Rhinovirus infection and house dust mite exposure synergize in inducing bronchial epithelial cell interleukin–8 release


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Summary

Background Human rhinoviruses (HRVs) and house dust mites (HDMs) are among the most common environmental factors able to induce airway inflammation in asthma. Although epidemiological studies suggest that they also synergize in inducing asthma exacerbations, there is no experimental evidence to support this, nor any information on the possible mechanisms involved.

Objective To investigate their interaction on the induction of airway epithelial inflammatory responses in vitro.

Methods BEAS-2B cells were exposed to activated HDM Dermatophagoides pteronyssinus major allergen I (Der p I), HRVs (HRV1b or HRV16) or both in different sequences. IL-8/CXCL8 release, intercellular adhesion molecule (ICAM)-1 surface expression and nuclear factor κB (NF-κB) translocation were evaluated. Complementary, primary human bronchial epithelial cells (HBECs) exposed to both Der p I and RVs and IL-8, IL-6, IFN-γ-induced protein (IP)-10/CXCL10, IFN-λ-1/IL-29, regulated upon activation normal T lymphocyte expressed and secreted (RANTES)/CCL5 release were measured.

Results RV and Der p I up-regulated IL-8 release, ICAM-1 expression and NF-κB translocation in BEAS-2B cells. Simultaneous exposure to both factors, as well as when cells were initially exposed to HRV and then to Der p I, resulted in further induction of IL-8 in a synergistic manner. Synergism was not observed when cells were initially exposed to Der p I and then to HRV. This was the pattern in ICAM-1 induction although the phenomenon was not synergistic. Concurrent exposure induced an early synergistic NF-κB translocation induction, differentiating with time, partly explaining the above observation. In HBECs, both HRV and Der p I induced IL-8, IL-6, IL-29 and IP-10, while RANTES was induced only by HRV. Synergistic induction was observed only in IL-8.

Conclusion HRV and enzymatically active Der p I can act synergistically in the induction of bronchial epithelial IL-8 release, when HRV infection precedes or is concurrent with Der p I exposure. Such a synergy may represent an important mechanism in virus-induced asthma exacerbations.

Keywords bronchial epithelium, house dust mite, human rhinovirus, IL-8, NF-κB

Introduction

Bronchial inflammation is a hallmark of asthma. Consequently, up-regulation of inflammatory factors after exposure to environmental stimuli is considered to be an important mechanism for the induction of disease exacerbations. Among such factors, respiratory viruses, mainly human rhinoviruses (HRVs), have been consistently shown to be a major trigger of acute asthma exacerbations [1, 2]. HRVs include more than 100 serotypes divided into two groups: major and minor, based on their cellular
receptors. The major subgroup (e.g. HRV14, HRV16) uses intercellular adhesion molecule 1 (ICAM-1, CD 54) while the minor subgroup (e.g. HRV1b, HRV2) uses the low-density lipoprotein receptor. HRVs are able to reach and replicate in the bronchial epithelium [3] and induce epithelial inflammation both in vitro [4] and in vivo [5–7].

On the other hand, allergens are also major inducers of both inflammation and clinical symptoms [8]. Among them, house dust mites (HDMs) represent a common world–wide–distributed allergen [9]. In addition to the IgE–mediated pathway, *Dermatophagoides pteronyssinus* major allergen I (Der p I), one of the major house mite allergens, has been shown to exert cysteine protease activity that can directly activate the bronchial epithelium to elicit an inflammatory response [10, 11].

It has been demonstrated in case–control studies that HRV infection synergizes with allergens in the induction of asthma exacerbations [12, 13]. Human experimental studies have shown that allergen exposure following HRV infection is able to induce allergen–specific airway hyper-responsiveness [14, 15] and increased airway inflammation. However, at least two well-designed studies have failed to demonstrate additive or synergistic effects when patients were exposed to allergen and subsequently infected with HRV [16, 17].

Among the mediators that both HRV and Der p I elicit from the bronchial epithelium, IL–8 (CXCL8) plays the main role [7, 10]. IL–8 is a CXC chemokine with the neutrophil–attractant Glu–Leu–Arg (ERL) motif. Both IL–8 and neutrophils are features of difficult–to–treat asthma phenotypes, similar to virus–induced acute asthma and severe asthma [18, 19].

Based on the above, we hypothesized that a synergistic effect may result from simultaneous or subsequent exposure of the epithelium to viruses and mites, acting as direct activators, and that timing of exposure may be important in this respect. To address this question, we combined well–characterized models of epithelial exposures to HRV and Der p I, using different timing regimes, and measured primarily IL–8 and also ICAM–1 and nuclear factor κB (NF–κB p65) nuclear translocation as inflammatory response indices. Subsequently, in order to evaluate our hypothesis further, we exposed primary human bronchial epithelial cells (HBECs) with both stimuli simultaneously, and IL–8, IFN–γ-induced protein 10 (IP–10/CXCL10), regulated upon activation normal T lymphocyte expressed and secreted (RANTES/CCL5), IL–6 and IFN–λI/IL–29 were measured.

**Materials and methods**

**Cell cultures**

BEAS–2B cells, an immortalized line of normal human bronchial epithelium derived by transfection of normal primary cells with SV40 early–region genes, were used in the majority of the experiments. BEAS–2B and Ohio–HeLa cells (initially obtained from ATCC and the MRC Cold Unit, UK, respectively) were cultured in Eagle's minimal essential medium (E–MEM) buffered with NaHCO₃ and supplemented with 10% (v/v) fetal bovine serum (FBS) and 40 μg/mL of gentamycin in a humidified 5% CO₂ incubator. All culture materials were purchased from Gibco–Invitrogen Corp. (Carlsbad, CA, USA) and Falcon (Becton Dickinson, Labware, NJ, USA). Biochemicals were from Sigma (St Louis, MO, USA), unless otherwise specified.

Primary HBECs, initially deriving from an adult non–asthmatic volunteer in the course of another ongoing study, were available frozen in liquid nitrogen. They were isolated as described earlier [4]. Cells were rapidly thawed and cultured on plates pre–coated with collagen type–I (Nutacon BV, Leimuden, the Netherlands) submerged in Clonetics BEGM (Cambrex, ML, USA). Medium was replaced daily. Cells were used at passage 2.

**Virus cultures and titration**

HRV types 1b (minor) and 16 (major) were propagated in Ohio–HeLa cells at 33 °C in a humified, 5% CO₂ incubator and titrated as described previously [20]. Briefly, on development of a full cytopathic effect (CPE), cells and supernatants were harvested after three cycles of freezing/thawing in order to rupture all membranes, clarified by centrifugation, aliquoted and stored at −70 °C. Then, HRV was titrated by making a logarithmic dilution (10⁻¹–10⁻⁸) and then inoculating Ohio–HeLa cells in 96–well plates. The end–point titre was defined as the highest dilution at which a CPE was detected in at least half of the wells and expressed as the inverse logarithm of this dilution. Lysates of parallel Ohio–HeLa cell cultures not infected with virus were used as controls.

In order to evaluate the specificity of HRV–mediated responses, several controls were included in initial experiments. HRV preparations were exposed to 58 °C for 1 h, in which successful inactivation was confirmed by lack of HRV replication in Ohio–HeLa cells. Furthermore, ultraviolet (UV) inactivation and filtration of virus were performed as described previously [7]. Lastly, HRV inactivation was also performed by pre–incubation with 1 mg/mL of soluble intercellular adhesion molecule–1 (sICAM–1), a kind gift from Pam Esmon (Bayer Corp., Berkley, CA, USA), for 45 min at room temperature.

**Dermatophagoides pteronyssinus major allergen I proteolytic activity determination**

Commercially available natural Der p I was purchased from Indoor Biotechnologies (Charlottesville, VA, USA). A 30–min pre–activation of Der p I with 5 mmol/L cysteine...
HCl, pH 7.0, at 37 °C was always performed before use in order to activate its thiol group [21, 22].

The catalytic activity of Der p I as cysteine protease was ascertained in a kinetic assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC (AMC, 7-amino-4-methylcoumarin; Boc, N-tert-butoxy-carbonyl) in a volume of 1 mL, as described [22, 23]. Cysteine-specific protease inhibitor E-64 was used to assess specificity. The fluorescence of AMC released from the substrate was measured by a fluorescence spectrophotometer (LS-2B Perkin-Elmer, Perkin - Elmer Instruments, Norwalk, CT, USA), with λ<sub>ex</sub> = 380 nm and λ<sub>em</sub> = 460 nm, and the results were related to an AMC standard curve.

**Effect of Dermatophagoides pteronyssinus major allergen I in rhinovirus replication**

In order to assess the effect of Der p I in HRV replication, we used the Ohio–HeLa cell titration system, a traditional and widely used assay especially for HRVs [6, 20, 24, 25]. Ohio–HeLa cell cultures were seeded in 96-well plates and allowed to reach a confluence of 60–70%. Cells were infected with RV 1b at a final concentration of one infection unit per cell (multiplicity of infection (MOI) = 1) and activated Der p I (1 μg/mL) was added to the culture. After 24 h of incubation, plates were fixed and stained with 5% formaldehyde, 5% ethanol and 0.1% crystal violet in phosphate-buffered saline (PBS). Substantially, plates were washed with water and air dried. The dye taken up by the cells was extracted with 16.6% v/v glacial acetic acid and 50% v/v methanol in ultra pure water, and the absorbance was read at 595 nm (Ceres 900C, Bio-Tec Instruments Inc., Winooski, VT, USA). The reported optical density (OD) was proportional to the remaining Ohio–HeLa cells, reflecting the HRV-induced cytotoxicity [26, 27].

**In vitro stimulation of epithelial cells**

BEAS-2B cells were plated in 48-well plates, allowed to grow until 80–90% confluence and were subsequently serum starved for 24 h in an E-MEM with 0.5% fetal calf serum (FCS). In order to evaluate the inflammation response of our system in initial experiments, BEAS-2B cells were incubated with 10 ng/mL lipopolysaccharide (LPS) (E. coli; Serotype 026:B6, Sigma). Further, BEAS-2B cells were incubated with 1 μg/mL poly (IC) (Sigma) in medium for 1 h on shaking cells, replaced with fresh medium, and E-MEM with 4% FCS [5].

For HRV infection, cells were exposed for 1 h at the desirable MOI in parallel to non-infected Ohio–HeLa cell lysates as negative controls with gentle shaking at room temperature as described previously [20, 27]. Subsequently, the HRV inoculum was replaced with fresh E-MEM with 4% FCS, 1% MgCl and 4% tryptase phosphate broth, and 40 μg/mL of gentamycin was added and plates were incubated at 33 °C.

For Der p exposure, activated Der p I or an equal amount of cysteine medium as a negative control was added to the cells without shaking during the whole incubation period.

Our model of exposure was designed in order to evaluate the following three different conditions: (a) both stimuli act simultaneously, (b) viral infection precedes allergen exposure and finally (c) allergen exposure precedes viral infection. In the first case, cells were cultured with conditioned medium (CM-HeLa cell lysates/cysteine medium) for 24 h, followed by washing and addition of CM, HRV, Der p or all three for the next 24 h. For the second case, cells were cultured with CM or infected with HRV cultured for 24 h, washed and then exposed to CM or Der p for the next 24 h. Finally, for the third case, cells were cultured with CM or exposed to Der p for 24 h, washed and then cultured with CM or infected with HRV for 24 h more. In each case, the supernatants were harvested for ELISA. For evaluation of ICAM-1 expression, the above schemes were followed by an 8-h incubation time based on our earlier studies [28].

HBECs were plated in 48-well plates until 90% confluence and then followed by 24-h starvation. Then, the scheme of simultaneous HRV and Der p I stimulation was followed and supernatants were collected as described above.

**Quantification of interleukin-8, induced protein-10, regulated upon activation normal T lymphocyte expressed and secreted, interleukin-6 and interleukin-29 levels in cell culture supernatants**

Supernatants clarified by centrifugation (10 min/3000 × g/4 °C) were stored at −70 °C until assayed. Levels of IL-8, IP-10, RANTES, IL-6 and IL-29 were measured by ELISA, with paired antibodies from R&D Systems (Abingdon, UK) according to the manufacturer’s recommendations. The sensitivities of the assays were 7.5, 3.9, 1.95, 0.78 and 7.8 pg/mL, respectively.

**Flow cytometry**

BEAS-2B cells were harvested non-enzymatically by incubation for 5 min at 37 °C with an enzyme-free, PBS-based cell dissociation buffer (Gibco-Invitrogen Corp.), followed by gentle pipette aspiration and washing (PBS with 1% FBS/200 g/10 min/4 °C) and finally they were resuspended at a density of 1 × 10<sup>5</sup> cells/100 μL in washing buffer.

For ICAM-1 analysis, cells were incubated with anti-ICAM, phycoerythrin-conjugated monoclonal antibody or its isotype control (Pharmingen, Becton Dickinson, Jan
Hose, CA, USA) for 30 min at 4 °C. After washing twice, cells were fixed with 0.5 mL of 1% paraformaldehyde in PBS and the mean fluorescence intensity (MFI) was assessed with a FACSort flow cytometer (Becton Dickinson, Jan Hose, CA, USA). Fluorescence data were collected from 10⁶ cells.

For NF-κB translocation, a flow cytometry assay was used with minor modifications [29]. Nuclei from 1 × 10⁵ BEAS-2B cells were prepared by incubating cells with 200 μL Pipes-Triton buffer (10 mM Pipes, 0.1 M NaCl, 2 mM MgCl₂ and 0.1% Triton × 100; Sigma) in PBS (30 min/4 °C), followed by two washes (in PBS-1% FBS, 500 g/5 min/4 °C). Then, nuclei were stained with mouse anti-human NF-κB p65 PE (F-6, sc-8008, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min at 4 °C or its isotype control. Nuclei were also stained with 7-aminoactinomycin D (7-AAD), (Pharmingen, Becton Dickinson). After washing twice, nuclei were fixed with 0.5 mL of 1% paraformaldehyde in PBS and analysed by FACS. Five thousand events were collected, in which nuclei were gated on the basis of 7-AAD staining (DNA-FL-3) and MFI was measured in the FL-2 (PE) gate.

Analysis was performed with the use of Cell Quest™ Becton Dickinson and Flow and FlowJo™ software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed with the SPSS 13.0 for Windows software (SPSS Inc., Chicago, IL, USA). The t-test for differences between the two groups for IL-8, IP-10, RANTES, IL-6, IL-29 and ICAM-1 or one-way ANOVA with Bonferroni’s multiple comparison test for NF-κB was used for comparison of means. P-values lower than 0.05 were considered to be significant.

Results

Dermatophagoides pteronyssinus major allergen I proteolytic activity

Using an assay based on a synthetic fluorogenic substrate, we confirmed that Der p I has a cysteine protease activity dependent fully on pro-activation. When activated Der p I was incubated with the substrate (2 μg for 10 min, 25 °C), an increased amount of fluorescence AMC was released compared with that released when not activated. Also, the phenomenon was totally abolished by the specific inhibitor E-64. E-64 did not have any effect in the blank (Fig. 1). Papain, used as a positive control, led to a massive release of AMC, which was abolished by E-64 (from 60.5 μM to 2.19), confirming the specificity of the reaction. Finally, Der p I proteolytic activity was dose and time dependent (data not shown).

Effect of Dermatophagoides pteronyssinus major allergen I on human rhinovirus replication and its subsequent ability to induce virus-mediated Ohio-HeLa cell death

In order to detect any direct effect of Der p I in HRV replication, we used Ohio-HeLa cells as they are extremely sensitive to the virus and any effect in its replication will directly decrease their HRV-induced cell death. HRV1b decreased, as expected, the Ohio-HeLa cell number significantly after 24 h (P<0.05 compared with medium). Activated Der p I alone has no effect on Ohio-HeLa by itself. Also, its addition did not effect the replication of HRV1b as there was no difference in its ability to induce cytotoxicity in Ohio-HeLa cells (Table 1).

Effect of human rhinovirus and Dermatophagoides pteronyssinus major allergen I on BEAS-2B interleukin-8 release

We performed a series of experiments in order to screen and validate our model. Firstly, we exposed BEAS-2B cells to a generically powerful inflammatory stimulus such as LPS for 24 h and measured the IL-8 released in the supernatant. LPS exposure led to an enhanced IL-8 release in comparison with control medium (6535 ± 353 vs. 439 ± 59 pg/mL, P < 0.001), indicating the ability of BEAS-2B to respond to an inflammatory stimulus.

Then, we used a more specific positive control, poly (IC), a synthetic analogue of dsRNA, which also causes an induction of IL-8 release [940.38 ± 33.09 vs. 350.9 ± 28.9 pg/mL, poly (IC) vs. control, P<0.001], revealing activation of TLR3.

After that, BEAS-2B cells were infected with HRVs at an MOI = 1 for 24 h and IL-8 was measured in supernatants...
Human rhinovirus and *Dermatophagoides pteronyssinus* major allergen I synergize on BEAS-2B interleukin-8 release in a sequence-dependent manner

Now, we move to the main question of our study: is there a synergy between HRVs and Der p I? We use the most potent serotype (HRV1b). When HRV1b infection and Der p I exposure were assessed simultaneously, a statistically significant increase of IL-8 release was observed in comparison with each stimulus alone (P<0.001). Also, there was a statistically significant increase between the combination of stimuli and the numerical sum of each stimulus alone (2452 ± 275 vs. 1722 ± 147 pg/mL, respectively, P<0.01), showing a synergistic effect (Fig. 2a). This effect was also present when cells were initially exposed to HRV1b and subsequently exposed to Der p I (6456 ± 847 vs. 4129 ± 377 pg/mL, combined and numerical sum stimuli, respectively, P<0.01) (Fig. 2b). In contrast, when cells were initially exposed to Der p I and subsequently infected with HRV1b, the resulting production of IL-8 was higher only compared with Der p I alone (Fig. 2c).

**Effect of human rhinovirus and *Dermatophagoides pteronyssinus* major allergen I on BEAS-2B intercellular adhesion molecule-1 expression**

In order to determine whether the exposure sequence effect is unique for IL-8 or observed in another inflammatory marker, we assessed ICAM-1 expression. For the infection, HRV16 was used as this serotype (major) uses ICAM-1 as its receptor and is up-regulated upon infection. BEAS-2B cells had a baseline expression of ICAM-1 382 ± 18 MFI, which was significantly up-regulated from both HRV16 (639 ± 23 vs. control, P<0.001) and Der p I (591 ± 18 vs. control, P<0.001) at 8 h, although in a modest way.

When both stimuli were added simultaneously, a statistically significant increase of ICAM-1 expression in comparison with either individual factor was observed (P<0.001) (Fig. 3a). Similarly, when BEAS-2B cells were initially infected with HRV16 and subsequently exposed to Der p I, there was a significant increase compared with each individual factor (P<0.001) (Fig. 3b). In contrast, no further increase in ICAM-1 expression was observed when cells were first exposed to Der p I and then infected with HRV16 (Fig. 3c).

**Human rhinovirus and *Dermatophagoides pteronyssinus* major allergen I synergize in nuclear factor-κB translocation in bronchial epithelial cells**

In order to investigate the role of NF-κB in the observed synergistic induction of IL-8, BEAS-2B cells were exposed to HRV16 (in order to evaluate both inflammatory

### Table 1. Effect of *Dermatophagoides pteronyssinus* major allergen I (Der p I) on human rhinovirus (HRV) 1b replication

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>OD</th>
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<tr>
<td>Medium</td>
<td>1090 ± 39</td>
</tr>
<tr>
<td>HRV1b</td>
<td>899 ± 35*</td>
</tr>
<tr>
<td>Der p I</td>
<td>1072 ± 45</td>
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<tr>
<td>HRV1b plus Der p I</td>
<td>918 ± 33**</td>
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Ohio-HeLa cells were cultured for 24 h with medium, Der p I (1 μg/mL), HRV 1b (MOI = 1) or HRV1b plus Der p I. Cells were fixed and stained with crystal violet followed by extraction and measurement of the dye at 595 nm. Optical density (OD) reflects the cell number. No difference was observed in the ability of HRV1b to induce cell death in the presence or absence of Der p I. The results are expressed as means ± SEM (n = 12).

*P<0.05, HRV1b compared with medium.

**P<0.05, HRV1b + Der p I compared with Der p I.

MOI, multiplicity of infection; SEM, standard error of mean.

by ELISA. We tested both a major (HRV16) and a minor (HRV1b) serotype as both will be used in different experiments. HRV16 infection led to a significant increase of IL-8 release in comparison with control medium (684.8 ± 56.26 vs. 392.1 ± 9.71 pg/mL, P=0.001). In order to evaluate the specificity of IL-8 induction, several controls were used. Filtration of HRV16 from live virus inoculum or exposure to high-temperature abrogate IL-8 release (filtrate 429.58 ± 16.92, heat 320.98 ± 29.24 pg/mL) confirmed that the observed response was specific to HRV16 and not to Ohio-HeLa-derived factors. Also, induction was dependent on both HRV16 entries into cells as well as on its replication, as pre-incubation with sICAM-1 (its receptor) and UV inactivation, respectively, also abolishes it (sICAM-RV16 329.8 ± 36.74 pg/mL, UV-RV16 431.8 ± 26.09 pg/mL). HRV1b infection resulted in higher induction of IL-8 (1313.6 ± 70.3 vs. control, P<0.001). Induction was also abolished after heat inactivation, and it was also dose and time dependent (data not shown).

Finally, exposure of BEAS-2B with activated Der p I (1 μg/mL for 24 h) also modulated IL-8 release (616.09 ± 19.48 vs. 329.14 ± 37.68 pg/mL, activated Der p I vs. control, P<0.001). This induction was fully dependent on its activation as plain Der p I did not have any effect (304.25 ± 53.53 pg/mL). This was also the case with addition of cysteine to medium (295.98 ± 22.94 pg/mL). Both these results also indicate that both reagents were LPS free. IL-8 induction was time (between 3 and 24 h) and dose dependent (data not shown).

In order to continue our experiments to facilitate the detection of any interactions between rhinovirus infection and allergen exposure, we use mid-range concentrations (HRV at an MOI = 1 and Der p I at 1 μg/mL) and 24 h as a time-point.
Fig. 2. Release of IL-8 from BEAS-2B after human rhinovirus (HRV) 1b (multiplicity of infection = 1) infection and Dermatophagoides pteronyssinus major allergen I (Der p I) (1 µg/mL) exposed in different sequences; BEAS-2B were first exposures to one stimulus or control medium (time = 0), incubated for 24 h and washed, followed by exposure to the next stimuli or their combination. In (a), HRV1b and Der p I act simultaneously, in (b) HRV1b-infected epithelium was exposed to Der p I and in (c), Der p I-exposed epithelial cells were infected with HRV1b. Data are from four independent experiments and results are presented as mean ± SEM. Dotted lines indicate the value corresponding to the numerical sum of the individual stimulus. Under all conditions, all stimuli induce increased IL-8 release compared with control medium *P<0.05. In (a) and (b), combined stimuli induce higher IL-8 release compared with each stimulus alone, **P<0.05, and with their numerical sum *P<0.05, in a synergistic manner. In (c), combined stimuli release significantly more IL-8 compared with Der p I alone ‰P<0.05. CM, conditioned medium; RV, rhinovirus; Der p, Dermatophagoides pteronyssinus.

Fig. 3. Intercellular adhesion molecule (ICAM-1) expression (mean fluorescence intensity) in BEAS-2B cells after human rhinovirus (HRV) 16 (multiplicity of infection = 1) infection and Dermatophagoides pteronyssinus major allergen I (Der p I) (1 µg/mL) exposure in different sequences. BEAS-2B were first exposed to one stimulus or control medium (time = 0), incubated for 8 h and washed, followed by exposure to the next stimuli or their combination. In (a), HRV16 and Der p I act simultaneously, in (b) HRV16-infected epithelium was exposed to Der p I and in (c) Der p I-exposed epithelial cells were infected with HRV16. Data are from four independent experiments and results are presented as mean ± SEM. Under all conditions, all stimuli induce increased ICAM-1 expression compared with control medium *P<0.05. In (a) and (b), combined stimuli increased ICAM-1 significantly in comparison with each stimulus alone ‰P<0.05. CM, conditioned medium; RV, rhinovirus; Der p, Dermatophagoides pteronyssinus.
response indices: IL-8 and ICAM-1) and NF-κB p65 translocation was detected by flow cytometry at baseline, 1, 2, 8 and 24 h. Both HRV16 and Der p I induce NF-κB translocation (Fig. 4a).

HRV16 induces NF-κB translocation at 2 (P<0.01), 8 (P<0.05) and 24 h (P<0.01) compared with the control medium. Der p I induced NF-κB translocation only at 2 h compared with medium (P<0.01). Simultaneous exposure significantly increased NF-κB translocation at all time-points of 1, 2, 8 and 24 h compared with medium (P<0.01). Importantly, at 1 h, the phenomenon was synergistic as the combined stimuli of HRV16 and Der p I

Fig. 4. Flow cytometric analysis of nuclear factor (NF)-κB translocation in BEAS-2B bronchial epithelial cells after infection with human rhinovirus (HRV) 16 (multiplicity of infection = 1) and exposure to Dermatophagoides pteronyssinus major allergen I (Der p I) (1 μg/mL). BEAS-2B nuclei were isolated and stained with 7-aminoactinomycin D (7-AAD) and NF-κB p65 PE. (a) HRV16 induces NF-κB translocation at 2, 8 and 24 h compared with medium *P<0.05. Der p I induced NF-κB only at 2 h compared with medium wP<0.05. Simultaneous exposure increased NF-κB translocation significantly at all time-points compared with medium or Der p I alone **P<0.05. Dotted line indicates the value corresponding to the numerical sum of the individual stimulus at 1 h, where there is a significant increase of combined stimuli compared with their sum *P<0.05, in a synergistic manner. Data are from four independent experiments and results are expressed as mean ± SEM of mean fluorescence intensity (MFI). (b) Representative plot of BEAS-2B nuclei stained for 7-AAD (DNA) in 1 h. Nuclei were gated and analysed further for expression of NF-κB p65 as shown in histogram (c). There is a clear induction of NF-κB translocation in combined stimuli. SSC-H; Side Scatter Height.

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were significantly higher than the numerical sum of each stimulus alone (430.5 ± 20.81 vs. 357.25 ± 20.17, MFI, combined and sum of stimuli, respectively, P < 0.05), which also occurred earlier, than either stimulus alone (Figs 4a and c). Finally, combined stimuli resulted in a significant increase of NF-κB translocation at all time-points at 2 (P < 0.01), 8 (P < 0.05) and 24 h (P < 0.01) compared with Der p I (Fig. 4a).

**Human rhinovirus and Dermatophagoides pteronyssinus major allergen I synergize in human bronchial epithelial cells interleukin-8 release but not on interleukin-6, induced protein-10, regulated upon activation normal T lymphocyte expressed and secreted or interleukin-29**

In order to show that the above results can be extended to primary cells, an experiment was carried out in such cells with simultaneous exposure to HRV16 and Der p I. In the supernatants, we evaluate IL-8, pro-inflammatory cytokine IL-6 as well as other chemokines as IP-10, RANTES and finally the new type III IFN IL-29.

Both HRV16 and Der p I induced IL-8 release compared with control (HRV16: 1579 ± 73 pg/mL, Der p I: 1894 ± 51 pg/mL vs. control: 644 ± 37 pg/mL, P < 0.01). The combined stimuli induced further IL-8 release in a synergistic manner (4175 ± 183 vs. 3472 ± 123 pg/mL, combined and sum of stimuli, respectively, P < 0.05), (Fig. 5a).

IL-8 was also induced by both HRV16 and Der p I (HRV16: 26.13 ± 1 pg/mL, Der p I: 32 ± 1.5 pg/mL vs. control: 13.95 ± 1.8 pg/mL, P < 0.05 and P < 0.01, respectively). When HRV16 and Der p I acted together, there was a significant increase compared with each stimulus alone but not in a synergistic or even an additive manner (39.4 ± 4 pg/mL, P < 0.05), (Fig. 5b).

IP-10 was also induced by each stimulus (HRV16: 112.3 ± 8.6 pg/mL, Der p I: 99.6 ± 3.8 pg/mL vs. control: 34.9 ± 5.6 pg/mL, P < 0.01); however, there was no further induction by their combination (Fig. 5c). RANTES was induced only by HRV16 compared with control (16.57 ± 1.8 vs. 7.87 ± 0.5, respectively, P < 0.05), (Fig. 5d).

Finally, IL-29 release was induced by each stimulus alone compared with control (HRV16: 328 ± 11 pg/mL, Der p I: 359 ± 17 pg/mL vs. control: 151 ± 8 pg/mL, P < 0.001). The combination of both stimuli elicited no further induction of IL-29, (Fig. 5e).

**Discussion**

The findings of this study provide new information and evidence for interaction between two major environmental stimuli associated with asthma exacerbations. First, it is shown that HRV and Der p I synergize in the induction of IL-8, a phenomenon occurring in both the BEAS-2B cell line as well in primary bronchial epithelial cells. Second, this synergy depends on the sequence of exposure and is only present when the viral stimulus precedes or acts in parallel to the enzymatic one. It is also shown that combined stimuli induce an early synergistic induction of NF-κB.

Finally, although not its main goal, this study reveal new data about the inflammatory response of bronchial epithelial cells after Der p I exposure. Hence, we observe...
that, in addition to its previously described pro-inflammatory activities, Der p I is also capable of up-regulating ICAM-1. As this occurs through a direct effect on the epithelium, it may offer an additional mechanistic insight into clinical observations of up-regulated ICAM-1 in nasal epithelial cells during natural HDM allergen exposure or challenge, which, until now, has been attributed solely to an IgE-mediated pathway [30, 31]. Also, we observe for the first time that Der p I can induce the release of IP-10 and IL-29 from bronchial epithelial cells.

Interactions between HRV or Der p I and other pro-inflammatory stimuli have been reported previously. Spannhake et al. [32] demonstrated a synergistic effect of HRV and NO2 in IL-8 release and an additive effect in ICAM-1 expression, possibly explaining the increased HRV and NO2 in IL-8 release and an additive effect in clinical observations of up-regulated ICAM-1 in nasal epithelial cells during natural HDM allergen exposure or challenge, which, until now, has been attributed solely to an IgE-mediated pathway [30, 31]. Also, we observe for the first time that Der p I can induce the release of IP-10 and IL-29 from bronchial epithelial cells.

Interactions between HRV or Der p I and other pro-inflammatory stimuli have been reported previously. Spannhake et al. [32] demonstrated a synergistic effect of HRV and NO2 in IL-8 release and an additive effect in ICAM-1 expression, possibly explaining the increased severity of virus-induced exacerbations in children exposed previously to high levels of NO2 shown by Chauhan et al. [33]. A synergistic effect between Der p I and respiratory syncytial virus (RSV), during the induction of IL-8 by epithelial cells, has also been reported [34]. Furthermore, a synergy was shown in IL-8 production by peripheral blood mononuclear cells after stimulation with Der p I and diesel exhaust particles [35]. However, interactions between HRV and Der p I have not been addressed previously at the cellular level, although these represent factors that are most frequently implicated in asthma pathogenesis.

Epidemiological studies have documented a synergy between allergen and virus in asthma exacerbations, including HRV and Der p I [12, 13]. Human experimental HRV infection models have consistently shown that following viral infection, there is an augmented response to allergen [14, 36]. However, in two notable exceptions such a synergistic effect was not confirmed [16, 17], or there was evidence of a differential effect depending on the exposure sequence [37, 38]. Interestingly, and in line with the findings of the present report, these studies used models in which experimental viral infection followed rather than preceded allergen exposure.

It is known that both HRVs [27, 39, 40] and allergens [8, 9] can induce both local and systemic immune responses, which probably was the case in the above studies. However, the fact that similar inflammatory responses can be reproduced in vitro without requiring the participation of the systemic immune response underlines the capacity of airway epithelium as a disease modulator [41].

The fact that the inflammatory response does not increase when Der p I is the initial stimulus, although ICAM-1 is up-regulated, suggests that an increase in ICAM-1 alone is not by itself sufficient to increase the effects of HRV infection by increasing viral entry, as had been postulated [42]. However, we must note that we only measured the membrane form of ICAM-1, while it has been shown that soluble (s) ICAM-1, which may significantly affect HRV infectivity, is down-regulated by HRV itself [43].

The possibility that Der p I may interfere with virus replication, as has been suggested for influenza virus [44] or RSV [34], was directly examined in our model using a sensitive Ohio-HeLA assay, commonly used for HRV titration. Our data suggest that this phenomenon did not occur in our model.

Our study was primarily focused on measurement of IL-8/CXCL8 as it is one of the major chemokines that can be produced in large amounts from airway epithelium in response to several stimuli including pro-inflammatory cytokines, viruses, microbe, allergens or other environmental factors [45]. IL-8 is a CXCR3 chemokine with the neutrophil-attractant Glu–Leu–Arg (ELR) motif that acts as a key mediator in neutrophil-mediated acute inflammation [46]. In asthma, although eosinophil is the characteristic cell, cross-sectional studies suggest that neutrophils may gradually replace eosinophils in proportion to the severity and/or the duration of the disease [47]. Both neutrophils and IL-8 are found in severe asthma [19], as well as in exacerbations of acute asthma induced by HRV [18], representing two asthma phenotypes that are difficult to treat [19]. Interestingly, both neutrophils and IL-8 were found by Lopuhaa when allergic patients were challenged with Der p I [9]. Furthermore, neutrophil elastase has been shown to induce eosinophil degranulation in vitro [48].

Another remarkable property of IL-8 is the variation in its level of expression. This resulted from a complex network that controlled its expression at both transcriptional and post-transcriptional levels. This includes firstly derepression of the gene promoter, then activation of NF-κB and JNK pathways to induce transcription and finally rapid stabilization of the resulting mRNA by the p38 MAPK pathway. Although the transcription step is essential and adequate for IL-8 production, in order to attain maximal IL-8 amounts, all steps are required [49].

Hence, although exploration of the molecular mechanisms leading to the observed sequence of exposure-dependent activation is beyond the scope of this paper, we try to address a possible one by measuring the central transcription step, NF-κB translocation. This, as both HRV and Der p I exert their pro-inflammatory activity through activation of NF-κB, is, among others, a transcription factor. However, Der p action is short lived, showing its effect during the first hours of exposure [50, 51], in contrast to HRV, whose transcriptional activation depends to a large extent on replication and takes more time [7, 28]. In fact, we were able to confirm the above observations; HRV induced an NF-κB induction that was still present at 24 h, while Der p induced it only for the first 2 h. Importantly, combined stimuli induce an early synergistic induction in NF-κB translocation. The above data, although not new, based on time schema, explain our
results partly regarding IL-8. When both stimuli act together, there is an early synergistic increase in activation of the transcriptional pathway. In the schema, when HRV leads, activation is still present at 24 h when the new signal is added, leading to further enhancement. However, when Der p precedes, at 24 h, the transcriptional activity returns to baseline levels, and so the next stimulus HRV acts as a totally new one. However, new experiments addressing the above questions more extensively are required as other transcription factors or pathways participate as AP-1, p38 or even the newly described epidermal growth factor receptor/extracellular signal-regulated kinase signalling pathway [25]. Hence, i.e. Liu et al. found that p38 MAPK inhibitor SB203580 blocked IL-8 secretion from RV16-infected BEAS-2B cells, while Sohn et al. [52] found that the same inhibitor did not effect IL-8 release from the D. farinae (another major dust mite)-exposed human NCI-H292 lung cell line. This indicates the participation of the p38 MAPK pathway in HRV infection but not in Der p exposure and consequently underlines the importance of post-transcriptional IL-8 regulation. This can also explain our results and simultaneously shows the complexity of the system.

In our model, we chose doses and time-points found in the literature [4, 5, 7, 10, 11, 32], and confirmed them further in our initial experiments. Moreover, the Der p I dose is relevant to the one that Green et al. [12] found on the house floors (0.64–1.75 \textmu g/dust). However, we cannot exclude the possibility that other doses and time-points could modify our results.

In order to verify our results further, we repeated the main experiment of simultaneous exposure in HBECs, confirming the synergistic induction of IL-8 release. Then, we further investigated whether this synergy applies only in IL-8 or whether it occurs in other cytokines and chemokines as well. We proceeded with IL-6, a major pro-inflammatory cytokine that both HRV and Der p can induce from airway epithelium [10, 27]. However, while each stimulus alone induced IL-6, their combination resulted only in a small further increase. Here, there is a discrepancy as IL-6 is also regulated by NF-xB. A possible explanation could be that Der p I, through its protease activity, can degrade IL-6, but not IL-8 as shown by King et al. [10]. This could also be the case when, in our attempt to determine the effect of synergy in other chemokines, we detected RANTES/CCL5 only in HRV-infected samples and not Der p or combined HRV and Der p. RANTES is a chemokine that belongs to the CC family and, by its preferential effect on the CCR3 chemokine receptor, plays an important role in asthma by inducing selective recruitment of Th2-type T cells and eosinophils. Of course, other factors can play a role in the above discrepancies, as i.e. post-transcriptional regulation. However, there are earlier studies that also failed to detect RANTES after Der p exposure [21], or used other end-points such as Western blotting [51]. Consequently, we measure IP-10/CXCL10, another chemokine of the CXC family as IL-8 but not with the ELR motif, produced by several cells, particularly epithelial cells, and that serves as a selective chemotactant for both activated Th1 lymphocytes and natural killer (NK) cells. Spurrell et al. [5] have shown recently that HRV infection induces its production from bronchial cells both in vitro and in vivo. We the confirm above data and we also showed for the first time that Der p can also induce IP-10 release from bronchial cells. However, there was no synergistic or even an additive effect of both stimuli together.

Finally, there are other possible explanations for our results. Der p I may induce anti-inflammatory mediator production, such as nitric oxide, IFNs, IL-10 or suppressors of cytokine signalling, effective only or mainly before virus infection [53, 54]. In this context, we addressed the question of whether a recently discovered type III IFN−λ1/IL-29 found to have a direct anti-viral effect in HRV infection of bronchial epithelial cells can be one of these [24]. As expected, HRV induces IL-29 in HBECs; importantly, Der p I also induces it, while their combination has no further effect. This IL-29 induction from Der p I could be a mechanism though which allergens do not induce synergy of IL-8 when they proceed. More importantly, in combination with induction of IP-10, a potent chemotactant for TH1 and NK cells, an anti-viral network can be induced that could explain the failure of the two clinical studies [16, 17].

Our observations are not restricted to asthma. The fact that HRVs are the main virus responsible for the common cold, and dust mites are present in almost all houses [12], suggests that such effects may occur in the expression of common colds, but also potentially in chronic obstructive pulmonary disease (COPD) exacerbations in which IL-8 plays an important role [55]. Also, increased number of neutrophils and IL-8 were found in sputum of patients with stable COPD in comparison with healthy subjects [56], which increased even more during exacerbations [57]. Moreover, Bhowmik et al. [58] found that COPD patients with increased baseline levels of IL-8 experience more frequent exacerbations, indicating IL-8 and IL-6 as predictors.

Finally we have to emphasize that here we attempted to construct a possible in vitro model of asthma exacerbation, which of course cannot completely reconstitute all the parameters of a ‘clinical asthma exacerbation’ or, as we hypothesize above, even an COPD exacerbation. In ‘clinical asthma,’ patients are usually exposed chronically to allergen and then infected with virus, especially if we discuss about allergens as HDMs, which are constantly present. However, as the allergic response is dependent on the time and amount of exposure and as our time of exposure is rather short (24 h), we believe that we have provided some insights into the model where a patient is
firstly exposed to an allergen, i.e. to Der p I due to house cleaning during a common cold, which may induce further release of mediators and may augment the existing exacerbation.

In conclusion, our study is the first to show that HRV and Der p I, two major environmental stimuli, synergize in the induction of pro-inflammatory airway epithelial responses and especially in the induction of IL-8. Furthermore, the sequence of exposure determines the robustness of the response. Also, here we report for the first time that HDM major allergen Der p I can induce new mediators from bronchial epithelial cells as IP-10 and IL-29, which may play an important role in the anti-viral immune response. These data provide new insights into the mechanisms of exacerbations as well as severity in airway diseases like asthma and COPD. However, it should be noted that the above study is mainly observational and new, more detailed studies investigating the underlying mechanisms are needed that should investigate them not only in the terms of interaction of virus with HDMs but also with other common allergens with protease activity, such as aspergillus and cockroach. This eventually will give us the opportunity for a future therapeutic intervention in terms of the above respiratory diseases.

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