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Respiratory syncytial virus infection influences tight junction integrity

Jeannette I. Kast\textsuperscript{1,2*}, Amanda J. McFarlane\textsuperscript{3}, Anna Głobińska\textsuperscript{1,2,6}, Milena Sokolowska\textsuperscript{1,2}, Paulina Wawrzyniak\textsuperscript{1,2}, Marek Sanak\textsuperscript{5}, Jürgen Schwarze\textsuperscript{3,4}, Cezmi A. Akdis\textsuperscript{1,2}, Kerstin Wanke\textsuperscript{1,2*}

\textsuperscript{1} Swiss Institute of Allergy and Asthma Research, University of Zurich, Davos, Switzerland.

\textsuperscript{2} CK-CARE, Christine Kühne Center of Allergy Research and Education, Davos, Switzerland.

\textsuperscript{3} Medical Research Council Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom

\textsuperscript{4} Child Life and Health, University of Edinburgh, Edinburgh, United Kingdom

\textsuperscript{5} Department of Medicine, Jagiellonian University Medical College, Cracow, Poland

\textsuperscript{6} Department of Immunology, Rheumatology and Allergy, Chair of Clinical Immunology and Microbiology, Medical University of Lodz, Poland

Corresponding author:
Kerstin Wanke, PhD
Swiss Institute of Allergy and Asthma Research (SIAF)
Obere Strasse 22
7270 Davos Platz
wanke.kerstin@gmail.com
Tel.: +41 (0) 76 740 74 96

Condensed title: Regulation of tight junction barrier by RSV

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Abstract:

Background  Respiratory syncytial virus (RSV) is an important risk factor of asthma development and is responsible for severe respiratory tract infections. However, the influence of RSV infection on barrier function of bronchial epithelial cells in vitro and in vivo is still unclear.

Objective  The aim of this study was to analyze the role of RSV in tight junction (TJ) regulation and to compare epithelial integrity between asthmatic and healthy individuals upon RSV infection.

Methods  Healthy and asthmatic human bronchial epithelial cells (HBECs) were differentiated at air-liquid interface (ALI) and infected with RSV and UV-irradiated RSV. TJ expression and their integrity were analysed by qPCR, transepithelial resistance (TER) and paracellular flux. To determine the effect in vivo, Balb/c mice were intranasally infected with RSV or UV-irradiated RSV A2. Bronchoalveolar lavage and TJ integrity were analysed on days 1, 2, 4 and 6 post infection by qPCR, bioplex and confocal microscopy.

Results  RSV increased barrier integrity in ALI cultures of HBEC from healthy subjects, but no effect was found in HBECs from asthmatics. This was not associated with an increase in TJ mRNA expression. In vivo, RSV induced lung inflammation in mice and downregulated claudin-1 and occludin mRNA expression in the whole lungs. Surprisingly, RSV infection was not observed in bronchial epithelial cells, but was found in the lung parenchyma. Decreased
expression of occludin upon RSV infection was visible in mouse bronchial epithelial cells in confocal microscopy. However, there was no regulation of claudin-1 and claudin-7 at protein level.

**Keywords** tight junctions, respiratory syncytial virus, asthma, airway epithelial cells
Introduction

Human respiratory syncytial virus (RSV) is a negative-sense, single-stranded virus, which belongs to the *Paramyxoviridae* family. RSV is not only infecting epithelial cells in the upper airways of adults, but is also the leading cause of lower respiratory tract infections in infants under the age of 2 years. Infections in children result in more severe, lower respiratory tract inflammation, leading in some cases to severe bronchiolitis, pneumonia and resulting in hospitalization. In addition, RSV infection in childhood has been linked to recurrent wheeze, induction of childhood asthma and sensitization to allergens. However, it is unclear how RSV and other respiratory viruses are propagating *in vivo* and how RSV is linked to the development of asthma and allergies. In case of rhinovirus infection, asthmatics display impaired interferon (IFN) production in response to infection, but on the other hand no IFN deficiency was observed in well-controlled asthma. In addition, a recent study suggested that there is no difference in IFN response to RSV between asthmatic and non-asthmatic individuals. However, a study on hospitalized infants infected with RSV demonstrated diminished IFN-γ production at the time of bronchiolitis. Reduced IFN-γ production was related to development of asthma after bronchiolitis in infants.

Infection with RSV induces secretion of cytokines and chemokines from epithelial cells and resident leukocytes, which in turn leads to recruitment of circulating leukocytes to the infected lungs. It has been demonstrated that RSV stimulates release of chemokines i.e. RANTES (regulated upon activation, normal T-cell expressed and secreted), MIP-1α, MIP-1β (macrophage inflammatory protein 1), MCP-1 (monocyte chemoattractant protein-1), KC (neutrophil chemoattractant), as well as cytokines, such as G-CSF (granulocyte-colony stimulating factor), IL-6 and IL-1β.
Tight junctions (TJ) form an essential part of the barrier between the mucosa or skin and the environment by connecting adjacent epithelial cells. A continuous band of TJ surrounds every epithelial cell and builds the most apical, extracellular cell-cell adhesion complex. TJ consist of large, transmembrane protein complexes with members of the claudin family, the MAL and related proteins for vesicle trafficking and membrane link (MARVEL) family, and the junctional adhesion molecule (JAM) family, which connect adjacent cells. (19, 20) Cell types from different human tissues express a unique pattern of TJ proteins according to their specialization. (21) TJ are important in barrier formation. They prevent particles and pathogens from penetrating the tissue, control extracellular fluid, paracellular flux of molecules, and provide the apico-basolateral axis of differentiation. (22) Epithelial barrier defects due to disruptions in TJ have been reported in several allergic and inflammatory diseases, such as atopic dermatitis, asthma, and chronic rhinosinusitis, and a role of TJ in smooth muscle cells in asthma has been described. (23-28) The connection between RSV infections, barrier function, and the impact of the virus on asthma development has not been thoroughly studied. So far, RSV-induced disruption of barrier function in a bronchial epithelial cell line and contradictory results of induction of TJ molecules in human nasal epithelial cells have been reported. (29, 30) Although there is some evidence of epithelial barrier dysfunction in asthma (31) (32), it is still not clear whether RSV infection may have an influence on TJ in asthma.

Therefore, the aim of this study was to identify the role of RSV in TJ regulation and function of epithelial integrity in vitro in asthmatic and healthy bronchial epithelial cell cultures. We also sought to explore the course of RSV infection in vivo using mouse model to mimic the situation in which RSV infection precedes asthma development and analyze here the inflammatory response and the regulation of TJ.
Material and Methods

RSV propagation

5 x 10^6 ml HEP2 cells were incubated overnight at 37°C, 5 % CO₂ in complete DMEM/F12 medium. After 24 hours, the cells were washed with serum free DMEM/F12 medium. The cultures were infected with human RSV (type A, strain A2 (ATCC, Middlesex UK)) at a virus concentration of 0.1 pfu/cell and cultured in serum free DMEM/F12 at 37°C, 5 % CO₂ for 48 hours. After this time, the cytopathic effect was assessed and the cells were harvested using a cell scraper. Cultures were centrifuged and the resultant supernatant was snap frozen as viral stock in liquid nitrogen. Viral stocks were kept at −80°C until use. Control flasks of uninfected HEP2 cells were treated in the same way as control supernatants.

Isolation of primary bronchial epithelial cells and ALI cultures

Primary human bronchial epithelial cells (HBECs) were obtained from asthmatic patients undergoing bronchoscopy and bronchial brushings after written informed consent by each patient. Healthy HBECs were bought from Lonza. Cells were removed from brushes, seeded into collagen and fibronectin coated 25 cm² T flasks and cultured in basal epithelial growth medium (BEGM, Lonza) at 37°C, 5 % CO₂. 1.5 x 10^5 cells of passage 3 or 4 were seeded into collagen-precoated (Sigma) 24-well, polyester, 6.5 mm transwell plates (Corning) with 0.4 µm pore size in ALI medium (DMEM (Gibco) 1:1 mixed with BEGM without triiodothyronine and freshly substituted with transretinoic acid (Sigma)). Apical medium was removed after 4 days. Since our previous experiments (33) have demonstrated lower transepithelial resistance (TER) values in HBECs from asthmatic donors compared to control subjects, cells were used for the experiment when ALI cultures from healthy donors reached at least 330 Ω*cm² and asthmatic HBECs at least 200 Ω*cm² in TER measurement with electrodes.
and a Millicell ERS Volt-Ohm Meter (Millipore). The 6 healthy and 6 asthmatic cultures were apically infected with RSV (MOI of 1.5 or 0.15) or UV-irradiated RSV (UV-RSV) (MOI of 1.5) or incubated with control medium (u.s.). After 2h the cultures were washed 3 times and continued to be cultured at ALI.

**RSV mouse model**

Wild-type Balb/c mice, 8-12 weeks old, were maintained under specific pathogen-free conditions. Mice were housed at the Medical Research Council Centre for Inflammation Research, University of Edinburgh, United Kingdom, in individually ventilated cages for the duration of the study, and all experimental procedures were carried out in accordance with Home Office regulations. Mice were infected intranasally with $7.4 \times 10^5$ PFU RSV A2 or UV-irradiated RSV, or left naïve. Mice were sacrificed at day 1, 2, 4 and 6 after infection (Figure S1A). Group size was n=8 and the experiment was repeated 3 times.

**BAL protein concentration and bioplex analysis**

Bronchoalveolar lavage (BAL) was obtained by injecting 1 ml of PBS containing protease inhibitor (Roche) into the mouse lung and retrieving usually 650-900 µl of injected fluid. The BAL supernatant was collected and total protein concentration was measured by a standard Bradford assay (BioRad). Bioplex analysis of the BAL has been done with Bio-Plex Pro™ Mouse Cytokine 23-plex Assay with the Bio-Plex 200 System (Bio Rad).

**mRNA isolation and quantitative RT-PCR**

Lung samples were directly stored in RNALater (Qiagen) and frozen for later analyses. Lungs were then shredded with ceramic beads in RLT buffer (Precellys®24 tissue
homogenizer, Bertin technologies). Cells from ALI cultures at day 6 after infection were
directly lysed in RLT buffer, and RNA was subsequently isolated with the RNeasy Mini Kit
(Qiagen) according to the manufacturer’s protocol. cDNA was prepared with reverse
transcription reagents containing random hexamer primers (Fermentas). For relative
quantification, cDNA was amplified in the presence of SYBR Green and detected by an ABI
PRISM 7000 Sequence Detection System (Applied Biosystems). Primer sequences for human
and mouse primers can be found in supplementary Table 1. For human samples elongation
factor 1α (EF1α) and for mouse samples glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) or EF1α were used as internal control. Relative expression of mRNA was calculated
using the comparative ΔΔCT-method.(34)

**Lung histology and immunofluorescence staining of ALI and lung sections**

For confocal microscopy, lung samples were frozen in Tissue Tek (Sakura, Finetek) and cut
into 30 µm thick sections with a cryomicrotome (Microm; Carl Zeiss). Lung sections were
fixed with 4 % paraformaldehyde, blocked and permeabilized with PBS containing 10 %
donkey serum (Sigma-Aldrich), 1 % BSA (Sigma-Aldrich), 0.1 % Triton-X 100 (Acros Organics),
and 0.02% SDS (Sigma-Aldrich). Samples were stained with Alexa488-labelled mouse anti-
occludin, polyclonal rabbit anti-claudin-1, polyclonal rabbit anti-claudin-7 and polyclonal
goat anti-RSV (AbD serotec) antibodies and detected with secondary antibodies labelled with
Alexa-488, 546, or 633 (Invitrogen). Stainings were mounted in ProLong-Gold with DAPI
(Invitrogen) and analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems).
Statistical analysis

Non-parametric data were analyzed using the Mann Whitney U test, while paired data sets were analyzed using the Wilcoxon matched pair test. Spearman’s correlation test was used between RSV load and chemokines, cytokines and TJ. All statistical analysis was conducted using the GraphPad Prism 5c software. A p-value <0.05 was considered statistically significant.
Results

RSV increased barrier integrity in healthy, but not in asthmatic bronchial epithelial cells

To determine the direct role of RSV on TJ of healthy and asthmatic HBECs, cells were cultured under ALI conditions and infected with RSV MOI of 0.15 or 1.5. Controls were treated either with medium or UV-RSV. As reported previously, we also noted that the baseline epithelial integrity is compromised in asthmatics compared to controls. (33) We demonstrated here that RSV increased transepithelial resistance (TER) and decreased paracellular flux in healthy, but not in asthmatic subjects (Figure 1A). As a control, we confirmed RSV infection of healthy and asthmatic HBECs (Figure 1B). On day 6 post infection, HBEC culture were harvested and analysed. Interestingly, virus load in asthmatic HBECs seemed to be lower than that observed in healthy individuals, yet this difference was not statistically significant. The expression of the TJ genes – occludin, claudin-1 and claudin-7 – were not significantly regulated on mRNA level in both groups (Figure 1C). Thus, our findings suggest that healthy HBECs’ response to RSV leads to an increase in epithelial barrier integrity reflected by decreased paracellular flux and enhanced transepithelial resistance, which does not occur in asthmatic patients. However, this effect was not due to a change in the expression of TJ molecules mRNA in HBECs.

RSV infected mouse lungs and induced inflammation

Since in the clinic, RSV infections in infants usually precede asthma development, we aimed to investigate the effect on lung epithelial barrier function in vivo. For this reason, mice were inoculated intranasally with RSV or UV-RSV. Mouse lungs and BAL were harvested on days 1, 2, 4 and 6 post infection (Figure S1A). Confirming the RSV infection in the lungs, we found high viral gene expression in RSV-infected mice, whereas there was no infection in
control naïve mice or in the UV-RSV-receiving control group already at day 1 after infection, persisting up to day 4 (Figure 2A). To investigate the influence of viral infection on barrier integrity and to get an insight into the inflammatory process in the lung, we performed a standard \textit{in vivo} bronchoalveolar lavage (BAL) procedure and analysis. An increased protein influx from the lung parenchyma to the BAL on days 2, 4 and 6 indicated a reduction of the epithelial barrier integrity (Figure 2B). Furthermore, we found an increased expression of MUC5AC mRNA on days 1, 4 and 6 upon RSV infection, which suggested an induction of innate immune response in the epithelium (Figure 2C). In parallel, MUC5AC mRNA expression and overall increase in BAL protein content correlated with the viral load (Figure S1B). Among the BAL proteins there were proinflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-12 p40, MCP-1, MIP-1α and MIP-1β, G-CSF, KC (neutrophil chemoattractant) and RANTES, of which most increased significantly upon RSV infection at day 1, 2, 4 and 6 (Figure 2D). Released chemokines and cytokines positively correlated with RSV load in the lung (Figure S1C). In conclusion, RSV infection \textit{in vivo} induced active lung inflammation and reduced airway epithelial barrier function.

**RSV infection downregulated TJ in mouse lungs**

To further characterize the mechanisms of barrier dysfunction upon RSV infection \textit{in vivo}, detailed qPCR analysis of TJ in RSV and UV-RSV-infected mice was performed. In mouse lungs, a distinct set of TJ was found. All claudin family members, except claudin-2 and claudin-6, the MARVEL family members – occludin, tricellulin and marvelD3 splice variant 2 (MarvelD3.2), as well as the junctional adhesion molecules – JAM-A, -B and -C and plaque protein zonula occludens (ZO) 1 genes were expressed (Figure S2A). Claudin-1 was significantly downregulated on days 1 and 6 post infection and there was a trend
towards the decrease on days 2 and 4. Occludin was significantly downregulated only on day 1 and showed a tendency to be downregulated on days 4 and 6 (Figure 3A). In addition, claudin-1 and occludin negatively correlated with RSV load in the lungs of infected mice (Figure 3B). In contrast, claudin-7 was upregulated in RSV-infected compared to naïve mice on day 1, but did not correlate with the RSV load (Figure S2B). Other TJ, such as claudin-10b and tricellulin were significantly downregulated compared to naïve at day 1 after infection (Figure S2C). In conclusion, RSV infection led to the downregulation of claudin-1 and occludin mRNA in mouse lungs, which correlated with barrier dysfunction and viral load. 

RSV was only found in the lung parenchyma and did not directly regulate TJ in mouse bronchial epithelial cells

To determine the regulation of TJ and location of RSV infection in mouse lungs, immunofluorescence staining of TJ and RSV envelope proteins was performed. Interestingly, we demonstrated that already at day 1 post infection, RSV was located in the lung parenchyma, but was not found in the bronchial epithelial cells (Figure 4, Figure S3). Moreover, we found that occludin expression in the bronchial epithelium was lower in the RSV-infected mouse as compared to UV-RSV at day 2 and day 4 (Figure 4) and corresponded to occludin mRNA expression level, correlation with viral load and increased protein leak to the BAL at day 2. However, there was no visible regulation of the protein expression of claudin-1 (Figure S3A) or claudin-7 (Figure S3B), suggesting that the stability of these proteins might be greater than occludin and that they are additionally highly regulated at the post-transcriptional level. To conclude, RSV infection correlated with the decreased occludin expression in the bronchial epithelium, while the expression of other TJ proteins was influenced at the transcriptional level in the whole lungs.
Discussion

The aim of this study was to investigate the role of RSV infection in TJ regulation. We first sought to investigate the effect of RSV on TJ in asthmatic HBECs and healthy controls, and secondly, to study RSV effects on TJ integrity in mouse model of infection, which usually precedes asthma development.

We found that RSV infection of primary HBECs led to an increase in barrier function in healthy, but not in asthmatic subjects. This was reflected by the change in barrier function parameters (TER, flux), but not by TJ gene expression level, suggesting very early innate response to RSV infection only in healthy epithelium. It is possible that Th2-type inflammation ongoing in allergic asthmatic patients alters epithelial cell function and precludes barrier tightening in response to RSV. This hypothesis is supported by the fact that normal bronchial epithelial cells stimulated with IL-4 and IL-13 demonstrate significant decrease in barrier integrity similar to that observed in HBECs from asthmatic patients. (33) Additionally, Th2 environment may interfere with the viral replication. Virus load at day 6 post infection in asthmatic HBECs was lower compared to healthy individuals HBECs. Similarly, significantly diminished proliferation of parainfluenza virus (another Paramyxoviridae family member) has been demonstrated in nasal epithelial cells from allergic individuals. (35) These results suggest that already compromised barrier function in asthmatic is unable to react efficiently to RSV infection and provide natural defence mechanisms, however decrease in barrier function do not result in the increase of virus load in asthmatic HBECs.

Thus, we observed here an additional dysfunction of asthmatic epithelium, which extends other observation of impaired innate mechanisms in response to viral infections (36, 37). These data are in line with previous observations that RSV infection of human nasal...
epithelial cells results in enhanced expression of claudin-4 and occludin, inducing cell polarity, which may facilitate virus budding. (30) (38)

The next aim of our study was to examine the influence of RSV on barrier function in vivo in a mouse model of RSV infection. We found that RSV was present only in the lung parenchyma, whereas the murine bronchial epithelial cells did not appear to be infected. We hypothesise that RSV might influence TJ expression in lung parenchyma cells either directly upon replication or indirectly via activation of inflammatory mediators. Similar RSV infection pattern has been found in children with severe RSV infection. (2, 39) This effect may be associated with the immature immune response and smaller physical dimensions of child’s airways, which may be a potential explanation of the viral location in the infant’s lungs. (40) Therefore, our results suggest that mouse model of RSV infection may mimic the course of infection in infants, which is related to disruption of the distal airway epithelium. On the other hand, RSV-induced barrier response in primary bronchial epithelial cells from adults might not be fully reflected in mouse model of infection. It has been demonstrated that BALB/c mice used in our study are semi-permissive to RSV infection and develop only limited inflammation. (41) In view of differences in the immune response and physical differences of lung structure between adults and infants, localization of the infection in adults is not limited to the lung parenchyma, but rather located in the upper airway epithelium. Therefore, being aware of the fact that mouse model of RSV infection may not reflect the range of pathologies observed in humans, we do not compare directly the results obtained in vitro with in vivo mouse model. It is possible that using another rodent model of RSV infection, i.e. cotton rats, we would be able to detect virus in epithelial cells of the lungs. (42) (43). On the reverse note, in vitro experiments with one type of cells might reflect only the early response of epithelium to the replication of virus and probably do not fully reflect
other events during on-going RSV-induced inflammation. Thus, we believe the results obtained from both experimental models complement each other and give different information: (i) in contrast to healthy controls, adult asthmatic epithelium is not able to provide resistance to RSV infection by tightening already compromised TJs barrier; and (ii) in vivo RSV-infection and following RSV-induced inflammation decrease whole lung barrier function, which possibly might contribute to further development of asthma.

In summary, our data demonstrated that RSV infection increased barrier integrity in healthy, which mechanism was impaired in asthmatic bronchial epithelial cells. We also demonstrated that in in vivo mouse model RSV is located in lung parenchyma and RSV infection dysregulated TJ expression and function in the whole lung. However, further studies are needed to elucidate the link between RSV infection and barrier function.

**Conflict of Interest Statement**

The authors declare that there are no conflicts of interest.


Figure legend

Figure 1. Infection of human healthy and asthmatic NHBE with RSV and its effect on TJ.
(A) TER analysis and paracellular flux analysis of healthy (n=6) and asthmatic (n=6) bronchial epithelial cell cultures infected with UV-RSV, RSV MOI 0.15, RSV MOI 1.5 and medium control (u.s.). (B) qPCR analysis of RSV N gene to determine the viral load of these cultures at day 6 after infection. (C) qPCR analysis of occludin, claudin-1 and claudin-7 of these cultures at day 6 after infection.

Figure 2. RSV infection leads to inflammation in the lungs.
(A) qPCR analysis of RSV N gene in the mouse lungs after the infection with RSV to determine the viral load. (B) Bradford assay of BAL to show protein influx into the lung lumen and (C) MUC5AC mRNA expression in the lung in naïve and post infection with RSV. (D) Level of IL-1β, IL-6, IL-12 p40, MCP-1, MIP-1α, MIP-1β, G-CSF, KC and RANTES in BAL. n=8, experiments were repeated 3 times.

Figure 3. TJ gene expression is regulated by RSV in mouse lungs.
(A) qPCR analysis of claudin-1 and occludin in the lung post infection with RSV compared to naïve and (B) their correlation with the RSV load. n=8, experiments were repeated 3 times.

Figure 4. RSV is located in the mouse lung parenchyma.
Representative confocal staining of occludin (green) and RSV (red) post infection with RSV and naïve control mouse lung. DAPI is stained in blue. Experiments were repeated 3 times.
Supplementary data

Figure S1: (A) Experimental protocol. Balb/c mice were infected intranasally with $7.4 \times 10^5$ PFU RSV A2 or UV-irradiated RSV, or left naïve, and lung tissue was analysed at 1, 2, 4, 6 days post infection with RSV A2, n=8. Experiments were repeated 3 times. (B) Correlations of protein influx and MUC5AC mRNA with the RSV load. (C) Correlation of IL-1β, IL-6, IL-12p40, MCP-1, MIP-1α, MIP-1β, G-CSF, KC, RANTES with the viral load. All detectable values of the each experiment are included in the graphs.

Figure S2: (A) qPCR analysis of the mRNA expression of the claudin, Marvel, JAM and ZO families in naïve mouse lungs. (B) Claudin-7 gene expression and its correlation with the viral load. (C) Claudin-4, claudin-10b and tricellulin gene expression.

Figure S3: Confocal microscopy of (A) claudin-1 (green) and (B) claudin-7 (green) and RSV (red) in naïve, UV-RSV and RSV-inoculated mice.
Table S1: Primer sequences used in this study:

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Figure 1