22BP were amplified from concanavalin A (Con A)-stimulated splenocytes and liver, respectively. The freshly synthesized full-length ChIL22 and ChIL22BP were directly inserted into pCR2.1-TOPO vector (Invitrogen, CA), followed by sub-cloning into either a prokaryotic or an eukaryotic vector using *EcoR* I and *Not* I for ChIL-22 and *BamH* I and *Hind* III (New England Biolabs, MA) for ChIL22BP.

Recombinant ChIL-22 and rChIL22BP were expressed in *E. coli* BL21 cells (New England Biolabs, MA) and purified using Ni+-resin (Bioline, MA) as described previously (Kim et al., 2009). Endotoxin was removed using ProteoSpin Endotoxin Removal Micro Kit (Norgenbiotek, ON, Canada) resulting in levels <0.06 EU/mg protein as indicated by the Limulus Amebocyte Lysate assay (Lonza, MD).

*Transfection and expression of rChIL-22 and rChIL22BP in DF-1 cells*

One day prior to transfection, DF-1 cells were seeded at 4 x $10^5$ cells/well in a 6-well plate, and incubated for 24 h at 40°C and 5% $CO_2$. The cells were gently washed once with DMEM and incubated with serum- and antibiotics-free DMEM for 30 minutes (min). Five micrograms of plasmid DNA were prepared with Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instruction. The prepared DNA mixture was added and cells were incubated overnight. The medium was changed and the cells were cultured for an additional 48 h. The supernatant was collected and concentrated using 10,000 MW cutoff membrane. The cells were directly treated with lysis buffer for isolation of total RNA.

*Cell proliferation assay*

The cell proliferation assay was performed using CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega, WI). The isolated PBMCs and lymphocytes were cultured at 2 x $10^4$ cells/mL and 6 x $10^4$ cells/mL in a 96-well plate, respectively, and then treated with media alone, rChIL-22, rChIL22BP and pre-incubated rChIL-22 with rChIL22BP (0.01 μg/mL of each) in the presence or absence of LPS (5 μg/mL) for PBMCs or Con A (5 μg/mL) for lymphocytes. The Dye Solution (15 μL) was directly added into each well, followed by 3h

incubation at 39°C with 5% CO2. Then, 100 μL of the Solubiliztion Solution/Stop Mix were added and mixed with a multi-channel pipette. After 1 h incubation at 39°C, the absorbance was measured at 595 nm using a microplate reader. Background value was measured at 630 nm, subtracted, and the results were analyzed in Excel (Microsoft Corp, WA).

*In vitro cell stimulation for biological function analysis*

The isolated PBMCs, splenocytes, CEKCs and CELCs were seeded onto either 12-well plates or 6-well plates, and cultured for 24 h for PBMCs and splenocytes and for 48 h for CEKCs and CELCs. After the cells were gently washed, the cells were treated with medium alone, rChIL-22, rChIL22BP, or rChIL-22 with rChIL22BP (0.01 μg/mL of each) in the presence or absence of Con A for splenocytes or LPS for PBMCs, CEKCs, and CELCs for 6 h. To inhibit the function of rChIL-22, it was pre-incubated with rChIL22BP before the mixture was added to the cells. Cell lysis buffer was directly added into the wells and total RNA was extracted using RNeasy Mini Kit (Qiagen, CA).

*Quantitative real-time PCR analysis of the cytokine transcripts*

To analyze the transcripts of various cytokines, primers were designed using Primer Express (Ver 3.0; Applied Biosystems, CA) (Table 5.1), and qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, CA) as described in Kim et al. (2010). Target gene expression in this study was normalized against the expression of chicken GAPDH mRNA, followed by analysis of the results using 7500 Software (Ver 2.0; Applied Biosystems,

CA). However, relative expression of ChIL-22 and its receptors in various tissues and cells was calculated as fold difference using GAPDH as an internal control.

*Statistical analysis*

All data were analyzed by either Student's t-test or Analysis of Variance (ANOVA) using JMP (Ver 8.0) software, and significant differences among groups were tested by the Tukey-Kramer Honestly Significant Difference post-hoc procedure.

## Results

*Sequence analysis of ChIL-22 and ChIL22BP*

While pursuing the identification of avian IL-10 and IL-19 (Kim et al., 2009), we also identified a putative avian IL-22 that bears similarity to avian IL-10 as well as human IL-22 using bioinformatics. ChIL-22 is located on Chromosome 1 along with IFN-γ and IL-26 (Figure 5.1A). The sequence analysis reveals ChIL-22 consists of 197 amino acids (Figure 5.2A) that are 35% similar to chicken IL-10 (Figure 5.3A), and 39% and 37% identical to human and mouse IL-22, respectively (Figure 5.3B). Among avian species including chicken, turkey, duck and zebra finch, ChIL-22 showed over 75% identity. The first 28 amino acids are predicted as a signal sequence, resulting in serine as the first amino acid of mature form of ChIL-22 (Figure 5.2A). There is only one *N*-linked glycosylation site predicted in ChIL-22 at the 60th amino acid residue (NRTY) (Figure 5.2A).

Chicken IL-22 binding protein, encoded in the same cluster with interferon gamma receptor 1 on chromosome 3 (Figure 5.1B), consists of 735-bp open reading frame that encodes a 244 amino acid protein of 28 kDa (Figure 5.2B). Sequence analysis revealed ChIL22BP has 2

potential *N*-linked glycosylation sites; 25$^{th}$ (NSTI) and 53$^{rd}$ (NNTL) amino acid residues (Figure 5.2B). Soluble receptor ChIL22BP showed 41% similarity with the extracellular domain of surface receptor ChIL22RA1 (Figure 5.3D). Also, it has 42% and 40% similarity with human and mouse IL22BP, respectively (Figure 5.3C).

*Tissue distribution of ChIL-22 and its soluble receptor ChIL22BP*

Based on sequence analysis, a primer set was designed to measure the expression of ChIL-22 in various tissues. Results of qRT-PCR showed that ChIL-22 showed little or no expression in most tissues tested in this study, except thymus, spleen, and small intestine (Figure 5.4A). The soluble receptor of IL-22, IL22BP, was highly expressed in liver, but not in other tested tissues (Figure 5.4B). Stimulating lymphocytes with Con A did not enhance IL-22 expression as shown in Figure 5.4C. Interestingly, lymphocytes incubated with LPS showed approximately 87% reduction in ChIL-22 transcript expression (Figure 5.4C).

*Effect of ChIL-22 on immune cells*

To determine the biological effect of ChIL-22 on immune cells, the transcripts of soluble mediators expressed in PBMCs and lymphocytes were measured by qRT-PCR. As shown in Figure 5.5A, addition of rChIL-22 to PMBCs culture did not induce significant expression of IL-1β, IFN-γ, and serum amyloid A (SAA). In contrast, LPS induced expression of all three cytokines. Similarly, incubation of lymphocytes with rChIL-22 showed no effect on transcript expression of Th1/Th2 cytokines including IL-2, IFN-γ, IL-6 and IL-13, while Con A led to significant induction of those cytokines (Figure 5.5B). Additionally, incubation of PBMCs or lymphocytes with rChIL-22 did not affect cell proliferation (Figure 5.5C).

To investigate the lack of response of immune cells toward ChIL-22, we measured mRNA level of IL-22 receptors by qRT-PCR. Figure 5.5D showed very little expression of ChIL22RA1 in both PBMCs and lymphocytes, although constant mRNA level of ChIL10RB was observed.

*Effect of ChIL-22 on non-immune cells*

Since rChIL-22 showed no effect on immune cells, we examined whether rChIL-22 affects epithelial cells and hepatocytes using chicken embryo kidney cells (CEKCs) and liver cells (CELCs). Freshly cultured CEKCs or CELCs were incubated with rChIL-22 in the presence or absence of LPS for 6 h. First, using the CEKCs, the effect of different doses of rChIL-22 on epithelial cells was determined by measuring the transcript of different immune genes, ChIL-10, IL-8 and gallinacin 2 (GAL2). The increased dose of rChIL-22 led to the increased expression of ChIL-10 (Figure 5.6A). Moreover, high rChIL-22 dose (1 μg/mL) induced expression of anti-microbial peptides (AMPs), GAL2 and a chemokine, IL-8 (Figure 5.6A). Next, the effect of rChIL-22 on epithelial cells was evaluated in the presence and absence of LPS. rChIL-22 alone did not affect epithelial cells, except it increased expression of ChIL-10. However, co-stimulation of rChIL-22 with LPS enhanced transcripts of pro-inflammatory mediators, IL-1β, IL-6, SAA, IL-8 and GAL2 (Figure 5.6B). This enhancement by rChIL-22 was abolished by pre-incubation of rchIL-22 with rChIL22BP (Figure 5.6B).

In comparison with epithelial cells, incubation of hepatocytes with rChIL-22 alone affected and induced acute phase reactants, including IL-1β, IL-8, Leap2 and SAA (Figure 5.7). Besides, co-stimulation of rChIL-22 with LPS enhanced the same transcripts. Similar with epithelial cells, pre-incubation of rChIL-22 with rChIL22BP abolished the effect of rChIL-22

(Figure 5.7). Since ChIL22BP was highly expressed in liver, the transcripts of ChIL-22 and ChIL22BP were measured in resting and activated epithelial cells and hepatocytes. While induction of ChIL-22 mRNA level was only observed in activated hepatocytes, ChIL22BP transcripts were induced by rChIL-22 and enhanced by co-stimulation with LPS (Figure 5.8). The transcripts of both ChIL-22 and ChIL22BP did not show any differences on epithelial cells.

## Discussion

The aim of this study was to characterize the biological role of the recently discovered cytokine IL-22 in the avian immune system. Analysis of the chicken genome showed there are 4 members of the IL-10 family on 2 clusters of the chicken chromosomes; IL-10 and IL-19 are syntenic on chromosome 26, and IL-22 and IL-26 are syntenic with IFN-γ on chromosome 1. By comparison, humans have 6 members of IL-10 family with IL-20 and IL-24, and the mouse has five members, lacking IL-26. Phylogenetic analysis of IL-10 family shows that ChIL-22 is evolutionarily closer to human and mouse IL-22, and is closely located in the IL-26 group. Sequence analysis revealed the presence of a pro-ChIL-22 which includes 5 exons and is composed of 197 amino acids. First 28 amino acids were predicted as signal sequence, indicating the cleavage site between $28^{th}$ and $29^{th}$ amino acid residues, resulting in a 169 amino acid mature form of ChIL-22. In comparison with mouse and human, which have 3 *N*-linked glycosylation sites, chicken and other avian IL-22 contain only one *N*-linked glycosylation site. Although there is one glycosylation site on ChIL-22, non-glycosylated rChIL-22 produced from *E. coli* functioned as same as one produced from eukaryotic system, like rChIL-19.

ChIL-22 is recognized by two different receptors; surface receptor complex of ChIL10R2 (GenBank Accession #: NM_204857) and ChIL22RA1 (GenBank Accession #: XM_417840) –

though ChIL22RA1 is only predicted, not identified, and soluble receptor ChIL22BP. In mammals, IL22RA1 is a specific for IL-22 (Kotenkot et al., 2001a; Xie et al., 2000). Similar to mammals, ChIL-22 is not recognized and does not activate target cells in the absence of ChIL22RA1 expression, even though ChIL10R2 is expressed constitutively. Chicken PBMCs and lymphocytes showed constant mRNA level of ChIL10R2, but lack of expression of ChIL22RA1, resulting in lack of biological effect of ChIL-22. These results suggest that ChIL22RA1 is specific chain of receptor complex for ChIL-22 and is required for ChIL-22 signal transduction. Additionally, absence of ChIL22RA1 expression explains the insensitivity of immune cells toward ChIL-22. The other receptor, IL22BP is a soluble receptor that prevents binding of IL-22 to the functional cell surface IL-22 receptor complex, and neutralizes activity of IL-22 (Dumoutier et al., 2001b; Kotenko et al., 2001b; Xu et al., 2001). Chicken IL22BP resides in the same cluster with IFNGR1 and IL20RA on chromosome 3, which is syntenic with human IL22BP on chromosome 6. Sequence analysis revealed ChIL22BP, consisting of 244-amino acid peptide, contains 2 potential *N*-linked glycosylation sites, whereas human and mice have five. Though we could not see the clear protein-protein interaction between ChIL-22 and ChIL22BP, the activity of ChIL-22 on epithelial cells and hepatocytes was abolished by pre-incubation of ChIL-22 with ChIL22BP, indicating ChIL22BP as a highly specific natural antagonist that is capable of neutralizing ChIL-22 activity.

IL-22 is mainly produced by Th17 cells (Liang et al., 2006; Zheng et al., 2007) and Th22 cells (Duhen et al., 2009; Trifari et al., 2009) in mammals. It is also expressed by NK cells (Wolk et al., 2002; Cella et al., 2009), LTi cells (Cupedo et al., 2009), LTi-like cells (Satoh-Takayama et al., 2008; Takatori et al., 2009) and NK-like cells (Satoh-Takayama et al., 2008). Lymphoid tissue induce (LTi) cells and NKp46 were demonstrated to be the major innate

sources of IL-22, especially in the intestine (Cella et al., 2009; Satoh-Takayama et al., 2008; Takatori et al., 2009). Additionally, $NKp44^{+}$ cells, which are predominantly located in the mucosa surrounding the lymphoid follicles, are a major innate source of IL-22 contributing to mucosal homeostasis and inflammation (Cella et al., 2009). Together, IL-22 has an important role as an innate immune mediator in various epithelial cells, including skin, colon, and lung epithelial cells. Due to the limited commercial availability of studying avian Th cells subsets, we could not identify major cellular sources of IL-22. However, tissue distribution revealed that high mRNA level of ChIL-22 in the thymus and spleen, which are the main source of T cells; however, it is also mainly expressed in jejunum and ileum. Additionally, specific receptor chain of ChIL-22, ChIL22RA1 is mainly expressed in small intestinal section, especially jejunum and ileum, implicating an important role of ChIL-22 in intestinal epithelial cells.

IL-22 not only shares structural homology with IL-10, but also uses the IL10R2 chain as one component in its receptor complex. Moreover, both IL-10 and IL-22 activate STAT3, turning on strong negative feedback loops by enhancing the expression of SOCS3 (O'Shea and Murray, 2008). Despite its relation to ChIL-10, which represents one of the most important interleukins, ChIL-22 does not appear to affect immune cells *in vitro*, mainly because ChIL22RA1 is not expressed by immune cells. This conclusion is based on the following observations. First, neither resting nor stimulated chicken PBMCs nor lymphocytes showed expression of IL222RA1. In contrast, the other chain of IL-22 receptor complex, IL10RB was highly expressed and regulated in these cells. The "isolated" expression of IL10RB is not surprising since this molecule functions as an accessory chain also for the receptor complexes for IL-10 and IL-26 (Langer et al., 2004). Second, rChIL-22 did not have any influence on any of the tested immune cells populations in vitro with respect to the release of cytokines and SAA, the

expression of variety of surface proteins and the GAL2 mRNA expression. An alternative hypothesis is that ChIL-22, as in mammal, exerts its inflammatory/anti-inflammatory functions on non-immune cells, such as epithelial cells and hepatocytes. This hypothesis was supported by observation of relatively high expression of IL22RA1 in a range of chicken tissues including skin and tissues of the digestive and the respiratory system. In this study, we used CEKCs, which are the best available chicken epithelial cell model and CELCs, which are hepatocytes. Both CEKCs and CELCs showed significant expression of ChIL22RA1 as well as ChIL10RB. The observed biological role of ChIL-22 in CEKCs and CELCs implicate following important avian aspect. First, relatively high expression of ChIL22RA1 indicates potential ChIL-22 signaling pathway. This signaling pathway was capable of inducing of pro-inflammatory cytokines and AMPs, which implicates the involvement of ChIL-22 in local inflammation. Co-stimulation of rChIL-22 with LPS enhanced pro-inflammatory cytokines and AMPs, suggesting positive feed-back effect of ChIL-22 in avian immune system. Incubation of rChIL-22 led to induction of acute phase proteins and their expression was increased during co-stimulation of rChIL-22 with LPS, implicating the major role of ChIL-22 during inflammation and tissue injury.

In summary, following the successful cloning and expression of ChIL-22 and ChIL22BP, the molecular function of the first described avian IL-22 is demonstrated. Our data indicate that avian IL-22 is another member of the IL-10 family and induces pro-inflammatory and AMPs in epithelial cells and hepatocytes, but not immune related cells. Characterization of ChIL-22 provides additional insight into the function of the avian immune system.

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**Table 5.1. Primer sequences for cloning ChIL-22 and ChIL2BP, and qRT-PCR analyses of cytokine transcript expressions.**

| Primer Name | Accession No. | Sense sequence | Anti-sense sequence |
|---|---|---|---|
| Cloning | | | |
| IL-22 | XM_416079 | gatcgaattcATGGCCACCCTGCACACCCT | gatcgcggccgcTCACAGATCCTCTTCTGAGATGAGTT TTTGTTCGTTTTTCTTCTTGTTCCCTCCCTT |
| IL22BP | XM_001233761 | gatcatatgATGGGAGGAGCTGCCGCCAGC | actgctagcTCACAGCAGCACGGTGCACTG |
| qRT-PCR | | | |
| GAPDH | NM_204305 | AGGGTGGTGCTAAGCGTGTTA | TCTCATGGTTGACACCCATCA |
| GAL2 | NM_204992 | GTCCCAGCCATCTAATCAAAGTC | AGGCCATTTGCAGCAGGAA |
| IFN-γ | NM_205149 | GCTCCCGATGAACGACTTGA | TGTAAGATGCTGAAGAGTTCATTCG |
| IL-1β | NM_204524 | CCCGCCTTCCGCTACA | CACGAAGCACTTCTGGTTGATG |
| IL-2 | NM_204153 | CGAGCTCTACACACCAACTGAGA | CCAGGTAACACTGCAGAGTTTGC |
| IL-6 | NM_204628 | GAACGTCGAGTCTCTGTGCTAC | CACCATCTGCCGGATCGT |
| IL-8 | NM_205498 | TCCTGGTTTCAGCTGCTCTGT | CGCAGCTCATTCCCCATCT |
| IL-10 | NM_001004414 | CGCTGTCACCGCTTCTTCA | CGTCTCCTTGATCTGCTTGATG |
| IL-13 | NM_001007085 | CATGACCGACTGCAAGAAGGA | CCGTGCAGGCTCTTCAGACT |
| IL-22 | XM_416079 | TGTTGTTGCTGTTTCCCTCTTC | CACCCCTGTCCCTTTTGGA |
| IL22BP | XM_001233761 | TGCGACCTGACAGAGGAGACT | AGCTTGCACCCTGCCATAGT |
| IL10RB | NM_204857 | CACCCAGTGTGAATGTGAAGTCT | AGCCGGGCCTGTGAAGT |
| IL22RA1 | Xm_417840 | CTCAGACCTCCGAGCAAAGC | GTGGTCTATGCCATCGACACA |
| Leap2 | AY534899 | TGTGCTTCCCTGCACCAA | GGCGTCATCCGCTTCAGT |
| SAA | GU929209 | GACATGCGTGAGGCAAACTACA | GTAATTGCCACGAGCATGGA |

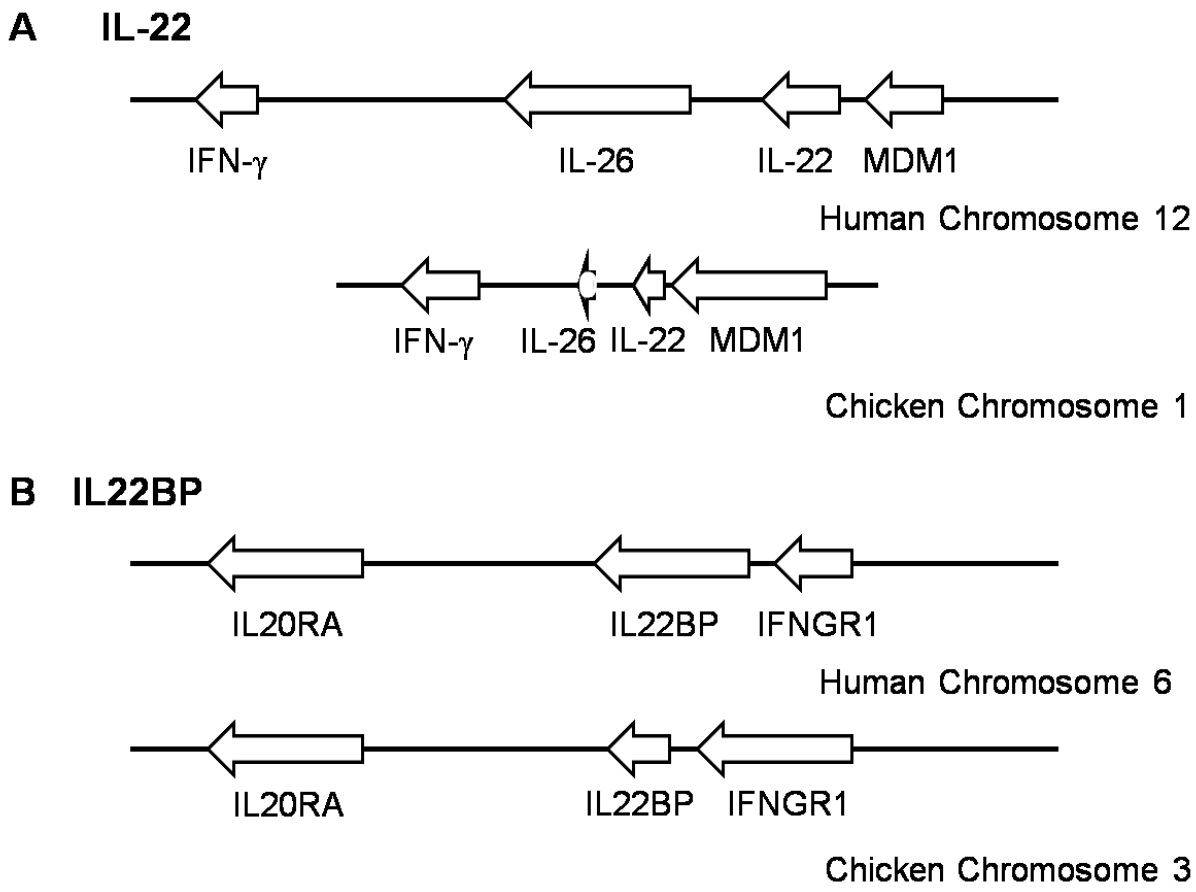


**Figure 5.1. Schematic drawings comparing the human and chicken IL-22 and IL22BP. A**. Similar synteny with human, ChIL-22 is located on chromosome 1 along with IFN-γ and IL-26. **B**. ChIL22BP is syntenic with IL20RA and IFNGR1 on chicken chromosome 3, which is similar synteny with human chromosome 6.

A

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001  ATGGCCACCCTGCACACCCTGACCAGGAGCTTCTCAGGATGGGTTGTCTTCTGCTGTTGT
001   M  A  T  L  H  T  L  T  R  S  F  S  G  W  V  V  F  C  C  C
061  TGCTGTTTCCCTCTTCTTCTCACCAGCCCCCTACCTCCAAAAGGGACAGGGGTGGTTTCC
021   C  C  F  P  L  L  L  T  S  P  L  P  P  K  G  T  G  V  V  S
121  AATGCCCATCAAGCCTGCAGGCTCAGGAAGATCAACTTCCAGCAGCCCTACATCAGGAAT
041   N  A  H  Q  A  C  R  L  R  K  I  N  F  Q  Q  P  Y  I  R  N
181  CGCACCTACACCTTGGCTGAAATGGCCAGGCTCTCAGATCAGGACACTGACAACAGACTC
061   R  T  Y  T  L  A  E  M  A  R  L  S  D  Q  D  T  D  N  R  L
241  ATCGGTCAGCAAATCTACGTCAACATCAGGGAGAACAACCGCTGCTACATGATGAAGAGA
081   I  G  Q  Q  I  Y  V  N  I  R  E  N  N  R  C  Y  M  M  K  R
301  ATTACAGAGATTATAGTGAAGGACATCCTTCTCACAGAGGCCAAGGAGAGGTACCCGTAT
101   I  T  E  I  I  V  K  D  I  L  L  T  E  A  K  E  R  Y  P  Y
361  GCTGAGGATGTGGCACGGTTCCTGGCATCCCTGACCTCGGAGCTGAGCAGATGTAAATAC
121   A  E  D  V  A  R  F  L  A  S  L  T  S  E  L  S  R  C  K  Y
421  TCAGGAAACAGAGAACATATTGAAAAGAACCTGGAAGAGATGAAGAGCAAAATGAAAGAG
141   S  G  N  R  E  H  I  E  K  N  L  E  E  M  K  S  K  M  K  E
481  TTGGGAGAGAATGGGAAGAACAAAGCCATCGGAGAGCTGGATTTACTGTTTGACTACATA
161   L  G  E  N  G  K  N  K  A  I  G  E  L  D  L  L  F  D  Y  I
541  GAGAATGCTTGTACTGATGCCCCAAAGAAGGGAGGGAACAAGAAGAAAAACTGA
181   E  N  A  C  T  D  A  P  K  K  G  G  N  K  K  K  N  *
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B

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001  ATGGGAGGAGCTGCCGCCAGCAGTGATTCTGGCAAAGCTCCACACTTGGGTCCTGCTAAG
001   M  G  G  A  A  A  S  S  D  S  G  K  A  P  H  L  G  P  A  K
061  GTGTGTTGGTACAATTCAACCATTTTAGTCCTGGAAAATCAAGACCTGCGAGATGCAATC
021   V  C  W  Y  N  S  T  I  L  V  L  E  N  Q  D  L  R  D  A  I
121  AAGCCACAGGAGGTACGGTTTTACTCACTGAACTTCAACAACACCCTGCGCTGGCAGCCT
041   K  P  Q  E  V  R  F  Y  S  L  N  F  N  N  T  L  R  W  Q  P
181  GGGAGGGCTGGAGAGGGAGAAACCACGCTCTACTTTGTGCAGTATAAAGTGTATGGGCAG
061   G  R  A  G  E  G  E  T  T  L  Y  F  V  Q  Y  K  V  Y  G  Q
241  AGCAAGTGGCATAACAAAGAAGAGTGCTGGGGGATTCAGAGCCTTTTCTGCGACCTGACA
081   S  K  W  H  N  K  E  E  C  W  G  I  Q  S  L  F  C  D  L  T
301  GAGGAGACTTCTGACGCCTATGAGCCCTACTATGGCAGGGTGCAAGCTGCTTCGGACGGT
101   E  E  T  S  D  A  Y  E  P  Y  Y  G  R  V  Q  A  A  S  D  G
361  GTCCACTCCAACTGGAGCCTCAGCTCCAGATTCACTCCCTGGCGAGAAACTATGATAGGA
121   V  H  S  N  W  S  L  S  S  R  F  T  P  W  R  E  T  M  I  G
421  CCTCCAACAATAAAGGTGCTTCACAGCAACAAGTTCATAATACTAAAGCTCCAGGCTCCA
141   P  P  T  I  K  V  L  H  S  N  K  F  I  I  L  K  L  Q  A  P
481  CGTTCAGCATATAAAAGGAAGAGAGGCAGCATGATACCAATGACAAATTATTACGATCTT
161   R  S  A  Y  K  R  K  R  G  S  M  I  P  M  T  N  Y  Y  D  L
541  CTGTACCAAGTCTTCATAATTAACAACTTGCTAGATGAGCAACACAGAGTGCTGGTGTAT
181   L  Y  Q  V  F  I  I  N  N  L  L  D  E  Q  H  R  V  L  V  Y
601  GAAGGAAAAGACAAGGTGATCAAAATAGAAGATCTGAGGCCGGGAGTCAGCTACTGCATT
201   E  G  K  D  K  V  I  K  I  E  D  L  R  P  G  V  S  Y  C  I
661  GTGGCTAGAACATCAGTGCTGGTGCTGGGCCGCAGCAGTGCCTACAGCAGCAGGCAGTGC
221   V  A  R  T  S  V  L  V  L  G  R  S  S  A  Y  S  S  R  Q  C
721  ACCGTGCTGCTGTGA
241   T  V  L  L  *
```

**Figure 5.2. Nucleotide and deduced amino acid sequences of ChIL-22 (A) and ChIL22BP (B)**. The primers used to amplify the full-length ChIL-22 and ChIL22BP are single underlined,

and double underlined sequences were used as primers for qRT-PCR. The predicted signal sequences are underlined under amino acid sequences, and "∇" marked site indicates cleavage site for the mature form. The boxed amino acid sequence represents potential *N*-linked glycosylation sites.

**A**

```
ChIL-10         -----MQTCCQALLLLLAACTLPAHCLEPTCLHFS-ELLPARLRELRVKFEEIKD-YFQS 53
ChIL-19         ----MLGSRVLLCLCSMTCCLTMLPAAGNTILHFGPCRISMSMSEIRAGFTAIKT-NIQA 55
ChIL-22         MATLHTLTRSFSGWVVFCCCCCFPLLLTSPLPPKGTGVVSNAHQACRLRKINFQQPYIRN 60
ChIL-26         -MKVYSIFRSGHLLVLLCLFTVEGKKSPTGKHTCRKGLLSQVTENLYTKASSLKS----S 55
                                :                       :.            ::
ChIL-10         RD-DELNIQLLSS-ELLDEFKGTFGCQSVSEMLRFYTDEVLPRAMQTSTSHQQSMG---- 107
ChIL-19         RD-PIRTLSILSHPHSLHRVQPSDKCCIVHKVFNFYVDKVFKHCQTENSYINRKIS---- 110
ChIL-22         RTYTLAEMARLSDQDTDNRLIGQQIYVNIRENNRCYMMKRITEIIVKDVLLTEAKERYPY 120
ChIL-26         VPKDLIKNTRLLKKTTKMLFMTN--CNVRDQLLSFYMKNVFSHLGMESEKLFVISA---- 109
                          *        .          :    *  : : .    .
ChIL-10         --DLGNMLLGLKATMRRCH---RFFTCEKRSKAIKQIKETFEKMDEN-GIYKAMGEFDIF 161
ChIL-19         --SIANSFLSIKRKLEQCHDENKCLCGQEPTERFKQILVNYEGLNVTSAAMKSLGELDIL 168
ChIL-22         AEDVAQFLASLTSELSRCK---YSGNREHIEKNLEEMKSKMKELGEN-GKNKAIGELDLL 176
ChIL-26         -------FRVLQENMNACLP---CAPSTRLTSAVKNIKKTFLKLGEK-GVYKAINELDIL 158
                      :  :   :  *          .  . .:::  .   :. . .  *::.*:*::
ChIL-10         INYIEEYLLMRRRK------- 175
ChIL-19         LDWMEKSP------------- 176
ChIL-22         FDYIENACTDAPKKGGNKKKN 197
ChIL-26         LPWIQAYIQTIV--------- 170
                : :::
```

**B**

```
tkIL-22          ------------MATLHTMTRSFSVWVIFCCCCC-------LPLLLTSPLPPKGTGMVSS 41
chIL-22          ------------MATLHTLTRSFSGWVVFCCCCC-------FPLLLTSPLPPKGTGVVSN 41
zfIL-22          ------------MASLHTLSKGFPGWVFFCCCCC-------LPLLLTSSLPLKG---AST 38
hIL-22           ------------MAALQKSVSSFLMGTLATSC-----------LLLLALLVQGGA--AAP 35
mIL-22           ------------MAVLQKSMSFSLMGTLAASC-----------LLLIALWAQEAN--ALP 35
zIL-22           MGDYAKGEKTTTVTYRHDIKAPEPQDALQVSTSRNNGVHKRTDTRIHSSTCDMKCFTLIA 60
                             ::  :         ..  .               : :
tkIL-22          AHQACRLRKINFQRPYIRNRTY---TLAEMV--SDQDTDNRFIGQQIYVNIRENNRCYMM 96
chIL-22          AHQACRLRKINFQQPYIRNRTY---TLAEMARLSDQDTDNRLIGQQIYVNIRENNRCYMM 98
zfIL-22          AHHACRLRKINFQQPYIRNRTY---TLAKTASASDTDTDNRLIGQQLFVNIRENNRCYMM 95
hIL-22           ISSHCRLDKSNFQQPYITNRTF---MLAKEASLADNNTDVRLIGEKLFHGVSMSERCYLM 92
mIL-22           VNTRCKLEVSNFQQPYIVNRTF---MLAKEASLADNNTDVRLIGEKLFRGVSAKDQCYLM 92
zIL-22           LLCSCFLSGCARPTPLDSSATWNDLAAMTDTARNEDDHETRLLPYFSHDMLQEEGSCCIN 120
                     * *       *   . *:         .   : : : *::    .  :  .  * :
tkIL-22          KRITEIILKDVLLTE--AKERYPYAEDVAQFLASLTSELG-RCKYSGNREHIEKNLEEMK 153
chIL-22          KRITEIIVKDVLLTE--AKERYPYAEDVAQFLASLTSELS-RCKYSGNREHIEKNLEEMK 155
zfIL-22          KRVVELVVKDVLLAE--VKNQYPYVEQVAQFLASLTSELS-GCQFLGKRDHIEKNLEQMK 152
hIL-22           KQVLNFTLEEVLFPQ--SDRFQPYMQEVVPFLARLSNRLS-TCHIEGDDLHIQRNVQKLK 149
mIL-22           KQVLNFTLEDVLLPQ--SDRFQPYMQEVVPFLTKLSNQLS-SCHISGDDQNIQKNVRRLK 149
zIL-22           ARILKYYVNHVLESDEHTDMKYPMIRNVREGLHRVEQELQNHCKHDYSSHPLVKQFKRNY 180
                  :: :  ::.** .:    .   *  .:*   *  : ..*   *:    .   : ::...
tkIL-22          SKMKELGENGK-NKAIGELDLLFDYIENACTDAPKKGGNKKKN 195
chIL-22          SKMKELGENGK-NKAIGELDLLFDYIENACTDAPKKGGNKKKN 197
zfIL-22          NKMEQLGENGK-LKAIGELDLLFDYMENACTDAPKKGGNKKKN 194
hIL-22           DTVKKLGESGE-IKAIGELDLLFMSLRNACI------------ 179
mIL-22           ETVKKLGESGE-IKAIGELDLLFMSLRNACV------------ 179
zIL-22           HASAIMDLAAARNKAIGETNTLYHYLFESCTPK---------- 213
                      :.  .   ***** : *:  : ::*
```

**C**

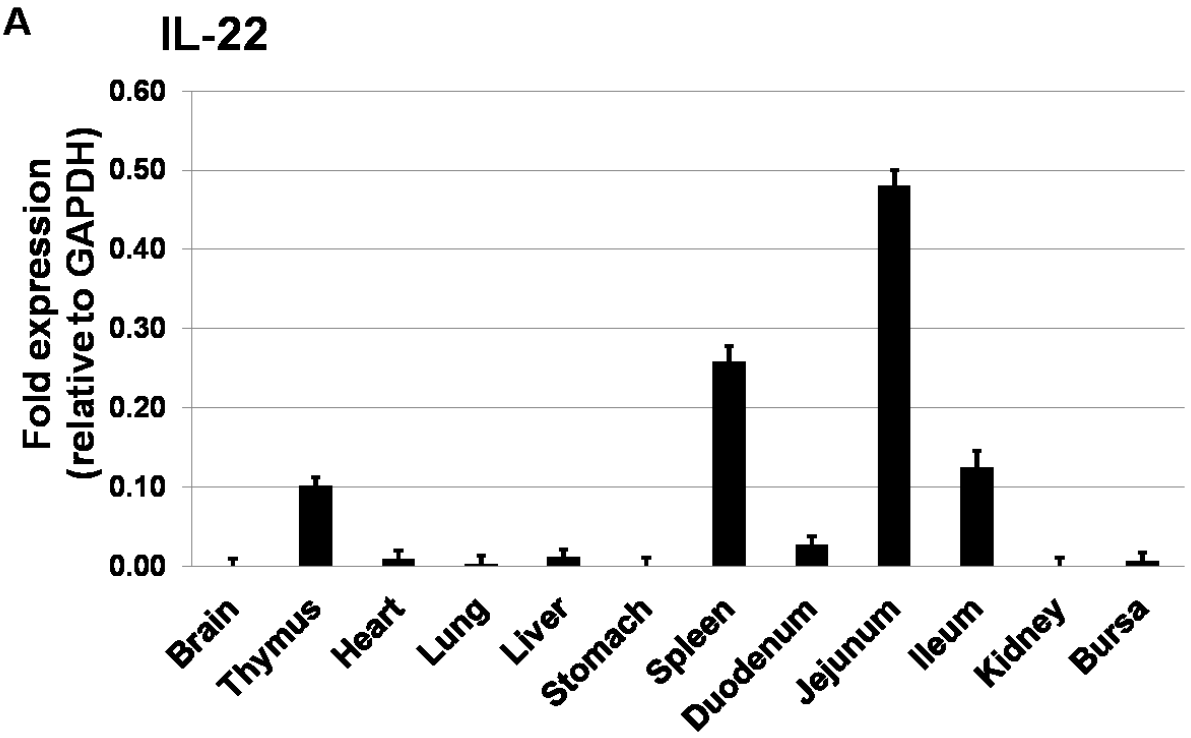
```
hIL22BP         --------MMPKHCFLGFLISFFLTG---VAGTQSTHESLKPQRVQFQSRNFHNILQWQP 49
mIL22BP         --------MMPKHCLLGLLI-ILLSS---ATEIQPARVSLTPQKVRFQSRNFHNILHWQA 48
chIL22BP        MGGAAASSDSGKAPHLGPAKVCWYNSTILVLENQDLRDAIKPQEVRFYSLNFNNTLRWQP 60
tkIL22BP        MGGAVASSDSGRAPYLGPGKVCWYNSTILVLENRDLRDAIKPQGVRFYSLNFNNTLRWLP 60
zfIL22BP        ----------------------------VLENQDLQDSIKPQKVEFHSLNFNTTLHWQP 31
zIL22BP         ----MPFNMLTLTGHQCLDIVFAISLLCWAECSPTQQKEMAPTEVQFQSLDFRNVLHWKH 56
                                                .      :  : *  *.* * :*.. *:*
hIL22BP         GRALTGNSSVYFVQYKIMFSCSMKSSHQKPSGCWQHISCNFPGCRTLAKYGQRQWKNKED 109
mIL22BP         GSSLPSNNSIYFVQYKM-------------------------------YGQSQWEDKVD 76
chIL22BP        GRAGEGETTLYFVQYKV-------------------------------YGQSKWHNKEE 88
tkIL22BP        GRAGEGETTLYFVQYKV-------------------------------YGQSKWQNKEE 88
zfIL22BP        GWAREARDALYFVQYKV-------------------------------YGQSTWQNKDE 59
zIL22BP         QHKATKN-LKYFVQHKI-------------------------------HGDKEWSNSKH 83
                       .   ****:*:                                 :*:  * :. .
hIL22BP         CWGTQELSCDLTSETSDIQEPYYGRVRAASAGSYSEWSMTPRFTPWWETKIDPPVMNITQ 169
mIL22BP         CWGTTALFCDLTNETLDPYELYYGRVMTACAGRHSAWTRTPRFTPWWETKLDPPVVTITR 136
chIL22BP        CWGIQSLFCDLTEETSDAYEPYYGRVQAASDGVHSNWSLSSRFTPWRETMIGPPTIKVLH 148
tkIL22BP        CWGIQSHFCDLTEETSDAYEAYYGRVQAASTDIRSDWSLSCRFTPWRETMIGPPTIKVVH 148
zfIL22BP        CWGISSCVCDLTHETSDIQEPYYSRVRAALAGVYSNWSLSCRFTPWRETMIGPPLVTVAH 119
zIL22BP         CQGIRTLQCDLTQETSDPREWYYARVRSLSPEGFSSWAISHRFYPQWETNFSPPQIKVTV 143
                 * *     **** ** *  * **.** :      * *: : ** *  ** :.** :.:
hIL22BP         VN-GSLLVILHAPNLPYRYQKEKNVSIEDYYELLYRVFIINNSLEKEQK--VYEGAHRAV 226
mIL22BP         VN-ASLRVLLRPPELPNRNQSGKNASMETYYGLVYRVFTINNSLEKEQK--AYEGTQRAV 193
chIL22BP        SN-KFIILKLQAPRSAYKRKRGSMIPMTNYYDLLYQVFIINNLLDEQHRVLVYEGKDKVI 207
tkIL22BP        SN-KFVVLKLRAPRSAYKRKRGSMIPMTNYYDLLYQVFIINNLLDEQHRVLVYEGKDKVI 207
zfIL22BP        SN-KSITVKLQAPRSPYRRKRGSKISMTNYYDLLFQVFIINNLLDEQNRVLVYEGKNKVI 178
zIL22BP         AGGQTIKVQIKPPRTPLRGHNGNRIRVTKLHKLTFRIFLMHNDVEEEVH--ETDSCSKEL 201
                  .   : : ::.*. . : :  .   :   : * :::* ::* :::: :    :.  : :
hIL22BP         EIEALTPHSSYCVVAEIYQPMLDRRSQRSEERCVEIP 263
mIL22BP         EIEGLIPHSSYCVVAEMYQPMFDRRSPRSKERCVHIP 230
chIL22BP        KIEDLRPGVSYCIVARTSVLVLGRSSAYSSRQCTVLL 244
tkIL22BP        KIEDLRPGISYCIVARTSVLVLGRSSAYSSRQCTVLL 244
zfIL22BP        KIEDLRPGVSYCIVAKMYMPMLDHSSAYSSRQCTVL- 214
zIL22BP         VIEALRPKTTYCLQALSVTPRSGRISSRSPRTCITTH 238
                  ** * *  :**: *       .: *  * . *
```

**D**

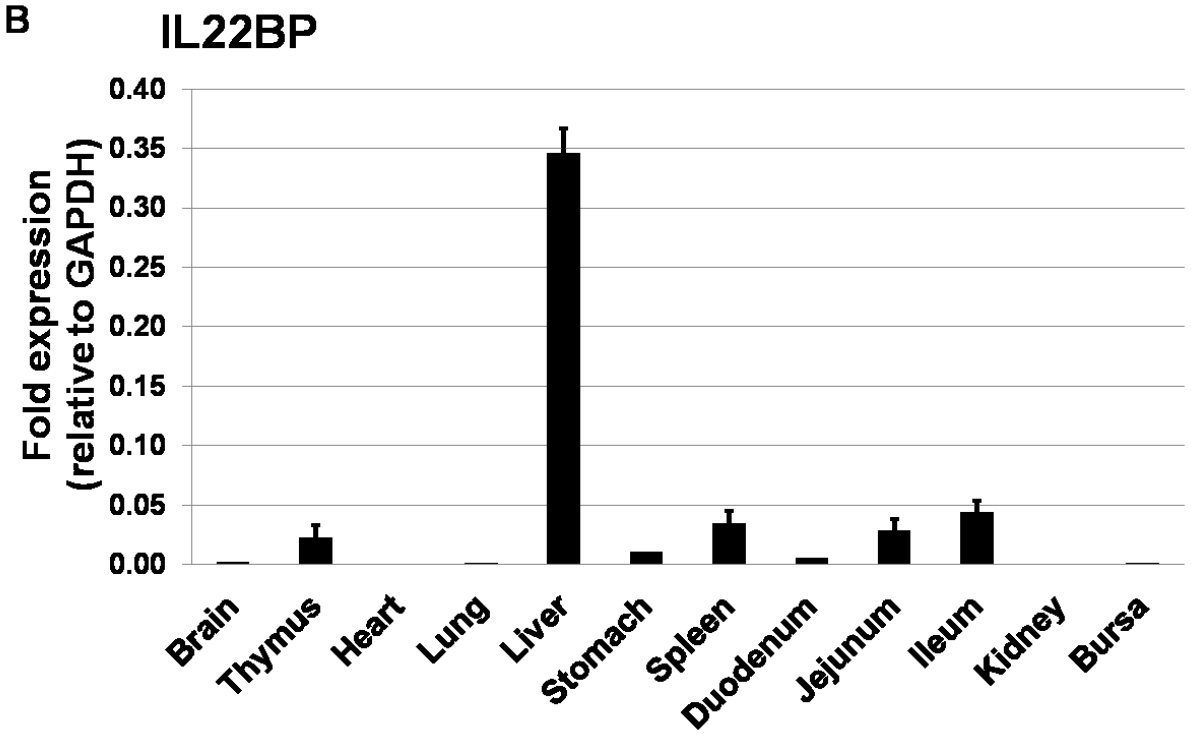
```
ChIL22RA1-Ex    ---------------------------------ERSSCLKR--VAFSSTNFENILTWET 24
chIL22RA2       MGGAAASSDSGKAPHLGPAKVCWYNSTILVLENQDLRDAIKPQEVRFYSLNFNNTLRWQP 60
                                                :  ..:*   * * * **:* * *:.
ChIL22RA1-Ex    -EADILPGTVFDVQYKQYGEKAWLNKRECQSITQPFCNLTHETENFTEHYYARVRATGHR 83
chIL22RA2       GRAGEGETTLYFVQYKVYGQSKWHNKEECWGIQSLFCDLTEETSDAYEPYYGRVQAASDG 120
                 .*.    *:: **** **:. * **.** .* . **:**.**.:  * **.**:*:..
ChIL22RA1-Ex    CSSNWVRSERFEPRKETIIGAPEVECIPHVRSIKFLIHSPYTPLRGEDGHQLNIEDIYSK 143
chIL22RA2       VHSNWSLSSRFTPWRETMIGPPTIKVLHSNKFIILKLQAPRSAYKRKRGSMIPMTNYYDL 180
                  ***  *.** * :**:**.* :: :   : * : :::* :. : : *  : : : *.
ChIL22RA1-Ex    FSTVDYHLTIFNQRTHQKWKKNEHNKEFEVSNLDPDTEYNGTVHLYLLERSSKSQVFWVK 203
chIL22RA2       LYQVFIINNLLDEQ-HRVLVYEGKDKVIKIEDLRPGVSYCIVARTSVLVLG-RSSAYSSR 238
                :  *    .:::::: *:    : ::* :::.:* *...*  ..:  :*  . :*..:  :
ChIL22RA1-Ex    TLPDNTWLFYCFVALGFCAGLVFAAL 229
chIL22RA2       ---------QCTVLL----------- 244
                         * * *
```

**Figure 5.3. Sequence comparison of IL-22 and IL22BP. A.** Using the reported and predicted sequences of ChIL-10 family members, sequences were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). **B and C.** The identified ChIL-22 sequences were aligned and compared with that of other species including human (Accession #: NP_065386 for IL-22; NP_443194 for IL22BP), mouse (Accession #: NP_058667 for IL-22; NP_839989 for IL22BP), zebrafish (Accession #: NP_001018628 for IL-22/NP_001038744 for IL22BP), turkey and zebra finch. **D.** Encoded ChIL22BP was aligned with the extracellular domain of predicted ChIL22RA1 (Accession #: XP_417840). Asterisks (*) indicate identical residues among sequences.

A


IL-22
Fold expression (relative to GAPDH)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Brain
Thymus
Heart
Lung
Liver
Stomach
Spleen
Duodenum
Jejunum
Ileum
Kidney
Bursa

B


IL22BP
Fold expression (relative to GAPDH)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Brain
Thymus
Heart
Lung
Liver
Stomach
Spleen
Duodenum
Jejunum
Ileum
Kidney
Bursa

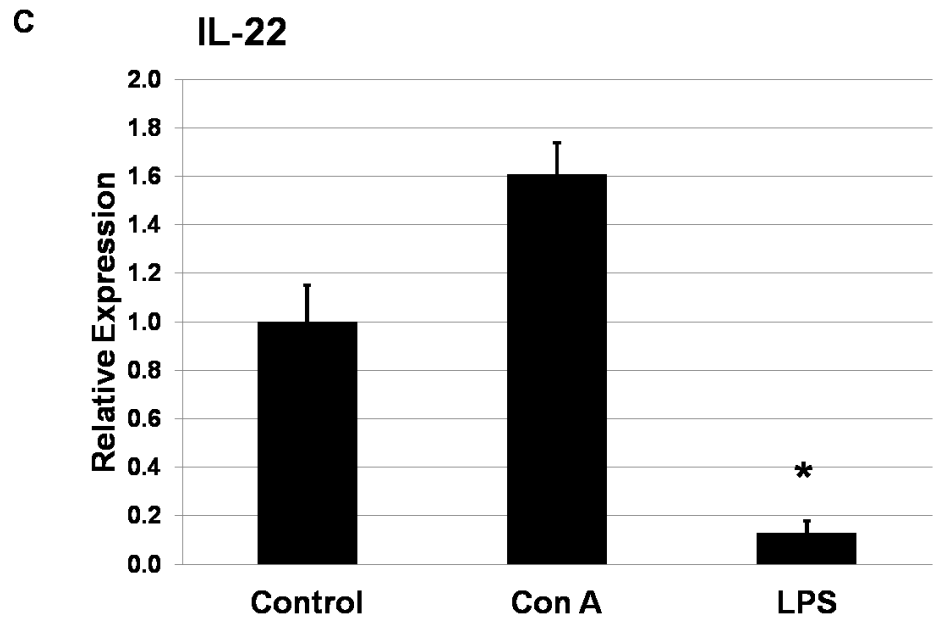
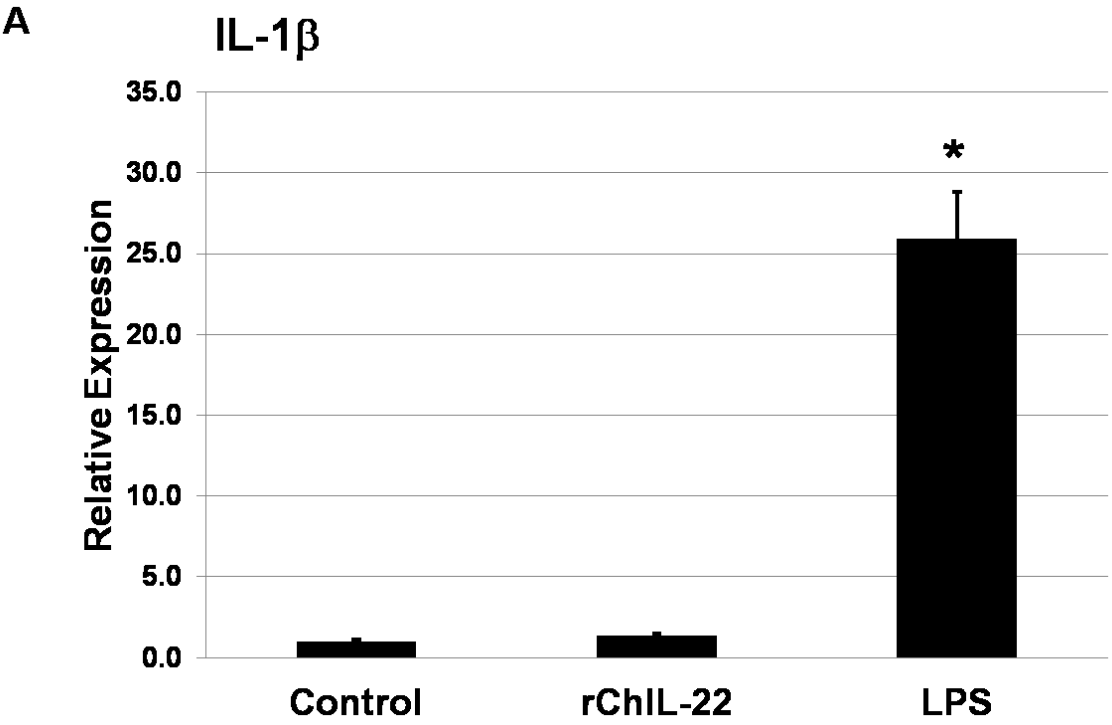
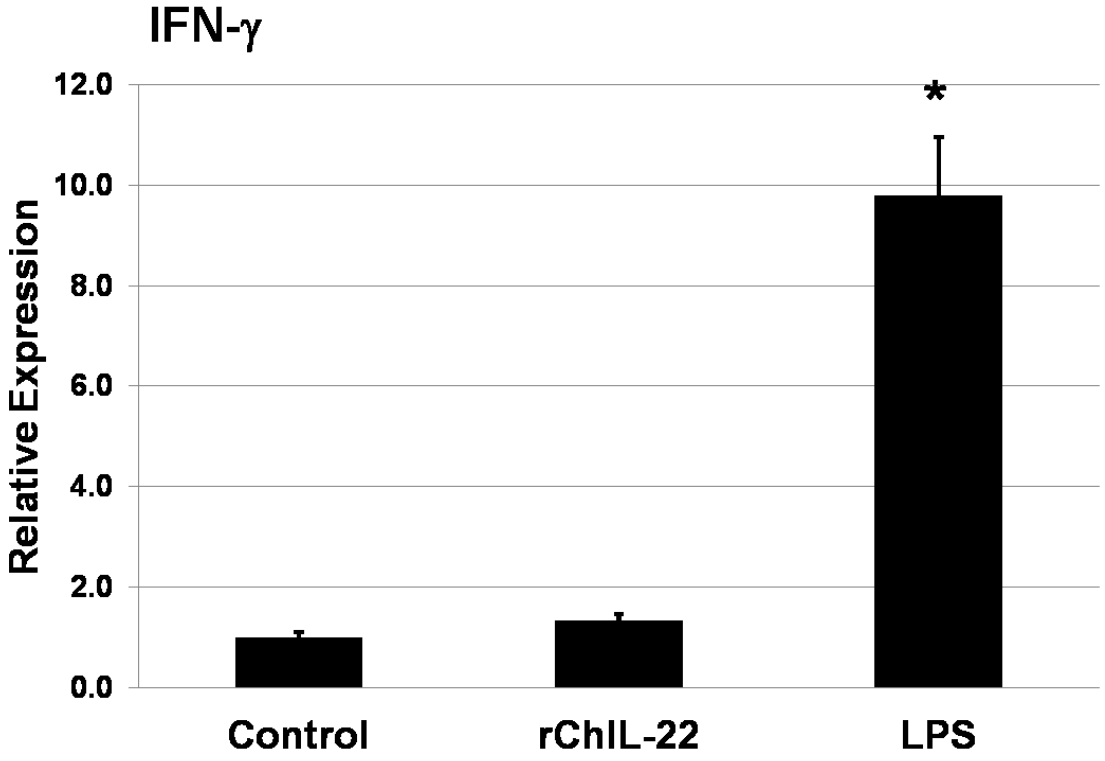
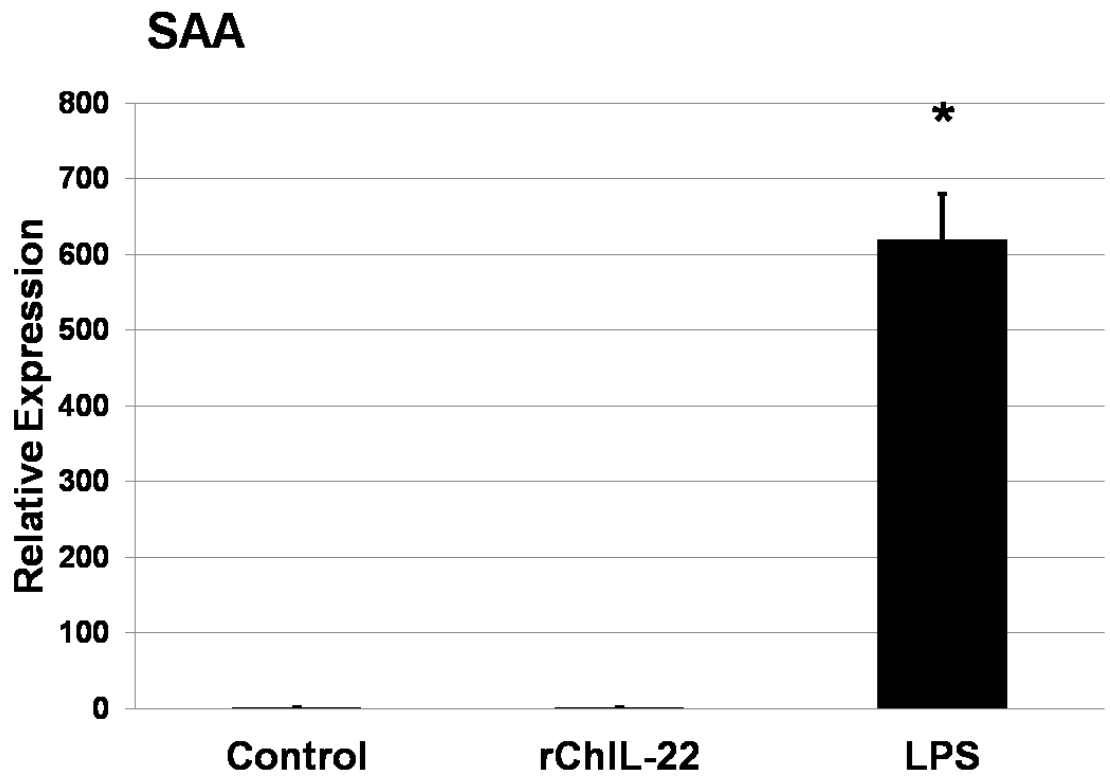



**Figure 5.4. Tissue distribution and expression of ChIL-22 and its soluble receptor IL22BP. A and B,** Tissue distribution of ChIL-22 and ChIL22BP. Relative ChIL-22 and ChIL22BP expression was calculated relative to the expression of GAPDH, following describe in fold expression using the $2^{-\Delta CT}$ method. **C.** Expression of ChIL-22 on lymphocytes during the stimulation with Con A or LPS. Isolated lymphocytes were incubated with media alone, Con A or LPS (5 μg/mL of each) for 12 h. Transcript of ChIL-22 was measured by qRT-PCR.

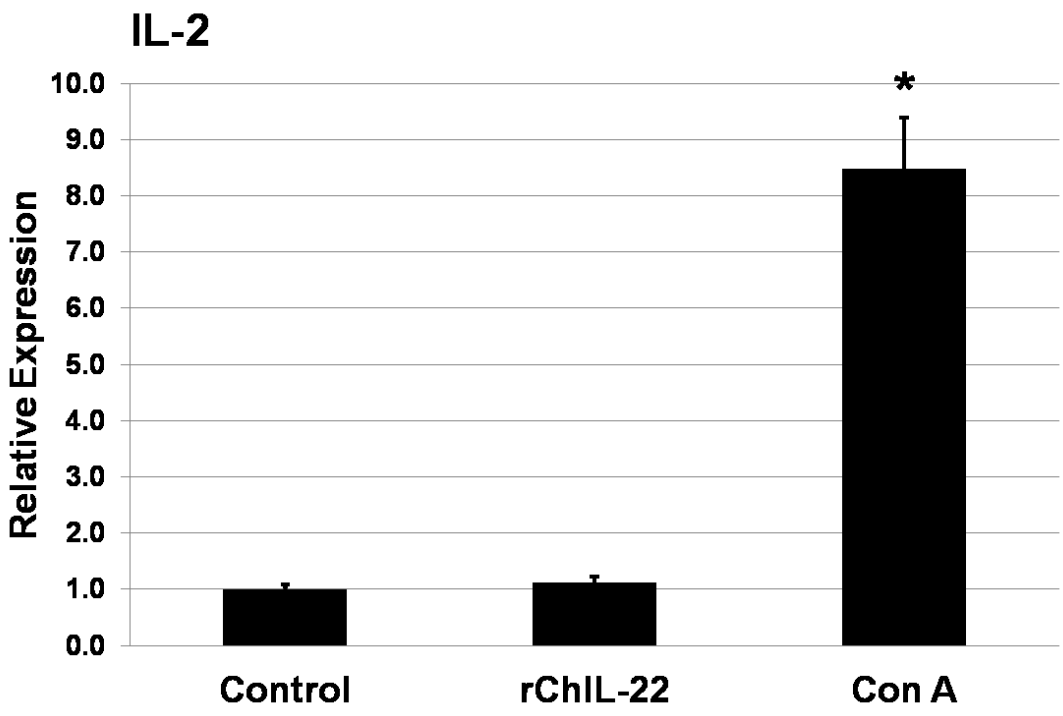
A

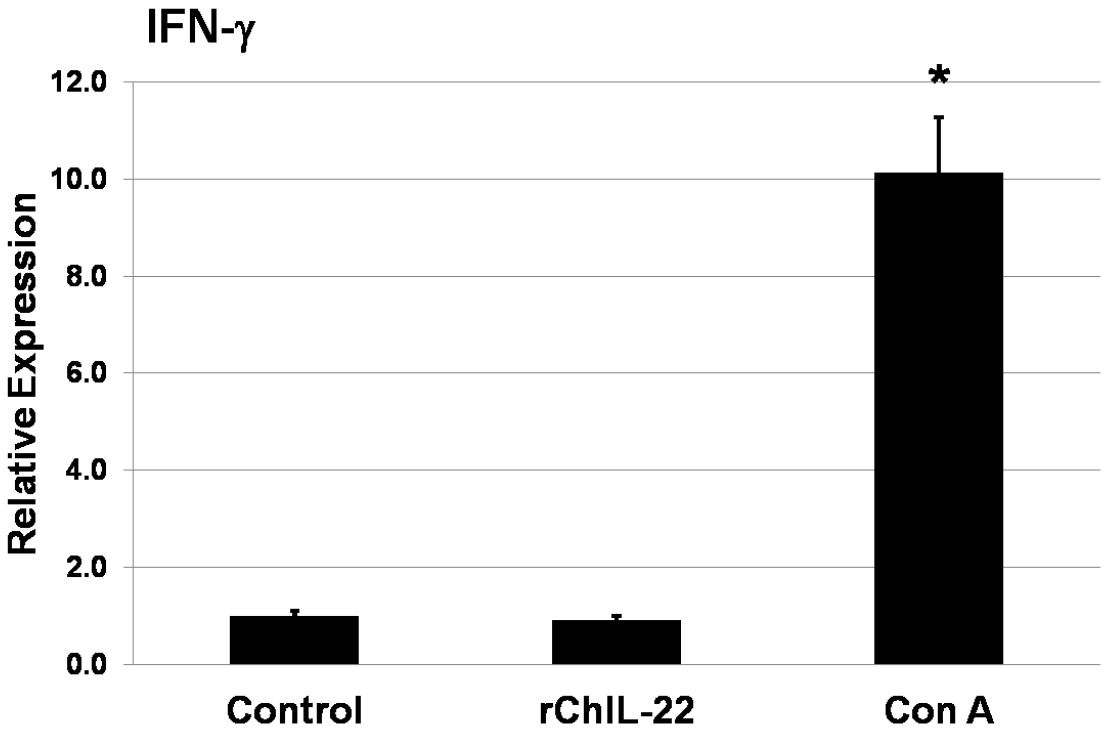

IL-1β
Relative Expression
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
Control
rChIL-22
LPS

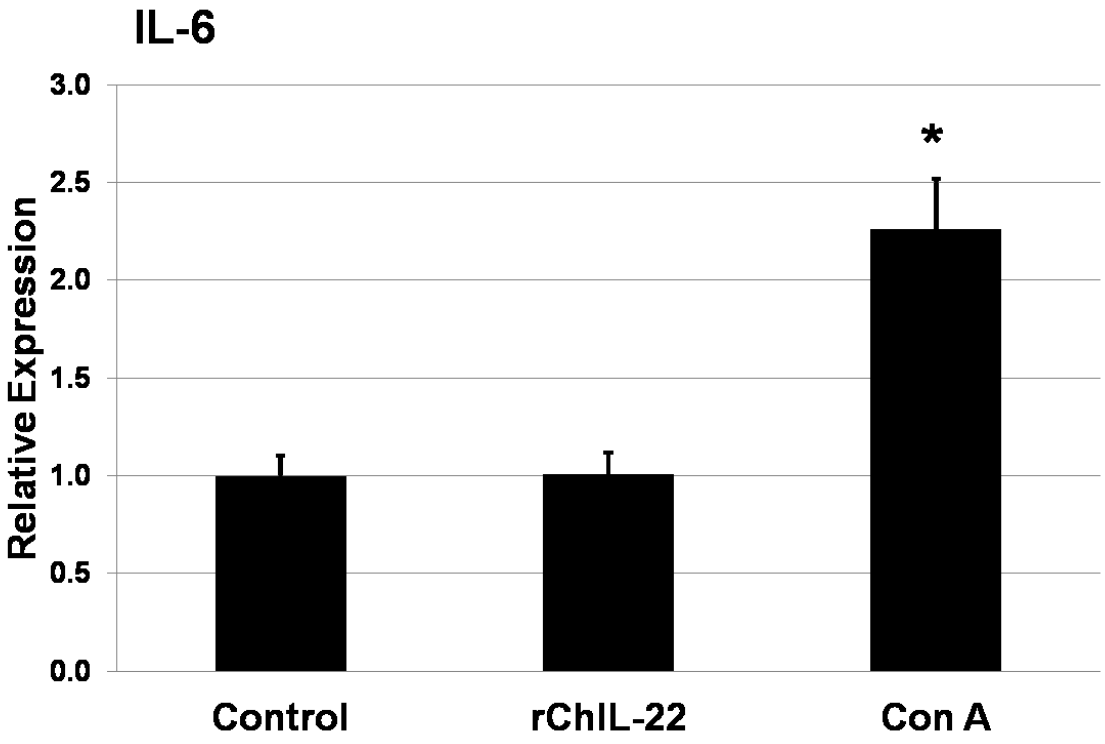

IFN-γ
Relative Expression
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
Control
rChIL-22
LPS

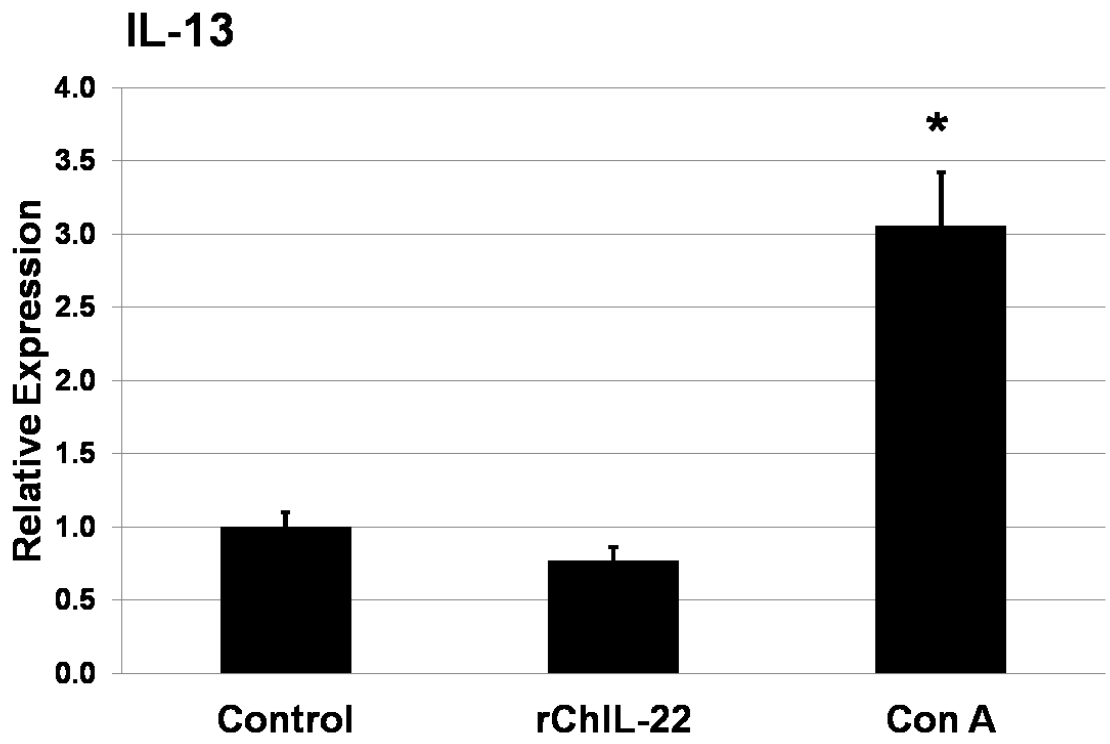

SAA
800
700
600
500
400
300
200
100
0
Relative Expression
*
Control
rChIL-22
LPS

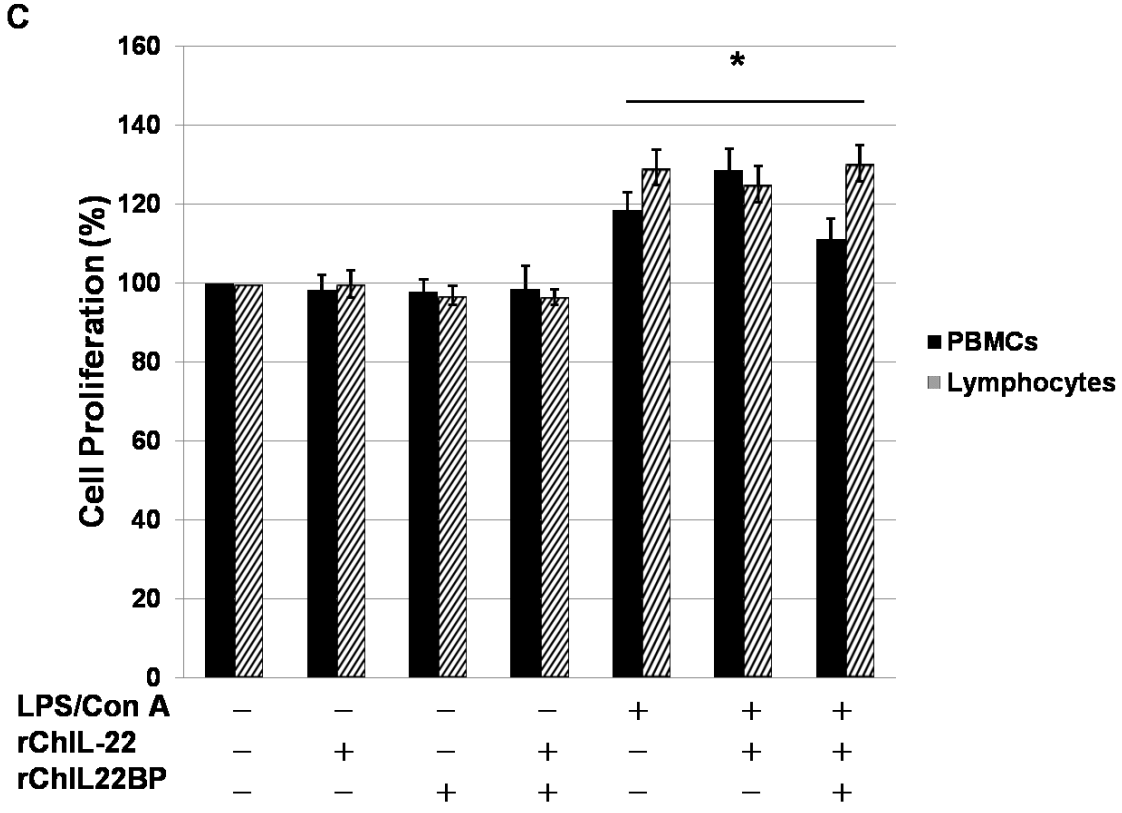
B


IL-2
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Relative Expression
*
Control
rChIL-22
Con A


IFN-γ
Relative Expression
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
Control
rChIL-22
Con A


IL-6
Relative Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
rChIL-22
Con A


IL-13
Relative Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
rChIL-22
Con A


C
Cell Proliferation (%)
160
140
120
100
80
60
40
20
0
*
PBMCs
Lymphocytes
LPS/Con A – – – – + + +
rChIL-22 – + – + – + +
rChIL22BP – – + + – – +

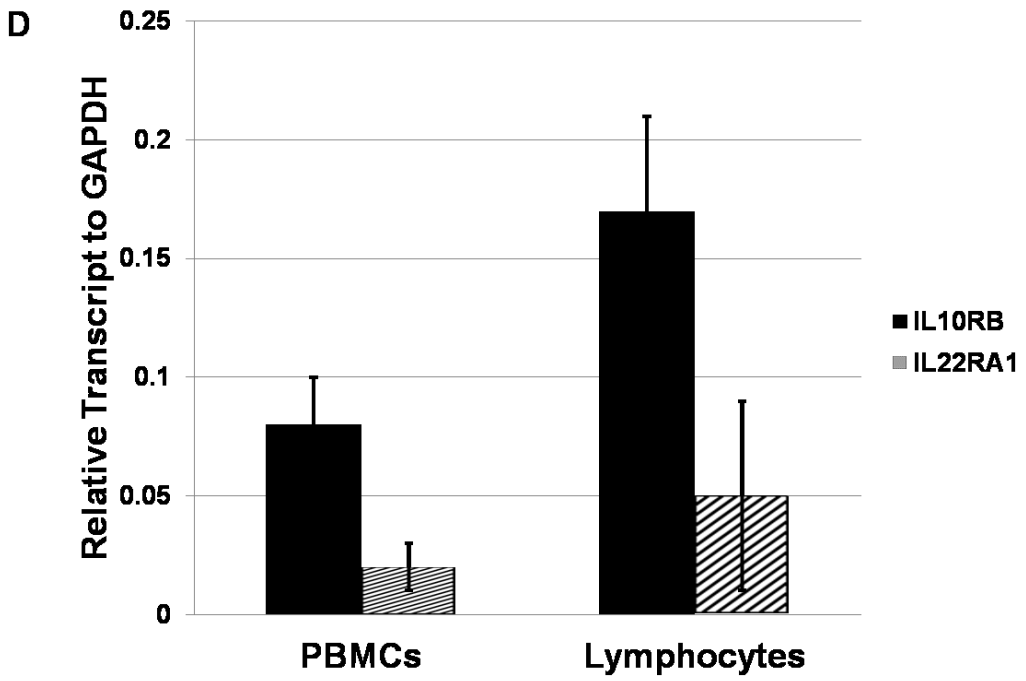
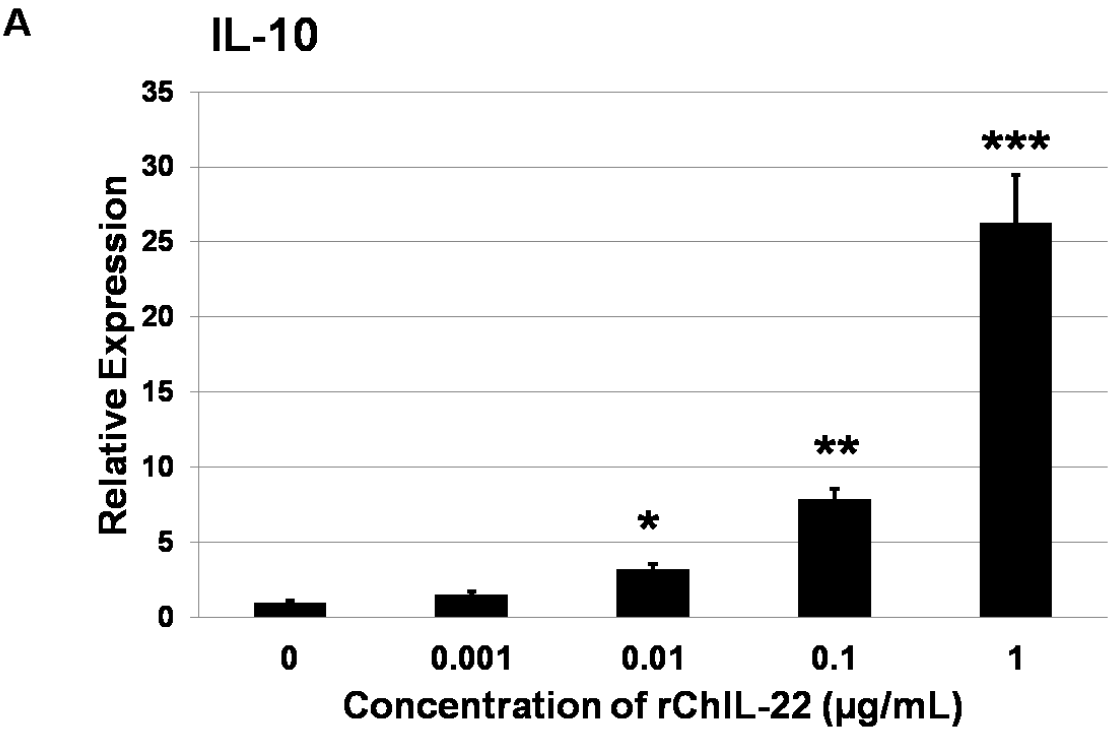
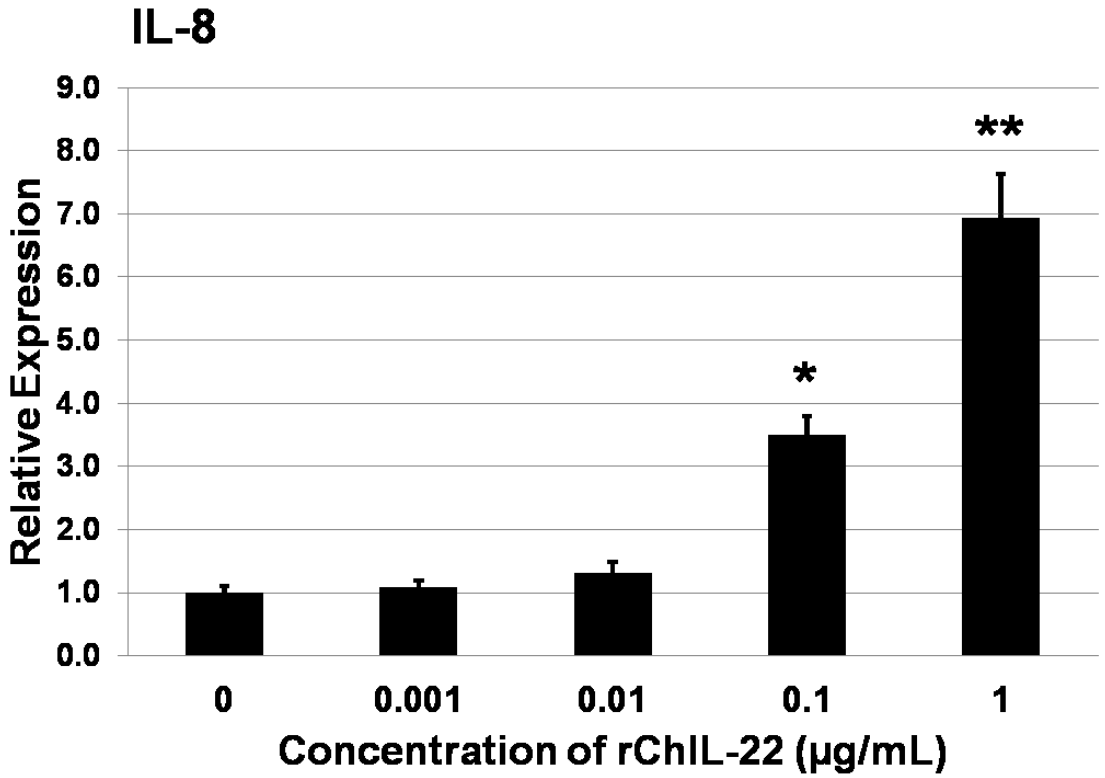
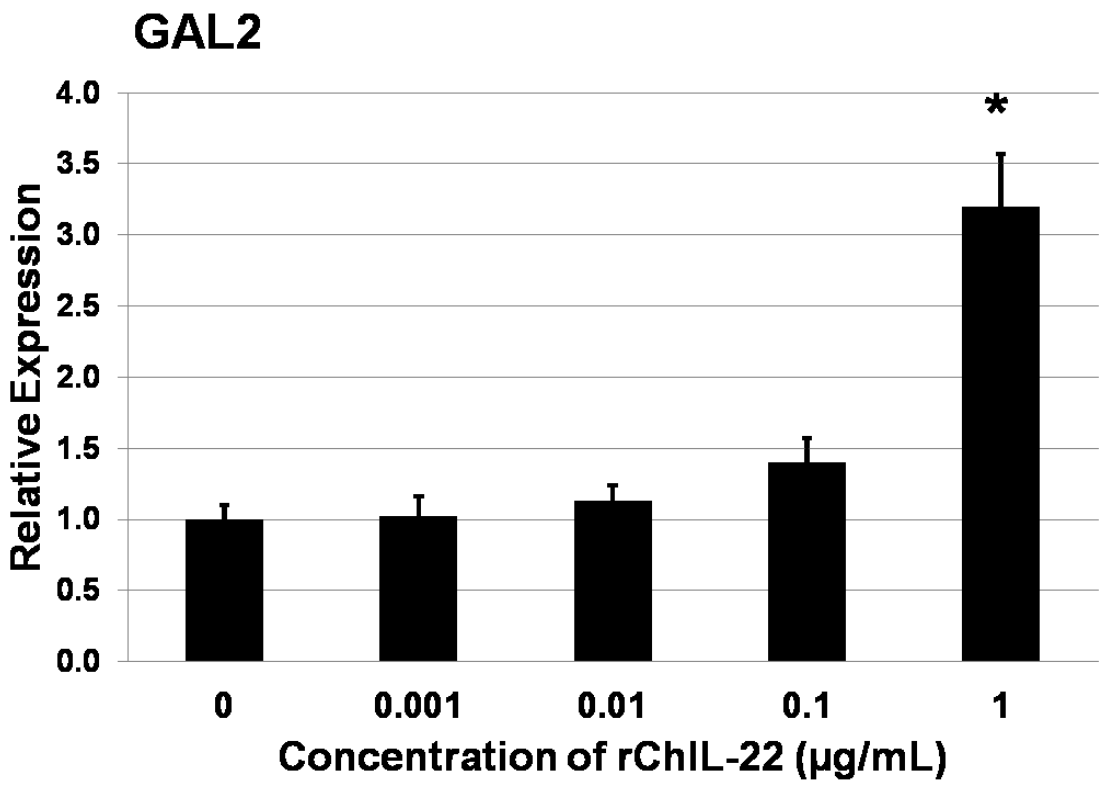


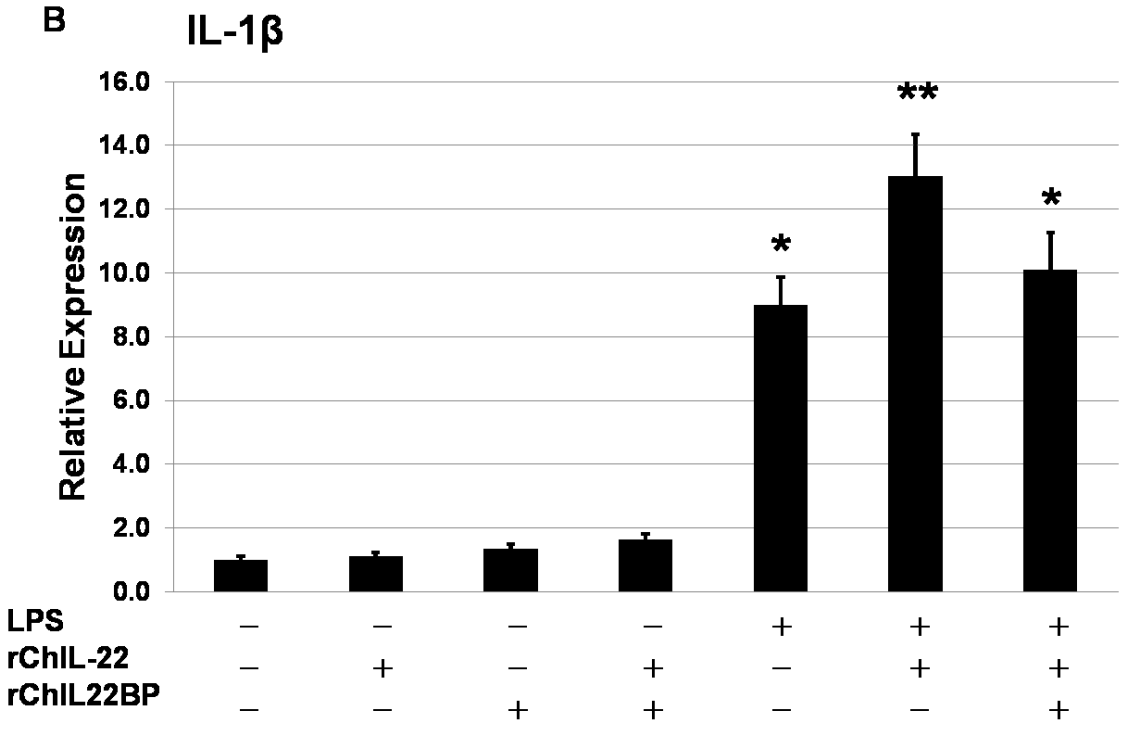

**Figure 5.5. No effect of ChIL-22 on immune cells. A.** The biological effect of rChIL-22 on PBMCs. Isolated PBMCs were treated with media alone, rChIL-22 (0.01 μg/mL) or LPS (5 μg/mL) for 6 h. The expression of IL-1β, IFN-γ and SAA were measured by qRT-PCR and compared to control media alone. **B.** The biological effect of rChIL-22 on lymphocytes. Freshly isolated lymphocytes were incubated with media alone, rChIL-22 (0.01 μg/mL) or Con A (5 μg/mL) for 12 h. The expression of Th1/2 cytokines was measured by qRT-PCR, and their transcripts were compared with control media alone. **C.** The effect of rChIL-22 on cell proliferation. Isolated chicken PBMCs or lymphocytes were incubated on a 96-well plate with rChIL-22 incubated for 12 h in the presence or absence of LPS or Con A (5 μg/mL). Then, cell proliferation was measured using CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega) followed by manufacturer's instruction. Induced cell proliferation was converted and shown in the percentage (%). **D.** Transcript of ChIL-22 receptor chains. Transcripts of IL10RB and IL22RA1 were measured at PBMCs and lymphoyctes and their fold expression was shown relative to GAPDH. Each bar represents the mean of 3 different experiments. The asterisk (*) indicates statistically significant difference ($p < 0.05$).

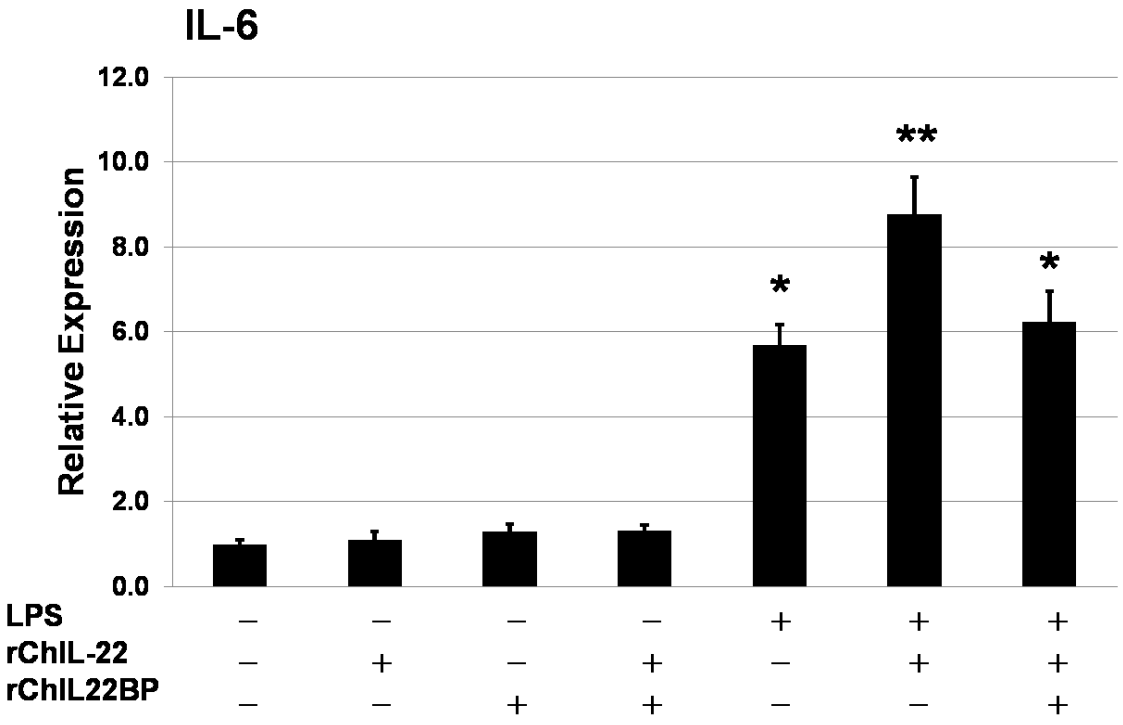
A

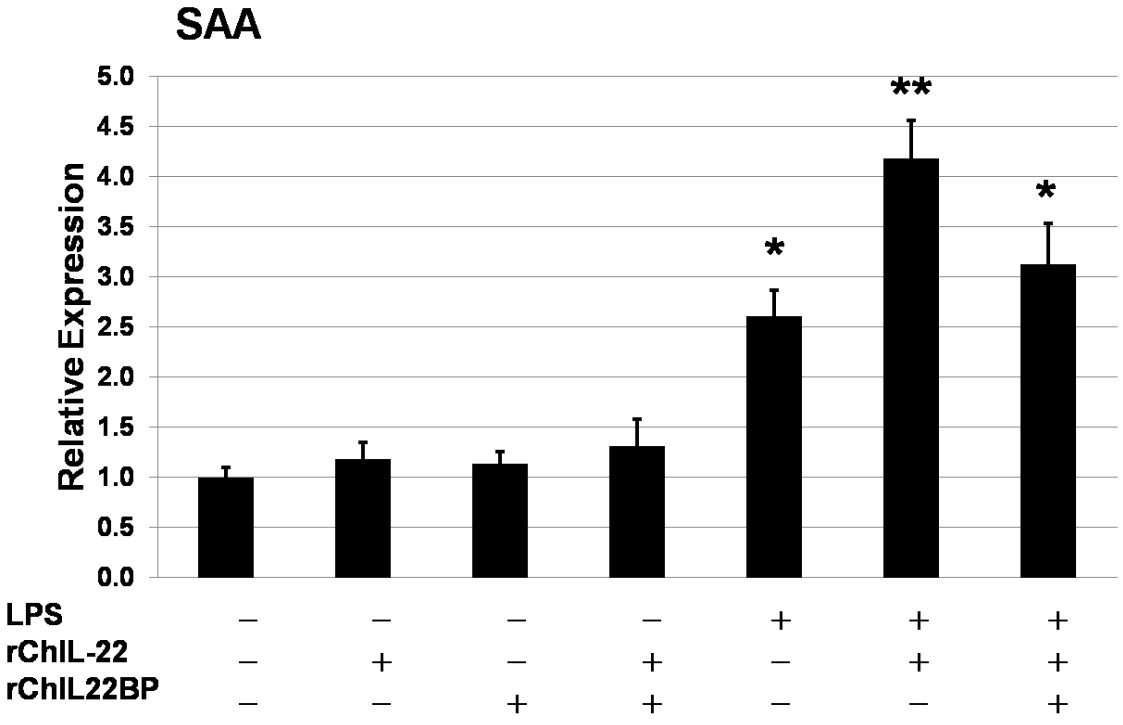

IL-10
Relative Expression
35
30
25
20
15
10
5
0
***
**
*
0
0.001
0.01
0.1
1
Concentration of rChIL-22 (μg/mL)


IL-8
Relative Expression
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
*
0
0.001
0.01
0.1
1
Concentration of rChIL-22 (μg/mL)


GAL2
Relative Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
0
0.001
0.01
0.1
1
Concentration of rChIL-22 (μg/mL)


B
IL-1β
Relative Expression
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
**
*
LPS – – – – + + +
rChIL-22 – + – + – + +
rChIL22BP – – + + – – +


IL-6
Relative Expression
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
**
*
LPS – – – – + + +
rChIL-22 – + – + – + +
rChIL22BP – – + + – – +


SAA
Relative Expression
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
*
LPS – – – – + + +
rChIL-22 – + – + – + +
rChIL22BP – – + + – – +

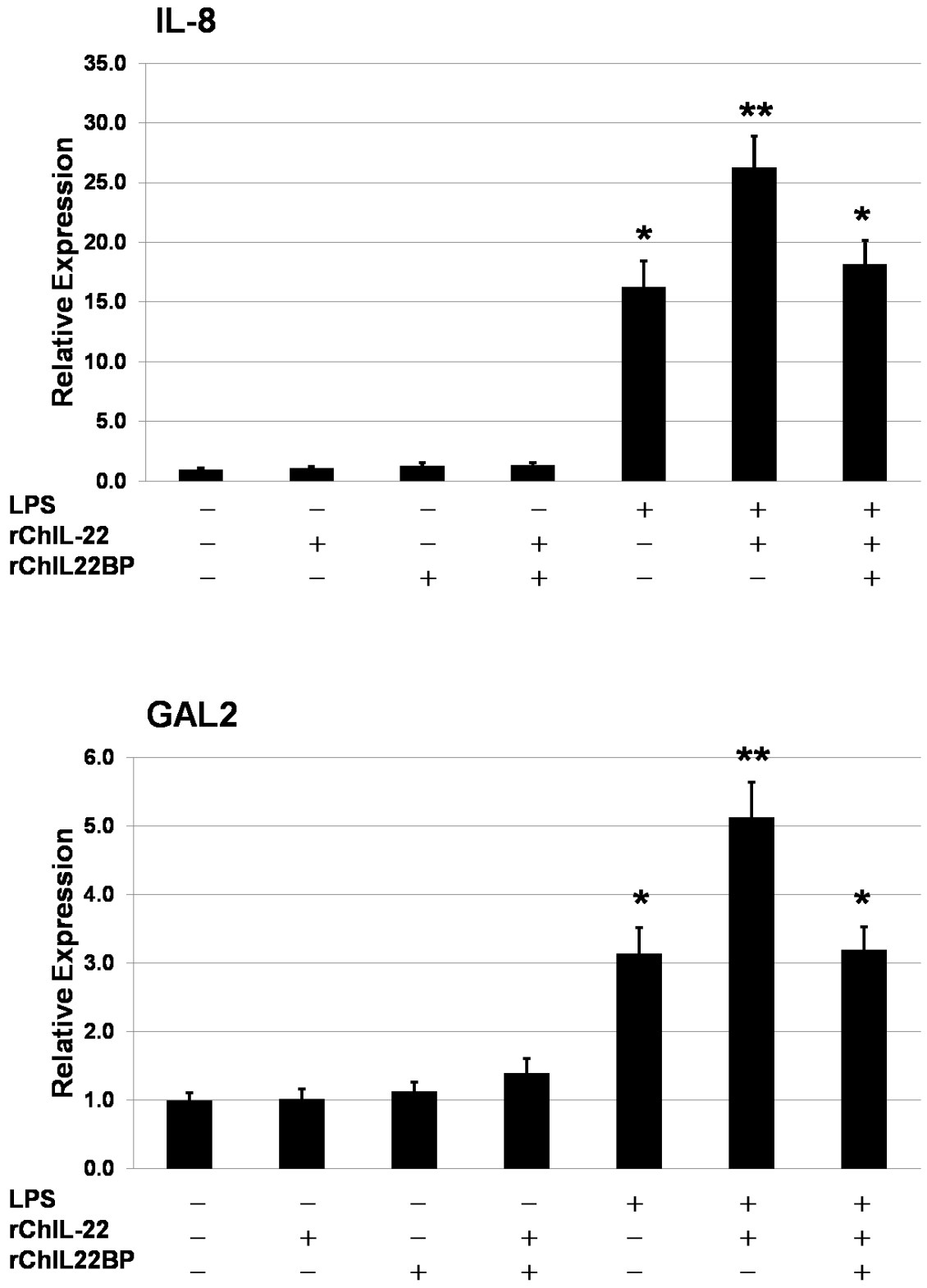
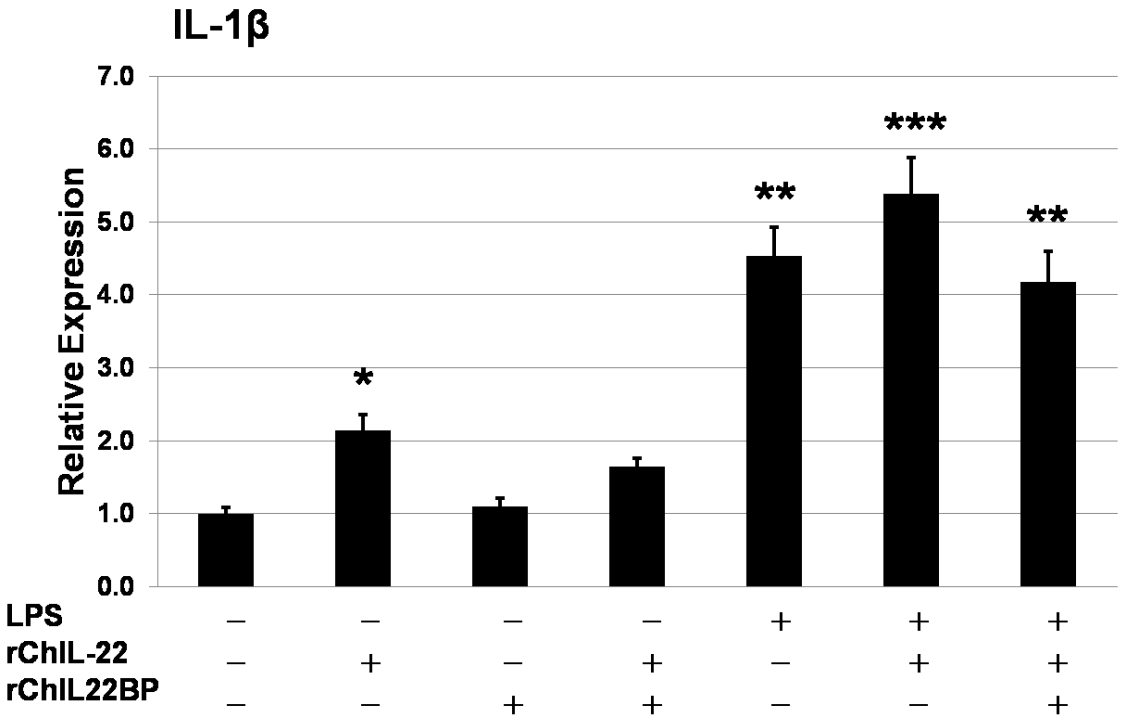
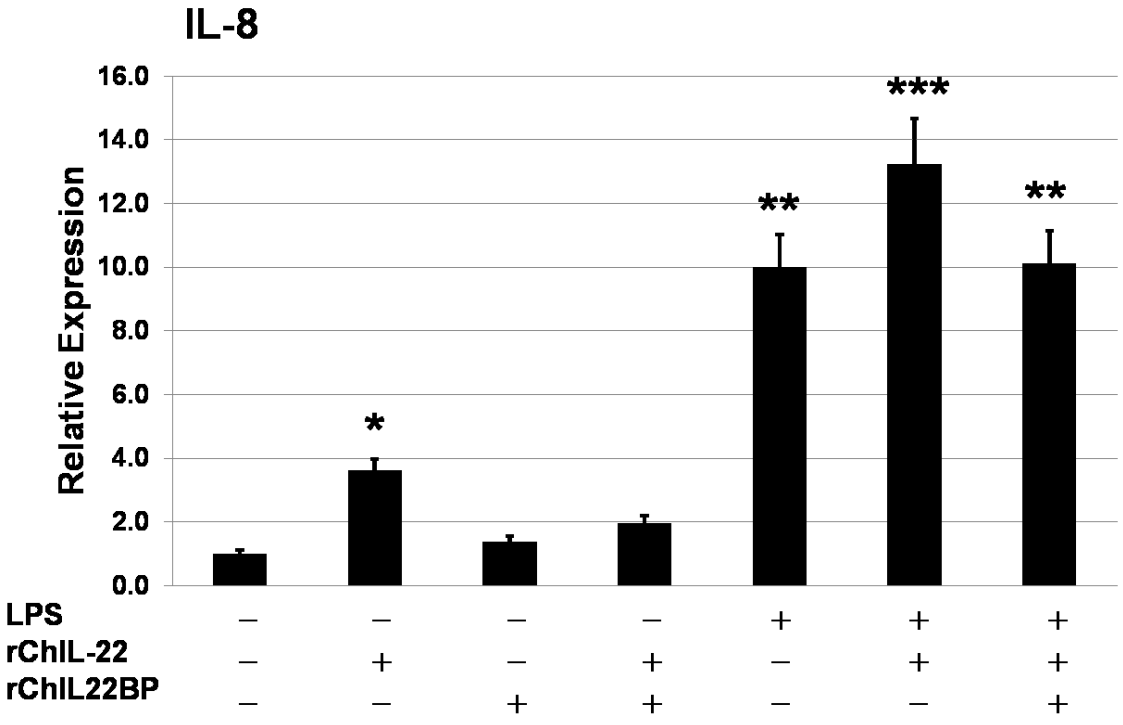



**Figure 5.6. Analysis of biological function of ChIL-22 on epithelial cells. A.** The effect of rChIL-22 on epithelial cells in a dosage-dependent manner. Freshly isolated CEKCs were

incubated with 0, 0.001, 0.01, 0.1, and 1 μg/mL of rChIL-22 for 6 h. Then, cells were lysed directly, total RNA was extracted and qRT-PCR was performed to measure expression of IL-10, IL-8 and GAL2. **B and C.** Enhanced transcript of pro-inflammatory cytokines, chemokine and AMP by rChIL-22 on epithelial cells. Cultured CEKCs were incubated with rChIL-22 in the presence or absence of LPS (5 μg/mL) for 6 h. rChIL22BP (0.01 μg/mL) was used to block the activity of rChIL-22. Cells were directly lysed and total RNA was extracted, following qRT-PCR. Each bar represents the mean of 3 different experiments. The asterisk (*) indicates statistically significant difference ($p < 0.05$).


IL-1β
Relative Expression
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
**
***
**
LPS – – – – + + +
rChIL-22 – + – + – + +
rChIL22BP – – + + – – +


IL-8
Relative Expression
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
**
***
**
LPS – – – – + + +
rChIL-22 – + – + – + +
rChIL22BP – – + + – – +

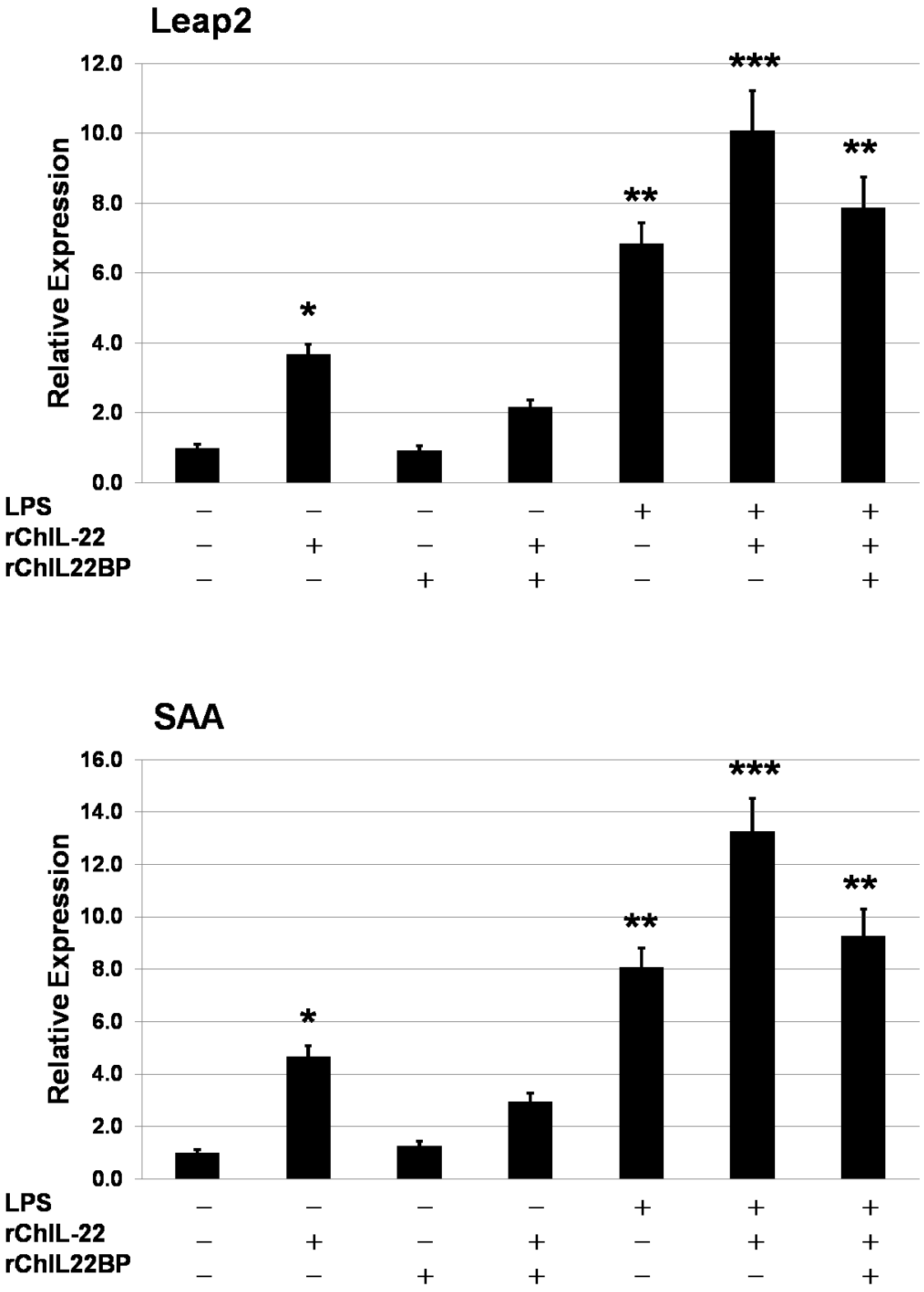



**Figure 5.7. Analysis of biological function of ChIL-22 on hepatocytes.** The cultured liver hepatocytes were incubated with rChIL-22 (0.01 μg/mL) in the presence or absence of LPS (5

μg/mL). After total RNA was extracted, qRT-PCR was used to measure expression of acute phase proteins. rChIL-22 activity was blocked by pre-incubation with rChIL22BP. Each bar represents the mean of 3 different experiments. The asterisk (*) indicates statistically significant difference ($p < 0.05$).

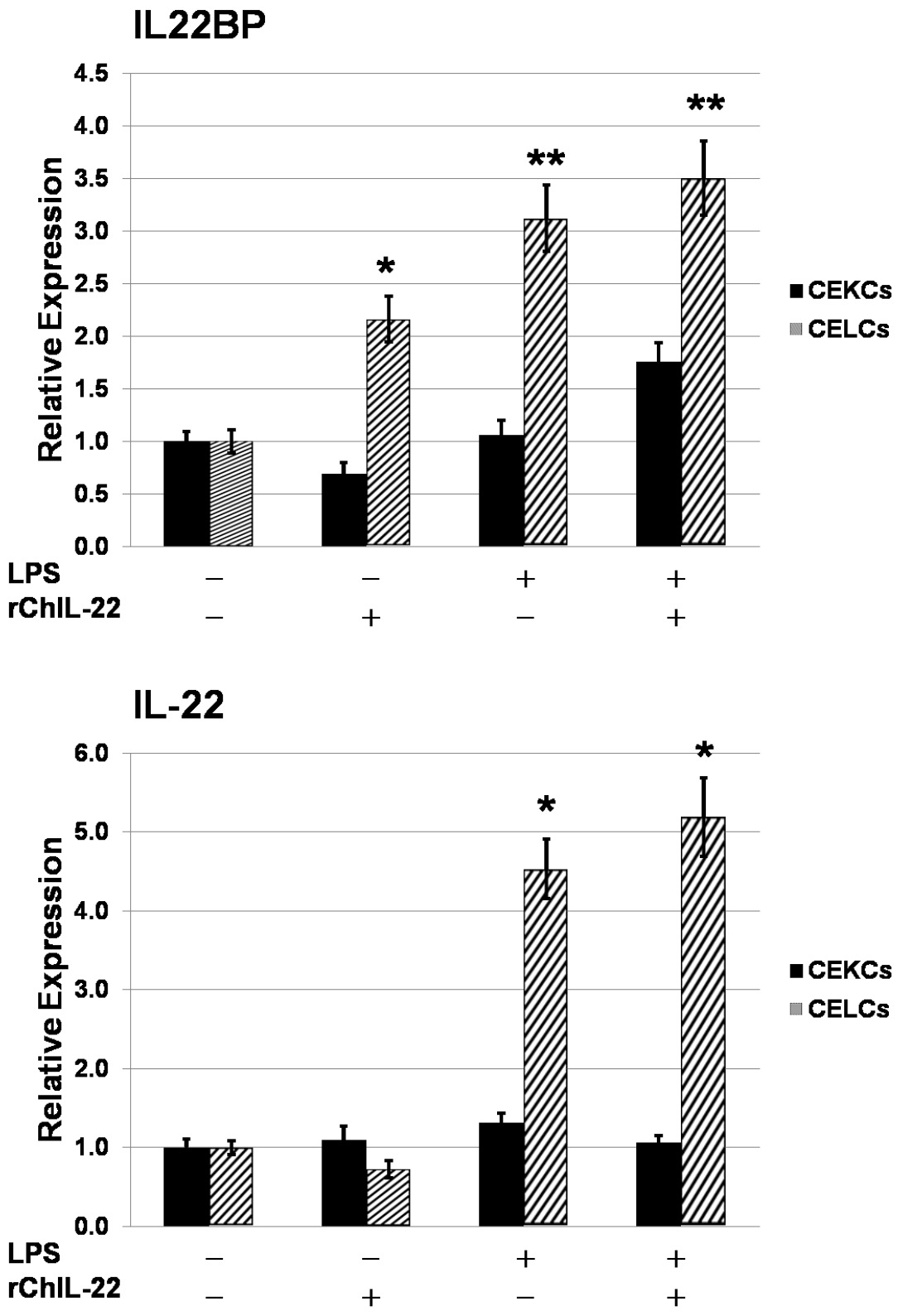



**Figure 5.8. Expression of ChIL-22 and ChIL22BP in kidney epithelial cells and hepatocytes.** Relative expression of ChIL22BP and ChIL-22 was measured during the

stimulation of epithelial cells or hepatocytes with LPS (5 μg/mL). Each bar represents the mean of 3 different experiments. The asterisk (*) indicates statistically significant difference ($p < 0.05$).

# CHAPTER VI

## The Effect of *Eimeria* Macrophage Migration Inhibitory Factor (MIF) on Chicken Mononuclear cells

**ABSTRACT:** Macrophage migration inhibitory factor (MIF) was the first cytokine to be identified almost 40 years ago as a soluble factor produced by sensitized T lymphocytes and inhibits the random migration of macrophages. Homologues of MIF have been identified and characterized recently from invertebrates, making it an interesting molecule from evolutionary as well as functional perspectives. The present study represents the first report of the biological effects of *Eimeria* MIF, which is homologous to avian MIFs. Quantitative real-time PCR (qRT-PCR) was performed to examine gene expression of inflammatory mediators from small intestines of *Eimeria*-challenged chickens. During *Eimeria* sp. infection, mRNA level of ChMIF was decreased, whereasChIL-22 mRNA level was upregulated. The effect of *Eimeria* MIF was tested on avian monocytes/macrophages using recombinant EaMIF. The chemotaxis assay revealed inhibitory function of *Eimeria* MIF on chicken macrophage migration. In vitro stimulation showed EaMIF enhanced expression of pro-inflammatory cytokines and chemokines in the presence of LPS. Furthermore, treatment of PBMCs with EaMIF, ChMIF and LPS sequentially in a 2 h interval led to the highest levels of IL-1β, K203, IL-18 and IFN-γ mRNA. To our knowledge, this study is the first to characterize the effects of *Eimeria* MIF on host avian immune cells, demonstrating the inhibition of chicken macrophage migration, similar to ChMIF, and the mediation of inflammatory responses during antigenic stimulation.

## Introduction

Coccidiosis is an economically important parasitic disease in the poultry industry for both chickens and turkeys. It is caused by protozoa of the genus *Eimeria* that infect the intestinal tract, resulting in an estimated loss of $3 billion annually worldwide (Dalloul and Lillehoj, 2006). Historically, seven species have been recognized to infect the chicken host: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella* (Allen and Fetterer, 2002; McDonald and Shirley, 2009). Infections (usually mixed strains) occur due to ingestion of oocysts, leading to digestive disorders resulting from damage to the intestinal epithelium, malabsorption of nutrients, changes in protein metabolism after absorption, reduced feed conversion efficiency, and reduction in weight gain (Conway et al., 1993; Shirley et al., 2005). The disruption of the intestinal epithelial layer naturally leads to the diminished ability of the intestine to absorb nutrients, resulting in reduced performance and higher susceptibility to other diseases such as necrotic enteritis (Yegani and Korver, 2008).

Macrophage migration inhibitory factor (MIF) was originally identified as a soluble factor produced by activated T cells that inhibited the random migration of macrophages (Bloom and Bennett, 1966; David, 1966; Weiser et al., 1989). Recent research revealed an essential role of MIF in adaptive immune responses as well as innate immunity. Mammalian MIF is an immunomodulator that controls macrophage function, resulting in the promotion of pro-inflammatory cytokine expression including TNF-$\alpha$, IL-1$\beta$, IL-2, IL-6, IL-8 and IFN-$\gamma$ (Calandra et al., 1994; Bacher et al., 1996; Donnelly et al., 1997), nitric oxide (NO) release (Bernhagen et al., 1994), and COX-2 activity (Mitchell et al., 2002). MIF also up-regulates the expression of TLR4, a well known innate immune sensor that recognizes LPS and induces the activation of monocytes/ macrophages, suggesting potential involvement of MIF early in innate immune responses (Roger et al., 2001). In adaptive immunity, MIF is constitutively expressed by T

lymphocytes but can also be induced by mitogens, CD3-specific antibody, and glucocorticoids (Bacher et al., 1996; Calandra et al., 1998; Abe et al., 2001). Produced primarily by activated Th2 cells, MIF appears to have a possible autocrine function, resulting in activation and proliferation of T cells and IL-2 production (Bacher et al., 1996). Moreover, MIF inhibits regulatory effects on cytotoxic $CD8^+$ T cells and regulates lymphocyte trafficking (Abe et al., 2001). Unlike mammalian MIF, ChMIF alone does not promote the expression of pro-inflammatory cytokines and NO release (Kim et al., 2010). However, ChMIF induces the expression of mRNA of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and IL-12) as well as NO, when chicken PBMCs are first primed with LPS. ChMIF also enhances Th1/Th2 cytokines in previously stimulated lymphocytes (Kim et al., 2010).

In recent years, MIF orthologues have been isolated from non-vertebrate organisms, such as nematodes, *Caenorhabditis elegans* (Marson et al., 2001), *Brugia* sp. (Pastrana et al., 1998; Zang et al., 2002), *Trichinella* sp., *Trichuris* sp. (Tan et al., 2001; Wu et al., 2003), *Wuchereria bancrofti* and *Onchocerca volvulus* (Pastrana et al., 1998). In these organisms, MIF appears to have tautomerase activity as well as the ability to chemotactically induce macrophage migration (Tan et al., 2001; Zang et al., 2002; Wu et al., 2003). It is unknown why a pro-inflammatory cytokine is actively expressed by parasitic nematodes, since this would not seem to be advantageous to parasites that induce a counter-inflammatory phenotype in the host (Maizels et al., 1995; Lawrence, 1996). However, there is some evidence that high MIF expression can produce anti-inflammatory effects (Bucala, 2000; Kleemann et al., 2000), hypothetically resulting in MIF-mediated inflammatory suppression and subsequent immune evasion.

Miska and colleagues (2007) recently characterized MIF from *E. acervulina* (Ea) and *E. tenella* (Et). The study reported that EaMIF and EtMIF are expressed in the highest mRNA level

in merozoites, while decreasing amounts of transcripts were detected in unsporulated and sporulated oocysts, respectively, with transcripts falling to undetectable levels in sporozoites, implicating an important role of *Eimeria* MIF in the developmental regulation. The study also showed cytosolic localization of *Eimeria* MIF with greater concentrations associated with the apical end of the merozoites (Miska et al., 2007). However, to date, no biological function of *Eimeria* MIF has been reported especially its effects on chicken cells. The present study mainly describes the effect of *Eimeria* MIF on chicken macrophages using expressed recombinant EaMIF and EtMIF. Observed biological effects of EaMIF and EtMIF included inhibitory function of chicken macrophage migration and enhancement of inflammatory response in monocytes.

## Materials and Methods

*Cells and Eimeria MIFs*

Peripheral blood mononuclear cells (PBMCs) were isolated from twenty-week old healthy broiler chickens (donated by Dr. Paul Siegel, Virginia Tech) and cultured as described by Kim et al. (2010). All birds had been housed and reared according to the Institutional Animal Care and Use Committee of Virginia Tech.

The full-length EaMIF and EtMIF were amplified, cloned and expressed in *E. coli*. The expressed EaMIF and EtMIF were purified as described in Kim et al. (2010).

*Total RNA from Eimeria-challenged birds*

Total RNA from small intestinal segments were obtained from *Eimeria*-challenged and non-challenged birds (Cox et al., 2010). Using 1 μg of total RNA, the first-strand cDNA was

synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA) following the manufacturer's instruction (Kim et al., 2010), and the cDNA was stored at -20°C.

*Chemotaxis assay using modified Boyden Chamber*

The purified recombinant *Eimeria* MIF was prepared by 10-fold serial dilutions (0.001 μg/mL to 1 μg/mL) with DMEM supplemented with 2 mM $_{L}$-Glutamine and 10% FCS. The medium supplemented with 10% FCS was used as positive control to induce cell migration, while serum-free medium was used as negative control. Recombinant protein and controls (25 μL) were pipetted into the bottom wells of the chamber, separated from the top wells by 5-μm pore of polycarbonate filter membrane. Isolated PBMCs were adjusted to 1 x $10^6$ cells/mL and 50 μL of suspended cells were loaded into the top wells of the chamber. Migration was allowed to continue for 4 hr at 39°C in humidified air containing 5% $CO_2$. The cells at the lower surface of the membrane were fixed and stained with Diff-Quick Staining (Fisher Scientific, NJ). The stained cells were counted and the percentage of migration inhibition was determined by applying the following formula (Weiser et al., 1989; Jin et al., 2007):

$$\text{Percent migration inhibition} = \left( 1 - \frac{\text{Mean area of migration in experimental group}}{\text{Mean area of migration in control group}} \right) \times 100$$

*In vitro cell stimulation for biological function analysis*

The isolated PBMCs were seeded onto 12-well plates and cultured for 24 h. After the cells were gently washed, they were treated for 6 h in two different groups as shown in Table

6.1. Cell lysis buffer was directly added into the wells and total RNA was extracted using RNeasy Mini Kit (Qiagen, CA).

*Quantitative real-time PCR analysis of the cytokine transcripts*

To analyze the transcripts of various cytokines, primers were designed using Primer Express (Ver 3.0; Applied Biosystems, CA) (Table 6.2), and qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, CA) as described by Kim et al. (2010). Target gene expression in this study was normalized against the expression of chicken GAPDH mRNA, followed by analysis of the results using 7500 Software (Ver 2.0; Applied Biosystems, CA).

*Statistical analysis*

All data were analyzed by either Student's t-test or Analysis of Variance (ANOVA) using JMP (Ver 8.0) software, and significant differences among groups were tested by the Tukey-Kramer Honestly Significant Difference post-hoc procedure.

## Results

*Transcripts of inflammatory mediators Nod1, MIF and IL-22 during Eimeria infection*

In previous studies, we found that ChNod1, ChMIF and ChIL-22 are important inflammatory mediators, especially during microbial infections. To evaluate the role of these inflammatory mediators during parasite infection, we examined the transcripts of ChNod1, ChMIF and ChIL-22 in the duodenum and jejunum of *Eimeria*-challenged birds (Figure 6.1). There was no significant difference in mRNA levels of all three genes in the *Eimeria*-infected

jejunum in comparison with those of non-challenged group (Figure 6.1A). However, expression of ChIL-22 was slightly increased in the duodenum, while expression of ChMIF was reduced (Figure 6.1A). Since ChIL-22 mRNA level was increased, expression of its receptors was measured. Both surface receptor (ChIL22RA1) and soluble receptor (ChIL22BP) showed no significant differences in expression in the jejunum in comparison with those of control (non-challenged) birds, like ChIL-22. However, expression of ChIL22BP was increased in *Eimeria*-infected duodenum similar to ChIL-22 (Figure 6.1B).

*Chemotaxis of EaMIF or EtMIF on chicken macrophage*

To examine chemotaxis of EaMIF or EtMIF on chicken macrophages, isolated chicken PBMCs were incubated with different concentrations of recombinant EaMIF or EtMIF (Figure 6.2). Similar to ChMIF, both EaMIF and EtMIF showed inhibitory function of macrophage migration. The lowest concentration (0.001 μg/mL) of EaMIF tested in this study showed approximately 60% inhibition of chicken macrophage migration and the highest concentration (1 μg/mL) showed approximately 90% inhibition. Percentage of inhibition by EtMIF exhibited very similar pattern to that of EaMIF, except EtMIF showed approximately 78% inhibition of chicken macrophage migration at the lowest concentration.

*The effect of Eimeria MIF on macrophage function*

To determine the effect of *Eimeria* MIF on chicken macrophage function, freshly cultured chicken PBMCs were incubated with different combinations of EaMIF, ChMIF and LPS. Similar to ChMIF, EaMIF alone had no effect on the expression of IL-1β, IL-18, IFN-γ and the chemokine K203, whereas LPS induced expression of IL-1β, IFN-γ and K203 (Figure

6.3). In addition, chicken PBMCs were not influenced by treatment of ChMIF and EaMIF together. However, incubation of PBMCs with EaMIF and LPS together led to significantly enhanced mRNA levels of IL-1β and K203. In addition, 2 h pre-treatment of PBMCs with EaMIF, followed by incubation with LPS, resulted in 200-fold enhanced expression of IL-1β, while expression of IFN-γ was similar to that of LPS treatment alone. Addition of LPS in the presence of ChMIF and EaMIF showed enhanced expression of IL-1β, K203 and IFN-γ in comparison with those treated with LPS alone or EaMIF with LPS. Two-hour interval sequential treatment of PBMCs with ChMIF and then EaMIF did not affect the tested immune mediators; however, sequential addition of ChMIF, EaMIF and LPS greatly enhanced IL-1β, K203, and IFN-γ mRNA levels. Interestingly, expression of ChIL-18 was only affected when the cells were treated with ChMIF, EaMIF and LPS sequentially in a 2 h interval.

## Discussion

In this study, we first measured the expression of inflammatory mediators ChNod1, ChMIF and ChIL-22 in *Eimeria*-infected intestinal samples. The biological function of *Eimeria* MIF on chicken mononuclear cells was also examined. qRT-PCR results revealed no difference in mRNA levels of ChNod1 in both duodenum and jejunum on day-14 post-hatch. Since we could not observe differences in mRNA levels of ChNod1 following treatment with its known ligands (CHAPTER III), these results were not totally surprising. In addition, whether or not ChNod1 is involved in immune responses during *Eimeria* infection was not conclusive based on the current results. Expression of ChMIF was reduced in the duodenum, but not in the jejunum. Increased ChIL-22 mRNA level in *Eimeria*-challenged duodenum on day-14 post-hatch indicates increased inflammation in the duodenum. However, the host immune system seems to neutralize

this condition by inducing ChIL22BP expression rather than stimulating IL-22 signal via IL22RA1. Further, induced ChIL22BP instead of ChIL22RA1 as influenced by *Eimeria* infection may have blocked ChIL-22 signaling pathway, thus leading to failure of further production of inflammatory mediators, as well as homeostasis of intestinal epithelial cells. Unfortunately, there is no clear answer for this phenomenon yet and further investigations are warranted.

Similar to avian MIFs, both EaMIF and EtMIF showed inhibitory function of chicken macrophage migration. We could not observe a decreased inhibition by *Eimeria* MIF on chicken macrophage migration based on tested concentrations (0.001 to 1 μg/mL). Unlike *Eimeria* MIF, *Trichinella spiralis* MIF showed the highest inhibitory ability at 50 pg/mL and its inhibitory ability was reduced at the higher concentration (Tan et al., 2001).

As to the effect of *Eimeria* MIF on chicken mononuclear cells, EaMIF alone or together with ChMIF did not influence the expression of cytokines and chemokines in chicken PBMCs. However, combination of EaMIF (and/or ChMIF) and LPS led to enhanced production of pro-inflammatory immune elements. These results follow a similar pattern to those of ChMIF (Kim et al., 2010). Furthermore, sequential treatment of EaMIF and LPS showed the greater enhancement of inflammatory mediators at the mRNA levels than any other condition. These results indicate that *Eimeria* MIF may induce host anti-inflammatory cytokines by enhancing the expression of inflammatory mediators over a short term period. On the other hand, *Eimeria* MIF itself may inhibit production of pro-inflammatory mediators. Kleemann et al. (2000) reported that absence of MIF leads to activation of the transcription factor AP-1 via Jab1, resulting in activation of pro-inflammatory genes. Jab1 is inactivated in the presence of MIF, resulting in a negative impact on inflammation and cell growth (Kleemann et al., 2000). Thus, similar amino

acid sequences and structure of *Eimeria* MIF with ChMIF may function as ChMIF to inhibit host inflammatory response and cell proliferation. Unfortunately, this hypothesis cannot explain why EaMIF enhances the production of pro-inflammatory cytokines and chemokines in the presence of LPS.

In summary, the results indicate that *Eimeria* MIF inhibits migration of chicken macrophages. Additionally, the effect of *Eimeria* MIF on host mononuclear cells showed that it functionally enhances pro-inflammatory cytokines and chemokines in pre-stimulated monocytes. This outcome suggests an important role of *Eimeria* MIF in potential secondary (e.g. bacterial) infections.

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**Table 6.1. Treatment of chicken PBMCs with ChMIF, EaMIF and LPS**

| Treatment | Group 1 | | | | | | | Group 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ChMIF (0.01 μg/mL) | − | + | − | − | + | − | + | + | − | + | + | +[a] |
| EaMIF (0.01 μg/mL) | − | − | + | − | + | + | + | +[a] | + | + | +[a] | + |
| LPS (5 μg/mL) | − | − | − | + | − | + | + | − | +[a] | +[a] | +[b] | +[b] |

Note

1) "a" indicates that the marked reagent was added 2 h later the first treatment ("+") was added.

2) "b" indicates that the marked reagent was added 2 h later "a" reagent was added.

**Table 6.2. Primer sequences for qRT-PCR analyses of cytokine transcripts**

| Primer Name | Accession No. | Sense sequence | Anti-sense sequence |
|---|---|---|---|
| GAPDH | NM_204305 | AGGGTGGTGCTAAGCGTGTTA | TCTCATGGTTGACACCCATCA |
| IFN-γ | NM_205149 | GCTCCCGATGAACGACTTGA | TGTAAGATGCTGAAGAGTTCATTCG |
| IL-1β | NM_204524 | CCCGCCTTCCGCTACA | CACGAAGCACTTCTGGTTGATG |
| IL-18 | NM_204608 | AGGTGAAATCTGGCAGTGGAAT | TGAAGGCGCGGTGGTTT |
| IL-22 | XM_416079 | TGTTGTTGCTGTTTCCCTCTTC | CACCCCTGTCCCTTTTGGA |
| IL22RA1 | Xm_417840 | CTCAGACCTCCGAGCAAAGC | GTGGTCTATGCCATCGACACA |
| IL22BP | XM_001233761 | TGCGACCTGACAGAGGAGACT | AGCTTGCACCCTGCCATAGT |
| K203 | Y18692 | CCTGCTGCACCACTTACATAACA | TGCTGTAGTGCCTCTGGATGA |
| MIF | M95776 | GCCCGCGCAGTACATAGC | CCCCCGAAGGACATCATCT |
| Nod1 | XM_418777 | TGAGGAACCACCCCAGGTT | CTCTGTCGTGATGCCATTGAA |
| EaMIF | DQ323515 | ATTGAGCAAGCTTCTCGGAAA | TCCCCCCGTGTCAAGCTAA |
| EtMIF | DQ323515 | TGCGATCCTGCAGCTAGTGT | GTTGGTGCGGCTGCTGAT |

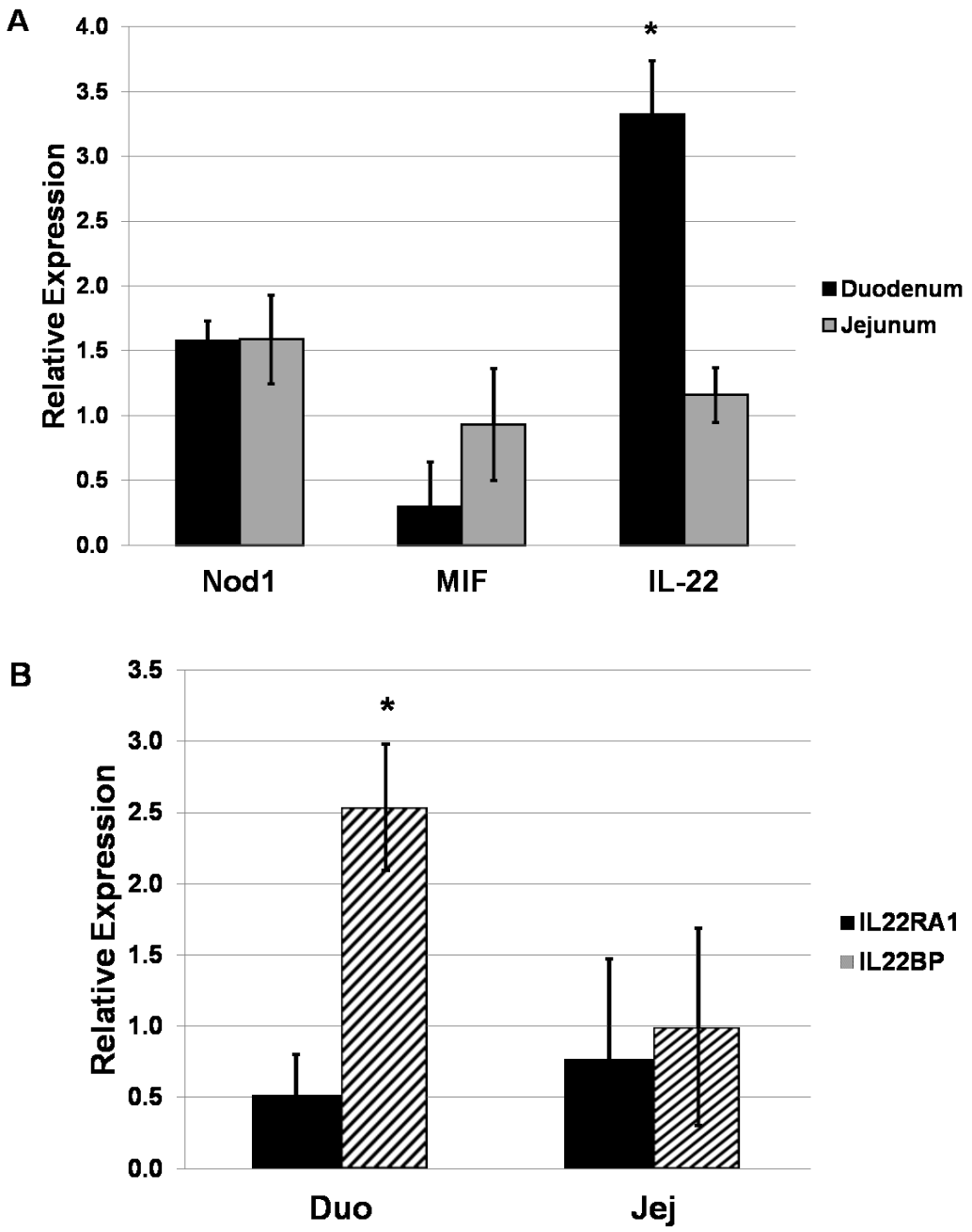



**Figure 6.1. Transcripts of inflammatory mediators during *Eimeria* infection.** Total RNA from small intestinal segments were obtained from *Eimeria*-challenged and non-challenged birds (Cox et al., 2010). Briefly, on day 8 post-hatch, the chicks were orally gavaged with 1 mL of mixed inoculums containing 50,000 *E. acervulina*, 10,000 *E. maxima*, and 2,500 *E. tenella* sporulated oocysts. On day 14 post-hatch, the small intestine segments (duodenum and jejunum) were collected, following total RNA extraction, and qRT-PCR.

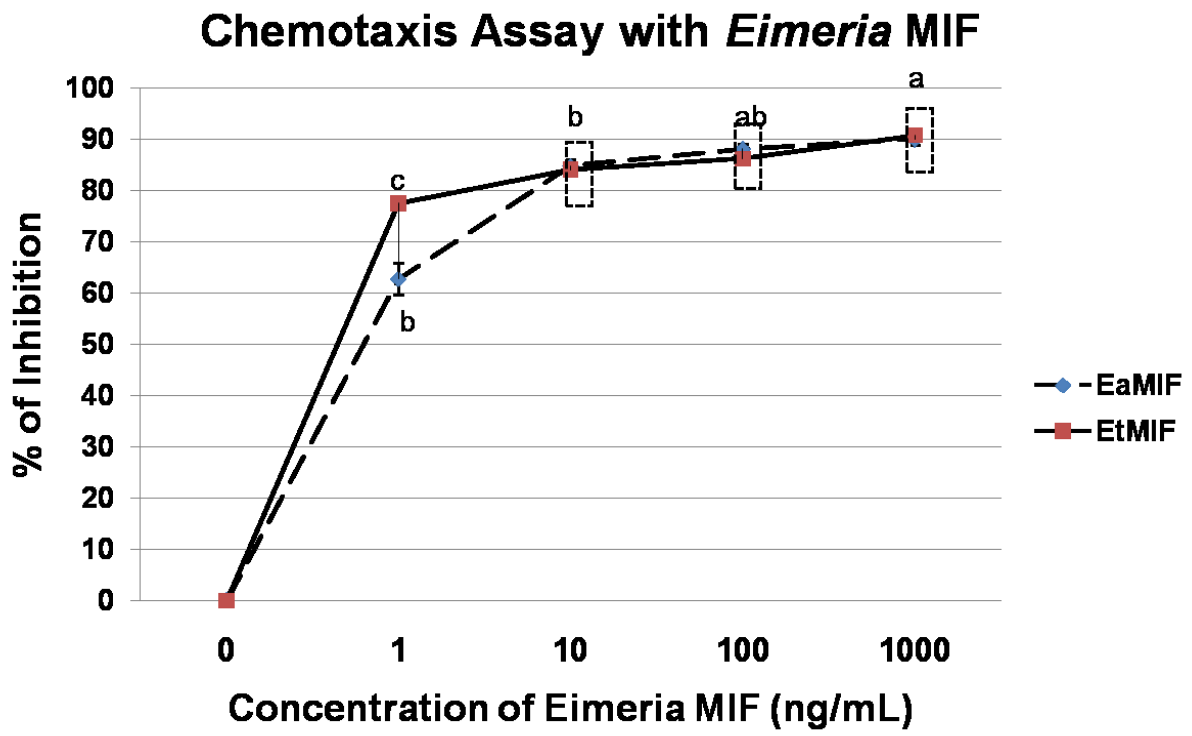
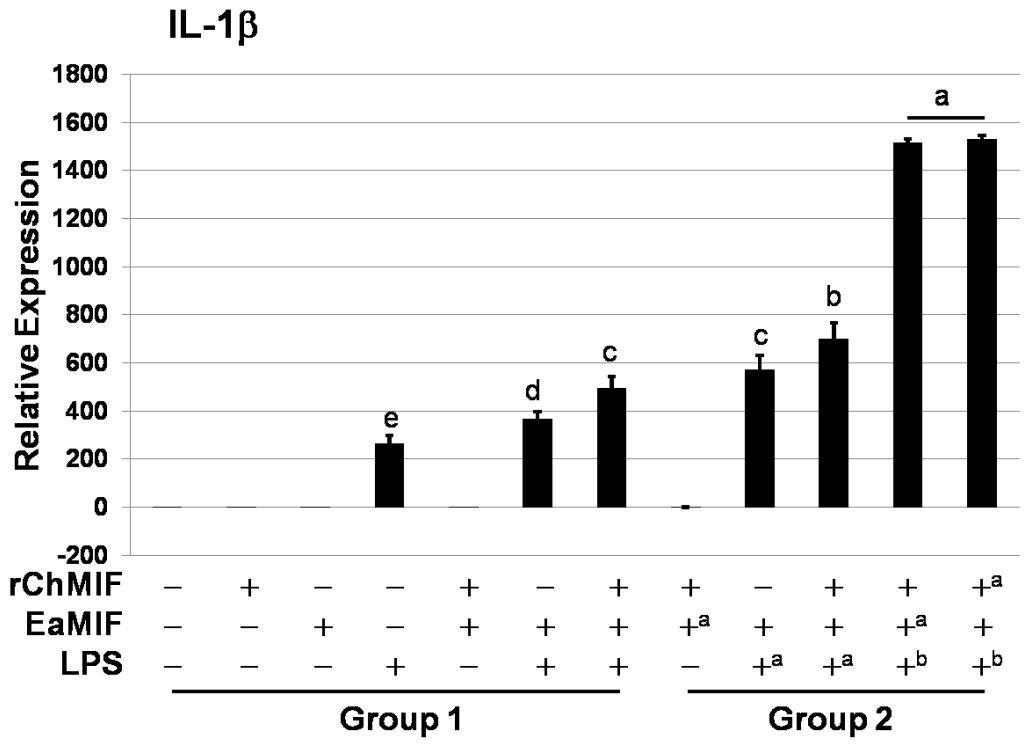
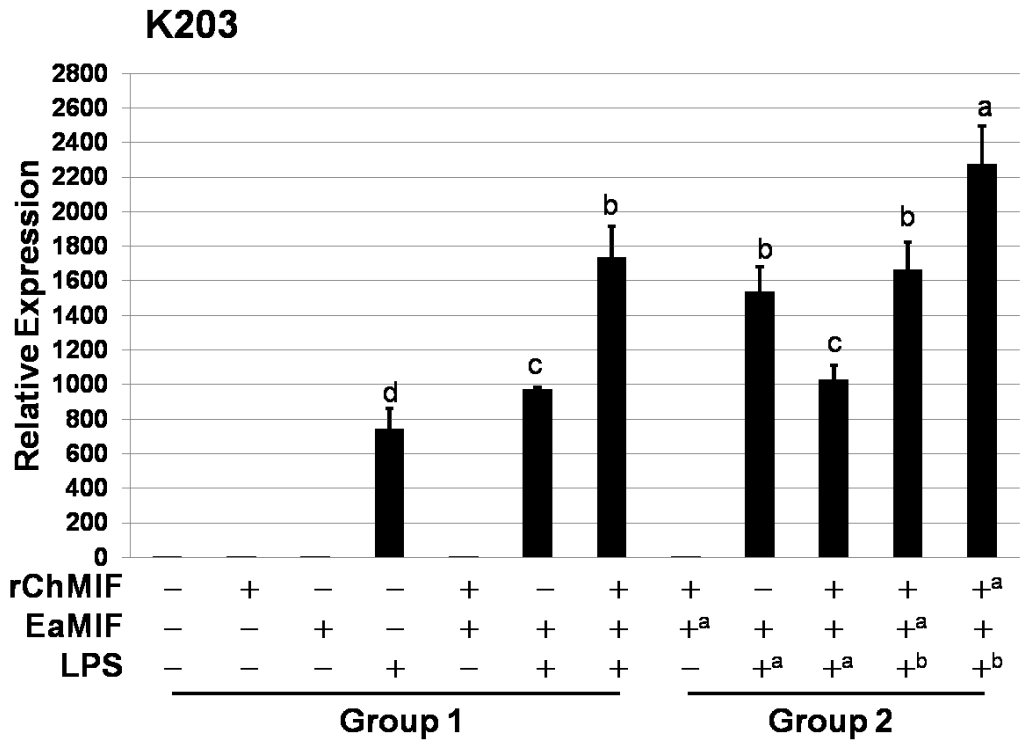



**Figure 6.2. Chemotaxis Assay in modified 48-well Boyden Chambers.** Migration of PBMCs was examined in the presence of different concentration of EaMIF ($10^{-3}$, $10^{-2}$, $10^{-1}$, and $10^{0}$ µg/ml). Isolated PBMCs were placed into the top chamber well separated by 5-µm pores membrane from Eimeria MIF in the bottom wells. The Chemotaxis chamber was incubated for 4 hr at 39°C in humidified air containing 5% $CO_2$. The cells were stained with DF staining method and the number of cells was counted. Each experiment was set up triplicate and the results represented mean of three individual experiments. Solid line with rectangle markers represents percentage of inhibition by EaMIF, while dash line with empty circle markers represents percentage of inhibition by EtMIF. Each letter indicates statistically significant difference ($p < 0.05$).


IL-1β
Relative Expression
1800
1600
1400
1200
1000
800
600
400
200
0
-200
a
b
c
c
d
e
rChMIF – + – – + – + + – + + +a
EaMIF – – + – + + + +a + + +a +
LPS – – – + – + + – +a +a +b +b
Group 1
Group 2


K203
Relative Expression
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
b
b
c
c
d
rChMIF – + – – + – + + – + + +a
EaMIF – – + – + + + +a + + +a +
LPS – – – + – + + – +a +a +b +b
Group 1
Group 2

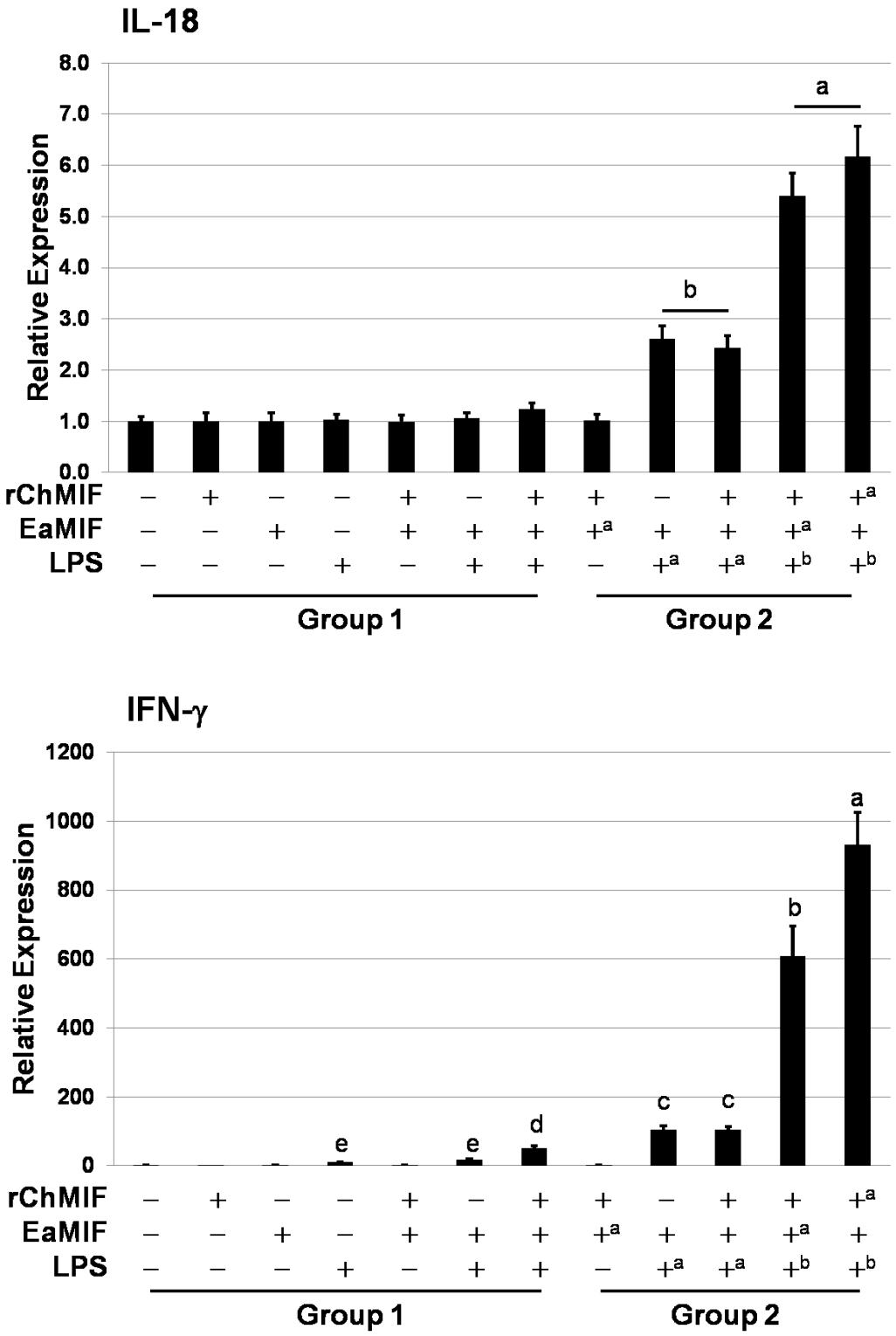



**Figure 6.3. Transcripts of pro-inflammatory cytokines and chemokines as influenced by EaMIF.** Freshly cultured chicken PBMCs were incubated for 6 h in two different groups as shown in Table 6.1. Cell lysis buffer was directly added into the wells and total RNA was

extracted using RNeasy Mini Kit (Qiagen, CA), following qRT-PCR analysis to measure mRNA level of chicken IL-1β, K203, IL-18 and IFN-γ. Each letter indicates statistically significant difference ($p < 0.05$).

## Epilogue

Inflammation is part of the complex immune response to pathogens and damaged cells. Many cellular and humoral immune components are involved in inflammatory responses, with the major players being signal starters (or sensors), modulators, and regulators. The members of NLR family Nod1 and Nod2 are intracellular innate sensors that initiate inflammatory responses. Based on its ligand in mammalian studies, Nod2 recognizes broader species of microorganisms than Nod1. However, there is currently no evidence of avian Nod2, based on avian genomic analyses including chicken, turkey and zebra finch, for which genome sequences have been published. Therefore, we hypothesized that avian Nod1 may recognize and be stimulated by both mammalian Nod1 and Nod2 ligands. To examine our hypothesis, the full-length ChNod1 and its mutant forms were amplified, cloned and expressed in HEK293T cells. Two mutants were cloned; one without the CARD domain resulting in failure of signal transduction, and the other in absence of LRR domain, which would result in failure of MAMPs recognition. However, sefl-activated ChNod1 molecule in HEK293T cells led to induced NF-κB activity. In addition, stimulation of chicken PBMCs with ligands for mammalian Nod1 or Nod2 did not affect mRNA levels of ChNod1 and its signal molecules, as well as pro-inflammatory mediators. Chicken PBMCs incubated with synthetic PGNs, which are agonists of both mammalian Nod1 and Nod2, led to induction of pro-inflammatory mediators, suggesting that ChNod1 recognizes a component of PGN, but not the same motif as in mammals.

During the characterization of ChNod1, we were interested in a mediator to enhance Nod1activation and signaling, and help convert signals from innate to adaptive immune response. Though there has been no direct connection between Nod1 and MIF in mammals, studies suggest that MIF functions as an immune mediator to transfer signals to adaptive

immunity from innate immune response initiated by Nod1. In avians, the chicken MIF nucleotide and deduced amino acid sequences were identified in 1993; however, there have been no further publications that characterized its biological function, though some reports showed differential RNA expression of ChMIF during early *Eimeria* infection. In our study, further characterization revealed that ChMIF enhanced expression of pro-inflammatory cytokines and chemokines in PBMCs when pre-stimulated with LPS. In addition, chicken lymphocytes stimulated with Con A showed enhanced expression of Th1 cytokines in the presence of ChMIF. Together, our results indicate that ChMIF boosts innate immunity as well as adaptive immune responses.

IL-10 and its subfamily members are well known inflammatory regulators. There are six members in the human IL-10 family; IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26. IL-22 has been studied intensively over the last decade and its biological function and signaling pathway has been reported. IL-22 is an inflammatory regulator produced by $CD4^+$ Th17, Th22, and NK-22 cells. Study of gene expression profiles showed induction of IL-22 mRNA levels as a result of Nod1 stimulation. In addition, activated Nod1 signaling induces proliferation of Th17 cells. Avian species have four members of the IL-10 family with the absence of IL-20 and IL-24 in comparison to mammals. Similar to mammalian IL-22, ChIL-22 did not affect immune cells due to the lack of ChIL22RA1, a specific receptor chain of ChIL-22 receptor complex. ChIL-22 is most likely involved in local inflammatory responses, where it showed positive feedback in stimulated epithelial cells. Furthermore, ChIL-22 stimulation of hepatocytes resulted in induction of acute phase reactants. Our data indicate that avian IL-22 induces pro-inflammatory mediators in epithelial cells and hepatocytes. Unfortunately, we could not observe negative feedback of ChIL-22 during local inflammatory response, an area of future research interest.

In this project, we could not find conclusive evidence that activated ChNod1 affected ChMIF and ChIL-22 during the inflammatory response since we could not effectively stimulate Nod1 signaling. In addition, expression of ChMIF, as well as ChNod1, was not changed under various tested conditions. Thus, in future studies, we first have to identify specific ligand(s) of ChNod1 to activate its signaling, and then characterize its role in the inflammatory response. Since no significant differences were observed in ChNod1 mRNA levels using synthetic ligands, one should examine how bacterial infections (e.g. *Salmonella*) affect the expression of ChNod1. Then, ChNod1 signaling and its impact on ChMIF and ChIL-22 should be studied in the context of epithelial cells because 1) ChIL-22 clearly influences epithelial cells, and 2) published mammalian research has shown important roles of Nod1 and IL-22 in mucosal immune responses. Furthermore, this approach would eventually expand avian immunology research to evaluate the functional roles of ChNod1, ChMIF and ChIL-22 in the mucosal immune system during *Eimeria* and other enteric infections. Nonetheless, the study of these avian inflammatory mediators constitutes a major first step in better understanding and characterizing avian immune responses during inflammation and pathogenic infecitons.

# Appendix A

## Molecular cloning and functional characterization of avian interleukin-19

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# Molecular cloning and functional characterization of avian interleukin-19

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ABSTRACT

The present study describes the cloning and functional characterization of avian interleukin (IL)-19, a cytokine that, in mammals, alters the balance of Th1 and Th2 cells in favor of the Th2 phenotype. The full-length avian IL-19 gene, located on chromosome 26, was amplified from LPS-stimulated chicken monocytes, and cloned into both prokaryotic (pET28a) and eukaryotic (pcDNA3.1) expression vectors. The confirmed avian IL-19 amino acid sequence has 66.5% homology with human and murine IL-19, with a predicted protein sequence of 176 amino acids. Analysis of avian IL-19 amino acid sequence showed six conserved, structurally relevant, cysteine residues as found in mammals, but only one N-glycosylation residue. The recombinant IL-19 (rChIL-19) expressed in the prokaryotic system was purified by $Ni^{+}$-resin column followed by endotoxin removal. Using purified avian rChIL-19, expression of Th2 cytokines was measured in splenocytes using quantitative real-time PCR (qRT-PCR). In the presence of rChIL-19, expression levels of IL-4 and IL-13, as well as IL-10, were significantly increased after 6- and 12 h treatments. This was confirmed by treating splenocytes with supernatants from IL-19 transfected cells. Also, avian monocytes incubated with rChIL-19 displayed increased expression of IL-1β, IL-6, and IL-19. This study represents the first report for the cloning, expression, and functional characterization of avian IL-19. Taken together, avian IL-19 function seems to be conserved and similar to that of mammals and may play an important role in responses to intracellular poultry pathogens like bacteria and protozoa.


## 1. Introduction

Interleukin (IL)-19, a recently discovered cytokine, belongs to the IL-10 family that includes IL-20, IL-22, IL-24 (MDA-7), and IL-26 (AK155) (Gallagher et al., 2000; Conti et al., 2003). It was initially identified by searching sequence databases for potential IL-10 homologs (Gallagher et al., 2000). Despite amino acid similarity between these cytokines, biological function of each member is different. Unfortunately, little is known about the biological function and gene regulation of IL-19. Mammalian IL-19 is secreted as a monomer that consists of seven helices, able to bind to its two-chain receptor (Chang et al., 2003; Gallagher et al., 2004). Mammalian IL-19 binds and signals through the IL-20R1/IL-20R2 heterodimer, like IL-20 and IL-24, although IL-20 and IL-24 are also ligands of IL-20R2/IL-22R1. Major sources of IL-19 are monocytes and B cells, and its expression can be up-regulated in monocytes following stimulation with lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, or tumor-necrosis factor (TNF)-alpha (Gallagher et al., 2000; Hsing et al., 2006). Although the biological effects of IL-19 are not clearly known, enhanced production of IL-19 elicits secretion of IL-6 and TNF-α in monocytes (Liao et al., 2002), and increases Th2 cytokine expression in activated $CD4^{+}$ T cells (Liao et al., 2004). In a dose-dependent manner, IL-19 induced IL-4 production and reduced IFN-γ production in activated human peripheral blood monocyte cells (PBMCs), suggesting that IL-19 altered the balance of Th1 and Th2 cells in favor of Th2 cells (Gallagher et al., 2004). IL-19 also induces apoptosis in monocytes and in lung epithelial cells, as a result of increased production of TNF-α (Liao et al., 2002; Hsing et al., 2008a,b). IL-19 stimulates monocytes and liver cells to produce reactive oxygen species (ROS) and promotes neutrophil chemotaxis (Hsing et al., 2008a,b). Clinically, IL-19 production is induced in post-cardiopulmonary bypass inflammatory response (Hsing et al., 2006) and severe sepsis (Hsing et al., 2008a,b), indicating that this cytokine may be involved in the pathogenesis of systemic inflammatory diseases.

A putative avian IL-19 nucleotide sequence was found during chicken genome analysis (Kaiser et al., 2005; Kaiser, 2007). There are only four avian members of the IL-10 family; IL-10, IL-19, IL-22, and IL-26. Similar to humans, chicken IL-19 (ChIL-19) is encoded in the same cluster with IL-10 on chromosome 26 in a region with synteny with human chromosome 1. Phylogenetic analysis suggests that ChIL-19 is basal to mammalian IL-19, while IL-20 and

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IL-24 were created by duplication of ChIL-19 (Kaiser et al., 2005). Although there is no research reporting the biological function of ChIL-19, increased expression of IL-19 transcripts was reported along with IL-4 and IL-13 in the chicken ileum following infections with extracellular pathogens (Kaiser, 2007).

In this study, the molecular cloning and functional characterization of avian IL-19 are reported. Recombinant ChIL-19 was cloned and expressed in both eukaryotic and prokaryotic systems. Observed biological effects of rChIL-19 included increased inflammatory response in monocytes and Th2 cytokines in lymphocytes, and increased apoptosis and production of nitric-oxide (NO) from monocytes.

## 2. Materials and methods

### 2.1. Birds, RNA source for cloning

Fifty-week old healthy Leghorn chickens were donated by Dr. Paul Siegel; birds had been housed and reared according to the Institutional Animal Care and Use Committee of Virginia Tech. Various tissues were collected including thymus, spleen, bursa, brain, lung, heart, intestine, and skin. Briefly, 50 mg tissues were mixed with 1 ml of TRI Reagent and homogenized using VWR PowerMax AHS 200 (VWR, PA). By addition of 0.2 ml of chloroform and centrifugation, total RNA was separated from DNA and proteins, and precipitated with 0.5 ml isopropanol, followed by centrifugation at 12,000 × *g* for 5 min at 4 °C. Precipitated total RNA was dissolved with RNase-free water and possible genomic DNA was removed by incubation with RNase-free DNase treatment (Qiagen, CA) at 37 °C for 1 h.

### 2.2. Isolation of lymphocytes and peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma, MO). Briefly, 10 ml of blood was collected by cardiac puncture from a single bird. The collected blood was diluted with equal volume of HBSS (HyClone, UT) and centrifuged at 50 × *g* for 10 min. The supernatant and buffy coat was collected and carefully overlaid on Histopaqe-1077. By centrifugation at 400 × *g* for 30 min, mononuclear cells were separated from plasma and red blood cells. The collected mononuclear cells were washed twice with DMEM (Mediatech, VA) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Amphotericin B, and 10% fetal calf serum (FCS) (all supplied by Atlanta Biologicals, GA), and incubated for 24 h at 40 °C with 5% $CO_2$ humidified air. The non-adherent cells were then removed by washing three times with warm DMEM.

Chicken lymphocytes were prepared from spleens using 0.22 μm cell strainer (BD, CA) and by serial centrifugation. Lymphocytes were cultured in RPMI-1640 (Mediatech, VA) containing 20% FCS and 1% penicillin/streptomycin and Amphotericin B for 24 h at 40 °C and 5% $CO_2$. Non-adherent cells were collected and seeded onto 24-well plates.

### 2.3. Cloning and expression of recombinant ChIL-19 (rChIL-19) in E. coli

For amplification of the full-length ChIL-19, a candidate sequence was identified from the chicken genome database. Using the predicted ChIL-19 sequence (GenBank Accession # XM_425824), primer sets were designed (Table 1). Total RNA was isolated from LPS-stimulated PBMCs using RNeasy Mini Kit (Qiagen, CA), followed by synthesis of the first-strand cDNA using iScript (Bio-Rad, CA). Polymerase chain reaction (PCR) was performed to amplify the full-length ChIL-19 using the following conditions; initial denaturation at 92 °C for 2 min, 35 cycles of denaturation at 92 °C for 15 s, annealing at 54 °C for 15 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 7 min. The freshly synthesized full-length ChIL-19 was directly inserted into pCR2.1-TOPO vector (Invitrogen, CA), followed by transformation into *E. coli* TOP10 (Invitrogen, CA). Transformed *E. coli* TOP10 were cultured in Luria-Bertani media (Fisher Scientific, NJ) at 37 °C overnight. A transformant was selected by a combination of PCR screening and endonuclease digestion with *EcoR* I (New England Biolabs, MA), and the sequence confirmed by sequencing (Virginia Bioinformatics Institute at VT, VA). For sub-cloning into eukaryotic or prokaryotic expression vectors, ChIL-19 was digested with endonucleases *BamH* I and *Not* I (New England Biolabs, MA). The digested ChIL-19 fragment was purified from an agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, WI), and ligation was performed with digested pcDNA3.1 (Invitrogen, CA) for eukaryotic expression or pET28a (Novagen, CA) for prokaryotic expression at 4 °C overnight. After transformation of ligated vectors into *E. coli* TOP10,

**Table 1**
Primer sequences for cloning chicken IL-19 and real-time PCR analyses of cytokine transcript expressions.

| Name | Accession no. | Nucleotide sequence (5′ → 3′) | Application |
|---|---|---|---|
| chIL-19_F | XM_425824 | GATCGGATCCATGCTGGGCTCCCGTGT | Cloning of recombinant ChIL-19 |
| chIL-19_R | | GATCGCGGCCGCCTACTTATCGTCGTCATCCTTGTAATCTGGGGATTTCTCCATCCAGTCC | |
| IL-1b_F | NM_204524 | GCTCTACATGTCGTGTGTGATGAG | Real-time PCR |
| IL-1b_R | | TGTCGATGTCCCGCATGA | |
| IL-4_F | NM_001007079 | GCTCTCAGTGCCGCTGATG | |
| IL-4_R | | GAAACCTCTCCCTGGATGTCAT | |
| IL-6_F | NM_204628 | GAACGTCGAGTCTCTGTGCTAC | |
| IL-6_R | | CACCATCTGCCGGATCGT | |
| IL-13_F | NM_001007085 | CATGACCGACTGCAAGAAGGA | |
| IL-13_R | | CCGTGCAGGCTCTTCAGACT | |
| IL-19_F | XM_425824 | AGCCGGGAACACGATCCTCCACTT | |
| IL-19_R | | TGCAGAGAGTGTGGGTGGGACAGG | |
| IFNgamma_F | NM_205149 | GCTCCCGATGAACGACTTGA | |
| IFNgamma_R | | TGTAAGATGCTGAAGAGTTCATTCG | |
| iNOS_F | D85422 | CCTGTACTGAAGGTGGCTATTGG | |
| iNOS_R | | AGGCCTGTGAGAGTGTGCAA | |
| GAPDH | NM_204305 | AGGGTGGTGCTAAGCGTGTTA | |
| GAPDH | | TCTCATGGTTGACACCCATCA | |

transformants were screened and nucleotide sequences were confirmed.

To express rChIL-19 in *E. coli*, purified ChIL-19-ET28a plasmid was introduced into *E. coli* BL21 (New England Biolabs, MA). A single colony was isolated and pre-cultured into 10 ml of 2TY media (16 g of tryptone, 10 g of yeast extract and 5 g of NaCl per liter) containing kanamycin (Fisher Scientific, NJ). For the main culture, 50 μl of pre-culture solution were inoculated into 50 ml of 2TY media containing kanamycin, and bacteria were incubated at 30 °C overnight on a rotating shaker at 180 rpm. The expression of rChIL-19 was induced by adding of 1 mM IPTG (Gold Biotechnology, MO) and bacteria were cultured for an additional 5.5 h at 25 °C at 200 rpm. The culture solution was transferred into 50 ml centrifuge tubes and incubated on ice for 10 min. The bacteria were pelleted by centrifugation at 3,500 × *g* for 1.5 h at 4 °C. The supernatant was removed and the pellet treated with 10 ml denaturation buffer (8 M Urea, 0.1 M $NaH_2PO_4$, and 0.01 M Tris, pH 8.0) after sonication. Recombinant ChIL-19 was purified using $Ni^+$-resin (Invitrogen, CA), the purified protein concentrated and buffer changed by ultrafiltration using a 10,000 molecular weight cutoff membrane (Amicon Ultra-15 Centrifugal Filter Unit; Millipore, MA). Endotoxin was removed using ProteoSpin Endotoxin Removal Micro Kits (Norgenbiotek, ON, Canada) resulting in levels <0.06 EU/mg protein as indicated by the Limulus Amebocyte Lysate assay (Lonza, MD).

### 2.4. Transfection and expression of rChIL-19 in HTC cells

One day prior to transfection, HTC cells (avian macrophage cell line provided by Dr. Rath, USDA) were counted using a hemacytometer and seeded at $5 \times 10^5$ cells/well in a 6-well plate, and incubated for 24 h at 40 °C and 5% $CO_2$. The cells were gently washed once with DMEM and incubated with serum- and antibiotics-free DMEM for 30 min. Five micrograms of plasmid DNA were prepared with Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instruction. The prepared DNA mixture was added and cells were incubated overnight. After changing medium the next day, the cells were cultured for an additional 48 h. The supernatant was collected and concentrated using a 10,000 MW cutoff membrane. The cells were directly treated with lysis buffer for isolation of total RNA.

### 2.5. Western blot

Concentration of both purified rChIL-19 and concentrated supernatant were measured using a BCA Protein Assay Kit (Pierce, IL). For SDS-PAGE, 2 μg and 4 μl of the purified rChIL-19 and the concentrated supernatants, respectively, were mixed with 10 μl of SDS sample buffer. Then, the samples were heated on a 100 °C hot plate for 10 min and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane (Millipore, MA) and the membrane incubated with anti-FLAG mouse antibody (Sigma, MO; 1/1,000) in blocking buffer containing 1% BSA (Sigma, MO) and 0.05% Tween-20 (Sigma, MO) in PBS for 1 h. After washing the membrane three times (wash buffer: 0.05% Tween-20 in PBS), anti-mouse IgG goat antibody (Sigma, MO; 1/5,000) was added and incubated for 1 h. Using SuperSignal West Pico Chemiluminescent Substrate (Pierce, IL), HRP signal was enhanced and detected on CL-XPosure film (Thermo Scientific, IL).

### 2.6. In vitro biological function analysis

The PBMCs were cultured at $2 \times 10^6$ cells/well in 12-well plates, and then treated with media alone, LPS (5 μg/ml), or rChIL-19 (0.1 μg/ml) for either 6 h or 12 h. After incubation, the supernatants were collected for NO assay and total RNA was extracted using RNeasy Mini Kit.

The spleen lymphocytes were cultured at $4 \times 10^6$ cells/well in 12-well plates and then treated with media alone, Concanavalin A (Con A; Sigma, MO; 10 μg/ml), rChIL-19 (0.1 μg/ml or 1 μg/ml) or Con A with rChIL-19 (0.1 μg/ml) for 6 h or 12 h. The supernatants were collected and production of IFN-γ was measured by ELISA (Invitrogen, CA). To analyze the induction of Th2 cytokine transcripts by IL-19, total RNA was isolated from the treated lymphocytes.

```
001   ATGCTGGGCTCCCGTGTGCTGCTCTGCCTCTGCTCCATGACCTGCTGCCTCACCATGCTG
001    M  L  G  S  R  V  L  L  C  L  C  S  M  T  C  C  L  T  M  L

061   CCGGCAGCCGGGAACACGATCCTCCACTTTGGCCCCTGTAGGATTTCAATGAGCATGAGT
021    P  A  A  G  N  T  I  L  H  F  G  P  C  R  I  S  M  S  M  S

121   GAGATCAGGGCTGGCTTCACTGCCATCAAAACCAACATCCAAGCCCGGGACCCCATCAGG
041    E  I  R  A  G  F  T  A  I  K  T  N  I  Q  A  R  D  P  I  R

181   ACGCTGAGCATCCTGTCCCACCCACACTCTCTGCACAGGGTCCAGCCTTCAGATAAATGC
061    T  L  S  I  L  S  H  P  H  S  L  H  R  V  Q  P  S  D  K  C

241   TGCATCGTCCACAAAGTCTTCAACTTCTACGTGGACAAAGTCTTCAAGCACTGCCAGACT
081    C  I  V  H  K  V  F  N  F  Y  V  D  K  V  F  K  H  C  Q  T

301   GAGAATTCCTACATCAACCGAAAGATCAGCAGCATTGCCAACTCCTTCCTCAGCATCAAA
101    E  N  S  Y  I  N  R  K  I  S  S  I  A  N  S  F  L  S  I  K

361   AGGAAACTCGAGCAATGTCATGATGAAAATAAGTGCTTGTGTGGACAGGAACCCACGGAG
121    R  K  L  E  Q  C  H  D  E  N  K  C  L  C  G  Q  E  P  T  E

421   AGATTTAAGCAGATCCTTGTGAACTACGAAGGGCTGAACGTCACATCAGCAGCAATGAAA
141    R  F  K  Q  I  L  V  N  Y  E  G  L  N  V  T  S  A  A  M  K

481   TCCCTGGGTGAGCTGGACATCCTGCTGGACTGGATGGAGAAATCCCCATAG
161    S  L  G  E  L  D  I  L  L  D  W  M  E  K  S  P  *
```

**Fig. 1.** Nucleotide and deduced amino acid sequences of ChIL-19. The primers used to amplify the full-length ChIL-19 are underlined. The double underlined sequences were used as primers for qRT-PCR. The boxed sequences represent putative glycosylation site.

(A)

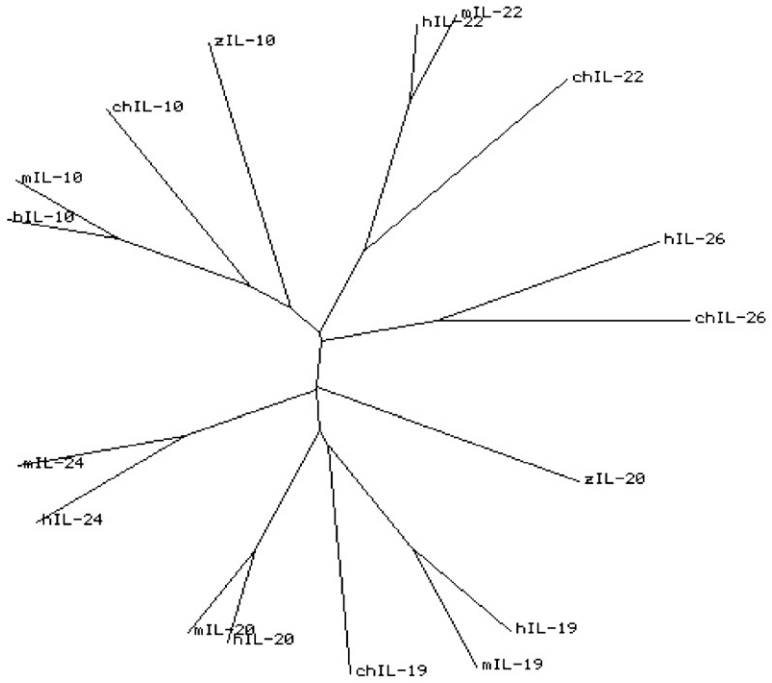
```
hIL-19          MKLQCVSLWLLGTILILCSVDNHG-----LRRCLISTDMHHIEESFQEIKRAIQAKDTFP 55
mIL-19          MKTQCASTWLLGMTLILCSVHIYS-----LRRCLISVDMRLIEKSFHEIKRAMQTKDTFK 55
chIL-19         MLGSRVLLCLCSMTCCLTMLPAAGNTILHFGPCRISMSMSEIRAGFTAIKTNIQARDPIR 60
                *        *      *               * **  *  *   *  **   *  *

hIL-19          NVTILSTLETLQIIKPLDVCCVTKNLLAFYVDRVFKDHQEPNPKILRKISSIANSFLYMQ 115
mIL-19          NVTILS-LENLRSIKPGDVCCMTNNLLTFYRDRVFQDHQERSLEVLRRISSIANSFLCVQ 114
chIL-19         TLSILSHPHSLHRVQPSDKCCIVHKVFNFYVDKVFKHCQTENSYINRKISSIANSFLSIK 120
                   ***    *    * * **       ** * **   *       * *********

hIL-19          KTLRQCQEQRQCHCRQEATNATRVIHDNYDQLEVHAAAIKSLGELDVFLAWINKNHEVMF 175
mIL-19          KSLERCQVHRQCNCSQEATNATRIIHDNYNQLEVSSAALKSLGELNILLAWIDRNHLETP 174
chIL-19         RKLEQCHDENKCLCGQEPTERFKQILVNYEGLNVTSAAMKSLGELDILLDWMEKSP---- 176
                  *  *     * * ** *     *  **  * *  ** ******   * *

hIL-19          SA 177
mIL-19          AA 176
chIL-19         --
```

(B)




(C)

```
                Helix A                         Helix B
chIL-10         -MQTCCQALLLLLAACTLPAHCLEPTCLHFS-ELLPARLRELRVKFEEIKDYFQSRDDEL 58
chIL-19         MLGSRVLLCLCSMTCCLTMLPAAGNTILHFGPCRISMSMSEIRAGFTAIKTNIQARDPIR 60
                   Helix A                         Helix B

                Helix C          Helix D                    Helix E
chIL-10         NIQLLSS-ELLDEFKGTFGCQSVSEMLRFYTDEVLPRAMQTSTSHQQSMGDLGNMLLGLK 117
chIL-19         TLSILSHPHSLHRVQPSDKCCIVHKVFNFYVDKVFKHCQTENSYINRKISSIANSFLSIK 120
                   Helix C          Helix D                    Helix E

                                Helix F
chIL-10         ATMRRCH---RFFTCEKRSKAIKQIKETFEKMDEN-GIYKAMGEFDIFINYIEEYLLMRR 173
chIL-19         RKLEQCHDENKCLCGQEPTERFKQILVNYEGLNVTSAAMKSLGELDILLDWMEKSP---- 176
                                Helix F                    Helix G

chIL-10         RK 175
chIL-19         --
```

**Fig. 2.** Sequence comparison and phylogenetic tree of amino acids encoded by ChIL-19. (A) Sequences were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2 ). Asterisks (*) indicate identical residues among sequences. Six conserved cysteine residues were highlighted yellow, and underlined sequences represent putative glycosylation sequences. (B) Phylogenetic tree was constructed by Phylodendron program using amino acid sequences aligned

### 2.7. Quantitative real-time PCR (qRT-PCR) analysis of the cytokine transcripts

To analyze the transcripts of various cytokines, primers were designed using Primer Express (Ver 3.0; Applied Biosystems, CA) (Table 1), and qRT-PCR was performed using FAST SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions in an ABI 7500 FAST Real-Time PCR System. The cDNA was diluted (1/25) with nuclease-free water, and 2 μl of the solution were used for FAST SYBR Green Master Mix; 100 nM primers and 10 μl of SYBR Green Master Mix in nuclease-free water in a final volume of 20 μl. Samples were heated to 95 °C for 20 s as initial denaturation and 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 57 °C for 30 s. Melt curve stage was added to analyze individual PCR products. GAPDH was used as an internal control gene to normalize for RNA quantity. The results were analyzed using 7500 Software (Ver 2.0; Applied Biosystems).

### 2.8. Apoptosis assay

The isolated monocytes were treated with media alone, LPS (5 μg/ml) or rChIL-19 (0.1 μg/ml) for 12 hr and cell apoptosis was determined using Annexin-V FITC Apoptosis Kit (Invitrogen). Treated cells were harvested by trypsinization and washed twice with PBS. They were resuspended at $2 \times 10^6$ cells/ml in 1X Annexin-V binding buffer. After transferring 5 μl of cells, 5 μl of Annexin-V FITC and 10 μl of propidium iodide were added and incubated for 15 min in the dark. The samples were analyzed by flow cytometry (BD FACS Aria, CA).

### 2.9. Nitric oxide (NO) assay

Using collected supernatant from treated monocytes, NO assay was performed using the Griess reagent system (Promega, WI). Fifty microliters of sample and nitrite standards were added in a 96-well plate, and then 50 μl of sulfanilamide solution and NED solution were added sequentially. The absorbance was measured at 540 nm and production of NO was determined by nitrite amounts in the media.

### 2.10. Statistical analysis

All data were analyzed by either Student's *t*-test or Analysis of Variance (ANOVA) using JMP (Ver 7.0) software, and significant differences among groups were tested by the Tukey-Kramer Honestly Significant Difference post-hoc procedure.

## 3. Results

### 3.1. Sequence analysis of ChIL-19

Sequence analysis showed that the cloned ChIL-19 consists of 176 amino acids (aa) with a predicted isoelectric point of 8.78 and a molecular weight of 19.9 kDa (Fig. 1). There is an IL-10 related domain stretching from aa 140 to the C terminus. In comparison with human and mouse IL-19, ChIL-19 has six conserved cysteine residues (Fig. 2A) and one potential glycosylation site, Asn153-Val-Thr155 (Fig. 1), while human IL-19 has two and mouse IL-19 has three glycosylation sites (Liao et al., 2002). Multiple sequence alignment showed 39% and 36% amino acid identity with human and mouse IL-19, respectively, and 66.5% similarity with both human and mouse. Phylogenetic analysis showed that ChIL-19 is evolutionarily close to the mammalian IL-19 group, not the IL-20 group (Fig. 2B). Analysis of secondary structure shows six highly conserved helix structures between ChIL-10 and ChIL-19 (Fig. 2C).

### 3.2. Expression of ChIL-19

The expression of ChIL-19 gene was examined in various tissues, including thymus, spleen, heart, lung, intestine and skin using qRT-PCR (Fig. 3A). The LPS-stimulated monocytes were also included for comparison. ChIL-19 expression was normalized with GAPDH, and then relative expression of ChIL-19 in various tissues was compared to that of the spleen. In comparison with the spleen, skin tissue showed the highest expression of ChIL-19, as did the LPS-stimulated monocytes. The heart, lung and spleen were also good sources for ChIL-19 expression, but not the thymus and brain.

### 3.3. Production and purification of rChIL-19

Recombinant ChIL-19 was expressed with a FLAG tag from both *E. coli* and HTC cells to allow detection by Western blot using anti-FLAG mouse antibody. Molecular weight at 24.4 kDa and 20.9 kDa bands were detected for rChIL-19 from *E. coli* and HTC cells, respectively (Fig. 3B).

### 3.4. Modulation of pro-inflammatory cytokines by ChIL-19 in monocytes

To determine the biological effects of ChIL-19 on the production of cytokines in monocytes, chicken PBMCs were incubated with media alone, 5 μg/ml of LPS or 0.1 μg/ml of rChIL-19 for either 6 h or 12 h. Total RNA was isolated from the treated monocytes and transcripts of cytokines were measured by qRT-PCR. The presence of rChIL-19 led to significantly increased expression of the pro-inflammatory cytokines IL-1β (4.3- and 2.6-fold) and IL-6 (7.1- and 2.6-fold) at 6 h and 12 h, respectively (Fig. 4). Transcripts of IL-1β and IL-6 showed higher expression in the 6 h treatment with rChIL-19 than in the 12 h treatment, suggesting that rChIL-19 stimulates monocytes earlier than 6 h. In an autocrine fashion, rChIL-19 induced ChIL-19 transcripts at 2.4-fold at 12 h post-treatment of PBMCs. Additionally, qRT-PCR showed 3.4-fold increase of the transcript of the inducible nitric oxide synthase (iNOS) gene 12 h post-treatment with rChIL-19. IL-4 transcripts were measured as a negative control, showing no significant change in the presence or absence of rChIL-19. LPS-treated PBMCs (positive control) showed significantly increased pro-inflammatory cytokines, IL-1β and IL-6, as well as iNOS transcripts, but not IL-4 (data not shown). PBMCs were also treated by concentrated supernatants of transiently over-expressed rChIL-19 in HTC cells. However, the addition of supernatants in PBMCs did not show any significant induction of pro-inflammatory cytokines after 6 h or 12 h (data not shown). Together, rChIL-19 induced pro-inflammatory cytokines including IL-19 itself, suggesting a role of ChIL-19 in inflammation.

with ClustalW2 (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html ). (C) Chicken IL-10 and ChIL-19 amino acid sequences were aligned and secondary helices of IL-10 and IL-19 were shown in vertical and horizontal lined box, respectively. Red bold cysteine indicates the structurally conserved cysteine residues. Human IL-10 (Accession # NP_000563 ); mouse IL-10 (Accession # NP_034678 ); chicken IL-10 (Accession # NP_001004414 ); zebrafish IL-10 (Accession # NP_001018621 ); human IL-19 (Accession # NP_037503 ); mouse IL-19 (Accession # NP_001009940 ); ChIL-19 (Accession # XP_425824 ); human IL-20 (Accession # NP_061194 ); mouse IL-20 (Accession # NP_067355 ); zebrafish IL-20 (accession no. NP_001076424 ); human IL-22 (Accession # NP_065386 ); mouse IL-22 (Accession # NP_058667 ); chicken IL-22 (Accession # XP_416079 ); human IL-24 (Accession # NP_006841 ); mouse IL-24 (Accession # NP_444325 ); human IL-26 (Accession # NP_060872 ); chicken IL-26 (Accession # NP_060872 ).

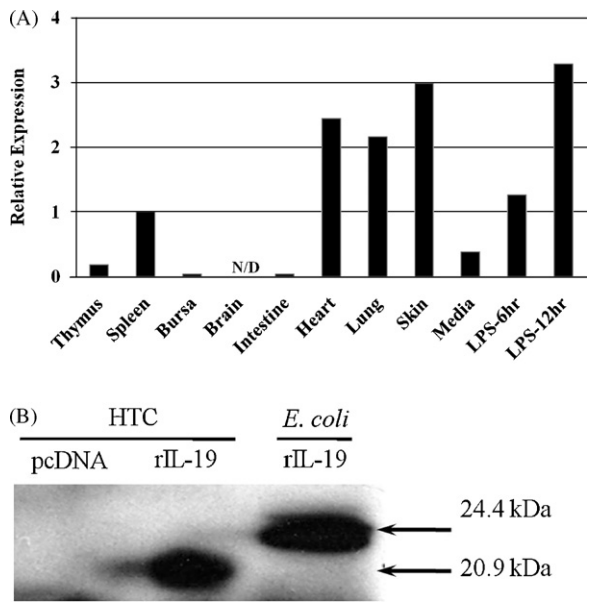



**Fig. 3.** Expression of ChIL-19. (A) ChIL-19 expression was measured by qRT-PCR. The transcript level of ChIL-19 was normalized against that of GAPDH, and the relative expression of ChIL-19 in various tissues was compared against spleen. As control, ChIL-19 expression was shown from stimulated monocytes with LPS for 6 h and 12 h (N/D = not detected). (B) The expression of rChIL-19 was detected by Western blot with anti-FLAG antibody.

### 3.5. Modulation of Th2 cytokines by rChIL-19 in lymphocytes

To determine the function of rChIL-19 in lymphocytes, chicken splenocytes were isolated and treated with Con A (10 μg/ml) or two concentrations of rChIL-19 (0.1 μg/ml or 1 μg/ml) for either 6 h or 12 h. qRT-PCR was performed to measure the transcripts of the Th2 cytokines IL-4, IL-13, and IL-5 (Fig. 5). IL-4 transcripts were significantly increased in the 6 h treatment by both concentrations of rChIL-19 (1.9- and 3.3-fold, respectively), but no significant difference was found in the 12 h treatment, although Con A induced IL-4 transcripts in both 6 h and 12 h treatments (3.8- and 2.3-fold, respectively) (Fig. 5A). Recombinant ChIL-19 induced IL-13 transcripts, especially in the 1 μg/ml rChIL-19 treatment (2.2- and 4.2-fold at 6 h and 12 h, respectively) (Fig. 5B). Induced IL-10 transcripts were also observed (data not shown). IL-5 transcripts were undetectable in the presence of rChIL-19. The increased IL-4 and IL-13 transcripts were confirmed by treatment of concen-

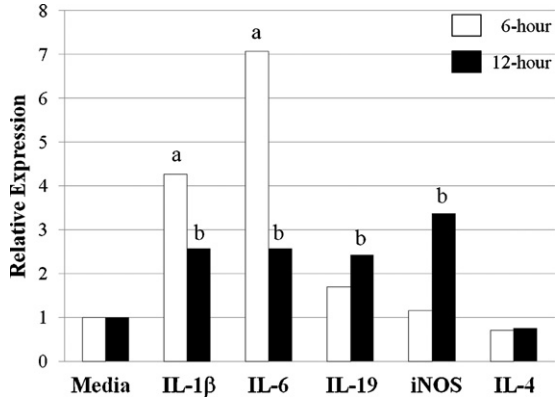



**Fig. 4.** The biological effect of rChIL-19 in PBMCs. Isolated PBMCs were treated with media alone, LPS (5 μg/ml) or rChIL-19 (100 ng/ml) for either 6 h or 12 h. The expression of pro-inflammatory cytokines were measured by qRT-PCR and compared to control media alone. Each bar represents the mean of three different experiments. Bars with no common letters are significantly different ($P < 0.05$).

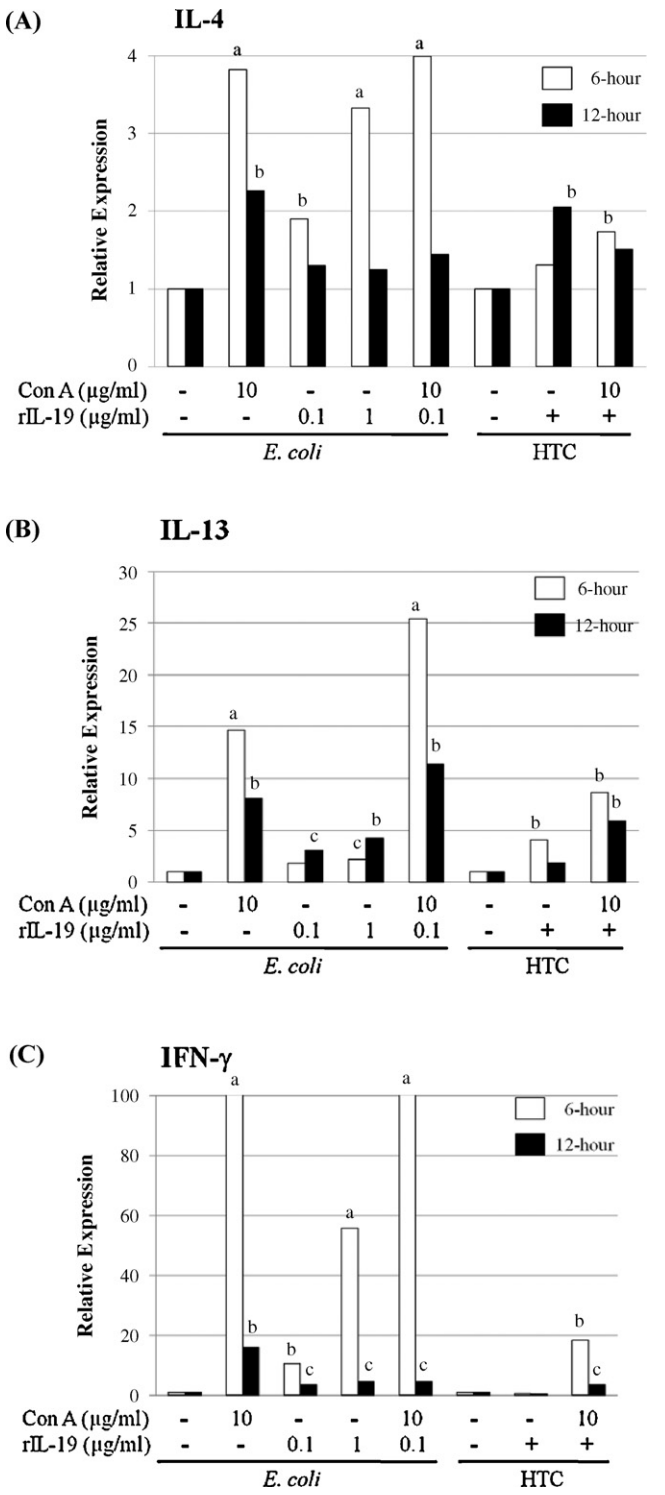



**Fig. 5.** The biological function of rChIL-19 in lymphocytes. Splenocytes were treated with media alone, Con A (10 μg/ml), rChIL-19 (0.1 μg/ml or 1 μg/ml), concentrated supernatant from vector only-transfected HTC cells, or supernatants from rChIL-19-transfected HTC cells. Also, splenocytes were pre-activated with Con A (10 μg/ml) for 3 h, followed by stimulation of rChIL-19 either from *E. coli* or HTC cells. The expression of Th2 cytokines and IFN-γ were measured by qRT-PCR and their transcripts were compared with control media alone or vector only-transfected HTC cells. Bars with no common letters are significantly different ($P < 0.05$).

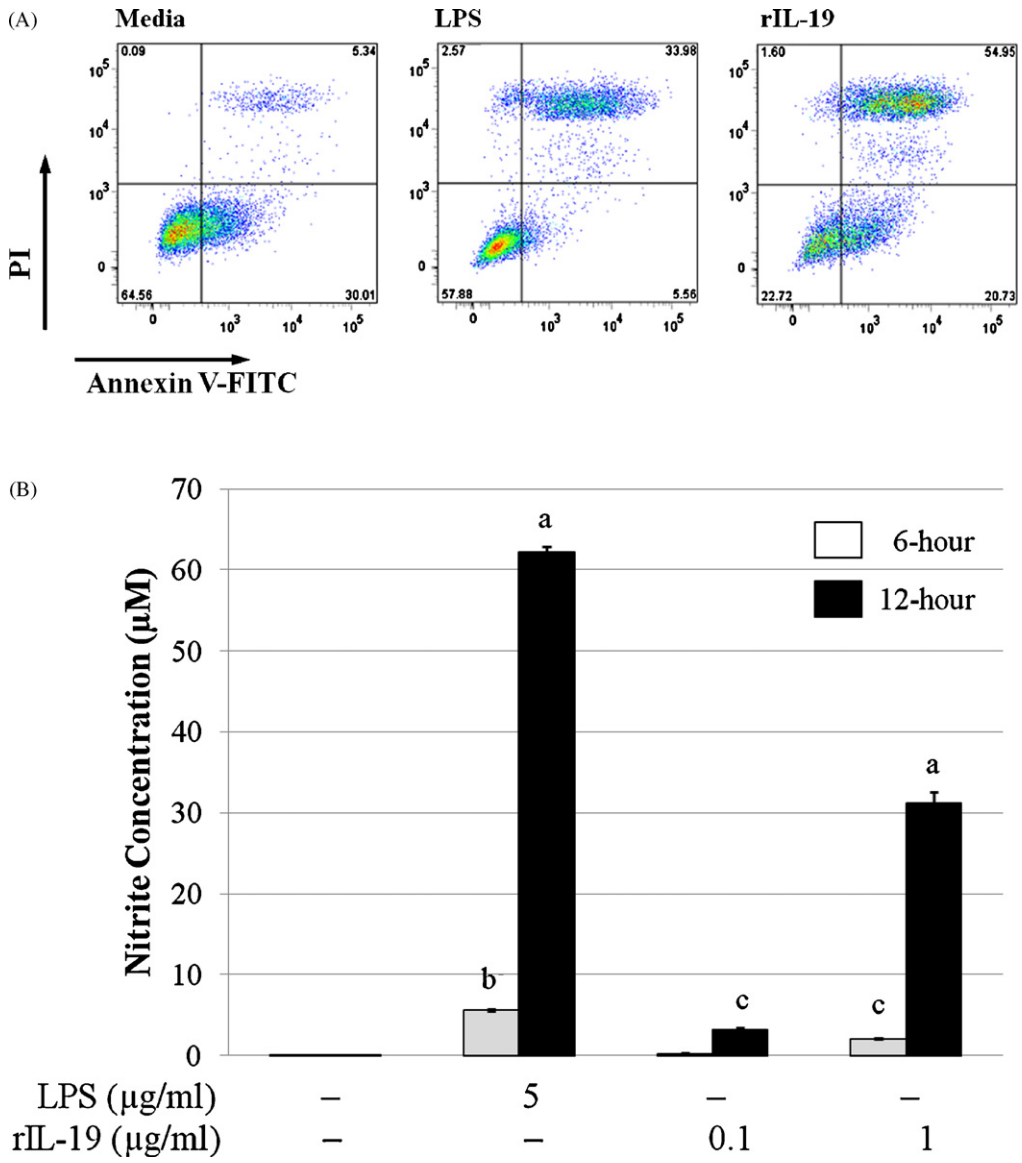



**Fig. 6.** The effect of rChIL-19 on cell apoptosis and NO production in monocytes. (A) PBMCs were treated with media alone, LPS (5 μg/ml), or rChIL-19 (0.1 μg/ml) for 12 h and apoptosis assay was performed using Annexin V-FITC and propidium iodide. (B) After PBMCs were treated with media alone, LPS (5 μg/ml), or rIL-19 (0.1 μg/ml or 1 μg/ml), the supernatants were collected and NO production was measured by Griess reagent system. Each bar represents the mean of three different experiments. Bars with no common letters are significantly different ($P < 0.05$).

trated supernatants of transiently over-expressed rChIL-19 in HTC cells. To examine the effect of rChIL-19 on activated lymphocytes, isolated splenocytes were stimulated with Con A (10 μg/ml) for 3 h. Recombinant ChIL-19 was then added to the media and the cells incubated for either 6 h or 12 h. Pre-activation of splenocytes showed significant difference of IL-4 transcripts only at the 6 h post-treatment (4-fold) by rChIL-19, but transcript level of IL-13 was induced by rChIL-19 in pre-activated lymphocytes at both time points (25.4- and 11.3-fold at 6 h and 12 h, respectively). We also observed significantly increased IFN-γ transcripts in the presence of rChIL-19 at both time points.

### 3.6. Monocyte apoptosis induced by rChIL-19

To examine the apoptotic effect of rChIL-19, an apoptosis assay was performed in treated PBMCs with either LPS (5 μg/ml) or rChIL-19 (0.1 μg/ml) for 12 h, followed by FACS analysis. In comparison to media alone (5.34% of cell population), both LPS (33.98%) and rChIL-19 (54.95%) treated cells showed increased ratio of dead cells (Fig. 6A; upper right quadrant of each histogram). Although we could not clearly detect the apoptotic population (bottom right quadrant) in LPS treated group (5.56%), rChIL-19 treated monocytes (20.73%) showed an increased population of apoptotic cells, suggesting an rChIL-19 induced apoptosis in monocytes.

### 3.7. Induced production of NO by rChIL-19

During gene expression analysis, iNOS transcripts were significantly induced by rChIL-19 at 12 h post-treatment. Thus, the effect of rCIL-19 in NO production was examined by measuring nitrite using Griess reagent as described above. Monocytes were treated with two different concentrations of rChIL-19 for 6 h or 12 h, and nitrite concentration in the supernatant was measured. In comparison with media alone, LPS treated PBMCs produced 5.58 μM and 62.2 μM of NO for 6 h and 12 h, respectively (Fig. 6B). At a low concentration of rChIL-19 (0.1 μg/ml), little NO production was induced (3.15 μM) after 12 h. At a higher concentration of rChIL-19 (1 μg/ml) significant production of NO after 6 h (2.08 μM) and

12 h (31.22 μM) treatments was observed, suggesting that ChIL-19 induces intracellular production of reactive oxygen species in the form of NO.

## 4. Discussion

The present study is the first report of molecular cloning and functional analysis of an avian IL-19. The gene consists of five exons on chicken chromosome 26, in the same cluster as chicken IL-10. Sequence analysis results showed 176 amino acids with six structurally conserved cysteine residues. Although analysis of the chicken genome has not revealed genes encoding chicken IL-20, ChIL-19 was assigned to the mammalian IL-20 group based on sequence homology in NCBI database. However, phylogenetic analysis shows ChIL-19 is evolutionarily closer to mammalian IL-19 than to IL-20. Additionally, ChIL-19 contains one potential N-glycosylation site, not found in any mammalian IL-20. Chicken IL-19 appears to be basal to mammalian IL-19 based on bootstrap analysis, with the IL-20 clade forming a sister clade with the IL-19 clade. Also, it appears that IL-24 clade is more basal to both IL-19 and IL-20, but the zebrafish IL-20 is basal to that whole group, suggesting a duplication event that led to the formation of IL-19, probably prior to the divergence of birds and mammals.

In mammals, the major sources are monocytes and B-cell (Gallagher et al., 2000; Hsing et al., 2006), and an immunostaining study showed that human IL-19 is expressed in specific tissue types, including epithelial cells, endothelial cells, and macrophages (Hsing et al., 2008a,b). Results of RT-PCR indicate that human IL-19 is expressed in the heart, lung, spleen, and placental tissues (Hsing et al., 2008a,b). We found that in chickens, IL-19 was mainly expressed in the skin, spleen, heart, and lung. Interestingly, ChIL-19 expression in skin tissues is equivalent to that of LPS-stimulated monocytes, suggesting that skin tissues, more specifically skin epithelial cells, are a major source of this cytokine. These results implicate an important role of ChIL-19 in skin epithelial cells, with potential application in studying the human skin autoimmune disease, vitiligo, using the Smyth line chickens (Wang and Erf, 2004; Sabat et al., 2007).

For functional characterization of ChIL-19, the gene for recombinant ChIL-19 was cloned and expressed in either *E. coli* or transformed chicken macrophage cells, HTC. There is no anti-ChIL-19 antibody commercially available, so rChIL-19 was expressed with a FLAG tag at the C-terminus. The expression of rChIL-19 was detected by Western blot with anti-mouse FLAG antibody and expression of rChIL-19 from both *E. coli* and HTC cells was observed. The expressed rChIL-19 from *E. coli* had a larger molecular weight (25 kDa) than that of expressed in HTC cells (21 kDa), because the former contains 6-histidine residues in the upstream region of IL-19 gene inserted for purification. Gallagher's report shows multiple bands of human IL-19 in Western blotting caused by N-linked glycosylation sites (Gallagher et al., 2000). Although sequence analysis showed one potential glycosylation site, Asn154-Val-Thr155 in ChIL-19, neither multiple bands nor a molecular shift of rChIL-19 from HTC cells in Western blotting was observed.

Mammalian IL-19 induces production of IL-6 and TNF-α in monocytes, unlike IL-10 which has a suppressor function (Liao et al., 2002). The results of qRT-PCR show that rChIL-19 stimulated the production of pro-inflammatory cytokines, IL-1β and IL-6, suggesting a role of ChIL-19 in inflammation similar to mammalian IL-19. Recombinant ChIL-19 also induced expression of ChIL-19 itself, albeit later than those of IL-1β and IL-6, suggesting that ChIL-19 first induces pro-inflammatory cytokines, then turns on its own expression. In monocytes, iNOS transcripts were also induced by rChIL-19, indicating a role of ChIL-19 in production of reactive oxygen species.

Study of asthmatic patients showed an elevated IL-19 production in serum, resulting in the up-regulation of Th2 cytokines (Liao et al., 2004). Their study observed increased levels of Th2 cytokines by IL-19 from (1) IL-19 cDNA-injected normal and asthmatic mice, (2) stimulated $CD4^+$ T cells with IL-2, and (3) Con A-activated Jurkat T cells. In our study, primary lymphocytes were stimulated with either rChIL-19 alone or Con A and rChIL-19. Recombinant ChIL-19 alone also induced the Th2 cytokines IL-4 and IL-13, and pre-stimulated primary lymphocytes with Con A showed significantly increased IL-13 transcripts in the presence of rChIL-19. Interestingly, IL-4 transcripts were significantly increased 6 h post-treatment, whereas IL-13 transcripts were increased 12 h post-treatment. The increased IL-5 transcript was not detected because chicken IL-5 is apparently a pseudogene, and does not encode a functional protein (Kaiser et al., 2005). Liao et al. (2004) showed that IFN-γ production was not detected in activated $CD4^+$ T cells treated with IL-19. Our study contradicts that observation showing a significant induction in IFN-γ transcripts by rChIL-19, even though the production of IFN-γ was not detected by ELISA (data not shown), perhaps due to lack of assay sensitivity. Further, since freshly isolated splenocytes were used, there is potential of monocyte contamination that can be stimulated by rChIL-19 and consequently activated T cells, resulting in induction of IFN-γ gene. Additionally, NO production is a result of cytokines produced upon T cell activation, most notably IFN-γ, suggesting that activated monocytes by rChIL-19 induce production of IFN-γ by activated T cells. Human IL-19 cDNA was first cloned from Epstein-Barr virus transformed human B cells (Gallagher et al., 2000), with the potential that viral infection can induce IL-19 expression in certain cells, more likely B cells, suggesting a role of IL-19 in IFN-γ production.

Further, mammalian IL-19 induced production of IL-6 and TNF-α, and increased TNF-α production leading to apoptosis in monocytes. Neutralization of TNF-α by its antibody abolished the apoptotic effect of IL-19 (Liao et al., 2002). A similar effect by ChIL-19 was observed; with rChIL-19 causing apoptosis in monocytes albeit more necrotic cells than apoptotic cell population were observed in the LPS-treated monocytes. Additionally, media alone showed high apoptotic cell population, most likely due to longer treatment time of primary monocytes.

In summary, following the successful cloning and expression of ChIL-19, the molecular function of the first described avian IL-19 was demonstrated. Our data indicate that avian IL-19 is another member of the IL-10 family and induces pro-inflammatory cytokines in monocytes and Th2 cytokines in lymphocytes.

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