**Online Supplement**

# Both viral and host factors determine innate immune responses in airway epithelial cells from children with wheeze and atopy

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Original Article

## Additional Methods and Results

### Preparation of RSV and hMPV for infection

RSV A2 (ATCC) was generated in HEp-2a cells (OptiMEM/2% fetal calf serum [FCS]; life technologies), and concentrated by centrifugation at 50,000 x *g* through a 30%/60% (w/v) sucrose cushion. The interface was removed, diluted 10-fold in OptiMEM, and virus pelleted at 13,000 x g for 4 hours. The final virus pellet was resuspended in OptiMEM without FCS and the amount of viable virus in the stock preparation quantified by immune-plaque assay as described below. hMPV CAN97-83 was generated in LLC-MK2 cells and concentrated in the same manner as RSV. The amount of viable virus in the stock preparation was quantified using $TCID_{50}$ assay as described below.

### Quantification of shed virus by titration

Culture supernatants of AECs infected with RSV or hMPV at a MOI of 0.1 were collected used to infect monolayers of either HEp-2a cells in a plaque assay (for quantification of RSV)

or LLC-MK2 cells in a $TCID_{50}$ assay (for quantification of hMPV). HEp-2a cells were exposed to a 10-fold dilution series of RSV-infected supernatants for 2 h, then overlayed with 0.8% methyl cellulose / OptiMEM / 2% FCS, and incubated for 6 days at 37$^{o}$C. Monolayers were then fixed and RSV-positive plaques identified using anti-RSV polyserum (Virostat). The quantity of RSV in cell culture supernatants was then calculated as pfu / ml. LLC-MK2 cells were exposed to a 10-fold dilution series of hMPV-infected supernatants for 2 h, then the cells washed and incubated for 8 days at 37$^{o}$C, after which time they were fixed and hMPV-positive monolayers identified using anti-hMPV antiserum (Ursula Buchholz, NIAID, NIH). The quantity of hMPV in cell culture supernatants was then calculated as a $TCID_{50}$ value using the Karber formula.

**RNA extraction and qPCR**

Total RNA was extracted from cells infected at a MOI of 3, and RT-qPCR performed to quantify intracellular viral RNA as a measure of viral replication, and also to quantify cellular gene expression. Total RNA was extracted using TRIzol (Life Technologies)/chloroform phase separation and isopropanol precipitation. RNA was DNase treated (Ambion), and RT performed using random hexamers (Applied Biosystems). RSV and hMPV specific primers were used to quantify vRNA copy number against control plasmids containing the RSV or hMPV nucleoprotein (N). vRNA was normalized to β-actin copy number, also quantified using specific primers and an expression plasmid. The expression of IRF7, Mx1 and IL-33 mRNA was quantified relative to β-actin, and expressed as fold induction over uninfected cells using the $2^{-\Delta\Delta Ct}$ equation. Primer sequences are listed in Table S1.

**Additional cytokine assays**

IL-6, IL-8, TNF-α, MIP-1α, MIP-1β, eotaxin-2 and eotaxin-3 secreted 24h following either RSV or HMPV infection at a MOI 3, or uninfected were quantified by Milliplex assays (Millipore). No differences in expression associated with disease status were identified (data not shown).

**Table S1**: Primer pairs used for qPCR detection of gene expression.

| Gene | Forward | Reverse |
|---|---|---|
| **β-actin** | TACGCCAACACAGTGCTGTCT | TCTGCATCCTGTCGGCAAT |
| **RSV** | AAGGGATTTTTGCAGGATTGTTT | CTCCCCACCGTAGCATTACTTG |
| **hMPV** | CAGAGAGAGTACAGCAGATTCTAA | TTCTCTACTCCGTGTATGTCTAAC |
| **IRF-7** | CCACTGTTTAGGTTTCGCTTTC | AGTCACAGGTGTTGAACCAG |
| **Mx-1** | GGATTGGAACCATAGCTCTACC | CCTCCCAGAGGAGTAGGATTAT |
| **IL-33** | CCACTGAGGAAAGAGCCATAG | TGAGCCTATCGTTTGGAACTG |

**Additional Figures:**

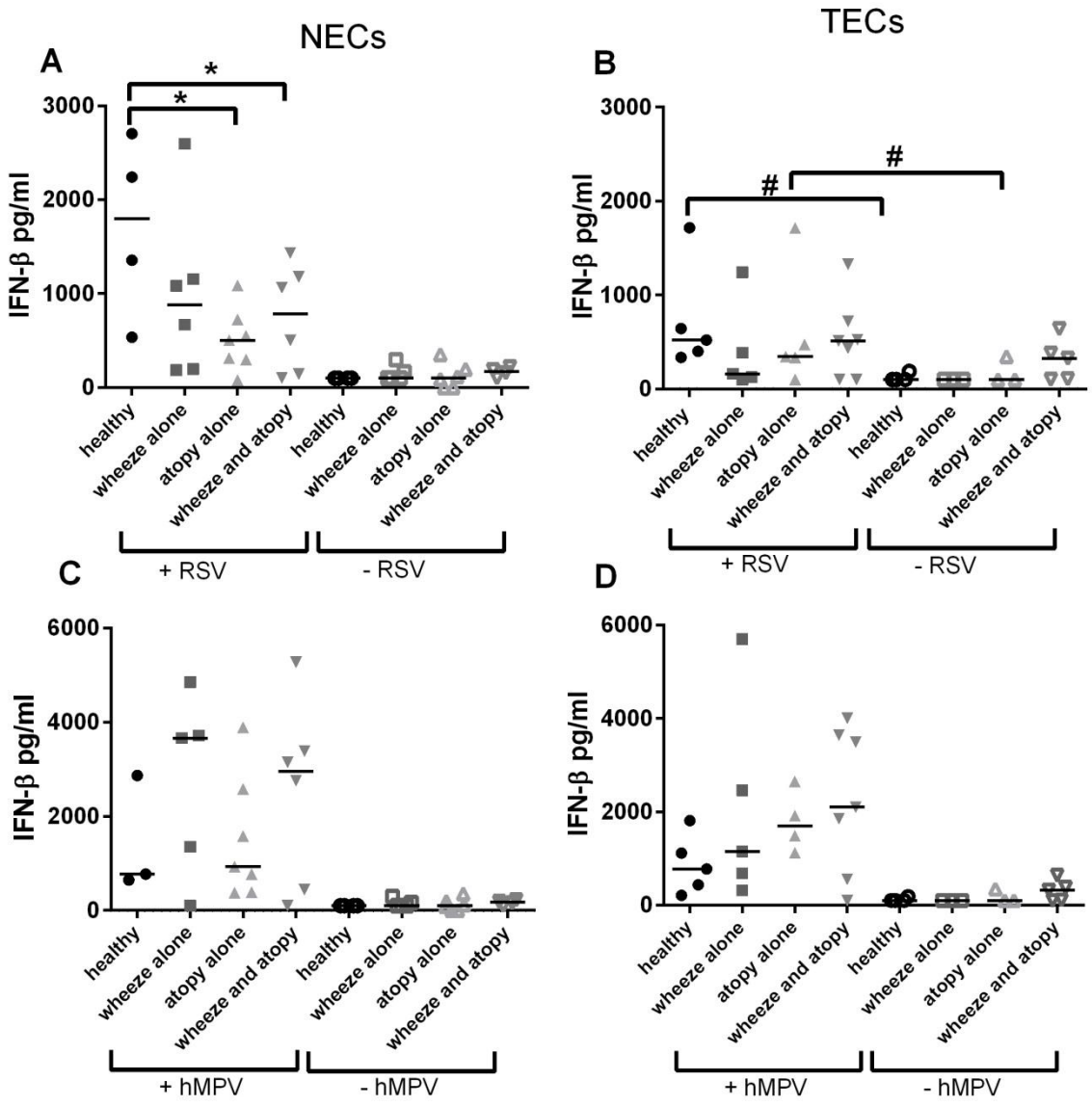


**Figure S1:** IFN-β secreted from nasal epithelial cells (NECs) (**A and C**) and tracheal epithelial cells (TECs) (**B and D**) on day 5, following infection with 0.1 pfu / cell RSV (**A and B**), or 0.1 $TCID_{50}$ units / cell hMPV (**C and D**), or uninfected. Comparison between groups was analysed using STATA software and tests for non-parametric data. median ± range, *$p < 0.05$. $^{\#}p < 0.01$ compared to uninfected control.

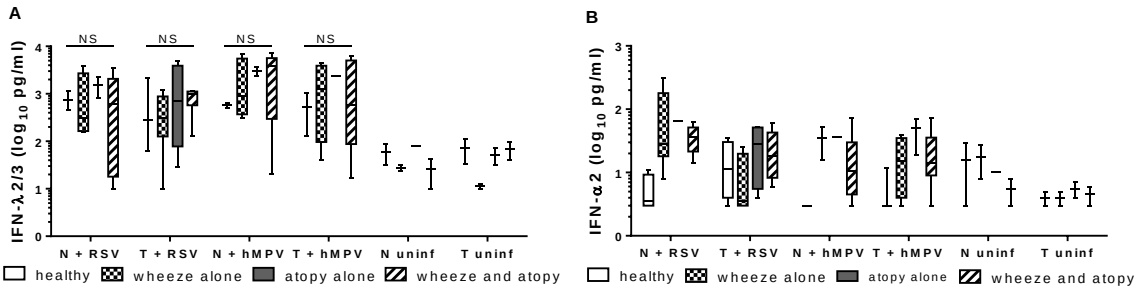


**Figure S2:** Secreted (**A**) IFN-λ2/3 and (**B**) IFN-α2 from nasal epithelial cells (NECs) and tracheal epithelial cells (TECs) on day 5 following infection with 0.1 pfu / cell RSV or 0.1 $TCID_{50}$ units / cell hMPV, or uninfected. Comparison between groups was analysed using STATA software and tests for non-parametric data. Bottom and top of box plots represent the $5^{th}$ and $95^{th}$ percentiles, bar represents the median and whiskers represent minimums and maximums.

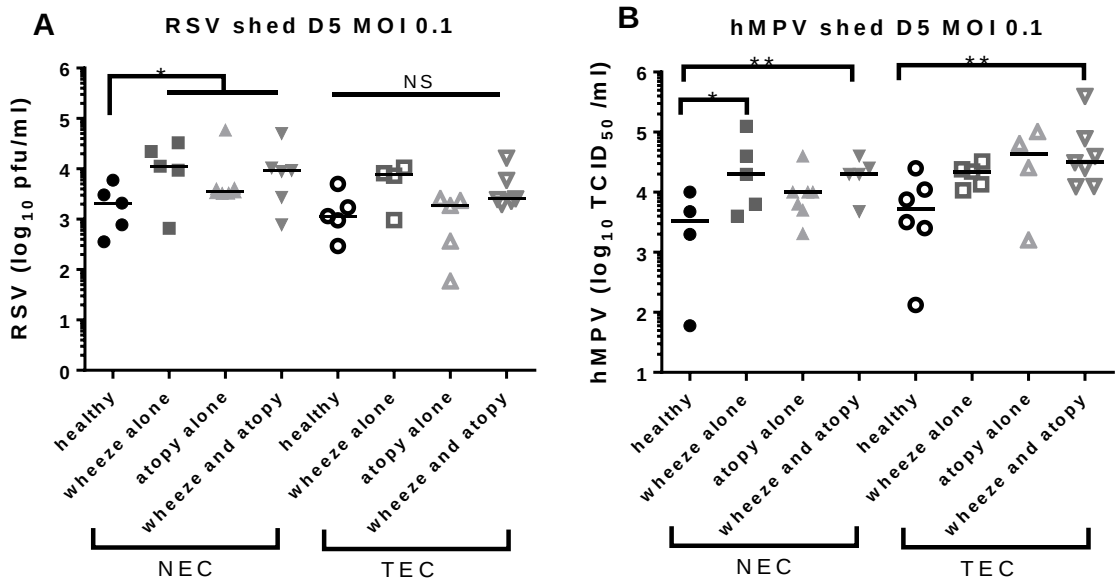


**Figure S3: (A**) RSV and **(B)** hMPV shed from nasal epithelial cells (NECs) and tracheal epithelial cells (TECs) on day 5 following infection with 0.1 pfu / cell RSV or 0.1 TCID50 units / cell hMPV, or uninfected. Comparison between groups was analysed using STATA software and tests for non-parametric data. median ± range, *$p < 0.05$, **$p < 0.01$.


**Figure S4:** RSV or hMPV N gene copy number was quantified by RT-qPCR using total RNA from nasal epithelial cells (NECs) and tracheal epithelial cells (TECs) infected at a high MOI of 3 pfu / cell RSV or 3 $TCID_{50}$ units / cell hMPV. Control plasmid for RSV and hMPV N gene and β-actin housekeeping gene were used for direct quantification of copy number. Comparison between groups was analysed using STATA software and tests for non-parametric data. Bottom and top of box plots represent the $5^{th}$ and $95^{th}$ percentiles, bar represents the median and whiskers represent minimums and maximums.

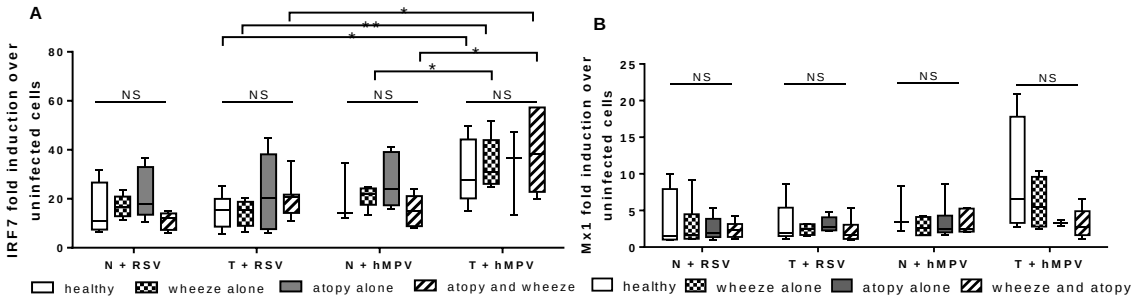


**Figure S5:** Fold induction of (**A**) IRF7 and (**B**) Mx1 genes was quantified by RT-qPCR for nasal epithelial cells (NECs) and tracheal epithelial cells (TECs) 24 h after infection with 3 pfu / cell RSV or 3 TCID50 units / cell hMPV, or uninfected. Fold induction was calculated using $2^{-\Delta\Delta Ct}$ normalized to the expression of β-actin gene. Comparison between groups was analysed using STATA software and tests for non-parametric data. Bottom and top of box plots represent the $5^{th}$ and $95^{th}$ percentiles, bar represents the median and whiskers represent minimums and maximums. *$p < 0.05$, **$p < 0.01$.

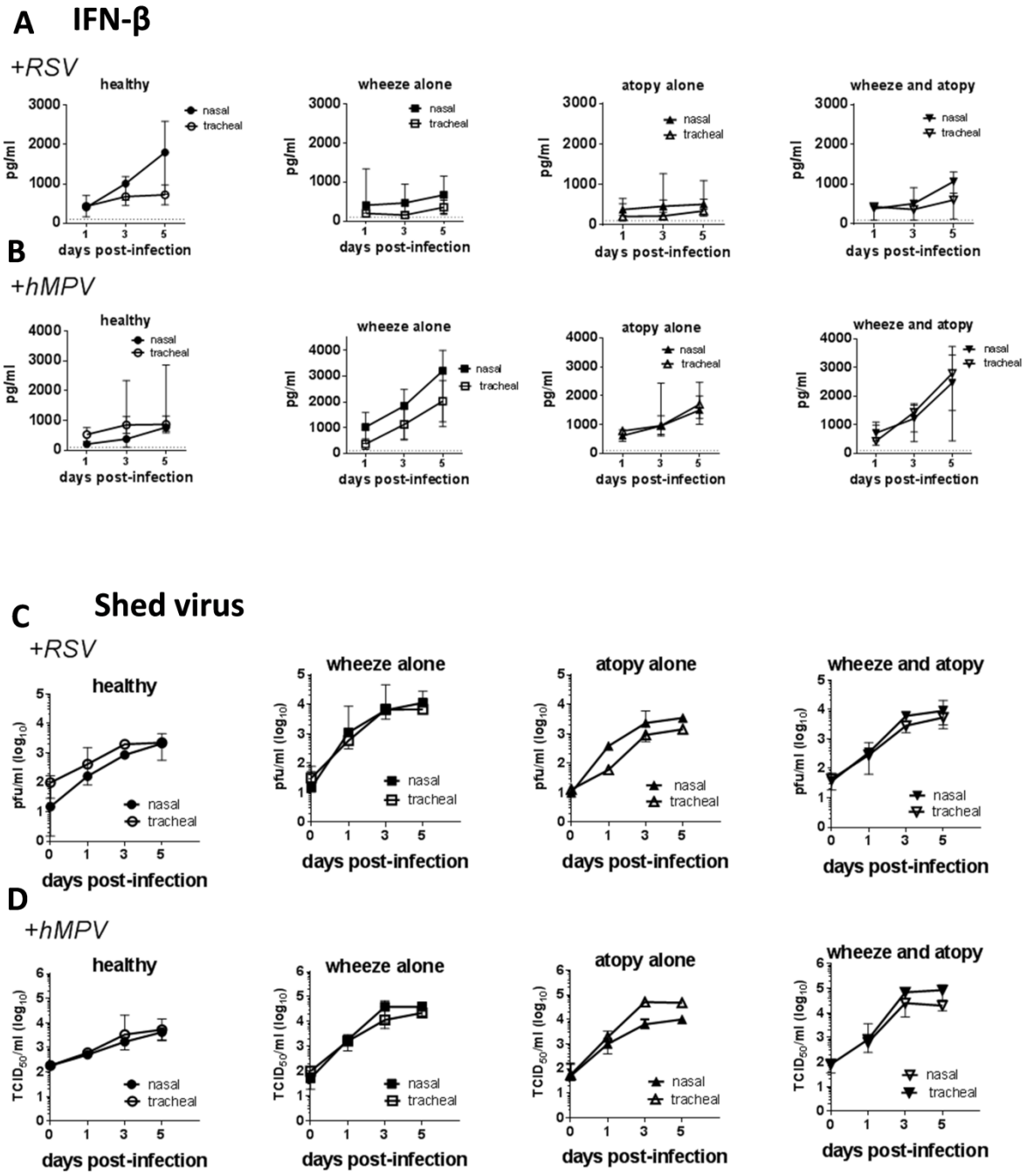


**Figure S6:** Data from figures 1 and 3, in which cells were infected with RSV (**A and C**) or hMPV (**B and D**) at a MOI of 0.1, or uninfected were used to compare the kinetics of IFN-β secretion (**A and B**) and shed virus (**C and D**) between NECs and TECs for each subject group. Median ± range.