CX3CR1 is an important surface molecule for respiratory syncytial virus infection in human airway epithelial cells

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Respiratory syncytial virus (RSV) is a major cause of severe pneumonia and bronchiolitis in infants and young children, and causes disease throughout life. Understanding the biology of infection, including virus binding to the cell surface, should help develop antiviral drugs or vaccines. The RSV F and G glycoproteins bind cell surface heparin sulfate proteoglycans (HSPGs) through heparin-binding domains. The G protein also has a CX3C chemokine motif which binds to the fractalkine receptor CX3CR1. G protein binding to CX3CR1 is not important for infection of immortalized cell lines, but reportedly is so for primary human airway epithelial cells (HAECs), the primary site for human infection. We studied the role of CX3CR1 in RSV infection with CX3CR1-transfected cell lines and HAECs with variable percentages of CX3CR1-expressing cells, and the effect of anti-CX3CR1 antibodies or a mutation in the RSV CX3C motif. Immortalized cells lacking HSPGs had low RSV binding and infection, which was increased markedly by CX3CR1 transfection. CX3CR1 was expressed primarily on ciliated cells, and ~50 % of RSV-infected cells in HAECs were CX3CR1+. HAECs with more CX3CR1-expressing cells had a proportional increase in RSV infection. Blocking G binding to CX3CR1 with anti-CX3CR1 antibody or a mutation in the CX3C motif significantly decreased RSV infection in HAECs. The kinetics of cytokine production suggested that the RSV/CX3CR1 interaction induced RANTES (regulated on activation normal T-cell expressed and secreted protein), IL-8 and fractalkine production, whilst it downregulated IL-15, IL1-RA and monocyte chemotactic protein-1. Thus, the RSV G protein/CX3CR1 interaction is likely important in infection and infection-induced responses of the airway epithelium, the primary site of human infection.

INTRODUCTION

Respiratory syncytial virus (RSV) is a major cause of severe pneumonia and bronchiolitis in infants and young children, and causes repeated respiratory infections throughout life (Falsey et al., 2005; Hall et al., 2009; Nair et al., 2010; Shay et al., 1999). The two RSV surface proteins, F and G, are key to the biology of infection, and are involved in RSV attachment and entry into cells, viral assembly, and induction of protective host immune responses. Both proteins bind to the cell surface through heparin-binding domains interacting with glycosaminoglycans (GAGs), particularly heparin sulfate proteoglycans (HSPGs) (Feldman et al., 1999, 2000). The F protein
initiates virus–cell fusion and reportedly also binds to cell surface nucleolin (Tayyari et al., 2011). The G protein is not required for replication (Karron et al., 1997), but facilitates infection, and its presence increases virus infectivity in vitro and in vivo (Heminway et al., 1994). RSV binding to the cell surface appears to be most efficient through the G protein (Techaarponkul et al., 2002), possibly because the G protein has higher affinity to GAGs, i.e. HSPG, than the F protein (Feldman et al., 2001). The G protein also has a fractalkine-like CX3C chemokine motif located at aa 182–186 and can bind to the fractalkine receptor CX3CR1 (Tripp et al., 2001).

CX3CR1, a G-coupled transmembrane chemokine receptor, previously identified as GPR13 or V28 (Combadiere et al., 1998b; Imai et al., 1997), is expressed in various cell types, including epithelial (Jamieson et al., 2008), microglia (Harrison et al., 1998), smooth muscle (Perros et al., 2007) and different immune cells, such as monocytes, dendritic, NK- and T-cells (Bar-On et al., 2010; Imai et al., 1997; Nishimura et al., 2002). CX3CR1 expression was also documented in human and mouse lung tissues (Combadiere et al., 1998a; Song et al., 2008; Suzuki et al., 2012), although in mouse lungs the majority of CX3CR1+ cells were found to be of leukocyte origin (Kim et al., 2011). In addition, it has been shown recently that G protein binding to CX3CR1 is an important contributor to RSV infection of human primary airway epithelial cells, unlike conventional cell lines such as HeLa or Vero cells, which have abundant GAGs on their surface, but very low or no expression of CX3CR1 (Johnson et al., 2013).

As airway epithelial cells are the primary site of RSV infection in humans, understanding the role of the G protein/CX3CR1 interaction in infection and host response in these cells is important to understanding human RSV disease pathogenesis. It has been shown that RSV infection of human airway epithelial cells (HAECs) induces strong cytokine/chemokine responses, including production of IL-1β, IL-6, IL-8, IP-10, macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein (MCP)-1 and RANTES (regulated on activation normal T-cell expressed and secreted) (Fonseca et al., 2012; Ioannidis et al., 2012; Olszewska-Pazdrak et al., 1998; Oshansky et al., 2010; Tristram et al., 1998; Villenave et al., 2011), and these cytokines and released viral proteins influence the host immune response to RSV (Chirkova et al., 2013; Ioannidis et al., 2012; McNamara et al., 2013; Qin et al., 2011). RSV G protein has been shown to impair innate and memory immune responses, such as suppressing production of IFN-β (Shingai et al., 2008), lymphoproliferation of T-cells (Ray et al., 2001), and responsiveness of monocytes/macrophages and dendritic cells (Johnson et al., 2012; Polack et al., 2005). Some of these effects on the immune response were found to be a consequence of RSV G protein interaction with CX3CR1, including suppressed T-cell trafficking and cytotoxicity in the lungs of RSV-infected mice (Harcourt et al., 2006), and enhanced pulmonary inflammation in RSV-challenged, formalin-inactivated RSV-vaccinated mice (Haynes et al., 2003). A decrease in respiratory rates in mice has also been linked to G binding to CX3CR1 (Tripp et al., 2003). In our previous work using an in vitro model of RSV infection, we showed RSV G protein/CX3CR1 interaction suppressed some human dendritic and memory T-cell responses (Chirkova et al., 2013).

In our present study, we explored the role of the G/CX3CR1 interaction in RSV infection of primary HAECs and demonstrated that unlike in immortalized cell lines, in HAECs this interaction is important for infection and cellular response to RSV.

RESULTS

RSV binding to CX3CR1 is HSPG-independent, and increases virus internalization and infectivity

To study the role of CX3CR1 in HSPG-independent RSV binding and internalization, HSPG-expressing (CHO-K1 and BEAS-2b) and HSPG-deficient CHO-K1ΔHSPG (pgsD-677) cell lines were transfected with human CX3CR1 (Fig. 1a). About 25–35 % of cells transfected and expressed moderate- to high-density surface CX3CR1. Endogenous CX3CR1 expression was detected in 1.2–4.8 % of mock-transfected cells.

A RSV binding assay showed that, as had been reported previously (Martínez & Melero, 2000), lack of HSPG on the cell surface significantly decreased RSV binding (Fig. 1b). Transfection with human CX3CR1 substantially increased the percentage of CHO-K1ΔHSPG cells with bound virus, from 1.7 % in mock-transfected to 11 % in CX3CR1-transfected cells. To confirm the HSPG-independent RSV binding to CX3CR1 in HAECs, CX3CR1-transfected BEAS-2b cells were treated with heparinase I before the assay (Fig. 1c). Treatment with heparinase I has been shown to be the most efficient way to cleave heparin sulfate chains from cell surface HSPG (Martínez & Melero, 2000). In these experiments, in addition to RSV A2, we included two relatively low-passage group A strains and two group B strains. For all five strains, heparinase I dramatically decreased RSV binding to mock-transfected BEAS-2b. CX3CR1 transfection significantly increased RSV binding to heparinase-treated cells. The strain-specific increase of RSV binding with CX3CR1-transfected cells correlated with binding in untreated cells, with 37–58 % of binding activity being restored.

A virus internalization assay was performed with carboxyfluorescein diacetate succinimidyl ester (CFDASE)-labelled RSV. Upon internalization, the intracellular esteras cleave diacetate groups in CFDASE, thus converting it to the fluorescent carboxyfluorescein succinimidyl ester (CFSE) form (Drobní et al., 2003). Cells exposed to RSV-CFDASE at 4 °C (Fig. 1d, dotted lines) exhibited low fluorescence (similar to levels at 0 h), whilst cells exposed to the virus at 37 °C had a progressive increase in percentage of CFSE-positive cells from 0 to 4 h post-infection (p.i.)
CX3CR1 facilitates RSV infection in HAECs

Fig. 1. Association of RSV binding and internalization with presence of HSPG and CX3CR1 on the cell surface. HSPG-expressing cells (CHO-K1 and BEAS-2b) and HSPG-deficient cells (CHO-K1ΔHSPG or BEAS-2b cells pre-treated with heparinase I) transfected or mock-transfected with human CX3CR1 were exposed to RSV. (a) Percentage of CX3CR1-expressing cells after CX3CR1 or mock transfection. CHO-K1 and CHO-K1ΔHSPG FACS plot: grey area, CHO-K1; dotted line, untreated CHO-K1ΔHSPG; dashed line, vector-transfected CHO-K1ΔHSPG; solid line, CX3CR1-transfected CHO-K1ΔHSPG. BEAS-2b FACS plot: grey area, untreated; dotted line, vector-transfected; solid line, CX3CR1-transfected cells. (b) Percentage of cells with bound RSV after exposure to virus at m.o.i. 5. *P<0.05 versus pgsD-677; unpaired t-test. (c) Percentage of cells with bound RSV in BEAS-2b cells transfected with human CX3CR1 (hCX3CR1-tr), treated with heparinase I and exposed to different RSV strains (exposure dose equal to 15 μg RSV G protein, m.o.i. 0.05–4.5). *P<0.05 versus heparinase-I-treated cells; unpaired t-test. (d) Cells were exposed to CFDASE-labelled RSV for different times at 37 (solid lines) or 4 °C (dotted line) as a negative control. The percentage of CFSE⁺ cells (cells with internalized viral particles) was measured by flow cytometry. CHO-K1, ●; CX3CR1-transfected CHO-K1, ○; CHO-K1ΔHSPG, △; CX3CR1-transfected CHO-K1ΔHSPG, Δ. *P<0.05 versus 4 °C exposure, ΔP<0.05 versus untransfected cells.
Transfection with human CX3CR1 significantly increased internalization of RSV in CHO-K1ΔHSPG cells.

**Characterization of primary human airway epithelial cultures with differential percentage of CX3CR1⁺ cells**

Seven different donor human airway epithelial cultures, two cystic fibrosis (CF) and five non-CF, were tested in the present study. To account for possible variations in the distribution of major cell types (i.e. ciliated, goblet and basal cells) and surface molecules that might affect RSV infection of HAECs, we determined the percentage of ciliated cells (β-tubulin IV⁺ or FoxJ1⁺), secretory cells (mucin-5AC⁺ or positive by Jacalin lectin binding) and basal cells (CD151⁺), and expression of HSPG (syndecan-1) and CX3CR1.

We found HAECs from different donors had similar distributions of the major airway epithelial cell subtypes. The majority of both CF and non-CF HAECs (~74–85%) had characteristics of ciliated cells, i.e. expressed both β-tubulin IV, a constituent of motile cilia (Jensen-Smith et al., 2003; Million et al., 1999), and FoxJ1, a forkhead box (f-box) transcription factor required for ciliogenesis and cilia assembly (Blatt et al., 1999; You et al., 2004). It has been noted previously that ciliated cells are the major target for RSV infection (Zhang et al., 2002). About 13–27% of HAECs had a secretory phenotype based on binding of Jacalin, a plant lectin which binds to O-glycosylated proteins such as mucins (Tachibana et al., 2006). Some of these secretory cells (9–18%) also stained positive for mucin-5AC. A very small percentage of cells (0.15–2.5%) were positive for CD151, a tetraspanin specifically expressed on keratinocytes, including airway basal cells (Sincock et al., 1997). Thus, CF and non-CF HAEC cultures were composed mainly of fully differentiated airway epithelial cells of a ciliated or secretory phenotype.

HAECs from different donors also had similar levels of surface HSPG expression as indicated by levels of syndecan-1 (Fig. 2a), a transmembrane HSPG family member and a major source of heparin sulfates on the cell surface (Bernfield et al., 1999; Park et al., 2000). The percentage of CX3CR1⁺ cells was, however, different amongst donors. CX3CR1 was predominantly detected on the surface of ciliated cells (FoxJ1⁺ subset; Fig. 2c), and the percentage of CX3CR1⁺ cells varied significantly between donors (Fig. 2b; two representative donors). We divided HAEC donors into two
groups, i.e. CX3CR1\textsuperscript{high} (two CF and three non-CF donors with the percentage of CX3CR1\textsuperscript{+} cells ranging from 10 to 26 \%) and CX3CR1\textsuperscript{low} (two non-CF donors with 2–7 \% CX3CR1-expressing cells). With similar levels of surface HSPG and different percentages of CX3CR1\textsuperscript{+} cells, CX3CR1\textsuperscript{high} and CX3CR1\textsuperscript{low} HAECs provide an opportunity to study the effect of CX3CR1 on RSV infection in HAECs.

**Percentage of CX3CR1 cells correlates with RSV infection in HAECs**

The level of RSV infection in HAECs increased in a dose-dependent manner (Fig. 3a) without obvious signs of cytopathology (data not shown). RSV was only detected in ciliated cells and 62–76 \% of RSV\textsuperscript{+} cells were also CX3CR1\textsuperscript{+} (Fig. 3c). Imaging flow cytometry showed localization of CX3CR1 and RSV antigens on the apical surface of the ciliated cells, i.e. the cell surface with cilia. RSV antigens have previously been noted to be located close to the apical surface in HAECs (Zhang et al., 2002). These data correlated with the finding that infectious progeny virus (Fig. 3b) and viral RNA (not shown) were detected only in the apical washes of infected HAECs.

CX3CR1\textsuperscript{high} HAECs had a higher percentage of RSV-infected cells (Fig. 4a), a higher relative amount of viral RNA (data not shown) and more cell death than CX3CR1\textsuperscript{low} HAECs (Fig. 4b). However, the percentages of RSV\textsuperscript{+} cells within the subset of CX3CR1\textsuperscript{+} cells were similar between both HAEC cultures (25–30 \%; Fig. 4a). Overall, in all seven tested HAEC donors, the percentages of RSV-infected cells correlated with percentages of CX3CR1-expressing cells with a correlation coefficient of 0.76 (Fig. 4c).

**Alteration of the RSV G protein CX3C motif decreases virus binding and replication**

To confirm the role of the RSV G protein CX3C motif in binding and infection of HAECs, we assessed the effects of the virus with a mutation in the CX3C motif (RSV CX4C) and anti-CX3CR1 antibody on binding and infection.

In the RSV attachment assay, anti-syndecan-1 antibody treatment showed the greatest decrease in RSV binding to HAECs (Fig. 5). Anti-CX3CR1 antibody treatment also decreased RSV binding, although less than anti-syndecan-1 treatment. CX4C showed impaired attachment to HAECs and treatment with anti-CX3CR1 antibody did not significantly change the binding of CX4C. Note that neither blocking with anti-CX3CR1 nor anti-syndecan-1 antibody eliminated all binding. These findings suggested that both HSPG and CX3CR1 contributed to RSV attachment to HAECs, but may not have been the only binding molecules for the virus.

Analysis of the kinetics of RSV infection in CX3CR1\textsuperscript{high} HAECs at day 2–8 p.i. showed significantly less replication with the CX4C compared with the CX3C virus (Fig. 6a).

We found that most of the difference in CX3C and CX4C replication occurred in the CX3CR1\textsuperscript{+} but not the CX3CR1\textsuperscript{–} population (Fig. 6a, compare panels 2 and 3). The percentage of CX3CR1\textsuperscript{+} cells remained stable through days 2–6 and decreased at day 8 p.i., but the difference was not statistically significant. Compared with mock and CX4C samples, cells infected with CX3C virus had a slight increase in the percentage of CX3CR1\textsuperscript{+} cells on day 4 and 6 p.i., but the difference was not statistically significant (data not shown). In addition, the percentage of RSV\textsuperscript{+} cells in CX3CR1\textsuperscript{–} cells was similar for the two viruses at days 2, 6 or 8 p.i. The correlation coefficient between RSV and CX3CR1\textsuperscript{+} cells was 0.20 for the CX4C virus and 0.76 for the CX3C virus. The percentage of RSV\textsuperscript{+} cells for the CX4C virus did not differ significantly for CX3CR1\textsuperscript{high} and CX3CR1\textsuperscript{low} HAEC cultures (Fig. 6b). In addition, the percentage of RSV\textsuperscript{+} cells in CX3C-infected but not CX4C-infected cultures was significantly decreased at day 4 p.i., by blocking CX3CR1 with a polyclonal anti-CX3CR1 antibody (Fig. 6c).

**RSV G protein CX3C motif affects cytokine responses in HAECs**

CX3CR1 interaction with its ligand fractalkine induces downstream signalling leading to NF\kappa B activation followed by production of fractalkine itself and some other pro-inflammatory cytokines (Chandrasekar et al., 2003). As the RSV G protein CX3C motif has structural similarity to the receptor-binding site of fractalkine and has been shown to mimic other fractalkine functions (Tripp et al., 2001), we also looked at the effect of G protein binding to CX3CR1 on the production of certain cytokines in airway epithelial cells.

RSV infection induced multiple cytokines and chemokines in HAECs (Table 1). Consistent with their inhibition by the viral NS1 and NS2 proteins, neither CX3C or CX4C RSV induced IFN-\alpha production in the HAECs. In contrast, both viruses induced significant production of IFN-\lambda1 (IL-29) and IFN-\lambda2 (IL-28x). Overall, production of cytokines was higher for CX3C than CX4C RSV. Some cytokines [e.g. IL-8, MIG (monokine induced by IFN-\gamma), fractalkine] were released only during CX3C virus infection, suggesting that RSV/CX3CR1 interaction induced their production. This conclusion was supported by a significant decrease of the production of these cytokines after anti-CX3CR1 antibody treatment of HAECs before CX3C RSV infection (Table 1). For other cytokines and chemokines, the higher levels in CX3C-infected cultures appeared to be related to greater replication of CX3C than CX4C RSV in the HAECs (Fig. 6a). To account for the potential effect of virus replication on differences in cytokine production, we calculated the ratio (\text{R}_{CV}) between the rate of increase in cytokine production and rate of increase in virus replication over time (see Methods). The \text{R}_{CV} values for CX3C and CX4C viruses (Fig. 7) suggested that RANTES was likely induced more effectively by
the CX3C virus (i.e. higher $R_{CV}$ value for CX3C than CX4C virus), whilst IL-1RA, IL-15 and MCP-1 were likely more effectively induced by CX4C RSV (higher $R_{CV}$ values for CX4C).

**DISCUSSION**

Our data show that in vitro the RSV G protein interaction with CX3CR1 on the cell surface of HAECs is more...
important to the pathogenesis of human RSV than anticipated from its role in immortalized cell lines used to study RSV infection. The RSV G protein has been shown to bind to cells through HSPGs in epithelial cell lines as well as to bind to CX3CR1 in CX3CR1-transfected cell lines (Feldman et al., 1999; Tripp et al., 2001). In in vivo studies in animal models, the G protein/CX3CR1 interaction has been shown to affect a number of host immune responses.

**Fig. 4.** Levels of CX3CR1+ cells correlate with RSV infection in HAECs. CX3CR1high (two CF and three non-CF) and CX3CR1low (two non-CF) HAECs were infected with RSV at m.o.i. 2 (grey bars) or mock-infected (white bars) and harvested from inserts on day 8 p.i. (a) Percentage of RSV-infected cells within total cell population (left panel) and CX3CR1+ subset (right panel). (b) Mean fluorescent intensity (MFI) of Fixable Viability Dye for three HAEC donors: one CF, one non-CF (CX3CR1high) and one non-CF (CX3CR1low). (c) Correlation between percentages of CX3CR1+ cells and RSV infection. Data represent individual insert measurements of HAECs (data from two to four inserts per donor).
to infection (Harcourt et al., 2006; Haynes et al., 2003), likely through immune cells expressing high levels of CX3CR1 (Imai et al., 1997). The importance of G protein/CX3CR1 interactions has, however, not yet been determined for human RSV infection. Recently, it was noted that human primary airway epithelial cells express CX3CR1 and that G protein binding to CX3CR1 increases RSV replication in these cells (Johnson et al., 2013). Our data support and expand these observations. We show that G protein/CX3CR1 interaction contributes to 50% of RSV infectivity in HAECs, whilst other molecules (including HSPGs) contribute to the remaining RSV infectivity in these cells. This conclusion is based on the correlation between levels of CX3CR1 cells and RSV infectivity, and the marked decrease in RSV infection of HAECs when RSV/CX3CR1 interaction is blocked by a mutated CX3C motif or by anti-CX3CR1 antibody. These findings are consistent with CX3CR1 being one of several RSV receptors on HAECs.

Cell surface HSPGs are defined as syndecans or glypicans based on their protein core (Bernfield et al., 1999; Park et al., 2000). HSPGs contain heparin sulfate chains to which RSV binds (Hallak et al., 2000). Amongst the several HSPGs, syndecan-1 is the one primarily expressed on epithelial cells, including immortalized HAEC lines such as A549 (Hayashida et al., 2009; Pruessmeyer et al., 2010) and BEAS-2b (Chen et al., 2009), and has been shown to support RSV infection (Park et al., 2000). This suggested to us that syndecan-1 is likely involved in the binding and infection of RSV in airway epithelial cells. Although some authors have reported the lack of HSPGs on the surface of respiratory epithelium (Duan et al., 1998; Keswani et al., 2012), others have noted the presence of syndecan-1 on human normal bronchial epithelial cells (Zhang et al., 2011). Zhang et al. (2011) noted that only well-differentiated HAECs exhibited abundant expression of syndecan-1. We detected expression of syndecan-1 on HAECs, which was associated with RSV binding (Figs 2 and 5). It has been shown also that airway epithelial cells shed syndecan-1 upon injury and bacterial infection (Chen et al., 2009; Li et al., 2002; Park et al., 2001; Popova et al., 2006). It is possible that the contribution that CX3CR1 plays in RSV infection of HAECs is even greater with bacterial co-infection. We noticed that the percentage of CX3CR1 cells varied amongst HAEC donors. This difference could be genetically determined, or result from a chronic pulmonary inflammation and lower levels of oxygenation known to affect expression of CX3CR1 in lung tissues (Hasegawa et al., 2005; McComb et al., 2008; 2009).

Fig. 5. Assessment of RSV binding to the HAECs. CX3CR1 high HAECs were exposed to RSV at m.o.i. 10 for 1 h at 4 °C. Some HAECs were pre-treated with anti-CX3CR1 or anti-syndecan-1 antibody before incubation with RSV. (a) RSV-specific ELISA. HEp-2 cells grown on membrane inserts and exposed to CX3C virus (black bar, HEp-2) were used as a positive control for RSV binding. *P<0.05; Mann–Whitney test. (b) Percentage of RSV+ cells measured by flow cytometry.
Fig. 6. Altering the CX3C motif in the RSV G protein decreases RSV replication in HAECs. CX3CR1<sup>high</sup> and CX3CR1<sup>low</sup> HAECs were infected with RSV with an intact CX3C motif (CX3C) or mutated motif (CX4C) and maintained up to day 8 p.i. (a) Percentage of RSV-infected cells in CX3CR1<sup>high</sup> HAECs on days 2–8 p.i., measured within total cells, and CX3CR1<sup>+</sup> and CX3CR1<sup>–</sup> subsets. *P < 0.05 versus Mock, ΔP < 0.05 versus CX4C, unpaired t-test, two-way ANOVA. (b) Percentage of RSV-infected cells and relative amount of viral RNA [real-time PCR, inverse cycle threshold (C<sub>t</sub>) values]. *P < 0.05 versus CX4C, ΔP < 0.05 versus CX3CR1<sup>low</sup> HAECs, unpaired t-test. Data from six HAEC donors: one CF, three non-CF CX3CR1<sup>high</sup> and two non-CF CX3CR1<sup>low</sup>. (c) Percentage of RSV-infected cells in CX3CR1<sup>high</sup> HAECs pre-treated with anti-CX3CR1 antibody. *P < 0.05 versus CX4C, ΔP < 0.05 versus CX3C pre-treated with anti-CX3CR1 antibody; Mann–Whitney test. Data from one CF and three non-CF HAECs.
Table 1. Cytokine and chemokine responses to RSV infection in HAECs

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration at day 4 p.i. (pg ml⁻¹)</th>
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<tr>
<td></td>
<td>Mock</td>
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<tr>
<td>IL-1RA</td>
<td>44 ± 5</td>
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<tr>
<td>IL-6</td>
<td>46 ± 3</td>
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<tr>
<td>IL-8</td>
<td>4859 ± 293</td>
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<tr>
<td>IL-15</td>
<td>12 ± 2</td>
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<tr>
<td>Fractalkine</td>
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<tr>
<td>IP-10</td>
<td>11 ± 2</td>
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<tr>
<td>MIG</td>
<td>7 ± 2</td>
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<tr>
<td>MIP-1β</td>
<td>14 ± 2</td>
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<tr>
<td>MCP-1</td>
<td>120 ± 20</td>
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<tr>
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<tr>
<td>IFN-α</td>
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<tr>
<td>IFN-λ1</td>
<td>5 ± 1</td>
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<tr>
<td>IFN-λ2</td>
<td>59 ± 3</td>
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*P<0.05 versus mock.
†P<0.05 versus CX3C; one-way ANOVA, Bonferroni’s multiple comparison test.

Perros et al., 2007; Rimaniol et al., 2003; Song et al., 2008) or other factors.

Our data also suggest that the G protein/CX3CR1 interaction, similar to the fractalkine/CX3CR1 interaction, affects the cellular inflammatory response to infection. Chandrasekar et al. (2003) showed that CX3CR1, being a G-coupled transmembrane chemokine receptor, conducts the signal through a cascade of kinases, including phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, Akt and IκB kinase, that leads to NFκB activation and fractalkine production (Chandrasekar et al., 2003). Interestingly, the fractalkine/CX3CR1 interaction has been linked to both pro- and anti-inflammatory effects depending on the cell type. For example, stimulation of CX3CR1 induced production of IL-1β and TNF-α in microglia cells and neurons (Johnston et al., 2004; Zhang et al., 2012), whilst loss of CX3CR1 intensified production of TNF-α, MCP-1, MIP-1α and RANTES in mouse liver macrophages (Aoyama et al., 2010). Our results showed that only WT RSV, but not CX4C virus, induced production of fractalkine, suggesting that RSV G protein works as a ligand-agonist for the epithelial CX3CR1 in HAECs (Table 1). The higher replication of CX3C in HAECs confounds the interpretation of differences in cytokine production compared with the CX4C virus. We addressed this concern by relating the rate of cytokine increase to the rate of virus replication (Fig. 7). This analysis showed qualitative differences likely related to the G protein/CX3CR1 interaction and not just virus replication; this difference merits further study. Our observations are consistent with Oshansky et al. (2010), who reported that purified RSV G protein elicited production of IL-1α and RANTES, and virus with a deleted G gene had lower IL-8 and higher IP-10 and MCP-1 levels in HAECs. Elevation levels of IL-1RA, IL-8, MCP-1, MIPs, IP-10 and RANTES have also been reported in nasopharyngeal aspirates of infants with RSV bronchiolitis, and higher levels correlated with the severity of disease (Bermejo-Martin et al., 2007; Welliver et al., 2002). Unlike our previous studies with A549 cells in which the CX3C virus produced significantly less IFN type III than the CX4C RSV (Chirkova et al., 2013), production of IFN-λ in these HAECs seemed to be independent of the G protein CX3C/CX3CR1 interaction.

![Fig. 7. Cytokine responses in RSV-infected HAECs. CX3CR1<sup>high</sup> HAECs were infected with RSV with an intact CX3C motif (CX3C) or mutated motif (CX4C). Graph represents ratios (RC<sub>V</sub>) between cytokine production curve slope and viral replication (amount of viral RNA) curve slope. The slopes were calculated for the day 2–4 p.i. segment when both cytokine production and viral replication represented stable exponential growth. *P<0.05 versus CX4C; unpaired t-test and two-way ANOVA.](image-url)
As with infection of cells, the role of CX3C in type III IFN production may vary with cell type.

In conclusion, RSV G protein interaction with CX3CR1, expressed on airway ciliated cells, facilitates virus attachment and infection of HAECs, and also modulates cell responses to infection. These findings, in conjunction with previous reports, suggest that CX3CR1 is one of several receptors for RSV, and along with GAGs and other molecules, such as nucleolin, provides RSV with multiple ways of attachment and infection of respiratory epithelium. As airway epithelial cells are an important site for human RSV replication, the RSV G protein/CX3CR1 interaction is likely an important contributor to human RSV infection and disease.

METHODS

Cell cultures and transfection. Primary human bronchial epithelial cells from CF and normal patients were purchased from ChanTest and Lonza Group or kindly provided by Dr C. U. Cotton (Case Western Reserve University, Cleveland, OH, USA), and cultured as described previously (Chen et al., 2008; Ziady et al., 2012). Briefly, cells were grown on Costar Transwell or Snapwell inserts (Corning) until confluent, then transferred in the air/liquid interface where cells were grown on Costar Transwell or Snapwell inserts (Corning) for surface markers, cells were stained with fluorochrome-labelled anti-CD138; BioLegend). Membranes were cut out of inserts, washed with PBS containing 0.5 % BSA, fixed with BD FACS Lysing Star). Positive signal and percentage of cells expressing markers were performed on ice. Flow cytometry analysis was performed using a PerCP-Cy5.5 anti-mouse IgG (H+L) antibody (Invitrogen) or donkey anti-goat IgG (H+L) antibody (Abcam). All washes and incubations were performed on ice. Flow cytometry analysis was performed using a four-laser BD LSR-II (BD Biosciences) and FlowJo software (Tree Star). Positive signal and percentage of cells expressing markers were assessed against cells stained with isotype control antibody. For the Jacalin-binding test, HEP-2 cells stained with FITC-labelled Jacalin were used as a negative control.

Expression of syndecan-1 on HAECs was determined by fluorescence microscopy. Without disrupting the cell sheet, cells were thoroughly washed with PBS containing 0.5 % BSA, fixed with BD FACS Lysing Solution, washed with PBS/0.5 % BSA, blocked with PBS containing 5 % BSA and stained with anti-syndecan-1 antibody (phycoerythrin-labelled anti-CD138; BioLegend). Membranes were cut out of inserts, placed on microscope slides, mounted with DAPI-containing Fluoromount-G (eBioscience) and analysed using an EVOS FL Cell Imaging System (Life Technologies).

Viruses, binding, internalization and infection. RSV strains group A (A/) and group B (B/) included; A/A2 and B/WV/14617/85 (acquired through ATCC), strain B/8/60 (kindly supplied by Dr U. Power, Queen’s University, Belfast, UK), and clinical strains A/1997/12-35 (passage 12) and A/TN/13/2-26 (passage 4) (isolated from children seen at Vanderbilt University, Nashville, TN, USA). The amount of G protein in these virus stocks was quantified by ELISA (see below). Two RSV A2-derived strains, WT and CX3C mutant, were generated with a reverse genetics system as described previously (Mitra et al., 2012). The WT strain (designated CX3C) has an intact CX3C motif (182CWAIC186) in the G protein and is parent to a mutant strain (designated CX4C) which has an alanine A186 insertion in the CX3C motif.

Viruses were grown in HEP-2 cells, purified by pelleting through 20 % sucrose cushion at 16 000 g for 2 h and stored at −80 °C. For the RSV internalization studies, virus was labelled with CFSEDAse (eBioscience) as described previously (Drobnik et al., 2003) by incubating for 20 min at room temperature with CFSEDAse at a final concentration of 5 μM; labelled virus was purified through a 20 % sucrose cushion and stored at −80 °C.

For the RSV-binding assay, CHO-K1, pgD-677 and BEAS-2b cells were harvested from plates and exposed to virus in suspension at 4 °C for 1 h. For heparinase I pre-treatment, cells were resuspended in heparinase I solution (1 U ml−1) (Sigma-Aldrich) and incubated for 1 h at room temperature before addition of RSV. For the RSV-binding assay with HAECs, cells were either left on plate/inserts or treated with trypsin/EDTA and harvested in suspension, untreated or pre-treated with CX3CR1 or syndecan-1-blocking antibody and exposed to RSV at 4 °C for 1 h. The cells were then washed extensively with cold PBS and virus binding was assessed: for cells left on the plate/inserts, by RSV-specific ELISA after fixation with BD FACS Lysing Solution (BD Biosciences); for cells in suspension, by flow cytometry (see below).

For the RSV entry assay, cells were inoculated with CFSEDAse-labelled RSV at m.o.i. 0.5 and incubated for up to 4 h at 37 °C. After incubation, the cells were harvested from plates with trypsin/EDTA and the fluorescence of intracellular CFSE was measured by flow cytometry. Cells exposed to CFSEDAse-labelled RSV at 4 °C were used as negative control for viral internalization.

For RSV infection, cells were inoculated with different RSV strains at the indicated m.o.i. determined by virus titration in HEP-2 cells; m.o.i. 2.0 was used to compare RSV strains and anti-CX3CR1 antibody treatment. HAECs were washed with PBS and incubated for 2 h at 37 °C with viral inoculum diluted in PBS, or plain PBS as a mock infection, added to the apical surface of the cells. After the incubation, the virus inoculum was aspirated, the apical surface was washed with PBS and the basolateral media was changed. RSV-infected HAECs were incubated for up to 8 days at 37 °C and 5 % CO2. For the experiments with anti-CX3CR1 or anti-syndecan-1 antibody treatment, cells were incubated for 2 h with polyclonal anti-human CX3CR1 antibody (100 μg ml−1) (eBioscience) or anti-syndecan-1 (20 μg ml−1) (BioLegend) antibody. Before using the antibody, sodium azide was removed from the solution by buffer exchange through Zeba Spin Desalting Columns (Fisher Scientific).

RSV titres in HAEC apical washes and bottom supernatants. For apical wash collection, 100 μl PBS was added to the apical surface of cell cultures, incubated for 15 min at 37 °C, and then collected and stored at −80 °C. The TCID50 was determined in a microtitre infectivity assay using an ELISA to detect virus replication as described previously (Anderson et al., 1985). Briefly, HEP-2 cells were grown in...
a 96-well microtitre plate, infected with 10-fold serial dilutions of the HAEC apical washes, and incubated at 37 °C and 5 % CO₂ for 5 days. After incubation, plates were washed with PBS, fixed with 70 % ethanol, dried and stored at 4 °C until testing by the RSV-specific ELISA.

**RSV antigen ELISA.** For the ELISA, fixed cells were washed with PBS containing 0.05 % Tween-20 (PBS/Tw), blocked with PBS/Tw containing 5 % BSA, incubated for 2 h at 37 °C with goat anti-RSV antibody (Millipore), and for 1 h at 37 °C with HRP-conjugated donkey anti-goat antibody (Jackson ImmunoResearch), and developed using o-phenylenediamine (OPD) substrate. A₄₉₀/₆₄₀ was determined. For RSV G-protein-specific ELISA, plates were coated with RSV G protein (BEI Resources) at serial dilutions and virus stocks at 1:100 dilution in carbonate/bicarbonate buffer at 4 °C overnight, and then plates were blocked with PBS/Tw containing 5 %BSA, incubated for 1 h at 37 °C with human anti-G antibody (Trellis Bioscience), and with HRP-conjugated goat anti-human antibody (Jackson ImmunoResearch), and developed with OPD.

**Flow cytometry of RSV-infected cells.** Cells harvested from plate/inserts as described above were transferred in a 96-well plate, washed with PBS, stained on ice with Fixable Viability Dye (eBioscience), washed with cold staining buffer (BD Biotics), incubated with anti-CX3CR1 antibody (BioLegend), fixed with BD FACs Lysing Solution, permeabilized with BD FACs Permeabilization Solution 2, blocked with PBS containing 5 % BSA, incubated with anti-RSV F protein human mAb (kindly provided by MedImmune) and Alexa Fluor 488-labelled goat anti-human IgG (H + L) antibody (Invitrogen). Flow cytometry analysis was performed using a four-laser BD LSR-II and FlowJo software. Imaging flow cytometry was performed using ImageStreamX Mark II and IDeAS data analysis software (AMNIIS; Millipore).

**RSV RNA detection in HAECs.** The amount of viral RNA was determined by a real-time (RT)-PCR assay. Total RNA was extracted from supernatants using a Qiagen Total RNA Extraction kit (Qiagen) according to the manufacturer’s instruction and stored at −80 °C. RT-PCR was performed by using an AgPath-ID OneStep RT-PCR kit (Applied Biosystems) and an ABI 7700 sequence detector system (PE Applied Biosystems) as described previously (Chirkova *et al.*, 2013). The cycle threshold (Ct) values, i.e. the number of cycles required to exceed the background level, were calculated.

**Cytokine analysis.** The supernatants from the bottom chamber were harvested at different time points post-infection, stored at −80 °C and analysed by Luminex magnetic bead kits (Invitrogen and Millipore).

**Data analysis.** Statistical analysis and graph production used Prism software (GraphPad). Data are expressed as mean ± SEM of three or more independent experiments with three replicates for each experimental condition. The Mann–Whitney test, unpaired t-test and two-way ANOVA were used for the comparison of the different treatments of HAECs and multiple cytokine responses. P < 0.05 was considered to be statistically significant. Cytokine kinetics analysis was performed by calculating the ratios (RCₜ) of slopes of cytokine production curves (pg cytokine ml⁻¹) and replication curves (relative amount of viral RNA in HAECs found by RT-PCR) between days 2 and 4 p.i., the exponential phase of both cytokine production and virus replication. The slopes were calculated using logarithmic transformation of the cytokine and relative RNA levels.

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