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

To the Editor:

Rhinovirus-induced wheezing in early childhood is a major risk factor for later asthma [1, 2] and rhinovirus infections have been identified as a main reason for asthma exacerbations [3–5]. While rhinovirus infections can manifest with a severe clinical course, rhinoviruses can also colonise the respiratory tract asymptomatically [6–8]. Thus, additional factors seem to determine which children develop a symptomatic rhinovirus infection and are at increased risk for asthma development, and which remain asymptomatic. For example, there is a genetic susceptibility, since rhinovirus-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]. Furthermore, the age of rhinovirus infection affects the clinical course differently, suggesting different time windows of susceptibility [1]. For example, rhinovirus-induced wheezing at ages 2–3 years is associated with a higher risk of 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 rhinovirus infections have not yet been studied comprehensively.

Therefore, we investigated age-dependency of anti-rhinovirus response in primary nasal epithelial cells (NECs), which are the main target for rhinovirus infections and act as a switchboard to initiate and regulate immune responses [11, 12]. We hypothesise that the initial response of NECs to rhinoviruses 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 rhinovirus (RV)-16 and RV-1b of re-differentiated NECs of children and adults.

NECs were obtained *via* nasal brushings (interdental brush; Top Caredent, Zurich, Switzerland) 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 PneumaCult Expansion Plus and ALI media (Stemcell Technologies, Vancouver, BC, Canada), NECs were infected with RV-16 or RV-1b (multiplicity of infection (MOI) 1 and 4; 1 h, 37°C; polyinosinic:polycytidylic acid (10 µg·mL^{-1}) and PBS with magnesium/calcium were used as controls). 20 h post-infection, we harvested basolateral supernatants and cell lysates (using TRizol; ThermoFisher Scientific, Waltham, MA, USA).

mRNA was isolated (RNA Clean & Concentrator-5 w/ Zymo-Spin IC Columns; Zymo Research, Irvine, CA, USA) and converted into cDNA (GoScript Reverse Transcription System; Promega, Madison, WI, USA). Real-time reverse-transcriptase 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, Darmstadt, Germany).

We subdivided the paediatric population into three groups: infancy (age 0–12 months, group 1, mean age (range) 8.0 (2.5–12.7) months), early childhood (age ⩾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 (range) age 3.4 (0.21–7.7) years) *versus* adults (n=12, 35.1 (24.9–65.6) years) was performed using the Wilcoxon rank-sum test. A p-value <0.05 defined statistical significance; analysis was performed using Stata (release 15; StataCorp, College Station, TX, USA).

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 (figure 1a). In the paediatric population, levels of viral loads after RV-16 infection increased with increasing age, albeit not significantly (figure 1b); RV-1b infection showed a similar pattern (data not shown). In children and adults separately, we could
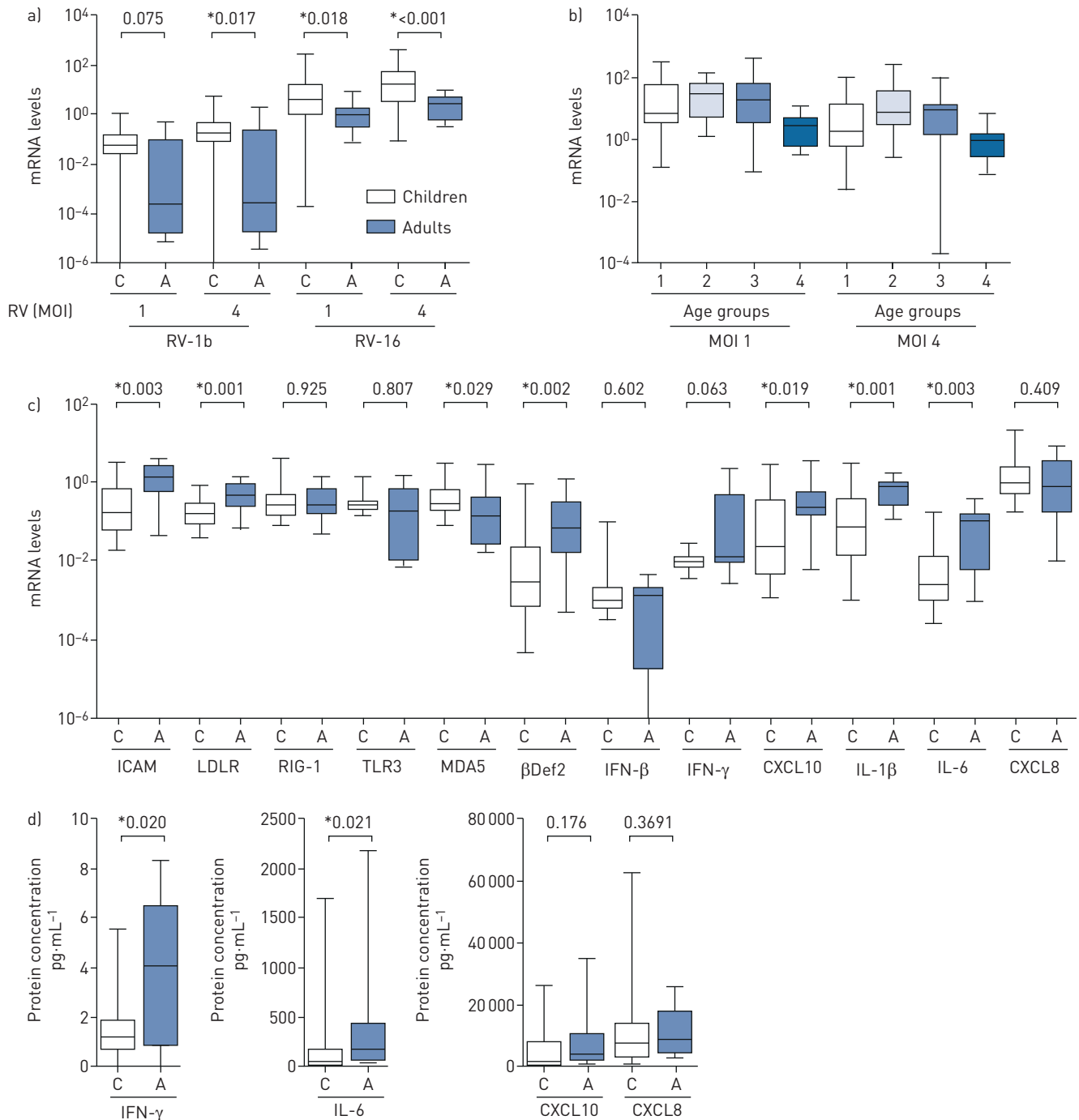

FIGURE 1 Viral loads, baseline immune factor expression and baseline protein concentrations in primary nasal epithelial cells (NECs) 20 h post-infection. a) Comparison of viral loads of rhinovirus (RV)-1b and RV-16 in NECs of children (C; n=48) and adults (A; n=12) measured by quantitative real-time reverse-transcriptase PCR. 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 rhinovirus infection). mRNA levels are presented as dCt values, normalised to the housekeeping gene phosphoglycerate kinase 1. d) Baseline protein concentrations assessed using multiplex bead-based immunoassays. Data are shown as box and whisker plots with line at median, interquartile range (box) and range (line). MOI: multiplicity of infection; ICAM: intercellular adhesion molecule; IFN: interferon; IL: interleukin. *: p<0.05, Wilcoxon test.

show a robust, dose-dependent antiviral response induced by rhinovirus infection (increase of intercellular adhesion molecule (*ICAM-1*), low-density lipoprotein receptor (*LDLR*), *RIG-1*, *MDA5*, *TLR3*, β-*Def2*, interferon (*IFN*)-β, *IFN*-λ (only for RV-16), *CXCL10*, interleukin (*IL*)-*1*β (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-rhinovirus response between adults and 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-1*β, *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 or *IFN*-λ (figure 1c). 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 (figure 1d). 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 [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. In a study including 29 adults (age 42–85 years), there was no association with age and ICAM-1 levels [15]. The age-dependent expression of *ICAM-1* and *LDLR* reported here 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 rhinovirus infection has prognostic value for subsequent asthma risk. Children who had rhinovirus-induced wheezing during their first year of life had a 2.7-fold asthma risk at 6 years, while rhinovirus-induced wheezing during the second year was associated with a 6.5-fold asthma risk, and rhinovirus-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 reported previously [1], are important for future intervention studies addressing rhinovirus infections as age-dependent differences in antiviral responses in epithelial cells need to be considered.

Our study is the first to investigate immune factors following rhinovirus 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 rhinovirus 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 antiviral response to rhinovirus represented by the respiratory epithelium, but more comprehensively the innate and late immune response.

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

Jakob Usemann [1,2,3], **Marco P. Alves**[4,5], **Nicole Ritz** [2], **Philipp Latzin** [1,2,6] and **Loretta Müller** [1,2,6]
[1]Paediatric Respiratory Medicine, Dept of Paediatrics, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland. [2]University Children's Hospital Basel (UKBB), Basel, Switzerland. [3]Division of Respiratory Medicine, University Children's Hospital Zurich, Zurich, Switzerland. [4]Institute of Virology and Immunology, Bern, Switzerland. [5]Dept of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland. [6]These authors contributed equally to this project.

Correspondence: Loretta Müller, Paediatric Pulmonology, Inselspital Bern, Murtenstrasse 50, 3008 Bern, Switzerland. E-mail: loretta.mueller@insel.ch

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