PATHOGENOMIC APPROACHES TO CHARACTERIZING THE AVIAN HOST INNATE IMMUNE RESPONSE TO MICROBIAL INFECTION

by

Michele Nancy Maughan

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

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Michele Nancy Maughan

Approved: _____
Jack Gelb Jr., Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: _____
Robin W. Morgan, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
Charles G. Riordan, Ph.D.
Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Calvin L. Keeler Jr., Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Carl J. Schmidt, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Kenneth L. van Golen, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Hyun S. Lillehoj, Ph.D.
Member of dissertation committee

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ABSTRACT

The avian innate immune response is activated within hours of infection. While it is difficult to prevent infection by a pathogen, clinical signs of disease can be ameliorated once the host-pathogen interactions are elucidated. Host-pathogen interaction research has predominantly focused on the adaptive immune response and cell signaling events later in infection; recently however, the innate immune response and early signaling events have garnered increased attention as another area worthy of investigation and intervention. Cells of innate immunity serve as the first-responders to infection, their signaling and antigen presentation is critical to the development of a protective adaptive immune response, and the cellular products of their activity (cytokines, reactive oxygen species, complement, etc.) are responsible for many of the clinical signs associated with disease.

Avian immunology research is expanding quickly due to the growing knowledge base of the chicken cytokines, Toll-Like Receptors (TLRs) and their immune signaling pathways. Our aim was characterize the avian innate immune response to microbial infection by utilizing a pathogenomics approach. By performing microarray experiments using our Avian Innate Immune Microarray (AIIM), we were able to measure the transcriptional host immune response to several important avian pathogens. Furthermore, by performing immunotherapeutic interventions using TLR agonists prior to challenge with highly pathogenic avian influenza virus, we were able to extend survival time of treated birds by 14% ($p<0.01$). This project has led to the characterization of the avian innate immune in

different avian species, to different pathogens, at early time points throughout infection, and with and without the aid of a pre-treatment.

Chapter 1

GENERAL INTRODUCTION

Avian Immune System

Introduction

The immune system represents a collection of mechanisms that function to protect an organism against a spectrum of pathogens and other disease-causing agents. There are two branches of the immune system, the innate immune responses and the adaptive, or acquired, immune responses. Innate immune responses are engaged immediately after an infection begins and are independent of the host's prior exposure to the pathogen, whereas, the adaptive immune responses operate later in infection and are highly specific for the pathogen that induced them (3). The innate immune responses are the first line of defense against pathogens and are required to initiate the specific adaptive immune responses. Despite having distinct roles in immunity, there is interplay and cross-talk between innate and adaptive immunity, resulting in a complex relationship (36, 79).

Immune Organs

The avian immune system is comprised of both primary (central) and secondary (peripheral) immune organs. The primary immune organs are the bursa of Fabricius and the

thymus from which B and T lymphocytes develop, respectively. The secondary immune organs are comprised of the spleen, lymph node and gut-, bronchus- and skin-associated lymphoid tissues (76). In many of our in vivo experiments, we harvest tissue samples from the lung, representing the bronchus-associated lymphoid tissue (BALT) and the spleen, representing a classical peripheral immune organ. The significance of these organs related to innate immunity is described herein.

Lung

The lung is an important site of entry for respiratory pathogens and consequent innate immune responses. The avian lung differs morphologically and functionally from the mammalian lung in several important ways. Functionally, the avian lung is anastomotic, meaning the bronchial branches eventually reconnect, leading to unidirectional airflow. Secondly avian lung ventilation occurs via air sacs (rather than a diaphragm, present in mammals) that maintain constant airflow (86). BALT is primarily located at the entrances to the secondary bronchi, which are comprised of ciliated and non-ciliated modified mucosal tissue. Respiratory macrophages rapidly transmigrate into the epithelium of the respiratory surface upon inflammatory conditions elicited by pathogens (50). Circulating dendritic cells, heterophils, and resident respiratory macrophages, as well as phagocytic lung epithelial tissue comprise the majority of the innate immune cells present in the lung (86). In chickens, the lung is the primary site of entry and replication for AIV. The chicken lung innate immune response to AIV has been described by Reemers *et al.* (85), Degen *et al.*, 2006 (23), and Rebel *et al.*, 2011 (84). These studies, while divergent in scope and thesis, described the unique avian aspects of the lung tissue, morphology, immune cell populations, and regional specializations, which contribute to a chicken-specific localized immune response to AIV. Their work and the studies described herein, highlight the importance of characterizing tissue-specific immune responses to AIV and the use of the avian animal model in AIV studies.

Spleen

The spleen is another organ often harvested and analyzed in order to characterize the avian immune response to pathogens. It has been postulated that the role of the spleen in the immune system of aves may be more important than in mammals because of the poorly developed avian lymphatic vessels and lack of distinct lymph nodes (76). The avian spleen functions chiefly in the following ways related to innate immunity and disease resistance: 1) formation of parts of the complement system, 2) development of macrophages from peripheral blood mononuclear cells, 3) phagocytosis and destruction of antigens, immune complexes, and damaged blood cells, and 4) production of a phagocyte-stimulating protein, tuftsin, which facilitates immunogenesis (38). For these reasons, the spleen is involved in mounting an innate (and adaptive) immune response to nearly all systemic diseases and therefore provides insight into host-pathogen interactions. The chicken spleen innate immune response to AIV has been described by Ewald *et al.* (25), Karpala *et al.* (45), and Degen *et al.* (23), Moulin *et al.*(69), thus establishing the spleen as an important organ in the evaluation of AIV pathogenesis and system infection.

Innate Immune Cells – Macrophages

Several cell types have distinct roles in the innate immune responses. Macrophages (MØ) and neutrophils are considered professional phagocytes. Chickens have a reduced repertoire of polymorphonuclear cells, wherein neutrophils, basophils, and eosinophils are replaced by a single cell type, the heterophil (40). The heterophil functions most like the human/murine neutrophil, a first responder at the site of infection, engaging in phagocytosis, superoxide production, and cytokine release (31).

MØs originate from hematopoietic bone marrow stem cells that initiate myeloid differentiation to form multipotent precursor monocytes which can further differentiate into either MØs or dendritic cells (DCs) (92). Monocytes enter the blood stream where they

constitute a major phagocytic cellular component in the chicken's blood (83). Monocyte differentiation results in different subpopulations of MØs and DCs depending on if the host is under normal homeostatic conditions or inflammatory conditions and in the presence of colony stimulating factors. In vitro culture of monocytes with colony stimulating factor 2 (OMIM: **CSF2**), alias granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin 4 (OMIM: **IL4**) leads monocytes to differentiate into DCs (92). Microbial infection shifts monocyte differentiation to form specialized subsets of DC populations that augment and boost microbial clearance (92).

MØs demonstrate the most robust phagocytosis, able to engulf up to 200% of their surface area per hour, and can internalize almost any form of antigen, either specifically via receptor recognition or nonspecifically (110). This unique ability contributes to the MØs major role in innate immunity: microbial clearance (110). MØs exhibit great heterogeneity depending on their maturation and activation states, and tissue microenvironment. They are present in the liver as Kupffer cells, lung (alveolar MØ), nervous system (microglial cells), epidermis (Langerhans cells), and various other nonlymphoid organs (108). MØs are the best characterized of the avian innate immune cell types and have been shown to be primary responders to AIV due to their presence in lung tissue (alveolar MØ) and their dominant presence in the spleen. In avian research, immortalized macrophage cell lines (MQ-NCSU, IN24, and LSCC-NP1) are often used to study immune function (48).

DCs are considered professional antigen presentation cells (APCs) and also display heterogeneity. During microbial invasion, immature DCs capture antigen and subsequently migrate to lymphoid organs, where they initiate primary adaptive immune responses by presenting antigen to CD4+ T-helper cells. This action stimulates CD8+ cytotoxic T cells, and B cells, as well as cells in the innate immune system – macrophages, heterophils, and natural killer (NK) cells (7).

Other innate immune cell types play critical roles in host defense, such as mast cells and NK cells. NK cells represent a unique subpopulation of lymphocytes that when

activated produce high levels of cytotoxic activity, inflammatory cytokines, and chemokines. Interferons, produced in response to viral infection, stimulate NK cell-mediated cytotoxicity and stimulate NK cells to produce interferon-gamma (OMIM: **IFNG**) (12). Mast cells, located in epithelial barrier mucosa, blood vessels, and in connective tissues contain histamine and other immune mediators that cause inflammation and recruit leukocytes to sites of tissue injury (27).

Innate Immunity

The innate immune system functions by recognizing and distinguishing between self and non-self and is comprised of three broad categories of defense systems: anatomical, cellular, and humoral proteins (72).

The anatomical defenses are comprised of three sub-sections: mechanical, chemical, and biological. The mechanical defenses are comprised of the integumentary system and the epithelial membranes that form a relatively impermeable physical barrier, respiratory cilia and the mucociliary escalator, and the gastrointestinal (GI) peristaltic movements that keep the GI tract free of pathogenic microbes. The processes of tear production, the salivary response to chewing, and the movement of mucous, also aid in the mechanical defenses (72). The chemical defenses are comprised of biochemical proteins that are produced by epithelia and glands, such as lysozyme and phospholipase found in tears, lung surfactants (i.e. opsonins), fatty acids and the low pH found in sweat that inhibits the growth of bacteria, and antimicrobial proteins (e.g. defensins, cathelicidins) produced by surface epithelia found in the lung, genitourinary, and GI tract (64). The biological defenses are represented by the commensal microorganisms that inhabit the host. These microbes comprise the normal flora that protects the host from pathogenic microbes through either resource competition (for nutrients or attachment sites) or by secreting toxic proteins (72).

The cellular defenses are composed of bone marrow derived common lymphoid and myeloid origin progenitor cells, the former yielding natural killer (NK) cells, while the latter

yields granulocytic neutrophils (avian heterophils), basophils, eosinophils, and mast cells, and monocytic (agranulocytic) monocytes, macrophages, and dendritic cells (72). Cellular defenses also include antimicrobial peptides produced by polymorphonuclear (PMN) leukocytes and the complement pathway proteins produced by a variety of cell types that results in bacterial opsonization (increased uptake of bacteria by phagocytic cells) (64).

Finally, the humoral defenses include the serum proteins/enzymes that aid in the inflammatory process and somewhat overlap with the cellular defenses, such as the complement cascade. Also included in the humoral defenses are the serine proteases of the coagulation system, which contain antimicrobial proteins such as beta-lysin, whose overall effect increases vascular permeability thereby allowing extravasation of immune cells. Iron-binding proteins and transporters, lactoferrin and transferrin, which limit bacterial growth by sequestering iron, an essential element for bacterial growth also contribute to humoral protection (72).

The cellular and humoral immune defenses overlap when cellular molecules work indirectly to activate extracellular matrix proinflammatory mediators (88). Examples of these molecules are fibrinogen, fibronectin, heparan sulfate, and collagen- elastin- laminin-derived peptides (88). These molecules have a variety of functions, but in general, they stimulate chemotaxis and cytokine production/release from various innate immune cell types leading to inflammation - an immune response.

One of the hallmark mechanisms of host-pathogen interactions of the innate immune response is phagocytosis. Most innate immune cell types are phagocytic to varying degrees, however, monocytes/macrophages, dendritic cells, and neutrophils (heterophils) are considered, “professional phagocytes” and are the most phagocytic (112). Microbes are met with a variety of humoral defenses like opsonins, IgG, complement, and other serum proteins that bind to the microbial surface and signal immune cells for phagocytosis. Phagocytosis begins when microbe-phagocyte contact occurs and phagocyte receptors (Fc, complement, scavenger receptors, integrin, lectin, and toll-like receptors) trigger intracellular signaling pathways to become activated (112). This interaction results in a wide range of cellular processes being stimulated,

including membrane trafficking, cytoskeleton rearrangement, microbial killing, production of pro- and anti-inflammatory cytokines, apoptosis, and antigen presentation (112). These cellular responses are diverse; however, our focus is on pathogen recognition, the resulting intracellular signaling pathways, and the end products that are major immune regulators.

Pattern Recognition Receptors

The innate immune responses recognize pathogens via germline-encoded pattern recognition receptors (PRRs) (2, 36, 63). PRRs can be secreted into the bloodstream and tissue fluids, expressed on immune cell surfaces, or contained within intracellular compartments (36). PRRs recognize conserved/invariant microbial components called pathogen-associated molecular patterns (PAMPs) (2). Their main functions consist of activating complement and coagulation cascades, opsonization, induction of phagocytosis and apoptosis, and activating immune signaling pathways (36).

There are many functionally distinct classes of PRRs, of which the best characterized class is the Toll-like receptors (63). The other classes of PRRs include protein kinase PKR, dead/h box 58 (OMIM: **DDX58**), a.k.a. the retinoic-acid-inducible gene I (RIG-I)-like receptor (RLR) family, mannan-binding lectin (MBL), C-reactive protein (CRP), Nod (nucleotide-binding oligomerization domain)-like receptors (NLR), TREM (triggering receptors expressed on myeloid cells), and C-type lectin receptors (CLR) (11, 36, 63, 90).

Toll-like Receptors

Toll-like receptors (TLRs) are type I transmembrane glycoprotein receptors characterized structurally by the presence of leucine-rich repeat (LRR) motifs in their extracellular domain and cytoplasmic signaling domains homologous to the interleukin 1 receptor, called the Toll/IL-1R homology (TIR) domain (2, 103). Type I transmembrane

receptors are a family of signaling molecules that contain an extracellular domain, a single-membrane-spanning α-helix, and a cytoplasmic domain (28). The TIR domain is an α/β structure found in Toll receptors and adaptors and is involved in post-receptor signaling (28).

TLRs are expressed on all of the innate immune cell types as well as B cells, certain subsets of T cells, fibroblasts, and epithelial cells (2). There are approximately 11 mammalian TLRs (TLRs 1-11) (2, 54) and 10 chicken TLRs (TLRs 1A, 1B , 2A, 2B, 3, 4, 5, 7, 15, 21) (14). Several chicken TLRs have clear orthologues in mammalian TLRs 3, 4, 5, and 7 (14). TLRs can be categorized by their subcellular location and ligand specificity: TLRs 1, 2, 4, and 5 are expressed on the cell surface and recognize microbial membrane components, while TLRs 3, and 7 are expressed intracellularly, in the endosome, and recognize microbial nucleic acids (2, 67). The chicken TLRs that recognize viral nucleic acids are TLRs 3 and 7, which recognize dsRNA and ssRNA, respectively (37). Both chTLR3 and chTLR7 have been shown to respond to avian influenza virus infection, TLR3 most likely recognizing the dsRNA intermediates during the viral nucleic acid replication and TLR7 recognizing ssRNA viral nucleic acid (44, 119). TLRs, in general, recognize a wide variety of PAMPs and the most recent data on their respective ligands are summarized in **Table 1** (16, 28, 41, 54, 87).

Table 1.1 Chicken Toll-like Receptors, Major Ligands, and Associated Tissue/Cell Types.

Chicken TLR	Ligand(s)	Cell/Tissue
TLR1LA (TLR1/6/10)	Lipopeptides	Heterophils, macrophages / spleen, bursa, thymus, liver, blood, intestine, oviduct, testis
TLR1LB		
TLR2A	Peptidoglycan, lipopeptides	Heterophils, macrophages / spleen, bursa, thymus, liver, blood, intestine, oviduct, testis
TLR2B		
TLR3	dsRNA	Heterophils, macrophages / spleen, bursa, thymus, liver, blood, lung, intestine, oviduct, testis
TLR4	Lipopolysaccharide (LPS)	Heterophils, monocytes, macrophages / spleen, tonsil, bursa, thymus, liver, brain, kidney, muscle, lung, heart, intestine, oviduct, testis
TLR5	Bacterial flagellin	Heterophils, macrophages / spleen, tonsil, bursa, thymus, blood, liver, lung, kidney, heart, intestine, testis
TLR7	ssRNA	Heterophils, macrophages / spleen, tonsil, bursa, thymus, bone marrow, blood, liver, lung, kidney, heart, intestine, oviduct, testis
TLR15	Lipopeptides	Macrophages / bursa, spleen, liver, intestine, tongue
TLR21	Umethylated CpG DNA	Heterophils / blood

Table 1 lists the biological recognition molecules as well as some of their synthetic ligands. The chickens TLRs, as previously mentioned, share homology with some mammalian, or human TLRs. **Figure 1** illustrates predicted domain organization of chicken TLRs as well as

their human and Pufferfish (*Takifugu rubripres*) orthologues (14). There are two chTLR1 and two chTLR2 receptors, chTLR1.1 and chTLR1.2, and chTLR2.1 and chTLR2.2, respectively. chTLR8 is a nonfunctional, fragmented receptor, disrupted by a chicken repeat-1 (CR1) retrovirus-like element (82), while chTLR9 has not been identified as of yet. chTLR15 is the most recently discovered chicken TLR and no orthologues have yet been identified (32). chTLR21 has also recently been identified, however, more research is necessary to confirm its identity (87).

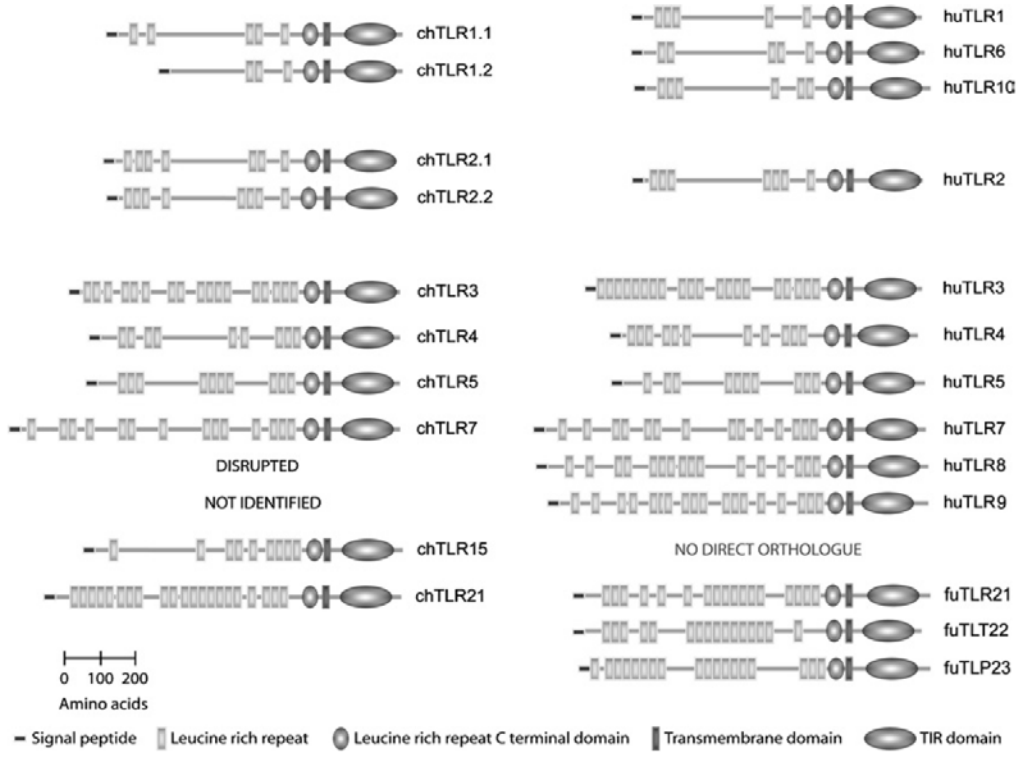

Figure 1.1 Chicken TLRs (chTLR) and their identified orthologues (14).

TLR Signaling

Once ligand binding has occurred, postreceptor signaling is initiated. Due to the fact that the cytoplasmic TIR domains do not possess enzymatic activity, signaling initiation must begin with dimerization or oligomerization such that the TIR domains recruit downstream adaptor proteins (28). Adaptor molecules are cytoplasmic proteins that connect elements within a signaling pathway. The TLRs recruit adaptor proteins containing a TIR domain to engage in TIR-TIR dimerization (46). The best characterized adaptor proteins are the TIR-domain containing adaptor molecule 1 (OMIM: **TICAM1**) (also known as TRIF – TIR domain containing adaptor protein inducing IFNβ (OMIM: IFNB)), TIR-domain containing adaptor molecule 2 (OMIM: **TICAM2**, also known as TRAM – TRIF-related adaptor molecule), TIR domain-containing adaptor protein (OMIM: **TIRAP**) (also known as MAL – MyD88 adaptor-like), and myeloid differentiation primary response gene (OMIM: **MYD88**) (2, 11, 46). Two pathways form from the aforementioned adaptor molecules, the TICAM1/TICAM2 (TRIF/TRAM)-dependent pathway and the MYD88/TIRAP-dependent pathway. A generalized diagram of the relationship between the functional groups of TLRs and their respective adaptor proteins is represented by **Figure 2** (46).

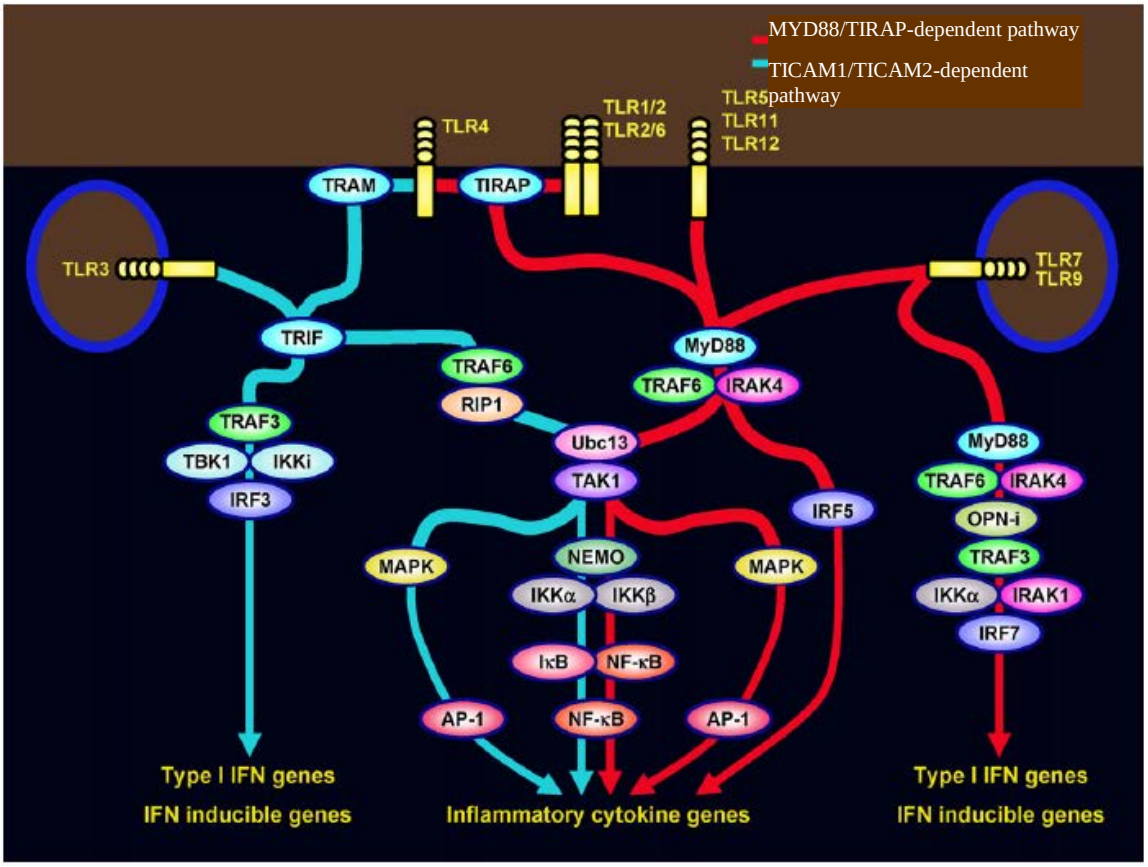

Figure 1.2. TLR Signaling through adaptor proteins (46).

It is clear from the figure that there is a great deal of convergence between the two adaptor-protein pathways. Several of the TLRs use one or more adaptor molecules: MYD88 is used by all of the TLRs, except for TLR3, and it is used exclusively by TLR5, 7, 8, and 9; TIRAP (MAL) is used by TLR2 and TLR4; TICAM (TRIF) is used by TLR3 and TLR4; and TICAM2 (TRAM) is exclusively by TLR4 (28, 46, 54).

The postreceptor signaling pathway continues after recruitment of the specific adaptor protein(s), and the downstream signaling cascade is dependent upon the initial adaptor protein(s). The MyD88 and TICAM1 pathways both lead to the production of inflammatory cytokines and type I interferons (IFN) (2).

The MyD88-dependent pathway, utilized by TLR7, associates with the cytoplasmic portion of the TLR and recruits two protein kinases, interleukin 1 receptor (IL-1R)-associated kinase 4 (OMIM: **IRAK4**) and IRAK-1 (OMIM: **IRAK1**) via a homophilic interaction of the N-terminal death domains (2). Upon IRAK1 and MyD88 associating, IRAK1 becomes phosphorylated by activated IRAK4 and associates with tumor necrosis factor receptor-associated factor 6 (OMIM: **TRAF6**) (2). TRAF6 functions as a ubiquitin protein ligase (E3) and recruits the ubiquitination conjugating E2 enzyme complex, UBC13 and UEV1A, which catalyzes the formation of a polyubiquitin chain on TRAF6 and on inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (OMIM: **IKBKG**)/ nuclear factor kappa-B (OMIM: **NFKB1**), alias NFKB essential modulator (NEMO) (2). TRAF6 also recruits a complex of mitogen activated protein kinase kinase kinase 7 (OMIM: **MAP3K7**), alias TGF-β-activated kinase (TAK1), and TAK1 binding proteins, mitogen activated protein kinase kinase kinase 7 interacting protein 1 (OMIM: **MAP3K7IP1**), alias TAK1 binding protein (TAB1), (OMIM: **MAP3K7IP2**) TAB2, and (OMIM: **MAP3K7IP3**) TAB3. MAP3K7 then phosphorylates inhibitor of kappa light chain gene enhancer in B cells kinase beta (OMIM: **IKBKB**), alias IKK-β, and mitogen activated protein kinase kinase 6 (OMIM: **MAP2K6**), alias MKK6. This phosphorylation activates proinflammatory transcriptions factors: nuclear factor kappa-B (OMIM: **NFKB1**), alias NF-κB, interferon regulatory factor 5 (OMIM: **IRF5**), and the activator protein 1 (AP1) family of transcriptions factors, (OMIM: **JUN, FOS, MAF, ATF**) (2). Collectively, these transcription factors form a stereospecific multi-protein complex called the enhanceosome, which has chromatin remodeling capabilities and assembles at the IFNB promoter resulting in initiation of IFNB transcription (93).

The MyD88-independent pathway, also known as the TICAM1/TICAM2 pathway, utilized by TLR3, proceeds as follows. Once ligand binding occurs, the adaptor protein TICAM1 binds to the TIR domain of TLR3 and dimerizes (TICAM2 is recruited specifically during TLR4 signaling) (2). The N- and C-terminal domains of TICAM1 have distinct functions. The C-terminal region activates TICAM1 to interact with TRAF6 and receptor-

interacting serine/threonine kinase 1 (OMIM: **RIPK1**), alias receptor-interacting protein 1 (RIP1) (46). This association causes RIPK1 to become polyubiquitinated and form a complex with TRAF6 and MAP3K7 (TAK1), which leads to the activation of NFKB1 and the AP1 transcription factors (46). The N-terminal region of TICAM1 activates both the NFKB1 and IFNB promoters by recruiting a complex of TNF-receptor associated factor 1 (OMIM: **TRAF3**), TANK-binding kinase 1 (OMIM: **TBK1**), interferon regulatory factor 3 (OMIM: **IRF3**), and inhibitor of kappa light polypeptide gene enhancer in B cells kinase epsilon (OMIM: **IKBKE**). This complex results in the phosphorylation of the IRF3 serine/threonine domains, which leads to an IRF3 dimer that translocates from the cytoplasm to the nucleus and induces expression of IFNB and Type I IFN inducible genes (46).

Antiviral TLR Signaling

Although there is a repertoire of possible signaling pathways, the focus here will be the MyD88-dependent and TICAM-dependent pathways related to TLR7 and TLR3 signaling respectively. As previously mentioned, the subgroup of mammalian TLRs that are responsible for the antiviral innate immune response are TLR3, 7, 8, and 9 (11). Of the four aforementioned mammalian viral recognition TLRs, only TLR3 and TLR7 have been identified in chicken, thus our focus will be on chTLR3 and chTLR7 in order to study the chicken antiviral innate immune response (11, 37).

chTLR3 and chTLR7 recognize viral nucleic acids and are contained intracellularly within the endosomal compartment. TLR3 specifically recognizes double-stranded RNA (dsRNA) nucleic acid, commonly found in dsRNA viruses and ssRNA viruses during viral replication (10). TLR3 also has a synthetic ligand, polyinosine/polycytosine (polyIC), a dsRNA analog (10). TLR7 specifically recognizes single-stranded RNA (ssRNA) nucleic acid, commonly found in ssRNA viruses such as influenza virus and vesicular stomatitis virus (10). Specifically, TLR7 responds to the frequency and spacing of uridine residues within ssRNA to discriminate between self-derived RNA and viral RNA (10). The synthetic ligands for TLR7 are

the, imidazoquinolines (R848), a family of ssRNA analogs (10, 82). chTLR3 and chTLR7 have both been shown to respond to the aforementioned ligands/agonists by upregulating the production of IFNA and IFNB, and IL1B and IL-8, respectively (82).

Antiviral TLR signaling results in the expression of proinflammatory cytokines/chemokines, activation of antigen presenting cells, NK cell activation, and induction of adaptive immunity (10). The hallmark of antiviral TLR signaling is the production of type I interferons (IFNA and IFNB). Once interferons are produced, signaling pathways amplify and promote interferon production in an autocrine and paracrine manner resulting in a positive feedback loop (22, 93). Activated interferon alpha/beta receptor (OMIM: **IFNAR**) triggers the activation of janus kinase 1 (OMIM: **JAK1**), and protein-tyrosine kinase 2 (OMIM: **TYK2**), which then bind, phosphorylate and activate signal transducer and activator of transcription, (OMIM: **STAT1**) and **STAT2** (22, 93). A heterotrimeric transcription factor complex then forms, consisting of STAT1, STAT2, and IRF9, collectively called the IFN-stimulated transcription factor 3 gamma (OMIM: **ISGF3G**). ISGF3G initiates the transcription of interferon-stimulated genes (ISGs) by binding to interferon-stimulated response elements in their promoter regions (22, 93). ISGs are responsible for perpetuating and maintaining the host antiviral state. Collectively, these genes inhibit virus replication, affect the rate at which cells undergo apoptosis, and aid in viral clearance (93, 106).

While interferon production occurs through TLR pathway signaling, studies show that IFN production still occurs even when all TLR signaling has been eliminated, pointing to the importance of the non-TLR pathways (e.g. DDX58/RIG-I pathway) in accomplishing the antiviral state (104). Plasmacytoid dendritic cells (pDCs) are considered professional interferon producing cells through the TLR pathway, however non-professional interferon-producing cells such as conventional dendritic cells (cDCs) and epithelial cells produce type I interferons via non-toll signaling pathways (e.g. RIG-I) (111).

Cytokines and Chemokines

While the avian immune system is similar to that of the higher order vertebrates, aves are considered to have a reduced repertoire of immune mediators. However, there are unique features of the avian immune physiology, such as the Bursa of Fabricius and the heterophil. Since the sequencing of the chicken genome in 2004, much progress has been made in identifying chicken immune genes via in silico and bioinformatic analysis (35). To date, there have been 12 interferon genes, 26 interleukins, 3 transforming growth factors, 12 tumor necrosis factors superfamily (TNFSF) genes, 2 colony-stimulating factors, and 24 chemokines identified in chicken (39).

Interferons

Chickens have a single gene of each of the three subgroups of type I interferons, **IFNA** (IFN-α), **IFNB** (IFN-β), and **IFNL3** (IFN-λ), formerly a triad of IL28A, IL28B, and IL29. Chickens also have a single **IFNG** (IFN-γ) gene (39).

Interleukins

Chickens have **IL1B** (IL-1 family), **IL10, IL19, IL22, IL26** (IL-10 family), **IL17 – A, B, D, F** (IL-17 family), **IL23** (Th1 family), **IL3, IL4, IL5, IL13**, KK34 (Th2 family), and **IL6, IL7, IL9, IL16** (40).

Transforming growth factors

Chickens have three TGF-β genes, TGF-β2 (OMIM: **TGFB2**), TGF-β3 (OMIM: **TGFB3**), and TGF-β4 (OMIM: **LEFTY**) (39).

Tumor necrosis factors

Chickens have 12 TNF superfamily members: **TNFSF8**, alias CD30 ligand (CD30L), **TNFSF10**, alias TNF-related apoptosis-inducing ligand (TRAIL), **TNFSF13B**, alias B cell activating factor of TNF superfamily (BAFF), **TNFSF4**, alias OX40L, TNFSF5 (OMIM:

CD40LG), TNFSF6, alias Fas Ligand (FASL), LPS-induced TNF-α factor (OMIM: **LITAF**), **TNFSF9**, **TNFSF11**, alias receptor activator of NF-kappa-B ligand (RANKL), **TNFSF18**, alias AITR ligand (AITRL), and **TNFSF15,** alias vascular endothelial growth inhibitor (VEGI) (80).

Colony stimulating factors

Chickens have two CSFs, colony-stimulating factor 2 (OMIM: **CSF2**), alias granulocyte-macrophage colony-stimulating factor (GM-CSF), and colony-stimulating factor 3 (OMIM: **CSF3**), alias granulocyte colony-stimulating factor (G-CSF) (39).

Chemokines

Chemokines are divided into four groups on the basis of the position and spacing of the first two conserved cysteine residues at the amino-terminus: XC chemokines have one cysteine, CC chemokines have two adjacent cysteines, CXC chemokines have an amino acid separating two cysteines, and CX3C chemokines have three amino acids separating two cysteines (92). Some of the major chemokines are listed below. The avian XC chemokine is XCL1, alias lymphotactin (LTN). The CC chemokines are CCL17, alias thymus and activation-regulated chemokine (TARC), CCL19, alias macrophage inflammatory protein 3 beta (MIP-3β), CCL20, aliases macrophage inflammatory protein 3 alpha (MIP-3α) and chicken ah189, CCL21, alias secondary lymphoid tissue chemokine (SLC), CCL5, aliases regulated upon activation normally T-expressed and presumably secreted (RANTES) and chicken ah294, CCL7, aliases monocyte chemotactic protein 3 (MCP3) and chicken ah221, CCL4, alias macrophage inflammatory protein 1 beta (MIP-1β), and CCL16, alias chicken K203. The CXC chemokines are IL8, aliases CXCL8 and chicken K60, CXCL12, alias stromal cell-derived factor 1 (SDF-1), and CXCL14, alias BRAK (39, 40, 115). The CX3C chicken chemokine is CX3CL1, alias neurotactin (NTT). In summary, chickens have one XC chemokine, 14 CC, 8 CXC, and 1 CX3C chemokines identified to date (39).

Microarrays

Schena *et al.*, 1995 defines microarray technology as an ordered array of nucleic acids, that enables parallel analysis of complex biochemical samples (91). The microarray has proven itself to be a powerful tool for the study of global gene expression, and specifically, immune system function. Microarrays have been used to study differences in human macrophage gene expression in a wide variety of applications: between resident macrophage populations (56), in response to mycobacterium virulence genes (30), and during hypoxia due to advanced atherosclerosis (97). However, the most powerful and utilized role for microarray analysis of immune gene expression has been seen in human cancer research, which has dominated journal publications (26, 73, 117).

In avian species, a number of chicken cDNA based microarrays have been developed in the last several years and include tissues/cells including intestine, liver, pineal gland, heart, lymphocyte, and macrophage (1, 13, 17, 19, 20, 49, 58, 60, 66, 68, 70, 74, 75, 98, 114). In addition to these custom microarrays, there are also commercial arrays available such as the Affymetrix® GeneChip 33K Chicken Genome Array (6), the Agilent® 44K Chicken Genome CGH Microarray (57), the Operon® 20K Oligonucleotide *Gallus gallus* (chicken) Roslin/ARK CoRe Array V1.0 (113). Chicken immune-related microarrays have been used to study host-pathogen interactions involving oncogenes in lymphomagenesis (74), Marek's disease virus (55), herpes virus of turkeys (43), and *Escherichia coli* and LPS (13).

Recently, a 5K chicken immune-microarray was used to characterize the early immune response of broiler chickens to infection with H9N2 LPAI (23). Through microarray and histological analysis, the authors were able to identify the host genes responding to avian influenza and to correlate the observed gene expression patterns with clinical data and lung histopathology. The authors found that vaccinated/immune potentiated birds showed fewer clinical signs, gross lesions, and histological damage, and showed insignificant immune gene expression changes in response to virus challenge. Another recent study by Xing *et al.*, 2008, utilized the Affymetrix GeneChip 33K chicken genome array to characterize the chicken

immune response the H9N2 LPAI and correlated the downregulation of immune gene expression to antibody suppression (119). It was found that MHC gene expression was severely repressed, which most likely caused the antibody suppression.

Microarrays have proven to be a critical tool for studying the global immune response to pathogens. Host immune responses can be measured transcriptionally to elucidate the how and why of host mortality, morbidity, and survival. Potential therapeutics, antivirals, resistant genetic lineages, nutritional states, and prophylactics have all been identified with the help of microarray analysis.

Avian Influenza

Avian influenza (AI) is a type A influenza. Influenza A viruses are the most common and widespread of the influenza viruses, infecting a wide range of animal species, including birds and humans. Migratory waterfowl are the most common reservoir for AI and they transmit the virus to domestic poultry via the fecal-oral route and inhalation (4). AI was first identified as "fowl plague" in Italy in 1878 by Perroncito (100). The virus is subtyped based on the composition of its two main surface proteins, the hemagglutinin (HA) and neuraminidase (NA) antigens. There are 16 HA subtypes and 9 NA subtypes, all of which have been isolated from birds. The H2, H3, H4, H5, H6, H7, and H9 hemagglutinin subtypes and N1, N2, N3, N4, N6, N7, and N9 neuraminidase subtypes have been isolated from chickens (94). In the field, influenza A viruses infecting poultry flocks are divided into two groups based on their apparent pathogenicity: high pathogenicity avian influenza (HPAI) and low pathogenicity avian influenza (LPAI). HPAI can result in flock mortality as high as 100%. Avian HPAI viruses are composed of the H5 and H7 hemagglutinin subtypes although not all viruses of these subtypes cause HPAI.

LPAI viruses can belong to any hemagglutinin subtype and usually present as a much milder respiratory disease, causing depression and a decrease in egg production, but in conjunction with secondary viral and/or bacterial infections or poor environmental conditions, severe disease with high mortality may be seen.

Influenza A viruses are enveloped, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family (53). The influenza genome is comprised of eight negative-sense ssRNA segments, encoding 10-11 viral proteins. The ten viral proteins can be divided into three categories based on their location within the virus. The surface proteins include the hemagglutinin (HA), the neuraminidase (NA), and matrix 2 (M2) proteins. The internal proteins include the proteins that comprise the RNA-dependent RNA transcriptase complex: PA, PB1, and PB2, the nucleoprotein (NP), matrix protein 1 (M1), and nonstructural protein 2 (NS2). Finally, NS1 is a nonstructural protein that is the only protein not packaged into the virion but it is produced in large quantities in infected host cells. NS1 is an RNA binding protein responsible for inhibiting the processing of host mRNA. It also is responsible for regulating viral pre-mRNA splicing, translation, and polymerase activity, and inhibiting host antiviral responses via interferon pathways (101). Recent studies using RNA interference have been focused on targeting the NS1 gene for knockdown in order to limit influenza viral replication (65). Viral NP and PA have also been targets for RNAi studies (29), as well as the matrix gene (122).

The virion, 50-120 nm in diameter, is enveloped and pleomorphic. The envelope has surface projections embedded within its membrane, comprised of the antigenic determinants, HA and NA. The HA antigen is a homotrimeric protein that is proteolytically cleaved into the HA1 and HA2 subunits (15). It is the receptor that binds the virus to 5-*N*-acetyl neuraminic acid (sialic acid) residues on the surface of host cells thus allowing attachment of the virus to the cell

(15). Some influenza viruses bind preferentially to terminal sialic acids containing α-(2,6) linkages and others prefer α-(2,3) linkages (99). Receptor binding specificity is correlated to amino acid position 226 of the HA protein. HAs containing a leucine at position 226 specifically bind α-(2,6) linkages, preferentially binding to human host cell receptors. HAs containing a glutamine at that position, specifically bind α-(2,3) linkages, preferentially binding avian and equine host cell receptors (121). The NA antigen is a tetrameric protein, which serves as a receptor destroying enzyme (15). These receptor-destroying enzymes cleave a terminal sialic acid residue from host cells and virion glycoproteins to prevent clumping of released virions due to HA binding (15). This activity is the target for influenza antivirals such as the neuraminidase inhibitors, oseltamivir (Tamiflu®) and zanamivir (Relenza®), which function by disrupting NA. These antivirals result in the clumping of virions at the cell surface, which are unable to detach and infect other cells (95). NA also digests mucin, which enables the virus to reach target epithelium (62). Mucins are heavily glycosylated proteins secreted on mucosal surfaces that are saturated with oligosaccharides, thereby making them resistant to proteolysis and consequently providing a protective barrier at the mucosal surface.

Most influenza viruses are detected and destroyed by the innate immune response within hours (71). The virus is met with mucus at the epithelial lining of the lung, alveolar macrophages, interferons, cytokines, NK cells, complement, and a febrile response along with many other innate immune defenses. If the influenza virus is able to escape these immune mechanisms, the adaptive immune responses comprised of T and B cells and their subsets, target the virus specifically for neutralization by antibodies and antigen specific memory by T lymphocytes (105).

AIV enters the host cell via receptor mediated endocytosis, followed by low-pH dependent fusion with the endosome (81). It is at this step that AIV first encounters TLR7, which activates the aforementioned MYD88-dependent signaling cascade (24). AIV can also be recognized by cytoplasmic receptor DDX58/ RIG-I and the nucleotide binding oligomerization domain-like receptor (NLR) Cryopyrin/Nalp3. Recent studies suggest that innate immune responses to influenza A viruses are not regulated by a single receptor or signaling pathway; rather, they appear to be regulated by multiple receptors and signaling pathways in an orchestrated manner involving several cell types and distinct waves of signaling molecules (42, 51, 104). AIV readily infects chicken macrophages (119), heterophils (9), and human dendritic cells (59, 109). Typically, avian influenza infected cells up-regulate a wide variety of immune genes: IL6, interferon regulatory factors (IRFs), TLR3 and TLR7, macrophage inflammatory proteins, interleukins, proteases, chemokines, cytokines, heat shock proteins (HSPs), complement (e.g. C3), cluster of determinants (CDs) and T cell markers, immunoglobulins, apoptosis related factors, and TNF related factors (e.g. TRAF6) (23). However, it should be noted that different viral subtypes, titers, and time courses produce different gene expression profiles.

It has been widely hypothesized that the pronounced elevation of cytokines during highly pathogenic H5N1 infection, is caused by hypercytokinemia or a “cytokine storm”. In a manner beneficial to the host, cytokines promote leukocyte and lymphocyte activation and infiltration to sites of infection and have antiviral effects. However, during hypercytokinemia, cytokines can have deleterious effects. Hypercytokinemia was noted during lung histopathology obtained from the victims of the 1918 Spanish Flu (107). Several studies have reported a significant increase in proinflammatory cytokines due to infection with H5N1 influenza, supporting evidence of an

H5N1 HPAI induced cytokine storm (5, 18, 78, 89, 102, 118). The cytokine storm is thought to be the main cause of shock, pulmonary edema, acute respiratory distress syndrome (ARDS), and mortality (78).

AI in Ducks

Ducks are a natural and primordial reservoir for avian influenza viruses, and are a critical part of the transmission cycle between mammals and all other avian species (116). Ducks are an ecologically and epidemiologically significant species due to their global migratory patterns (77), asymptomatic presentation of AIV infection (34), and their close phylogenetic relationship to economically important avian species (domestic poultry) (21, 47, 96). The molecular basis for the resistance of ducks to AIV infection is a current area of study, with several potential mechanisms under investigation. One study examined the role of a gene, retinoic acid-inducible gene 1 protein (or RIG-I), a cytoplasmic RNA sensor, that when triggered by influenza virus produces interferon and stimulates antiviral signaling cascades (8, 61, 120). Barber *et al.* 2010, demonstrated that RIG-I decreases influenza viral replication, that RIG-I is present in ducks and absent in chickens, and that when duck RIG-I was transfected into chicken cells, an antiviral response was elicited by the transfected chicken cells in response to AIV infection and AIV replication decreased (8). In another recent study, Kuchipudi *et al.* 2012 found that that duck cells underwent rapid cell death following infection with LPAI H2N3, classical swine H1N1, and HPAI H5N1 viruses, while chicken cells did not (52). These findings suggest that induction of rapid death in duck cells may be a host resistance mechanism employed by ducks to fight influenza infection (52). In summary, mechanisms of duck resistance to influenza are being explored. A wide variety of research opportunities will be available once the duck genome is published and annotated. This will allow researchers to directly compare the molecular differences between resistant (duck) and susceptible (chicken) avian species, thereby identifying key regulators of the immunity responsible for host survival.

Research Objectives

Host-pathogen interactions involve many cell types, receptors, signaling pathways, and end products. Due to the growing knowledge base of the chicken cytokines, TLRs and their immune signaling pathways, antiviral research is expanding quickly. Our aim was to better understand the avian innate immune response to avian influenza viral infection by utilizing a pathogenomics approach. Due to the Asian strains of H5N1 HPAI and their public health concerns, and the economic significance of AI outbreaks, there was a clear need to study this specific host-pathogen interaction. By performing microarray experiments using our Avian Innate Immune Microarray (AIIM), we were able to measure the transcriptional host immune response to AIV. In the same vein, by studying AIV infected chickens and ducks (avian species critical to the transmission of AI) and LP- and HP- AIVs, we were able to characterize their differential immune responses and better understand how these viruses were interacting with the innate immune system. Furthermore, by exploring the use of TLR agonists in order to alter the host transcriptome prior to and during AIV infection, we were able to critically evaluate the innate immune signaling pathways responsible for the different clinical outcomes associated with in vivo HPAIV infection. This research project had three specific objectives related to characterizing the avian innate immune response to avian influenza virus:

1) Construct and evaluate an avian cDNA microarray (AIIM) emphasizing pathways (TLR, macrophage activation, antiviral response, etc) and clusters of genes (apoptosis, antigen presentation, cell signaling, etc) important for avian immunity.
2) Transcriptomic analysis of the avian in vivo immune response to avian influenza.
3) Determine the mechanism(s) by which the avian innate immune response (TLR pathway) is activated by and responds to avian influenza.

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Chapter 2

A FUNCTIONAL GENOMICS APPROACH TO THE STUDY OF AVIAN INNATE IMMUNITY

Calvin L. Keeler, Jr.[a], Travis W. Bliss[a], Miha Lavrič[b], **Michele M. Maughan**[a]

[a]Department of Animal and Food Sciences, College of Agriculture and Natural Resources, University of Delaware, Newark, Delaware 19716-2150

[b]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia

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Abstract

A second-generation 4,959 element cDNA microarray has been created and evaluated for its potential use in examining the avian innate immune response. The elements in this array were obtained from EST libraries of stimulated avian PMNC-derived monocytes/macrophages and supplemented by genes of interest from several specific innate immune pathways. The elements are spotted in triplicate resulting in 14,877 total spots per slide. The avian innate immunity microarray (AIIM) contains 25 avian interleukin, chemokine, and cytokine elements. The array also contains elements for several innate immune pathways, including genes involved in the Toll-like receptor (TLR) pathway (including six of the currently known avian TLR receptors), avian interferon/antiviral response pathway genes, and genes involved in apoptosis, antigen presentation and the oxidative burst. The AIIM can be used to evaluate global gene expression patterns in a number of immunologically relevant tissues and in chickens, turkeys and ducks. The array has also been evaluated for its ability to monitor the avian immune response to both bacterial (avian pathogenic *Escherichia coli*) and viral (avian influenza) avian pathogens.

Introduction

Infectious diseases influence all aspects of poultry production. As with other vertebrates, successful defense against infection is dependent on the ability of the animal to detect the presence of the invading pathogen. The innate immune system recognizes and reacts to microbes and also distinguishes commensals from true pathogens (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997, 2000). Innate host defenses are dedicated to the containment of the pathogens, holding infections to a level that can be resolved by the ensuing development of acquired immune mechanisms.

Infectious microbes contain conserved molecular structures, or pathogen-associated molecular patterns (PAMPs) (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997, 2000).

These structures are required for the growth and survival of the microbe and are distinct from any host antigens. Several well known examples include the lipopolysaccharides of gram-negative bacteria, the lipoteichoic acids of gram-positive bacteria, lipoproteins and peptidoglycans of all bacteria, the glycolipids of mycobacteria, the mannans found in yeast cell walls, double stranded RNA of RNA viruses, and the unmethylated CpG motifs found in bacterial DNA but not mammalian DNA (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997; Medzhitov *et al.*, 1997).

The cells of the innate immune system utilize many receptors in the process of microbial recognition and internalization. These include Fc and complement receptors, integrins, lectins, mannose receptor, CD14, and the Toll-like receptors (TLR) (Daeron, 1997; Stossel, 1999; Underhill and Odinsky, 2002). The TLRs are a family of evolutionarily conserved type I integral membrane glycoproteins (Akira and Takeda, 2004). They function as direct receptors for microbial and viral PAMPs and they control many aspects of both the innate and adaptive immune responses (Akira, 2003). PAMP recognition activates effector mechanisms of innate host defenses, including phagocytosis, the synthesis of antimicrobial peptides and interferons, and the induction of the respiratory burst and nitric oxide synthase. Furthermore, recognition of PAMPs induces the expression and production of pro-inflammatory cytokines and chemokines. PAMP recognition also induces the development of immune memory and effector cells (Medzhitov and Janeway, 1997, 2000; Medzhitov *et al.*, 1997; Kopp and Medzhitov, 1999).

The microarray has become a powerful tool for the study of immune system function. In avian species, a number of low-density and high-density cDNA based microarrays have been developed in the last few years (Liu *et al.*, 2001; Morgan *et al.*, 2001; Neiman *et al.*, 2001, 2003; Cogburn *et al.*, 2003, 2004; Koskela et al, 2003; Min *et al.*, 2003; Munir and Kapur, 2003; Bliss *et al.*, 2005; Burnside *et al.*, 2005; Degan *et al.*, 2006; Smith *et al.*, 2006). Recently, a consortium of research groups has developed a comprehensive 13,000 element chicken cDNA microarray (http://www.fhcrc.org/shared_resources/genomics/chicken_13k.pdf) for use by the avian research community.

We have previously reported on the construction and evaluation of an avian PMNC-derived monocyte/macrophage cDNA microarray (Bliss *et al.*, 2005). To improve our understanding of the avian innate immune response to bacterial and viral pathogens we have expanded this microarray into a 4,959 element (14,877 spot) cDNA avian innate immunity microarray (AIIM). This expanded array contains additional TLR, antiviral response, and chemokine/cytokine genetic elements. The array has been used to examine the transcriptional response of the avian Toll-like receptor pathway (TLR) to avian pathogenic *Escherichia coli* (APEC) and the interferon/antiviral response pathway to avian influenza (H7N2).

Materials and Methods

Preparation of PMNC-derived adherent cells

Heparinized peripheral blood was collected and pooled from six > 3-month-old commercial broiler chickens. Peripheral mononuclear cells (PMNC), composed of thrombocytes, lymphocytes and monocytes, were obtained by centrifugation through Histopaque 1077 and monocytes were selected through adherence to 60 mm plastic tissue culture dishes (Martin *et al.*, 1993). Primary PMNC-derived adherent cells (5×10^8 cells/60 mm dish or 8×10^7 cells/60 mm vented cap flask) were grown in RPMI (Invitrogen Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (complete RPMI) at 37 °C, 5% CO_2 . After overnight incubation, non-adherent cells (95% of the culture) were removed by washing the monolayers with RPMI medium. Previous experiments have shown that these preparations are highly enriched for mononuclear phagocytes. Utilizing flow cytometry, approximately 60% of the cells are positive with the K1 anti-chicken mononuclear phagocyte antibody (data not shown) and microscopic analysis indicates that thrombocytes make up 5–10% of the adherent cell population.

Microarray construction

The avian innate immunity microarray (AIIM) was constructed from a total of 4,959 genetic elements. The majority of the clones (4,772) were derived from four avian macrophage EST libraries (Bliss *et al.*, manuscript in preparation). Additional EST clones (134) were kindly provided from the chickEST project by Dr. Robin Morgan and thirteen full-length cDNA clones were provided by Dr. Pete Kaiser. Plasmid DNA was prepared using R.E.A.L. Prep 96 kits (Qiagen Inc., Valencia, CA). cDNA inserts were amplified using PCR SuperMix (Invitrogen Inc.) in 50 µl reactions using 1 µl of plasmid DNA as template and using primers specific for the various plasmid vectors used in the construction of the cDNA libraries or full-length clones. In addition, 40 genetic elements were obtained by direct amplification from cellular RNA using gene-specific primers and the Qiagen One-Step RT-PCR kit (Qiagen Corp.). Details of these genes and the primers used in their amplification are provided as supplemental data on a web database at http://www.aviangenomics.udel.edu/. Purified PCR products were dried, resuspended to a concentration of ~150 ng/µl in 10 µl of spotting solution (3 X SSC, 0.01% SDS), and spotted in triplicate (14,877 total spots). Spotting quality was evaluated as described previously (Bliss *et al.*, 2005).

Experimental design

Avian pathogenic Escherichia coli (APEC) strain V-G (Skyberg *et al.*, 2003) was grown in Luria-Bertani (LB) medium to an absorbancy at 600 nm of 1.0. Bacteria were then pelleted and resuspended in complete RPMI. Bacterial infection was initiated by replacing the growth medium in primary PMNC-derived adherent cell cultures with fresh medium containing bacteria at an MOI of 100. To prevent bacterial replication, tetracycline was added at a pre-determined bacteriostatic concentration of 7 µg/ml. Cultures exposed to *E. coli* were incubated at 37 °C, 5% CO 2 for 1, 6, or 24 h. Total cellular RNA was isolated using the RNeasy Midi RNA Purification Kit according to the manufacturer's instructions (Qiagen Inc.) and amplified and

labeled as described below. Gene expression was analyzed at control (0), 1, 6 and 24 h time points by setting up a two-color (Cy3 and Cy5) four slide time-loop.

A low pathogenic strain of avian influenza (LPAI), CK/Maryland/Hobo/2003 (H7N2), was propagated in embryonated eggs. Viral infection was initiated by replacing the growth medium in 60 mm vented flasks with fresh medium containing 250 HA units of virus for each flask. Virus infected cultures were incubated at 37 °C, 5% CO_2 for 4 h. Total cellular RNA was isolated using the RNeasy Midi RNA Purification Kit. The 0 h and 4 h samples were hybridized in a dye-swap format on two slides in a single replicate.

Avian tissues (air sac, lung, liver, spleen, embryonic spleen, thymus, duodenum) were aseptically recovered from healthy 6-month-old Single Comb White Leghorn chickens after necropsy. Total cellular RNA was isolated from 250 µg of tissue using the RNeasy Midi RNA Purification Kit (Qiagen Inc.), amplified, and indirectly fluorescently-labeled with Cy3. Hybridizations were performed on seven individual slides as described below.

Total cellular RNA isolated from turkey and duck spleens was generously provided by Dr. Erica Spackman. Total cellular RNA was isolated from 250 µg of tissue using the RNeasy Midi RNA Purification Kit (Qiagen Inc.), amplified, and indirectly fluorescently-labeled with Cy3. Hybridizations were performed on two separate slides as described below.

Microarray hybridization and data analysis

Two microgram of total RNA from each sample was amplified into amino allyl modified RNA (aRNA) using the Ambion Amino Allyl MessageAmp II aRNA Amplification kit (Ambion Inc., Austin, TX). aRNA (15 µg) was fluorescently labeled with AlexaFluor 555 (Cy3) or AlexaFluor 647 (Cy5). Concentration and labeling efficiencies of aRNA were determined spectrophotometrically. Single color and two color microarray hybridizations (60 µl) were performed in Mica hybridization chambers (Genomic Solutions, Ann Arbor) at 50 °C overnight. After hybridization, slides were rinsed in 0.5 X SSC, 0.01% SDS at room temperature and then washed for 15 min in 0.2 X SSC, 0.2% SDS at 50 °C, three times for 1 min in 0.2 X SSC at room

temperature, and finally three times for 1 min in water at room temperature. Washed slides were scanned with an ArrayWoRx scanner (Applied Precision, Issaquah, WA) using Cy3 and Cy5 filters. Spot and background intensities were acquired using SoftWoRx tracker (Applied Precision) and data analysis was performed using GeneSpring v7.0 (Silicon Genetics, Redwood City, CA) as described previously (Bliss *et al.*, 2005). Background intensity was determined using the SoftWorx tracker cell method. Abnormal spots (dust, bubbles), spots with intensities not greater than background + 2 standard deviations and elements that were not represented by at least two replicate spots on each slide were removed from further analysis.

In the *E. coli* and avian influenza experiments, those elements exhibiting > 2-fold changes in signal intensity compared to the 0 h time point were analyzed by ANOVA using the Benjamini and Hochberg (1995) False Discovery Rate (FDR) multiple testing correction with a P value of < 0.01 to determine which biologically significant changes were also statistically significant. In compliance with the MIAME guidelines, information on the AIIM and additional supplemental data is available on a web database at http://www.aviangenomics.udel.edu and at the NCBI GenBank Gene Expression Omnibus (GEO) repository, platform number GPL1461.

Results

AIIM design

The cDNA-based avian innate immunity microarray (AIIM) has been constructed from 4,959 avian genetic elements. Most of the elements (4,772) are derived from four avian PBMC-derived monocyte/macrophage EST libraries and have been described previously (Bliss *et al.*, 2005). These clones were supplemented by 134 clones generously provided by Dr. Robin Morgan and the University of Delaware chickEST project (http://www.chickest.udel.edu/). In addition, 13 full-length cDNA clones were kindly provided by Dr. Pete Kaiser (Institute of Animal Health, Compton, UK). Finally, 40 elements were amplified directly by RT-PCR from

targeted genes of interest identified on the avian genome. As described in Materials and methods, these 4,959 elements were spotted in triplicate to create the 14,877 spot AIIM. The resulting cDNA microarray contains 28 elements from each of two critical innate immune response pathways, the TLR pathway and the viral (interferon) response pathway (Table 1). These elements include pathway-specific receptor molecules (including six of the TLR receptors), many internal signaling elements, and several transcription factors. In addition, the AIIM contains a significant number of avian cytokine and chemokine elements, including several CXCL and CCL chemokines and 13 interleukin elements (Table 2).

Table 2.1. TLR and viral response pathway genes present on the avian innate immunity cDNA microarray (AIIM)

TLR Pathway

Symbol	Accession Number
FOS	CK607079
JUN	CK606834
ECSIT	BU209678
NFKB1A/IKBα	NM_001001472
CHUK/IKKα	BM486156
IKBKB/IKKβ	BM489568
IRAK2	CK610881
MAPK8/JNK1	BI390952
LBP	BQ484391
MAP3K1/MEKK1	XM_424734
MAP2K3/MKK3	NM_001012787
MAP2K4/MKK4	XM_415583
NFKB	CK608565
NIK/MAP4K4	BI392233
p38	BQ038538
PKR/EIF2AK2	NM_204487
TAB1/MAP3K7IP1	NM_001006240
TAB2	CK611620
TAK1	BM426610
TIRAP	DQ019929
TLR1/6/10	CK611396
TLR2	CK607640
TLR3	NM_001011691
TLR4	NM_001030693
TLR5	BI066471
TLR7	BM440145
TOLLIP	AJ720279
TRAF6	CK607050

Viral Response Pathway

Symbol	Accession Number
FOS	CK607079
CIITA	XM_425250
COX2	NP_006918
GCH1	CK609195
IRF8/ICSBP	NM_205416
IFNAR1	CK611371
IFNAR2	CK610289
IFNG	AY163160
IFNGR2	BG710035
IKBA	NM_001001472
IKKE	CK614834
IRF1	CK612627
IRF2	CK609283
ISGF3	CK610684
JAK1	CK614545
JAK2	NM_001030538
MKK3/MAP2K3	NM_001012787
MKK4/MAP2K4	XM_415583
PTGES	XM_415475
CEBPB/NF-IL6	CK614519
NFKB	CK608565
MAPK14/p38	BQ038538
EIF2AK2/PKR	NM_204487
SP1	CK606789
STAT1A/p91	CK609556
STAT1B/p84	XM_419149
TYK2	AF041801
ZFP91	CK609556

Table 2.2. Cytokine and chemokine genetic elements present on the avian innate immunity cDNA microarray (AIIM)

Gene Family	Gene Name	GenBank Accession Number
CXCL	chCXCLi1 (K60, IL8, CXCL8b)	CK610286
CCL	chCCLi1 (ah294)	CK609464
	chCCLi3 (K203)	CK613216
	chCCLi7 (ah221)	CK610423
	ChCCL20 (ah189, MIP3-α)	CK613680
Interferon Family	IFNA	AM049251
	IFNB	AY974089
	IFNG	DQ470471
Interleukin Family	IL1B	CK607391
	IL2	AJ224516
	IL3	AJ621740
	IL4	AJ621249
	IL5	AJ62125
	IL6	CK613692
	IL10	AJ621254
	IL12A	AY262751
	IL12B	AJ564201
	IL13	AJ621250
	IL15	BM489119
	IL16	CK614630
	IL18	CK613996
TNF Superfamiy	CD40LG	AI982044
	TNFSF13B/BAFF	CK608618
TGF-Beta Family	TGFB3	BI064554
	LEFTY2/TGF-β4	M31160
Other	GMCSF	AJ621740

Array utility

Two experiments were performed in order to evaluate the utility of the AIIM. First, total RNA was isolated from seven avian tissues with relevance to the avian immune response (**Table 3**). These tissues were collected from normal non-infected birds. In all instances the fluorescently-labeled aRNA derived from these tissues hybridized to the vast majority of the elements present on the array (72% - 92%). In a separate experiment fluorescently-labeled aRNA was synthesized from total RNA isolated from the normal spleens of chicken, turkey, and duck and hybridized to the array. As shown in **Fig. 1** a significant number of elements hybridized to turkey spleen-derived aRNA (1,365 or 27.5%) and duck spleen-derived aRNA (1,763 or 35.6%). As expected, no elements providing hybridization signals (> 2 times spot-normalized background intensity) were found to be unique to the turkey or duck samples.

Table 2.3. Number (percent) of the 4,959 elements on the AIIM which produce positive signals (>background + 2 SD) upon hybridization with fluorescently-labeled RNA derived from avian tissues involved in the immune response

Tissues	Number (percent)
Air Sac	4,253 – 86%
Lung	3,969 – 80%
Liver	3,585 – 72%
Spleen	4,520 – 92%
Spleen (20 d embryo)	3,698 – 75%
Thymus	3,549 – 72%
Duodenum	4,100 – 83%

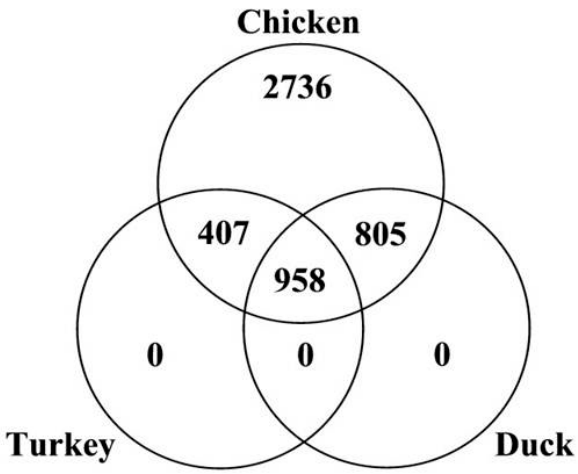
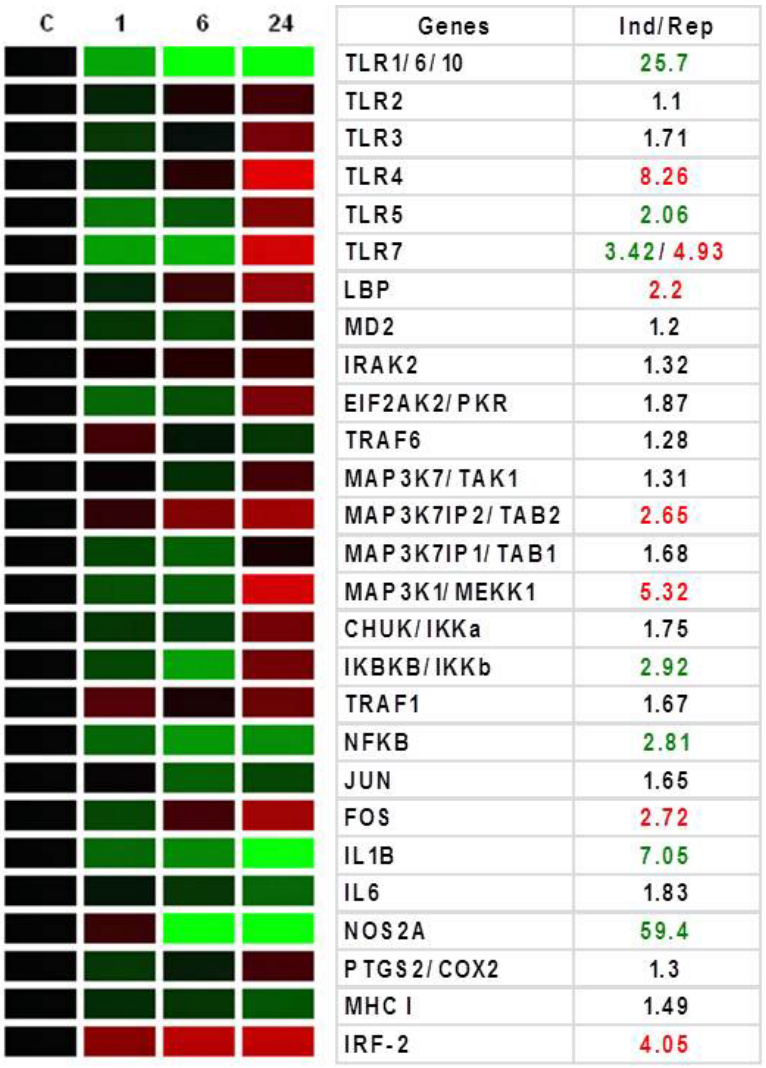


Figure 2.1. Number of AIIM elements exhibiting significant levels of expression in chicken, duck, or turkey spleen. The number of common or shared elements exhibiting a significant (greater than two times background) level of expression is indicated.

Bacterial and viral immune responses

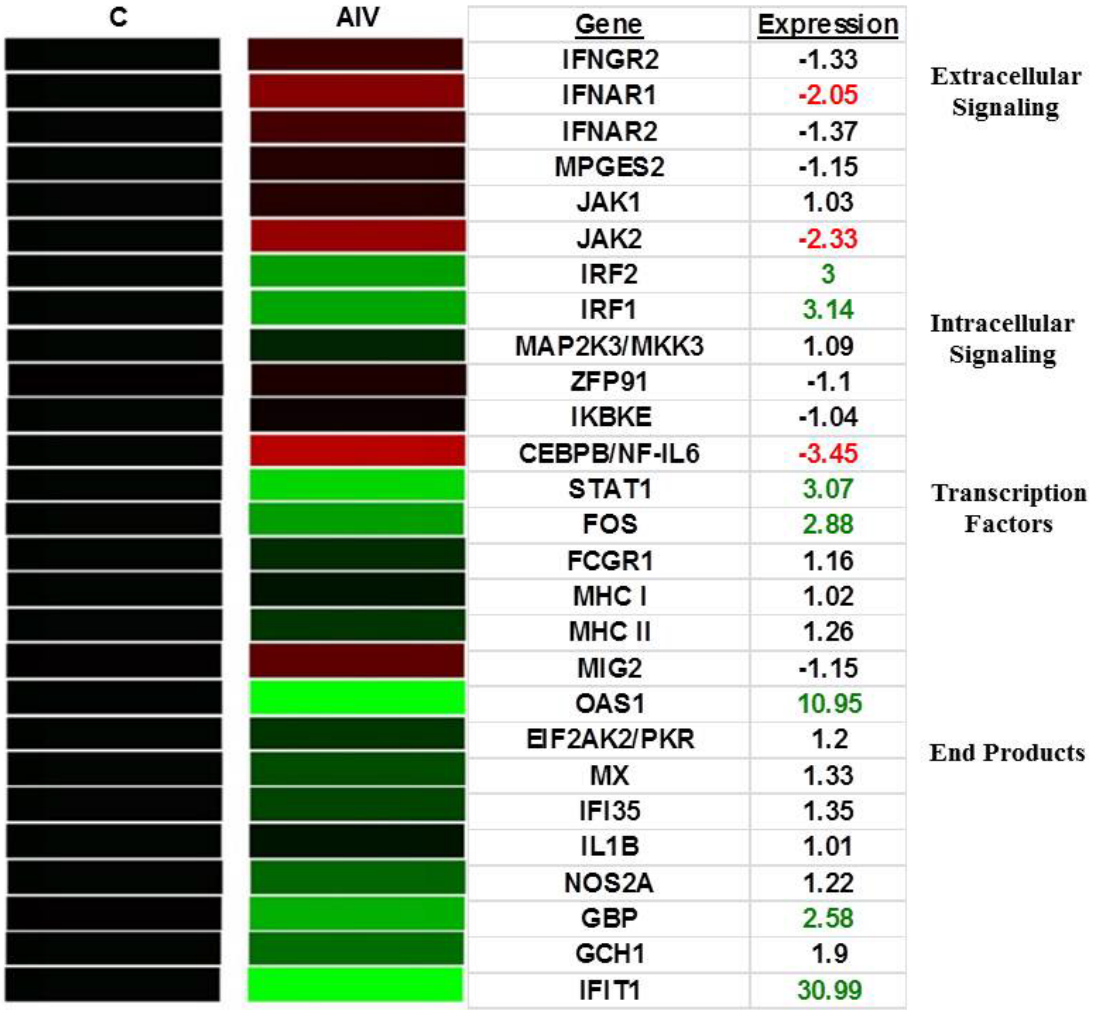
The ultimate purpose of the AIIM is to determine the transcriptional response of avian species upon exposure to various pathogens. To that end, PMNC-derived avian monocytes/macrophages were exposed to APEC *E. coli* strain V-G. Over a 24 h period, total cellular RNA was extracted, transcribed, amplified, fluorescently labeled and hybridized to the array. The transcriptional response of those genes on the array known to be involved in the signaling process of the TLR pathway is shown in **Fig. 2**. From the gene tree it can be observed that during the first 6 h a number of genes involved in PAMP signaling, both TLR receptors (TLR 1/6/10, TLR5, TLR7) and intracellular signaling molecules and transcription factors (IKKB, NFKB), are modestly induced. However, by 24 h, with the exception of TLR 1/6/10 and the transcription factor NFKB, the majority of these genes are repressed in their transcriptional levels. Meanwhile end products of the TLR pathway generally remain induced throughout the course of exposure.



Genes	Ind/Rep	
TLR1/6/10	25.7	Extracellular Receptors
TLR2	1.1	
TLR3	1.71	
TLR4	8.26	
TLR5	2.06	
TLR7	3.42/4.93	
LBP	2.2	
MD2	1.2	
IRAK2	1.32	Intracellular Signaling
EIF2AK2/PKR	1.87	
TRAF6	1.28	
MAP3K7/TAK1	1.31	
MAP3K7IP2/TAB2	2.65	
MAP3K7IP1/TAB1	1.68	
MAP3K1/MEKK1	5.32	
CHUK/IKKa	1.75	
IKBKB/IKKb	2.92	
TRAF1	1.67	
NFKB	2.81	Transcription Factors
JUN	1.65	
FOS	2.72	
IL1B	7.05	
IL6	1.83	
NOS2A	59.4	End Products
PTGS2/COX2	1.3	
MHC I	1.49	
IRF-2	4.05	

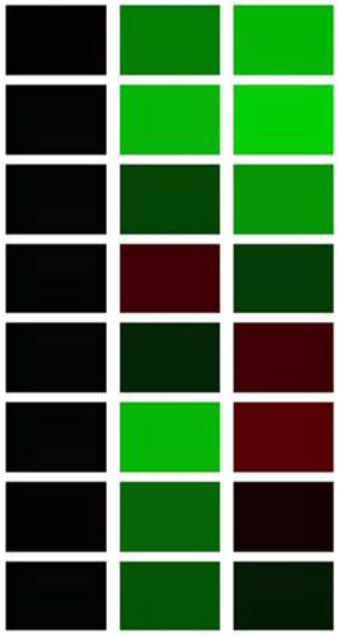
Figure 2.2. Regulation of avian TLR pathway gene expression. Pathway genes present on the AIIM (Table 1) which passed the initial slide quality evaluation were arranged according to their location in the TLR signaling pathway from the extracellular components to the eventual end products of the pathway. Time points evaluated by microarray are 0, 1, 6, and 24 h after exposure of avian PMNC-derived monocytes/macrophages to *E. coli* strain V-G. Expression levels were compared to the control (0 h) time point. A green color indicates gene induction, red indicates gene repression, black indicates no change (control or 0 h value). Fold values of induction or repression (Ind/Rep) are indicated, with expression levels two fold or greater than the control (0 h) level indicated by green or red accordingly.

After a 4 h exposure of PMNC-derived avian monocytes/macrophages to avian influenza (H7N2) most of the elements present on the microarray which are involved in extracellular signaling of the viral response pathway have been repressed (interferon receptors) while transcription factors (c-fos) and many potential end products are modestly induced (**Fig. 3**). The gene tree in **Fig. 4** illustrates differences observed in the expression of specific cytokines and chemokines in response to a bacterial or viral pathogen relatively early after exposure (6 or 4 h respectively). Expression of both chCXCLi1 (K60) and chCCLi3 (bK203) are induced upon exposure to both the bacterial and viral pathogens. However, while chCCLi1 (ah294) is induced only in the presence of the APEC strain of E. coli, transcription of IL16, IL18, and chCCLi7 (ah221) is either significantly or moderately induced only upon exposure to avian influenza.



Gene	Expression	
IFNGR2	-1.33	Extracellular Signaling
IFNAR1	-2.05	
IFNAR2	-1.37	
MPGES2	-1.15	
JAK1	1.03	
JAK2	-2.33	
IRF2	3	
IRF1	3.14	Intracellular Signaling
MAP2K3/MKK3	1.09	
ZFP91	-1.1	
IKBKE	-1.04	
CEBPB/NF-IL6	-3.45	
STAT1	3.07	Transcription Factors
FOS	2.88	
FCGR1	1.16	
MHC I	1.02	
MHC II	1.26	
MIG2	-1.15	
OAS1	10.95	
EIF2AK2/PKR	1.2	End Products
MX	1.33	
IFI35	1.35	
IL1B	1.01	
NOS2A	1.22	
GBP	2.58	
GCH1	1.9	
IFIT1	30.99	

Figure 2.3. Regulation of avian viral response pathway gene expression. Pathway genes present on the AIIM (Table 1) which passed initial slide quality evaluations were arranged according to their location in the viral response signaling pathway from the extracellular components to the eventual end products of the pathway. The single time point evaluated by the experiment was 4 h after exposure of avian monocytes/macrophages to an H7N2 strain of avian influenza (AIV). Gene expression levels were compared to the control (0 h) time point. A green color indicates gene induction, red indicates gene repression, black indicates no change.



Gene	AIV	EC
chCXCLi1	2.26	3.59
chCCLi3	3.59	4.3
chCCLi1	1.33	2.79
IL6	-1.29	1.29
TNFSF13B/BAFF	1.09	-1.29
chCCLi7	3.6	-1.51
IL18	1.75	-1.09
IL6	1.54	1.05

Figure 2.4. Differential responses of avian chemokines and cytokines to *E. coli* and avian influenza. Selected chemokine and cytokine elements from the AIIM are indicated. Time points for expression evaluated by microarray are 6 h after monocyte/macrophage exposure to *E. coli* (EC) and 4 h after exposure to avian influenza (AIV). Expression levels relative to the matched control (0 h) are indicated.

Discussion

In a previous report we described the development and initial evaluation of an avian macrophage microarray (Bliss *et al.*, 2005). Although useful for monitoring the transcriptional activity of avian macrophages during the phagocytic response, we found that this cDNA microarray was limited in its usefulness in monitoring the avian immune response to viral infection. The 187 specifically targeted genetic elements which were added to this array beyond the initial 4,772 macrophage EST clones have significantly improved the capabilities of this microarray platform. The elements were chosen in order to enhance the capability of the array to monitor the avian innate immune response. More specifically, these elements were chosen in order to comprehensively include genes involved in the TLR signaling (28 elements) and in the viral immune response (28 elements) pathways (Table 1) and to include the majority of the known avian chemokines, cytokines, and lymphokines (Table 2), important effector molecules of these immune pathways.

In characterizing the avian innate immunity microarray we endeavored to determine its broader utility for the study of avian immunity. First, we have clearly demonstrated that the array not only effectively monitors monocyte/macrophage gene expression but that it can monitor gene expression from tissues important for determining avian immunity (Table 3). This determination enables this platform to be used for in vivo studies aimed at determining the response and signaling patterns of different tissues and cell types. Intriguingly, and not surprisingly, the AIIM was found to be effective in determining gene expression in embryonic chicken spleens. The United States poultry industry has been revolutionized in the past decade by the application and acceptance of *in ovo* vaccination. However, this comprehensive change in vaccination strategy needs to be supported by studies on the ability of the avian immune response to develop and function pre-hatch in the face of an antigen challenge. We expect to use this microarray in future efforts to answer these questions.

Additional experiments determined that the AIIM may have utility in monitoring the transcriptional response of immune cells in turkey and duck as well as in chicken. There are few

studies which use microarray techniques to study turkey or duck gene expression (Dar *et al.*, 2005; Munir and Kapur, 2003). A number of elements on the AIIM (27%– 35%) produced hybridization signals above background levels when hybridized to turkey- or duck-derived aRNA. Although significant, we believe that a greater number of elements are capable of cross-species hybridization than observed in this experiment. This experiment was performed under relatively stringent hybridization conditions and changes in hybridization and/or wash conditions may increase the number of cross-hybridizing elements.

Lastly, experiments in which chicken PMNC-derived adherent cells (monocytes/macrophages) were infected with either a strain of avian pathogenic E. coli or avian influenza confirmed that the AIIM can be used to examine the transcriptional response to both bacterial and viral pathogens. The ability to detect differences in the chemokine and cytokine response to different pathogens will permit the development of hypothesis driven experiments designed to evaluate and understand the complexity of the avian immune signaling response. With tools such as the AIIM we are now able to compare and contrast at the transcriptional level the avian immune response to different pathogens. In addition, we can systematically follow the temporal response in vivo and we can evaluate the response to different strains and pathotypes. Finally, we not only have the capability to examine the complex interactions between the various innate immune cell types and pathogens but we can also study the complex signaling interactions between specific avian cell types.

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Chapter 3

GENE EXPRESSION MODULATION IN CHICKEN MACROPHAGES EXPOSED TO *MYCOPLASMA SYNOVIAE* OR *ESCHERICHIA COLI*

Miha Lavric[a,b], **Michele N. Maughan**[b], Travis W. Bliss[b], John E. Dohms[b], Dusan Bencina[a], Calvin L. Keeler Jr[b], and Mojca Narat[a]

[a]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domzale, Slovenia

[b]Department of Animal and Food Sciences, College of Agriculture and Natural Resources, University of Delaware, 040 Townsend Hall, Newark, DE 19717-1303, USA

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Abstract

Mycoplasma synoviae and *Escherichia coli* are two avian pathogens that exhibit markedly different mechanisms for infection and pathogenicity and may be expected to manipulate the host innate immune response differently. The aim of this study was to determine the extent of modulated genes and make a comparison between the transcriptomes of chicken macrophages exposed to either *M. synoviae* type strain WVU 1853 (MS) or avian pathogenic *E. coli* strain V-G (APEC). To analyze temporal gene expression profile of monocyte-derived macrophages (MDM) and HD11 cell line macrophages after each exposure, two avian immunity microarrays were used: the avian macrophage microarray (AMM) and the avian innate immunity microarray (AIIM). The quantity of MS-modulated genes was estimated in three experiments, using both microarrays. A cross-section revealed 14 AMM/AIIM genetic elements that were modulated in both types of macrophages. Additionally, to compare immunomodulatory activity of MS and APEC, MDM were exposed to each pathogen and gene modulation was detected by AIIM microarray. This study revealed 157 elements uniquely modulated by MS and 1603 elements uniquely modulated by APEC. AIIM microarray analysis also revealed a core set of 146 elements modulated by both pathogens, with generally higher induction/repression levels after APEC exposure. Validation of selected gene expression was done by quantitative real time RT-PCR. The study shows higher transcription levels of IL-1b, IL-6, iNOS, NCF1, peroxiredoxin 1 and cathepsin L genes after MDM exposure to APEC than after exposure to MS. Surprisingly, complement component C3 gene was repressed after MDM exposure to APEC, while being induced after exposure to MS.

Introduction

It is common for poultry pathogens to enter the host through the lung surface and subsequently disseminate to their target organs (Reese *et al.*, 2006). Cells belonging to the mononuclear phagocytic system lineage (monocytes–macrophages) are considered as the first

line of defence against infective agents in the lung as well as other tissues (Reese *et al.*, 2006; Qureshi, 1998). When present and in contact with live bacteria or bacterial products, macrophages utilize a variety of receptors (i.e. Fc and complement receptors, integrins, lectins, mannose receptor, CD14, and Toll-like receptors (TLR)) for the process of microbial recognition, phagocytosis or cytokine production (Underhill and Ozinsky, 2002; Qureshi, 2003).

Mycoplasma synoviae (MS) is a chicken and turkey pathogen, most frequently causing a subclinical respiratory tract infection, which can progress to respiratory disease, to infectious synovitis, or to systemic disease causing pathological changes in numerous organs and autoimmune processes (Kleven, 2003).

Strains of avian pathogenic *Escherichia coli* (APEC) cause extraintestinal diseases in chickens, turkeys, and other avian species. APEC strains are capable of surviving inside macrophages (Bastiani *et al.*, 2005) and some virulence factors protect them from phagocytosis and from bactericidal effects (Mellata *et al.*, 2003). Lipopolysaccharide, the major outer membrane constituent of gram negative bacteria, is capable of inducing gene expression and secretion of a number of cytokines from avian splenocytes, heterophils and macrophages (Bliss *et al.*, 2005; Kogut *et al.*, 2005, 2006; Lavric *et al.*, 2007; Schwarz *et al.*, 2007).

In our in vitro experiments, chicken peripheral blood monocyte-derived macrophages (MDM) or HD11 chicken macrophage cell line were exposed to MS WVU 1853. Separately, MDM were infected with field isolate APEC V-G (Skyberg *et al.*, 2003) and a comparison of macrophage gene expression was made by using two cDNA microarrays representing genes of the avian innate immunity. The arrays used were the avian macrophage microarray (AMM) (Bliss *et al.*, 2005) with 4906 elements and its extended version, the avian innate immunity microarray (AIIM) with all 4906 elements from AMM microarray enlarged with newly added 53 elements for a total of 4959 elements (Keeler *et al.*, 2007). Gene expression of selected elements was evaluated with quantitative real time RT-PCR.

Materials and Methods

Macrophage cell culture

Blood was collected from four adult broiler breeder chickens into heparinized syringes. Blood samples were pooled and MDM were prepared from blood mononuclear cells by Histopaque®1-1077 (Sigma–Aldrich Corp., St. Louis, MO) density gradient centrifugation (Peck *et al.*, 1982), washed three times with phosphate buffered saline at pH 7.2 (PBS) and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Life Technologies Inc., Gaithersburg, MD), 100 U/ml of penicillin (Life Technologies Inc.) and 100 µg/ml of streptomycin (Life Technologies Inc.). Approximately 5 x 10^8 cells were seeded on 100 mm plastic tissue culture dishes (BD Biosciences, San Jose, CA). After overnight incubation, non-adherent cells (95%) were removed by washing the monolayers two times with PBS and the remaining adherent cells were then overlaid with RPMI containing 10% FBS. The chicken macrophage cell line HD11 (Beug *et al.*, 1979) was cultured in same media as MDM cells.

M. synoviae and *E. coli* growth and macrophage exposure

MS WVU 1853 was grown at 37 8C to mid-logarithmic phase on modified Frey's medium containing 12% porcine serum (Life Technologies Inc.) and 0.1 g NAD/l (Merck & Co. Inc., Whitehouse Station, NJ) of broth (Kleven, 2003). APEC V-G (Skyberg *et al.*, 2003) was grown to mid-logarithmic phase at 37 8C in Luria–Bertani (LB) medium. The number of MS WVU 1853 and *E. coli* cells is approximately equal to the number of colony forming units (CFU) and was determined by seeding agar plates with an aliquot of MS or *E. coli* culture used in experiment. After counting the colonies the CFU/ml for each sample was defined. Bacteria were pelleted and resuspended in RPMI 1640 medium containing 10% FBS and no antibiotics (Bliss *et al.*, 2005). Macrophages were exposed to bacteria by replacing the macrophage growth medium with fresh medium containing bacteria at a multiplicity of approximately 100 bacterial cells per macrophage. Experimental design of chicken macrophage exposure to bacterial agents is presented in **Table 1**. Non-exposed MDM or HD11 were used as negative (0 h) controls. To

prevent rapid bacterial replication, tetracycline (Life Technologies Inc.) was added at a pre-determined bacteriostatic concentration of 7 µg/ml for APEC. This enabled a more reliable comparison of interaction with macrophages, because MS cells are much smaller (~one order of magnitude) and replicate considerably slower than APEC in in vitro conditions. Macrophage cultures exposed to bacteria were incubated at 37 °C, 5% CO_2 before harvesting cells for RNA. At 0 h (control) and at 1, 6, 12 or 24 h of exposure to bacteria, growth medium was aspirated from plates, RLT lysis buffer (Qiagen Corp., Valencia, CA) was added and cells were harvested with a rubber policeman.

Table 3.1. Experimental design of chicken macrophage exposure to bacterial agents and time-course sample hybridization to microarrays

Infectious agent[a]	Cell line[b]	Time points (h)[c]	Microarray used[d]	Sample hybridization format[d]
MS WVU 1853	HD11	NE, 6, 12, 24	AMM	Time loop
MS WVU 1853	MDM	NE, 6	AMM	Dye swap
MS WVU 1853	MDM	NE, 1, 6, 24	AIIM	Time loop
APEC V-G	MDM	NE, 1, 6, 24	AIIM	Time loop

[a] Chicken macrophages were exposed to either *M. synoviae* type strain WVU 1853 or avian pathogenic *E. coli* strain V-G.
[b] Cell lines used were either HD11 chicken macrophage cell line or peripheral blood monocyte derived macrophages (MDM).
[c] Time points at which RNA samples were harvested, NE= non-exposed macrophages.
[d] RNA Samples were hybridized to either a 4906 element avian macrophage microaray (AMM) or to a 4959 element avian innate immunity microarray (AIIM) in a time loop or dye swap format (Bliss *et al.* 2005).

RNA preparation and microarray hybridization

Total RNAwas extracted from harvested cells using Qiagen RNeasy Mini/Midi kits (Qiagen Corp.) and 2 µg of total RNA from each sample was purified and amplified into amino allyl modified RNA(aRNA) using the Ambion Amino Allyl MessageAmp™ II aRNA

Amplification kit (Ambion Inc., Austin, TX). aRNA (15 µg) was fluorescently labelled with AlexaFluor 555 (Cy3) or AlexaFluor 647 (Cy5) (Life Technologies Inc.). Concentration and labelling efficiency of aRNA was determined spectrophotometrically. Fluorescently labelled aRNA was hybridized to either AMM or AIIM. Both cDNA arrays contain three sub-arrays of 4,906 (AMM) or 4,959 (AIIM) elements, giving 14,718 or 14,877 spots total, respectively. The AMM was designed for analysis of avian macrophage specific gene expression (Bliss *et al.*, 2005). The AIIM is an upgraded version of the AMM, with 53 new elements added, to include a more complete list of genes in signalling pathways related to innate immunity (Keeler *et al.*, 2007). Two colour microarray hybridizations (65 µl) were performed in ArrayBooster (Advalytix AG, Munich, Germany) hybridization chambers at 50 °C overnight. Samples were hybridized to AMM or AIIM array slides in a time loop or dye swap format as shown in Table 1. After hybridization, unbound dye was removed from slides as described by Bliss *et al.* (2005). Briefly, slides were rinsed in 0.5 X SSC, 0.01% SDS at room temperature and then washed for 15 min in 0.2 X SSC, 0.2% SDS at 50 °C, three times for 1 min in 0.2 X SSC at room temperature, and finally three times for 1 min in water at room temperature. Washed slides were scanned with an ArrayWoRx scanner (Applied Precision, Issaquah, WA) using Cy3 and Cy5 filters.

Microarray data acquisition, processing, and analysis

Microarray data was acquired, processed and analyzed as described by Bliss *et al.* (2005). Briefly, spot and background intensities were acquired using SoftWoRx tracker (Applied Precision) and data analysis was performed using GeneSpring GX 7.3 (Agilent Technologies, Inc., Santa Clara, CA). Background intensity was determined using the Gene-Spring cell method. Elements not represented by at least two replicate spots, due to low intensity versus background, dust or bubbles, were removed from further analysis, resulting in at least four replicates per slide

(two per dye used) for statistical analysis. On each slide, spot intensities were normalized to that slide's median background subtracted spot intensity.

The normalized values of each sample treated with Cy3 were compared to the normalized values of samples treated with Cy5. An arbitrary restriction was set at which more than 80% of elements should not exceed more than two-fold change between both dye treatments. Samples/slides showing less quality were excluded. Values for quality passing samples in both dye treatments were combined and then normalized to the control (0 h) channel value so that fold change from control could be determined. Relevant to the arbitrary restriction set at the dye bias quality control step, those elements exhibiting at least greater than two-fold change in signal intensity during at least one time point were analyzed by ANOVA using the Benjamini and Hochberg (1995) false discovery rate (FDR) < 0.05 to determine which biologically significant changes were also statistically significant. In compliance with the MIAME (Minimum Information About a Microarray Experiment) guidelines, supplemental data is available on a web-based database at http://www.aviangenomics.udel.edu and array data has been deposited at the NCBI GenBank gene expression omnibus (GEO) (to be submitted under GEO accession number GSE1794).

Quantitative real-time RT-PCR (qRT-PCR)

To confirm and validate gene expression changes quantitative real-time RT-PCR (qRT-PCR) was performed on seven genes: interleukin-1b (IL-1b) and interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), neutrophil cytosolic factor-1 (NCF1), peroxiredoxin (PRDX1), complement component C3 (C3) and cathepsin L (CTSL). qRT-PCR primers were designed using Primer Express v2.0 (Applied Biosystems, Foster City, CA) and the TIGR GgGI database (http://compbio.dfci.harvard.edu/tgi/gi/gggi/searching/reports.html) tentative consensus sequences (**Table 2**). qRT-PCR was performed with aliquots of RNA from the same samples that were used in the AIIM microarray analysis. RNA samples (3–10 µg) were treated with DNase-I

following the manufacturer's protocol (Life Technologies Inc.). Quantitative PCR was performed for each sample in triplicate on an ABI 7900HT Sequence Detection System (Applied Biosystems). The two-step amplification procedure utilizing 2 X SYBR Green Master Mix (Stratagene Corp., La Jolla, CA) was performed in a 50 µl reaction volume containing 300 nM of each primer and 200 pg of cDNA. Data were analyzed using SDS2.1 (Applied Biosystems). The relative quantities of amplified gene products were determined by comparing the results from stimulated samples to the mean data from control macrophage RNA samples using the $2^{-(\Delta\Delta C\,t)}$ method (Bliss *et al.*, 2005).

Table 3.2. Real-time quantitative RT-PCR primers

RNA target		Primer sequence	TC[c] and Genbank accession numbers
IL-1ß	F[a]	5'ATGACCAAACTGCTGCGGAG3'	TC126176[c]
	R[b]	5'GTCGCTGTCAGCAAAGTCCC3'	
IL-6	F	5'CAGGACGAGATGTGCAAGAAG3'	NM_204628
	R	5'CCCTCACGGTCTTCTCCATA3'	
iNOS	F	5'GCATTCTTATTGGCCCAGGA3'	TC99103[c]
	R	5'CATAGAGACGCTGCTGCCAG3'	
NCF1	F	5'TGGAACGACCTCTCCGAAAA3'	NM_00130709
	R	5'CAGTGCTTTATGGAACTCGTAAATG3'	
PRDX1	F	5'ACGATCTTCCTGTTGGCCG3'	XM_422437
	R	5'CTGTAAACTGGAAGGCCTGCA3'	
C3	F	5'TGGGAGACCTTCGGTATTAACC3'	NM_205405
	R	5'GGTATGCAAGTTGTTGGGTGTAAC3'	
CTSL	F	5'CCAGGGTCAGTGTGGCTCTT3'	XM_425038
	R	5'TCTGAAGTGCTGGCCTTCAA3'	

IL-1β, interleukin 1 beta; IL-6, interleukin 6, iNOS, inducible nitric oxide synthase, NCF1, nuclear cytosolic factor 1; PRDX1, peroxiredoxin 1; C3, complement component C3; CTSL, cathepsin L.

[a] Forward.
[b] Reverse.
[c] TIGR GgGI database (http://compbio.dfci.harvard.edu/tgi/gi/gggi/searching/reports.html) tentative consensus sequences.

Results

Initial analysis of microarray data

After exposure to either MS or APEC, chicken macrophages were subjected to initial gene expression analysis. The quality control steps and elements left after each quality control step are summarized in Table 3. Two cross-sections were made amongst the gathered data: (i) amongst the three experiments of exposure to MS analyzed with AMM/AIIM microarray and (ii) between the experiments of exposure to either MS or APEC which were analyzed with the AIIM. As seen from Table 3, 14 elements showed modulation after exposure to MS considering both macrophage types (MDM/HD11), both microarrays (AMM or AIIM) and all three experimental designs (Table 1). When comparing the MS and APEC AIIM time-loop analyses (Table 1), 146 elements were modulated after macrophage exposure to both pathogens, MS or APEC (**Table 3**).

Table 3.3. Initial analysis of gene expression after exposure of chicken macrophage cell line HD11 or chicken monocyte-derived macrophages (MDM) to either *M. synoviae* WVU 1853 or *E. coli* V-G

	MS WVU 1853 on HD11 (NE[a], 6, 12, 24 h)	MS WVU 1853 on MDM (NE[a], 6 h)	MS WVU 1853 on MDM (NE[a], 1, 6, 24 h)	APEC V-G on MDM (NE[a], 1, 6, 24 h)
Elements with two good replicate spots in all samples	3479	3812	4540	4204
Elements with >2-fold changes in expression	940	53	396	1928
Elements with statistically significant (FDR < 0.05) changes in expression	760	53	303	1749
Core set of elements modulated after exposure to MS WVU 1853[b]		14		
Core set of elements modulated after exposure to MS WVU 1853 or APEC[c]			146	

[a] NE, non-exposed macrophages.
[b] Number of elements that pass all three criteria, indicated above and were modulated after exposure to MS WVU 1853 in each experiment (cross-section of 760, 53 and 303 elements modulated by MS WVU 1853 in three experiments) irrespective of macrophage cells (HD11 or MDM).
[c] Number of elements that pass all three criteria, indicated above and were modulated in PBM irrespective of pathogen (MS WVU 1853 or APEC); cross-section of 303 elements modulated by MS WVU 1853 and 1749 elements modulated by APEC.

Chicken macrophage genes modulated by exposure to *M. synoviae*

As shown in Table 3, there were 14 chicken macrophage genetic elements shown to be significantly (FDR < 0.05) modulated consistently during all experimental models of macrophage exposure to MS. These 14 elements, representing actually 11 distinct genes, create a core set of genes used by both type of cells. Seven of those genes, IL-1b, iNOS, CXCLi1, CCL5, CCL4L1, avidin and IL-1 receptor type 2 are presented in **Fig. 1**. Last three genes (CCL4L1, avidin and IL-1 receptor type 2) were amongst the ‘‘top 10’’ induced genes in microarray analysis of two, MDM (non-exposed (NE), 1, 6, 24 h) and HD11 (NE, 6, 12, 24 h) exposure experiments, while failing to pass quality controls in the MDM (NE, 6 h) (Table 1) exposure experiment. Except for iNOS, other six genes, presented in Fig. 1, showed higher level of transcription in HD11 cell line than in MDM.

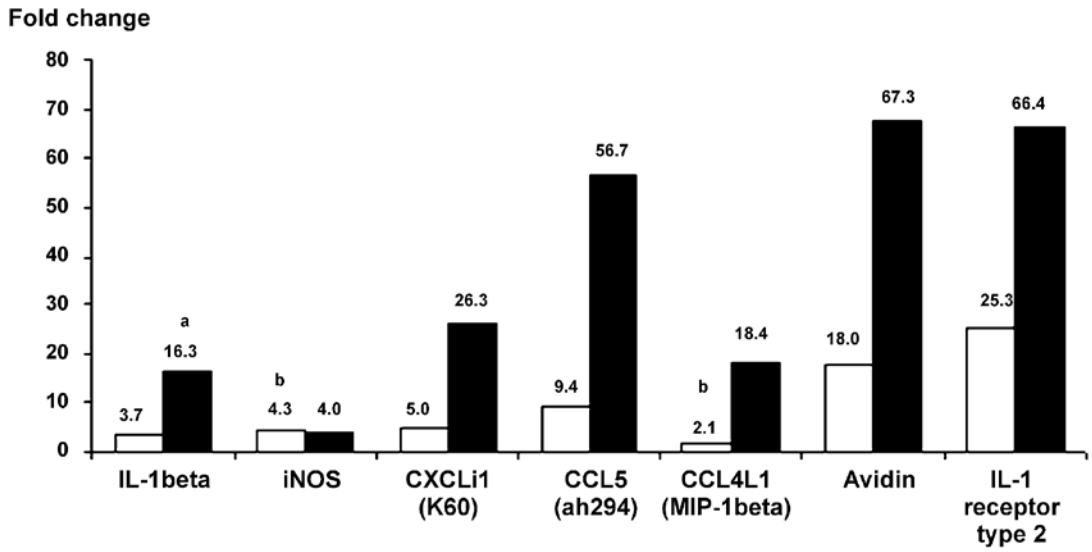

Figure 3.1. Microarray analysis of selected genes induced after exposure of chicken macrophage cell line HD11 or chicken monocyte-derived macrophages (MDM) to *Mycoplasma synoviae* WVU 1853. Maximum induction values of genes for IL-1b, iNOS, CXCLi1, CCL5, CCL4L1, avidin and IL-1 receptor type 2 after exposure to *M. synoviae* WVU 1853. White columns indicate the induction values of MDM genes, black columns indicate the induction values of HD11 genes. Unless noted otherwise, numbers indicate relative induction values after 24 h of exposure. Superscripts indicate: a, induction at 1 h after exposure; b, induction at 6 h after exposure.

A core set of macrophage genetic elements modulated by exposure to *M. synoviae* and *E. coli*

To compare immunomodulatory activity of MS and APEC, MDM were exposed to either MS or APEC for 24 h. In this AIIM microarray study, 303 elements were significantly (FDR < 0.05) modulated by MS and 1749 elements were significantly (FDR < 0.05) modulated by APEC (Table 3). A cross-section of both sets of modulated elements showed 146 elements which were modulated by both, MS and APEC (Table 3) and 157 elements uniquely modulated by MS and 1603 elements uniquely modulated by APEC. In the core set of 146 elements, 87 elements were induced, while 20 elements were repressed. Macrophage genes represented by

core set of elements were placed into relevant functional groups, and genes showing induction are presented in **Table 4**, while genes showing repression are presented in **Table 5**. Tables 4 and 5 do not list cases where multiple elements represent single genes nor do they list elements with no assigned function. The remaining 39 elements represented two subgroups: 7 elements which were induced by MS/repressed by APEC and 32 elements which were repressed by MS/induced by APEC. A complete list of all the commonly induced/repressed elements is found on http://www.aviangenomics.udel.edu.

Table 3.4. Functional groupings of elements commonly induced in chicken monocyte derived macrophages (MDM) exposed to *M. synoviae* WVU 1835 or *E. coli* V-G

Induced elements	Max. induction level		GenBank accession no.
	MS	APEC	
Anti-bacterial (lysozyme)	8.7 (6)[a]	13.3 (24)	XM_416896
Apoptosis (inhibitor of T-cell apoptosis)	3.5 (6)	5.6 (6)	U27466
Cell surface (CD83 antigen precursor)	3.7 (6)	6.0 (24)	XM_418929
Cytokines (interleukin-1β)	3.7 (1)	6.2 (24)	NM_204524
Chemokines			
CCL5 (ah294)	9.4 (24)	10.0 (24)	AY037859
CCLi7 (ah221)	7.7 (24)	4.0 (24)	XM_415781
CXCLi1 (K60)	2.8 (1)	5.8 (24)	AF277660
Enzymes			
Acyl-CoA synthetase	6.2 (24)	29.7 (24)	NM_001012578
Beta-hexosaminidase	3.1 (1)	3.4 (24)	XM_424791
Deoxyribonuclease I	3.1 (24)	2.1 (24)	NM_001037838
L-Amino acid oxidase	16.1 (24)	30.2 (24)	XM_415327
GalNAc kinase	3.4 (24)	2.9 (24)	NM_001030557
Glutamate-cysteine ligase	3.7 (24)	9.4 (24)	NM_001007953
GTP cyclohydrolase 1	2.8 (6)	2.5 (6)	NM_205223
L-Lactate dehydrogenase	4.8 (24)	4.8 (24)	NM_205284
N-Acetylglucosamine-6-sulfatase	2.2 (24)	2.3 (24)	XM_416070
Phosphoglycerate kinase 1	2.1 (24)	3.0 (24)	NM_204985
Delta 9 desaturase	2.2 (6)	6.8 (6)	NM_204890
Oxidative burst			
Cytochrome b-245 heavy chain	3.4 (24)	4.5 (24)	XM_416783
Inducible nitric oxide synthase	3.2 (24)	47.2 (24)	NM_204961
Peroxiredoxin 1	5.3 (24)	25.1 (24)	XM_422437
Receptors			
IL-13 receptor alpha 2	9.2 (6)	16.4 (24)	NM_001048078
Porimin	2.3 (24)	6.1 (24)	NM_001006279
Primary C-C chemokine receptor cluster	11.3 (24)	24.4 (24)	AJ627213
Toll-like receptor 1/6/10	6.2 (24)	18.3 (24)	NM_001037835
Signalling/trafficking/transcription/transport			
Na^+/K^+ ATPase	3.2 (24)	4.2 (24)	NM_205521
ATP-binding cassette transporter 1	6.5 (24)	5.9 (24)	NM_204145
Ch 21 protein	3.4 (24)	2.1 (24)	M80580
Interferon regulatory factor 1	2.3 (6)	2.7 (6)	NM_205415
Kruppel like factor 2	2.6 (24)	3.4 (24)	XM_418264
Rel-associated pp40	2.4 (1)	2.4 (6)	NM_001001472
Sorting nexin 10	4.8 (1)	10.8 (24)	NM_001030986
Tandem PH domain containing protein-1	10.9 (24)	11.6 (24)	XM_421799
Transcription factor BTF3	2.8 (24)	2.5 (24)	XM_423823
TAP2	2.8 (6)	3.0 (6)	AJ843262
Miscellaneous			
Avidin	18.0 (24)	12.6 (24)	NM_205320
IFIT 1	7.3 (6)	8.8 (24)	XM_421662
Ornithine decarboxylase antizyme 1	2.6 (24)	4.2 (24)	NM_204916

MS, *M. synoviae* WVU 1853; APEC, E. coli V-G.

[a] Maximal increase of gene transcription. The relevant time points (hours after exposure) are in parentheses.

Table 3.5. Functional groupings of elements commonly repressed in chicken monocyte-derived macrophages (MDM) exposed to *M. synoviae* WVU 1835 or *E. coli* V-G

Repressed elements	Max. repression level MS	Max. repression level APEC	Genebank Acc.No.
Anti-bacterial (ovotransferrin)	4.0 (6)[a]	7.5 (24)	AB222603
Enzymes (Glutathione S-transferase)	2.6 (6)	3.7 (1)	XM_416409
Signaling/Trafficking/Transcription/Transport (guanylate binding protein)	7.1 (1)	2.5 (24)	NM_204652
Miscellaneous (TIMP)	2.0 (24)	3.1 (24)	NM_205487

MS, *M. synoviae* WVU 1853; APEC, *E. coli* V-G.
[a] Maximal decrease of gene transcription. The relevant time points (hours after exposure) are in parentheses.

The exposure of chicken macrophages to *E. coli* induced higher transcription of cytokine genes and genes involved in oxidative burst than exposure to *M. synoviae*

Quantitative real-time PCR (qRT-PCR) was performed on seven selected genes listed in Table 2 in order to validate AIIM generated results (Fig. 2). qRT-PCR revealed increased transcription of IL-1b, IL-6 and iNOS in at least one time point after exposure toeither MS or APEC V-G, however higher levels of transcription occurred after exposure to APEC. While IL-1b and iNOS induction was observed by AIIM and qRT-PCR, increased transcription of IL-6 after exposure to either MS or APEC could only be observed in qRT-PCR. In the AIIM analysis of chicken macrophages exposed to APEC, NCF1 showed the highest induction of expression, 222.5-fold. The qRT-PCR analysis of NCF1 showed less dramatic increase in its transcription after exposure to MS and APEC (Fig. 2). In the case of CTSL and PRDX1, the expression patterns seen in AIIM analysis were similar to those in qRT-PCR (Fig. 2). qRT-PCR analysis of C3 gene expression, showed a five-fold increase in transcription after exposure to MS in comparison with no significant modulation shown by AIIM analysis. However, repression of

C3 after exposure to APEC, observed by AIIM analysis, was validated through repression (10-fold) shown by qRT-PCR.

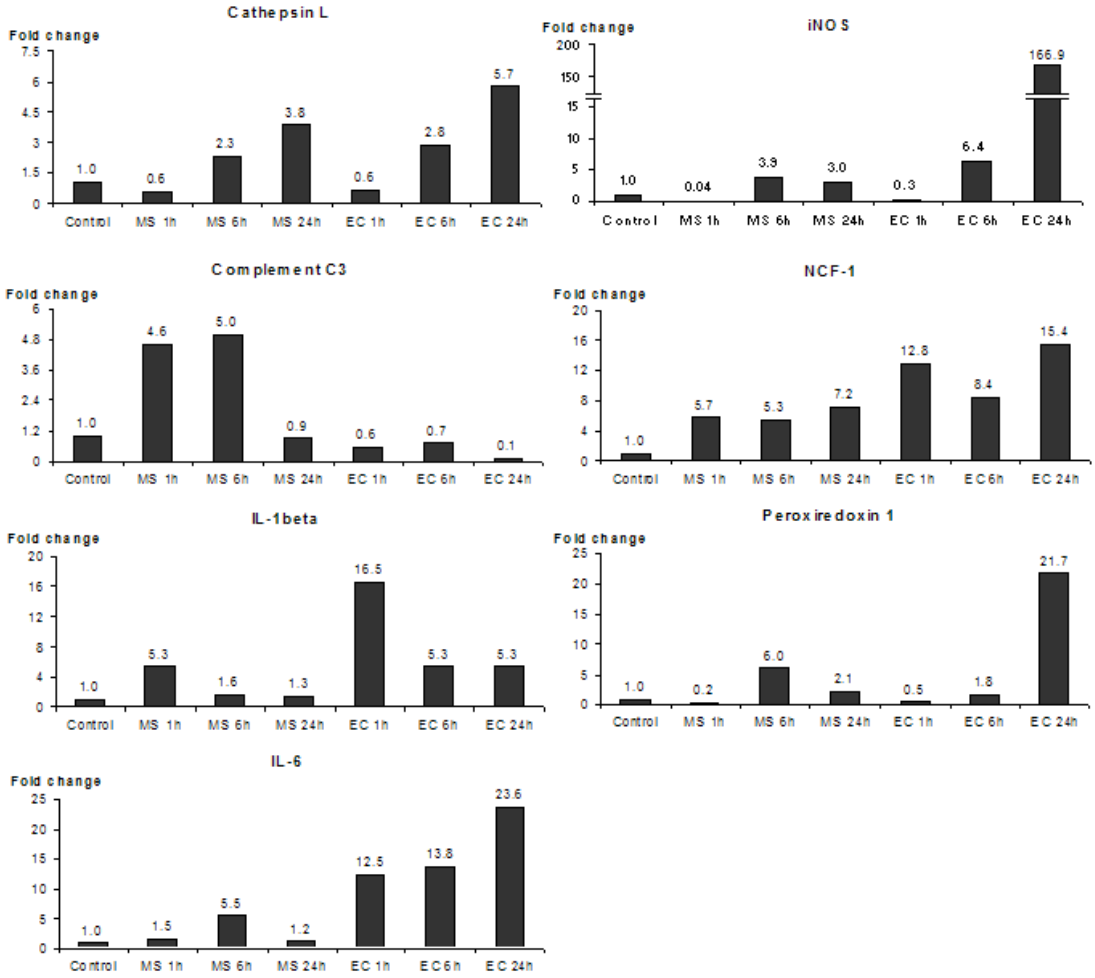

Figure 3.2. Quantitative real time reverse transcription PCR (qRT-PCR) of selected genes after exposure of chicken monocyte-derived macrophages (MDM) to *M. synoviae* WVU1853 or *Escherichia coli* V-G. The microarray expression patterns of seven genes: IL-1b, IL-6, iNOS,NCF1, PRDX1, C3 and CTSL, were evaluated by qRT-PCR as described. Relative macrophage gene induction values are indicated above the column, with the relevant pathogen (MS,APEC) and exposure time (in hours) indicated below the column. Control in all graphs is for non-exposed macrophages (0 h).

Discussion

Upon exposure to bacteria and other inducing agents, professional phagocytes, heterophils and macrophages, are the first immune cell responders. Therefore, we examined the initial response of the chicken immune system by using AMM and AIIM microarrays. Gene transcription changes in macrophages exposed to MS WVU 1853 or APEC V-G were evaluated. Amongst macrophage genes significantly induced after exposure to MS WVU 1853, we found (Fig. 1) and confirmed by qRT-PCR (Fig. 2) iNOS andIL-1b. The induction of the IL-6 gene by MS, shown in this study by qRT-PCR, is consistent with our previous observations that MS proteins, including MSPB, the lipoprotein subunit of MS haemagglutinin VlhA, induce secretion of IL-1b, IL-6 and nitric oxide in chicken macrophages (Lavric *et al.*, 2007). However, it seems that genes for IL-1b, IL-6 and iNOS are much more induced when macrophages are exposed to APEC than to MS, which might explain a more exacerbated inflammatory response during APEC infections than during *M. synoviae* infections (Barnes *et al.*, 2003; Kleven, 2003). Mycoplasmas do contain a large number of lipoproteins, which are strongly immunogenic like MSPB of *M. synoviae* (Narat *et al.*, 1998). E. coli cell surface contains a number of pathogen-associated molecular patterns (PAMP's), like LPS, flagellin and triacylated lipoproteins, each capable of interacting with its own Toll-like receptor (TLR) on the macrophage surface (Werling and Jungi, 2003; Rodriguez-Siek *et al.*, 2005). The observed induction of the proinflammatory cytokines IL-1b and IL-6 as well as iNOS can be explained by activation of the TLR signal transduction pathway, through either MS or APEC PAMP's (Iqbal *et al.*, 2005; Kogut *et al.*, 2005; He *et al.*, 2006). A higher induction of NCF1, PRDX1 and iNOS genes after exposure of macrophages to APEC compared to MS, suggests a more volatile oxidative burst response to an APEC infection, with more reactive oxygen and nitrogen species being formed, and consequently enhancing the inflammatory effect.

We also observed the induction of three chemokines, CXCLi1 (K60), CCL5 (ah294) and CCL4L1 (MIP-1b), in macrophages exposed to MS (Fig. 1). Interestingly, Lam (2002) showed in his report that supernatants of MG-infected monocytes, macrophages, heterophils and

thrombocytes were able to exert chemotactic activity towards heterophils and lymphocytes. Moreover, *M. gallisepticum*-infected HD11 cells revealed enhanced transcription of the IL-6 gene (Lam, 2004), as is seen in this study for MDM exposed to *M. synoviae* (Fig. 2). Indeed, in our previous studies *M. synoviae* proteins induced nitric oxide secretion in MQ-NCSU macrophage cell line, as well as in HD11 macrophages (Lavric *et al.*, 2007; unpublished data). Higher gene modulation in HD11 cells might be related to the viral transformation of this cell line. It is known that HD11 cells are hyper-responders to different ligands when compared to MDM cells. Although the different levels of gene modulation were observed for HD11 cells and for MDM, the trend of gene modulation was the same for both cell types.

Transcription of genes encoding complement component C3 and CTSL, were analyzed by qRT-PCR because of their induction patterns detected by AIIM. C3 plays a central role in the activation of the complement system. The proteolytic degradation product of complement component C3 is a mediator of the local inflammatory process, inducing smooth muscle contraction, increasing vascular permeability and causing histamine release from mast cells and basophilic leukocytes (de Bruijn and Fey, 1985). By AIIM analysis we were not able to see any significant change in transcription of the macrophage C3 gene after exposure to MS, we only observed the repression of C3 in macrophages exposed to APEC V-G. However, through qRT-PCR we were able to demonstrate induction of C3 gene after exposure to MS, as well as confirming repression after exposure to APEC.

Cathepsin L (Fig. 2), which is important for the overall degradation of proteins in lysosomes (Wada and Tanabe, 1986) and thereby contributing to pathogen killing in phagocytes, was induced after exposing macrophages to either MS or APEC, but higher levels were detected in exposure to APEC.

Although some chicken macrophage genes respond in a similar fashion when exposed to either MS or APEC, a set of differentially regulated core set of elements and a vast number of uniquely expressed genes suggest that macrophages are capable of distinguishing between these two pathogens by using similar as well as different signal transduction pathways.

Exposure of macrophages to MS caused a much milder reaction than exposure to APEC. A reasonable assumption would be that a lesser number of chicken macrophage signal transduction pathways were activated by MS than by APEC. We are aware that our results do not reflect the complex events occurring in tissues infected by MS or APEC, where not only macrophages, but a mixed population of cells is present. We are also well aware of microarray technology limitations, with some being based upon specific statistical assumptions. Nevertheless, our results revealed until now unidentified highly altered transcription of individual macrophage genes as a consequence of either MS or APEC infection. We think that this should provide a solid basis for more targeted future research.

Conclusions

This study describes a novel approach for detecting pathogen induced gene modulation in certain immunocompetent cells and a comparison of two unrelated avian pathogens in their immunomodulation capability. MS and APEC showed similarities and differences concerning global gene induction and/or repression in chicken macrophages. Certain genes become highly transcribed in order to effectively eliminate the pathogen, but we also detected some changes in macrophage gene modulation that might enable survival of the pathogen. We believe that our data could be a valuable contribution to intensive host–pathogen interaction studies.

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Chapter 4

INNATE IMMUNE RESPONSE OF DUCKS TO DIFFERENT SPECIES-OF-ORIGIN LOW PATHOGENICITY AVIAN INFLUENZA VIRUSES

Michele N Maughan[1], Lorna S Dougherty[1], Lauren A Preskenis[1], Brian S Ladman[1], Jack Gelb Jr.[1], Erica Spackman[2], and Calvin L Keeler Jr.[1]

[1] Department of Animal and Food Sciences, University of Delaware, Newark, DE, 19716, USA.

[2] Southeast Poultry Research Laboratory, USDA-ARS, 934 College Station Rd. Athens, GA, 30605, USA

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Abstract

Background: Wild waterfowl, including ducks, represent the classic reservoir for low pathogenicity avian influenza (LPAI) viruses and play a major role in the worldwide dissemination of AIV. AIVs belonging to the hemagglutinin (H) 7 subtype are of epidemiological and economic importance due to their potential to mutate into a highly pathogenic form of the virus. Thus far, however, relatively little work has been conducted on elucidating the host-pathogen interactions of ducks and H7 LPAIVs. In the current study, three H7 LPAIVs isolated from either chicken, duck, or turkey avian species were evaluated for their comparative effect on the transcriptional innate immune response of ducks.

Results: Three H7 LPAIV isolates, chicken-origin (A/chicken/Maryland/MinhMa/2004), duck-origin (A/pintail/Minnesota/423/1999), and turkey-origin (A/turkey/Virginia/SEP-67/2002) were used to infect Pekin ducks. At 3 days post-infection, RNA from spleen tissue was used for transcriptional analysis using the Avian Innate Immune Microarray and quantitative real-time RT-PCR. Microarray analysis revealed a core set of 61 genes differentially regulated in response to all three LPAIVs tested and 101, 135, and 628 differentially expressed genes unique to infection with the chicken-, duck-, or turkey-origin LPAIV isolates respectively. qRT-PCR results revealed significant ($p<0.05$) induction of IL-1β, IL-2, and IFNγ transcription, especially with the chicken-origin isolate. Several key innate immune pathways were activated in response to LPAIV infection including the toll-like receptor and RIG-I-like receptor pathways.

Conclusions: Pekin ducks elicite a unique innate immune response to different species-of-origin H7 LPAIV isolates, however, a subset of genes are differentially expressed regardless of isolate origin. This core set of genes expressed in response to H7 LPAIVs represent several innate immune pathways critical to the duck immune response to AI. These

data provide insight into the potential mechanisms employed by ducks to tolerate AI viral infection.

Introduction

As the natural reservoir for an epidemiologically and economically important pathogen, the study of host pathogen interactions between ducks and avian influenza virus (AIV) is vital to an understanding of the global transmission of avian influenza (AI). The two pathotypes of AI – low pathogenicity (LP) and high pathogenicity (HP) are classified based on their pathogenicity in chickens and the amino acid sequence at the hemagglutinin cleavage site [1]. Of particular interest are the H5 and H7 subtypes of AIV, the two hemagglutinin subtypes that have historically mutated from the LP to HP forms [2].

The experiments described herein were part of a larger study published by Spackman *et al.* [3] in which the pathogenesis of 12 North American H7 LPAIV isolates were evaluated in three avian species: specific pathogen free (SPF) white leghorn chickens (*Gallus gallus domesticus*), broad breasted white turkeys (*Meleagris galopova*) and Pekin ducks (*Anas platyrhynchos domesticus*). The Spackman *et al.* [3] study concluded that the severity of disease and the degree of virus shed relied on specific combinations of species and isolates. Additionally, they concluded that turkeys may be more susceptible to clinical disease from the H7 LPAI than either chickens or ducks.

This *in vivo* experiment provides new insight into the transcriptional response of ducks to AIV. Our 4,959 element avian innate immunity microarray (AIIM) has been successfully used to evaluate the transcriptomic response of several avian species to various microbial challenges, including ducks and avian influenza [4]. In the present study, we utilized the AIIM to characterize the global host immune response of ducks to three H7 low

pathogenicity avian influenza (LPAIV) isolates. The aim of this study was to evaluate the consequences of H7 LPAIV infection in ducks with viruses isolated from chickens, ducks, and turkeys.

To elucidate the host mechanisms employed in response to LPAIV infection, we evaluated gene expression changes of the natural host (ducks) to different isolates of LPAIV. We hypothesized that the species-of-origin of an isolate would induce different gene expression patterns related to the innate immune response in Pekin ducks. Gene expression in response to LPAIV infection has been studied in duck: peripheral blood mononuclear cells (PBMC) [5], lung cell cultures [6], intestine [7], and lung, spleen, and lymphatic tissues [8]. In support of the growing research interest in the duck transcriptional immune response, Crowley *et al.* [9] performed a proof-of-concept microarray study of Pekin ducks infected with high pathogenicity avian influenza virus (HPAIV) H5N1 (A/MuscovyDuck/Vietnam/453/2004).

Adams *et al.* studied the effects of an H11N9 LPAIV on duck PBMC [5]. In their studies, they noted consistent up-regulation of interleukin 6 (IL6), interferon-alpha (IFNA), interferon gamma (IFNG), and interleukin 2 (IL2) at 8, 24, and 36 hours post-infection (hpi), minimal gene expression changes in toll-like receptor 7 and MHC I and II gene expression (<3.0 fold), and down-regulation of interleukin 1-beta (IL1B). The authors concluded that the cytokine responses demonstrate a skew towards a weak Th1 response in duck PBMC and the absence of signs of disease in ducks correlated with low pro-inflammatory cytokine levels. Additionally, Adams *et al.*, concluded that, in comparison to the chicken response to LPAIV, the lower overall expression of IFNs by duck PBMC in response to AIV infection results in a longer viral shedding duration (persistence) and weaker viral clearance.

Fleming-Capua *et al.* 2011 [8] studied the duck splenic immune response to LPAIV (A/mallard/BC/500/05 (H5N2)) and observed no gene expression changes in cytokines important

in the signaling and extravasation of dendritic cells and naïve lymphocytes to secondary lymphoid tissues (CCL19 and CCL21). This finding led the authors to conclude that ducks experience a weakened adaptive immune response to LPAIV versus HPAIV. Our study compares immune related gene expression of ducks infected with different species-of-origin LPAIV isolates.

Materials and Methods

Viruses

Three H7 LPAI viruses were selected to represent different species of origin (Table 1). Viruses were propagated and titrated in 9 to 11 day-old embryonated chicken eggs by standard procedures [30]. The chicken-origin isolate (A/chicken/Maryland/MinhMa/2004) was described in 2004 by Ladman *et al.* [31] during an outbreak in 6-wk-old commercial broilers on the Minh Ma Farm in Wicomico County, Maryland. The duck-origin isolate (A/pintail/Minnesota/423/1999) was described in 2005 by Spackman *et al.* [32] during an evaluation of North American AIV natural reservoirs (free-flying waterfowl). The turkey-origin isolate (A/turkey/Virginia/SEP-67/2002) was described in 2002 by Spackman *et al.* [33] during a commercial turkey farm outbreak in Virginia, West Virginia, and North Carolina.

Table 4.1. Low pathogenicity avian influenza virus isolates evaluated for pathogenesis in Pekin ducks.

Isolate	Subtype	Source	Abbreviation
A/chicken/Maryland/MinhMa/2004	H7N2	Broiler chickens*	CK/MD/MinhMa
A/pintail/Minnesota/423/1999	H7N3	Wild Pintail ducks	PT/MN/423
A/turkey/Virginia/SEP-67/2002	H7N2	Meat-type turkeys*	TK/VA/67

*Live bird market (LBM) lineage

Animals

Pekin ducks (*Anas platyrhynchos domesticus*) were obtained from commercial hatcheries at day of age and were housed in negative pressure glove-port isolators (Allentown Caging, Allentown, NJ) under biosafety level 3 containment conditions in the Charles C. Allen Biotechnology Laboratory at the University of Delaware. Ducks were obtained from flocks with no antibody or prior exposure to AI virus. The ducks were provided with *ad libitum* access to feed and water before and after exposure to the viruses. Ducks were cared for in accordance with established humane procedures and University of Delaware biosecurity guidelines.

Evaluation of Viral Pathogenicity in Pekin Ducks

Fifteen Pekin ducks were separated into four treatment groups: Group 1 – Non-infected controls, Group 2 - CK/MD/MinhMa inoculated, Group 3 - PT/MN/423 inoculated, and Group 4 - TK/VA/67 inoculated. At 2 weeks of age, each duck was inoculated with 10^6 EID_{50} per bird in 0.1 ml by the intrachoanal (cleft palate) route. Birds were monitored daily for clinical disease signs which were scored as follows: 0 = no clinical signs, 1 = mild depression, 2 = moderate to severe (i.e. depressed, not eating, neurological signs), 3 = dead. Oral-pharyngeal (OP) and cloacal (CL) swabs were collected at 2, 4, 7, 10 and 14 days post-inoculation (d.p.i.) to

evaluate virus shed by quantitative real-time RT-PCR (qRT-PCR) [3]. Three d.p.i., 3 birds from each treatment group were euthanized and necropsied to evaluate gross lesions and collect spleens. One hundred mg of spleen tissue was collected from each bird, and stored in 5-10 volumes of RNAlater at -80°C for RNA isolation and subsequent microarray and qRT-PCR analysis.

RNA Isolation

Spleen samples from each of the three birds selected for necropsy were pooled according to treatment group. Total cellular RNA was isolated from 100 mg of spleen tissue using the RNeasy Midi RNA Purification Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols. The optional DNaseI on-column digestion step was employed to remove any trace or contaminating duck genomic DNA from the samples. RNA quantity was determined using a Nanodrop 1000 (Nanodrop, Wilmington, DE), and RNA quality was assessed using the Agilent RNA 6000 Nano Assay Protocol in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA Integrity Numbers (RINs) were obtained for each sample to confirm sample quality.

RNA Amplification, Fluorescent Labeling, and Hybridization

One μg of total cellular RNA from each treatment group pool was amplified into amino allyl modified RNA (aRNA) using the Ambion Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion Inc., Austin, TX) using two rounds of amplification and following the manufacturer's instructions. Ten ug of aRNA mixed with 9 μL of coupling buffer was fluorescently labeled with Alexa Fluor 555 (Invitrogen, Carlsbad, CA) and resuspended in 11 μL of DMSO. The labeling reaction was performed at room temperature for 3 hours in the dark. Post-labeling aRNA purification, post-hybridization washes, and microarray slide scanning were

performed as previously described [4] and hybridization to the AIIM was conducted at 42°C overnight.

Microarray Data Analysis

Spot and background intensities were acquired using GenePix Pro 4.1 Software (Molecular Devices, Sunnyvale, CA). Abnormal spots (dust, bubbles in the hybridization solution) were removed from further analysis. Spot intensity was determined using a local background subtraction method. Data from analyzed slides was imported to GeneSpring v7.3 (Agilent Technologies, Santa Clara, CA). Each experimental slide was compared to the control slide (non-infected duck spleen) to determine relative spot intensities, and differential gene expression. A gene list was created from those elements that appeared in two of the three replicate spot locations in each slide, in all three experimental conditions (i.e. infections with either the chicken-, duck-, or turkey- species-of-origin LPAI isolates). Subsets of this gene list consisting of two-fold differentially regulated genes from each infected treatment group were exported for further pathway and gene ontology (GO) analysis. Lists of differentially expressed (DE) genes were created using GeneSpring v7.3. The corresponding Entrez Gene IDs were imported to AgBase v2.0 GORetriever to obtain GO IDs [34]. The GORetriever GO ID output was then analyzed in The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [35, 36]. Functional annotation and gene functional annotation analyses were performed using DAVID, which provided batch annotation and GO term enrichment analysis to highlight the most relevant GO terms associated with the input gene list. Further DAVID analysis yielded the significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways represented in the data set.

Quantitative real-time RT-PCR (qRT-PCR)

To further investigate the splenic innate immune response, qRT-PCR targeting select duck immune genes was performed on the splenic RNA samples (Table 2). Primer sequences were kindly provided by Dr. Darrell Kapczynski (DK, personal communication) and Dr. Carol Cardona as referenced. qRT-PCR was performed with aliquots of RNA from the same samples that were used in the AIIM microarray analysis. Gene expression levels of mRNA transcripts were determined by qRT-PCR using a QuantiTect SYBR Green RT-PCR kit (Qiagen). qRT-PCR was performed for each sample in triplicate on an ABI 7900HT Sequence Detection System (Life Technologies Corp., Carlsbad, CA). The amplification procedure was performed in a 20 µL reaction volume containing 300 nM of each primer and 100 ng of RNA. The following thermal-cycling conditions were used: reverse transcription (30 min at 50°C), PCR initial activation (15 min at 95°C), and 40 cycles of denaturation (15 sec at 94°C), annealing (30 sec at 55°C), and extension (30 sec at 72°C). Data were analyzed using SDS2.3 (Life Technologies Corp.).

Table 4.2. Real-time quantitative RT-PCR primers.

RNA Target	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Genbank Accession #	Ref.
IFNA	GACAGCCAACGCCAAAGC	AATGCTTGAGCAGCAGCGAC	EF053034	(1)
IFNB	CCTCAACCAGATCCAGCATT	GGATGAGGCTGTGAGAGGAG	AY831397	(1)
IFNG	CAACGCTCAACTACTCTC	TGTGGTTAATCTGTCCTTAG	AJ012254	DK
IL1B	TCGACATCAACCAGAAGTGC	GAGCTTGTAGCCCTTGATGC	DQ393268	(1)
IL2	GCCAAGAGCTGACCAACTTC	ATCGCCCACACTAAGAGCAT	AF294323	(1)
IL6	TTCGACGAGGAGAAATGCTT	CCTTATCGTCGTTGCCAGAT	AB191038	(1)
MHCI	GAAGGAAGAGACTTCATTGCCTTGG	CTCTCCTCTCCAGTACGTCCTTCC	AB115246	(1)
MHCII	CCACCTTTACCAGCTTCGAG	CCGTTCTTCATCCAGGTGAT	AY905539	(1)
TLR7	CCTTTCCCAGAGAGCATTCA	TCAAGAAATATCAAGATAATCACATCA	AY940195	(1)
GAPDH	ATGTTCGTGATGGGTGTGAA	CTGTCTTCGTGTGTGGCTGT	AY436595	(1)

qRT-PCR Data and Statistical Analysis

Average cycle threshold (Ct) values for each target gene were normalized by the Ct value of an endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative gene expression data were analyzed using the Livak and Schmittgen $2^{-\Delta\Delta Ct}$ method [37] and ΔCt values were calculated by subtracting average GAPDH Ct values from average target gene Ct values. Normalized Ct values (ΔCt) from LPAIV infected samples was compared to the ΔCt from non-infected control duck spleen samples, the difference (ΔΔCt) being transformed into $2^{-\Delta\Delta Ct}$ value as the estimated fold change of the experimental sample (infected) over the control (non-infected) sample. The three replicate Ct values for each gene were analyzed by one-way ANOVA ($p<0.05$) to determine the statistical significance between means of individual

genes. A post-hoc statistical test, Tukey-Kramer minimum significant differences (MSD), was utilized to analyze the differences amongst means of genes grouped by LPAIV isolate ($p<0.05$).

Results

Pathogenesis of LPAIV in Pekin Ducks

Clinical disease signs, depression, anorexia, neurological signs, and death, were not observed in Pekin ducks infected with any of the three LPAIV isolates from days 2 through 14 days post-infection (d.p.i.). Three days after infection with LPAIV, three birds from each treatment group were sampled for detection of gross lesions. Gross lesions were observed in infected ducks, specifically in the nasal cavity, trachea, and pulmonary and renal systems, though there were no statistically significant differences in gross lesions among the LPAIV isolates.

Viral Shed

Absolute quantification qRT-PCR was performed by Spackman *et al.* [3] in order to quantify the amount of virus genomic material (AIV matrix gene) present in the OP and CL swabs and determine viral shed and relative viral titers. The duration of viral shedding was used to determine viral persistence, that is, how long each virus isolate was maintained within the sampled areas (oral-pharyngeal or cloacal). The three LPAIV isolates in this experiment demonstrated different virus recovery and persistence characteristics. As shown in Figure 1a, the duck-origin LPAIV (PT/MN/423/99) virus had the highest recovery in the OP swabs throughout the experiment, while the chicken-origin (CK/MD/MinhMa) and turkey-origin (TK/VA/67) viruses did not display significantly different virus shedding (except on day 10). Significant differences ($p<0.05$) among the virus isolates were observed in persistence and recovery when examining CL swabs, as shown in Figure 1b. There was both greater recovery and longer persistence of the duck-origin LPAIV (PT/MN/423/99) with virus being recovered throughout

the 14 day time course, when compared to the chicken- and turkey- origin viruses, in which virus recovery was only demonstrated on 2 and 7 d.p.i..

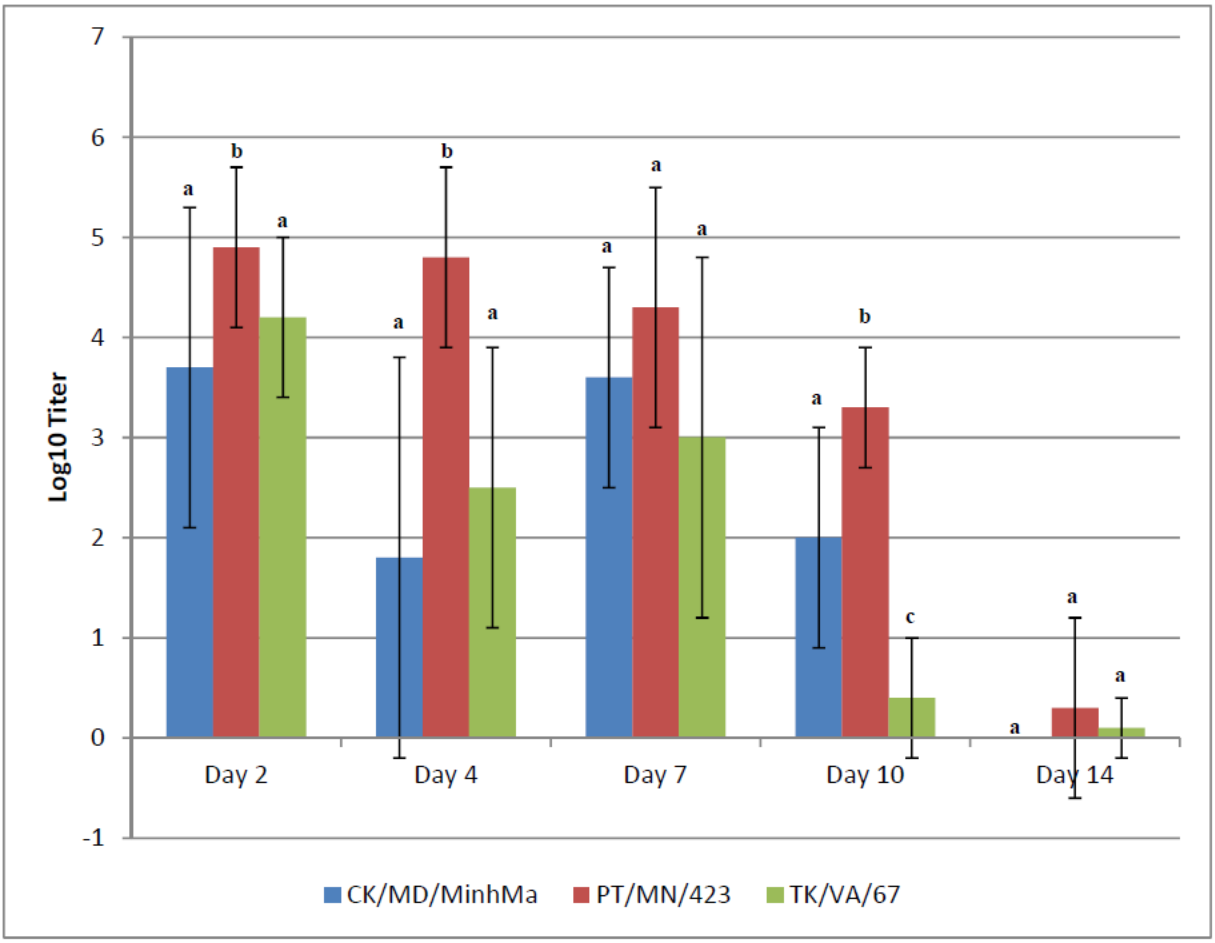

Figure 4.1a. Mean oral-pharyngeal (OP) virus titers from Pekin ducks. OP virus titers by day post-infection as determined by quantitative real-time RT-PCR for the influenza M gene. Average qRT-PCR titers expressed as exponents (e.g. a titer value of 4.2 is 104.2). Error bars indicate standard error of titers. Different superscript letters represent statistically significant differences ($p<0.05$) among titers within a given day.

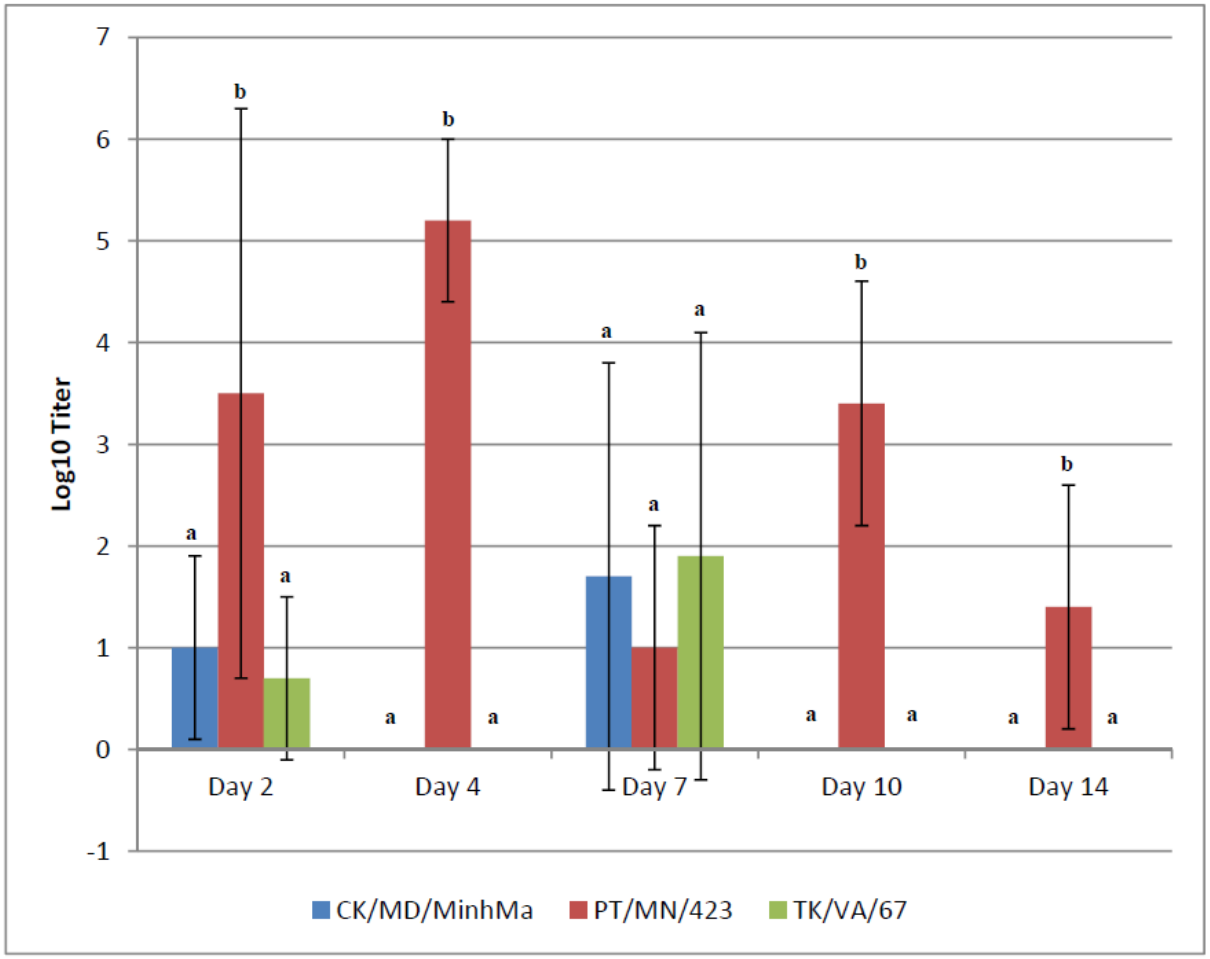

Figure 4.1b. Mean cloacal (CL) virus titers from Pekin ducks. CL virus titers by day post-infection as determined by quantitative real-time RT-PCR for the influenza M gene. Average qRT-PCR titers expressed as exponents (e.g. a titer value of 4.2 is 104.2). Error bars indicate standard error of titers. Different superscript letters represent statistically significant differences ($p<0.05$) among titers within a given day.

Modulation of Gene Expression in Ducks by LPAIVs

At 3 d.p.i. with the selected H7 LPAIVs, duck spleen was harvested and RNA was extracted for use in a microarray experiment utilizing the AIIM. To characterize the transcriptional immune response to LPAIV, we analyzed all of the two-fold differentially regulated genes in each of the three LPAIV infections to find genes unique to a specific species-of-origin isolate, common to all isolates, and the various permutations thereof. Combining all three LPAIV-infected treatment groups, there was more down-regulation (1198) than up-regulation (559) of duck splenic genes. There were 101, 135 and 628 2-fold differentially expressed genes unique to infection with the chicken-, duck-, and turkey-origin LPAIV isolates respectively (Figure 2). The number of elements that were up- or down-regulated in response to infection with the chicken-origin virus (CK/MD/MinhMa) was approximately evenly distributed between up- and down-regulated genes (108 and 133, respectively). Additionally, infection with CK/MD/MinhMa yielded the smallest number of differentially expressed genes (241/1757, or 14% of the differentially expressed genes). The number of elements that were down-regulated (352) in response to infection with the duck-origin virus (PT/MN/423) was greater than the number of up-regulated elements (142). The proportion of differentially expressed genes responding to the duck-origin virus (PT/MN/423) was 28% (494/1,757). Finally, the greatest number of differentially expressed genes (1,022) were observed in response to infection with the turkey-origin virus (TK/VA/67), comprising 58% of all differentially expressed genes. Furthermore, 70% (712/1,022) of these differentially expressed genes were down-regulated, and only 30% were up-regulated (310).

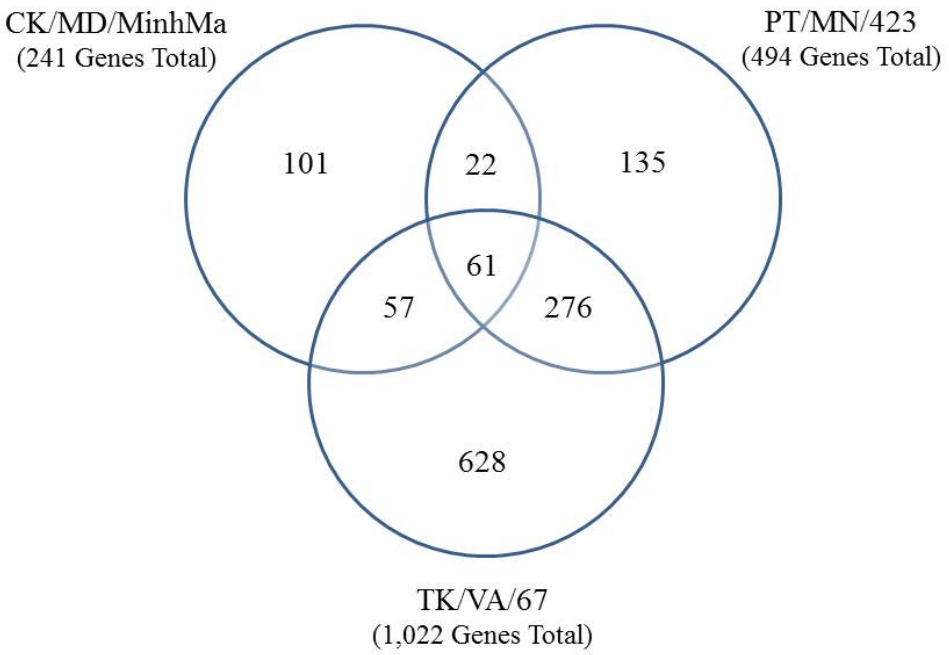

Figure 4.2. Genes displaying a two-fold change in expression in response to infection with LPAIV isolates derived from chickens, ducks, and turkeys. The universe is all genes that were detected in two of three replicates on each AIIM slide in each of the experimental condition slides (3,697 genes total). 1757 genes are differentially regulated (up- or down-regulated) at least 2 fold over the pooled control samples.

Gene List and Gene Ontology Analysis

Gene Ontology (GO) analysis was conducted in order to examine overall trends in the microarray data, and the subset of differentially expressed genes common to all three LPAIV infections. To identify the biological pathways activated in response to LPAIV infection, we submitted a total of 1,757 Entrez Gene IDs (1,198 2-fold down- and 559 2-fold up-regulated) to GORetriever to obtain GO IDs. GORetriever output was then analyzed in DAVID's functional annotation tools. Out of the 1,757 genes, 621 genes had DAVID IDs and 10 statistically

significant ($p<0.05$) canonical signaling pathways found in the Kyoto Encyclopedia of Genes and Genomes (KEGG). Functional analyses of the GO terms associated with these gene lists revealed differences in KEGG pathways that were either stimulated or repressed in response to LPAIV infection (Table 3).

To analyze the commonality of the innate immune response amongst all three LPAIV infections, we compared DE (2 fold up- or down-regulated) genes from each infection and identified the union of these gene lists. Sixty-one genes were differentially expressed in response to all three LPAIV infections (Figure 2), indicating that ducks differentially regulated the same 61 genes regardless of the H7 LPAIV avian-origin isolate. Due to the current completeness of annotation of the chicken genome and the mammalian-bias in functional annotation software, of the 61 DE genes, our bioinformatics pipeline identified 13 genes for functional annotation.

Functional analyses of the GO terms analyzed in DAVID are summarized in Table 4. AIIM data confirms a consistent, amongst all three species-of-origin LPAIV isolate infections, down-regulation of JUN (jun oncogene) and PMM2 (phosphomannomutase 2). JUN is a key regulator of several innate immune pathways and PMM2 functions in several metabolic pathways. The 13 genes were categorized according to their representation in one or more canonical KEGG pathways. Of the 13 genes, 69% (9/13) belong to innate immune pathways (highlighted in Table 4), illustrating an unsurprising enrichment of genes involved in the immune response to avian influenza. Evidence exists for an association between influenza infection and the subsequent differential regulation of several genes in our list, such as cadherin 1 [10], ATPase [11], mago-nashi homolog [12], proteasome 26S subunit [13], and ribosomal protein L35a [14].

Table 4.3. Functional Gene Ontology Annotation using DAVID. The gene list containing the 559 up-regulated and 1198 down-regulated differentially expressed genes in duck spleen common to all three LPAIV infections at 3 dpi was entered into the DAVID functional annotation software. The following KEGG pathways are enriched in our dataset. The percentage column indicates percentage of DE genes that mapped to the DAVID database with a corresponding significance value ($p<0.05$).

Up-Regulated			**Down-Regulated**		
Pathway	**%**	**P value**	**Pathway**	**%**	**P value**
gga04142:Lysosome	3.32	0.0155	gga04520:Adherens junction	2.97	0.0000
gga00190:Oxidative phosphorylation	3.32	0.0420	gga04060:Cytokine-cytokine receptor interaction	2.97	0.0456
gga04620:Toll-like receptor signaling pathway	2.90	0.0213	gga03010:Ribosome	2.75	0.0004
gga04621:NOD-like receptor signaling pathway	2.07	0.0259	gga04514:Cell adhesion molecules (CAMs)	2.54	0.0153
			gga04142:Lysosome	2.33	0.0300
			gga04350:TGF-beta signaling pathway	1.91	0.0483

Table 4.4. Functional Gene Ontology Annotation using DAVID. The gene list containing the 61 differentially expressed genes in duck spleen common to all three LPAIV infections at 3 d.p.i. was entered into the DAVID functional annotation software. The following genes and their cognate KEGG pathways are enriched in our dataset. Influenza-associated genes are highlighted in green.

Gene Name	KEGG Pathway
ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c	gga00190:Oxidative phosphorylation, gga04142:Lysosome
F-box protein 4	gga04120:Ubiquitin mediated proteolysis
cadherin 1, type 1, E-cadherin (epithelial)	gga04514:Cell adhesion molecules (CAMs), gga04520:Adherens junction
erbb2 interacting protein	gga04621:NOD-like receptor signaling pathway
etoposide induced 2.4 mRNA	gga04115:p53 signaling pathway
interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	gga04060:Cytokine-cytokine receptor interaction, gga04620:Toll-like receptor signaling pathway, gga04622:RIG-I-like receptor signaling pathway, gga04630:Jak-STAT signaling pathway
jun oncogene	gga04010:MAPK signaling pathway, gga04012:ErbB signaling pathway, gga04310:Wnt signaling pathway, gga04510:Focal adhesion, gga04620:Toll-like receptor signaling pathway, gga04912:GnRH signaling pathway
mago-nashi homolog, proliferation-associated (Drosophila)	gga03040:Spliceosome
phosphomannomutase 2	gga00051:Fructose and mannose metabolism, gga00520:Amino sugar and nucleotide sugar metabolism
proteasome (prosome, macropain) 26S subunit, non-ATPase, 7 (Mov34 homolog)	gga03050:Proteasome
ribosomal protein L35a	gga03010:Ribosome
secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	gga04510:Focal adhesion, gga04512:ECM-receptor interaction, gga04620:Toll-like receptor signaling pathway
thioredoxin reductase 1	gga00240:Pyrimidine metabolism

Gene Expression Modulation by LPAIVs (qRT-PCR)

Since the AIIM is a chicken-transcriptome-based microarray used in a cross-species hybridization experiment and a more qualitative than quantitative tool, qRT-PCR using primers derived from duck-specific gene sequences was performed. Select publicly available duck immune gene sequences were analyzed using qRT-PCR to obtain quantitative levels of gene expression of interferon-α (IFNA), interferon-β (IFNB), interferon-γ (IFNG), interleukin-1β (IL1B), interleukin-2 (IL2), interleukin-6 (IL6), major histocompatibility complex class I (MHCI), major histocompatibility complex class II (MHCII), and toll-like receptor 7 (TLR7). These genes were selected for their known role in the response to AIV and their function in innate immunity.

Figure 3 illustrates the changes in gene expression of the interleukins, MHCs, and TLR7. IL2 demonstrated the greatest level of gene expression induction in response to all three LPAIV infections, especially during infection with CK/MD/MinhMa (19.7 fold up-regulation). IL2 was up-regulated by 7.8 and 9.1 fold for the PT/MN/423 and TK/VA/67 infections respectively. IL1B gene expression was up-regulated in response to infection with all three LPAIV isolates as well, with the greatest gene expression changes in the CK/MD/MinhMa infection at 8.2 fold. Minimal gene expression changes (<2.5 fold up-regulated) were observed for IL6, MHCI, MHCII, and TLR7.

Gene expression changes in the interferon genes are illustrated in Figure 4. The results for IFNA were not statistically significant at $p<0.05$, however, the results for IFNB and IFNG were statistically significant and demonstrated a 4.3 fold increase in IFNB expression in ducks infected with the turkey-origin LPAIV isolate (TK/VA/67). Large up-regulation of IFNG was seen in ducks infected with the chicken-origin isolate (8.9 fold) and in ducks infected with the turkey-origin isolate (7.1 fold).

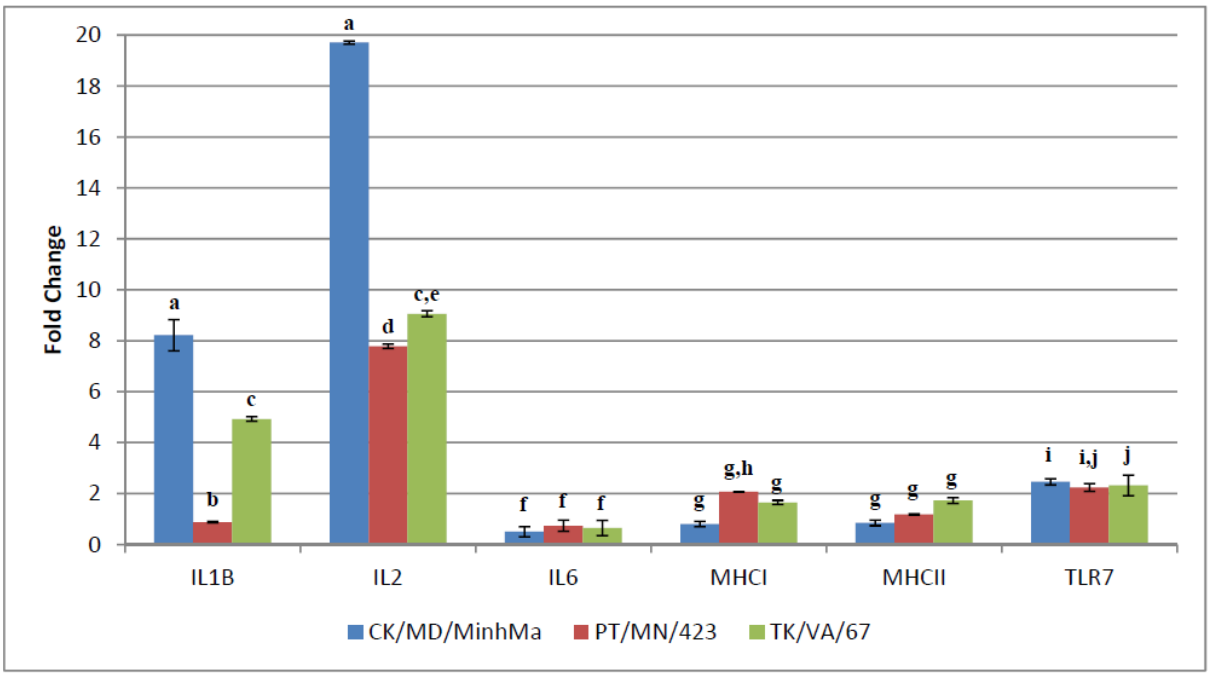

Figure 4.3. Expression of selected cytokines and immune genes in response to infection with CK/MD/MinhMa (chicken-origin), PT/MN/423 (duck-origin), or TK/VA/67 (turkey-origin) LPAIV isolates. qRT-PCR relative quantification results are represented as fold-change of the infected 3 d.p.i. duck spleens over the time-matched control (non-infected) duck spleen. Error bars represent standard error of the mean. Means with different letters are significantly different (Tukey-Kramer MSD, $p<0.05$).

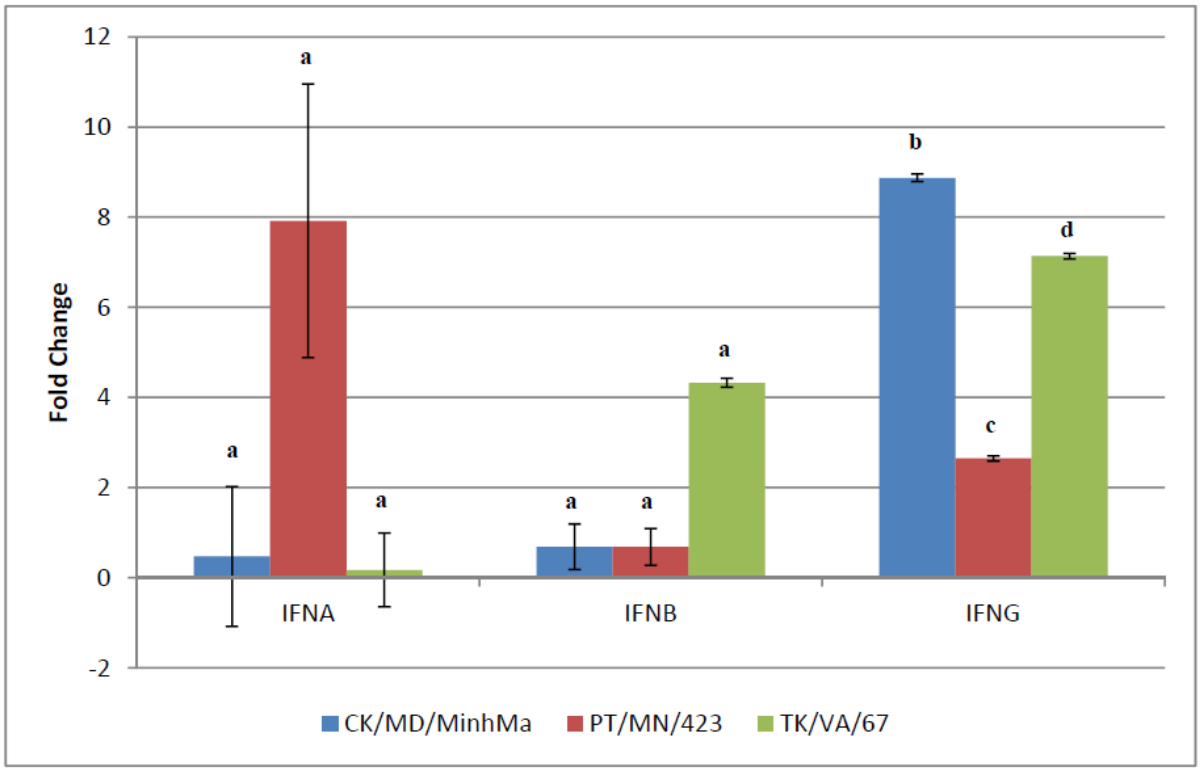

Figure 4.4. Expression of interferon genes in response to infection with CK/MD/MinhMa (chicken-origin), PT/MN/423 (duck-origin), or TK/VA/67 (turkey-origin) LPAIV isolates. qRT-PCR relative quantification results are represented as fold-change of the infected 3 d.p.i. duck spleens over the time-matched control (non-infected) duck spleen. Error bars represent standard error of the mean. Means with different letters are significantly different (Tukey-Kramer MSD, $p<0.05$).

Discussion

In the current study, we aimed to characterize the pathogenomic host response of ducks to different species-of-origin low pathogenicity avian influenza isolates. Spackman *et al.* [3] evaluated the comparative pathogenesis of twelve isolates of H7 LPAIV on chickens, ducks, and turkeys. Specifically, they assessed pathogenesis by measuring clinical signs, viral replication titers, immunohistochemistry, and seroconversion. These methods provided insight into the pathogenesis of H7 LPAIV isolates, revealing that turkeys may be more susceptible to

clinical disease than chickens or ducks, and that disease severity and the degree of virus shed was dependent on specific species and isolate combinations. To build upon the Spackman *et al.* study and investigate the molecular mechanisms of innate immunity in Pekin ducks, we utilized microarrays and qRT-PCR in order to qualify and quantify gene expression changes in response to LPAIV. Three H7 LPAI viruses were evaluated for their effect on the transcriptional activity of the duck spleen at 3 d.p.i.. Pekin ducks were infected with H7 LPAI viruses isolated from chickens, ducks, or turkeys, representing three different species-of-origin influenza isolates.

Based on the pathobiology of LPAI viruses and as previously reported, ducks exhibited no clinical signs in response to LPAIV infection [3, 15]. With respect to the pathology of the H7 LPAIV isolates used in this study, the highest AIV qRT-PCR titers were observed in both the OP and CL swabs of ducks in response to the duck-origin virus (PT/MN/423). OP titers were highest at 2 d.p.i. ($10^{4.9}$ EID_{50}), while cloacal titers peaked at 4 d.p.i. ($10^{5.2}$ EID_{50}). Both OP and CL titers remained positive through 14 d.p.i.. This finding demonstrates the adaptation of PT/MN/423 to the duck host. Given the absence of clinical signs and limited observance of gross and microscopic lesions, AIV replication is indicative of active AI infection and demonstrates that ducks are managing viral pathogenesis in ways other than decreasing viral replication, suggesting they use alternate strategies to prevent disease signs.

In order to evaluate gene expression changes caused by the different species-of-origin LPAIV isolates, we utilized our avian innate immune microarray (AIIM) to characterize the transcriptomic response of ducks to LPAIV. By hybridizing RNA from infected duck spleens to our 4,959 element microarray, we were able to survey the transcriptional profiles of a critical immune organ during LPAIV infection. In general, more genes were down-regulated (1197) than up-regulated (558) (Figure 2). One hypothesis for this finding is that perhaps ducks, as asymptomatic carriers and the natural reservoir for AIV, tolerate infection due in part to down-regulation of their immune system in order to remain subclinical. The overall down-

regulation of immunity-related genes observed in our microarray data adds to a possible mechanistic explanation of how ducks tolerate AIV infection. In fact, disease tolerance is now being considered a distinct host defense strategy, employed by a wide variety of species [16]. Perhaps ducks are able to fine-tune their innate immune response, differentially regulating the TLR, NOD-like receptor (NLR), cytokine-cytokine receptor interaction, and TGF-beta signaling pathways (Table 3), bypassing the negative consequences associated with AIV infection. Regardless of the origin of the LPAIV isolate, infected ducks induce both the TLR and NLR pathways, while they repress cytokine-cytokine receptor interaction pathways. Immune regulation represents one probable mechanism ducks consistently use to tolerate LPAIV infections.

AIIM data revealed two genes, JUN and PMM2, of the core set of 61 differentially expressed genes that were consistently down-regulated and found to be the most highly repressed genes (data not shown). JUN is a cellular component of the activating protein 1 (AP-1) transcription factor complex and is also a key regulator of the mitogen activated protein kinase (MAPK), influenza A, and Toll-like receptor signaling pathways [17]. JUN has also been shown to play role in both the negative and positive regulation of viral transcription according to the curated gene expression studies in the NextBio database (Santa Clara, CA). Recently, JUN has been demonstrated to be differentially regulated, and specifically down-regulated during LPAIV infection, in a gene expression study of avian influenza infected lung cell lines [18]. A plausible role for the down-regulation of JUN could be the host's manipulation of its own transcriptional machinery in order to prevent tissue damage or unchecked influenza virus replication. Another gene exhibiting consistent down-regulation is phosphomannomutase 2 (PMM2), a gene found in metabolic pathways such as amino sugar and nucleotide sugar metabolism (gga00520) and fructose and mannose metabolism (gga00051) [17]. Interestingly, differential regulation of PMM2 has been associated with virus infection of chicken embryo fibroblast cell cultures, thus providing an additional line of evidence supporting PMM2 down-regulation in our study [19].

The cellular pathways activated in response to LPAIV infection in ducks confirmed an innate immune response at the transcriptional level (Table 4). Activation of the nucleotide oligomerization domain (NOD), TLR, and retinoic acid inducible gene-I (RIG-I) pathways is noteworthy, as these are primary signaling pathways in the innate immune response to AIV. Specifically, NLR signaling regulates inflammation and apoptotic cascades, while TLR signaling activates the NFKB, MAPK, and type I interferon pathways [20]. RIG-I has recently gained attention due to the fact that it is absent in chickens and present in ducks, providing a potential explanation for the differential immune responses and susceptibility between these two birds [21]. These pathways intersect at critical signaling molecules and also trigger other immune pathways (apoptosis, lymphocyte recruitment, proteolysis, MAPK signaling) and the production of interferons, cytokines, and chemokines [22], pathways and proteins critical in combating influenza infection.

An emphasis on the innate immune response of the ducks to AIV is warranted given that the strength of the innate response largely determines the strength of the subsequent adaptive immune response [23]. Additionally, it has been demonstrated that ducks lack a substantial humoral immune response to AIV [23-25], inferring an increased reliance on innate immune mechanisms. Furthermore, a robust innate immune response has been correlated to increased mean death time and decreased morbidity in Pekin ducks in response to HPAIV challenge [26].

Our qRT-PCR findings provide insight into possible host defense mechanisms in LPAIV-infected ducks. There were some overall similarities between this study and the results described by Adams *et al.* [5] with respect to disease pathogenesis and the cytokine responses of ducks to LPAIV despite the fact that our studies used different tissues (PBMC versus spleen), time points (8, 12, 36 hpi versus 72 hpi), and LPAIV subtypes (H11N9 versus H7N2 or H7N3). Specifically, the lack of clinical signs and low-level expression of IL6 is supported by the Adams

et al. [5] study and human studies in which a positive correlation between the severity of clinical signs and IL6 plasma levels was demonstrated [27]. It was interesting to note the up-regulation of the pro-inflammatory cytokine, interleukin 2 (IL2) (Figure 3). IL2 has been implicated in the protective role of the mouse host against lethal influenza virus challenge [28] and is highly expressed in duck embryonic fibroblasts in response to HP H5N1 avian influenza infection [29]. Inferences regarding a type II interferon response can also be made since the type II interferon (IFNG) was up-regulated in response to the chicken- and turkey-origin LPAIV isolates (8.9 and 7.1 fold, respectively) **(**Figure 4). IFNG up-regulation has been demonstrated in duck PBMC in response to a duck-origin H11N9 LPAIV infection [5]. Taken together, these results point to a type II-mediated IFN response that ducks utilize to combat LPAIV infections caused by isolates that are not duck-origin. Modulation of these critical innate immune genes provides further evidence of duck immune system fine-tuning of the innate immune response to different isolates of H7 LPAIV.

Conclusions

In conclusion, we have identified several immune pathways that are activated in response to LPAIV infection of ducks. While many of these pathways have been previously associated with influenza virus infection, this study identified new cellular pathways associated LPAIV infected ducks, such as the fructose and mannose metabolism (gga00051) and amino sugar and nucleotide sugar metabolism (gga00520) pathways. Additionally, we have gained further insight into the differences and similarities among innate immune responses based on the avian species from which the LPAIV was isolated. A core set of 61 genes was differentially expressed during all three LPAIV infections while 101, 135, and 628 genes were uniquely differentially expressed in response to the chicken-, duck-, and turkey-origin isolates respectively, indicating the importance of host-adaptation of LPAIV on transcriptional immune responses. Further studies will be required to elucidate the virus and host mechanisms

controlling gene expression during infection and to understand what factors contribute to the differential host immune response.

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Chapter 5

PRE-TREATMENT WITH TOLL-LIKE RECEPTOR AGONISTS AUGMENTS THE TRANCSRIPTIONAL INNATE IMMUNE RESPONSE AND INCREASES SURVIVAL TIME AFTER CHALLENGE WITH A HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS

Michele N. Maughan[1], Cynthia M. Boettger[1], and Calvin L. Keeler Jr.[1]

[1] Department of Animal and Food Sciences, University of Delaware, Newark, DE, 19716, USA.

Introduction

Avian influenza (AI) is caused by a type A influenza virus with a wide host range. AIV primarily infects birds, however some strains have been shown to infect horses, mink, cats, dogs, ferrets, stone martens, palm civets, and marine mammals [1]. The natural reservoirs for AIV are wild waterfowl and shore birds. According to the World Organisation for Animal Health (OIE), the world is currently experiencing the most extensive highly pathogenic avian influenza virus (HPAIV) outbreak ever recorded [1]. These recent AIV outbreaks have been caused by the H5 subtype, which are of particular interest as H5 and H7 are the two hemagglutinin subtypes that have historically mutated from the low pathogenicity (LP) to high pathogenicity (HP) forms [2]. HPAIV strains are unique in that the amino acid sequence at the hemagglutinin gene cleavages site is polybasic (RXR/KR) making them sensitive to ubiquitous host proteases, leading to virus replication in multiple host organs and eventually severe disease and mortality [3]. Given the mutability and zoonotic potential of AIV, it is a major concern for global public health.

The innate immune response to AIV is an area of active investigation. The innate immune response is particularly important because the rapid onset of morbidity and mortality associated with HPAIV infections does not lend itself to a proper adaptive immune response. Toll-like receptors (TLR) are an evolutionarily conserved group of pathogen recognition receptors that are critical sensors of pathogen-associated molecular patterns. TLR signaling pathways are critical to the innate immune response and the subsequent formation of adaptive immunity. There are 10 confirmed chicken TLRs (1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15, 21) [4]. Of relevance to AIV infection, are TLR3 and TLR7, which recognize exogenous double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) respectively [5]. Due to recent findings that pre-stimulation of innate immunity via TLRs has been shown to confer resistance against lethal

influenza infection in mice [6], we hypothesized that administration of TLR agonists would stimulate the innate immunity of chickens and improve the outcome of HPAIV infection.

In mammals, the therapeutic application of TLR agonists has been widely reviewed and several candidate therapeutics are currently being evaluated [7-11]. The *in vivo* application of TLR agonists to alter the immune system and combat infection in the avian system is less well studied, however there are a few reports suggesting the potential of this approach [12-14]. Immunomodulation strategies have been employed to decrease morbidity and mortality associated with influenza infections, however, there two reports that utilize the *in ovo* or *vivo* avian model [12, 15, 16]. Recently, Stewart *et al.* 2011were the first to implement the TLR agonist strategy using the avian model [12]. Stewart *et al.* demonstrated the effects elicited by TLR7 agonist, 7-allyl-8-oxoguanosine (loxoribine) which inhibited influenza A replication *in vitro* (HD11 cells) and *in ovo* in a dose-dependent manner. Loxoribine also stimulated transcription of eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2, a.k.a.PKR), myxovirus (influenza virus) resistance 1 (MX1), interferon-alpha (IFNA), interferon-beta (IFNB), and interferon-gamma (IFNG), all factors associated with establishing an antiviral innate immune response [12]. St. Paul *et al.* 2012 [16], evaluated whether TLR ligands could be used prophylactically in chickens to enhance host immunity to LPAIV. Chickens received intramuscular injections of either low or high doses of TLR3, TLR4, and TLR21 ligands and 24 hours post-treatment, chickens were infected with the LPAIV strain A/Duck/Czech/56 (H4N6). St. Paul *et al.* found that all of the TLR ligand pre-treatments induced a significant reduction in virus shedding, with the TLR3 ligand (poly I:C) conferring the greatest immunity to LPAIV compared to control birds and transcriptional analysis of gene expression in the spleen and lungs suggested IFNA and interleukin-8 (IL8) as correlates of immunity [16].

The present study was designed to investigate the ability of a TLR agonist cocktail to pre-stimulate mucosal and splenic innate immunity in chickens subsequently subjected to

HPAIV challenge. At the tissue/cellular level, the goal was to abrogate host-mediated inflammation in order to better prepare the target organ for virus infection and regulate, in a coordinated fashion, an appropriate inflammatory response. In studying host-pathogen interactions, the outcome of influenza virus infection is determined by both host and viral factors. In an effort to elucidate the host mechanisms employed to combat influenza virus infection, we investigated a therapeutic strategy that targets the host innate immune system. From our study we conclude that altering the host immune response using TLR agonists before infection with HPAIV can have beneficial effects as measured by increased survival time.

Materials and Methods

Chickens

Fertile specific-pathogen-free (SPF) white leghorn chicken eggs (Sunrise Farms, Inc., Catskill, NY) were hatched and housed at the Charles C. Allen Laboratory, University of Delaware, Newark, Delaware. Poultry were maintained under BSL-3 containment in negative-pressure glove-port isolation units with dip tanks. Birds were fed commercial diets and water *ad libitum* and were cared for in accordance with established humane procedures and University of Delaware biosecurity guidelines.

Virus

A highly pathogenic strain (A/Chicken/Pennsylvania/1370/1983 (H5N2)) of AIV was obtained from the United States Department of Agriculture (USDA) National Veterinary Services Laboratory (NVSL) in Ames, Iowa. Virus was cultivated in 9- to 11-day old embryonated chicken eggs to prepare seed stocks. Virus stocks were titrated in 9- to 11-day old SPF chicken embryos inoculated via the chorioallantoic sac. Stock virus titers were determined by the median egg infectious dose (EID_{50}) [17].

TLR Agonists and Pre-Treatment of Chickens

The following TLR agonists (Invivogen; San Diego, California USA) were combined to create a cocktail administered to the appropriate birds 36h pre-challenge: TLR3 agonist, poly I:C (Cat. No. tlrl-pic), a synthetic dsRNA analog (1.25 mg/kg), TLR4 agonist, LPS (Cat. No. tlrl-eklps), the lipopolysaccharide portion of E. coli K12 bacterial cell wall (2.5 mg/kg), and TLR7 agonist, loxoribine (Cat. No. tlrl-lox), a guanosine analog (2.5 mg/kg). Birds were inoculated intranasally with 100 µL volume of the TLR agonist cocktail resuspended in a mix of sterile water and DMSO.

Infections

Chickens were divided into four treatment groups, containing 18 animals per group: control birds that were not challenged with HPAIV nor pre-treated with the TLR agonists (NC); TLR agonist pre-treated birds (TLR); TLR agonist pre-treated birds and 36 hours later, HPAIV challenged (TLR+HPAIV); birds challenged with HPAIV but not pre-treated (HPAIV). At 2 weeks of age, birds in the HPAIV-challenged groups were inoculated via the intrachoanal route with $10^{4.9}$ EID_{50} of HPAIV. Birds were observed daily for clinical signs associated with HPAIV. At 0, 12, 24, and 48 hours post-infection (hpi) birds were weighed, rectal body temperatures were measured, and oral-pharyngeal (OP) swabbings were performed. Additionally, at each time point, two birds from the TLR+HPAIV and HPAIV treatment groups were humanely euthanized and spleen and lung tissues were harvested for histopathology and RNA extraction.

Histopathology

Lung and spleen specimens collected for histopathology were placed in 10% neutral buffered formalin. Fixed tissues were processed routinely, sectioned into 3–5-mm sections, and stained with hematoxylin and eosin.

RNA Isolation and qRT-PCR for Host Gene Expression Analysis

Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Valencia, CA), employing an on-column DNase-treatment. RNA was quantified using a UV-VIS spectrophotometer (Nanodrop, Wilmington, DE) and RNA quality was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). Quantitative real-time RT-PCR (qRT-PCR) primers were either designed using Primer3 [18] or from the published literature as noted in Table 1. Gene expression levels of mRNA transcripts were determined by qRT-PCR using a QuantiTect SYBR Green RT-PCR kit (Qiagen). qRT-PCR was performed for each sample in triplicate on an ABI 7900HT Sequence Detection System (Life Technologies Corp., Carlsbad, CA). The amplification procedure was performed in a 20 µL reaction volume containing 300 nM of each primer and 200 ng of cDNA. Data were analyzed using SDS2.4 (Life Technologies Corp.).

The average of each individual sample (run in triplicate), presented as a critical threshold cycle (C_T) value was used for data analysis. The C_T values of target genes were normalized by the C_T value of an internal control gene (GAPDH). Normalized C_T value (ΔC_T) from TLR pre-stimulated group was compared to the ΔC_T from the untreated group, the difference ($\Delta\Delta C_T$) being transformed into a $2^{-\Delta\Delta CT}$ value as the estimated fold change of the TLR pre-stimulation effect. Relative gene expressions were represented by fold change at each time point post-infection. Effects of TLR agonist pre-treatment were analyzed by ANOVA and differences between groups of genes and timepoints were determined by Tukey-Kramer minimum significant differences (MSD) ($p < 0.05$). Error bars were used to display standard error of the mean.

Table 5.1. Real-time quantitative RT-PCR primers. The source of the primer sequences are referenced and † indicates that the primers were developed using Primer3 [18].

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	GenBank Accession #	Ref.
TLR3	GCAACACTTCATTGAATAGCCTTGAT	GCCAAACAGATTTCCAATTGCATGT	DQ780341	[19]
TLR4	AGCTCCTGCAGGGTATTCAAGTGT	TGCACAGGACAGAACATCTCTGGA	AY064697	[20]
TLR7	GCACACCGGAAAATGGTACAT	AGCATTGGAAATAAGAAGAGCAAGA	AJ720504	†
MYD88	AAGTTGGGCCACGACTACCT	CTGCTGCTTCCTTCGTAAGT	AJ851640	[20]
TICAM1 (TRIF)	TCAGCCATTCTCCGTCCTCTTC	GGTCAGCAGAAGGATAAGGAAAGC	EF025853	[21]
IRF3	CGTATCTTCCGCATCCCTTGG	TCGTCGTTGCACTTGGAGCG	U20338	[21]
TRAF6	CGCCCTTGGAAAGTAAATACG	CATGGCGTCTGCACTGCTT	XM_421089	[21]
NFKB	GAAGGAATCGTACCGGGAACA	CTCAGAGGGCCTTGTGACAGTAA	NM_205134	[20]
SOCS3	GCCCCAGGTGATGGTGTA	CTTAGAGCTGAACGTCTTGAGG	AF424806	[22]
IFNA	CCAGCACCTCGAGCAAT	GGC GCT GTA ATC GTT GTC T	AB021154	[20]
IFNB	CCTCAACCAGATCCAGCATT	GGATGAGGCTGTGAGAGGAG	AY974089	[23]
IFNG	GTGAAGAAGGTGAAAGATATATCATGG	GCTTTGCGCTGGATTCTCA	Y07922	[24]
IL1B	ATGACCAAACTGCTGCGGAG	GTCGCTGTCAGCAAAGTCCC	Y15006	[23]
IL6	CAGGACGAGATGTGCAAGAAG	CCCTCACGGTCTTCTCCATA	AJ309540	[23]
NOS2	GCATTCTTATTGGCCCAGGA	CATAGAGACGCTGCTGCCAG	NM_204961	[23]
OASL	CACGGCCTCTTCTACGACA	TGG GCC ATA CGG TGT AGA CT	AB037592	[25]
TNFA-like	TGCTGTTCTATGACCGCC	CTTTCAGAGCATCAACGCA	AY765397	[21]
MX1	GTTTCGGACATGGGGAGTAA	GCATACGATTTCTTCAACTTTGG	Z23168	[26]
GAPDH	CCTCTCTGGCAAAGTCCAAG	CATCTGCCCATTTGATGTTG	AF047874	[23]

RNA Isolation and qRT-PCR for HPAIV Replication Analysis

OP swabs were obtained from individual birds using rayon tipped plastic shafted swabs at 0, 12, 24, 48, 72, 96 hpi. Individual swabs were placed in separate tubes containing 1.5 ml of brain heart infusion (BHI) broth supplemented with antibiotics (10,000 mg/ml

streptomycin and 10,000 international units/ml penicillin). RNA was extracted from OP swabs using the MagMAX 96 AI/ND Viral RNA isolation kit (Ambion, Inc., Austin, TX) with the KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA) according to the manufacturer's protocols. qRT-PCR was conducted with a primer-probe set that targeted the matrix gene as described previously [27] using the AB 7500 FAST (Applied Biosystems, Foster City, CA) instrument and the AgPathID (Ambion) one-step RT-PCR kit according to the manufacturer's protocols. Standard curves for virus quantification were established with RNA extracted from dilutions of the same titrated stock of the virus being evaluated.

Results and Discussion

Chickens tolerate TLR agonist administration without deleterious effects

TLR agonist pre-treatment did not cause any clinical signs, change in body weight (data not shown), or change in body temperature 36 hours post-treatment. The normal body temperature of SPF leghorn chickens has been reported for 4-week-old birds (39.9°C to 41.4°C) [28] and 42°C in adult white leghorn hens [29]. Control and TLR agonist pre-treated birds both had an average body temperature of 42.08°C (107.74°F) and maintained steady body temperatures of 42.01°C ±0.872 (107.61°F) and 42.04°C ±0.938 (107.68°F), respectively throughout the experimental time course (Figure 1). The HPAIV challenged birds in the TLR+HPAIV (43.3 °C) and HPAIV (43.1 °C) treatment groups both responded with a rise in body temperature at 48 hpi, followed by a steep drop in temperature thereafter or until death.

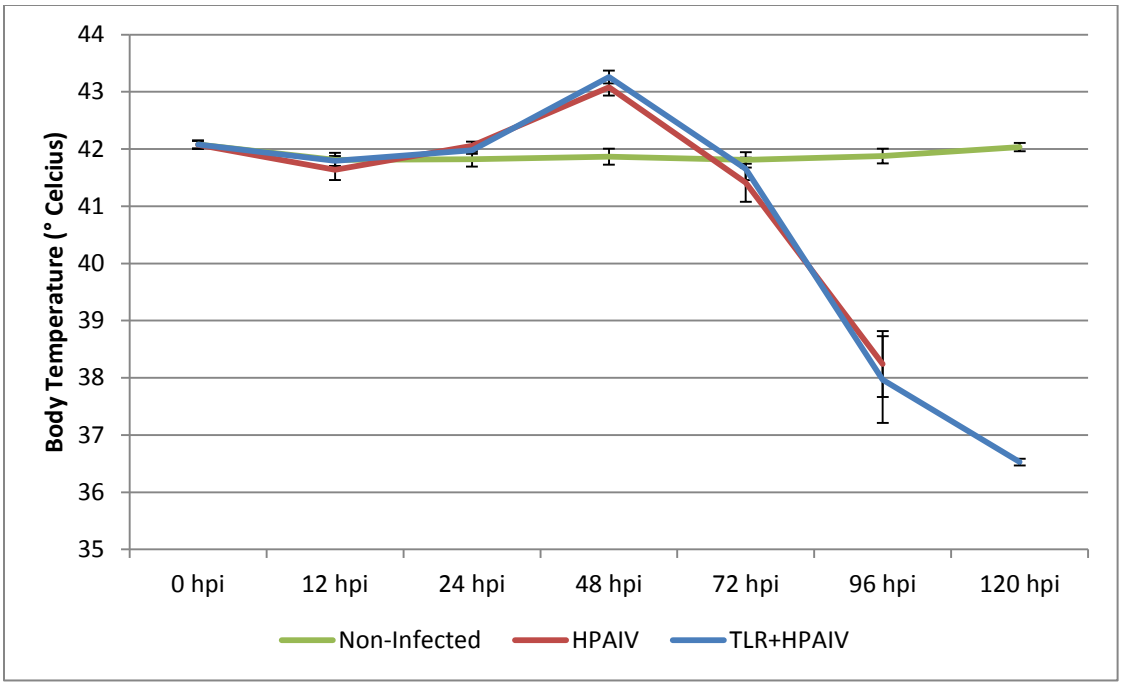

Figure 5.1. Mean Body Temperature Measurements. All treatment groups (NC, TLR, TLR+HPAIV, and HPAIV) consisted of 18 birds each. Rectal body temperature of each bird was recorded at the times indicated. Error bars represent standard error of the mean.

TLR agonist cocktail pre-treatment increased survival time in chickens challenged with HPAIV

A TLR agonist cocktail was developed specifically for the TLRs that respond to infection with an RNA virus (TLR3 and TLR 7) and a highly immunostimulatory agonist for TLR4 which was recently demonstrated to protect mice from HPAIV challenge [6]. To investigate the potential of TLR agonists to abrogate the morbidity and mortality associated with HPAIV infection, SPF chickens were inoculated intranasally with a TLR agonist cocktail consisting of polyI:C (TLR3 agonist), LPS (TLR4 agonist), and loxoribine (TLR7 agonist) 36 hours before challenge with HPAIV isolate A/Chicken/Pennsylvania/1370/1983 (H5N2). By 168 hpi, all HPAIV infected birds succumbed to infection, becoming febrile, lethargic, and

depressed, and showing signs of inappetance, swelling of the head and neck region, cyanotic (blue color) and necrotic combs and wattles, subcutaneous hemorrhage of leg shanks, and ruffled feathers. However, TLR agonist pre-treated chickens (TLR+HPAIV) survived lethal influenza virus challenge with HPAIV 14% longer than untreated chickens with a mean death time of 112 hpi vs. 97.37 hpi, $p < 0.01$ (Figure 2).

Upon necropsy, the following gross lesions were observed: coagulative necrosis, vascular thrombosis/ischemia, edemic and cyanotic combs and wattles, petechial hemorrhages on internal membrane surfaces, and diffuse hemorrhage and edema of internal organ surfaces and pleura. Cause of death was attributed to excessive inflammatory infiltrates, acute respiratory distress, and multi-system organ failure related to HPAIV infection. These finding are in accordance with previous studies that identified factors implicated in the high morbidity and mortality from influenza virus infection; These include robust cytokine production (hypercytokinemia), excessive inflammatory infiltrates, and virus-induced tissue destruction [30].

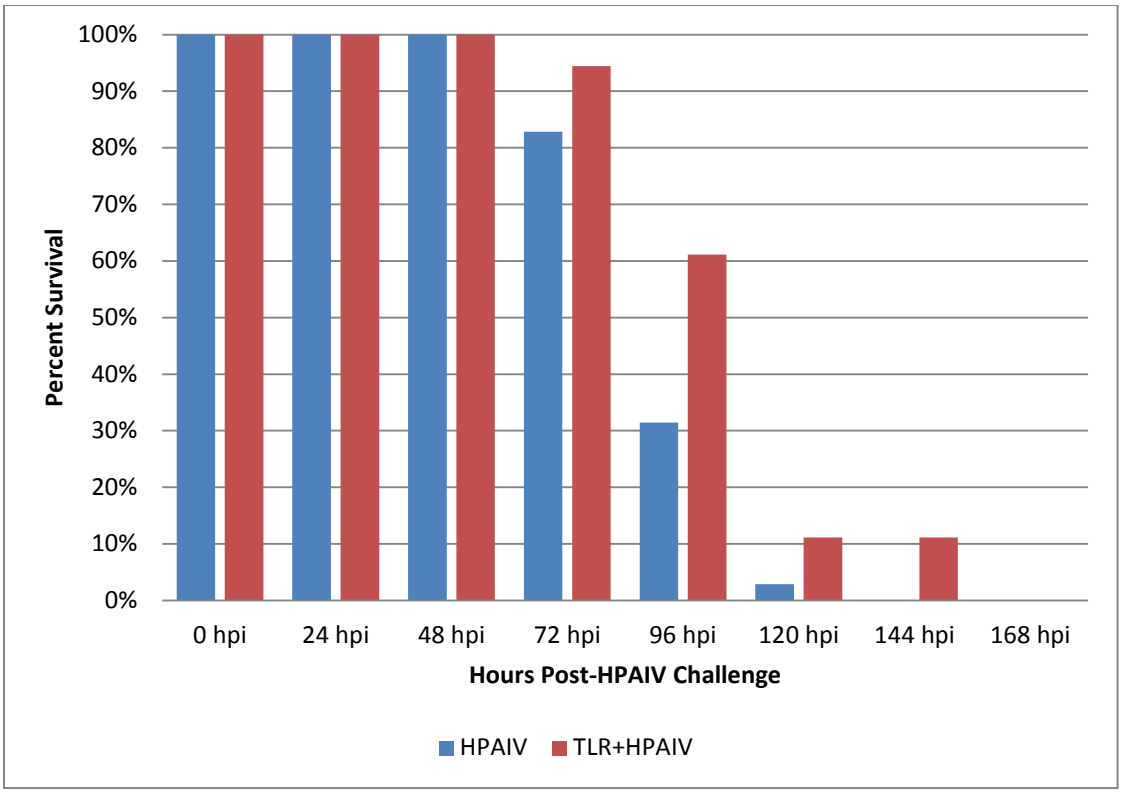

Figure 5.2. Survival of Birds Challenged with HPAIV or Birds Pre-Treated with a TLR3/4/7 Agonist Cocktail and then Challenged with HPAIV. Treatment groups (TLR agonist cocktail pre-stimulated and subsequently HPAIV challenged (TLR+HPAIV), and only HPAIV challenged (HPAIV)) consisted of 18 birds each.

Oral-pharyngeal HPAIV titers increase during the early time points of infection

At 12, 24, 48, 72, and 96 hpi, oral-pharyngeal swabs were collected from each surviving bird in each treatment group (NC, HPAIV, and TLR+HAPIV) and the amount of viral nucleic acid from the influenza matrix gene was quantified using qRT-PCR (Figure 3). The virus titers represented in Figure 3 are the mean $\log_{10}$ titers for each treatment group at each time point. The OP swabs from all NC birds were all negative for virus. In general, virus titers steadily increased over the course of infection in non-TLR pre-treated birds (HPAIV group), with the highest titers at 72 and 96 hpi, 6.5 and 6.4 $\log_{10}$ respectively. A correlation between

influenza virus titers and mean death time has been previously reported in the literature [31-35] and supports our findings. The TLR+HPAIV group did not show a statistically significant difference among the five post-infection sampling time points. A possible explanation for this finding was that bird-to-bird variation, while representative of the entire group, led to heterogeneity of viral titers.

A difference in viral titers, however, was observed between treatment groups. The TLR+HPAIV treatment group had statistically significantly higher viral titers at 12 ($p = 0.021$) and 24 hpi ($p = 0.004$) over the HPAIV treatment group. While there were no observable differences in clinical signs and gross lesions between the HPAIV and TLR+HPAIV groups, the TLR+HPAIV birds lived 14% longer than the HPAIV birds. In agreement with a study by de Jong *et al.* in 2008, there was a correlation between the administration of a TLR1/2 agonist (Pam3CSK4) and increased human immunodeficiency virus (HIV-1) transmission and replication [36]. This finding could perhaps explain a potential negative effect (increased HPAIV replication) of the TLR agonist cocktail used in our experiment but that was not outweighed by the positive effect of increased survival time by a not yet determined mechanism. Regardless of the difference in viral replication observed at 12 and 24 hpi, there were no statistically significant differences in viral titers from 48 hpi on. Several experiments conducted in mice have also shown that morbidity and mortality were reduced by immunomodulatory agents and without any decrease in virus replication, suggesting that modulation of host innate immunity alone may be sufficient to improve the outcome associated with influenza virus infections [37].

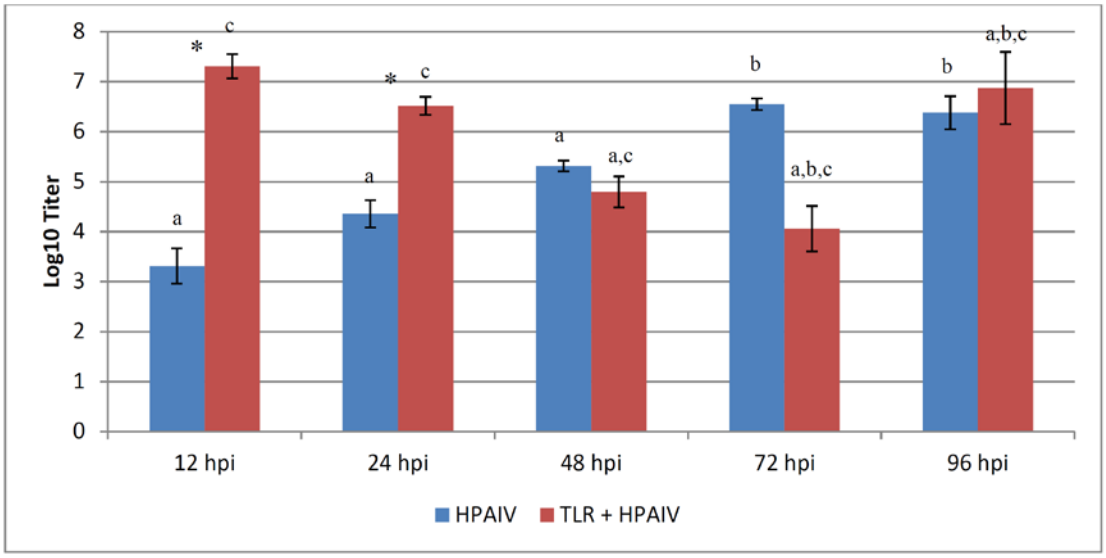

Figure 5.3: Mean oral-pharyngeal virus shed titers by hours post-infection as determined by quantitative real-time RT-PCR for the influenza M gene. Error bars represent standard error of the mean. *Differences between treatment groups (HPAIV versus TLR+HPAIV) are statistically significant ($p < 0.05$) as determined by a one-way ANOVA. Means with the same letter are not significantly different from each other (Tukey–Kramer test, $p < 0.05$).

Differential effects of TLR agonist pre-treatment on the transcription of TLR pathway genes

Thirty-six hours after chickens were treated intranasally with a TLR agonist cocktail, spleen and lung tissues were harvested and RNA was extracted in order to measure gene expression changes elicited by TLR agonist treatment and to determine if there was an immunomodulatory effect of the treatment. Figures 4 and 5 illustrate the fold change of TLR pathway genes in response to TLR agonist pre-treatment in lung and spleen tissue, respectively. In the lungs of TLR agonist pre-treated birds, 11 out of the 18 TLR pathway genes were significantly differentially expressed ($p < 0.05$) compared to the control birds (Figure 4). Eight genes were up-regulated: MYD88 (1.17 fold, $p = 0.0019$), TICAM (1.57 fold, $p = 0.0043$), TRAF6 (2.17 fold, $p = 0.007$), NFKB (2.28 fold, $p = 0.0004$), IFNB (1.54 fold, $p = 0.00002$), IL6

(1.38 fold, $p = 0.0026$), NOS2 (1.80 fold, $p = 0.0002$), and TNFA-like (2.44 fold, $p = 0.0002$). Three genes were down-regulated: IFNA (1.14 fold, $p = 0.0001$), MX1 (1.85 fold, $p = 0.00032$), SOCS3 (1.13 fold, $p = 0.00007$). Overall, there was an effect of TLR agonist administration on gene expression of TLR pathway elements in the lung tissue of chickens 36 hours post-treatment.

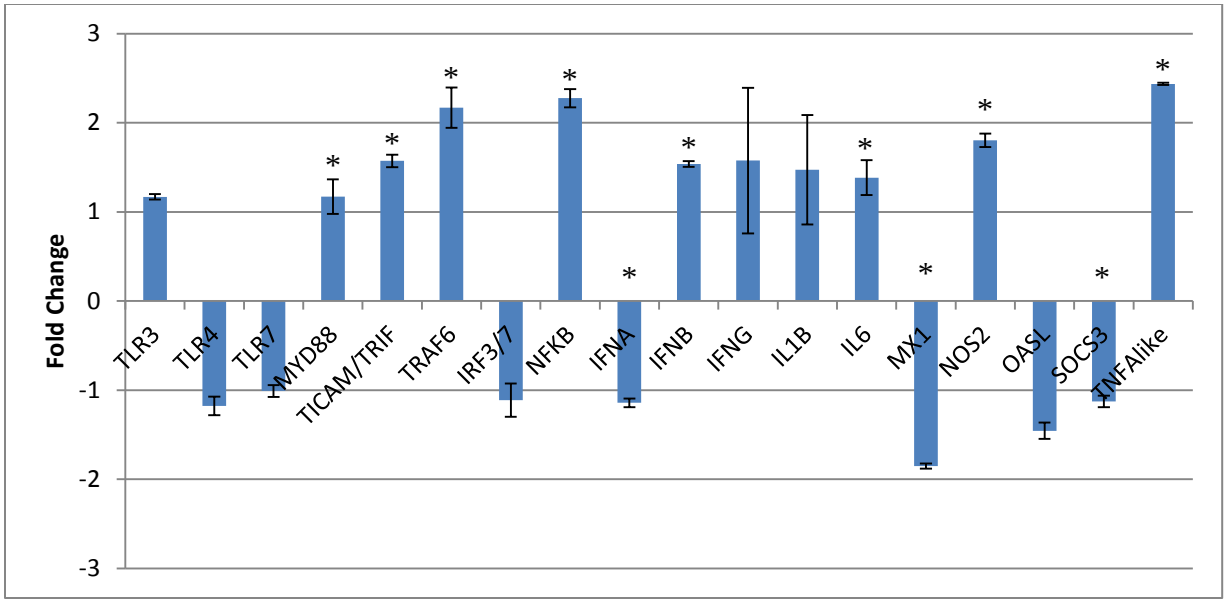

Figure 5.4: Lung Gene Expression after 36h of TLR Agonist Pre-Treatment. Relative gene expression as determined by qRT-PCR of TLR pathway genes of the TLR pre-treated birds (TLR) over the untreated control birds (NC) at time zero. Error bars represent standard error of the mean. *Differences between treatment groups (NC versus TLR) are statistically significant ($p < 0.05$) as determined by a one-way ANOVA.

In the spleens of TLR agonist pre-treated birds, 12 of the 18 TLR pathway genes were significantly differentially expressed ($p < 0.05$) compared to the control birds (Figure 5). Seven genes were up-regulated: TLR3 (1.07 fold, $p = 0.0004$), TLR7 (1.62 fold, $p = 0.0078$), TRAF6 (1.49 fold, $p = 0.0006$), IRF3/7 (1.51 fold, $p = 0.0004$), MX1 (3.53 fold, $p = 0.0002$), OASL (3.14 fold, $p = 0.00003$), and TNFA-like (1.28 fold, $p = 0.0041$). Five genes were down-regulated: TLR4 (1.29 fold, $p = 0.014$), MYD88 (1.27 fold, $p = 0.014$), TICAM (1.44 fold, $p =$

0.00002), NFKB (1.11 fold, $p = 0.0005$), and IFNA (3.72 fold, $p = 0.035$). Overall, there was a definite effect of TLR agonist administration on gene expression of TLR pathway elements in the spleen tissue of chickens 36 hours post-treatment.

The lung and spleen displayed unique tissue-specific TLR pathway gene expression profiles in response to TLR agonist pre-treatment. Of the statistically significant differentially regulated genes in the lung (11/18 genes, 61%) and spleen (12/18 genes, 67%), only TRAF6, IFNA, and TNFA-like demonstrated the same gene expression patterns in terms of directionality. The other four genes (MYD88, TICAM, NFKB, and MX1) displayed tissue-specific differential expression.

Myxovirus resistance gene 1 (MX1) is an interferon-stimulated gene expressed in response to viral infection and MX1 protein is the main effector molecule in the establishment of the interferon-induced anti-viral state. In chickens, MX1 is highly polymorphic and amino acid position 631 of the MX1 protein has been correlated with anti-viral activity. Presence of an asparagine (Asn) residue at position 631 of the MX1 protein has been shown to confer anti-viral activity in both in vitro and in vivo systems, while a serine (Ser) residue at that location renders the MX1 protein inactive [38, 39]. While these finding have been difficult to replicate in other systems, a recent study demonstrated the association between an MX1 Asn631 variant allele with decreased morbidity, early mortality, viral shedding, and cytokine responses in broiler chickens infected with a HPAIV [40]. The significance of the differential regulation of MX1 in response to the TLR agonist pre-treatment as well as during HPAIV infection is controversial as we do not know the MX1 amino acid sequence of the SPF single comb white leghorn chickens used in this study nor do we know if MX1 protein is active these birds.

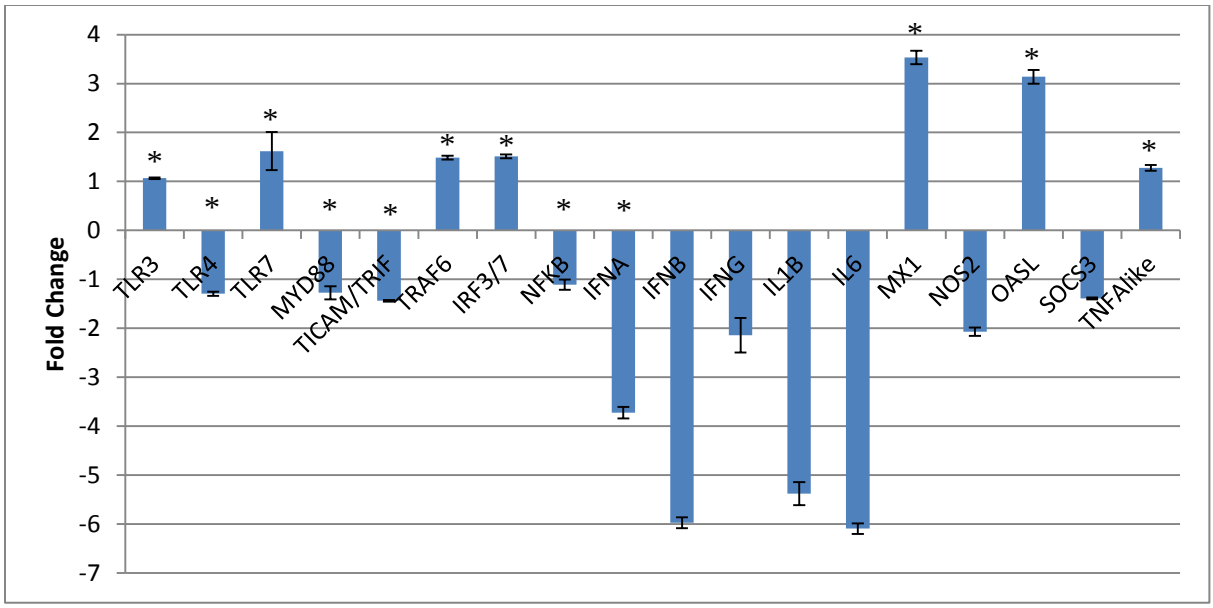

Figure 5.5: Spleen Gene Expression after 36h of TLR Agonist Pre-Treatment. Relative gene expression as determined by qRT-PCR of TLR pathway genes of the TLR pre-treated birds (TLR) over the untreated control birds (NC) at time zero. Error bars represent standard error of the mean. *Differences between treatment groups (NC versus TLR) are statistically significant ($p < 0.05$) as determined by a one-way ANOVA.

Tissue specific responses to HPAIV Challenge

Thirty-six hours after pre-treatment with a TLR agonist cocktail, TLR+HPAIV and HPAIV treatment group chickens were inoculated via the intrachoanal route with $10^{4.9}$ EID_{50} of A/Chicken/Pennsylvania/1370/1983 (H5N2) HPAIV. At 12, 24, and 48 hpi tissue and spleen samples were harvested from two birds from each treatment group (HPAIV and TLR+HPAIV) and RNA was extracted for qRT-PCR analysis of TLR signaling pathway genes and genes selected for their role in AIV pathogenesis. Genes thought to be involved in the development of hypercytokinemia are including IP10, OASL, SOCS3, NOS2, PKR, RANTES, MIG, MCP1, IL1B, IL2, IL6, IL8, IL10, IL18, IFNA, IFNB, IFNG, TNFA, MIP1A, and [37, 41, 42], hence the inclusion of several of these signaling molecules in our qRT-PCR panel.

qRT-PCR revealed tissue-specific gene expression in response to HPAIV. When analyzing lung genes that demonstrated at least 5 fold induction or repression, 5 genes (TLR3, IRF3/7, IFNG, MX1, OASL) were up-regulated and one gene (TICAM/TRIF) was down-regulated (Table 2). The majority of these large expression changes occurred at 24 hpi, highlighting the importance of investigating early time points in the infection cycle. The differential regulation of these genes indicates signaling through the TLR3 pathway due to the up-regulation of TLR3, its adaptor protein TICAM, and downstream effector molecule, IRF3, a transcription factor which binds an interferon-stimulated response element (ISRE) that causes the activation of MX1 transcription.

Eight splenic genes exhibited at least 5 fold induction or repression: 2 genes (MX1 and OASL) were up-regulated and 6 genes (TLR4, TICAM/TRIF, IFNA, IFNB, IL1B, and IL6) were down-regulated (Table 3). These large gene expression changes were nearly equally distributed between the 24 and 48 hpi time points, indicating that splenic transcription was modulated to a greater degree and temporal extent than lung tissue transcription.

The differential expression of the interferon genes was of particular interest in that the spleen differentially expressed type I interferons (IFNA and IFNB), while the lung differentially expressed type II interferon (IFNG). Perhaps these are unique tissue-specific mechanisms employed to alter innate immune signaling pathways to establish an antiviral state.

In response to HPAIV challenge spleen and lung tissue induced three genes in common (TICAM/TRIF, MX1, and OASL). TICAM/TRIF is the adaptor protein for TLR3 and its differential expression indicates TLR3-mediated signaling. MX1 protein is associated with the establishment of the antiviral state [43]. OASL transcription is induced by IFN, and activation of the OAS enzymes results in the synthesis of 2’-5’-linked oligoadenylates that can

bind to RNase L, which then degrades viral and cellular RNAs. This cascade of events suppresses protein synthesis and viral growth [44]. As interferon response genes (ISGs), MX1 and OASL up-regulation has been observed during AIV infections of avian hosts [45, 46].

In influenza pathogenesis there is a direct correlation between cytokine production, viral replication, and disease [47], hence the importance of quantifying cytokine transcription resulting from the stimulation of a critical innate immune response pathway such as the TLR pathway.

Table 5.2: Lung Genes Eliciting Major (> 5 fold) Induction/Repression. Differentially expressed (5 fold up-regulated or 5 fold down-regulated at any time point) genes are represented in bolded font. The non-infected zero hour control lung sample was used as the calibrator to determine gene expression changes of TLR+HPAIV and HPAIV treatment group birds.

	12 hpi TLR+HPAIV	12 hpi HPAIV	24 hpi TLR+HPAIV	24 hpi HPAIV	48 hpi TLR+HPAIV	48 hpi HPAIV	Induction (↑) or Repression (↓)
TLR3	1.08	1.60	**15.63**	**14.85**	2.00	3.12	↑
TICAM/TRIF	-1.08	1.16	1.03	1.25	**-6.80**	**-6.38**	↓
IRF3/7	-1.21	1.51	**10.79**	**12.25**	1.23	-1.01	↑
IFNG	-1.55	-1.34	3.83	**5.02**	**9.71**	**7.72**	↑
MX1	1.08	1.38	**43.61**	**42.07**	**14.41**	**21.84**	↑
OASL	1.44	1.06	**17.10**	**20.92**	2.66	3.56	↑

Table 5.3: Splenic Genes Eliciting Major (> 5 fold) Induction/Repression. Differentially expressed (5 fold up-regulated or 5 fold down-regulated at any time point) genes are represented in bolded font. The non-infected zero hour control spleen sample was used as the calibrator to determine gene expression changes of TLR+HPAIV and HPAIV treatment group birds.

	24 hpi TLR+HPAIV	24 hpi HPAIV	48 hpi TLR+HPAIV	48 hpi HPAIV	Induction (↑) or Repression (↓)
TLR4	**-230.13**	**-9.39**	-2.33	**-10.44**	↓
TICAM/TRIF	**-45.33**	**-7.25**	-4.29	**-12.94**	↓
IFNA	**-5.84**	**-5.10**	**-15.44**	**-13.43**	↓
IFNB	**-24.98**	**-14.36**	**-20.11**	**-18.37**	↓
IL1B	-2.98	-3.31	-3.98	**-7.71**	↓
IL6	**-7.44**	-2.92	-2.53	**-5.35**	↓
MX1	**52.15**	**35.61**	**28.90**	**25.82**	↑
OASL	**71.02**	**43.66**	**29.08**	**30.61**	↑

TLR signaling pathway activation in response to HPAIV and TLR agonists

In the current study, we chose to investigate the transcriptional response of two organs, lung and spleen, critical to the innate immune response to influenza. The lung is an epithelial surface that has a low baseline level of innate immunity activity due to the efficiency of the mucociliary escalator to eliminate microbes from inhaled air. When exposed to pathogens, however, the highly specialized lung epithelium rapidly responds by secreting antimicrobial effector molecules, enhancing barrier function, and recruiting macrophages, dendritic cells, and lymphocytes to areas of infection or damage [48]. Lung tissue was chosen for analysis, as it is the primary site of entry for AIV, represents immune events early in infection, and is an ideal location for stimulating innate immunity using immunotherapeutics given that the lungs' antimicrobial defenses have been shown to be therapeutically inducible [48]. Additionally, the avian lung differs in structure, function, and immunologically from the mammalian lung, highlighting the importance of studying HPAIV in the avian host [49].The spleen is a tissue replete with circulating and resident innate immune cells and provides insight into the events of

AIV pathogenesis later in infection, as the virus establishes a systemic and disseminated infection.

In the lung (Figure 6) and spleen (Figure 7), several genes were significantly ($p < 0.05$) differentially expressed between the HPAIV and TLR+HPAIV treatment groups either in directionality (induction versus repression) or in magnitude. In lung tissue, TLR7 was repressed in HPAIV samples and induced in TLR+HPAIV samples at 12 and 24 hpi, although only to a small degree in either direction (12 hpi HPAIV: -1.01, TLR+HPAIV: 1.11; 24 hpi HPAIV: -1.03, TLR+HPAIV: 1.73). IRF3/7 also demonstrated differential expression at 12 hpi and was induced in HPAIV birds (1.51 fold) and repressed in TLR+HPAIV birds (-1.21 fold). At 24 hpi, IL6 was repressed -1.18 fold in HPAIV birds and induced 1.29 fold in TLR+HPAIV birds and at 48 hpi, TNFA-like was repressed -2.21 fold in HPAIV birds and induced 1.42 fold in TLR+HPAIV birds. While these gene expression changes were small in magnitude, they were nonetheless significantly different in these two treatment groups, indicating that the TLR agonist effected TLR pathway expression throughout the course of infection.

In lung, three other genes were notably significantly ($p < 0.05$) differentially expressed between the HPAIV and TLR+HPAIV treatment groups in magnitude. At 24 hpi, SOCS3 was induced by 20.92 fold in HPAIV birds and 17.10 fold in TLR+HPAIV birds. MX1, at 48 hpi, was induced by 21.84 fold in HPAIV birds and 14.41 fold in TLR+HPAIV birds. Finally, at 24 hpi OASL was induced by 20.92 fold in HPAIV birds and 17.1 fold in TLR+HPAIV birds.

In spleen tissue, only one gene, TLR7 (at 48 hpi), significantly differed ($p < 0.05$) in directionality between the HPAIV and TLR+HPAIV treatment groups. In the former, TLR7 was repressed 1.40 fold and in the latter, TLR7 was induced 1.08 fold. Several genes were significantly ($p < 0.05$) differentially expressed between the HPAIV and TLR+HPAIV treatment

groups in magnitude, however, one gene, TLR4 (at 24 hpi) differed over 2,000%, with a repression of -9.39 fold in HPAIV birds and -230.13 fold in TLR+HPAIV birds. This result was so striking that it almost over powered the fold differences of TLR4 at 48 hpi (HPAIV: -10.44, TLR+HPAIV: -2.33), MX1 at 24 hpi (HPAIV: 35.61, TLR+HPAIV: 52.15), OASL at 24 hpi (HPAIV: 43.66, TLR+HPAIV: 71.02), and TICAM/TRIF (HPAIV: -7.25, TLR+HPAIV: -45.33).

In comparing tissues, expression of TLR pathway genes was differentially regulated to a greater magnitude in the spleen while lung tissue differentially expressed more TLR pathway genes in terms of directionality. There were no observable patterns when comparing the two treatment groups, HPAIV and TLR+HPAIV. At the post-infection time points, each group demonstrated differential expression and often switched between time points. For example in the lung tissue, IRF3/7 was induced in HPAIV and repressed in TLR+HPAIV birds at 12 hpi, then at 24 hpi IRF3/7 was induced in both groups to nearly the same magnitude, and finally at 48 hpi, IRF3/7 was repressed in HPAIV and induced in TLR+HPAIV birds. Many of the genes assayed displayed similarly inconsistent gene expression changes or there were no significant differences between the two treatment groups.

It remains to be determined if these small gene expression changes, in their totality, were enough to alter the course of AIV pathogenesis or the host innate immune response and antiviral state. On the other hand, is it possible for the differential expression of one gene, in one tissue, at one time point (e.g. TLR4 in the spleen), but to an enormous magnitude, to augment the innate immune response to HPAIV and have the clinical effect of extending survival time?

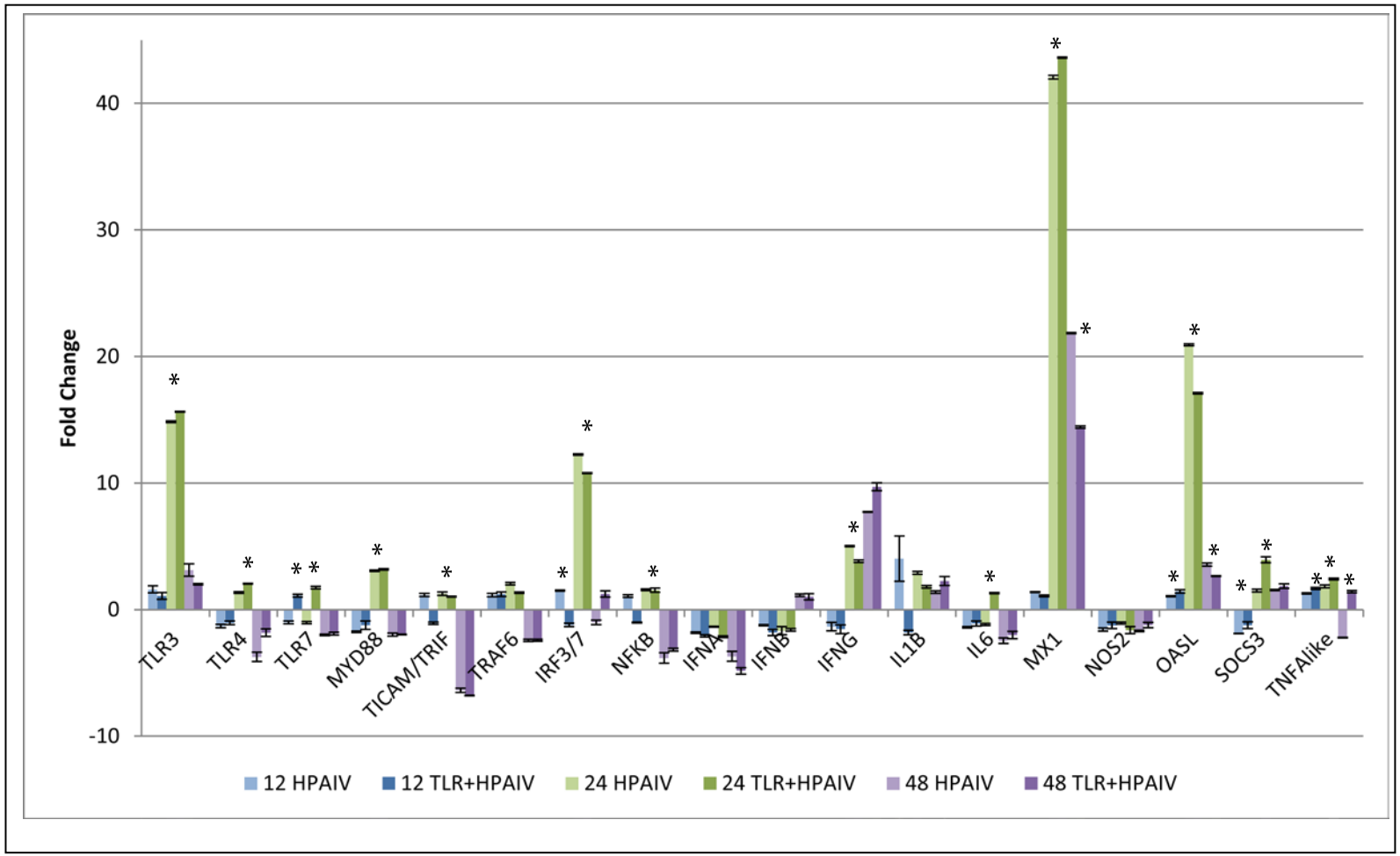

Figure 5.6: Gene Expression of Lung Tissue in Response of TLR+HPAIV and HPAIV Treatment Groups. Relative quantification of the HPAIV and TLR+HPAIV birds over the untreated control birds at time zero. Error bars represent standard error of the mean. *Differences between treatment groups (HPAIV versus TLR+HPAIV) are statistically significant ($P < 0.05$).

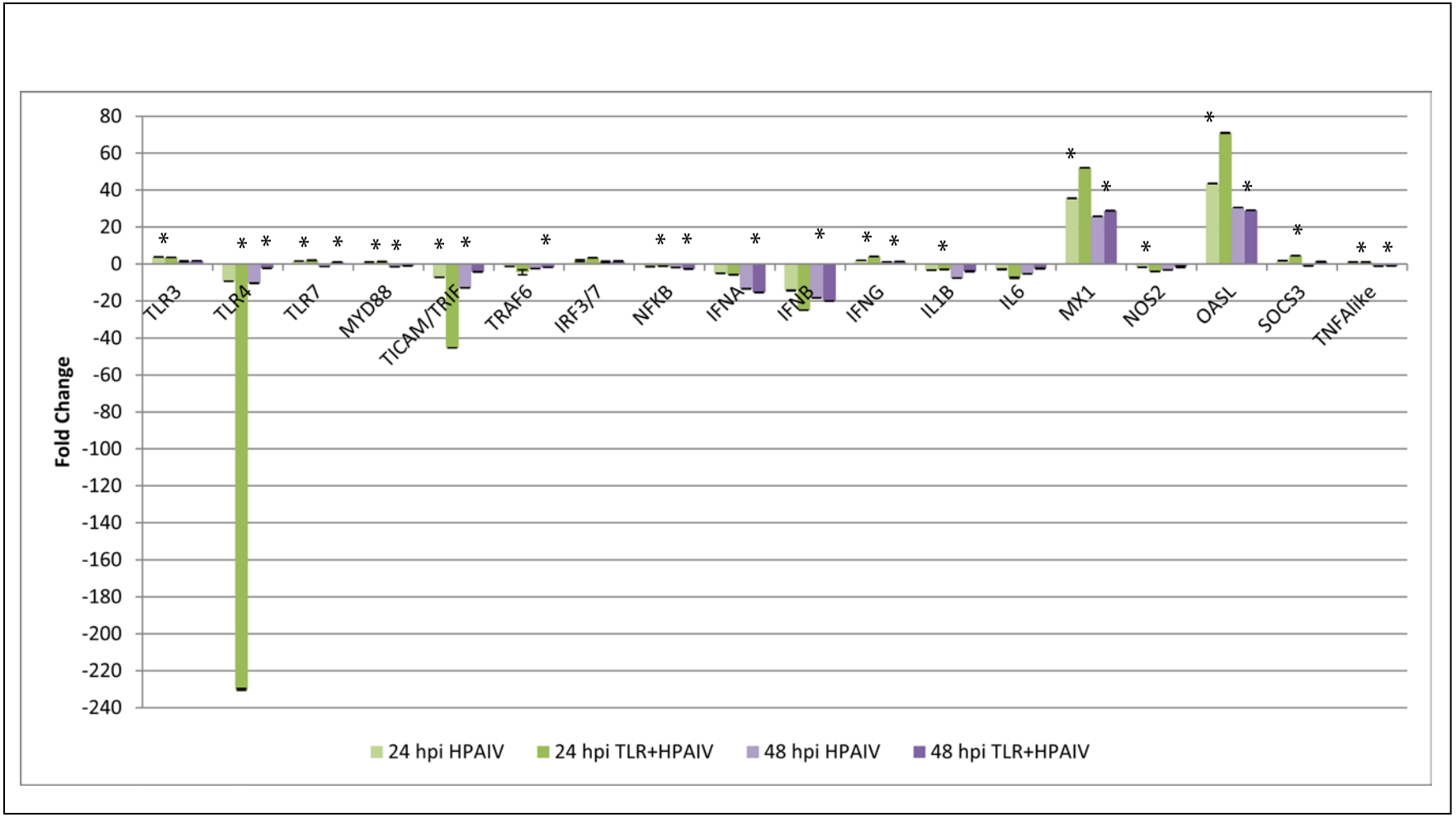

Figure 5.7: Gene Expression of Splenic Tissue in Response of TLR+HPAIV and HPAIV Treatment Groups. Relative quantification of the HPAIV and TLR+HPAIV birds over the untreated control birds at time zero. Error bars represent standard error of the mean. *Differences between treatment groups (HPAIV versus TLR+HPAIV) are statistically significant ($P < 0.05$). A splenic RNA sample from the 12 hpi time point became degraded and did not pass our quality control criterion and was therefore eliminated from the qRT-PCR assay.

Conclusion

During HPAIV infection chickens, up-regulate TLR3 and IFNB mRNA expression in lung and spleen 24 hpi [19], therefore we targeted the TLRs involved in the innate immune response to AIV and measure the downstream transcriptional changes in the TLR pathway. Additionally, there are several demonstrations of protective effects elicited by bacterial lysates and TLR4 agonists (LPS and synthetic lipid A mimetic, aminoalkyl glucosaminide phosphates) administered pre-influenza challenge in order to stimulate innate immunity [48, 50, 51], hence the inclusion of the TLR4 agonist, LPS, in our TLR agonist cocktail. Recent reports have also shown a beneficial and synergistic effect of using multiple TLR agonists, thus providing a solid rationale for using a TLR agonist cocktail comprised of three TLR agonists [48, 52-54].

The rationale for utilizing a pre-treatment with TLR agonists has several lines of documentation. In mammalian literature, several reports have indicated that mice intranasally pretreated with TLR ligands exhibit enhanced survival when challenged with influenza virus [55-57]. In developing the experimental design for our work, we aimed to incorporate many of the ideas in the mammalian literature noted here.

Wong *et al.* 2007 found that a liposome-encapsulated TLR3 agonist, polyriboinosinic-polyribocytidylic acid (poly I:C) stabilized with poly- l-lysine and carboxymethyl cellulose (LC), administered to mice 48 hours before challenge, provided 100% protection against low pathogenicity virus A/PR/8/34 and 63-75% protection against HPAI strain A/H5N1/chicken/Henan [57]. Similarly in 2009, Wong

et al. demonstrated the effectiveness of different TLR agonists for TLR3 and TLR9 (CpG ODN) to protect mice from seasonal influenza strain A/Aichi/2 (H3N2) [56, 58].

Seo *et al.* 2007, assessed the contribution of innate immunity to early protection by employing a TLR3 (poly I:C) and/or TLR7 (imiquimod) agonist 24 hour pre-stimulation to examine if this resulted in protection against H1N1 human influenza challenge [55]. Poly I:C treated mice survived (62.5%) while mice received PBS without any TLR agonists (PBS control group) succumbed to infection and when poly I:C was combined with imiquimod (a TLR3/7 cocktail) it enhanced survival (100%). These results demonstrated that the innate immune responses induced by TLR 3 and TLR 7 agonists can provide protection from lethal influenza challenge.

Hammerbeck *et al.* 2007 explored the use of a dual TLR7 and TLR8 agonist (3M-011) and found that intranasal administration of 3M-011 significantly inhibited H3N2 influenza viral replication in the nasal cavity and lungs when administered from 72 h before viral inoculation to 6 h after inoculation [59]. Hammerbeck *et al.* found a correlation between viral inhibition and the ability of the TLR7/8 agonist to stimulate type I IFNs and other cytokines such as TNFA, IL12, and IFNG in rat PBMCs. Three-fold increases in the aforementioned cytokines were observed 4-6 hours post-treatment.

Lao *et al.* 2009 found that a TLR3 ligand exhibited potent inhibition of influenza virus replication and had strong adjuvant activity [60]. The TLR3 agonist, PIKA, provided broad-spectrum prophylaxis against a number of influenza A viruses

and when admixed with influenza vaccine preparations, a significant adjuvanting effect leading to accelerated viral clearance was observed in a murine model.

Norton *et al.* 2010 discovered that through diverse immunomodulatory mechanisms, three bacterially-derived immune-enhancing agents (cholera toxin, a mutant form of cholera toxin-related *Escherichia coli* heat-labile enterotoxin, and CpG ODN) induced an initial inflammatory process and subsequently enhanced the immune response to primary influenza virus challenge [61]. Specifically, pre-treatment with the various immunomodulators prevented or delayed mortality and weight loss, and significantly reduced initial lung viral loads.

Shinya *et al.* 2011 showed that TLR2 and TLR4 agonist pre-stimulation protected mice from lethal challenge with HPAIVs [6]. The authors noted that different TLR agonists were more or less effective depending on the influenza challenge strain. Pre-stimulation TLR2 agonist, synthetic mycoplasmal lipoprotein FSL-1, was most effective against a highly pathogenic H1N1 reassortant virus and TLR4 agonist, LPS from *E. coli* K12 msbB, protected against lethality induced by a highly pathogenic A/Vietnam/1203/04 (H5N1) strain. These results suggest that the most efficacious method of stimulating innate immunity may be dependent upon the particular virus in question.

The St. Paul *et al.* 2012 [16] study was the only study that utilized the *in vivo* chicken model and thus some comparisons can be made despite the different TLRs targeted (TLR3/4/21 versus TLR3/4/7), pathotypes of AIV (LPAIV versus

HPAIV) used for virus challenge, and immune genes assayed. We similarly discovered that several immune genes were up-regulated in the lungs while being down-regulated in the spleen. Additionally, we also noted a tissue-specific response of type I interferons differential regulation in the spleens and type II interferon differential regulation in the lungs of HPAIV challenged birds.

In our study, the IFNA was slightly down-regulated (-1.14 fold change) and IFNB was slightly up-regulated (1.54 fold change) in lung while both IFNA and IFNB were down-regulated in spleen, with fold changes of -3.72 and -5.97, respectively, 36 hours post-treatment with the TLR agonist cocktail. In future studies, it will be helpful to investigate the effect of TLR agonist treatment at several time points before HPAIV challenge to better evaluate at which point cytokines demonstrate the greatest levels of up- or down-regulation and if HPAIV challenge at those times correlates with increased or decreased morbidity and mortality.

Given the growing body of literature demonstrating the positive outcomes associated with the prophylactic administration of TLR agonists and influenza challenge, a solid rationale existed for performing similar experiments using the *in vivo* avian model. A potential application for prophylactic administration of TLR agonists would be to boost innate immunity prior to a known influenza outbreak and better prepare a population for an imminent pandemic in the time frame in which vaccines would not yet be available. Altered gene expression of TLR pathway associated cytokines due to the TLR agonist pre-treatment and a clinical response was demonstrated in this experiment and several anti-inflammatory therapeutics, including

TLR agonists/antagonists, are currently being explored for their ability to treat inflammation-mediated illnesses such as influenza infection [11, 62-67].

While thought to be highly immunostimulatory, at 36 hours post-treatment with the TLR agonist cocktail, some important antiviral TLR pathway end-products assayed were down-regulated and other inflammatory cytokines were weakly up-regulated. Perhaps there was a TLR agonist-induced immunostimulatory phase characterized by the up-regulation of TLR pathway genes that was simply not observed in the timeframe selected. Another possibility is that there was either a synergistic effect of the three TLR agonists combined that resulted in an immunosuppression rather than an immunostimulation. A likely explanation is that neither a complete up- or down-regulation of TLR pathway signaling is a reasonable expectation as a TLR agonist cocktail would cause augmented cell signaling, a fine-tuning.

A number of questions remain from this work. How do we define cytokine storm in the avian host and is it the same as in the mammalian host? Is this response tissue specific and if so how do we interpret transcriptome changes at the tissue level? Was abrogation of TLR pathway enough to effect survival time? In what ways can we predict, based on cytokine gene expression alone, patient outcomes? What is the potential for inducible resistance to noncognate pathogens or heterotypic viral strains afforded by innate immune augmenting therapeutics? Finally, what contribution does the host immune response and AIV pathogenesis play in the morbidity and mortality associated with HPAIV infections?

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Chapter 6

GENERAL DISCUSSION

Economic Importance of Poultry

Poultry production is an economically important industry. The United States is the world's largest poultry producer, the second largest egg producer, and the second largest exporter of poultry meat. The combined value of production from broilers, eggs, turkeys, and the value of sales from chickens in 2011 was $35.6 billion (United States Department of Agriculture (USDA) National Agricultural Statistics Service (NASS) Poultry - Production and Value 2011 Summary, April 2012). Broiler production accounts for the majority of this value, followed by eggs, turkey, and other chicken. When poultry become ill due to microbial infection, a cascade of economic consequences follows. Poultry are often condemned at the processing plant and therefore do not make it to sale, however, this is the least financially damaging of the consequences. During outbreaks of influenza, the U.S. will depopulate all the birds on a farm and depending on the pathotype (LP or HP), a perimeter around the contaminated farm will be established and all of the poultry within that radius will be euthanized. Influenza outbreaks also affect international trade (i.e. exports) as countries will embargo poultry products from the U.S. during AI outbreaks. To

maintain the global supply of affordable protein sources in the form of meat and eggs, we must therefore protect poultry health.

Significance of Chickens as a Model Organism

The chicken has now been established as a model organism due to the variety of discoveries made in chickens that serve as a disease model for several other applications (1, 3). Due to the short (21 day) incubation period of eggs and rapid development of the embryo, research conducted on embryonated chicken eggs has greatly affected embryology and study of vertebrate development (1). Several of the early classical genetics studies by Charles Darwin were carried out by studying the morphological traits of chickens and their feathering (1). The discovery of the bursa of Fabricius by Hieronymus Fabricius of Aquapendente (1537–1619) and then the elucidation of the role of the bursa in B-cell development in 1955 led immunologists to investigate the mammalian equivalent of the bursa of Fabricius (2). It was later discovered that bone marrow in mammals provides the B-cell or antibody-mediated immunity (4). Another immunology-related discovery occurred over 100 years ago in 1908, when Oluf Bang and Vilhelm Ellerman demonstrated that avian erythroblastosis, a chicken leukemia cancer, could be transmitted by cell-free extracts. This finding was confirmed for solid tumors in chickens in 1910-1911 by Peyton Rous, thus providing the first evidence of an oncovirus, a virus capable of causing cancer (6). These important discoveries and many more have led to the establishment of the chicken as a model organism for several diseases.

Project Aims

The immunology research with respect to microbial infections has historically focused on adaptive immunity, the robustness of the antibody response of the host to infection, or the T cell subsets activated in response to a particular pathogen. While the information garnered from this research currently dominates immunology clinical practice and has led to the development of most of the preventatives (vaccinations) and treatments (pharmaceutical), there remains much to be learned about the critical early steps of immunity, specifically innate immunity. Formation of an adaptive immune response relies exclusively on signaling from innate immunity; therefore, it is warranted that we pay equal attention to innate immunity and the steps that advance infection to disease.

The aim of this dissertation was to characterize the innate immune response of birds to infection with various microbes to:

1. determine which receptors and signaling pathways are activated during infection and if there is any discernible pattern that correlates the pathogenic microorganism with its consequential transcriptional immune response,
2. compare the gene expression profiles of different avian species and different bacterial and viral strains in order to understand the host's unique ability to either tolerate infection or succumb to it,
3. explore the use of immunotherapeutics to alter gene expression, the host immune response, and morbidity and mortality.

Summary

A progression of publications led to the accomplishment of these research objectives. Conducting research using the avian model, and most often chickens, has been limited (in comparison to the mammalian models) by a lack of immunological reagents, annotated genome information, and genetically engineered "knockout" chickens, however, since the publication of the chicken genome in 2004 (5), the field of functional genomics and highly advanced avian research has been made possible. Our laboratory capitalized on the characterization of large chicken expressed sequence tag (EST) libraries, the publication of the chicken genome, and the sophisticated analytical tools and assays such as microarrays, DNA/RNA sequencing, and quantitative RT-PCR, and created an immunity-focused microarray in order to characterize the avian immune response to microbial infections.

Chapter 2 described several experiments to evaluate the utility of the avian innate immunity microarray (AIIM) to elucidate the gene expression profiles of tissues derived from chickens, ducks, and turkeys in response to viral and bacterial pathogens, a low pathogenicity avian influenza (A/Chicken/Maryland/Hobo/2003 (H7N2)) and an avian pathogenic *Escherichia coli* (APEC) strain V-G. Firstly, we established that RNA derived from several immunologically-relevant tissues (air sac, lung, liver, spleen, embryonic spleen, duodenum, and thymus) were all able to be hybridized to the AIIM and gene expression profiles for each tissue were created and analyzed. Secondly, we found that the AIIM, a microarray comprised on chicken cDNA, was able to measure the transcriptional activity of non-homologous avian

species, specifically ducks and turkeys. The significance of this finding greatly expands the use of species-specific microarrays to closely related species and has applications beyond the avian research community. Thirdly, we were able to identify core sets of genes that were differentially regulated in response to either bacterial or viral infection. The ability to detect differences in the chemokine and cytokine response to different pathogens permitted the development of hypothesis driven experiments designed to evaluate and understand the complexity of the avian immune signaling response, such as those described in chapters 3 and 4.

Chapter 3 described the first full use of the AIIM to compare two different bacteria (*Mycoplasma synovia* (MS) and an avian pathogenic *Escherichia coli* (APEC)) in two cell types (HD11 avian macrophage immortalized cell line, and monocyte-derived macrophage (MDM) primary cells). The aim was to determine the extent of modulated genes and make comparisons between the transcriptomes of chicken macrophages exposed to either bacteria. This study revealed 157 macrophage genes that were uniquely modulated by MS, 1603 genes uniquely modulated by APEC, and a core set of 146 genes modulated by both pathogens. The relevance of these findings was that APEC infection elicited greater gene expression induction/repression levels, which corresponds to the severe clinical signs associated with APEC infection *in vivo*, while MS caused lesser gene expression changes, altering the transcription of only 157 genes (3% of the AIIM elements), which corresponds to the primarily subclinical infection caused by MS infection. APEC infection also caused greater expression of genes involved in oxidative burst (IL1B, NOS2A, IL6), a pathway that contributes to tissue inflammation. These results demonstrated that a relationship exists between the magnitude and quality of gene

expression and the severity of disease caused by these two bacteria, thus establishing an important paradigm for the understanding of host-pathogen interactions.

Once we tested the AIIMs ability to perform a robust gene expression study of chicken cells in response to bacteria, we decided to explore the full utility of the microarray to evaluate the *in vivo* gene expression of ducks in response to different strains of low pathogenicity avian influenza virus. In **Chapter 4**, we investigated how ducks, the natural reservoir for all avian influenza subtypes responded to three H5 strains of AIV isolated from different avian species, namely a duck, chicken, and turkey. AIIM analysis revealed a core set of 61 genes differentially regulated in response to all three LPAIVs tested. The significance of these findings was that regardless of influenza strain, 61 genes were expressed, identifying a pool of potential biomarkers for disease diagnosis or identification of a pathogen by evaluating the host transcriptome as opposed to the classical method of disease diagnosis that involves isolating the microorganism or detection of the pathogen's genomic material. AIIM analysis also revealed 101, 135, and 628 differentially expressed genes unique to infection with the chicken-, duck-, or turkey-origin LPAIV isolates respectively. This result highlighted the importance of host-adaptation of LPAIV on transcriptional immune responses. Finally, canonical pathway analysis revealed several key innate immune pathways that were activated in response to LPAIV infection including the toll-like receptor and RIG-I-like receptor pathways. This result set the groundwork for comparing duck versus chicken host responses to AIV and characterizing the different pathways each bird species utilizes to combat influenza infection. Given infection with the same strain of AIV, ducks tolerate infection, remaining subclinical, while chickens often experience severe morbidity and mortality, in future work, we aim to

determine why.

Chapter 5 demonstrated the first *in vivo* application of a TLR agonist pre-treatment to alter the immune response of chickens to highly pathogenic avian influenza virus. The aim was to investigate the efficacy of TLR agonist cocktail to pre-stimulate innate immunity in chickens subsequently subjected to HPAIV challenge. At the tissue/cellular level, the goal was to abrogate host-mediated inflammation in order to better prepare the target organ (lung) for virus infection and regulate, in a coordinated fashion, an appropriate inflammatory response. In this experiment, we analyzed morbidity and mortality, viral replication titers, and expression of 18 genes within the TLR pathway. We determined that we were indeed able to alter the gene expression of the lung and spleen 36 hours after administering a TLR agonist cocktail intranasally without any adverse effects. Furthermore, the TLR agonist pre-treated birds survived HPAIV challenge 14% longer than untreated birds, indicating that the TLR agonist was able to change the host immune response significantly and in a way that prolonged survival time. Interestingly, viral replication titers at 12 and 24 hours post-infection were higher in the TLR agonist pre-treated birds, however, there was no statistically significant difference in titers at 48 through 96 hours post-infection. qRT-PCR analysis of the TLR pathway genes revealed large up-regulation of MX1 and OASL, genes critical to the innate immune response and establishment of the antiviral state. The significance of these findings from a basic research perspective is that we have been able to characterize many of the gene expression changes that occur early in infection due to HPAIV infection. In terms of broader applications, we have demonstrated that altering host immunity fundamentally changes the outcome of the subsequent host-pathogen interaction and consequential

morbidity and mortality. Most pharmaceutical treatments developed to combat microbial infection focus on targeting the microorganism, however, the potential afforded by altering the host immune system to fight infection is enormous as it is not strain- or pathogen-specific. In this case, we used the chicken as a model organism for prophylactic preparation of a population during an influenza epidemic.

Future Perspectives

The AIIM has made an important contribution to the field of avian immunology by allowing for the rapid elucidation of gene expression changes during various immunological challenges. Using the molecular biological tools described in this dissertation many interesting discoveries were made in a wide array of settings: bacterial and viral, *in vivo* and *in vitro*, in chicken, duck, and turkey hosts, and in several immunologically-relevant organs/tissues.

It will be crucial to the sustained progress of this work to perform high throughout next-generation sequencing to expand transcriptomic profiling of host-pathogen interactions, perform side-by-side comparative studies on chickens and ducks in order qualify their differential immune responses, and follow up on the promise of immunomodulatory agents to prevent morbidity and mortality, such as the TLR agonists employed in chapter 5. Future work in microbial pathogenomics in the avian host may uncover many interesting and novel mechanisms employed by the pathogen and host during the complex interplay of infection.

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