

Retinoic Acid–Inducible Gene I Activation Inhibits Human Respiratory Syncytial Virus Replication in Mammalian Cells and in Mouse and Ferret Models of Infection

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Infections caused by human respiratory syncytial virus (RSV) are associated with substantial rates of morbidity and mortality. Treatment options are limited, and there is urgent need for the development of efficient antivirals. Pattern recognition receptors such as the cytoplasmic helicase retinoic acid–inducible gene (RIG) I can be activated by viral nucleic acids, leading to activation of interferon-stimulated genes and generation of an “antiviral state.” In the current study, we activated RIG-I with synthetic RNA agonists (3pRNA) to induce resistance to RSV infection *in vitro* and *in vivo*. *In vitro*, pretreatment of human, mouse, and ferret airway cell lines with RIG-I agonist before RSV exposure inhibited virus infection and replication. Moreover, a single intravenous injection of 3pRNA 1 day before RSV infection resulted in potent inhibition of virus replication in the lungs of mice and ferrets, but not in nasal tissues. These studies provide evidence that RIG-I agonists represent a promising antiviral drug for RSV prophylaxis.

Keywords. RSV; RIG-I agonist; antiviral; innate immunity; ferret.

Human respiratory syncytial virus (RSV) is an enveloped virus of the Pneumoviridae family with a single-stranded, negative-sense RNA genome consisting of 10 genes that encode for 6 internal and 2 nonstructural proteins (M, NS1, NS2, M2-1, M2-2, P, L, and N) and 3 surface glycoproteins (SH, G, and F) (reviewed in [1]). RSV infects most children within the first 2 years of life but also reinfects older children and adults, including elderly adults. While most infections are associated with upper airway disease, RSV infections can result in acute lower respiratory tract infection, and RSV is among the most frequent causes of

pediatric bronchiolitis and pneumonia [2]. Limiting the impact of RSV infection is important, but treatment options are limited, and supportive care remains the mainstay of treatment. Of note, there are only 2 Food and Drug Administration–approved antivirals available: aerosolized ribavirin, a guanosine analogue with broad-spectrum antiviral activity [3], and palivizumab, a humanized monoclonal antibody (mAb) to RSV F [4]. Both antivirals exhibit clinical benefit; however, their use is limited by several factors, which include cost and safety concerns [5].

Host-directed therapy approaches are an attractive approach to circumvent the necessity for drugs targeting viral structures directly. Viral nucleic acids are recognized by pattern recognition receptors such as Toll-like receptors, retinoic acid–inducible gene I (RIG-I)–like receptors [6, 7], and nucleotide-binding oligomerization domain–like receptors [8]. Viral sensing by different by pattern recognition receptors triggers intracellular signaling cascades to activate transcription factors, which ultimately induce production of types I and III interferons (IFNs) [9]. Secreted IFNs bind to their corresponding receptors in an autocrine and/or paracrine manner to induce expression of hundreds of IFN-stimulated genes (ISGs) [10], resulting in an “antiviral state” in virus-infected and uninfected neighboring cells.

Prophylactic and therapeutic treatments using synthetic agonists to target RIG-I have shown promising results as broad-spectrum antivirals. *In vitro*, treatment of human cells with RIG-I agonists resulted in protection from subsequent

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infection with different RNA viruses such as influenza A virus (IAV), chikungunya virus (CHIKV), dengue virus, hepatitis C virus, and human immunodeficiency virus type 1 [11–14]. Moreover, prophylactic administration of RIG-I agonists to mice before infection with CHIKV [15] and IAV [11, 13–17] resulted in significant protection from disease. Recent studies also demonstrated that prophylactic and therapeutic treatment with RIG-I agonists reduced lung virus titers and enhanced survival of mice infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [18, 19].

In the current study, we demonstrated that pretreatment (ie, treatment before infection) of human, mouse, or ferret airway cell lines with a synthetic RIG-I agonist (3pRNA) resulted in reduced susceptibility to subsequent RSV infection and replication. Moreover, a single intravenous injection of mice or ferrets with 3pRNA 1 day before RSV infection resulted in potent inhibition of virus growth in the lung but not in the upper airways. Together, these *in vitro* and *in vivo* findings highlight the potential of RIG-I agonists as an effective antiviral prophylaxis against RSV.

METHODS

Cells and Viruses

Human lung epithelial (A549) cells (American Type Culture Collection [ATCC]), human epithelial type 2 (HEp-2) cells (ATCC), ferret lung epithelial (FRL) cells (described below), or primary mouse lung fibroblasts (isolated as described elsewhere [20]) were maintained and passaged in Dulbecco modified Eagle medium (Gibco) containing 10% (vol/vol) fetal calf serum (Sigma-Aldrich) and supplemented with 2 mmol/L L-glutamine (Gibco), 1 mmol/L sodium pyruvate (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). FRL cells were immortalized by transformation with adenovirus serotype 5 (ATCC). Single cells were obtained by limiting dilution with subsequent passage to establish the cell line that has been used in other studies [21]. Mouse lung epithelial (LA-4) cells (ATCC) were cultured in Ham's F-12K (Kaighn's) medium (Gibco), supplemented as described above. FRL and LA-4 RIG-I knockdown cells were generated using a pLKO.1 lentiviral plasmid system [22]. RIG-I^{-/-} A549 cell lines have been described elsewhere [23].

The RSV A2 strain was obtained from the Department of Microbiology and Immunology, University of Melbourne, and the Long strain (VR-26) was purchased from ATCC. Recombinant human RSV (HRSV) Long viruses expressing firefly luciferase (rHRSV-Luc) or mCherry (rHRSV-Cherry) have been described elsewhere [24]. RSV stocks were grown in HEp-2 cells and titers of infectious virus were quantified on HEp-2 cells by means of plaque assay and/or ViroSpot assay [25].

Oligonucleotides and Transfection

Synthetic 3pRNA, consisting of a 5'-triphosphorylated, double-stranded hairpin RNA, was chemically synthesized by solid-phase synthesis using product-specific labelling, as described elsewhere [16]. Control (ctrl) RNA was also generated [16]. For *in vitro* studies, Lipofectamine 2000 (Invitrogen) was used to transfect mammalian cells with 3pRNA or ctrl RNA (both 100 ng/mL) in OptiMEM (Gibco-BRL), according to the manufacturer's instructions.

Cytokine Detection

IFN- α was measured in murine cell supernatants by enzyme-linked immunosorbent assay, using a rat anti-mouse IFN- α mAb for coating (RMMA-1; PBL Assay Science), followed by incubation with cell culture supernatants and subsequent incubation with a rabbit polyclonal anti-mouse IFN- α Ab (PBL Assay Science). Expression was visualized using a mouse anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology) and TMB Substrate (BD OptEIA, BD Bioscience).

In Vitro Virus Infection and Growth Assays

After overnight culture, RSV was added to cell monolayers in serum-free medium at the indicated multiplicity of infection and incubated for 1 hour at 37°C. Cells were washed and further incubated in serum-free medium. Supernatants were harvested at indicated time points and virus titers determined in clarified supernatants by ViroSpot assay [20]. For immunostaining, cells were harvested 17 hours after infection and then fixed, permeabilized and stained with a mAb specific for the RSV nucleoprotein (130-12H, Merck) and analyzed using flow cytometry. Alternatively, cells were stained with a mAb against the RSV fusion protein (F) (133-1H, Merck), in conjunction with fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (A32766; ThermoFisher), fixed, and analyzed using flow cytometry. Fixable viability dye eFluor 780 (eBioscience) was used to exclude dead cells.

Plaque and ViroSpot Assays to Determine Titers of Infectious RSV

Titers of infectious RSV in culture supernatants and samples from RSV-infected mice or ferrets were determined with ViroSpot assay [25] or plaque assay [26] on HEp-2 cells.

Animal Models

All research complied with the University of Melbourne's Animal Experimentation Ethics guidelines and policies, in accordance with the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes.

In Vivo Treatment, Infection and Bioluminescence Imaging of Mice

BALB/c, C57BL/6 (B6), and interferon regulator factor (IRF)3/7^{-/-} [27], or type I interferon receptor (IFNAR)2^{-/-} [28] mice on a B6 background were bred and maintained in specific pathogen-free

conditions at the Bioresources Facility at The Peter Doherty Institute for Infection and Immunity, Melbourne, Australia. For treatment of mice, 12.5 µg of 3pRNA or ctrl RNA was formulated in in vivo jetPEI transfection reagent (Polyplus-transfection) at an N/P ratio of 8 (referring to the number of nitrogen residues [N] in the reagent per phosphate [P] of nucleic acid) and injected via the intravenous route. Then, 24 hours later, mice were infected with the indicated dose of rHRSV-Luc in 50 µL of phosphate-buffered saline (PBS) via the intranasal route. For bioluminescence imaging, mice were injected intraperitoneally with VivoGlo Luciferin (Promega) (15 mg/mL in PBS, at a dose of 150 mg/kg) 5 minutes before imaging. Mice were anesthetized by means of isoflurane inhalation. Bioluminescence signals were measured using the Lumina XRMS Series III In Vivo Imaging System (IVIS; Perkin Elmer). Living Image software (version 4.0; Caliper Life Sciences) was used to measure luciferase activity. Bioluminescence images were acquired for 1 minute ($f/\text{stop} = 1$; binning = 8).

In Vivo Treatment and RSV Infection of Ferrets

Adult outbred ferrets (600–1500 g) were housed in the Bioresources Facility at The Peter Doherty Institute for Infection. Ferrets received intravenous injections of 0.3 mg/kg 3pRNA or ctrl RNA formulated in in vivo jetPEI at an N/P ratio of 8, following manufacturer's instructions. At 24 hours after 3pRNA or ctrl RNA delivery, ferrets were intranasally inoculated with RSV Long by dropwise delivery of 500 µL (5×10^5 infectious particles).

Ferret Tissue Collection and Processing

After RSV infection, daily nasal wash samples were collected. Ferrets were lightly sedated (5 mg/kg Ilium Xylazil-20; Troy Laboratories), and 1 mL of PBS was flushed through the nostrils. Expelled liquid was collected, snap frozen, and stored at -80°C . At experimental end points, ferrets were anesthetized with ketamine and xylazine before intracardiac injection of sodium pentobarbitone (Lethabarb, 0.5 mL/kg; Troy Laboratories) before removal of respiratory tissues. At relevant time points, peripheral blood was collected, and peripheral blood mononuclear cells (PBMCs) were isolated by means of density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare).

Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from PBMCs or cell lines using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, including on-column DNase treatment (Qiagen), and then reverse-transcribed into complementary DNA using the Omniscript RT Kit (Qiagen). Real-time (RT) polymerase chain reaction (PCR) reactions were performed using Sensifast Lo-ROX SYBR Green (Bioline) on the ABI7500 Real Time PCR System (Applied Biosystems) and specific primers for ferret Mx1, OAS1, ISG15, CCL5 (all in [29]), and glyceraldehyde

3-phosphate dehydrogenase (GAPDH) [30]. Gene expression was normalized to housekeeping gene GAPDH and graphed as fold change compared with untreated controls, using $2^{-\Delta\Delta\text{CT}}$ method [31].

Statistical Analysis

Statistical analyses were conducted using Prism software, version 8.0, and are described in the figure legends.

RESULTS

Effect of RIG-I Agonist Treatment on RSV Infection and Replication in Human and Mouse Airway Cells

Treatment of human A549 airway epithelial cells with RIG-I agonists has been shown to inhibit infection and growth of different RNA and DNA viruses [11–15, 18, 19]. However, its activity against RSV has not been reported to date. Our initial studies showed that pretreatment of A549 cells with the RIG-I agonist (3pRNA) for 24 hours before RSV infection significantly reduced the percentage of cells infected with RSV when compared with ctrl RNA-treated cells (Figure 1A). Moreover, 3pRNA treatment was associated with significant reductions in viral replication, as determined by measuring titers of infectious virus in clarified cell supernatants at 48 and 72 hours after infection (Figure 1B). RIG-I knockout A549 cells confirmed the importance of RIG-I for 3pRNA-mediated inhibition of RSV infection, as demonstrated by the increased susceptibility of 3pRNA-treated RIG-I knockout cells to infection (Figure 1A). Moreover, 3pRNA treatment in RIG-I knockout cells did not result in reduced viral replication over time (mean [standard deviation (SD)] titer, at 24 and 72 hours, 2.3 [0.4] and 5.1 [0.2] \log_{10} virospot (VS)/mL, respectively, for 3pRNA and 2.1 [0.4] and 5.4 [0.1] \log_{10} VS/mL for ctrl RNA).

Because mice represent an important preclinical animal model for RSV, we next confirmed that pretreatment of mouse LA-4 epithelial cells with 3pRNA resulted in IFN- α release (Figure 1C). Pretreatment also reduced the percentage of RSV-infected parental (wild-type [WT]) LA-4 cells (Figure 1D), as well as RSV replication over time (Figure 1E). To confirm the importance of mouse RIG-I, LA-4 cells were transduced with control short hairpin RNA (shRNA) (LA-4 kd CTRL) or shRNA to knockdown RIG-I (LA-4 kd RIG-I). When assessed with quantitative RT (qRT) PCR at 24 hours after treatment, the time point when LA-4 cells were infected in Figure 1D and 1E, 3pRNA induced ISG15 expression in LA-4 kd CTRL cells; however, this was markedly reduced in LA-4 kd RIG-I cells (mean fold induction compared with untreated cells and normalized to GAPDH, 66.6 [SD, 11.4] and 6.8 [1.9] for LA-4 kd CTRL and LA-4 kd RIG-I, respectively), consistent with effective knockdown of RIG-I signaling. 3pRNA treatment was also less effective at inhibiting RSV infection in LA-4 kd RIG-I (Figure 1D), resulting in a 1.5-fold reduction in infection (mean [SD], 48.0% [1.5%] for ctrl RNA versus 32.8% [3.4%] for

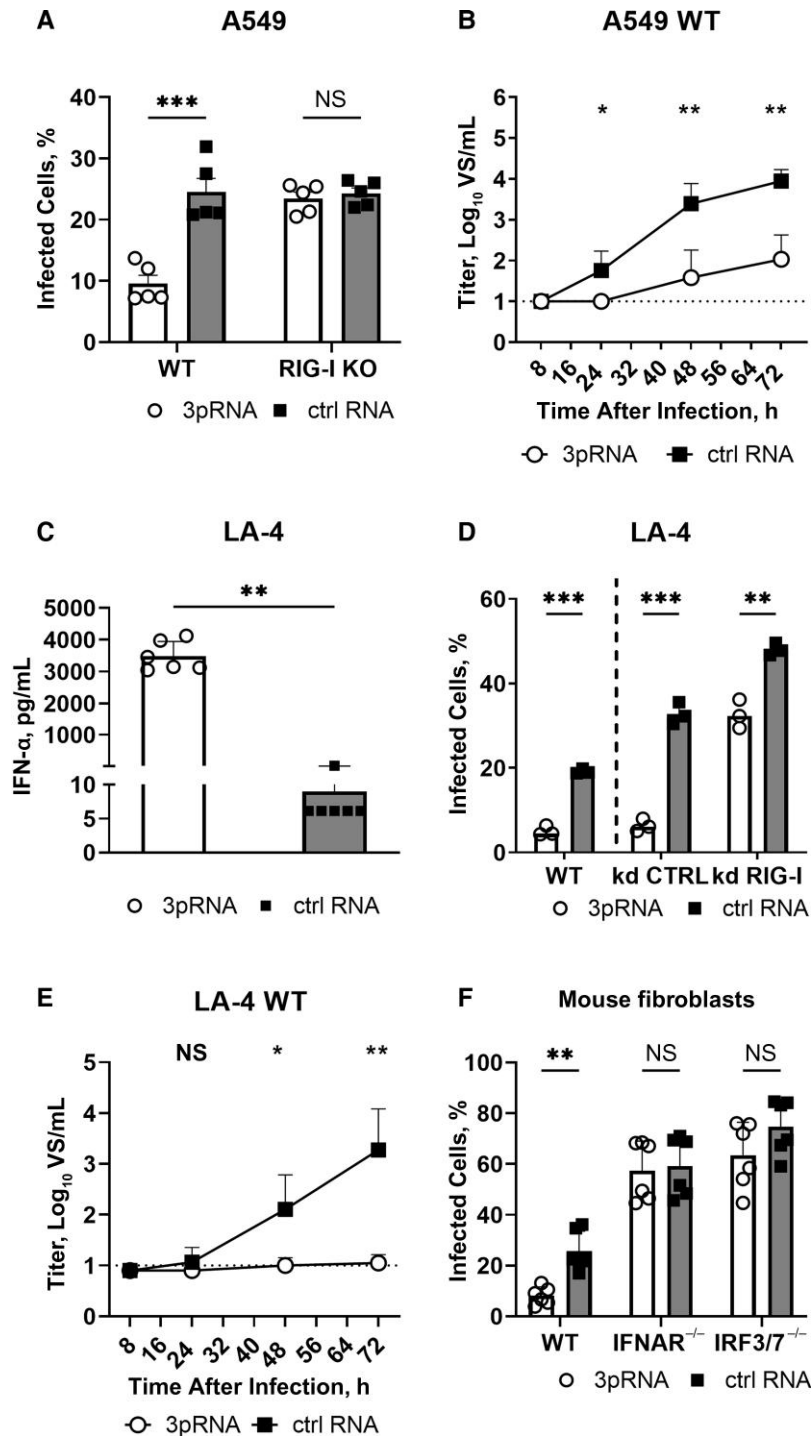


Figure 1. Pretreatment of human or mouse cells with retinoic acid-inducible gene I (RIG-I) agonists inhibits respiratory syncytial virus (RSV) infection and growth. Parental (wild-type [WT]) or RIG-I knockout (KO) human lung epithelial (A549) cells, mouse lung epithelial (LA-4) cells, or mouse lung fibroblasts were transfected with 3pRNA or control (ctrl) RNA and incubated for 24 hours. **A**, A549 cells were then infected with RSV Long (multiplicity of infection [MOI], 1), fixed and stained for expression of RSV F protein at 18 hours after infection. **B**, WT cells were infected with RSV Long (MOI, 0.01) and harvested at indicated time points, and titers of infectious virus determined. **C**, LA-4 supernatants were harvested and assessed for interferon (IFN) α with enzyme-linked immunosorbent assay. **D**, WT, knockdown (kd) control (CTRL), and kd RIG-I cells were infected with RSV Long expressing green fluorescent protein (RSV Long-GFP) (MOI, 1), fixed at 18 hours after infection, and analyzed by means of flow cytometry. **E**, WT cells were infected with RSV Long (MOI, 0.01), and titers of infectious virus were determined. Dashed lines represent the limit of detection. Samples below the detection limit ($<1 \log_{10}$ virospot (VS)/mL) were assigned values of 0.9 for statistical analysis. **F**, Mouse lung fibroblasts from C57BL/6 (WT), IRF3/7-deficient (IRF3/7^{-/-}), or IFNAR2-deficient (IFNAR2^{-/-}), mice were infected with RSV Long (MOI, 10), fixed, and stained for intracellular RSV nucleoprotein 18 hours after infection. All data represent means (standard deviations) of pooled samples ($n = 5-6$) from ≥ 2 independent experiments, except in **D**, which represents a single experiment ($n = 3$). Data were assessed for normal distribution, and samples in **A** and **D**, which showed normal distributions, were analyzed using multiple Student *t* tests; data in **B**, **C**, **E**, and **F** were assessed with nonparametric Mann-Whitney *U* tests. * $P < .05$; ** $P < .01$; *** $P < .001$; NS, not significant.

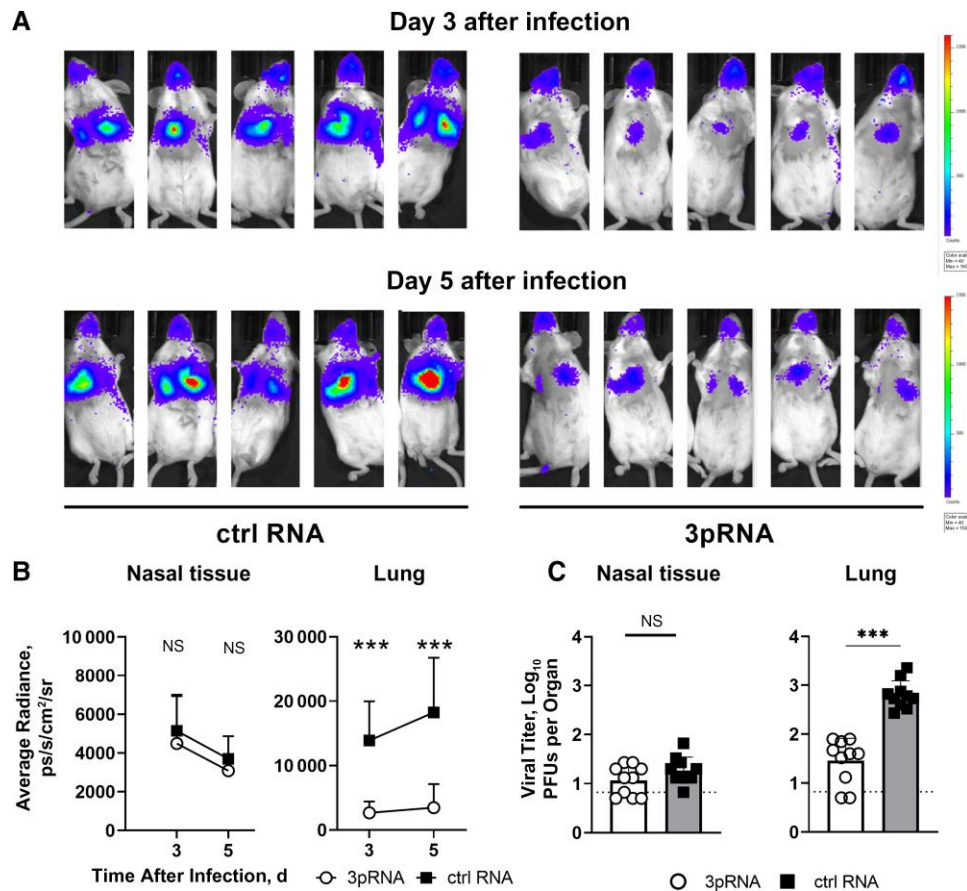


Figure 2. Retinoic acid–inducible gene I agonist treatment of mice 24 hours before respiratory syncytial virus (RSV) infection results in reduced virus titers in the lungs, but not in nasal tissues. BALB/c mice received a single intravenous injection with 12.5 μg of 3pRNA or control (ctrl) RNA and, 24 hours later, were infected by the intranasal route with 10^5 virospot (VS) of rHRSV-Luc in 50 μL of phosphate-buffered saline. *A*, Bioluminescence was assessed at 3 and 5 days after infection. *B*, Bioluminescence quantified as the average radiance per sum of the photons per second from each pixel inside the region of interest/number of pixels ($\text{p/s/cm}^2/\text{steradian}$). *C*, At 5 days after infection, virus titers were determined in clarified nasal tissues and lungs by means of plaque assay on human epithelial type 2 cells. Data represent means with standard deviations ($n = 10$ per group) from 1 of 2 independent experiments, and dashed lines represent the limit of detection. Samples below the detection limit ($<0.8 \log_{10}$ plaque-forming units [PFUs]/mL), were assigned values of $0.7 \log_{10}$ PFUs/mL for statistical analysis. Data were assessed for normal distribution, and samples in *B* and *C* showed normal distribution and were analyzed using unpaired Student *t* tests. *** $P < .001$; NS, not significant.

3pRNA), compared with a 5-fold reduction (32.8% [2.6%] for ctrl RNA vs 6.4% [1.5%] for 3pRNA), for LA-4 kd RIG-I and LA-4 kd CTRL cells, respectively.

We also examined the effectiveness of 3pRNA treatment at inhibiting RSV infection using primary mouse lung fibroblasts from C57BL/6 (WT), IRF3/7^{-/-} and IFNAR2^{-/-} mice. As seen in Figure 1F, 3pRNA pretreatment of WT mouse lung fibroblasts significantly reduced the percentage of RSV-infected cells. Of interest, mouse lung fibroblasts from IRF3/7^{-/-} and IFNAR2^{-/-} mice were markedly more susceptible to RSV infection, indicating that both functional type I IFN signaling and IRF3/7 expression are required to restrict RSV infection. Moreover, pretreatment with 3pRNA did not affect the percentage of RSV-infected cells in cells from IRF3/7^{-/-} or IFNAR2^{-/-} mice (Figure 1F). Together, these data highlight the importance of functional type I IFN signaling for RIG-I agonist-mediated inhibition of RSV in mouse cells.

RIG-I Agonist Injection Before RSV Infection in Mice: Effect on Virus Replication in the Lung

BALB/c mice received a single intravenous injection of 3pRNA or ctrl RNA and, 24 hours later, were infected with RSV Long engineered to express firefly luciferase (rHRSV-Luc). While 3pRNA treatment did not reduce luciferase activity in the nasal tissues at 3 or 5 days after infection, it was associated with reduced bioluminescence in the lungs at both time points (Figure 2A and 2B). Consistent with bioimaging results, 3pRNA treatment led to significantly (10-fold) reduced viral titers in the lungs but not in the nasal tissues, at 5 days after infection (Figure 2C). Mice did not show any clinical signs or weight loss after RSV infection (data not shown).

Effect of RIG-I Agonist Treatment on RSV Infection in Ferret Airway Cells

Pretreatment of a ferret airway cell line (FRL) with 3pRNA also resulted in potent inhibition of RSV infection (Figure 3A) and

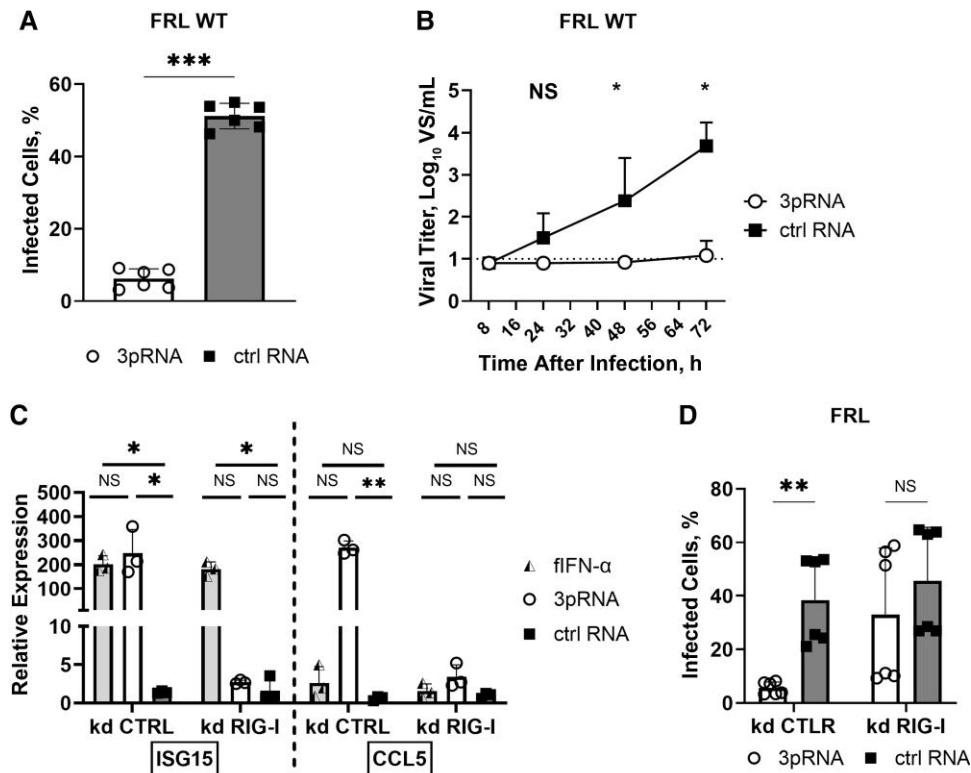


Figure 3. Pretreatment of ferret airway cells with retinoic acid-inducible gene I (RIG-I) agonist inhibits respiratory syncytial virus (RSV) infection and growth. Ferret lung epithelial (FRL) cells were transfected with 3pRNA or control (ctrl) RNA or treated with 0.5 µg/mL ferret interferon (IFN) α (IFN-α) for 24 hours. *A, B*, Cells were then infected with RSV Long-GFP (multiplicity of infection [MOI], 1) and analyzed at 18 hours after infection (*A*) or infected with RSV Long (MOI, 0.01) (*B*), and infectious virus titers were determined and expressed as virospot (VS)/ml. *C*, Total RNA was isolated from FRL knockdown (kd) control (CTRL) or kd RIG-I and expression of interferon-stimulated gene (ISG) 15 and CCL5 was determined, normalized to GAPDH and expressed relative to untreated cells. *D*, FRL kd CTRL or kd RIG-I cells were infected with RSV Long (MOI, 2), fixed, and stained for RSV F protein at 18 hours after infection. All data are means (standard deviations) of pooled samples (n = 5–6) from ≥2 independent experiments, except in *C*, which represents a single experiment. Data were assessed for normal distribution. Samples showing normal distribution (*A*) were analyzed using Student *t* tests; other samples were analyzed using nonparametric Mann-Whitney *U* (*B, D*) or Kruskal-Wallis (*C*) tests. **P* < .05; ***P* < .01; ****P* < .001; NS, not significant.

growth (Figure 3B). To confirm the importance of ferret RIG-I, FRL cells were transduced with control shRNA (FRL kd CTRL) or shRNA to knockdown RIG-I (FRL kd RIG-I). As seen in (Figure 3C), 3pRNA treatment induced ISG15 and CCL5 from FRL kd CTRL; however, induction was significantly reduced in FRL kd RIG-I cells, consistent with knockdown of RIG-I signaling. Of note, ferret IFN-α also induced ISG15, but not CCL5, in both cell lines, consistent with reported studies that CCL5 is induced by RIG-I stimulation but not by IFN-α [32, 33]. Moreover, while pretreatment with 3pRNA potently reduced RSV infection in FRL kd CTRL cells, its inhibitory effects were modest in FRL kd RIG-I cells (Figure 3D). Together, these data confirm that pretreatment of cells with 3pRNA results in a RIG-I-dependent reduction in RSV infection of FRL cells.

RIG-I Agonist Injection Before RSV Infection in Ferrets: Effect on Virus Replication in the Lung

Ferrets represent an additional animal model used to study the pathogenesis, transmission and immunity to human RSV

infections [25]. Ferrets received a single intravenous injection of 3pRNA or ctrl RNA and, 24 hours later, were infected intranasally with RSV. Whole-blood samples collected from each animal before, as well as 24 hours after, 3pRNA or ctrl RNA treatment, noting that collection of the latter sample occurred before RSV challenge. As seen in (Figure 4A), qRT-PCR confirmed up-regulation of ferret ISGs Mx1, ISG15, and OAS1 in PBMCs in response to 3pRNA.

After RSV infection, no significant differences were observed in body weight and temperature between ctrl RNA- and 3pRNA-treated animals up to 3 days after infection (data not shown). RSV titers in daily nasal wash samples confirmed infectious RSV in samples from all animals at ≥1 time point, and area under the curve analysis confirmed no significant difference in viral loads between 3pRNA- and ctrl RNA-treated animals (Figure 4B). At the experimental end point (3 days after infection), no differences were observed in virus titers recovered from nasal tissues of ctrl RNA or 3pRNA-treated animals (Figure 4C). For 3pRNA-treated

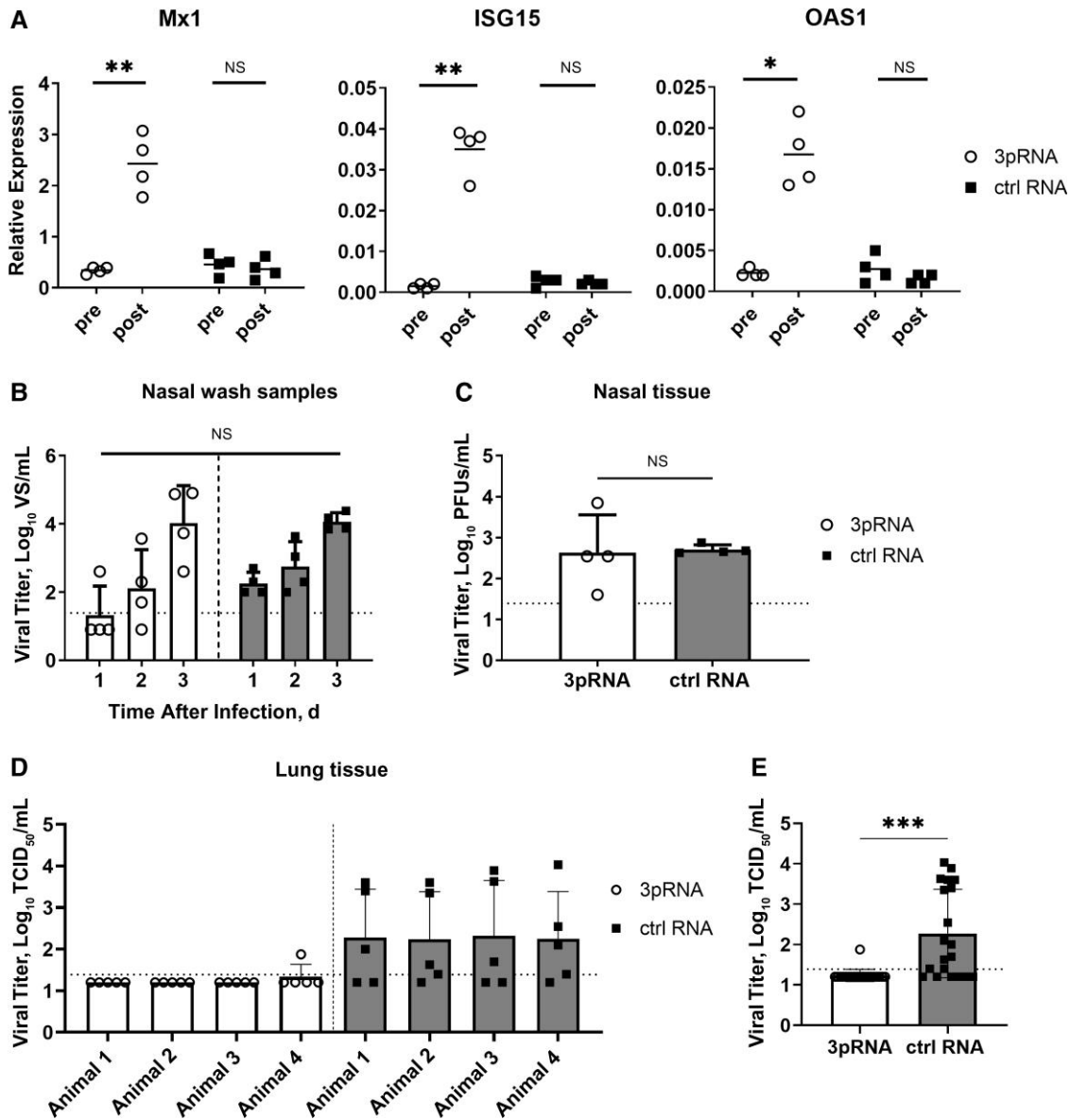


Figure 4. Retinoic acid-inducible gene I (RIG-I) agonist treatment of ferrets 24 hours before respiratory syncytial virus (RSV) infection results in systemic interferon-stimulated gene (ISG) induction and reduced virus titers in the lungs. Animals ($n = 4$ /group) received a single intravenous injection of 3pRNA or control (ctrl) RNA (0.3 mg/kg) 24 hours before inoculation with 5×10^5 virospot (VS) of RSV Long in 500 μ L. **A**, Whole blood was collected and peripheral blood mononuclear cells isolated before (Pre) and 24 hours after (Post) intravenous injection of 3pRNA or ctrl RNA. After RNA isolation, quantitative real-time polymerase chain reaction was used to determine Myxoma protein (Mx)1, interferon stimulated gene (ISG)15, and 2'-5'-oligoadenylate synthetase (OAS)1 levels, expressed relative to GAPDH. **B**, RSV titers in nasal wash samples determined by means of ViroSpot assay. **C–E**, At 3 days after infection, ferrets were killed. Viral titers in clarified nasal tissue and lung homogenates were determined by plaque assay. Data points in **D** represent individual lung lobes from individual 3pRNA- or ctrl RNA-treated animals; for data in **E**, individual lung lobes from all 3pRNA- or ctrl RNA-treated animals are pooled. Dashed lines represent limits of detection. Data in **A** represent median values, and data in **B–E**, means with standard deviations. Data were assessed for normal distribution, and samples in **C** were analyzed using Student *t* tests. Other samples were analyzed using nonparametric a Kruskal-Wallis (**A**) or Mann-Whitney *U* (**E**) tests. Abbreviations: PFUs, plaque-forming units; TCID₅₀, median tissue culture infective dose. * $P < .05$; ** $P < .01$; *** $P < .001$; NS, not significant.

ferrets, virus was not detected in any lung lobes from 3 of 4 animals, with low titers detected in 1 of 5 lung lobes from 1 animal (1 of 20 lobes in total) (Figure 4D and 4E). After ctrl RNA treatment, virus was recovered in at least 3 of 5 lobes from each animal (14 of 20 lobes in total). Virus titers from 3pRNA-treated animals were significantly reduced compared with pooled lung lobes from

all ctrl RNA-treated animals ($P = .03$ for animals 1, 2, and 3) except animal 4, which had 1 lung lobe with infectious virus ($P = .10$). Together, these data demonstrate that a single intravenous injection of ferrets with 3pRNA 24 hours before subsequent RSV challenge resulted in systemic induction of ISGs and reduced virus replication in the lung but not the upper airways.

DISCUSSION

Activation of nucleic acid-sensing innate immune receptors such as RIG-I has proved a promising strategy to combat viral infections. In vitro studies have used human cells to demonstrate the efficacy of RIG-I agonist treatments in inhibiting diverse viruses [11–13, 15, 17], with complementary in vivo data obtained in mouse models of IAV [11, 13, 15–17], CHIKV [15], and SARS-CoV-2 infections [18, 19]. Our results support the clinical development of RIG-I ligands for the prophylaxis of RSV infections, given that pretreatment of human, mouse, and ferret airway cells with 3pRNA resulted in reduced susceptibility to subsequent RSV infection and reduced replication in vitro. Moreover, a single intravenous injection of mice or ferrets with RIG-I agonist before RSV infection resulted in potent inhibition of RSV growth in the lungs, but not in nasal tissues.

The ability of RIG-I agonist treatment to ameliorate disease in mouse models of IAV [11, 13, 15–17] and SARS-CoV-2 [18, 19], and in mouse and ferret models of RSV infection in the current study, highlight their potential as broad-spectrum antivirals for respiratory virus infections. While our studies focused on a single prophylactic administration, future studies should assess the effectiveness of multiple RIG-I agonist treatments before and during RSV infection. Already, evidence suggests that RIG-I agonist treatment during an established viral infection can be effective in mice.

For example, Coch et al [16] demonstrated that a single RIG-I agonist injection 18 hours after IAV infection reduced viral titers in the lungs of mice, and that this was associated with protection from a lethal infection. Mao et al [18] also demonstrated that a single RIG-I agonist injection 4 hours after SARS-CoV-2 infection resulted in clearance of virus from the lungs and substantially improved survival rates. When considering development toward clinical use, it is of note that repeated 3pRNA treatment of mice (up to 4 doses) before and/or after IAV challenge did not result in desensitization, nor were any adverse reactions reported [11, 13, 16]. Moreover, Coch et al [16] also reported that a single 3pRNA treatment of mice up to 7 days before IAV challenge resulted in enhanced survival. While similar studies are yet to be performed in the context of RSV, the potential for long-lasting protection and the flexibility to use multiple treatments highlight the need for further studies to assess the potential of RIG-I agonists as prophylactic and/or therapeutic treatments for RSV infection.

Compared with other respiratory viruses, human RSV is generally considered to be a poor inducer of type I IFNs [34, 35] and the virus-encoded NS1 and NS2 proteins, as well as the G glycoprotein, are well-described suppressors of type I IFN induction and production [36]. Of note, type III IFNs have been reported to be the predominant IFNs induced by RSV infection of human nasal epithelial cells [37]. While the relative contribution of type I and type III IFN signaling in

RIG-I agonist-mediated protection against RSV infection remains to be elucidated, Coch et al [16] and Mao et al [18] demonstrated the critical role of type I IFNs during therapeutic or prophylactic RIG-I agonist treatment of IAV- and SARS-CoV-2-infected mice, respectively. Given that IAV and SARS-CoV-2 also exert mechanisms to antagonize type I IFN signaling [38, 39], these findings suggest that RIG-I agonist treatment, at least in mouse models, enhances IFN induction in the lungs to promote ISGs and other downstream antiviral effectors. Therefore, prophylactic administration is likely to induce IFN-induced antiviral effectors before, and independently of, virus-induced antagonism of IFN responses.

Robust protection against RSV growth in the lungs, but not the upper airways, of mice and ferrets suggests that intravenous administration of RIG-I agonists does not result in efficient delivery, and therefore induction of local antiviral immunity, in nasal tissues. Of interest, the intravenous route has been used in all published studies to date reporting a protective role for RIG-I agonists in mouse models of IAV [11, 13–17] or SARS-CoV-2 infections [18, 19], while intraperitoneal inoculation (also resulting in systemic administration) was used to demonstrate RIG-I agonist-mediated protection in a mouse model of CHIKV infection [15]. We suggest that intravenous 3pRNA results in ISG induction in PBMCs, which could promote recruitment of activated leukocytes to the lung. Moreover, given that intravenous 3pRNA primarily localizes to the liver, spleen and lung [40], direct delivery to parenchymal cells in the lung might also induce ISGs and therefore protection against RSV.

In subsequent studies, intranasal delivery could be investigated as an approach to induce local ISG induction and protection in the airways of mice and ferrets. In addition to timing and dosage, identifying appropriate delivery systems for effective intracellular delivery to the airways remains a key challenge in the clinical development of nucleic acid therapeutics. While our findings suggest that an intravenous RIG-I agonist injection before RSV infection might not be effective in limiting subsequent viral shedding and transmission, its ability to potentially inhibit RSV in the lungs highlights its potential utility in treating or preventing disease associated with RSV infection of the lower airways.

Notes

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Potential conflicts of interest. G. H. is an inventor on a patent about RIG-I ligands. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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