

# Avian Toll-like receptor 3 isoforms and evaluation of Toll-like receptor 3–mediated immune responses using knockout quail fibroblast cells

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**ABSTRACT** Toll-like receptor 3 (TLR3) induces host innate immune response on recognition of viral double-stranded RNA (dsRNA). Although several studies of avian TLR3 have been reported, none of these studies used a gene knockout (KO) model to directly assess its role in inducing the immune response and effect on other dsRNA receptors. In this study, we determined the coding sequence of quail TLR3, identified isoforms, and generated TLR3 KO quail fibroblast (QT-35) cells using a CRISPR/Cas9 system optimized for avian species. The TLR3-mediated immune response was studied by stimulating the wild-type (WT) and KO QT-35 cells with synthetic dsRNA or polyinosinic:polycytidylic acid [poly(I:C)] or infecting the cells with different RNA viruses such as influenza A virus, avian reovirus, and vesicular stomatitis virus. The direct poly(I:C) treatment significantly increased IFN- $\beta$  and IL-8 gene expression along with the cytoplasmic dsRNA receptor,

melanoma differentiation–associated gene 5 (MDA5), in WT cells, whereas no changes in all corresponding genes were observed in KO cells. We further confirmed the antiviral effects of poly(I:C)-induced TLR3-mediated immunity by demonstrating significant reduction of virus titer in poly(I:C)-treated WT cells, but not in TLR3 KO cells. On virus infection, varying levels of IFN- $\beta$ , IL-8, TLR3, and MDA5 gene upregulation were observed depending on the viruses. No major differences in gene expression level were observed between WT and TLR3 KO cells, which suggests a relatively minor role of TLR3 in sensing and exerting immune response against the viruses tested in vitro. Our data show that quail TLR3 is an important endosomal dsRNA receptor responsible for regulation of type I interferon and proinflammatory cytokine, and affect the expression of MDA5, another dsRNA receptor, most likely through cytokine-mediated communication.

**Key words:** Toll-like receptor 3, CRISPR/Cas9, quail fibroblast cell, gene knockout, immune response

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## INTRODUCTION

Toll-like receptor 3 (TLR3) is a key receptor for sensing double-stranded RNA (dsRNA) that is also generated during virus replication (Thompson et al., 2011; Son et al., 2015). On recognition of viral dsRNA or polyinosinic:polycytidylic acid [poly(I:C)] (synthetic dsRNA), the TLR3 activates downstream signaling

resulting in transcription of interferons and proinflammatory cytokine genes (Kumar et al., 2006; Wong et al., 2009). Several studies in mammalian hosts have shown that the TLR3 is involved in viral infection, clearance and pathogenesis (Le Goffic et al., 2006; Hutchens et al., 2008; Daffis et al., 2009; Leung et al., 2014). Other studies have shown the coordination between the endosomal TLR3 and cytoplasmic dsRNA receptors such as retinoic acid–inducible gene-1 (RIG-I) and melanoma differentiation–associated gene-5 (MDA5) in virus recognition and induction of a wide range of interferons and proinflammatory cytokines (Slater et al., 2010; Nasirudeen et al., 2011; Wang et al., 2011). In the study by Slater et al. (2010), the TLR3 stimulated the expression of RIG-I and MDA5 genes to induce maximal innate

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immune responses during rhinovirus infection in human bronchial epithelial cells. In another study by Loo et al. (2008), using gene knockout (KO) murine embryonic fibroblast cells, the *MDA5* gene was found to be dispensable for induction of interferon regulatory factor 3-dependent genes in paramyxovirus infection, whereas both MDA5-dependent and RIG-I-dependent immune responses were observed in dengue virus and reovirus infection. Because the RIG-I receptor is absent in birds of order Galliformes (including quail, chickens, and turkeys) (Barber et al., 2010; Zheng and Satta, 2018), it is possible that the roles played by the TLR3 and MDA5 in regulating immune responses in these birds are different than those reported in humans and other mammalian species.

The TLR3 genes have been determined in various avian species including chicken, turkey, duck, goose, and pigeon (Hayashi et al., 2011; Chen et al., 2013; Zhang et al., 2015; Yong et al., 2018). The functions of TLRs are reported to be conserved among the vertebrates (Roach et al., 2005), and the degree of similarity of TLR3 coding sequences is high among avian species (Chen et al., 2013). Using small-interfering RNA to knock down the TLR3 gene expression in chicken fibroblast cells treated with poly(I:C), Karpala et al. (2008) demonstrated that activation of the avian TLR3 results in upregulation of IFN- $\beta$  production. Other more recent studies have shown that poly(I:C) treatment of chickens or chicken fibroblast cells can reduce the replication of avian influenza virus, Newcastle disease virus, and Marek's disease virus (St. Paul et al., 2012; Cheng et al., 2014; Hu et al., 2016). However, none of the studies used a TLR3 KO avian model to directly assess the role of the TLR3-mediated immune response, and a TLR3 KO model can greatly enhance our understanding of the TLR3 function in avian species.

Japanese quail (*Coturnix japonica*) have become the focus of many biological studies because of their relatively small size, fast sexual maturation, and abundant egg production (Huss et al., 2008; Seidl et al., 2013; Choi et al., 2014; Shin et al., 2015). In addition, Japanese quail have been used as an animal model for studying susceptibility, transmission, and pathogenesis of various avian viruses and are highly susceptible to influenza A viruses (IAV) and many other viruses including Newcastle disease virus and avian leucosis virus (Thontiravong et al., 2012; Sharawi et al., 2015; Susta et al., 2018; Zhang et al., 2019). Thus, quail can serve as a great avian transgenesis model to study gene functions in relation to viral pathogenesis. In this study, we determined the coding sequence of the quail TLR3 (qTLR3) and compared it with TLR3 sequences available for other avian species. In addition, TLR3 KO quail fibroblast (QT-35) cells were generated using the poultry-specific CRISPR/Cas9 genome editing system that we developed previously (Ahn et al., 2017). The KO cells were used to investigate the role of the TLR3 in mediating the immune responses in response to synthetic dsRNA treatment and infection with different RNA viruses.

## MATERIALS AND METHODS

### Sequence Analysis and Identification of TLR3 Isoforms in Avian Species

Total RNA was extracted from quail lung tissue, and complementary DNA (cDNA) was synthesized using oligo (dT) primer as described previously (Shin et al., 2015). A primer set of the TLR3 F1/R1 (Table 1) was used to amplify the full-length coding sequence of the qTLR3 using the cDNA. The PCR was performed with Taq DNA polymerase (Invitrogen, Carlsbad, CA) with the following conditions: one-time denaturation at 95°C for 1 min, and then 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 40 s, and extension at 72°C for 2 min 30 s, with the final extension at 72°C for 10 min. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Germantown, MD), and the extracted DNA were cloned into pCR2.1-TOPO vector (Invitrogen). The transformation and propagation of the plasmids were performed as previously described (Ahn et al., 2014). The plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen), and the miniprep products were Sanger-sequenced at the Plant-Microbes Genomics Facility, The Ohio State University (Columbus, OH).

To determine the alternative splicing forms and expression levels of the TLR3 in different tissues, total RNAs were isolated from different tissues (abdominal fat; the lung, liver, and kidney; thigh, breast, and heart muscles) of chicken (Ross 708 broiler), turkey (random-bred turkey line maintained in the university animal facility, Wooster, OH), and Japanese quail maintained in our laboratory as described previously (Shin et al., 2015). Tissues were collected from 3 birds per avian species as described previously (Shin et al., 2015). The total RNAs were converted into cDNA and amplified using the primer set TLR3 F2/R2 for quail, TLR3 F3/R3 for chicken, and TLR3 F1/R3 for turkey that cover the splicing-occurring region in exon 1 (Figure 1 and Table 1). The PCR was performed under the conditions described above, with annealing at 55°C for 40 s and extension at 72°C for 50 s. The PCR products were purified and sequenced as described above. Sequence assemblies, alignments, and analyses were performed using DNASTAR Lasergene, version 10 (Madison, WI). All sequence data from this study have been deposited in GenBank under accession numbers MN959450 (qTLR3-A), MN959451 (qTLR3-B), MN959452 (qTLR3-C), MN959453 (tTLR3-A), MN959454 (tTLR3-B), and MN959455 (tTLR3-C).

### Customization of CRISPR/Cas9 Vector for Generating TLR3 Knockout QT-35 Cell Lines

The construction of the avian-specific CRISPR/Cas9 vector was previously described (Ahn et al., 2017). A single guide RNA (gRNA) required was designed (Figure 2A) to specifically target the minus strand on exon 2 of the qTLR3 using an online gRNA design tool (<http://crispr.mit.edu>).

edu)(Hsu et al., 2013). Based on the target sequence of gRNA, 2 oligonucleotides containing gRNA expression sequences (underlined) were designed: oligo-I, 5'CTCTGTGTAGAACACAAGCTCACGA3' and oligo-II, 5'-AAACTCGTGAGCTTGTGTTCTACAC3'. A CTCTG overhang was added at the 5' end of oligo-I while AAAC and C overhangs were, respectively, added at 5' and 3' ends of oligo-II to facilitate cloning into the CRISPR/Cas9 vector. Annealing of the oligonucleotides and cloning of annealed oligos into the customized CRISPR/Cas9 construct was performed as previously described (Ahn et al., 2017). Proper insertion of gRNA was confirmed by sequencing the target region of the CRISPR/Cas9 construct.

### Cell Culture, Transfection, and Cell Sorting

Quail fibroblast cells were purchased from the Sigma Aldrich (St. Louis, MO) and maintained in the Minimum Essential Medium (MEM, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 10- $\mu$ g/mL gentamicin (Life Technologies). The cells were grown in 6-well plates for 24 h to a confluency of about 70% before transfection with 2.5  $\mu$ g of the avian-specific CRISPR/Cas9 vector with gRNA expression sequences using lipofectamine 3,000 (Invitrogen) following the manufacturer's protocol. A CRISPR/Cas9 vector without gRNA expression sequences was also included for transfection as a negative control. The cells were monitored for green fluorescent protein (GFP) expression under a fluorescent microscope (Olympus IX70-S1F2, Olympus America, Center Valley, PA). After 48 h of post-transfection, the cells were trypsinized and suspended in 1% FBS in the PBS solution (Life technologies). The GFP-positive single cells were sorted into 96-well plates using the fluorescence-activated cell sorting (FACS) machine (FACS Aria III, BD Biosciences, CA) at the Analytical Cytometry Services, The Ohio State University. The GFP-positive single cells were propagated and sequentially transferred from 96-well plates to 24-well plates, and into 6-well plates.

### TLR3 Knockout Confirmation

Mutation of the TLR3 was confirmed by sequencing genomic DNA and mRNA-derived cDNA. Genomic DNA was extracted from each GFP-positive single cell colonies according to the established procedure (Shin et al., 2014). Mutation at the target region in the *TLR3* gene was confirmed by sequencing amplified genomic DNA using forward and reverse primers as follows: qTLR3-gDNA-F: 5'-CATCTTCTCTGCA-GAAGTTGAATATTTTG-3' and qTLR3-gDNA-R: 5'-GCTTCCTACCTCTTTCAGGGGATT-3'. We also confirmed mutation induced in all the cell lines by sequencing cDNA prepared from total mRNA as described below.

### Viruses, Poly(I:C), and Recombinant Chicken IFN $\alpha$

RNA viruses that differ in genome structure and sensitivity to interferons were selected (Ellis et al., 1983). Avian reovirus (ARV) is a dsRNA virus that is highly resistant to interferons. Influenza A virus and vesicular stomatitis virus (VSV) are ssRNA viruses with different sensitivities to interferons: IAV is moderately sensitive, whereas VSV is highly sensitive. The ARV O'Neil strain was reported previously (Ngunjiri et al., 2019). The IAV A/WSN/33 (H1N1) strain replicates efficiently in both avian and mammalian cells. Influenza A virus A/WSN/33-delNS1 was reverse genetically created by replacing the NS gene segment of the wild-type (WT) WSN virus with that of A/TK/OR/71-delNS1 virus, which expresses a severely truncated NS1 protein and induces high levels of interferons (Marcus et al., 2005; Cauthen et al., 2007; Wang et al., 2008). Recombinant VSV-expressing reporter GFP was described previously (Ma et al., 2014). The median tissue culture infectious dose (TCID<sub>50</sub>) of each virus was determined in 96-well plates based on the Reed-Muench method (Reed and Muench, 1938). The synthetic dsRNA ligand for the TLR3, poly(I:C) high molecular weight, was purchased from InvivoGen (San Diego, CA). Recombinant chicken interferon alpha (rChIFN $\alpha$ ) was prepared as previously described (Sekellick et al., 1994; Jang et al., 2016).

### Experimental Design for Evaluation of TLR3-Mediated Immune Gene Regulation and Antiviral Immunity

To compare gene expression levels, WT cells and TLR3 KO QT-35 cells (KO#3) were seeded in 6-well plates at  $1 \times 10^6$  cells/well and incubated at 37°C for 24 h. The cells were treated in triplicate by either directly adding poly(I:C) at a concentration of 20  $\mu$ g/mL for 12 h or transfecting poly(I:C) at 0.5  $\mu$ g/mL using lipofectamine 3,000 (Invitrogen) for 4 h in the MEM without FBS. The rChIFN- $\alpha$  was directly added at a concentration of 50 units/mL in the MEM for 4 h. The control received only the MEM. The cells were harvested for RNA extraction to perform transcriptional analysis.

To compare poly(I:C)-induced antiviral activities between WT and KO cells, 10  $\mu$ g/mL of poly(I:C) was added into the media and incubated for 24 h before virus infection at the multiplicity of infection (MOI) 0.1. The supernatant was collected at 12 and 24 h post infection (hpi) for virus titration.

To compare virus replication and immune response between WT and TLR3 KO cells, cells were plated into 6-well plates at  $1 \times 10^6$  cells/well and incubated at 37°C for 24 h. The cells were either infected with each virus at 0.1 MOI or kept uninfected in triplicate. The cells and supernatant were collected at 12 and 24 hpi for ARV and IAVs and 12 and 16 hpi for VSV for transcription analysis and virus titration, respectively.

**Table 1.** Primer sequences for TLR3 PCR amplification and real-time quantitative PCR.

Name	Sequence (5' -> 3')	Product (bp)	Reference
Primers for amplification of <i>TLR3</i> gene fragments			
TLR3-F1	TCCCAATGAAAGCATAAAACA	2,812	XM_003205774.2
TLR3-R1	ATTCAGCGCACTTTACTATTAGATT		XM_003205774.2
TLR3-F2	ATGGGATGTTCTATTCTTTGCTGGA	689	MN959450
TLR3-R2	TTGTTCAAGTATAAGGCCAAACAGA		MN959450
TLR3-F3	AAACTATGGGATGCTCTATTCCCTT	683	FJ915480.1
TLR3-R3	AGGCCAAACAGATTTCCAATTG		EF137861.1
Primers for quantitative PCR			
qTLR3-F	ATCCGTGGTGCAGGAAGTTTA	168	MN959450
qTLR3-R	GCCAGTTCAAGATGCAGCGA		
qIFN- $\beta$ -F	ACAACCTCCTACAGCACAACTA	61	Karpala et al., 2011
qIFN- $\beta$ -R	GCTGGAGGCGGCACATG		
qIL-8-F	GCCCTCCTCCTGGTTTCAG	74	Ferro et al., 2004
qIL-8-R	TGGCACCGCCAGCTCATT		
qMDA5-F	GGACGACCACGATCTCTGTGT	79	Liniger et al., 2012
qMDA5-R	CACCTGTCTGGTCTGCATGTTATC		
qGAPDH-F	CCCCAATGTCTCTGTTGTTGAC	83	Karpala et al., 2011
qGAPDH-R	CAGCCTTCACTACCCTCTTGAT		

Abbreviation: TLR3, Toll-like receptor 3.

## Measurement of Gene Expression by Real-Time Quantitative PCR

The cells were homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted using Direct-zol RNA miniprep plus kits (Zymo Research, Irvine, CA). The expression of cytokines and dsRNA receptors was measured by real-time quantitative PCR (qPCR) following a previously established protocol (Jang et al., 2016). Briefly, equal (800 ng) amount of mRNA was converted into cDNA by RT-PCR with oligo(dT) primer (Promega, Madison, WI). The cDNA was diluted (1:5) and subjected to qPCR using the SYBR GREEN system (Quanta, Gaithersburg, MD) in Applied Biosystems 7,500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). The primer sequences used for the amplification of each gene are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase gene was used as a housekeeping gene to normalize the gene expression. The  $\Delta\Delta C_t$  method (Livak and Schmittgen, 2001) was used to determine differential gene regulation and gene expression levels in the treated/infected samples and expressed as fold change over the control sample.

## Virus Titration

The collected supernatants from the virus-infected cells were titrated in QT-35 cells, and the TCID<sub>50</sub> was determined as described previously (Jang et al., 2016).

## Statistical Analysis

Statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). Comparison of gene expression in poly(I:C)-treated and virus-infected cells was analyzed by Student's *t*-test. The virus titers in infected WT and TLR3 KO cells at different time points were analyzed by one-way ANOVA followed by Tukey's post hoc test.

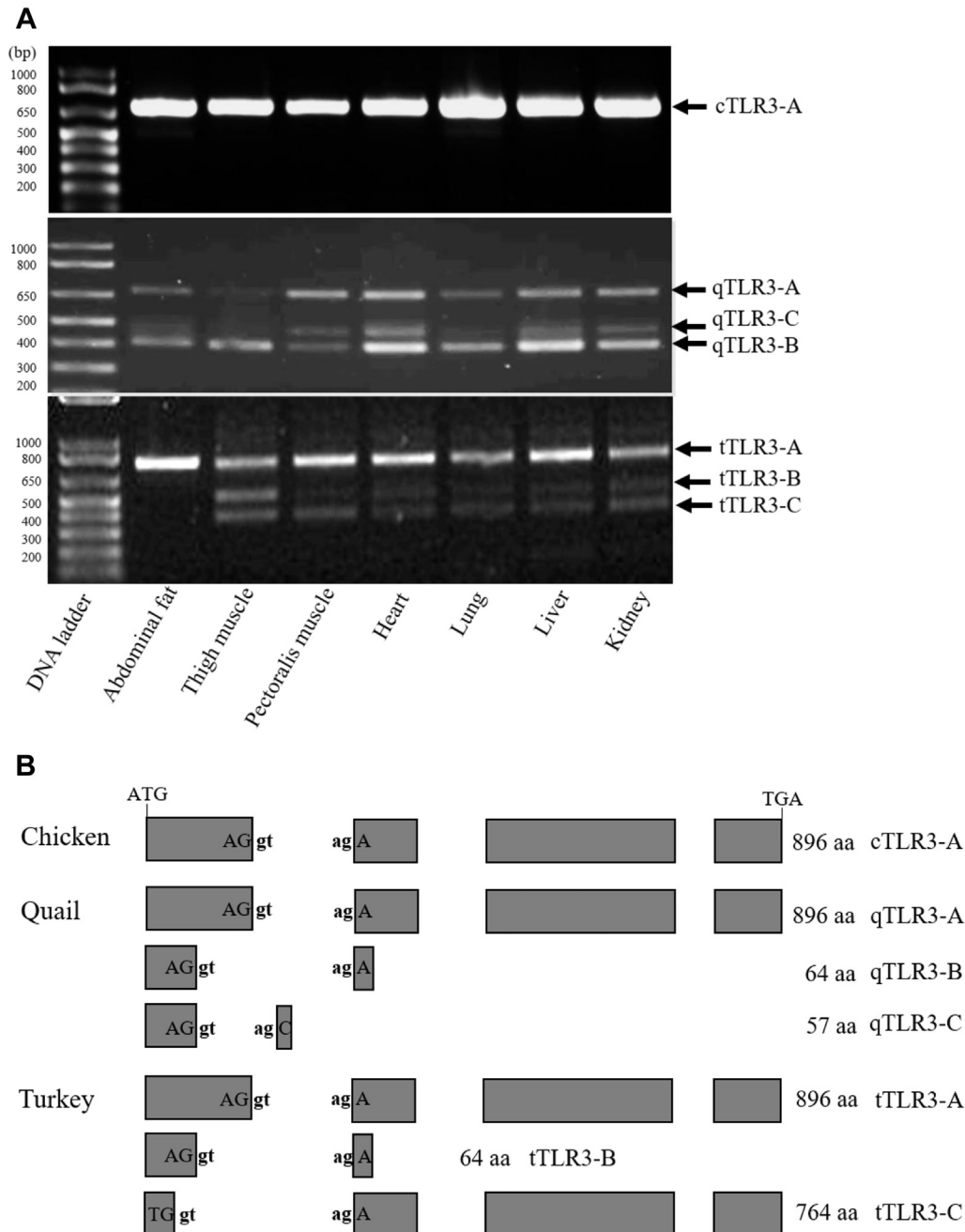
## RESULTS

### Coding Sequence of qTLR3

The coding sequence of the qTLR3 was determined to be 2691 nucleotides base pairs (bp) encoding a protein of 896 amino acids (aa) in length. Although qTLR3 cloning and sequencing was underway during this study, another qTLR3 sequence was predicted through automated computational analysis of quail genome and published in the GenBank (accession number: XM\_015862120). Interestingly, the predicted qTLR3 (948 aa) has 52 additional aa at the N-terminus relative to the 896 aa qTLR3 sequence determined in this study and the expected TLR3 sequences of other poultry species (chicken, turkey, duck, and goose) (Zhang et al., 2015; Yong et al., 2018). The qTLR3 sequence determined in this study is 99% identical to the published sequence in the corresponding region. The qTLR3 shares a higher sequence similarity (93–94%) with the chicken and turkey TLR3 compared with the duck and goose TLR3 (86–88%) (Table 2). Avian TLR3 aa sequences including those reported in this study are ~60% identical to the human and mouse TLR3 (data not shown). All avian TLR3 have several conserved aa residues (His39, His61, His 539, and Asn541) (data not shown) that are associated with dsRNA binding (Bell et al., 2006; Pirher et al., 2008).

### Alternative Splicing of TLR3 Gene

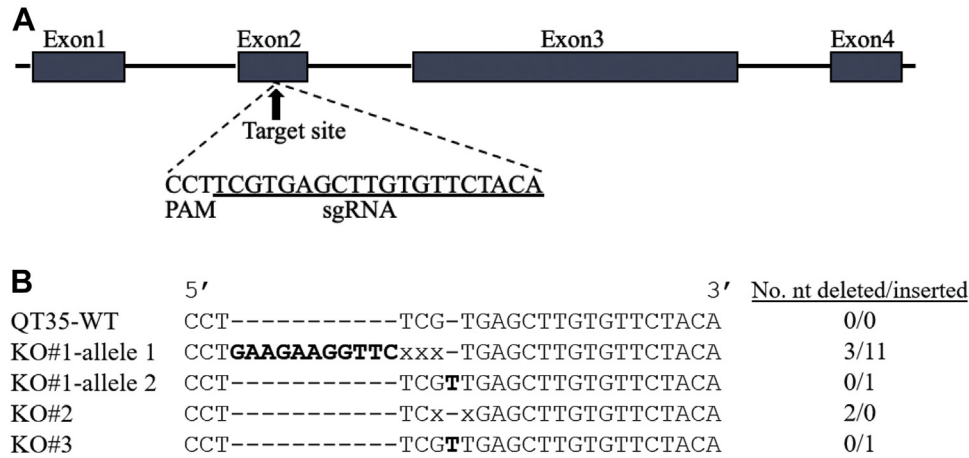
During the PCR amplification of the full-length qTLR3 with the TLR3-F1/R1 primer set (Table 1), we observed different sizes of the bands smaller than the expected size. Sequencing of different-size bands identified a canonical splicing sequence (Mount, 1982; Bursat et al., 2000), and new primers were designed to detect TLR3 mRNA isoforms in different tissues from quail, chicken, and turkey (Table 1). Interestingly, multiple TLR3 fragments of different sizes were detected in



**Figure 1.** Alternative splicing of TLR3 gene in chicken, quail, and turkey. (A) Complementary DNA synthesized from different tissues of chicken, turkey, and quail was PCR-amplified with TLR3 primer sets covering the alternative splicing occurring region. A full-length TLR3 (TLR3-A) and TLR3 fragments of smaller sizes, TLR3-B/C, were detected. (B) Schematic representation of TLR3 isoforms in chicken, turkey, and quail. Alternative splicing forms in quail and turkey TLR3 are shown with shorter sequences and the presence of canonical dinucleotides GT and AG for the donor and acceptor sites, respectively. The coding region of the TLR3 isoforms is shown in gray shading on the exons. Abbreviation: TLR3, Toll-like receptor 3.

tissues derived from quail and turkey tissues, but not in chicken tissues (Figure 1A). The full-length TLR3 coding sequence from quail (isoform qTLR3-A), turkey (isoform tTLR3-A), and chicken (cTLR3) was as expected based on published avian TLR3 sequences (Zhang et al., 2015; Yong et al., 2018). Both qTLR3-B and tTLR3-B isoforms shared the same alternative splicing donor and acceptor sites, resulting in the deletion of 275 nt (Figure 1B). The other 2 isoforms differed: tTLR3-C had the same acceptor site as qTLR3-B and tTLR3-B but a different donor site, leading to a 396 nt deletion, whereas the qTLR3-C isoform had the same

donor site as qTLR3-B and tTLR3-B but a different acceptor site, which caused a 205 nt deletion (Figure 1B). Sequences of the qTLR3-B, qTLR3-C, and tTLR3-B isoforms have premature stop codons that are predicted to produce smaller proteins of 64 aa (qTLR3/tTLR3-B) and 57 aa (qTLR3-C) in length. The tTLR3-C isoform has no premature stop codon and is predicted to encode a 764-aa protein. The predicted proteins of qTLR3-B, qTLR3-C, and tTLR3-B isoforms lack the C-terminal dsRNA binding domain, whereas that of tTLR3-C isoform lacks the N-terminal dsRNA binding site (Figure 1B) (Pirher et al., 2008).



**Figure 2.** Generation and confirmation of TLR3 KO QT-35 cell lines. (A) Schematic representation of the quail *TLR3* gene. The 20-bp target specific sequence of sgRNA is designed at exon 2 adjacent to the protospacer adjacent motif (PAM). (B) Three TLR3 KO QT-35 cell lines were generated using the poultry-specific CRISPR/Cas9 system. “-” represents absence of nucleotides and was used for sequence alignment. Nucleotides in bold letter represent insertion mutation, and “x” represents deletion mutation in the KO cell lines. Abbreviations: KO, knockout; QT-35, quail fibroblast; TLR3, Toll-like receptor 3.

### Generation of TLR3 Knockout Quail Cells

Three KO cell QT-35 lines (KO#1, #2, and #3) were obtained after transfection with the avian-specific CRISPR/Cas9 vector with gRNA expression sequences, screening, propagation, and genomic DNA sequencing. Different indel mutations were observed at the TLR3 target region (Figures 2A and 2B). The KO#1 cell line had heterogeneous mutations: 3 nt deletion and 11 nt insertion in one allele, and 1 nt insertion in the other allele. Both KO#2 and KO#3 cell lines had homogeneous mutations in both alleles: 2 nt deletions in KO#2 and 1 nt insertions in KO#3. The indel mutations were further confirmed by sequencing cDNA reverse-transcribed from total mRNA of the corresponding cell line. These frame-shift mutations resulted in early stop codons and severe truncation of the TLR3 at the C-terminus. The mRNA from the KO cells may be translated to N-terminal TLR3 protein fragments that are 174/189-, 183-, and 174-aa long for KO#1, KO#2, KO#3 cell lines, respectively. However, these protein fragments cannot be functional because of lack of the C-terminal dsRNA binding site (Bell et al., 2006). Through stimulation of the cells with poly(I:C) and subsequent assessment of downstream signaling by transcription analysis, we directly confirmed that none

of the KO cell lines produced a functional TLR3 protein (Figure 3A, data not shown for KO#1 and KO#2). Therefore, we randomly chose one of the KO cells (KO#3) and used it in all subsequent experiments.

### Differential Immune Gene Expression in WT and TLR3 KO Cells Treated With Poly(I:C)

Stimulation of TLR3 receptors in the endosome was examined by adding poly(I:C) in the growth medium (20 µg/mL) for 12 h. This treatment significantly increased the expression of IFN-β (186-fold) and IL-8 (78-fold) genes in WT cells but not in KO cells (Figure 3A). A significant increase in expression of the *TLR3* gene (145-fold) was also observed in the treated WT cells. Interestingly, the treated WT cells also overexpressed *MDA5*, a gene encoding a cytoplasmic dsRNA receptor, by ~295-folds compared with the nontreated control. Endosomal stimulation with poly(I:C) (in the growth medium) did not affect TLR3 and *MDA5* gene expression in KO cells.

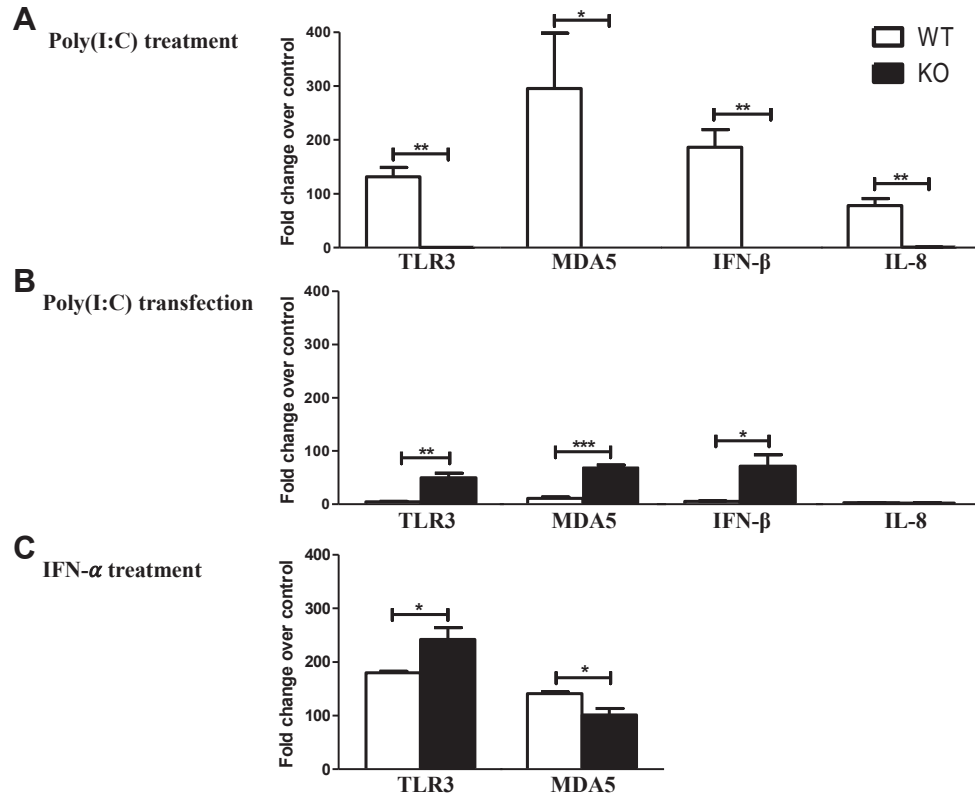
To directly stimulate the MDA5 receptor in the cytoplasm, cells were transfected with poly(I:C) (0.5 µg/mL of the transfection medium) for 4 h, and gene expression levels were measured. The poly(I:C) transfection led to significant upregulation of *MDA5* gene expression in both WT and KO cells compared with untransfected control cells, with the level of expression in KO cells being significantly higher than in WT cells (Figure 3B). Interestingly, the poly(I:C) transfection also induced significant overexpression of the *TLR3* gene in KO cells compared with WT cells. We confirmed by sequencing that the upregulated TLR3 mRNA in KO cells contained the expected mutation for KO#3 cells as shown in Figure 2. The IFN-β gene was overexpressed in transfected cells in a similar manner as dsRNA receptor upregulation. A minor (~2-fold) but significant upregulation of IL-8 was observed only in WT cells.

**Table 2.** Percent identities among avian TLR3 nucleotide (above diagonal) and amino acid sequences (below diagonal).<sup>1</sup>

Species	Quail	Chicken	Turkey	Duck	Goose
Quail		95	95	89	89
Chicken	93		95	90	90
Turkey	94	94		90	90
Duck	86	87	88		97
Goose	87	88	88	96	

Abbreviation: TLR3, Toll-like receptor 3.

<sup>1</sup>GenBank accession numbers for TLR3: quail (MN959450), chicken (EF137861.1), turkey (XM\_003205774.3), duck (NM\_001310782.1), and goose (XM\_013179768.1).



**Figure 3.** Effect of poly(I:C) and chicken IFN- $\alpha$  treatment on wild-type (WT) and TLR3 KO QT-35 cells. (A) The cells were treated by adding poly(I:C) at 20- $\mu$ g/mL concentration in the medium for 12 h. (B) The cells were transfected with poly(I:C) at 0.5- $\mu$ g/mL for 4 h. (C) The cells were treated by adding 50U/mL of IFN- $\alpha$  for 4 h. The gene expression level was measured by real-time quantitative PCR and normalized by GAPDH. The values represent the average of triplicate samples of each cell line with average SEs and expressed as fold change over nontreated control. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001 as determined by Student's  $t$ -test. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KO, knockout; MDA5, melanoma differentiation-associated gene 5; poly(I:C), polyinosinic:polycytidylic acid; QT-35, quail fibroblast; TLR3, Toll-like receptor 3.

To explore a possible mechanism by which TLR3 and MDA5 receptors might upregulate the expression of each other, we stimulated the cells with type I IFN (rChIFN $\alpha$ ) for 4 h and performed transcription analysis. In both WT and TLR3 KO cells, IFN treatment significantly upregulated the *TLR3* and *MDA5* gene expression relative to untreated cells (Figure 3C).

### Antiviral Effects of TLR3-Mediated Immune Responses on ARV, IAV, and VSV Replication

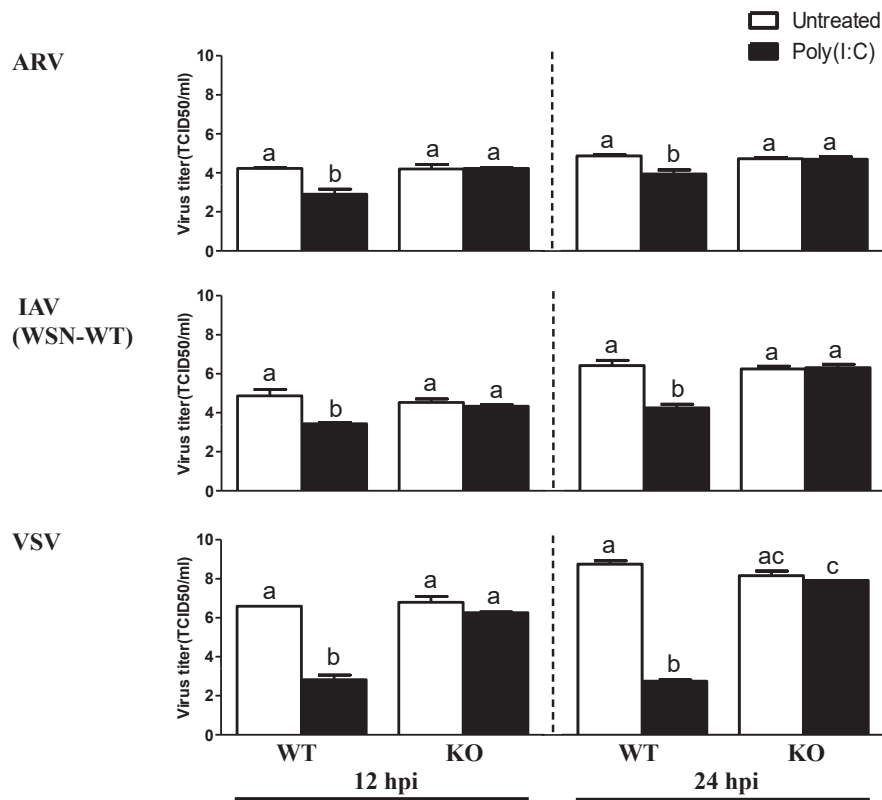
Wild-type and KO cells were incubated with the poly(I:C)-treated medium (10  $\mu$ g/mL) for 24 h to induce IFN production (Figures 3A and 3B) and, subsequently, trigger an IFN-induced antiviral state. There was significant reduction in replication of all 3 viruses in poly(I:C)-treated WT cells at 12 and 24 hpi compared with the corresponding untreated cell controls (Figure 4). The poly(I:C)-induced, TLR3-mediated, antiviral effect in WT cells was the greatest on VSV followed by IAV and ARV. In contrast, the viral titers in treated and untreated KO cells were statistically indistinguishable. Another noteworthy observation is that without treatment, each virus replicated to high titers in both the

WT and KO cells and there were no statistical differences between the cell lines (Figure 4).

### Virus-Induced Immune Responses in WT and KO Cells

Because viral titers were statistically indistinguishable between untreated WT and KO cells (Figure 4), we examined whether the levels of virus-induced innate immune responses were also similar between the cell lines. Infection with ARV, WSN, and WSN-delNS1 significantly increased TLR3 expression in both WT and KO cells relative to uninfected control cells, whereas no change in TLR3 expression was observed with VSV infection (Figure 5). In WT cells, TLR3 expression level was the highest with WSN-delNS1 virus infection followed by ARV and WSN, respectively. In KO cells, TLR3 expression was the highest with ARV infection followed by WSN-delNS1 and WSN-WT infection, respectively. Similarly, MDA5 expression was significantly upregulated in virus-infected WT and KO cells, with the highest expression level observed with ARV infection followed by WSN-delNS1, WSN, and VSV infection, respectively.

The interferon beta (IFN- $\beta$ ) and interleukin 8 (IL-8) expression was also significantly upregulated in both



**Figure 4.** Antiviral response of poly(I:C) pretreatment against ARV, IAV (WSN-WT), and VSV replication in wild-type (WT) and TLR3 KO QT-35 cells. The WT and TLR3 KO QT-35 cells were treated with 10- $\mu$ g/mL poly(I:C) for 24 h and infected with the viruses at 0.1 MOI. The supernatants were collected at indicated hours post infection (hpi), and 50% tissue culture infectious dose (TCID<sub>50</sub>) was measured. The values represent the average of triplicate samples of each cell lines with average SEs. Different letters indicate statistically significant difference ( $P < 0.05$ ) between the groups determined by one-way ANOVA test followed by Tukey's post hoc test. Abbreviations: ARV, avian reovirus; IAV, influenza A virus; KO, knockout; MOI, multiplicity of infection; poly(I:C), polyinosinic:polycytidylic acid; QT-35, quail fibroblast; TLR3, Toll-like receptor 3; VSV, vesicular stomatitis virus.

WT and KO cells after virus infections. In both WT and TLR3 KO cells, WSN-delNS1 infection significantly upregulated expression of most of the tested genes (except IL-8 in KO cells) compared with WSN-WT infection. Infection with ARV and VSV generally induced lower levels of IFN- $\beta$  and IL-8 mRNA in WT cells than WSN-delNS1 infection. This was not the case with KO cells where IFN- $\beta$  and IL-8 mRNA levels were similar among the viruses (Figure 5).

In line with the observation in untreated cells in the previous experiment (Figure 4), all viruses including A/WSN/33-delNS1 replicated to high titers at the tested time points, and there was no difference in virus titers between WT and KO cells (Figure 6).

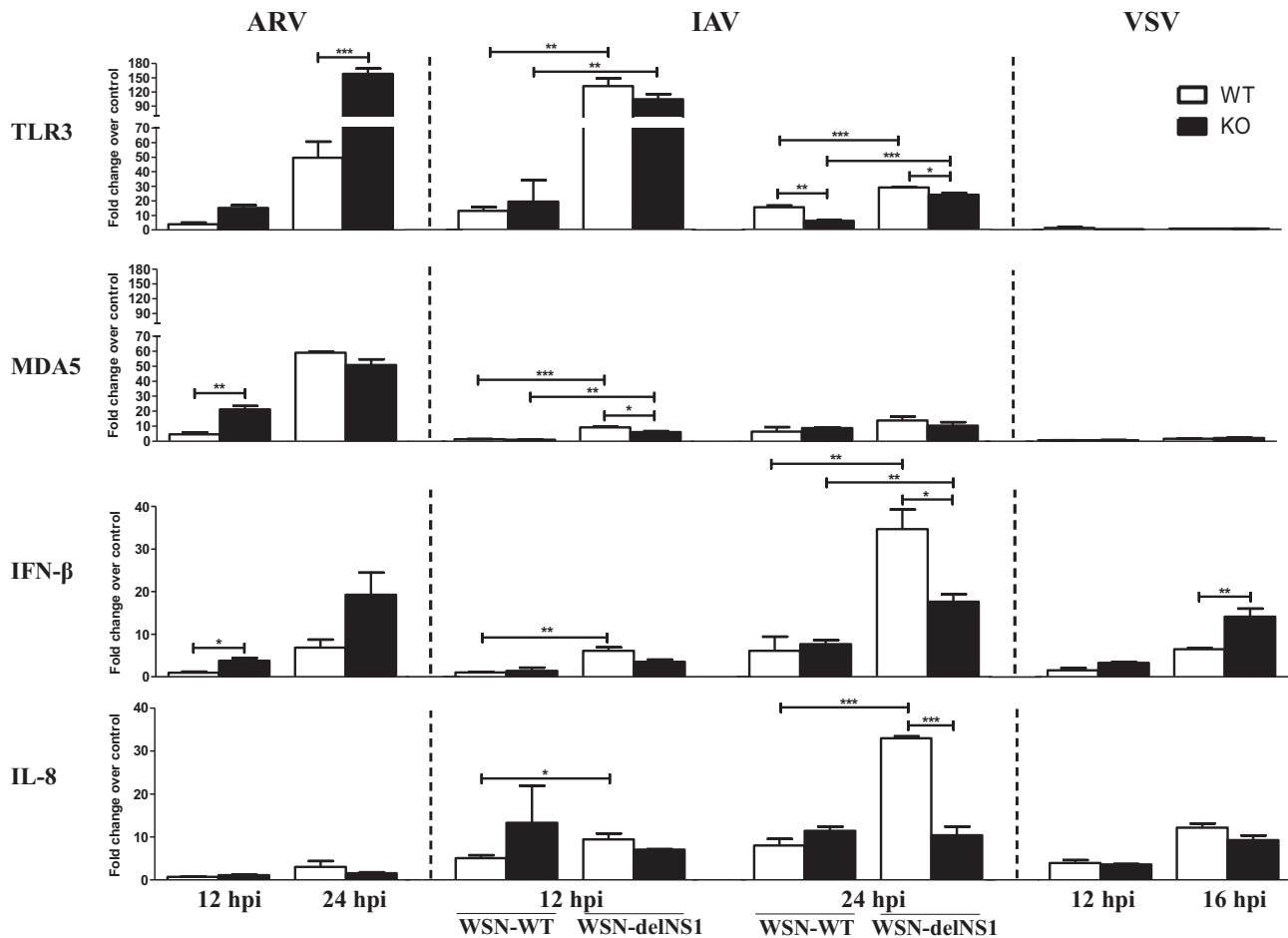
## DISCUSSION

The avian TLR3 remains poorly characterized despite being associated with upregulation of innate immune responses during virus infection (Karpala et al., 2008; Zhang et al., 2015; Kang et al., 2016). In this study, we have determined the coding sequence of qTLR3 and characterized its biological function using knockout QT-35 cells. The coding sequences of qTLR3 mRNA are highly identical to published chicken and turkey TLR3 sequences (Table 2). Based on high sequence homology, it

is possible that the TLR3 is functionally indistinguishable between quail, turkeys, and chickens. However, this study also provides novel insights into post-transcriptional processing of precursor TLR3 mRNA in different avian species. We have identified alternative splice forms of the TLR3 in quail and turkeys and used that information to predict their presence in ducks through bioinformatic analysis. Such isoforms were absent in chicken tissues (Figure 1) and are predicted to be absent in geese. However, it is possible that other isoforms are generated in avian species that are not readily detectable by PCR. Two of the splice forms in quail and one in turkey can be translated to severely truncated TLR3 protein lacking the C-terminal dsRNA binding sites required to signal innate immune responses (Funami et al., 2004; Bell et al., 2006). The truncated proteins may have detrimental effects on TLR3 signaling. For example, small proteins translated from mRNA isoforms of human TLR3 and grouper fish TLR9 have been shown to negatively regulate downstream signaling of the full-length proteins (Smith and Valcárcel, 2000; Lee et al., 2015; Seo et al., 2015). We will investigate if the alternative splice forms of quail and turkey TLR3 can hamper or augment downstream signaling by full-size TLR3 in a future study.

To our knowledge, this study is the first to use KO cells to investigate the biological function of the avian





**Figure 5.** Immune responses induced by ARV, IAVs, and VSV infection in wild-type (WT) and TLR3 KO QT-35 cells. The WT and TLR3 KO QT-35 cells infected with viruses at 0.1 MOI. The cells were collected at indicated hours post infection (hpi). The gene expression levels were measured by quantitative PCR and normalized by GAPDH. The values represent the average of triplicate samples of each cell line with average SEs and expressed as fold change over non-infected control. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  as determined by Student's  $t$ -test. Abbreviations: ARV, avian reovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IAV, influenza A virus; KO, knockout; MOI, multiplicity of infection; QT-35, quail fibroblast; TLR3, Toll-like receptor 3; VSV, vesicular stomatitis virus.

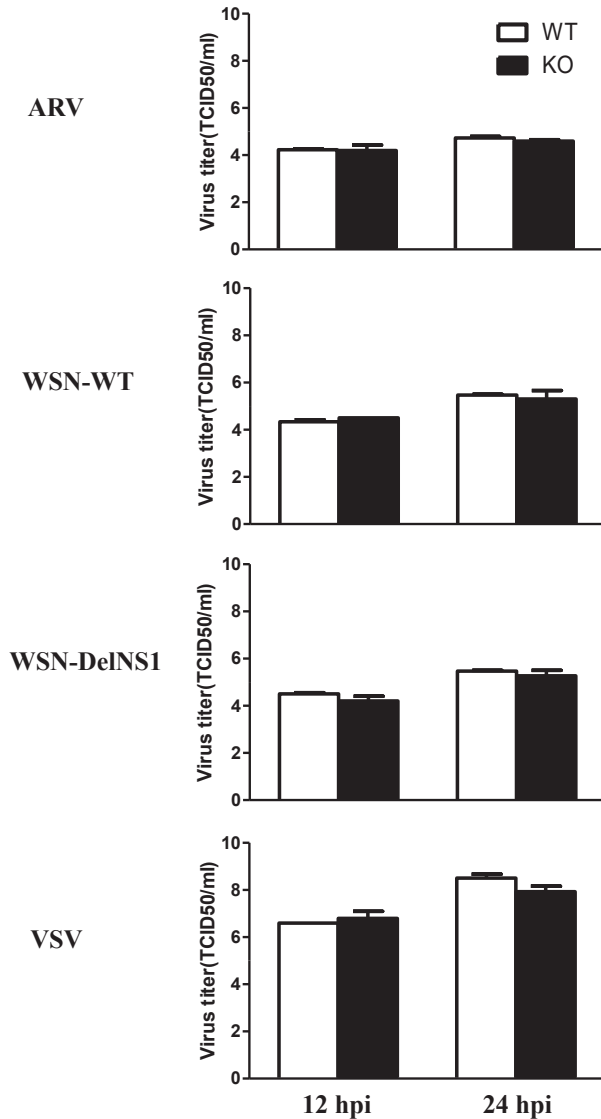
TLR3. This would not be possible without our customized poultry-specific CRISPR/Cas9 system (Ahn et al., 2017). The TLR3 KO was confirmed by sequencing of genomic DNA (Figure 2) and mRNA-derived cDNA (data not shown) and functionally validated by the lack of gene upregulation or inhibition of virus replication when the KO cells were exogenously treated with poly(I:C) (Figures 3A and 4).

Upregulation of IFN- $\beta$  and IL-8 gene expression in poly(I:C)-treated QT-35 WT cells (Figure 3A) is in agreement with observations made in chicken and mammalian cells (Kumar et al., 2006; Karpala et al., 2008; Haunshi and Cheng, 2014; Kang et al., 2016; Ahmed-Hassan et al., 2018). The clean negative results showing no change in expression of TLR3 or cytokine genes in poly(I:C)-treated TLR3 KO cells (Figure 3A) suggest that the TLR3 is the only functional dsRNA-sensing endosomal receptor in quail. Besides that, the qTLR3 KO cells have enabled the detection of a reciprocal interaction between *TLR3* and *MDA5* (compare Figures 3A and 3C) through a mechanism that is yet to be determined. We speculate this interaction to be indirectly mediated by a positive cytokine feedback loop as demonstrated through rChIFN $\alpha$

treatment (Figure 3B). Further investigation with a combination of KO cells (TLR3 $^{-/-}$ , MDA5 $^{-/-}$ , and TLR3 $^{-/-}$  + MDA5 $^{-/-}$ ) and cytokine inhibitors will uncover how these spatially separated dsRNA receptors coordinate to signal immune responses in avian species.

The inhibition of the virus replication in poly(I:C)-treated WT cells (Figure 4) is in agreement with previous reports showing poly(I:C)-induced block of IAV and Marek's disease virus replication in chicken cells (Ahmed-Hassan et al., 2018; Bavananthasivam et al., 2018). Polyinosinic:polycytidylic acid also has shown promising results in live birds when used as a prophylactic agent or an adjuvant in vaccines against influenza and Marek's disease (Parvizi et al., 2012; St. Paul et al., 2012; Zhang et al., 2017). Although the level of poly(I:C)-mediated TLR3-dependent virus reduction (Figure 4) corresponded well with the expected sensitivity to type I interferons (high, moderate, and low sensitivity for VSV, IAV, and ARV, respectively) (Sekellick et al., 2000), other cytokines and antiviral mechanisms may have been involved.

On virus infection, the level of *TLR3* and *MDA5* gene upregulation was generally higher in cells infected with



**Figure 6.** ARV, IAVs (WSN-WT and WSN-delNS1), and VSV replication in wild-type (WT) and TLR3 KO QT-35 cells. The WT and TLR3 KO QT-35 cells were infected with viruses at 0.1 MOI. The supernatants were collected at indicated hours post infection (hpi), and the 50% tissue culture infectious dose (TCID<sub>50</sub>) was measured. The values represent the average of triplicate samples of each cell lines with average SEs. No statistical difference in virus replication was observed between the WT and KO cell lines. Abbreviations: ARV, avian reovirus; IAV, influenza A virus; KO, knockout; MOI, multiplicity of infection; QT-35, quail fibroblast; TLR3, Toll-like receptor 3; VSV, vesicular stomatitis virus.

dsRNA virus (ARV) than those infected with ssRNA viruses (WSN-WT and VSV) (Figure 5). Upregulation of *TLR3* expression has been reported in reovirus-infected duck fibroblast cells (Zhang et al., 2015) and in IAV-infected human and chicken cells and chicken tissues (Karpala et al., 2008; Meng et al., 2016; Ranaware et al., 2016; Cao et al., 2018). Likewise, *MDA5* expression in mouse fibroblast cells was upregulated by several RNA viruses including reoviruses and influenza viruses (Loo et al., 2008). The relatively lower expression of *TLR3* and *MDA5* with VSV infection may be due to relatively minimal role of *TLR3* in recognition of the VSV (Hüsser et al., 2011).

The levels of *TLR3* mRNA expressed in KO cells infected with virus or treated with IFN or poly(I:C) were generally similar to or higher than those observed in the corresponding WT cells (Figures 3B and 3C, and 5). Transcription of nonfunctional mRNAs from KO genes is not uncommon (Liu et al., 2016; Dabrowska et al., 2018). The mutant mRNAs containing premature stop codons undergo degradation through an RNA surveillance mechanism known as nonsense-mediated mRNA decay (Shyu et al., 2008). Still, the fact that not all mutated mRNA undergo nonsense-mediated mRNA decay (Reber et al., 2018) may explain the accumulation of high levels of qTLR3 KO mRNAs after infection with viruses (such as ARV and WSN-delNS1), which induce large amounts of interferons.

Despite stimulation of varying degrees of innate immune gene (IFN- $\beta$  and IL-8) upregulation depending on the infecting virus (Figure 5), there was no difference in viral titers between WT and KO cells (Figure 6). This finding is quite interesting considering that the WT cells are capable of developing an antiviral state and block virus replication (Figure 4). We speculate that the conditions (MOI, sampling time points, etc.) used in that experiment were suboptimal for sequential induction of cytokine and antiviral state in the cell monolayer before the virus could undergo multiple replication cycles. In previous studies, reovirus infection was shown to be detected by all 3 dsRNA receptors, TLR3, RIG-I, and MDA5, in human and mouse fibroblasts cells (Loo et al., 2008; Maitra et al., 2017). The TLR3 was also able to recognize reovirus infection in duck cells (Zhang et al., 2015). On the contrary, in swine cells, VSV and IAV were mainly detected by RIG-I with no or minor contribution of the *TLR3* or *MDA5* (Hüsser et al., 2011). In chicken cells, it was shown that IAV infection is recognized by *MDA5* (Liniger et al., 2012). Because quail lack RIG-I, the generation and use of the TLR3 and MDA5 KO and double-KO cells will be useful in understanding the relevance of each dsRNA receptor in pathogenesis of different viruses in future studies.

It is important to note that the in vitro infection data obtained in this study may not reflect the complex viral pathogenesis observed in vivo. For example, using the KO mouse model, one study reported that the TLR3 is required to resist West Nile virus infection (Daffis et al., 2009), whereas other studies suggested that the TLR3 can exacerbate viral pathogenesis by signaling the production of inflammatory cytokines and enhancing virus dissemination in the infected host (Le Goffic et al., 2006; Hutchens et al., 2008). The in vivo role of the avian TLR3 and other pattern recognition receptors will be addressed using a KO quail model (Lee et al., 2019).

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## DISCLOSURES

The authors declare no conflicts of interest.

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