EFFECT OF HUMAN RHINOVIRUS INFECTION ON AIRWAY EPITHELIUM TIGHT JUNCTION PROTEIN DISASSEMBLY AND TRANSEPITHELIAL PERMEABILITY

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ABSTRACT

Rationale: No studies have assessed the effects of HRV infection on epithelial tight junctions (TJ) and resultant barrier function.

Aim: To correlate viral infection with TJ disassembly, epithelial barrier integrity and function.

Methods: Human airway epithelial cells were infected with HRV-1B at various Tissue Culture Infectivity Doses (TCID₅₀) over 72 hours. HRV replication was assessed by qPCR while cell viability and apoptosis were assessed by proliferation and apoptotic assays respectively. Protein expression of claudin-1, occludin and zonula occludens-1 (ZO-1) was assessed using In-Cell™ Western assays. Transepithelial permeability assays were performed to assess effects on barrier functionality. RT² Profiler focused qPCR arrays and pathway analysis evaluating associations between human TJ and anti-viral response were performed to identify potential interactions and pathways between genes of interests.

Results: HRV-1B infection affected viability that was both time and TCID₅₀-dependant. Significant increase in apoptosis and viral replication post infection correlated with viral titre. Viral infection Significant decrease in claudin-1 protein expression at the lower TCID₅₀ while significant decrease in all 3 TJ protein expression occurred at higher TCID₅₀. Decrease in protein expression was concomitant with significant increase in epithelial permeability of FITC-dextran 4 and 20 kDa. Analysis of focused qPCR arrays demonstrated significant decrease in ZO-1 gene expression. Furthermore, network analysis between human TJ and anti-viral response genes revealed possible interactions and regulation of TJ genes via IL-15 in response to HRV-1B infection.

Conclusion: HRV-1B infection directly alters human airway epithelial TJ expression leading to increased epithelial permeability potentially via an anti-viral response of IL-15.

Keywords: Airway epithelial cells, tight junctions, human rhinovirus, epithelial barrier

INTRODUCTION

A pseudostratified mucosal barrier consisting of a multitude of cell types functions as the initial protective interface between the internal milieu of the lung and the external environment of micro-organisms, aeroallergens and noxious gases within the human respiratory tract. A range of junctional complexes including tight junctions, adherens junctions, GAP junctions, desmosomes and connexins maintain this protective barrier. Tight junctions (TJ) are typically located at the apical borders of adjacent epithelial cells where they serve to regulate the movement of ions and solutes as well as to prevent unwanted migration of pathogens and their products to the sub-epithelial space (1). A compromised epithelial barrier function, including disruption in the TJ protein, zonula occludens protein-1 (ZO-1) has been shown to increase the paracellular traffic of pathogenic molecules into the lung interstitium (2). Furthermore, a compromised barrier integrity has also been correlated to the release of pro-inflammatory cytokines including interleukin-8 (CXCL8), eotaxin (CCL11), regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5), interferon-inducible protein-10 (CXCL10) and macrophage inflammatory protein 1 α (CCL3) (3).

Airway barrier integrity may become compromised after exposure to injurious stimuli including airborne pollutants, aeroallergens, bacteria and respiratory viruses. Respiratory viruses such as human rhinovirus (HRV) are common insults to the airway epithelium. Despite evidence suggesting that a significant reduction in TJ protein expression and a disassembly of the apical junction proteins is associated with an attenuated barrier function (2, 4, 5), the pathways involved in the regulation of airway epithelial TJ proteins following HRV infection and the effects on barrier function are not well understood. Furthermore, altered expression of a single TJ is commonly reported within the literature and most studies rarely assess the interactions and expression of multiple TJs following airway insults. Hence, translation of such research findings often remains a challenge due to the multitude of junctional proteins and the potential compensatory effects in response to airway insults. In addition, there is evidence demonstrating the direct expression relationships between different TJs and

epithelial integrity (6, 7). Three of note include; claudin-1, which is ubiquitously expressed within the human airways (8); occludin and ZO-1, both of which have been demonstrated to being integral in the maintenance of epithelial integrity (9, 10).

Hence, this investigation was conducted to investigate any direct correlation between HRV infection, altered TJ expression and transepithelial permeability by utilizing hTERT modified human airway epithelial cells (AEC). Specifically, this study tested the hypothesis that TJ protein expression of claudin-1, occludin and ZO-1 would be significantly reduced following HRV infection resulting in increased epithelial permeability. We performed all experiments on undifferentiated cultures grown in monolayer culture, since it has previously been shown that differentiated cultures are more resistant to HRV infection (11) and that basal cells are the primary target of HRV (12). This allowed us to clearly identify the dissociative effects of HRV infection on multiple epithelial junctional proteins as well as correlating these effects with attenuation of barrier functionality.

Our results show a time and TCID₅₀-dependant effect of viral infection on cell viability, while infection resulted in significant increases in both cellular apoptosis and viral replication that correlated to viral titres. In addition, protein expression for claudin-1, occludin and ZO-1 was significantly reduced following HRV-1B infection. Concomitant with this decrease, epithelial permeability was significantly increased following infection. Furthermore, expression of 58 genes encoding epithelial tight junctional proteins such as claudin-1, occludin and ZO-1 were down-regulated following HRV infection. Gene network reconstruction identified a signalling pathway in which gene expression of TJ were potentially mediated via an anti-viral response of IL-15. Collectively, these observations show that infection with HRV-1B in healthy AEC directly alters TJ protein expression, resulting in a disruption of epithelial barrier integrity through junctional disassembly, subsequently leading to an attenuated barrier function.

MATERIALS AND METHODS

Patient and sample collection

Airway epithelial cells were obtained from 3 children with no history of asthma (Table 1) undergoing elective surgery for non-respiratory-related conditions. Children with an existing bacterial or viral chest infection were excluded from the study. Primary AECs were isolated and established as previously described (13-16). The study was approved by both Princess Margaret Hospital for Children and St John of God Hospital's Human Ethics Committee.

Cells

Human airway epithelial cells, modified by hTERT to express exogenous telomerase, (NuLi-1) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in bronchial epithelial basal medium (BEBM) supplemented with SingleQuot additives (Lonza) and maintained at 37°C in an atmosphere of 5% CO₂ / 95% air. For expansion, cells were washed with HEPES buffered saline solution (HBSS) (Lonza) and detached from the culture vessels by incubating with Trypsin-Ethylenediaminetetraacetic acid (T-EDTA) (Lonza) solution for 4 minutes (min) at 37°C. Culture vessels were then washed with Trypsin Neutralizing Solution (Lonza) and culture media and the resulting cell suspension collected and centrifuged at 500 g for 7 min at 4°C. The supernatant was then aspirated and the cell pellet re-suspended in the growth medium. A total cell count and viability stain was also performed. Cells were plated at 2×10^4 cells/cm² into new tissue culture vessels and maintained as described above.

Rhinovirus and titrations

Human rhinovirus minor serotype 1B (HRV-1B) was provided by Professor Peter Wark (Hunter Medical Research Institute, Newcastle, NSW, Australia). To determine viral titers, MRC-5 cells were grown in 96-well flat bottom tissue culture plates and infected with 10-fold serial dilutions of virus

in 8 replicates. Culture plates were incubated for 5 days and the wells assessed daily for cytopathic effect (CPE) by microscopic evaluation. The tissue culture infectivity dose (TCID₅₀) of the virus was calculated based upon the 5 day CPE utilizing the Reed Muench method. All HRV-mediated responses were confirmed to be specific to active virus, since UV-inactivation of viral stocks for 120 min resulted in a lack of replication in MRC-5 cells (data not shown).

Infection of cell cultures

Cells were grown on tissue culture plates or inserts, were infected with HRV-1B or an equivalent volume of UV-inactivated sham control and incubated over 72 hours depending on the assay. Briefly, growth media was replaced with fresh basal media and subsequently infected with HRV-1B at a 50% Tissue Culture Infectivity Dose (TCID₅₀) of 2.5, 10, 20, 40 or 80 x 10⁴ TCID₅₀/ml. After each incubation period, media was collected and stored at -80°C and cells utilized in various downstream functional assays. Primary AECs were similarly grown on tissue culture plates or inserts and infected with HRV-1B at a 50% Tissue Culture Infectivity Dose (TCID₅₀) of 10 x 10⁴ TCID₅₀/ml for 24 hours. We have previously demonstrated a dose and time dependent effect of HRV-1B on cell viability above this dose on primary AECs (17). At the end of the incubation period, media was similarly collected, stored at -80°C and cells used for membrane tight junction protein and epithelial permeability assessment.

Cell viability assay

To determine the effects of HRV-1B on viability, cells were seeded into 96-well plates and grown to 80% confluence. HRV-1B was added to the wells between 2.5 x 10⁴ and 80 x 10⁴ TCID₅₀/ml for 24, 48 and 72 h. Following exposure, cell viability was assessed using a 3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium inner salt (MTS) assay (Promega) (18).

Single-stranded DNA (ssDNA) apoptosis assay

To determine the percentage of cells that underwent apoptosis during HRV exposure, a ssDNA apoptosis ELISA kit was utilized as previously described (19). This procedure is based upon the selective denaturation of DNA in apoptotic cells by formamide and the detection of denatured DNA with a specific monoclonal antibody for ssDNA. Briefly, cells were plated onto 96-well microplates pre-coated with fibronectin (10 mM) at a seeding density of 6×10^4 cells/cm² and incubated at 37°C in an atmosphere of 5% CO₂/ 95% air in BEBM containing growth supplements as described earlier. Cells were then infected with HRV-1B at three viral concentrations: 2.5×10^4 TCID₅₀/ml, 10×10^4 TCID₅₀/ml and 80×10^4 TCID₅₀/ml for 24 hours. Cells were fixed and the assay as performed in accordance with the manufacturer's instruction (Chemicon).

Quantification of HRV viral copy number

Viral copy number was determined quantitatively via two-step RT-PCR reactions using a HRV-1B advanced kit (PrimerDesign™ Ltd) in combination with MultiScribe™ Reverse Transcriptase and Taqman® Universal Master Mix (Thermo Fisher Scientific) as previously described (19).

In-Cell Western™ Assay

Cells were plated at 1.2×10^5 cells/cm² onto 96-well plates pre-coated with fibronectin (10 mM) and maintained as described earlier. When confluent, growth media was aspirated and cell monolayer immediately fixed using 150 µl of 3.7% formaldehyde for 20 min 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 per wash with gentle shaking. However, this was only performed on half of the experimental setup to determine total protein expression. Concurrently, non-

permeabilized cells of the experimental setup was incubated with only 1X PBS. This allowed for the determination of membrane protein expression. Blocking was performed for 90 min 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 per wash and incubated with near-infrared (NIR) fluorescent dye conjugated-secondary antibody (1:800, Licor Biosciences) containing cellular nuclear stains DRAQ5 (1:10000, 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 five 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).

Transepithelial permeability

Cells were plated at a seeding density of 1.2×10^5 cells/cm² onto rat-tail collagen coated culture inserts (BD Biosciences) and maintained as described earlier. When confluent, transepithelial permeability was assessed using a transepithelial permeability assay with fluorescein isothiocyanate labeled dextran (FITC-dextran) to determine apparent permeability of HRV-1B infected and non-infected control inserts. Growth media within the apical and basolateral compartments was replaced with HEPES buffered Hank's balanced salt solution (HBSS-HEPES). FITC-dextran of molecular weight 4kDa or 20kDa (2 mg/ml) was added to the apical chamber and 50 μ l of this solution immediately sampled. A 500 μ l volume of solution was removed from the basolateral compartment

at hourly intervals for a period of 6 hours. The volume of the basolateral compartment was maintained by addition of fresh buffered HBSS-HEPES. All experiments were performed at 37°C and on a calibrated orbital shaker at 300 rpm to minimize the unstirred water layer. Fluorescence of FITC-dextran was detected using an Enspire multilabel fluorescence counter (PerkinElmer) at an excitation wavelength of 492 nm and emission wavelength of 520 nm. The apparent permeability of the epithelial monolayer to FITC-dextran from the apical to basolateral compartment (P_{app}) was then calculated as previously described (20).

Human tight junction and anti-viral response RT² Profiler focused quantitative-polymerase chain reaction (qPCR) analysis

Cells were infected with a viral titer of 10×10^4 TCID₅₀/ml for 9 h (to encompass the reproductive cycle of the virus) and subsequently collected for RNA. Transcript levels of specific genes associated with human TJ and the anti-viral response were analysed as part of two separate RT² Profiler focused qPCRs, each consisting of 84 key genes encoding proteins forming the epithelial barrier and the anti-viral responses respectively (QIAGEN). Total RNA was extracted using a RNeasy mini kit (QIAGEN) 9 hours post HRV-1B infection. After generating cDNA from 1 µg total RNA using RT² PCR First Strand Kit (QIAGEN), quantitative PCR was performed on an ABI Prism[®] 7300 (Thermo Fisher Scientific) in accordance with the manufacturer's protocol.

To gain insight into the relationship between human TJ genes and genes involved in the anti-viral response, the wiring network of these genes was reconstructed in the context of known biological response and regulatory networks using Ingenuity[®] Pathways Analysis (IPA[®]).

Statistics

Before statistical evaluation, all results were tested for population normality and homogeneity of variance. Experiments were performed in at least triplicates and all data was considered non-parametric. For these data, analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. Data was presented as whisker box plots (median, Min – Max) and all p values less than 0.05 were considered to be significant.

RESULTS

Effect of human rhinovirus infection on airway epithelial viability, apoptosis and viral replication

Although HRV has been reported to have a cytotoxic effect on primary airway epithelial cells (21), the effects on epithelial tight junction and barrier function together, remains largely unanswered. Hence cytotoxicity assays were performed initially to determine the effects of HRV-1B infection on cell viability. Figure 1 shows that infection with HRV-1B reduced viability in a dose and time dependent manner. Specifically, infection of AEC with HRV-1B between 2.5×10^4 and 10×10^4 TCID₅₀/ml had a minor effect on viability (93.2 - 100%) (Figure 1A). However, when infected for 24 hours with the viral titer of $20 - 80 \times 10^4$ TCID₅₀/ml, a significant loss of viability was observed (Figure 1A; $p < 0.05$). Incubation for 48 – 72 hours produced a significant loss in AEC viability at all TCID₅₀.

Apoptotic responses in virally infected cells are key protective mechanisms to prevent and minimize viral replication and release. Hence, we assessed apoptosis 24 hours following infection with HRV-1B. A significant dose-dependent apoptotic response was observed post infection with all viral titers, ranging from 55 – 88% over control levels (Figure 1B; $p < 0.05$). Furthermore, increasing amounts of HRV RNA was detected, concomitant with the increase in viral titer used in the infection. (Figure 1C; $p < 0.05$).

Effect of human rhinovirus infection on membrane tight junction disassembly

In-Cell™ Western assays were utilized to corroborate membrane protein disassembly of claudin-1, occludin and ZO-1. There was high endogenous occludin expression ($0.038 \text{AU} \pm 0.007$) within the airway epithelial cells, while, endogenous claudin-1 and ZO-1, which demonstrated similar levels of expression were significantly lower ($0.015 \text{AU} \pm 0.002$ and $0.016 \text{AU} \pm 0.002$ respectively) (Figure 2A and C – non-infected; $p < 0.05$). Following HRV-1B infection at a low viral titer to simulate chronic infectivity (2.5×10^4 TCID₅₀/ml), a significant decrease in claudin-1 expression ($0.007 \text{AU} \pm 0.0002$;

$p < 0.05$) was observed and although both occludin and ZO-1 expression was reduced ($0.02\text{AU} \pm 0.001$ and $0.012\text{AU} \pm 0.0004$ respectively), this did not reach statistical significance. When infected at a higher viral titer, similar to an acute infection (20×10^4 TCID₅₀/ml), significant decreases in all membrane protein expressions of claudin-1 ($0.007\text{AU} \pm 0.0001$; $p < 0.05$), occludin ($0.016\text{AU} \pm 0.002$; $p < 0.05$) and ZO-1 ($0.007\text{AU} \pm 0.00009$; $p < 0.05$) were observed compared to controls (Figure 2A and C; $p < 0.05$).

When membrane protein disassembly was assessed in primary AECs, similar high endogenous occludin expression was demonstrated followed by claudin-1 and ZO-1 respectively (Figure 2B and D). When infected with HRV-1B at a viral titre of 10×10^4 TCID₅₀/ml, significant decrease in all 3 TJ membrane protein expression was observed compared to controls (Figure 2B and D; $p < 0.05$).

Effect of human rhinovirus infection on transepithelial permeability

Epithelial paracellular permeability is a key functional indicator of cellular junctional integrity. Hence, to correlate HRV-1B induced disassembly of membrane TJ with barrier function, a transepithelial permeability assay was performed. Following infection with 2.5×10^4 TCID₅₀/m of HRV-1B, a significant increase in permeability to FITC-dextran of 4kDa ($292.6 \pm 2.4 \times 10^{-4}$ cm/sec; $p < 0.05$) and 20kDa ($249.8 \pm 10.33 \times 10^{-4}$ cm/sec; $p < 0.05$) was observed compared to controls ($221.9 \pm 8.5 \times 10^{-4}$ cm/sec and $115 \pm 4.5 \times 10^{-4}$ cm/sec respectively) (Figure 2E). Interestingly, when cells were infected with a higher viral titer (20×10^4 TCID₅₀/ml), significant increases in transepithelial permeability of FITC-dextran 4kDa ($357.9 \pm 12.2 \times 10^{-4}$ cm/sec; $p < 0.05$) and 20kDa ($302.2 \pm 7.2 \times 10^{-4}$ cm/sec; $p < 0.05$) was observed when compared to controls ($221.9 \pm 8.5 \times 10^{-4}$ cm/sec and $115 \pm 4.5 \times 10^{-4}$ cm/sec respectively) (Figure 2E).

Moreover, elevated permeability to FITC-dextran 4kDa was observed to be significantly higher ($292.6 \pm 2.4 \times 10^{-4}$ cm/sec and $357.9 \pm 12.2 \times 10^{-4}$ cm/sec) when compared to 20kDa ($249.8 \pm 10.33 \times$

10^{-4} cm/sec and $302.2 \pm 7.2 \times 10^{-4}$ cm/sec) post-infection with both viral titres respectively (Figure 2E; $p < 0.05$).

When transepithelial permeability was assessed in primary AECs, similar significant increase in epithelial permeability to FITC-dextran 4kDa was observed post-infection when compared to controls (Figure 2F; $p < 0.05$). In addition, increased permeability to FITC-dextran 4kDa was similarly observed to be significantly higher when compared to 20kDa, at non-infected and post-infection levels (Figure 2F; $p < 0.05$).

Effects of HRV-1B infection on mRNA expression of tight junction and anti-viral responses

In order to directly correlate HRV infection with altered expression in tight junction associated proteins, a RT² Profiler focused qPCR was performed. Following infection with HRV-1B for 9 hours, 58 genes encoding for epithelial tight junctional proteins were down-regulated with the most down-regulated being *CLDN8* (14.2-fold). Conversely, 26 genes were up-regulated following HRV-1B infection, with the most up-regulated being *Crb3* (1.9-fold). From our data, significant down-regulation was observed for *CLDN8* (14.2-fold), *PTEN* (3.5-fold), *CLDN12* (2.3-fold), *ASH1L* (1.9-fold), and *TJP1* (*ZO-1*) (1.3-fold) (Table 2; $p < 0.05$). Our data demonstrated down-regulation of mRNA for *claudin-1* (1.1-fold), *occludin* (1.2-fold) and *ZO-1* (1.3-fold) following HRV-1B infection. However, infection did not have a significant effect on the transcript expression of both *claudin-1* and *occludin* but was only observed for *ZO-1* (Table 2 – TJP1; $p < 0.05$). Down-regulation of *ZO-1* transcripts levels following HRV-1B infection was concomitant with a significant decrease in protein expression levels (Figure 2C – ZO-1; $p < 0.05$). Beyond the genes of interest, we assessed a number of the more differentially expressed genes including *PTEN* and corroborated that down-regulated transcript levels were paralleled with significant decreases in protein expression levels following HRV-1B infection (data not shown).

Infection with HRV-1B resulted in the down-regulation of 41 genes encoding for anti-viral responses with the most significant being *CXCL9* (2.9-fold) (Table 3; $p<0.05$). Conversely, 43 genes were shown to be up-regulated post infection, with the most significant being *IFNA2* (6.1-fold) (Table 3; $p<0.05$). Of these, 36 genes were determined to be significantly up-regulated (Table 3; $p<0.05$).

Network interactions of tight junction genes and anti-viral responses

Utilizing the molecular interaction data from the Ingenuity Systems Knowledge base to construct a network interaction diagram, our analysis revealed that tight junction protein 1 (*TJPI*) or ZO-1 was the most highly interactive gene in the human TJ network, exhibiting 26 functional interactions with other TJ genes while occludin and claudin exhibited 12 and 9 functional interactions with other TJ genes respectively (Figure 3A). Additional construction of network interaction diagrams between tight junction and anti-viral response genes illustrated multiple, complex interactions and identified *IL15* as a hub gene with high connectivity within the anti-viral network (33 connections). Furthermore, *IL15* was connected to 7 genes within the TJ network including both *TJPI* and *OCLN*, revealing a potential regulatory mechanism of *IL15* on the TJ network. (Figure 3B).

DISCUSSION

The mechanisms that explain how HRV infection impact on the expression and regulation of tight junction genes and subsequent disassembly of TJ proteins resulting in impairment of epithelial barrier functions are poorly understood. Previous studies have used cell lines or primary AECs for understanding the effects of viral infection on membrane TJ dissociation alone or disassembly in membrane TJ proteins leading to an observed increase in epithelial permeability (2, 4, 22). However, no single study has yet incorporated both and at present, this is the first in which the effects of HRV on multiple TJ gene expression, disassembly of membrane TJ proteins and subsequent impairment

on transepithelial permeability have been examined. Findings from this study also demonstrated the dynamic relationships between various TJs as well as provide evidence for the need to include multiple junctional proteins rather than a single protein for the assessment of epithelial barrier integrity.

Airway epithelial cells are of vital importance during viral infections as they serve as the host cell for viral replications 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 (19, 23-25). However, limited access to primary AECs as well as variability between different donor epithelia often restricts and confounds the ability to accurately interpret the generated data. Hence, utilizing modified human AECs, this study, in addition to providing proof of principle of the direct associations between HRV infection with changes in multiple TJ genes, proteins expression and resulting functional effect manifested as alterations in transepithelial permeability also demonstrates potential pathways for the regulation of epithelial junctional proteins via anti-viral responses.

Our data confirmed previously reported associations between viral titers of HRV-1B with cell viability, apoptosis and viral replication (19, 21). This demonstrates the suitability of the modified human AEC as an intermediary culture model bridging immortalized cell lines and primary human derived AEC for the assessment of HRV-1B induced effects on airway epithelial mechanics such as barrier integrity via junctional protein complex expression.

Tight junction complexes which are the most apical of the junctional complex family not only serve to provide intercellular adhesion but also form a continuous permeability barrier which regulates the trafficking of molecules across the epithelial layer (26). Numerous junctional complexes interact to provide structural support for the regulation and integrity of airway epithelium. Recent evidence has

demonstrated the coordinated involvement of multiple domains of the TJ protein, ZO-1 in the assembly of a normal and functioning epithelial barrier (7). Moreover, studies have also demonstrated the integral role of ZO-1 as a scaffolding protein necessary for the localization of both claudin-1 and occludin (6, 7) for the formation of cell to cell contacts. Hence, perturbations in these integral TJ complexes would likely result in increased epithelial permeability, facilitating the traffic of aeroallergens or pathogens as well as increased exposure of basolateral receptors to the inhaled air.

We demonstrated significantly reduced protein expression of claudin-1, occludin and ZO-1 following infection with HRV-1B indicating the disassembly of membrane claudin-1, occludin and ZO-1 junctions post infection. In addition, disassembly of these TJ protein complexes results in increased transepithelial permeability of macromolecules, in particular, small sized macromolecules. Importantly, these observations were subsequently corroborated in a series of experiments using primary AECs, confirming our findings. Collectively, our observations demonstrate the effects of HRV-1B infection on the dissociation of membrane claudin-1, occludin and ZO-1, which corresponds with an elevated epithelial permeability. Observations from this study suggest that an altered epithelial permeability towards different sized macromolecules could possibly result in increased exposure of basolateral receptors thereby facilitating elevated viral entry and eventual dissemination throughout the respiratory system. Increased transepithelial permeability following viral infection can result in transmigration of bacteria into the sub-epithelial space, an observation reported by Sajjan and co-workers (2008). This would have serious implications for individuals with asthma (27) as the consequence of such an increased epithelial permeability to respiratory viruses or bacteria could enhance airway inflammation. This could lead to recurrent disease exacerbations through generation of a variety of pro-inflammatory cytokines including IL-1 β , IL-6, GM-CSF and IL-11 (28-30) as well as chemokines such as CXCL8, CXCL10 and CCL5 (23, 31-33) ultimately resulting in chronic airway inflammation and eventually, structural remodelling (34-36).

We performed RT² Profiler focused qPCRs of HRV infected AECs and using the data obtained, constructed network interaction pathways to elucidate the interactions between junctional proteins and anti-viral responses and also to propose potential regulatory mechanisms of TJ by anti-viral responses. Our data showed, the down-regulation of 58 genes and up-regulation of 26 genes encoding for epithelial barrier junction proteins; and the down-regulation of 29 genes and up-regulation of 36 genes encoding for anti-viral responses. Expression of the ubiquitous claudin-1 (*CLDN1*), was down-regulated. In a study by Coyne and colleagues, the authors characterized claudin expression in normal human airways and determined that claudin 1 and 3 interacted to contribute towards decreased epithelial permeability while expression of claudin 5 resulted in a leakier epithelium (8). Our data demonstrates that following HRV-1B infection, there is a decrease in the expression of the associated TJ genes of the studied TJ proteins, namely claudin-1, occludin and ZO-1, although significance was only observed for *ZO-1* (*TJPI*). In a study by Yeo and colleague (37), they demonstrated the effects of HRV-1B on modulating TJ gene expression and illustrated the effects on *claudin-1*, *occludin* and *ZO-1* gene expression. However, as demonstrated in our focused qPCR data, infection with HRV-1B did not only affect the TJ genes of interest, but other genes responsible for epithelial barrier integrity.

Our unique observation that gene networks associated with HRV-induced disassembly of tight junctions and anti-viral responses are linked, has implications for diseases such as asthma and cystic fibrosis in which impaired innate immune responses have been observed (38-40). Innate immune responses to viral infections are triggered when the viral proteins and nucleic acids are detected by pattern recognition receptors, which in turn activate the intracellular signalling cascades, converging on the IRF and NF- κ B families of transcription factors (41). These transcription factors then translocate to the nucleus where the activation of anti-viral and inflammatory processes is initiated. Our findings identified an up-regulation of the transcription factor interferon regulatory factor-3 (*IRF3*) with HRV-1B infection. *IRF3* normally resides within the cytoplasm in an inactive form but undergoes a conformational change through phosphorylation and dimerization, leading to nuclear

translocation upon viral infection. Nuclear localization of *IRF3* subsequently forms a complex with co-activators CBP and / or p300, resulting in the binding to target DNA sequence in type I interferon, cytokine and chemokine genes and subsequent activation following viral infection (42-44). Our data identifies a potential causal regulatory mechanism, where an up-regulation of *IRF3* would lead to increased expression of type I interferons (IFN) such as IFN- α and - β as well as cytokines such as IL-15, which in turn result in the down-regulation of TJ expression. *IRF7*, which has also been implicated in the regulation of antiviral responses, was similarly identified in our data but was down-regulated upon virus infection. This was in contrast to the data reported by Bosco and colleagues, where they identified an up-regulation of *IRF7* following virus infection (45). This dissimilarity could be attributed to several factors differing between the experimental designs in these studies including, (1) HRV strains utilized; (2) viral titers used for infection and (3) length of infection before collection of total RNA for gene expression analysis. Interestingly, low or diminished *IRF7* expression has been previously associated with an elevated expression of genes involved in the response to oxidative stress (46-49). These observations, together with a study conducted by Comstock and colleagues (22) where they demonstrated impairment of epithelial barrier functions due to reactive oxygen species, further implicates the probable indirect associations of *IRF7* in regulating barrier integrity. Follow-up studies are not underway to elucidate the relationship of the IRF-family of transcription factors in modulating antiviral responses as well as airway epithelial barrier integrity and function.

This study, having established the associations between TJ protein expression and barrier functionality in response to HRV infection within healthy AECs as well as the possible mechanisms behind the regulation of epithelial junctional proteins, provides the opportunity to further investigate the gene and protein expression levels in AECs of diseased airways. However, we acknowledge a number of limitations. The study only assessed the protein expression of three TJs, claudin-1, occludin and ZO-1 following HRV-1B infection. As evident from the RT² Profiler qPCR data and network analysis, other genes such as *PTEN*, which are also involved in epithelial barrier integrity,

were similarly affected following infection. Thus, future investigations utilizing the high-throughput methodology of In-Cell™ Western could assess the expression of additional junctional proteins. Also, this study utilized a modified human airway epithelial cell culture, thus, will need to be corroborated in primary human AEC to determine the association of HRV infection on modulating TJ gene, protein expression and barrier functionality. Additionally, the potential influence of atopy in tight junction expression and function post infection could not be investigated due to the utilization of airway epithelial cells where this information is not routinely collected and provided. Future studies involving primary human AEC stratified according to atopy could address this. Also, this study only assessed the effects of HRV-1B on TJ disassembly. As demonstrated by Nakagome and colleagues (50), other serotypes of HRV would induce different epithelial responses following infection. Hence, the effects of other HRV serotypes on TJ responses could also be addressed in future studies.

In summary, this study is the first to demonstrate the direct associations between alterations in a number of TJ protein expression and the impaired functionality of the epithelium in regulating passage of macromolecules of varying size following HRV infection. In addition, our study also demonstrates the down-regulation of a range of epithelial junctional genes, *CLDN-1*, *OCN* and in particular, *ZO-1*. The down-regulation of these epithelial junctional genes following HRV-1B infection was concomitant with a loss in junctional proteins and a subsequent increase in epithelial permeability. Thus, demonstrating the relationship between airway epithelial gene expression, protein and functionality. Furthermore, through network analysis, we are able to identify potential regulatory pathways between anti-viral response and TJ gene expression following HRV-1B infection. When interpreted collectively, our findings strongly suggest the capacity of HRV to effectively disrupt epithelial integrity and attenuating barrier function.

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DECLARATIONS OF INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: AK, GRZ, ANL, KL, SMS and DAK. Performed the experiments: KL. Analyzed the data: KL, AK, DAK and SMS. Technical assistance and analysis: NMT, AB and AGB. Drafted the manuscript: KL. Helped critically revised the manuscript: LWG, TI, KML, KMM EKS, NCS, ENS, GRZ, PJR, ANL, DAK, AK and SMS. All authors have read and approved the manuscript.

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Figure 1 Reduction in cell viability, increased apoptotic response and viral replication in NuLi-1 after viral infection: (A) After infection with a range of HRV-1B titres ($2.5 - 80 \times 10^4$ TCID₅₀/ml), cell viability was assessed at 24, 48 and 72 h post infection via a 3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl] 2H-tetrazolium inner salt (MTS) assay. Results presented as whisker box plots (median, Min – Max) in percentages from at least three different experiments with each data assayed in triplicate and normalized to non-infected control cells (---). Infection with HRV-1B caused a time and dose-dependent cytotoxic effect on NuLi-1 cells. (B) After infection with a range of HRV-1B titres ($2.5 - 40 \times 10^4$ TCID₅₀/ml) for 24 h, apoptotic response of the cells was determined using a colorimetric assay ssDNA apoptosis kit; and it was observed overall apoptotic response increased significantly at all viral titres ($p < 0.05$). Data presented as whisker box plots (median, Min – Max) in percentages from at least three different experiments with each data assayed in triplicate relative to non-infected control cells. (C) NuLi-1 cells were established and infected with a range of HRV-1B titres ($2.5 - 40 \times 10^4$ TCID₅₀/ml) for 24 h. Cells were harvested to extract RNA and HRV-1B RNA measured using qPCR. Viral copy number increased with increasing viral titre and was significant for all viral titres used ($p < 0.05$). Data were normalized to microgram RNA and presented as whisker box plots (median, Min – Max) from at least three different experiments with each data assayed in triplicates. Kruskal-Wallis with Dunn's multiple comparison post-hoc tested for statistical significance. *denotes significance relative to control ($p < 0.05$).

Figure 2 Effect of HRV-1B infection on membrane TJ protein expression and transepithelial permeability in NuLi-1 and primary airway epithelial cells (AEC): (A – B) Representative images of In-Cell™ Western assay of NuLi-1 (n=5) and primary AECs (n=3) following 24 h HRV-1B infection illustrating signal intensity of TJ proteins and cell and nuclear stains. (TJ: TJ protein stain [OCLN: occludin; CLDN1: claudin-1; ZO-1: zonula occluden-1]; CN: Cell and nuclear stain; M: Merged) (C) After infection with HRV-1B titres (2.5 and 20×10^4 TCID₅₀/ml) for 24 h, membrane TJ protein expression as assessed via In-Cell™ Western assay was quantified. As observed, overall

membrane claudin-1, occludin and ZO-1 protein expression decreased following exposure to HRV-1B at both viral titres. (D) Significant decrease in all 3 TJ membrane protein was similarly observed in primary AECs following HRV-1B infection with a viral titre of 10×10^4 TCID₅₀/ml for 24 h. Kruskal-Wallis with Dunn's multiple comparison post-hoc analysis tested for statistical significance for all three TJ proteins ($p < 0.05$). Data were normalized to cell numbers and whisker box plots (median, Min – Max); $n = 5$ for NuLi-1; $n = 3$ for primary AECs individual experiments each performed in triplicates. (E) After infection with HRV-1B titres (2.5 and 20×10^4 TCID₅₀/ml) for 24 h, epithelial permeability was assessed via a transepithelial permeability assay. As observed, overall transepithelial permeability to FITC-dextran 4 kDa and 20 kDa was significantly increased at both viral titres ($p < 0.05$). Results are presented as whisker box plots (median, Min – Max); $n = 5$ individual experiments each performed in triplicates. (F) After infection with HRV-1B titre of 10×10^4 TCID₅₀/ml for 24 h, significant increase in transepithelial permeability was only observed for FITC-dextran 4 kDa in primary AECs ($p < 0.05$). Results are presented as whisker box plots (median, Min – Max); $n = 3$ individual experiments each performed in triplicates. Kruskal-Wallis with Dunn's multiple comparison post-hoc tested for statistical significance. * denotes significance relative to non-infected control ($p < 0.05$). # denotes significance relative to 20 kDa ($p < 0.05$).

Figure 3: Interaction network of human tight junction and anti-viral response in airway epithelial cells obtained by Ingenuity® Pathway Analysis (IPA®). Molecular interaction between genes was illustrated through the construction of network interaction diagrams utilizing the Ingenuity Systems Knowledge Base. (A) Interactions between the 3 tight junctions of interest in airway epithelial cells identified *TJP1* (*ZO1*) being the most interactive gene followed by occludin (*OCLN*) and claudin-1 (*CLDN1*), exhibiting 26, 12 and 9 functional interactions with other TJ respectively. (B) Network interactions between genes encoding for human tight junction and anti-viral responses in airway epithelial cells obtained by IPA® illustrated multiple complex interactions and identifying *IL15* as a hub gene with interactions to 7 genes within the TJ network. A potential regulatory pathway

for TJ expression through *IL15* and *IRF3* was also identified from the network interaction analysis. Up- and down-regulated genes in red and green, respectively.

Table 1: Demographic data for participants recruited to the study

Table 2: Human tight junction genes that were up- or down-regulated in modified human airway epithelial cells following HRV-1B infection as determined by RT² profiler focused qPCR array.

Table 3: Human anti-viral response genes that were up- or down-regulated in modified human airway epithelial cells following HRV-1B infection as determined by RT² profiler focused qPCR array.

Table 1 Demographic data for participants recruited to the study

	Number	Gender (M/F)	Mean Age (Range)	Mean IgE (IU/L)	Allergies	Hay Fever / Eczema / Both
Non-asthmatic	3	2/1	4.6 (3.4 – 6.4)	17	0	0

M –male; F - Female

Table 2: Human tight junction genes that were up- or down-regulated in modified human airway epithelial cells following HRV-1B infection as determined by RT² profiler focused qPCR array.

Gene Symbol	Gene Description	Fold Up/Down	P Value
CLDN8	Claudin 8	-14.30	0.05077
PECAM1	Platelet/endothelial cell adhesion molecule	-4.31	0.07098
SPTA1	Spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	-3.65	0.08105
PTEN	Phosphatase and tensin homolog	-3.51	0.04063
CLDN19	Claudin 19	-3.46	0.10757
MAGI2	Membrane associated guanylate kinase, WW and PDZ domain containing 2	-3.27	0.29596
IGSF5	Immunoglobulin superfamily, member 5	-3.21	0.2049
ESAM	Endothelial cell adhesion molecule	-2.73	0.39208
CLDN12	Claudin 12	-2.31	0.03772
ICAM2	Intercellular adhesion molecule 2	-2.11	0.34791
ARHGEF2	Rho/rac guanine nucleotide exchange factor (GEF) 2	-2.06	0.13813
ASH1L	Ash1 (absent, small, or homeotic)-like (Drosophila)	-1.95	0.03865
ZAK	Sterile alpha motif and leucine zipper containing kinase AZK	-1.93	0.08563
CLDN14	Claudin 14	-1.90	0.27774
CLDN3	Claudin 3	-1.89	0.16849
SPTB	Spectrin, beta, erythrocytic	-1.84	0.07891
MLLT4	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	-1.82	0.10406
CLDN9	Claudin 9	-1.81	0.15737
CRB1	Crumbs homolog 1 (Drosophila)	-1.81	0.11888
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa	-1.73	0.06933
PARD3	Par-3 partitioning defective 3 homolog (C. elegans)	-1.70	0.1556
TJP3	Tight junction protein 3 (zona occludens 3)	-1.64	0.20543
CLDN15	Claudin 15	-1.62	0.18782
CLDN5	Claudin 5	-1.58	0.16883
CSNK2A2	Casein kinase 2, alpha prime polypeptide	-1.52	0.16144
CTNNA3	Catenin (cadherin-associated protein), alpha 3	-1.51	0.1226
PARD6B	Par-6 partitioning defective 6 homolog beta (C. elegans)	-1.48	0.36591
JAM2	Junctional adhesion molecule 2	-1.45	0.10841
ACTN2	Actinin, alpha 2	-1.39	0.18307

MARK2	MAP/microtubule affinity-regulating kinase 2	-1.38	0.25732
EPB41	Erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)	-1.36	0.15507
CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)	-1.32	0.22956
F11R	F11 receptor	-1.31	0.32857
TJP1	Tight junction protein 1 (zona occludens 1)	-1.30	0.02726
GNAI1	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	-1.28	0.29824
CD99	CD99 molecule	-1.25	0.31641
MPDZ	Multiple PDZ domain protein	-1.23	0.45559
MAGI1	Membrane associated guanylate kinase, WW and PDZ domain containing 1	-1.22	0.4117
OCLN	Occludin	-1.21	0.51138
JAM3	Junctional adhesion molecule 3	-1.17	0.54812
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	-1.16	0.53607
LLGL2	Lethal giant larvae homolog 2 (Drosophila)	-1.15	0.61568
CGN	Cingulin	-1.14	0.55527
CLDN1	Claudin 1	-1.11	0.60746
RHOA	Ras homolog gene family, member A	-1.10	0.62207
CTTN	Cortactin	-1.10	0.58774
CLDN18	Claudin 18	-1.08	0.6287
TJAP1	Tight junction associated protein 1 (peripheral)	-1.08	0.47969
INADL	InaD-like (Drosophila)	-1.07	0.49042
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	-1.07	0.69523
SYMPK	Symplekin	-1.06	0.85782
MPP6	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	-1.05	0.72462
ACTN4	Actinin, alpha 4	-1.04	0.76078
LLGL1	Lethal giant larvae homolog 1 (Drosophila)	-1.04	0.72833
CLDN6	Claudin 6	-1.02	0.81897
ACTN1	Actinin, alpha 1	-1.02	0.89235
ILK	Integrin-linked kinase	-1.02	0.8028
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	-1.00	0.87173
ACTN3	Actinin, alpha 3	1.01	0.86424
ICAM1	Intercellular adhesion molecule 1	1.02	0.71733
CLDN7	Claudin 7	1.03	0.96286
CDK4	Cyclin-dependent kinase 4	1.04	0.60435
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	1.04	0.80054

AMOTL1	Angiomotin like 1	1.04	0.91476
CLDN10	Claudin 10	1.06	0.54588
CTNNA2	Catenin (cadherin-associated protein), alpha 2	1.07	0.90389
TJP2	Tight junction protein 2 (zona occludens 2)	1.19	0.57443
CLDN11	Claudin 11	1.20	0.25665
PRKCI	Protein kinase C, iota	1.23	0.44612
CSDA	Cold shock domain protein A	1.23	0.43812
MPP5	Membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	1.25	0.43665
HCLS1	Hematopoietic cell-specific Lyn substrate 1	1.28	0.20818
CLDN17	Claudin 17	1.32	0.73856
SPTAN1	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	1.33	0.39112
CSNK2A1	Casein kinase 2, alpha 1 polypeptide	1.33	0.35265
CLDN2	Claudin 2	1.35	0.22708
PRKCZ	Protein kinase C, zeta	1.39	0.29967
TIAM1	T-cell lymphoma invasion and metastasis 1	1.41	0.17262
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	1.51	0.24028
CLDN16	Claudin 16	1.72	0.73347
CLDN4	Claudin 4	1.73	0.1021
CSNK2B	Casein kinase 2, beta polypeptide	1.76	0.10652
PARD6A	Par-6 partitioning defective 6 homolog alpha (C. elegans)	1.81	0.17462
CRB3	Crumbs homolog 3 (Drosophila)	1.93	0.07219

Table 3: Human anti-viral response genes that were up- or down-regulated in modified human airway epithelial cells following HRV-1B infection as determined by RT² profiler focused qPCR array.

Gene Symbol	Gene Description	Fold Up/Down	P Value
CXCL9	Chemokine (C-X-C motif) ligand 9	-2.93	0.00000047
CARD9	Caspase recruitment domain family, member 9	-2.86	0.00004748
IRAK1	Interleukin-1 receptor-associated kinase 1	-2.75	0.00001615
DAK	Dihydroxyacetone kinase 2 homolog (S. cerevisiae)	-2.29	0.00020652
NOD2	Nucleotide-binding oligomerization domain containing 2	-2.18	0.00000001
SPP1	Secreted phosphoprotein 1	-2.17	0.00001152
TLR7	Toll-like receptor 7	-2.05	0.00051151
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	-1.97	0.00000001
IRF7	Interferon regulatory factor 7	-1.83	0.00002603
TRADD	TNFRSF1A-associated via death domain	-1.67	0.00909072
MAVS	Mitochondrial antiviral signaling protein	-1.67	0.00000035
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	-1.52	0.00008138
CASP10	Caspase 10, apoptosis-related cysteine peptidase	-1.52	0.00006931
FADD	Fas (TNFRSF6)-associated via death domain	-1.43	0.00000001
MAPK14	Mitogen-activated protein kinase 14	-1.43	0.03774484
MAPK3	Mitogen-activated protein kinase 3	-1.43	0.01045563
JUN	Jun proto-oncogene	-1.41	0.00029771
CTSB	Cathepsin B	-1.34	0.00071302
MAP2K1	Mitogen-activated protein kinase kinase 1	-1.33	0.00072620
IRF5	Interferon regulatory factor 5	-1.32	0.33829921
CD40	CD40 molecule, TNF receptor superfamily member 5	-1.30	0.00051747
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	-1.20	0.00000423
MAPK1	Mitogen-activated protein kinase 1	-1.19	0.30268804
IFNA1	Interferon, alpha 1	-1.18	0.00023519
FOS	FBJ murine osteosarcoma viral oncogene homolog	-1.17	0.03137685
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	-1.17	0.13164653
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	-1.10	0.02094787
CHUK	Conserved helix-loop-helix ubiquitous kinase	-1.09	0.03574090
TRAF6	TNF receptor-associated factor 6	-1.09	0.17437058
NLRP3	NLR family, pyrin domain containing 3	-1.08	0.00040454

NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-1.08	0.00507649
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	-1.07	0.00784655
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	-1.07	0.00004979
MAPK8	Mitogen-activated protein kinase 8	-1.06	0.09300370
ATG5	ATG5 autophagy related 5 homolog (S. cerevisiae)	-1.02	0.89788235
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	-1.02	0.72534872
TLR3	Toll-like receptor 3	-1.02	0.87838850
IL1B	Interleukin 1, beta	-1.01	0.78185343
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	-1.01	0.79643923
TRAF3	TNF receptor-associated factor 3	1.01	0.96555616
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	1.07	0.03711316
IFNAR1	Interferon (alpha, beta and omega) receptor 1	1.07	0.03577578
AZI2	5-azacytidine induced 2	1.08	0.51377372
CASP8	Caspase 8, apoptosis-related cysteine peptidase	1.11	0.80836990
ISG15	ISG15 ubiquitin-like modifier	1.12	0.00554821
MAP2K3	Mitogen-activated protein kinase kinase 3	1.13	0.12194074
CYLD	Cylindromatosis (turban tumor syndrome)	1.14	0.01209585
STAT1	Signal transducer and activator of transcription 1, 91kDa	1.16	0.24645673
MYD88	Myeloid differentiation primary response gene (88)	1.18	0.00002183
TICAM1	Toll-like receptor adaptor molecule 1	1.18	0.00492646
CCL3	Chemokine (C-C motif) ligand 3	1.18	0.00000032
PYDC1	PYD (pyrin domain) containing 1	1.18	0.00000032
AIM2	Absent in melanoma 2	1.19	0.44973387
IL18	Interleukin 18 (interferon-gamma-inducing factor)	1.21	0.00049428
CD80	CD80 molecule	1.24	0.00105612
IRF3	Interferon regulatory factor 3	1.24	0.00991388
PYCARD	PYD and CARD domain containing	1.25	0.00141023
CTSL1	Cathepsin L1	1.27	0.35211584
TRIM25	Tripartite motif containing 25	1.28	0.10898547
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	1.29	0.00143938
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	1.30	0.00000021
IFIH1	Interferon induced with helicase C domain 1	1.30	0.00012226
IFNB1	Interferon, beta 1, fibroblast	1.31	0.00129833
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	1.33	0.23093048

IL15	Interleukin 15	1.36	0.00006954
SUGT1	SGT1, suppressor of G2 allele of SKP1 (<i>S. cerevisiae</i>)	1.49	0.00505288
CD86	CD86 molecule	1.50	0.00493762
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	1.51	0.00459035
CTSS	Cathepsin S	1.56	0.00000325
MEFV	Mediterranean fever	1.57	0.00000002
TNF	Tumor necrosis factor	1.59	0.01890268
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	1.71	0.04202078
CXCL11	Chemokine (C-X-C motif) ligand 11	1.75	0.00000834
CXCL10	Chemokine (C-X-C motif) ligand 10	1.80	0.00007272
TBK1	TANK-binding kinase 1	1.82	0.00000658
CCL5	Chemokine (C-C motif) ligand 5	1.85	0.00006885
IL8	Interleukin 8	2.05	0.00003576
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	2.08	0.00000066
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2.32	0.00370093
IL6	Interleukin 6 (interferon, beta 2)	2.56	0.00000002
TLR9	Toll-like receptor 9	2.65	0.03062186
TLR8	Toll-like receptor 8	3.27	0.00000000
IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	3.29	0.00044460
IFNA2	Interferon, alpha 2	6.13	0.00000001