Avian immunology, immunogenetics, and host immune response to Salmonella enterica serovar Enteritidis infection in chickens

Jennifer Helen Cheeseman
Iowa State University

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Avian immunology, immunogenetics, and host immune response to *Salmonella enterica* serovar Enteritidis infection in chickens

by

**Jennifer Helen Cheeseman**

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

Major: Immunobiology

Program of Study Committee:
Susan J. Lamont, Major Professor
D. L. (Hank) Harris
Eileen Thacker
Christopher Tuggle
Michael J. Wannemuehler

Iowa State University
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Investigation of immune function in chickens demonstrated a role for genetics on the percentage of immune cell subsets in peripheral blood and cytokine expression. Higher cell surface expression of CD8 was observed for Egyptian Fayoumi MHC congenic lines, M15.2, and M5.1 compared to the other chicken lines examined. A two-fold lower expression of CD3 positive T cells in peripheral blood for the Leghorn MHC congenic layer lines, G-B1 and G-B2, was observed and both Leghorn lines have increased cell surface density for CD3. Examination of early gene expression in young chicks demonstrated a clear pattern in which the Leghorn line showed higher levels of splenic mRNA for IL-10, IL-12α, CXCLi2 (IL-8 like), and CCLI2 (MIP family member) in comparison to broilers and Fayoumis. The Leghorn line had higher IL-18, but lower CCLi2, IL-12α, and IL-12β, mRNA expression in the cecum compared to broilers. Infection with Salmonella enteritidis (SE) induced the up-regulation of IL-18 and IFN-γ mRNA in the spleen and CXCLi1 and CXCLi2 (both IL-8 like) mRNA in the cecum in young chickens. Using IHC, it was noted that SE infection resulted in a decreased appearance of apoptotic cells and increased numbers of macrophages in the cecum of young birds one-week after oral inoculation. Using RNA interference (RNAi), we demonstrated siRNA-mediated knock-down of the iNOS gene in chicken macrophages. When transfected with iNOS siRNAs and later stimulated with IFN-γ, HD-11 chicken macrophages produced significantly lower nitric oxide (NO) and had decreased iNOS mRNA transcripts compared to macrophages treated with a non-sense iNOS siRNA and IFN-γ, or HD-11 macrophages stimulated with only IFN-γ. Understanding the role of genetics in shaping poultry immunology, the chicken immune response to SE infection, and use of RNAi technology to investigate avian macrophage function are the purpose of this dissertation.
Chapter 1. General Introduction

Organization of Dissertation

This dissertation arranged in the alternative format contains four chapters (Chapters 2, 3, 4, and 5) each representing individual manuscripts, which have been published, submitted for publication, or will be submitted for publication. Figures and tables included in each manuscript chapter directly follow the references section. Immediately preceding these chapters is a review of relevant literature (Chapter 1). The final chapter (Chapter 6) contains a discussion of general conclusions raised in this body of work future directions of research. Reference citations in individual chapters, although published or submitted for publication in different scientific journals, were formatted identical for continuity purposes.

Introduction

Salmonella infections, salmonellosis, are a leading cause of food-borne illness and death in the United States (Mead et al., 1999). Approximately 40,000 cases of non-typhoidal salmonellosis are reported to the Centers for Disease Control (CDC) annually (Rabsch et al., 2001); however, these infections often go unreported and current estimates of over 1 million cases may be a more accurate representation of the true incidence of infection (Rabsch et al., 2001; Schroeder et al., 2005). In the last two decades, Salmonella enteritidis replaced S. typhimurium as the leading serovar responsible for non-typhoidal Salmonella infections (Guard-Petter 2001; Rabsch et al., 2001).

As a major cause of food borne illnesses, S. enteritidis is largely associated with contaminated eggs (Humphrey 2006). Additionally, 80% of S. enteritidis (SE) outbreaks in the United States are linked to eggs and products containing eggs (Schroeder et al., 2005). Although many different serovars of Salmonella have been isolated from egg production systems, SE is the only human pathogen to routinely infect and contaminate chicken eggs (Guard-Petter 2001). According to Ebel and Schlosser 2000, 35% of U.S. flocks are SE positive, based upon data on spent hens sampled in 1991 and 1995. While generally not a
fatal illness, *Salmonella* infections do kill about 500 Americans each year, and hospitalize 15,000 (Mead *et al.*, 1999).

These statistics are compelling and have increased concerns over food safety on a national level. Additional concerns have been raised over the use of antimicrobial additives in animal feed and how this practice has increased the antimicrobial resistance of bacterial pathogens to drugs routinely used in human medicine (Anderson *et al.*, 2003; Molbak 2005).

Antimicrobial resistance of nontyphoidal *Salmonella* species has increased steadily since the 1980s, with evidence supporting the link between agricultural uses of antibiotics and antimicrobial resistance of isolates recovered from humans (Hohmann 2001; Angulo *et al.*, 2000). It has been estimated that more than 95% of all *Salmonella* infections result from ingestion of contaminated food products, and, thus, are considered food-borne (Mead *et al.*, 1999). An early 1990s survey of antimicrobial-resistance of *Salmonella* isolated from slaughtered broilers found that 57% of all isolates were resistant to one antimicrobial agent, and 45% were resistant to more than two antimicrobials (Lee, 1993 *et al.*, 1993). A more recent study of antimicrobial resistance in SE isolates from various types of sources found that approximately 90% were resistant to at least one antimicrobial drug (De Oliveira *et al.*, 2005). Furthermore, these authors found that more than 50% were multi-drug resistant, and all poultry-related samples were resistant to at least one antimicrobial agent tested.

To combat the problem of SE, which includes antimicrobial resistance to drugs, the United States has implemented a no tolerance plan for poultry production (USDA Fresh and frozen poultry products – GB16869-2005). This mandate calls for the destruction (slaughter) of entire flocks that test positive for the bacterium, regardless of the incidence rate at which a flock is colonized. These measures, while seemingly drastic, reflect the true nature of this international problem and demonstrate just one method explored to reduce (perhaps eliminate) SE from commercial poultry and the human food chain.

Other methods aimed at reducing SE in poultry include improved sanitation practices, vaccine development, breeding of genetically resistant animals, and investigating host immune responses to the infection. The latter would best describe the major direction and
focus of the current dissertation and experiments. Understanding immunological functions of poultry and their relation to SE infection in young chickens encompasses a general theme of the thesis. This work seeks to answer specific questions regarding the role of host immunity and host genetics on early SE infections in chickens. The use of varied technologies such as flow cytometry, immunohistochemistry, quantitative PCR adds depth to this dissertation. Additionally, this dissertation presents the first reported use of RNAi methods to investigate avian macrophage function.

**Literature Review**

**The Immune System of Chickens: An Overview**

Scientific research on poultry immunology and the diseases affecting avian species is not a new concept. Marek’s disease research has been well documented for many years since the early twentieth century in effort to understand this virally induced cancer (Osterrier *et al.*, 2006). The groundbreaking discovery that host genetics, namely the MHC, greatly influenced Marek’s disease susceptibility is a highlight of the many contributions of poultry immunogenetics research to the general body of immunological knowledge. Additionally, the studies of B cell development in birds and of the role the thymus in T cell immunity helped to lay the foundation for understanding the dual nature of the immune system (Glick *et al.*, 1956; Cooper *et al.*, 1966).

More recently, the chicken was the first agricultural species for which a genome sequence map was published (Wallis *et al.*, 2004). This information has allowed for an in depth analysis and identification of immunological genes in the chicken such as cytokines and chemokines (Kaiser *et al.*, 2005). When compared to mammals, chickens generally have fewer genes for the various classes of interleukins, interferons, and chemokines. For example chickens have alpha, beta, and gamma interferons, and IL-1β, but not IL-1α or any of the other IL-1 family genes. Birds have four IL-10 family members, four IL-17 family members, GM-CSF, and two TGF-β genes. The T cell proliferation cytokines, IL-2, IL-15, and IL-21 are present as well as members of both Th1 and Th2 gene families. Although
defining the precise function of chicken chemokines will require more experimentation, genes representing the major classes (CXCL, CCL, XCL, and CX3CL) have been identified. Of special interest, chickens do not have well-developed lymph nodes or functional eosinophils and the genes lymphotaxin and eotaxin(s), respectively, which are involved with these tissues/cell types have not been identified in the chicken genome (Kaiser et al., 2005). Additionally, the chicken genome contains more B cell chemokines than have been identified in mice or humans. This may reflect the inherent differences and greater complexity in B cell development between mammals and avian species.

Chickens, like mammals have both humoral and cell mediated arms of the adaptive immune system (Erf 2004; Scott 2004; Sharma 1991). The bursa of Fabricius is essential for normal B cell development and humoral immunity in birds, as evident in the pivotal work by Glick et al., 1956 where bursectomized chickens failed to produce antibody responses to immunization. Embryonic stem cells migrate to the bursa and undergo rapid proliferation, which persists for several weeks after hatching. These precursor B cells have already rearranged their immunoglobulin genes prior to entering the bursa. Compared to mammalian species, the chicken contains a very limited number of variable genes. Chickens use a process termed gene conversion to create antibody diversity, where the variable heavy and light chains are replaced with upstream pseduogenes (Benatar et al., 1992). While only one variable (V) light chain, one joining (J) light chain, one V heavy chain, one J heavy chain, and sixteen D heavy chains are initially available for gene rearrangement, approximately 25 V light and 80 V heavy pseudogenes can be inserted to create antibody diversity, which occurs in the bursa.

The humoral immune system of the chicken functions in many of the same ways as in mammals. Affinity maturation and secondary responses are evident (Maldonado et al., 2005) as well as antibody mediated complement activation (Parmentier et al., 2002). Chicken antibodies are involved in agglutination reactions (Gast 1997) and are passed from hen to chick via the egg (Hamal et al., 2006). Class switching occurs (Yasuda et al., 2003) and secretory IgA helps to protect the gastrointestinal tract (Wieland et al., 2004) as in mammals.
The cell mediated immune system in poultry includes many of the well known mammalian counterparts. Chickens have both αβ and γδ T cells and natural killer (NK) cells (Erf 2004; Sowder et al., 1988; Myers and Schat 1990; Chai and Lillehoj 1988). Chicken natural killer cells have been shown to be potent killers of tumors and virally infected cells (Chai and Lillehoj 1988; Myers and Schat 1990). The γδ T cells isolated from IL-2 stimulated splenic cultures also show anti-tumor activity (Choi and Lillehoj 2000). Both CD8 and CD4 T cell subsets are present in the chicken, and it has been well established that Th1 CD4 T cells in the chicken function similarly as in humans and mice (Gobel et al., 2003; Chan et al., 1988). Until recently, the existence of Th2 cells had been speculative; however, the discovery of the Th2 cytokine gene cluster (Avery et al., 2004; Kaiser et al., 2005) provided evidence for the functional existence of these cells in poultry as well. These observations are further supported by the observations that chickens are able to induce Th1-biased immune responses important in intracellular infections as well as produce a Th2 directed response to extracellular pathogens like helminths (Degen et al., 2005a).

**Avian Immune Function: Cytokines**

Knowing what specific immune molecules are encoded in the chicken genome provides an excellent framework to build and expand our knowledge on the functional roles of these molecules. As stated previously, chickens possess many of the same cytokines, chemokines, found in mammals. Type 1 interferons, which include IFN-α and IFN-β, in chickens, are important in viral immune responses (Schultz et al., 2004). In chickens, both molecules have been found to be potent antiviral cytokines, but lack the ability to induce macrophage activation (Schultz et al., 1995). This MAF (macrophage-activating factor) activity is attributed to Type 2 IFN or IFN-γ. Chicken IFN-γ activates macrophages to upregulate MHC class I & II expression and enhances NO production through de-novo synthesis of iNOS (Weining et al., 1996; Song et al., 1997; Kaspers et al., 1994). Chicken IFN-γ mRNA is upregulated in response to Marek’s disease virus indicating a role for this cytokine in viral and intracellular diseases (Kaiser et al., 2003; Liu et al. 2001).
Additionally, IFN-γ mRNA expression was enhanced in the intestines of chickens after challenge with *Eimeria maxima* (Hong *et al*., 2006).

Despite a low level of sequence identity to mammalian cytokines, chicken cytokines display similar biological activity in functional studies. Chicken macrophages rapidly produce IL-1β in response to lipopolysaccharide (LPS) stimulation (Weining *et al*., 1998). The secreted IL-1β proinflammatory protein was found to stimulate production of CXC chemokine (later determined to be IL-8 like) mRNA by chicken fibroblasts, and IL-β mRNA expression is upregulated following *E. maxima* infection in the intestines of chickens (Hong *et al*., 2006). Chicken IL-1β has also been implicated in Marek’s disease with enhanced mRNA expression in infected birds one to two weeks after infection (Xing and Schat 2000).

Another cytokine with proinflammatory activity is IL-18, which has been shown to induce IFN-γ production in chicken spleen cells and acts as a CD4 T cell growth factor (Puehler *et al*., 2003; Gobel *et al*., 2003; Schneider *et al*., 2000). Chicken IL-18, when injected with an immunogen, also appears to play a role in antibody-mediated immune responses as it acts as an adjuvant and enhances humoral response to various vaccines (Degen *et al*., 2005b). Chicken macrophages stimulated with infectious bursal disease virus have elevated levels of IL-18 mRNA transcripts, which suggest chicken macrophages play a role in proinflammatory responses to viral pathogens by producing IL-18 (Palmquist *et al*., 2006). Interleukin-18 may also play a role in the pathogenesis of Marek’s disease as mRNA for this cytokine is upregulated in brain tissues of infected birds, and animals showing obvious clinical signs of the disease (paralysis) had higher levels of IL-18-specific mRNA when compared to infected birds with no clinical disease (Abdul-Careem *et al*., 2006).

Interleukin-12 through the induction of IFN-γ promotes Th1 immune responses and links adaptive and innate immunity (Trinchieri 2003). A heterodimeric cytokine, chicken IL-12, requires both the subunits (p35 and p40) for biological activity like mammalian counterparts (Degen *et al*., 2004). Recombinant chicken IL-12 stimulates the release of IFN-γ by spleen cells as measured by the production of NO, and is able to induce proliferation in chicken T cells (Degen *et al*., 2004). IL-12 mRNA was produced in several different cell
types in response to various stimulating agents, such as macrophages responding to LPS or CpG, splenocytes responding to ConA, B cell responding to LPS, and T cells responding to PMA (Degen et al., 2004). Interleukin-12 is involved in the immune responses to several poultry diseases. Enhanced IL-12 mRNA was observed in the brain tissues of chickens infected with Marek’s disease (Abdul-Careem et al., 2006) and in intestinal intraepithelial lymphocytes after infection with *E. maxima* (Hong et al., 2006).

Interleukin-6 (IL-6) has many immunological functions including hematopoiesis, induction of acute phase reactions, and regulation of immune responses (Taga and Kishimoto, 1997). It has also been established that IL-6 can modulate Th1/Th2 responses by inhibition of Th1-induced differentiation (Diehl et al., 2000). Recombinant chicken IL-6 was found to increase serum corticosterone levels at 2 and 4 hours after injected into chickens (Schneider et al., 2001), thus showing IL-6 has a role in acute phase responses in the chicken. After LPS stimulation, chicken macrophages (HD-11 line) produced IL-6 mRNA with optimal IL-6 mRNA level obtained at 4 and 8 hours post-stimulation, and recombinant IL-6 induced proliferation in IL-6 dependent hybridoma cells in a dose dependant manner (Nishimichi et al., 2005). Using the murine IL-6 7TD1 bioassay, serum from *Eimeria* infected mice and chickens both exhibited IL-6 activity (Lynagh et al., 2000). Additional studies have provided a role for IL-6 in chicken immune responses to bacterial and viral pathogens. When stimulated with a hemagglutinin from *Mycoplasma synoviae*, chicken macrophages produced IL-6 and IL-1β (Lavric et al., 2006). Immunization with a chicken IL-6 plasmid increased protection against infectious bursal disease virus challenge in chickens (Sun et al., 2005).

Interleukin-10 is generally characterized as a Th2 promoting cytokine because it inhibits IL-12 transcription and suppresses proinflammatory responses of antigen presenting cells (Mocellin et al., 2003; Trinchieri, 2003). Chicken IL-10 appears to function in a similar manner. Recombinant chicken IL-10 was able to suppress IFN-γ production in lymphocytes activated with concanavalin A (ConA) or phytohaemagglutinin (PHA) mitogen (Rothwell et al., 2004). Infections with both *E. maxima* and *E. acervulina* resulted in increased expression of IL-10 mRNA (Hong et al., 2006; Rothwell et al., 2004).
The chicken genome contains three IL-8 like CXC chemokines that are denoted as CXCLi1, CXCLi2, and CXCLi3 (Kaiser et al., 2005). CXCLi1, also known as K60, and CXCLi2, previously known as IL-8 or CAF, have been studied in more detail than CXCLi3. Although the precise activity of the IL-8 like chemokines remains to be elucidated, there appears to be some similarity in function to mammalian IL-8 such as cell recruitment and promoting inflammatory processes (Gangur et al., 2002). Heterophils produce IL-8 in response to both opsonized and nonopsonized SE (Kogut et al., 2003a). Additionally, IL-8 mRNA was expressed in a chicken macrophage line, HD-11, after stimulation with either IL-1β or IFN-γ (Sick et al., 1999). Chicken IL-8 (CXCLi2) has also been demonstrated to induce NO production and iNOS mRNA expression in osteoclast-like cells (Sunyer et al., 1996). Interleukin-8 expression appears to be influenced by the type of cell that is stimulated, as Mycoplasma gallisepticum inhibits IL-8 mRNA expression in MSB-1 cells (a lymphoblastoid cell line), but enhances gene transcription in the chicken macrophage line, HD-11 (Lam 2004). Interestingly, CXCLi2 (IL-8) but not CXCLi1 (K60) expression was enhanced in the intestinal tissues following *E. maxima* infection, suggesting different regulatory pathways and or function for these similar chemokines (Hong et al., 2006).

Mammalian IL-8 acts on primarily neutrophils to recruit them towards the site of infection and inflammation (Gangur et al., 2002). Chicken IL-8 (CXCLi2) appears to function in a similar manner, but targets cells of the monocytes and macrophage lineage instead of heterophils (the avian neutrophil) as the N-terminus region is similar in structure to mammalian MCP-1 which attracts human monocytes (Martins-Green 2001). Chicken CXCLi2 also is involved in angiogenesis and wound healing (Martins-Green and Feugate 1998).

Initially described as MIP-1β, based upon sequence similarity to human MIP-1β, chicken CCLi2 is now postulated to be one of several MIP family chemokines found in the chicken genome (Kaiser et al., 2005). The exact function of CCLi2 and if it is the chicken homologue of human MIP-1β require further investigation; however, an experiment has shown that this chemokine is produced in response to *M. gallisepticum* infection and induces
migration of chicken heterophils and lymphocytes (Lam 2002). Chicken CCLi2, also known as K203, is induced in macrophages and fibroblasts with the proinflammatory cytokine IL-1β or the Th1 molecule IFN-γ (Sick et al., 1999). S. typhimurium infection of young chickens induced MIP-1β mRNA expression in the liver and intestinal tissues (Withanage et al., 2004), indicating a role for this chemokine in bacterial infections of birds.

Chicken antimicrobial peptides, Gallinacins, are small cysteine rich molecules produced by heterophils and epithelial cells (Sugiarto and Yu 2004; Brockus et al., 1998; Harwig et al., 1994; Lynn et al., 2007). Biologically active against many microbes, such as Escherichia coli, Staphylococcus aureus, Campylobacter jejuni, and Listeria monocytogenes, chicken antimicrobial peptides are believed to play a role in innate immunity in avian species (Harwig et al., 1994; Evans et al., 1994; Evans et al., 1995). Unlike mammalian neutrophils, chicken heterophils lack myeloperoxidases and it has been suggested that Gallinacins are more important in innate avian immunity because of this absent mechanism (Sugiarto and Yu 2004).

**Cytokines and Salmonella Infections: Mice and Men**

Protection against and ultimate clearance of intracellular pathogens, like Salmonella, requires a strong cellular Th1-mediated immune response (Stoycheva et al., 2004; Mastroeni 2002). The prototypical cytokine involved in Th1 immune responses is IFN-γ which activates macrophages for more effective immune responses such as phagocytosis and antigen processing, production of proinflammatory cytokines, reactive oxygen and nitrogen species production, and enhanced MHC class II cell surface expression (Paulnock 1992; Gordon and Taylor 2005). In addition to IFN-γ, IL-12 and IL-18 play essential roles in promoting Th1 cellular immune responses to intracellular pathogens (Garcia et al., 1999; Chehimi and Trinchieri 1994). Activated macrophages produce both IL-12 and IL-18 which induces T cell production of IFN-γ, thereby creating a loop-back amplification mechanism that promotes the activation of other macrophages and Th1 immune responses (Sugawara 2000; Trinchieri 2003).
Other molecules such as proinflammatory cytokines and chemokines also play a role in controlling intracellular bacterial infections by recruiting immune cells to the site of infection and promoting effective lymphocyte reactions (Gangur et al., 2002; Kunkel et al., 2003; Matsukawa et al., 2000; Peters and Ernst 2003; Imhof and Dunon 1997). These molecules provide an essential communication bridge between the innate and adaptive immune systems. In addition to connecting both the adaptive and innate aspects of the immune system, these molecules provide the initial warning signs to alert the immune system to potential threats.

Interleukin-1β, produced by macrophages and epithelial cells in response to various stimuli like LPS, is an inducer of both T cell and macrophage activation (Janeway et al., 2001; Murtaugh and Foss 2002). Interleukin-6, known to be produced by macrophages, T cells, and endothelial cells, acts to promote both B and T cell differentiation and acute phase responses. TNF-α, produced by macrophages, T cells, and NK cells, promotes localized inflammation and endothelial cell activation that allows greater access of immune cells and other mediators like antibodies to tissues at the site of infection. As part of the acute-phase response to infection IL-1β, along with IL-6 and TNF-α, promotes fever, which is thought to aid immune responses by reducing pathogen replication, stimulating acute phase protein production important in complement responses, and enhancing adaptive immune responses (Janeway et al., 2001; Gabay 2006; Gruys et al., 2005).

Chemokines are small molecules that are categorized based upon the spacing of one or more cysteine residues at their amino-terminal end, and, in a broad sense, function to recruit various immune cells to the site of infection or tissue damage. Cells produce chemokines in response to numerous extrinsic (bacteria, viruses) or intrinsic (cytokines) stimuli. While chemokines can be produced by many different cell types, including monocytes, T cells, B cells, fibroblasts, dendritic cells, and stromal cells, their target cell types are generally very specific. Interleukin-8 is produced by monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells, and epithelial cells to attract neutrophils and naïve T cells. The MIP family of chemokines, which include MIP-1α and MIP-1β, is
produced by monocytes, macrophages, T cells, neutrophils, mast cells, and T cells and attract dendritic cells, monocytes, NK cells, and T cells.

The involvement of cytokines during *Salmonella* infections is well established from studies utilizing knock-out mouse strains, clinical observations of cytokine and/or cytokine receptor deficient humans, and established cell lines (Eckmann and Kagnoff 2001; Lalmanach and Lantier 1999; van de Vosse et al., 2006; Rosenzweig and Holland 2005; Picard and Casanova 2004; Kovanen and Leonard 2004). Briefly, intestinal epithelial cells produce both mRNA and protein for TNF-α, IL-8, IL-6, GM-CSF, and G-CSF following exposure to various *Salmonella* species such as *S. dublin* and *S. typhi* (Eckmann et al., 2000; Jung et al., 1995; McCormick et al., 1993; Weinstein et al., 1997). Mouse and human macrophages have been shown to produce mRNA and secrete IL-1β, TNF-α, IL-6, and IL-12 in response to *S. typhimurium* (ST), *S. dublin*, or LPS (Bost and Clements 1997; Rosenberger et al., 2000; Galdiero et al., 1993; Ciacci-Woolwine et al., 1998; Yamamoto et al., 1996; Wyant et al., 1999).

In animal models of disease, which more accurately reflect the dynamic host-pathogen interactions than in-vitro cell studies, numerous cytokines have been determined to be intimately involved in the immune response to *Salmonella* (Eckmann and Kagnoff 2001; Lalmanach and Lantier 1999). For example, mice infected with ST then treated with IFN-γ have lower bacterial levels in the spleen and liver as well as higher survival rates than untreated mice (Eckmann and Kagnoff, 2001; Matsumura et al., 1990). Other studies have shown that neutralization of IFN-γ reduces survival rates in mice (Gulig et al., 1997; Nauciel and Espinasse-Maes 1992) and IFN-γ knock-out mice have higher bacterial burdens in both the spleen and liver compared to wild-type mice (Bao et al., 2000).

Interleukin-12 and IL-18, both part of the classical Th1-mediated immune response, play very important roles in *Salmonella* infections in mice models (Eckmann and Kagnoff 2001). Cytokine treatment of infected mice with IL-12 or IL-18 reduced mortality and IL-18 also reduced bacterial numbers within the spleen and liver (Eckmann et al., 1996; Mastroeni et al., 1999; Kincy-Cain et al., 1995; Dybing et al., 1999). Neutralization of either cytokine
resulted in increased bacterial colonization of the spleen and decreased animal survival rates (Mastroeni et al., 1999; Dybing et al., 1999; Kincy-Cain et al., 1996).

Chemokine production in response to Salmonella infection has been established in both epithelial cell lines and macrophages (Eckmann and Kagnoff, 2001). Interleukin-8 mRNA expression and secretion of the protein in response to Salmonella infection has been demonstrated in human epithelial cell lines (Eckmann and Kagnoff, 2001; Eckmann et al., 1993; Jung et al., 1995). Other chemokines, including members of both C-C and C-X-C families, such as macrophage inflammatory protein 1 beta (MIP-1β), monocyte chemotactic protein 1 (MCP-1), growth related oncogene alpha (GROα), and growth related oncogene gamma (GRO-γ) are implicated in Salmonella infections as mRNA expression for these molecules is up-regulated in infected epithelial cell lines (Jung et al., 1995; Eckmann et al., 2000; Yang et al., 1997). Mouse macrophages have also been shown to produce chemokines like MIP-1α, MIP-1β, and MIP-2 in response to ST infection (Rosenberger et al., 2000; Yamamoto et al., 1996).

Cytokines and the Immune Response to Salmonella Infections: Chickens

Numerous studies have clearly illustrated a role for cytokines and chemokines in Salmonella infections in mammals, but somewhat less clearly understood is the role of avian cytokines on the host response to intracellular pathogens such as SE. However, recent investigations do show an important role for these immune molecules in poultry diseases (Abdul-Careem et al., 2007; Dalloul et al., 2007; Li et al., 2007; Hong et al., 2006). Whether evaluating gene expression with QPCR, global gene expression using microarray, in-vitro cell lines, or use of recombinant avian cytokines to stimulate in vitro cellular immune responses, it is clear that cytokines are intimately involved in the avian host immune response to Salmonella infection.

Expression of IL-1β, IL-2, IL-6, and IFN-γ cytokines and IL-6 and IFN-γ protein activity were evaluated following in-vitro exposure of chicken kidney cells (CKC) to S. enteritidis, S. gallinarum, and ST (Kaiser et al., 2000). Culture with any of the three
Salmonella species did not influence mRNA expression of IFN-γ in chicken kidney cells, however, IFN-γ-like activity was inferred based upon the production of nitric oxide by HD-11 macrophage cell line. As measured by proliferation of 7TDI cells, IL-6 mRNA expression and IL-6-like activity in the culture supernatants was increased in response to all of the Salmonella species investigated compared to supernatants collected from uninfected CKC controls (Kaiser et al., 2000). S. enteritidis infected chicken kidney cells (CKC) produced significantly less IL-2 compared to control CKC cultures, and neither S. gallinarum nor ST elicited differences in IL-2 production. In comparison to control cell cultures, differential induction of IL-1β expression was only observed in response to the addition of ST to the culture medium (Kaiser et al., 2000). The chicken kidney cell culture models epithelial cell interactions with Salmonella bacteria and demonstrated cytokine expression in response to various Salmonella species and illustrated an important aspect that cytokine production responses appeared species specific.

Examination of the early cytokine expression in the internal organs of newly hatched chicks infected with ST demonstrates the involvement of avian cytokines in local immune responses to Salmonella infections (Withanage et al., 2004). Within 6 to 12 hours following Salmonella infection, IL-8 and K60 (both avian CXCLi chemokines) mRNA expression is up-regulated in the intestines (jejunem and ileum), liver, and cecal tonsils in the young birds. Enhanced expression of the MIP family chemokine, MIP-1β was also shown at 12 and 48 hours post-infection (PI) in the cecal tonsils and ilea, and at 12 and 24 hours PI in the liver of infected chicks. The proinflammatory cytokine IL-1β was demonstrated to be involved in the acute phase immune response of young chicks to Salmonella infection (Withanage et al., 2004). Interleukin-1β mRNA expression was increased, compared to uninfected chicks, in the ilea at 12, 24, and 48 hours and in the cecal tonsils throughout the duration of the study (6 to 48 hours). Enhanced IL-1β mRNA expression in the liver and the spleen of infected chicks at latter time points 24 and 48 hours PI, respectively. The expression of chemokines and IL-1β, a proinflammatory cytokine, in the intestines and other internal organs like the
spleen, liver, and cecal tonsils of young chicks indicate these molecules are important in the early immune responses of birds to ST infection.

Primary and secondary immune responses to ST infection in young chickens indicated that cytokines, chemokines, antibody production, and Th1 cellular immune responses played a critical role in controlling gastrointestinal Salmonella infections of poultry (Withanage et al., 2005). Primary immune responses to Salmonella infection generally followed three stages. In the first two weeks of ST infection, increased IFN-γ mRNA expression was observed in the liver, ileum, and cecal tonsils of young chicks, and in response to ST infection, both the cecal tonsil and ileum expressed IL-6 mRNA from 3 to 4 weeks post infection in young chickens. Compared to non-infected animals, expression of transforming growth factor β4, an anti-inflammatory cytokine, increased at one-week post-infection in the liver, spleen, ileum, and cecal tonsils likely indicating a mechanism to control local inflammation and tissue damage. Salmonella specific antibody production (IgM, IgG, and IgA serotypes) in serum increased from 10 days post infection and reached maximum levels at approximately 30 days after oral ST infection.

Secondary immune responses to ST infection in young chicks were characterized by more robust and rapid production of IgG and IgA anti-Salmonella antibodies compared to antibody production observed during the primary immune response (Withanage et al., 2005). Interleukin-6 mRNA expression during the primary immune response was only observed at two weeks post infection in the ileum and cecal tonsils, whereas during the secondary immune response to ST infection, IL-6 expression was rapidly observed from one to three days post infection. The MIP-1β mRNA expression in the cecal tonsil and ileum during the secondary immune response was more substantial and occurred more rapidly than during the primary immune response to ST infection in young chicks.

Although not as extensively studied compared to mice and mammalian cell culture models, recombinant chicken cytokines have been utilized in experiments including Salmonella infections. Recombinant chicken IFN-γ has been shown to induce NO production in the chicken macrophage cell line, HD-11 (Lillehoj and Li 2004). Chicken
heterophils, akin to the mammalian neutrophil, have been shown to produce IL-1β, IL-6, and IL-8 (proinflammatory) mRNA and IFN-γ and IL-18 (Th1) mRNA in response to SE phagocytosis when primed with recombinant chicken IFN-γ (Kogut et al., 2005). In a similar experiment, chicken heterophils primed with recombinant chicken IL-2 were observed to produce IL-8 and IL-18 mRNA in response to SE phagocytosis (Kogut et al., 2003b).

Intraperitoneal (IP) injection of *S. enteritidis*-immune lymphokines (SEILK) was shown to be protective in very young chickens and turkeys by increasing resistance to SE organ invasion in the birds (Genovese et al., 1998). Additional experiments demonstrated the activity of these SEILK’s was not limited to SE, but also reduced liver invasion by ST indicating the non-specific generalized protection against *Salmonella* infection (Ziprin and Kogut 1997). Resistance to SE organ invasion conferred by the SEILKs correlated to an influx of heterophils to the site of administration (Kogut et al., 1994), demonstrating the ability of SEILKs to attract inflammatory cells through the production of IL-8, as antibody mediated neutralization of this chemokine abrogated heterophilic chemotaxis (Kogut 2002).

*Salmonella enteritidis* vaccinated chickens have been shown to produce higher serum levels of IL-2 and IFN-γ after both primary and secondary SE immunizations (Okamura et al., 2004). The enhanced serum cytokine production was observed in both young chicks vaccinated at 4 week of age and mature chickens vaccinated at 8 months. Enhanced serum IFN-γ levels in both age groups were observed at 7 to 14 days following primary immunization, while IFN-γ serum levels were significantly higher as early as 3 days after secondary immunization. Interleukin-2 serum levels were elevated from 11 to 14 days post-immunization, occurring later in the immune response compared to IFN-γ (Okamura et al., 2004). Chicks vaccinated at three weeks of age demonstrated higher serum levels of IL-1β, IL-6, IL-8, and IFN-γ after primary immunization relative to uninfected control birds, suggesting a role for these cytokines, along with IL-2, in the avian immune response to *S. enteritidis* infection and to SE vaccination.

**Genetics and Immune Function**
Considerable evidence suggests that genetic variation in immune cell compartments exists and may contribute to differences observed in disease resistance. The MHC of mice has been associated with controlling the size of the CD4 compartment in the thymus (Duarte et al., 2001). The BALB/c strain of mice was found to have a larger proportion of CD4 cells in the thymus compared to the C57BL/6 strain, and this was attributed to decreased efficacy of T cell maturation from double positive (CD4 CD8) to single positive (CD4) T cell lymphocytes in the latter strain (Duarte et al., 2001). Another study determined that genetic differences in lineage commitment of thymocytes and not selection caused the variation in CD4 and CD8 T cell populations in mice (van Meerwijik et al., 1998). Additionally, MHC genes are known to determine the peripheral T cell ratio (CD4/CD8) in rats (Damoiseaux et al., 1999).

Genetics is also involved in determining the T cell populations of poultry. Congenic lines of chickens, which differ only in the MHC region, have been found to have striking differences in the T cell compartments of peripheral blood lymphocytes (Hala et al., 1991). The CB line had more CD4$^{+}$ T-cells but fewer CD8$^{+}$ T-cells than the CC line of chickens. These results were later extended to splenic cell populations in both lines (Hala et al., 1992). While genetically similar, except for their respective MHC regions, the CB and CC lines respond very differently to Rous Sarcoma virus (RSV) infections. The CB line has been described as a tumor regressing line, while in the CC line tumors induced by the virus progress (Hala et al., 1991; Hala et al., 1992). Although not clearly understood, the differences in the T cell populations, due to genetic variation in the MHC region, may contribute to the observed differences in RSV tumor progression in the lines.

Because of production-oriented genetic selection in broiler chickens, unintended changes in basic immune responses have been observed (Qureshi and Havenstein 1994; Cheema et al., 2003). A comparison made between a 1991 commercial broiler line to a random-bred 1957 strain found that genetic selection for broiler performance traits negatively influenced the antibody response to sheep red blood cells (SRBC), but did not affect macrophage or NK cell functions (Qureshi and Havenstein 1994). A more recent comparison
made between the same 1957 random-bred strain with a 2001 commercial broiler line found similar results. The 2001 broiler line, selected for production traits of economic importance, had diminished antibody titers in response to SRBC indicating the adaptive arm of the chicken immune system was negatively influenced by the imposed genetic selection pressures (Cheema et al., 2003). Cell-mediated and innate immune responses, measured by lymphoproliferation and phagocytosis, respectively, were actually more robust in the modern 2001 commercial broiler line suggesting that genetic selection for certain production traits can have both negative and positive influences on immune responses of poultry.

Additional work utilizing turkeys has suggested that selection for increased body weight, which mirrors the selective pressures applied in modern poultry breeding programs, has also influenced immune system parameters of these animals (Li et al., 1999). At 16 weeks of age, turkeys from a line selected for increased body weight (F line) were observed to have a larger percentage of CD4 T cells in the peripheral blood when compared to a randombred control line and two commercial sire lines. Because the F line birds are more susceptible to infectious diseases, the authors speculated that this may be due to the increased CD4 T cell population observed in this turkey line (Li et al., 1999).

The chicken MHC as well as non-MHC genes are known to influence B cell-mediated antibody responses to various antigens (Lamont 1991). The magnitude of the antibody responses induced by a vaccine consisting of killed infectious bursal disease virus was influenced by specific MHC haplotype of inbred chicken lines (Juul-Madsen et al., 2006). Selection over five generations for antibody responses (high versus low antibody production) to SRBC skewed the MHC haplotype composition in the final generation compared to the heterozygous starting population such that one haplotype (B21) was associated with higher antibody titers and the other haplotype (B13) consistently had lower antibody titers (Dunnington et al., 1996). Additionally, two Smyth lines that differ in MHC haplotype and the onset and severity of depigmentation were found to have differing secondary anti-SBRC titers (Sreekumar et al., 1995).
Smyth line chickens spontaneously develop an autoimmune-mediated loss of feather pigment cells, melanocytes, termed vitiligo (Erf and Smyth 1996; Erf et al., 1997). Comparisons of immune cell populations in the peripheral blood and dermal lymphoid aggregates between Smyth line birds and normally pigmented Brown line birds have revealed striking differences in immune cell composition in regard to the disease and to a lesser extent, how genetics influence these observations as both sample populations have a common genetic ancestry (Erf and Smyth 1996; Erf et al., 1997). The Smyth line depigmented birds had consistently higher levels of monocytes and heterophils isolated from peripheral blood samples from 4 to 16 weeks of age during the study compared to the age matched normal brown line birds (Erf and Smyth 1996), however there were no observed differences in the total peripheral lymphocyte population for the two groups. Additionally, the same authors have determined that dermal lymphoid aggregates in Smyth line chickens contain a higher percentage of \( \gamma \delta \) T cells and a lower ratio of CD4+ to CD8+ T cells (Erf et al., 1997), indicating that disease pathology influences the local immune cell composition in chickens.

Genetic influences on avian diseases have been extensively studied. Probably the most well known example is the association of MHC and Marek’s disease virus in which specific MHC haplotypes have been observed to be less susceptible to infection and tumor formation (Schierman and Collins 1987; Kaufman 2000; Bumstead 1998). Additional evidence supports the role of the chicken MHC in immune responses to other viral pathogens such as RSV and lymphoid leucosis viruses (Schierman and Collins 1987), notably with specific genes mapping to the B-F region located inside the chicken MHC.

Along with the association of both MHC and non-MHC genes in chickens for specific immune responses and resistance to various pathogens, genetic background also influences fundamental immunological parameters in poultry. Layer hens have been shown to produce a stronger cellular immune response and an IgG dominant antibody response when compared to broilers, which produced an IgM antibody response to trinitrophenyl (TNP) antigen (Koenen et al., 2002). Additionally, these authors speculated that the enhanced cellular and humoral immune responses in the layer chickens compared to the broilers reflects their
longer life expectancy and the selective pressures placed upon each of the line types. Investigation of the inflammatory immune responses in broiler and layer chickens also demonstrated the apparent differences between the two poultry types (Leshchinsky and Klasing 2001). Layer-type chickens had a more robust febrile response to LPS injection and higher in vitro proliferation of splenocytes cultured with LPS compared to broilers. Additionally, the mRNA expression of the inflammatory molecules IFN-γ, IL-1β, and MGF in spleen cells was observed to be higher in the layer-type birds than in the broilers. The authors suggest that a decreased inflammatory immune responsiveness in the broilers may be reflective of the enhanced growth demands placed upon them through genetic selection processes, as decreased food intake often occurs during acute inflammatory responses (Leshchinsky and Klasing 2001).

Single nucleotide polymorphisms or SNPs in genes known to be involved in immune responses have been associated with antibody responses in chickens (Zhou et al., 2002; Zhou and Lamont 2003a; Zhou and Lamont 2003b, Zhou and Lamont 2003c). A correlation between higher serum IFN-γ protein and higher SE-specific antibody levels SE following vaccination was observed with a SNP in the promoter region of the IFN-γ gene, indicating a possible role for this cytokine in antibody responses in poultry (Zhou et al., 2002). The magnitude or vigor of primary antibody responses to SRBC or Brucella abortus (BA) antigen are associated with SNPs in IL-15Rα and ZOV3, and IAP-1 and ChB6 genes, respectively (Zhou and Lamont 2003a). A SNP in transforming growth factor β2 (TGF-β2) also has been associated with antibody responses to SRBC, BA, and SE (Zhou and Lamont 2003b). Similarly, chicken MHC class I and II genes are also associated with antibody responses to these same antigens (Zhou and Lamont 2003c).

**Genetics and SE Infection in Chickens**

Resistance against SE infections by chickens has been established in studies that examined mortality rates as well as the recovery of and enumeration of bacteria in various organs over time. Resistance to *Salmonella* infections in poultry is not mediated by a single
trait or immune measurement, but reflects a spectrum of host responses to the invading pathogen. All of these individual examinations to characterize the immune response add to the growing body of information available on *Salmonella* infection in the chicken.

Outbred lines of chickens, which are more reflective of the animals found in commercial production settings, were observed to have very different mortality rates after oral or intramuscular SE inoculation (Guillot *et al.*, 1995). The Y11 line, a meat type line, was found to have a much lower rate of mortality compared to eight other lines examined; however, there was no correlation between mortality and cecal carriage of SE. This would suggest that determination of resistance based solely upon the rate of mortality provides an incomplete picture of infections without a measurement of other relevant traits such as bactericidal activity in organs, phagocytic potential of MØ and heterophils, and antibody production.

A comparison of the immune responses to SE infection in four broiler (meat type) lines found an inverse relationship between cecal colonization by SE and the level of bacterial burden in the spleen and liver (Kramer *et al.*, 2001). Lines 2 and 4 were observed to have a higher systemic SE infection as they consistently had higher colonization of the spleen and liver, but a lower rate of cecal colonization. Lines 1 and 3 had higher colonization in the cecum compared to the systemic organs, the liver and the spleen. These results are explained by differential antibody production and phagocytic ability among the four lines. High titer antibody responses were found in lines 1 and 3 which had a lower level of systemic colonization than lines 2 and 4, reflecting the ability of SE-specific antibodies to eliminate SE from the birds system. Higher phagocytic activity was found in lines 2 and 4 compared to lines 1 and 3, which correlates to reduced cecal colonization observed for lines 2 and 4.

Cytokine and chemokine gene expression after macrophage culture with ST or *S. gallinarum* has been found to differ significantly between cells recovered from two lines of chickens previously determined to be resistant (W1) or susceptible (7) to systemic *Salmonella* infections (Wigley *et al.*, 2006). Macrophages from the resistant W1 line in
response to both *Salmonella* strains produced more IL-1β and IL-6 cytokines and CXCL1 (K60) and CCL2 (MIP family member) chemokines compared to macrophages isolated from the susceptible strain (7) under identical conditions. The enhanced mRNA gene expression in the W1 line occurred early in the assay time, within 20 minutes to 1 hour after bacterial infection. IL-18 mRNA expression by macrophages was also enhanced in the resistant (W1) line compared to the susceptible line (7), although this response occurred later at 4 hours post-infection (Wigley et al., 2006).

Heterophils, from distinctly different genetic lines, have been shown to differentially express cytokine-specific mRNA patterns in response to SE infection (Ferro et al., 2004; Swaggerty et al., 2004; Swaggerty et al., 2006). Lines A and D, more resistant to SE infection compared to B and C, have heterophils that produce more pro-inflammatory cytokine mRNA (IL-6, IL-8, and IL-18) in response to SE infection. Heterophils from the resistant lines, A and D, also show decreased expression of the anti-inflammatory cytokine, TGF-β4. The pattern of higher pro-inflammatory cytokine and lower anti-inflammatory mRNA expression in the resistant lines (A and D) persist from days 1, 14, and 28 post-hatch, suggesting these attributes are likely reflective of the genetic background and are not age related.

By following the burden of cecal colonization of young chicks by SE beginning at one week of age, it was noted that the duration of colonization within four separate outbred chicken lines differed greatly (Duchet-Suchaux et al., 1997). Lines L2 and B13, both layer type lines, were observed to have higher SE cecal colonization levels and these increased bacterial burdens remained consistently higher over the course of the experiment when compared to lines PA12 (layer) and Y11 a meat type chicken line. Earlier studies that examined resistance to SE colonization and contamination of lain eggs from infected hens from four outbred lines mirror the results of SE colonization and cecal carriage in young chicks from the same chicken lines (Protais et al., 1996). Compared to the other chicken lines, the L2 line was more susceptible to SE infection based on the observation that infected hens laid the highest number of SE contaminated eggs and internal organs were more culture
positive for SE. The four lines, L2, B13, PA12, and Y11, have been tested for anti-SE antibody production in both the serum and intestinal secretions 9 weeks after infection of day old chicks (Berthelot-Herault et al., 2003). The L2 and B13 lines, previously determined to be susceptible to SE infection, had higher IgG and IgA anti-SE antibodies in the serum compared to the resistant lines, PA12 and Y11. Additionally, the L2 and B13 lines also had higher levels of IgA anti-SE antibodies found in the intestinal secretions indicating a negative correlation between antibody production and resistance to SE infection (Berthelot-Herault et al., 2003). Investigation of inbred and partially inbred lines of chickens has demonstrated differences in mortality of newly hatched chicks with ST infection (Bumstead and Barrow 1988). Resistant lines, W, 6(1), and N consistently had lower mortality rates than susceptible lines, C and 15I when challenged with several ST strains differing in virulence, or challenged by different routes (oral and intramuscular) of inoculation. The observed resistant and susceptible phenotypes were not ST-specific (Bumstead and Barrow 1993). The susceptible lines, C and 15I, and the resistant lines, N, W, and 6(1) demonstrated similar mortality rates when infected with SE, *Salmonella pullorum*, and *Salmonella gallinarum* as when infected with ST. In 2001, Kaiser and Lamont reported that the observed differences in survival and bacterial colonization of the cecum in young layer chicks representing four genetic lines, provides additional evidence to support the role of genetics in resistance to SE.

Resistant and susceptible lines, 6(1) and 15I, respectively, have been analyzed for expression patterns of immune response related genes (Sadeyen et al., 2004; Sadeyen et al., 2006). While expression of IL-1β, IL-8, IL-18, NRAMP1, and iNOS mRNA was not correlated with resistance or susceptibility to SE infections of one-week old chicks, the susceptible line had significantly lower IFN-γ mRNA expression in the cecal tonsil compared to the resistant line and healthy uninfected control animals (Sadeyen et al., 2004). Adult hens 30 weeks of age at SE infection from the resistant line, 6(1), and the susceptible line, 15I, in contrast to their younger counterparts had very different expression levels of immune response genes (Sadeyen et al., 2006). Hens from line 6(1), which are resistant to *Salmonella* infections and have reduced cecal colonization levels of SE compared to the 15I
line, had consistently higher mRNA expression for IL-8, IL-18, iNOS, TLR4, MIM 1, Gal1, Gal2, and IFN-γ genes at 1, 2, and 3 weeks post bacterial inoculation. This data suggested that regulation of these genes may beneficially influence the avian immune response to SE infection.

Polymorphisms (i.e., SNPs) in genes known to be involved in immune function have been associated with resistance to SE challenge or vaccination have been demonstrated in chickens (Lamont *et al.*, 2002; Liu and Lamont 2003; Malek *et al.*, 2004; Hasenstein *et al.*, 2006). The level of SE burden in the spleen has been associated with MHC class I, IAP-1, and NRAMP-1 (Lamont *et al.*, 2002) and MD-2 gene polymorphisms (Malek *et al.*, 2004). Additionally, Malek *et al.*, 2004, demonstrated associations of the CD28 gene with SE bacterial colonization in the cecum and antibody responses to an SE vaccine. Caspase-1 gene polymorphisms have been associated with cecal colonization and SE vaccine antibody production, and an SNP in the IAP-1 gene correlated with splenic SE bacterial burden (Liu and Lamont 2003). Gallinacins, or chicken β-defensins, are part of the innate immune system, and have been shown to be influential in the avian immune response to SE. SNPs in two gallinacin genes, Gal3 and Gal7, were associated with vaccine antibody responses to SE in young chicks (Hasenstein *et al.*, 2006).

**Colonization and Persistence of Salmonella in Chickens**

Examination of the early immune response to *Salmonella* infection in young chicks provides a model that accurately reflects the initial exposure of birds to pathogens in the production setting. After oral inoculation, *Salmonella* is readily isolated from the cecum within the first 12 hours; however, colonization of the liver and the spleen is only detected at 24 hours or later (Withanage *et al.*, 2004; van Immerseel *et al.*, 2002). Changes in gene expression and histology were observed during the early phase of *Salmonella* infection in young chicks (less that a week old). From approximately 2 days post-infection, the numbers of macrophages, T cells, and B cells in the cecum were elevated in response to SE (van Immerseel *et al.*, 2002) and remained elevated in the organ for 6 to 8 days PI. An increased
influx of avian heterophils is present in the liver and intestinal tissue of ST infected chicks at 2 days following oral inoculation (Withanage et al., 2004). Subsequent to Salmonella infection, the tissues are characterized by inflammation (i.e., increased heterophils, edema) and increased expression of 2 CXCLi chemokines (IL-8 and K60) and IL-1β (Withanage et al., 2004). These observations of cellular infiltration of heterophils, macrophages, T cells, and B cells along with the tissue edema and increased cytokine and chemokine expression demonstrates that chickens respond to a Salmonella infection with a characteristic inflammatory response similar to that of mammals.

The liver, spleen, and ceca of day old chicks infected with SE will remain colonized for the first four weeks after receiving the initial inoculum (Gast and Holt 1998). S. enteritidis persisted in the cecum of infected birds throughout the course of one study (16 weeks) and, at 24 weeks post-infection, more than half of the birds were still shedding SE in feces. Another study examined SE infection in day old chicks, measuring antibody responses to the bacteria and to Newcastle disease virus vaccination or SE bacterin (Holt et al., 1999). As in the previous study, 50 % of the chicks remained infected (i.e., culture positive) for as long as 23 weeks PI, indicating that very young chicks have substantial difficulties in responding to and clearing the infection. The humoral immune responses of young chicks one week after SE inoculation were low but increased over time reaching peak levels at approximately 8 weeks PI (Holt et al., 1999). Although IgA antibody production in the intestinal tissues of infected birds was measurable as early as one week after infection, these mucosal antibodies were not effective in reducing bacterial colonization. Another important feature of the study was how SE infection of very young birds seemed to influence immune response to vaccines. Infected chicks produced a weaker antibody response to SE bacterin and to a Newcastle disease virus vaccine, which suggests that SE infection in the very young chick is immunosuppressive. These effects of decreased response to antigenic challenge were observed throughout the 23 weeks of the study indicating the immunoresponsiveness in these birds is compromised long after the initial infection began (Holt et al., 1999).
Age of first exposure to *Salmonella* will greatly influence the course of infection and the bird’s ability to clear the bacterial pathogen (Beal *et al.*, 2004; Gast and Beard 1989; Desmidt *et al.*, 1997). Chicks infected with ST at one day after hatching had a higher incidence of mortality than chicks infected just one week later (Gast and Beard 1989). Approximately 80% of surviving day old chicks were *Salmonella* positive when examined at 7 weeks post-infection compared to 60% that were infected at 8 days of age. In addition to a greater frequency in the rate of colonization, inoculation of day old chicks resulted in maintenance of a greater ST load in the cecum than that observed when older chicks were infected (Gast and Beard 1989; Desmidt *et al.*, 1997). Oral inoculation of white Leghorn SPF chicks at one day of age and at four weeks with SE showed similar results (Gast and Beard 1989; Desmidt *et al.*, 1997). Mortality was only observed in the younger chicks that also displayed symptoms of clinical disease, lethargy, and diarrhea. In the first week post-infection, both the young and older birds had similar percentage of organ infection, however, over the next few weeks the older birds were observed to have lower incidence of SE positive organs which indicates these mature animals were better at controlling the colonization and SE infection and suggests that the immune response must mature in order to effectively protect the birds from an otherwise lethal infection.

Although older chicks are better equipped to control *Salmonella* infections as evidenced by the lower incidence of organ colonization, birds of all ages are readily colonized. Bacteria can be isolated from the internal organs of adult hens infected with SE by either oral inoculation or horizontal transfer methods (Gast and Beard 1990). Within the first 5 weeks PI, SE was isolated from approximately 50% of the livers, ceca, and spleens and about 20% of the ovaries and oviducts of experimentally infected hens. Over time, the incidence of SE positive isolates generally decreased, but SE could be recovered at low levels from internal organs for more than 20 weeks PI (Gast and Beard 1990).

*S. typhimurium* has been shown to persist in the gastrointestinal tract of infected birds for over 8 weeks post-inoculation regardless of the age at primary exposure (1, 3, or 6 weeks) (Beal *et al.*, 2004). The birds infected at three and six weeks of age were noted as having a
more rapid antibody response compared to the one week old animals; however, the older birds remained infected even though they exhibited higher titer antibody responses. Upon examination of the secondary immune responses of re-challenged infected birds, fewer \textit{Salmonella} were found in the gut, liver, and spleen of all ages compared to age-matched individuals receiving an initial bacterial administration (Beal et al., 2004). The birds initially exposed at three or six weeks of age were more efficient at clearing the \textit{Salmonella} re-challenge compared to similarly treated one week old birds, which correlated with higher T cell proliferation and higher anti-salmonella-specific IgY antibody in circulation. This study would suggest that younger birds are more susceptible to long-term \textit{Salmonella} infections and less capable at producing an effective protective immune response compared to birds infected at a later age.

Colonization of reproductive tracts by SE in laying hens is well established (De Buck et al., 2004; Withanage et al., 2003; Withanage et al., 1998). Systemic infection with SE has been shown to lead to bacterial colonization of the ovaries and oviduct in laying hens (Miyamoto et al., 1997; Okamura et al., 2001a; Okamura et al., 2001b). Compared to other \textit{Salmonella} species such as ST, SE colonizes the reproductive organs of laying hens more frequently despite similar ability in colonization of internal organs (Okamura et al., 2001a; Okamura et al., 2001b). In addition to the predilection of SE to colonize reproductive organs of adult hens, SE was also the main species found in eggs laid from the experimentally infected hens. These studies would suggest that SE is uniquely able to colonize the ovaries and oviducts leading to the transmission of the bacteria into developing eggs.

\textit{S. enteritidis} infection of the reproductive organs in mature hens evokes an immune response from the bird, which includes both humoral and cellular immunity. After infection, hens produced SE specific antibodies at similar levels in both serum and oviducts, obtained with oviductal washing (Withanage et al., 1998). Compared to uninfected control hens, SE infection resulted in the increased secretion of antibody subclasses, IgA, IgG, and IgM, which lasted throughout the duration of the experiment. Maximal IgA production was obtained 1 week after inoculation in both the serum and oviducts, and following a secondary
bacterial challenge (Withanage et al., 1999). SE-specific antibody production correlated with a reduction in bacterial load in the oviducts, indicating local humoral immune responses do play an important role in *Salmonella* infection of reproductive organs in chickens.

Changes in the cellular composition of immunocytes have been observed in the ovaries and oviducts of SE infected hens. Increases in T cells in both ovaries and the oviducts one-week post-infection remained elevated at 10 days followed by an increase in B cells at 14 days PI (Withanage et al., 1998). Macrophage numbers decreased upon SE infection, but returned to pre-infection levels within 21 days PI at which time both T cell and B cell numbers similarly returned to basal levels. Similar observations of altered lymphocyte and macrophage cell populations have been reported (Withanage et al., 2003). T cell subsets including CD4 and CD8 cells were increased in the reproductive organs of SE infected hens during the first two weeks post-inoculation. Following a T cell influx into the ovaries and oviducts, B cell population numbers also increased. Expansion of lymphocytes correlated with a persistent SE infection of the organs (Withanage et al., 2003). Increased numbers of macrophages were also observed in the reproductive organs of infected hens, implicating the involvement of innate and cell-mediated immunity in the avian host immune response to SE infection of chickens.

**Macrophages – *Salmonella* and Apoptosis, Innate Immune Function**

Cells of the monocyte/macrophage lineage play many important roles in protecting the avian host from bacterial infections such as *Salmonella* (Qureshi et al., 2000). Macrophages, in the broadest sense, provide the host with surveillance and protection against foreign microbial invaders, and, by producing cytokines and chemokines, macrophages can activate and direct other immune cells to the sites of infection (Crawford et al. 1994; Murtaugh and Foss 2002). Macrophages, as part of the innate immune system, are also very adept in killing pathogenic microbes by phagocytosis and through the production of antimicrobial products like nitric oxide (MacMicking et al., 1997). Macrophages perform these functions and many more which are beneficial to the host during the course of an
immune response to various pathogens; however, these cells can be exploited and manipulated by the very same pathogens (Rosenberger and Finlay 2003; DeLeo 2004). As a target of intracellular pathogens like Salmonella, macrophages and the essential immune functions performed by them can be subverted to protect the pathogen from the host’s immune system.

Macrophages, as part of the innate immune system, contributes to an early defensive team that patrols and protects the host from invading pathogens, potential insults, or tissue damage (Qureshi et al., 2000). Monocytes, the precursors of mature macrophages, circulate throughout the host via blood. Upon entry into tissues, monocytes differentiate and mature into tissue-specific macrophages with different phenotypes and functional characteristics shaped by the conditions of the local microenvironment they encounter (Stout and Suttles 2004; Tacke and Randolph 2006). Macrophages display great flexibility in the range of immune responses they perform, reflective of the conditions they encounter during activation. In this regard, three types of macrophage activation have been described: classical, alternative, and type II (Mosser 2003).

Classically activated macrophages (stimulated by IFN-γ and microbial triggers such as LPS), in contrast to type II activated macrophages and alternatively activated macrophages, facilitate pro-inflammatory, Th1 cellular immune responses important in viral and intracellular bacterial infections (Goerdt et al., 1999a; Goerdt and Orfnos 1999b; Gordon 2003; Anderson and Mosser 2002). Classically activated macrophages up-regulate MHC class II surface expression and secrete IL-1, IL-6, IL-12, and TNF, which promote Th1 immune responses by inducing T cells to produce IFNγ. Classically activated macrophages, compared to alternatively or type II activated macrophages, are more efficient killers of intracellular microbes as they produce nitric oxide and reactive oxygen species (MacMicking et al., 1997). Because of the pathogenesis of salmonellosis, the ability of classically activated macrophages to promote Th1 mediated immune responses and kill intracellular pathogens make them a critical component of host resistance against Salmonella infections.
Cell death and delayed phagosome maturation leading to prolonged host cell colonization are two pathogenic mechanisms associated with *Salmonella* invasion of macrophages (Brumell and Grinstein 2004; Hueffer et al., 2004; Knodler and Finlay 2001). As a facultative intracellular bacterial pathogen, *Salmonella* species are adept at invading various host cell types like gastrointestinal epithelial cells, dendritic cells, and neutrophils; however, macrophages appear to be their primary target cell (Pietila et al., 2005; Yrlid et al., 2001; Jones and Falkow 1996; Garcia-del 2001; Amer and Swanson 2002; Linehan and Holden 2003).

Bone marrow-derived murine macrophages when cultured with *Salmonella* exhibit the classical features of apoptosis, DNA fragmentation and the formation of cytoplasmic vacuoles (Monack et al., 1996, Chen et al., 1996). This cellular death, which has features resembling apoptosis, including chromatin fragmentation and membrane blebbing, does not require bacterial invasion or uptake into the affected macrophages.

Using human monocyte-derived macrophages, ST has been shown to rapidly kill these cells by inducing apoptosis displaying fragmented chromatin in a similar manner as in the murine studies (Zhou et al., 2000). Additionally the study demonstrated that when pretreated with an inhibitor of caspase-3, a pro-apoptotic molecule, ST was less efficient in killing human macrophages. The involvement of other caspase family members, most notably caspase-1, in cellular apoptosis responses has been documented (Los et al., 1999; Monack et al., 2001). Caspase-1, also known as IL-1β-converting enzyme or ICE, cleaves the inactive forms of IL-18 and IL-1β cytokines in macrophages (Monack et al., 2001; Jarvelainen et al., 2003).

The activation of caspase-1 in macrophages by *Salmonella* or *Shigella* result in host cell death and the initiation of inflammatory processes (Monack and Falkow 2000; Haimovich et al., 2006; Navarre and Zychlinsky 2000). Although there remains the question of whether caspase-1 mediated cell death of macrophages in response to *Salmonella* infections accurately reflects true apoptotic phenomena, necrosis, or a combination of both processes, nevertheless cellular death of macrophages and the production of pro-
inflammatory cytokines IL-1β and IL-18 appears to be required for the establishment of murine salmonellosis (Monack et al., 2001; Boise and Collins 2001). It is tempting to speculate that the rapid killing of host macrophages and the resulting inflammatory process would likely recruit more phagocytic cells to the site of the initial infection. This scenario could result in an ideal situation for the Salmonella bacteria to then infect more host cells but not to kill, to hide and replicate inside avoiding the host animal immune system. Alternatively, the inflammatory process could attract neutrophils capable of killing Salmonella and resolving the infection. While Salmonella efficiently destroys host macrophages in-vitro and in-vivo, the bacteria are also quite capable of residing inside the very same cell type, thus establishing persistent colonization of the infected host animal (Brumell and Grinstein 2004). After bacterial invasion, Salmonella are able to remain inside the phagosomal vacuoles of macrophages. Salmonella are able to subvert the normal process of phagosomal maturation in the infected macrophages allowing the bacteria ample time to replicate (Brumell and Grinstein 2004).

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CHAPTER 2. GENETIC LINE EFFECT ON PERIPHERAL BLOOD LEUKOCYTE CELL SURFACE MARKER EXPRESSION IN CHICKENS

A paper published in *Poultry Science* 1
J. H. Cheeseman 2, 3, M. G. Kaiser 4, and S. J. Lamont 2, 5

Abstract

To determine the role of genetics in baseline lymphocyte parameters, several distinct lines of chickens were examined for differences in peripheral blood leukocyte populations. Four highly inbred chicken lines (MHC congenic Fayoumi lines M15.2 and M5.1, and MHC congenic Leghorn lines G-B1 and G-B2), two advanced intercrosses [F₅(Broiler x G-B2) and F₅(Broiler x M15.2)], and an outbred population of broilers were used. Leukocytes isolated from healthy adult birds were labeled with monoclonal antibodies: chCD3, chCD4, chCD8, chBu-1, and hCD14. Flow cytometry was used to determine the total percentage of positively labeled cells for each surface marker in a sample as well as the mean fluorescent intensity, or surface marker density, of a labeled subset. Significant line differences for percent positive of CD3 T cells and the ratio of B cells: T cells (represented by a Bu-1: CD3 ratio) were found. The effect of line was also significant for both CD3 and CD8 T cell receptor density. Effects of sex and MHC on peripheral blood leukocyte cell surface marker expression were not significant in the lines examined. This study demonstrates the effect of genetic line on resting leukocyte composition of peripheral blood in the chicken lines examined. Observed peripheral blood leukocyte differences add to our growing knowledge of the varied roles that immune system status, defined by specific cell populations, and genetic backgrounds have in determining susceptibility and disease progression in chickens.

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2 Graduate student and Professor, respectively, Department of Animal Science, Iowa State University.
3 Primary researcher and author.
4 Associate researcher and author.
5 Author for correspondence.
Introduction

There are many ways to distinguish the several leukocyte populations from each other. Location, activity assays, size, and surface markers are some of the most common means. Surface markers are probably the most utilized because of the wide availability of specific antibody reagents (Chung et al., 1991; Veromaa et al., 1988). Lymphocytes arise from progenitor stem cells that, through the complex process of differentiation, produce each of the many cell types of the immune system. T cells generally express the CD3 complex early in cell development and expression remains throughout the life of each individual T cell (Chan et al., 1988: Janeway et al., 2001). T cells can be further classified by cell development and the expression of co-receptor molecules CD4 and CD8. Cytotoxic T cells typically express CD8 compared to helper T cells that express CD4 (Chan et al., 1988: Janeway et al., 2001). CD14 along with TLR4 molecules are expressed on the surface of many phagocytic cell types such as: monocytes, macrophages, neutrophils, and dendritic cells (Janeway et al., 2001). B cells can be distinguished by the expression of IgM and/or other surface molecules like Bu-1 (Janeway et al., 2001: Veromaa et al., 1988).

Significant PBL composition differences exist in the MHC congeneric chicken lines designated CB and CC. Hala et al., 1991, found a higher percentage of CD4+ PBL in the CB line compared to a higher number of CD8+ PBL in the CC line, and concluded that MHC played an important role in determining T cell PBL composition. These two lines were later shown to have markedly different CD4, CD8, and CD4: CD8 ratio populations in peripheral blood samples obtained at 7 day intervals over a six-week post-hatch time period (Hala et al., 1992).

Another study that examined PBL populations of two inbred chicken lines using flow cytometry found differences in CD4, CD8, and B cell numbers, but not CD4: CD8 ratios (Burgess and Davidson, 1999). Line 72, compared to line 61, had higher numbers of CD4 and CD8 T cells and B cells in peripheral blood samples, but significant differences for CD4:
CD8 ratios, T:B cell ratio, or MHC class I surface molecule expression could not be demonstrated.

Chicken lines divergently selected based upon their antibody response to sheep red blood cells (SRBC), had significant differences in CD4, CD8, and B cell composition for splenic and thymic tissues, but not in PBL (Parmentier et al., 1995). In general, the high antibody line had a larger proportion of CD4 and B cells, compared to the low antibody line that had a higher proportion of CD8 cells. When examined at several time points from week 2 through 14, PBL samples analyzed by flow cytometry showed no differences in CD3, CD4, CD8, and CD4:CD8 cell ratios.

In light of these varied results with different chicken lines, it is important to continue the examination additional well-defined genetic lines. Examination of total leukocyte percent positive cells and mean cell marker density of PBL in mature animals of several well-characterized chicken lines was the objective of this study.

**Materials and Methods**

**Animals**

The G-B1 and G-B2 are MHC congenic lines of Leghorn chickens that originated in 1965 and the MHC congenic lines M15.2 and M5.1 are Egyptian Fayoumi and were established in 1954 (Zhou and Lamont, 1999). The G-B1 and G-B2 Leghorn congenic pair and the M15.2 and M5.1 Egyptian Fayoumi congenic pair each share an identical genetic background and differ only in the microchromosome bearing the MHC. These four lines are highly inbred, each with an inbreeding coefficient of 0.99 (Zhou and Lamont, 1999). Outbred broiler breeder males (Kaiser and Lamont, 2002) were mated to G-B2 and M15.2 females and the resulting progeny were intermated (within dam line) for four generations to produce the advanced intercross lines. A total of ten birds were sampled from each of the two advanced intercross lines, F5 (Br x G-B2) and F5 (Br x M15.2). Nine broilers, six G-B1, five G-B2, five M5.1, and four M15.2 birds were also sampled. Similar numbers of males and females per line were used. All animals appeared healthy and were individually caged.
with ad libitum access to water and feed meeting or exceeding National Research Council (1994) requirements.

**Sample Collection, Preparation, and Cell Surface Labeling**

Individual animals were assayed on one of six collection dates over a period of eight days. To minimize the potential effect of assay date on study results, each line was tested on each of the six collection dates.

Peripheral blood samples (approximately 5-10 mls) from adult chickens 38-40 weeks in age were collected from either wing or jugular veins in 10 ml syringes preloaded with 0.5 ml of sterile 0.5 M EDTA to prevent clotting. Leukocytes were separated from red blood cells using Histopaque 1077 (Sigma Diagnostics INC., St. Louis, MO) by centrifugation for 30 min at 400 x g. The leukocytes, contained in the buffy coat, were initially washed three times in PBS by centrifugation for 10 min at 400 x g, pelleted and resuspended in PBS. Leukocyte cell counts and viability were determined by using Trypan Blue (Sigma Diagnostics INC., St. Louis, MO) exclusion.

Isolated chicken leukocytes were incubated with the following monoclonal antibodies: chCD3 Fluorescein Isothiocyanate (FITC), chBu-1 (FITC), chCD4 (FITC), chCD8 Phycoerythrin (PE), and hCD14 (FITC) (Southern Biotech, Birmingham, AL). All antibodies are chicken specific with the exception of CD14, which has been shown to label chicken CD14 cells (Dil and Qureshi, 2002). A total of 1x10^6 cells from each individual chicken were incubated for 1h at room temperature with antibody concentrations as follows: chCD3 (FITC) 4.0 µg/10^6 cells, chBu-1 (FITC) 2.0 µg/10^6 cells, chCD4 (FITC) 1.0µg/10^6 cells, chCD8 (PE) 1.0 µg/10^6 cells, and hCD14 (FITC) 1:4 volume ratio. Leukocyte samples were dual labeled with CD4 and CD8 antibodies in a two-step manner, while all other samples were singularly labeled with the remaining antibodies. All samples were washed post antibody incubation three times in PBS by centrifugation for 10 min at 400 x g, pelleted and resuspended in PBS with 0.1% sodium azide. Following the final wash cycle, samples were re-suspended in 0.5 ml PBS with 1% formaldehyde and held at 4^0 C overnight before
Fluorescence Activated Cell Sorter (FACS) processing. FACS (FACScan Becton Dickinson, Franklin Lakes, NJ) is a method used to count and sort cells based upon size and fluorescence. Flow cytometric analysis on all lymphocyte samples was performed at the Iowa State University Cell and Hybridoma Facility. Samples were analyzed for both percent of total population positive for the cell surface marker examined and fluorescent intensity. Fluorescent intensity is the relative degree of antibody binding to the cell surface on leukocytes that are positive for the specific marker.

**Statistical Analysis**

All data were analyzed using JMP software (SAS Institute, 2000) to determine least squares mean (LS means) and \( P \) values for parameters tested. The GLM procedure of SAS (SAS Institute, 2000) was used for the following model:

\[
y = u + line + sex + assay date + e
\]

Line and sex were considered fixed effects and assay date was a random effect. Statistically significant differences between individual line data (LS means) were determined using Student’s t test (\( P < 0.05 \)). Two-way interactions, line x sex, sex x assay date, and line x assay date, were initially tested and found insignificant (\( P > 0.1 \)). These two-way interactions were excluded from the final model.

**Results and Discussion**

**Peripheral Blood Leukocyte Percentages**

The LS mean percentages for the effects of line, sex, and assay date on tested cell surface markers are shown in Table 1. The effect of sex was not significant (Bu-1, CD3, CD14, CD4, CD8, and CD4 CD8), and is consistent with previous work by Hala et al., 1991, in their investigation of MHC congenic chickens for CD4 and CD8 PBL differences. The effect of assay date was significant for the tested cell surface markers with the exception of CD4 and CD8 percent positive cells. Incorporated into this study’s experimental design was
the sampling of each line on the six assay dates as a means to minimize confounding of potential assay date and genetic line effects.

In comparing the LS mean percentages of leukocytes that expressed the tested cell surface receptors, there was a significant line difference ($P = 0.04$) in the percent of CD3 positive but not for the other cell surface markers examined. For the CD3 marker, G-B1 and G-B2 birds had lower percentages of observed leukocyte populations compared to the other lines examined. The MHC congenic Leghorn lines had CD3 population percentages that were two-fold lower than the other lines (Table 1). These data suggest that the aforementioned Leghorn lines have a lower resident CD3 T cell population as indicated by the lower percent of peripheral CD3 positive cells. Another explanation could be that, although peripheral blood CD3 T cell populations were observed to be two-fold lower in these congenic lines, a higher percentage of the whole CD3 T cell population might be found residing in specific lymphoid organs such as the spleen or lymph nodes.

In general, peripheral blood leukocyte composition differences were not observed in this study with the exception of CD3. Erf and Smyth, 1996, found no differences in PBL composition of Smyth line chickens for CD3, CD4, CD8, and B cell types. Although the Smyth line study conflicts with our current observation of a significant line effect for CD3 leukocyte percentages among the chicken lines examined in this study, it does however agree with the overall findings of the present study that indicate no differences in peripheral blood leukocytes for the other cell surface markers. Parmentier et al., 1995, found no significant PBL subset differences in percentages of CD3, CD4, and CD8 cells in two chicken lines divergently selected for antibody response to SRBC. However, the same study found line differences in CD4 and CD8 cell populations of spleen and thymus tissue samples. In light of these findings, it is quite plausible that the seven chicken lines of the current study may have measurable lymphocyte differences in splenic and thymic tissues that were not reflected in our study of peripheral blood leukocytes.

Closer examination of percentages of cells positive for the surface markers CD3, CD4, and CD8 show interesting phenomena (Table 1). The percentage of CD3 positive cells
is lower than the sum of the CD4, CD8, and CD4$^+$ CD8$^+$ T cell percentages (data not shown). This discrepancy is highest for the two Leghorn inbred lines, G-B1 and G-B2. There is no clear explanation for the observed inconsistency in CD3 cell percentages compared to those obtained when CD4, CD8, and CD4$^+$ CD8$^+$ T cells are added, but CD4 can be expressed on monocytes and macrophages (Janeway et al., 2001). The monoclonal antibody used to stain CD3 cells may have had a lower affinity for the targeted surface marker compared to the two other antibodies, CD4 and CD8. The same T cell population labeling inconsistencies have been observed in other studies. Maslak and Reynolds (Maslak and Reynolds, 1995) examined lymphocyte concentrations in the Harderian gland of chicks at 1, 4, and 8 weeks in age. Lymphocyte subset profiles of that study at all three time points showed lower CD3 cell numbers, compared to the sum of CD4 and CD8 positive cells. More recently, Withanage et al., 1998, also found similar lower CD3 cell numbers compared to the sum of CD4 and CD8 positive cells in the ovaries, uterus, and vagina of laying hens after experimental exposure to *Salmonella enteritidis*. Although Withanage et al., 1998, examined tissue samples for changes in T cell subsets and the current study used peripheral blood cells, both research studies utilized the same antibody clones from the same vendor. In light of this, it is likely that the choice of monoclonal antibody, especially CD3, has an influence on labeling percentages measured for T cells.

**Lymphocyte Surface Antigen Intensity**

The effect of sex on fluorescent intensity of peripheral blood leukocytes positive for a specific cell surface antigen was not significant (Table 2). The effect of line on fluorescent intensity on surface marker expression for the Bu-1 and CD4$^+$ CD8$^+$ double positive T cells approached significance with $P$ values of 0.06 and 0.09 respectively.

Genetic line had a significant effect on LS mean intensity of leukocytes expressing the surface markers CD8 and CD3 (Table 2). Intensity for CD8, which corresponds to the cytotoxic T lymphocyte population in the peripheral blood sampled, was highly significant ($P < 0.001$). The M5.1 line had the highest level of CD8 co-receptor fluorescent intensity and
was twice the measured level for the G-B1, G-B2, and F₅ (Br x G-B2) lines (Table 2). The broiler, M15.2, and F₅ (Br x M15.2) lines had similar LS mean fluorescent intensity values for CD8. The G-B1 and F₅ (Br x G-B2) lines had the lowest observable LS mean fluorescent intensity for the CD8 cell surface co-receptor.

The effect of line, therefore genetic background, on fluorescent intensity of leukocytes expressing the CD8 surface co-receptor was of the greatest statistical significance in this study (Table 2). Among the lines, there were vast differences in cytotoxic T cells in regard to their surface receptor density. The recognition of MHC presented peptide antigens and cytolytic activity is shown to be greatly enhanced by the presence of CD8 surface proteins on T cells (Cho et al., 2001). Human CD8 is a co-receptor that intimately interacts with the MHC-peptide structure and enhances antigen sensitivity for T cell cytolytic activity (Purbhoo et al., 2001). Lines with a higher density of CD8 surface receptors, such as the M15.2, M5.1, and the F₅ (Br x M15.2) birds, could be the result of enhanced transcriptional mechanisms or through selective pressures favoring the maturation of CD8 T cells that express high levels of this surface receptor.

The effect of chicken line on CD3 cell fluorescent intensity was also significant (Table 2). The G-B2 line had the highest observed fluorescent intensity whereas the F₅ (Br x M15.2) line had the lowest measured CD3 surface antigen density of all the lines. In general, the two MHC congenic Leghorn lines, G-B1 and G-B2 had a similar level of CD3 intensity compared which was approximately 1.5 fold greater than that of all the other lines.

The effect of genetic line was significant on both percent positive and fluorescent intensity of the CD3 surface receptor (Tables 1&2). This suggests that genetic line has a strong affect on CD3 positive T cells, not only on percentage in peripheral blood samples, but in surface receptor density on these cells as well. The CD3 surface protein is intimately associated with the T cell receptor (TCR), which recognizes MHC class I and II presented peptides (Janeway et al., 2001). It would be of interest to determine if TCR density expression is also controlled by genetic line. Use of another T cell surface marker such as
CD45, would add additional information about total T cell population measurements in future studies.

In studies of the immune system, inbred strains are often employed to limit the potential effects of genetic variation (Knippels et al., 1999; Yamakawa et al., 1996). Two mouse strains with immune system response differences are BALB/c and C57BL/6. BALB/c mice have more dominant humoral immune responses; whereas in C57BL/6 mice the cell mediated immune response is dominant (Karp et al., 1994). The C57BL/6 mice have lower percentages of thymic CD4 T cells than BALB/c mice (Duarte and Penha-Goncalves, 2001).

The two sets of MHC congenic pairs had similar within-pair CD3 surface receptor density (Table 2), in agreement with the general findings of the present study that MHC had little influence on the composition of peripheral blood leukocytes of adult chickens. The non-MHC genes involved in leukocyte composition are likely to be multi-factoral in nature and remain to be fully elucidated.

**Cell Population Ratios**

The primary flow cytometric data were used to examine ratios of specific leukocyte populations that were positively labeled with the surface marker. The ratio of CD4:CD8 T cells, was used to estimate the helper T cell: cytotoxic T cell ratio in chicken peripheral blood. Genetic line shows a strong influence in determining the CD4:CD8 ratio in different strains of mice (Duarte and Penha-Goncalves, 2001; van Meerwijk et al., 1998). In the present study, however, the effect of genetic line on CD4:CD8 ratios were not significant. The ratio of CD14:CD3+Bu-1 cells, which correlates to the heterophil: B + T cell ratio, was also examined. The effect of line on the ratio of CD14:CD3+Bu-1 leukocyte cells were not significant in this study.

Line effects on the Bu-1: CD3 ratio, which estimates B cell: T cell ratios, were observed (Table 3). The G-B2 line had the highest Bu-1: CD3 ratio, whereas the F5 (Br xM15.2) line had the lowest Bu-1: CD3 ratio (Table 3). The five remaining lines had Bu-1: CD3 ratios that were not significantly different and ranged from 0.56 to 0.91. Significant
differences in B: T cell population, as estimated by the Bu-1: CD3 ratio, suggests that further examination into whether these lines are predisposed to respond to a pathogen either with a humoral or cell-mediated based immunity, reflective of the overall peripheral blood lymphocyte composition, is warranted.

The differences in the B: T cell ratios in this study are a result of varied levels of CD3 cells, not B cells, in the peripheral blood (Table 1). Observed B cell percentages were very consistent, among lines, in comparison to the more widely distributed T cell numbers for peripheral blood leukocytes (Table 1). Although Burgess and Davison, 1999, found no B: T cell ratio differences in their chicken lines, our experiment used a CD3 receptor as an indicator of the whole T cell population in PBL samples, compared to their combination of CD4 and CD8 positive cells to estimate the same leukocyte subset.

In the present study, we report small percentages of CD4+CD8+ double positive T cells in all seven chicken lines. These data agree with a study that found this novel T cell subset in peripheral blood and tissues such as the spleen and intestinal epithelium of chickens (Luhtala et al., 1997). Associations of age and dominant Mendelian inheritance patterns of double positive T cell abundance have also been reported (Erf et al., 1998; Luhtala et al., 1997). The presence of CD4+CD8+ T cells is well established, but no clear function has yet been established in the chicken. Analysis of double positive T cells in the peripheral blood of humans and pigs suggest their role as memory T cells (Zuckermann 1999).

The genetic lines studied allowed the opportunity to contrast the effect of both MHC and non-MHC genetic variation on lymphocyte subsets. Based on the two MHC congenic pairs in this study, the results suggest that the non-MHC genes, in total, had a larger effect on the lymphocyte characteristics than did the variation in the MHC haplotypes. Of all the assays conducted, only one single contrast within a congenic pair (fluorescent intensity for CD8, Table 2) was significant. This result may be specific to the individual MHC haplotypes examined, or may be a more general phenomenon reflecting the multigenic control of cell-surface protein receptor expression. Results of this flow cytometric study suggest that
genetic line influences specific subsets of peripheral blood leukocyte composition in chickens.

References


TABLE 1. Least squares means ± standard error and P-values for percent positive leukocytes expressing cell surface antigens for Bu-1, CD14, CD3, CD4, CD8, and CD4+CD8+ double positive

<table>
<thead>
<tr>
<th>Line (LS Mean)</th>
<th>Bu-1</th>
<th>CD14</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4+CD8+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler</td>
<td>7.30 ± 0.90</td>
<td>25.07 ± 3.13</td>
<td>14.88 ± 1.98&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.05 ± 1.86</td>
<td>7.12 ± 1.64</td>
<td>1.12 ± 0.21</td>
</tr>
<tr>
<td>F&lt;sub&gt;5&lt;/sub&gt; (Br x G-B2)</td>
<td>6.79 ± 0.86</td>
<td>21.15 ± 2.97</td>
<td>16.32 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.63 ± 1.76</td>
<td>12.96 ± 1.57</td>
<td>1.25 ± 0.20</td>
</tr>
<tr>
<td>F&lt;sub&gt;5&lt;/sub&gt; (Br x M15.2)</td>
<td>5.69 ± 0.84</td>
<td>21.72 ± 2.98</td>
<td>16.71 ± 1.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.70 ± 1.75</td>
<td>7.75 ± 1.54</td>
<td>1.31 ± 0.20</td>
</tr>
<tr>
<td>G-B1</td>
<td>6.15 ± 1.09</td>
<td>14.99 ± 3.75</td>
<td>9.17 ± 2.40&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>11.84 ± 2.26</td>
<td>11.45 ± 1.99</td>
<td>0.86 ± 0.26</td>
</tr>
<tr>
<td>G-B2</td>
<td>6.39 ± 1.21</td>
<td>17.19 ± 4.28</td>
<td>7.71 ± 2.67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.02 ± 2.51</td>
<td>11.53 ± 2.21</td>
<td>1.13 ± 0.29</td>
</tr>
<tr>
<td>M15.2</td>
<td>7.29 ± 1.26</td>
<td>21.75 ± 4.64</td>
<td>12.28 ± 2.95&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.70 ± 2.77</td>
<td>7.58 ± 2.44</td>
<td>1.12 ± 0.32</td>
</tr>
<tr>
<td>M5.1</td>
<td>7.44 ± 1.23</td>
<td>21.02 ± 5.29</td>
<td>16.14 ± 2.72&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.96 ± 2.55</td>
<td>10.77 ± 2.25</td>
<td>1.47 ± 0.29</td>
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</table>

P-value

<table>
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<tr>
<th>Cell Surface Antigens</th>
<th>0.83</th>
<th>0.58</th>
<th>0.04</th>
<th>0.17</th>
<th>0.12</th>
<th>0.75</th>
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<tr>
<td>Line</td>
<td>0.32</td>
<td>0.96</td>
<td>0.49</td>
<td>0.91</td>
<td>0.62</td>
<td>0.08</td>
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<tr>
<td>Sex</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.62</td>
<td>0.46</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1 Leukocytes expressing both CD4 and CD8 cell surface antigens

<sup>a-d</sup> Means within a column that do not share a common superscript differ significantly (P < 0.05, Student’s t test)
TABLE 2. Least squares means ± standard error and P-values for fluorescent intensity of leukocytes expressing cell surface antigens for Bu-1, CD14, CD3, CD4, CD8, and CD4+CD8+ double positive

<table>
<thead>
<tr>
<th>Line (LS Mean)</th>
<th>Bu-1</th>
<th>CD14</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4+CD8+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler</td>
<td>31.37 ± 2.78</td>
<td>13.58 ± 3.03</td>
<td>21.45 ± 3.43&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.45 ± 0.03</td>
<td>32.17 ± 2.44&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>117.29 ± 14.09</td>
</tr>
<tr>
<td>F5 (Br x G-B2)</td>
<td>36.33 ± 2.66</td>
<td>17.34 ± 2.90</td>
<td>21.66 ± 3.28&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.40 ± 0.03</td>
<td>21.09 ± 2.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>145.45 ± 13.48</td>
</tr>
<tr>
<td>F5 (Br x M15.2)</td>
<td>39.83 ± 2.63</td>
<td>14.03 ± 2.86</td>
<td>19.11 ± 3.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.39 ± 0.03</td>
<td>34.77 ± 2.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>132.41 ± 13.30</td>
</tr>
<tr>
<td>G-B1</td>
<td>33.12 ± 3.83</td>
<td>23.17 ± 3.69</td>
<td>31.77 ± 4.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.48 ± 0.04</td>
<td>20.69 ± 2.97&lt;sup&gt;d&lt;/sup&gt;</td>
<td>150.84 ± 17.13</td>
</tr>
<tr>
<td>G-B2</td>
<td>38.95 ± 3.76</td>
<td>15.94 ± 4.09</td>
<td>38.20 ± 4.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36 ± 0.04</td>
<td>25.50 ± 3.30&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>172.21 ± 19.03</td>
</tr>
<tr>
<td>M15.2</td>
<td>39.62 ± 4.15</td>
<td>10.33 ± 4.52</td>
<td>20.90 ± 5.11&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.38 ± 0.05</td>
<td>33.90 ± 3.65&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>104.73 ± 21.03</td>
</tr>
<tr>
<td>M5.1</td>
<td>39.62 ± 4.15</td>
<td>10.33 ± 4.52</td>
<td>20.90 ± 5.11&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.38 ± 0.05</td>
<td>33.90 ± 3.65&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>104.73 ± 21.03</td>
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<table>
<thead>
<tr>
<th>P-value</th>
<th>Line</th>
<th>Sex</th>
<th>Assay Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.06</td>
<td>0.25</td>
<td>0.02</td>
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<tr>
<td></td>
<td>0.33</td>
<td>0.32</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>0.03</td>
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</tr>
</tbody>
</table>

<sup>1</sup> Leukocytes expressing both CD4 and CD8 cell surface antigens
<sup>a-d</sup> Means within a column that do not share a common superscript differ significantly ($P < 0.05$, Student’s t test)
### TABLE 3. Least squares means ± standard error and P-values for percent positive cell population ratios in chicken peripheral blood, by line

<table>
<thead>
<tr>
<th>Line</th>
<th>CD4:CD8</th>
<th>Bu-1:CD3</th>
<th>CD14:CD3+Bu-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler</td>
<td>1.66 ± 0.20</td>
<td>0.56 ± 0.13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.24 ± 0.25</td>
</tr>
<tr>
<td>F&lt;sub&gt;5&lt;/sub&gt; (Br x G-B2)</td>
<td>1.09 ± 0.19</td>
<td>0.56 ± 0.12&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.02 ± 0.24</td>
</tr>
<tr>
<td>F&lt;sub&gt;5&lt;/sub&gt; (Br x M15.2)</td>
<td>1.09 ± 0.19</td>
<td>0.35 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.07 ± 0.23</td>
</tr>
<tr>
<td>G-B1</td>
<td>1.18 ± 0.24</td>
<td>0.77 ± 0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.24 ± 0.30</td>
</tr>
<tr>
<td>G-B2</td>
<td>1.40 ± 0.27</td>
<td>1.10 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.51 ± 0.33</td>
</tr>
<tr>
<td>M15.2</td>
<td>0.97 ± 0.30</td>
<td>0.62 ± 0.19&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.21 ± 0.37</td>
</tr>
<tr>
<td>M5.1</td>
<td>1.16 ± 0.27</td>
<td>0.91 ± 0.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.13 ± 0.34</td>
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**P-value**

<table>
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<tr>
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<tr>
<td></td>
<td>0.39</td>
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<td>0.71</td>
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<td>0.02</td>
<td>0.55</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>0.71</td>
<td>0.04</td>
<td>0.00</td>
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</tbody>
</table>

<sup>a-c</sup> Means within a column that do not share a common superscript differ significantly (P < 0.05, Student’s t test)
CHAPTER 3. BREED EFFECT ON EARLY CYTOKINE mRNA EXPRESSION IN SPLEEN AND CECUM OF CHICKENS WITH AND WITHOUT SALMONELLA ENTERITIDIS INFECTION

A paper published in Developmental and Comparative Immunology

Jennifer H. Cheeseman, Michael G. Kaiser, Ceren Ciraci, Pete Kaiser, and Susan J. Lamont

Abstract

We examined mRNA expression of 11 genes: BAK, Bcl-x, Interferon [IFN] -γ, Interleukin [IL]-1β, IL-6, IL-10, IL-12α, IL-12β, IL-18, CXCLi2 [IL-8/CAF], and a MIP family chemokine, CCLi2, in the spleen and cecum of day-old chicks after oral inoculation with Salmonella enteritidis (SE) or medium. Three distinct chicken breeds (broiler, Fayoumi, and Leghorn) were evaluated for mRNA expression levels at 2 and 18 hours post-inoculation using quantitative RT-PCR. SE exposure significantly increased splenic IL-18 and IFN-γ expression. Breed effect was significant (P < 0.05) for CXCLi2, IL-10, IL-12α, and CCLi2 mRNA expression in the spleen, and for IL-12α, IL-12β, IL-18, and CCLi2 mRNA expression in the cecum. Generally, mRNA expression levels were higher in the spleen, and lower in the cecum, of Leghorns versus broilers. These results support a role for breed genetics influencing cytokine mRNA expression in young chickens and may potentially explain some generalized immune response differences between breeds.
Introduction

Genetic resistance to disease in the chicken has been identified for various types of pathogens, most notably in the association of MHC with Marek’s disease resistance (DiFronzo and Scheirman, 1989; Zekarias et al., 2002). Additional genetic disease resistance for viral pathogens in poultry has been established for Newcastle disease, infectious bursal disease, and avian leukosis (Bumstead 1998; Hassan et al., 2004). Varied resistance to intestinal parasites such as Ascaridia galli and Eimeria tenella has been shown in different commercial and outbred chicken lines, demonstrating a role of genetics in disease resistance for this class of pathogen (Pinard-Van Der Lann et al., 1998; Schou et al., 2003). Studies of response to bacteria, such as Salmonella, in the chicken show that a strong genetic component is involved (Bumstead and Barrow, 1988; Guillot et al., 1995; Kramer et al., 2001; Kramer et al., 2003). Differences in host resistance to Salmonella is observed among commercial broiler lines (Kramer et al., 2001; Kramer et al., 2003), inbred or experimental lines (Bumstead and Barrow, 1988; Guillot et al., 1995), and with individual bacterial species (Bumstead and Barrow, 1988; Kramer et al., 2001).

Cytokines are an integral part of the host immune response to Salmonella in mammalian species and in cell culture models of infection. Specifically, increased mRNA expression and protein secretion of chemokines, proinflammatory and Th1 cytokines such as IFN-γ, IL-1, IL-6, IL-8, IL-12, and MIP-1β are observed following infection with various Salmonella species (Eckmann and Kagnoff, 2001). Production of IL-12 and IFN-γ are critical to host defense against intra-cellular pathogens such as mycobacteria and salmonella (Jouanguy et al., 1999). By promoting IFN-γ production, IL-18 supports host resistance and clearance of bacterial infections (Bohn et al., 1998; Garcia et al., 1999; Mastroeni et al., 1999). Avian cytokines, like their mammalian counterparts, are influential in the host immune response to infection. Lymphokines produced from immunized spleen cells of adult hens or from a T cell line have protective properties, reducing bacterial counts when injected into young birds challenged with SE (Genovese et al., 1998; Ziprin and Kogut, 1997). Young chicks defined as SE susceptible to cecal carrier state had significantly lower expression of
IFN-γ compared to resistant and healthy control animals, indicating a positive role for this cytokine in the reduction of pathogen burden (Sadeyen et al., 2004). Recombinant chicken IFN-γ, when co-administered with inactivated SE, reduced intestinal colonization, thus providing additional evidence for a protective role of IFN-γ in Salmonella infections (Takehara et al., 2003).

*Salmonella*-induced gene expression profiles suggest the importance of additional cytokines and chemokines in the avian host defense response (Kaiser et al., 2000; Sadeyen et al., 2004; Withanage et al., 2004; Withanage et al., 2005). While Withanage et al., 2005, did not observe changes in gastrointestinal expression of IL-1β, IL-6, IL-8, and K60 following *S. typhimurium* infection of one week-old chicks at 1, 3 or 7 days post-infection, Kaiser et al., 2000, determined IL-6 mRNA is upregulated in chicken kidney cells infected with various *Salmonella sp.* (Kaiser et al., 2000), IL-1β mRNA expression was upregulated by *S. typhimurium* and infection with *S. enteritidis* resulted in decreased IL-2 mRNA expression thus showing differences in the pathogenicity of both *Salmonella sp.* and the importance of tissue or cell choice in evaluating gene expression. An additional study showed the spatial/temporal dynamics of chemokine and pro-inflammatory cytokine expression (Withanage et al., 2004). In general, increased mRNA gene expression for the jejuna, ilea, and cecal tonsil was first observed between 6 and 12 hours post-infection, while similar changes were not observed until later time points in the liver (12 to 24 hours) and spleen (48 hours) (Withanage et al., 2004).

Functional studies of chicken cytokines suggest similar properties as in their mammalian counterparts. Lipopolysaccharide (LPS) stimulates chicken macrophages to secrete IL-1β, which induced CXC mRNA transcripts in a chicken embryonic fibroblast cell line (Weining et al., 1998). Chicken IL-12 requires both subunits (p35 and p40) for biological activity and the recombinant protein stimulates proliferation and IFN-γ production in isolated chicken splenocytes (Degen et al., 2004). Interleukin-18 strongly induces IFN-γ production in chicken splenocytes and serves as a growth factor for CD4+ T cells (Gobel et al., 2003). Recombinant chicken IL-10 inhibited IFN-γ synthesis in activated lymphocytes...
Chicken MIP-1β has demonstrated chemotactic properties having induced heterophil and lymphocyte migration (Lam 2002); however, current literature would suggest that a direct comparison of this gene to the mammalian MIP-1β chemokine is incorrect (Kaiser et al., 2005). Proposed is a new nomenclature for chicken inflammatory chemokines numbered according to genomic organization, where CXCl2 corresponds to CAF/IL-8 and CCl2 denotes the MIP-like family member previously known as chicken MIP-1β (Kaiser et al., 2005).

*Salmonella enteritidis* (SE) is the main serovar responsible for non-typhoidal *Salmonella* infections in poultry food products in the US (Patrick et al., 1999). An average of 35% of US chicken flocks sampled were reported as SE positive (Ebel and Schlosser, 2000), and 90.1% of SE isolates from food, human, or poultry sources were resistant to one or more antimicrobial drugs (De Oliveria et al., 2005). Understanding the genetics of host-immune response to SE would provide non-drug-based options to reduce pathogen contamination of poultry products.

Associations of single nucleotide polymorphisms in chicken genes with spleen bacterial load (Lamont et al., 2002; Liu et al., 2003; Liu and Lamont, 2003; Malek et al., 2004), and TRAIL, TGF-β3, and CD28 with cecum bacterial burden (Malek et al., 2004) have been identified for MHC class I, natural resistance-associated macrophage protein 1 (NRAMP1), MD-2, and the inhibitor of apoptosis protein-1 (IAP-1). Having shown association of sequence variation in genes involved in various immunological and apoptosis-related networks with SE bacterial load, it was of interest to investigate early gene expression levels in young chicks orally challenged with SE.

An important aspect of the current study was to examine the role of genetic breed on mRNA expression in proinflammatory, Th1/Th2 induction, and apoptosis-related genes early in the post-hatch period of chicks. With the significance of host genetics in avian disease resistance (Zekarias et al., 2002), and general immunological differences between layer and broiler breeds (Koenen et al., 2002; Leshchinsky and Klasing, 2001) firmly established, we examined mRNA expression in three genetically diverse chicken breeds. The outbred broiler
birds, which originated from a commercial broiler breeder line (Kaiser et al., 1998), are reflective of commercial genetic selection for rapid growth and high muscle percentage. The highly inbred Leghorn (G-B2) layer line has been used in disease resistance and MHC-restricted immune response studies (Dil and Qureshi, 2002a: Dil and Qureshi, 2002; Lakshmanan et al., 1996) The highly inbred Egyptian Fayoumi line, which was imported to the US because of reported resistance to leukosis, is genetically distant from the broiler and Leghorn lines (Lakshmanan et al., 1996; Zhou and Lamont, 1999). It represents a native breed that was not subjected to the genetic selection history of commercial broiler and layer chickens. Thus, the three breeds utilized in the current study are distinct, both genetically and immunologically, and serve as a strong contrast of the variation that exists within the species.

**Materials and Methods**

**Salmonella Enteritidis**

Nalidixic acid resistant virulent SE phage type 13a (gift of H.M. Opitz, University of Maine, Orono, ME) was propagated as described previously (Kaiser and Lamont, 2002). Briefly, bacteria were cultured in Luria-Bertani (LB) broth at 37°C with agitation until reaching an exponential growth phase. *Salmonella enteritidis* concentration within the inoculum was estimated using optical density at 600 nm. At one-day of age, chicks were intraesophageally inoculated with $1 \times 10^4$ cfu in 0.25 ml LB broth. Chicks not exposed to SE were mock inoculated in a similar manner with 0.25 ml LB broth.

**Experimental Animals**

Seventy-two chicks, 24 each of broiler, Fayoumi, and Leghorn breeds, were used. Within each breed, half of the chicks received SE inoculation and half were mock inoculated. The broiler birds originated from a commercial broiler breeder line (Kaiser et al., 1998). The Fayoumi and Leghorn animals are highly (>99%) inbred (Zhou and Lamont, 1999). Hatched chicks were wing-banded and placed in pens in biosafety level-2 animal rooms, and they
were not fed prior to inoculation. Throughout the duration of the experiment the chicks were housed on wood chip bedding and given access to water *ad libitum*.

**Organ Harvest**

At 2 or 18 hours post-inoculation, half of the chicks of each breed were euthanized. Spleens were aseptically removed and immediately placed in 1.5 ml label snap cap tubes and placed into liquid nitrogen. Prior to quick freezing of the cecum in liquid nitrogen, cecal contents were gently removed by lightly squeezing the excised organ with an application of light pressure. Tissue samples were stored at -70°C until processed for RNA isolation.

**Total RNA Isolation**

Total RNA (tRNA) was isolate from homogenized spleen and cecum tissues using RNAqueous (Ambion, Austin, TX) according to manufacturer’s instructions. RNA concentration and purity were determined using the spectrophotometric absorbance at 260 nm and 280 nm. Undiluted tRNA samples were stored at -70°C. Individual sample dilutions (50 ng/µl) were prepared for use in quantitative RT-PCR gene expression assays and stored at -20°C.

**Quantitative Real-Time RT-PCR**

The mRNA expression levels of BAK, Bcl-x, IL-1β, IL-6, IL-10, IL-12α, IL-12β, IL-18, IFN-γ, CCLi2, and CXCLi2 were determined by quantitative real-time RT-PCR, using QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA). Each RT-PCR reaction, run in triplicate, consisted of either 50 ng or 75 ng total RNA, 12.5µl QuantiTect SYBR Green master mix, 0.25 µl QuantiTect RT mix, forward and reverse primers, and RNAse-free water for a final volume of 25 µl. Primer sequences have been previously reported for all genes except BAK, IL-12α, and IL-12β (Degen *et al.*, 2004; Kogut *et al.*, 2003; Koskela *et al.*, 2003; Withanage *et al.*, 2004). Primer sequences for these genes are: BAK (F) 5’ GGGCTGGGCGACCCGGGAG 3’ (R) 5’ CGAACAAGCTGGAGGCGAT 3’, IL-12α (F)
5’ TGGCCGCTGCAAACG 3’ (R) 5’ ACCTCTTCAAGGGTGCACTCA 3’, and IL-12β (F)
5’ TGTCTCACCTGCTATTTGCCTTAC 3’  (R) 5’
CATACACATTCTCTCTAAAGTTTCCACTGT 3’. The q-RT-PCR reactions were performed on an Opticon 2 (MJ Research Inc., Waltham, MA) with the following program: 1 cycle at 50°C for 30 min., 95°C for 15 min. followed by 40 cycles of 94°C for 15 sec., 59°C for 30 sec., and 72°C for 30 sec followed by reading of the plate. Gene slopes were determined with a series of 10-fold plasmid dilutions. A melting curve from 60-90°C with a reading at every 1°C was also performed for each individual RT-PCR plate. Cycle threshold (Ct) values were calculated as follows:

\[
\frac{(40-\text{Ct sample mean})(\text{Gene slope})}{(28S \ 40-\text{Ct sample mean} / \text{Overall 40-Ct sample mean})(28S \text{ slope})}
\]

**Statistical Analysis**

Individual animal mRNA data are represented as the mean of triplicate measurements. Expression levels of mRNA for each gene were analyzed by using JMP software with an ANOVA model (SAS Institute, 2004). Breed, SE challenge status, and post inoculation sample time (2 or 18 hours) were considered fixed main effects. All interactions for the fixed main effects were tested. All were non-significant (P-value ≥ 0.1) and excluded from the final model with the exception of the single instance of significant interaction, Breed x SE exposure on IL-6 mRNA expression in the spleen.

Student’s T test was used to determine individual rankings of classes within the significant main effect (Breed) and the interaction of Breed x SE on splenic IL-6 mRNA expression. Because sample collection time and SE exposure status, as well as all two-way interactions, were generally not significant factors on mRNA expression level, data of all tests for each individual gene within each breed were pooled for analysis of breed effect, so that there were 24 individuals per breed, each tested in triplicate, for each gene’s mRNA measurement.
Results

Gene Expression in the Spleen

Breed was the only consistent main effect that significantly impacted mRNA expression in the spleen (Table 1). Breed effect occurred for CXCLi2 (CAF/IL-8), IL-10, IL-12α, and the MIP-like family chemokine CCLi2. There were very few (3/22) highly significant effects due to either SE exposure or sample time. Two of these were the effect of SE exposure on IL-18 and IFN-γ mRNA expression. Challenge with SE increased IL-18 mRNA expression ($P = 0.007$) with LS means Ct values of 17.8 ± 0.2 for SE exposed versus 17.0 ± 0.2 for unexposed birds [regardless of breed?]. Challenge with SE also increased IFN-γ mRNA expression ($P = 0.032$) with LS means Ct values of 13.6 ± 0.2 for SE exposed versus 12.9 ± 0.2 for unexposed birds. Higher IL-18 mRNA expression was observed for the 18-hour sample time compared to 2 hours with LS means Ct values of 17.9 ± 0.2 and 16.9 ± 0.2, respectively. A significant interaction ($P = 0.006$) of breed X SE exposure was observed for IL-6 (Table 1), which comes from the differential IL-6 mRNA expression in only the Leghorn breed after SE exposure. The Leghorn breed upregulated IL-6 mRNA levels following SE challenge, with LS means Ct values for SE-exposed birds of 22.2 ± 0.4 and those unexposed to SE with a Ct value of 20.7 ± 0.3.

For the cytokine genes for which breed had a significant effect on mRNA expression levels, a consistent pattern can be observed. The Leghorn birds show the highest mRNA gene expression for CXCLi2, IL-10, and IL-12α (Fig. 1), while the broiler breed had the lowest expression, which differed significantly (p value) from Leghorns. The trend of higher mRNA expression of the Leghorns compared to broilers extends to the chemokine CCLi2 as well; however the Fayoumi breed had the lowest level of CCLi2 expression. Breed effect was not significant on the expression of the apoptosis-related genes BAK and Bcl-x, Th1/Th2 genes IL-12β and IL-18, or the proinflammatory cytokines IL-1β and IL-6 in the spleen.

Gene Expression in the Cecum
Breed was the only consistent main effect that was significant on mRNA expression in the cecum for IL-12α, IL-12β, IL-18, and CCLi2 genes (Table 1), with the exception of sample time on IL-6 mRNA gene expression in the cecum. The IL-6 mRNA transcript was higher at 18 hours than the 2 hour sample period. The LS means Ct values of IL-6 cecal mRNA expression at 18 hours versus 2 hours were 21.0 ± 0.3 and 20.0 ± 0.3, respectively.

In contrast to the pattern of higher mRNA expression in the spleen for the Leghorn breed, we observed the opposite trend for cecal tissue. CCLi2, IL-12α, and IL-12β cytokine mRNA expression were consistently and significantly higher in the broiler chicks compared to the Leghorns (Fig. 2). Additionally, the broilers were significantly higher than the Fayoumis in IL-12β mRNA expression. Although the broilers had the highest level of cecal mRNA expression in IL-12α, IL-12β, and CCLi2 genes, this breed had a significantly lower level of IL-18 expression than both the Fayoumi and Leghorn birds. No effect of breed was observed for the apoptosis related genes, BAK and Bcl-x, the proinflammatory cytokines IL-1β, IL-6, and CXCLi2, as well as the Th2-inducing cytokine IL-10.

Discussion

We investigated early mRNA expression of cytokine and apoptosis-related genes in day-old chicks of three diverse breeds (Leghorn, Fayoumi, and broiler), from two tissue sources (spleen and cecum), with and without SE challenge, and at two post-inoculation harvest times (2 and 18 hours). Splenic IL-18 and IFN-γ mRNA expression was higher in SE-exposed chicks. These results suggest that avian spleen cells may rapidly respond to Salmonella pathogen exposure, within hours of inoculation, likely influencing the subsequent host immune response.

IL-18 promotes IFN-γ expression, the hallmark of Th1 immune responses (Gobel et al., 2003). Previous studies of chicken cells in vitro have shown increased IL-18 mRNA expression in spleen cell cultures in response to LPS stimulation (Sijben et al., 2003), and in heterophils from chicken lines cultured with either opsonized or non-opsonized SE (Swaggerty et al., 2004; Weining et al., 1998). Results of SE-enhanced mRNA expression of
IL-18 in the whole, in situ spleen provide additional evidence for the role of this Th1-promoting, proinflammatory, cytokine in the avian immune response to intracellular pathogens like *Salmonella*. Additionally, we observed IFN-γ mRNA upregulated in the spleen of SE-exposed chicks. Taken together, our results show that exposure of live young chicks to SE induces the in situ production of splenic Th1-promoting mRNA gene transcripts. Our results of SE-induced up-regulation of splenic IL-18 and IFN-γ mRNA expression do not agree with a previous study (Withanage *et al.*, 2004) that found IFN-γ mRNA expression increased at 7 days post-infection but not at either 1 or 3 day time points. These conflicting results may be due to choice of *Salmonella* species, as the current experiment examined the effect of SE on mRNA expression, while Withanage *et al.*, 2004, examined mRNA gene expression after *S. typhimurium* inoculation, or to different genetics of the chickens studied.

A significant interaction (p value) between SE exposure and breed was observed for expression of IL-6 mRNA in the spleen in the current study. Leghorns that were exposed to SE produced significantly more IL-6 mRNA compared to their unexposed counterparts, while no difference was observed for the broiler or Fayoumi animals exposed or unexposed to SE. These results are in accordance with other studies and demonstrate breed, or genetic line, influences the immune response to pathogens in chickens. Heterophils from SE resistant lines isolated at one-day of age produced more IL-6 mRNA than heterophils from susceptible lines when cultured with opsonized and non-opsonized SE (Swaggerty *et al.*, 2004). Splenocytes from Line 7 and Line P, lines defined as being susceptible to Marek’s disease virus, each were shown to produce higher levels of IL-6 mRNA at 3-5 days post-infection compared to resistant lines 6 and N under similar conditions (Kaiser *et al.*, 2003).

The present study was designed to examine early (2 and 18 hours) mRNA expression changes associated with in vivo oral inoculation of SE in three genetically diverse breeds of chickens. With the exception of IL-18 and IFN-γ mRNA expression in the spleen, SE inoculation did not significantly affect early mRNA expression in the present study. The limited SE-induced mRNA gene expression changes detected in the present study may be a
result of the early sampling times. SE reaches the cecum in young chicks within 6 to 12 hours of oral inoculation, whereas bacteria require 1 to 2 days to colonize the spleen and liver (van Immerseel et al., 2002; Withanage et al., 2004). In our experimental design, the cecum has likely been exposed to SE and various environmental antigen sources within 18 hours, while the spleen has not. Therefore, gene mRNA expression in the spleen may represent a constitutive level of mRNA expression for this organ. Other studies have shown Salmonella-induced mRNA expression changes at 48 hours to several weeks post-exposure (Sadeyen et al., 2004; Withanage et al., 2004; Withanage et al., 2005). Expression of CXCLi2 and IL-1β mRNA was enhanced in the cecum of SE-exposed birds at 2 and 6 weeks post-bacterial inoculation (Sadeyen et al., 2004). In S. typhimurium infected birds, CCLi2 and IL-1β mRNA expression in the spleen was upregulated at 48 hours post-inoculation (Withanage et al., 2004). Enhanced IL-10 mRNA expression in the spleen and small intestine was observed after 6 days post-infection with Eimeria maxima (Rothwell et al., 2004). Another study found that mRNA expression of pro-inflammatory cytokines IL-1β, CXCLi2, and CCLi2 was upregulated in the cecum and jejunum of chickens starting at 3-4 days post-infection with either E. tennela or E. maxima (Laurent et al., 2001).

The role of host genetics on cytokine mRNA expression in the chicken has been investigated primarily in the context of pathogen-induced changes (Kaiser et al., 2003; Sadeyen et al., 2004; Swaggerty et al., 2004). In the current study, we observed some consistent patterns of mRNA expression among the three chicken breeds and in the two organs examined. Leghorns had a higher level of mRNA expression than the broilers for CXCLi2, IL-10, IL-12α, and CCLi2 in the spleen. In the cecum, however, broilers had a higher level of mRNA expression than the Leghorns for IL-12α, IL-12β, and CCLi2. These organ-specific mRNA expression patterns are likely a result of several factors including: the natural route of oral antigen exposure, resident cell populations within an organ, and differences in immune function between layer (Leghorn) and broiler chickens.

Histological examinations of the cell populations in the spleen and cecum have been previously reported. The spleens of seven-day old broilers contain approximately 30% T
cells, 20% B cells, and 12% macrophages (Gomez et al., 1998). Therefore, in this experiment, total lymphocytes vastly outnumbered (approximately 4:1) the population of macrophages. In another study that examined the area percentage of various cells in the cecum of 2-day old chicks, this organ contained 3% macrophages, 1% T cells, and 0.2% B cells (van Immerseel et al., 2002). Furthermore, upon oral exposure to SE a large influx of macrophages, but not T or B cells, occurred within 10 hours. Different cell compositions of these organs may explain the organ-specific mRNA expression levels observed in the present experiment.

Macrophages are an important immunological cell type likely to respond upon pathogenic bacterial exposure in the cecum of young chicks and genetic background influences macrophage effector function, measured as nitric oxide production (Dil and Qureshi, 2002a; Dil and Quershi, 2002b). In comparison of macrophages from two Leghorn lines, K-strain and G-B2, the MØ from the G-B2 birds consistently produced less nitric oxide (NO) after stimulation with LPS from a variety of bacterial species. The authors postulate that the G-B2 “hypo-responder” macrophages have weaker “LPS-mediated signaling via CD14, TLR4, and NF-κB” than the K-strain birds. Thus, in an organ that uses macrophages as the primary responding cell, as the cecum, having low macrophage LPS responsiveness may significantly skew cytokine mRNA expression levels. This may be especially relevant as the “hypo-responder” G-B2 birds are from the same highly inbred line of Leghorn chicks used in the present study. While showing the Leghorn G-B2 birds have muted macrophage responses, which correlate with the present observation of decreased cecal mRNA levels for IL-12α, IL-12β, and CCLi2 in these animals, specific cytokine mRNA or protein production was not examined in the prior studies (Dil and Qureshi, 2002a; Dil and Quershi, 2002b). Perhaps not all macrophage effector pathways or responses are equally diminished in the G-B2 Leghorns, and although this remains to be fully elucidated, the present data of higher cecal IL-18 mRNA expression in Leghorns (compared to broilers) suggests that a more detailed investigation of G-B2 macrophage function is warranted. Alternatively, broiler macrophages may represent an extreme hypo-responsive type.
Toll-like receptor (TLR) activation on antigen presenting cells results in the production of numerous cytokines such as IL-12 and IL-10, and chemokines such as CXCL12 and CCL12, and critical to this innate immune response is the signal transduction molecule, NF-κB (Li and Stark, 2002; Luster, 2002). Fundamental immunological differences between broiler and layer (Leghorn) chickens are well established (Koenen et al. 2002; Leshchinsky and Klasing, 2001). Gene-expression profiles of broiler and layer birds showed that NF-κB was expressed at lower levels in broilers pre- and post-LPS injection compared to layers (Zhu 2005). Toll-like receptor (TLR) activation on antigen presenting cells results in the production of numerous cytokines such as IL-12 and IL-10, and chemokines such as CXCL12 and CCL12, and critical to this innate immune response is the signal transduction molecule, NF-κB (Li and Stark, 2002; Luster, 2002). The lower expression levels of NF-κB activation may help to explain our results of lower splenic mRNA expression, of broilers versus layers for proinflammatory chemokines, CXCL12 and CCL12, and the Th1 (IL-12α) or Th2 (IL-10) promoting cytokines observed in the current experiment.

Oral inoculation with SE had limited effects on early mRNA expression in the spleen and cecum of day-old chicks from the three breeds examined. The current study, however, provides strong evidence of genetic influence on immunological gene expression, as well as providing additional information on the biological differences between broiler, layer (Leghorn), and Fayoumi chickens.

**Acknowledgements**

The authors thank Jason Hasenstein and Huaijun Zhou for excellent technical assistance and sample preparation. This work was supported by National Research Initiative Grant no. 2004-35205-14234 from the USDA Cooperative State Research, Education, and Extension Service; Research Grant IS-3021-98CR from BARD, the Binational Agriculture Research and Development Fund; and Animal Health, State of Iowa and the Iowa State University Center for Integrated Animal Genomics Funds.
References


Table 1
Breed, SE exposure, and time effects on mRNA expression in the spleen and cecum of day-old chicks (P values, ANOVA)

<table>
<thead>
<tr>
<th>Variables</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>CXCL2</th>
<th>CCL2</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>IL-12α</th>
<th>IL-12β</th>
<th>IL-18</th>
<th>BAK</th>
<th>Bel-x</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breed</td>
<td>0.107</td>
<td>0.136</td>
<td>0.003</td>
<td>0.044</td>
<td>0.258</td>
<td>0.044</td>
<td>0.033</td>
<td>0.059</td>
<td>0.943</td>
<td>0.703</td>
<td>0.564</td>
</tr>
<tr>
<td>SE Exposure</td>
<td>0.183</td>
<td>0.248</td>
<td>0.388</td>
<td>0.216</td>
<td>0.032</td>
<td>0.318</td>
<td>0.719</td>
<td>0.757</td>
<td>0.007</td>
<td>0.429</td>
<td>0.927</td>
</tr>
<tr>
<td>Time post-SE</td>
<td>0.397</td>
<td>0.259</td>
<td>0.924</td>
<td>0.375</td>
<td>0.933</td>
<td>0.101</td>
<td>0.398</td>
<td>0.564</td>
<td>&lt;0.001</td>
<td>0.318</td>
<td>0.417</td>
</tr>
<tr>
<td><strong>Cecum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breed</td>
<td>0.174</td>
<td>0.871</td>
<td>0.339</td>
<td>0.045</td>
<td>0.202</td>
<td>0.354</td>
<td>0.042</td>
<td>0.014</td>
<td>0.014</td>
<td>0.692</td>
<td>0.476</td>
</tr>
<tr>
<td>SE Exposure</td>
<td>0.873</td>
<td>0.455</td>
<td>0.580</td>
<td>0.639</td>
<td>0.218</td>
<td>0.932</td>
<td>0.162</td>
<td>0.960</td>
<td>0.454</td>
<td>0.378</td>
<td>0.179</td>
</tr>
<tr>
<td>Time post-SE</td>
<td>0.263</td>
<td>0.022</td>
<td>0.367</td>
<td>0.115</td>
<td>0.735</td>
<td>0.909</td>
<td>0.161</td>
<td>0.072</td>
<td>0.463</td>
<td>0.823</td>
<td>0.758</td>
</tr>
</tbody>
</table>

*Variables in the ANOVA model include Breed (Broiler, Fayoumi, and Leghorn), SE exposure or media, and Time post-SE (2 or 18 hours).*
Fig. 1. Mean mRNA expression levels, by breed, in the spleen of day-old chicks. Bars, within gene, not sharing a letter (when letters are shown) are significantly different by LS Means Student’s t Test.
Fig. 2. Mean mRNA expression levels, by breed, in the cecum of day-old chicks. Bars, within gene, not sharing a letter (when letters are shown) are significantly different by LS Means Student’s t Test.
CHAPTER 4. MACROPHAGE POPULATION DYNAMICS, APOPTOSIS, AND INFLAMMATORY CXCLi CHEMOKINE mRNA EXPRESSION IN THE CECUM OF CHICKS WITH SALMONELLA ENTERITIDIS INFECTION

A manuscript submitted to Avian Diseases

Jennifer H. Cheeseman, Nyssa A. Levy, Pete Kaiser, Hyun S. Lillehoj, and Susan J. Lamont

Abstract

To better understand the avian host immune response to Salmonella enteritidis (SE), we examined mRNA expression for 8 genes: CXCLi1[K60], CXCLi2 [IL-8/CAF], interferon [IFN] -γ, interleukin [IL] -1β, IL-6, IL-12α, IL-12β, and gallinacin [Gal] -2 in the cecum of young chicks one week post-inoculation with SE. Cecal tissue sections were stained and evaluated for the presence of macrophages, lymphocytes, heterophils, and apoptotic cells following SE infection. Using quantitative RT-PCR, SE infection was associated with a significant ($P < 0.01$) up-regulation of cecal CXCLi1 and CXCLi2 mRNA expression. Infection with SE was also associated ($P < 0.05$) with increased staining for macrophages and decreased apoptosis (ssDNA) in cecal tissue sections when compared to uninfected animals. Changes in chemokine expression and cell population dynamics are a direct result of SE infection, as uninfected animals do not show these alterations. Thus, these SE induced changes reflect the host immune response to SE in young chickens.

12 Submitted to Avian Diseases
13 Graduate student and Professor, respectively, Department of Animal Science, Iowa State University.
14 Primary researcher and author.
15 Associate researcher and author.
16 Collaborator, Department of Animal Health, Compton, Berkshire RG20 7NN, UK.
17 Collaborator, Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, USDA-ARS, East Beltsville, MD 20705, USA.
18 Author for correspondence.
**Introduction**

Poultry eggs and meat contaminated with *Salmonella enterica* serovar *enteritidis* continue to be very significant sources of human food poisoning in the United States (Patrick *et al.*, 2004; Schroeder *et al.*, 2005) with approximately 35% of US flocks testing positive for *Salmonella enteritidis* (SE) in 2000 (Ebel and Schosser, 2000). Antimicrobial resistance in SE strains is widespread, with more than 90% being resistant to at least one antimicrobial drug, and over 50% of SE strains isolated from poultry have been found to be multi-drug resistant (De Oliveria *et al.*, 2005). A more thorough understanding of the avian immune response to SE may provide novel insights for vaccine development as well as enhancing host immunity to this problematic bacterial pathogen with an overall goal of reducing human illness.

Once ingested, SE rapidly travels to the cecum within about 12 hours and later becomes a systemic infection reaching the spleen and liver in 24 to 48 hours (van Immerseel *et al.*, 2002). Bacterial colonization of the spleen and liver is undetectable after four weeks post-inoculation indicating chickens are able to resolve infection in these organs; however, SE can persist in the cecum for at least 16 weeks (Gast and Holt, 1998; Sadeyen *et al.*, 2004). Additionally, half of infected hens were shedding SE in feces from 18 to 24 weeks (Gast and Holt, 1998) suggesting long-term cecal colonization and fecal shedding contributes to the risk of SE positive poultry products contaminating human foods.

The cecum, part of the avian gut-associated lymphoid tissue, contains cecal tonsils which are considered secondary lymphoid tissues similar to the spleen and bone marrow (Mathew *et al.*, 2002). Cecal tonsils are diffuse lymphoid tissues that contain germinal centers with distinct B and T cell zones, and are believed to be functionally similar to mammalian Peyer’s patches (Mathew *et al.*, 2002). The development of the cecal tonsils is characterized by dynamic changes in immune cell populations that ultimately result in the complex and highly organized structure of an adult cecal tonsil...
(Del Moral et al., 1998). Already present in the cecum on the day of hatch are well defined leukocytes, T cells, and B cells. These populations continue to expand in number over the following 2 to 6 weeks. TCR2+ (αβ) T cells outnumber TCR1+ (γδ) T cells from 4 days post-hatch, with relatively equal numbers of CD4+ and CD8+ T cells until 2 weeks post-hatch, where CD8+ T cells become the dominant T cell type (Del Moral et al., 1998). As the development and maturation of the cecum, specifically the cecal tonsil, occurs over the first weeks of life in the young chick, it is important to investigate how microflora and pathogen load can influence this process.

Induction of cytokines is a well established consequence of the host immune response to Salmonella infections (Eckmann and Kagnoff, 2001). In response to various Salmonella species, epithelial cell lines produce increased amounts of mRNA and secreted IL-6 and IL-8 protein (Eckmann et al., 1993; Weinstein et al., 1997). Additionally, mouse macrophages upregulated mRNA expression and protein production of interleukin [IL]-1β, IL-6, and IL-12 in response to S. typhimurium (Galdiero et al., 1993; Rosenberger et al., 2000). In mouse models of Salmonella infection, treatment with interferon [IFN]-γ, IL-6, and IL-12 proteins resulted in enhanced survival and decreased bacterial colonization of the spleen and liver (Eckmann et al., 1996). Furthermore, these protective effects are abrogated in animals treated with the corresponding neutralizing antibodies (Eckmann and Kagnoff, 2001).

Although less clearly understood, cytokines also appear to be involved in the avian host immune response to Salmonella (Wigley et al., 2006; Withanage et al., 2004; Withanage et al., 2005). Intraperitoneally injected lymphokines, generated from splenic T cells of SE immunized hens, provide protection against organ invasion of Salmonella in young chicks and turkey poults (Genovese et al., 1998; Zipring and Kogut, 1997). Peripheral blood leukocytes isolated from adult chickens responded to SE in culture by downregulating IL-6, IL-8, and TGF-β4 mRNA transcription (Kaiser et al., 2006). Additionally, we observed an increase in splenic IL-18 and IFN-γ mRNA expression in day-old chicks exposed to SE (Cheeseman et al., 2007).
In macrophages, *Salmonella* can reside within phagocytic vacuoles or can cause apoptosis (Knodler and Finlay, 2001; Navarre and Zychlinsky, 2000). Apoptosis occurs through several well defined stages that represent a mechanism of programmed cell death (Hueffer and Galan, 2004). An early event in apoptosis is the breakdown in the phospholipid bilayer where phosphatidylserine, usually located on the inner layer, becomes externalized to the outer or external layer of the cell membrane (Hanshaw and Smith, 2005). DNA fragmentation, mediated by caspase-activated DNases, is a classical feature of late stage apoptosis (Stadelmann and Lassmann, 2000). Our laboratory has previously demonstrated associations of sequence variation in candidate genes in apoptosis pathways with organ colonization by SE. Caspase-1 was associated with cecal bacterial load (Lamont *et al.*, 2002) and inhibitor of apoptosis protein-1 (IAP-1) was associated with bacterial load in the spleen (Lamont *et al.*, 2002; Liu and Lamont, 2003). These associations and the established role of apoptosis in *Salmonella* infections led us to investigate what involvement apoptosis may have in SE infections in chickens.

The current study examined mRNA expression of cytokines and an antimicrobial peptide, along with changes in immune cell populations and apoptosis, in the cecum of young chicks infected with SE. Understanding the ceca-specific immune response of young chicks infected with SE may provide insights to explain the persistent colonization of this organ, and open avenues to reduce the incidence of contamination in eggs and meat products.

**Materials and Methods**

**Growth and Inoculation of *Salmonella enteritidis***

Nalidixic acid-resistant SE phage type 13a was cultured in Luria-Bertani (LB) broth at 37°C as previously described (Cheeseman *et al.*, 2007). Inoculum concentration was estimated using an optical density measurement at 600 nm. Chicks were intraesophageally inoculated with $1 \times 10^4$ cfu in 0.25 ml LB broth at one day post-hatch. Unexposed chicks were mock inoculated with 0.25 ml LB broth in a similar manner.
Experimental Animals

Two advanced intercross lines (F₈ generation) of chicks initially produced by a cross of a broiler line (meat-type) to two highly inbred light-bodied lines (Leghorn and Fayoumi) were used in the present study. Approximately 90 one day-old chicks (45 per cross) were assigned wing bands and housed in pens in biosafety level-2 animal rooms. Following bacterial (60 chicks) or mock (30 chicks) inoculation, all chicks were given *ad libitum* access to both food and water throughout the duration of the experiment.

Organ Harvest and Bacterial Counts

One week post-inoculation, chicks were euthanized by cervical dislocation. Both ceca were aseptically removed, one placed into a sterile labeled snap cap tube and the other into OTC medium, and quick frozen in liquid nitrogen. Prior to freezing, a sample of cecal contents was obtained using a sterile cotton swab and used for bacterial culture and quantification. Each swab was cultured overnight at 37°C in 10 mL of selenite enrichment broth. To determine SE colony-forming units per milliliter, 10-fold serial dilutions of enrichment broth were plated and cultured overnight at 37°C on brilliant green agar plates that contained 100 µg/mL of nalidixic acid (Kaiser and Lamont, 2001). Cecal tissue samples were stored at -70°C until processed for RNA isolation.

Isolation of RNA

Total RNA was isolated from homogenized frozen cecal tissues using RNAqueous (Ambion, Austin, TX) and DNase treated with DNA-free (Ambion, Austin, TX) according to manufacturer’s instructions. Spectrophotometric absorbance at 260 nm and 280 nm was used to determine total RNA concentration and sample purity. From these stock total RNA isolates, aliquots containing 50 ng/µl were prepared and stored at -20°C until use in gene expression assays.
Quantification of mRNA Expression in Tissue Samples

Quantitative real-time RT-PCR, using QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA), determined the mRNA expression levels of CXCL1[K60], CXCL2 [IL-8/CAF], IFN-γ, IL-1β, IL-6, IL-12α, IL-12β, and gallinacin [Gal] -2. Primer sequences have been previously published for all genes (Cheeseman et al., 2007; Kaiser et al., 2000; Smith et al., 2005; Wigley et al., 2006; Withanage et al., 2004; Withanage et al., 2005). Quantitative real-time RT-PCR reactions, run in triplicate for each sample and gene, were performed on an Opticon 2 (MJ Research Inc., Waltham, MA), as previously described (Cheeseman et al., 2007). Data were transformed and expressed as the adjusted Ct (cycle threshold) value using the following formula:

\[
40 - [(\text{mean test gene Ct}) + (\text{median 28S Ct} - \text{mean 28S Ct}) \times (\text{test gene slope/28S slope})]
\]

Where slopes are determined with a series of 10-fold dilutions of plasmids encoding each target gene to account for PCR efficiency, and median 28S (28S rRNA) Ct represents the median Ct value of all individual samples for this housekeeping reference gene.

Preparation and Staining of Tissue Slides

Individual cecal lobes were placed into OTC medium and quick frozen in liquid nitrogen. Samples were stored at -70°C prior to cutting. Approximately 6 µm thick frozen sections were cut, placed on new slides, and fixed in chilled acetone. Following each staining step, a wash for 5 min in PBS pH 7.5 was performed. Endogenous peroxidase activity was quenched by an initial incubation of 0.3% hydrogen peroxide for 30 min. Using Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) slides were blocked for 20 min with normal horse serum, after which excess serum was blotted. Tissue sections were incubated with the primary antibodies [K1 (macrophages), K55 (lymphocytes), HB2 (human T cells), anti-ssDNA, and normal mouse IgM, or
biotinylated annexin V protein] for 60 min. The K1 and K55 monoclonal antibodies are specific for avian macrophages and pan-lymphocytes, respectively, although the exact molecules recognized have not been determined (Chung and Lillehoj, 1991; Chung et al., 1991). Annexin V binds to phosphatidylserine on B cells with a high level of affinity (Hanshaw and Smith, 2005), and the ssDNA monoclonal antibody binds single-stranded DNA (Stadelmann and Lassmann, 2000). Both annexin V and the ssDNA antibodies are not species-specific reagents. Concentrations used were: K1 (1:10 dilution), K55 (1:25 dilution), HB2 (1:100 dilution), ssDNA (1.0 µg/mL), annexin V (1.0 µg/mL), and normal mouse IgM (5.0 µg/mL) in diluted blocking serum. The HB2 (Hong et al., 2006) and normal mouse IgM antibodies were used as negative controls. Sections were then incubated with biotinylated secondary antibody (anti-mouse IgG or IgM) for 30 min, excluding those stained with Annexin V. Next, slides were incubated 30 min with Vectastatin ABC reagent. Sections were incubated for 5 min in Vector NovaRed peroxidase substrate (Vector Laboratories, Inc., Burlingame, CA) and rinsed with tap water. Following a counter stain with Vector Hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA), slides were air-dried overnight and cover slips mounted with VectaMount mounting medium (Vector Laboratories, Inc., Burlingame, CA). For histological examination of heterophils, tissues were cut, placed on slides, and fixed in a similar manner as reported above and then stained with Vector Hematoxylin QS and Accustain eosin Y solution (Sigma-Aldrich, Inc., St. Louis, MO). Primary staining reagents were obtained from the following sources: biotinylated annexin V protein (BioVision, Mountain View, CA), IgM anti ssDNA isotype (Alexis Corp., Lausen, Switzerland), normal mouse IgM (Bethyl Laboratories Inc., Montgomery, TX).

**Slide Imaging, Staining Analysis, and Heterophil Counts**

Ceca from 24 chicks were stained and analyzed, 12 per genetic cross, representing 8 infected and 4 uninfected with SE. Tissue sections were stained in duplicate per individual chick and staining reagent. Two separate images per slide were obtained on a
Zeiss Axiophot bright field microscope (50x). Data are represented as an average of four measurements and presented as a ratio of red-stained area (positive) to the area of blue-staining (negative). Any unstained (white) area was subtracted from the total image area and excluded from analysis. Heterophils, observed by H & E staining with a Spencer confocal microscope (40x), appeared round or oval in shape, had bi-lobulated nuclei that were bluish purple in color, and an orange cytoplasm (Lucas and Jamroz, 1961; Petrone et al., 2002).

Statistical Analysis

Gene mRNA expression and tissue staining levels were analyzed with an ANOVA model using JMP software (JMP) (SAS Institute, 2004). Individual bird mRNA data are represented as the average of triplicate measurements. Duplicate slides were prepared (on different days) for each tissue stain. Data for cecal tissue staining levels are represented as the average of four images (two per slide).

Results

Increased CXCLI Gene mRNA Expression in the Cecum of Young Chicks at One Week Post-Infection with Salmonella enteritidis

In the current study, expression of cytokine-specific mRNA was measured in response to bacterial infection. From the cecum of infected and uninfected chicks, total RNA was harvested and assayed for expression of CXCLi1 [K60], CXCLi2 [IL-8/CAF], interferon [IFN] -γ, interleukin [IL] -1β, IL-6, IL-12α, IL-12β, and gallinacin [Gal] -2 by RT-PCR. Infection with SE induced a significant increase (p value) in inflammatory CXCL chemokine mRNA expression. Both CXCLi1 (also known as K60) and CXCLi2 (previously referred to as IL-8 and CAF) mRNA transcripts were upregulated in SE infected animals compared to uninfected birds (Fig. 1). SE infection did not induce differential gene expression for IFN-γ, IL-1β, IL-6, IL-12α, IL-12β, or the antimicrobial
peptide Gal-2. No significant differences in gene expression were observed between genders or genetic lines (data not shown).

**Cecal Gene Expression is Not Correlated with Salmonella enteritidis Bacterial Load**

To confirm bacterial colonization of the cecum, we cultured cecal contents in selenite enrichment broth. All SE inoculated chicks were positive for the presence of the bacterium one week after initial exposure, while none of the uninfected birds cultured positive. A broad range of bacterial colonization levels (19-22 natural log) were observed for the SE infected chicks, however there was no significant correlation of bacterial count from the samples of cecal content and individual gene expression level for cecal tissue (data not shown).

**Salmonella enteritidis Infection Induces Recruitment of Macrophages and Decreases Apoptosis in the Chicken Cecum**

To investigate the changes in the numbers of local cell populations in the cecum of neonatal chicks are influenced by SE infection, we used IHC to determine changes in macrophage, lymphocyte, and heterophil numbers in cecal tissue sections. Additionally, both early and late stages of apoptosis were assayed on the tissue sections. A significant increase [p value] in total area staining positive for macrophages (K1) in the cecum was associated with SE infection, suggesting an influx (Fig. 2 & 3). SE infection was additionally associated with a four-fold decrease in the area positive for late apoptotic cells (i.e., stained positive with anti-ssDNA) (Fig. 4). Bacterial infection did not influence the total area positive for lymphocytes (K55) or early apoptotic cells (annexin V) in the cecum (Fig. 2). Heterophil counts of SE infected and control animals did not differ significantly and no associations of gender or genetic line with changes in the numbers of cecal macrophages, lymphocytes, heterophils, or apoptotic cell populations were observed (data not shown).
Discussion

Infection of young chicks with SE in the current study resulted in enhanced mRNA transcription of two chemokines, CXCL1 and CXCL2, which are similar to mammalian CXCL8 (IL-8), and have a role in inflammation (Kaiser et al., 2005). Although the exact function of these chicken chemokines remains to be determined, it is reasonable to postulate that these proteins recruit avian heterophils, as CXCL8 preferentially attracts mammalian neutrophils (Gangur et al., 2002). However, evidence from other studies suggests that macrophages, monocytes, and lymphocytes are chemotactic targets for these CXCLi chemokines, not the avian heterophil (Martins-Green and Feugate, 1998; Martins-Green, 2001; Zipirin and Kogut, 1997). Our results provide additional support for CXCL1 and CXCL2 in macrophage recruitment, as we observed an increase in area of tissue staining for this cell population that coincided with an enhanced expression of these two chemokines. An increase in macrophages (K1 positive) and upregulation of CXCL1 (K60) and CXCL2 (IL-8) mRNA expression was also observed for intestinal intraepithelial lymphocytes 7 days post-infection with E. maxima (Hong et al., 2006), indicating these responses are broadly induced by different classes of intracellular pathogens (i.e., bacteria and parasite) and in different areas of the gastrointestinal tract and may represent generalized responses to pathogens in the avian gut.

Salmonella enteritidis infection did not influence IFN-γ, IL-1β, IL-6, IL-12, or Gal-2 gene expression in the cecum of young chicks (Fig. 1). These results seem to contradict published reports of the involvement of these genes in immune responses to Salmonella in poultry (Kaiser et al., 2000; Kogut et al., 2005; Okamura et al., 2004), mice, or humans (Eckmann and Kagnoff, 2001); however, this may be because of the specific time points measured. Our study examined only one time point, one week after SE infection, and we did not determine the kinetics of cytokine gene expression. Heterophils, which produce antimicrobial peptides such as Gallinacin-2 (Brookus et al., 1998; Evans et al., 1994), did were not significantly increased in ceca of SE-infected
chicks; therefore, a lack of Gal-2 mRNA upregulation in the tissue of these infected animals is not surprising. Infection with SE did not alter IL-1β or IL-6 mRNA expression and this may reflect both the young age at infection (one day) and the tissue harvest time (1 week post-infection). Primary infection in chickens with *S. typhimurium* induced an upregulation of IL-6 mRNA expression at 14 to 28 days pi, but upon secondary infection, IL-6 mRNA was rapidly expressed between one and seven days PI (Withanage *et al.*, 2005), suggesting that IL-6 mediates a later and predominantly secondary immune response in the cecum of *Salmonella*-infected chickens.

Chicken IL-1β mRNA is expressed in heterophils (Kogut *et al.*, 2005), macrophages (Rath *et al.*, 2003; Weining *et al.*, 1998; Wigley *et al.*, 2006) and epithelial cells (Kaiser *et al.*, 2000) in response to various stimuli including LPS and *Salmonella*. The upregulation of chicken IL-1β mRNA in these cell types occurred early, often reaching detectable levels in one to four hours PI. IL-1β mRNA expression was enhanced in the cecal tonsil of *S. typhimurium* infected birds from 6-48 hours pi, and the highest level of expression was reached at 12 PI (Withanage *et al.*, 2005). These studies suggest that IL-1β functions in acute or early immune responses and our one week post-infection sample time is perhaps outside this window, and, therefore, differential IL-1β expression was not detectable.

Activation of macrophages from various external stimuli and cytokine production results in enhanced effector functions such as reactive oxygen intermediate production, phagocytosis, and cytotoxicity towards microbes and tumor cells (Ross and Auger, 2002). Cytokines such as IL-6, IL-12, and IFN-γ promote macrophage activation and are also produced by these cells, and other cell types such as NK and T cells for IFN-γ (Taga and Kishimoto, 1997; Trinchieri, 2003). Both IL-12 and IFN-γ also promote Th1 immune responses that are critical for the induction of protective immune responses against intracellular pathogens like viruses and some bacterial species (Eckmann and Kagnoff, 2001; Trinchieri, 2003). The current study on SE infection in the cecum of young chickens found no significant differences in gene expression for IL-6, IL-12, and IFN-γ.
in response to pathogen challenge (Fig. 1). Although an unexpected result, we suggest that this could reflect the activation state of macrophages in the cecum. Although we do observe an increase of macrophage cells (as measured by total area staining positive for these cells) in the cecum following SE infection (Fig. 2 & 3), the actual source (influx of recruited cells or proliferation in situ) is not known. The activation state of these cells would greatly influence the local immune response. Activated macrophages, rather than rapidly migrating or proliferating cells, would be better equipped to control and eliminate the bacterial challenge presented by *Salmonella enteritidis* in the chicken cecum.

Additional evidence to support our hypothesis of decreased macrophage activation in SE infected chicks is provided by the observation of increased cecal IL-10 mRNA expression one week after SE infection in these infected animals (M. G. Kaiser, personal communication). IL-10 has many influences on the host immune response, most notably that it down regulates IL-12 and IFN-γ gene expression and deactivates macrophages (Mocellin *et al.*, 2003). Enhanced IL-10 mRNA expression, as observed, would favor an environment where immature and non-activated macrophages predominate.

Macrophages are a target cell of *Salmonella* in which bacteria invade and successfully replicate, allowing for spread of infection (Eckmann and Kagnoff, 2001; Knodler and Finlay, 2001; Navarre and Zychlinsky, 2000; Vazquez-Torres and Fang, 2001). The ability to influence host macrophage responses would be very beneficial to the invading microbe, and *Salmonella* is well known to do this by delaying phagosome maturation in macrophages (Vazquez-Torres and Fang, 2001). Infection with SE was associated with a decreased level of late stage apoptosis (ssDNA staining) in the current study (Fig. 2 & 4). Although the exact cells that are affected by the observed reduction in late stage apoptosis are unknown, it would be of benefit to the pathogen to have access to non-activated and long-lived macrophages in which to infect and rapidly replicate. *Salmonella* rapidly cause macrophage apoptosis, however it has been suggested that during a systemic infection this would be detrimental to the pathogen and delaying...
apoptosis of macrophages would allow for the time required by the bacteria to replicate in sufficient numbers, escape, and eventually invade other macrophages (Knodler and Finlay, 2001). This strategy is yet to be fully investigated, especially in non-mammalian species such as chickens, but, we hypothesize that our novel observation of decreased late apoptosis may be a mechanism contributing to persistent cecal colonization in poultry.

Our results in the present study show that SE infection in the young chick causes up-regulation of two chemokines, CXCL1 and CXCL2, which actively recruit cells of the monocyte/macrophage lineage. Increased macrophage staining in the cecum of SE infected chicks was observed and is likely the result of up-regulation of these chemokines. The lack of differential mRNA expression of IL-6, IL-12, and IFN-γ and increased IL-10 mRNA expression suggests that these macrophages display an immature non-activated phenotype. SE infection in these young birds decreased staining for a marker of late-stage apoptosis in the cecum. These SE-induced changes could provide an ideal environment for the bacterium to infect and replicate in the cecum without evoking a protective host immune response to clear the infection and eliminate the presence of this zoonotic pathogen.

Acknowledgements

The authors thank Jason Hasenstein, Michael Kaiser and Huaijun Zhou for excellent technical assistance and sample preparation, and H. M. Opitz for the gift of nalidixic acid-resistant SE phage type 13a.

References


Fig. 1. Differential CXCLi1, CXCLi2, Gal-2, IFN-γ, IL-1β, IL-6, IL-12α, and IL-12β mRNA expression in the cecum of week-old chicks with or without Salmonella enteritidis infection. CXCLi1 and CXCLi2 mRNAs were significantly upregulated in Salmonella enteritidis infected chicks compared to uninfected chicks, with $P$ values less than 0.01 (**).
Fig. 2. Differential staining of Annexin V, Lymphocyte, Macrophage, and ssDNA in frozen cecal tissue samples of one week-old chicks with (gray) or without (open) *Salmonella enteritidis* infection. Data presented as a ratio of area stained to the area counterstained with hematoxylin. A significant increase in macrophage staining and decrease in ssDNA staining of SE infected chicks compared to uninfected chicks is shown, with $P$ values less than 0.05 (*).
Fig. 3. Staining for the presence of macrophages in frozen cecal tissue samples of week-old chicks with (A) or without (B) *Salmonella enteritidis* infection. Increased staining for macrophages in infected animals (A) compared to those uninfected (B). Staining is expressed as a ratio of red-stained area (positive) to the area of blue-staining area (negative). Unstained (white) area was subtracted from the total image area and excluded from analysis. Anti-K1 immunostaining, counterstaining with Hematoxylin QS; original magnification, X50.
Fig. 4. Staining for the presence of apoptotic cells in frozen cecal tissue samples of week-old chicks with (A) or without (B) *Salmonella enteritidis* infection. Increased staining for ssDNA in uninfected animals (B) compared to those infected (A). Staining is expressed as a ratio of red-stained area (positive) to the area of blue-staining area (negative). Unstained (white) area was subtracted from the total image area and excluded from analysis. Anti-ssDNA immunostaining, counterstaining with hematoxylin QS; original magnification, X50.
CHAPTER 5. REDUCED NITRIC OXIDE PRODUCTION AND iNOS mRNA EXPRESSION IN IFN-γ STIMULATED HD-11 CHICKEN MACROPHAGES TRANSFECTED WITH iNOS siRNAs

A paper to be submitted to Veterinary Immunology and Immunopathology\textsuperscript{19}

Jennifer H. Cheeseman\textsuperscript{20, 21}, and Susan J. Lamont \textsuperscript{2, 22}

Abstract

Utilizing RNA interference technology with siRNA in HD-11 cells, we investigated how the inhibition or knock-down of the iNOS (inducible nitric oxide synthase) gene would affect IFN-γ induced macrophage production of nitric oxide (NO) and mRNA expression of genes involved in this biological pathway in the chicken. Chicken macrophages produce NO when stimulated with recombinant chicken IFN-γ, however, when transfected with iNOS siRNAs, the production of NO is significantly decreased. We observed a 14-28% reduction in NO production by IFN-γ stimulated HD-11 cells at 48 hours after initial siRNA transfection compared to non-transfected IFN-γ-stimulated macrophages. Significant knock-down of iNOS mRNA expression was observed for each of four iNOS siRNAs, when compared to non-transfected IFN-γ-stimulated macrophages and to those treated with a negative control siRNA. The IFN-γ-stimulated chicken macrophages transfected with iNOS siRNAs did not show altered levels of mRNA expression for genes involved in IFN-γ signaling and iNOS pathways (IL-1β, IL-6, IFN-γ, TGF-β4, or SOCS-3) suggesting that the observed decrease in NO production is a direct result of siRNA mediated knock-down of iNOS, rather than IFN-γ-induced changes in the other genes tested.

\textsuperscript{19} To be submitted for publication.
\textsuperscript{20} Graduate student and Professor, respectively, Department of Animal Science, Iowa State University.
\textsuperscript{21} Primary researcher and author.
\textsuperscript{22} Author for correspondence.
Introduction

RNA interference or RNAi is a powerful tool to examine the function of specific genes and potential role(s) in biological pathways (McManus and Sharp, 2002; Tuschl 2001). When used to knock-down or silence a target gene of interest, the resulting loss of function can illuminate intricate gene interactions involved in fundamental biological processes such as growth and development, reproduction, cellular homeostasis, and immune responses. RNAi technology is an especially powerful tool for studying deleterious or lethal knock-out genes or for experiments with animal species not readily manipulated with current transgenic or knock-out procedures, such as the chicken.

Silencing of specific genes using RNA interference (RNAi) is accomplished by a process of double-stranded RNA-dependant post-transcriptional silencing (Hannon 2002; Novia and Sharp, 2004; Meister and Tuschl, 2004). Double-stranded RNA is digested by Dicer resulting in the production of small interfering RNAs or siRNAs that measure 21-23 nucleotides in length (Hammond et al., 2000; Elbashir et al., 2001). The siRNAs generated then become incorporated into RISC (RNA-induced silencing complex) a multicomponent nuclease complex that destroys the targeted cognate mRNAs (Hammond et al., 2000; Hammond et al., 2001).

RNA interference has been utilized in numerous studies investigating the role of specific gene(s) in biological pathways and disease processes including many related to immune function. Using RNA interference, TLR2 and TLR3 molecules were shown to be involved in IFN-γ stimulated macrophage recognition of Leishmania donovani (Flandin et al., 2006). Silencing of IL-10 in human dendritic cells promoted Th1 responses in naïve CD4 T-cells via production of IL-12 and IFN-γ while decreasing IL-4 cytokine production (Liu et al., 2004). Inhibition of the chemokine receptor, CCR5, induced IL-6 and IL-8 protein production and blocked replication of HIV in a CD4 T-cell line (Pauls et al., 2006). RNA interference of viral replication, developmentally controlled genes, and myostatin in avian cells has also been demonstrated (Chen et al., 2007; Dai et al., 2005; Sato et al., 2006).
Gene silencing technology has been extended to include the exploration of *in vivo* gene function (Gao and Zhang, 2007; de Fougerolles *et al.*, 2007). Inhibition of TLR9 by siRNA decreased inflammation and the influx of polymorphonuclear leukocytes to the corneas of BALB/c mice infected with *Pseudomonas aeruginosa* (Huang *et al.*, 2005). Additionally, decreased IL-1β and MIP-2 protein, and mRNA levels of IL-12 and IFN-γ were observed in the mice receiving TLR9 siRNA treatment. Caspase-3 and caspase-8 silencing increased survival and prevented vascular endothelial cell injury in mice experimentally induced microbial septic shock (Matsuda *et al.*, 2007). Gene interference of metastasis-associated gene 1 in melanoma cells abrogated the ability of the cancer cells to proliferate and metastasize in mice (Qian *et al.*, 2007).

Inducible nitric oxide synthase (iNOS), also known as NOS-2, is an enzyme that produces nitric oxide (NO) from the amino acid L-arginine (Bogdan 2001; Bogdan *et al.*, 2000; Alderton *et al.*, 2001). Produced by macrophages stimulated with cytokine and/or microbial components), NO plays a powerful role in immune responses due to its antimicrobial and anti-tumor functions (Bogdan 2001; Blanchete *et al.*, 2003; Bogdan *et al.*, 2000; MacMicking *et al.*, 1997). iNOS activity is primarily regulated at the transcriptional level, although translational and posttranslational events such as protein dimerization and stability along with phosphorylation have been shown to influence iNOS activity (Aktan 2004; Kleinert *et al.*, 2004).

The activation of transcription factors such as NF-κβ by LPS, TNF-α, and IL-1β induces iNOS expression and NO production in macrophages (Aktan 2004; Kleinert *et al.*, 2004). Additional cytokines and cellular signaling molecules have been implicated in the regulation and induction of iNOS-mediated NO production (Bogdan 2001). Stimulation of murine, rat, and human macrophages with LPS, IFN-γ, IL-6, IL-1β, TNFα, or a combination of these molecules induces iNOS expression (Kleinert *et al.*, 2003; Kleinert *et al.*, 2004). Interleukin-6 and IFN-γ activate members of the Jak/Stat pathway of intracellular signaling and transcription factors, namely Jak1 and Jak2, and Stat1 and Stat5 (Paukku and Silvennoinen, 2004; Schindler and Bogdan, 2001). Suppressor of
cytokine signaling-3 (SOCS-3), intimately associated with Jak1, is induced by various cytokines such as IL-1, IL-6, IFN-γ, and TNF-α and inhibits the signaling of IL-6 and IFN-γ and other cytokine signaling pathways as well (Paukku and Silvennoinen; Tan and Rabkin, 2005). Transforming growth factor-β1 (TGF-β1) negatively regulates iNOS expression and NO production at many levels and is considered the most important negative regulator of iNOS-mediated NO production in macrophages (Vodovotz 1997; Vodovotz et al., 1999).

Murine (Blanchette et al., 2003) and human macrophages (Bogdan 2001) stimulated with IFN-γ produce high levels of NO. The production of NO by IFN-γ stimulated chicken macrophages and monocytes has also been established (Okamura et al., 2005; Withanage et al., 2005; Crippen et al., 2003). In a similar manner as IFN-γ, LPS has been shown to induce NO production in chicken macrophages (Hussain and Qureshi, 1997; Dil and Qureshi, 2003). Increased NO production by chicken macrophages infected with various Salmonella and Eimeria species indicates a role of NO and therefore likely iNOS activity, in avian immunity to disease (Babu et al., 2006; Lillehoj and Li, 2004). Using siRNA methodology, we investigated how knock-down of the iNOS gene would alter nitric oxide production in the chicken macrophage line, HD-11, and the potential effects on mRNA expression of several genes involved in IFN-γ-iNOS-NO pathways.

Materials and Methods

**Macrophage Culture, Transfection, and IFN-γ Stimulation**

The chicken macrophage cell line, HD-11 (Beug et al., 1979), was maintained in RPMI 1640 medium (Sigma) supplemented with 10% newborn calf serum (heat-inactivated), 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100µg/ml streptomycin, and 5 x 10⁻⁵ M 2-mercaptoethanol (pH 7.3) at 41°C and 5% CO₂. Cells were cultured in 75 cm² tissue flasks (Corning INC., Corning, NY) and split approximately every 3 days at a 1:5 ratio.
into fresh media and new sterile culture flasks. HD-11 macrophages (1 x 10^5 cells in 200 µl media) were cultured overnight in sterile 96 well plates. Prior to transfection, the RPMI 1640 media was gently aspirated from the wells and cells were rinsed with 100 µl Opti MEM(R) I reduced-serum medium (Gibco Invitrogen, Carlsbad, CA) to remove any residual of RPMI 1640 medium. siPORT NeoFX (Ambion, Austin, TX) transfection reagent was used according to manufacturer’s instructions to deliver a 100 nM concentration of siRNA in a final volume of 100 µl. Cells not treated with siRNA were incubated in a similar manner with 100 µl Opti MEM(R) I reduced-serum medium alone. Following four hours of incubation at 41°C and 5% CO2, all medium was removed and cells were rinsed with 100 µl Opti MEM(R) I reduced-serum medium. 200 µl of RPMI 1640 supplemented as above with recombinant chicken IFN-γ at a dilution of 1:25 was added to each individual well and cultured for 48 hours at 41°C and 5% CO2. Following 48 hours of IFN-γ stimulation, assay plates were frozen at –20°C until thawed for RNA isolation and quantification of NO production. HD-11 cells not treated with siRNA or stimulated with recombinant chicken IFN-γ were given 200 µl of RPMI 1640 plus supplements alone and incubated under identical conditions. A total of four replicate experiments consisting of six identical wells per each treatment (unstimulated and untreated/untransfected with siRNA, IFN-γ stimulated and untreated with siRNA, stimulated and treated with siRNA #1, 2, 3, 4, or a non-sense negative control, and stimulated and treated with a combination of siRNA #1, 2, 3, and 4) per plate were performed.

**iNOS siRNA Construction and Sequence**

Four siRNA targeted towards iNOS and one non-sense negative control were designed with siRNA Target Finder software (Ambion, Austin, TX) and were custom synthesized and HPLC purified by Ambion. The iNOS siRNA sequences used are as follows:

siRNA #1  5’ GUGUGGAGUUCACAAAGUUt  3’
siRNA #2 5’ GAUUCUGUGCAUGGAUGAGtt 3’
siRNA #3 5’ UUCCCAUGAAGCUGAAAUUtt 3’
siRNA #4 5’ GCCGUGCAUUCUUAUUGGtt 3’
siRNA neg. 5’ CUUGAUGACUAUAAGAUGGtt 3’

Quantification of Nitric Oxide (NO)

Thawed aliquots of 50 µl culture supernatants mixed with 50µl Griess reagent (5% phosphoric acid, 1% sulfanilamide, 0.1% N-naphthylethlenediamine) were incubated at room temperature for approximately 10 minutes and then were read on an ELISA Microplate plate reader (Bio-Rad, Hercules, CA) at 570nm. A standard curve produced from 0-50 nmoles of NaNO2 was prepared for calculation of NO production in test samples.

RNA Isolation and Gene Expression

Total RNA was isolated from pooled samples (6 individual wells per treatment, 4 replicates per each treatment performed on different days) using RNAquous© (Ambion, Austin, TX) according to manufacturer’s instructions. Gene expression levels of mRNA transcripts were analyzed by quantitative real-time RT-PCR using QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA) as previously reported (Cheeseman et al., 2007). Primer sequences for 28S, IL-1β, IL-6, IFN-γ, and TGF-β4 have been previously reported and do not amplify genomic DNA as they span an intron-exon boundary (Kogut et al., 2003). Because the primers for iNOS (Xing and Schat, 2000) have been reported to amplify both RNA and genomic DNA, samples were DNase treated with DNA-Free (Ambion, Austin, TX) according to manufacturer’s instructions before amplification. Chicken TGF-β4 is generally recognized as the avian counterpart of mammalian TGF-β1, having similar functions in immunity in birds (Jakowlew et al., 1997). The SOCS-3 primer sequences are as follows:

F 5’ GCCCCAGGTGATGGTGA 3’
Quantitative real-time RT-PCR reactions, run in triplicate for each sample and gene, were performed as previously described (Cheeseman et al., 2007). Briefly, the q-RT-PCR reactions were performed on an Opticon 2 (MJ Research Inc., Waltham, MA) with the following program: 1 cycle at 50°C for 30 min., 95°C for 15 min. followed by 45 cycles of 94°C for 15 sec., 59°C for 30 sec., and 72°C for 30 sec followed by reading of the plate. Additionally, a melting curve from 60-90°C with a reading every 1°C was performed on all RT-PCR 96-well plates. Data were transformed and expressed as the adjusted Ct (cycle threshold) value using the following formula:

\[ 40 - [(\text{mean test gene Ct}) + (\text{median 28S Ct} - \text{mean 28S Ct}) \times (\text{test gene slope/28S slope})] \]

Slopes were determined with a series of 10-fold dilutions of plasmids encoding each target gene to determine PCR efficiency, and median 28S Ct represents the median Ct value of all individual samples for this housekeeping reference gene.

**Statistical Analysis**

Individual mRNA levels for each treatment and the four replicates are shown as the mean of triplicate well measurements. Analysis of gene mRNA expression and analyzed NO production levels was performed with an ANOVA model using JMP software (JMP) (SAS Institute, 2004).

**Results**

**Reduced Nitric Oxide (NO) Production in siRNA iNOS Transfected HD-11 Cells**

HD-11 chicken macrophages produce nitric oxide (NO) when stimulated with recombinant chicken IFN-γ (Lillehoj and Li, 2004). To determine the role of iNOS expression in NO production in chicken macrophages, we cultured HD-11 cells with one
of four iNOS siRNAs, a combination of the four iNOS siRNAs, and a non-sense (negative) siRNA then stimulated the cells with chicken IFN-γ. After 48 hours of IFN-γ stimulation we measured NO using the Griess assay. Using a standard curve produced from 0-50 nmoles of NaNO$_2$, we determined the amount of NO (in uM) produced in HD-11 macrophages.

HD-11 chicken macrophages transfected with iNOS siRNAs and stimulated with IFN-γ produced significantly lower levels of nitric oxide than macrophages transfected with a non-sense iNOS siRNA and stimulated with IFN-γ, or HD-11 macrophages stimulated with IFN-γ alone (Table 1). The non-sense (negative) iNOS siRNA induced a modest % but significant decrease in NO production by the HD-11 macrophages. Compared to IFN-γ stimulated macrophages, treatment with the non-sense siRNA resulted in a reduction of only 4.5% while the other iNOS specific siRNAs showed significantly lower levels of NO production compared to the non-sense siRNA or no siRNA transfected macrophages.

All iNOS siRNAs induced decreased NO production in the HD-11 chicken macrophages to varying degrees (Table 1) and the location of iNOS siRNA primers is shown (Figure 1). Macrophages treated with iNOS siRNA #1 showed the largest decrease in NO production (28.6%) compared to non-transfected IFN-γ stimulated macrophages. The lowest reduction of NO production was observed in siRNA #2 treated macrophages corresponding to a 14.5% reduction in NO production compared to non-transfected IFN-γ stimulated chicken macrophages. On average, we observed a 22% reduction in NO production by the cells with iNOS siRNAs.

**siRNA-Mediated Knock-Down of iNOS mRNA Expression in Chicken**

**Macrophages**

To investigate iNOS siRNA-mediated changes in gene expression, we determined mRNA levels for several genes known to be involved in the IFN-γ-induced iNOS biological pathways. Transfection with iNOS siRNAs did not alter mRNA expression
levels for IFN-γ, IL-1β, IL-6, TGF-β4, or SOCS-3 in HD-11 chicken macrophages
(Figure 2). However, we observed a significant decrease or “knock-down” of iNOS
mRNA expression in macrophages treated with all iNOS siRNAs compared to HD-11
cells stimulated with IFN-γ alone (Figure 2). No difference in iNOS mRNA expression
was observed for HD-11 macrophages treated with a non-sense or negative siRNA
compared to those stimulated with only IFN-γ.

As shown in Figure 1, we did not observe any iNOS siRNA-mediated alteration in
gene expression for IFN-γ, IL-1β, IL-6, TGF-β4, or SOCS-3, indicating that this set of
genes was not responsible for our observed decreases in iNOS mRNA expression (Figure
2). Although we cannot exclude other genes not examined in this study, the iNOS
siRNA-mediated knock-down of iNOS mRNA levels in HD-11 chicken macrophages
appears to be target-sequence specific.

**Discussion**

In this paper we report the first usage of siRNAs to knock-down gene expression
in avian macrophages. Specifically we demonstrate that HD-11 chicken macrophages
when treated with iNOS siRNAs and stimulated with recombinant chicken IFN-γ
produced significantly less nitric oxide and had lower iNOS mRNA levels compared to
IFN-γ stimulated HD-11 cells untreated with siRNAs. No alterations in mRNA levels for
several other genes known to be involved in iNOS and IFN-γ pathways such as: IFN-γ,
IL-1β, IL-6, TGF-β4, and SOCS-3 were observed, suggesting that the lower NO
production and decreased iNOS mRNA presented in this study are the direct result of
siRNA-mediated inhibition of the iNOS gene in chicken macrophages.

Previous studies have reported the use of RNA interference technology in the
chicken embryo and primarily explored genes involved in developmental or
differentiation networks (Chesnutt and Niswander, 2004; Sato *et al.*, 2004; Sato *et al*.,
2006). Recently, inhibition of the cytokine genes IL-10 and IFN-γ with siRNAs was
reported in the pig (Sidahmed and Wilkie, 2007) demonstrating the feasibility of RNA
interference in studies involving agricultural animal species. As knock-outs are not readily available for most agricultural species, such as the chicken, siRNA technology to reduce gene expression could prove to be a powerful tool in advancing basic knowledge of avian immune function and immune responses to infection. Our novel demonstration of siRNA-mediated knock-down of iNOS mRNA and nitric oxide production in HD-11 macrophages establishes the validity and feasibility of using RNAi technology in the avian immune system and provides a foundation for future investigations in avian immune function and chicken immune responses to disease.

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References


Table 1. Nitric oxide production in HD-11 chicken macrophages treated with IFN-γ and siRNAs. OD readings not sharing a letter are significantly different by LS Means Student’s t Test ($P < 0.05$). A standard curve was used to calculate uM concentration of NaNO$_2$ (*).

\[(uM \text{ NO} = 142.06(\text{OD 570nm}) - 5.09)\]

<table>
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<tr>
<th>Treatment</th>
<th>OD 570nm</th>
<th>uM NaNO$_2$</th>
<th>% Reduction Compared to IFN-γ Alone</th>
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</thead>
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<tr>
<td>IFN-γ</td>
<td>0.1032$^a$</td>
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<td>0</td>
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<tr>
<td>Neg. siRNA + IFN-γ</td>
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<td>4.5</td>
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<td>siRNA #2 + IFN-γ</td>
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</table>
Figure 1. Location of iNOS siRNAs. Nucleotides shown in bold type denote the location of each of four iNOS siRNAs. Based upon the chicken iNOS mRNA sequence (Accession # NM204961).
Gene Expression in HD-11 Chicken Macrophages Transfected with siRNAs

Figure 2. mRNA expression of iNOS, IFN-γ, IL-1β, IL-6, TGF-β4, and SOCS-3 in HD-11 chicken macrophages treated with iNOS siRNAs and stimulated with IFN-γ. Reduction of iNOS mRNA expression in HD-11 chicken macrophages treated with iNOS siRNAs and IFN-γ. Bars not sharing a letter are significantly different by LS Means Student’s t Test ($P = 0.03$).
CHAPTER 6. GENERAL CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

Genetic Influence on Immune Cell Subsets and Cytokine Expression

Fundamental immunological differences in several chicken lines representing meat type, layer type, and non-commercial (indigenous) birds has been demonstrated for both cell subset composition of peripheral blood leukocytes in adults and for mRNA expression of cytokines in the spleen and cecum of newly hatched chicks, as presented in Chapters 2 and 3. Comparison of the two MHC congenic Leghorn layer lines also known as G-B1 and G-B2, an outbred meat type broiler line, and the exotic Egyptian Fayoumi MHC congenic lines, denoted M5.1 and M15.2, demonstrated consistent patterns in immune related profiles specific to each genetic line or breed.

Significant differences for CD3 T cell population, ratio of B cells: T cells and T cell receptor density of CD8 and CD3 molecules were reported amongst the genetic lines examined using flow cytometric analysis of peripheral blood samples from adult birds (Chapter 2). The two MHC congenic Leghorn layer type lines, G-B1 and G-B2, had markedly lower numbers of CD3 positive T cells compared to the other lines examined. This two fold reduction in CD3 positive T cells in these Leghorn lines suggests they have a smaller T cell population compartment compared to the other lines, or alternatively a lower peripheral CD3 T cell population but larger resident tissue specific CD3 T cell population. Either way, the peripheral and circulating population of CD3 positive T cells is significantly lower in the Leghorn lines.

Interestingly, although the total percent of cells positive for the CD3 marker is lower in the Leghorn lines, the fluorescent intensity or number of CD3 molecules on the surface of the T cells is much denser than the other lines investigated in Chapter 2. This indicates that the Leghorn layer lines, G-B1 and G-B2 have a smaller proportion of CD3 positive T cells that express more surface CD3 molecules. CD3, which is a complex of
three invariant chains, CD3γ, CD3δ, and CD3ε, is intimately involved in T cell receptor signaling through their ITAMs (immunoreceptor tyrosine-based activation motifs) (Janeway et al., 2005; Underhill and Goodridge 2007; Fodor et al., 2006; Pitcher and van Oers 2003). As the Leghorn lines exhibit increased surface density of CD3 complexes, they may have increased sensitivity or more finely tuned T cell receptor signaling compared to the other lines investigated which compensates for their lower percentage of circulating CD3 positive cells. This hypothesis may provide some insight to understanding generalized breed or line specific changes in immune response and function. Immunological differences between broiler and Leghorn layers have been established (Koenen et al. 2002; Leshchinsky and Klasing, 2001). Layer-type chickens demonstrated a stronger cellular immune response compared to broilers and the layers showed a longer secondary humoral immune response as well (Koenen et al. 2002).

Increased cell surface expression of CD8 (representing cytotoxic T cells) on the Egyptian Fayoumi MHC congenic lines, M15.2 and M5.1, is also observed (Chapter 2). The M5.1 line, in particular, had the highest level of CD8 cell surface expression of all the lines examined and measured about twice the fluorescent intensity for this molecule compared to both Leghorn MHC congenic lines. The Egyptian Fayoumi lines were imported to the US a half-century ago due to reported resistance to avian leukosis complex, later determined to be two separate viral diseases, Marek’s disease and avian leukosis virus (Lakshmanan et al., 1996; Zhou and Lamont, 1999). Higher cell surface expression of the cytotoxic T cell receptor molecule, CD8, on the Fayoumi lines is interesting, because these birds have shown increased resistance to viral diseases (Lakshmanan et al., 1996) and CD8 T cells are critically important in cellular immune responses to viruses and cancers (Alexander-Miller 2005; Lin et al., 2000; Melief and Kast 1990; Schat 1991).

Examination of early gene expression in the spleen and cecum of young chicks demonstrated a clear pattern of influence by breed (Chapter 3). In the spleen, the Leghorn line showed increased mRNA message for IL-10, IL-12α, CXCLi2 (IL-8 like),
and CCLi2 (MIP family member) compared to the broilers and or the Fayoumi lines. Additionally, the Leghorn’s cecum also demonstrated higher IL-18 mRNA expression compared to the broiler line. Alternatively, the Leghorn line showed significantly lower levels of gene expression compared to the broilers for CCLi2, IL-12α, and IL-12β in the cecum. These breed and organ specific immune-related gene expression patterns support results presented in Chapter 2 of genetic line influence on peripheral blood leukocyte composition, and add additional details for previously published work on general differences in immune response or immune cell composition between breeds and lines in the chicken (Koenen et al. 2002; Leshchinsky and Klasing, 2001; Hala et al., 1991; Hala et al., 1992; Parmentier et al., 1995; Burgess and Davidson, 1999).

**Salmonella enteritidis Infection-Induced Modulation of Immune Responses**

While Chapter 3 reports a demonstration of the role of chicken breed on early cytokine mRNA expression in the spleen and cecum in young chicks, *Salmonella enteritidis* (SE) infection also significantly influenced gene expression. Both IL-18 and IFN-γ mRNA transcripts are up-regulated in the spleen of infected chicks within the first 18 hours following inoculation, indicating Th1 cytokine expression occurs early in the host immune response to SE. Effective Th1 or cellular immune responses including IL-18 and IFN-γ production are required for response against intracellular pathogens like viruses and bacteria such *Salmonella* and *Mycobacteria* (Garcia et al., 1999; Mastroeni 2002; Stoycheva et al., 2004). Increased IL-18 and IFN-γ mRNA transcripts in the spleen, as reported in Chapter 3, provides evidence for an early Th1 directed chicken immune response in young birds towards SE infection. An early Th1 directed immune response towards intracellular pathogens for chickens infected with SE (Chapter 3), mirrors those firmly established in mammalian systems. The early response of IL-18 and IFN-γ mRNA expression by avian spleen cells to SE exposure may contribute to subsequent host immune responses to this pathogen.
Expression of mRNA for CXCLi1 (K60) and CXCLi2 (IL-8/CAF), both chicken IL-8 like chemokines, is increased in the cecum one week after oral infection with SE in young chicks (Chapter 4). The up-regulation of mRNA expression for these two chemokines, which are similar to mammalian IL-8, in the cecum in response to SE infection suggests a role for these molecules in inflammation (Kaiser et al., 2005). Mammalian neutrophils are preferentially targeted for recruitment by IL-8 (Gangur et al., 2002); however this function may not extend into the chicken immune system. Evidence from studies on chicken IL-8 chemokines, CXCLi1 and CXCLi2, suggests macrophages, monocytes, and lymphocytes, but not heterophils, may be the target cells of choice for recruitment to the site(s) of tissue infection and/or inflammation (Martins-Green and Feugate, 1998; Martins-Green, 2001; Zipirin and Kogut, 1997). The study reported in Chapter 4 provides additional support for CXCLi1 and CXCLi2 function in recruitment of avian macrophages, as an increase in the total area staining positive for the macrophages coincided with up-regulated expression of these chemokines in the cecum one-week post infection.

Chapter 4 reports that SE infection in the cecum of young chicks one-week post infection decreases apoptosis. Staining for late stage apoptosis with an antibody directed at ssDNA showed a four-fold decrease of area stained in the cecum of infected animals compared to uninfected birds. Salmonella species can readily invade and rapidly induce cellular death via apoptosis in host macrophages, however it has been postulated that early death of the host cells during systemic infection could be detrimental to the bacteria by killing off large numbers of targeted cells (macrophages) and that if the Salmonella bacteria could delay or reduce apoptosis of infected macrophages, then ample time for bacterial replication and escape to infect other host cells would occur (Knodler and Finlay, 2001). Although speculative, the decreased apoptosis in the cecum of SE infected chicks could be a mechanism for persistent colonization of the cecum (Gast and Holt, 1998), increasing the potential of poultry products such as eggs to be sources of food poisoning.
Knockdown of Nitric Oxide Production and iNOS mRNA Expression in IFN-γ Stimulated Chicken Macrophages Using iNOS siRNAs

Chicken macrophages from the HD-11 cell line produce NO when stimulated with recombinant chicken IFN-γ (Lillehoj and Li, 2004). At 48 hours after siRNA treatment, reduced NO production is observed in the chicken macrophages (Chapter 5). This reduced production of NO by macrophages occurred when cells treated with any one of four siRNAs directed towards the chicken iNOS (inducible nitric oxide synthase) gene were subsequently stimulated with IFN-γ compared to cell stimulated with IFN-γ alone and non-sense siRNA treated and IFN-γ stimulated cells. Additionally, a combination of all four iNOS siRNAs shows a similar level of knockdown in NO production, the end product of iNOS. A negative control siRNA, not specific for the iNOS gene but similar in nucleotide composition, does not show the same marked decrease in NO production.

Analysis of genes known to be involved in IFN-γ stimulated NO production by macrophages such as iNOS, IFN-γ, SOCS-3, IL-6, IL-1β, and TGF-β4 demonstrated significant knock-down of iNOS mRNA expression directly related to transfection with iNOS specific siRNAs (Chapter 5). All four iNOS siRNAs induced decreased iNOS mRNA expression in HD-11 chicken macrophages. Data presented in Chapter 5 demonstrated proof of principle in the utilization of RNA interference technology to analyze the knock-down of immune genes in chicken macrophages and represents the first report of siRNA-mediated decreases in NO production and iNOS mRNA expression in chicken macrophages.

Use of Multiple Techniques and Technologies to Investigate Avian Immunology, Immunogenetics, and Host Immune Responses to *Salmonella enteritidis* Infection

When investigating scientific or biological questions the use of appropriate and multiple techniques or technologies can provide insightful answers and future directions
of research. More complete and comprehensive answers to hypotheses are likely to result from multiple and varied approaches undertaken by researchers. By using several analytical techniques or different technologies, each method can provide a unique piece of information creating a more holistic “picture” of the question(s) under investigation.

The current dissertation employs several different technological strategies to answer questions regarding the role of genetics in shaping immune profiles in both adult and young chickens, how Salmonella enteritidis infection alters cytokine expression in the spleen and cecum as well as cellular populations in the cecum, and the potential use of siRNA technology in probing gene function and genes involved in similar biological pathways in chickens.

In Chapter 2 of the dissertation, flow cytometry revealed genetic line, or breed, induced specific differences in the composition of immune cells in peripheral blood. Additional evidence corroborating the immune related differences among chicken breeds/lines is presented in Chapter 3. Measurement of mRNA expression in several cytokine and apoptosis related genes using quantitative PCR (qPCR) (Chapter 3 of this dissertation) showed breed-specific early expression profiles in both the spleen and cecum of young chicks.

To investigate the immune response to infection with SE in young chicks, complimentary techniques of qPCR and immunohistochemistry were employed (Chapter 4). Very early in infection with SE, within the first 24 hours post-inoculation, an increase in Th1 cytokine (IL-18 and IFN-γ) mRNA expression in the spleen is shown (Chapter 3). One week after SE infection an up-regulation in two IL-8 (CXCLi1 and CXCLi2) chemokines is induced (Chapter 4). Adding to the comprehensive understanding of SE-induced immune changes in the cecum of infected chicks, immunohistochemical analysis reported in Chapter 4 reveals an increase in the population of macrophages and decreased occurrence of late-stage apoptotic cells within the organ.

Chapter 5 reports studies that utilized cutting edge technology of RNAi or RNA interference to investigate NO production by iNOS and several genes known to be
involved in the biological pathway in cultured chicken macrophages. Transfection of iNOS siRNAs decreased nitric oxide production and iNOS mRNA expression in HD-11 cells. Chapter 5 demonstrates that RNAi is a viable technique showing future promise to other investigators wanting to examine gene pathways and gene interactions in chicken cells.

**Potential Impact of Current Dissertation and Future Research Directions**

Research presented in this dissertation provides new information on the role of genetic background in shaping immune parameters in chickens such as peripheral blood leukocyte composition and early immune gene expression in the spleen and cecum. These results provide a greater understanding of how genetics influences the avian immune system and its response(s) to disease. Knowledge of immune system parameters inherent to a specific chicken breed or population may facilitate targeted vaccine strategies against avian diseases, gene discovery, or breeding programs aimed at enhancing immunity towards economically important pathogens.

Additionally, this dissertation provides evidence for a role for CXC
ci chemokines in SE infection and in the recruitment of macrophages. Discovering a novel decrease in apoptosis in the cecum of SE infected chickens may indicate a pathogen strategy to create a persistent colonization of this organ. Understanding how a host animal responds to microbial infection provides initial clues valuable for later measures such as: vaccines, breeding strategies, natural flora, and environmental management, aimed at reducing and possibly eliminating the problem. This dissertation provides new information on the immune response of young chicks to a critical pathogen, *Salmonella enteritidis*.

Finally this dissertation presents the first reported use of siRNA-mediated gene knock-down in chicken macrophages. RNAi technology has successfully demonstrated the function of developmental genes in a chicken embryo model, however this
dissertation provides the first report of gene knock-down by siRNAs in the avian immune system. As gene knock-out models are not readily available in poultry as in mammalian models, such as mice, this technology will likely be utilized in future experiments investigating avian immune function and diseases of chickens.

The observed decrease in apoptosis in the ceca of SE-infected chickens warrants future investigation. An examination of organ apoptosis over time in relation to bacterial colonization and persistence of several Salmonella species could show if lower or delayed apoptosis actually provides the bacteria with an advantage to promote persistent host colonization. In addition to the potential role of delayed apoptosis in host colonization, other causes of persistent Salmonella enteritidis colonization of poultry must be explored, as the persistent colonization of chickens with SE represents a major source of bacterial contamination and food poisoning to the human consumer.

Because many of the tools and reagents utilized in studies of mammalian immunology and diseases are not routinely available for examination in the chicken immune system, progress will likely occur slower for avian immunologists. Although gene knock-outs are not readily available in chickens, siRNA technology to reduce gene expression could prove to be a powerful tool in advancing basic knowledge of avian immune function and immune responses to infection. Our novel demonstration of siRNA-mediated knock-down of iNOS mRNA and nitric oxide production in HD-11 macrophages establishes the validity and feasibility of using RNAi technology in studies of the avian immune system. A natural and logical direction of future studies could probe the role of candidate genes, like cytokines and chemokines, in modulation of immune function. Additionally, exploration of immune responses to specific avian diseases could benefit from siRNA technology in a similar manner as in knockout mice models.
References


APPENDIX A. VITA

Education and Professional Experience:

**Iowa State University**
Department of Animal Science
Major: Immunobiology
Ph.D. September 2002 – Present (December 2007 Graduation)

  Graduate Research Assistant (Dr. S. J. Lamont)
  Iowa State University, Department of Animal Science
  December 2002 - Present

  Graduate Student Rotation Mentor
  Ceren Ciraci (Interdepartmental Genetics)
  Nyssa Levy (Immunobiology)

**R & D Systems**
Research Associate (Level II)
Department of Polyclonal Antibodies
April 1999 - August 2002

**ViroMed**
Research Assistant (Level I)
Department of Lab Services
Department of In-Vivo Animal Testing (Medical Devices)
May 1997 – April 1999

**St. Olaf College**
Major: Biology
B.S. May 1997

Meetings and Posters:


Professional Skills:

- **RNA Techniques**
  Total RNA isolation, Gel Electrophoresis, QPCR, and siRNA

- **Immunohistochemistry**
  Frozen tissue staining

- **DNA Techniques**
  Gel Electrophoresis and PCR

- **Flow Cytometry**
  Single and Dual Color Analysis

- **Protein Techniques**
  Protein purification, cleavage, and conjugation (biotin). ELISA, Western Blot, and Immunoprecipitation

- **Cell Culture**
  Primary (PBL) and Cell Lines – propagation, stimulation assays, Griess assay (NO production)

- **Antibody Techniques**
Monoclonal (mouse, rat, hamster) and Polyclonal (rabbit, goat, sheep) antibody purification – Protein A & G and Antigen Affinity. Antibody cleavage and conjugation (biotin)

- **Animal Experience**
  - Chicken – blood collection and tissue isolation
  - Mouse – IV tail vein and SC injections
  - Rabbit – blood collection, IV ear vein and SC injections

- **Bacterial Techniques**
  - General growth, staining, and identification techniques

- **Computer Experience**
  - General use programs – Microsoft Word, Power Point and Excel
  - Statistical Software – JMP

**Honors and Awards:**

- Gamma Sigma Delta
- Honor Society of Agriculture
- April 2004