

Virus-induced eosinophil mediator release requires antigen-presenting and CD4⁺ T cells

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Background: The most frequent trigger of asthma exacerbation is infection with common airway viruses; however, the precise mechanism regulating such severe reactions is not understood. The presence of airway eosinophil products is a unique feature detected in asthmatic airways. Using an animal model, we previously demonstrated that T cells play an important role in regulating an eosinophil-dependant pathway of virus-induced airway hyperreactivity. We hypothesize that human eosinophils respond to viruses, although only after interaction with T cells. **Objectives:** We sought to determine whether eosinophils can respond to airway viruses *in vitro* and determine the mechanism of response.

Methods: An *in vitro* coculture model of human eosinophils, antigen-presenting cells, and T cells was used with parainfluenza virus, respiratory syncytial virus, or rhinovirus. We measured release of eosinophil peroxidase (EPO) in concert with T-cell proliferation, cytokine release, and changes in T-cell phenotype.

Results: The viruses induced release of EPO when cocultured in the presence of antigen-presenting cells (dendritic cells or macrophages) and T cells. Virus-mediated release was associated with proliferation of CD3⁺CD4⁺ T cells and release of cytokines. UV inactivation of the virus did not prevent virus-induced EPO release or T-cell proliferation. Proliferating CD4⁺ T cells show increased expression of CD25 and CD45RO. CD8⁺ T cells were not activated.

Conclusion: Virus-induced EPO release can occur in the context of antigen presentation to CD4⁺ T cells. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Eosinophil, virus infection, asthma exacerbation, eosinophil peroxidase, leukotriene, T cell, macrophage, dendritic cell

Asthma exacerbation is a deterioration in lung function that can result in hospitalization and even death. The most common trigger for exacerbation is infection by one of the common cold-like viruses.^{1,2} Why patients with asthma have such severe reactions to viruses is not understood. The degree of airway obstruction before infection does not fully predict an asthma attack.³ Atopy is a strong risk factor predicting hospitalization caused by virus infections.^{4,5} Airway eosinophilia and the presence of major basic protein during exacerbations is well established,^{6,7} and thus monitoring sputum eosinophils can prevent impending exacerbation.^{8,9} The prevention and treatment of asthma exacerbation relies on the use of corticosteroid therapy,^{10,11} for which eosinophils are particularly sensitive.^{12,13}

Viral infections typically cause an influx of neutrophils into the airway with interferon-producing mononuclear cells.^{14,15} The ability to generate airway neutrophilia⁸ and clear virus infection is similar between asthmatic and nonasthmatic patients.^{16,17} Thus the unique feature of viral infections in asthmatic patients is the presence of airway eosinophils. In atopic individuals experimental rhinovirus (RV) infection increases the recruitment of eosinophils to the airways after antigen challenge¹⁸ and causes increased airway reactivity compared with that seen in nonallergic individuals.¹⁹ Furthermore, biopsy specimens of the lower airways of asthmatic subjects show increased eosinophil numbers after RV infection that persist even into convalescence.²⁰ RV infection of atopic individuals causes airway eosinophilia and airway hyperreactivity (AHR), but nonatopic patients show only mild lower airway narrowing and inflammation without eosinophilia.²¹ During naturally acquired viral infection, children with asthma show increases in eosinophils and major basic protein in their airway secretions.²² Whether eosinophils are responding specifically to the virus or what the precise regulatory pathway or pathways for this response might be remains unknown.

In animal models of allergen sensitization before virus infection, we and others have demonstrated that eosinophils together with T cells play a critical role in the development of virus-induced AHR.²³⁻²⁵ To extend our previous *in vivo* observations, we adapted an allergen-based *in vitro* coculture model to study the interaction of human eosinophils with common airway virus infections. We hypothesized that eosinophils respond to virus infection with release of mediators in the presence of T cells. Although we considered that T cells might recognize viruses directly, the model was designed to also study virus antigen presentation to T cells.

In this report we demonstrate a novel *in vitro* system to study virus-induced eosinophil mediator release, which occurs in

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Abbreviations used

AHR:	Airway hyperreactivity
APC:	Antigen-presenting cell
Cys-LT:	Cysteinyl leukotriene
DC:	Dendritic cell
EPO:	Eosinophil peroxidase
LCMV:	Lymphocytic choriomeningitis virus
LT:	Leukotriene
PFU:	Plaque-forming unit
PI3:	Phosphoinositide 3
PIV:	Parainfluenza virus type 1
RSV:	Respiratory syncytial virus
RV:	Rhinovirus

response to virus infection, but only through interaction with activated CD25⁺CD45RO⁺ T cells and antigen-presenting cells (APCs). Understanding the mechanism of virus-induced mediator release from eosinophils *in vitro* has implications for the management of virus-induced asthma exacerbations and might explain why asthmatic patients respond so poorly to infection with common cold viruses.

METHODS

A complete version of these methods is available in the Methods section of the Online Repository at www.jacionline.org.

Lymphocyte isolation

Blood was purified from adults after obtaining written informed consent, as approved by the faculty's health research ethics board. After erythrocyte sedimentation, the supernatant was centrifuged in Ficoll (Pharmacia Biotech, Uppsala, Sweden). Mononuclear cells were collected and incubated in 6-well plates (12×10^6 cells/well in RPMI plus 10% FBS for 2 hours at 37°C). Non-adherent cells were run through a nylon wool column to enrich for T cells (>95% purity by means of FACS) and stored at -80°C (FBS with 10% dimethyl sulfoxide).

Macrophage/dendritic cell preparation

Adherent cells (>95% pure by means of FACS) were cultured in 6-well plates with either GM-CSF (40 ng/mL) or GM-CSF plus IL-4 (8 ng/mL) for 7 days (37°C).

Eosinophil isolation

Purified eosinophils (>99%) were obtained by means of negative immunomagnetic selection with a mix of CD16-, CD14-, and CD3-coated beads.²⁶

Coculturing of T cells, macrophages/dendritic cells, and virus

Lymphocytes were placed in aliquots in 96-well plates (2×10^5 cells/well). Autologous macrophages or dendritic cells (DCs) were added (5×10^3 cells/well). The plates containing individual cell types (T cells, macrophages, and DCs alone) or in combination (T cells plus either macrophages or DCs) were incubated overnight. To this was added either parainfluenza virus type 1 (PIV; ATCC, Sendai virus, 1:1000 dilution of 10^6 mol/L tissue culture infectious dose₅₀ (TCID₅₀) grown in CMK cells with LHC8 media, Bioscience), respiratory syncytial virus (RSV; ATCC, strain A2, 1:1000 of 6.5×10^6 plaque-forming units [PFU] grown in Hep-2 cells with RPMI), RV-16 (1:1000 of 1×10^6 TCID₅₀ U/mL, generously provided by Dr David Proud,²⁷ University of Calgary), lymphocytic choriomeningitis virus (LCMV; strain

Armstrong, 1:5 of 5×10^5 PFU/mL grown in BHK-21 cells with RPMI), or sham materials. Some experiments used UV-inactivated PIV.

Virus-induced eosinophil mediator release in coculture

After 6 days, eosinophils (2×10^5 cells/well) were added to wells overnight. Lysine-coated cyanogen bromide-coupled Sepharose beads (PharMin-gen, San Diego, Calif) were added (for 2 hours at 37°C) to improve eosinophil mediator release.²⁸ Some eosinophils were preincubated with the phosphoinositide 3 (PI3)-kinase inhibitor wortmannin (Sigma-Aldrich, St Louis, Mo).

Measurement of eosinophil peroxidase and leukotriene release

Purified human eosinophil peroxidase (EPO; a generous gift from Dr G. J. Gleich, University of Utah) served as the standard, allowing calculation of EPO. *o*-Phenylenediamine was the peroxidase substrate (optical density, 490 nm).²⁶ For cysteinyl leukotriene (Cys-LT) release, cell-free supernatants were assayed by means of ELISA (Cysteinyl-Leukotriene EIA Assay Kit; Cayman Chemical, Ann Arbor, Mich).

Virus-induced T-cell proliferation assay by means of tritiated thymidine incorporation

DCs and macrophages were irradiated (2000 rad) before incubation with lymphocytes. After 6 days, with or without virus, tritiated thymidine (1 μ Ci/well) was added for 24 hours before harvesting cells for radioactivity measurements (in counts per minute). PHA (5 μ g/mL) served as a positive control.

Measurement of cytokines in supernatant of coculture

IFN- γ , IL-5, IL-13, and GM-CSF were measured in supernatants by means of ELISA (eBioscience, San Diego, Calif).

Surface marker expression by means of flow cytometry

T cells were measured for CD3 (peridinin-chlorophyll-protein), CD4 (Alexa488), CD8 (R-phycoerythrin [rPE]), CD25 (APC), and CD45RO (rPE) expression; DCs and macrophages for HLA-DR and CD11c expression (BD Biosciences, Mississauga, Ontario, Canada); and CD3⁺CD4⁺CD25⁺ cells for FoxP3 expression (in permeabilized cells, eBioscience). Confirmation of RSV uptake by DCs was performed by using the mouse anti-RSV G protein MAB858 (Chemicon International, Temecula, Calif). Flow cytometry was performed with a BD FACSCanto Flow Cytometer (BD Biosciences), and analysis was performed with WinMDI 2.8 software.

Statistical analysis

Data are expressed as means $\pm$ SEMs, except for cytokine release, which was shown as the range of individual values with the median. Comparisons of EPO release, tritiated thymidine incorporation, and expression of CD25 and CD45RO were performed by means of ANOVA (Statview 5.0; SAS Institute, Inc, Cary, NC). Mann-Whitney analysis was used for cytokine and leukotriene (LT) data. A *P* value of less than .05 was considered statistically significant. Linear correlation between optical density and EPO molarity was performed with Microplate Manager Software (Bio-Rad, Hercules, Calif).

RESULTS**PIV induces release of EPO from eosinophils cocultured with T cells and APCs**

EPO release was measured in wells containing various combinations of cultured cells in the presence or absence of PIV.

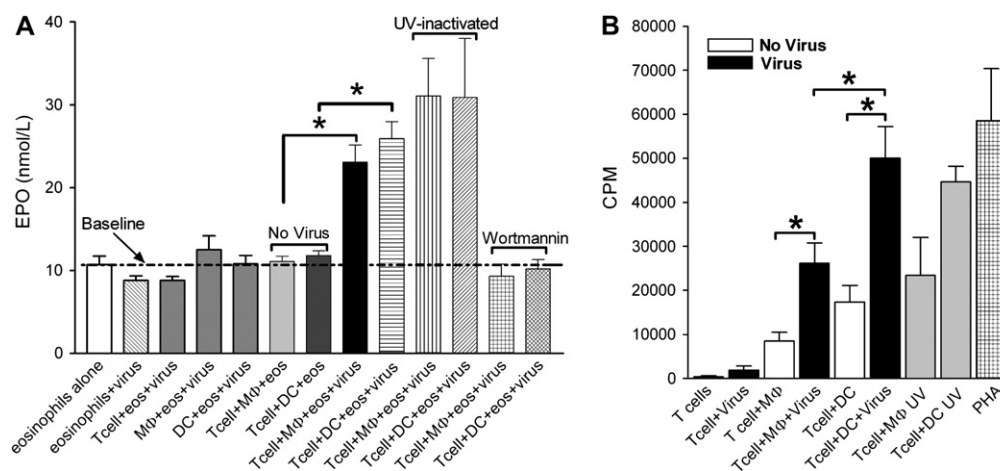


FIG 1. PIV induces EPO release and T-cell proliferation when incubated with APCs. **A**, Adding PIV to cultures of T cells with macrophages or DCs induced EPO release ($n = 36$, black bars, $P < .0001$). UV inactivation did not prevent EPO release ($n = 3$, $P < .05$). **B**, T-cell proliferation was observed only with PIV, macrophages, or DCs (black bars, $n = 19$). T-cell proliferation with DCs was greater than that with macrophages ($P < .0001$). PHA served as a positive control (netted bar, $n = 24$).

Eosinophils released a negligible but consistent background amount of EPO (10.8 nmol/L; Fig 1, A). Adding PIV to eosinophils alone (8.8 nmol/L) did not induce EPO release greater than baseline release. Eosinophils cultured individually with T cells, macrophages, or DCs with or without PIV did not release EPO (8.6, 13.1, and 10.8 nmol/L, respectively). In the absence of PIV, eosinophils in combination with T cells and APCs, either macrophages or DCs, did not release EPO (10.9 and 11.6 nmol/L, respectively). The addition of virus to this final combination of T cells with macrophages or DCs induced EPO release from eosinophils that was significantly greater than that seen in the nonvirus controls (23.2 and 25.9 nmol/L, respectively; $P < .0001$ each). Virus replication did not appear necessary for EPO release because UV-inactivated PIV induced equal amounts of EPO release from eosinophils incubated with T cells and APCs (macrophages, 31.1 nmol/L; DCs, 30.9 nmol/L) compared with those with active virus. Finally, some eosinophils were blocked with the PI3-kinase inhibitor wortmannin to help determine whether the eosinophil response was a physiologically regulated event rather than just spontaneous necrosis. Wortmannin prevented virus-induced EPO release from eosinophils incubated with T cells and APCs (macrophages, 9.3 nmol/L; DCs, 10.2 nmol/L). No significant differences in EPO measurements were detected in eosinophils pretreated with wortmannin in all other coculture combinations with or without virus (data not shown). We confirmed that the effect was not due to MHC class differences by performing experiments with autologous eosinophils, which showed no appreciable differences from homologous cells.

PIV induces proliferation of T cells in coculture with APCs

T cells incubated alone with PIV (1908 cpm; Fig 1, B) did not proliferate more than cells without virus (355 cpm). In contrast, T cells cultured with either macrophages or DCs showed proliferation in wells when virus was added (25,139 cpm [$P = .005$] and 49,220 cpm, respectively). The induction of T-cell proliferation by virus was greater in cultures with DCs than that in cultures with macrophages ($P < .0001$). In addition, UV-inactivated PIV

induced similar proliferation from T cells incubated with both macrophages and DCs compared with proliferation induced by active virus (23,408 and 44,662 cpm, respectively).

RSV induces similar EPO release and T-cell proliferation in coculture with APCs

We determined that eosinophil mediator release was not PIV specific because RSV induced a similar amount of EPO release from eosinophils incubated with T cells and APCs (macrophages, 25.2 nmol/L; DCs, 28.0 nmol/L) compared with PIV (Fig 2, A). Here, too, EPO release was not increased above baseline values when RSV was incubated with eosinophils alone or eosinophils in coculture with either T cells or APCs used individually (data not shown). RSV also induced a similar pattern of proliferation from T cells incubated with both macrophages and DCs (Fig 2, B). T cells incubated alone with or without RSV showed no proliferation. Again, the induction of T-cell proliferation by virus was greater in wells containing DCs than in wells containing macrophages ($P < .009$).

Characterization of APCs by means of flow cytometry

At the final time point of coculture experiments, we stained the cells in the wells representing DCs and macrophages for expression of CD3, HLA-DR, and CD11c (Fig 3). Approximately 90% of the cells were monocytes based on size, granularity, and CD11c expression (Fig 3, A). Of the monocyte-gated population, $91\% \pm 3\%$ were CD11c⁺ compared with their isotype control. Exposure of DCs to PIV or RSV induced an increase in the percentage of HLA-DR expression in the CD11c⁺ cells ($n = 8$; Fig 3, E).

PIV, RSV, and RV induce CD25⁺CD45RO⁺ expression only on CD4⁺ T cells

We determined the phenotype of the T-cell population by staining for CD3, CD4, and CD8 expression. Fig 4 displays the typical flow cytometric dot plot depicting CD4 and CD25 expression in T cells alone in control media (Fig 4, A), T cells and

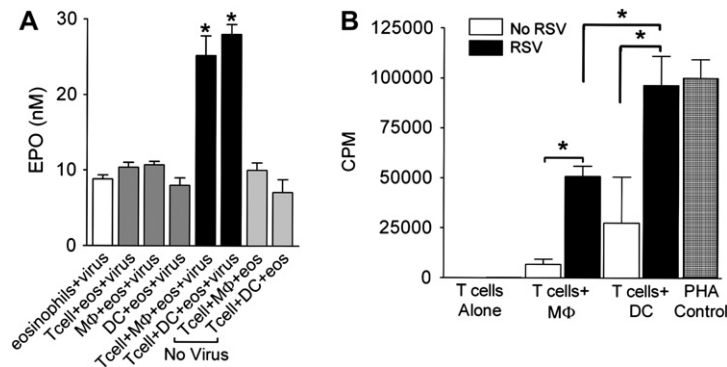


FIG 2. RSV induces similar EPO release and associated T-cell proliferation. **A**, RSV induced EPO release from eosinophils incubated with T cells and either macrophages or DCs (black bars, $n = 4$) compared with appropriate controls (gray bars, $n = 4$, $P < .01$). **B**, RSV induced proliferation from T cells incubated with either macrophages or DCs ($n = 3$, $P < .01$). T-cell proliferation was greater in wells containing DCs than in those containing macrophages ($P < .009$). PHA served as a positive control.

DCs (Fig 4, B), T cells and DCs with LPS (Fig 4, C), and T cells and DCs with PIV (Fig 4, D). Without virus, $CD3^+CD4^+$ cells alone or with DCs have low expression of the IL-2 receptor CD25 ($2.2\% \pm 1.1\%$ and $5.2\% \pm 1.1\%$, respectively; Fig 4, E). In the absence of DCs, the addition of PIV, RSV, RV, or LCMV to T cells did not induce CD25 expression greater than that induced with T cells alone ($1.7\% \pm 0.7\%$, $2.3\% \pm 0.9\%$, $2.3\% \pm 0.4\%$, and $4.3\% \pm 2.4\%$ for PIV, RSV, RV, and LCMV, respectively). In contrast, induction of CD25 was demonstrated on $CD3^+CD4^+$ cells when cocultured with DCs and virus (PIV, $16\% \pm 2\%$; RSV, $28.4\% \pm 5\%$; and RV, $7.8\% \pm 1.7\%$; $P < .05$ each). LCMV is a common mouse pathogen, and under normal circumstances, human-to-human transmission is rare.²⁹⁻³¹ Immune memory was expected to be low, and in contrast to the common human viral pathogens, LCMV was unable to induce CD25 expression in $CD4^+$ T cells with DCs. As a positive control, LPS induced robust proliferation from T cells cocultured with DCs and stimulated expression of CD25 on both $CD4^+$ and $CD8^+$ T cells (Fig 4, C). LPS did not induce T-cell proliferation in the absence of an APC (data not shown). There was no change in the CD4/CD8 ratio before or after virus infection (2.0 ± 0.6 , $n = 10$). $CD3^+CD8^+$ cells did not express CD25 after any virus infection, even when incubated with DCs. Selective isolation of $CD4^+$ or $CD8^+$ T cells was performed ($n = 2$). Although $CD4^+$ T cells cultured with DCs show upregulation of CD25 expression, the $CD8^+$ cells did not (data not shown).

As a marker of T-cell memory, CD45RO expression was determined in all wells of T cells. Fig 5 displays the typical flow cytometric dot plot of T cells and DCs (Fig 5, A) and T cells, DCs, and RSV (Fig 5, B). Without virus, $CD3^+CD4^+$ cells alone or with DCs have low expression of CD45RO and CD25 ($3.3\% \pm 2.8\%$ and $3.1\% \pm 1.8\%$, respectively; Fig 5, C). In the absence of DCs, the addition of PIV, RSV, or RV to T cells did not induce greater CD45RO expression than did T cells alone ($1.1\% \pm 0.9\%$, $1.6\% \pm 1.6\%$, and $4.0\% \pm 1.9\%$ for PIV, RSV, and RV, respectively). In contrast, induction of CD45RO was increased on $CD4^+CD25^+$ cells when cocultured with DCs and PIV, RSV, or RV ($13.3\% \pm 6.8\%$, $14.6\% \pm 3.5\%$, and $11.4\% \pm 5.2\%$, respectively; $P < .05$ each) compared with control values. LCMV was unable to induce CD45RO expression in $CD4^+$ T cells with DCs. Incubation with PHA (positive control) induced proliferation of T cells with CD45RO expression on all $CD3^+$ T cells,

including the $CD8^+$ cells (data not shown). No significant CD45RO was demonstrated on $CD4^+$ cells ($CD8^+$) in virus-exposed conditions (ie, Fig 5, A, vs Fig 5, B; CD8 values not shown). FoxP3 staining was performed to determine whether the $CD25^+$ cells were T regulatory in nature. FoxP3 expression was absent in $CD4^+CD25^+$ cells ($n = 3$, data not shown).

PIV induces the release of cytokines from T cells in culture with APCs

Production of IFN- γ , IL-5, and GM-CSF was measured in the supernatants from the cells in coculture with or without PIV. T cells, macrophages, or DCs incubated individually with PIV did not release significant levels of IFN- γ or GM-CSF (data not shown). Similarly, in the absence of PIV, T cells cultured with either macrophages or DCs showed no significant release of IFN- γ or GM-CSF compared with nonvirus levels (Fig 6, A and B). In contrast, the addition of PIV to T cells cultured with macrophages or DCs induced IFN- γ and GM-CSF production ($n = 13$, $P < .001$ for IFN- γ and $P < .001$ for GM-CSF). The induction of GM-CSF production by virus was greater in wells containing DCs than in wells containing macrophages ($P < .001$). Although the trend for IFN- γ production also appeared to be greater in wells containing DCs than macrophages, this did not reach statistical significance. Both IFN- γ and GM-CSF concentrations appeared to increase when eosinophils were added to the coculture of T cells, virus, and DCs ($n = 20$), although this did not reach statistical significance ($P = .2$). Eosinophils cultured alone did not release cytokines in response to virus, nor was there cytokine release when eosinophils were added to either T cells or APCs (used individually) with or without virus (data not shown). IL-5 release was not detected.

RV induces the release of cytokines and LTs

Production of IFN- γ and IL-13 was measured in the supernatants from the cells in coculture with or without RV. T cells or DCs incubated individually with or without RV showed low levels of IFN- γ or IL-13 (data not shown). Similarly, in the absence of RV, T cells cultured with DCs showed low levels of IFN- γ or IL-13 (Fig 6, C and D). In contrast, the addition of RV to T cells cultured with DCs induced IFN- γ and IL-13 production, although given the low sample size, this only reached statistical significance for

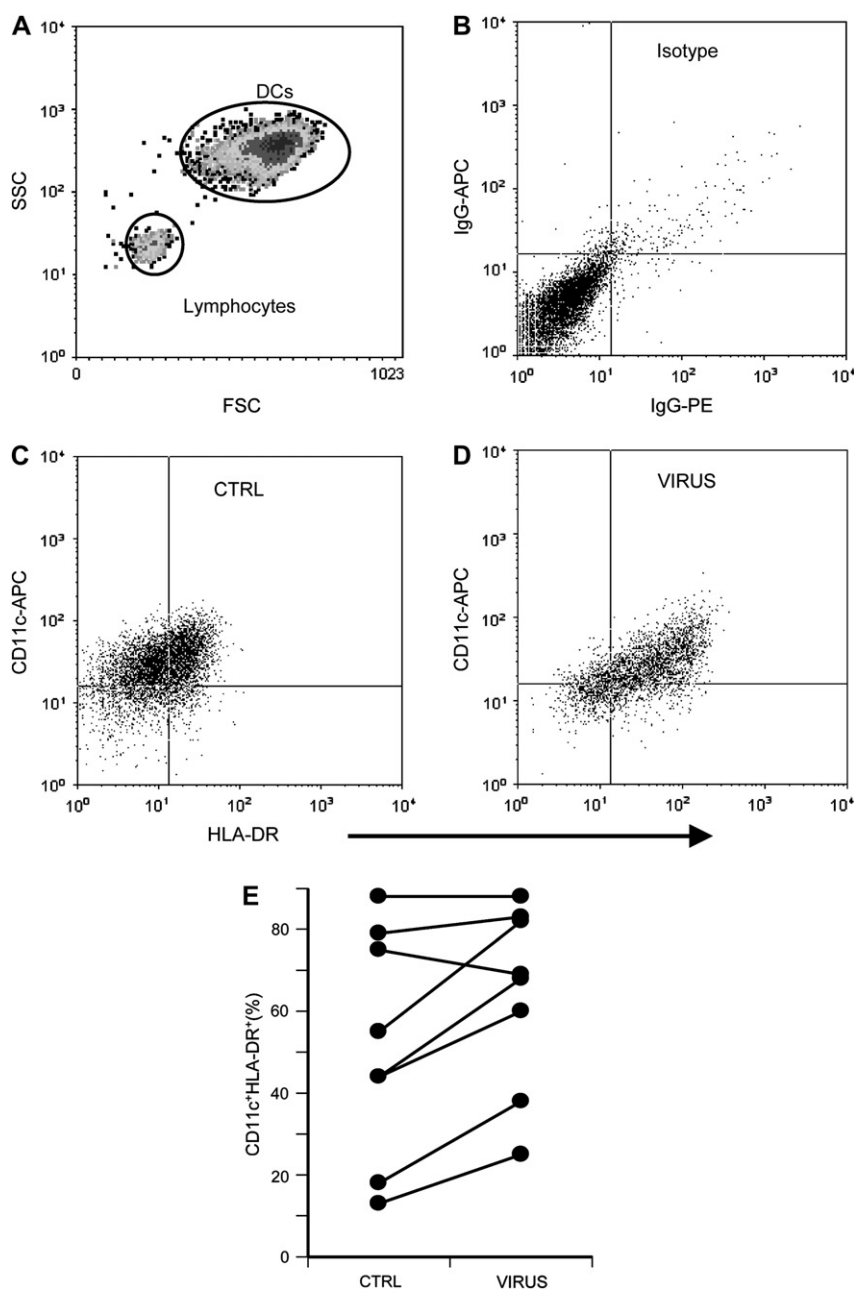


FIG 3. Virus-induced CD11c and HLA-DR expression on DCs. **A**, Representative forward-scatter (*FSC*) and side-scatter (*SSC*) plots of DCs and T cells in coculture. **B**, Isotype control for HLA-DR (APC) and CD11c (PE). **C** and **D**, Typical flow cytometric dot plot depicting HLA-DR and CD11c expression on DCs in control condition (Fig 3, C) versus virus-inoculated media (Fig 3, D). **E**, Results of 8 experiments show increased HLA-DR and CD11c double-positive DCs after incubation with RSV ($P < .01$). *CTRL*, Control.

IFN- γ ($n = 3$ each). LT release was measured in wells containing various combinations of cultured cells in the presence or absence of RV (Fig 6, E). Similar to the EPO data, eosinophils released a consistently low background amount of LT, which was not increased by the addition of RV alone or T cells, DCs, or a combination thereof without virus (data not show). In contrast, RV did induce LT release from eosinophils cultured with T cells and DCs compared with release in nonvirus wells (53.8 ± 5.1 vs 10.7 ± 1.6 pg/mL, respectively; $P = .004$).

DISCUSSION

The experiments in this report were designed to determine whether human eosinophils release their mediators in response to airway viruses and to understand the mechanism of the response. Our studies and those of other groups have demonstrated that virus infection of allergen-sensitized animals induces AHR, which is eosinophil mediated, but through a process involving interaction with CD8⁺ T cells.^{24,25} These animals do not reflect the human experience because they had no previous exposure

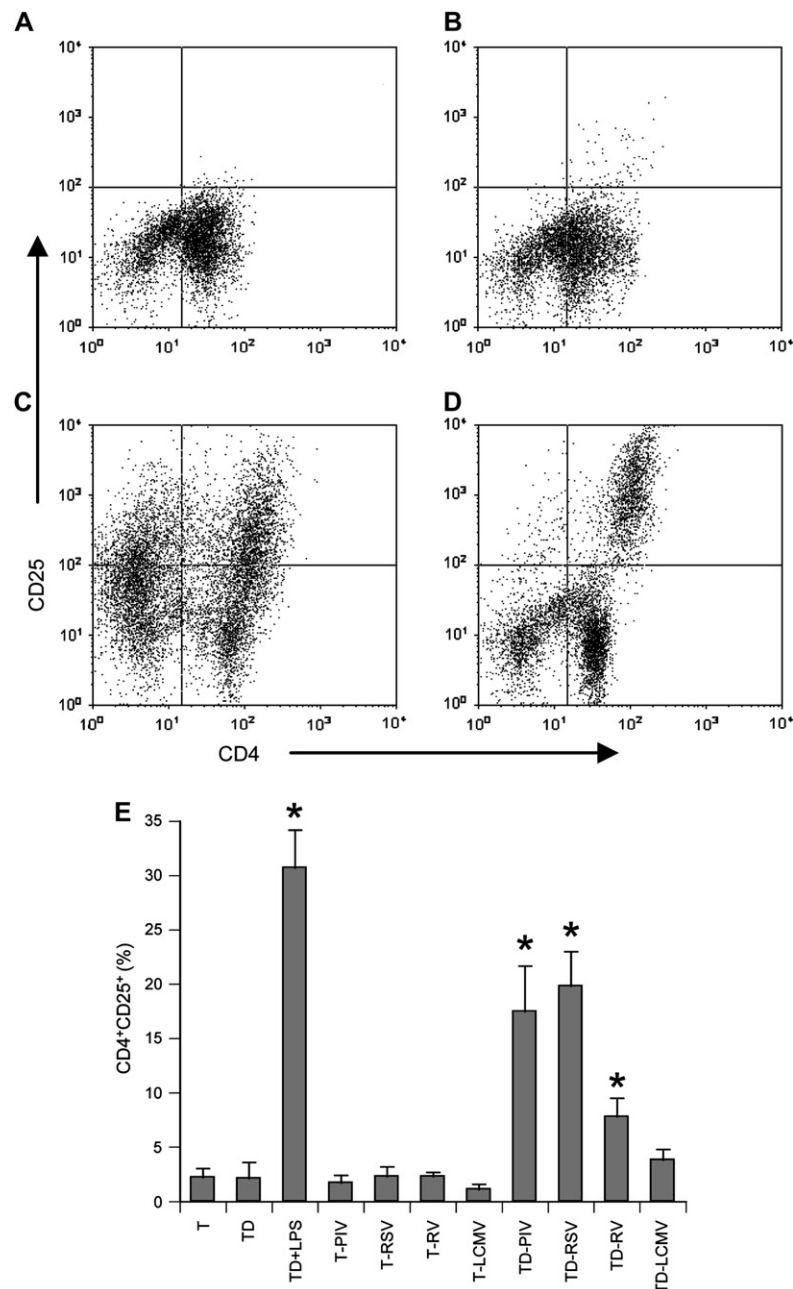


FIG 4. RSV-, PIV-, and RV-treated DCs induce expression of CD25 on CD4⁺ T cells compared with LCMV. Typical flow cytometric dot plot: **A**, T cells alone in control media (*T*); **B**, T cells and DCs (*TD*) in control media; **C**, T cells and DCs with LPS (*TD+LPS*); **D**, T cells and DCs and PIV (*TD-PIV*). **E**, Mean results of independent experiments (PIV, *n* = 6; RSV, *n* = 3; RV, *n* = 6; LCMV, *n* = 3). **P* < .05.

to the virus and thus had no immune memory. In contrast, even young children have begun developing immune memory from exposure to common cold viruses.³²⁻³⁵ Given the importance of T cells in mediating eosinophil effector function, we designed a virus/eosinophil coculture system with or without T cells. We hypothesized that because our adult donors have acquired immune memory, the role of APCs might also be a vital element to consider in the model.

For immune effector cells like eosinophils or T cells to release their mediators, there must be primary recognition of the virus. We found no evidence of virus-induced EPO or LT release from

eosinophils. In the absence of APCs, the viruses also failed to trigger lymphocyte activation directly, as measured based on the lack of T-cell proliferation, CD25 expression, or cytokine release.

T cells recognize viruses after antigen presentation, either through infected cells (MHC I-dependent fashion) or through professional APCs, including macrophages or DCs (an MHC II-dependent fashion). Macrophages or DCs incubated with viruses on their own could not induce release of EPO or LT from eosinophils. In contrast, APCs were able to facilitate EPO or LT release from eosinophils, although only when T cells were cocultured with virus. Eosinophil mediator release corresponded

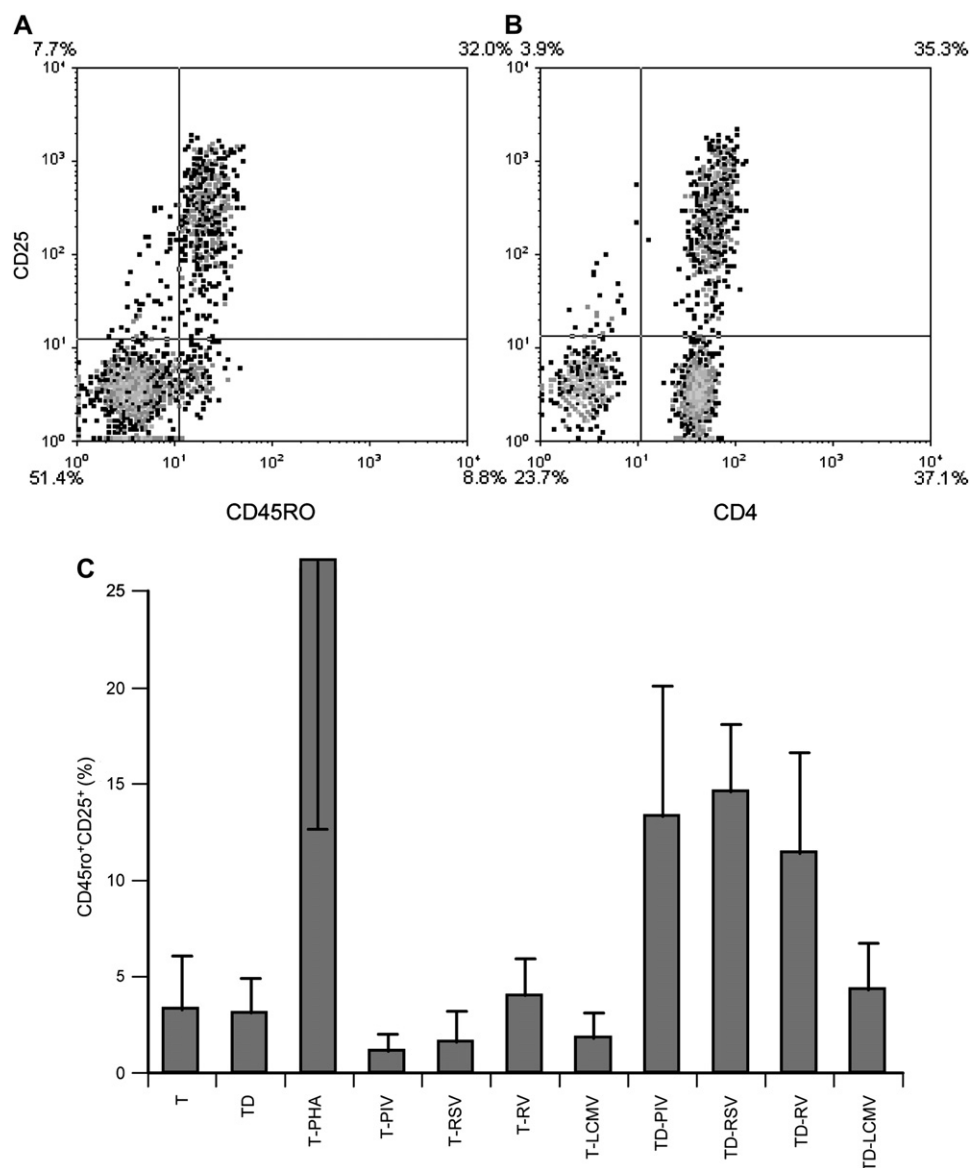


FIG 5. RSV-, PIV-, and RV-treated DCs induce expression of CD45RO on CD25⁺CD4⁺ T cells compared with LCMV. Typical flow cytometric dot plot: **A**, T cells with DCs alone in control media (TD); **B**, T cells, DCs, and RSV (TD-RSV). **C**, Mean results of independent experiments (PIV, n = 6; RSV, n = 6; RV, n = 4; LCMV, n = 3). **P* < .05. PHA-treated cells served as a positive control (T-PHA).

with induction of T-cell proliferation. Inhibition of the eosinophil PI3-kinase signaling pathway blocked the virus-induced release of EPO, further supporting the physiologic nature of the response rather than simple induction of eosinophil necrosis. We also observed release of IL-13, IFN- γ , and GM-CSF, the last 2 known for influencing eosinophil viability and mediator release *in vitro*.^{26,36,37} Whether cytokines are directly responsible for eosinophil stimulation requires further investigation.

Lymphocyte expression of CD25 (IL-2 receptor) and CD45RO was increased on CD4⁺, but not CD8⁺, T cells. In keeping with this, we found that the ability of virus to infect and replicate was not necessary for the induction of eosinophil mediator release because UV-inactivated virus induced a similar amount of EPO release and T-cell proliferation compared with active virus. These

data further highlight the importance of virus antigen presentation in an MHC II fashion rather than an MHC I fashion. Recent work with mice with immune memory to RSV show both CD4⁺ and CD8⁺ T-cell responses.³⁸ Likely such CD8⁺ T cells are responding to the infected tissues (ie, epithelium). We would also expect that memory CD8⁺ T cells could induce eosinophil mediator release, but our *in vitro* model was not designed to determine this.

Although we were aware of studies showing *in vitro* inhibitory effects of viruses on DC-induced T-cell proliferation,³⁹⁻⁴¹ these studies cocultured monocyte-derived DCs with allogeneic rather than autologous T cells, as we report here. In allogeneic models T cells have a significant degree of proliferation even before the virus is added, likely through MHC I differences. We designed our model to study the ability of T cells to respond to virus antigen

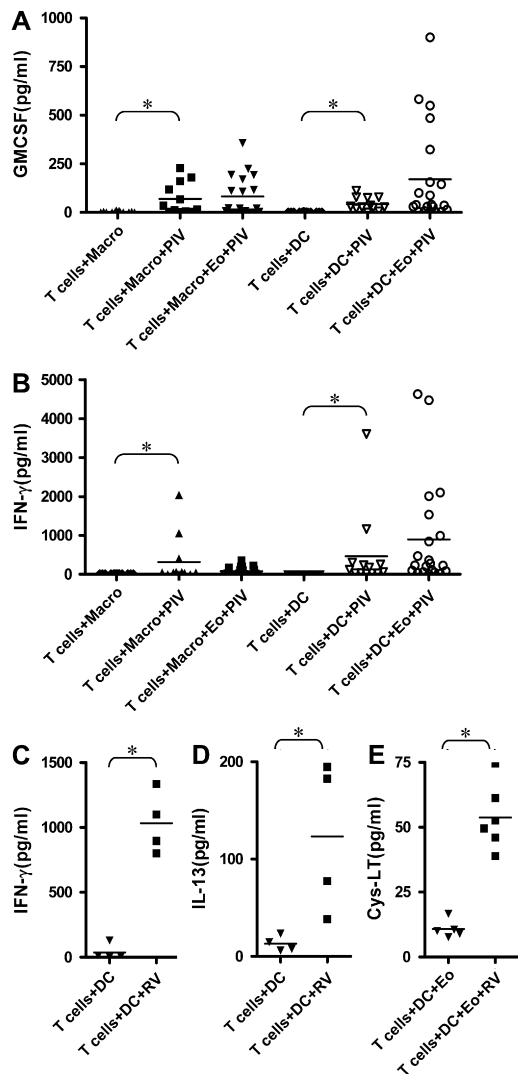


FIG 6. Virus-induced cytokine and LT release in supernatants. PIV cocultured with macrophages or DCs and T cells induced release of GM-CSF (A) and IFN-γ (B; n = 20 each) compared with cultures without PIV (n = 13). RV cocultured with T cells and DCs induced IFN-γ and IL-13 release compared with non-RV cultures (C and D; n = 3 each). After adding eosinophils, RV cocultured with T cells and DCs induced LT release compared with non-RV cultures (E; n = 5 each). Each subject's values are shown, with the median as the line. *P < .05.

presentation. To support this, our DCs appeared more efficient than macrophages in all measured parameters. Although there was evidence of some T-cell proliferation in the absence of virus, this did not induce significant release of cytokines or eosinophil mediators. Similar proliferation in the absence of antigen has been described, although the mechanism remains unclear.⁴²

Although there are numerous common cold viruses, we embarked on this project as a pediatric focused laboratory, studying RSV and Sendai virus (antigenically similar to PIV⁴³). Both are single-stranded RNA viruses known for recurrent infection in most children and bronchiolitis or asthma exacerbation¹ in some. Either virus would be an immune stimulus for adult donor T cells. In contrast, LCMV was used to support our notion of a lack of immune memory in this model. Finally, we added RV for its greater importance in asthma exacerbation.^{1,44}

RSV plays such an important role in hospitalization for infants, whereas RV is the leading cause in older children and adults.^{4,44} The strong effect that atopy plays in virus-induced hospitalization is largely observed in children older than 3 years.^{4,45} Before this age, hospitalization caused by RSV-induced wheezing illness is similar between atopic and nonatopic children. A key factor predicting hospitalization from RSV-induced bronchiolitis in infants is the lower immunity to RSV.⁴⁶ Thus RSV might not induce bronchiolitis and hospitalization in older children or adults because virus infectivity is better contained by immune memory. Unlike RSV or PIV, RV is the most frequent airway virus infection year round. RV is less cytotoxic⁴⁷ and tends to present more with upper airway symptoms. Although there are reports of RV-related bronchiolitis,⁴⁸ the infants tend to be older and have signs suggestive of early asthma.^{4,49,50} We suggest that viruses cause hospitalization in the younger child not because of their antigenicity but for their ability to kill epithelium and obstruct the airways. In contrast, once adequate antiviral immunity develops, virus antigen is the remaining factor able to induce asthma exacerbation in older children and adults, stimulating effector cells, such as eosinophils.

Both asthmatic and healthy adults show similar responses to common airway viruses in terms of frequency of infection, initial cold symptoms, and ability to clear infection.^{16,17} The difference is in the development of lower airway narrowing of greater severity and duration. Even when clinically stable, patients with asthma exhibit increased airway eosinophilia.⁵¹ Animal and human asthma data suggest that airway eosinophils are positioned in close proximity to parasympathetic nerves.^{52,53} We propose that immune memory to an airway virus triggers eosinophils to release their biologically active mediators. As such, the underlying common cold virus sensitivity of asthmatic patients might not relate to abnormal perception or processing of viruses but rather to the aberrant increase in the presence and position of secondary effector cells (eg, eosinophils) in asthma. This would be in keeping with the strong protective effect of corticosteroid therapy,⁵⁴ which we are currently investigating in our model. We suggest that the increased frequency of infection with RV and its subsequent eosinophilic immune response could explain why RV is the most frequent virus associated with asthma exacerbation.⁴⁴ Our report is the first to describe the regulation of virus-induced eosinophil mediator release in the context of T cells and APCs *in vitro*.

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Clinical implications: The knowledge of immune regulation of virus-induced exacerbation of asthma will expand our understanding of this clinically relevant occurrence.

REFERENCES

- Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995;310:1225-9.
- Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. *BMJ* 1993;307:982-6.
- O'Byrne PM, Barnes PJ, Rodriguez-Roisin R, Runnerstrom E, Sandstrom T, Svensson K, et al. Low dose inhaled budesonide and formoterol in mild persistent asthma: the OPTIMA randomized trial. *Am J Respir Crit Care Med* 2001;164:1392-7.

4. Heymann PW, Carper HT, Murphy DD, Platts-Mills TA, Patrie J, McLaughlin AP, et al. Viral infections in relation to age, atopy, and season of admission among children hospitalized for wheezing. *J Allergy Clin Immunol* 2004;114:239-47.
5. Murray CS, Poletti G, Kebadze T, Morris J, Woodcock A, Johnston SL, et al. Study of modifiable risk factors for asthma exacerbations: virus infection and allergen exposure increase the risk of asthma hospital admissions in children. *Thorax* 2006;61:376-82.
6. Frigas E, Gleich GJ. The eosinophil and the pathophysiology of asthma. *J Allergy Clin Immunol* 1986;77:527-37.
7. Bousquet J, Chané P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
8. Hargreave FE. Quantitative sputum cell counts as a marker of airway inflammation in clinical practice. *Curr Opin Allergy Clin Immunol* 2007;7:102-6.
9. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;360:1715-21.
10. Barnes PJ. Efficacy of inhaled corticosteroids in asthma. *J Allergy Clin Immunol* 1998;102:531-8.
11. Rowe BH, Spooner CH, Ducharme FM, Bretzlaff JA, Bota GW. Corticosteroids for preventing relapse following acute exacerbations of asthma. *Cochrane Database Syst Rev* 2007;CD000195.
12. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 1996;156:4422-8.
13. Sivertson KL, Seeds MC, Long DL, Peachman KK, Bass DA. The differential effect of dexamethasone on granulocyte apoptosis involves stabilization of Mcl-1L in neutrophils but not in eosinophils. *Cell Immunol* 2007;246:34-45.
14. Yamaya M, Sasaki H. Rhinovirus and asthma. *Viral Immunol* 2003;16:99-109.
15. Ada GL, Jones PD. The immune response to influenza infection. *Curr Top Microbiol Immunol* 1986;128:1-54.
16. van Elden LJ, Sachs AP, van Loon AM, Haarman M, van de Vijver DA, Kimman TG, et al. Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract. *J Clin Virol* 2008;41:116-21.
17. Corne JM, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate ST, et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002;359:831-4.
18. Calhoun WJ, Dick EC, Schwartz LB, Busse WW. A common cold virus, rhinovirus 16, potentiates airway inflammation after segmental antigen bronchoprovocation in allergic subjects. *J Clin Invest* 1994;94:2200-8.
19. Grunberg K, Smits HH, Timmers MC, de Klerk EP, Dolhain RJ, Dick EC, et al. Experimental rhinovirus 16 infection. Effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am J Respir Crit Care Med* 1997;156:609-16.
20. Bardin PG, Fraenkel D, Sanderson G, Dorward M, Johnston S, Holgate S. Increased sensitivity to the consequences of rhinoviral infection in atopic subjects. *Chest* 1995;107(suppl):157S.
21. de Kluijver J, Grunberg K, Sont JK, Hoogeven M, van Schadewijk WA, de Klerk EP, et al. Rhinovirus infection in nonasthmatic subjects: effects on intrapulmonary airways. *Eur Respir J* 2002;20:274-9.
22. Baker DG, Don HF, Brown JK. Direct measurement of acetylcholine release in guinea pig trachea. *Am J Physiol Lung Cell Mol Physiol* 1992;263:L142-7.
23. Adamko DJ, Yost BL, Gleich GJ, Fryer AD, Jacoby DB. Ovalbumin sensitization changes the inflammatory response to subsequent parainfluenza infection. Eosinophils mediate airway hyperresponsiveness, M2 muscarinic receptor dysfunction, and antiviral effects. *J Exp Med* 1999;190:1465-78.
24. Adamko D, Odemuyiwa SO, Moqbel R. The eosinophil as a therapeutic target in asthma: beginning of the end, or end of the beginning? *Curr Opin Pharmacol* 2003;3:227-32.
25. Schwarze J, Cieslewicz G, Joetham A, Ikemura T, Hamelmann E, Gelfand EW. CD8 T cells are essential in the development of respiratory syncytial virus-induced lung eosinophilia and airway hyperresponsiveness. *J Immunol* 1999;162:4207-11.
26. Adamko DJ, Wu Y, Gleich GJ, Lacy MP, Moqbel R. The induction of eosinophil peroxidase release: improved methods of measurement and stimulation. *J Immunol Methods* 2004;291:101-8.
27. Gern JE, Dick EC, Lee WM, Murray S, Meyer K, Handzel ZT, et al. Rhinovirus enters but does not replicate inside monocytes and airway macrophages. *J Immunol* 1996;156:621-7.
28. Adamko DJ, Wu Y, Ajamian F, Ilarraza R, Moqbel R, Gleich GJ. The effect of cationic charge on release of eosinophil mediators. *J Allergy Clin Immunol* 2008. In press.
29. Fischer SA, Graham MB, Kuehnert MJ, Kotton CN, Srinivasan A, Marty FM, et al. Transmission of lymphocytic choriomeningitis virus by organ transplantation. *N Engl J Med* 2006;354:2235-49.
30. Emonet S, Retornaz K, Gonzalez JP, de Lamballerie X, Charrel RN. Mouse-to-human transmission of variant lymphocytic choriomeningitis virus. *Emerg Infect Dis* 2007;13:472-5.
31. Jamieson DJ, Kourtis AP, Bell M, Rasmussen SA. Lymphocytic choriomeningitis virus: an emerging obstetric pathogen? *Am J Obstet Gynecol* 2006;194:1532-6.
32. Glezen WP, Frank AL, Taber LH, Kasel JA. Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. *J Infect Dis* 1984;150:851-7.
33. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child* 1986;140:543-6.
34. Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. *Scand J Immunol* 2004;59:1-15.
35. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med* 2001;7:719-24.
36. Davoine F, Labonte I, Ferland C, Mazer B, Chakir J, Laviolette M. Role and modulation of CD16 expression on eosinophils by cytokines and immune complexes. *Int Arch Allergy Immunol* 2004;134:165-72.
37. Lacy P, Mahmudi-Azer S, Bablitz B, Hagen SC, Velazquez JR, Man SF, et al. Rapid mobilization of intracellularly stored RANTES in response to interferon-gamma in human eosinophils. *Blood* 1999;94:23-32.
38. Lee YM, Miyahara N, Takeda K, Prpich J, Oh A, Balhorn A, et al. IFN-gamma production during initial infection determines the outcome of reinfection with respiratory syncytial virus. *Am J Respir Crit Care Med* 2008;177:208-18.
39. Roberts NJ Jr. Different effects of influenza virus, respiratory syncytial virus, and Sendai virus on human lymphocytes and macrophages. *Infect Immun* 1982;35:1142-6.
40. Stockl J, Vetr H, Majdic O, Zlabinger G, Kuechler E, Knapp W. Human major group rhinoviruses downmodulate the accessory function of monocytes by inducing IL-10. *J Clin Invest* 1999;104:957-65.
41. Guerrero-Plata A, Casola A, Suarez G, Yu X, Spetch L, Peebles ME, et al. Differential response of dendritic cells to human metapneumovirus and respiratory syncytial virus. *Am J Respir Cell Mol Biol* 2006;34:320-9.
42. Woodhead VE, Stonehouse TJ, Binks MH, Speidel K, Fox DA, Gaya A, et al. Novel molecular mechanisms of dendritic cell-induced T cell activation. *Int Immunol* 2000;12:1051-61.
43. Granoff A, Webster RG. *Encyclopedia of Virology*. Academic Press; 1999.
44. Johnston SL. Natural and experimental rhinovirus infections of the lower respiratory tract. *Am J Respir Crit Care Med* 1995;152(suppl):S46-52.
45. Korppi M, Kotaniemi-Syrjanen A, Waris M, Vainionpää R, Reijonen TM. Rhinovirus-associated wheezing in infancy: comparison with respiratory syncytial virus bronchiolitis. *Pediatr Infect Dis J* 2004;23:995-9.
46. Welliver RC. Respiratory syncytial virus immunoglobulin and monoclonal antibodies in the prevention and treatment of respiratory syncytial virus infection. *Semin Perinatol* 1998;22:87-95.
47. Winther B, Gwaltney JM, Hendley JO. Respiratory virus infection of monolayer cultures of human nasal epithelial cells. *Am Rev Respir Dis* 1990;141:839-45.
48. Andreoletti L, Lesay M, Deschildre A, Lambert V, Dewilde A, Wattré P. Differential detection of rhinoviruses and enteroviruses RNA sequences associated with classical immunofluorescence assay detection of respiratory virus antigens in nasopharyngeal swabs from infants with bronchiolitis. *J Med Virol* 2000;61:341-6.
49. Mansbach JM, McAdam AJ, Clark S, Hain PD, Flood RG, Acholonu U, et al. Prospective multicenter study of the viral etiology of bronchiolitis in the emergency department. *Acad Emerg Med* 2008;15:111-8.
50. Carlsen KH, Orstavik I, Halvorsen K. Viral infections of the respiratory tract in hospitalized children. A study from Oslo during a 90 months' period. *Acta Paediatr Scand* 1983;72:53-8.
51. Gibson PG, Saltos N, Borgas T. Airway mast cells and eosinophils correlate with clinical severity and airway hyperresponsiveness in corticosteroid-treated asthma. *J Allergy Clin Immunol* 2000;105:752-9.
52. Costello RW, Schofield BH, Kephart GM, Gleich GJ, Jacoby DB, Fryer AD. Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function. *Am J Physiol Lung Cell Mol Physiol* 1997;273:L93-103.
53. Adamko DJ, Fryer AD, Bochner BS, Jacoby DB. CD8+ T-lymphocytes in viral hyperreactivity and M2 muscarinic receptor dysfunction. *Am J Respir Crit Care Med* 2003;167:550-6.

METHODS

Culture medium and reagents

Cells were cultured in complete RPMI-1640 (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 2 mmol/L L-glutamine, 10% FBS, 100 U/L penicillin, and 100 µg/L streptomycin. Recombinant human IL-4 and GM-CSF were purchased from R&D Systems (Minneapolis, Minn).

Lymphocyte isolation

Peripheral blood was purified from adult donors. Donors were excluded if they had received oral corticosteroids in the last 6 months. The study conformed to the Helsinki Agreement, and all subjects provided written informed consent, as approved by the University of Alberta Health Research Ethics Board. After red cell sedimentation in 6% dextran (T500, Sigma-Aldrich; 30 minutes at room temperature), the leukocyte-rich supernatant was subjected to density gradient centrifugation on Ficoll. The mononuclear cell layer was collected and incubated in 6-well plates (12×10^6 cells/well in RPMI-10% FBS for 2 hours at 37°C in a 5% CO₂ atmosphere). The nonadherent cells were run through a nylon wool column to yield a T-cell suspension (>95% purity confirmed by means of flow cytometry). The cells were counted and stored at -80°C in FBS with 10% dimethyl sulfoxide for future use.

Macrophage/DC preparation

Remaining adherent cells in 6-well plates from the previous step were washed with HBSS before adding GM-CSF (40 ng/mL) or both GM-CSF and IL-4 (8 ng/mL) to mature the monocytes to macrophages or DCs, respectively. Cells were incubated for 7 days (at 37°C in a 5% CO₂ atmosphere). Purity (>95%) was confirmed by means of flow cytometry.

Eosinophil isolation

From the Ficoll step, eosinophils were purified from the granulocyte pellet by means of negative immunomagnetic selection with a mix of CD16-, CD14-, and CD3-coated iron beads (MACS system; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), resulting in a highly purified population of CD16⁺ eosinophils (>99%).^{E1}

Coculturing of T cells, macrophages/DCs, and virus

Lymphocytes were thawed (50% to 80% recovery), washed (color-free RPMI-10% FBS), and added to 96-well plates precoated and washed to block nonspecific activation of eosinophils (2.5% human serum albumin for 2 hours at 37°C, 2×10^5 cells/well).^{E2} Macrophages or DCs (from the same lymphocyte donor) were added to the 96-well plates (5×10^3 cells/well). The 96-well plates with various cell types individually (T cells alone, macrophages alone, and DCs alone) or in combination (T cells with either macrophages or DCs) were incubated overnight (150 µL of medium per well at 37°C in a 5% CO₂ atmosphere) before the addition of the viruses or their respective sham controls: PIV (ATCC, Sendai virus, tissue culture infectious dose₅₀ (TCID₅₀) of 1×10^6 grown in CMK cells with LHC8 media, Bioscience, diluted 1:1000 in RPMI or PIV sham 1:1000 of LHC-8 supernatant from uninfected CMK cells), RSV (ATCC, strain A2, 6.5×10^6 PFU grown in Hep-2 cells with RPMI and then diluted 1:1000 in RPMI or RSV sham media 1:1000 RPMI from uninfected Hep-2 cells), RV-16 (generously provided by Dr David Proud,^{E3} University of Calgary, 1×10^6 TCID₅₀ U/mL, diluted 1:1000 in RPMI), or LCMV (strain Armstrong, 5×10^5 PFU/mL grown in BHK-21 cells with RPMI and diluted 1:5 in RPMI). Some experiments used UV-inactivated PIV (UV light for 30 minutes with inactivation confirmed by the loss of virus-induced hemagglutination in CMK cells).

Virus-induced eosinophil mediator release in coculture

The various combinations of cell groups with or without virus were cultured for 6 days (at 37°C in a 5% CO₂ atmosphere). Eosinophils (2×10^5 cells/well) were added to the wells overnight (at 37°C in a 5% CO₂ atmosphere). Lysine-coated cyanogen bromide-coupled Sepharose beads (1:40 bead

to well volume, PharMingen) were added to the wells (2 hours at 37°C in a 5% CO₂ atmosphere) with the eosinophils to improve eosinophil mediator release, with the rationale being that eosinophils have a greater release of EPO in response to cytokines when they adhere to cationic-charged lysine-coated beads.^{E4}

Measurement of EPO and LT release

As previously described,^{E1} purified human EPO (125 nmol/L; provided through a generous gift from Dr G. J. Gleich, University of Utah) served as the standard and allowed the calculation of EPO in molarity (in nanomoles). Peroxidase substrate solution for the measurement of EPO was prepared by adding 800 µL of 5 mmol/L *o*-phenylenediamine HCl (Sigma-Aldrich) to 4 mL of 1 mol/L Tris buffer (pH 8.0) and 1.25 µL of 30% hydrogen peroxide (Sigma). Some eosinophils were preincubated with the PI3-kinase inhibitor wortmannin (25 nmol/L for 30 minutes at room temperature, Sigma-Aldrich). The reaction was stopped with the addition of 4 mol/L H₂SO₄ (2:1 sample to H₂SO₄) and read at a wavelength of 490 nm for optical density. The reaction is linear within this EPO concentration range. Regarding Cys-LT release, 50 µL of cell-free supernatants from each cell culture were recovered and assayed in duplicate by means of ELISA with the Cysteinyl-Leukotriene EIA Assay Kit (Cayman Chemical), a competitive ELISA used for measuring all Cys-LTs (including LTC₄, LTD₄, and LTE₄). Plates were developed at room temperature and read at 415 nm for up to 90 minutes at 15-minute intervals. Best standard-curve fit readings, usually at 90 minutes, are selected and used for determination of Cys-LT concentrations.

Virus-induced T-cell proliferation assay by means of tritiated thymidine incorporation

Before coculture, DCs and macrophages were irradiated (2000 rad) to prevent their proliferation. After 6 days with virus or controls, tritiated thymidine (1 µCi/well) was added to the wells. The next day, cells were harvested for radioactivity measurements (in counts per minute) by using a cell harvester (Tomtec, Hamden, Conn). The tritiated thymidine was detected with a scintillation counter (Wallac, Milton Keynes, United Kingdom). PHA (5 µg/mL) with T cells alone served as the positive control.

Measurement of cytokines in supernatants of coculture

IFN-γ, IL-5, IL-13, and GM-CSF were measured in supernatants by means of ELISA as per kit protocol (eBioscience). Some plates were generously donated by Dr P. Man (University of British Columbia, Canada).

Surface marker expression by means of flow cytometry

After incubation, T cells were harvested and incubated with a mix of antibodies specific for human CD3 (peridinin-chlorophyll-protein), CD4 (Alexa488), CD8 (rPE), CD25 (APC), and CD45RO (rPE). DCs and macrophages were incubated with antibodies against HLA-DR and CD11c. All antibodies were purchased from BD Biosciences. Intracellular detection of FoxP3 in CD3⁺CD4⁺CD25⁺ cells and confirmation of RSV uptake by DCs was done with the mouse anti-RSV G protein MAB858 (Chemicon). Flow cytometry was performed with a BD FACSCanto Flow Cytometer (BD Biosciences), and analysis was performed with WinMDI 2.8 software.

Statistical analysis

All data are expressed as means ± SEM, except for cytokine release, which was shown as the range of individual values with the median. Comparison of EPO release, tritiated thymidine incorporation, and expression of CD25 and CD45RO was performed by means of ANOVA (Statview 5.0; SAS Institute, Inc). A *P* value of less than .05 was considered statistically significant. Given the sample size and nongaussian distribution, Mann-Whitney analysis was used for cytokine and LT data. Calculation of values for linear correlation

between optical density and EPO molarity was performed with Microplate Manager Software (Bio-Rad).

REFERENCES

- E1. Adamko DJ, Wu Y, Gleich GJ, Lacy P, Moqbel R. The induction of eosinophil peroxidase release: improved methods of measurement and stimulation. *J Immunol Methods* 2004;291:101-8.
- E2. Kato M, Abraham RT, Okada S, Kita H. Ligation of the beta2 integrin triggers activation and degranulation of human eosinophils. *Am J Respir Cell Mol Biol* 1998;18:675-86.
- E3. Gern JE, Dick EC, Lee WM, Murray S, Meyer K, Handzel ZT, et al. Rhinovirus enters but does not replicate inside monocytes and airway macrophages. *J Immunol* 1996;156:621-7.
- E4. Adamko DJ, Wu Y, Ajamian F, Ilarraza R, Moqbel R, Gleich GJ. The effect of cationic charge on release of eosinophil mediators. *J Allergy Clin Immunol* 2008. In press.