MANCHESTER 1824
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DOI:
10.1111/all.12931

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citation for published version (APA):
Aab, A., Wirz, O., van de Veen, W., Söllner, S., Stanic, B., Rückert, B., Aniscenko, J., Edwards, M. R., Johnston, S. L., Papadopoulos, N., Rebane, A., Akdis, C. A., & Akdis, M. (2017). Human rhinoviruses enter and induce proliferation of B lymphocytes. *Allergy: European Journal of Allergy and Clinical Immunology*, *72*(2), 232-243. https://doi.org/10.1111/all.12931

Published in:
Allergy: European Journal of Allergy and Clinical Immunology

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Download date:30. Jun. 2022

Human Rhinoviruses Enter and Induce Proliferation of B Lymphocytes

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17

Abstract

Background: Human rhinoviruses (HRV) are one of the main causes of virus induced asthma exacerbations. Infiltration of B lymphocytes into the subepithelial tissue of the lungs has been demonstrated during rhinovirus infection in allergic individuals. However, the mechanisms through which HRVs modulate the immune responses of monocytes and lymphocytes are not yet well described.

Objective: To study the dynamics of virus uptake by monocytes and lymphocytes, and the ability of HRVs to induce activation of *in vitro* cultured human peripheral blood mononuclear cells.

Methods: Flow cytometry was used for the enumeration and characterization of lymphocytes. Proliferation was estimated using ^{3}H-thymidine or CFSE labelling and ICAM-1 blocking. We used bead based multiplex assays and quantitative PCR for cytokine quantification. HRV accumulation and replication inside B lymphocytes was detected by a combination of *in situ* hybridation (ISH), immunofluorescence and with PCR for positive strand and negative strand viral RNA. Cell images were acquired with imaging flow cytometry.

Results: By means of imaging flow cytometry, we demonstrate a strong and quick binding of HRV types 16 and 1B to monocytes, and slower interaction of these HRVs with CD4+ T cells, CD8+ T cells and CD19+ B cells. Importantly, we show that HRVs induce the proliferation of B cells while addition of anti-ICAM-1-antibody partially reduces this proliferation for HRV16. We prove with ISH that HRVs can enter B cells, form their viral replication centers and the newly formed virions are able to infect HeLa cells. In addition, we demonstrate that similarly to epithelial cells, HRVs induce the production of pro-inflammatory cytokines in PBMCs.

Conclusion: Our results demonstrate for the first time that HRVs enter and form viral replication centers in B lymphocytes and induce the proliferation of B cells. Newly formed virions have the

capacity to infect other cells (HeLa). These findings indicate that the regulation of human rhinovirus induced B cell responses could be a novel approach to develop therapeutics to treat the virus-induced exacerbation of asthma.

Keywords: Asthma; B lymphocyte; Human rhinovirus.

Abbreviations

CFSE	carboxy-fluorescein succinimidyl ester
DAPI	4', 6-diamidino-2-phenylindole
HRV	human rhinovirus
ICAM-1	intercellular Adhesion Molecule 1 also known as CD54
IFN	interferon
IL	interleukin
LDLR	low-Density Lipoprotein Receptor
LRT	lower respiratory tract
MOI	multiplicity of infection
PBMC	peripheral blood mononuclear cell
TCID	tissue culture infective dose
Th	T helper cell
URT	upper respiratory tract
UV HRV	ultraviolet inactivated HRV
vRNA	viral RNA

62 **Introduction**

63 Virus-induced exacerbations are the major cause of morbidity and mortality among asthma patients.
64 Human rhinoviruses (HRVs) are the most common triggering factors of acute asthma exacerbations
65 (1, 2); however, evidence demonstrating a causal relationship between HRV infection and asthma
66 exacerbation is mostly limited to epidemiological studies and the immunological mechanisms are
67 poorly understood. T helper (Th) 2 cells are considered the main initiating effector cell type in atopic
68 asthma in general, and asthma exacerbations in particular. However, exaggerated Th2 cell activities
69 alone do not explain all aspects of asthma and exacerbations. In addition, they cannot explain
70 susceptibility to viral infection, shared between asthma and chronic obstructive pulmonary disease
71 (3).

72 HRV was the first identified member of the *Picornaviridae* family. The picornaviruses are non-
73 enveloped viruses with a single stranded, positive sense RNA genome. The family consists of twelve
74 genera, including the Enterovirus genus, to which HRV belongs (4). On nucleic acid identity this genus
75 can be subdivided further into ten species, from which three are HRV-A,-B and -C. ICAM-1
76 (intercellular adhesion molecule 1, also known as CD54) is the virus entry receptor for the major
77 group of rhinoviruses, such as HRV16 and HRV14 serotypes, and LDLR (low-density lipoprotein
78 receptor) is the receptor for minor group rhinoviruses, such as HRV1B and HRV29 (5). It has been
79 recently found that HRV-C enters the cells using Cadherin-related family member 3 (CDHR3) (6, 7).
80 ICAM-1 and LDLR are expressed on the surface of many different cell types, including those that are
81 active in respiratory immune responses, such as monocytes and airway macrophages, dendritic cells,
82 airway eosinophils, mast cells, B lymphocytes and activated T lymphocytes (8, 9). Less is known about
83 expression of CDHR3 but it is highly expressed in human lung tissue (10) and bronchial epithelium (7).

84 Among terminally differentiated bronchial epithelial cells, HRV replication has been documented
85 predominantly in ciliated epithelial cells (11). The HRV-infected epithelial cells release a variety of

86 inflammatory factors, which orchestrate proliferation, chemotaxis, and activation of immune cells,
87 resulting in an amplification of inflammation. It is generally believed that HRVs are not able to infect
88 white blood cells. In contrast, it has been recently shown that HRVs are able to infect human
89 macrophages (12), T cells (13-15) and Ramos cells (human B lymphocyte cell-line) (16). Even then,
90 and similar to bronchial epithelial cells, only a small fraction of cells are positive for virus replication
91 (12). Innate activation of monocytes upon a viral infection(17), and close contact of upper and lower
92 airway epithelium cells with infiltrating inflammatory leukocytes, granulocytes, dendritic cells and
93 monocytes suggests that these cells might influence immune responses to HRV *in vivo*, and be
94 important to explain processes during asthma exacerbations (18).

95 In addition to induction of asthma exacerbations, there is likely a causative link between HRV
96 infections and the development of childhood asthma (19), but yet fundamental questions persist
97 about mechanisms linking this common pathogen to the disease. As it is generally thought that
98 asthma results from over-zealous responses to innocuous antigens or poor regulation of the immune
99 system, the details of how HRV infection interacts with the host immune system could be the key in
100 uncovering the underlying mechanism of the development of asthma. Understanding disease
101 pathophysiology is important for the development of effective and evidence-based treatments that
102 are currently a major unmet need for both asthma exacerbations and asthma onset.

103 In the present study, we investigated the ability of HRVs to bind and activate lymphocytes using *in*
104 *vitro* cultured human peripheral blood mononuclear cells (PBMCs). We investigated the dynamics of
105 infection in monocytes and lymphocytes and demonstrate for the first time that HRVs enter, form
106 viral replication centers and induce strong proliferation of B cells.

107

108 **Methods**

109 **Study subjects**

110 Sixteen subjects (13 female, 3 male) adult volunteers participated in the study. All subjects were
111 healthy in the moment of drawing blood. The status of possible infections was followed three days
112 before and three days after drawing the venous blood and confirmed not to be other than healthy.

113 **Generation of HRV stocks**

114 HRV serotypes 14, 16, 1B and 29 were propagated in Ohio HeLa cells using standard protocol (20).
115 Virus stocks were titrated by infecting HeLa monolayers with serially diluted HRV and assessing
116 cytopathic effect to estimate their 50% tissue culture infective dose (TCID50)/ml by Kremser method.
117 The identities of all HRVs were confirmed by neutralization using serotype-specific antibodies (ATCC).
118 TCID50/ml was expressed as multiplicity of infection (MOI) based on TCID50 0.1 mL of a TCID50 virus
119 of 1e7 per mL on 1e6 cells would be an MOI of 1.

120 **Ultraviolet inactivation of virus and "Mock" solution**

121 UV-inactivated virus was prepared by irradiating the virus suspensions in a 24-well tissue culture dish
122 on ice for 10 minutes with a 75 W UV source (254 nm) at a distance of 5 cm. Treatment resulted in
123 complete loss of infectious titer as estimated by titration assay. To produce "mock" solution, Ohio
124 HeLa cells were cultured as for virus production without viruses to the culture.

125 **Isolation and culturing of PBMCs**

126 PBMCs were obtained from heparinized whole blood by Biocoll (Biochrom KG, Berlin, Germany)
127 density gradient centrifugation. Cells were washed three times with PBS (0.02% EDTA added) and
128 suspended in RPMI 1640 medium supplemented L -glutamine (2 mmol/L), MEM vitamin, penicillin

129 (100 U/mL), streptomycin (100 µg/mL), kanamycin, nonessential amino acids, sodium pyruvate (Life
130 Technologies, Carlsbad, Calif), and with 10% heat inactivated fetal calf serum (FCS; Invitrogen).
131 Further we call the RPMI 1640 medium together with supplements as cRPMI (complete RPMI).

132 **Culturing of PBMC with virus**

133 PBMC were exposed to HRV at MOI = 1.0 or 10 or other when indicated. As controls, UV-treated
134 HRVs or no virus ("mock"), were used.

135 **Proliferation assay with ^{3}H-thymidine**

136 After 5 day of stimulation with unlabelled active or UV inactivated or without viruses (mock) in
137 triplicates, PBMCs were pulsed for 8 hours with ^{3}H-thymidine, harvested with a Tomtec plate washer,
138 and counted on a Betaplate counter. Results were expressed as a stimulation index (SI).

139 **CFSE labelling**

140 PBMC were washed in PBS twice, suspended in 10 mL PBS containing 5% FBS. CSFE (from 10 mM
141 stock and at the final concentration 5µM) was added to the tube and immediately shaked vigorously.
142 Labelling of PBMCs was done promptly 5 minutes at room temperature in the dark. Cells were
143 washed three times in PBS supplemented with 5% FCS. Thereafter, cRPMI media was added and cells
144 were transferred to cell culture incubator (37°C under 5% CO_2) for further experiments.

145 **Flow cytometry and cell sorting**

146 For the expression of surface markers, cells were stained with the following antibodies: CD3–FITC,
147 CD16-FITC, CD19- ECD, CD19- PC5, CD4-PE/Cy7, CD14-APC/Cy7, CD19-Brilliant Violet 510, CD19-
148 APC/Cy7. Matching isotype controls were used as negative controls. Samples were measured with a
149 FACSAria II instrument (Beckton Dickinson) or Galios flow cytometer and analyzed using Kaluza

150 software (Beckman Coulter). Dead cell were excluded based on staining with the eFluor 450 dye.

151 Before imaging flow cytometry CD14+ cells were sorted out from rest of the cells accordingly to the

152 CD14-APC/Cy7 signal. To get purified CD14+ cells we excluded from this cell population eFluor 450

153 positive cells as dead cell population.

154 **Detection of infectious virions**

155 PBMCs were infected with unlabelled-HRV16 at MOI 10 and incubated for 24 h at 37°C in tissue

156 culture incubator using regular cell culture conditions. Then the PBMCs were washed three times

157 with pre-warmed PBS and added to HeLa cells at a number of 800000 cells per well in a 48-well plate

158 (500 ul volume). Uninfected HeLa cells were seeded in these wells 24 h before and were growing in

159 log-phase when PBMCs were added. In the ICAM-1-blocking condition, anti-ICAM1-Ab was added to

160 HeLa cells at the concentration of 1ug/ml 2h before PBMCs were added. Three days after the

161 addition of PBMCs, HeLa cells were washed thoroughly to remove non-adherent PBMCs and RNA

162 was isolated from HeLa cells using the RNAeasy Micro Plus kit (Qiagen). The expression of negative

163 and positive strands of viral RNA was analyzed by RT-qPCR.

164 **Labelling the virus with DyLight650**

165 Labelling of viruses with DyLight650 was performed according to manufacturer - Thermo Scientific –

166 protocol. Shortly – media component in the virus (both active or UV inactivated) solution was

167 replaced with PBS dialyzing it 2 times for 4 hours with cold PBS (at pH 7.0 and 4°C). For dialyzes,

168 membrane MWCO of 6-8 kDa Spectra/Por ® Dry Standard RC Dialysis Tubing (Spectrumlabs) was

169 used. To the 15 mL samples was added 1 mg DyLight650 NHS Ester, mixed and stained at RT for 1

170 hour protected from light. Labelled and starting non-labelled virus solution were titrated by end-

171 point titration assay in parallel and stored at -80°C. Labelling caused the drop of virus infectivity 5

172 times when to compare with the starting non-labelled virus.

173 **Imaging flow cytometry**

174 Cells were labelled and promptly measured by cell imaging flow cytometer “ImageStream”. To avoid
175 overlapping of the APC/Cy7 signal with the side scatter signal on the “ImageStream”, monocytes
176 were sorted out with the cell sorter FACS Aria before taking images on the “ImageStream”. Signal
177 strength was compensated between different channels according to the manufacturer protocols.
178 Flow speed was stabilized before collecting 30 000 events and 40x magnification was used for taking
179 images. Data were analyzed using “Ideas” software (Amnis).

180 **QuantiGene ViewRNA *in-situ* hybridization**

181 ISH was performed according to manufacturer -Affymetrix, Santa Clara, CA - protocol. Shortly –
182 PBMCs were added on top of the polylysine coated microscopic slides. HRV or controls were added
183 to the wells. Before cell fixation, media was carefully replaced with PBS. Thereafter, cells were fixed
184 with 4% paraformaldehyde and washed with PBS and dehydrated with ethanol at increasing
185 concentrations (50%, 70%, and 100%). Slides were submerged in 100% ethanol at −20° C for storage.
186 Before further analysis, cells were rehydrated using decreasing concentrations of ethanol (70% and
187 50%) and washed with PBS. ISH was performed using QuantiGene ViewRNA protocols designed to
188 detect specifically 20 nucleotide long sequences in HRV vRNA – 15 sites are for amplifying the signal
189 and 5 sites are for blocking the nonspecific binding sites. Cells on the slides were permeabilized with
190 Working Detergent Solution (Affymetrix, Santa Clara, CA) and hybridized for 3 hours at 40° C with
191 custom-designed QuantiGene ViewRNA probes against positive strand HRV1B and human actin-beta.
192 Unbound probes were flushed out with Wash Buffer (Affymetrix, Santa Clara, CA). The bound probes
193 were amplified through PreAmp (Affymetrix, Santa Clara, CA) hybridization for 1 hour at 40° C,
194 followed by Amp (Affymetrix, Santa Clara, CA) hybridization for 1 hour at 40° C. Label Probes
195 (Affymetrix, Santa Clara, CA) targeting the individual probe types were added for 1 hour at 40° C. The

196 slides were covered in 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium and
197 examined using an LSM 510 confocal microscope (Zeiss).

198 **Immunofluorescence**

199 Microscope slides with PBMC after ISH were further incubated 30 minutes with anti-human CD20
200 (MS4A1 RabMAb®, rabbit anti-human, Epitomics 1632-1) or with anti-rabbit polyclonal antibody (IC
201 control), incubated 30 minutes at RT in dark with secondary antibody (Alexa Fluor® 488 Goat Anti-
202 Rabbit IgG (H+L)(Invitrogen, A11034). The slides were covered in DAPI-containing mounting medium
203 and examined using "Leica TCS SPE confocal microscope" (Leica Microsystems).

204

205 **Cytokine measurement**

206 PBMCs were cultured in cRPMI with or without HRV ("mock"). After incubation for 5 days, the cells
207 were pelleted, and supernatants were frozen (–80°C). Cytokines were measured with MILLIPLEX®
208 customized panel of following cytokines - TNF-α, IL-7, IL-27, IL-1β, IL-9, IL-28, IL-6, IL-10, IL-12p70, IL-
209 13, IL-8, IL-17a, RANTES, IFN-γ, MIP-1α, IP-10, MIP-1β, IL-17e/IL-25, IFN-α2, IL-23, IL-5, IL-33.
210 Fluorescent signals were read and analyzed using the Bio-Plex 200 System (Bio-Rad Laboratories).

211 **Statistical analysis**

212 Unless otherwise indicated, data show mean $\pm$ confidential interval (CI) 95%. Statistical tests were
213 performed using GraphPad Prism 5.0 and ggplot2. Statistical significance was determined using the 2-
214 tailed Student's t test or one-way ANOVA with Bonferroni's Multiple Comparison Test to pinpoint the
215 difference between two groups in multiple selections. Statistical significance was retained for P-value
216 < 0.05. The number of donors indicated in the figure legends includes all donors analyzed in the
217 relevant experiment.

218 **Results**

219 **HRV1B induces proliferation of PBMCs**

220 While most of the studies in the HRV field have focused on the relationship between HRVs and
221 airway epithelial cells, we were interested in PBMCs; investigating how HRVs and human
222 mononuclear cells interact. First, we cultured PBMCs from healthy donors with different
223 concentrations of HRV1B and determined that HRV1B is able to induce the proliferation of PBMCs at
224 a concentration range MOI = 1.0 ÷ 10 *in vitro*. At lower and higher viral concentration the
225 proliferation was not detectable or not significantly different from baseline – “mock” – condition (Fig.
226 1 A).

227 Next we studied, which cell type(s) proliferate in response to HRVs. To measure cell proliferation, we
228 added carboxy-fluorescein succinimidyl ester (CFSE) to the cells on day 0 and cultured PBMCs with
229 HRV1B, HRV14, HRV16 or HRV29 at MOI = 1.0 or MOI = 10 for 3 and 5 days, and then stained the
230 CFSE labeled cells with antibodies recognizing CD19, CD4 and CD8 and performed flow cytometry. On
231 the 5th day after infection, 6 - 13 % or 14 - 33% of CD19+ B cells were detected as proliferating when
232 PBMCs were infected with HRV1B, HRV14, HRV16 or HRV29 at MOI = 1.0 or MOI = 10, respectively.
233 The remarkable exception was HRV16: using higher viral concentration MOI = 10 caused less
234 proliferation of B lymphocytes even when compared with “mock” induced background proliferation.
235 Only a small fraction of CD4+ or CD8+ T cells proliferated with statistically non-significant difference
236 when compared to the “mock” (Fig. 1 B). The proliferation of B cells was noticeable on day 3, but the
237 difference from “mock” condition was statistically insignificant on day 5 (Fig. 1 C).

238 To confirm that HRV induced B lymphocyte proliferation is not caused by unspecific stimulation of
239 immune responses caused by the presence of virus components in the medium, we inactivated virus
240 with ultraviolet (UV) radiation. No significant proliferation of PBMCs was observed when UV-treated

241 HRV1B in comparison with non-treated HRV1B and HRV16 at MOI 1 and MOI 10 (Fig. 1 D). ICAM-1
242 blocking partially decreased the proliferation of PBMCs app %40 (%14-%80) during virus stimulation
243 (Fig. 1 E). This suggests that HRV may directly bind to PBMCs and induce B cell proliferation.

244 These data suggest HRVs are able to induce B cell proliferation at certain viral concentration and
245 timepoint *in vitro*.

246 **HRVs can attach or enter to monocytes, CD4+ and CD8+ T cells and B cells**

247 Next we studied the HRV ability to attach and enter to the lymphocytes as the first events needed for
248 the infection. We labelled HRV1B and HRV16 with fluorescent dye DyLight650 and performed flow
249 cytometry analysis at different timepoints between 30 min to 24 hours to estimate whether different
250 cell populations take up HRVs. As controls, we used either UV-inactivated HRV1B and HRV16 or
251 “mock” condition. Fig. 2 A and B demonstrate that HRV16 and HRV1B attached and probably also
252 entered into the monocytes already 30 minutes and to CD4+ T and B lymphocytes 8 hours after the
253 addition of the virus. Monocytes were almost 100% positive for both UV-treated and untreated
254 HRV16 and HRV1B at 24h point of time. This indicates that in monocytes the virus was at least
255 partially taken up via passive internalization and not an active infection. Remarkably, 3.3% (±0.2%) of
256 CD4+ T cells and 2.9% (±0.4%) B cells at 8h and 14.0% (±1.5%) of CD4+ T cells, 5.9% (±1.5%) of B cells
257 and 7.4% (±2.3%) of CD8+ T cells at 24h timepoint were detected to be positive for HRV1B and
258 HRV16 when the higher virus concentration was used. No positive signal of DyLight650 labeled HRV
259 was detected in B cells, CD4+ T cells and CD8+ T-cell at lower virus concentrations and at higher
260 concentration of UV-inactivated virus (Fig. 2 A and B).

261 To further characterize the interactions of HRVs with different PBMC subsets, we performed imaging
262 flow cytometry. PBMCs were cultured with higher concentration (MOI = 10) of DyLight650 labelled
263 HRV1B and analyzed at different time-points chosen according to the flow cytometry analysis results.

264 Monocytes were analyzed 1h, while B cells and CD3+/CD4+ and CD3+/CD4- T cells 24h after the
265 infection. Consistent with the flow cytometry results, monocytes were detected to be positive for
266 HRV1B 1h after the infection and CD4+ T cells and CD19+ B cells 24h after infection (Fig. 2 C, S1-3
267 Fig.). CD3+/CD4- T cells, representing CD8+ cytotoxic T cell subset, did not give a positive signal in
268 imaging flow cytometry (data not shown). In CD4+ T cells, DyLight650 labelled HRV1B was seen on
269 cell surface where it co-localized with anti-CD4 staining (Fig. 2C, S2 Fig.), while in monocytes and
270 CD19+ B cells, DyLight650 staining pattern indicates that DyLight650 labelled HRV1B was located
271 inside the cells (Fig. 2 C, S1 and S3 Fig.).

272 **HRV forms viral replication centers in B cells and infectious virions in PBMCs**

273 Next, we used *in situ* hybridization (ISH) for HRV1B to study whether we are capable of detecting
274 viral RNA (vRNA) that would indicate that HRV could uncoat and infect PBMCs. PBMCs were cultured
275 with infectious HRV1B and ISH was performed at 5th day (Fig. 3 A, S4 Fig.) and as positive control,
276 HeLa cells, were cultured with infectious or UV-treated HRV1B and ISH was performed 1 day after
277 infection (Fig. 3 B). As an additional positive control, we used ISH probes recognizing beta-actin
278 mRNA that is strongly expressed during the activation or division of the cells. In PBMCs, vRNA signal
279 was detected as an intracellular vesicle-like formation with the size around 200 – 500 nm, which is 10
280 – 25 times the size of the single viral particle, suggesting that we were able to detect viral replication
281 centers, but not single vRNA with ISH (Fig. 3 A, S4 Fig.). Similar vRNA signal pattern was detected in 4
282 different donors. More intensive dotted intracellular signal was seen in HeLa cells (Fig. 4 B, S6 Video).
283 Moreover, immunofluorescence analysis demonstrated that the vRNA signal often localized in the
284 cells that stained positively with anti-CD20, a marker for non-terminally differentiated B cells. In
285 conclusion, these data demonstrate that HRV1B can enter into B cells *in vitro* in cultured PBMCs. To
286 demonstrate that HRV can replicate in PBMCs, we pre-infected PBMCs with HRV16 and seeded them
287 over uninfected HeLa cells after washing then HRV RNA was analysed in stringently washed HeLa

288 cells after 72h. Our data demonstrate that HRV can replicate efficiently in PBMCs and that newly
289 produced virions may have the capacity to infect HeLa cells, as demonstrated by measuring viral RNA
290 in the HELA cells. Figure 3 C shows the expression of positive and negative strand viral RNAs in HeLa
291 cells after all of the above procedures. UV irradiated HRV was not able to infect PBMCs and therefore
292 virus was not able to replicate and reinfect HELA cells. Blocking of ICAM-1 on HeLa cells was leading
293 to a reduced infection rate in HELA cells to about %57 (%24-%84) of the virus load in average that
294 was observed for the non-blocked infection condition. The same results were obtained in both
295 positive and negative strand mRNA detections.

296 **The cytokine profile of HRV-infected PBMCs**

297 To explore whether and how different HRVs stimulate immune responses that possibly support the
298 observed B cell proliferation, we next determined the profile of chemokines/cytokines produced by
299 PBMCs. We infected cultured PBMCs using the same experimental settings as in Fig. 2-5, collected
300 the supernatants on day 5 after HRV infection and measured the panel of 27 cytokines. All used virus
301 strains upregulated IL-6, IFN-α, IL-10, IFN-γ and RANTES. MIP1β, IL-1beta, IL-6, and TNF-α were
302 stimulated more strongly upon HRV16 infection (Fig. 4). All viruses induced IFN-α production, but
303 minor group rhinoviruses, HRV1B and HRV29, induced significantly stronger expression of IFN-α at
304 MOI=10. For major group viruses, HRV16 and HRV14, there was no significant concentration
305 dependent difference in the induction of IFN-α. IFN-γ was significantly stronger expressed at all virus
306 types compared to “mock” with the exception HRV16 MOI=10 and HRV29 MOI=1, where there was
307 no significant difference. Interestingly, HRV16 MOI=10 concentration did not increase the IFN-γ
308 production and at the same time IL-10 production was strongly upregulated. IL-27 was upregulated
309 by major HRV types, HRV16 and HRV14 and was observed to be more strongly expressed at MOI=1.
310 These results show that HRVs induce immune responses in cultured PBMCs with moderate
311 differences between the virus strains.

312 **Discussion**

313 The mechanisms of the development of immune responses to HRVs and the causal relationship
314 between HRV infection and asthma exacerbation are not completely understood. In the current
315 study, we demonstrate that monocytes, CD4+ T cells, CD19+ B cells, and CD8+ T cells are able to
316 interact with HRVs in *in vitro* cultured PBMCs. More importantly, we show that HRVs enter and can
317 induce the proliferation of CD19+ B-cells. In addition, we demonstrate that HRVs activate PBMCs and
318 induce the production of inflammatory cytokines MIP-1β, IL-1β, IL-6, TNF-α, IFN-α, IL-10, RANTES,
319 IFN-γ and IL-27.

320 During the last several decades, strong evidence have accumulated supporting the idea that HRV
321 infections enhance allergic inflammation in the airway leading often to increased asthmatic symptom
322 – asthma exacerbation (2, 21-23). Most commonly, HRVs infect only URT epithelial cell lining, and for
323 most of us, HRV infection causes only a relatively mild illness with upper respiratory symptoms as
324 seen during the common cold (24). Remarkably, more than 50% of bronchial epithelial biopsies
325 collected from patients during virus induced asthma exacerbation have found to be HRV positive
326 (25).

327 While the research has been focused mostly on viral infection in bronchial epithelia as the principal
328 site of viral replication, the capacity of HRVs to directly infect immune cells and to modulate immune
329 responses has been studied less. Still, Levandowski et al. demonstrated that the total lymphocyte
330 count of infected individuals was significantly reduced on day 3 after the challenge with a rhinovirus
331 of the minor group(26). In addition, they showed that specifically T lymphocyte numbers were
332 reduced, while B lymphocyte levels were constant. Decreased cell populations positively correlated
333 with disease severity. In addition, virus shedding was the highest in individuals with the greatest
334 reduction in lymphocytes. The reduced number of lymphocytes in the circulation might be at least
335 partly explained by a massive lymphocytic and eosinophilic infiltration into the infected tissues. This

336 cell infiltration to bronchial mucosa might be responsible for changes in airway hyper-responsiveness
337 and asthma exacerbations(27). Viremia may occur during HRV respiratory infections, suggesting that
338 rhinoviremia may be involved in asthma exacerbation pathogenesis(28). In addition, Gern et al.
339 showed that the activation of T cells estimated by CD69 was detectable at HRV16 MOI of 0.03, with
340 maximum CD69 expression occurring with a MOI of 1 (29). UV-irradiation did not significantly
341 increase T cell CD69 expression. The narrow range of viral concentration was similar to what was
342 observed in our experiments with B cells. B cell proliferation reached its highest values on days 3 to 5
343 while the HRV induces no statistically significant proliferation of B lymphocytes during first 48 h as it
344 has been noticed already by Papadopoulos et al. but still they observed a suboptimal Th1 response in
345 PBMC from atopic asthmatic subjects and differential expression of costimulatory molecules(30). It
346 was also dependent on the virus strain and concentration, which was optimum around MOI 1 and 10
347 showing difference between the viral strains.

348 As a novel approach, we followed the dynamics of virus attachment and uptake by different PBMC
349 populations using virus particles labelled with DyLight650. Uptake of the HRV by all leukocytes was
350 observed 20 years ago using [^{35}S]HRV16 and from these experiments was known that HRV16
351 attaches to all type of leukocytes and has fourfold higher binding effect to monocytes (29). We
352 directly visualized the attachment by imaging flow cytometry and describe here first time the kinetics
353 of it by conventional flow cytometry. DyLight650 dye contains N-hydroxysuccinimide (NHS) esters,
354 what react with the amines on the virus capsid proteins and thereby labelling the viral capsid
355 proteins with red fluorescent dye. One should understand that labelling is present only on the parent
356 generation of viruses and it will be lost during viral replication. We observed that monocytes
357 internalize HRV and UV-inactivated viruses. This indicates that the uptake of HRV particles by
358 monocytes is at least partially a passive event and not only via receptor mediated infection. B and T
359 lymphocytes had distinct attachment and/or internalization pattern in case of functional virus and no
360 signal with UV-treated viruses detected. There was a lag time around 8h before HRV was detected in

361 B and CD4+/CD8+ T cells. In case of CD4 T cells, the DyLight650 labelled virus signal overlapped CD4
362 signal that points to the attachment of virus to CD4. Our results were concordant with the previously
363 reported results about attachment of HRV to different types of leukocytes(29) but give better
364 understanding of the kinetics of the process what allows better to plan similar experiments to
365 further study HRV leukocytes interactions.

366 In addition to DyLight650 labelling, we used ISH for viral RNA as the second visualization method. We
367 were able to detect accumulation of vRNA on the surface and inside of B cells. The size of the vRNA
368 signal, which is found on B cells indicates that these clusters are viral replication centers. Additionally
369 we demonstrate that HRV infected PBMCs were able to transfer the infectious particles to
370 other cells, as shown by measuring virus RNA-expression in HELA cells after co-culture with
371 pre-infected PBMCs. These finding indicate strongly that HRV is able to replicate inside the
372 peripheral blood mononuclear cells. It might seem to contradict with the study conducted 20
373 years ago where it was shown that the viral titers decrease over time when PBMCs were exposed to
374 HRV16 (MOI = 10, 72h) suggesting that no type of cells in PBMCs develops effective infection(29). Still
375 the authors found HRV RNA in the cytoplasm of monocytes as we did, but no viral RNA synthesis was
376 detected. But other studies showed later that under certain conditions (especially timing and choice
377 of “right” viral concentration are important) HRV is still able to infect alveolar macrophages(12), T
378 cells(13) and Ramos cells (B cell line)(16). Definitely the discussion stays open while the studies are
379 conducted in vitro and the topic needs further studies to clarify if the infection of lymphocytes
380 (beside alveolar macrophages) is relevant in vivo.

381 We observed that HRVs are able to induce B-cell proliferation. The rate of proliferation was time,
382 virus type and dose dependent. HRV-induced lymphocyte proliferation was slightly detectable at
383 lower (MOI < 1.0) viral concentrations, heavily dampened at higher concentrations (MOI > 10) and
384 clearly visible in the range of MOI = 1.0 ÷ 10. HRV16 was exceptional as the proliferation dampening

385 happened earlier at lower viral concentration (MOI = 10) suggesting that its virulence might be
386 stronger. Similar to our results with HRVs, other picornaviruses, such as poliovirus and coxsackievirus
387 efficiently induce proliferation of B cells, which start to produce IgM independently from T-cell help
388 (31). As it is known that T cell independent antibody response results from extensive B-cell receptor
389 cross-linking by the highly organized, repetitive picornavirus virion structure and is postulated to be
390 generally characteristic of antibody-controlled cytolytic viruses (31).

391 It has been shown that major group rhinoviruses significantly inhibit T-cell proliferation (32, 33). We
392 did not observe induction of neither CD4+ nor CD8+ T-cell proliferation in PBMCs with any of used
393 virus strains even in the conditions where B lymphocytes proliferated. T cell proliferation upon HRV
394 was confirmed couple of years ago, but as authors pointed out the viral concentration needed to be
395 very low – 1000 more diluted – and the T cell proliferation was detected later on the day 5 – 7 when
396 compare with our experiments and it explains why we did not detect the T cell proliferation, but
397 these two studies show how sensitive is the event of lymphocyte proliferation to the chosen
398 condition. The mechanism how HRV inhibits antigen-specific CD4+ or CD8+ T cell proliferation is
399 known to be ICAM-1-dependent (32). Our results indicate that there might be a similar regulatory
400 mechanism through the LDL receptor as minor group HRVs, HRV1B and HRV29, also did not induce T-
401 cell proliferation.

402 A recent study demonstrated that B-lymphocytes play an important role in the induction of asthma-
403 like inflammation in mice (34). It has been also suggested that B-lymphocytes may become
404 increasingly relevant as antigen-presenting cells when antigen load is low even without the help of
405 CD4+ T-lymphocytes (35). It is possible that in allergic/asthmatic individuals, HRVs infect not only LRT
406 epithelial cells but also underlining infiltrating immune cells in the inflamed epithelium, which
407 ultimately may lead to a strong nonspecific induction of B cell proliferation in bronchial tissue. This

408 might lead to a rapid increase in the total serum IgE, as observed previously (36) and thereby can

409 provide synergistic link between allergy, HRV infection and exacerbation of asthma (20).

410 In conclusion, this study provides first time evidence that HRVs can be taken up and induce the

411 proliferation of B lymphocytes and suggests that the mechanism how HRV induces B lymphocyte

412 proliferation and activation can be promising target for new therapeutic intervention strategies for

413 asthma exacerbation.

414 **Figures**

415 **Figure 1** The capacity of HRV to induce proliferation of PBMCs and B cells. (A) Proliferation rate was

416 measured by using ^{3}H-thymidine incorporation assay. PBMCs were incubated with HRV1B at

417 indicated concentrations for 5 days. Stimulation index is a ratio of counts per minute (CPM) in virus

418 induced versus "mock" induced condition. Data are mean (line) with CI 95% (grey area) from 11

419 different donors. (B, C, D) Proliferation rate of B cells and CD8+ and CD4+ cells was measured using

420 CFSE labelling assay and anti-CD4, anti-CD8 and anti-CD19 antibodies as discrimination markers.

421 PBMCs were incubated with mock, HRV1B, HRV16 (MOI=1.0 or 10.0) and with UV inactivated HRV1B

422 or HRV16 for 3 and/or 5 days. Number of samples was 6. Data are mean with **P<0.01, *P=

423 0.01÷0.05 from 6 different donors. (E) Addition of anti-ICAM-1-antibody to PBMCs leads to a marked

424 reduction in PBMC proliferation compared to isotype control. PBMCs were infected with MOI 10 of

425 HRV16. Number of samples was 4.

426 **Figure 2** Analysis of PBMCs incubated with DyLight650 labelled HRV1B and HRV16. PBMCs were

427 cultured with DyLight650 labelled HRV1B or HRV16 at MOI = 1.0 and MOI = 10 or "mock" and

428 analyzed at the indicated time-points by flow cytometry (A, B) or imaging flow cytometry (C). The UV

429 treated viruses were used at MOI = 10. (A) A representative of the flow cytometry. (B) Data are

430 mean with CI 95% of 6 donors. (C) Imaging of HRV interactions with monocytes, T and B cells.

431 Brightfield (BF) image shows the shape of cells. CD14+ monocytes were sorted out 1h after
432 incubation with the virus by cell sorter and then analyzed for DyLight650-labelled HRV1B (red). T
433 lymphocytes were analyzed for surface markers using anti-CD3 FITC (green) and anti-CD4 PE/Cy7
434 (purple) and for DyLight650-labelled HRV1B (red) 24h after incubation with the virus. B lymphocytes
435 were analyzed with anti-CD19 FITC (green) for DyLight650-labelled HRV1B (red) after 24h incubation
436 with the virus.

437 **Figure 3** Visualization of HRV1B vRNA with *in situ* hybridization (ISH). HRV1B vRNA is designated with
438 green, human beta-actin mRNA with red, DAPI with blue, and anti-CD20 (purple arrow) as the marker
439 for B-cells with purple color. (A) PBMCs were cultured with HRV1B and subjected to ISH analysis on
440 5th day. Representative images of 4 donors on 5th day are presented. White arrows indicate the vRNA
441 signal. Bars indicate 10 μm. (B) HeLa cells were infected with HRV1B or HRV1B UV. After 24 hours
442 cells were fixed and subjected to ISH analysis. Bars indicate 10 μm. (C) HRV can successfully replicate
443 in PBMCs and produce infectious virions, which are able to infect HELA cells. Expression of positive
444 strand and negative strand viral RNA in HELA cells after co-culture with pre HRV incubated PBMCs for
445 three days are shown.

446 **Figure 4** HRV-induced cytokine responses of PBMCs. PBMCs were incubated with HRV1B, HRV16,
447 HRV14 and HRV29 at MOI = 1.0 and MOI = 10 concentrations or cultured in “mock” condition. Cell
448 culture supernatants were collected at day 5. Data are mean with CI 95% of 8 donors. **P<0.01, *P=
449 0.01÷0.05

450 **Author contributions**

451 AA had primary responsibility for framework of the study, outcome assessment and manuscript
452 preparation. OW produced substantial amount of data during revising the manuscript and deserves
453 to share first author ship together with AA. AA, WV, AR, ME, SJ and CA contributed to the conception

454 and design of the study, and MA supervised the project. AA, OW, SS, BS, BR and JA performed the
455 experiments. AA, OW, WV, BS, AR, CA and MA conducted data analysis and interpretation of the
456 results. NGP provided help for the planning of additional experiments and interpretation of the data
457 during revising the manuscript. All authors contributed to revision of the manuscript.

458 **Funding**

459 M. Akdis has received research support from the Swiss National Science Foundation No. 320030-
460 159870 and the European Commission's Seventh Framework Programme MeDALL (No: 261357) and
461 PREDICTA (No: 260895). C.A. Akdis has consultant arrangements with Actellion, Aventis, Stallergenes,
462 Allergopharma, and Circacia; is employed by the Swiss Institute of Allergy and Asthma Research,
463 University of Zurich; and has received research support from Novartis, the Swiss National Science
464 Foundation No. 310030_156823, and the Christine Kühne-Center for Allergy Research and Education
465 (CK-CARE). A. Rebane has received research support from European Regional Fund with Archimedes
466 Foundation, EU structural assistance grant SARMP12219T, personal research grant PUT214 from
467 Estonian Research Council and European Union through the European Regional Development Fund
468 (Project No. 2014-2020.4.01.15-0012).

469 **Conflicts of interest**

470 The authors declare that they have no potential conflict of interest.

471 **Supporting Information**

472 **Figure S1** Imaging of HRV uptake by monocytes. Brightfield (BF) image shows the shape of cells.
473 CD14+ monocytes were sorted out 1h after incubation with the virus by cell sorter and then analyzed
474 for DyLight650-labelled HRV1B (red) (A) and DyLight650-labelled and UV-treated HRV1B (B).

475 **Figure S2** Imaging of HRV uptake by CD4+ T-cells. Brightfield (BF) image shows the shape of cells.
476 CD4+ T lymphocytes were analyzed for surface markers using anti-CD3 FITC (green), anti-CD4 PE/Cy7

477 (purple) and for DyLight650-labelled HRV1B (red) 24h after incubation with the virus. PBMCs were

478 cultured with DyLight650-labeled HRV1B (A) and DyLight650-labelled and UV-treated HRV1B (B) and

479 CD4+ T-cells were sorted out 24h after incubation with the virus by cell sorter.

480 **Figure S3** Imaging of HRV uptake by CD19+ B-cells. Brightfield (BF) image shows the shape of cells. B

481 lymphocytes were analyzed for surface markers using anti-CD19 FITC (green) and for DyLight650-

482 labelled HRV1B (red) 24h after incubation with the virus. PBMCs were cultured with DyLight650-

483 labeled HRV1B (A) and DyLight650-labelled and UV-treated HRV1B (B) and B lymphocytes were

484 sorted out 24h after incubation with the virus by cell sorter.

485 **Figure S4** Donor 5 (D5) – HRV1B is inside the B cell. HRV1B vRNA is designated with green, human

486 beta-actin mRNA with red, DAPI with blue, and anti-CD20 as the marker for B-cells with purple color.

487 PBMCs were cultured with HRV1B and subjected to ISH analysis on 5th day. Cells images are different

488 slices to visualize the localization of HRV replication centers.

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