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Effects of *Salmonella enteritidis* Vaccination on the Expression of Innate Immune Molecules and Histone Modifications in the Follicular Theca of Laying Hens

Ye Kang[1], Takahiro Nii[1,2], Naoki Isobe[1,2] and Yukinori Yoshimura[1,2]

[1] Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan
[2] The Research Center for Animal Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

The aim of this study was to examine whether *Salmonella enteritidis* (SE) vaccination affects innate immune function and histone modifications responsible for epigenetic reprogramming in the follicular theca of laying hens. White Leghorn laying hens were administered the SE vaccine or phosphate buffered saline (PBS; control) one week before sample collection. The largest follicles (F1) were collected for total RNA and histone protein extraction. Gene expression levels of immune molecules (Toll-like receptors [TLRs], cytokines, and avian β-defensins [AvBDs]), and histone modifications in the follicular thecal tissues, were examined using real-time PCR and western blot, respectively. The results showed that the expression levels of *TLR1-1*, *2-1*, *4*, and *15* were upregulated by SE vaccination. Although vaccination caused no significant change in cytokine expression, *AvBD1*, *2*, *4*, and *7* expression levels were significantly upregulated in the vaccinated group. In addition, the relative density of histone H3-lysine9 dimethylation (H3K9me2) was increased by the vaccination. These results suggest that SE vaccination enhances innate immune functions in the ovary of laying hens, including upregulating TLR and AvBD expression, and is also associated with an increase in histone H3K9me2 in thecal cells.

Key words: histone modification, innate immunity, laying hen, ovary, *Salmonella enteritidis* vaccine

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Introduction

The chicken ovary is susceptible to various pathogenic microorganisms such as *Salmonella*. Ovarian infections in chickens can result in functional disorders in egg formation, as well as egg contamination. The innate immune system in the ovary may play significant roles in maintaining ovarian functions and producing hygienic eggs. Toll-like receptors (TLRs) can recognize microbe-associated molecular patterns (MAMPs) and induce an innate immune response, which includes the production of cytokines and avian β-defensins (AvBDs). Cytokines are responsible for regulating cellular functions and are secreted by specific immune cells to affect the behavior of other cells, making them key factors in immune responses and inflammatory processes. The AvBDs are antimicrobial peptides (AMPs) that play important roles in the innate immune response by killing a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, enveloped viruses, and fungi (Yoshimura, 2015). Previous studies reported that 10 TLRs, proinflammatory cytokines, and AvBDs are expressed in the chicken follicular theca (Subedi *et al*., 2007; Yoshimura and Barua, 2017; Kang *et al*., 2018). The expression of interleukin-6 (*IL-6*) and/or *IL-1β* was shown to be upregulated by TLR2, 3, 4, and 21 ligands, and, in turn, elevated levels of IL-1β may stimulate the expression of *AvBD12* in the theca (Abdelsalam *et al*., 2012). These results suggest that the innate immune system, including the recognition of bacterial and viral patterns by TLRs and synthesis of cytokines and AvBDs, is active in the follicular thecal tissue of laying hens.

In higher vertebrates, the adaptive immune system is known to generate immunological memory mediated by memory lymphocytes, and that vaccinations induce this immunological memory to strengthen immunity. It was originally believed that innate immune memory did not exist; however, recent studies suggest that innate immune memory, namely trained immunity, can also develop in invertebrates

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Correspondence: Yukinori Yoshimura, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima 739-8528, Japan. (E-mail: yyosimu@hiroshima-u.ac.jp)
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(Kurtz, 2005) and mammals (Netea *et al*., 2011, 2016; van der Meer *et al*., 2015). In mammals, vaccinations can induce immunological memory in innate immune cells such as monocytes and natural killer (NK) cells (Kleinnijenhuis *et al*., 2012, 2014; Töpfer *et al*., 2015). We recently reported that routine multiple vaccinations positively or negatively affected the expression of innate immune molecules in the chick ovary, including that of TLRs, TNFSF15, and AvBDs, and may be associated with epigenetic reprogramming through histone modifications in ovarian cells (Kang *et al*., in press). However, it is still unclear whether innate immune memory occurs in follicular thecal cells of laying hens.

Innate immune memory is associated with epigenetic reprogramming regulated by histone methylation and acetylation. Recent studies in mammals suggested that H3K4 trimethylation (H3K4me2/3), H3K27 acetylation (H3K27ac), and H3K9 dimethylation (H3K9me2) were responsible for the epigenetic reprogramming of some innate immune cells following vaccination (Kleinnijenhuis *et al*., 2012; Netea and Crevel, 2014; Töpfer *et al*., 2015; Yoshida *et al*., 2015; de Bree *et al*., 2018). Acetylation of histone H3 at Lys9 (H3 K9ac) was switched to methylation in the cochlea during aging (Watanabe and Bloch, 2013) and there is evidence that other histone modifications may also occur during aging (Kawakami *et al*., 2009; Larson *et al*., 2012; McCauley and Dang, 2014). Our study in chicks showed that the densities of H3K9me2 and H3K9ac were significantly higher in the vaccinated group than in the control, whereas H3K4me2/3 and H3K27ac did not show differences between the groups (Kang *et al*., in press). If vaccination can enhance innate immune functions in the hen ovary, it may also be able to enhance the defense system in the ovaries of matured laying hens by activating innate immunity. However, it has not yet been determined whether vaccination modulates innate immune functions and histone modifications in ovarian cells of laying hens. Moreover, if the histone modification profiles in ovarian cells are altered by changes in the levels of innate immunity proteins, it may partially explain the mechanism by which innate immune functions are modulated by vaccination.

Salmonella is one of the most important pathogens that can infect the chicken ovary. Administration of the *Salmonella enteritidis* (SE) vaccine to the chicken may protect not only ovarian functions but also the eggs against Salmonella infection. If SE vaccination is found to affect the expression of TLRs, cytokines, and AvBDs in the follicular theca, new strategies for enhancing the defense system through innate immunity in the ovary of matured laying hens may then be considered, thus extending the laying cycle of these hens.

The aim of this study was to examine whether SE vaccination in laying hens affected innate immune function in the follicular theca. Specifically, we asked whether SE vaccination affected the expression of innate immune molecules (TLRs, cytokines, and AvBDs) and whether it induced changes in histone modification profiles in the follicular theca.

Materials and Methods

Experimental Birds and Treatment

White Leghorn laying hens, approximately 350 d old, laying five or more eggs in a sequence were used. The hens were purchased from a local poultry farm (Akita Co., Ltd, Fukuyama, Japan) at the pullet stage (120 d old), which had received routine vaccinations before 75 d of age at the farm, including the SE vaccine. After acquisition, the birds were kept in individual cages under a lighting regimen of 14 h light: 10 h dark and provided with feed and water *ad libitum* at the Hiroshima University farm until use. They were then injected intramuscularly with 0.5 mL of inactivated SE vaccine (1×10^9 cfu; Salenvac; Inter Vet Co., Kasumigaura, Japan) (vaccination group), or 0.5 mL of sterile phosphate buffered saline (PBS; control group) one week before sample collection ($n=5$ each group). All the hens were euthanized under anesthesia with sodium pentobarbital (Somnopentyl; Kyoritsu Pharmaceutical Co., Tokyo, Japan) for sample collection. The largest preovulatory follicles (F1) were collected approximately 18–22 h before ovulation to obtain the maximum concentrations of sample total RNA and histone proteins in one follicle. This study was approved by the Hiroshima University Animal Research Committee (no. C15–17).

RNA Extraction and cDNA Preparation

Thecal layers of F1 follicles were isolated and washed in PBS. Briefly, the superficial tissues were removed, followed by cutting the stigma to release the yolk and granulosa layer. The obtained thecal layer was separated into two pieces by cutting vertically, i.e., from the basal (where the stalk was located) to the apical stigma region. Total RNA was extracted from half of each theca using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan) and a Polytron™ homogenizer (Polytron™ PT1200ci Kinematica AG, Luzern, Switzerland). The RNA samples were then dissolved in TE buffer (10 mM Tris, pH 8.0, with 1 mM EDTA). The concentration of RNA in each sample was measured using Gene Quant™ Pro (Amersham PharmaciaBiotech, Cambridge, UK). The RNA samples were mixed with RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) and incubated in a PTC-100 programmable thermal controller (MJ Research, Inc., Waltham, MA, USA) at 37℃ for 45 min. The RNA samples were then reverse-transcribed using ReverTra Ace® (Toyobo Co., Ltd, Osaka, Japan) at 42℃ for 30 min, followed by heat inactivation at 99℃ for 5 min on the PTC-100 programmable thermal controller (MJ Research, Inc.). The reaction mixture (10 μL) contained 1 μg of purified RNA, 1 × RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor, 0.5 mM oligo(dT)20 primer, and 50 U ReverTra Ace.

Real-time PCR

The expression of 10 TLRs (*TLR1* [type 1 and 2], *2* [type 1 and 2], *3*, *4*, *5*, *7*, *15*, and *21*), cytokines (*IL-1β*, *IL-6*, tumor necrosis factor (ligand) superfamily 15 (*TNFSF15*), C-X-C motif chemokine ligand 2 (*CXCLi2*), interferon-alpha (*IFN-α*), and (*IFN-β*), and AvBDs (*AvBD1*, *2*, *4*, *7*, and *12*) in thecal tissues was analyzed by real-time PCR using a Roche

Table 1. **Primer sequences of *TLRs*, cytokines, *AvBDs* and *RPS17* for PCR**

Target genes	Sequences 5′-3′	Accession No.	Annealing temperature (℃) (time, sec)
TLR1-1	F: TTACTGCCAATTGCTTGCAC R: GGTTAGGAAGACCGTGTCCA	NM_001007488	58 (20)
TLR1-2	F: AGTCCATCTTTGTGTTGTCGCC R: ATTGGCTCCAGCAAGATCAGG	NM_001081709	58 (20)
TLR2-1	F: CTGCAACGGTCATCTCAGCTA R: CCGGGGGAATGAAGTCCAAA	NM_204278	61 (30)
TLR2-2	F: TACAGATGCTACTGTGCCTGA R: CCACTTTCCAGTGCCCAAGA	AB046533	58 (20)
TLR3	F: TCAGTACATTTGTAACACCCCGCC R: GGCGTCATAATCAAACACTCC	NM001011691	58 (20)
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	NM_001020693	58 (20)
TLR5	F: CCACATCTGACTTCTGCCTTT R: TGCACATGTTTTCTCCTAGGT	NM_001024586	58 (20)
TLR7	F: CCTGACCCTGACTATTAACCATT R: CGTAAAGTAGCAGGAAGACCC	NM_001011688	55 (20)
TLR15	F: GTTCTCTCTCCCAGTGGGTGAAATAGC R: GTGGTTCATTGGTTGTTTTTAGGAC	NM_001037835	55 (20)
TLR21	F: TGCCCCTCCCACTGCTGTCCACT R: AAAGGTGCCTTGACATCCT	NM_001030558	55 (20)
IL-1β	F: GTGAGGCTCAACATTGCGCTGTA R: TGTCCAGGCGGTAGAAGATGAAG	NM_204524	63 (30)
IL-6	F: AGAAATCCCTCCTCGCCAAT R: AAATAGCGAACGGCCCTCA	NM_204628.1	62 (30)
TNFSF15	F: CCTGAGTTATTCCAGCAACGCA R: ATCCACGAGCTTGATGTCACTAAC	NM_001024578	64 (30)
CXCLi2	F: GGCTTGCTAGGGGAAATGA R: AGCTGACTCTGACTAGGAAACTGT	AJ009800	60 (30)
IFN-α	F: ATCCTGCTGCTCACGCTCCTTCT R: GGTGTTGTGGTGTCCAGGATG	XM_004937096	60 (30)
IFN-β	F: GCTCTCACCACCACCTTCTC R: GCTTGCTTCTTGTCCTTGCT	NM_001024836	58 (30)
AvBD1	F: GATCCTCCCAGGCTCTAGGAAG R: GCCCCATATTCTTTTGC	NM_204993.1	55 (20)
AvBD2	F: GTTCTGTAAAGGAGGGTCCTGCCAC R: ACTCTACAACACAAAACATATTGC	NM_204992	55 (20)
AvBD4	F: ATCGTGCTCCTCTTTGTGGCAGTTCA R: CTACAACCATCTACAGCAAGAATACT	NM_ 001001610	58 (20)
AvBD7	F: ACCTGCTGCTGTCTGTCCTC R: TGCACAGCAAGAGCCTATTC	NM 001001194.1	55 (20)
AvBD12	F: GGAACCTTTGTTTCGTGTTCA R: GAGAATGACGGGTTCAAAGC	AY534898	55 (20)
RPS17	F: AAGCTGCAGGAGGAGGAGAGG R: GGTTGGACAGGCTGCCGAAGT	NM_204217	62 (30)

F, forward; R, reverse

Light Cycler Nano System (Roche Applied Science, Indianapolis, IN, USA). A reaction mixture (10 μL) containing 0.5 μL of cDNA, 5 μL of Thunderbird® SYBR® qPCR mix (Toyobo Co., Ltd, Osaka, Japan), and 0.5 μM of each primer was added to the PCR tubes (Roche Diagnostics GmbH, Mannheim, Germany). Primer sequences used for the analysis are shown in Table 1. The amplification was carried out for 50 cycles (for TLRs and AvBDs) or 45 cycles (for cytokines) at 95℃ for 10 s, followed by annealing at the temperatures shown in Table 1. Quantitative real-time PCR data

were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of each gene in each sample and were expressed as a ratio in relation to the ribosomal protein S17 (*RPS17*) housekeeping gene (Livak and Schmittgen, 2001). A sample of follicular theca from a control group hen was used as a standard for each analysis.

Protein Extraction

Total histone protein was extracted from the other half of the F1 follicular theca using a histone extraction kit (ab 113476; Abcam Inc., Cambridge, MA, USA) according to the manufacturer's instructions. Each tissue was placed in a 2-mL tube with 1× Pre-Lysis buffer, disaggregated by 50–60 strokes, and centrifuged at 10,000×*g* for 1 min at 4℃. After removing the supernatant, tissues were resuspended in lysis buffer (approximately 200 μL/100 mg of tissue), homogenized using a Polytron homogenizer (Polytron PT 1200ci), and incubated on ice for 30 min. Samples were centrifuged at 12,000×*g* for 5 min at 4℃ and the supernatant fraction was transferred into a new tube. Finally, 0.3 volumes of balance-DTT buffer was immediately added to the supernatants and the samples stored at −80℃ until use.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

Protein concentrations in the extracted histone samples were measured using a Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin (BSA; Sigma-Aldrich Japan K.K., Tokyo, Japan) was used to generate a standard curve. The optical density was measured at 620 nm using a Thermo Scientific Multiskan™ FC instrument (Thermo Fisher Scientific Inc., USA). Samples were mixed with Laemmli buffer (30% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 4% [w/v] SDS, 0.06% [w/v] bromophenol blue, and 150 mM Tris-HCl, pH 7.0) and boiled for 5 min. Samples containing 5 μg of histone protein were separated by SDS-PAGE (4% stacking gel and 15% separating gel) at 90 V for the stacking gel and 180 V for the separating gel. The protein bands on some gels were confirmed by Coomassie brilliant blue staining.

After SDS-PAGE, the protein samples in the gels were electrophoretically transferred onto PVDF membranes (Bio-Rad Laboratories) at 60 mA for 3.5 h using a PowerPac™ 300 power supply (Bio-Rad Laboratories). The transfer buffer consisted of 0.03% (w/v) Tris base, 0.14% (w/v) glycine, pH 8.3, and 10% (v/v) methanol. The membranes were washed with Tris-buffered saline with Tween 20 (TBST) (20 mM NaCl, 2 mM Tris-base, 0.1% Tween 20, pH 7.6) for 30 min (10 min×3) and incubated with 3% (w/v) BSA in TBST for 1 h.

The membranes were washed with TBST for 30 min (10 min×3) and incubated with 3% BSA in TBST for 1 h, then incubated overnight at 4℃ with the following primary antibodies: mouse anti-di-methyl histone H3 (Lys9) (H3K9me2) antibody (ab1220; Abcam Inc.), mouse anti-di-methyl and anti-tri-methyl histone H3 (Lys4) (H3K4me2/3) antibody (ab6000; Abcam Inc.), rabbit anti-acetyl-histone H3 (Lys9) (H3K9ac) antibody (cat. no. ABE18; Merck Millipore Co., Darmstadt, Germany), and rabbit anti-acetyl-histone H3 (Lys 27) (H3K27ac) antibody (ab4729; Abcam Inc.). The antibodies were diluted in TBST at a concentration of 1:4,000 (0.25 μg/mL), 1:1,000 (2.9 μg/mL), 1:50,000 (0.02 μg/mL), or 1:1,000 (1.0 μg/mL), respectively. As an internal control, total histone H3 content was also examined by incubating membranes with a goat anti-histone H3 antibody (ab12079; Abcam Inc.) diluted in TBST at a concentration of 1:10,000 (0.1 μg/mL). After washing with TBST for 30 min (10 min ×3), the membrane was incubated with peroxidase-conjugated anti-mouse IgG (Bio-Rad Laboratories; for H3K9me 2 and H3K4me2/3 staining), peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories; for H3K9ac and H3K27 ac staining), or peroxidase-conjugated anti-goat IgG (Bio-Rad Laboratories; for H3 staining) diluted in TBST at a concentration of 1:5,000 for 1 h. The membranes were then washed with TBST for 30 min (10 min×3) and the immunoreactivity of the blots was examined using an ImmunoStar® Zeta (cat. no. 295-72404, Wako Pure Chemical Industries, Ltd, Osaka, Japan). The band signals were imaged with Ez Capture Ⅱ (ATTO Co., Tokyo, Japan). The relative expression level in each sample was determined by standardizing H3K9me2, H3K4me2/3, H3K9ac, or H3K27ac expression to the H3 band using the CS Analyzer 3.0 system (ATTO Co., Tokyo, Japan).

Statistical Analysis

The results of real-time PCR for the relative expression levels of TLRs, cytokines, and AvBDs and of band densities in the western blots corresponding to H3K9me2, H3K4me 2/3, H3K9ac, and H3K27ac levels are shown as means± SEM ($n=5$). Significant differences between the control and vaccinated groups were assessed with the Student's *t*-test. Differences were considered statistically significant at $P<0.05$.

Results

Gene Expression Analysis of Immune Molecules

The changes in TLR expression levels in the follicular theca with or without SE vaccination are shown in Fig. 1. The expression of *TLR1-1*, *2-1*, *4*, and *15* in the vaccinated group was significantly higher than that in the control group (Fig. 1a, c, f, and i). There were no differences in the expression of *TLR1-2*, *2-2*, *3*, *5*, *7*, and *21* between the vaccinated and control groups (Fig. 1b, d, e, g, h, and j).

Figure 2 shows the expression of cytokines in the thecal layer of the vaccinated and control groups. The expression levels of all the examined cytokines (*IL-1β*, *IL-6*, *TNFSF15*, *CXCLi2*, *IFN-α*, and *IFN-β*) were not different between the two groups.

The expression levels of AvBDs in the thecal layer of the vaccinated and control groups are shown in Fig. 3. The expression of four AvBDs, *AvBD1*, *2*, *4*, and *7*, was significantly higher in the vaccinated group relative to the control (Fig. 3a–d). However, there was no difference in the expression of *AvBD12* between the vaccinated and control groups (Fig. 3e).

Histone Modification Analysis

The western blot images of histone methylation and acety-

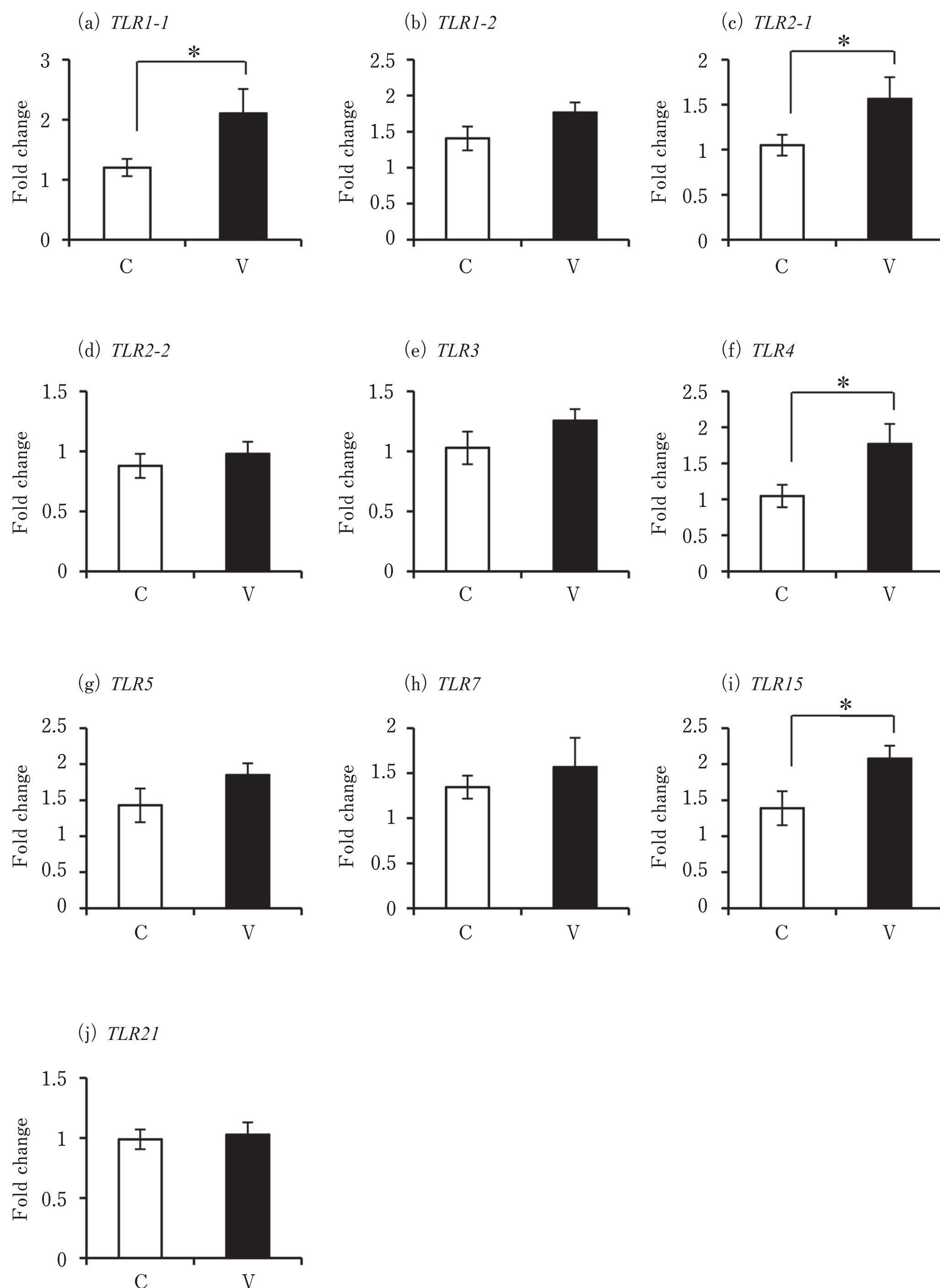

Fig. 1. **Changes in the expression of TLRs in F1 follicular thecal tissues from chickens treated with (V) or without (C) *Salmonella enteritidis* vaccine.** White bars (□)=control group; black bars (■)= vaccinated group. Values are expressed as means± SEM ($n=5$). Asterisks (*) indicate significant differences between the control and vaccinated groups ($P<0.05$).

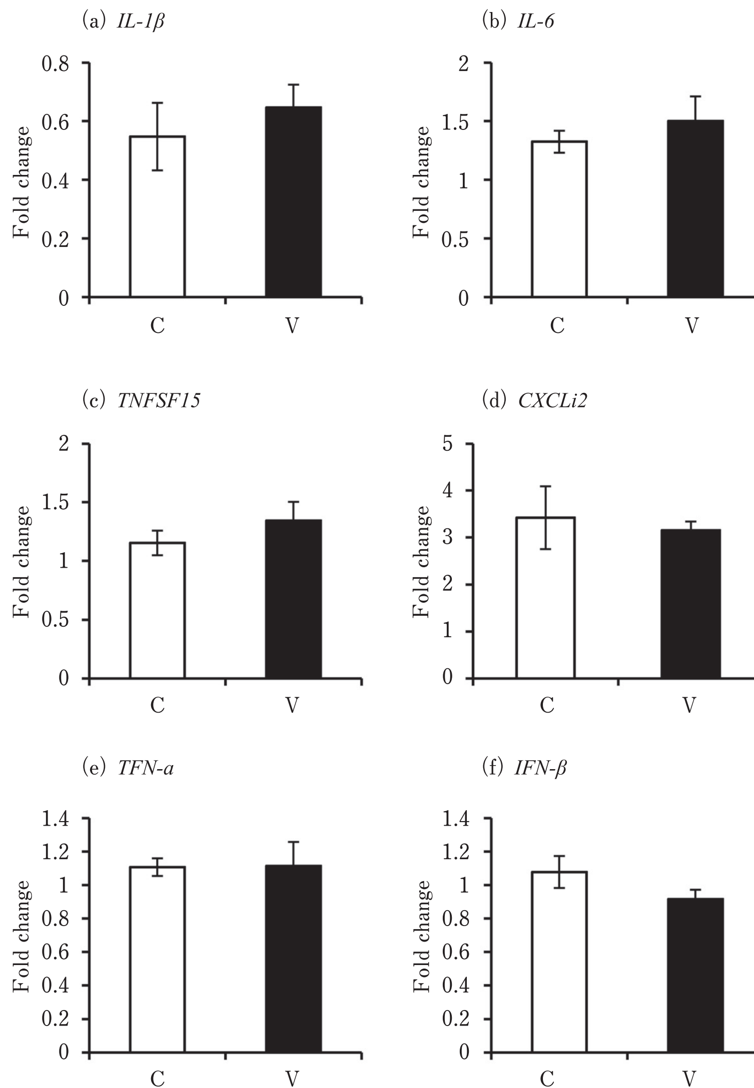

Fig. 2. **Changes in the expression of cytokines in F1 follicular thecal tissues from chickens treated with (V) or without (C) *Salmonella enteritidis* vaccine.** White bars (□)=control group; black bars (■)= vaccinated group. Values are expressed as means±SEM ($n=5$). Asterisks (*) indicate significant differences between the control and vaccinated groups ($P<0.05$).

lation show the presence of histone H3K9me2, H3K4me2/3, H3K9ac, H3K27ac, and histone H3 in follicular thecae of laying hens from both the control and vaccinated groups with band sizes of approximately 15 kDa (Fig. 4a–e). Only the relative density of H3K9me2 was greater for hens in the vaccinated group than those in the control group (Fig. 4f). The vaccination did not affect the relative densities of H3K4me2/3, H3K9ac, and H3K27ac (Fig. 4g–i).

Discussion

In this study, we examined the effects of SE vaccination on the expression of innate immune molecules (TLRs, cytokines, and AvBDs). In addition, we also evaluated whether histone modifications in the follicular theca of laying hens were affected by SE vaccination. The major findings were: (1) the expression levels of *TLR1-1*, *2-1*, *4*, and *15* were upregulated by SE vaccination; (2) the expression levels of *AvBD1*, *2*, *4*, and *7* were also upregulated by the same vaccination; and (3) the level of H3K9me2 was significantly higher in the SE vaccination group than in the control group.

In the present study, SE vaccination resulted in increased expression of *TLR1-1*, *2-1*, *4*, and *15*, but no effects of the vaccination were found on the expression of all the examined cytokines. The results suggest that SE vaccination may

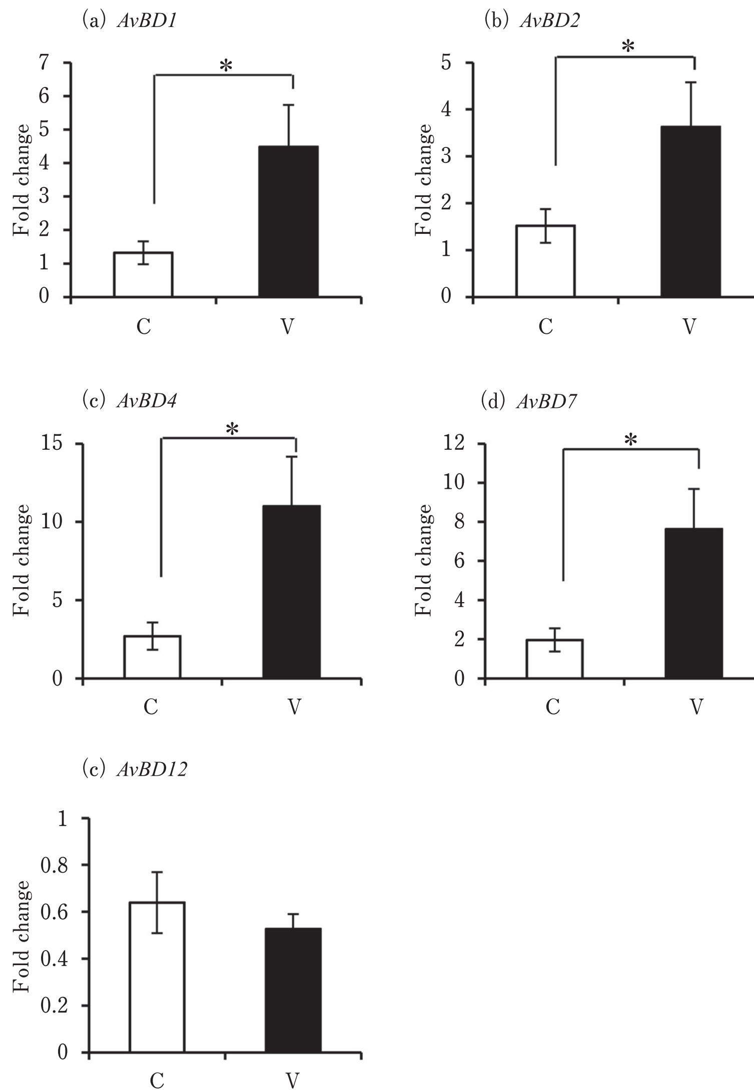

Fig. 3. **Changes in the expression of AvBDs in F1 follicular thecal tissues from chickens treated with (V) or without (C) *Salmonella enteritidis* vaccine.** White bars (□)=control group; black bars (■)=vaccinated group. Values are expressed as means±SEM (n=5). Asterisks (*) indicate significant differences between the control and vaccinated groups (P<0.05).

enhance microbe pattern recognition by upregulation of TLR expression, mainly for Gram-positive (by TLR2), Gram-negative (by TLR4), and bacterial secretory substances (by TLR15). However, the mechanism by which the expression levels of four TLRs were selectively upregulated by the SE vaccination remains unknown.

The current study showed higher expression for four TLRs and four AvBDs in the vaccinated group compared to the control group, but no significant differences were found in the expression of proinflammatory cytokines between the two groups. This suggests that the SE vaccination used in the current study does not affect constitutive proinflammatory cytokine expression in the theca 7 d after vaccination. Bacille de Calmette et Guérin (BCG) vaccination was shown to increase the production of proinflammatory cytokines (*IL-1β*, *IL-6*, and *TNF*) by NK cells in response to mycobacteria and unrelated bacterial and fungal pathogens (Kleinnijenhuis *et al*., 2014). We assume that the effects of vaccination on innate immune function may vary depending on the vaccine type and target tissue, and the effects of SE vaccination on the expression of proinflammatory cytokines in the theca become negligible by d 7 after vaccination.

The expression of *AvBD1*, *2*, *4*, and *7* in thecal tissue was upregulated following SE vaccination. This result is dif-

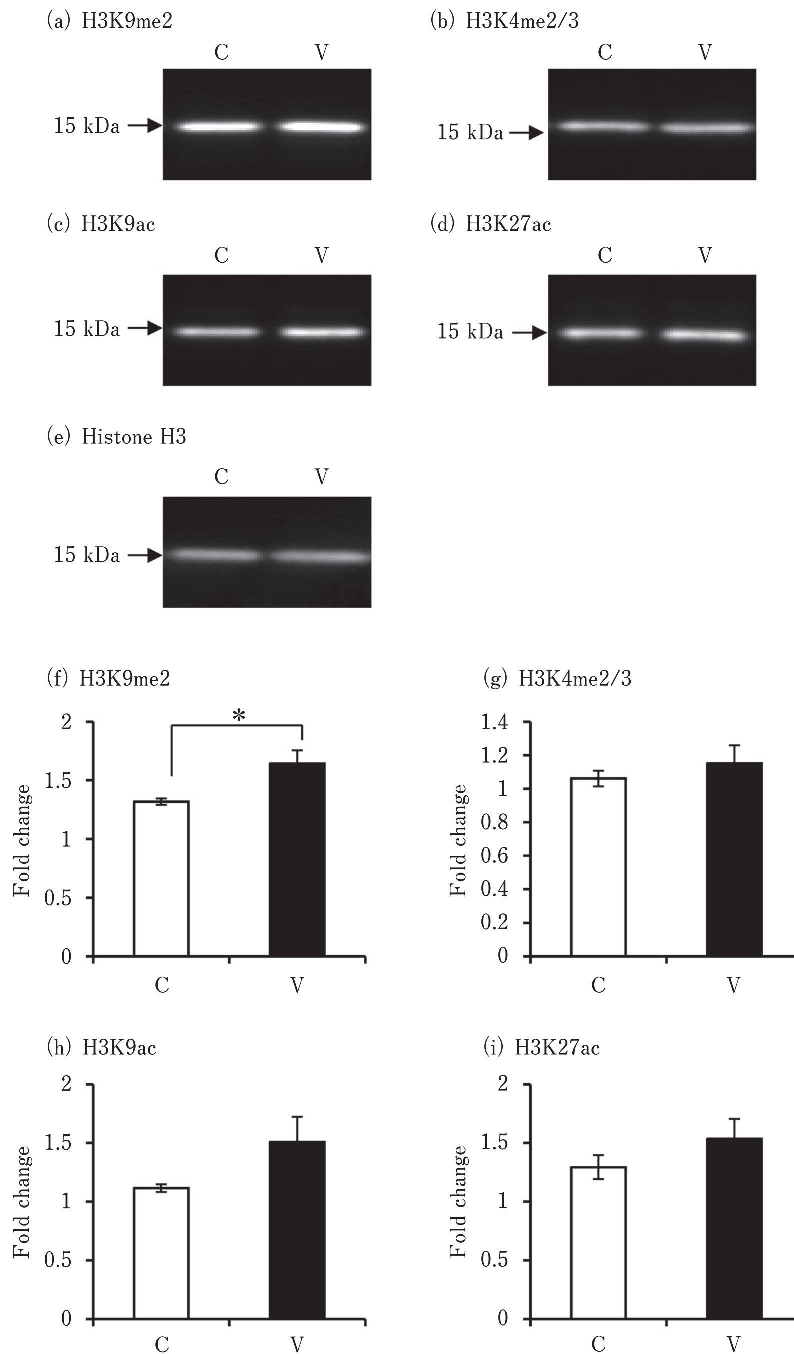

Fig. 4. **Western blot analysis of di-methyl histone H3 (Lys9) (H3K9 me2), di-methyl and tri-methyl histone H3 (Lys4) (H3K4me2/3), acetyl-histone H3 (Lys9) (H3K9ac), and acetyl-histone H3 (Lys27) (H3K27ac) in the follicular theca of laying hens with (V) or without (C) *Salmonella enteritidis* vaccination.** (a, f) H3K9me2; (b, g) H3K4 me2/3; (c, h) H3K9ac; (d, i) H3K27ac; and (e) histone H3. Values represent the means±SEM of the band densities relative to histone H3 ($n=5$). Asterisks (*) indicate significant differences between the control and vaccinated groups ($P<0.05$).

ferent from our previous study in chicks that received multiple vaccinations, including against infectious bronchitis, Marek's disease, Newcastle disease, and infectious bursal disease, where vaccination resulted in reduced expression of *AvBD1*, *2*, *4*, and *7* in the ovaries (Kang *et al*., in press). This was likely due to differences in the vaccines and in the responses of the cells between immature and matured ovaries. We do not know why SE vaccination elicited different effects among the AvBDs, namely, that the vaccination led to the increased expression of four AvBDs but not *AvBD12*; however, there may be differences in the responsiveness of AvBD-producing cells to SE vaccination for the different AvBDs. The expression of some AvBD genes, including *AvBD12*, in preovulatory follicles was reportedly upregulated by intravenous LPS injection in laying hens (Subedi *et al*., 2007; Abdelsalam *et al*., 2010). Moreover, *AvBD12* expression was also increased by oral Salmonella inoculation in hen ovarian tissues (Michailidis *et al*., 2012). Abdelsalam *et al*. (2012) reported that LPS did not significantly affect the expression of *AvBD10* and *12*, but upregulated the expression of *IL-1*β in thecal tissue *in vitro*. They then showed that IL-1β stimulated *AvBD12* expression, suggesting that LPS could indirectly induce *AvBD12* expression. Since the levels of all the examined AvBDs, except for *AvBD12*, were increased in the follicular theca following SE vaccination, an appropriate dose of the SE vaccine may be able to directly upregulate the expression of AvBDs in the theca, even 7 d after vaccination. The role of IL-1β in this process may be less distinct since its expression was not different between the vaccinated and control groups.

This study showed that the relative H3K9me2 density increased with SE vaccination, suggesting that the vaccination induces histone modifications in the thecal tissues of laying hens. In contrast, we recently reported that the densities of H3K9me2 and H3K9ac increased significantly, whereas the densities of H3K4me2/3 and H3K27ac in the chick ovary were not affected by multiple vaccinations (Kang *et al*., in press). The reason why the H3K9ac density in the current study was not increased, unlike in the previous study where chicks received multiple vaccinations, is not known. However, we assume that it may also be due to differences in the vaccines or in ovarian maturational stages, similar to the vaccination effects observed for the expression of AvBDs. It has been reported that H3K9 methylation is a primary signal that is sufficient to initiate a gene repression pathway *in vivo* (Snowden *et al*., 2002). Previous studies in mammals have suggested that vaccination induces histone modifications, including H3K4 trimethylation (Kleinnijenhuis *et al*., 2012; Netea and Crevel., 2014; Töpfer *et al*., 2015; de Bree *et al*., 2018) and H3K27 acetylation (Töpfer *et al*., 2015) in circulating monocytes, which may be responsible for the epigenetic reprogramming that enhances their activity. Although the modification induced by SE vaccination at H3K9me2 that was observed in the current study may negatively regulate the transcription of as yet unidentified genes, it may also induce epigenetic reprogramming that affects the functions of thecal cells. Thus, vaccines that enhance innate immune defense functions should be developed. The improved vaccines should exert positive effects or diminish any negative effects on the expression of innate immune molecules. The effects of vaccination on histone modifications may be one of the mechanisms by which vaccination can modulate innate immune functions in the theca since it may enable vaccines to be effective for extended durations. In human monocytes, trained innate immunity following BCG vaccination was identified more than 3 months after vaccination (Netea *et al*., 2016).

In conclusion, we suggest that the SE vaccination used in this study resulted in upregulated expression of TLRs and AvBDs in the follicular theca of laying hens, and these processes may be associated with histone modifications through an increase in the relative density of H3K9me2. Thus, appropriate vaccinations may be effective in enhancing partial innate immune functions in the ovary, including in matured hens.

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