

No exacerbation but impaired anti-viral mechanisms in a rhinovirus-chronic allergic asthma mouse model

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Abstract

Severe asthma and viral-induced asthma exacerbations represent a high unmet medical need as no therapy is currently available for these patients. HRV (human rhinovirus) is prominently associated with asthma exacerbations in humans. The aim of the present study was to establish a mouse model of severe asthma with additional rhinovirus infection to investigate the interplay between chronic allergic airway inflammation and acute respiratory viral infection. Balb/c mice were sensitized with HDM (house dust mite) extract (25 µg in 50 µl of saline) by i.n. (intranasal) delivery to the lung over 7 weeks. HRV1B (HRV serotype 1B) inoculation was performed i.n. on the last 3 days. Therapeutic treatment with FP (fluticasone propionate) was performed to assess steroid efficacy. Lung resistance was measured invasively to assess AHR (airway hyper-responsiveness). BAL (bronchoalveolar lavage) differential cell count, cytokines, lung histology and the proliferative and cytokine response of MLN (mediastinal lymph node) cells upon *in vitro* restimulation were analysed. Chronic HDM application induced a strong Th2-skewed eosinophilic airway inflammation and AHR, which was not exacerbated by superimposed HRV1B infection. Therapeutic steroid intervention in the chronic HDM model reduced BAL eosinophil cell counts, cytokine levels and AHR, while neutrophil numbers were unaffected. Steroid efficacy against inflammatory readouts was maintained during additional HRV1B infection. Animals with chronic allergic airway inflammation exhibited a diminished immune response towards superimposed HRV1B infection compared with HRV1B alone, as induction of the anti-viral and pro-inflammatory cytokines IFN (interferon)-α, IFN-γ and IL (interleukin)-12 were suppressed. Although superimposed HRV1B infection did not provoke asthma exacerbation in this severe model, a deficient anti-viral immune response to HRV1B was present under chronic allergic airway inflammatory conditions. Thus, this model is able to reflect some aspects of the complex interplay of respiratory virus infection in chronic allergic asthma.

Key words: allergic asthma, chronic model, exacerbation, rhinovirus, superimposed viral infection

INTRODUCTION

The link between viral respiratory infection and asthma exacerbation has become increasingly apparent over the past years [1]. Clinical evidence accumulates that respiratory virus infection accounts for up to 80% of exacerbation of symptoms in asthmatic children [2,3] and adults [4]. HRV (human rhinovirus) has emerged as the most prominently associated respiratory tract virus among these patients [2–4]. As the primary causative pathogen for the common cold, in asthmatics HRV also triggers lower

airway disease [2] frequently leading to hospitalization due to the severity of symptoms [5].

Despite this obvious link it is yet unclear how rhinovirus infection interacts with ongoing allergic inflammation to trigger severe conditions during asthma exacerbations.

HRV is a ubiquitously occurring respiratory virus of the *Picornaviridae* family. HRV is a non-enveloped positive single strand RNA virus with more than 150 known serotypes [6–10]. The first encounter of the host with the viral pathogen takes place at the airway mucosal site and HRV invades the epithelial cells as primary

Abbreviations: AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; BALF, BAL fluid; BEC, bronchial epithelial cells; CPE, cytopathic effects; FBS, fetal bovine serum; FP, fluticasone propionate; HDM, house dust mite; HPA, Health Protection Agency; HRV, human rhinovirus; HRV1B, human rhinovirus serotype 1B; IFN, interferon; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; i.n., intranasal(y); LDLR, low-density lipoprotein receptor; MCh, methacholine; MEM, minimal essential medium; MLN, mediastinal lymph nodes; NEAA, non-essential amino acids; OCT, optimal cutting temperature; RL, pulmonary resistance; TCID₅₀, 50% tissue culture infective dose; TLR, Toll-like receptor.

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target [11]. HRV uses different pathways for entry into the host cells and serotypes usually are divided into subgroups depending on their receptor [7,12]. Major group rhinoviruses constituting about 90% of HRV serotypes A and B use the human ICAM-1 (intercellular adhesion molecule 1) [12]. Minor group rhinoviruses use receptors of the LDLR (low density lipoprotein receptor) family [7,13–16]).

Upon successful entry into the host cell the viral RNA is released into the cytosol (uncoating). In contrast to major group HRV, the uncoating of minor group HRV is not catalysed by the receptor and therefore is receptor independent. Upon uncoating and release of the viral RNA into the cytosol, replication is initiated leading ultimately to the release of new virions from the cell without inducing direct CPE (cytopathic effects) [17,18]. HRV is able to replicate in epithelial cells both of the upper and lower airways [19–21]. The epithelium however is not only the host cell reservoir for viral replication, but immediately initiates the innate immune response against the invading virus. Several pattern recognition receptors such as TLR (Toll-like receptor)-3, -7 and -8, RIG-I and MDA (methylene dianiline)-5 recognize viral RNA and activate diverse signalling pathways resulting in the secretion of type I IFN (interferons) (IFN- α , IFN- β) as well as type III IFN- λ 2, IFN- λ 3, IFN- λ 1 also known as IL (interleukin)-28A, B and IL-29 respectively [11,22–25] by epithelial cells, but also by monocytes/macrophages [26]. Furthermore, proinflammatory cytokines such as IL-1 β , IL-6, IFN- γ and IL-12, chemokines such as IL-8, eotaxins, IFN- γ , IP (inducible protein)-10 and RANTES (regulated upon activation, normal T-cell expressed and secreted) as well as growth factors such as GM-CSF (granulocyte/macrophage colony-stimulating factor) are released [11]. These cytokines and chemokines elicit the anti-viral immune response of the host resulting in direct interference with viral replication, apoptosis of infected cells and recruitment of primarily neutrophil granulocytes but also cytotoxic and NK (natural killer) cells producing IFN- γ [27–29].

The causal link between HRV infection and asthma exacerbation is still poorly understood until now. Dissecting the underlying mechanisms will be even more complex as asthma today is understood as a heterogeneous disease comprising variable phenotypes (reviewed in [30–32]).

New therapies, especially ‘personalized’ medicine tailored to the particular needs of different asthma phenotypes, are urgently needed, as exacerbations relate to a severe condition and the morbidity and mortality of asthma [33] which are associated with high economic burden.

In this context, there is an essential need of mouse models that not only represent different asthma phenotypes, especially those with severe and difficult to treat asthma conditions, but furthermore allow the study of the complex interaction between chronic allergic airway inflammation and acute respiratory viral infection. Such models would be a valuable tool not only to elucidate the mechanisms of asthma exacerbations but also for efficacy studies of new therapeutics with acceptable predictivity. For this purpose, a mouse model of chronic allergic airway inflammation with superimposed acute rhinovirus infection was established to investigate rhinovirus infection in severe asthma.

MATERIALS AND METHODS

Animals

Wild-type Balb/c mice (6–8 weeks old) were purchased from Charles River. All animals were held in regular 12 h dark/light cycles at 22°C and received laboratory food and tap water *ad libitum*. The animals were acclimatized for at least 2 weeks and randomized 6 days before the experiments started. The health condition of the animals was monitored daily. The study was performed in strict concordance with the German animal protection law and approved by the appropriate governmental authority (10/0207). All virus experiments including viral infection of mice, subsequent animal housing and all experimental procedures for sample analysis were conducted in biosafety level 2 laboratories.

Generation of HRV1B (HRV serotype 1B) stocks

Minor group HRV1B was obtained from HPA (Health Protection Agency) (NCPV #624). The virus was propagated in HeLa Ohio cells purchased from HPA (#84121901). HeLa cells were grown in the cell culture medium [1×MEM (minimal essential medium), 10% (v/v) FBS (fetal bovine serum), 2% penicillin/streptomycin, 1×NEAA (non-essential amino acids) and 2 mM glutamine] to 80–90% confluence at 37°C, 5% CO₂. Viral stocks were generated by infection of HeLa cells with HRV1B in serum-free cell culture medium and incubated at 33°C, 5% CO₂ until CPE were observed in approximately 90% of the cells. As a control HeLa cells were sham-infected with the serum-free medium alone. HeLa cell lysates were harvested and cell debris was separated by centrifugation (3000 g, 10 min). The supernatant was used as inoculum for further infection cycles. For *in vivo* experiments, virus purification was performed adapting a published method [34]. Briefly, the supernatants were sterile filtered (0.2 µm) and concentrated by centrifugation with 100 kDa Amicon Ultra filtration devices (Millipore). HRV1B was precipitated by incubation with a final concentration of 7% PEG [poly(ethylene glycol)] and 0.5 M NaCl for 1 h on ice. The precipitated virus was pelleted by centrifugation (4000 g, 1 h, 4°C) and thoroughly resuspended in PBS. A second filtration and concentration step was performed and the concentrated virus suspension washed twice in PBS. The titre of the purified virus stock was assessed with TCID₅₀ (50% tissue culture infective dose) and plaque assays. UV-inactivated virus was used as a control. The quality of the virus batches for *in vivo* infection of mice was verified by infection of naïve mice.

Measurement of viral titres

For virus titration, TCID₅₀ and plaque assays were performed. For plaque assay, HeLa cells were seeded in 6 well microtitre plates to reach about 80% confluence. Serial dilution of virus samples was performed and 100 µl per well were inoculated. After 1 h incubation, the inoculum was removed and 4 ml per well overlay medium [final concentration 1×MEM, 2% (v/v) FBS, 2% penicillin/streptomycin, 1×NEAA, 2 mM glutamine, 0.18% sodium bicarbonate, 20 mM MgCl₂, 100 µg/ml DEAE-Dextran and 0.6% LMP (low-melting point) agarose] were added. Upon solid gelling of the overlay the cells were incubated for

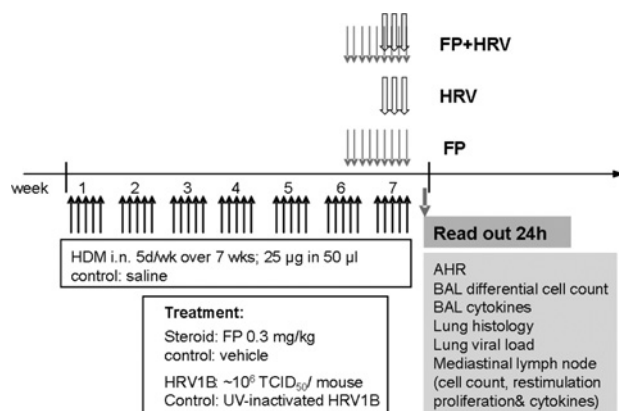


Figure 1 Treatment protocol for chronic HDM model + secondary acute HRV infection

Balb/c mice were i.n. treated with 50 μ l saline or 25 μ g HDM in 50 μ l of saline 5 days per week for 7 weeks. Secondary HRV infection was performed by i.n. inoculation of 1×10^6 TCID₅₀ HRV1B 30 min prior to HDM challenge during the final 3 days of week 7. Steroid-treated animals received FP at 0.3 mg/kg of body weight i.n. in a volume of 50 μ l daily on the final 9 days. $n = 16$ animals per group. Lung function (AHR to MCh) was measured 24 h after the last treatment. Directly afterwards, BAL was performed and lungs and MLN were obtained for further analysis.

3 days at 33 °C and 5 % CO₂. After fixation with 2 % formalin/5 % methanol for 1 h, the agarose overlay was removed and the cell layer stained with 0.1 % Crystal Violet. Plaques were counted and the titre calculated from the appropriate virus dilution.

The TCID₅₀ was determined by infection of HeLa cells in 96-well microtitre plates using serial dilution of virus samples using 25 μ l inoculum per well. After incubation for 1 h, serum-free medium was added to a total of 200 μ l per well and the cells were incubated at 33 °C, 5 % CO₂ for 3 days. The cells were fixed and stained as described above and the CPE was determined for each dilution. The titre was calculated according to the Spearman–Karber method.

HRV1B inoculation

Inoculation of the virus was performed by i.n. (intranasal) delivery to the lung using 50 μ l per mouse under light inhalative isoflurane anaesthesia. The infectivity of the virus batch was previously determined by the degree of neutrophilia induced in naïve mice. Purified virus was inoculated during the study with a titre of 1×10^6 TCID₅₀ per mouse on the final 3 days 30 min before HDM (house dust mite) challenge. Control groups were infected with UV-inactivated virus of the same batch.

Induction of chronic allergic airway inflammation by i.n. delivery of HDM (7 week HDM model)

Chronic allergic airway inflammation was induced by chronic HDM application (first published by Johnson et al. [35]) (Figure 1). Naïve Balb/c mice, 6–8 weeks old, received 25 μ g HDM i.n. in 50 μ l of saline under light inhalative isoflurane anaesthesia on 5 days per week over 7 weeks. Animals of the negative control group received 50 μ l of saline i.n. for sham sensitization.

Steroid treatment

To assess steroid efficacy in HDM-sensitized HRV1B infected and non-infected animals, animals of two groups were treated i.n. with FP (fluticasone propionate) at 0.3 mg/kg of body weight in a volume of 50 μ l 1 h before HDM application daily during the final 9 days before necropsy. Animals of non-treated groups received 50 μ l of vehicle i.n. instead. The body weights of steroid-treated animals were monitored daily.

Measurement of AHR (airway hyper-responsiveness)

24 h after the final HDM challenge, pulmonary function was measured invasively in anaesthetized, spontaneously breathing mice to assess AHR as described previously [36,37]. Briefly, RL (pulmonary resistance) was calculated from the measured differences in transpulmonary pressure and tidal respiratory flow using body plethysmography (type 871; HSE-Harvard apparatus) under isoflurane anaesthesia in orotracheally intubated animals (HEM 4.2 software; Notocord Systems). After recording baseline values for 2 min, AHR to aerosolized MCh (methacholine chloride, from 5 % solution; Sigma) was determined by using a Bronchy type III generator (Fraunhofer ITEM) under continuation of lung function measurement. Aerosol concentrations were determined by a gravimetrically calibrated photometer. The total dose inhaled via the orotracheal tube in μ g was calculated and controlled by a computerized feedback dose-control system (Fraunhofer ITEM) based on the continuously measured respiratory minute volume and aerosol concentration [37]. Mice were exposed to MCh dose steps of 0.031, 0.063, 0.125, 0.3, 0.5, 1, 2 and 4 μ g. From dose–effect plots (Δ % RL against MCh dose) the effective inhalation dose in μ g of MCh required to produce a 100, 150 and 200 % increase in RL was evaluated for each animal (ED₁₀₀, ED₁₅₀ and ED₂₀₀ RL).

Animal dissection

Necropsy was performed 24 h after the last HDM challenge by intraperitoneal application of an overdose of pentobarbital. Collection of samples and subsequent processing was performed as described before [38]. Briefly, mice were bled via the *vena cava caudalis*. BAL (bronchoalveolar lavage) was performed by twice instillation of 0.8 ml ice-cold sterile PBS. MLN (mediastinal lymph nodes) were collected in ice-cold RPMI and kept on ice until further processing. The left lung lobe was filled with 1:3 diluted OCT (optimal cutting temperature), embedded in OCT, snap frozen and stored at –70 °C. The right lobes were removed and homogenized in 1 ml ice-cold PBS with an automated tissue homogenizer (Fast Prep®; MP Biomedicals). The supernatants were stored at –70 °C until further processing.

BAL differential cell count

The BALF (BAL fluid) was centrifuged (320 g, 10 min, 4 °C) and the supernatant immediately aliquoted and stored at –70 °C. The cells were resuspended in 0.5 ml of PBS and counted automatically with a Casy® cell counter. Cytospots were prepared and stained by Pappenheim stain to evaluate differential cell counts.

Lung histology

Cryosections (8 μm) of the OCT-embedded lungs were performed and stained with H&E (haematoxylin and eosin) to assess histopathological changes. The slides were manually examined under a light microscope.

BAL and lung viral load

BAL and lung homogenate supernatant were obtained as described above. The supernatants were titred by duplicate plating of serial dilutions on HeLa cell monolayers and analysis of CPE (TCID_{50}).

MLN restimulation

MLN were homogenized by mashing with a plunger of a syringe through a 70 μm cell strainer (BD Biosciences) to yield a single-cell suspension. 10^5 cells per well were seeded in 96-well round-bottom plates and restimulated *in vitro* with 50 $\mu\text{g/ml}$ HDM for 3 days. Supernatants were collected for cytokine measurement. Proliferation was measured by ^3H -thymidine incorporation.

Cytokine measurement

Cytokine concentrations in BALF and supernatants of MLN restimulation were determined using mouse Th1/Th2 9-plex Ultra-Sensitive Kits according to the manufacturer's instructions (Meso Scale Discovery). The detection limit was 2.4 pg/ml. BAL IFN- α and IFN- λ were measured by ELISA (eBioscience) with a detection limit of 30 pg/ml.

Statistics

Data in the Figures are given as means + S.E.M. or medians + interquartile range for normally distributed or non-normally distributed data respectively. Statistical analysis was performed as follows: for comparison of two groups a Student's *t* test in case of normally distributed data or else a Mann–Whitney *U* test was performed. Accordingly, for multiple group data ANOVA or Kruskal–Wallis with parametric (Bonferroni) or non-parametric (Dunn's) post-test respectively was performed for comparison of groups (GraphPad Prism 4, version 4.03). Differences between the groups were considered as statistically significant at the level of $P < 0.05$.

RESULTS

Rhinovirus infection during established chronic allergic airway inflammation does not induce exacerbation of asthma symptoms

In the present study a combined mouse model of chronic allergic airway inflammation induced by HDM and superimposed acute respiratory virus infection using HRV1B was established to mimic rhinovirus infections observed in human patients with severe asthma (Figure 1).

The chronic application of HDM i.n. for 7 weeks induced the characteristic features of allergic airway inflammation as previously described [35]. HDM sensitized and challenged mice (further designated as HDM group) developed strong airway inflammation reflected by the increase in total BAL cell counts

compared with the saline group (see Supplementary Figure S1 at <http://www.clinsci.org/cs/126/cs1260055add.htm>). The cellular infiltrate primarily comprised of eosinophils, but neutrophils and lymphocytes were also induced (Figure 2A). Notably, the saline group (non-sensitized) responded to the frequent i.n. treatment with a slight neutrophil influx (Figure 2A). Therapeutic steroid treatment with FP at 0.3 mg/kg of body weight resulted in diminished airway inflammation with regards to total and eosinophil cell counts. However, neutrophil and lymphocyte numbers were refractory to FP treatment (Figure 2A).

To evaluate whether an acute respiratory viral infection with HRV1B can exacerbate pre-existing severe asthma, three consecutive inoculations with HRV1B were performed on top of the established chronic allergic airway inflammation (HDM + HRV group) and compared with a non-infected asthma group (HDM). Additional HRV infection did not elicit increased inflammatory cell influx in BAL (Figure 2A). Eosinophil cell counts were actually slightly decreased in the HDM/HRV1B group compared with the sham-infected HDM group (Figure 2A). Furthermore, steroid efficacy was maintained in viral infected HDM mice, indicated by the decrease in eosinophil numbers, which was comparable with the non-infected HDM group (Figure 2A).

The inflammatory response elicited by chronic HDM application was primarily Th2-type as increased concentrations of Th2 cytokines IL-4, IL-5 and IL-10 were detected in BALF (Figure 2B). Th1-type cytokines were only partially induced, as IL-12 concentrations were increased (Figure 2B), whereas IFN- γ and IL-1 β were under the detection limit (results not shown). BAL cytokine levels were efficiently reduced by steroid treatment regarding IL-4, IL-10 and IL-12 (Figure 1B). Interestingly, IL-5 levels remained unresponsive to steroid. Superimposed HRV1B infection did not alter the BAL cytokine profile or the steroid efficacy against inflammatory readouts compared with the non-infected asthma group (HDM) (Figure 2B).

Histological evaluation of the lungs confirmed an exaggerated inflammation induced by chronic HDM delivery, which was characterized by multifocal peribronchial and perivascular infiltration, while in the negative control group no inflammation was present (Supplementary Figure S2 at <http://www.clinsci.org/cs/126/cs1260055add.htm>). Within histological sections of the HDM/FP group, only a moderate inflammatory infiltration in the lung tissue was observed. The HRV/HDM group displayed a strong peribronchial and perivascular infiltration, whereas the HRV/HDM/FP group showed only moderate inflammation (Supplementary Figure S2).

The immune response during chronic allergic airway inflammation with or without superimposed HRV infection was further characterized not only locally in the lung compartment, but also in the draining lymph nodes (MLN). In HDM-sensitized animals MLN cell counts were increased about 2-fold due to the ongoing allergic immune response (Figure 3A). *In vitro* restimulation with HDM allergen extract accordingly resulted in an increased proliferative response in the HDM group compared with the non-sensitized saline group (Figure 3B). The recall response revealed the Th2-biased immune response as enhanced secretion of Th2 cytokines (IL-4, IL-5) and IL-10 was observed, while levels of Th1 cytokines IFN- γ or IL-12 remained low

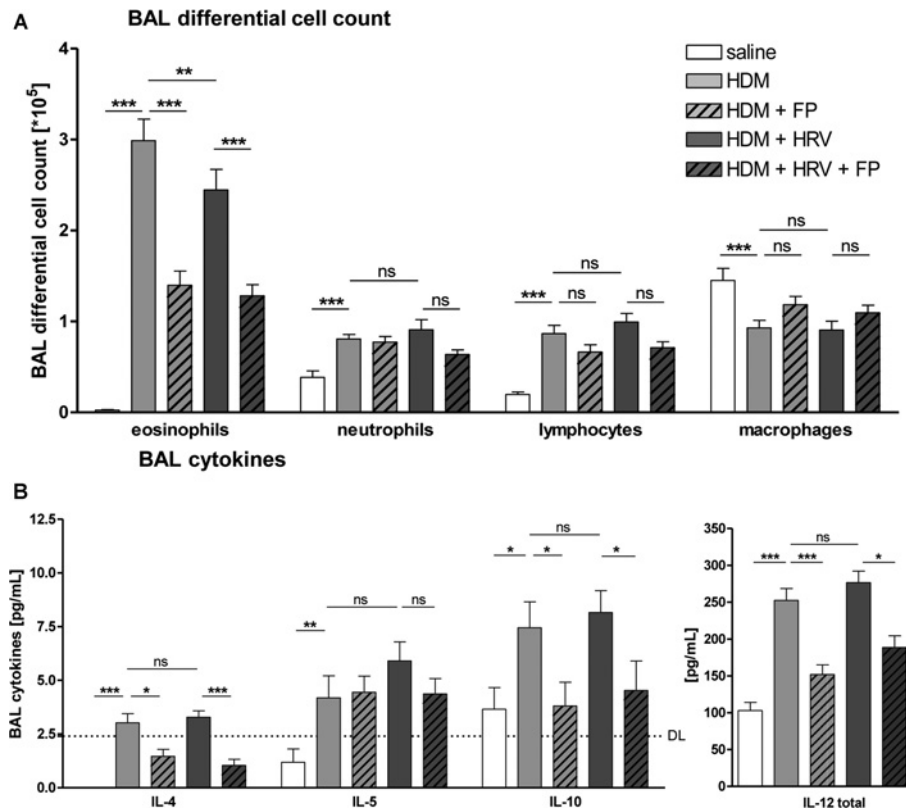


Figure 2 Effect of HRV infection during late phase chronic airway inflammation on BAL cellular infiltrate and cytokine profile

(A) BAL differential cell count, (B) BAL cytokines in chronic HDM + HRV model. Balb/c mice were treated with saline (white bars) or HDM (light grey bars) for 7 weeks. Additional HRV inoculation was performed on the final 3 days of week 7 (dark grey bars). Steroid-treated animals received FP on the final 9 days (striped light and dark grey bars). $n = 16$ animals per group. Values are medians + interquartile range (A) or means + S.E.M. (B). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to Kruskal–Wallis and Dunn's post-test (A) or ANOVA and Bonferroni post-test (B). ns, not significant.

(results not shown) or even were decreased compared with saline control group, respectively (Figure 3C) and IL-1 β was under the detection limit (results not shown). The lymphocyte response was efficiently suppressed in steroid-treated animals, as lower MLN cell counts were observed, accompanied by a diminished proliferative response upon allergen restimulation (Figures 3A and 3B). Interestingly, the cytokine profile remained unchanged and secretion of Th2 cytokines upon allergen restimulation was not suppressed (Figure 3C). The superimposed HRV infection did not modify the ongoing immune response in the draining lymph nodes. MLN cell counts upon HRV infection were not statistically significantly increased and consistently the proliferation and cytokine profile upon HDM restimulation was similar to the non-infected HDM group (Figures 3A–3C). Steroid effects in HRV- and non-infected HDM groups were comparable.

The severe allergic airway inflammation induced by chronic HDM application was associated with strong AHR, as indicated by the increased lung resistance in response to MCh compared with non-sensitized animals (saline group) (Figure 4). Steroid treatment with FP resulted in partially improved lung function. In the HRV-infected HDM group no further increase in AHR to MCh was detected compared with the non-infected HDM group.

Notably, although steroid treatment during HRV infection maintained the trend towards improvement of AHR, this effect was less pronounced and no longer statistically significant. As the lung function and BAL inflammatory cell count and cytokines, as the main physiological parameters measured in the diagnosis of asthma patients, failed to show a clear effect of rhinovirus with respect to exacerbation, the assessment of further molecular end points was not regarded as valuable.

Ongoing chronic allergic airway inflammation impairs anti-viral immune response to acute superimposed rhinovirus infection

As the superimposed HRV1B infection did not provoke asthma exacerbation, whereas obvious signs of viral infection were induced in naïve mice during pretesting of the virus batch, the question of different host susceptibility in healthy compared with chronically diseased animals was investigated. Therefore the anti-viral immune response elicited by HRV was evaluated in the chronic HDM HRV infected mice and compared with a satellite group of healthy, age-matched only HRV infected mice. For this purpose, untreated naïve mice were infected by three consecutive HRV i.n. inoculations after 7 weeks without any prior treatment (naïve control group). Interestingly, these mice developed AHR

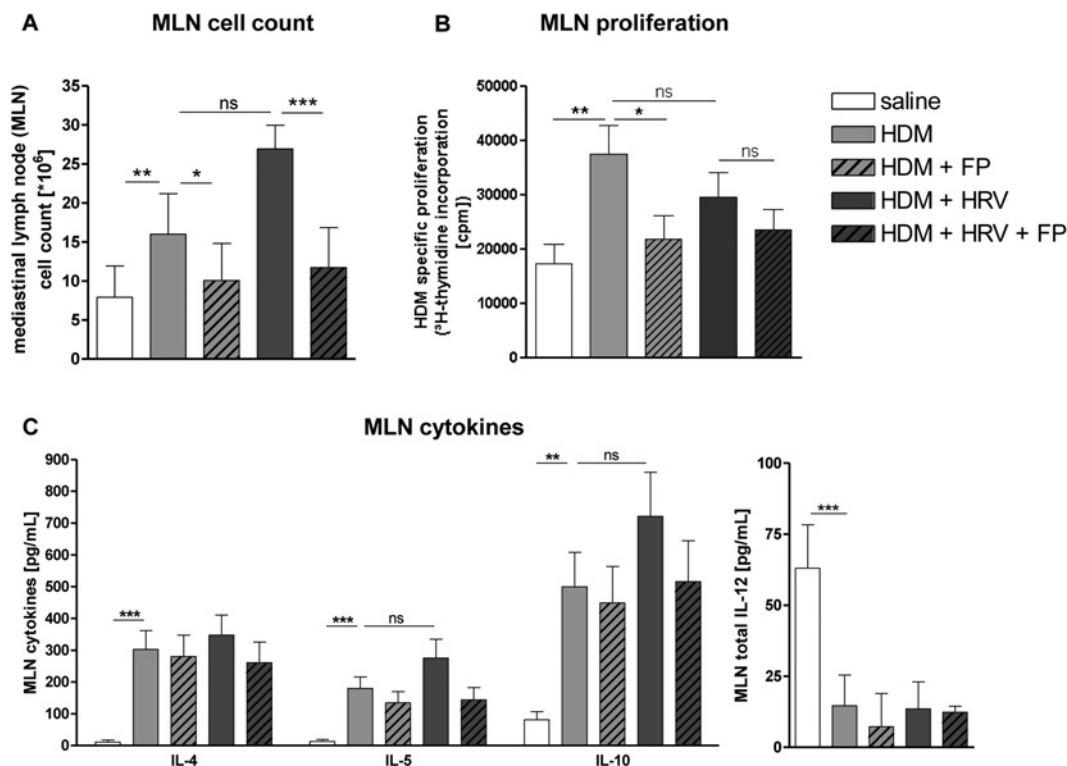


Figure 3 Effect of HRV infection during late phase chronic airway inflammation on MLN response (A) MLN total cell count. (B) MLN proliferation upon *in vitro* restimulation with 50 $\mu\text{g}/\text{ml}$ HDM for 3 days measured by ^3H -thymidine incorporation indicated as HDM specific proliferation (increase over basal proliferation upon restimulation with medium). (C) MLN cytokine profile upon HDM restimulation. Bars represent saline group (white bars) or HDM challenged group (light grey bars), additionally HRV infected mice (dark grey bars) and steroid-treated groups (striped light and dark grey bars). $n = 16$ animals per group. Values are medians + interquartile range (A) or means + S.E.M. (B, C). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to Kruskal–Wallis and Dunn's post-test (A) or ANOVA and Bonferroni post-test (B, C). ns, not significant.

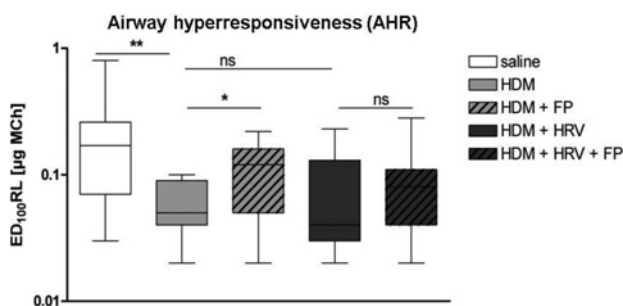


Figure 4 Effect of HRV infection during late phase chronic airway inflammation on AHR

Balb/c mice were treated with saline (white bar) or HDM (light grey bar) for 7 weeks. Additional HRV inoculation was performed on final 3 days of week 7 (dark grey bars). Steroid-treated animals received FP on the final 9 days (striped light and dark grey bars). AHR was measured 24 h after final HDM challenge. Graph represents the effective inhalational dose of MCh in μg required to increase RL by 100 % above baseline (ED₁₀₀RL) measured in anaesthetized, orotracheally intubated and spontaneously breathing mice. $n = 16$ animals per group. Box and whisker plot show median and interquartile range. * $P < 0.05$ and ** $P < 0.01$ according to Kruskal–Wallis and Dunn's post-test. ns, not significant.

comparable with the AHR induced by chronic allergic airway inflammation (Supplementary Figure S3 at <http://www.clinsci.org/cs/126/cs1260055add.htm>). The induced neutrophilic airway

inflammation (Figure 5A) confirmed the activity of the used virus batch as expected from pre-tests in naïve mice (Supplementary Figure 4A at <http://www.clinsci.org/cs/126/cs1260055add.htm>). HDM-HRV mice displayed lower neutrophil numbers while the dominant eosinophilic infiltration was maintained (Figure 5A). Furthermore, naïve mice developed a Th1-driven anti-viral immune response upon HRV1B infection as indicated by increased IFN- γ , IL-1 β and IL-12 concentrations measured in BAL, which in contrast were nearly absent in mice with chronic HDM background (Figure 5B). MLN cell counts were lower in the naïve HRV than in the HDM/HRV group (Figure 6A). Interestingly, naïve HRV MLN showed a massive unspecific proliferation *in vitro*, while HDM/HRV MLN cells were only activated after HDM restimulation (Figure 6B). Concordantly, MLN of the naïve HRV group produced high levels of IFN- γ , IL-1 β and IL-12 while MLN of HRV-infected HDM mice failed to respond to the HRV infection (Figure 6C).

To more precisely define the anti-viral response in naïve compared with chronic HDM allergic mice, type I and type III IFN secretion upon HRV infection was measured in BAL of both groups. Increased levels of type-I-IFN- α and a trend towards higher type-III-IFN- $\lambda 3$ secretion were observed in naïve HRV infected compared with HDM/HRV mice (Figure 7), confirming impaired anti-viral response during established allergic airway

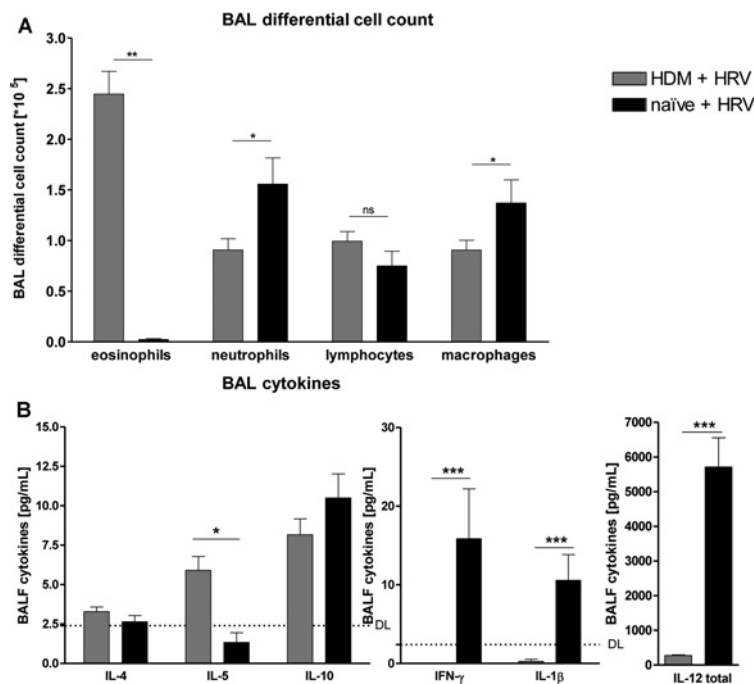


Figure 5 Impaired immune response towards HRV infection in mice with chronic allergic airway inflammation compared with naive mice

(A) BAL differential cell count and (B) Th1/Th2 cytokines from Balb/c mice treated with HDM 7 weeks (grey bars) or age-matched untreated, naive mice (black bars) were infected in parallel with HRV1B using three consecutive i.n. inoculations of the same virus batch. Group size $n=16$ (HDM + HRV) and $n=4$ (naive + HRV). Values are means + S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to Mann–Whitney U test (two-tailed). ns, not significant.

inflammation. To investigate a possible impact on viral replication, virus titre assays were performed using BAL and lung homogenate supernatant, but due to the high background signal induced by the samples of animals with severe chronic inflammation could not confirm replication (results not shown). Despite viral replication not being confirmed by titre assays, the control used in the study provides evidence of *in vivo* replication, as UV-inactivated virus did not result in neutrophilic inflammation, verifying that the induced immune response is replication dependent. In a pre-study in naïve mice to test HRV1B infectivity, viral titre from lung tissue could be measured at 6 h post-inoculation but was not detectable at 24 h as measured by TCID₅₀ assay (Supplementary Figure 4C). Thus viral titre seems to be rapidly decreasing *in vivo*, while a strong neutrophilic infiltration followed by lymphocyte influx is induced (Supplementary Figures 4A and 4B). Owing to the rapidly decreasing viral titres observed in the present study in naïve mice, the viral inoculation in the asthma model was performed repetitively on three consecutive days to gain considerable virus load.

DISCUSSION

In the present study we aimed to establish a mouse model of chronic allergic airway inflammation with superimposed acute HRV1B infection to investigate the complex interplay of respiratory viral infection in severe asthma, a condition described as

being associated with asthma exacerbation. As clinical evidence points towards rhinovirus being the most prominent respiratory virus associated with asthma exacerbation, we selected HRV1B as model virus. As an underlying model of severe asthma, the chronic HDM mouse model of allergic airway inflammation was chosen, as this has been described to reflect several clinical signs of chronic asthma very close to the human pathology [35]. For viral infection in this mouse asthma model HRV1B was used. This serotype belongs to the minor group rhinoviruses using the LDLR family for entry into the host cell. In contrast with major group HRV using ICAM-1, this LDLR-mediated infection with minor group serotypes has been described to be applicable in mouse models [34,39,40]. However, HRV infection efficacy in mice remains a matter of debate as viral replication was found to be very limited but possible to overcome using ICAM-1 transgenic mice or a higher inoculum of minor group HRV [23,34,41].

Within the present study replicating virus could only be detected at 6 h but not 24 h after inoculation in naïve mice. Previous reports on HRV titres in mouse models *in vivo* detected infectious virus at 24 h and longer, but also observed the same tendency of decreasing viral titre over time [34,39] rather than a replication-induced increase of virus load. Regarding the rapidly decreasing viral titre a repetitive infection regimen with three consecutive inoculations on top of the asthma model was chosen to gain considerable virus load. However, in the chronic HDM + HRV model, it was not possible to evaluate viral titre in BAL or lung tissue. Thus the virus levels at the time point analysed (24 h after last HRV inoculation) were still below the detection limit

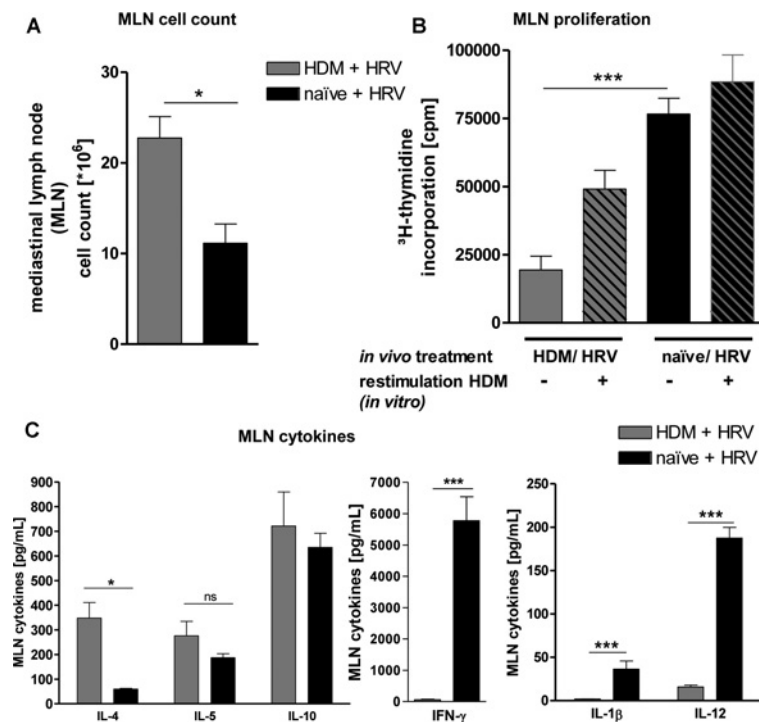


Figure 6 Impaired MLN response upon HRV infection in mice with chronic allergic airway inflammation compared with naive mice

(A) MLN total cell count, (B) MLN proliferation upon *in vitro* restimulation with 50 $\mu\text{g}/\text{ml}$ HDM for 3 days measured by ^3H -thymidine incorporation, and (C) MLN cytokine profile upon HDM restimulation. Balb/c mice treated with HDM 7 weeks (grey bars) or age-matched untreated, naive mice (black bars) were infected in parallel with HRV1B using three consecutive i.n. inoculations of the same virus batch. Group size $n = 16$ (HDM + HRV) and $n = 4$ (naïve + HRV). Values are means $\pm$ S.E.M. * $P < 0.05$ and *** $P < 0.001$ according to Student's *t* test (two-tailed) (A, C) or ANOVA with Bonferroni post-test (B). ns, not significant.

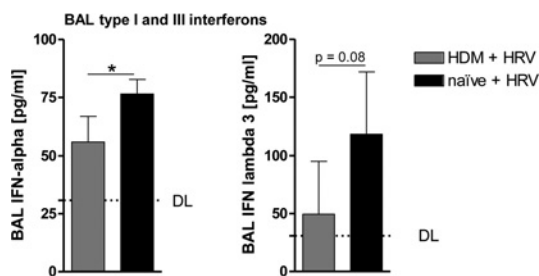


Figure 7 Impaired type I and III IFN production upon HRV infection in mice with chronic allergic airway inflammation compared with naive mice

Left-hand panel, BAL IFN- α (type I) and right-hand panel BAL IFN- λ 3 (type III) levels in mice treated with HDM for 7 weeks (grey bars) or age-matched untreated naive mice (black bars) upon infection with HRV1B using three consecutive i.n. inoculations of the same virus batch. Group size $n = 16$ (HDM + HRV) and $n = 4$ (naïve + HRV). Values are medians $\pm$ interquartile range. * $P < 0.05$ according to Mann-Whitney *U* test. DL, detection limit.

of the assays. Furthermore, due to the high level of inflammation the BAL and lung tissue matrix interfered with the assay and a clear discrimination from viral-induced CPE was not possible. While replication remained unclear, viral infection reproducibly induced a strong neutrophilic inflammation. This immune response was not present in mice receiving UV-inactivated virus,

showing that the induced immune response was replication dependent.

Despite the clear immune response elicited by the virus in naive mice, exacerbation upon infection superimposed onto the background of chronic allergic airway inflammation was not observed. This might be an issue of the severity of the induced immune response. The chronic HDM model elicits a massive eosinophilic airway infiltration, accompanied by impaired lung function manifested as severe AHR. It might be speculated that upon this profound response a superimposed rather moderate infection induced by HRV was not able to exaggerate the immune response any further. Previous studies in acute models nicely showed that acute allergic asthma can be exacerbated by rhinovirus [34]. The aim of this study was to proceed further towards the chronic situation of patients with severe and difficult to treat asthma. Thus, we combined a severe chronic asthma model with acute rhinovirus infection, as clinical evidence suggests this combination of severe asthma and innocuous rhinovirus infection to be associated with exacerbation and severe symptoms. The present study demonstrates that in the chronic condition exacerbation by rhinovirus cannot be mimicked like in acute models. However, with the objective of establishing models that are highly clinically predictive, models reflecting the chronic, perpetuating immune response present in humans will have to be advanced in future studies to develop models of asthma exacerbation.

Although HRV infection did not induce any exacerbation of the end points analysed, the therapeutic effect of steroid treatment on HDM-induced AHR in animals with superimposed HRV infection seemed to be diminished. This potentially could reflect a relative steroid insensitivity resulting from the viral infection and thus may be clinically important, as episodes of viral associated wheezing and impaired lung function were often observed to be relatively insensitive to steroid treatment. However, further studies need to be performed to address the question of steroid efficacy in HRV infected animals.

Interestingly, clinical evidence is accumulating that asthmatics are more susceptible to rhinovirus infections, developing more frequent and more severe lower respiratory tract symptoms [42]. This has previously been linked to a deficient innate immune response to viral infection observed in asthmatic conditions. Type I IFN- β has been shown to be reduced in asthmatic BEC (bronchial epithelial cells) *in vitro* which was associated with diminished apoptosis of infected cells and hence an increase in viral replication [43]. Following up this important finding, Contoli et al. [28] also showed that type III IFN- λ are crucial in anti-viral immunity towards rhinovirus infection in BEC. They further demonstrated a deficient IFN- λ production in BEC but also macrophages derived from atopic asthmatics, with decreased IFN levels inversely correlating not only with increased virus load, but importantly also with aggravation of the severity of symptoms [28]. Other studies described impaired capacity for rhinovirus-induced type I IFN secretion in BAL and peripheral blood mononuclear cells of asthmatics [44,45], further supporting the notion of an impaired response to rhinovirus in asthma. Recent studies emphasized the importance of these observations as impaired IFN production was linked to severe therapy-resistant asthma [46]. The results obtained in the current study suggest that this deficient type I and III IFN response could be reflected in the herein established model, as mice with chronic airway inflammation exhibited an impaired IFN- α and IFN- λ response upon HRV infection compared with 'healthy' untreated mice. Thus, although no exacerbations of any end points measured were observed at the time point analysed, the present model closely reflected the human situation regarding this aspect of impaired anti-viral immune response described for asthmatic patients.

The discrepancy regarding exacerbation of symptoms might be a matter of timing. In the present study, HRV infection was performed upon 7 weeks of HDM application and the inflammatory response was analysed directly (24 h) after the last combined HDM/HRV application. However, the impaired anti-viral response observed in chronic HDM mice might lead to an altered progression of the disease subsequent to the given time point of analysis. Interestingly, Contoli et al. [28] showed deficient IFN- λ production *in vitro* correlated with increased severity upon HRV infection *in vivo* performed 2 weeks later. As we demonstrated impaired IFN production in HDM-mice compared with healthy mice without exacerbation, it remains to be elucidated if this affects further progression of the disease, especially in terms of promoting asthma exacerbation.

The time course of viral induced immune responses is described to be not only limited to the initial initiation of the innate and adaptive response to the virus but furthermore is followed

by post-viral acute and chronic disease [47]. The development of post-viral disease, however, takes 3–7 weeks, thus much later than the analysis time point used in the present study which focused on the initiation of innate and adaptive response 3 days after first viral inoculation [47,48]. Thus future studies following up viral infection in asthma models for a longer time course are needed to evaluate if viral infection ultimately translates into asthma exacerbation. Furthermore, the use of other respiratory viruses such as respiratory syncytial virus and influenza might be of value for establishing exacerbation models of asthma.

In summary, the model established within the present study shows that the situation observed in the subpopulation of patients with severe chronic asthma and rhinovirus-induced exacerbation cannot directly be transferred to mouse models. Despite this, the current model provides a tool to exploit the interplay of severe chronic allergic airway inflammation and superimposed respiratory viral infection with regards to the altered anti-viral immune response, reflecting the human situation in this aspect.

CLINICAL PERSPECTIVES

- Severe asthma and viral-induced asthma exacerbations represent a high unmet medical need as no therapy is currently available for these patients. HRV (human rhinovirus) is prominently associated with asthma exacerbations in humans
- The aim of the present study was to establish a mouse model of asthma with additional rhinovirus infection to investigate the interaction of chronic allergic airway inflammation and acute respiratory viral infection.
- Although no exacerbation of the characteristic features of asthma was induced by superimposed HRV1B infection, an impaired anti-viral immune response was observed in chronically allergic compared with healthy mice, reflecting the situation described for humans. Thus this model provides a valuable tool to investigate the viral response in chronic asthma condition.

AUTHOR CONTRIBUTION

All experiments were conceived by Sabine Rochlitz, Meike Müller and Armin Braun. All experiments were conducted by Sabine Rochlitz. Heinz-Gerd Hoymann contributed by conceiving and analysing the invasive lung function measurements. Sabine Rochlitz wrote the paper. Meike Müller and Armin Braun critically reviewed the paper and provided substantial intellectual contribution.

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conducted on behalf of the U-BIOPRED Study Group with input from the U-BIOPRED Patient Input Platform, Ethics Board and Safety Management Board (<http://www.ubiopred.european-lung-foundation.org/18043-full-list-of-consortia-members.htm>).

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SUPPLEMENTARY ONLINE DATA

No exacerbation but impaired anti-viral mechanisms in a rhinovirus-chronic allergic asthma mouse model

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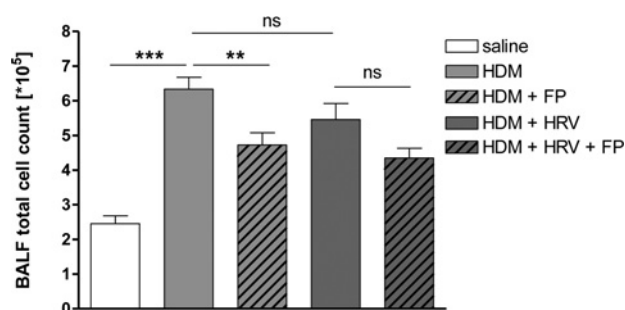


Figure S1 Effect of HRV infection during late phase chronic airway inflammation on BAL total cell count

BAL total cell count depicted for Balb/c mice treated with saline (white bar) or HDM (light grey bar) for 7 weeks. Additional HRV inoculation was performed on final 3 days of week 7 (dark grey bars). Steroid-treated animals received FP on the final 9 days (striped light and dark grey bars). $n = 16$ animals per group. Values are means $\pm$ S.E.M. $**P < 0.01$ and $***P < 0.001$ according to ANOVA and Bonferroni post-test. ns, not significant.

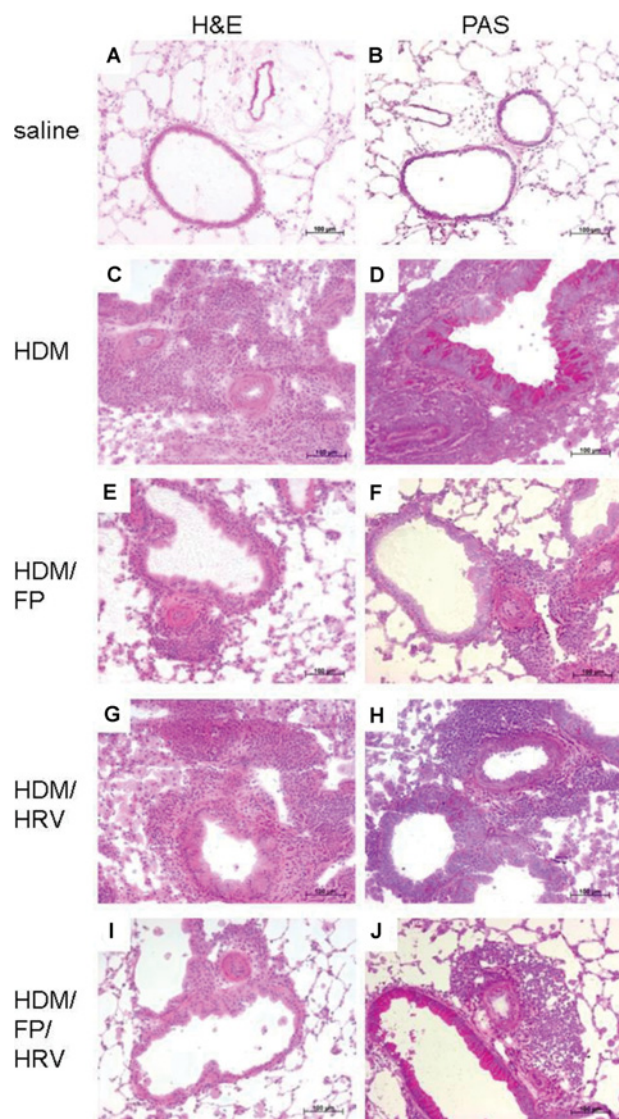


Figure S2 Effect of HRV infection during late phase chronic airway inflammation on lung inflammation

Representative photomicrographs of either H&E (haematoxylin and eosin)-stained (left-hand panel) or PAS (periodate–Schiff)-stained (right-hand panel) lung cryosections of mice after 7 weeks of saline (A, B) or HDM challenge (C, D) and additional steroid treatment with FP (E, F). Two groups received secondary HRV1B infection on top of chronic HDM challenge (G, H) and additional steroid treatment (I and J). Scale bar, 100 μm.

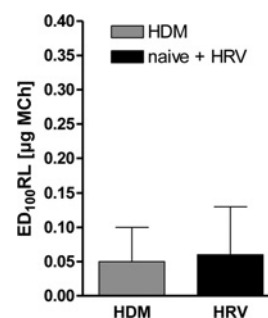


Figure S3 AHR induced by HRV in naive mice compared to AHR induced in chronic HDM asthma model

Balb/c mice treated with HDM 7 weeks (light grey bar) or age-matched untreated, naive mice (black bar) were infected in parallel with HRV1B using three consecutive i.n. inoculations of the same virus batch. Columns represent the effective inhalational dose of MCh in μg required to increase RL by 100% above baseline (ED₁₀₀RL) measured in anaesthetized, orotracheally intubated and spontaneously breathing mice. Values are medians + range for $n=16$ (HDM) or $n=3$ (naive + HRV) animals.

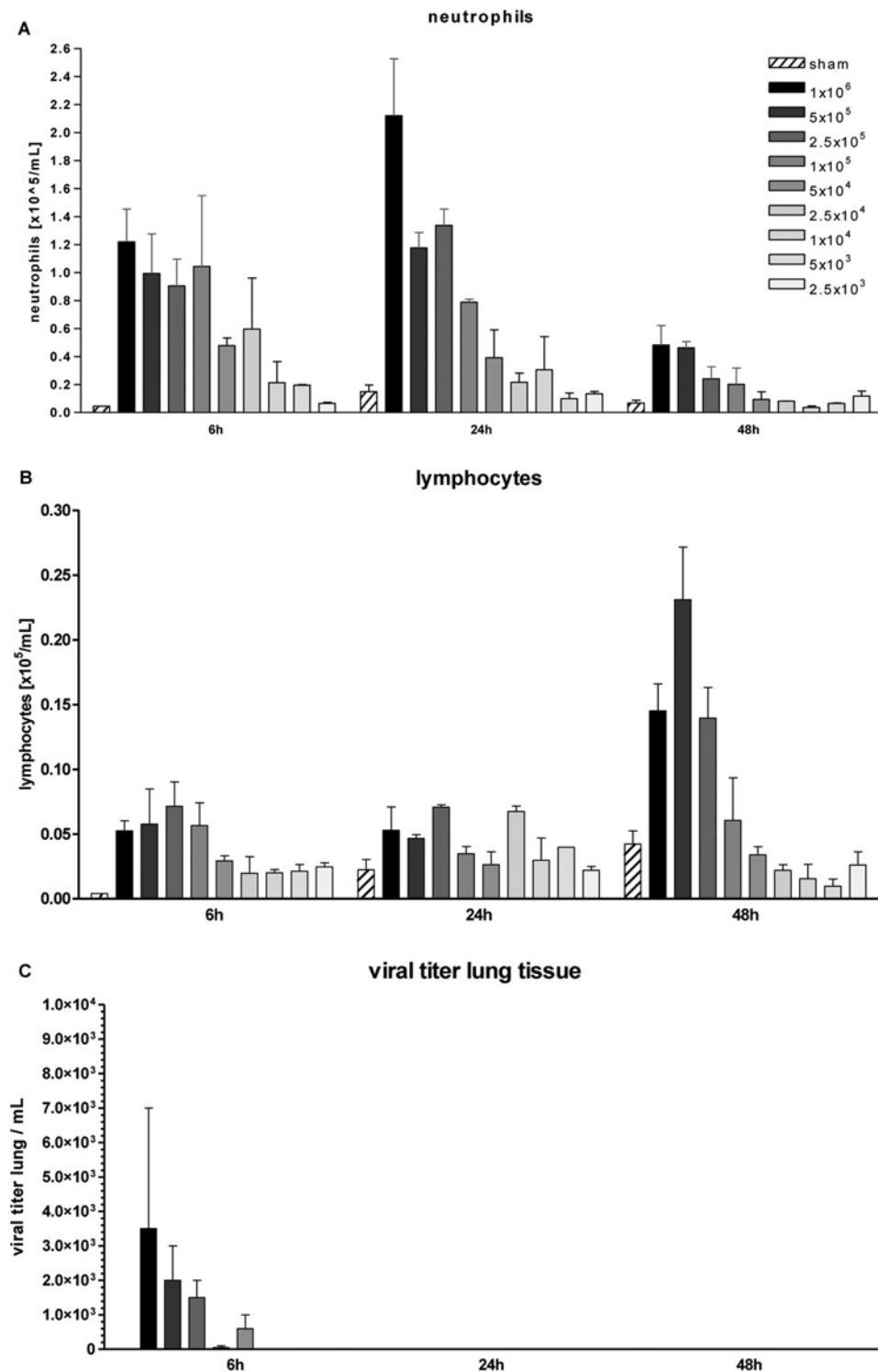


Figure S4 Kinetic analysis of HRV-induced immune response in naïve mice

Naïve Balb/c, 6–8 weeks, were either sham-infected (striped white bar) or infected with serial dilutions of HRV1B starting with 1×10^6 TCID₅₀/mouse (black bars) until 2.5×10^3 TCID₅₀/mouse (light grey bars). $n = 2$ animals for each inoculum and analysis time point. **(A)** Neutrophil cell count in BAL, **(B)** lymphocyte cell count in BAL, and **(C)** viral titre in lung as measured by TCID₅₀ assay using lung homogenisate supernatant plated on HeLa cells and subsequent CPE analysis.

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