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Innate Immunity in Chickens: In Vivo Responses to Different Pathogen Associated Molecular Patterns

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Innate Immunity in Chickens:
In Vivo Responses to Different Pathogen Associated Molecular Patterns

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Poultry Science

by

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University of Arkansas
Bachelor of Science in Poultry Science, 2011

August 2016
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This dissertation is approved for recommendation to the Graduate Council

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Abstract

Pattern recognition receptors (PRRs) on host cells recognize motifs known as pathogen associated molecular patterns (PAMPs) that are common to groups of microbes. Examples include LPS on Gram-negative bacteria, the structural motif PGN common to all bacteria, MDP the smallest immunostimulatory unit of PGN, and poly I:C the dsRNA analog. PAMP recognition by and stimulation of the innate immune system is crucial to an individual’s ability to quickly limit microbial growth and stimulate the adaptive immune system. Characterization of the in vivo immune responses initiated by PAMPs has not been directly addressed. Using growing feathers (GF) as a novel intradermal test site along with concurrent blood sampling, we examined the time-course, phenotype, and severity of PAMP (LPS, PGN, MDP, or poly I:C) elicited leukocyte and cytokine responses in both the peripheral blood circulation (automated hematology analysis) and at the local injection-site (pulp of GF; immunofluorescent staining of pulp cell suspensions). Intradermal injection of LPS or PGN in the pulp of GF resulted in increased heterophil levels in the blood and local tissue as well as smaller increases in macrophage proportions in the tissue. However, injection of PGN also resulted in the rapid recruitment and sustained presence of high levels of T and B lymphocytes at the local tissue site. Unlike PGN, its derivative MDP was not found to be highly immunostimulatory. Poly I:C injection also initiated a unique leukocyte infiltration profile with rapid (4-8 h) increases in macrophages and B cell levels in the pulp of injected GF. Injection of PAMPs (except MDP) in GF was shown to stimulate mRNA expression of IL-1β, IL-6, IL-10, and CXCL8, and at lower expression levels IL-4 and IFNγ. As the first comprehensive investigation into the immunostimulatory effects of PAMPs in a complex tissue, we show that intradermal administration of PAMPs initiates an elaborate cascade of responses not seen in single cell in
vitro studies. Future studies building upon the research reported here will continue to evaluate and dissect innate immune responses in poultry and will find direct application in the development of immunomodulatory treatments, vaccine adjuvants, and breeding/management strategies to improve poultry health.
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*used with permission by Pat Byrne text conversation Feb 22, 2016 6:06PM CST (told you I would work it in somewhere Dad!)
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Introduction
Recognition of foreign pathogens by the innate immune system occurs through pattern recognition receptors (PRRs) located on the cells’ outer membrane, endosomal membranes, and in the cytosol (Abbas et al., 2014; Lester and Li, 2014; Schat et al., 2014). The ligands for stimulation of PRRs are pathogen associated molecule patterns (PAMPs); motifs common to groups of pathogens, vital for continued survival of the pathogens, and foreign to the host organism (Seya, 2010; Kumar et al., 2011; Portou et al., 2015). Common PAMPs in immunology and disease research include lipopolysaccharide (LPS), the cell wall component of Gram-negative bacteria; peptidoglycan (PGN), a structural component of all bacteria; muramyl-dipeptide (MDP), the minimal immunostimulatory portion of PGN; and polyinosinic-polycytidylic acid (poly I:C), a structural analog to viral double stranded RNA (Ki et al., 1994; Dziarski, 2003; Traub et al., 2006; Matsumoto and Seya, 2008). These PAMPs are important immunostimulatory components in vaccine adjuvants (Kumar et al., 2010; Seya, 2010; Martins et al., 2015). In chickens there is a paucity of information regarding local and systemic cellular leukocyte recruitment and gene expression profiles following in vivo injection of PAMPs.

Often referred to as endotoxin, LPS is one of the most well studied PAMPs in both avian and mammalian immunology research (Sultzzer and Alerts, 1969; Ki et al., 1994). A fatty carbohydrate located on the outer membrane of Gram-negative bacteria, LPS retains a slight negative charge that helps the bacterium retain its shape and prevent some forms of chemical attack (Ki et al., 1994). The amino acid-sugar polymer, PGN, is a vital component of all bacterial species, although it is present in much higher quantities on Gram-positive bacteria compared to Gram-negative bacteria (Stewart-Tull, 1980; Winn et al., 2006). The smallest immunostimulatory unit of PGN is MDP, and since it was identified in the 1970’s as a part of the Gram-positive bacterium, *Mycobacterium*, included in Freund’s Complete Adjuvant, MDP has
been an important immunostimulatory molecule (Girardin et al., 2003). Many forms of viral genetic material (dsRNA, ssDNA, CpG DNA, etc.) are recognized by PRR, and one of the more common viral PAMPs used in research is the structural dsRNA analog, poly I:C (Kogut et al., 2005).

The innate immune system is home to multiple cell types that work together to initiate inflammatory or anti-viral responses, present antigen to adaptive immune cells, and produce signals to drive and hone recruitment and effector functions of both innate and adaptive cells (Abbas et al., 2014). Each individual T and B cell (cells of adaptive immunity) recognizes one specific part of a pathogen and undergoes clonal expansion before beginning effector functions and destroying the pathogen (Abbas et al., 2014). Unlike adaptive immunity, cells of the innate immune system recognize many different pathogens and begin containment and elimination of the infection within minutes to hours of first exposure (Kumar et al., 2011). In mammals and birds, PAMP stimulation initiates differing immune responses (i.e. inflammatory, anti-viral, humoral, cell-mediated, etc.) depending on the PAMP and cell type.

Heterophils are granulocytes with a polymorphous nucleus and are the first cell type recruited to the site of infection. Similar to their mammalian counterpart, the neutrophils, heterophils are capable of killing phagocytosed pathogens via generation of reactive oxygen species or release of toxic factors into the endosomal compartment (Abbas et al., 2014). Although not a prolific producer of cytokines and chemokines, binding of PAMP ligands to PRR on heterophils induces downstream signaling cascades, producing chemokines and cytokines ultimately resulting in recruitment of other immune cells to the site of infection (Ellis and Beaman, 2004; Schat et al., 2014).
Macrophages are professional phagocytes with the largest array of PRR of any leukocyte and have special capabilities in eliminating intracellular microbes, particularly if stimulated by IFNγ (Qureshi, 2003; Abbas et al., 2014). Upon PAMP recognition, macrophages produce cytokines and chemokines such as interleukin-1beta (IL-1β), IL-6, CXCL8, interferons (IFN) α, β, and γ that direct immune responses toward inflammation, anti-viral, or T helper type 1 (Th1) responses. Macrophages are also active in cleanup of cellular debris, tissue repair, and down regulation of the inflammatory response. Macrophages mediate adaptive immune responses via antigen presentation to T helper cells and production of activation signals (Abbas et al., 2014). A portion of the macrophage population resides in the tissue along with other resident cells such as dendritic cells and mast cells. Little is known in birds regarding their function beyond an important role of dendritic cells in antigen presentation to naïve T cells and the pro-inflammatory activities of activated mast cells which include vascular changes aiding in leukocyte recruitment from blood into infected tissue (Schat et al., 2014).

Lymphoid cells of the adaptive immune system (T and B cells) recognize pathogens through T cell receptors (TCR) or B cell receptors (BCR; i.e. surface antibodies). Each T and B cell has a homogenous set of TCR and BCR, respectively, with specificity to small molecular features of a complex antigen (Abbas et al., 2014). When an individual lymphoid cell recognizes its specific antigen, it undergoes clonal expansion and develops into different types of T helper cells, cytotoxic T cells, or antibody producing B cells (Schat et al., 2014). Recent studies suggest that avian, like mammalian, T and B cells also express PRR, and B cells in particular are capable of responding to PAMPs (Lester and Li, 2014; Schat et al., 2014; Egbuniwe et al., 2015). Although of lymphoid origin, natural killer (NK) cells function as innate immune cells in that they recognize abnormal host cells through a variety of receptors, including PRRs, but do not
need to undergo clonal expansion or differentiation in order to kill host cells that harbor endogenous antigens (e.g. viruses; tumor antigens). In chickens, IL-2 (Sundick and Gill Dixon, 1997), IL-15 (Lillehoj et al., 2001), and IFNγ (Merlino and Marsh, 2002) have been shown to stimulate NK cell growth (IL-2 and IL-15) and cytotoxicity (IFNγ).

The pro-inflammatory cytokines/chemokines IL-1β, IL-6, and CXCL8 are produced by chicken leukocytes after stimulation with PAMPs (Wigley and Kaiser, 2003). Local and systemic inflammatory responses are induced by IL-1β and IL-6 while IL-1β also causes pyrogenesis and IL-6 induces hematopoiesis and influences activation of B and T lymphocytes (Wigley and Kaiser, 2003). As inflammatory cytokines, IL-1β and IL-6 activate numerous cell types including macrophages and heterophils, initiating and honing effector functions such as endocytosis of pathogens and recruitment of leukocytes to the site of infection (Wigley and Kaiser, 2003; Abbas et al., 2014; Schat et al., 2014). The cytokines IL-1β and IL-6 stimulate heterophil effector function, and the chemokine CXCL8 is important for heterophil recruitment (Kogut et al., 2005; Genovese et al., 2013; Schat et al., 2014). Initially referred to as IL-8, CXCL8 is the primary chemokine associated with avian heterophils chemotaxis (Wigley and Kaiser, 2003; Wigley, 2013; Abbas et al., 2014; Schat et al., 2014).

Cytokines produced during innate immune response can influence the type of adaptive immune response to a microbe. Vice versa, effector cells of adaptive immunity help innate leukocytes focus their response on the microbe and enhance their functional abilities. For example, production of IFNγ by NK cells during antigen presentation provides signals to CD4+ T cells to differentiate into Th1 cells. Once fully activated and differentiated effector Th1 cells produce their signature cytokine IFNγ which enhances antimicrobial activities in macrophages. Similarly, depending on the cytokines present during antigen presentation, CD4+ T cells may
instead become type 2 T helper cells. Th2 cells support B cell or antibody mediated responses and express IL-4 as one of their hallmark cytokines (Romagnani, 1999; He et al., 2011a; Straub et al., 2013; Abbas et al., 2014; Schat et al., 2014).

Characterization of the immune response to PAMPs in chickens has primarily occurred through in vitro examination of changes in cytokine gene expression and effector functions such as phagocytosis, nitric oxide production, generation of reactive oxygen species, and degranulation (Kogut et al., 2003, 2005; He et al., 2007, 2011a; b; Nerren et al., 2010; Arsenault et al., 2013). Very few studies have examined the effect of PAMPs in vivo. The most common in vivo methods to study the avian immune responses to PAMPs included administration of PAMPs to mucosal membranes (i.e. lungs or gastrointestinal tracts) and skin derivatives such as wing webs, wattles, and food pads or intravenous (i.v.) injections (Awadhiya et al., 1981; Cotter et al., 1987; Pillai et al., 1988). Typical measurements in these types of studies include markers of inflammation such as swelling, redness, or development of a febrile response; excision of injected tissue; blood sampling; and sacrifice of birds to study immune organ infiltration (Smith et al., 1975; Itho et al., 1989; Parmentier et al., 1998; Zhu et al., 1999; Bowen et al., 2006).

However, these in vivo methods are limited in either the measurements that can be taken (i.e. swelling, redness) or the amount of information that can be gathered from an individual over time (Erf and Ramachandran, 2016). As described in Erf and Ramachandran (2016), the avian growing feather (GF) presents a unique opportunity to examine local tissue responses to injected materials via ex vivo analyses using a minimally invasive procedure.

GFs arise from living integumental tissue in avian species. The living portion (pulp) of a GF is a column approximately 8-10 mm in height with a 2-3 mm diameter. The inner dermal layer of GF is completely surrounded by an epidermal layer and an outer connective tissue
sheath (Lucas and Stettenheim, 1972). Each GF has its own network of blood vessels supplying nutrients and access to damaged or infected cells by the immune system (Lucas and Jamroz, 1961). A method developed in our laboratory involves micro-injection of test materials into the dermis of GFs and collection of individual feathers minutes, hours, or days after injection (Erf and Ramachandran, 2016). Along with continuous monitoring of immune cell infiltration patterns and activities in the local tissue (injected GF), a blood sample can be taken at the same time points as tissue samples allowing for concurrent monitoring of local and systemic immune responses.

The overall aim of this dissertation project is to examine the cellular and cytokine responses to various PAMPs in chickens. Specifically, we utilize the growing feather as a dermal test site to address three main objectives:

**Objective 1:** To determine the cellular infiltration and recruitment patterns in the local tissue (GF pulp) and systemic blood over a 24 h time frame following injection of endotoxin free PBS (vehicle control) or different doses of LPS into the dermis of GF. Levels of heterophils, macrophages, total lymphocytes, and various lymphocyte subpopulations were determined via immunofluorescent staining of pulp cell suspensions or automated hematology analysis at 0 h (before injection), 2, 4, 8, and 24 h post GF injections. Preliminary gene expression analyses at the transcriptome level were also carried out using LPS or PBS injected GF with focus on key genes of innate immunity: IL-1β, IL-10, CXCL8, and inducible nitric oxide synthase (iNOS).

**Objective 2:** To determine avian cellular immune cell infiltration patterns to three doses of PGN and three doses of MDP in the local injected tissue (pulp of GF) and the peripheral blood over a 7 d time course in layer type chickens. Heterophil, macrophage, total lymphocyte, and lymphocyte subpopulations were determined via immunofluorescent staining of pulp cell
suspensions or automated hematology analysis at 0 h (before injection), 4 h, 6 h, 8 h, and 1, 2, 3, 5, and 7 d post PGN, MDP, or control injections.

Objective 3: To compare the immune responses of age and line matched layer type chickens to various bacterial and viral PAMPs in vivo. The same concentration of LPS, PGN, or poly I:C (1 μg/GF) was injected in the pulp of GF and cellular infiltration and gene expression profiles in the injected pulp were followed over a 3 d time course. Relative proportions of heterophils, macrophages, NK cells, total lymphocytes, and lymphocyte subpopulations in pulp tissue were determined by immunofluorescent staining of pulp cell suspensions. To gain insight into functional activities initiated by PAMPs in injected pulp, the expression of interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-12α, IL21, CXCL8, IFNα, IFNβ, IFNγ, and LITAF were examined.
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Literature Review
Commercial chickens are exposed to bacterial and viral pathogens which have the potential to cause both morbidity and mortality. Selection pressure and genetic mutations allow pathogens to change and acquire the genetic information necessary to escape vaccines and drugs the poultry industry uses to prevent and treat disease (Stewart et al., 2013). Pressure from both customers and consumers of poultry products is driving the industry away from the use of antibiotics and other drugs to improve performance and prevent disease. Modulation of the chicken’s own innate immune system presents an interesting opportunity to prevent disease and potentially improve flock performance.

Vaccine companies rely on materials known as adjuvants to improve the response to a vaccine’s antigenic component. Adjuvants can be organic or inorganic molecules that stimulate an innate immune response, recruiting innate and adaptive immune cells to the site of injection. Ultimately this increases the opportunities for T and B cells to recognize their specific antigen and development into long-lived, antigen-specific memory cells; preventing morbidity or mortality upon exposed to the fully pathogenic or wild-type pathogen. Modulation of current vaccines to include organic, immunostimulatory molecules such as those naturally occurring on bacteria or viruses have been shown to improve the efficacy of vaccines (Brown, 2010; Seya, 2010; Martins et al., 2015).

Current research is considering the use of natural immunostimulatory molecules from bacteria, viruses, and fungi as an avenue for prophylactic treatment of a flock prior to microbial challenge (St. Paul et al., 2013). While these immunostimulatory molecules would not protect against a specific disease or provide protection as long lasting as a vaccine, they could prime the animal to respond faster or stimulate the development of immature, innate immune cells into mature cells (Kumar et al., 2010; St. Paul et al., 2013). However, research is needed to
understand how these microbial immunostimulatory molecules interact with the avian immune system and could be used to prevent disease.

**Introduction to the Innate Immune System**

Innate immunity is found in virtually all life forms including bacteria and plants. Unlike the adaptive immune system, which is capable of differentiating between closely related species of bacteria, viruses, or fungi (e.g. *Salmonella enterica* subsp. Typhimurium as different from *Salmonella enterica* subsp. Heidelberg), the innate immune system recognizes broad features common to groups of pathogens known as pathogen associated molecular patterns or PAMPs (Abbas et al., 2014). Recognition of PAMPs occurs through pattern recognition receptors (PRRs) which are located on the host cell surface as well as in endosomal vesicles and in the cytosol, allowing the innate immune system to come in contact with pathogens that may reside outside of the cells, have been endocytosed by a phagocyte, or reside within a host cell’s cytosol (Keestra et al., 2013). Interactions between PAMPs and PRRs initiate the production and secretion of cytokines, chemokines, and other downstream pathways that drive the innate immune system toward an inflammatory or antiviral response (Xagorari and Chlichlia, 2008; Kumar et al., 2011). Unlike adaptive immunity’s T and B cells where each cell has a homogenous set of antigen-receptors that recognize one specific pathogen, innate immune cells present a wide range of PRRs that recognize and respond to a variety of pathogens.

As physical barriers, the skin and mucosal tissues serve as the first line of defense against pathogens (Abbas et al., 2014). Specialized immune cells (e.g. dendritic cells, mast cells, tissue-resident macrophages, Langerhans cells, B1-B cells, etc.) reside in the skin and mucosal tissues and are capable of recognizing and initiating immune responses to invading pathogens (Egbuniwe et al., 2015). These specialized tissue resident cells respond to the pathogens in part
by releasing proteins to stimulate a variety of responses including recruitment of immune cells to the site of infection.

A polymorphonuclear (PMN) cell, known as a heterophil in avian species, is functionally similar to the mammalian neutrophil and is the first cell type to be recruited to the site of infection (Kogut et al., 2005; Genovese et al., 2013). Avian heterophils destroy both pathogens as well as microbial infected host cells through wide array of techniques including phagocytosis, release of toxic granules, and production of immunostimulatory molecules (Genovese et al., 2013). As reviewed in Genovese et al. (2013), upon heterophil recognition of a PAMP through a PRR, downstream signals induce the heterophil to phagocytose opsonized or non-opsonized pathogens. Once the microbe has been internalized in the phagosome compartment by the cells, the phagosome fuses with the lysosome compartment which contains toxic granules. Fusion of the two compartments results in the destruction of the pathogen by the generation of reactive oxygen species and activation of bactericidal factors and enzymes originating from the heterophil’s granules (Kogut et al., 1994; Genovese et al., 2013). In some cases heterophils can be triggered to release toxic granules outside of the cell surface membrane killing non-phagocytosed pathogens, but also causing damage to the host tissue.

The classic neutrophil attractant chemokine in humans is CXCL8 (previously known as interleukin (IL-) 8), and has two functional analogs in chickens: CXCLi1 and CXCLi2 which have been shown to induce heterophils (CXCLi1) and monocytes (CXCLi2) to leave the blood, enter the tissue and migrate to the site of infection (Wigley and Kaiser, 2003; Abbas et al., 2014; Schat et al., 2014). Interferon-gamma (IFNγ) enhances the phagocytic abilities of chicken heterophils and mammalian neutrophils including an increase in oxidative burst and degranulation activities as well as increased cytokine production (Kogut et al., 2001; Farnell et
al., 2003; Ellis and Beaman, 2004). A range of PAMPs were found to stimulate in vitro gene expression of key inflammatory cytokines such as IL-1β (inflammation and pyogenesis inducing cytokine), IL-6 (acute inflammatory cytokine), and IL-10 (anti-inflammatory cytokine) in peripheral heterophils from a commercial layer line (Kogut et al., 2005). In vitro stimulation of heterophils from commercial broiler or layer lines with live Salmonella enteriditis bacteria did not upregulate mRNA expression of IL-10 or IL-6 in heterophils [expression of IL-1β was not examined (Redmond et al., 2009)].

Upon encounter with a pathogen, dendritic cells, macrophages, or other tissue resident cells release chemotaxis signals recruiting monocytes from the blood into the tissue where they differentiate into macrophages (Chu and Dietert, 1988; Qureshi et al., 2000; Abbas et al., 2014). In both mammalian and avian species, macrophages preform several functions necessary for the recognition and destruction of invading pathogen and recruitment and stimulation of the T and B lymphocytes (Wigley, 2013). In chickens and turkeys, macrophages were shown to be functional at day of hatch (Qureshi et al., 2000).

Macrophages constantly sample the local environment via pinocytosis, endocytosis, and receptor mediated phagocytosis (Abbas et al., 2014). These processes allow PRR on macrophage surface and endosomal membranes to come into contact with microbes. PRR recognition of PAMPs stimulates lysozyme vesicles to fuse with the phagosome or endosome and generate nitric oxide or reactive oxygen species to destroy the pathogen inside the combined phagolysosome (Sung et al., 1991; Bowen et al., 2009). Multiple studies have shown that avian macrophages are capable of phagocytosis of bacteria (Campylobacter jejuni, E. coli, Pasteurella multocida, and Salmonella enterica), fungi (Candida albicans), and several immunomodulators including beta-1,3-1,6-glucan, beta 1-4 mannobiose, and LPS (Rossi and
Macrophages are one of the most potent producers of cytokines and chemokines early in the immunological response to pathogens (Abbas et al., 2014). Depending on specific PAMP-PRR interactions, macrophages are stimulated to enter either inflammatory or antiviral pathways of gene transcription and cytokine and chemokine production (Abbas et al., 2014; Schat et al., 2014). PAMPs have been shown to increase macrophage gene expression and production of the key pro-inflammatory cytokines (IL-1β and IL-6) when added to macrophages in culture. (Weining et al., 1998; Gyorfy et al., 2003; Rath et al., 2003; Smith et al., 2005; Schneider et al., 2011). In mammals, tumor necrosis factor (TNF)-α is another key pro-inflammatory cytokine; however, the avian homolog of TNF-α has not been identified in chickens (Schat et al., 2014). In chicken HD11 macrophage cell cultures, LPS-induced TNFα factor (LITAF) is reported to have similar biological properties as mammalian TNF-α and may serve as the avian equivalent to TNF-α (Lillehoj et al., 2001). Similar to mammals, expression of IL-10 (anti-inflammatory cytokine) and IL-18 (part of the IL-1 family of inflammatory cytokines) were upregulated in avian macrophages cultures (De Lima et al., 2014; Schat et al., 2014). Together these and other secreted factors function to recruit and control the influx of innate and adaptive cells to the site of infection.

Constant sampling of the surrounding environment via pinocytosis and receptor mediated endocytosis allows macrophages to bind, process, and display foreign antigens on their cell surface to cells of the adaptive immune system (Harmon and Glisson, 1989; Qureshi, 2003; Schat et al., 2014). Macrophages also express MHC class II molecules which enables them to function as antigen presenting cells and activators of adaptive immunity. The process of antigen
presentation involves expression of antigen/peptide/MHC class II complexes on the surface of the antigen presenting cell (e.g. macrophages). Binding of this complex by specific TCR on CD4+ T cells, is the first signal for T cell activation. Interactions with other molecules on the T cell and macrophage provides the second signal required for activation of CD4+ T cells and differentiation into cytokine secreting T helper cells. Antigen presentation by macrophages also drives CD4+ Th cells to differentiate into Th1 or Th2 subtypes, which produce a number of cytokines involved in leukocyte recruitment, B cell production of high-affinity antibodies, and development of fully functional cytotoxic T lymphocytes (CTLs; Abbas et al., 2014). T helper cells are the orchestrator of adaptive immunity, therefore the ability of macrophages to present antigen to CD4+ T cells is an important link between the two arms of the immune system.

Macrophages also produce cytokines which aide in the maturation of T and B cells into their effector cell functions such as release of T cell cytokines by T helper cells, cytotoxic killing by CD8+ T cells, and antibody production by B cells (Qureshi et al., 2000; Abbas et al., 2014; Schat et al., 2014).

Unlike other innate immune cells which develop from a common myeloid progenitor cell, natural killer (NK) cells along with T and B cells are of lymphoid origin (Abbas et al., 2014). However, unlike T and B cells in which each individual cell expresses a homogenous set of antigen receptors, NK cells do not recognize one specific antigen, but instead respond to a variety of pathogenic molecules (i.e. PAMPs) and do not need to undergo clonal expansion to perform their effector functions (Abbas et al., 2014). Functionally NK cells belong to the innate immune system and were first described to kill virally infected host cells and since have been shown to kill cells infected or damaged by either viruses or bacteria (Rogers et al., 2008; Abbas et al., 2014; Schat et al., 2014). Research with NK cells in avian species has focused on the in
vivo response of NK cells to diseases caused by viruses. Reports are contradictory as to the recruitment of NK cells into the spleen or intestinal epithelial lumen in response to infectious bursal disease virus and Marek’s disease virus (Rogers et al., 2008). NK cells have shown increased activity in vitro to IFNγ (Merlino and Marsh, 2002), but not after in vivo oral administration of IFNα (Jarosinski, K. W. et al., 2001).

Eosinophils and basophils have been described in avian systems (Lucas and Jamroz, 1961), and in mammals play an important role in defense against helminths and in allergy responses (Abbas et al., 2014). However, compared to other leukocytes such as heterophils and macrophages the functionality of eosinophils and basophils in chickens and other avian species has not been well determined (Schat et al., 2014). Using a range of intravenous injected immunostimulatory materials (e.g. horse serum, material from spontaneously occurring eosinophilia chicken, etc.), Maxwell (1980) was unable to increase the levels of eosinophils in chickens. However intra-dermal injection of chickens challenged with dinitrochlorobenzene (DNCB) or citraconic anhydride (CA) increased eosinophil and heterophil levels in the skin indicating a potential role of eosinophils in early inflammatory responses (Maxwell, 1984).

Dendritic cells (DCs) along with macrophages and B cells are professional antigen presenting cells (APCs) which play a vital role in antigen presentation to the adaptive immune system as well as secretion of cytokines and chemokines to recruit immune cells to the site of infection (Olah et al., 1979; Olah and Glick, 1995; Schat et al., 2014). It is only recently that avian immunologists have gained the necessary tools (i.e. recombinant cytokines, DC specific primary antibodies, and sequencing of the avian genome) to isolate avian dendritic cells. Indeed it was only in 2009 that the functional DCs were described for the first time in an avian system (Wu et al., 2009).
Heterophils, macrophages, NK cells, and other cells of the innate immune system work together to provide early warning and defense against bacterial, viral, or fungal infection. These cells are able to respond within minutes to hours of first exposure to pathogens by expression of a wide range of receptors that recognize features common across groups of pathogens.

**Pathogen Associated Molecular Patterns**

It is well established that the innate immune system recognizes pathogens through soluble and cell-associated (cytosolic, endosomal, and plasma membrane) pattern recognition receptors [PRRs (Abbas et al., 2014; Schat et al., 2014)]. The ligand for PRRs are proteins, nucleic acids, carbohydrates, lipids, or a combination of these molecular motifs that remain invariant within a class of microbes and are known as pathogen associated molecular patterns (PAMPs). Several common features are found among PAMPs including: foreign to the host (i.e. double stranded RNA), evolutionarily conserved among a kingdom or family of microbes (i.e. β-glucans from fungi), and vital for the survival of the pathogen such as peptidoglycan, an important structural component of bacteria (Sorrell and Chen, 2009; Abbas et al., 2014; Fieber and Kovarik, 2014). Binding of a PAMP to its specific PRR can initiate a variety of immune responses depending upon the PAMP and the cell involved.

* Lipopolysaccharide (LPS)

As the primary endotoxin in Gram-negative bacteria, lipopolysaccharide (LPS) forms a constitutive layer in the outer membrane of Gram-negative bacteria. The negative charge generated by the lipid and polysaccharide components of LPS is critical to maintaining membrane structure and repelling certain types of chemical attacks (Ki et al., 1994; Varbanets, 2014). Recognition of PAMPs by leukocytes occurs through a variety of PRRs, but the largest and most well studied family of PRRs is the toll-like receptor (TLR) family. In both mammalian
and avian species, the innate immune receptor for LPS is TLR 4 (Karnati et al., 2014; Portou et al., 2015). Binding of LPS to TLR 4 has been determined to induce a significant inflammatory response in the host, characterized by fever and leukocyte (heterophil and macrophage) infiltration at the site of infection (Richardson et al., 1989; Islam and Pestka, 2006; Gray et al., 2013).

In vivo examination of cellular recruitment profiles after exposure to LPS or Gram-negative bacteria in chickens are rare. Oral challenge studies in broiler chickens revealed that heterophil and mononuclear cells infiltrate the mucosal layers of the intestinal lumen after challenge with Gram-negative Salmonella bacteria (Henderson et al., 1999). Intravenous injection of LPS in layer-type chickens decreased peripheral blood heterophil and monocyte concentrations 1 h after exposure to LPS, followed by a rapid, 3.5- and 2-fold increase in heterophils and monocytes concentration at 24 and 48 h post injection, respectively (Bowen et al., 2009). In the same study, blood monocytes from peripheral blood mononuclear cells (PBMC; monocytes, thrombocytes, and lymphocytes) increased expression of nitric oxide upon re-exposure to LPS in vitro. Similar studies in mice and humans have reported drops in neutrophil and monocyte concentrations followed by rapid increases in respective concentrations within 24 h of intravenous injection of LPS (Sultzer and Alerts, 1969; Richardson et al., 1989).

Similar to reported studies with mammalian cells, LPS induces changes in cytokine and chemokine expression in avian leukocytes which can be measured using targeted gene-expression analyses at the transcriptome level (Fan and Cook, 2004; Abbas et al., 2014; De Lima et al., 2014). Primary circulatory macrophages (mononuclear cells which adhered to plastic cell culture plates) from 3 to 5 month old broiler chickens exposed to LPS in culture for 2 h increased expression of IL-1β by 21-fold (Bliss et al., 2005). Cells from the HD11 chicken macrophage
cell line increased expression of IL-6, IFNα, and IFNB genes 2 hours after LPS exposure (Lian et al., 2012). Chicken PBMCs (monocytes, thrombocytes, and lymphocytes) from various breeds showed increased transcriptional expression of interleukin (IL)-1β, -2, -6, CXCL8 and IFNB after treatment with LPS in vitro (Karnati et al., 2014).

*Peptidoglycan (PGN) and Muramyl Dipeptide (MDP)*

Peptidoglycan (PGN), is an important structural motif common to all bacteria, recognized by several PRR, including TLR 2, nucleotide-binding oligomerization domain (NOD) 1 and 2, and peptidoglycan receptor proteins (PGRPs; Girardin and Philpott, 2004; Dziarski and Gupta, 2005). Depending upon the species of bacteria, PGN makes up roughly 40-80% of the dry weight of all Gram-positive bacteria. It comprises several layers of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) sugars crosslinked via 3-5 amino acids attached to the NAM sugars, providing bacteria with the membrane rigidity necessary for survival (Stewart-Tull, 1980; Winn et al., 2006).

As a critical structural component of all bacteria, PGN is recognized by the innate immune systems of many life forms including birds, mammals, insects, and plants (Girardin and Philpott, 2004). Recognition of the whole PGN structure occurs through the cell surface receptor, TLR 2 (Martinon et al., 1930; Keestra et al., 2013; Wigley, 2013). However, smaller units of PGN are capable of stimulating an immune response in both mammals and birds through receptors found in the cytosol of leukocytes (Cirardin and Tedin, 2003; Girardin et al., 2003). Muramyl dipeptide (MDP) is recognized by the NOD2 receptor and is the smallest subunit of PGN capable of initiating an immune response (Dziarski and Gupta, 2005; Schat et al., 2014). This receptor has not been annotated in the chicken genome (Schat et al., 2014).
In the 1970’s, PGN and MDP were recognized as active immunostimulatory components of Freund’s Complete Adjuvant and continue to remain important components of adjuvant research (Stewart-Tull, 1980; Traub et al., 2006). Avian studies on immunostimulatory properties of PGN and MDP are rare. However, in vitro work with chicken peripheral blood heterophils and macrophage cell lines suggests that PGN increases transcriptional expression of the inflammatory genes IL-1β, IL-6, and CXCL8 (Kogut et al., 2005; He et al., 2011a). Intra-dermal injection of Gram-positive bacteria (*Mycobacterium butyricum*) in growing feathers (GF) of *Mycobacterium butyricum* sensitized chickens showed that leukocytes infiltrated the GF pulp (injection site) by 6 h p.i., the earliest time-point examined. Heterophils were the primary leukocyte type recruited early in the response; however, monocytes/macrophages and lymphocytes also infiltrated the injected GF, reaching peak levels 2 and 3 d p.i. before returning to pre-injection levels (Erf and Ramachandran, 2016).

Research with many other animal species reveal strong immune cell recruitment after MDP treatment (Girardin et al., 2003; Traub et al., 2006; Schaffler et al., 2014). In chickens and quail, MDP has been shown to have little immunostimulatory effect in vivo or in vitro (Neumann et al., 1982; Rojs et al., 2000; Koutsos and Klasing, 2001). However, in chickens immunized with live infectious bursal disease virus and then treated with the MDP synthetic analog (LK415) or a commercial immunostimulatory control, the LK415-treated chickens showed less morbidity and a faster recovery as well as increased humoral immune responses when compared to the immunostimulatory control (Rojs et al., 2000). Also previous research in our laboratory showed that when peripheral blood mononuclear cells were stimulated with high doses of MDP, expression of the inflammatory gene IL-1β was upregulated (unpublished observations).
Together these results suggest that MDP is recognized by avian leukocytes and capable of stimulating an immune response.

*Polyinosinic-Polycytidylic Acid (poly I:C)*

The double stranded RNA (dsRNA) analog polyinosinic-polycytidylic acid (poly I:C) is commonly used in host immune response and vaccine adjuvant research as a substitute for viral genetic material (Martins et al., 2015). In mammalian and avian species, poly I:C is recognized by multiple PRRs including TLR 3, melanoma differentiation-associated factor 5 (MDA5), and retinoic acid-inducible gene I [RIG-I (Takeuchi and Akira, 2007; Chen et al., 2013; Schat et al., 2014)]. In mammals, receptor mediated signaling of poly I:C recognition occurs primarily through TLR 3 (an endosomal receptor) and MDA5 (a cytosolic receptor) and to a lesser extent through RIG-I recognition with high-molecular weight poly I:C (Martins et al., 2015). Unlike other avian species including ducks and geese, chickens lack the cytoplasmic RIG-I receptor, which may play a role in the susceptibility of chickens to avian influenza (Chen et al., 2013).

In vitro studies with poly I:C indicate that monocytes are stimulated to increase transcriptional expression of both inflammatory (IFNγ and IL-10) and anti-viral (IFNα and IFNβ) cytokines (He et al., 2011b). The HD11 macrophage cell line increased production of nitric oxide upon pre-exposure to IFNγ in culture followed by stimulation with poly I:C (He et al., 2011a). In heterophils, oxidative burst and degranulation were induced after in vitro stimulation with poly I:C; however, expression of IL-1β, IL-6, and CXCL8 were downregulated compared to controls (Kogut et al., 2005).

Mammalian and avian immunologists are beginning to understand the important role the innate immune system plays in early control of an infection and in the stimulation of and interaction with the adaptive immune system. Research in this field has increased in recent years,
but there remains a lack of general knowledge regarding the cellular and molecular responses of the innate immune system to PAMPs in vivo.

**Growing feathers as a Dermal Injection Site to Study the Avian Immune System**

Primary characterization of the avian immune response to PAMPs has occurred through *in vitro* examination of cytokine gene expression and phagocytosis, nitric oxide production, generation of reactive oxygen species, degranulation, and other effector functions (Kogut et al., 2003, 2005; He et al., 2007, 2011a; b; Nerren et al., 2010; Arsenault et al., 2013). These *in vitro* studies provided information on the ability of a single cell type to recognize and respond to PAMPs. However, during an infection in a living bird, immune cells function in concert with many different cell types and soluble factors in manners that can only be understood with *in vivo* studies. Relatively few studies have characterized the *in vivo* immune response to individual PAMPs in chickens.

The simplest *in vivo* methods to study the avian immune responses to PAMPs included presentation of PAMPs to skin derivatives such as wing webs, wattles, and food pads (Awadhiya et al., 1981; Cotter et al., 1987; Pillai et al., 1988). Measurements can range from simple markers of inflammation such as swelling, redness, and fever, to excision of injected tissue to study leukocyte infiltration patterns (Smith et al., 1975; Itho et al., 1989; Zhu et al., 1999). Examination of markers of inflammation (swelling, redness, and fever) allow researchers to determine whether a test material initiates an immune response and the degree of the response, but gives little information regarding the specific types and activities of cells recruited to the injection site. Sampling of the injected tissue via biopsy or sacrifice of the animal for tissue collection provides information regarding recruited cell types and their interactions, but is
limited by the number of samples taken from one animal. As a result, it is difficult to observe the complete immune response from initiation to resolution.

One of the best methods to characterize an immune reaction from initiation to resolution in a single animal is via the skin window. Utilization of this method requires an animal to be denuded of hair or feathers in the region to be examined. After a 24-48 h recovery period, the region is gently abraded and a sterile or test material soaked glass cover slip is placed over the abraded skin and periodically removed and replaced with a fresh sterile cover slip (Fekety Jr., 1969; Smith et al., 1975). Recruited leukocytes adhere to the slide and by removal and replacement of the cover slip, researchers can characterize the recruited leukocytes at predetermined intervals [i.e. minutes, hours, days after initiation of immune response; (Fekety Jr., 1969; Smith et al., 1975)]. However, this method can be relatively painful for the test animal due to abrasion of the skin and partial immobilization required to keep the glass coverslip in place, and results are confounded by injury associated with the abrasion and repeat removal of the cover slips. Utilization of the skin window method in chickens is particularly difficult as the wing web is the normal test area and requires complete immobilization of the wing to keep the glass slide attached (Smith et al., 1975). Nevertheless, the skin window method has remained one of the best methods for examination of immune responses in the same individual over time. A novel method developed in our laboratory utilizes the natural characteristics of avian growing feathers (GF) to serve as a window into in vivo tissue/cellular activities initiated by intra-dermal injection of test material without the need to immobilize the animal or denude and abrade the skin (Erf and Ramachandran, 2016).

Growing feathers are modified integument tissue with the same epidermal and dermal layers as smooth avian or mammalian skin, but form a column similar to hair or fur embedded in
a follicle that is surrounded by a hard outer connective tissue sheath (Lucas and Stettenheim, 1972). The living portion (pulp) of a GF is a column approximately 8-10 mm in height with a 2-3 mm diameter (Lucas and Jamroz, 1961; Lucas and Stettenheim, 1972). A network of blood vessels are located throughout the GF and provide nutrients for continued growth and a way for immune cells to access damaged or infected tissue (Lucas and Jamroz, 1961).

As described by Erf and Ramachandran (2016), the avian growing feather (GF) presents a unique opportunity to examine both local tissue responses as well as systemic blood responses to injected test materials. Eighteen days before injection of the test material, feathers along the breast tract are plucked and allowed to regrow to insure uniform size and age at the beginning of the experiment. On the first day of the experiment, GFs are prepared for injection by removing the emerging barbs and portion of the sheath above the epidermal cap with scissors, and injected with 10 μL of the test material using 0.3 mL syringes with 0.01 mL gradation and 31 x 8 mm gauge needles. By injecting several GF at the same time, and collecting one or more injected GFs at the desired time points (minutes, hours, or days after injection) thereafter, in vivo responses in the GF initiated by injection of PAMPs can be examined ex vivo. Collection of GF can be achieved by gently pulling the loosely attached growing feather from its follicle, providing a tissue sample for ex vivo analysis with minimal trauma to the bird.

Utilizing a variety of ex vivo analyses, a great deal of information can be gained from the tissue sample. Changes in leukocyte profiles can be monitored via preparation of a single cell suspension of the GF followed by immunofluorescent staining and flow cytometric analysis to determine individual cell populations. Location, distribution, relative amounts, and cellular interactions among leukocyte present in the injected GF can be examined using conventional histology procedures or immunohistochemistry. Collected GF can also be used for gene-
expression studies to gain insight into functional activities initiated in the injected tissue. Along with monitoring the immune response at the local tissue injection site (GF), the systemic response to the injected material can also be monitored by concurrent blood sampling. Collected blood can be used to examine the effects of the GF injection on blood leukocyte profiles and gene expression, levels of soluble factors such as antibodies, cytokines, nitric oxide and reactive oxygen species production, acute phase proteins, etc. The avian growing feather provides a unique opportunity to monitor local and systemic response to test materials in vivo and specifically provides an exciting opportunity to examine the cellular recruitment and gene expression profiles of chickens to PAMPs.
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CHAPTER I

Characterization of the in vivo inflammatory response in blood and local tissue following intra-dermal injection of varying doses of lipopolysaccharide.
Introduction

The innate immune system recognizes pathogens through a diverse system of germline encoded protein receptors (pattern recognition receptors; PRRs). The PRR family is one of the earliest forms of pathogen recognition as indicated by similar receptors found across kingdoms and phyla, including plants, vertebrate and invertebrate animals (Neyen and Lemaitre, 2016). Unlike the adaptive immune system which utilizes gene rearrangement to recognize small portions of molecular features unique to a specific pathogen, the innate immune system recognizes motifs that are common across groups of microbes. When these pathogen associated molecular patterns (PAMPs) are recognized by PRR on innate immune cells, divergent immune responses such as inflammation or antiviral responses are initiated.

In both mammalian and avian species, the most well characterized PAMP-PRR interaction is lipopolysaccharide (LPS) and toll-like receptor 4 (TLR 4). As a negatively-charged structural component of the outer membrane of Gram-negative bacteria, LPS is critical for survival of these bacteria (Winn et al., 2006). Recognition of LPS by TLR 4 on tissue resident leukocytes, such as macrophages, induces an inflammatory response characterized by chemokine and cytokine induced vascular changes and rapid recruitment of phagocytes from the blood to the infected tissue (Sultzer and Alerts, 1969; Richardson et al., 1989; Bowen et al., 2009; Abbas et al., 2014).

Stimulation and recruitment of innate leukocytes is critical to early control of microbes. Recruited avian heterophils and monocytes/macrophages phagocytose microbes which are then killed by oxygen-dependent and oxygen-independent mechanisms in a membrane bound compartment inside the cell (Qureshi et al., 2000; Wigley, 2013). Macrophages display the resulting microbial fragments on their cell surface for stimulation and recruitment of other
leukocytes such as T and B lymphocytes (Qureshi, 2003). Macrophages are one of the most potent producers of cytokines and chemokines early in the immunological response to pathogens and depending on the cytokine milieu, induce cells to enter inflammatory or antiviral pathways, recruit leukocytes to the site of infection, and hone adaptive immune responses (Abbas et al., 2014).

Studies examining the cellular recruitment of leukocytes after exposure to LPS or Gram-negative bacteria in chickens are rare. Oral challenge studies with Salmonella in broiler chicks showed heterophil and mononuclear cell infiltration into the mucosal layers of the intestinal lumen (Henderson et al., 1999). Intravenous (i.v.) injection of LPS in chickens resulted in an initial decrease in heterophil and monocyte concentrations in the blood at 1 h post injection followed by a rapid 3.5 fold increase in heterophil concentrations and 2 fold increase in monocyte concentrations at 24 and 48 h post injection respectively (Bowen et al., 2009). Bowen et al. (2009) also showed increased production of nitric oxide by peripheral blood mononuclear cells (PBMC; monocytes, thrombocytes, and lymphocytes) upon re-exposure to LPS in vitro.

Similar to observations in mammalian cells (Fan and Cook, 2004; Abbas et al., 2014; De Lima et al., 2014), changes in chemokine and cytokine expression after LPS stimulation of avian leukocyte could be demonstrated by targeted gene-expression analyses at the transcriptome level. Exposure of primary macrophage cultures (blood mononuclear cells which adhered to plastic cell culture plates) from 3 to 5 month old broiler chickens to LPS for 2 h increased expression of interleukin (IL-) 1β by 21 fold (Bliss et al., 2005). Cells from the HD11 chicken macrophage cell line exhibited increased expression of IL-6, interferon (IFN) α, and IFNβ genes 2 h after LPS exposure (Lian et al., 2012). Similarly, chicken PBMCs from various breeds showed increased
transcriptional expression of IL-1β, -2, -6, -8, and IFNβ after in vitro LPS treatment (Karnati et al., 2014).

To date, no studies have been reported in which the inflammatory response triggered by tissue injection of LPS was examined in the peripheral circulation as well as at the local tissue site in the same individual birds from initiation to resolution of the response. As described in Erf and Ramachandran (2016), using growing feathers (GF) as a novel skin test site provides a unique opportunity to examine and monitor in vivo effects of LPS. Using this minimally invasive method we can monitor the immune response at the local tissue injection site and, when used in conjunction with blood sampling, in the systemic circulation of the same individual. With the growing feather (GF) as the dermal tissue injection site, we conducted a 24 h time-course study examining the local (GF dermal tissue) and systemic (blood) leukocyte responses as well as local cytokine responses to three different concentrations of LPS.

MATERIALS AND METHODS

Experimental Animals Non-vaccinated male and female layer-type chickens were raised in HEPA filtered rooms in the Poultry Health Laboratory (Arkansas Experiment Station, University of Arkansas, Fayetteville, AR) in floor pens on wood shaving litter. Food and water were available ad libitum and standard light and temperature protocols were followed (Shi and Erf, 2012). Animal use was approved by the University of Arkansas Institutional Animal Care and Use Committee (approval #15021).

Injection of Growing Feathers with 1, 0.1, or 0.01 µg of Lipopolysaccharide Eighteen days before injection, ten growing feathers at various stages of development from the right and left breast tracts of 16 experimental animals were plucked to ensure regenerating growing feathers (GF) were of uniform size and age at injection day when the chickens were 9-wks of
age. To prepare GF for injection, the emerging barbs and portion of the sheath above the epidermal cap were cut off with scissors as described in Erf and Ramachandran (2016). Using 0.3 mL syringes with 0.01 mL gradation and 31 x 8 mm gauge needles (BD, Franklin Lakes, NJ), prepared GF were injected with 10 µL of endotoxin free saline control (Dulbecco’s phosphate buffered saline; PBS; Sigma, St Louis, MO) or 10 µL of 100, 10, or 1 µg/mL of LPS from *Salmonella typhimurium* (Sigma, St Louis, MO). These treatments will be referred to as PBS, LPS 1, LPS 0.1 and LPS 0.01, respectively, reflecting the actual amount of LPS injected into each GF (0, 1, 0.1, or 0.01 µg). Each treatment group consisted of 2 male and 2 female chickens (n = 4 per treatment). Two injected GF were collected from each chicken before injection (0 h) and at 2, 4, 8, and 24 h post injection. Collected GF were placed in cold Dulbecco’s PBS (PBS; Sigma, St Louis, MO) or in room temperature RNA preservation buffer (RNAlater® Thermofisher, Waltham MA) and maintained at their respective temperatures until processing. Additionally, heparinized syringes (3 mL with 25 x 1 gauge needles) were used to collect 0.5 to 1 mL of blood from the wing vein of each bird at each GF collection time point. Blood samples were used for same day blood profile analysis via automated hematology (Cell-Dyn, Abbott Diagnostics, Abbott Park, IL).

**Preparation of Pulp Cell Suspensions, Immunofluorescent Staining, and Cell Population Analyses by Flow Cytometry** Feather pulp suspensions were prepared on day of collection as described in Erf and Ramachandran (2016). Briefly the living epidermal and dermal tissue (pulp) was removed from the GF and stored in ice cold PBS. The isolated pulp tissue was then incubated in 0.1% collagenase/dispace (Collagenase type IV, Life Technologies, Carlsbad, CA; Dispase II, Sigma-Aldrich, St. Louis, MO) at 40°C for 15 min and pushed through a 60 µm nylon mesh to prepare single cell suspensions. Pulp cell suspensions were washed and
resuspended in PBS+ buffer (PBS, 1% bovine serum albumin and 0.1% sodium azide; VWR, Radnor, PA) for immunofluorescent staining with a panel of fluorescently labeled (FITC, PE, or SPRD) mouse-monoclonal antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) using a three-color direct staining procedure. Specifically, all suspensions were labeled with a pan leukocyte marker (CD45-SPRD) to identify and gate around all leukocytes before determination of individual leukocyte subpopulations. Individual leukocyte subpopulations determined included: macrophages (MHCII-FITC+KUL01-PE+), B cells (Bu-1-FITC+IgM-PE+), CD4+ lymphocytes (CD4-FITC+), CD8+ lymphocytes (CD8-PE+), γδ T cells (T cell receptor (TCR)1-PE+), αβ1 T cells (TCR2-FITC+), and αβ2 T cells (TCR3-PE+). Total lymphocyte populations were determined by addition of B cells, γδ T cells, αβ1 T cells, and αβ2 T cells estimates for each cell suspension. Due to the lack of chicken heterophil specific antibodies, heterophil populations in pulp cell suspensions were determined based on size (FSC) and granularity (SSC) characteristics of CD45+ leukocytes as described in Seliger et al. (2012). A pool of all cell suspensions was incubated with a cocktail of FITC, PE, and SPRD labeled mouse IgG1 isotype controls to confirm absence of non-specific binding of labeled antibodies (all mouse IgG) and to set the cut-off between fluorescence positive and negative populations. Pooled cells suspensions were also single stained with either CD45-FITC, CD45-PE or CD45-SPRD to set compensation for three color analysis. Cell population analysis was carried out using a Becton Dickinson FACSort flow cytometer equipped with a 488-nm argon laser (BD Immunocytometry Systems, San Jose, CA) and the percentage of each leukocyte population in the pulp cell suspensions was determined using CellQuest software (BD, Franklin Lakes, NJ).

**RNA Isolation, Quantification, and cDNA Synthesis** Pulp tissue from GF stored in RNAlater® (Ambion, Waltham, MA) were removed as described above and stored at -20°C until
RNA isolation following a gradual decrease in temperature as described in the RNAlater® protocol manual. Pulps from injected GF were homogenized by Tissue Tearor™ (BioSpec Products, Inc, Bartlesville, OK, Model: 985370-395) in lysis buffer provided in the Qiagen RNeasy® Mini kit (Qiagen Inc., Valencia, CA) and total RNA was isolated from homogenates using the same kit. Following the procedure described in Hamal et al. (2010) RNA integrity and concentration were determined and RNA (1.5 µg/sample) was transcribed to cDNA using a High Capacity cDNA reverse transcription kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA).

**Relative Expression of Cytokines** Target gene primers and probes used in this study are listed in Table 1. Real-time PCR was performed according to Hamal et al. (2010). A pool of cDNA from non-injected feather pulps was used as the calibrator sample. The relative gene expression was determined by the delta delta Ct (ΔΔCt) method (Wong and Medrano, 2005) and data were expressed as fold change relative to the calibrator sample.

**Blood Profile Analysis via Automated Hematology (CellDyn)** Blood cell concentrations for total WBC, heterophils, monocytes, lymphocytes, basophils, eosinophils, and thrombocytes were determined using an automated hematology analyzer (Cell-Dyn; Abbott Diagnostics, Abbott Park, IL) optimized for chicken blood analyses.

**Statistical Analysis** The experimental unit is the individual chicken with 4 chickens per treatment (PBS, LPS 1, LPS 0.1, and LPS 0.01). Using Sigma Plot 13 Statistical Software (Systat Software, Inc., San Jose, CA), a two-way Repeated Measures Analysis of Variance (RM ANOVA) was conducted to determine significant effects of time, treatment, and treatment by time interaction and followed by a multiple means comparison (Holm-Sidak method) of main effects of time and treatment where no significant interactions were found. When significant
interactions were determined, a protected Student’s t-test of time was performed for each treatment group (PBS, LPS 1, LPS 0.1, and LPS 0.01) using JMP 12 software (SAS Institute Inc., Cary, NC). For all analyses, differences were considered significant at $P \leq 0.05$.

**RESULTS**

Following a two-way repeat measure analysis of variance (ANOVA), significant interactions ($P \leq 0.05$) were determined for heterophil, monocyte, and lymphocyte peripheral blood concentrations as well as heterophil and macrophage pulp proportions. Therefore, a protected Student’s t-test was conducted for each treatment within in each cell type to determine significant effects of time. Where no significant interactions were identified, data were analyzed using the main effect of time or treatment by a multiple means comparison with the Holm-Sidak method.

*Changes in peripheral blood concentrations and proportions of pulp cells after intra-dermal injection of PBS*

Injection of the sterile saline control (PBS) in GF significantly increased heterophil GF pulp proportions at 2 and 8 h post injection (p.i.). Tissue proportions returned to pre-injection levels by 24 h. No significant changes in neither heterophil GF proportions, monocyte/macrophage blood concentrations and GF proportions, nor in total lymphocyte blood concentrations were found following PBS injection into GF. Total lymphocyte proportions in GF were lower following intra-dermal (i.d.) injection of PBS (main effect of time $P = 0.001$; Figure 1).

*Intra-dermal injection of LPS induced changes in peripheral blood concentrations and proportions of pulp cells*
Before GF injection (0 h) no significant differences among LPS treatments were observed in heterophil, monocyte, total lymphocyte, or lymphocyte subpopulations in either the peripheral blood or pulp cell suspensions. A significant time by treatment interaction effect \((P \leq 0.002)\) was observed in all populations measured except with GF pulp proportions of total lymphocytes and lymphocyte subpopulations (B cells, CD4+, CD8+, and all T cell receptor (TCR) defined cells). GF pulp proportions of total lymphocyte and all lymphocyte subpopulation were however affected by time (main effect \(P < 0.01\); Figure 1).

**Heterophils**

Peripheral blood heterophil concentrations for the lowest dose (LPS 0.01) were significantly increased at 2 h compared to pre-injection levels and continued to increase until reaching maximum concentrations at 8 h p.i. Heterophil concentrations in the blood returned to pre-injection levels by 24 h p.i. following LPS 0.01 injection into GF.

Injection of the highest dose of LPS in GF resulted in a drop in peripheral blood heterophil concentrations immediately (2 h) after injection. The medium dose of LPS (LPS 0.1) induced an observable but not significant drop in heterophil concentrations at 2 h p.i. At 4 h p.i., the drop in peripheral heterophil concentrations for both LPS 1 and LPS 0.1 had reversed and heterophil levels were significantly increased compared to pre-injection levels reaching maximal concentrations at 8 h p.i. before returning to at or near pre-injection levels at 24 h (Figure 1).

In GF, injection of the lowest dose of LPS resulted in significantly elevated levels (% pulp cells) of heterophils at 2, 4 and 8 h p.i., whereby maximal levels were observed at 2 h and 8 h, with slightly lower levels \((P \geq 0.05)\) at 4 h p.i. (Figure 1). Proportions of heterophils in the GF pulp returned to near pre-injection levels by the end of the study (24 h p.i.).
Injection of the higher LPS doses (LPS 0.1 and 1) into GF resulted in significantly elevated heterophil proportions at 2 (LPS 0.1) and 4 (LPS 1) h p.i. Proportions of heterophils continued to increase and reached maximum levels at 8 h post LPS 1 (highest dose) injection. Proportions of heterophils reached maximum levels at 4 h post LPS 0.1 injection in GF and did not significantly change between 4 and 8 h. Heterophil proportions returned to pre-injection levels by 24 h p.i. for LPS 0.1 and near pre-injection levels for LPS 1 (Figure 1).

Monocytes/Macrophages

No significant changes occurred in peripheral blood monocyte concentrations after pulp injection of LPS 0.01 compared to pre-injection levels. Peripheral blood monocyte concentrations increased slightly at 8 h post LPS 0.1 injection in GF (1.27 ± 0.24 K/µL at 0 h to 2.93 ± 0.88 at 8 h). A significant drop in monocyte concentrations was observed at 2 h p.i. with the highest dose of LPS (LPS 1). Monocyte concentrations then increased at 4 and 8 h p.i., and returned to pre-injection levels at 24 h (Figure 1).

Increase proportions of macrophages in the pulp of GF were observed beginning at 2 h post LPS 0.01 injection and 8 h post LPS 0.1 and LPS 1 injection. Macrophage proportions in injected GF reached maximal levels at 8 h p.i. for all three LPS dosages and remained elevated at the end of the experiment (24 h p.i.).

Total Lymphocytes and Lymphocyte Subpopulations

Following GF injection, significant decreases in peripheral blood lymphocyte concentrations were observed with all three LPS dosages. The time and duration of the decreases in blood lymphocyte concentrations varied with the dose of LPS injected into GF. Specifically, for LPS 0.01 the drop was observed at 4 h p.i., for LPS 0.1 at 2 and 4 h p.i., and for the highest LPS dose (LPS 1) at 2, 4, and 8 h p.i., with lymphocyte concentrations reaching the lowest levels.
at 4 h for LPS 0.01 and LPS 0.1 and at 2 h for LPS 1. For all LPS treatments, lymphocyte concentrations had returned to pre-injection by the 24 h time-point (Figure 1).

Proportions of total lymphocytes in the pulp of injected GF exhibited no treatment by time interaction effect. The observed significant main effect ($P = 0.001$) of time suggested an overall decrease in total lymphocyte concentrations beginning at 2 h p.i. and remaining until the end of the experiment for all treatments (PBS and LPS injections). The decrease in total lymphocytes could not be attributed to any one subpopulation of lymphocytes (B cell, CD4+ lymphocytes, CD8+ lymphocytes, or all TCR defined populations; Figure 2).

**Gene Expression Analysis**

The overall qualitative and quantitative aspects of leukocyte infiltration into injected GF were similar for the two highest doses of LPS (LPS 0.1 and LPS 1), therefore only RNA isolated from GF pulps injected with LPS 0.1, LPS 0.01, and PBS were processed for gene expression analysis. Two-way repeated measures ANOVA revealed a significant main effect of time, no effect of treatment and no treatment by time interactions regarding the expression of any target genes assessed [interleukin (IL-) 1β, IL-10, CXCL8, or inducible nitric oxide synthase (iNOS)].

Multiple means comparisons revealed greatly increased expression (fold change) of IL-1β at 4 h p.i., which was numerically highest with LPS 0.1 (mean fold change in gene expression of $3573.70 \pm 2419.58$; Figure 3). Expression of the chemoattractant, CXCL8, was also upregulated at 4 h p.i. for all treatments (main effect of time $P = 0.001$) with the greatest change in gene expression observed with LPS 0.1 ($10003.5 \pm 4925.8$). No significant differences in IL-10 expression were determined, although observable results indicate a several hundred-fold increase for all treatments at various time points. At 4 h p.i., expression of iNOS was upregulated compared to 0, 8, and 24 h (main effect of time $P = 0.006$).
DISCUSSION

The current study is the first to simultaneously monitor the effects of intra-dermally administered LPS on leukocyte levels in the blood as well as leukocyte levels and cytokine/chemokine activities at the site of injection (i.e. dermis of GF) in the same chicken. Observations made here also confirm similarities between the avian and mammalian inflammatory response initiated by LPS (Sultzer and Alerts, 1969; Moeller et al., 1978; Richardson et al., 1989; Bowen et al., 2009; Abbas et al., 2014). Notably, as shown in Figure 1, heterophils were the first cell type recruited into the LPS injected tissue with all LPS dosages (2-4 h p.i.). Heterophil recruitment was accompanied or followed by increases in macrophages. Heterophils remained the predominant phagocyte early in the LPS response (2-8 h p.i.), while macrophage levels (% pulp cells) remained near maximal infiltration levels at 8 and 24 h for all LPS doses. Heterophils are the first leukocyte recruited to a site of infection and only live for a short time in inflamed tissues. Macrophages however are considered the more professional phagocyte, with a diverse array of pattern recognition receptors, and better ability to respond to and produce a variety of inflammatory mediators as well as initiating tissue healing and restoring tissue homeostasis (Abbas et al., 2015).

Using different dosages of LPS, we were able to identify distinct leukocyte recruitment profiles in both the blood and at the local injection site with a low dose of LPS versus 10 to 100 fold higher doses. Heterophils in the blood and at the local injection site increased within 2 h following i.d. GF injection of the low LPS dose (LPS 0.01 = 0.01 µg/GF), remained elevated at 4 and 8 h p.i. and returned to normal within 24 h. Whereas with the higher dosages of LPS, heterophil concentration in the blood dropped by 2 h p.i. before increasing substantially above pre-injection levels by 4 h, reaching maximal levels at 8 h and decreasing to normal (LPS 0.1) or
near normal (LPS 1) by 24 h p.i. (Figure 1). Compared to the low dose LPS, infiltration of heterophils into the injected pulp was delayed by 2 h, but increased substantially by 4 h p.i. and remained elevate at 8 h (LPS 0.1) or increased above pre-injection levels at 8 h p.i. (LPS 1) and both doses of LPS returned to near pre-injection levels by 24 h (Figure 1).

Based on heterophil profiles alone, it would appear that the low dose resulted in a local inflammatory response where local events signaled recruitment of heterophils from the blood into the affected tissue and stimulated more heterophil production and/or their release into the peripheral circulation. The higher LPS doses on the other hand resulted in heterophil blood concentration changes similar to those described in chickens (Bowen et al., 2009) and humans (Richardson et al., 1989) as a result of intravenous (i.v.) injection of LPS. Specifically, Bowen et al. (2009) reported a significant decrease in the peripheral blood concentration of heterophils at 1 h post LPS i.v. injection before a rapid and high increase (2.5 fold) in heterophils concentrations a few hours later. Thus it appears that the higher LPS doses used for intra-dermal injection likely allowed for some of the injected LPS to enter the blood stream causing a response similar to that seen with i.v. injection.

In our study, macrophage infiltration in GF was already evident by 2 h p.i. when the low dose of LPS (LPS 0.01) was injected into the pulp while significant levels of macrophage infiltration were not evident until 8 h p.i. with the higher LPS dosages. Independent of LPS dose used, macrophage levels in injected pulp remained elevated at 24 h. However, while heterophil infiltration into LPS injected pulp was accompanied with increased levels of heterophils in the blood, macrophage infiltration was not accompanied or preceded by increased levels of monocytes in the blood. In fact, especially for the high LPS doses, a drop in monocyte concentrations in the blood was still evident at the time macrophage levels in the injected tissue
increased. This drop in circulating monocytes may be explained by vascular adhesion of monocytes (i.e. adherent monocytes would not be included in the blood sample), followed by gradual extravasation of monocytes into injected tissues.

For lymphocytes, concentrations in the blood and proportions in the GF decreased 2 to 4 h after pulp injection of any LPS dose. While levels of lymphocytes in the blood recovered by 8 and 24 h, normal lymphocyte levels in injected pulp were not restored by 24 h. The reason for the lower lymphocyte levels in blood and LPS injected pulp are not clear, but cytokines produced at sites of inflammation are known to stimulate expression of T and B cell chemokines in secondary lymphoid organs in mice and humans (Abbas et al., 2016). Recruitment of lymphocytes to lymphoid organs would explain their lower circulating levels. An overall main effect of time revealed a decrease in total lymphocytes in the pulp of PBS and all LPS injected GF that could not be attributed to any one specific lymphocyte subpopulation (Figure 2), not even IgM+ B cells or γδ T cells, which are known to have barrier and innate functions. This drop in tissue lymphocytes likely is also due to lymphocyte recruitment to the secondary lymphoid organs initiated by inflammatory signals generated in response to sterile wounding (PBS) and the pro-inflammatory effects of LPS.

To gain preliminary insight into functional activities initiated by LPS administration in the local tissue, the transcription of four target genes expected to be differentially expressed in response to LPS was examined. These included the pro-inflammatory cytokine IL-1β, anti-inflammatory cytokine IL-10, chemoattractant CXCL8, and inducer of nitric oxide synthesis (iNOS; Abbas et al., 2014; Schat et al., 2014). While substantial numerical increases in the fold expression of these target genes could be observed post LPS injection into the pulp of GF (Figure 3), statistical analyses revealed only increased expression of CXCL8 and iNOS at 4 h p.i (main
effect of time; $P \leq 0.01$). However, the numerical changes observed here in vivo are in agreement with increased transcription of these target genes in avian heterophils and monocytes/macrophages stimulated with LPS in vitro and seem biologically relevant (Kogut et al., 2001, 2005; Farnell et al., 2003; Qureshi, 2003; Bliss et al., 2005; He et al., 2011; Genovese et al., 2013). The extensive variation observed for the gene expression data in our in vivo study may reflect the low number of experimental animals ($n = 4$ per treatment) and/or the inherent variation in extent and time-course of an individual’s up and down regulation of gene expression. Future analysis of gene expression using GF as a dermal test site may benefit from an increase in the number of experimental animals.

Together the data presented here reveal that i.d. injection of LPS in the pulp of GF is primarily mediated by an increase of heterophils in the blood and local tissue as well as smaller increases in macrophage proportions in the tissue. Dose of LPS injected affects the time course and quantitative aspects of the cellular response in blood and local tissue, which may be due in part to LPS entering the circulatory system when high doses of LPS are injected (0.1 and 1 µg of LPS/GF). Collectively, the data reported here confirm that the avian GF can be used as a cutaneous test site to monitor the cellular immune responses to PAMPs in the local injected tissue (GF) and, when used in combination with blood sampling, leukocyte concentrations in the peripheral blood. Moreover, considering that monocyte concentrations in the blood exhibited different alterations in response to tissue injection of LPS than those observed in the injected tissue (Figure 1), the data emphasize that the blood is not always a clear window into events occurring in affected tissues.
REFERENCES


Available at http://dx.doi.org/10.1016/j.dci.2013.04.008.


Table 1: Primer and probe sequences\(^1\) for target genes

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<th>Target</th>
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<th>Accession NO.</th>
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<td>Reverse</td>
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\(^1\) Primer and probe oligos were synthesized by Eurofins MWG Operon LLC, Huntsville, AL.

\(^2\) Sequences were from Smith et al. (2005)
Figure 1. Heterophil, macrophage, and total lymphocyte blood cell concentrations and pulp cell proportions after intra-dermal (i.d.) injection of lipopolysaccharide (LPS) or endotoxin free PBS (control) into growing feathers. Twenty growing feathers (GF) of sixteen 9-wk-old male and female chickens were injected with PBS (control), 0.01, 0.1, or 1 µg of LPS per GF. Blood (0.5-1 mL) and one injected GF from each chicken was collected at 0 (before injection), 2, 4, 8, and 24 h post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to identify chicken macrophages and total lymphocytes. Analysis of cell populations was carried by flow cytometry and percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+). An automated hematology analyzer (Cell-Dyn) was used to determine heterophil, monocyte, and lymphocyte blood cell concentrations (10³ cells/µL). Data shown are mean ± SEM; n = 4 per time point and treatment group.
Figure 2. Infiltration of T and B cell subpopulations after intra-dermal (i.d.) injection of lipopolysaccharide (LPS) or endotoxin free PBS (control). Twenty growing feathers (GF) of sixteen 9-wk-old male and female chickens were injected with PBS (control), 0.01, 0.1, or 1 µg of LPS per GF. One (1) injected GF from each chicken was collected at 0 (before injection), 2, 4, 8, and 24 h post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to chicken lymphocyte populations: B cells, CD4+ cells, CD8+ cells, γδ T cells, αβ1 T cells, and αβ2 T cells. Analysis of cell populations was carried by flow cytometry. Data shown are mean ± SEM; n = 4 per time point and treatment group.
Figure 3. Relative expression of IL-1β, IL-10, CXCL8, and iNOS from the pulp of growing feathers injected with endotoxin free PBS (control) or lipopolysaccharide (LPS). Twenty growing feathers (GF) of sixteen 9-wk-old male and female chickens were injected with PBS (control), 0.01, or 0.1 µg of LPS per GF. RNA was isolated from one injected GF from each chicken collected at 0 (before injection), 2, 4, 8, and 24 h post injection and reverse transcribed into 1.5 µg of cDNA/sample. Relative expression of IL-1β, IL-10, CXCL8, and iNOS was calculated based on the delta delta CT method, using cDNA from non-injected GF pulp as the calibrator and chicken 28S as the endogenous control. Data shown are mean ± SEM; n = 4 per time point and treatment group.
CHAPTER II

Assessment of local and systemic in vivo cellular responses initiated by intra-dermal injection of peptidoglycan or muramyl dipeptide, cell wall products of Gram-positive bacteria.
Introduction

Peptidoglycan (PGN) is an protein and sugar polymer forming several layers of a Gram-positive bacterium’s cell wall and is directly responsible for 40-80% of the bacterial dry weight depending upon the species (Winn et al., 2006). The cell wall of Gram-negative bacteria also contains PGN, but only in a single layer. Alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) sugars crosslink via 3-5 amino acids attached to the NAM sugar to form a mesh-like structure, providing bacteria with the structural strength required for survival (Winn et al., 2006).

Unlike the adaptive immune system which recognizes minute differences between specific pathogens, the innate immune system recognizes features common to groups of pathogens known as pathogen associated molecular patterns (PAMPs). PAMPs are foreign to the host (i.e. double stranded RNA), evolutionarily conserved among a kingdom or family of microbes (i.e. β-glucans from fungi), and vital for the survival of the pathogen [i.e. PGN, the structural component of bacteria (Sorrell and Chen, 2009; Abbas et al., 2014; Fieber and Kovarik, 2014)]. Recognition of PAMPs by the host’s innate immune system occurs through pattern recognition receptors (PRRs). PRRs are expressed on the surface of endosomal and plasma membranes as well as in the cytosol, ensuring that the immune system can respond to pathogens located both externally as well as inside host cell compartments (Abbas et al., 2014; Schat et al., 2014). Binding of PAMPs to PRRs initiates an immune response specific to the PAMP and the leukocytes expressing the PRR (i.e. an inflammatory vs anti-viral response).

In both mammalian and avian species, PGN is recognized by the PRR toll-like receptor 2 (TLR 2; Martinon et al., 1930; Keestra et al., 2013; Wigley, 2013; Abbas et al., 2014), and in mammals CD14 has also been shown to be a receptor for PGN (Dziarski and Gupta, 2005). In
mammals, small fragments of PGN such as γ-glutamyl diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) are recognized by the cystolic PRRs, nucleotide-binding oligomerization domain 1 and 2 (NOD1 and NOD2) respectively (Cirardin and Tedin, 2003; Girardin et al., 2003; Dziarski and Gupta, 2005; Schat et al., 2014). Muramyl dipeptide (MDP) is the smallest subunit of PGN capable of stimulating an immune response, but recognition of MDP is inconclusive in chickens as the NOD2 receptor has not been annotated in the chicken genome (Schat et al., 2014).

In the 1970’s PGN and thus MDP were discovered to be key immunostimulatory components in Freund’s Complete Adjuvant (Stewart-Tull, 1980). Both PGN and MDP remain important components of adjuvants and adjuvant research to this day (Traub et al., 2006). However little is known regarding direct PGN or MDP stimulation of the innate immune system in chickens. Previous research in our laboratory with MDP stimulation of peripheral blood mononuclear cells in culture, showed that high doses of MDP upregulated the expression of the inflammatory IL-1β gene (unpublished observations).

While no in vivo studies on the effect of PGN in birds were found, a recent study by Erf and Ramachandran (2016) examined the local tissue response to injection of Gram-positive bacteria in chickens. Using the pulp of growing feathers as a novel skin test-site, Erf and Ramachandran (2016) examined local leukocyte infiltration patterns in response to intra-dermal injection of *Mycobacterium butyricum* during a 7 d time course in the same individual. Heterophil infiltration into the pulp was evident within 6 h (the earliest time course examined) of *M. butyricum* injection. The increase in heterophils was accompanied by mononuclear cell infiltration (monocytes/macrophages and lymphocytes) which reached peak levels on day 2 and 3, while heterophil levels returned to pre-injection levels. Intravenous injection of MDP was
found in Japanese quail (Koutsos and Klasing, 2001) and chickens (Neumann et al., 1982) to elicit little to no immunostimulatory activity. However, chickens immunized with live infectious bursal disease virus and then treated with the MDP analog (LK415) showed less morbidity and faster recovery time as well as increased humoral immune responses when compared to injection of a commercially available immunostimulatory control (Rojs et al., 2000). Together these results suggest that Gram-positive bacteria (and thus potentially PGN) stimulate a heterophil, macrophage, and lymphocyte response in chicken. The immune response to MDP is however still unconfirmed, but it is possible that chickens are responsive to MDP.

The use of the growing feather (GF) as a dermal test-site, presents unique opportunity to examine the cellular recruitment patterns of PGN and MDP in chickens using minimally invasive methods. When combining the GF injection method with concurrent sampling of the blood, insight will be gained into innate immune activities initiated by PAMPs both at the local tissue site and in the systemic circulation. The purpose of the current study is to use this novel approach to examine and monitor the immunostimulatory effects of intra-dermal administered PGN and MDP on the local injected tissue and the peripheral blood.

**Materials and Methods**

*Experimental Animals* Twenty-eight (28) male and female layer-type chickens (11 to 12 wks of age) were raised in HEPA filtered rooms in the Poultry Health Laboratory (Arkansas Experiment Station, University of Arkansas, Fayetteville, AR) in floor pens on wood shaving litter. Food and water was available ad libitum and standard light and temperature protocols were followed (Shi and Erf, 2012). Animal use was approved by the University of Arkansas Institutional Animal Care and Use Committee (approval #15021).
**Injection of Growing Feathers with PBS, PGN, or MDP**

Eighteen days before injection, 10 growing feathers at various stages of development from the right and left breast tracts of all experimental animals were plucked to ensure uniform size and age of regenerating growing feathers (GF) at time of injection. To prepare GF for injection, the emerging barbs and portion of the sheath above the epidermal cap were removed with scissors as described in Erf and Ramachandran (2016). Prepared GF were injected with 10 µL of the endotoxin free saline control (Dulbecco’s phosphate buffered saline; PBS; Sigma, St Louis, MO) or 10 µL of 5, 50, or 500 µg/mL of soluble PGN (Invivogen; San Diego, CA) or 10 µL of 5, 50, or 500 µg/mL of MDP Invivogen; San Diego, CA). These treatment groups will hence be referred to as PBS, PGN 0.05, PGN 0.5, PGN 5, MDP 0.05, MDP 0.5, and MDP 5, respectively, reflecting the actual amount of control or test material injection into each GF (0 (PBS), 0.05, 0.5, or 5 µg of PGN or MDP).

Each treatment group consisted of 4 male (PBS and MDP) or female (PGN) chickens. One PBS and PGN injected GF was collected at 0 (before injection), 0.17 (4 h), 0.33 (8 h), 1, 2, 3, 5, and 7 d post injection for PBS and PGN injected GF. One MDP injected GF was collected at the same time points as PBS and PGN except a 6 h time point was included instead of the 4 and 8 h time points. Injected feathers were stored in cold PBS (Sigma, St Louis, MO) or room temp RNAlater® (Ambion, Waltham, MA) until processing. Additionally, heparinized syringes (3 mL with 25 x 1 gauge needles) were used to collect 0.5 to 1 mL of blood from the wing vein of each bird at each GF collection time point. Blood samples were used for same day blood profile analysis via automated hematology (Cell-Dyn, Abbott Diagnostics, Abbott Park, IL).

**Preparation of Pulp Cell Suspensions**

Feather pulp suspensions were prepared on day of collection as described in (Erf and Ramachandran, 2016). Briefly the living epidermal and
dermal tissue (pulp) was removed from the GF and stored in ice cold PBS. The isolated pulp
tissue was then incubated in 0.1% collagenase/dispase solution (Collagenase type IV, Life
Technologies, Carlsbad, CA; Dispase II, Sigma-Aldrich, St. Louis, MO) at 40°C for 15 min and
pushed through a 60 µm nylon mesh to prepare single cell suspensions.

**Immunofluorescent Staining and Cell Population Analyses by Flow Cytometry**

Pulp cell suspensions were washed and resuspended in PBS+ buffer (PBS, 1% bovine serum albumin
and 0.1% sodium azide; VWR, Radnor, PA) for immunofluorescent staining with a panel of
fluorescently labeled (FITC, PE, or SPRD) mouse-monoclonal antibodies (Southern
Specifically, all suspensions were labeled with a pan leukocyte marker (CD45-SPRD) to identify
and gate around all leukocytes before determination of individual leukocyte subpopulations.
Individual leukocyte subpopulations determined included: macrophages (MHCII-FITC+KUL01-
PE+), B cells (Bu1-FITC+IgM-PE+), CD4+ lymphocytes (CD4-FITC+), CD8+ lymphocytes
(CD8-PE+), γδ T cells (T cell receptor (TCR)1-PE+), αβ1 T cells (TCR2-FITC+), and αβ2 T
cells (TCR3-PE+). Total lymphocyte populations were determined by addition of B cells, γδ T
cells, αβ1 T cells, and αβ2 T cells estimates for each cell suspension. Due to the lack of chicken
heterophil specific antibodies, heterophil populations in pulp cell suspensions were determined
based on size (FSC) and granularity (SSC) characteristics of CD45+ leukocytes as described in
Seliger et al. (2012). A pool of all cell suspensions was incubated with a cocktail of FITC, PE,
and SPRD labeled mouse IgG1 isotype controls to confirm absence of non-specific binding of
labeled antibodies (all mouse IgG) and to set the cut-off between fluorescence positive and
negative populations. Pooled cells suspensions were also single stained with either CD45-FITC,
CD45-PE or CD45-SPRD to set compensation for three color analysis. Cell population analysis
was carried out using a Becton Dickinson FACSort flow cytometer equipped with a 488-nm argon laser (BD Immunocytometry Systems, San Jose, CA) and the percentage of each leukocyte population in the pulp cell suspensions was determined using CellQuest software (BD, Franklin Lakes, NJ).

**Blood Profile Analysis via Automated Hematology (Cell-Dyn)** Blood cell concentrations for total WBC, heterophils, monocytes, lymphocytes, basophils, eosinophils, and thrombocytes were determined using an automated hematology analyzer (Cell-Dyn; Abbott Diagnostics, Abbott Park, IL) optimized for chicken blood analyses.

**Statistical Analysis** The experimental unit is the individual chicken with 4 chickens per treatment (PBS (control), PGN 0.05, PGN 0.5, PGN 5, MDP 0.05, MDP 0.5, and MDP 5). For statistical analysis, leukocyte population data were separated into different groups based on treatment [i.e. PBS (1 dose), PGN (3 doses), or MDP (3 doses)]. Using Sigma Plot 13 Statistical Software (Systat Software, Inc., San Jose, CA), a one-way Repeated Measures Analysis of Variance (RM ANOVA) of time was performed on endotoxin free PBS samples. Sigma Plot 13 Statistical Software was also used to conduct a two-way RM ANOVA for PGN and MDP treatments to determine significant effects of time, dose, and dose by time interaction. The two-way RM ANOVA for PGN and MDP was followed by a multiple means comparison (Holm-Sidak method) of main effects of time and treatment where no significant interactions were found. In the presence of significant interactions, the effect of time for each dose (PGN 0.05, PGN 0.5, PGN 5 or MDP 0.05, MDP 0.5, MDP 5) was determined by a protected Student’s t-tests using JMP 12 software (SAS Institute Inc., Cary, NC). For all analyses, differences were considered significant at $P \leq 0.05$.

**RESULTS**
Endotoxin free PBS (control) injected growing feathers

Compared to pre-injection levels (0 d), injection of endotoxin free PBS (vehicle-control) into the pulp of GF resulted in increased heterophil levels in the blood (2.65 ± 0.19 K cells/µL at 0 h and 5.30 ± 0.45 K cells/µL at 4 h) and in the injected pulp (0.68 ± 0.11 % pulp cells at 0 h and 3.78 ± 0.42 % pulp cells at 4 h). In both blood and injected GF, heterophils returned to pre-injection levels within 1 d (Figure 1). Injection of PBS into GF did not affect the blood concentrations or the pulp proportions of other leukocytes examined (Figure 1).

Peptidoglycan (PGN) injected growing feathers

Dose by time interactions were observed for heterophil and lymphocyte blood concentrations and for pulp proportions of heterophils and macrophages following intra-dermal (i.d.) injection of PGN into the GF. For all other cell types and measurements, significant main effects of time were observed in the absence of dose by time interactions (e.g. blood monocyte concentrations; proportions (% pulp cells) of total lymphocytes and all lymphocyte subpopulations: B cells, CD4+ cells, CD8+ cells, γδ T cells, αβ1 T cells, and αβ2 T cells). Changes in the levels of these cell populations as a result of GF injection are described across all PGN dosages.

Heterophils

Concentrations of heterophils in the blood increased 8 h after injection of the highest dose of PGN (PGN 5) into GF (a 2.5-fold increase). The lowest dosages of PGN (PGN 0.5 and PGN 0.05) did not increase the concentration of heterophils in the blood; instead a small drop in peripheral heterophil concentrations was observed at 1, 2, and 3 d post PGN 0.05 injection into GF (Figure 2). Heterophil proportions in the pulp of GF increased after injection of all concentrations of PGN; specifically, 15-fold with PGN 5, 8-fold with PGN 0.5, and 4-fold with
PGN 0.05 at the height of response (8 h p.i.). Heterophil proportions increased only at 8 h p.i. for the two highest doses; however, after injection of the lowest dose of PGN (PGN 0.05) heterophil proportions increased at 4 h and remained elevated until 24 h p.i. (Figure 2).

**Monocytes/Macrophages**

There was no dose by time interaction in peripheral blood monocyte concentrations; however, there was a significant main effect of time \((P = 0.001)\) indicating a small drop in monocyte concentrations at 2 d p.i. \((1.22 \pm 0.17 \text{ K cells/µL})\) compared to pre-injection levels \((2.08 \pm 0.17 \text{ K cells/µL})\). In the pulp of PGN 0.5 and PGN 5 injected GF, proportions of macrophages increased 5-fold at 4 and 8 h p.i. before returning to pre-injection levels by 1 d p.i. and stayed at this level throughout the remainder of the study (Figure 2). Proportions of macrophages in the pulp of GF also increased after PGN 0.05 (lowest dose) injection at 4 and 8 h but only a relatively minor 2-fold increase. Numerically macrophage proportions remained elevated at 24 h post PGN 0.05 injection in GF, but statistically were not significantly different from pre-injection levels. By 48 h p.i. macrophage proportions following PGN 0.05 injection were not different from pre-injection levels, numerically and statistically.

**Total Lymphocytes and Lymphocyte Subpopulations**

Overall no changes in peripheral blood concentrations of total lymphocytes were observed except for a 2-fold decrease 8 h after GF injection of PGN 5 and a 1.3-fold decrease 3 d after injection of PGN 0.05. Lymphocyte levels in the blood returned to and remained at pre-injection levels by 1 and 5 d post PGN 5 and PGN 0.05 injections respectively. For all doses of PGN (main effect \(P < 0.05\)), in the local tissue (pulp of GF) proportions of total lymphocytes increased 10- to 15-fold by 1 d p.i. compared to pre-injection levels, remained elevated at 2 d, and began a gradual decrease thereafter back to near pre-injection levels by 7 d p.i. (Figure 2).
Further examination of the contributions of the various lymphocyte subpopulations to the more than 10-fold increase in total lymphocyte levels (% pulp cell) following PGN injection into GF, revealed significantly higher proportions of all lymphocyte subsets [B cells, CD4+ cells, CD8+ cells, and all T cell receptor (TCR) subtypes (γδ, αβ1, and αβ2)] within 1 d p.i. compared to 0 h levels. Only levels of CD8+ lymphocytes were significantly elevated within 8 h p.i. By the end of the experiment (7 d p.i.) levels of all lymphocyte subsets had begun to approach pre-injection levels, although only γδ T cells had fully returned to normal levels (Figure 3).

**Muramyl dipeptide (MDP) injected growing feathers**

For MDP treatments (MDP 0.05, 0.5 and 5), no significant dose by time interactions or main effects of dose were found for any measured parameter (Figure 4). A main effect of time was observed for all peripheral blood and GF measurements except macrophage proportions in the pulp of GF.

**Heterophils**

In the peripheral blood, heterophil concentrations decreased from pre-injection levels at 2, 3, and 5 d p.i., but returned to normal by the end of the experiment. Heterophil proportion in single cell suspensions of MDP injected GF pulps increased 11-fold at 6 h p.i. and returned and stayed at normal levels 1 d p.i. and onwards. Although heterophil proportions from MDP 5 injected GF remained elevated but they were not different from pre-injection levels until 3 d p.i. (Figure 4)

**Monocytes/Macrophages**

Peripheral blood monocyte concentrations decreased 1.5-fold 6 h post MDP injection in GF and returned to pre-injection levels by 1 d p.i. No changes in the GF proportions (% pulp cells) of macrophages were observed after i.d. pulp injection of MDP.
Total Lymphocytes and Lymphocyte Subpopulations

Total lymphocytes concentrations in the blood decreased 6 h after MDP injection in GF, while proportions of total lymphocytes in the pulp increased at the same time point compared to pre-injection levels. Both lymphocyte blood concentrations and pulp proportions returned to normal by 1 d p.i. (Figure 4). Further analysis of lymphocyte subpopulations present in MDP injected GF pulps revealed that proportions (% pulp cells) of all TCR populations (γδ T cells, αβ1 T cells, and αβ2 T cells) were elevated at 6 h p.i. and those of B cells at 1 d p.i. All populations had returned to pre-injection levels by the next time point measured and remained at that level for the remainder of the study (Figure 5).

DISCUSSION

Information on the immunostimulatory effects of cell wall products from Gram-positive bacteria is sparse. Although Gram-positive bacteria, such as mycobacteria, are often included in adjuvants, the in vivo effects of PGN and MDP have not been established in poultry. The current study is the first to describe the leukocyte recruitment patterns initiated by the inflammatory response to these cell wall products both in a complex tissue such as the dermis of growing feathers (GF) and the peripheral blood over the course of 7 days. Using the GF as a cutaneous test site made it possible to examine tissue samples from multiple time points in the same individuals (Erf and Ramachandran, 2016).

Peptidoglycan (PGN)

The in vivo effects of i.d. injected PGN was examined at 3 dosages ranging from 0.05 µg to 5 µg of PGN/GF. Considering the large range in PGN concentrations tested, it was surprising to find no or only minor differences in the leukocyte recruitment responses, both in the injected pulp and in the peripheral blood (Figure 2). Independent of PGN dose, pulp injection resulted in
increased levels of heterophils reaching peak levels in both the blood and injected pulp at 8 h p.i. and returning to pre-injection levels by 24-48 h. In the pulp of GF, 15-, 8-, and 4-fold increases in heterophil proportions were observed after injection PGN 5, PGN 0.5, and PGN 0.05 respectively at the height of the response. Similar temporal and quantitative observations were reported in in vivo mice studies examining leukocyte recruitment into lungs exposed to PGN. Inhalation of PGN resulted in polymorphonuclear cells (assumed to be neutrophils) recruitment into the lungs at 8 times the pre-injection levels 4 h after stimulation. (Leemans et al., 2003).

Heterophil infiltration into PGN injected GF was accompanied by increased levels of macrophages which returned to near pre-injection levels by 2 d. This influx of macrophages into the pulp was not reflected by increased monocyte levels in the blood. Rather, monocyte concentrations dropped gradually following injection of PGN, reaching significantly lower levels of macrophages in the blood on day 2 before gradually increasing again to normal levels by 7 d p.i. The mismatch between increases in macrophage levels in the tissue, but decreases in the blood may be explained by vascular adhesion followed by gradual extravasation of monocytes into injected tissues as previously reported in Chapter 1.

One of the most striking observation was the substantial and rapid infiltration of lymphocytes into injected pulp and their sustained presence at high levels throughout the 7 d study (Figure 2). Infiltrating lymphocytes included similar proportions of B and T cells (Figure 3). The increased proportion (% pulp cells) of lymphocytes in GF was accompanied by a small drop in blood lymphocyte concentrations with the highest dose of PGN by 8 h p.i., but otherwise blood lymphocyte concentrations were at normal levels throughout the 7 d study.

The qualitative, quantitative, and temporal aspects of the response initiated by PGN observed here are substantiated by similar results reported in in vivo studies with cutaneous (pulp
of GF, wing web, and wattle) injection of Gram-positive bacteria (*Mycobacterium butyricum;* Erf and Ramachandran, 2016). The only difference between the *M. butyricum* and PGN responses was a prolonged and heightened macrophage presence during the later part (2 to 7 d) of the response to *M. butyricum* in injected pulps. This difference may be attributable in part to the prior sensitization of chickens with *M. butyricum* in the Erf and Ramachandran study, before injecting *M. butyricum* into GF and other skin derivatives.

The rapid and sustained increase in lymphocyte population after PGN injection in GF is striking, because it is not a response typically associated with PAMP research. However, examination of the literature does hint at the potential for PGN to induce a strong lymphocyte response. Studies with Freund’s Complete Adjuvant (FCA; of which PGN as part of *Mycobacterium* is a major immunostimulatory component) reveals that FCA recruits large numbers of lymphocytes to the injection site (Stewart-Tull, 1980). Mammalian literature reveals that TLR 2 is expressed on B cells and therefore these cells may be directly stimulated by PGN and other TLR 2 agonists (Dziarski and Gupta, 2005). Also as T cells recognize protein antigens (Abbas et al., 2014), it is possible that a subset of T cells specifically recognize the peptide component of PGN, triggering an adaptive immune response. However, with an antigen-specific adaptive immune response, lymphocytes must travel to the lymph nodes and undergo various forms of stimulation and clonal expansion before large numbers of lymphocytes would be recruited to the site of infection (Abbas et al., 2014). Moreover the participation of B cells in a response to *Mycobacterium* and PGN, also suggests that the lymphocyte recruitment and possible local activation and proliferation of B and T lymphocytes in injected GF is likely mediated by toll-like receptors (TLRs) present on both cell types (Szczepanik et al., 2003; Abbas et al., 2014; Schierloh et al., 2014).
In both avian and mammalian species, PGN is thought to stimulate leukocytes by interaction with the plasma membrane bound TLR 2 receptor (Dziarski, 2003; Dziarski and Gupta, 2005; Kogut et al., 2005; Schat et al., 2014). However, the role of PGN as a stimulator of TLR 2 has been questioned in recent years as studies have shown that insoluble (ultra-pure) PGN is incapable of stimulating TLR 2 in mammals (Girardin and Philpott, 2004; Travassos et al., 2004). Traditional methods to purify PGN result in a soluble product that contains both PGN and lipid molecules such as lipoteichoic acid (LTA) which are also capable of stimulating an innate immune response through TLR 2 (Kogut et al., 2005; Abbas et al., 2014; Schat et al., 2014). Ultra-pure (insoluble) PGN is incapable of initiating a TLR 2 response and appears to exert its immunostimulatory effects via the NOD1 or NOD2 pathways (i.e. the MDP pathway; Dziarski, 2003; Girardin et al., 2003; Dziarski and Gupta, 2005). However, studies of PGN stimulation in chickens either make no mention of the solubility of the PGN used or report the use of soluble PGN. The variation in immunostimulatory ability of different PGN products (ultra-pure vs soluble) is not widely known and at the time of purchase, the differences between soluble vs ultra-pure PGN was not identified by the manufacturer of the PGN used in this study (Invivogen, San Diego, CA). Further studies are needed to examine the different immunostimulatory properties of soluble and ultra-pure PGN on the chicken immune system.

*Muramyl Dipeptide (MDP)*

Overall intra-dermal injection of MDP in GF did not change the cellular recruitment profiles in the blood or local tissue beyond the changes observed with vehicle injection (Figure 1, 4, and 5), with the exception of early B and γδ T cell infiltration. The increase of heterophils in the blood and local tissue following injection of vehicle (PBS) is indicative of a wound healing response. The physical act of inserting a needle through the living epidermal and into the dermal
layers of cutaneous tissue causes trauma to the tissue, triggering a wound healing response, characterized by rapid recruitment of neutrophils/heterophils to the site of injury (Singer and Clark, 1999; Dovi et al., 2004; Eming et al., 2014; Portou et al., 2015). Repeat blood sampling has also been shown to increase levels of neutrophils in the peripheral blood without any further stimulation (Abbas et al., 2014).

The observed significant (MDP main effect $P = 0.07$) increases in B cells and $\gamma\delta$ T cells at 1 d p.i. in the current study do suggest some immunostimulatory activity of MDP in chickens. Further phenotyping of the GF infiltrating B cells describes them as Bu-1+ IgM+ lymphocytes which implies a naïve phenotype but also potential classification as a B1-B cell type. B1-B cells as well as $\gamma\delta$ T cells express B and T cell antigen receptors and hence are considered cells of adaptive immunity. However, due to their limited antigen specificity and greatly reduced activation requirements compared to follicular B cells (B2-B cells) and $\alpha\beta$ T cells, B1-B cells and $\gamma\delta$ T cells are considered lymphocytes with functions intermediate to innate and adaptive immunity and as first line defenders against frequently encountered microbes in barrier tissues such as skin and mucosa.

While the recruitment and presence of these B and T cells in MDP injected GF is relatively short lived it does suggest immunostimulatory activity of MDP in chickens. Whether this is a direct effect of MDP is not clear from the current study. In mammals MDP is recognized by the cytosolic NOD2 receptor (Girardin et al., 2003; Abbas et al., 2014; Schaffler et al., 2014). However, NOD2 has not been annotated in the chicken genome (Schat et al., 2014) which may explain the minimal MDP stimulatory effects observed here. Chickens may possess other cytosolic receptors such as peptidoglycan receptor proteins (PGRP) capable of sensing MDP. Another explanation for the minor leukocyte recruitment effects of MDP observed in the current...
study is the administration of MDP in a saline solution without additional components that would facilitate uptake of MDP into the cytosol and provide opportunity for MDP to interact with cytosolic receptors. A strong, direct immunostimulatory effect of MDP may be revealed by further studies facilitating the internalization of MDP by cells (i.e. via liposomes, niosomes, dimethyl sulfoxide, etc.; Colas et al., 2007; Marren, 2011; Pierre and Santos, 2011; Rahimpour and Hamishehkar, 2012).
REFERENCES


Figure 1. Leukocyte peripheral blood concentrations and growing feather (GF) pulp proportions after intra-dermal (i.d.) injection of endotoxin free PBS control. Twenty growing feathers (GF) of four, 12-wk-old chickens were injected with 10 µL of saline control (PBS) per GF. Blood (0.5-1 mL) and one injected GF from each chicken was collected at 0 (before injection), 4 h, 1, 2, 3, 5, and 7 d post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to chicken macrophages, B cells, CD4+ cells, CD8+ cells, γδ T cells, αβ1 T cells, and αβ2 T cells. Analysis of cell populations was carried by flow cytometry and percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+). An automated hematology analyzer (Cell-Dyn) was used to determine heterophil, monocyte, and lymphocyte blood cell concentrations (10^3 cells/µL). Data shown are mean ± SEM; n = 4 per time point and treatment group.
Figure 2. Infiltration of heterophil, macrophage, and total lymphocyte into the peripheral blood and growing feathers after intra-dermal (i.d.) injection peptidoglycan (PGN). Twenty growing feathers (GF) of twelve, 12-wk-old chickens were injected with 0.05, 0.5, or 5 µg of PGN per GF. Blood (0.5-1 mL) and one injected GF from each chicken was collected at 0 (before injection), 4 h, 8 h 1, 2, 3, 5, and 7 d post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to identify chicken macrophages and total lymphocytes. Analysis of cell populations was carried by flow cytometry and percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+). An automated hematology analyzer (Cell-Dyn) was used to determine heterophil, monocyte, and lymphocyte blood cell concentrations (10^3 cells/µL). Data shown are mean ± SEM; n = 4 per time point and treatment group.
Figure 3. Infiltration of lymphocyte subsets into growing feathers after intra-dermal (i.d.) injection of peptidoglycan (PGN). Twenty growing feathers (GF) of twelve, 12-wk-old chickens were injected with 0.05, 0.5, or 5 µg of PGN per GF. One injected GF from each chicken was collected at 0 (before injection), 4 h, 8 h 1, 2, 3, 5, and 7 d post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to chicken lymphocytes: B cells, CD4+ cells, CD8+ cells, γδ cells, αβ1 T cells, and αβ2 T cells. Data shown are mean ± SEM; n = 4 per time point and treatment group.
Figure 4. Infiltration of heterophil, macrophage, and total lymphocyte into the peripheral blood and growing feathers after intra-dermal (i.d.) injection of muramyl dipeptide (MDP).

Twenty growing feathers (GF) of twelve 12-wk-old chickens were injected with 0.05, 0.5, or 5 µg of MDP per GF. Blood (0.5-1 mL) and one injected GF from each chicken was collected at 0 (before injection), 6 h, 1, 2, 3, 5, and 7 d post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to identify chicken macrophages and total lymphocytes. Analysis of cell populations was carried by flow cytometry and percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+). An automated hematology analyzer (Cell-Dyn) was used to determine heterophil, monocyte, and lymphocyte blood cell concentrations (10^3 cells/µL). Data shown are mean ± SEM; n = 4 per time point and treatment group.
Figure 5. Infiltration of lymphocyte subsets into growing feathers after intra-dermal (i.d.) injection of muramyl dipeptide (MDP). Twenty growing feathers (GF) of twelve, 12-wk-old chickens were injected with 0.05, 0.5, or 5 µg of MDP per GF. One injected GF from each chicken was collected at 0 (before injection), 6 h, 1, 2, 3, 5, and 7 d post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to chicken lymphocytes: B cells, CD4+ cells, CD8+ cells, γδ T cells, αβ1 T cells, and αβ2 T cells. Analysis of cell populations was carried by flow cytometry. Data shown are mean ± SEM; n = 4 per time point and treatment group.
CHAPTER III

Cutaneous in vivo responses to varying pathogen associated molecular patterns differ in kinetics, type and amount of cellular contents, and local cytokine gene-expression.
Introduction

With the recent push to reduce antibiotic use in animal agriculture, new mechanisms are needed to prevent and treat disease outbreaks. Modulation of an animal’s own innate immune system presents an interesting opportunity to stimulate the host cells to prevent disease by inducing an inflammatory or anti-viral response. Unlike the adaptive immune system which takes days to weeks to mount a full response, the innate immune system is capable of fully responding to a pathogen within hours of first exposure, preventing and limiting the spread of disease. The innate immune system has evolved to provide the host with early warning of pathogenic invasion by recognizing and responding to molecular features common to groups of pathogens known as pathogen associated molecular patterns (PAMPs). The innate immune system recognizes PAMPs that are common amongst a microbial family or species, critical for the survival of the pathogen, and foreign to the host (Winn et al., 2006; Kumar et al., 2011).

Common PAMPs used in research and medicine include: the outer membrane component of Gram-negative bacteria, lipopolysaccharide (LPS); a structural component of all bacteria, peptidoglycan (PGN); and viral genetic material such as double stranded RNA (dsRNA). Recognition of PAMPs occurs through pattern recognition receptors (PRRs; Abbas et al., 2014) which along with various co-receptors and downstream signaling pathways, upregulate the transcriptional and subsequent protein expression of multiple immune genes including, interleukin (IL-) 1, IL-6, IL-10, CXCL8, interferons (IFN) α, β, γ, and many other cytokines. The in vivo cellular quantitative and qualitative responses to different doses of LPS and PGN after intra-dermal injection in GF have previously been reported in Chapters I and II respectively. Other literature relating to the immunostimulatory effects of LPS and PGN is summarized briefly below.
Both oral challenge studies with *Salmonella* in broiler chicks (Henderson et al., 1999) and intravenous injection of LPS in layer type chickens (Bowen et al. 2009) report increased levels of heterophil and mononuclear leukocyte infiltration into the mucosal layers of the intestinal lumen or the blood, liver, and spleen by 24-48 h post stimulation. Similar increases in neutrophils (avian heterophils) and monocytes have been reported in human and mouse studies after intravenous injection of LPS (Sultzer and Alerts, 1969; Richardson et al., 1989). At the transcriptome level, LPS upregulated the expression of key inflammatory cytokines including IL-1β in primary macrophage cultures (Bliss et al., 2005); IL-6, IFNα, and IFNβ in macrophages from the HD11 macrophage cell line (Lian et al., 2012); and IL-1β, -2, -6, CXCL8, and IFNβ in peripheral blood mononuclear cells (Karnati et al., 2014).

In the 1970’s, PGN was recognized as an active immunostimulatory component of Freund’s Complete Adjuvant and remains an important component of adjuvants and adjuvant research (Stewart-Tull, 1980; Traub et al., 2006). Recognition of PGN by the innate immune system is important as PGN is common to all species of bacteria and as such, is recognized by multiple PRR including TLR 2, nucleotide-binding oligomerization domains 1 and 2 (NOD1 and NOD2), various peptidoglycan recognition proteins [PGRPs; (Girardin and Philpott, 2004; Dziarski and Gupta, 2005; Winn et al., 2006)]. In vitro research with chicken peripheral blood heterophils and macrophage cell lines indicates that transcriptional expression of the inflammatory genes IL-1β, IL-6, and CXCL8 is upregulated upon exposure to PGN (Kogut et al., 2005; He et al., 2011a). Intra-dermal injection of Gram-positive bacteria (*Mycobacterium butyricum*) in growing feathers (GF) showed that leukocytes infiltrated the injection site (pulp of GF) beginning at 6 h post injection (p.i.). Heterophils were the first leukocyte recruited; however, monocytes/macrophages and lymphocytes also infiltrated the *Mycobacterium*
butyricum injected GF, reaching peak levels on 2 and 3 d p.i. before returning to pre-injection levels (Erf and Ramachandran, 2016).

Polyinosinic-polycytidylic acid (poly I:C) is a dsRNA analog, commonly used in vaccine adjuvant and PAMP based innate immunity research (Martins et al., 2015). As an analog to the genetic material of dsRNA viruses, poly I:C is recognized in mammals by multiple PRRs including TLR 3, melanoma differentiation-associated factor 5 (MDA5), and retinoic acid-inducible gene I [RIG-I (Takeuchi and Akira, 2007; Chen et al., 2013; Schat et al., 2014)]. However these three poly I:C receptors are not found in all poultry species. Of particular note, chickens unlike ducks and geese lack the RIG-1 receptor which may contribute to the susceptibility of chickens to avian influenzas (Chen et al., 2013). In vitro studies with poly I:C indicate that peripheral blood monocytes are stimulated to increase expression of both inflammatory (IFNγ and IL-10) and anti-viral (IFNα and IFNβ) cytokines at the transcriptome level (He et al., 2011b). In heterophils, oxidative burst and degranulation were induced after in vitro stimulation with poly I:C; however, expression of IL-1β, IL-6, and CXCL8 was downregulated compared to controls (Kogut et al., 2005).

Stimulation of the innate immune system with PAMPs may provide a way to increase the host’s sensitivity to foreign pathogens, allowing the animal to respond faster or more efficiently to the presence of a disease causing agent. By understanding how the immune system responds to broad categories of pathogens (i.e. Gram-negative bacteria, Gram-positive bacteria, viruses, etc.) we can develop vaccine adjuvants or drug treatments to mimic these immune responses, stimulating the host’s own immune system to kill instead of relying on antibiotics or antivirals, to which pathogens can eventually become resistant. However, first it is necessary to understand how PAMPs interact with the innate immune system and the differences if any in cellular and
molecular changes induced by different PAMPs. This study seeks to understand the local immune responses of chickens to three common PAMPs (LPS, PGN, and poly I:C) using the growing feathers as a dermal test site (Erf and Ramachandran, 2016) to monitor and assess leukocyte infiltration profiles and cytokine/chemokine expression in vivo over the course of 3 day time course.

**Materials and Methods**

**Experimental Animals** Twenty-four (24) non-vaccinated male layer-type chickens (between 12 and 17 wks of age) were raised in HEPA filtered rooms at the Poultry Health Laboratory (Arkansas Experiment Station, University of Arkansas, Fayetteville, AR) in floor pens on wood shavings. Food and water was available ad libitum and standard light and temperature protocols were followed (Shi and Erf, 2012). Animal use was approved by the University of Arkansas Institutional Animal Care and Use Committee (approval #15021).

**Injection of Growing Feathers with Test Material** To ensure uniform size and age, ten growing feathers (GF) at various stages of development from the right and left breast tracts of all chickens were plucked eighteen days before injection and allowed to regrow. To prepare GF for injection, the emerging barbs and portion of the sheath above the epidermal cap were cut off with scissors as described in (Erf and Ramachandran, 2016). Using 0.3 mL syringes with 0.01 mL gradation and 31 x 8 mm gauge needles (BD, Franklin Lakes, NJ), prepared GF were injected with either an endotoxin free saline control (Dulbecco’s phosphate buffered saline; PBS; Sigma, St Louis, MO) or one of three test materials [LPS from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO), soluble PGN from *Staphlococcus aureus* (InvivoGen, San Diego, CA), and low molecular weight poly I:C (InvivoGen, San Diego, CA)]. Each treatment group (PBS, LPS, PGN, and poly I:C) consisted of 6 chickens. The test materials (LPS, PGN, and poly
I:C) were suspended in endotoxin free PBS at a concentration of 0.01 mg/mL and 10 µL of each test material was injected per GF (a final dose of 0.1 µg of test material per GF). At 0h (before injection), and at 4, 8, 24, 48, and 72 h post injection two (injected) GF were collected. One GF was placed in ice cold PBS (Sigma, St Louis, MO) for same day processing. The pulp from the other GF was stored in RNA preservation buffer (RNAlater®; Ambion, Waltham, MA) at -20°C until processing.

**Preparation of Pulp Cell Suspensions, Immunofluorescent Staining, and Cell Population Analyses by Flow Cytometry** Feather pulp suspensions were prepared on the day of collection as described in Erf and Ramachandran (2016); briefly the living epidermal and dermal tissue (pulp) was removed from the GF and stored in ice cold PBS. The isolated pulp tissue was then incubated in a 0.1% collagenase/dispase solution (Collagenase type IV, Life Technologies, Carlsbad, CA; Dispase II, Sigma-Aldrich, St. Louis, MO) at 40°C for 15 min and pushed through a 60 µm nylon mesh to prepare single cell suspensions. Pulp cell suspensions were washed and resuspended in PBS+ buffer (PBS, 1% bovine serum albumin and 0.1% sodium azide; VWR, Radnor, PA) for immunofluorescent staining with a panel of fluorescently labeled (FITC, PE, or SPRD) mouse-monoclonal antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) using a three-color direct staining procedure. Specifically, all suspensions were labeled with a pan leukocyte marker (CD45-SPRD) to identify and gate around all leukocytes before determination of individual leukocyte subpopulations. Individual leukocyte subpopulations determined included: macrophages (MHCII-FITC+KUL01-PE+), natural killer cells (NK cells; CD8α-PE+CD3-FITC-), B cells (Bu-1-FITC+IgM-PE+), CD4+ lymphocytes (CD4-FITC+), CD8+ lymphocytes (CD8+PE-), γδ T cells (T cell receptor (TCR1)-PE+), αβ1 T cells (TCR2-FITC+), and αβ2 T cells (TCR3-PE+). The total lymphocyte population was determined by
addition of B cells, γδ T cells, αβ1 T cells, and αβ2 T cells estimates for each cell suspension. Due to the lack of chicken heterophil specific antibodies, heterophil populations in pulp cell suspensions were determined based on size (FSC) and granularity (SSC) characteristics of CD45+ leukocytes as described in Seliger et al. (2012). A pool of all cell suspensions was incubated with a cocktail of FITC, PE, and SPRD labeled mouse IgG1 isotype controls to confirm absence of non-specific binding of labeled antibodies (all mouse IgG) and to the set cut-off between fluorescence positive and negative populations. Pooled cells suspensions were also single stained with either CD45-FITC, CD45-PE or CD45-SPRD to set compensation for three color analysis. Cell population analysis was carried out using a Becton Dickinson FACSort flow cytometer equipped with a 488-nm argon laser (BD Immunocytometry Systems, San Jose, CA) and the percentage of each leukocyte population in the pulp cell suspensions was determined using FlowJo software (FlowJo, LLC, Ashland, OR).

**RNA Isolation, Quantification, and cDNA Synthesis** Pulps from GF placed into RNALater® were removed as described above and stored at -20°C until RNA isolation following a gradual decrease in temperature described in the RNALater® manual. Pulps from injected GF were homogenized by Tissue Tearor™ (BioSpec Products, Inc, Bartlesville, OK, Model: 985370-395) in TRIzol® provided with the Direct-Zol RNA MiniPrep Kits (Zymo Research, Irvine, CA) and total RNA was isolated from homogenates using the same kit. Following Hamal et al. (2010), RNA integrity and concentration were determined and RNA (1.5 µg/sample) was transcribed to cDNA using a High Capacity cDNA reverse transcription kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA).

**Relative Expression of Cytokines** Target gene primers and probes used in this study are listed in Table 1. Real-time PCR was performed according to Hamal et al. (2010). A pool of
cDNA from non-injected feather pulps was used as the calibrator sample. The relative gene expression was determined by the delta delta Ct (ΔΔCt) method (Wong and Medrano, 2005) and data expressed as fold change relative to the calibrator sample.

**Statistical Analysis** The experimental unit is the individual chicken with 6 chickens per treatment (PBS, LPS, PGN, and poly I:C). Using Sigma Plot 13 Statistical Software (Systat Software, Inc., San Jose, CA 95110), a two-way Repeated Measures Analysis of Variance (RM ANOVA) was conducted to determine significant effects of time, treatment, and treatment by time interaction. The two-way RM ANOVA was followed by a multiple means comparison (Holm-Sidak method) of main effects of time and treatment where no significant interactions were found. In the presence of significant interactions, the effect of time was determined within each treatment group (PBS, LPS, PGN, and poly I: C) by a protected Student’s t-test of time using JMP 12 software (SAS Institute Inc., Cary, NC). For all analyses, differences were considered significant at \( P \leq 0.05 \).

**RESULTS**

Following a two-way repeated measures analysis of variance (RM ANOVA), significant \( (P \leq 0.005) \) treatment by time interactions were observed for all cell populations and most mRNA expression data analyzed except IL-6, IL-21, CXCL8, and IFNβ. Where no significant interactions were identified, data were analyzed using the main effect of time or treatment. In the presence of significant treatment by time interactions, data were analyzed by a protected Student’s t-test for each treatment within each cell type to determine significant effects of time. Before injection (0 h) no differences among treatments were observed for any of the measured cell population or mRNA expression data.
Changes in proportions of pulp cells after intra-dermal injection of PBS, LPS, PGN, and poly I:C

Heterophils

In growing feathers (GF) proportions of heterophils increased after injection of LPS, PGN, or poly I:C beginning at 4-8 h post injection (p.i.) and returned to pre-injection levels by 48 h. Vehicle control (endotoxin free PBS) injection did not alter the proportions of heterophils in the pulp. Of the four treatments (PBS, LPS, PGN, and poly I:C), injection of LPS into GF recruited the highest proportions of heterophils into the local tissue (pulp of GF), an almost 50-fold increase in heterophil proportions compared to pre-injection levels at the height of response (8 h p.i.; Figure 1). After injection of LPS, proportions of heterophils began increasing at 4 h post injection (p.i.), reached maximum levels at 8 h, decreased beginning at 24 h, and returned to pre-injection levels by 48 h p.i.

Proportions of heterophils in the local tissue increased 9- and 8-fold after injection of PGN and poly I:C (respectively) at the height of the response. As indicated, the relative change in heterophil proportions after PGN and poly I:C injection were similar, but the absolute number of heterophils in the tissue were quite different at the height of respective responses: 8.79 ± 2.25 % of pulp cells with PGN injection and 3.85 ± 0.56 % with poly I:C injection. As with LPS injected GF, PGN induced recruitment of heterophils into the pulp beginning at 4 h p.i.; heterophil proportions (% pulp cells) then increased to maximal levels at 8 h p.i. and returned to pre-injection levels by 24 h. Recruitment of heterophils into the tissue reached maximal levels at 4 h post injection of poly I:C (earliest time point measured), began decreasing at 8 h, and returned to pre-injection levels by 48 h p.i. (Figure 1).

Macrophages
Injection of LPS, PGN, and poly I:C in GF significantly increased the proportions of macrophages in the local tissue, although macrophage proportions did not differ between LPS, PGN, or poly I:C treatments at the height of response (8 h; \( P > 0.05 \); Figure 1). Injection of PBS did not alter the proportions of macrophages in the pulp of GF at any time point.

Macrophage proportions (% pulp cells) were elevated 4 h after injection of PGN and poly I:C in the pulp of GF, but not until 8 h after injection of LPS. Macrophage levels peaked at 8 h p.i. of LPS, PGN, and poly I:C, respectively reaching 6-, 3-, and 4-fold increases compared to pre-injection levels. Beginning at 24 h p.i. of LPS and poly I:C, macrophage proportions in GF began decreasing and returned to pre-injection levels at 72 and 48 h respectively. Proportions of macrophages in the pulp of PGN injected GF had returned to normal by 24 h p.i. (Figure 1).

**Natural Killer (NK) Cells**

While the overall percentage of NK cells (CD3-CD8+) in the pulp of GF is small (mean = 0.06 % pulp cells) at 0 h in all experimental animals (n = 24), proportions of NK cells in PGN injected GF increased 4-fold (mean = 0.30 % pulp cells) at 48 h p.i. (n = 6). Proportions of NK cells were elevated at 48 and 72 h after PGN injection into GF. For NK cell proportions, no other differences across time within PGN or PBS, LPS, and poly I:C treatments were found (Figure 1).

**Total Lymphocytes and Lymphocyte Subpopulations**

No changes in the proportions of total lymphocytes were observed in PBS injected GF. Injection of poly I:C induced a small (2-fold) increase in total lymphocytes at 4 h p.i. (Figure 1). This increase was due to a significant (\( P = <0.001 \)) 7-fold increase in B cells proportions 4 h after intra-dermal (i.d.) injection of poly I:C compared to pre-injection levels, which remained heightened at 8 and 24 h p.i., and returned to pre-injection levels by 48 h (Figure 2). A decrease in
CD8+ lymphocytes compared to pre-injection levels was observed at 8 h p.i. which returned to normal levels at 24 h p.i. and remained there until the end of the experiment.

Intra-dermal injection of LPS resulted in a small 2- to 3-fold increase of total leukocytes (% pulp cells) in the GF at 48 and 72 h p.i. All lymphocytes subpopulations were elevated at various time points except γδ T cells (TCR1+ cells); however, the changes in lymphocyte subpopulations were small compared to changes observed with poly I:C (B cell) and PGN (all lymphocyte subpopulations).

Following injection of PGN in GF, total lymphocyte proportions increased at 24 h p.i., continued to increase and reached maximum levels at 48 h p.i., a 5-fold increase compared to pre-injection levels (Figure 1). Thereafter, the levels (% pulp cells) of total lymphocytes decreased slightly but remained elevated by the end of the experiment (72 h p.i.).

Injection of PGN into the pulp of GF increased the proportions of all lymphocyte subpopulations in the local tissue at various time points, typically beginning at 24-48 h p.i. The greatest change in lymphocyte subpopulations following PGN injection in GF was a 10-fold and 11-fold increase in B cells and CD4+ T cells respectively. Compared to pre-injection levels, proportions of B cells were elevated at 24 h post PGN injection, continued to increase reaching maximum proportions at 48 h and 72 h (Figure 2). Proportions (% pulp cells) of CD4+ T cells also increased at 24 h p.i., remained heightened at 48 and 72 h following i.d. PGN injection. An increase in proportions of CD8+ lymphocytes compared to pre-injection levels was only observed at 48 h p.i. and levels of CD8+ lymphocytes in the pulp returned to normal at 72 h.

The only subpopulation of lymphocytes to significantly (P ≤ 0.05) increase earlier than 24 h after PGN injection was TCR1+ γδ T cells. The pulp proportions of non-MHC restricted γδ T cells were already elevated 4 h (earliest time-point examined) after PGN injection into GF and
remained at the same 5-fold increase for the remainder of the experiment (Figure 2). The proportions of the MHC restricted αβ1 T cells and αβ2 T cells were elevated by 24 h p.i. of PGN, continued to increase to maximum proportions at 48 h and decreased to near pre-injection levels by the end of the experiment (72 h p.i.). As αβ1 T cells make up a greater proportion of the total αβ T cell population, the similar 4-fold increase in both αβ1 and αβ2 T cell proportions appears to be larger in αβ1 T cells due to the innate differences in tissue levels of the two cell types.

*Changes in transcriptional expression of cytokines and chemokines in pulp cells after intra-dermal injection of PBS, LPS, PGN, and poly I:C*

Where significant interactions were observed, time differences within individual treatment groups (PBS, LPS, PGN, and poly I:C) were examined by protected Student’s t-tests. Of the 11 genes (Table 1) related to chicken inflammatory and anti-viral responses examined in this study, only four did not have significant time by treatment interactions (IL-6, IL-21, CXCL8, and IFNβ). For genes without significant time by treatment interactions, a main effect of time was observed for IL-6 and CXCL8 but not for IL-21 (data not shown) or IFNβ. Further analysis of the main effect of time observed for the pro-inflammatory cytokine IL-6 and the chemoattractant CXCL8 revealed increased mRNA expression of both at 4 h p.i. and a return to pre-injection levels by 8 h p.i. (Figure 3). A main effect of treatment was observed for IFNβ where transcriptional expression was overall higher after injection of poly I:C in GF compared to PBS, LPS, and PGN GF injections. No differences in time, treatment, or time by treatment interactions were observed with transcription expression of IL-21.

*Saline control (PBS)*
Overall, endotoxin free PBS injection in GF did not altered mRNA expression of the genes examined in this study. However, at 72 h post PBS injection, transcription expression of the inducible form of IL-12 (IL-12α) increased. This was the only point and treatment at which increased expression of IL-12α was observed (results not shown).

*Lipopolysaccharide*

After intra-dermal injection of LPS in GF, mRNA expression of the inflammatory cytokines (IL-1β, IL-6, IL-10, CXCL8, and LITAF) were significantly \((P \leq 0.05)\) upregulated. Transcriptional expression of the pro-inflammatory cytokine interleukin (IL) -1β increased roughly 2,000 to 4,000 fold at 4 and 8 h p.i. respectively in LPS injected tissue before returning to pre-injection levels at 24 h p.i. (Figure 3). Beginning at 4 h p.i. mRNA expression of the inflammatory cytokine IL-6 was elevated, decreased at 8 h, but remained above pre-injection levels until 24 h p.i. Following LPS injection in the pulp of GF, both CXCL8 (chemoattractant) and IL-10 (anti-inflammatory cytokine) were upregulated (5,000 and 800 fold respectively) at 4 h p.i and remained significantly upregulated at 8 h before returning to pre-injection levels at 24 h. The LPS-induced transcriptional expression of TNF-α like factor (LITAF) increased 20-fold at 4 h p.i. and returned to pre-injection levels by the next time point measured (8 h).

Expression (mRNA) of IFNγ (key cytokine for development of Th1 cell-mediated immunity) was upregulated in the pulp at 24 and 48 h p.i. compared to pre-injection levels and returned to normal by the end of the experiment (72 h). Transcriptional expression of the anti-viral cytokine, IFNα was upregulated 17-fold at 24 h p.i. compared to pre-injection levels. Expression levels (fold change) of IL-4, IL-12α, IFNβ, and IL-21 were not altered after injection of LPS in GF. Overall fold change in IFNγ and IFNα expression was much lower than
expression of the primarily innate inflammatory cytokines (IL-1β, IL-6, IL-10, CXCL8, and LITAF).

*Peptidoglycan*

Injection of PGN in GF altered the transcription expression of several immune genes including IL-1β, IL-6, CXCL8, and IL-4 (Figure 3). Transcriptional expression of IL-1β, IL-6, and CXCL8 was upregulated 49-, 19-, 69-fold (respectively) at 4 h p.i. and returned to pre-injection levels thereafter. Expression (fold change in mRNA levels) of IL-4 was elevated at 24 h p.i., remained elevated at 48 h, and returned to normal levels by the end of the experiment (Figure 3).

*Polyinosinic-polycytidylic acid*

Expression of the IL-6 was upregulated 4 h after injection of poly I:C in GF, and returned to pre-injection levels at 8 h p.i. \( (P = 0.079; \text{Figure 3}) \). Poly I:C also upregulated expression of the anti-inflammatory gene IL-10 at 4 h p.i. Following the same trend as the other PAMP treatments (main effect of time; \( P \leq 0.05 \)), CXCL8 expression levels were elevated 4 h p.i. and gradually returned to pre-injection levels by 48 h (Figure 3). Expression of IL-4 was upregulated 4 h after poly I:C injection in GF before returning to pre-injection levels at 8 h p.i. Expression of LITAF and IFNγ increased at 4 h p.i., reaching a maximum elevation of 8- and 3-fold respectively. IFNγ expression remained elevated at 8 h p.i. and both LITAF and IFNγ mRNA expression levels returned to pre-injection levels by 24 h p.i.

**DISCUSSION**

In the drive to find new ways to prevent and treat poultry diseases, modulation of the innate immune system provides a unique opportunity to stimulate the bird’s inherent defenses to fight pathogens. Innate immunity is critical to early control of infection and for stimulation and
modulation of the slower responding adaptive immune system. Studying the effects of microbial PAMPs in vivo provides a unique opportunity to better understand of the bird’s innate immune responses as well as an opportunity to identify future disease treatments and prevention therapies.

In a 72 h time course, the local immune response of individual chickens to common bacterial and viral PAMPs was examined by injection of LPS, PGN, poly I:C, or vehicle control (endotoxin free PBS) in the dermis of growing feathers (GF) using the method described in Erf and Ramachandran (2016). Specifically, test materials (PAMPs or vehicle) were injected in GF of 6 chickens per treatment group and GF were collected at key time points after injection. The local responses initiated by PAMPs in vivo, i.e. leukocyte infiltration and cytokine/chemokine mRNA expression, were then examined ex vivo in the pulp of collected GF.

In the current study a moderate dose of LPS or PGN (0.1 µg/GF) was injected into GF; this dose of LPS and PGN is known to induce divergent leukocyte recruitment responses in injected GF (Chapters I and II). The leukocyte infiltration response to i.d. injection of poly I:C into GF was not previously tested; however, chicken leukocytes have been shown to respond to poly I:C in vitro. He et al. (2011a; b) showed that in vitro poly I:C stimulation altered transcription in chicken monocytes as indicated by increased expression of IFNγ, IFNα, and IFNβ mRNA. Using macrophages from the HD11 chicken cell line He et al. also found increased nitric oxide production when macrophages were stimulated with poly I:C and synergistic enhancement of nitric oxide production when macrophages were first stimulated with IFNγ and then with poly I:C. In heterophils, oxidative burst and degranulation were induced upon in vitro stimulation with poly I:C; however, expression of the inflammatory cytokines, IL-1β, IL-6, and CXCL8 in the cultured heterophils were downregulated compared to controls (Kogut et al.,
For the current in vivo study, poly I:C was injected at the same dose (0.1 µg/GF) as LPS and PGN to compare its immunomodulatory activities to those induced by the bacterial PAMPs at the same concentration. Responses to the injected PAMPs were examined over a 72 h period to cover the time period when LPS and PGN exhibited maximal activities (Chapter I and II).

Both LPS and PGN injection into the pulp of GF resulted in significant influx of heterophils into the local tissue (pulp of GF). At the height of respective responses (8 h p.i.), the proportions of heterophils in the pulp was 2-fold higher with LPS injection compared to PGN injection in GF. Considering that both LPS and PGN are bacterial cell wall products, recruitment of phagocytes such as heterophils is expected as they are most effective in the early, rapid removal of extracellular bacteria. Furthermore, the heterophil recruitment effects of LPS, although not those of PGN, are well documented in the scientific literature for both mammals and birds (Moeller et al., 1978; Richardson et al., 1989; Bowen et al., 2009; Abbas et al., 2014; Schat et al., 2014). Only minor increases in heterophil proportions were observed with poly I:C or the PBS injections (Figure 1). The lack of strong heterophil recruitment to poly I:C injection in GF may in part be due to the location of poly I:C PRRs. The TLR3 and MDA5 receptors that recognize poly I:C in chickens are endosomal membrane and cytosolic receptors respectively (Schat et al., 2014). Without the aid of a delivery vehicle (i.e. liposomes), the poly I:C injected in this study may not have efficiently reached and stimulated these receptors. More importantly, however, the intracellular pathways initiated by the interaction of poly I:C, an analog for viral genomic material, with its PPRs are expected to generate an antiviral response which is not known to involve heterophils to a great extent.

Macrophages infiltration into the injected tissue accompanied and followed heterophil recruitment with all three PAMPs. Macrophage proportions in GF returned to normal levels by
24, 48, and 72 h post PGN, poly I:C, and LPS injection respectively. In addition to being professional phagocytes, able to efficiently phagocytose extracellular microbes and produce an extensive repertoire of antimicrobial responses, macrophages are also able to interact with and stimulate other leukocytes through production of cytokines and chemokines, killing infected host cells, and repairing tissue (Qureshi et al., 2000; Qureshi, 2003; Abbas et al., 2014). Therefore, participation of macrophages in responses initiated by the various PAMPs can be explained by the multitude of responses macrophages are known to support. The sustained presence of macrophages in the pulp of LPS injected GF may be responsible for the increased mRNA expression of inflammatory cytokines including IFN\(\gamma\), IL-1\(\beta\), LITAF, and IL-10 with LPS injection compared to other treatment groups (Figure 3).

Lymphocyte recruitment was highest and sustained over the three-day period in PGN injected GF, confirming observations reported in Chapter II. Levels of lymphocyte subpopulations (B cells, CD4+, CD8+, \(\gamma\delta\) T cells, \(\alpha\beta1\) T cells, and \(\alpha\beta2\) T cells) were elevated early (4-24 h) after injection of PGN and with the exception of CD8+ lymphocytes, remained elevated at the end of the experiment (72 h). Populations of B cells and CD4+ lymphocytes increased the most (10-fold) following injection of PGN. It should be noted that the PGN used in this study was the same soluble product (Invivogen, San Diego, CA) used for the Chapter II PGN injection study, and as such may contain both PGN and lipid molecules such as lipoteichoic acid (Dziarski, 2003; Travassos et al., 2004; Dziarski and Gupta, 2005; Chapter II).

The rapid response time (4-24 h p.i.) of lymphocytes observed for PGN in this study and the previous study (Chapter II) is unusual as most T and B cells are antigen specific cells which typically take days to weeks to be recruited at high levels to the site of infection. Mammalian literature reveals that TLR 2 is expressed on B cells and therefore these cells may be directly
stimulated by PGN and other TLR 2 agonists (Dziarski and Gupta, 2005). The presence and early participation of B and T cells in the response to Mycobacterium and PGN also suggests that lymphocyte are recruited to participate in a non-antigen-specific manner in the local response to Gram-positive bacteria. In this scenario lymphocytes likely are activated by PAMPs through the toll-like receptors (TLRs) present on both cell types and their products (IFNγ and other cytokines, complement factors, natural antibodies, etc) are needed for effective elimination of the bacterial infection (Szczepanik et al., 2003; Abbas et al., 2014; Schierloh et al., 2014). Further studies are needed to examine chemokine expression in PGN injected complex tissue such as the GF pulp to determine mechanisms involved in lymphocyte recruitment during an innate immune response.

The 4 h increase in total lymphocytes after poly I:C injection could be attributed to a 7-fold increase in B cell proportions. Previous research in mammals with poly I:C as an adjuvant has indicated a strong presence of Th1 immunity which along with macrophage activation promotes antibody class switching in B cells (Abbas et al., 2014; Martins et al., 2015). In this study the B cells recruited to the pulp of GF in response to poly I:C injection were shown to be Bu-1+IgM+ and may be B1-B cells that respond in an innate fashion to frequently encountered microbial antigens (Swanson et al., 2010; Egbuniwe et al., 2015). B cells have been shown to be activated by PAMPs, specifically unmethylated CpG DNA in chickens and other viral genetic material in mammals (He et al., 2007; Xagorari and Chlichlia, 2008; Ciraci and Lamont, 2011). Moreover, B cells are known to secrete IL-6 and can be a major source of IL-6 during polyclonal activation of B cells, which can be achieved by TLR stimulation. The parallel increases in B cell infiltration (Figure 2) and IL-6 RNA levels (Figure 3) observed in poly I:C injected GF suggests
that the B cells may be expressing the IL-6 target gene (Barr et al., 2012; Abbas et al., 2014; Corneth et al., 2016).

NK cell proportions (CD8+CD3- lymphocytes) increased 4-fold (Figure 1) after PGN injection in GF at 24, 48, and 72 h p.i. No changes in NK cell levels in the pulp were observed with any of the other treatments. Considering that poly I:C is an analog of viral genetic material, NK cell recruitment to the site of PGN rather than poly I:C injection is surprising as NK cells play an important role in early anti-viral responses (Abbas et al., 2014). However, early production of IFNγ by NK cells is known to be important in stimulating cell-mediated immune activity, particularly responses to intracellular bacteria (Abbas et al., 2014).

Overall the cytokine mRNA expression data gathered in this study tended to agree with the cellular recruitment patterns described above. For example, the strong inflammatory heterophil and macrophage recruitment observed after LPS injection was marked by increased transcription of inflammatory cytokine genes. Injection of LPS in GF recruited the highest levels of heterophils into injected tissue and similar levels of macrophages compared to PBS, PGN, or poly I:C injected GF. Correspondingly, compared to the other treatments, LPS showed the highest numerical expression of IL-1β and LITAF (pro-inflammatory cytokines), CXCL8 (chemoattractant), IL-10 (anti-inflammatory cytokine, stimulates alternative macrophage activation), and IFNγ (stimulates and hones macrophage functions). The gene expression data and the heterophil and macrophage infiltration profiles observed here are in full agreement with the classical LPS inflammatory response described in immunology textbooks, and confirm the similarities between mammalian and avian innate immune system defenses.

Recruitment of heterophils and macrophages was observed after injection of PGN, another bacterial PAMP, and corresponded with an increase in inflammatory cytokines.
However, unlike LPS, poly I:C, or PBS, PGN also recruited and sustained a lymphocyte presence in the GF. While this study did not examine transcriptional expression of many cytokines associated with lymphocyte responses, expression of the key cytokines produced by effector CD4+ T helper (Th) cells was examined, specifically the Th1 cytokine, IFNγ and the Th2 cytokine, IL-4. These cytokines may be produced by other cells (e.g. NK cells, other innate lymphoid cells, and mast cells) influencing the differentiation path of Th cells to Th1 or Th2 during antigen presentation and activation of the adaptive immune responses, thereby forming a link between innate and adaptive immunity (Abbas et al., 2014; Schat et al., 2014). After PGN injection in GF transcriptional expression of both IL-4 and IFNγ was elevated toward the end of the experiment (24-48 h p.i.). Interestingly, LPS also increased the expression of IL-4 and IFNγ indicating that PGN and LPS activated both classical macrophage microbial killing activities as well as macrophage wound repair mechanisms (Abbas et al., 2014; Portou et al., 2015).

While LPS can initiate an inflammatory pathway of chemokine and cytokine production in LPS activated cells, it is also capable of initiating a classical anti-viral pathway resulting in degradation of viral RNA and an causing neighboring cells to enter an anti-viral state, limiting the spread of the virus (Goossens et al., 2013; Abbas et al., 2014). Transcriptional expression of both of the anti-viral cytokines examined in this study (IFNα and IFNβ) were shown to be elevated 24 h post LPS injection in GF (IFNβ not significant due to high variability within gene expression). The analog to viral dsRNA, poly I:C, also increased the transcriptional expression of IFNβ (4 h p.i.); however, IFNα expression was not significantly alter over time. In vitro studies with chickens have shown that silencing of the chicken MDA5 gene (PRR of poly I:C) and subsequent challenge with avian influenza virus decreases the transcription expression of
cytokine production of IFNβ indicating that IFNβ plays an important role in the regulation of the immune system to viral infections (Karpala et al., 2011; Liniger et al., 2012).

Regardless of material injected, the bacterial and viral PAMPs as well as sterile injury induced by endotoxin free PBS elevated the levels of inflammatory cytokines in the pulp of injected GF. These results challenge the dogma of viral PAMPs initiating solely an anti-viral response and bacterial PAMPs initiating an inflammatory response typically reported for in vitro studies where the responses of a single cell type were examined. This study highlights the complexity of the innate immune system response to microbes in vivo. While the interaction between PAMPs and their PPR is specific, introduction of a single PAMP into a complex living tissue will result in the activation of multiple immunological components, both soluble factors and cells, needed to active the optimal mechanisms to defend against pathogens in vivo. In the future both in vitro and in vivo studies are needed to understand the complex processes initiated by PAMPs. For this, the GF can serve as a minimally invasive procedure to study immune responses in vivo from initiation to resolution.
REFERENCES


macrophages alters response to LPS and poly (I:C) stimulation. BMC Vet. Res. 8:23
Available at http://www.biomedcentral.com/1746-6148/8/23.


Table 1: Primer and probe sequences\(^1\) for target genes

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\(^1\) Primer and probe oligos were synthesized by Eurofins MWG Operon LLC, Huntsville, AL.

\(^2,4,5\) Sequences were from Avery et al., 2004; Kogut et al., 2005; Shi, 2011; He et al., 2012, respectively.
Infiltration of heterophils, macrophages, total lymphocytes, and natural killer (NK) cells into growing feathers after intra-dermal (i.d.) injection of various pathogen associated molecular patterns. Twenty growing feathers (GF) of twenty-four, 12- to 17-wk-old chickens were injected i.d. with 10 µL of endotoxin free saline control (PBS) or test material (0.1 µg of LPS, PGN, or poly I:C per GF). One injected GF from each chicken was collected at 0 (before injection), 4, 8, 24, 48, and 72 hours post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to chicken macrophages (KUL01+), NK cells (CD3-C8+), and total lymphocytes (addition of individual Bu1+IgM+, TCR1+, TCR2+, and TCR3+ populations). Analysis of cell populations was carried by flow cytometry and percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+). Data shown are mean ± SEM; n = 6 per time point and treatment group.
Figure 2. Infiltration of lymphocyte subsets into growing feathers after intra-dermal (i.d.) injection of various pathogen associated molecular patterns. Twenty growing feathers (GF) of twenty-four, 12- to 17-wk-old chickens were injected i.d. with 10 μL of endotoxin free saline control (PBS) or test material (0.1 μg of LPS, PGN, or poly I:C per GF). One injected GF from each chicken was collected at 0 (before injection), 4, 8, 24, 48, and 72 h post injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to chicken lymphocytes: B cells, CD4+ cells, CD8+ cells, γδ1 T cells, αβ1 T cells, and αβ2 T cells. Data shown are mean ± SEM; n = 6 per time point and treatment group.
Figure 3. Relative expression of cytokines and chemokines from the pulp of growing feathers injected with endotoxin free PBS control or various pathogen associated molecular patterns. Twenty growing feathers (GF) of twenty-four, 12- to 17-wk-old chickens were injected intradermally with 10 μL of saline control (PBS) or test material (0.1 μg of LPS, PGN, or poly I:C per GF). RNA was isolated from one injected GF from each chicken collected at 0 (before injection), and at 2, 4, 8, and 24 h post injection and reverse transcribed into 1.5 μg of cDNA/sample. Relative expression of interleukin (IL) 1β, IL-4, IL-6, IL-10, IL-12α, CXCL8, IFN-α, IFN-β, IFN-γ, and LITAF was calculated based on the delta delta CT method, using a cDNA pool from non-injected GF pulp as the calibrator and chicken 28S as the endogenous control gene. Data shown are mean ± SEM of fold change in expression compared to the calibrator sample; n = 6 per time point and treatment group.
Conclusions

Collectively, the data reported here confirm that the avian GF is a valuable cutaneous test site to monitor the cellular immune responses to PAMPs in the local injected tissue (GF) and, when used in combination with blood sampling, leukocyte concentrations in the peripheral blood. Specifically, we show that intra-dermal (i.d.) injection of LPS in the pulp of GF results in increased heterophil levels in the blood and local tissue as well as smaller increases in macrophage proportions in the tissue. The dose of i.d. injected LPS but not PGN affected the time course and quantitative aspects of the cellular inflammatory response in blood and pulp of GF (Chapter I). As with LPS injected GF, PGN recruited heterophils and macrophages into the pulp of GF; however, one of the most striking observation with PGN i.d. injection was the substantial and rapid infiltration of lymphocytes into the injected pulp and their sustained presence at high levels throughout the 7-day study (Chapter II). The rapid infiltration of B cells and CD4+ cells along with the generation of lymphocyte attracting chemokines (IL-4, IL-10, and IFNγ) in the injected issue suggest the presence of innate pattern recognition receptors on B and CD4+ T lymphocytes. Unlike PGN, its derivative MDP was not found to be highly immunostimulatory, possibly due the lack of the MDP sensing, cytosolic NOD2 receptor in chickens and/or the inability of local tissue resident dendritic cells or macrophages to take up MDP into the cytosol when administered in a saline solution. Direct comparisons of LPS, PGN, and poly I:C confirmed the LPS and PGN data reported in Chapters I & II and showed that poly I:C injection also initiates a unique leukocyte infiltration profile with rapid (4 and 8 h) increases in macrophage and B cell levels in the pulp of injected GF (Chapter III). Analysis of cytokine RNA expression profiles in GF injected with the various PAMPs was associated with high sample variability possibly due to the low number of experimental animals and/or inherent
variation of an individual’s responsiveness to PAMP stimulation. Nevertheless, injection of PAMPs in GF was shown to primarily stimulate expression of inflammatory associated cytokines (IL-1β, IL-6, IL-10, and CXCL8), and at lower expression levels IL-4 and IFNγ. The expression of inflammatory cytokines even with poly I:C (analog of viral genetic material expected to induce anti-viral cytokine expression), emphasizes that administration of PAMPs in vivo initiates a complex cascade of responses not observed with single cell in vitro studies. Research conducted in this dissertation project constitutes a first comprehensive investigation into the immunostimulatory effects of PAMPs in a complex tissue where networks of cells and soluble factors work together to initiate responses focused on eliminating microbial infections and reestablishing homeostasis in the affected tissue. Future studies will build on observations reported here to further evaluate and dissect innate immune responses in poultry. New knowledge gained from this and future studies will find direct application in development of breeding and management strategies to improve poultry health.
MEMORANDUM

TO: Dr. Gisela Erf
FROM: Craig N. Coon, Chairman
    Institutional Animal Care and Use Committee (IACUC)
DATE: November 7, 2014
SUBJECT: IACUC APPROVAL
    Expiration date: November 7, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your to
protocol 15021 Avian innate immune function: responses to pathogen associated
molecular patterns.

In granting its approval, the IACUC has approved only the information provided. Should
there be any further changes to the protocol during the research, please notify the
IACUC in writing (via the Modification form) prior to initiating the changes. If the study
period is expected to extend beyond November 7, 2017 you must submit a new protocol
prior to that date to avoid any interruption. By policy the IACUC cannot approve a study
for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal
guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian