

ORIGINAL ARTICLE

Asthma and Rhinitis

Effects of human rhinovirus on epithelial barrier integrity and function in children with asthma

K. Looi^{1,2} | A. G. Buckley³ | P. J. Rigby³ | L. W. Garratt^{1,2} | T. Iosifidis^{1,4} |
G. R. Zosky⁵ | A. N. Larcombe^{2,6} | F. J. Lannigan^{1,7} | K.-M. Ling² | K.
M. Martinovich² | E. Kicic-Starceovich^{2,8} | N. C. Shaw² | E. N. Sutanto^{2,8} |
D. A. Knight^{9,10,11} | A. Kicic^{1,2,4,6,8}  | S. M. Stick^{1,2,4,8}

¹School of Paediatrics and Child Health, University of Western Australia, Nedlands, WA, Australia

²Telethon Kids Institute, University of Western Australia, Subiaco, WA, Australia

³Centre for Microscopy, Characterisation and Analysis (CMCA), University of Western Australia, Crawley, WA, Australia

⁴Centre for Cell Therapy and Regenerative Medicine, University of Western Australia, Nedlands, WA, Australia

⁵School of Medicine, Faculty of Health, University of Tasmania, Hobart, TAS, Australia

⁶Occupation and Environment, School of Public Health, Curtin University, Perth, WA, Australia

⁷School of Medicine, Notre Dame University, Fremantle, WA, Australia

⁸Department of Respiratory Medicine, Princess Margaret Hospital for Children, Perth, WA, Australia

⁹School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, NSW, Australia

¹⁰Priority Research Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, Newcastle, NSW, Australia

¹¹Department of Anaesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, BC, Canada

Correspondence

A. Kicic, Telethon Kids Institute, Subiaco, Perth, WA, Australia.
Email: Anthony.Kicic@telethonkids.org.au

Summary

Background: Bronchial epithelial tight junctions (TJ) have been extensively assessed in healthy airway epithelium. However, no studies have yet assessed the effect of human rhinovirus (HRV) infection on the expression and resultant barrier function in epithelial tight junctions (TJ) in childhood asthma.

Objectives: To investigate the impact of HRV infection on airway epithelial TJ expression and barrier function in airway epithelial cells (AECs) of children with and without asthma. Furthermore, to test the hypothesis that barrier integrity and function is compromised to a greater extent by HRV in AECs from asthmatic children.

Methods: Primary AECs were obtained from children with and without asthma, differentiated into air-liquid interface (ALI) cultures and infected with rhinovirus. Expression of claudin-1, occludin and zonula occluden-1 (ZO-1) was assessed via qPCR, immunocytochemistry (ICC), in-cell western (ICW) and confocal microscopy. Barrier function was assessed by transepithelial electrical resistance (TER; R_T) and permeability to fluorescent dextran.

Results: Basal TJ gene expression of claudin-1 and occludin was significantly upregulated in asthmatic children compared to non-asthmatics; however, no difference was seen with ZO-1. Interestingly, claudin-1, occludin and ZO-1 protein expression was significantly reduced in AEC of asthmatic children compared to non-asthmatic controls suggesting possible post-transcriptional inherent differences. HRV infection resulted in a transient dissociation of TJ and airway barrier integrity in non-asthmatic children. Although similar dissociation of TJ was observed in asthmatic children, a significant and sustained reduction in TJ expression concurrent with both a significant decrease in TER and an increase in permeability in asthmatic children was observed.

Conclusion: This study demonstrates novel intrinsic differences in TJ gene and protein expression between AEC of children with and without asthma. Furthermore, it

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correlates directly the relationship between HRV infection and the resultant dissociation of epithelial TJ that causes a continued altered barrier function in children with asthma.

KEYWORDS

asthma, epithelial permeability, human rhinovirus, tight junction

1 | INTRODUCTION

Recent evidence supports the concept that airway epithelial cells in children with asthma have impaired responses following viral infections,¹ which can lead to increased susceptibility to further injury.² In addition, early studies have also demonstrated that the epithelium in asthma is damaged.^{3,4} Resultant disruption of the epithelial barrier would then present the opportunity for infectious bacteria, virus and environmental particles to penetrate the airway epithelium. This in turn triggers subsequent immune and inflammatory responses that would cause further damage to the airway epithelium. Several studies have identified potential causes of epithelial barrier disruptions in asthma such as proteolytically active allergens and respiratory viruses.⁵⁻⁷ However, it is also apparent that TJ disruption can occur in fully differentiated epithelial cell cultures from adults with asthma in the absence of exogenous stimuli.⁸ Hence, it remains unclear whether the observed TJ abnormality in the asthmatic epithelium is intrinsic or a consequence of external stimuli. Furthermore, it is unknown whether these potential differences extend to children with asthma for whom the asthma burden is the greatest.⁹

Respiratory viruses such as respiratory syncytial virus (RSV), influenza and human rhinovirus (HRV) have all been shown to cause disruption of the epithelial barrier and altered innate immunity defences.¹⁰⁻¹² In this context, HRV has been identified as a primary trigger in the majority of asthma exacerbations in children.^{6,7,13} Although HRV typically affects the upper airways, evidence also suggests the possibility of lower airway infection and subsequent increased bronchial responsiveness in patients with asthma.^{14,15} Previous *in vitro* and *in vivo* studies have shown elevated HRV replication in the setting of asthma¹⁶⁻¹⁹ despite no significant differences in viral load being observed between asthmatics and non-asthmatic cohorts following a natural cold.²⁰

We have previously identified intrinsic biochemical and functional differences between AECs derived from children with and without asthma and have also shown that these differences were maintained over consecutive passages, which indicates that the differences observed are not dependant on an *in vivo* environment.²¹ We have also demonstrated impaired responses of AECs from children with asthma to HRV¹ and have recently explored the effects of HRV on TJ expression, protein disassembly and epithelial permeability in modified non-diseased human airway epithelial cells and demonstrated a direct association between HRV infection with a loss in junctional protein expression and a subsequent increase in airway epithelial permeability.²² In this study, we wanted to extend the

observations to both diseased and non-diseased airway epithelium and hypothesized that there are baseline differences in asthmatic airway TJ expression that result in a higher permeability. Furthermore, we hypothesized that HRV would result in an exaggerated change in airway TJ, which in turn would affect barrier integrity and permeability to different sized molecules. Using cell cultures obtained from our paediatric cohorts, we show that there is significantly lower TJ protein expression concomitant with lower epithelial resistance in AECs from children with asthma compared to non-asthmatic controls. We also demonstrate for the first time, that HRV causes prolonged TJ dissociation and the resultant increase in permeability and loss of epithelial resistance in AECs from asthmatic children.

2 | MATERIALS AND METHODS

2.1 | Reagents

Bovine serum albumin, foetal calf serum, bovine hypothalamus acetone power, hydrocortisone, recombinant human epidermal growth factor, epinephrine hydrochloride, fibronectin, triiodothyronine, transferrin, trans retinoic acid, trypsin and gentamicin were obtained from Sigma (St. Louis, MO, USA). Bronchial epithelium basal medium (BEBM®), Clonetics™ B-ALI™ air-liquid interface BulletKit™ was purchased from LONZA (Walkersville, MD, USA). Penicillin G, RPMI-1640 media, streptomycin sulphate, amphotericin B and L-glutamine were purchased from Invitrogen (Melbourne, VIC, Australia). Ultrosor G was supplied from CIPHERGEN (Cergy-Saint-Christophe, France). Normal goat serum (NGS) was purchased from Life Technologies (CA, USA). Bovine serum albumin (BSA), Hoechst 33342 and Fluorescent mounting media containing DABCO were purchased from Sigma-Aldrich (MO, USA). Collagen type I and IV was purchased from BD (Franklin Lakes, New Jersey, USA), and all tissue culture plastic wares were purchased from Corning (Corning, New York, USA).

2.2 | Patients and sample collection

The study was approved by both Princess Margaret Hospital for Children and St John of God Hospital's Human Ethics Committee (1903/EP and 1402/EP), and written consent was obtained from each study participant's legal guardian after being fully informed about the premise and purpose of the study. Airway epithelial cells were obtained through trans-laryngeal, non-bronchoscopic brushings of the tracheal mucosa of children via an endotracheal tube, isolated and fractionated out as previously described for downstream RNA,

protein and functional assessments.^{21,23-25} Bronchial brushings were obtained from 96 children with no history of asthma and 38 children with asthma undergoing elective surgery for non-respiratory-related conditions (Table 1). Children which were diagnosed as having an existing bacterial or viral infection were excluded from the study. Asthma was defined as clinician diagnosed asthma with a documented wheeze in the past 12 months and confirmed with positive responses to related questions on both the International Study of Asthma and Allergies in Childhood (ISAAC) and American Thoracic Society (ATS) questionnaires.^{26,27} All children with asthma had mild disease and although receiving either inhaled or oral glucocorticosteroids or β -agonists, none were taken for at least 1 month before surgery.

2.3 | Airway epithelial cell cultures

Airway epithelial cells were cultured in monolayers, as previously described^{1,21,28} as well as on transwells pre-coated with human placental collagen type IV.²⁹ Cells were grown in Bronchial-Air-Liquid Interface (B-ALI™, LONZA) growth media until confluent then changed to B-ALI™ differentiation media at air-liquid interface (ALI) for 28 days by which beating cilia as well as mucus production were well established.

2.4 | Rhinovirus and infection

Human rhinovirus minor serotype 1B (HRV-1B) was propagated in HeLa cells and viral titre determined as previously described.¹ Cells were infected with HRV-1B (2.5, 10 or 20×10^4 TCID₅₀/mL) or UV-inactivated sham control depending on the assay after which media was collected and cells utilized in downstream functional assays.

2.5 | Quantitative PCR (qPCR)

Gene expression of claudin-1, occludin and zonula occludens-1 (ZO-1) was determined via a two-step RT-PCR reaction as previously described²¹ using gene-specific primers (Table 2). *PPIA* was chosen as the housekeeping gene of choice as we have previously shown its uniformity of expression in human epithelial cells derived from healthy and disease phenotypes.³⁰

TABLE 1 Demographic data for participants recruited to the study

	Number	Gender (M/F)	Mean age (Range)	Allergies	Hay fever/Eczema/Both
Non-asthmatic	96	59/37	5.7 (1.9-16.4)	39	39
Asthmatic	38	24/14	6.9 (2.9-14.7)	38	25

M, male; F, Female.

TABLE 2 Oligonucleotide primer sequences used in the study

Gene	Primer	Sequence
Claudin	Forward	5'-GGCAGATCCAGTGCAAAGTC-3'
	Reverse	3'-TCTTCTGCACCTCATCGTCTT-5'
Occludin	Forward	5'-AGGCCTGATGAATTCAAACCG-3'
	Reverse	3'-CTGGGTAAAAAGAGTAGGCTGGC-5'
ZO-1	Forward	5'-GCCCCGTCCCCGCTCGCTCTC-3'
	Reverse	3'-CCGCCCGCTCCTCACGCCACAG-5'
PPIA	Forward	5'-TGAGCACTGGAGAGAAAGGA-3'
	Reverse	3'-CCATTATGGCGTGTAAGTCA-5'

2.6 | In cell western™ assay

Confluent cell monolayers which were infected with HRV-1B (2.5 and 20×10^4 TCID₅₀/mL) over 24-48 hours were subsequently fixed and assessed for TJ protein expression as previously described.²² Briefly, when confluent, growth media was aspirated and cell monolayer immediately fixed using 150 μ L of 3.7% formaldehyde for 20 minutes at room temperature (RT). Permeabilization of the cells was achieved by washing five times with 1X phosphate-buffered saline (PBS) containing 0.1% Triton-X-100 for 5 min/wash with gentle shaking. However, this was only performed on half of the experimental set-up to determine total protein expression. Concurrently, non-permeabilized cells of the experimental setup were incubated with only 1X PBS. This allowed for the determination of membrane protein expression. Blocking was performed for 90 minutes at RT using 150 μ L of Odyssey Blocking Buffer and subsequently, cells were then incubated with primary antibodies specific to zonal occludin-1 (1:200, Invitrogen), claudin-1 (1:200, Invitrogen) and occludin (1:200, Invitrogen) for 24 hours at 4°C. After this period, cells were washed five times at RT in 1X PBS containing 0.1% Tween-20 for 5 min/wash and incubated with near-infrared (NIR) fluorescent dye conjugated-secondary antibody (1:800, Licor Biosciences) containing cellular nuclear stains DRAQ5 (1:10 000, Biostatus Limited) and Sapphire700 (1:1000, Licor Biosciences) for 1 hour at RT with gentle shaking within a dark room. Cells were then washed for an additional 5 times in 1X PBS containing 0.1% Tween-20. Specific antibody staining for protein expression was then immediately visualized using a two NIR laser imaging system for the excitation of NIR fluorescent dye conjugated-secondary antibodies bound onto the target proteins. The obtained fluorescence is then converted to an integrated intensity values through the accompanying image analysis software which can then be normalized to cell numbers stained by the cellular nuclear stains (Licor Biosciences).

2.7 | Cytokine assays

Cytokine concentrations were measured in collected supernatants using commercial ELISA kits. Proteins measured included IL-4 and IL-13 (R & D Systems, Minneapolis, MN, USA). Briefly, each kit was a solid phase sandwich ELISA utilizing monoclonal antibodies specific for the target protein. Biotinylated secondary antibodies were used

to detect the immobilized capture antibodies and Streptavidin-Peroxidase used at the detection agent. Standard curves were generated using serial dilutions of recombinant IL-4 and IL-13 between the ranges of 31.2–2000 pg/mL and 93.8–6000 pg/mL, respectively, and sample concentrations determined from triplicate values.

2.8 | Immunocytochemistry

Primary AECs cytopun onto slides ($20\text{--}50 \times 10^3$ cells/slide) were stained for claudin-1, occludin and zonal occludin-1 (1:100, Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, United States) as previously described.²¹

2.9 | Immunofluorescence confocal microscopy

When confluent, ALI cultures were infected with HRV-1B (10×10^4 TCID₅₀/mL) for 24 hours and fixed using ice-cold methanol: acetone for 10 minutes at -20°C for occludin and zonal occludin-1 staining. Cultures were washed 3 times at 5 minutes each wash with 1X TBS followed by non-specific binding in buffer containing 10% normal goat serum (v/v), 10% foetal calf serum (v/v) and 1% bovine serum albumin (w/v) in 1X TBS for 30 minutes at room temperature. Cultures were incubated with primary antibodies for 1 hour, followed by washing with 1X TBS, 6 times at 10 minutes each wash. Cultures were then incubated with secondary antibodies for 1 hour in the dark and subsequently washed with 1X TBS for 1 hour, followed by counterstaining with Hoechst 33 342 for 5 minutes. All culture samples were mounted with fluorescent mounting medium containing DABCO. Control samples were included where the primary antibody was omitted to eliminate any non-specific secondary antibody binding from analysis.

Cultures were imaged using a Nikon A1 inverted confocal microscope (Nikon, Japan), with a Nikon Plan Apo VC 60x NA 1.4 oil immersion objective (Nikon, Japan) and NIS-AR Elements software (v4.2.22, Nikon, Japan), with six random fields acquired per sample. Individual channels were captured sequentially, where 405 nm laser was used for Hoechst 33 342 with collection through a 450/50 bandpass filter, whilst AF488 and AF568 were imaged with 488 nm and 561 nm lasers, and 525/50 and 585/50 bandpass filters, respectively. Z-stack images with step size of 0.5 μm were collected with a pinhole of 35.8 μm (1.2 A.U. for 488 nm laser), where the top and bottom of the stacks were determined visually due to the uneven nature of the membrane.

2.10 | Transepithelial electrical resistance (TER) measurement and paracellular permeability

Transepithelial electrical resistance (TER; R_T) measurement was performed on days 7, 14, 21 and 28 to ensure the formation and integrity of epithelial TJ between cells using an epithelial volt ohmmeter with silver chloride “chopstick” electrodes. Prior to measurement, the apical layer of the ALI cultures was washed with tissue-culture sterile 1X PBS and HEPES buffered Hank’s Balanced Salt Solution (HEPES-HBSS) added to both apical and basolateral sides to equilibrate the cultures. Triplicate measurements per well were made, and

the mean resistance was calculated. The resistance obtained from a cell-free culture insert was subtracted from the resistance measured across each cell monolayer and corrected for the surface area of the culture insert to yield the TER of the epithelial cells with values expressed in Ωcm^2 .

When confluent, ALI cultures were infected with HRV-1B (10×10^4 TCID₅₀/mL) for 24 hours and paracellular permeability subsequently assessed using a transepithelial permeability assay using fluorescein isothiocyanate labelled dextran (FITC-dextran) to determine apparent permeability of HRV-1B infected and non-infected control inserts as previously described.²² The apparent permeability of the epithelial monolayer to FITC-dextran from the apical to basolateral compartment (P_{app}) was then calculated following the general equation: $P_{app} = (dQ/dt) \times (1/AC_0)$ where dQ/dt is the steady-state flux, A is the surface area of the membrane and C_0 is the initial concentration in the donor compartment as previously described.³¹

2.11 | Statistics

Experiments were performed in at least triplicates, and all parametric data were analysed using Student’s t-test. For all non-parametric data, analysis was performed using a Mann-Whitney test. Data presented as mean $\pm$ SD and all P values less than .05 were considered to be significant.

3 | RESULTS

3.1 | Cohort characteristics

The clinical characteristics and demographics for study participants are provided in Table 1. Of the 134 study participants recruited, 56 were non-asthmatic, had no response to any of the allergens tested while 39 participants without asthma tested positive to one or more allergens. A further 38 participants were classified as asthmatic which was doctor diagnosed, with 25 showing one or more positive responses to the panel of tested allergens. All asthmatics had a history of documented wheeze in the 12 months preceding recruitment and were receiving glucocorticosteroids or β -agonists, although none were taken (inhaled or oral) for at least a month prior to surgery. All asthmatics had mild disease and had one or more positive response to a panel of allergens tested. We conducted an initial series of experiments on a subset of children with and without asthma, to assess whether atopy had any influence on paracellular permeability to FITC-dextran (20 kDa). Results showed no significant difference in the observed barrier permeability between non-atopic and atopic subjects with and without mild asthma (Figure 1). Thus, all recruited subjects were classified as “non-asthmatic” or “asthmatic” irrespective of atopy and all analysis performed comparing these two groups.

3.2 | Basal tight junction gene expression—Ex vivo

We first determined the basal expression levels of three sentinel TJs, *claudin-1*, *occludin*, which are pivotal in the regulation of barrier integrity

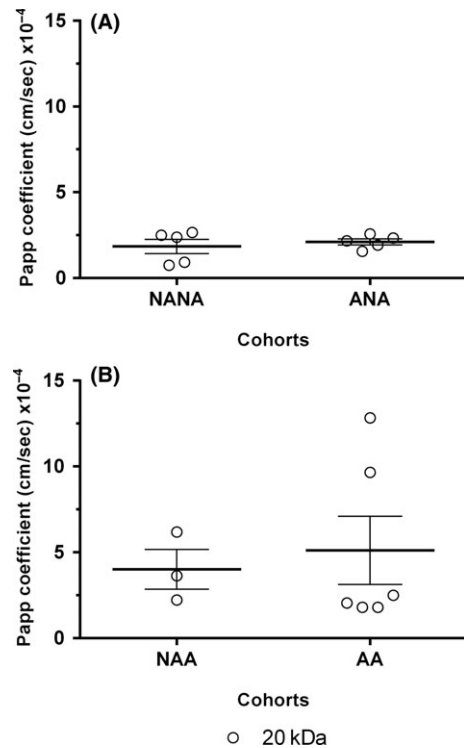


FIGURE 1 Transepithelial permeability in ALI cultures from AECs of children with and without asthma further categorized based on atopy: A, Epithelial permeability to FITC-dextran 20 kDa was not significantly different between AEC ALI cultures of children who were non-atopic non-asthmatic (NANA) and atopic non-asthmatic (ANA). B, No significant difference in transepithelial permeability towards FITC-dextran 20 kDa was similarly observed in AEC ALI cultures of children who were non-atopic asthmatic (NAA) and atopic asthmatic (AA). Data presented as mean $\pm$ SD from 3 to 6 independent experiments

and ZO-1 which is central in supporting the formation of TJs. Interestingly, results showed significantly higher levels of basal TJ mRNA expression of *claudin-1* (1.4-fold) and *occludin* (2.6-fold) in AECs of asthmatic children compared to their non-asthmatic counterparts (Figure 2A & B; $P < .05$) but ZO-1 mRNA expression was not statistically different (Figure 2C). In comparing the three TJ, *claudin-1* was the most highly expressed (non-asthmatic, $7.86\text{AU} \pm 0.95$; asthmatic, $11.28\text{AU} \pm 1.03$), followed by *occludin* (non-asthmatic, $2.26\text{AU} \pm 0.18$; asthmatic, $6.04\text{AU} \pm 0.34$) and ZO-1 being the least expressed (non-asthmatic, $1.07\text{AU} \pm 0.12$; asthmatic, $1.16\text{AU} \pm 0.1$) (Figure 2).

3.3 | Basal tight junction protein expression—Ex vivo

We next investigated whether increased levels of ex vivo TJ mRNA led to increased protein expression in ex vivo cytospun cells. Of significance, we found that in contrast to mRNA, staining intensities of membrane bound *claudin-1*, *occludin* and ZO-1 were higher in AECs of children without asthma compared to AECs of asthmatic children (Figure 3). In comparing relative expression of TJs, staining intensities of *claudin-1* and *occludin* expression were similar and greater

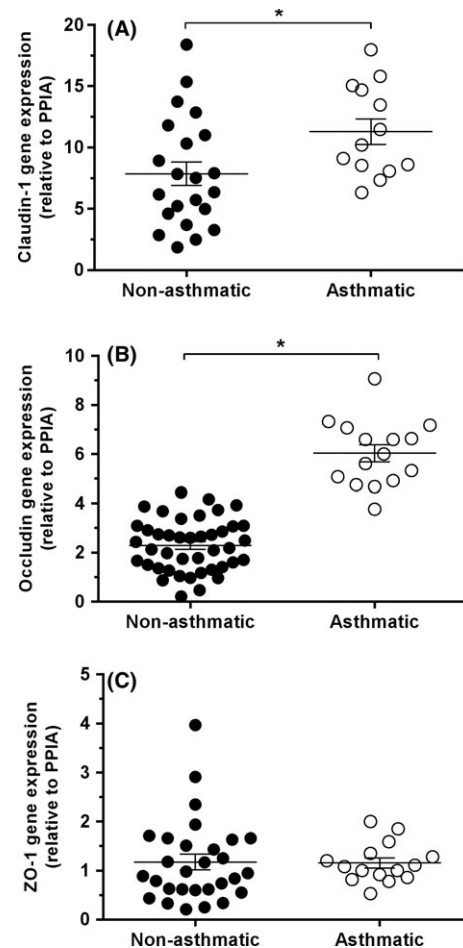


FIGURE 2 Ex vivo mRNA expression of TJs from AECs of children with and without asthma: mRNA expression of *claudin-1* (A), *occludin* (B) and ZO-1 (C) in AECs of non-asthmatic and asthmatic cohorts was quantified by RT-qPCR. (A) mRNA expression of *claudin-1* was significantly higher in children with asthma compared to the non-asthmatics. (B) mRNA expression of *occludin* was observed to be significantly higher in children with asthma. (C) No significant differences were observed between the two cohorts for expression of ZO-1. (A–C) mRNA expression of *claudin-1* was most abundantly expressed compared to *occludin* and ZO-1. *Statistical significance relative to non-asthmatics ($P < .05$)

than ZO-1 expression (Figure 3—non-asthmatic). In AEC from asthmatic children, greatest staining intensity was observed for *occludin*, followed by ZO-1 and *claudin-1* showing similar diminished staining intensities respectively (Figure 3—asthmatic).

3.4 | Basal tight junction protein expression—In vitro

Having established that AECs from children with asthma showed marked decrease in TJ protein expression ex vivo, we assessed TJ protein expression in cultured cells. Consistent with our observations ex vivo, total protein expression of *claudin-1*, *occludin* and ZO-1 was lower in AECs of asthmatic children compared to non-asthmatic children (data not shown). When basal membrane protein expression was assessed, *claudin-1*, *occludin* and ZO-1 membrane expression

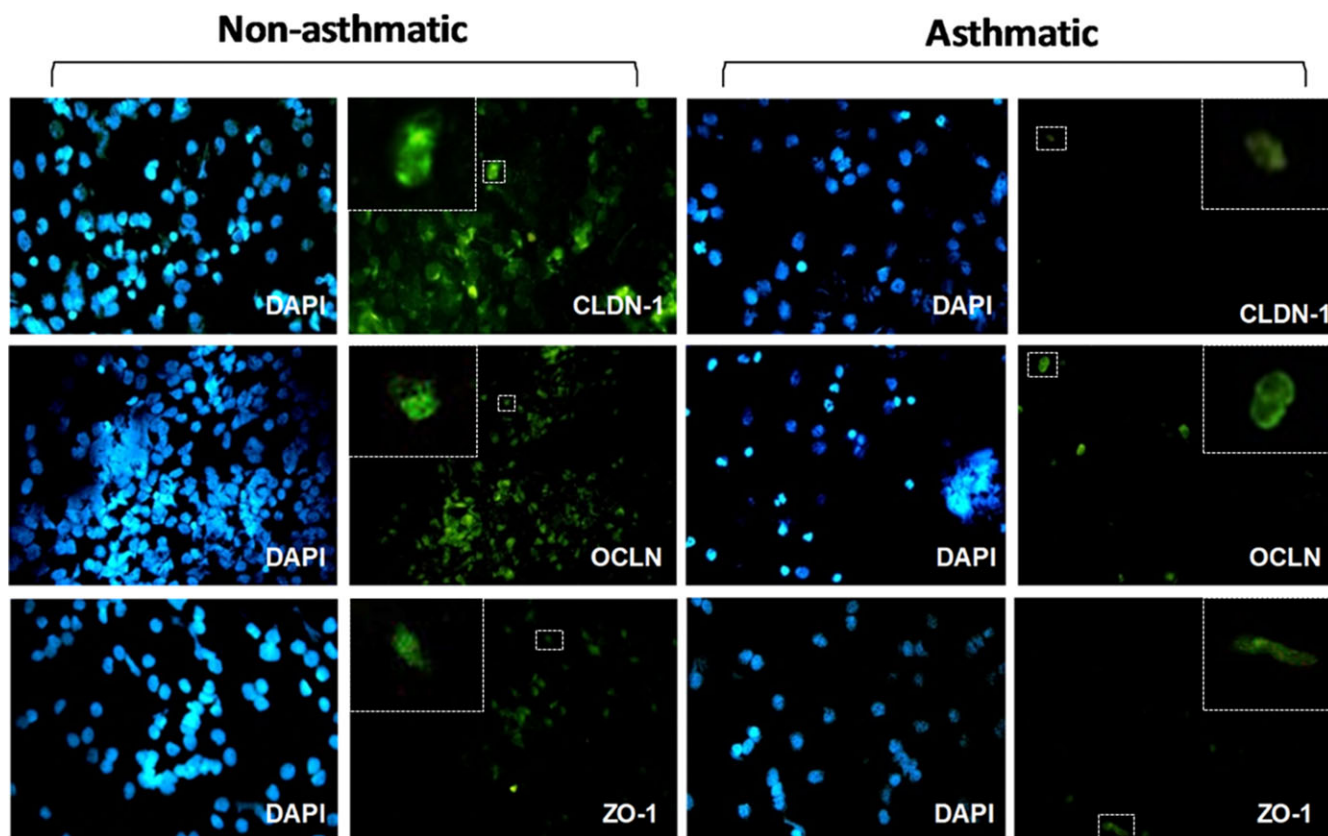


FIGURE 3 Ex vivo membrane protein expression of TJs are lower in AECs from children with asthma: Cytopspins were prepared from cells obtained from children with and without asthma. Immunocytochemical staining of claudin-1, occludin and ZO-1, respectively, in AECs from children without asthma demonstrates strong membrane staining intensity of each tight junction protein. In contrast, immunocytochemical staining of claudin-1, occludin and ZO-1, respectively, within the asthmatic cohort demonstrates a marked decrease in membrane staining intensity of each tight junction

was significantly lower in AECs of asthmatic children compare to non-asthmatic counterparts (Figure 4 A-D; $P < .05$). When we extended this over 72 hours, basal membrane TJ protein expression of claudin-1, occludin and ZO-1 in AECs of asthmatic children was not significantly different (Figure 4 E,F) and was concomitant with epithelial permeability (data not shown).

3.5 | HRV infection causes tight junction dissociation

Twenty-four hours after HRV-1B infection, we saw a significant decrease in the expression of all 3 TJ proteins in AECs of children with and without asthma (Figure 5A-C; 24 hours). However, after 48 hours, expression of TJ proteins recovered in AECs of non-asthmatic children, with levels approaching those seen in non-infected cells (Figure 5). In contrast, expression of all TJ protein in AECs of asthmatic children remained low for up to 48 hours after infection (Figure 5A-C; 48 hours).

3.6 | Effect of HRV infection on cytokine release

Triplicate measurements were performed to assess the production of IL-4 and IL-13 following HRV-1b infection in AECs obtained from

five non-asthmatic and asthmatic children, respectively. There was no significant IL-4 and IL-13 release detected at the titre used in this investigation (data not shown).

3.7 | Effect of HRV infection on membrane tight junction expression in ALI cultures

We performed immunofluorescence confocal microscopy to examine whether a decrease in ZO-1 and occludin expression was similarly observed in ALI cultures after HRV infection. The presence of a continuous, uninterrupted membrane protein expression of both ZO-1 (Figure 6A and I) and occludin (Figure 6B and J) was observed in the apical regions of differentiated ALI cultures of non-asthmatic and asthmatic children, respectively (Figure 6D and L; white arrows). Interestingly, expression was uniform and abundant in ALI cultures from non-asthmatic children (Figure 6A-D) but was less even, with regions of lower intensity for some tight junctions, particularly occludin, in asthmatic children (Figure 6I-L). Although tight junction expression remained unchanged in many areas following HRV infection in non-asthmatic children, expression did alter in specific regions where a loss of staining was observed at a defined cell edge, typically between two cells (Figure 6E-H; white and red arrows). This disruption was far greater in cultures of AECs from children with

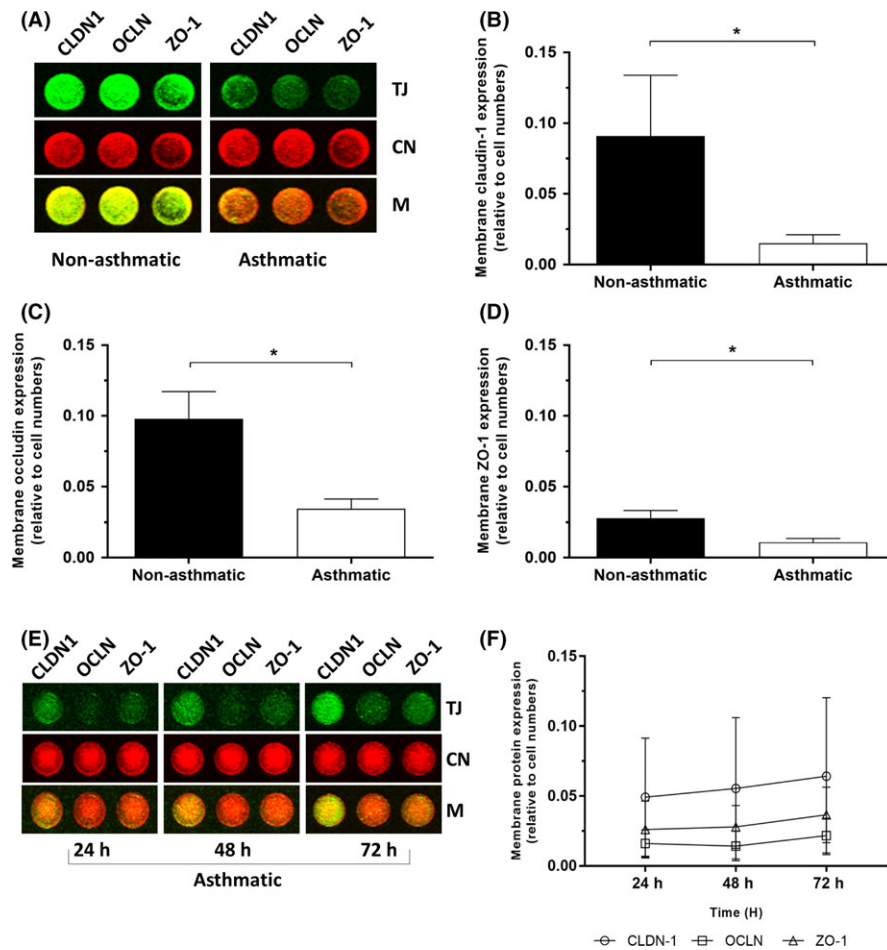


FIGURE 4 Diminished basal membrane TJ protein expression in AECs from children with asthma: A, Representative images of In-Cell Western assay in non-asthmatic ($n = 22$) and asthmatic ($n = 11$) AECs illustrating signal intensity of TJ proteins and cell and nuclear stains. (TJ: TJ protein stain [CLDN1: claudin-1; OCLN: occludin; ZO-1: zonula occluden-1]; CN: cell and nuclear stain; M: merged). B, Airway epithelial cells from children with asthma expressed significantly lower levels of membrane TJ claudin-1. C, Membrane occludin was also significantly lower in AECs of children with asthma compared to non-asthmatics. D, Similarly, AECs from children with asthma also showed lower levels of membrane ZO-1 expression when compared to AECs of non-asthmatics. (B-D) Similar levels of membrane protein expression were observed for claudin-1 and occludin in AECs of children without asthma and were significantly elevated compared to membrane ZO-1. Interestingly, AECs of children with asthma demonstrated significantly elevated level of basal membrane occludin protein expression compared to claudin-1 and ZO-1. No significant difference in membrane protein expression was observed between claudin-1 and ZO-1 in AECs of children with asthma. E, Representative images of In-Cell Western assay in asthmatic ($n = 11$) AECs illustrating signal intensity of TJ proteins and cell and nuclear stains over 72 h. (TJ: TJ protein stain [CLDN1: claudin-1; OCLN: occludin; ZO-1: zonula occluden-1]; CN: cell and nuclear stain; M: merged). F, Membrane protein expression for claudin-1, occludin and ZO-1 in asthmatic AECs was not significantly different when assessed over 72 h. Data were normalized to cell numbers and presented as mean $\pm$ SD. *Statistical significance relative to non-asthmatic cohort ($P < .05$)

asthma with loss of tight junction expression over a region of cells (Figure 6M-P; white and red arrows). Interestingly in other areas, tight junction expression remained unaltered in response to virus and exhibited similar intensity of staining as healthy controls.

3.8 | Effect of HRV infection on TER and permeability in well-differentiated air-liquid interface (ALI) cultures

Having shown that HRV infection induced TJ dissociation, we next sought to determine the effect on barrier function. In addition to 20 kDa FITC-dextran, we also assessed a smaller, 4kDa sized FITC-dextran. We demonstrated higher R_T in non-infected ALI cultures

from children without asthma ($851.4 \Omega\text{cm}^2 \pm 156.6$) and which, following HRV infection, was halved ($402.8 \Omega\text{cm}^2 \pm 54.6$) (Figure 7A-TER; $P < .05$). Consistent with the decrease in R_T , 24 hours after HRV infection, ALI cultures from non-asthmatic children demonstrated a significant increase in permeability to the smaller, 4 kDa ($22.4 \times 10^{-4} \text{ cm/s} \pm 8.4$) but not the larger, 20 kDa ($3.1 \times 10^{-4} \text{ cm/s} \pm 0.5$) FITC-dextran compared to non-infected controls ($9.2 \times 10^{-4} \text{ cm/s} \pm 2.4$ and $2.6 \times 10^{-4} \text{ cm/s} \pm 0.4$, respectively) (Figure 7A).

We also showed higher R_T in non-infected ALI cultures of children with asthma ($611.5 \Omega\text{cm}^2 \pm 350.7$) which, following infection, decreased threefold ($183.7 \Omega\text{cm}^2 \pm 112.8$) (Figure 7A-TER; $P < .05$). Concomitant with a decrease in R_T , 24 hours after infection ALI

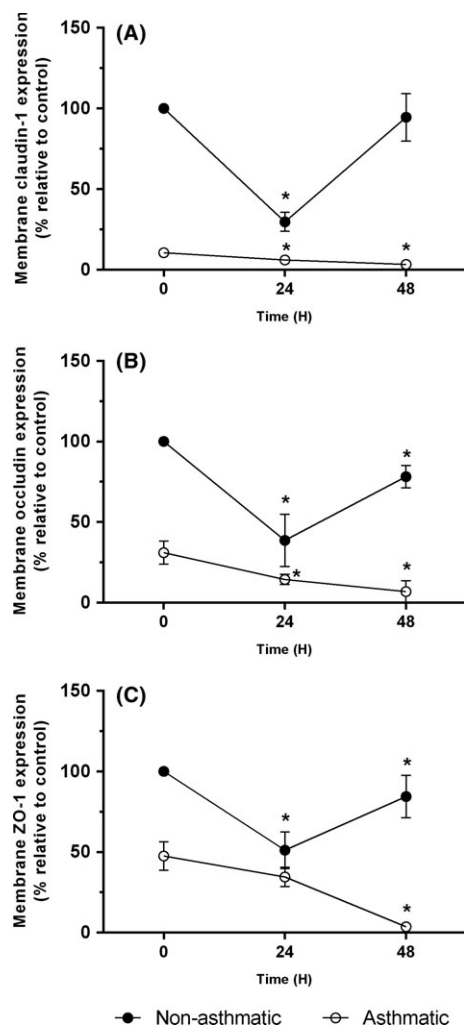


FIGURE 5 Membrane TJ protein expression over time in AECs from non-asthmatic and asthmatic children following viral infection: Decreased membrane protein expression of claudin-1 (A), occludin (B) and ZO-1 (C) was observed following infection with HRV-1B at 24 h in the non-asthmatic cohort (●). However, at 48 h post-infection, a significant increase towards non-infected levels was observed in non-asthmatic airway epithelial cells. Significant difference in membrane protein expression of all 3 TJs was observed in asthmatic airway epithelial cells (○) at 24 h post-infection compared to 0 h. Further significant decrease in expression was observed at 48 h following infection compared to 0 h. Data presented as mean $\pm$ SD percentage relative to control from 4 to 8 independent experiments. *Statistical significance relative to non-infected controls ($P < .05$)

cultures from children with asthma showed significant increase in permeability only to the 4 kDa (31.4×10^{-4} cm/s $\pm$ 5.5) but not the 20 kDa (6.2×10^{-4} cm/s $\pm$ 2.7) FITC-dextran compared to non-infected controls (15.4×10^{-4} cm/s $\pm$ 3.2 and 6.1×10^{-4} cm/s $\pm$ 1.8, respectively) (Figure 7A; $P < .05$).

When comparing between cohorts, significantly lower R_T was observed in ALI cultures of asthmatic children compared to non-asthmatics. Following infection, significantly lower R_T was shown in ALI cultures from children with asthma compared to those without (Figure 7A; R_T). Epithelial permeability to the smaller, 4 kDa FITC-

dextran at non-infected and infected states was significantly different between cohorts. Moreover, permeability to the larger, 20 kDa FITC-dextran was significantly greater in ALI cultures from children with asthma following HRV infection (Figure 7A; Papp coefficient). When we extended the infection period to 48 hours, we observed a significant decrease in transepithelial permeability in ALI cultures of non-asthmatic children (Figure 7B; $P < .05$) while epithelial permeability to the smaller, 4 kDa FITC-dextran in ALI cultures of asthmatic children remained significantly elevated at 48 hours following infection (Figure 7C; $P < .05$). This was concomitant with the expression levels of membrane TJ proteins in AECs of non-asthmatic approaching non-infected levels and a sustained decrease in membrane TJ proteins in AECs of asthmatic children 48 hours following HRV infection (Figure 7B, C).

4 | DISCUSSION

We investigated TJ gene and protein expression as well as functional differences between AECs of children with or without asthma and in response to HRV. Our data demonstrated that, at baseline, AECs of children with asthma expressed significantly higher levels of *claudin-1* and *occludin* mRNA but lower levels of claudin-1, occludin and ZO-1 protein compared to non-asthmatic children. Infection with HRV led to decreased expression of TJs in AECs of children with and without asthma, although the effect was sustained in AECs from children with asthma. Epithelial resistance was also significantly lower in AECs from children with asthma and HRV infection resulted in further loss of resistance concomitant with increased permeability to larger-sized molecules. These data support the hypotheses that the epithelium is intrinsically more permeable in asthma and that HRV leads to decreased expression of TJs which in turn reduces epithelial resistance and increases permeability.

The molecular mechanisms involved in the regulation of epithelial permeability are poorly understood. The apico-lateral junctional complexes consisting of tight and adherens junctions, contribute significantly to barrier function as well as providing a link to the cellular cytoskeleton via various adaptor proteins. Although previous studies have investigated levels of TJ gene and protein expression following various insults,^{11,32,33} these studies were performed using either established epithelial cell lines or AECs from adults. Hence, there is a paucity of data on the intrinsic gene and protein expression of epithelial tight junction complexes in asthmatic children. This is of critical importance as asthma often occurs early in life and is a critical determinant of disease progression into adulthood.³⁴⁻³⁷

Our observations of TJ gene and protein expression in primary AECs of non-asthmatic children was concomitant with our earlier findings of a direct association between TJ gene and protein expression in modified non-asthmatic human airway epithelial cells.²² We extended our investigation here to include asthmatic children and made the significant observation of differences between baseline expression of TJ gene and protein in AEC of children with and without asthma suggesting an intrinsic alteration in epithelial barrier

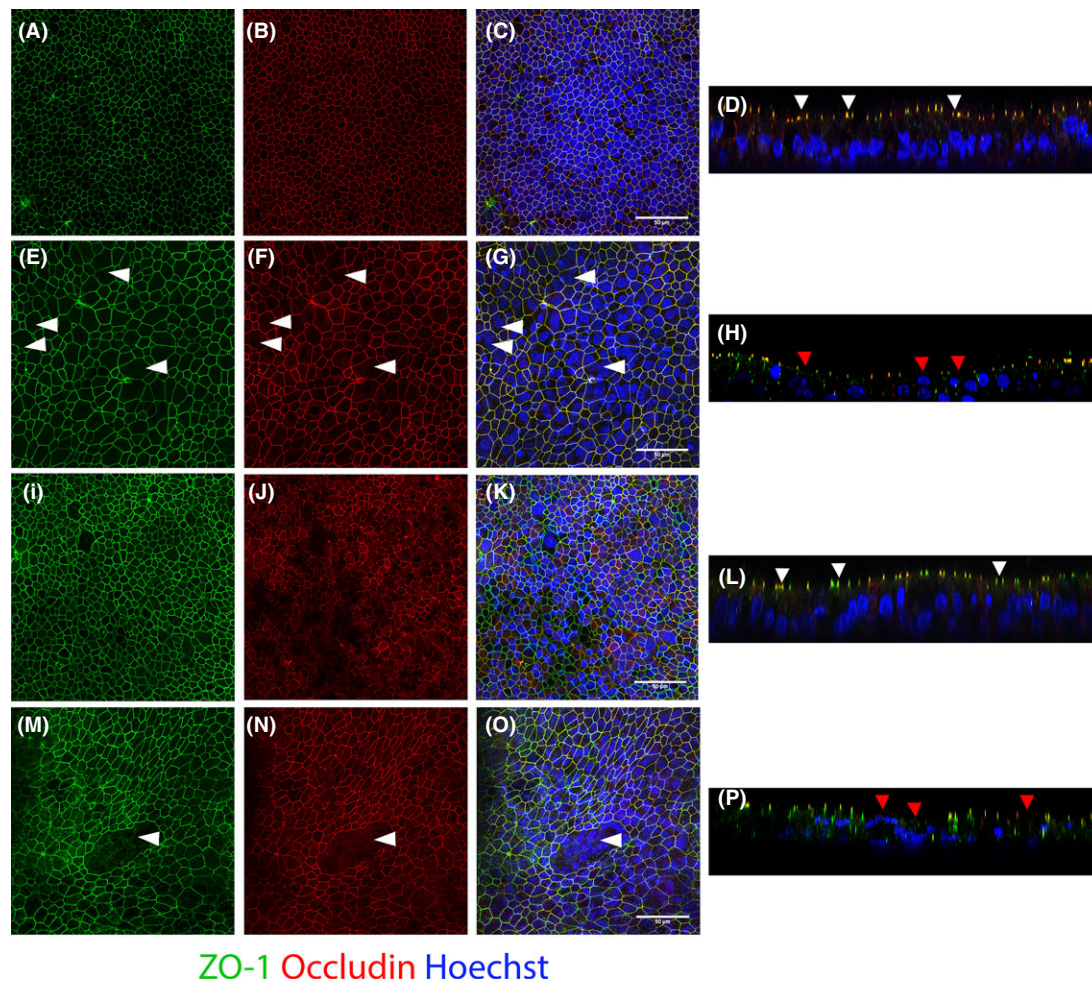
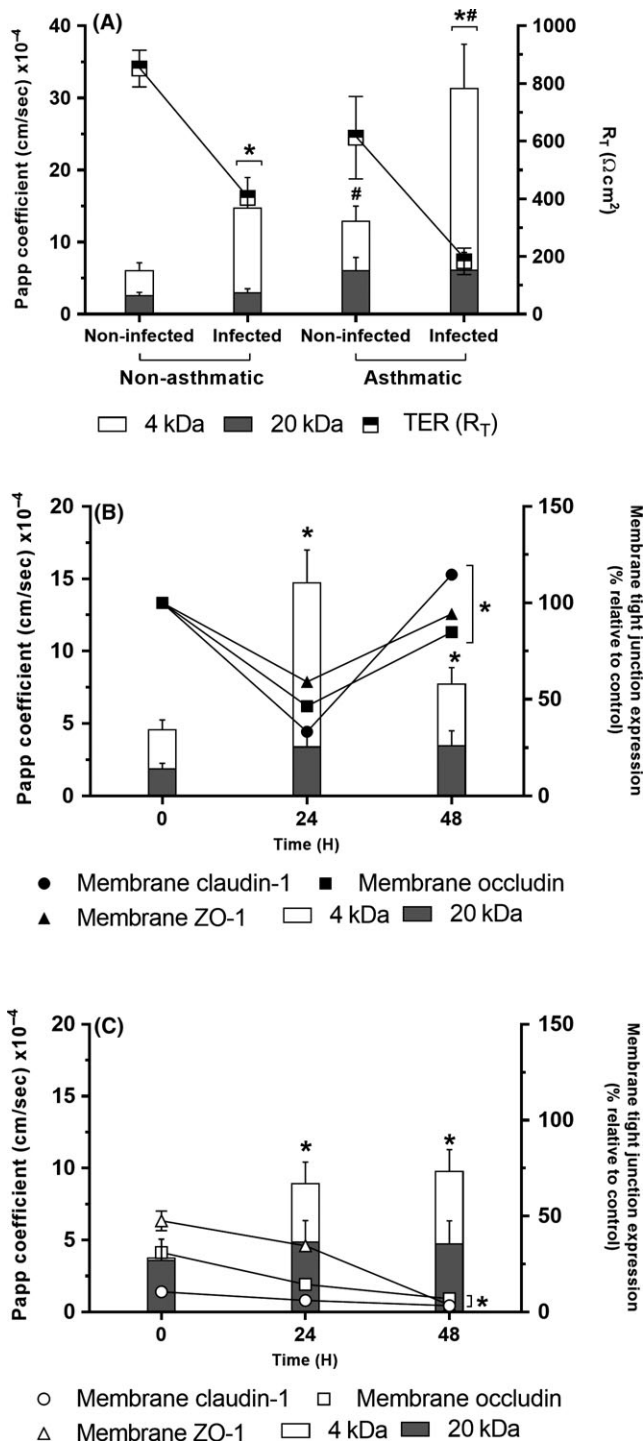


FIGURE 6 Membrane TJ protein expression in ALI cultures generated from AECs of children with and without asthma following viral infection: Air-liquid interface cultures of airway epithelial cells derived from non-asthmatic (A-H) and asthmatic (I-P) children were assessed for zonula occluden-1 (ZO-1) (green; A,E,I and M) and occludin (red; B, F, J and N). Merged image of ZO-1 and occludin (C,G,K and O) demonstrates the areas of tight junction expression. Confocal imaging of ZO-1 and occludin in representative ALI samples of AECs from non-infected non-asthmatic (A-D) and asthmatic (I-L) children demonstrates continuous uninterrupted membrane protein expression of both TJ proteins at the apical region of the differentiated culture (white arrows; D and L). Confocal imaging of ZO-1 and occludin in representative ALI samples of AECs from infected non-asthmatic (E- H) and asthmatic (M- P) children demonstrates loss in expression of membrane protein expression of both TJ proteins at the apical region of the differentiated culture (white and red arrows; E-H and M-P) with asthmatic cohorts showing greater areas of junctional loss (Objective 60x N.A 1.49 Oil Immersion)

integrity in these children. Specifically, our observations of increased TJ gene expression in AEC of children with asthma did not translate into increased protein levels, which we found to be significantly decreased. The reasons for this discordance are presently unclear and could be attributed to different factors such as the pleiotropic transcriptional repressor Snail, which has been shown to be involved in post-transcriptional events resulting in decreased synthesis of claudin-1 protein but having no effect on transcription of claudin-1 mRNA.³⁸ In addition, T-helper type-2 cytokines such as IL-4 and IL-13, which are associated with asthma pathogenesis,^{39,40} have also been shown to inhibit cellular migration and wound healing, respectively,⁴¹ thereby potentially altering epithelial barrier integrity and consequently, decreased barrier function. Although IL-4 and IL-13 levels were previously demonstrated to be elevated in stimulated bronchoalveolar lavage cells obtained from asthmatic patients,¹⁷ our

observation of a lack of IL-4 and IL-13 release by asthmatic AECs following HRV infection in vitro further implicates the possible interactive and contributory role of both the innate and adaptive immune cells in regulating airway epithelial barrier integrity. Interpreted collectively, the lack of IL-4 and IL-13 release by AECs observed along with augmented release in stimulated bronchoalveolar lavage cells of asthmatic patients shown previously, suggests a novel approach of inhibiting T-helper type-2 cytokines in generating an effective epithelial barrier integrity as a therapeutic potential in the treatment of HRV induced asthma exacerbations.

Airway epithelial cells serve as the primary host cell for viral replication as well as initiating the innate and adaptive immune responses. Previous studies have demonstrated that primary AECs are susceptible to viral infections and that HRV is able to successfully replicate resulting in a cytotoxic effect.^{1,28,42-44} Human



rhinovirus is the most common cause of acute exacerbations of asthma^{13,45-48}; however, very little is known about the effects of HRV on TJ complex expression and function in AECs, particularly in children with asthma. We have recently demonstrated the effects of HRV on epithelial TJ disassembly and function in modified non-diseased human AEC²² and that AECs from asthmatic children have impaired responses to HRV.¹ Collectively and in this context, this investigation has extended our initial assessment of AEC responses to specifically assess barrier integrity and function in this study. In

FIGURE 7 Transepithelial electrical resistance (R_T) and permeability in ALI cultures from AECs of children with and without asthma: A, Corroborating the reduced R_T , an increase in transepithelial permeability towards both FITC-dextran 4 and 20 kDa was observed in AEC ALI cultures of children without asthma following 24 h HRV-1B infection. Epithelial permeability towards FITC-dextran 4 kDa was significantly higher when compared to FITC-dextran 20 kDa in non-infected and infected AEC ALI cultures of children without asthma. Significant reduction in R_T was concomitant with significant difference in transepithelial permeability towards FITC-dextran 4 kDa but not 20 kDa in AEC ALI cultures of children with asthma following infection. However, permeability towards FITC-dextran 4 kDa was significantly higher when compared to FITC-dextran 20 kDa in non-infected and infected AEC ALI cultures of children with asthma. Data presented as mean $\pm$ SD from 6-8 independent experiments. B, Significant decrease in membrane protein expression of claudin-1 (●), occludin (■) and ZO-1 (▲) was observed following infection with HRV-1B at 24 h in the non-asthmatic cohort. However, at 48 h post-infection, a significant increase towards non-infected levels was observed in non-asthmatic airway epithelial cells. This was concomitant with a significant increase in transepithelial permeability to FITC-dextran 4 kDa at 24 h following infection and a subsequent decrease in epithelial permeability at 48 h. C, Significant difference in membrane protein expression of claudin-1 (○), occludin (□) and ZO-1 (△) was observed in asthmatic AECs at 24 h post-infection compared to 0 h. Further significant decrease in expression was observed at 48 h following infection compared to 0 h and was concomitant with a significantly sustained decrease in epithelial permeability at the same time points. *Statistical significance relative to non-infected control ($P < .05$)

the present study, we demonstrated significantly reduced protein expression of claudin-1, occludin and ZO-1 24 hours post-infection suggesting a dissociation of TJs complexes. Moreover, while TJ protein expression returned to baseline levels in AECs from children without asthma, this decreased expression was sustained over 48 hours in AECs from asthmatic children. This is further supported by a recent study in adults demonstrating similar observations of reduced junction expression following HRV infection.⁴⁹ We also demonstrate reduced basal epithelial resistance in AEC of asthmatic children compared to non-asthmatics concomitant with a significant increase in transepithelial permeability, suggesting that in asthma, HRV infection could contribute to the sustained functional disorganization of TJ complexes. Moreover, the increases in permeability to the larger-sized molecules suggest that HRV infection would also facilitate increased passage across the epithelium by allergens or pathogens and their interaction with the internal milieu of the airways, thus further enhancing airway inflammation and structural remodelling.^{1,50-52} Our ongoing studies indicate that epithelial permeability to small-sized molecules in AECs from children without asthma decreases at 48 hours post-infection and were concomitant with TJ protein levels returning to baseline levels. Based on these findings, we further demonstrated that in AECs from asthmatic children, a prolonged decrease in expression of TJ following HRV infection as shown in this study, ultimately resulted in a sustained increase in epithelial permeability. This sustained elevation in

permeability could potentially increase epithelial susceptibility towards the trafficking of pathogens into the subepithelial space.

In summary, this work has been the first to characterize and demonstrate that the epithelial barrier integrity is intrinsically altered in AECs from asthmatic children compared to non-asthmatic children which is indicative of an impaired epithelial barrier function. Furthermore, this study has provided new data on the direct effects HRV infection has on junctional protein expression and disruption in barrier function, with infection robustly reducing barrier integrity in AECs of children with asthma. In a broader context, a compromised barrier would facilitate the movement of more pathogens across the epithelium and their interaction with the basolateral receptors, thus potentially enhancing airway inflammation and prolonging exacerbation episodes in paediatric asthma.

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AUTHORS' CONTRIBUTIONS

AK, GRZ, ANL, KL, DAK and SMS conceived and designed the experiments. KL and AGB performed the experiments. KL, AK, DAK and SMS analysed the data. AGB and PJR provided technical assistance and analysis. KL drafted the manuscript. PJR, LWG, TI, GRZ, ANL, KML, KMM, EKS, NCS, ENS, DAK, AK and SMS performed critical evaluation of manuscript. FJL performed sample collection. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

None of the authors have conflicts of interests financially or otherwise to declare.

ORCID

A. Kicic  <http://orcid.org/0000-0002-0008-9733>

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