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ORIGINAL ARTICLE

Azithromycin reduces airway inflammation induced by human rhinovirus in lung allograft recipients

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ABSTRACT

Background and objective: **Human rhinovirus (RV) is a common upper and lower respiratory pathogen in lung allograft recipients causing respiratory tract exacerbation and contributing towards allograft dysfunction and long-term lung decline. In this study, we tested the hypothesis that RV could infect both the small and large airways, resulting in significant inflammation.**

Methods: **Matched large and small airway epithelial cells (AEC) were obtained from five lung allograft recipients. Primary cultures were established, and monolayers were infected with RV1b over time with varying viral titre. Cell viability, receptor expression, viral copy number, apoptotic induction and inflammatory cytokine production were also assessed at each region. Finally, the effect of azithromycin on viral replication, induction of apoptosis and inflammation was investigated.**

Results: **RV infection caused significant cytotoxicity in both large AEC (LAEC) and small AEC (SAEC), and induced a similar apoptotic response in both regions. There was a significant increase in receptor expression in the LAEC only post viral infection. Viral replication was elevated in both LAEC and SAEC, but was not significantly different. Prophylactic treatment of azithromycin reduced viral replication and dampened the production of inflammatory cytokines post-infection.**

Conclusion: **Our data illustrate that RV infection is capable of infecting upper and lower AEC, driving cell death and inflammation. Prophylactic treatment with azithromycin was found to mitigate some of the detrimental responses. Findings provide further support for the prophylactic prescription of azithromycin to minimize the impact of RV infection.**

SUMMARY AT A GLANCE

We highlight the ability of human rhinovirus (RV) to infect both large and small airway epithelial cells of lung allograft recipients resulting in cell death and inflammation. Importantly, we show that prophylactic treatment with azithromycin predominantly mitigates viral replication and the resulting inflammatory effects of viral infection.

Key words: airway epithelium, cell biology, epithelial cells, infection and inflammation, viral infection.

INTRODUCTION

Allograft lung infections from respiratory viruses are a major cause of morbidity and mortality in lung transplant recipients[1,2] and the association of respiratory virus infections and the development of chronic lung allograft dysfunction (CLAD) or bronchiolitis obliterans syndrome (BOS) has been well established.[1,3–8] Investigations have identified human rhinovirus (RV) as one of the more common viral pathogens detected in lung allograft recipients that contribute towards allograft dysfunction and permanent damage.[9–11]

Although exposure to RV specifically may not be a major risk factor of morbidity, they are known to cause serious respiratory tract exacerbations in immunocompromised individuals.[12] Previous investigations have illustrated that the duration of RV-induced viral shedding is approximately 1–2 weeks in immunocompetent individuals[12–14] but is prolonged for up to 12 months in lung allograft recipients.[10,12,13] RV infection in allograft lung is also likely to trigger a secondary infection with other pathogens due to impaired mucociliary function, altered lymphatic drainage as well as absence of cough reflex,[15] increasing the severity of RV infections. To date, a number of clinical studies have associated lower respiratory tract infection with acute rejection and the

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development of BOS with long-term declined lung function.[10,16,17] Although data supporting this outcome is inconclusive, effective prevention or care is essential due to limited antiviral agents on the market.

In this study, we hypothesized that RV infection is not regionally specific, and can infect both the small and large airways, with infection leading to inflammation. To do this, we assessed the susceptibility of primary airway epithelial cell (AEC) of lung transplant recipients to RV infection. We characterized the effects of RV on large AEC (LAEC) and small AEC (SAEC), and assessed viral replication post-infection in each region as well as the effect of the macrolide azithromycin commonly administered post-transplant on the inflammatory response of both LAEC and SAEC to RV infection.

METHODS

Patient and sampling procedures

This study was approved by the Royal Perth Hospital, Ethics Committee (Registration: EC2006/021) and written consent was obtained from each participant after being fully informed about the premise and purpose of the study. LAEC and SAEC were obtained from lung allograft recipients without BOS, infection or rejection during their routine surveillance bronchoscopy as previously described.[18] Airway cells were then grown to a confluent monolayer and seeded for viral infection.[19,20]

Viral infection

Confluent primary cultures were infected with RV1b (provided by Dr Peter Wark, Hunter Medical Research Institute, Newcastle, NSW, Australia) at a low [2.5×10^4 Tissue Culture Infections Dose 50% (TCID50/mL)] and high (10×10^4 $TCID_{50}$/mL) titre over 48 h at 37°C. RV1b was selected from all available serotypes as it is commonly contracted during the flu season and is more virulent. Cells, supernatants and RNA were then collected at various time points post-infection for subsequent downstream analysis.

Cell viability and apoptosis

To assess the effect of RV1b infection on AEC viability, a CellTiter 96 assay (Promega, Madison, WI, USA) was utilized. Similarly, apoptotic induction was measured by a single-stranded (ss) DNA apoptosis kit (Millipore, Billerica, MA, USA). Both were performed as per the manufacturer's instructions and as previously described.[19,20]

Quantification of LDLR gene expression

Low-density lipoprotein receptor (LDLR) gene expression was determined by quantitative polymerase chain reaction (qPCR). Here, total RNA was extracted from infected and non-infected LAEC and SAEC using the PureLink RNA Mini Kit (Life Technologies, Mulgrave, Australia), quantified and reverse transcribed to cDNA using the Multiscribe Reverse Transcriptase (Life Technologies). LDLR-specific primers (forward: 5′-GACATGAGCGATGAAGTTGG-3′; reverse: 3′-CCATT ATGGCGTGTAAAGTCA-5′) from GeneWorks (Hindmarsh, Australia) relative to the house keeping gene peptidylprolyl isomerase A (PPIA) (forward: CCT TGG GCC GCG TCT CCT TT, reverse: CAC CAC CCT GAC ACA TAA ACC CTG G) (Life Technologies) were used in conjunction with SYBR Green PCR Master Mix (Life Technologies).

Viral replication

Viral replication was quantitated via qPCR using the RV1B Advanced Kit (PrimerDesign Ltd., Southampton, UK) as described previously.[19,20] Briefly, after infection, cells were collected, RNA extracted, quantified and reverse transcribed into cDNA using provided RV-specific primers. Viral copy was then detected via Taqman qPCR (Applied Biosystems, Carlsbad, CA, USA) using RV primer-probe mix (PrimerDesign Ltd., Southampton, UK). A supplied RV-positive control was serially diluted to generate a standard curve from which viral copy number was then calculated.

Cytokine assays

Production of inflammatory cytokines was measured from supernatants collected prior to and following viral infection. The expression of IL-8 and Chemokine (C-C motif) ligand 5 (RANTES) was measured by commercially available ELISA kits (IL-8: Becton Dickinson, Biosciences, San Diego, CA, USA; RANTES: R&D, Minneapolis, MN, USA). IL-6 was measured using a time-resolved fluorometry detection system as previously described[20] (PerkinElmer, Waltham, MA, USA).

Azithromycin treatment

The effect of azithromycin on viral infection was also assessed. Here, azithromycin (Pfizer Inc., Groton, CT, USA) was prepared (1 μg/mL) in MiliQ water and added to LAEC and SAEC 24 h prior to viral infection. Cell supernatant and RNA were then collected, and downstream experiments conducted to assess cytokine production and viral replication capacity.

Statistical analysis

Experiments were performed in triplicate, with five patients per experiment. Data were initially tested for population normality and homogeneity of variance prior to analysis, using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). All data were assumed to be non-parametric, with analysis performed using the Wilcoxon signed rank test. Data are presented as median ± interquartile range (IQR) or minimum to maximum where indicated, with P-values <0.05 were considered significant.

RESULTS

Effect of RV on cell viability

To determine any cytopathic effect of RV1b on LAEC and SAEC, viability assays were performed post-infection. Exposure of LAEC to both low (2.5×10^4 $TCID_{50}$/

mL) and high (10×10^4 $TCID_{50}$/mL) titres of RV1b induced cytotoxicity over time. Viral infection at low viral titre caused significant cytotoxicity at 24 h post-infection, with cell viability reduced by ~20% (92.51; IQR = 87.02–80.07%, $P < 0.05$; Fig. 1A). Viability was reduced further when infection was extended to 48 h (50.28; IQR = 51.09–35.22%, $P < 0.05$; Fig. 1A). Cytotoxic effects were enhanced further when cells were infected at higher virus titre, with cell viability reduced significantly at both 24 h (65.35; IQR = 68.74–64.610%, $P < 0.05$) and 48 h (32.83; IQR = 44.9–29.66%, $P < 0.05$; Fig. 1A). A less pronounced effect was observed on SAEC. Here, cell viability was only marginally reduced at low titre at 24 h (92.51; IQR = 97.13, 91.64%, $P > 0.05$; Fig. 1B) but was significantly reduced with longer infection (67.30; IQR = 76.05–34.93%, $P < 0.05$; Fig. 1B). Similar to LAEC, effects of viral infection on SAEC viability were significantly greater when higher viral titres were used at both points (24 h, 72.48; IQR = 74.46–62.53%; 48 h, 37.95; IQR = 42.34–22.59%, both $P < 0.05$; Fig. 1B). When we compared the effects of viral infection between LAEC and SAEC, no differences were observed ($P > 0.05$).

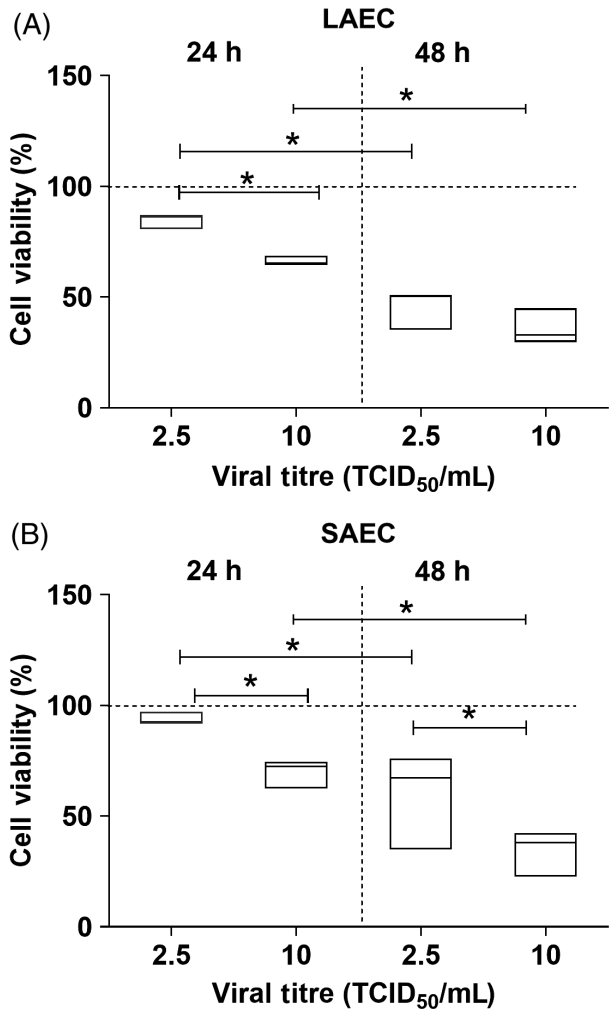


Figure 1 Dose-dependent cytotoxic effects of rhinovirus (RV) 1b on airway epithelial cell (AEC) viability at 2.5×10^4 and 10×10^4 $TCID_{50}$/mL. The CellTitre 96 aqueous non-radioactive cell proliferation assay was used to assess the number of metabolically active cells post-RV1b infection. The percentage cell viability in comparison to uninfected cells was then assessed at 24 and 48 h. (A) Exposure of large AEC (LAEC) to RV1b resulted in significant cell death that was viral titre and time dependent. (B) Infection of matched small AEC (SAEC) with RV1b also resulted in marked cell death that was both viral titre and time dependent. No significant difference was found when viral effects were compared between LAEC and SAEC. *A significant reduction in cell viability from unexposed cells ($P < 0.05$). Experiments were performed on matched LAEC and SAEC derived from five lung allograft recipients and presented as box and whisker plots.

RV receptor expression and replication in LAEC and SAEC

Basal expression of the LDLR was then compared between LAEC and SAEC. Results demonstrated that basal LDLR expression was not significantly different between LAEC and SAEC ($P > 0.05$; Fig. 2A). Interestingly, receptor expression was significantly elevated in LAEC post viral infection ($P < 0.05$; Fig. 2A) but no change was seen in SAEC ($P > 0.05$; Fig. 2A). Furthermore, there was a significant increase in RV1b copy number in AEC from both LAEC and SAEC post-infection ($P < 0.0001$; Fig. 2B).

Effect of viral infection on LAEC and SAEC apoptotic responses

Apoptosis, or programmed cell death, is an essential internal cellular mechanism to combat and limit virus infection[21,22] and thus we assessed its induction in both LAEC and SAEC in response to infection. Specifically, apoptosis was induced in LAEC following 24 h of infection at both low (165.26; IQR = 201.32–106.04%, $P < 0.05$) and high viral titres (310.11; IQR = 368.77–132.25%, $P < 0.05$; Fig. 2C). Although programmed cell death was also induced after 48 h of infection (206.28; IQR = 251.73–147.74% and 339.15; IQR = 413.26–186.63% for low and high viral titres, respectively, $P < 0.05$), this was not significantly different than that seen at 24 h (Fig. 2C). The effect of viral infection on apoptotic induction in SAEC was similar to that seen in LAEC (Fig. 2D). Here, a significant apoptotic response was evident after 24 h of infection (121.08; IQR = 195.22–103.67% and 218.11; IQR = 314.95–114.98% for low and high RV1b titres, respectively, $P < 0.05$) which was elevated after 48 h of infection (175.79; IQR = 202.27–125.19% and 283.49; IQR = 308.35–135.40% for low and high viral titres, respectively, $P < 0.05$; Fig.2D). When both regions were compared, there were no differences in apoptotic induction at any viral titre or infection period. Infection with RV1b was found to drive the apoptotic response in both LAEC and SAEC and this was directly associated with reduced viability (LAEC; $R^2 = 0.6965$, SAEC; $R^2 = 0.733$; Fig. 2E, F).

Effects of azithromycin treatment on apoptosis, viral replication and inflammation

Given that RV1b induces a highly cytotoxic response on AEC from lung transplant recipients and that this

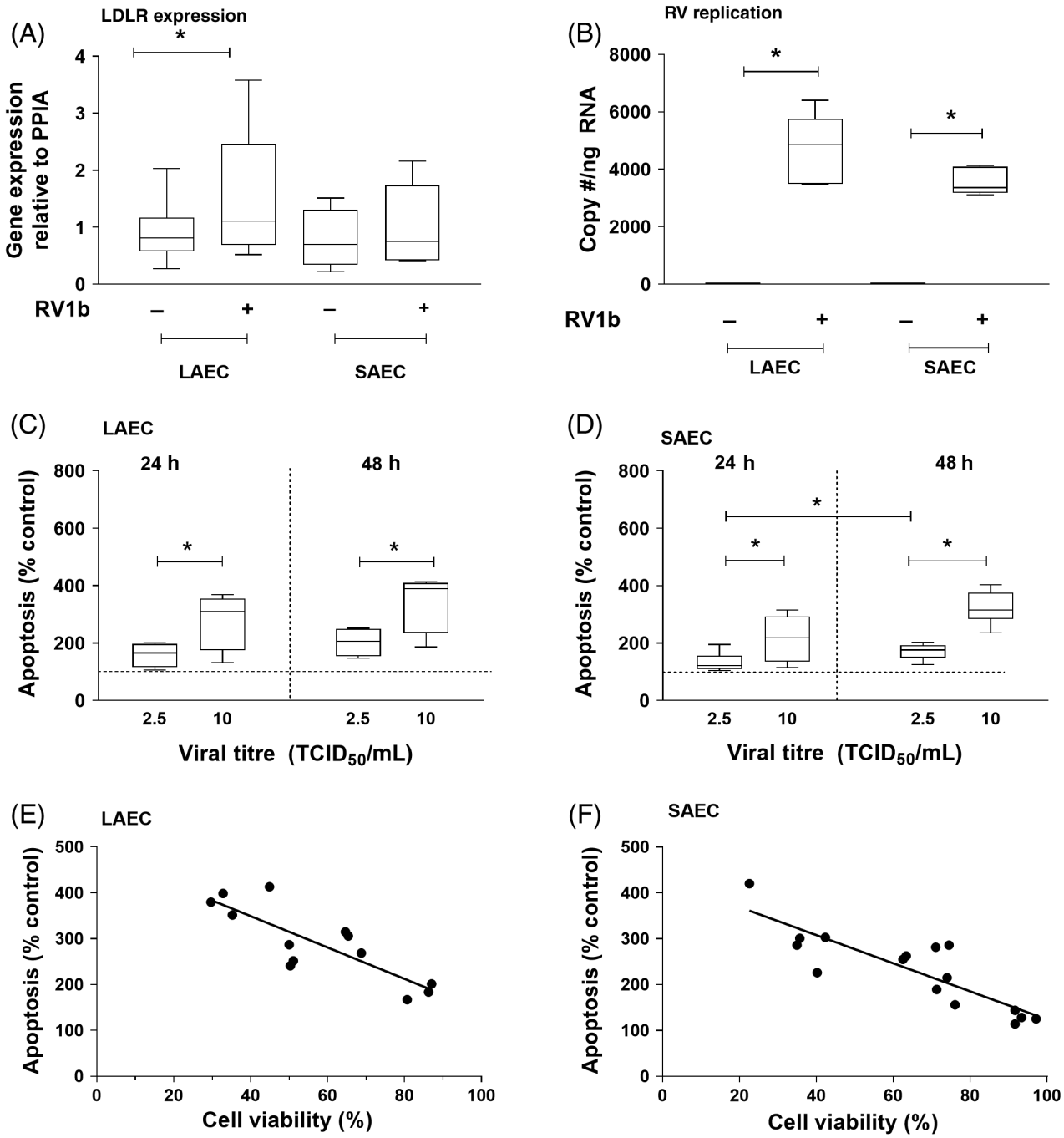


Figure 2 Rhinovirus (RV) 1b receptor (low-density lipoprotein receptor, LDLR), replication and apoptotic responses by airway epithelial cell (AEC). Matched large AEC (LAEC) and small AEC (SAEC) from allograft recipients were grown to confluence, infected with 10×10^4 $TCID_{50}$/mL for 24 h, and viral receptor, replication and programmed cell death were determined via quantitative PCR and a commercial single-stranded (ss) DNA apoptosis kit, respectively. (A) LAEC and SAEC expressed LDLR at similar levels prior to infection, which was elevated post-infection; however, this was only significant in LAEC. (B) LAEC and SAEC were found to facilitate RV1b replication to a similar extent, with no significant difference observed between the two regions. (C) Apoptosis was induced in LAEC post RV1b infection that was titre dependent; however, no significant differences were observed between time points. (D) Similarly, apoptosis was induced in SAEC post-infection that was both viral and time dependent. (E) Using regression analysis, we observed a strong association between apoptosis and cell viability in LAEC ($R^2 = 0.6965$). (F) A similar, although slightly stronger, association was observed between apoptosis and cell viability in SAEC ($R^2 = 0.733$). *Significant differences were observed at $P < 0.05$. Experiments were performed on matched LAEC and SAEC derived from five lung allograft recipients and presented as box and whisker plots. PPIA, peptidylprolyl isomerase A.

could be mitigated by pretreatment with azithromycin, we next performed experiments to assess its effects on apoptosis, viral replication and inflammation. Pretreatment of both LAEC and SAEC with azithromycin had no effect on inducing apoptosis above that than observed without treatment at either

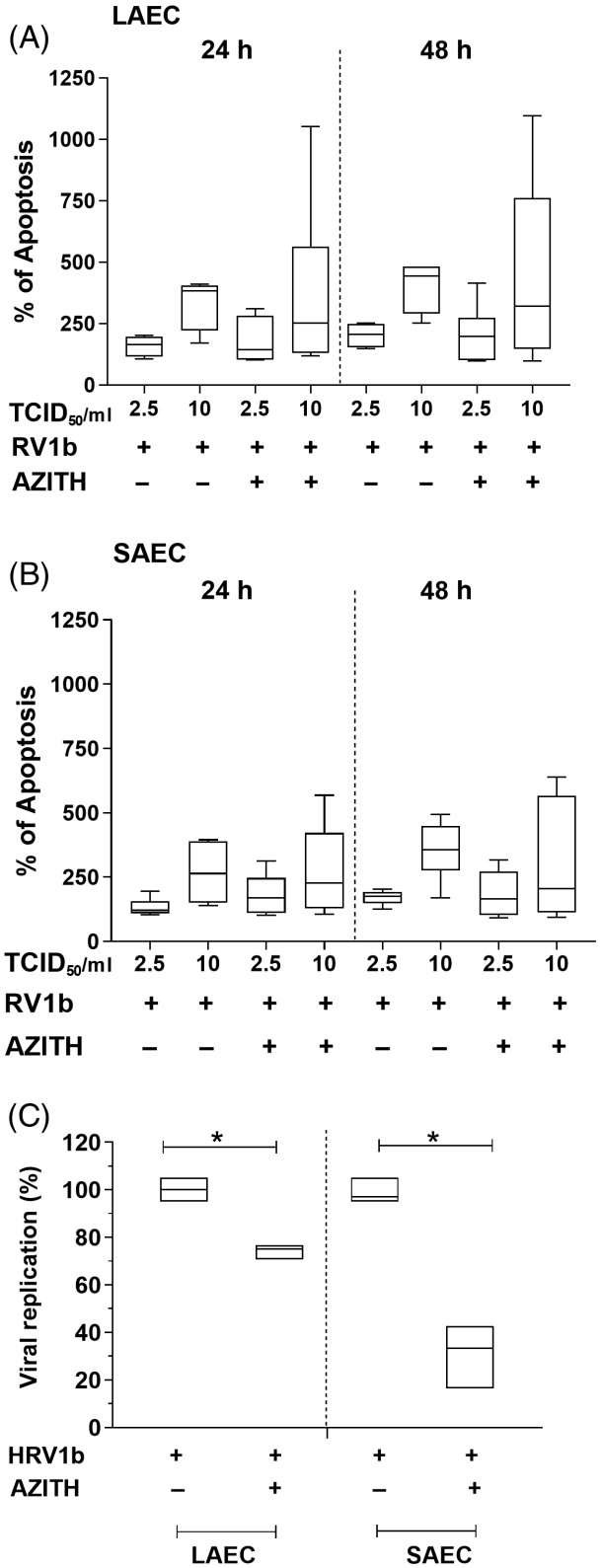


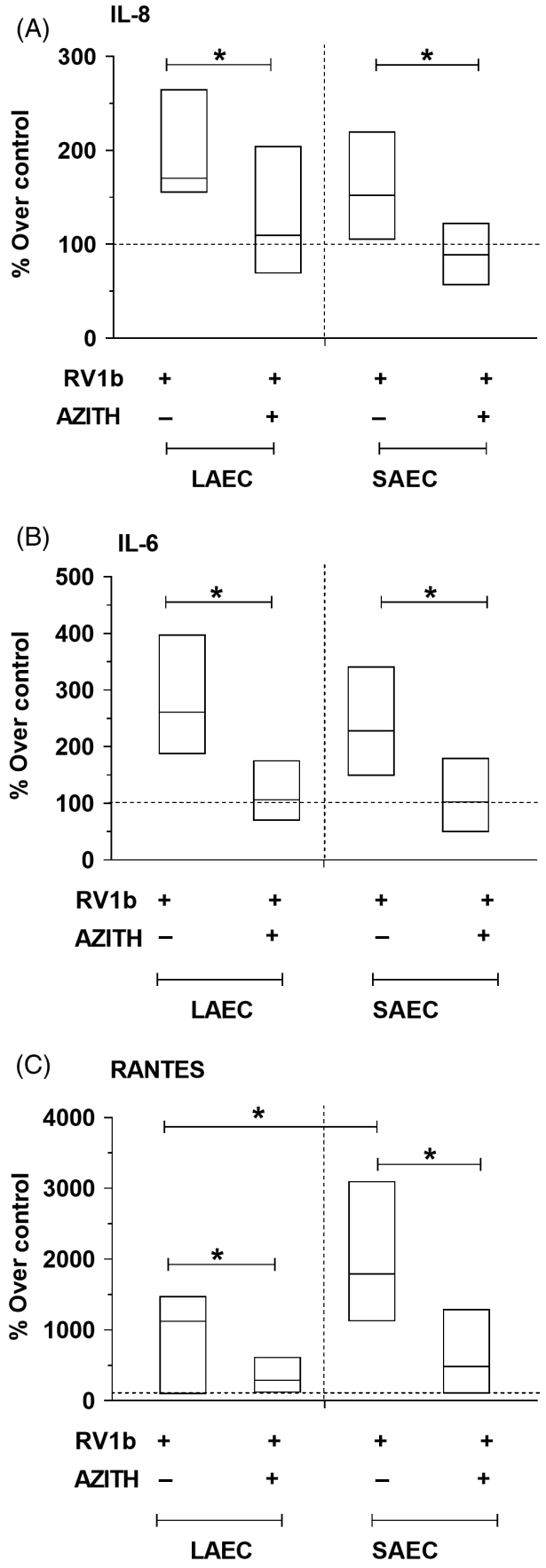
Figure 3 Effect of azithromycin treatment on apoptosis and viral replication in large airway epithelial cell (LAEC) and small airway epithelial cell (SAEC) following rhinovirus (RV) 1b infection. Matched LAEC and SAEC from allograft recipients were grown to confluence, infected with either 2.5 or 10×10^4 $TCID_{50}$/mL and co-treated with azithromycin (1 μg/mL) for 24 and 48 h. Apoptosis was then determined using a commercially derived ssDNA apoptosis kit. Matched LAEC and SAEC were also pretreated with azithromycin (1 μg/mL) for 24 h prior to infection with RV1b (10×10^4 $TCID_{50}$/mL). Viral replication was then assessed via two-step reverse transcription polymerase chain reaction (RT-PCR) using a commercially derived RV1b advanced kit. (A) Co-treatment of infected LAEC with azithromycin for either 24 or 48 h did not significantly induce apoptosis. (B) Similarly, apoptosis was not significantly induced when infected SAEC were co-treated with azithromycin at either time point. (C) Pretreatment with azithromycin significantly reduced viral replication in LAEC (30%) and SAEC (65%) with the reduction more pronounced in SAEC. *Significant differences were observed at $P < 0.05$. Experiments were performed on matched LAEC and SAEC derived from five lung allograft recipients and presented as box and whisker plots.

viral titre or infection period (Fig. 3A,B). However, we saw a significant reduction in viral replication following azithromycin pretreatment in both LAEC and SAEC ($P < 0.05$; Fig. 3C). Effects were greatest in SAEC (~60% reduction) compared to their large airway counterparts (~25% reduction). Finally, we also saw that azithromycin pretreatment significantly reduced inflammation caused by viral infection (Fig. 4). Here, azithromycin significantly reduced IL-8 production by 64% in LAEC and 67% in SAEC (both $P < 0.05$; Fig. 4A) with a similar reduction observed for IL-6 (154% reduction in LAEC and 130% reduction in SAEC; both $P < 0.05$), to baseline levels measured pre-infection. Finally, RANTES production was reduced by 593% in LAEC and 1465% in SAEC following azithromycin pretreatment, with a greater magnitude of reduction seen in SAEC ($P < 0.05$; Fig. 4C). However, levels produced post-infection remained elevated above pre-infection baseline production.

DISCUSSION

This study is one of the first to assess the impact of RV infection on AEC from lung allograft recipients in vitro. In this study, we have shown that RV has the capacity to infect LAEC and SAEC of the lower respiratory system. RV infection was found to cause significant cytotoxicity in both LAEC and SAEC, and induced a similar apoptotic response in both regions of the airway. Of interest was the observation that prophylactic treatment of azithromycin reduced viral replication and dampened the production of inflammatory cytokines. Collectively, we show that RV infection drives cell death and inflammation in the lung, and that prophylactic treatment with azithromycin can mitigate these detrimental responses.

The nasopharyngeal region is easily infected by RV[9]; however, it can also migrate to the lower airways resulting in potent effects for at-risk populations including hospital-admitted acute asthmatic exacerbations.[12,23] In lung allograft recipients, RV infection periods have been reported to last up to 12 months, which in turn increases the risk of secondary infections and subsequent exacerbations.[10,12,13] AEC play an important role during viral infection as they act as the primary site of viral attachment and replication. AEC in other respiratory setting including cystic fibrosis and asthma have also demonstrated a time- and dose-



dependent cytotoxic response to RV infection.[19,20] Data generated in our study showed a similar reduction in AEC viability (~65%) in lung allograft recipients that was time and viral load dependent.

LDLR, the cell receptor for RV serotype 1b, was found not significantly different at baseline between LAEC and SAEC. However, post RV infection, LDLR expression in LAEC expression significantly increased indicating enhanced attachment and entry into these cells. Interestingly, viral replication was similar between LAEC and SAEC, suggesting that no regional differences exists in their susceptibility to RV infection. The resulting viral load (LAEC: $4.67 \pm 1.23 \times 10^3$ #copy/ng RNA, SAEC: $3.58 \pm 0.48 \times 10^3$ #copy/ng RNA) was found to be comparable to other clinical observations including the one conducted by Costa and colleagues[24] ($4.10 \pm 0.16 \times 10^5$ RNA copies/mL) determined from BAL fluid specimens of lung allograft recipients and another by Ambrosioni *et al.* (1.25×10^4–1.99×10^8 RNA copies/mL) obtained from the upper respiratory tract of lung transplant recipients.[9]

To mitigate viral spread, the host typically induces an apoptotic innate immune response. A study conducted by Wark *et al.* investigated the response of AEC of adult asthmatic to RV infection and observed a significant reduction in cell viability and induction of apoptosis post-infection.[25] We also observed a significant apoptotic induction post-infection that correlated with a reduction in cell viability in both LAEC and SAEC. Such an increase in apoptosis reduces the efficiency of viral replication in the host cell, which may explain the lack of variation in viral replication between the non-allograft controls and allograft recipients.

Azithromycin is an antibiotic commonly used in respiratory diseases due to its anti-inflammatory and immunomodulatory properties in addition to its traditional antibacterial properties.[26–28] The current clinical practice guidelines for lung allograft recipients suggest that introduction of prophylactic azithromycin treatments in patients with suspected BOS reduces disease progression[27] With its reported antiviral and anti-inflammatory properties,[28] we investigated what effect prophylactic azithromycin treatment had on viral replication, apoptosis and inflammation. Our results demonstrate that pretreatment with azithromycin was effective in reducing viral replication and the

Figure 4 Effect of azithromycin pretreatment on inflammatory cytokine production in matched large airway epithelial cell (LAEC) and small airway epithelial cell (SAEC) following rhinovirus (RV) 1b infection. Confluent LAEC and SAEC monolayers were infected with RV1b (10×10^4 $TCID_{50}$/mL), treated with azithromycin (1 μg/mL) and the resultant IL-8, IL-6 and RANTES were measured via commercially derived ELISA. (A) Pretreatment with azithromycin prior to infection significantly reduced the production of IL-8 in both LAEC (64%) and SAEC (67%). (B) In addition, IL-6 production was also significantly reduced in both LAEC (154%) and SAEC (130%) with azithromycin pretreatment. (C) Similarly, RANTES production was reduced in both LAEC (593%) and SAEC (1465%) with a greater magnitude of reduction seen in the small airway. *Significant differences were observed at $P < 0.05$. Experiments were performed on matched LAEC and SAEC derived from five lung allograft recipients and presented as box and whisker plots.

production of sentinel inflammatory cytokines including IL-6, IL-8 and RANTES. Although the antiviral activity of azithromycin has been suggested to occur via its activation of the interferon-beta (IFN-β) pathway,[29,30] the fact that it had no effect on apoptosis in this study, a process typically induced by IFN-β, suggests an alternative mode of action, possibly via lung microbiome modification.[31] Our results appear consistent with that of others, who have demonstrated its antiviral and anti-inflammatory nature[26–28] suggesting that prophylactic azithromycin may aid in protecting the lungs against viral infection and the resultant inflammatory-associated damage.

Our study acknowledges the limitation of only using one viral type. Although recognized as a common cause of exacerbation and resultant damage in immunocompromised individuals, RV is not the only viral risk factor in allograft recipients. Work by others have identified respiratory syncytial virus (RSV) and influenza as prominent viral pathogens[24,29,30,32] in allograft recipients and hence further research is necessary in order to gain a better understanding of the effects of these respiratory viruses in the lower airways of lung allograft recipients.

In conclusion, we have identified that RV infection occurs in both LAEC and SAEC to a similar extent and induces a number of host responses including induced AEC death, elevated viral replication and inflammation. We also showed that pretreatment with azithromycin is effective in reducing viral replication and the production of inflammatory cytokines. Collectively, these findings provide supportive evidence for the potential prescription of azithromycin as prophylactic treatment in lung allograft patients to mitigate the impact of RV infection and improve longevity of the allograft.

Acknowledgements

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Abbreviations: AEC, airway epithelial cell; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; ELISA, enzyme-linked immunosorbent assay; IFN-β, interferon-beta; IL, interleukin; IQR, interquartile range; LAEC, large AEC; LDLR, low-density lipoprotein receptor; qPCR, quantitative PCR; RANTES, Chemokine (C-C motif) ligand 5; PPIA, peptidylprolyl isomerase A; RV, rhinovirus; SAEC, small AEC; ss, single-stranded; TCID, Tissue Culture Infections Dose 50%.

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Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

Visual Abstract Prophylactic treatment with Azithromycin minimizes impact of human rhinovirus induced airway inflammation.

