Ruxolitinib inhibits poly (I:C) and IL-13-induced CCL5 production in bronchial epithelial cells: A potential therapeutic agent for severe eosinophilic asthma.

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Abstract

Rationale Severe eosinophilic asthma is characterized by airway eosinophilia and corticosteroid-resistance, commonly overlapping with type-2 inflammation. It has been reported that CCL5 is involved in asthma exacerbation due to RNA virus infections. We hypothesized that treatment with a virus-associated ligand and a Th2-cytokine can synergistically stimulate CCL5 production in bronchial epithelial cells. We also aimed to evaluate the mechanisms underlying CCL5 production in this in vitro model and to assess the potential of JAK1 as a novel therapeutic target via the use of ruxolitinib. Methods We stimulated primary normal human bronchial epithelial (NHBE) cells and BEAS-2B cells with poly (I:C) along with IL-13 or IL-4, and assessed CCL5 production. We also evaluated the signals involved in virus- and Th2-cytokine-induced CCL5 production and explored a therapeutic agent that attenuates the CCL5 production. Results Poly (I:C) stimulated NHBE and BEAS-2B cells to produce CCL5. Poly (I:C) and IL-13 increased CCL5 production. Poly (I:C)-induced CCL5 production occurred via the TLR3-IRF3 and IFNAR/JAK1-PI3K pathways, but not the IFNAR/JAK1-STATs pathway. In addition, IL-13 did not augment poly (I:C)-induced CCL5 production via the canonical IL-13R/IL-4R/JAK1-STAT6 pathway but likely via subsequent TLR3-IRF3-IFNAR/JAK1-PI3K pathways. JAK1 was identified to be a potential therapeutic target for severe eosinophilic asthma. The JAK1/2 inhibitor, ruxolitinib, was demonstrated to more effectively decrease CCL5 production in BEAS-2B cells than fluticasone propionate. Conclusion We have demonstrated that JAK1 is a possible therapeutic target for severe corticosteroid-resistant asthma with airway eosinophilia and persistent Th2-type inflammation, and that ruxolitinib has potential as an alternative pharmacotherapy.

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Running title: RUXOLITINIB INHIBITS CCL5 PRODUCTION IN BRONCHIAL EPITHELIUM

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Abstract Rationale

Severe eosinophilic asthma is characterized by airway eosinophilia and corticosteroid-resistance, commonly overlapping with type-2 inflammation. It has been reported that CCL5 is involved in asthma exacerbation due to RNA virus infections. We hypothesized that treatment with a virus-associated ligand and a Th2-cytokine can synergistically stimulate CCL5 production in bronchial epithelial cells. We also aimed to evaluate the mechanisms underlying CCL5 production in this in vitro model and to assess the potential of JAK1 as a novel therapeutic target via the use of Ruxolitinib.

Methods

We stimulated primary normal human bronchial epithelial (NHBE) cells and BEAS-2B cells with poly (I:C) along with IL-13 or IL-4, and assessed CCL5 production. We also evaluated the signals involved in virus- and Th2-cytokine-induced CCL5 production and explored a therapeutic agent that attenuates the CCL5 production.

Results

Poly (I:C) stimulated NHBE and BEAS-2B cells to produce CCL5. Poly (I:C) and IL-13 increased CCL5 production. Poly (I:C)-induced CCL5 production occurred via the TLR3-IRF3 and IFNAR/JAK1-PI3K pathways, but not the IFNAR/JAK1-STATs pathway. In addition, IL-13 did not augment poly (I:C)-induced CCL5 production via the canonical IL-13R/IL-4R/JAK1-STAT6 pathway but likely via subsequent TLR3-IRF3-IFNAR/JAK1-PI3K pathways. JAK1 was identified to be a potential therapeutic target for severe eosinophilic asthma. The JAK1/2 inhibitor, ruxolitinib, was demonstrated to more effectively decrease CCL5 production in BEAS-2B cells than fluticasone propionate.

Conclusion

We have demonstrated that JAK1 is a possible therapeutic target for severe corticosteroid-resistant asthma with airway eosinophilia and persistent Th2-type inflammation, and that ruxolitinib has potential as an alternative pharmacotherapy. (249 words)

Introduction

Bronchial asthma is characterized by chronic airway inflammation, leading to expiratory airflow limitation and presentation of respiratory symptoms (e.g., dyspnea and wheezing) (1). A small proportion of patients with asthma (5–10%) can be classified as having severe asthma, in which symptoms remain uncontrolled despite the administration of high-dose inhaled corticosteroids (i.e. fluticasone propionate) in combination with a second long-term controller medication (2). These patients represent a substantial economic burden owing to their symptoms, disease exacerbation, and medication-induced side effects, accounting for >60% of the medical costs associated with asthma (3). While eosinophilic asthma with persistent type-2 inflammation constitutes the most common phenotype of severe asthma, there is limited knowledge regarding the pathophysiology of refractory eosinophilic asthma.

Many reports have suggested that respiratory viral infections are associated with the onset and/or exacerbation of asthma. This is termed virus-induced asthma, and infection with a respiratory virus may be associated with >80% of asthma cases (4). Especially human rhinovirus (HRV), respiratory syncytial virus (RSV), and enteroviruses (EV) may cause virus-induced asthma (4-6). In general, the primary immune response against viruses is innate immunity (7, 8). Innate immunity in the host against RNA viruses reportedly involves various Toll-like receptors (TLR) including TLR3 and TLR4 (9), which are activated by viral RNA (7). Immune responses through the TLRs are associated with the pathophysiology of asthma, although the precise mechanisms are not fully understood.

Th2 cytokines, including interleukin (IL)-4 and IL-13, are closely related to various allergic diseases including asthma (10). Numerous reports have shown that IL-13 causes exacerbation of asthma (11-14). This cytokine is produced by Th2 lymphocytes, which act on the allergic immune cells (e.g., eosinophils and mast cells), inducing their migration from the vessel (15). Moreover, IL-13 can induce a chemokine, Chemokine (C-C motif) ligand 5 (CCL5), from various cells including airway epithelial cells (16, 17). CCL5 attracts T cells, its expression is regulated by activated T cells, and it has strong chemotactic activity for eosinophils (18). Thus IL-13 and CCL5 may be associated with asthma exacerbation, although this relationship remains unclear (10, 19, 20).

Furthermore, ruxolitinib, a Janus kinase (JAK) 1 and JAK2 subtype inhibitor, is used as a molecular targeted agent for the treatment of osteofibrosis (21, 22). TLR3, TLR4, and IL-13 may induce the phosphorylation of JAK1, resulting in allergic reactions. Thus, ruxolitinib may regulate the allergic reaction induced by TLRs and IL13 in the airway cells, leading to asthma remission.

Based on the available evidence, the objective of this study was to clarify the relationships among innate immunity induced by poly (I:C) (surrogate of RNA virus infection), allergic cytokine (IL-13), and chemokine (CCL5) in airway epithelial cells (BEAS-2B). We also examined whether ruxolitinib intervenes in these reactions.

Materials and Methods.

Reagents

It is well known that poly (I:C) stimulates innate immunity such as TLR3 (23). Thus, it was used for surrogate viral RNA such as rhinovirus (9). We also used CpG oligonucleotides (CpG-ODN), TLR9 ligand replacement of viral DNA. Both nucleic acid compounds were purchased from Novus Biologicals (Littleton, CO, United States). IL-13 and IL-4 were purchased from PeproTech (Rocky Hill, NJ, United States). IL-33 was purchased from Wako Pure Chemical (Osaka, Japan). IL-37 and CC16 (Clara cell secretory protein; a marker of bronchial lung epithelial cells) were purchased from ProSpec (East Brunswick, NJ, United States) and R&D Systems (Minneapolis, MN, United States), respectively. BAY 11-7082 (an inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB) was purchased from InvivoGen (San Diego, CA, United States). Ruxolitinib, stattic (an inhibitor of signal transducer and activator of transcription 3 [STAT3]), and LY294002 (a phosphatidylinositol kinase-3 inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI, United States). The chemotherapy agent, fludarabine, was purchased from Wako Pure Chemical. The corticosteroid, fluticasone propionate (FP), was purchased from Sigma (Saint Louis, MO, United States). A Type I interferon (IFNs) neutralizing antibody mixture was purchased from PBL Assay Science (Piscataway, NJ, United States). Small interfering RNAs (siRNAs) for TLR3, interferon regulatory factor (IRF) 3, RelA (a NF-κB subunit), JAK1, STAT6, extracellular signal-regulated kinase (ERK) 1, ERK2, Stealth RNAi siRNA Negative Control Med GC Duplex #2, and Lipofectamine RNA iMAX Reagent were purchased from Invitrogen (Carlsbad, CA, United States).

Cell Culture

BEAS-2B cells and primary normal human bronchial epithelial cells (NHBE cells) were purchased from

ATCC (Manassas, VA, United States) and Lonza (Tokyo, Japan), respectively, and were cultured according to manufacturer recommendations. BEAS-2B cells are virus-transformed human bronchial epithelial cells. Briefly, BEAS-2B cells (2.0×10^5 cells/well) and NHBE (1.0×10^4cells/well) cells were cultured with serum-free Bronchial Epithelial Growth Media (BEGM^{TM}, Lonza) to a confluence of 80-100%, which usually took 3 days. Cells were afterwards stimulated with cytokines and/or TLR ligands for 24 hours in a 24-well culture plate unless otherwise specified. For inhibitory assays, BEAS-2B cells were co-incubated with inhibitors for 2 hours prior to cytokine stimulation. To evaluate the activity of inhibitors, percent (%) inhibition at each concentration of the inhibitor was calculated using the following equation:

% inhibition = (1-A/B) x 100,

where A and B were culture-media CCL5 concentrations of BEAS-2B cells grown with poly (I:C) after pre-incubation treatment with an inhibitor and a vehicle, respectively. We then performed a curve fitting analysis using the following equation:

Y = Bottom + X * (Top - Bottom) / (EC_{50} + X)

and calculated the maximal inhibitory effect of inhibitors.

Transfection of small interfering RNAs (siRNAs)

BEAS-2B cells, grown to 60-80% confluence, were transfected with siRNAs or negative controls using the transfection reagent Lipofectamine RNA iMAX for 2-3 days. This was done according to the manufacturer's instructions and was followed by cytokine stimulation.

ELISA

CCL5 was measured using an ELISA kit (Duoset, R&D Systems), following the manufacturer's instructions.

RNA isolation and real-time PCR

Total RNA was isolated and purified by using a QIA shredder (QIAGEN, Tokyo, Japan) and RNeasy Mini kit (QIAGEN). Complementary DNA (cDNA) was synthesized using the following protocol. One microgram (μg) of total RNA, a random primer (Takara Bio, Shiga, Japan), dNTP mix (Invitrogen), and distilled water were mixed and heated to 65 for 5 minutes using the Gene Atlas 485 (ASTEC, Fukuoka, Japan). SuperScript III RT (Invitrogen), 5× First-Strand Buffer (Invitrogen), 0.1 M DTT (dithiothreitol, Invitrogen), and RNaseOUT (Invitrogen) were then added to the mixture, which was allowed to incubate at 55 for 60 minutes and again at 75 for 15 minutes using Gene Atlas 485. cDNA, QuantiTect SYBR Green PCR (QIAGEN), a specific primer, and distilled water were mixed and incubated as follows: 45 cycles at 95 for 15 seconds, 55degC for 15 seconds, and 75 for 20 seconds. The [?][?]CT (delta-delta CT) relative value method, using GAPDH as the housekeeping gene, was utilized to calculate gene expression, after which the threshold cycle numbers were obtained using Stratagene Mx3000p (Agilent Technologies, Tokyo, Japan). The sequences of the specific primers were: CCL5 forward: 5'-TGA CCA GGA AGG AAG TCA GC-3', reverse: 5'-AGC CGA TTT TTC ATG TTT GC-3', GAPDH forward: 5'-TGA ACG GGA AGC TCA CTG G-3', reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3', TLR3 forward: 5'-CTC AGA AGA TTA CCA GCC GCC-3', reverse: 5'-CCA TTA TGA GAC AGA TCT AAT G-3', ICAM-1 forward: 5'-GGC TGG AGC TGT TTG AGA AC-3', reverse: 5'-ACT GTG GGG TTC AAC CTC TG-3'.

The tetrazolium salt assay

The cytotoxic effect of ruxolitinib and FP over 24 h was evaluated using a tetrazolium salt assay, which involved 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT; Wako Pure Chemical). This was dissolved in BEBM at a concentration of 0.5 mg/mL and incubated with the cells for 2 h. After the supernatant had been removed, DMSO was added and the cells were placed on a shaking platform for 30 min. Absorbance at 570 nm was then measured for duplicate 100 μL samples using an iMark Microplate Reader.

Statistical Analysis

All data are shown as mean ± standard error of three or more replicates. When comparing two or more groups, we used the student t -test or one-way ANOVA with post-hoc Holm-Sidak's multiple tests for comparing selected pairwise measurements. P- values of less than 0.05 were considered statistically significant. All experiments were repeated at least twice, with similar results. For statistical analysis, GraphPad Prism ver. 7.00 (San Diego, CA) was used.

Results

Poly (I:C) potentiates CCL5 production in human bronchial epithelial cells

Initially, we examined whether bronchial epithelial cells produced CCL5 when stimulated with poly (I:C), a ligand for TLR3. We found that poly (I:C) enhanced CCL5 production in primary bronchial epithelial cells (Fig. 1A). In BEAS-2B cells, poly (I:C) stimulated both CCL5 protein release (Fig. 1B) and mRNA expression (Fig. 1C). For better reproducibility we used BEAS-2B cells instead of primary bronchial cells for all subsequent culture experiments. We also stimulated BEAS-2B cells with CpG-ODN, another viral ligand for TLR9, but this treatment did not augment cellular CCL5 production (Fig. 1D). These results demonstrated that viral infection enhanced CCL5 production in bronchial epithelial cells.

Poly (I:C)-induced CCL5 production is further enhanced by the presence of Th2 cytokines

Next, we examined whether the presence of Th2-type cytokines further stimulated poly (I:C)-induced CCL5 production in bronchial epithelial cells. IL-13 enhanced poly (I:C)-induced CCL5 production (Fig. 1E) and mRNA expression (Fig. 1F) in BEAS-2B cells. IL-4 also augmented poly (I:C)-induced CCL5 production (Fig. 1G), even though neither IL-13 nor IL-4 alone stimulated CCL5 production in BEAS-2B cells (Fig. 1E, G). IL-33, IL-37, and CC16 in combination with poly (I:C) all failed to stimulate CCL5 production (Supp. Fig. 1). We also examined whether IL-13 enhanced poly (I:C)-induced CXCL8 production, but it did not (Supp. Fig. 2). Together, these data show that poly (I:C) plus IL-13 or IL-4 synergistically induced CCL5 production in bronchial epithelial cells. This experimental setup thus provides an *in vitro* model of severe eosinophilic asthma associated with viral infection and persistent type-2 inflammation.

Signal transduction mechanisms in BEAS-2B cells stimulated with poly (I:C) and IL-13

Using si-RNA techniques and inhibitors, we investigated the signal transduction mechanisms that may be involved in CCL5 production of BEAS-2B cells after stimulation with poly (I:C) and IL-13.

We first examined molecular signals for CCL5 production as induced by poly (I:C) alone. TLR3- and IRF3-knockdown strongly inhibited poly (I:C)-induced CCL5 production (Fig. 2A, B), whereas Rel A-knockdown and NF-κB inhibitor (BAY11-7082) did not affect CCL5 production (Fig. 2C, D). The neutralizing antibody against type I IFNs mixture inhibited poly (I:C)-induced CCL5 production (Fig. 2E). Next, we assessed pathways that were associated with the interferon receptor. We found that si-JAK1 and the JAK1 inhibitor, ruxolitinib, attenuated poly (I:C)-induced CCL5 production (Fig. 2F, G). However, inhibitors for STAT1 and STAT3 did not affect poly (I:C)-induced CCL5 production (Fig. 2H, I). These findings suggest that canonical type I interferon receptor (IFNAR)/JAK-STAT-associated pathways were not involved in the activation of poly (I:C)-induced CCL5 production.

We subsequently assessed alternative pathways, including PI3K and Erk1/2, and we found that PI3K, but not Erk1/2, is involved in poly (I:C)-induced CCL5 production (Figs 2J, K). Therefore, the TLR3-IRF3-

IFNAR/JAK1-PI3K cascade played an important role in poly (I:C)-induced production of CCL5 in bronchial epithelial cells.

Next, we evaluated the role of IL-13 receptor-associated signals in cells treated with both IL-13 and poly (I:C). Although the canonical IL-13 receptor signal activated the IL-4Rα/IL-13Rα1/JAK1-STAT6 pathway, STAT6-knockdown failed to inhibit the synergistic effect observed from poly (I:C) and IL-13 (Fig. 3A), suggesting that an alternative pathway was involved. Since IL-13 also activated the IL-13Rα2-PI3K-AKT pathway (24), and we noted that the PI3K inhibitor attenuated CCL5 production (Fig. 3B), it is likely that the IL-13Rα2-PI3K-AKT pathway is implicated in bringing about the synergistic effect observed. IRF3- and JAK1- knockdowns (Fig. 3C, D) and treatment with the JAK1/2 inhibitor ruxolitinib (Fig. 3E) also attenuated CCL5 production, further confirming that the TLR3-IRF3-IFNAR/JAK1-PI3K pathway is involved.

Together, these data demonstrate that IL-13 plus poly (I:C) synergistically induced the production of CCL5 in BEAS-2B cells via the TLR3-IRF3-IFNR/JAK1-PI3K-AKT and IL-13Rα2-PI3K pathways (Supp. Fig. 3).

Ruxolitinib is a potential therapeutic agent for severe eosinophilic asthma.

Based on our data, the IFNAR/JAK1-PI3K pathway was a key regulator of CCL5 production in our *in vitro* model of severe eosinophilic asthma with persistent type-2 inflammation. Ruxolitinib, a JAK1 inhibitor, is already clinically available. Hence, we assessed the therapeutic potential of ruxolitinib in comparison with FP, as measured by the ability of both drugs to inhibit CCL5 production after induction with both poly (I:C) and IL-13.

We first conducted a dose-response-inhibition experiment, followed by a curve-fitting analysis. These experiments estimated that the maximal inhibitory effects of ruxolitinib and FP against poly (I:C) induced-CCL5 production were 73.4% and 41.7% (Fig. 4A, B), respectively. We then conducted a head-to-head comparison between ruxolitinib (10μM) and FP (1μM). The selected concentrations of these drugs were based on the maximal non-toxic concentrations as determined by preliminary cell-toxicity experiments (Supp. Fig.4). We found that ruxolitinib (10μM) was a stronger inhibitor of CCL5 production than FP (1μM) (Fig. 4C). The ruxolitinib activity in this *in vitro* model supports the hypothesis that ruxolitinib is a better therapeutic option than FP for managing eosinophilic asthma with type-2 inflammation.

Discussion

In the present study, we found that poly (I:C) and IL-13 synergistically enhanced the production of CCL5 in bronchial epithelial cells. We regarded this experimental setup as a characteristic *in vitro*model of airway disease, because CCL5 production is clinically observed in patients with repeated viral infections, eosinophilic asthma, and persistent Th2-type inflammation. The production of CCL5, induced by poly (I:C) plus IL-13, was regulated by the TLR3-IRF3-IFNAR/JAK1-PI3K pathway; this may be due to activation of the IL-13Rα2-PI3K pathways. This prompted our trial of ruxolitinib, a clinically available JAK1 inhibitor. We confirmed that ruxolitinib was a better inhibitor than FP for decreasing the synergistic production of CCL5 *in vitro* . Hence, ruxolitinib is a potential therapeutic agent for corticosteroid-resistant severe eosinophilic asthma with a persistent type-2 inflammation phenotype. This is the first observation of the potential role of ruxolitinib in this setting.

Exacerbation of asthma is strongly associated with various RNA virus infections, including rhinovirus, RSV, and EV (4, 22). Clinical and experimental data demonstrate that asthmatics have deficient immune responses to viruses and show higher viral loads and greater airway inflammation after viral infections than healthy subjects (25-27). The airway viral load in asthmatics correlates strongly with the severity of symptoms,

hyperresponsiveness, and airflow limitations (26, 28), and is associated with airway eosinophilia. Furthermore, latent infection resulting from rhinovirus has been observed in 73% of stable asthmatics, in whom viral infection has been associated with eosinophilic lung infiltration and decreased lung function (29). In this study, we confirmed that poly (I:C), an RNA virus-related TLR3 ligand, stimulated CCL5 production in bronchial epithelial cells. Taken together, it can be stated that patients with asthma are highly susceptible to viral infections, and both active and latent virus infection enhance airway eosinophilia and increase disease severity.

Eosinophilic airway infiltration plays a major role in the pathogenesis of asthma. Airway eosinophilia is associated with recurring bronchial hyperresponsiveness and airflow limitations that account for the pathogenesis and the severity of asthma, respectively (30). Airway eosinophil counts in asthmatics are higher than in healthy subjects, and are further elevated after viral infection (26). Moreover, viral infections cause the release of IL-33 from bronchial epithelial cells, which enhances IL-13 and IL-5 production in Th0 cells and ILC-2s (26), providing a mechanism by which eosinophilic and Th2-type asthma commonly overlap. We have demonstrated that the virus-associated TLR3 ligand, along with either IL-13 or IL-4, enhanced CCL5 production in bronchial epithelial cells. Because a genome-wide association study and experiments using mouse asthma models have indicated that IL-13 contributes more strongly to the pathogenesis of arising asthma than does IL-4 (31, 32), we used IL-13 instead of IL-4 for the rest of the experiments. These findings demonstrate that viral infection in Th2-type asthma results in a prominent increase in airway CCL5 production, which offers a potential mechanism through which severe eosinophilic asthma develops.

CCL5 is a major eosinophil chemotactic molecule that exists in greater volumes in the lungs of asthmatics than in those of non-asthmatics (33, 34). Asthmatics show higher CCL5-mRNA expression in bronchial mucosal cells than in control non-asthmatic subjects (35). Experimental allergen challenge in asthmatics also causes an increase in the airway CCL5 level, which correlates positively with airway eosinophil counts (36). Airway CCL5 levels correlate positively with asthma severity, as measured by the percent predicted forced expiratory volume in 1 second (FEV1) (34). CCL5 levels are higher in severe asthmatics than in untreated mild asthmatics (37). Additionally, in a mice model, viral infection enhances airway CCL5 levels (38). Our *in vitro* study has demonstrated that poly (I:C) plus Th2-type cytokines synergistically stimulates CCL5 production in bronchial epithelial cells. The simulation of viral infection and Th2-type inflammation results in increased CCL5 production in bronchial epithelial cells, as is observed in humans. As such, we consider this experimental setup a representative *in vitro* model of severe eosinophilic asthma.

Multiple signal-transduction pathways are involved in virus-triggered CCL5 production in bronchial epithelial cells. ds-RNA binds to TLR3 and activates downstream NF-κB and IRF3 cascades, leading to CCL5 production (39). NF-κB directly activates a variety of genes associated with inflammation (40), whereas IRF3 induces gene expression of IFNs, which in turn leads to the expression of ISGs via receptors for type I IFN (41). Our data demonstrate that IRF3-knockdown strongly inhibited CCL5 production while Rel A-knockdown and BAY11-7082 failed to inhibit CCL5 production in BEAS-2B cells. These results show that the TLR3 ligand stimulated the BEAS-2B cells to produce CCL5 via IRF3 but not NF-κB.

Øvrevik et al. have also reported that Rel A-knockdown did not attenuate poly (I:C)-induced CCL5 production in BEAS-2B cells (42). However, other reports showed that poly (I:C) stimulated BEAS-2B cells to produce CCL5 via both the NF-κB and IRF3 pathways (43, 44). The reason for the discrepancy between our data and the existing literature remains unclear. There are two possible explanations for these differences: (1) we and Øvrevik et al.'s group used serum-free medium for culturing BEAS-2B cells, whereas others have used medium containing fetal bovine serum (42-44); and (2) the quality or purity of the poly (I:C) used may have differed between studies. The former is the more likely explanation, because the presence of fetal bovine serum leads to NF-κB activation (45). The latter is less likely, since poly (I:C) is a synthesized molecule. Nevertheless, our data agreed with previously demonstrated findings that show that the TLR3-IRF3 axis plays an important role in poly (I:C)-induced CCL5 production in bronchial epithelial cells.

TLR3-induced CCL5 production is mediated by type I IFNs. IRF3 drives the expression of the gene encoding IFN and subsets of ISGs(46). Type I IFNs bind to the interferon-α/β receptor (IFNAR), which is

composed of IFNAR1 and IFNAR2 (bearing JAK1 and TYK2, respectively), and activates multiple canonical pathways (i.e. STAT1-STAT2-IRF9, STAT1-STAT1, and STAT3-STAT3 mediated) (47). In addition, it has been also reported that IFNAR activates alternative cascades mediated by the PI3K-AKT and Erk 1/2 pathways (48), and another study has reported that type I IFN activates PI3K in JAK1-dependent manner (49). Our results have demonstrated that poly (I:C)-induced CCL5 production was attenuated by the neutralizing antibody against type I IFNs, JAK1-knockdown, the JAK1/2 inhibitor ruxolitinib, and the PI3K inhibitor LY294002, but not by inhibitors for STAT1, STAT3, or Erk 1/2. These findings indicate that poly (I:C)-induced CCL5 production is stimulated by TLR3-IRF3-IFNAR/JAK1-PI3K pathway but not by the canonical IFNAR/JAK-STAT pathway in BEAS-2B cells.

IL-13 and IL-4 both have augmented poly (I:C)-induced CCL5 production in BEAS-2B cells. The canonical receptor for IL-13 is a type II receptor complex that consists of IL-4Rα and IL-13Rα1 anchoring JAK1 and TYK2, respectively (50). The receptor for IL-4 receptor is a type I receptor complex that includes IL-4Rα and a common γ-chain that bears JAK1 and JAK3 (50), which also activates the PI3K pathway in JAK1-dependent manner (49, 51). The non-canonical IL-13 receptor is IL-13Rα2, which lacks JAKs and TYK2 (50) but activates the PI3K and Erk 1/2 pathways (52, 53).

Contrary to expected findings, IL-13 and poly (I:C)-induced CCL5 production was independent of STAT6 but was dependent on PI3K, indicating that the synergy of the two stimulating cytokines was possibly induced by the IL-13Rα2-PI3K pathway. This is consistent with a previous report whereby PI3K was found to bind to TLR3, which subsequently phosphorylated IRF3: an essential step for TLR3-IRF3-mediated gene induction (54). This signaling cascade can explain our observation that IL-13-augmented poly (I:C)-induced CCL5 production, while IL-13 alone did not stimulate CCL5 production. Moreover, the IFNAR/JAK1-PI3K pathway is also involved downstream of the TLR3-IRF3 pathway (49). Therefore, the JAK1-PI3K pathway is a key regulator for synergistic CCL5 production, which is a potential therapeutic target for severe asthma.

In our *in vitro* model of severe eosinophilic asthma that used IL-13 and poly (I:C), the JAK1-PI3K pathway played a pivotal role in CCL5 production. The JAK1 inhibitor, ruxolitinib, is already clinically available for treating myelofibrosis (21, 22). We demonstrated that ruxolitinib could more strongly decrease poly (I:C) and IL-13-induced CCL5 production compared with the corticosteroid, FP. These findings suggested that ruxolitinib may be used for treating severe asthma and is thus a potential therapeutic agent for corticosteroid-resistant severe eosinophilic asthma.

Despite recent advances in medication for bronchial asthma, approximately 10% of those diagnosed have uncontrolled symptoms (3). A common phenotype of severe asthma includes persistent type-2 inflammation, which is characterized by sputum eosinophilia, high doses of inhaled corticosteroids, severe airflow limitations, and airway hyperresponsiveness (3). We demonstrated, using an *in vitro*model, that ruxolitinib is potentially beneficial for treating severe eosinophilic asthmatics who require high doses of inhaled corticosteroids. Previous studies have shown that JAK1 is involved in many signaling cascades of IFNs, growth factors, and cytokines (55). This suggests that ruxolitinib has a possible advantage, given its ability to inhibit multiple pathways, versus monoclonal antibody therapies that target only a single molecule. Furthermore, we demonstrated that ruxolitinib is a more effective inhibitor of CCL5 production than FP, which is also used in inhaled corticosteroids. This indicates that ruxolitinib inhalation therapy may have therapeutic potential, since it carries a lower risk of systemic toxicity than FP, although further research is required to confirm this.

Our study has some limitations. First, we used a cell line, BEAS-2B, in most experiments, although we initially used primary bronchial cells derived from healthy subjects and not asthmatic patients. As such, it is important to verify the efficacy of ruxolitinib in animal models of asthmatic disease. Second, although we found that the IL-13Rα2- PI3K pathway was implicated in the observed effects of poly (I:C) and IL-13, we did not investigate the impact of IL-13R α2 si-RNAs or inhibitors on this *in vitro* system. The effectiveness of ruxolitinib, however, in this cellular model of eosinophilic asthma is novel and encouraging and warrants additional exploration. In future we plan to conduct the following investigations: (1) evaluate the efficacy of ruxolitinib in a mouse asthma model; and (2) investigate the potential of ruxolitinib inhalation therapy,

using intratracheal administration in animal models.

In conclusion, by treating BEAS-2B cells with poly (I:C) and IL-13, we developed an *in vitro* model of severe eosinophilic asthma with persistent type-2 inflammation, as evidenced by increased CCL5 production. The efficacy of the JAK1 inhibitor ruxolitinib in inhibiting CCL5 production in this *in vitro* model suggests that ruxolitinib has therapeutic potential in severe eosinophilic asthma, which requires high-dose inhaled corticosteroids. Further evaluation in animal models and clinical studies are necessary to confirm the suitability of ruxolitinib in patients.

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Conflict of Interest

There were none of conflict of interest in this study.

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Figure legends

Figure 1. Poly (I:C) stimulates CCL5 production in bronchial epithelial cells.

(A) NHBE cells were stimulated with poly (I:C) for 24h, and CCL5 levels were measured in the culture supernatant. (B–D) BEAS-2B cells were stimulated with poly (I:C) or CpG-ODN as indicated for 24 hours (B, D) or 12 hours (C), and the CCL5 concentration in the culture supernatant (B, D) and CCL5 mRNA expression (C) were evaluated.

(E–G) BEAS-2B cells were stimulated with poly (I:C), IL-13, and IL-4, as indicated, for 24 hours (E, G) or 12 hours (F), and the CCL5 concentration in the culture supernatant (E, G) and CCL5 mRNA expression (F) were evaluated.

$^{*}P < 0.05$, $^{**}P < 0.01$, as compared to medium alone, using one-way ANOVA with post-hoc Holm-Sidak's multiple tests to conduct selected pairwise comparisons.

pIC, poly (I:C).

Figure 2. Signal transduction mechanisms in poly (I:C)-induced CCL5 production in BEAS-2B cells.

(A–D) Of the TLR3-related signals, si-TLR3 (A) and si-IRF3 (B), but neither NF-ϰb inhibitor BAY117082 (C) nor si-RelA (D) inhibited poly (I:C)-induced CCL5 production. (E–I) In type I IFN-related signals, neutralizing anti-type I IFN antibody mixture (E), si-JAK1 (F), and JAK1/2 inhibitor ruxolitinib (G), but neither STAT1 inhibitor fludarabine (H) nor STAT3 inhibitor Stattic (I) attenuated poly (I:C)-induced

CCL5 production. (J, K) In alternative signals, PI3K inhibitor LY294002 (J) but not si-Erk1/2 (K) reduced poly (I:C)-induced CCL5 production. For all experiments, BEAS-2B cells were transfected with si-RNAs for 2 days (A–B, D, F, K) or pre-incubated with inhibitors for 2 hours (C, E, G–I). Afterwards, these were stimulated with poly (I:C) (0.1 μg/ml) for 24 hours, followed by measurement of CCL5 concentrations in the culture supernatant (A–I)

*$P < 0.05$, **$P < 0.01$, as compared to medium alone. We used student$t$ -tests (A, B, D, F, K) or one-way ANOVA with post-hoc Holm-Sidak's multiple tests to conduct selected pairwise comparisons of treatments (C, E, G-I).

pIC, poly (I:C).

Figure 3. Signal transduction mechanisms in poly (I:C) and IL-13-induced CCL5 production in BEAS-2B cells.

(A–E) Poly (I:C) and IL-13-induced CCL5 production was not reduced with si-STAT6 (A) but was inhibited with the PI3K inhibitor, LY294002 (5μM, B). (C–E) si-IRF3 (C), and si-JAK1 (D). The JAK1/2 inhibitor, ruxolitinib (10μM, E), also reduced poly (I:C) and IL-13-induced CCL5 production. BEAS-2B cells were pre-incubated with siRNA for 2 days (A, C, D) or with inhibitors for 2 hours (B, E), followed by stimulation with poly (I:C) (0.1 μg/ml) for 24 hours.

*$P < 0.05$, **$P < 0.01$, as compared to medium alone. We used student t -tests (A, C, D) or one-way ANOVA with post-hoc Holm-Sidak's multiple tests to conduct selected pairwise comparisons of treatments (B, E).

pIC, poly (I:C).

Figure 4. Ruxolitinib is a stronger inhibitor than fluticasone propionate for reducing CCL5 in BEAS-2B cells treated with poly (I:C) and IL-13.

(A-B) BEAS-2B cells were pre-incubated with ruxolitinib (A) or fluticasone propionate (B) for 2 hours, followed by stimulation with poly (I:C) (0.1 μg/ml). The maximal percentage (%) inhibition of ruxolitinib and fluticasone propionate against poly (I:C)-induced CCL5 production was 73.4% and 41.7%, respectively. (C) BEAS-2B cells were pre-incubated with medium alone (control), fluticasone (FP, 1μM), ruxolitinib (Ruxo, 10μM), or both, followed by stimulation with poly (I:C) with and without IL-13.

$P < 0.05$, **$P < 0.01$, as compared to medium alone. We used one-way ANOVA with post-hoc Holm-Sidak's multiple tests to conduct selected pairwise comparisons of treatments.

pIC, poly (I:C).