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Short communication

Evaluation of the Calu-3 cell line as a model of *in vitro* respiratory syncytial virus infection☆

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Respiratory syncytial virus (RSV) replication is primarily limited to the upper respiratory tract epithelium and primary, differentiated normal human bronchial epithelial cells (NHBE) have, therefore, been considered a good system for *in vitro* analysis of lung tissue response to respiratory virus infection and virus–host interactions. However, NHBE cells are expensive, difficult to culture, and vary with the source patient. An alternate approach is to use a continuous cell line that has features of bronchial epithelial cells such as Calu-3, an epithelial cell line derived from human lung adenocarcinoma, as an *in vitro* model of respiratory virus infection. The results show that Calu-3 fully polarize when grown on permeable supports as liquid-covered cultures. Polarized Calu-3 are susceptible to RSV infection and release infectious virus primarily from the apical surface, consistent with studies in NHBE cells. The data demonstrate that polarized Calu-3 may serve as a useful *in vitro* model to study host responses to RSV infection.

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Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract illness in infants and children worldwide, and infection can also result in serious disease in elderly and immune-compromised patients (Falsey et al., 1995; Hall et al., 1991; Panitch, 2001; Shay et al., 1999). RSV enters the respiratory tract primarily through fomite or hand-to-nasal epithelium transmission following contact with infectious secretions (Hall and Douglas, 1981; Hall et al., 1980). Infection is usually limited to the upper respiratory tract, and progression into the lower respiratory tract can result in serious complications of infection, including hospitalization for pneumonia and bronchiolitis. In humans, similar to other paramyxoviruses, RSV infection and replication primarily occurs in ciliated airway epithelial cells (Wright et al., 1997). As infection progresses, perivascular and peribronchiolar mononuclear cell infiltration is followed by necrosis of the airway epithelium (Aherne et al., 1970; Downham et al., 1975; Ferris et al., 1973). The mechanisms of cellular responses to RSV infection have been studied extensively *in vitro* in a variety of immortalized epithelial cell lines grown in monolayer cultures, including but not limited to Vero, Hep-2, A549, and Madin–Darby canine kidney (MDCK) cells (Kwilas et al., 2009; Li et al., 2010; Lupfer and Pastey, 2010; Roberts et al., 1995; Stark et al., 1996; Swedan et al., 2009; Wright et al., 2005).

In contrast to observations made in RSV infected non-polarized epithelial cell lines, the *in vivo* response to RSV infection is directional. One *ex vivo* model, differentiated, polarized cell cultures of primary normal human bronchial epithelial cells (NHBE), has provided some insights into the mechanism of RSV infection and the cellular response to infection. This model system more closely mimics the airway epithelium structure than monolayer-cultured cells, and likely provides a better *in vitro* model of RSV infection and the associated cellular responses. In NHBE cells, RSV primarily infects ciliated lumenal columnar cells, and infectious virus is released primarily from the apical surface of NHBE (Zhang et al., 2002). These data from the polarized, differentiated NHBE model demonstrate the insights that can be gained using cell systems that more closely mimic the airway epithelium. However, NHBE cell systems are time- and labor-intense, costly to maintain, and require access to primary lung tissue samples. Additionally they require substantial expertise in isolating and culturing epithelial cells from the lung tissue, and significant amount of time to first polarize and then differentiate at an air–liquid interface.

Given these limitations, an alternative to NHBE cells as an *in vitro* model of polarized airway epithelium cells was identified and evaluated. Calu-3 cells originate from a human bronchus adenocarcinoma of a mixed phenotype, believed to be derived from submucosal gland serous cells (Yoshikawa et al., 2009). When grown on trans-well inserts, Calu-3 polarize into apical and baso-

☆ The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of CDC.

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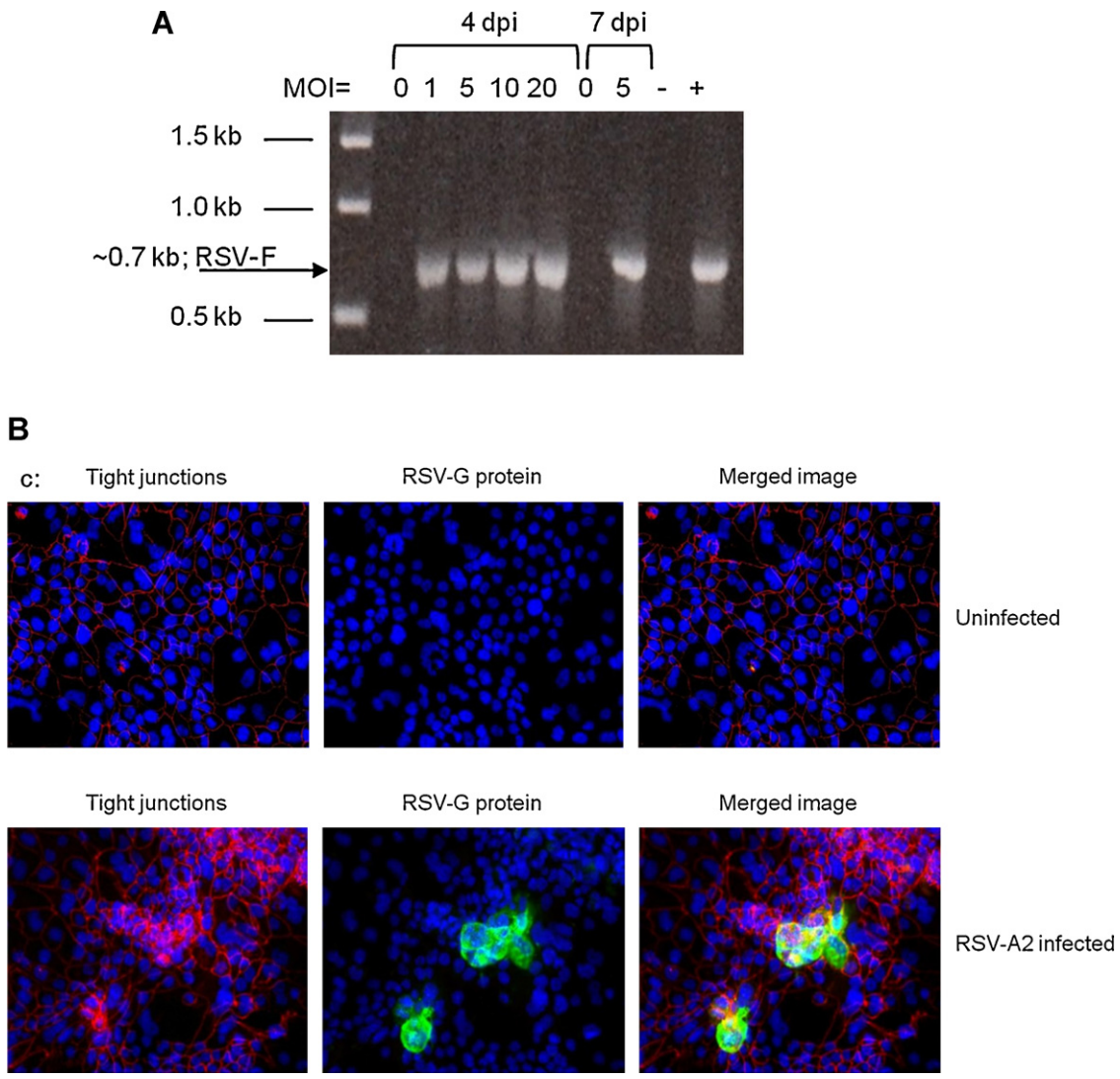

Fig. 1. Calu-3 susceptibility to RSV infection. Calu-3 cells were cultured as monolayers and infected with RSV strain A2 at MOI 1, 5, 10 or 20 (a). At days 4 and 7 post-infection, the presence of RSV-F message RNA was analyzed by one-step RT-PCR (a). Tight junction formation and RSV G protein gene expression at 7 days post-infection (MOI = 10) was evaluated by immunofluorescence assay (b). Tight junction formation was evaluated by staining cells with anti-zona occludens – 1 (ZO-1) antibody (Novus Biologics) and 594-AlexaFluor conjugated anti-ms IgG (Invitrogen; red), RSV-G protein expression was evaluated by staining cells with 488-AlexaFluor conjugated anti-RSV-G protein monoclonal antibody 131-2G (green), and nuclei were stained with DAPI (blue). Stained cells were visualized using a Zeiss AxioImager microscope at 10× magnification.

lateral surfaces, but do not differentiate into multiple cell types and layers as observed with cultured NHBE (Grainger et al., 2006). Polarized Calu-3 are susceptible to infection by respiratory viruses, including influenza virus A, rhinovirus and severe acute respiratory syndrome – associated coronavirus (SARS-CoV) (Grantham et al., 2009; Saedisomeolia et al., 2009; Yoshikawa et al., 2009, 2010). The studies presented here evaluate polarized Calu-3 as an *in vitro* model of RSV infection.

To determine whether RSV infects and replicates in non-ciliated respiratory epithelial cells, monolayers of non-polarized Calu-3 were infected with RSV strain A2 at a multiplicity of infection (MOI) between 1 and 20, and viral RNA expression was determined. RSV-F viral mRNA was detectable at both 4 and 7 days post-infection by RT-PCR, demonstrating that Calu-3 cells can be infected by RSV (Fig. 1A). The expression of RSV surface proteins attachment, G, and fusion, F, was examined by immunofluorescence assay using anti-RSV-G (131-2G) and anti-RSV-F (131-2A) monoclonal antibodies. At seven days post-infection, there was little evidence of syncytia formation and RSV G (Fig. 1B) and RSV F proteins were detected in a few small clusters of cells within the Calu-3 monolayer (Fig. 1B). Interestingly, tight junction formation, as indicated by immunostaining for zona occludens (ZO)-1 (Fig. 1B), was maintained during 7 days of infection. RSV surface protein expression co-localized with tight junctions (Fig. 1B), consistent with the lack of any obvious cytopathology in differentiated, RSV-infected NHBE cells (Zhang et al., 2002).

Polarized Calu-3 cultures may be generated by growing cells at an air–liquid interface (ALI) or as liquid-covered cultures (LCC). Calu-3 cells were evaluated in liquid-covered culture (LCC), in which they are primarily non-ciliated (Grainger et al., 2006). The polarization of Calu-3 in LCC is dependent on several factors including growth medium, cell density and trans-well membrane pore size and composition. For these studies, Calu-3 were seeded at a density of 6.0×10^5 cells/cm^2 onto polyester trans-well inserts (0.33 cm) with 3 μ pores (Corning) in Eagles Modified Essential Medium (EMEM, Gibco) supplemented with 10% fetal bovine serum (EMEM-10% FBS, complete medium), and polarization was demonstrated by trans-epithelial electrical resistance (TEER) development and by a passive sodium fluorescein equilibration assay. Following seeding into trans-well culture, the development of TEER by Calu-3 cultures was monitored using an epithelial voltohmmeter (World Precision Instruments). The resistance of Calu-3

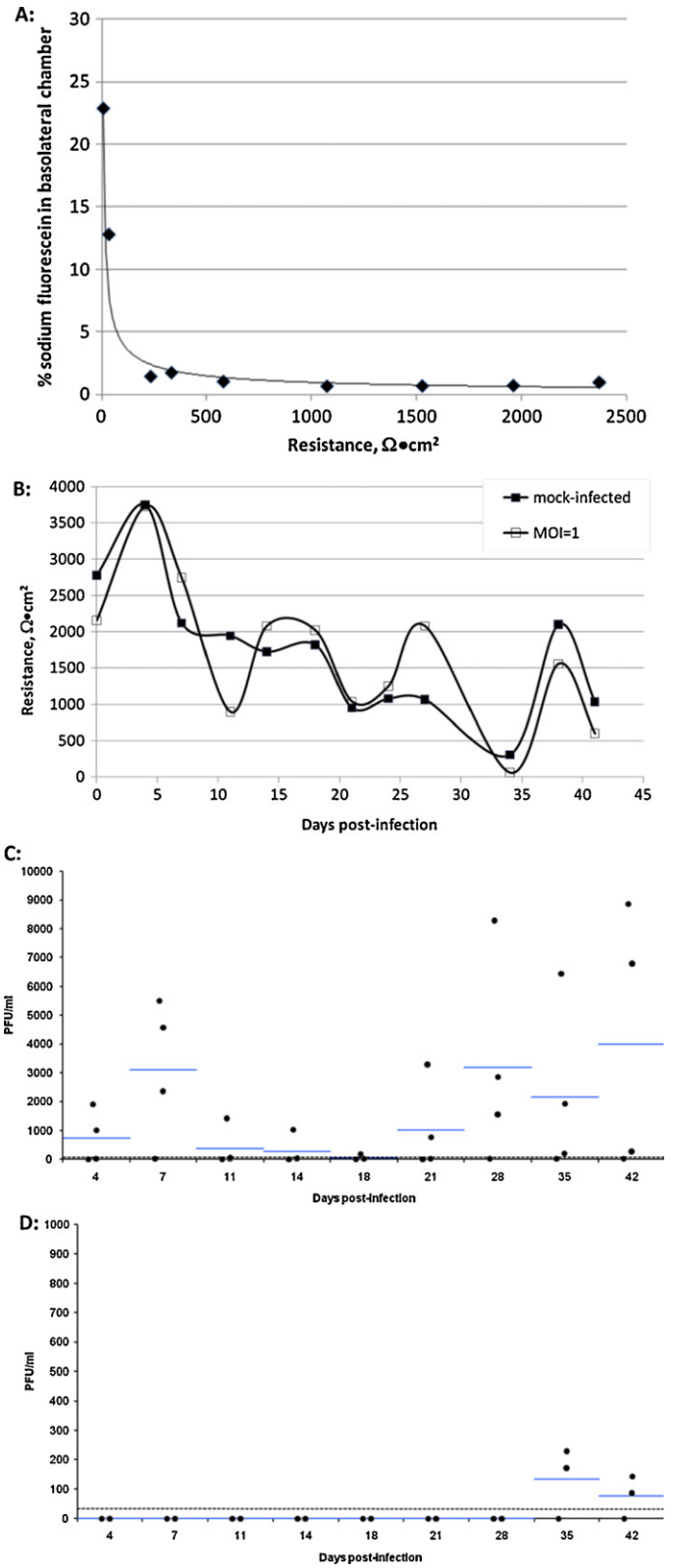

Fig. 2. Directional release of RSV from polarized Calu-3. Apical and basolateral compartment media of trans-well cultured Calu-3 was replaced every 3–4 days. Resistance development was confirmed by a passive sodium fluorescein equilibration assay as previously described (A, (Geys et al., 2006, 2007)), and is representative of 3 independent experiments. Resistance development of Calu-3 infected with RSV-A2 (MOI = 1) at the apical surface (B), and apical (C) and basolateral (D) release of infectious virus were monitored for 6 weeks post-infection. For resistance development, each value represents the median ± SEM measurements from 3 individual wells (3 measurements/insert) and is presented as Ω cm^2. The amount of infectious virus released from the apical (C) and basolateral (D) media of Calu-3 infected at the apical surface was evaluated by plaque assay on Vero cells from three individual samples, and is presented in PFU/ml for each sample. The lower limit of detection for the plaque assays is indicated with a dashed line. Data is representative of 3 independent experiments.

gradually increased after seeding into trans-wells, and peaked between 1500 and 2100 Ω cm^2 by 21 days post- seeding (data not shown). Calu-3 polarization was confirmed by measuring the passive diffusion of sodium fluorescein through the cell monolayer as previously described (Geys et al., 2006, 2007). Briefly, polarized cells were washed with non-fluorescent buffer, and sodium fluorescein (1 mg/ml) was added to the apical compartment and non-fluorescent buffer to the basolateral compartment of cells. After an hour incubation, the amount of dye that diffused into the basolateral compartment was determined by spectrophotometric analysis of the basolateral sample compared to a sodium fluorescein standard curve. Polarized Calu-3 cultures do not allow equilibration of sodium fluorescein between apical and basolateral compartments. As the measurable resistance of polarized cells increased, the percent of sodium fluorescein that diffused into the basolateral compartment declined from 25% of the initial amount of dye added to the apical compartment when cells were non-polarized, to less than 1% when TEER was ≥1200 Ω cm^2 (Fig. 2A). Calu-3 were considered to be completely polarized once the TEER was ≥1200 Ω cm^2 and the amount of sodium fluorescein that diffused into the basolateral compartment was less than 1% of the initial amount in the apical compartment, the threshold of polarization (Geys et al., 2006, 2007).

Polarized cells were infected at either the apical or basolateral surface with RSV strain A2 at an MOI of 1, based on the concentration of cells initially seeded into the trans-well inserts. Apical infections were performed by overlaying virus on the apical surface of polarized cells for 2 h at 37 °C. For basolateral surface infections, trans-well inserts were inverted and virus was applied directly to the basolateral surface and incubated in similar conditions. For both infections, inoculum was removed after incubation, wells that were infected at the basolateral surface were re-inverted into their original locations, and growth medium was added to all wells. At the indicated times post-infection, TEER was measured, apical and basolateral media were collected, and virus was quantitated by standard plaque assay on Vero cell monolayers as previously described (Sullender, 1995). There was little variability in the TEER of Calu-3 prior to apical infection (data not shown), but greater variability between individual trans-wells in the TEER of RSV-A2 – infected and mock-infected Calu-3 following infection (Fig. 2B). This decline in the stability of TEER measurements was observed in both mock- and RSV-A2 – infected Calu-3, and occurred following infection at either the apical (Fig. 2B) or basolateral (data not shown) surface. The fluctuations in resistance observed following infection may in part be due to a disruption in the polarized state of cells caused by the infection process. Despite resistance fluctuations following RSV infection, the overall TEER of polarized Calu-3 was not significantly decreased compared to mock-infected cells for at least 6 weeks post-infection, suggesting that apical or basolateral RSV infection of polarized Calu-3 does not significantly alter tight junction formation. These observations are consistent with RSV-infection in Calu-3 monolayer cultures (Fig. 1B), and with RSV infection of polarized, differentiated NHBE (Zhang et al., 2002).

In vitro and *ex vivo* studies have shown that RSV is released apically from infected, polarized epithelial cells (Batonick et al., 2008; Brock et al., 2003; Roberts et al., 1995; Wright et al., 2005; Zhang et al., 2002). Virus release into the apical compartment of Calu-3 infected at the apical surface was detectable as early as 4 days post-infection (Fig. 2C). While the amount of virus release varied between time points and between individual samples at each time point, infectious virus was detectable for at least six weeks

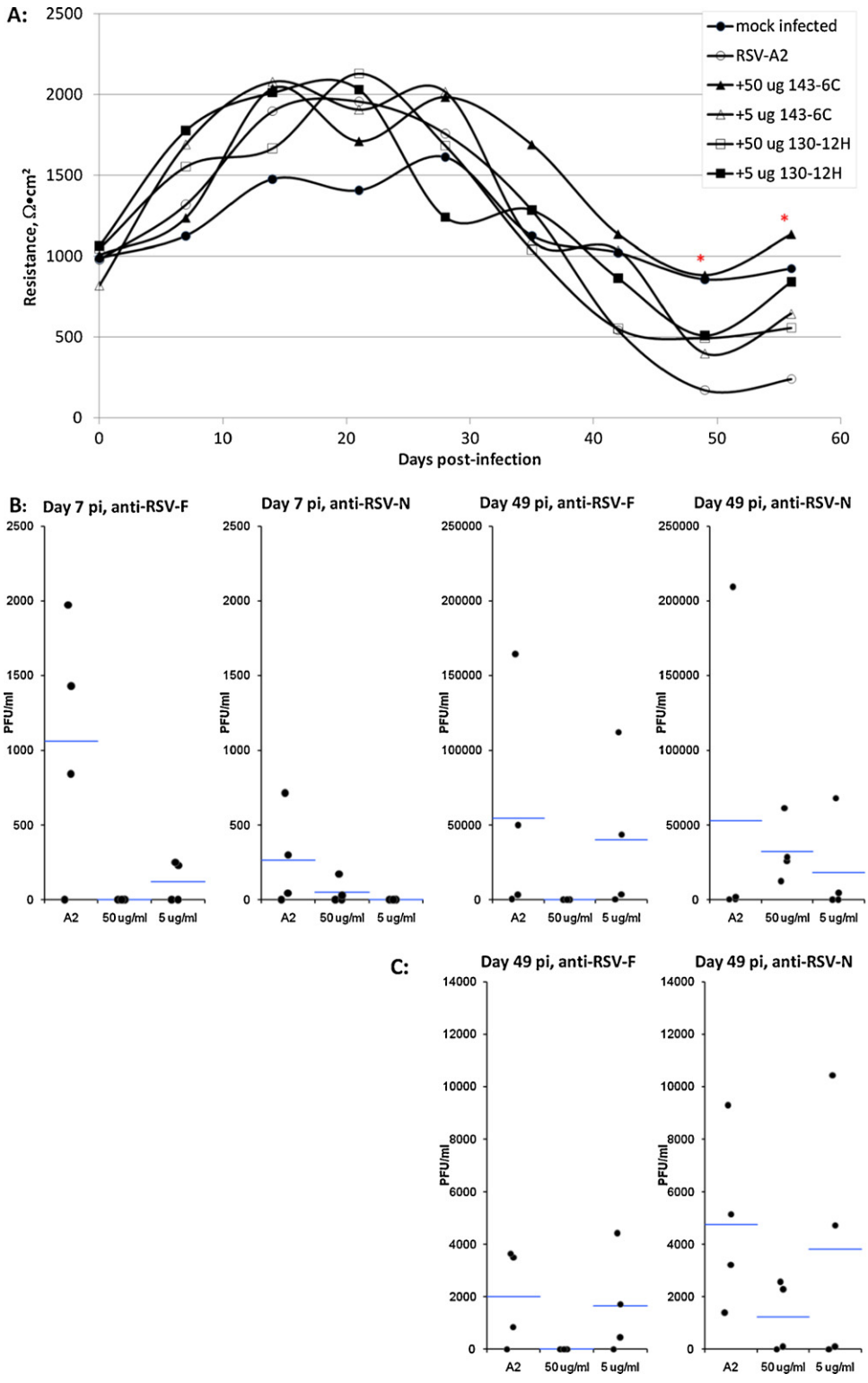

Fig. 3. Anti-RSV-F antibody neutralizes RSV infection of polarized Calu-3. Resistance development of Calu-3 infected at the apical surface (a), and apical (b) and basolateral (c) release of infectious virus from apically-infected Calu-3 (MOI = 1) were monitored for 8 weeks post-infection. Prior to infection, virus was incubated with anti-RSV-F (143-6C) or anti-RSV-N (130-12H) antibody at either 50 μg/ml or 5 μg/ml for 1 h at 37 °C. Each TEER value represents the median ± SEM measurements from 4 individual wells (3 measurements/insert) and is presented as Ω cm². The amount of infectious virus released from the apical (b) and basolateral (c) media of Calu-3 infected at the apical surface was evaluated by plaque assay on Vero cells from four individual samples, and is presented in PFU/ml for each sample. No virus was detectable in basolateral media (c) at day 7 pi. *Statistically different values between RSV-A2 – infected wells and wells which were infected with antibody-preadsorbed virus, as determined by two-tailed unpaired analysis, when $p < 0.05$.

post-infection (day 42 pi, Fig. 2C). This persistent RSV infection of polarized Calu-3 is consistent with findings in RSV-infected differentiated NHBE cultures, in which infection was detectable for at least 36 days post-infection (Zhang et al., 2002). Similar to apical infection, virus release after basolateral infection was detected as early as 4 days post-infection, primarily from the apical sur-

face, and was detectable for at least six weeks post-infection (data not shown). After either apical or basolateral infection, virus was detected in the basolateral media of cells only after day 35 pi, corresponding with decreased TEER in infected Calu-3 (Fig. 2D, and data not shown). The detection of virus in the basolateral media may be due to basolateral release of virus or to the mixing of apical and basolateral media in cultures due to decreased polarization. Whether cells were infected at the apical or basolateral surface, greater than 90% of the virus release occurred into the apical compartment, demonstrating directionality in the response of Calu-3 to RSV infection.

In order to determine whether RSV entry into Calu-3 was mediated by RSV-F surface protein, anti-RSV-F or anti-RSV-N monoclonal antibody (mAb 143-6C and mAb 130-12H, respectively) were incubated with virus for 1 h at 37 °C, prior to infecting polarized Calu-3 cells. There was sample-to-sample variability in the amount of infectious virus released from infected cells at each time point examined. Infection of Calu-3 was inhibited by the neutralizing anti-RSV-F antibody (mAb 143-6C), a murine mAb that recognizes the same epitope as Palivizumab (Anderson et al., 1988; Johnson et al., 1997), at 50 μg/ml but not 5 μg/ml while a control anti-RSV-N mAb failed to inhibit infection at either 50 or 5 μg/ml (Fig. 3). The anti-RSV-F mAb completely neutralized apical (Fig. 3B) and basolateral (Fig. 3C) release of RSV at 50 μg/ml at day 7 ($p < 0.05$) and at day 49 pi ($p = 0.07$). Treatment with a control anti-RSV N mAb did not significantly neutralize apical or basolateral RSV infection at the timepoints examined (Fig. 3B and C). Consistent with protection from RSV infection, treatment with 50 μg/ml anti-RSV-F mAb, but not of anti-RSV-N mAb, protected against the accelerated decline in polarization observed in RSV-A2 infected cells (Fig. 3A). The TEER of anti-F mAb treated Calu-3 was comparable to mock-infected controls as late as day 56 pi.

The data presented in this report demonstrate that Calu-3 cells are susceptible to RSV infection when cultured as monolayers. Infected cells expressed viral message and viral protein, but did not form obvious cytopathology, and infection did not appear to alter tight junction formation. The lack of obvious cytopathology and limited viral spread following RSV infection of Calu-3 is in contrast to observations made in non-polarized epithelial cell lines used to study RSV infection, including HEp-2, where infection becomes widespread across the monolayer of cells. However, these findings are consistent with those made in cultured NHBE (Zhang et al., 2002) and in human adenoidal epithelial cells (HAE) and human adenoid organ culture (Wright et al., 2005), in which RSV spread is restricted and few cells appear to be infected with RSV. Consistent with previous studies in polarized MDCK (Roberts et al., 1995) and in differentiated NHBE, polarized Calu-3 released infectious virus primarily from the apical surface, and infection was persistent, detectable for at least 6 weeks post-infection. Persistent RSV infection of cells in culture has been reported for at least 36 days post-infection of NHBE cells (Zhang et al., 2002). The persistent infection and release of infectious virus for at least 6 weeks post-infection of Calu-3 cells is consistent with observations of persistent infection in *ex vivo*, cultured NHBE, in animal models of RSV infection, and in some RSV-infected individuals (Couch et al., 1997; Dakhama et al., 1997; Hegele et al., 1994; Isaia et al., 1985; Mejias et al., 2008; Sikkel et al., 2008; Zhang et al., 2002). Finally, consistent with previous reports in other cell lines, RSV infection in Calu-3 could be inhibited by an antibody directed against the attachment protein of RSV. Although Calu-3 do not differentiate, the results indicate that polarized Calu-3 respond to infection similar to polarized, differentiated NHBE cells, and more closely reflect *in situ* RSV infection than non-polarized epithelial cells. Calu-3 are more readily available than NHBE cells, polarize into stable cultures more rapidly than NHBE cells, and demonstrate restricted, persistent infection and directional viral maturation similar to NHBE cultures, demonstrating that Calu-3 are a useful *in vitro* model of polarized human lung epithelium-derived cells for studying cellular responses to RSV infection.

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