GENOMIC APPROACHES TO STUDY INNATE IMMUNE RESPONSE TO *SALMONELLA* ENTERITIDIS INFECTION IN CHICKENS

A Dissertation

by

HSIN-I CHIANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Huaijun Zhou
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	Sanjay Reddy
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ABSTRACT

Genomic Approaches to Study Innate Immune Response to *Salmonella* Enteritidis Infection in Chickens. (December 2008)

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Chair of Advisory Committee: Dr. Huaijun Zhou

Salmonella enterica serovar Enteritidis (SE) is one of the most common food-borne pathogens that cause human salmonellosis. Contamination of consumed poultry products continues to be a global threat to public health. Genetic resistance using genomic approach provides a promising solution to controlling SE infection in poultry. The mechanism of SE resistance in chickens remains elusive. Three different approaches, microarray techology, gene silencing, and computational gene analysis, have been utilized to study SE-induced transcriptional changes of host immune response in the chicken.

A whole genome chicken 44K microarray was used to analyze the transcriptome of heterophils from SE-resistant (line A) and SE-susceptible chickens (line B) with/without *in vitro* SE stimulation. Many differentially expressed immune-related genes were found in the SE-infected to non-infected comparison, where more immune-related genes were down-regulated in line B than line A. These results suggested a similar Toll-like receptor (TLR) regulatory network might exist in heterophils of both lines, and provided strong candidates for further investigating SE resistance and susceptibility in chickens. In the gene silencing study, small interfering RNAs (siRNA) were used to specifically inhibit

the expression of NFkB1 in the chicken HD11 macrophage cell line with SE challenge. Genes related to the NF-kB signaling pathway were selected to examine the effect of NFkB1 inhibition on TLR pathway. With 36% inhibition of NFkB1 expression, the results showed an increased expression of TLR4 and interleukin (IL)-6 following SE challenge and suggested a likely inhibitory regulation of NFkB1 on TLR signal pathway. Finally, two novel chicken C-type lectin-like receptors were identified and annotated to chicken CD69 and CD94/NKG2-like with multiple evidences generated by computational (in-silico) sequence analysis. Both genes located in a region on chicken chromosome 1 that is syntenic to mammalian Nature Killer Receptor Complex (NKC) region, which may have existed before the divergence between mammals and aves.

While siRNA lays the foundation of using loss-of-function approach on testifying gene-gene interactions, in-silico analysis aids in gathering information of unknown genes of great interest. Both approaches provide great potential to use for down-stream analysis following microarray study.

DEDICATION

To my wife, my family and friends, for their support and encouragement throughout the course of this work.

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CHAPTER I

INTRODUCTION

Salmonellosis is an infection with bacteria called *Salmonella.* Most people infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to72 hours after infection. Every year, approximately 40,000 cases of salmonellosis are reported in the United States (data from CDC, Centers for Disease Control and Prevention, United States., Nov. 2006). However, the actual number of infections may be underestimated because many milder cases are not diagnosed or reported. Within >2500 different serotypes, four serotypes, namely *Salmonella* Typhimurium (19%), *Salmonella* Enteritidis (14%), *Salmonella* Newport (9%) and *Salmonella* Javiana (5%) account for almost half of all human isolates in United States.

In many countries *Salmonella enterica* serovar Enteritidis (SE) has become the first or second most common *Salmonella* serotype (Braden 2006a). A rapid increase of incidence of SE in human began in the Northeast of United States in 1970s, and then spread to the mid-Atlantic states by the mid-1980s. The epidemiology of SE accounts for the main sources of salmonellosis in human through the consumption of contaminated poultry meat or shell eggs. In the U.S., an estimated 1.4 million non-typhoidal *Salmonella* infections cost $ 3 billion annually (Data from WHO, World Health Organization, Apr. 2005).

This dissertation follows the style and format of Immunogenetics.

SE can persist in the cecal or ovaries of adult birds for months without triggering clinical signs. The asymptomatic *Salmonella* carrier status in poultry has serious consequences on public health. The colonized SE can be excreted in feces (horizontal transmission) or through the yolk (vertical transmission) to contaminate other birds in the flock as well as the poultry products such as meat (after slaughtering) and eggs (Tilquin et al. 2005a). Consumption of contaminated meat, eggs or other poultry products may trigger food poisoning, which is particularly dangerous for susceptible persons such as young children, elderly people and immuno-suppressed patients.

Current control of salmonellosis in poultry is mainly through hygiene measures combined with vaccination programs. However, the current killed vaccines or non-characterized live attenuated vaccines are only partially effective (Wigley, 2004a). Selection for genetic resistance to systemic salmonellosis offers an alternative control measure. It has been reported that the level and duration of bacterial colonization of gut are under genetic control of chicken host (Sadeyen et al. 2004; Wigley 2004a). Previous studies showed that innate immunity, especially in macrophages and heterophils, was associated with the difference between *Salmonella*-resistant and -susceptible inbred chickens (Rosenberger et al. 2000; Swaggerty et al. 2004a; Wigley et al. 2006). *Salmonella*-resistant inbred birds perform greater and more rapid expression of proinflammatory cytokines which are associated with the NFkB signal transduction pathway (Wigley et al. 2006). Although the study of genetic resistance in the chicken has rapidly progressed, the mechanism of intestinal colonization with SE on chicken remains elusive. It may be due to the limited knowledge on genes associated with

intestinal colonization of SE in chikcens, or compared to mammalian system, there is a different signal transduction pathway engaged in chicken innate immunity (Lynn et al. 2004b).

The commercial chicken lines may vary in their susceptibility to infectious diseases including salmonellosis. The determination of the role of genes involved in host resistance to Salmonella in chickens is essential to the control of salmonellosis in chicken and human. The recognition of invaded microorganisms can cause the changes in the gene expression levels in particular host cells, and these changes in gene expression level can be different between susceptible and resistant chickens. We hypothesized that the genes with significant expressional changes are associated with the control of immune response such as initiation, regulation and termination. To identify and understand the role of these genes is the key to unveil the mechanism of disease susceptibility in poultry.

The traditional one by one gene study such as Northern or quantitative real-time PCR (qRT-PCR) has its limits on detecting multiple gene expressions for whole tissue or genome. Furthermore, these methods can only examine gene interaction via indirect comparison with an internal standard. In order to identify genes involved in the genetic disease resistance and to get insight into the mechanisms that determine differences in susceptibility, two advanced genomics tools, a newly developed 44K whole genome chicken DNA microarray (Li et al. 2008) and RNA interference (RNAi) gene silencing, were used in this study. In this research project, the 44K DNA microarray enables for a parallel monitoring of transcriptional change induced by SE infection at the whole

genome level, and have provided global information of candidate genes for further analysis. The technology of RNAi gene silencing was used to generate a temporary knockout, a so called knockdown, on NF-kB 1 gene for directly studying its function in the SE pathogenesis and host response. In addition, given that the current chicken genome was only partially annotated, the bioinformatics will be able to annotate functions of unknown genes differentially expressed from microarray analysis. The overall goal of this project is to study genes or signal pathways associated with SE infection in chickens. Subsequently, these genes can be utilized in poultry breeding program to improve poultry health after the validation.

CHAPTER II

LITERATURE REVIEW

Salmonella Serotypes

Salmonella, named after Dr. Daniel E. Salmon (Salmon 1884), is a genus of gram negative bacteria belonging to the family of Enterobacteriaceae, usually in rods shape. The genus *Salmonella* contains more than 2,500 serotypes. Serotyping is usually based on the identification of somatic (O) and flagellar (H) antigens using specific antisera. The organisms occur worldwide in water, soil, animal feeds, raw meat and offal, and in vegetables (Quinn and Markey 2003). Several *Salmonella* species are pathogenic, some producing mild gastroenteritis, others producing a severe and often fatal food poisoning, which is called salmonellosis. Salmonellosis is caused by the infection with *Salmonella* mainly through the route of ingestion. The common syndromes include diarrhea, fever, and abdominal cramps. Although most patients can recover from illness without antibiotic treatment in 4 to 7 days, the illness can be severe to elderly, infants and those with impaired immune systems and cause death unless the person is treated promptly with hospitalization (CDC, Centers for Disease Control and Prevention, http://www.cdc.gov/ncidod/dbmd/diseaseinfo/salment_g.htm.). The reported incidences of human infections by *Salmonella* have dramatically increased since 1980, and at present there are approximately 1,400,000 cases every year in the United States. Although outbreaks usually attract media attention, more than 80% of all salmonellosis cases occur individually rather than as outbreaks, which causes a significant economical

loss due to medical costs and loss of productivity (Economic Research Service, http://www.ers.usda.gov/data/Foodborneillness/).

The virulence of *Salmonella* is related to its ability to invade host cells, replicate and resist both digestion and destruction against host defense system. *Salmonella* are often classified according to the adaptation to animal hosts. A few serotypes have a limited host-spectrum (affect only one or a few animal species) such as *Salmonella* Typhi in primates, *Salmonella* Pullorum in poultry, *Salmonella* Choleraesuis in pigs, and *Salmonella* Dublin in cattle. These serotypes are relatively host –specific. When these stains cause disease in humans, it is often invasive and can be life threatening. In contrast, most serotypes have a broad host-spectrum such as *Salmonella* Enteriditis and *Salmonella* Typhimurium. Typically the infection with such strains in humans cause uncomplicated gastroenteritis and does not need treatment. However the syndrome can be severe in the young and elderly and patients with weakened immunity. The *Salmonella* serotypes of importance in domestic animal and the consequences of infection are indicated in **Table 2.1** (Quinn and Markey 2003).

Table 2.1 *Salmonella* serotypes of clinical importance and the consequences of infection

Salmonella Serotype	Hosts	Consequences of infection
Salmonella Typimurium	Many animal species	Enterocolitis and septicaemia
		Food poisoning
	Humans	
Salmonella Dublin	Cattle	Many disease conditions
	Sheep, horses, dogs	Enterocolitis and septicaemia
Salmonella Choleraesuis	Pigs	Enterocolitis and septicaemia
Salmonella Pullorum	Chicks	Pullorum disease (bacillary white diarrhoea)
Salmonella Gallinarum	Adult birds	Fowl typhoid
Salmonella Arizonae	Turkeys	Arizona or paracolon infection
Salmonella Enteritidis	Poultry	Often subclinical in poultry
	Many other species	Clinical disease in mammals
	Humans	Food poisoning
Salmonella Brandenburg	Sheep	Abortion

Salmonella Enteritidis

The evolution of specific *Salmonella* serotypes in intensive animal husbandry or humans has been observed over the three decades. During the 1990s, a rapid increase of incidence in *Salmonella enterica* serovar Enteritidis (SE) surpassed *S*. Typhimurium as the predominant *Salmonella* serotypes from humans in the United States (Altekruse et al. 2006; Patrick et al. 2004). Poultry products account for the main sources of SE-salmonellosis in human through the consumption of contaminated meats or shell eggs (Altekruse et al. 2006; Braden 2006a). The accumulating evidence suggested that eggs are the most important source of human SE infection, and this raises questions about how eggs are contaminated. The hypothesis that SE might be transmitted directly to the

internal contents of the egg prior to the laying of egg was confirmed by experimental studies performed at the US department of Agriculture (USDA) (Braden 2006b). Subsequent studies of inoculated hens indicated that ovary and oviduct are primary internal organs account for the vertical transmission of SE to eggs. On the other hand, the colonized SE in ceca can be excreted in feces and easily spread to other birds in the flock (Tilquin et al. 2005a).

Unlike other host-specific *Salmonella* spp. which causes systemic disease after intestinal colonization in birds, SE belongs to the broad-host-range *Salmonella* spp. and its infection in chickens is often silent, with the exception of very young chickens (< three days), among which high mortality rates were observed (Sadeyen et al. 2004). The asymptomatic status of SE-infected chicken (also known as healthy carrier) has made it more difficult to control SE from spreading, therefore causes serious consequences on public health.

Current Control of Salmonellosis

The control of *Salmonella* infection in poultry is associated with different factors including good hygienes on a farm, enhanced biosecurity, monitoring the flocks, and taking appropriate protective mechanisms such as the usage of vaccination. In United States, the National Poultry Improvement Plan, a cooperative program administered by the USDA, was initiated in 1989 to enhance the health of poultry and safety of poultry products regarding SE infection or contamination (Braden 2006a). Although these programs have been shown to be effective in reducing rates of human SE illnesses

mostly by improving egg-quality assurance, the incidence of SE contaminated broiler carcass was increased from 2000 to 2005 (Altekruse et al. 2006). Since SE can shed in the feces and lead to infection on birds in the same flock, the horizontal transmission of SE by healthy (asymptomatic) carrier is the major problem of controlling SE from spreading (Beinke and Ley 2004).

Salmonellae are intracellular parasites. A live-attenuated vaccine is therefore expected to confer better protection than killed vaccines since the former stimulate both cell-mediated and humoral immunity. The drawbacks of using killed vaccines, primary the variable efficacy, have accelerated the increasing usage of more effective live vaccine. However, from the point of view of customer safety, the current developments in live *Salmonella* vaccines are hindered by fears associated with the releases of live vaccines whose pathogenicity could be genetically recovered from the mutants.

The widespread usage of antibiotics has led to the emergence of multiple antibiotic-resistant bacteria. In 1995, the use of low dose antibiotics such as fluoroquinolones, enrofloxicin and sarafloxicin was approved in poultry in United States for disease prevention and growth promotion (Braden 2006a). Since then the antibiotic-resistant *Salmonella* has caused an increasing public health concern. Selective pressure from the use of antimicrobials is the major driving force behind the emergence of antibiotic resistance (Angulo et al. 2000). Although in most cases, multi-drug resistance (resistance to multiple antimicrobials) is transferred through a coherent plasmid DNA in organism, some variants of *Salmonella* have developed multi-drug resistance as an integral part of genetic material of the organism (chromosomally encoded) and may

remain the drug-resistance everlastingly regardless the use of antimicrobials (Rowe et al. 1997). It is of paramount importance to limit the unnecessary usage of antimicrobials in food animals, so that the spread of drug-resistant *Salmonella* should not be further jeopardized.

Genetic Control of Resistance to *Salmonella* Infection

With increasing consumer demands for safe poultry products, public concerns are prompting government regulations on the use of antibiotics in animal production, and the ability of current vaccines to protect against emerging hyper-virulent strains of pathogens is also becoming an issue (Lillehoj et al. 2007). Selective breeding of chickens for genetic resistance to systemic salmonellosis offers an additional possible control measure (Wigley, 2004a). Disease susceptibility is a large part heritable and therefore genetically determined. Genetic differences in disease susceptibility can be due to the diversity of the immune system and ineffective (too low, too high or misdirected) immune reactivity during bacteria infection. It has been reported that different inbred chicken lines had different degree of susceptibility to *Salmonella* infection (Bumstead and Barrow 1993), and the level and duration of bacterial colonization of gut are under genetic control of chicken host (Sadeyen et al. 2004; Wigley 2004a). Genetic resistance can be investigated by linkage analysis such as quantitative trait loci (QTL) mapping and association studies. Both approaches can help identifying loci or markers associated with disease resistance traits, where the genes physically linked to these loci/markers might be functionally controlling the disease resistance. In chickens, both QTL and association

studies have been conducted for investigating resistance to salmonellosis (Kramer et al. 2003; Liu and Lamont 2003; Tilquin et al. 2005b). However, these linkage studies are very labor-intensive as extensive breeding is needed for establishing inbred lines with contrasting phenotypes. Furthermore, the resolution obtained in a QTL region is usually limited which contains too many genes and therefore hampers the efficiency for identifying trait genes within a QTL region.

Most reported candidate genes (trait genes) for disease resistance are involved in immune mechanisms. The genetic difference in disease susceptibility could also be studied at a molecular level such as by measuring transcriptome (mRNA) changes of candidate genes. Recognition of pathogens can cause changes in the gene expression levels in particular immune-related cells of the host, and these changes can be different between susceptible and resistant chickens. Candidate genes that influence *Salmonella* resistance in domestic animals have been well described by Wigley previously (Wigley 2004b). However, although a number of factors were found associated with *Salmonella* resistance in chicken, very few of them has shown consistent differences of resistance between susceptible and resistant lines by experimental investigations (Lamont et al. 2002; Wigley 2004b; Wigley et al. 2002).

Chicken Immune System

The immune system is the host defense system against infectious diseases, including innate and adaptive immune responses. Innate immunity is an ancient and universal mechanism utilized by many organisms, while the adaptive immune is an

evolutionarily newer system which induces a delayed antigen-specific immune response increased with successive exposure to the same microbes (Abbas and Lichtman 2005). Functionally, both systems are working complementary and highly interrelated in a host defense system. The activation of innate immunity leads to a series of signaling events resulting in the induction of dendritic cell maturation, which is responsible and necessary for initiation of adaptive immune responses (Pasare and Medzhitov 2004; Werling and Jungi 2003).

The innate immunity system is evolutionally conserved and is the first line of the defensive mechanisms which protects the host from invading microbial pathogens (Akira et al. 2001). Elements of the innate immunity include: barriers (e.g. epithelial layers, defensins and intraepithelial lymphocytes), circulating effector cells (e.g. neutrophil (heterophils in birds), machophages and Natural Killer cells), circulating effector proteins (opsonins) (e.g. complements, Mannose-binding lectin and C-reactive proteins), and cytokines.

The innate immunity is capable of removing the infectious agents shortly after the infection. One of the mechanisms by which the innate immune system senses the invaded pathogenic micro-organism is through recognizing highly conserved sets of molecular structure specific to the microbes (pathogen-associated molecular patterns, PAMP) using a set of germ line encoded receptors named pattern-recognition receptors (PRRs) (Pasare and Medzhitov 2004). These PRRs could effectively recognize constitutive structures of foreign pathogens such as lipopolysaccharide (LPS) and peptidoglycan (PDG), or chemicals expressed only in pathogens instead of the host self,

by which this mechanism allows innate immune system to detect microorganisms without complicated diversity on receptors (Abbas and Lichtman 2005).

The reported PRRs include non-phagocytic receptors, such as Toll-like receptors, Nucleotide Oligomerization Domain (NOD) proteins and receptors that induce phagocytosis, such as Scavenger receptors, mannose receptors and β-glucan receptors (Lata and Raghava 2008).These PRRs play an important role on mediating antimicrobial effects by activating the complement system, assisting phagocytosis and killing of foreign microorganisms. Recent studies have demonstrated strong interactions between signaling through these receptors, where the signaling pathways downstream of PRRs and their cross talk control immune responses in effective manners (Lee and Kim 2007). Although little is known regarding these receptors in farm animals, the knowledge about PRRs can unlock the key to animal diseases and aid in the understanding of pathogenesis (Werling and Coffey 2007).

Adaptive immunity is the second line of defense evolved in vertebrates exclusively. This system uses a diverse set of somatically rearranged receptors, T-cell receptors and B-cell receptors, with the ability to recognize a large spectrum of antigens. The adaptive immune response is also called specific immunity since its extraordinary capacity to distinguish among different, even closely related microbes and molecules.

The components of adaptive immunity are lymphocytes (B cells and T cells) and their products. Two types of adaptive immune responses are regulated by different lymphocyte groups, respectively. One is based on the formation of immunoglobulins (antibodies) by B-cells and is called humoral immunity. Antibodies recognize microbial

antigens, neutralize the infected microbes and target microbes for elimination by other effector mechanisms. The other one named cell-mediated immunity (also called cellular immunity) is mediated by T-cells. The cell-mediated immunity can induce the destruction of microbes residing in phagocytes or the killing of infected cells to eliminate reservoirs of infections. This is effective against microbes that are inaccessible to circulating antibodies such as viruses, some intracellular bacteria (Abbas and Lichtman 2005).

Both arms of the adaptive immune response, humoral and cell-mediated immune imunity, are interactive yet distinct in functions. On the perspective of antigen preference, the humoral immunity may be more effective in controlling bacterial infections whereas cell-mediated immunity would be desirable in eliminating virus-infected cells. It was reported that genetic selection for improved broiler performance (e.g. carcass weight and growth rate) has had a negative impact on the humoral immune response, but an increase in the cell-mediated and inflammatory responses (Cheema et al. 2003). It is possible the consequence of decreased humoral immunity may elevate the risk of bacterial infection on current broiler strains, which have been intensively selected for growth related traits.

Salmonella Pathogenesis

Microbial pathogens have evolved a huge diversity of virulence factors to engage in target hosts in very complex interactions. These interactions are aimed at: (1) gaining access to the host, (2) avoiding host defense mechanisms, (3) multiplying, and (4)

ultimately moving on to a new host (Galan 1998). *Salmonella* enterica has been a good example of studying well-adapted microbial pathogens. Two principal clinical syndromes associated with *Salmonella* infection are enteric (typhoid) fever and gastroenteritis. Enteric fever is a protracted systemic illness that results from infection with typhoidal *Salmonella* stains such as *S. typhi* and *S. paratyphi*. The pathological characteristics of enteric fever are mononuclear cell infiltration and hypertrophy of the reticuloendothelial system, including Peyer's patches, mesenteric lymph nodes, spleen and bond marrow. In contrast, the nontyphoidal *Salmonella* such as *S. enteriditis* and *S. typhimurium*, infect a wide range of animal hosts, and usually cause a self-limited enteritis in the adapted hosts.

All salmonellosis begin with the ingestion of organisms via the contaminated food or water. Although low pH of gastric acidity was thought as the initial barrier to the infection (Giannella et al. 1972), some strains of *Salmonella* express acid tolerance response genes in acidic host environments, which can promote the infection of *Salmonella* (Garcia-del Portillo et al. 1993). After entering the small bowel, the invasion of *Salmonellas* preferentially occurs at microfold cells (M cells) of the intestinal epithelium. M cells are the specialized non-professional phagocytes that sample intestinal antigen through pinocytosis and transport antigens to epithelial lymphoid cells located at Peyer's patches. However, the role of M cells and enterocyted invasion may be different in different host animals (Paulin et al. 2002).

Salmonella must traverse the intestinal mucus layer before encountering and adhering to cells of the intestinal epithelium. To do so, *Salmonella* express several

fimbriae that contribute to their ability to adhere to intestinal epithelial cells (Baumler et al. 1996). Shortly after bacteria adhere to the apical epithelial surface, *Salmonella* invade epithelial cells through a complicated process called 'bacterial-mediated endocytosis'. This process resembles the host membrane ruffling and macropinocytosis induced by growth factors in many cells, but it is morphologically and functionally distinct from 'receptor-mediated endocytosis' by which many other pathogens enter non-phagocytics cells. The process of bacteria-mediated endocytosis includes the profound cytoskeletal rearrangements occur in the host cell, disrupting the normal epithelial brush border and inducing the subsequent formation of membrane ruffles that reach out and enclose adherent bacteria in large vesicles(Ohl and Miller 2001). It is still unclear about these *Salmonella*-containing vesicles (SCV) or how bacteria exist inside. Following bacteria-mediated endocytosis, a fraction of the SCV translocates to the basolateral membrane, and the apical epithelial brush border reconstitutes (Santos et al. 2003).

The events of *Salmonella* invasion are clinically associated with the illnesses they cause. After the crossing of the intestinal epithelial barrier, *Salmonella* serotypes that cause enteritis will induce a secretary response in the intestinal epithelium and initiate recruitment and transmigration of neutrophil (or heterophils in birds) into the intestinal lumen. The recruitment of neutrophil across the epithelial surface requires protein synthesis in both bacteria and epithelial cell and is associated with production of several cytokines including chemokine interleukin-8. Once across the intestinal epithelium, *salmonella* encounters another barrier of innate immunity, the submucosal macrophage. *Salmonella* serotypes that cause systemic infection will enter macrophages

by induced macropinocytosis, and subsequently activate virulence mechanisms that allow evasion of the microbicidal functions of the pathgocyte, permitting survival and replication in the intracellular environment (Santos et al. 2003). The migration of infected macrophages to other organs of the reticuloendothelial system probably facilitates dissemination of *Salmonella* in the host (Alpuche-Aranda et al. 1994).

Salmonella Virulence Mechanisms

Many of *Salmonella* virulence genes are clustered in certain areas of the chromosome known as *Salmonella* pathogenicity islands (SPI). To date, five SPI have been reported and two of them, SPI-1 and SPI-2, are found playing critical roles in *Salmonella* pathogenesis. In murine model, the S. typhimurium strains with mutations in SPI-1 and SPI-2 have shown 50-fold and >10,000-fold attenuation, respectively (Galan and Curtiss 1989; Ochman et al. 1996).

Both SPI-1 and SPI-2 encode a subset of secreted proteins that form a translocation complex called type III secretion system (TTSS), a “molecular syringe” for *Salmonella* to deliver virulent factors into the host cell cytoplasm (Galan 1998; Zhang et al. 2003). Functionally, the SPI-1-encoded type III secretion system (TTSS-1) is required for the initial invasion of intestinal epithelium (non-professional phagocytes) (Galan and Curtiss 1989) and enteropathogenesis (Tsolis et al. 1999), while the SPI-2 encoded type III secretion system (TTSS-2) is required for systematic infection such as intracellular survival and replication in macrophages (Ochman et al. 1996).

Previous studies indicated that TTSS-1 is the prime virulence determinant of *Salmonella*-induced diarrhea. Distinct from secretory diarrheas which are caused by cholera toxin, *Salmonella*-induced diarrheas are caused by inflammatory response and neutrophil -induced tissue injury (necrosis) followed by the loss of protein-rich fluid (contains intestinal epithelium, discernible villi and crypt structure) into the intestinal lumen. It is reported that an intact TTSS-1 is essential for eliciting inflammatory response and recruiting neutrophil through two possible mechanisms: (1) During the invasion and transmigration of *Salmonella* through epithelial cells, the effector proteins in TTSS-1 may facilitate recognition by Nods or Toll-like receptors, thereby triggering proinflammatory signaling events (Inohara et al. 2002; Sieling and Modlin 2002); (2) the effector proteins in TTSS-1 may stimulate proinflammatory signaling events through direct binding such as sipB (in TTSS-1) and caspase 1 (in host cells) on the release of proinflammatory cytokine interleukin-1β (Hersh et al. 1999; Zhang et al. 2003). The selected events in *Salmonella* pathogenesis are summarized in **Figure 2.1**.

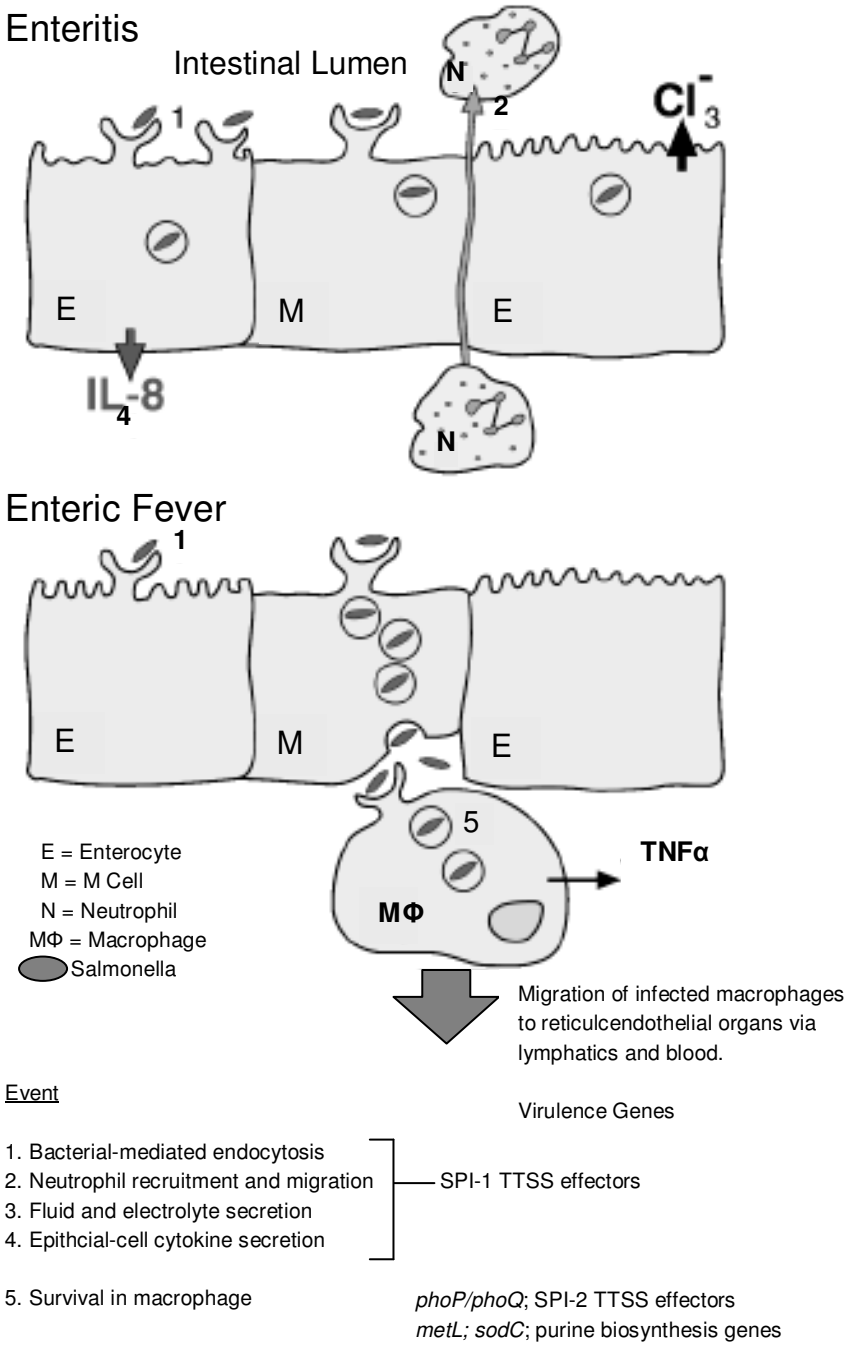

Figure 2.1 Selected events in *Salmonella* pathogenesis and associated virulence genes (Ohl and Miller 2001).

Host Response during *Salmonella* Infection

Neutrophil migration. Following oral ingestion, intestinal epithelial cells are the first barrier encountering bacteria invasion, which plays an important role in the outcome of infection by mediating the host inflammatory response. *Salmonella* invasion to epithelial cells by TTSS-1 system results in an increase in cytosolic concentration of calcium. The elevated calcium concentration is required for activation of the NFkB pathway and mitogen-activated protein kinase pathway, which lead to the expression and secretion of interleukin 8 (IL8), a chemoattractant for neutrophil (Gewirtz et al. 2000). Further experiments have led to the identification of pathogen-elicited epithelial chemoattractant (PEEC), another chemotractant secreted on the apical side of the epithelial cell. Although the molecular nature of PEEC is not well characterized, the secreted PEEC was reported to induce direct migration of neutrophil across monolayers of intestinal epithelial cells (McCormick et al. 1998).

It is known that IL8 and PEEC act in concert to promote neutrophil migration. While the role of IL8 is primarily recruitment of neutrophil to the lamina propria, the role of PEEC is to help the migration of neutrophil across epithelia to intestinal lumen (Gewirtz et al. 1999). An event similar to neutrophil migration in mammalian is also found in birds. It has been reported that a large influx of heterophils, the avian counterpart of mammalian neutrophil, is observed in the intestines of *Salmonella* infected chickens. This finding indicates that an increase in heterophils to the infection site is essential for defending against microbial infection (Kogut et al. 1994; Swaggerty et al. 2005).

***Salmonella*-induced macrophage apoptosis.** Host response during the later stages of *Salmonella*-macrophage interactions is critical to controlling infection. In the macrophages, *Salmonella* is able to replicate and stay in the areas of the spleen, liver, and bone marrow as systematic infection (Daigle et al. 2001). Additionally, *Salmonella* can also trigger cell death on resided macrophages or macrophage-like cells (Monack et al. 1996). Previous reports indicated that *Salmonella* invasion protein (SipB) as the bacterial effectors responsible for induction of *Salmonella*-induced macrophage apoptosis. During the infection of macrophages, SipB protein is translocated into the host cell cytosol via the TTSS-1 secretion system. The secreted SipB protein leads to the binding and activation of caspase-1, an intracellular cysteine protease which subsequently triggers apoptosis. On the other hand, SipB protein can also induce the degradation of the host protein v-raf-1, a murine leukemia viral oncogene homolog 1 (Raf-1), which favors apoptosis since Raf-1 acts by antagonizing the caspase-1-mediated apoptosis (Jesenberger et al. 2001).

Since caspase-1 also functions as a converting enzyme for IL1β, *Salmonella*-induced macrophage apoptosis is also associated with substantial release of active IL1β, a potent pro-inflammatory cytokine. In general, the apoptosis is defined as a form of cell death unrelated to inflammatory reaction. The release of IL1β provides a important link between *Salmonella*-elicited apoptosis and inflammation (Santos et al. 2003).

Toll-like receptors. During the evolution, multi-cellular organisms have developed various mechanisms to discriminate between self and non-self in order to

protect themselves from the invasion by infectious pathogens. Toll receptors or Toll-like receptors (TLRs) are groups of pattern recognizing receptors (PRRs) specifically recognizing different pathogen associated molecular patterns on foreign microbes (Akira et al. 2001). Toll receptors were first identified in Drosophila, and later on TLRs were found widely existing in vertebrates including birds and fish. In mammals, at least eleven different TLRs were identified from various tissue/cell types (Fukui et al. 2001; Medzhitov et al. 1997; Oshiumi et al. 2003).

The Toll-like receptor family is characterized by the presence of an extracellular domain containing leucine-rich repeats (LRRs), and a Toll/Interleukin-1 receptor (TIR) homology domain in intracytoplasmic region. Based on amino acid sequence and genomic structure, mammalian TLRs could be divided into five subfamilies: TLR2, -3, -4, -5, and -9. The TLR2 subfamily contains TLR1, -2, -6, and -10, whereas the TLR9 subfamily includes TLR7, -8, and -9 (Takeda et al. 2003). The cytoplasmic domains of these TLRs are relatively conserved, while the extra-cellular parts vary among different TLRs, which confer specific binding abilities to different compounds or chemicals of foreign pathogens.

The locations of TLRs are also related to different types of binding ligands, in which the surface-expressed TLRs (TLR1, -2, -4, -5 and -6) mainly respond to the cell wall components of foreign pathogens, whereas the intracellular membrane-expressed TLRs (TLR3, -7, -8, and -9) recognize nucleic acids such as RNA or DNA (Dunne and O'Neill 2005). Specifically, TLR1, -2 and -6 respond to various bacterial components including lipopeptide or peptidoglycan from gram-positive bacteria, TLR3 recognizes

double-stranded RNA (dsRNA) from viruses during their replication, TLR4 mainly recognizes LPS from gram-negative bacteria, TLR5 responds to bacterial flagellin, TLR7 and -8 can recognize single-stranded RNA (ssRNA) as well as imidazoquinolines, and TLR9 responds to un-methylated CpG DNA motif from bacterium or virus and hemozoin from malaria (Iqbal et al. 2005; Werling and Jungi 2003). Although the exact ligand for TLR10 remains unknown, it has been reported that TLR10 shares a similar structure with TLR1 and -6, and can heterodimerize with TLR1 or -2 (Hasan et al. 2005). At last, the mouse TLR11 was thought to recognize profilin-like ligands from some parasites (Yarovinsky et al. 2005).

Binding between ligands and TLRs can trigger signal transduction pathways and activate transcription factors within the host cells. Two primary pathways involved in TLR signaling transduction are nuclear factor kappa-B (NFkB) pathway and the interferon regulatory factor (IRF) pathway (Akira et al. 2001; Yamamoto et al. 2004). The first step in signal transduction requires the interaction between a diversity of adaptor proteins and their corresponding TLRs. Different TLRs may recruit different adaptor proteins to induce different signaling cascades. Common adaptor proteins include MyD88 (myeloid differentiation primary-response protein 88), Mal (MyD88-adaptor-like protein, also known as TIR-domain-containing adaptor protein or TIRAP), TRIF (TIR-domain-containing adaptor protein inducing IFN-beta), and TRAM (TRIF-related adaptor molecule) (O'Neill 2003).

During the transduction pathways, a series of cytoplasmic intermediates, such as IRAK (IL1 receptor-associated kinase) and TRAF (TNF receptor-associated factor), are

also recruited and phosphorated in turns. Finally, the transcription factors, such as NFkB and IRF-3, are activated and translocated in host cells, in which they can bind to the transcription factor binding sites (TFBSs) on target genes and induce their expressions. The induced genes include pro-inflammatory cytokines, chemokines and other immune-related factors, such as interleukin-6 (IL6), IL1, tumor necrosis factor-alpha (TNF-alpha) and interferon (IFN) (Akira 2003).

Chicken TLRs (chTLRs) were the first identified non-mammalian vertebrate TLRs. To date, ten chicken TLR genes, namely TLR1LikeA (TLR1LA), -1LikeB (TLR1LB), -2A, -2B, -3, -4, -5, -7, -15, and -21, have been reported (Temperley et al. 2008). A recent phylogenetic analyses showed that six of these genes have orthologs in mammals and fish, while only TLR21 is shared by fish and three chicken TLRs (TLR1LA, -1LB and -15) appear to be unique to birds. In addition to the identified chicken TLRs, a series of chicken genes involved in TLR signaling pathway were also found using *in silico* bioinformatic approach, which raises the question whereas a similar regulation system also exists in chickens TLR signaling pathway (Lynn et al. 2003b). However, more efforts are needed on chicken TLRs compared to mammalian TLRs.

Notably, two chicken TLRs, TLR4 and TLR15, have been reported involved in host defense with *Salmonella* infection (Wigley 2004a, Higgs, 2006 #119). The role of chicken TLR4 in *Salmonella* infection has been contradictory. Although TLR4 shows substantial differences in response to LPS, no significant response of TLR4 in cecum was observed following *in vivo S.* Typhimurium infection. TLR15 is a chicken specific

TLR which shows increased expression with the infection of heat-killed or live *S.* Typhimurium (Higgs et al. 2006a). However, the ligand of TLR15 has not been fully identified (Temperley et al. 2008). Further studies of chicken TLRs will eventually promote poultry health and benefit both the research and the industry.

Nucleotide-binding oligomerisation domain (NOD) protein. Besides TLRs, more recently, NOD molecules, NOD1 and NOD2, have also been shown to be involved in the innate immune response toward bacterial infection. The NOD proteins are cytoplasmic proteins structurally similar to a protein family in plants named R protein, which is associated in disease resistance against pathogen infection (Gomez-Gomez 2004). Similar to R proteins, NOD proteins have a C-terminal series of leucine-rich repeats (LRRs) and caspase-activating and recruitment domain (CARD) at N-terminus(Fritz et al. 2006a). Both NOD1 and NOD2 recognize peptidoglycan (PGN), a major constituent of the cell wall of Gram-positive bacteria, while it is found in the periplasmic space between the outer and cytoplasmic membranes areas in Gram-negative bacteria (Inohara et al. 2002; Takada and Uehara 2006). Since NOD proteins are cytosolic, the PGN ligands need to find a way of entering the cell in order to be recognized by these receptors and to trigger a defense response. Although the mechanism remains largely unknown, it is suggested that PGN may gain entry into cells through the type IV secretion system with bacterial infection, or through the phagocytic vacuole with the endocytosis and degradation of professional phagocytic cells (Kufer et al. 2005; McDonald et al. 2005).

After detecting PGN, NOD1 and NOD2 rapidly form oligomers and then transiently recruit receptor-interacting protein 2 (RIP2) through CARD-CARD interactions (Kufer et al. 2005). The NOD-RIP2 complex then recruits the inhibitors of NFkB kinase complex, which leads to activation of NFkB through the uniquitin-proteasome-dependent degradation of inhibitory chaperone IKB-α (Fritz et al. 2006b).

The discovery of TLRs and NOD proteins has opened up the field of innate immune system on recognizing microorganisms. More and more evidences show that TLR and NOD ligands act in synergy to trigger diverse cellular responses (Werts et al. 2006). It is believed that NOD proteins play a role as the cytoplasmic counterparts of TLR, which is cross-talking with TLRs and constitute a more complete cellular defense both at the plasma membrane and from within the cell. The synergistic effect of TLR and NOD could induce higher innate immune response **(Figure 2.2)**, and subsequently induce higher Th1-type immune responses **(Figure 2.3)** (Takada and Uehara 2006).

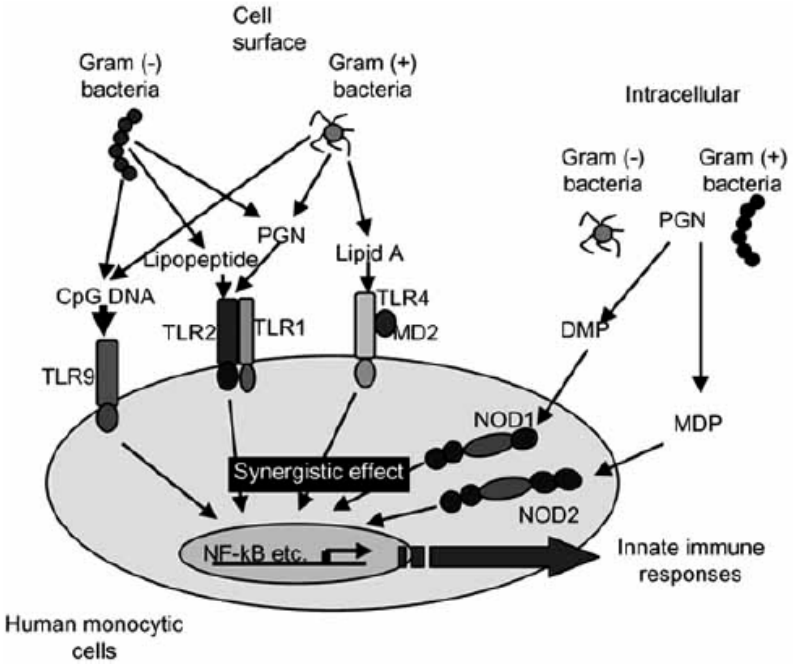

Figure 2.2 Synergistic effects of bacterial TLR ligands and NOD ligands to induce higher innate immune responses in human monocytic cells (Takada and Uehara 2006).

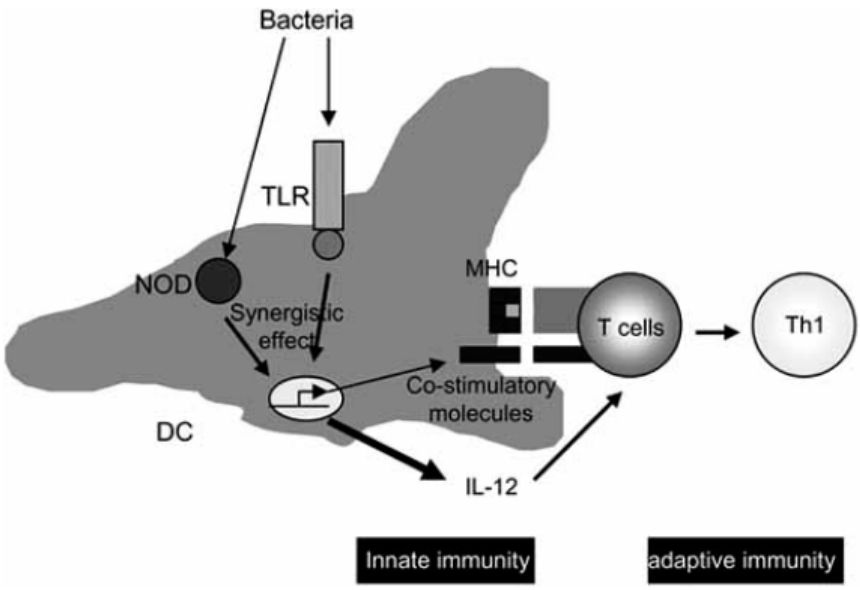

Figure 2.3 Synergistic effects of bacterial TLR ligands and NOD ligands on DCs induce higher Th1-type immune responses (Takada and Uehara 2006).

Candidate Genes Associated with Salmonella Resistance

Genetic approaches have many benefits to be included in a comprehensive program for disease management and production enhancement. Genetic enhancement of the immune response can increase vaccine efficacy and disease resistance, and thereby reduce the drug use in farm animals and economic losses due to the high mortality rate. Besides, the control of microbes colonization in domestic animal can prevent subsequent transmission of certain food-borne pathogen through consumption of meat or eggs (Wigley 2004a). Currently, lack of knowledge about the specific genes controlling resistance traits is the primary limit for applying molecular genetic approach to improve animal health. Compared to experimental animal models, the genetic resistance to *Salmonella* infection in domestic animal has been relatively poorly described. Fortunately, the study in chickens has progressed rapidly due to several advantages such as small size of body, rapid growth, short generation time and the availability of several well-defined inbred chicken lines with difference in susceptibility to *Salmonella* infection.

Most of candidate genes associated with disease resistance are involved in immune mechanisms. In the chicken, several genes were associated with *Salmonella* resistance such as SAL1 (Mariani et al. 2001), Slc11a1 (formerly known as Nramp1 [natural-resistance-associated macrophage protein 1] (Hu et al. 1996), Tnc (Hu et al. 1997), MHC class 1 and IAP1 (inhibitor of apotosis 1), PSAP (prosaposin) (Lamont et al. 2002), and TLR4 (Wigley 2004a).

Traditionally the identification of these candidate genes were based on homologous sequence searching through the known ortholog previously identified in other species, or by mapping disease resistance loci through the linkage analysis in inbred lines with contrasting phenotypes of Salmonella resistance trait. The resistance to *Salmonella* infection in chickens is a complex trait (Hu et al. 1997). Although some genes are supported by evidence such as the existence of single-nucleotide polymorphism (SNP) mutation within the gene, or *in vivo* study of *Salmonella* challenging, the control of *Salmonella* resistance at the molecular level is not quite clear so far. There has little or no functional difference was found in these genes between chicken lines that showed great difference in susceptibility (Wigley 2004a). Additionally, certain genes have some effects on systemic disease from salmonellosis, but appeared to play little role in gastrointestinal colonization, which indicated the survival strategies of different *Salmonella* strains is a critical factor against host defense system (Wigley 2004a; Wigley et al. 2001). Since the *Salmonella* resistance is probably the consequence of multiple-factor events, the traditional 'one-gene at a time' study is time consuming and is biased on choosing candidate genes by known biological properties of the gene products.

Identification of Candidate Genes through Gene Expression

Pathogens can modulate transcriptional expression of host cells during the infection. The genes targeted by pathogens frequently belong to the immune system or are involved in host cellular processes to infection. Jenner and Young have reported a

cluster of 511 genes that are designated ‘the common host-transcriptional response’, as these genes are induced in many different cell types in response to exposure to several different pathogens. The gene ontology analysis showed that these genes cover several functional groups such as mediating inflammation, response to interferon, activating/limiting immune response, and lymphocyte activation (Jenner and Young 2005).

Gene expression array. Transcriptional profiling has provided a wealth of information about host-pathogen interactions (Hossain, 2006). With the feasibility of more advanced molecular technologies, the potential candidate genes can be efficiently identified through appropriate experimental approaches. Through the functional analysis or pathway-based analysis, information from different candidate genes can be integrated and might lead us to elucidate molecular mechanisms that control *Salmonella* resistance.

Large-scale expression profiling such as gene expression array technology is a powerful tool that has been successfully used to determine host responses to invasive bacteria in the chicken. Previous studies monitor the changes in gene expression that take place in host epithelial cells (van Hemert et al. 2007), lymphocytes (Sarson et al. 2007), spleen tissues (Zhou and Lamont 2007), and macrophages (Bliss et al. 2005) after the stimulation with LPS or the contact with a specific pathogen. The advantages of using microarray technology were well reviewed by Rosenberger and his collegues (Rosenberger et al. 2001). Briefly, microarray technology is able to provide a more comprehensive, relatively unbiased knowledge of all gene networks including members

of gene families, ligands, receptors, and transcription factor. Additionally, it also allows for the discovery of new genes and/or pathways previously not known to be involved in a specific host-pathogen interaction.

Although most gene expression profiling assasys were focused on the change of transcriptome, similar array technology has been applied on studying proteomics. Protein arrays, also known as antibody array, are solid-phase ligand binding assay systems using immobilised proteins (usually antibodies) on surfaces which include glass, membranes, microtiter wells or beads (Eickhoff et al., 2002). Different resource of proteins for comparison are directly/indirectly conjugated with different fluorophores (e.g. Cy-3, Cy-5), and then mixed, bound to antibodies on the array. Through the measuremet of fluoricent colour that acts as a readout for changes in target abundance, the protein array is capable to identify protein-protein interactions, the targets of biologically active small molecules, or to monitor their expression levels and functions. The protein array is rapidly becoming a powerful means in a wide-ranging applications such as Diagnostics, protein isolation, and Protein functional analysis (Eickhoff et al., 2002). It can also serves as a potential tool to compensate the nucleotide arrays on studying proteomics.

There are also limitations to the use of microarray technology. One limitation is potential variability in sample preparation, array platforms and data analysis that obstructs true comparison between studies. Since array experiments typically generate more data than can be published in a paper or studied by one laboratory, it is important and more economic to facilitate the data mining of hidden information by comparative

analysis between studies. To do that, it is hoped that standard protocols and complete raw data set will be more accessible for normalization and comparisons between studies. Secondly, although microarray rapidly generates numerous hypotheses, many of them could be fallen into false-positive error due to multiple testing error (Pounds 2006). In order to pursue the true biological relevance of array data, it is essential to improve statistical analysis and confirm differential expression by other robust techniques (e.g quantitative PCR, Northern, or proteomic analysis) before drawing significant conclusions.

RNA interference (RNAi). RNAi is the process of sequence-specific post-transcriptional gene silencing, which was first described in the beginning of 1990s in plants (Duxbury and Whang 2004). It has become clear that RNAi is the oldest and most ubiquitous antiviral system that appeared before the divergence of plants and animals (Sharp 2001). This ancient process allowed the host to have defensive system against parasitic sequence, and use epigenetic regulation mechanisms to defend transposable elements TE and viral sequence (Matzke et al. 2001).

Out of the many types of RNA-mediated silencing process, RNAi has become relatively well known due to its usefulness as a molecular biology tool, an alternative choice for loss-of-function approach (Matzke and Birchler 2005). The mediator of messenger RNA degradation are 21~26 nucleotide small interfering RNA (siRNA), which could be generated by artificially chemical synthesis, or through ribonuclease III-like dicer by cleaving the longer double strand RNA (dsRNA). Since knocking down'

genes is infinitely easier than knocking them out in perspectives of time and demanded technology, RNAi was commonly used for *in vitro* validation of gene function by selectively inhibiting the expression of specific gene (Aigner 2006a).

The combination of microarray and RNAi, especially siRNA gene silencing, has being successfully used in many previous studies (Kuldell 2006; Mittal 2004). Especially in the study of gene pathway, RNAi could be used to create mutant phenotype for microarray study (Gorreta et al. 2005) or to identify and validate the components of a signaling pathway (after microarray study) that are associated with the silenced gene (Ramos-Nino et al. 2003). RNAi technology may be particularly useful in the chicken model, since the technology of generating knock-out chicken is very difficult to achieve. Although currently there is a lack of information regarding RNAi experiments in chickens, it is hoped this technology could be further developed and integrated with other approaches to dissect specific pathway in the near future.

Bioinformatics. Also known as in-silico analysis, bioinformatics is an interdisciplinary field that blends computer science and molecular biology. An important goal of bioinformatics is to facilitate the management, analysis, and interpretation of data from biological experiments and observational studies (Moore, 2007). In general, bioinformatics consists of two primary components: (1) the computerized annotation of genomic and biological data (databases); (2) the transformation and manipulation of these data (software tools). Bioinformatics emerged as an important discipline shortly

after the development of high-throughput DNA sequencing technologies in the 1970s (Boguski 1994).

The most pressing tasks in bioinformatics involve the analysis of nucleotide/protein sequence information. Since the speed of data discoverying is faster than data mining under current molecular technologis, the need of gene annotation become more indispensable for accelerating these types of studies especially for genome-wide study or microarray study. The common methods of sequence analysis include: (1) Finding the genes in the DNA sequences of various organisms, (2) Developing methods to predict the structure and/or function of newly discovered proteins and structural RNA sequences, (3) Clustering protein sequences into families of related sequences and the development of protein models, and (4) Aligning similar proteins and generating phylogenetic trees to examine evolutionary relationships.

Although the whole chicken genome was first released in 2004, the unknown function expressed sequence tags (ESTs) still account for a huge portion of genome. According to the latest statistics, within about 40,000 genes predicted from current chicken genome, less than 5000 genes are known protein-coding genes, and about 7500 genes (projected protein-coding genes) have been predicted their functions using bioinformtic approaches, which suggests there still has a large space for gene annotation tasks to be finished (http://www.ensembl.org/Gallus_gallus/index.html). A major problem for gene annotation in chicken using orthologs sequences, in particular from mammals, is the lack of sequence homology between these genes due to their high rate of evolution (Burt 2005). In the case of annotating a gene with weak sequence homology,

a large amount of accumulated evidence is critical for improving the accuracy of gene annotation (Smith, 2004).

Bioinformatics was also successfully used in chicken immunology study. Smith and colleagues have identified 185 immune-related genes using *in silico* from a large scale searching from 460,000 chicken ESTs sequence (Smith et al, 2004). These genes include chicken cytokines, chemokines, antigens, cell surface proteins, receptors and MHC-associated genes, in which 95 of these genes are previously not present in the GenBank/EMBL nucleotide and protein databases (Smith et al. 2004). Similarly, chicken TLRs and several critical adaptor proteins, namely Toll-interacting protein (Tollip), IL1 receptor-associated kinase 4 (IRAK-4), myeloid differentiation factor 88 adapter-like (Mal), TGFbeta-activated kinase 1 binding protein 1 (TAB1) and myeloid differentiation factor 88 (MyD88), were identified by through the homology searching of assembled ESTs sequence, which confirmed the evolutionary conservation of the TLR pathway in chicken and showed its essential homology to the TLR pathway in mammals (Lynn et al. 2003b; Yilmaz et al. 2005). Besides giving new insight into avian evolutionary studies, the identification of these genes has accelerated the further study of avian immunology and paved the way for large-scale immune-related microarray experiments.

CHAPTER III

MICROARRAY ANALYSIS OF CHICKEN HETEROPHILS WITH *SALMONELLA* ENTERITIDIS INFECTION

Overview

Salmonella enterica serovar Enteritidis (SE) is one of the most common food-borne pathogens that cause human salmonellosis and usually results from the consumption of contaminated poultry products. The mechanism of SE resistance in chickens remains largely unknown. Previously, heterophils isolated from broilers with different genetic backgrounds (SE-resistant [line A] and -susceptible [line B]) have been shown to be important in defending against SE infections. To dissect the interplay between heterophils and SE infection, we utilized large-scale gene expression profiling.

The results showed more differentially expressed genes were found between different lines than between infection (SE-treated) and non-infection (control) samples within line. However, the numbers of expressed immune-related genes between these two comparisons were dramatically different. More genes related to immune function were down-regulated in line B than line A.

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*Reprinted from "Gene expression profiling in chicken heterophils with *Salmonella* enteritidis stimulation using a chicken 44 K Agilent microarray" by Chiang, et al., 2008, BMC Genomics. 9: 526. Copyright 2008 of BioMed Central Ltd.

The analysis of the immune-related genes indicated that SE infection induced a stronger, up-regulated gene expression of line heterophils A than line B, and these genes include several components in the Toll-like receptor (TLR) signaling pathway, and genes involved in T-helper cell activation. We found: (1) A divergent expression pattern of immune-related genes between lines of different genetic backgrounds. The higher expression of immune-related genes might be more beneficial to enhance host immunity in the resistant line; (2) a similar TLR regulatory network might exist in both lines, where a possible MyD88-independent pathway may participate in the regulation of host innate immunity; (3) the genes exclusively differentially expressed in line A or line B with SE infection provided strong candidates for further investigating SE resistance and susceptibility. These findings have laid the foundation for future studies of TLR pathway regulation and cellular modulation of SE infection in chickens.

Introduction

Salmonellosis in humans often results from consuming foods contaminated with *Salmonella*. The reported incidences of human infections by *Salmonella* have dramatically increased since 1980, and at present are approximately 1,400,000 cases every year in the United States, which indirectly caused a significant economical loss due to medical costs and loss of productivity (Economic Research Service, http://www.ers.usda.gov/data/Foodborneillness/). *Salmonella* enterica serovar Enteritidis (SE) is one of the most common *Salmonella* serotypes in many countries including the US, and is the main source of human salmonellosis through the consumption of

contaminated poultry or shell eggs (Braden 2006b). SE is a zoonotic pathogen and persists in the chicken cecum or ovaries without triggering clinical signs in the host. Salmonellosis in young chickens may cause high mortality as a result of severe diarrhea and dehydration (Barrow et al. 1987; Sadeyen et al. 2004).

The outcome of an encounter with *Salmonella* is dependent on multiple factors including genetic background (Mastroeni and Sheppard 2004; van Hemert et al. 2006). Although several studies have focused on the pathogenesis of SE in infected chickens, the mechanism of SE resistance in healthy-carrier chickens remains unknown. Heterophils, the avian counterpart of mammalian neutrophil , are the most abundant leukocytes in the peripheral blood and are essential for initiating and modulating innate immunity (Kogut et al. 2001). It has been reported that a large influx of heterophils is observed in the intestines of SE-infected chickens, indicating an increase in heterophils to the infection site contributes to defending against microbial infection (Kogut et al. 1994; Swaggerty et al. 2005). Both studies of *in vivo* and *in vitro* SE-infected heterophils from different chicken lines also revealed that the up-regulated mRNA expression levels of interleukin (IL)-1β, IL6, IL8 (CXCLi2), IL18, and anti-inflammatory cytokines transforming growth factor-β4 (TGF-β4) might be responsible for determining overall immune competence (Ferro et al. 2004; Swaggerty et al. 2006).

Heterophils play an important role in providing increased resistance against SE infections in poultry. The objective of the present study was to examine SE resistance by dissecting the interplay between heterophils and SE. Large-scale expression profiling technology including microarrays has been successfully used to achieve this goal

(Sarson et al. 2007; van Hemert et al. 2006; van Hemert et al. 2007; Zhao et al. 2006; Zhou and Lamont 2007). Microarray technology provides a more comprehensive, unbiased knowledge of all gene networks including members of gene families, ligands, receptors, and transcription factors (Rosenberger et al. 2001). Additionally, microarray analysis allows for the discovery of new genes and/or pathways previously not known to be involved in a specific host-pathogen interaction. In the present study, a chicken genome Agilent microarray (Li et al. 2008) was used to profile differential gene expression in heterophils from two genetically distinct parental broiler lines (SE-susceptible [line B] and –resistant [line A]) following *in vitro* stimulation with SE. The objectives of this study were to discover genes or gene networks associated with SE resistance and to examine the genetic effects on defending against SE infections in chicken heterophils.

Material and Methods

Experimental chickens. The two distinct parental broiler lines used in this study were obtained from a commercial company. To maintain confidentiality, the lines were designated A and B. At the day of hatch, chickens were placed in floor pens (8 feet × 8 feet) containing wood shavings, provided supplemental heat, water, and a balanced, un-medicated corn and soybean meal based chick starter diet *ad libitum*. The feed was calculated to contain 23% protein and 3200 kcal of metabolized energy/kg of diet, and all other nutrient rations met or exceeded the standards established by the National Research Council (1994).

Bacteria. A poultry isolate of SE (#97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA) and approved by the United States Department of Agriculture (USDA). SE was cultured in tryptic soy broth (Difco Laboratories, Becton Dickinson Co., Sparks, MD) overnight at 41°C. Stock SE (1×10^9 colony forming units [cfu]/ml) was prepared as previously described (Swaggerty et al. 2003).

Heterophil isolation. Heterophils were isolated from the peripheral blood of 100 chickens per line six days post-hatch. Following blood collection, heterophils were isolated as previously described (Swaggerty et al. 2006). Briefly, blood from chickens was collected in vacutainer tubes containing disodium ethylenediaminetetraacetic acid (EDTA) (BD vacutainer, Franklin Lakes, NJ) and mixed thoroughly. The blood and EDTA for each line was pooled and diluted 1:1 with RPMI 1640 media containing 1% methylcellulose and centrifuged at 40 *g* for 15 min at 4°C. The supernatant was transferred to a new conical tube and diluted with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (1:1), layered onto discontinuous Histopaque® gradients (specific gravity 1.077 over 1.119) and centrifuged at 190 *g* for one h at 4°C. The histopaque layers were collected, washed with RPMI 1640 (1:1) and pelleted at 485 *g* for 15 min at 4°C. The cells were then re-suspended in fresh RPMI 1640, counted on a hemacytometer, and diluted to 1×10^7/ml in RPMI. All tissue culture reagents and chemicals obtained from Sigma Chemical Company, St. Louis, MO, unless noted otherwise.

Total RNA isolation. Heterophils (1×10^7) were treated with 300 µl RPMI or SE, for 1 h at 39°C on a rotary shaker. Treated heterophils were pelleted, washed with RPMI (485×*g* for 15 min at 4°C), the supernatant discarded, the cells re-suspended in lysis buffer (Qiagen RNeasy mini RNA extraction kit, Qiagen Inc., Valencia, CA), and frozen. The lysed cells were transferred to QIAshredder homogenizer columns and centrifuged for 2 min at ≥ 8000 × *g*. Total RNA was extracted from the homogenized lysate according to the manufacturer's instructions, eluted with 50 µl RNase-free water and stored at -80°C.

Microarray experiment design. A dual color, balanced design was used to provide four different comparisons: AI/AN, BI/BN, AN/BN and AI/BI. Four biological replicates were conducted in each comparison and the dye balance was used throughout in order to prevent the dye-bias during the sample labeling (**Figure 3.1**).

Labeling and hybridization. The integrity of total RNA samples was confirmed using Agilent Bioanalyzer 2100 Lab-on-chip system (Agilent Technologies, Palo Alto, CA, USA). Five hundred nanograms (ng) of total RNA were reverse-transcribed to cDNA during which a T7 sequence was introduced into cDNA. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labeling of RNA with Cy3 (or Cy5) dye.

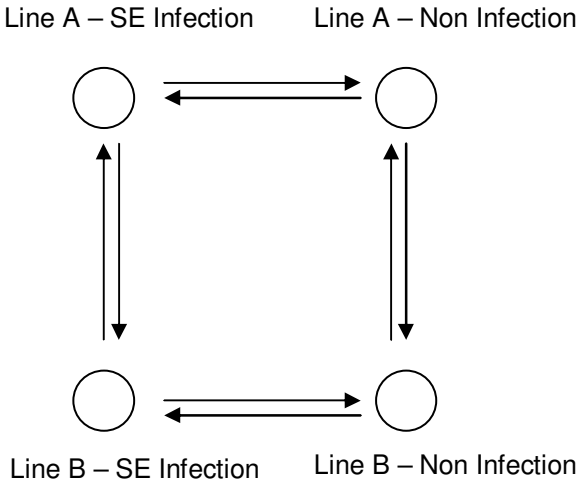


Figure 3.1 Balanced design of the microarray experiment. Each circle represents a single sample. Arrows represent an individual slide in hybridization. Arrowheads indicate samples labeled with Cy5, and arrowends represent samples labeled with Cy3. Dye balance was achieved for each biological type.

The fluorescent cRNA probes were purified using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and an equal amount (825 ng) of Cy3 and Cy5 labeled cRNA probes were hybridized on a 44K chicken Agilent array (GEO accession: GSE9416). The hybridized slides were washed using a commercial kit package (Agilent Technologies, Palo Alto, CA, USA) and then scanned using Genepix 4100A scanner (Molecular Devices Corporation, Sunnyvale, CA) with the tolerance of saturation setting of 0.005%.

Microarray data collection and analysis. For each channel, the median of the signal intensity and local background values were used. A Locally Weighted Linear Regression (LOWESS) normalization was applied to remove signal intensity-dependent dye bias for each array using R program. The normalized data was analyzed using commercial SAS 9.1.3 program (SAS Institute Inc. Cary, NC) with mixed model analysis. The mixed model used to identify significantly differentially expressed genes was:

$$Y_{ijklm} = \mu + T_i + L_j + D_k + S_l + T*L_{ij} + e_{ijklm}$$

Where Y_{ijklm} represents each normalized signal intensity; μ is an overall mean value; T_i is the main effect of treatment (SE infection) i; L_j is the main effect of chicken line j; D_k is the main effect of dye k; S_l is the random effect of slide l; $T*L_{ij}$ is the interaction between treatment and line; and e_{ijklm} is a stochastic error (assumed to be

normally distributed with mean 0 and variance σ^2). An approximate F test on least-square means was used to estimate the significance of difference for each gene in each comparison where $P < 0.001$ was considered to be statistically different. The false discovery rate (Q value) was calculated for each *P* value using R program according to the Storey and Tibshirani method (Storey and Tibshirani 2003).

Quantitative real-time PCR. Total RNA (300 ng) from each sample (AI, AN, BI and BN) was used for cDNA synthesis with random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. The cDNAs were quantified by qRT-PCR using ABI prism 7900HT system (Applied Biosystems, Foster, CA) with SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA). The specific oligonucleotide primers (**Table 3.1**) were designed by PRIMER3 program (Rozen and Skaletsky 2000). The conditions of qRT-PCR amplification were: 1 cycle at 95ºC for 10 min, 40 cycles at 95 ºC for 15 s and 59 ºC for 1 min. The chicken β-actin gene was used as the internal control. Dissociation curves were performed at the end of amplification for validating data quality. Each individual sample was run in triplicate and the average critical threshold cycle (Ct) was used for calculating relative quantification by fold-change and statistical significance.

Bioinformatics. An unreleased version of the High Throughput Gene Ontology Functional Annotation Toolkit (HTGOFAT, http://liru.ars.usda.gov/mainbioinformatics.html) was utilized to assign updated Gene Ontology numbers (Ashburner et al. 2000), Enzyme Commission (Shah and Hunter 1998) numbers, mappings to Kyoto Encylopedia of Genes and Genomes (KEGG) Pathways (Ogata et al. 1999) and updated definitions. Statistics related to over representation of functional categories were performed using a Fisher Exact statistic methodology similar to that described by Al-Shahrour et al (Al-Shahrour et al. 2004). In brief, differentially expressed genes ($P < 0.001$) were selected and separated based on regulation directions (up or down) in each comparison. Data mining to PubMed IDs was performed using a beta version module within HTGOFAT that searched PubMed abstracts using upon experimental conditions or terms (e.g. chicken and *Salmonella*) that co-occur with gene names and symbols that are represented within a given dataset. Additionally, differentially regulated genes were mapped to Protein Information Resource (PIR) keywords (Wu et al. 2002) and COG (Tatusov et al. 2003) functional annotations through the use of primary mappings with HTGOFAT and subsequent mapping and clustering using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al. 2003).

Table 3.1 Primers used for qRT-PCR

Accession No.	Gene Name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	PCR Product Size (bp)*
NM_205518	β-actin	ACGTCTCACTGGATTTCGAGCAGG	TGCATCCTGTCAGCAATGCCAG	298
L34553	CCL4	TACTACGAGACCAACAGCCAGT	TGTCTCCACATCCATTCTATCC	153
AJ309540	Interleukin 6 (IL6)	AGGACGAGATGTGCAAGAAGTTC	TTGGGCAGGTTGAGGTTGTT	78
AJ851659	CD80 antigen	CAGAGTCTCCAGTCTTCACCAG	GGAAAACCTCCATGAGAAGAAC	173
Y14971	CXC chemokine K60 (K60)	AGACTCATTCCAAGTTCATCCA	TTTGTTCTTTGCTTTAGGATGC	216
M64990	Prostaglandin-endoperoxide synthase 2 (PTGS2)	TCGAGATCACACTTGATTGACA	TTTGTGCCTTGTGGGTCAG	230
AF176086	Similar to NUMB protein (NUMB)	CAGCAGACGTTCCCTCAGTA	ATCACTTGAGAAGGGGTTGG	213
U20338	Interferon regulatory factor 7 (IRF7)	ATCCCTTGGAAGCACAACGCC	CTGAGGCAACCGCGTAGACCTT	223
AF082329	Caspase 6, apoptosis-related cysteine peptidase (CASP6)	CAGAGGAGACAAGTGCCAGA	CCAGGAGCCGTTTACAGTTT	250

*All of primers are designed with melting temperature around 59 ºC

Results

Identification of differentially expressed genes. The genome-wide expression profiling of each element (probe) was assigned to four different comparisons as AI/AN (line A infection vs. non-infection), BI/BN (line B infection vs. non-infection), AN/BN (non-infection line A vs. line B) and AI/BI (infection line A vs. line B).

In this context, the word infection refers to *in vitro* stimulation with SE. In the microarray analysis (**Figure 3.2A**), genes differentially expressed at $P < 0.001$ were considered statistically significant. The estimated false discovery rates at this level were controlled as 20%, 20%, 5%, and 5% in each comparison of AI/AN, BI/BN, AN/BN, and AI/BI, respectively. The biological significance (**Figure 3.2B**) for each comparison was analyzed using the assigned cut-off expression ratio of 1.5 fold-change, and the direction of regulation.

In general, more differentially expressed genes were found in the comparison between different lines than between infected and non-infected cells within line by both statistical- and biological-based analyses. The regulation direction of these differentially expressed genes showed an adverse tendency in the comparisons of infected vs. non-infected cells between two lines.

There were more genes up-regulated in the AI/AN (115 out of total 152 differentially expressed genes in AI/AN, and 48 out of total 173 differentially expressed genes in BI/BN). However, more genes were down-regulated in the BI/BN (37 out of total 152 differentially expressed genes in AI/AN, and 125 out of total 173 differentially expressed genes in BI/BN). A similar pattern of infected vs. non-infected cells was observed between two lines in terms of the number of up-regulated and down-regulated genes. In general, line B had stronger gene expression than line A on both control and SE-treated cells.

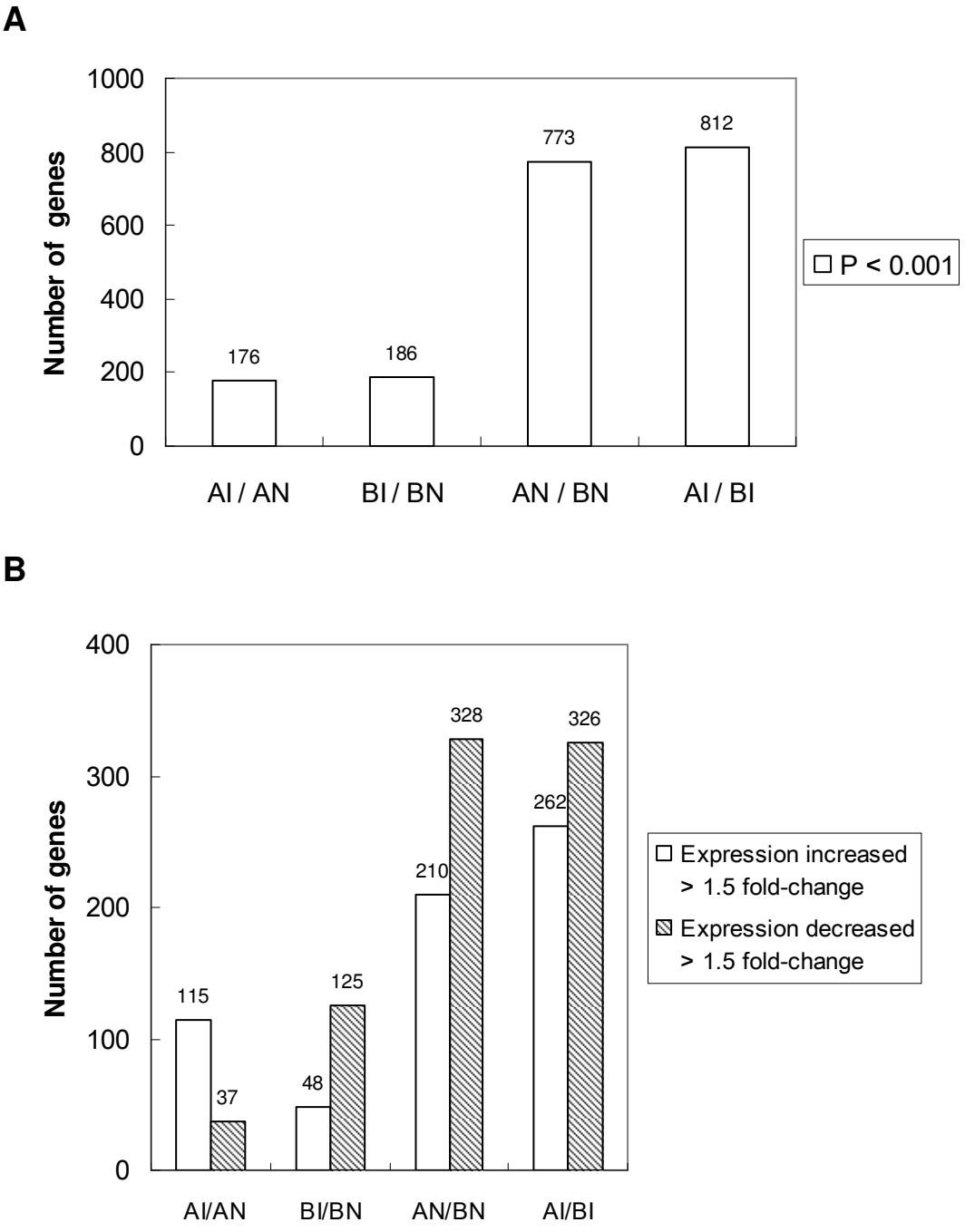


Figure 3.2 Number of differentially expressed genes at four different comparisons. A: P value smaller than 0.001. B: 1.5 fold-change of increased or decreased expression with P value smaller than 0.001.

Gene ontology analysis. The functional analysis was performed by identifying gene ontology (GO) terms (biological processes) of genes whose expression were significantly enriched among the pool of all differentially expressed genes. A Fisher-exact test was used to determine the enrichment of associated GO terms. Only significantly enriched ($P < 0.05$) GO terms are presented. In general, fewer significantly enriched GO terms were found in the comparison between infected and non-infected cells within line (**Figure 3.3A**) than between genetic lines (**Figure 3.3B**). In the comparisons between infected and non-infected cells, many functional terms were enriched in line B, while none of the functional terms were found significantly enriched in the same comparison for line A. For the down-regulated genes in the BI/BN comparison, many significantly enriched functional terms, including defense and immune response, and response to stress, were associated with the host defense system according to the GO term annotation (Ashburner et al. 2000). For the comparison between lines, there were more enriched functional terms in the down-regulated genes than in the up-regulated genes. Interestingly, many enriched terms were overlapped between AN/BN and AI/BI with the similar abundance (%) of genes.

A

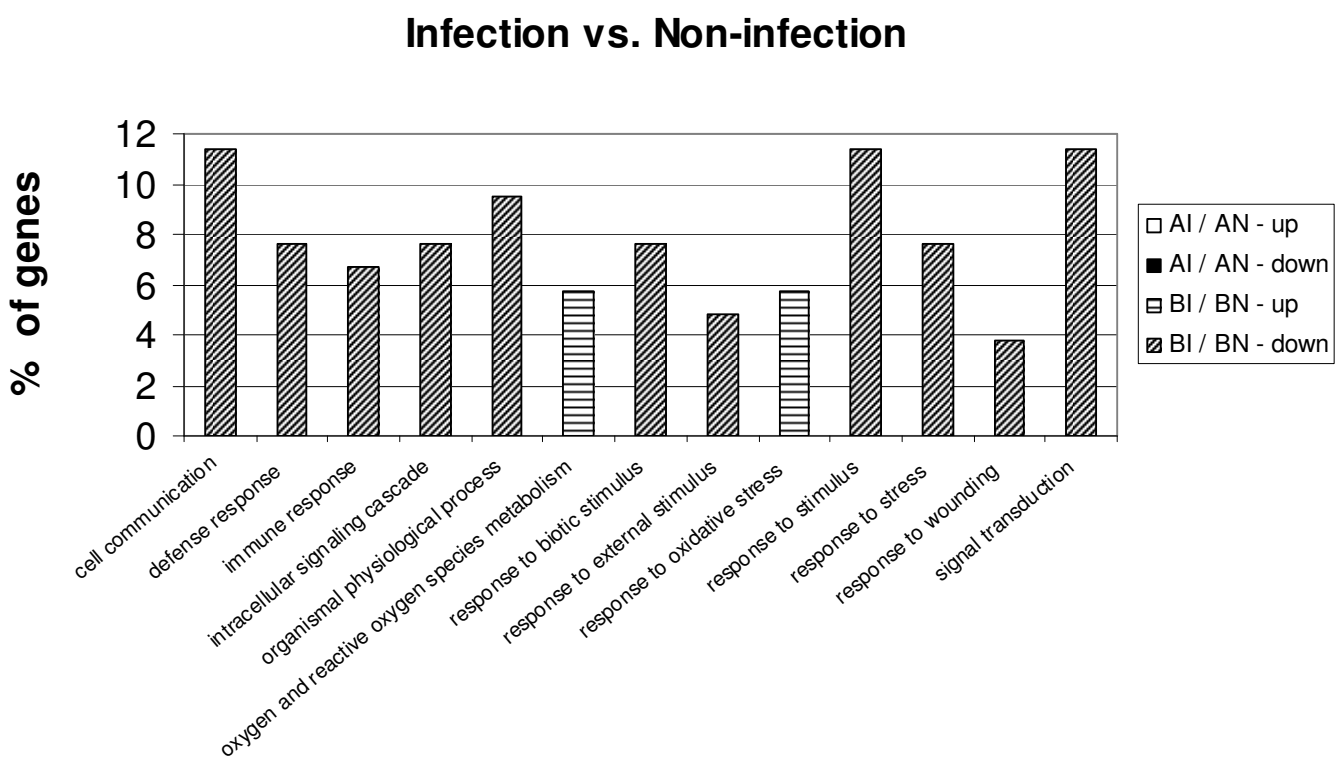


Figure 3.3 Gene ontology (GO) annotation of differentially expressed genes ($P < 0.001$). A: Biological processes of up- or down-regulated genes between infection and non-infection treatments (I/N). B: Biological processes of up- or down-regulated genes between line A and line B heterophils (A/B). The percentage represents the enriched-intensity of each term. The percentage represents the enriched-intensity of each term.

B

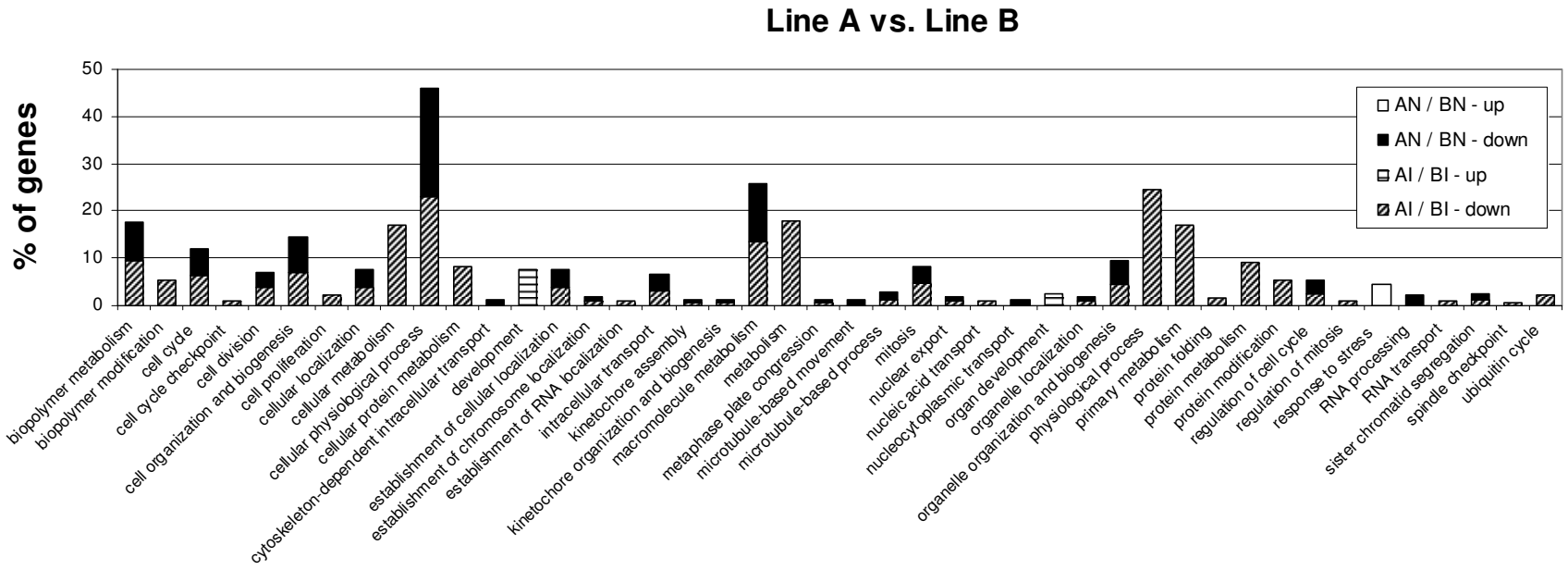


Figure 3.3 Continued.

Quantitative real-time PCR. Validation of the microarray data was performed using quantitative real-time PCR (qRT-PCR). This allowed us to: (1) confirm the microarray results across different comparisons, and (2) validate selected immune-related genes associated with *Salmonella* infection of heterophils. Eight significantly expressed genes were selected for qRT-PCR confirmation using specific primers (**Table 3.1**). The samples used in qRT-PCR were not the same as in the microarray study but were obtained according to the same experimental design. The results showed that most of the genes selected for qRT-PCR (17 incidences) analysis were consistent with the results obtained from the microarray (21 incidences) (**Table 3.2**). For the four inconsistencies, the fold-changes from three were very close to the microarray results.

Immune-related genes. According to the information of the Gene Ontology Consortium's annotation (Ashburner et al. 2000), 426 immunologically-related genes (represented 542 probes in the array) were identified in this array. In order to study the host response to *Salmonella* infection and the role of genetic differences between the two lines, the list of immune-related genes were used to narrow down those previously identified differentially expressed genes ($P < 0.001$). Using the designated cut-off of 1.5

fold-change, 20 genes were found differentially expressed with SE infection (**Table 3.3**), where 13 genes were found in the comparison of line A and line B (**Table 3.4**). Several genes have duplicate probes in the array with consistent expression among comparisons. The number and regulation direction of immune-related genes showed a similar tendency to that of overall differentially expressed genes (**Figure 3.2**), in which there were more up-regulated genes in the AI/AN comparison, and more down-regulated genes in the BI/BN comparison.

In the comparison between the genetic lines, fewer immune-related genes were identified with only a few genes that had differential expression overlapped between IA/IB and NA/NB. Since most immune-related genes showed positive fold-change ratio these data indicate that these genes have a stronger expression in line A than line B regardless of the SE infection.

Table 3.2 Expression differences found with the microarray compared with the qRT-PCR

Gene Name	AI/AN fold-change[a]		BI/BN fold-change[a]		AI/BI fold-change[a]		AN/BN fold-change[a]	
	Microarray	qRT-PCR	Microarray	qRT-PCR	Microarray	qRT-PCR	Microarray	qRT-PCR
Chemokine (C-C) ligand 4 (CCL4)	**7.30**	**3.76**	**3.59**	**1.73**	2.48	2.09	1.22	1.04
Interleukin 6 (IL6)	**11.39**	**6.59**	**8.96**	**5.02**	-1.11.	1.01	-1.41.	1.29
CD80 antigen	**3.85**	**4.17**	1.53	**2.31**	1.60	1.25	-1.57	1.45
CXC chemokine K60 (K60)	**8.93**	**17.14**	**4.12**	**21.46**	2.15	1.67	-1.01	2.09
Prostaglandin-endoperoxide synthase 2 (PTGS2)	**3.14**	**4.90**	**3.19**	**7.87**	2.12	**1.52**	2.15	**2.44**
Similar to NUMB protein (NUMB)	-1.08	1.07	**-2.29**	**-2.51**	**2.18**	**1.87**	1.02	1.15
Interferon regulatory factor 7 (IRF7)	-1.16	-1.31	1.01	-1.59	**2.03**	**1.59**	**2.40**	1.31
Caspase 6, apoptosis-related cysteine peptidase (CASP6)	1.32	1.68	1.06	1.42	-2.15	-1.48	**-2.68**	**-1.74**

a,: Bold are differences in expression levels found with the microarray ($P < 0.001$) as well as the qRT-PCR ($P < 0.05$).

Table 3.3 List of immune genes with differential expression ($P < 0.001$) between infection (I) and non-infection (N) treatment

Accession No.	Gene Name	AI/AN [a] fold-change	BI/BN [b] fold-change
AJ720630	Antizyme inhibitor 1 (AZIN1)	2.39	-
AJ851659	CD80 antigen	3.85	-
Y15006	Interleukin-1beta (IL1β)	3.40	-
AJ309540	Interleukin 6 (IL6)	8.30	6.95
AJ564201	Interleukin 12B (IL12B)	3.87	4.31
AJ720504	Toll-like receptor 7 (TLR7)	-3.62	-4.78
BX930367	PREDICTED: bactericidal/permeability-increasing protein	-2.08	-2.39
CR390308	Glioma Amplified Sequence 41	-1.90	-1.88
L34553	Chemokine (C-C) ligand 4 (CCL4)	5.96	3.31
M16199	Interleukin 8 (IL8)	6.60	3.82
M64990	Prostaglandin-endoperoxide synthase 2 (PTGS2)	3.12	3.06
Y14971	CXC chemokine K60 (K60)	8.02	4.02
AF176086	Similar to NUMB protein (NUMB)	-	-2.32
AF335427	Nuclear factor,interleukin 3 regulated	-	-1.97
AJ639839	Similar to immunoglobulin-like receptor B4	-	-5.55
AJ851768	Interleukin-1receptor-associated kinase 2 (IRAK2)	-	-1.97
AY621307	Beta-defensin 5 (GAL9)	-	-1.80
BU133261	Similar to Inhibitor of nuclear factor kappa-B kinase epsilon subunit (IKBKE/KK-ε)	-	-5.18
BU265026	TIR domain containing adaptor inducing interferon-beta (TRIF/TICAM1)	-	-1.96
CR338732	TNF receptor-associated factor 7 (TRAF7)	-	-1.62

a,: The dash ("-") means that the expression differences were less than 1.5 fold. When the ratio (AI/AN) is smaller than 1, the ratio –(AN/AI) is given.

b,: The dash ("-") means that the expression differences were less than 1.5 fold. When the ratio (BI/BN) is smaller than 1, the ratio –(BN/BI) is given.

Table 3.4 List of immune genes with differential expression (P < 0.001) between chicken lineages A and B

Accession No.	Gene Name	AI/BI [a] fold-change	AN/BN [b] fold-change
AF176086	Similar to NUMB protein (NUMB)	2.18	-
AJ719428	TNF receptor-associated protein 1 (TRAP1)	-1.60	-
TC207578	Immunoglobulin-like receptor Ig1-37	1.93	-
U97157	Lunatic fringe homolog (LFNG)	1.89	-
CD735422	B-G V-region-like B-G antigen (B-G)	1.80	1.57
L34552 [a]	CC Chemokine (CCL1L2)	1.63	1.62
U20338	Interferon regulatory factor 7 (IRF7)	2.03	2.40
AF082329	Caspase 6, apoptosis-related cysteine peptidase (CASP6)	-	-2.68
AF320331	Interferon regulatory factor 4 (IRF4)	-	2.15
CV858509	TNF receptor-associated factor 2 (TRAF2)	-	1.66
L34552 [a]	CC Chemokine (CCL1L2)	-	1.66
L39766	Interferon regulatory factor 1 (IRF1)	-	1.93
Y12012	CD4 antigen (CD4)	-	1.71

a,: The dash ("-") means that the expression differences were less than 1.5 fold. When the ratio (AI/BI) is smaller than 1, the ratio –(BI/AI) is given.

b,: The dash ("-") means that the expression differences were less than 1.5 fold. When the ratio (AN/BN) is smaller than 1, the ratio –(BN/AN) is given.

Utilization of the array. All microarray results from this study were deposited in NCBI's Gene Expression Omnibus (GEO) database (Barrett et al. 2007). The accession numbers are: Platform, GPL4993; Series, GSE9416; Samples, GSM239322, GSM239330, GSM239336, GSM239337, GSM239347, GSM239349-GSM239352, GSM239354-GSM23935456, GSM239358, GSM239370, GSM239372 and GSM23935473.

Discussion

Evaluation of host responses to bacterial infections *in vitro* using microarray technology has become one of the major research areas in the study of functional genomics. This technology allows us to characterize the comprehensive host response(s) to complex pathogen stimuli under different experimental conditions. There has been a rapid increase in studies reporting the host response to *Salmonella* in chickens (Sadeyen et al. 2006; Sadeyen et al. 2004). While most of the recent studies focus on profiling gene expression in immunologically-related tissues (Sarson et al. 2007; Smith et al. 2006; van Hemert et al. 2007; Zhou and Lamont 2007), the present study is the first to examine the response of a chicken innate immune leukocyte, the heterophil, to SE using microarray technology. Based on earlier experimental results, one hour post infection was chosen as the point at which the *in vitro* SE infection induced the biggest difference of host responses between the two lines. More time points following SE exposure to heterophils would provide interesting information regarding the kinetics of the host-pathogen interaction.

The microarray experimental design used in this study provided direct comparisons to identify differentially expressed genes due to SE infection (infection or non-infection) or genetic differences (line A or B). More differentially expressed genes were detected in the comparisons between the lines compared to that observed between SE infected and non-infected cells. These data indicate there may be an intrinsic genetic difference between line A and B chickens. Line A chickens have a stronger immune response against *in vivo* bacteria challenge than line B chickens (Swaggerty et al. 2005), however, no further study has been conducted. In addition, fewer differentially expressed genes in the comparison between infected and non-infected cells within line may also be due to the limited variance contributed by host response associated with SE infection at one time point (1 h post infection). The direction of gene regulation revealed that line A (AI/AN) had less down-regulated genes, but more up-regulated genes compared to line B (BI/BN). Interestingly, similar patterns were observed on the expressional direction of immune-related genes (Table 3). Given that line A chickens are more resistant to SE than line B, it is possible that the enhanced SE-resistance is associated with a different host response in terms of both a higher number of up-regulated immune-related genes accompanied by fewer down-regulated genes.

GO terms enrichment analysis. The analysis of enriched GO terms allowed us to discover significant categories that could be overlooked when evaluating individual genes. The enriched GO terms could aid in interpreting the dominant functions controlled by differentially expressed genes. Although the higher number of identified GO terms might be positively correlated to more differentially expressed genes

identified, the regulation direction of genes showed a remarkable difference as most enriched GO terms were composed of down-regulated genes in all comparisons.

No specific functional term (biological process) was significantly enriched with SE infection in the comparison of AI/AN, while several functional terms associated with defense systems were found from down-regulated genes in the BI/BN comparison. The annotations of these terms suggest that line B may be more vulnerable to SE infections due to the suppressed functions on prevention or recovery from damages caused by infection. The results of the functional analysis further supported that down-regulated genes (functions) with SE infection might be associated with the immuno-inefficiency observed in line B.

The comparison between different lines showed that most enriched functions had higher expression in line B than line A on both infected and non-infected cells. This suggested that these functions are not immune-related, and therefore the higher expression in line B might not benefit the host defense system. Although only three functions showed higher expression in line A, one of these functions named 'response to stress' might benefit line A by remaining normal under exposure to infections. There is a complex interface between the immune system and metabolism (Matarese and La Cava 2004). It is possible that the highly expressed functions in line B may serve as an advantage over other performances in different desired traits. More studies are needed in order to understand the effects of the line differences on other parameters.

Analysis of immune-related genes. One of the key objectives of this study was to identify novel candidate genes associated with *Salmonella* resistance in chickens. The genetic variance contributing to the immune function only consist of a part of the whole genetic variance which is the overall genetic difference between line A and B. There were more differentially expressed genes in the comparisons between the two lines (773 and 812 genes in AN/BN and AI/BI, respectively) than observed in the comparisons between the infected and non-infected cells (176 and 186 genes in AI/BI and AN/BN, respectively). However, more immune-related genes were found in the comparisons between the infection and non-infection pairings (12 and 17 genes in AI/BI and AN/BN, respectively) than between the two lines (7 and 9 genes in AI/BI and AN/BN, respectively).

Numerous genes associated with immune function were found in both AI/AN and BI/BN pairing with a slightly higher fold-change in gene expression in AI/AN than BI/BN. These genes included the cytokines IL1β and IL6, and the chemokines IL8 (also known as CXCLi2), CCL4 and K60 (CXCLi1). Cytokines and chemokines are essential for an effective innate immune response (Kaiser et al. 2005). These data confirm and support earlier studies showing the higher expression of these signaling molecules in resistant line A are more important for their role in recruiting heterophils to the site of SE infection and pathogen clearance (Kogut et al. 1995), and initiating the signaling cascades that promote a pro-inflammatory cytokine/chemokine response (Zhou and Lamont 2007).

The effect of the MHC on determining immunity to *Salmonella* is described in the chicken (Cotter et al. 1998; Lamont et al. 2002). There is a polymorphism

(Lys148→Met148) in the MHC I α2 domain that is associated with bacterial load in the spleen following an SE challenge (Liu et al. 2002). Interestingly, several genes involved in MHC II system were differentially expressed in the present study. These genes included CD80, MHC II β chain (accession no. U02881), c-KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene, accession no. D13225), B-G and CD 4. CD80, MHC II β chain ($0.001 < P < 0.05$) and c-KIT ($0.001 < P < 0.05$) were up-regulated in the comparisons of AI/AN, and the expression of B-G and CD 4 were higher in line A than line B on both infected and non-infected cells. Functionally, the CD80 antigen is a surface molecule that co-regulates with another surface molecule, CD86, to provide a co-stimulating signal for T- helper cell activation (O'Regan et al. 1999; Tizard 2004). On the other hand, c-KIT is a stem cell factor receptor that is co-expressed with MHC II to sustain T-helper cell development (Ody et al. 2000). Heterophils have never been shown to have a role in antigen presentation and subsequent development of an acquired immune response; however, these data are indicative that heterophils may actually have such a role and future experiments will be conducted to assess this possibility.

Defensins are small peptides composed of cysteine-rich cationic molecules with broad-spectrum antimicrobial activity against bacteria, fungi and enveloped viruses (Milona et al. 2007). One of the families, namely β-defensins, is widely accepted as an important component for the hosts' immune system. It has been suggested that avian β-defensins play a significant role in the avian innate defense system since heterophils lack an oxidative killing mechanisms (Sugiarto and Yu 2004). To date, 14 β-defensin genes, known as gallinacins (GAL) 1, 1A, 2-13, are described in chickens (Lynn et al. 2004b;

Xiao et al. 2004). In the present study, SE infection suppressed the gene expression of β-defensin 5 (GAL 9) on line B heterophils, while no significant effect was observed on line A heterophils. GAL 9 has stronger antimicrobial activity against *Salmonella* serovars than GAL 4 and 7 (Milona et al. 2007). It is possible that repression of GAL 9 is related to the impaired SE-resistance in the susceptible line and lends itself as a potential candidate gene for selecting poultry with increased resistance against SE.

The TLR signaling pathway plays a critical role for elevating host immune responses by sensing pathogen-associated molecular patterns (PAMPs). Several genes associated with the TLR pathway are reported to respond to *Salmonella* infection (Kaiser et al. 2000; Kogut et al. 2003; Kogut et al. 2006; Swaggerty et al. 2006; Wigley et al. 2006). In the present study, two novel candidate adaptors, IKK-ε (inhibitor of nuclear factor kappa-B kinase epsilon subunit) and TRIF (TIR domain containing adaptor inducing interferon-beta), were found repressed exclusively in BI/BN. IKK-ε (also know as IKK epsilon or IKK-i) is an IKK homolog but not in components of IKK complex (Kishore et al. 2002; Peters and Maniatis 2001). Although the underlying mechanisms remain elusive, it suggests that IKK-ε plays a role in the activation of IRF3 and NFkB by involving TANK-binding kinase 1 (TBK1) (Chariot et al. 2002; Fitzgerald et al. 2003; Li and Verma 2002; Nomura et al. 2000). TRIF is an adaptor of the MyD88-independent pathway that leads to interferon (IFN)-β production, and the downstream cascade of TRIF is directly regulated by the adaptors IKK-ε and TBK1 (Yamamoto et al. 2003). Interestingly, the co-repression of TRIF and IKK-ε in line B observed in the current study suggests an important role for MyD88-independent pathway in host defense. A few genes involved in the TLR pathway were not significant since a stringent cut-off *P*-

value established, even though the *P*-values of these genes approached 0.001. While controlling false discovery rate is one of major objectives for microarray analysis, false negative might be an issue. In reality, it is possible that the lack of TLRrelated expressed genes may in fact be one of the findings lost using stringent FDR criteria. Specific genes include receptors (TLR4, TLR15) and adaptors (MD-2 like, MKK3, NFkB-1) and all showed higher expression in line A than line B with SE infection ($0.001 < P < 0.05$). Collectively, these findings support our assumption that the TLR pathway is, but probably not the only one, involved in altering host defense system to SE infection through a response of releasing signaling molecules differently as seen in cytokines and chemokines.

Most immune-related genes showed stronger expression in line A heterophils than in line B heterophils regardless of the SE infection. It is unclear if these genes are responsible for the stronger induction of immune response in the resistant line. Numb is an inhibitor of the notch signaling pathway that maintains normal cell-to-cell communication, cell fate specification and tissue regeneration (Katoh and Katoh 2006; Lai 2004). In the current study, the expression of numb was suppressed with SE infection in line B, while there was a significant up-regulation in the AI/BI. Given that line B showed down-regulation in the function of cell communication with SE infection, it is possible that the suppressed numb in line B indirectly retards the host immune network through impaired cell communication.

Conclusions

In summary, the results from this study demonstrate that higher expression of immune-related genes is more beneficial to enhance the host response against SE infection. The immune deficiency in the susceptible line is likely due to suppressed functions in recovering from cellular changes induced by SE infection. The genes exclusively differentially expressed in the AI/AN or BI/BN in the study has provided strong candidates for further investigation of disease resistance and susceptibility to SE infection in chickens, respectively. The identified immune-related genes also suggested a similar TLR regulatory network might exist in both lines, where a possible MyD88-independent pathway may participate in the regulation of host innate immunity in line B. Finally, the MHC II system might be important to initiate T-helper cell activation for the host defense.

To our knowledge, this is the first report to profile global gene expression in chicken heterophils with *in vitro Salmonella* infection. It is also expected that candidate genes discovered from this study along with the increasing information will add more genes to the chicken immune gene database. The findings in this study have made an indispensable contribution to characterizing the role of heterophils in the host immune system, and laid a solid foundation to further study the role of host genetics and resistance against *Salmonella*.

CHAPTER IV

ANALYSIS OF CHICKEN TOLL-LIKE RECEPTORS PATHWAY IN HETEROPHILS WITH *SALMONELLA* ENTERITIDIS INFECTION

Overview

Toll-like receptors (TLRs) are germ-line-encoded receptors and play an important role in innate immunity. Previously we demonstrated a large-scale gene expression profiling on heterophils isolated from broilers with different genetic backgrounds (*Salmonella*-resistant line A and -susceptible line B). Many immune-related genes showed significantly differential expression with *Salmonella enteritidis* (SE) infection, which included genes involved in TLR pathway. To expand our knowledge of TLR pathway on chicken heterophils, an approach of pathway analysis with extensive mRNA quantification was used to analyze genes expressions associated with TLR pathway. Results: an inferred chicken TLR pathway consisting of 72 chicken genes was constructed based on homology mapping to the reference TLR pathway. Numerous subtle, but consistent regulation expressions were identified in the genes with related functions in the pathway. The co-expression of these genes in TLR pathway suggested the existence of biological inferences previously not seen by the statistical screening analysis. Our results also indicated that MAPKs may be associated with the biological difference of heterophils between line A and line B chickens. To our knowledge, this study is the first one that provides most complete information of chicken TLR pathway. These findings in the regulatory network have laid the foundation for

future studies of TLR pathway regulation and cellular modulation of SE infection in chickens.

Introduction

Salmonella enterica serovar Enteritidis (SE) has become one of the most common *Salmonella* serotype in many countries (Braden 2006b). The epidemiology of SE accounts for the main source of salmonellosis in humans through the consumption of contaminated poultry or shell eggs. SE can persist in the cecum or ovaries of adult birds for months without triggering clinical signs. Colonized SE can continuously be excreted in feces (horizontal transmission) or through the yolk (vertical transmission) to contaminate other birds in the flock as well as the poultry products such as meats and eggs (Tilquin et al. 2005b). Current control of salmonellosis in poultry is mainly through hygiene measures combined with vaccination programs, however, the current vaccines are only partially effective (Wigley et al. 2002). An alternative approach is to select chickens for genetic resistance to *Salmonella* infection as it is reliable, permanent and environment-friendly. The studies of *Salmonella* resistance on different farm animals have been fully described in Wigley's review (Wigley 2004b). The progress in chickens was comparatively rapid due to their small size, rapid growth, short generation time, and availability of several well-defined inbred chicken lines with *Salmonella* resistant/susceptible trait (Bumstead 1998; Bumstead and Barrow 1993). Several studies in chickens have been conducted in immunologically-related tissues to identify candidate genes associated with resistance against SE infection (Lamont et al. 2002; Sadeyen et al. 2006; van Hemert et al. 2006; van Hemert et al. 2007; Wigley 2004b).

Although many candidate genes, including SAL1, NRAMP1 (natural resistance associated macrophage protein 1/SLC11A1), MHC class I, PSAP and IAPI, were found to be associated with the host's susceptibility, the mechanism of SE resistance remains unknown due to the inconsistent gene expressions in different inbred chicken lines (Lamont et al. 2002; Sadeyen et al. 2004; Wigley 2004b; Wigley et al. 2002).

Toll-like receptors (TLRs) are germ-line-encoded receptors that play an important role connecting innate and acquired immunity (Fearon and Locksley 1996). TLRs expressed primarily on antigen-presenting cells and function as the mammal's pattern-recognition receptors (PPRs) in recognition of microbial components (Janeway and Medzhitov 2002; Medzhitov and Janeway 1999). TLRs' response are stimulated by binding to pathogen-associated molecular patterns (PAMP) that are exclusively found in microbial (bacterial, viral, fungal and protozoan) components or their products but not in mammals. Therefore, TLRs provide an unique mechanism to trigger innate immune responses against a broad-spectrum of microbial invasion (Takeda and Akira 2004).

To date, thirteen mammalian TLRs (1-13) have been reported with at least one ligand that has been identified for each different TLR except TLR10 (Gerold et al. 2007; Temperley et al. 2008; West et al. 2006). TLRs have been identified in many animal lineages, but the majority of researches were carried on human and mice. The mechanism of TLR pathways in lower vertebrates is still largely unknown although several signaling proteins in TLR pathways were conserved across species (Kogut et al. 2006; Lynn et al. 2003a). Previous studies on the identification of avian TLRs were primarily depending on bioinformatics (*in silico*) approaches (Lynn et al. 2004a; Lynn et al. 2003a; Temperley et al. 2008; Yilmaz et al. 2005). Limited information is available

regarding avian TLRs' functions and mechanisms of regulation. The fact that only five chicken TLRs are human orthologs in a total of ten identified chicken TLRs suggested a considerable difference of TLRs exists between birds and mammals due to evolutionary processes (Temperley et al. 2008).

Heterophils, the avian counterpart of mammalian neutrophil , are the most abundant leukocytes in the peripheral blood and are essential for initiating and modulating innate immunity (Kogut et al. 2001; Kogut et al. 2003; Kogut et al. 1994). Studies of heterophils from both *in vivo* and *in vitro* SE-infection showed that there is a significant genetic control in different function of heterophils for determining overall immune competence (Ferro et al. 2004; Swaggerty et al. 2005; Swaggerty et al. 2006; Swaggerty et al. 2003). Previously, Farnell *et al.* observed TLRs-mediated oxidative burst in heterophils with stimulation of lipopolysaccharide (LPS) or lipoteichoic acid (LTA) (Farnell et al. 2003). Furthermore, a broad TLR expression profile was found in heterophils which suggested that heterophils may play a major role as first-line effector cells through the TLRinduced signaling transduction (Kogut et al. 2005). Taken together, these findings raised up a question whether a different TLRs signaling regulation is associated with the different immuno-efficiency in heterophils from two genetically distinct parental broiler lines (line A and line B). A previous study of global gene expression profiling in our lab has shown that heterophils from line A and line B chickens response differently with *in vitro* SE infection (here insert reference of MA *Salmonella* paper). Several immune-related genes showed higher expression in the resistant line than susceptible lines including genes involved in TLR pathway. The identification of significant expression of TLR pathway-related genes in array analysis

prompted us to investigate (1) if there are more genes expressing differentially in the TLR pathway with SE infection, and (2) if these differences are associated with SE-resistance via the TLR pathway regulation in heterophils of chickens from different genetic background. To do that, data of chicken orthologs of mammalian genes in TLR pathway were retrieved from our chicken whole genome Agilent array database, and analyzed with less stringent statistical threshold and revalidated with more sensitive real-time PCR quantification technology. The objectives of this study were to construct chicken TLR pathway using SE-infected heterophils as an example, and to examine the genetic effects on defending against SE infections in chicken heterophils.

Material and Methods

Experimental chickens. The two distinct parental broiler lines used in this study were obtained from a commercial company. To maintain confidentiality, the lines were designated as A and B. At the day of hatch, chickens were placed in floor pens (8 feet × 8 feet) containing wood shavings, provided supplemental heat, water, and a balanced, unmedicated corn and soybean meal based chick starter diet *ad libitum*. The feed was calculated to contain 23% protein and 3200 kcal metabolized energy/kg of diet, and all other nutrient rations met or exceeded the standards established by the National Research Council (1994).

Bacteria. A poultry isolate of SE (#97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA) and approved by the United States Department of Agriculture (USDA). SE was cultured in tryptic soy broth (Difco

Laboratories, Becton Dickinson Co., Sparks, MD) overnight at 41°C. Stock SE (1×10^9 colony forming units (cfu)/ml) was prepared as previously described (Swaggerty et al. 2003).

Heterophil isolation. Heterophils were isolated from the peripheral blood of 100 chickens per line six days post-hatch. Following blood collection, heterophils were isolated as previously described (Swaggerty et al. 2006). Briefly, blood from chickens was collected in vacutainer tubes containing disodium ethylenediaminetetraacetic acid (EDTA) (BD vacutainer, Franklin Lakes, NJ) and mixed thoroughly. The blood and EDTA for each line was pooled and diluted 1:1 with RPMI 1640 media containing 1% methylcellulose and centrifuged at 40 *g* for 15 min at 4°C. The supernatant was transferred to a new conical tube and diluted with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (1:1), layered onto discontinuous Histopaque® gradients (specific gravity 1.077 over 1.119) and centrifuged at 190 *g* for 1 h at 4°C. The histopaque layers were collected, washed with RPMI 1640 (1:1) and pelleted at 485 *g* for 15 min at 4°C. The cells were then re-suspended in fresh RPMI 1640, counted on a hemacytometer, and diluted to 1×10^7 cfu/ml in RPMI. All tissue culture reagents and chemicals obtained from Sigma Chemical Company, St. Louis, MO, unless noted otherwise.

Total RNA isolation. Heterophils (1×10^7 cfu) were treated with 300 µl RPMI or SE, for 1 h at 39°C on a rotary shaker. Treated heterophils were pelleted, washed with RPMI (485×*g* for 15 min at 4°C), the supernatant discarded, the cells re-suspended in lysis buffer (Qiagen RNeasy mini RNA extraction kit, Qiagen Inc., Valencia, CA), and

frozen. The lysed cells were transferred to QIAshredder homogenizer columns and centrifuged for 2 min at ≥ 8000 × *g*. Total RNA was extracted from the homogenized lysate according to the manufacturer's instructions, eluted with 50 µl RNase-free water and stored at -80°C.

Identification of chicken genes associated with TLR pathway. To identify chicken genes associated with TLR pathway (TLR pathway-related genes), the protein sequences of components in the reference TLR pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Aoki and Kanehisa 2005)were used to (1) blat against chicken genome using UCSC genome browser (Karolchik et al. 2003), or (2) to search against NCBI homologous genes database. The identified chicken genes were used to retrieve corresponding microarray results previously developed in our lab (accession numbers: GSE9416, NCBI Gene Expression Omnibus).

Quantitative real-time PCR. Genes having more than one probe with inconsistent regulation expression were further confirmed by quantitative real-time PCR (qRT-PCR). Total RNA (300 ng) from each sample, line A infection (AI), line A non-infection (AN), line B infection (BI) and line B non-infection (BN), was used for cDNA synthesis with random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. The cDNAs were quantified by qRT-PCR using ABI prism 7900HT system (Applied Biosystems, Foster, CA) with SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA). The specific oligonucleotide primers (**Table 4.1**) were designed by PRIMER3 program

(Rozen and Skaletsky 2000). The conditions of qRT-PCR amplification were: 1 cycle at 95ºC for 10 min, 40 cycles at 95 ºC for 15 s and 59 ºC for 1 min. The chicken β-actin gene was used as the internal control. Dissociation curves were performed at the end of amplification for validating data quality. Each individual sample was run in triplicate and the average critical threshold cycle (Ct) was used for calculating relative quantification by fold-change and statistical significance.

Table 4.1 Primers used for qRT-PCR

Gene Name	Accession No.	Primer Sequence (5'-3')	PCR Product Size (bp)[c]
β-actin	NM_205518	F[a]: ACGTCTCACTGGATTTCGAGCAGG	298
		R[b]: TGCATCCTGTCAGCAATGCCAG	
MD-2	BX932484	F: CGTGCTGAATTTTTGTTCCTCT	231
		R: TGCCAGATGGATTTGTTCAGTA	
CD14-like	TC202210	F: AATCGTACAGCTTTCAGGATCA	181
		R: CAGAAAGGGTTGTTAAGCACTG	
TLR1B	BU405042	F: CCCAGAAGACTTGAGCGGAA	151
		R: CCACGGCACATCCAGGTAG	
TLR3	CR407213	F: CCTTCGTGAGCTTGTGTTGT	160
		R: GCCAAACAGATTTCCAATCG	
TLR4	NM_001030693	F: ACTGCAGTTTCTGGATCTTTCA	215
		R: TATTCAAGTGTCCGATGGGTAG	
TLR5	CR353090	F: CTCACCTCTCTCTCAGGGTTTT	224
		R: TGGGTACACACAGTACCTGTCA	
TLR7	AJ720504	F: CCTCGATCTCAACCCTACTTCT	150
		R: CAGTATCTTTTCCTCACCACACA	
FADD	BX931327	F: GGAGTTCATAGAGGAGGGAGAA	187
		R: ATTATTTGGCTTGGCTACCATT	
IRAK4	AJ720408	F: AATTGCTTGGTTTCTCAAGTG	138
		R: GCAATTTCACACCTTGTGTTC	
TRIF	BU265026	F: CACAGGACAGTGAGGAAGAATC	165
		R: CGTGCTCAAGAAATAGAGGCTA	
AKT1	AF039943	F: GTCACCTGAAGCAAAATCTCT	173
		R: ACTTGTGGCTTAAATGGAGGT	
CASP8	AY057939	F: AGTCTGCCTGATCCTGAATAA	225
		R: GTCTTTGTTGTTGTGGTCCAT	
IKKA	AJ720520	F: CTTTCATCTATGGCAACTCCTG	244
		R: ATGTCCAAACCAAGACGTGAT	

Table 4.1 continued

Gene Name	Accession No.	Primer Sequence (5'-3')	PCR Product Size (bp)[c]
NFkB1	BU479586	F: TTGCTGTGTGCTTTACCTCTTT	150
		R: CAGCCAATTTGTTTCATTTCAT	
MKK3	TC206869	F: GCGCAGTGTTTAAGGAAGAAC	
		R: CCATGTCAGTCTCTTTGGTGTC	100
MKK4	BU111907	F: TTCTTCTGGACAGAAATGGAAA	
		R: AACTGGTCAAACACACTGTTCC	
IRF7	U20338	F: ATCCCTTGGAAGCACAACGCC	223
		R: CTGAGGCAACCGCGTAGACCTT	
STAT1	BX933123	F: CAGAAAATCTGCTGCCTATGTC	193
		R: ATAGCCTTCCCAAAGAATTGAA	
AP-1	NM_001031289	F: GCCTCAGACTGTACCTGAAATG	107
		R: TTCTGTTTCTCATGCGTTTTCT	
IL1B	Y15006	F: GCTCTACATGTCGTGTGTGATGAG	80
		R: TGTCGATGTCCCGCATGA	
IL6	AJ309540	F: AGGACGAGATGTGCAAGAAGTTC	78
		R: TTGGGCAGGTTGAGGTTGTT	
IL12A	BX258839	F: GCAGAGATGTAAGAAGCAAACG	188
		R: AAAACACTGAAGACAAACAGCAG	
IL12B	AJ564201	F: GACCCACCTCAATGTCAGTATG	205
		R: GCACAGAGATCTTGTCTTTTGG	
CCL4	CR389044	F: TACTACGAGACCAACAGCCAGT	153
		R: TGTCTCCACATCCATTCTATCC	
CD80	AJ851659	F: CAGAGTCTCCAGTCTTCACCAG	173
		R: GGAAAACCTCCATGAGAAGAAC	

a,:Forward primer
b,:Reverse primer
c,:All of primers are designed with melting temperature around 59 ºC.

Construction of chicken TLR pathway. A reference TLR pathway from KEGG database was used as a framework to construct an inferred chicken TLR pathway. The data from qRT-PCR were used to replace the corresponding data of same genes from microarray analysis. The corrected expression profiles of 72 chicken TLR pathway-related genes were integrated into the inferred chicken TLR pathway, and the expression fold-changes of AI/AN and BI/BN were represented in arrows for each of genes.

Results

Chicken homologous genes associated with TLR pathway. Based on the homology search using 69 components in the reference TLR pathway, 72 chicken homologous genes were identified from our chicken microarray which contained several gene families such as JNK family, p38 family, and P13K family. These genes include 2 secreted proteins, 8 receptors, 50 adaptors, 9 cytokines/chemokines, and 3 costimulatory proteins (CD markers). Ten genes, lipopolysaccharide binding protein (LBP), CD14, TLR9, TRIF-related adaptor molecule (TRAM), Interleukin-1 receptor-associated kinase 1 (IRAK1), IRF3, TNF-alpha and MIP-1alpha, from the reference TLR pathway are missing in chicken genome according to NCBI GenBank, while two of them, CD14 and TRAM, were found having putative chicken genes on the chicken array based on the sequence homology search (**Table 4.2**).

Visualization of gene expressions form chicken microarray analysis. The gene expressions of 72 chicken TLR pathway-related genes were retrieved from previous microarray database and shown in the comparison between infected vs. non-infected cells within line (Supplementary file 1). The range of *P*-value and fold-changes of these gene expressions were characterized using volcano plot (**Figure 4.1**), while upper corners of the plot represent genes that showed both statistical significance and large fold change. The results indicated that most genes had fold-changes between -1 and 1 of log2 ratio on both AI/AN and BI/BN comparisons, and the higher fold-change the smaller *P*-value regardless regulation direction. Based on the range of *P*-value and fold-changes shown in the plot, an arbitrary cut-off, fold-change larger than 1.2 (up- or down-regulated) with *P*-value smaller than 0.05, was used to define differential expressions.

Table 4.2 Chicken homologous genes associated with TLR pathway[a]

KEGG symbol	Chicken homologous gene	Chicken Gene ID assigned by KEGG	Corresponding Target on microarray		Gene type
			NCBI Accession no.	Chromosal location	
MD-2	MD-2	GGA: 420189	BX932484	chr2:122832311-122838396	Secreted protein
CD14	CD14	none	TC202210	chr13:863920-864549	Secreted protein
TLR1	TLR1-type1	GGA: 426274	AJ720806	chr4:71,563,594-71,566,050	Receptor
TLR6	TLR1-type2	GGA: 771173	BU405042	chr4:71549140-71553127	Receptor
TLR2	TLR2-type 1	GGA: 374141	AB050005	chr4:21,101,196-21,108,303	Receptor
	TLR2-type 2	GGA: 769014	AB046533	chr4:21105737-21107563	Receptor
TLR3	TLR3	GGA: 422720	CR407213	chr4:63164487-63166087	Receptor
TLR4	TLR4	GGA: 417241	NM_001030693	chr17:4,062,994-4,068,447	Receptor
TLR5	TLR5	GGA: 554217	CR353090	chr3:18975827-18978465	Receptor
TLR7/8	TLR7	GGA: 418638	AJ720504	chr1:126,823,957-126,830,698	Receptor
IFN (a/b) R	IFNAR1	GGA: 395665	AF082664	chr1:108,723,811-108,741,081	Adaptor
	IFNAR2	GGA: 395664	AF082665	chr1:108,690,430-108,701,715	Adaptor
RAC1	RAC1	GGA: 395871	U79755	chr14:9,036,810-9,050,145	Adaptor
TOLLIP	TOLLIP	GGA: 423099	AJ720279	chr5:15,766,715-15,791,782	Adaptor
MYD88	MYD88	GGA: 420420	AJ851640	chr2:4730082-4742682	Adaptor
TIRAP	TIRAP	GGA: 419715	BX933959	chr24:425631-430175	Adaptor
TRAM	TRAM	none	BX950451	chr10:22,158,828-22,161,432	Adaptor
PIK3 (gene family)	PIK3CA	GGA: 424971	AF001076	chr9:18,774,942-18,800,202	Adaptor
	PIK3CB	GGA: 424826	AJ851398	chr9:6651428-6734668	Adaptor
	PIK3CD	GGA: 419444	AJ851606	chr21:3457205-3480864	Adaptor
	PIK3CG	GGA: 417706	TC224633	chr1:15391797-15397267	Adaptor
	PIK3R1	GGA: 427171	CR389387	chrZ:21057437-21064169	Adaptor
	PIK3R2	GGA: 771142	BM491349	chr28:3553364-3554008	Adaptor
	PIK3R5	GGA: 417319	AJ720866	chr18:1938528-1987805	Adaptor
FADD	FADD	GGA: 423146	BX931327	chr5:19045916-19063437	Adaptor
IRAK4	IRAK4	GGA: 417796	AJ720408	chr1:31,853,699-31,865,151	Adaptor
TRIF	TRIF	GGA: 100008585	BU265026	chr28:4,422,103-4,422,781	Adaptor
AKT	AKT1	GGA: 395928	AF039943	chr5:54,122,987-54,193,913	Adaptor
	AKT3	GGA: 421497	BX950472	chr3:36,136,489-36,449,793	Adaptor
CASP8	CASP8	GGA: 395284	AY057939	chr7:12,508,765-12,514,865	Adaptor
TRAF6	TRAF6	GGA: 423163	BU445218	chr5:21187316-21188080	Adaptor
TRAF3	TRAF3	GGA: 423471	BX935958	chr5:52320785-52369130	Adaptor
TAB1	TAB1	GGA: 418014	AJ720321	chr1:52567547-52575977	Adaptor
TAB2	TAB2	GGA: 421622	BX933932	chr3:49957287-49963174	Adaptor
TAK1	TAK1	GGA: 421808	CR524033	chr3:77807497-77834655	Adaptor
RIP1	RIP1	GGA: 378921	AB108485	chr2:67527226-67547158	Adaptor

Table 4.2 continued

KEGG symbol	Chicken homologous gene	Chicken Gene ID assigned by KEGG	Corresponding Target on microarray		Gene type
			NCBI Accession no.	Chromosal location	
IKKE	IKKE	GGA: 430480	BU133261	chr26:2,290,243-2,292,642	Adaptor
TBK1	TBK1	GGA: 417825	TC206677	chr1:30578618-30578677	Adaptor
IKKA	IKKA	GGA: 423669	AJ720520	chr6:10,408,241-10,431,602	Adaptor
IKKB	IKKB	GGA: 426792	AJ720901	chr22:2772157-2784184	Adaptor
NFKB	NFkB1	GGA: 396033	BU479586	chr4:62669998-62674146	Adaptor
	NFkB2	GGA: 386574	D16367	chr6:18,022,427-18,025,184	Adaptor
Tp12	Tp12	GGA: 420479	BU419009	chr2:14938789-14939495	Adaptor
MKK3/6	MKK3	GGA: 416496	TC206869	chr14:4510951-4511609	Adaptor
MKK4/7	MKK4	GGA: 417312	BU111907	chr18:746333-748124	Adaptor
MEK1/2	MEK1	GGA: 415549	AJ720834	chr10:20632188-20664052	Adaptor
	MEK2	GGA: 396349	L28703	chr28:2108066-2116998	Adaptor
OPN	OPN	GGA: 395210	CR353415	chr4:47107503-47110546	Adaptor
IKB-alpha	IKB-alpha	GGA: 396093	M74544	chr5:38574319-38577806	Adaptor
ERK	ERK	GGA: 373953	AY033635	chr15:520665-531338	Adaptor
P38 (gene family)	P38-MAPK11	GGA: 417739	AJ720776	chr1:21806996-21828380	Adaptor
	P38-MAPK12	GGA: 769763	CR339030	chr1:21761267-21765358	Adaptor
	P38-MAPK14	GGA: 421183	CO635361	chr26:139880-149908	Adaptor
JNK (gene family)	JNK-MAPK8	GGA: 423778	BX934173	chr6:19,340,849-19,355,571	Adaptor
	JNK-MAPK9	GGA: 395983	CR406144	chr13:14,110,644-14,111,792	Adaptor
	JNK-MAPK10	GGA: 422592	TC224553	chr4:47391377-47391944	Adaptor
IRF5	IRF5	GGA: 430409	AJ720409	chrUn_random:19690743-19694138	Adaptor
IRF7	IRF7	GGA: 396330	U20338	chr5:16950071-16954584	Adaptor
STAT1	STAT1	GGA: 424044	BX933123	chr7:8886125-8889016	Adaptor
AP-1	AP-1	GGA: 424673	NM_001031289	chr8:27,141,995-27,143,699	Adaptor
IL1B	IL1B	GGA: 395196	Y15006	chr22:3,876,886-3,878,491	Cytokine
IL6	IL6	GGA: 395337	AJ309540	chr2:30,893,617-30,896,304	Cytokine
IL12A	IL12A	GGA: 407090	BX258839	chr9:23,816,856-23,817,522	Cytokine
IL12B	IL12B	GGA: 404671	AJ564201	chr13:7,914,587-7,926,488	Cytokine
IFN-alpha	IFN-alpha	GGA: 396398	BU319434	chrZ:6,896,666-6,897,474	Cytokine
IFN-beta	NF-beta	GGA: 554219	AY831397	chrZ:6,888,979-6,889,589	Cytokine
IL8	IL8	GGA: 396495	M16199	chr4:52,446,739-52,449,903	Chemokine
RNATES	CCL5	GGA: 417465	CD739895	chr19:379,128-380,651	Chemokine
MIP-1-beta	CCL4	GGA: 395551	CR389044	chr19:366403-367507	Chemokine
CD86	CD86	GGA: 427944	AM050135	chr1:79,726,248-79,736,927	costimulatory protein

a,: Based on reference TLR pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

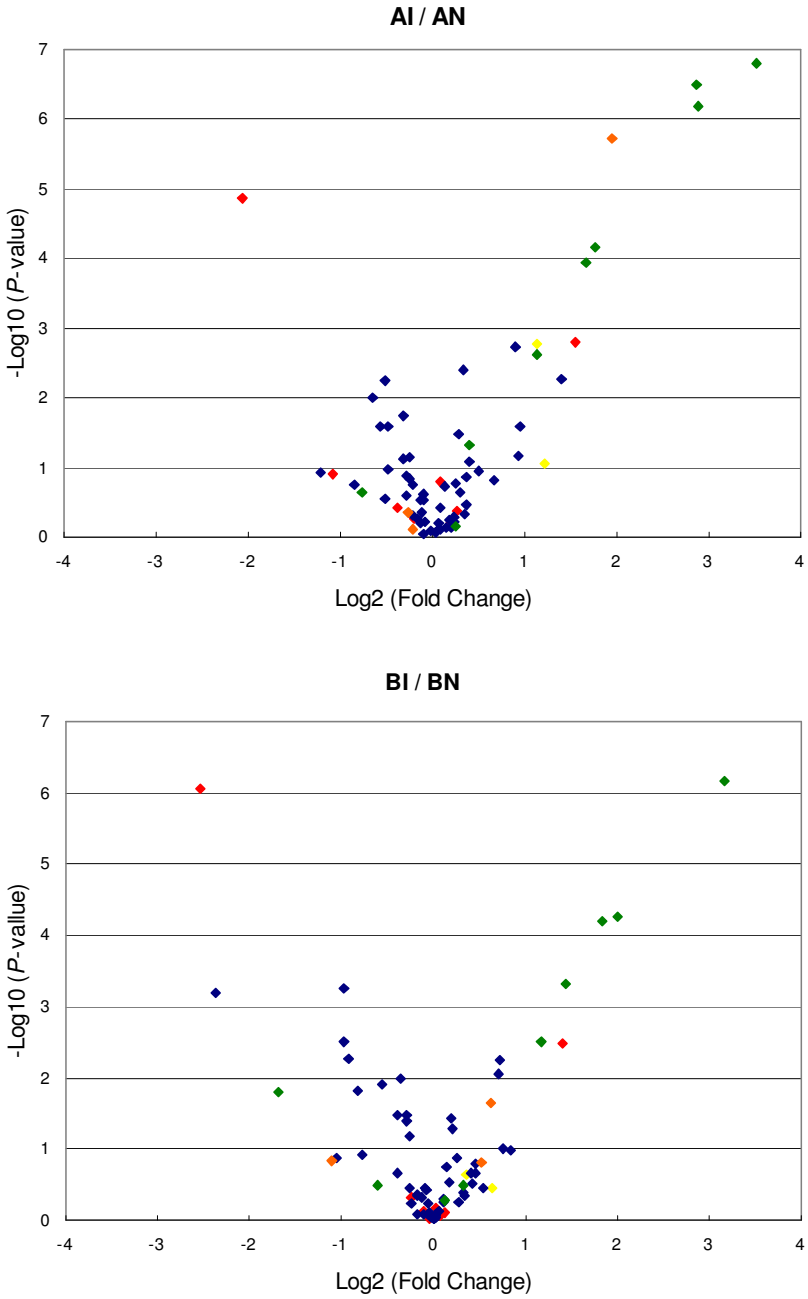


Figure 4.1 The volcano plot analysis of fold change comparison of gene expression. AI/AN (infected line A heterophils to non-infected line A heterophils), and BI/BN (infected line B heterophils to non-infected line B heterophils). Genes classified in different categories (secreted protein, receptor, adaptor, cytokine/chemokines and costimulatory protein) are shown in distinct colors (yellow, red, blue, green and orange, accordingly).

A different pattern of gene expression changes in volcano plot was observed between AI/AN and BI/BN. In general, AI/AN showed more genes with >2 fold-change up-regulated expression, but fewer genes with > 2 fold-change down-regulated expression than BI/BN.

Validation of microarray results by real-time PCR. Twenty-five genes from microarray analysis were found having inconsistent expression direction shown in more than one probes. These genes are chosen to exam the gene expressions using qRT-PCR. The comparison of microarray and qRT-PCR results was shown in **Table 4.3**. Most genes showed consistent results between Microarray approach and qRT-PCR except TLR7, and IL12A. qRT-PCR detected much higher fold-change than microarray on TLR4 and IL12B, and has more incidences of significant expressions than microarray (12 and 16 incidence in microarray and qRT-PCR, respectively for AI/AN comparison; 11 and 13 incidences in microarray and qRT-PCR, respectively for BI/BN comparison).

Table 4.3 Quantitative RT-PCR validation for gene expression changes within infection and non-infection heterophils

Chicken homologous gene	Accession no.	AI/AN fold-change[a]		BI/BN fold-change[b]	
		Microarray	qRT-PCR	Microarray	qRT-PCR
MD-2	BX932484	**2.20**	2.85	1.29	2.20
CD14	TC202210	2.33	1.79	1.56	1.01
TLR1B	BU405042	1.21	**2.23**	-1.08	1.52
TLR3	CR407213	-1.31	-1.07	1.05	1.57
TLR4	NM_001030693	**2.93**	**21.47**	**2.65**	**15.69**
TLR5	CR353090	1.15	**1.97**	1.09	1.46
TLR7	AJ720504	**-4.21**	1.61	**-5.78**	-1.00
FADD	BX931327	1.10	1.62	**1.16**	**1.65**
IRAK4	AJ720408	1.05	**1.86**	**1.15**	**1.52**
TRIF	BU265026	**-1.57**	1.67	**-1.96**	1.16
AKT1	AF039943	1.28	**1.92**	1.78	**1.79**
CASP8	AY057939	**-1.43**	**-1.89**	-1.08	-1.75
cIKKA	AJ720520	1.13	1.32	-1.32	-1.00
NFkB1	BU479586	**1.94**	**1.50**	1.34	1.21
MKK3	TC206869	**2.63**	**1.74**	1.70	**1.75**
MKK4	BU111907	1.22	**2.09**	-1.02	1.43
IRF7[c]	U20338	-1.16	-1.31	1.02	**-1.59**
STAT1	BX933123	1.19	**2.48**	1.07	**1.82**
AP-1	NM_001031289	1.29	**1.69**	-1.13	**1.44**
IL1B	Y15006	**3.40**	**7.12**	**2.25**	**7.55**
IL6[c]	AJ309540	**11.39**	**6.59**	**8.96**	**5.02**
IL12A	BX258839	-1.71	2.27	**-3.21**	1.51
IL12B	AJ564201	**3.19**	**15.95**	**2.71**	**14.49**
CCL4[c]	CR389044	**7.30**	**3.76**	**3.59**	**1.73**
CD80[c]	AJ851659	**3.85**	**4.18**	**1.53**	**2.31**

a,: Bold are differences in expression levels found with the microarray ($P \leq 0.05$) as well as the qRT-PCR ($P \leq 0.05$). When the ratio (AI/AN) is smaller than 1, the ratio –(AN/AI) is given.

b,: Bold are differences in expression levels found with the microarray ($P \leq 0.05$) as well as the qRT-PCR ($P \leq 0.05$). When the ratio (BI/BN) is smaller than 1, the ratio –(BN/BI) is given.

c,: Data adapted from Chiang, *et. al.,* 2008.

Expressional changes of genes in chicken TLR pathway. The fold-change results of qRT-PCR were used to replace that of microarray analysis for corresponding genes (**Table 4.4**). The combined expression profiles of 72 chicken TLR pathway-related genes were visualized on the inferred chicken TLR pathway (**Figure 4.2**). Ten missing TLR pathway-related genes in chickens were shaded in gray, and only two of them (CD14 and TRAM) have data presented using putative genes found in our array. Most differentially expressed adaptors were found involved in the TLR4-induced My88-dependent pathway. These genes include TIRAP, IRAK4, TAB1 and gene families in the downstream signaling cascade such as NFkB family and p38 family. The downstream signals were passed down through AP-1 for the induction of cytokines and chemokines relevant in pro-inflammatory responses.

Instead shown thoroughly in the whole signaling cascade, similar pattern of expression fold-change between AI/AN and BI/BN were found in a group of genes with related function (e.g. PI3K, NFkB and p38 gene family) or with close regulation among them (e.g. CD-14, MD-2 and TLR4; MyD88, TOLLIP and TIRAP).

Table 4.4 Combined results (microarray and qRT-PCR) of gene expression changes within infection and non-infection heterophils

KEGG symbol	Chicken homologus gene	Accession no.	AI/AN fold-change[a]	BI/BN fold-change[b]	Data resource: microarray (M) or qRT-PCR (Q)
MD-2	MD-2	BX932484	2.85	2.20	Q
CD14	CD14	TC202210	1.79	-	Q
TLR6	TLR1A	AJ720806	-2.13	-	M
TLR1	TLR1B	BU405042	**2.23**	1.52	Q
TLR2	TLR2A	AB050005	-	-	M
	TLR2B	AB046533	-	-	M
TLR3	TLR3	CR407213	-	1.57	Q
TLR4	TLR4	NM_001030693	**21.47**	**15.69**	Q
TLR5	TLR5	CR353090	**1.97**	1.46	Q
TLR7/8	TLR7	AJ720504	1.61	-	Q
IFN-alpha	IFN-alpha	AF082664	**1.32**	-	M
IFN-beta	INF-beta	AF082665	-	-1.52	M
IFN (a/b) R	IFN-R1	U79755	-	-1.20	M
	IFN-R2	AJ720279	-	-	M
RAC1	RAC1	AJ851640	-	-	M
MYD88	MYD88	BX933959	-	-2.07	M
TOLLIP	TOLLIP	BX950451	-	-1.72	M
TIRAP	TIRAP	AF001076	-	**-1.22**	M
TRAM	TRAM	AJ851398	-	-	M
PIK3 (gene family)	PIK3CA	AJ851606	-	-	M
	PIK3CB	TC224633	-	**-1.95**	M
	PIK3CD	CR389387	**-1.40**	**-1.47**	M
	PIK3CG	BM491349	-	-	M
	PIK3R1	AJ720866	-1.22	-	M
	PIK3R2	BX931327	-	1.37	M
	PIK3R5	AJ720408	-	**-1.90**	M
FADD	FADD	BU265026	1.62	**1.65**	Q
IRAK4	IRAK4	AF039943	**1.86**	**1.52**	Q
TRIF	TRIF	BX950472	1.67	-	Q
AKT	AKT1	AY057939	**1.92**	**1.79**	Q
AKT	AKT3	BX935958	-1.40	-1.20	M
CASP8	CASP8	BU445218	**-1.89**	-1.75	Q
TRAF3	TRAF3	AJ720321	1.92	-	M
TRAF6	TRAF6	BX933932	1.29	1.26	M
TAB1	TAB1	CR524033	**1.26**	-	M
TAB2	TAB2	AB108485	-	1.45	M
TAK1	TAK1	BU133261	-	-	M

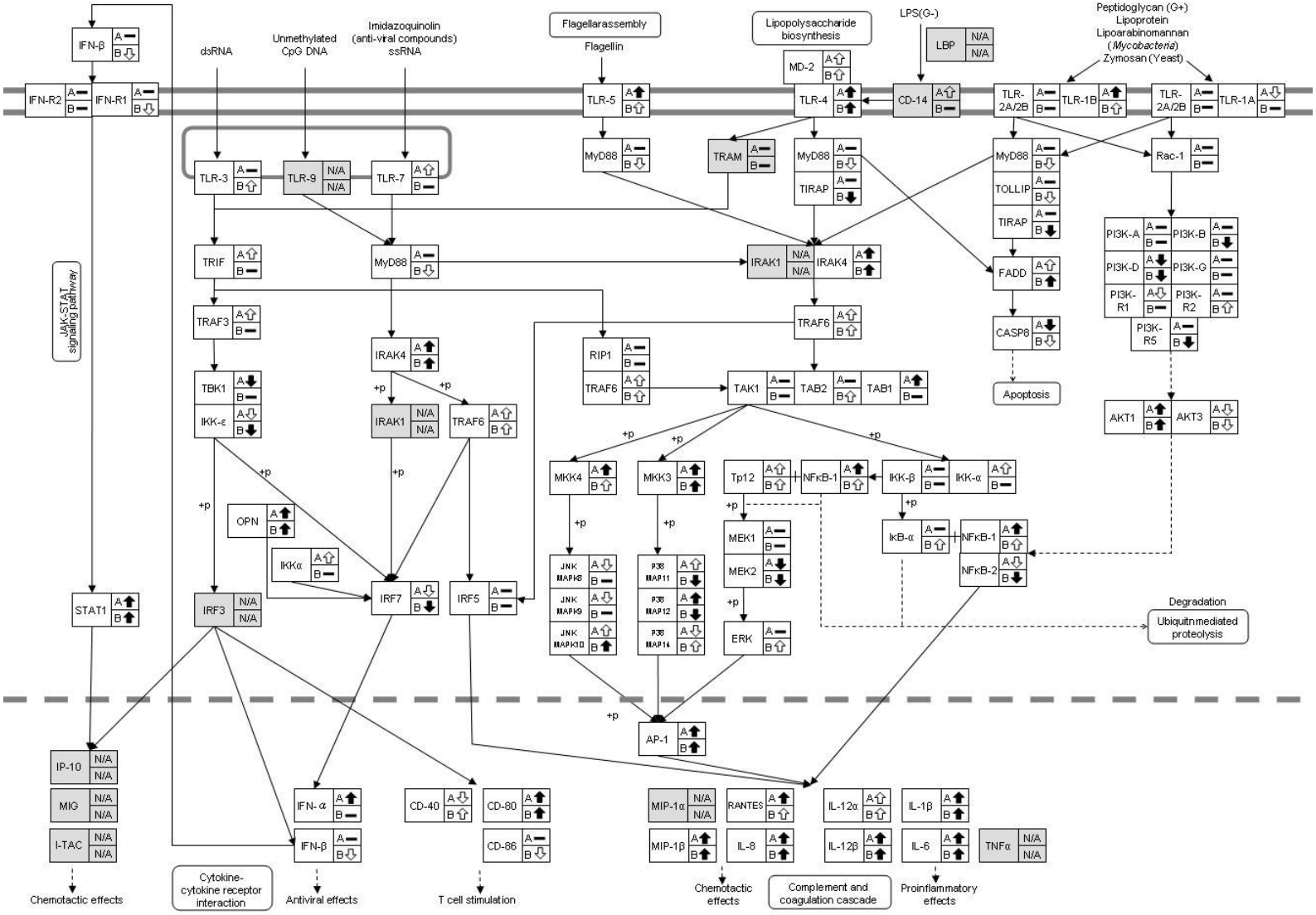
Table 4.4 continued

KEGG symbol	Chicken homologus gene	Accession no.	AI/AN fold-change[a]	BI/BN fold-change[b]	Data resource: microarray (M) or qRT-PCR (Q)
TBK1	TBK1	AJ720520	**-1.48**	-	M
IKKE	IKKE	AJ720901	-1.81	**-5.18**	M
IKKA	IKKA	BU479586	1.32	-	Q
IKKB	IKKB	D16367	-	-	M
NFKB	NFkB1	BU419009	**1.50**	1.21	Q
	NFkB2	TC206869	-1.25	**-1.31**	M
Tp12	Tp12	BU111907	1.59	1.27	M
MKK3/6	MKK3	AJ720834	**1.74**	**1.75**	Q
MKK4/7	MKK4	L28703	**2.09**	1.43	Q
MEK1/2	MEK1	CR353415	-	-	M
MEK1/2	MEK2	M74544	**-1.25**	**-1.28**	M
OPN	OPN	AY033635	**1.86**	**1.64**	M
IKB-alpha	IKB-alpha	AJ720776	-	1.32	M
ERK	ERK	CR339030	-	1.38	M
P38 (gene family)	P38-MAPK11	CO635361	1.42	**-1.77**	M
	P38-MAPK12	BX934173	**1.22**	**-1.22**	M
	P38-MAPK14	CR406144	-1.44	1.21	M
JNK (gene family)	JNK-MAPK8	TC224553	-2.31	-	M
	JNK-MAPK9	AJ720409	-1.22	-	M
	JNK-MAPK10	U20338	1.33	**1.65**	M
IRF5	IRF5	BX933123	-	-	M
IRF7	IRF7	NM_001031289	-1.31	**-1.59**	Q
STAT1	STAT1	Y15006	**2.48**	**1.82**	Q
AP-1	AP-1	AJ309540	**1.69**	**1.44**	Q
IL1B	IL1B	BX258839	**7.12**	**7.55**	Q
IL6	IL6	AJ564201	**6.59**	**5.02**	Q
IL8	IL8	BU319434	**7.40**	**4.03**	M
IL12A	IL12A	AY831397	2.27	1.51	Q
IL12B	IL12B	M16199	**15.95**	**14.49**	Q
RNATES	CCL5 (RNATES)	CR389044	**2.21**	1.25	M
MIP-1B	CCL4 (MIP-1B)	CD739895	**3.76**	**1.73**	Q
CD40	CD40	AJ293700	-1.21	1.43	M
CD80	CD80	AJ851659	**4.18**	**2.31**	Q
CD86	CD86	AM050135	-	-2.15	M

a,: Bold are differences in expression levels found with the microarray ($P \leq 0.05$) as well as the qRT-PCR ($P \leq 0.05$). When the ratio (AI/AN) is smaller than 1, the ratio –(AN/AI) is given.

b,: Bold are differences in expression levels found with the microarray ($P \leq 0.05$) as well as the qRT-PCR ($P \leq 0.05$). When the ratio (BI/BN) is smaller than 1, the ratio –(BN/BI) is given.

Figure 4.2 Chicken homologous TLR pathway with expression changes against SE infection. A or B represents the comparison AI/AN or BI/BN. Up- or down- arrows represent up- or down-regulated expression with SE infection, respectively. The arrow filled in black represent significant (P<0.05) results. Genes shaded in gray are missing in chicken genome. The notation of protein-protein interactions refers to http://www.genome.jp/kegg/document/help_pathway.html


IFN-β
IFN-R2
IFN-R1
dsRNA
Unmethylated CpG DNA
Imidazoquinolin (anti-viral compounds) ssRNA
Flagellarassembly
Flagellin
Lipopolysaccharide biosynthesis
LPS(G-)
LBP
MD-2
Peptidoglycan (G+)
Lipoprotein
Lipoarabinomannan (Mycobacteria)
Zymosan (Yeast)
TLR-5
TLR-4
CD-14
TLR-2A/2B
TLR-1B
TLR-2A/2B
TLR-1A
TLR-3
TLR-9
TLR-7
MyD88
TRAM
MyD88
TIRAP
MyD88
TOLLIP
TIRAP
Rac-1
JAK-STAT signaling pathway
TRIF
MyD88
IRAK1
IRAK4
FADD
PI3K-A
PI3K-B
PI3K-D
PI3K-G
PI3K-R1
PI3K-R2
PI3K-R5
TRAF3
TRAF6
CASP8
IRAK4
RIP1
TRAF6
TAK1
TAB2
TAB1
Apoptosis
AKT1
AKT3
TBK1
IKK-ε
IRAK1
TRAF6
MKK4
MKK3
Tp12
NFκB-1
IKK-β
IKK-α
OPN
IKKα
MEK1
MEK2
IκB-α
NFκB-1
NFκB-2
JNK MAPK8
JNK MAPK9
JNK MAPK10
P38 MAP11
P38 MAP12
P38 MAP14
ERK
Degradation
Ubiquitn mediated proteolysis
STAT1
IRF3
IRF7
IRF5
AP-1
IP-10
MIG
I-TAC
IFN-α
IFN-β
CD-40
CD-80
CD-86
MIP-1α
MIP-1β
RANTES
IL-8
IL-12α
IL-12β
IL-1β
IL-6
TNFα
Chemotactic effects
Cytokine-cytokine receptor interaction
Antiviral effects
T cell stimulation
Chemotactic effects
Complement and coagulation cascade
Proinflammatory effects

Discussion

The TLRs constitute a multi-gene family which recognize distinct PAMP and use a variety of intracellular adaptors to initiate a response to PAMP recognition. TLR were found conserved in many animal lineages and play a key role in defense against both viral and bacterial infections (Akira 2003; Akira et al. 2001). Recent analyses of chicken ESTs and genome annotation have revealed many homologous TLRs in chickens (Higgs et al. 2006b; Iqbal et al. 2005; Roach et al. 2005; Smith et al. 2004; Yilmaz et al. 2005), and their expressions have been reported on heterophils and monocytes/macrophages (Bliss et al. 2005; He et al. 2006; Keeler et al. 2007; Kogut et al. 2005). TLRs provide an example of multi-gene family whose members have diversified functionally during the evolutionary process such as gene duplication and deletion (Hughes 1994; Hughes and Piontkivska 2008). Although TLRs have been identified in many animal lineages, a nomenclature issue has been existed in chicken TLRs due to the distinct evolutionary processes within species (Leulier and Lemaitre 2008; Roach et al. 2005; Yilmaz et al. 2005). Therefore, the nomenclature of chicken TLRs used in this study were following the suggestion by Temperley and his colleagues (Temperley et al. 2008). To this date, ten TLRs (not including pseudogenes) have been reported in chicken genome (Temperley et al. 2008). A huge difference was observed when comparing chicken TLRs to mammalian TLRs. Chicken has no TLR10 and TLR6 which are established by gene duplication 300 million years ago (Mya) and 160 Mya, respectively, in eutherian mammals. Instead, chicken gains genes by two other duplications occurred 147 Mya and 65 Mya which lead to chicken TLR1A, TLR1B and TLR2A, TLR2B respectively (Temperley et al. 2008). Besides, chicken does not have TLR8 and TLR9 which are

present in most mammals, but has TLR21 which is conserved in Teleostei and Amphibia only (Roach et al. 2005). Chicken also has a newly identified TLR15 which is exclusively existed in chicken and associated with triggering host response against bacteria infection (Higgs et al. 2006b; Temperley et al. 2008). Although the property of ligands for TLR15 and TLR21 are still unknown, chicken TLR15 appeared to be associated with triggering host response against to *Salmonella* infection (Higgs et al. 2006b). A previous study of phylogenetic analysis has shown that chicken TLR15 shares a clade with TLR1, -2, -6, -10 and -14, where TLRs in this clade are found to respond to bacterial pathogens (Temperley et al. 2008). It would be interesting to investigate if TLR15 also share a similar ligand-binding specificity with other TLRs in the same clade.

Very few studies were examined on adaptors involved in chicken TLR pathway. Although many adaptors were identified in chicken genome, they are poorly understood in terms of the expression and regulation pattern with bacterial infection. With array technology, it is capable to characterize the complicated interactions among numbers of adaptors simultaneously. The expression profiling of a total of 72 representative chicken TLR pathway-related genes from microarray were used in current study for two purpose: (1) To unveil the genes regulations under the stimulation of SE infection; (2) To compare the difference of signal transduction between line A and line B heterophils.

The volcano plot is commonly used to look at fold-change and statistical significance simultaneously for interpreting gene expression of microarray results (Allison et al. 2006). By visualizing gene expressions using volcano plot, a shift of gene expression from right to left on adaptors (blue diamond) was observed between AI/AN and BI/BN, which implied that there are more down-regulated adaptors in line B

heterophils than line A heterophils with SE infection. We previously reported that heterophils from SE-susceptible line B has a less effective functional responsiveness on producing end-product signaling molecules such as pro-inflammatory and chemomatic cytokines (Swaggerty et al. 2004b). It is likely the reduced functional responsiveness from line B is associated with the modest expression of adaptors seen in the current study.

The expression fold-change (up- or down-regulation) of TLR pathway-related genes were varied in different protein types. The standard deviation of fold-changes was the lowest in adaptor genes (1.38 and 1.52 for AI/AN and BI/BN, respectively (n = 40)) but the highest in cytokines/chemokines molecules (4.02 and 3.47 for AI/AN and BI/BN, respectively, (n = 9)). The results indicated that although adaptor genes have relatively subtle gene expressions during TLR pathway signaling, the end-products of pathway were still highly produced in terms of signaling molecules. It may be more appropriate to think of these adaptors as members in multiple teams, but not as solo actors dedicated to a single mission (Ben-Shaul et al. 2005). In the biological network, the adaptation to various stimuli of SE infection might involve subtle adjustments in a large set of elements, rather than extreme changes in a few selected members.

Due to the subtle expression fold-changes in majority of genes in chicken TLR pathway, a traditional screening process to identify a list of differentially expressed genes might be more likely to fall into false negative error if the impropriate stringent criteria were used. It has been reported that pathway analysis can detect more subtle changes than gene lists that are generated from univariate statistical analysis (Curtis et al. 2005). Therefore the strategy by characterizing individual genes from a single pathway

could be more suited for studying genes association of chicken TLR pathway in the present study. Many databases such as the KEGG contain information on biological pathways. The combination of microarray data and pathway analysis may highlight the biological process taking place in the heterophils and provide novel information on identifying critical genes in chicken TLR signaling system.

The precision of gene expression microarray is often considered to be relatively limited despite the continually improving sensitivity and dynamic range of microarrays. This limitation has prompted genomics researchers to validate their microarray results with other independent technologies (Chuaqui et al. 2002). qRT-PCR is the choice of many for quantitatively measuring specific mRNA, which is commonly used to validate array data as this method is rapid, relatively inexpensive and requires less RNA template then conventional assays such as Northern (Rajeevan et al. 2001a; Rajeevan et al. 2001b). However the strategies of choosing genes for validation are usually debatable and aims-dependent in most array experiments (Allison et al. 2006). In the present study, we found that most chicken TLR pathway-related genes on the array have multiple probes designed on separated regions of each gene, and array results from these probes were consistent within the same gene, which is known as 'up-front validation' (Chuaqui et al. 2002). Therefore, the genes chosen for qRT-PCR validation were genes that primarily have inconsistent array results from multiples probes within a gene (for the validation against random measurement error of array technology).

More than 90% of genes were consistent between qRT-PCR and array analysis except TLR7 and TRIF. An additional six genes (TLR1B, TLR5, IRAK4, MKK4, STAT1 and AP1) with no significant expression differences in the microarray analysis

showed significant in qRT-PCR. It has been reported that qRT-PCR has larger dynamic range and increased sensitivity compared with microarray analyses (Carter et al. 2003; Zhao et al. 2006). For genes that are not declared differentially expressed in microarray, it is possible that random measurement error has reduced the ability of detecting true differences and created an erroneous inference (Allison et al. 2006). This could be the explanation for our observation that qRT-PCR can detect significant difference with greater sensitivity of gene expression for those five genes; however, qRT-PCR did also identify three microarray false positives (MD-2, TLR7 and TRIF).

Despite many homologous genes information are available for various species, pathway information is generally not available for some less well-studied species such as chicken. The use of homology mapping has become the critical tool to extend the knowledge on biological processes across species (Salomonis et al. 2007; Vastrik et al. 2007). To do that, we incorporated all reported chicken homologous genes into the existing reference TLR pathway in KEGG database. It is important to note that this inferred chicken TLR pathway is not a genuine species-specific pathway, but rather a translation of reference pathway where chicken genes have been mapped based on homology. Although the TLR pathway is extremely conserved across species (Takeda et al. 2003), an accurate pathway inference requires more knowledge that particular biological process and molecular interactions are also conserved and endowed with the same biological functions between organisms (Salomonis et al. 2007). To our knowledge, this is the first analysis that includes the most complete chicken homologous genes in TLR pathway. The study presented herein provides important information required for dissecting the complex nature of the innate response to *Salmonella* in heterophils.

Salmonella has at leas four TLR ligands: lipopolysaccaride (LPS), bacterial lipoproteins, flagellin and CpG DNA, which activate TLR4, TLR2, TLR5 and TLR9, respectively. In mammals, it is clear that macrophages and dendritic cells can sense *Salmonella* through these TLRs and induce the production of cytokines relevant in host responses (Gerold et al. 2007). Birds are lack of TLR9 due to the gene deletion, while TLR4 and TLR5 were relatively conserved in avian genome and both showed significant up-regulation with *Salmonella* infection in the present study. It has been suggested that TLR4 is the dominant TLR involved in the host response to *Salmonella* infection, as TLR2 responses are relevant only if TLR4 is active (Weiss et al. 2004). Opposed to TLR4, the activation of TLR5 appears to be deleterious for the host in a *Salmonella* infection by which it facilitates the migration of invaded *Salmonella* from the intestinal tract to the lymph nodes in mammals (Uematsu and Akira 2006). The chicken TLR2 has two variants, TLR2A and TLR2B, but none of them showed discernible (fold-change larger than 1.2) expression with *Salmonella* infection in the current study. Alternatively, one of TLR1 variants, TLR1B (also known as TLR6 in mammals), showed significant up-regulation with *Salmonella* infection in AI/AN. It is suggested that evolutionarily mammalian TLR1, TLR2, and TLR6 have arisen from the same ancestor and shared a conserved homology on structure and ligand-binding specificity. Functionally, these TLRs act by cross-linking two molecules of TLRs to form heterodimers and recognizes PAMP such as bacteria lipoproteins (Hughes and Piontkivska 2008; Zhou et al. 2007). It is increasingly clear that gene function is a multi-dimensional variable (Hughes and Piontkivska 2008). During the evolution, gene duplication permits duplicate genes to explore different aspects of the multi-dimensional functional space, which might explain

the dissimilar response of chicken TLR2 and TLR1B to mammalian TLRs in the present study.

Although several adaptors gene showed significant expressions, most of them are scattered in different signaling cascades of the pathway. It is reasonable to assume that a pathway contains more genes with significant expression induced by *Salmonella* infection may have higher probability to be a true signaling transduction in host responses. Notably, the current pathway analysis successfully identified many subtle, but consistent changes in expression of a group of genes with related function or close regulation among them. One of the examples shown in our results is the MyD88-dependent pathway. MyD88 is the central adaptor molecule interacting with all TLRs except TLR3. Upon ligand activation, MyD88 recruit members of the IRAK family. IRAKs then become phosphorylated and dissociates from MyD88, which results in TRAF6 being activated, and following by TAK1-TAB1-TAB2 kinase complex formation and activation (Miggin and O'Neill 2006) (**Figure 4.2**). Similar expression regulation were found within the group of MyD88, TOLLIP and TIRAP (down-regulation in BI/BN), and within the signaling cascade from IRAK4 to downstream adaptors such as Tp12, MKK3 and MKK4 (up-regulation in AI/AN or BI/BN or both).

Intracellular signaling transduction via the adaptor molecules may be regulated in a number of ways, including protein phosphorylation, degradation, sequesteration and interaction with inhibitory adaptor molecules (Miggin and O'Neill 2006). A pathway might be both up-regulated and down-regulated, perhaps due to a block in the pathway where gene above and below the block respond differently (Curtis et al. 2005), or due to the negative regulation of TLR signaling (Dunne and O'Neill 2005). In the current study,

all TLR4, TLR5 and TLR1B interact directly with down-stream adaptors MyD88 and TIRAP, which showed consistent down-regulation of gene expression in BI/BN while TLR4,-5 and -1B are significantly up-regulated with *Salmonella* infection. It is possible the down regulation of MyD88 is associated with the negative regulation by suppressor of cytokine signaling 1 (SOCS-1), as a significant increase of chicken SOCS-1-like (BX933215) expression was observed in BI/BN from our array results. Likewise, as opposed to MyD88, the up-regulation of IRAK4 could be due to other inhibitory molecules such as splice variant of MyD88 (MyD88s), IRAK-M or IRAK1-c (Dunne and O'Neill 2005; Janssens et al. 2003; Miggin and O'Neill 2006; Wang et al. 2001). Unfortunately, the gene IDs for these molecules are currently unavailable in chicken genome at this time.

In mammals, the stimulation of TLRs results in the downstream activation of NFkB, p38 and JNK kinase pathway through the phosphorylation of activated TAK1 (Kanayama et al. 2004), which in turn activates AP-1 and leads to the expression of genes that participate in the innate immune response. Both p38 and c-Jun amino terminal kinases (JNK) are gene families belonging to mitogen activated protein kinase (MAPK) superfamiy, which controls a vast array of physiological processes by mediating signals triggered by cytokines, growth factors, and environmental stress (Johnson and Lapadat 2002). The function of MAPKs has been characterized on chicken heterophils, which shows that MAPKs (especially p38) plays an important role on mediating distinct cellular response with stimulations of different TLR agonists (Kogut et al. 2005). It was suggested the difference in the activation of MAPK signaling pathways might be associated with the dissimilar functional responsiveness between heterophils from

resistant and susceptible line chickens (Kogut et al. 2006). The results in this study agreed with that members of p38 family showed most differential expressions, and these genes expressed divergently in regulation direction between line A and line B heterophils with *Salmonella* infection. These findings indicated that, besides canonical NFkB pathway, MAPKs pathway may be involved in the immune variability in these genetically divergent chickens. Further studies will be needed to elucidate the functional difference of MAPK pathway using alternative approaches such as analysis at protein level or genomic level.

In summary, the results from this study indicated that the expression of adaptor genes in TLR pathway was involved in the different host response between heterophils from two genetically distinct broiler lines. The resistene (A) line had more adaptors with up-regulated expression than susceptible (B) line, and most differentially expressed adaptors were found involved in the downstream signaling cascades of TLR4-induced My88-dependent pathway.

The inferred chicken TLR pathway identified several groups of genes that had related-functions and co-expressed in consistent directions with SE infection. The signaling cascades involved in these genes provided potential biological inferences which controlled avian immune response against SE infection through TLR pathway. It is of note that while most knowledge of chicken TLRs are broadly adopted from that found in mammalian TLRs, some difference do appear between species, and nonetheless to say between mammalian and non-mammalin systems (Iqbal et al. 2005; Rehli 2002). More detailed functional analysis of the regulation on chicken TLR pathway-related genes may help to improve the accuracy of current inferred chicken TLR pathway. Nevertheless, the current study will help deep our understanding of the induction of avian immune response via TLR pathway regulation.

Table 4.5 Microarray results for gene expression changes within infection and non-infection heterophils

Chicken homologous gene	Chicken Gene ID assigned by KEGG	Probe index	Accession no.	AI/AN fold-change[a]	BI/BN fold-change	Gene Tyep
MD-2	GGA: 420189	18206	BX932484	**2.20**	1.29	Secreted protein
CD14	none	29557	TC202210	2.33	1.56	Secreted protein
TLR1-type1	GGA: 426274	33193	AJ720806	-2.13	-1.03	Receptor
TLR1-type2	GGA: 771173	4222	BU405042	1.21	-1.08	Receptor
TLR2-type 1	GGA: 374141	11718	AB050005	-1.15	-1.18	Receptor
TLR2-type 2	GGA: 769014	730	AB046533	1.06	1.02	Receptor
TLR3	GGA: 422720	17987	CR407213	-1.31	1.05	Receptor
TLR4	GGA: 417241	5323	NM_001030693	**2.93**	**2.65**	Receptor
TLR5	GGA: 554217	820	CR353090	1.15	1.09	Receptor
TLR7	GGA: 418638	32488	AJ720504	**-4.21**	**-5.78**	Receptor
IFNAR1	GGA: 395665	25462	AF082664	-1.10	-1.20	Adaptor
IFNAR2	GGA: 395664	9406	AF082665	-1.07	-1.07	Adaptor
RAC1	GGA: 395871	378	U79755	1.04	1.01	Adaptor
TOLLIP	GGA: 423099	39706	AJ720279	1.11	-1.72	Adaptor
MYD88	GGA: 420420	23268	AJ851640	-1.07	-2.07	Adaptor
TIRAP	GGA: 419715	16189	BX933959	-1.19	**-1.22**	Adaptor
TRAM	none	4949	BX950451	1.02	1.19	Adaptor
PIK3CA	GGA: 424971	27707	AF001076	1.03	1.05	Adaptor
PIK3CB	GGA: 424826	6450	AJ851398	-1.10	**-1.95**	Adaptor
PIK3CD	GGA: 419444	31936	AJ851606	**-1.40**	**-1.47**	Adaptor
PIK3CG	GGA: 417706	6318	TC224633	-1.02	-1.04	Adaptor
PIK3R1	GGA: 427171	16849	CR389387	-1.22	-1.03	Adaptor
PIK3R2	GGA: 771142	2051	BM491349	-1.16	1.37	Adaptor
PIK3R5	GGA: 417319	41463	AJ720866	-1.13	**-1.90**	Adaptor
FADD	GGA: 423146	31680	BX931327	1.10	**1.16**	Adaptor
IRAK4	GGA: 417796	30479	AJ720408	1.05	**1.15**	Adaptor
TRIF	GGA: 100008585	23504	BU265026	**-1.57**	**-1.96**	Adaptor
AKT1	GGA: 395928	31645	AF039943	1.28	1.78	Adaptor
AKT3	GGA: 421497	37601	BX950472	-1.40	-1.20	Adaptor
CASP8	GGA: 395284	32224	AY057939	**-1.43**	-1.08	Adaptor
TRAF3	GGA: 423471	16279	BX935958	1.92	-1.08	Adaptor
TRAF6	GGA: 423163	23381	BU445218	1.29	1.26	Adaptor
TAB1	GGA: 418014	18014	AJ720321	**1.26**	1.10	Adaptor
TAB2	GGA: 421622	2910	BX933932	1.15	1.45	Adaptor
TAK1	GGA: 421808	10979	CR524033	-1.19	1.13	Adaptor
RIP1	GGA: 378921	42351	AB108485	1.17	-1.18	Adaptor
IKKE	GGA: 430480	29969	BU133261	-1.81	**-5.18**	Adaptor

Table 4.5 continued

Chicken homologous gene	Chicken Gene ID assigned by KEGG	Probe index	Accession no.	AI/AN fold-change[a]	BI/BN fold-change	Gene Tyep
TBK1	GGA: 417825	42686	TC206677	**-1.48**	-1.13	Adaptor
IKKA	GGA: 423669	8235	AJ720520	1.13	-1.32	Adaptor
IKKB	GGA: 426792	1981	AJ720901	-1.06	1.01	Adaptor
NFkB1	GGA: 396033	10475	BU479586	**1.94**	1.34	Adaptor
NFkB2	GGA: 386574	31088	D16367	-1.25	**-1.31**	Adaptor
Tp12	GGA: 420479	37463	BU419009	1.59	1.27	Adaptor
MKK3	GGA: 416496	43539	TC206869	**2.63**	1.70	Adaptor
MKK4	GGA: 417312	39018	BU111907	1.22	-1.02	Adaptor
MEK1	GGA: 415549	3758	AJ720834	-1.09	1.08	Adaptor
MEK2	GGA: 396349	8112	L28703	**-1.25**	**-1.28**	Adaptor
OPN	GGA: 395210	2195	CR353415	**1.86**	**1.64**	Adaptor
IKB-alpha	GGA: 396093	8792	M74544	1.06	1.32	Adaptor
ERK	GGA: 373953	6264	AY033635	1.18	1.38	Adaptor
P38-MAPK11	GGA: 417739	18274	AJ720776	1.42	**-1.77**	Adaptor
P38-MAPK12	GGA: 769763	14763	CR339030	**1.22**	**-1.22**	Adaptor
P38-MAPK14	GGA: 421183	12389	CO635361	-1.44	1.21	Adaptor
JNK-MAPK8	GGA: 423778	13292	BX934173	-2.31	-1.13	Adaptor
JNK-MAPK9	GGA: 395983	8229	CR406144	-1.22	1.02	Adaptor
JNK-MAPK10	GGA: 422592	40303	TC224553	1.33	**1.65**	Adaptor
IRF5	GGA: 430409	36902	AJ720409	-1.07	-1.05	Adaptor
IRF7	GGA: 396330	22785	U20338	-1.16	1.02	Adaptor
STAT1	GGA: 424044	28865	BX933123	1.19	1.07	Adaptor
AP-1	GGA: 424673	14626	NM_001031289	1.29	-1.13	Adaptor
IL1B	GGA: 395196	32934	Y15006	**3.40**	**2.25**	Cytokine
IL6	GGA: 395337	43474	AJ309540	**11.39**	**8.96**	Cytokine
IL12A	GGA: 407090	19858	BX258839	-1.71	**-3.21**	Cytokine
IL12B	GGA: 404671	17736	AJ564201	**3.19**	**2.71**	Cytokine
IFN-alpha	GGA: 396398	35729	BU319434	**1.32**	1.09	Cytokine
INF-beta	GGA: 554219	21734	AY831397	1.18	-1.52	Cytokine
IL8	GGA: 396495	18282	M16199	**7.40**	**4.03**	Chemokine
CCL4	GGA: 395551	20444	CR389044	**7.30**	**3.59**	Chemokine
CCL5	GGA: 417465	18946	CD739895	**2.21**	1.25	Chemokine
CD40	GGA: 395385	20966	AJ293700	-1.21	1.43	costimulatory protein
CD80	GGA: 768950	18731	AJ851659	**3.85**	**1.53**	costimulatory protein
CD86	GGA: 427944	20287	AM050135	-1.17	-2.15	costimulatory protein

a,: Bold are differences in expression levels found with the microarray ($P < 0.05$) as well as the qRT-PCR ($P < 0.05$). When the ratio (AI/AN) is smaller than 1, the ratio –(AN/AI) is given.

CHAPTER V

INHIBITION OF NFkB1 (NFkBp50) BY RNA INTERFERENCE IN CHICKEN MACROPHAGE HD11 CELL LINE CHALLENGE WITH *SALMONELLA* ENTERITIDIS

Overview

The NFkB pathway plays an important role in the regulation of immune response in animals. In this study, small interfering RNAs (siRNA) were used to specifically inhibit the expression of NFkB1 and to elucidate the role of NFkB1 in the signal transduction pathway of a *Salmonella* challenge in the chicken HD11 macrophage cell line. The cells were transfected with either NFkB1 siRNA, glyceraldehyde 3-phosphate dehydrogenase siRNA (positive control) or negative control siRNA for 24 h, followed by *Salmonella enteritidis* (SE) challenge or non-challenge for 1 h and 4 h. Eight candidate genes related to the signal pathway of SE challenge were selected to examine the effect of NFkB1 inhibition on their expressions by real-time quantitative PCR. The results indicated that, with 36% inhibition of NFkB1 expression, the gene expression of Toll-like receptor (TLR) 4 and interleukin (IL)-6 was consistently and significantly increased at both 1 h and 4 h following SE challenge, whereas the gene expressions of MyD88 and IL1β were increased at 1 h and 4 h, respectively. These findings suggest a likely inhibitory regulation of NFkB1 on TLR signal pathway. This study will lay the foundation for further investigation of the gene network of the innate immune response to SE infection in chickens.

Introduction

Salmonella enterica serovar Enteritidis (SE) has become one of the most common *Salmonella* serotype in many countries (Braden 2006b). The epidemiology of SE accounts for the main source of salmonellosis in humans through the consumption of contaminated poultry or shell eggs. The SE can persist in the cecum or ovaries of adult birds for months without triggering clinical signs. The colonized SE can continue to be excreted in feces (horizontal transmission) or through the yolk (vertical transmission) to contaminate other birds in the flock as well as the poultry products such as meat (after slaughtering) and eggs (Tilquin et al. 2005b).

Current control of salmonellosis in poultry is mainly through hygiene measures combined with vaccination programs; however, the current vaccines are only partially effective (Wigley et al. 2002). Selection of chickens for genetic resistance to *Salmonella* infection offers an alternative environment-friendly control measure. Macrophages are critical components of the immune system and play significant roles in both innate and acquired immune responses during SE infection. Through the process of phagocytosis, the macrophage is responsible for the clearance and destruction of both intracellular and extracellular pathogens (Ohl and Miller 2001). In chickens, previous studies have shown that macrophages from a *Salmonella*-resistant line have a stronger capability to clear colonized *Salmonella* than macrophages from susceptible lines (Wigley et al. 2002). The different biological of macrophages might be associated with greater and more rapid expression of proinflammatory cytokines generated through the NFkB signal pathway (Wigley et al. 2006).

Although gene expression profiling of chicken macrophages has been conducted using an avian macrophage-specific cDNA microarray with LPS stimulation (Bliss et al. 2005), the role of NFkB in the immune response to SE infection in chickens is still unknown. Given that NFkB is the central regulator of the innate immune response to invasive bacteria and is activated by the MyD88-dependent signaling of TLR pathway (Elewaut et al. 1999; Moynagh 2005), it is essential to elucidate the role of NFkB in the TLR pathway in macrophages and to improve our knowledge of SE pathogenesis in the chicken. To do that, RNAi technology using chemically synthesized siRNA was applied to specifically inhibit expression of NFkB in a chicken macrophage cell line (HD11). Expressions of selected genes including receptors, adaptors and cytokines associated with NFkB pathway were evaluated before and after SE challenge in the siNFkB1-treated cells. In the present study, gene expressions of several candidate genes were influenced with the inhibition of NFkB1.

Material and Methods

Culture of chicken macrophage HD11 cells. Chicken macrophage HD11 cells, an established chicken myelomonocytic line transformed by the *myc*-encoding MC29 virus (Van 1996), were used in this study. The HD11 cells were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 1% sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), 1% glutamax (Invitrogen, Carlsbad, CA), 5% chicken serum (Sigma-Aldrich, St. Louis, MO), and 5% fetal bovine serum (Atlanta biologicals,

Lawrenceville, GA) at 37°C in a 5% CO_2 incubator following routine cell culture procedures.

siRNA synthesis and transfection. Messenger RNA sequences of chicken NFkB1 (NM_205134) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_204305.1) were used to design siRNA primers as target gene and positive control, respectively. For each mRNA sequence, three different siRNA primers were designed targeting distinct sites in order to compare the silencing efficacy. All of the siRNA primers were synthesized and annealed by Ambion (Ambion, Austin, TX) (**Table 5.1**). A universal negative control siRNA primer (Ambion, Austin, TX) with no specific targeting to the chicken genome was used to normalize the relative gene inhibition of the target gene. Twenty-four hours before transfection, 450 μL HD11 cells were transferred on a 24-well plate (pre-plating) to reach a 50 to 80% confluency, and were transfected with NFkB1 siRNA (siNFkB1) or GAPDH siRNA (siGAPDH) or scrambled siRNA negative control (siNC) using the chemical transfection reagent siPORT-Amine (Ambion, Austin, TX). The transfection was conducted according to the manufacturer's manual with minor modification. Briefly, 1.5 μL of 10 μM annealed siRNA were mixed thoroughly and incubated with 4 μL of siPORT-Amine in a volume of 50 μL of Opti-mem medium (Invitrogen, Carlsbad, CA) at room temperature for 20 min, this was then added to 450 μL of HD11 cell culture with gentle agitation for mixing. The optimal conditions for siRNA transfection were optimized by adjusting different transfection parameters including cell number ($2x10^5$, $3x10^5$ and $4x10^5$ cells/mL), siPORT-Amine concentration (3, 4, and 5 uL/50 uL of total reaction volume of siRNA

mixture), siRNA concentration (20 nM, 30 nM and 40 nM), and incubation time (12 h, 24 h, 36 h and 48 h). After incubation, the total RNA of siRNA- (siNFkB1, siGAPDH or siNC) treated cells was extracted for the first strand cDNA synthesis, and the relative gene inhibition was measured by real-time quantitative PCR (qRT-PCR) using SYBR green master mix and ABI prism 7900HT system (Applied Biosystems, Foster, CA).

Table 5.1 List of chemically synthesized small interfering RNAs for specific gene silencing

Primer name	mRNA target	Sense / Anti-sense	Primer sequence	Accession no. in GenBank
siGAPDH	GAPDH	Sense	5'-GGUGCUGAGUAUGUUGUGGtt-3'	NM_204305
		Anti-sense	5'-CCACAACAUACUCAGCACCtg-3'	
siNFkB1	NFkB1	Sense	5'-GGAGAGGAUCCGUAUAUUAtt-3'	NM_205134
		Anti-sense	5'-UAAUAUACGGAUCCUCUCCtg-3'	

SE challenge. A SE isolate (no. 97-11771, kindly provided by Dr. Kogut at College Station, USDA-ARS) was used in the present study. The SE was maintained in glycerol stocks at -80℃, and grown overnight in Luria-Bertani broth at 37℃ to recover the fresh culture. The recovered SE was adjusted in sterile PBS to a concentration of $1x10^9$ CFU per milliliter using spectrophotometric absorbance as previously described (Ferro et al. 2004). Before challenge, the HD11 culture medium was replaced by antibiotic-free medium and the cells were cultured for 2 h prior to challenge. The number of siRNA-treated HD11 cells was calculated from synchronized duplicates from the siNC transfected group. Both siNFkB1 and siNC transfected HD11 cells were stimulated with non-opsonized SE at multiplicity of infection (MOI) of 100, or with sterilized PBS (non-challenged). The treated culture plate was then centrifuged at 1000 x

g for 5 min to maximize the contact between bacteria and cells, and incubated at 37℃, 5% CO_2 for 1 h or 4 h as previously described (Wigley et al. 2002).

Quantitative RT-PCR. Total RNA was extracted from the SE-challenged cells with RNAqueous kit (Ambion, Austin, TX) following the manufacturer's manual. The cDNA was synthesized from equal amounts (300 ng) of total RNA with random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. The cDNAs from different treatments were quantified by qRT-PCR using ABI prism 7900HT system (Applied Biosystems, Foster, CA) with the software setting at relative quantification mode. Briefly, the 20 μL reaction mixtures contained 10 μL of SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA), 0.3 μM of each specific oligonucleotide primer **(Table 5.2**) and 1 μL of non-diluted first strand cDNA synthesized from 300 ng of total RNA. The condition of qRT-PCR amplification was set up as: 1 cycle at 95℃ for 10 min, 40 cycles at 95℃ for 15 s and 59℃ for 1 min. Dissociation curves were performed for each amplified product at the end of amplification. Each individual sample was run in triplicate and the averaged critical threshold cycle (Ct) was used for the data analysis. The Ct values of target genes were normalized by the Ct value of internal control (chicken β-actin gene).

Table 5.2 List of primers for quantitative real-time RT-PCR analysis

mRNA target		Primer sequence	Accession no. in GenBank	PCR product size (bp)
β -actin	F[a]	5'-ACGTCTCACTGGATTTCGAGCAGG-3'	NM_205518	298
	R[b]	5'-TGCATCCTGTCAGCAATGCCAG-3'		
GAPDH	F	5'-GAGGGTAGTGAAGGCTGCTG-3'	NM_204305	113
	R	5'-CATCAAAGGTGGAGGAATGG-3'		
NFkB1	F	5'-GAAGGAATCGTACCGGGAACA-3'	NM_205134	131
	R	5'-CTCAGAGGGCCTTGTGACAGTAA-3'		
IL1β	F	5'-GCTCTACATGTCGTGTGTGATGAG-3'	NM_204524[c]	80
	R	5'-TGTCGATGTCCCGCATGA-3'		
IL6	F	5'-AGGACGAGATGTGCAAGAAGTTC-3'	NM_204628	78
	R	5'-TTGGGCAGGTTGAGGTTGTT-3'		
IL18	F	5'-CACTGTTACAAAACCACCGC-3'	NM_204608d[d]	213
	R	5'-CTTAAAAGCCTTGGAGCTGC-3'		
TL1A	F	5'-CCTGAGTTATTCCAGCAACGCA-3'	NM_001024578[e]	292
	R	5'-ATCCACCAGCTTGATGTCACTAAC-3'		
TLR4	F	5'-TGCACAGGACAGAACATCTCTGGA-3'	NM_001030693[f]	347
	R	5'-AGCTCCTGCAGGGTATTCAAGTGT-3'		
TLR15	F	5'-TGCTGCCACATTTGGAAGATC-3'	NM_001037835	131
	R	5'-GATCGGTGCTCCACACAAGTC-3'		
TRAF6	F	5'-AGTAAATACGAGTGCCCGATCT-3'	CK607050	176
	R	5'-TTAGCGAAGTTGTCTGGAAAAA-3'		
MyD88	F	5'-AAGTTGGGCCACGACTACCT-3'	NM_001030962	216
	R	5'-CTGCTGCTTCCTTCGTAAGT-3'		

[a]Forward primer
[b]Reverse primer
[c]From Ferro et al.(Ferro et al. 2004)
[d]From Sadeyen et al.(Sadeyen et al. 2004)
[e]From Takimoto et al.(Takimoto et al. 2005)
[f]From He et al.(He et al. 2006a; He et al. 2006b)

Within the group of same siRNA treatment (siNFkB1 or siNC), the normalized Ct value (△Ct) from SE-challenged HD11 cells was compared to the △Ct from non-challenged cells, and the difference (△△Ct) was transformed into $2^{-\triangle\triangle Ct}$ value as the estimated fold change of siNFkB1 effect. The relative gene expressions (SE-challenged to non-challenged) were represented by fold change at both 1 h and 4 h post-infection, and the comparisons of fold change between siNC and siNFkB1 treated HD11 cells were conducted at different time points.

Statistical analysis. Data analysis was conducted by the mean of △△Ct from six individual data in two biological replicates, and the difference between them was evaluated with two tailed, paired Student's *t*-test using Microsoft® Excel 2003 version (Microsoft Corporation, 2003). The $P < 0.05$ was considered significant. Data displayed in the Figures were expressed as means of fold change ± standard error from six individual data points.

Results

RNAi efficacy of siGAPDH and siNFkB1 in HD11 cells. Since the transfection efficacy of siRNA delivery is varied in different target cell types, to establish a protocol for siRNA in HD11 cell line, a positive control siRNA (chicken siGAPDH) was first used to optimize the transfection conditions. Three distinct siGAPDH primers (data not shown) were transfected with different primer concentrations, transfection reagent concentrations, HD11 cell concentrations, and incubation times. The optimal transfection condition was defined as the condition under which siGAPDH could induce the highest inhibition of chicken GAPDH expression. In the current study, the optimized condition was as follows: cell concentration of 2.4 x 10^5/mL, siPORT-Amine (non-diluted): siRNA (10 μM) at 2.6:1 (v/v), and an incubation time of 24 h, in which the expression of GAPDH was reduced by 45% compared to the mock transfected group using negative control siRNA primers (**Figure 5.1**). The same transfection conditions were then used for delivering siNFkB1 primers and a similar efficacy of inhibition (36%) was observed on NFkB1 gene. A cross test was conducted by measuring GAPDH expression with siNFkB1 treated sample and NFkB1 expression with siGAPDH treated sample. Only small reductions of gene expression (8 % for GAPDH and 5% for NFkB1) were observed within the cells treated with target-unrelated siRNAs. The siRNA primers with highest inhibitory efficacy (one out of three from siGAPDH primers and one out of six from siNFkB1 primers, data not shown) were selected for silencing the target genes in SE-challenged HD11 cells (**Table 5.1**).

The effects of reduced NFkB1 expression on SE induced immune response in the HD11 cells. Both siNFkB1 treated and negative control (siNC treated) HD 11 cells were followed by SE challenge. The expressions of several candidate genes, including receptors, adaptors, and cytokines, were measured before and after SE challenge (1 and 4 h postinfection). Both TLR4 and TLR15 were receptor candidate genes due to their critical functions of microbial recognition by binding pathogen-associated molecular patterns (PAMPs). With SE challenge, the expression of TLR4 was downregulated at both 1 h (-1.75 fold) and 4 h (-3.45 fold) postinfection (**Figure 5.2A**).

Interestingly, the inhibition of siNFkB1 was found to significantly elevate the downregulated gene expression of TLR4 at both time points ($P < 0.05$). Unlike TLR4, the expression of TLR15 was consistently upregulated with SE challenge (2.44 fold at 1 h and 3.52 fold at 4 h postinfection), whereas no significant change was observed with the inhibition of siNFkB1. Myeloid differentiation primary response gene 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6) genes are important adaptors that assist the signal transduction in the canonical TLR pathway. Within siNC treated groups, both

MyD88 and TRAF6 were consistently downregulated with the SE challenge (-2.34 fold at 1 h and -2.37 fold at 4 h postinfection for MyD88, and -1.60 fold at 1 h and -1.45 fold at 4 h postinfection for TRAF6) (**Figure 5.2B**). However, with the NFkB1 inhibition, MyD88 was significantly ($P = 0.0047$) upregulated at 1.28 fold at 1 h postinfection, while no significant change was found on the expression of TRAF6.

Four cytokine genes were chosen to examine the effects of inhibited NFkB1 on SE induced immune response. All other cytokines' expressions were up regulated after SE challenge except that a down regulated expression was observed at 1 h postinfection in IL6, and the gene expressions were consistently higher in 4 h than in 1 h postinfection (**Figure 5.3**). The comparison between siNC and siNFkB1 treated samples indicated that the expression of IL1β was significantly ($P = 0.001$) upregulated by the siNFkB1 inhibition at 4 h postinfection only, while the expression of IL6 was significantly upregulated at both 1 h and 4 h postinfection ($P = 0.009$ and $P = 0.0004$, respectively). No significant effect of siNFkB1 inhibition on IL18 or TL1A was observed.

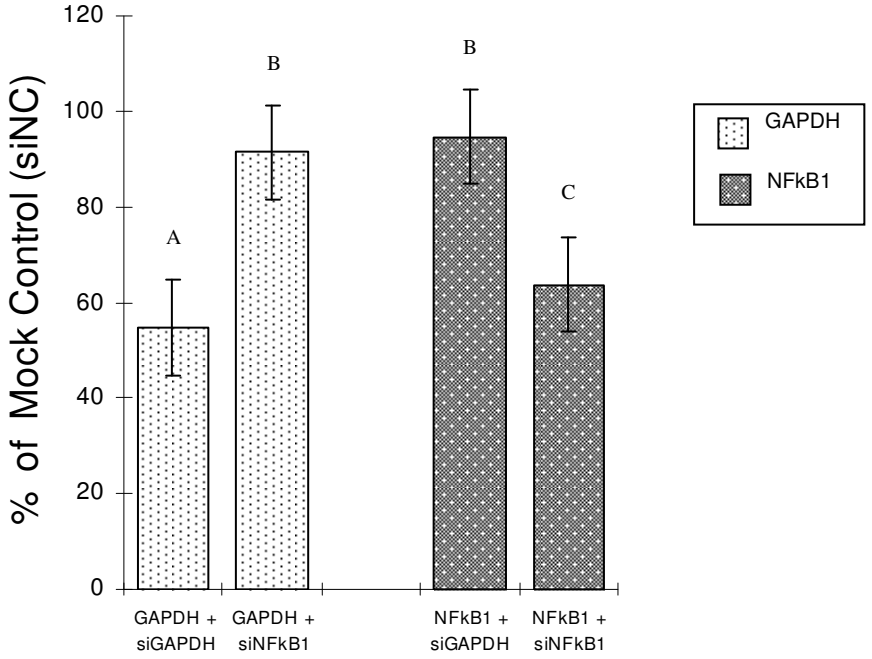
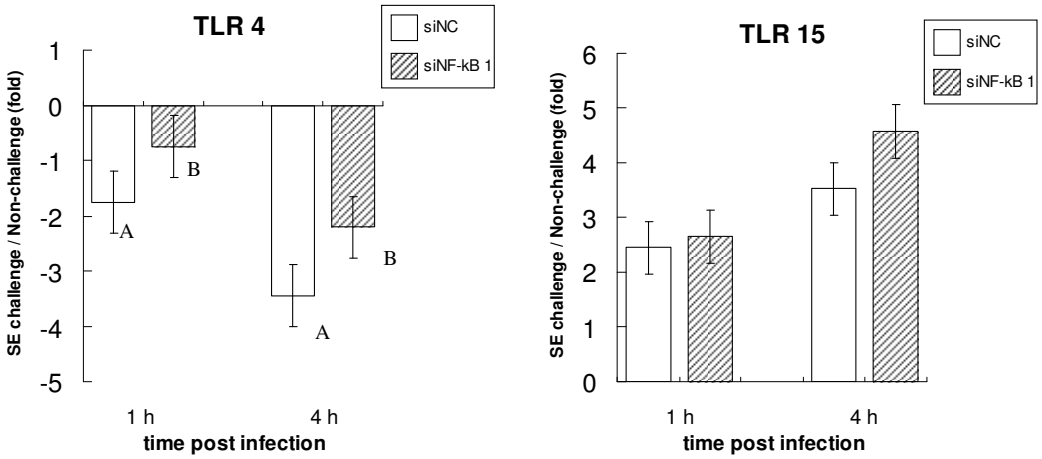


Figure 5.1 Reduced mRNA expression (GAPDH and NFkB1) of chicken HD11 cells after siGAPDH or siNFkB1 treatments. The data are presented as the mean (± standard error) from two replicate experiments. Data with different superscripts are statistically different ($P < 0.05$).

A)



B)

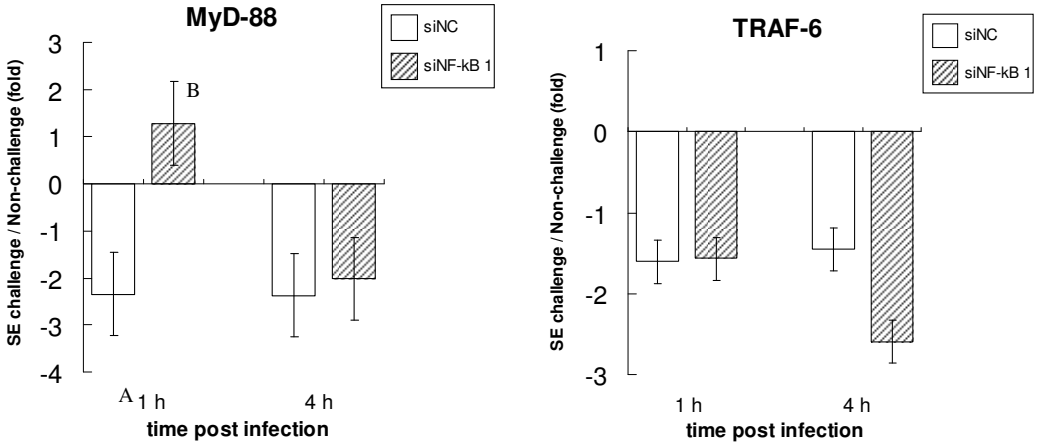


Figure 5.2 The effects of NFkB1 inhibition on mRNA expression of (A) receptors (TLR4, TLR15) and (B) adaptors (MyD88, TRAF6) from HD11 cells at different time point post SE infection. The genes with positive fold change are upregulated. The genes with negative fold change are downregulated. The data are presented as the mean (± standard error) from two replicate experiments. Data with different superscripts at same time point are statistically different ($P < 0.05$).

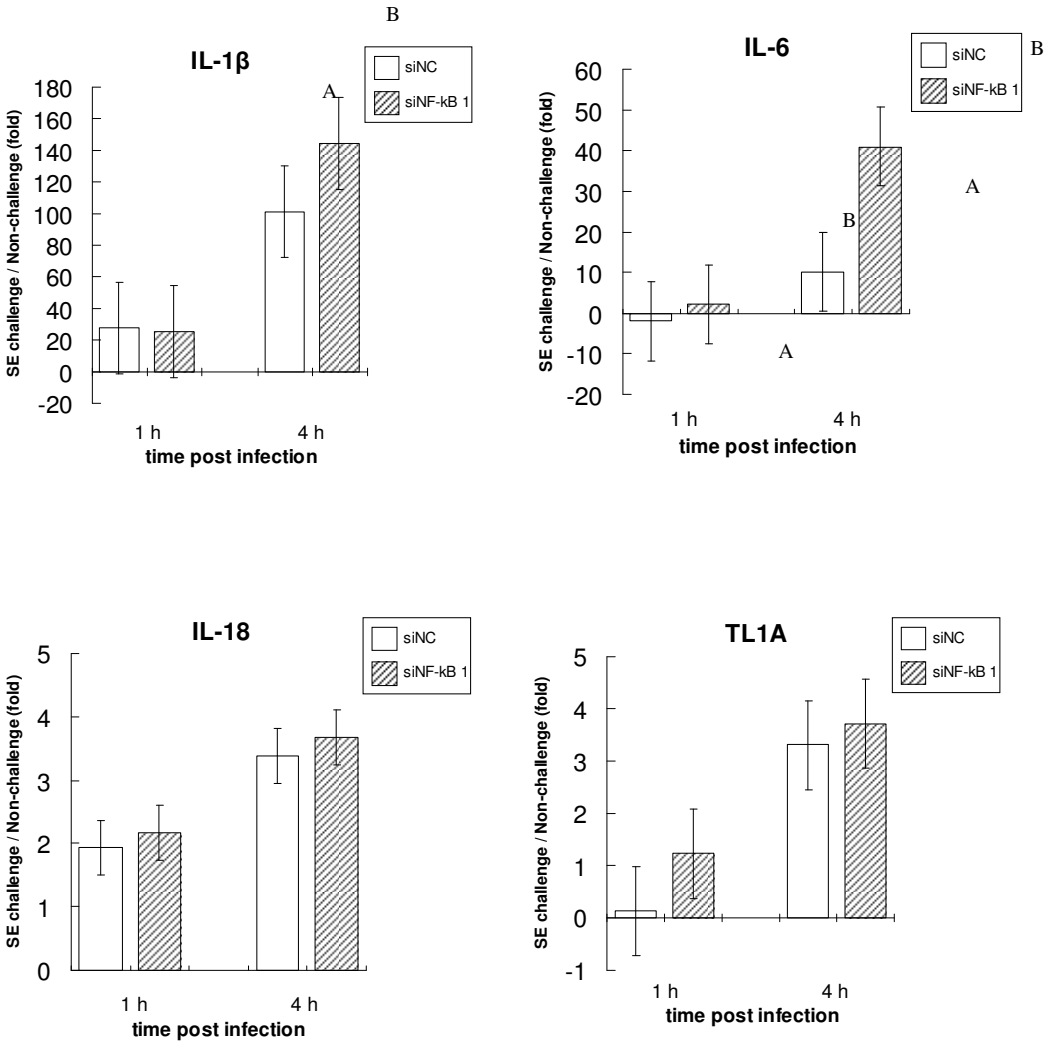


Figure 5.3 The effects of NFkB1 inhibition on mRNA expression of cytokines (IL1β, IL6, IL18 and TL1A) in HD11 cells at different time point post SE infection. The genes with positive fold change are upregulated. The genes with negative fold change are downregulated. The data are presented as the mean (± standard error) from two replicate experiments. Data with different superscripts at same time point are statistically different ($P < 0.05$).

Discussion

RNA interference (RNAi) is the process of sequence-specific post-transcriptional gene silencing. The mediator of messenger RNA degradation are 21~26 nucleotide siRNA generated by ribonuclease III-like dicer by cleaving the longer double strand RNA (dsRNA) (Duxbury and Whang 2004; Finnegan and Matzke 2003). RNAi offers great potential for both *in vitro* target validation and novel therapeutic strategies based on its mechanism that allows selective inhibition of expression of specific gene (Aigner 2006b). One of the key experimental approaches to elucidate gene function is to selectively ablate its expression or activity by the loss-of-function (LOF) approach. The gene knockout approach is not currently applicable in chickens. The RNA interference using siRNA has more favorable efficiency, efficacy, and cost compared to the other LOF techniques such as antisense DNA oligonucleotides, small molecular inhibitors, and dominant-negative mutants technology (Aigner 2006; Dees et al. 2000; Roth 1986). Along with these features, siRNA has less chance to trigger the interferon response than long double-stranded RNA (dsRNA) (Stark et al. 1998). The siRNA, therefore, became the first option for LOF selected in the current study.

Most of currently reported RNAi experiments in chickens were focused on studying embryogenesis using vector-based RNAi transfection (Chesnutt and Niswander 2004; Das et al. 2006; Kudo and Sutou 2005), and very few studies reported using chemically synthesized short interfering RNAs (siRNAs) on chicken cells (Hu et al. 2002; Sato et al. 2006). The lack of information regarding validated positive and negative control siRNA in chickens makes it more difficult to conduct an effective siRNA experiment using the chicken as a model. Since siRNA gene silence is a

transfection-dependent technology, optimization of siRNA transfection was considered the first step to set up a successful siRNA experiment (Duxbury and Whang 2004). We found that the 24-well plate was the most suitable for condition optimization and was able to provide enough RNA extracted from HD11 cells for mRNA quantification using qRT-PCR. It has been reported that the efficiency of a siRNA transfection is related to the intrinsic thermodynamic properties and the accessibility of the target site of the siRNA duplex (Kurreck 2006). Despite the fact that many successful RNAi mediated approaches have been reported, the design of highly potent siRNAs still remains an obstacle. Currently, the approach using multiple (three or more) primer design targeted to different sites in the target sequence is widely used to overcome this hurdle (Duxbury and Whang 2004). By using Ambion's design algorithms, our results showed that about 30% of tested siRNA primers could achieve a reproducible gene knockdown efficacy around 40 % compared to the siNC groups, which implies that designing three pairs of siRNA primer for one mRNA sequence might be an efficient and economic approach. Although siRNAs are not thought to trigger general translational attenuation through the interferon response like long double-stranded RNA do, the specificity of the silencing ability and potential side effects (e.g. transfection reagent toxicity) were tested by measuring the expression changes of target-unrelated genes. The small reductions on target-unrelated genes in the current study (data not shown) suggested that there was no considerable general translational attenuation, indicating that the observed effects were not biased. In contrast, both target gene and positive control showed significant reduction in gene expression with the corresponding siRNA treatments. Since the expression of all candidate genes in the SE-challenged cells was measured within 6 h

after the inhibition of NFkB1, the recovery of repressed NFkB1 in the present study was unlikely due to the very short time span allowing HD 11 cell division.

The macrophage is one of the primary leukocytes defending epithelium cells against *Salmonella* infection (Ohl and Miller 2001). Macrophages recognize different microbial patterns by many receptors including Fc and complement receptors, integrins, lectins, the mannose receptor, CD14, and the TLRs (Underhill and Ozinsky 2002). One of the most extensively described pathways is the TLR pathway. The predominant signaling pathway used by the TLRs results in the activation of NFkB, a transcription factor that is involved in T-cell activation and the production of cytokines promoting the killing invaded microbes (Moynagh 2005). According to the mammalian model, NFkB was assumed to participate in the signal transduction of TLR (MyD88- dependent) pathway and in cytokine release in response to bacterial infection (Lynn et al. 2003a). However, no direct connection has been confirmed between NFkB and associated genes involved in the TLR pathway in chickens. In the current study, the role of NFkB in TLR pathway were studied by combining NFkB1 silencing and SE stimulation of the HD11 cells. Eight candidate genes involved in TLR pathway were analyzed after NFkB1 repression. It is assumed that genes with significant change of expression might have stronger connection with NFkB signal pathway.

The role of chicken TLR4 in *Salmonella* infection has been contradictory (Wigley 2004b). Allelic variation in TLR4 was associated with susceptibility to *S.* ser. Typhimurium in chickens (Leveque et al. 2003), while Higgs and his colleagues have reported that there was no upregulation of TLR4 in cecum following *S.* Typhimurium infection (Higgs et al. 2006b). Very few studies have reported gene expression of TLR4

in response to SE infection of chicken macrophages or HD11 cells. Our results showed a down regulation of TLR4 after the SE challenge at both 1 h and 4 h postinfection, which was significantly attenuated following the inhibition of NFkB1 expression. For TLR15, a novel chicken TLR associated with *S.* Typhimurium infection (Higgs et al. 2006b), there was no statistical significance at both 1 h and 4 h postinfection, although a slight upregulation with the inhibition of siNFkB1 was observed. The results implied that NFkB1 might be involved in feedback mediating TLR4 expression through an inhibitory effect, but with no or minor effects on mediating TLR15 expression.

MyD88 is a universal adaptor for all TIR-domain-containing receptors except TLR3, based on a MyD88 knock-out mice study (Janssens and Beyaert 2002; O'Neill 2003). After the TLRs are activated by their ligand, MyD88 recruits IL1 receptor-associated kinases (IRAKs) to interact with TLRs. The activated IRAKs then associate with TRAF6 to activate the IkB kinase (IKK) complex, which finally releases the NFkB by degrading the IkBα molecule (Takeda and Akira 2004b; Yamamoto et al. 2004). Both MyD88 and TRAF6 are downstream adaptors of TLR4, and interestingly, they both showed a consistent downregulation with SE challenge. However, with inhibition of siNFkB1, a dramatic upregulation of MyD88 at 1 h postinfection was observed, while no significant change was found for TRAF6, except for an even lower expression at 4 h postinfection with unknown reason. Few studies have reported the detailed regulation of MyD88 or TRAF6 in chickens. In human monotypic THP-1 cell, no significant change of mRNA expression on MyD88 was observed between 0 to 4 h after LPS stimulation (Tamai et al. 2003). The stimulation of LPS on murine macrophage also showed no significant change on protein level of TRAF6 (Chen et al. 2006). It is unclear if the

minor down regulation of both MyD88 and TRAF6 in the present study is due to variant stimulation (SE), cell type or different time point tested. However, this is the first study to demonstrate the gene expression change of adaptors in chicken macrophages as a consequence of SE challenge. Our results also indicate a potential feedback regulation between MyD88 and NFkB1.

The inflammatory cytokines such as IL1β, IL6, IL12, IL18, and TNF-alpha are the end products of the MyD88-dependent TLR pathway (Takeda and Akira 2004a; Yamamoto et al. 2004). The production of inflammatory cytokines would be expected in macrophage where an inflammatory response occurs (Kaiser et al. 2004). In the current study, all cytokines showed significantly upregulated expression at 4 h postinfection, which indicated that SE challenge was sufficient to induce the innate immune response in HD11 cells. More rapid and dramatic expressional fold changes have been observed for IL1β (25 to 44 fold) and IL6 (-1.9 to 40 fold), which were similar to the previous study reporting on chicken macrophage with *Salmonella* challenge (Wigley et al. 2006). Although the synthesis of inflammatory proteins is assumedly related to NFkB binding to their cognate promoter, our results showed the repressed NFkB has significant up-regulatory effects on both IL1β and IL6. In mammals, both IL1β and IL6 are critical for activating the immune response and synthesizing acute-phase proteins (Giansanti et al. 2006). It is speculated that these two pro-inflammatory cytokines might be essential in the early phase of inflammatory stage against *Salmonella* infection.

The function of IL18 is associated with enhancing Th1-type response and activating PMN (polymorphonuclear) cells such as neutrophil in mammal (Lee et al. 2004). Swaggerty and her colleagues reported that a higher IL18 mRNA expression in

heterophils (a counterpart of neutrophil in birds) of chickens was associated with the resistance to SE infection. This suggested that IL18 also play a protective role against *Salmonella* infections (Swaggerty et al. 2006). A previous study in human has reported that IL18 has weak or absent repression with competitive inhibition of NFkB compared to IL1β(Lee et al. 2004). In the present study, a rapid increase of IL18 was observed on chicken macrophage after SE challenge. However, there is no significant effect on gene expression of IL18 with the inhibition of NFkB1.

Chicken TL1A (also known as TNF superfamily 15) was suggested to function as a substitute for mammalian TNF-α induced by LPS via NFkB pathway (Hong et al. 2006; Takimoto et al. 2005) and very few studies have been reported about this novel chicken cytokine. Although no significant effect on TL1A expression was observed after NFkB1 repression, our results showed that SE challenge could also induce the TL1A expression in chicken macrophage especially at 4h postinfection. The increased TL1A may fulfill an inflammatory function by enhancing the nitric oxide (NO) production and heterophil phagocytosis (Takimoto et al. 2005).

Notably, all of the regulatory directions of siNFkB1 on cytokines were similar to its effects on TLR genes, which support the possible function of NFkB1 as an inhibitory component in the TLR pathway. In mammals, three pathways were reported to activate NFkB's function through different dimeric complexes (p65-p50, p50-p50 and p52-RelB). The canonical pathway via the p65-p50 heterodimer was essential for the immune

response, while homodimers of p50-p50 may function as repressors by competing for the DNA binding site of other NFkB dimers (Lernbecher et al. 1993). It was also reported that the adenovirus-mediated induced p50-p50 dimer showed a suppressed IL1β activation but had no effect on TNF-alpha in colon-derived HT-29 cells (Tong et al. 2004). The new findings in the present study have provided new insights into how NFkB1 can regulate the TLR signal transduction pathway as a possible inhibitory factor in chickens. Further studies focusing on RelA protein may help reveal the roles of NFkB in this sophisticated TLR signal pathway.

CHAPTER VI

CHICKEN CD69 AND CD94-LIKE GENES IN A CHROMOSOMAL REGION SYNTENIC TO MAMMALIAN NATURAL KILLER CELL RECEPTOR GENE COMPLEX*

Overview

In mammals, natural killer (NK) cell C-type lectin receptors are encoded in a gene cluster called nature killer receptor complex (NKC). NKC is not reported in chicken yet. Instead, NK receptors were found in the major histocompatibility complex. In this study, two novel chicken C-type lectin-like receptors were identified in a region on Choromosome 1 that is syntenic to mammalian NKC region. Based on phylogenetic analysis, one receptor is highly homologous to mammalian CD69 and the other to CD94. Unlike mammalian NKC, these two chicken C-type lectin receptors are not closely linked but separated by 42 million bp according to the chicken draft genome sequence. The arrangement of several other genes that are located outside the mammalian NKC is conserved among chicken, human and mouse. The chicken NK C-type lectin like receptors in the NKC syntenic region indicate that this chromosomal region existed before the divergence between mammals and aves and there were probably no or much less gene duplication of NK receptor genes in the region in chickens after the divergence.

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*Reprinted from "Chicken CD69 and CD94/NKG2-like Genes in a Chromosomal Region Syntenic to Mammalian Natural Killer Gene Complex" by Chiang, et al., 2007, Immunogenetics. 59(7): 603-11.

Introduction

Nature killer (NK) cells are lymphocytes that have natural killing effects on tumor cells and infected cells. Unlike T and B cells, NK cells can develop into functional cytotoxic cells in absence of previous antigen stimulation and do not express *de novo* antigen-specific receptors as T and B lymphocytes. NK cells are a crucial component of the innate immune system. Once activated, NK cells became potent cytotoxic cells that are capable of lysing tumor cells, cells infected with intracellular pathogens, and antibody-coated cells. The cytotoxic activity of NK cells is regulated by signals from activating and inhibitory NK cell receptors (Lanier 2005). NK cells use their receptors to recognize and bind to potential target cells. After binding to ligands available on target cells, both NK activating and inhibitory receptors transmit signals to determine whether the NK cells detach from the cells or respond by releasing cytotoxic granules and secreting cytokines. The major histomopatibility complex class I and class I-like (MHC-I) molecules are known ligands recognized by NK inhibitory receptors.

Functionally, NK cell receptors are divided into inhibitory receptors and stimulating receptors based on their effects on cytotoxic activity. All inhibitory NK cell receptors have one or more copies of immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains. On the other hand, several activating NK receptors, like T cell and B cell antigen receptors, use adapter proteins containing an immunoreceptor tyrosine-based activation motif (ITAM) to transmit their signals. Structurally, NK cell receptors are also classified into two main groups: one group called NK C-type lectin receptor consisting of a dimeric type II transmembrane domain and a domain very similar to C-type lectin, and the other group named NK immunoglobulin

(Ig)-like receptors (KIR) possessing a type I transmembrane domain and immunoglobulin-like domains. In mammals, all C-type lectin-like NK receptors are encoded in a chromosomal region called NK cell gene complex (NKC), e.g. human chromosome12p13 and mouse chromosome 6qf3 (Trowsdale et al. 2001), whereas all Ig-like NK receptors are located in a gene cluster called leukocyte receptor complex (LRC), located on human chromosome 19 and mouse chromosome 7 (Trowsdale et al. 2001). Genes in NKC include NK cells-specific Ly49 family, Nkrpl family, NKG2 family, CD94, killer cell lectin-like receptor subfamily 1, and others are immune-related, such as CD69 and mast cell function associated antigen.

Most C-type lectin-like NK cell receptors were identified from mammals, specifically human and mouse. In fish, 28 killer cell C-type lectin receptors (KLR) have been reported in bony fish and at least 26 genes occupy a single chromosomal region called KLR complex, a region similar to NKC in mammalian (Kikuno et al. 2004). A putative KLR gene has also been characterized in teleostean fishes, which has a similar structure to human CD94/NKG2 subfamily (Sato et al. 2003). In chicken, a chromosomal region syntenic to mammalian NKC region was reported on chicken chromosome 1 (Bumstead 1998), but no C-type lectin-like receptors has been annotated in this chromosomal region. Instead, four C-type lectin-like NK receptors were identified in chicken chromosome 16, chicken major histocompatibility complex (MHC) B-F/B-L region (Kaufman et al. 1999; Rogers et al. 2005) and Rfp-Y region (Rogers et al. 2003). The NK receptors in chicken MHC suggest that NKC and MHC might evolve from a common ancestor. The NKC has been found to be associated with disease resistance to viral infections in mammalian (Hasan et al. 2005; Scalzo et al. 2005). There were also

three disease resistance QTLs mapped to this chromosomal region in chickens (Bumstead 1998a; Yonash et al. 1999; Zhu et al. 2003). It would be important to identify similar NK cell receptors in the chicken chromosomal region syntenic to mammalian NKC. In this study, we present the evidence of chicken NKC-like chromosomal region containing two C-type lectin receptors genes homologous to mammalian CD69 and CD94.

Material and Methods

***In silico* gene analysis.** Chicken CD69 and CD94 expressed sequences were identified by searching chicken expression sequence databases in National Center of Biotechnology Information (NCBI) (http://www.ncbi.nih.gov/Genbank/) and The Institute of Genomic Research using the TBLASTN program. The genomic locations of the hit sequences were determined using UCSC chicken genome browser (Karolchik et al. 2003) or assembled into longer contigs using CAP3 program (Huang and Madan 1999) if necessary. Candidate mRNA sequences were amplified with gene specific primers designed from predicted and/or homologus gene sequence. The PCR products were sequenced using BigDye terminator cycle sequencing reaction kits and an ABI prism 377XL DNA sequencer (Applied Biosystems Inc., CA). Protein sequences of chicken CD69 and CD94 were predicted based on the most likely open reading frame (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The functional domains or motif sequences were predicted using SMART program (Letunic et al. 2004) and ELM program (Puntervoll et al. 2003).

For protein sequence homology comparison, complete protein sequences of known CD69 and CD94 from different species (orthologs) were retrieved from Genbank protein database. The sequence homology was determined by multiple sequence alignment result using ClustalW program (Thompson et al. 1994) and Pairwise-alignments by BLAST2 (Tatusova and Madden 1999).

In order to get a unanimous result, only C-type lectin domains (CTLD), the most conserved region of CD69 and CD94 orthologs were applied in phylogenetic analysis as reported in previous studies (Rogers et al. 2005). The phylogenetic analysis of CTLD domain of chicken, human and mouse NKC genes was performed by Neighbor-Joining method using MEGA3 program (Kumar et al. 2004), with options selected for 500-replica bootstrap test, pairwise deletion and passion correction. The 3D structures of chicken CD69 and CD94 were predicted using GETATOMS followed by evaluation using Verify3D program (Eisenberg et al. 1997).

RNA isolation and reverse transcription (RT). Total RNA was isolated from nine different tissues, including white blood cells, kidney, liver, lung, oviduct, small intestine, large intestine, and spleen of laying White Leghorn hens (Gallus domesticus), using TriZol reagent (Invitrogen, Carlsbad, Calif.). The isolated total RNA were quantified with a UV spectrophometer (Brinkmann Instruments, Westbury, N.Y.) and treated with RNase-free DNAse I (Invitrogen, Carlsbad, Calif). Approximately 200 ng of each RNA sample was used directly as a template for PCR, using primers for chicken cytoplasmic β-actin (GenBank accession number X00182) to confirm the absence of genomic DNA in the RNA samples after DNAse I digestion. The first-strand cDNA was

synthesized from 2 μg total RNA, using the ThermoScript® reverse transcriptase system (Invitrogen, Carlsbad, Calif) and random hexamers as primers according to the manufacturer's protocol.

Tissue expression profile and DNA sequencing. Tissue expression screening was performed by Polymerase Chain Reaction (PCR) using first-strand cDNA as template. Approximately 50 ng of the first-strand cDNA was amplified in a 10-μl PCR reaction carried out using a thermocylcer (MJ Researc, Watertown, Mass.) and Taq DNA polymerase (New England Biolabs; Beverly, Mass.). The reaction was performed as follows: An initial denaturation step at 94 cfor 2 min and 35 cycles of denaturation, annealing, and extension at 94℃ for 40 sec, 59℃ for 40 sec, and 72 for 1 min. The final extension step was carried out at 72℃ for 10 min. Primers for PCR amplification were designed based reported mRNA or chicken expressed sequence tags (ESTs): Forward 5'-CGACTGAGTCATTATTGCGAGA-3' and the reverse primer 5'-CCACTGGAGTGATAAAAAGGAG-3' for chicken CD69, where as forward 5'-TTACGACTCCCAGCTTCAGAT-3' and reverse 5'- AGCCTAGGTGCAGGCAAAG-3' for chicken CD94. PCR products were examined using agarose gel electrophoresis and recovered for nucleotides sequencing. Unincorporated nucleotides were removed from amplified PCR product using BioMax spin-50 mini-columns (Millipore, Billerica, MA). The DNA sequencing was performed using BigDye terminator cycle sequencing reaction kits and an ABI prism 377XL DNA sequencer (Applied Biosystems).

Plasmid DNA isolation and fluorescent *in situ* hybridization (FISH) mapping. Bacterial artificial chromosome clone (BAC) screening was performed by BACPAC Resources Center (BPRC). 17 BACs were retrieved from chicken BAC library CHORI-261 (BACs containing chicken CD69: 106H9, 26B10, 48B13, 138J24, 58P11, 165H14, and 169D9; BACs containing chicken CD94: 6J7, 122O9, 35H4, 124J13, 72I22, 77B23, 81L24, 82F13, 180N9 and 84H15) using primers designed in genomic region: (chicken CD94 forwadr: 5'-AGGAAAGAAGCTGCAGGCCATGAG -3'; reverse: 5'-TGCTGGACAGAGAGCGCATTCTG-3' and for chicken CD69 forward: 5'-GGGGGAACTACTAGCCAAGG -3', reverse: 5'-GCTCTTACCCCCGTAACCTC-3'). Two BACs (26B10 and 72I22 for chicken CD69 and CD94, respectively) were chosen for plasmid DNA isolation using QIAprep kit (QIAGEN, Valencia, CA) following the product manual. The quality and concentration were evaluated by 0.8% agarose gel electrophoresis and Nanodrop (company name) before FISH mapping.

Chicken metaphase chromosome spreads were prepared from short term bone marrow cultures as described by Christidis (Christidis 1983). The CD69 and CD94 containing BAC clones were individually labeled with biotin (Bio-Nick translation Mix, Roche). The hybridization mixture contained 1 μg of labeled probe DNA, 10 μg chicken genomic DNA, 10% dextran sulphate, 50% formamide and 2xSSC. Overnight *in situ* hybridization, post-hybridization washing and signal detection with two layers of avidin-FITC (Vector) and one layer of biotinylated anti-avidin (Vector) was carried out as described elsewhere in details (Raudsepp et al. 1999). Twenty metaphase spreads for each hybridization were analyzed under Zeiss Axioplan2 fluorescent microscope, and images were captured and processed using the CytoVision®/Genus™ software version 2.7 (Applied

Imaging). Chicken chromosomes were identified according to the nomenclature proposed by Ladjali-Mohammedi et al. (Ladjali-Mohammedi et al. 1999).

Results and Discussion

The conserved CTLD region of protein sequence was used as the most informative sites in our phylogenetic studies, which generated a more consistent topology result than using whole length of protein sequence. Although the root region of phylogenetic tree has lower boos value in a 500-replica bootstrap test, which may caused by high homology between members of NKC, in the taxa region the phylogenetic topology still shows that the CTLD of chicken CD69 is closely related to human and mouse CD69, whereas the CTLD of chicken CD94 was closely aligned to fish KLR, though both of them are still involved in the same root of mCD94, hCD94 sub-cluster (**Figure 6.1**). In addition, chicken CD69 is related to a group of activation-induced lymphocyte receptors, which includes activation-induced C-type lectin, lectin-like transcript-1, and C-type lectin related receptors. Several chicken C-type lectin receptors identified in the MHC were also reported to be related to this receptor group (Rogers et al. 2005).

The comparison protein sequence homology was performed by multiple sequence alignment using predicted protein sequences of chicken CD69 and CD94 and retrieved known protein sequences of orthologous CD69 and CD94 genes. A conserved C-type lectin domain (CTLD) was found within extracellullar region in both predicted chicken CD69 and CD94 protein sequences which contains six canonical conservative cysteine residues (marked as C1 to C6) as reported in all known C-type lectin proteins

(**Figure 6.2**). Two typical N-linked glycosylation sites (N-X-S/T) were also found in chicken CD69 CTLDs (data not shown). As reported in previous studies, different numbers of N-glycosylation sites were found in CD69 sequences from different species. These glycosylation sites are probably responsible for the different sizes of glycoprotein chains for CD69 protein (Testi et al. 1994; Vance et al. 1999).

The comparison of chicken CD69 and c17.5, one of the identified chicken C-type lectin proteins in chromosome 16 indicated a major difference in cytoplasmic region. Chicken CD94 was additionally compared with NKG2 gene family using BLAST2 program **(Table 6.1)**, since functionally CD94 forms heterodimers with these receptors and shares a extremely high similarity in amino acid sequence in mammals (Boyington et al. 1999). The predicted chicken CD94 protein sequence is 233 amino acids in length, which is longer than mammalian CD94 receptors (179 amino acids) and the same length as human NKG2A.

Pairwise-alignments show that chicken CD94 has the highest similarity to mouse CD94 (64% overall). Compared to human NK C-type lectin receptors, chicken CD94 is similar to both human NKG2A (52%) and human CD94, however no ITIM or ITAM motifs were found in chicken CD94 as a functional evidence which was exited in NKG2 gene family. Interestingly, a similar molecule that possesses the characteristics of both the mammalian CD94 and NKG2 protein was also reported in Teleostean fish (Sato et al. 2003). The results implied that phylogenetically the gene found in chicken and fish may be the primitive gene of this receptor family. 3D crystal structure analysis has become a more powerful approach for annotating novel protein molecule. Unlike traditional linear amino acids profile comparison, 3D crystal structure counts the possible interaction between amino acids or motifs which is suitable on detecting a subtle similarity within distantly related orthorlogs. A finely overall review of NK receptors protein 3D structures have been reported by Sawicki and his colleagues which provided a useful reference in our study (Sawicki et al. 2001).

Table 6.1 Comparison of amino acid homology of representative C-type lectins expressed on mouse, human and rat NK cells with cCD94

	Amino acid identity (%) / similarity (%)[1]	
	Whole sequence	C-type lectin domain
hNKG2A	31% / 52%	32% / 56%
mNKG2A	23% / 46%	23% / 47%
hNKG2C	28% / 51%	29% / 55%
mNKG2C	22% / 44%	22% / 45%
hNKG2D	29% / 46%	29% / 46%
mNKG2D	31% / 50%	31% / 50%
hCD94	30% / 51%	31% / 50%
mCD94	33% / 62%	32% / 60%
fKLR[2]	32% / 45 %	32% / 44%

[1]The percentage of similarity is based on NCBI Blast2 program using matrix BLOSUM62.
[2]Teleostean fish nature keller cell C-type lectin receptor.

Figure 6.1 Phylogenetic analysis of CTLD sequences of human (h), mouse (m), fish (f) and chicken (c) genes in NKC. The distance given in the scale represents 0.2-aa substitutions per site. All of the accession numbers of retrieved sequences are listed below: mKlra1 (NM_016659), mLy49A (AF074456), mLy49D (L78247), mLy49G (U12890), mLy49E (AY620247), mLy49C (U34891), mLy49H (U12889), hKLRG1 (NM_005810), mKlrg1 (NM_016970), hDECTIN1 (AY026769), mDectin1 (NM_020008), mNkrp1b (X64723), mNkrp1a (M77753), hNKG2D (AF461811), mNkg2d (AF054819), mNkg2a (AF095447), mNkg2c (AF109785), mNkg2e (AF195779), hCLEC2 (NM_016509), mClec2 (NM_019985), hNKG2E (AF461157), hNKG2A (AF461812), hNKG2C (AJ001684), hKLRB1 (NM_002258), mNkrp1f (AY029597), hCD94 (NP_002253), mCD94 (NP_034784), fKLR(AY297060), cB-NK (AJ634338), hCD69 (NM_001781), mCD69 (P37217), cB-LEC (NP_998747), c17.5 (NM205429), cY-lec2(CAD61336), cY-lec1 (CAD61337), hLLT1 (AF133299), hAICL (X96719), mClrf (AF350410), mClrg (AF350411), mClrb (AF350409).

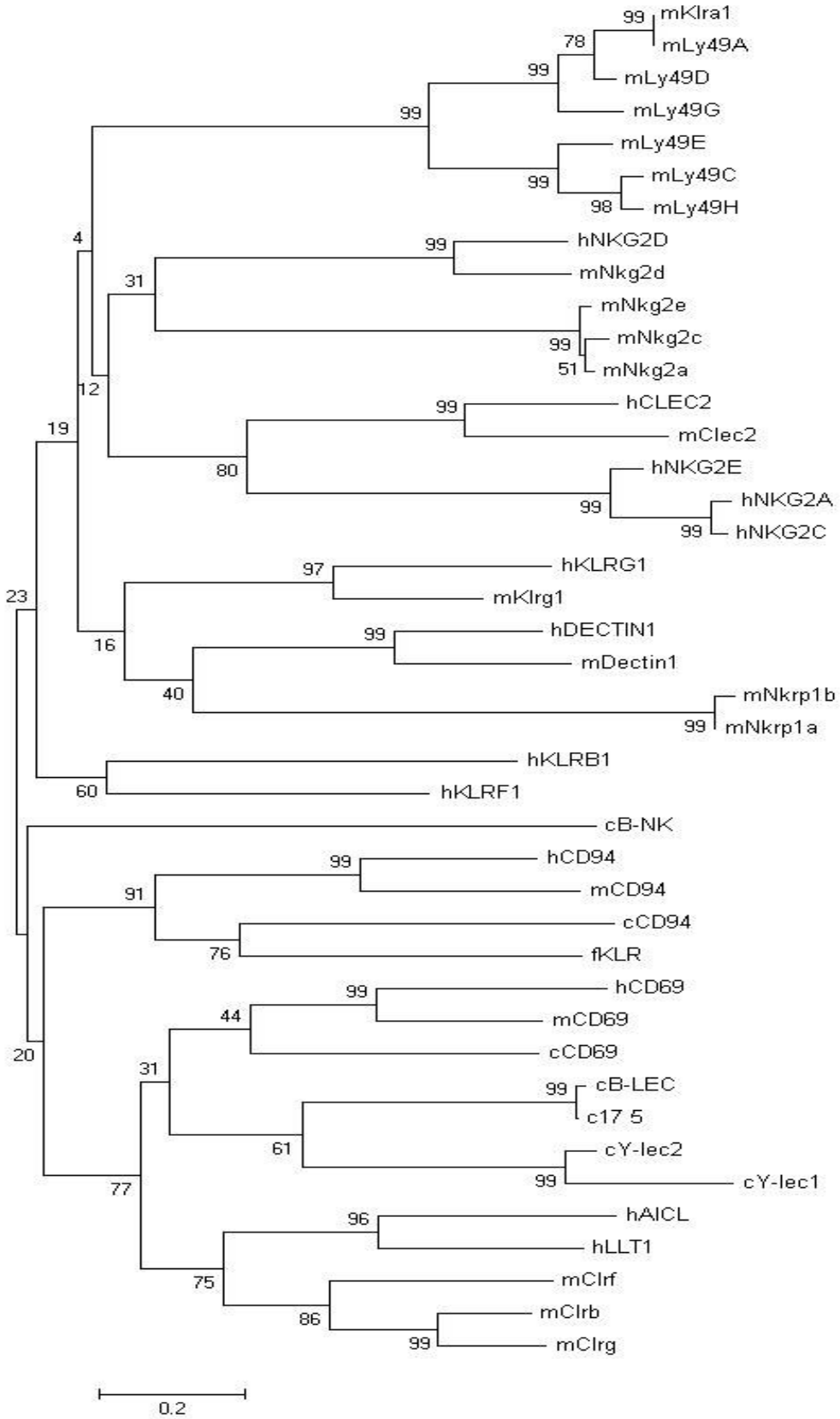

mKlra1
mLy49A
mLy49D
mLy49G
mLy49E
mLy49C
mLy49H
hNKG2D
mNkg2d
mNkg2e
mNkg2c
mNkg2a
hCLEC2
mClec2
hNKG2E
hNKG2A
hNKG2C
hKLRG1
mKlrg1
hDECTIN1
mDectin1
mNkrp1b
mNkrp1a
hKLRB1
hKLRF1
cB-NK
hCD94
mCD94
cCD94
fKLR
hCD69
mCD69
cCD69
cB-LEC
c17 5
cY-lec2
cY-lec1
hAICL
hLLT1
mClrf
mClrb
mClrg
0.2

Figure 6.2 a. Amino acid sequence comparison of CD69 of cow (b), pig (s), human (h), mouse (m), rat (r) and chicken (c). b. Amino acid sequence comparison of human (h) NKG2 representatives, CD94 of human (h), mouse (m), chicken (c) and chicken CD94 splicing variant (cCD94v). Alignment gaps are indicated by dashes. The consensus residues with identical amino acid more than 50% of all sequences are highlighted in gray and conserved residues in dark gray. The superscripts C1 to C6 indicated six conserved Cysten residues.

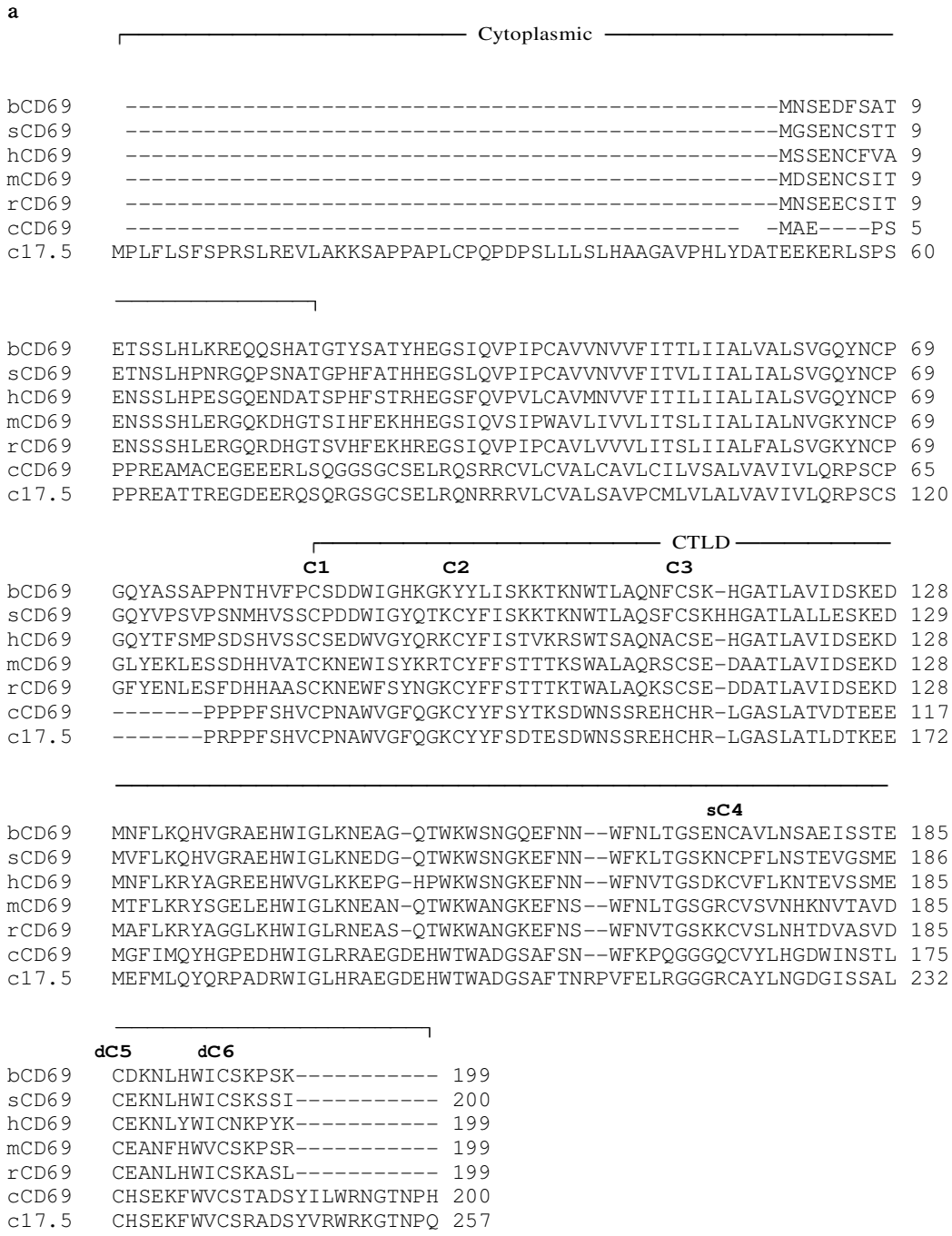
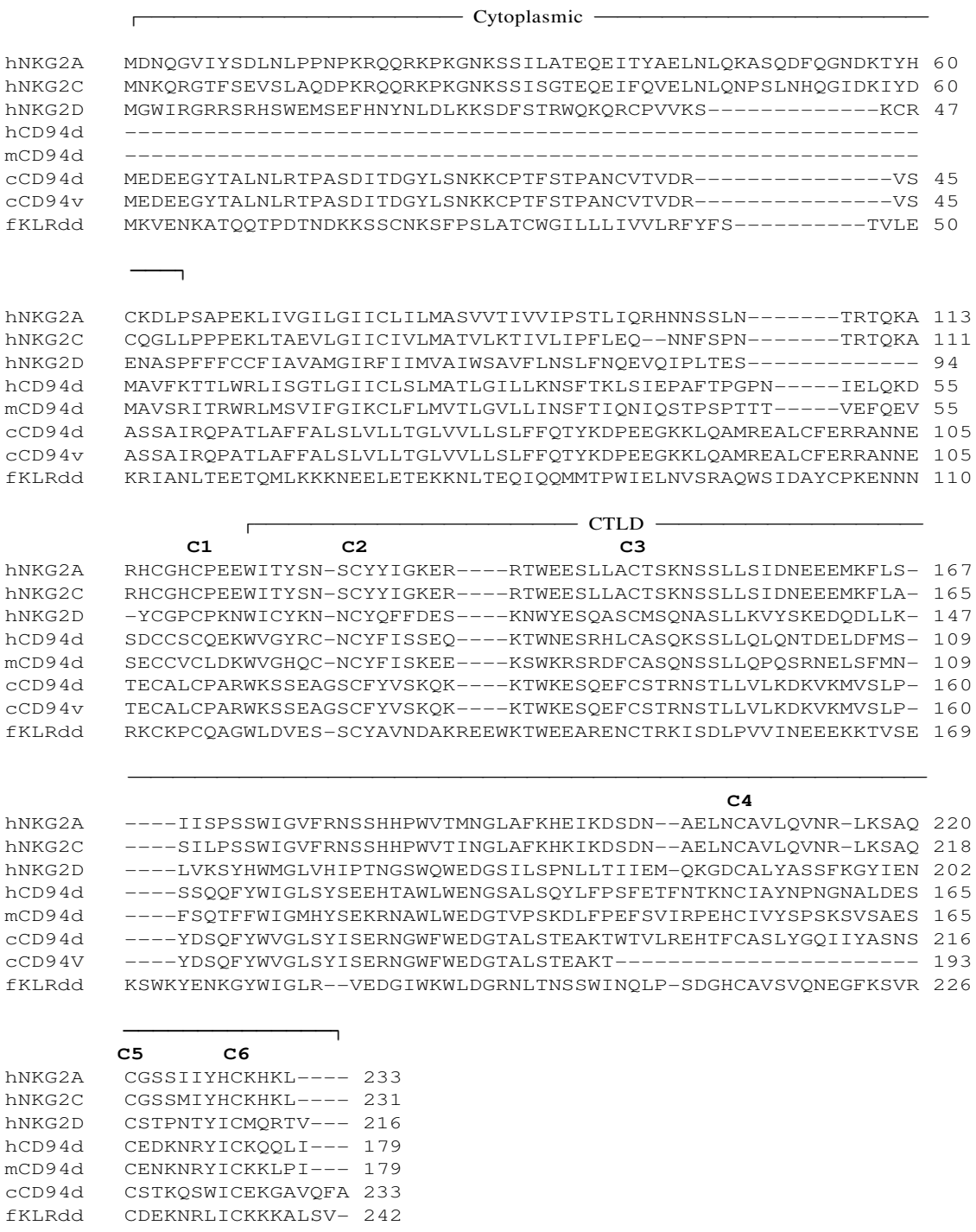


Figure 6.2 Continued.

b




```
                 Cytoplasmic
hNKG2A   MDNQGVIYSDLNLPPNPKRQQRKPKGNKSSILATEQEITYAELNLQKASQDFQGNDKTYH 60
hNKG2C   MNKQRGTFSEVSLAQDPKRQQRKPKGNKSSISGTEQEIFQVELNLQNPSLNHQGIDKIYD 60
hNKG2D   MGWIRGRRSRHSWEMSEFHNYNLDLKKSDFSTRWQKQRCPVVKS-------------KCR 47
hCD94d   ------------------------------------------------------------
mCD94d   ------------------------------------------------------------
cCD94d   MEDEEGYTALNLRTPASDITDGYLSNKKCPTFSTPANCVTVDR---------------VS 45
cCD94v   MEDEEGYTALNLRTPASDITDGYLSNKKCPTFSTPANCVTVDR---------------VS 45
fKLRdd   MKVENKATQQTPDTNDKKSSCNKSFPSLATCWGILLLIVVLRFYFS----------TVLE 50

hNKG2A   CKDLPSAPEKLIVGILGIICLILMASVVTIVVIPSTLIQRHNNSSLN-------TRTQKA 113
hNKG2C   CQGLLPPPEKLTAEVLGIICIVLMATVLKTIVLIPFLEQ--NNFSPN-------TRTQKA 111
hNKG2D   ENASPFFFCCFIAVAMGIRFIIMVAIWSAVFLNSLFNQEVQIPLTES------------- 94
hCD94d   MAVFKTTLWRLISGTLGIICLSLMATLGILLKNSFTKLSIEPAFTPGPN-----IELQKD 55
mCD94d   MAVSRITRWRLMSVIFGIKCLFLMVTLGVLLINSFTIQNIQSTPSPTTT-----VEFQEV 55
cCD94d   ASSAIRQPATLAFFALSLVLLTGLVVLLSLFFQTYKDPEEGKKLQAMREALCFERRANNE 105
cCD94v   ASSAIRQPATLAFFALSLVLLTGLVVLLSLFFQTYKDPEEGKKLQAMREALCFERRANNE 105
fKLRdd   KRIANLTEETQMLKKKNEELETEKKNLTEQIQQMMTPWIELNVSRAQWSIDAYCPKENNN 110

                 CTLD
         C1          C2                  C3
hNKG2A   RHCGHCPEEWITYSN-SCYYIGKER----RTWEESLLACTSKNSSLLSIDNEEEMKFLS- 167
hNKG2C   RHCGHCPEEWITYSN-SCYYIGKER----RTWEESLLACTSKNSSLLSIDNEEEMKFLA- 165
hNKG2D   -YCGPCPKNWICYKN-NCYQFFDES----KNWYESQASCMSQNASLLKVYSKEDQDLLK- 147
hCD94d   SDCCSCQEKWVGYRC-NCYFISSEQ----KTWNESRHLCASQKSSLLQLQNTDELDFMS- 109
mCD94d   SECCVCLDKWVGHQC-NCYFISKEE----KSWKRSRDFCASQNSSLLQPQSRNELSFMN- 109
cCD94d   TECALCPARWKSSEAGSCFYVSKQK----KTWKESQEFCSTRNSTLLVLKDKVKMVSLP- 160
cCD94v   TECALCPARWKSSEAGSCFYVSKQK----KTWKESQEFCSTRNSTLLVLKDKVKMVSLP- 160
fKLRdd   RKCKPCQAGWLDVES-SCYAVNDAKREEWKTWEEARENCTRKISDLPVVINEEEKKTVSE 169

                                                          C4
hNKG2A   ----IISPSSWIGVFRNSSHHPWVTMNGLAFKHEIKDSDN--AELNCAVLQVNR-LKSAQ 220
hNKG2C   ----SILPSSWIGVFRNSSHHPWVTINGLAFKHKIKDSDN--AELNCAVLQVNR-LKSAQ 218
hNKG2D   ----LVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEM-QKGDCALYASSFKGYIEN 202
hCD94d   ----SSQQFYWIGLSYSEEHTAWLWENGSALSQYLFPSFETFNTKNCIAYNPNGNALDES 165
mCD94d   ----FSQTFFWIGMHYSEKRNAWLWEDGTVPSKDLFPEFSVIRPEHCIVYSPSKSVSAES 165
cCD94d   ----YDSQFYWVGLSYISERNGWFWEDGTALSTEAKTWTVLREHTFCASLYGQIIYASNS 216
cCD94V   ----YDSQFYWVGLSYISERNGWFWEDGTALSTEAKT---------------------- 193
fKLRdd   KSWKYENKGYWIGLR--VEDGIWKWLDGRNLTNSSWINQLP-SDGHCAVSVQNEGFKSVR 226

         C5      C6
hNKG2A   CGSSIIYHCKHKL---- 233
hNKG2C   CGSSMIYHCKHKL---- 231
hNKG2D   CSTPNTYICMQRTV--- 216
hCD94d   CEDKNRYICKQQLI--- 179
mCD94d   CENKNRYICKKLPI--- 179
cCD94d   CSTKQSWICEKGAVQFA 233
fKLRdd   CDEKNRLICKKKALSV- 242
```

Figure 6.2 Continued.

Compared to human NK C-type lectin receptors **(Figure 6.3)**, our results indicated that chicken CD69 shares a very similar structure with human CD69. Both are lack of Loop 4 between Loop 1 and 3. The most unique feature observed in human CD94 is the absence of one of the two canonical major alpha helices presented in most C-type lectin protein structures (Sawicki et al. 2001). Interestingly the same α2 helix is absent in predicted chicken CD94 crystal structure, although the region of loop 1 and 3 are slightly variant from reference human CD94. To determine the tissue expression profile of the genes, the total RNA was isolated from different immune related tissues/organs including whole blood cells, kidney, liver, lung, oviduct, small intestine, large intestine, and spleen. The first-strand cDNA was synthesized from the tissue-specific total RNA and then served as template in regular PCR test. The results showed that chicken CD69 was highly expressed in all eight tissues tested **(Figure 6.4)**, which agrees with the previous observation in pig (Yim et al. 2002). In contrast, chicken CD94 was expressed highly in white blood cells and lungs, weakly in the spleen, liver, and kidney, but not in the large and small intestine and oviduct. A longer alternative splicing variant (DQ156497) of chicken CD94 was found to be expressed in the lung and white blood cells. The analysis of sequence result shows that the chicken CD94 variant contains an additional exon located between the fifth and sixth exon of chicken CD94 and therefore results in a shorter open reading frame. As mentioned previously, the similar CD94/NKG2 gene found in teleostean fishes was also expressed in white blood cells and gills (Sato et al. 2003). The results suggest that chickens and fishes may have a large number of NK cells in respiratory system and blood.

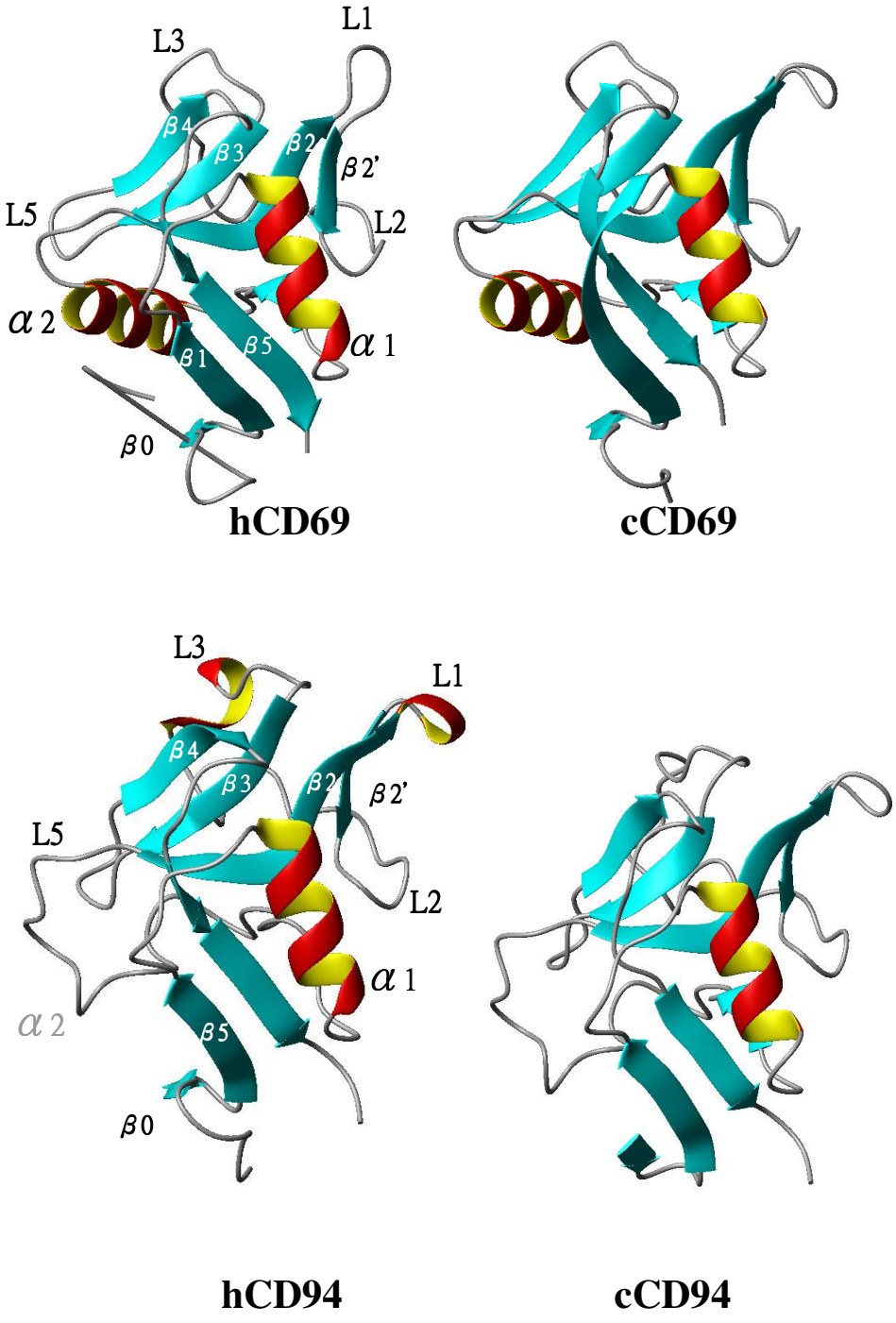


Figure 6.3 Ribbon diagrams of hCD69, cCD69, hCD94 and cCD94. Loop regions (L) are shown as silver rope, β-strands as blue, α-helices (α) as yellow/red. The number indicated the order in secondary structure from NH2 end to COOH end. PDB accession code: hCD69 (1FM5), hCD94 (1B6E).

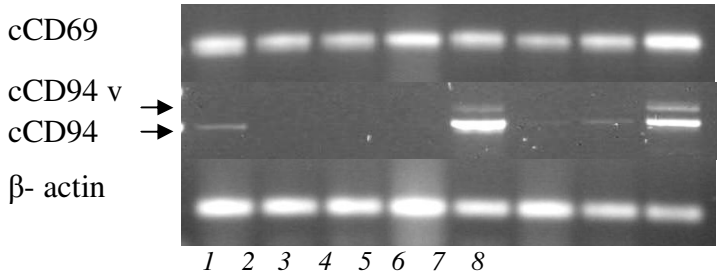


Figure 6.4 Tissue expression patterns of chicken CD69 and CD94, CD94 variant and β-actin genes. Lane1, spleen; 2, large intestine; 3, small intestine; 4, oviduct; 5, lung; 6, liver; 7, kidney; and 8, white blood cells.

The comparison of the syntenic chromosomal region among chicken, human, and mouse shows that the gene arrangement in the region was conserved **(Figure 6.5)**. However, unlike human and mouse, the two novel chicken C-type lectin-like receptors were not closely linked but separated by 42,389k bp in chicken chromosome 1 base on chicken genome draft. Both of the genes location and separated distance were further confirmed by fish mapping result **(Figure 6.6)**. Several genes located outside of NKC in human chromosome 12 and in mouse chromosome 6, 10 and 15 are also located in the chicken syntenic region. The comparison of these three species indicated that the chromosome rearrange in this region occurred more frequently within chicken to mouse than mouse to human, since human and mouse are more closely related to each other than to chicken in evolution. Based on the finding of this syntenic region, it can be concluded that chicken C-type lectin-like receptors are probably distributed in two chromosomal regions, one similar to mammalian NKC and another in the MHC on Chromosome 16 (Rogers et al. 2003) that is unique in chicken.

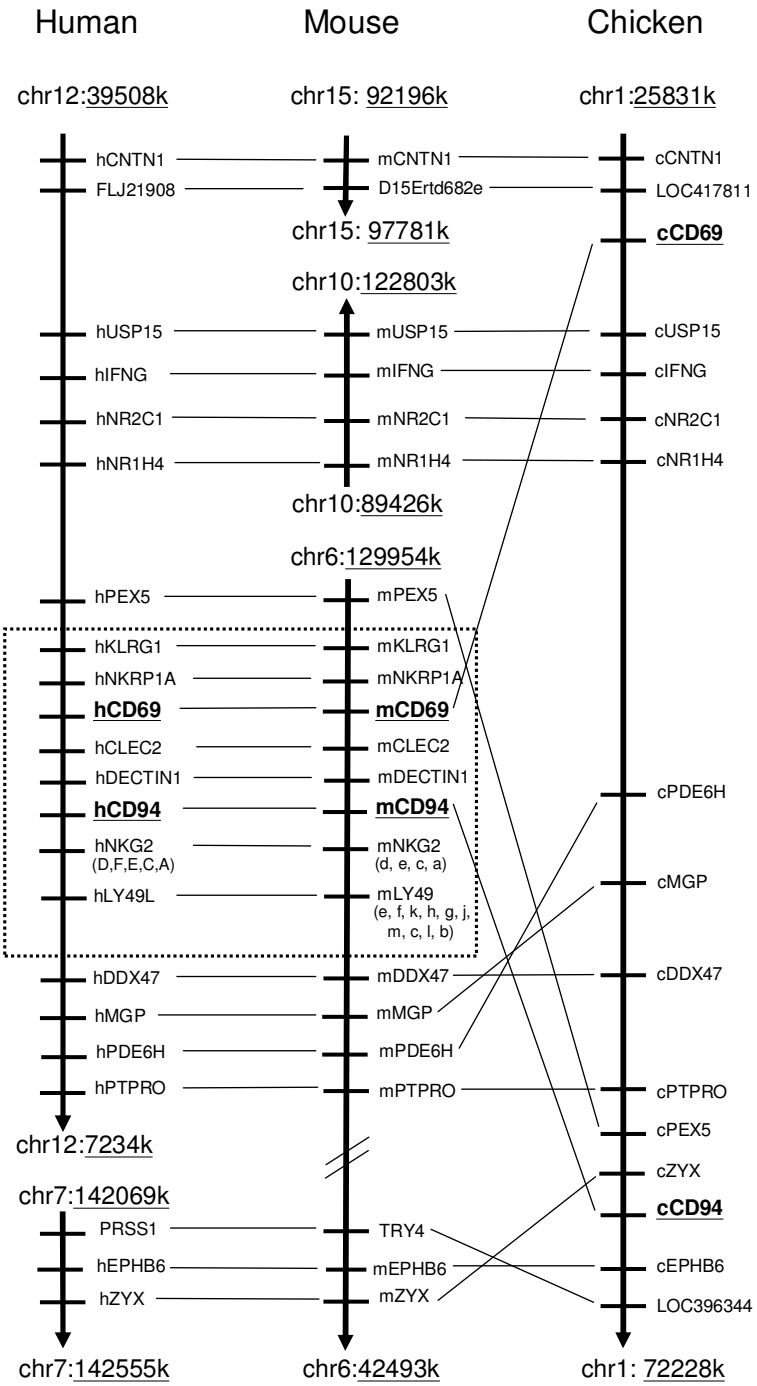


Figure 6.5 Genomic organization of human, mouse, and chicken NKC regions. The direction of arrow represents the orientation of chromosomes. The dotted line box represents the NKC region in human and mouse.

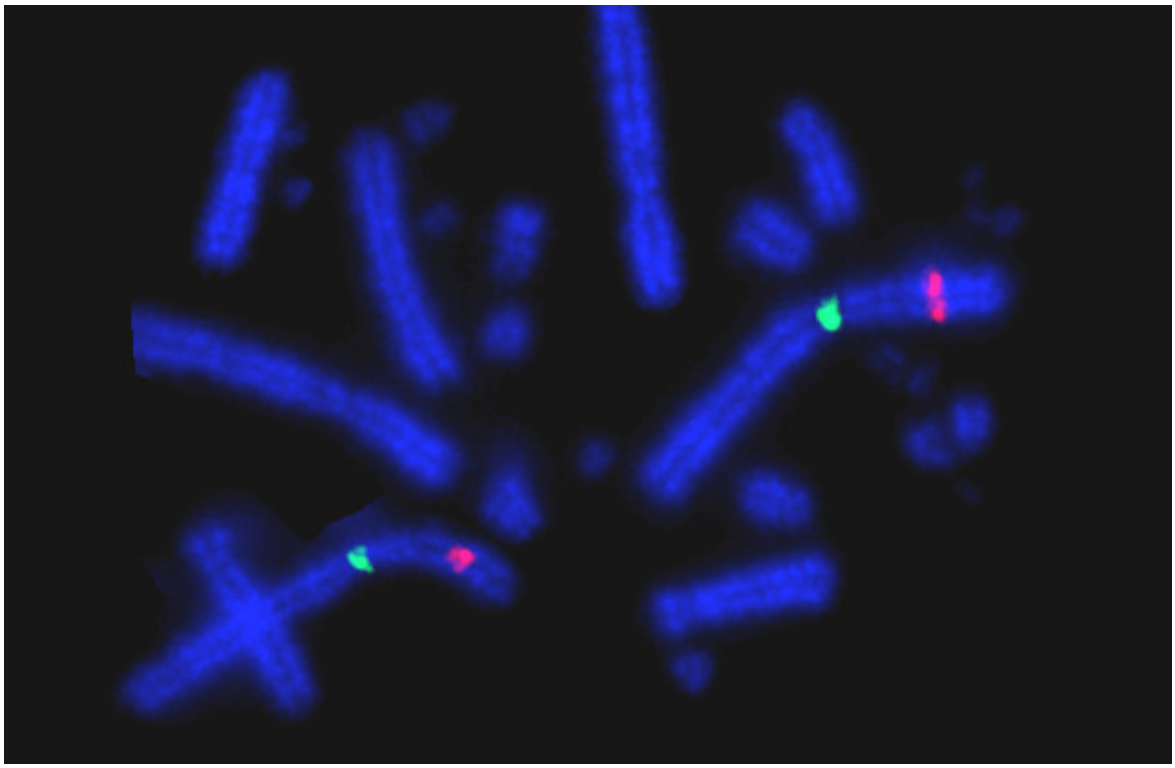


Figure 6.6 Fluorescent *in situ* hybridization with BACs containing chicken CD69 (red) and CD94 (green) genes produced distinct signals on chicken chromosome 1. CD69 mapped to 1p21-p14 and CD94 to 1q11, very close to the centromere.

Conclusion

A series of *in silico* analysis doesn't just provide the putative annotation to an unknown gene, but it also provides other implicated information for the further studying *in vitro* (or *in vivo*). Besides, applying multiple approaches on annotation work is necessary because the accumulated evidence from each different approach provide a more clear picture for us to define a novel gene, and also save us tons of time and money than processing it *in vitro*.

A study reported that 28 distinct C-type lectin-like receptor loci are identified in bony fish Oreochromis niloticus and most of them are located in a single chromosomal region named KLR complex, which is more compact compared to NK complex (NKC) in humans or mice (Kikuno et al. 2004). The finding of KLR complex in fish and the chicken C-type lectin-like receptors in the syntenic region of mammalian NKC indicate that this chromosomal region is primitive. The NKC is highly polymorphic and allelic variability of various NKC loci has been demonstrated to be associated with resistance to Malaria in mice (Hansen et al. 2005) and MCMV (Scalzo et al. 2005). Interestingly, this region was also found to be associated with resistance to avian coccidiosis and Marek's disease (Pifer et al. 2002; Zhu et al. 2003). Although previous C-type lectin-like NK receptors were identified in the chicken major histocompatibility complex (MHC) in chromosome 16 (Kaufman et al. 1999; Rogers et al. 2003; Rogers et al. 2005), our identifications of C-type lectin receptors in the chromosome 1 region provide new genetic information to study the association of these genes with disease resistance in chickens.

CHAPTER VII

SUMMARY

The selection of *Salmonella* resistance in chicken is one of the primary apporaches to prevent the outbreak of food-borne associated diseases in human. With the available chicken genome information and updated technologies, the study of genetic resistance in the chicken has been rapidly progressed in recent years. Although microarray offered great potential in providing information on elucidating genetic control mechanisms for disease resistance, it still require more follow-up data mining processes with different analysis to validate microarray results. By using SE pathogen as an example, the current dissertation demonstrated the applications of different genomic approaches for studying innate immune response to *Salmonella* infection in the chicken. The results from these studies are summarized below:

Microarray Analysis of Chicken Heterophils with *Salmonella* Enteritidis Infection

The results from this study indicate that higher expression of immune-related genes is more beneficial to enhance the host response against SE infection. The immune deficiency in the susceptible line is likely due to suppressed functions in recovering from cellular changes induced by SE infection. In the results of infection vs. non-infection comparison, the genes exclusively differentially expressed in the resistant (A) line or susceptible (B) line with SE infection has provided strong candidates for further investigation of disease resistance and susceptibility to SE infection in chickens, respectively. The identified immune-related genes also suggested a similar TLR

regulatory network might exist in both lines, where a possible MyD88-independent pathway may participate in the regulation of host innate immunity in line B. Finally, the MHC II system might be important to initiate T-helper cell activation for the host defense.

Analysis of Chicken Toll-like Receptors Pathway in Heterophils with *Salmonella* Enteritidis Infection

The results from this study indicated that the expression of adaptor genes in TLR pathway was involved in the different host response between heterophils from two genetically distinct broiler lines. The resistene line had more adaptors with up-regulated expression than susceptible line, and most differentially expressed adaptors were found involved in the downstream signaling cascades of TLR4-induced My88-dependent pathway.

The inferred chicken TLR pathway identified several groups of genes that had related-functions and co-expressed in consistent directions with SE infection. The signaling cascades involved in these genes provided potential biological inferences which controlled avian immune response against SE infection through TLR pathway.

Inhibition of NFkB1 (NFkBp50) by RNA Interference in Chicken Macrophage HD11 Cell Challenged with *Salmonella* Enteritidis

By RNA interference approach, the inhibition of target gene expressions on chicken GAPDH and NFkB could achieve 45% and 36% expression reduction compared to the negative (mock) control, respectively, without substantial general translational attenuation and biased effects.

With the inhibition of NFkB, the gene expression of IL6 was consistently and significantly increased at both 1 hour and 4 hours with *Salmonella* infection, whereas the gene expression of IL1b and TLR 15 were increased at 4 hours only. MyD88 was up-regulated by siNFkB treatment at 1 hour of Salmonella infection only.

The regulatory directions of siNFkB1 on cytokines were found similar to its effects on TLR genes. It is possible that NFkB1 can regulate the TLR signal transduction pathway as a potential inhibitory factor in chickens.

Chicken CD69 and CD94-like Genes in a Chromosomal Region Syntenic to Mammalian NKC Receptor Gene Complex

Two novel chicken C-type lectin-like receptors were identified in a region on Choromosome 1 that is syntenic to mammalian NKC region. Based on phylogenetic analysis, one receptor is highly homologous to mammalian CD69 and the other to CD94. Unlike mammalian NKC, these two chicken C-type lectin receptors are not closely linked but separated by 42 million bp according to the chicken draft genome sequence. The chicken NK C-type lectin like receptors in the NKC syntenic region indicate that this chromosomal region existed before the divergence between mammals and aves.

General Discussion

The main scope of this dissertation was to delineate genetic mechanisms involved in *Salmonella* resistance in chickens. The microarray study in heterophils indeed identified several genes and pathways that varied in the regulation between chicken lines with different susceptibility, and this data is largely confirmatory of previous work on innate responses with SE infection. It is noticed that the host resistance against

Salmonella was divided in two ways: resistance against systemic infection and resistance against carrier state. While the former induced noticeable immune response with *Salmonella* infection, the latter develops unnoticeable immune response unfavorable for *Salmonella* colonization. Since the RNA samples used for comparisons were obtained from heterophils of 1-wk old chickens whose immune system were not fully developed, the microarray studies described in current dissertation were focused on resistance against systemic infection. However, it would also be interesting to investigate the transformation of host immune response within a series time points, and to identify the connection between two types of host resistances. It is possible the resistance against chicken carrier state is more important to address food safety issue.

It was shown in our studies that TLR pathway plays an important role in mediating innate response of heterophils in chickens. The signaling transductions of TLR pathway were regulated in a complicated mechanism which may include both positive and negative regulations. It is noticed in the current study that most adaptor proteins in TLR pathway had subtle gene express on signal intensity, although the end products such as cytokines and chemokines were highly expressed. It may be more appropriate to think of these adaptors genes not as solo actors dedicated to a single mission, but rather as members in multiple teams. Therefore, the adaptation of biological networks to various stimuli might involve subtle adjustments in a large set of elements, rather than significant changes in a few selected members. Alternatively, proteomic assay might provide more insights to characterize the change of signaling transduction, since these changes may occur in the post-transcription level.

The siRNA technology has laid the foundation of using loss-of-function approach on validating gene-gene interactions, especially those involved in a complicated signaling pathway. It is shown in this dissertation that siRNA interference with chemical transfection has potential to be used on gene function studies. The transfected siRNA in cytoplasma can induce specific degradation of mRNA of target gene, which is more economical and feasible than using gene-knock out animal as a model. However, there are several limitations that could hamper the use of chemical-transfected siRNA, such as the cell toxicity of transfection reagent, low transfection efficiency and unstable siRNA potency. Above all, there is a lack of availability of commercial chicken antibodies to be used for the following-up quantification of reduced protein. The results shown in this dissertation also suggested that to achieve considerable knock-down efficiency (e.g. 60%~80%) of target mRNA will be the primary goal in the future. It is expected that with rapidly developing technology, the use of siRNA in chicken will become as mature as it is in mammals in the near future.

The general definition of bioinformatics is regarding all kinds of biological data processing using computers and networks (Xiong 2006). Nowadays bioinformatics has become an indispensible tool for molecular biology studies. As seen in this dissertation, bioinformatics plays a critical role in data mining of microarray studies such as: gene ontology analysis (functional analysis), pathway analysis and gene annotation for those unknown differentially expressed genes. Within them, the gene function annotation might be the most needed, since currently the coverage of gene annotations in a whole genome were still low for many of newly supported species including chicken. It is possible a number of biological insights are still unveiled in our array data due to the

imcomplete annotation for those differentially expressioned ESTs in the array results. Most unknown ESTs are either due to lack of the sufficient nucleotide length for homology searching by blast, or lack of distinct feature to be distinguished from other paraloges within the same gene family. Whereas these unknown ESTs are difficult to annotate by automatic programming, the manual annotation using multiple approaches are more suitable for the annotation.

In this dissertation, the identification of two C-type lectin proteins, chicken CD69 and CD94-like, have demonstrated that how to utilize manual annotation to integrate different consensus evidences into the inferred conclusion. Although there was no differential expression of these two chicken genes in our array results, the similar approach of annotating process would be useful in identifying other unknown genes of interests.

Selection for immune function has been very challenge for commercial breeding companies. It is increasing clear that improvements in one trait achieved by selective breeding are often associated with losses in other economically important traits. Immune response is only one of many traits that are under intensive selection, thus selection pressure needs to be carefully balanced across multiple traits. Although the use of marker-assisted selection has considerable value to overcome this limitation, the better

understanding of genetic differences in susceptibility and resistance to various diseases is needed before this information can be fully utilized by the poultry breeding industry.

It is noticed that the gene expression study in this dissertation are only focused on the change of transcriptions (mRNAs). The knowledge of these identified differential expressions in our study should be moved to DNA level, thereby, DNA marker can be utilized in poultry breeding for selecting the desired traits. It is known that the most effective markers are the functional mutations within the trait genes. The next challenge for molecular geneticists is to identify markers for candidate genes that control the phenotypic variation in the target traits.

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Chiang HI, Swaggerty CL, Kogut MH, Dowd SE, Li X, Pevzner IY, Zhou H. 2008. Gene Expression Profiling in Chicken Heterophils with *Salmonella* enteritidis Stimulation Using a Chicken 44K Agilent Microarray. BMC Genomics. 9: 526.

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