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Time course investigation of the dermal leukocyte response to lipoteichoic acid in chickens

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Honors Thesis

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Contents

Abstract

Lipoteichoic acid (LTA) is component of the cell wall of Gram-positive bacteria that stimulates inflammation during bacterial infection. However, few studies have investigated the *in vivo* immune response to LTA, and none of the *in vivo* studies done have been performed in birds. For this project, the pulp (a skin-derivative) of growing feathers (GFs) of chickens were used as a test site to investigate the *in vivo* effects of intradermally injected LTA. In Study 1, the pulp of 12 GFs of 11-week-old Light-brown Leghorn (LBL) males were injected with 10 μL of differing concentrations of LTA (0.1, 1.0, 10, 100 or 250 μg LTA/mL; 3 chickens/dose). For each chicken, 2 GF were plucked before injection and at 6, 24, 48, and 72 h post-injection, flash frozen, and stored at -80°C. Frozen pulp sections were stained using immunohistochemistry for visual inspection of leukocyte infiltration in response to LTA. Based on this study, the 10 μg/mL LTA solution was found to be the optimal concentration to stimulate inflammation. In Study 2, GFs of 12, 15-week-old LBL males were then injected with either 10 μL of 10 μg/mL LTA (0.1 μg LTA/GF, 12 GF/bird, n = 8), or 10 μL of PBS (vehicle; n = 4). GFs were collected before injection (0 h) and at 6, 24, 48, and 72 h post-injection. At each time-point, pulp cell suspensions were prepared and immunofluorescently stained with a panel of chicken-leukocyte-specific monoclonal antibodies, and cell population analysis was carried out by flow cytometry. Analyses revealed elevated levels (% pulp cells) in total leukocytes, monocytes/macrophages, and class II MHC expressing cells in GFs injected with LTA when compared to the control. Infiltration of lymphocytes and heterophils was not different between treatment groups. This study suggests that the inflammatory response to LTA in chickens is characterized primarily by recruitment of monocytes/macrophages to the site of inflammation.

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Introduction

Background and Need

The problem of antibiotic resistance is ever increasing in the modern world. In agriculture, as in medicine, there is a growing need to reduce the usage of antibiotics so that they may continue to be effective in the future (Laloučková & Skřivanová, 2019). This requires the development of new methods of preventing and fighting bacterial infection in livestock. However, in order to find new, better ways of fighting pathogens, a greater understanding of the immune system is required.

The immune system in vertebrates is often considered to be divided into two main components that protect against invading pathogens: the innate and acquired immune system. The innate immune system is the first line of defense, designed to confine and combat infectious agents. The innate immune system is genetically determined and consists of general responses that are effective against groups of pathogens. This contrasts with the acquired immune system, which is slower to produce an effective response but targets specific pathogens. The acquired immune system is mediated primarily by lymphocytes, while the innate immune system consists of diverse elements such as certain leukocytes, complement proteins, and mucous secretions (Abbas et al., 2015). However, there is not a hard line between innate and acquired immunity, as many cells play a role in both types of immunity.

Lipoteichoic acid (LTA) is a surface-associated component of the Gram-positive bacterial cell wall. Gram-positive bacteria release LTA during bacteriolysis caused by various factors such as neutrophils, complement proteins, or antibiotics. LTA elicits an immune reaction that causes inflammation. In animal studies, high doses of LTA have been shown to cause septic shock and multiorgan failure (Ginsburg, 2002). LTA plays a role in immune response to all Gram-positive

bacterial infections, so understanding how the immune system responds to LTA is critical to immunology and medicine as a whole.

Problem Statement

LTA and its role in immune response has been researched by previous studies. However, there are few studies looking at local inflammatory responses to LTA in a complex tissue, and no *in vivo* studies have been conducted in chickens. In chickens, intradermal injection of LTA into the pulp of growing feathers (GFs) allows for the observation of *in vivo* immune response over time in the same individual (Erf & Ramachandran, 2016). This approach is minimally invasive because injected GF can easily be collected for *ex vivo* analyses while dermal investigation of immune responses in other animals would require invasive biopsy or sacrificing the animal. A better understanding of the progression of the innate immune response to LTA is important to understanding the natural defenses of chickens. More research needs to be conducted in order to find new, more effective ways of preventing infection, which requires a better understanding of immune function.

Purpose of the Study

The purpose of this study is to observe *in vivo* how the inflammatory response initiated by intradermal LTA injection progresses over time. Simultaneous intradermal injection of multiple GFs of a chicken with LTA and subsequent periodic sampling of the GF for laboratory analysis will provide a profile of the local tissue response to LTA. No previous study has been conducted that observes local immune response to LTA in an individual over time. In this study, a time course experiment will be conducted in which differences in individuals' immune systems are not a factor. Looking into the progression of the LTA induced inflammatory response in an

individual will yield information critical to understanding Gram-positive bacterial infections in chickens as well as in humans.

Objectives

- Measure the type and relative number of leukocytes that infiltrate the growing feather in response to LTA
- Determine how leukocyte presence changes over time as the inflammatory response to LTA progresses

Literature Review

Introduction

The innate and acquired immune system are two halves that comprise a vertebrate's system of defense against pathogens. The first barrier against invading pathogens is the epithelial cell layer that covers the external body (skin) and lines the digestive, respiratory, and genitourinary tract (mucosal epithelium) (Günther & Seyfert, 2018). However, if the epithelium is compromised through injury, pathogens are able to enter and colonize the body. As a first response to injury and infection, wounds will produce an inflammatory response which allows important elements of the immune system such as leukocytes and plasma proteins to access the site of infection (Medzhitov, 2008).

Innate Immunity

Inflammation is the primary response of the innate immune system to microbial infection or tissue damage and also plays a role in viral infection (Abbas et al., 2018). During inflammation, increased vascular permeability allows leukocytes, also known as white blood cells which are the cells of the immune system, and plasma proteins to access the site of infection

(Medzhitov, 2008). These changes are mediated by cytokines released by resident cells in the affected tissue such as macrophages, mast cells, and endothelial cells (Abbas et al., 2018). Initially, neutrophils, or the analogous heterophils in birds, are the most abundant leukocytes recruited from the blood, but the number of monocytes increases more slowly over time until they are more prominent. Infections are initially recognized by a group of molecules called pattern recognition receptors (PRRs) on sentinel cells in tissues (i.e., macrophages, dendritic cells, and mast cells) (Günther & Seyfert, 2018). PRRs recognize a limited number of molecules that are shared by large groups of pathogens, known as pathogen-associated molecular patterns (PAMPs) (Medhitov, 2008). Also important are a related class of molecules known as damage-associated molecular patterns (DAMPs). These are endogenous molecules which are not normally released in healthy cells and their presence signals the immune system that the cell is abnormal and needs to be removed (Abbas et al., 2018). PAMPs and DAMPs bind to PRRs which signal cells to respond in several ways, often activating defenses against infection and tissue-repair mechanisms (Günther & Seyfert, 2018).

Types of Leukocytes

Leukocytes are the primary cellular components of the immune system. Acquired, or adaptive, immunity is carried out by T and B lymphocytes, while innate immunity relies on a variety of leukocytes, such as granulocytes, monocytes/macrophages, dendritic cells, and innate lymphocytes, such as natural killer cells and innate lymphoid cells (Abbas et al., 2018).

Lymphocytes of adaptive immunity consist primarily of B cells and T cells (Abbas et al., 2018). B cells and T cells are unique in that they display antigen receptors on their plasma membranes (Treanor, 2012; Rojo et al., 2008). These receptors detect specific antigens such as proteins and polysaccharides that come from pathogens. The specificity of the antigen receptors

on B cells and T cells is randomly determined during development so that each cell will have a different receptor specificity (Nemazee, 2006). When one of these cells is activated by binding with an antigen, it proliferates, creating more lymphocytes with receptors of the same unique antigen specificity (Abbas et al., 2018). Lymphocytes that have never encountered an antigen are called 'naïve', whereas those that have been activated are called 'effector cells'.

Effector B cells produce antibodies, which are large proteins that have the same specificity as the B cell's antigen receptor but are secreted to bind to extracellular antigens. Antibodies have numerous functions; these include marking a pathogen for destruction by leukocytes and blocking cellular binding sites on a pathogen in order to neutralize it (Abbas et al., 2018). B-cell receptors are able to bind a wide range of antigens, but many effector functions of B cells require activation by T cells via antigen presentation. This is the process by which a B cell is able to present antigen to helper T cells with receptors for that antigen, activating both the T cell and the B cell to perform effector functions. However, while B-cell receptors are able to bind lipids, carbohydrates, nucleic acids, proteins, and other molecules, helper-T-cell receptors are only able to bind peptide antigens in association with presentation molecules called MHC molecules on antigen-presenting cells, such as dendritic cells, B cells, and macrophages. With T-cell help, the B-cell activation is greatly enhanced and leads to production of better-quality antibodies by switching the antibody isotype and increasing the antibodies' affinity for the antigen. Moreover, with T-cell help, activated B cells can differentiate into long-lasting cells that quickly respond to known antigens the next time it is encountered (memory cells).

There are two primary types of T-cell receptor (TCR): the αβ TCR present on CD4+ and CD8+ T cells and the γδ TCR, which may be expressed on CD8+ or CD8- T cells (Abbas et al., 2018). CD4+ cells, named for the CD4 protein expressed on their cell membranes, are also

known as helper T cells; these cells release cytokines, cell-signaling proteins of the immune system, which play a role in activating other leukocytes and stimulating inflammation. CD4+ cells may differentiate into a number of known effector-cell subtypes designated Th1, Th2, and Th17 cells. However, this differentiation requires antigen presentation, which can be performed by any cell expressing the class II major histocompatibility complex (MHCII), including macrophages, dendritic cells, and B cells. CD8+ cells, or cytotoxic T cells, have antigen receptors that allow them to detect and kill cells infected by intracellular pathogens such as viruses or antigens generated inside cells (e.g., tumor antigens). γδ T cells are T lymphocytes that have a unique receptor composed of a γ chain and a δ chain; this receptor is not limited to recognition of antigen peptide in association with MHC molecules on antigen-presenting cells; rather, it can bind to and be activated by various types of molecules. While γδ T cells are relatively rare in humans and mice, they make up a high proportion of T cells in chickens, cattle, and other production animals. Moreover, their specificity is limited to frequently encountered antigens, and, although they are cells of adaptive immunity, they are more innate-like in their function and most abundant in barrier tissues (mucosa and skin).

Myeloid lineage leukocytes are cells of innate immunity and include granulocytes and monocytes. Granulocytes are so named because they have many lysosomes containing microbicidal substances that appear as small granules under a microscope when stained (Abbas et al., 2018). In avian species, heterophils are the most abundant granulocyte; they are analogous to neutrophils in mammalian species. Both neutrophils and heterophils play an important role in phagocytizing pathogens in the earliest stages of infection. Heterophils only function for 1 to 2 days before dying. The other types of granulocytes are mast cells, basophils, and eosinophils. Mast cells reside in tissues and release histamine which promotes inflammation. They play an

important role in allergic reactions. Basophils are functionally and structurally similar to mast cells but circulate in the blood. Eosinophils release enzymes that combat parasites, particularly parasitic worms known as helminths. Mononuclear phagocytes are the other main type of myeloid leukocyte. These cells are known as monocytes when circling in the blood stream. However, outside of the blood stream they become tissue residents known as macrophages where their role is to phagocytize pathogens. Lastly, dendritic cells are also phagocytes, excellent antigen-presenting cells and function as sentinel cells in tissue. These myeloid lineage leukocytes are members of the innate immune system and play an important role in inflammation.

Lipoteichoic Acid Structure and Function

Lipoteichoic acid (LTA) is a cell wall component found in Gram-positive bacteria composed of a variable polymer chain attached to a glycolipid anchor (Reichmann & Gründling, 2011). The glycolipid anchor generally consists of one to three sugars linked to a diacylglycerol which embeds the molecule in the plasma membrane (Percy & Gründling, 2014). Percy and Gründling (2014) describe five types of LTAs that have been identified in various bacterial species. Type I LTA is the most well studied; its polymer chain consists of repeating units of glycerol phosphate. This type of LTA is found in many bacteria of the phylum *Firmicutes*, including *Bacillus subtilis*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The physiological function of LTA in bacteria is not entirely understood (Reichmann & Gründling, 2011). However, various studies have found that strains of *Bacillus* lacking LTA are filamentous in shape; LTA-negative bacterial mutants also seem to be highly sensitive to osmolality (Reichmann & Gründling, 2011; Percy & Gründling, 2014). These findings suggest that LTA plays an important role in bacterial growth and division as well as osmoregulation.

Innate Immune Response to Lipoteichoic Acid

The exact role of LTA in innate immune responses is not fully understood. As LTA is not a peptide antigen, it is unable to be presented to CD4+ cells to trigger their differentiation into effector T helper cells. This means that LTA is unable to activate T-cell-dependent responses, and that leukocyte infiltration in response to LTA is primarily a result of innate immune signaling pathways. Many older studies questioned whether LTA played a significant role in immune response due to questions of contamination of LTA samples (Ginsburg, 2002). Seo et al. (2008) used Gram-positive culture supernates (GPCSs) of four bacterial species on RAW 264.7 cells (a commonly used line of murine macrophages) and selectively inactivated LTA using human platelet activating factor-acetylhydrolase (PAF-AH). GPCSs caused RAW 264.7 cells to release tumor necrosis factor (TNF) alpha, while addition of PAF-AH caused a reduction in TNF alpha from 50% to more than 90%, concluding that LTA is a significant immune stimulating factor in the innate immune response to Gram-positive bacteria (Seo et al., 2008).

Multiple studies have confirmed that the cellular immune response to LTA is toll-like receptor (TLR) 2 dependent (Schröder et al., 2003; Dessing et al. 2008). LTA has been identified as a major TLR2 ligand (Oliveira-Nascimento et al., 2012). Schröder et al. (2003) also found that the presence of both LPS binding protein (LBP) and CD14 greatly increase release of tumor necrosis factor (TNF) alpha, an inflammatory cytokine. A later study Schröder et al. (2004) confirmed that extracellular LBP can bind LTA and deliver it to the membrane bound CD14. CD14 transfers LTA to TLR2. CD36, a membrane-bound scavenger receptor, is also able to capture and transfer LTA to TLR2 (Oliveira-Nascimento et al., 2012). Ranoa et al. (2013) later confirmed that LBP and CD14 are both able to deliver LTA to TLR2 independent of each other.

TLRs are membrane-associated receptors, found on the plasma and endosomal membranes, where they bind extracellular and vesicular ligands, triggering a variety of inflammatory and antiviral responses (Abbas et al., 2018). TLR2 is expressed on the exterior of plasma membrane and forms a heterodimer with either TLR1 or TLR6. As summarized by Oliveira-Nascimento et al. (2012), LTA binding to either of the TLR2 heterodimers results in the activation of the same intracellular pathways. The intracellular domain of TLR2 activates MyD88 (myeloid differentiation primary-response gene 88) which eventually leads to activation of the transcription factors NF-κB (nuclear factor κB) and AP-1 (activator protein 1). NF-κB and AP-1 cause inflammation by increasing transcription of pro-inflammatory genes, notably TNF and interleukin 1 (IL-1).

TLR2 has previously been shown to trigger a number of inflammatory reactions in mammalian and avian models. In cultured chicken heterophils, it was found that LTA was able to stimulate an oxidative burst, a sudden release of reactive oxygen species (ROS) which kill phagocytized bacteria, in a TLR2- and CD14-dependent manner (Farnell et al., 2003). In murine macrophages, it was shown that LTA can trigger the NLRP6 inflammasome (Hara et al., 2018). An inflammasome is a multiprotein complex that forms in the cytosol from three different types of subunits: an NLRP (nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing) receptor, an ASC (apoptosis-associated speck like protein containing a caspase recruitment domain) adaptor, and a caspase protein. The NLRP6 inflammasome contains the NLRP6 receptor, which was found to be activated by LTA. The activated NLRP6 protein binds to ASC which binds and activates caspase-11. Caspase-11 then activates caspase-1, which leads to cleavage of pro-IL-1β and pro-IL-18 into the active IL-1β and IL-18 forms. Activation of the inflammasome complex can also trigger pyroptosis in macrophages and dendritic cells, a

type of inflammatory cell death (Abbas et al., 2018). In chicken macrophages, LTA has been found to upregulate IL-1β and iNOS (inducible nitric oxide synthase), a producer of nitric oxide, which can form microbicidal peroxinitrites when combined with ROS (Haddadi et al., 2015). In human keratinocytes, LTA was found to increase cytokines IL-1α, IL-1β, IL-36α, and IL-8 and chemokines CXCL1 and CXCL2 (Brauweiler et al., 2019a).

γδ T cells are a poorly understood subset of T cells that function in both innate and adaptive responses (Abbas et al., 2018). Uniquely, their receptors contain γ and δ subunits instead of the α and β subunits on other subtypes of T cells. γδ TCRs are unique in that they have the ability to recognize non-protein antigens and can do so independently of MHC molecules (Abbas et al., 2018; Wesch et al., 2011). γδ T cells are known to express TLRs, including TLR2, and have been shown to respond to LTA (Wesch et al., 2011). γδ T cell recognition of LTA by TLR2 is facilitated by LTA capture by CD36 (Lubick & Jutila, 2006). While γδ TCRs recognize a number of glycolipid and phospholipid antigens, no research has investigated whether the γδ TCR is able to directly bind LTA.

Few studies have previously looked at the *in vivo* effects of LTA. Brauweiler et al. (2019a) investigated the effects of intradermal LTA in mice. This study found that 50 μg LTA injected intradermally resulted in a 500-fold increase in mRNA of the neutrophil marker Ly-6G as well as increased expression of CXCL1 and CXCL2 at 48 hours after injection. IL-1β and IL-36 also increased in response to LTA. IL-1α expression was not significantly different from the control *in vivo*, even though the same study did find an increase in IL-1α in human keratinocytes in response to LTA. LTA injection also caused hyperproliferation and epidermal thickening. A later study by Brauweiler et al. (2019b) found that intradermal LTA in mice after 48 hours resulted in increased mRNA levels of TSLP (thymic stromal lymphopoietin) and IL-4 by 60-fold

and 40-fold respectively, as well at IL-13 and TNF by around 10-fold. Furthermore, PCR analysis of mRNA markers specific to helper T cells, mast cells, eosinophils, and basophils found that only basophil-specific mRNA expression was elevated at 48 hours after intradermal injection with LTA. No research, however, has investigated dermal LTA response in chickens. Haddadi et al. (2015) found that chicken embryos injected with LTA had an increased number of macrophages in the lungs and increased IL-1β expression.

Function of LTA-Associated Cytokines and Chemokines

Several cytokines and chemokines expressed in response to LTA have been identified. TNF and IL-1 are two of the most common inflammatory cytokines, and both have been identified as transcripts resulting from activation of the MyD88 pathway by TLRs (Oliveira-Nascimento et al., 2012). TNF produces a number of inflammatory effects including activation of inflammation in endothelial cells, activation of neutrophils, and fever (Abbas et al., 2018). IL-1 is another inflammatory mediator that activates inflammation in endothelial cells and fever. It also activates synthesis of acute-phase proteins, proteins produced by the liver that function in inflammation. Additionally, IL-18 is expressed in response to LTA (Hara et al., 2018). IL-18 is involved in stimulating interferon γ (IFN-γ) production by natural killer cells and T helper cells. IFN-γ is a potent activator of macrophages and stimulator of MHCII expression (Abbas et al., 2018).

Brauweiler et al. (2019a) found a significant increase in IL-8 and IL-36 as well as CXCL1 and CXCL2 in mice injected intradermally with LTA. IL-8 is also known as CXCL8. It is a chemokine that causes recruitment of neutrophils to the target area (Abbas et al., 2018). IL-36 is less understood, but it is a cytokine that is believed to contribute to inflammatory response in the skin and enhance Th17 response (Zhou & Todorovic, 2021). CXCL1 and CXCL2 are

important chemokines that, like IL-8, are associated with activation and recruitment of neutrophils to the targeted area (Abbas et al., 2018). Brauweiler et al. (2019b) later found that TSLP, IL-4, and IL-13 are also upregulated in response to LTA in mice. TSLP is known to be produced by epithelial cells in response to signals from helminths and causes Th2 activation, leading to an anti-helminth or allergic response (Abbas et al., 2018). IL-4 and IL-13 are produced by several cells in response to TSLP including eosinophils, mast cells, and Th2 cells (Brauweiler et al., 2019b). IL-4 and IL-13 have very similar functions including further stimulation of Th2 development, production of IgE type antibodies by B cells, and activation and recruitment of a number of leukocytes, notably eosinophils and mast cells, but also macrophages involved in tissue repair.

Methods and Materials

Experimental Animals

Two experimental groups of male Light-brown Leghorn (LBL) reared and maintained by Dr. Gisela Erf at the University of Arkansas System Division of Agriculture (UADA) Poultry Research Farm in Fayetteville, Arkansas, were used for in this experiment. Study 1 involved 15, 11-week-old male LBL chickens to examine the dose response and time course of the local inflammatory response initiated by intradermal injection of LTA into the pulp of GF. Study 2 involved 12, 15-week-old male LBL chickens to examine the type, quantity, and time course of the leukocyte recruitment in response to injection of 10 μg/mL LTA into the pulp of GFs. All experimental animals were raised in floor pens on wood shaving litter with standard light and temperature protocols as described by Shi and Erf (2012). Food and water were given *ad libitum*.

This research was approved by the University of Arkansas Department of Agriculture Institutional Animal Care and Use Committee (UADA-IACUC approval #21035).

Study 1. LTA-Dose Response Study

GF-injection and Sample Collection for Immunohistological Staining

When the chickens were 11 weeks of age, the pulp of 18-day-old regenerating GFs were injected with 10 µL of LTA suspension per GF as described in French et al. (2020). Different concentrations of LTA (*Staphylococcus aureus*; Sigma-Aldrich, St. Louis, MO) in endotoxin-free Dulbecco's phosphate buffered saline (EF-DPBS; Sigma-Aldrich) were used for GF-pulp injection; 0.1, 1.0, 10, 100, and 250 µg LTA/mL was administered to 3, 3, 4, 3, and 2 chickens, respectively, with 12 GFs injected per bird. Before injection (0 h), and at 6-, 24-, 48-, and 72-hours post GF-pulp injection, GFs were collected from each chicken for analysis. The pulp of a GF was isolated from the sheath, placed in OCT freezing medium in a labeled tissue cryo-cassette, flash-frozen in liquid nitrogen, and stored at -80°C until use for immunohistochemical staining to identify and localize leukocytes responding to the LTA injection.

Immunohistological Staining of Frozen Pulp Sections

Frozen pulp tissue sections (6 µm thick) were cut at -23°C using a cryostat, placed on positively charged glass microscope slides, fixed in acetone for 5 minutes, and immunochemically stained as described in Sullivan and Erf (2017). To prevent non-specific binding of staining reagents, a solution of phosphate buffered saline (0.01 M; PBS) and 10% horse serum (HS) was added to the section, and the sections were incubated overnight in a humidified chamber at room temperature. After overnight incubation in PBS/10% HS and washing with PBS, pulp sections were incubated 30 minutes with a panel of primary mouse-anti-

chicken (mac) monoclonal antibodies (mAb) specific for cell surface proteins of various leukocyte populations or with mouse IgG1 mAb with irrelevant specificity (isotype control). The primary mac-mAb (all mouse IgG1 isotype) used included CD45 (pan-leukocyte marker), KUL-01 (macrophage marker), CD3 (pan-T-cell marker), Bu-1 (B-cell marker), CD4 (T-helper-cell marker), CD8 (cytotoxic-T-cell marker), TCR1 (γδ-TCR marker), MHCII, and MCAM (endothelial cell marker). All mAbs were purchased from Southern Biotech (Southern Biotech, Birmingham, AL). After the incubation, sections were washed with PBS and incubated with biotinylated horse anti-mouse (ham) IgG secondary antibody (Vektor Laboratories, Inc, Burlingame, CA). Following the 30-minute incubation, the sections were washed again and incubated 30 minutes with a mixture of avidin and horseradish-peroxidase-labeled biotin (Vekta-stain Elite reagents; Vector Laboratories). After this incubation, peroxide-charged diaminobenzidine tetrahydrochloride (DAB), a colorogenic substrate for the peroxidase, was added to the section. This enzyme-substrate reaction forms a brown precipitate which the cells with the antibody complexes brown. The immunochemically stained pulp sections were then counter stained with Methyl green nuclear stain.

Stained tissue sections were observed using a bright field microscope and photographed in order to visualize leukocyte infiltration and complement data obtained by flow cytometry. Observations of leukocyte infiltration were used to inform decisions on the concentration of LTA to be used in Study 2.

Study 2. Leukocyte Infiltration Profile Analysis by Fluorescence-Based Flow Cytometry

LTA-injection and Sample Collection

In Study 2, 12, 15-week-old male LBL chickens were used. The pulp of 12, 18-day-old GFs per chicken were injected with either 10 μL of 10 μg/mL LTA (0.1 μg LTA/GF; n = 8) or 10

μL/GF of EF-DPBS (vehicle-control) as described in French et al. (2020). Before injection (0 hour), and at 6-, 24-, 48-, and 72-hours post injection a GF was collected from each chicken for analysis, placed in Dulbecco's PBS (DPBS), and kept on ice until preparation of pulp suspensions.

Immunofluorescent Staining and Flow Cytometry

Pulp cell suspensions were prepared and immunofluorescently stained as described by French et al. (2020). Briefly, the sheath of the GF was cut longitudinally, and the pulp was removed with forceps, and placed in 0.1% collagenase-dispase solution for 10 minutes at 40°C. The pulp was then gently pushed through a 60 μm nylon mesh with extra DPBS. Cells were washed twice by centrifugation at 250 x *g* for 8 minutes at 4°C, and the final pellet was resuspended in 0.25 mL of PBS+ (0.1 M DPBS, 1% bovine serum albumin, and 0.1% sodium azide).

Cells were stained using a panel of fluorescently labeled mouse IgG1 monoclonal antibodies (mAb) against known leukocyte markers in chickens (Southern Biotech). The mouse anti-chicken (mac) antibodies were used in two-color and three-color direct staining combinations. These are the specific staining combinations used:

1. mac-CD45 mAb conjugated to spectral red (CD45-SPRD; pan-leukocyte marker) and mac-KUL-01 mAb conjugated to phycoerythrin (KUL-01-PE; macrophage marker)
2. mac-TCR1 mAb conjugated to fluoroisothiocyanine (TCR1-FITC; γδ-TCR marker), CD4-PE, and CD8α-SPRD
3. KUL-01-FITC, MHCII-PE, and CD3-SPRD
4. TCR2&TCR3-FITC (αβ TCR marker) and Bu-1-PE (B cell marker)

For immunofluorescent staining, 50 µL of cell suspension was incubated with 50 µL of each staining combination in separate wells of a 96-well round-bottom plate and incubated for 30 minutes at 4°C. Samples of each cell suspension were also pooled and incubated with a mixture of fluorescently-labeled mouse IgG1 mAb of irrelevant specificity to determine non-specific binding of FITC, PE, and SRPD antibodies (isotype control) and the cut-off between negative and positive fluorescence, or single stained with CD45-FITC, CD45-PE, or CD45-SPRD to set compensation. After incubation, cells were washed twice via centrifugation at 250 x *g* for 4 minutes at 4°C. Finally, cells were resuspended in 200 µL PBS+ and acquired on the flow cytometer (Becton Dickinson Accuri C6 Plus; BD Biosciences, San Jose, CA). Flow cytometry data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR) and leukocyte infiltration was expressed as % of total pulp cells in the cell suspension.

Statistical Analysis

Two-way analysis of variance (ANOVA) was conducted to determine the effect of Time (0, 6, 24, 48, and 72 hours post-GF injection), Treatment (LTA, EF-DPBS), and Time by Treatment interaction. Multiple means comparisons were made using Fisher's Least Significant Difference (LSD) method. All difference were considered significant at $P \leq 0.05$.

Results

Study 1. LTA-Dose Response Study

Pulp tissue sections stained using IHC were inspected by bright-field microscopy for the extent of leukocyte infiltration in response to varying doses of LTA. Overall, there was not an appreciable difference in leukocyte infiltration profiles between 10 µg/mL and 100 µg/mL LTA injection, though at 0.1 µg/mL, 1.0 µg/mL, and 250 µg/mL, overall infiltration seemed to be

lower. Representative pictures of IHC staining for MHCII+ cells, T cells (CD3+), and macrophages (Kul-01+) before injection and 24 h after intradermal injection of 10 µL of 10 µg/mL LTA or 100 µg/mL LTA attest to the local inflammatory activity in response to LTA-injection (**Figure 1)**. Based on the IHC staining evaluation, the 10 µg LTA/mL concentration was chosen as the optimal dose for Study 2.

Study 2. Leukocyte Infiltration Profile Analysis by Fluorescence-Based Flow Cytometry

Overall, injection of LTA in the GF resulted in pulp-infiltration of leukocytes (CD45+) reaching maximal levels 6 h post injection (p.i.) and remained elevated throughout the 72-hour examination (**Figure 2**). However, of the infiltrating leukocytes, only levels (% pulp cells) of macrophages and MHCII+ cells were higher ($P \leq 0.05$) in LTA- than in PBS-injected GFs (**Figure 3**).

Specifically, for total leukocytes there was a time x treatment interaction ($P = 0.048$), hence time and treatment effects are described for each treatment (LTA, PBS) at each time point (0, 6, 24, 48 and 72 h). Before injection (0 h), levels of total leukocytes were not different in LTA- and PBS-injected GFs (5% vs. 6%, respectively; Note: percentage pulp cell values are rounded to the nearest whole number). At 6 h p.i., the proportion of leukocytes increased in LTA-injected GFs to 19% and in PBS-injected GFs to 15% of pulp cells, and this increase was higher ($P \leq 0.013$) for LTA. In PBS-injected GFs, leukocyte levels dropped to 10% at 24 h, 8% at 48 h, and returned to baseline levels at 72 h. However, in LTA-injected GFs, leukocyte levels remained elevated at 24 h (17%) before decreasing ($P \leq 0.05$) to 10% at 48 h and remained elevated (9 %) at 72 h. Leukocyte levels were higher in LTA- than PBS-injected GFs at 6, 24, and 72 h p.i. (**Figure 2A**). Heterophil levels changed over time ($P < 0.001$) with both treatments increasing from 2% before injection to peak levels (5%) at 6 h p.i. and returning to baseline

levels by 24 h and then remaining at this level thereafter. There were no treatment differences at any of the time points examined (**Figure 2B**).

A time x treatment interaction effect also was observed for the macrophage population ($P < 0.001$). Before injections (0 h), macrophage levels were similar (near 2%) in both LTA and PBS treatment groups. Macrophage levels did not change over the time course examined in response to PBS injection. However, following injection of LTA, the proportion of macrophages increased ($P = 0.005$) to 3% at 6 h, then increased further ($P < 0.001$) reaching peak levels of 6% at 24 h before dropping ($P < 0.001$) to above pre-injection levels (3%) at 48 h and remaining at this elevated level at 72 h. Macrophage levels in LTA-injected GFs were higher than in PBS-injected GF at 24, 48, and 72 h p.i. ($P < 0.001$, $P = 0.010$, and $P < 0.001$, respectively) (**Figure 2A**).

A time-treatment interaction effect was not observed for MHCII+ cells ($P = 0.064$). However, there was a main effect of treatment, with overall higher ($P = 0.021$) levels of MHCII+ cells in LTA- than PBS-injected GFs (5.44 ± 0.28 % vs 4.37 ± 0.03 %), and a main effect of time, with elevated levels of MHCII+ cells at 6, 24, and 48 h p.i. In **Figure 2B**, the time course of the PBS and LTA responses are shown separately, rather than the main effect means, to high-light that the LTA MHCII+ cell profile parallels that observed for macrophages.

There were no time x treatment interactions or treatment effects for all T cell populations examined (i.e., all T cells (CD3+), and CD4+, CD8+, αβ TCR+, γδ, TCR+ T cell subsets) and the CD4 to CD8 T cell ratio (**Table 1 & 2**). The only treatment effect noted is a lower ratio between γδ T cells and αβ T cells in LTA- compared to PBS-injected GFs (0.41 ± 0.02 % vs 0.50 ± 0.03 %; $P = 0.031$). With the exception of γδ T cell, which were elevated at 6 and 24 h p.i., all other T cell subset, the CD4 to CD8 T cell ratio and the γδ TCR to αβ TCR ratio were elevated at

6, 24, and 48 h p.i. (**Table 1 & 2**). Similarly, for B cells (Bu-1+) there was only a main effect of time ($P < 0.001$), with elevated levels at 6 h that reach a peak at 24 and 48 h and drop to 6 h levels at 72 h (**Table 2**).

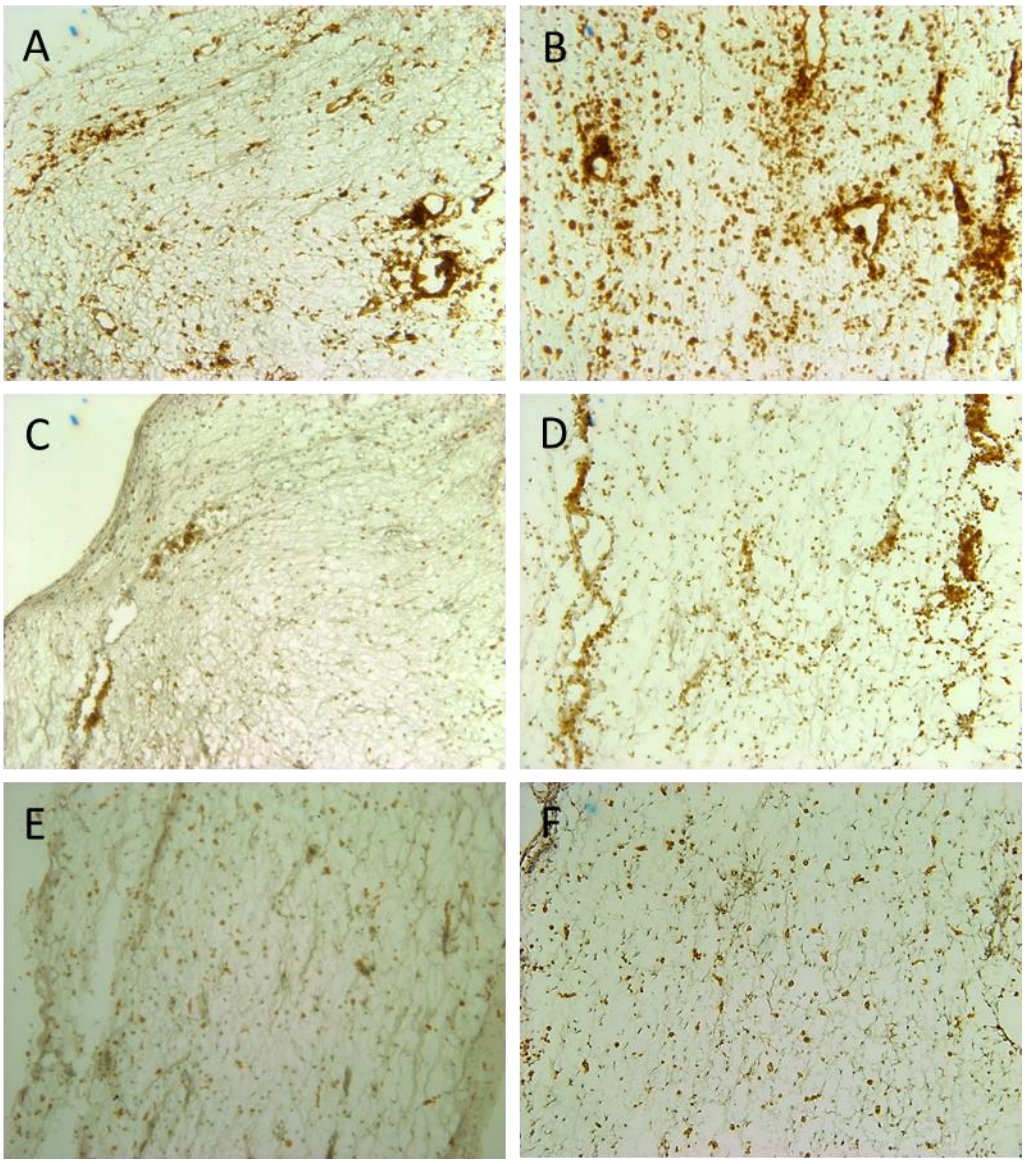

Figure 1. Immunohistochemical staining of MHC II+ cells, T cells, and macrophages in the pulp of chicken growing feathers (GFs) injected with lipoteichoic acid (LTA) solution in endotoxin free phosphate buffered saline. Growing feathers of 11-week-old chickens were injected with 10 µL of 100 µg/mL of LTA (B, D, E) or 10 µg/mL of LTA (F) per GF. GF pulp was isolated from the sheath and flash frozen in liquid nitrogen. Histological slides were cut using a cryostat and stained with mouse-monoclonal primary antibodies against chicken MHCII (A, B), CD3 (C, D; T cells), and Kul-01 (E, F; macrophages), before (A, C) and at 24 hours post-LTA injection (B, D, E, F). Photos were taken at 100x magnification on a bright-field microscope; brown cells are expressing the markers detected by the primary antibodies.

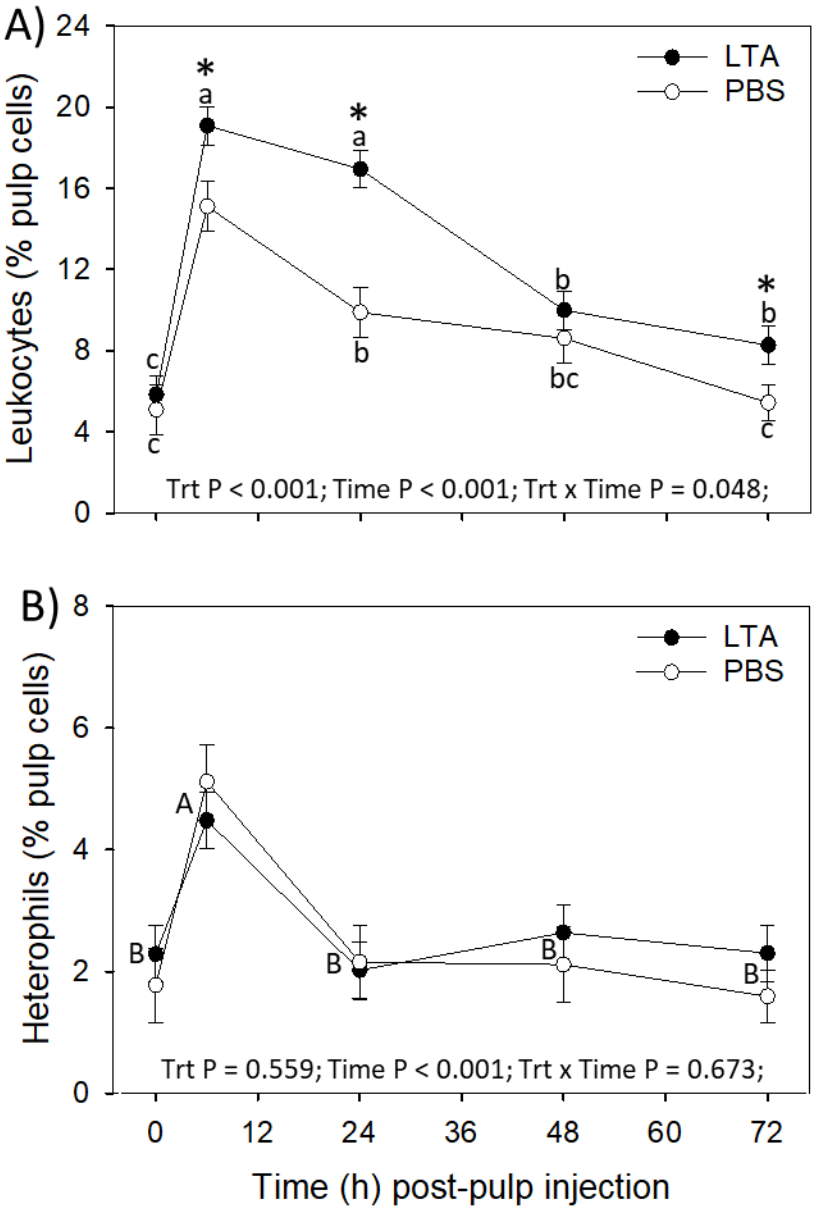

Figure 2. Total leukocyte pulp cell proportion after intradermal injection of lipoteichoic acid (LTA) or endotoxin-free phosphate buffered saline (PBS; vehicle control) into growing feathers (GFs). Growing feathers of 12, 18-day-old chickens were injected with 10 µL PBS (control) or 10 µg/mL of LTA per GF. GFs were collected before injection (0 hours), and at 6-, 24-, 48-, and 72-hours post-injection. Pulp cell suspensions were created from GFs and immunofluorescently stained using a panel of fluorescence-conjugated mouse monoclonal antibodies against chicken CD45 (pan-leukocyte marker). Cell populations were analyzed by flow cytometry; heterophils were identified based on forward and side scatter characteristics of CD45+ cells. Data shown are mean ± SEM; LTA n = 8; PBS n = 4. Data shown are mean ± SEM. $^{a\text{-}c}$Within a treatment (Trt), means without a common letter are different ($P \leq 0.05$); *Trt means within a time-point are different ($P \leq 0.05$); $^{A\text{-}C}$Time main effect means without a common letter are different ($P \leq 0.05$).

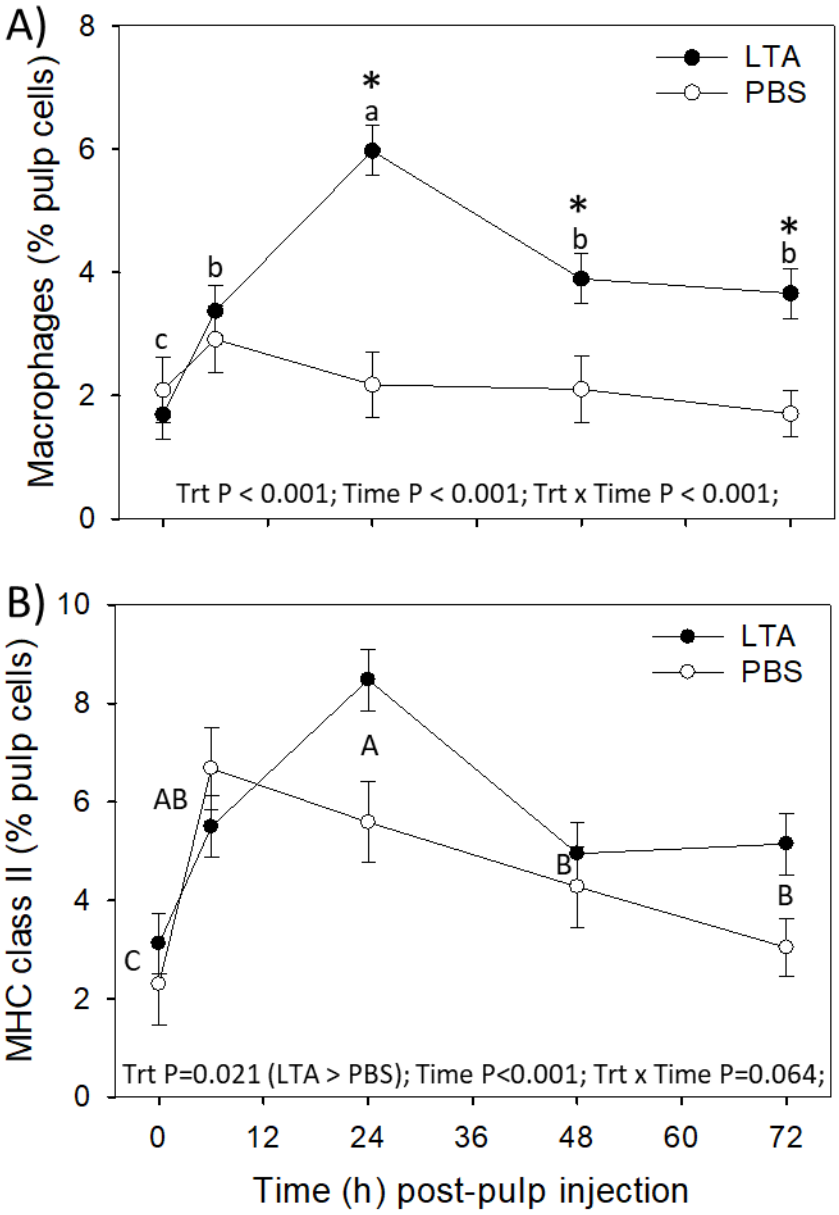

Figure 3. Macrophage and MHC class II-expressing pulp cell proportions after intradermal injection of lipoteichoic acid (LTA) or endotoxin-free phosphate buffered saline (PBS; vehicle control) into growing feathers (GFs). Growing feathers of 12, 18-day-old chickens were injected with 10 µL PBS (control) or 10 µg/mL of LTA per GF. GFs were collected before injection (0 hours), and at 6-, 24-, 48-, and 72-hours post-injection. Pulp cell suspensions were prepared from GFs and immunofluorescently stained using a panel of fluorescence-conjugated mouse monoclonal antibodies against chicken Kul-01 (macrophage marker) or MHCII. Cell populations were analyzed by flow cytometry. Data shown are mean ± SEM; LTA n = 8; PBS n = 4. $^{a\text{-}c}$Within a treatment (Trt), means without a common letter are different ($P \leq 0.05$); *Trt means within a time-point are different ($P \leq 0.05$); $^{A\text{-}C}$Time main effect means without a common letter are different ($P \leq 0.05$).

Table 1. Pulp cell proportions of T cells after intradermal injection of lipoteichoic acid (LTA) or phosphate buffered saline (PBS; vehicle control) into growing feathers of 18-day-old chickens.[1]

Treatment	CD3	CD4	CD8	CD4:CD8[2]
LTA	4.71 ± 0.24[3]	1.45 ± 0.13	2.63 ± 0.10	0.54 ± 0.04
PBS	4.61 ± 0.32	1.42 ± 0.16	2.70 ± 0.13	0.51 ± 0.06
Time (h)				
0	2.67 ± 0.45**c**	0.47 ± 0.24**c**	2.38 ± 0.19**c**	0.19 ± 0.08**d**
6	6.28 ± 0.45**a**	2.42 ± 0.24**a**	3.00 ± 0.19**b**	0.80 ± 0.08**a**
24	6.05 ± 0.45**ab**	1.96 ± 0.24**ab**	3.71 ± 0.19**a**	0.53 ± 0.08**bc**
48	4.89 ± 0.45**b**	1.49 ± 0.24**b**	2.23 ± 0.19**c**	0.67 ± 0.08**ab**
72	3.40 ± 0.45**c**	0.83 ± 0.20**c**	2.01 ± 0.19**c**	0.43 ± 0.08**c**
Effects (P-value)				
Treatment	0.798	0.894	0.687	0.633
Time	<0.001	<0.001	<0.001	<0.001
Treatment x Time	0.470	0.334	0.531	0.529

[1]Growing feathers of 12, 18-day-old chickens were injected with 10 µL PBS (control) or 10 µg/mL of LTA per GF. GFs were collected before injection (0 hours), and at 6-, 24-, 48-, and 72-hours post-injection. Pulp cell suspensions were created from GFs and immunofluorescently stained using a panel of fluorescence-conjugated mouse monoclonal antibodies against chicken CD3 (T cells), CD4 (helper T cells), and CD8 (cytotoxic T cells). Cell populations were analyzed by flow cytometry.

[2]CD4:CD8 is the ratio of CD4+ cells to CD8+ cells.

[3]Data shown are mean ± SEM.

a, b, c, d: means within a column without a common letter are different based on $P \leq 0.050$ based on multiple means comparisons.

Table 2. Pulp cell proportions of lymphocytes after intradermal injection of lipoteichoic acid (LTA) or phosphate buffered saline (PBS; vehicle control) into growing feathers of 18-day-old chickens.[1]

Treatment	αβ TCR	γδ TCR	Bu-1	γδ TCR:αβ TCR[2]
LTA	3.42 ± 0.18[3]	1.29 ± 0.08	0.57 ± 0.07	0.41 ± 0.02**b**
PBS	3.13 ± 0.22	1.47 ± 0.11	0.56 ± 0.09	0.50 ± 0.03**a**
Time (h)				
0	1.79 ± 0.33**b**	0.88 ± 0.15**b**	0.11 ± 0.13**c**	0.52 ± 0.05**a**
6	4.37 ± 0.33**a**	1.91 ± 0.15**a**	0.45 ± 0.13**b**	0.43 ± 0.05**a**
24	3.99 ± 0.33**a**	2.06 ± 0.15**a**	0.86 ± 0.13**a**	0.56 ± 0.05**a**
48	3.87 ± 0.33**a**	1.02 ± 0.15**b**	0.93 ± 0.13**a**	0.27 ± 0.05**b**
72	2.36 ± 0.27**b**	1.04 ± 0.15**b**	0.47 ± 0.11**b**	0.47 ± 0.05**a**
Effects (P-value)				
Treatment	0.326	0.192	0.921	0.031
Time	<0.001	<0.001	<0.001	<0.001
Treatment x Time	0.605	0.295	0.104	0.750

[1]Growing feathers of 12, 18-day-old chickens were injected with 10 µL PBS (control) or 10 µg/mL of LTA per GF. GFs were collected before injection (0 hours), and at 6-, 24-, 48-, and 72-hours post-injection. Pulp cell suspensions were created from GFs and immunofluorescently stained using a panel of fluorescence-conjugated mouse monoclonal antibodies against chicken αβ T-cell receptor (TCR), γδ TCR, and Bu-1 (B cells). Cell populations were analyzed by flow cytometry.

[2] γδ TCR:αβ TCR is the ratio of γδ T cells to αβ T cells.

[3]Data shown are mean ± SEM.

a, b, c: means within a column without a common letter are different based on $P \leq 0.050$ based on multiple means comparisons.

Discussion

The type of leukocytes that respond to a specific immunogen can reveal a lot about the immune response to certain types of pathogens. In chickens, the GF can be used as a minimally invasive dermal test site to investigate leukocyte responses to immunogenic substances (Erf & Ramachandran, 2016). An understanding of how the immune system responds to LTA *in vivo* can help paint a clearer picture of the progression of infection by Gram-positive bacteria. In this experiment, LTA injection into multiple GFs of a chicken and collection of GFs at regular time points thereafter provided insight into the type of local cellular responses that LTA is able to initiate.

The local cellular response to LTA in dermal tissue of chickens is dominated by macrophages. Total leukocytes, macrophages, and MHCII+ cells were the only groups of cells whose concentrations increased significantly in the pulp following injection of LTA when compared to the PBS control. Given that macrophages are included in both total leukocytes and MHCII+ cells, it is likely that the increase in these two groups can be explained at least partially by the increase in macrophages. As illustrated in **Figure 3**, the response to LTA of MHCII+ cells over time closely reflects the response of macrophages, suggesting that macrophages make up the major portion of the MHCII+ cells. Other MHCII+ cells likely are B cells and endothelial cells of venules.

Surprisingly, heterophils were not recruited at significantly higher levels in response to LTA compared to the PBS control. This means the increased levels of heterophils seen at 6 h p.i. are only a result of tissue damage associated with the injection. Heterophils, the avian equivalent of neutrophils, are the most abundant and primary responders in an inflammatory reaction (Abbas et al., 2018). French et al. found that heterophils dominated the immune response to

intradermal lipopolysaccharide (LPS), a major immunogen and TLR4 ligand found in Gram-negative bacteria (French et al., 2020). Furthermore, Brauweiler et al. (2019a) found that intradermal LTA increased mRNA concentration of the neutrophil marker Ly-6G in mice when compared to a PBS control. As macrophages were recruited at significantly higher levels compared to the PBS control, it appears that the macrophage-dominated local leukocyte response to LTA in chickens involves the expression of a different set of cytokines and chemokines compared to mice, as well as a different set of signaling molecules than those expressed in response to LPS. Chicken TLR2 is known to cause expression of CXCL8, a heterophil attracting chemokine (Keestra et al., 2013). However, it appears that this pathway was not activated at a sufficient level to significantly increase heterophil infiltration beyond the level induced by the vehicle injection control.

Lymphocytes were not recruited at levels significantly higher after LTA injection when compared to the PBS control. Interestingly, the ratio of γδ T cells to αβ T cells was significantly lower in response to LTA injection when compared to PBS. GFs injected with LTA averaged higher levels of αβ T cells and lower levels of γδ T cells than those injected with PBS, though these values were not statistically significant. It is unclear why T cell infiltration in response to LTA may include lower levels of γδ T cells relative to αβ T cells, and more research needs to be done to investigate this.

While macrophages levels peaked at 24 h p.i., total leukocytes infiltration peaked at 6 h p.i. At 6 h p.i. macrophage infiltration was not significantly different between treatments, while total leukocyte infiltration was. This suggests that some leukocyte other than macrophages, heterophils, or lymphocytes accounted for the difference in total leukocyte levels. Given that Brauweiler et al. (2019b) found that LTA stimulated recruitment of basophils in the murine

dermis, it is possible that basophil recruitment contributed to the increase in total leukocytes observed at 6 h p.i.

While a number of studies have investigated *in vitro* effects of LTA, there is a lack of research investigating the effects of this molecule *in vivo*, and none of these studies have been performed in chickens. Future research should investigate changes in cytokine and chemokine levels in response to intradermal LTA injection in order to paint a clearer picture of why LTA-stimulated leukocyte infiltration is dominated by macrophages and lacking in heterophils and lymphocytes. It will also be important to investigate infiltration of other subsets of leukocytes, specifically granulocytes.

References

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2018). *Cellular and molecular immunology*. (9th ed.). Philadelphia, PA: Elsevier Saunders.

Brauweiler, A. M., Goleva, E., & Leung, D. Y. M. (2019). *Staphylococcus aureus* lipoteichoic acid damages the skin barrier through an IL-1–mediated pathway. *Journal of Investigative Dermatology*, *139*(8), 1753-1761. https://doi.org/10.1016/j.jid.2019.02.006

Brauweiler, A. M., Goleva, E., & Leung, D. Y. M. (2019). *Staphylococcus aureus* lipoteichoic acid initiates a TSLP-basophil-IL4 axis in the skin. *Journal of Investigative Dermatology, 140*(4), 915-917. https://doi.org/10.1016/j.jid.2019.09.004

Dessing, M. C., Schouten, M., Draing, C., Levi, M., von Aulock, S., & van der Poll, T. (2008). Role played by toll-like receptors 2 and 4 in lipoteichoic acid–induced lung inflammation and coagulation. *Journal of Infectious Diseases*, *197*(245), 245-252. https://doi.org/10.1086/524873

Erf, G. F., & Ramachandran, I. R. (2016). The growing feather as a dermal test site: Comparison of leukocyte profiles during the response to *Mycobacterium butyricum* in growing feathers, wattles, and wing webs. *Poultry Science*, *95*(9), 2011-2022. https://doi.org/10.3382/ps/pew122

French, C. E., Sales, M. A., Rochell, S. J., Rodriguez, A., & Erf, G. F. (2020). Local and systemic inflammatory responses to lipopolysaccharide in broilers: new insights using a two-window approach. *Poultry Science, 99*(22), 6593-6605. https://doi.org/10.1016/j.psj.2020.09.078

Farnell, M. B., He, H., & Kogut, M. H. (2003). Differential Activation of Signal Transduction Pathways Mediating Oxidative Burst by Chicken Heterophils in Response to Stimulation

with Lipopolysaccharide and Lipoteichoic Acid. *Inflammation*, *27*(4), 225-231. https://doi.org/10.1023/A:1025088514676

Ginsburg, I. (2002). Role of lipoteichoic acid in infection and inflammation. *The Lancet Infectious Diseases*, *2*(3), 171-179. https://doi.org/10.1016/S1473-3099(02)00226-8

Günther, J., & Seyfert, H. M. (2018). The first line of defence: insights into mechanisms and relevance of phagocytosis in epithelial cells. *Seminars in immunopathology*, 40(6), 555–565. https://doi.org/10.1007/s00281-018-0701-1

Haddadi, S., Thapa, S., Kameka, A. M., Hui, J., Czub, M., Nagy, E., Muench, G., & Abdul-Careem, M. F. (2015). Toll-like receptor 2 ligand, lipoteichoic acid is inhibitory against infectious laryngotracheitis virus infection *in vitro* and *in vivo*. *Developmental and Comparative Immunology, 48*(1), 22-32. https://doi.org/10.1016/j.dci.2014.08.011

Hara, H., Seregin, S. S., Yang, D., Fukase, K., Chamaillard, M., Alnemri, E. S., Inohara, N., Chen, G. Y., & Núñez, G. (2018). The NLRP6 Inflammasome Recognizes Lipoteichoic Acid and Regulates Gram-Positive Pathogen Infection. *Cell*, *175*(6), 1651-1664. https://doi.org/10.1016/j.cell.2018.09.047

Keestra, A. M., de Zoete, M. R., Bouwan, L. I., Vaezirad, M. M., & van Putten, J. P. M. (2013). Unique features of chicken Toll-like receptors. *Developmental & Comparative Immunology, 41*(3), 316-323. https://doi.org/10.1016/j.dci.2013.04.009

Laloučková, K., & Skřivanová, E. (2019). Antibiotic Resistance in Livestock Breeding: A Review. *Scientia Agriculturae Bohemica*, *50*(1), 15-22. https://doi.org/10.2478/sab-2019-0003

Lubick, K., & Jutila, M. A. (2006). LTA recognition by bovine γδ T cells involves CD36. *Journal of Leukocyte Biology, 79*(6), 1268-1270. https://doi.org/10.1189/jlb.1005616

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature, 454*, 428-425 (2008). https://doi.org/10.1038/nature07201

Nemazee, D. (2006). Receptor editing in lymphocyte development and central tolerance. *Nature Reviews Immunology, 6*, 728-740. https://doi.org/10.1038/nri1939

Oliveira-Nascimento, L., Massari, P., & Wetzler, L. M. (2012). The role of TLR2 in infection and immunity. *Frontiers in Immunology, 3*(79). https://doi.org/10.3389/fimmu.2012.00079

Percy, M. G., & Gründling, A. (2014). Lipoteichoic acid synthesis and function in gram-positive bacteria. *Annual Review of Microbiology, 68,* 81-100. https://doi.org/10.1146/annurev-micro-091213-112949

Ranoa, D. R. E., Kelley, S. L., & Tapping, R. I. (2013). Human lipopolysaccharide-binding protein (LBP) and CD14 independently deliver triacylated lipoproteins to toll-like receptor 1 (TLR1) and TLR2 and enhance formation of the ternary signaling complex. *Journal of Biological Chemistry, 288*(14), 9729-9741. https://doi.org/10.1074/jbc.M113.453266

Reichmann, N. T., & Gründling, A. (2011). Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in gram-positive bacteria of the phylum *Firmicutes*. *FEMS Microbiology Letters, 319*(2), 97-105. https://doi.org/10.1111/j.1574-6968.2011.02260.x

Rojo, J. M., Bello, R., & Portolés, P. (2008). T-Cell Receptor. In A. B. Sigalov (Eds.), Multichain Immune Recognition Receptor Signaling (pp. 1-11). Springer, New York, NY. https://doi.org/10.1007/978-0-387-09789-3_1

Schröder, N. W. J., Heine, H., Alexander, C., Manukyan, M., Eckert, J., Hamann, L., Göbel, U. B., & Schumann, R. R. (2004). Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. *The Journal of Immunology, 173*(4), 2683-2691. https://doi.org/10.4049/jimmunol.173.4.2683

Schröder, N. W. J., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U. B., Weber, J. R., & Schumann, R. R. (2003). Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *The Journal of Biological Chemistry*, *278*(18), 15587-15594. https://doi.org/10.1074/jbc.M212829200

Seo, H. S., Michalek, S. M., & Nahm, M. H. (2008). Lipoteichoic acid is important in innate immune responses to gram-positive bacteria. *Infection and Immunity, 76*(1), 206-213. https://doi.org/10.1128/IAI.01140-07

Shi, S., & Erf, G. F. (2012). IFN-γ, IL-21, and IL-10 Co-Expression in Evolving Autoimmune Vitiligo Lesions of Smyth Line Chickens. *Journal of Investigative Dermatology, 132*(3), 642-649. https://doi.org/10.1038/jid.2011.377

Sullivan, K. A., and Erf, G. F. (2017). CD4+ T cells dominate the leukocyte infiltrations response initiated by intra-dermal injection of phytohemagglutinin into growing feathers in chickens. *Poultry Science, 96*(10), 3574-3580. https://doi.org/10.3382/ps/pex135

Treanor, B. (2012). B-cell receptor: from resting state to activate. *Immunology, 136*(1). https://doi.org/10.1111/j.1365-2567.2012.03564.x

Wesch, D., Peters, C., Oberg, H. H., Pietschmann, K., & Kabelitz, D. (2011). Modulation of γδ T cell responses by TLR ligands. *Cellular and Molecular Life Sciences, 68*(14), 2357-2370. https://doi.org/10.1007/s00018-011-0699-1

Zhou, L., & Todorovic, V. (2021). Interleukin-36: Structure, Signaling and Function. *Advances in Experimental Medicine and Biology, 21*, 191–210. https://doi.org/10.1007/5584_2020_488