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CELLULAR RESPONSE TO INFECTION

Released Tryptophanyl-tRNA Synthetase Stimulates Innate Immune Responses against Viral Infection

Hyun-Cheol Lee,[a] Eun-Seo Lee,[a] Md Bashir Uddin,[a,b] Tae-Hwan Kim,[a] Jae-Hoon Kim,[a,c] Kiramage Chathuranga,[a] W. A. Gayan Chathuranga,[a] Mirim Jin,[d,e] Sunghoon Kim,[f,g] Chul-Joong Kim,[a] Jong-Soo Lee[a]

[a]College of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea
[b]Faculty of Veterinary & Animal Science, Sylhet Agricultural University, Sylhet, Bangladesh
[c]Laboratory Animal Resource Center, KRIBB, University of Science and Technology (UST), Daejeon, Republic of Korea
[d]Laboratory of Microbiology, College of Medicine, Gachon University, Incheon, Republic of Korea
[e]Department of Health Science and Technology, GAIHST, Gachon University, Incheon, Republic of Korea
[f]Medicinal Bioconvergence Research Center, College of Pharmacy, Seoul National University, Gwanak-gu, Seoul, Republic of Korea
[g]Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Gwanak-gu, Seoul, Republic of Korea

ABSTRACT Tryptophanyl-tRNA synthetase (WRS) is one of the aminoacyl-tRNA synthetases (ARSs) that possesses noncanonical functions. Full-length WRS is released during bacterial infection and primes the Toll-like receptor 4 (TLR4)-myeloid differentiation factor 2 (MD2) complex to elicit innate immune responses. However, the role of WRS in viral infection remains unknown. Here, we show that full-length WRS is secreted by immune cells in the early phase of viral infection and functions as an antiviral cytokine. Treatment of cells with recombinant WRS protein promotes the production of inflammatory cytokines and type I interferons (IFNs) and curtails virus replication in THP-1 and Raw264.7 cells but not in TLR4$^{-/-}$ or MD2$^{-/-}$ bone marrow-derived macrophages (BMDMs). Intravenous and intranasal administration of recombinant WRS protein induces an innate immune response and blocks viral replication *in vivo*. These findings suggest that secreted full-length WRS has a noncanonical role in inducing innate immune responses to viral infection as well as to bacterial infection.

IMPORTANCE ARSs are essential enzymes in translation that link specific amino acids to their cognate tRNAs. In higher eukaryotes, some ARSs possess additional, noncanonical functions in the regulation of cell metabolism. Here, we report a novel noncanonical function of WRS in antiviral defense. WRS is rapidly secreted in response to viral infection and primes the innate immune response by inducing the secretion of proinflammatory cytokines and type I IFNs, resulting in the inhibition of virus replication both *in vitro* and *in vivo*. Thus, we consider WRS to be a member of the antiviral innate immune response. The results of this study enhance our understanding of host defense systems and provide additional information on the noncanonical functions of ARSs.

KEYWORDS alarmin, innate immunity, virus infection, WRS

The innate immune system is the first line of host defense against invading pathogens that possess conserved pathogen-associated molecular patterns (PAMPs) (1). The PAMPs are detected by host pattern recognition receptors (PRRs), leading to the activation of downstream signaling molecules such as tank-binding kinase 1 (TBK1), interferon (IFN)-regulatory factor 3 (IRF3), and nuclear factor-κB (NF-κB) (2–4). This cascade of reactions eventually results in the secretion of proinflammatory cytokines and type I IFNs, which trigger inflammation or convert neighboring cells to an infection-

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Address correspondence to Jong-Soo Lee, jongsool@cnu.ac.kr.

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resistant state by inducing IFN-stimulated genes (ISGs) (5, 6). The hallmark of innate immunity is the immediate response to pathogen invasion, which is rapidly initiated by PRRs such as retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and Toll-like receptors (TLRs) (7–9), each of which recognizes specific pathogenic ligands (1, 2, 10).

TLR4 is an extracellular receptor that, together with its accessory proteins myeloid differentiation factor 2 (MD2) and cluster of differentiation 14 (CD14), recognizes lipopolysaccharide (LPS) (10–12). Once activated by the LPS-binding protein (LBP) complex (13), TLR4 signals to downstream effectors via the myeloid differentiation primary response 88 (MyD88)-dependent pathway or the TIR domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway (14, 15). The MyD88-dependent pathway is a conserved signaling pathway among TLRs that is mediated by the adaptor molecule MyD88. This pathway results in the activation of NF-κB and mitogen-activated protein kinase (MAPK) and the secretion of proinflammatory cytokines. The TRIF-dependent pathway activates NF-κB and IRFs to produce proinflammatory cytokines and type I IFNs (2, 7). These functions of TLR4 in the inflammatory response not only are closely related to pathogen invasion but also might be involved in autoimmunity, neurological diseases, and cancer (16–19). Due to the important roles of TLR4, its ligands have also been studied in detail. In addition to LPS from Gram-negative bacteria, teichuronic acid from Gram-positive bacteria, the F protein of syncytial viruses, and the NS1 protein of the dengue virus are also known pathogenic ligands of TLR4 (4, 20, 21).

In addition to these pathogenic ligands, a number of endogenous ligands are reported to activate TLR4 (22, 23). Several endogenous molecules, including high-mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), and defensin, have been suggested as endogenous ligands of TLR4 (22, 24). Such endogenous ligands that activate the immune system are referred to as alarmins (25). There is still controversy regarding the exact definition, but generally, alarmins are proteins that are quickly released in response to pathogen infection or the resulting tissue damage and stimulate innate and adaptive immune responses (25, 26). Molecules such as HMGB1, interleukin-1 (IL-1), IL-33, and galectins have been characterized as alarmins and shown to protect against pathogenic infection (27, 28). For example, HMGB1 activates TLR2, TLR4, and receptor for advanced glycation end products (RAGE) to induce innate immune responses (29–33).

Aminoacyl-tRNA synthetases (ARSs) are essential enzymes that catalyze the ligation of specific amino acids to their cognate tRNAs (34). In addition to their canonical role in translation, ARSs in higher organisms possess additional domains or novel motifs that mediate diverse noncanonical functions in processes such as cell metabolism, tumorigenesis, angiogenesis, and innate immunity (35–38). These noncanonical functions of ARSs are not confined to the cytosol, where their role in linking amino acids to tRNAs is carried out, but occur in the nucleus or in the extracellular space after secretion. Human tryptophanyl-tRNA synthetase (WRS) also carries out additional functions. Inside the nucleus, WRS stimulates DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), activity via its WHEP domain, leading to the phosphorylation and activation of p53 (39). In addition, the miniature form of WRS, which lacks the 47 N-terminal amino acids, is secreted into the extracellular space and inhibits angiogenesis by interacting with VE-cadherin (40). Recently, a role for WRS in innate immunity against bacterial infection was reported. However, its specific role in the response to viral infection has not been clarified in detail (41).

In the present study, we show that WRS is rapidly secreted by virus-infected immune cells, and secreted WRS can induce the secretion of antiviral cytokines, including type I IFNs. Consequently, secreted WRS inhibits virus replication *in vitro* and *in vivo*. These findings suggest a novel role for WRS as an enhancer of innate immune responses against viral infection.

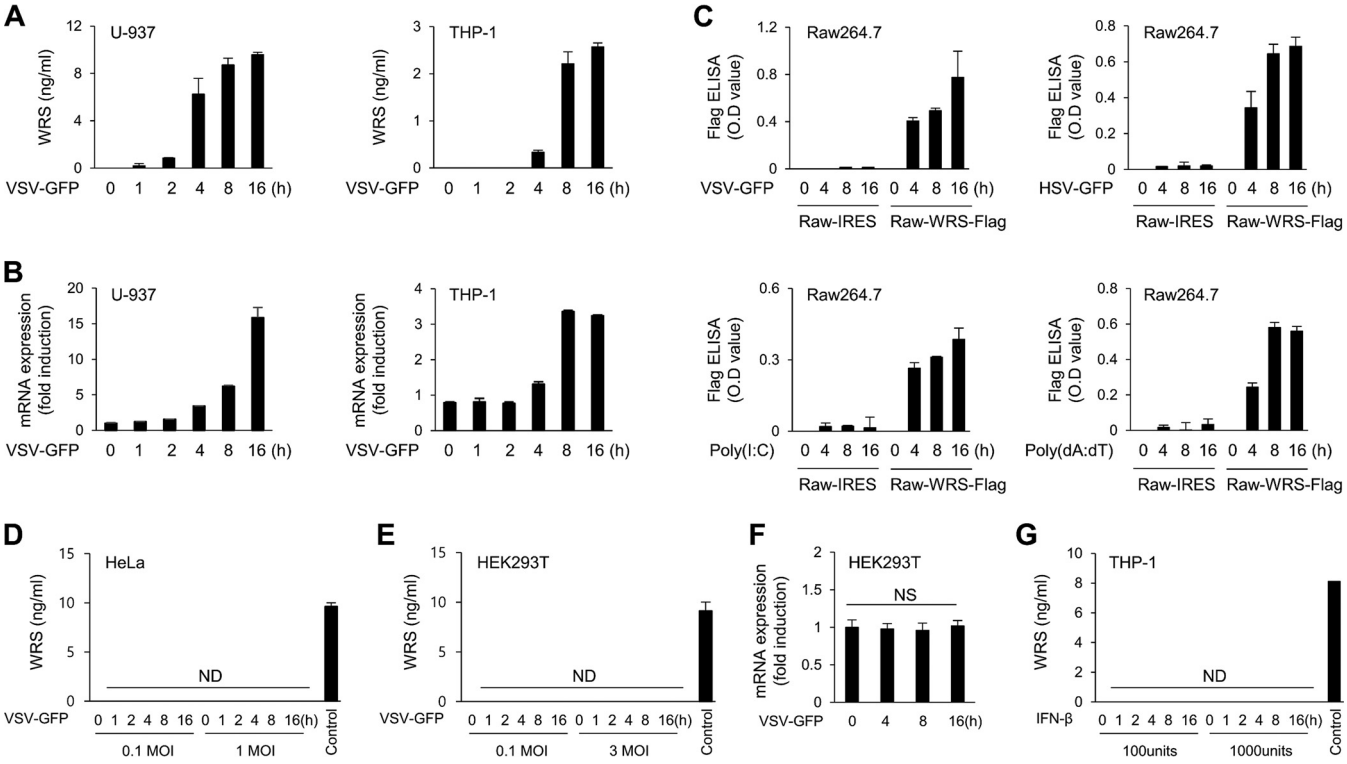

FIG 1 WRS is secreted in response to virus infection. (A) ELISA of WRS levels in the supernatant of U-937 and THP-1 cells infected with VSV-GFP at a multiplicity of infection (MOI) of 3 at indicated time points. (B) mRNA expression level of WRS shown in panel A was determined by qRT-PCR. (C) Flag-tagged mWRS expressing stable Raw264.7 cells and control cells were infected with VSV-GFP (upper, left) or HSV-GFP (upper, right) at an MOI of 1. Secreted levels of Flag-tagged mWRS were assessed by anti-Flag ELISA in the supernatant at the indicated time points. Shown is anti-Flag ELISA of the supernatant in the same cell line with treatment of 40 μg poly(I-C) (lower left) or transfection of 1 μg poly(dA-dT) (lower right). OD, optical density. (D) ELISA of WRS levels in the supernatant of HeLa cells infected with VSV-GFP at an MOI of 0.1 or 1 for indicated time points. ND, not determined. (E) ELISA of WRS levels in the supernatant of HEK293T cells infected with VSV-GFP at an MOI of 0.01 or 3 for indicated time points. (F) mRNA expression level of WRS in HEK293T cells infected with VSV-GFP at an MOI of 0.01, as determined by qRT-PCR. NS, not significant. (G) THP-1 cells were treated with 100 or 1,000 U of recombinant IFN-β. Secreted levels of WRS were assessed by ELISA; 1,000 ng/ml of recombinant WRS standard was used as a positive control for the experiment. ND, not detected; NS, not significant. Error bars, means ± SD.

RESULTS

Viral infection induces secretion of WRS on immune cells. To examine whether viral infection triggers WRS secretion, human immune cell lines were infected with green fluorescent protein-tagged vesicular stomatitis virus (VSV-GFP). WRS levels in the supernatant increased in a time-dependent manner after infection (Fig. 1A) and the transcription of WRS also increased as viral infection progressed (Fig. 1B), suggesting that WRS is related to the response to viral infection.

To further investigate the characteristics of WRS secretion, various cells and viruses were tested. Raw264.7 cells stably expressing Flag-tagged murine WRS were infected with viruses with various types of genomes. Raw264.7 cells infected with VSV-GFP (an RNA virus) or herpes simplex virus (HSV)-GFP (a DNA virus) secreted WRS into the supernatant (Fig. 1C). In addition, cells stimulated with poly(I-C) (a viral RNA ligand) or poly(dA-dT) (a viral DNA ligand) also released WRS into the supernatant (Fig. 1C). To investigate the effects of WRS on epithelial cells, HeLa or HEK293T cells next were infected with VSV-GFP, and WRS secretion and transcription was assessed. In contrast to infected immune cells, WRS production was not observed in infected epithelial cells at the protein or the mRNA level (Fig. 1D to F).

In response to viral infection, cells secrete antiviral cytokines, particularly IFNs, that alert neighboring cells to the infection. This IFN-mediated signaling induces secondary antiviral factors for host cell protection. Based on the fact that WRS is secreted at early time points, we hypothesized that WRS was secreted directly in response to viral infection, not mediated by IFNs. To confirm this, THP-1 cells were treated with 100 or

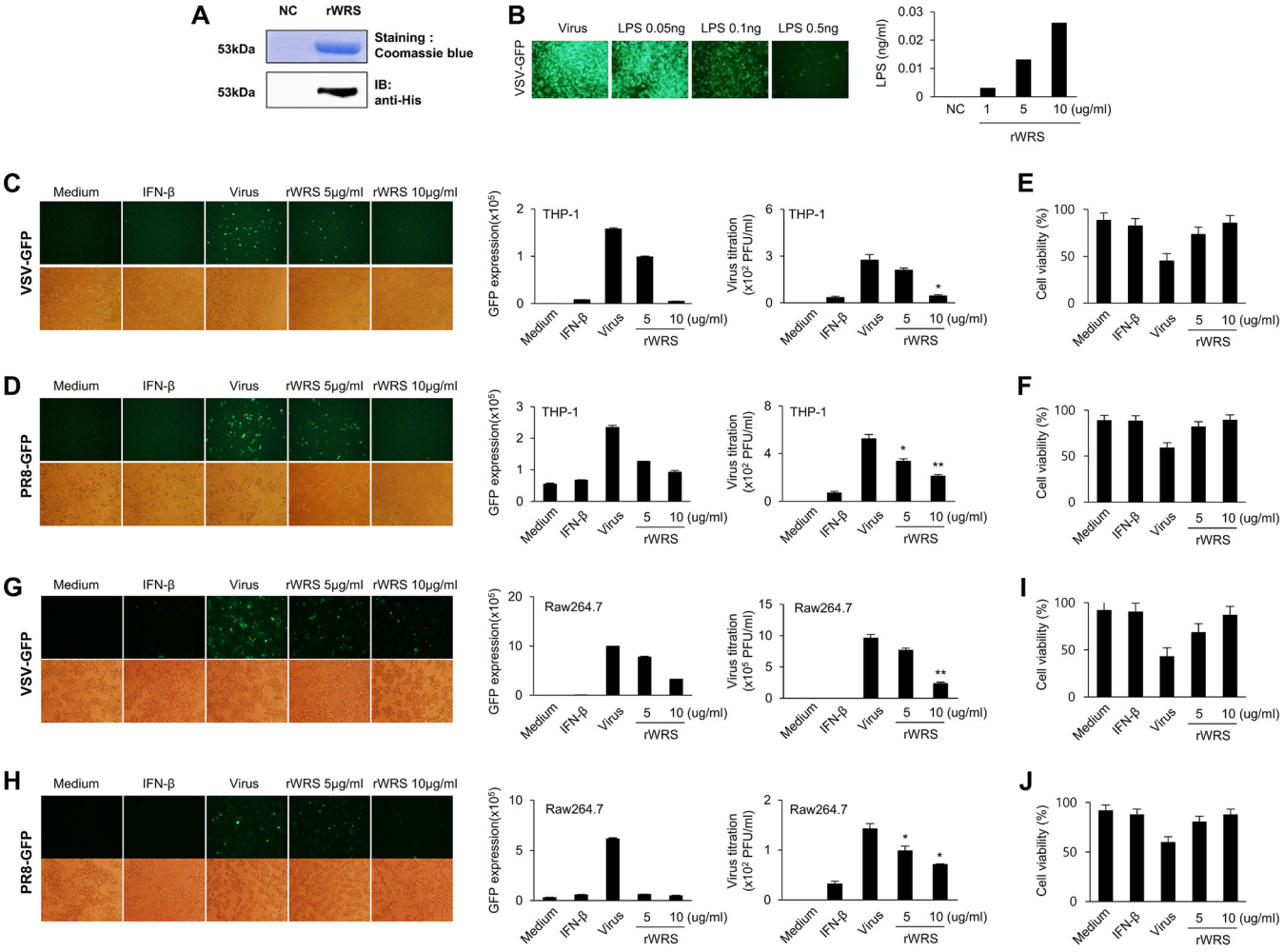

FIG 2 WRS reduces virus replication in immune cells. (A) Expression of rWRS (purified protein) confirmed by SDS-PAGE. NC, negative control; IB, immunoblot. (B) Determination of endotoxin concentrations in the purified recombinant protein rWRS by the LAL assay. (C and D) THP-1 cells were treated with medium alone, 5 and 10 μg/ml of rWRS, or 1,000 U/ml recombinant human IFN-β 12 h prior to infection with VSV-GFP (C) or PR8-GFP (D) at an MOI of 3.0. GFP expression (left) and GFP absorbance (middle) were obtained at 24 hpi. Virus titers were determined by standard plaque assay (right). (E and F) Cell viability in panels C and D was measured by trypan blue assay. (G and H) Raw264.7 cells were treated with medium alone, 5 and 10 μg/ml of rWRS, or 1,000 U/ml recombinant mouse IFN-β 12 h prior to infection with VSV-GFP (G) or PR8-GFP (H) at an MOI of 1. GFP expression (left) and GFP absorbance (middle) were obtained at 24 hpi. (Right) Virus titers were determined by standard plaque assay. (I and J) Cell viability from results shown in panels G and H was measured by trypan blue assay. Error bars, means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

1,000 U of IFN-β, and WRS secretion was measured. However, treatment with IFN-β did not induce the release of WRS (Fig. 1G). Taken together, these findings suggest that WRS is one of the primary signals released by immune cells in response to viral infection.

WRS mediates antiviral effects on immune cells. To assess the function of WRS, recombinant human WRS (rWRS) was produced in *Escherichia coli* and purified with His tag affinity chromatography. Purified protein was confirmed by SDS-PAGE and immunoblotting (Fig. 2A). As recombinant proteins from bacterial expression systems contain by-products, such as LPS, that can cause immune stimulation, the LPS was removed from the recombinant protein solution by extraction with Triton X-114. Furthermore, the minimum dose of LPS that inhibited the replication of VSV-GFP in Raw264.7 cells was determined to be 0.1 ng/ml. The rWRS used throughout this study was confirmed by *Limulus* amebocyte lysate (LAL) assay to have an endotoxin concentration of less than 0.03 ng/ml (Fig. 2B).

Because WRS was secreted by virus-infected immune cells, we investigated the effect of WRS on the antiviral activity of the cells. THP-1 cells were pretreated with rWRS

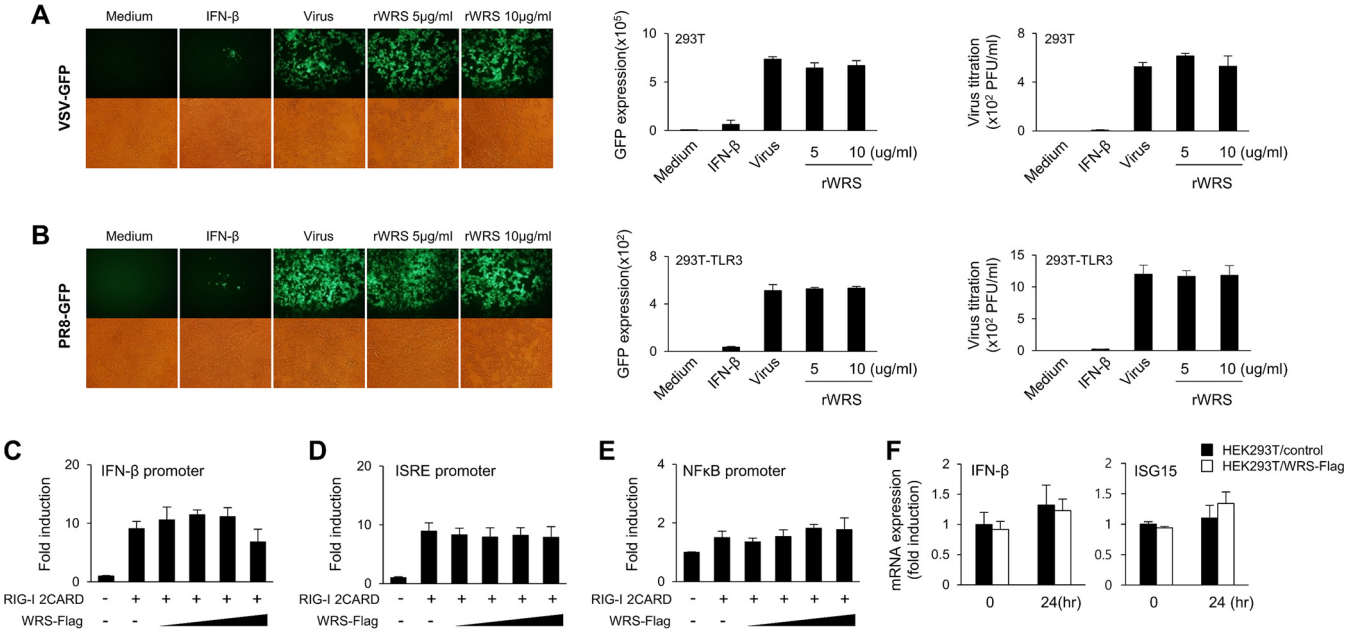

FIG 3 WRS does not induce antiviral effect on HEK293T. (A and B) HEK293T cells (A) or TLR3-expressing stable HEK293T cells (B) were treated with medium alone, 5 and 10 μg/ml of rWRS, or 1,000 U/ml recombinant human IFN-β 12 h prior to infection with VSV-GFP at an MOI of 0.01. GFP expression (left) and GFP absorbance (middle) were obtained at 24 hpi. Virus titers were determined by standard plaque assay (right). (C to E) HEK293T cells were transfected with 400 ng of IFN-β (C), 800 ng of ISRE (D), 800 ng of NF-κB promoter reporter gene (E), 10 ng of TK-renilla, and 5 ng of RIG-I 2CARD, together with 100, 200, 400, and 800 ng of Flag-tagged WRS expression vector. Luciferase activity was analyzed in a luminometer. (F) HEK293T cells were transfected with Flag-tagged WRS expression vector and control vector. mRNA expression of IFN-β and ISG15 was determined by qPCR at 24 h. Error bars, means $\pm$ SD.

for 12 h, washed, and then infected with VSV-GFP or PR8-GFP (a strain of influenza A virus) for 2 h. At 24 h postinfection (hpi), rWRS-treated cells showed markedly reduced levels of GFP expression. The replication of VSV-GFP and PR8-GFP and cell death were also decreased compared with that in control cells (Fig. 2C to F). Likewise, the antiviral effect of WRS on Raw264.7 cells was tested in a similar manner. Consistent with the results in THP-1 cells, rWRS-treated Raw264.7 cells were more resistant to VSV-GFP and PR8-GFP infection (Fig. 2G to J).

Collectively, these results demonstrate that extracellular stimulation of immune cells with rWRS prior to viral infection had antiviral effects. These results suggest that WRS contributes to viral clearance and that WRS functions as an antiviral signaling molecule that is regulated by immune cells in response to viral infection.

WRS has no effect on epithelial cells or intracellular innate immune signaling pathways. IFNs signal through various IFN receptors, which are expressed by diverse cell types. Therefore, we asked whether WRS activates antiviral signaling in epithelial cells as well as immune cells. HEK293T cells were treated with rWRS for 12 h and then infected with VSV-GFP for 24 h. However, rWRS-treated cells were similar to the control cells with respect to GFP expression and viral titer (Fig. 3A), indicating that the antiviral function of rWRS is confined to immune cells. Additional analyses were performed on HEK293T cells overexpressing TLR3, which recognizes double-stranded RNA. However, TLR3-overexpressing HEK293T cells were also unaffected by rWRS treatment (Fig. 3B). These results suggest that WRS interacts with a membrane receptor only expressed by immune cells.

During viral infection, recognition of PAMPs by PRRs results in the activation of specific intracellular signaling pathways. We therefore examined the intracellular signaling pathways activated in innate immune cells in response to stimulation with WRS. HEK293T cells were transfected with Flag-tagged WRS, together with the two caspase recruitment domains (2CARD) of RIG-I and an IFN-β luciferase promoter. About a 10-fold increase in IFN-β promoter expression was observed in response to expression of the RIG-I CARD, an inducer of the RLR-mediated pathway. However, intracellular

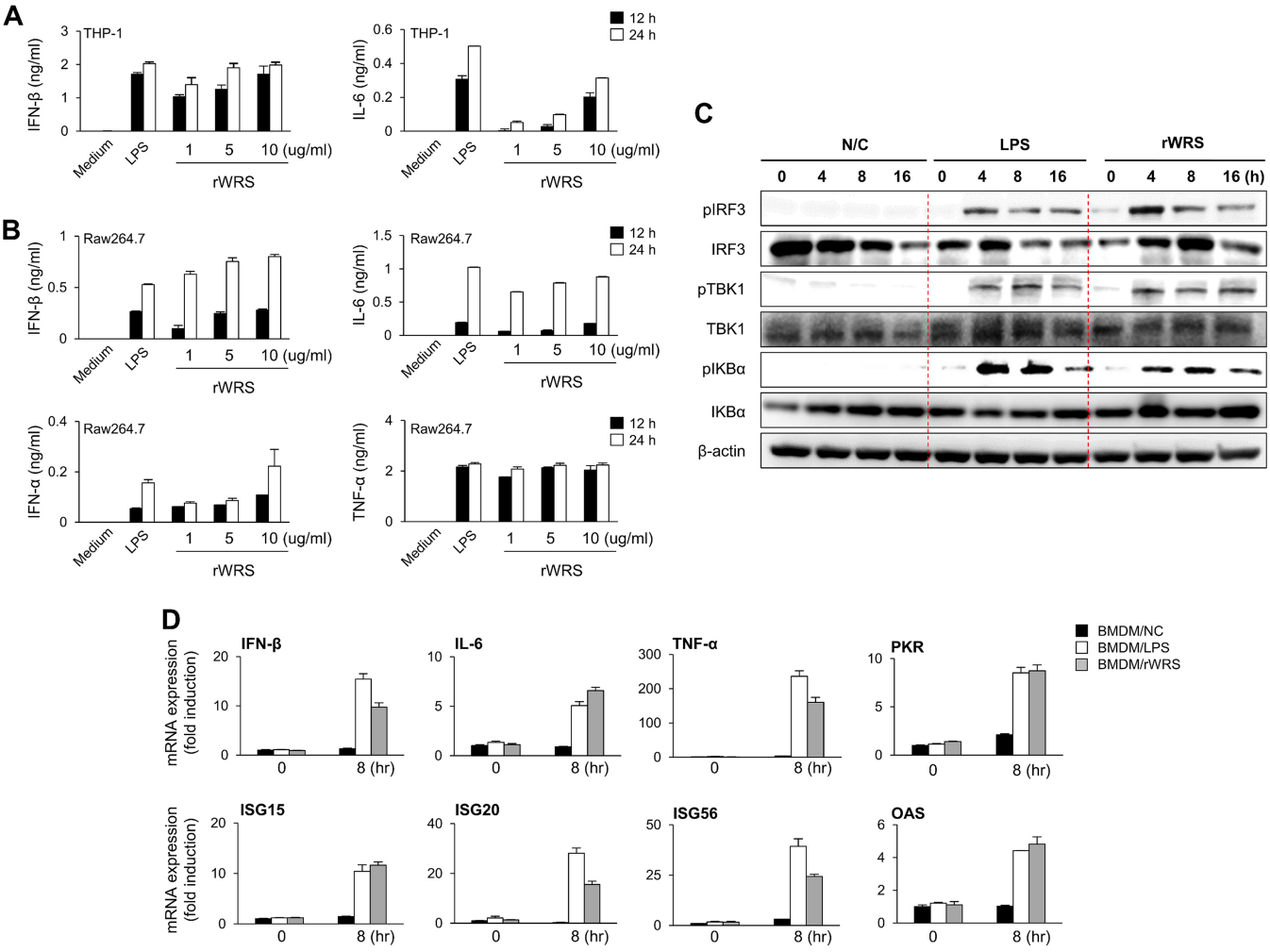

FIG 4 Extracellular stimulation of WRS elicits innate immune responses. (A) ELISA of IFN-β (left) and IL-6 (right) levels in the supernatant of THP-1 cells treated with the indicated dose of rWRS for 12 or 24 h. LPS (100 ng) was used as a positive control. (B) ELISA of IFN-β (upper left), IL-6 (upper right), IFN-α (lower left), and TNF-α (lower right) levels in the supernatant of Raw264.7 cells treated with the indicated dose of rWRS for 12 or 24 h. LPS (100 ng/ml) was used as a positive control. (C) Raw264.7 cells were treated with rWRS for 4, 8, and 16 h. Samples were immunoblotted with normal and phosphorylated forms of IRF3, TBK1, IκB-α, and β-actin. LPS (100 ng/ml) was treated as a positive control. (D) mRNA expression level of IFN-β, IL-6, and other IFN-related antiviral genes in BMDMs treated with 10 μg of rWRS for 8 h. LPS (100 ng/ml) was used as a positive control. Error bars, means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

expression of WRS had no effect on IFN-β promoter expression (Fig. 3C). The activities of an IFN-stimulated response element (ISRE) promoter and an NF-κB luciferase promoter were also tested in the same system. Although the ISRE and NF-κB promoters showed approximately 10- and 5-fold induction in expression, respectively, in response to RIG-I 2CARD expression, coexpression of WRS had no additional effect (Fig. 3D and E). In addition, we found that overexpression of WRS also did not induce gene expression of IFN-β or ISG-15 (Fig. 3F). Collectively, these results, together with those shown in Fig. 1E, suggest that WRS mediates antiviral effects as a secreted factor but not as an intracellular stimulus.

WRS elicits innate immune responses and induces antiviral cytokines. To further characterize the antiviral functions of WRS, antiviral cytokine secretion in response to rWRS treatment was evaluated. In these experiments, THP-1 cells were treated with rWRS for 12 or 24 h, and IFN-β and IL-6 levels in the supernatant were analyzed by enzyme-linked immunosorbent assay (ELISA). In response to rWRS treatment, THP-1 cells secreted large amounts of IFN-β and IL-6 in a dose-dependent manner (Fig. 4A). This experiment was next repeated using Raw264.7 cells. Similarly, Raw264.7 cells treated with rWRS showed a dose-dependent increase in the secretion

of IFN-β, IFN-α, IL-6, and tumor necrosis factor alpha (TNF-α) (Fig. 4B). These data indicate that WRS enhances the secretion of cytokines involved in the innate immune response to viral infection.

IFN-β and IL-6 induce signaling cascades that result in the phosphorylation of IFN-related signaling molecules such as TBK1, IRF3, and IKBα, the latter of which results in NF-κB activation. To analyze the activation of these signaling molecules, phosphorylation-specific immunoblotting was performed after treatment of Raw264.7 cells with rWRS. In response to rWRS stimulation, Raw264.7 cells showed higher levels of phosphorylated TBK1, IRF3, and IKBα (Fig. 4C). Moreover, the effect of rWRS on the gene expression of IFN-β and IL-6 was evaluated by real-time quantitative PCR (qRT-PCR). Raw264.7 cells treated with rWRS showed increased levels of *IFNB1* and *IL-6* mRNA. The expression of other IFN-induced antiviral factors, such as *ISG15* and *ISG20*, was also increased (Fig. 4D). These data demonstrate that stimulation of innate immune cells with WRS triggers the production of antiviral cytokines as well as IFN-related antiviral factors.

TLR4 and MD2 mediate the antiviral function of WRS. A recent study reported that secreted WRS is a primary defense factor against bacterial infection and demonstrated that WRS acts via TLR4-MD2 by measuring inflammatory cytokine production from TLR4$^{-/-}$ bone marrow-derived macrophages (BMDMs) (41). To assess the interaction between WRS and TLR4-MD2 in viral infection, BMDMs were isolated from wild-type (WT), TLR2$^{-/-}$, TLR4$^{-/-}$, MD2$^{-/-}$, and MyD88$^{-/-}$ mice. Cultured BMDMs were treated with rWRS for 12 h and then infected with VSV-GFP. WT and TLR2$^{-/-}$ BMDMs showed reduced virus replication following treatment with rWRS. IFN-β and IL-6 levels in the supernatant at 12 and 24 h posttreatment were also increased (Fig. 5A and B). On the other hand, there was no antiviral effect of rWRS on TLR4$^{-/-}$ or MD2$^{-/-}$ BMDMs. Moreover, rWRS failed to stimulate antiviral cytokine secretion in these cells, demonstrating that TLR4-MD2 is the receptor for WRS (Fig. 5C and D).

MyD88 is an adaptor molecule that transmits signals to downstream molecules that interact with the intracellular region of TLR4 (7). BMDMs isolated from MyD88$^{-/-}$ mice were also evaluated for their response to rWRS treatment. WRS did not inhibit viral replication in MyD88$^{-/-}$ BMDMs and induced lower levels of IFN-β and IL-6 than in WT BMDMs (Fig. 5E). The low levels of IFN-β and IL-6 secreted by WRS-stimulated MyD88$^{-/-}$ BMDMs are likely induced through another adaptor molecule, TRIF, which also transmits signals downstream from TLR4-MD2. Taken together, these data suggest that WRS enhances antiviral activity and cytokine secretion via TLR4-MD2.

WRS inhibits virus replication *in vivo*. We next addressed the antiviral effect of WRS *in vivo*. To assess whether rWRS induces antiviral cytokine secretion in mice, rWRS was intravenously injected into mice via the tail vein. Mice treated with rWRS showed elevated serum levels of IFN-β and IL-6, which peaked at 3 and 6 h postinjection, respectively (Fig. 6A). Mice next were treated with rWRS and then intravenously infected with VSV-GFP. Consistent with the serum cytokine results, there was less VSV replication in rWRS-treated mice at 12 h postinfection (Fig. 6B). We also examined the effects of intranasal administration of WRS, and cytokine levels in the bronchoalveolar lavage fluid (BALF) were measured. IFN-β and IL-6 levels in the BALF increased and peaked at 6 h postinfection (Fig. 6C). In the serum, cytokine levels peaked at 3 h after intranasal administration of rWRS (Fig. 6D). A respiratory syncytial virus (RSV)-GFP infection model next was used to evaluate the antiviral effect of intranasal WRS. Based on detection of viral genes by qPCR, WRS inhibited replication of RSV in the lung at 3 days postinfection (Fig. 6E).

Cross-reactivity of WRS. Potential stimulators of innate immunity can be used as adjuvant therapies to enhance immune responses to vaccination (42). In addition to traditional adjuvants like alum and emulsion oil, which help to promote continuous antigen presentation, immune stimulators enhance the efficacy of vaccines by increasing host immune responses. To validate WRS as a potential adjuvant in the context of animal husbandry, we assessed the cross-reactivity of WRS across species.

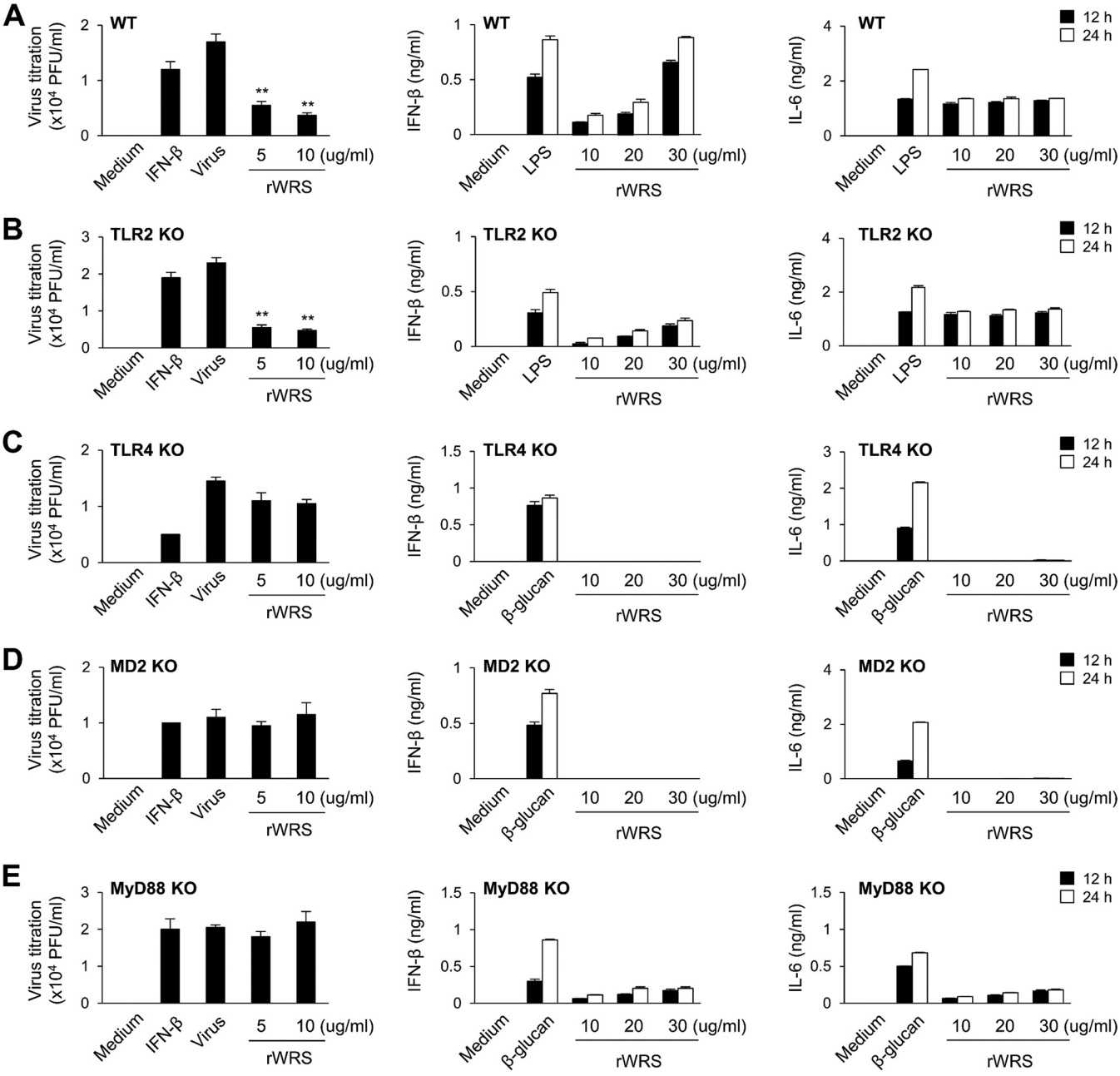

FIG 5 TLR4 and MD2 are indispensable for effect of WRS. (A) BMDMs isolated from WT mice were treated with rWRS for 12 h, followed by VSV-GFP infection at an MOI of 3 (left) for 24 h. ELISA of IFN-β (middle) and IL-6 (right) levels in the supernatant of cells treated by rWRS was also performed at 12 and 24 hpt. BMDMs isolated from TLR2$^{-/-}$ (B), TLR4$^{-/-}$ (C), MD2$^{-/-}$ (D), and MyD88$^{-/-}$ (E) mice were used for the same analysis. β-Glucan (100 μg/ml) was used as a positive control. Error bars, means $\pm$ SD.

Human WRS showed about 90.02% homology with mouse WRS and had activity on the mouse immune cells used throughout this study (Table 1 and Fig. 7A). Homology between human WRS and porcine WRS was about 92.99% (Table 1 and Fig. 7A). On the basis of these data, we hypothesized that WRS has activity across mammalian species. To confirm this, we stimulated the porcine alveolar macrophage (PAM) cell line with rWRS. PAM cells secreted IL-6 and showed increased IL-6 mRNA expression following stimulation with rWRS (Fig. 7B and C). Meanwhile, human WRS has about 77.49% homology to chicken WRS (Table 1 and Fig. 7A). To determine the cross-reactivity between human and chicken WRS, we tested the activity of purified recombinant chicken WRS (rcWRS) on mammalian cells (Fig. 7D). In contrast to our results with

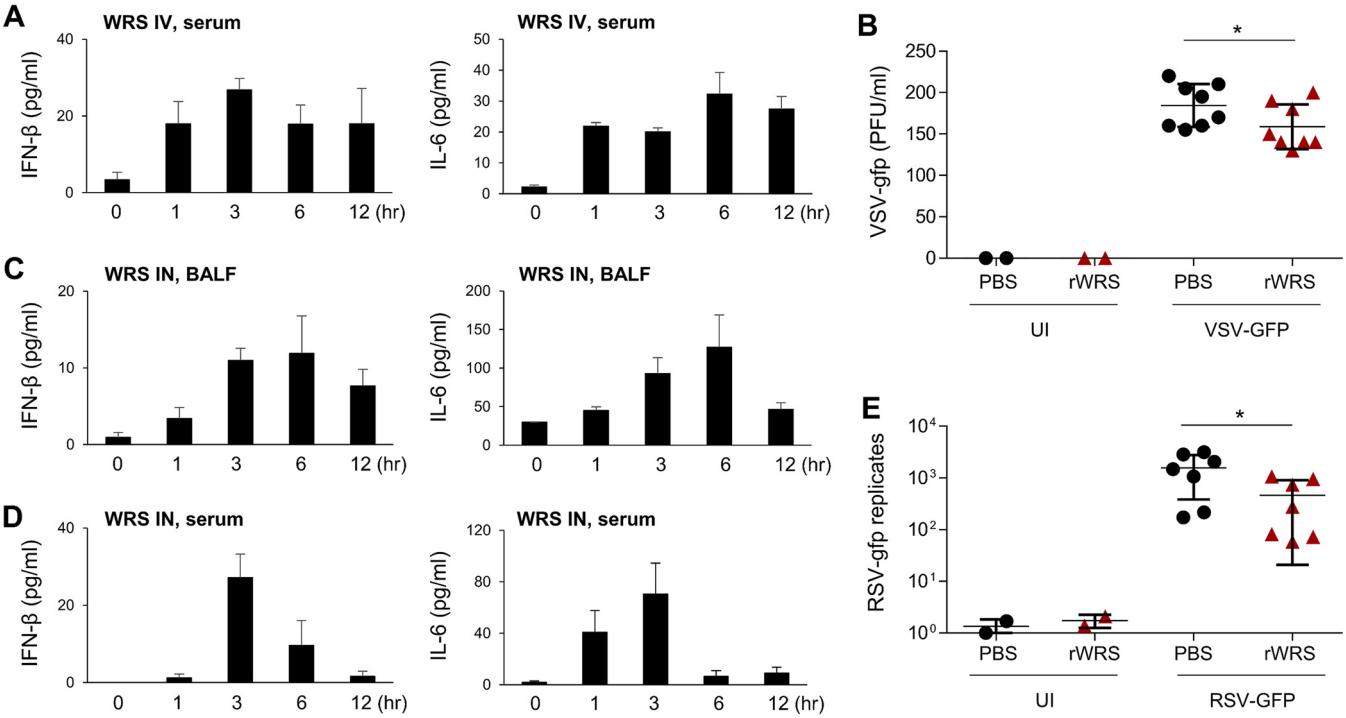

FIG 6 WRS induces cytokine secretion and antiviral effect *in vivo*. (A) ELISA of IFN-β (left) and IL-6 (right) levels in the serum of mice intravenously injected with 60 μg of rWRS at the indicated time points. (B) Determination of viral load by plaque assay in the serum of mice 12 h after VSV-GFP infection (2×10^8 PFU/head). The mice were intravenously injected with 60 μg of rWRS 2 times for 6 and 12 h before virus infection. (C and D) ELISA of IFN-β (left) and IL-6 (right) levels in the BALF (C) and the serum (D) of mice intranasally injected with 30 μg of rWRS at indicated time points. (E) Determination of viral load by real-time qPCR of RSV-G gene in the serum of mice 3 days after RSV-GFP infection (1×10^6 PFU/head). The mice were intranasally injected with rWRS 2 times for 3 and 6 h before virus infection. Error bars, means $\pm$ SD. *, $P < 0.05$ (Mann-Whitney U test).

mouse cells, rcWRS had no effect on Raw264.7 cells (Fig. 7E). Chicken BMDMs (cBMDMs) next were isolated and treated with rWRS for 12 or 24 h. As expected, rWRS did not induce IL-6 expression from cBMDMs (Fig. 7F). Interestingly, rcWRS also failed to induce IL-6 production from cBMDMs, indicating that chicken WRS does not have the ability to elicit innate immune responses. These data provide evidence that human WRS is only cross-reactive to mammalian species, not poultry, but suggest that WRS could be used in applications in other mammalian species.

DISCUSSION

Host cells possess several defensive mechanisms against virus invasion. In the early stages of infection, recognition of PAMPs by PRRs expressed on host innate immune cells results in the secretion of antiviral cytokines that prime host cells to mediate antiviral responses (1, 2, 5). These initial responses of host cells are important to successfully protect the host against viral infection. In addition to cytokines, host cells secrete other factors to prime and alert the immune system, called alarmins (25). Alarmins are endogenous molecules released from stressed host cells that act as danger signals to the innate immune system and promote antipathogenic responses (25–27). A number of molecules have been suggested to act as alarmins. HMGB1 is an alarmin that activates receptors including TLR2, TLR4, and RAGE (28, 29, 33). Galectin-3 and -9

TABLE 1 Homology of WRS between species

	% Identity to *Homo sapiens* WRS	
Species	Protein	DNA
Porcine	92.99	88.28
Mouse	90.02	87.01
Chicken	77.49	73.38

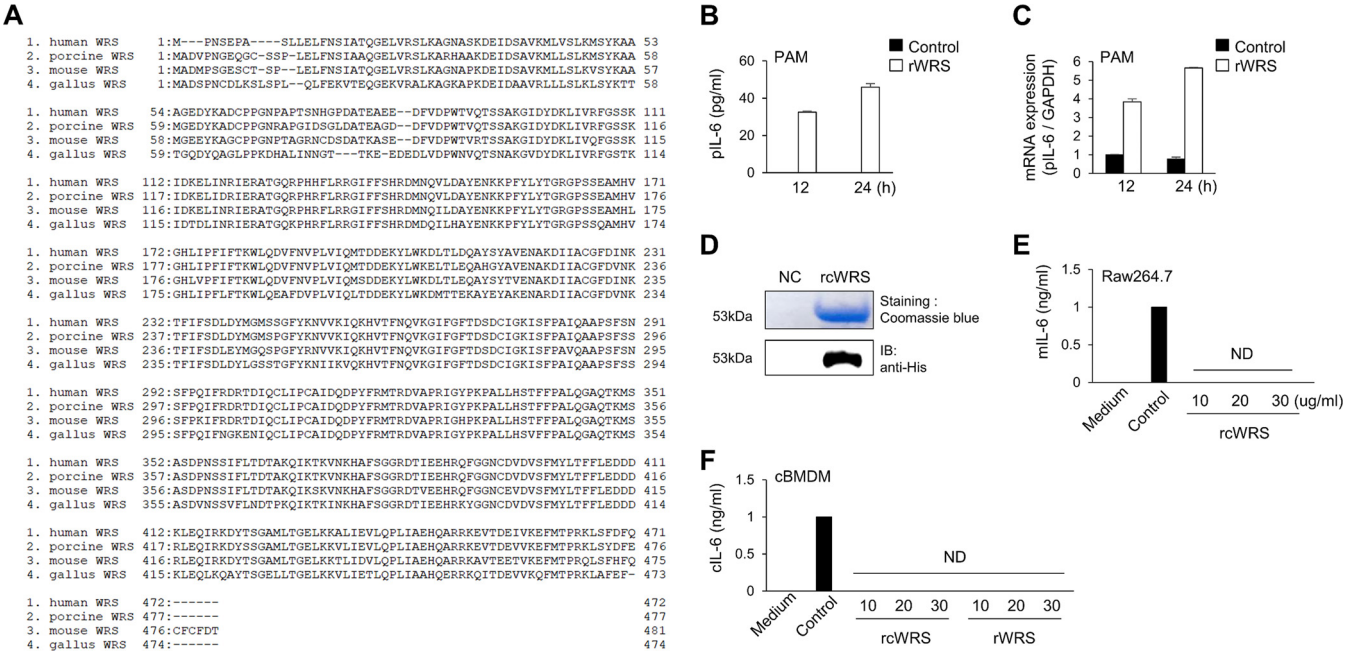

FIG 7 Cross-reactivity is completely valid between mammalians and chicken. (A) Sequence alignment showing homology of WRS between species. (B) ELISA of porcine IL-6 levels in the supernatant of PAM cells treated with 30 μg of rWRS at 12 and 24 hpi. (C) mRNA expression level of porcine IL-6 shown in panel B, determined by real-time qPCR. (D) Immunoblot and Coomassie blue staining of purified rcWRS. (E) ELISA of mouse IL-6 levels in the supernatant of Raw264.7 cells treated with the indicated dose of rcWRS at 24 hpt. (F) ELISA of chicken IL-6 levels in the supernatant of chicken BMDMs treated with the indicated dose of rWRS or rcWRS at 24 hpt. Error bars, means ± SD.

are reported to function as alarmins mediating inflammatory responses during bacterial infection (43, 44). In addition, hepatoma-derived growth factor (HDGF), HSPs, S-100 proteins, and annexins are known to function as alarmins (25, 26).

In the present study, we present evidence suggesting that WRS acts as an alarmin to stimulate innate immune responses against viral infection. First, immune cells rapidly secreted WRS in response to infection with RNA and DNA viruses. Secretion of WRS occurred directly in response to viral infection and was not mediated by IFN signaling (Fig. 1). Second, extracellular treatment with rWRS inhibited virus replication *in vitro* and *in vivo* (Fig. 2, 3, and 6). Third, WRS enhanced the expression of antiviral cytokines and other antiviral genes by innate immune cells. WRS also induced the activation of intracellular signaling cascades within innate immune cells (Fig. 4). Finally, studies using BMDMs isolated from various knockout (KO) mice showed that the antiviral effects of WRS were dependent on the interaction between WRS and the TLR4-MD2 complex. Taken together, these data suggest that WRS is released by innate immune cells in the early stages of viral infection and acts as an alarmin to activate antiviral immune responses.

The findings reported here suggest a new role for WRS as an alarmin, in addition to its canonical role in translation. One of the properties of alarmins is that they are rapidly secreted in response to damage-associated molecular patterns (DAMPs), which collectively refers to danger signals released during viral infection. As shown in Fig. 1A, WRS is rapidly secreted and can be detected in the supernatant as soon as 1 h postinfection. Additionally, the transcription of WRS increased beginning at 4 h postinfection. This observation suggests that intracellular WRS is released into the extracellular space immediately upon infection, and then additional WRS is synthesized to compensate for the loss. As shown in Fig. 1G, WRS secretion is not mediated by IFN signaling, which is an additional property of alarmins. Another characteristic of alarmins is that they induce host immune responses after secretion into the extracellular space. Our results, especially those shown in Fig. 4, demonstrate the ability of WRS to stimulate innate immune cells to secrete antiviral cytokines.

Recently, Ahn et al. reported that secreted WRS is a primary defense against pathogen invasion (41). They showed that WRS was secreted rapidly, was secreted in larger amounts than HMGB1 or HSP70, enhanced immune responses, and protected mice from bacterial infection. These findings agree with our data showing that WRS reduced virus replication *in vitro* and *in vivo*. They also suggested that these effects of WRS were dependent on the TLR4-MD2 complex and partially on TLR2. As shown in Fig. 5, the antiviral effects of WRS were inhibited in TLR4$^{-/-}$ or MD2$^{-/-}$ BMDMs. However, the effects of WRS were preserved in TLR2$^{-/-}$ cells. It is possible that the affinity of WRS for the TLR4-MD2 complex is higher than its affinity for TLR2. Collectively, data from this recent report and from the present study demonstrate that WRS is an important host factor that promotes innate immunity against pathogen invasion by interacting with the TLR4-MD2 complex. On the basis of these collective data, we believe that WRS should be considered an alarmin that contributes to host defense during pathogen invasion.

The importance of discovering endogenous factors, like WRS, that stimulate host immune responses is that these factors can be developed as adjuvants for vaccination (42). In addition to the classical adjuvants, which promote consistent antigen exposure in the host, several novel immune stimulants have been used to enhance the efficacy of vaccination (45). For example, monophosphoryl lipid A (MPL) is a form of LPS that has been modified to prevent toxicity. MPL has been combined with alum and developed as an adjuvant for vaccination against hepatitis B virus (HBV) and human papillomavirus (HPV). CpG oligodeoxynucleotide, an agonist for TLR9, is also being tested for use as a novel adjuvant. Other agonists of PRRs, including RIG-I, stimulator of IFN genes (STING), and C-type lectin, are also being assessed as candidates for novel adjuvants (46, 47).

We investigated the cross-reactivity of human WRS on the basis of this potential use. In these experiments, human rWRS promoted cytokine secretion from human, mouse, and porcine immune cells. However, human WRS was not active on chicken cells and vice versa. This evidence suggests that cross-reactivity of human WRS is conserved among mammals but not poultry (Fig. 7). It was previously reported that human and mouse TLR4 and MD2 can form a functional complex with each other but not with the chicken receptors (48, 49). Thus, differences between the species could come not only from differences in WRS but also structural differences in the TLR4 and MD2 receptors, although further investigation is needed. Taken together, these data suggest that WRS has applications as an immune stimulator in humans as well as in other mammals.

In conclusion, we report that WRS is secreted by immune cells during the early phase of viral infection. Immune cells stimulated with WRS showed the enhanced secretion of antiviral cytokines and activation of major signaling pathways that promote antiviral immune responses, ultimately resulting in reduced viral replication *in vitro* and *in vivo*. The results of this study enhance the understanding of host defense systems against virus infection and provide additional information on the noncanonical functions of ARSs.

MATERIALS AND METHODS

Cell culture. A human acute monocytic leukemia (THP-1) cell line, human acute myeloid leukemia (U-937) cell line, and porcine alveolar macrophages (PAM) cell line were cultured in RPMI (PAN-Biotech). A mouse leukemic monocyte macrophage (Raw264.7) cell line and human embryonic kidney 293 (HEK293T) cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) (PAN-Biotech). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAN-Biotech) and 1% antibiotics/antimycotics (GIBCO), and cells were maintained in a 5% CO_2 incubator at 37°C.

Viruses. PR8-GFP was amplified in specific-pathogen-free embryonated chicken eggs. VSV-GFP and HSV-GFP were amplified on Vero cells, and RSV-GFP was amplified on HEp-2 cells. Virus concentration for *in vivo* experiments was performed using a polyethylene glycol (PEG) virus precipitation kit (K904-50; BioVision) in accordance with the manufacturer's protocols. Briefly, 40 ml of original virus stock was incubated with 10 ml of PEG solution at 4°C overnight. After centrifugation, pellets were resuspended and virus titer was determined by standard plaque assay.

ELISA. The levels of cytokine in cell culture supernatant or serum samples were determined using a commercially available, specific ELISA kit by following the instructions provided by the manufacturer. Human IFN-β (CSB-E09889h; Cusabio), human IL-6 (555240; BD Bioscience), human WRS (CSB-E11789h; Cusabio), mouse IFN-β (CSB-E04945m; Cusabio), mouse IL-6 (550950; BD Bioscience), porcine IL-6

(P6000B; R&D Systems), and chicken IL-6 (CSB-E08549ch; Cusabio) ELISA kits were used for analysis. Flag ELISA was conducted using anti-Flag-coated plates (P2983; Sigma). Supernatant samples from a mouse WRS (mWRS)-Flag-expressing Raw264.7 cell line were incubated for 2 h, followed by incubation with anti-mWRS antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies at a ratio of 1:1,000 for 1 h. The plate was then washed with phosphate-buffered saline-Tween 20 3 times after every step. Anti-mWRS antibodies were generated from rabbit immunized with the purified mWRS recombinant protein and Freund's adjuvant (complete F5881 and incomplete F5506; Sigma) mixture.

Plasmid construction. Human WRS plasmid was received from Sunghoon Kim (Seoul National University, Seoul, South Korea). mWRS plasmid was received from the Korea Human Gene Bank, Genome Research Center, KRIBB (Daejeon, South Korea). The chicken WRS (cWRS) gene was synthesized according to the codon preference of *Escherichia coli* by Bioneer Corp. (Daejeon, South Korea). Genes were cloned into the mammalian expression vector (pIRES-Flag) or the bacterial expression vector (pHis-parallel).

Purification of proteins. To purify the human recombinant WRS and chicken recombinant WRS, *E. coli* Rosetta-gami (DE3) competent cells (Novagen) were transformed with each plasmid. Colonies were seeded into 5 ml of LB broth supplemented with ampicillin at 37°C with shaking at 200 rpm overnight. The bacterial cell culture was scaled in large volumes of LB medium until the optical density (OD) reached 0.6 and then was supplemented with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Bio Basic) at 25°C overnight. The cell suspension was centrifuged at 6,000 $\times$ *g* for 20 min, and the cell pellets were resuspended in cold phosphate-buffered saline supplemented with a protease inhibitor (1 mM phenylmethylsulfonyl fluoride; Sigma) and sonicated 3 times repeatedly (10-s pulses at 40% amplitude). The homogeneity of purified target proteins was determined by Coomassie blue staining of SDS-PAGE gel. A large amount of recombinant protein was purified by a Bio-Rad fast protein liquid chromatography system in accordance with the manufacturer's protocol (Bioprogen Co.). Immobilized metal affinity chromatography was performed, followed by dialysis using permeable cellulose membrane.

Endotoxin removal. Endotoxin removal from protein solutions was performed with a previous protocol (50). Briefly, Triton X-114 (Sigma) was added to the protein preparation to a final concentration of 2%. The mixture was incubated at 4°C for 20 min with constant stirring and incubated at 37°C in a heat block for 10 min, followed by centrifugation (12,000 rpm, 10 min) at 25°C. The upper aqueous layer containing protein was carefully removed and subjected to Triton X-114 phase separation for at least three more cycles. The remaining endotoxin level was determined using the commercially available LAL endotoxin detection assay kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. The measurements were conducted in duplicated wells and measured at an absorbance of 405 nm.

Luciferase assay. HEK293T cells were transfected with 400 ng of luciferase reporter construct (IFN-β, ISRE, and NF-κB) and 10 ng of Renilla plasmid (pRL-TK), together with hWRS-Flag plasmid. The 2CARD domain of RIG-I was cotransfected to stimulate cells. Transfected cells were harvested 24 h after transfection and used for the Dual-Luciferase reporter assay system (Promega) in accordance with the manufacturer's protocols.

Immunoblotting and antibodies. Phosphorylation of IFN-related signaling molecules was evaluated by immunoblot analysis. Raw264.7 cells were cultured in 6-well plates (1×10^6 cells/well) and treated with 100 ng/ml LPS or 10 μl/ml rWRS. Cells were harvested at 0, 4, 8, and 16 h posttransfection (hpt) and subjected to immunoblot analysis. Cell pellets were lysed by radioimmunoprecipitation assay (RIPA) lysis buffer, and the lysates were mixed with sample buffer (Sigma) at a 1:1 ratio for separation by SDS-PAGE gel. The protein sample was then electroblotted to an Immun-blot polyvinylidene difluoride (PVDF) Western blotting membrane (Bio-Rad). The membrane was blocked in 5% bovine serum albumin (BSA; Sigma) for 1 h and incubated with specific antibodies at 4°C overnight on a rocking platform. The membrane blot was carefully washed and rinsed with 1$\times$ Tris-buffered saline containing 0.05% Tween 20 (TBST) and incubated with 1:3,000 dilutions of HRP-conjugated secondary antibodies for 1 h at room temperature. The membrane was then washed and the target protein detected using enhanced chemiluminescence (ECL) detection as a luminescent substrate according to the manufacturer's instructions (Bio-Rad) using a Las-4000 mini-lumino image analyzer. Antibodies used in immunoblotting were anti-IRF3 (ab25950; Abcam), anti-phospho IRF3 (4947; Cell Signaling), anti-TBK1 (3504S; Cell Signaling), anti-phospho TBK1 (5483S; Cell Signaling), anti-phospho IKBα (2859S; Cell Signaling), anti-IKBα (9242S; Cell Signaling), and β-actin (sc47778; Santa Cruz).

qRT-PCR. Bone marrow-derived macrophages (BMDMs) were isolated from wild-type mice and stimulated with rWRS. Total RNA was extracted according to the protocol of the RNeasy minikit (Qiagen), and cDNA was synthesized using a ReverTra Ace kit (Toyobo). The quantification of selected mRNA transcripts in a particular cDNA was performed using gene-specific primer pairs from a QuantiTect SYBR green PCR kit (Toyobo) on a Rotor-Gene Q (Qiagen) after normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Relative mRNA expression levels of those genes were calculated using the delta-delta threshold cycle method.

Mice and BMDM isolation. TLR2$^{-/-}$, TLR4$^{-/-}$, MD2$^{-/-}$, and MyD88$^{-/-}$ mice on C57BL/6 background were kindly provided by Chul-Ho Lee (Laboratory Animal Resource Center, KRIBB). Isolation of mouse BMDMs was performed as described previously (38). Briefly, femurs and tibias were aseptically isolated from euthanized C57BL/6 mice and flushed with DMEM only. Cells were temporarily incubated with ACK lysing buffer (Gibco), resuspended, and placed in petri dishes with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) for 5 days at 37°C in a 5% CO_2 atmosphere. Chicken BMDMs were isolated in accordance with previous studies (51). Flushed cells were cultured in petri dishes with RPMI 1640 medium containing 10% FBS and 350 ng/ml of recombinant chicken GM-CSF at 41°C for 7 days.

TABLE 2 Primers for qRT-PCR

Gene[a]	Forward	Reverse
hWRS	AAAGGCATTTTCGGCTTCACTG	GCACATGGGATAAGGCACTGG
mIFN-β	TCCAAGAAAGGACGAACATTCG	TGCGGACATCTCCCAACGTCA
mIL-6	TCCATCCAGTTGCCTTCTTGG	CCACGATTTCCCAGAGAACATG
mTNF-α	AGCAAACCACCAAGTGGAGGA	CCA CGATTTCCCAGAGAACAT
mPKR	GCCAGATGCACGGAGTAGCC	GAAAACTTGGCCAAATCCACC
mISG15	CAATGGCCTGGGACCTAAA	CTTCTTCAGTTCTGACACCGTCAT
mISG20	AGAGATCACGGACTACAGAA	TCTGTGGACGTGTCATAGAT
mISG56	AGAGAACAGCTACCACCTTT	TGGACCTGCTCTGAGATTCT
mOAS	GAGGCGGTTGGCTGAAGAGG	GAGGAAGGCTGGCTGTGATTGG
mGAPDH	TGACCACAGTCCATGCCAT	GACGGACACATTGGGGGTAG
RSV-G	CCAAACAAACCCAATAATGATTT	GCCCAGCAGGTTGGATTGT

[a]h, human; m, mouse.

***In vivo* experiments.** Mice were intravenously treated with 60 μg of rWRS for 6 and 12 h before VSV-GFP intravenous infection (2×10^8 PFU/head). Serum VSV titration was performed using standard plaque assay at 12 hpi. For RSV-GFP, mice were intranasally inoculated with 30 μg of rWRS for 3 and 6 h, followed by intranasal infection (1×10^6 PFU/head). Lung RSV titration was measured by RSV-G gene quantification using a specific primer (Table 2).

Statistical analysis. Data are presented as means ± standard deviations (SD) unless stated otherwise. Normality test of data was performed using Kolmogorov-Smirnov test. According to the results of the normality test, significance between the groups was determined using nonparametric Mann-Whitney test. *P* values of less than 0.05 were considered significant and are indicated in the figure legends.

Ethics statement. Animal experiments were conducted following approval from the institutional animal care and use committee of Chungnam National University (reference no. CNU-00677).

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H.-C.L., E.-S.L., and M.B.U. performed most of the experiments, with help from T.-H.K., J.-H.K., K.C., and W.A.G.C. M.J., S.K., and C.-J.K. contributed to the discussion and provided critical reagents. H.-C.L. and J.-S.L. designed the study and wrote the manuscript. J.-S.L. supervised the study. All authors helped with data analysis.

We have no conflicts of interest to declare.

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