Effects of avian infectious bronchitis with Newcastle disease and Marek's disease vaccinations on the expression of toll-like receptors and avian β-defensins in the kidneys of broiler chicks

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ABSTRACT The aim of this study was to determine the effect of vaccinations for avian infectious bronchitis with Newcastle disease (**IB/ND**) and Marek's disease (**MD**) on the expression of toll-like receptors (**TLR**) that recognize viral RNA and microbial DNA, and AvBD in chick kidneys. Day-old chicks were vaccinated with MD or IB/ND vaccines or received no treatment (control group). The gene expression of TLR and AvBD in the kidneys of 3-day-old chicks and 10-day-old chicks was examined using real-time PCR. The localization of AvBD2 and AvBD4 was examined by immunohistochemistry at day three only. At 3 days of age, the expression of TLR7 and TLR21 was significantly higher in the IB/ND group (but not in the MD group) than in the control group. Conversely, at 10 days of age there was no significant difference in the expression of the three TLR between groups. In the 3-day-old chicks the expression levels of AvBD4, 5, 6, and 7 were higher in the MD group than in the control group. Furthermore, at this age, the expression levels of other AvBD were not significantly different between the control and vaccination (MD and IB/ND) groups. At 10 days of age, no AvBD expression was affected by MD and IB/ND vaccinations. Immunohistochemistry results localized AvBD2 in the leukocytes in the interstitial tissue and AvBD4 in the surface of microvillus epithelial cells of renal tubules, and in the epithelial cells of the collecting ducts and ureter. The localization of AvBD2 and AvBD4 was identified in all chicks. We suggest that the expression of innate immune molecules (including TLR and AvBD) in kidneys could be modulated by MD and IB/ND vaccination when performed at the day-old stage. Although the effects of both vaccinations may subside within 10 days, the enhanced expression of those innate immune molecules may support the innate immunodefense function in the kidneys of young chicks.

Key words: broiler chick kidney, toll-like receptors, AvBD, vaccination

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INTRODUCTION

Many reports have demonstrated that the avian infectious bronchitis (**IB**) virus and Newcastle disease (**ND**) virus infect the kidneys of immature and mature chickens, causing pathological changes and disease (Chen and Itakura, 1996; Bande et al., 2016; El-Bahrawy et al., 2017). Inoculation of chickens with Marek's disease (**MD**) virus isolated from CVI988/Rispens-vaccinated chickens caused tumors, mainly in the spleen, liver, and kidney (Cui et al., 2016). Experimentally inoculated pathogenic *Escherichia coli* and *Salmonella typhimurium* infected various organs, including the kidneys (Barrow et al., 1987; Pourbakhsh et al., 1997). Thus, the kidney is an organ susceptible to many pathogenic microbes. Because the adaptive immune functions are not mature during the first few weeks of a chicken's life, the innate immune system plays an essential role in defense against infection by pathogenic microorganisms.

Toll-like receptors (**TLR**) recognize microbe-associated molecular patterns to initiate the innate immune response, such as the synthesis of proinflammatory cytokines and antimicrobial peptides (Yoshimura, 2015). TLR3 and TLR7 recognize the dsRNA and ssRNA of viruses, whereas TLR21 recognizes unmethylated CpG-oligo DNA of microbes, including the DNA of viruses. TLR2, 4, 5, and 15 recognize bacterial patterns such as peptidoglycans, lipopolysaccharide, flagellin, and bacterial secretory proteinase.

Avian β-defensins (**AvBD**) are antimicrobial peptides that have a broad spectrum of antimicrobial activity against enveloped viruses, bacteria, and fungi. Fourteen AvBD have been identified to date and their expression has been shown in many tissues such as leukocytes and reproductive, digestive, and respiratory systems (Abdel

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Mageed et al., 2008, 2009; Cuperus et al., 2013; Yoshimura, 2015; Yoshimura and Barua, 2017). Although previous reports identified the expression of AvBD in chicken kidneys (Xu et al., 2015; Mowbray et al., 2018), the cells responsible for the expression of these proteins have not been identified.

Enhancing the innate immune-defense system is important for the production of healthy chicks. We reported that treating chicks with probiotics affects the innate immune system by modulating the expression of TLR, proinflammatory and anti-inflammatory cytokines, and AvBD (Terada et al., 2020a). Kang et al. (2019) reported that the routine multiple vaccination procedure positively or negatively affected the expression of innate immune molecules (including TLR, cytokines, and AvBD), with changes in the frequency of histone modification because of acetylation and methylation in the chick ovary. However, an effective treatment for enhancing innate immune functions in chick kidneys remains to be established.

The aim of this study was to determine the effects of 2 different vaccines (MD and IB/ND vaccines) on the expression of TLR and AvBD in the chick kidney. The MD and IB/ND viruses in the vaccines are DNA virus and ssRNA virus, respectively (Jackwood, 2012; Couteaudier and Denesvre, 2014). These vaccinations are routinely performed on chicks. Specifically, the following were investigated: (1) whether MD and IB/ND vaccinations have different effects on the gene expression of TLR recognizing virus patterns and AvBD in the chick kidney and (2) the identity of renal cells expressing AvBD2 and AvBD4 proteins.

MATERIALS AND METHODS

Treatment of Birds and Tissue Collection

Fertilized eggs (Chunky broiler) purchased from a local hatchery (Fukuda Breeders Co., Okayama, Japan) were incubated in a humidified incubator at 37.5°C. The day-old female chicks were divided into 3 groups: control, MD, and IB/ND. Chicks in the MD group received the MD vaccine containing MD virus CVI988 strain (Poulvac MDcvi; Kyoritsu Seiyaku Co., Tokyo, Japan) by intramuscular injection. Chicks in the IB/ND group were given mixed vaccines of IB and ND containing IB virus H120 strain and ND virus B1 strain (Poulvac COMBI; Kyoritsu Seiyaku Co.) through eye drops. The vaccinations were performed following the instruction of manufacturers. The chicks in the control group received no treatment. All chicks were maintained in a brooding room with a lighting schedule of 23 h light:1 h dark. They were given a commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water ad libitum. Tissue collection was performed at 2 different ages, 3- and 10-day-old (2 d and 9 d after vaccination, respectively). Chicks were euthanized with carbon dioxide before tissue collection (only the caudal renal division was collected to make the sample tissue uniform). The left kidney was used for gene expression analysis, and the right kidney was collected for histology. The number of 3-day-old chicks totaled 20, including 6 chicks in the control, 7 in the MD, and 7 in the IB/ND groups. The number of 10-day-old chicks used in the analysis also totaled 20, involving 7 chicks in control, 6 in MD, and 7 in IB/ND group. This study was approved by the Hiroshima University Animal Research Committee (No. C15-16).

RNA Isolation and cDNA Preparation

Total RNA was extracted using Sepasol RNA Super (Nacalai Tesque, Inc., Kyoto, Japan), following the manufacturer's instructions. The extracted total RNA samples were dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and stored at −80°C until required. Samples were treated with RQ1 RNase-free DNase mixture (Promega Co., Madison, WI; 1-μg total RNA, 1 × DNase buffer, and 1 unit DNase in 10 μL) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA), programmed at 37°C for 30 min, followed by incubation at 65°C for 10 min with 1 U RQ1 DNase Stop Solution (Promega Co.). The concentration of RNA in each sample was measured using NanoDrop Lite (Thermo Fisher Scientific., Waltham, MA). The RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) as per manufacturer's instructions. The reaction mixture (10 μL) comprised 0.5-μg total RNA, 1 × reverse transcription buffer (Toyobo Co., Ltd.), 1-mM deoxyribonucleotide triphosphate (**dNTP**) mixture (Toyobo Co., Ltd.), 5 units RNase inhibitor (Toyobo Co. Ltd.), 2.5 pmol oligo (dt) 20 (Toyobo Co., Ltd.), and 50 units ReverTra Ace. Reverse transcription was performed at 42°C for 30 min, followed by heat inactivation at 99°C for 5 min using a programmable thermal controller. Finally, the cDNA samples were stored at −30°C until use.

Real-Time PCR

Real-time PCR was performed using the AriaMx Real-time PCR system (Agilent Technologies Japan Ltd., Tokyo, Japan). The reaction mixture (10 μL) consisted of 1-μL cDNA, 1 × Brilliant III SYBR Green QPCR Mix (Agilent Technologies Japan, Ltd.), 0.25 μM of each primer, and water. The primer sequences used in this study are shown in Table 1. Two different PCR protocols were used for the amplification. The first PCR protocol was 50 cycles at 95°C for 5 s and 60°C (RPS17, TLR3, 7 and 21), 62°C (AvBD2, 4, 6, 9, and 12) or 63°C (AvBD5 and 10) for 10 s. The second protocol was 50 cycles at 95°C for 5 s and 55°C (AvBD1 and 7), 56°C (AvBD3), 60°C (AvBD11, 13, and 14), or 62°C (AvBD8) for 10 s, followed by 72°C for 10 s each. The real-time PCR products using the samples from 3-day-old chicks were examined by electrophoresis on 2% (w/v) agarose gel containing

Table 1. Primer sequences of TLR, AvBD, and RPS17 for PCR.

Target genes	Primer sequences 5′–3′	Accession no.
TLR3	F: TCAGTACATTTGTAACACCCCGCC R: GGCGTCATAATCAAACACTCG	NM_001011691.3
TLR7	F: CCTGACCCTGACTATTAACCAT R: CGTAAAGTAGCAGGAAGACCC	NM_001011688.2
TLR21	F: TGCCCCTCCCACTGCTGTCCACT R: AAAGGTGCCTTGACATCCT	NM_001030558.1
AvBD1	F: GATCCTCCCAGGCTCTAGGAAG R: GCCCCATATTCTTTTGC	NM_204993.1
AvBD2	F: GTTCTGTAAAGGAGGGTCCTGCCAC R: ACTCTACAACACAAAACATATTGC	XM_015285091.2
AvBD3	F: CCACTCAGTGCAGAATAAGAG R: AATTCAGGGCATCAACCTC	NM_204650.2
AvBD4	F: ATCGTGCTCCTCTTTGTGGCAGTTCA R: CTACAACCATCTACAGCAAGAATACT	NM_001001610.2
AvBD5	F: GCTGTCCCTTGCTCGAGGATT R: GGAATACCATCGGCTCCGGC	NM_001001608.2
AvBD6	F: GATCCTTTACCTGCTGCTGTCT R: TCCTCACACAGCAAGATTTTAGTC	NM_001001193.1
AvBD7	F: ACCTGCTGCTGTCTGTCCTC R: TGCACAGCAAGAGCCTATTC	NM_001001194.1
AvBD8	F: TTCTCCTCACTGTGCTCCAA R: AAGGCTCTGGTATGGAGGTG	NM_001001781.1
AvBD9	F: GCTTACAGCCAAGAAGACGCT R: GGAGCTAGGTGCCCATTTGCA	NM_001001611.2
AvBD10	F: TGGGGCACGCAGTCCACAAC R: CATGCCCCAGCACGGCAGAA	NM_001001609.2
AvBD11	F: ACTGCATCCGTTCCAAAGTCTG R: TCGGGCAGCTTCTCTACAAC	NM_001001779.1
AvBD12	F: GGAACCTTTGTTTCGTGTTCA R: GAGAATGACGGGTTCAAAGC	NM_001001607.2
AvBD13	F: GATCCTCCAGCTGCTCCTTG R: AGTGGCCATGGTTGTTCCT	NM_001001780.1
RPS17	F: AAGCTGCAGGAGGAGGAGAGG R: GGTTGGACAGGCTGCCGAAGT	NM_204217.1

0.025% (w/v) ethidium bromide to confirm the products of amplification. For that electrophoresis, 5-μL real-time PCR product solutions was loaded equally in each sample.

In the real-time PCR analysis, expression of TLR3, 7, and 21 was examined as they are the receptors recognizing RNA and DNA virus molecular patterns. The expression of AvBD2, 4, 5, 6, 7, 9, 10, and 11 was examined because their real-time PCR products showed dense bands on the agarose gel electrophoresis. Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method to calculate the relative level of gene expression in each sample and were expressed as ratios to the ribosomal protein S17 (RPS17) housekeeping gene (Livak and Schmittgen, 2001). An RNA sample from the control group was used as a standard.

Immunohistochemistry for AvBD

Samples from the right side caudal renal division from chicks in each group (control, MD, and IB/ND) were fixed in 10% (v/v) formalin in PBS and processed for paraffin sections (4 μm in thickness). After deparaffinization, they were autoclaved at 110°C for 15 min in 10 mM Tris buffer (pH 10.0) for antigen retrieval. Then, the sections were incubated with 1% (w/v) blocking reagent (Roche Co., Basel, Switzerland) for 1 h, followed by an overnight incubation with AvBD2 antibody diluted at 10 μg/mL or AvBD4 antibody diluted at 20 μg/mL in PBS at 4°C. The AvBD2 and AvBD4 were selected for examination because they were the 2 of eight AvBD showing dense PCR products. Sections were then washed with PBS (5 min × 3 times) and incubated with the biotin-conjugated antirabbit IgG (1:200) and avidin-biotin-peroxidase complex (1:50) for 1 h using a VECTASTAIN ABC Kit (Vector Laboratories, Inc., Burlingame, CA). The immunoreaction products were visualized using a reaction mixture of 0.02% (w/v) 3,3′-diaminobenzidine-4HCl and 0.05% (v/v) H_2O_2. Then, these sections were counterstained with hematoxylin and covered after dehydration.

The antibody to AvBD2 had been used to localize that protein in the chick intestine (Terada et al., 2020b). Briefly, antiserum to AvBD2 raised in rabbits by immunization with KLH-conjugated AvBD2 synthetic peptides (CPSHLIKVGS) was supplied by Medical & Biological Laboratories, Nagoya, Japan. The AvBD2 antibody in the antiserum was purified using an affinity column (HiTrap NHS-activated HP, GE Healthcare Japan, Tokyo, Japan) conjugated with AvBD2 synthetic peptide according to manufacturer's instruction. For the preparation of AvBD4 antibody, rabbit antiserum to AvBD4 prepared by immunization with KLH-conjugated AvBD4 synthetic peptides (C-Ahx-PYGNAYLGL) was obtained from Sigma-Aldrich Japan Co., Tokyo, Japan. The AvBD4 antibody in the antiserum was purified using an affinity

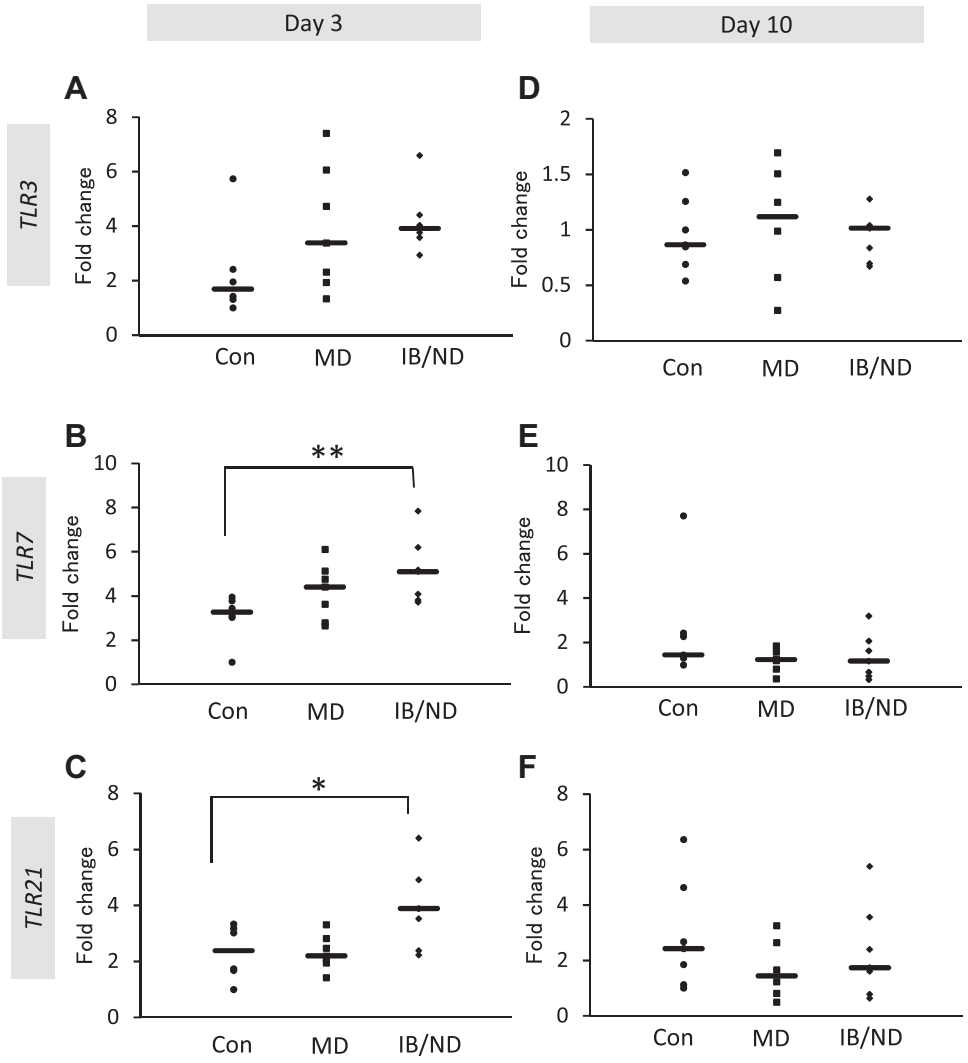

Figure 1. Effects of Marek's disease (MD) and avian infectious bronchitis with Newcastle disease (IB/ND) vaccinations on the expression of toll-like receptors (TLR) 3, 7, and 21 (A−C: 3-day-old; D−F: 10-day-old) in the chick kidneys. Day-old chicks were vaccinated with IB/ND or MD vaccines, and the TLR expression was examined at 3 and 10 d of age using real-time PCR. Chicks in the control group (Con) received no vaccines. Values are the fold changes in the expression of target genes calculated using the $2^{-\Delta\Delta CT}$ method and expressed as a ratio to the ribosomal protein S17. The solid bar represents the median value within each group. *, **Significantly different at $P < 0.05$ and 0.01, respectively (one-way ANOVA and Dunnett's test). The numbers of chicks in the control, MD, and IB/ND groups were 6, 7, and 7 in the 3-day-old analysis and 7, 6, and 7 in the analysis performed on 10-day-old chicks, respectively.

column (HiTrap NHS-activated HP, GE Healthcare Japan) conjugated with AvBD4 synthetic peptide as described above.

Negative-control staining was performed to confirm the specificity of immunostaining by replacing the primary antibodies with absorbed antibodies, which were prepared by incubating AvBD2 or AvBD4 antibodies with corresponding peptides at a ratio of 1:5 by weight.

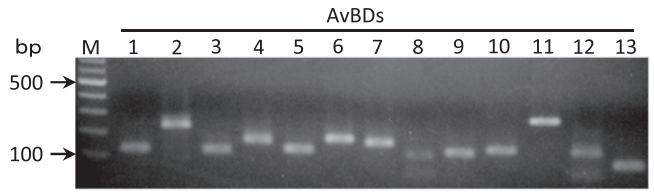

Figure 2. Real-time PCR products of avian β-defensins (AvBD) in the kidneys of 3-day-old chicks. Equal amount of PCR product solution (5 μL) for each AvBD was loaded. The amplified products of AvBD2, 4–7, and 9–11 show dense bands.

Statistical Analysis

The significance of differences in the real-time PCR data between the control and vaccine (MD and IB/ND) groups was examined using one-way ANOVA followed by Dunnett's test. Differences were considered significant when the P-value was <0.05.

RESULTS

Figure 1 shows the effects of an MD or IB/ND vaccination on the expression of TLR3, 7, and 21 in 3- and 10-day-old chicks. In the 3-day-old chicks the expression

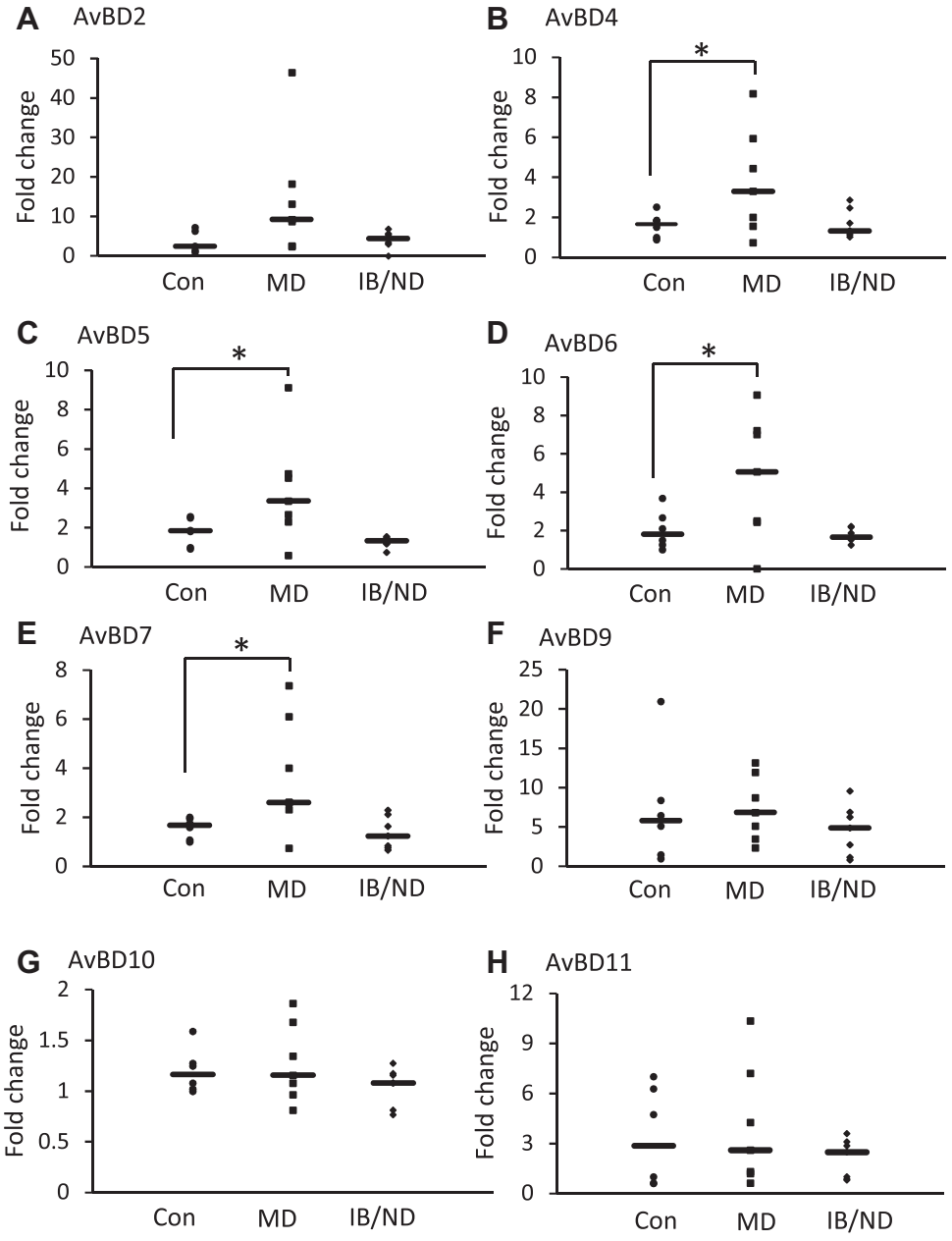

Figure 3. Effects of MD and IB/ND vaccinations on the expression of avian β-defensins (AvBD) in the kidneys of 3-day-old chicks. (A–H) These panels show AvBD2, 4−7, and 9−11. Values are the fold changes in the expression of target genes (n = 6, 7, and 7 in the Con, MD, and IB/ND groups, respectively). *Significantly different at $P < 0.05$ (one-way ANOVA and Dunnett's test). See Figure 1 for further explanations. Abbreviations: Con, control; IB/ND, infectious bronchitis with Newcastle disease; MD, Marek's disease.

levels of TLR7 and TLR21 were significantly higher in the IB/ND group than in the control group (Figures 1A–1C). The expression levels of all 3 TLR showed no difference between the control and MD groups. In the 10-day-old chicks there was no significant difference in expression levels between groups for all 3 TLR (Figures 1D–1F).

The real-time PCR products of AvBD of the kidneys from a 3-day-old chick are shown in Figure 2. The bands of AvBD1 to 13 were identified, whereas the amplified products of AvBD2, 4–7, and 9–11 showed dense bands. Figures 3 and 4 show the effects of MD and IB/ND vaccinations on the expression of selected AvBD in the kidneys of 3- and 10-day-old chicks. In the 3-day-old chicks, the expression levels of AvBD4, 5, 6, and 7 were higher in the MD group than in the control group (Figures 3A–3E). The expression levels of other AvBD were not significantly different between the control and vaccination (MD and IB/ND) groups (Figures 3F–3H). In contrast, the MD and IB/ND vaccinations showed no effect on AvBD expression in 10-day-old chicks (Figures 4A–4H).

Figure 5 shows the localization of immunoreactive AvBD2 and 4. In the control group, AvBD2 was identified in the leukocytes in the interstitial tissue among the renal tubules and renal corpuscles (Figure 5a). The AvBD4 was localized on the surface of microvillus epithelial cells of renal tubules (Figure 5d). Immunoreaction

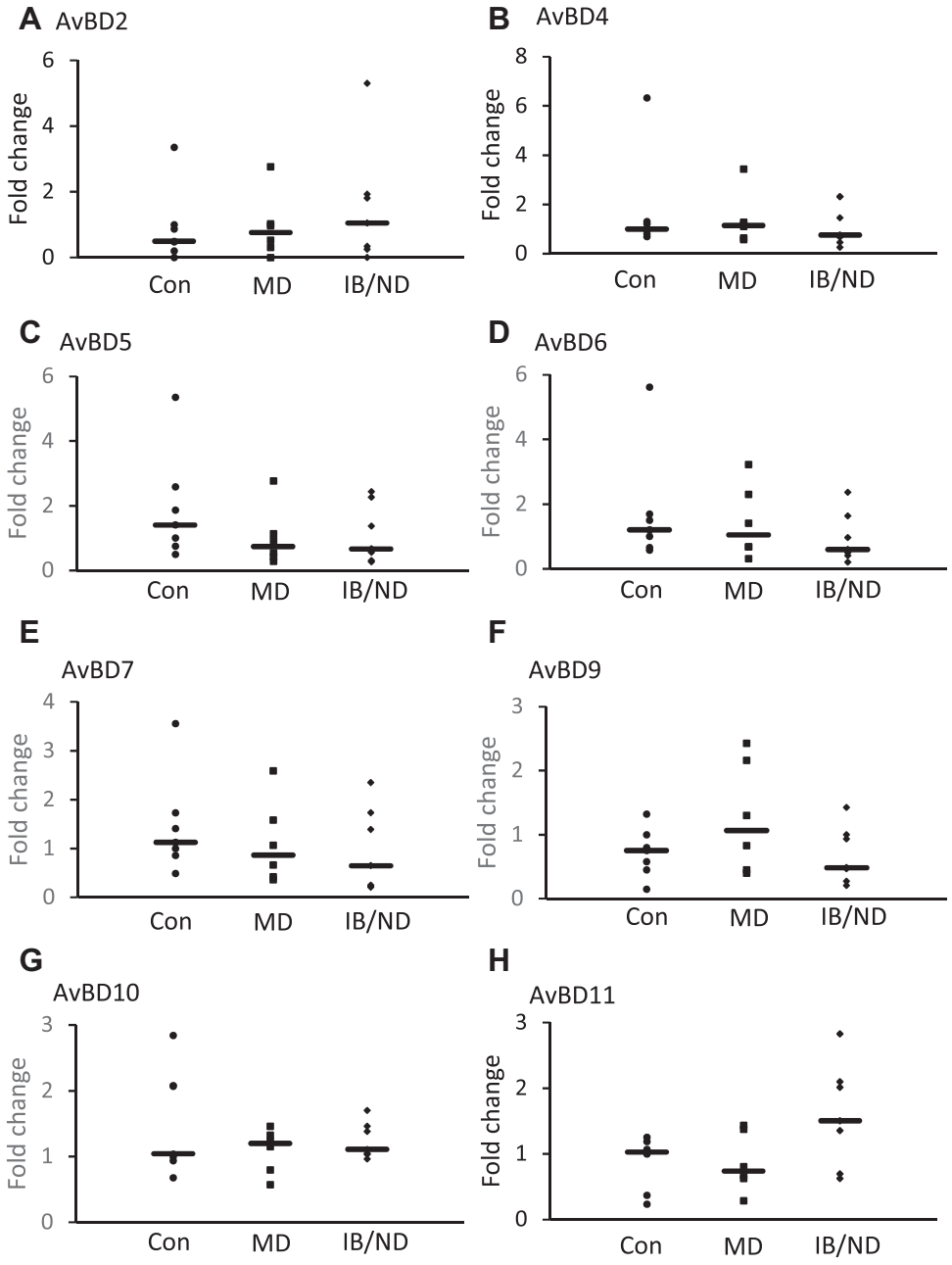

Figure 4. Effects of MD and IB/ND vaccination on the expression of avian β-defensins (AvBD) in the kidneys of 10-day-old chicks. (A–H) These panels show AvBD2, 4−7, and 9−11. Values are the fold changes in the expression of target genes (n = 7, 6, and 7 in the Con, MD, and IB/ND groups, respectively). *Significantly different at $P < 0.05$ (one-way ANOVA and Dunnett's test). See Figure 1 for further explanations. Abbreviations: Con, control; IB/ND, infectious bronchitis with Newcastle disease; MD, Marek's disease.

signals for AvBD4 was also localized in the cytoplasm of epithelial cells of the collecting ducts and ureter, whereas the epithelial cells in the ureter generally showed strong staining (Figure 5g and i). These localization of AvBD2 and AvBD4 was also identified in the MD group (Figure 5b, e, h and k) and IB/ND group (Figure 5c, f, i and l). Negative-control staining using absorbed antibodies for AvBD2 showed no positive immunoreaction signal (Figure 5m). In the negative control for AvBD4, staining in the surface of microvillus epithelial cells of renal tubules and in the cytoplasm of epithelial cells of the collecting ducts and ureter was disappeared (Figure 5n and o).

DISCUSSION

We report here that TLR and AvBD are expressed in the chick kidney, and their expression is affected by MD and IB/ND vaccination performed at 1 d of age. The major findings were, at 3 d (2 d after vaccination), (1) the expression of TLR7 and 21 was higher in the chicks that received IB/ND vaccination than in

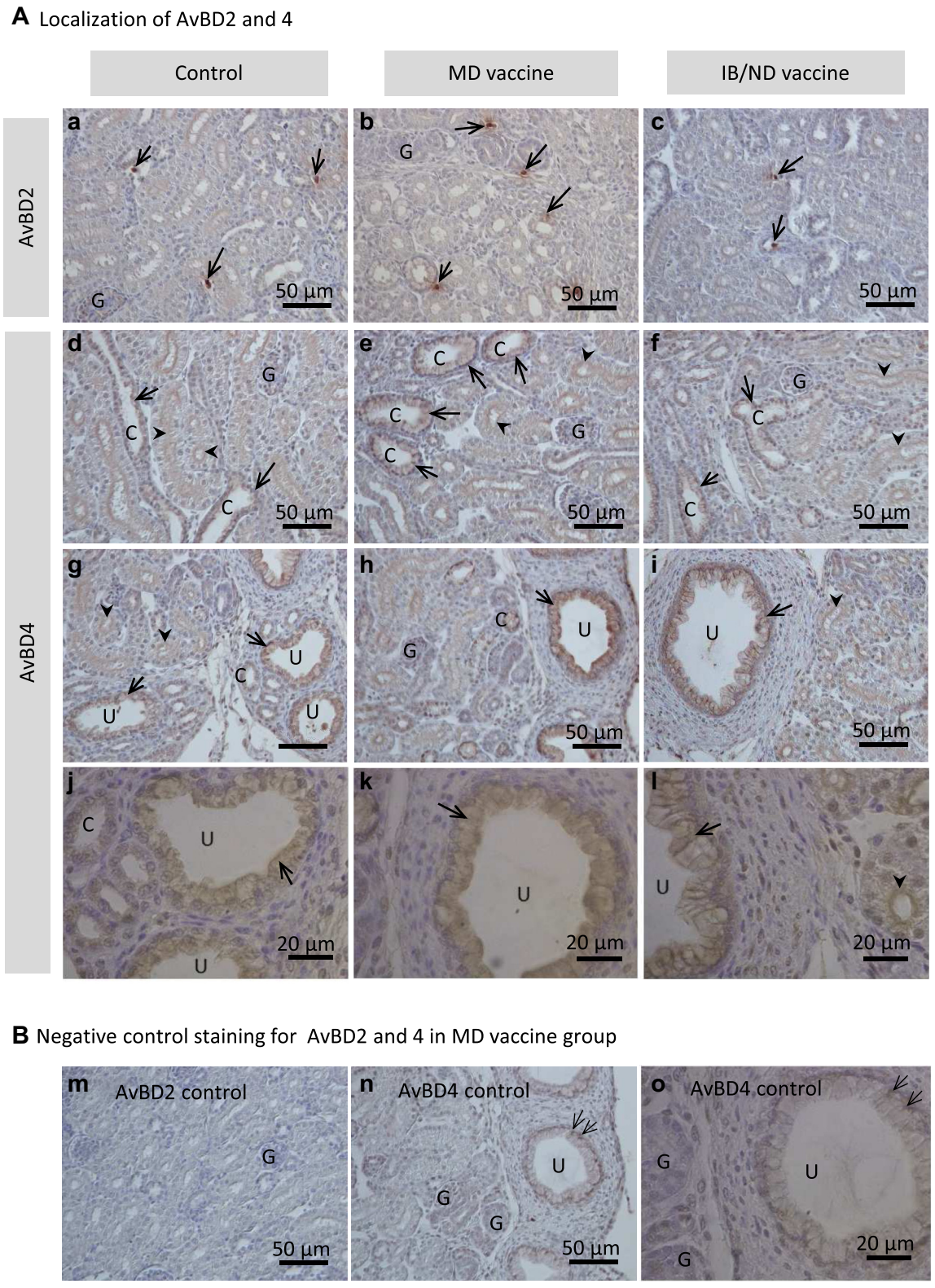

Figure 5. Immunolocalization of avian β-defensin 2 and 4 (AvBD2 and 4) in the kidneys of 3-day-old chicks with or without MD and IB/ND vaccinations. See Figure 1 for the explanation of vaccination. (A) Localization of AvBD2 and 4. [a–c] Immunoreactive AvBD2 is localized in the leukocytes (arrows) in the interstitial tissues of control and vaccinated (MD and IB/ND groups) chicks. [d–f] Immunoreactive AvBD4 is localized on the surface of microvillus epithelial cells of renal tubules (arrowheads) and in the cytoplasm of epithelial cells of collecting ducts (arrows) in all chick groups. [g–i] Strong immunoreaction signals for AvBD4 is localized in the epithelial cells in the ureter (arrows) in all chick groups. [j–l] A magnified view of AvBD4 staining confirms the presence of AvBD4 in the cytoplasm of the epithelial cells in the ureter (arrows) in all chick groups, and on the surface of microvillus epithelial cells of the renal tubule (arrowhead on Figure 5l). (B) Negative control staining for AvBD 2 and 4 in MD vaccine group. [m–o] Negative-control staining for AvBD2 and AvBD4 does not show positive staining, except for only a faint staining in the nucleus of some ureter epithelial cells on the AvBD4 negative-control sections (thin arrows in Figure 5o), suggesting that staining of AvBD2 in the leukocytes and AvBD4 in the surface of microvillus epithelial cells of renal tubules and in the cytoplasm of epithelial cells of the collecting ducts and ureter was specific. Abbreviations: IB/ND, infectious bronchitis with Newcastle disease; MD, Marek's disease.

control, (2) the expression of AvBD4, 5, 6, and 7 was higher in the chicks vaccinated by MD than in control, and (3) AvBD2 was localized in the leukocytes, and AvBD4 was in the epithelium of renal tubules and ducts.

The current results for the expression of TLR3, 7, and 21 support the report by Xu et al. (2015) who showed the expression of TLR1 to 5, 7, 15, and 21 in kidneys of 22-day-old White Leghorn chicks. The expression of these TLR suggests that different viral and bacterial molecular patterns could be recognized to initiate the innate immune response in the kidney. Xu et al. (2015) also reported that, among AvBD1 to 13, all AvBD (except for AvBD2 and 8) were expressed in the kidneys of layer chicks. Mowbray et al. (2018) showed the expression of AvBD6 to 8 in the kidneys of broiler chicks from hatching to 21 d of age. The present study showed the expression of AvBD1 to 13 in the kidney of 3- and 10-day-old chicks. There are differences in the expression profiles (which may be due to the breed and age of chicks), but all 3 studies suggest that AvBD are expressed in the chick kidney.

Immunohistochemical results suggest that AvBD2 was synthesized by the leukocytes in the interstitial tissues and AvBD4 was synthesized by the epithelial cells of renal tubules and ducts in all chicks vaccinated with or without MD and IB/ND vaccines. The leukocytes containing AvBD2 may be heterophils because the synthesis of AvBD2 by chick heterophil-like cells has been reported in previous studies (Terada et al., 2018, 2020b). It is likely that the synthesis of AvBD4 was more developed in the epithelial cells of the caudal renal tract than in the proximal renal tubules because the collecting ducts and ureter showed intense immunostaining. Chen and Itakura (1996) reported that epithelial cells of the lower nephron and ducts are the primary target cells in the IBV-infected chick kidney. The better developed AvBD synthesis capability in the caudal renal tract may play an important role in the defense of these tissues. Furthermore, AvBD4 in the distal ducts (namely the lower part of the renal tract) may play a role in suppressing the pathogenic microbes ascending from the cloaca. Although the reason for differences in the AvBD4 density between the proximal and distal parts of the duct remains unknown, we assume that the cell differentiation of epithelial cells to express AvBD4 and stimuli by luminal contents in the ducts may affect the AvBD4 expression.

The IB/ND vaccination upregulated the expression of TLR7 and 21, whereas MD vaccination upregulated the expression of 4 AvBD in the kidney of 3-day-old chicks (day 2 of vaccination), but not in 10-day-old chicks. Thus, the effects on innate immune functions are likely different between IB/ND and MD vaccines; namely, IB/ND vaccination may modulate the functions to recognize viral ssRNA and microbial DNA by TLR7 and 21, whereas MD vaccination may enhance the potential synthesis of AvBD in the chick kidney for a few days after vaccination. The IB virus is a gamma coronavirus with single-stranded positive-sense RNA genome (Jackwood, 2012), whereas MD virus is a herpesvirus with a double-stranded DNA genome (Couteaudier and Denesvre, 2014). Although we are not aware of the mechanism by which the 2 vaccinations caused different effects on the TLR and AvBD, it may be possible that the differences in the viral antigens in MD and IB/ND vaccines affected expression of the innate immune molecules differently. Xu et al. (2015) reported a different expression response of AvBD when provided the pathogenic IB virus or attenuated IB virus in the chick kidney. In their study, pathogenic IB virus infection tended to upregulate AvBD expression, whereas attenuated IB virus tended to downregulate AvBD expression. Because we have not performed the challenge test using pathogenic microbes, it remains to be determined whether vaccinations affect the function of TLR to recognize microbe patterns and the ability to express AvBD in response to different pathogens. However, the modulation of TLR and AvBD expression by IB/ND and MD vaccinations suggests that those vaccinations may strengthen the prerequisite immunodefense function in the chick kidney under noninfected status.

It has been reported that in humans a vaccination with attenuated vaccines (including Bacillus Calmette–Guerin vaccine, measles vaccine, and oral polio vaccine) induced trained innate immunity through epigenetic and metabolic reprograming of innate immune cells (Netea et al., 2016; De Bree et al., 2018). We also demonstrated that routine multiple vaccinations (vaccines for IB, MD, ND, and infectious bursal disease) caused positive or negative regulation of TLR and AvBD expression in association with epigenetic reprogramming by histone modification (Kang et al., 2019). However, the effects of IB/ND and MD vaccinations for modulating the expression of TLR and AvBD in the kidneys may not be sustained for long periods because differences in expression were not identified between the control and vaccinated groups in 10-day-old chicks. Thus, the effects of vaccination on innate immune functions may differ between kidney and ovarian cells. The increase in TLR and AvBD expression in kidneys may be due to the temporal stimulatory effects of antigens in vaccines, and the effects might decline with decreasing the circulating antigen levels in the kidney.

In conclusion, we suggest that the expression of innate immune molecules, namely TLR and AvBD, in the kidneys may be modulated by MD and IB/ND vaccination when performed on day-old chicks. Although the effects of both vaccinations may not persist after 10 d, the enhanced expression of those innate immune molecules may support the innate immunodefense functions in kidneys of young chicks. Because we found the possibility that vaccinations could modulate the expression of TLR and AvBD, it is expected to develop more effective vaccines for enhancing the expression of innate immune functions in the future studies.

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Conflict of Interest Statement: The authors declare that they have no conflict of interest.

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