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Toll-like Receptor 3 Activation Decreases Porcine Arterivirus Infection

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus that initiates infection in pulmonary alveolar macrophages (PAMs), elicits weak immune responses, and establishes a persistent infection. To understand the role of dsRNA intermediates in eliciting host immunity, we sought to determine if toll-like receptor-3 (TLR3), a well-known dsRNA sensor, is involved in the regulation of PRRSV infection. TLR3 gene expression was increased in PAMs of congenitally infected 2-wk-old pigs. Stimulation of PAMs with dsRNA increased gene expression for TLR3 and interferon-β and suppressed PRRSV infectivity. To investigate activation and signaling parameters, expression constructs of wild-type and functional-domain-truncated porcine TLR3 were used in cell transfection studies. When cells that overexpressed porcine TLR3 were stimulated with dsRNA a rapid and robust calcium influx was induced. Moreover, ligand activation of porcine TLR3 expressed in MARC-145 cells elicited an antiviral response to PRRSV. Conversely, transfection of PAMs with small-interfering RNA targeting porcine TLR3 resulted in up to 80% suppression of TLR3 mRNA expression and an increase in PRRSV infectivity. These data provide fundamental genetic and molecular information for porcine TLR3, and implicate its involvement in PRRSV infection, findings that may suggest new strategies to limit this costly pandemic disease.

Introduction

ALTHOUGH PIGS ARE INCREASINGLY USED AS ANIMAL MODELS FOR HUMAN DISEASES and for xenotransplantation (7,21), information about porcine receptors that recognize disease agents, particularly viruses, is limited. The porcine arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV), causes an economically significant pandemic viral disease in pigs (14,28). This virus is one of the primary pathogens involved in the porcine respiratory disease complex, which causes severe respiratory disease in young pigs (35,48,54). During late gestation, PRRSV infection results in reproductive failure including aborted, stillborn, and weak-born pigs, followed by decreases in conception and fertilization rates (28,35,48,54,59). Although much has been learned about PRRSV since its initial appearance as "mystery swine disease" in the late 1980s, several aspects of the interaction of PRRSV with the host immune system remain unresolved (26–28,49).

Immunity to PRRSV begins with the interaction between the virus and porcine cells, predominantly pulmonary alveolar macrophages (PAMs), and intravascular macrophages of the placenta and umbilical cord (29,33,50). Recent studies have made progress in identifying a receptor for PRRSV (2,10,18,45); however, how porcine cells interact with PRRSV at early stages of innate immunity is largely unknown. PRRSV is an enveloped, positive-sense, single-stranded RNA virus (59). During replication, genomic and subgenomic RNAs participate in the formation of several double-stranded intermediate structures (59). Interaction between PRRSV and macrophages likely alters the production of cytokines, including interferon (IFN)-α, IFN-β, IFN-γ and interleukin (IL)-10 (3,5,38,51). Replication of PRRSV in monocyte-derived dendritic cells also leads to suboptimal induction of adaptive immunity (55). Although early upregulation of IFN-γ production, activation of NK cells and $\gamma\delta$ T cells, and stimulation of protective antibody were found in PRRSV-infected pigs (30,57), optimal immune protection was not achieved (55,58). Despite significant efforts to identify immunogenic viral epitopes (9,32,61) and to develop and optimize vaccines with various adjuvants, effective means to control this disease have not been achieved (4,34,38).

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Host cells use various receptors to perceive viral infections by recognizing pathogen-associated molecular patterns (PAMPs), and subsequently induce an antiviral response (16,56). Prominent among these are toll-like receptors (TLRs). Currently, more than 10 TLRs have been identified in humans and mice (16,25,56). Several TLRs perceive viral PAMPs, including TLR3, which detects double-stranded RNA (dsRNA) derived from viral replication; TLR7 and TLR8, which recognize single-stranded RNA (ssRNA) fragments derived from viral genomes; and TLR9, which senses unmethylated cytosine-phosphate-guanine (CpG) motifs common to both bacterial and viral DNA. A non-TLR cytosolic receptor, retinoic acid inducible gene-I (RIG-I) that was originally thought to recognize dsRNA, binds to 5′ triphosphate ssRNA (15,31). TLR7, TLR8, and TLR9, likely form a functional subgroup within the TLR family that recognizes viral PAMPs in endosomal or lysosomal compartments (16,25). In contrast, the location of TLR3 varies depending on the viral infection and cell type, being expressed intracellularly or on the cell surface (13,23,43). After perceiving the presence of viral nucleic acids, these TLRs, such as TLR3, mediate the induction of type I IFNs through a signaling pathway involving adaptor proteins, myeloid differentiation factor 88, or toll/IL-1R domain-containing adapter-inducing IFN-β protein, and elicit a rapid increase of intracellular calcium influx, activate the intermediate protein kinase cascade, and activate transcription factors including nuclear factor-κB and interferon regulatory factor (IRF)-3 and IRF-7. Although several porcine TLRs have been identified (24,46,47,52,53), identification and detailed studies of porcine TLRs responding to viral PAMPs are limited. We have recently reported the molecular identification and functional expression of porcine TLR3 (42). Here we report the involvement of porcine TLR3 in PRRSV infection. Our findings show that activation of porcine TLR3 signaling is important in stimulating effective responses to PRRSV infection, a property that may be exploited by the virus to avoid eliciting effective immune responses and may suggest new strategies to limit this costly pandemic disease.

Materials and Methods

Virus strains and titration

The North American macrophage-tropic PRRSV strain, SDSU-23983-P6 (P6), was used to infect pigs as previously described (17,36,37). All animal and virus procedures were approved by the Kansas State University Institutional Animal Care and Use, and Biosafety Committees. MARC-145 cells, an African green monkey kidney cell line sensitive to PRRSV infection, was used to test PRRSV infectivity and for virus titration. Virus stocks [SDSU-23983-P7 (P7)] collected from supernatant of P6-infected MARC-145 cells were used to infect cell cultures. The tissue culture 50% infectious dose ($TCID_{50}$) of P7 stocks was $10^{7.25}$/mL. Cells were infected at a standard multiplicity of infection (MOI) of 0.1 $TCID_{50}$/cell. This MOI was chosen because quantities of the SDSU-23983 virus greater than 0.1 MOI do not enhance infectivity of MARC-145 cells (35). For titration, MARC-145 cells were cultured in MEM supplemented with 8% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 IU penicillin and 100 μg/mL streptomycin (Chemicon International, Inc., Temecula, CA) in a humidified 5% CO_2 95% air atmosphere at 37°C. Monolayers of MARC-145 cells in 96-well tissue culture plates were fixed with 80% cold acetone and incubated with fluorescent-labeled monoclonal antibodies to PRRSV nucleocapsid (N) protein (SDOW17; Rural Technologies, Inc., Brookings, SD). PRRSV-positive cells were identified by fluorescent microscopy.

PRRSV infection and tissue sample collection

Fetal and young pig lung samples from an earlier study were used in which seronegative pregnant females were infected at 85–90 d of gestation with wild type P6 as previously described (36,37). Infection of late gestation females is a standard model for the study of fetal and congenital PRRSV infection. For the pigs that survive birth, the outcome is productively infected pigs with more severe disease (36). Animals were allowed to give birth and live-born pigs were euthanized at 14 d of age or fetuses were obtained at 107 and 112 days of gestation. Tissue samples were immediately placed in RNAlater (Ambion, Inc., Austin, TX) and stored at −20°C until used (36,37).

PAM collection and culture

Porcine pulmonary alveolar macrophages (PAMs) were obtained by bronchoalveolar lavage from healthy 5-wk-old pigs (6,58). Lungs were lavaged with PBS and recovered lavage fluid was centrifuged at 400 × g for 15 min. After washing with PBS, the cells were resuspended in culture medium (RPMI 1640, 10% fetal bovine serum, 5 mM HEPES, 1 mM glutamine, antibiotic-antimycotic, and 50 mg/mL gentamicin; Invitrogen Life Technologies, San Diego, CA). Cells were cultured in T75 flasks for 2 h in a humidified 5% CO_2 95% air atmosphere at 37°C. Adherent PAMs were collected by washing with cold (4°C) PBS, and counted and cryopreserved until used.

Cell cultures and treatments

Two days before treatments or infection with PRRSV, PAMs were thawed from stocks and plated in 24- or 48-well tissue culture plates (7×10^4 or 4×10^4 cells/well) in supplemented RPMI 1640 medium, and cultured in a humidified 5% CO_2 95% air atmosphere at 37°C. After one change of fresh medium 2 h later to remove non-adherent cells, the PAMs were cultured for 16 h with replenished medium containing the TLR3 ligand, synthetic dsRNA (polyinosinic-polycytidilic acid; Invitrogen Life Technologies) at 5 μg/mL. This concentration was chosen after dose-response tests using ranges of 1–25 μg/mL. Supernatants were collected for interferon assays and RNA was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO). Cells in duplicate wells were infected with PRRSV-P7 and replenished with fresh medium containing the indicated stimulators for 18 h. PAMs were directly stained for PRRSV N protein with a monoclonal antibody (SDOW17) and visualized with TRITC-conjugated goat anti-mouse IgG. The PRRSV-positive cells were examined and counted using fluorescent microscopy.

RT-PCR analysis

Total RNA was extracted with TRI reagent (Sigma-Aldrich) from cells or from frozen tissues ground in liquid nitrogen. Real-time and one-step RT-PCR assays (Qiagen,

Inc., Valencia, CA) were used to detect expression of mRNA transcripts. Briefly, total RNA was treated with RQ1 RNase-free DNAse I (Promega Co., Madison, WI) to remove possible genomic DNA contamination. For real-time RT-PCR a SYBR-green-based assay (Qiagen) was performed on a SmartCycler (Cepheid, Sunnyvale, CA) as previously described (40,41). Relative gene-expression data were normalized against Ct values of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase and the relative index ($2^{-\Delta\Delta Ct}$) was determined in comparison to the average expression levels of control samples with the index defined as 1.000 (19).

Semi-quantitative RT-PCR was performed using one-step RT-PCR (Qiagen) at 32, 35, 38, or 40 cycles. RNA samples (50–250 ng) were run in a 15- or 25-μL RT-PCR reaction mixture with 0.1 μM of each sense and antisense primer derived from cDNA sequences as previously described (42). Generation of PCR primers, PCR data acquisition, and standardization were conducted as previously described (40–42).

Interferon bioassay

Concentrations of type I IFN were measured using a bovine kidney cell line (MDBK) stably expressing a human MxA-promoter (11) in a luciferase reporter vector pGL4.14 [luc2/Hygro] (Promega). Luminescence from the activated luciferase in IFN-treated cells was measured with a Steady-Glo® luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Standard curves were calculated using recombinant human IFN-β (R&D Systems, Minneapolis, MN) at a concentration range of 0–1000 U/mL, and regression analysis between luminescence intensity and IFN concentration was conducted with an exponential growth model (SigmaPlot 9.0; Systat Software, Inc., San Jose, CA).

Construction and expression of porcine TLR3 and TLR3N cDNAs

Full-length cDNA of porcine TLR3 was obtained using RT-PCR and rapid amplification of cDNA ends as previously described (41,42). In addition, the 3′-terminal region of TLR3 at 612 nt long, which is predicted to encode the cytoplasmic TIR/IL-1 domain, was truncated using PCR to create mutated TLR3 (i.e., TLR3N). Purified plasmids were used to transfect HEK293A cells for expression of EGFP-tagged proteins using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) as previously described (40). Transformation efficiency was determined by enhanced green fluorescent protein (EGFP) expression using inverted fluorescence microscopy. After transformation (24 h), cells were collected and transformants were sorted by fluorescence-activated flow cytometry (FACSVantage SE; Becton Dickinson, Franklin Lakes, NJ). Overexpression of control EGFP and EGFP-fused TLR proteins was confirmed by both RT-PCR and immunoblotting. For immunoblotting, proteins were extracted with a mammalian protein extraction reagent (Pierce Inc., Rockford, IL), separated on 4–20% precast sodium dodecyl sulfate (SDS)-polyacrylamide gels (Pierce), and transferred onto polyvinylidene difluoride blots. EGFP and EGFP-fused proteins were then detected with monoclonal anti-EGFP antibodies (1:8000; Clontech, Palo Alto, CA) and visualized using a color development reaction catalyzed by alkaline phosphatase-conjugated secondary antibodies (39,41).

MARC-145 cells were transfected with pEGFP-TLR3 or pEGFP-TLR3N constructs using Fugene HD transfection reagent (Roche Diagnostics). Cells were cultured in 24-well plates or 8-well chamber slides (Electron Microscopy Sciences, Hatfield, PA), and transfection was performed according to the manufacturer?s instructions. After transfection (24 h), cells (>20% of EGFP-positive cells) in different wells were treated with dsRNA (poly [I:C], 5 μg/mL), or incubated with monoclonal antibodies to EGFP (Jl-8, 10 μg/mL; Clontech, Palo Alto, CA) for 30 min, then cross-linked with goat anti-mouse IgG (5 μg/mL; Sigma). Cells were then infected with PRRSV (P7) for 48 h (17,36), and collected for RNA extraction and detection of viral RNA replication using RT-PCR. Cells in chamber slides were fixed and permeabilized with Fix/Perm solution (BD Biosciences, San Jose, CA). The slides were then treated with a monoclonal antibody (SDOW17) to PRRSV N protein and treated with TRITC-conjugated goat anti-mouse IgG. After counterstaining nuclei with Hoechst 33342, the slides were examined using fluorescent microscopy for EGFP (transformed TLR3 or TLR3N) and TRITC (PRRSV). The influence of porcine TLR3 overexpression in mediating PRRSV infectivity was evaluated by comparison with TLR3N-transfected and normal MARC-145 cells.

Intracellular calcium measurement

TLR3-mediated calcium flux in transfected HEK293A cells was measured (1) using the calcium dyes Fluo-3-AM and Fura Red-AM (Molecular Probes, Eugene, OR). HEK293A cells transfected with pEGFP-C3 constructs were sorted (41) and suspended at 5×10^6/mL in 1× Hanks' balanced salt solution (HBSS) with Fluo-3 and Fura Red (2.6 μM Fluo-3 and 5.5 μM Fura Red). Cells were incubated at 37°C for 30–45 min, washed once with 1× HBSS, and resuspended at 1×10^6/mL. Aliquots (250 μL) were warmed to 37°C prior to measurement on a FACSCalibur flow cytometer (Becton Dickinson) equipped with argon and red diode lasers. Fluo-3 was detected at 530/30 nm and Fura Red at 610/20 nm. Cells were analyzed at a rate of ~1000 events/sec. After establishment of the baseline for about 20 sec, dsRNA was added to achieve a final concentration of 10 μg/mL. Recording commenced after replacing the tubes with the stimulator and continued for up to 204 sec. Fluorescence signals of Fluo-3 and Fura Red were collected and the data were processed using flow cytometry analysis software (WinList 5.0; Verity Software House, Inc., Topsham, ME) to obtain the geometric mean fluorescence intensity every 2 sec. The ratio of fluorescence intensity of Fluo-3 to Fura Red was plotted against time using SigmaPlot 9.0 (Systat Software, Inc., San Jose, CA).

siRNA

Gene-specific siRNA for porcine TLR3 was designed (siRNA Target Finder: http://www.ambion.com/techlib/misc/siRNA_finder.html) and matched to position 1088–1108 in the TLR3 cDNA sequence (5′-UGUCAAACUGAGCCCCAGUtt-3′; GenBank accession number DQ647698). Sense and antisense sequences of the siRNA were synthesized (Qiagen). To facilitate the selection of efficient transfection reagents, an Alexa Fluor-488 (AF 488)-labeled

scrambled siRNA (Qiagen), which was designed to not suppress any mammalian gene, also was used. PAMs were seeded in a 24-well plate at 7×10^4 cells/well/0.5 mL, and transfected with different reagents after 24 h according to the manufacturer's instructions. Transfection efficiency was estimated using the AF 488-labeled scrambled siRNA and evaluated by inverted fluorescence microscopy after 24 h. The transfection reagent HiPerfect™ (Qiagen) was used for transfection of TLR3 siRNA into PAMs (60). Twenty-four hours after siRNA transfection, cells were infected with PRRSV for an additional 24 h. Cells in different wells were collected for RNA extraction at 24 and 48 h, or fixed for PRRSV immunostaining as previously described. Infection (%) was obtained by calculation of PRRSV-positive cells in ~500 randomly examined cells in each treatment.

Statistical analysis

Data are presented as means ± SD. Experimental data were analyzed by Student?s *t*-test and statistical significance was set at $p < 0.05$.

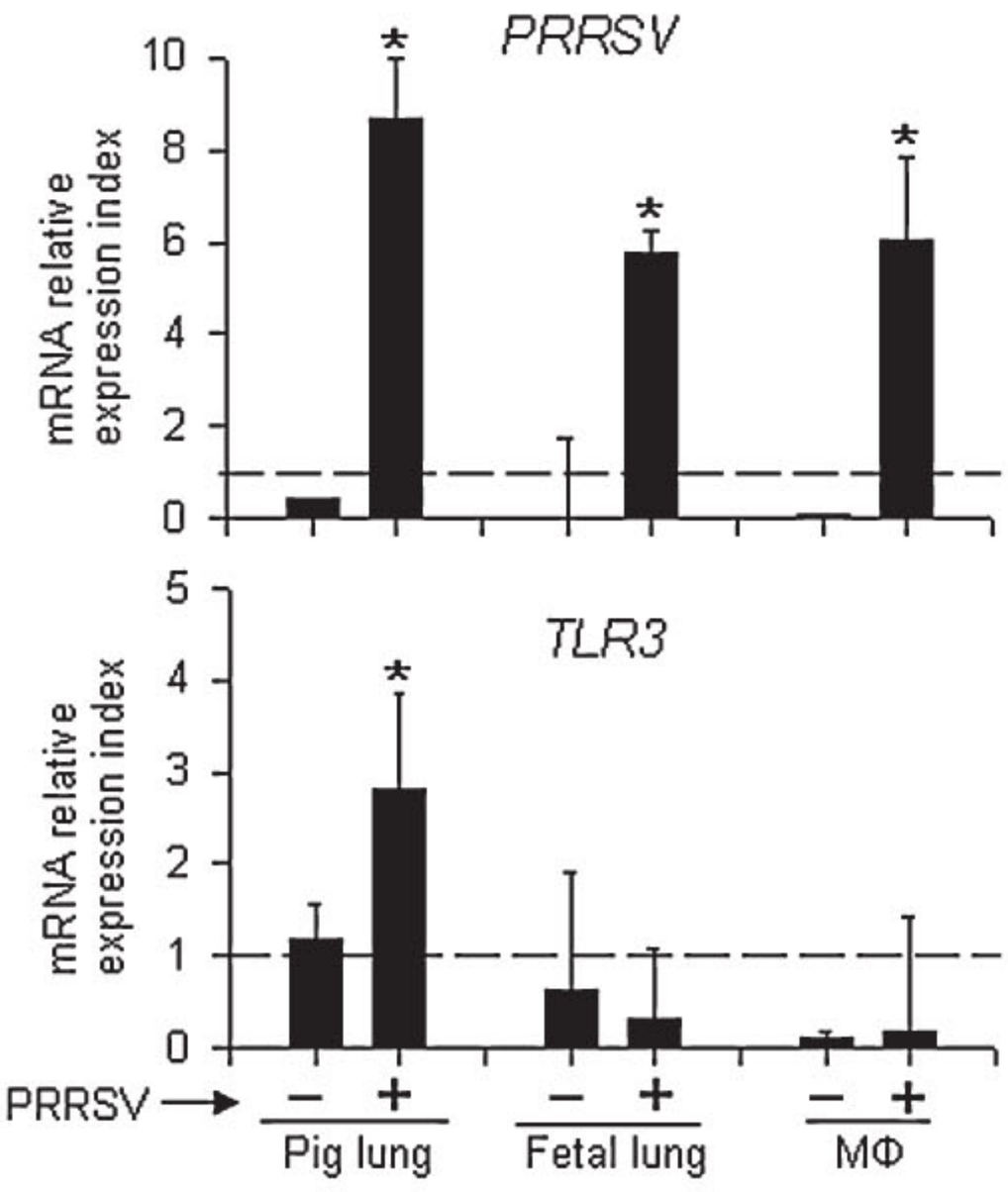
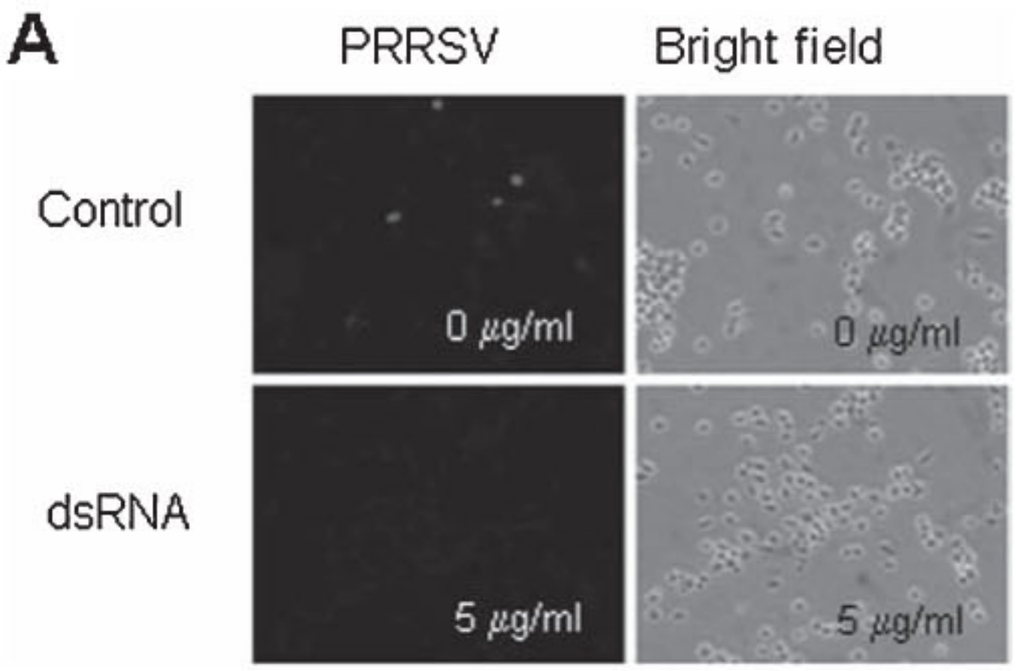


FIG. 1. Expression of porcine TLR3 in PRRSV-infected lungs and pulmonary alveolar macrophages (PAMs). Lungs from fetal and 14-d-old pigs from pregnant females infected with PRRSV, and PAMs infected *in vitro* with PRRSV for 16 h were evaluated for mRNA expression of TLR3. Real time RT-PCR was conducted with gene-specific primers using total RNA (200 ng in 25 μL PCR reaction). Relative gene-expression data were normalized against Ct values of the housekeeping gene, GAPDH, and the relative index ($2^{-\Delta\Delta Ct}$) was determined in comparison to the average expression levels of control samples with the index defined as 1.000 (indicated by the horizontal line). Data are means ± SD, n = 3. *Different from control ($p < 0.05$).



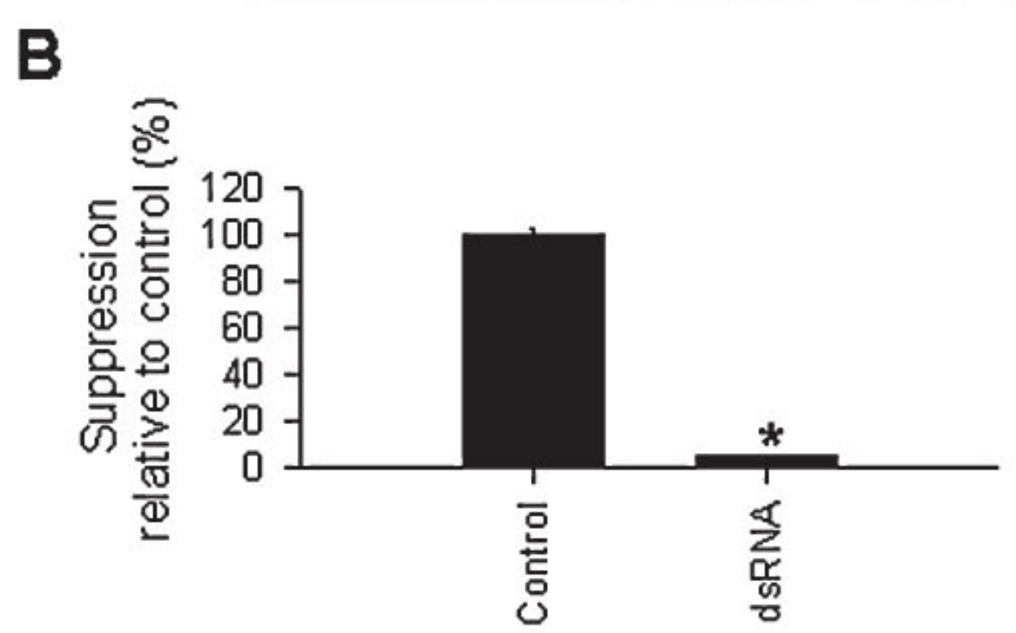


FIG. 2. TLR3 ligand, dsRNA, decreases PRRSV infection in porcine pulmonary alveolar macrophages (PAMs). (**A**) PAMs were cultured in 48-well plates, treated with dsRNA, and infected with PRRSV. Eighteen hours after infection, PAMs were stained for PRRSV N protein with a monoclonal antibody and visualized with TRITC-conjugated goat anti-mouse IgG. The PRRSV-positive cells were examined using fluorescent microscopy. (**B**) Data are means ± SD, n = 3 of 500 cells counted for each repeat. *Different from control ($p < 0.001$).

Results

Expression of porcine TLR3 in PRRSV-infected lungs and PAMs

Our initial approach in studying porcine TLR3 aimed to determine if it was expressed and regulated in PRRSV-infected animals and cells. Indeed, porcine TLR3 was expressed in porcine lungs and PAMs. Upon PRRSV infection, expression of TLR3 mRNA was increased up to threefold in lungs of infected 2-wk-old pigs from pregnant females that had been exposed to PRRSV in late gestation; however, TLR3 mRNA expression was not altered in fetuses from PRRSV-infected sows or in PAMs infected with PRRSV *in vitro* (Fig. 1). The absence of upregulated TLR3 in PAMs and fetuses does not reflect a lack of infection, as PRRSV replication was clearly evident (Fig. 1).

TLR activation induces anti-PRRSV activity in PAMs and MARC-145 cells

Engagement of TLR3 with dsRNA caused a clear decrease in PRRSV infectivity in PAMs, indicated by almost no

PRRSV-positive cells in the dsRNA-treated PAMs (Fig. 2). In addition, the influence of TLR3 activation in MARC-145 cells on PRRSV infectivity also was evaluated. Consistent with the PAM data, dsRNA treatment suppressed PRRSV infectivity in MARC-145 cells. The effective concentration range of dsRNA tested on MARC-145 to suppress PRRSV infectivity was 0.5–25 μg/mL. More than 60% infectivity of PRRSV was inhibited by dsRNA at concentrations higher than 2.5 μg/mL, with the optimal suppressive dose at 5–10 μg/mL (data not shown).

PRRSV infection and dsRNA treatment increases TLR3 and IFN-β gene expression and IFN-β activity

Treatment of PAMs with dsRNA for 6 h significantly increased expression of TLR3 mRNA three- to fourfold (Fig. 3A). The increase in TLR3 mRNA expression was accompanied by a similar increase in IFN-β gene expression; however, IFN-β was not altered (Fig. 3A). Similarly to the gene expression data, dsRNA increased the production of type I IFNs in PAMs as early as 5 h after treatment, and the activity was equivalent to 40- and 158-fold increases in IFN-β at 5 and 10 h, respectively (Fig. 3B). PRRSV-infected PAMs exhibited delayed and lower IFN-β activity compared to cells treated with dsRNA. Supernatants from PRRSV-treated PAMs had 30- and 60-fold increases in IFN-β activity at 10 and 24 h, respectively, compared to controls.

Overexpression of porcine TLR3 and its truncated mutant

We recently reported the molecular identification and functional expression of porcine TLR3 (42). Using those findings to facilitate gain-of-function studies, we expressed whole or partial ORFs of porcine TLR3 fused to EGFP at their amino termini. The partial ORFs were designed to express N-terminal extracellular and transmembrane domains after truncation of the 3′-cDNA regions encoding the C-terminal TIR domains (named TLR3N). Thus, this truncated mutant was designed as a functional control that lacks the ability to induce signal transduction upon perceiving ligands (16,56). Expression of fused proteins was first achieved by introducing TLR3 plasmid constructs into HEK293A cells. Shown in Fig. 4A are the expressed whole and truncated proteins encoded by the whole and truncated ORFs of TLR3. Estimated molecular weight of porcine TLR3 is 103.5 kDa and TLR3N is 24.3 kDa less than its intact forms.

Location and expression of functional porcine TLR3 in HEK293A cells

Before examining potential antiviral responses mediated by cells overexpressing porcine TLR3, we first identified its location and functional properties in HEK293A cells. The transfection efficiency of HEK293A cells was consistently >50% with lipid-formulated reagents (Fugene 6 or HD;

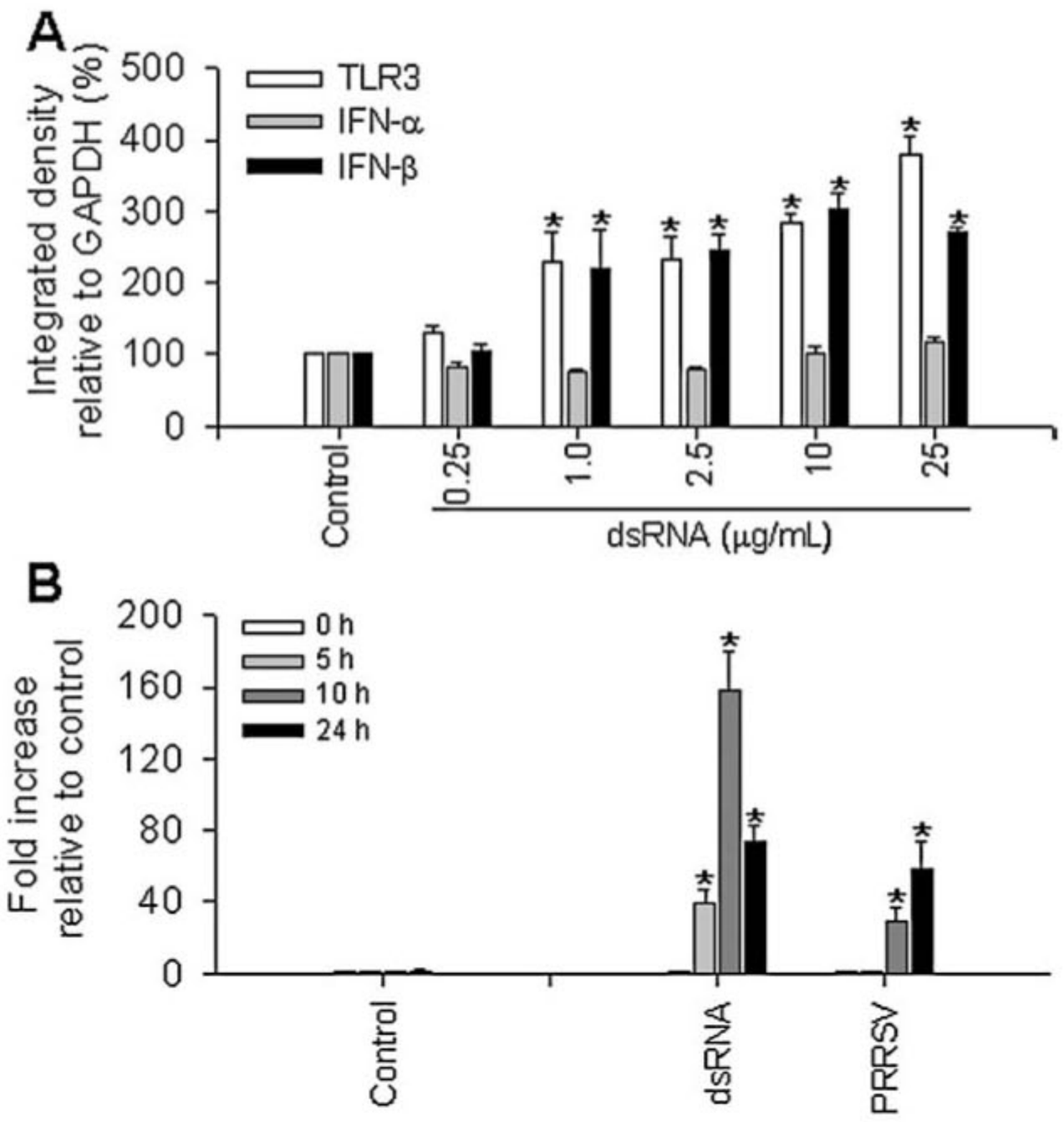
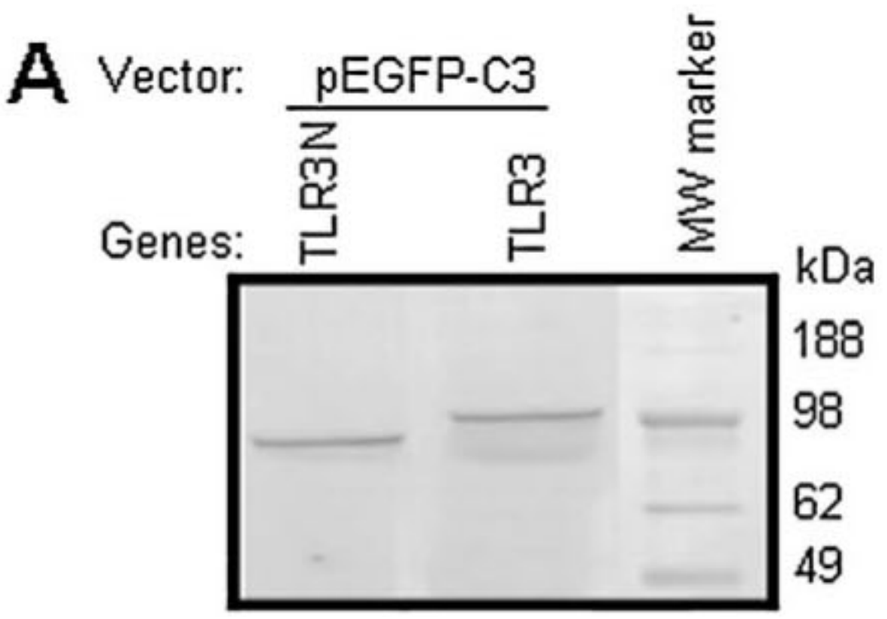
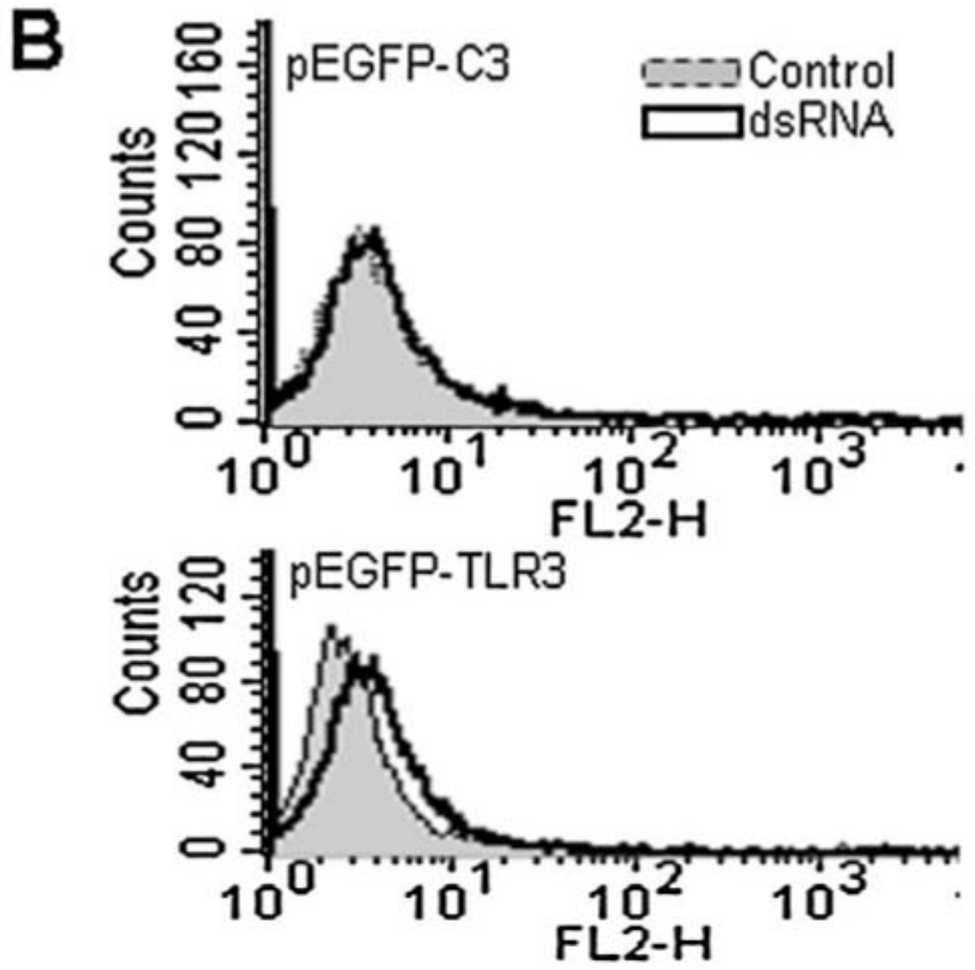
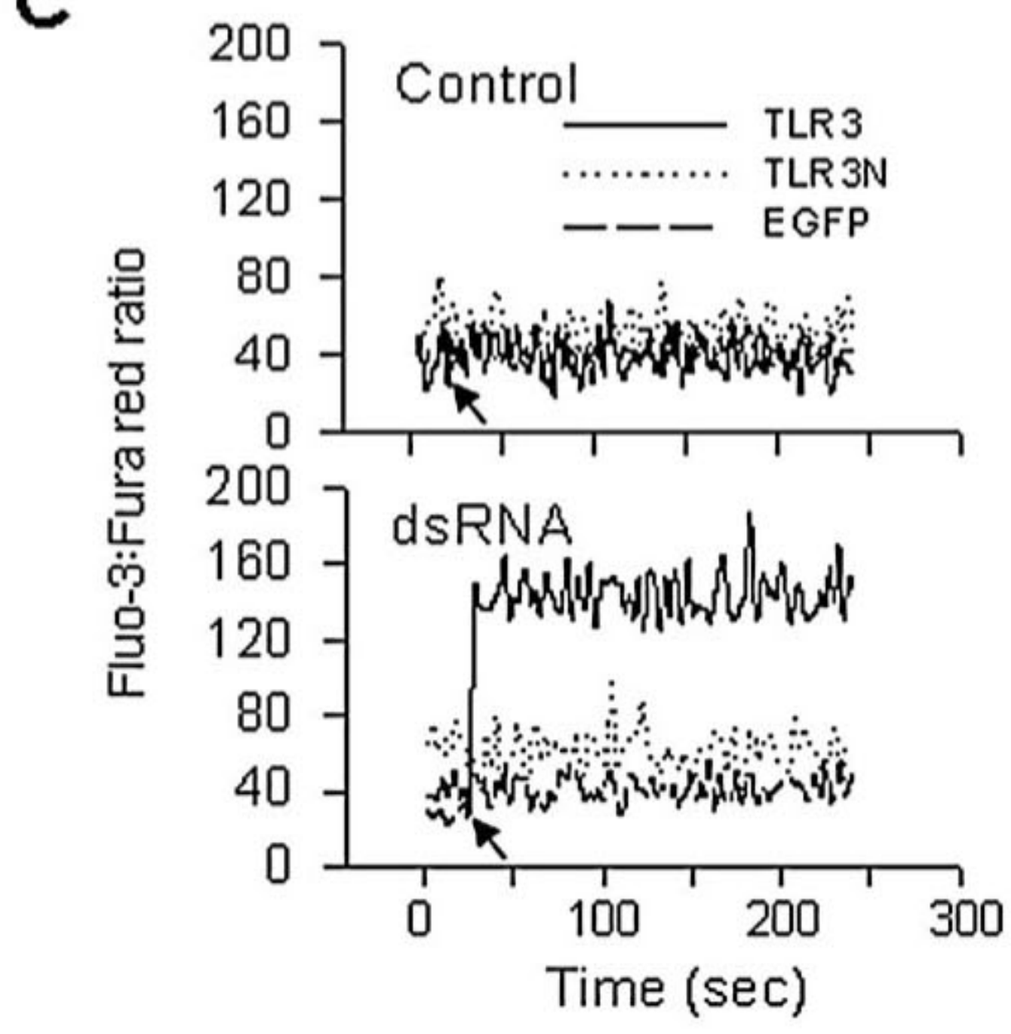


FIG. 3. Stimulation of TLR3 and type I IFN expression by synthetic dsRNA, poly (I:C). (**A**) PAMs were cultured in 48-well plates and treated with dsRNA for 6 h and cells were collected for RNA detection with RT-PCR. (**B**) Bioassay of porcine type I IFNs in PAM supernatants after stimulation with the TLR3 ligand, dsRNA, and infection with PRRSV. Data are activity equivalent to IFN-β. Data are means ± SD, n = 3. *Different from control ($p < 0.05$).







Roche). Cells treated with dsRNA displayed a small but authentic proportion of porcine TLR3 that localized on the cell surface (Fig. 4B). Importantly, porcine TLR3 expressed in HEK293A cells was biologically relevant, as cells stimulated with dsRNA (10 μg/mL) exhibited a rapid and robust calcium influx (Fig. 4C). Before stimulation, cells transformed to express EGFP, EGFP-TLR3N, and EGFP-TLR3 all had similar basal levels of calcium influx.

Activation of porcine TLR3 suppresses PRRSV infectivity

To evaluate the role of TLR3 in mediating anti-PRRSV activity, MARC-145 cells, an established cell line sensitive to PRRSV infection, were transfected with EGFP-TLR3, or EGFP-TLR3N constructs; control cells were mock transfected. Treatment of cells with dsRNA suppressed PRRSV infectivity in transformed and non-transformed cells (Fig. 5A), a finding that likely resulted from activation of endogenous TLR3. To address this issue, anti-EGFP antibody plus secondary antibody was used to cross-link surface-expressed N-terminal EGFP fused to TLR3 in transformed cells (8). Similarly to dsRNA, aggregation of surface-expressed porcine TLR3 with anti-EGFP antibody also significantly decreased PRRSV infectivity. This response was specific, as antibody aggregation of TLR3 was only able to induce an antiviral response in EGFP-TLR3 transformed cells, not in mock- or EGFP-TLR3N-transformed cells (Fig. 5A). When PRRSV replication was evaluated by fluorescent microscopy, similar results were obtained (i.e., cross-linking TLR3 with the anti-EGFP significantly decreased PRRSV infectivity) (Fig. 5B).

Silencing porcine TLR3 decreases IFN-β mRNA expression and increases PRRSV infectivity in PAMs

To further investigate TLR3 involvement in PRRSV infection in PAMs, siRNA was used to silence endogenous TLR3. Optimal transfection conditions were established with an AF-488-labeled control siRNA (Qiagen). At 50 h after transfection, approximately 80% of cultured PAMs loaded with siRNA were successfully transfected as estimated with the control

FIG. 4. Expression of functional porcine TLR3 in HEK293A cells. (**A**) Immunoblot of EGFP-fused TLR3 and TIR-domain truncated TLR3 (TLR3N) in lysates of HEK293A cells transfected with pEGFP-C3 constructs. Cell lysates (50 μg) were resolved on a 4–20% SDS-PAGE gel, transferred to a PVDF membrane, and detected with a primary anti-EGFP monoclonal antibody and a secondary alkaline phosphatase-conjugated anti-mouse IgG. MW, prestained molecular mass markers. (**B**) Cell surface translocation of EGFP-tagged porcine TLR3 was stimulated by dsRNA (poly [I:C], 10 μg/mL) in transformed HEK293A cells. Surface located EGFP-TLR3 was labeled with anti-EGFP mAb and detected with phycoerythrin (R-PE)-conjugated goat anti-mouse IgG. The ratio of R-PE positive cells (FL2) in EGFP-positive cells (FL1) was quantified by flow cytometric analysis. (**C**) Stimulation of calcium influx in TLR3-transformed HEK293A cells. Cells were loaded with calcium dyes Fluo-3 and Fura Red, stimulated with dsRNA (poly [I:C]), and fluorescence was detected with FL1 and FL2 detectors of a flow cytometer in the EGFP-positive cells, and the fluorescence ratio of Fluo-3:Fura red was analyzed with WinList 5.0.

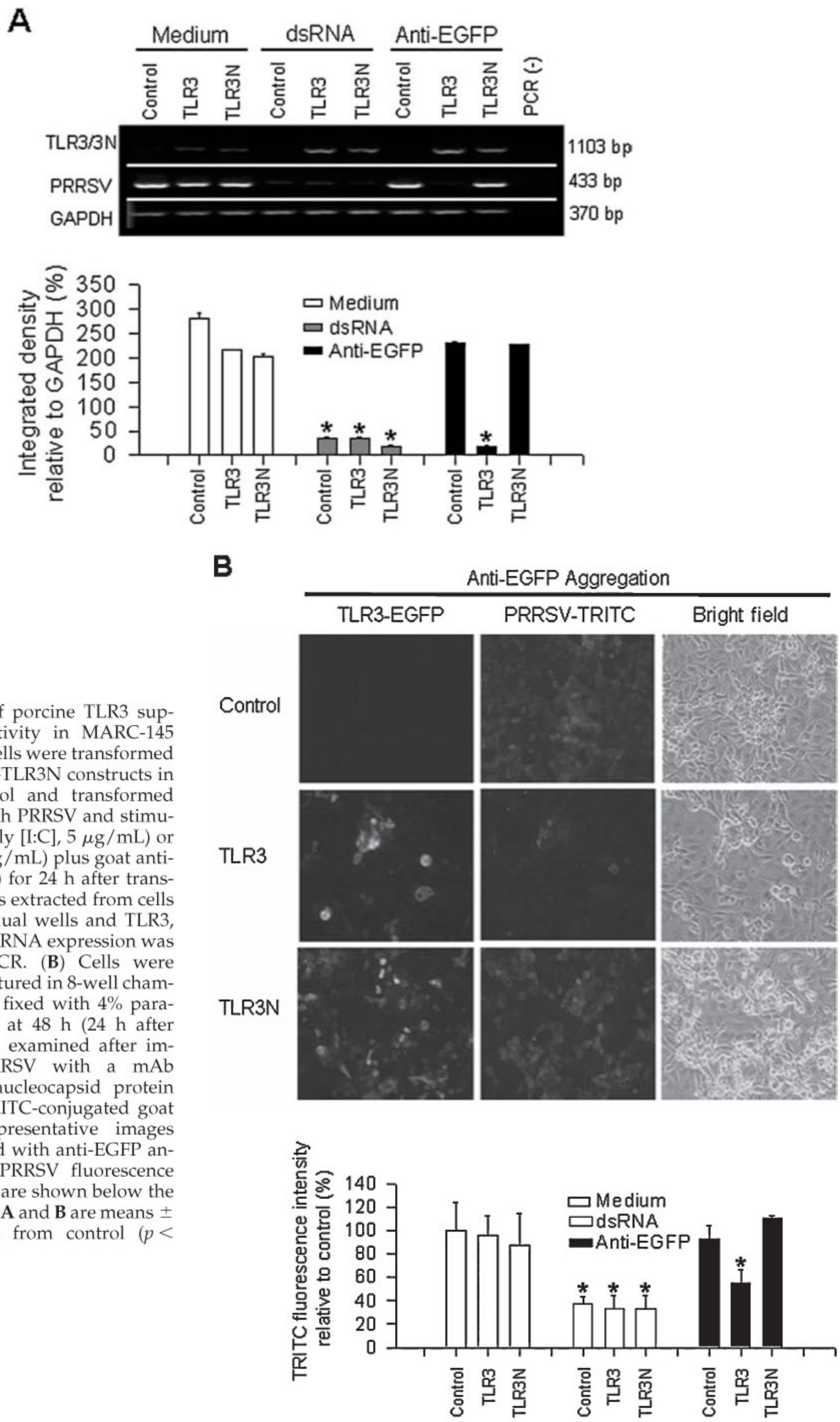


FIG. 5. Activation of porcine TLR3 suppresses PRRSV infectivity in MARC-145 cells. (**A**) MARC-145 cells were transformed with pEGFP-TLR3 or -TLR3N constructs in 24-well plates. Control and transformed cells were infected with PRRSV and stimulated with dsRNA (poly [I:C], 5 μg/mL) or anti-EGFP mAb (10 μg/mL) plus goat anti-mouse IgG (5 μg/mL) for 24 h after transfection. Total RNA was extracted from cells collected from individual wells and TLR3, TLR3N, and PRRSV mRNA expression was detected using RT-PCR. (**B**) Cells were treated as in **A,** but cultured in 8-well chamber slides. Cells were fixed with 4% paraformaldehyde in PBS at 48 h (24 h after PRRSV infection) and examined after immunostaining of PRRSV with a mAb (SDOW17) to viral nucleocapsid protein and detected with TRITC-conjugated goat anti-mouse IgG. Representative images from the group treated with anti-EGFP antibodies are shown. PRRSV fluorescence data for all treatments are shown below the images. Data in panels **A** and **B** are means ± SD, n = 3. *Different from control ($p < 0.05$).

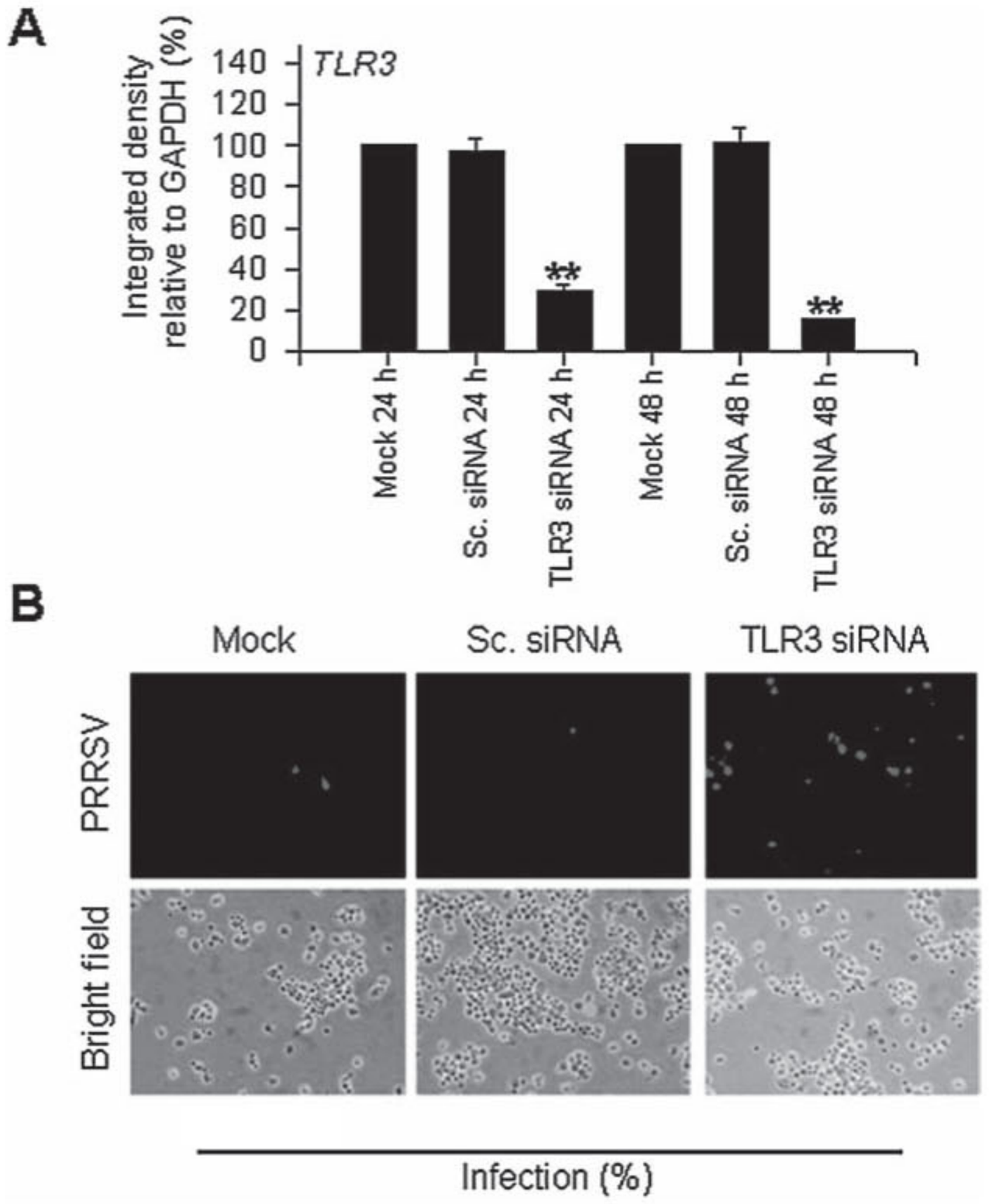


FIG. 6. Silencing TLR3 increases PRRSV infectivity in porcine alveolar macrophages (PAMs). (**A**) PAMs were cultured in 24-well plates and transfected with siRNA dissolving buffer (Mock), Alexa Fluor 488 (AF 488)-labeled scrambled (Sc) siRNA, or gene-specific siRNA to porcine TLR3 (TLR3 siRNA) for 48 h. Cells in individual wells were collected for RNA extraction at 24 h and 48 h. Gene expression of TLR3 and GAPDH was assayed using RT-PCR and quantified as described in Fig. 5. Data are means ± SD, n = 3. **Different from mock-transfected cells ($p < 0.01$). (**B**) Silencing TLR3 increases PRRSV infection. PAMs were transfected with siRNA and infected with PRRSV at 30 h after transfection. Twenty hours after infection, PAMs were fixed and immunostained for PRRSV as in Fig. 5. Infection (%) was obtained by calculation of PRRSV-positive cells in ~500 randomly examined cells in each treatment.

AF-488 siRNA. Transfection with siRNA for TLR3 decreased endogenous TLR3 mRNA expression 50–70% at 24 h, and 80% at 48 h (Fig. 6A). When compared to mock and scrambled siRNA-transfected cells, suppression of TLR3 in PAMs by gene-specific siRNA caused six- to eightfold increases in PRRSV-positive cells at 20 h after infection (Fig. 6B).

Discussion

This study provides experimental support for three new findings concerning the involvement of TLR3 in porcine viral infections. First, it provides the first molecular and functional characterization of porcine TLR3, data that are critical for comparative studies using porcine models for infection and immunity. Second, it shows that activation of TLR3 produced significant antiviral responses to PRRSV and decreased replication of this economically significant porcine virus. Third, it shows that cells with mutated or diminished TLR3 function exhibit increased PRRSV infection. These findings suggest that TLR3 is important for effective innate immunity to PRRSV.

Similar to other mammals, pigs have an extensive repertoire of TLRs, and at the amino acid sequence level, generally show about 80% similarity to TLR orthologs from other mammalian species (24,46,47,52,53). Here we provide the first functional characterization of porcine TLR3, an innate immune receptor well characterized for its function in perceiving viral molecules. TLR3 recognizes dsRNA originating from viruses or host cells. Other viral-sensing TLRs, TLR7, TLR8, and TLR9, recognize viral ssRNA and unmethylated CpG DNA in viral genomes (16,25,43). When we examined viral TLR gene expression in response to PRRSV infection in porcine lungs and PAMs, we found that TLR3 mRNA expression was increased in lungs of young pigs, but not in lungs of fetal pigs or PAMs. The absence of upregulation of TLR3 in the fetal pig may reflect downregulation of *in utero*

TH1 responses or the immaturity of the fetal immune system. Expression of other TLR mRNA was not significantly influenced by PRRSV infection (data not shown). Several possibilities might explain the finding that PRRSV infection selectively influenced expression of these virus-responsive TLRs. First, these innate immune receptors may be constitutively expressed in various tissues, and thus are not susceptible to significant upregulation. Second, an increase in TLRs elicited by PRRSV infection may have occurred at an earlier time than when the samples were collected. Third, it is possible that PRRSV has a mechanism to mitigate upregulation of TLRs.

Because PAMs are a primary cell type for PRRSV infection, we examined expression of porcine TLR3 in PAMs and evaluated the influence of activating TLR3 on innate viral protection. Poly (I:C), a synthetic dsRNA TLR3 ligand, induced significant effects in PAMs, yielding protective responses against PRRSV infection. Porcine TLR3 and IFN-β were significantly increased by poly (I:C) treatment, which suggests that TLR3 was involved in the stimulation of IFN-β expression and the subsequent suppression of PRRSV infection. After extensively comparing poly (I:C)–induced cytokine profiles in porcine PAMs and peritoneal macrophages, Loving *et al.* (20) concluded that dsRNA induced cytokine expression in PAMs mainly via mediation of TLR3, and in peritoneal macrophages via PKR. Similar to our findings in PAMs, treating MARC-145 cells with poly (I:C) also suppressed PRRSV infection, suggesting that poly (I:C) stimulated similar antiviral responses at least partially via TLR3 in MARC-145 cells (22).

To evaluate the contribution of the TLR3 pathway in anti-PRRSV responses, we conducted gain-of-function and loss-of-function studies. In gain-of-function experiments, EGFP-tagged porcine TLR3 was functionally expressed in both HEK293A cells and MARC-145 cells. Selective activation of transfected EGFP-TLR3 using antibody aggregation resulted in protective activity against PRRSV infection. Because only about 10% of the cells had EGFP-TLR3 located on the surface and thus available for cross-linking by antibodies, the cells may exploit a communication pathway to transmit and enhance the signal from stimulated cells that have activated TLR3, perhaps analogous to the cross-priming function of TLR3 in dendritic cells to cytotoxic T cells (44). Overexpression of the null mutant, TLR3N, only slightly enhanced PRRSV infectivity in MARC-145 cells. We reason that this finding likely resulted because PRRSV attains nearly saturated infectivity in MARC-145 cells. The loss-of-function study using siRNA to TLR3 confirmed that TLR3 plays an irreplaceable role in perceiving dsRNA in PAMs, and probably in inducing expression of IFN-β. Importantly, silencing endogenous TLR3 caused a clear increase of PRRSV infectivity, further implicating the importance of TLR3 in antiviral responses to PRRSV. In addition to TLR3, PKR, RIG-I, and melanoma differentiation-associated gene-5 (Mda-5) also have been implicated as dsRNA sensors (12). During preparation of this paper, a study reported that PRRSV suppresses IFN-β production, primarily by interfering with the RIG-I signaling pathway, and partially by suppression of TLR3 signaling in MARC-145 cells (22). It is unknown whether PRRSV adopts a similar mechanism to escape innate immunity in porcine cells such as PAMs. Our data, generated mostly in PAMs, show that TLR3 signaling is important for PAM antiviral responses to PRRSV. It is possible that the TLR3-signaling pathway could be augmented in response to RIG-I signaling attenuation by PRRSV. Further investigation of the signaling pathways mediated by TLR3, RIG-I-like receptors, and Mda-5 in innate immune responses to PRRSV is needed.

Conclusion

Taken together, our findings suggest that activation of porcine TLR3 stimulates significant protective activity against PRRSV infection. The TLR3-mediated responses include production of type I IFNs. Because production of type I IFNs has been reported to be compromised during PRRSV infection (29,33,38), it is tempting to speculate that this virus may possess mechanisms to evade TLR3 activation in host immune cells. This possibility warrants further investigation.

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Author Disclosure Statement

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