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Early View

Research letter

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Age-dependent response of the human nasal epithelium to rhinovirus infection

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Take home message (255 of 256 character incl. spaces): After rhinovirus (RV) infection, RV viral loads are higher and immune factor expressions lower in nasal epithelial cells from children versus adults in vitro. This may explain age-dependent severity of RV infections and association with asthma development.

Keywords

Airways, children, adults, air-liquid interface, respiratory infection

To the editor:

Rhinovirus (RV)-induced wheezing in early childhood is a major risk factor for later asthma (1, 2) and RV-infections have been identified as a main reason for asthma exacerbations (3-5). While RV-infections can manifest with a severe clinical course, RVs can also colonize the respiratory tract asymptomatically (6-8). Thus, additional factors seem to determine which children develop a symptomatic RV-infection and are at increased risk for asthma development, and which remain asymptomatic. For example, there is a *genetic susceptibility*, since RV-induced wheezing *and* specific genetic variants increase the risk for asthma (9). *Environmental factors*, such as farming and air pollution, have been shown to influence the risk for asthma development (10). Further, the age of RV-infection differently affects the clinical course, suggesting different time windows of susceptibility (1). For example, RV-induced wheezing at ages 2-3 years is associated with a higher risk for asthma development compared to wheezing during the first year of life (1), indicating age-dependent immune responses. However, underlying mechanisms responsible for age-dependent severity of RV-infections have not yet been studied comprehensively.

Therefore, we investigated age-dependency of anti-RV response in primary epithelial cells (NECs), which are the main target for RV-infections and act as a switchboard to initiate and regulate immune responses (11, 12). We hypothesize that the initial response of NECs to RVs is weaker at younger ages. To test this, we investigated the age-dependency of viral loads and the early epithelial immune response to *in vitro* infection with RV-16 and RV-1b of re-differentiated NECs of children and adults.

NECs were obtained via nasal brushings (interdental brush, Top Caredent) from healthy adult volunteers and healthy children undergoing elective surgery (13) (study approved by the Ethics Committee Nordwest- und Zentralschweiz, Switzerland, reference number 250/13, written informed consent obtained from all donors). After cultivation and re-differentiation using the PneumCult Expansion Plus and ALI media (Stemcell Technologies), NECs were infected with RV-16 or RV-1b (multiplicity of infection (MOI) 1 and 4; 1h, 37°C; polyinosinic: polycytidylic acid (pI:C) (10µg/ml) and phosphate-buffered saline with Mg/Ca were used as controls). 20h post-infection, we harvested basolateral supernatants and cell lysates (using TRizol).

mRNA was isolated (RNA Clean & Concentrator-5 w/ Zymo-Spin IC Columns, Zymo Research) and converted into cDNA (GoScript Reverse Transcription System, Promega). Real-time RT-PCR was performed using the GoTaq qPCR Master Mix system (Promega). Protein concentrations were measured in the basolateral media using a Milliplex MAP kit (Merck).

We subdivided the paediatric population into three groups: infancy (0-12 months, group 1, mean age (range) 8.0 (2.5-12.7) months), early ($\geq$13-36 months, group 2, 26.4 (13.3-35.5) months), and late childhood (>36-93 months, group 3, 62.2 (36.3-92.7) months). Comparison of outcomes between children (N=49, (mean age (range)) (3.4 (0.21-7.7) years) versus adults (N=12, (35.1 (24.9-65.6) years) was done using the Wilcoxon rank-sum test. A p-value <0.05 defined statistical significance, analysis was performed with Stata®, release 15.

Re-differentiated, air-liquid-interface (ALI) exposed NECs of children showed higher viral loads after RV-1b and RV-16 infection than NECs of adults. This finding was more pronounced in cells infected with RV-16 compared to RV-1b and those infected

at higher MOI (Fig.A). In the paediatric population, levels of viral loads after RV-16 infection increased with increasing age, albeit not significantly (Fig.B), RV-1b infection showed a similar pattern (data not shown). In children and adults separately, we could show a robust, dose-dependent anti-viral response induced by RV-infection (increase of *ICAM-1, LDLR, RIG-1, MDA5, TLR3, β-Def2, IFN-β, IFN-λ* (only for RV-16), *CXCL10, IL-1β* (only for RV-16), *CXCL8* (only for RV-16), *IL-6* mRNA and CXCL10, CXCL8 and IL-6, but not IFN-γ protein, data not shown), and observed differences of the anti-RV response between adults children. Interestingly, those differences were already present at baseline (in uninfected controls). We found significantly lower mRNA levels of *ICAM-1, LDLR*, *β-DEF2*, *CXCL10*, *IL-1β*, *IL-6* and *IFN-λ* (only borderline significant) in children compared to adults, while mRNA levels of *MDA5* were significantly higher in children compared to adults. We did not find any differences between children and adult NECs in mRNA levels of *RIG1, TRL3, IFN-β, IFN-λ* (Fig.C). These differences persisted after infection (data not shown). Compared to children, protein concentrations of IFN-γ and IL-6 were higher in adults at baseline, but not after infection. We did not see differences in CXCL10 or CXCL8 protein concentrations (Fig.D). Within the paediatric age groups, expression levels and protein release did not differ significantly.

This is the first study to investigate RV-1b and RV-16 infection in an ALI cell culture model. We found lower viral loads in cells infected with RV-1b compared to RV-16. Cell entry of RV-16 occurs via the ICAM-1 cell surface receptor, while RV-1b enters via the LDLR receptor (14). We found that compared to the highly expressed ICAM-1 levels, LDLR expression levels were much lower, possibly explaining the reduced RV-1b mRNA levels found in this study. Expression levels of *ICAM-1* and *LDLR*

mRNA levels were higher in adults compared to children. Association between age and the expression of *ICAM-1* or *LDLR* has scarcely been studied. Within a study including 29 adults (age 42-85 years), there was no association with age and ICAM-1 levels (15). The here reported age-dependent expression of *ICAM-1* and *LDLR* could be relevant for epidemiological and experimental studies addressing a large age-range.

The childhood origins of asthma (COAST) study investigated the timing and specific viral aetiology of wheezing illnesses during early childhood and the impact on asthma development within a high-risk population. These data suggest that the age of RV-infection has prognostic value for subsequent asthma risk. Children who had RV-induced wheezing during their first year of life had a 2.7-fold asthma risk at six years, while RV-induced wheezing during the second year was associated with a 6.5-fold asthma risk, and RV-induced wheezing during the third year was associated with a dramatic 31.7-fold asthma risk (1). Interestingly, we found lower viral load in infants compared to older children potentially reflecting the age-dependent associations reported previously (1). Our findings, along with those previously reported (1), are important for future intervention studies addressing RV-infections as age-dependent differences in ant-viral responses in epithelial cells.

Our study is the first to investigate immune factors following RV-infection using an ALI cell model within a large population across a broad age range. Previous studies investigated either RV-16 or RV-1b, while we investigated both virus types in parallel, allowing comparison of immune response to both RV types. The study is limited by the cross-sectional assessment of the immune response, precluding the analysis of individual immune development or course of infection. Other cell types and viruses should be studied to investigate not only the anti-viral response to RV represented by

the respiratory epithelium, but more comprehensively the innate and late immune response.

We report on age-dependent anti-viral response in NECs after infection with major and minor group RVs. Children had higher viral loads than adults, likely the result of lower levels of immune factors. These findings may explain age-dependent severity of RV-infections and age-dependent associations between RV-induced wheezing and asthma development and indicate that early immune function priming is relevant for later chronic airway disease.

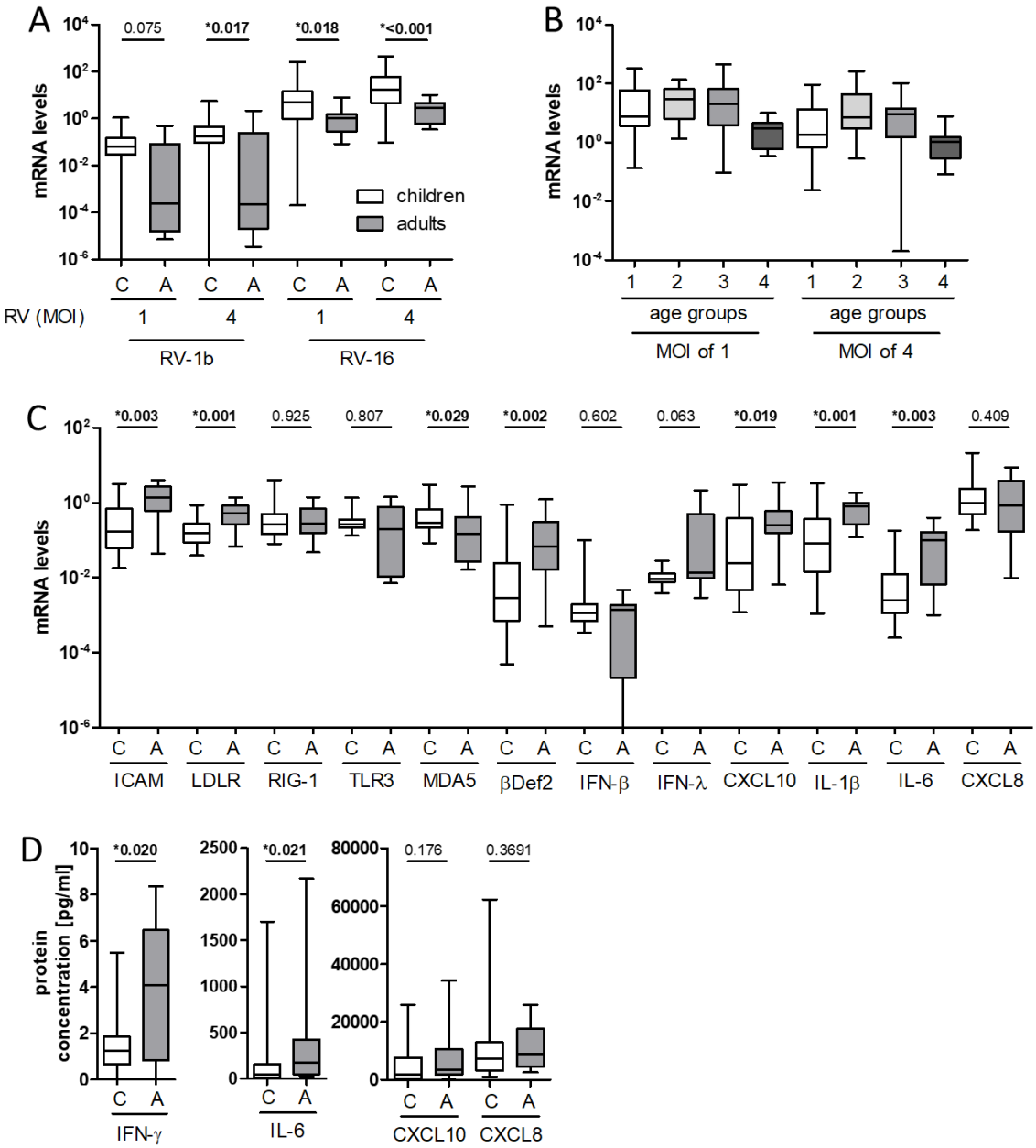

Figure. (A&B) Viral loads of RV-1b and RV-16 in NECs 20 h post-infection measured by quantitative real-time RT-PCR. (A) Comparison of viral loads in NECs of children (n=48) and adults (n=12). **(B)** Comparison of viral loads after RV-16 infection in different paediatric age groups and adults. Group 1 n=10, group 2 n=14, group 3 n=25, group 4 adults n=12. **(C) mRNA levels of immune factors at baseline (without RV infection).** mRNA levels are presented as dCt values, normalized to the house-keeping gene phosphoglycerate kinase 1. **(D) Baseline**

protein concentrations assessed using multiplex bead-based immunoassays. Data are shown as box and whisker plot with line at median, interquartile range (box) and range (line). *p<0.05, tested with Wilcoxon test.

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Conflict of interest

The authors have no conflicts of interest to disclose. Dr. Latzin reports personal fees from Gilead, Novartis, Polyphor, Roche, Santhera, Schwabe, Vertex, Vifor, and Zambon, outside the submitted work. Dr. Usemann reports personal fees from Vertex outside the submitted work.

Additional Information

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

Author contribution: JU performed RT-PCR measurements, analysed the data and wrote the main manuscript text. MPA provided stock of RV-16 and RV1B and helped with data interpretation. NR supported the cytokine analysis and helped with data

interpretation. PL developed the project idea and helped with data interpretation. LM designed the study, performed experiments and analysis, prepared the figures and the main manuscript text. All authors reviewed and approved the manuscript.

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