Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene

Presentation by
Niraanjana Sathananthan, Frances Vu and Jahan Parsa
The article investigates the effects of using siRNA targeting the viral NS1 gene encapsulated in nanometer sized chitosan particles, in inhibiting respiratory syncytial virus infection.

Previous experiments have indicated that NS1 gene of bovine and human RSV are antagonists of type-1 IFN mediated antiviral response.

Blocking of the NS1 gene expression might attenuate RSV replication and provide an effective antiviral and immune enhancement therapy.
Why consider designing effective antiviral treatment for RSV infection?

Respiratory syncytial virus is a member of the genus *Pneumovirus* and is a negative stranded RNA virus that is enveloped and non-segmented.
Genome size is approximately 15kb and is transcribed into 10 transcripts encoding 11 distinct proteins including the nonstructural NS1 and NS2.

NS1 and NS2 are encoded from separate mRNAs so there is no splicing involved which explains why RSV replication occurs in cytoplasm.

**CLINICAL MANIFESTATIONS OF RSV**

RSV is considered a major viral respiratory pathogen and produces an annual epidemic of bronchiolitis in children and pneumonia in adults and the elderly.

Individuals who are immunocompromised or with congenital heart defects are at higher risk for developing more severe diseases with RSV infection.

Owing to the large immunosuppressed state of the high risk population, vaccine treatment of RSV infection may not be practical or even effective.
Furthermore RSV infections are common due to incomplete immunity caused by multiple RSV infections.

Multiple RSV infections have been known to be associated with Th2 like response in infants thereby predisposing them for the development of allergic diseases and asthma.
Why Use the siRNA Strategy?

The siRNA strategy has proven effective in silencing a number of specific target genes by promoting mRNA degradation in cultured mammalian cells and mice.

The siRNA duplex behaves like dsRNA so there is the potential to elicit immune responses via interactions with Toll-like receptor 3 that triggers mainly IFN responses.

The paper by Davis et al addresses this issue by examining the response of mice to naked siRNAs.
They examined serum levels of IL-12 and IFN-α in response to stimulation with varying doses of poly I:C (analog of dsRNA) and siRNA in mice as well as cultured monocytes labeled RAW264.7.

Results revealed that poly I:C significantly evoked strong IL-12 and IFNα production while siRNAs failed to induce production of above cytokines.

Co-injection of luciferase expressing plasmid and siRNA targeting the luciferase gene followed by whole body imaging of live mice at various timepoints, further confirmed the uptake and sequence specific function of injected synthetic siRNAs.

Results from their experiments concluded that siRNAs were capable of down regulating both exogenous and endogenous targets without eliciting and IFN type immune response which is a critical parameter to consider if siRNA is ever to be used in human therapeutics.
siNS1 Inhibition of recombinant RSV infection

Fig 1(a) shows immunoblot analysis of NS1 protein expression at 24 hours after infection with rgRSV in A549 cells. Cells pretreated with siNS1 upon RSV infection showed no NS1 protein expression compared to untreated cells that were challenged with RSV infection.

Fig 1(b) shows results for the flow cytometer analysis of rgRSV positive uninfected cells and Vero cells. siNS1 transduced A549 showed significant decrease in viral infection as compared to IFN deficient Vero cells.
Fig 1 (c) further elaborates on results from figure 1(b) showing a plaque assay conducted to determine viral titer in culture supernatants.

Results reiterate that virus replication was significantly attenuated by siNS1 treatment in A549 cells but not in Vero cells.

This suggests that NS1 protein of RSV promotes virus replication by inhibiting type IFN pathway.
Mechanism of siNS1-mediated up-regulation of type-1 IFN pathway

- **Immunoblotting**: A549 cells transfected with siNS1 or siNS1a produced increased amounts of IFN-B compared to controls (Fig. 2a,b).

- **Microarray analysis**: isolated RNAs from cells and found that siRNA-transduced cells increased expression of 25 IFN-inducible genes (Table 1).

- Further examined these genes by western blotting: found that p-STAT1, STAT1, IRF1, IRF3, ISGF-3γ and MxA protein were upregulated in siNS1-transduced cells.

- **Immunoblot analysis** to determine if NS1 affected STAT1 and IRF1 translocation: found higher nuclear localization of p-STAT1 and IRF1 in cells treated with siNS1 compared to controls. (Fig. 2d,e).

- **NS1 protein blocks trafficking of p-STAT1 and IRF1 to nucleus.**
## Mechanism of siNS1-mediated up-regulation of type-1 IFN pathway

### Table 1 IFN-inducible genes change more than sixfold in RSV-infected A549 cells

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Gene</th>
<th>Function</th>
<th>Fold change (FC)a</th>
<th>rgRSV</th>
<th>rgRSV + siNS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_007315</td>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_002198</td>
<td>IRF1</td>
<td>interferon regulatory factor 1</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_001571</td>
<td>IRF3</td>
<td>interferon regulatory factor 3</td>
<td>6</td>
<td>NC</td>
<td>1</td>
</tr>
<tr>
<td>NM_004030</td>
<td>IRF7</td>
<td>interferon regulatory factor 7</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_006084</td>
<td>IRF9</td>
<td>ISGF3G (p48)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_005531</td>
<td>IFI16</td>
<td>interferon gamma-inducible protein 16</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_005532</td>
<td>IFI27</td>
<td>interferon, alpha-inducible protein 27</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_006332</td>
<td>IFI30</td>
<td>interferon gamma-inducible protein 30</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>BF338947</td>
<td>IFITM2</td>
<td>interferon induced transmembrane protein 2</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>AL121994</td>
<td>1-8U</td>
<td>contains a pseudogene similar to IFITM3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BE049439</td>
<td>IFI44</td>
<td>interferon-induced, hepatitis C-associated microtubular aggregate protein (44kD)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_004509</td>
<td>IFI41</td>
<td>SP110 nuclear body protein (interferon-induced protein 75, 52kD)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_004509</td>
<td>IFI41</td>
<td>SP110 nuclear body protein (interferon-induced protein 75, 52kD)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_003641</td>
<td>PTS</td>
<td>6-pyruvoyl tetrahydropterin synthase - interferon induced transmembrane protein 1/2 (IFITM1)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_005101</td>
<td>ISG15</td>
<td>interferon alpha-inducible protein (clone IFI-15K)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_005201</td>
<td>ISG20</td>
<td>interferon stimulated gene (20kD) (ISG20)</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_022147</td>
<td>IFRG28</td>
<td>28kDa interferon responsive protein</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_002176</td>
<td>IFNB1</td>
<td>interferon beta 1, fibroblast 8</td>
<td>D</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>NM_002462</td>
<td>MxA</td>
<td>interferon-regulated resistance GTP-binding protein</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_002463</td>
<td>MXB</td>
<td>interferon-regulated resistance GTP-binding protein</td>
<td>7</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_016817</td>
<td>OAS2</td>
<td>2'-5'-oligoadenylate synthetase 2, 69/71 kDa</td>
<td>8</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_037333</td>
<td>OAS1</td>
<td>2'-5'-oligoadenylate synthetase-like</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_037333</td>
<td>OAS1</td>
<td>2'-5'-oligoadenylate synthetase 1, 40/46 kDa</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_006187</td>
<td>OAS3</td>
<td>2'-5'-oligoadenylate synthetase 3, 100 kDa</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_001550</td>
<td>IFRD1</td>
<td>interferon-related developmental regulator 1</td>
<td>6</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>NM_001547</td>
<td>IFIT2</td>
<td>interferon-induced protein with tetratricopeptide repeats 2</td>
<td>8</td>
<td>D</td>
<td>1</td>
</tr>
</tbody>
</table>

aValue for the fold change in expression calculated by the Microarray Suite 5.0 (MAS 5.0) program.

bThe data were compared to arrays of rgRSV-infected A549 cells either with or without siNS1 treatment. I, increased; NC, not changed; D, decreased.
Mechanism of siNS1-mediated up-regulation of type-1 IFN pathway

- **Immunoblotting**: A549 cells transfected with siNS1 or siNS1a produced increased amounts of IFN-B compared to controls (Fig. 2 a,b)

- **Microarray analysis**: isolated RNAs from cells and found that siRNA-transduced cells increased expression of 25 IFN-inducible genes (Table 1).

- Further examined these genes by **western blotting**: found that p-STAT1, STAT1, IRF1, IRF3, ISGF-3γ and MxA protein were upregulated in siNS1-transduced cells.

- **Immunoblot analysis** (determine if NS1 affected STAT1 and IRF1 translocation): found higher nuclear localization of p-STAT1 and IRF1 in cells treated with siNS1 compared to controls. (Fig. 2d,e).

- **NS1 protein blocks trafficking of p-STAT1 and IRF1 to nucleus.**
Silencing NS1 polarizes human DCs toward a Th1-promoting phenotype

- Measured concentration of IFN-α and IFN-β in supernatants of cultured, infected, monocyte-derived DCs transfected with siNS1 or control siRNA by ELISA: Results show that siNS1 treatment indeeded a higher production of both type-1 IFNs in infected DCs than control (Fig. 3a).

- To assess effect of siNS1 treated DCs on T-cell function, allogenic naïve CD4+ T cells with RSV-infected DCS +/- siNS1 were stained for intracellular cytokines: Results showed increase in IFNγ and decrease in IL-4 in siNS1 treated RSV infected DCs compared to controls (Fig. 3b).

- Infected DCs treated with siNS1 produce much more type-1 IFNγ and also drive naïve T-cells towards Th1 type lymphocytes (generates more IFN-γ and less IL-4).
Nanoparticle-complexed siNS1 (nano-siNS1) significantly attenuates RSV infection and pulmonary pathology in mice

- To determine if siNS1 exerts an antiviral response in vivo, they complexed siNS1 plasmid with nanochitosan polymer and administered nanoparticles as nasal drop 2 days before viral inoculation.

- **RT-PCR**: showed siNS1 knocked down expression of RSV NS1 gene but not F gene. (Fig. 4a).

- **Plaque assays** on A549: showed viral titer in supernatants of homogenized lungs was decreased in siNS1 treated mice compared to control.

- Mice were challenged with methylcholine (causes airways to constrict) followed by RSV infection (measure airway reactivity and lung function in response to infection). Results showed RSV-infected cells had >400% increase in enhanced pause values. siNS1 group only had 300% increase (protection from airway reactivity).
Nanoparticle-complexed siNS1 (nano-siNS1) significantly attenuates RSV infection and pulmonary pathology in mice

- **Histology:** siNS1 treated mice had lower AHR and reduced pulmonary inflammation (decreased goblet cells hyperplasia of bronchi and number of infiltrating inflammatory cells compared to control). (Fig. 4d)

- **RT-PCR:** increased Ifnb1 gene expression in lung of siNS1 treated mice compared to controls (Fig. 4e,f).

- **ELISA:** 2-fold increase in IFN-α in bronchoalveolar lavage fluid in siNS1 treated mice compared to control.
Potential of Nano-siNS1 for prophylaxis and treatment of RSV infection

- Mice treated with NG042-siNS1 or controls 2, 4 or 7 days before RSV inoculation
- Analysis of viral titers 5 days after inoculation showed prophylactic effect of siNS1 can last for at least 4 days
- There are even lower titers of virus if siNS1 given 7 days prior to inoculation
Prophylactic blocking of NS1 activity can induce anti-RSV immunity

- Treat mice with NG042-siNS1 or controls and inoculate with RSV 2 days later, followed by a reinoculation of RSV 16 days later.

- Intracellular cytokine staining of splenocytes show increase in IFN-gamma in both CD4+ and CD8+ T cells, and an increase in IL-4 in CD4+ T cells compared to control mice.
Prophylactic blocking of NS1 activity can induce an anti-RSV response and protect the host against reinfection.

NG042-siNS1 treated mice show a significant decrease in viral titers compared to control mice upon secondary infection.
Therapeutic potential of siNS1

- Inoculate mice with RSV and at 0, 2 and 3 days after inoculation treat with NG042-siNS1
- Mice treated the same day or 2 days after showed significantly lower titers of virus

- Lung sections show that siNS1 treated mice (2 days after RSV inoculation) showed a decrease in:
  - Lung inflammation
  - Goblet cell hyperplasia
  - Infiltration of inflammatory cells
Discussion

- Previously, other studies have shown antisense oligonucleotide-mediated attenuation of RSV infection.

- Both antisense and siRNA are thought to work the same way mechanistically: both reduce expression of a target gene post-transcriptionally.

If this is the case, and the antisense oligonucleotide can be used, why bother with making the siRNA? What are the advantages to using the siRNA?
Advantages of siRNA over antisense oligonucleotides

1) Antisense oligonucleotides accumulate in the nucleus where they may alter splicing of precursor mRNA

   **BUT**

   RSV replication, like many other viruses, occurs in the cytoplasm. The cytoplasm is where the siRNA accumulates

2) Intracellular expression from promoters such as RNA pol III promoter allow for stable siRNAs to be produced in the cell, and hence lower concentrations of siRNA vector are needed than the antisense oligonucleotide to achieve a silencing of a target gene.
siNS1 expression can attenuate RSV replication and allow for expression of IFN genes and signaling

- Normally, NS1 antagonizes type-1 IFN responses, allowing for RSV replication
- siNS1 inhibited rgRSV production by about 90-97%
- siNS1 allows for proper modulation of the type-1 IFN pathway
  - 3 fold increase in IFN-beta expression
  - Allows for such events as interfering with viral replication, and modulation of the host immune response
- In A549 cells see an increase in IFN-inducible genes:
  - IRF3
  - MxA
  - STAT1 (siNS1 also promotes phosphorylation of STAT1)
  - IRF1
  - ISGF-3gamma
siNS1 used by Dendritic Cells to generate anti-RSV immunity

- NS1 decreases type-1 IFN production which affects the activation and antigen presentation ability of DCs
- Due to the lowered ability of DC to induce IFN-gamma production in naïve T cells, a specific immune response to RSV is delayed and subsequent reinfections of RSV can occur
- siNS1 allows for DCs to produce more type-1 IFN and drive naïve T cells to $T_{H}1$-type T cells which can activate macrophages

Why is a $T_{H}1$ response favoured over a $T_{H}2$ response in this case?
Prophylactic and therapeutic uses of siRNA

- Use of oligomeric nanometer-size chitosan particles, NG042 for *de novo* expression of siNS1
- siNS1 induces protection from rgRSV infection, infection induced inflammation and airway reactivity, and can protect for at least 4 days
- Prophylaxis with siNS1 can inhibit subsequent reinfection of mice when inoculated with RSV again 16 days after the first inoculation
- NG042 attenuates established RSV infections by decreasing viral titers in the lung, improving pulmonary function and attenuating inflammation in the lungs of rgRSV infected mice
NS1 protein of the RSV is known to promote viral infection of human epithelial cells and dendritic cells by inhibiting the type-1 IFN pathway.

Using the NG042-siNS1 as either a prophylactic or therapeutic drug is effective in reducing viral titers and pulmonary pathology in mice infected with RSV.

Hence the use of siRNA nanoparticles seem to show promise in therapeutic and prophylactic drug design, not only for RSV infections, but possibly for other types of infections.
Summary

- RSV is a respiratory pathogen that encodes 11 distinct proteins, 2 of which are nonstructural proteins (NS1 and NS2) that play a role in viral replication.
- NS1 antagonizes the type-1 IFN pathway, which allows for viral replication.
- siNS1 can inhibit recombinant RSV infection.
- siNS1 allows for the upregulation of type-1 IFNs via a gene specific silencing.
- Silencing NS1 also allows for production of type-1 IFNs to allow DCs to promote a TH1 phenotype.
- The upregulation of several IFN-stimulated genes coincides with siNS1 treatment, such as STAT1, IRF3, IRF1, and MxA.
- Nano-siNS1 was found to significantly attenuate RSV infection and its pathological effects both in a prophylactic and therapeutic manner and can prevent reinfection by RSV.