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Rhinovirus induced CCL17 and CCL22 in asthma exacerbations and differential regulation by STAT6

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SLJ, MRE and NWB agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

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Abstract

Rationale: The interplay of type-2 inflammation and anti-viral immunity underpins asthma exacerbation pathogenesis. Virus infection induces type-2 inflammation-promoting chemokines CCL17 and CCL22 in asthma, however mechanisms regulating induction are poorly understood.

Objectives: To use a human rhinovirus (RV) challenge model in human airway epithelial cells *in vitro* and mice *in vivo* to assess mechanisms regulating CCL17 and CCL22 expression.

Methods: Subjects with mild-to-moderate asthma and healthy volunteers were experimentally infected with RV and airway CCL17 and CCL22 protein quantified. *In vitro* airway epithelial cell- and mouse-RV infection models were then employed to define STAT6- and NF-κB-mediated regulation of CCL17 and CCL22 expression.

Main Results: Following RV infection, CCL17 and CCL22 expression was higher in asthma, which differentially correlated with clinical and immunological parameters. Air-liquid interface (ALI) differentiated primary epithelial cells from donors with asthma also expressed higher levels of RV-induced CCL22. RV infection boosted type-2 cytokine-induced STAT6 activation. In epithelial cells, type-2 cytokines and STAT6 activation had differential effects on chemokine expression: increasing CCL17 and suppressing CCL22, whereas NF-κB promoted expression of both chemokines. In mice, RV infection activated pulmonary STAT6 which was required for CCL17, but not CCL22 expression. STAT6-knockout mice infected with RV expressed increased levels of NF-kB-regulated chemokines, which was associated with rapid viral clearance.

Conclusions: RV-induced upregulation of CCL17 and CCL22 was mediated by NF-κB activation, whereas expression was differentially regulated by STAT6. Together, findings suggest

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therapeutic targeting of type-2-STAT6 activation alone will not block all inflammatory pathways during RV infection in asthma.

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Introduction

Rhinoviruses (RV) are the most frequent virus infections associated with asthma exacerbations(1). Preventing or treating RV-induced asthma exacerbations remains a challenge, as there are currently no effective treatments for RV infection. An improved understanding of the immune response to RV infection is necessary to aid development of new therapies.

The role of type-2 immune responses in the pathogenesis of asthma is well documented(2) and RV infection augments type-2 inflammation(3-5). For example, in experimental RV infections of subjects with atopic asthma, exacerbation severity correlated with T_H2 cell cytokine production and viral load(3). In type-2 inflammatory airways diseases (e.g. allergic asthma), recruitment of CCR4-expressing immune cells to the airways is regulated by production of chemokines, including CCL17(6) and CCL22(7). The relevance of these CCR4 ligands to disease is supported by their increased levels in serum and sputum from patients with asthma(8).

CCR4 is expressed by type-2 inflammatory cells (including T_H2 cells(9) and innate lymphoid cells (ILC2s)(10)), which produce IL-4, IL-5 and IL-13 and underpin the pathogenesis of allergic asthma(11). Genetic ablation of either CCR4 ligand (CCL17 or CCL22) reduced lung inflammation, decreased T_H2 infiltration and reduced airway hyper-reactivity in mouse models of allergic airways disease(6, 12). Monoclonal antibodies targeting CCR4 (e.g. mogamulizumab) have been approved in Japan for the treatment of adult T-cell leukaemia-lymphoma(13). While phase I trials have also assessed CCR4-targeting monoclonal treatment in asthma, trials were discontinued and have not been further pursued for asthma or viral-induced exacerbations.

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Promoter analyses of CCL17 and CCL22 have identified binding sites for NF-κB and STAT transcription factors, consistent with regulation by multiple inflammatory pathways(14-16). Co-stimulation of human lung epithelial cells with the STAT6-activating cytokine IL-4, and with TNFα (which activates NF-κB), synergistically induced CCL17 production(17). This highlighted the potential for RV infection-induced NF-κB activation(18) to augment CCL17 in asthma, modulating airway inflammation and disease exacerbation.

There has been long-standing interest in CCL17 and CCL22 as treatment targets for type-2 inflammatory diseases, including asthma. Despite this, there is a limited understanding of whether pulmonary CCL17 and CCL22 production is regulated by STAT6 and/or NF-κB in the context of RV infection and asthma. Further, there is evidence to suggest that the regulation of CCL17 and CCL22 by STAT6 is context-dependent(19). We previously reported that RV infection augments CCL17 and CCL22 expression in a mouse model of allergic airways disease(18) and recently reported both chemokines are induced by RV infection in humans, and further increased following infection in people with asthma(20).

In the current study, we investigate relationships between human CCL17 and CCL22 protein expression in the context of allergic inflammation *in vivo* following experimental RV infection of healthy volunteers and patients with atopic asthma. We also employed mouse RV infection models to assess functional roles of STAT6 and NF-κB using transgenic- and pharmacological-interventions. As airway epithelial cells are the primary site of RV infection, we also utilised *in vitro* human airway epithelial cell RV infection models to assess transcriptional regulation of CCL17 and CCL22. This is the first study to identify correlations between CCL17 and CCL22 airway protein expression and disease parameters associated with RV infection in asthma. We

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also define the role of key transcription factors (NF-κB and STAT6) in RV- and type-2 cytokine-induction of these chemokines *in vivo* and by human airway epithelial cells *in vitro*.

Materials & Methods

Human participants

We studied non-smoking subjects with mild to moderately severe asthma and healthy volunteers aged 18-55 years (confirmed serum RV-A16-neutralising antibody negative), as previously reported(5). The study received Research Ethics Committee approval (09/H0712/59) and subjects provided informed consent(5). Inclusion/exclusion criteria for subjects with asthma included doctor diagnosis of asthma, airway hyper-responsiveness, evidence of atopy (by skin prick test) and a history of infection-induced asthma exacerbation(5). Subjects with a history of severe asthma, >5 pack-year history or recent smoking, current symptoms of allergic rhinitis, or who experienced an asthma exacerbation or respiratory virus illness within the previous 6 weeks were excluded. Non-smoking, non-asthmatic healthy controls were recruited based on: no history of asthma, atopy or allergic disease and no objective airway hyper-responsiveness. For patient demographics and additional information see reference(5).

Human rhinovirus challenge

Participants underwent bronchoscopy approximately 14 days prior to inoculation with RV-A16(21) and on day 4 post-infection. On day 0, RV-A16 was delivered to both nostrils (100 $TCID_{50}$) using an atomiser, as previously described(5). Protein mediators in nasal secretions were assessed by nasosorption (days 0, 2-5 and 7)(5). On day 4 post-infection,

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bronchosorption was conducted as previously described(20). Diary cards of upper and lower respiratory tract symptoms were commenced prior to baseline sampling and continued for 6 weeks after RV-A16 infection, as previously described(3, 5). CCL17 and CCL22 proteins in nasal and bronchial secretions were quantified by Meso Scale Discovery multi-spot human cytokine assay, as described previously(20).

Mouse rhinovirus infection

Female BALB/c mice, 6-8 weeks, were purchased from Harlan UK. *Tnfrsf1a*$^{-/-}$-*Rela(p65)*$^{+/-}$ mice (129/Sv - C57BL/6 background) were bred to generate TNFR1-deficient mice of *p65*$^{+/+}$ (wildtype) or *p65*$^{+/-}$ (heterozygous)(22). Genotype was determined by PCR. As TNFR1/p65-deficient mice are susceptible to bacterial infections, antibiotics were included in drinking water(23). STAT6 knockout (ko) mice (*Stat6*$^{tm1/Gru}$ – Balb/c background) were purchased from Jackson Laboratory (Bar Harbor, USA).

RV-A1 (American Tissue Culture Collection) was purified and titrated, as described previously(24). Where indicated, UV-inactivated RV-A1 (1,200 mJ/cm^2; 30 min) was used. Mice were infected by intranasal (i.n.) instillation RV-A1 (5 x 10^6 $TCID_{50}$), as previously described(24, 25). Infectious RV titre in homogenised lung tissue was determined in HeLa H1 cells via TCID50 assay as previously described (24). To inhibit Iκβ-kinase-β (IKK-β) mice PS-1145 (40mg/kg in 10% DMSO) was administered by intraperitoneal injection (Millenium Pharmaceuticals(26)). At indicated times after infection, broncho-alveolar lavage (BAL) was performed and CCL17, CCL22, IFN-λ2/3, CXCL1, and CXCL2 protein levels quantified by ELISA, according to manufacturer instructions (R&D Systems). CCL17 and CCL22 expression in airway epithelium was determined by immunohistochemistry staining of tissue samples.

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STAT6 protein quantification in mouse lung

Lung tissue cell cytoplasmic and nuclear protein extracts were prepared using CelLytic NuCLEAR extraction kit (Sigma), according to manufacturer's instructions. Cytoplasmic fraction proteins were resolved by denaturing SDS-PAGE and immunoblotted using antibodies against STAT6-phosphor Y641 (Abcam). EMSA was performed, as previously described(27). Lung tissue nuclear protein extracts were incubated with labelled STAT6-binding DNA probe and binding specificity demonstrated using 100x excess (20 pmoL) unlabelled DNA. DNA-protein complexes were resolved on native acrylamide gel and visualised by autoradiography.

CCL22 and CCL17 protein immunostaining in mouse lung tissue

Single immunohistochemical staining was performed as previously described (28), using goat anti-mouse CCL22 (sc-12288; Santa Cruz Biotechnology) and goat anti-mouse CCL17, respectively (AF529; R&D Systems). Staining was scored by a single, blinded investigator (PC) using microscopy. CCL22 and CCL17 epithelial staining was scored as: 0 = no staining; 1-4 based on 1-24%, 25-49%, 50-74% or 75-100%(28, 29).

Human cell culture

A549 lung epithelial cells (ECACC) were cultured in DMEM containing L-glutamine, HEPES, sodium bicarbonate and 10% FCS. Cells were pre-treated with IL-4 (10ng/mL) 16 h before infection with RV-A1 (multiplicity of infection (MOI) = 20). Knock-down of NF-κB p65, IKK-β and STAT6 was achieved using ON-TARGET plus SMART pool siRNA (4 siRNAs per target; Dharmacon/ThermoFisher) and Lipofectamine 2000 transfection reagent (Invitrogen), as previously described(30). Reduction of targeted protein expression was confirmed by western blot(30).

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BEAS-2B airway epithelial cells (ECACC) were cultured in RPMI 1640 medium with 10% FCS. Transient transfection of BEAS-2B cells with a STAT6-responsive-luciferase reporter plasmid c/EBP-N4-luc (19, 31) was performed, as described previously(18). Briefly, cells were transfected with c/EBP-N4-luc and pRL Renilla luciferase control reporter vector (Promega) in Superfect (Qiagen) transfection reagent, based on manufacturer recommendations. At 24 hours post-transfection, cells were treated with IL-4 (10 ng/mL) and / or infected with RV-A1 (MOI = 1). At 24 h post-treatment/infection luciferase activity in cell lysates were measured using a dual luciferase protocol (Promega).

Bronchial epithelial cells (BECs) from healthy and asthmatic donors were cultured at air-liquid interface (ALI), as previously described(32). Briefly, BECs were cultured on placental collagen-coated flasks until confluent, then seeded onto transwell inserts at passage 2 in 1:1 mix of Dulbecco's modified Eagle medium and BEBM plus supplements (Lonza). Apical media was removed once cells were confluent and cultures differentiated for 24 days, where evidence of mucus and cilia was present. At day 24 post-ALI, basal media was replaced with minimal BEBM media supplemented with human recombinant IL-4 and IL-13 (10ngmL; Peprotech). On day 25, cells were infected with RV-A1 (MOI 0.1) (33) and harvested 4 days post-infection.

Human CCL22 and CCL17 protein in epithelial cell cultures was quantified using DuoSet ELISAs, according to manufacturer instructions (R&D Systems). For STAT6, JAK1, and JAK2 protein expression and phosphorylation analysis, BEC protein lysates were diluted with Laemmli loading buffer, denatured, and run on a TGX 4-12% stain-free gel (Biorad). Membranes were subsequently transferred onto nitrocellulose, blocked in bovine serum albumin/skimmed milk and stained overnight for pSTAT6 (CST), STAT6 (Santa Cruz), JAK1 (CST), JAK2 (CST) or B-actin

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(Abcam). Blots were then washed in TBS-T and stained with anti-rabbit and anti-mouse secondary antibodies (Abcam), imaged using a Chemidoc imager (BioRad).

Quantitative PCR (qPCR)

CCL17, CCL22, SOCS1, and SOCS3 mRNA levels were measured by qPCR, normalised to 18S reference gene levels (primer sequences in Table 1). RNA was extracted using miRNeasy mini kit (Qiagen) and quantified using nanodrop spectrophotometer. cDNA was generated using Taqman reverse transcription reagents and quantitative qPCR performed on an ABI7500 with Quantstudio software (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using GraphPad Prism v8.2.1 software. Wilcoxon matched pairs test was used to assess effect of treatment within a group and Mann-Whitney test to compare between different groups. For comparisons between multiple groups of non-normally distributed data Friedman test with Dunn's multiple comparison was used. Log-transformed viral load data was compared by two-way ANOVA with Sidak's multiple comparisons test. Transformed data was fitted to a straight line in a QQ plot confirming normal distributions for comparison. For time-course protein data, comparisons were performed by two-way ANOVA with Sidak's multiple comparisons test. *P* values <0.05 were considered statistically significant.

Results

RV induction of CCL17 and CCL22 in asthma correlated with markers of disease

Time-course nasal expression of CCL17 and CCL22 from a human RV challenge study in subjects with mild to moderate asthma and healthy volunteers has previously been reported(20). In the current study we reanalysed this data by plotting baseline (day 0) nasal

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CCL17 and CCL22 protein expression and peak expression during the infection to determine correlations with immunological and clinical outcomes, as previously described(5). At baseline, CCL17 protein was higher in nasosorption samples from asthmatic subjects compared to healthy controls. Peak CCL17 was also higher in asthma during RV infection. For CCL22, baseline levels were non-significantly (p=0.072) higher in asthma vs healthy and peak levels were significantly higher in subjects with asthma compared to healthy volunteers during RV infection (**Fig.1A**). Peak nasal CCL17 and CCL22 concentrations during infection were positively correlated with each other in both healthy subjects and subjects with asthma (**Fig.1B**). We previously reported that RV infection induced type-2 cytokines (IL-4, IL-5 and IL-13) and that IL-5 and IL-13 levels during infection in asthma positively correlated with respiratory symptom severity(5). In the current study, IL-4, IL-5 and IL-13 were positively correlated with peak nasal CCL17 (but not CCL22) production during RV infection (**Fig.1C**). Further, CCL17 and CCL22 levels during RV infection correlated with peak upper respiratory symptom scores (**Fig.1D**) and at day 4 post-infection bronchial CCL22 protein levels correlated with peak lower respiratory symptom scores in subjects with asthma (**Fig.1E**). These data show that RV-induced CCL17 and CCL22 levels are differentially associated with clinical and immunological asthma exacerbation parameters and CCL17 (but not CCL22) positively correlated with type-2 cytokine expression.

RV infection in bronchial epithelial cells from donors with asthma induces higher levels of CCL22 compared to CCL17

Airway epithelial cells are a source of CCL17 and CCL22(17, 34) and the primary site of RV infection(35). We next quantified CCL17 and CCL22 expression in RV-infected ALI-cultured BECs from donors with asthma (donor characteristics in Table 1). Infection with either major

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group RV-A43 or minor group RV-A1 did not significantly increase CCL17 mRNA expression (**Fig.2A**). However, RV infection did increase CCL22 mRNA expression. RV-A1 infection rapidly induced CCL22 expression which peaked at day 2 post-infection, whereas induction by RV-A43 infection peaked at day 4 (**Fig.2B**). We noted peak RV-induced CCL22 mRNA levels were approximately 10-fold higher than CCL17. CCL17 protein in apical supernatants were below the limit of detection, whereas CCL22 protein was detectable and significantly increased by RV-A1 infection (with a non-significant (*P*=0.088) increase after RV-A43 infection) at day 4 post-infection (**Fig.2C**). Based on this time-course data, we selected baseline and day 4 post-infection as relevant timepoints to compare RV-induced CCL22 protein expression in ALI-cultured BECs from a separate cohort of donors with asthma (AS) and non-asthmatic, healthy control subjects (HC; n=7; donor characteristics in Table 2). To increase sample size, we combined the CCL22 protein data generated with the previous day 4 CCL22 protein data from asthma (AS) BECs, providing n=14 asthma donors infected with RV-A1 (**Fig.2D**)**.** Infection of BECs from donors with asthma significantly increased CCL2 expression, which was higher (p=0.055) than RV-induced CCL22 in BECs from healthy control donors (**Fig.2D**). We noted variability in RV-induced CCL22 levels between BEC cultures from asthmatic donors, but note that variability did not correlate with atopic status determined by skin prick test positivity (**Supplementary Figure 2**). These data are consistent with observed RV induction of CCL22 *in vivo* and indicate that airway epithelial cells from patients with asthma are primed to express higher levels of CCL22, compared to cells from non-asthmatic, healthy control subjects.

RV infection augments type 2 cytokine-induced STAT6 activation

We next characterised transcriptional regulation of CCL17 and CCL22 during RV infection. Previous studies identified NF-κB and STAT6 binding sites in CCL17 and CCL22 promoters(14-

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16). Further, STAT6 binding can have differential effects on target gene expression depending on the cellular context(19). Whether (and how) these transcription factors regulate expression of these chemokines in human airway epithelial cells during RV infection is unknown. We previously reported RV-induced epithelial cell NF-κB activation during RV infection(18). Here we investigated STAT6 activation following RV-A1 infection, in the presence of cytokine (IL-4/IL-13) induced STAT6 phosphorylation in ALI-differentiated BEC cultures from healthy donors (n=6). Type 2 cytokine treatment alone induced a near-significant (p=0.067) increase in pSTAT6 levels, which was further increased with RV-A1 infection (**Fig.3A**).

To gain insight into potential mechanisms underlying increased STAT6 phosphorylation in ALI cultures following RV infection and stimulation with type-2 cytokines, we quantified expression of two Janus kinases (JAK1and JAK2) that are activated by viral infection and have been shown to phosphorylate STAT6 (36, 37). IL-4/13 treatment significantly reduced constitutive expression of JAK1, whereas RV infection alone did not significantly deplete JAK1 expression (**Fig. 3A**). RV infection reduced the suppressive effect of type-2 cytokines such that IL-4/13 treatment did not significantly reduce the expression of JAK1 compared to RV infection alone or compared to untreated cells (**Fig. 3A**). For JAK2, RV infection increased expression above levels in type-2 cytokine-treated cells (**Fig. 3A**). Again, RV infection partially reversed the suppressive effect of type 2 cytokine treatment such that the combination did not significantly reduce JAK2 compared to RV infection alone (**Fig. 3A**). The data suggests that RV infection partially restores type-2 cytokine-suppressed JAK1 expression, which could contribute to increased STAT6 phosphorylation observed following RV infection of type-2 cytokine-treated cells.

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In addition to assessing expression of RV-induced kinases that phosphorylate STAT6 we also quantified negative regulators of STAT activation, namely SOCS1 and SOCS3(38). RV infection alone as well in combination with type 2 cytokine treatment significantly increased SOCS1 and SOCS3 mRNA expression compared to untreated cells (**Fig. 3B**). IL-4/IL-13 treatment with RV infection also increased SOCS3 expression compared to type-2 cytokine-treated BECs (**Fig. 3B**). This data identified RV infection as the primary driver of SOCS3 expression. However, this induction of SOCS3 was not sufficient to block RV-augmented type-2 cytokine-induced STAT6 phosphorylation, as shown in **Fig 3A**.

To determine if observed STAT6 phosphorylation led to increased transcriptional activity we developed an *in vitro* assay system by treating BEAS-2B cells with IL-4 and/or infecting with RV. We initially confirmed expression of CCL17 and CCL22 protein by BEAS-2B cells. As in primary cells, RV infection alone did not induce detectable levels of CCL17 protein. However, combined IL-4 and RV treatment/infection significantly increased CCL17 protein (**Sup Fig.1A**). RV infection alone increased CCL22 protein, while pre-treatment with IL-4 reduced RV induction of CCL22 protein levels (**Sup. Fig.1B**).

Having established the utility of this BEAS-2B cell assay system to investigate the interaction between RV infection and type 2 cytokine treatment on CCL17 and CCL22 expression we also used this system to examine STAT6 promoter activation following transfection of a STAT6-driven luciferase reporter plasmid. Compared to untreated cells, promoter activity was increased 2-3-fold following RV infection (**Fig.3C**). IL-4 treatment significantly induced STAT6 promoter activation, compared to RV infection alone (approximately 7-fold), and combined IL-4 and RV exposure additively increased activation (>10 fold) (**Fig.3C**). These data provide

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evidence that RV infection alone is sufficient for low level STAT6 activation in airway epithelial cells, and RV infection augments type-2 cytokine-induced-STAT6 transcriptional activation.

STAT6 and NF-κB activation have differential effects on RV- and type-2 cytokine-induced CCL17 and CCL22 expression

Experiments in BEAS2B cells indicated that type-2 cytokine/STAT6 differentially regulate CCL17 and CCL22. To further investigate transcriptional regulation of CCL17 and CCL22 expression, we assessed the effects of administering small interfering RNAs (siRNAs) targeting NF-κB (p65 or IKK-β) and STAT6 in A549 cells treated with IL-4 and/or infected with RV. We confirmed that siRNA administration reduced protein expression of the respective targets (**Supplementary Fig.3**). As observed previously, highest CCL17 levels were induced by a combination of IL-4 and RV-A1 exposure (**Fig.4A**). SiRNAs targeting p65, IKK-β or STAT6 all reduced IL-4/RV-A1-induced CCL17 production, compared to control siRNA treatment (**Fig.4A**). This was not the case for CCL22 expression, where the highest level of CCL22 expression was observed following RV-A1 infection alone, and significantly reduced by IL-4 treatment (**Fig.4B**). SiRNA targeting p65 or IKK-β reduced CCL22 expression in RV-infected cells. In contrast, CCL22 expression increased following STAT6 knockdown in IL-4-treated cultures infected with RV (**Fig.4B**). Inhibition of STAT6 expression also increased RV (alone) induced CCL22 expression providing further evidence of direct STAT6 activation by RV (**Fig. 4B**). These data indicate that unlike CCL17, type-2 cytokine-induced STAT6 activation negatively regulates CCL22 expression in RV-infected airway epithelial cells. We confirmed this observation in a more physiologically relevant system, observing that type-2 cytokine treatment (IL-4 and IL-13) suppressed RV-induced CCL22 protein expression in ALI-differentiated BEC cultures derived from healthy donors (**Fig.4C**).

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Differential kinetics of expression of RV-induced CCL17 and CCL22 in vivo

We also investigated pulmonary expression of CCL17 and CCL22 *in vivo* in a mouse model of RV infection(24) and observed s CCL17 and CCL22 protein expression in airway epithelium (**Fig.5A**). RV infection induced strong CCL17 staining exclusively in the bronchial epithelium (**Fig.5A**), with negligible staining in lung tissue from mock-infected or UV-inactivated RV inoculated mice**.** Constitutive CCL22 expression was apparent in lung tissue following mock infection or inoculation with UV-inactivated RV-A1. CCL22 was more broadly expressed in the lung than CCL17, with expression also detected in endothelial cells (**Supplementary Fig.4**). CCL17 and CCL22 were also significantly elevated in BAL samples during RV-A1 infection, with CCL17 levels peaking at 1-day post-infection (p.i.) and CCL22 peaking 2 days p.i., with both chemokines significantly elevated through 4 days p.i. (**Fig.5B**).

RV-induced CCL17 and CCL22 expression are differentially regulated by STAT6 *in vivo*

We next determined when STAT6 phosphorylation occurred in the lung during RV infection. We previously reported that RV infection induced NF-κB activation(18), so in the current study we focused on STAT6. Increased levels of cytoplasmic phosphorylated-STAT6 (pSTAT6) in lung tissue were observed 8 hours post RV-A1 infection (**Fig.6A**). Increased STAT6-DNA binding was observed in the nuclear fraction of lung protein samples from RV-infected mice (**Fig.6B**). This finding was confirmed in samples from four additional mice at 8 hours p.i. and binding specificity was confirmed using excess (100X) unlabelled probe (**Fig.6B**).

To determine the functional role of STAT6 activation on CCL17 and CCL22 production, we utilized a RV-A1 time course to identify 1-day p.i. for assessment in STAT6ko mice. At 1-day p.i., lower levels of CCL17 were detected in BAL samples from STAT6ko mice, compared to wild-type (WT) controls (**Fig.6C**). In contrast, but in agreement with our *in vitro* human data,

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STAT6 deficiency had no effect on CCL22 levels, indicating that STAT6 is not required to drive CCL22 expression in the context of RV infection (**Fig.6C**). We also examined the role of NF-κB *in vivo,* using pharmacological IKK-β inhibition (**Fig.6D**) and genetically deficient $p65^{+/-}$ mice (**Fig.6E**). Administration of the IKK-β inhibitor (PS1145) significantly reduced both CCL17 and CCL22 BAL protein levels following RV-A1 infection, compared with vehicle-treated controls (**Fig.6D**). Similarly, $p65^{+/-}$ mice had reduced CCL17 BAL protein levels after RV-A1 infection, compared to homozygous wild-type $p65^{+/+}$ mice, following RV infection (**Fig.6E**). In $p65^{+/-}$ mice a trend (*P*=0.08) towards reduced levels of CCL22 was also observed (**Fig.6E**). These data confirm that unlike CCL17, STAT6 is not required for RV-induced CCL22 expression. In contrast, NF-κB was required for expression of both chemokines following infection.

STAT6 expression inhibits viral clearance and expression of NF-κB-regulated chemokines

To determine whether perturbation of STAT6 altered the RV-induced lung inflammatory milieu, we performed an RV-infection time course analysis in wild-type (WT) and STAT6ko mice. At 8 hours post-infection viral load was not different between WT and STAT6ko mice. In contrast, by 24 hours post-infection lung viral load was approximately 10-fold lower in STAT6ko mice compared to WT controls (**Fig. 7A**). RV infection induces airway neutrophilic inflammation and we noted a consistent trend for increased neutrophils in BAL samples isolated from STAT6ko mice (**Fig. 7B**). Further, this was associated with significantly increased expression of the NF-κB-regulated neutrophil-recruiting chemokines CXCL1 and CXCL2 in BAL fluid from STAT6ko mice. In contrast, IFN-λ levels were not increased in STAT6ko mice compared to WT mice (**Fig. 7C)**.

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Discussion

This study interrogated the regulation of RV-induced CCL17 and CCL22 as a framework to better understand the interplay between two key inflammatory pathways (i.e. STAT6 and NF-κB), which collectively underpin the pathogenesis of asthma exacerbations. To do this we utilized a human RV experimental infection model, *in vitro* airway epithelial cell- and mouse RV infection models to show that blocking STAT6 activation alone (particularly in the context of viral infection and NF-κB activation), will not completely inhibit inflammatory cell trafficking via chemokines such as CCL22 (where STAT6 activation may restrict expression).

We have previously reported time-course of CCL17 and CCL22 protein expression in nasal and bronchial secretions in a human RV experimental infection model, in healthy volunteers and subjects with asthma(20). We reanalysed this data to identify the peak expression level for each subject so that it could be correlated to type-2 cytokine production. This approach has been used previously to define clinical and immunological correlates of expression of other mucosal-epithelial expressed inflammatory cytokines such as IL-33(5). Peak expression of CCL17 (and not CCL22) correlated with IL-4, IL-5 and IL-13 expression in the nasal mucosa. This indicated that type 2 inflammation in combination with viral infection increased CCL17 expression. This conclusion is supported by in vitro experiments in human airway epithelial cells. In A549 cells the combination of IL-4 treatment and RV infection was required for detectable expression of CCL17 protein. In primary human BECs differentiated at ALI, adding multiple type 2 cytokines (IL-4 and IL-13) with RV infection did not induce detectable CCL17 protein expression suggesting that signals in addition to direct epithelial cell STAT6 activation, potentially mediated by immune cells, are needed for maximal expression by mucosal tissues. Immune cell augmentation of CCL17 production has been documented in other systems. For

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example, CCL17-expessing dendritic cells have been identified in intestinal mucosa and drive inflammation in a mouse colitis model(39).

Limited research has assessed the expression and regulation of CCL17 and CCL22 during respiratory virus infection in the context of asthma exacerbations (40-42). In a mouse model of RV infection, Nagarkar et al. assessed the lung inflammatory transcriptome and reported CCL17 as one of the most highly induced genes (>14 fold). Further, CCL22 gene expression was significantly increased (>3 fold)(43). Here we extended these results to report increased chemokine protein expression following RV infection in both human and mouse RV infection models. Our human *in vitro* BEC data demonstrated that RV subtypes binding different receptors: major group (ICAM-1 receptor) and minor group (low density lipoprotein receptor) induced CCL22 expression, evidence that CCL22 expression is a general response to RV infection. While we noted 2-3 fold higher secretion of CCL22 and CCL17 within our *in vitro* and *in vivo* systems compared to the human experimental model, we attribute this as likely due to the higher RV infection doses and direct infection of the lower respiratory tract for mouse infection model.

To look more broadly at RV infection and activation of NF-κB and STAT6 signalling pathways, we transfected siRNA into an epithelial cell line. Whilst both NF-κB- and STAT6 appear to be involved in regulating CCL17 production by alveolar epithelial cells, STAT6 negatively regulated CCL22 expression. This was further confirmed in $STAT6^{-/-}p65^{+/-}$ mice and following IKK-β inhibitor PS1145 treatment, where CCL17 production was abrogated in the absence of NF-κB or STAT6, while CCL22 secretion was unaffected by altering STAT6. STAT6 has previously been reported to directly inhibit p65 translocation to the nucleus by inhibiting IKBα phosphorylation in HeLa cells(44). Differential and direct interaction between NF-κB and STAT6 have previously

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been reported, with the two transcription factors synergistically enhancing luciferase expression by a synthetic inducible IL-4 reporter gene construct(45). Other studies have reported that IL-4-induced STAT6 activation reduced NF-κB DNA binding and IL-6 expression in certain cell types(46, 47). Ohmori and colleagues reported that STAT6 trans-activating domain competed for the transcriptional coactivator CBP, and overexpression of CBP relieved the suppressive effect of STAT6 on TNF-α-induced NF-κB activation(26). Future studies will investigate STAT6 transactivation and interference with the activity of CBP co-activator on CCL17 versus CCL22 expression in the context of RV infection and type-2 cytokine stimulation in the lung.

STAT6 has been shown to either activate or repress type-2 cytokine-induced gene expression in different contexts. Georas and colleagues reported that IL-4 stimulation highly activated a synthetic multimerized STAT6-response element contained within the c/EBP-N4-luc reporter(19). However, IL-4-induced STAT6 activation negatively regulated native IL-4 promoter-driven IL-4 expression leading the authors to conclude that the transcriptional function of STAT6 is dependent on promoter-context(19). We previously reported that in a mouse model of RV-induced exacerbation, RV infection increased allergen (OVA)-induced expression of both CCL17 and CCL22(18). Increased type-2 cytokine expression is a feature of this model(24), so our data is consistent with STAT6 promoting both CCL17 and CCL22 expression. This further highlights the context-dependent transcriptional activity of STAT6 in the interplay between type-2 allergic inflammation and RV infection. It is noteworthy that CCL17 (and not CCL22) protein expression significantly correlated with type-2 cytokine (IL-4, IL-5 and IL-13) expression in the nasal mucosa. This is consistent with type-2 cytokine-STAT6 activation preferentially driving CCL17 expression in the context of respiratory virus infection and acute exposure to type-2 cytokines. This raises the question of the net effect on CCR4-

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binding chemokine expression if STAT6 selectively suppresses expression of CCL22, while promoting expression of CCL17. We also need to consider cell-specific expression profile – as BEC expression of CCL22 was higher than CCL17 and tissue expression *in vivo* (mouse lung) was also different. This highlights that the system is complex and that direct counter-regulation by STAT6 may proportionally affect expression of CCL17 vs CCL22 such that the net effect on CCR4 mediated immune cell recruitment is zero depending on the specific circumstances.

In BEC cultures, CCL17 expression was synergistically increased by type-2 cytokines and RV infection, as has previously been noted following respiratory syncytial virus infection(41). However, the current study found that addition of IL-4 repressed RV-induced CCL22 and that siRNA-mediated knockdown of STAT6 relieved IL-4-induced suppression. Upon investigating upstream mediators of STAT6 signalling we found that type-2 cytokines negatively regulated RV-induction of both JAK1 and JAK2. A previous study in Schwann cells found that inhibition of JAK2/STAT pathways downregulated CCL22 gene expression(48), which compliments the reduction in CCL22 secretion we observed when type-2 cytokines suppress JAK2 protein expression. Despite the upregulation in SOCS1 and SOCS3 gene expression that we observed following RV infection, RV infection still promoted JAK2 expression.

In summary, we identify differential transcriptional regulation and relationships with asthma disease parameters of RV-induced CCL17 and CCL22. Our data show that RV-challenged individuals with asthma had increased bronchial CCL22 associated with asthma symptoms, whilst peak CCL17 in the nose was associated with type-2 cytokine expression and upper respiratory tract symptoms. We demonstrated distinct STAT6-mediated transcriptional regulation of CCL17 and CCL22 during RV infection, while both chemokines were regulated by

For Review Only

NF-κB. Thus, our findings provide new insight into the context-dependent expression of these often interchangeable CCR4 ligands. Our findings suggest that clinical trials targeting CCR4 (or downstream pathways) will be useful in asthma exacerbations, particularly those triggered by virus infection given the interplay of STAT6 and NF-κB in this clinical disease setting. In support of this, CCL17 and CCL22 were detectable in the nasal mucosa and RV-induced expression was higher in asthma compared to viral induction in healthy volunteers. Furthermore, bronchial CCL22 protein levels at day 4 post experimental RV infection positively correlated with severity of lower respiratory tract symptoms in asthma. Further studies with selective inhibitors of CCR4/CCL17/CCL22 are needed to support therapeutically targeting this pathway in virus induced asthma exacerbations.

For Review Only

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For Review Only

References

1. Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Josephs LK, Cunningham A, Robinson BS, Myint SH, Ward ME, Tyrrell DA, Holgate ST. The relationship between upper respiratory infections and hospital admissions for asthma: a time-trend analysis. *Am J Respir Crit Care Med* 1996; 154: 654-660.
2. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol* 2015; 15: 57-65.
3. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebadze 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.
4. Beale J, Jayaraman A, Jackson DJ, Macintyre JD, Edwards MR, Walton RP, Zhu J, Ching YM, Shamji B, Edwards M, Westwick J, Cousins DJ, Hwang YY, 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.
5. 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.
6. Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, Imai T, Yoshie O, Homma I, Yamamoto K, Matsushima K. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* 2001; 166: 2055-2062.
7. Pilette C, Francis JN, Till SJ, Durham SR. CCR4 ligands are up-regulated in the airways of atopic asthmatics after segmental allergen challenge. *Eur Respir J* 2004; 23: 876-884.
8. Sekiya T, Yamada H, Yamaguchi M, Yamamoto K, Ishii A, Yoshie O, Sano Y, Morita A, Matsushima K, Hirai K. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy* 2002; 57: 173-177.
9. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998; 187: 129-134.
10. Weston CA, Rana BMJ, Cousins DJ. Differential expression of functional chemokine receptors on human blood and lung group 2 innate lymphoid cells. *J Allergy Clin Immunol* 2019; 143: 410-413 e419.
11. Lambrecht BN, Hammad H, Fahy JV. The Cytokines of Asthma. *Immunity* 2019; 50: 975-991.
12. Gonzalo JA, Pan Y, Lloyd CM, Jia GQ, Yu G, Dussault B, Powers CA, Proudfoot AE, Coyle AJ, Gearing D, Gutierrez-Ramos JC. Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J Immunol* 1999; 163: 403-411.
13. Subramaniam JM, Whiteside G, McKeage K, Croxtall JC. Mogamulizumab: first global approval. *Drugs* 2012; 72: 1293-1298.

For Review Only

14. Nakayama T, Hieshima K, Nagakubo D, Sato E, Nakayama M, Kawa K, Yoshie O. Selective induction of Th2-attracting chemokines CCL17 and CCL22 in human B cells by latent membrane protein 1 of Epstein-Barr virus. *J Virol* 2004; 78: 1665-1674.
15. Liddiard K, Welch JS, Lozach J, Heinz S, Glass CK, Greaves DR. Interleukin-4 induction of the CC chemokine TARC (CCL17) in murine macrophages is mediated by multiple STAT6 sites in the TARC gene promoter. *BMC Mol Biol* 2006; 7: 45.
16. Wirnsberger G, Hebenstreit D, Posselt G, Horejs-Hoeck J, Duschl A. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *Eur J Immunol* 2006; 36: 1882-1891.
17. Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamaguchi M, Fujisawa T, Pawankar R, Sano Y, Ohta K, Ishii A, Morita Y, Yamamoto K, Matsushima K, Yoshie O, Hirai K. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* 2000; 165: 2205-2213.
18. Bartlett NW, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P, Clarke DL, Message SD, Aniscenko J, Kebadze T, Zhu J, Mallia P, Mizgerd JP, Belvisi M, Papi A, Kotenko SV, Johnston SL, Edwards MR. Defining critical roles for NF-kappaB p65 and type I interferon in innate immunity to rhinovirus. *EMBO Mol Med* 2012; 4: 1244-1260.
19. Georas SN, Cumberland JE, Burke TF, Chen R, Schindler U, Casolaro V. Stat6 inhibits human interleukin-4 promoter activity in T cells. *Blood* 1998; 92: 4529-4538.
20. 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.
21. Bardin PG, Sanderson G, Robinson BS, Holgate ST, Tyrrell DA. Experimental rhinovirus infection in volunteers. *Eur Respir J* 1996; 9: 2250-2255.
22. Alcamo E, Mizgerd JP, Horwitz BH, Bronson R, Beg AA, Scott M, Doerschuk CM, Hynes RO, Baltimore D. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. *J Immunol* 2001; 167: 1592-1600.
23. Quinton LJ, Jones MR, Simms BT, Kogan MS, Robson BE, Skerrett SJ, Mizgerd JP. Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia. *J Immunol* 2007; 178: 1896-1903.
24. Bartlett NW, Walton RP, Edwards MR, Aniscenko J, Caramori G, Zhu J, Glanville N, Choy KJ, Jourdan P, Burnet J, Tuthill TJ, Pedrick MS, Hurle MJ, Plumpton C, Sharp NA, Bussell JN, Swallow DM, Schwarze J, Guy B, Almond JW, Jeffery PK, Lloyd CM, Papi A, Killington RA, Rowlands DJ, Blair ED, Clarke NJ, Johnston SL. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. *Nat Med* 2008; 14: 199-204.
25. Bartlett NW, Singanayagam A, Johnston SL. Mouse models of rhinovirus infection and airways disease. *Methods Mol Biol* 2015; 1221: 181-188.
26. Ohmori Y, Hamilton TA. Interleukin-4/STAT6 represses STAT1 and NF-kappa B-dependent transcription through distinct mechanisms. *J Biol Chem* 2000; 275: 38095-38103.

For Review Only

27. Birrell MA, Hardaker E, Wong S, McCluskie K, Catley M, De Alba J, Newton R, Haj-Yahia S, Pun KT, Watts CJ, Shaw RJ, Savage TJ, Belvisi MG. Ikappa-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *Am J Respir Crit Care Med* 2005; 172: 962-971.
28. Varani K, Caramori G, Vincenzi F, Adcock I, Casolari P, Leung E, Maclennan S, Gessi S, Morello S, Barnes PJ, Ito K, Chung KF, Cavallesco G, Azzena G, Papi A, Borea PA. Alteration of adenosine receptors in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006; 173: 398-406.
29. Jayaraman A, Jackson DJ, Message SD, Pearson RM, Aniscenko J, Caramori G, Mallia P, Papi A, Shamji B, Edwards M, Westwick J, Hansel T, Stanciu LA, Johnston SL, Bartlett NW. IL-15 complexes induce NK- and T-cell responses independent of type I IFN signaling during rhinovirus infection. *Mucosal Immunol* 2014; 7: 1151-1164.
30. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, Sykes A, Dahdaleh S, Clarke DL, Belvisi MG, Kon OM, Fujita T, Jeffery PK, Johnston SL, Edwards MR. Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. *PLoS Pathog* 2010; 6: e1001178.
31. Mikita T, Campbell D, Wu P, Williamson K, Schindler U. Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. *Mol Cell Biol* 1996; 16: 5811-5820.
32. Hackett TL, Shaheen F, Johnson A, Wadsworth S, Pechkovsky DV, Jacoby DB, Kicic A, Stick SM, Knight DA. Characterization of side population cells from human airway epithelium. *Stem Cells* 2008; 26: 2576-2585.
33. Singanayagam A, Glanville N, Girkin JL, Ching YM, Marcellini A, Porter JD, Toussaint M, Walton RP, Finney LJ, Aniscenko J, Zhu J, Trujillo-Torralbo MB, Calderazzo MA, Grainge C, Loo SL, Veerati PC, Pathinayake PS, Nichol KS, Reid AT, James PL, Solari R, Wark PAB, Knight DA, Moffatt MF, Cookson WO, Edwards MR, Mallia P, Bartlett NW, Johnston SL. Corticosteroid suppression of antiviral immunity increases bacterial loads and mucus production in COPD exacerbations. *Nat Commun* 2018; 9: 2229.
34. Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, Arrigoni G, Fabbri LM, Sinigaglia F. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001; 107: 1357-1364.
35. Reid AT, Sutanto, E. N., Chander-Veerati, P., Looi, K., Fung Li, N., Iosifidis, T., Loo, S-L., Garratt, L. W., Kicic, A. Ground zero-the airway epithelium. In: Bartlett NW, Wark, P. A. B., Knight, D., editor. Rhinovirus Infections: Rethinking impact on human health and disease, 1 ed. London: Academic Press; 2019. p. 314.
36. Cho W, Jeoung DI, Kim YM, Choe J. STAT6 and JAK1 are essential for IL-4-mediated suppression of prostaglandin production in human follicular dendritic cells: opposing roles of phosphorylated and unphosphorylated STAT6. *Int Immunopharmacol* 2012; 12: 635-642.
37. Palmer-Crocker RL, Hughes CC, Pober JS. IL-4 and IL-13 activate the JAK2 tyrosine kinase and Stat6 in cultured human vascular endothelial cells through a common pathway that does not involve the gamma c chain. *J Clin Invest* 1996; 98: 604-609.
38. Gielen V, Sykes A, Zhu J, Chan B, Macintyre J, Regamey N, Kieninger E, Gupta A, Shoemark A, Bossley C, Davies J, Saglani S, Walker P, Nicholson SE, Dalpke AH, Kon OM, Bush A, Johnston SL, Edwards MR. Increased nuclear suppressor of cytokine

For Peer Review Only

signaling 1 in asthmatic bronchial epithelium suppresses rhinovirus induction of innate interferons. *J Allergy Clin Immunol* 2015; 136: 177-188 e111.
39. Heiseke AF, Faul AC, Lehr HA, Forster I, Schmid RM, Krug AB, Reindl W. CCL17 promotes intestinal inflammation in mice and counteracts regulatory T cell-mediated protection from colitis. *Gastroenterology* 2012; 142: 335-345.
40. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995; 310: 1225-1229.
41. Monick MM, Powers LS, Hassan I, Groskreutz D, Yarovinsky TO, Barrett CW, Castilow EM, Tifrea D, Varga SM, Hunninghake GW. Respiratory syncytial virus synergizes with Th2 cytokines to induce optimal levels of TARC/CCL17. *J Immunol* 2007; 179: 1648-1658.
42. Zhang Y, Luxon BA, Casola A, Garofalo RP, Jamaluddin M, Brasier AR. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. *J Virol* 2001; 75: 9044-9058.
43. Nagarkar DR, Wang Q, Shim J, Zhao Y, Tsai WC, Lukacs NW, Sajjan U, Hershenson MB. CXCR2 is required for neutrophilic airway inflammation and hyperresponsiveness in a mouse model of human rhinovirus infection. *J Immunol* 2009; 183: 6698-6707.
44. Nelson G, Wilde GJ, Spiller DG, Kennedy SM, Ray DW, Sullivan E, Unitt JF, White MR. NF-kappaB signalling is inhibited by glucocorticoid receptor and STAT6 via distinct mechanisms. *J Cell Sci* 2003; 116: 2495-2503.
45. Shen CH, Stavnezer J. Interaction of stat6 and NF-kappaB: direct association and synergistic activation of interleukin-4-induced transcription. *Mol Cell Biol* 1998; 18: 3395-3404.
46. Donnelly RP, Crofford LJ, Freeman SL, Buras J, Remmers E, Wilder RL, Fenton MJ. Tissue-specific regulation of IL-6 production by IL-4. Differential effects of IL-4 on nuclear factor-kappa B activity in monocytes and fibroblasts. *J Immunol* 1993; 151: 5603-5612.
47. Takeshita S, Gage JR, Kishimoto T, Vredevoe DL, Martinez-Maza O. Differential regulation of IL-6 gene transcription and expression by IL-4 and IL-10 in human monocytic cell lines. *J Immunol* 1996; 156: 2591-2598.
48. Fletcher JS, Springer MG, Choi K, Jousma E, Rizvi TA, Dombi E, Kim MO, Wu J, Ratner N. STAT3 inhibition reduces macrophage number and tumor growth in neurofibroma. *Oncogene* 2019; 38: 2876-2884.

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Tables

Table 1: Clinical characteristics of time course donors

	Asthma
Number, n	6
Age, years (SD)	58.3 (15.3)
Gender (M/F)	(1/5)
Atopy (SPT positive)	(1/6)
Severity	Mild-Moderate (5)
	Severe (1)
FEV1, % predicted (SD)	82.5 (11.9)
FVC, % predicted (SD)	89 (8.3)
Daily ICS dose, beclomethasone equivalent, ug (SD)	218.3 (37.6)

ICS: Inhaled corticosteroid

FVC: Forced vital capacity

FEV1: Forced expiratory volume in one second

Table 2: Clinical characteristics of healthy and donors with asthma

	Healthy	**Asthma**
Number, n	8	8
Age, years (SD)	61.5 (13.6)	61.5 (8.8)
Gender (M/F)	(1/7)	(3/5)
Atopy (SPT positive)	(1/8)	(5/8)**
Severity	NA	Moderate (1)
	NA	Severe (7)
FEV1, % predicted (SD)	89.1 (12.4)	70.6 (21.5)*
FVC, % predicted (SD)	97.5 (15.9)	87.7 (12.6)
Daily ICS dose, beclomethasone equivalent, ug (SD)	NA	287 (95.7)***

***** significantly different compared with healthy controls

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Supplemental Table 1: Primer sequences

Gene	Forward	Reverse	Probe
18S	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG	FAM-ACCGGCGCAAGACGGACCAGA-TAMRA
huCCL17	GGATGCCATCGTTTTTGTAACTG	TTAACTGCATTCTTCACTCTCTTGTTG	FAM-AGGGCCATCTGTTCGGACCCCA-TAMRA
huCCL22	CTACAGACTGCACTCCTGGTTGTC	GGGCCTGCCTCAGTTGCT	FAM-TCGTCCTCCTTGCTGTGGCGCT-TAMRA

All sequences presented in 5’ to 3’ orientation.

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Figure legends

Figure 1. Increased in vivo rhinovirus induced CCL17 and CCL22 in asthma is correlated with type 2 inflammation and disease severity. Individuals with asthma and healthy volunteers were infected with RV-A16 and nasal samples were obtained. A Protein levels of peak CCL17 and CCL22 in the nose. Median +/- IQR, *p<0.05, **p<0.01, Healthy vs Asthma at baseline or peak of infection analysed by Mann-Whitney U test. B Correlation of peak nasal CCL17 and CCL22 levels. C Correlations of peak nasal CCL17 protein with peak nasal type-2 cytokine proteins (IL-4, IL-5 and IL-13). D Correlation of peak nasal CCL17 and CCL22 proteins with upper respiratory symptom scores. E Correlation of bronchial CCL22 protein with lower respiratory symptom scores.

Figure 2. CCL22 induction by rhinovirus and increased expression by bronchial epithelial cells from donors with asthma. ALI-differentiated bronchial epithelial cells from donors with asthma were infected with either RV-A1 or RV-A43. Time course expression of A CCL17- and B CCL22-mRNA and C CCL22 protein. n = 6 donors for each time-point. Median +/- IQR, analyzed by two way ANOVA with Dunnett's multiple comparison test. **** P < 0.001 RV-A43 vs mock, # P < 0.05 RV-A1 vs mock. D CCL22 protein expression at Baseline and four days after RV-A1 infection in separate cohort of healthy control (HC) and asthmatic (AS) bronchial epithelial cells. n=7 HC donors, n=14 AS donors, median +/- IQR analyzed by Mann-Whitney U test (HC vs AS) or Wilcoxon matched pairs test (baseline vs RV-A1) * P < 0.05.

Figure 3. Rhinovirus infection increased type 2 cytokine-induced STAT6 activation. Differentiated primary bronchial epithelial cells from n=6 healthy donors were treated with

For Review Only

type 2 cytokines or infected with RV. A Total STAT6, phosphorylated STAT6 (pSTAT6), total JAK1, JAK2 and β-actin protein expression was measured by immunoblot, representative blot, M molecular weight marker, and densitometry normalised to β-actin. B Fold change gene expression for SOCS1 and SOCS3 normalized to 18S RNA. Median +/- IQR analysed by Friedman test with Dunn's multiple comparisons test *p<0.05, **p<0.01, ***p<0.001 for indicated comparisons. C Luciferase activity relative light units (RLU) in BEAS-2B cells transfected with STAT6 reporter plasmid and infected with RV-A1 and or treated with IL-4, n=7-8 replicate wells. Mean +/- STDEV analysed by 1 way ANOVA with Bonferroni multiple comparisons test **p<0.01.

Figure 4. Differential STAT6 and NF-κB regulation of type 2 cytokine- and RV induced-CCL17 and CCL22 expression. A549 cells were transfected with NF-κB p65, IKK2 or STAT6- targeting siRNA or scrambled non-targeting control siRNA. Untreated cells (medium) or cells treated with IL-4 and/or RV-A1 were assessed for A CCL17- B CCL22-protein expression. Data representative of at least 3 replicate wells. Median +/- IQR analysed by Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001. C CCL22 protein expression by differentiated primary bronchial epithelial cells from n = 6 healthy donors untreated or treated with IL-4/IL-13 and/or infected with RV-A1, 96 h post-infection. Median +/- IQR analysed by Friedman test with Dunn's multiple comparisons. *P<0.05, ***P<0.001 and Wilcoxon matched-pairs test #p<0.05.

Figure 5. Rhinovirus infection induces airway expression of CCL17 and CCL22. Mice were infected intranasally with RV-A1 (RV) or UV-inactivated RV-A1 (UV). Mock-infected mice were dosed intranasally with PBS A Expression of CCL17 and CCCL22 in the bronchi at 2 days post-

For Review Only

infection with IHC staining score. Specificity of staining confirmed with non-targeting isotype control antibody. n = 7-8 mice per group (for RV- and UV-treated mice). Mean +/- SDEV, P values calculated using Welch's t test. B Time-course of CCL17 and CCL22 protein in BAL. n = 4 mice per group, representative of 2 independent experiments. Median +/- IQR analysed by 2 way ANOVA with Sidak's multiple comparison test ***p<0.001, ****p<0.0001.

Figure 6. STAT6 and NF-κB differentially regulate rhinovirus induced CCL17 and CCL22 expression in vivo. RV-A1-infected (RV)- or UV-inactivated RV (UV)-treated mice. Mock – uninfected mice. A Representative western blot time course analysis of STAT6 phosphorylation in lung tissue cytoplasmic protein from n =4 mice. B Time-course STAT6 DNA binding in lung tissue nuclear protein fraction measured by electrophoretic mobility shift assay. N = 4 mice shown for 8 h post-infection, 100X unlabelled probe C Expression of CCL17 and CCL22 protein in BAL at 24 h post-infection of n = 5 wild type (WT) and STAT6ko mice, pooled data from 2 independent experiments. Expression of CCL17 and CCL22 protein in BAL of RV infected mice D treated with vehicle (DMSO) or IKK2 inhibitor (PS1145), E in p65 homozygous positive (p65+/+)- and p65 heterozygous (p65+/-)-mice. n = 4 per group, pooled data from 2 independent experiments mean+/-STDEV *p<0.05, **p<0.01, ***p<0.001 Mann-Whitney test.

Figure 7. STAT6 knockout mice have reduced lung viral load associated with increased expression of NF-κB-regulated chemokines. RV infected wild-type (WT) or STAT6ko mice. Time course analysis of A titre of infectious virus in lung tissue, B neutrophils in BAL and C CXCL1, CXCL2 and IFN-λ2/3 proteins in BAL at baseline (0 hours) and 8, 24 and 48 hours after

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i.n. infection with RV-A1 . N = 5 per group, representative of 2 independent experiments. Mean +/-STDEV **p<0.05, **p<0.01, ***p<0.001 Two way ANOVA with Sidak's multiple comparison test.

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Supplementary Figure Legends

Supplementary Figure 1. IL-4 and RV differentially regulate CCL17 and CCL22 expression *in vitro.* A549 cells were pre-treated with IL-4 (10 ng/mL) 16 hours pre-infection with RV-A1 (MOI of 20). IL-4 and RV regulation of **A** CCL17 and **B** CCL22 protein secretion. Data representative of at least 3 replicate wells. *p<0.05, ** p<0.01, ***p<0.001 analysed by Mann-Whitney test.

Supplementary Figure 2. RV-induced CCL22 and atopic status are not correlated *in vitro*. Atopic status (as determined by positive skin-prick test) of ALI-differentiated BECs were compared with CCL22 secretion levels from RV-A1 infected BECs.

Supplementary Figure 3. Confirmation of mRNA and protein expression knockdown following NF-κB p65, IKK-β and STAT6 siRNA administration. A549 cells were either non-transfected or transfected with control (non-targeting), NF-κB p65-, IKK-β- or STAT6-targeting siRNA in duplicate wells. Messenger RNA for **A** p65 or **B** IKK-β was expressed as percentage of non-transfected cells. Verification of protein reduction following **C** p65, IKK-β and **D** STAT6 siRNA-mediated knockdown by western blot.

Supplementary Figure 4. RV infection induces CCL22 expression in airway vessels. Mice were infected intranasally with RV-A1 or UV-inactivated RV-A1. Mock-infected were dosed intranasally with PBS. Expression of CCL22 in the airway vessels at 2 days post RV-infection, assessed by immunohistochemical staining.

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Rhinovirus induced CCL17 and CCL22 in asthma exacerbations and differential regulation by STAT6

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Abstract

Rationale: The interplay of type-2 inflammation and anti-viral immunity underpins asthma exacerbation pathogenesis. Virus infection induces type-2 inflammation-promoting chemokines CCL17 and CCL22 in asthma, however mechanisms regulating induction are poorly understood.

Objectives: To use a human rhinovirus (RV) challenge model in human airway epithelial cells *in vitro* and mice *in vivo* to assess mechanisms regulating CCL17 and CCL22 expression.

Methods: Subjects with mild-to-moderate asthma and healthy volunteers were experimentally infected with RV and airway CCL17 and CCL22 protein quantified. *In vitro* airway epithelial cell- and mouse-RV infection models were then employed to define STAT6- and NF-κB-mediated regulation of CCL17 and CCL22 expression.

Main Results: Following RV infection, CCL17 and CCL22 expression was higher in asthma, which differentially correlated with clinical and immunological parameters. Air-liquid interface (ALI) differentiated primary epithelial cells from donors with asthma also expressed higher levels of RV-induced CCL22. RV infection boosted type-2 cytokine-induced STAT6 activation. In epithelial cells, type-2 cytokines and STAT6 activation had differential effects on chemokine expression: increasing CCL17 and suppressing CCL22, whereas NF-κB promoted expression of both chemokines. In mice, RV infection activated pulmonary STAT6 which was required for CCL17, but not CCL22 expression. STAT6-knockout mice infected with RV expressed increased levels of NF-kB-regulated chemokines, which was associated with rapid viral clearance.

Conclusions: RV-induced upregulation of CCL17 and CCL22 was mediated by NF-κB activation, whereas expression was differentially regulated by STAT6. Together, findings suggest

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therapeutic targeting of type-2-STAT6 activation alone will not block all inflammatory pathways during RV infection in asthma.

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Introduction

Rhinoviruses (RV) are the most frequent virus infections associated with asthma exacerbations(1). Preventing or treating RV-induced asthma exacerbations remains a challenge, as there are currently no effective treatments for RV infection. An improved understanding of the immune response to RV infection is necessary to aid development of new therapies.

The role of type-2 immune responses in the pathogenesis of asthma is well documented(2) and RV infection augments type-2 inflammation(3-5). For example, in experimental RV infections of subjects with atopic asthma, exacerbation severity correlated with T_H2 cell cytokine production and viral load(3). In type-2 inflammatory airways diseases (e.g. allergic asthma), recruitment of CCR4-expressing immune cells to the airways is regulated by production of chemokines, including CCL17(6) and CCL22(7). The relevance of these CCR4 ligands to disease is supported by their increased levels in serum and sputum from patients with asthma(8).

CCR4 is expressed by type-2 inflammatory cells (including T_H2 cells(9) and innate lymphoid cells (ILC2s)(10)), which produce IL-4, IL-5 and IL-13 and underpin the pathogenesis of allergic asthma(11). Genetic ablation of either CCR4 ligand (CCL17 or CCL22) reduced lung inflammation, decreased T_H2 infiltration and reduced airway hyper-reactivity in mouse models of allergic airways disease(6, 12). Monoclonal antibodies targeting CCR4 (e.g. mogamulizumab) have been approved in Japan for the treatment of adult T-cell leukaemia-lymphoma(13). While phase I trials have also assessed CCR4-targeting monoclonal treatment in asthma, trials were discontinued and have not been further pursued for asthma or viral-induced exacerbations.

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Promoter analyses of CCL17 and CCL22 have identified binding sites for NF-κB and STAT transcription factors, consistent with regulation by multiple inflammatory pathways(14-16). Co-stimulation of human lung epithelial cells with the STAT6-activating cytokine IL-4, and with TNFα (which activates NF-κB), synergistically induced CCL17 production(17). This highlighted the potential for RV infection-induced NF-κB activation(18) to augment CCL17 in asthma, modulating airway inflammation and disease exacerbation.

There has been long-standing interest in CCL17 and CCL22 as treatment targets for type-2 inflammatory diseases, including asthma. Despite this, there is a limited understanding of whether pulmonary CCL17 and CCL22 production is regulated by STAT6 and/or NF-κB in the context of RV infection and asthma. Further, there is evidence to suggest that the regulation of CCL17 and CCL22 by STAT6 is context-dependent(19). We previously reported that RV infection augments CCL17 and CCL22 expression in a mouse model of allergic airways disease(18) and recently reported both chemokines are induced by RV infection in humans, and further increased following infection in people with asthma(20).

In the current study, we investigate relationships between human CCL17 and CCL22 protein expression in the context of allergic inflammation *in vivo* following experimental RV infection of healthy volunteers and patients with atopic asthma. We also employed mouse RV infection models to assess functional roles of STAT6 and NF-κB using transgenic- and pharmacological-interventions. As airway epithelial cells are the primary site of RV infection, we also utilised *in vitro* human airway epithelial cell RV infection models to assess transcriptional regulation of CCL17 and CCL22. This is the first study to identify correlations between CCL17 and CCL22 airway protein expression and disease parameters associated with RV infection in asthma. We

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also define the role of key transcription factors (NF-κB and STAT6) in RV- and type-2 cytokine-induction of these chemokines *in vivo* and by human airway epithelial cells *in vitro*.

Materials & Methods

Human participants

We studied non-smoking subjects with mild to moderately severe asthma and healthy volunteers aged 18-55 years (confirmed serum RV-A16-neutralising antibody negative), as previously reported(5). The study received Research Ethics Committee approval (09/H0712/59) and subjects provided informed consent(5). Inclusion/exclusion criteria for subjects with asthma included doctor diagnosis of asthma, airway hyper-responsiveness, evidence of atopy (by skin prick test) and a history of infection-induced asthma exacerbation(5). Subjects with a history of severe asthma, >5 pack-year history or recent smoking, current symptoms of allergic rhinitis, or who experienced an asthma exacerbation or respiratory virus illness within the previous 6 weeks were excluded. Non-smoking, non-asthmatic healthy controls were recruited based on: no history of asthma, atopy or allergic disease and no objective airway hyper-responsiveness. For patient demographics and additional information see reference(5).

Human rhinovirus challenge

Participants underwent bronchoscopy approximately 14 days prior to inoculation with RV-A16(21) and on day 4 post-infection. On day 0, RV-A16 was delivered to both nostrils (100 $TCID_{50}$) using an atomiser, as previously described(5). Protein mediators in nasal secretions were assessed by nasosorption (days 0, 2-5 and 7)(5). On day 4 post-infection,

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bronchosorption was conducted as previously described(20). Diary cards of upper and lower respiratory tract symptoms were commenced prior to baseline sampling and continued for 6 weeks after RV-A16 infection, as previously described(3, 5). CCL17 and CCL22 proteins in nasal and bronchial secretions were quantified by Meso Scale Discovery multi-spot human cytokine assay, as described previously(20).

Mouse rhinovirus infection

Female BALB/c mice, 6-8 weeks, were purchased from Harlan UK. *Tnfrsf1a*$^{-/-}$-*Rela(p65)*$^{+/-}$ mice (129/Sv - C57BL/6 background) were bred to generate TNFR1-deficient mice of *p65*$^{+/+}$ (wildtype) or *p65*$^{+/-}$ (heterozygous)(22). Genotype was determined by PCR. As TNFR1/p65-deficient mice are susceptible to bacterial infections, antibiotics were included in drinking water(23). STAT6 knockout (ko) mice (*Stat6*$^{tm1/Gru}$ – Balb/c background) were purchased from Jackson Laboratory (Bar Harbor, USA).

RV-A1 (American Tissue Culture Collection) was purified and titrated, as described previously(24). Where indicated, UV-inactivated RV-A1 (1,200 mJ/cm^2; 30 min) was used. Mice were infected by intranasal (i.n.) instillation RV-A1 (5 x 10^6 $TCID_{50}$), as previously described(24, 25). Infectious RV titre in homogenised lung tissue was determined in HeLa H1 cells via TCID50 assay as previously described (24). To inhibit Iκβ-kinase-β (IKK-β) mice PS-1145 (40mg/kg in 10% DMSO) was administered by intraperitoneal injection (Millenium Pharmaceuticals(26)). At indicated times after infection, broncho-alveolar lavage (BAL) was performed and CCL17, CCL22, IFN-λ2/3, CXCL1, and CXCL2 protein levels quantified by ELISA, according to manufacturer instructions (R&D Systems). CCL17 and CCL22 expression in airway epithelium was determined by immunohistochemistry staining of tissue samples.

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STAT6 protein quantification in mouse lung

Lung tissue cell cytoplasmic and nuclear protein extracts were prepared using CelLytic NuCLEAR extraction kit (Sigma), according to manufacturer's instructions. Cytoplasmic fraction proteins were resolved by denaturing SDS-PAGE and immunoblotted using antibodies against STAT6-phosphor Y641 (Abcam). EMSA was performed, as previously described(27). Lung tissue nuclear protein extracts were incubated with labelled STAT6-binding DNA probe and binding specificity demonstrated using 100x excess (20 pmoL) unlabelled DNA. DNA-protein complexes were resolved on native acrylamide gel and visualised by autoradiography.

CCL22 and CCL17 protein immunostaining in mouse lung tissue

Single immunohistochemical staining was performed as previously described (28), using goat anti-mouse CCL22 (sc-12288; Santa Cruz Biotechnology) and goat anti-mouse CCL17, respectively (AF529; R&D Systems). Staining was scored by a single, blinded investigator (PC) using microscopy. CCL22 and CCL17 epithelial staining was scored as: 0 = no staining; 1-4 based on 1-24%, 25-49%, 50-74% or 75-100%(28, 29).

Human cell culture

A549 lung epithelial cells (ECACC) were cultured in DMEM containing L-glutamine, HEPES, sodium bicarbonate and 10% FCS. Cells were pre-treated with IL-4 (10ng/mL) 16 h before infection with RV-A1 (multiplicity of infection (MOI) = 20). Knock-down of NF-κB p65, IKK-β and STAT6 was achieved using ON-TARGET plus SMART pool siRNA (4 siRNAs per target; Dharmacon/ThermoFisher) and Lipofectamine 2000 transfection reagent (Invitrogen), as previously described(30). Reduction of targeted protein expression was confirmed by western blot(30).

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BEAS-2B airway epithelial cells (ECACC) were cultured in RPMI 1640 medium with 10% FCS. Transient transfection of BEAS-2B cells with a STAT6-responsive-luciferase reporter plasmid c/EBP-N4-luc (19, 31) was performed, as described previously(18). Briefly, cells were transfected with c/EBP-N4-luc and pRL Renilla luciferase control reporter vector (Promega) in Superfect (Qiagen) transfection reagent, based on manufacturer recommendations. At 24 hours post-transfection, cells were treated with IL-4 (10 ng/mL) and / or infected with RV-A1 (MOI = 1). At 24 h post-treatment/infection luciferase activity in cell lysates were measured using a dual luciferase protocol (Promega).

Bronchial epithelial cells (BECs) from healthy and asthmatic donors were cultured at air-liquid interface (ALI), as previously described(32). Briefly, BECs were cultured on placental collagen-coated flasks until confluent, then seeded onto transwell inserts at passage 2 in 1:1 mix of Dulbecco's modified Eagle medium and BEBM plus supplements (Lonza). Apical media was removed once cells were confluent and cultures differentiated for 24 days, where evidence of mucus and cilia was present. At day 24 post-ALI, basal media was replaced with minimal BEBM media supplemented with human recombinant IL-4 and IL-13 (10ngmL; Peprotech). On day 25, cells were infected with RV-A1 (MOI 0.1) (33) and harvested 4 days post-infection.

Human CCL22 and CCL17 protein in epithelial cell cultures was quantified using DuoSet ELISAs, according to manufacturer instructions (R&D Systems). For STAT6, JAK1, and JAK2 protein expression and phosphorylation analysis, BEC protein lysates were diluted with Laemmli loading buffer, denatured, and run on a TGX 4-12% stain-free gel (Biorad). Membranes were subsequently transferred onto nitrocellulose, blocked in bovine serum albumin/skimmed milk and stained overnight for pSTAT6 (CST), STAT6 (Santa Cruz), JAK1 (CST), JAK2 (CST) or B-actin

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(Abcam). Blots were then washed in TBS-T and stained with anti-rabbit and anti-mouse secondary antibodies (Abcam), imaged using a Chemidoc imager (BioRad).

Quantitative PCR (qPCR)

CCL17, CCL22, SOCS1, and SOCS3 mRNA levels were measured by qPCR, normalised to 18S reference gene levels (primer sequences in Table 1). RNA was extracted using miRNeasy mini kit (Qiagen) and quantified using nanodrop spectrophotometer. cDNA was generated using Taqman reverse transcription reagents and quantitative qPCR performed on an ABI7500 with Quantstudio software (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using GraphPad Prism v8.2.1 software. Wilcoxon matched pairs test was used to assess effect of treatment within a group and Mann-Whitney test to compare between different groups. For comparisons between multiple groups of non-normally distributed data Friedman test with Dunn's multiple comparison was used. Log-transformed viral load data was compared by two-way ANOVA with Sidak's multiple comparisons test. Transformed data was fitted to a straight line in a QQ plot confirming normal distributions for comparison. For time-course protein data, comparisons were performed by two-way ANOVA with Sidak's multiple comparisons test. *P* values <0.05 were considered statistically significant.

Results

RV induction of CCL17 and CCL22 in asthma correlated with markers of disease

Time-course nasal expression of CCL17 and CCL22 from a human RV challenge study in subjects with mild to moderate asthma and healthy volunteers has previously been reported(20). In the current study we reanalysed this data by plotting baseline (day 0) nasal

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CCL17 and CCL22 protein expression and peak expression during the infection to determine correlations with immunological and clinical outcomes, as previously described(5). At baseline, CCL17 protein was higher in nasosorption samples from asthmatic subjects compared to healthy controls. Peak CCL17 was also higher in asthma during RV infection. For CCL22, baseline levels were non-significantly (p=0.072) higher in asthma vs healthy and peak levels were significantly higher in subjects with asthma compared to healthy volunteers during RV infection (**Fig.1A**). Peak nasal CCL17 and CCL22 concentrations during infection were positively correlated with each other in both healthy subjects and subjects with asthma (**Fig.1B**). We previously reported that RV infection induced type-2 cytokines (IL-4, IL-5 and IL-13) and that IL-5 and IL-13 levels during infection in asthma positively correlated with respiratory symptom severity(5). In the current study, IL-4, IL-5 and IL-13 were positively correlated with peak nasal CCL17 (but not CCL22) production during RV infection (**Fig.1C).** Further, CCL17 and CCL22 levels during RV infection correlated with peak upper respiratory symptom scores (**Fig.1D**) and at day 4 post-infection bronchial CCL22 protein levels correlated with peak lower respiratory symptom scores in subjects with asthma (**Fig.1E**). These data show that RV-induced CCL17 and CCL22 levels are differentially associated with clinical and immunological asthma exacerbation parameters and CCL17 (but not CCL22) positively correlated with type-2 cytokine expression.

RV infection in bronchial epithelial cells from donors with asthma induces higher levels of CCL22 compared to CCL17

Airway epithelial cells are a source of CCL17 and CCL22(17, 34) and the primary site of RV infection(35). We next quantified CCL17 and CCL22 expression in RV-infected ALI-cultured BECs from donors with asthma (donor characteristics in Table 1). Infection with either major

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group RV-A43 or minor group RV-A1 did not significantly increase CCL17 mRNA expression (**Fig.2A**). However, RV infection did increase CCL22 mRNA expression. RV-A1 infection rapidly induced CCL22 expression which peaked at day 2 post-infection, whereas induction by RV-A43 infection peaked at day 4 (**Fig.2B**). We noted peak RV-induced CCL22 mRNA levels were approximately 10-fold higher than CCL17. CCL17 protein in apical supernatants were below the limit of detection, whereas CCL22 protein was detectable and significantly increased by RV-A1 infection (with a non-significant (*P*=0.088) increase after RV-A43 infection) at day 4 post-infection (**Fig.2C**). Based on this time-course data, we selected baseline and day 4 post-infection as relevant timepoints to compare RV-induced CCL22 protein expression in ALI-cultured BECs from a separate cohort of donors with asthma (AS) and non-asthmatic, healthy control subjects (HC; n=7; donor characteristics in Table 2). To increase sample size, we combined the CCL22 protein data generated with the previous day 4 CCL22 protein data from asthma (AS) BECs, providing n=14 asthma donors infected with RV-A1 (**Fig.2D**)**.** Infection of BECs from donors with asthma significantly increased CCL2 expression, which was higher (p=0.055) than RV-induced CCL22 in BECs from healthy control donors (**Fig.2D**). We noted variability in RV-induced CCL22 levels between BEC cultures from asthmatic donors, but note that variability did not correlate with atopic status determined by skin prick test positivity (**Supplementary Figure 2**). These data are consistent with observed RV induction of CCL22 *in vivo* and indicate that airway epithelial cells from patients with asthma are primed to express higher levels of CCL22, compared to cells from non-asthmatic, healthy control subjects.

RV infection augments type 2 cytokine-induced STAT6 activation

We next characterised transcriptional regulation of CCL17 and CCL22 during RV infection. Previous studies identified NF-κB and STAT6 binding sites in CCL17 and CCL22 promoters(14-

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16). Further, STAT6 binding can have differential effects on target gene expression depending on the cellular context(19). Whether (and how) these transcription factors regulate expression of these chemokines in human airway epithelial cells during RV infection is unknown. We previously reported RV-induced epithelial cell NF-κB activation during RV infection(18). Here we investigated STAT6 activation following RV-A1 infection, in the presence of cytokine (IL-4/IL-13) induced STAT6 phosphorylation in ALI-differentiated BEC cultures from healthy donors (n=6). Type 2 cytokine treatment alone induced a near-significant (p=0.067) increase in pSTAT6 levels, which was further increased with RV-A1 infection (**Fig.3A**).

To gain insight into potential mechanisms underlying increased STAT6 phosphorylation in ALI cultures following RV infection and stimulation with type-2 cytokines, we quantified expression of two Janus kinases (JAK1and JAK2) that are activated by viral infection and have been shown to phosphorylate STAT6 (36, 37). IL-4/13 treatment significantly reduced constitutive expression of JAK1, whereas RV infection alone did not significantly deplete JAK1 expression (**Fig. 3A**). RV infection reduced the suppressive effect of type-2 cytokines such that IL-4/13 treatment did not significantly reduce the expression of JAK1 compared to RV infection alone or compared to untreated cells (**Fig. 3A**). For JAK2, RV infection increased expression above levels in type-2 cytokine-treated cells (**Fig. 3A**). Again, RV infection partially reversed the suppressive effect of type 2 cytokine treatment such that the combination did not significantly reduce JAK2 compared to RV infection alone (**Fig. 3A**). The data suggests that RV infection partially restores type-2 cytokine-suppressed JAK1 expression, which could contribute to increased STAT6 phosphorylation observed following RV infection of type-2 cytokine-treated cells.

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In addition to assessing expression of RV-induced kinases that phosphorylate STAT6 we also quantified negative regulators of STAT activation, namely SOCS1 and SOCS3(38). RV infection alone as well in combination with type 2 cytokine treatment significantly increased SOCS1 and SOCS3 mRNA expression compared to untreated cells (**Fig. 3B**). IL-4/IL-13 treatment with RV infection also increased SOCS3 expression compared to type-2 cytokine-treated BECs (**Fig. 3B**). This data identified RV infection as the primary driver of SOCS3 expression. However, this induction of SOCS3 was not sufficient to block RV-augmented type-2 cytokine-induced STAT6 phosphorylation, as shown in **Fig 3A**.

To determine if observed STAT6 phosphorylation led to increased transcriptional activity we developed an *in vitro* assay system by treating BEAS-2B cells with IL-4 and/or infecting with RV. We initially confirmed expression of CCL17 and CCL22 protein by BEAS-2B cells. As in primary cells, RV infection alone did not induce detectable levels of CCL17 protein. However, combined IL-4 and RV treatment/infection significantly increased CCL17 protein (**Sup Fig.1A**). RV infection alone increased CCL22 protein, while pre-treatment with IL-4 reduced RV induction of CCL22 protein levels (**Sup. Fig.1B**).

Having established the utility of this BEAS-2B cell assay system to investigate the interaction between RV infection and type 2 cytokine treatment on CCL17 and CCL22 expression we also used this system to examine STAT6 promoter activation following transfection of a STAT6-driven luciferase reporter plasmid. Compared to untreated cells, promoter activity was increased 2-3-fold following RV infection (**Fig.3C**). IL-4 treatment significantly induced STAT6 promoter activation, compared to RV infection alone (approximately 7-fold), and combined IL-4 and RV exposure additively increased activation (>10 fold) (**Fig.3C**). These data provide

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evidence that RV infection alone is sufficient for low level STAT6 activation in airway epithelial cells, and RV infection augments type-2 cytokine-induced-STAT6 transcriptional activation.

STAT6 and NF-κB activation have differential effects on RV- and type-2 cytokine-induced CCL17 and CCL22 expression

Experiments in BEAS2B cells indicated that type-2 cytokine/STAT6 differentially regulate CCL17 and CCL22. To further investigate transcriptional regulation of CCL17 and CCL22 expression, we assessed the effects of administering small interfering RNAs (siRNAs) targeting NF-κB (p65 or IKK-β) and STAT6 in A549 cells treated with IL-4 and/or infected with RV. We confirmed that siRNA administration reduced protein expression of the respective targets (**Supplementary Fig.3**). As observed previously, highest CCL17 levels were induced by a combination of IL-4 and RV-A1 exposure (**Fig.4A**). SiRNAs targeting p65, IKK-β or STAT6 all reduced IL-4/RV-A1-induced CCL17 production, compared to control siRNA treatment (**Fig.4A**). This was not the case for CCL22 expression, where the highest level of CCL22 expression was observed following RV-A1 infection alone, and significantly reduced by IL-4 treatment (**Fig.4B**). SiRNA targeting p65 or IKK-β reduced CCL22 expression in RV-infected cells. In contrast, CCL22 expression increased following STAT6 knockdown in IL-4-treated cultures infected with RV (**Fig.4B**). Inhibition of STAT6 expression also increased RV (alone) induced CCL22 expression providing further evidence of direct STAT6 activation by RV (**Fig. 4B**). These data indicate that unlike CCL17, type-2 cytokine-induced STAT6 activation negatively regulates CCL22 expression in RV-infected airway epithelial cells. We confirmed this observation in a more physiologically relevant system, observing that type-2 cytokine treatment (IL-4 and IL-13) suppressed RV-induced CCL22 protein expression in ALI-differentiated BEC cultures derived from healthy donors (**Fig.4C**).

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Differential kinetics of expression of RV-induced CCL17 and CCL22 in vivo

We also investigated pulmonary expression of CCL17 and CCL22 *in vivo* in a mouse model of RV infection(24) and observed s CCL17 and CCL22 protein expression in airway epithelium (**Fig.5A**). RV infection induced strong CCL17 staining exclusively in the bronchial epithelium (**Fig.5A**), with negligible staining in lung tissue from mock-infected or UV-inactivated RV inoculated mice**.** Constitutive CCL22 expression was apparent in lung tissue following mock infection or inoculation with UV-inactivated RV-A1. CCL22 was more broadly expressed in the lung than CCL17, with expression also detected in endothelial cells (**Supplementary Fig.4**). CCL17 and CCL22 were also significantly elevated in BAL samples during RV-A1 infection, with CCL17 levels peaking at 1-day post-infection (p.i.) and CCL22 peaking 2 days p.i., with both chemokines significantly elevated through 4 days p.i. (**Fig.5B**).

RV-induced CCL17 and CCL22 expression are differentially regulated by STAT6 *in vivo*

We next determined when STAT6 phosphorylation occurred in the lung during RV infection. We previously reported that RV infection induced NF-κB activation(18), so in the current study we focused on STAT6. Increased levels of cytoplasmic phosphorylated-STAT6 (pSTAT6) in lung tissue were observed 8 hours post RV-A1 infection (**Fig.6A**). Increased STAT6-DNA binding was observed in the nuclear fraction of lung protein samples from RV-infected mice (**Fig.6B**). This finding was confirmed in samples from four additional mice at 8 hours p.i. and binding specificity was confirmed using excess (100X) unlabelled probe (**Fig.6B**).

To determine the functional role of STAT6 activation on CCL17 and CCL22 production, we utilized a RV-A1 time course to identify 1-day p.i. for assessment in STAT6ko mice. At 1-day p.i., lower levels of CCL17 were detected in BAL samples from STAT6ko mice, compared to wild-type (WT) controls (**Fig.6C**). In contrast, but in agreement with our *in vitro* human data,

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STAT6 deficiency had no effect on CCL22 levels, indicating that STAT6 is not required to drive CCL22 expression in the context of RV infection (**Fig.6C**). We also examined the role of NF-κB *in vivo,* using pharmacological IKK-β inhibition (**Fig.6D**) and genetically deficient $p65^{+/-}$ mice (**Fig.6E**). Administration of the IKK-β inhibitor (PS1145) significantly reduced both CCL17 and CCL22 BAL protein levels following RV-A1 infection, compared with vehicle-treated controls (**Fig.6D**). Similarly, $p65^{+/-}$ mice had reduced CCL17 BAL protein levels after RV-A1 infection, compared to homozygous wild-type $p65^{+/+}$ mice, following RV infection (**Fig.6E**). In $p65^{+/-}$ mice a trend (*P*=0.08) towards reduced levels of CCL22 was also observed (**Fig.6E**). These data confirm that unlike CCL17, STAT6 is not required for RV-induced CCL22 expression. In contrast, NF-κB was required for expression of both chemokines following infection.

STAT6 expression inhibits viral clearance and expression of NF-κB-regulated chemokines

To determine whether perturbation of STAT6 altered the RV-induced lung inflammatory milieu, we performed an RV-infection time course analysis in wild-type (WT) and STAT6ko mice. At 8 hours post-infection viral load was not different between WT and STAT6ko mice. In contrast, by 24 hours post-infection lung viral load was approximately 10-fold lower in STAT6ko mice compared to WT controls (**Fig. 7A**). RV infection induces airway neutrophilic inflammation and we noted a consistent trend for increased neutrophils in BAL samples isolated from STAT6ko mice (**Fig. 7B**). Further, this was associated with significantly increased expression of the NF-κB-regulated neutrophil-recruiting chemokines CXCL1 and CXCL2 in BAL fluid from STAT6ko mice. In contrast, IFN-λ levels were not increased in STAT6ko mice compared to WT mice (**Fig. 7C)**.

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Discussion

This study interrogated the regulation of RV-induced CCL17 and CCL22 as a framework to better understand the interplay between two key inflammatory pathways (i.e. STAT6 and NF-κB), which collectively underpin the pathogenesis of asthma exacerbations. To do this we utilized a human RV experimental infection model, *in vitro* airway epithelial cell- and mouse RV infection models to show that blocking STAT6 activation alone (particularly in the context of viral infection and NF-κB activation), will not completely inhibit inflammatory cell trafficking via chemokines such as CCL22 (where STAT6 activation may restrict expression).

We have previously reported time-course of CCL17 and CCL22 protein expression in nasal and bronchial secretions in a human RV experimental infection model, in healthy volunteers and subjects with asthma(20). We reanalysed this data to identify the peak expression level for each subject so that it could be correlated to type-2 cytokine production. This approach has been used previously to define clinical and immunological correlates of expression of other mucosal-epithelial expressed inflammatory cytokines such as IL-33(5). Peak expression of CCL17 (and not CCL22) correlated with IL-4, IL-5 and IL-13 expression in the nasal mucosa. This indicated that type 2 inflammation in combination with viral infection increased CCL17 expression. This conclusion is supported by in vitro experiments in human airway epithelial cells. In A549 cells the combination of IL-4 treatment and RV infection was required for detectable expression of CCL17 protein. In primary human BECs differentiated at ALI, adding multiple type 2 cytokines (IL-4 and IL-13) with RV infection did not induce detectable CCL17 protein expression suggesting that signals in addition to direct epithelial cell STAT6 activation, potentially mediated by immune cells, are needed for maximal expression by mucosal tissues. Immune cell augmentation of CCL17 production has been documented in other systems. For

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example, CCL17-expessing dendritic cells have been identified in intestinal mucosa and drive inflammation in a mouse colitis model(39).

Limited research has assessed the expression and regulation of CCL17 and CCL22 during respiratory virus infection in the context of asthma exacerbations (40-42). In a mouse model of RV infection, Nagarkar et al. assessed the lung inflammatory transcriptome and reported CCL17 as one of the most highly induced genes (>14 fold). Further, CCL22 gene expression was significantly increased (>3 fold)(43). Here we extended these results to report increased chemokine protein expression following RV infection in both human and mouse RV infection models. Our human *in vitro* BEC data demonstrated that RV subtypes binding different receptors: major group (ICAM-1 receptor) and minor group (low density lipoprotein receptor) induced CCL22 expression, evidence that CCL22 expression is a general response to RV infection. While we noted 2-3 fold higher secretion of CCL22 and CCL17 within our *in vitro* and *in vivo* systems compared to the human experimental model, we attribute this as likely due to the higher RV infection doses and direct infection of the lower respiratory tract for mouse infection model.

To look more broadly at RV infection and activation of NF-κB and STAT6 signalling pathways, we transfected siRNA into an epithelial cell line. Whilst both NF-κB- and STAT6 appear to be involved in regulating CCL17 production by alveolar epithelial cells, STAT6 negatively regulated CCL22 expression. This was further confirmed in $STAT6^{-/-}p65^{+/-}$ mice and following IKK-β inhibitor PS1145 treatment, where CCL17 production was abrogated in the absence of NF-κB or STAT6, while CCL22 secretion was unaffected by altering STAT6. STAT6 has previously been reported to directly inhibit p65 translocation to the nucleus by inhibiting IKBα phosphorylation in HeLa cells(44). Differential and direct interaction between NF-κB and STAT6 have previously

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been reported, with the two transcription factors synergistically enhancing luciferase expression by a synthetic inducible IL-4 reporter gene construct(45). Other studies have reported that IL-4-induced STAT6 activation reduced NF-κB DNA binding and IL-6 expression in certain cell types(46, 47). Ohmori and colleagues reported that STAT6 trans-activating domain competed for the transcriptional coactivator CBP, and overexpression of CBP relieved the suppressive effect of STAT6 on TNF-α-induced NF-κB activation(26). Future studies will investigate STAT6 transactivation and interference with the activity of CBP co-activator on CCL17 versus CCL22 expression in the context of RV infection and type-2 cytokine stimulation in the lung.

STAT6 has been shown to either activate or repress type-2 cytokine-induced gene expression in different contexts. Georas and colleagues reported that IL-4 stimulation highly activated a synthetic multimerized STAT6-response element contained within the c/EBP-N4-luc reporter(19). However, IL-4-induced STAT6 activation negatively regulated native IL-4 promoter-driven IL-4 expression leading the authors to conclude that the transcriptional function of STAT6 is dependent on promoter-context(19). We previously reported that in a mouse model of RV-induced exacerbation, RV infection increased allergen (OVA)-induced expression of both CCL17 and CCL22(18). Increased type-2 cytokine expression is a feature of this model(24), so our data is consistent with STAT6 promoting both CCL17 and CCL22 expression. This further highlights the context-dependent transcriptional activity of STAT6 in the interplay between type-2 allergic inflammation and RV infection. It is noteworthy that CCL17 (and not CCL22) protein expression significantly correlated with type-2 cytokine (IL-4, IL-5 and IL-13) expression in the nasal mucosa. This is consistent with type-2 cytokine-STAT6 activation preferentially driving CCL17 expression in the context of respiratory virus infection and acute exposure to type-2 cytokines. This raises the question of the net effect on CCR4-

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binding chemokine expression if STAT6 selectively suppresses expression of CCL22, while promoting expression of CCL17. We also need to consider cell-specific expression profile – as BEC expression of CCL22 was higher than CCL17 and tissue expression *in vivo* (mouse lung) was also different. This highlights that the system is complex and that direct counter-regulation by STAT6 may proportionally affect expression of CCL17 vs CCL22 such that the net effect on CCR4 mediated immune cell recruitment is zero depending on the specific circumstances.

In BEC cultures, CCL17 expression was synergistically increased by type-2 cytokines and RV infection, as has previously been noted following respiratory syncytial virus infection(41). However, the current study found that addition of IL-4 repressed RV-induced CCL22 and that siRNA-mediated knockdown of STAT6 relieved IL-4-induced suppression. Upon investigating upstream mediators of STAT6 signalling we found that type-2 cytokines negatively regulated RV-induction of both JAK1 and JAK2. A previous study in Schwann cells found that inhibition of JAK2/STAT pathways downregulated CCL22 gene expression(48), which compliments the reduction in CCL22 secretion we observed when type-2 cytokines suppress JAK2 protein expression. Despite the upregulation in SOCS1 and SOCS3 gene expression that we observed following RV infection, RV infection still promoted JAK2 expression.

In summary, we identify differential transcriptional regulation and relationships with asthma disease parameters of RV-induced CCL17 and CCL22. Our data show that RV-challenged individuals with asthma had increased bronchial CCL22 associated with asthma symptoms, whilst peak CCL17 in the nose was associated with type-2 cytokine expression and upper respiratory tract symptoms. We demonstrated distinct STAT6-mediated transcriptional regulation of CCL17 and CCL22 during RV infection, while both chemokines were regulated by

For Review Only

NF-κB. Thus, our findings provide new insight into the context-dependent expression of these often interchangeable CCR4 ligands. Our findings suggest that clinical trials targeting CCR4 (or downstream pathways) will be useful in asthma exacerbations, particularly those triggered by virus infection given the interplay of STAT6 and NF-κB in this clinical disease setting. In support of this, CCL17 and CCL22 were detectable in the nasal mucosa and RV-induced expression was higher in asthma compared to viral induction in healthy volunteers. Furthermore, bronchial CCL22 protein levels at day 4 post experimental RV infection positively correlated with severity of lower respiratory tract symptoms in asthma. Further studies with selective inhibitors of CCR4/CCL17/CCL22 are needed to support therapeutically targeting this pathway in virus induced asthma exacerbations.

For Review Only

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For Review Only

References

1. Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Josephs LK, Cunningham A, Robinson BS, Myint SH, Ward ME, Tyrrell DA, Holgate ST. The relationship between upper respiratory infections and hospital admissions for asthma: a time-trend analysis. *Am J Respir Crit Care Med* 1996; 154: 654-660.
2. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol* 2015; 15: 57-65.
3. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebadze 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.
4. Beale J, Jayaraman A, Jackson DJ, Macintyre JD, Edwards MR, Walton RP, Zhu J, Ching YM, Shamji B, Edwards M, Westwick J, Cousins DJ, Hwang YY, 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.
5. 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.
6. Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, Imai T, Yoshie O, Homma I, Yamamoto K, Matsushima K. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* 2001; 166: 2055-2062.
7. Pilette C, Francis JN, Till SJ, Durham SR. CCR4 ligands are up-regulated in the airways of atopic asthmatics after segmental allergen challenge. *Eur Respir J* 2004; 23: 876-884.
8. Sekiya T, Yamada H, Yamaguchi M, Yamamoto K, Ishii A, Yoshie O, Sano Y, Morita A, Matsushima K, Hirai K. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy* 2002; 57: 173-177.
9. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998; 187: 129-134.
10. Weston CA, Rana BMJ, Cousins DJ. Differential expression of functional chemokine receptors on human blood and lung group 2 innate lymphoid cells. *J Allergy Clin Immunol* 2019; 143: 410-413 e419.
11. Lambrecht BN, Hammad H, Fahy JV. The Cytokines of Asthma. *Immunity* 2019; 50: 975-991.
12. Gonzalo JA, Pan Y, Lloyd CM, Jia GQ, Yu G, Dussault B, Powers CA, Proudfoot AE, Coyle AJ, Gearing D, Gutierrez-Ramos JC. Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J Immunol* 1999; 163: 403-411.
13. Subramaniam JM, Whiteside G, McKeage K, Croxtall JC. Mogamulizumab: first global approval. *Drugs* 2012; 72: 1293-1298.

For Review Only

14. Nakayama T, Hieshima K, Nagakubo D, Sato E, Nakayama M, Kawa K, Yoshie O. Selective induction of Th2-attracting chemokines CCL17 and CCL22 in human B cells by latent membrane protein 1 of Epstein-Barr virus. *J Virol* 2004; 78: 1665-1674.
15. Liddiard K, Welch JS, Lozach J, Heinz S, Glass CK, Greaves DR. Interleukin-4 induction of the CC chemokine TARC (CCL17) in murine macrophages is mediated by multiple STAT6 sites in the TARC gene promoter. *BMC Mol Biol* 2006; 7: 45.
16. Wirnsberger G, Hebenstreit D, Posselt G, Horejs-Hoeck J, Duschl A. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *Eur J Immunol* 2006; 36: 1882-1891.
17. Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamaguchi M, Fujisawa T, Pawankar R, Sano Y, Ohta K, Ishii A, Morita Y, Yamamoto K, Matsushima K, Yoshie O, Hirai K. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* 2000; 165: 2205-2213.
18. Bartlett NW, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P, Clarke DL, Message SD, Aniscenko J, Kebadze T, Zhu J, Mallia P, Mizgerd JP, Belvisi M, Papi A, Kotenko SV, Johnston SL, Edwards MR. Defining critical roles for NF-kappaB p65 and type I interferon in innate immunity to rhinovirus. *EMBO Mol Med* 2012; 4: 1244-1260.
19. Georas SN, Cumberland JE, Burke TF, Chen R, Schindler U, Casolaro V. Stat6 inhibits human interleukin-4 promoter activity in T cells. *Blood* 1998; 92: 4529-4538.
20. 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.
21. Bardin PG, Sanderson G, Robinson BS, Holgate ST, Tyrrell DA. Experimental rhinovirus infection in volunteers. *Eur Respir J* 1996; 9: 2250-2255.
22. Alcamo E, Mizgerd JP, Horwitz BH, Bronson R, Beg AA, Scott M, Doerschuk CM, Hynes RO, Baltimore D. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. *J Immunol* 2001; 167: 1592-1600.
23. Quinton LJ, Jones MR, Simms BT, Kogan MS, Robson BE, Skerrett SJ, Mizgerd JP. Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia. *J Immunol* 2007; 178: 1896-1903.
24. Bartlett NW, Walton RP, Edwards MR, Aniscenko J, Caramori G, Zhu J, Glanville N, Choy KJ, Jourdan P, Burnet J, Tuthill TJ, Pedrick MS, Hurle MJ, Plumpton C, Sharp NA, Bussell JN, Swallow DM, Schwarze J, Guy B, Almond JW, Jeffery PK, Lloyd CM, Papi A, Killington RA, Rowlands DJ, Blair ED, Clarke NJ, Johnston SL. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. *Nat Med* 2008; 14: 199-204.
25. Bartlett NW, Singanayagam A, Johnston SL. Mouse models of rhinovirus infection and airways disease. *Methods Mol Biol* 2015; 1221: 181-188.
26. Ohmori Y, Hamilton TA. Interleukin-4/STAT6 represses STAT1 and NF-kappa B-dependent transcription through distinct mechanisms. *J Biol Chem* 2000; 275: 38095-38103.

For Review Only

27. Birrell MA, Hardaker E, Wong S, McCluskie K, Catley M, De Alba J, Newton R, Haj-Yahia S, Pun KT, Watts CJ, Shaw RJ, Savage TJ, Belvisi MG. Ikappa-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *Am J Respir Crit Care Med* 2005; 172: 962-971.
28. Varani K, Caramori G, Vincenzi F, Adcock I, Casolari P, Leung E, Maclennan S, Gessi S, Morello S, Barnes PJ, Ito K, Chung KF, Cavallesco G, Azzena G, Papi A, Borea PA. Alteration of adenosine receptors in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006; 173: 398-406.
29. Jayaraman A, Jackson DJ, Message SD, Pearson RM, Aniscenko J, Caramori G, Mallia P, Papi A, Shamji B, Edwards M, Westwick J, Hansel T, Stanciu LA, Johnston SL, Bartlett NW. IL-15 complexes induce NK- and T-cell responses independent of type I IFN signaling during rhinovirus infection. *Mucosal Immunol* 2014; 7: 1151-1164.
30. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, Sykes A, Dahdaleh S, Clarke DL, Belvisi MG, Kon OM, Fujita T, Jeffery PK, Johnston SL, Edwards MR. Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. *PLoS Pathog* 2010; 6: e1001178.
31. Mikita T, Campbell D, Wu P, Williamson K, Schindler U. Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. *Mol Cell Biol* 1996; 16: 5811-5820.
32. Hackett TL, Shaheen F, Johnson A, Wadsworth S, Pechkovsky DV, Jacoby DB, Kicic A, Stick SM, Knight DA. Characterization of side population cells from human airway epithelium. *Stem Cells* 2008; 26: 2576-2585.
33. Singanayagam A, Glanville N, Girkin JL, Ching YM, Marcellini A, Porter JD, Toussaint M, Walton RP, Finney LJ, Aniscenko J, Zhu J, Trujillo-Torralbo MB, Calderazzo MA, Grainge C, Loo SL, Veerati PC, Pathinayake PS, Nichol KS, Reid AT, James PL, Solari R, Wark PAB, Knight DA, Moffatt MF, Cookson WO, Edwards MR, Mallia P, Bartlett NW, Johnston SL. Corticosteroid suppression of antiviral immunity increases bacterial loads and mucus production in COPD exacerbations. *Nat Commun* 2018; 9: 2229.
34. Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, Arrigoni G, Fabbri LM, Sinigaglia F. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001; 107: 1357-1364.
35. Reid AT, Sutanto, E. N., Chander-Veerati, P., Looi, K., Fung Li, N., Iosifidis, T., Loo, S-L., Garratt, L. W., Kicic, A. Ground zero-the airway epithelium. In: Bartlett NW, Wark, P. A. B., Knight, D., editor. Rhinovirus Infections: Rethinking impact on human health and disease, 1 ed. London: Academic Press; 2019. p. 314.
36. Cho W, Jeoung DI, Kim YM, Choe J. STAT6 and JAK1 are essential for IL-4-mediated suppression of prostaglandin production in human follicular dendritic cells: opposing roles of phosphorylated and unphosphorylated STAT6. *Int Immunopharmacol* 2012; 12: 635-642.
37. Palmer-Crocker RL, Hughes CC, Pober JS. IL-4 and IL-13 activate the JAK2 tyrosine kinase and Stat6 in cultured human vascular endothelial cells through a common pathway that does not involve the gamma c chain. *J Clin Invest* 1996; 98: 604-609.
38. Gielen V, Sykes A, Zhu J, Chan B, Macintyre J, Regamey N, Kieninger E, Gupta A, Shoemark A, Bossley C, Davies J, Saglani S, Walker P, Nicholson SE, Dalpke AH, Kon OM, Bush A, Johnston SL, Edwards MR. Increased nuclear suppressor of cytokine

For Peer Review Only

signaling 1 in asthmatic bronchial epithelium suppresses rhinovirus induction of innate interferons. *J Allergy Clin Immunol* 2015; 136: 177-188 e111.
39. Heiseke AF, Faul AC, Lehr HA, Forster I, Schmid RM, Krug AB, Reindl W. CCL17 promotes intestinal inflammation in mice and counteracts regulatory T cell-mediated protection from colitis. *Gastroenterology* 2012; 142: 335-345.
40. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995; 310: 1225-1229.
41. Monick MM, Powers LS, Hassan I, Groskreutz D, Yarovinsky TO, Barrett CW, Castilow EM, Tifrea D, Varga SM, Hunninghake GW. Respiratory syncytial virus synergizes with Th2 cytokines to induce optimal levels of TARC/CCL17. *J Immunol* 2007; 179: 1648-1658.
42. Zhang Y, Luxon BA, Casola A, Garofalo RP, Jamaluddin M, Brasier AR. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. *J Virol* 2001; 75: 9044-9058.
43. Nagarkar DR, Wang Q, Shim J, Zhao Y, Tsai WC, Lukacs NW, Sajjan U, Hershenson MB. CXCR2 is required for neutrophilic airway inflammation and hyperresponsiveness in a mouse model of human rhinovirus infection. *J Immunol* 2009; 183: 6698-6707.
44. Nelson G, Wilde GJ, Spiller DG, Kennedy SM, Ray DW, Sullivan E, Unitt JF, White MR. NF-kappaB signalling is inhibited by glucocorticoid receptor and STAT6 via distinct mechanisms. *J Cell Sci* 2003; 116: 2495-2503.
45. Shen CH, Stavnezer J. Interaction of stat6 and NF-kappaB: direct association and synergistic activation of interleukin-4-induced transcription. *Mol Cell Biol* 1998; 18: 3395-3404.
46. Donnelly RP, Crofford LJ, Freeman SL, Buras J, Remmers E, Wilder RL, Fenton MJ. Tissue-specific regulation of IL-6 production by IL-4. Differential effects of IL-4 on nuclear factor-kappa B activity in monocytes and fibroblasts. *J Immunol* 1993; 151: 5603-5612.
47. Takeshita S, Gage JR, Kishimoto T, Vredevoe DL, Martinez-Maza O. Differential regulation of IL-6 gene transcription and expression by IL-4 and IL-10 in human monocytic cell lines. *J Immunol* 1996; 156: 2591-2598.
48. Fletcher JS, Springer MG, Choi K, Jousma E, Rizvi TA, Dombi E, Kim MO, Wu J, Ratner N. STAT3 inhibition reduces macrophage number and tumor growth in neurofibroma. *Oncogene* 2019; 38: 2876-2884.

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Tables

Table 1: Clinical characteristics of time course donors

	Asthma
Number, n	6
Age, years (SD)	58.3 (15.3)
Gender (M/F)	(1/5)
Atopy (SPT positive)	(1/6)
Severity	Mild-Moderate (5)
	Severe (1)
FEV1, % predicted (SD)	82.5 (11.9)
FVC, % predicted (SD)	89 (8.3)
Daily ICS dose, beclomethasone equivalent, ug (SD)	218.3 (37.6)

ICS: Inhaled corticosteroid

FVC: Forced vital capacity

FEV1: Forced expiratory volume in one second

Table 2: Clinical characteristics of healthy and donors with asthma

	Healthy	Asthma
Number, n	8	8
Age, years (SD)	61.5 (13.6)	61.5 (8.8)
Gender (M/F)	(1/7)	(3/5)
Atopy (SPT positive)	(1/8)	(5/8)**
Severity	NA	Moderate (1)
	NA	Severe (7)
FEV1, % predicted (SD)	89.1 (12.4)	70.6 (21.5)*
FVC, % predicted (SD)	97.5 (15.9)	87.7 (12.6)
Daily ICS dose, beclomethasone equivalent, ug (SD)	NA	287 (95.7)***

***** significantly different compared with healthy controls

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Supplemental Table 1: Primer sequences

Gene	Forward	Reverse	Probe
18S	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG	FAM-ACCGGCGCAAGACGGACCAGA-TAMRA
huCCL17	GGATGCCATCGTTTTTGTAACTG	TTAACTGCATTCTTCACTCTCTTGTTG	FAM-AGGGCCATCTGTTCGGACCCCA-TAMRA
huCCL22	CTACAGACTGCACTCCTGGTTGTC	GGGCCTGCCTCAGTTGCT	FAM-TCGTCCTCCTTGCTGTGGCGCT-TAMRA

All sequences presented in 5’ to 3’ orientation.

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Figure legends

Figure 1. Increased in vivo rhinovirus induced CCL17 and CCL22 in asthma is correlated with type 2 inflammation and disease severity. Individuals with asthma and healthy volunteers were infected with RV-A16 and nasal samples were obtained. A Protein levels of peak CCL17 and CCL22 in the nose. Median +/- IQR, *p<0.05, **p<0.01, Healthy vs Asthma at baseline or peak of infection analysed by Mann-Whitney U test. B Correlation of peak nasal CCL17 and CCL22 levels. C Correlations of peak nasal CCL17 protein with peak nasal type-2 cytokine proteins (IL-4, IL-5 and IL-13). D Correlation of peak nasal CCL17 and CCL22 proteins with upper respiratory symptom scores. E Correlation of bronchial CCL22 protein with lower respiratory symptom scores.

Figure 2. CCL22 induction by rhinovirus and increased expression by bronchial epithelial cells from donors with asthma. ALI-differentiated bronchial epithelial cells from donors with asthma were infected with either RV-A1 or RV-A43. Time course expression of A CCL17- and B CCL22-mRNA and C CCL22 protein. n = 6 donors for each time-point. Median +/- IQR, analyzed by two way ANOVA with Dunnett's multiple comparison test. **** P < 0.001 RV-A43 vs mock, # P < 0.05 RV-A1 vs mock. D CCL22 protein expression at Baseline and four days after RV-A1 infection in separate cohort of healthy control (HC) and asthmatic (AS) bronchial epithelial cells. n=7 HC donors, n=14 AS donors, median +/- IQR analyzed by Mann-Whitney U test (HC vs AS) or Wilcoxon matched pairs test (baseline vs RV-A1) * P < 0.05.

Figure 3. Rhinovirus infection increased type 2 cytokine-induced STAT6 activation. Differentiated primary bronchial epithelial cells from n=6 healthy donors were treated with

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type 2 cytokines or infected with RV. A Total STAT6, phosphorylated STAT6 (pSTAT6), total JAK1, JAK2 and β-actin protein expression was measured by immunoblot, representative blot, M molecular weight marker, and densitometry normalised to β-actin. B Fold change gene expression for SOCS1 and SOCS3 normalized to 18S RNA. Median +/- IQR analysed by Friedman test with Dunn's multiple comparisons test *p<0.05, **p<0.01, ***p<0.001 for indicated comparisons. C Luciferase activity relative light units (RLU) in BEAS-2B cells transfected with STAT6 reporter plasmid and infected with RV-A1 and or treated with IL-4, n=7-8 replicate wells. Mean +/- STDEV analysed by 1 way ANOVA with Bonferroni multiple comparisons test **p<0.01.

Figure 4. Differential STAT6 and NF-κB regulation of type 2 cytokine- and RV induced-CCL17 and CCL22 expression. A549 cells were transfected with NF-κB p65, IKK2 or STAT6- targeting siRNA or scrambled non-targeting control siRNA. Untreated cells (medium) or cells treated with IL-4 and/or RV-A1 were assessed for A CCL17- B CCL22-protein expression. Data representative of at least 3 replicate wells. Median +/- IQR analysed by Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001. C CCL22 protein expression by differentiated primary bronchial epithelial cells from n = 6 healthy donors untreated or treated with IL-4/IL-13 and/or infected with RV-A1, 96 h post-infection. Median +/- IQR analysed by Friedman test with Dunn's multiple comparisons. *P<0.05, ***P<0.001 and Wilcoxon matched-pairs test #p<0.05.

Figure 5. Rhinovirus infection induces airway expression of CCL17 and CCL22. Mice were infected intranasally with RV-A1 (RV) or UV-inactivated RV-A1 (UV). Mock-infected mice were dosed intranasally with PBS A Expression of CCL17 and CCCL22 in the bronchi at 2 days post-

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infection with IHC staining score. Specificity of staining confirmed with non-targeting isotype control antibody. n = 7-8 mice per group (for RV- and UV-treated mice). Mean +/- SDEV, P values calculated using Welch's t test. B Time-course of CCL17 and CCL22 protein in BAL. n = 4 mice per group, representative of 2 independent experiments. Median +/- IQR analysed by 2 way ANOVA with Sidak's multiple comparison test ***p<0.001, ****p<0.0001.

Figure 6. STAT6 and NF-κB differentially regulate rhinovirus induced CCL17 and CCL22 expression in vivo. RV-A1-infected (RV)- or UV-inactivated RV (UV)-treated mice. Mock – uninfected mice. A Representative western blot time course analysis of STAT6 phosphorylation in lung tissue cytoplasmic protein from n =4 mice. B Time-course STAT6 DNA binding in lung tissue nuclear protein fraction measured by electrophoretic mobility shift assay. N = 4 mice shown for 8 h post-infection, 100X unlabelled probe C Expression of CCL17 and CCL22 protein in BAL at 24 h post-infection of n = 5 wild type (WT) and STAT6ko mice, pooled data from 2 independent experiments. Expression of CCL17 and CCL22 protein in BAL of RV infected mice D treated with vehicle (DMSO) or IKK2 inhibitor (PS1145), E in p65 homozygous positive (p65+/+)- and p65 heterozygous (p65+/-)-mice. n = 4 per group, pooled data from 2 independent experiments mean+/-STDEV *p<0.05, **p<0.01, ***p<0.001 Mann-Whitney test.

Figure 7. STAT6 knockout mice have reduced lung viral load associated with increased expression of NF-κB-regulated chemokines. RV infected wild-type (WT) or STAT6ko mice. Time course analysis of A titre of infectious virus in lung tissue, B neutrophils in BAL and C CXCL1, CXCL2 and IFN-λ2/3 proteins in BAL at baseline (0 hours) and 8, 24 and 48 hours after

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i.n. infection with RV-A1 . N = 5 per group, representative of 2 independent experiments. Mean +/-STDEV **p<0.05, **p<0.01, ***p<0.001 Two way ANOVA with Sidak's multiple comparison test.

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Supplementary Figure Legends

Supplementary Figure 1. IL-4 and RV differentially regulate CCL17 and CCL22 expression *in vitro.* A549 cells were pre-treated with IL-4 (10 ng/mL) 16 hours pre-infection with RV-A1 (MOI of 20). IL-4 and RV regulation of **A** CCL17 and **B** CCL22 protein secretion. Data representative of at least 3 replicate wells. *p<0.05, ** p<0.01, ***p<0.001 analysed by Mann-Whitney test.

Supplementary Figure 2. RV-induced CCL22 and atopic status are not correlated *in vitro*. Atopic status (as determined by positive skin-prick test) of ALI-differentiated BECs were compared with CCL22 secretion levels from RV-A1 infected BECs.

Supplementary Figure 3. Confirmation of mRNA and protein expression knockdown following NF-κB p65, IKK-β and STAT6 siRNA administration. A549 cells were either non-transfected or transfected with control (non-targeting), NF-κB p65-, IKK-β- or STAT6-targeting siRNA in duplicate wells. Messenger RNA for **A** p65 or **B** IKK-β was expressed as percentage of non-transfected cells. Verification of protein reduction following **C** p65, IKK-β and **D** STAT6 siRNA-mediated knockdown by western blot.

Supplementary Figure 4. RV infection induces CCL22 expression in airway vessels. Mice were infected intranasally with RV-A1 or UV-inactivated RV-A1. Mock-infected were dosed intranasally with PBS. Expression of CCL22 in the airway vessels at 2 days post RV-infection, assessed by immunohistochemical staining.

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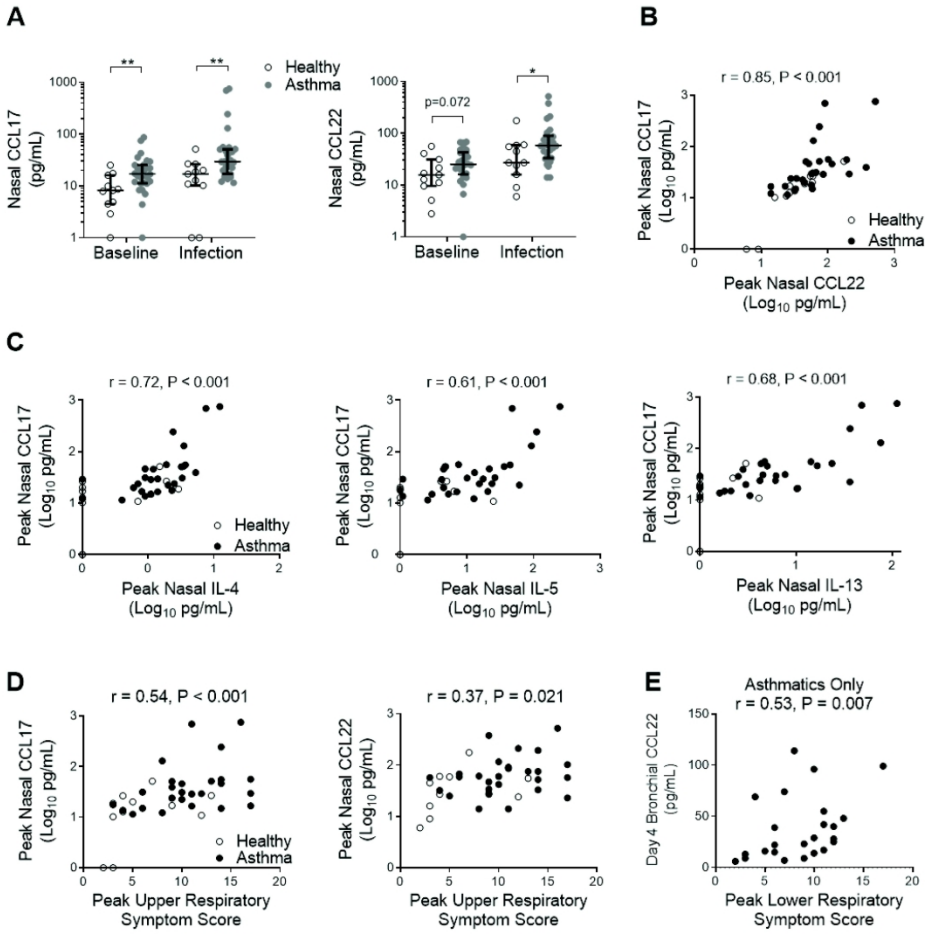


Figure 1. Increased in vivo rhinovirus induced CCL17 and CCL22 in asthma is correlated with type 2 inflammation and disease severity. Individuals with asthma and healthy volunteers were infected with RV-A16 and nasal samples were obtained. A Protein levels of peak CCL17 and CCL22 in the nose. Median +/- IQR, *p<0.05, **p<0.01, Healthy vs Asthma at baseline or peak of infection analysed by Mann-Whitney U test. B Correlation of peak nasal CCL17 and CCL22 levels. C Correlations of peak nasal CCL17 protein with peak nasal type-2 cytokine proteins (IL-4, IL-5 and IL-13). D Correlation of peak nasal CCL17 and CCL22 proteins with upper respiratory symptom scores. E Correlation of bronchial CCL22 protein with lower respiratory symptom scores.

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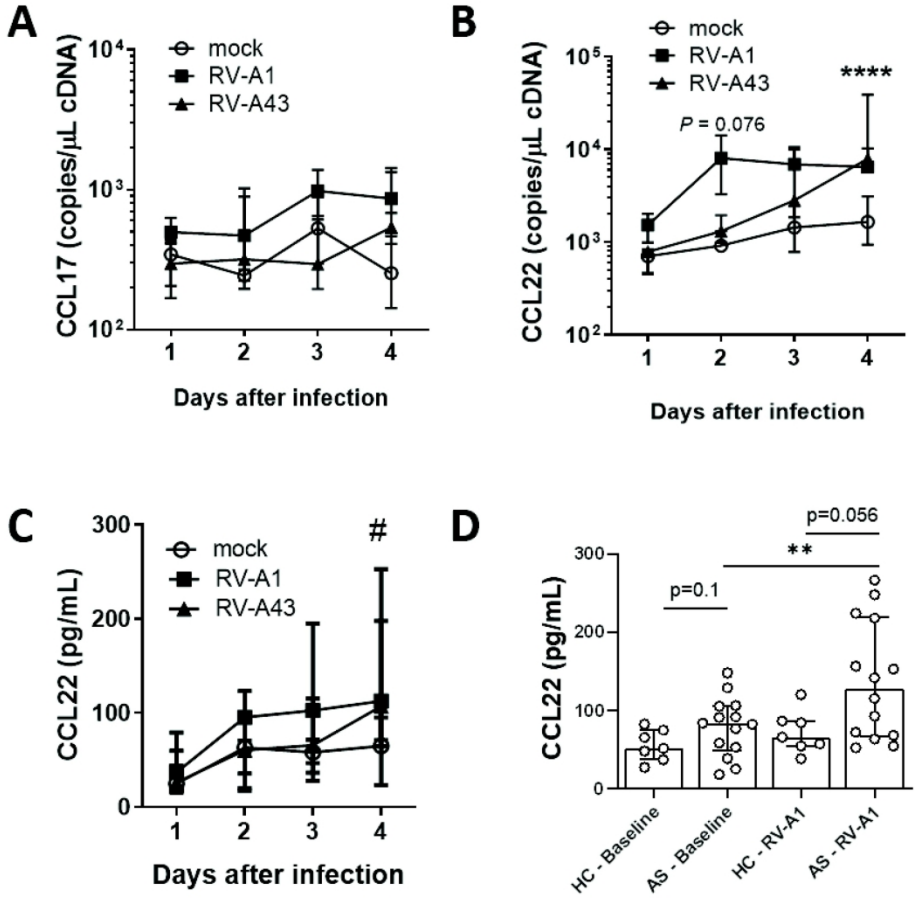


Figure 2. CCL22 induction by rhinovirus and increased expression by bronchial epithelial cells from donors with asthma. ALI-differentiated bronchial epithelial cells from donors with asthma were infected with either RV-A1 or RV-A43. Time course expression of A CCL17- and B CCL22-mRNA and C CCL22 protein. n = 6 donors for each time-point. Median +/- IQR, analyzed by two way ANOVA with Dunnett's multiple comparison test. **** P < 0.001 RV-A43 vs mock, # P < 0.05 RV-A1 vs mock. D CCL22 protein expression at Baseline and four days after RV-A1 infection in separate cohort of healthy control (HC) and asthmatic (AS) bronchial epithelial cells. n=7 HC donors, n=14 AS donors, median +/- IQR analyzed by Mann-Whitney U test (HC vs AS) or Wilcoxon matched pairs test (baseline vs RV-A1) * P < 0.05.

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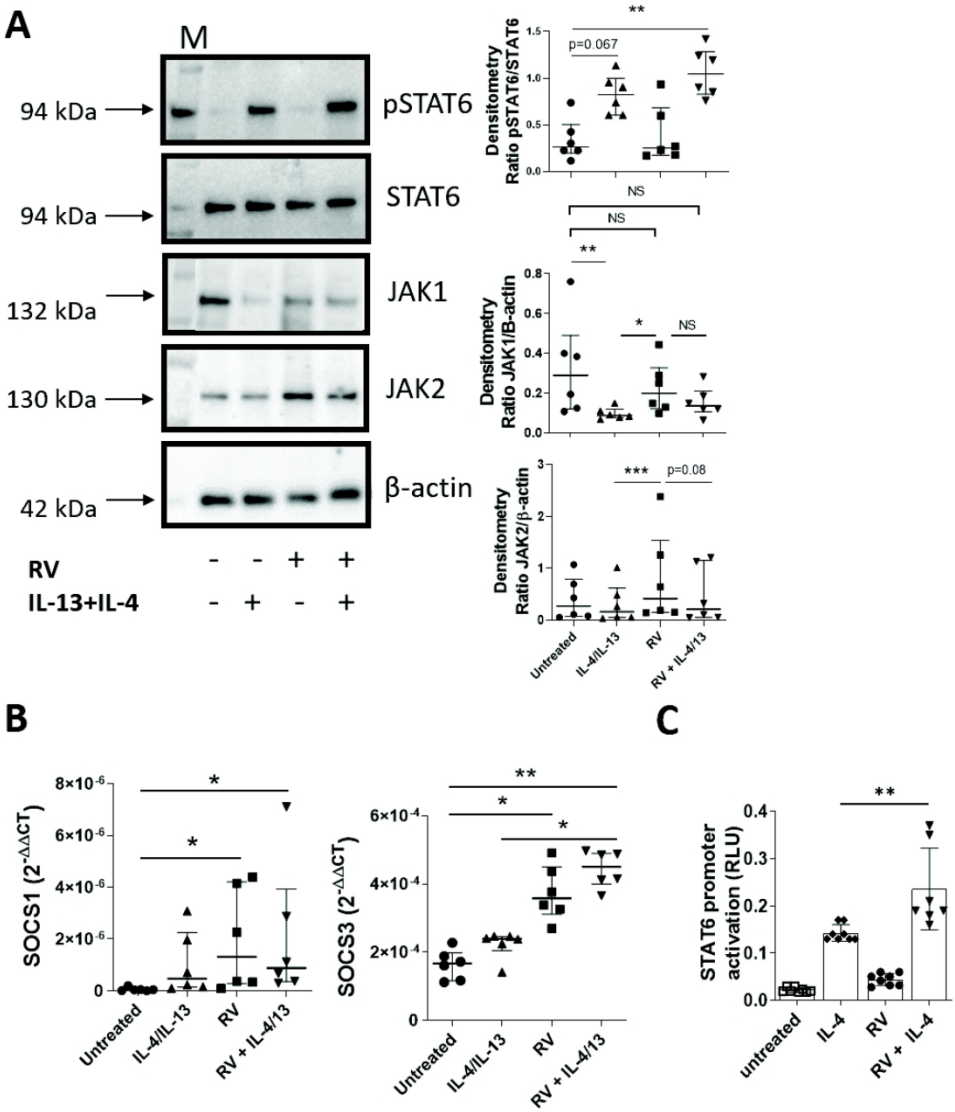


Figure 3. Rhinovirus infection increased type 2 cytokine-induced STAT6 activation. Differentiated primary bronchial epithelial cells from n=6 healthy donors were treated with type 2 cytokines or infected with RV. A Total STAT6, phosphorylated STAT6 (pSTAT6), total JAK1, JAK2 and β-actin protein expression was measured by immunoblot, representative blot, M molecular weight marker, and densitometry normalised to β-actin. B Fold change gene expression for SOCS1 and SOCS3 normalized to 18S RNA. Median +/- IQR analysed by Friedman test with Dunn's multiple comparisons test *p<0.05, **p<0.01, ***p<0.001 for indicated comparisons. C Luciferase activity relative light units (RLU) in BEAS-2B cells transfected with STAT6 reporter plasmid and infected with RV-A1 and or treated with IL-4, n=7-8 replicate wells. Mean +/- STDEV analysed by 1 way ANOVA with Bonferroni multiple comparisons test **p<0.01.

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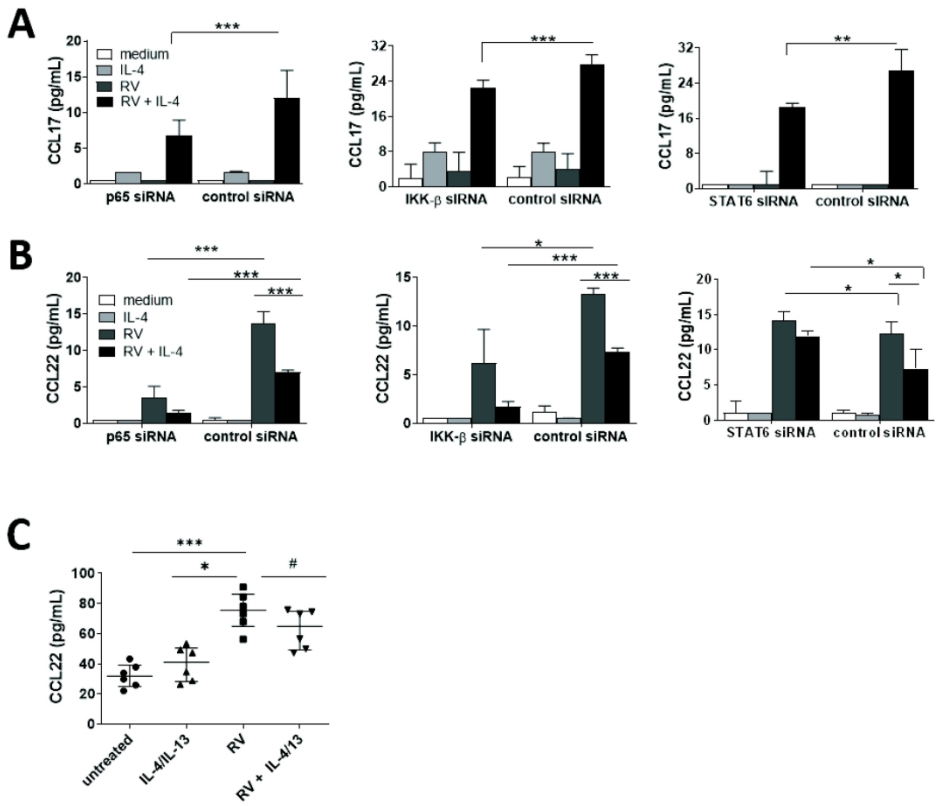


Figure 4. Differential STAT6 and NF-κB regulation of type 2 cytokine- and RV induced-CCL17 and CCL22 expression. A549 cells were transfected with NF-κB p65, IKK2 or STAT6- targeting siRNA or scrambled non-targeting control siRNA. Untreated cells (medium) or cells treated with IL-4 and/or RV-A1 were assessed for A CCL17- B CCL22-protein expression. Data representative of at least 3 replicate wells. Median +/- IQR analysed by Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001. C CCL22 protein expression by differentiated primary bronchial epithelial cells from n = 6 healthy donors untreated or treated with IL-4/IL-13 and/or infected with RV-A1, 96 h post-infection. Median +/- IQR analysed by Friedman test with Dunn's multiple comparisons. *P<0.05, ***P<0.001 and Wilcoxon matched-pairs test #p<0.05.

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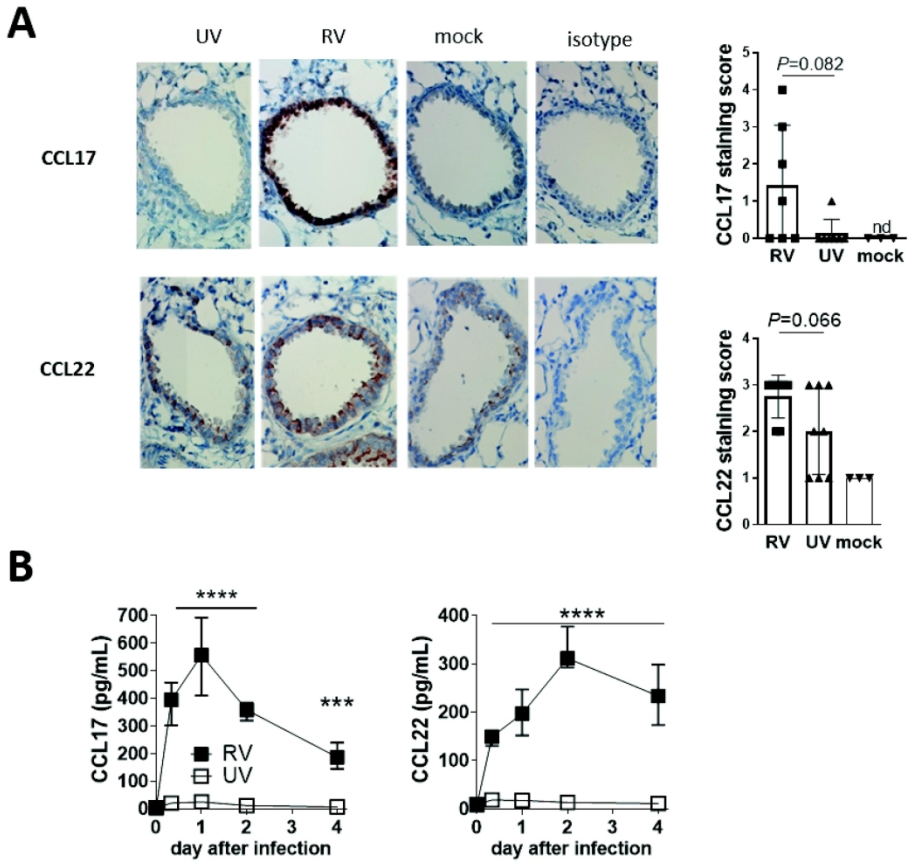


Figure 5. Rhinovirus infection induces airway expression of CCL17 and CCL22. Mice were infected intranasally with RV-A1 (RV) or UV-inactivated RV-A1 (UV). Mock-infected mice were dosed intranasally with PBS A Expression of CCL17 and CCCL22 in the bronchi at 2 days post-infection with IHC staining score. Specificity of staining confirmed with non-targeting isotype control antibody. n = 7-8 mice per group (for RV- and UV-treated mice). Mean +/- SDEV, P values calculated using Welch's t test. B Time-course of CCL17 and CCL22 protein in BAL. n = 4 mice per group, representative of 2 independent experiments. Median +/- IQR analysed by 2 way ANOVA with Sidak's multiple comparison test ***p<0.001, ****p<0.0001.

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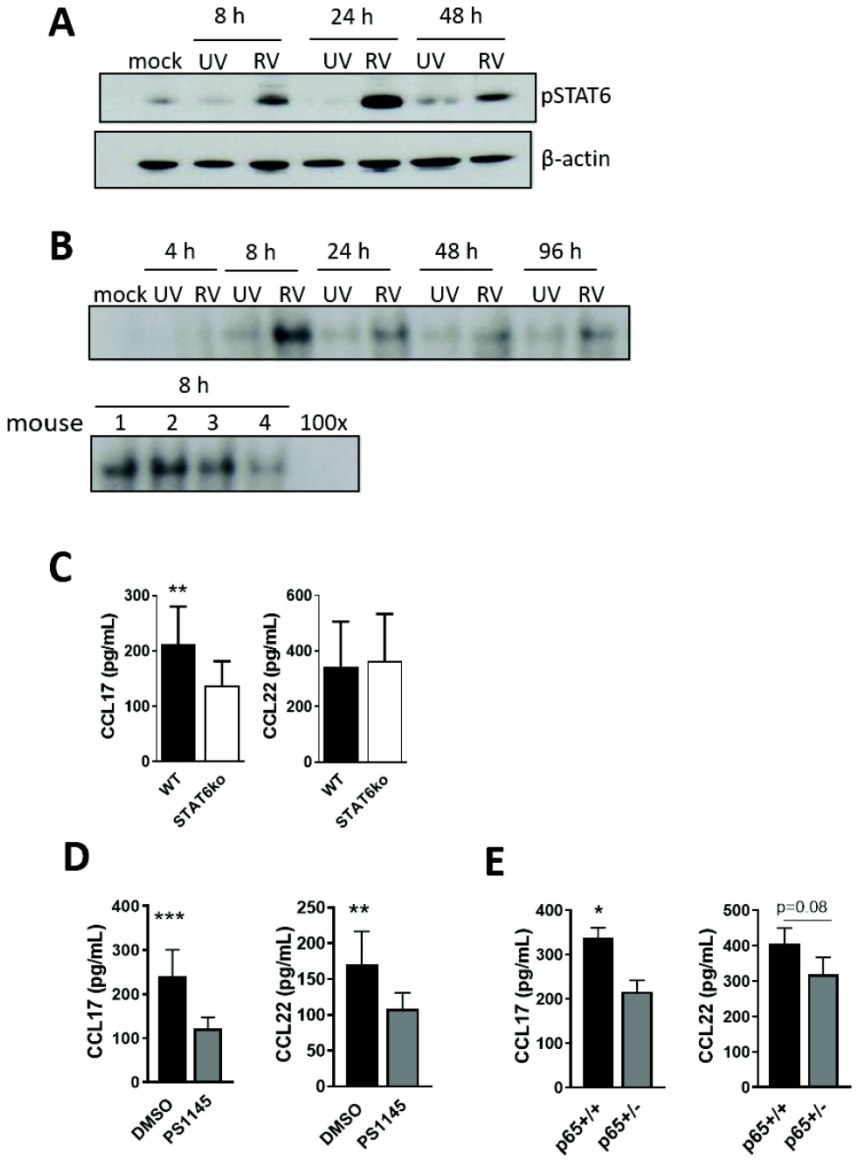


Figure 6. STAT6 and NF-κB differentially regulate rhinovirus induced CCL17 and CCL22 expression in vivo. RV-A1-infected (RV)- or UV-inactivated RV (UV)-treated mice. Mock – uninfected mice. A Representative western blot time course analysis of STAT6 phosphorylation in lung tissue cytoplasmic protein from n =4 mice. B Time-course STAT6 DNA binding in lung tissue nuclear protein fraction measured by electrophoretic mobility shift assay. N = 4 mice shown for 8 h post-infection, 100X unlabelled probe C Expression of CCL17 and CCL22 protein in BAL at 24 h post-infection of n = 5 wild type (WT) and STAT6ko mice, pooled data from 2 independent experiments. Expression of CCL17 and CCL22 protein in BAL of RV infected mice D treated with vehicle (DMSO) or IKK2 inhibitor (PS1145), E in p65 homozygous positive (p65+/+)- and p65 heterozygous (p65+/-)-mice. n = 4 per group, pooled data from 2 independent experiments mean+/-STDEV *p<0.05, **p<0.01, ***p<0.001 Mann-Whitney test.

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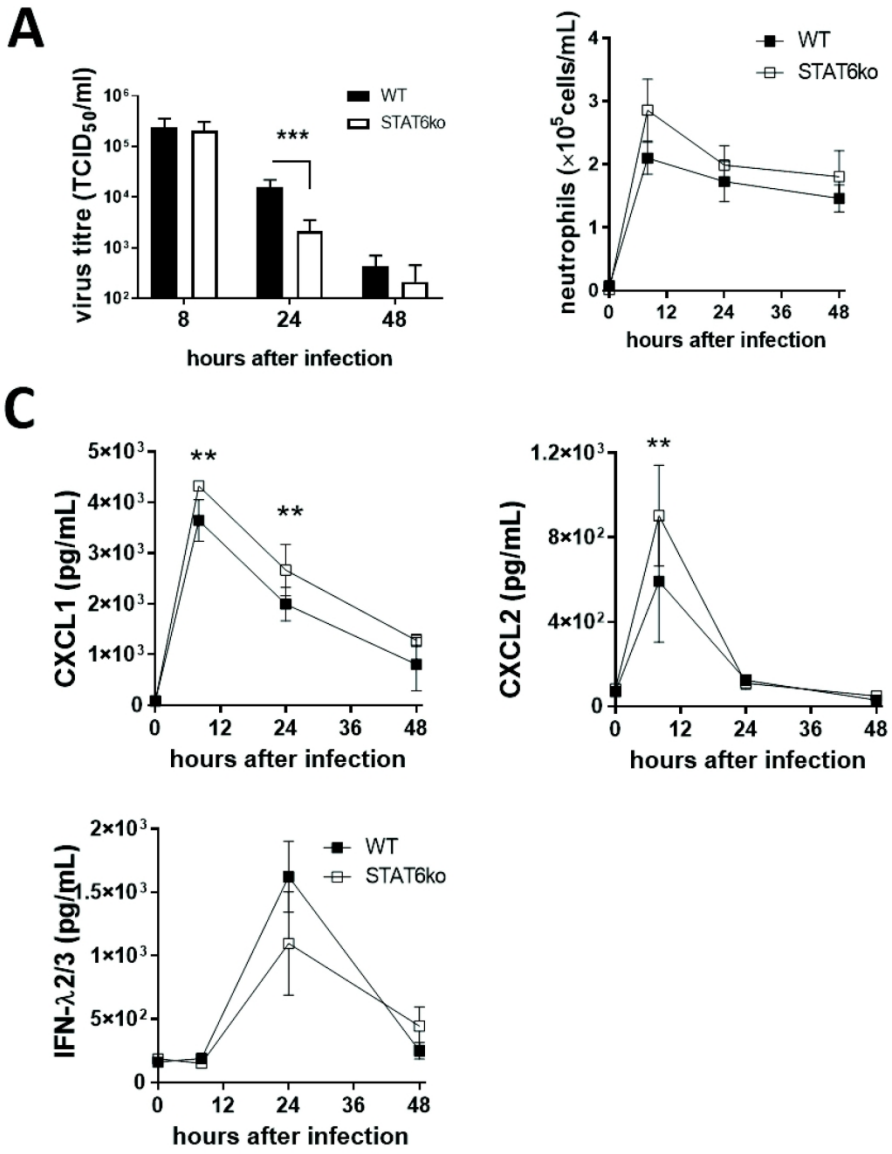
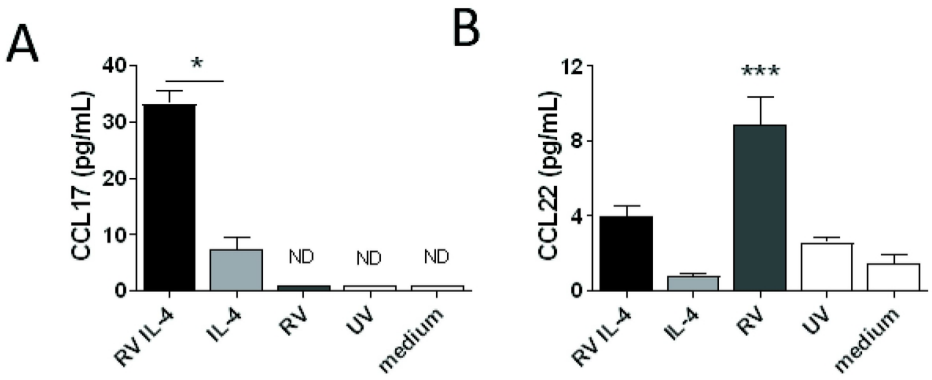


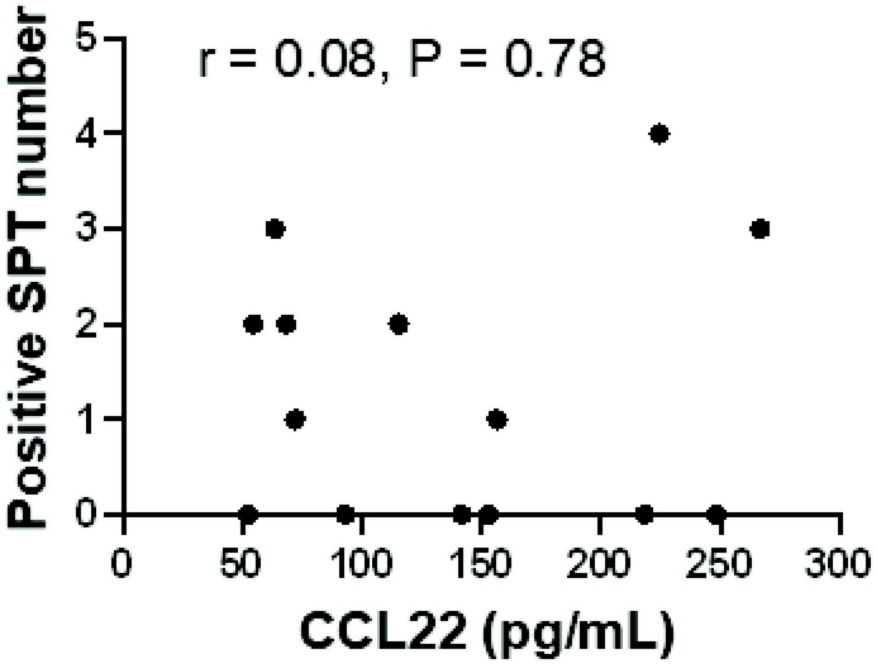
Figure 7. STAT6 knockout mice have reduced lung viral load associated with increased expression of NF-κB-regulated chemokines. RV infected wild-type (WT) or STAT6ko mice. Time course analysis of A titre of infectious virus in lung tissue, B neutrophils in BAL and C CXCL1, CXCL2 and IFN-λ2/3 proteins in BAL at baseline (0 hours) and 8, 24 and 48 hours after i.n. infection with RV-A1 . N = 5 per group, representative of 2 independent experiments. Mean +/-STDEV **p<0.05, **p<0.01, ***p<0.001 Two way ANOVA with Sidak's multiple comparison test.

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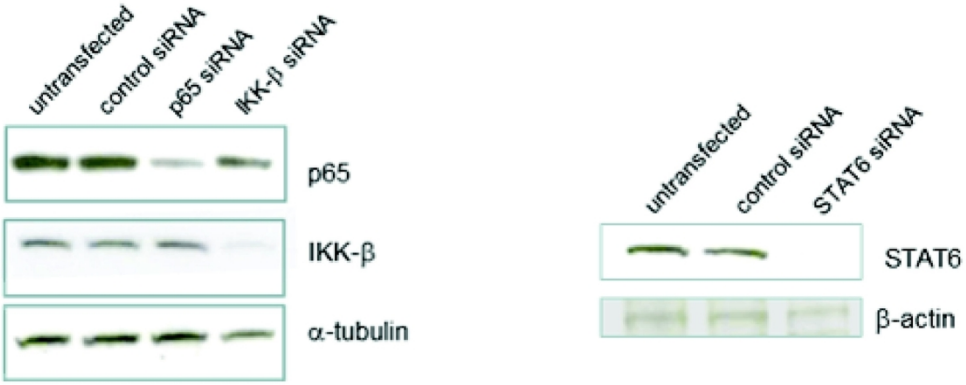
Supplementary Figure 1. IL-4 and RV differentially regulate CCL17 and CCL22 expression in vitro. A549 cells were pre-treated with IL-4 (10 ng/mL) 16 hours pre-infection with RV-A1 (MOI of 20). IL-4 and RV regulation of A CCL17 and B CCL22 protein secretion. Data representative of at least 3 replicate wells. *p<0.05, ** p<0.01, ***p<0.001 analysed by Mann-Whitney test.

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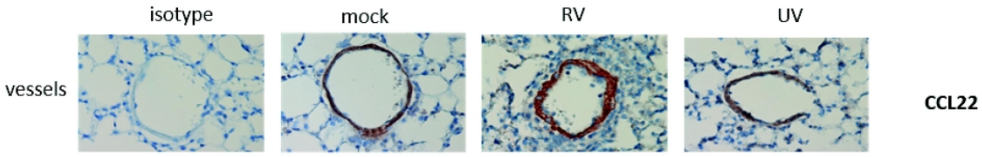
Supplementary Figure 2. RV-induced CCL22 and atopic status are not correlated in vitro. Atopic status (as determined by positive skin-prick test) of ALI-differentiated BECs were compared with CCL22 secretion levels from RV-A1 infected BECs.

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Supplementary Figure 3. Confirmation of mRNA and protein expression knockdown following NF-κB p65, IKK-β and STAT6 siRNA administration. A549 cells were either non-transfected or transfected with control (non-targeting), NF-κB p65-, IKK-β- or STAT6-targeting siRNA in duplicate wells. Messenger RNA for A p65 or B IKK-β was expressed as percentage of non-transfected cells. Verification of protein reduction following C p65, IKK-β and D STAT6 siRNA-mediated knockdown by western blot.

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Supplementary Figure 4. RV infection induces CCL22 expression in airway vessels. Mice were infected intranasally with RV-A1 or UV-inactivated RV-A1. Mock-infected were dosed intranasally with PBS. Expression of CCL22 in the airway vessels at 2 days post RV-infection, assessed by immunohistochemical staining.

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