AVIAN TOLL-LIKE RECEPTOR 7 (TLR7) AND INTERFERON REGULATORY FACTOR 7 (IRF7) FUNCTION IN THE PRO-INFLAMMATORY RESPONSE TO SINGLE-STRANDED RNA

by

Randall J. Derstine

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Fall 2013

AVIAN TOLL-LIKE RECEPTOR 7 (TLR7) AND INTERFERON REGULATORY FACTOR 7 (IRF7) FUNCTION IN THE PRO-INFLAMMATORY RESPONSE TO SINGLE-STRANDED RNA

by

Randall J. Derstine

Approved: _____
Calvin L. Keeler, Jr. Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Randall L. Duncan, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Calvin L. Keeler, Jr. Ph.D. for providing me with the opportunity to pursue my research interests in his laboratory. Also, my fellow research group members have provided invaluable intellectual input to the development of my project, and I wish them the best in their future careers. And of course, I would like to thank my family for their support over the last two years as I complete my master's degree at the University of Delaware.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

For animals, the innate immune system is the first line of defense against foreign pathogens. It is a non-specific response to infectious challenge that is independent of antigen recognition, utilizing a combination of anatomical, physiological, and chemical factors to eliminate a pathogen before it can colonize its target host. The toll-like receptor (TLR) family of proteins are a critical group of pattern recognition receptors (PRRs) which are present on multiple cell types, such as neutrophils, natural killer (NK) cells, dendritic cells, and macrophages. In mammals, one of these PRRs, toll-like receptor 7 (TLR7), has been demonstrated to bind single-stranded RNA (ssRNA) of viral and synthetic origin. The toll-like receptor pathway consists of multiple types of transmembrane receptor proteins that recognize unique microbial structures, also known as pathogen associated molecular patterns (PAMPs). The binding of a PAMP to its respective TLR leads to signal transduction to the cell nucleus by the use of multiple adaptor proteins and signal mediators, resulting in the transcription of pro-inflammatory cytokines and chemokines. In mammals, a transcription factor, interferon regulatory factor 7 (IRF7), is known to mediate the transcription of type I interferons and helps to induce an anti-viral response. Interferons play a critical role in the anti-viral response by inhibiting protein translation in virally infected cells, as well as by enhancing antigen presentation to cytotoxic T lymphocytes.

The goal of this study was to clarify the functional homology of avian TLR7 and IRF7 to mammalian TLR7 and IRF7, and to determine if their respective roles in

mediating the pro-inflammatory response to ssRNA are conserved. It was hypothesized that avian TLR7 is functionally homologous to mammalian TLR7 in binding ssRNA to mediate a pro-inflammatory response. We further hypothesized that avian IRF7 functions in a manner similar to mammalian IRF7 in regulating the transcription of type I interferons. To test these hypotheses, a siRNA-mediated RNAi method for transient gene knockdown in the immortalized macrophage cell line HD11 of *Gallus gallus* was developed. With this method, TLR7 and IRF7 were silenced in HD11s by an average of 60% at 24 hours post-transfection with TLR7 and IRF7 specific siRNAs. These cultures were subsequently exposed to the ssRNA chemical analog R848. The roles of avian TLR7 and IRF7 in mediating the pro-inflammatory response to ssRNA were elucidated by measuring the transcription levels of two pro-inflammatory genes, iNOS and IFN-α. The iNOS and IFN-α genes were selected based on their ability to be induced by 25.3 fold at 2 hours and 15.6 fold at 8 hours, respectively, in response to a 10 ug/mL R848 treatment. With TLR7 silenced, HD11 cultures displayed a 23.7% reduction in iNOS expression and a 23.7% reduction in IFN-α expression when treated with R848 ($p = < .0001$). With IRF7 silenced, HD11 cultures displayed a 14.3% reduction in iNOS expression ($p = < .0022$) and a 34% reduction in IFN-α expression ($p = < .0001$). The combined knockdown of TLR7 and IRF7 resulted in an additive reduction in iNOS and IFN-α expression in response to R848, with a 42.3% reduction in iNOS ($p = < .0001$) and a 46.7% reduction in IFN-α ($p = < .0001$). The results of this study provide strong evidence for the roles of avian TLR7 and IRF7 in mediating the pro-inflammatory response to ssRNA.

Chapter 1

INTRODUCTION

1.1 The Innate Immune System

The innate immune system constitutes the first line of defense against foreign pathogens. It is an immediate, non-specific response to infectious challenge that is independent of antigen recognition [31]. In contrast to the adaptive immune system, the innate immune system utilizes a combination of anatomical, physiological, and chemical factors to eliminate a pathogen before it can effectively colonize the host system [7,31]. These factors are evolutionarily conserved between multiple species and much of what is known about avian innate immunity has been extrapolated from mammalian studies. While these factors are described independently, it is critical to view them as working cooperatively and simultaneously to prevent or inhibit microbial pathogenesis. Humans are continuously exposed to foreign pathogens from the environment, yet do not display observable pathology; evidently the mechanisms of the innate immune response are highly effective in preventing microbial infection. Defining the molecular mechanisms of the innate immune response is paramount for the development of improved therapeutic strategies which may mitigate loss to infectious disease.

1.1.1 Anatomical Barriers to Microbial Infection

Upon initial contact with the internal or external surfaces of their target host, a bacterial or viral pathogen will encounter multiple elements of the innate immune system, collectively leading to its elimination. Anatomically, many species are designed to make entry and colonization by foreign pathogens difficult. The internal and external surfaces

of the human body are sealed by tightly packed epithelial cells, creating a physical barrier between internal tissues and environmental pathogens [31,34]. The skin itself is dry, cool, and slightly acidic; these conditions do not facilitate microbial growth [35]. The continuous sloughing of dead, keratinized skin cells removes microorganisms that may have colonized the skin surface [31]. In avian species, feathers are a natural protection against direct contact of pathogens with the skin surface.

The respiratory, gastrointestinal, and urogenital tracts are also optimally designed to prevent pathogenic infection. Each of these tracts are lined with a mucus-secreting epithelial layer; this mucus layer not only traps microbes but is rich in lysozyme, a bactericidal enzyme that degrades bacterially-produced peptidoglycan [65,66]. Comprising the surface of epithelial cell membranes are cilia; hairlike protrusions that create synchronous directional movement to flush microbes trapped in the mucus layer [34]. The natural contraction and relaxation of smooth muscles within these tracts, known as peristalsis, also contributes to the flushing action and thus the removal of pathogens from the host.

Another important anatomical component of the innate immune system are the bony encasements, such as the skull and thoracic cage, that protect vital internal organs from direct exposure to environmental microbes [31]. While these bony encasements help maintain the structural integrity of the host, they are also effective in preventing direct entry of foreign pathogens, even in the event of traumatic injury to external surfaces. It is also the boney encasements that gives rise to hematopoietic stem cells (HSC) which circulate the blood stream and ultimately differentiate into all of the myeloid and

lymphoid cell lineages. Among the myeloid cell lineages are macrophages, neutrophils, and dendritic cells, all of which are critical innate immune cell that serves critical functions in both innate immunity and activation of the adaptive immune response [34,35].

1.1.2 Physiological Barriers to Microbial Infection

One function of the innate immune system is to create a physiological environment that does not facilitate microbial growth. Two important physiological factors are pH and temperature. Human skin is maintained at a slightly acidic pH of 5. Sebaceous glands secrete an oily substance known as sebum, which is composed of approximately 45% fatty acid and contributes to the low pH and thus the antimicrobial properties of the skin [36]. In the gastrointestinal tract, the stomach has a pH between 1.35-3.5, the result of hydrochloric acid secretion by parietal cells of the stomach lining [36].

Just as the level of acidity can prevent or inhibit microbial growth, so can alterations in body temperature. Microbes have an optimal temperature at which they grow; a rise in body temperature can either kill the microbe or slow its growth so that the body's innate and adaptive immune defenses can eliminate it from the host [34]. One hallmark of microbial infection in humans is fever, a physiological response triggered by the secretion of chemical mediators from innate immune cells that signal to the body the presence of foreign pathogens [35]. These particular chemical mediators, known as pyrogens, circulate in the bloodstream and stimulate the anterior hypothalamus of the brain; the result is a systemic increase in body temperature [66]. This increase in

temperature functions to inhibit microbial growth, as well as to increase the activity of other cellular processes designed to eliminate the pathogen from the host.

1.1.3 Chemical Barriers to Microbial Infection

The chemical elements of the innate immune system are diverse and highly effective in preventing microbial colonization. Some of these elements are specific to bacteria, while others work to inhibit viral replication inside host cells. Lysozyme is one such element, and is produced by paneth cells of the intestinal epithelium. This anti-bacterial enzyme is found in tears, saliva, and in the mucosal secretions of internal epithelium and it specifically degrades bacterial peptidoglycan, a structural component of the bacterial cell wall [55,65]. Besides lysozyme, animals express a diverse repertoire of anti-microbial peptides that are chemically designed to inhibit or destroy bacteria, viruses, and fungi [11]. The epidermal layers are characterized by two broad groups of anti-microbial peptides; cathelicidins and defensins [11]. Cathelicidins were first discovered in human skin keratinocytes at sites of inflammation; they are secreted by activated neutrophils, mast cells, and keratinocytes and function primarily as protease inhibitors [34,35]. Cathelicidins also function as chemoattractants for inactive neutrophils and monocytes circulating in proximity to the site of infection, and can even act directly on bacterial cells by punching holes in the bacterial cell wall. [13]. Defensins are expressed primarily by epithelial cells of the gastrointestinal and pulmonary tracts and can stimulate the expression of pro-inflammatory chemical mediators, collectively known as cytokines and chemokines, from macrophages and other cell types [11].

Examples of these chemical mediators include tumor necrosis factor-α (TNF-α) and interleukin 1-beta (IL-1β); TNF-α and IL-1β have the ability to induce apoptosis of virally infected cells. Interleukins constitute a large family of cytokines, and are expressed primarily by leukocytes and endothelial cells [13]. The secretion of pro-inflammatory cytokines and chemokines from leukocytes and other cell types is a defining element of the innate immune response. These small proteins, roughly 25 kDa, can induce cellular responses in an autocrine or paracrine fashion [14,15]. The effects of cytokines and chemokines can be local or systemic. Local effects can include increased vascular permeability at the site of infection to allow greater access of complement effectors and immune cells, as well as activation of phagocytic cells such as natural killer (NK) cells and macrophages that will engulf and degrade microbes along with the infected cells [14,63]. A major systemic effect of cytokine and chemokine release is fever; activated macrophages can secrete Interleukin 6 (IL-6) which will induce prostaglandin E2 (PGE_2) production in the hypothalamus, leading to an increase in body temperature and inhibition of microbial pathogenesis [15]. This increase in body temperature can serve as an environmental cue that activates key anti-microbial enzymes and cellular processes that function to eliminate the pathogen from the host [34,35].

Apart from antimicrobial peptides, enzymes, and chemical effectors, another major chemical component of innate immunity is the production of reactive oxygen and nitrogen species such as nitric oxide (NO), that have potent cytotoxic effects [16]. An activated macrophage can secrete TNF-α and interferon gamma (IFN-γ), which will act synergistically to activate naïve macrophages. This activation leads to a massive

expression of the iNOS gene, or inducible nitric oxide synthase [19]. This enzyme is a critical anti-microbial weapon used by cells, particularly macrophages, to generate reactive nitrogen oxide species that can damage or destroy pathogens that have been internalized in the phagolysosome [15,16]. The exact mechanism by which nitric oxide destroys pathogens is unclear, but is has been shown to destabilize DNA and RNA. The induction of the iNOS gene is carefully regulated, as a prolonged exposure to reactive oxygen species at high concentrations can damage healthy tissue and ultimately the host as well [17]. When a known suppressor of iNOS, transforming growth factor- β1 (TGF-β1), has been knocked out in mice, a systemic over-production of nitric oxide in multiple organs led to chronic inflammation and tissue deterioration [17].

When a host cell is infected, for example by a virus, the organism must have a method for detecting and eliminating not only the virus but also any cells that may have been infected. This is made possible by a family of cytokines known as interferons. As the name suggests, their role is to interfere with viral replication [14]. Viruses are characterized by differences in envelope glycoproteins and their genomic make-up, which can be single-stranded RNA or DNA (ssRNA/DNA) or double-stranded RNA or DNA (dsRNA/DNA) [18]. These molecular constituents, known as Pathogen Associated Molecular Patterns (PAMPs), will trigger specific receptors in different cell types, known as Pattern Recognition Receptors (PRRs); the interaction of a PAMP with its respective PRR leads to signal transduction to the cell nucleus and transcription of pro-inflammatory genes [32,36]. There are multiple families of PRRs that can lead to downstream activation of pro-inflammatory genes, for example the Toll-Like Receptors (TLRs) [58].

There are two general classifications of interferons, type I, which includes IFN-α and IFN-β, and type II, which includes IFN-γ [62,73]. Transcription of these genes is regulated by a family of transcription factors known as Interferon Regulatory Factors (IRF) [18]. In humans, these are represented by IRF3 and IRF7; sequence analysis of *Gallus gallus* indicates there is no avian IRF3, which may have significance in understanding the pro-inflammatory response in the avian system. Secretion of interferons from an activated cell, for example a macrophage, can lead to several events. A primary effect is inhibition of protein synthesis in a virally infected cell. This occurs when the interferon, for example IFN-α or IFN-β, binds to the interferon α/β receptor (IFNAR) on a neighboring cell [20]. This interaction leads to downstream signaling in the Janus-Kinase Signal Transducer and Activator of Transcription (JAK-STAT) pathway. This signaling cascade leads to transcription of protein kinase R (PKR) which phosphorylates eIF-2 (eukaryotic initiation factor 2) to form an inhibitory complex with eIF2B [20,62]. These proteins are critical players in the cell's translational machinery. The result is a sharp decrease in protein synthesis and a reduced ability of the virus to sustain its replication inside the host cell. Another effect of interferon-mediated stimulation is the transcription of genes coding for MHCI/II (Major Histocompatibility Complex I/II) which leads to expression of MHC I/II on the infected cell surface [36,53]. These complexes present viral peptides to cytotoxic T cells and helper T cells, targeting the infected cell for destruction [20]. Cytotoxic T cells and helper T cells can also secrete pro-inflammatory cytokines that activate neighboring macrophages and natural killer (NK) cells.

1.1.4 Microbial Antagonism by Commensal Flora

Another major component of the innate immune response, though not the focus of this study, is the commensal flora that naturally inhabits the external and internal surfaces of the body. The gastrointestinal tract is home to trillions of commensal microbes that have diverse functions in metabolism, homeostasis, and innate immunity [21,22]. Commensal bacteria of the intestinal tract have been described as the interface between metabolism and regulation of the immune system. The dynamic signal transduction and transcription networks of bacteria can have direct and indirect effects on the function, differentiation, proliferation, and migration of immune cells [22,23]. A prime example is the synthesis of short-chain fatty acids (SCFA) by bacteria, and their regulation of host immune cells [25,26]. Immune cells such as neutrophils, macrophages, and monocytes express the SCFA receptor FFA2 (Free Fatty Acid Receptor 2) that, upon binding bacterial SCFA, can mediate a chemotactic response by causing an influx of intracellular calcium and a decrease in extracellular signal regulated kinase 2 (ERK2) activity [26]. Commensal bacteria also have the ability to synthesize essential vitamins and organic nutrients that are recognized and utilized directly by select immune cells [25]. There are 13 essential vitamins within the A, B, C, D, E, and K groups. Commensal bacteria have been shown to synthesize vitamins B and K, which are utilized in biosynthetic pathways that can regulate immune cells [28,29]. The monomorphic major histocompatibility complex class I-related protein (MR1) presents vitamins from the riboflavin biosynthetic pathway to mucosal T cells, which produce pro-inflammatory cytokines such as IL-17 and IFN-γ in response to these metabolites [29]. Furthermore, commensal bacteria can

assist the host in the extraction and/or synthesis of key amino acids. Obviously, amino acids are essential for protein synthesis yet they can also act as direct substrates in biosynthetic and metabolic pathways [22,23]. The amino acids arginine, leucine, glutamine, and tryptophan are associated with regulation of the immune system. Tryptophan can influence the proliferation of T cells by regulating passage through the G1 phase of the cell cycle [24,27]. Studies involving germ-free mice have shown that there is a significant loss of tryptophan from the gastrointestinal tract, suggesting that commensal bacteria play a role in retention of this key amino acid [24,30]. Commensal bacteria also inhabit the skin, outcompeting foreign pathogens for nutrients and space [23,25,28].

1.2 Receptor Mediated Innate Immune Pathways

The innate immune system can be described as fast acting and non-specific, yet the adaptive immune response is highly dependent on the effective triggering of the innate immune response. There are multiple families of innate immune receptors that function to bind microbial structures and that mediate signal transduction to the cell nucleus, leading to increased transcription of pro-inflammatory genes [15]. These receptors are expressed on many cell types, and are classified based on cellular localization, ligand specificity, and evolutionary relationship to other gene families [32].

Studies involving the characterization of avian immune receptors are commonly extrapolated from mammalian studies and the advent of high-throughput sequencing technologies, along with robust computational tools, has allowed for wide scale genomic mapping to determine sequence homology of gene families within or between a species

[66]. The next step is to determine functional homology of the corresponding gene products, whether it is RNA or protein. There are three general families of innate immune receptors that recognize microbial structures and can mediate a pro-inflammatory response; the Toll-Like Receptors (TLRs), Nucleotide-Binding Oligomerization Domain (NOD)-Like Receptors (NLRs), and the Retinoic Acid Inducible Gene I (RIG-I)- Like Receptors (RLRs) [32,63]. A common theme in functional biology is redundancy, these receptor pathways can be separated based on sequence differences at the genomic level, yet their functional role in the cell may overlap. A macrophage can have multiple receptors that recognize single-stranded or double-stranded RNA (ss/dsRNA), all of which may lead to downstream activation and transcription of pro-inflammatory genes [36,54]. In this regard, targeting a single gene for a functional study does have limitations in understanding the signaling mechanisms of the pro-inflammatory response.

1.2.1 Pattern Recognition Receptors (PRRs) and Pathogen Associated Molecular Patterns (PAMPs)

Triggering of a receptor mediated innate immune response begins with the binding of a Pattern Recognition Receptor (PRR) to its respective Pathogen Associated Molecular Pattern (PAMP). A PRR can be defined as an evolutionarily ancient anti-microbial sensor that recognizes conserved molecular constituents of foreign pathogens. These PRRs are expressed on almost all cell types, however it is the innate immune cells such as dendritic cells, macrophages, and neutrophils that are potent producers of pro-inflammatory cytokines and chemokines upon PRR activation. The binding of a PAMP to a PRR leads to transcription of pro-inflammatory genes and release of cytokines and

chemokines [24,31]. There are two primary types of PRRs; membrane bound and cytoplasmic. The membrane bound PRRs are represented by the TLR family, which are present on extracellular or endosomal membranes [4]. The Toll-Like Receptor (TLR) family of proteins was first discovered in *Drosophila melanogaster*. There are 10 TLR family members in humans, and 12 in mice and their ligands can include bacterial, viral, fungal, and parasitic structures [6,7,38]. Phylogenetic analysis of the chicken (*Gallus gallus*) has revealed a total of 10 TLR genes, five of which have mammalian orthologs [8,38].

Cytoplasmic PRRs are represented by the Nucleotide-Binding Oligomerization Domain (NOD)-Like Receptors (NLR) and the Retinoic Acid Inducible Gene I (RIG-I)-Like Receptors (RLR) [55,62]. The NLRs recognize a wide variety of microbial PAMPs, particularly viral and bacterial nucleic acids, with 23 known NLRs in humans and 34 in mice [31,32]. There is still controversy about the total number and function of the NLRs present in *Gallus gallus*, as well as to their exact function. The RLR family contains 3 major components which are responsible for detecting viral nucleic acids in the cytoplasm; RIG-I, Melanoma Differentiation-Associated Gene 5 (MDA5), and Laboratory of Genetics and Physiology (LGP2). While all three of these components can be found in avian systems, only MDA5 and LGP2 are present in the *Gallus gallus* genome [38].

1.2.2 Inflammatory Response

The inflammatory response is a complex biological process that occurs within vascular tissues in response to invasion by foreign pathogens; the process begins with the triggering of receptor-mediated pro-inflammatory pathways in a variety of cell types including dendritic cells, macrophages, lymphocytes, endothelial cells, mucosal epithelial cells, neutrophils, and fibroblasts [32,33]. The binding of a microbial PAMP to a PRR present on any of these cell types (to be discussed below) can mediate signal transduction events resulting in the transcription of pro-inflammatory cytokines and chemokines. These chemical mediators can have a variety of effects, ranging from direct targeting of the invading pathogen to the recruitment of other cellular effectors to the site of infection [31,32]. The inflammatory response can also lead to systemic effects such as fever, increased blood pressure, decreased sweating, and malaise. These physiological changes result from an increase in acute-phase proteins present in the blood plasma. Examples of these acute-phase proteins include c-reactive protein (CRP), fibrinogen, transferrin, and hemopexin [33]. These proteins are produced by hepatocytes of the liver in response to pro-inflammatory cytokines being released from activated immune cells such as macrophages and natural killer (NK) cells [24]. The acute-phase proteins also fall within the realm of the complement system. The complement system is considered a peripheral yet essential element to the innate immune response in that it assists with internalization and destruction of pathogens by phagocytic cells, along with the further recruitment of complement effectors and secretion of pro-inflammatory cytokines from activated immune cells [24].

Expression of c-reactive protein (CRP) by hepatocytes occurs in response to interleukin 6 (IL-6). Interleukin 6 is a potent pro-inflammatory cytokine secreted by macrophages and plays a major role in activating the fever response, as well as the complement system. This cytokine travels through the bloodstream to cross the blood brain barrier, leading to secretion of prostaglandin E2 (PGE_2) from the hypothalamus to induce fever. CRP binds to phosphocholine found in microbes and enhances their phagocytosis by binding to CRP receptors present on the macrophage surface [33]. This is an example of opsonin mediated phagocytosis [66]. Fibrinogen is another acute phase protein that is highly expressed by hepatocytes in response to pro-inflammatory cytokines. Fibrinogen plays a critical role in the coagulation cascade and allows for the formation of a fibrin clot. The ability to form fibrin clots is essential in sealing off damaged blood vessels and for the sequestration of invading pathogens [24]. The acute phase protein transferrin sequesters free iron, a critical element required for the survival of many bacterial pathogens. Hemopexin serves a similar role to transferrin by regulating the amount of free heme, an organic compound that transports iron throughout the body [61]. Given these highly efficient pro-inflammatory mechanisms, it is important for the host system to maintain negative feedback mechanisms necessary to prevent chronic inflammation [36]. This condition can be highly detrimental to the host system. In humans, a chronic inflammatory response can lead to atherosclerosis, rheumatoid arthritis, periodontitis, and certain types of cancer [33].

1.2.3 Nucleotide-Binding Oligomerization Domain (NOD) Pathway

The Nucleotide-Binding Oligomerization Domain Like Receptors (NLRs) are composed of several domains; a C-terminal leucine-rich repeat (LRR) domain that recognizes a given PAMP, a n-terminal death effector domain (DED), a Pyrin domain (PYD), a Caspase Activation and Recruitment Domain (CARD), a Baculovirus Inhibitor Repeat (BIR) domain, and an acidic domain which is necessary for binding to downstream signaling proteins [39]. A critical CARD domain containing adaptor protein known as Receptor-Interacting Serine/Threonine Protein Kinase 2 (RIPK2) is recruited upon activation of a NLR; this leads to activation of NF-Kβ and transcription of pro-inflammatory cytokines [41]. NLRs also contain a nucleotide-binding and oligomerization domain (NACHT), an ATP-dependent domain that mediates the formation of receptor complexes for downstream signaling [39]. Induction of proinflammatory cytokines by activation of an NLR occurs through the activation of NF-Kβ or Mitogen Activated Protein (MAP) Kinase signaling pathways. This can lead to the formation of a multi-protein complex known as the "inflammasome" [40]. Within this complex are various caspases that assist with post-translational modifications of the cytokine proteins, as seen with IL-1β and IL-18 [62]. The inflammasome can also trigger apoptosis if the infected cell has sustained significant damage [50].

The NLR family of PRRs are present in the cytoplasm of any given cell and can recognize a wide variety of microbial ligands. Two well characterized NLRs are NOD1 and NOD2; NOD1 recognizes the bacterial peptidoglycan of *Bacillus subtilis, Listeria monocytogenes, Escherichia coli, Shigella flexneri,* as well as several other species [45].

NOD2 is known to bind bacterial derived peptides, for example the muramyl dipeptide (MDP) of *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* [46]. Another NLR, NOD-Like Receptor CARD Domain Containing 5 (NLRC5), has recently been elucidated in *Gallus gallus*, and is highly expressed in lymphocytic and macrophage cell lines [9]. NLRC5 can be induced by IFN-γ, synthetic dsRNA (Poly I:C), and LPS. It is known to regulate the anti-viral innate immune response by the induction of type I interferons and MHC I molecules in lymphoid and epithelial cells [45]. NLRC5 is also known to interact with IKK-α and IKK-β (IKb Kinase α/β) and can negatively regulate NF-Kβ. NLRC5 has also been shown to work cooperatively with RIG-I and MDA5 to mediate the anti-viral response [48]. In a mouse model in which NLRC5 has been knocked out, NLRC5 deficient mice were still able to induce production of type I interferons through activation of NF-Kβ [46,48].

The discovery of the "inflammasome" has led to the elucidation of three major inflammasome complexes, NLRP3, NLRC4, and NLRP1 [49, 50]. Stimulation of immune cells such as macrophages and dendritic cells leads to the assembly of these complexes, although there are several non-NLR proteins that are essential for this complete assembly. The NLRP3 inflammasome is the best characterized inflammasome, and can recognize viral, bacterial, and fungal PAMPs, as well as DAMPs (Danger Associated Molecular Patterns) which include heat shock proteins and even sense specific environmental pollutants such as silica and asbestos [50]. The NLRC4 inflammasome is composed of several CARD-domain containing proteins, and has been linked to programmed cell death in response to *Shigella flexneri*. It has also been

suggested that bacterial Type-III/IV Secretion Systems are potent activators of the NLRC4 inflammasome [50]. The NLRP1 inflammasome is highly responsive to microbial DNA, specifically from DNA viruses [51]. Contained within the NLRP1 complex is AIM2 (Absent In Melanoma 2), a protein that can directly detect cytoplasmic DNA and activates caspase-1. Caspase-1 is essential for cleavage of pro-IL-1β to its active IL-1β form, as described earlier [51].

1.2.4 Retinoic Acid Inducible Gene I (RIG-I) Pathway

The Retinoic Acid Inducible Gene I (RIG-I)- Like Receptors (RLRs) are responsible for recognizing viral RNA in the cytoplasm of infected cells. Among the RLRs are Retinoic Acid Inducible Gene I (RIG-I), Melanoma Differentiation-Associated Gene 5 (MDA5), and Laboratory of Genetics and Physiology (LGP2) [57]. The RLRs contain a CARD domain, as well as a Repressor Domain (RD), which is required for the regulation of downstream signaling [56]. A critical domain in these proteins is the DExD/H-Box RNA Helicase domain, which gives the RLRs their characteristic RNA binding capability. This domain also functions as an ATPase. Mice with a mutation in this domain lack the ability to induce type I interferons in response to viral challenge [56,62]. Between RIG-I, MDA5, and LGP2, the RLRs can recognize a wide variety of viral RNA structures in the cytoplasm; RIG-I binds short dsRNA as well as positive and negative sense ssRNA, MDA5 binds long length dsRNA as well as positive and negative sense ssRNA, and LGP2 binds both ss/dsRNA [57]. The diversity of the ligands recognized by these RLRs is a major consideration in this present study, as it highlights the functional redundancy that exists among the pattern recognition receptors. Activation

of the RLRs occurs when viral nucleic acids are directly introduced into the cytoplasm, or upon release of viral nucleic acids from the endosome [57,63]. Binding of RIG-I, MDA5, or LGP2 to viral RNA leads to a conformational change that will expose the CARD domains, allowing for the binding of CARD-domain containing adaptor proteins [56]. One of these RLR specific adaptor proteins is Interferon Promoter Stimulator 1 (IPS-1). Interestingly, IPS-1 localizes to the mitochondria upon stimulation and activates IRF3, allowing for transcription of anti-viral effectors such as IFN-α and IFN-β [56].

1.2.5 Toll-Like Receptor (TLR) Pathway

The Toll-Like Receptors (TLRs) are the most widely studied of the innate immune receptors, first identified in *Drosophila melanogaster* [37]. There are 10 distinct TLRs in humans, and 12 in mice. There are 10 distinct avian TLRs, with 5 of these (TLR2a, TLR2b, TLR4, TLR5, TLR7) having clear sequence orthologs with humans and mice [4,6]. Although the avian and mammalian lineages diverged roughly 300 million years ago, the conservation of the protein adaptors, receptors, kinases, and downstream signaling genes between birds and mammals is significant [4,38]. The differences arise in the organization of lymphoid tissues, the repertoire of effector cells, the genes they express, and the organization of these genes within their respective genomes [7]. Complete genome sequencing of the Red Junglefowl, the ancestor of *Gallus gallus*, has allowed for an analysis of the avian and mammalian genomes to determine sequence orthologs of TLR related genes [6,7].

Toll-like Receptors (TLR) are type I transmembrane glycoproteins. The N-terminal domain contains a leucine rich repeat (LRR) that bind to microbial PAMPs

while the cytoplasmic C-terminal domain contains an interleukin-1 receptor domain (IL-1) that will bind the adaptor proteins needed for signal transduction. Toll-like receptors are characterized based on the type of PAMP they bind, as well as on their sub-cellular location. The TLRs are found on the extracellular membrane or within endocytic compartments (Figure 1). Stimulation of a TLR results in downstream activation of transcription factors such as NF-Kβ and the Interferon Regulatory Factors (IRF). The precise pathway and genes transcribed are dependent on the agonist as well as on the cell type [63,68]. There are four critical adaptor proteins in the TLR pathway that are conserved between mammalian and avian genomes: MyD88, TRIF, TRAM, and TIRAP [61,67]. All mammalian TLRs utilize MyD88 with exception of TLR3, although it is common for MyD88 and TIRAP to work cooperatively in mediating the activation of the transcription factors NF-Kβ, IRF3, and IRF7 [38,42]. The adaptors TRIF and TRAM function as a complex to mediate downstream signaling upon TLR3 stimulation [8].

The TLRs can recognize PAMPs derived from mycobacteria, bacteria, virus, fungi, and parasites. Toll-Like Receptor 1 (TLR1) is present on the extracellular membrane and recognizes bacterial triacyl lipopeptides, acting through MyD88 dependent signaling to activate NF-Kβ. TLR1 is represented by the duplicate genes TLR1a and TLR1b, both of which recognize bacterial lipoproteins [38,64]. Toll-Like Receptor 2 (TLR2) recognizes bacterial peptidoglycan, a major component of the bacterial cell wall, and mediates downstream signaling by binding TIRAP and MyD88 cooperatively to induce MAP-Kinase signaling [65,66]. Similar to TLR1, TLR2 exists as a duplicate in the avian

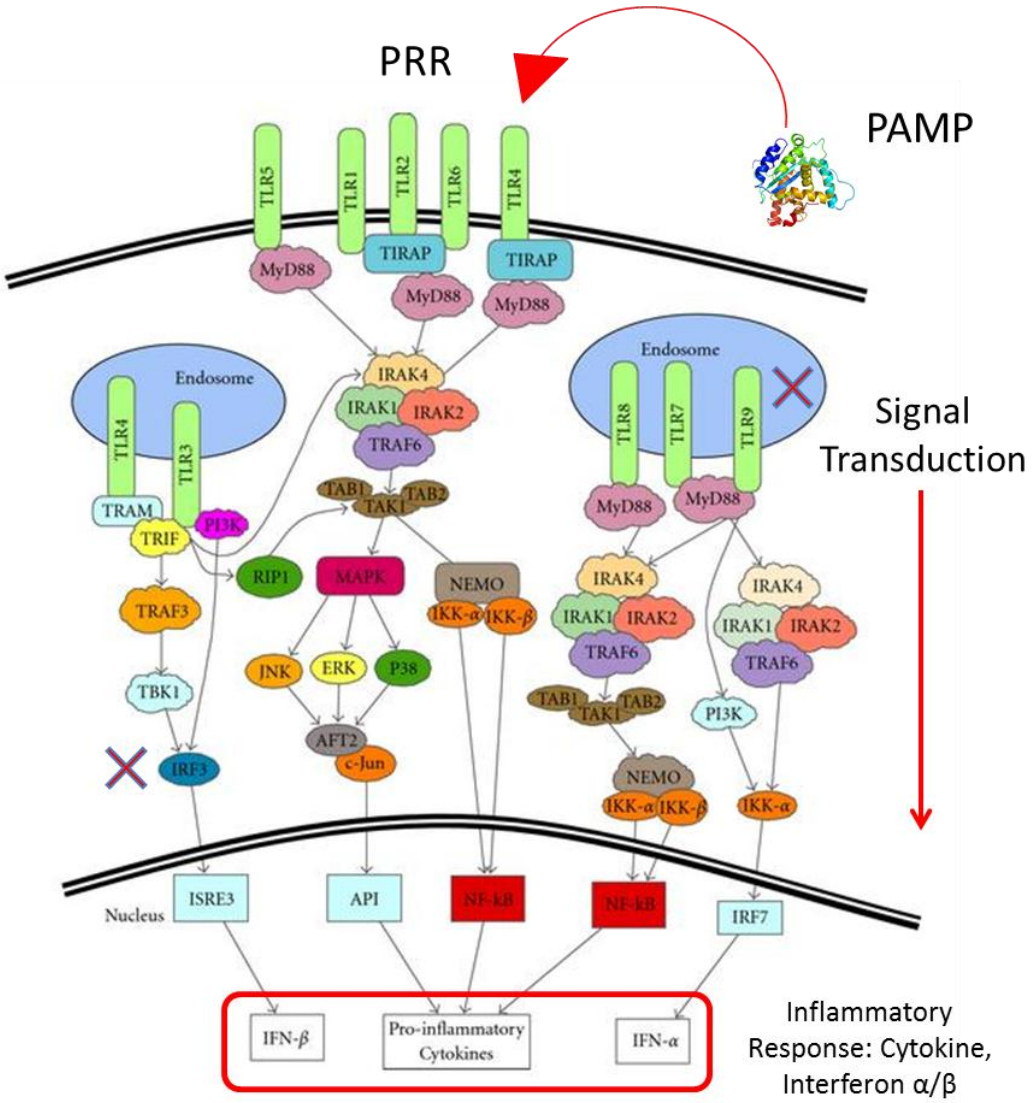

Figure 1: Mammalian Toll-Like Receptor (TLR) Signaling Pathway. Binding of a specific microbial PAMP to an extracellular or endocytic TLR leads to recruitment of adaptor proteins that will mediate signal transduction to the cell nucleus; transcription factors such as NF-Kβ and IRF will induce transcription of pro-inflammatory cytokines and chemokines. IRF3 and TLR9 (Red X) are absent in the avian genome. Not shown is the avian TLR9 ortholog TLR21, as well as avian TLR15. Image modified from Hindawi Immunology (2008).

genome, known as TLR2a and TLR2b, both of which can recognize bacterial lipoproteins [61].

The anti-viral TLRs, TLR3 and TLR7, are highly conserved between mammals and birds and are strictly localized in the endosome. Mammalian TLR3 and TLR7 recognize viral dsRNA and ssRNA, respectively, and stimulation of either induces the expression of type I interferons [20,64]. Chicken TLR3 exhibits 48% amino acid identity to human TLR3, and has recently been shown to mediate the expression of IFN-β upon stimulation with the dsRNA chemical analog Poly I:C (1.3.3.1) [42,43]. Chicken TLR7 has 63% amino acid homology to human TLR7 and treatment of mammalian tissues with synthetic ssRNA compounds such as R848 (Imidazoquinoline) leads to TLR7/MyD88 signaling to induce the expression of type I interferons IFN-α and IFN-β. Toll-Like Receptor 4 (TLR4) is perhaps the most widely recognized anti-bacterial TLR. This TLR is present on the cell surface and binds the lipopolysaccharide (LPS) of gram-negative bacteria [38]. Chicken TLR4 has 43% amino acid homology to human TLR4. The primary cytokines expressed in response to TLR4 stimulation include IL-8 and IL-1β. Toll-Like Receptor 5 (TLR5) is also an anti-bacterial TLR present on the cell surface. This receptor recognizes bacterial flagella [69]. Chicken TLR5 has 50% amino acid homology to human TLR5, acting through MyD88 dependent signaling to induce expression of IL-1β and IL-6 [69] (Table 1).

Toll-Like Receptor 9 (TLR9) is absent in the avian genome, however a functional ortholog of TLR9, known as TLR21, has been identified in *Gallus gallus* and also recognizes CpG oligodeoxynucleotides (CpG-ODNs) and can lead to the expression of

TLR	Putative Ligand	
	Avian	Mammalian
TLR1a	Lipopeptide	Lipopeptide
TLR1b	Lipopeptide	Lipopeptide
TLR2a	Peptidoglycan	Peptidoglycan
TLR2b	Peptidoglycan	Peptidoglycan
TLR3	dsRNA	dsRNA
TLR4	LPS	LPS
TLR5	Flagella	Flagella
TLR7	?	ssRNA
TLR8	Absent	ssRNA
TLR9	Absent	CpG-ODN
TLR15	Lipopeptide	Absent
TLR21	CpG-ODN	Absent

Table 1: Avian Versus Mammalian Toll-Like Receptors

IL-1β, IL-6, IL-8, and IFN-γ through MyD88-dependent signaling [38,68]. Toll-Like Receptor 15 (TLR15) is a TLR unique to avian species, and is highly expressed in various tissues of *Gallus gallus* including bursa, bone marrow, and spleen [70]. A functional study in *Gallus gallus* macrophages in which MyD88 was knocked down (1.3.3.2) revealed that TLR15 is MyD88-dependent, binding bacterial lipoproteins and inducing the expression of IL-1β. Another study of TLR15 demonstrated that chicken TLR15 can induce expression of iNOS upon stimulation with *Mycoplasma synoviae* and diacylated lipopeptides (1.3.3.4) [61,70].

Toll-Like receptor signaling is characterized as either MyD88-dependent or TRIF-dependent. The MyD88-dependent pathway utilizes a series of kinases that function to induce NF-Kβ transcription. These kinases are collectively known as IRAKs (Interleukin-1 Receptor-Associated Kinases) [62,63]. An active MyD88 adaptor molecule will form a complex with IRAK1, IRAK2, and IRAK4, followed by recruitment of tumor necrosis factor receptor associated factor 6 (TRAF6); the result is downstream activation of NF-Kβ and transcription of pro-inflammatory cytokines and chemokines [66,67]. Conversely, the TRIF-dependent signaling pathway can activate multiple pathways and lead to the expression of pro-inflammatory genes, as well as transcription factors such as IRF3, IRF7, and NF-Kβ. The avian genome lacks a sequence ortholog for IRF3, although another critical IRF, IRF7, is present [73]. The TRIF-dependent pathway utilizes TRAF3, as opposed to TRAF6, as well as several Iκβ Kinase (IKK) molecules to mediate downstream signaling by activating IRF3 and NF-Kβ for the transcription of pro-inflammatory genes [64].

1.2.6 Anti-Viral Toll-Like Receptors

Understanding the role of TLRs in mediating the anti-viral response requires a detailed understanding of those receptors that facilitate the expression of anti-viral effector proteins [61,62]. TLR7 and TLR3 recognize ssRNA and dsRNA, respectively, and are localized in the endosome [38,42]. It is proposed that TLR3 and TLR7 work synergistically in the induction of type I interferons and other anti-viral proteins based on the understanding that many viral species will generate ssRNA and dsRNA intermediates as they proceed through their replicative cycle, or are degraded, in the endosome [10]. These intermediates will therefore trigger both TLR3 and TLR7. One example is influenza A virus of the *Orthomyxoviridae* family. Binding of the viral glycoprotein hemagglutinin to a sialic receptor on the cell surface initiates receptor-mediated endocytosis into the host cell [10]. As the influenza viral particle sheds its protein coat in the endosome, the virus begins synthesizing its own viral RNA transcripts by use of its own RNA polymerase [58] . This generates a pool of ssRNA transcripts along with dsRNA intermediates. The resident endosomal anti-viral receptors, TLR3 and TLR7, will be activated and can mediate the downstream expression of pro-inflammatory cytokines and chemokines [44].

1.2.6.1 Toll-Like Receptor 3 (TLR3)

Toll-Like Receptor 3 (TLR3) has been established in mammals and birds to bind dsRNA, and is a critical anti-viral receptor leading to expression of type I interferons [43,58]. As described, TLR3 is present in the endosome and operates through TRIF-dependent signaling to induce IFN-β. Human and chicken TLR3 share 48% amino acid

homology; mammalian TLR3 has been shown through *in vitro* and *in vivo* modeling to be a critical anti-viral receptor, while chicken TLR3 has been shown *in vitro* to be an active anti-viral dsRNA receptor (1.3.3.1) [42,43].

1.2.6.2 Toll-Like Receptor 7 (TLR7)

Toll-Like Receptor 7 (TLR7) has been established in mammalian systems to bind ssRNA and mediate a MyD88-dependent induction of type I interferons such as IFN-α and IFN-β [10]. Human and chicken TLR7 have 63% amino acid homology and is highly expressed in immune related tissues [38]. Studies have shown mammalian TLR7 to be highly responsive to synthetic ssRNA compounds, leading to induction of IL-1β, IL-6, IL-8, IFN-α, and IFN-β [72]. A recent study performed in HD11 chicken macrophage cells as well as chicken splenocytes revealed that synthetic ssRNA compounds induce the expression of pro-inflammatory cytokines, including type- I - interferons, and infection of HD11 cells with avian influenza strain H9N2 led to significant TLR7 upregulation [72]. While this data suggests that avian TLR7 is a functional ortholog of mammalian TLR7, experimental evidence linking avian TLR7 stimulation to an anti-viral response remains to be presented [59].

1.2.7 Interferon Regulatory Factor 7 (IRF7)

Among the transcription factors mediating the pro-inflammatory response, the interferon regulatory factors (IRFs) are critical for transcription of interferons; the ability of IRFs to mediate transcription of type I and II interferons has made them a priority in understanding the signaling mechanisms of the anti-viral response [63,73]. The mammalian genome codes for 9 IRFs; two of these, IRF3 and IRF7, have been elucidated

in mammals as the primary transcription factors regulating type-I-interferon production [20]. These IRFs reside in the cytoplasm and are maintained at low levels; upon stimulation by an activating kinase, such as IKK (Iκβ Kinase) or PI3K (Phospho-Inositol 3 Kinase), IRF3 and IRF7 are translocated to the nucleus to transcribe IFN responsive genes [73]. The avian genome lacks a sequence ortholog for mammalian IRF3, although IRF7 is present and is postulated to be a functional ortholog of mammalian IRF7. Similar to avian TLR7, the goal of this study has been to understand the role of avian IRF7 in mediating the anti-viral immune response in HD11 chicken macrophages. Despite the lack of an IRF3 sequence ortholog, it is possible that an IRF3 functional ortholog exists and works cooperatively with other avian transcription factors to mediate the pro-inflammatory response.

1.3 RNA-Interference (RNAi): Overview

In 1998, two scientists and their affiliates, Craig C. Mello of the University of Massachusetts and Andrew Z. Fire of Stanford University, found that injection of double stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* resulted in a down-regulation of gene expression and an altered phenotype [5]. Ironically, *C. elegans* researchers had been using RNAi as a tool several years before the full characterization of the RNAi pathway by Craig Mello and Andrew Fire [77,78]. The pioneering work by Mello and Fire determined three characteristics of RNAi; it is induced by dsRNA, the effects of RNAi are systemic, and RNAi is heritable [78]. It was discovered that soaking *C. elegans* in dsRNA led to knockdown phenotypes, and that injection of dsRNA into the body cavity or gonads created an equal RNAi response [5]. It was evident that the

molecular machinery of RNAi was germ-line encoded and therefore inherent to each cell. It was observed through *in vitro* studies of *D. melanogaster* that the injected dsRNA was somehow being processed by cytoplasmic machinery to create small RNA intermediates, also known as small-interfering RNAs (siRNAs), that were guided to a complementary mRNA transcript; this allowed for a targeted mRNA reduction and the corresponding knockdown phenotype [74,75].

1.3.1 Mechanism

The RNAi pathway is an endogenous mechanism by which cells regulate gene expression at the post-transcriptional level [5]. The RNAi pathway is responsible for regulating cell growth, tissue differentiation, chromatin remodeling, and cell division. There are two general classes of small RNAs that regulate gene expression; microRNAs (miRNAs) and small interfering RNAs (siRNAs) [74,76]. These two types of RNA can be endogenous or exogenous to the cell; however a majority of genomically encoded gene regulatory RNAs are miRNAs, with roughly 5% of the human genome coding for miRNAs that regulate 30% of the genes [76,78]. While siRNAs can be endogenously encoded, siRNAs are most commonly derived from viral or synthetic sources. Transcription of genes coding for miRNAs require several processing steps in the nucleus before being exported to the cytoplasm for incorporation into a multi-protein complex known as the RNA-Induced Silencing Complex (RISC) [76,77]. Within the nucleus are a series of enzymes that will process the pri-miRNA transcript; this pri-miRNA transcript forms a characteristic stem-loop structure, which is further modified by a nuclear RNase III enzyme known as Drosha [79]. This mature pri-miRNA is exported to the nucleus for

incorporation into the RISC complex; it is at this step that the pathways for miRNA and siRNA processing converge [76,78] (Figure 2). A cytoplasmic RNase III enzyme known as Dicer can recognize and process long dsRNA molecules, whether they are miRNAs or siRNAs, and facilitate their loading into the RISC complex [78,79]. Dicer also works in conjunction with the Argonaute class of proteins to unwind the miRNA or siRNA duplex; the guide strand of the unwound miRNA or siRNA is then targeted to its complementary mRNA transcript. A key difference between miRNAs and siRNAs is the degree of complementarity between the miRNA/siRNA duplex and the target mRNA [77,78]. A miRNA typically will not have perfect complementarity to its mRNA target, and the mRNA is sequestered into cytoplasmic entities known as P-bodies [5]. Conversely, siRNAs can have perfect complementarity to their target mRNA, resulting in endonucleolytic cleavage of the mRNA [5]. Despite these differences, miRNAs and siRNAs both play a critical role in post-transcriptional regulation and have a significant impact on gene expression in a given cell.

1.3.2 RNAi: Reverse Genetic Approach to Studying Gene Function

In the early days of studying gene function, researchers were limited by their inability to target a single gene for functional study; elucidating gene function was accomplished using a methodology known as "forward genetics" and relied on the generation of random mutations through chemical or radioactive treatments. The resulting mutant offspring of the model organism, for example *Drosophila melanogaster* or *Caenorhabditis elegans*, could be screened based on phenotypic variation, a reflection of mutations in different gene sequences [37]. These unique mutations could be mapped

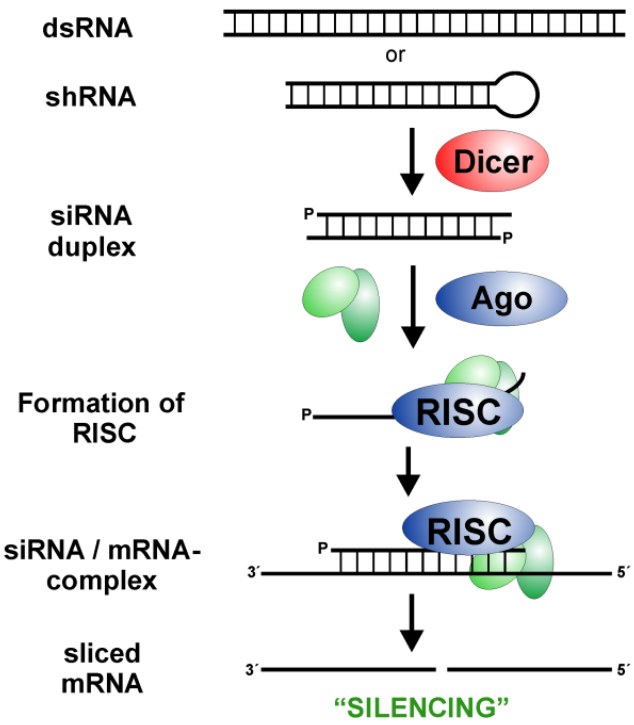

Figure 2: Mechanism of siRNA Mediated RNA-interference (RNAi). Gene silencing by siRNA mediated RNAi begins with precursor dsRNA that is processed by endoribonuclease Dicer; the siRNA duplex is unwound by a catalytic Argonaute protein and loaded into the RNA-Induced Silencing Complex (RISC); the Argonaute protein will guide the RISC complex to its complementary target mRNA, leading to cleavage of the mRNA and translational repression of the corresponding protein.

to their genetic loci through selective cross breeding with known chromosomal markers [70]. Quantitative analysis of the inheritance patterns of a particular phenotype allowed researchers to determine the genetic loci of a mutation relative to the chromosomal marker. The discovery of technologies such as site-directed mutagenesis, siRNA mediated gene silencing (RNAi), and targeted transgene insertion has allowed researchers to target a single gene without major disturbance to the host system or other DNA sequences [60]. This methodology is appropriately termed “reverse genetics”, and is a powerful alternative to classic methods in forward genetics.

1.3.3 Previous RNAi Studies of *Gallus gallus* Receptor Mediated Innate Immune Pathways

Several functional studies have been performed in *Gallus gallus* in an effort to understand the signaling mechanisms of the avian innate immune response. A critical tool for this effort has been RNA- interference (RNAi), and allows researchers to target a single mRNA transcript corresponding to a single protein. Currently, five innate immune related genes have been knocked down using RNAi in *Gallus gallus*; TLR3, MyD88, TLR15, iNOS, and NLRC5. Each of these studies have utilized the immortalized chicken macrophage cell line HD11, indicating that HD11s are a practical model for functional gene studies of *Gallus gallus* using RNAi technology.

1.3.3.1 Toll-Like Receptor 3 (TLR3)

A study on *Gallus gallus* Toll-Like Receptor 3 (TLR3) conducted at the Cooperative Research Centre for the Australian Poultry Industry was able to create a link between chicken TLR3 and its regulation of IFN-β in the context of an anti-viral immune

response to avian influenza [42]. This study utilized the synthetic dsRNA chemical analog known as Poly I:C to understand the regulatory mechanisms of type-I interferon production and TLR3 induction. Poly I:C has been used extensively in mammalian studies as a dsRNA agonist to stimulate TLR3 and subsequent transcription of type I interferons [3]. In this study, chickens were infected with highly pathogenic avian influenza (H5N1) and analyzed for pro-inflammatory gene expression in various tissues. Using qRT-PCR, it was found that TLR3 and IFN-β were strongly upregulated at 24 hours in the spleen, lung, and brain; highest IFN-β induction was 25-fold in the spleen, while the highest TLR3 induction was 66-fold in the brain [3]. In conjunction with this experiment, HD11 chicken macrophage cells were transfected with a cocktail of three siRNAs targeting TLR3 mRNA for a transient knockdown, followed by treatment with Poly I:C to mimic a dsRNA challenge. A significant fold decrease in IFN-β expression was observed in TLR3 knockdown cell cultures relative to the scrambled siRNA control, providing evidence for the importance of avian TLR3 in recognizing dsRNA molecules to induce a type- I- interferon response [3].

1.3.3.2 Myeloid Differentiation Primary Response Gene 88 (MyD88)

A study conducted by researchers at Iowa State University determined that a novel TLR of *Gallus gallus*, TLR15, recognizes bacterial lipopeptides and functions through MyD88-dependent signaling to induce pro-inflammatory genes [70]. Utilizing HD11 chicken macrophage cells, it was determined that multiple agonists, including lipopeptides, can upregulate the expression of IL-1β as well as TLR15 [2]. To determine whether TLR15 functions through MyD88-dependent signaling, a cocktail of three

siRNAs were designed to target MyD88 of *Gallus gallus* for a transient MyD88 knockdown; HD11 cell cultures with a 70% reduction in MyD88 were unresponsive to lipopeptide treatment as measured through quantitative analysis of IL-1β by quantitative-real time polymerase chain reaction (qRT-PCR) [2]. The strong induction of IL-1β and TLR15 in HD11 cell cultures by lipopeptides, combined with a loss of IL-1β induction after MyD88 knockdown, provides evidence for TLR15 as a MyD88-dependent receptor recognizing bacterial lipopeptide [2].

1.3.3.3 Toll-Like Receptor 15 (TLR15)

In a study conducted by researchers from the University of Ljubljana, Slovenia, the role of TLR15 in recognizing bacterial lipoproteins was validated through a siRNA-mediated knockdown of TLR15 in HD11 chicken macrophage cells; HD11 cultures with a 70% knockdown of TLR15 showed a significant decrease in iNOS induction upon treatment with diacylated lipopeptide derived from *Mycoplasma synoviae* [71]. Treatment of normal HD11 cultures with bacterial lipoproteins led to a strong induction of the pro-inflammatory gene iNOS, or inducible nitric oxide synthase. This enzyme catalyzes the production of reactive oxygen species that will destroy pathogens by inducing oxidative damage to pathogenic RNA or DNA. The connection between a TLR15 knockdown and a reduction in iNOS expression upon lipoprotein treatment was validated using the Griess Reagent System for measuring nitric oxide (NO). Cell supernatants were quantified for the presence of NO; it was found that TLR15 knockdown cultures had significantly less NO in the supernatant relative to the control [71].

1.3.3.4 Inducible Nitric Oxide Synthase (iNOS)

Mammalian iNOS has been established to be a potent anti-microbial effector gene, coding for the inducible nitric oxide synthase enzyme that will catalyze the synthesis of reactive oxygen species such as nitric oxide (NO). Macrophages treated with microbial components or stimulatory cytokines such as IFN-γ leads to a strong induction of iNOS, providing evidence for iNOS as a pro-inflammatory gene [1]. A study conducted by researchers at Iowa State University served to demonstrate avian iNOS to be a critical anti-microbial effector gene; HD11 cell cultures treated with iNOS siRNAs showed a significant decrease in iNOS mRNA when stimulated with IFN-γ relative to control HD11 cultures [1]. Furthermore, genes that are responsive to iNOS expression such as IL-1β, IL-6, TGF-β4, and SOCS-3 were measured in siRNA treated HD11 cultures and did not show any alterations upon IFN-γ treatment [1]. This data demonstrates avian iNOS to be a critical anti-microbial pro-inflammatory gene.

1.3.3.5 NOD-Like Receptor Family CARD Domain Containing 5 (NLRC5)

In another study performed by researchers at Iowa State University, a member of the NLRs, NLRC5 (NOD-Like Receptor Family CARD Domain Containing 5) was targeted for knockdown in the HD11 chicken macrophage cell line. Previous studies of human NLRC5 have demonstrated NLRC5 to be a positive regulator in the innate immune response, being highly expressed in lymphoid tissues and in macrophages and inducing IFN-α, IFN-β, and IL-6 upon binding to bacterial ligands [9]. In this study, siRNAs targeting *Gallus gallus* NLRC5 in HD11 cells were transfected to create a transient NLRC5 knockdown, followed by treatment with bacterial LPS to measure

expression of pro-inflammatory genes [9]. It was found that after stimulation of NLRC5 knockdown HD11 cultures with LPS, the cells had a diminished ability to express IFN-α and IFN-β, although IL-6 induction did not change significantly between control and experimental cultures [9]. These results suggest that NLRC5 is a positive regulator of the pro-inflammatory response in *Gallus gallus*.

1.4 Conclusion

Understanding the mechanisms of the avian innate immune response is critical for mitigating the loss to avian infectious disease. Each year, hundreds of millions of poultry are slaughtered in an effort to contain or eradicate avian infectious diseases, a testament to the current lack of therapeutic counter-measures necessary to prevent infection. The innate immune system utilizes a combination of anatomical, physiological, and chemical barriers to microbial infection to eliminate a pathogen before it can effectively colonize the host system. The hallmark of the innate immune response, inflammation, occurs when a Pathogen Associated Molecular Pattern (PAMP) binds to a Pattern Recognition Receptor (PRR) on the cell surface, in the endosome, or in the cytoplasm of a host cell. There are three classes of PRRs; the NOD-Like Receptors (NLRs), RIG-I Like Receptors (RLRs), and Toll-Like Receptors (TLRs). The objective of this study is elucidate the function of *Gallus gallus* Toll-Like Receptor 7 (TLR7) as a potential anti-viral innate immune receptor recognizing single-stranded RNA (ssRNA); mammalian TLR7 has been established as an anti-viral receptor binding ssRNA, and mediates the expression of type I interferons. In addition, this study serves to elucidate the function of *Gallus gallus* Interferon Regulatory Factor 7 (IRF7), a transcription factor regulating the expression of

type I interferons. The *gallus gallus* genome has a sequence ortholog for mammalian IRF7, but not IRF3, and presents an interesting candidate for therapeutic targeting. The application of RNAi technology to the study of avian innate immunity has proven to be an effective methodology for understanding gene function, and this study has successfully applied RNAi for the study of *Gallus gallus* TLR7 and IRF7.

1.5 Hypothesis

It is hypothesized that avian Toll-Like Receptor 7 (TLR7) is functionally homologous to mammalian Toll-Like Receptor 7 (TLR7) as a pattern recognition receptor (PRR) recognizing single-stranded RNA (ssRNA), and is responsible for mediating the transcription of pro-inflammatory genes, specifically those coding for type I interferons and reactive oxygen species. Given that Interferon Regulatory Factor 7 (IRF7) has been identified in mammals as a critical transcription factor regulating type I interferon expression, it is hypothesized that the *Gallus gallus* sequence ortholog for IRF7 plays a similar role in regulating type I interferon transcription downstream of a TLR7 stimulation with ssRNA. While it is hypothesized that a ssRNA challenge will lead to transcription of genes coding for reactive oxygen species, for example iNOS, the role of IRF7 in mediating transcription of iNOS in collaboration with TLR7 is unclear.

1.6 Objectives

The first objective of this study was to demonstrate a siRNA-mediated knockdown of *Gallus gallus* Toll-Like Receptor 7 (TLR7) and Interferon Regulatory Factor 7 (IRF7) in the immortalized chicken macrophage cell line HD11. The second objective was to understand the function of avian TLR7 and IRF7 in mediating the pro-

inflammatory response to a ssRNA challenge in the avian system. To accomplish this, siRNA mediated RNA-interference (RNAi) was employed to target TLR7 and IRF7 mRNA transcripts for a transient protein knockdown in HD11s, followed by a ssRNA challenge with a synthetic chemical agonist known as R848 to understand the functional consequence of a TLR7, IRF7, and a TLR7 + IRF7 knockdown. By measuring fold changes in pro-inflammatory gene expression upon R848 treatment under these circumstances, it was possible to bring some functional understanding to TLR7 and IRF7 and their roles in mediating the innate immune response of *Gallus gallus*, specifically to a ssRNA challenge.

Chapter 2

MATERIALS AND METHODS

2.1 Culture and Transfection of HD11 Chicken Macrophage Tissue Culture

The immortalized chicken (*Gallus gallus*) macrophage cell line HD11 was cultured in Gibco's Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) in T25 flasks (Corning, Inc., Corning, New York) passaged at a 1:3 or 1:4 ratio every 3 days. Cells were allowed to reach 80-90% confluence before splitting. Upon splitting, old media was aspirated and the flask was washed with 5 mL of phosphate buffered saline (PBS), followed by aspiration and treatment with 1 mL of trypsin reagent to detach adhered cells; after 1 minute, the trypsin was neutralized with the appropriate dilution volume of complete DMEM. The Gibco's DMEM was supplemented with 5% fetal bovine serum (FBS) and 5% chicken serum (CS), along with 1% Penicillin/Streptomycin (PS). The seeded T25 flasks were incubated at 37 ° C and 5% CO_2 in a HERA-Cell CO_2 Incubator (Thermo Scientific, Waltham, MA) maintained at a constant humidity.

Experiments were conducted in 24-well tissue culture plates (Corning, Inc., Corning, New York) with a working volume of 500 uL per well. On the day of splitting, HD11 cells were detached with 1 mL of trypsin reagent, followed by neutralization with 2 mL of complete DMEM. From this suspension, 100 uL was diluted in 100 uL of Trypan Blue Exclusion Dye (Life Technologies, Carlsbad, CA, USA) and loaded onto a hemocytometer for counting. Cells were visualized under a light microscope; the four

corner squares of the hemocytometer were quantified. After calculating the number of cells in the original suspension, the appropriate volume of suspension was seeded into the 24-well plate to achieve a starting density of $6x10^4$ cells in 500 uL of complete DMEM. The plate was incubated overnight in the HERA-Cell incubator under standard conditions of 37 ° C and 5% CO_2. At 24 hours post-seeding, the HD11 culture was approximately 50% confluent with the cells displaying extended pseudopods, characteristic of macrophage cells.

A mock transfected well was trypsinized, counted, and measured for viability using Trypan Blue Exclusion Dye (Life Technologies, Carlsbad, CA, USA). Cells were consistently shown to be ≥ 95% viable at 24-hours post transfection. Transfection efficiency was measured using the fluorescently labeled control siRNA TYE-563 (Integrated DNA Technologies, Coralville, IA) and visualized under a fluorescent microscope (Nikon Eclipse 80i Fluorescence Microscope, Nikon, Tokyo, Japan) measured at 560 nm. Both fluorescent and bright phase images were taken of the TYE-563 transfected cultures and merged to allow visual counting of fluorescent cells in the captured frame. A transfection efficiency of ≥ 95% was consistently achieved in each TYE-563 transfected HD11 culture.

2.2 Agonist Treatment of HD11 Macrophage Cells

Gene expression profiles for 9 avian genes were determined for HD11 macrophages treated with the agonists R848 or Poly I:C. A 24 well plate was seeded at $1x10^5$ cells/well in 500 uL of complete DMEM; at 24 hours post seeding the HD11 cultures had reached 90% confluence. For each agonist, R848, a ssRNA agonist, and Poly

I:C, a dsRNA agonist, four time points were measured; 0,1,2, and 8 hours post treatment. This corresponded to a total of 8 wells; four wells for R848 and four wells for Poly I:C. At 24 hours post seeding, the wells were aspirated and replaced with 500 uL of specialized maintenance media (1% chicken serum/1% fetal bovine serum/1% Penicillin Streptomycin) containing 10 ug/mL of each respective agonist. Total RNA was harvested using Qiagen's RNeasy Mini Kit according to manufacturers instructions. For each RNA sample, a total of nine genes were measured by Quantitative Real Time- Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR); GAPDH, TLR3, TLR7, NF-Kβ, iNOS, IL-1β, IL-6, IFN-α, and IFN-β. The mRNA expression levels of each gene were normalized to GAPDH and calculated relative to time point 0 using the Livack Method.

Total RNA isolation from HD11 cell cultures was accomplished using Qiagen's RNeasy Mini Kit specific for total RNA isolation from animal tissues. This kit allows for a maximum of 100 ug of total RNA extract in a 30 or 50 uL elution volume; at 48 hours post-seeding of $6x10^4$ cells/well in a 24-well plate, an average of 200 ng/uL of total RNA in 50 uL RNase free water (10 ug) was extracted. A critical step in this RNA isolation protocol was the DNase I treatment of the RNeasy Mini Column; a special solution containing the enzyme DNase I was applied to the column for 15 minutes, which would specifically degrade any DNA that might contaminate the final RNA elution. A 260/280 ratio between 2.1-2.3, indicating pure RNA, was consistently achieved. Quantity and purity of total RNA extract was measured by nanodrop (Thermo Fisher Scientific, Waltham, MA). Measurement of gene expression was accomplished using Qiagen's QuantiTect SYBR Green RT-PCR Kit (Qiagen, Venlo, Netherlands) which allows for

one-step reverse transcription and PCR in a single reaction volume. The qRT-PCR was performed in a 20 uL reaction volume using a 384-well plate. For each gene assayed, three replicates containing 200 ng of RNA was used, along with two negative controls wells; the first negative control had no RNA template and the second was pure RNase free water. Forward and reverse primers for each respective gene were combined in a volume of 4 uL containing 0.5 uM of each primer. Each reaction contained 10 uL of 2x QuantiTect SYBR Green RT-PCR Master Mix, along with 0.1 uL QuantiTect RT Mix containing Omniscript and Sensiscript Reverse Transcriptase in a total reaction volume equalized to 20 uL using RNase free water. Prior to thermo-cycling, the 384-well plate was lightly centrifuged to ensure each 20 uL reaction was completely settled in the plate. The thermo-cycler was adjusted according to manufacturers instructions as well as to the melting temperatures of the DNA primers.

All genes assayed by qRT-PCR were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) using the Livak Method (Washington University, 2001). This method calculates the ΔΔCt value can be applied to the equation 2^-(ΔΔCt) which will give the fold increase or decrease in gene expression. The fold decrease in gene expression is expressed as 1/2^-(ΔΔCt), while fold increase is simply 2^-(ΔΔCt). To determine relative gene expression levels in normal versus TLR7/IRF7 knockdown HD11 cultures when treated with R848, the – R848 NC1 wells at each specific time point were normalized to + R848 NC1 and + R848 TLR7/IRF7 knockdown HD11 cultures. In this case, the Livak Method was applied such that the NC1 wells served as control ΔCt values and the TLR7/IRF7 wells served as experimental ΔCt values.

Gene	5'-Forward-3'	5'-Reverse-3'
GAPDH	CCTCTCTGGCAAAGTCCAAG	CATCTGCCCATTTGATGTTG
HPRT	ACGACCTGGACTTGTTCTGCATAC	GTGTCCTCCCATGCCCTTCATAAT
TLR3	GCAACACTTCATTGAATAGCCTTGAT	GCCAAACAGATTTCCAATTGCATGT
TLR7	GCACACCGGAAAATGGTACAT	AGCATTGGAAATAAGAAGAGCAAGA
NF-κβ	GAAGGAATCGTACCGGGAACA	CTCAGAGGGCCTTGTGACAGTAA
IRF7	CGTATCTTCCGCATCCCTTGG	TCGTCGTTGCACTTGGAGCG
iNOS	GCATTCTTATTGGCCCAGGA	CATAGAGACGCTGCTGCCAG
IL-6	CAGGACGAGATGTGCAAGAAG	CCCTCACGGTCTTCTCCATA
IL-1β	ATGACCAAACTGCTGCGGAG	GTCGCTGTCAGCAAAGTCCC
IFN-α	CCA GCA CCT CGAGCAAT	GGCGCTGTAATCGTTGTCT
IFN-β	CCTCAACCAGATCCAGCATT	GGATGAGGCTGTGAGAGGAG

Table 2: Forward and reverse primers for quantitative measurement of gene expression using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

Final values were calculated as percent reduction in inflammatory gene expression upon R848 treatment when TLR7/IRF7 were knocked down.

2.3 siRNA Transfection of HD11 Macrophage Cells

Transfection of siRNAs into HD11 cells was accomplished using the Roche X-tremeGene siRNA Transfection Reagent (Roche Diagnostics, Basel, Switzerland). RNA-interference targeting TLR7 and IRF7 mRNA was performed using TriFECTa RNAi Kit (Integrated DNA Technologies, Coralville, IA). This kit provides a cocktail of three target specific double-stranded siRNAs for TLR7 and IRF7 were designed by IDT using the National Center for BioTechnology Information (NCBI) RefSeq Genbank sequences specific for *Gallus gallus*. These siRNAs are designed to target the splice-common exon of the target mRNA. When transfected as a cocktail into cell culture at ≥ 90% transfection efficiency, these siRNAs are projected to give a ≥ 70% knockdown when assayed at 24 hours post-transfection. The TriFECTa RNAi Kit utilizes a novel Dicer-Substrate methodology in which a 27-mer dsRNA molecule is processed by Dicer and subsequently loaded into the cytoplasmic RISC complex. This kit also provides three control siRNAs; a positive control siRNA targeting the highly conserved HPRT gene, a fluorescently labeled siRNA (TYE-563) to validate transfection efficiency, and a scrambled sequence negative control siRNA (NC1) to ensure no off-target effects on gene expression in the HD11 cell by the siRNAs. Each TLR7 and IRF7 specific duplex was provided as a 2 nM lyophilized pellet and was suspended in 100 uL of molecular biology grade nuclease free water provided by IDT to create a 20uM master stock. The control duplexes came as 1 nM lyophilized pellets to be suspended in 50 uL of molecular

biology grade nuclease free water to create a 20 uM master stock. The suspended siRNAs were stored at -20 °C for storage and thawed on ice on the day of transfection.

On the day of transfection, a series of sterile 1.5 mL microfuge tubes were arranged such that a 25 uL media suspension containing the desired concentration of siRNA could be prepared and pipetted into a corresponding 1.5 mL microfuge tube containing 3 uL X-tremeGene siRNA Transfection Reagent suspended in 25 uL of media. Both the siRNA and transfection reagent were prepared in Gibco's Opti-MEM I Reduced Serum Media (Life Technologies, Carlsbad, CA). The use of reduced serum media in transfection was critical for ensuring high transfection efficiency. The HPRT, TYE-563, and NC1 control siRNAs were added (150 nanomoles each) to their respective microfuge tube containing 25 uL media. Likewise, each of the 3 siRNAs for TLR7 and IRF7 were added to their respective 1.5 mL microfuge tube containing 25 uL Opti-MEM. After 5 minutes of incubation at room temperature, the siRNAs were added to the corresponding microfuge tube containing X-tremeGene siRNA Transfection Reagent. The resulting 50 uL solution was incubated for 20 minutes at room temperature to allow liposomes to encapsulate the siRNAs for delivery into the HD11 cells. During this incubation time, the original growth media from the 24-well plate containing the HD11 cell cultures was aspirated and replaced with 200 uL of Gibco's Opti-MEM I Reduced Serum Media. After 20 minutes, the 50 uL transfection mixture was pipetted drop wise into its appropriate well on the 24-well plate to create a final volume of 250 uL. The plate was incubated for 4 hours at 37 °C and 5 % CO_2; after 4 hours, an additional 250 uL of 2x fetal bovine serum/chicken serum complete DMEM was added to the 250 uL volume in each well to

create a 500 uL total volume. The plate was incubated overnight and at 24 hours post-transfection, the wells were aspirated, followed by RNA extraction or agonist treatment as described.

To mimic ssRNA challenge to the HD11 cell cultures, a ssRNA chemical agonist known as R848 (Imidazoquinoline) (Invivogen, San Diego, CA) was prepared in 500 uL of specialized maintenance DMEM media containing 1% Chicken Serum/1% Fetal Bovine Serum/1% Penicillin Streptomycin. The R848 stock vial was suspended in sterile-endotoxin free water to create a 1 mg/mL solution; from this stock, 5 uL was added to 500 uL of maintenance media to create a 10 ug/mL working solution to be added to each respective well of the 24-well plate at 24 hours post transfection. Two 24-well plates were seeded at $6x10^4$ cells/well for the R848 agonist experiments; one 24-well plate would serve as the (-) R848 control to validate successful transfection and knockdown of the positive gene control HPRT as well as TLR7 and IRF7. The (-) R848 plate also served to show no off-target effects on gene expression by introduction of siRNAs with the use of the scrambled negative control siRNA NC1. The second 24-well plate received a 10 ug/mL treatment of R848 and contained NC1, TLR7, IRF7, TLR7 + IRF7 siRNA transfected wells which were then compared to the (-) R848 plate for inflammatory gene expression profiles. Each time point measured had a corresponding NC1 transfected well with and without R848 treatment, which was necessary for accurate normalization of inflammatory gene expression profiles between experimental and control samples of the -/+ R848 treated plates.

siRNA ID/Gene Target	Duplex
TYE-563 Fluorescent siRNA	Sense /5TYE563/T*CrC rUrUrC rCrUrC rUrCrU rUrUrC rUrCrU rCrCrC rUrUrG rUG*A Antisense /5TYE563/T*CrA rCrArA rGrGrG rArGrA rGrArA rArGrA rGrArG rGrArA rGG*A
HPRT Positive Control	Sense /5Phos/rGrCrC rArGrA rCrUrU rUrGrU rUrGrG rArUrU rUrGrA rArAT T Antisense -rArArUrUrUrCrArArArUrCrCrArArCrArArArGrUrCrUrGrGrCrUrU
NC1 Scrambled Negative Control	Sense rCrGrU rUrArA rGrCrG rCrGrU rArUrA rArUrA rCrGrC rGrUA T Antisense -rArUrA rCrGrC rGrUrA rUrUrA rUrArC rGrCrG rArUrU rArArC rGrArC
TLR7 Duplex 1	5'- GCUAUGGAGAUAAUGUGAAAGUGGA -3' 3'- AACGAUACCUCUAUUACACUUUCACCU -5'
TLR7 Duplex 2	5'- CCUUUACAACCUAGAAAUUCUUGAT -3' 3'- UUGGAAAUGUUGGAUCUUUAAGAACUA -5'
TLR7 Duplex 3	5'- CCUUUAGUCAAUGGAGAUUGCAUGA -3' 3'- UUGGAAAUCAGUUACCUCUAACGUACU -5'
IRF7 Duplex 1	5'- ACCGAGGAAAGAUGGUCUACCAGGA -3' 3'- GAUGGCUCCUUUCUACCAGAUGGUCCU -5'
IRF7 Duplex 2	5'- GCUGUCUUGCAAAGAGCUUUCUGGC -3' 3'- GGCGACAGAACGUUUCUCGAAAGACCG -5'
IRF7 Duplex 3	5'- AGCAAUACCACAUGCAGACAGACTG -3' 3'- GCUCGUUAUGGUGUACGUCUGUCUGAC -5'

Table 3: Control and experimental siRNA sequences used in HPRT, TLR7, and IRF7 knockdown in HD11 tissue culture.

2.4 Statistics

To determine significance of siRNA mediated HPRT, TLR7, and IRF7 gene knockdown as well as reduction of iNOS and IFN-α expression upon R848 treatment, the Tukey-Kramer All-Pairs Honestly Significant Difference (HSD) test was employed. This statistical tool compares the mean fold expression between control and experimental treatments, and can compare all means with the assumption that the sample sizes to be compared are the same. For gene knockdown, the mean residual mRNA levels in siRNA treated HD11 cultures were compared to scrambled siRNA (NC1) treated HD11 cultures, as well as to mock-transfected containing X-tremeGene siRNA Transfection Reagent. The Tukey-Kramer test was also applied to determine significance of iNOS and IFN-α reduction in siRNA treated HD11 cultures. Three replicates for TLR7, IRF7, and TLR7 + IRF7 treated cultures were compared to maximum iNOS and IFN-α induction at 2 or 8 hours post R848 treatment, respectively. Statistical analysis was performed using JMP Version 10.0 (SAS Institute, Chesterbrook, PA, USA) licensed by the University of Delaware.

Chapter 3

RESULTS

3.1 siRNA Mediated Knockdown of Avian TLR7 and IRF7

Developing a protocol for siRNA transfection of HD11 cells that could maximize siRNA delivery into the HD11 cell while minimizing cytotoxicity was the initial objective of this project. Transfection of HD11 cells was developed for a 24-well format, with a starting cell density of 6×10^4 cells per well as described in the Materials and Methods. At 24 hours post-seeding, the cell cultures had reached 40-50% confluence and were transfected with 150 nanomoles of a specific siRNA. To determine transfection efficiency, TYE-563, a fluorescently labeled siRNA (Integrated DNA Technologies, Coralville, IA) was transfected into HD11 cell culture using 3 uL of Roche X-tremeGene siRNA Transfection Reagent (Roche Diagnostics, Basel, Switzerland) (Figure 3). Transfection efficiency was consistently ≥ 95% (Fluorescent Cells Counted/Total Cells Counted= 395/400), as determined through a merged bright phase and rhodamine overlay of the TYE-563 transfected HD11 culture. The TYE-563 fluorescent siRNA was visualized under the rhodamine excitation wavelength of 560 nm using a Nikon Eclipse 80i Fluorescence Microscope (Nikon, Tokyo, Japan).

Once it was established that siRNAs could be efficiently delivered into the HD11 tissue culture in a 24-well format, the next step was to target avian TLR7 and IRF7 for knockdown. The seeded 24-well plate was transfected for 4 hours, and at 24-hours post transfection the wells were harvested for total RNA using Qiagen's RNeasy

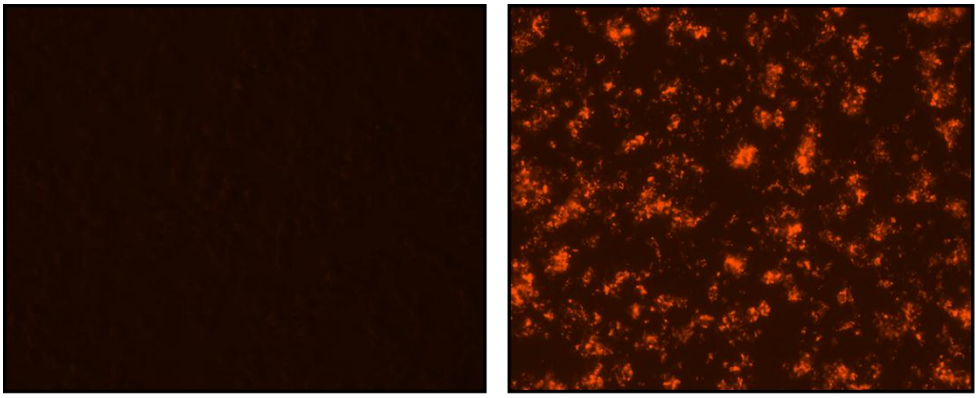

Figure 3: Transfection of fluorescent siRNA control TYE-563 into HD11 chicken macrophages. HD11 macrophage cells were transfected with 150 nanomoles of TYE-563 siRNA using 3 uL X-tremeGene siRNA Transfection Reagent. At 24 hours post transfection, the cells were visualized at 560 nm (Rhodamine) using a Nikon Eclipse 80i Fluorescence Microscope.

Mini Kit. Measurement of TLR7 and IRF7 mRNA levels was accomplished by qRT-PCR, with each gene measured being normalized to GAPDH. For TLR7 siRNA treated cultures, the GAPDH and TLR7 mRNA levels were measured using GAPDH and TLR7 specific primers and compared to the non-treated negative control HD11 cultures. This same method was applied to the measurement of IRF7 knockdown in IRF7 siRNA treated HD11 cultures.

Each siRNA transfection utilized 150 nanomoles of siRNA. The control siRNA HPRT (Hypoxanthine-guanine phosphoribosyltransferase) was incorporated as a positive control siRNA that targets the highly conserved HPRT gene (Figure 4). When transfected at 150 nanomoles in a 24-well format, the HPRT siRNA gave an average knockdown of 58.3% (n = 9). Statistical analysis using the Tukey-Kramer All-Pairs Honestly Significant Difference (HSD) test was utilized to determine the significance between HPRT siRNA treated HD11 cultures and NC1 siRNA treated cultures and mock transfected HD11 cultures. The difference between HPRT siRNA treated HD11 cultures and NC1 mock transfected cultures was found to be highly significant ($p = < .0001$).

The mRNA transcripts of the TLR7 and IRF7 genes were targeted with a cocktail of 3 siRNAs. Each siRNA was delivered at 150 nanomoles, making a total of 450 nanomoles of target specific siRNA. Each replicate knockdown of TLR7 and IRF7 was completed alongside NC1 and a mock transfected HD11 culture containing the X-tremeGene transfection reagent. With a TLR7 specific cocktail of 3 siRNAs, an average knockdown of 68.7% was achieved (n = 9) with a significance of $p = < .0001$ as calculated by the Tukey-Kramer All-Pairs Honestly Significant Difference (HSD) test

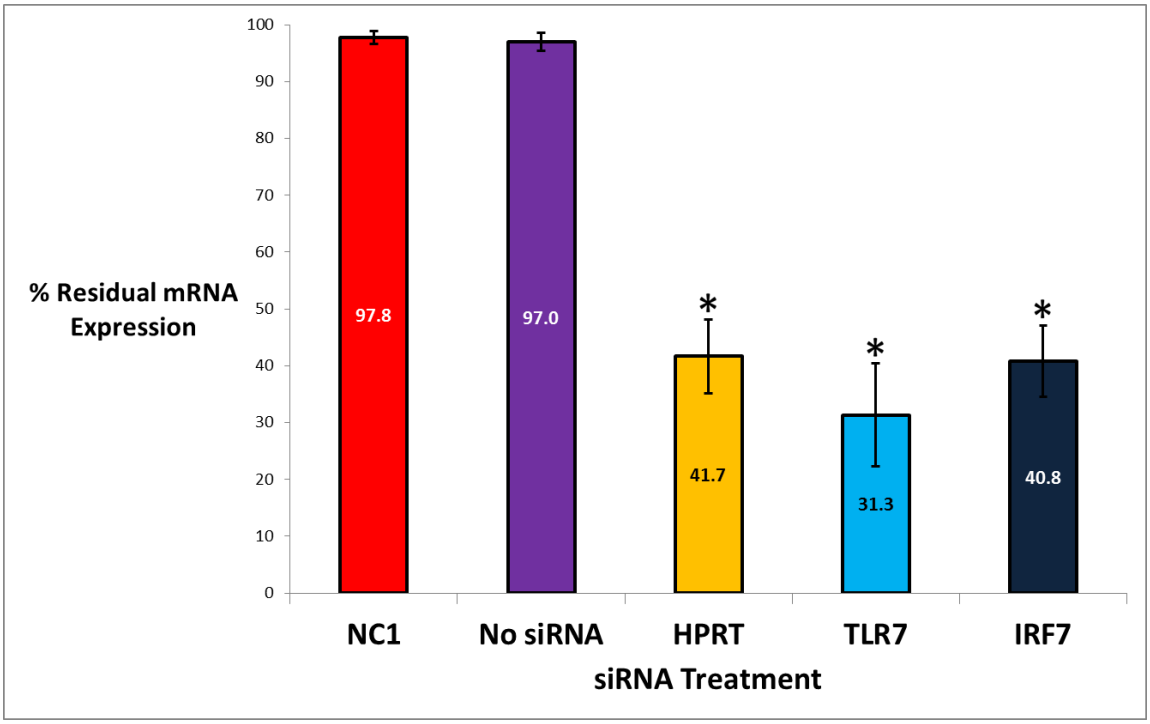

Figure 4: Residual mRNA expression upon treatment of HD11 tissue cultures with 150 nanomoles of HPRT, TLR7, and IRF7 siRNAs. Measured at 24 hours post-transfection, each HPRT, TLR7, and IRF7 siRNA treated HD11 culture was compared to NC1 and mock transfected cultures to determine significance of knockdown. The NC1 negative control is a scrambled sequence siRNA to show no off target effects on HPRT, TLR7, or IRF7 expression. The HPRT siRNA treated culture showed an average knockdown of 58.3% (n=9, p = < .0001). The TLR7 siRNA treated culture showed an average knockdown of 68.7% (n=9, p = < .0001). The IRF7 siRNA treated culture showed an average knockdown of 59.2% (n=7, p = < .0001).

(Figure 4). Knockdown of IRF7 also utilized a cocktail of 3 siRNAs (Table 2), transfected at 150 nanomoles per siRNA for a total of 450 nanomoles. With a cocktail of 3 IRF7 specific siRNAs, an average knockdown of 59.2% was achieved (n = 7) with a significance of p = < .0001 (Figure 4).

3.2 Induction of Pro-Inflammatory Genes in HD11 Chicken Macrophage Cells by R848 (ssRNA) and Poly I:C (dsRNA) Chemical Analogs

Before proceeding with a functional knockdown study of TLR7 and IRF7 in HD11 chicken macrophages, it was necessary to develop a quantitative and qualitative gene expression profile in response to synthetic chemical agonists, specifically to ssRNA and dsRNA. A series of pro-inflammatory and TLR pathway associated genes were selected for a time course study in which HD11 cultures were treated with 10 ug/mL of the respective agonist, followed by RNA extraction at 0,1,2 and 8 hours post treatment for quantitative analysis by qRT-PCR. The ssRNA agonist R848 is widely used in mammalian studies to simulate ssRNA challenge, while Poly I:C is utilized as a dsRNA agonist. The R848 agonist is considered a TLR7 agonist based on the observation that mammalian TLR7 binds ssRNA to mediate a pro-inflammatory response. Poly I:C is considered a TLR3 agonist since Poly I:C binds mammalian TLR3 to mediate a pro-inflammatory response. Both the R848 and Poly I:C time course studies measured the same set of 8 genes; TLR3, TLR7, NF-Kβ, iNOS, IL-1β, IL-6, IFN-α, and IFN-β. The TLR3 and TLR7 genes were included as TLR pathway associated receptors, while NF-Kβ is a critical transcription factor in the TLR pathway. The remaining five genes (iNOS, IL-1β, IL-6, IFN-α, IFN-β) were selected as pro-inflammatory genes and provided

an effective panel of stimulatory genes for comparison between R848 and Poly I:C agonists in HD11 macrophage cells.

The treatment of HD11 macrophage cells with 10 ug/mL of R848 had a stimulatory effect on the transcription of TLR3, TLR7, and NF-Kβ (Figure 5). Interestingly, TLR3 showed an induced expression of 15.8 fold at 2 hours post-R848 treatment. In mammals, R848 is a TLR7 agonist, yet in *Gallus gallus* it appears that a ssRNA agonist can also strongly induce the dsRNA receptor TLR3. TLR7 and NF-Kβ were induced between 4 and 6 fold within 2 hours, and these induced levels of transcripts remained steady throughout the 8 hour time course.

Treating HD11 cells with R848 had a significant impact on the expression of pro-inflammatory genes, specifically iNOS, IL-1β, and IFN-α (Figure 6). At 1 hour post-R848 treatment, IL-1β had a clearly defined peak level of 31.9 fold. The iNOS gene was induced 25.3 fold at 2 hours post-R848 treatment, followed by a sharp decrease similar to IL-1β. Also induced was IFN-α, demonstrating a gradual increase in expression until reaching maximum level of induction of 15.6 fold at 8 hours post-R848 treatment. The induction of iNOS, IL-1β, and IFN-α provided solid evidence for the stimulatory effect of the synthetic ssRNA compound R848 on HD11 chicken macrophage cells. The R848 treatment was not able to induce IL-6 transcription, however IFN-β was induced 5 fold at 8 hours post treatment.

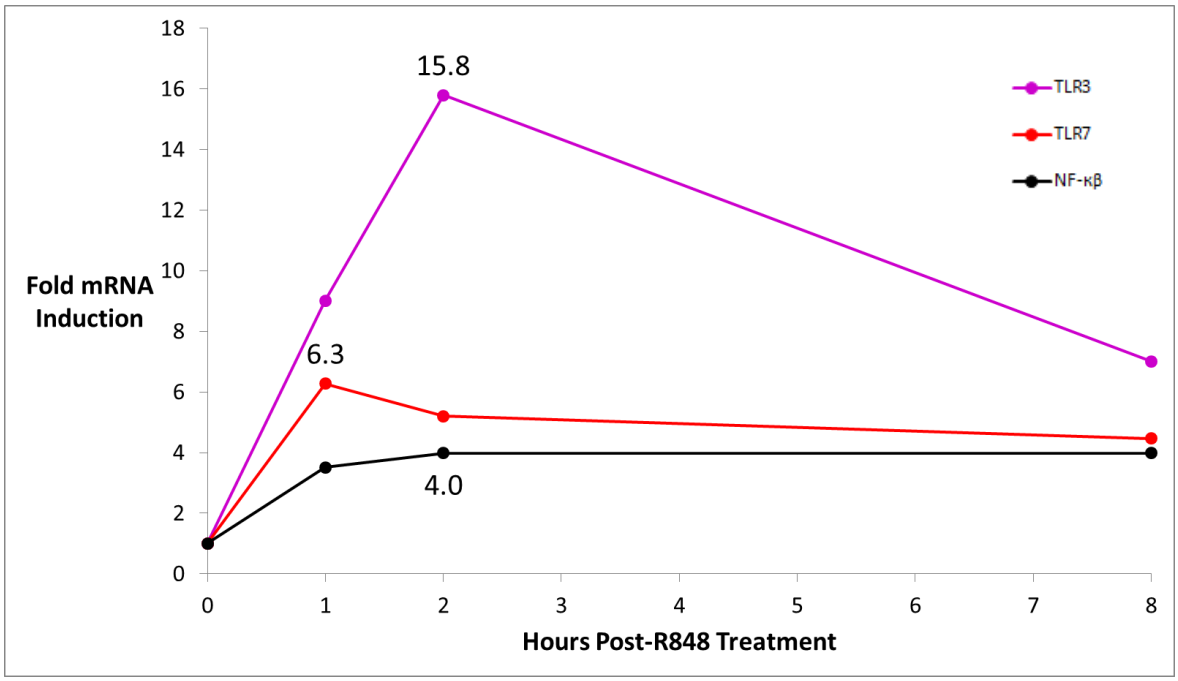

Figure 5: Induction of TLR3, TLR7, and NF-Kβ genes in HD11 tissue culture at 0, 1, 2, and 8 hours post 10 ug/mL R848 treatment. Total RNA samples were extracted from R848 treated HD11 cultures at 0, 1, 2, and 8 hours post-treatment. The ssRNA agonist R848 was able to induce TLR3 (Purple) transcription by 15.8 fold at 2 hours, while TLR7 (Red) and NF-Kβ (Black) only reached between 4 and 6 fold within 2 hours.

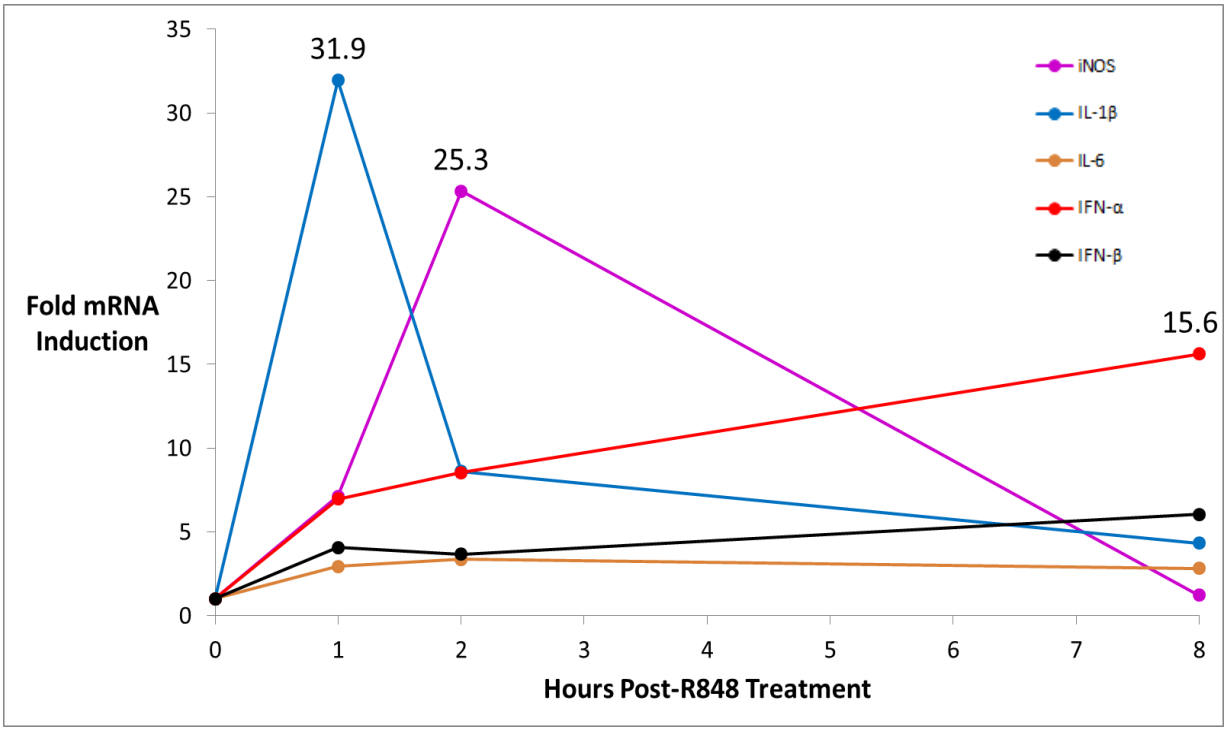

Figure 6: Induction of iNOS, IL-1β, IL-6, IFN-α, and IFN-β genes in HD11 tissue culture at 0, 1, 2, and 8 hours post 10 ug/mL R848 treatment. Total RNA samples were extracted from R848 treated HD11 cultures at 0, 1, 2, and 8 hours post-treatment. R848 treatment induced IL-1β (Blue) 31.9 fold at 1 hour post-treatment, while iNOS (Purple) reached 25.3 fold at 2 hours post-treatment. A gradual increase of IFN-α (Red) transcription was observed, reaching 15.6 fold by 8 hours. IFN-β (Black) was induced approximately 5 fold by 8 hours, while IL-6 (Orange) showed no significant induction.

The treatment of HD11 chicken macrophages with the synthetic dsRNA chemical agonist Poly I:C showed a different response profile when compared to R848. With regards to TLR3, TLR7, and NF-Kβ, only NF-Kβ showed a significant stimulation of 7.7 fold 2 hours post-Poly I:C treatment (Figure 7). The TLR7 gene was not induced throughout the time course, while TLR3 displayed a strong reduction in mRNA levels. At 8 hours post-Poly I:C treatment, TLR3 had dropped 12.2 fold relative to time zero. This is in sharp contrast to the R848 treated HD11 cultures, which showed a 15.8 fold induction of TLR3 at 2 hours post-R848 treatment. Induction of pro-inflammatory genes by Poly I:C showed significantly different patterns compared to R848 treatment (Figure 8). Transcription of IL-1β was significantly induced by Poly I:C to 40.3 fold at 2 hours post treatment, iNOS was not as strongly induced as it was with R848 treatment, reaching 6 fold at 2 hours. Another gene that was significantly induced was IL-6, induced 25.6 fold at 2 hours post-Poly I:C treatment. This is a critical difference between R848 and Poly I:C treated cultures, as R848 did not induce the transcription of IL-6. Interestingly, IFN-β was strongly induced by Poly I:C with a maximum of 7 fold 2 hours post-Poly I:C treatment. IFN-α was not induced in response to Poly I:C. These results demonstrate the stimulatory effect on HD11 cells upon exposure to the synthetic dsRNA chemical agonist Poly I:C.

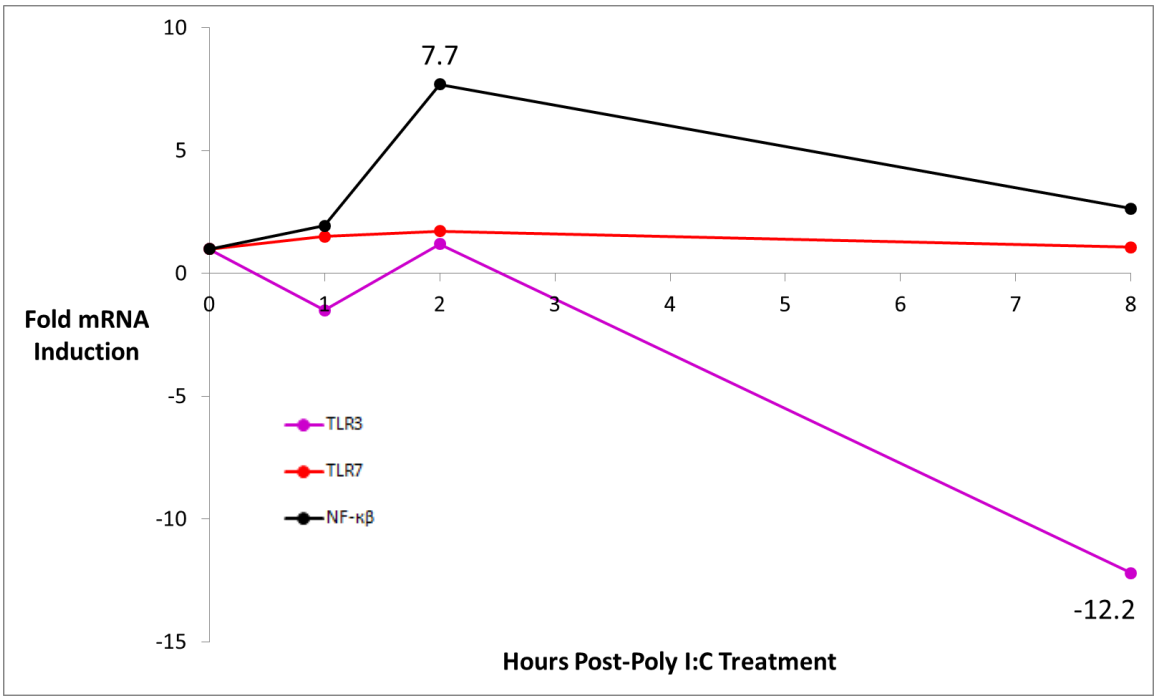

Figure 7: Induction of TLR3, TLR7, and NF-Kβ genes by Poly I:C in HD11 tissue culture at 0, 1, 2, and 8 hours post 10 ug/mL Poly I:C treatment. Total RNA samples were extracted from Poly I:C treated HD11 cultures at 0, 1, 2, and 8 hours post-treatment. NF-Kβ (Black) was induced 7.7 fold by 2 hours, while TLR3 (Purple) was strongly reduced 12.2 fold by 8 hours post-treatment. There was no significant stimulation of TLR7 (Red).

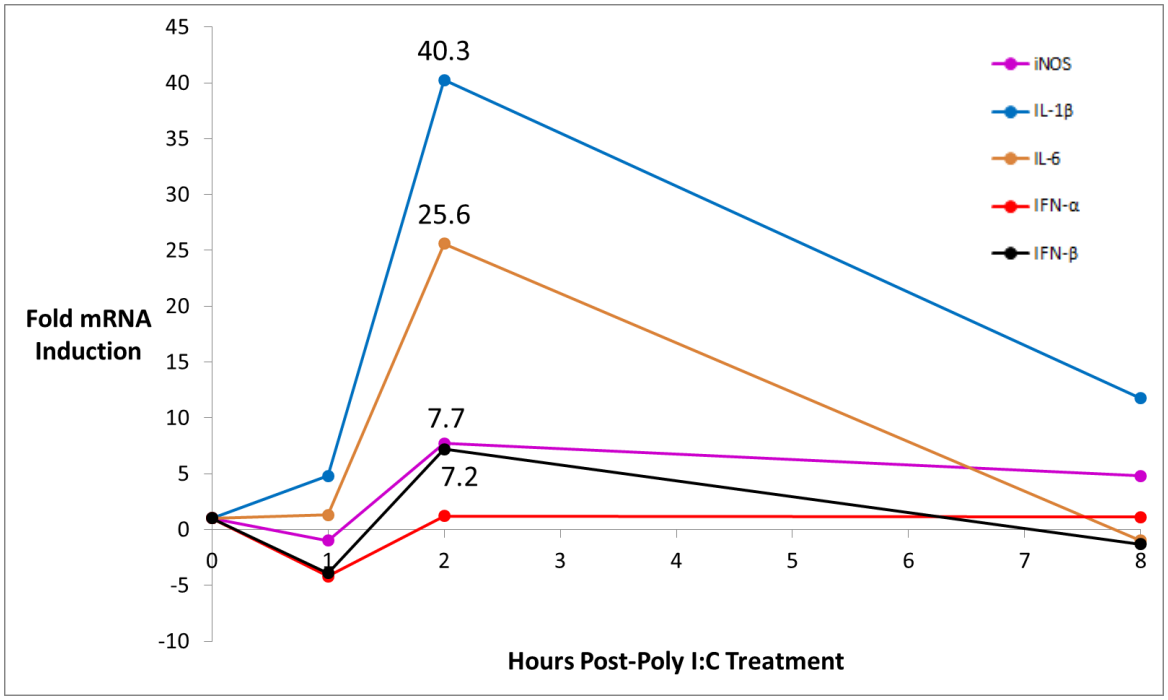

Figure 8: Induction of iNOS, IL-1β, IL-6, IFN-α, and IFN-β genes by Poly I:C in HD11 tissue culture at 0, 1, 2, and 8 hours post 10 ug/mL Poly I:C treatment. Total RNA samples were extracted from Poly I:C treated HD11 cultures at 0, 1, 2, and 8 hours post-treatment. IL-1β (Blue) was strongly induced to 40.3 fold at 2 hours post-treatment, while IL-6 (Orange) was also strongly induced 25.6 fold by 2 hours. The iNOS (Purple) gene was induced 7.7 fold at 2 hours, and IFN-β (Black) was induced 7.2 fold by 2 hours. IFN-α (Red) was not induced by Poly I:C.

3.3 Characterization of iNOS and IFN-α Transcription After Knockdown of TLR7, IRF7, and TLR7 + IRF7 Levels

The ultimate goal of this project was to understand the functions of avian TLR7 and IRF7 in mediating the pro-inflammatory response to ssRNA. By knocking down TLR7 and IRF7 in HD11 macrophage cells of *Gallus gallus*, it was possible to understand the functional role of each of these proteins by measuring the functional output of two pro-inflammatory genes, iNOS and IFN-α. Given that mammalian TLR7 is an anti-viral receptor recognizing ssRNA, it was hypothesized that a knockdown of avian TLR7 would correspond to a reduction in the pro-inflammatory response upon treatment with a ssRNA chemical agonist (R848). Furthermore, mammalian IRF7 functions downstream of TLR7 in mediating the expression of type I interferons, and it is hypothesized that this functional relationship is conserved in the avian system. Therefore, a knockdown of TLR7 and IRF7 should result in a synergistic reduction in the pro-inflammatory response to ssRNA in HD11 macrophage cells.

The use of iNOS and IFN-α as the pro-inflammatory genes to be measured in response to ssRNA in HD11s was determined through the R848 time course study (Section 3.2) in which it was shown that iNOS is induced 25.3 fold by 2 hours post-treatment with R848, while IFN-α is induced 15.6 fold by 8 hours post-treatment. With the ability to knockdown TLR7 and IRF7 in HD11 macrophages, and knowing the pro-inflammatory response to R848 in HD11s, an experiment could be designed in which TLR7, IRF7, and TLR7 + IRF7 are knocked down and treated with a ssRNA chemical agonist. The roles of avian TLR7 and IRF7 in mediating the pro-inflammatory response

to ssRNA could then be determined by measuring iNOS and IFN-α induction at 2 and 8 hours, respectively, post R848 treatment.

This experiment was performed using two 24-well plates seeded at 6 x 10^4 cells per well. The first plate served as the control plate necessary to verify knockdown of TLR7 and IRF7, as well as to verify successful siRNA transfection using fluorescent siRNA TYE-563 and targeted gene knockdown using the positive control siRNA HPRT. Also on this plate was a well treated with NC1, which is critical for ensuring no off target effects on gene expression by siRNA transfection. The first plate did not receive any R848 treatment, however the second plate received a 10 ug/mL R848 treatment for 2 or 8 hours depending on whether iNOS or IFN-α was being measured. The second plate contained wells transfected with NC1, TLR7, IRF7, and TLR7 + IRF7 siRNAs, which were then treated with R848 to measure the capacity of these HD11 cultures to mediate a pro-inflammatory response to ssRNA, specifically with induction of iNOS and IFN-α. Measurement of iNOS and IFN-α induction in TLR7, IRF7, and TLR7 + IRF7 knockdown wells was calculated relative to the maximum induction of iNOS or IFN-α in the NC1 (+) R848 control sample. By this method, induction of iNOS or IFN-α could be expressed relative to a maximum induction of 100% in the NC1 (+) R848 well. The iNOS and IFN-α experiments were repeated 3 times each (n = 3).

A successful analysis of TLR7 and IRF7 and their roles in mediating iNOS and IFN-α induction in response to ssRNA (R848) was dependent on a successful and efficient knockdown of TLR7 and IRF7. As stated, the iNOS and IFN-α experiments were repeated 3 times to give average values for HPRT, TLR7, and IRF7 knockdown

(Table 4). The wells for TLR7, IRF7, and TLR7 + IRF7 knockdown of the (-) R848 plate were analyzed by qRT-PCR at either 2 or 8 hours depending on whether iNOS or IFN-α was being analyzed. Knockdown of HPRT showed an average between 50-60% at 2 and 8 hours post R848 treatment, while TLR7 knockdown averaged between 55-65% at 2 and 8 hours post R848 treatment. Knockdown of IRF7 at 2 and 8 hours averaged roughly 60%. The combined knockdown of TLR7 + IRF7 had average knockdowns of 60-70% at 2 and 8 hours for TLR7, and 60-65% at 2 and 8 hours for IRF7 (Table 4).

With TLR7 knocked down at 2 hours post-R848 treatment, iNOS displayed a 23.7% reduction in expression in response to R848 (Figure 9). Using the Tukey-Kramer All-Pairs Honestly Significant Difference (HSD) test, it was determined that this was statistically significant with $p = < .0001$. When IRF7 was knocked down, iNOS showed a 14.3% reduction in expression, with a significance of $p = < .0022$. For the TLR7 + IRF7 knockdown sample, a synergistic effect of TLR7 and IRF7 knockdown was observed, with a 42.3% reduction in iNOS induction by R848, with a significance of $p = < .0001$ (Figure 9). Overall, it appears that a knockdown of TLR7, IRF7, or TLR7 + IRF7 does have a significant impact on the pro-inflammatory response with regards to iNOS induction, and the combined knockdown of TLR7 + IRF7 functions synergistically in reducing the pro-inflammatory response to ssRNA.

To understand the effects of TLR7, IRF7, and TLR7 + IRF7 knockdown on IFN-α expression, HD11 cultures of the (+) R848 plate were harvested for total RNA at 8 hours post-R848 treatment and analyzed by qRT-PCR. With TLR7 knocked down at 8 hours post-R848 treatment, IFN-α displayed a 23.7% reduction in expression relative to the

	Average % Knockdown	
Gene	**iNOS (2 Hour)**	**IFN-α (8 Hour)**
HPRT	59.8%	53.5%,
TLR7	57.5%	63.2%
IRF7	59.7%	58.5%
TLR7 + IRF7	57.7% + 64.3%	68.1% + 65.6%

Table 4: Average knockdown values for HPRT, TLR7, and IRF7 at 2 and 8 hours post-R848 treatment. The first plate, (-) R848, of each iNOS and IFN-α experiment was used to verify successful knockdown of HPRT, TLR7, and IRF7.

NC1 (+) R848 control sample. Statistical analysis by the Tukey-Kramer All-Pairs Honestly Significant Difference (HSD) test indicated a strong significance of $p = < .0001$ (Figure 10). A knockdown of IRF7 resulted in a 34% reduction in IFN-α expression, with a significance of $p = < .0001$. A combined knockdown of TLR7 and IRF7 led to a synergistic reduction in IFN-α expression by 46.7%, with a significance of $p = < .0001$ (Figure 10).

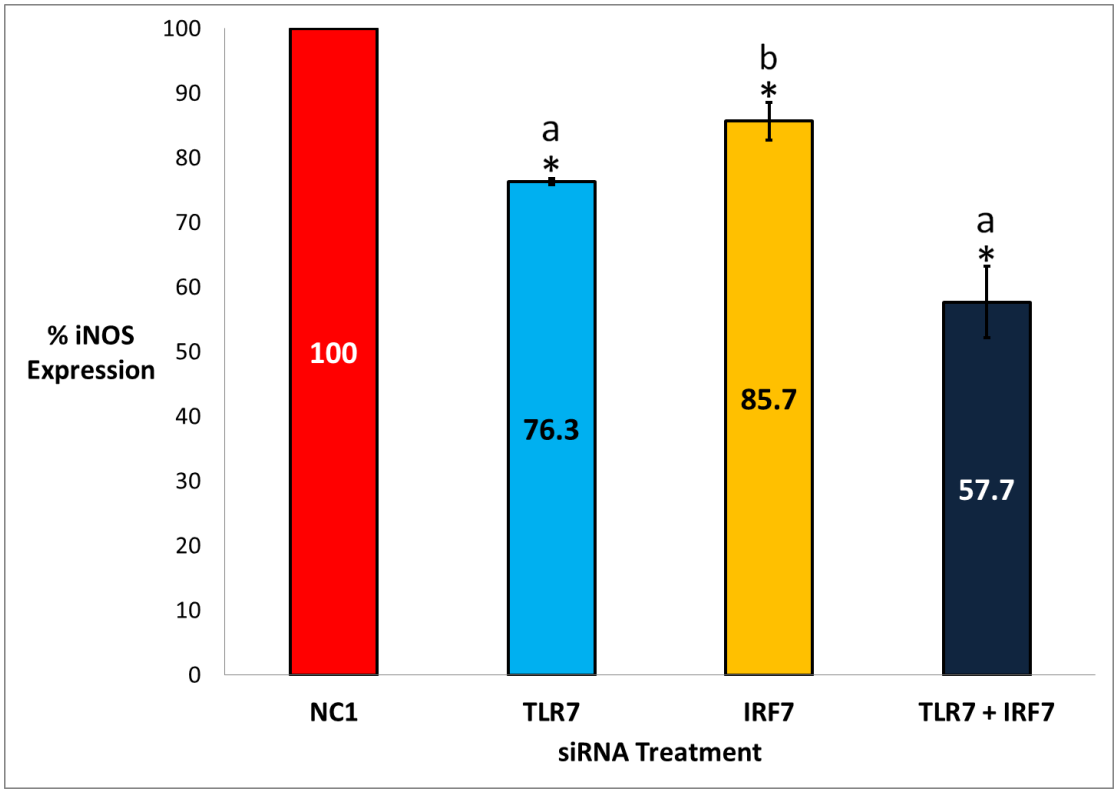

Figure 9: Expression levels of iNOS in HD11 tissue culture after a 2 hour R848 treatment when TLR7, IRF7, or TLR7 + IRF7 have been knocked down. TLR7, IRF7, and TLR7 + IRF7 knockdown cultures were treated with R848 for 2 hours and measured for iNOS expression relative to NC1 (+) R848. A knockdown of TLR7 resulted in a 23.7% reduction in iNOS levels upon R848 treatment, while a knockdown of IRF7 led to a 14.3% reduction in iNOS. A combined knockdown of TLR7 and IRF7 resulted in a 42.3% reduction in iNOS. (n=3, **a** = p= < .0001, **b** = p= < .0022).

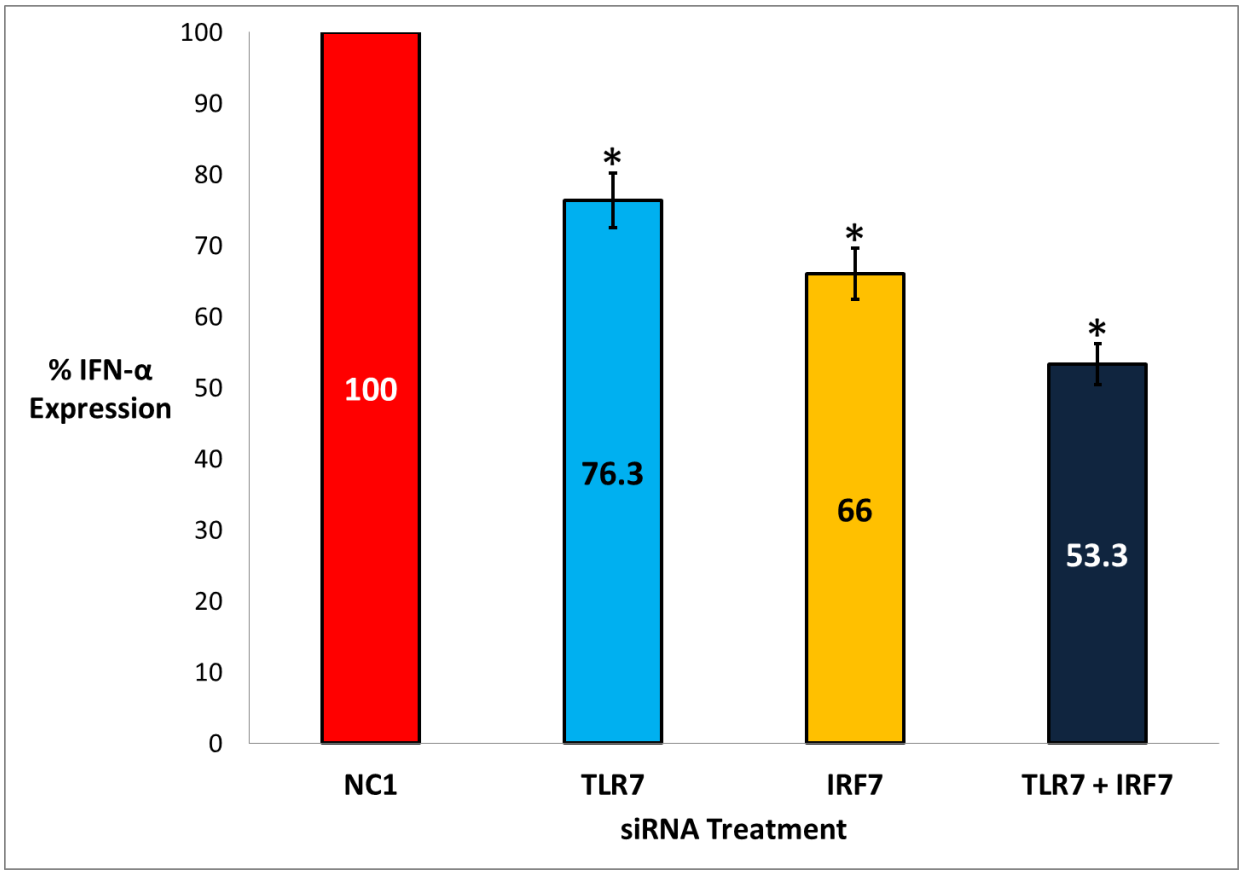

Figure 10: Expression levels of IFN-α in HD11 tissue culture after an 8 hour R848 treatment when TLR7, IRF7, or TLR7 + IRF7 have been knocked down. TLR7, IRF7, and TLR7 + IRF7 knockdown cultures were treated with R848 for 8 hours and measured for IFN-α expression relative to NC1 (+) R848. A knockdown of TLR7 resulted in a 23.7% reduction in IFN-α, while an IRF7 knockdown resulted in a 34% reduction in IFN-α expression. A combined TLR7 + IRF7 knockdown led to a 46.7% reduction in IFN-α expression (n=3, p= < .0001).

Chapter 4

DISCUSSION

4.1 Developing a siRNA-Mediated RNAi System in *Gallus gallus* Macrophage (HD11) Tissue Culture

The first step to knocking down TLR7 and IRF7 in HD11 chicken macrophages was to establish a platform in which cells could be efficiently treated with the siRNA transfection reagent containing the target siRNAs. Initial experiments involved determining the growth properties of HD11 cells in 6,12, and 24 well plates. Transfection guidelines provided by Roche suggested a starting cell culture confluence between 40-60% for siRNA treatment. By seeding a 24 well plate with $6x10^4$ cells/well, a starting confluence of 50% could be achieved at 24 hours post-seeding. Another consideration was ensuring healthy HD11 cell culture before experimental treatment. Macrophages have an extended morphology reflective of the pseudopodia used in phagocytosing of surrounding debris. Unhealthy cells were observed to be rounded and detached from the plate surface, and HD11 maintenance was perfected to ensure that unhealthy cells were eliminated from the cell culture. It was expected, and observed, that transfection comes at a cost to cell viability as the cell membrane fuses with the liposome carrying the siRNA. The objective was to maximize transfection efficiency while minimizing cytotoxicity. Using the fluorescent control molecule TYE-563 siRNA, it was determined that 3 uL of X-tremeGene siRNA Transfection Reagent mixed with 150 nanomoles of siRNA

provided ≥ 95% transfection efficiency. Fluorescence was observed to be dispersed throughout the nucleus and cytoplasm of the HD11 cells.

It is unknown what the exact consequence of siRNA transfection may have on the internal organization of the cell, or how it may impact gene expression. This emphasizes the importance of using positive and negative controls to ensure that mRNA knockdown is targeted and not an off-target effect of siRNA transfection. The scrambled negative control siRNA NC1 and the positive control siRNA targeting HPRT were used to address this concern. In addition, a mock transfected well containing only 3 uL of transfection reagent was used to observe any off-target effects by the reagent alone. Transfection of HD11 cells with NC1 and mock treatment with the transfection reagent demonstrated no significant impact on HPRT, TLR7, or IRF7 mRNA levels. This provided the confidence necessary to knock down TLR7 and IRF7 in HD11 cells, which could then be used in a functional agonist study with an ssRNA agonist (R848).

4.1.1 Technical Issues for Establishing siRNA-Mediated RNAi in HD11 Tissue Culture

The two major obstacles to establishing an siRNA-mediated RNAi system in HD11 cells was maximizing transfection efficiency, as well as ensuring that the siRNAs were being effectively dispersed into cytoplasm of the HD11 macrophage cell. Initial transfection experiments utilized a relatively large transfection volume of 200 uL, with 100 uL of Gibco's Opti-MEM I Reduced Serum Media containing 150 nanomoles of each individual siRNA combined with 100 uL of the same media containing 3 uL of transfection reagent. This mixture, when added to a 24-well plate containing HD11 cells

at 50% confluency, only gave a 40-50% transfection efficiency. This was not ideal given that even a 100% TLR7 or IRF7 knockdown of only a 50% sub-population of the cells would still leave 50% of the cells normal, making functional studies impractical. It was postulated that by reducing the volume of the transfection mixture, the siRNAs and transfection reagents would be more concentrated, resulting in a greater number of siRNA-containing liposomes. Consequently, the media volumes were reduced to 50 uL by combing 25 uL of Gibco's Opti-MEM I Reduced Serum Media containing 150 nanomoles of siRNA combined with 25 uL of Gibco's Opti-MEM I Reduced Serum Media containing 3 uL of transfection reagent. The 50 uL mixture was added to corresponding wells of the 24-well plate containing 200 uL Gibco's Opti-MEM I Reduced Serum Media. This change proved to be beneficial, resulting in successful siRNA delivery into ≥ 95% of the HD11 cell culture as verified by fluorescent TYE-563 siRNA. After this was established, an experiment was performed in which 150 nanomoles of HPRT siRNA was transfected into HD11 cell cultures seeded on a 24-well plate, resulting in 65% reduction of HPRT mRNA levels as measured by qRT-PCR.

In the early stages of developing a transfection protocol, significant experimentation was done with the widely used transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Interestingly, this reagent proved ineffective for siRNA transfection into HD11 cells. When fluorescent TYE-563 siRNA was transfected into HD11 cells using Lipofectamine 2000, fluorescence appeared to be trapped in a cytoplasmic vacuole, showing no diffusion throughout the cell. Despite a high transfection efficiency, an experiment in which 150 nanomoles of HPRT siRNA was

transfected into HD11 cells resulted in no detectable knockdown of HPRT mRNA levels. This was not surprising given that the siRNA appeared to be trapped in a cytoplasmic vacuole, and there was little visual evidence to suggest that the siRNAs were being processed by dicer and effectively loaded into the RISC complex for targeted mRNA silencing. This observation raised awareness to the biochemical differences between transfection reagents and their compatibility to different cell lines of different species, as well as to the type of nucleic acid being transfected. The Lipofectamine 2000 reagent worked well with a positive control DNA plasmid expressing cyan fluorescent protein (CFP), with a large percentage of the HD11 cells showing diffuse expression throughout the cytoplasm.

4.1.2 Understanding the Factors Impacting Efficiency of Gene Knockdown

Targeting a gene for knockdown requires two major events. First, the siRNA must be efficiently delivered to the entire cell population, with the expectation that the more cells that are transfected, the greater efficiency of delivery of the siRNA to its mRNA target. Second, the siRNA must be efficiently processed by the cytoplasmic enzyme Dicer and loaded into the RNA-Induced Silencing Complex (RISC). One consideration for understanding knockdown efficiency is the health of the HD11 cell culture, addressed in the early phases of this work. The method of transfection can also effect the efficiency of gene knockdown. This study utilized forward transfection, with the transfection mixture being added to an already seeded HD11 cell culture plate. A reverse transfection functions by mixing a cell suspension with siRNAs, followed by plating. Whether forward or reverse transfection works better in gene knockdown is dependent on the cell

type, and specifically whether the cell line is adherent or grown in free suspension. Forward transfections are generally used for adherent cell lines, and in this study the forward transfection method has provided strong gene knockdown efficiency. Another factor impacting knockdown efficiency is the type of siRNA being introduced. The TriFECTa RNAi Kit provided by Integrated DNA Technologies utilizes a novel Dicer-Substrate methodology in which a 27-mer siRNA duplex, not a traditional 21-mer, is introduced into the cytoplasm and processed by Dicer. It is hypothesized that Dicer plays a major role in the loading of the siRNA into the RISC complex, and by making the siRNA a substrate of the Dicer enzyme, gene knockdown becomes more efficient.

4.1.3 Alternative Methods to Transient siRNA-Mediated RNAi for Gene Knockdown Studies

While transient siRNA transfections are useful for functional gene studies, there are alternative methodologies that can be used. This study was conducted based on the assumption that the targeted protein, either TLR7 or IRF7, was substantially knocked down at 24 hours post transfection of target specific siRNAs. After this time period, however, the effect of knockdown may wane and functional studies become irrelevant. An alternative to this is to develop a stable HD11 cell line by introduction of a plasmid construct expressing an siRNA that can integrate into the host cell genome. This construct would also contain a selectable drug resistant marker, such as the neomycin resistance gene (neo) that confers resistance to the antibiotic G418. Constitutive expression of the siRNA would lead to a stable, heritable knockdown of TLR7 or IRF7. Another alternative is the use of plasmid vectors coding for short-hairpin RNAs

(shRNA). These vectors can be used to drive the expression of short hairpin RNAs that are quickly processed in the cytoplasm by dicer and loaded into the RISC complex for targeted mRNA knockdown.

4.2 Comparing the Roles of Toll-Like Receptors TLR3 and TLR7 of *Gallus gallus* in Mediating the Pro-Inflammatory Response to Viral Challenge

It is known that many viral genomes produce ssRNA and dsRNA intermediates throughout their life cycle, and viral particles internalized in the cell endosome will trigger the endosomal PRRs TLR3 and TLR7. Mammalian studies have confirmed that TLR3 as an endosomal dsRNA receptor, recognizing synthetic dsRNA compounds such as Poly I:C. The TLR3 ortholog of *Gallus gallus* has been shown to interact with Poly I:C, and a siRNA mediated knockdown of TLR3 in HD11 macrophages has confirmed that a reduction in TLR3 corresponds to a reduction in IFN-β expression. In this study, treatment of HD11 cells with Poly I:C led to a 7 fold increase in IFN-β levels at 2 hours post-treatment. Curiously, Poly I:C did not significantly induce IFN-α expression. This explains why the TLR3 study used IFN-β to monitor the anti-viral response in HD11 cells treated with Poly I:C.

The role of TLR7 in mediating the pro-inflammatory response to ssRNA has also been well established in mammalian systems, and our study has successfully shown TLR7 to be a ssRNA receptor in *Gallus gallus*. By treating HD11 cell cultures with a ssRNA chemical analog (R848), we determined a gene expression profile which could then be applied to a functional knockdown study of TLR7 and IRF7. The reduction of iNOS and IFN-α gene expression in HD11 cells when TLR7 was knocked down supports

the hypothesis that avian and mammalian TLR7 are functionally conserved. Furthermore, the reduction of both iNOS and IFN-α gene expression in TLR7 + IRF7 knockdown HD11 cultures indicates that avian TLR7 utilizes IRF7 as a transcription factor regulating type I interferon expression.

4.3 Understanding the Pathway Interaction of TLR7 and IRF7 in Mediating the Pro-Inflammatory Response to ssRNA in *Gallus gallus*

Classic functional biology views signaling pathways as independent systems regulating a specific molecular process, for example in the study of Toll-Like Receptors (TLRs) and how they mediate the expression of pro-inflammatory genes. This study has provided evidence for the function of avian TLR7 and IRF7, however there is also evidence to support a functional overlap of TLRs with NLRs and RLRs. Two genes, iNOS and IFN-α, were utilized in a functional knockdown study of TLR7 and IRF7. A separate and combined knockdown of TLR7 and IRF7 suggests that these two proteins both mediate the transcription of iNOS and IFN-α. However, there is functional redundancy with other ssRNA receptors such as MDA-5 of the RIG-I receptors.

4.3.1 Role of TLR7 and IRF7 in Mediating iNOS Induction

Two hours post-R848 treatment of HD11 cell cultures, iNOS displayed a 25.3 fold induction. Therefore, R848 has a stimulatory effect on the expression of nitric oxide as a response to ssRNA. When TLR7 was knocked down, iNOS induction was reduced by 23.7% relative to control HD11 cultures. A knockdown of IRF7 resulted in a 14.3% reduction in iNOS expression in response to R848. From a statistical standpoint, a knockdown of TLR7 has a greater effect on iNOS expression when compared to an IRF7

knockdown, suggesting that IRF7 may have greater functional redundancy with other pro-inflammatory transcription factors in regulating iNOS expression. It is likely that NF-Kβ is utilized in response to a TLR7 stimulation with R848 (Figure 11). The synergistic reduction in iNOS induction when TLR7 and IRF7 were knocked down concurrently suggests a cooperative interaction between these two proteins, however the HD11 cell still remains highly responsive to ssRNA challenge.

4.3.2 Role of TLR7 and IRF7 in Mediating IFN-α Induction

Measuring IFN-α expression in response to R848 was ideal in this experiment because it has direct implications to an anti-viral response. It is evident in this study that a stimulation of HD11 cells with R848 leads to induction of IFN-α, with a maximum of 15.6 fold at 8 hours post-R848 treatment. As the name suggests, Interferon Regulatory Factor 7 (IRF7) is a critical transcription factor regulating type I interferons. Avian IRF7 presented an interesting target for RNAi in *Gallus gallus* HD11s due to its strong sequence homology to mammalian IRF7, also because the avian genome lacks a sequence ortholog for mammalian IRF3. When TLR7 was knocked down in HD11s, a 23.7% reduction in IFN-α expression upon R848 treatment was observed, suggesting that TLR7 functions in the anti-viral response to ssRNA. A knockdown of IRF7 resulted in a 34% reduction in IFN-α levels, which is significantly greater than the 14.3% reduction in iNOS expression in response to ssRNA when IRF7 was knocked down. A combined knockdown of TLR7 and IRF7 led to a synergistic reduction in IFN-α expression of 46.7%. This near 50% loss of IFN-α expression provides strong evidence for a

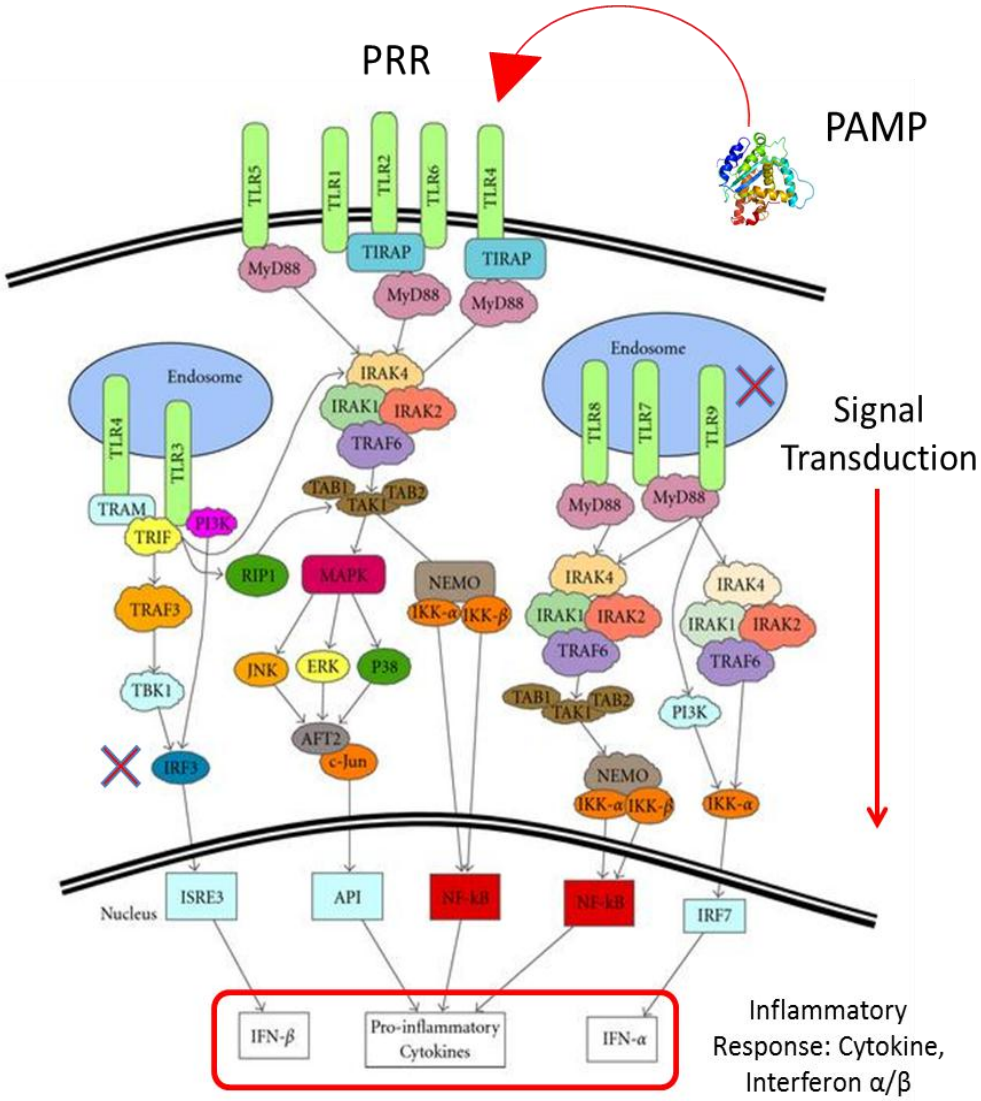

Figure 11: The mammalian toll-like receptor pathway (Hindawi Immunology, 2008).

cooperative relationship of *Gallus gallus* TLR7 and IRF7 in mediating the pro-inflammatory, anti-viral response to ssRNA.

4.4 Contributions by NOD-Like Receptors (NLRs) and RIG-I Like Receptors (RLRs) to the Pro-Inflammatory Response to ssRNA in *Gallus gallus*

A common theme in biology is redundancy, and it is expected that there are multiple pathways by which a cell can recognize ssRNA and mediate a pro-inflammatory response. In the *Gallus gallus* system, there are two PRRs besides TLR7 that may have implications for ssRNA recognition; MDA-5 of the RIG-I pathway and NLRC2 of the NOD pathway. It is known that MDA-5 exists in mammals and birds, acting as a cytoplasmic ssRNA receptor that can mediate the expression of pro-inflammatory cytokines and type I interferons. In mammals, MDA-5 utilizes NF-Kβ, IRF3, and IRF7 to mediate expression of pro-inflammatory cytokines and type I interferons. Avian MDA-5 utilizes NF-Kβ and IRF7 to mediate the pro-inflammatory response. The robust induction of iNOS and IFN-α in HD11 cultures even with TLR7 knocked down can be explained by the presence of MDA-5. Another potential ssRNA receptor recently discovered in *Gallus gallus* is NLRC2 of the NOD pathway, a sequence ortholog of mammalian NLRC2 that can bind ssRNA to mediate a pro-inflammatory response.

4.5 Correlations Between Avian and Mammalian TLR7 and IRF7 in Mediating the Pro-Inflammatory Response to ssRNA

This study was performed with the knowledge that avian TLR3 and mammalian TLR3 are functional orthologs, with 48% amino acid homology. Mammalian TLR7 recognizes ssRNA, with 63% amino acid homology to avian TLR7. Knowing that mammalian TLR3 and TLR7 are anti-viral endosomal receptors binding dsRNA and

ssRNA, respectively, it was reasonable to believe that avian TLR3 and TLR7 are functionally conserved and play a significant role in mediating the anti-viral pro-inflammatory response. Furthermore, avian and mammalian TLR7 have an even greater amino acid homology than TLR3, suggesting a high probability that their functions are conserved. It appears that avian and mammalian IRF7 are also functionally homologous with the observation that a knockdown of *Gallus gallus* IRF7 leads to a significant reduction in IFN-α expression. Mammalian IRF7 is known to regulate the expression of type I interferons IFN-α and IFN-β, and this study has confirmed a similar relationship in *Gallus gallus*.

4.6 Future Work

With the development of siRNA-mediated RNAi in HD11s, there are multiple studies that can be performed to further understand the pro-inflammatory response of *Gallus gallus*. Virtually any gene in the TLR, NLR, or RLR pathway can be targeted in HD11s for knockdown, followed by treatment with a variety of agonists. These responses can be measured in multiple ways at the level of RNA and/or protein, for example by the Griess Assay to measure nitric oxide production (4.6.6) or by western blot to visualize protein levels (4.6.5).

4.6.1 Characterizing Other Pro-Inflammatory Genes in HD11 TLR7, IRF7, and TLR7 + IRF7 Knockdown Cultures in Response to ssRNA

This study utilized two genes as a functional readout for the pro-inflammatory response to ssRNA (R848); IFN-α and iNOS. These genes were chosen based on their ability to be induced by R848, and they have strong relevance to an anti-viral response.

Another pro-inflammatory gene induced by R848 was IL-1β, displaying a 31.9 fold induction at 1 hour post treatment. This cytokine is highly expressed by macrophages and can act as a chemoattractant for neighboring monocytes, and can also trigger apoptosis of virally infected cells. Considering that IL-1β is highly expressed by macrophages and is a universal pro-inflammatory mediator, it is difficult to suggest whether a TLR7 or IRF7 knockdown will have a significant impact on IL-1β induction. The IL-1β gene of *Gallus gallus* does not fall under the control of the IRF family of transcription factors, and other ssRNA receptors such as MDA-5 may compensate for the loss of TLR7. Another interesting pro-inflammatory gene with implications to an anti-viral response are the MHC I/II (Major Histocompatibility Complex I/II) proteins, which a cell will display as distress signals to surrounding cytotoxic T cells. By binding and presenting viral peptides, the MHC complexes mark the infected cell for destruction (1.1.3).

4.6.2 Novel Pro-Inflammatory Related Gene Targets for RNAi in *Gallus gallus*

Besides TLR7 and IRF7, there are many other potential targets within the complex network of innate immune signaling that can be knocked down. This study examined the result of a TLR7 knockdown as well as the transcription factor IRF7; from a signal transduction standpoint, these knockdowns targeted early (TLR7) and late stage (IRF7) signaling proteins. Another major element to TLR signaling are the adaptor proteins, represented by MyD88, TRIF, TRAM, and TIRAP. A knockdown of MyD88 was used to establish TLR15 of *Gallus gallus* as a MyD88-dependent receptor recognizing bacterial lipopeptides (1.3.3.2). Other adaptor molecules, for example TIRAP, can be targeted for knockdown in HD11s using the established protocol. This

experiment would determine whether MyD88 of *Gallus gallus* is dependent on TIRAP for signal transduction upon agonist treatment with the understanding that MyD88 and TIRAP often work cooperatively in the mammalian pro-inflammatory response. Another target for RNAi that is relevant to *Gallus gallus* is TRAF6, which in mammals is an intermediary between MyD88 and IRF7 signal transduction upon TLR7 stimulation.

4.6.3 Characterizing the *in vivo* Response to Synthetic Chemical Agonists R848 and Poly I:C

The use of chemical agonists *in vitro* can provide valuable information regarding gene expression profiles in response to various biological entities, however chemical agonists such as R848 are commonly used as immune response modifiers to induce antibody production and can even have potent anti-tumor properties. In this study, *Gallus gallus* TLR7 has been shown to interact with ssRNA and can mediate expression of type I interferons and other pro-inflammatory mediators; this suggests that R848 can be used in *Gallus gallus* to modulate an anti-viral response to RNA viruses such as avian influenza. Because of its immunostimulatory properties, R848 is often used as a co-stimulant with various antigens to create a stronger antibody response. This study has also demonstrated Poly I:C to be an immunostimulant, however it is not widely used as a co-stimulant. It is interesting to note that different agonists can trigger distinct signaling mechanisms leading to expression of different effector molecules. This property highlights the potential for chemical agonists to be used as immuno-modulators *in vivo*, triggering specific cellular responses directed towards specific pathogens.

4.6.4 Viral Challenge by *Influenza A* Virus in HD11 TLR7, IRF7, and TLR7 + IRF7 Knockdown Cultures

The use of chemical agonists to mimic biological molecules can be very useful, however the ultimate goal of any *in vitro* study is to provide understanding to the mechanisms that occur *in vivo*, which in many cases is considerably different from an *in vitro* analysis. A major step in this study would be to challenge a HD11 TLR7, IRF7 and TLR7 + IRF7 knockdown culture with a live virus relevant to *Gallus gallus*, for example *Influenza A* virus. This virus causes significant mortality amongst domestic poultry, and is of scientific and commercial interest in terms of developing therapeutics that can mitigate its global impact. It is likely that a viral challenge by *Influenza A* would cause a broad spectrum pro-inflammatory response in HD11 knockdown cultures, specifically with anti-viral effectors such as IFN-α and IFN-β. A knockdown of TLR7 alone would be unlikely to significantly reduce the pro-inflammatory response, as other PRRs such as MDA-5 of the RLRs can also bind ssRNA to induce a pro-inflammatory response. Also to be considered is that the influenza virus will produce dsRNA intermediates while in the endosome, leading to a TLR3 stimulation and induction of pro-inflammatory cytokines such as iNOS, IL-1β, IFN-β, and IL-6 as seen with TLR3 stimulation with Poly I:C in HD11 cell culture. In this study, a knockdown of IRF7 led to a diminished expression of IFN-α in response to R848, however the loss of IRF7 can be compensated for by NF-Kβ and MAP-K signaling cascades. The transcription factor NF-Kβ can be utilized by NLRs, RLRs, and TLRs to mediate the expression of pro-inflammatory cytokines and chemokines, and a simultaneous stimulation of multiple receptors by

influenza virus will likely overshadow an IRF7 knockdown. A combined TLR7 and IRF7 knockdown can be described in a similar manner, given that multiple receptors within the NOD, RIG-I, and TLR pathways can mediate a pro-inflammatory response. In this study, only IRF7 and NF-Kβ have been described as primary transcription factors of *Gallus gallus* in mediating the pro-inflammatory response. It is possible that novel transcription factors regulating pro-inflammatory gene expression have yet to be discovered. While *Gallus gallus* may lack a sequence ortholog for mammalian IRF3, it is possible that a functional ortholog exists and can also induce interferon expression.

4.6.5 Measuring TLR7 and IRF7 Protein Levels by Western Blot Analysis

A major limitation that exists in the field of avian research is the lack of commercially available antibodies, a critical tool for protein diagnostics. In this study, it would be ideal to perform a western blot in conjunction with qRT-PCR to confirm a reduction in TLR7 and IRF7 protein levels, as opposed to measuring mRNA levels. Based on the reduction in iNOS and IFN-α mRNA levels in HD11 TLR7, IRF7, and TLR7 + IRF7 knockdown cultures in response to R848, there is reason to believe that the knockdown levels of TLR7 and IRF7 do correlate to a reduction in the corresponding protein levels.

4.6.6 Griess Assay to Quantify Nitric Oxide (NO) Output in HD11 TLR7, IRF7, and TLR7 + IRF7 Knockdown Cultures Treated with R848

This study has utilized qRT-PCR to measure the pro-inflammatory response to ssRNA (R848) in HD11 TLR7, IRF7, and TLR7 + IRF7 knockdown cultures. It was demonstrated that a knockdown of TLR7 in HD11 macrophages led to a 23.7% reduction

in iNOS mRNA expression in response to ssRNA (R848). A knockdown of IRF7 led to a 14.3% reduction in iNOS mRNA levels, and a combined knockdown of TLR7 + IRF7 led to an iNOS mRNA reduction of 42.3%. While measuring mRNA levels of iNOS by qRT-PCR does provide strong evidence for a reduced ability of these knockdown cultures to induce iNOS in response to R848, an alternative would be to use the Griess Assay, a colorimetric assay that can measure the presence of nitric oxide in solution. The Griess reagent is a sulfanilic acid solution that reacts with nitric oxide (NO) to form a diazonium salt, producing a pink color that can be measured by UV spectroscopy. By taking supernatants from HD11 knockdown cultures treated with R848, a relative measurement of NO from different samples can be made. It is expected that the differences in iNOS mRNA should be proportional to the amount of NO in the measured supernatants.

4.7 Conclusion

Understanding the signaling mechanisms of the pro-inflammatory response of *Gallus gallus* is crucial for the development of therapeutic strategies necessary to mitigate loss to avian infectious disease. The innate immune response is powerful in its ability to counteract microbial infection, however the receptor-mediated components of the avian innate immune response still require elucidation in how they facilitate the pro-inflammatory response. This study has successfully employed RNA-interference technology for an analysis of Toll-like receptor 7 (TLR7) and interferon regulatory factor 7 (IRF7) in *Gallus gallus*. Using the immortalized chicken macrophage cell line HD11, it was determined that avian TLR7 functions in the recognition of ssRNA to mediate a pro-inflammatory response, while IRF7 regulates the expression of type I interferons as well

as other pro-inflammatory genes. A combined reduction of these two proteins in *Gallus gallus* correlates to an increased reduction in the pro-inflammatory response, as observed by an increased reduction in iNOS and IFN-α mRNA levels in HD11 macrophage cells.

REFERENCES

1. Cheeseman, J. H., Lillehoj, H. S., Lamont, S. J. Reduced nitric oxide production and iNOS mRNA expression in IFN-γ-stimulated chicken macrophages transfected with iNOS siRNAs. Veterinary Immunology and Immunopathology 2008: 125, 375-380

2. Ciraci, C., Lamont, S. J. Avian-specific TLRs and downstream effector responses to CpG-induction in chicken macrophages. Developmental and Comparative Immunology 2011: 392-398

3. Karpala, A. J., Lowenthal, J. W., Bean, A. G. Activation of the TLR3 pathway regulates IFNβ production in chickens. Developmental and Comparative Immunology 2008: 32, 435-444

4. Takeda, K., Akira, S. TLR signaling pathways. Seminars in Immunology 2004: 3-9

5. Grishok, A. RNAi mechanisms in *Caenorhabditis elegans*. FEBS Letters 2005: 5932-5939

6. Yu, H., Wang, Z., Sun, G., Yu, Y. Recognition of Nucleic Acid Ligands by Toll-Like Receptors 7/8: Importance of Chemical Modification. Current Medicinal Chemistry 2012: 19, 1365-1377

7. Hong-Geller, E., Chaudhary, A., Lauer, S. Targeting Toll-Like Receptor Signaling Pathways for Design of Novel Immune Therapeutics. Current Drug Discovery Technologies 2008: 5, 29-38

8. Alcaide, M., Edwards, S.V. Molecular Evolution of the Toll-Like Receptor Multigene Family in Birds. Molecular Biology and Evolution 2011: 1703-15

9. Lian, L., Ciraci, C., Chang, G., Hu, J., Lamont, S.J. NLRC5 knockdown in chicken macrophages alters response to LPS and poly (I:C) stimulation. BMC Veterinary Research 2012: 1746-6148/8/23

10. Takeuchi, O., Akira, S. Recognition of viruses by innate immunity. Immunological Reviews 2007 Vol. 220:214-224

11. Gallo, R.L., Izadpanah, A. Antimicrobial peptides. Journal of the American Academy of Dermatology 2005 Vol. 52:381-90

12. Livak, K.J., Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. METHODS 25, 402-408, 2001.

13. De Y., Chen Q., Schmidt A.P., Anderson G.M., Wang J.M., Wooters J., et al. LL-37, the neutrophil granule- and epithelial cell-dervided cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 2000; 192:1069-74

14. Chaly Y.V., Paleolog E.M., Kolesnikova T.S., Tikhonov II, Petratchenko E.V., Voitenok N.N. Neutrophil alpha-defensin human neutrophil peptide modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells. Eur Cytokine Netw 2000; 11:257-66

15. Friedlander, R.M., Gagliardini, V., Rotello, R.J., Yuan, J. Functional Role of Interleukin 1β (IL-1β) in IL-1β-converting Enzyme-mediated Apoptosis. J. Exp. Med. 1996; Vol. 184 717-724

16. Patel, J.D., Krupka, T., Anderson, J.M. iNOS-mediated generation of reactive oxygen and nitrogen species by biomaterial-adherent neutrophils. Journal of Biomedical Materials Research 2006: 10.1002/jbm.a.30907

17. Zamora, R., Vodovotz, Y., Billiar, T.R. Inducible Nitric Oxide Synthase and Inflammatory Diseases. Molecular Medicine 2000; 6(5): 347-373

18. Malmgaard, L. Induction and Regulation of IFNs During Viral Infections. Journal of Interferon & Cytokine Research 2004; 24:439-454

19. Nathan, C., Xie, Q.W. Regulation of biosynthesis of nitric oxide. Journal of Biol. Chem. 1994: 269: 13725-13728

20. Sen, G.C. Viruses and interferons. Annu. Rev. Microbiol 2001: **55,** 255-281

21. Brestoff, J.R., Artis, D. Commensal bacteria at the interface of host metabolism and the immune system. Nature Immunology. The Microbiota (2013); Vol. 14, No 7, 676-684

22. Backhed, F. et. al. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. (2004); 101, 15718-15723

23. Musso, G., Gambino, R., Cassader, M. Interactions between gut microbiota and host metabolism predisposing to obesity (2011) 62, 361-380

24. Nicholson, J.K. et. al. Host-gut microbiota metabolic interactions (2012) Science; 336: 1262-1267

25. Le Poul, E. et al. Functional characterization of human receptors for short chan fatty acids and their role in polymorphonuclear cell activation (2003) J. Biol. Chem; 278: 25481-25489

26. Cox, M.A. et. al. Short-chain fatty acids act as anti-inflammatory mediators by regulating prostaglandin E(2) and cytokines. World J. Gastroenterol (2009); 15: 5549-5557

27. Resta, S.C. Effects of probiotics and commensals on intestinal epithelial physiology: implications for nutrient handling. J. Physiol. (2009) 587: 4169-4174

28. Bhaskaram, P. Micronutrient malnutrition, infection, and immunity: an overview. Nutr. Rev. (2002) 60: S40-S45

29. Kjer-Nielsen, L. et. al. MR1 presents microbial vitamin B metabolites to MAIT cells. Nature (2012) 491: 717-723

30. Matsumoto, M. et. al. Impact of intestinal microbiota on intestinal luminal metabolome. Scientific Reports (2012) 2: 233

31. Janeway, C.A., Medzhitov, R. Innate immune recognition. Annu Rev Immunol (2002) 20: 197-216

32. Kawai, T., Akira, S. The role of pattern-recognition receptors in innate immunity: Update on Toll-Like receptors. Nat Immunol (2010) 11:373-384

33. Takeuchi, O., Akira, S. Pattern recognition receptors and inflammation. Cell (2010) 140:805-820

34. Medzhitov, R. Recognition of microorganisms and activation of the immune response. Nature (2007) 449: 819-826

35. Blasius, A.L., Beutler, B. Intracellular Toll-Like Receptors. Immunity (2010) 32: 305-315

36. Kumar, H., Kawai, T., Akira, S. Pathogen recognition in the innate immune response. Biochem J (2009) 420: 1-16

37. Lemaltre B., Nicolas, E., Michaut, L., et. al. The dorsoventral regulatory gene cassette spatzle/toll/cactus controls the potent antifungal response in drosophila adults. Cell (1996) 86: 973-983

38. Burt, D.W., Bumstead, N., Bitgood, J.J., Ponce de Leon, F.A., Crittenden, L.B. Chicken genome mapping: a new era in avian genetics. Trends Genet. (1995) 11: 190-194

39. Shaw M.H., Reimer, T., Kim, Y.G., Nunez, G. Nod-like receptors (NLRs): Bona fide intracellular microbial sensors. Curr Opin Immunol. (2008) 20: 377-382

40. Kanneganti, T.D., Lamkanfi, M., Nunez, G. Intracellular Nod-like receptors in host defense and disease. Immunity (2007) 27: 549-559

41. Franchi, L., Warner, N., Viani, K., Nunez, G. Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev. (2009) 227: 106-128

42. Alexopoulou L., Holt, A.C., Medzhitov, R., Flavell, R.A. Recognition of of double-stranded RNA and activation of NF-Kβ by toll-like receptor 3. (2001) 413: 732-738

43. Sanghavi, S.K., Reinhart, T.A. Increased expression of TLR3 in lymph nodes during simian immunodeficiency virus infection: implications for inflammation and immunodeficiency. J Immunol (2005) 175: 5314-5323

44. Kumar, A., Zhang, J. Yu, F.S. Toll-like receptor 3 agonist poly (I:C) induced antiviral response in human corneal epithelial cells. Immunology (2006) 117: 11-21

45. Kufer, T.A., Kremmer, E., Adam, A.C. The pattern-recognition molecular NOD1 is localized at the plasma membrane at sites of bacterial interaction. Cell Microbiol (2008) 10: 477-486

46. Barnich, N., Aguirre, J.E., Relnecker, H.C. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor – {kappa} b activation in muramyl dipeptide recognition. J Cell Biol (2005) 170: 21-26

47. McDonald, C., Inohara, N., Nunez, G. Peptidoglycan signaling in innate immunity and inflammatory disease. J Biol Chem (2005) 280: 20177-20180

48. Melssner, T.B., Li, A., Biswas, A. NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. Proc Natl Acad Sci (2010) 107 13794-13799

49. Kumar, H., Pandey, S., Zou, J. NLRC5 Deficiency Does Not Influence Cytokine Induction by Virus and Bacteria Infection. J Immunol (2010) 4049

50. Franchi, L., Eigenbrod, T., Munoz-Planillo, R., Nunez, G. The inflammasome: A caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol (2009) 10: 241-247

51. Schroder, K., Tschopp, J. The inflammasomes. Cell (2010) 140: 821-832

52. Suzuki, T., Franchi, L., Toma, C. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via IPAF and ASC in shigella-infected macrophages. PLoS Pathog (2007) 3:e111

53. Suzuki, T., Nunez, G. A role for Nod-like receptors in autophagy induced by shigella infection. Autophagy (2008) 4: 73-75

54. Miao, E.A., Ernst, R.K., Dors, M. Pseudomonas aeruginosa activates caspase 1 through IPAF. Proc Natl Acad Sci (2008) 105: 2562-2567

55. Wright, E.K., Goodart, S.A., Growney, J.D. NAIP5 affects host susceptibility to the intracellular pathogen legionella pneumophila. Curr Biol (2003) 13: 27-36

56. Wilkins, C., Gale, M. Recognition of viruses by cytoplasmic sensors. Curr Opin Immunol (2010) 22: 41-47

57. Yoneyama, M., Fujita, T. Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity (2008) 29: 178-181

58. Sun, Q., Sun, L., Liu, H.H. The specific and essential role of MAVS in antiviral innate immune responses. Immunity (2006) 24: 633-642

59. Dixit, E., Boulant, S., Zhang, Y. Peroxisomes are signaling platforms for antiviral innate immunity. Cell (2010) 141: 668-681

60. Okabe, Y., Sano, T., Nagata, S. Regulation of the innate immune response by threonine-phosphatase of eyes absent. Nature (2009) 460: 520-524

61. Consortium. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature (2004) 432: 695-716

62. Jin, M.S., Lee, J.O. Structures of the Toll-like receptor family and its ligand complexes. Immunity (2008) 29: 182-191

63. Carpenter, S., O'Neill, L.A. Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins. Biochem J (2009) 422: 1-10

64. Kumar, H., Kawai, T., Akira, S. Toll-like receptors and innate immunity. Biochem Biophys Res Commun (2009) 388: 621-625

65. Schwandner, R., Dziarski, R., Wesche, H. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J Biol Chem (1999) 274: 17406-17409

66. Iqbal, M., Philbin, V.J., Smith, A.L. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. Vet Immunol Immunopathol (2005) 104: 117-127

67. Heil, F., Ahmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S., Wagner, H., Bauer, S. The toll-like receptor (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol (2003) 33: 2987-2997

68. Uematsu, S., Akira, S. Toll-Like receptors (TLRs) and their ligands. Handb Exp Pharmacol (2008) 1-20

69. Uematsu, S., Jang, M.H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., Kato, H., Sougawa, N., Matsui, H., Kuwata, H., Hemmi, H., Coban, C., Kawai, T., Ishii, K.J., Takeuchi, O., Miyasaka, M., Takeda, K., Akira, S. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c + lamina propria cells. Nat Immunol (2006) 7: 868-874

70. Ramasamy, K.T., Reddy, M.R., Verma, P.C., Murugesan, S. Expression analysis of turkey (*Meleagris gallopavo*) toll-like receptors and molecular characterization of avian specific TLR15. Mol Biol Rep (2012) 39: 8539-8549

71. Oven, I., Resman, R., Dusanic, D., Bencina, D., Keeler, C.L., Narat, M. Diacylated lipopeptide from *Mycoplasma synoviae* mediates TLR15 induced innate immune response. Veterinary Research (2013) 44: 99

72. Xing, Z., Cardona, C.J., Li, J., Dao, N., Tran, T., Andrada, J. Modulation of immune responses in chickens by low pathogenicity avian influenza virus H9N2. J Gen Virol (2008) 89: 1288-1299

73. Honda, K., Takaoka, A. Tye I Interferon Gene Induction Review by the Interferon Regulatory Factor Family of Transcription Factors. Immunity (2006). 25: 349-360

74. Ross, C.W., Jennifer, A.D. Molecular Mechanisms of RNA Interference. Annu Rev. Biophys. (2013) 42:217-239

75. Jinek, M., Doudna, J.A. A three-dimensional view of the molecular machinery of RNA interference. Nature (2009) 457: 405-412

76. MacFarlane, L.A., Murphy, P.R. MicroRNA: biogenesis, function and role in cancer. Curr. Genomics (2010) 11: 537-561

77. Grishok, A. RNAi mechanisms in *Caenorhabditis elegans*. Fed. of Europ. Biochem. Societies. (2005) 579: 5932-5939

78. Fire, A., Xu, S. Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature (1998) 391: 806-811

79. Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., Mello, C.C. The rde-1 gene, RNAi interference and transposon silencing in *C. elegans*. Cell (1999) 123-132