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Title:

Increased type 2 inflammation post rhinovirus infection in patients with moderate asthma.

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Abstract:

Rhinovirus (RV) infections are a major cause of exacerbations in patients with asthma. Experimental RV challenges can provide insight into the pathophysiology of viral exacerbations. Previous reports, investigating mild or moderate asthma patients, have shown an upregulation in type 2 inflammation post RV infection, however, studies specifically involving asthma patients taking inhaled corticosteroids have concentrated on symptoms and lung function, rather than the inflammatory response.

Eleven moderate asthma patients were inoculated with RV. Cold symptoms and asthma control were assessed at baseline and post infection. Nasal epithelial lining fluid and bronchial alveolar lavage (BAL) fluid were collected at baseline and 4 days post infection for assessment of inflammatory proteins.

Patients suffered increased cold symptoms and decreased asthma control within 7 days of infection. Antiviral mechanisms were induced following inoculation, with increases in interferon α , β , γ and λ , as well as CXCL10 and CXCL11. Type 2 inflammatory cytokines were also significantly elevated post RV infection in both nasal and bronchial samples. In BAL, epithelial derived IL-25 and IL-33 levels strongly correlated with Th2 cytokines, IL-4, IL-5 and IL-13.

We show how experimental rhinovirus challenge regulates lung and nasal biomarkers in asthma patients taking inhaled corticosteroids. These biomarkers could be used to evaluate the effects of novel drugs for asthma.

Keywords:

Asthma; Rhinovirus; Type 2 inflammation; inhaled corticosteroid; IL-25; IL-33;

Abbreviations:

BAL: bronchoalveolar lavage; CXCL: chemokine (C-X-C motif) ligand; FEV₁: forced expiratory volume in one second; ICS: inhaled corticosteroids; IL: interleukin; ILC2: type 2 innate lymphoid cells; IFN: Interferon; PEF: peak expiratory flow rate; LABA: long acting beta agonist; NELF: nasal epithelial lining fluid; RV: rhinovirus; RV16: rhinovirus type 16; T2: type 2 inflammation; TNF: tumour necrosis factor; TSLP: thymic stromal lymphopoietin

1. Introduction

Viral exacerbations in asthma are associated with increased symptoms, airflow obstruction and airway inflammation (1). Host anti-viral immune defence mechanisms include the interferons (IFN- α , β , γ and λ , recently reviewed in (2)). The experimental rhinovirus (RV) infection model has been used to characterise host immune response to viral infection in asthma, showing upregulation of type 2 (T2) inflammatory responses (3). Epithelial derived cytokines including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) are secreted in response to viral infection and activate T2 responses in lymphocytes, including type 2 innate lymphoid cells (ILC2s) (4-7). Experimental RV infection increases IL-25 and IL-33 in nasal secretion (4, 5), however, results for bronchoalveolar lavage (BAL) have not been reported. Murine and in vitro models show that RV infection induces TSLP production (6, 8), but in vivo human evidence showing RV infection upregulates TSLP secretion is lacking.

In steroid naïve mild asthma patients, RV infections trigger a decrease in lung function (9), but no changes in lung function are observed in inhaled corticosteroid (ICS) using patients (10). RV infection studies comprising of mild or moderate asthma patients have reported both clinical and immune responses (3-5), however, studies involving only patients using ICS have concentrated on clinical and antiviral responses, rather than the inflammatory response (10). The aim of this study was to comprehensively characterise the host immune response to RV infection in moderate asthma patients taking ICS. We performed nasal and lung sampling to measure T2 immunity, epithelial derived cytokines including IL-25, IL-33 and TSLP, and anti-viral mechanisms.

2. Methods

2.1. Patients:

Sixty two moderate asthma patients were screened, of which 51 failed to meet the eligibility criteria (see supplementary material); the principle reasons for failing were a high RV16 antibody titre or an Asthma Control Questionnaire score <1.0 . Eleven patients (Global Initiative for Asthma treatment steps 2 to 4; see Table 1 for clinical characteristics) passed screening and were nasally inoculated with RV16 (1000 times the 50% tissue culture

infective dose; gift from Professor J. Gern, University of Wisconsin, USA). All patients were aged 18-65 years, had a history of worsening asthma symptoms following the development of an upper respiratory tract infection within the previous 5 years, an Asthma Control Questionnaire score of ≥ 1 at screening (11), and had a low serum RV16 antibody titre at screening (assessed by Hvido, London, UK). Patients were on a stable daily dose of inhaled corticosteroid of ≥ 200 mcg Beclomethasone or equivalent, with 7/11 patients prescribed long-acting beta agonists (LABA). All patients were never smokers. Atopy was assessed by skin prick test against cat dander, grass pollen and house dust mite extract (Soluprick SQ, Alk-Abello, UK); positive atopy was defined by development of a wheal ≥ 3 mm to any allergen. The study was approved by the local research ethics committee (NRES Committee North West – Preston; REC Ref: 14/NW/0121NRES). All subjects provided written informed consent.

2.2. Study design

The study timeline is summarised in Figure 1. Daily peak expiratory flow (PEF), forced expiratory volume in one second (FEV₁) and cold symptoms (12, 13) were recorded, and the Asthma Control Questionnaire was performed weekly (11); these measurements were performed on Day 1 before inoculation and for 2 weeks post inoculation. Questionnaires were completed in the morning and occurred prior to collection of any samples. BAL was collected before and 4 days after inoculation; baseline bronchoscopies were performed 6 weeks prior to RV inoculation to ensure carry-over of bronchoscopy-induced inflammation was minimal. Nasal epithelial lining fluid (NELF) samples were collected on Day 1 prior to inoculation and Day 5 post-inoculation. Nasal lavage was collected on Day 1 prior to inoculation, and on Days 2-7 and 14. RV16 levels were measured in nasal lavage and BAL to confirm infection. For full methods, see Supplementary Material.

A subject was defined as a clinical responder to RV if they met the following criteria: subject had a total Jackson symptom score of at least 6 over the 5 days following inoculation and either (1) reported rhinorrhoea for at least 3 days over the 5 days post inoculation, or (2) the subject believed that he or she has had or currently has a cold (based on the response to the cold question on Day 6). (13)

2.3. Cytokine Analysis

Cytokine levels in NELF and BAL were assessed by multiplex assays (Meso Scale Diagnostics, Maryland, USA); lower levels of quantification (LLOQ) are listed in Supplementary Table 1. Where cytokine levels were detected, but were below the LLOQ, samples were assigned the arbitrary value of 0.5 x the LLOQ to allow analysis.

2.4. Serum RV16 antibody titre assay

RV16 neutralisation assay was performed on serum samples to quantify the amount of RV16 neutralising antibodies present. This analysis was performed by Hvido, London, UK. Serially diluted sera were mixed with RV16 ($3.3 \log_{10}$ 50% Tissue culture Infective Dose/ml) for 30 minutes, before incubating with MRC-5 lung fibroblast-like cells (European Collection of Authenticated Cell Cultures, Salisbury, UK) for 5-7 days. Each sample dilution was tested in duplicate. Viral cytopathic effect (CPE) was assessed for each well by microscopy and compared to the virus on control wells; a cell stain was not used. Results with a $\geq 50\%$ reduction in CPE from the no sera, virus only controls were scored as antibody positive. A patient was defined as having a low antibody titre if their serum failed to neutralise $\geq 50\%$ RV16-induced killing of MRC-5 cells when diluted ≤ 8 -fold.

2.5. Virology confirmation

Viral loads in BAL and nasal lavage were quantified by RV16 target sequence copy number qPCR assay. This analysis was performed by Hvido, London, UK. RV RNA was extracted from samples using a Qiagen DSP Virus/Pathogen Midi kit on a QIASymphony SP module (Qiagen, Manchester, UK). The PCR reaction for each extracted RNA sample was loaded in triplicate by the QIASymphony AS module into Qiagen OneStep QuantiFAST mastermix containing a RV-16 Taqman hydrolysis probe and PCR primers targeting the RV-16 genome. The one-step RT-qPCR reaction is performed using a ViiA7 Dx (Applied Biosystems, Altrincham, UK). The quantity of viral RNA is determined by the comparison of cycle threshold (Ct) values to calibration standard curves. These curves are generated from *in vitro* generated nucleic acid copies of the target RV-16 gene sequence. No template and extraction negative controls are included in the assay. The lower level of quantification (LLOQ) for cycle threshold (ct) was 36.3. The LLOQ for viral load was 60.0 copies/ml of sample. The

LLOQ and lower level of determination for the assay was determined during assay validation. The assay was validated by Hivivo as per guidelines provided by the Food and Drug Administration (FDA) (14), the European Agency for the Evaluation of Medicinal Products (15) and the International Conference on Harmonisation (ICH) (16). Where viral load was detected, but was below the LLOQ, samples were assigned the arbitrary value of 0.5 x the LLOQ i.e. 30 copies/ml. Samples were defined as Non detectable, if their ct was above 36.3 and these were assigned the arbitrary value of 0.1 x the LLOQ i.e. 6 copies/ml. A patient was defined as not being infected if their viral load levels in both BAL (4 day post inoculation) and nasal lavage (peak level 1-13 days post inoculation) were <60 copies/ml.

2.6. Statistical Analysis

For lung function and questionnaires, comparisons between pre-inoculation and post-inoculations were by 1-way ANOVA with Dunnett post-test or Friedman test with Dunns post-test. Analysis of cytokine levels in baseline BAL or pre-inoculation NELF and levels 4 days post inoculation were by Wilcoxon match pairs test, with Spearman's rank analyses used to assess correlations between IL-25 and IL-33 with T2 cytokines. All analysis was carried out using Graphpad Prism version 7.04 (La Jolla, California, USA).

3. Results

3.1. Clinical Characteristics

Of the 11 patients inoculated, 3 patients were excluded from the analysis as they lacked quantifiable levels of RV16 in either nasal or BAL samples post-inoculation (Supplementary Table 2). All 8 patients with detectable levels of RV16, showed a positive clinical response to the virus (Figure 2A); these 8 patients comprised of 2 females and 6 males. Rhinovirus infection did not affect peak expiratory flow or lung function (Figure 2B and C). Cold symptoms increased within three days of infection, while asthma control decreased (Figure 2D-F); all returned to baseline levels by Day 14.

3.2. Inflammatory Responses

Of the 8 patients who were infected with RV, paired pre and post-RV BAL samples were collected from 7 patients. Antiviral mechanisms were significantly upregulated ($p < 0.05$); IFN γ , CXCL10 and CXCL11 increased in both NELF (mean fold changes 4.9, 16.8 and 150.4 respectively) and BAL (mean fold changes 2.0, 4.4 and 6.5 respectively) after infection. IFN α , β and λ were increased in at least one of these media (Table 2).

IL-4, IL-5, IL-13 and IL-25 were significantly increased ($p < 0.05$) in both NELF (mean fold changes: 1.6, 3.8, 1.7 and 13.7 respectively) and BAL (mean fold changes: 1.6, 2.3, 1.8 and 2.4 respectively) (Figure 3; Table 2). IL-33 levels showed a non-significant trend towards an increase in NELF ($p = 0.055$). BAL TSLP levels were undetectable both pre and post RV-infection, while TSLP was measurable in $< 40\%$ of NELF samples. No other cytokines measured in NELF and BAL showed significant changes after RV infection (Table 2).

BAL IL-25 and IL-33 concentrations after infection correlated with IL-4 (both $p = 0.012$), IL-5 ($p = 0.003$ and 0.024 , respectively) and IL-13 (both $p = 0.007$) (Figure 4A and B). IL-25 and IL-33 levels in NELF did not correlate with Th2 cytokines (Figure 4C-D).

Levels of nasal and BAL IL-4, IL-5, IL-13, IL-25 and IL-33 did not correlate with CSS or WURSS symptom scores taken on day 5 prior to sample collection (data not shown).

4. Discussion

RV infection in moderate asthma patients induced cold symptoms, reduced asthma control and increased both anti-viral and T2 inflammatory responses. It is known that RV infection increases nasal and bronchial IL-4, IL-5 and IL-13 levels in mild to moderate asthma patients, but not healthy subjects (3, 4). We provide evidence of comparable findings in a cohort comprising only moderate asthma patients taking ICS, indicating that ICS use does not completely inhibit RV-induced T2 inflammation. The upregulation of T2 inflammation was observed with only $n = 8$, suggesting potential for this challenge method to be used in early phase clinical trials of novel drugs using comparatively small numbers of ICS using patients.

RV infection did not alter lung function, which confirms results from a previous RV study involving only asthma patients using ICS (10). ICS (and/or LABA)-use may stabilise lung function during RV infection; FEV₁ and PEF decreases were reported in studies involving steroid naïve asthma patients (4, 9, 17).

Previous studies have reported increased IL-25 (5) and IL-33 (4) in nasal samples of asthma patients following RV infection, which is supported by our own findings. Hilvering et al commented that Jackson et al's comparison of a single pre-inoculation cytokine level with the peak level observed post infection was likely to obtain a positive result because "*an increase is to be expected as a result of regression to the peak*" (18). We believe that our results reflect RV-induced increases in nasal IL-25 and IL-33 as we compared single pre and post-infection samples, and our results match those of Jackson et al when their data was reanalysed comparing cytokine concentrations for individual days post-inoculation with baseline values (19). We also demonstrated increased BAL IL-25 levels. Bronchial IL-33 levels were not increased post infection, which matches the findings of Jackson et al. Bronchoscopy samples were collected 4 days post inoculation, and perhaps this was not the optimal time point for measuring IL-33 induction.

Increased IL-25 and IL-33 may drive T2 cytokine production post RV infection. Jackson et al showed that airway IL-33 levels correlate with IL-5 and IL-13 post infection (4). Our results expand on this by showing that both IL-33 and IL-25 correlate with BAL IL-4, IL-5 and IL-13 levels. The lack of correlation between IL-4 and IL-33 in the Jackson study may be due to a difference between BAL and bronchoabsorption samplings. Ex vivo studies have shown that RV infection induces IL-33 production in bronchial epithelial cells and that conditioned media from such cultures drives differentiation of naïve t-cells into IL-4, IL-5 and IL-13 producing Th2 cells (4). RV infection in mice increases airway epithelial production of IL-25, with blocking of IL-25 signalling reducing T2 cytokine production (5). The relationship between IL-25 and IL-33 with T2 cytokines may be strongest in the lower airways, as no correlations were apparent in NELF. Animal studies suggest TSLP is active in RV infections (6), but in this study BAL and NELF levels were too low to observe any changes in TSLP.

Jackson et al showed correlations between peak levels in nasal IL-5, IL-13 and IL-33 with total symptom severity score, which was calculated as the sum of the daily symptom scores. We found no correlations between infection symptoms, taken on the day of sample collection, with BAL or NELF IL-25, IL-33 or T2 cytokine levels; this may be due to peak symptoms occurring before the day of sample collection. Alternatively, the differences in correlation could be due to the Jackson study using a mixture of steroid naïve and moderate asthma patients or the smaller number of patients used in our study.

Collection of BAL in asthma patients can lead to a fever-like symptoms and a short term decline in lung function (20). To more accurately measure the viral specific response, some recent RV16 infection studies have subtracted symptom scores, collected following baseline bronchoscopies, from those reported after the post-inoculation bronchoscopy (4). We did not collect cold symptom and asthma control data post baseline bronchoscopy. However, we are confident that patients suffered an increase in cold symptoms as all 8 patients met the definition of clinical responder to RV when only pre-bronchoscopy data was considered (Jackson score based on just first 4 days post inoculation results; data not shown) and the peak score for the Wisconsin Upper Respiratory Symptom Survey predated the bronchoscopy. As FEV₁ levels did not alter post inoculation, the increases in ACQ scores were driven by raised symptom levels and the related increase use in short acting beta agonists; potentially, these may have been affected by the bronchoscopy, as well as the RV infection.

The study population had more males, which may have influenced the study results. While efforts were made for gender balance during recruitment, the high screen failure rates impeded this.

Three patients did not develop a detectable infection following RV inoculation; this is a similar proportion to other studies (4) highlighting the need to measure virus levels post-inoculation. Nasal samples appear to be good surrogates for bronchoscopy samples in RV

studies, as changes in proteins were similar in the two matrices. Nasal samples are also simpler to collect and allow repeat samplings.

Our study shows the effects of RV challenge on specific biomarkers in moderate asthma patients taking ICS. These biomarkers may be used in future RV challenge studies to evaluate the effects of novel drugs for asthma.

5. **Declarations:**

5.1. Ethics approval and consent to participate: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Favourable ethical opinion was obtained from Committee North West – Preston (14/NW/0121NRES). Participants were recruited from a research database of primary and secondary care patients from Northwest England (The Medicines Evaluation Unit, Manchester, UK). Written informed consent was obtained before any study specific assessments were performed

5.2. Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

5.3. Competing interests: DS has received sponsorship to attend international meetings, honoraria for lecturing or attending advisory boards and research grants from various pharmaceutical companies including Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi, Genentech, GlaxoSmithKline, Glenmark, Johnson and Johnson, Merck, NAPP, Novartis, Pfizer, Skypharma, Takeda, Teva, Therevance and Verona. SFM, RMS and VJE are employees of Novartis. TS, CP and NK are employees of the Medicines Evaluation Unit.

5.4. Funding: This work was supported by Novartis Pharma AG, Basel, Switzerland and The Medicines Evaluation Unit, Manchester, UK.

5.5. Authors' contributions: TS participated in the design of the study, performed the data analysis and drafted the manuscript. SFM devised and performed the protein analysis. VJE and DS conceived the study, CP, NK, NK, RMS, VJE and DS participated in the study's design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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6. References:

1. Lewis TC, Henderson TA, Carpenter AR, Ramirez IA, McHenry CL, Goldsmith AM, Ren X, Mentz GB, Mukherjee B, Robins TG, Joiner TA, Mohammad LS, Nguyen ER, Burns MA, Burke DT, Hershenon MB. Nasal cytokine responses to natural colds in asthmatic children. *Clin Exp Allergy* 2012; 42: 1734-1744.
2. Edwards MR, Strong K, Cameron A, Walton RP, Jackson DJ, Johnston SL. Viral infections in allergy and immunology: How allergic inflammation influences viral infections and illness. *J Allergy Clin Immunol* 2017; 140: 909-920.
3. Hansel TT, Tunstall T, Trujillo-Torralbo MB, Shamji B, Del-Rosario A, Dhariwal J, Kirk PDW, Stumpf MPH, Koopmann J, Telcian A, Aniscenko J, Gogsadze L, Bakhsoliani E, Stanciu L, Bartlett N, Edwards M, Walton R, Mallia P, Hunt TM, Hunt TL, Hunt DG, Westwick J, Edwards M, Kon OM, Jackson DJ, Johnston SL. A Comprehensive Evaluation of Nasal and Bronchial Cytokines and Chemokines Following Experimental Rhinovirus Infection in Allergic Asthma: Increased Interferons (IFN-gamma and IFN-lambda) and Type 2 Inflammation (IL-5 and IL-13). *EBioMedicine* 2017; 19: 128-138.
4. Jackson DJ, Makrinioti H, Rana BM, Shamji BW, Trujillo-Torralbo MB, Footitt J, Jerico D-R, Telcian AG, Nikonova A, Zhu J, Aniscenko J, Gogsadze L, Bakhsoliani E, Traub S, Dhariwal J, Porter J, Hunt D, Hunt T, Hunt T, Stanciu LA, Khaitov M, Bartlett NW, Edwards MR, Kon OM, Mallia P, Papadopoulos NG, Akdis CA, Westwick J, Edwards MJ, Cousins DJ, Walton RP, Johnston SL. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *Am J Respir Crit Care Med* 2014; 190: 1373-1382.
5. Beale J, Jayaraman A, Jackson DJ, Macintyre JDR, Edwards MR, Walton RP, Zhu J, Man Ching Y, Shamji B, Edwards M, Westwick J, Cousins DJ, Yi Hwang Y, McKenzie A, Johnston SL, Bartlett NW. Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation. *Sci Transl Med* 2014; 6: 256ra134.
6. Han M, Rajput C, Hong JY, Lei J, Hinde JL, Wu Q, Bentley JK, Hershenon MB. The Innate Cytokines IL-25, IL-33, and TSLP Cooperate in the Induction of Type 2 Innate Lymphoid Cell Expansion and Mucous Metaplasia in Rhinovirus-Infected Immature Mice. *J Immunol* 2017; 199: 1308-1318.
7. Hong JY, Bentley JK, Chung Y, Lei J, Steenrod JM, Chen Q, Sajjan US, Hershenon MB. Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells. *J Allergy Clin Immunol* 2014; 134: 429-439.
8. Mehta AK, Duan W, Doerner AM, Traves SL, Broide DH, Proud D, Zuraw BL, Croft M. Rhinovirus infection interferes with induction of tolerance to aeroantigens through OX40 ligand, thymic stromal lymphopoietin, and IL-33. *J Allergy Clin Immunol* 2016; 137: 278-288 e276.
9. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebabdz T, Contoli M, Sanderson G, Kon OM, Papi A, Jeffery PK, Stanciu LA, Johnston SL. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci U S A* 2008; 105: 13562-13567.
10. Adura PT, Reed E, Macintyre J, Del Rosario A, Roberts J, Pestridge R, Beegan R, Boxall CB, Xiao C, Kebabdz T, Aniscenko J, Cornelius V, Gern JE, Monk PD, Johnston SL, Djukanovic R. Experimental rhinovirus 16 infection in moderate asthmatics on inhaled corticosteroids. *Eur Respir J* 2014; 43: 1186-1189.
11. Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999; 14: 902-907.
12. Barrett B, Locken K, Maberry R, Schwamman J, Brown R, Bobula J, Stauffacher EA. The Wisconsin Upper Respiratory Symptom Survey (WURSS): a new research instrument for assessing the common cold. *J Fam Pract* 2002; 51: 265.

13. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. *AMA Arch Intern Med* 1958; 101: 267-278.
14. Guidance for Industry: Bioanalytical Method Validation: U.S. Department of Health and Human Services, Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM) May 2018 (<https://www.fda.gov/media/70858/download>).
15. European Agency for the Evaluation of Medicinal Products, 2011, Guideline on Validation of the Bioanalytical Methods. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf.
16. International Conference on Harmonisation (ICH) of technical requirements for technical registration of pharmaceuticals for human use; Guideline validation of analytical procedures: Text and methodology Q2(R1) Current Step 4 version. Parent Guideline dated 27 October 1994. Complementary Guideline on Methodology dated 6 November 1996 incorporated in November 2005. (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf).
17. Grunberg K, Timmers MC, de Klerk EP, Dick EC, Sterk PJ. Experimental rhinovirus 16 infection causes variable airway obstruction in subjects with atopic asthma. *Am J Respir Crit Care Med* 1999; 160: 1375-1380.
18. Hilvering B, Xue L, Pavord ID. IL-33-dependent Th2 response after rhinovirus infection in asthma: more information needed. *Am J Respir Crit Care Med* 2015; 191: 237.
19. Jackson DJ. Reply: IL-33-dependent type 2 inflammation in asthma exacerbations. *Am J Respir Crit Care Med* 2015; 191: 238.
20. Jarjour NN, Peters SP, Djukanovic R, Calhoun WJ. Investigative use of bronchoscopy in asthma. *Am J Respir Crit Care Med* 1998; 157: 692-697.

n	11
Male/Female	9/2
Age*	43.6 ± 13.2
Age of asthma diagnosis [#]	6 (1-58)
Atopic	9 of 11
FEV ₁ % predicted*	89% ± 15%
ACQ*	1.54 ± 0.91
Blood Eosinophil (x10 ⁹ /L)*	0.12 ± 0.06
SABA usage	11 of 11
LABA usage	7 of 11
ICS usage	11 of 11
Daily beclomethasone equivalent dosage (mcg)*	618 ± 260

Table 1: Patients' clinical characteristics at screening. Data presented as *mean ± standard deviation or [#] median (range). Abbreviations: FEV₁ %: Percent predicted forced expiratory volume in one second; ACQ: Asthma control questionnaire; SABA: short acting beta agonist; LABA: Long acting beta agonist; ICS: inhaled corticosteroid.

	NELF		BAL	
	mean fold-change	p	mean fold-change	p
IL-4	1.6 ± 1.0	0.0156*	1.6 ± 0.4	0.0313*
IL-5	3.8 ± 3.2	0.0156*	2.3 ± 1.1	0.0469*
IL-13	1.7 ± 0.9	0.0156*	1.8 ± 0.4	0.0156*
IL-25	13.7 ± 11.0	0.0234*	2.4 ± 1.2	0.0469*
IL-33	3.2 ± 2.0	0.0547	2.0 ± 1.9	0.4688
IFN α	6.2 ± 12.5	0.0156*	1.5 ± 1.3	0.4688
IFN β	13.0 ± 16.9	0.3125	1.7 ± 0.5	0.0469*
IFN γ	4.9 ± 3.4	0.0156*	2.0 ± 0.8	0.0469*
IFN λ	23.1 ± 39.1	0.0391*	9.1 ± 9.0	0.2188
CXCL10	16.8 ± 25.3	0.0156*	4.4 ± 3.6	0.0313*
CXCL11	150.4 ± 218.2	0.0078*	6.5 ± 5.6	0.0313*
IL-1 β	2.0 ± 2.3	0.4609	3.1 ± 3.7	0.5781
IL-2	2.4 ± 1.6	0.25	2.1 ± 2.7	0.375
CXCL8	2.2 ± 1.6	0.1484	11.7 ± 26.7	0.4688
IL-10	2.5 ± 1.8	0.218	2.4 ± 1.3	0.25
IL-12	3.4 ± 3.1	0.1094	1.5 ± 0.9	0.375
IL-17	25.1 ± 39.6	0.0781	5.8 ± 9.9	0.1094
IL-22	2.1 ± 1.4	0.375	2.2 ± 1.7	0.1563
TNF	2.0 ± 1.2	0.1953	8.7 ± 19.4	0.8125

Table 2: Effects of rhinovirus infection on nasal epithelial lining fluid (NELF) and bronchoalveolar lavage (BAL) cytokine levels. NELF samples were collected on Day 1 prior to inoculation and 5 days post-inoculation. BAL samples were collected 6 weeks before and 5 days after RV inoculation. Cytokine levels were measured by Meso Scale Discovery multiplex assays. Data is presented as mean fold-change ($\pm$ sd) in cytokine concentration post-infection. Comparisons between pre- and post-infection levels were by Wilcoxon matched pairs test: * $p < 0.05$. Abbreviations: CXCL: chemokine (C-X-C motif) ligand; IFN: Interferon; IL: Interleukin; TNF: tumour necrosis factor.

Figure 1: Study Timeline

Figure 2: Effects of rhinovirus infection on clinical characteristics in asthma patients (n=8). (A) Clinical response; (B) Peak flow; (C) Pulmonary function test; (D) Wisconsin upper respiratory symptom survey; (E) Cold symptom score and (F) asthma control questionnaire. For Jackson score (A) dotted line illustrates cut-off for positive response. Data are presented as mean $\pm$ sd (B, C and F) or median $\pm$ range (D and E). Comparisons between pre-inoculation Day 1 and postinoculations Days 2-14 were by 1-way ANOVA with Dunnett post-test (B, C and F) or Friedman test with Dunns post-test (D and E): * $p < 0.05$; ** $p < 0.01$.

Figure 3: Effects of rhinovirus infection on Type 2 inflammation in asthma patients (n=7). Levels of cytokines in BAL fluid from pre and post RV-inoculation bronchoscopies were measured by Meso Scale Discovery multiplex assay (A-E). Comparisons between levels were assessed by Wilcoxon match pairs test: *p<0.05.

Figure 4: Correlations between post inoculation levels of T2 cytokines and IL-25 and IL-33. BAL (n=7) (A-B) and NELF (n=8) (C-D) T2 cytokine levels (IL-4, 5 and 13) were compared to IL-25 (A and C) and IL-33 (B and D) Correlations were assessed by Spearman's rank analysis, with results presented to the right of each graph.

SUPPLEMENTARY MATERIAL

Study Methodology

This was an open-label, allocation, exploratory study to assess the effect of RV-challenge in patients with asthma. Favourable ethical opinion was obtained from Committee North West – Preston (14/NW/0121NRES). Participants were recruited from a research database of primary and secondary care patients from Northwest England (The Medicines Evaluation Unit, Manchester, UK). Written informed consent was obtained before any study specific assessments were performed.

1. Inclusion criteria

- Aged 18-65 years inclusive
- Clinician diagnosis for asthma
- Use of inhaled short acting beta-agonist (SABA)
- Use of stable daily dose of inhaled corticosteroid of ≥ 200 mcg Beclomethasone or equivalent.
- Pre-bronchodilator FEV1 $\geq 65\%$ prior to RV challenge
- Asthma Control Questionnaire score of ≥ 1 at screening
- Have experienced worsening of asthma symptoms following the development of an upper respiratory tract infection within the previous 5 years.
- Low serum antibody titre to RV-16.

2. Exclusion criteria

- Current or former smoker
- Any concurrent medical condition(s) that may interfere with the performance of spirometry or pose a potential hazard when performing spirometry.
- Contact with those whose health may be at risk if exposed to RV16, including but not limited to premature infants and the immunocompromised.
- Male and Female patients without adequate contraception or unwilling to use an effective form of contraception from Screening until 1 month after the RV challenge.

- History of serious adverse reaction, severe hypersensitivity or allergy to any drug or in any other circumstance (e.g. anaphylaxis).
- Vulnerable patients (e.g. persons kept in detention).
- Inability to understand the protocol requirements, instructions and study-related restrictions, the nature, scope, and possible consequences of the study.
- Significant illness (other than respiratory illness) within the 2 weeks prior to Day 1.
- History of drug or alcohol abuse within the 12 months prior Screening
- Current symptoms of allergic rhinitis at screening or predicted symptoms during the study period.
- Current treatment with systemic steroids or previous treatment in the 3 months prior to Screening.
- Have ever had an RV challenge at any time prior to Screening.
- Have received an investigational drug treatment within the preceding 30 days of screening.
- Respiratory tract infection or asthma exacerbation within 4 weeks of Screening or at any time prior to RV challenge on Day 1.
- Asthma exacerbation requiring treatment with systemic corticosteroids in the past 3 months prior to Screening.
- Hospitalisation (including emergency room visits) for the treatment of asthma within 3 months of screening or history of life-threatening asthma.

3. Study design

Following consent, patients underwent screening to assess for suitability for inclusion against the above criteria. Eligible subjects had a baseline bronchoscopy, post-screening, which was performed 6 weeks prior to the rhinovirus challenge (Day 1), followed by a second bronchoscopy 4 days post-inoculation. Patients visited the Medicines Evaluation Unit on Days 1-7, as well as Day14. Nasal lavage samples were collected pre-inoculation on Day 1 and for two weeks post-inoculation, while NELF was collected pre-inoculation Day 1 and Day 5 post-inoculation. Bronchoalveolar lavage (BAL) and nasal lavage were used to assess viral loads, with BAL and NELF samples used to assess protein levels.

Patients completed daily diary cards to record changes in their symptoms (see below), and completed Asthma Control Questionnaires pre-inoculation on Day 1, and on Days 7 and

14 post-innoculation. Peak flow measurements were performed, in the morning, by the patients at home pre-inoculation on Day 1 and post-inoculation of Days 2-14, and lung function measured pre-inoculation of Day 1 and on Days 2-7, as well as Day 14.

4. Questionnaires

a. Wisconsin Upper Respiratory Symptom Survey (WURSS)

The negative impact of upper respiratory infection was assessed using the WURSS-21 quality of life instrument (1). Patients were asked to complete the questionnaire at baseline and for 2 weeks post viral inoculation.

b. Cold Symptom Score

Cold symptoms were assessed at baseline and for 2 weeks post viral inoculation using the Jackson scoring method (2). The various symptoms, comprising of cough, rhinorrhea, sneezing, nasal obstruction, laryngitis, headache, malaise and chilliness, were graded from 0 to 3 (absent, mild, moderate, or severe symptoms). The sum of the individual symptom scores gave the patients daily total symptom severity score.

c. Asthma Control Questionnaire

Asthma characteristics were assessed using the Asthma Control Questionnaire (3) at screening, pre-inoculation, Day 7 and Day 14 post viral inoculation. Briefly, the ACQ consists of 7 questions (5 cardinal symptoms, FEV₁ % predicted and daily rescue bronchodilator use). Patients are asked to recall how their asthma has been during the previous week and to respond to the symptom and bronchodilator use questions. FEV₁% predicted is measured on the day of questionnaire. Each component is scored on a 7-point scale (0 to 6) and the ACQ score is calculated as the mean value, resulting in a score between 0 (totally controlled) and 6 (severely uncontrolled).

5. Lung function

a. Peak flow

Peak Expiratory Flow (PEF) was measured by the subjects at home daily using a Wright peak flow meter. The highest of three technically acceptable measurements was recorded at each time point in the diary provided.

b. Spirometry

Spirometry (FEV₁) was performed at specified time points throughout the study. The highest of at least three technically acceptable measurements were recorded at each time point. Patients were required to withhold their rescue medication (SABA) for at least 6 hours prior to lung function testing.

6. Virus inoculation

Infection with RV16 was performed on Day 1 of the infection period. Each subject was inoculated while lying in a recumbent position with a target dose of 1000 TCID₅₀ in a total administration volume of 1 mL.

All RV were prepared in a designated Biological Safety Cabinet by appropriately trained personnel. Stock RV virus was stored in a secure -80°C freezer with restricted access to designated personnel. RV16 was thawed in a 37°C water bath by gently swirling, aliquots were maintained on wet ice until inoculation. Using a sterile Pasteur pipette, 0.5 mL RV16 from the vial was administered into each nostril with approximately 2-5 minutes interval between each administration. Subject remained in the recumbent position for approximately 2-3 minutes following administration of RV16. They were instructed to wear a face mask for at least 30 minutes post inoculation and to refrain from rubbing or blowing their nose for at least 30 min.

A subject was defined as a clinical responder to RV16 if they met the following criteria (2):

The subject has a total Jackson symptom score of at least 6 over the 5 days following inoculation AND

1) The subject has reported rhinorrhoea for at least 3 days over the 5 days following inoculation

OR

2) The subject believes that he or she has had or currently has a cold (based on the response to the cold question on Day 6).

7. Nasal lavage

Nasal lavage involved instillation of an iso-osmotic salt solution into the nasal cavity.

A syringe was pre-filled with a 5ml sterile iso-osmotic solution (0.9% sodium chloride at room temperature). A nasal adaptor/olive of an appropriate size for the patient's nostril was selected which was then attached to the syringe to make a close fitting (tight seal) and prevent leakage of lavage fluid. The subject sat over a desk facing the physician with head forward-flexed (approximately 60° from the upright position) to prevent fluid from reaching the nasopharynx.

The pre-filled syringe with an adaptor was inserted into nasal cavity forming a tight seal with an adaptor. The iso-osmotic solution was then introduced into the nasal cavity; the fluid was flushed and aspirated repeatedly a maximum of 20 times over a 1-minute period. The recovered lavage was centrifuged and supernatants frozen for viral load analysis.

8. Nasoabsorption

Nasal secretion sampling is used as a harmless method for studying nasal airway inflammation by sampling the NELF from the nose which has a strong functional and immunological relationship with the bronchi. Nasal secretion sampling was performed by introducing the Synthetic absorptive material (SAM) into the nasal cavity.

With the patient sitting comfortably upright, and the head extended backwards, a nasosorption SAM strip was placed on the lateral wall of the nasal cavity, onto the lateral/inferior surface of the inferior turbinate using blunt-ended forceps. Light pressure with the subject's finger was used in order to prevent the strips from slipping out of the nasal cavity. SAM was left to absorb nasal secretions for 2 min. The recovered SAM was placed in a cryotube and then centrifuged. The collected sample was analysed for inflammatory mediators.

9. Bronchoscopy

Bronchoscopy was performed after the subjects had been sedated. Bronchoalveolar lavage (BAL) was collected from the right bronchus. The bronchoscope was wedged in the bronchus and a maximum of 4 × 60 ml aliquots of pre-warmed sterile 0.9 % saline solution were instilled. The aspirated fluid was stored on ice before filtration (100 µm filter, Becton Dickenson, Oxford, UK). The filtrate was centrifuged (400 g/10 min at 4°C) and the BAL fluid was stored at -80°C in 1ml aliquots for biomarker and viral load analysis.

10. Protein analysis

Baseline and post-inoculation BAL and nasoabsorption samples were assessed for anti-viral and Type 2 inflammatory cytokines by multiplex assay (Meso Scale Discovery, Rockville, Maryland, USA)

Supplementary References

1. Barrett B, Locken K, Maberry R, Schwamman J, Brown R, Bobula J, Stauffacher EA. The Wisconsin Upper Respiratory Symptom Survey (WURSS): a new research instrument for assessing the common cold. *J Fam Pract* 2002; 51: 265.
2. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. *AMA Arch Intern Med* 1958; 101: 267-278.
3. Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999; 14: 902-907.

Supplementary Table 1: Lower level of quantification (LLOQ) for Meso Scale Discovery multiplex assay

Cytokine	LLOQ (pg/ml)
CXCL11	0.431
IFN- α 2A	46
IFN- β	114.3
IFN- γ	20
IL-13	2.24
IL-25	10.574
IL-33	11.89
IL-4	2.24
IL-5	4.687
CXCL10	10.334
IL-10	0.932
IL-12p70	1.36
IL-17A	4.49
IL-1 β	0.95
IL-2	0.48
IL-22	0.83
CXCL8	0.56
TNF	92
TSLP	2.47
IFN- λ /IL-29	2.88

Abbreviations: CXCL: chemokine (C-X-C motif) ligand;; IFN-: interferon; IL-: interleukin; LLOQ: lower level of quantification; TNF: tumour necrosis factor; TSLP: Thymic stromal lymphopoietin.

Supplementary Table 2: BAL and nasal lavage viral loads

Patient	Viral Load (copies/ml sample)				Evidence of Infection (Yes/No)
	Baseline BAL	Post inoculation BAL	Pre-inoculation Nasal lavage	Highest post-inoculation Nasal lavage	
1	6.0	5740.2	6.0	15312.9	Yes
2	6.0	6.0	6.0	6.0	No
3	6.0	17528.0	6.0	4292.2	Yes
4	6.0	30.0	6.0	514.5	Yes
5	6.0	9037.8	6.0	24068.1	Yes
6	6.0	6634.8	6.0	23240.9	Yes
7	6.0	6.0	6.0	1754.3	Yes
8	6.0	6.0	6.0	12263.8	Yes
9	6.0	6.0	6.0	6.0	No
10	No Sample		6.0	26101.1	Yes
11	6.0	6.0	6.0	30.0	No

Abbreviations: BAL: bronchoalveolar lavage