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A sustained antiviral host response in respiratory syncytial virus infected human nasal epithelium does not prevent progeny virus production

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ABSTRACT

Respiratory syncytial virus infection was examined using a human nasal epithelial cell model. Maximum levels of shed-virus were produced at between 3 and 5 days post-infection (dpi), and the infectivity of the shed-virus was stable up to 10 dpi. The highest levels of interferon signalling were recorded at 2dpi, and infection induced a widespread antivirus response in the nasal epithelium, involving both infected cells and non-infected cells. Although these cellular responses were associated with reduced levels of progeny virus production and restricted virus spread, they did not inhibit the infectivity virus that is shed early in infection. In the clinical context these data suggest that although the host cell response in the nasal epithelium may restrict the levels of progeny virus particles produced, the stability of the shed-virus in the nasal mucosa may be an important factor in both disease progression and virus transmission.

1. Introduction

Respiratory syncytial virus (RSV) is the most important viral cause of lower respiratory tract infection in young children and neonates, leading to high levels of mortality and morbidity (Nair et al., 2010). In permissive cell lines the mature RSV particles assemble at the apical cell surface as cell-associated filamentous structures called virus filaments (Roberts et al., 1995), and these structures are capable of mediating virus transmission (Gower et al., 2005; Ravi et al., 2013). Virus filaments are also detected in epithelial cells obtained directly from patients with severe RSV infection, suggesting that these structures have a clinical relevance (Parham et al., 1993). The virus polymerase complex is formed by the nucleocapsid (N) protein, the phosphoprotein (P protein), the M2-1 protein and the large (L) protein, and it is packaged within the virus filaments. The virus filaments are surrounded by a protein layer formed by the matrix (M) protein, which forms beneath the virus lipid envelope in which the virus fusion (F) and attachment (G) glycoproteins are inserted.

The nasopharyngeal cavity is the primary route of RSV infection, and the ciliated epithelium of the upper respiratory airway is a major site of RSV replication (Henderson et al., 1978; Wright et al., 2005; Zhang et al., 2002). The CX3CR1 protein is the putative host cell receptor for RSV (Tripp et al., 2001), and its expression on the cilia of airway epithelial cells (Jeong et al., 2015; Chirkova et al., 2015; Johnson et al., 2015) provides a molecular basis for the selectivity of ciliated cells to support RSV replication. It is expected that efficient virus production in the nasopharyngeal cavity would normally precede infection in the lower airway where the severe disease symptoms are manifested. Although the nasal epithelium is the primary barrier to infection, it is still poorly understood how RSV interacts with the nasal epithelium during the initial phase of infection. An improved understanding of the interaction between RSV and the nasopharyngeal epithelium may lead to a better understanding of the disease progression following RSV infection. In this context, *in vitro* cell systems that are able to reproduce many of the features of the human nasal epithelium should lead to an improved understanding of this process in human infection, and may lead to the development of novel strategies to mitigate RSV transmission to the lower airway.

Several studies have described RSV infections that are performed using ciliated epithelial cells that were derived from the cells in the lower airway (e.g. (Villenave, 2012)). However, it is likely that ciliated cells derived from the upper and lower airway will respond differently

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to RSV infection (Yaghi and Dolovich, 2016). In this context, we have previously described an alternative human primary nasal epithelium 3-D cell culture system to examine RSV infection in the nasal epithelium (Jumat et al., 2015a). It is derived from human nasal epithelial stem/progenitor cells, and similar to nasal epithelium, it forms a properly stratified mucociliary airway epithelium containing ciliated columnar cells and goblet cells (Zhao et al., 2012). The apical surface is exposed to humidified air and it replicates many of the physiological properties of the nasal epithelium, e.g. mucus production and cilia beat function. Using the nasal epithelium (NE) cell model we have previously demonstrated that RSV infection leads to the formation of virus filaments. This process is accompanied by cilia dysfunction and cilia loss (Jumat et al., 2015a), which is a robust feature of RSV-infected ciliated epithelial cells (Jumat et al., 2015a; Smith et al., 2014; Mata et al., 2012). In our earlier analysis we had hypothesised that RSV infection may induce host cellular responses that may be a factor in promoting cilia dysfunction. It is expected that robust antiviral responses could also potentially limited or prevent virus infection. In this study we have extended our previous analysis by using the NE cell system to examine the host response to RSV infection, and to determine if this process can limit the spread of infection in the nasal epithelium.

2. Materials and methods

2.1. Infection of fully differentiated nasal epithelial cells with RSV

The RSV A2 strain was prepared in HEp-2 cells as described previously (Radhakrishnan et al., 2010), and the nasal epithelial cells were prepared from mucosa biopsies were cultured in Transwell™ inserts as described previously (Zhao et al., 2012). Infection of the fully differentiated NE cell model with RSV was performed as described previously (Jumat et al., 2015a). Briefly, the differentiated nasal epithelial cells grown on Transwell™ inserts were washed once with PBS at 33 °C and the cells were infected with RSV in the apical chamber using the desired multiplicity of infection (MOI) and incubated in a humidified chamber at 33 °C with 5% CO_2. Unless otherwise specified a MOI of 3 was used. After 2 h the virus inoculum was removed and washed twice using PBS and the incubation continued in a humidified chamber at 33 °C with 5% CO2. In mock-infected cells an identical volume of DMEM was used.

2.2. Measuring cell-associated and cell-free RSV infectivity

At each time of infection the cell-associated and released virus infectivity was measured as described previously (Huong et al., 2016). Briefly, the cell-free virus infectivity was recovered by adding 100 μL of pre-warmed DMEM to the apical side of the Transwell™ insert and after 10 min the medium was gently aspirated over the cell surface and the virus containing medium harvested. The cell-associated virus was recovered by scrapping the cells from the membrane support into 100 μL DMEM, and the virus infectivity released from the cells by two rounds of freeze-thaw cycles. Contaminating cellular material was removed from each virus preparation by centrifugation at 5000 g for 10 min at 4 °C, and the recovered viral infectivity in each fraction was assessed on HEp-2 cells by immuno-microplaque assay using anti-RSV MAb (Novacastra).

2.3. Antibodies and specific reagents

The following antibodies were purchased: anti-G (Abcam), anti-RSV (Novacastra Laboratories), anti-β4 Tubulin (Abcam), Apoptosis Antibody sampler kit (Cell Signalling Technology), anti-mouse and anti-rabbit IgG conjugated to Alexa 488 and Alexa 555 (Molecular Probes), anti-actin (Sigma Aldrich), the anti-pMAPKp38, anti-MAPKp38, anti-STAT1, anti-pSTAT1 (Transduction laboratories). The RSV N, P, and M2-1 antibodies have been described previously (Brown et al., 2004; McDonald et al., 2004). The anti-viperin was a gift from Peter Cresswell (Yale) or purchased from Transduction laboratories. The ARL13b antibody was a gift from Dr Lu Lei (NTU, Singapore).

2.4. Immunofluorescence microscopy

This was performed as described previously (Jumat et al., 2015a). Briefly, the cells in the Transwell™ insert were fixed using 4% (w/v) paraformaldehyde (PFA) in PBS at 25 °C for 20 min and washed using PBS at 4 °C. The membrane from each Transwell™ insert was excised and stained using the primary and secondary antibody combinations and visualized using a Nikon eclipse 80i fluorescence microscope (Nikon ECLIPSE TE2000-U) or Zeiss Axioplan 2 LSM510 confocal microscope as indicated.

2.5. Immunoblotting analysis

This was performed as described previously (Jumat et al., 2015a). Briefly, the cells on the Transwell™ support membrane were washed twice using sterile PBS (at 4 °C), and extracted directly into Boiling Mix (1%(w/v) SDS, 5% mercaptoethanol (v/v) in 20 mM Tris/HCL, pH 7.5). The extracted cells were immediately heated at 100 °C for 2 min and then clarified by centrifugation (13,000 × *g* for 2 min). The proteins were separated by SDS-PAGE and transferred by Western blotting onto nitrocellulose membranes. Protein bands were then probed with the relevant primary antibody and corresponding secondary antibody conjugated to HRP, and visualized using the ECL detection system (GE Healthcare). The immunoblotting data were further quantified by using ImageJ (ver IJ1.46r) to analyse the protein bands on autoradiograhs. Protein profiles were obtained or protein bands to be quantified were delineated, and the relative intensities determined and compared with the background intensity in control lanes.

2.6. Annexin V binding assay

This was performed according to the manufacturer's instructions (MACS, Miltenyi Biotech). Briefly, cells in Transwell™ inserts were washed using the annexin V binding buffer (ABB) and then annexin V-FITC in ABB was added to the apical side for 15 min at 18 °C. The cells were washed with ABB, and either imaged directly or PFA-fixed and then co-stained with relevant additional primary rabbit polyclonal antibody and anti-rabbit IgG conjugated to Alexa555. For anti-AL13b co-staining the cells were also permeabilised with 0.1% (v/v) triton X100 after PFA treatment. The stained cells were visualized using a Nikon eclipse 80i fluorescence microscope (Nikon ECLIPSE TE2000-U).

2.7. qPCR

The total RNA was extracted using the mirVanaTM miRNA isolation kit (Life Technologies) and subjected to cDNA synthesis using the Maxima first-strand cDNA synthesis kit (ThermoScientific). The primers for SYBR green-based qPCR were designed, pre-optimized and synthesized by Sigma Aldrich (STable 1). The qPCR reactions were set up in duplicates using GoTaq-qPCR Master Mix kit (Promega), and thermal cycling was performed with a Viia 7 PCR machine (Life Technologies). Relative gene expression was calculated using the comparative method 2-ΔΔCt, i.e. 2(ΔCt of gene - ΔCt of PGK1), and normalized against the housekeeping PGK1 mRNA level. All data were analysed using GraphPad Prism 6 software (San Diego, California, US) and SPSS software (SPSS Inc, Chicago, US). The group ANOVA *t*-test was performed to compare mRNA levels between the serial time points vs. the mock (0 dpi) infection control. Data was presented as fold change. P-values < 0.05 were considered significant.