

Immunological Response to *Clostridium perfringens* in Two Genetically Divergent Lines of Chickens as Influenced by Major Histocompatibility Complex (MHC) Genotype

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Keywords: necrotic enteritis, *Clostridium perfringens*, chicken, genetic resistance,
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Lindsay H. Sumners

ABSTRACT

Chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) displayed a correlated change in major histocompatibility complex (MHC), so that LA chickens were 96% B¹³ and HA chickens were 96% B²¹. During a clinical outbreak of necrotic enteritis, B²¹B²¹ genotypes experienced significantly less mortality (6% vs. 13 %) compared to B¹³B¹³ genotypes. A study was carried out to assess immunological differences between LA and HA lines during exposure to *Clostridium perfringens*. In Experiment 1, chickens were orally gavaged with a low (10⁷ CFU/mL) or high (10⁹ CFU/mL) dose of *C. perfringens*. In Experiment 2, chickens were orally gavaged with live coccidia oocysts on experiment d 1, followed by 10⁷ CFU/mL *C. perfringens* on d 5. Unfortunately, establishment of necrotic enteritis infection was unsuccessful in both experiments as evidenced by lack of significant intestinal lesions, as well as no negative effect on bird performance. In an ex vivo study, peripheral blood mononuclear cells (PBMCs) were isolated from each genetic line, cultured, stimulated with LPS (4 h), and exposed to varying concentrations of *C. perfringens* α -toxin (1, 10, 100, 1000 U/L) for 2 and 4 h. Evaluation of cellular proliferation, percent cytotoxicity and immunological gene expression was carried out in a variety of experiments. Genetic lines were found to be highly divergent in all analyses.

Keywords: necrotic enteritis, *Clostridium perfringens*, chicken, genetic resistance, immune response

Dedication

I would like to dedicate this thesis to my grandmother, Martha Hart. Two full generations of Hart college graduates, all because of you. I will love you forever and always.

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Mom and Dad – You have both always been there through everything, and more than that, you have always believed in me. Mom, you taught me to ride a bike, sewed my prom dress, handmade tablecloths for my wedding, and showed me how to be a strong and successful woman. Dad, you taught me to sail, made me an M&M costume for Halloween, built an arbor and cupcake stand for my wedding, and showed me what to look for in a good husband. You both mean more to me than you will ever know, and I can only hope that Austin and I will be half as good parents as you were, and still are.

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List of Abbreviations

- AGPs:** Antibiotic growth promoters
- ANOVA:** Analysis of variance
- APCs:** Antigen presenting cells
- BW:** Body weight
- BWG:** Body weight gain
- CFU:** Colony forming unit
- ConA:** Concanavalin A
- CP:** *Clostridium perfringens*
- CTL:** Cytotoxic T lymphocytes
- d:** Day
- D:** Diversity
- DMEM:** Dulbecco's Modified Eagle's Medium
- DOH:** Day of hatch
- Duo:** Duodenum
- FBS:** Fetal bovine serum
- FCR:** Feed conversion ratio
- FI:** Feed intake
- GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase
- h:** Hours
- HA:** High antibody
- HBSS:** Hank's Balance Salt Solution
- HSD:** Honestly significant differences
- IFN:** Interferon
- Ig:** Immunoglobulin
- IL:** Interleukin
- Ile:** Ileum
- iNOS:** Inducible nitric oxide synthase
- J:** Joining
- Jej:** Jejunum

LA: Low antibody
LDH: Lactose dehydrogenase
LITAF: Lipopolysaccharide induced tumor necrosis factor- α factor
LPS: Lipopolysaccharide
LS Means: Least squares means
MHC: Major histocompatibility complex
NED: *N*-1-naphthylethylenediamine dihydrochloride
NK: Natural killer
NO: Nitric oxide
PAMPs: Pathogen associated molecular patterns
PBS: Phosphate buffered saline
PGY: Peptone glucose yeast
PMNs: Polymorphonuclear cells
PRRs: Pathogen recognition receptors
qRT-PCR: Quantitative real-time polymerase chain reaction
SRBC: Sheep red blood cell
Tc: Cytotoxic T cell
TCR: T cell repertoire
Th: Helper T cell
Th0: Naïve T cell
Th1: Type 1 helper T cell
Th2: Type 2 helper t cell
TLR: Toll-like receptor
TNF: Tumor necrosis factor
V: Variable

Chapter I

Introduction

Necrotic enteritis is a common enteric disease experienced by several avian species including broilers, laying hens, turkeys, and quail, and is found in most poultry producing countries (McDevitt et al., 2006). Chickens may suffer from two forms of the disease; clinical (acute necrotic enteritis) or subclinical infection. Clinical infection is typically characterized by development of necrotic intestinal lesions, predominantly confined to the distal portion of the small intestine. Birds may experience depression, ruffled feathers, inappetance, closed eyes, immobility, and dark color diarrhea. In addition to lesion development, intestines often become friable, inflamed, and appear distended. The intestinal wall is usually coated with a thick intestinal mucosa, ranging in color from yellow to dark brown. The clinical illness is usually extremely short in duration and birds are most often found dead before any clinical symptoms are observed (Wages and Opengart, 2003). Chickens suffering from subclinical necrotic enteritis are negatively affected in terms of low body weight gain and poor feed efficiency. Due to the lack of clinical symptoms, subclinical necrotic enteritis often goes unnoticed and is considered to be even more financially taxing than the acute form (Keyburn et al., 2008). As a whole, necrotic enteritis has been estimated to cost the United States poultry industry \$2 to 3 billion annually (McDevitt et al., 2006; Zekarias et al., 2008).

The causative agent of necrotic enteritis is the anaerobic bacterium *Clostridium perfringens*. *C. perfringens* is a Gram-positive, spore-forming, rod shaped bacterium found ubiquitously in the environment, as well as in the intestinal tracts of most animals, including poultry and humans. There are five main types of *C. perfringens* (A, B, C, D, E) that produce four main toxins (alpha, beta, epsilon, iota), as well as a common enterotoxin encoded by the *cpe*

gene (Niilo, 1980; Sheedy et al., 2003). Type A is responsible for producing avian necrotic enteritis, as well as gas gangrene and some types of human food poisoning (Wages and Opengart, 2003; Weiduo et al., 2007). The *C. perfringens* α -toxin is produced by all five types; however, type A is unique in that it is the only strain known to exclusively secrete α -toxin. Research conducted over the past several decades has consistently implicated α -toxin as one of the main virulence factors of *C. perfringens* in the pathogenesis of avian necrotic enteritis (Al-Sheikhly and Truscott, 1976a, b, c; Fukata et al., 1988; Kulkarni et al., 2007, 2008; Zekarias et al., 2008; Cooper et al., 2009).

A wide array of predisposing factors can result in increased susceptibility to infection, including diets consisting of wheat, rye, oats or barley, large amounts of dietary animal byproducts, such as fish meal, meat meal and animal fat, dietary zinc, low intestinal acidity, intestinal stasis, climate and time of year, intestinal mucosa damage, particularly due to coccidial infection, and immunosuppression (Williams, 2005). Taking care to avoid exposure to predisposing factors is a critical initial step in preventing necrotic enteritis outbreak in a flock. One of the more common prevention and treatment options involves dietary inclusion of antibiotic growth promoters (AGPs) or ionophores (Williams, 2005). However, with the recent consumer concern regarding antibiotic resistance, coupled with the European Union ban on the use of antibiotics in poultry feed, there is increased need for unique prevention and treatment options. One such option involves development of chickens genetically resistant to necrotic enteritis infection.

In a selective breeding experiment carried out by geneticist Dr. Paul B. Siegel, White Leghorn chickens were genetically selected for low (LA) or high (HA) antibody response five days following intravenous injection of 0.1 mL (0.25% suspension) sheep red blood cells

(SRBC; Siegel and Gross, 1980). Individuals from the two lines were crossed and then backcrossed to each parental line for three consecutive generations. Sublines were closed following each generational backcross. Selective breeding allowed for the development of two sublines containing a minimum of 93% background genome (LA or HA) and possessing one of two MHC genotypes (B^{13} or B^{21}). Over the next several generations, genetic lines became increasingly divergent so that by the 12th generation of selection, chickens exhibited a correlated change in MHC; LA birds were comprised of 96% B^{13} , while HA birds were 96% B^{21} (Dunnington et al., 1992). Selection is currently in the 38th generation, during which the lines have been extensively utilized in a multitude of experiments and studies. LA and HA lines continue to be increasingly divergent in regards to production traits, immune response, and disease resistance.

When chickens were in their 5th generation after backcrossing ceased, a flock containing birds from each background genome (LA and HA), as well as both MHC genotypes (B^{13} and B^{21}) were diagnosed with necrotic enteritis at 214 days of age (Siegel et al., 1993). Following examination of daily records, there was no real difference in mortality between background genomes; however, chickens possessing $B^{13}B^{13}$ genotypes experienced higher mortality (13% vs. 6%) beginning at an earlier time point. The fact that chickens possessing the MHC genotype $B^{21}B^{21}$ may experience decreased susceptibility to necrotic enteritis could serve as a valuable tool in developing genetically resistance flocks.

The objective of this study was to evaluate immunological differences between LA ($B^{13}B^{13}$) and HA ($B^{21}B^{21}$) lines during exposure to *C. perfringens*. A better understanding of immune response to *C. perfringens* and its secreted toxins, as influenced by MHC genotype, will provide important knowledge in the battle against avian necrotic enteritis.

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CHAPTER II

Literature Review

In the constant battle against common poultry diseases, like necrotic enteritis, the role of infectious immunity is an important and ongoing topic of research. Breeding for decreased disease susceptibility through genetic selection, particularly at the major histocompatibility complex (MHC), is a unique and effective way of incurring immunity to infection.

Avian Immune System

The immune system is responsible for mounting defense against foreign antigens. The avian immune system is comparable to its mammalian counterpart in terms of both structure and function, with some exceptions. Two key components of the immune system are innate and adaptive immunity; the latter can be further broken down to include humoral and cell-mediated immune responses.

Innate Immunity

The innate immune system serves as the first line of defense against invasion by foreign antigens. The most basic form of protection lies in the ability of mucosal layers lining the respiratory and gastrointestinal tracts to prevent physical entrance of elements. The high temperature maintained by avian species, as well as the intestinal microflora and acidic enteric environment, also aid in preventing colonization by infectious agents (Goldsby et al., 2000; Butcher and Miles, 2001; Sharma, 2003).

Innate immune responses are characterized as rapid and immediate reactions to non-specific invaders. The innate immune system is indiscriminate between self and non-self, resulting in a lack of immunologic memory and the inability to provide long-term immunity to

infectious diseases. The fundamental concept behind eliciting a proper immune response is the ability to determine self from non-self. Due to lack of specificity, the innate immune system utilizes a unique mechanism for detecting invading pathogens involving recognition of molecular structures present in foreign organisms. These structures, known as pathogen associated molecular patterns (PAMPs), are critical for pathogen survival and can be found in microbial membranes, cell walls, proteins, and nucleic acids (Jenkins et al., 2007). Pattern recognition receptors (PRRs) are expressed on the surface of host immune cells and allow for PAMP detection.

Immune cells involved in innate immune response include macrophages, heterophils, dendritic cells, and natural killer (NK) cells (Zekarias et al., 2002; Fairbrother et al., 2004). Non-specific immune processes include phagocytosis, inflammatory response, acute phase reaction, and initiation of the complement system (Zekarias et al., 2002; Fairbrother et al., 2004). Almost immediately following detection of an invading pathogen, the innate immune system begins initiating a response (Kogut, 2009). Specifically, heterophils have been observed to exhibit adhesion, chemotaxis, phagocytosis, production of cytokines and chemokines, degranulation, and respiratory burst (Swaggerty et al., 2009). Toll-like receptors (TLRs) also play an important role in this rapid response by triggering a series of signal transduction networks, including activation of microbicidal activities, cytokine/chemokine secretion, production of co-stimulatory molecules, and antigen presentation (Kogut, 2009; Swaggerty et al., 2009). In this way, the innate immune system plays a critical role in initiating an acquired immune response.

Adaptive Immunity

The adaptive immune system is capable of exerting a highly evolved and specialized type of immune response only found in vertebrates. In contrast to innate immunity, the adaptive

immune system possesses specificity for antigen receptors and, in comparison, executes a much slower response. It is capable of retaining specific antigenic memory and therefore plays an important role in the differences in disease resistance among individuals (Zekarias et al., 2002). Mediator proteins associated with adaptive immunity include MHC molecules, T and B cell receptors, antibodies, and cytokines (Zekarias et al., 2002). The adaptive immune system can be further broken down to include cell-mediated and humoral immunity.

Cell-mediated Immunity

The cell-mediated immune system is predominantly involved in elimination of intracellular pathogens through direct cell-cell interactions. The main cell responsible for performing the majority of cell-mediated functions is the T cell. T cells are antigen-specific but are capable of recognizing a variety of antigens through the T-cell receptor (TCR) repertoire (Erf, 2004). In other words, each TCR is specific for a particular antigen, but each T cell expresses a large number of different TCRs on its surface. In addition to the TCR, all T cells also express CD3 complexes. T cells that express CD4 molecules are called T helper (Th) cells, which typically possess a regulatory role.

There are two main types of Th cells, type-1 (Th1) and type-2 (Th2). Th1 cells favor a cell-mediated response, while Th2 cells are predominantly involved in a humoral response. CD4⁺ cells are MHC class II-restricted, meaning they can only interact with antigen presenting cells (APCs) expressing both an antigen-derived peptide and an MHC class II molecule. The main APCs capable of presenting to Th cells include dendritic cells, macrophages and B cells (Erf, 2004). All helper cells are considered naïve (Th0) until an antigen is successfully presented to them, resulting in activation. Upon activation, Th cells proliferate and differentiate into either effector cells or memory cells. Memory cells allow for immunity to infectious agents through

immediate recognition of an antigen during subsequent exposure. Effector cells are capable of activating other immune cells by producing cytokines and expressing membrane-bound cell-surface molecules (Erf, 2004). Effector cells can be divided into two categories based on cytokine production and effector functions. Th1 effector cells predominantly secrete interferon (IFN)- γ and interleukin (IL)-2, while Th2 cells are involved in production of IL-4, IL-5, IL-10, and tumor-necrosis factor (TNF)- α (Erf, 2004).

Another important T cell is the cytotoxic T cell (Tc) which, in addition to CD3 and the TCR, predominantly expresses CD8 molecules. Like Th cells, Tc cells are also MHC-restricted, but can only interact with cells expressing MHC class I molecules. Since all nucleated cells are considered MHC class I, Tc cells do not require the help of a specialized APC. Tc cell activation is a three-step process in which, (1) the TCR must bind to the antigen-peptide-MHC class I complex, (2) CD8 must bind to the MHC class I molecule, and (3) Th1 cells must provide signaling through direct contact or production of IFN- γ or IL-2 (Erf, 2004). Once activated, Tc cells also proliferate and differentiate into either memory cells or effector cells (CTL).

Due to their lack of specific antigen recognition, NK cells are often considered mediators of innate immunity; however, they are also often involved in aTh1 response. Interaction with Th1 cytokines results in enhanced proliferation and function of NK cells (Erf, 2004). NK cells are not MHC-restricted and in fact, exhibit increased preference for destruction of cells lacking MHC class I presentation (Erf, 2004). Macrophages, also typically associated with an innate immune response, can also be considered effector cells of cell-mediated immunity. IFN- γ is known to specifically interact with macrophages, resulting in activation of capabilities that far exceed those typically associated with an innate immune response (Erf, 2004).

Humoral Immunity

The predominant mediators of a humoral immune response are B cells and antibodies, also known as immunoglobulins (Fairbrother et al., 2004). In mammals, B cells are produced primarily in bone marrow, while in avian species, B cell development occurs in the bursa of Fabricius, an organ unique to birds. Immunoglobulins are glycoproteins produced by B cells that are either secreted or are attached to B cells as membrane-bound receptors (Zekarias et al., 2002). In either situation, immunoglobulins act as specific receptors for binding pathogens (Fairbrother et al., 2004). B cells are considered naïve precursor cells until stimulated by an antigen, at which time they proliferate and differentiate into plasma cells capable of producing antibodies (Fairbrother et al., 2004). There are only three different antibody classes found in chickens; immunoglobulin M (IgM), immunoglobulin Y (IgY, also referred to as IgG) and immunoglobulin A (IgA; Fairbrother et al., 2004). IgM is predominantly involved in a primary immune response, while IgG is typically associated with a secondary response. IgA is often considered a mucosal antibody.

While B cells are capable of producing all three antibody classes, a single B cell is only able to secrete a single type of immunoglobulin. Each antibody molecule has two main functions, (1) specifically bind antigen, and (2) recruit other cells that aid in elimination of the bound target (Davison, 2003). These two roles are carried out by separate locations on the antibody. The variable region is responsible for antigen binding, while the constant region is associated with effector cell recruitment (Davison, 2003). An individual's antibody repertoire is due in part to interaction between these two distinct antibody regions. The basic structure of an immunoglobulin includes two light and two heavy chains connected by disulfide bonds (Davison, 2003). Both the light chains and heavy chains contain a variable (V) region and a

joining (J) region (V_L and J_L vs. V_H and J_H , respectively); rearrangement of these segments results in light chain diversity. Heavy chain diversity is generated in a similar fashion, with the addition of a diversity (D) region, or D_H . Although important, the amount of diversity produced by these events is minimal compared to that generated in mammals. Chickens are unique in that diverse antibody repertoire is mostly a result of somatic gene conversion (Ratcliffe, 2006). Gene conversion, which only occurs in the bursa, involves the interaction of upstream pseudogenes with rearranged VJ_L or VDJ_H genes (Davison, 2003; Ratcliffe, 2006).

The main functions of antibodies include neutralizing viruses, aiding in phagocytosis by coating bacteria (opsonization), binding to target T cells, and enhancing cell lysis through activation of the complement system (Fairbrother et al., 2004). Humoral immunity involves the generation of a primary and a secondary immune response. A primary response is generated by naïve B cells activated during initial exposure to an antigen, and involves differentiation into plasma cells capable of producing immunoglobulins, as well as generation of memory B cells (Fairbrother et al., 2004). Upon future encounter with the same antigen, memory cells initiate a secondary response that is both quicker and more effective compared to the primary response.

Major Histocompatibility Complex (MHC)

The MHC consists of a group of genes that encode transmembrane proteins primarily responsible for antigen presentation (Zekarias et al., 2002; Davison, 2003). In the chicken, this group of genes is referred to as the B complex and codes for three main classes of molecules; class I (B-F), class II (B-L), and class IV (B-G; Zekarias et al., 2002). MHC class I molecules are expressed by all nucleated cells and are involved in Tc cell activation, while MHC class II molecules are only expressed by APCs and are essential for presenting antigen to Th cells. The

class IV MHC is unique to avian species, is involved in antibody response, and is expressed on erythrocytes, liver, bursal, thymic, and stromal cells (Zekarias et al., 2002).

Compared to mammals, the chicken MHC is composed of relatively few genes and occupies a small, 92-kb region (Zekarias et al., 2002; Davison, 2003). The mammalian MHC gene region is ~95% larger, yet chickens possess a higher degree of selection on individual alleles (Zekarias et al., 2002). This large amount of variation explains the MHC's role in disease resistance, as well as the high diversity of MHC molecules possessed by different individuals. Chickens express 29 different MHC haplotypes, each expressing one dominant class I MHC molecule at different levels (Zekarias et al., 2002). The role of the MHC in disease resistance and susceptibility is suspected to be dependent on the peptide-binding specificity of the dominant class I MHC molecule (Zekarias et al., 2002). A wealth of information exists in regards to disease resistance conferred by differences in MHC haplotype, specifically concerning Rous sarcoma virus and Marek's disease virus (Zekarias et al., 2002; Davison, 2003). The MHC is involved in antibody responses, cytokine production, Tc and NK cell activity, T cell proliferation, serum complement proteins, chemotactic activity of mononuclear leukocytes, and macrophage effect functions such as phagocytosis, bacterial killing, respiratory burst, and nitric oxide synthesis (Zekarias et al., 2002).

Necrotic Enteritis

Overview

Necrotic enteritis is a bacterial intestinal infection commonly experienced by a variety of species. In regards to avians, the disease has been identified in broilers, laying hens, turkeys, and quail (McDevitt et al., 2006). The anaerobic bacterium *Clostridium perfringens* acts as the

causative agent of necrotic enteritis, which exists ubiquitously in the environment and is often found in soil, sediment, vegetation, and decaying organic matter (Sheedy et al., 2003). The bacterium also typically colonizes the gut of most animals, including humans, and is non-pathogenic at low population levels (Sheedy et al., 2003; McDevitt et al., 2006). A shift in intestinal microflora is thought to result in rapid proliferation of bacteria, which in turn produces large amounts of extracellular toxins that damage the intestinal wall and lead to development of necrotic lesions (Keyburn et al., 2006; Weiduo et al., 2007).

Necrotic enteritis may exist in the clinical or sub-clinical form. The majority of chickens affected by the clinical form of the disease, known as acute necrotic enteritis, are between two and five weeks old (Wages and Opengart, 2003). Clinical symptoms include depression, ruffled feathers, inappetance, closed eyes, immobility, dark color diarrhea, and intestinal lesions. The observance of intestinal lesions upon post mortem inspection is usually the determining factor in diagnosing the disease. Intestines often become friable, inflamed, and appear distended. The intestinal wall is usually coated with a thick intestinal mucosa, ranging in color from yellow to dark brown. The clinical illness is usually extremely short in duration and birds are most often found dead before any clinical symptoms are observed (Wages and Opengart, 2003). Chickens suffering from subclinical necrotic enteritis are negatively affected in terms of low body weight gain and poor feed efficiency. Due to the lack of clinical symptoms, subclinical necrotic enteritis often goes unnoticed and is considered to be even more financially taxing than the clinical form (Keyburn et al., 2008). As a whole, necrotic enteritis has been estimated to cost the United States poultry industry \$2 to 3 billion annually (McDevitt et al., 2006; Zekarias et al., 2008).

Prevention and Treatment

Over the years, several factors have been identified that predispose birds to necrotic enteritis infection. Common predisposing factors include diets consisting of wheat, rye, oats or barley, large amounts of dietary animal byproducts, such as fish meal, meat meal and animal fat, dietary zinc, low intestinal acidity, intestinal stasis, climate and time of year, intestinal mucosa damage, particularly due to coccidial infection, and immunosuppression (Williams, 2005). The simplest forms of prevention involve avoiding exposure to aforementioned predisposing factors. It is also important to maintain proper litter conditions, including adequate depth of material and reduced moisture (Williams, 2005). In terms of dietary supplements, an extensive amount of research has been conducted in hopes of determining supplements successful in treating and/or preventing necrotic enteritis. Feed additives that have shown promise, only to name a few, include β -glucanase, pentosanase, cellulose, xylanase, pectinase, and amylase (Williams, 2005). Due to their protective effects against intestinal mucosa damage, anticlostridial drugs such as antibiotic growth promoters (AGPs) and ionophores are sometimes effective in preventing development of the disease (Williams, 2005). Administration of probiotics and prebiotics, as well as diets containing high lipid concentrations, has also been successful in reducing infection severity (Williams, 2005; McDevitt et al., 2006). The concept of 'competitive exclusion' has been investigated by the oral administration of live bacteria, which has shown some promise (McDevitt et al., 2006). The extensive variation of *C. perfringens* isolates has made vaccine development particularly challenging. However, birds with high maternal antibody titers against *C. perfringens* α -toxin have exhibited decreased mortality compared to those with low titers (McDevitt et al., 2006). Some success has been demonstrated with vaccines based on *C. perfringens* type A and C toxoids (Lovland et al., 2004), as well as vaccines developed using

recombinant *C. perfringens* proteins (Kulkarni et al., 2007). Most recent advances in preventing and treating necrotic enteritis infection include Entegard REV, a lysozyme-based antimicrobial blend (Zhang et al., 2010); NetvaxTM, a *C. perfringens* type A α -toxoid vaccine (Crouch et al., 2010); the use of *Bacillus licheniformis* spores as a probiotic or direct-fed microbial (Knap et al., 2010); and butyric acid, medium-chain fatty acids and/or essential oils as feed additives (Timbermont et al., 2010).

Host Immune Response

Unfortunately, little is known about immunity to necrotic enteritis. The fact that *C. perfringens* exists naturally in the gut, coupled with the ambiguous nature of virulent vs. non-virulent strains, creates a particular challenge in determining host immune responses during infection. Elements involved in assembly and processing of MHC class I molecules, as well as cytotoxic T cells, are thought to be involved in the host immune response to necrotic enteritis (Zhou et al., 2009). In addition, MHC class II molecules and CD45⁺ cells are evidenced to play a role during infection (Zhou et al., 2009). The initiation of cell-mediated immunity, particularly the involvement of T cell responses, is speculated to initiate a humoral response as evidenced by an increase in IgA and IgG (Lovland et al., 2003; Zhou et al., 2009). *C. perfringens* infection also induces a mucogenic response as evidenced by an increase in mucin production by goblet cells (Collier et al., 2008). Few studies have analyzed immune related gene expression profiles associated with necrotic enteritis infection; however, there appears to be great inconsistency in terms of results (see Table 2.1). This variation could be a result of several factors, namely differing *C. perfringens* strains, as well as more general factors such as experimental model, and breed and age of chickens. Regardless, such inconsistencies create additional challenges in ascertaining knowledge of host immune response during necrotic enteritis infection.

Some of the more commonly investigated immune related genes that appear to play a role in necrotic enteritis infection include, IFN- γ , IL-2, IL-8, IL-10, IL-13, IL-15, IL-16, IL-18, inducible nitric oxide synthase (iNOS), CXCLi1, and lipopolysaccharide-induced tumor necrosis factor- α factor (LITAF). IFN- γ is an important pro-inflammatory cytokine produced by T and B lymphocytes, as well as NK cells, is highly regulated by IL-12 and IL-18 (Schneider et al., 2000; Schroder et al., 2004; Giansanti et al., 2006), and is known to possess antiviral properties against intracellular pathogens (Schroder et al., 2004). Mammalian IL-2 has been identified as a cytokine cell growth factor that plays a role in the proliferation and differentiation of T and B lymphocytes, as well as NK cells (Giansanti et al., 2006). Chicken IL-2 is expressed by activated T cells (Kolodsick et al., 2001) and is capable of inducing chicken splenocyte proliferation in vitro (Stepaniak et al., 1999), as well as priming heterophils to elicit a phagocytic response during bacterial infections (Kogut et al., 2002). IL-8 is a CXC chemokine produced by macrophages, which functions predominantly as a chemoattractant and therefore plays an important role in inflammation (Strober, 1998; Shahzad et al., 2010). Expression of IL-10 cytokines favors a Th2 immune response by downregulating the production of pro-inflammatory cytokines, and chicken IL-10 has been observed to specifically inhibit IFN- γ expression (Giansanti et al., 2006). In mammals, IL-13 shows a predisposition towards a Th2 immune response by specifically inhibiting production of pro-inflammatory modulators, as well as the NF κ B pathway (Miyoshi et al., 2007). Mammalian IL-15 plays an important role in cell-mediated immunity by regulating the proliferation and activation of T and NK cells (Giansanti et al., 2006), and chicken IL-15 is structurally homologous to its mammalian counterpart (Lillehoj et al., 2001). In humans, IL-15 has been specifically cited as an important immunomodulator during invasion by intracellular organisms (Jullien et al., 1997; Waldmann and Tagaya, 1999).

IL-16 functions as a mediator of inflammation by possessing strong chemotactic activity for CD4⁺ T lymphocytes (Cruikshank et al., 2000). Chicken IL-16 is highly expressed in the bursa of Fabricius, suggesting a possible role in the development of B lymphocytes (Min and Lillehoj, 2004). IL-18 is expressed by a number of immune cells and is responsible for the induction of IFN- γ synthesis (Schneider et al., 2000; Giansanti et al., 2006). iNOS is one of three forms of NOS and is predominantly produced by macrophages (Lin et al., 1996). NOS functions as a catalytic enzyme in the production of NO which plays an important role in the immune system acting as a cytotoxic and tumoricidal agent (Lin et al., 1996). CXCLi1, also known as K60, is a chemokine that acts as a chemoattractant for neutrophils in mammals (Giansanti et al., 2006). Expression is highly upregulated in chicken HD-11 cells exposed to IFN- γ and IL-1 β , but not IFN- α or IFN- β (Sick et al., 2000). LITAF is predominantly expressed in the spleen of chickens, as well as in intestinal intraepithelial lymphocytes, and has been found to be upregulated in macrophages exposed to *Escherichia coli*, *Salmonella typhimurium*, *E. acervulina*, *E. maxima*, and *E. tenella* (Hong et al., 2006).

Clostridium perfringens

Types and Toxins

The causative agent of necrotic enteritis is the anaerobic bacterium *C. perfringens*. There are five main types of *C. perfringens* (A, B, C, D, E) that produce four main toxins (alpha, beta, epsilon, iota), as well as a common enterotoxin encoded by the *cpe* gene (Niilo, 1980; Sheedy et al., 2003). Type A is responsible for producing avian necrotic enteritis, as well as gas gangrene and some types of human food poisoning (Wages and Opengart, 2003; Weiduo et al., 2007). The *C. perfringens* α -toxin is produced by all five types; however, type A is unique in that it is the

only strain known to exclusively secrete α -toxin. α -toxin is a zinc-metalloenzyme with phospholipase C and sphingomyelinase activities (Songer, 1997). Research conducted over the past several decades has consistently implicated α -toxin as one of the main virulence factors of *C. perfringens* in the pathogenesis of avian necrotic enteritis (Al-Sheikhly and Truscott, 1976a, b, c; Fukata et al., 1988; Kulkarni et al., 2007, 2008; Zekarias et al., 2008; Cooper et al., 2009).

C. perfringens α -toxin disrupts membrane phospholipids by hydrolyzation, and also possesses hemolytic activity (Petit et al., 1999; Van Immerseel et al., 2004; Zekarias et al., 2008). Membrane degradation is accomplished through the calcium-dependent catalytic action of α -toxin, which allows for binding of its carboxy-terminal tail to membrane phospholipids (Petit et al., 1999; Van Immerseel et al., 2004). *C. perfringens* is unable to produce 13 essential amino acids, which are obtained in vivo through exotoxin secretion (Cooper and Songer, 2009). These exotoxins, most of which are hydrolytic enzymes, not only provide the bacterium with growth nutrients, but also act as additional virulence factors (Petit et al., 1999). Production of hydrolytic enzymes, including collagenase, DNase, hyaluronidase, neuraminidases, protease, and urease, can lead to diacylglycerol formation through hydrolysis of lecithin (Petit et al., 1999). This process results in protein kinase C activation, triggering the arachidonic acid cascade, which induces production of various inflammatory mediators like leukotrienes, thromboxane, platelet-agglutinating factor, and prostacyclin (Petit et al., 1999).

Although α -toxin is likely involved in necrotic enteritis pathogenesis, it is probably not the only mediator of infection. All *C. perfringens* strains secrete a wide range of toxins, the profile of which varies greatly among strains (Gholamiandekhordi et al., 2006; Lanckriet et al., 2010), making it difficult to elucidate a single virulence factor.

Involvement in Necrotic Enteritis

As mentioned previously, *C. perfringens* naturally colonizes the intestinal tract of chickens, where it exists in a non-pathogenic state. Both virulent (necrotic enteritis causing) and non-virulent strains of the bacterium are commonly isolated from poultry flocks. It appears that even in the presence of virulent strains, chickens must be exposed to one of the many predisposing factors causing intestinal mucosa damage, for development of necrotic enteritis to occur (Cooper and Songer, 2009). The precise mechanism behind necrotic enteritis pathogenesis has yet to be established; it is unknown if commensal non-virulent strains are capable of converting to virulent strains (and what factors would initiate this), or whether a mechanism promoting colonization by virulent strains is to blame.

Even healthy chickens may possess 2 to 5 different *C. perfringens* type A bacteria (Cooper and Songer, 2009). However, only a single genotype, which is usually different from those colonizing healthy birds, is typically isolated from infected chickens (Cooper and Songer, 2009). After infection, either by natural recovery or treatment, birds are once again colonized by multiple genetic types and the necrotic enteritis causing strain is no longer present (Cooper and Songer, 2009). Virulent *C. perfringens* strains actually inhibit growth of non-virulent strains in vitro, suggesting that necrotic enteritis causing strains may establish dominance through bacteriocin production (Cooper and Songer, 2009).

In addition to intestinal mucosa damage, development of necrotic enteritis is also typically characterized by the presence of a large number of *C. perfringens* bacteria (Williams, 2005). It is possible that small numbers (undetectable) of virulent *C. perfringens* strains colonize intestines of healthy birds, and an unknown mechanism(s) then initiates rapid bacterial proliferation (Cooper and Songer, 2009). *C. perfringens* regulates population numbers through

quorum sensing, in which bacteria secrete molecules called autoinducers that accumulate in the extracellular environment in proportion to cell density (McDevitt et al., 2006). It is possible that an unidentified element or mechanism interferes with proper quorum sensing abilities, leading to an unnecessary increase in bacterial numbers.

Effect on Immune Cells

Although little has been reported on the effect of *C. perfringens* toxins on avian immune cells during necrotic enteritis infection, research conducted on cellular cytotoxicity during gas gangrene infection could provide important insight. Like necrotic enteritis, gas gangrene is caused by *C. perfringens* type A and α -toxin also plays a major role in infection pathogenesis. The cytotoxic effect of α -toxin on leukocytes appears to be somewhat varied, with some reports of leukotoxic effect, but little effect of purified α -toxin on polymorphonuclear cells (PMNs) in vitro (O'Brien and Melville, 2004). It has been suggested that α -toxin is not cytotoxic to macrophages when acting alone, as evidenced by the lack of cytotoxicity conferred by a *Bacillus subtilis* strain engineered to express the *C. perfringens* gene encoding α -toxin, *plc* (Ninomiya et al., 1994).

However, *C. perfringens* is cytotoxic to macrophages under both aerobic and anaerobic conditions (O'Brien and Melville, 2000). O'Brien and Melville (2000) observed *C. perfringens* is capable of residing in the cytoplasm of macrophages following phagocytosis, perhaps by escaping the phagosome. Due to the inherent membrane degrading abilities of α -toxin, the toxin may play a role in actively lysing the phagosomal membrane. α -toxin is necessary for the persistence of *C. perfringens* in the presence of mouse peritoneal macrophages, as well as the survival of *C. perfringens* in vivo at sublethal doses, and also mediates the phagosomal escape in

a macrophage cell line and mouse peritoneal macrophages (O'Brien and Melville, 2004). It is highly likely that α -toxin may play similar roles during necrotic enteritis infection in poultry.

Genetic Lines of Chickens

Genetic Selection

In a selective breeding experiment carried out by geneticist Dr. Paul B. Siegel, White Leghorn chickens were genetically selected for low (LA) or high (HA) antibody response five days following intravenous injection of 0.1 mL (0.25% suspension) sheep red blood cells (SRBC; Siegel and Gross, 1980). Individuals from the two lines were crossed and then backcrossed to each parental line for three consecutive generations. Sublines were closed following each generational backcross. Selective breeding allowed for the development of two sublines containing a minimum of 93% background genome (LA or HA) and possessing one of two MHC genotypes (B^{13} or B^{21}). Over the next several generations, genetic lines became increasingly divergent so that by the 12th generation of selection, chickens exhibited a correlated change in MHC; LA birds were comprised of 96% B^{13} , while HA birds were 96% B^{21} (Dunnington et al., 1992). Selection is currently in the 38th generation, during which the lines have been extensively utilized in a multitude of experiments and studies.

Differences in Production Traits

A wealth of information exists in regards to divergent traits possessed by the two genetic lines. The LA line exhibited greater body weight at 4 and 24 weeks in generations 8 and 14 (Siegel et al., 1982; Martin et al., 1990). LA chickens commence egg production at younger ages and at lower body weights (Siegel et al., 1982; Martin et al., 1990). In generations 8 and 14, LA birds had greater hen-day egg production by 300 days of age, but there was no difference

between the lines at 364 to 386 days of age (Siegel et al., 1982; Martin et al., 1990). Hens from line LA produced more defective eggs at generation 7, but not at generation 14 (Siegel et al., 1982; Martin et al., 1990). LA chickens also produced more double-yolk eggs at generation 14 (Martin et al., 1990).

Differences in Immune Response

In addition to differences in production traits, LA and HA lines are also divergent in terms of disease resistance and specifically, B and T cell responses. Line HA initiates a secondary response predominantly consisting of IgG antibodies, whereas both primary and secondary responses initiated by line LA mainly involve IgM antibodies (Martin et al., 1989). HA birds also produce higher total and IgG antibody titers in response to *Brucella abortus* (Dunnington et al., 1992; Scott et al., 1994). Peripheral lymphocytes isolated from HA chickens exhibit higher T cell activity during in vitro response to ConA and PHA-M mitogens (Scott et al., 1991). Line HA appears to be more resistant to invasion by intracellular pathogens, including *Mycoplasma gallisepticum* (Gross et al., 1980), *Eimeria necatrix* and *E. tenella* (Gross et al., 1980; Martin et al., 1986), Newcastle disease virus (Gross et al., 1980), splenomegaly virus (Gross et al., 1980), as well as fowl mites (Gross et al., 1980). Line HA also exhibits lower lifetime mortality due to non-specific causes (Siegel et al., 1982). In contrast, LA chickens are generally less susceptible to infection by extracellular organisms such as *E. coli* (Gross et al., 1980; Dunnington et al., 1991) and *Staphylococcus aureus* (Gross et al., 1980). Resistance to *C. perfringens* is one instance in which the lines do not follow their established trend of pathogen susceptibility. During a clinical outbreak of necrotic enteritis, HA chickens experienced significantly decreased mortality compared to LA chickens (Siegel et al., 1993). It is clear that

the lines are divergent in regards to both genetic background, as well as a variety of production traits and immune responses.

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Table 2.1. Varying Gene Expression Profiles in Response to *Clostridium perfringens* Infection in Chickens

Gene	Park et al. 2008	Sarson et al. 2009	Zhou et al. 2009
AH294	↓		
Apoptosis-associated protein	↓		
B2m	↑		
B-F	↑		
Calnexin	↑		
Calreticulin	↑		
Caspase 8	↓		
Caspase 9	↓		
CD45	↑		
CD8β	↑		
Chemokine receptor 8	↓		
CXCR1	↓		
Fas	↓		
FLIP	↓		
Galectin 3		↑	
GlcNAc		↑	
Granzyme A		↑	↓
IFN-α	↑		
IFN-γ	↑		↓
IgA	↑		
IgG	↑		
IgY-R		↑	
IL-1β	↑		↓
IL-2	↑		↓
IL-8	↓		↓
IL-10	↓		
IL-12	↑		
IL-13	↑		
IL-15	↓		
IL-16	↓		
IL-17	↑		
IL-18		↑	↓
CXCLi1	↑ d1, ↓ d2		
LITAF	↓		
Lymphocyte function associated antigen-1	↑		
Macrophage inflammatory protein 1β	↓		
TAP2	↑		
TCR-γ		↑	
TFGβ4	↑		
TNFSF15	↑ d1, ↓ d2		

CHAPTER III

Evaluation of Genetic Resistance to Necrotic Enteritis in Chickens as Influenced by Genetic Background and Major Histocompatibility Complex (MHC)

ABSTRACT: Chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) displayed a correlated change in major histocompatibility complex (MHC), so that LA chickens were 96% B¹³ and HA chickens were 96% B²¹. A multitude of studies over the past several decades have evaluated exceptional differences between the two genetic lines. The LA line appears to be less susceptible to invasion by extracellular pathogens, while HA chickens are more resistant to infection by intracellular organisms. Resistance to *Clostridium perfringens* is one instance in which the lines do not follow their established trend of pathogen susceptibility. During a clinical outbreak of necrotic enteritis, B²¹B²¹ genotypes experienced significantly less mortality (6% vs. 13 %) compared to B¹³B¹³ genotypes. A study was carried out to assess immunological differences between LA and HA lines during necrotic enteritis infection. In Experiment 1, chickens were orally gavaged with a low (10⁷ CFU/mL) or high (10⁹ CFU/mL) dose of *C. perfringens*. In Experiment 2, chickens were orally gavaged with live coccidia oocysts (50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts) on experiment d 1, followed by 10⁷ CFU/mL *C. perfringens* on d 5. Unfortunately, establishment of necrotic enteritis infection was unsuccessful in both experiments as evidenced by lack of intestinal lesions, as well as no negative effect on bird performance.

Introduction

Disease resistance as influenced by genetic selection has been extensively studied for numerous decades in a variety of species (Warner et al., 1987; Gogolin-Ewens et al., 1990;

Hirschhorn et al., 2002). Specifically, the major histocompatibility complex (MHC) is known to play a vital role in immune response and disease resistance to autoimmune, viral, bacterial, and parasitic diseases (Lamont et al., 1989). In a selective breeding experiment carried out by geneticist Dr. Paul B. Siegel, White Leghorn chickens were genetically selected for low (LA) or high (HA) antibody response 5 days following intravenous injection of 0.1 mL (0.25% suspension) sheep red blood cells (SRBC; Siegel and Gross, 1980). Individuals from the two lines were crossed and then backcrossed to each parental line for three consecutive generations. Sublines were closed following each generational backcross. Selective breeding allowed for the development of two sublines containing a minimum of 93% background genome (LA or HA) and possessing one of two MHC genotypes (B^{13} or B^{21}). Over the next several generations, genetic lines became increasingly divergent so that by the 12th generation of selection, chickens exhibited a correlated change in MHC; LA birds were comprised of 96% B^{13} , while HA birds were 96% B^{21} (Dunnington et al., 1992). Selection is currently in the 38th generation, during which the lines have been extensively utilized in a multitude of experiments and studies. LA chickens have been observed to possess larger thymuses, but smaller spleens and bursa of Fabricius (Ubosi et al., 1985). During the first 250 days of life, LA chickens exhibit greater body weight and feed efficiency, and also reach maturity earlier, lay a larger number of eggs, and maintain fertility for a longer period of time (Siegel et al., 1982). HA chickens possess larger but less active macrophages (Biozzi et al., 1982) and in regards to disease resistance, they have generally been found to be less susceptible to intracellular pathogens. The HA line is more resistant to Newcastle disease, *Mycoplasma gallisepticum*, *Eimeria necatrix*, feather mites (Gross et al., 1980), *E. tenella* (Martin et al., 1986), and Marek's disease (Dunnington et al., 1996). In contrast, LA chickens generally display increased resistance to extracellular

pathogens, such as *Escherichia coli* and *Staphylococcus aureus* (Gross et al., 1980). The lifetime mortality due to non-specific causes has also been recorded as higher for the LA line (Siegel et al., 1982).

Resistance to *Clostridium perfringens* is one instance in which the lines do not follow their established trend of pathogen susceptibility. When chickens were in their 5th generation after backcrossing ceased, a flock at 209 days of age experienced 10 days of significant mortality (Siegel et al., 1993). The flock contained birds from each background genome (LA and HA), as well as both MHC genotypes (B¹³ and B²¹). Birds were diagnosed with necrotic enteritis on day 214, at which time litter was immediately replaced, and 0.05% bacitracin was added to the feed. Following examination of daily records, there was no real difference in mortality between background genomes; however, chickens possessing B¹³B¹³ genotypes experienced higher mortality (13% vs. 6%) beginning at an earlier time point. B¹³B¹³ genotypes also exhibited an earlier decrease in hen-day egg production, but recovered more quickly than B²¹B²¹ birds.

Necrotic enteritis is a common disease of poultry reported to occur in nearly every poultry producing country (Keyburn et al., 2006). The bacterial infection was first described in domestic chickens by Parish in 1961 and is currently the most financially devastating bacterial disease in broilers (Keyburn et al., 2006; Cooper et al., 2008). In fact, it has been estimated to cost the poultry industry 0.05 cents per bird, averaging \$2 billion annually (Zekarias et al., 2008). The majority of birds affected by the clinical form of the disease, known as acute necrotic enteritis, are between two and five weeks old (Wages and Opengart, 2003). Predisposing factors include a sudden change in diet, diets containing viscous cereal grains, fish and meat meal, low intestinal acidity, intestinal stasis, damage to intestinal mucosa by enteric pathogens, specifically coccidial infection, and immunosuppression (Williams, 2005; Cooper and Songer, 2009).

Clinical symptoms include depression, ruffled feathers, inappetance, closed eyes, immobility, dark color diarrhea, and presence of intestinal lesions. The clinical illness is usually extremely short in duration, and birds are most often found dead before any clinical symptoms are observed (Wages and Opengart, 2003). The observance of intestinal lesions upon post mortem inspection is usually the determining factor in diagnosing the disease. Lesion development is predominantly confined to the distal portion of the small intestine (Wages and Opengart, 2003). The intestines often become friable, inflamed, and appear distended. The intestinal wall is usually coated with a thick intestinal mucosa, ranging in color from yellow to dark brown.

The causative agent of necrotic enteritis is the anaerobic bacterium *C. perfringens*. There are five main types of *C. perfringens* (A, B, C, D, E) that produce four main toxins (alpha, beta, epsilon, iota), as well as a common enterotoxin encoded by the *cpe* gene (Niilo, 1980; Sheedy et al., 2003). Type A is responsible for producing avian necrotic enteritis, as well as gas gangrene and some types of human food poisoning (Wages and Opengart, 2003; Weiduo et al., 2007). The *C. perfringens* α -toxin is produced by all five types; however, type A is unique in that it is the only strain known to exclusively secrete α -toxin. α -toxin is a zinc-metalloenzyme with phospholipase C and sphingomyelinase activities (Songer, 1997). Research conducted over the past several decades has consistently implicated α -toxin as one of the main virulence factors of *C. perfringens* in the pathogenesis of avian necrotic enteritis (Al-Sheikhly and Truscott, 1976a, b, c; Fukata et al., 1988; Kulkarni et al., 2007, 2008; Zekarias et al., 2008; Cooper et al., 2009).

The purpose of this study was to evaluate immunological differences between LA and HA lines during exposure to *C. perfringens*. Chickens used in the following experiments were from the 37th generation of selection. All birds from the LA background genome were homozygous for the B¹³ MHC genotype, while all HA birds were B²¹ homozygous. All

procedures were carried out in accordance with guidelines established by the Virginia Tech Institutional Animal Care and Use Committee.

Materials and Methods

Birds and Treatment Groups

White Leghorn chickens from both LA and HA genetic lines (generation 37) were hatched and reared at the Virginia Tech Poultry Farm. In Experiment 1, a total of forty 12-week-old White Leghorn chickens (20 from each genetic line) were transferred from floor pens at the Virginia Tech Poultry Farm to battery cages located in a BSL-2 facility. A total of 45 chickens were transferred in Experiment 2. Birds from each line were randomly assigned to one of three treatment groups in Experiment 1 and one of two treatment groups in Experiment 2 (see Tables 3.1 and 3.2). Chickens were housed 2 birds per cage, feed and water were provided *ad libitum* and chickens were allowed to acclimate for several days prior to the start of each experiment.

C. perfringens Culture

Bacteria were isolated from chickens experiencing a clinical outbreak of necrotic enteritis in the field. Isolates were delivered to our lab in vials of cooked meat broth. *C. perfringens* cultures were streaked onto peptone glucose yeast (PGY) medium and incubated under anaerobic conditions overnight at 39 °C with 5% CO₂. To ensure a pure culture, a single colony forming unit (CFU) was streaked onto a new PGY plate and incubated overnight. Following incubation, a single CFU was inoculated into liquid PGY for culturing.

Coccidia Oocyst Preparation

Sporulated *E. acervulina*, *E. maxima* and *E. tenella* oocysts (stored in potassium dichromate) were obtained from the Animal Parasitic Diseases Laboratory ARS-USDA in Beltsville, MD. Oocysts were washed several times in cold tap water and live oocyst counts were obtained using Trypan blue and a hemacytometer.

Experiment 1

On experiment d 1, CP challenge birds (groups B, C, E, F) were orally gavaged with either a low (10^7 CFU/mL) or high (10^9 CFU/mL) dose of *C. perfringens* (see Table 3.1 for explanation of treatment groups). Control birds (groups A and D) were gavaged with an equal volume of PBS (1 mL). Additionally, individual bird and feed weights were recorded three days prior to the start of the experiment (d -3), as well as on d 1, 3 and 5. Feed conversion ratio (FCR) was calculated by dividing feed intake by body weight gain. On d 3 and 5, two birds from each control group and four birds from each experimental group (20 birds/day) were euthanized by cervical dislocation. Each section of the small intestine (duodenum, jejunum, ileum) were examined and scored for the presence of lesions indicative of necrotic enteritis (Cooper and Songer, 2009; see Table 3.3 for scoring system). Immediately following lesion scoring, all three intestinal sections were separately collected for RNA extraction. Approximately 1 inch piece of intestine was finely minced using razor blades and/or dissection scissors, packaged in aluminum foil and immediately snap frozen in liquid nitrogen. Ceca from each bird were also collected, sealed in Whirl-pak bags and placed on dry ice. All samples were kept at -80 °C for long-term storage.

On d 5 only, spleens were collected and placed in 15 mL tubes containing Dulbecco's Modified Eagle's Medium (DMEM; Mediatech Inc., Manassas, VA) supplemented with 2 mM

L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B, and 10% fetal bovine serum (FBS; all from Atlanta Biologicals Inc., Lawrenceville, GA) and kept on ice. Lymphocytes were isolated from spleens as previously described by Kim et al. (2010). Splenocytes were seeded into a 96-well cell culture plate at a concentration of 10^5 cells/well with a total volume of 100 µL/well. One hundred microliters of Concanavalin A (ConA; 10 µg/mL) were added to half the wells, while 100 µL supplemented DMEM were added to the other half. Cells were incubated at 39 °C with 5% CO₂ for 24 h. Following incubation, media were removed and cells were washed with 100 µL plain DMEM. Cell proliferation was quantified using a commercially available cell proliferation kit (CellTiter 96 Non-radioactive Cell Proliferation Assay; Promega, Madison, WI) following the manufacturer's protocol.

Experiment 2

On experiment d 1, groups B and D were orally gavaged with 1 mL PBS containing 50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts (see Table 3.2 for explanation of treatment groups). Control birds (groups A and C) were orally gavaged with 1 mL plain PBS. On d 5, groups B and D were orally gavaged with 10^7 CFU/mL *C. perfringens* and groups A and C were orally gavaged with an equal volume (1 mL) of PBS. Feed was initially weighed twelve days prior to the start of the experiment (d -12), as well as on d 1, 5 and 9. Chickens were weighed on d 1, 5 and 9. Chickens were euthanized by cervical dislocation on d 7 and 9 (3 birds/day from groups A and B; 8 birds/day from groups C and D; unequal numbers due to limited bird availability) and all three sections of small intestine were scored for presence of lesions indicative of necrotic enteritis (see Table 3.3 for scoring system). As described in Experiment 1, intestinal samples were collected for RNA extraction, as well as spleen samples. Small portions of each intestinal section (duodenum, jejunum, ileum) were also

collected for histology. Approximately 1 inch piece of intestine was carefully flushed with PBS using a needle and syringe, a small snip was made at each end (allowing for formalin to more easily enter inside the intestinal section), and the sample was placed in a sealed container filled with 10% buffered formalin.

Statistical Analysis

Data were analyzed using the Fit Model platform in JMP 8.0 (SAS Institute Inc., Cary, NC). Effects of treatment were analyzed by ANOVA and difference detected by Tukey honestly significant differences (HSD). Each pen served as the experimental unit, and feed intake was calculated on a per bird basis. Significance was accepted at $P < 0.05$ and results are reported as least squares (LS) means.

Results

Experiment 1

Unfortunately, as evidenced by lack of intestinal lesions (Figure 3.1), a proper infection model was not successfully established. Only five birds (n = 40 total) were given a lesion score of +1 for the duodenum. These results were quite unexpected; given the fact that *C. perfringens* is known to predominantly infect the distal portion of the small intestine. Due to the lack of infection, the majority of planned experimental parameters were not carried out.

There were no significant differences observed in body weight gain (Figure 3.2). Due to the small number of birds used in this experiment, there was a high degree of variability among birds, resulting in high standard error. Less overall variability was observed for feed intake; however, there were no significant differences (Figure 3.3). During d 3 through 5, LA chickens infected with a high dose of *C. perfringens* experienced significantly decreased feed efficiency

compared to HA chickens (Figure 3.4). No significant differences were observed in terms of lesion scores (Figure 3.1). Splenocyte proliferation differed significantly between LA and HA chickens infected with a low dose of *C. perfringens* (Figure 3.5).

Experiment 2

Compared to the first experiment, Experiment 2 resulted in a slightly more successful necrotic enteritis infection model. As shown in Figure 3.6, higher lesion scores were recorded; however, the majority of lesions were once again observed in the proximal portion of the small intestine. Although birds seemed to experience a decrease in weight from d 5 through 9, no overall significant differences were observed (Figure 3.7). There were also no significant differences in feed intake or feed efficiency (Figures 3.8 and 3.9). Figure 3.6 depicts a main effect of infection on lesion scores in the duodenum and jejunum on both d 7 and 9; however, there was no significant difference between the two genetic lines.

Discussion

Experiment 1

As mentioned previously, the lack of infection made it impossible to ascertain any real differences between LA and HA lines. Reasons for our limited success are purely speculative; however, we thought they may have been partly due to the fact that chickens suffered no predisposing factors to necrotic enteritis infection. For this reason, we chose to introduce a coccidiosis challenge in addition to a necrotic enteritis challenge in the second experiment.

Experiment 2

Based on lesion score results, it appears chickens experienced a more severe infection in Experiment 2, compared to Experiment 1. Unfortunately, due to the location of intestinal

infection (proximal vs. distal), as well as lesion appearance, it was concluded the majority of tissue damage was a result of coccidial infection, as opposed to infection by *C. perfringens*. Once again, lack of proper infection caused us to abort the majority of experimental analysis. Our lab recently obtained a new virulent strain of *C. perfringens*, and plans to conduct further studies are currently underway.

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Table 3.1. Experiment 1: Treatment Groups

Treatment Group	Description	No. of Birds
A	HA – Control	4
B	HA – CP challenge, Low Dose	8
C	HA – CP challenge, High Dose	8
D	LA – Control	4
E	LA – CP challenge, Low Dose	8
F	LA – CP challenge, High Dose	8

Chickens from high (HA) and low (LA) antibody lines were randomly assigned to one of six treatment groups, A through F. Groups A and D served as the negative control and were orally gavaged with 1 mL PBS. Groups B and E were challenged with a low dose (10^7 CFU/mL) of *Clostridium perfringens* (CP), while groups C and F were challenged with a high dose (10^9 CFU/mL) of CP. Unequal number of birds within each genetic line due to bird availability.

Table 3.2. Experiment 2: Treatment Groups

Treatment Group	Description	No. of Birds
A	HA – Control	6
B	HA – Infected	6
C	LA – Control	16
D	LA – Infected	17

Chickens from high (HA) and low (LA) antibody lines were randomly assigned to one of four treatment groups, A through D. Groups A and C served as the negative control and were orally gavaged with 1 mL PBS. Groups B and D were infected with 10^7 CFU/mL *Clostridium perfringens*. Unequal bird numbers within genetic lines due to bird availability.

Table 3.3. Lesion Scoring System (Cooper and Songer, 2009)

Score	Description
0	Normal
+1	Thin-walled, friable intestine
+2	Focal necrosis, ulceration
+3	Large patches of necrosis
+4	Severe/extensive necrosis – field cases
+5	Birds dying with +4 lesions

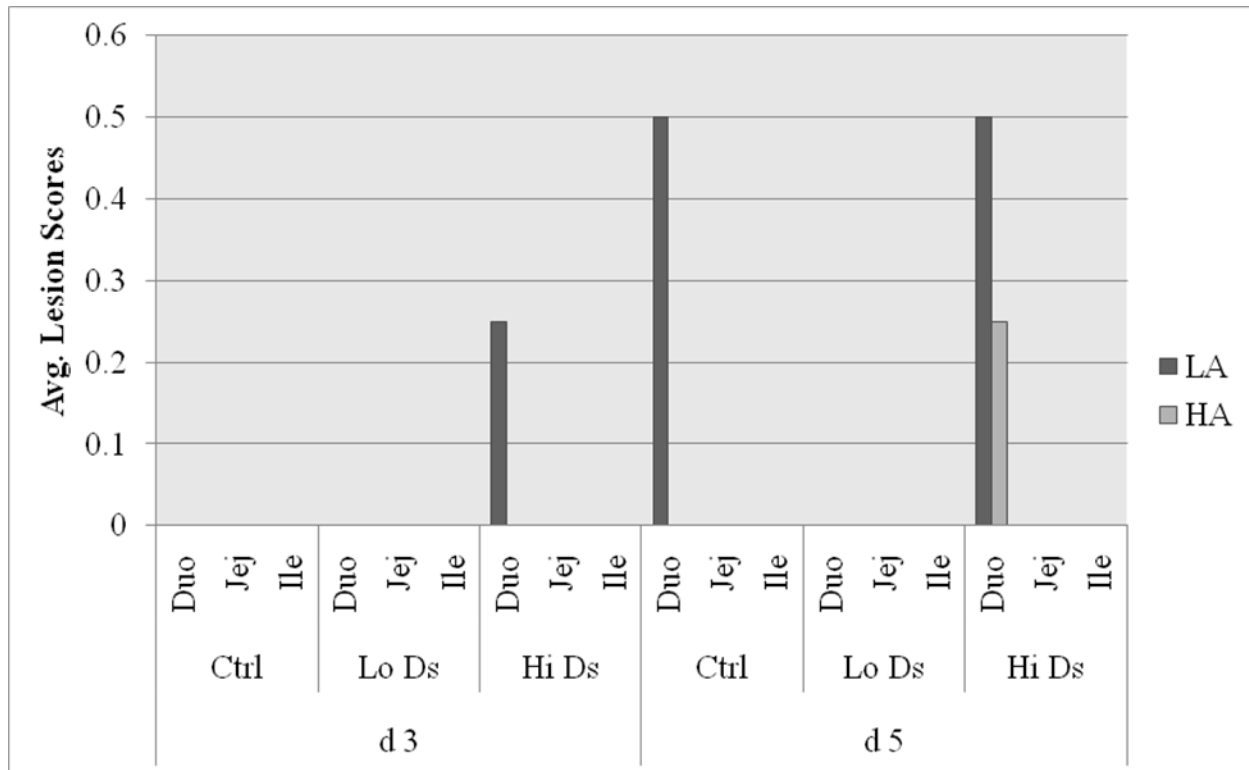


Figure 3.1. Effect of *Clostridium perfringens* infection on lesion scores in Experiment 1.

Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with a Lo Ds (10^7 CFU/mL) or Hi Ds (10^9 CFU/mL) of *C. perfringens* on experiment d 1. Small intestinal segments, duodenum (duo), jejunum (jej) and ileum (ile) were examined for the presence of lesions on experiment d 3 and 5 (see Table 3.3 for lesion scoring system). To determine differences in lesion scores between the two genetic lines, data were analyzed at two separate time points (d 3 and 5). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. No statistically significant differences were observed.

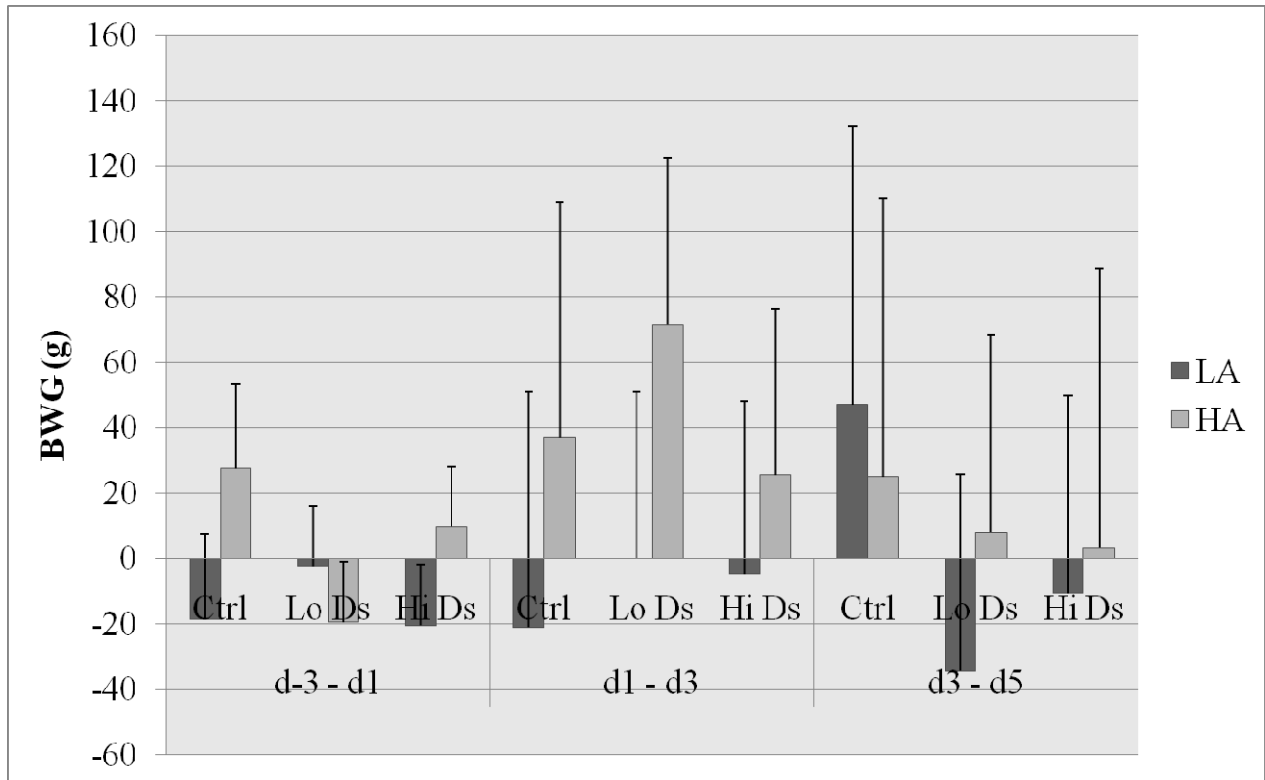


Figure 3.2. Effect of *Clostridium perfringens* infection on body weight gain (BWG) in Experiment 1. Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with a Lo Ds (10^7 CFU/mL) or Hi Ds (10^9 CFU/mL) of *C. perfringens* on experiment d 1. Individual body weights were recorded three days prior to the start of the experiment (d -3), as well as on d 1, 3 and 5. To determine differences in BWG between the two genetic lines, data were analyzed at three separate time intervals (d -3 – d 1, d 1 – d 3, d 3 – d 5). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. No statistically significant differences were observed.

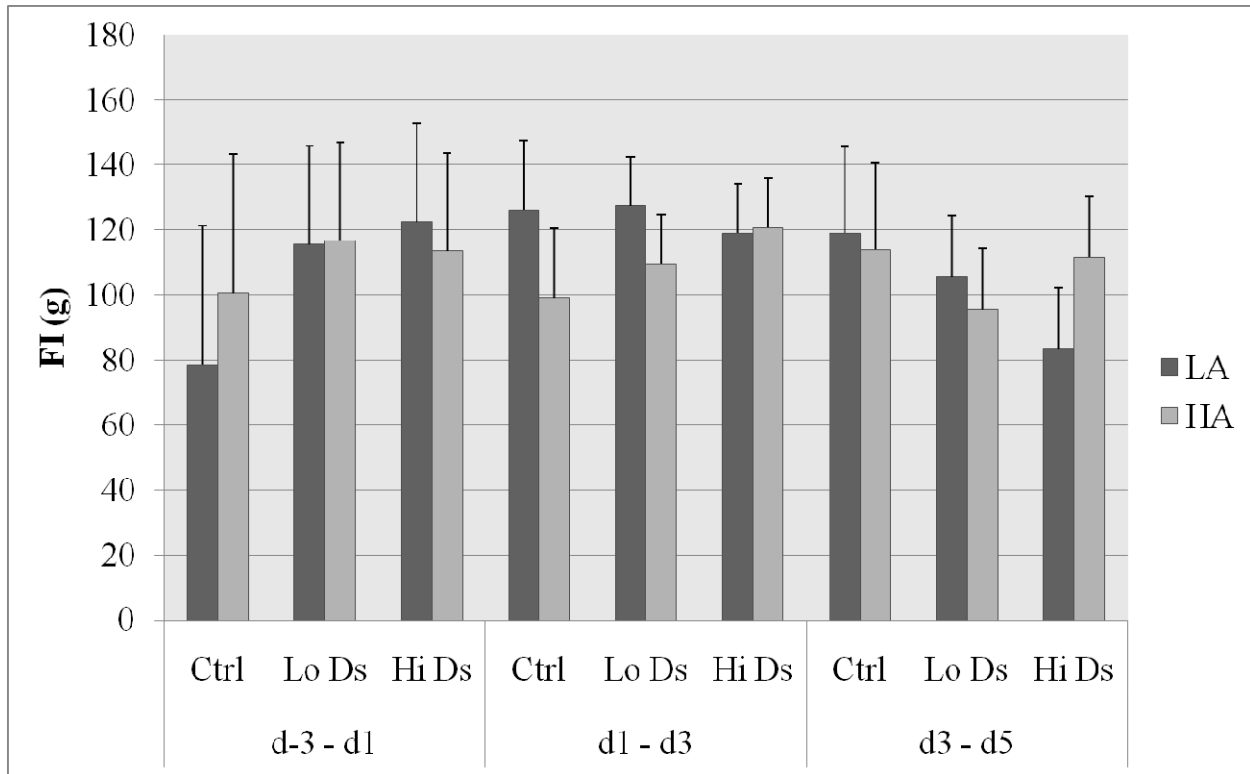


Figure 3.3. Effect of *Clostridium perfringens* infection on feed intake (FI) in Experiment 1.

Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with a Lo Ds (10^7 CFU/mL) or Hi Ds (10^9 CFU/mL) of *C. perfringens* on experiment d 1. Feed weights were recorded three days prior to the start of the experiment (d -3), as well as on d 1, 3 and 5. To determine differences in FI between the two genetic lines, data were analyzed at three separate time intervals (d -3 – d 1, d 1 – d 3, d 3 – d 5). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. No statistically significant differences were observed.

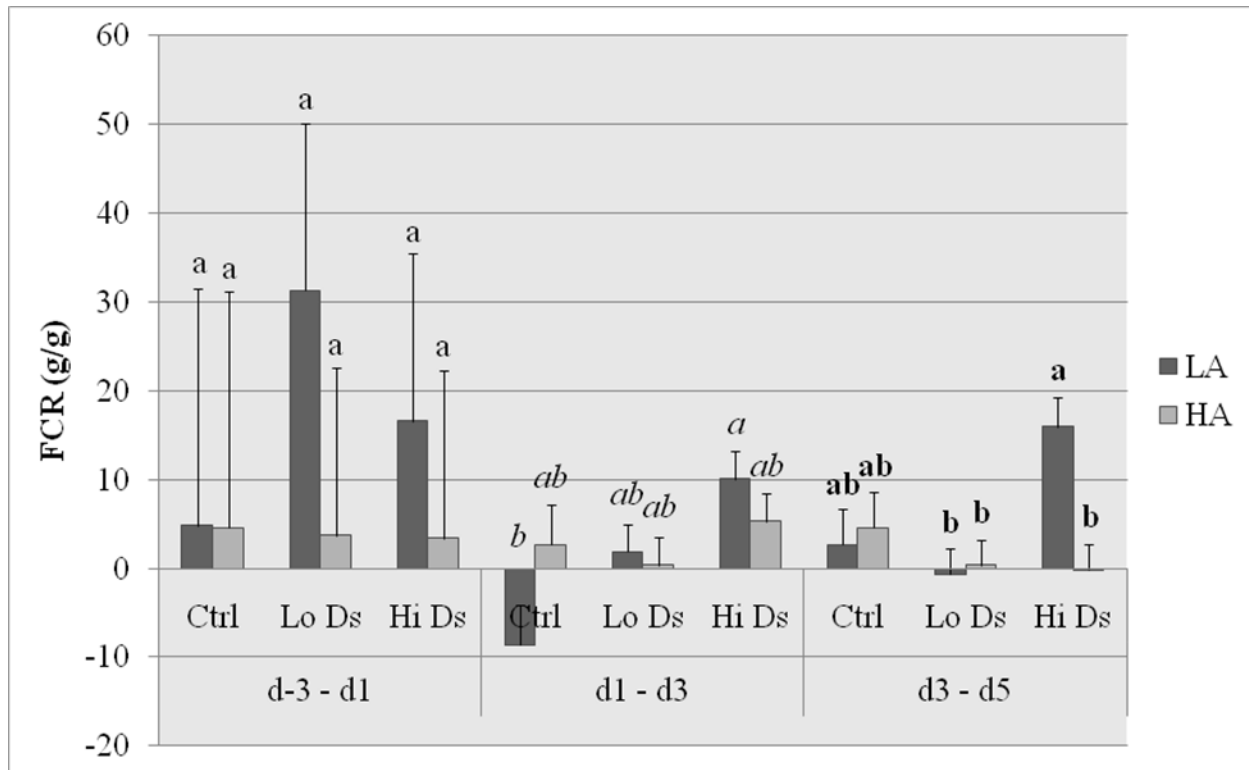


Figure 3.4. Effect of *Clostridium perfringens* infection on feed conversion ratio (FCR) in Experiment 1. Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with a Lo Ds (10^7 CFU/mL) or Hi Ds (10^9 CFU/mL) of *C. perfringens* on experiment d 1. Individual body weights and feed weights were recorded three days prior to the start of the experiment (d -3), as well as on d 1, 3 and 5. FCR was calculated by dividing feed intake by body weight gain. To determine differences in feed efficiency between the two genetic lines, data were analyzed at three separate time intervals (d -3 – d 1, d 1 – d 3, d 3 – d 5), as indicated by different lettering styles (regular, italicized, bold). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. Bars represented by different letters, within each time interval, differ significantly.

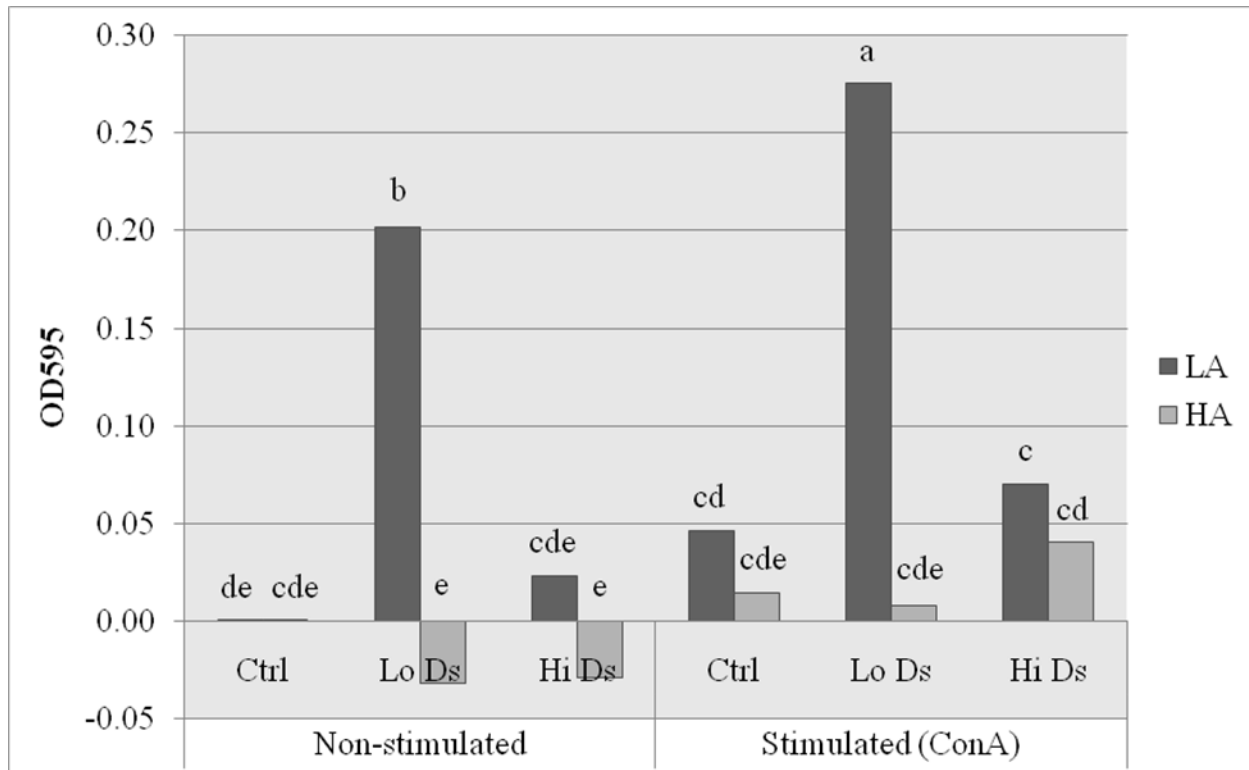


Figure 3.5. Effect of *Clostridium perfringens* infection on splenocyte proliferation in

Experiment 1. Chickens (12 weeks of age) genetically selected for low (LA) or high (HA)

antibody response to sheep red blood cells (SRBC) were orally gavaged with a Lo Ds (10^7

CFU/mL) or Hi Ds (10^9 CFU/mL) of *C. perfringens* on experiment d 1. Lymphocytes were

isolated from spleens on d 5 and cell proliferation was quantified using CellTiter 96 Non-

radioactive Cell Proliferation Assay (Promega, Madison, WI). Treatment averages for the non-

stimulated control of each genetic line were set to zero by subtracting the average OD₅₉₅ for the

control from each individual value. Data are represented as least squares (LS) means (n = 3

wells/treatment) and significance was accepted at $P < 0.05$. Bars represented by different letters

differ significantly.

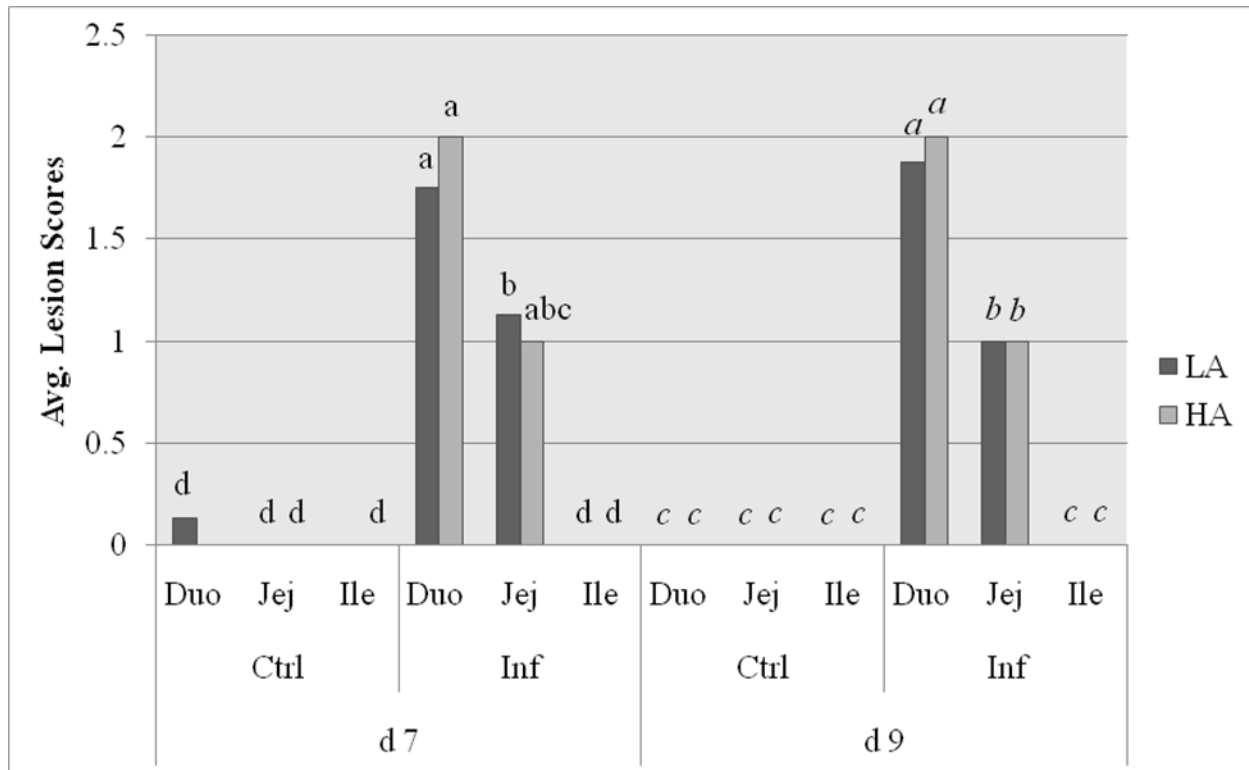


Figure 3.6. Effect of *Clostridium perfringens* infection on lesion scores in Experiment 2.

Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with either 1 mL PBS (Ctrl) or 1 mL live coccidia oocysts (50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts; Inf) on experiment d 1. On d 5, chickens were orally gavaged with 10^7 CFU/mL *C. perfringens*. Small intestinal sections, duodenum (duo), jejunum (jej) and ileum (ile) were examined for the presence of lesions on experiment d 7 and 9 (see Table 3.3 for lesion scoring system). To determine differences in lesion scores between the two genetic lines, data were analyzed at two separate time points (d 7 and 9), as indicated by separate lettering styles (normal, italicized). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. Bars represented by different letters, within each time interval, differ significantly.

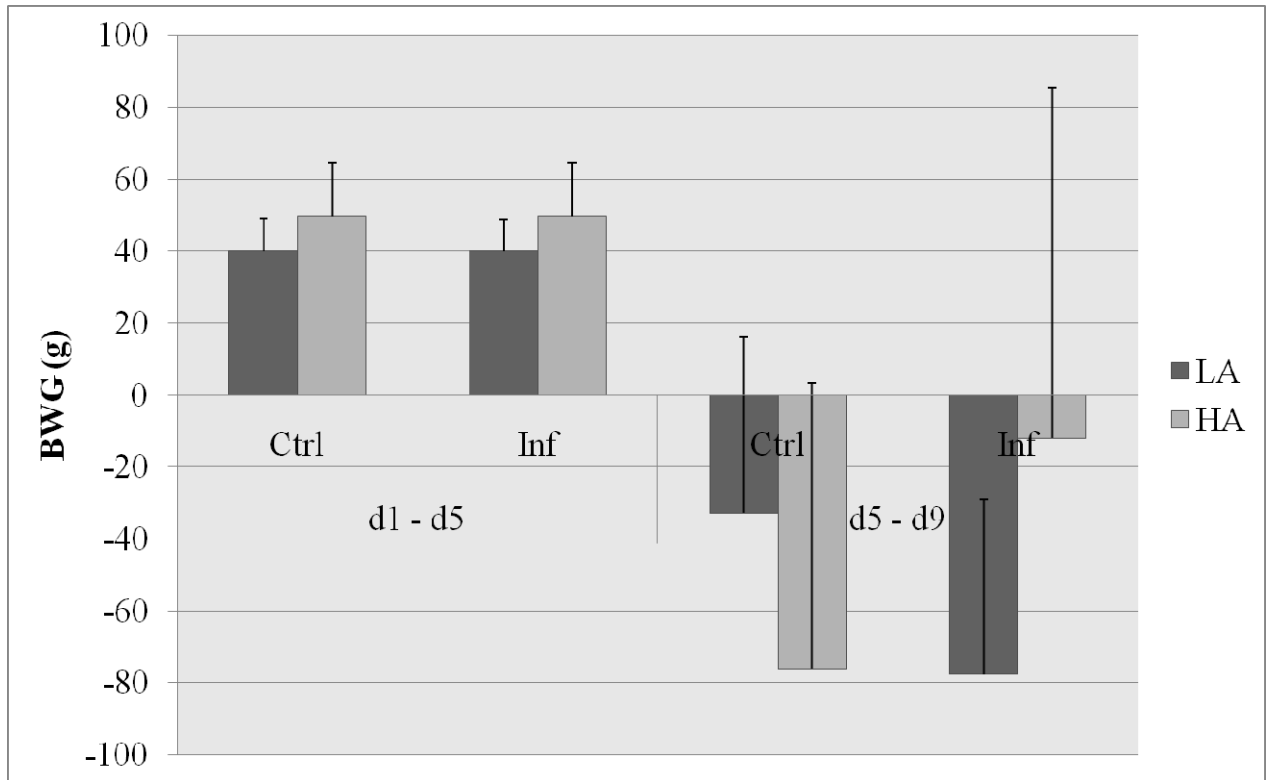


Figure 3.7. Effect of *Clostridium perfringens* infection on body weight gain (BWG) in Experiment 2. Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with either 1 mL PBS (Ctrl) or 1 mL live coccidia oocysts (50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts; Inf) on experiment d 1. On d 5, chickens were orally gavaged with 10^7 CFU/mL *C. perfringens*. To determine differences in BWG between the two genetic lines, data were analyzed at two separate time intervals (d 1 – d 5 and d 5 – d 9). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. No statistically significant differences were observed.

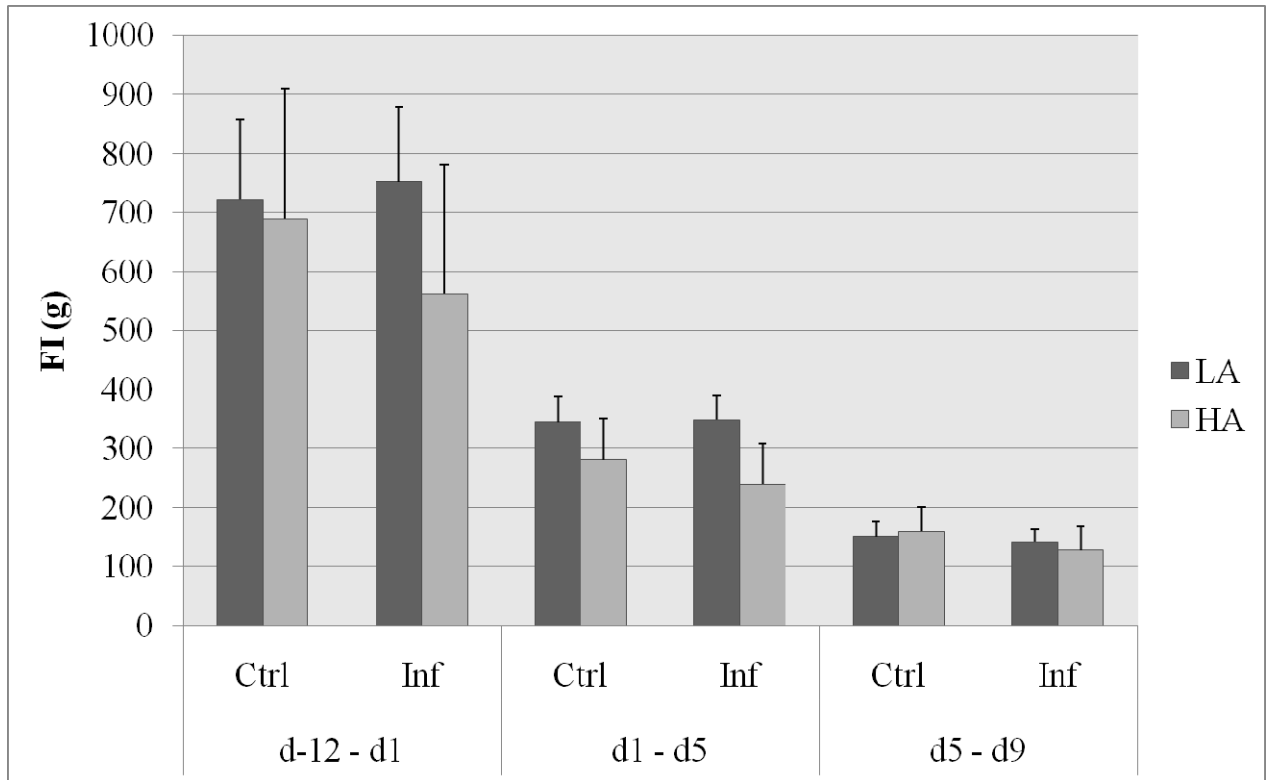


Figure 3.8. Effect of *Clostridium perfringens* infection on feed intake (FI) in Experiment 2.

Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with either 1 mL PBS (Ctrl) or 1 mL live coccidia oocysts (50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts; Inf) on experiment d 1. On d 5, chickens were orally gavaged with 10^7 CFU/mL *C. perfringens*. Feed weights were recorded twelve days prior to the start of the experiment (d -12), as well as on d 1, 5 and 9. To determine differences in FI between the two genetic lines, data were analyzed at three separate time intervals (d -12 – d 1, d 1 – d 5, d 5 – d 9). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. No statistically significant differences were observed.

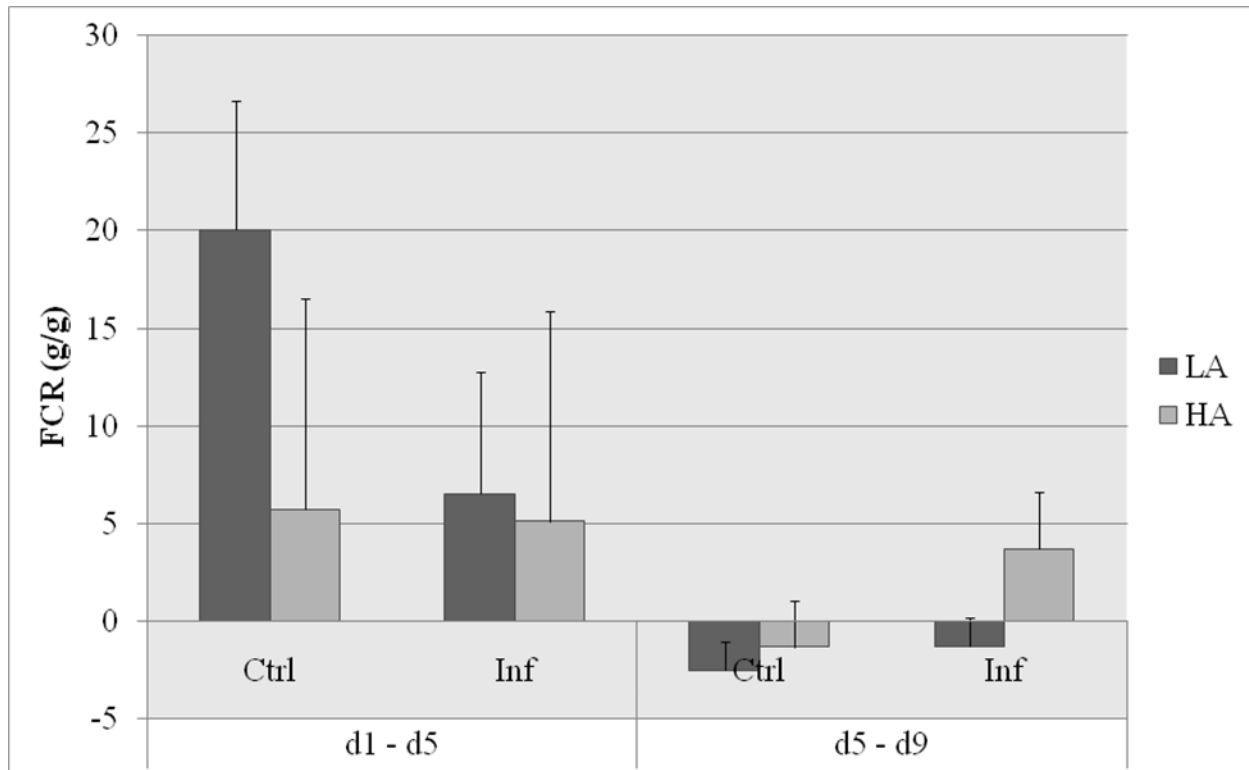


Figure 3.9. Effect of *Clostridium perfringens* infection on feed conversion ratio (FCR) in Experiment 2. Chickens (12 weeks of age) genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were orally gavaged with either 1 mL PBS (Ctrl) or 1 mL live coccidia oocysts (50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts; Inf) on experiment d 1. On d 5, chickens were orally gavaged with 10^7 CFU/mL *C. perfringens*. Individual body weights and feed weights were recorded on d 1, 5 and 9. FCR was calculated by dividing feed intake by body weight gain. To determine differences in feed efficiency between the two genetic lines, data were analyzed at two separate time intervals (d 1 – d 5 and d 5 – d 9). Data are represented as least squares (LS) means and significance was accepted at $P < 0.05$. No statistically significant differences were observed.

CHAPTER IV

Immunological Response to *Clostridium perfringens* α -toxin in Two Genetically Divergent Lines of Chickens as Influenced by Major Histocompatibility Complex (MHC) Genotype

ABSTRACT: Chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) displayed a correlated change in major histocompatibility complex (MHC), so that LA chickens were 96% B¹³ and HA chickens were 96% B²¹. A multitude of studies over the past several decades have evaluated exceptional differences between the two genetic lines. The LA line appears to be less susceptible to invasion by extracellular pathogens, while HA chickens are more resistant to infection by intracellular organisms. Resistance to *Clostridium perfringens* is one instance in which the lines do not follow their established trend of pathogen susceptibility. During a clinical outbreak of necrotic enteritis, B²¹B²¹ genotypes experienced significantly less mortality (6% vs. 13 %) compared to B¹³B¹³ genotypes. A study was carried out to assess immunological differences between LA and HA lines during exposure to *C. perfringens* α -toxin. Peripheral blood mononuclear cells (PBMCs) were isolated from each genetic line, cultured, stimulated with LPS (4 h), and exposed to varying concentrations of α -toxin (1, 10, 100, 1000 U/L) for 2 and 4 h. Evaluation of cellular proliferation, percent cytotoxicity and immunological gene expression was carried out in a variety of experiments. Cells isolated from HA chickens showed significantly increased proliferation compared to LA cells at low toxin levels (1 and 10 U/L), but exhibited significantly decreased proliferation at high toxin levels (100 and 1000 U/L). Following exposure to LPS, LA cells exhibited higher percent cytotoxicity compared to HA cells. In both assays, HA cells displayed superior performance following LPS-stimulation, suggesting a more advanced secondary immune

response to *C. perfringens* infection. Gene expression analysis of immune transcripts by quantitative real-time polymerase chain reaction (qRT-PCR) revealed significantly upregulated expression of interferon (IFN)- γ , interleukin (IL)-8, IL-13 (2 h), IL-15, and CXCL11 (4 h) in the HA line, compared to the LA line. Cells isolated from the LA line displayed significantly elevated expression of IL-2, IL-10, IL-13 (4 h), IL-16, IL-18, inducible nitric oxide synthase (iNOS), CXCL11 (2 h), and lipopolysaccharide-induced tumor necrosis factor- α factor (LITAF), compared to the HA line. It is clear that the two genetic lines display highly divergent immune responses in regards to *C. perfringens* exposure.

Introduction

Disease resistance as influenced by genetic factors has been extensively studied for numerous decades in a variety of species (Warner et al., 1987; Gogolin-Ewens et al., 1990; Hirschhorn et al., 2002). Specifically, the major histocompatibility complex (MHC) is known to play a vital role in immune response and disease resistance to autoimmune, viral, bacterial, and parasitic diseases (Lamont et al., 1989). In a selective breeding experiment conducted at Virginia Tech, White Leghorn chickens were genetically selected for low (LA) or high (HA) antibody response five days following intravenous injection of 0.1 mL (0.25% suspension) sheep red blood cells (SRBC; Siegel and Gross, 1980; Keuhn et al., 2006). Chickens exhibited a correlated change in MHC so that by the 10th generation of selection, LA birds were comprised of 99% B¹³, while HA birds were 80% B²¹ (Dunnington et al., 1984). Selection is currently in the 38th generation, during which the lines have been extensively utilized in a multitude of experiments and studies. LA chickens have been observed to possess larger thymuses, but smaller spleens and bursa of Fabricius (Ubosi et al., 1985). During the first 250 days of life, LA

chickens exhibit greater body weight and feed efficiency, and also reach maturity earlier, lay a larger number of eggs, and maintain fertility for a longer period of time (Siegel et al., 1982). HA chickens possess larger but less active macrophages (Biozzi et al., 1982) and in regards to disease resistance, have generally been found to be less susceptible to intracellular pathogens. The HA line is more resistant to Newcastle disease, *Mycoplasma gallisepticum*, *Eimeria necatrix*, feather mites (Gross et al., 1980), *E. tenella* (Martin et al., 1986), and Marek's disease (Dunnington et al., 1996). In contrast, LA chickens generally display increased resistance to extracellular pathogens, such as *Escherichia coli* and *Staphylococcus aureus* (Gross et al., 1980). The lifetime mortality due to non-specific causes has also been recorded as higher for the LA line (Siegel et al., 1982).

Resistance to *Clostridium perfringens* is one instance in which the lines do not follow their established trend of pathogen susceptibility. A flock containing sublimes from each background genome (LA and HA), as well as both MHC genotypes (B¹³ and B²¹) were diagnosed with necrotic enteritis on day 214, at which time litter was immediately replaced and 0.05% bacitracin was added to the feed (Siegel et al., 1993). Following examination of daily records, there was no real difference in mortality between background genomes; however, chickens possessing B¹³B¹³ genotypes experienced higher mortality than B²¹B²¹ birds (13% vs. 6%) beginning at an earlier time point. B¹³B¹³ genotypes also exhibited an earlier decrease in hen-day egg production, but recovered more quickly than B²¹B²¹ birds.

The causative agent of necrotic enteritis is the anaerobic bacterium *C. perfringens*. There are five main types of *C. perfringens* (A, B, C, D, E) that produce four main toxins (alpha, beta, epsilon, iota), as well as a common enterotoxin encoded by the *cpe* gene (Niilo, 1980; Sheedy et al., 2003). Type A is responsible for producing avian necrotic enteritis, as well as gas gangrene

and some types of human food poisoning (Wages and Opengart, 2003; Weiduo et al., 2007). The *C. perfringens* α -toxin is produced by all five types; however, type A is unique in that it is the only strain known to exclusively secrete α -toxin. α -toxin is a zinc-metalloenzyme with phospholipase C and sphingomyelinase activities (Songer, 1997). Research conducted over the past several decades has consistently implicated α -toxin as one of the main virulence factors of *C. perfringens* in the pathogenesis of avian necrotic enteritis (Al-Sheikhly and Truscott, 1976a, b, c; Fukata et al., 1988; Kulkarni et al., 2007, 2008; Zekarias et al., 2008; Cooper et al., 2009).

The purpose of this study was to evaluate immunological differences between LA and HA lines during exposure to *C. perfringens* α -toxin. Chickens used in the following experiments were from the 38th generation of selection. An ex vivo experiment was designed to investigate the effects of *C. perfringens* α -toxin (1, 10, 100, 1000 U/L) on peripheral blood mononuclear cells (PBMCs) isolated from both genetic lines. Incubation with lipopolysaccharide (LPS; 4 h) was used to simulate bacterial infection prior to incubation with α -toxin (2 and 4 h). LPS-priming was expected to result in heightened immune response during α -toxin exposure, compared to non-stimulated cells. Immune response was evaluated through cell proliferation and cytotoxicity assays, as well as relative expression analysis of immune gene transcripts by quantitative real-time polymerase chain reaction (qRT-PCR). We hypothesized that cells isolated from HA chickens would exhibit increased cell proliferation and decreased cytotoxicity compared to cells isolated from LA chickens. All procedures were carried out in accordance with guidelines established by the Virginia Tech Institutional Animal Care and Use Committee.

Materials and Methods

Blood Collection and PBMC Isolation

Blood was extracted from both HA and LA birds using 12 or 20 mL syringes filled with sodium citrate to prevent coagulation. After collection, the needle was removed from the syringe (to prevent cell lysis) and blood was carefully transferred from the syringe into 50 mL conical tubes. Blood from each genetic line was kept separate, but pooled among birds within each line. Blood was kept on ice until it was brought back to the lab for PBMC isolation. For each assay, approximately 200 mL of blood were collected from each line (about 7 to 10 birds/line). For gene expression analysis, 500 mL of blood were collected from each line.

All PBMC isolation procedures were carried out under sterile conditions inside a certified biological safety cabinet. New 50 mL conical tubes were filled with 20 mL of cold Histopaque 1077 (Sigma Aldrich, St. Louis, MO) and 20 mL of blood was carefully layered on top of the Histopaque in each tube by slowly pipeting down the side of the tube. Tubes were centrifuged for 30 minutes at 400 x g with acceleration and deceleration both set at 1 (Sorvall Legend RT, rotor no. 7500 6445). Following centrifugation, 4 distinct layers could be easily visualized (from top to bottom; plasma, mononuclear cells/buffy coat, Histopaque 1077, red blood cells). PBMCs were carefully collected from each tube using a Pasteur pipet and were pooled (within each genetic line) together in new 50 mL tubes. Each tube was only filled to a maximum volume of 20 mL. 20 mL of Hank's Balanced Salt Solution (HBSS; Hyclone, South Logan, UT) were added to each tube and centrifuged for 10 minutes at 250 x g with acceleration and deceleration both set at 9. Without disturbing the cell pellet, supernatant was carefully collected via serological pipet and discarded, and the cells were washed with HBSS 2x. For the final wash, cells were transferred to 15 mL tubes (one tube for each genetic line) and the volume was

adjusted to 10 mL with HBSS before centrifugation. After aspirating the supernatant for the final time, an appropriate amount of Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Inc., Manassas, VA) supplemented with 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B, and 10% fetal bovine serum (FBS; all from Atlanta Biologicals Inc., Lawrenceville, GA) was added to each tube depending on the size of the cell pellet (approximately 2x the size of the pellet). Total number of cells was determined using Trypan blue and a hemacytometer. Supplemented DMEM was added to each tube to obtain the desired final concentration of cells.

Treatment Groups and Plate Set-up

For each assay/experiment, wells containing cells were assigned to one of four main treatment groups (see Table 4.1). Experiments were run in triplicate for each time point (2 and 4 h), as well as each concentration of α -toxin (1, 10, 100, and 1000 U/L). Commercially available α -toxin was obtained from Sigma Aldrich (Phospholipase C from *C. perfringens*, Type 1). Toxin concentrations used were based on previous experiments (Rehman et al., 2009; Tumurkhuu et al., 2009).

Quantification of Cell Proliferation

PBMCs were seeded into 96-well cell culture plates (one plate for each genetic line) at a concentration of 10^3 cells/well with a total volume of 100 µL/well. Cells were incubated overnight at 39 °C with 5% CO₂ to allow for proper adherence. The following day, media were removed from each well and cells were gently washed with 100 µL plain DMEM. One hundred microliters of LPS (Sigma Aldrich) were added to groups 3 and 4 at a concentration of 5 µg/mL; 100 µL of supplemented DMEM were added to groups 1 and 2, and plates were incubated for 4 h at 39 °C with 5% CO₂. Following incubation, LPS and media were removed and cells were

washed with 100 μ L plain DMEM. α -toxin was added to groups 2 and 4 at concentrations of 1, 10, 100, and 1000 U/L with a total volume of 100 μ L/well. Supplemented DMEM was added to groups 1 and 3 (100 μ L/well) and plates were incubated for 2 h at 39 °C with 5% CO₂. Following this incubation, cell proliferation was quantified in half of the wells (2 h time point) using a commercially available cell proliferation kit (CellTiter 96 Non-radioactive Cell Proliferation Assay; Promega, Madison, WI) following the manufacturer's protocol. Plates were immediately returned to the incubator for an additional 2 h. After a total of 4 h incubation, cell proliferation was similarly quantified in the remaining wells.

Following the manufacturer's protocol, a dye solution containing a tetrazolium component was added to each well. During incubation, living cells convert tetrazolium into a formazan product. After a stop solution was added, the amount of formazan product produced was detected by a microplate reader (OD₅₉₅), which is proportional to the number of living cells. To calculate the amount of cell proliferation, the treatment average for the negative control (group 1) of each genetic line was set to zero. This was done by subtracting the average OD₅₉₅ for group 1 from each individual value.

Quantification of Percent Cytotoxicity

PBMCs were seeded into 96-well cell culture plates (one plate for each genetic line) at a concentration of 2×10^4 cells/well with a total volume of 200 μ L/well. Cells were treated in the same manner as described previously. In this experiment, cell cytotoxicity was quantified using a commercially available cytotoxicity kit (LDH Cytotoxicity Detection Kit; Clontech, Mountain View, CA) following the manufacturer's protocol.

Lactose dehydrogenase (LDH), an enzyme present in the cytoplasm of cells, is quickly released upon damage of the plasma membrane. Following the manufacturer's protocol, a

reaction mixture was added to cell-free supernatant and incubated at room temperature for 30 minutes. During incubation, lactic acid was converted to pyruvic acid by LDH, and tetrazolium was converted to formazan by diaphorase, a catalyst present in the reaction mixture. The number of dead cells is therefore directly proportional to both an increase in LDH production, as well as the amount of formazan product produced. The amount of formazan dye present was quantified by measuring the OD₄₉₂ using a microplate reader. Percent cytotoxicity was calculated using the following formula: [(triplicate absorbance – low control)/(high control – low control)] x 100.

Gene Expression Analysis

PBMCs were seeded into 6-well cell culture plates at a concentration of 5×10^5 cells/well with a total volume of 2 mL/well. Cells were treated in the same manner as described previously. Following the 2 and 4 h incubation time points, α -toxin and media were removed and 600 μ L RLT buffer were added directly to the wells. RNA extraction was performed using Qiagen RNeasy Mini Kit (Valencia, CA) following the cell spin method and using QIAshredder columns. RNA concentration and quality were determined using a NanoDrop (ND-1000) spectrophotometer (NanoDrop products, Wilmington, DE); OD_{260/280} ratio was examined to insure RNA quality. Complementary DNA was transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA) following the manufacturer's protocol, diluted 1:30 and stored at -20 °C until use. Primers were designed using Primer Express 3.0 (Applied Biosystems) software and synthesized by Eurofins MWG Operon (Huntsville, AL). A list of primer sequences is provided in Table 4.2. Real-time polymerase chain reaction (quantitative $\Delta\Delta$ Ct) was performed on an Applied Biosystems 7500 Fast RT-PCR system using Fast SYBR green (Applied Biosystems); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Samples were run in triplicate in

96-well plates. Relative gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) and the average ΔC_t values of group 1 served as the calibrator within each time point (2 or 4 h incubation with α -toxin).

Statistical Analysis

Data were analyzed using the Fit Model platform in JMP 8.0 (SAS Institute Inc., Cary, NC). Effects of treatment were analyzed by ANOVA and difference detected by Tukey honestly significant differences (HSD). Significance was accepted at $P < 0.05$ and results are reported as least squares (LS) means.

Results

Cell Proliferation

Following 2 h incubation with α -toxin, non-stimulated HA cells (group 2) had significantly less proliferation at 100 and 1000 U/L toxin concentration, compared to non-stimulated LA cells (Figure 4.1). Toxin-free, LPS-stimulated HA cells (group 3) proliferated significantly more than similarly treated LA cells. HA cells in group 4 also exhibited significantly increased proliferation compared to LA cells at 1 and 10 U/L toxin concentrations. However, group 4 HA cells subjected to 100 and 1000 U/L toxin concentrations proliferated significantly less than LA cells. After 4 h incubation with α -toxin, observed trends were very similar to those exhibited by cells after 2 h toxin incubation (Figure 4.2). At 100 and 1000 U/L toxin concentrations, HA cells in both groups 2 and 4 proliferated significantly less than LA cells. However, group 4 HA cells proliferated significantly more than LA cells at the 1 U/L toxin concentration.

Cytotoxicity

Although cell cytotoxicity data were not statistically significant, an interesting trend was observed (2 h, $P = 0.6461$; 4 h, $P = 0.0568$). After both 2 and 4 h incubations with α -toxin, percent cytotoxicity for HA and LA cells was directly proportional to the amount of toxin present (Figures 4.3 and 4.4). Following 2 h toxin incubation, cells in groups 2 and 4 performed similarly, with LA cells exhibiting slightly higher cytotoxicity than HA cells (Figure 4.3). Very similar results were observed after 4 h incubation with toxin. According to Figures 4.3 and 4.4, cells in group 3 appear to have exhibited negative cytotoxicity; however, it is important to note that the formula used to calculate percent cytotoxicity resulted in group 1 values set to zero. Therefore, the graphs (Figures 4.3 and 4.4) depict that group 3 cells simply experienced less cytotoxicity compared to group 1 cells.

Gene Expression

When exposed to 1000 U/L toxin for 2 h, cells isolated from HA chickens exhibited significantly higher interferon (IFN)- γ expression than cells isolated from LA chickens (Figure 4.5). It is important to note that we were unable to obtain a significant amount of high-quality RNA from cells incubated with 1000 U/L α -toxin for 4 h, presumably due to extreme cell death, as evidenced by cell cytotoxicity results. IFN- γ was also significantly increased in HA cells exposed to 100 U/L toxin for 4 h (Figure 4.6). Following 2 h incubation with 10 and 100 U/L toxin, interleukin (IL)-2 expression was significantly increased in group 4 LA cells compared to HA cells (Figure 4.7). While after 4 h incubation with α -toxin, group 2 LA cells showed increased IL-2 expression when exposed to 10 and 100 U/L toxin (Figure 4.8). IL-8 expression was significantly upregulated in group 4 LA cells subjected to 100 U/L α -toxin for 2 h, as well as both group 2 and 4 cells subjected to 1000 U/L toxin (Figure 4.9). However, following 4 h

incubation with α -toxin, IL-8 expression was significantly higher in group 4 HA cells exposed to 10 U/L (Figure 4.10). When subjected to α -toxin for 2 h, IL-10 was upregulated ($P < 0.0001$) in group 4 HA cells at 100 U/L and group 4 LA cells at 1000 U/L (Figure 4.11). IL-10 was also significantly upregulated in LA cells exposed to 100 U/L toxin for 4 h in the presence or absence of LPS (Figure 4.12). Group 2 HA cells subjected to 1000 U/L toxin, as well as group 4 cells subjected to 100 U/L toxin, exhibited increased ($P < 0.0001$) IL-13 expression following 2 h incubation (Figure 4.13). In contrast, following 4 h toxin incubation, LA cells exposed to 100 U/L in the presence or absence of LPS showed significantly upregulated IL-13 expression compared to HA cells (Figure 4.14). After 2 h, compared to LA cells, IL-15 expression was significantly higher in group 2 HA cells incubated with 1 U/L toxin, as well as in group 4 HA cells incubated with all four toxin concentrations (Figure 4.15). After 4 h, IL-15 expression was still increased ($P < 0.0001$) in group 4 HA cells subjected to 1 and 100 U/L, compared to LA cells (Figure 4.16). When exposed to 1000 U/L α -toxin for 2 h, LA cells showed significantly upregulated IL-16 expression compared to HA cells; however, HA cells exhibited higher ($P < 0.0001$) IL-16 expression than LA cells at 100 U/L in the presence of LPS (Figure 4.17). Following 4 h incubation, IL-16 was significantly elevated in group 2 LA cells exposed to 1, 10 and 100 U/L; group 4 cells exposed to 100 U/L, as well as group 3 cells (Figure 4.18). Group 4 LA cells subjected to 100 and 1000 U/L toxin for 2 h exhibited significantly higher IL-18 expression than HA cells (Figure 4.19). After 4 h incubation with 1, 10 and 100 U/L toxin, group 2 LA cells also exhibited upregulated ($P < 0.0001$) IL-18 expression (Figure 4.20). Inducible nitric oxide synthase (iNOS) was significantly elevated in group 4 LA cells exposed to 10, 100 and 1000 U/L toxin for 2 h (Figure 4.21). iNOS was also significantly upregulated in group 4 LA cells exposed to 100 U/L for 4 h; however, expression was higher in group 4 HA

cells exposed to 10 U/L for 4 h (Figure 4.22). In the presence of LPS, LA cells exhibited increased ($P < 0.0001$) CXCLi1 expression when subjected to 1000 U/L toxin for 2 h (Figure 4.23). However, when exposed to 100 U/L toxin for 4 h, group 4 LA cells showed decreased CXCLi1 expression compared to HA cells (Figure 4.24). Lipopolysaccharide-induced tumor necrosis factor- α factor (LITAF) expression was significantly upregulated in LA cells incubated with 1000 U/L for 2 h in the presence or absence of LPS; however, expression was higher in group 2 HA cells incubated with 100 U/L for 2 h (Figure 4.25). Following 4 h incubation, LITAF expression was elevated ($P < 0.0001$) in group 2 LA cells exposed to 10 and 100 U/L, group 4 LA cells exposed to 100 U/L, as well as group 3 cells (Figure 4.26).

Discussion

Cell Proliferation

As shown in Figures 4.1 and 4.2, cells performed similarly when incubated with α -toxin for either 2 or 4 h. Group 4 cells isolated from HA birds showed greater proliferation compared to LA cells at the lower toxin levels (1 and 10 U/L). However, when subjected to high levels of α -toxin (100 and 1000 U/L) HA cells exhibited significantly less proliferation than LA cells. This particular observation is further elucidated during discussion of cytotoxicity results. After 2 h, HA cells that were LPS-stimulated but not exposed to toxin (group 3) also exhibited significantly increased proliferation compared to LA cells. At low toxin concentrations (1 and 10 U/L), group 4 HA cells exhibited an increase in proliferation compared to LA cells, as well as group 2 HA cells. It is clear that cells isolated from the HA line displayed a greater response when primed with LPS. These results provide evidence that HA chickens may possess a more advanced secondary immune response to *C. perfringens* infection, compared to the LA line.

Perhaps the HA line is equipped with better pathogen recognition and memory, allowing birds to mount an exceptionally efficient immunological defense against invasion by *C. perfringens*.

It is also critical to note the increased cell proliferation displayed by both genetic lines (groups 2 and 4) at low toxin concentrations (1 and 10 U/L), compared to control cells (group 1). This suggests exposure to α -toxin resulted in the activation of macrophages, thus alluding to an enhanced immune response during low infection levels. In terms of bacterial numbers and/or toxin production, these results may indicate the existence of a cutoff point in which the immune system is no longer capable of operating and responding efficiently during severe infection.

Cytotoxicity

As indicated in Figures 4.3 and 4.4, data were not statistically significant ($P = 0.6461$ and $P = 0.0568$, respectively); however, cells exhibited a definite trend. Following both 2 and 4 h incubation with α -toxin, LA and HA cells showed increased cytotoxicity with increasing toxin concentration. Non-stimulated cells (group 2) displayed a somewhat more variable trend, while LPS-stimulated cells (group 4) revealed an increase in cellular cytotoxicity exhibited by LA birds compared to HA birds at all toxin levels. Regarding LPS-stimulated cells (group 4), it is important to point out that at both time points (2 and 4 h), percent cytotoxicity of cells isolated from the HA line began to level off around 100 and 1000 U/L, while cytotoxicity exhibited by LA cells continued to increase. These observations are similar to results obtained during cell proliferation studies, in that HA cells appeared to benefit greatly from LPS-priming, perhaps allowing HA chickens to mount a superior secondary immune response against *C. perfringens*. Cytotoxicity results may also offer a possible explanation for the significantly greater proliferation of LA cells compared to HA cells at higher toxin levels. PBMCs isolated from LA chickens exhibited severe cell death at high toxin concentrations, perhaps leading to increased

cell proliferation to compensate for decreased cell numbers. As mentioned previously, cytotoxicity of HA cells leveled off at high toxin concentrations, meaning it may not have been necessary for birds to allocate energy towards producing a large number of cells.

Gene Expression

The expression of eleven gene transcripts were analyzed by qRT-PCR with the goal of obtaining a better understanding of immune competence possessed by the two genetic lines of chickens, LA and HA, during exposure to *C. perfringens* α -toxin. The immune transcripts investigated were chosen based on previous gene expression studies during *C. perfringens* infections in chickens (Park et al., 2008; Sarson et al., 2009; Zhou et al., 2009). A summary of general gene expression results is provided in Table 4.3.

Elevated Expression in HA Compared to LA

As mentioned previously, chickens from the HA genetic line have been observed to be more resistant to infection by *C. perfringens*. IFN- γ is an important pro-inflammatory cytokine produced by T and B lymphocytes, as well as NK cells, and is highly regulated by IL-12 and IL-18 (Schneider et al., 2000; Schroder et al., 2004; Giansanti et al., 2006). IFN- γ is known to possess antiviral properties against intracellular pathogens (Schroder et al., 2004), an observation that coincides with the highly upregulated expression exhibited by HA cells, compared to LA cells. Although an increase in IFN- γ may not be necessarily beneficial during exposure to *C. perfringens*, it may partly explain the innate resistance of HA chickens to infections caused by intracellular pathogens. It is important to note that IFN- γ production appears to have been somewhat delayed in non-stimulated HA cells, whereas group 4 HA cells responded to LPS-stimulation with increased IFN- γ expression at an earlier time point. IL-8 is a CXC chemokine produced by macrophages, which functions predominantly as a chemoattractant and therefore

plays an important role in inflammation (Strober, 1998; Shahzad et al., 2010). IL-8 displayed significantly upregulated expression in HA cells, compared to LA cells. Previous studies have also reported a significant upregulation of IL-8 in primed chicken heterophils, following exposure to the intracellular bacterium *Salmonella enteritidis* (Kogut et al., 2003). IL-15 was also significantly upregulated in cells isolated from HA birds, compared to cells isolated from LA chickens. It appears that α -toxin exposure resulted in a shutdown of IL-15 in the LA line, since expression in LA cells was downregulated compared to the control. Mammalian IL-15 plays an important role in cell-mediated immunity by regulating the proliferation and activation of T and NK cells (Giansanti et al., 2006), and chicken IL-15 is structurally homologous to its mammalian counterpart (Lillehoj et al., 2001). In humans, IL-15 has been specifically cited as an important immunomodulator during invasion by intracellular organisms (Jullien et al., 1997; Waldmann and Tagaya, 1999), which once again, may point to the reason behind the upregulated expression of IL-15 observed in HA cells. IL-13 is unique in that it was the only interleukin to exhibit differences in expression between LA and HA cells at the two different time points. IL-13 expression was increased in HA cells, compared to LA cells, following 2 h incubation with α -toxin, but not after 4 h. In mammals, IL-13 shows a preference towards a Th2 immune response by specifically inhibiting production of pro-inflammatory modulators, as well as the NF κ B pathway (Miyoshi et al., 2007). Compared to the control, IL-13 was upregulated at higher toxin concentrations in both LA and HA cells; however, it is interesting that HA cells show a greater preference towards a Th2 response at an earlier time point, and LA cells at a later time point.

Elevated Expression in LA Compared to HA

Cells isolated from LA chickens appeared to have significantly increased expression of IL-2, compared to cells isolated from HA chickens. Mammalian IL-2 has been identified as a

cytokine cell growth factor that plays a role in the proliferation and differentiation of T and B lymphocytes, as well as NK cells (Giansanti et al., 2006). Chicken IL-2 is expressed by activated T cells (Kolodsick et al., 2001) and is capable of inducing chicken splenocyte proliferation in vitro (Stepaniak et al., 1999), as well as priming heterophils to elicit a phagocytic response during bacterial infections (Kogut et al., 2002). The fact that IL-2 plays an important role in priming the immune system during invasion by extracellular pathogens may partly explain the upregulated expression in isolated LA cells. It is also important to point out the difference in IL-2 expression between 2 and 4 h incubation with α -toxin. While IL-2 transcripts were more elevated in LA cells compared to HA cells at both time points, upregulated expression was only observed in LPS-stimulated cells at 2 h, while expression was only increased in non-stimulated LA cells at 4 h. These results suggest that while IL-2 expression may have been somewhat delayed in non-stimulated cells, LPS-priming was successful in boosting IL-2 expression early on; however, the effect appears to have worn off by the 4 h time point. IL-10 expression was also mostly elevated in LA cells compared to HA cells. Expression of IL-10 cytokines favors a Th2 immune response by downregulating the production of pro-inflammatory cytokines (Giansanti et al., 2006). Chicken IL-10 has been observed to specifically inhibit IFN- γ expression (Giansanti et al., 2006), which may partly explain the decreased IFN- γ expression in LA cells compared to HA cells. Conversely, the pro-inflammatory cytokine IL-18 was also significantly upregulated in LA cells compared to HA cells. IL-18 is expressed by a number of immune cells and is responsible for the induction of IFN- γ synthesis (Schneider et al., 2000; Giansanti et al., 2006). Its role in immunity is seemingly antagonistic to the function of IL-10, which makes it difficult to explain the lower IFN- γ expression in LA cells compared to HA cells. However, it is important to note that the included figures are presented in such a way as to

specifically show the significant difference between LA and HA cells. Although not statistically significant, LA cells incubated with α -toxin did exhibit upregulated IFN- γ expression compared to the control (group 1). Another pro-inflammatory cytokine that exhibited significantly elevated levels in LA cells compared to HA cells was IL-16. IL-16 also functions as a mediator of inflammation by possessing strong chemotactic activity for CD4⁺ T lymphocytes (Cruikshank et al., 2000). Chicken IL-16 is highly expressed in the bursa of Fabricius, suggesting a possible role in the development of B lymphocytes (Min and Lillehoj, 2004). CXCLi1, also known as K60, is a chemokine that acts as a chemoattractant for neutrophils in mammals (Giansanti et al., 2006). Expression is highly upregulated in chicken HD-11 cells exposed to IFN- γ and IL-1 β , but not IFN- α or IFN- β (Sick et al., 2000). In this experiment, CXCLi1 expression was significantly increased in LA cells treated with α -toxin for 2 h, compared to HA cells. Following 4 h incubation with toxin, CXCLi1 expression was higher in HA cells at the 100 U/L concentration. Unfortunately, we do not have gene expression data for cells exposed to 1000 U/L α -toxin for 4 h, which would be important for comparison since CXCLi1 expression was consistently low at the lower toxin concentrations (1, 10, 100 U/L). iNOS is one of three forms of NOS and is predominantly produced by macrophages (Lin et al., 1996). NOS functions as a catalytic enzyme in the production of NO which plays an important role in the immune system acting as a cytotoxic and tumoricidal agent (Lin et al., 1996). We found that iNOS expression was significantly upregulated in cells isolated from LA chickens, compared to HA cells. This increase in expression may aid LA birds in mounting a more effective immune response during invasion by extracellular pathogens. Hussain and Qureshi (1997) have previously observed a difference in the ability of chicken macrophages isolated from different genetic sources to produce nitrite. They reported that LPS-stimulated macrophages from GB1 (B¹³B¹³) and GB2

(B⁶B⁶) chickens produced a significantly less amount of nitrite compared to Cornell K-strain (B¹⁵B¹⁵) chickens, as well as MQ-NCSU cells. This information provides additional perspective in terms of our study; while LA chickens (B¹³B¹³) exhibited increased iNOS expression compared to HA chickens (B²¹B²¹), Hussain and Qureshi found GB1 (B¹³B¹³) chickens were actually hyporesponsive to LPS in terms of nitrite production, compared to other genetic lines. LITAF is predominantly expressed in the spleen of chickens, as well as in intestinal intraepithelial lymphocytes (Hong et al., 2006). LITAF expression was found to be significantly elevated in LA cells, compared to HA cells, particularly at the 4 h time point. In fact, HA cells exposed to α -toxin for 4 h showed downregulated expression compared to the control. In previous in vitro studies, LITAF was found to be upregulated in macrophages exposed to *E. coli*, *S. typhimurium*, *E. acervulina*, *E. maxima*, and *E. tenella* (Hong et al., 2006). It is possible that increased LITAF expression may play a role in the resistance of LA chickens to *E. coli* and *S. typhimurium*.

The results of this study have cultivated an exceptional appreciation for the complexities of immune responses possessed by these two genetically divergent lines of chickens. There certainly appears to be a distinct difference in the immune repertoire maintained by LA and HA birds, specifically in response to *C. perfringens* α -toxin.

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Table 4.1. Experimental Treatment Groups

Treatment Group	LPS^a (5 µg/ml)	α-toxin^b
1	-	-
2	-	+
3	+	-
4	+	+

^aLPS – lipopolysaccharide (Sigma Aldrich, St. Louis, MO)

^bα-toxin – Phospholipase C from *Clostridium perfringens*, Type 1 (Sigma Aldrich)

Table 4.2. Chicken Primer Sequences. The listed oligonucleotides were used to analyze gene expression via quantitative real-time polymerase chain reaction (qRT-PCR). Sequences were generated using Primer Express 3.0 (Applied Biosystems, Foster City, CA) software and synthesized by Eurofins MWG Operon (Huntsville, AL).

Gene	Primers	Accession No.
IFN-γ	For: 5' – GCTCCCGATGAACGACTTGA – 3' Rev: 5' – TGTAAGATGCTGAAGAGTTCATTCG – 3'	NM_205149
IL-2	For: 5' – CGAGCTCTACACACCAACTGAGA – 3' Rev: 5' – CCAGGTAACACTGCAGAGTTTGC – 3'	NM_204153
IL-8	For: 5' – TCCTGGTTTCAGCTGCTCTGT – 3' Rev: 5' – CGCAGCTCATTCCCCATCT – 3'	NM_205498
IL-10	For: 5' – CGCTGTCACCGCTTCTTCA – 3' Rev: 5' – CGTCTCCTTGATCTGCTTGATG – 3'	NM_001004414
IL-13	For: 5' – CATGACCGACTGCAAGAAGGA – 3' Rev: 5' – CCGTGCAGGCTCTTCAGACT – 3'	NM_001007085
IL-15	For: 5' – GACTAACCATCTTCTTCCTATGTGCTT – 3' Rev: 5' – AGAACGTCTGACCACTTACAGTGATT – 3'	AF139097
IL-16	For: 5' – GGAACAAAGCAGCCCAGTTC – 3' Rev: 5' – GGCTGTGGTGTGCACCTGTA – 3'	NM_204352
IL-18	For: 5' – AGGTGAAATCTGGCAGTGGAAAT – 3' Rev: 5' – TGAAGGCGCGGTGGTTT – 3'	NM_204608
iNOS	For: 5' – CCTGTACTGAAGGTGGCTATTGG – 3' Rev: 5' – AGGCCTGTGAGAGTGTGCAA – 3'	D85422
CXCLi1	For: 5' – GGCTGGAGCAAAAGGTATGG – 3' Rev: 5' – GCACTGGCATCGGAGTTCA – 3'	NM_205018
LITAF	For: 5' – TGTTCTATGACCGCCCAGTTC – 3' Rev: 5' – AGACGTGTCACGATCATCTGGTTA – 3'	AY765397

Table 4.3. Summary of Results. Changes in relative gene expression between low (LA) and high (HA) antibody chickens following exposure of isolated peripheral blood mononuclear cells (PBMCs) to *Clostridium perfringens* α -toxin

LA \uparrow	HA \uparrow
CXCLi1 (2 h)	CXCLi1 (4 h)
IL-2	IFN- γ
IL-10	IL-8
IL-13 (4 h)	IL-13 (2 h)
IL-16	IL-15
IL-18	
iNOS	
LITAF	

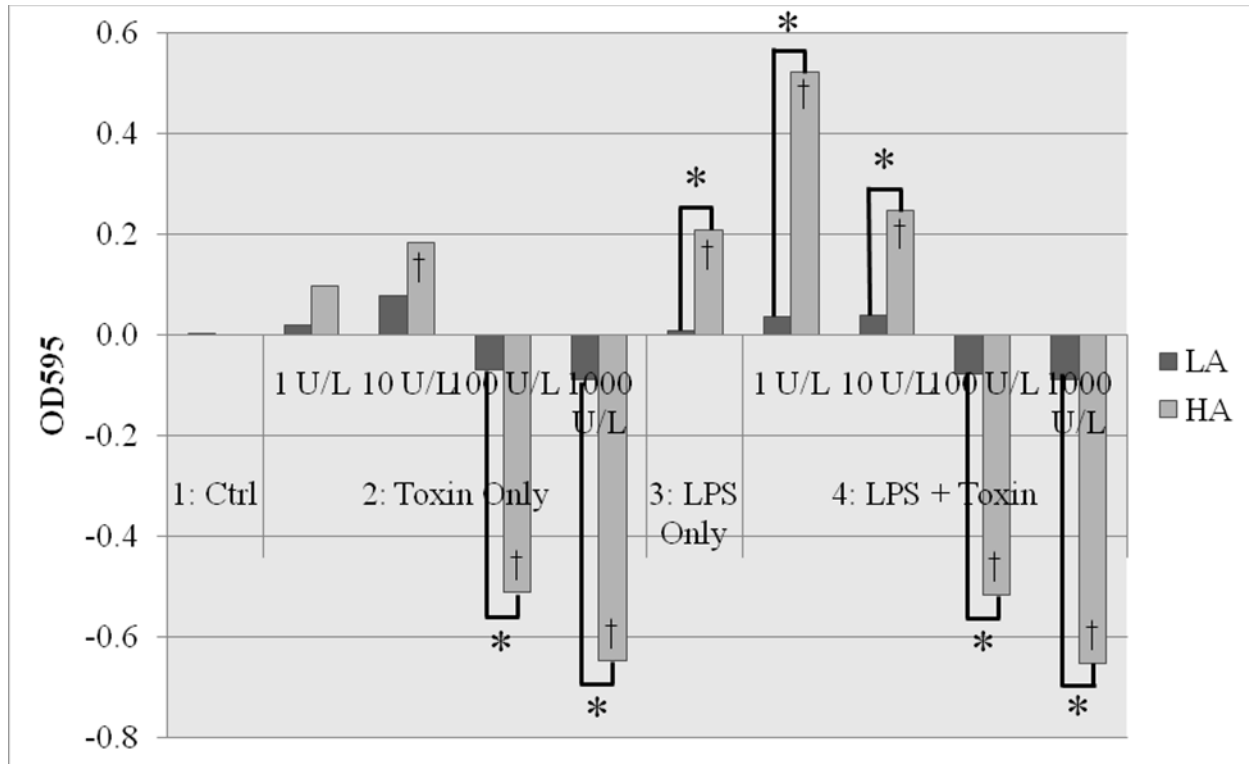


Figure 4.1. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on cell proliferation. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 96-well cell culture plates (10^3 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. Cell proliferation was quantified using CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega, Madison, WI). Treatment averages for the negative control (group 1) of each genetic line was set to zero by subtracting the average OD₅₉₅ for group 1 from each individual value. Data are represented as least squares (LS) means (n = 3 wells/treatment) and significance was accepted at $P < 0.05$.

Asterisks indicate significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

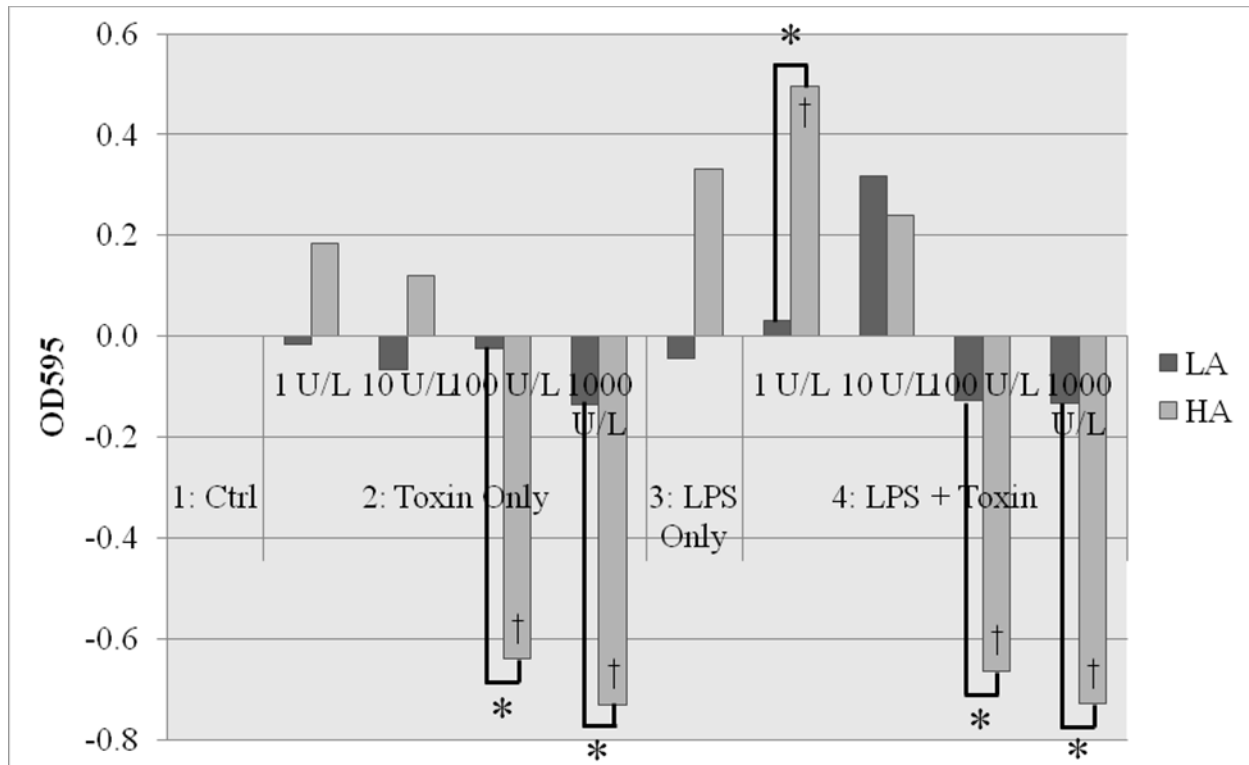


Figure 4.2. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on cell proliferation. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 96-well cell culture plates (10^3 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. Cell proliferation was quantified using CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega, Madison, WI). Treatment averages for the negative control (group 1) of each genetic line was set to zero by subtracting the average OD₅₉₅ for group 1 from each individual value. Data are represented as least squares (LS) means (n = 3 wells/treatment) and significance was accepted at $P < 0.05$.

Asterisks indicate significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

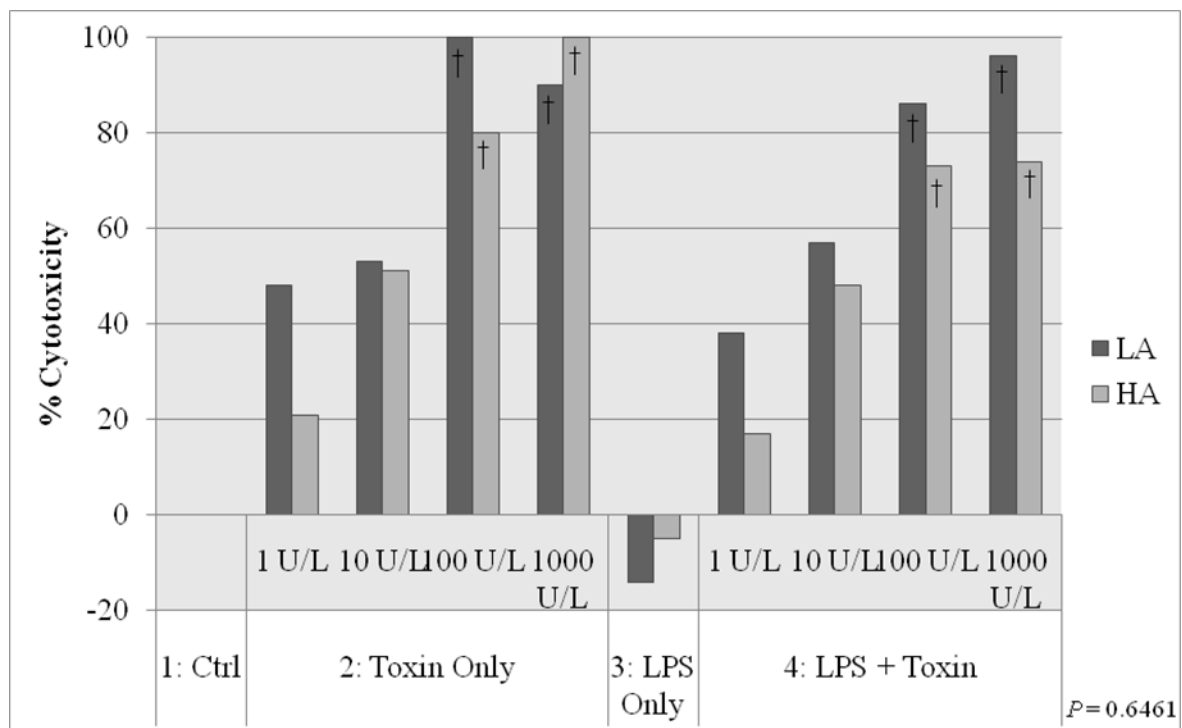


Figure 4.3. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on cell cytotoxicity. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 96-well cell culture plates (2×10^4 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. Cellular cytotoxicity was quantified using LDH Cytotoxicity Detection Kit (Clontech, Mountain View, CA). Percent cytotoxicity was calculated by normalizing values against the negative control (group 1) of each genetic. Data are represented as least squares (LS) means ($n = 3$ wells/treatment) and significance was accepted at $P < 0.05$. Crosses indicate significant difference from negative control (group 1).

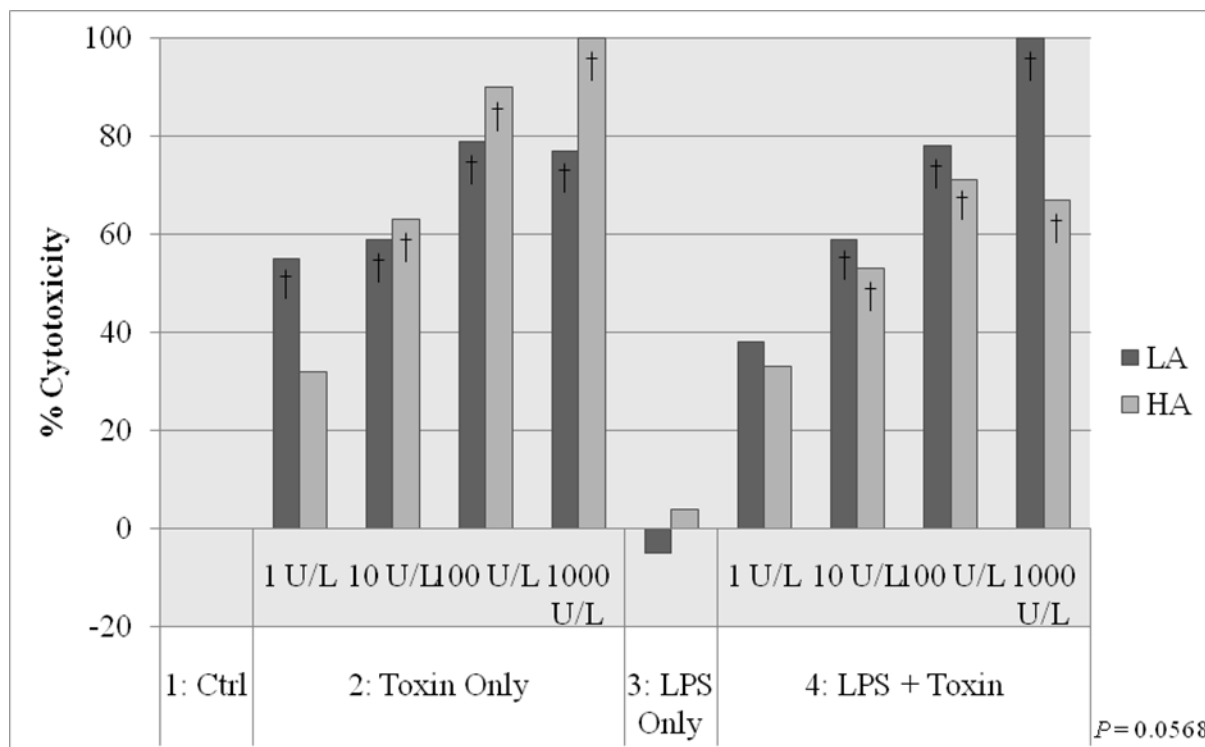


Figure 4.4. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on cell cytotoxicity. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 96-well cell culture plates (2×10^4 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. Cellular cytotoxicity was quantified using LDH Cytotoxicity Detection Kit (Clontech, Mountain View, CA). Percent cytotoxicity was calculated by normalizing values against the negative control (group 1) of each genetic. Data are represented as least squares (LS) means ($n = 3$ wells/treatment) and significance was accepted at $P < 0.05$. Crosses indicate significant difference from negative control (group 1).

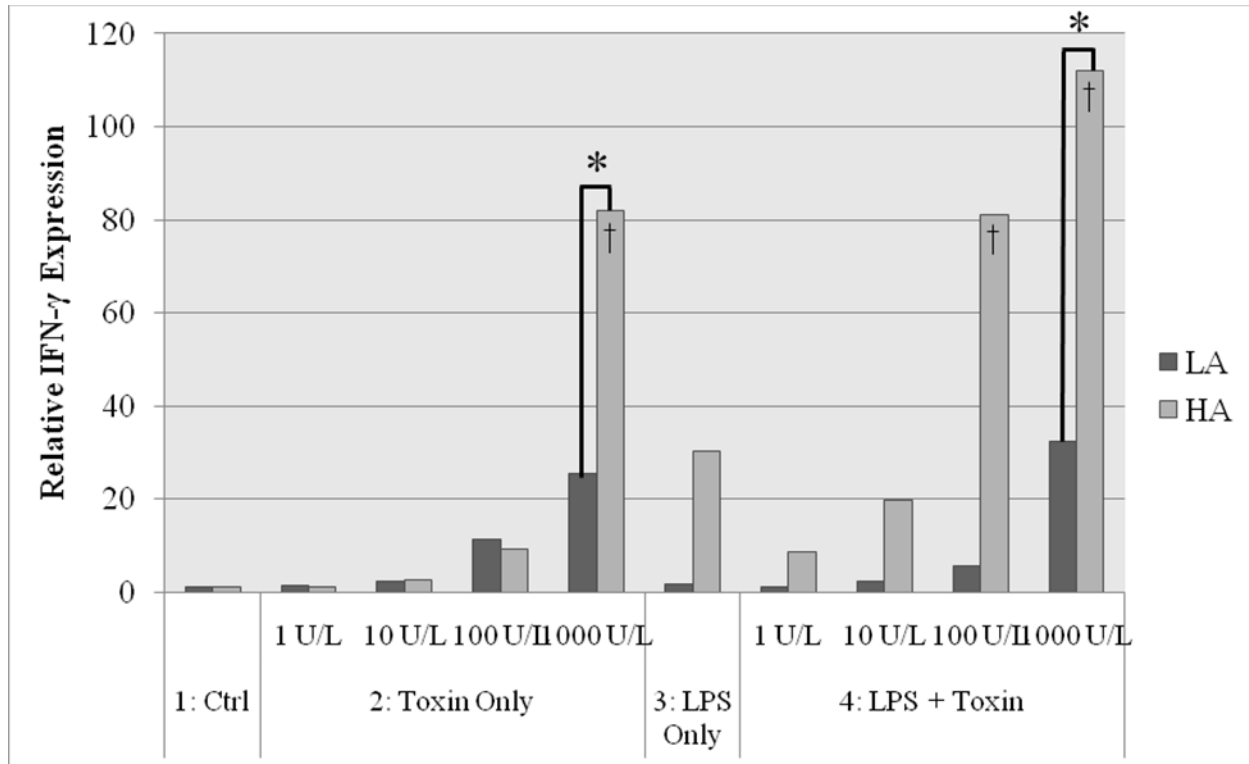


Figure 4.5. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interferon (IFN)- γ expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IFN- γ expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks indicate significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

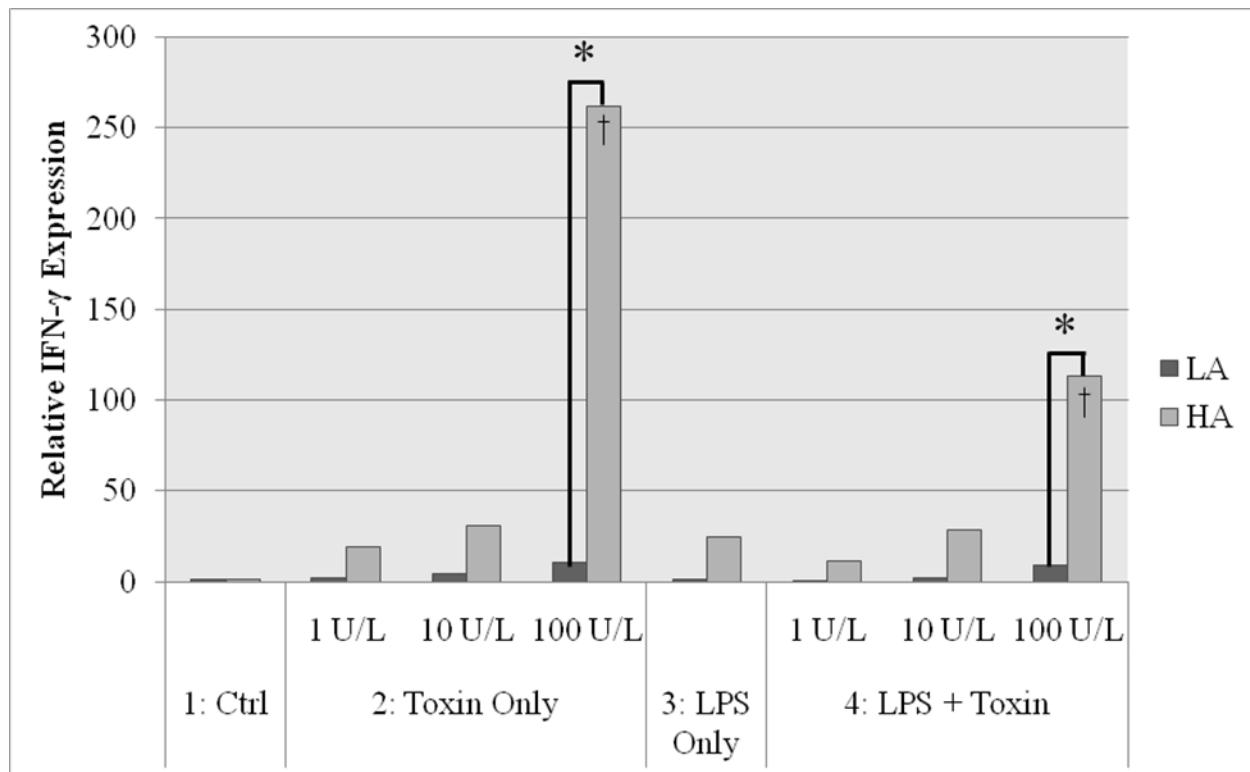


Figure 4.6. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interferon (IFN)- γ expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IFN- γ expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

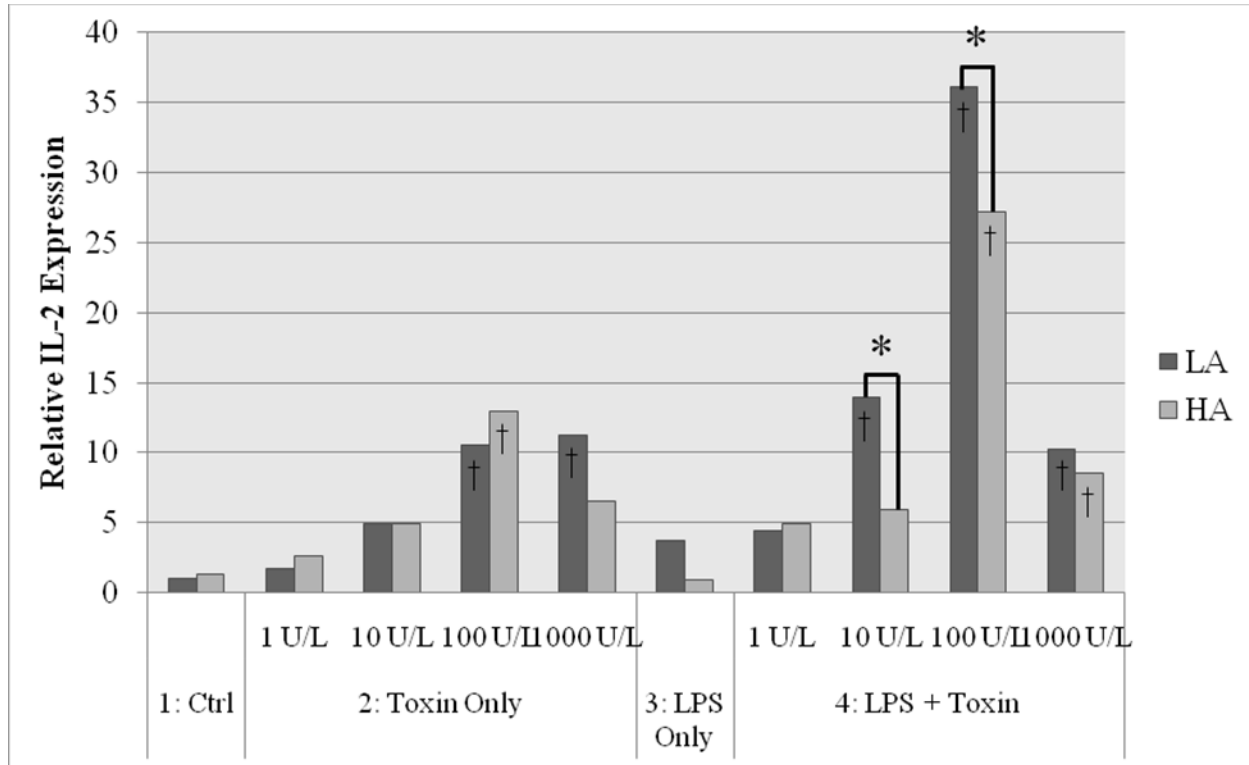


Figure 4.7. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-2 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-2 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

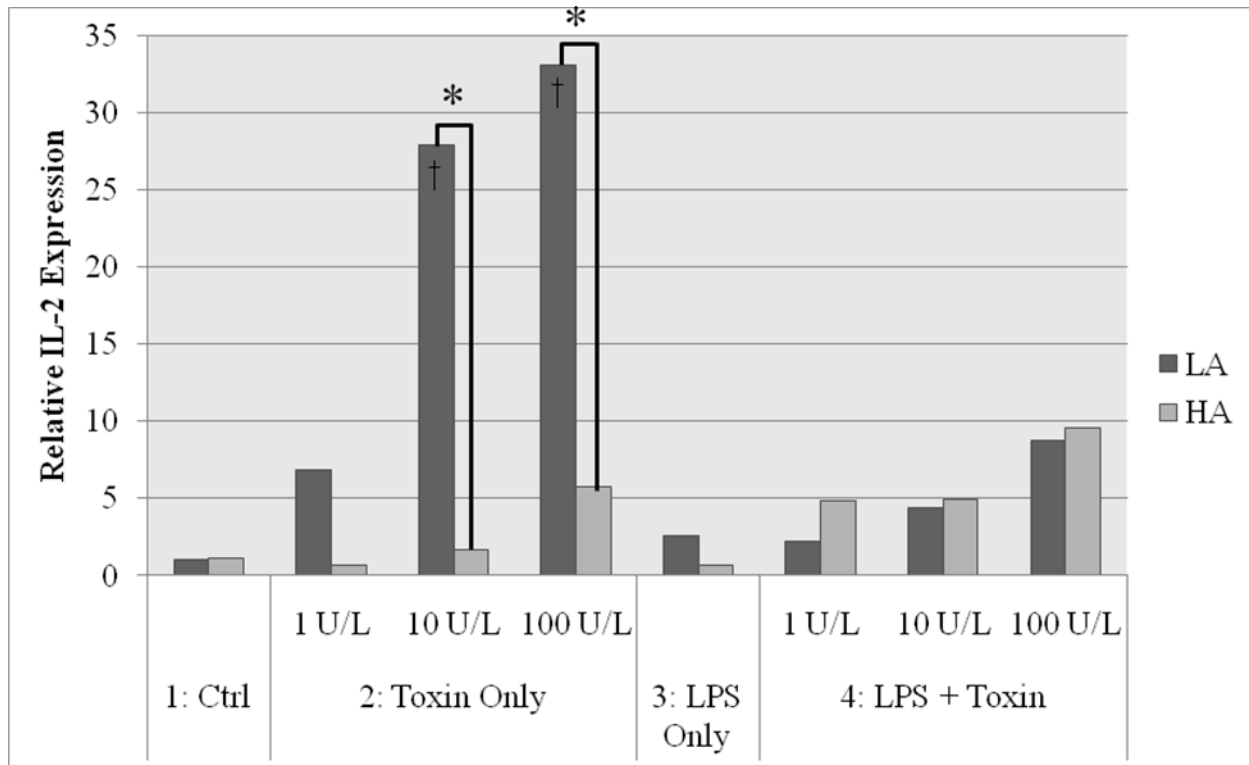


Figure 4.8. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-2 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-2 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

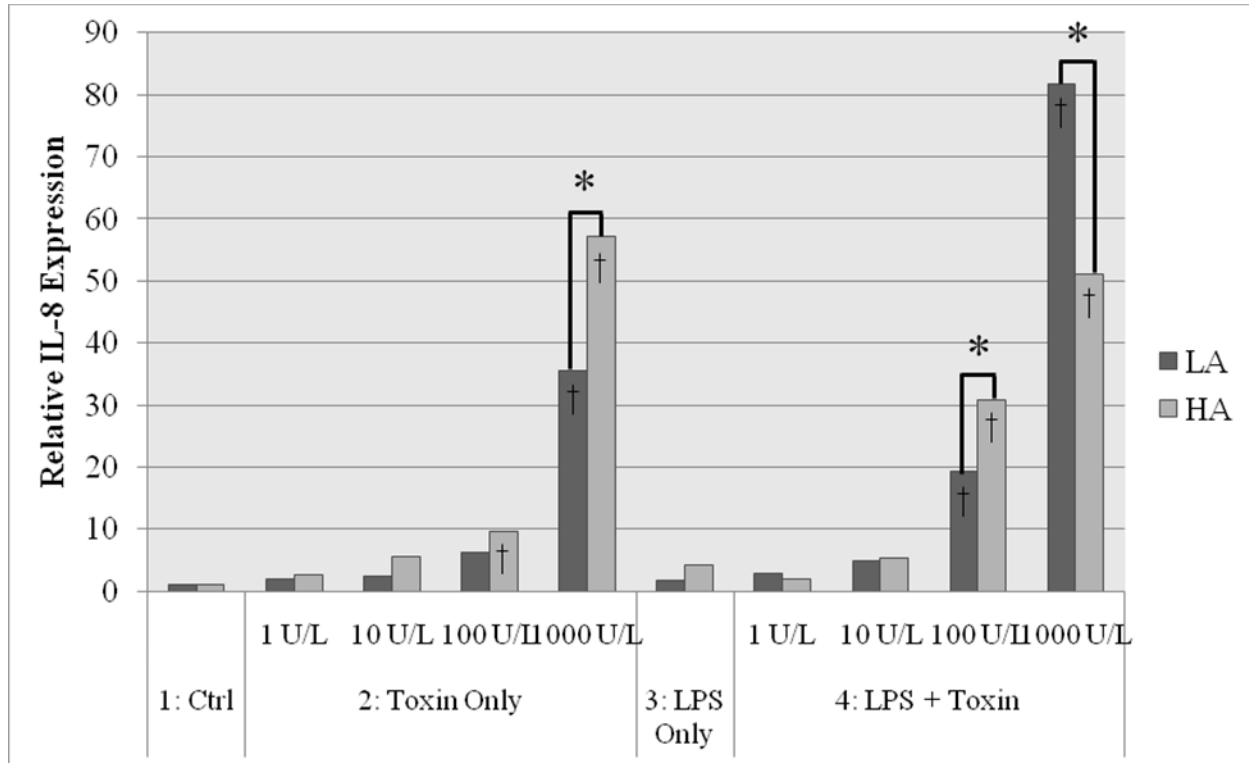


Figure 4.9. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-8 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-8 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

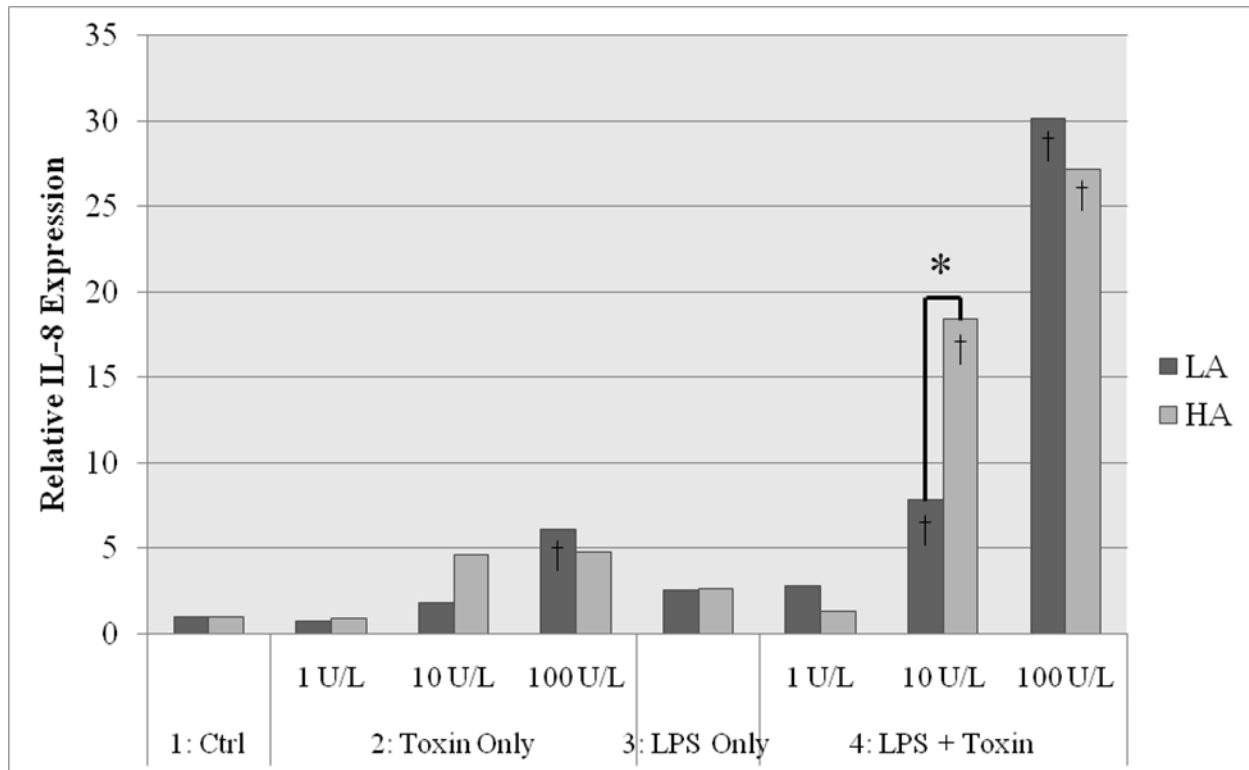


Figure 4.10. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-8 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-8 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

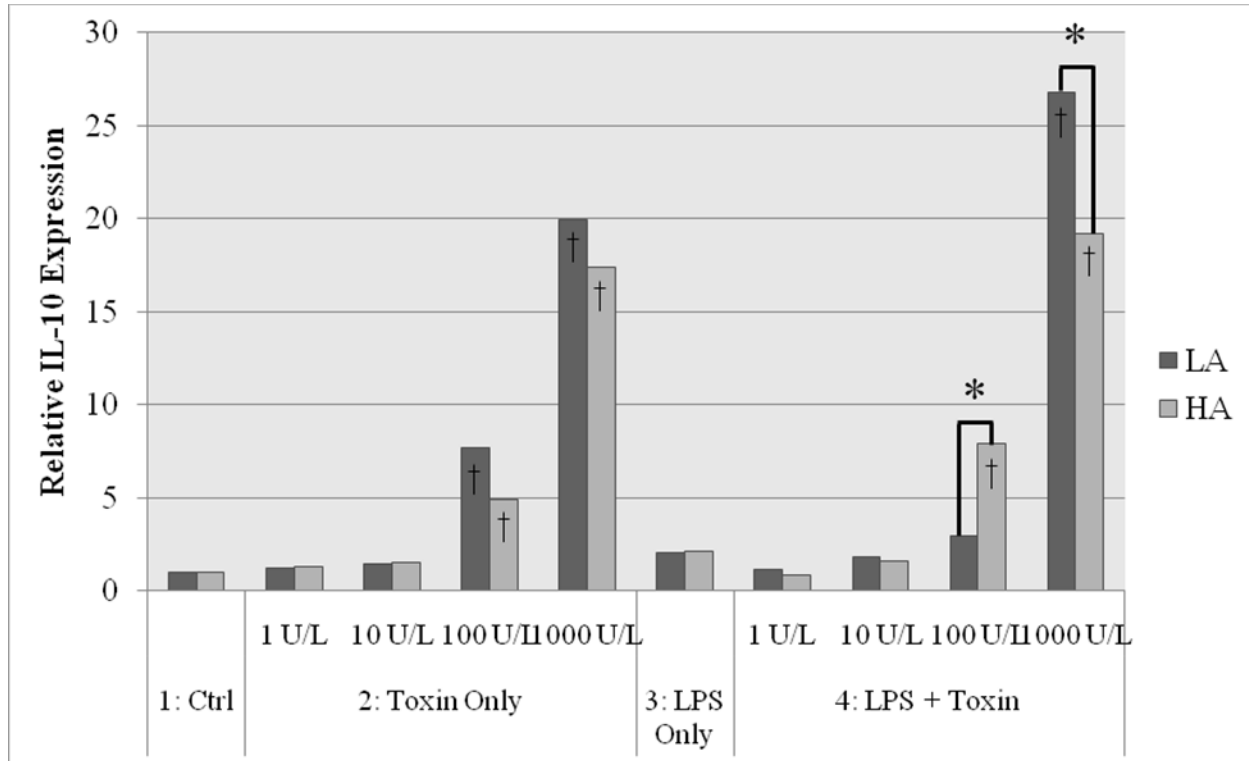


Figure 4.11. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-10 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-10 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

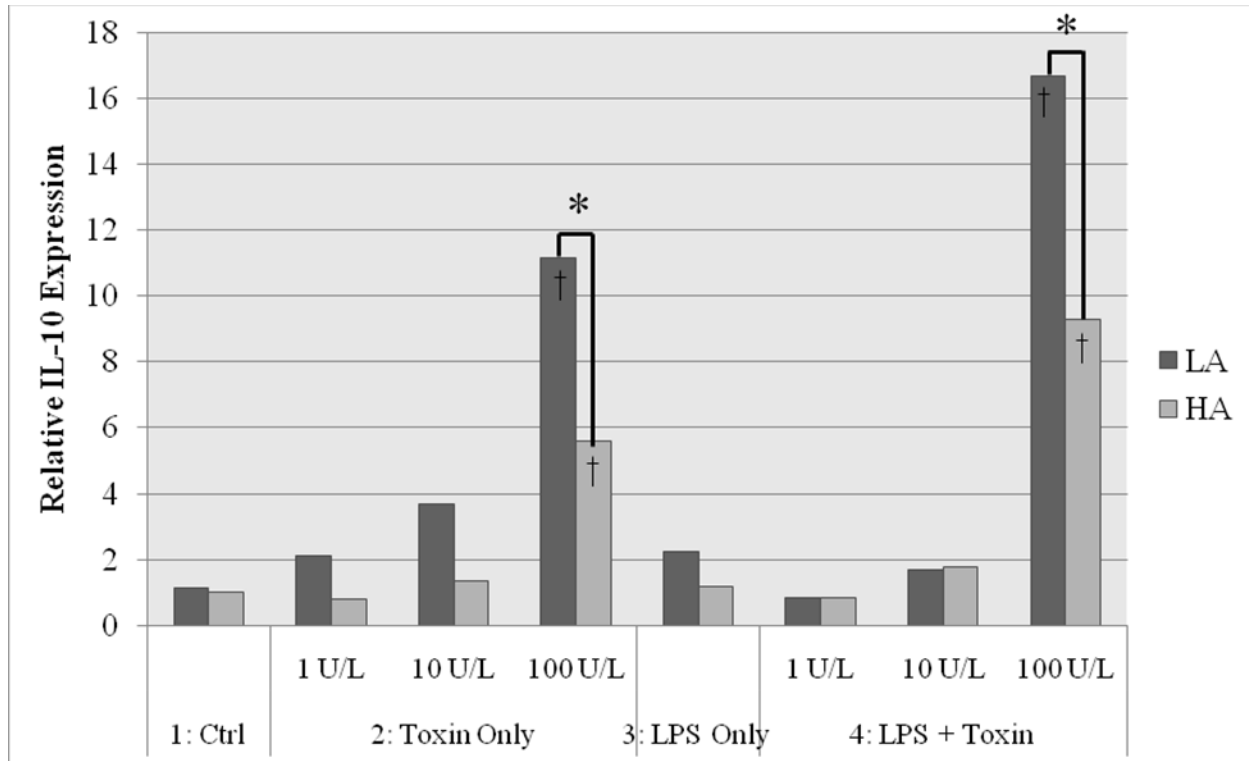


Figure 4.12. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-10 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-10 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

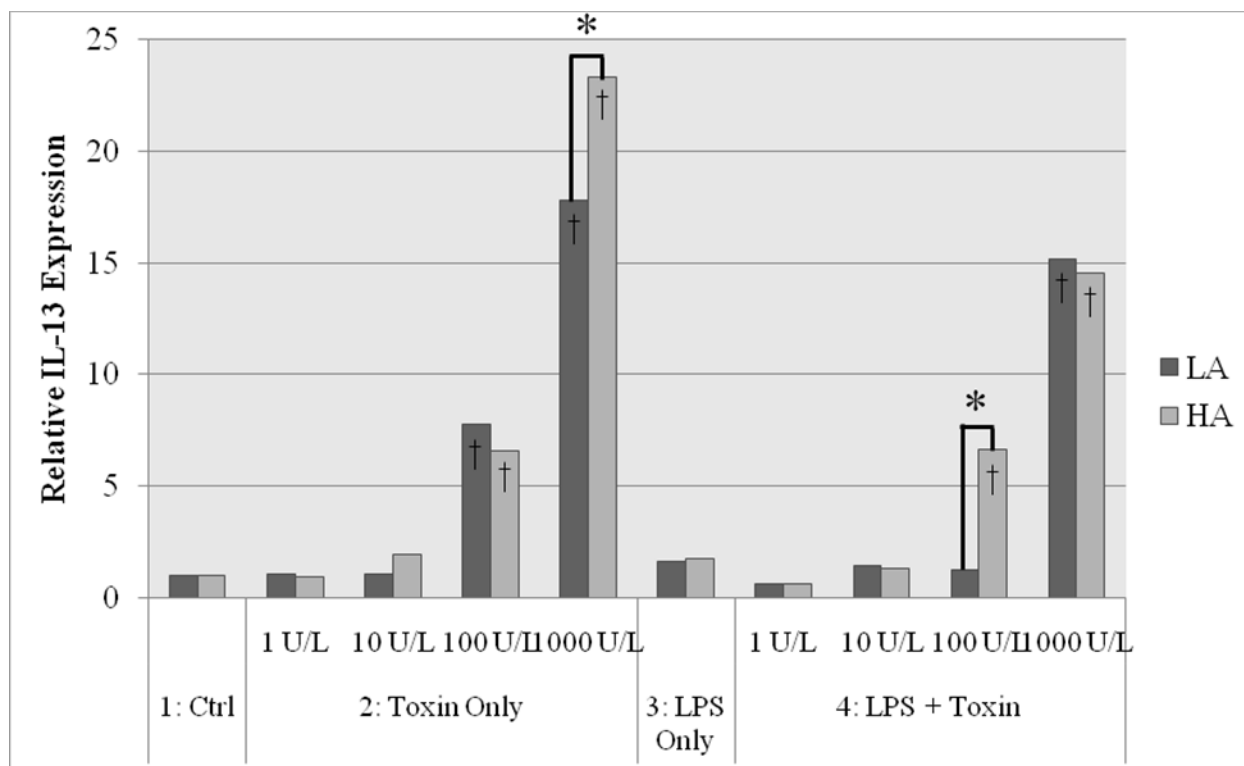


Figure 4.13. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-13 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-13 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

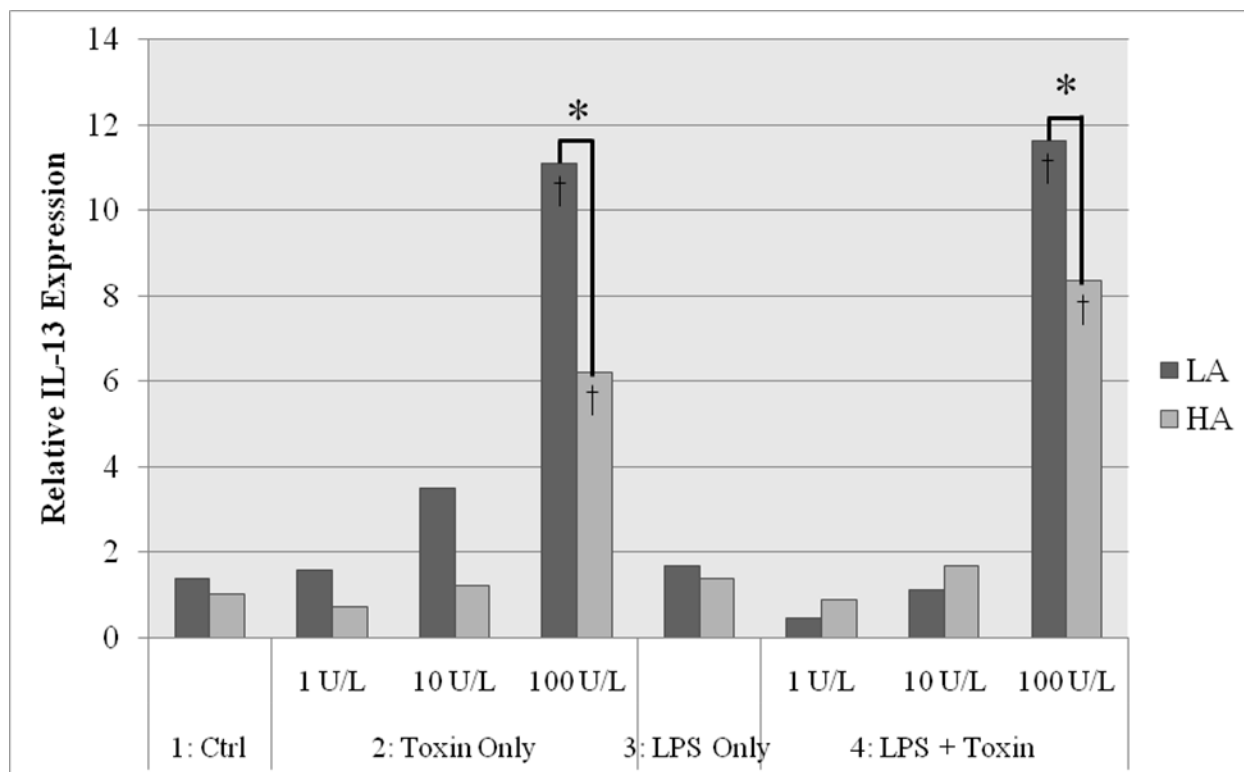


Figure 4.14. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-13 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-13 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

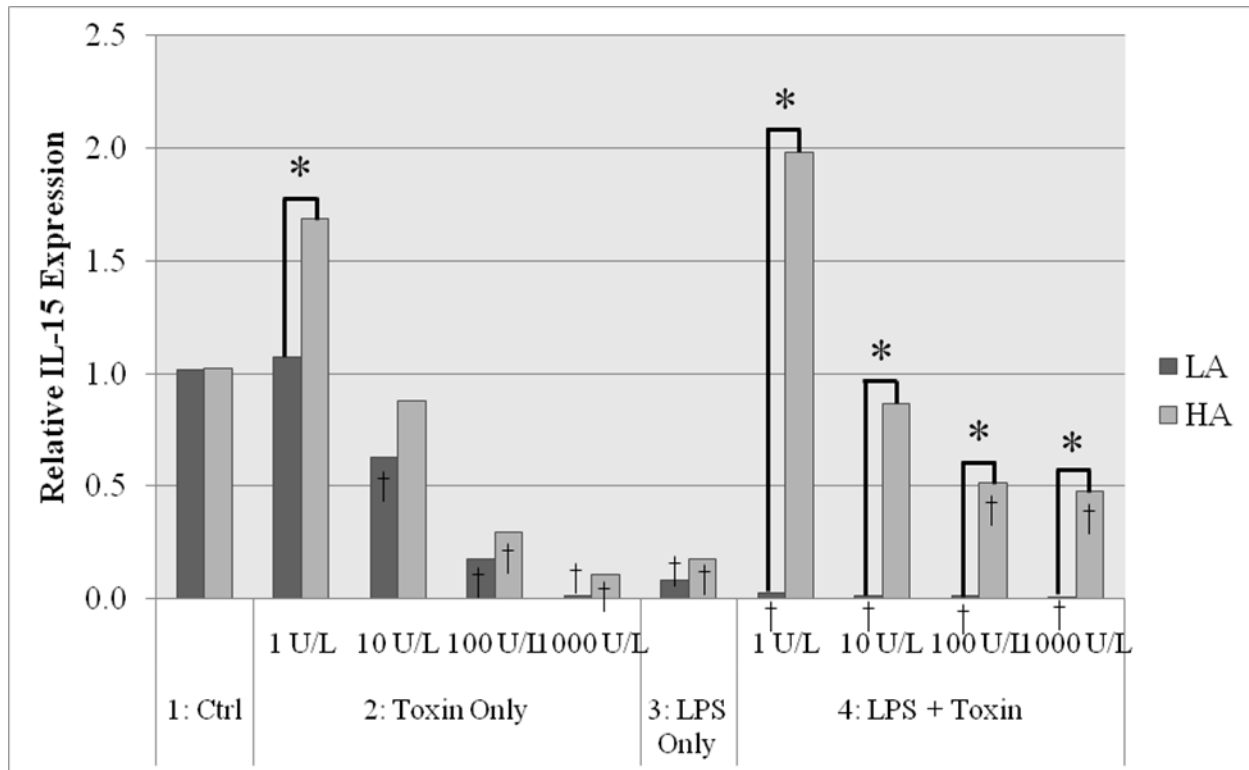


Figure 4.15. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-15 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-15 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

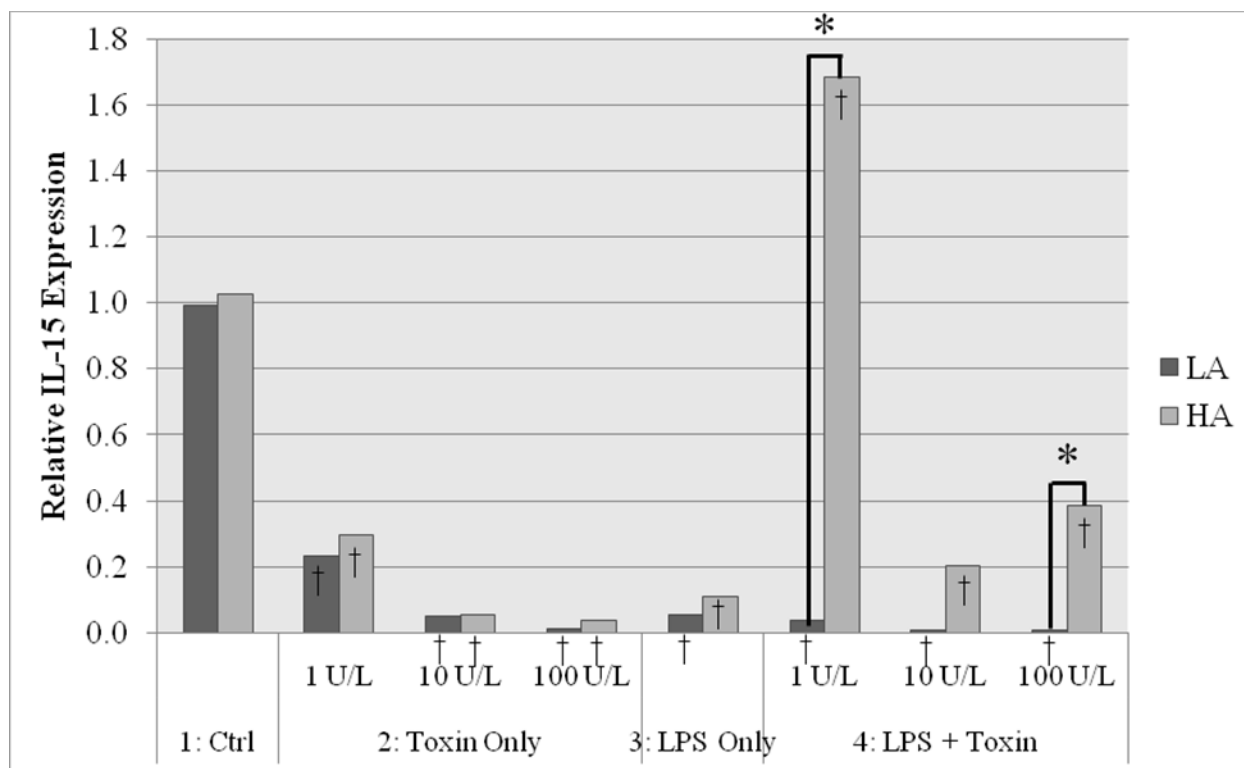


Figure 4.16. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-15 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-15 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

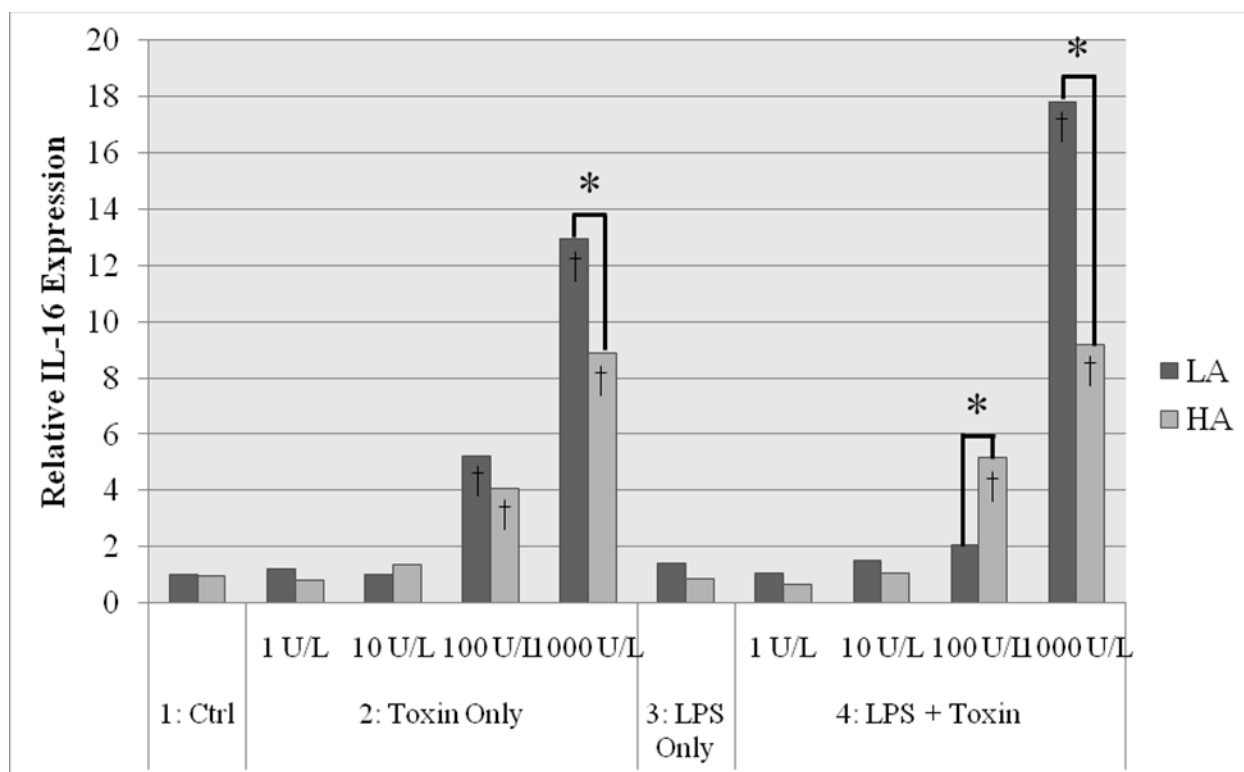


Figure 4.17. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-16 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-16 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

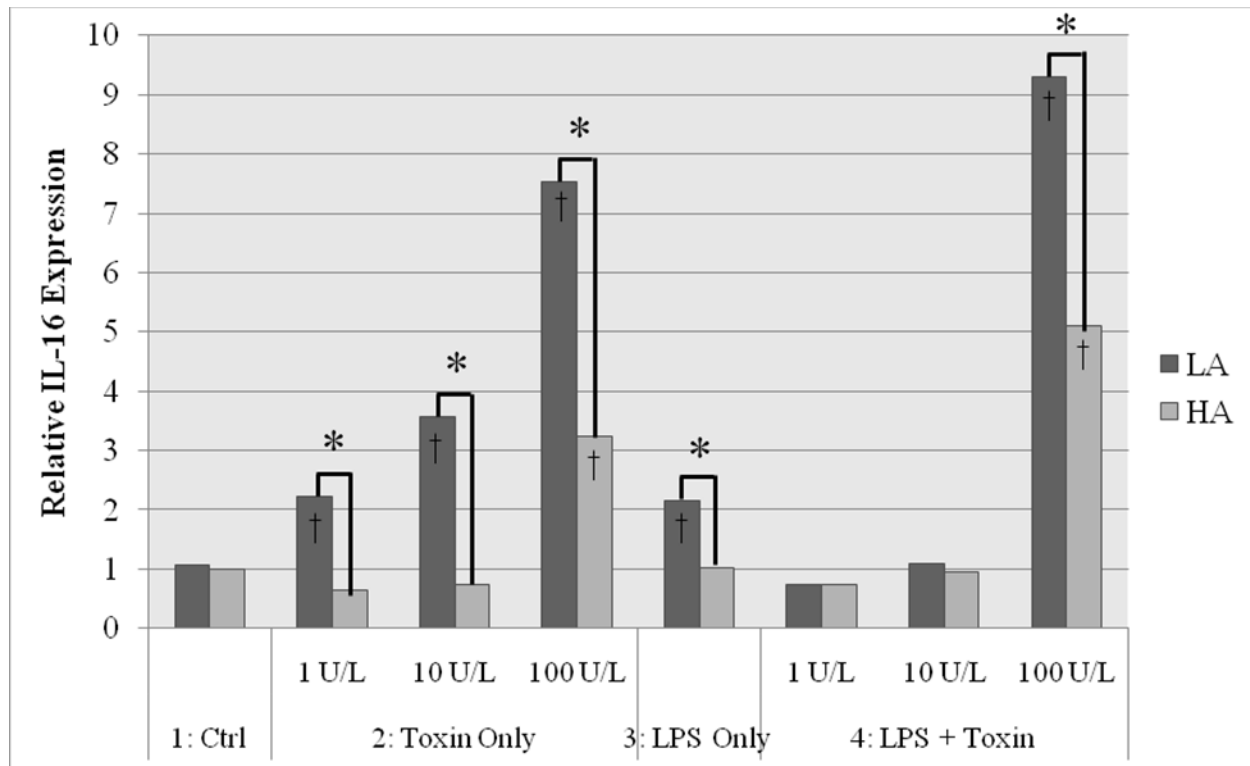


Figure 4.18. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-16 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-16 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

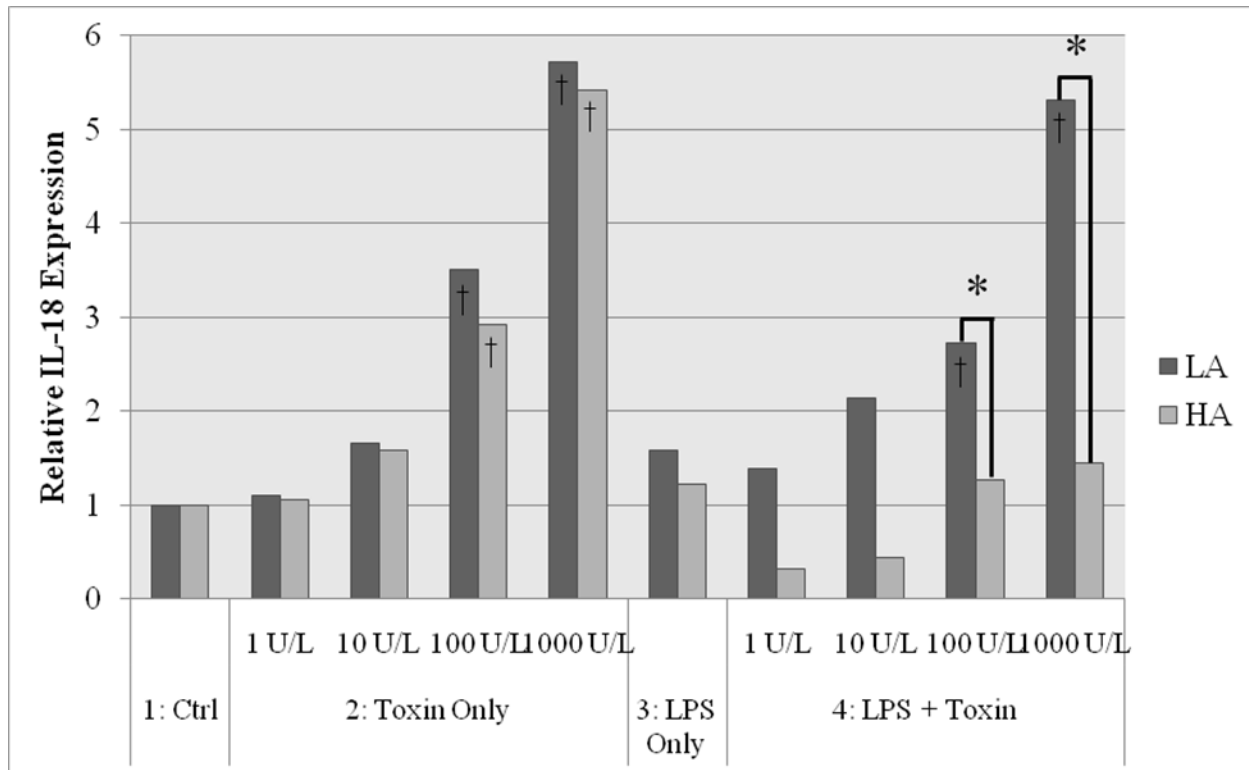


Figure 4.19. Effect of 2 h incubation with *Clostridium perfringens* α-toxin on interleukin (IL)-18 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α-toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. IL-18 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

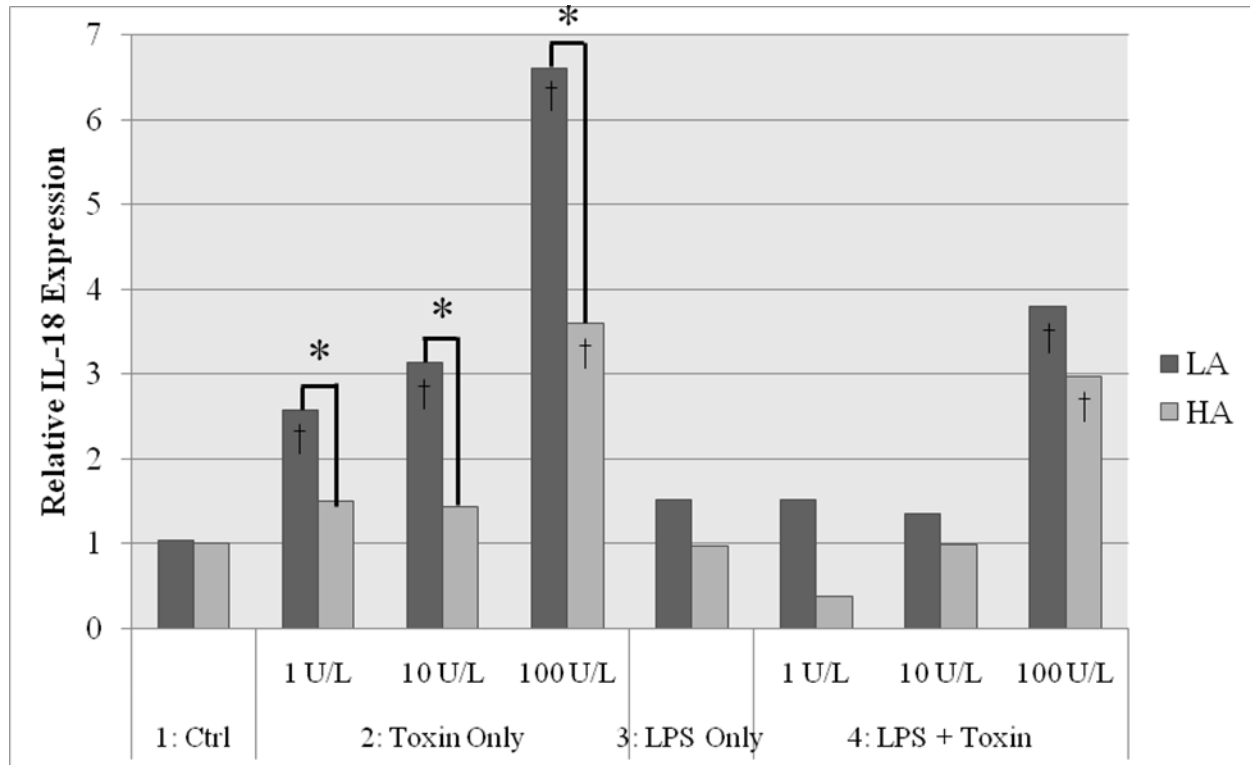


Figure 4.20. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on interleukin (IL)-18 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. IL-18 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

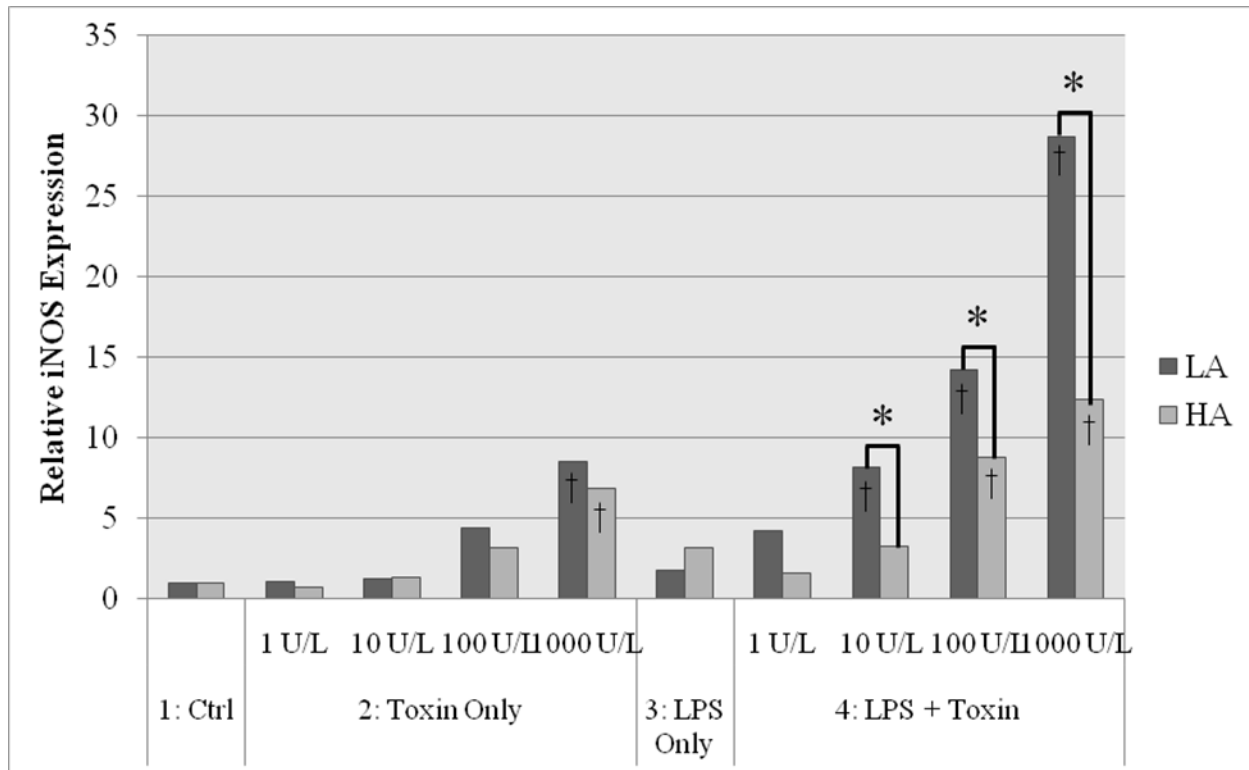


Figure 4.21. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on inducible nitric oxide synthase (iNOS) expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. iNOS expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares

(LS) means (n = 3 wells/treatment) and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

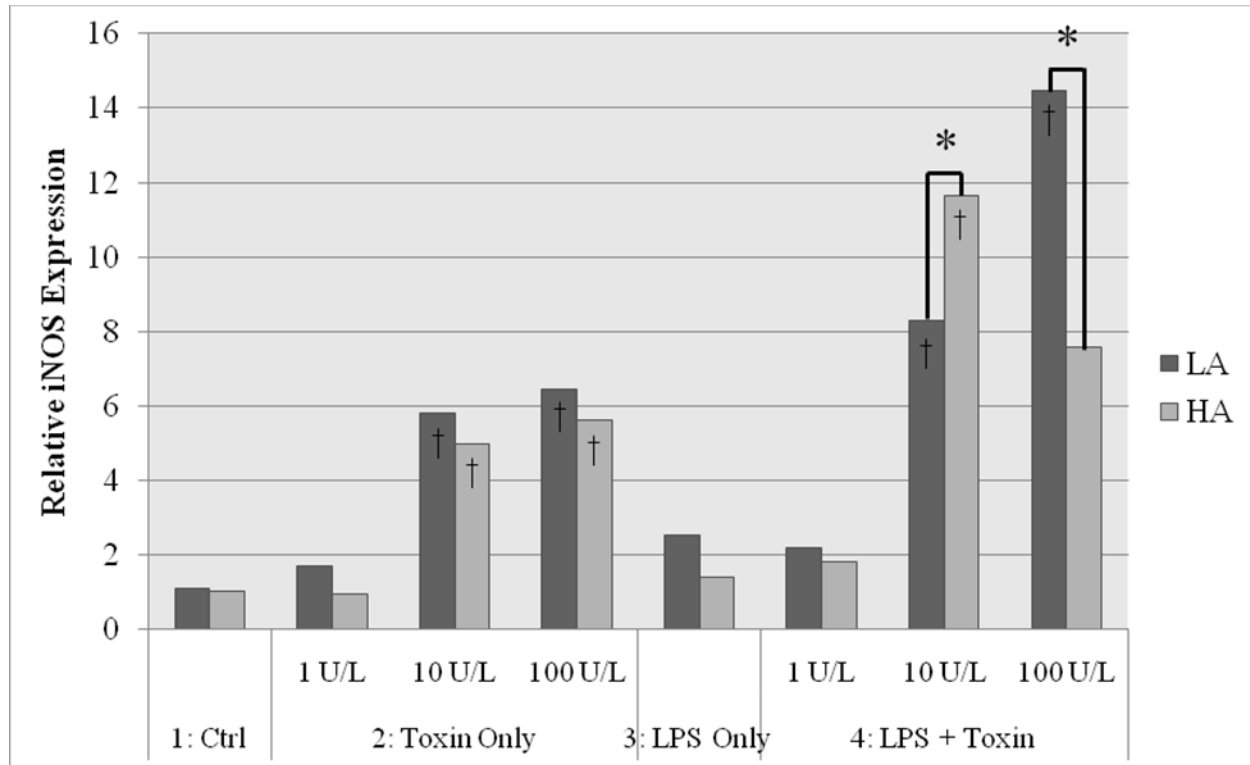


Figure 4.22. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on inducible nitric oxide synthase (iNOS) expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. iNOS expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares

(LS) means (n = 3 wells/treatment) and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

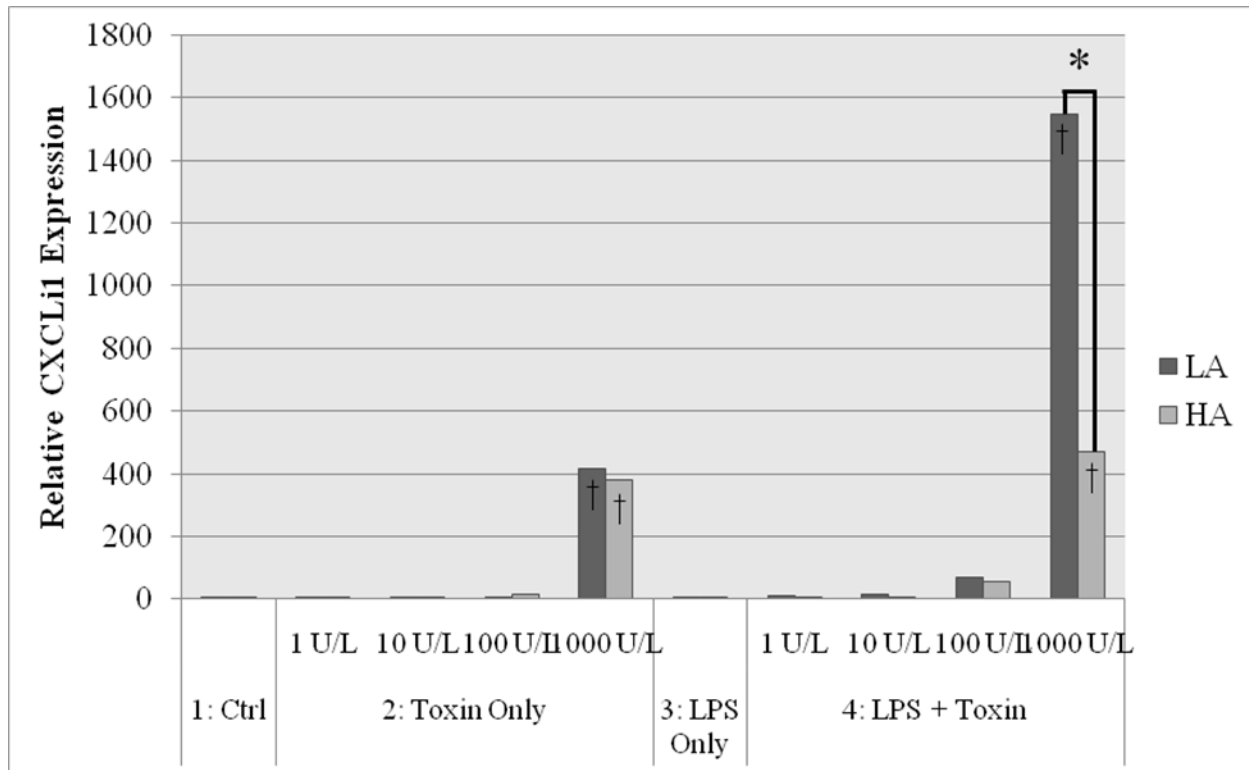


Figure 4.23. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on CXCLi1

expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. CXCLi1 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

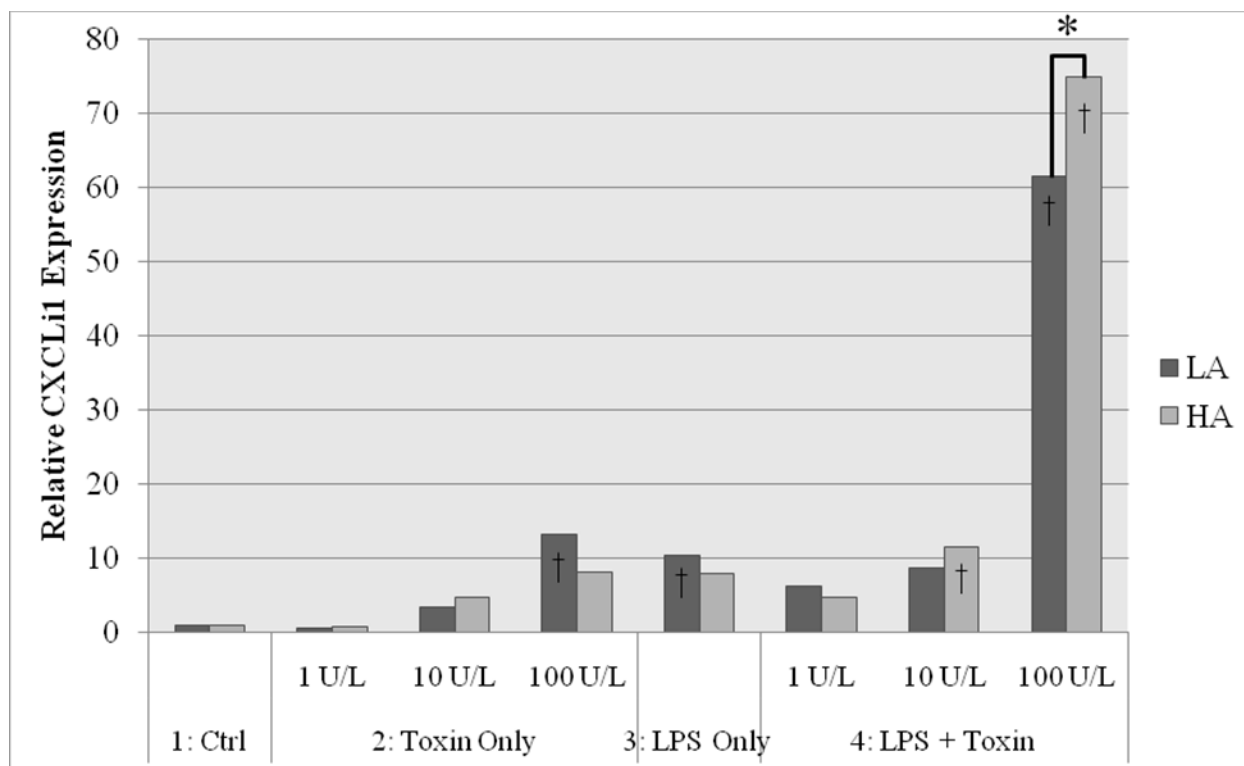


Figure 4.24. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on CXCLi1 expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. CXCLi1 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data are represented as least squares (LS) means ($n = 3$ wells/treatment)

and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

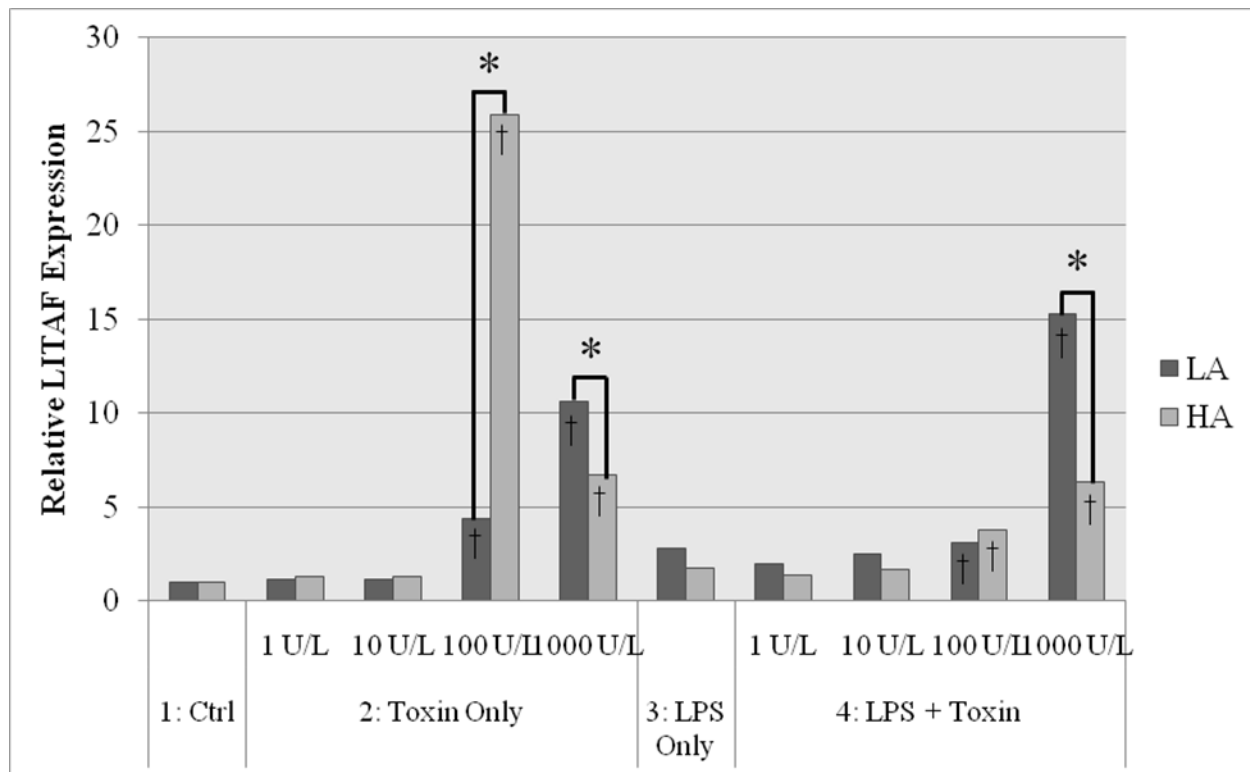


Figure 4.25. Effect of 2 h incubation with *Clostridium perfringens* α -toxin on lipopolysaccharide-induced tumor necrosis factor- α factor (LITAF) expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 2 h. LITAF expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data

are represented as least squares (LS) means ($n = 3$ wells/treatment) and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

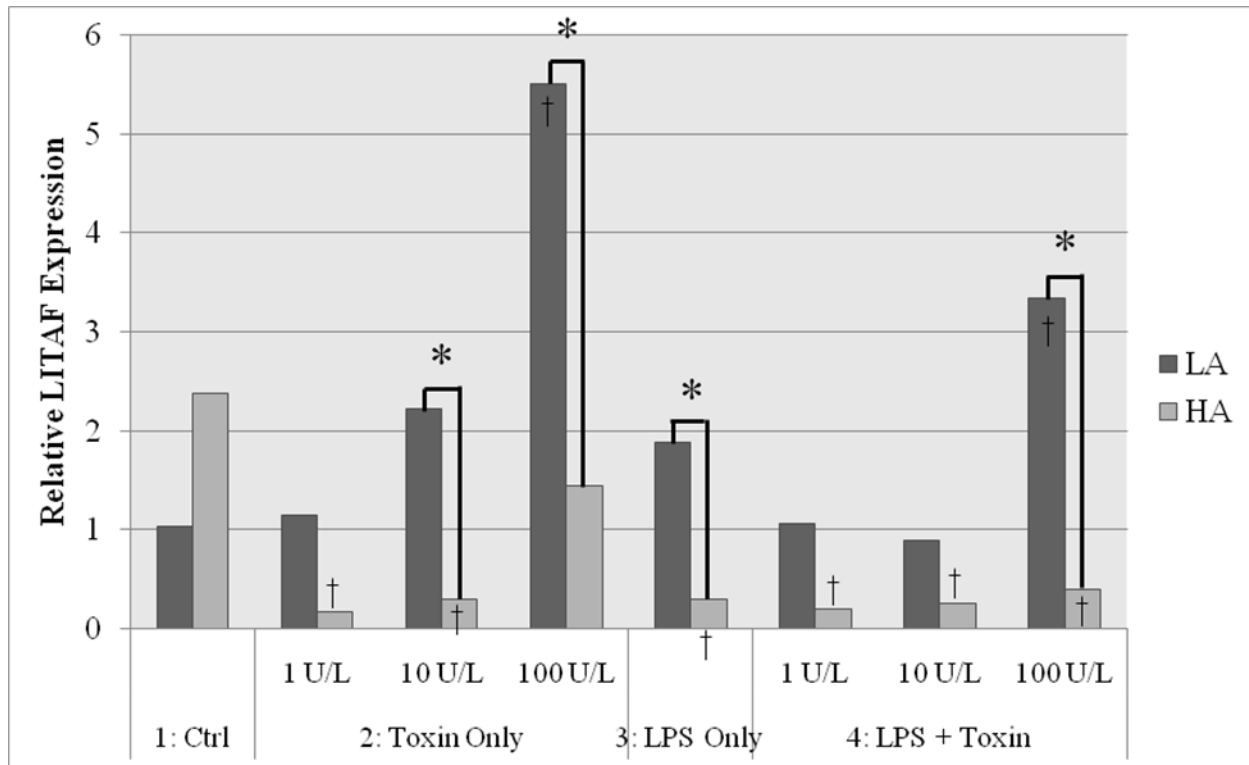


Figure 4.26. Effect of 4 h incubation with *Clostridium perfringens* α -toxin on lipopolysaccharide-induced tumor necrosis factor- α factor (LITAF) expression. Peripheral blood mononuclear cells (PBMCs) isolated from chickens genetically selected for low (LA) or high (HA) antibody response to sheep red blood cells (SRBC) were seeded into 6-well cell culture plates (5×10^5 cells/well) and assigned to one of four treatment groups: group 1, Control; group 2, Toxin Only; group 3, lipopolysaccharide (LPS) Only; group 4, LPS + Toxin. Groups 3 and 4 were stimulated with LPS for 4 h, and groups 2 and 4 were incubated with varying concentrations (1, 10, 100, 1000 U/L) of *C. perfringens* α -toxin (Phospholipase C, Type 1; Sigma Aldrich, St. Louis, MO) for 4 h. LITAF expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Relative quantitation (RQ) of gene expression was calculated using the $\Delta\Delta C_t$ method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Average ΔC_t values of group 1 served as the calibrator. Data

are represented as least squares (LS) means ($n = 3$ wells/treatment) and significance was accepted at $P < 0.05$. Asterisks represent significant difference between LA and HA cells, while crosses indicate significant difference from negative control (group 1).

Chapter V

Epilogue

Although first described 40 years ago, necrotic enteritis remains a challenging concern in the poultry industry. Even today, little is known regarding the virulence factors involved in pathogenesis of the disease, making both prevention and treatment particularly difficult. The idea of reduced susceptibility through genetic selection, particular at the MHC, is an attractive option for disease control. Breeding chickens resistant to necrotic enteritis could potentially reduce the need for AGPs, thereby lowering production costs, as well as appeasing consumers concerned about antibiotic resistance.

The results of this thesis provide further evidence of decreased susceptibility of HA chickens possessing the B²¹B²¹ MHC genotype to *C. perfringens*. Although experimental necrotic enteritis infection was not successfully established during live bird trials, ex vivo experiments using PBMCs isolated from the genetic lines were helpful in elucidating differences in immune response to *C. perfringens* α -toxin. When examining results of the cell proliferation assay, it is clear that cells isolated from the HA line displayed a superior response when primed with LPS. Similar results were seen in terms of α -toxin cytotoxicity, in that HA chickens showed decreased cell death compared to LA chickens when primed with LPS. These results provide evidence that HA chickens may possess a more advanced secondary immune response to *C. perfringens* infection, compared to the LA line. Perhaps the HA line is equipped with better pathogen recognition and memory, allowing birds to mount an exceptionally efficient immunological defense against invasion by *C. perfringens*. Although highly divergent between lines, gene expression profiles were more difficult to interpret in terms of susceptibility to *C. perfringens*. This is not surprising considering the varying results presented in recent literature.

It would be interesting to compare gene expression profiles produced by exposure to different strains of *C. perfringens*, as well as different *C. perfringens* toxins, in an attempt to explain the extreme variation commonly seen among studies.

Since *C. perfringens* α -toxin is most likely not the only mediator of necrotic enteritis infection, it would be helpful to conduct similar ex vivo experiments using additional *C. perfringens* toxins, or perhaps cell-free culture supernatant. Additionally, successfully inducing necrotic enteritis in a live bird trial would provide invaluable insight to further understanding divergent immune responses possessed by the two lines. Plans are currently underway to conduct an in vivo experiment using a recently acquired virulent strain.