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Differential modulation of avian β-defensin and Toll-like receptor expression in chickens infected with infectious bronchitis virus

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Abstract The host innate immune response either clears invading viruses or allows the adaptive immune system to establish an effective antiviral response. In this study, both pathogenic (passage 3, P3) and attenuated (P110) infectious bronchitis virus (IBV) strains were used to study the immune responses of chicken to IBV infection. Expression of avian β-defensins (AvBDs) and Toll-like receptors (TLRs) in 16 tissues of chicken were compared at 7 days PI. The results showed that P3 infection upregulated the expression of AvBDs, including AvBD2, 4, 5, 6, 9, and 12, while P110 infection downregulated the expression of AvBDs, including AvBD3, 4, 5, 6, and 9 in most tissues. Meanwhile, the expression level of several TLRs showed a general trend of upregulation in the tissues of P3-infected chickens, while they were downregulated in the tissues of P110-infected chickens. The result suggested that compared with the P110 strain, the P3 strain induced a more pronounced host innate immune response. Furthermore, we observed that recombinant AvBDs (including 2, 6, and 12) demonstrated obvious anti-viral activity against IBV in vitro. Our findings contribute to the proposal that IBV infection induces an increase in the messenger RNA (mRNA) expression of some AvBDs and TLRs, which suggests that AvBDs may play significant roles in the resistance of chickens to IBV replication.

Keywords Chicken · Avian β-defensins · Toll-like receptors · Infectious bronchitis virus

Introduction

Respiratory pathogens, including infectious bronchitis virus (IBV), continue to have serious health and economic impacts on the poultry industry (Cavanagh 2003; Fang et al. 2007) despite the existence of vaccination programs. IBV is a coronavirus that causes an acute, highly contagious respiratory disease, and some strains can also cause nephritis. In addition, the reproductive tract of layer and breeder birds can be affected, causing decreased egg quality and production. As well as being an economically relevant pathogen in poultry, IBV also bears close resemblance to human coronaviruses, such as the pathogen severe acute respiratory syndrome coronavirus and Middle East respiratory syndrome coronavirus. Up to date, while much progress has been made in understanding the adaptive immune response to these respiratory pathogens (Ignjatovic and Galli 1995; Gelb et al. 2005), the innate immune response remains poorly characterized. The innate immune system is the first line of host defense against an invading viral pathogen, and the outcome of an infection is dependent on the ability of host cells to recognize the invading pathogen and activate appropriate signaling pathways (Liao et al. 2011). If the innate immune response fails, pathogens are susceptible to triggering what is referred to as a cytokine storm (Tisoncik et al 2012). The innate immune responses activated

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as a result of virus interaction with host cells play an essential role in the outcome of viral infection. The response is induced by recognition of specific components of the infecting pathogenic microorganisms known as pathogen-associated molecular patterns (PAMPs) by special receptors (Garcia et al 2007; Takeuchi and Akira 2009; Liao et al 2011). Toll-like receptors (TLRs) are the members of such receptors. To date, ten TLRs have been identified in chickens, namely TLR1-1 (TLR1A), TLR1-2 (TLR1B), TLR2-1 (TLR2A), TLR2-2 (TLR2B), TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21. Interaction of TLRs with their specific ligands leads to an innate immune response, which leads to the expression and secretion of both pro-inflammatory cytokines and defensins as well (Abdel-Mageed et al. 2014).

Cytokines and chemokines are key mediators that initiate immune responses and ultimately shape the adaptive immune responses via chemoattraction of adaptive immune cells, activation of macrophages, differentiation of Th1 from T cells and control of intracellular pathogens, promoting protective adaptive immunity, upregulation of co-stimulatory molecules required for antigen presentation, and prevention of fatal immunopathology. Furthermore, they may be involved in both clearance of pathogens and pathological tissue damage (Carpenter and O'Neill 2007; Quintana et al. 2011). Defensins are small polypeptides that play an important role in the innate host defense against bacteria, fungi, and some viruses (Ganz and Lehrer 1999; Lehrer and Ganz 2002; Wang et al. 2010; Ma et al. 2011, 2012a, b, c, 2013, 2014; Barabas et al. 2013). In addition to their antimicrobial effect, defensins have been shown to activate monocyte-derived dendritic cells and modulate interferon (IFN)-γ production in antigen-presenting cells, suggesting that they may also play a role in the shaping of adaptive immunity (Presicce et al. 2009; Nijnik et al. 2012; Quintana et al. 2011). IBV infection of chickens causes an acute and highly contagious respiratory disease (Fang et al. 2007; Shen et al. 2004). It initially infects the upper respiratory tract, where it is restricted to the ciliated and mucus-secreting cells (Raj and Jones 1997). Following infection of chickens by IBV, IFN was detected in the trachea and lungs (Otsuki et al. 1987). Meanwhile, the production of pro-inflammatory cytokines IL-6 and IL-8 were also greatly increased during IBV infection of cultured cells (Liao et al. 2011). While the induction of cytokines has recently been observed in chickens infected with IBV (Pei et al. 2001; Liao et al. 2011), the regulation of defensins in the tissues of chicken, and their significance in the pathogenesis of IBV, remains poorly described. In this study, we demonstrate that prestimulation with a pathogenic IBV strain (passage 3 (P3)) results in a beneficial expression of several avian β-defensins (AvBDs) in the tissues of chicken compared with an attenuated strain (P110). Our results further provide evidence that this regulation of AvBD expression may be ascribed to their antiviral effect against IBV and seems to be mediated by TLRs.

Materials and methods

Viral infection and virus detection in kidney tissues of the infected chickens

One-day-old, specific pathogen-free (SPF) chickens were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Harbin, China).

The pathogenic IBV strain CK/CH/LHLJ/04 V (P3) and attenuated (P110) strain (Liu et al. 2009) were used in this study. The P3 and P110 viruses were propagated once in 9-day-old embryonated SPF chicken eggs, as described previously (Liu et al. 2013), to obtain titers of 10^6–10^8 50 % median embryo infectious doses (EID_{50}) per 0.1 mL. Before use, viruses isolated from the allantoic fluids of inoculated eggs were confirmed by negative-contrast electron microscopy, real-time PCR (RT-PCR), and DNA sequencing (Liu et al. 2009).

Forty-five 1-day-old SPF White Leghorn chicks were housed in separate isolators and divided into three groups of 15 chicks each at 15 days of age. Chickens in groups 1 and 2 were inoculated either with P3 or with P110 respectively, by intraocular application, with a dose of 10^6 EID_{50} per chick. Birds in group 3, which served as the negative control, were mock-inoculated with sterile allantoic fluid. Five birds from each of the three groups were killed humanely at 7 days post-inoculation (dpi). The proventriculus, liver, spleen, lung, kidneys, duodenum, pancreas, small intestine, large intestine, cecum, cecal tonsil, rectum, bursa of Fabricius, trachea, Harderian gland, and thymus of each bird were collected, rinsed in cold sterile saline, snap-frozen in liquid nitrogen, and stored at −70 °C until further use. Serum samples were collected at 12 dpi from groups 1 and 2, as well as from control birds, and assayed in triplicate using a commercial enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories, Inc., Westbrook, MA, USA) according to the manufacturer's instructions. The remaining birds in groups 1 and 2 were killed humanely 15 dpi, and blood was collected for antibody tests, as described above. Serum-to-positive ratios were calculated as described previously (Liu et al. 2006). Individual serum titers were expressed as absorption values at an optical density (OD) at 650 nm according to the manufacturer's instructions.

Real-time PCR

For RNA extraction, equal amounts of tissues (1 g) were excised in cold, RNase-free phosphate-buffered saline (PBS) as described previously (Ma et al. 2012a, b, c; Liu et al. 2013). Briefly, tissue samples were homogenized, ground, or cut into small pieces before being adjusted to a 10 % (*w*/*v*) suspension

in PBS. Nucleic acid extractions were prepared from 50 μL of homogenized samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was air-dried for 2–10 min, redissolved in 40 μL of RNase-free water, and stored at −70 °C until use. To evaluate RNA quality, the ODs of RNA at 260 and 280 nm were examined. The OD260 to OD280 ratios were within 1.8 to 2.2 (data not shown). All of the processes were performed under RNase-free conditions.

One-step RT-PCR was performed using the One-step Real-time PrimeScript® RT-PCR kit (TaKaRa Biotechnology, Dalian Co., LTD.) on a LightCycler® 480 II RT-PCR system (Roche, Basel, Switzerland) according to previous studies (Ma et al. 2012a, b, c; Liu et al. 2013). Serial tenfold dilutions of plasmids containing chicken 18S ribosomal RNA (18S rRNA), AvBDs 1–14; TLRs 1–5, 7, 15, and 21; and inducible nitric oxide synthase (iNOS) were used as controls. Chicken TLRs 1–5 and 7 are the functional orthologs of mammalian TLRs 1–5 and 7, and chicken TLR15 and 21 have no orthologs in mammals (Keestra et al. 2013). Plasmids containing these genes were stored in our laboratory. 18S rRNA was selected as the most stable reference gene across all samples from a panel of potential genes, including 18S rRNA, β-actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), from chickens (data not shown). The initial DNA concentrations were quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The optimal concentrations of forward and reverse primers were determined by titrating 1-, 2.5-, 5-, and 10-μmol concentrations (data not shown). As a result, the real-time RT-PCR reactions (25 μL) contained 1 μL (2.5 μmol) of each primer, 12.5 μL of 2× One Step SYBR RT-PCR Buffer 4, 1 μL of Primer Script 1 Step Enzyme Mix 2, 7.5 μL of nuclease-free water, and 2 μL of either a template RNA sample or a known concentration of standard plasmid. The primers are shown in Table 1.

The presence of IBV in tissue samples of the infected birds was confirmed by detection of the viral RNA using the One-step Real-time PrimeScript® RT-PCR kit (described above) according to the following steps: reverse transcription at 42 °C for 10 min, denaturation at 95 °C for 10 s, and 40 cycles at 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 10 s, followed by a cooling step at 40 °C for 10 s (Cong et al. 2013).

Standard curves were obtained by plotting the crossing cycle number (the threshold or crossing point) as a function of the log plasmid DNA concentration for each target sequence. The concentration of target complementary DNA (cDNA) in a sample was deduced from the crossing point obtained and from the corresponding standard curve. The presence of IBV was expressed for each sample as the viral RNA copy number. The results for the other genes were expressed for each sample as the copy numbers of each target cDNA normalized to 10^9 times the copy number of the reference gene, 18S rRNA, using the following formula: (target gene cDNA copy number/18S rRNA cDNA copy number)$\times 10^9$. All real-time RT-PCR products were confirmed by electrophoresis on 2.0 % agarose gels, followed by ethidium bromide staining. Furthermore, the specificities of the reactions were checked by cloning and sequencing three independent PCR products. This experiment was performed in three independent experiments, with five replicates per experiment.

Protein expression and purification

The DNA fragments that encoded AvBD2, 6, and 12 were amplified by PCR from their respective plasmids (stored in our laboratory) using the primers (shown in Table 2). Amino acid sequences of these recombinant AvBDs are listed in Fig. 5a.

The PCR products, which contained the coding sequence of AvBD2, AvBD6, or AvBD12 flanked by *Eco*RI/*Xho*I sites, were inserted into the same sites of the pProEX-HTa expression vector (Invitrogen). The resultant plasmids were designated as recombinant AvBD2, 6, and 12, respectively, and were sequenced again. The constructs that were confirmed to contain these AvBDs were transformed into competent *Escherichia coli* BL21 (DE3) cells and induced subsequently with 0.6 mmol/L isopropyl-β-D-1-thiogalactopyranoside (IPTG). The proteins were purified and refolded using a purification and refolding kit (Novagen, Darmstadt, Germany), according to the manufacturer's instructions. The proteins were quantified by the Bradford assay (Bradford 1976). The soluble fractions were recovered, analyzed on 15 % (*w*/*v*) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V using the Mini-protean III system (Bio-Rad, Beijing, China), and stained with Coomassie brilliant blue R-250 (Schägger and von Jagow 1987).

Antiviral activity of recombinant His-tagged AvBDs against IBV in vitro

The antiviral activities against IBV strain CK/CH/LHLJ/04V P3 of a recombinant 6× His-tagged peptide and recombinant His-tagged AvBD2, 6, and 12 were determined according to methods described for neutralization assays (OIE 2004). A dose of 10^6 EID_{50} of IBV was serially diluted tenfold and treated with equal volumes of 6× His-tagged peptide, or His-tagged AvBDs (final concentration, 300 μg/mL in PBS), or PBS at 37 °C for 60 min. The mixture was inoculated into 11-day-old SPF chicken embryo (200 μL administered per egg). The inoculated chicken embryos were incubated at 37 °C. Fifty microliters of allantoic fluid were collected from each

Table 1 Real-time PCR primer sequences used for profiling AvBDs, TLRs, iNOS, IBV strain P3, and 18S rRNA

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number	Amplicon size (bp)	Reference
AvBD1	GATCCTCCCAGGCTCTAGGAAG	GCCCCATATTCTTTTGC	NM_204993	137	a
AvBD2	GGTTGTCTTCGCCCCGGCGGGA	TTATGCATTCCAAGGCCATTTG	NM_204992	137	a
AvBD3	GAACTGCCACTCAGTGCAGAAT	ATGGGGGTTGTTTCCAGGAGC	NM_204650	182	a
AvBD4	TCATCGTGCTCCTCTTTGTG	AATACTTGGGACGGCATAGC	NM_001001610	153	a
AvBD5	GCTGTCCCTTGCTCGAGGATT	GGAATACCATCGGCTCCGGC	NM_001001608	139	a
AvBD6	GTCAGCCCTACTTTTCCAGC	GCCCACCTGTTCCTCACAC	NM_001001193	143	a
AvBD7	ACCTGCTGCTGTCTGTCCTC	TGCACAGCAAGAGCCTATTC	NM_001001194	173	a
AvBD8	TTCTCCTCACTGTGCTCCAA	AAGGCTCTGGTATGGAGGTG	NM_001001781	124	a
AvBD9	GCTTACAGCCAAGAAGACGCT	GGAGCTAGGTGCCCATTTGCA	NM_001001611	145	a
AvBD10	GGCTCAGCAGACCCACTTTTCC	CTGCGCCGGAATCTTGGCAC	NM_001001609	146	a
AvBD11	GGTCTCGGCTTGCCCAGAGAC	ATGGAAGTCTGATGTAGTGTC	AY621313	150	a
AvBD12	GGAACCTTTGTTTCGTGTTCA	GAGAATGACGGGTTCAAAGC	AY534898	155	a
AvBD13	GATCCTCCAGCTGCTCTTTG	AGTGGCCATGGTTGTTCCT	AY701473	104	a
AvBD14	CATATTCCTCCTGTTTCTTGTTCTC	GCCAGTCCATTGTAGCAGGT	AM402954	150	a
TLR1	AGTCCATCTTTGTGTTGTCGCC	ATTGGCTCCAGCAAGATCAGG	NM_001081709	127	a
TLR2	GATTGTGGACAACATCATTGACTC	AGAGCTGCTTTCAAGTTTTCCC	XM_001232192	294	a
TR3	TCAGTACATTTGTAACACCCCGCC	GGCGTCATAATCAAACACTCC	NM_001011691	256	b
TLR4	AGTCTGAAATTGCTGAGCTCAAAT	GCGACGTTAAGCCATGGAAG	NM_001030693.1	190	a
TLR5	CCTTGTGCTTTGAGGAACGAGA	CACCCATCTTTGAGAAACTGCC	NM_001024586	124	a
TR7	TTCTGGCCACAGATGTGACC	CCTTCAACTTGGCAGTGCAG	NM_001011688	219	b
TLR15	GTTCTCTCTCCCAGTTTTGTAAATAGC	GTGGTTCATTGGTTGTTTTTAGGAC	NM_001037835	262	a
TLR21	TGCCCCTCCCACTGCTGTCCACT	AAAGGTGCCTTGACATCCT	NM_001030558	112	a
iNOS	GAACAGCCAGCTCATCCGATA	CCCAAGCTCAATGCACAACTT	GGU46504	103	–
18S rRNA	TCAGATACCGTCGTAGTTCC	TTCCGTCAATTCCTTTAAGTT	AF173612	154	–
IBV	CTATCGCCAGGGAAATGTC	GCGTCCTAGTGCTGTACCC	FJ641062	174	c

References for the primers are as follows: a = Meade et al. (2009), b = Villanueva et al. (2011), and c = Liu et al. (2013)

egg at 48 and 72 hpi, using a 26-g needle, through a small hole created in the shell near the air sac (Hewson et al. 2012). Viral RNA was extracted from the allantoic fluid and subjected to real-time RT-PCR as described above. The embryos were incubated at 37 °C for 7 days and then chilled at 4 °C and examined for characteristic IBV lesions such as the dwarfing, stunting, or curling of embryos. Embryo mortality recorded in the first 24 h post-inoculation was considered nonspecific. Samples were considered positive if the real-time RT-PCR was positive and embryos show lesions after three blind passages of 7-day duration. This experiment was performed in three independent experiments.

Statistics

Data are expressed as the mean ± SD. Statistical analyses, where appropriate, were performed using one-way analysis of variance (ANOVA) followed by Tukey's test to identify differences between observations and groups using the generalized linear model (GLM) procedure of SAS software (SAS 1996). A P value <0.05 was considered to be statistically significant. Correlations between relative gene expressions of AvBDs and TLRs in the tissue samples of chickens in response to IBV infection were performed using Pearson's tau of SAS software (SAS 1996). P value <0.01 was considered to be statistically significant.

Table 2 PCR primer sequences used for protein expression of the recombinant AvBDs (Ma et al. 2014)

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)
AvBD2	GAATTCATGCCCCGGCGGGACATGCTG	CTCGAGTTATGCATTCCAAGGCCATTTG
AvBD6	GAATTCATGCCCTACTTTTCCAGCCCTATTC	CTCGAGTCAGGCCCACCTGTTCCTCACAC
AvBD12	GAATTCATGGACAGCTGTAACCACG	CTCGAGTCAGGTCTTGGTGGGAGTTG

Results

Clinical signs and viral detection

Similar to uninfected control chickens, no obvious clinical signs were observed in the CK/CH/LHLJ/04V P110-inoculated chickens during the experiment. In contrast, chicks inoculated with strain CK/CH/LHLJ/04V P3 showed obvious clinical signs, such as ruffled feathers and dark, shrunken combs, at 3–13 dpi. Two chicks died during the experiment, and nephritis was observed. The clinical signs of the survived birds tended to gradually disappear after 13 dpi. All chickens inoculated with strain CK/CH/LHLJ/04V P3 showed a positive serum antibody response at 12 and 15 dpi, respectively; however, four and five chickens inoculated with strain CK/CH/LHLJ/04V P110 showed a positive serum antibody response at 12 and 15 dpi, respectively, whereas those in the control group were negative for serum antibody response (Fig. 1a). To confirm the presence of IBV in the infected birds, viral loads in 16 tissue samples of chickens inoculated with the P3 and P110 strains were compared at 7 days PI. Viral RNA was not detected in the proventriculus, liver, spleen, lung, pancreas, or thymus of all five chickens inoculated with the P110 strain (Fig. 1b). In contrast, viral RNA was found in these same tissues in all five chickens inoculated with the P3 strain. Furthermore, the amount of P3 viral RNA was significantly greater than that of P110 viral RNA in the kidneys, duodenum, and large intestine. Similar to the P3 virus, the P110 virus showed an affinity for the bursa of Fabricius and gastrointestinal tract, especially the small intestine, cecal tonsil, and rectum, as reflected by high viral RNA copy numbers demonstrated by real-time RT-PCR. Collectively, these results confirmed the successful IBV infection of SPF chickens.

Virus-induced expression of AvBDs in tissues

AvBDs have been shown to play vital roles in the immune response of birds against viral pathogens. To analyze the expression of AvBDs 1–14 in the tissues of chicken in response to IBV infection, both highly pathogenic (P3) and attenuated (P110) IBV strains were used in this study. Sixteen tissues mentioned above were collected from chickens inoculated with the aforementioned viruses, as well as from control chickens. Compared with the control, both the P3 and P110 strains induced the expression of most of the AvBDs (Fig. 2). Compared with control chickens, the expression of AvBD2 in the large intestine, cecum, cecal tonsil, and thymus; AvBD4 in the trachea and thymus; AvBD5 in the small intestine and thymus; AvBD6 in the kidneys, duodenum, small intestine, large intestine, bursa of Fabricius, and thymus; AvBD9 in the small intestine and thymus; and AvBD12 in the pancreas, small intestine, bursa of Fabricius, and Harderian glands of P3-infected chickens was upregulated. Interestingly, in contrast to the upregulation of expression observed in the tissues of P3-infected chickens, for P110-infected chickens, the expression of nearly all of the AvBDs investigated in this study was downregulated in most of the tissues. In addition, compared with the high level of expression in most of the tissues in both control and P3-infected chickens, the expression of AvBD10 was undetectable in the lung, pancreas, small intestine, and cecum of P110-infected chickens. Similarly, little expression of AvBD11 was detected in the proventriculus, liver, pancreas, small intestine, and Harderian gland in P110-inoculated chickens. In addition, no significant differences in the expression of the other AvBDs were found in response to IBV infection. Because of their marked differences in expression in the tissues of chickens inoculated with IBV P3, as well as in P110-infected and control chickens, AvBD2, 6, and 12 were chosen for further analysis in the study.

Expressions of Toll-like receptors and inducible nitric oxide synthase

TLR gene expression data are illustrated in Fig. 3. The expression level of TLRs showed a general trend of upregulation in the tissues of P3-infected chickens and downregulation in the tissues of P110-infected chickens. Compared with control chickens, we observed an increase in the expression of TLR1 in the small and large intestines, TLR2 in the kidneys and small intestine, and TLR4 in the large intestine and bursa of Fabricius of P3-infected chickens. However, in P110-infected chickens, the expression of TLRs 1–3, 5–7, 15, and 21 was downregulated in most tissues, except for the rectum, when compared with both control and P3-infected chickens.

In contrast to the expression patterns of TLRs, no obvious changes in the expression of iNOS were observed in the tissues of P3-infected chickens, as compared with controls, whereas in P110-infected chicks, iNOS gene expression was downregulated in the proventriculus, pancreas, large intestine, and trachea (Fig. 4).

Relationship between gene expressions of AvBDs and TLRs

It was shown that gene expression of AvBD2 correlated positively with TLRs (1–5, 7, and 21) from P110-infected chicks ($P<0.01$ or $P<0.001$). For P3-infected chickens, significant correlations were observed between AvBD4 expression and expressions of both TLR4 and 5 ($P<0.001$), expression of AvBD5 and expressions of both TLR2 and 7 ($P<0.01$), both AvBD6 and 9 expression and TLR3 ($P<0.01$), expressions of AvBD10 and TLR21 ($P<0.01$), AvBD11 expression and expressions of both TLR1 and 7 ($P<0.01$), and expressions of AvBD12 and TLR4 ($P<0.01$) (Table S1).

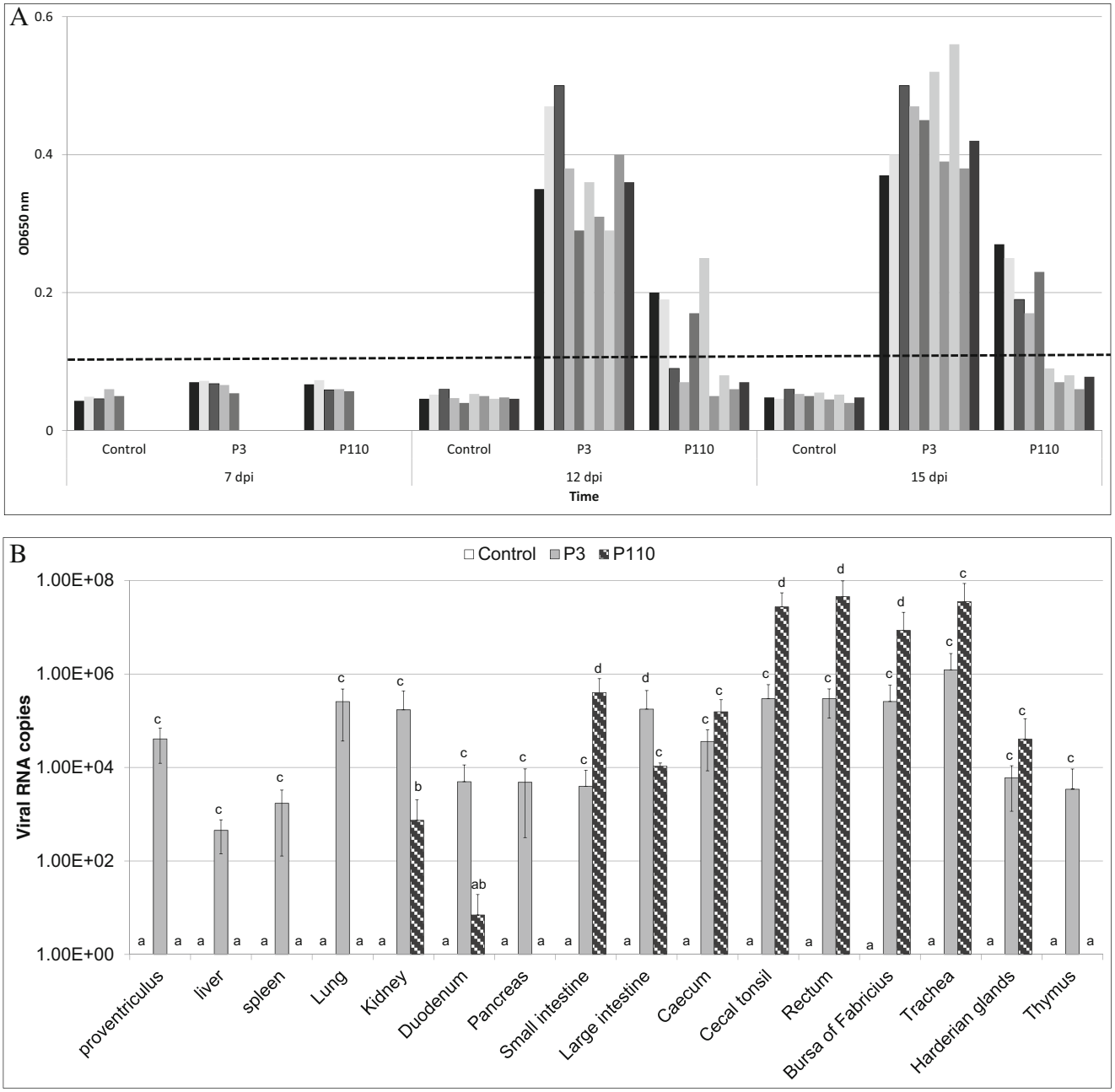

Fig. 1 Humoral immune responses and viral RNA copies in the tissue samples of chickens inoculated with CK/CH/LHLJ/04V P3 or P110. **a** Humoral immune responses in SPF chickens inoculated with IBV CK/CH/LHLJ/04V passages evaluated by indirect ELISA. Five chickens were tested in each inoculated group on day 7 post-inoculation, and ten were tested at 12 and 15 days after inoculation. *Dashes* show the S/P ratios, calculated as described in "Materials and methods" section. Serum samples with S/P ratios equal or above the *dashes* were considered positive, and those below were considered negative. The serum sample S/P ratios of chickens in the negative control group were all below the *dashes* and are not indicated in the figures. **b** Viral RNA copy numbers in the tissue samples from five chickens in each group were measured by real-time RT-PCR on day 7 post-inoculation. All assays were performed in triplicate, with five replicates per experiment, and *each bar* is the mean ± SD. The statistical significance of differences between groups of the control, P3, and P110 was assessed using the generalized linear model (GLM) procedure of SAS software (SAS 1996). The *values with different letters* are significantly different ($P<0.05$)

Expression, purification, and antiviral activities of recombinant AvBD2, 6, and 12 against IBV in vitro

The cDNAs encoding AvBD2, 6, and 12 were produced as His-tagged AvBDs (molecular weights 10–15 kDa) in *E. coli* (Fig. 5b). The His-tagged AvBDs were purified and refolded by using a purification and refolding kit mentioned above.

Viral RNA loads in the allantoic fluids of P3-infected chicken embryos inoculated with 6× His peptide (which served as the control) or His-tagged AvBDs were investigated

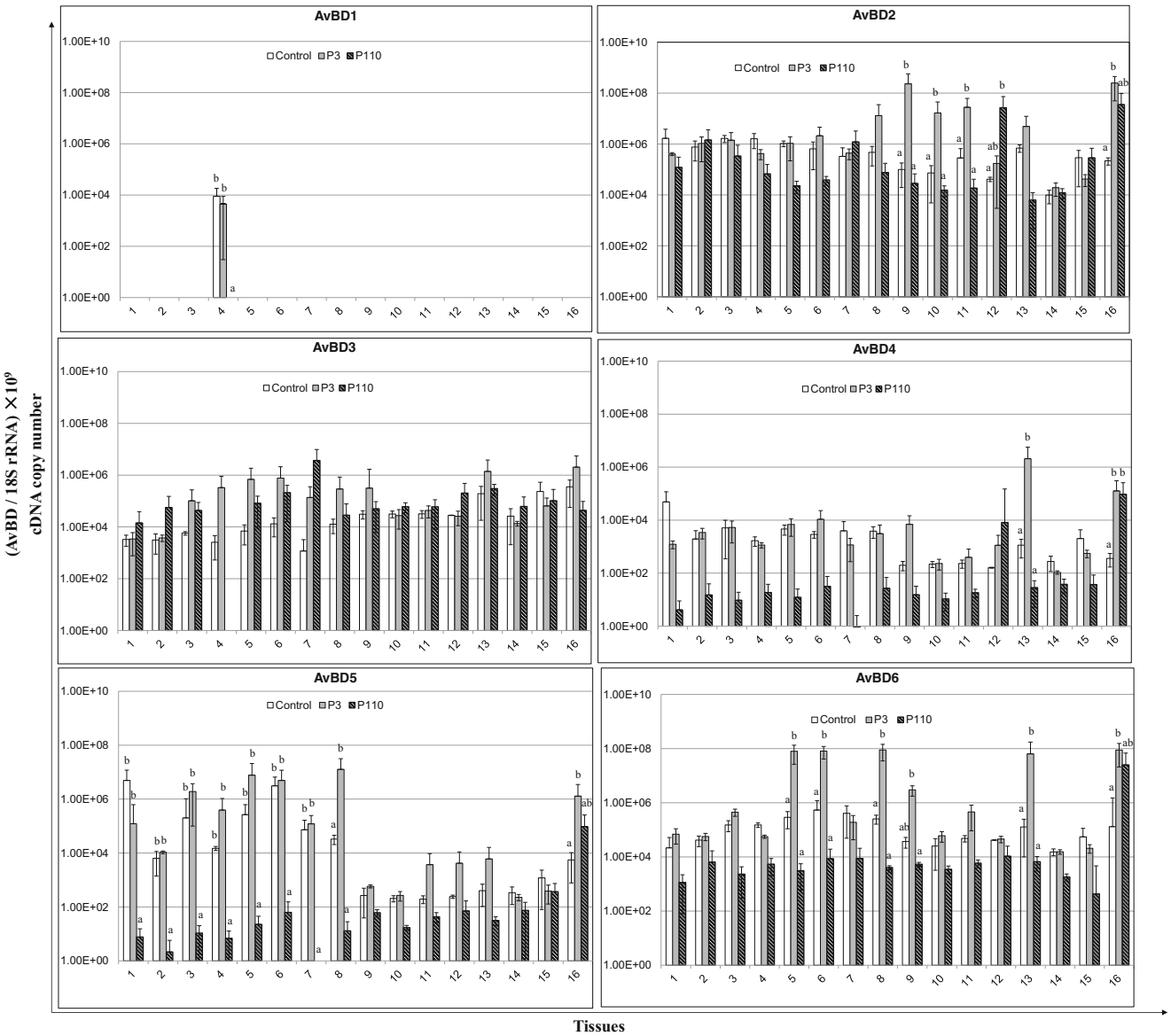

Fig. 2 Relative mRNA expression of avian β-defensins (AvBDs) in the tissues of chickens in response to IBV infection. *1* Proventriculus, *2* liver, *3* spleen, *4* lung, *5* kidneys, *6* duodenum, *7* pancreas, *8* small intestine, *9* large intestine, *10* cecum, *11* cecal tonsil, *12* rectum, *13* bursa of Fabricius, *14* trachea, *15* Harderian gland, *16* thymus. cDNA copy numbers in the tissue samples from five chickens of each group were measured by real-time PCR on day 7 post-infection. Results are shown for each sample as the copy number of each target cDNA normalized to 10^9 times the copy number of the reference gene, 18S rRNA, using the following formula: (target gene cDNA copy number/18S rRNA cDNA copy number)$\times 10^9$. All assays were performed in triplicate, with five replicates per experiment, and *each bar* is the mean ± SD. The statistical significance of differences between groups of the control, P3, and P110 was assessed using the generalized linear model (GLM) procedure of SAS software (SAS 1996). The *values with different letters* are significantly different ($P<0.05$)

by real-time RT-PCR at 48 and 72 h post-infection (Fig. 6). Compared with the control, the viral load of AvBD2-inoculated embryos was significantly decreased at 48 h post-infection ($P<0.05$). Furthermore, low viral loads were observed in the chicken embryos inoculated with AvBD6 or AvBD12 at 48 h post-infection ($P<0.01$). However, no significant change was observed at 72 h post-infection for AvBD2. In contrast, only little viral RNA loads were detected for both AvBD6- and AvBD12-inoculated embryos at 72 h post-infection. The results showed that both AvBD6 and AvBD12 exhibit stronger antiviral activity against IBV (P3) than AvBD2 ($P<0.01$).

Discussion

IBV exhibits mortality rates as high as 30 % in chickens less than 4 weeks old, and it causes losses in egg production (Kameka et al. 2014). Live attenuated viral vaccines are available to control IBV, and they have been very reliable

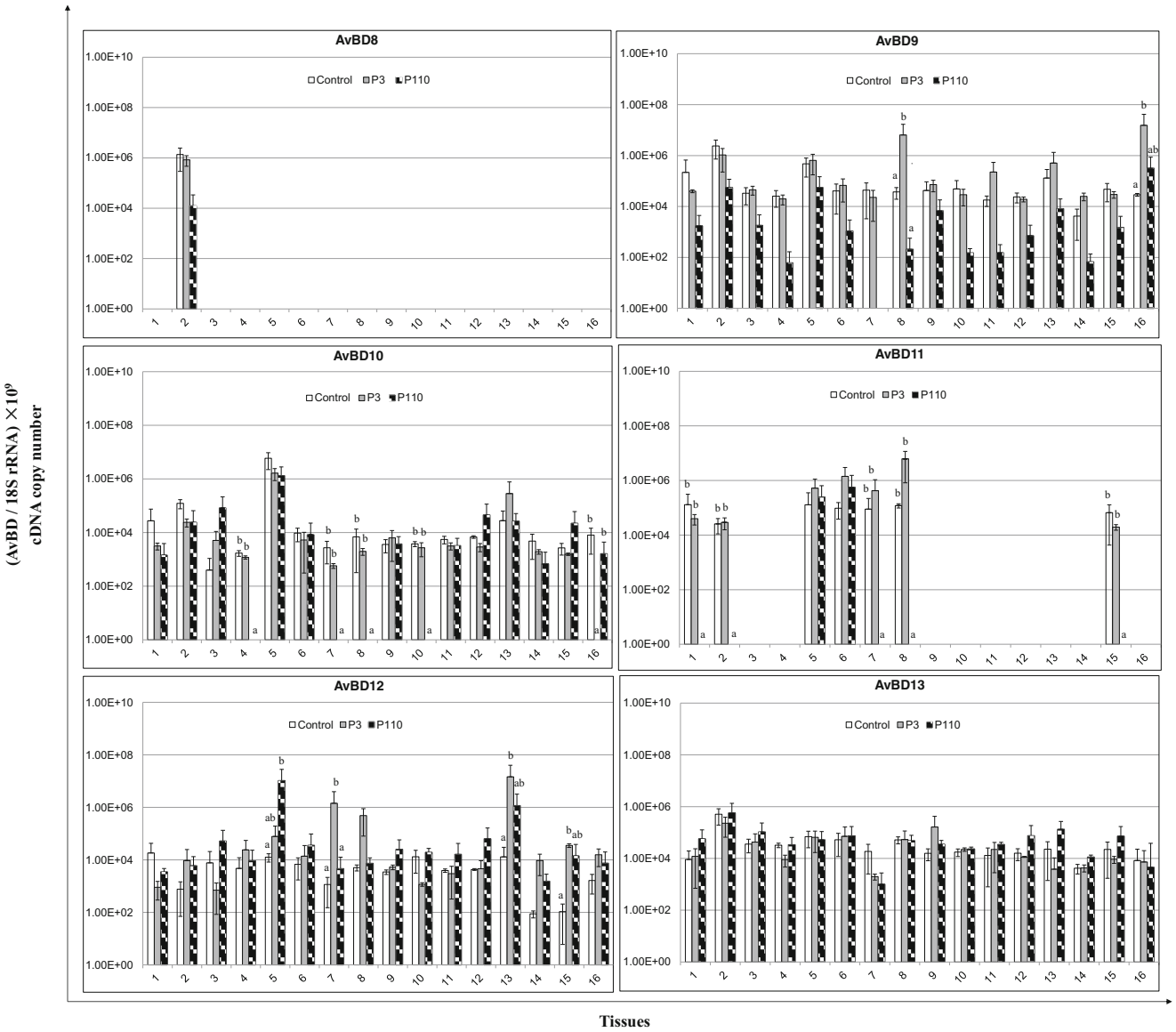

Fig. 2 (continued)

(Cavanagh 2003, 2007; Kameka et al. 2014). However, the emergence of new IBV variants, which led to infectious bronchitis outbreaks in vaccinated flocks, resulted in significant production losses (Xu et al. 2007; Shimazaki et al. 2009; Kameka et al. 2014). Therefore, a novel approach that could be used as an alternative to, or in addition to, the existing means of control is urgently needed. One such approach may be the use of innate immune mediators to empower the innate immune system.

Generally, innate immunity induced by IBV occurs during the early phase of infection (e.g., 3–5 days after infection) (Wang et al. 2006). Our previous report showed that the immunogenicity of IBV strain CK/CH/LHLJ/04V decreased and that the immune response of chickens to a CK/CH/LHLJ/04V strain that was passaged 110 times in embryonated chicken eggs also delayed (Liu et al. 2009). None of the chickens showed seroconversion at 7 dpi with CK/CH/LHLJ/04V P110, and only 40 % of the P110-inoculated chickens showed seroconversion at 12 dpi (Liu et al. 2009). Hence, we investigated the differential modulation of AvBD and TLR expression in chickens infected with CK/CH/LHLJ/04V highly pathogenic P3 at 7 dpi in comparison with P110-infected and control chickens.

Our findings showed that chickens are likely to respond differently to various pathogenic IBV strains. We found that chickens infected with the P3 strain showed an increased production of AvBDs, including AvBD2, 4, 5, 6, 9, and 12, in comparison with the P110 strain, which downregulated the expression of AvBDs, including AvBD3, 4, 5, 6, and 9, in several tissues, including digestive and immune organs. The expression of AvBD2, 6, and 12 was particularly upregulated in P3-infected chickens. Consistent with the present study,

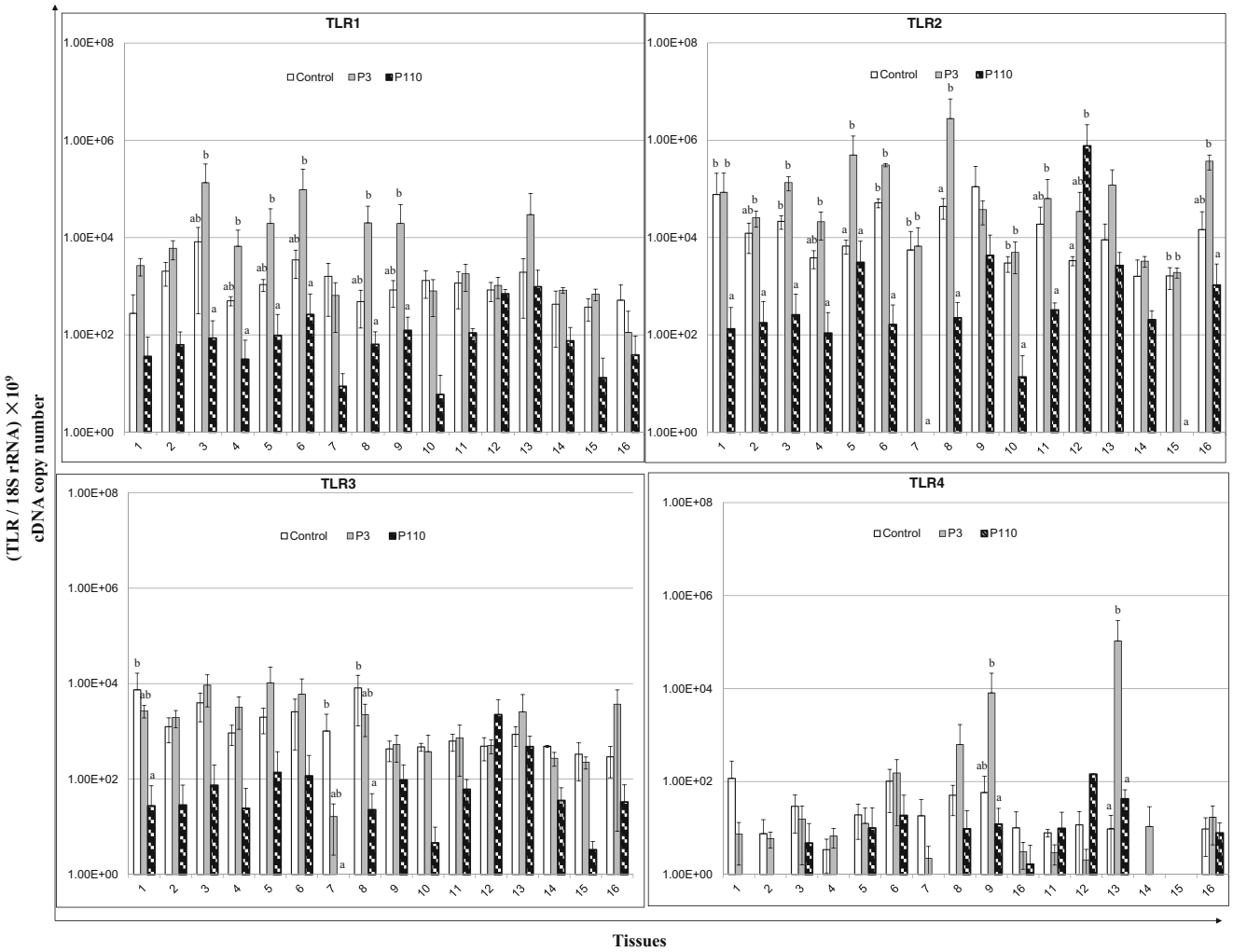

Fig. 3 Relative mRNA expression of Toll-like receptors (TLRs) in the tissue samples of chickens in response to IBV infection. *1* Proventriculus, *2* liver, *3* spleen, *4* lung, *5* kidneys, *6* duodenum, *7* pancreas, *8* small intestine, *9* large intestine, *10* cecum, *11* cecal tonsil, *12* rectum, *13* bursa of Fabricius, *14* trachea, *15* Harderian gland, *16* thymus. cDNA copy numbers in the tissue samples from five chickens of each group were measured by real-time RT-PCR on day 7 post-infection. Results are shown for each sample as the copy number of each target cDNA normalized to 10^9 times the copy number of the reference gene, 18S rRNA, using the following formula: (target gene cDNA copy number/18S rRNA cDNA copy number)$\times 10^9$. All assays were performed in triplicate, with five replicates per experiment, and *each bar* is the mean ± SD. The statistical significance of differences between groups of the control, P3, and P110 was assessed using the generalized linear model (GLM) procedure of SAS software (SAS 1996). The *values with different letters* are significantly different ($P<0.05$)

two recent studies found that the expression of AvBDs in the livers of ducks was significantly upregulated in response to duck hepatitis virus (DHV) infection (Ma et al. 2011, 2012b). Similarly, increased levels of β-defensins in tissues or cells of other animal species, including lung homogenates of lambs and lungs of mice, and even human epithelial cells, have also been observed in vitro or in vivo following viral infections (Duits et al. 2003; Grubor et al. 2004; Proud et al. 2004; Chong et al. 2006, 2008). These results suggested that β-defensins, including AvBDs, play an essential role in the animal defense against viral infection. However, in contrast to the upregulation of expression that was observed in the tissues of P3-infected chickens, several AvBDs were downregulated in P110-infected chickens. It is likely that different signaling pathways of host were activated by the different IBVs with variant pathogenicity though the exact mechanism needed to be further investigated. Similar results were found that TLR7 gene expression was downregulated in variant infectious bursal disease virus (IBDV)-infected bursa; however, in classic IBDV-infected bursas, TLR7 gene expression was upregulated (Rauf et al. 2011).

Similar to the expression pattern of AvBDs, the expression level of TLRs showed a general trend toward upregulation in the tissues of P3-infected chickens, while they were downregulated in the tissues of P110-infected chickens. We observed a significant increase in TLR1 messenger RNA (mRNA) expression in both the small and large intestines, TLR2 mRNA expression in both the kidneys and small intestine, and TLR4

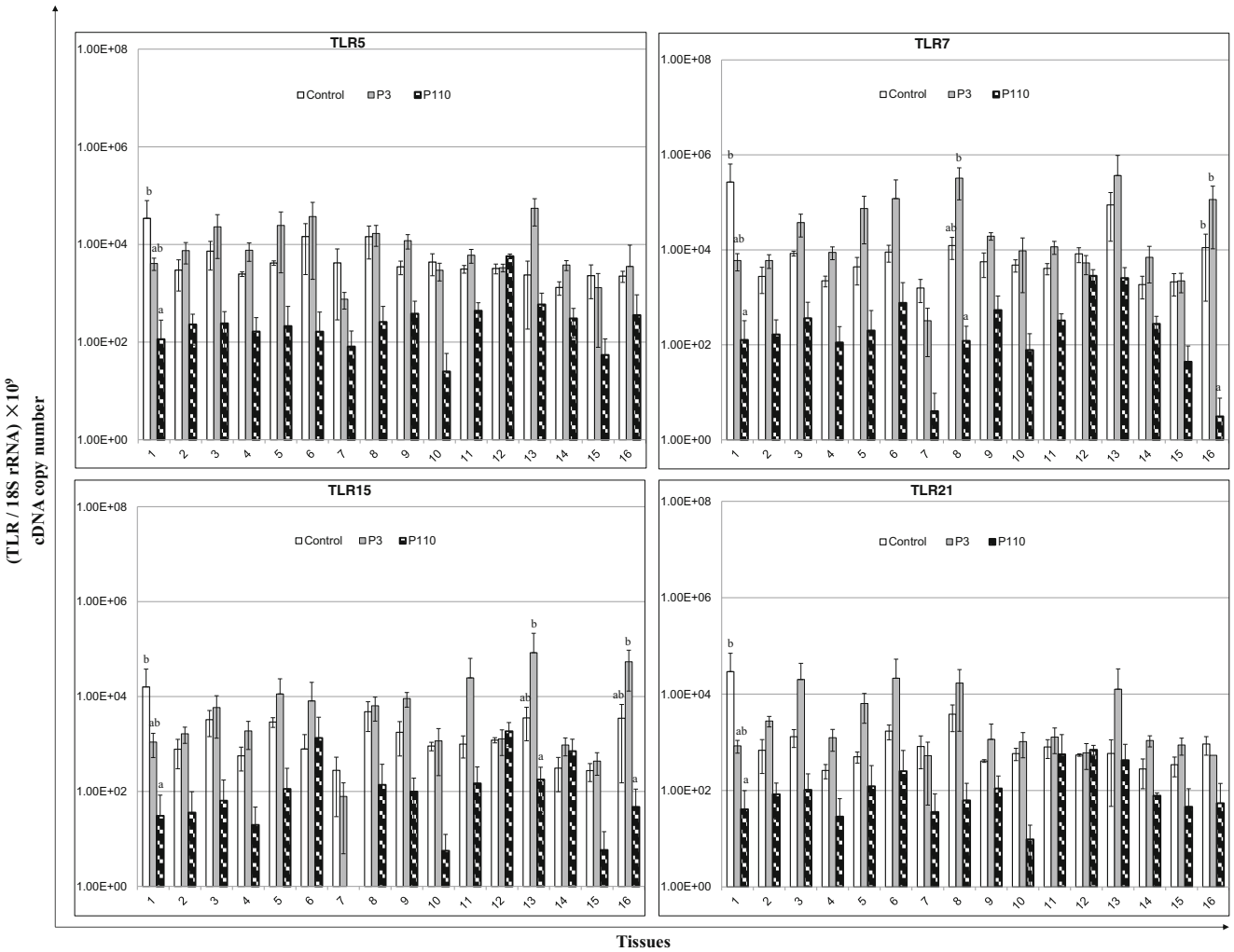

Fig. 3 (continued)

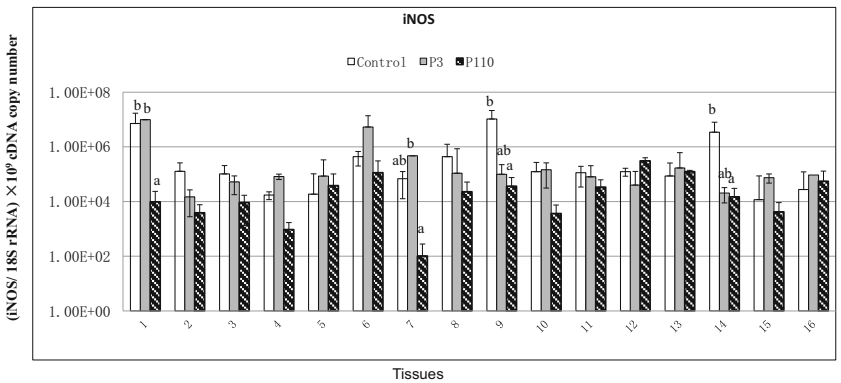

Fig. 4 Relative mRNA expression of inducible nitric oxide synthase (iNOS) in the tissue samples of chickens in response to IBV infection. *1* Proventriculus, *2* liver, *3* spleen, *4* lung, *5* kidneys, *6* duodenum, *7* pancreas, *8* small intestine, *9* large intestine, *10* cecum, *11* cecal tonsil, *12* rectum, *13* bursa of Fabricius, *14* trachea, *15* Harderian gland, *16* thymus. cDNA copy numbers in the tissues from five chickens of each group were measured by real-time RT-PCR on day 7 post-infection. Results are shown finally for each sample as the copy number of each target cDNA normalized to 10^9 times the copy number of the reference gene, 18S rRNA, using the following formula: (target gene cDNA copy number/18S rRNA cDNA copy number)$\times 10^9$. All assays were performed in triplicate, with five replicates per experiment, and *each bar* is the mean ± SD. The statistical significance of differences between groups of the control, P3, and P110 was assessed using the generalized linear model (GLM) procedure of SAS software (SAS 1996). The *values with different letters* are significantly different ($P<0.05$)

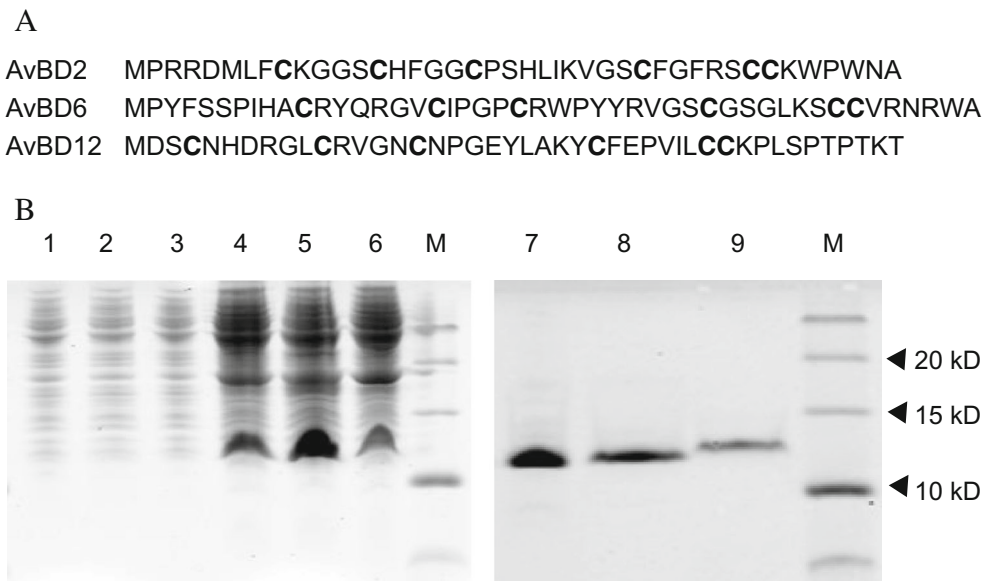

Fig. 5 Amino acid sequences of the recombinant AvBDs and SDS-PAGE analysis of His-tagged recombinant AvBD (His-rAvBD) proteins expressed in *E. coli* BL21 (DE3) cells. **a** Amino acid sequences of the recombinant AvBDs. The conserved six cysteines (*C*) are in *bold*. **b** SDS-PAGE analysis of His-tagged recombinant AvBD (His-rAvBD) proteins expressed in *E. coli* BL21 (DE3) cells. *Lanes*: *1–3*, total protein from BL21 containing AvBD2, 6, and 12 without IPTG induction; *lanes 4–6*, total protein from BL21 containing AvBD2, 6, and 12 with IPTG induction; *lanes 7–9*, purified protein of AvBD2, 6, 12, with IPTG induction; *M*, protein molecular weight marker. *IPTG* isopropyl-β-D-thiogalactoside

mRNA expression in both the large intestine and bursa of Fabricius in P3-infected chickens. These results paralleled the above findings that chickens infected with the P3 strain exhibited an increased production of AvBDs in digestive and immune organs. These observations are in accordance with earlier reports demonstrating that mouse β-defensin 2 activates immature dendritic cells through an interaction with TLR4 (Biragyn et al. 2001) and that human β-defensin 3 activates antigen-presenting cells via TLR1 and TLR2 in an nuclear factor kappa B (NF-κB)-dependent manner (Funderburg et al. 2007, 2011). Previous studies have also shown an increase in the mRNA expression of TLR2, TLR3, TLR6, and TLR7 following IBV immunization (Guo et al. 2008; Wang et al. 2006). The nature and the extent of the innate host responses elicited against IBV are not known. Surprisingly, we did not detect significant changes in TLR3, 5, 7, 15, and 21 expression following infection with the P3 strain. In contrast, a previous study detected a significant increase in both TLR3 and TLR7 mRNA expression in the tracheas of Massachusetts-type IBV-infected chickens (Kameka et al. 2014), which could be due to the use of different virulent vaccine strains in their study. However, because of the downregulation of mRNA expression that was observed for several AvBDs in most tissues in P110-infected chickens compared with P3-infected or control chickens, we were not surprised to see a similar decrease in the expression of TLRs in most of these tissues as well, though the reason was not known and needed to be further investigated. Furthermore, relationship between AvBD and TLR expression analysis showed that AvBDs (2, 4–6, 9–12) correlated positively with different TLRs (including 1–5, 7, and 21) from chickens infected by IBV.

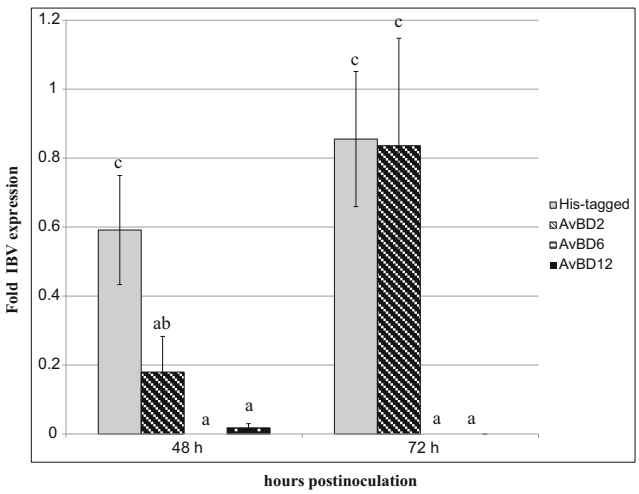

Fig. 6 Antiviral activities of His-rAvBDs against IBV in chicken embryo. Values are indicated as the fold viral RNA copies compared with that of the control (PBS). All assays were performed in three independent experiments, with five replicates per experiment, and *each bar* is the mean±SD. The statistical significance of differences between the His-tagged, AvBD2, AvBD6, and AvBD12 was assessed using the generalized linear model (GLM) procedure of SAS software (SAS 1996). The *values with different letters* are significantly different ($P<0.05$)

Findings obtained in mammals showed that the microbial ligand-mediated induction of TLR signaling in cells, via activation of the transcription factor NF-κB, induces the

expression and secretion not only of pro-inflammatory cytokines (Galvan-Moroyoqui et al. 2011), but also of defensins, such as HBD2 and HBD3, into the extracellular milieu (Abreu et al. 2002; Yoon et al. 2010). In a recent study, it is shown that the expression of AvBD10 can be downregulated by TLR ligands in the uterus of laying hens (Abdel-Mageed et al. 2014). The present results about correlation between TLR expression and AvBD expression in chickens infected with IBV further confirm these findings.

Of the 14 AvBDs detected in the present study, the mRNA expressions of AvBD2, 6, and 12 were found to be upregulated extensively in the tissues following infection with the IBV pathogenic P3 strain and were chosen for further discussion in this study. His-tagged AvBD2, 6, and 12 were produced and purified to further characterize their antiviral activities. In contrast to the extensive studies on the antimicrobial activities of AvBDs, reports on the antiviral activity of such peptides, especially AvBDs, are limited. In avian species, Apl-AvBDs exhibited significant antiviral activity against DHV in vitro (Ma et al. 2011, 2012b). Similarly, our experiment showed that AvBD2, 6, and 12 demonstrated obvious anti-viral activities against IBV in vitro. The results further confirm that AvBDs might play significant roles in the resistance of chickens to IBV replication.

It has been shown that viruses, including IBV, NDV, and AIV H9 subtype infection, can elicit inflammatory responses characterized by recruitment of myeloid or lymphoid cells. Consequently, the cellular types of tissues harvested from pathogenic and attenuated virus-inoculated chickens for gene expression analysis may be different due to immune cell infiltration. As a result, the changes revealed by gene expression analysis of some genes such as AvBDs and TLRs that could also be expressed in immune cells in the course of viral infection may be caused or partly caused by cellular heterogeneity occurring during the inflammatory response to viral infection (including infiltration by innate and adaptive immune cells). This needed to be further investigated.

In spite of the novel aspect our work on the evaluation of differential modulation of AvBD, TLR, and iNOS expression in response to IBV infection in vivo, analysis of expression of these molecules may not be sufficient to completely understand IBV immunologic mechanisms. In addition, the underlying mechanism of the natural host's response to attenuated IBV strain (P110) could not be explained and remained unknown. Furthermore, whether known chicken TLR ligands can induce AvBD expression in vivo or in vitro in chickens needs to be studied further. Therefore, further analysis of the above mentioned concerns is warranted.

In conclusion, this study reports striking difference in the activation of host responses by a pathogenic IBV strain (P3) and an attenuated IBV strain (P110). Compared with the P110 strain, the P3 strain induced a more production of AvBDs and TLRs. The present study highlights some of the underlying mechanisms of induction of host responses by IBV in chickens. Further studies may employ these findings to select AvBDs for enhancing immune responses in chickens.

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Ethics statement The animals had received humane care throughout all the procedures in accordance with the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/openbook.php?record_id=5140&page=R1). All animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang province, China (License no. SQ20130507).

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