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CCL7 and IRF-7 Mediate Hallmark Inflammatory and IFN Responses following Rhinovirus 1B Infection

Jason Girkin,*† Luke Hatchwell,*† Paul Foster, † Sebastian L. Johnston,‡ Nathan Bartlett, ‡ Adam Collison,*† and Joerg Mattes*, ‡§

Rhinovirus (RV) infections are common and have the potential to exacerbate asthma. We have determined the lung transcriptome in RV strain 1B–infected naïve BALB/c mice (nonallergic) and identified CCL7 and IFN regulatory factor (IRF)-7 among the most upregulated mRNA transcripts in the lung. To investigate their roles we employed anti-CCL7 Abs and an IRF-7–targeting small interfering RNA in vivo. Neutralizing CCL7 or inhibiting IRF-7 limited neutrophil and macrophage influx and IFN responses in nonallergic mice. Neutralizing CCL7 also reduced activation of NF-κB p65 and p50 subunits, as well as airway hyperreactivity (AHR) in nonallergic mice. However, neither NF-κB subunit activation nor AHR was abolished with infection of allergic mice after neutralizing CCL7, despite a reduction in the number of neutrophils, macrophages, and eosinophils. IRF-7 small interfering RNA primarily suppressed IFN-α and IFN-β levels during infection of allergic mice. Our data highlight a pivotal role of CCL7 and IRF-7 in RV-induced inflammation and IFN responses and link NF-κB signaling to the development of AHR.
Mice were sensitized and challenged to HDM followed by infection with RV, as previously reported (10, 11). Briefly, mice were treated intranasally under light isoflurane anesthesia with HDM (50 μg/50 μl in sterile saline) daily for 3 d during sensitization. Mice were then intranasally challenged 12 d later, with HDM (5 μg/50 μl) during 4 d to induce AAD. Nonsensitized mice (saline) received sterile endotoxin-free saline only. Allergic (HDM) mice were intranasally infected with RV (50 μl containing 1 × 10⁷ virions) or UV-inactivated RV 24 h after the last HDM challenge to exacerbate preexisting AAD. Mice were euthanized 24 h after the RV infection at the peak of host response. Twenty-four hours postinfection was chosen for our analyses, as innate immune responses induced by RV1B in BALB/c mice peaks 24 h postinfection and RV infection is quickly resolved (12) by 4 d postinfection.

AHR measurement

AHR was measured as previously described (13–15). Briefly, mice were anesthetized with ketamine-xylene combination (Illum) and total lung resistance and dynamic compliance were measured invasively (Buxco). Mice were mechanically ventilated and increased lung resistance to nebulized methacholine was expressed as percentage change from control (baseline).

Analysis of bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea of euthanized mice and flushing the airways twice with 1 ml HBSS. BALF was centrifuged at 800 x g for 10 min at 4˚C. Pelleted cells were

![Figure 1](http://www.jimmunol.org/) Neutralization of CCL7 suppresses RV-induced AHR and lung inflammation. BALB/c mice infected with RV were assessed for CCL7 expression 24 h postinfection. Mice were then treated with anti-CCL7 Abs and subsequently infected with RV and sacrificed 24 h postinfection. (A) CCL7 mRNA upregulation validated by qPCR. (B) Levels of chemokines in whole-lung homogenates as assessed by ELISA. (C) Total lung resistance presented as percentage change in response to methacholine (n = 6–8). (D) Cell populations present in BALF as assessed by May–Grünewald–Giemsa staining. Results are means ± SEM at 1 d postinfection (n = 3–5 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001 as compared with isotype control group.

![Figure 2](http://www.jimmunol.org/) Neutralization of CCL7 alters RV-induced MAPK, NF-κB activity, and IFN production. BALB/c mice were infected with RV following administration of anti-CCL7 Abs and sacrificed 24 h postinfection. (A) Levels of p-ERK1 and (B) p65 and p50 in lung homogenates as assessed by ELISA. (C) mRNA expression of IRF-7, IRF-5, and IRF-3 and (D) positive-strand RV1B RNA in blunt dissected airway tissue as assessed by qPCR. (E) IFN-α and IFN-β in lung homogenates as assessed by ELISA. (F) Numbers of pDCs and alveolar macrophages present in mouse lungs determined by flow cytometry. All results are means ± SEM at 1 d postinfection (n = 3–6 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as compared with isotype control group. *p < 0.05 (one-tailed) as compared with isotype control.
treated with RBC lysis buffer (12 mM NaHCO3, 0.1 mM EDTA, 155 mM NH4Cl [pH 7.35]) for 5 min and centrifuged as above. Cell pellets were resuspended in 100 μl HBSS and total number of viable cells was determined by trypan blue exclusion in a hemocytometer. Following a cytopsin, slides were stained with May–Grünewald–Giemsa, blinded, and differential cell counts were determined from a count of 200 cells per slide.

**Histology**

An excised lung lobe was fixed in 10% neutral buffered formalin for 24 h and then stored in 70% ethanol. Carbol’s chromotrope-hematoxylin and periodic acid–Schiff (PAS) stains of 5-μm slices of fixed lungs were used for semiquantitative histopathologic scoring (range, 0–10) as previously described (16). Scores are a sum of perivascular edema (0, absent; 1, mild to moderate, present in <25% of the perivascular spaces; 2, moderate to severe, in 25–75% of perivascular spaces; or 3, severe, present in >75% of perivascular spaces), perivascular/peribronchial acute inflammation (0, absent; 1, perivascular edematous inflammation with fewer than five neutrophils per high-power field; 2, moderate perivascular inflammation, involving some peribronchial spaces, with more than five neutrophils per high-power field; or 3, severe, acute perivascular and peribronchial inflammation with numerous neutrophils encircling >50% bronchioles), bronchiole goblet cell metaplasia (0, absent; 1, two or fewere bronchioles with fewer than five goblet cells; or 2, large numbers of goblet cells present), and inflammation in alveolar spaces (0, absent; 1, present in 25–75% of alveolar spaces; or 2, present in >25% of alveolar spaces). Images representative of all mice in a group were taken from tissue sections using Image-Pro Plus software and enhanced in Adobe Photoshop CS5.1 to reduce brightness (~30) and enhance contrast (+100).

**Quantitative RT-PCR**

Thoracic contents were extracted from euthanized mice and forceps were used to separate airways from the parenchyma by blunt dissection (17), resulting in the isolation of several generations of airway tissue. TRIzol (Ambion, Carlsbad, CA) was used to extract airway mRNA and concentrations were determined by spectrophotometry (NanoDrop) for reverse transcription with BioScript (Bioline, Alexandria, NSW, Australia) to generate cDNA. Quantitative PCRs (qPCRs) were performed according to manufacturer’s instructions with SYBR Green (Invitrogen, Mulgrave, VIC, Australia) using the primer sequences in Supplemental Table I. Copy numbers of target genes were obtained by referencing CTS to a standard curve of known concentration. All values were normalized to the endogenously expressed control gene, hypoxanthine phosphoribosyltransferase.

**Flow cytometry**

Mouse lung cells were mechanically extracted using gentleMACS tubes as per manufacturer’s instructions and stained with anti–CD11c-FITC, anti–CD11b-PerCP (BD Pharmingen), anti–MHC class II-PE, and anti–F4/80-allophycocyanin (eBioscience) to determine myeloid dendritic cell and macrophage numbers or anti–mPDCA-1-allophycocyanin to determine plasmacytoid DC (pDC) numbers (Miltenyi Biotec). We determined numbers of positive cells by flow cytometry (FACSCanto, Becton Dickinson).

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**FIGURE 3.** Inhibiting IRF-7 suppresses RV-induced IFN responses and BALF cell recruitment. BALB/c mice infected with RV following administration of IRF-7 targeting siRNA and sacrificed 24 h postinfection. (A) mRNA expression of IRF-7, IRF-5, and IRF-3 in blunt dissected airway tissue as assessed by qPCR. (B) IFN-α and IFN-β in lung homogenates as assessed by ELISA. (C) Copy numbers of positive-strand RV1B RNA from blunt dissected airway tissue as assessed by qPCR. (D) Total lung resistance presented as percentage change in response to methacholine (n = 6). (E) Cell populations present in the BALF, as assessed by May–Grünwald–Giemsa staining. (F) Levels of chemokines, (G) p-ERK1, and (H) active NF-κB p65 and p50 as assessed by ELISA. All results are means ± SEM at 1 d postinfection (n = 3–6 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001 as compared with nonsense control group.
Quantification of lung chemokines, p-ERK1, and active NF-κB subunits

A single excised lung lobe from each mouse was snap frozen before homogenization in buffers and protease inhibitors as recommended in manufacturers‘ instructions. Levels of CCL7 (eBioscience), CCL11, CCL20, CXCL2 (R&D Systems), and p-ERK1 were determined in clarified lung lysates by ELISA. Quantification of activated NF-κB subunits was performed with a TransAM NF-κB transcription factor assay (Active Motif). All concentrations were normalized to lung lobe weight.

Statistical analyses

All graphs in figures were created in GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA) are expressed as means ± SEM. A Student t test or two-way ANOVA was used as appropriate with an α value of 0.05 for all analyses. All significantly different results shown are a comparison with the isotype or nonsense control, unless otherwise stated.

Results

CCL7 is the most significantly upregulated gene induced by RV infection, and inhibition of CCL7 reduces inflammation and AHR

CCL7 is the most significantly upregulated gene at the peak of RV infection in vivo (www.ebi.ac.uk/arrayexpress, access no. E-MTAB-2826), which was confirmed by qPCR (Fig. 1A) and ELISA (Fig. 1B). Therefore, we administered a neutralizing Ab against CCL7 and subsequently infected BALB/c mice with RV. Twenty-four hours after infection, the peak of viral load, and host response, mice were sacrificed for analyses. Anti-CCL7 treatment did not affect CXCL2 (also known as MIP2-α) release, demonstrating that the observed effects were specifically due to CCL7 inhibition (Fig. 1B). Anti-CCL7 suppressed AHR induced by RV (Fig. 1C), concurrent with a decrease in neutrophil and macrophage numbers in BALF (Fig. 1D).

CCL7 mediates proinflammatory transcription factors and antiviral responses

Intrapleural administration of anti-CCL7 24 h prior to RV infection suppressed levels of the phosphorylated MAPK ERK1 and active NF-κB p65 and p50 (Fig. 2A, 2B). Anti-CCL7 treatment reduced expression of IFN-γ and IFN-5 mRNA, but not IFN-3 mRNA (Fig. 2C), and it dampened IFN-β release (Fig. 2E). Anti-CCL7 did not affect viral titer as determined by qPCR of positive-strand RV1B RNA (Fig. 2D). To explore the relationship between CCL7, IRF-7 and IFN-β, we assessed pDCs and macrophage populations in the lung by flow cytometry. Flow cytometric analysis of pDCs revealed that anti-CCL7 did not reduce the number of pDCs recruited by RV (Fig. 2F). However, alveolar macrophage numbers in lung tissue were reduced to baseline by aCCL7 (Fig. 2F).

IRF-7 mediates type 1 IFN production and inflammation in response to RV infection

To explore the role of IRF-7 upregulation during RV infection, we administered IRF-7 siRNA intranasally 24 h prior to RV infection. IRF-7 siRNA successfully suppressed IRF-7 mRNA expression at 24 h postinfection without affecting other IRFs, such as IRF-5 or IRF-3 (Fig. 3A). This resulted in complete suppression of IFN-α and IFN-β production, but, similar to inhibition of CCL7 and subsequent impaired IRF-7 expression, it had no effect on RV titer...
IRF-7 siRNA treatment reduced macrophage and neutrophil numbers in the BALF (Fig. 3E), but this did not result in a significant reduction in AHR compared with RV-infected mice that received nonsense siRNA control (Fig. 3D). IRF-7 siRNA treatment did not affect CCL7 or CXCL2 production or reduce levels of p-ERK1 or active NF-κB subunits (Fig. 3F–H).

CCL7 mediates airways inflammation during RV-induced exacerbation of AAD

To investigate the role of CCL7 in RV-induced exacerbations of AAD, we sensitized and challenged mice to HDM and on the day of final challenge, 24 h prior to RV infection, mice received anti-CCL7 i.p. CCL7 was upregulated in mice treated with an isotype control Ab and was successfully neutralized in the lung after anti-CCL7 treatment (Fig. 4A) and resolved 4 d postinfection along with clearance of RV infection (Supplemental Fig. 1). CCL7 inhibition also downregulated CCL20 (also known as MIP3-α), but not CXCL2 (Fig. 4A) or CCL11 (also known as eotaxin-1) (Fig. 4B). BALF neutrophils, macrophages, and eosinophils were reduced with anti-CCL7 (Fig. 4C). However, anti-CCL7 treatment did not ameliorate AHR (Fig. 4D). Furthermore, NF-κB p65 and p50 activity remained elevated in anti-CCL7–treated allergic mice (Fig. 4E). CCL7 neutralization during RV infection of allergic mice did not affect IRF-7, type I IFN mRNA, or RV1B copy numbers (Supplemental Fig. 2).

IRF-7 promotes IFN production during RV-induced exacerbation of AAD

Next, we investigated the effects of IRF-7 inhibition in RV-induced exacerbation of AAD. We sensitized and challenged mice to HDM and on the day of final challenge, 24 h prior to RV infection, mice received IRF-7 siRNA intranasally. IRF-7 mRNA expression was significantly induced 24 h following RV infection and resolved 4 d postinfection (Supplemental Fig. 1). IRF-7 siRNA effectively reduced IRF-7 mRNA, resulting in abrogated type 1 IFN responses with no effect on viral titer (Fig. 5A–C). Unlike anti-CCL7, IRF-7 siRNA treatment did not suppress inflammation or chemokine production in the context of an RV-induced exacerbation (Fig. 5D–H).

Neither CCL7 neutralization nor IRF-7 inhibition affected lung histopathology

We employed histopathological scoring of 5-μm sections of fixed mouse lungs to evaluate pulmonary inflammation in allergic mice based on the presence of perivascular edema, perivascular and peribronchiolar inflammation, mucus production, and alveolar inflammation. Allergic mice displayed high numbers of periodic acid–Schiff+ cells as well as perivascular and peribronchiolar inflammatory infiltrates and, in some cases, alveolar inflammation. Representative images of RV exacerbation groups are shown in Fig. 6A. RV infection of allergic mice did not increase pulmonary histopathological scores compared with allergic mice given UV control. Furthermore, inhibiting IRF-7 or neutralizing CCL7 during RV exacerbation did not affect pulmonary histopathology scores, although all allergic mice were scored significantly higher than saline controls (Fig. 6B, 6C).

Discussion

Our study has dissected the role of CCL7 and IRF-7 in RV-induced inflammatory and antiviral responses.

CCL7 activates CCR2, which is expressed on neutrophils to mediate chemotaxis (18). It has previously been shown that CCL7 also binds to CCR3 on eosinophils to induce chemotaxis and activation (19). Airway epithelial cells and macrophages produce...
CCL7, which may orchestrate cell recruitment in the context of oxidative stress (20, 21). Induction of CCL7 during viral infections has been observed in asthmatic patients (22) and in models of RV infection dissecting the role of CCL2 (23). Our study significantly extends these findings by showing that CCL7 inhibition limited macrophage and neutrophil influx into the lung and the development of AHR in nonallergic mice, which was associated with suppression of the MAPK signaling factor ERK1 and NF-κB subunits p50 and p65. In contrast, AHR and NF-κB activation was not significantly reduced by CCL7 inhibition in allergic mice although inflammation was limited. Thus, our data suggest an association between NF-κB activation and AHR, which has been reported previously (10, 11, 24). NF-κB promotes Th2 polarization and subsequent IL-13 production as well as IgE production from B cells, resulting in mast cell degranulation after allergen-specific IgE cross-linkage, both of which directly result in propagation of AHR in the context of allergy (24–28). In the context of RV exacerbations, we have shown previously that by restoring protein phosphatase 2A (PP2A) activity, levels of NF-κB subunits were reduced, which protected against AHR (11). Furthermore, when we inhibited PP2A activity with an siRNA targeted to PP2Aα (the active or catalytic subunit of the polypeptide) in these experiments, both NF-κB activity and AHR were restored (10).

We showed that CCL7-mediated inflammation is not essential for the development of RV-induced AHR in allergic airways disease. Dissociation between AHR and inflammation has been previously reported in other models of allergic airways disease (29) and in subjects with asthma (24, 30).

Interestingly, CCL7 neutralization in RV infection also suppressed IRF-7 and IFN-β expression, which was associated with reduced macrophage but not pDC numbers in the lung. A role of CCL7 in macrophage recruitment is further highlighted by the observation of a correlation between nasal CCL7 production and the recruitment of macrophages in children with virus-induced asthma (31). A previous report showed IFN-α and IFN-β production by macrophages upon norovirus infection, which was abolished in IRF-3 and IRF-7 double knockout cells (32). It is therefore possible that CCL7 increases IRF-7 expression and IFN-β production in the lung by regulation of macrophage inflammation upon RV infection.

To elucidate the role of IRF-7 in RV infection, we employed siRNA-mediated inhibition. IRF-7 is thought to be a master regulator of type 1 IFN production (33–36), and induction of IRF-7 mRNA has been reported previously (37) in asthmatic children during periods of respiratory viral infections. As expected, IRF-7 silencing suppressed IFN-α and IFN-β responses. Interestingly, IRF-7 inhibition also reduced neutrophil and macrophage numbers in a similar fashion to CCL7 antagonism but did not reduce AHR or NF-κB activity. Although our results therefore support an important role of IRF-7 in RV-induced antiviral responses through induction of IFN production, they also reveal an unexpected role of IRF-7 in modulating the inflammatory response upon in vivo RV exposure. Recently, IRF-7 was identified to control pro-to
anti-inflammatory (M1-to-M2) macrophage phenotype switch, and IRF-7 expression reduced proinflammatory macrophage activity (38). This may be relevant to RV infection, and the synergistic effects of CCL7 on IRF-7 expression observed in our study may regulate the balance between the proinflammatory activity of lung macrophages and their anti-inflammatory capabilities.

Collectively, we dissect the role of CCL7 and IRF-7 in RV-induced antiviral and inflammatory responses and show an important collaborative function for the recruitment and activation of inflammatory cells in the lung and for mounting an IFN-β response to RV.

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Disclosures

S.L.J. has received consultancy fees from Centocor, Sanofi Pasteur, Synairgen, GlaxoSmithKline, Chiesi, Boehringer Ingelheim, and Novartis; has patents pending, planned, or issued (U.K. patent application no. 02 167 294.9 and international patent application no. PCT/EP2003/007939, U.K. patent application no. GB 0405634.7, and U.K. patent application no. 0518425.4); and has stock/stock options in Synairgen.

References


**Supplementary Table 1.**

**Forward and reverse primers used for all qRT-PCR analyses**

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PCR analyses conducted using SYBR Green chemistry in a total volume of 6µl per reaction on cDNA generated from RNA extracted from blunt dissected airway tissue mentioned in the methods section.
Supplementary Figure 1. IRF-7 and CCL7 peak at 24hrs post rhinovirus infection of allergic mice and are resolved by four days post infection. BALB/c mice were sensitised and challenged with house dust mite to induce allergic airways disease, subsequently infected with RV1B and sacrificed at 24hrs and 4days post infection. Positive strand rhinovirus RNA and mRNA expression of IRF-7 and CCL7 were assessed by qPCR in blunt dissected airway tissue. CCL7 protein were assessed by ELISA in whole lung homogenates. All results are Mean±SEM at 1 dpi (n=5-6 mice per group). **, p < 0.01. ***, p < 0.001 as compared to HDM+RV1B group of the relevant timepoint.
Supplementary Figure 2. CCL7 neutralisation does not affect IFNs or viral titre during RV induced exacerbations. mRNA expression of IRF-7, -5 and -3 and positive strand RV1B RNA assessed by qPCR in blunt dissected airway tissue. All results are Mean±SEM at 1 dpi (n=5-6 mice per group). **, p < 0.01. ***, p < 0.001 as compared to Isotype control group.