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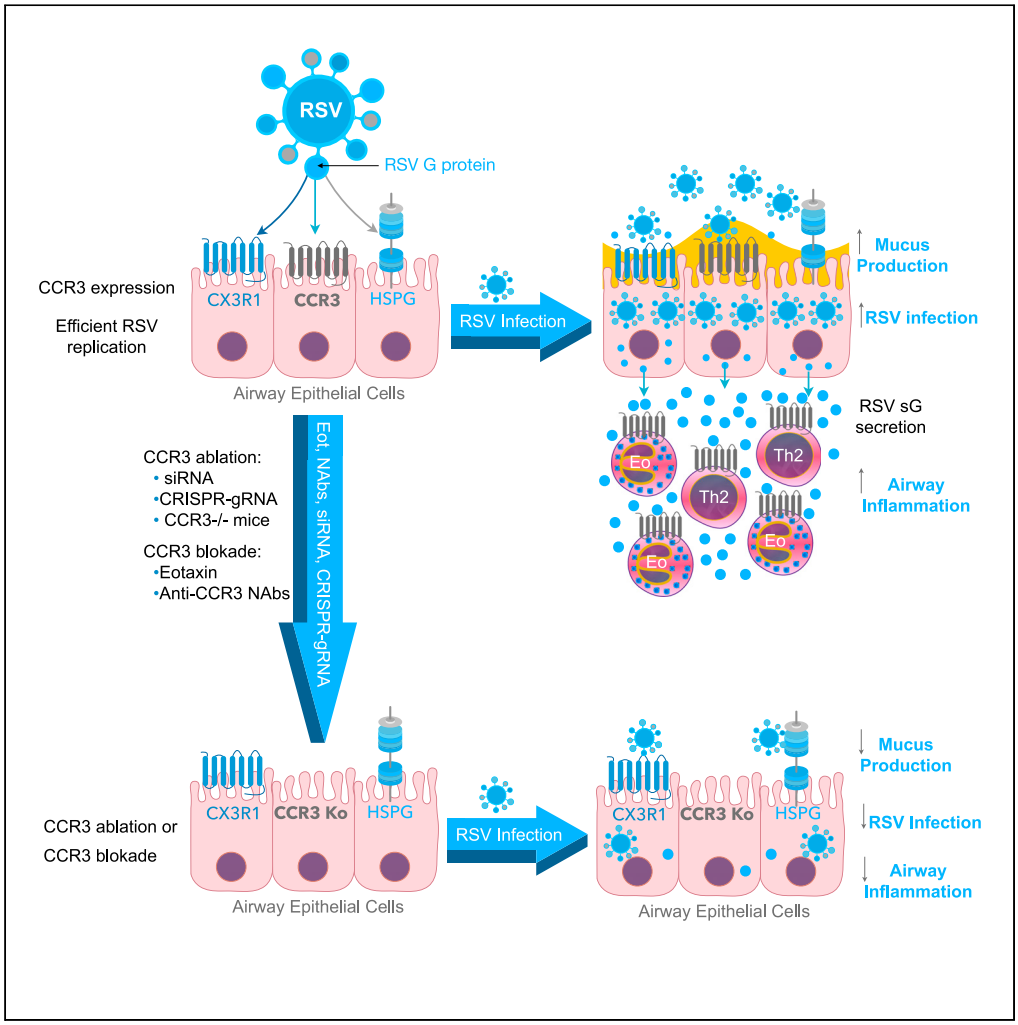

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Highlights

CCR3 mediates RSV infection of human airway epithelial cells

Eotaxin-1 blocks RSV-G binding to CCR3 and significantly decreases RSV infection

RSV-G secreted protein (sG) attracts human eosinophils and Th_2 cells through CCR3

RSV infection of mice lacking CCR3 exhibited reduced inflammation and mucus secretion

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Role of CCR3 in respiratory syncytial virus infection of airway epithelial cells

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SUMMARY

Respiratory syncytial virus (RSV) infection is the principal cause of severe lower respiratory tract disease and accounts for a significant risk for developing asthma later in life. Clinical studies have shown an increase in airway responsiveness and a concomitant Th_2 response in the lungs of RSV-infected patients. These indications suggest that RSV may modulate aspects of the immune response to promote virus replication. Here, we show that CCR3 facilitates RSV infection of airway epithelial cells, an effect that was inhibited by eotaxin-1/CCL11 or upon CCR3 gene silencing. Mechanistically, cellular entry of RSV is mediated by binding of the viral G protein to CCR3 and selective chemotaxis of Th_2 cells and eosinophils. In vivo, mice lacking CCR3 display a significant reduction in RSV infection, airway inflammation, and mucus production. Overall, RSV G protein-CCR3 interaction may participate in pulmonary infection and inflammation by enhancing eosinophils' recruitment and less potent antiviral Th_2 cells.

INTRODUCTION

Respiratory syncytial virus (RSV) is a common cold virus that causes severe pneumonia and bronchiolitis of which newborns require treatment and often hospitalization (McIntosh and Chanok, 1985; Kim et al., 1969). Currently, there are still no licensed vaccines for RSV, and although most children are infected with RSV by 2 years of age and produce neutralizing antibodies, life-long immunity is not induced (Kim et al., 1969). Early attempts at vaccination with formalin-inactivated and attenuated viruses failed to induce protective immunity and, in some infants, increased the severity of the disease after subsequent natural infections (Kim et al., 1969; Belshe et al., 1982). Therefore, the design of alternative vaccines requires detailed characterization of RSV antigenicity, understanding the biology of infection, the immune responses, and disease pathogenesis that occur during the course of infection.

Two major structural glycoproteins (G and F) are involved in RSV attachment, entry into cells, and represent presumptive sites for the protective immune response. RSV G is a unique viral membrane protein that contains approximately 60% carbohydrates by weight (Gruber and Levine, 1985; Satake et al., 1985) and is expressed as membrane-anchored (mG) and secreted (sG) forms. Besides its role in initiating a new infectious cycle, RSV G protein appears to be linked to the pathogenesis of RSV disease (Widjojoatmodjo et al., 2010) and airway eosinophilia (Hancock et al., 1996; Tebbey et al., 1998). G protein central conserved region (residues 149–197) contains an antigenic determinant responsible for atypical enhanced pulmonary eosinophilia (Tebbey et al., 1998). Mice infected with RSV lacking sG form show reduced replication accompanied by a reduced amount of MCP-1/CCL3 and RANTES/CCL5 and decreasing numbers of pulmonary T cells and eosinophils (Tebbey et al., 1998; Price et al., 1999). The G protein promotes the activation of Th_2 $CD4^+$ T cells and induces eosinophilic infiltrates in the lung following the RSV challenge (Elliott et al., 2004a; Tripp et al., 1999; Johnson et al., 1998). Attempts to promote Th_1-like responses rather than Th_2 responses minimally decreased eosinophilia and IL-5 production induced by G priming (Alwan et al., 1992, 1994). These data suggest that the primary or secondary antigenic structure of G protein influence the composition of subsequent RSV-specific immune responses, and that sG predispose to Th_2-like immune responses.

RSV G has been shown to strongly over-ride the antiviral innate and memory immune responses to limit immunity against RSV reinfection (Johnson et al., 2012; Polack et al., 2005; Shingai et al., 2008; Chirkova

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et al., 2013). Besides, the recruitment and activation of CX3CR-1$^+$ T cells, a major component of the cytotoxic response to RSV infection appear to be controlled by G protein and its CX3C motif (Tripp et al., 2001; Ha et al., 2019; Harcourt et al., 2006) most probably by antagonizing the activities of the Frk/CX3CL1 (Tripp et al., 2001). Furthermore, sG was shown to act as an antigen decoy to help RSV escape the antibody-dependent restriction of replication and its clearance by macrophages and complement (Bukreyev et al., 2008; Johnson et al., 2012). In addition, by promoting a Th_2-type inflammatory response in the lung, early infection with RSV has been reported to impair regulatory T cell function and increase susceptibility to allergic asthma (Krishnamoorthy et al., 2012). G protein can also increase levels of IL-1α, and many chemokines including eotaxin-1/CCL11(Haeberle et al., 2001) suggest that RSV proteins alone may affect the early immune response before and/or during infection (Oshansky et al., 2010).

Eotaxin-1/CCL11 is a critical chemokine for the recruitment and retention of eosinophils in airway inflamed tissue (Baggiolini, 1996). In addition to being a major eosinophil chemoattractant, eotaxin-1/CCL11 activates eosinophil effector functions, and enhances eosinophil differentiation (Lamkhioued et al., 2003). CCR3 mediates the potent chemotactic and activating effects of eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, CCL5, MCP-3/CCL7, and MCP-4/CCL13 on eosinophils (Daugherty et al., 1996). CCR3 is also expressed by Th_2 cells (Sallusto et al., 1997), mast cells (de Paulis et al., 2001), and structural cells including airway smooth muscle cells (Joubert et al., 2005) and airway epithelial cells (Stellato et al., 2001). Recent studies suggest that pulmonary eosinophils have dual outcomes, one linked to RSV-induced airway inflammation and pulmonary pathology (Matthews et al., 2005) and one with innate features that contribute to the antiviral defense and may thus limit virus-induced lung dysfunction (Johnson et al., 2008; Phipps et al., 2007; Su et al., 2015). CCR3 has been shown to facilitate infection of primary HIV-1 isolates, and binding of the CCR3 ligand, eotaxin-1/CCL11, inhibited infection ability (Choe et al., 1996). The association of CCR3 and its ligands with allergic airway diseases has also been studied in patients and in murine models of allergic diseases. However, the importance of CCR3 and its ligands in RSV pathogenesis has not been examined.

Recently, studies performed on epithelial cell lines demonstrated that chemokines play a critical role in preventing viral infection and act as major virus-suppressive factors (Pelchen-Matthews et al., 1999). Precisely, it has been proposed that CCL5 has a direct anti-viral effect against RSV by blocking RSV F protein interactions with epithelial cells (Elliott et al., 2004b). Moreover, a family-based genetic association analysis showed that genetic variants of the CCL5 major receptor, CCR5, are associated with severe RSV bronchiolitis (Hull et al., 2003). In mice, infection with RSV results in various chemokines production, in amounts that parallel the intensity of inflammation in the lung (Haeberle et al., 2001; Miller et al., 2004). Besides, increased levels of CCL5, MIP-1α/CCL3, CXCL8, and eotaxin-1/CCL11 have been reported in airway epithelial cells after RSV infection and in nasal aspirate samples taken from children during proven RSV-induced exacerbations (Harrison et al., 1999). The importance of chemokines in the pathophysiology of RSV infection became apparent when it was demonstrated that CX3CL1 inhibits RSV infection of target cells and that RSV G protein binds CX3CL1 receptor, CX3CR-1. The latter mediates leukocyte chemotaxis, as well as facilitates RSV infection (Tripp et al., 2001). The interaction between chemokine and viruses may be part of a more general strategy employed by many micro-organisms to elude their hosts' immune system. Therefore, a single virus may engage different receptors to infect different cell types or host species (Murphy, 2001).

During a thorough reexamination of various human chemokines on their antiviral activity on the panel of Ghost cell line stably expressing human chemokine receptors (CCR1 to 5, CXCR4, and CX3CR1), we unexpectedly found that eotaxin-1/CCL11 inhibits RSV infection by binding to CCR3. This report provides evidence that binding and uptake/entry of RSV into airway epithelial cells depends on interactions between the G protein and chemokine receptor CCR3. This study also demonstrates that sG protein binds to CCR3 and attracts Th_2 cells and eosinophils. The finding that anti-CCR3 is beneficial in this setting suggests that therapies designed to interfere with RSV G protein effects might be useful for such conditions.

RESULTS

Inhibition of CCR3-dependent respiratory syncytial virus infection by CLL11

It has been reported that CCL5 and CX3CL1, the ligands for CCR-5 and CX3CR1, respectively, inhibit the infection of host cells by RSV (Tripp et al., 2001; Elliott et al., 2004b). Whether it is the case for eotaxin-1/CLL11, which solely binds to CCR3 is not known. To study the effect of eotaxin-1/CLL11 on virus production, Hep-2 cells were treated with or without recombinant eotaxin-1/CLL11 prior, during and after inoculation

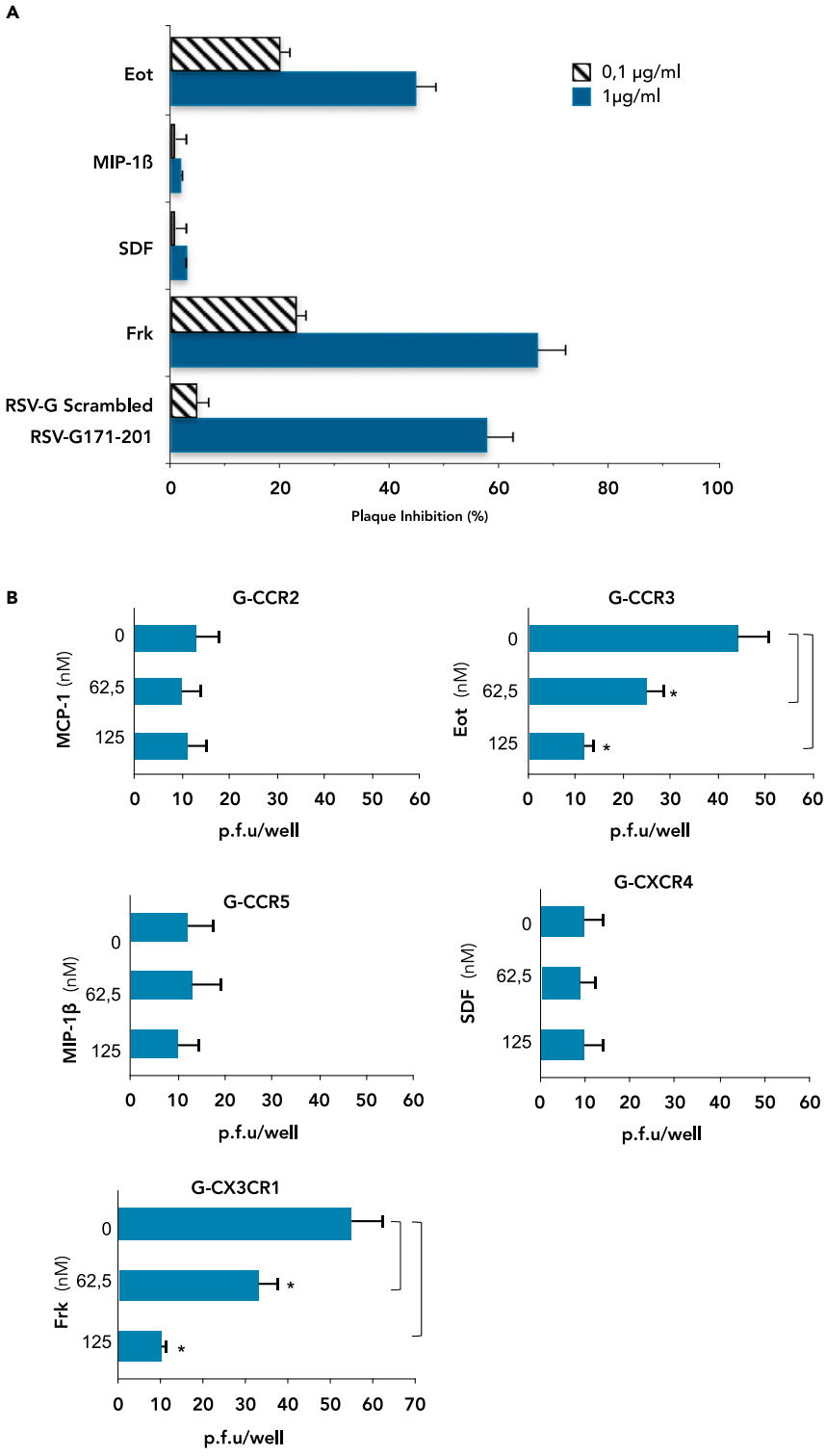

Figure 1. CCR3-dependent infection by RSV and inhibition by eotaxin-1/CLL11

(A) RSV plaque reduction by chemokines in Hep-2 cells inoculated with RSV. Three-days postinfection, the plaques were counted and the percent inhibition of virus infectivity of treated cells was determined versus untreated control wells.

Figure 1. *Continued*

(B) RSV plaque-forming units per well detected 3-days postinfection with or without chemokines (MCP-1/CCL3, eotaxin/CLL11, MIP-1β/CCL4, SDF-1/CXCL12, and Frk/CX3CL1) in Ghost cells expressing CCR2, CCR3, CCR5, CXCR4, or CX3CR-1. The number of parental cells that were infected with RSV was always less than in CCR3 transfected cells ($p < 0.005$). Eotaxin-1/CLL11 exhibited a dose-dependent inhibition of infection of G.CCR3$^+$ cells as compared to PBS-treated cells ($p < 0.05$).

with RSV and compared its effect to other chemokines including MIP-1β/CCL4, SDF-1/CXCL12, and Frk/CX3CL1 by examining RSV plaque reduction in Hep-2 cells (Figure 1A). Eotaxin-1/CLL11 reduced RSV infection of Hep-2 cells to a greater extend, compared to CCL4 and CXCL12. CX3CL1and RSV $G_{171-201}$ peptide that contains the CX3C domain of CX3CL1were able to inhibit Hep-2 infection with RSV (Figure 1A).

Ghost cells expressing CX3CR1, a chemokine receptor reported to facilitate RSV infection of airway primary epithelial cells (Johnson et al., 2015), and Ghost cells expressing CCR2, CCR3, CCR5, and CXCR4 were included in these experiments (Figure 1B). When CCR3-transfected Ghost-cells (G.CCR3+) were inoculated with RSV, the expression of CCR3 molecules resulted in significantly enhanced infection (Figure 1B). To confirm that CCR3 was sufficient for efficient RSV infection, G.CCR3$^+$ cells were incubated with eotaxin-1/CLL11, the major CCR3 ligand, prior to and during infection with RSV. The data in Figure 1B indicate that eotaxin-1/CLL11 exhibited a dose-dependent inhibition of infection of G.CCR3$^+$ cells as compared to phosphate-buffered saline (PBS) treated cells at each concentration of eotaxin-1/CLL11 tested ($p < 0.05$). No effect of eotaxin-1/CLL11 was observed on G.CCR5$^+$ cells or G.CX3CR1$^+$ cells (not shown). As expected, CX3CL1 has been shown to inhibit RSV infection (Tripp et al., 2001) was able to inhibit the RSV infection of G.CX3CR1$^+$ cells compared with PBS treated cells at each concentration of CX3CL1 tested ($p < 0.05$). No effects of CCL3, CCL4, or CXCL12 were observed on the infection of G.CCR2$^+$, G.CCR-5$^+$, or G.CXCR4$^+$ cells by RSV. These findings demonstrate that the enhancement of RSV infection by CCR3 appears to be specific and is not merely a result of another receptors' expression.

Association of respiratory syncytial virus G and CCR3

Based on the above results, we hypothesized that RSV G protein associate with CCR3 as an initial step in virus attachment during the infection of target cells. To test this hypothesis, we investigated whether RSV secreted protein (sG) associates with the CCR3 molecule. RSV sG protein was prepared and after the final purification step, only the RSV G band was detected by western blotting (Figure 2A). Thereafter, purified sG protein was examined for binding to CCR3 using Western blot detection of immunoprecipitated proteins, a commonly used technique to study protein–protein interactions.

Immunoblot analysis with CCR3-specific antibodies showed a 50 kDa band, which corresponds to human CCR3, coimmunoprecipitated with the sG molecules (Figure 2B). Similar results were obtained when immunoprecipitation was performed with human eotaxin-1/CLL11 instead of sG protein and G.CCR3 extract proteins (Figure 2B). This interaction between CCR3 and sG protein was specific, as no association was seen in immunoprecipitation performed with control IgG isotype Ab (Figure 2B), in the absence of sG or eotaxin-1/CLL11 proteins. Similarly, when membranes were blotted with control Abs, no interaction can be detected (data not shown). Because epithelial cells produce chemokines, the eluate was also examined by western blotting with anti-eotaxin-1/CLL11 antibodies. No band was detected using these antibodies (not shown).

A549 and G.CCR3 cells were then used to determine whether sG and RSV F proteins were able to bind CCR3 on these cells (Figure 2). We preliminarily analyzed CCR3 expression by G.CCR3$^+$ cells and A549 cells using flow cytometry based on an eotaxin-1/CLL11-biotin/avidin-FITC detection system (Figures 2C and 2D, respectively). Flow cytometry analysis shows the binding of human eotaxin-1/CLL11 to G.CCR3$^+$ cells (75–95%) and A549 cells (25–67%) (Figures 2C and 2D, respectively) that was inhibited by neutralizing antibodies directed against eotaxin-1/CLL11. Our data also show that sG protein inhibited 45–75% of eotaxin-1/CLL11 binding to G.CCR3$^+$ cells (Figure 2E), but did not inhibit MIP-1β/CCL4 binding to G.CCR5$^+$ cells (0–4%) (Figure 2G) or A549 (Figure 2H) suggesting that sG protein interact specifically with CCR3. Similarly, binding of eotaxin-1/CLL11 to A549 cells was inhibited by sG protein (40–46%) (Figure 2F), while RSV F protein shows no interference with eotaxin-1/CLL11 binding to G.CCR3$^+$ cells (Figure 2E) or A549 (Figure 2F).

CCR3 is expressed on human airway epithelial cells

Because airway epithelial cells provide the first line of defense in the respiratory tract and are the main target of respiratory viruses, we anticipated that RSV receptors might be expressed by these cells in human lungs.

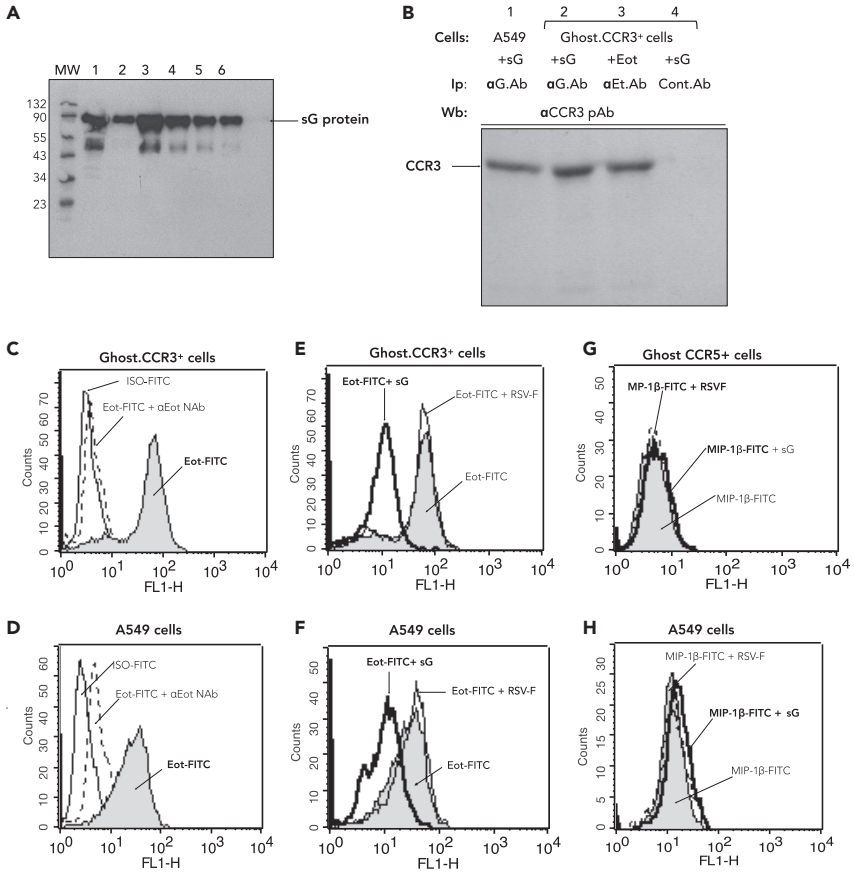

Figure 2. CCR3 associates with RSV G protein

(A) Western blot of purified RSV sG using rabbit anti-sG glycoprotein Abs. Fractions were collected, separated by electrophoresis, and sG protein was identified through their binding to specific anti-sG antibodies.

(B) A549 cells and G.CCR3$^+$ lysates were incubated with sG protein (50 μg/mL) and the complexes formed by RSV G-protein and CCR3 were immunoprecipitated with anti-RSV G-protein mAb (5 μg/mL) and then analyzed with western blotting using goat anti-CCR3 polyclonal antibodies. mAb isotype controls were included as negative controls in these experiments. In B, eotaxin-1/CLL11 (2 μg/mL) and anti-Eotaxin-1/CLL11 mAb (5 μg/mL) were used as a positive control to immunoprecipitate CCR3.

(C and D) Flow cytometry results showing Eotaxin-1/CLL11-FITC binding to G.CCR3$^+$ or A549 (D) and inhibition of binding by anti-eotaxin-1/CLL11 NAbs.

(E and F) Eotaxin-1/CLL11-FITC binding to G.CCR3$^+$ cells and A549 and inhibition of binding by sG and RSV-F proteins.

(G and H) sG, and RSV-F proteins had no effect on the binding of MIP-1β/CCL4 to G.CCR5$^+$ and A549 cells.

Therefore, endobronchial biopsies specimens collected from both control and asthmatic subjects were used to assess CCR3 and CX3CR1 protein expression by immunocytochemistry. As previously reported (Stellato et al., 2001), positive staining of airway epithelial cells was observed in lung tissue specimens from asthmatic subjects (Figure 3A). Epithelial cells appeared as the primary cells expressing CCR3 (Figure 3A) and CX3CR1 (Figure 3A) in the lung tissue. No immunoreactivity was found in cells when the control antibody was used instead of the primary antibodies (not shown). Human bronchial tissue from patients with COPD and normal controls, as well as primary nasal epithelial cells, expressed high levels of CCR3 on their surface as revealed by antibody immunoreactivity (Figures S1A and S1B, respectively), whereas airway epithelial cells displayed low-level expression of CX3CR1 (Figure S1A) and nasal epithelial cells did not show any surface expression of CX3CR1 (Figure S1B). The relevance of these observations was enforced by the finding that both CCR3 and CX3CR1 could be expressed together or separately (Figure S1A) suggesting that both receptors essentially contribute to the infection of airway epithelial cells.

CCR3 and CX3CR1 expression was further confirmed using A549, Hep-2 cells, and eosinophils used as a positive control (Figure 3B). PCR amplification products (both CCR3 and CX3CR1) from the A549, Hep-2 cells displayed

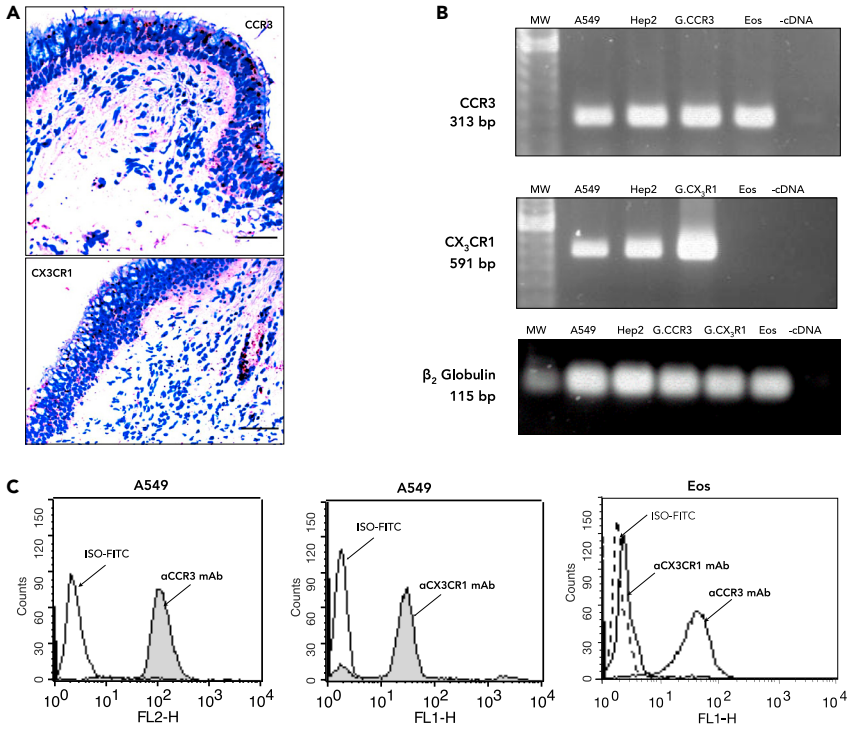

Figure 3. CCR3 and CX3CR-1 protein and mRNA expression in epithelial cells and eosinophils

(A) Detection of CCR3 and CX3CR1 immunoreactivity on airway epithelium. Airway sections cells from an asthmatic donor showed positive immunoreactivity for CCR3 and CX3CR1. Lung sections were stained with goat anti-CCR3 polyclonal antibodies and rabbit anti-CX3CR1 polyclonal antibodies. No staining was detected with the negative control antibodies (data not shown). Similar results were obtained in 5 other sections.

(B) RNA purified from epithelial cell lines (A549 and Hep-2) and eosinophils was examined for the presence of CCR3 and CX3CR1 transcripts by RT-PCR. For positive controls, RNA from Ghost cells transfected with CCR3 or CX3CR1 were used. Results are representative of three independent experiments performed under the same conditions.

(C) Surface expression of CCR3 and CX3CR-1 on A549 and eosinophils using flow cytometry and mouse anti-human CCR3 and anti-CX3CR1 mAbs or IgG isotype control mAbs (R&D Systems). Results are representative of three independent experiments performed under the same conditions. Scale bars indicate 125 μm.

See also Figure S1

the presence of a CCR3-specific 313-bp amplicon and CX3CR1-specific 591-pb amplicon (Figure 3B). In contrast, cultured eosinophils show only CCR3 gene expression but no CX3CR1. cDNA from G.CCR3 and G.CX3CR1 cells was used as a positive control for the presence of CCR3 and CX3CR1, respectively.

Furthermore, surface expression of CCR3 was significantly increased in cultured A549 cells and eosinophils (Figure 3C) as expected from the previous study (Rosenberg et al., 2009). Anti-CX3CR1 mAb was reactive on A549 cells; however, there is no evidence for CX3CR1expression in human eosinophils (Figure 3C).

RSV-GFP (green fluorescent protein) infection of human airway epithelial cells expressing CCR3

Having demonstrated that sG protein binding to CCR3 appears to facilitate the infection of the epithelial cell line, we decided to validate the functional role of CCR3 on human primary airway epithelial cells (PAEC). Therefore, we inoculated PAEC cultures with RSV-GFP in the presence or absence of eotaxin-1/CLL11 and fluorescence was measured by flow cytometry (FACSCalibur) (Figure 4A). After 3 days, fluorescence intensity was lower in eotaxin/CLL11 treated and RSV-GFP infected PAEC than in a medium with only RSV-GFP (Figure 4A). CCR3 expression in G.CCR3$^+$ cultures resulted in an increase in fluorescence compared to the infected parental G cells or non-infected G.CCR3$^+$ cells (Figures 4B and 4C), whereas fluorescence was lower when G.CCR3$^+$ cells were inoculated with RSV-GFP in the presence of eotaxin-1/CLL11 (Figure 4D). Our results also show that fluorescence did not differ between parental-Ghost cultures

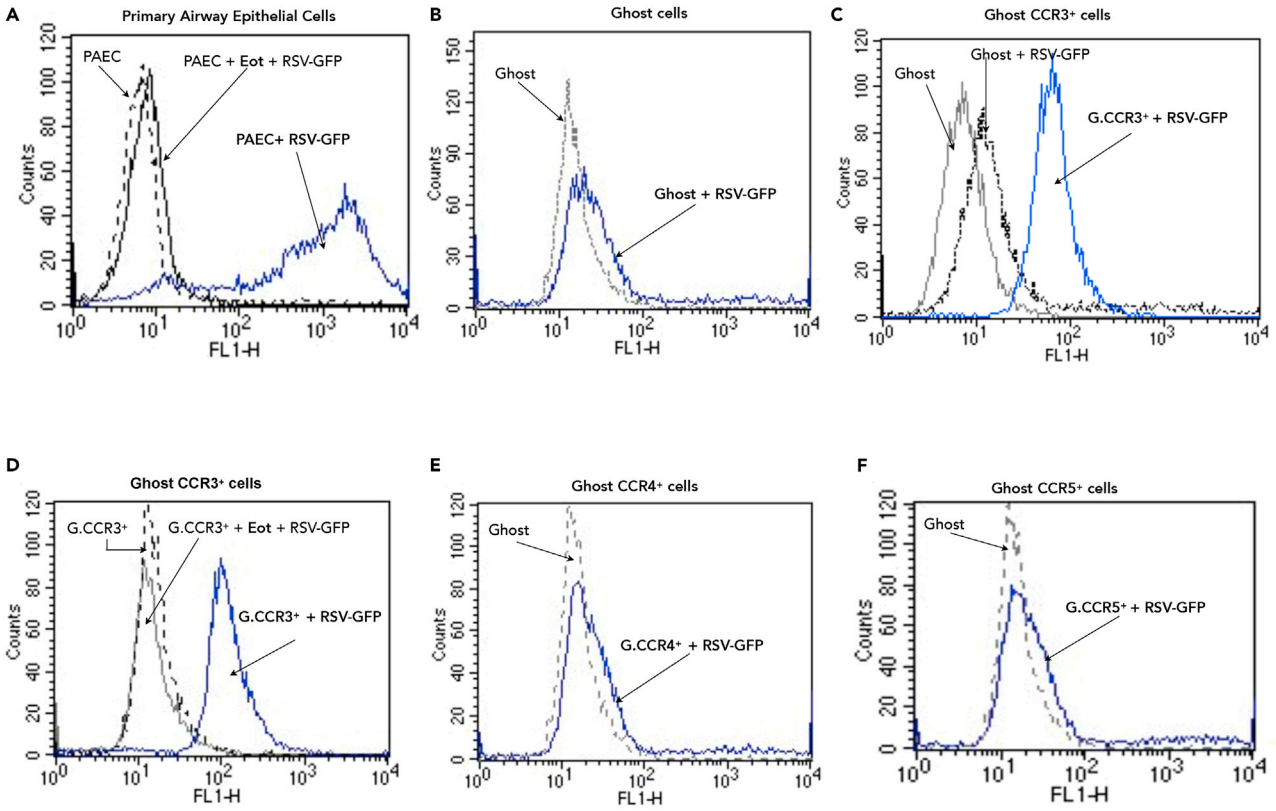

Figure 4. Inhibition of CCR3-dependent RSV-GFP infection by eotaxin-1/CLL11 using flow cytometry

Human primary airway epithelial cells (PAEC) and G.CCR3$^+$ cells were inoculated with RSV-GFP in the presence or absence of eotaxin-1/CLL11 and 3 days post-infection, cells were trypsinized and the percentage of infected cells was determined by assessing GFP fluorescence via flow cytometry. A representative flow cytometry plot of the mean percentage of GFP-positive PAEC and Ghost transfected cells for each of the conditions tested is shown in (A–F).

(A) Human PAEC were infected with RSV-GFP in virus medium with 0 and 1 μg/mL of eotaxin-1/CLL11 after 3 days of infection (neg Ctl: no RSV and no eotaxin-1/CLL11).

(B) Fluorescence measured by flow cytometry in parental Ghost cells inoculated or noninoculated with RSV-GFP.

(C) GFP fluorescence in parental Ghost and G.CCR3$^+$ cells inoculated with or without RSV-GFP during 3 days.

(D) G.CCR3$^+$ cells after 3 days infection with RSV-GFP in the absence or presence of eotaxin-1/CLL11 (1 μg/mL).

(E and F) G.CCR4$^+$ and G.CCR5$^+$ cells 3-days postinfection with RSV-GFP, respectively.

inoculated with RSV (infected cells) and negative (non-infected) cultures (Figure 4B) nor between infected and non-infected G.CCR4$^+$ and G.CCR5$^+$ (Figures 4E and 4F, respectively). Together, these data suggest that CCR3 is a receptor for RSV on PAEC cultures and that eotaxin-1/CLL11 inhibits the G protein interaction with CCR3.

Silencing CCR3 reduces respiratory syncytial virus infection

We then analyzed the down-modulation of CCR3 in Hep-2 cells and its subsequent impact on RSV infection. CCR3 expression in Hep2 cells transduced by CCR3-siRNA decreased by 82% ($p < 0.001$) with no change in CX3CR1 or CXCR4 surface expression when analyzed by FACS. Transduction of Hep2 cells with CXCR4 and CX3CR1 siRNA led to decreased CXCR4 and CX3CR1 expression by 87 and 89%, respectively (data not shown). When these cells were infected with RSV, CCR3-siRNA, and CX3CR1-siRNA significantly reduced the infection of Hep2 cells (by 53% ($p < 0.001$) and 78% ($p < 0.001$), respectively) (Figure 5A). In contrast, RSV infection of Hep2 cells was not affected by transduction targeting CCR5, CXCR-4 receptors, or by control si-RNA (Figure 5A). These data were confirmed in Ghost cells using siRNA for CCR3, CCR5, CXCR4, and CX3CR1 receptors. As shown in Figure 5B, significant protection from infection with RSV was seen in G.CCR3$^+$ and G.CX3CR1$^+$ cells transduced with CCR3-siRNA and CX3CR1-siRNA, respectively.

We also studied the potential interaction/synergy of binding to CCR3 and CX3CR1 and subsequent infection with RSV. Therefore, we deleted CCR3 and CX3CR1 receptors in airway epithelial cells using

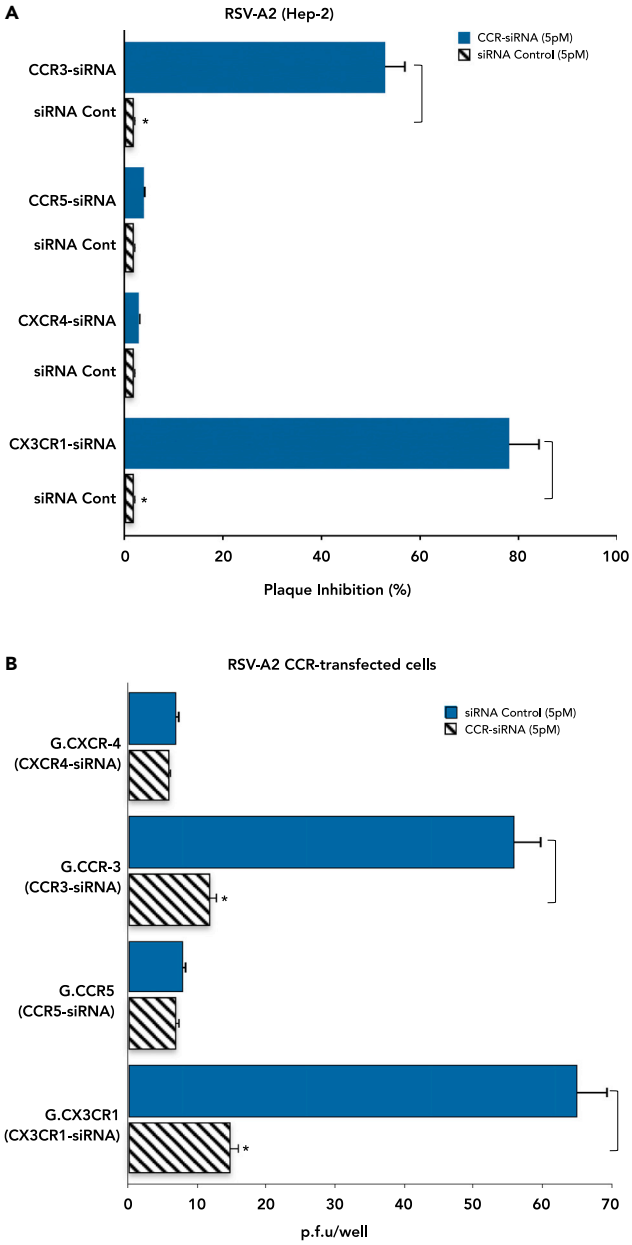

Figure 5. Silencing CCR3 reduces RSV infection in Hep-2 cells

(A) Chemokine receptors-targeting and control siRNA were transfected into Hep-2 cells and harvested 24h after transfection. Cultures were challenged with RSV and 3 days post-infection the percentage inhibition was determined by dividing the mean plaque-forming units (PFU) of siRNA-treated Hep-2 cells by the number of PBS-treated Hep-2 cells. Each CCR siRNA was compared to its siRNA control. Error bars represent the standard deviation of triplicates.

(B) siRNA compounds were assessed to block RSV infection of Ghost transfected cells. The compounds were added to cells followed by infection with RSV. Three-days postinfection, the PFU were counted in siRNA-treated cells versus PBS-treated control wells. Error bars represent the standard deviation of three independent experiments with triplicates for each experimental condition.

See also Figures S2 and S3

CRISPR-Cas-9, a specific and efficient gene-editing technology (see STAR Methods). CCR3 and CX3CR1 expression was measured in A549 wild-type (WT) and CRISPR-Cas9 edited (A549) cell lines. Using confocal microscopy and FACS analysis, CCR3 and CX3CR1 expression proved to be reduced efficiency in the edited cell line (Figures S2A and S2B). These cells were thereafter used for RSV-GFP infection (Figure S2C).

Corroborating their role of entry receptors for RSV in epithelial cells, ablation of CCR3 and CX3CR1 significantly reduced the amount of RSV-GFP$^+$ A549 cells as compared to nonedited control cells (Figure S2C). More importantly, analysis of RSV infection in CCR3/CX3CR1 simultaneous editing cells (double knockout cells) at 2- and 5- days p.i showed significantly less replication than single CCR3 or CX3CR1 knockout cells infected with RSV (Figures S2C and S3D). These findings are consistent with CCR3 being a functional receptor among several RSV receptors on airway epithelial cells.

Because RSV binding to cells also involves an initial interaction between the basic amino acids present on the viral envelope G protein and heparin sulfate proteoglycans (HSPG), we have also treated A549 cells with heparinase-I to remove HSPG (Figures S3A and S3B). It has been noted previously that Heparinase-I treatment is the most efficient way to cleave heparin sulfate chains from cell surface HSPG (Martinez and Melero, 2000). Among the several HSPGs, syndecan-1 is the one primarily expressed on airway epithelial cells including A549 (Hayashida et al., 2009; Chirkova et al., 2015) and has been shown to support RSV infection (Chirkova et al., 2015). Treatment with heparinase-1 dramatically decreased syndecan-1 expression in airway epithelial cells (Figures S3A and S3B) (Chirkova et al., 2015). As reported previously (Chirkova et al., 2015), lack of HSPG on the cell surface significantly decreased RSV infection of heparinase-I treated cells (Figure S3C). Ablation of either CCR3 or CX3CR1 and treatment with heparinase-I showed significantly less virus replication compared with infected CCR3 or CX3CR1 single knockout cells (Figures S3C and S3D). More importantly, CCR3/CX3CR1 double knockout cells and treated with heparinase-I showed significantly less RSV replication when compared to infected CCR3/CX3CR1 double knockout cells (Figures S3C and S3D). Note that neither deletion of CCR3, CX3CR1 nor heparinase-I treatments eliminated all binding and infection of target cells suggesting that other receptors may be involved in RSV-G binding and infection (Figure S3D). Taken together, our results demonstrate that CCR3 is a critical host factor for RSV G attachment and entry in airway epithelial cells, and this interaction is dependent on the RSV G protein.

CCR3 knockout mice are less susceptible to respiratory syncytial virus infection than wild-type mice

To elucidate the in vivo contribution of CCR3 in RSV infection of the airways, we used CCR3-deficient and their CCR3-deficient littermates and infected them intranasally with live RSV (Figure 6). Compared to WT mice, CCR3$^{-/-}$ mice showed reduced titers of RSV infection at 5 days postinoculation as demonstrated by plaque assay and ELISA (Figure 6A). The number of goblet cells staining positively with periodic acid–Schiff for mucin was significantly decreased in the airway epithelium of CCR3$^{-/-}$ mice, compared with WT mice ($p < 0.01$) (Figure 6B). Histological analysis also revealed less inflammation in the lungs of CCR3$^{-/-}$ mice than WT mice (Figure 6B). These data show that CCR3 is required for optimal disease development and supports the idea that this receptor may act as an RSV receptor in the airways.

Respiratory syncytial virus -G protein mediates eosinophils and Th_2 cells chemotaxis

Because activated CCR3$^+$ eosinophils are known to be important effector cells in bronchiolitis disease and abnormalities in their immune response may even result in RSV induced exacerbation of asthma (Rosenberg et al., 2009), we tested the migratory behavior of eosinophils in response to sG protein and eotaxin-1/CLL11 (Figure 7A). As expected eotaxin-1/CLL11 and sG protein were found to increase the chemotaxis of these cells in a modified Boyden chamber chemotaxis assay (Figures 7A and 7B). The addition of anti-CCR3 Ab to eosinophils markedly decreased their migratory response to these molecules (Figures 7A and 7B), whereas anti-CX3CR1 Ab did not affect the chemotaxis of eosinophils toward sG protein (Figure 7B).

Histopathological findings show that the inflammatory infiltrate in RSV bronchiolitis comprises eosinophils and Th_2 cells (Rosenberg et al., 2009; Peebles and Graham, 2005). Given that the RSV G protein has been shown to mediate both human and mouse lymphocyte chemotaxis (Tripp et al., 2001; Harcourt et al., 2004), we investigated whether sG protein could induce Th_1 and Th_2 chemotaxis in vitro and compare its effect to eotaxin-1/CLL11, which is known to attract Th_2 cells (Figure 8). Thus, we generated Th_1 and Th_2 lines (Figure 8A) and we observed that the highest migration was achieved with CCL4 (a specific agonist of CCR5) for Th_1 cells and eotaxin-1/CLL11 (a specific agonist of CCR3) for Th_2 cells (Figure 8B) (Lamkhioued et al., 1997). sG protein-induced minimal migration of Th_1 cells that were not affected by the addition of anti-CCR3 nor anti-CCR5 Abs (Figure 8B). Further, sG protein was more effective in attracting polarized Th_2 lymphocytes compared to Th_1 cells that were inhibited in the presence of anti-CCR3 Abs (Figure 8B), but retained a significant migration in the presence of anti-CCR5 and anti-CXCR3 mAbs

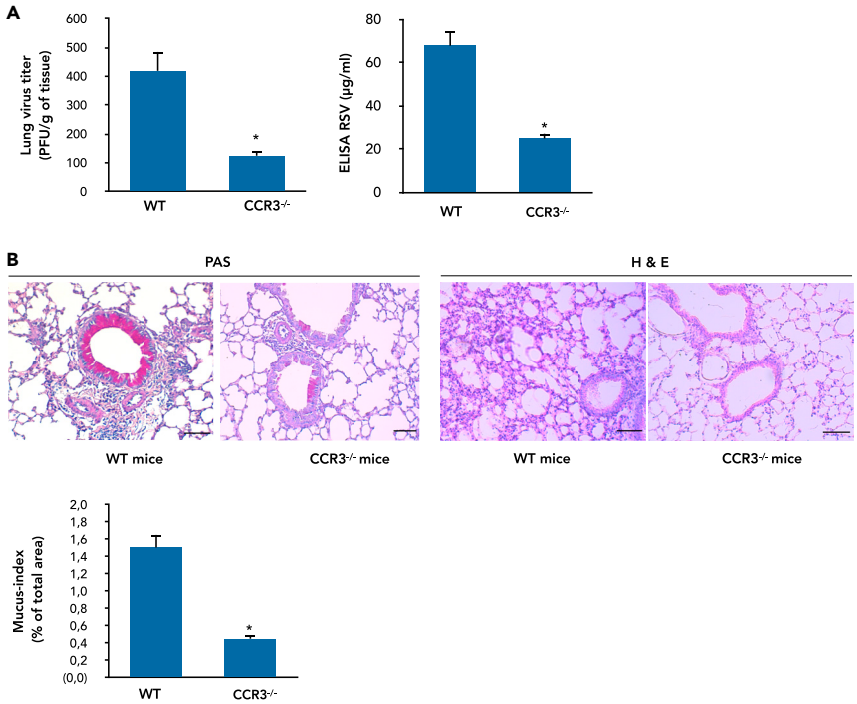

Figure 6. CCR3 deficiency prevented lung RSV-induced inflammation and mucus production

(A) Effect of CCR3 deficiency on lung viral titer 5 days after infection. Bar graphs show quantitative plaque assays and ELISA of RSV in lung homogenates. $p < 0.001$, $n = 7$, and $n = 8$ per group for experiments in RSV plaque assay and ELISA, respectively.

(B) Paraffin-embedded lung tissues obtained 5-days post-infection from RSV-infected wild type and CCR3$^{-/-}$ mice were stained by periodic acid-Schiff (PAS) and hematoxylin and eosin (H&E). B, show reduced inflammation in CCR3 deficient mice and mucus production as evidenced by significantly fewer PAS-positive cells. The significance of difference between selected groups was determined by unpaired t test. *, $p < 0.05$. Scale bars indicate 200 µm.

(Figure 8B) suggesting that CCR3 is involved in this process. The magnitude of Th_2 cell migration in response to sG protein was greater than that of eotaxin-1/CLL11 (Figure 8B).

DISCUSSION

In the last few years, studies have shown that chemokines play a critical role in the containment of viral infections and act as major virus-suppressive factors (Pelchen-Matthews et al., 1999) at least in the case of two important human pathogens, the malarial parasite plasmodium vivax(Horuk et al., 1993), and the human immunodeficiency virus (HIV) (Moore et al., 1997). Indeed, eotaxin-1/CLL11 has been shown to block the entry of HIV into cells (Cocchi et al., 1995; Bleul et al., 1996; He et al., 1997). A recent study has shown that the eotaxins, eotaxin-1/CLL11, eotaxin-2/CCL24, and eotaxin-3/CCL26 showed potent bactericidal activity against common airway pathogens (Gela et al., 2015). Therefore, it seems reasonable to assume that the functional consequences of increased levels of chemokines in nasal aspirate samples taken from children who have naturally occurring RSV infection (Harrison et al., 1999) may not only be related to the recruitment of inflammatory cells but may also be considered as a mechanism of viral infection clearance by competing with target receptors. We here provide evidence that CCR3 was efficiently utilized as a receptor by RSV to infect airway epithelial cells. Also, sG protein attaches directly to CCR3$^+$ eosinophils and Th_2 cells and may contribute significantly to the recruitment of these inflammatory cells to the airways.

The results presented herein indicate that in addition to GAG and CX3CR1, CCR3 may play a critical role in RSV infection. Therefore, the increase of eotaxin-1/CLL11 release during RSV-induced inflammation of the airways may have a suppressive effect that allows rapid elimination of RSV. Indeed, our data showing eotaxin-1/CLL11 inhibition of CCR3-mediated RSV infection (Figures 1, 2 and 4) raise the possibility that CCR3

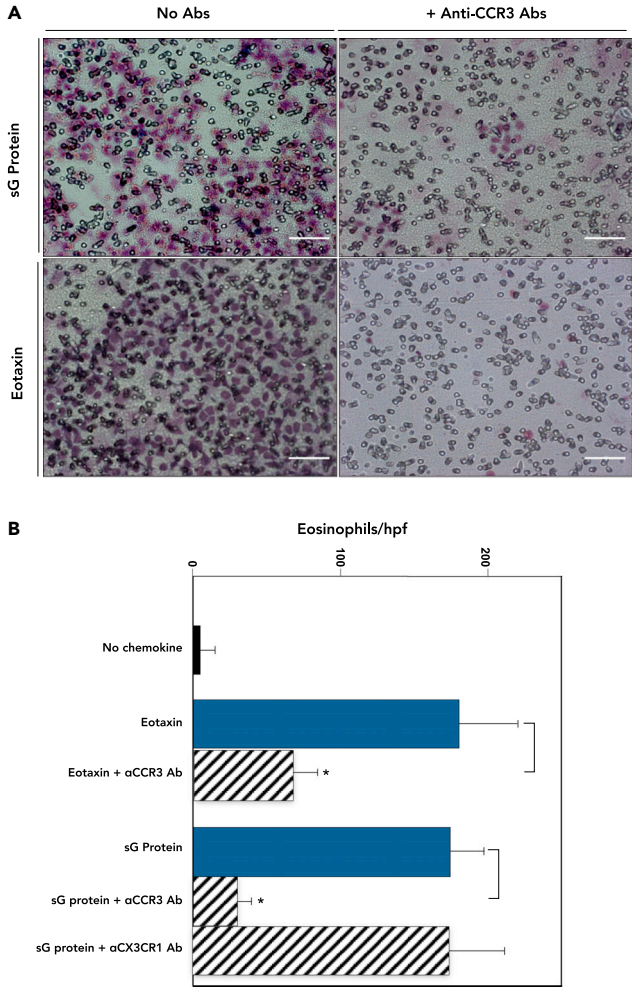

Figure 7. Eosinophil migration induced by sG protein and eotaxin-1/CLL11 (Eot) in filter assays

Eosinophils (Eos) were collected, purified, and incubated with or without anti-CCR3 or anti-CX3CR1 mAbs and a chemotaxis assay was performed in Boyden chambers as described in STAR Methods.

(A) Transmigration through polycarbonate filters showing chemotactic activity of purified human eosinophils to sG protein and eotaxin-1/CLL11 in the absence or presence of anti-CCR3 Abs.

(B) Eotaxin-1/CLL11 induced eosinophils chemotaxis and blockade of CCR3 with antibodies significantly inhibited eosinophil migration ($p < 0.01$ compared with chemotaxis to eotaxin-1/CLL11 alone). Incubation with anti-CCR3 mAb decreased the potency of sG protein to attract eosinophils markedly ($p < 0.001$ compared with sG protein alone). Whereas, preincubation of eosinophils with anti-CX3CR1 Ab or control antibodies had no effect on chemotaxis (not shown). Data are presented as the number of cells per field (magnification, X400). The results shown are representative experiments and are presented as the mean ± SD of 5 high power fields per well. Med, medium. Scale bars indicate 75 μm.

may represent important host components that specify susceptibility to RSV infection or in already infected individuals. Previous studies have shown that eotaxin-1/CLL11 has no effect on the RSV infection of epithelial cells whereas CCL5 has been able to inhibit RSV infection of A549 epithelial cells (Elliott et al., 2004b). Both CCR3 and CCR5 were responsive to CCL5 with CCR3 being localized to the airway epithelium (Beck S et al., 2001) (Figures 3A, S1 and S3) whereas no CCR5 was detected (data not shown). Additionally, the increased RSV inhibitory activity of eotaxin-1/CLL11 compared with CCL4 suggest that CCR5 may not be responsible for mediating this inhibition as CCR5 has been reported to exhibit greater sensitivity to CCL4 and low affinity to eotaxin-1/CLL11 (Ogilvie et al., 2001). The involvement of another chemokine receptor, such as CCR3, which is responsive to eotaxin-1/CLL11 and has a low affinity to CCL4, could explain these findings. As eotaxin-3/CCL26, a functional ligand for CCR3, competed with CX3CL1 for binding to

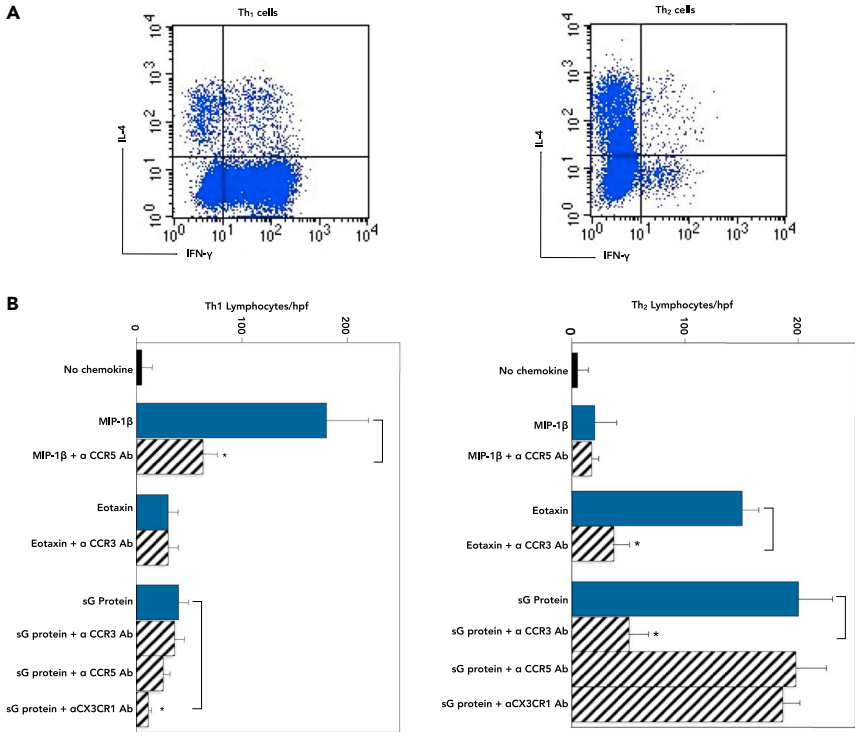

Figure 8. RSV sG protein attracts preferentially Th_2 cells

Th_1 and Th_2 cells were generated as described in the STAR Methods. As shown at the single-cell level by measuring intracellular cytokine production

(A) neonatal T cells primed under the Th_1 conditions differentiated into T cells producing IFNγ but little IL-4, (A, upper left), whereas naive T cells primed in the presence of IL-4 and anti-IL-12 resulted in a population of T cells producing mainly IL-4. (A, upper right).

(B) Chemotactic response of Th_1 cells (B, lower left) and Th_2 cells (B, lower right) to sG protein (50 ng/mL), eotaxin-1/CLL11 (100 ng/mL) or MIP-1β/CCL4 (100 ng/mL). The chemotactic activity of sG protein, eotaxin-1/CLL11, and MIP-1β/ CCL4 were compared to medium alone with the use of modified Boyden chambers. The anti-CCR3, anti-CCR5 or anti-CX3CR1 Abs were added to the upper chamber to examine the antagonism of cell migration toward the chemoattractants (sG protein, eotaxin-1/CLL11, or MIP-1β/CCL4) in the lower chamber. Data are presented as the number of cells per field (magnification, ×400). Isotype control mAbs had no effect on the chemotaxis (not shown). The results shown are representative experiments and are presented as the mean ± SD of 5 high power fields per well. Med, medium.

CX3CR1 (Nakayama et al., 2010), it remains to be determined whether eotaxin-1/CCL11 and eotaxin-2/CCL24 behave as agonists for CX3CR1 and inhibit G protein-CX3CR1 interaction and subsequent RSV infection. Furthermore, the mechanism by which chemokines exert their inhibitory effects on RSV cell entry may rely on many steps. These complex steps involved receptor blockade, desensitization, sequestration or internalization, phosphorylation, and change in affinity state through G-protein uncoupling. Further work will be required to distinguish among these possibilities.

The high expression of CCR3 in G.CCR3^{+} transfected cells significantly enhances infection by RSV than in non-transfected Ghost cells suggesting that CCR3 is important for efficient infection of these cells (Figures 1, 2 and 4). Using CCR3$^{-/-}$ mice infected with RSV, we found a significant decrease in viral titers, airway inflammation, and mucus secretion compared to WT mice. The airways of RSV-infected CCR3$^{-/-}$ mice displayed less inflammation and mucus-producing goblet cells as compared with those of WT mice (Figure 6). There are several possibilities for these observations. The CCR3$^{-/-}$ mice may have altered epithelial cell activation during RSV-induced disease that prevents goblet cell development. Thus, the direct activation of CCR3 on airway cells may influence the overproduction of mucus. Alternatively, the absence of CCR3 may lead to reduced infection of the airway epithelium and altered recruitment of particular immune cells like eosinophils within the airway that would otherwise lead to cellular damage and/or altered immune activation. This is supported by reduced eosinophils and lymphocyte accumulation and disease severity in the

airways of mice treated with a blocking antibody to eotaxin-1/CLL11 or shRNA to CCR3 (Matthews et al., 2005; Zhu et al., 2017). However, the eotaxin-1/CCL11 knockout mice had significantly higher lung RSV titers compared with wild-type mice, and when lung eosinophilia was restored by either intratracheal recombinant eotaxin-1/CCL11 administration or adoptive transfer of eosinophils this resulted in increased viral clearance (Shen et al., 2006). These studies suggest that pulmonary eosinophilia has dual outcomes: one linked to RSV-induced airway inflammation and pulmonary pathology and one with innate features that contribute to a reduction in the viral load (Phipps et al., 2007). It is likely a combination of all of these that affects the pulmonary responses during RSV infection (Su et al., 2015). Conditional CCR3 gene knockout in mice bronchial epithelial cells and/or eosinophils will be of great interest to better delineate the role of CCR3 in RSV infection and/or RSV-induced inflammation into the lungs of adult mice. The present results suggest that the detection of CCR3 in airway epithelial cells provides a potential mechanism for airway epithelial cells infection and subsequent airway inflammation. As the viral load was decreased in the mouse strain that is most susceptible to virus infection after the deletion of CCR3 activity, our results lead us to suggest that the clinical development of selective extracellular CCR3 inhibitors may have potential as a broad-spectrum antiviral therapeutic strategy. RSV retained, however, some of its infectivity for $CCR3^{-/-}$ mice suggesting that RSV may also use an alternative RSV G receptor such as CX3CR1 or GAGs (Tripp et al., 2001; Feldman et al., 1999).

In this study, we also address the contributions of sG protein and inflammatory cells to the induction of disease-enhancing immune responses. Our data show that natural sG protein has a selective chemotactic activity for eosinophils and Th_2 cells. Also, we have identified CCR3 as a critical contributor to cells chemotaxis (Figures 7 and 8). These findings define these inflammatory cells as target cells for sG protein and show that initial contact between RSV and the eosinophils or Th_2 cells may occur through binding to CCR3. Supernatants of RSV infected epithelial cells and nasal secretions obtained from patients with bronchiolitis contain several chemokines such as CCL3, CCL5, and eotaxin-1/CLL11 (Harrison et al., 1999), and the processes governing the recruitment of inflammatory cells are likely to be the result of a combination of chemokines and sG protein. These results are consistent with recent findings showing G protein CX3C chemokine mimicry, impairment of CX3CL1-mediated responses (Harcourt et al., 2006), and exaggerated Th_2-type cytokine expression by pulmonary leukocytes in RSV-infected mice. It is possible that sG protein binds to CCR3 and amplifies the local inflammation by recruiting more eosinophils and Th_2 type inflammatory cells. Thus, a potent and specific chemokine, such as eotaxin-1/CLL11, could combine with nonspecific but highly effective chemokine-like protein, such as sG protein, to promote a selective eosinophil and Th_2 accumulation and activation in sites of airway inflammation. These findings are consistent with RSV G protein-associated reduction of Th_1-type cytokine responses (Harcourt et al., 2006). They suggest that sG protein expression during RSV infection may favor less potent antiviral Th_2 cells and reduce the antiviral Th_1 response to promote RSV infection or replication. The expression of CCR3 appears to be restricted, with little expression in peripheral blood T lymphocytes with Th_2 phenotype (Sallusto et al., 1997) and high levels of expression in eosinophils (Ponath et al., 1996). The latter observation suggests that CCR3 could be the main factor in facilitating eosinophils infection, as they are not expressing CX3CR1 (Figures 3B and 3C). Indeed, eosinophils that express high levels of CCR3 have been reported to be infected by RSV (Rosenberg et al., 2009; Kimpen et al., 1996). The role of CCR3 in these and other potential RSV target cells (Th_2 cells and airway smooth muscle cells) merits further investigations.

In summary, these results implicate CCR3 as an essential host factor RSV infection and suggest that blocking CCR3 may be an important therapeutic intervention in the treatment of RSV infection.

Limitations of the study

Our study demonstrates an anti-viral role of eotaxin-1/CLL11, limiting viral infection of airway epithelial cells. Also, CCR3 facilitates RSV infection in vivo and in vitro. Although we show that the RSV infection of airway epithelial cells involves interaction with the cellular CCR3 and is independent of GAGs, the underlying mechanism of this interaction remains to be elucidated. Also, the precise role of CCR3 and/or CX3CR1 in mediating the anti-viral effect of eotaxin-1/CLL11 needs further study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103433.

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AUTHOR CONTRIBUTIONS

B.L. conceptualized the study. B.L. and A.S.G. designed the experiments, wrote the manuscript, and secured funding. V.W., H.A.B., E.F., and F.B. performed the experiments. R.L., S.D., and F.L. provided samples and analyzed the data. B.L. reviewed and edited the manuscript. The manuscript was submitted on behalf of all authors by B.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human IL-4 NAb	R&D Systems	Cat# MAB204; RRID: AB_2126745
Anti-human IL-12 NAb	R&D Systems	Cat# MAB219; RRID: AB_2123616
Anti-human CCR3-FITC	R&D Systems	Cat# FAB155F; RRID: AB_357052
Rat IgG2A FITC Isotype Control	R&D Systems	Cat# IC006F; RRID: AB_357255
Anti-human CCR5-FITC	R&D Systems	Cat# FAB180F; RRID: AB_357095
Anti-human CXCR4	R&D Systems	Cat# MAB172; RRID: AB_2089399
Anti-human CX3CR1-PE	R&D Systems	Cat# FAB5204P; RRID: AB_2276862
Anti-human CX3CR1	Torrey Pines Biolabs	Cat# TP502; RRID: AB_10889906
Goat anti-rabbit IgG-FITC	CEDARLANE	Cat# CL6100F; RRID: AB_10061168
Anti-human IFN-γ-FITC	BD Biosciences	Cat# 561053; RRID: AB_10561691
Anti-human IL-4-PE	BD Biosciences	Cat# 559333; RRID: AB_397230
Anti-human CCR3	Thermo-Fisher	Cat# PA1-21622; RRID: AB_557983
Horse anti-goat IgG-Biotin	Vector Laboratories	Cat# BA-8000; RRID: AB_2336140
Horse anti-rabbit IgG-Biotin	Vector Laboratories	Cat# BA-1100; RRID: AB_2336201
Anti-human CD138 Ab-APC	BioLegend	Cat# 352307; RRID: AB_10901175
Rat IgG2A APC Isotype control	R&D Systems	Cat# IC006A; RRID: AB_357254
Mouse IgG1 PE isotype Control	R&D Systems	Cat# IC002P; RRID: AB_357242
Anti-RSV G protein	SinoBiological	Cat# 11,070-RP01
Bacterial and virus strains		
RSV-A2	ATCC	Cat# VR-1401
RSV-GFP	Gift from Dr M.E. Peeples	N/A
Biological samples		
Healthy blood (n = 36)	Reims University Hospital (CHU), Reims. France	https://www.chu-reims.fr/offre-de-soins/prises-en-charge/service/pneumologie
Human bronchial biopsies from asthmatics, COPD patients and normal controls	Reims University Hospital (CHU), Reims. France	https://www.chu-reims.fr/offre-de-soins/prises-en-charge/service/pneumologie
Chemicals, peptides, and recombinant proteins		
rh IL-2	R&D Systems	Cat# 10453-IL
rh IL-4	R&D Systems	Cat# 204-IL
rh IL-12	R&D Systems	Cat# 10018-IL
rhEotaxin-1/CCL11	R&D Systems	Cat# 320-EO
rhMIP-1α/CCL3	R&D Systems	Cat# 270-LD
rhMIP-1β/CCL4	R&D Systems	Cat# 271-BME
MDC/CCL22	R&D Systems	Cat# 336-MD
SDF/CXCL12	R&D Systems	Cat# 350-NS
CX3CL1	R&D Systems	Cat# 365-FR
Critical commercial assays		
RSV ELISA	Bioo Scientific	Cat#5506-01
CCR3-siRNA	Santa Cruz	Cat# sc-39884
CCR5-siRNA	Santa Cruz	Cat# sc-35062

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CXCR4-siRNA	Santa Cruz	Cat# sc-35421
CX3CR1-siRNA	Santa Cruz	Cat# sc-39904
Gene Knockout Kit v2	Synthego	http://www.synthego.com
Experimental models: Cell lines		
A549	ATCC	Cat# CCL-185; RRID: CVCL_0023
Hep-2	ATCC	Cat# CCL-23; RRID: CVCL_1906
GHOST(3) cell line	NIH-ARP	Cat# 3679-234; RRID: CVCL_S489
GHOST(3).CCR1 cell line	NIH-ARP	Cat# 3680-235; RRID: CVCL_1E06
GHOST(3).CCR2b cell line	NIH-ARP	Cat# 3681-236; RRID: CVCL_1E07
GHOST(3).CCR3 cell line	NIH-ARP	Cat# 3682-237; RRID: CVCL_S490
GHOST(3).CCR4 cell line	NIH-ARP	Cat# 3683-238; RRID: CVCL_S497
GHOST(3).V28/CX3CR1 cell line	NIH-ARP	Cat# 3939-242; RRID: CVCL_1E09
GHOST(3).CCR5 cell line	NIH-ARP	Cat# 3944-343; RRID: CVCL_1E17
Oligonucleotides		
sgRNAs target sequence CCR3, G*G*U*CUCAACUGUAUCCUAGUG, C*U*A*GAUACAGUUGAGACCUU	This paper	http://www.synthego.com
sgRNAs target sequence CX3CR1, G*G*C*UGAGGCCUGUUAUAUUG, U*G*A*GUACGAUGAUUUGGCUG	This paper	http://www.synthego.com
Software and algorithms		
InStat Software 3.0	GraphPad software	https://www.graphpad.com/
ImageJ	-	https://imagej.net/software/fiji/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bouchaïb Lamkhioued (bouchaib.lamkhioued@univ-reims.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data are included in the published article and the supplemental information files or are available from the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECTS DETAILS

Mice

6 week-old male BALB/c WT and CCR3-deficient mice (BALB/c background: C.129S4-Ccr3^{tm1Cge}/J) were obtained from Jackson Laboratories (Bar Harbor, USA). BALB/C CCR3$^{-/-}$ mice and BALB/c W.T. mice were maintained in pathogen-free conditions. All experiments were approved by the institutional animal care and use committee of the University of Reims, following the guidelines of the federation of European laboratory animal science associations (FELASA) and the European directive 2010/63/EU on the protection

of animals used for scientific purposes. The mice were housed in microisolator cages and fed with sterilized water and food ad libitum.

Human samples

All biopsies were obtained from healthy donors (n=8), asthmatics (n=4, males, 3; female, 1) or COPD patients (n=4; males, 2; females, 2) after physical examination in the Department of Pulmonary Medicine, Reims University Hospital Center (CHU) (France). The biopsies were collected following the standards of the Ethics Committee of the University of Reims. All patients gave their written informed consent prior to inclusion in the study. A clinical diagnosis of asthma (n=4) was made based on evaluating the patient's medical file by a respiratory physician. Diagnostic criteria included prior diagnosis and treatment for asthma, documented evidence of variable airflow obstruction >15%, and bronchial hyperresponsiveness. COPD patients were enrolled on the basis of clinical and functional assessments with a forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) < 0.7 after bronchodilation. Diagnostic criteria included prior diagnosis and treatment for COPD with no acute exacerbation for 4 weeks.

Cell culture

Hep-2 (human laryngeal carcinoma) and A549 (human type II alveolar pneumocyte cell) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The human osteosarcoma cell lines (Ghost parental cell) transfected with CCR1 (G.CCR1), CCR2b (G.CCR2b), CCR3 (G.CCR3), CCR4 (G.CCR4), CCR5 (G.CCR5), CXCR4 (G.CXCR4) and CX3CR1 (G.CX3CR1) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (kindly provided by Dr. Vineet N. Kewal Ramani and Dr. Dan R. Littman). All cells, A549 and Hep-2 and chemokine receptors transfected, were cultured in Eagle's modified essential medium (EMEM, Life Technologies, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin at 37°C in 5% CO_2.

Human primary nasal epithelial cells were obtained from nasal polyps resected from patients without respiratory diseases (n=3, males, 2; female, 1), undergoing endoscopic surgery at the Department of otorhinolaryngology, University Hospital (CHU) of Reims, France. Cells were dissociated by overnight pronase incubation (0.5 mg/ml, Sigma-Aldrich) and then cultured on collagen type IV (0.3 mg/ml, Sigma-Aldrich) coated Petri-dishes and maintained in PneumaCult-EX (StemCell) culture medium.

METHOD DETAILS

Virus preparation and titration

The A2 strain of RSV was purchased from the American Type Culture Collection (ATCC, VR-1401). The recombinant strain of RSV expressing green fluorescent protein (rgRSV; here, RSV-GFP) was a gift from Dr. M.E. Peeples (Children's Research Institute, Columbus, OH, USA). Hep-2 cells were used to amplify the viruses, as described previously (Das et al., 2020). For RSV infection, cells with 80% confluency in 12-well plates, were inoculated with the RSV virus and the plates were shaken several times gently during the adsorption period. After adsorption for 2 h at 37°C, the cells were washed with phosphate buffered saline (PBS), and 10% EMEM was added. The infection was allowed to proceed for 3 to 5 days until the entire monolayer shows cytopathic effects. The contents were resuspended in 1 ml aliquots, snap-frozen and stored at −80°C. Virus titration was done by plaque assay using Hep-2 cells in 24-well tissue culture plates and titers were maintained in the range of 10^6 to 10^8 pfu/ml for more than 6 months at −70°C.

RSV infection of epithelial cells

Hep-2, parental Ghost cells and Ghost transfected cells with chemokine receptors (CCR-1, CCR-2b, CCR3, CCR4, CCR5 or CX3CR1) were seeded in 24 tissue culture plates at $3x10^5$/well. CCL3, eotaxin-1/CCL11, CCL22, CCL4, CX3CL1 and anti-chemokine receptors antibodies (R&D systems) were assayed for their abilities to inhibit RSV infectivity. RSV $G_{171-201}$ peptide that contains the CX3C domain of CX3CL1 and is demonstrated to inhibit RSV infection was used in these experiments as previously described (Tripp et al., 2001). For plaque reduction neutralization assays, cells were preincubated for 1 hour at 37°C with chemokines (various doses up to 1 μg/ml), anti-chemokine receptors Abs (5 μg/ml) or PBS followed by infection with RSV at a m.o.i. of 2. After 2 hours of incubation, cells were washed with PBS and overlaid with RPMI containing the same reagents (chemokines or antibodies), 10% FCS and 0,75% methylcellulose. Three days post-infection, the cells were fixed and stained with hematoxylin and eosin. Plaques were counted,

and percent inhibition of virus infectivity of treated cells was determined versus untreated control wells (Feldman et al., 1999).

For GFP fluorescence measurement, A549 WT and edited cells were infected with RSV-GFP at a m.o.i. of 1 and incubated at 37°C and 5% CO2 for 48 h. Fluorescence could be observed with a fluorescence microscope. For plaque reduction assays, the RSV-GFP virus was grown on A549 cells and plaques were counted using the plaque assay protocol described above.

Purification of RSV F and secreted G proteins

Monolayers of Hep2 cells were infected with RSV A2 at a m.o.i. of 2. After 3 days of growth, the culture supernatant was transferred to 50-ml centrifuge tubes, and RSV G secreted (sG) protein was prepared using a modified two-steps isolation procedure (Barr et al., 2000; Lamkhioued et al., 1995). Culture supernatant was overlaid on a cushion of 1.46 M sucrose–1 mM NaH2PO4 (pH7.2) and centrifuged at 62000 x g for 90 min in Beckman ultracentrifuge. The supernatant was removed, dialyzed overnight at 4°C against ConA binding buffer containing 0.2 M NaHCO3 and 0.5 M NaCl (pH 8.3) and then applied to a ConA-Sepharose lectin column (Pharmacia Biotech, Piscataway, NJ). Unbound proteins were removed from the column by washing with binding buffer until OD280 (optical density at 280 nm) readings were less than 0.02. Bound proteins were eluted by application of 0.5 M methyl-α-mannopyranoside in 20 mM Tris–HCl–0.5M NaCl (pH 7.4). The eluted fractions were collected throughout the chromatographic run. Fractions were screened by western blotting analysis, and G-containing fractions were pooled and concentrated 100-fold in Centricon-10 membranes (Amicon, Inc., Beverly, Mass.). According to a previously described technique (Lamkhioued et al., 1995), 100 mg of cyanogen bromide-activated sepharose-4B beads (Pharmacia) were coupled to 1 mg of anti-RSV G mAb. The eluted fraction from the previous column was then incubated with anti-RSV G mAb bound to sepharose for 16h at 4°C. The beads were then placed in a 1-ml syringe as a small column. After extensive washes, the bound material was eluted with 0.1 glycin buffer. Fractions were collected and sG protein was identified by western blot using a rabbit an anti-sG protein polyclonal antibodies (SinoBiological). G-containing fractions were pooled and concentrated using Centricon 10 membranes (Merck, France). The concentration of G protein was estimated using a molecular weight of 90 kD. The purified proteins were stored at −70°C. RSV-F glycoprotein was obtained from Hep-2 cells infected with the RSV. Following detergent lysis, F protein was purified by immunoaffinity chromatography using an anti-F protein mAb as described above.

Immunoprecipitation and Western blot

RSV G-protein interaction with CCR3 on epithelial cells was performed using a combination of immunoprecipitation and western blotting with minor modifications as described earlier (Redhu et al., 2009). Briefly, A549 cells or G.CCR3$^+$ cells were washed twice with PBS and lysed for 30 min at 4°C in NP-40 lysis buffer supplemented with a cocktail of protease inhibitors (2 mM sodium orthovanadate, 1 mM phenyl-methylsulfonylfluoride, 10 μg/ml leupeptin, 0.15 units/ml approtinin, 1 μg/ml pepstatin A) (Sigma-Aldrich) and centrifuged for 20 min to remove nuclei. The supernatant from the cell lysates was pre-cleared with protein G-sepharose-coated beads (Amersham-Pharmacia) for 2 hrs at 4°C in a rotating mixer, followed by incubation with 50 μg/ml of sG protein at 4°C for 2h. The mixture was then incubated with protein A/G sepharose-coated beads conjugated with 5 μg/ml of murine anti-RSV G protein mAb or isotype mouse monoclonal antibody (Chemicon International) for 16 h at 4°C. Immuno-complexes were then pelleted by centrifugation and washed six times with the wash buffer (PBS/1% NP40). For immunoblotting, samples were separated on SDS polyacrylamide gel and electrotransferred onto PVDF membrane (Immobilon, Millipore Canada). The membrane was blocked at RT for 2 hrs with 5% Blotto (Santa Cruz Biotechnology, CA, USA), incubated with goat anti-CCR3 polyclonal Ab (Thermo Fisher Scientific) (1 μg/ml) at room temperature for 2 h, followed by secondary antibody HRP-rabbit anti-goat IgG (H+L) prepared in TBST (1:5000). The blots were developed by enhanced chemiluminescence as recommended by the supplier (Life Science, Canada). As a positive control, 10 μg of eotaxin-1/CCL11 were directly added to G.CCR3$^+$ cells lysate. The immunoprecipitation was carried out with protein A/G sepharose-coated beads conjugated with 5 μg/ml of murine anti-eotaxin-1/CCL11 mAb and the blots were developed as described above.

Cell-binding assays using flow cytometry analysis

Cells were labeled with a Fluorokine kit for human chemokine receptors according to the manufacturer's instructions (R&D Systems). Briefly, adherent A549 and Ghost cells were first detached from the flask by adding a solution of PBS-EDTA (0.5 M) for 20 min at 37°C. A549 and Ghost cells were washed once with

PBS and resuspended at a 4 x10^6 cells/ml concentration. 10 μl of biotinylated recombinant chemokines (eotaxin-1/CCL11 or CCL4) reagent was added to 25 μl of the washed cells and incubated for 60 min on ice. As a negative staining control, an identical sample of cells was stained with 10 μl of biotinylated negative control reagent (soybean trypsin inhibitor, R&D Systems). Following the incubation period, 10 μl of Avidin-FITC reagent was added, and cells were incubated for an additional 30 min at 4°C in the dark. The specificity of the reaction was assessed by mixing 20 μl of blocking antibody with 10 ml of biotinylated chemokine and incubating for 15 min at RT. Cells were then washed twice, using the buffer provided to remove unreacted Avidin-FITC, resuspended in 200 μl of PBS. Cell-associated immunofluorescence was immediately analyzed using the FACScan flow cytometer (Becton Dickinson, Mississauga, Canada) to determine the surface expression level of chemokine receptors.

CCR3 and CX3CR1 were also identified using PE-labeled anti-CCR3 mAb (R&D systems) and rabbit anti-CX3CR-1 Ab (Torrey Pines Biolabs). Briefly, A549, eosinophils, G.CCR3 and G.CX3CR1, were recuperated from a confluent 75 cm^2 tissue culture flask with PBS-EDTA 0.5 M. Cells (10^6/ml) were washed with PBS-BSA 1% –Azide 0,1 % (FACS-buffer). A hundred microliters (10^6 cells/ml) were distributed in 96-well V-bottomed microplates and centrifuged. Then, cells were resuspended in 50 μl of anti-CCR3-PE conjugated mAb (2 μg/10^6) or control isotype antibodies (2 μg/10^6) (R&D Systems) at 4°C for 60 minutes. For the CX3CR1 staining, cells were resuspended in 50 μl of FACS-buffer and 50 μl of rabbit anti-CX3CR1-purified Igs for 30 minutes at 4°C, washed three times with FACS-buffer and then incubated for another 30 min on ice with 50 μl of a goat anti-rabbit FITC-conjugated IgG (Cedarlane, Burlington, Ontario, CA) diluted 1/50 in FACS-buffer. After extensive washing (FACS-buffer), cells were resuspended in PBS and stored at 4°C until analyses. Cell-associated immunofluorescence was analyzed by a FACscan flow cytometer (Becton Dickinson, Mississauga, Canada) to determine the level of surface expression of CCR3 and CX3CR1.

Immunostaining studies

Immunohistochemistry was performed on sections of the major airways (large bronchus) of four asthmatics and four normal subjects. Immediately following biopsies, lung specimens were prepared for immunohistochemistry as previously described (Lamkhioued et al., 1997). Briefly, fresh frozen sections (5μm) were fixed with methanol/acetone at −20°C for 10 minutes. After blocking with Dako blocking solution for 30 min, slides were incubated with goat anti-human CCR3 Abs (Thermo-Fisher, France) or rabbit anti-human CX3CR1 Abs (Torrey Pines Biolabs, France) (5μg/ml) overnight at 4°C, followed by incubation for 1 hour at 37°C with 5 μg/ml biotin-labeled horse anti-goat IgG or biotin-labeled horse anti-rabbit IgG respectively (Vector laboratories, Burlingame, USA) (Gounni et al., 2006; Lamkhioued et al., 1997). After incubation with antibodies, slides were extensively washed with TBS. Positive cells stained red after development with streptavidin-AP and fast red (Sigma Chemical Co., St. Louis, MO). Nuclei of cells were stained for 1 min with hematoxylin. Goat and rabbit normal serums were used at the same dilution as negative controls.

Confocal fluorescent microscopy

Immediately following biopsies, lung specimens were prepared for immunofluorescence as previously described (Lamkhioued et al., 1997). Briefly, Formalin-fixed paraffin-embedded (FFPE) lung tissues (5μm) were deparaffinized using xylene and then washed in serial dilutions of ethanol in order to rehydrate the tissue. Sections and primary nasal epithelial cells were fixed in PAF2% for 15 minutes. After blocking with Dako blocking solution for 30 min, slides were incubated with mixture of primary Abs; it consists of APC anti-human CCR3 mAb (1/100 dilution, R&D System) and PE anti-human CX3CR1 Ab (1/100 dilution, BioLegend) overnight at 4°C (Gounni et al., 2006). After incubation with antibodies, slides were extensively washed with TBS. Nuclei of cells were stained for 1 min with DAPI. Irrelevant mice IgG_{2a} isotype control mAbs were used at the same dilution as negative controls. Images were taken by Confocal Zeiss LSM710 microscope (Oberkochen, Germany). All images were analyzed using ImageJ (Version 1.53a, National Institutes of Health, USA).

Measurement of mRNA expression by reverse transcription-PCR

Total RNA was isolated from A549, Hep-2, G.CCR3, G.CX3CR1, parental Ghost cells and eosinophils with the TRIzol reagent (Invitrogen). Reverse transcription was performed by using 2 μg of total RNA in a first-strand cDNA synthesis reaction with the Moloney murine leukemia virus reverse transcriptase (life technologies, Gaithersburg, MD). β-2-globulin 5′ primer 5′GATGAGTATGCCTGCCGTGTG3′ and the 3′ primer 5′CAATCCAAATGCGGCATCT3′ were used as the standard to control for variations in RNA isolation, cDNA synthesis and PCR performance. A sample for cDNA was subjected to sequential cycles of

amplification (30 cycles). Samples were amplified at 94°C for 1 min, 60°C for 2 min and 72°C for 3 min. A 313-bp fragment was generated using the 5′ primer 5′AAGCTTACGCCAAA GCTCACACCT3′ and the 3′ primer 5′GAATTCTGGCTTTGGAGTTGGAGAT3′ specific for human CCR3 and a 591-bp fragment was generated using the 5′ primer 5′CGGACCGTGCAGCATGGCG3′ and the 3′ primer 5′GAACACTTCCATG CCTGCTCC3′ specific for human CX3CR1. The band intensities were obtained on ethidium bromide-stained agarose gels.

siRNA transduction in G.CCR$^+$ cells

For short-interfering RNA (siRNA)-induced gene silencing studies, a pool of three to five target-specific 20-25nt siRNAs designed to knockdown gene expression of CCR3, CCR5, CXCR4 and CX3CR1 were obtained from Santa Cruz Biotechnology. A control siRNA unrelated to chemokine receptors sequences (scramble siRNA with no specific degradation of any known cellular mRNA) was used as a negative control. Transient transfection of cells was performed according to the manufacturer's specifications. For each transfection, 80 pmols of siRNA in 8 μl of transfection medium were added to 8 μl of the transfection reagent and the mixture was incubated for 30 min at room temperature. The G-CCR$^+$ cells were washed with 2 ml of siRNA transfection medium and incubated for an additional 24 hours at 37°C in a CO_2 incubator, with the corresponding siRNA, control siRNA or PBS. After this period, cells were washed and overlaid with DMEM containing the same reagents and 10% FCS. CCR3, CCR5, CXCR4 and CX3CR1 expression was then determined by flow cytometry using FITC anti-CCR3, PE anti-CX3CR1, FITC anti-CCR5 and anti-CXCR4 (R&D Systems) antibodies at 1/100 dilution as described above. For RSV infection, cells with 80% confluency in 12-well plates, were inoculated with the RSV virus. Three days post-infection, the cells were fixed and stained with hematoxylin and eosin. Plaques were counted and percent inhibition of virus infectivity of treated cells was determined versus untreated control wells.

Generation of CCR3 and/or CX3CR1 knock out human airway epithelial cells

CRISPR editing experiments has been designed for targeting CCR3 and/or CX3CR1 using recombinant Cas9 protein with synthetic guide RNAs and introduced directly as ribonucleoprotein (RNP) complex into cells. Two sgRNAs were designed according to Synthego's multi-guide gene knockout (key resource table, see STAR Methods). To induce gene knockout in A549 cells, 20 pmol Synthego 2NLS-Cas9 nuclease from S. Pyogenes (Synthego) was combined with 36 pmol total synthetic sgRNA CCR3 or CX3CR1 (Synthego) to form ribonucleoproteins (RNPs) in 30 uL total volume with Nucleofector SF solution (Lonza). The RNP assembly reaction was mixed by pipetting up and down and incubated at room temperature for 10 min.

All cells were dissociated into single cells using trypsin-EDTA (GIBCO), washed and resuspended in culture media and counted. For A549 transfections, 1x10^5 cells per reaction were used for electroporation. Cells were pelleted by centrifugation at 500 x g for 5 min and each cell pellet was resuspended in 30 μL RNP-Nucleofection solution tube. Nucleofections were performed on a Lonza nucleofector II system using program x-001 for A549 and Amaxa Cell Line Nucleofector Kit T (Lonza). Immediately following nucleofection, the cells were resuspended in each well of the Nucleocuvette with 70 μL of pre-warmed growth media, and mixed gently by pipetting up and down 2-3 times. The cells were then transferred to the corresponding well of 12-well plate and incubated in 37°C/5%CO_2 incubator for 2 days. pMAXGFP vector has been used in the same conditions to assess transfection efficiency. Single cell derived clonal cell lines were generated using limiting dilution and clonal expansion procedure (Synthego). To confirm the KO phenotype of the cells, confocal microscopy analysis and flow cytometry were performed on clonal populations. Next, phenotypical confirmed human cells deficient for CX3CR1 were transfected with CRISPR gRNAs CCR3 to create human cells deficient for CX3CR/CCR3 (double KO cell lines). Lastly, phenotypical confirmed human cells deficient for CX3CR1/CCR3 were treated with Heparinase-I (1 U/ml) (Sigma-Aldrich) for 1 h at 37°C to create human cells deficient for CCR3/CX3CR1/Syndecan-1. CCR3, CX3CR1 and syndecan-1 expression on A549 cells was then determined by flow cytometry or confocal microscopy as described above using APC anti-CCR3 (R&D Systems), PE anti-CX3CR1 (R&D systems) and APC anti-CD138 (syndecan-1) (BioLegend) antibodies at 1/100 dilution. For RSV infection, A549 edited cells were seeded on the day before viral infection, after which, cells were infected with RSV-GFP as described above.

Viral load detection in lungs

Mice (n=8/genotype) were lightly anesthetized and infected intranasally (i.n) with 10^6 p.f.u RSV in 100 μl. Control mice (n= 8 mice/genotype) were inoculated with 100 μl of sterile PBS alone. Mice were monitored

daily, and on day 5, they were killed and the lungs were removed and processed as described below. The left upper lobe from each mouse was flash-frozen in liquid nitrogen and kept frozen at 80°C. Just before running RSV ELISA assays, the samples were weighed and homogenized in 1 ml of homogenization buffer (Bioo Scientific, Austin, USA) containing one Roche complete protease inhibitor cocktail tablet (Boehringer Mannheim, Germany) and 0.1% Triton-X in 50 ml of phosphate-buffered saline (PBS). Viral load in lungs were also detected by plaque assay on Hep cells as described above.

Lung fixation and histology

The right lung was removed, weighed, resuspended in DMEM and serial dilutions of clarified lung homogenates were plated for viral titer. The right ventricle was flushed with 5 ml clean PBS to flush the left lung vasculature. The trachea was cannulated and the lungs were inflated with phosphate-buffered formalin (10% formalin) at 20 cm H_2O pressure. We then removed the lung and submerged it in formalin for fixation and subsequent histological sectioning. Thin sections were cut from paraffin-embedded lungs and stained with hematoxylin and eosin (H&E). As a semiquantitative analysis of mucus induction, mucus-producing goblet cells were identified and quantified in lung sections stained with Periodic-acid Schiff (PAS) reagent. The mucus index was calculated using ImageJ software (NIH) on entire lung sections encompassing large and small bronchial airways. The goblet cell counts were calculated as the average of three airways in lung sections from each mouse and then reported as the mean and standard deviation for each treated group as described previously (Johnson et al., 2008).

Cell purification and *in vitro* generation of Th1 and Th2 cells

Mononuclear cells and granulocytes were purified from peripheral blood by Ficoll-Paque (Pharmacia) density centrifugation. Granulocytes were obtained by dextran sedimentation and human eosinophils were further purified by negative selection with anti-CD16 and anti-CD14-coated immunomagnetic microbeads using a Magnetic Cell Sorting system (Miltenyi Biotec, Auburn, USA) at 4°C (Lamkhioued et al., 1997). The degree of purity of eosinophil populations, estimated after staining with Giemsa, was between 92 and 100%. T cells were purified from cord blood by Ficoll Paque and Th_1 and Th_2 cell polarization was performed as previously described (Gounni et al., 2006). Briefly, cord blood T cells were stimulated with phytohaemagglutinin (PHA, 1 μg/ml) under Th_1 polarizing conditions [human rIL-12 (2 ng/ml) plus neutralizing mAb to IL-4 (200 ng/ml)] or Th_2 polarizing conditions [human recombinant IL-4 (200 U/ml) plus neutralizing mAb to human IL-12 (2 μg/ml)]. IL-2 (40 U/ml) was added on day 3. After 10 days, the cultures were washed and restimulated for 4h with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin (1μg/ml) in the presence of brefeldin A (1 μg/ml). Then the cells were fixed with 4% paraformaldehyde and permeabilized with saponin. Fixed cells were stained with FITC–anti-IFNγ and PE–anti-IL-4 mAbs (BD Pharmingen) and analyzed by FACScan flow cytometer (BD Biosciences) (Gounni et al., 2006).

Chemotaxis assay

Experiments were performed in a 48-well micro-chemotaxis chamber (NeuroProbe) and carried out as previously described (Lamkhioued et al., 1997). Chemotactic properties of each chemokine and sG protein were evaluated measuring the Th_1, Th_2 cells and eosinophils migration through a 5 μm pore polycarbonate filter. Cells were suspended at 1 x 10^6 cells/ml in complete RPMI plus 0.5% BSA, and were added to the top chamber. Various concentrations of different chemokines (eotaxin-1/CLL11, CCL4) or sG protein were added to the bottom chamber. After 2 h of incubation at 37°C with 5% CO_2, the filters were recovered and the cells were counted by microscopy in five selected high-power fields (magnification x 400). In some experiments, cells were preincubated at 4°C for 30 minutes with antibodies directed against chemokine receptors (CCR3, CCR5 or CX3CR1) or control antibodies at 10 μl prior to the chemotaxis assay and the number of migrated cells was determined as previously described (Lamkhioued et al., 1997; Gounni et al., 2006)

Ethics statement

Written informed consent for biopsies harvesting was obtained from all patients. The human research ethics board of the University of Reims, France, approved all the experimental procedures. All experimental procedures of animals were performed in Biosafety level 2 facilities at the University of Reims and were approved by the Animal Care and Use Committee at ''Structure Fédérative de Recherche''.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All statistical tests were performed using InStat Software 3.0 (GraphPad Software, San Diego, CA). Data are expressed as mean -± SD of three or more independent experiments with three replicates. Student's t test (unpaired, two-tailed) was performed when comparing two groups to each other. P values less than 0.05 were considered statistically significant.