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Gold Nanorods Inhibit Respiratory Syncytial Virus by Stimulating the Innate Immune Response

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Abstract

Respiratory syncytial virus (RSV) causes severe pneumonia and bronchiolitis in infants, children and older adults. The use of metallic nanoparticles as potential therapeutics is being explored against respiratory viruses like Influenza, Parainfluenza and Adenovirus. In this study, we showed that gold nanorods (GNRs) inhibit RSV in HEp-2 cells and BALB/c mice by 82% and 56%, respectively. The RSV inhibition correlated with marked upregulated antiviral genes due to GNR mediated TLR, NOD-like receptor and RIG-I-like receptor signaling pathways. Transmission electron microscopy of lungs showed GNRs in the endocytotic vesicles and histological analyses indicated infiltration by neutrophils, eosinophils and monocytes correlating with clearance of RSV. In addition, production of cytokines and chemokines in the lungs indicate recruitment of immune cells to counter RSV replication. To our knowledge, this is the first *in vitro* and *in vivo* report that provides possible antiviral mechanisms of GNRs against RSV.

Graphical Abstract

Gold nanorods (GNRs) inhibit respiratory syncytial virus (RSV) *in vitro* and *in vivo*. The antiviral gene expression study showed interplay of Toll-like receptor, NOD-like receptor and RIG-I-like receptor signaling pathways. Transmission electron microscopy, histological investigation and cytokine analysis indicate that GNRs stimulates innate immune response resulting in RSV inhibition.

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Keywords

Gold nanorods; Bronchiolitis; Pneumonia; nanomedicine; antiviral; nanoparticles

Background

Recent developments in the field of nanomedicine have led to development of novel nanomaterials with potential applications in diagnosis and treatment of infectious and non-infectious diseases.¹ Nanoparticles, unmodified or functionalized with biomolecules, can be used for anti-viral drug delivery,² virus detection^{3–6} and as anti-viral therapeutic agents^{7–12}. Metallic nanoparticles have shown anti-viral activity against respiratory viruses like Influenza,^{10, 13, 14} Parainfluenza¹⁵ and Adenovirus¹⁶. Gold nanomaterials are gaining popularity due to their surface structure, low toxicity and their potential for a wide range of biomedical applications. Gold nanorods (GNRs) are currently being investigated for treatment of cancer,^{17–19} HIV,²⁰ Respiratory Syncytial Virus (RSV),²¹ and for various biomedical applications.²²

RSV, a negative-sense, single-stranded RNA virus causes severe pneumonia and bronchiolitis resulting in an estimated annual global mortality of 160,000 – 600,000.²³ In the United States, almost all children below the age of two acquire RSV infection and over 100,000 children are hospitalized every year. The geriatric and immunocompromised populations are at the high risk end of developing severe RSV disease.^{24, 25} Nevertheless, there is no effective vaccine or treatment for RSV. There is limited success for treatment of RSV using palivizumab (prescribed for high-risk individuals) and ribavirin.^{26–29} Current research efforts to develop effective treatments against RSV are focused on using fusion inhibitors, subunit vaccine, attenuated RSV, DNA vaccine, siRNA molecules and virus-like particles.^{30, 31} Despite a high potential for use of nanomaterials as anti-RSV therapeutic, this option remains minimally explored.

In this study, we investigated the ability of GNRs to inhibit RSV infection in Hep-2 cells and in BALB/c mice. In addition we examine the impact of GNR on antiviral genes and cytokines expression levels both in Hep-2 cells and in mice. Our results show that GNRs are potent inhibitors of RSV primarily via inducing the innate immune response. Thus, GNRs have the potential for treatment against RSV and other viruses with similar infection mechanisms.

Methods

Cells virus and reagents

HEp-2 cells and RSV long strain (VR-26) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Minimum Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), PKS [penicillin (75 U/mL), kanamycin (100 µg/mL) and streptomycin (75 µg/mL)] designated as MEM-10. DMEM supplemented with 2% FBS (designated as DMEM-2) was also used in other experiments.

GNRs (45 nm × 10 nm) were purchased from Nanopartz Inc. (Loveland, CO, USA) (Supplementary Figure S1.). For *in vitro* assays, HEp-2 cells were incubated for 24 h at 37 °C, 5% CO₂. Next day, cells were treated with either 2.5 µg/mL GNR (**GNR**) and 100 PFU/mL RSV (**RSV**) or pre-incubation of GNRs (2.5 µg/mL) with 100 PFU/mL RSV for 30 min (**GNR-RSV**). The cells were incubated for 1 h at 37 °C and processed for immunofluorescence and other *in vitro* studies.

Immunofluorescence microscopy

RSV inhibition was studied using immunofluorescence microscopy as described previously.³² Briefly, following treatment with GNR and GNR-RSV, cells were incubated for another 48 h. After 48 h, cells were fixed with 10% trichloroacetic acid and washed successively with 70%, 90% and 100% ethanol. Cells were then blocked with 3% dry milk powder and incubated for 1 h with 1:500 goat anti-RSV antibody (Millipore, Temecula, CA, USA) followed by anti-goat antibody (FITC-conjugated IgG H+L, 1:1000). Finally, ProLong® Gold antifade mountant with DAPI was applied and visualized using fluorescence microscope.

Animal experiments

Animal experiments were performed according to the National Institutes of Health (NIH) guidelines for animal use and were approved by Alabama State University's Institutional Animal Care and Use Committee (IACUC). Female 4 to 6 week-old BALB/c mice (Charles River Laboratories Inc, Wilmington, MA, USA) were housed under standard approved conditions and provided daily with sterile food and water *ad libitum*.³³ Four groups of mice (n=3 per group) were administered intranasally either with PBS, RSV (6×10⁵ PFU), GNR (20 µg) or GNR-RSV (20 µg GNR and 6×10⁵ PFU of RSV). On day 5, mice were sacrificed and blood, bronchoalveolar lavage (BAL), lungs and spleens were collected. RSV titers were quantified from lungs (using plaque assay and qPCR) and BAL (plaque assay). Transmission electron microscopy (TEM), histopathology and antiviral PCR array analysis was done for lung tissues. Cytokine analysis of lung homogenate, BAL and serum was performed.

Plaque assay

The ability of GNRs to inhibit RSV *in vitro* was determined by plaque assay as described previously.³² HEp-2 cells were infected with RSV and treated with GNRs (1.25 and 2.5 µg/mL). Lungs were collected in DMEM and processed to determine the RSV titers using gentleMACS dissociator (Miltenyi Biotec Inc., Auburn, CA, USA) following the

manufacturer's procedures. Similarly, BAL samples collected from mice were used to determine RSV titers in BAL.

Real-time PCR

Lungs collected from control and treatment group mice were used to extract total RNA using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using SuperScript® II reverse transcriptase following the manufacturer's protocol (Invitrogen, Life Technologies, Carlsbad, CA, USA) using an oligo-dT primer. Master mix and PCR conditions were prepared as described previously.^{34, 35}

Transmission electron microscopy

Lungs from each mice group were collected and fixed with paraformaldehyde-glutaraldehyde and osmium tetroxide followed by ethanol washes. Tissue sections were polymerized in Embed812 resin and ultrathin sections were collected on copper grids. The tissue sections were stained with uranyl acetate, lead citrate and then imaged using a Zeiss EM10 TEM microscope.

Histopathology

Lungs were harvested from PBS, RSV, GNR and GNR-RSV groups of mice on day 5 after treatment and tissues were fixed in formalin, sectioned, stained with hematoxylin-eosin and analyzed at Nationwide Histology (Veradale, WA, USA).

Antiviral PCR array

Antiviral gene response was studied using HEp-2 cells either untreated (control) or treated with RSV or GNR-RSV for 24 h. Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) followed by cDNA synthesis and PCR reaction for human antiviral response PCR arrays (Qiagen SA Biosciences, Valencia, CA, USA) following the manufacturer's protocol. The RT² profiler PCR array data analysis version 3.5 (available on vendor's web portal) was used for data analysis. Fold changes in gene regulation were calculated for RSV and GNR-RSV treated cells by comparing them with untreated cells.

Similarly, for *in vivo* mouse anti-viral gene response, lungs (PBS, RSV, GNR and GNR-RSV groups) were harvested at day 5 after treatments and total RNA was extracted using RNeasy fibrous tissue mini kit. cDNA synthesis, PCR reaction and data analysis was performed for mouse antiviral response PCR arrays (Qiagen SA Biosciences, Valencia, CA, USA) as described above. Only genes that were significantly up- or down-regulated (p-value < 0.05) from both *in vitro* and *in vivo* experiments were included in the final analysis. The gene network displays were generated using gene regulation data to predict the functional protein associations for *in vitro* and *in vivo* experiments with the help of STRING version 10.³⁶

Isolation and stimulation of splenocytes

Spleens from each mice groups were harvested and splenocytes were processed following previously described method.³⁷ Splenocytes (1×10^6 /mL) were stimulated with

lipopolysaccharide (LPS, 1 µg/mL), RSV (100 PFU/mL) and GNR (2.5 µg/mL) in RPMI containing 10% FBS followed by incubation at 37 °C, 5% CO₂. Cell-free supernatants were collected after 24 h and stored at –80°C until used.

Cytokine assay

The BAL, lung homogenate and serum collected from mice groups were used to detect cytokines production using the Bio-Plex Pro Mouse Cytokine 23-Plex Immunoassay (BioRad, Hercules, CA, USA). Splenocytes collected from each mice group were re-stimulated with LPS, RSV, GNR and GNR-RSV. Cell-free supernatants from splenocytes were analyzed for the secreted cytokines, TNF-α, IFN-γ, IL-6, IL-12p40 and IL-10 using ELISA kits (BD OptEIA™, BD Biosciences, San Jose, CA, USA) following the manufacturer's protocols.

Statistical analysis

Results were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and represented as mean ± standard deviation. One- or two-way ANOVA with Bonferroni's post-test were performed as applicable and differences were expressed as significant at $p < 0.0001$ (***), $p < 0.001$ (**) or $p < 0.05$ (*).

Results

GNRs inhibit RSV

RSV inhibition with GNRs was assessed *in vitro* with immunofluorescence microscopy and plaque assay. Immunofluorescence microscopy showed that HEp-2 cells infected with RSV prominently display RSV infection, while cells treated with GNR-RSV show very minimal RSV infection (Figure 1,A). Effectiveness of GNRs was further evaluated by infecting HEp-2 cells with RSV followed by GNR treatment at 1.25 µg/mL and 2.5 µg/mL using plaque assay (Figure 1,B). Plaque assay shows RSV inhibition by 61% and 82% at 1.25 µg/mL and 2.5 µg/mL of GNRs, respectively, confirming dose-dependent GNR activity.

To corroborate the *in vitro* results of RSV inhibition by GNRs, we tested the effect of GNRs on RSV infection in mice. On day 5 of the infection, the lungs and BAL samples were used to determine the RSV titers using the plaque assay. Mice treated with GNR-RSV showed 56 % reduction in RSV infection when compared to the RSV group (Figure 1,C). Our real-time PCR data also confirmed the inhibition of RSV by GNRs (Figure 1,D). The RSV F gene copy numbers were reduced in the GNR-RSV-treated as compared to the RSV group, which is indicative of reduced RSV replication. BAL samples from the GNR-RSV group similarly showed no plaque formation (Figure 1,E) thus verifying GNRs ability to block RSV infection *in vivo*.

GNRs were localized in the cytoplasmic vesicles

Lungs harvested from four groups of mice were used to study possible morphological changes in lungs and localization of GNRs in lung tissues using TEM. Lung tissues from the PBS group (Figure 2,A) had intact cells in the alveolar region without any damage to the cell membrane. In contrast, RSV infected cells show extensive damage to the cell membranes

and thickened extracellular matrix (Figure 2,B). The GNR treated mice lungs did not show any cell membrane damage or any visible effect on the lung cells (Figure 2,C). Mice treated with GNR-RSV were protected from lung damage and showed ciliated and non-ciliated epithelial cells (Figure 2,D). Gold nanorods were localization in cytoplasmic vesicles in the GNR (Figure 2,E) and GNR-RSV (Figure 2,F) treated mice lungs.

RSV infection leads to infiltration of lungs with leukocytes

The histology analysis indicates no lung pathology in the PBS group (Figure 3,A). The lungs from RSV-infected mice (Figure 3,B) showed perivascular (PV) and peri-bronchiolar (PB) edema compared to the PBS group. Interstitial infiltration of monocytes, PV infiltration of neutrophils, eosinophils and monocytes, and PB infiltration with the monocytes were observed in RSV infected mice. A marked bronchiolar hyperplasia including syncytia and some PB smooth muscle hyperplasia was observed. Minimal to mild capillary congestion or alveolar hemorrhage was also observed. The GNR mouse group showed moderate PV and PB infiltration with edema (Figure 3,C–D). There were mild patchy alveolar and focal interstitial infiltrations with minimal to mild fibrinous intra-lesional changes as well as capillary congestion. The GNR-RSV group also showed minimal to mild PV/PB infiltration and the interstitial infiltration was diffuse (Figure 3,E–F). There were no fibrinous intra-lesional changes but mild patchy capillary congestion was observed. In addition, we confirmed the presence of GNRs in the extracellular matrix in GNR and GNR-RSV treated groups of mice (Figure 3,D–F).

In vitro RSV inhibition was mediated by TLR, NLR and RLR pathways

The *in vitro* anti-viral gene profiles for HEp-2 cells treated with RSV and GNR-RSV show up-regulation of various human anti-viral genes involved in signaling pathways (Table 1). RSV infection down-regulated majority of the genes analyzed while few anti-viral genes (*IL12B* and *IFNA2*) were up-regulated as compared to the GNR-RSV-treated cells. The GNR-RSV-treated cells significantly up-regulated anti-viral genes which are involved in the Toll-like receptor (TLR), NOD-like receptor (NLR) and RIG-I-like receptor (RLR) signaling pathways. In particular, the TLR (*CXCL11*, *CXCL10*, *CCL5*, *CTSL*, *CTSS*, *FOS*, *IL-6*, *SPP1* and *TLR3*), NLR (*OAS2*, *PYDC1*) and RLR (*DDX58*, *DHX58*, *IRF7*, *IFIH1*, and *IL8*) up-regulated genes played a role in innate immunity. The increased expression of TLR, NLR and RLR genes also resulted in increase in type-I interferon genes (*IFNB1* and *STAT1*). The network of predicted anti-viral functional protein association following RSV infection (Figure 4,A) show a limited number of gene interactions, while protein associations of GNR-RSV treatment group (Figure 4,B) showed many anti-viral gene responses.

Innate immune response and antiviral genes were significantly upregulated in mice

Anti-viral gene expression profile was analyzed using lungs cells from mice treated with RSV, GNR and GNR-RSV (Table 2). In the RSV-infected mice, TLR (*Cxcl9*, *Cxcl10* and *Cxcl11*) NLR (*Oas2*) and RLR (*Irf7*, *Isg15*) genes were highly expressed compared to control mice (PBS). Interleukin genes (*Il6* and *Il12b*) were also up-regulated which could be attributed to a high level of expression of the *Ifnb1* gene. However, the GNR-treated mouse group did not show significant change in the gene expression, except for the TLR (*Cxcl9* and

Il6) genes. The anti-viral response of the GNR-RSV-treated mice followed a similar pattern as the RSV group where TLR (*Cxcl9*, *Cxcl10* and *Cxcl11*) NLR (*Oas2*) and RLR (*Dak*, *Irf7* and *Isg15*) genes were highly expressed compared to the control group (PBS). Since a range of innate immune response anti-viral genes (*Ccl5*, *Ccl9*, *Ccl10*, *Ifnb1*, *Mx1*, *Stat1*, *Il1b*, *Il12b*, and *Traf3*) were highly expressed in RSV infected and GNR-RSV treated mice.

The network of predicted mouse anti-viral functional protein association following RSV infection (Figure 5,A) show involvement of many genes which belong to TLR, NLR and RLR pathways. The GNR treated group showed minimal protein associations (Figure 5,B). The protein associations of GNR-RSV treatment group (Figure 5,C) show an extensive interrelation of anti-viral genes which are associated with the innate immune response. This also shows that GNR may find its applications against RSV and similar pathogen to enhance innate immunity.

RSV and GNRs induced cytokines in the lungs and bronchoalveolar lavage

Severe RSV infection and its clearance are dependent on immune responses generated in lungs. Therefore, we analyzed cytokine response in lung homogenates and BAL of all animals groups. Our results revealed that lung homogenates from GNR-treated mice had higher levels of KC, MIP-1a, and IL-12p40 (Figure 6,A) in comparison to the PBS, RSV and GNR-RSV groups. The BAL cytokines (Figure 6,B) indicated higher levels of IL-17, IL-13 and IL-9 in both the GNR and GNR-RSV treatment groups, albeit more elevated in the latter. These findings suggest enhancement of cytokines which are essential for clearance of RSV.

RSV and GNRs induce production of inflammatory cytokines in the serum

The RSV-infected mouse serum showed elevated levels of IL-12p70, IL-17, IL-1B, TNF- α and IL-9, with moderate production levels of MCP-1, MIP-1b and GM-CSF (Figure 7). Animals treated with GNR and GNR-RSV exhibited similar cytokine patterns, albeit at lower concentrations, except for IL-1b which was significantly higher in the GNR-RSV group. Our results suggest differential induction of cytokines by RSV and GNR systemically (serum) and locally (lungs).

GNRs induce anti-inflammatory cytokines upon restimulation of RSV treated splenocytes

Recall experiments were conducted to test the ability of splenocytes from groups of treated mice to produce cytokines. Thus, pooled splenocytes from each mouse group were restimulated with LPS, RSV, GNR, and GNR-RSV to evaluate cytokine levels of IL-12p40 (Figure 8,A), IL-6 (Figure 8,B), IL-10 (Figure 8,C), TNF- α (Figure 8,D), and IFN- γ (Figure 8,E). Overall, re-stimulated splenocytes induced negligible cytokines levels. Interestingly, splenocytes from RSV-infected mice produced significantly more IL-6 than of the other cytokines (TNF, IL-10, IL-12p40 and IFN- γ) following re-stimulation by GNRs. This finding may imply that GNRs induction of IL-6 may function to control the inflammatory responses.³⁸

Discussion

Recently, metallic nanoparticle have been used in cell culture for viral inhibition of HIV,⁹ Influenza,^{39, 40} Parainfluenza and Herpes simplex viruses.¹⁵ Additionally, GNRs have been explored as a RSV vaccine delivery.²¹ Some studies with metallic nanoparticles have been performed in animals with respect to respiratory viruses. However, there is need to understand the molecular events associated with nanoparticle-based viral inhibition. Therefore, our study aimed to demonstrate the anti-RSV properties of GNRs and their molecular mechanisms responsible for anti-viral effects in HEp-2 cells and mice.

Here in our study we demonstrate, for the first time, the effectiveness of GNRs to inhibit RSV replication not only in HEp-2 cells but also in mice. TEM images show the presence of GNRs in the vesicles of the cells (macrophages), most likely in lysosomes and but not in the nucleus. This finding was further confirmed by the histopathology analyses demonstrating the presence of GNRs in the extracellular matrix. Our finding is in agreement with other reports showing accumulation of gold nanoparticles in lysosomes and extracellular vesicles following cellular uptake by phagocytosis can trigger TLR signaling pathway.^{41–43}

The human antiviral gene response in HEp-2 cells infected with RSV showed down-regulation of majority of the genes. Conversely, GNR-RSV treated cells showed upregulation of majority of the genes involved in TLRs, NLRs and RLRs signaling pathways, suggesting their upregulation as potential mediators of RSV inhibition by GNR. TLRs play an important role in inducing innate immune responses following recognition of antigens. Also, NLRs function in close coordination with TLRs due to their gene interactions and their role in innate immunity. RLRs induce innate immunity against RNA viruses by recognizing dsRNA. The role of RLRs up-regulated genes is particularly relevant in our studies since RSV uses dsRNA for its replication. Interestingly, the genes highly upregulated by GNR-RSV treatment in HEp-2 cells are involved in regulation of chemokines and interleukins leading to the induction of innate immunity. Both our TEM and histopathology results support the antiviral genes expression of cathepsin proteases (CTSL and CTSS) and TLR genes. These genes are expressed when antigens are accumulated in the lysosomes and endosomes to degrade the antigens and activation of TLR-mediated antiviral response.⁴³ Several genes belonging to type-I interferon response (such as *IFNB1*) upregulated in our studies work in association with interferon regulatory-factor 7 (*IRF7*) and induce innate immunity.⁴⁴

The mouse antiviral gene response revealed that many of the TLR, NLR and RLR signaling pathway genes were similarly up-regulated in both RSV and GNR-RSV treated mice. These similarities are most likely due to conserved surface proteins of RSV which are not altered by GNR treatment. Antiviral activity is modulated via JAK/STAT pathway in association with ISG15.⁴⁵ In addition, upregulation of NLR genes (*OAS2*, modulated by IFN) play a significant role in viral genome degradation (particularly degradation of RSV genes) resulting in viral inhibition.⁴⁶ Furthermore, upregulation of pro-inflammatory cytokine genes (*IL8*, *CCL5*, *CXCL10* and *CXCL11*) serve as chemoattractant for monocytes, macrophages, T cells, NK cells, and dendritic cells which are integral to the innate immunity response. As to why many TLR, NLR and RLR signaling pathway genes were down-

regulated in RSV infected HEp 2 cells genes is not clearly understood, but can be due to immunosuppression by RSV NS1 and NS2 proteins. It should be noted that the antiviral response in the lungs represents a mixed transcription profile of different tissues and infiltrating cell types. Nevertheless, our data strongly supports that the inhibitory effect of GNRs against RSV in HEp-2 cells and in mice correlates with its concomitant ability to induce potent innate immune responses.

RSV disease has been extensively studied and it is well established that cytokines in lungs and local milieu such as BAL play an important role in disease reduction or severity. The expression of KC/CXCL1 and MIP-1 α /CCL3 in the lungs are linked to the recruitment of leukocytes and T-cells to infiltrate the site to clear the infection and secrete more inflammatory cytokines.⁴⁷ The induction of IL-13 and IL-17 in BAL suggest a Th2 immune response since these cytokines exhibit pro/anti-inflammatory activities and also mediate production of other cytokines and chemokines.⁴⁸ Interestingly, GNR induced high levels of IL-6 in RSV-infected splenocytes which may, in part, explain the lower levels of other examined cytokines, given its role in controlling inflammation.⁴⁹

Although gold nanospheres and GNRs different in their geometry and surface structure, GNRs could find its applications against viruses including RSV in a similar fashion as reported for gold nanoparticles against dengue¹² and influenza⁵⁰ viruses. Also, ability of metallic nanoparticles to modulate B-cells, T-cells, NK cells, mast cells, neutrophils, dendritic cells and macrophages to mount innate immunity is very promising. Based on our study, GNR therapeutic application needs careful design, treatment regimen and delivery to be considered effective for RSV treatment. GNRs in combination with antiviral drugs and/or biocompatible molecules seem to be a promising therapeutic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Gold nanorods inhibit RSV *in vitro* and *in vivo*. **(A)** Immunofluorescence images of HEP-2 cells alone (cells), cells infected with RSV (RSV) and cells treated with pre-incubated GNR and RSV (GNR-RSV). Cells were stained with DAPI (blue) and FITC conjugated anti-RSV F antibodies (green). **(B)** HEP-2 cells infected with RSV and treated with 1.25 and 2.5 $\mu\text{g/mL}$ GNRs. **(C)** RSV titer of lungs harvested from mice treated with PBS, RSV and GNR-RSV. **(D)** Real-time PCR confirming RSV infection in the lungs (for mice groups) by quantifying the RSV F gene copy number. **(E)** RSV titer of BAL samples collected from PBS, RSV and GNR-RSV groups.

Figure 2.

Transmission electron microscope images of the mouse lung tissues of four groups of mice at 5000 $\times$ magnifications (A–D) and 10000 $\times$ magnification (E and F). (A) PBS group mice show normal lung cells (B) while mice infected with RSV show extensive lung cell damage. (C) The GNR treated mice were similar to their morphology with PBS group without any cell membrane damage. (D) The GNR-RSV group of animals also show normal cell membrane and no damage. The distribution of GNRs in lung cells were observed at 10000 magnification (E and F). (E) The lung cells of GNR treated mice show presence of GNRs in cytoplasmic vesicles (and inset image showing the vesicle). (F) Similarly, mice treated with GNR-RSV show GNRs in the vesicle of cytoplasmic vesicles (and inset image showing the vesicles). The nuclei of lungs in both animal groups (GNR and GNR-RSV) were free of GNRs. Green, black and red arrows indicate cell membrane, extracellular matrix and damaged cell membrane, respectively.

Figure 3.

Histopathology of mouse lung tissues of four groups of animals. **(A)** Lungs of animals treated with PBS (400×). **(B)** Animals infected with RSV (400×). **(C)** Animals treated with GNR at 400× and **(D)** 1000× magnification, **(E)** mice treated with GNR+RSV at 400× and **(F)** 1000× magnification. The scale bar represents 100 μm and PB (peri-bronchiolar) and PV (peri-vascular) structures in lungs. The inflammation of PB or PV structure is visible in the treatment groups, when compared to the PBS (control) group. The black arrows indicate GNR aggregation in the lung tissues.

Figure 4. The gene network displays of antiviral response by the HEp-2 cells. Anti-RSV gene expression upon **(A)** RSV infection and gene expression following **(B)** GNR-RSV treatment. The significantly regulated genes (p value < 0.05) predicted for various functions or interactions are shown with the colored lines and their corresponding functions. The gray lines indicate the high confidence.

Figure 5. The gene network display of the mouse antiviral gene response of the mice treated with (A) RSV, (B) GNR and (C) GNR-RSV. The significantly regulated genes (p value < 0.05) predicted for various functions or interactions are shown with the colored lines and their corresponding functions. The gray lines indicate the high confidence.

Figure 6. Cytokine response in the (A) lung homogenate and (B) BAL from PBS, RSV, GNR and GNR-RSV treated mice groups.

Figure 7.
Serum cytokine response of the PBS, RSV, GNR and GNR-RSV mice groups.

Figure 8.

Splenocytes ($1 \times 10^6/\text{mL}$) from each treated mice groups (PBS, RSV, GNR, GNR-RSV) were restimulated with LPS, RSV, GNR and GNR-RSV. Supernatants were collected and used to measure the levels of (A) IL-12p40, (B) IL-6, (C) IL-10, (D) TNF- α , and (E) IFN- γ cytokine production.

Table 1

Antiviral gene response in the HEp-2 cell lines upon RSV and GNR-RSV treatment after 24 hr. The gene expression for RSV, GNR and GNR-RSV is shown as the fold regulation with respect to (w.r.t) healthy HEp-2 cells (Control), negative sign denotes down-regulation and positive values denotes up-regulation of the gene w.r.t. corresponding control. The significant (p-value <0.05) is shown with an asterisk(*). Genes involved in two or more signaling pathways are represented by corresponding superscripted symbols for Toll-like receptor signaling(#), Nod-like receptor signaling(\$), RIG-I-like receptor signaling(†) or Type-I-Interferon signaling and response(+) as applicable.

Gene	Function of the gene product	RSV		GNR-RSV	
		Fold regulation	p-value	Fold regulation	p-value
Toll-like receptor signalling ^(#)					
<i>CXCL11</i>	Activated T-cell chemoattractant	-1.40	0.21	4.63	0.00*
<i>CXCL10[†]</i>	Chemoattractant for macrophages, NK, dendritic and T cells	-1.04	0.17	4.00	0.00*
<i>CCL5</i>	Chemoattractant for monocytes, TH cells and eosinophils	-1.27	0.14	5.08	0.00*
<i>CTSL</i>	Lysosomal cysteine proteinase- antigen processing	-1.12	0.29	3.90	0.00*
<i>CTSS</i>	Lysosomal cysteine proteinase- antigen processing	-1.14	0.38	4.63	0.00*
<i>FOS</i>	Interacts with JUN and regulates cell proliferation, differentiation, and transformation	-1.13	0.20	2.81	0.00*
<i>IL1B^{\$}</i>	Proinflammatory cytokine	1.08	0.63	2.19	0.00*
<i>IL12A[†]</i>	Stimulates IFN- γ production	-1.01	0.82	-1.99	0.00*
<i>IL12B[†]</i>	Stimulates IFN- γ production	4.53	0.12	1.72	0.42
<i>IL6</i>	Proinflammatory interleukin	1.24	0.11	5.26	0.00*
<i>JUN</i>	Interact with FOS to form AP-1 transcription factor	-1.18	0.05	1.02	0.86
<i>MAPK14[†]</i>	MAP kinase signal transduction	-1.10	0.19	-1.25	0.05
<i>MAP2K1</i>	MAP kinase signal transduction	-1.18	0.09	-1.19	0.05
<i>MAP2K3</i>	MAP kinase signal transduction	-1.31	0.10	-1.74	0.02*

Gene	Function of the gene product	RSV		GNR-RSV	
		Fold regulation	p-value	Fold regulation	p-value
<i>MAP3K1</i>	MAP kinase signal transduction and NF- κ B pathway	-1.18	0.12	-1.39	0.03
<i>MAPK8[†]</i>	MAP kinase signal transduction	1.03	0.81	-1.51	0.02*
<i>SPP1</i>	Upregulates expression of IFN- γ and IL-12	-1.24	0.11	7.46	0.05
<i>TLR3⁺</i>	Recognizes dsRNA	-1.03	0.75	4.11	0.00*
Nod-like receptor signalling ([§])					
<i>HSP90A4A1</i>	Molecular chaperon involved in cell cycle and signal transduction	-1.06	0.56	-1.74	0.02*
<i>IL18</i>	Proinflammatory cytokine	-1.13	0.39	-1.63	0.04*
<i>OAS2</i>	Activates latent RNase L	1.08	0.75	14.44	0.00*
<i>PYDC1</i>	Associates with apoptosis-associated speck like protein containing a CARD domain	4.72	0.03*	5.72	0.17
<i>SUGT1</i>	Ubiquitination and proteasomal degradation	-1.18	0.17	-1.63	0.01*
RIG-I-like receptor signalling ([†])					
<i>DDX58</i>	MDA5 mediated antiviral signalling	1.01	0.98	2.69	0.00*
<i>DHX58</i>	Antiviral activity via RIG-I/MDA5 mediated induction of IFN- α/β	-1.57	0.04*	2.40	0.01*
<i>FADD</i>	IFN mediated antiviral immune response, apoptosis	-1.41	0.04*	-1.43	0.05
<i>IRF3[‡]</i>	Activates transcription of IFN- α/β	-1.06	0.62	-1.93	0.01*
<i>IRF7[‡]</i>	Transcriptional regulator of IFN- α/β	1.33	0.04*	10.70	0.00*
<i>IFIH1</i>	Senses viral RNA and activates antiviral response	-1.11	0.17	3.94	0.00*
<i>IL8</i>	Proinflammatory cytokine; chemotactic for NK cells and granulocytes	2.36	0.00*	14.78	0.00*
<i>ISG15⁺</i>	Ubiquitin-like protein	-1.21	0.10	2.78	0.00*
<i>MAVS</i>	Innate antiviral response	-1.06	0.45	-1.30	0.04*

Gene	Function of the gene product	RSV		GNR-RSV	
		Fold regulation	p-value	Fold regulation	p-value
<i>NFKB1</i> [#]	Regulates cytokines and stress responses	-1.03	0.64	-1.44	0.01*
<i>NFKB1A</i> [#]	Promotes ubiquitination	-1.24	0.18	-1.74	0.01*
<i>PIN1</i>	Role in type-1 IFN mediated immunity	-1.43	0.02*	-1.31	0.09
<i>TRADD</i>	Apoptosis and activation of NF- κ B	-1.66	0.01*	-1.73	0.02*
<i>TRIM25</i>	Suppressing DDX58 signal transduction	-1.06	0.63	1.48	0.01*
Type-1-Interferon signalling and response (*)					
<i>IFNA</i> [#]	Interferon alpha	1.20	0.00*	1.08	0.18
<i>IFNA2</i> [#]	Interferon alpha	6.12	0.05	4.54	0.16
<i>IFNB</i> ^{#/†}	Interferon beta	1.88	0.00*	4.84	0.01*
<i>IL15</i> [#]	Activation and proliferation of T and NK cells	-1.62	0.06	-2.00	0.04*
<i>STAT1</i>	Mediates cellular responses to IFN	-1.16	0.17	2.16	0.00*

Table 2

Antiviral gene response in the BALB/c mice lung tissues of PBS, RSV, GNR and GNR-RSV groups. The gene expression for RSV, GNR and GNR-RSV is shown as fold regulation with respect to (w.r.t) PBS (Control), negative sign denotes down-regulation and positive values denotes up-regulation of the gene w.r.t. control. The significant (p-value <0.05) is shown with an asterisk (*). Genes involved in two or more signaling pathways are represented by corresponding superscripted symbols for Toll-like receptor signaling(#), Nod-like receptor signaling(\$), RIG-I-like receptor signaling([†]) or Type-I-Interferon signaling and response(+) as applicable.

Gene	Function of the gene product	RSV		GNR		GNR-RSV	
		Fold regulation	p-value	Fold regulation	p-value	Fold regulation	p-value
Toll-like receptor signalling ^(#)							
<i>Ccl5</i>	Chemoattractant for monocytes, TH cells and eosinophils	1.3	0.55	1.28	0.57	3.22	0.01*
<i>Cxcl9</i>	T-cell chemoattractant (IFN- γ induced)	775.65	0.26	19.99	0.17	732.64	0.00*
<i>Cxcl10[†]</i>	Proinflammatory; participate in T-cell effector function	27.86	0.00*	1.25	0.64	4.97	0.00*
<i>Cxcl11</i>	Activated T-cell chemoattractant	24.18	0.29	-1.32	0.47	31.98	0.02*
<i>Il1b^{\$}</i>	Proinflammatory cytokine	1.57	0.59	2.96	0.27	3.94	0.09
<i>Il12a[†]</i>	Stimulates IFN- γ production	-2.07	0.00*	-1.14	0.20	-1.27	0.03*
<i>Il12b[†]</i>	Stimulates IFN- γ production	3.18	0.01*	1.92	0.01*	1.7	0.02*
<i>Il6</i>	Proinflammatory interleukin	5.89	0.05	3.45	0.17	6.56	0.09
<i>Mapk14[†]</i>	MAP kinase signal transduction	-1.24	0.07	-1.22	0.01*	-1.33	0.02*
<i>Mapk3</i>	MAP kinase signal transduction	-1.45	0.01*	-1.21	0.05	-1.43	0.00*
<i>Tlr3[*]</i>	Recognizes dsRNA	1.69	0.01*	1.28	0.02*	1.43	0.00*
<i>Tlr7</i>	Recognises ssRNA	1.16	0.02*	-1.00	0.96	1.19	0.09
<i>Tlr9</i>	Recognizes unmethylated CpG DNA sequences	-1.06	0.41	1.38	0.00*	1.15	0.33
Nod-like receptor signalling ^([§])							

Gene	Function of the gene product	RSV		GNR		GNR-RSV	
		Fold regulation	p-value	Fold regulation	p-value	Fold regulation	p-value
<i>Aim2</i>	Recognizes cytosolic dsDNA	-1.12	0.66	-1.18	0.30	-1.41	0.03*
<i>Card9</i>	Caspase recruitment	-1.65	0.01*	-1.2	0.10	-1.56	0.01*
<i>Nod2</i>	Binds MAVS and involved in NF- κ B signalling	-1.28	0.08	1.09	0.48	-1.32	0.04*
<i>Oas2</i>	Activates latent RNase L	1.98	0.03*	1.49	0.03*	1.97	0.02*
<i>Tbkbp1</i>	binds TBK1	-1.12	0.28	-1.27	0.02*	-1.59	0.00*
RIG-I-like receptor signalling (*)							
<i>Atg5</i>	Involved in autophagy	-1.09	0.23	-1.23	0.02*	-1.23	0.04*
<i>Dak</i>	Suppressor of MDA5 but not RIG-I or TLR	-1.56	0.01*	1.11	0.26	1.99	0.01*
<i>Ddx58</i>	MDA5 mediated antiviral signaling	-1.07	0.39	-1.17	0.04*	-1.12	0.20
<i>Irf7</i> [#]	Transcriptional regulator of IFN- α/β	2.49	0.00*	1.19	0.07	2.21	0.00*
<i>Isg15</i> [†]	Ubiquitin-like protein	2.32	0.00*	1.21	0.08	2.18	0.00*
<i>Map3k7</i> [#]	MAP kinase signal transduction	-1.01	0.99	-1.01	0.97	1.56	0.03*
<i>Mavs</i>	Innate antiviral response	-1.09	0.46	-1.21	0.02*	-1.24	0.28
<i>Nfkbid</i> [#]	Promotes ubiquitination	-1.03	0.65	-1.18	0.12	-1.46	0.01*
<i>Tbk1</i> [#]	Regulates inflammatory antiviral responses	1.06	0.57	-1.12	0.08	-1.36	0.01*
<i>Tnf</i> [#]	Stimulates cell death and IL-1	1.61	0.01*	1.24	0.39	1.89	0.01*
<i>Traf6</i> [#]	Signal transducer of NF- κ B	-1.08	0.59	-1.63	0.35	-1.14	0.02*
Type-I-Interferon signalling and response (*)							
<i>Ifnar1</i>	Interferon alpha receptor	1.01	0.89	1.16	0.61	1.72	0.02*
<i>Ifnb1</i> ^{+/+}	Interferon beta receptor	10.14	0.00*	-1.43	0.84	8.44	0.01*

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Gene	Function of the gene product	RSV		GNR		GNR-RSV	
		Fold regulation	p-value	Fold regulation	p-value	Fold regulation	p-value
<i>Mx1</i>	Antiviral activity	2.44	0.04*	1.75	0.07	3.17	0.01*
<i>Stat1</i>	Mediates cellular responses to IFN	2.1	0.00*	1.12	0.15	1.68	0.00*