Epigenetic Modification of TLRs in Leukocytes Is Associated with Increased Susceptibility to *Salmonella enteritidis* in Chickens

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### Abstract

Toll-like receptors (TLRs) signaling pathways are the first lines in defense against *Salmonella enteritidis* (*S. enteritidis*) infection but the molecular mechanism underlying susceptibility to *S. enteritidis* infection in chicken remains unclear. SPF chickens injected with *S. enteritidis* were partitioned into two groups, one consisted of those from Salmonella-susceptible chickens (died within 5 d after injection, n = 6), the other consisted of six Salmonella-resistant chickens that survived for 15 d after injection. The present study shows that the bacterial load in susceptible chickens was significantly higher than that in resistant chickens and TLR4, TLR2-1 and TLR21 expression was strongly diminished in the leukocytes of susceptible chickens compared with those of resistant chickens. The induction of expression of pro-inflammatory cytokine genes, IL-6 and IFN-β, was greatly enhanced in the resistant but not in susceptible chickens. Contrasting with the reduced expression of TLR genes, those of the zinc finger protein 493 (ZNF493) gene and Toll-interacting protein (TOLLIP) gene were enhanced in the susceptible chickens. Finally, the expression of TLR4 in peripheral blood mononuclear cells (PBMCs) infected in *vitro* with *S. enteritidis* increased significantly as a result of treatment with 5-Aza-2-deoxycytidine (5-Aza-dc) while either 5-Aza-dc or trichostatin A was effective in up-regulating the expression of TLR21 and TLR2-1. DNA methylation, in the predicted promoter region of TLR4 and TLR21 genes, and an exonic CpG island of the TLR2-1 gene was significantly higher in the susceptible chickens than in resistant chickens. Taken together, the results demonstrate that ZNF493-related epigenetic modification in leukocytes probably accounts for increased susceptibility to *S. enteritidis* in chickens by diminishing the expression and response of TLR4, TLR21 and TLR2-1.

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### Introduction

Toll-like receptors (TLRs) signaling pathways are the first lines in defense against *Salmonella* infection. The TLRs are broadly distributed on a variety of leukocytes [1], where they function as the primary sensors to initiate innate immune responses by responding to pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, fungi or parasites [2,3,4]. The transcription factor NF-κB [5] is subsequently activated to induce the expression of immune and pro-inflammatory genes such as tumor necrosis factor alpha (TNF), interleukins 6, 1 beta, 8 and 12 (IL-6, IL-1β, IL-8, IL-12), and interferon (IFN), etc. [6,7,8].

Four TLRs (TLR4, TLR2, TLR9 and TLR5) are responsible for recognition of antigens from *S. enteritidis* in humans and mice. The dominant TLR involved in the host response to *Salmonella* infection is TLR4 [9]. Mutations in the TLR4 gene increase the risk of Gram-negative infections in humans and mice [10,11,12] and mice deficient in both TLR4 and TLR2, or TLR4 and TLR9, were highly susceptible to *Salmonella typhimurium* [13]. Several specific avian TLR genes have been described. Avian TLR2A (TLR2-1) and TLR2B (TLR2-2) seem to have arisen from a duplication of TLR2 found in other vertebrates [14] and avian TLR21 is a functional homolog of mammalian TLR9 [15].

Signaling pathways mediated by TLRs are tightly regulated to balance the activation and inhibition of inflammatory responses [16]. Multiple layers, involving many diverse factors, participate in negative regulation of TLR signaling. For example, suppressor of cytokine signaling 1 (SOCS1), phosphatidylinositol 3 kinase (PI3K), toll interacting protein (TOLLIP), and zinc finger protein A20 (A20) are intracellular negative regulators suppressing the signaling of TLR2, TLR4 and TLR9 in multiple pathways [17]. Transcriptional regulation of TLRs can also influence the inflammatory responses. In the clinical course of cystic fibrosis (CF), increased expression of TLR2 caused chronic inflammation [18]. Diminished expression and function of TLR1, TLR2 and TLR4 accounts for T cell hyporesponsiveness in human filarial infection [19].
Little is known about the underlying mechanisms of transcriptional regulation of TLRs beyond ZNF160-dependent epigenetic regulation decreasing the expression of TLR4 in intestinal epithelial cells [20,21,22]. While the ZNF160 gene has not been identified in chicken, a Blastn search identified an avian homolog (ZNF493). Whether or not the same mechanism plays a role in modulating the immune response of the host to S. enteritidis infection remains unclear. Hypermethylation of promoter CpG dinucleotides has been associated with decreased expression of the gene [23,24]. Some reports have indicated that methylation status of exonic CpG islands correlates with transcriptional activity [25]. In order to analyze the regulatory mechanism of TLRs, the methylation status in the promoter region and exonic CpG islands of TLRs were investigated.

Chickens are carriers of S. enteritidis that colonize the alimentary tract of chickens and, through excrement, can contaminate food products and water [26]. It was considered to be important to delineate part of the molecular mechanisms underlying differences in susceptibility of chickens to infection with S. enteritidis. The present study confirmed that the aberrant expression of TLR4, TLR2-1, and TLR2-1 in peripheral blood leukocytes was associated with the susceptibility to S. enteritidis infection in chickens. More interestingly, it was demonstrated that the dysregulation of TLR4, TLR2-1, and TLR2-1 was probably due to ZNF493-related epigenetic modification, including histone acetylation and DNA methylation.

**Results**

**Increased bacterial load in susceptible chickens**

The bacterial load in the blood at 0 h (before bacteria challenge), 8 h, 16 h, 24 h and 3 d post infection (TPI) were compared in six chickens that died within 5 d after infection with S. enteritidis (susceptible group) and six chickens that survived until 15 d TPI (resistant group). Results are presented in Table 1. S. enteritidis was not detected in any of the samples until 8 h TPI. From 16 h to 3 d TPI, the number of S. enteritidis in susceptible chickens was significantly higher (P<0.05) than that in resistant chickens. Notably, the bacterial load in susceptible chickens increased more dramatically at 16 h TPI and declined less significantly at 3 d TPI than that in resistant chickens. The results indicate that increased bacterial load is associated with susceptibility to S. enteritidis in chickens.

**Decreased expression of TLR4, TLR2-1 and TLR2-1 genes in susceptible chickens**

In order to explore the molecular mechanisms of susceptibility to S. enteritidis infection, the expression levels of Toll-like receptors (TLRs) were examined in susceptible chickens. The abundance of TLR4, TLR2-1, TLR2-1 and TLR2-2, and transcripts and changes at all times post-inoculation were compared by q-RT-PCR in susceptible and resistant chickens. There were no significant differences in the expression of TLRs at 0 h (data not shown) and 9 h TPI between these two groups (Fig. 1), but, at later times, susceptible chickens had depressed expression of TLRs genes compared with resistant chickens. This was most evident at 16 h TPI, when TLR4, TLR2-1 and TLR2-1 transcripts were all significantly lower in the susceptible group than in the resistant group. Only TLR2-2 mRNA did not differ between the two groups across all sampling times, whereas TLR4 expression in resistant chickens was persistently and significantly higher than in susceptible chickens from 16 h to 3 d (Fig. 1). The results suggest that higher susceptibility to S. enteritidis and increased bacterial load might result from depressed expression of TLR4, TLR2-1 and TLR2-1 at the early stage of infection.

**Partially diminished inflammatory response in susceptible chickens**

Four pro-inflammatory cytokine genes (IL-6, IFN-β, TNF-α, and IL-8) were used to investigate if the decreased expression of TLR4, TLR2-1 and TLR2-1 at 16 h in susceptible chickens resulted in a mitigated inflammatory response. Consistent with the expression of TLRs in the resistant and susceptible groups, the induction of IL-6 and IFN-β transcription was greatly enhanced in the resistant, but not in the susceptible chickens at 16 h post-infection (Fig. 2). These results indicate that diminished expression of TLR4, TLR2-1 and TLR2-1 in the susceptible chickens leads to a decreased inflammatory response. The similar levels of IL-8 in both groups demonstrated that only some of the pro-inflammatory cytokines showed down-regulation in susceptible chickens, perhaps because IL-8 was regulated by other TLRs. In contrast, there was no obvious difference in the expression of TNF-α at 16 h, indicating that not all pro-inflammatory cytokine genes are induced at this early stage of infection (Fig. 2). Collectively, the results were consistent with higher susceptibility to S. enteritidis in birds being due to the partially diminished inflammatory response associated with decreased expression of TLR4, TLR2-1 and TLR2-1.

**Enhanced expression of TOLLIP and ZNF493 genes in susceptible chickens**

An attempt was then made to identify the molecular mechanisms responsible for decreased expression of TLR4, TLR2-1 and TLR2-1 in the susceptible chickens. The expression of four negative regulators of TLR2, TLR4 and TLR2-1 signaling pathways (TOLLIP, PI3K, SOCS1 and ZNF493, a chicken homolog of mammalian ZNF160) was compared between susceptible and resistant chickens at 8 h and 16 h. There were no differences (P>0.05) between susceptible and resistant chickens in expression of TOLLIP, PI3K, SOCS1 and ZNF493 before infection or at 8 h TPI, when expression was increased in all birds. At 16 h, however, expression of TOLLIP and ZNF493 in susceptible chickens was pronounced and exceeded that in the resistant chickens (P<0.01), while the other genes were up-regulated to lesser degrees and there were no differences between the two groups of chickens. Note the substantial increase in ZNF493 transcripts between 8 h and 16 h in the susceptible chickens when those in resistant chickens changed in the opposite direction (Fig. 3).

**Table 1. Kinetics of Salmonella Enteritidis loads in inoculated SPF chickens determined by qPCR across all the times.**

|          | 0 h  | 8 h  | 16 h | 24 h | 3 d  | 12 d |
|----------|------|------|------|------|------|------|
| S        | 0.00±0.00 | 6.35±0.32 | 7.05±0.23 | 6.96±0.06 | 6.87±0.21 |
| R        | 0.00±0.00 | 6.30±0.08 | 6.37±0.59 | 6.49±0.10 | 5.75±0.32 | 6.09±0.17 |
| P value  | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |

Data are presented as the mean bacterial loads and is expressed as log10 of the bacterial genome copy number per ml of blood (± SD) obtained from susceptible (S) and resistant (R) chickens.

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DNA methyltransferase inhibitor 5-Aza-dc and/or the histone deacetylase inhibitor TSA increased expression of TLR4, TLR2-1 and TLR2-1

The possibility that TLR4, TLR2-1 and TLR2-1 gene expression was regulated by epigenetic modification (histone acetylation and/or DNA methylation) in leukocytes infected with S. enteritidis was
examined. Isolated PBMCs from SFP chickens were infected with *S. enteritidis* in the absence and presence of combinations of 5-Aza and TSA in the culture media. As shown in Fig. 4, 5-Aza-dc provoked a significant increase in TLR4 expression. Either 5-Aza-dc or TSA was effective in up-regulating the expression of TLR21 and TLR2-1; the effect of 5-Aza-dc was greater in the case of TLR21 and that of TSA was greater for TLR2-1. No cooperative effects of 5-Aza-dc and TSA on the expression of the genes were observed. These results indicate that histone acetylation and DNA methylation are involved in the repression of TLR4, TLR21 and TLR2-1 expression in PBMCs of chickens during *S. enteritidis* infection.

Higher methylation in the predicted promoter region of TLR4 and TLR21 gene, and an exonic CpG island of TLR2-1 gene in susceptible chickens

The possibility that diminished expression of TLR4, TLR21 and TLR2-1 at 16 h TPI might be due to differences in methylation was explored at multiple locations within each of these genes, using leukocyte DNA at 0 h and 16 h TPI. For both TLR4 (Fig. 5A) and TLR21 (Fig. 6A), 15 CpG motifs in the predicted promoter regions were assessed. In the case of TLR2-1, 10 CpG motifs within the promoter and 18 CpG motifs in an exonic CpG island (Fig. 7A) were evaluated. There were no differences (P>0.05) in the methylation of TLR4, TLR21 and TLR2-1 genes between the susceptible and resistant chickens at 0 h (data not shown), and the average methylation level of all the 12 chickens before infection is shown as the basic methylation status. Interestingly, the average methylation levels of TLR4 and TLR21 at 16 h rose dramatically from the basic level at 0 h in susceptible chickens whereas it fell slightly (around 1%) in resistant chickens. Thus, higher methylation in the predicted promoter region of the TLR4 and TLR21 genes, was evident in susceptible versus resistant chickens at 16 h (Fig. 5B, Fig. 6B). This trend was also evident in several CpG sites (5 sites for TLR4, 7 for TLR21). No significant differences were observed in the promoter region of the TLR2-1 gene between these two groups at 16 h (data not shown), but an exonic CpG island of the TLR2-1 gene showed higher methylation.

Figure 1. Decreased expression of TLR4, TLR21 and TLR2-1 genes in susceptible chickens. The relative expression of TLR4, TLR21, TLR2-1 and TLR2-2 in leukocytes of susceptible (□——□) and resistant (●——●) chickens at 8 h, 16 h, 24 h, 3 d, and 12 d after infection with *S. enteritidis* is shown. Relative values, normalized using β-actin mRNA levels and the average expression levels in both groups at 0 h, are shown. The data are means (SD shown by the vertical bars) of 6 birds (*P<0.05; **P<0.01). TPI is time post-infection. doi:10.1371/journal.pone.0033627.g001

Figure 2. Partially diminished inflammatory response in susceptible chickens. The relative expression of IL-6, IFN-β, TNF-α and IL-8 in susceptible (open bars) and resistant (filled bars) chickens at 8 h and 16 h after infection with *S. enteritidis* is shown. Data are means (n = 6), normalized to β-actin mRNA and the average expression at 0 h (**P<0.01). The vertical bar is the SD from the error mean square of the ANOVA. HPI is hours post-infection. doi:10.1371/journal.pone.0033627.g002
level in susceptible than resistant chickens, although the difference was not as great as that in TLR4 and TLR21 (Fig. 7B).

Collectively, the results presented here show that diminished expression and response of TLR4, TLR21 and TLR2-1 in peripheral blood leukocytes, due to epigenetic modification, likely account for increased susceptibility to S. enteritidis in chickens.

**Discussion**

Although the physiological importance of transcriptional regulation of TLRs is unclear, several reports indicate that it directly influences the immune response of the host. The expression of TLRs, specifically TLR2 and TLR4, is induced by various PAMPs from bacteria, viruses, fungi or parasites for inflammatory responses in macrophages, epithelia, cecum and spleen [27,28,29]. Dysregulated expression of TLRs can impair the immune response of the host, resulting in various diseases. In the clinical course of cystic fibrosis (CF), dysregulated expression of TLR2 caused chronic inflammation [18]. Diminished expression and function of TLR1, TLR2 and TLR4 accounts for T cell hyporesponsiveness in human filarial infection [19]. The fact that various expression patterns of TLRs appear in tissues with different immune responses and function demonstrates the important role of transcriptional regulation of TLRs in the signaling of TLRs. For example, in enterocytes, depressed expression of TLR4 contributes to maintenance of intestinal homeostasis [22]. The downregulation of TLR5 expression was observed in cecum by S. enteritidis infection, which might be beneficial to protect host cells from overstimulation by bacterial flagellin [29]. In addition, genetic line has significant effect on TLR expression, which may partly explain genetic variability in immune response to S. enteritidis [30].

While not previously described for S. enteritidis infection, epigenetic regulation of TLR4 and TLR21 involving ZNF493 in chickens, participates in the negative regulation of TLRs. The avian ZNF493 examined here (and the mammalian homolog ZNF160) are Kruppel-related zinc finger proteins with an N-terminal repressor domain, the Kruppel associated box (KRAB), a potent repressor of transcription [31]. The mechanism involves recruiting KRAB-associated protein 1 (KAP1), triggering de novo DNA methylation [32], and the forming of a multimolecular complex comprising histone deacetylases, which induces transcriptional repression through the formation of heterochromatin [33,34,35].

The present study shows a dramatic enhancement of ZNF493 expression in susceptible chickens at 16 h, contrasting with diminished expression of TLR4 and TLR21 (Fig. 3). This finding prompted the experiment using chicken PBMCs infected with S. enteritidis in vitro, which demonstrated that expression of TLR4 and TLR21 was significantly promoted by inhibitors of DNA methyltransferase and histone deacetylase (Fig. 4). In addition, the susceptible, but not the resistant, chickens had increased methylation of TLR4 and TLR21 genes at 16 h compared with their basal levels at 0 h, which is consistent with the increased expression of ZNF493 in the susceptible chickens at 16 h (Fig. 5, Fig. 6). All of these findings indicate that ZNF493-related epigenetic regulation of TLR4 and TLR21 in leukocytes plays a role in the negative regulation of TLRs in chickens. Two possibilities might explain the differences at the transcriptional level of ZNF493 gene in susceptible and resistant chickens: (1) polymorphisms in the regulatory regions, including promoter of the ZNF493 gene; (2) polymorphisms of regulatory genes of the ZNF493 gene. White Leghorn chickens are known to have genetic variability in resistance to S. enteritidis among different strains [36]. The SPF chicken used in the present study is a commercial Babcock® White Leghorn line, which is very likely to have multiple genetic origins and genetic variability in susceptibility to

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**Figure 3. Enhanced expression of TOLLIP and ZNF493 genes in susceptible chickens.** The relative expression of TOLLIP, PI3K, SOCS1 and ZNF493 genes in susceptible (open bars) and resistant (filled bars) chickens at 8 h and 16 h after infection with S. enteritidis is shown. Data are means (n=6), normalized to β-actin mRNA and the average expression at 0 h (**P<0.01). The vertical bar is the SD from the error mean square of the ANOVA. HPI is hours post-infection. doi:10.1371/journal.pone.0033627.g003

**Figure 4. DNA methyltransferase inhibitor 5-Aza-dc and/or the histone deacetylase inhibitor TSA increased TLR4, TLR21 and TLR2-1 expression.** Peripheral blood mononuclear cells were incubated with S. enteritidis without additions (controls) or in the presence of 5-Aza-dc, TSA or TSA plus 5-Aza-dc. Relative abundances of TLR4, TLR21 and TLR2-1 mRNA were analyzed by qPCR and normalized to β-actin mRNA. Data are means (n=3) and comparisons were made to expression in the controls (- -). The vertical bar is the SD from the error mean square of the ANOVA, * indicates P<0.05. doi:10.1371/journal.pone.0033627.g004
between susceptible and resistant chickens. The average of % methylation at each CpG site within all 15 CpGs in peripheral blood leukocytes of uninfected chicken and 6 birds for susceptible and resistant chickens, respectively) are shown and comparisons were made means of 12 birds for uninfected chicken and 6 birds for susceptible and resistant chickens, respectively. The frequency of methylated CpGs in each CpG site (data are dinucleotides from $-2443$ to $-1361$ in the upstream region of the $\text{TLR4}$ gene relative to the translation start site (+1)). (B) Genomic DNA from peripheral blood leukocytes of uninfected chickens at 0 h (−), susceptible (□) and resistant (●) chickens at 16 h TPI was modified with sodium bisulfite, amplified by PCR, cloned, and 12–16 independent clones were sequenced. The frequency of methylated CpGs in each CpG site (data are means of 12 birds for uninfected chicken and 6 birds for susceptible and resistant chickens, respectively) are shown and comparisons were made between susceptible and resistant chickens. The average of % methylation at each CpG site within all 15 CpGs in peripheral blood leukocytes of uncharged chickens (0 h, filled grey bars), susceptible (S, open bars) and resistant (R, filled black bars) chickens at 16 h after infection with $\text{S. enteritidis}$ are presented. The vertical bar is the SD from the error mean square of the ANOVA, * indicates $P<0.05$, ** indicates $P<0.01$.

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$\text{S. enteritidis}$. The present study, however, shows that no polymorphisms in the promoter region of avian $\text{ZNF493}$ gene were detected in susceptible and resistant chickens (data not shown). It implies that diminished expression of $\text{ZNF493}$ gene might result from the polymorphisms of its regulatory genes or other regulatory regions (introns, 3′-UTR…).

There is little known about the overall transcriptional regulatory mechanism of $\text{TLR}$s. Based on the known reports, it can be inferred that positive transcriptional regulation of $\text{TLR}$ by cytokines to augment $\text{TLR}$ signaling and negative feedback control from negative regulators to terminate activation of $\text{TLR}$s are the basic mechanisms of transcriptional regulation of $\text{TLR}$s [17,37,38]. Moreover, transcriptional regulation of $\text{TLR}$s varies in different tissues, indicating that tissue-specific genes modify the regulatory system [22,29]. In addition, the pathogen probably can also exploit and modulate the regulatory system, disturbing the regulatory system [22,29]. In the present study, the expression of $\text{TLR}$s showed a common trend in obviously rising to the maximal level at around 3 d, followed by a fall by 12 d (Fig. 1). This trend indicates positive regulation by cytokines played a role in the initial upregulation stage and negative feedback control in the later downregulation stage. The epigenetic modification of $\text{TLR}$s in this study seems to be driven by two opposite mechanism, methylation and demethylation, depending on the particular $\text{TLR}$, $\text{TLR4}$ and $\text{TLR21}$, but not $\text{TLR2-1}$ showed an obvious downregulation and increase in methylation level in susceptible chickens at 16 h TPI, which probably resulted from $\text{ZNF493}$-related negative epigenetic modification. For all the three genes, the abundance of mRNA increased significantly compared with that at 0 h and the methylation level in resistant chickens similarly declined slightly from the basic level (Fig. 5, Fig. 6, Fig. 7), indicating that demethylation was widely involved in the regulation of $\text{TLR}$s. This demethylation happened in resistant chickens with higher expression of inflammatory proinflammatory cytokines, indicating that it could be one of the positive regulatory mechanisms of the cytokines.

The role for $\text{ZNF493}$-related epigenetic regulation of $\text{TLR}$s in the response to infection with $\text{S. enteritidis}$, however, seems to be quite different from the basic negative regulatory mechanism of the $\text{TLR}$ signaling pathway. Immune signaling pathways mediated by $\text{TLR}$s are tightly regulated to avoid over-activation of inflammatory responses and most negative regulators use a mode of negative feedback to terminate $\text{TLR}$ activation. They are induced by the activation of $\text{TLR}$s, or are constitutively expressed, but could possibly exert their functions only when $\text{TLR}$s are over-activated [41,42]. Since the diminished expression of $\text{TLR}$s and induction of $\text{ZNF493}$ in the susceptible chickens occurred at the early stage of $\text{S. enteritidis}$ invasion when induction of inflammatory response genes was even lower than in the resistant chickens (Fig. 2), and expression of $\text{TLR4}$, $\text{TLR21}$ and $\text{TLR2-1}$ remained at low levels (Fig. 1), it is not reasonable to account for the induction of $\text{ZNF493}$ by negative feedback control from the host and, instead, it might have been provoked by $\text{S. enteritidis}$. $\text{S. enteritidis}$ almost certainly benefits from the diminished expression of $\text{TLR4}$, $\text{TLR21}$ and $\text{TLR2-1}$ for its successful invasion and colonization of the susceptible host. Indeed, $\text{S. enteritidis}$ secretes virulence factors
to temporarily turn off TLR signaling to aid in colonization of host cells by inactivating IRAK, a kinase in the signaling pathway [39,40].

All of the findings described here, comparing blood bacterial load, transcript abundance and DNA methylation in leukocytes of susceptible and resistant chickens, along with the effects of inhibitors for epigenetic modification on transcript abundance in isolated PBMCs, are consistent with *S. enteritidis* being able to provoke epigenetic regulation of the transcription of TLR4, TLR21 and TLR2-1 as an important strategy for weakening host defenses.

**Materials and Methods**

**Bacterial Strains and Infections**

*S. enteritidis* (50041) was obtained from the China Institute of Veterinary Drugs Control (IVDC, Beijing, China) and was used for all infections. Bacteria were resuscitated overnight in Luria–Bertani (LB) broth at 37°C in an orbital shaking incubator at 150 rpm. The number of CFU of *S. enteritidis* was determined by plating serial dilutions.

**SPF Chickens and In Vivo Infections**

Animal studies were performed according to protocols approved by the Beijing Laboratory Animal Use and Care office (approval number: SYXK 2006-0027). Specific-pathogen-free White Leghorn chickens were purchased from the Beijing Laboratory Animal Research Center (BLARC, Beijing, China). Birds were reared in separate cages in the SPF chicken experimental center of Beijing Academy of Agriculture and Forestry Sciences (Beijing, China) and given *ad libitum* access to water and a diet specifically designed for SPF chickens (BLARC). Birds were confirmed to be free of Salmonella by culturing faecal samples in buffered peptone water (BPW) overnight with shaking at 150 rpm followed by spreading and culture (37°C, 18–24 h) on brilliant green agar containing 100 mg/ml nalidixic acid [43].

Chickens (n = 20) aged 30 d, were blood sampled (0 h) then injected intramuscularly into the breast with 0.5 ml PBS containing 8.7 × 10^8 CFU *S. enteritidis* (50041) and additional blood samples were taken at 8 h, 16 h, 24 h, 3 d, and 12 d. Blood from the wing vein (0.5 ml) was taken into EDTA-coated syringes and held on ice for 1 h before lysing and isolating leukocytes (see below). Nine chickens died within 5 days, 4 died between the 5th and the 8th day after injection and the remaining 7 survived until 15 d. Before detailed analyses were performed, the chickens and their samples were partitioned into two groups, one consisted of those from Salmonella-susceptible chickens (died within 5 d after injection, n = 6), the other consisted of six of the total of seven Salmonella-resistant chickens that survived for 15 d.

**Quantitative RT-PCR (qPCR)**

Erythrocytes in blood samples were lysed with Red Blood Cell Lysis Buffer (Roche, Shanghai, China) to isolate peripheral blood leukocytes. Total RNA from leukocytes or cultured PBMCs was prepared using Trizol reagent (Invitrogen, USA) and purified with...
Determinations of bacterial load in blood

The bacterial load in the blood of chicken was estimated by serovar-specific qPCR assay as described previously [45,46]. The probe (5′-FAM-TGCACGGCATATCCCTCGAGAGC-TAMRA-3′) and primers set (the forward primer, 5′-TCCCTGAATCTGGAGAGACACTC-3′; the reverse primer, 5′-TGTAATGCTGTTGTTGTCTGTC-3′) were designed from the Sdf1 gene (Gen-Bank Accession No. AF370707.1). Bacterial DNA isolated from peripheral blood of chickens at 0, 8, 16, 24, 3d, and 12d was amplified using a real-time PCR core kit (R-PCR version 2.1, Takara, Dalian, China) in a 25μL reaction mixture containing 0.6μL of each primer (10μmol/L), 0.75μL of deoxyribonucleotide triphosphates (10mmol/L), 1.25 U of Ex Taq DNA Polymerase (Ex Taq Hot Start Version, Takara), 5μL of 5× PCR buffer (Mg2+ free), 0.3μL of TaqMan probe (5μmol/L), 0.5μL of Mg2+ (250mmol/L), and 5μL of templates. Each PCR run consisted of a 5(min hot start at 93°C, followed by 40 cycles consisting of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and a fluorescence read step. S. enteritidis DNA was isolated
from bacterial cultures and CFU of *S. enteritidis* in the bacterial cultures was quantified by serial dilutions in BPW and plating, as the number of genomic copies. To extrapolate the bacterial number in each blood sample, serial dilutions of the genomic DNA were amplified (copy number ranging from $10^2$ to $10^8$).

**Isolation and culture of peripheral blood mononuclear cells (PBMCs)**

Peripheral blood mononuclear cells (PBMCs) were isolated from a group of six 30 day-old SPF chickens using Ficoll-Hypaque, specific gravity 1.077 (Tian Jin Hao Yang Biological Manufacturing Co., Tianjin, China). Briefly, fresh, non-coagulated blood, diluted 1:1 in Ca$^{2+}$, Mg$^{2+}$-free Hanks’ balanced salt solution (Sigma, Shanghai) was overlaid and centrifuged at 1500 rpm for 30 min. To obtain the 1.077 band, the PBMCs were collected and washed twice in RPMI 1640 medium (Invitrogen, USA) and resuspended in fresh RPMI 1640. The cell concentration was adjusted to $1.5 \times 10^5$ cells/ml and 2 ml were cultured in 1640 medium containing 10% (v/v) fetal bovine serum (Biowest, Beijing, China). Cells were cultured at 37°C in a humidified incubator under 5% CO$_2$.

**Inhibition of histone deacetylase and DNA methyltransferase in PBMCs**

Cells, prepared as above, were inoculated with $1 \times 10^5$ CFU *S. enteritidis* in PBS and treated as follows: 10 μM 5-aza-2-deoxycytidine (5-Aza-dc; Sigma, Shanghai, China) for 3 d; 80 nM trichostatin A (TSA; Shanghai, Sigma) for 24 h, or with TSA plus 5-Aza-dc for an additional 24 h after initial treatment with just 5-Aza-dc for 2 d. Cells were then harvested to obtain total RNA. Transcripts of *TLR4*, *TLR21*, and *TLR21* were quantified by qPCR as described below.

**Bisulfite conversion reaction and DNA sequencing**

Genomic DNA from peripheral blood leukocytes of susceptible (*n* = 6) and resistant (*n* = 6) chickens at 16 h after infection with *S. enteritidis* was prepared using the phenol/chloroform method. To analyze methylation of CpG motifs, 500 ng of genomic DNA was denatured at 98°C for 10 min, modified by the conversion reagent (bisulfite) at 64°C for 2.5 h, and then purified using an EZ DNA MethylGold Kit™ (Zymo Research, Beijing, China).

The promoter region (including core promoter, proximal promoter and distal promoter) of the *TLR4* and *TLR21* genes were amplified by PCR from the sulfite-modified genomic DNA using two pairs of primers of *TLR4* (TLR4-P1, TLR4-P2) and TLR21 (TLR21-P1, TLR21-P2, TLR21-P3). The promoter region and a predicted CpG island in the exon of TLR21 were amplified using PCR primer pairs TLR21-P1 and TLR21-P2 (Table 3). CpG islands were found (http://www.uscnorris.com/cpgislands2/cpgaspx) using 50% GC; Obs CpG/Exp CpG; 0.60; length, 300 bp; and gap between adjacent islands, 100 bp. PCR amplifications were performed using the GoTaq® Hot Start Colorless Master Mix (Promega). Following purification of PCR products, they were cloned into the pMD-18T vector for sequencing; 12–16 clones from each sample were analyzed.

**Statistical analysis**

When needed for normality and homogeneity of variance, data were log-transformed. Analyses were by two-way GLM ANOVA in vivo study or one-way in vitro study ANOVA using SAS (version 8.0). The models were:

**Table 2. qPCR primers used in this study.**

| Gene name | Sequence | GenBank No. |
|-----------|----------|-------------|
| β-actin   | f-5'-GAGAATTTGCGGATATCA-3' | NM_001174871  |
| TLR4      | r-5'-CTTGGCTATCCTCTCCTGCA-3' | NM_001174871  |
| TLR2-1    | f-5'-TTACCGGGTCTTCAACATTCA-3' | NM_00489542  |
| TLR2-2    | r-5'-CATATCCATGCTTCCCTCC-3' | NM_00489542  |
| IL-6      | f-5'-AAAGGCGAAACAACACTTCTC-3' | NM_00557401  |
| TFIIB      | r-5'-AGGTGTTGAGAAGACCTAGAAT-3' | NM_00557401  |
| IL-8      | f-5'-GAGGACGGGACGGAGAAG-3' | NM_006286  |
| IFN-β     | r-5'-CCAGGTCCTTCCCTGAGC-3' | NM_006286  |
| P3K       | f-5'-AACACTCCGGAACCAACAGG-3' | NM_006286  |
| SOCS1     | r-5'-GCGGGTTGACCAATACCTCC-3' | NM_006286  |
| TOULIP    | f-5'-AGGAGGTTGTAGTGGCCACAG-3' | NM_006286  |
| A20       | r-5'-CAAGCCCGAACGCGTCATT-3' | NM_006286  |
| ZNF493    | f-5'-CGGAGCACACGAGCTGAGA-3' | NM_006286  |

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**Table 3. Primers for methylation detection.**

| Primer name | Sequence | Gene ID |
|-------------|----------|---------|
| TLR4-P1     | f-5'-AAAAGTACATTGATGTATTGGTTTCTTTGGA-3' | 417241  |
|             | r-5'-TGTTTTTCTTTGTGATTTTCCCTCCTTTGGA-3' | 417241  |
| TLR4-P2     | f-5'-AGAGTTTATGGTATTTGGTTTCTTTGGA-3' | 417241  |
|             | r-5'-GTATTGAGATTTGATTTGGTTTCTTTGGA-3' | 417241  |
| TLR21-P1    | f-5'-GGTTTGTGATTTGATTTGGTTTCTTTGGA-3' | 415623  |
|             | r-5'-ATATCTCCTCTTCTCTTCTTTGGA-3' | 415623  |
| TLR21-P2    | f-5'-GGTTTGTGATTTGATTTGGTTTCTTTGGA-3' | 415623  |
|             | r-5'-AAAGGTTTTTCTTTGGAAGGCTG-3' | 415623  |
| TLR21-P3    | f-5'-TAGAGTTTATGGTATTTGGTTTCTTTGGA-3' | 415623  |
|             | r-5'-ACTCAATAACACCACTCCTTCTTTGGA-3' | 415623  |
| TLR21-P1    | f-5'-AAATTTTCTTTGGAAGGCTG-3' | 374141  |
|             | r-5'-CTACACCTCTTCTTTGGAAGGCTG-3' | 374141  |

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where: $\gamma$ = relative mRNA expression (log-transformed); $L$ = bacterial load; $m$ = the frequency of methylated CpG; $T$ = the effect of 4 treatment combinations (5-Aza-dc, chimeras); $T = the effect of time (8 h, 16 h, 24 h, 3 d or 12 d after infection); $I =$ the effect of 4 treatment combinations ($\beta$-Aza-dc, cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 167: 1609–1616.

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