Host Avian Beta-Defensin and Toll-Like Receptor Responses of Pigeons following Infection with Pigeon *Paramyxovirus* Type 1

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The high morbidity and mortality in pigeons caused by pigeon paramyxovirus type 1 (PPMV-1) highlights the need for new insights into the host immune response and novel treatment approaches. Host defense peptides (HDPs) are key components of the innate immune system. In this study, three novel avian β -defensins (AvBDs 2, 7, and 10) were characterized in pigeons and shown to possess direct antiviral activity against PPMV-1 *in vitro*. In addition, we evaluated the mRNA expression of these AvBDs and other immune-related genes in tissues of 2-month-old infected pigeons at 3 and 7 days postinfection. We observed that the expression of AvBD2 in the cecal tonsil, lungs, and proventriculus, as well as the expression of AvBD10 in the spleen, lungs, proventriculus, and kidneys, was upregulated in infected pigeons. Similarly, the expression of both Toll-like receptor 3 (TLR3) and TLR7 was increased in the spleen, trachea, and proventriculus, while TLR15 expression was increased only in the lungs of infected pigeons. In addition, inducible nitric oxide synthase (iNOS) expression was upregulated in the spleen, the bursa of Fabricius, the trachea, and the proventriculus of infected pigeons. Furthermore, we observed a high correlation between the expression of AvBD2 and the expression of either TLR7 or TLR15, as well as between AvBD10 expression and either TLR3 or TLR7 expression in respective tissues. The results suggest that PPMV-1 infection can induce innate host responses characterized by the activation of TLRs, particularly TLR3 and TLR7, AvBDs (2 and 10), and iNOS in pigeons.

The paramyxoviruses are a subfamily of the *Paramyxoviridae* family (order *Mononegavirales*) of enveloped, nonsegmented negative-strand RNA viruses (NNSV), which also includes the *Pneumovirus* subfamily. Based largely on antigenic cross-reactivity and neuramidase activity, paramyxoviruses currently are classified into seven genera: *Rubulavirus*, *Avulavirus*, *Henipavirus*, *Morbillivirus*, *Respirovirus*, *Ferlavirus*, and *Aquaparamyxovirus* (1). Members of the paramyxovirus family show diverse tissue tropism and infect a variety of species in a fashion that does not appear to be specific to genus classification.

Newcastle disease virus (NDV), a member of the genus *Avulavirus* in the family *Paramyxovirus*, is the causative agent of Newcastle disease (ND), a highly contagious disease that may result in high morbidity and mortality in poultry (2). Pigeon paramyxovirus type 1 (PPMV-1) viruses are variant strains of NDV associated with infections of pigeons, and they have a worldwide distribution. Several ND outbreaks in chickens have been attributed to PPMV-1, which makes it a real threat to the poultry industry (3–5).

Despite control measures, including heightened biosecurity and intensive vaccination, ND is still a serious problem in the poultry industry around the world. Despite extensive research characterizing the pathogenesis of different isolates and pathotypes, limited data about the host response to NDV have been reported. NDV infection *in vitro* results in nitric oxide (NO) induction in chicken heterophils and peripheral blood mononuclear cells, alpha and beta interferon (IFN- α and IFN- β , respectively) mRNA detection in macrophages, and gamma interferon (IFN- γ) mRNA production in peripheral blood mononuclear cells (6, 7). Similarly, results regarding NDV infection *in vivo* showed that the mRNA expression of IFN- γ and interleukin-6 (IL-6) was enhanced at day 2 in the spleen of chickens (8). While useful, such data are insufficient to understand the nature of the

host response to NDV or the underlying host immune mechanisms related to viral pathogenesis. Despite decades of research characterizing the pathogenesis of different isolates, little is known about the molecular mechanisms of disease caused by NDV in the host.

Overall, the innate immune response to virus infection is an immediate reaction designed to control and inhibit virus multiplication and to aid the developing pathogen-specific protection through the adaptive immune response. Host cells use various receptors to detect viral infections by recognizing pathogen-associated molecular patterns (PAMPs) and subsequently inducing an antiviral response. Among these are Toll-like receptors (TLRs) (9, 10). The virus is first recognized by host sentinel proteins, including TLR proteins, which results in rapid signaling and transcription factor activation that lead to the production of soluble factors, such as cytokines and host defense peptides (HDPs), designed to limit and contain viral replication (7).

Although the importance of cytokines in the host response to NDV infection has been demonstrated, the production of HDPs,

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TABLE 1 PCR primer sequences and predicted product lengths

Target mRNA (purpose)	Primer sequence (5'-3') ^a		Product size (bp)	GenBank accession no.
	Sense	Antisense		
AvBD2 (RT-PCR and qPCR)	ACCATGAGGATCCTTTACCTGC	TTATGCATTCCAAGGCCATTG	198	KF951417
AvBD7 (RT-PCR and qPCR)	ACCATGAGGATCCTTTACCTGC	GAGGTGCTTGAGACATTGTTGTAGC	312	KF951416
AvBD10 (RT-PCR)	GGATCCATGAAGATCCTCTGCCTGCTCTTC	GAATTCCTGCGCCGGAATCTTGGCACAGC	216	KF951415
AvBD10 (qPCR)	GGTTCTGCAGATCCTATTTTCC	CCATCTCCTGCTCAAAATCCA	147	KF951415
TLR2 (qPCR)	GATTGTGACAACATCATTGACTC	AGAGCTGCTTCAAGTTTCC	294	XM_005500842
TLR3 (qPCR)	TCAGTACATTTGTAAACACCCGCC	GGCGTCATAATCAAACTCC	256	XM_005500210
TLR5 (qPCR)	CCTTGTGCTTTGAGGAACGAGA	CACCCATCTTTGAGAACTGCC	124	XM_005511337
TLR7 (qPCR)	TTCTGGCCACAGATGTGACC	CCTTCAACTTGGCAGTGCAG	219	XM_005512700
TLR15 (qPCR)	GTTCTCTCTCCAGTTTGTAAATAGC	GTGGTTCATGGTTGTTTTAGGAC	262	KR018385
iNOS (qPCR)	GAACAGCCAGCTCATCCGATA	CCCAAGCTCAATGCACAACCTT	103	XM_005503254
β-Actin (qPCR)	AGGCTACAGCTTCACCACCAC	CCATCTCCTGCTCAAAATCCA	95	AB618546
AvBD2 (protein expression)	GAATTCATGCCTCAGCGGAGATGT	CTCGAGTTATGCATTCCAAGGCCA	141	KF951417
AvBD7 (protein expression)	GAATTCATGCCTTACATTCTGGGCG	CTCGAGTCAGCCCGTCTCCACC	156	KF951416
AvBD10 (protein expression)	GGTACCATGCCTATTTTCTGACACCG	AAGCTTTTGAATTCCTGCGCCGG	159	KF951415

^a Sequences of the corresponding restriction sites are underlined.

originally called antimicrobial peptides, and the potential role of HDPs in avian immunity against NDV have not been described. HDPs are a diverse group of small, cationic, and antimicrobial polypeptides mainly expressed by epithelial barriers and immune cells that play an important role in the innate host defense against bacteria, fungi, and some viruses (11–14). It is well established that HDPs lead to the differentiation, activation, and chemotaxis of multiple types of leukocytes, inhibit lipopolysaccharide (LPS)-induced effects, and enhance phagocytosis, DNA uptake, and wound healing (15–17). It is clear that these peptides form a vital, but often overlooked, component of the innate immune system. For avian species, three main classes of HDPs have been described. These are β-defensins, cathelicidins, and liver-expressed antimicrobial peptide-2 (17–21). Until now, over 40 avian β-defensins (AvBDs) have been detected. They display a wide range of microbicidal or microbiostatic activities against bacteria, fungi, and viruses (17, 19, 21–28). In addition, our recent studies showed that AvBD expression was increased in the tissues of ducks in response to duck hepatitis virus (DHV) infection and in the tissues of chicken in response to infectious bronchitis virus (IBV) infection, and that the regulation of AvBD expression may be ascribed to their antiviral effects, which seem to be mediated by TLRs (14, 24, 26). However, to date, knowledge of the host immune responses in pigeons induced by PPMV-1, particularly regarding AvBD induction, still is lacking.

The scarce information on the host immune responses of pigeons to PPMV-1 infection motivated us to investigate the molecular mechanisms involved in these processes *in vivo*. The goal of the present study is to evaluate the antiviral activities of three recombinant AvBDs (2, 7, and 10) against PPMV-1 *in vitro*, as well as expression patterns of these AvBDs, TLRs, and inducible nitric oxide synthase (iNOS) in infected pigeons *in vivo*.

MATERIALS AND METHODS

Ethics statement. All animal work was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (29). All animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China (license no. SQ20130507).

Animals. A total of 45 pigeons, hatched in captivity, were used in the present study, and they were provided food and water *ad libitum*. All of them lacked NDV-specific hemagglutination inhibition (HI) antibodies.

Before the experiments were performed, their health status was observed for a period of nearly 2 months, and a histopathological examination was performed as well, which confirmed that the pigeons used in this study were clinically healthy.

Nucleotide sequencing of AvBD, TLR, and iNOS genes in pigeons.

Total RNA was prepared from the bone marrow of five healthy 2-month-old pigeons (mentioned above) using TRIzol reagent (Invitrogen, Beijing, People's Republic of China) according to the manufacturer's protocol. Total RNA was purified by precipitation in isopropyl alcohol and diluted in RNase-free water. The RNA was treated with DNase I (1 U per μg of RNA) (Promega, Madison, WI) at 37°C for 1 h, followed by inactivation of DNase I at 65°C for 10 min to remove residual genomic DNA. To evaluate RNA quality, the optical densities of RNA at 260 and 280 nm (OD₂₆₀ and OD₂₈₀, respectively) were examined. The OD₂₆₀/OD₂₈₀ ratios were within 1.8 to 2.0 (data not shown). All of the processes were performed under RNase-free conditions. cDNA was synthesized from RNA using oligo(dT) primers (Invitrogen) in a 40-μl reaction mixture by using a SuperScript III kit (Invitrogen) according to the manufacturer's instructions.

Specific cDNAs were amplified by PCR using *Ex Taq* polymerase (TaKaRa Bio, Inc., Otsu, Shiga, Japan). For the primer design, the nucleotide sequences of β-defensins, TLRs, and iNOS from birds, including chickens (*Gallus gallus*), pigeons (*Columba livia*), ducks (*Anas platyrhynchos*), and geese (*Anser anser*), were selected and aligned by using the Lasergene sequence analysis software package (DNASTar). The consensus sequences were selected and used for primer design (Table 1). The accession numbers of these genes are shown in Table 2. The PCR products were cloned into the pMD18-T vector (TaKaRa) to confirm the amplification, followed by sequencing using the Sanger method. Basic searches were conducted with the basic local alignment search tool (BLAST) using three entire *Columba*_AvBD cDNAs from pigeons. Sequences of the other known AvBDs were selected for sequence comparisons with the three novel *Columba*_AvBDs.

Protein expression, purification, and *in vitro* antiviral activity of *Columba*_AvBDs against PPMV-1. The cDNA fragments that encode the *Columba*_AvBDs were amplified by PCR from the respective plasmids described above using the primers for protein expression shown in Table 1. The PCR products, which comprised either the coding sequence of *Columba*_AvBD2 or *Columba*_AvBD7 flanked by EcoRI and XhoI sites, were inserted into the same sites of the pProEX-HTa vector, while the PCR product comprising the coding sequence of *Columba*_AvBD10 flanked by KpnI and HindIII sites was inserted into the same sites of the pProEX-HTb vector (Invitrogen, Beijing, People's Republic of China). The resulting plasmids were designated recombinant *Columba*_AvBD2,

TABLE 2 Accession numbers of AvBD, TLR, and iNOS genes from other avian species

Gene	Accession number			
	Chicken	Duck	Goose	Pigeon
AvBD2	NM_204992	FJ465147	HQ909024	KF951417
AvBD7	NM_001001194	JF831960		KF951416
AvBD10	NM_001001609	EU833478	HQ909025	KF951415
TLR2	AB046119.2	HQ166194		XM_005500842
TLR3	JF273967	KM434239	KC292270	XM_005500210
TLR4	KF697090	JN048668	KP993296	XM_005498384
TLR5	NM_001024586	KF316966	JN641303	XM_005511337
TLR7	NM_001011688	DQ888645	JN641303	XM_005512700
TLR15	HM773175		JQ014619	KR018385
iNOS	D85422	FJ966247		

*Columba*_AvBD7, and *Columba*_AvBD10 and sequenced again. The constructs that were confirmed to contain these *Columba*_AvBDs were transformed into competent *Escherichia coli* BL21(DE3) cells. Expression of the fusion proteins was induced with isopropyl β -D-1-thiogalactopyranoside (IPTG). The proteins were purified and refolded using a commercial purification and refolding kit (no. 70123-3; Novagen, Darmstadt, Germany) according to the manufacturer's instructions. The fusion proteins were resolved by 12% 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 (30). The concentration of the purified *Columba*_AvBDs was determined by the Bradford method using bovine serum albumin as the standard (31).

The antiviral activities of the three recombinant *Columba*_AvBDs against PPMV-1 strain PI/CH/LHLJ/110822 (32) were determined according to methods described for neutralization assays (<http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>).

Briefly, the optimal concentrations of *Columba*_AvBDs were determined by titrating them to 0, 50, 150, and 250 μ g/ml final concentrations (data not shown). PPMV-1 (10^6 median embryo infectious doses, or EID₅₀) was 10-fold serially diluted and treated with equal volumes of His peptide or the recombinant *Columba*_AvBDs (0 or 300 μ g/ml; final concentrations of 0 or 150 μ g/ml) at 37°C for 60 min, and 200 μ l of the mixture was inoculated into 12-day-old specific-pathogen-free (SPF) pigeon embryos. The inoculated pigeon embryos were incubated at 37°C and observed for 7 days. Sixty microliters of allantoic fluid was collected from each egg at 24, 36, and 48 h postinfection (hpi) via a 26-gauge needle that was inserted through a small hole created in the shell near the air sac (33), and the virus titers were evaluated by hemagglutination (HA) assays.

The embryos that died between 24 h and 7 days postinoculation and those that showed characteristic changes were recorded as positive for PPMV-1 infection. All of the antiviral activity assays against PPMV-1 were performed in three independent experiments with five replicates per experiment.

Hemolysis assay. The hemolytic activities of the recombinant *Columba*_AvBDs were determined as described previously (24–28).

Viral challenge in pigeons and specific gene expression analysis by qPCR. When 2 months old, the remaining 40 pigeons were separated randomly into two groups of 20 per group. Pigeons in one of the groups were inoculated intranasally with a PPMV-1 strain (10^6 EID₅₀ of PI/CH/LHLJ/110822). Pigeons in the control group were mock inoculated with phosphate-buffered saline (PBS). The pigeons were monitored for clinical signs for up to 21 days postinfection (dpi) and killed at 3 and 7 dpi (five individuals at each time point). Twelve tissues, including brain, spleen, bone marrow, Harderian glands, cecal tonsil, bursa of Fabricius, lungs, trachea, proventriculus, stomach muscle, small intestine, and kidneys, were collected from pigeons for quantitative PCR (qPCR) analysis of host genes. OneStep real-time PrimeScript reverse transcription-PCR (RT-PCR) was used to quantify the mRNA levels of the selected genes. The preparation of the qPCR followed the QIME requirement (54). Primers were designed using Primer Express. Briefly, RNA was extracted as de-

scribed above. The assays were performed using 2 μ l of total RNA and the OneStep real-time PrimeScript RT-PCR kit (TaKaRa Biotechnology, Dalian Co., Ltd.) in a 20- μ l reaction mixture on a LightCycler 480II real-time PCR system (Roche, Basel, Switzerland) according to previous studies (14, 34). Serial 10-fold dilutions of plasmids containing pigeon β -actin, AvBDs (2, 7, and 10), TLRs (2, 3, 5, 7, and 15), and iNOS were used as controls. Plasmids containing these genes were stored in our laboratory. The presence of PPMV-1 in the tissue samples of both infected and control pigeons was confirmed by detection of the viral RNA using the OneStep real-time PrimeScript RT-PCR kit (described above) according to a previous study (35; also data not shown). All amplifications were performed in triplicate. The concentration of target cDNA in a sample was deduced from the crossing point obtained and from the corresponding standard curve. The data are expressed for each sample as the copy number of each target cDNA normalized to that of the reference gene (β -actin), as described previously (24–28).

Statistics. Data are presented as means $\pm$ standard deviations (SD). The statistical significance of differences in gene expression was assessed using the generalized linear model (GLM) procedure of SAS software (36). Correlation between the relative gene expression of *Columba*_AvBDs and TLRs was performed using Pearson's tau using SAS software (36), and $P < 0.05$ was considered statistically significant.

Nucleotide sequence accession numbers. The nucleotide sequences of the three novel *Columba*_AvBDs are available from GenBank under the accession numbers KF951417 (*Columba*_AvBD2), KF951416 (*Columba*_AvBD7), and KF951415 (*Columba*_AvBD10).

RESULTS

Sequence analysis of AvBD, TLR, and iNOS genes in pigeons and recombinant expression and purification of *Columba*_AvBDs.

To examine the host immune responses of pigeons infected with PPMV-1, we identified full-length sequences of three novel AvBDs (2, 7, and 10), partial sequences of five TLRs (i.e., 2, 3, 5, 7, and 15), and sequences of iNOS genes from the bone marrow of pigeons. The sequences were determined by RT-PCR of RNA from pigeons with primer sets designed based upon a conserved sequence region in pigeons or orthologs in other species that were available in the database. The complete nucleotide sequences of these three novel AvBDs contained 195, 204, and 204 bp and encoded 64, 67, and 67 amino acids, respectively. The full-length sequences of these peptides shared high amino acid identities (more than 80%) with AvBD2 from turkeys, ducks, chickens, and geese, 64.7% identity with AvBD7 from chickens and ducks, and more than 80% identity with AvBD10 from quails, chickens, geese, and ducks (Fig. 1A). Furthermore, the predicted amino acid sequences of all three of these peptides had the six cysteine residues and the GXC motif that are conserved across all β -defensins.

FIG 1 Deduced amino acid sequence alignment of three novel avian β -defensins (AvBDs) from pigeons and SDS-PAGE analysis of His-tagged recombinant AvBD (His-rAvBD) proteins expressed in *E. coli* BL21(DE3) cells. (A) Deduced amino acid sequence alignment of three novel AvBDs from pigeons. The signal sequences of the AvBDs are italicized. The six conserved cysteines (C) are underlined. Dashes indicate that no identical or conserved residues were observed. (B) SDS-PAGE analysis of His-tagged recombinant AvBD (His-rAvBD) proteins expressed in *E. coli* BL21(DE3) cells. (Left panel) Lanes from left to right are as follows: total proteins from BL21 containing AvBD2, -7, and -10 (without IPTG induction), respectively; total proteins from BL21 containing AvBD2, -7, and -10 (with IPTG induction), respectively; protein molecular weight marker. (Right panel) Lanes from left to right are as follows: inclusion bodies of AvBD2, -7, and -10, respectively; purified proteins of AvBD2, -7, and -10, respectively; protein molecular weight marker. IPTG, isopropyl- β -D-thiogalactoside.

Thereafter, these three novel peptides were identified conclusively as β -defensin orthologs in the pigeon and named *Columba*_AvBD2, *Columba*_AvBD7, and *Columba*_AvBD10. Other than these three novel *Columba*_AvBDs, the partial sequences of TLRs and iNOS determined in this study shared more than 95% identities with the available TLR and iNOS sequences of pigeons or chickens that were available in a public database.

Antiviral and hemolytic activities of recombinant *Columba*_AvBDs against PPMV-1 *in vitro*. To determine whether these three *Columba*_AvBDs displayed antiviral activity against PPMV-1 *in vitro*, the 6 $\times$ His-tagged *Columba*_AvBDs (i.e., 2, 7, and 10), which had molecular masses of approximately 12 kDa, were expressed in an insoluble form in *E. coli* BL21(DE3) cells (Fig. 1B). These recombinant peptides then were purified and refolded using a commercial purification and refolding kit (described above).

To determine whether these three *Columba*_AvBDs displayed antiviral activity against PPMV-1 *in vitro*, PPMV-1 titers in the allantoic fluids of infected pigeon embryos inoculated with a 6 $\times$ His peptide (which served as the control) or His-tagged *Columba*_AvBDs were investigated at 24, 36, and 48 hpi by HA test. As illustrated in Fig. 2A, at 24 and 36 hpi, the HA titers in these three AvBD-inoculated embryos were significantly reduced compared to that of the control ($P < 0.05$). In contrast, no significant change was observed at 48 hpi for these three AvBDs compared to the level of the control (Fig. 2A).

The hemolytic activities of these three His-tagged *Colum-*

*ba*_AvBDs were determined using pigeon erythrocytes. No significant hemolytic activity of these His-tagged *Columba*_AvBDs was observed at concentrations of 100, 250, and 500 μ g/ml (Fig. 2B).

Host AvBD, TLR, and iNOS responses in the tissues of pigeons infected with PPMV-1. We recently showed that the mortality rate of pigeons after PPMV-1 (strain PI/CH/LHLJ/110822) infection exceeded 70%, and the onset of viral shedding via the oral and cloacal routes ranged from 3 to 7 dpi, as evidenced by the detection of viral loads in tissues (35). As a result, we used samples taken at 3 and 7 dpi to study the host immune response in the present study. We observed that AvBD2 was detectable in all 12 tissues investigated from both the control and infected pigeons. Furthermore, we detected significantly higher levels of AvBD2 expression in the cecal tonsil, lungs, and proventriculus of PPMV-1-infected pigeons at 7 dpi compared with the expression levels in both the control and infected pigeons at 3 dpi ($P < 0.05$). In contrast, AvBD7 was detectable in only two (bone marrow and trachea) of the 12 tissues investigated, and we did not observe any significant differences in AvBD7 expression in tissues between the control and PPMV-1-infected pigeons. Similar to the pattern of AvBD2 expression, AvBD10 also was found in all 12 tissues investigated, and the expression levels were significantly increased in the spleen, lungs, proventriculus, and kidneys of PPMV-1-infected pigeons at 7 dpi compared with the expression levels in the control or PPMV-1-infected pigeons at 3 dpi ($P < 0.05$) (Fig. 3).

We next evaluated the levels of TLR and iNOS expression. Unfortunately, we did not identify the TLR1, TLR4, and TLR21 genes

FIG 2 Antiviral activities of His-rAvBDs against PPMV-1 in pigeon embryos and hemolytic activities of His-rAvBDs. (A) Antiviral activities of His-rAvBDs. The virus titers were tested by hemagglutinin (HA) at 24, 36, and 48 hpi. All assays were performed in three independent experiments, with five replicates per experiment, and each bar is the mean $\pm$ SD. (B) Hemolytic activities of His-rAvBDs. Freshly isolated pigeon red blood cells were incubated with different concentrations of AvBDs (0 to 500 μ g/ml). The release of hemoglobin, as a measure of hemolysis, was measured at 405 nm. The release of hemoglobin upon the addition of 1% Triton X-100 was set at 100%. The percentage of hemolysis was calculated as $[(A_{405 \text{ nm, peptide}} - A_{405 \text{ nm, PBS}}) / (A_{405 \text{ nm, 1\% Triton X-100}} - A_{405 \text{ nm, PBS}})] \times 100\%$. All assays were performed in three independent experiments, with three replicates per experiment, and each point is the mean $\pm$ SD. Different letters above columns indicate values that are significantly different ($P < 0.05$).

in pigeons in this study. Therefore, we analyzed five TLRs (i.e., 2, 3, 5, 7, and 15) and found that all five were detected in the 12 tissues of pigeons. We did not observe any significant differences in the regulation of TLR2 or TLR5 expression in the 12 tissues investigated in infected pigeons compared to those of the control group (Fig. 4). In contrast, in PPMV-1-infected pigeons, the expression level of TLR3 was significantly increased in the spleen at both 3 and 7 dpi, as well as in the trachea at 7 dpi, compared to levels for control pigeons ($P < 0.05$) (Fig. 4). Similarly, we also detected significantly higher levels of TLR7 expression in the spleen (3 and 7 dpi) and proventriculus (7 dpi) of PPMV-1-infected pigeons than in control pigeons ($P < 0.05$). For TLR15, the expression level was increased only in the lungs of PPMV-1-infected pigeons at 7 dpi ($P < 0.05$). Interestingly, in the infected pigeons, levels of iNOS expression were significantly upregulated

in 4 of the 12 tissues investigated, the spleen and bursa of Fabricius at both 3 and 7 dpi, and the trachea and proventriculus at 7 dpi ($P < 0.05$) (Fig. 5).

Considering the upregulation of AvBD2 and AvBD10 expression in some tissues of pigeons in response to PPMV-1 infection, we plotted the levels of both of these AvBDs against TLRs in respective tissues, with the aim of examining the molecular mechanisms by which AvBDs respond to PPMV-1 infection. We observed a significantly positive correlation between AvBD2 and TLR7 expression in the proventriculus ($r = 0.99$, $P < 0.01$), between AvBD2 and TLR15 expression in the lungs ($r = 0.99$, $P < 0.01$), between AvBD10 and TLR3 and TLR7 expression in the spleen ($r = 0.98$ or $r = 0.99$, $P < 0.01$), and between AvBD10 and TLR7 expression in the proventriculus ($r = 0.99$, $P < 0.01$). Taken together, these results indicated that PPMV-1 infection induces an increase in the mRNA expression of both AvBD2 and AvBD10, which suggests that both AvBDs play significant roles in the resistance of pigeons to PPMV-1 replication, which seems to be mediated mainly by TLRs 3 and 7 (Fig. 6).

DISCUSSION

PPMV-1, which originated from pigeons, is an antigenic and host variant of NDV. Although some strains of PPMV-1 are nonpathogenic in chickens, they cause high morbidity and mortality in pigeons (32, 35). Pigeons are often sold in live-bird markets across Asia and also are commonly found in outdoor poultry (37). Together, our findings suggest that PPMV-1 infection of pigeons is a threat to animal health. However, a detailed analysis of the host immune responses of pigeons against PPMV-1 infection has not been performed due to difficulties attributed mainly to the fact that only a few host immune-related genes have been characterized in pigeons, and their sequences have not been disclosed (37). In this study, we identified full-length sequences of three novel AvBDs (i.e., 2, 7, and 10) from pigeons. Other than these three AvBDs, partial sequences of five TLRs (i.e., 2, 3, 5, 7, and 15) and iNOS genes were determined from the tissues of pigeons as well. Furthermore, we set up a quantitative RT-PCR analysis in pigeons and assessed how PPMV-1 induced these host immune-related genes, with the aim of understanding the host responses in pigeons against PPMV-1 infections.

AvBDs isolated from other avian species have been shown to play a vital role in the innate host defense against bacteria, fungi, and some viruses (14, 17, 19, 24–26, 28). To evaluate the antiviral activity of these three AvBDs against PPMV-1, recombinant proteins were produced. The data demonstrated that all three recombinant AvBDs displayed direct antiviral activity against PPMV-1 *in vitro*. Similarly, some AvBDs from other avian species (i.e., chickens and ducks), as well as HDPs from mammals, have been reported to show antiviral properties against viruses such as IBV, DHV, and influenza viruses (14, 24, 26, 38–40). These data further demonstrate that HDPs, including AvBDs, are potential alternatives for the control of pathogenic virus, including PPMV-1, possibly by increasing natural HDP expression in the virus-infected tissues or as a therapeutic treatment of virus-infected individuals using naturally occurring HDPs, synthetic analogues, or peptides modified to maximize the key antiviral functions and provide significant protection against disease.

In addition to their direct antimicrobial effect, certain HDPs, including AvBDs, are capable of promoting local (innate) and systemic (adaptive) immune responses (13, 14, 17, 24–28, 41, 42).

FIG 3 Relative gene expression of avian β -defensins (AvBDs) in the tissues of pigeons in response to PPMV-1 infection. cDNA copy numbers in the tissue samples from five pigeons of each group were measured by quantitative PCR (qPCR) at 3 and 7 dpi. AvBD levels were normalized to the levels of β -actin in the same samples. All assays were performed in triplicate, with five replicates per experiment, and each bar is the means $\pm$ SD. Different letters above columns indicate values that are significantly different ($P < 0.05$).

However, the immune response of pigeons to PPMV-1 infection has been poorly studied. In our recent study, it was observed that six tissues (i.e., kidneys, lungs, brain, trachea, Harderian glands, and proventriculus) were the main target tissues when pigeons

were inoculated with PPMV-1 strains (35). With this in mind, a total of 12 tissues, including the aforementioned six tissues, as well as six other immune and gastric tissues, were taken from experimental pigeons. It was demonstrated that both AvBD2 and -10

were widely distributed in the 12 tissues investigated in both the control and infected pigeons. In contrast, AvBD7 was detected only in the bone marrow and trachea of all pigeons. Similarly, both AvBD2 and AvBD10 were reported to be widely distributed in the tissues of other avian species (i.e., chickens, ducks, and geese), and AvBD7 was detected in a limited number of tissues in chickens and ducks (14, 24, 26). At this point, both AvBD2 and AvBD10 may contribute to host defense in these avian species.

Furthermore, we observed that AvBD2 in the cecal tonsil, lungs, and proventriculus and AvBD10 in the spleen, lungs, proventriculus, and kidneys were induced in PPMV-1-infected pigeons at 7 dpi. Interestingly, in our parallel study, viral loads were detected in the lungs, proventriculus, and kidneys, in gross lesions, such as a proventriculus hemorrhage and kidney enlargement, and by histopathology in the kidneys and lungs (degenerated kidney tubule epithelium and mild congestion in the lungs) in pigeons infected with PPMV-1 strains (35). The effect of pathogenic microorganisms on the expression of the AvBDs of other avian species has been investigated extensively in recent years, and it has been shown that infections can cause highly variable effects that are different for each AvBD, depending upon the organs examined and the breed or age of the birds (17). *Salmonella enteritidis*-infected chickens showed an upregulation of AvBD2, AvBD10, and AvBD12 *in vivo* (43). In another study, it was demonstrated that *S. enteritidis* infection caused the induction of AvBD2 and AvBD6, while the expression levels of AvBD4, AvBD9, AvBD10, and AvBD11 were repressed by the infection (44). Our recent study showed that *S. enteritidis* infection caused the upregulation of AvBD2, AvBD5, AvBD9, and AvBD10 expression in tissues of infected geese (28). Similarly, the expression of AvBDs (i.e., 1, 3 to 7, and 12) in the livers of ducks was significantly upregulated in response to DHV infection (24, 26). Although it remains unknown whether the target microorganisms differ between different AvBDs, the defense functions against pathogens may be stronger if different AvBDs are induced when a tissue is infected. Thus, higher levels of AvBD expression may partly contribute to protective immunity in tissues of infected pigeons.

The involvement of TLR signaling in defensin regulation has been described in mammals as well as birds (45, 46). Thus far, 10 TLRs have been identified in chickens, namely, TLR1-1, TLR1-2, TLR2-1, TLR2-2, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21 (47, 48). In the current study, we identified five TLRs in pigeons, namely, TLR2, TLR3, TLR5, TLR7, and TLR15. Our results demonstrated that both TLR3 and TLR7 were induced in the spleen and some target tissues, while TLR15 was induced only in a target tissue (lungs) of PPMV-1-infected pigeons. Our results revealed that these molecules are functionally active in recognizing the pathogen. Furthermore, we observed a high correlation between the expression of AvBD2 and TLR7 or TLR15, as well as between the expression of AvBD10 and TLR3 and TLR7, in respective tissues. In mammals, it has been reported that both TLR3 and TLR7 recognize viral PAMPs, and TLR3 detects double-stranded RNA (dsRNA) derived from viral replication, whereas single-stranded RNA (ssRNA) is detected by TLR7 (9, 10). Similarly, TLR3 and TLR7 are the only TLRs implicated in antiviral responses in birds (26, 37, 49). TLR15 is a unique TLR that was recently identified in chickens, and now in pigeons, and it may recognize nonsecreted, virulence-associated fungal and bacterial proteases (50, 51). Our results demonstrated that three TLRs (i.e., 3, 7,

FIG 4 Relative gene expression of Toll-like receptors (TLRs) in the tissue samples of pigeons in response to PPMV-1 infection. cDNA copy numbers in the tissue samples from five pigeons of each group were measured by quantitative PCR (qPCR) at 3 and 7 dpi. TLR levels were normalized to the levels of β-actin in the same samples. All assays were performed in triplicate, with five replicates per experiment, and each bar is the mean ± SD. Different letters above columns indicate values that are significantly different ($P < 0.05$).

FIG 5 Analysis of relative gene expression of iNOS in the tissue samples of pigeons in response to PPMV-1 infection. cDNA copy numbers in the tissue samples from five pigeons of each group were measured by qPCR at 3 and 7 dpi. iNOS levels were normalized to the levels of β-actin in the same samples. All assays were performed in triplicate, with five replicates per experiment, and each bar is the mean ± SD. Different letters above columns indicate values that are significantly different ($P < 0.05$).

and 15) may contribute to the antiviral defense of pigeons against PPMV-1, even though it is defined as an ssRNA virus. Therefore, pigeons may have developed specific immune mechanisms that are distinct from those of chickens and mammals. Further studies are required to elucidate the contribution

of TLRs to the host defense in pigeons. In addition, we observed that the expression of the iNOS gene was significantly upregulated in some lymphoid and other target tissues of PPMV-1-infected pigeons. Consistent with this finding, it has been demonstrated that the iNOS gene was upregulated in the

FIG 6 Correlation between AvBD and TLR expression in tissues of pigeons in response to PPMV-1 infection. cDNA copy numbers in tissue samples from five pigeons of each group were measured by qPCR at 3 and 7 dpi. Gene levels were normalized to the levels of β-actin in the same samples. All assays were performed in triplicate, with five replicates per experiment, and each bar is the mean ± SD.

lungs of Marek's disease virus-infected chickens (52). iNOS could produce NO, which has been shown previously to inhibit the replication of viruses (50–53).

In conclusion, this study demonstrates that three novel *Columba* AvBDs (2, 7, and 10) possess potent antiviral activity against PPMV-1. Furthermore, the results reported here suggest that PPMV-1 infection can induce innate host responses characterized by the activation of TLRs, particularly TLR3 and TLR7, AvBDs (2 and 10), and iNOS of pigeons. Further studies are needed to identify the role of the antiviral proteins responsible for mediating differential host responses against PPMV-1 in pigeons.

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