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Rhinovirus Infection of Allergen-Sensitized and -Challenged Mice Induces Eotaxin Release from Functionally Polarized Macrophages

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Human rhinovirus is responsible for the majority of virus-induced asthma exacerbations. To determine the immunologic mechanisms underlying rhinovirus (RV)-induced asthma exacerbations, we combined mouse models of allergic airways disease and human rhinovirus infection. We inoculated OVA-sensitized and challenged BALB/c mice with rhinovirus serotype 1B, a minor group strain capable of infecting mouse cells. Compared with sham-infected, OVA-treated mice, virus-infected mice showed increased lung infiltration with neutrophils, eosinophils and macrophages, airway cholinergic hyperresponsiveness, and increased lung expression of cytokines including eotaxin-1/CCL11, IL-4, IL-13, and IFN- γ . Administration of anti-eotaxin-1 attenuated rhinovirus-induced airway eosinophilia and responsiveness. Immunohistochemical analysis showed eotaxin-1 in the lung macrophages of virus-infected, OVA-treated mice, and confocal fluorescence microscopy revealed colocalization of rhinovirus, eotaxin-1, and IL-4 in CD68-positive cells. RV inoculation of lung macrophages from OVA-treated, but not PBS-treated, mice induced expression of eotaxin-1, IL-4, and IL-13 *ex vivo*. Macrophages from OVA-treated mice showed increased expression of arginase-1, Ym-1, Mgl-2, and IL-10, indicating a shift in macrophage activation status. Depletion of macrophages from OVA-sensitized and -challenged mice reduced eosinophilic inflammation and airways responsiveness following RV infection. We conclude that augmented airway eosinophilic inflammation and hyperresponsiveness in RV-infected mice with allergic airways disease is directed in part by eotaxin-1. Airway macrophages from mice with allergic airways disease demonstrate a change in activation state characterized in part by altered eotaxin and IL-4 production in response to RV infection. These data provide a new paradigm to explain RV-induced asthma exacerbations. *The Journal of Immunology*, 2010, 185: 000–000.

Viral infections trigger 80% of asthma exacerbations in children and nearly 50% in adults (1, 2), with human rhinovirus (RV) being the most common virus identified. RV infections were once thought to be restricted to upper airway tissues (3), but it is now clear that infections of the upper respiratory tract are accompanied by the entry of the virus into lower respiratory tract cells (4–7), although the quantity of viral replication is not known.

In normal subjects, RV causes airway narrowing in response to methacholine and increased airway neutrophils and submucosal CD3⁺ cells (8, 9). In theory, RV infection of airway cells elicits the production of chemokines, subsequently inducing recruitment of inflammatory cells to the airways. Inflammatory cells, in turn, elaborate cytokines and mediators capable of increasing airways

responsiveness. This paradigm, however, does not explain why asthmatic subjects experience exacerbations of lower airways disease after respiratory tract infection while control subjects do not.

Numerous clinical studies suggest a role for IL-8/CXCL8 in the pathogenesis of RV-induced asthma exacerbations. IL-8 and neutrophils are found in the nasal secretions, sputum or bronchoalveolar lavage (BAL) fluid of allergic subjects undergoing experimental RV infection (9–14). After RV16 infection, asthmatic patients show increased levels of IL-8 in their nasal lavage, which correlates with the level of airways responsiveness (11), in contrast to unaffected individuals in whom IL-8 does not increase (15). Eosinophils and eosinophil cationic protein have also been detected in the airways after experimental RV infection (10, 14, 16). Patients with asthma who are undergoing experimental RV infection demonstrate greater eosinophilic inflammation than do RV-infected control subjects (14). These data suggest that patients with asthma experience a different response to viral infection than do controls.

We recently showed that inoculation of C57BL/6 mice with RV1B, a minor group virus that binds to proteins of the highly conserved low-density lipoprotein receptor family, induces airway neutrophilic inflammation and methacholine hyperresponsiveness (17). In contrast, replication-deficient UV-irradiated virus did not cause lasting hyperresponsiveness. We also found positive- and negative-strand viral RNA in the lungs up to 4 d postinfection, suggesting replication of RV *in vivo*. It was recently shown that RV infection of BALB/c mice induces similar airway changes (18). Infection of OVA-sensitized and -challenged mice increased bronchoalveolar neutrophils, eosinophils and lymphocytes compared

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Abbreviations used in this paper: AF, Alexa Fluor; BAL, bronchoalveolar lavage; MCP, macrophage chemotactic protein; RV, rhinovirus.

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with allergen-challenged mice treated with UV-inactivated virus. However, the mechanism by which eosinophils are attracted to the airways after RV infection, and the requirement of eosinophils for the development of RV-induced airway hyperresponsiveness, were not examined.

In this study, we show that, in OVA-sensitized and challenged BALB/c mice, RV1B infection increased production of proinflammatory cytokines including eotaxin-1/CCL11, Th-2 cytokines IL-4, and IL-13. Bronchoalveolar and lung neutrophils, eosinophils, macrophages, and airway responsiveness were elevated in the RV-infected, OVA-treated mice. Neutralization of eotaxin-1/CCL11 reduced both airway eosinophilic inflammation and hyperresponsiveness. Eotaxin-1 and IL-4 were localized to RV-infected airway macrophages. Finally, macrophages from OVA-treated, but not PBS-treated, mice expressed eotaxin-1, IL-4, IL-13 in response to RV infection *ex vivo*, as well as the alternative activation markers arginase-1, Ym-1, MGL-2, and IL-10. Finally, depletion of macrophages from OVA-RV-treated mice significantly decreased eosinophil infiltration and airway responses compared with nondepleted controls. These results suggest that allergen sensitization and challenge skews a predominantly neutrophilic RV response in naive mice to a Th-2-dominant eosinophil response that is augmented, at least in part, by alternatively activated macrophages.

Materials and Methods

Generation of RV

RV1B (American Type Culture Collection, Manassas, VA) was concentrated, purified, and titered as described previously (19, 20). Fifty percent tissue culture infectivity doses (TCID₅₀) were determined by the Spearman-Kärber method. RV1B was UV-irradiated using a CL-1000 crosslinker (Ultraviolet Products, Upland, CA).

OVA sensitization and challenge and RV exposure

This study was approved by the Institutional Animal Care and Use Committee. Animal use followed guidelines set forth in the *Principles of Laboratory Animal Care* (National Association for Biomedical Research, Washington, DC). Female 8 wk-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were injected i.p. with 200 μ l of a 5-mg/ml solution of alum and endotoxin-free OVA or PBS (Sigma-Aldrich, St. Louis, MO) on days 1 and 7; they were treated intranasally with 50 μ l of a 20-mg/ml solution of OVA or PBS on days 14, 15, and 16. Immediately following the last OVA or PBS treatment, mice were inoculated intranasally with 45 μ l of 1×10^8 TCID₅₀/ml RV1B, UV-irradiated RV, or an equal volume of sham HeLa cell lysate (17).

Bronchoalveolar inflammatory cells and macrophage culture

BAL was performed using 1-ml PBS aliquots. Cytospins were stained with Diff-Quick (Dade Behring, Newark, DE), and differential counts determined from 200 cells. BAL fluid from PBS- and OVA-treated mice was seeded in 12-well plates. To partially purify macrophages, cells were allowed to adhere for 90 min, and nonadherent cells were removed by suction. Diff-Quick staining showed adherent cells to consist of >90% macrophages, with the rest of the cells being neutrophils. Remaining cells were resuspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA), stimulated for 2 h with sham or RV1B (multiplicity of infection, 5.0) and RNA harvested 8 h postinfection. In selected experiments, cells were pretreated with 30 ng/ml IL-4 and IL-13 (Peprotech, Rocky Hill, NJ).

Lung inflammation

To quantify inflammatory cells, lung digests were obtained by mincing the tissue, proteolysis in collagenase type IV (Invitrogen, Carlsbad, CA) and straining through a 70- μ m nylon mesh (BD Falcon, San Jose, CA) as described (21). The resulting pellet was treated with RBC lysis buffer (BD Pharmingen, San Diego, CA), and leukocytes were enriched by spinning the cells through 40% Percoll (Sigma-Aldrich). Lung leukocyte cytopins were stained and counted as described above.

Cytokine/chemokine expression

Lung RNA was extracted with Trizol (Sigma-Aldrich) and analyzed for cytokine and mucin gene expression by quantitative real-time PCR using

specific primers and probes. Signals were normalized to GAPDH and expressed as fold-increase. BAL fluid was spun for 15 min at $1500 \times g$, and the supernatants were analyzed for cytokine protein by multiplex immune assay (Bio-Rad, Hercules, CA).

Respiratory system resistance

Airway responsiveness was assessed by measuring changes in respiratory system resistance in response to increasing doses of nebulized methacholine (17).

Macrophage depletion

Depletion of alveolar macrophages was accomplished by intratracheal instillation of liposomes containing clodronate (dichloromethylenediphosphonic acid, disodium salt) as previously described (22). PBS-containing liposomes were used for control experiments. Eight milligrams cholesterol and 86 mg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform and slowly evaporated. The filmy layer was resuspended in 10 ml PBS or 0.6 M clodronate. The mixture was exposed to N₂ gas and incubated for 2 h at room temperature with gentle shaking. The mixture was then sonicated and incubated for another 2 h to allow liposome swelling. The solution was centrifuged at $10,000 \times g$ for 15 min, and the liposomes were collected and washed twice with sterile PBS. Liposomes were kept at 4°C under N₂ until use. Depletion was performed 24 h after the last OVA challenge by introducing 60 μ l clodronate- or PBS-containing liposomes intratracheally. Twenty-four hours later, mice were infected with RV1B, as described above. Differential cell counts were performed on lung digests, and respiratory resistance to methacholine was measured.

Immunohistochemistry and confocal fluorescence microscopy

Lungs were fixed with 10% formaldehyde overnight and embedded in paraffin. Blocks were sectioned at 500- μ m intervals at a thickness of 5 μ m, and each section was deparaffinized, hydrated, and stained with goat anti-mouse eotaxin-1 (Santa Cruz Biotechnology, Santa Cruz, CA). For immunohistochemical analysis, sections were incubated with biotinylated secondary goat-IgG, ABC reagent (Vector Laboratories, Burlingame, CA), diaminobenzidine (Sigma-Aldrich) and Gill's hematoxylin (Fisher Scientific, Kalamazoo, MI). For fluorescence microscopy, slides were incubated with Alexa Fluor (AF)-conjugated donkey anti-goat IgG (Molecular Probes, Portland, OR) and rat anti-mouse CD68 (AbD Serotec, Raleigh, NC) or isotype control IgG. In selected experiments, sections were costained with antiserum against RV1B (American Type Culture Collection). Antiserum was partially purified by incubation with nitrocellulose-bound HeLa cell proteins and passing through an affinity resin containing non-denatured mouse lung protein. Repurified Ab was directly conjugated to AF. Nuclei were stained with Hoechst 33258. Images were visualized using an LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY) and Axiovert 100M inverted microscope (Carl Zeiss). CD68-, eotaxin-1/CCL11-positive cells were counted at 500- μ m intervals and expressed as the number per field.

Data analysis

Data are represented as mean $\pm$ SEM. Statistical significance was assessed by one- or two-way ANOVA, as appropriate. Differences were pinpointed by Student Newman-Keuls' multiple range test.

Results

RV infection of OVA-sensitized and -challenged mice further increases airway inflammation

We previously showed that RV1B infection of naive C57BL/6 mice induces a state of modest airways hyperresponsiveness that lasts at least 4 d after viral inoculation (17). Hyperresponsiveness was associated with a short-lived increase in bronchoalveolar neutrophils. In the current study, we infected OVA-sensitized and -challenged BALB/c mice. Tissue eosinophils, macrophages, and neutrophils were elevated in OVA-treated mice up to 4 d after RV inoculation (Fig. 1), with maximal recruitment of macrophages and eosinophils occurring two days postinfection. In terms of absolute numbers, macrophages were the cell type most heavily recruited to the tissues. Lung neutrophil recruitment was a relatively brisk event, with a significant increase in OVA/RV mice on day 1 postinfection and a dramatic decrease on days 2 and 4.

Although OVA treatment significantly increased lung lymphocytes compared with naive mice, there was no significant difference in lymphocyte counts between OVA/RV and OVA/sham mice. In the BAL, RV infection increased neutrophils, eosinophils, and lymphocytes in both naive and OVA-sensitized and -challenged mice (Supplemental Fig. 1). As in the lung tissue, the largest absolute increase in BAL inflammatory cells after RV infection of OVA-sensitized and -challenged mice was observed in the macrophage line.

Effects of RV infection on lung proinflammatory cytokines

To determine changes in proinflammatory cytokines that might be responsible for the observed increase in eosinophilic inflammation, we measured lung cytokine levels by multiplex immunoassay. Compared with OVA-treated mice, OVA/RV mice demonstrated significantly higher levels of eotaxin-1/CCL11, IL-4, and IL-13, 1 d postinfection (Fig. 2). In contrast, there was no effect of RV infection on lung IL-5 levels. On day 4, lungs from RV-infected OVA-treated mice showed a sustained increase in eotaxin-1 and IL-4 levels (Fig. 3). Eotaxin mRNA was elevated in the OVA/RV treated groups on days 1, 2, and 4 postinfection.

RV infection increases the airway responsiveness of OVA-sensitized and -challenged mice

To determine whether the observed airway inflammation was functionally significant, all groups were tested for responsiveness to a bronchoconstrictor agonist 4 d after sham or RV1B treatment. Methacholine (0–20 mg/ml) was administered by nebulization and total respiratory system resistance values recorded. As expected, OVA treatment increased airway cholinergic responsiveness (Fig. 4A). However, RV-infected OVA mice demonstrated significantly higher airways responses, with significant differences noted at methacholine doses of 10 and 20 mg/ml ($p < 0.05$, two-way ANOVA). In contrast, UV-irradiated, replication-deficient RV had no effect on airway responses in OVA-sensitized and -challenged mice. To determine whether the observed elevated airway responses to methacholine were due to a higher viral load, we measured lung positive-strand RV RNA. Surprisingly, vRNA levels were significantly lower in the OVA/RV treatment group (Fig. 4B), suggesting

that allergen sensitization and challenge increases viral clearance. As shown previously (17), viral copy numbers were negligible by 4 d postinfection, consistent with the notion that airway hyperresponsiveness persists after viral clearance in this model.

Eotaxin-1 is required for maximal RV-induced eosinophilic airway inflammation and hyperresponsiveness in OVA mice

In OVA-treated mice, RV infection increased lung eosinophils (Fig. 1) and the protein level of eotaxin-1/CCL11 (Figs. 2, 3), an eosinophil-specific chemokine. We therefore sought to examine the contribution of eosinophils to RV-induced airway responsiveness by administering neutralizing Ab to mouse eotaxin-1. To ensure the suppression of augmented eosinophilic inflammation in RV-infected mice, a subset of OVA-treated mice was given two systemic injections of rabbit antiserum, the first on the day of RV inoculation and the second 2 d later. Control mice were treated with the isotype control. We found that, compared with IgG, anti-eotaxin treatment significantly reduced lung eosinophils in OVA-treated, RV-infection mice, but not OVA-treated, sham-inoculated mice (Fig. 4C). Anti-eotaxin-1 neutralizing Ab did not reduce the infiltration of neutrophils, macrophages, or lymphocytes (data not shown), suggesting that the Ab specifically targeted eosinophils. Furthermore, administration of anti-eotaxin to OVA/RV mice significantly reduced responsiveness to methacholine compared with IgG (Fig. 4D), suggesting that eotaxin-1 and eosinophils are required for maximal airway responses in RV-infected allergen-sensitized and -challenged mice. Anti-eotaxin had no effect on lung vRNA 1 d postinfection (data not shown).

Eotaxin-1 is mainly localized to CD68-positive macrophages

We examined lungs of PBS- and OVA-treated mice for eotaxin-1 localization (Fig. 5A–C). Immunohistochemical analysis of lungs from RV-infected, OVA-treated mice showed abundant eotaxin-1 staining, which appeared to be localized to airway and submucosal macrophages. There also appeared to be a small number of eotaxin-positive eosinophils and limited staining in the airway epithelium. Fluorescence confocal microscopy with anti-eotaxin-1 and anti-CD68 showed intense yellow-orange staining, consistent with colocalization of CD68 and eotaxin-1 in lung macrophages (Supplemental

FIGURE 1. OVA/RV-treated mice show increased tissue eosinophils and macrophages in response to RV infection. Wild type BALB/c mice were sensitized i.p. with endotoxin-free OVA and alum on days 1 and 7, and they were challenged intranasally on days 14, 15, and 16 with OVA. Controls were treated with PBS. Mice were inoculated with RV1B or sham (HeLa cell supernatant) on day 16. Mouse lungs were harvested 1, 2, and 4 d postinfection. Lungs were digested for 1 h in type IV collagenase in serum free RPMI 1640. Strained cells were treated with RBC lysis buffer, spun, and enriched for leukocytes with 40% Percoll. Resulting pellets were resuspended in PBS and total cell count was determined. Cytospins of leukocytes were stained with Diff-Quik, and differential cell count was determined for 200 cells. Time courses for tissue neutrophils (A), lymphocytes (B), eosinophils (C), and macrophages (D) are shown. $n = 4–5$ mice per group; bars represent mean $\pm$ SEM. *Different from respective sham group. †Different from respective PBS group; $p < 0.05$, one-way ANOVA.

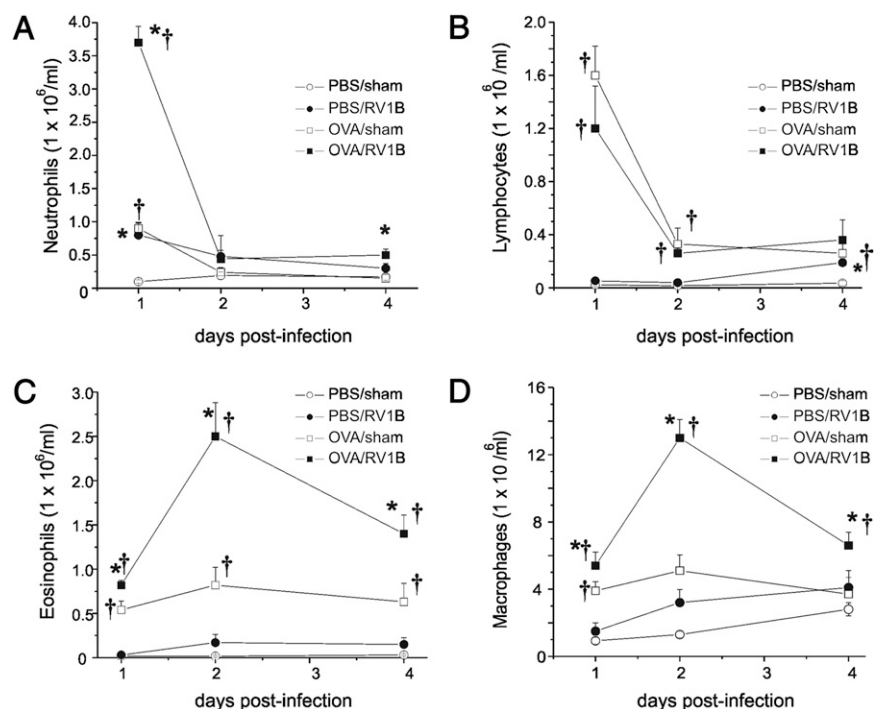


FIGURE 2. RV infection of OVA-sensitized and -challenged mice increases cytokine production. Twenty-four hours after sham or RV infection, lung BAL fluid was centrifuged at $1500 \times g$ and the resulting supernatant subjected to multiplex immunoassay. Results are shown for eotaxin-1/CCL-11 (A), IL-4 (B), IL-13 (C), and IL-5 (D). $n = 5$ mice per group; bars represent mean $\pm$ SEM. *Different from respective sham group, $p < 0.05$. †Different from respective PBS group; $p < 0.05$ one-way ANOVA.

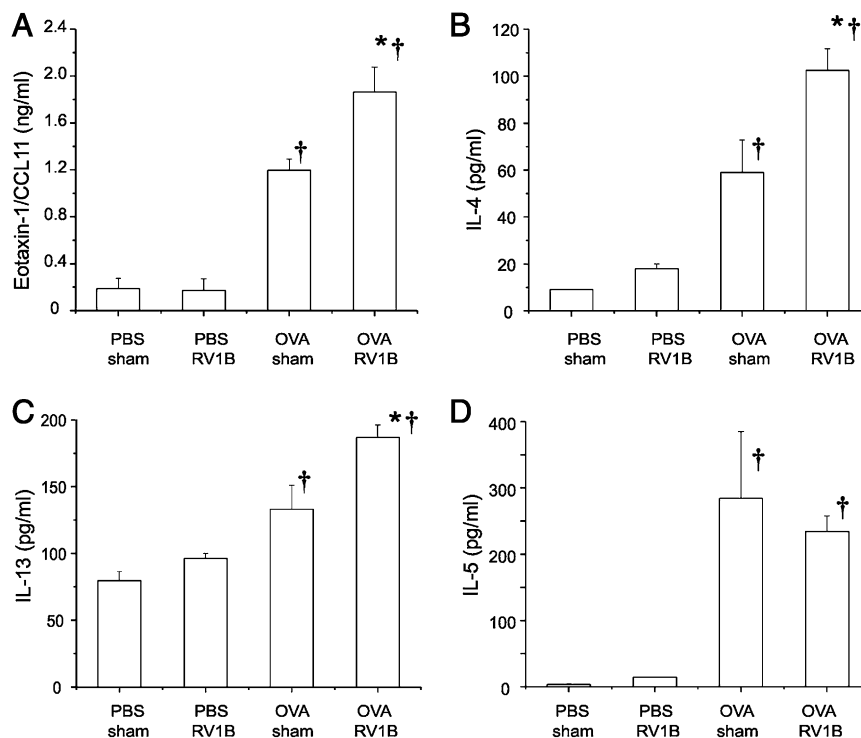


Fig. 2). When CD68⁺/eotaxin-1/CCL11-positive cells were counted, OVA/RV mice showed a significantly higher number of cells per field compared with the other groups. We also found colocalization of RV1B in CD68-, eotaxin-1/CCL11-positive cells (Fig. 5B–F), suggesting that RV infection initiates cytokine expression and/or secretion in airway macrophages. Most RV-infected macrophages were located in the submucosa of large airways, but others were found in the airways and epithelium (Fig. 5G). A minor amount of RV1B and eotaxin staining was also found in airway epithelial cells. Finally, we found colocalization of RV1B, IL-4,

and CD68 in the lungs of OVA-treated (Fig. 5I–M), but not IgG Ab-treated sections (Fig. 5H) or PBS-treated mice (data not shown), indicating that, after exposure to an allergic environment, lung macrophages produce Th2 cytokines in response to RV infection in vivo.

Macrophages are required for RV-induced eosinophil infiltration and airway hyperresponsiveness in OVA-sensitized and -challenged mice

We delivered clodronate- or PBS-containing liposomes to OVA-treated mice intratracheally. Twenty-four hours later, mice were

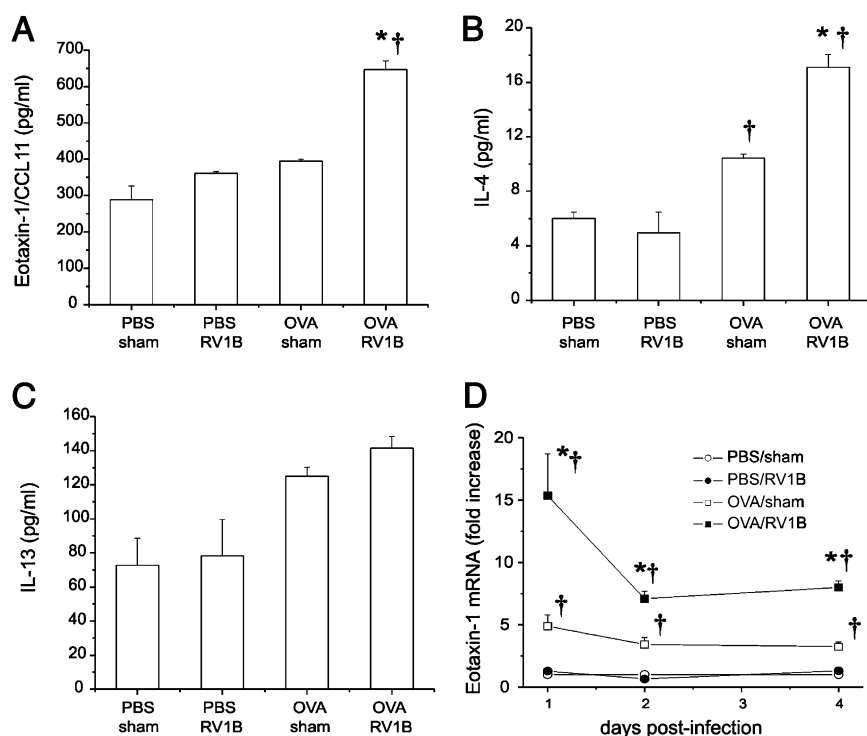


FIGURE 3. RV infection of OVA-sensitized and -challenged mice increases cytokine production 4 d postinfection. Lung BAL fluid was centrifuged at $1500 \times g$, and the resulting supernatant was subjected to multiplex immunoassay. Results are shown for eotaxin-1/CCL11 (A, D), IL-4 (B), and IL-13 (C). cDNA for eotaxin-1/CCL11 was synthesized using reverse transcriptase and subjected to quantitative real-time PCR using a Taqman probe. $n = 5$ mice per group; bars represent mean $\pm$ SEM. *Different from respective sham group, $p < 0.05$. †Different from respective PBS group; $p < 0.05$, one-way ANOVA.

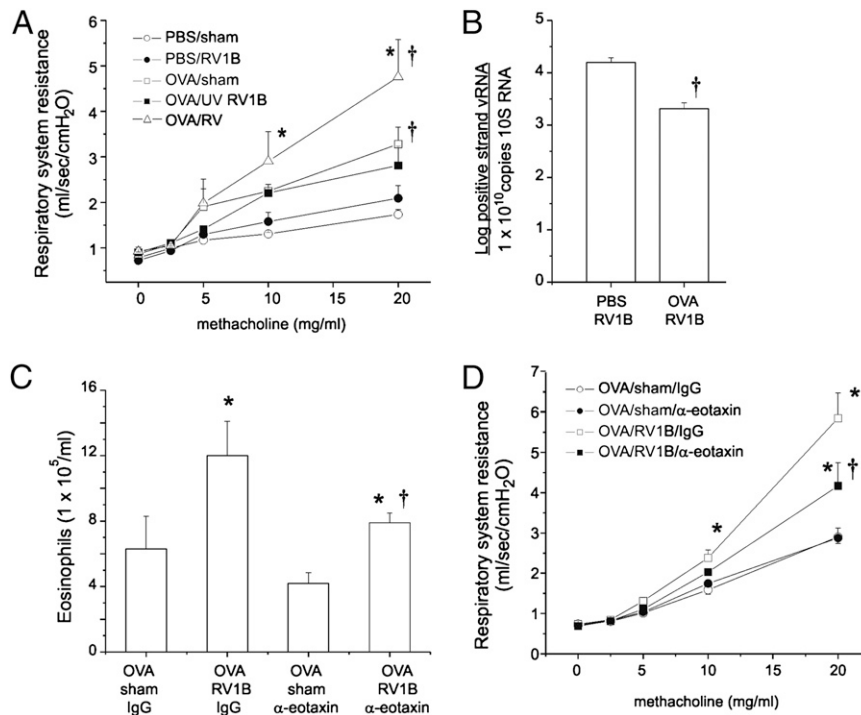


FIGURE 4. RV infection of OVA-sensitized and -challenged mice induces eotaxin-1-mediated airway cholinergic responsiveness. **A**, Mice were anesthetized and endotracheally intubated, and changes in respiratory system resistance to nebulized methacholine were measured using the FlexiVent system (Scireq, Montreal, CA). Four days postinfection, RV-infected OVA mice demonstrated significantly higher airway responses than all other groups at methacholine doses of 10 and 20 mg/ml. **B**, Measurement of viral copy number from lungs of PBS/RV and OVA/RV treated mice 1 d postinfection. OVA/RV treatment significantly reduced the viral copy number by 1 log. $n = 5$ mice per group; bars represent mean $\pm$ SEM. *Different from respective sham group; $p < 0.05$. †Different from respective PBS group; $p < 0.05$, one-way ANOVA. **C**, Selected RV-infected, OVA-sensitized and -challenged mice were given two systemic injections of rabbit anti-mouse eotaxin-1. Additional mice were treated with the isotype control Ab. Mice given anti-eotaxin displayed reduced tissue eosinophils 4 d postinfection. **D**, Neutralizing Ab and isotype control-treated mice were administered increasing doses of aerosolized methacholine. Treatment with anti-eotaxin-1 significantly reduced airway cholinergic responsiveness compared with the IgG-treated group. Bars represent mean $\pm$ SEM. *Different from respective sham group. †Different from IgG group; $p < 0.05$, one-way ANOVA.

inoculated with sham or RV1B. As expected, clodronate treatment depleted total lung macrophages (Fig. 6A). Differential cell counts revealed a markedly lower eosinophil influx in clodronate-treated mice (Fig. 6B). Compared with PBS-containing liposomes, no significant differences in neutrophil or lymphocyte accumulation were observed (Fig. 6C, 6D). OVA/RV mice receiving clodronate liposomes demonstrated a significantly reduced response to methacholine (Fig. 6E). We also observed significant reductions in the mRNA expression of eotaxin-1 and IL-13 (Fig. 7A, 7B), but not IL-4 (data not shown) in OVA/RV mice treated with clodronate, suggesting that depletion of macrophages decreases eosinophil influx by attenuating macrophage production of eotaxin-1. These data suggest that macrophages play an essential role in eosinophil infiltration and airways responsiveness in RV-infected mice with allergic airways disease.

OVA sensitization and challenge alters macrophage proinflammatory cytokine expression in response to RV and upregulates markers of alternative activation

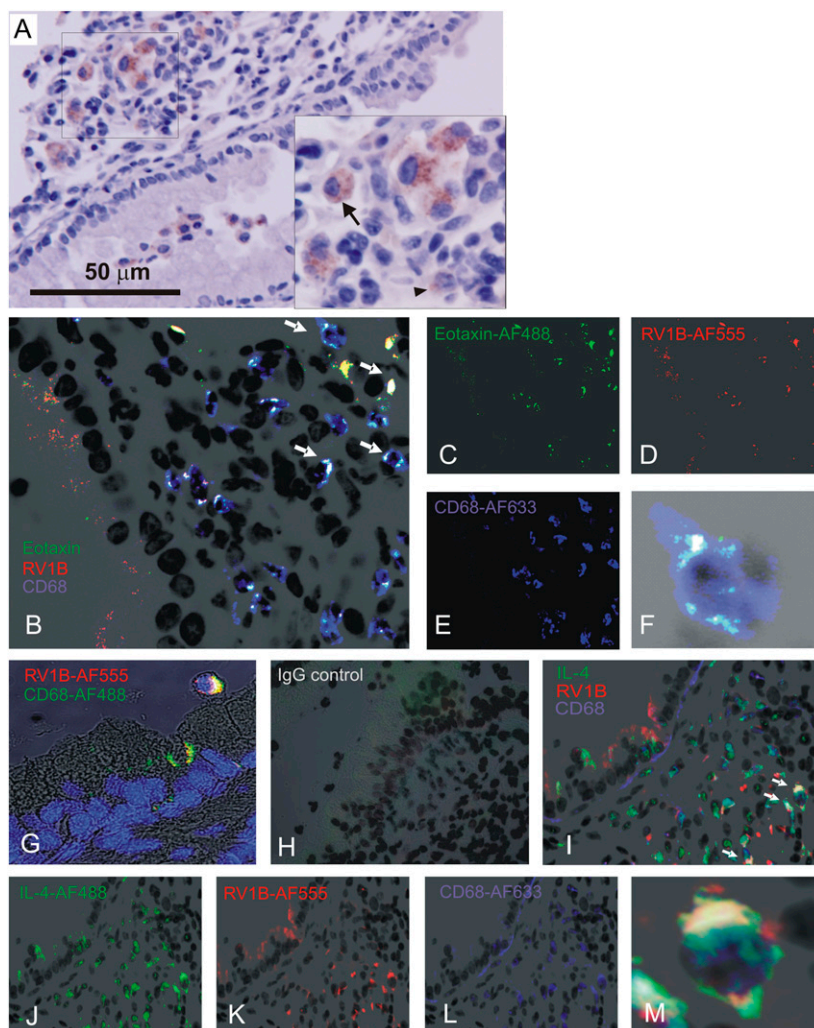
To determine the combined effects of allergen sensitization and RV on macrophage responses, adherent BAL cells were studied. Cells from PBS- or OVA-sensitized and -challenged mice were then stimulated with HeLa cell lysate (sham) or RV1B ex vivo. Eight hours after sham or RV exposure, cells were harvested for total RNA. Cytokine expression was determined by quantitative real-time PCR. Macrophages from control mice produced no eotaxin-1 ex vivo, either at baseline or in response to RV (Fig. 8). However, macrophages exposed to an allergic environment in vivo expressed

eotaxin-1 mRNA, and this level was significantly increased following RV1B stimulation. In addition, RV treatment of macrophages from OVA-sensitized mice induced expression of IL-4, IL-13, IL-10, and IFN- γ . UV-irradiation of RV abrogated the eotaxin, IL-10, and IFN responses, indicating that expression is dependent on viral replication. Because UV-irradiated virus also increased IL-4 and IL-13 mRNA levels, expression of these cytokines appeared to be independent of replication. Finally, in contrast to the above cytokines, the TNF- α response to RV infection was significantly decreased in macrophages isolated from OVA-sensitized and -challenged mice.

We also measured the production of selected cytokines in cell supernatants following ex vivo RV stimulation (Supplemental Fig. 3). IL-4 production was significantly increased in RV-stimulated macrophages from OVA-treated mice. We detected a small but significant increase in eotaxin-1/CCL11 production in macrophages from OVA mice exposed to RV 8 h postinfection. It is conceivable that, in contrast to eotaxin mRNA expression, the release of eotaxin-1 requires the coordinated action of other mediators which may not be present in vitro. In contrast, macrophages from OVA-sensitized and -challenged mice showed reduced levels of TNF- α and p70 IL-12 production after RV stimulation.

Based on the pattern of increased Th2 cytokine expression, we hypothesized that allergic sensitization induces macrophages to deviate from their classical pattern of activation and instead exhibit a functionally polarized phenotype. To test this hypothesis, we measured markers of macrophage activation in cells isolated from PBS- and OVA-treated mice. We also found significant upregulation of the M2 polarization markers Arg-1, MGL-2, Ym-1 (Fig. 9A), and IL-10.

FIGURE 5. RV colocalizes with CD68⁺ macrophages, eotaxin-1, and IL-4 in OVA-sensitized and -challenged mice. **A**, Lungs were formaldehyde fixed overnight, paraffin embedded, sectioned at 5 μ m, and incubated with a 1:2500 dilution of donkey-antimouse eotaxin-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or isotype control IgG (original magnification $\times 200$). Eotaxin was identified by diaminobenzidine staining. After OVA/RV treatment, eotaxin-1 localization is noted in macrophages (arrows) and eosinophils (arrowheads), but not in the airway epithelium (line segment, 50 μ m). **B–E**, OVA/RV lung sections were costained with antiserum against RV1B, which was directly conjugated to AF-594 (red), and CD68 was conjugated to AF-633 (far red optical spectrum, shown in blue). Secondary Ab to eotaxin-1 was conjugated to AF-488 (green). Cells with colocalization (white) are designated by arrows, and a high-magnification view is shown in **F** (original magnification $\times 600$). **G**, RV infection of CD68-positive cells in the airway lumen and epithelial layer. In this panel, RV antiserum was directly conjugated to AF-594 (red), and CD68 was conjugated to AF488 (green). Colocalization is yellow. Colocalization in the epithelium suggests infiltration by a macrophage. **H**, Sections incubated with secondary Abs alone showed no staining. **I–M**, RV colocalizes with IL-4 in CD68-positive cells. RV and CD68 were conjugated with AF-594 (red) and AF-633 (shown in blue), respectively. There is some blue background staining of elastin in the epithelial basement membrane. IL-4 was directly conjugated to AF-488 (green). **M**, Colocalization of RV, IL-4, and CD68 is white (original magnification $\times 400$).



IL-4 and IL-13 treatment has been shown to shift classically activated M1 macrophages to an M2 alternative activation phenotype (23–25). We therefore tested the effect of these cytokines on eotaxin mRNA expression in macrophages from naive mice. In vitro exposure of macrophages from PBS-treated mice to the Th2 cytokines IL-4 and IL-13 significantly increased RV-induced eotaxin mRNA expression (Fig. 9B). These data suggest that allergen sensitization and challenge alters the activation state and augments the cytokine response of lung macrophages to RV infection, contributing to enhanced recruitment of eosinophils to the airways.

Discussion

RV is responsible for majority of the common colds and $\sim 50\%$ of asthma exacerbations (1, 2). Previous studies have demonstrated that neutrophils are the predominant inflammatory cell in the airways of patients with acute asthma exacerbation (26–28). Experimental RV infection has been shown to increase airway neutrophilic inflammation in normal and asthmatic subjects (9–14). Eosinophils and cationic protein have also been detected in the airways following experimental RV infection (10, 14, 16). However the precise mechanism of RV-induced asthma exacerbations is not well understood. After experimental RV16 infection, patients with asthma show increased levels of IL-8 in their nasal lavage, which correlates with the level of airway responsiveness (11), in contrast to unaffected individuals in whom IL-8 does not increase (15). In a recent study, patients with asthma who are undergoing

experimental RV infection demonstrated greater neutrophilic, lymphocytic, and eosinophilic inflammation than did RV-infected control subjects, although only the number of eosinophils achieved statistical significance (14). These data suggest that patients with asthma experience a different response to viral infection than do control subjects.

Previously, we showed that RV infection of naive mice induces airway inflammation, marked predominantly by neutrophils and lymphocytes (20). RV infection also induced moderate airway hyperresponsiveness to methacholine. In this study, we delineate the response to RV in the context of allergic inflammation. We found that, after RV infection of allergen-sensitized and -challenged mice, the largest populations of cells elicited in the BAL fluid were, in fact, eosinophils and macrophages. The increase in eosinophils was associated with a concomitant rise in expression of the eosinophil chemoattractant eotaxin-1/CCL11, which was significantly greater in OVA/RV mice in comparison with all other groups. Eosinophil infiltration was also accompanied by a synergistic increase in the Th-2 cytokines IL-4 and IL-13, each of which were significantly higher in the OVA/RV-treated mice compared with all other groups. It is worth noting that RV infection alone failed to significantly increase airway eosinophils, eotaxin-1, IL-4, and IL-13. RV infection also enhanced airways responsiveness in allergen-sensitized and -challenged mice, with hyperresponsiveness persisting at least 4 d postinfection. These data confirm and extend a recent report (18), and they are consistent with the notion that the allergic environment qualitatively alters the response to RV.

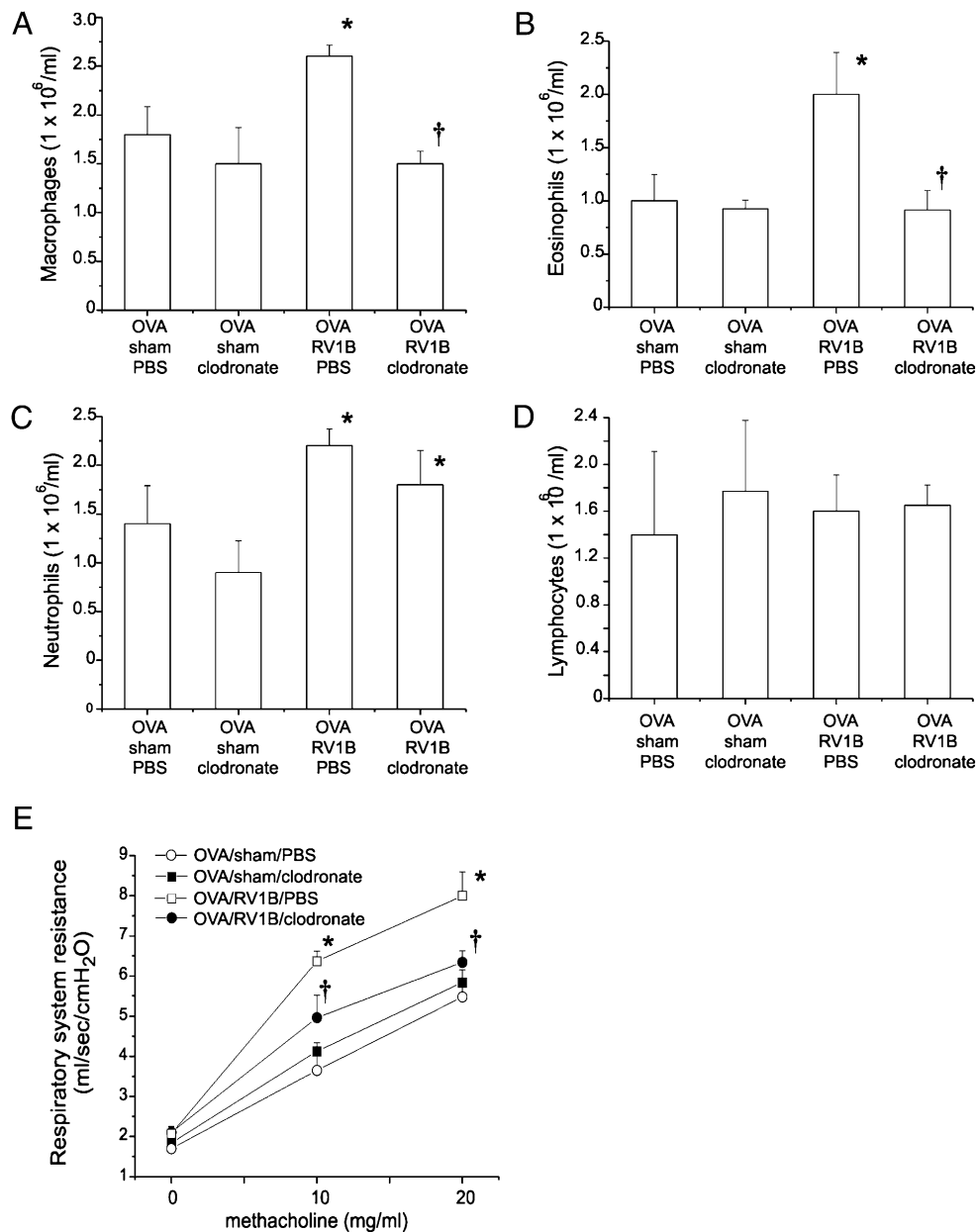


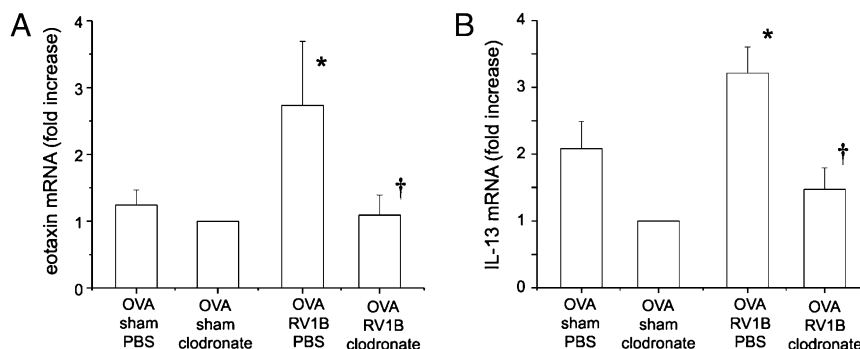
FIGURE 6. Macrophage-depleted OVA-treated mice show reduced airway eosinophils and hyperresponsiveness following RV infection. Clodronate or PBS-containing liposomes were instilled into the trachea 24 h after the last OVA challenge. Twenty-four hours after macrophage depletion, mice were inoculated with sham or RV and harvested 24 h postinfection. Lung digests were performed as described in Fig. 1. Differential counts were determined. Cytospins of leukocytes were stained with Diff-Quik, and differential cell count was determined for 200 cells. Macrophages (A), eosinophils (B), neutrophils (C), and lymphocytes (D) are shown. E, Airway resistance for each group was measured after treatment with 0, 10, and 20 mg/ml methacholine. $n = 4$ mice per group; bars represent mean $\pm$ SEM. *Different from respective sham group. †Different from OVA/RV/ PBS liposome group; $p < 0.05$, one-way ANOVA.

We measured viral copy number in the lungs of infected PBS- and OVA-treated mice. Viral load was not increased in allergen-sensitized and -challenged mice, demonstrating that the augmented airway inflammation and responsiveness was not due to an increase in the susceptibility to RV. Indeed, RV copy number was unexpectedly decreased in mice with allergic airways disease. These data are consistent with a previous report examining parainfluenza infection of OVA-treated animals (29), and they suggest that inflammatory cells play a role in viral clearance. More importantly, these data demonstrate an uncoupling of viral load and airway inflammation. This finding may seem surprising, but viral infection can set off a proinflammatory cascade that outlasts the presence of live virus. Consistent with this finding, we previously found that RV-infected,

naive mice demonstrate airway hyperresponsiveness 4 d after RV infection, when viral copy number is decreasing (20). We also found that replication-deficient virus is sufficient to induce moderate neutrophilic inflammation and airway responsiveness 1 d after inoculation.

In OVA-treated mice, RV infection increased lung eosinophils and expression of eotaxin-1/CCL11. Eosinophils and the eotaxin/CCR3 axis are known to play a critical role in chronic experimental allergic airway inflammation (30–32). To test for the requirement of eotaxin-1 for enhanced eosinophilic inflammation and airways hyperresponsiveness in allergic, RV-infected mice, we targeted eotaxin-1 production by administering an antimouse eotaxin-1 neutralizing Ab following the last OVA challenge and RV infection.

FIGURE 7. Clodronate-mediated depletion of macrophages reduces OVA/RV-induced cytokine expression. Lung mRNA was extracted, and corresponding cDNA was subjected to quantitative real-time PCR. $n = 4$ mice per group; bars represent mean $\pm$ SEM. *Different from sham group. †Different from OVA/RV/PBS liposome group; $p < 0.05$, one-way ANOVA.



Anti-eotaxin-1 significantly reduced the number of airway eosinophils, but not the neutrophils or lymphocytes, demonstrating that eotaxin-1 is required for homing of eosinophils to the airway following RV infection. Furthermore, administration of anti-eotaxin-1 decreased RV-induced airways hyperresponsiveness in allergen-sensitized and -challenged mice. We did not determine the precise mechanism by which eosinophils increase airways responses, but eosinophils are a known source of bronchoconstrictor agonists, including major basic protein and cysteinyl leukotrienes. When guinea pigs are sensitized to OVA and subsequently infected with parainfluenza, virus-induced hyperresponsiveness and M2 receptor

dysfunction are blocked by depletion of eosinophils with Ab to IL-5 or Ab to major basic protein (29). RV infection has also been shown to increase 5-lipoxygenase and cyclooxygenase-2 in bronchial biopsy specimens from nonatopic subjects (33). Furthermore, it should be noted that administration of anti-eotaxin-1 did not completely abolish RV1B-induced airways eosinophilic inflammation and hyperresponsiveness. It is conceivable that the neutralizing Ab was not fully effective in eliminating eotaxin function. It is also possible that other cytokines also contribute to airway narrowing. For example, our unpublished observations (D.S. and M.H.) indicate that RV1B infection also increases lung expression of macro-

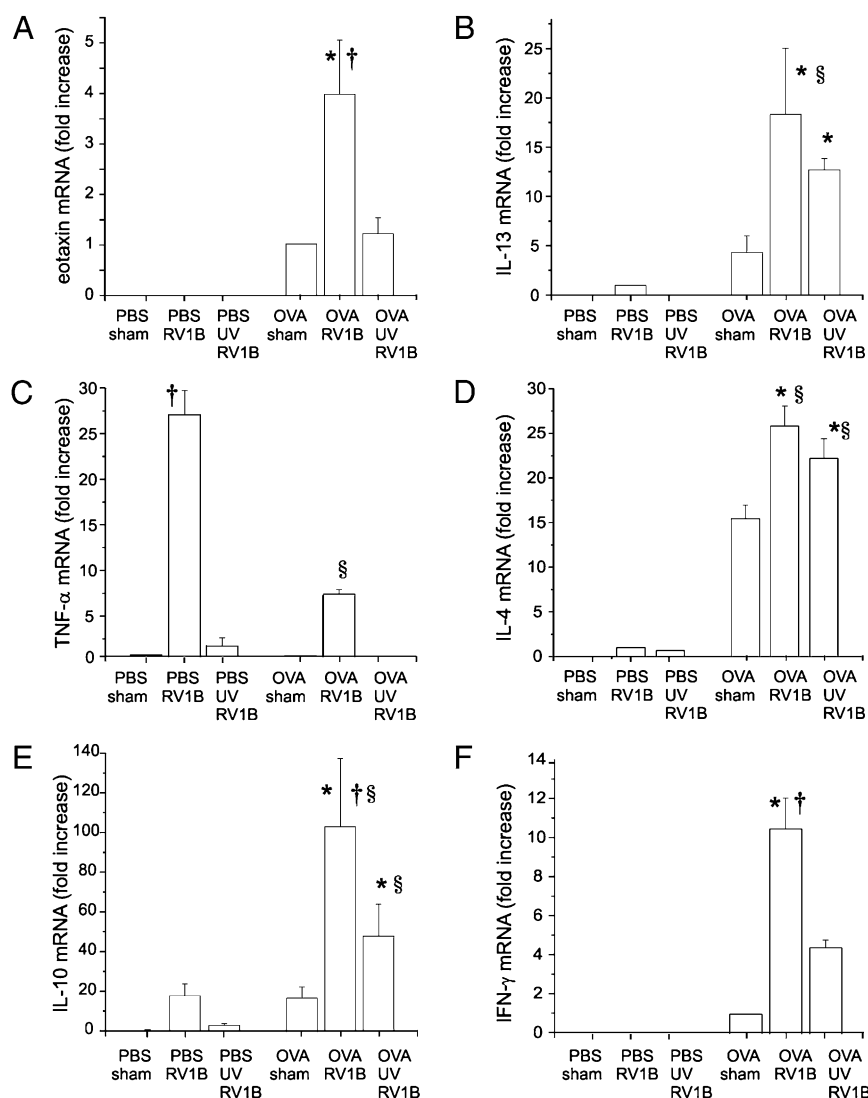


FIGURE 8. Macrophages from OVA-sensitized and -challenged mice show increased cytokine mRNA expression after RV stimulation ex vivo. BAL fluid was extracted from PBS-treated and OVA-sensitized and -challenged mice and was seeded in 12-well plates. Cells were allowed to adhere to plates for 90 min. Adherent cells were subsequently infected with RV1B, sham, or media (controls). A, Eotaxin-1 expression was observed in adherent BAL cells from OVA-treated but not PBS-treated mice. Eotaxin-1 significantly increased after RV stimulation. B, IL-13. C, TNF-α. D, IL-4. E, IL-10. F, IFN-γ. Because some treatment conditions yielded no detectable mRNA expression, data were normalized to the condition with the lowest detectable mRNA signal. $n = 3-4$; bars represent mean $\pm$ SEM. *Different from respective sham group. †Different from respective ultraviolet RV group. §Different from respective PBS group; $p < 0.05$, one-way ANOVA.

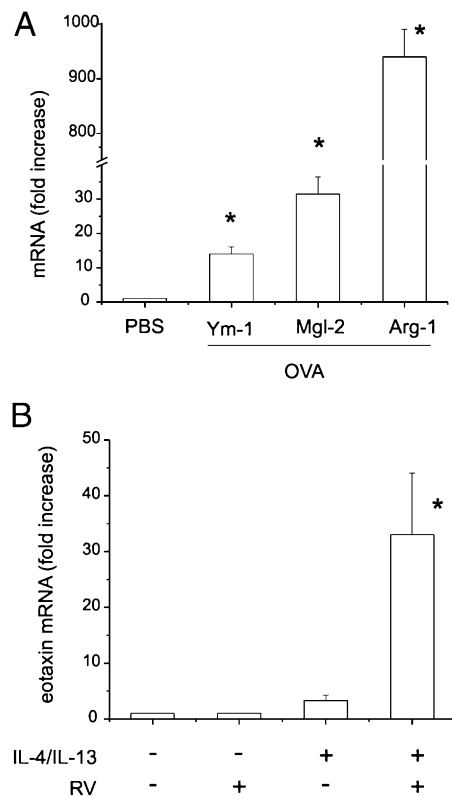


FIGURE 9. Effect of Th2 environment on macrophage polarization. *A*, OVA sensitization and challenge alters the mRNA expression of macrophage activation markers. Data are fold-increase compared with macrophages from PBS-treated mice $n = 3$ –4; bars represent mean $\pm$ SEM. *B*, Effect of IL-4/IL-13 incubation on the eotaxin response to RV infection in macrophages from PBS-treated naive mice. $n = 3$; bars represent mean $\pm$ SEM of fold-increase in mRNA expression compared with control cells; * $p < 0.05$, one-way ANOVA.

phage chemotactic protein (MCP)-1/CCL2, and that MCP-1/CCL2 partially contributes to RV-induced airways inflammation and hyperresponsiveness in OVA-sensitized and -challenged mice.

To determine the cells responsible for the observed increase in eotaxin-1 expression in response to RV, we performed immunohistochemical analysis on OVA/RV mice. Although previous reports demonstrated production of eotaxin-1/CCL11 by RV-infected, cultured airway epithelial cells (34–38), eotaxin-1 was only minimally localized to the airway epithelium. Instead, eotaxin-1 protein abundance was readily apparent in alveolar macrophages. These data are consistent with previous reports showing that, in airway inflammatory cells from asthmatic patients, eotaxin-1 immunoreactivity is colocalized predominantly to macrophages, with a lesser contribution from eosinophils (39–41). Furthermore, macrophages isolated from allergen-sensitized and -challenged mice demonstrated a significant eotaxin-1 response to RV stimulation *ex vivo*, in contrast to cells from naive mice, which showed no response. RV has been shown previously to induce cytokine responses in alveolar macrophages *in vitro*. Production of MCP-1/CCL2 and IP-10/CXCL10 not dependent on replication (42–45), whereas production of TNF- α may be dependent on replication (44). In the current study, we show for the first time that *ex vivo* macrophage responses to RV are augmented following allergen-sensitization and -challenge, and that macrophages produce cytokines in response to airway RV infection *in vivo*. However, we cannot determine from our images whether colocalization represents true replicative infection, endocytosis of virus, or phagocytosis of RV1B by airway macrophages.

Eotaxin production in response to RV infection has not been previously demonstrated *in vivo*. In the one study of which we are aware that examined eotaxin-1 expression in response to natural or experimental RV infection in subjects with asthma, mRNA transcripts for eotaxin-1 were not expressed at consistently detectable levels in induced sputum (46). However, our preliminary studies examining nasal washes from asthmatic children show that natural viral infection, as detected by PCR, is associated with a 6-fold increase in eotaxin-1 protein abundance compared with virus-negative weeks (T.L., T.H., and M.H., unpublished data).

In addition to eotaxin-1, we found that the combination of OVA treatment and RV infection increased production of IL-4 from alveolar macrophages, both *in vivo* and *ex vivo*. Macrophages from OVA-treated mice also expressed higher levels of IL-13 in response to RV *ex vivo*. The notion that a non-T cell source of Th2 cytokines may also act to enhance allergic inflammation by secreting IL-4 or IL-13 has not been well studied. IL-13 production has been noted in lung macrophages from Sendai virus-infected C57BL/6J mice (47). The role of macrophages in the pathogenesis of asthma and allergic inflammation is unresolved. Macrophage subsets are recruited into the lung following OVA sensitization and challenge of BALB/cJ mice, and transfer to naive mice increased airways responsiveness, eosinophilic inflammation, and Th-2 cytokine secretion (48). Alternatively, transfer of alveolar macrophages from OVA-exposed Sprague-Dawley rats protects against the development of airway hyperresponsiveness in macrophage-depleted OVA-treated Brown-Norway rats (49). In our study, depletion of macrophages resulted in a significant amelioration of eosinophil infiltration and airway responsiveness. Macrophage-depleted OVA/RV mice also showed significantly reduced eotaxin-1 expression, suggesting for the first time that RV-induced asthma exacerbations may be directed by eotaxin-producing lung macrophages. There are other possible explanations, however. It is conceivable that clodronate attenuated airway eosinophilic inflammation and hyperresponsiveness by directly killing phagocytic eosinophils. However, to our knowledge this effect of clodronate has not been reported previously. We should also note that, although clodronate treatment decreased macrophage infiltration in RV-infected OVA mice, there was no effect in sham-inoculated OVA mice. It is possible that administration of a fourth OVA challenge after clodronate treatment restored the macrophage response. Alternatively, the phagocytic response of macrophages from sham-inoculated OVA-sensitized and -challenged mice might have been poor, owing to their strict M2 polarization. In RV-infected OVA mice, this effect might have been partially mitigated by viral infection, which led to the submaximal induction of TNF- α , a prophagocytic M1 signal (50).

Our data demonstrating increased production of eotaxin-1 and IL-4 from alveolar macrophages, both *in vivo* and *ex vivo*, suggests an alteration in the phenotype of tissue macrophages in response to allergen sensitization and RV infection. In addition, RV treatment of macrophages from OVA sensitized mice, but not PBS-treated mice, induced expression of IL-13, IL-10 and IFN- γ . In contrast, TNF- α and p70 IL-12 were significantly decreased. Shift of classically activated M1 macrophages to an M2 alternative activation phenotype, under the influence of the Th2 cytokines IL-4 and IL-13, has been associated with an altered secretory repertoire and pattern of phagocytic receptors (reviewed in Ref. 25). IL-4 and IL-13 have been shown to induce alternative macrophage activation *in vitro* (23) and *in vivo* (24). In the latter study, IL-13-overexpressing transgenic mice infected with *Cryptococcus neoformans* showed the presence of alternatively activated macrophages expressing Arg-1, macrophage mannose receptor, and Ym-1, as well as lung eosinophilia, goblet cell metaplasia, elevated mucus production, and enhanced airways responsiveness. Consistent

with this finding, we found significant upregulation of M2 markers in OVA-sensitized and -challenged mice, including Arg-1, MGL-2, Ym-1, Fizz-1, and IL-10. Modulation of Arg-1, Ym-1, Fizz-1, MGL-1, and MGL-2 expression was previously noted following OVA sensitization and challenge (51). Upregulation of Arg-1 may be of particular physiologic importance. In patients with asthma, Arg-1 mRNA expression is increased in submucosal inflammatory cells (52). Arginase expression is increased in the lungs of allergen-sensitized and -challenged mice, and inhibition attenuates methacholine responsiveness in OVA-sensitized and -challenged mice (53).

In addition to changes in macrophage receptor and cytokine expression typically associated with M2 polarization including Arg-1, MGL-2, Ym-1, and IL-10, we also found that OVA treatment increased expression of the classical activation marker IFN- γ . Patterns of macrophage gene expression may not display a strict dichotomy between type 1 and type 2 responses. For example, it has been reported that exposure of macrophages to IL-4 prior to LPS stimulation strongly enhances inflammatory activity (TNF- α , IL-12 production) and Arg-1 expression (54). These data suggest the possibility that exposure to a Th2 environment induces a functional phenotypic change in airway macrophages, which does not strictly fit the M1/M2 model, leading to increased secretion of both type I and type II cytokines in response to RV stimulation.

In cultured macrophages, UV-irradiation of RV abrogated the eotaxin, IL-10, and IFN responses. The reduced cytokine expression following treatment with UV-irradiated virus is consistent with the notion that RV causes a replicative infection in macrophages. In vitro studies have noted attachment of human RV to peripheral blood monocytes and airway macrophages, with subsequent secretion of numerous proinflammatory cytokines, chemokines, and IFNs (42–45, 55, 56). A small amount of viral replication has been noted in human RV-infected peripheral blood monocyte-derived macrophages, but not in BAL-derived macrophages (42, 44).

Finally, we would like to add a few comments about our mouse combined model of asthma and RV infection. First, whereas experimental RV infection increased airways responsiveness of mice with allergic airways disease as it does in human asthmatics (11, 14), OVA sensitization and challenge of BALB/c mice may not recapitulate the severity and genetic background of many human patients with asthma. In regard to the RV infection model, we (20) and others (18) have found that a much higher viral titer is required to infect mice compared with humans. This finding is to be expected, because differences in the homology of viral receptors and intracellular signaling mechanisms are likely to restrict viral infection and replication in mice. Nevertheless, we have clearly shown that human RV1B replicates in mouse lungs, as evidenced by: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice, 2) transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells; and 3) the induction of a robust lung interferon response (20). Replication-deficient UV-irradiated virus has none of these effects. We therefore believe that our mouse model of human RV infection holds promise for the study of RV-induced exacerbations of chronic airways diseases such as asthma.

In conclusion, we have shown in allergen-sensitized and -challenged mice that lung macrophages participate in RV-enhanced airway eosinophilic inflammation and hyperreactivity. Macrophages from allergen-sensitized and -challenged mice, but not control animals, produce eotaxin-1 and IL-4 in response to RV infection, both in vivo and ex vivo. The altered response to RV infection is driven by a functional change in macrophage polarization state, likely a response to Th2 cytokines in the allergic environment. These data provide a new paradigm to explain RV-induced asthma exacerbations and identify the

macrophage as a potential therapeutic target for the treatment of virus-induced exacerbations of chronic airway disease.

Disclosures

The authors have no financial conflicts of interest.

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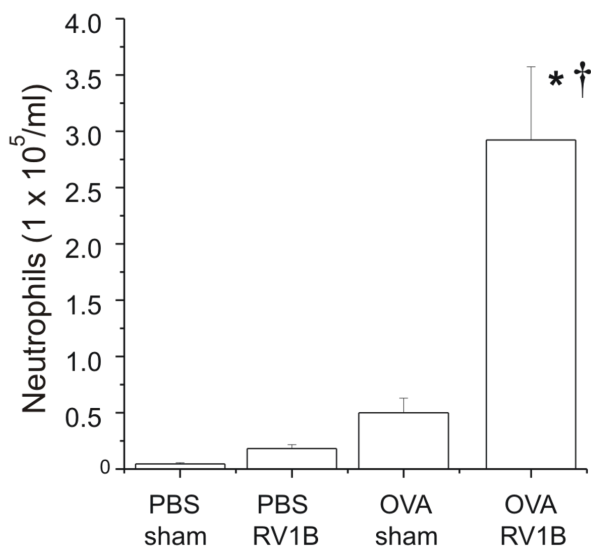
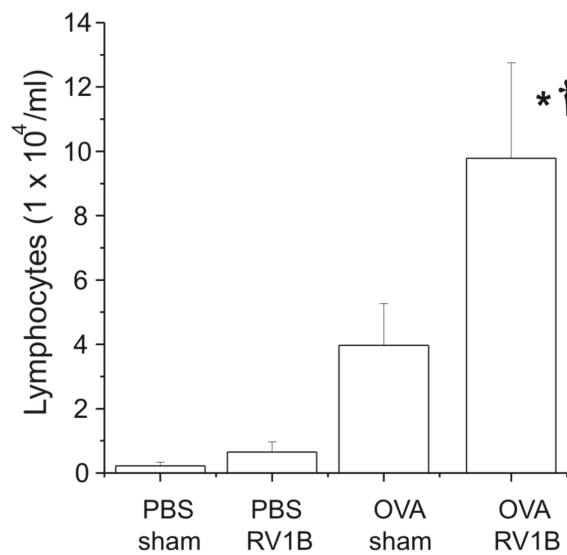
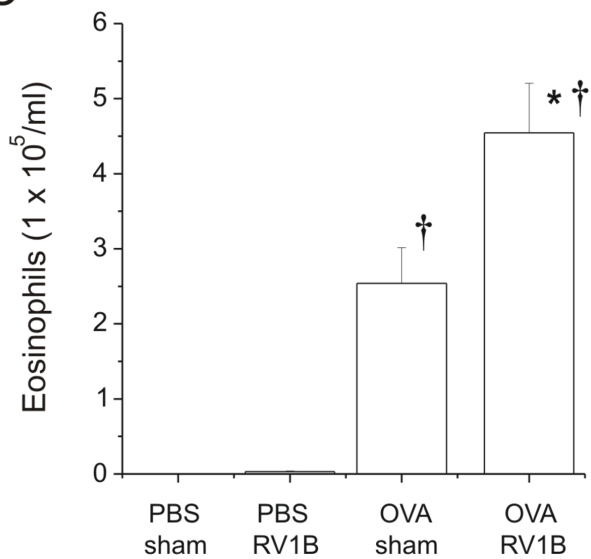
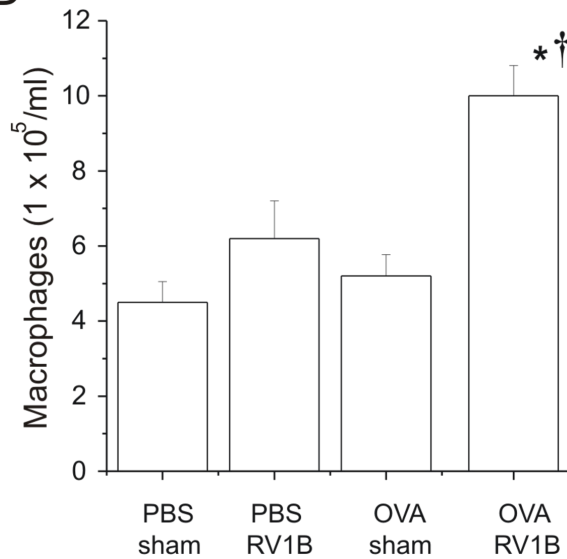
Supplemental Data

Figure Legends

Figure S1. *OVA-sensitized and -challenged mice show increased airway inflammation in response to RV infection 4 days after infection.* Bronchoalveolar lavage was performed with saline containing 5mM EDTA. BAL neutrophils (A), lymphocytes (B), eosinophils (C) and macrophages (D) are shown. ($N = 4-6$ mice per group, bars represent mean $\pm$ SEM, *different from respective sham group, $p < 0.05$; †different from respective PBS group, $P < 0.05$ one-way ANOVA.)

Figure S2. *Eotaxin-1 is produced by alveolar macrophages from OVA-sensitized and -challenged mice, but not cells from PBS-treated mice.* Lungs sections were stained with donkey-anti-mouse eotaxin-1 for 30 min and AF488-labeled donkey anti-goat IgG secondary overnight. Sections were costained with AF594-conjugated rat anti-mouse CD68 or isotype control IgG overnight. Lungs from PBS/sham (A), PBS/RV (B), OVA/sham (C) and OVA/RV (D) mice are shown. Intense yellow staining is seen in OVA/RV sections indicating colocalization of CD68 (red) and eotaxin-1 (green), while OVA/sham lungs show less intense staining. Sections incubated with secondary antibody alone showed no staining. E. OVA/RV lungs have increased CD68⁺/eotaxin-1⁺ cells per field compared to all other groups. ($N = 3$, bars represent mean $\pm$ SEM, *different from respective sham group, $P < 0.05$; †different from respective PBS group, $P < 0.05$ one-way ANOVA.)

Figure S3. Macrophages from OVA-sensitized and -challenged mice show increased cytokine secretion after RV stimulation *ex vivo*. Cell supernatants were spun and subjected to bioplex immuneassay or ELISA. A. Eotaxin-1. B IL-4. C. TNF- α . D. IL-12 p70. ($N=3$, bars represent mean $\pm$ SEM, *different from respective sham group, †different from respective UV RV group, §different from respective PBS group, $P<0.05$, one-way ANOVA.)

A**B****C****D****Figure S1**

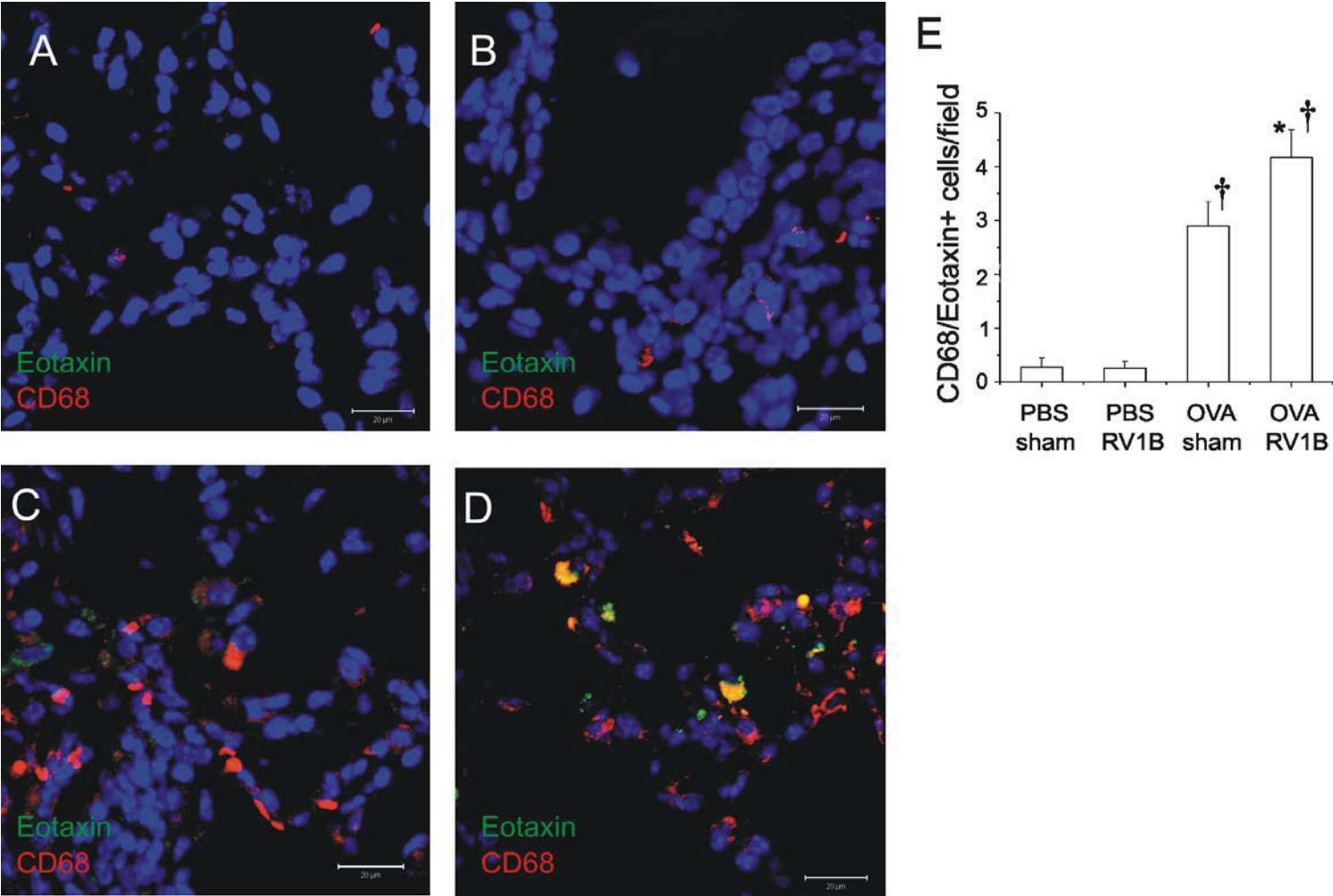


Figure S2

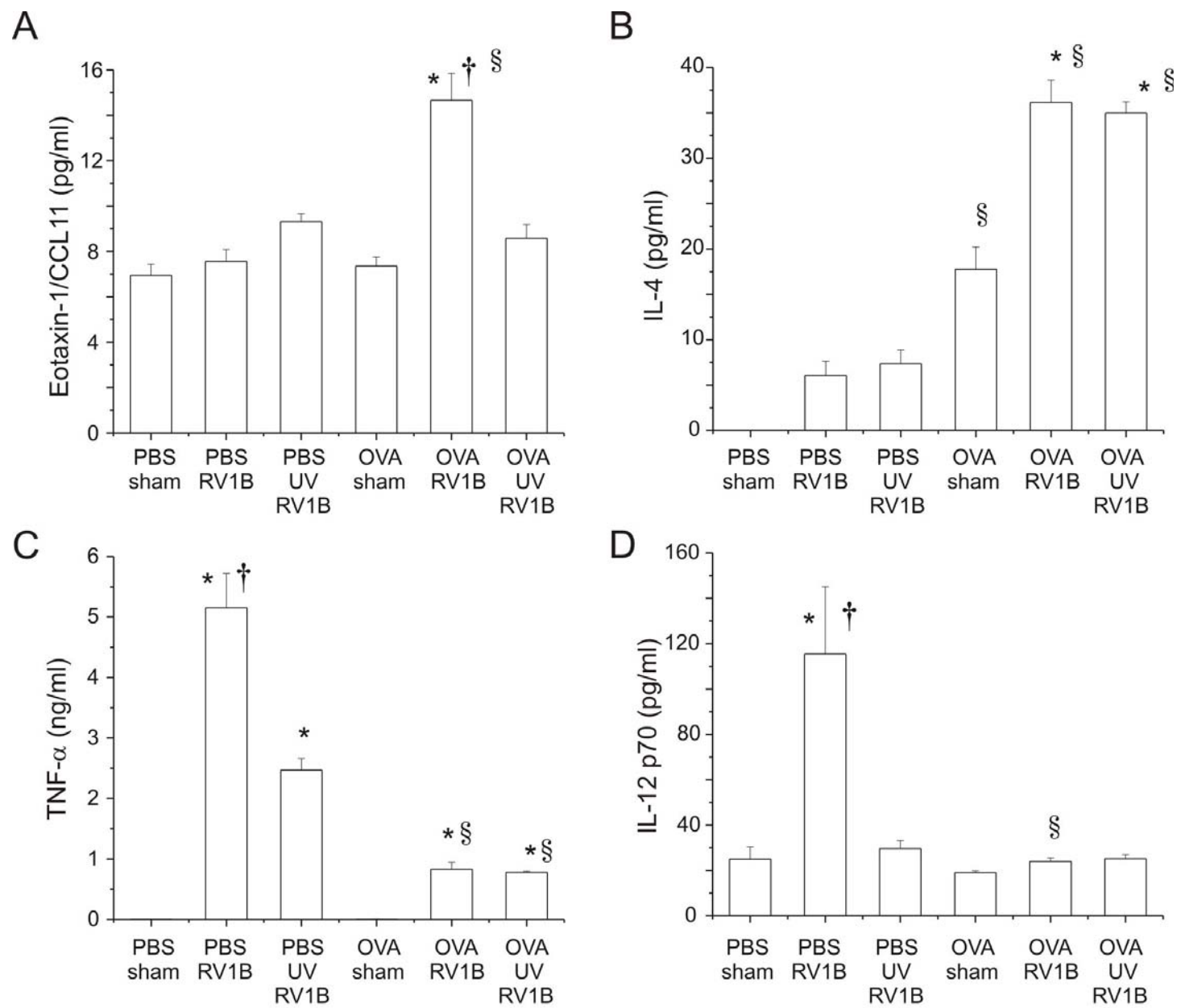


Figure S3