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Genetic lines differ in Toll-like receptor gene expression in spleens of chicks inoculated with Salmonella enterica serovar Enteritidis

B. Abasht
Iowa State University

Michael G. Kaiser
Iowa State University, mgkaiser@iastate.edu

J. van der Poel
Wageningen University

Susan J. Lamont
Iowa State University, sjlamont@iastate.edu

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Abstract

Toll-like receptors (TLR) recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns) of infectious microbes and initiate innate immune response upon activation with relevant pathogens. This study investigated the acute effect of *Salmonella* Enteritidis challenge on TLR mRNA expression in cecum and spleen of birds from 3 distinct genetic lines. Chicks from broiler, Leghorn, and Fayoumi lines were inoculated or mock-inoculated with *Salmonella* Enteritidis. The mRNA expression levels of TLR2, TLR4, and TLR5 genes were assessed by quantitative reverse transcription-PCR of cecum and spleen tissue harvested at 2 or 18 h postinoculation (PI). There were no significant genetic line effects on TLR mRNA expression in spleen or cecum of mock-infected birds, or in the cecum of infected birds. Genetic line effect was significant ($P < 0.05$) on TLR mRNA expression in the spleen of *Salmonella* Enteritidis- infected birds. The Fayoumi line had higher TLR2 and TLR4 expression than Leghorn, higher TLR2 mRNA expression than broiler, and the broiler line had higher TLR5 expression than Leghorn and Fayoumi. In *Salmonella* Enteritidis-infected birds, the TLR2 expression in both cecum and spleen and TLR4 expression in spleen were significantly higher at 18 h PI than 2 h PI. The results demonstrate a significant genetic line effect on TLR expression in the spleen of *Salmonella* Enteritidis-infected birds, which may partly explain genetic variability in immune response to *Salmonella* Enteritidis.

Keywords

chicken, Toll-like receptor, genetics, Salmonella Enteritidis

Disciplines

Agriculture | Animal Sciences | Genetics | Poultry or Avian Science

Comments

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Genetic lines differ in Toll-like receptor gene expression in spleens of chicks inoculated with *Salmonella enterica* serovar Enteritidis

B. Abasht,* M. G. Kaiser,* J. van der Poel,† and S. J. Lamont*[1]

*Department of Animal Science, Iowa State University, Ames 50011; and †Animal Breeding and Genetics Group, Wageningen University, 6700 AH, Wageningen, the Netherlands

ABSTRACT Toll-like receptors (TLR) recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns) of infectious microbes and initiate innate immune response upon activation with relevant pathogens. This study investigated the acute effect of *Salmonella* Enteritidis challenge on TLR mRNA expression in cecum and spleen of birds from 3 distinct genetic lines. Chicks from broiler, Leghorn, and Fayoumi lines were inoculated or mock-inoculated with *Salmonella* Enteritidis. The mRNA expression levels of TLR2, TLR4, and TLR5 genes were assessed by quantitative reverse transcription-PCR of cecum and spleen tissue harvested at 2 or 18 h postinoculation (PI). There were no significant genetic line effects on TLR mRNA expression in spleen or cecum of mock-infected birds, or in the cecum of infected birds. Genetic line effect was significant ($P < 0.05$) on TLR mRNA expression in the spleen of *Salmonella* Enteritidis-infected birds. The Fayoumi line had higher TLR2 and TLR4 expression than Leghorn, higher TLR2 mRNA expression than broiler, and the broiler line had higher TLR5 expression than Leghorn and Fayoumi. In *Salmonella* Enteritidis-infected birds, the TLR2 expression in both cecum and spleen and TLR4 expression in spleen were significantly higher at 18 h PI than 2 h PI. The results demonstrate a significant genetic line effect on TLR expression in the spleen of *Salmonella* Enteritidis-infected birds, which may partly explain genetic variability in immune response to *Salmonella* Enteritidis.

Key words: chicken, Toll-like receptor, genetics, *Salmonella* Enteritidis

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INTRODUCTION

Toll-like receptors (**TLR**) are members of a class of cellular receptors known as pattern-recognition receptors, which recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns) of infectious microbes. Activation of TLR with pathogen-associated molecular patterns can potentiate immune response by modulation of the innate and adaptive immune systems (Trinchieri and Sher, 2007). Recent structural and phylogenetic analyses by Temperley et al. (2008) confirmed the 10 TLR reported in chickens (Boyd et al., 2001; Fukui et al., 2001; Leveque et al., 2003; Lynn et al., 2003; Smith et al., 2004; Iqbal et al., 2005a,b; Philbin et al., 2005; Roach et al., 2005; Yilmaz et al., 2005; Higgs et al., 2006; Keestra et al., 2007) and found no other TLR. In vitro studies using chicken immune cells demonstrated that bacterial TLR agonists induce upregulation of mRNA expression of cytokines, nitric oxide production, and oxidative burst (Farnell et al., 2003; Bliss et al., 2005; Kogut et al., 2005a,b; He et al., 2006, 2007). However, there is limited information on the in vivo regulation of chicken TLR mRNA expression by TLR agonists, bacterial infection, or genetic line effect. Upregulation of TLR2 mRNA expression in spleen, TLR4 mRNA expression in both cecum and spleen, and downregulation of TLR5 RNA expression in cecum were previously shown after *Salmonella* Enteritidis infection in 2 advanced intercross lines (Abasht et al., 2008). In the same study, genetic line differences in TLR2 mRNA expression in spleen were observed between the Broiler × Fayoumi and the Broiler × Leghorn advanced intercross lines (Abasht et al., 2008). The current study was designed to determine whether the 3 foundation lines of the advanced intercrosses differ in TLR2, TLR4, and TLR5 mRNA expression levels in cecum and spleen tissue after infection with *Salmonella* Enteritidis. These specific TLR genes were selected because of their roles in recognizing bacterial components. The TLR2 recognizes lipoproteins and glycolipids, which are present in a variety of bacteria including gram-negative and gram-positive bacteria; TLR4 recognizes lipopolysaccharide from

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[1]Corresponding author: sjlamont@iastate.edu

gram-negative bacteria; and TLR5 recognizes flagella from both gram-negative and gram-positive bacteria (Akira et al., 2001).

MATERIALS AND METHODS

Experimental Animals, Salmonella Enteritidis Infection

One outbred broiler line originating from a broiler breeder male line and 2 highly inbred lines (inbreeding coefficient and genome homozygosity >99%), the Leghorn G-B2 and Fayoumi M 15.2 lines, were used (Zhou and Lamont, 1999; Abasht and Lamont, 2007). From each line, 24 chicks were equally divided into 2 animal biosafety level 2 animal rooms. At 1 d of age, as described previously (Kaiser and Lamont, 2001), the chicks in one room were intraesophageally inoculated with 1×10^4 cfu *Salmonella* Enteritidis in 0.25 mL of Luria-Bertani broth, whereas the chicks in the other room were mock-inoculated with 0.25 mL of Luria-Bertani broth.

At 2 or 18 h postinoculation (**PI**), chicks were killed by cervical dislocation. The spleen and 1 cecum from each chick were aseptically extracted and rinsed with sterile PBS and individually quick-frozen in liquid nitrogen. Contents of the cecum were removed before the freezing. Tissue samples were stored at −80°C until RNA extraction.

Real-Time Quantitative Reverse Transcription-PCR

Ribonucleic acid isolation and primer sequences for quantitative reverse transcription-PCR (**qRT-PCR**) and 1-step qRT-PCR of TLR2, TLR4, and TLR5 mRNA and 28S rRNA were as described previously (Abasht et al., 2008). Each qRT-PCR plate contained samples run in triplicate and a 10-fold serial dilution for the test gene standard. Data were adjusted for PCR efficiency and starting template amount with the following equations:

$$\text{Adjusted Ct value} = \{(40 - \text{Ct}_{\text{test gene}})/[(40 - \text{Ct}_{28S})/(40 - \text{Mean}_{28S})]\} \times (\text{Slope}_{\text{test gene}}/\text{Slope}_{28S})$$

where $\text{Ct}_{\text{test gene}}$ = mean of the triplicate cycle threshold (**Ct**) values of the gene being tested; Ct_{28S} = mean of the triplicate Ct value of the housekeeping gene 28S; Mean_{28S} = overall experimental mean of Ct_{28S}; $\text{Slope}_{\text{test gene}}$ = slope from 10-fold test gene standard regression equation; and Slope_{28S} = slope from 10-fold 28S standard regression equation (Kaiser et al., 2006; Abasht et al., 2008).

The adjusted Ct value was corrected for assay-specific PCR efficiency with the use of the slope ratio ($\text{Slope}_{\text{test gene}}/\text{Slope}_{28S}$) term of regression slope test gene-regression slope of 28S (Scott et al., 2008). The test gene standard was a PCR-amplified fragment containing the target segment, whereas the 28S standard was a pooled sample of multiple chicken RNA samples. The initial quantity of total RNA was normalized across samples by the correction of $[(40 - \text{Ct}_{28S})/(40 - \text{Mean}_{28S})]$ term (Kaiser et al., 2000).

Statistical Analysis

All analyses were performed using the GLM procedure of the JMP statistical program package (SAS Institute, 2005). Before the analyses, 2 adjusted Ct values outside of overall mean ± 4 SD were excluded (1 from TLR2 in mock-inoculated chicks and 1 from TLR5 in *Salmonella* Enteritidis-inoculated chicks from the cecum data set). Because an initial analysis with inoculation state in the model showed significant line effect on mRNA expression levels of TLR2, TLR4, and TLR5 in spleen; TLR4 in cecum; and nonsignificant *Salmonella* Enteritidis inoculation effect on mRNA expression of these genes in both organs, we separately analyzed the groups by inoculation state using the following model:

$$Y = \mu + \text{Line} + \text{PI Time} + \text{Sire (Line)} + \text{PCR Plate} + \text{Line} \times \text{PI Time}$$

where Y = adjusted Ct value; μ = population mean; Line = the effect of 3 different lines (broiler, Fayoumi, and Leghorn); PI Time = effect of PI sample time (2 or 18 h); and PCR Plate = PCR plate effect. Line and PI Time were treated as fixed effects; Sire (Line) and PCR plate effects were treated as random.

Two-way interactions of Line and PI Time effects were excluded if $P > 0.1$ for the interaction tests. Multiple comparisons of least squares (**LS**) means for line effects were performed by Tukey-Kramer honestly significant differences test of JMP (SAS Institute, 2005). Differences were considered significant at $P \leq 0.05$.

RESULTS

RNA Expression Levels

Spleen. In *Salmonella* Enteritidis-inoculated birds, but not in mock-inoculated birds, genetic line had a significant effect on TLR gene mRNA expression level in spleen (Table 1). The *Salmonella* Enteritidis-inoculated Fayoumi chicks had significantly higher spleen TLR2 mRNA expression level than the broilers and Leghorns, and expression level of broilers and Leghorns did not differ (LS means adjusted Ct value of 19.91, 20.48, and 21.14, respectively, in Leghorn, broiler, and Fayoumi lines, Figure 1). The *Salmonella* Enteritidis-inoculated Fayoumi chicks also had significantly higher spleen TLR4 mRNA expression level than the Leghorns; that of the broilers was intermediate and was not significantly different from the Leghorns and Fayoumis (LS means adjusted Ct value of 10.92, 12.65, and 13.18, re-

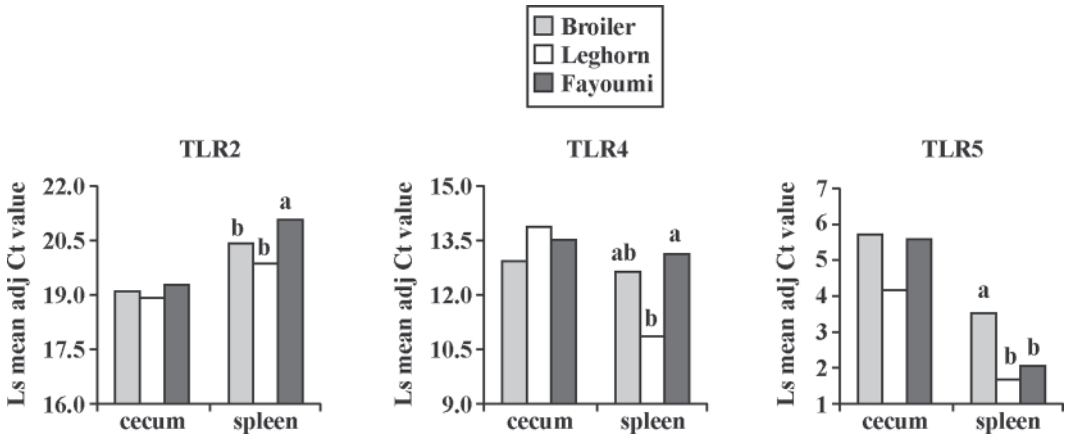

Figure 1. Line differences in Toll-like receptor (TLR)2, TLR4, and TLR5 mRNA expression level in cecum and spleen of *Salmonella* Enteritidis-inoculated birds, across 2 postinoculation times (2 and 18 h). Bars represent least squares (Ls) means of adjusted cycle threshold (adj Ct) values and error bars represent SE. Bars not sharing a common letter, within gene and tissue, are significantly different ($P < 0.05$, Tukey's test).

spectively, in Leghorn, broiler, and Fayoumi lines, Figure 1). The mRNA expression level of TLR5 was higher in spleen of *Salmonella* Enteritidis-inoculated broilers than in the Leghorns and Fayoumis, which did not differ from each other. (LS means adjusted Ct value of 1.75, 2.05, and 3.57, respectively, in Leghorn, Fayoumi, and broiler lines, Figure 1).

Fold changes showing the differences in gene expression in spleen, as a result of *Salmonella* Enteritidis inoculation compared with mock inoculation, are presented in Figure 2. The TLR5 mRNA expression was consistently decreased in the spleen of all 3 lines after infection with *Salmonella* Enteritidis, compared with mock-infected chicks. The mRNA expression levels of both TLR2 and TLR4 in spleen were increased in Fayoumis and decreased in Leghorns after *Salmonella* Enteritidis inoculation. In broilers, spleen TLR2 mRNA expression level was decreased and TLR4 was increased after *Salmonella* Enteritidis inoculation.

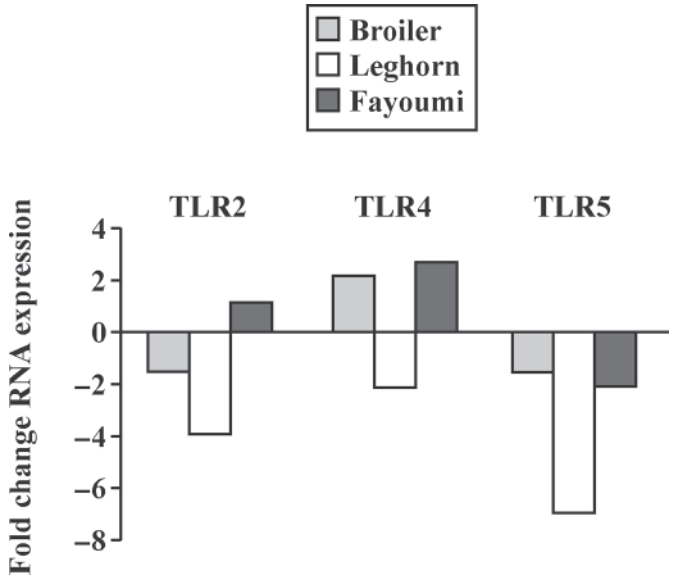

Figure 2. Fold changes in Toll-like receptor (TLR) mRNA expression in spleen of *Salmonella* Enteritidis-inoculated birds compared with mock-inoculated birds within 3 lines, across 2 postinoculation times (2 and 18 h).

There was no significant PI time effect on TLR2 and TLR4 mRNA expression levels in spleen of mock-inoculated birds or on TLR5 expression level in *Salmonella* Enteritidis-inoculated and mock-inoculated birds (Table 1). However, in the spleen of *Salmonella* Enteritidis-inoculated birds, both TLR2 and TLR4 mRNA expression levels were significantly higher at 18 h than 2 h PI time (LS means adjusted Ct value of 19.65 and 21.34, respectively, at 2 and18 h PI time for TLR2 and 11.30 and 13.06, respectively, at 2 and 18 h PI time for TLR4).

Random effects of sire and PCR plate on TLR mRNA expression were not significant in the spleen of either *Salmonella* Enteritidis-inoculated or mock-inoculated birds (data not shown).

Cecum. Genetic line had no significant effect on TLR2, TLR4, or TLR5 mRNA expression levels in cecum in either *Salmonella* Enteritidis-inoculated or mock-inoculated birds (Table 1). However, in *Salmonella* Enteritidis-inoculated chicks, the genetic line ranking for TLR mRNA expression levels in cecum generally agrees with those observed in spleen, except for the TLR4 Leghorn response (Figure 1).

The PI time had a significant effect only on TLR2 mRNA expression level in *Salmonella* Enteritidis-inoculated birds (Table 1), with higher expression levels at 18 h than 2 h PI (LS means adjusted Ct value of 18.32 and 20.32, respectively, at 2 and18 h PI time).

The random effects of sire and PCR plate on TLR mRNA expression were not significant in the cecum of either *Salmonella* Enteritidis-inoculated or mock-inoculated birds (data not shown).

DISCUSSION

Gene expression in response to bacterial infection can be the result of many factors that modulate the amount and timing of bacteria reaching each tissue or

Table 1. Line and postinoculation (PI) time effects on Toll-like receptor (TLR) mRNA expression in cecum and spleen of *Salmonella* Enteritidis-inoculated and mock-inoculated birds (P-value)[1]

	TLR2		TLR4		TLR5	
Item	Mock-inoculated	*Salmonella* Enteritidis-inoculated	Mock-inoculated	*Salmonella* Enteritidis-inoculated	Mock-inoculated	*Salmonella* Enteritidis-inoculated
Spleen						
Line	0.609	0.001	0.670	0.024	0.300	0.025
PI time	0.877	0.005	0.063	0.020	0.608	0.432
Cecum						
Line	0.821	0.765	0.371	0.239	0.626	0.082
PI time	0.089	0.001	0.434	0.268	0.297	0.880

[1]Two-way interactions of line and PI time effects were excluded from model if $P > 0.1$ for the interaction tests. P-values of line × PI time interaction were 0.073 and 0.054, respectively, for TLR2 and TLR4 RNA expression levels in the mock-inoculated group in cecum. In all other cases, P-values of the line × PI time interaction test were >0.10.

organ, and these factors may differ among individuals and genetic lines. The current study was designed to be an experimental model of a typical field challenge with *Salmonella*, which occurs at the time that chicks are first placed into the poultry house. Chicks, therefore, were orally inoculated with bacteria at 1 d of age to evaluate early gene expression in specific organs or tissues in the context of the biological environment of the whole host organism's complex response to the bacteria. Genetic line had a significant effect on early (2 to 18 h) TLR2, TLR4, and TLR5 mRNA expression in the spleen of *Salmonella* Enteritidis-inoculated birds in the present study. Previously, genetic line differences in TLR2 mRNA expression in spleen at 1 wk PI were observed between broiler × Fayoumi and broiler × Leghorn F8 advanced intercross lines, with higher TLR2 mRNA expression level in the broiler × Fayoumi cross (Abasht et al., 2008). Because the difference between the 2 F8 crosses is expected to be partly from the maternal inbred lines (Fayoumi or Leghorn), the higher spleen TLR2 mRNA level in the broiler × Fayoumi cross is in agreement with the higher spleen TLR2 in the Fayoumi line of the current study, even though mRNA expression was measured at different times PI.

Higher levels of cytokine (IL-6, IL-8, and IL-18) mRNA expression were observed in heterophils from *Salmonella* Enteritidis-resistant than *Salmonella* Enteritidis-susceptible lines after in vitro treatment with *Salmonella* Enteritidis (Swaggerty et al., 2004). Similarly, heterophils isolated from *Salmonella* Enteritidis-resistant chickens infected with *Salmonella* Enteritidis had higher levels of cytokine (IL-1β, IL-6, and IL-8) mRNA expression than susceptible chickens (Swaggerty et al., 2004). Wigley et al. (2006) reported that macrophages from a *Salmonella*-resistant chicken line had a rapid increase and greater levels of cytokine mRNA expression than a *Salmonella*-susceptible line, after in vitro challenge with *Salmonella* Typhimurium and *Salmonella* Gallinarum (Wigley et al., 2006). Higher cell-surface TLR4 expression in macrophages from the K-strain than from the Leghorn G-B2 line was reported and postulated as the reason for higher inducible nitric oxide synthase RNA expression and consequently higher nitric oxide production in lipopolysaccharide-treated macrophages from K-strain than from G-B2 chickens (Dil and Qureshi, 2002). The current study demonstrated that *Salmonella* Enteritidis-inoculated Fayoumi line chicks had higher TLR2 and TLR4 mRNA expression in the spleen than Leghorn chicks and higher TLR2 mRNA expression than broiler chicks, which may modulate antibacterial functions. However, cytokine (IL-1β, IL-6, CXCLi2, CCLi2, interferon-γ, IL-10, IL-12α, IL-12β, and IL-18) mRNA expression levels in the spleen of the Fayoumi line were not significantly higher than broiler and Leghorn lines (measured in *Salmonella* Enteritidis-inoculated and mock-inoculated birds at the same PI times as the current study; Cheeseman et al., 2007). Considering the important role of TLR2 and TLR4 in recognizing bacterial components and activating the immune response, the early higher level of TLR2 and TLR4 mRNA expression in the spleen of Fayoumi chicks inoculated with *Salmonella* Enteritidis in the current study may be associated with enhanced cytokine expression at a later PI time. This postulation is consistent with a previous study by Withanage et al. (2004), in which the inflammatory response (increase in IL-1β, IL-8, and macrophage inflammatory protein-1β) in spleen was observed only 48 h after PI with *Salmonella* Typhimurium.

In chickens, as in mammals and fishes, bacterial flagellin acts as a TLR5 agonist and upregulates cytokine RNA expression (Iqbal et al., 2005b). Chicken TLR5 recognizes and responds to *Salmonella* Enteritidis flagellin by activating nuclear factor-κB, a major cytokine expression regulator (Keestra et al., 2008). In the current study, *Salmonella* Enteritidis-inoculated Fayoumis and Leghorns had significantly lower spleen TLR5 RNA expression than broilers. The TLR5 mRNA expression in response to infection (fold change, *Salmonella* Enteritidis inoculation versus mock-inoculation) was decreased in the spleen of all 3 genetic lines. In a previous study, *Salmonella* Enteritidis infection was shown to downregulate TLR5 mRNA expression in the cecum (Abasht et al., 2008).

The current study profiled expression of TLR2, TLR4, and TLR5 mRNA in the cecum and spleen of day-old

chicks with and without *Salmonella* Enteritidis inoculation. Genetic line significantly affected TLR mRNA expression in the spleen of *Salmonella* Enteritidis-inoculated chicks. Because the focus of the current study was on very early (less than 24 h PI) gene expression changes, it was not possible to simultaneously evaluate correlations of TLR expression with bacterial colonization levels. A recent study, which extended observations to 1 wk PI, analyzed the correlation of TLR2, TLR4, and TLR5 mRNA expression with bacterial colonization level in the tested organs and found no significant correlation of gene expression and bacterial burden measured at the same time (Abasht et al., 2008). We hypothesize that the network of genes involved in controlling bacterial burden is complex in number and kinetics, and thus the simultaneous measurement of TLR gene expression and bacterial number may not be optimal for detecting the relationship of TLR expression and control of bacterial burden. This lack of correlation does not, however, eliminate the possibility that TLR expression changes may modulate *Salmonella* level at a subsequent time, through intermediate steps such as signal transduction.

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REFERENCES

Abasht, B., M. G. Kaiser, and S. J. Lamont. 2008. Toll-like receptor gene expression in cecum and spleen of advanced intercross line chicks infected with *Salmonella enterica* serovar Enteritidis. Vet. Immunol. Immunopathol. 123:314–323.

Abasht, B., and S. J. Lamont. 2007. Genome-wide association analysis reveals cryptic alleles as an important factor in heterosis for fatness in chicken F2 population. Anim. Genet. 38:491–498.

Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: Critical proteins linking innate and acquired immunity. Nat. Immunol. 2:675–680.

Bliss, T. W., J. E. Dohms, M. G. Emara, and C. L. Keeler Jr. 2005. Gene expression profiling of avian macrophage activation. Vet. Immunol. Immunopathol. 105:289–299.

Boyd, Y., M. Goodchild, S. Morroll, and N. Bumstead. 2001. Mapping of the chicken and mouse genes for toll-like receptor 2 (*TLR2*) to an evolutionarily conserved chromosomal segment. Immunogenetics 52:294–298.

Cheeseman, J. H., M. G. Kaiser, C. Ciraci, P. Kaiser, and S. J. Lamont. 2007. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without *Salmonella* Enteritidis infection. Dev. Comp. Immunol. 31:52–60.

Dil, N., and M. A. Qureshi. 2002. Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds. Vet. Immunol. Immunopathol. 84:191–207.

Farnell, M. B., T. L. Crippen, H. He, C. L. Swaggerty, and M. H. Kogut. 2003. Oxidative burst mediated by toll like receptors (TLR) and CD14 on avian heterophils stimulated with bacterial toll agonists. Dev. Comp. Immunol. 27:423–429.

Fukui, A., N. Inoue, M. Matsumoto, M. Nomura, K. Yamada, Y. Matsuda, K. Toyoshima, and T. Seya. 2001. Molecular cloning and functional characterization of chicken Toll-like receptors. A single chicken Toll covers multiple molecular patterns. J. Biol. Chem. 276:47143–47149.

He, H., K. J. Genovese, D. J. Nisbet, and M. H. Kogut. 2006. Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes. Mol. Immunol. 43:783–789.

He, H., K. J. Genovese, D. J. Nisbet, and M. H. Kogut. 2007. Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. Mol. Immunol. 44:3234–3242.

Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk, and C. O'Farrelly. 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infect. Immun. 74:1692–1698.

Iqbal, M., V. J. Philbin, and A. L. Smith. 2005a. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. Vet. Immunol. Immunopathol. 104:117–127.

Iqbal, M., V. J. Philbin, G. S. Withanage, P. Wigley, R. K. Beal, M. J. Goodchild, P. Barrow, I. McConnell, D. J. Maskell, J. Young, N. Bumstead, Y. Boyd, and A. L. Smith. 2005b. Identification and functional characterization of chicken Toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar Typhimurium. Infect. Immun. 73:2344–2350.

Kaiser, M. G., J. H. Cheeseman, P. Kaiser, and S. J. Lamont. 2006. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to *Salmonella enterica* serovar Enteritidis. Poult. Sci. 85:1907–1911.

Kaiser, M. G., and S. J. Lamont. 2001. Genetic line differences in survival and pathogen load in young layer chicks after *Salmonella enterica* serovar Enteritidis exposure. Poult. Sci. 80:1105–1108.

Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Gallinarum. Microbiology 146:3217–3226.

Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten. 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J. Immunol. 178:7110–7119.

Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten. 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol. Immunol. 45:1298–1307.

Kogut, M. H., H. He, and P. Kaiser. 2005a. Lipopolysaccharide binding protein/CD14/TLR4-dependent recognition of *Salmonella* LPS induces the functional activation of chicken heterophils and up-regulation of pro-inflammatory cytokine and chemokine gene expression in these cells. Anim. Biotechnol. 16:165–181.

Kogut, M. H., M. Iqbal, H. He, V. Philbin, P. Kaiser, and A. Smith. 2005b. Expression and function of Toll-like receptors in chicken heterophils. Dev. Comp. Immunol. 29:791–807.

Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow, J. C. Loredo-Osti, K. Morgan, and D. Malo. 2003. Allelic variation in *TLR4* is linked to susceptibility to *Salmonella enterica* serovar Typhimurium infection in chickens. Infect. Immun. 71:1116–1124.

Lynn, D. J., A. T. Lloyd, and C. O'Farrelly. 2003. In silico identification of components of the Toll-like receptor (TLR) signaling pathway in clustered chicken expressed sequence tags (ESTs). Vet. Immunol. Immunopathol. 93:177–184.

Philbin, V. J., M. Iqbal, Y. Boyd, M. J. Goodchild, R. K. Beal, N. Bumstead, J. Young, and A. L. Smith. 2005. Identification and characterization of a functional, alternatively spliced Toll-like re-

ceptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology 114:507–521.

Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem. 2005. The evolution of vertebrate Toll-like receptors. Proc. Natl. Acad. Sci. USA 102:9577–9582.

SAS Institute. 2005. JMP User Guide. Release 6. SAS Institute Inc., Cary, NC.

Scott, V. L., S. C. Burgess, L. A. Shack, N. N. Lockett, and K. S. Coats. 2008. Expression of CD134 and CXCR4 mRNA in term placentas of FIV-infected and control cats. Vet. Immunol. Immunopathol. 123:90–96.

Smith, J., D. Speed, A. S. Law, E. J. Glass, and D. W. Burt. 2004. In-silico identification of chicken immune-related genes. Immunogenetics 56:122–133.

Swaggerty, C. L., M. H. Kogut, P. J. Ferro, L. Rothwell, I. Y. Pevzner, and P. Kaiser. 2004. Differential cytokine mRNA expression in heterophils isolated from *Salmonella*-resistant and -susceptible chickens. Immunology 113:139–148.

Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt. 2008. Evolution of the chicken Toll-like receptor gene family: A story of gene gain and gene loss. BMC Genomics 9:62.

Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. Nat. Rev. Immunol. 7:179–190.

Wigley, P., S. Hulme, L. Rothwell, N. Bumstead, P. Kaiser, and P. Barrow. 2006. Macrophages isolated from chickens genetically resistant or susceptible to systemic salmonellosis show magnitudinal and temporal differential expression of cytokines and chemokines following *Salmonella enterica* challenge. Infect. Immun. 74:1425–1430.

Withanage, G. S., P. Kaiser, P. Wigley, C. Powers, P. Mastroeni, H. Brooks, P. Barrow, A. Smith, D. Maskell, and I. McConnell. 2004. Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with *Salmonella enterica* serovar Typhimurium. Infect. Immun. 72:2152–2159.

Yilmaz, A., S. Shen, D. L. Adelson, S. Xavier, and J. J. Zhu. 2005. Identification and sequence analysis of chicken Toll-like receptors. Immunogenetics 56:743–753.

Zhou, H., and S. J. Lamont. 1999. Genetic characterization of biodiversity in highly inbred chicken lines by microsatellite markers. Anim. Genet. 30:256–264.