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γδT cells suppress inflammation and disease during rhinovirus-induced asthma exacerbations

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Most asthma exacerbations are triggered by virus infections, the majority being caused by human rhinoviruses (RV). In mouse models, γδT cells have been previously demonstrated to influence allergen-driven airways hyper-reactivity (AHR) and can have antiviral activity, implicating them as prime candidates in the pathogenesis of asthma exacerbations. To explore this, we have used human and mouse models of experimental RV-induced asthma exacerbations to examine γδT-cell responses and determine their role in the immune response and associated airways disease. In humans, airway γδT-cell numbers were increased in asthmatic vs. healthy control subjects during experimental infection. Airway and blood γδT-cell numbers were associated with increased airways obstruction and AHR. Airway γδT-cell number was also positively correlated with bronchoalveolar lavage (BAL) virus load and BAL eosinophils and lymphocytes during RV infection. Consistent with our observations of RV-induced asthma exacerbations in humans, infection of mice with allergic airways inflammation increased lung γδT-cell number and activation. Inhibiting γδT-cell responses using anti-γδTCR (anti-γδT-cell receptor) antibody treatment in the mouse asthma exacerbation model increased AHR and airway T helper type 2 cell recruitment and eosinophilia, providing evidence that γδT cells are negative regulators of airways inflammation and disease in RV-induced asthma exacerbations.

INTRODUCTION

Respiratory virus infections are associated with around 85% of asthma exacerbations in both adults and children, and human rhinoviruses (RV) represent the majority of virus species detected.[1–3] Experimental infection studies have provided further support for a causative role for RV in asthma exacerbations.[4,5] Current therapies are inadequate for treating asthma exacerbations, thus there remains a need for further investigation into the mechanisms underlying disease to identify targets for more specific and effective therapies.

We previously compared normal and asthmatic subjects before and after experimental RV infection, reporting that conventional CD4 + T helper type 2 (Th2) cells in the airways positively correlated with increased lower airway symptoms in asthmatics.[4] Unlike conventional T cells, however, the role of innate lymphocytes in RV-induced asthma exacerbations is completely unknown despite studies in mouse asthma models having reported important functions for "unconventional" T cells in airways hyper-reactivity (AHR) and airways inflammation.[6,7] γδT cells, in particular, possess a range of functions that might make them key players in inflammatory airways diseases such as asthma, including maintenance of epithelial tissue homeostasis,[8,9] modulation of innate and adaptive immune responses,[10–12] and the ability to contribute to respiratory pathogen control.[13,14] γδT cells are reportedly enriched in asthmatic airways[15–17] and, in mouse studies, have been shown to influence AHR and/or airways inflammation in acute and chronic allergic asthma models.[18–20] However, because differing effects on AHR and allergic inflammation have been described depending on the model, method, timing,

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or subset of γδT cells manipulated, their function in asthma pathogenesis remains somewhat ambiguous.[18,21–25] Insight into γδT-cell responses to respiratory viral infections is limited, but airway γδT-cell responses to respiratory syncytial virus, sendai virus, and influenza infections have each been reported,[26–28] with both pro-inflammatory (respiratory syncytial virus) and pro-resolution (influenza) functions having been ascribed to γδT cells.[26,28]

The available evidence therefore indicates that γδT cells respond to respiratory viral infections and potentially have an important role in asthma, but to date, γδT-cell responses during RV-induced asthma exacerbations have not been investigated. To address this, we used human and mouse models, reporting that the magnitude of the γδT-cell response to experimental RV infection in humans was positively associated with the severity of airways obstruction and AHR. To determine whether γδT-cell responses were a consequence or cause of airways inflammation, we investigated γδT-cell deficiency in a mouse asthma exacerbation model. Inhibiting γδT-cell responses caused increased AHR and allergic airways inflammation in allergen- and RV-challenged mice, suggesting that γδT cells are important negative regulators of disease during RV-induced asthma exacerbations.

RESULTS

γδT-cell numbers are greater in asthmatic airways during RV infection and correlate with clinical illness severity, virus load, and airways inflammation

To determine whether γδT cells were associated with responses to experimental RV infection, we first measured γδT-cell numbers in the airways of allergic asthmatic and healthy control subjects at baseline (before infection), during RV infection (day 4), and at 6 weeks when infection had resolved.[4]

At baseline, there was a trend for increased numbers of γδT cells in the bronchoalveolar lavage (BAL) of asthmatics compared with healthy control subjects. By day 4 post infection, BAL γδT-cell numbers in asthmatics had increased such that they were significantly higher than in healthy controls (**Figure 1a**; asthmatics $0.90 \times 10^6 l^{-1}$ (0.63, 1.79), healthy controls 0.37 (0.185, 0.67), $P = 0.007$). At 6 weeks, γδT-cell number remained significantly elevated compared with healthy controls (**Figure 1a**; asthmatics $0.89 \times 10^6 l^{-1}$ (0.32, 1.34), healthy controls 0.40 (0.15, 0.60), $P = 0.04$). We next assessed the relationship between γδT cells and clinical measures of airways disease. BAL γδT-cell number during infection correlated with increased airways obstruction as assessed by maximum percentage fall in peak expiratory flow (PEF; **Figure 1b**, $r = -0.567$, $P = 0.006$) and maximum percentage fall in forced expiratory volume in 1 s (FEV_1) ($r = -0.423$, $P = 0.049$; not shown) following RV infection. BAL γδT-cell number also correlated with increased AHR as determined by fall in the provocative concentration of histamine required to cause a 10% decrease in FEV_1 (PC_{10}) from baseline to day 6 (**Figure 1c**, $r = -0.436$, $P = 0.038$). In addition, BAL γδT-cell number also correlated with BAL virus load on day 4 post infection (**Figure 1d**, $r = -0.674$, $P = 0.006$).

We have previously reported increased BAL eosinophil, neutrophil, and lymphocyte responses associated with reductions in PEF or chest symptoms in asthmatics during RV infection.[4] To determine whether increased γδT-cell numbers were also associated with cellular airways inflammation, we correlated γδT cells with neutorphils, lymphocytes, and macrophages in BAL. We found that γδT-cell numbers positively correlated with increased eosinophils (**Figure 1e**, $r = 0.49$, $P = 0.01$) and lymphocytes (**Figure 1f**, $r = 0.70$, $P = 0.0001$). We also observed a similar nearly statistically significant relationship between γδT cells and BAL neutrophils (data not shown, $r = 0.36$, $P = 0.09$). There was no association between γδT cell and macrophage number (data not shown).

Reduced γδT-cell number in peripheral blood of RV-infected asthmatics is associated with clinical illness severity

In contrast to BAL, flow cytometric analysis of peripheral blood mononuclear cells indicated a fall in γδT-cell concentration from baseline to day 4 after infection in asthmatic subjects, although this change was not statistically significant (**Figure 1g**, baseline 6.096 (5.321, 13.01) vs. $4.387 \times 10^7 l^{-1}$ (3.342, 7.647)). As with BAL cells, γδT-cell numbers in blood were associated with the severity of airways obstruction and AHR. The percentage of reduction in γδT cells in blood from baseline to day 4 correlated with maximum fall in FEV_1 (**Figure 1h**; asthmatics $r = 0.943$, $P = 0.017$; all subjects $r = 0.5035$, $P = 0.028$) and with change in PC_{10} in asthmatic subjects only (not shown; $r = 0.943$, $P = 0.017$).

Allergen-sensitized and challenged mice have similar RV-induced increases in airway γδT-cell number to RV-infected human asthmatics

Given the association of γδT-cell responses with RV-induced disease in human asthmatic subjects, we next determined whether γδT cells were similarly increased in a mouse model of RV-induced asthma exacerbation in which γδT cells could be manipulated. First, we assessed γδT-cell responses to RV infection in healthy mice, which is characterized by neutrophillic and lymphocytic airways inflammation and pro-inflammatory cytokine and mucus production.[29] RV infection caused a significant increase in the number of γδT cells in the lungs from day 2 until day 7 after infection (**Figure 2a**; RV vs. ultraviolet (UV)-RV $P < 0.001$). Numbers of γδT cells returned to baseline level by day 10 post infection, which corresponds with resolution of airways inflammation in this model.[29]

We next assessed the utility of a mouse model of RV-induced exacerbation of allergic airways inflammation (**Supplementary Figure S1a** online) to determine the role of γδT cells during asthma exacerbations. As previously reported for the human asthma RV infection model,[4] RV infection in mice with allergic airways inflammation caused increased neutrophil, lymphocyte, and eosinophil recruitment compared with RV-infected non-allergic controls (**Supplementary Figure S1b** online) and reported previously.[29] Increased IL-4 and IL-6 protein

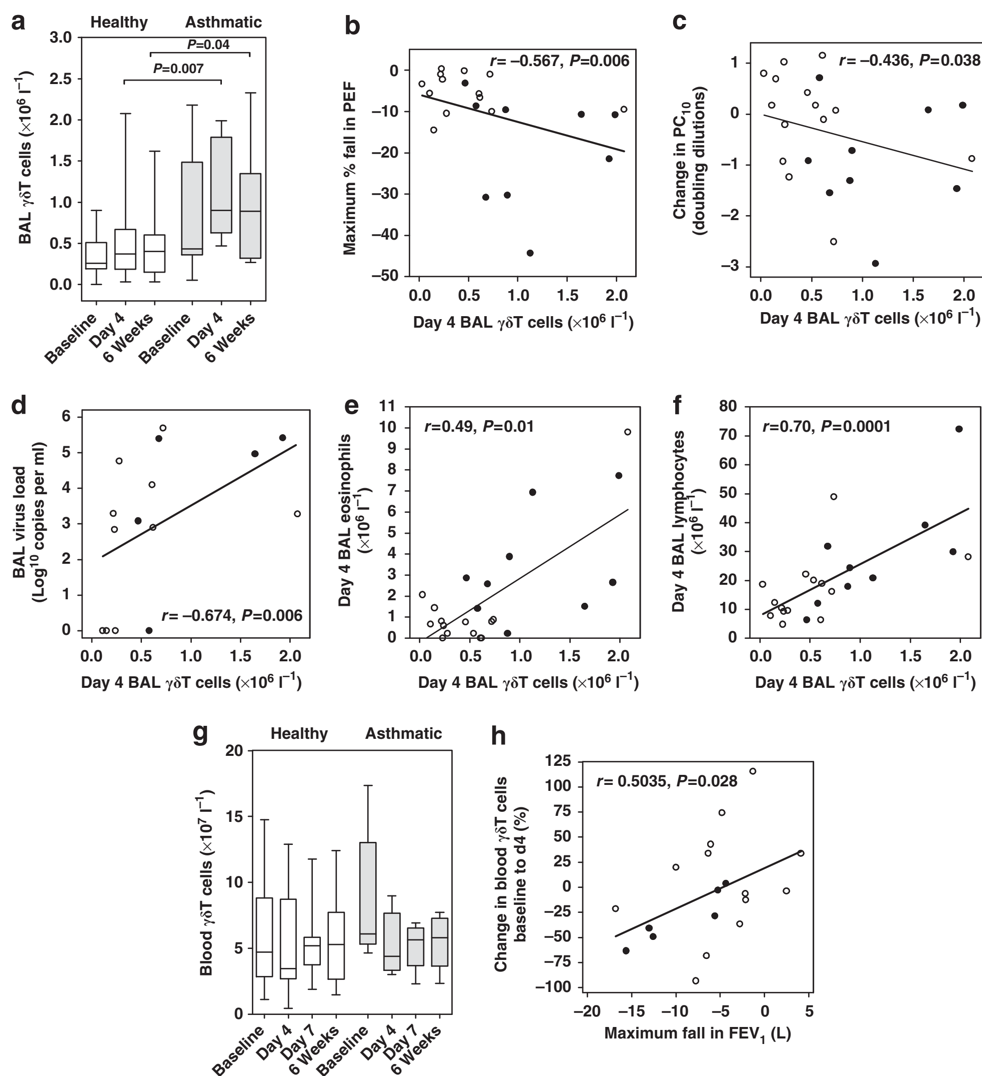

Figure 1 γδT-cell number is increased in asthma during RV infection and is associated with clinical illness severity, virus load, and inflammatory cell infiltration. (**a**) Numbers of γδT cells in bronchoalveolar lavage (BAL) of asthmatic and healthy control subjects were assessed by flow cytometry at baseline and at 4 days and 6 weeks after infection with rhinoviruses (RV). Shaded bars, asthmatics ($n=9$); open bars, healthy controls ($n=15$). (**b**) The number of γδT cells in BAL during acute infection (day 4 post infection) correlated with maximum percentage fall in peak expiratory flow (PEF) throughout the study, (**c**) change in histamine PC_{10} (provocative concentration of histamine required to cause a 10% decrease in FEV_1) from baseline to day 6, (**d**) day 4 BAL virus load, (**e**) day 4 BAL eosinophil number and (**f**) day 4 BAL lymphocyte number. Closed circles, asthmatics ($n=5$–9); open circles, healthy controls ($n=10$–14). (**g**) γδT cells in the peripheral blood of asthmatic and healthy control subjects measured by flow cytometry at baseline and at the indicated time points after infection. Shaded bars asthmatics ($n=6$), open bars healthy controls ($n=13$). (**h**) Percentage change in blood γδT-cell number from baseline to day 4 post infection correlated with maximum percentage fall in FEV_1 (forced expiratory volume in 1 s). Closed circles, asthmatics ($n=6$); open circles, healthy controls ($n=13$). Statistics indicated in correlations are for all the subjects.

production and increased AHR (**Supplementary Figure S1c–e** online) were further evidence of RV-exacerbated asthma-related responses in the mouse model, as we also observed increased IL-4 and IL-6 protein levels in human asthmatic airway samples during RV infection (**Supplementary Figure S1f,g** online). Increased AHR during RV infection in the human asthma exacerbation model has been reported previously.[4]

By day 2, ovalbumin (OVA) challenge alone had induced a significant increase in the number of γδT cells in the lungs (**Figure 2b**; UV-OVA vs. UV-PBS (phosphate-buffered saline) day 2 $P<0.001$). Lung γδT-cell numbers were significantly increased on day 1 in OVA-challenged, RV-infected (RV-OVA) mice compared with OVA or RV challenge alone (**Figure 2b**; RV-OVA vs. UV-OVA day 1 $P<0.05$; RV-OVA vs. RV-PBS days 1 and 2 $P<0.001$). RV infection similarly increased OVA-induced numbers of γδT cells expressing the Vγ4 T-cell receptor (TCR), as defined by Heilig *et al.*[30] (**Figure 2c**). The proportion of γδT cells expressing the early activation marker CD69 was also elevated in OVA-challenged mice and was further enhanced by RV on days 1 and 2 post infection (**Figure 2d**; RV-OVA vs. UV-OVA day 1 $P<0.01$, day 2 $P<0.05$). Thus, γδT-cell responses in the mouse modeled the situation in human allergic asthma. Mice

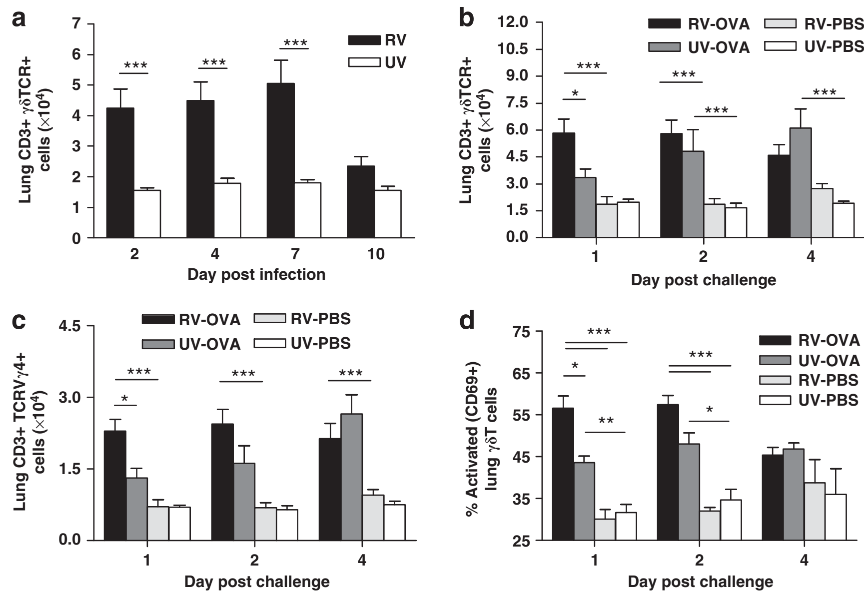

Figure 2 Lung γδT-cell responses in mouse models of rhinoviruses (RV) infection and RV-induced asthma exacerbation. (**a**) Mice were infected intranasally with RV1B and CD3 + γδTCR + (γδT-cell receptor) cells in lung tissue were enumerated by flow cytometry. (**b–d**) Alternatively, ovalbumin (OVA)-sensitized mice were challenged with OVA or phosphate-buffered saline (PBS) and infected with RV (RV-OVA, RV-PBS) or ultraviolet (UV)-inactivated RV (UV-OVA, UV-PBS) control as described. (**b**) Total CD3 + γδTCR +, (**c**) CD3 + TCRVγ4 + and (**d**) CD69-expressing γδT cells in lung tissue assessed by flow cytometry. $n = 4$ mice/group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

with allergic airways inflammation exhibited increased numbers of γδT cells in the lungs similarly to allergic asthmatic subjects, and γδT-cell number was further increased during RV-induced exacerbated lung inflammation.

γδT cells suppress allergic airways inflammation during RV-induced asthma exacerbations

Although associated with clinical disease during RV-induced asthma exacerbations, the functional role of γδT cells had not been defined. To do this, we determined the effect of γδT-cell deficiency achieved via the systemic administration of an anti-γδTCR antibody during the challenge phase of the mouse asthma exacerbation model (**Figure 3a**). Anti-γδTCR antibody treatment caused a > 90% reduction in the number of γδT cells detectable in the lungs and BAL by flow cytometry, until at least 7 days post challenge (**Supplementary Figure S2** online).

AHR is an important feature of disease during asthma exacerbations. We measured AHR at 24 and 48 h post infection. Inhibition of γδT-cell responses had no effect at 24 h, with both the RV-OVA groups (anti-γδTCR and isoptype control) exhibiting similar levels of significantly enhanced AHR compared with UV-PBS negative controls. By 48 h, the anti-γδTCR-treated RV-OVA group continued to demonstrate significantly enhanced AHR, whereas in RV-OVA-isotype-treated mice AHR had diminished (**Figure 3b**; RV-OVA-anti-γδTCR vs. RV-OVA-isotype, $P < 0.05$).

We also examined the effect of anti-γδTCR treatment on airways inflammation. BAL leukocytes were differentially counted on days 1–7 post challenge to examine both innate and adaptive immune responses. Neutrophil and macrophage numbers were increased in BAL of anti-γδTCR vs. isotype control–treated RV-OVA mice on day 2, coinciding with the enhanced AHR induced by anti-γδTCR treatment (**Figure 3c**; neutrophils $P < 0.05$, macrophages $P < 0.05$). On day 7, eosinophil and lymphocyte numbers were also increased in the BAL of anti-γδTCR antibody–treated mice (**Figure 3c**; eosinophils $P < 0.05$, lymphocytes $P < 0.01$). In addition, γδTCR-negative CD4 + T-cell responses were also assessed in the lung on day 7 post challenge using intracellular flow cytometry to determine whether increases in lymphocytes in BAL represent specific increases in allergy-associated Th2 responses. Anti-γδTCR treatment caused a significant increase in Th2 (CD3 + CD4 + IL-4 +) cell number (**Figure 3d**; $P < 0.01$). Differences in cellular lung inflammation were not evident by hematoxylin and eosin staining (**Supplementary Figure S3** online). The increase in Th2 cell number in the lung was associated with a trend for increased production of the Th2 cell recruiting chemokine CCL17 (C-C motif chemokine ligand 17) in BAL on day 7 (**Figure 3e**) but not in the levels of Th2 cytokines IL-4 and IL-13 on days 1 and 2 when they are detectable in this model (**Supplementary Figure S3** online). Anti-γδTCR treatment also had no effect on BAL mucin protein levels or lung PAS (periodic-acid Schiff) staining scores (**Supplementary Figure S3** online).

Immunoglobulin E (IgE) is another marker of allergic disease and was measured in serum on day 7 post challenge. OVA-specific IgE levels were significantly increased in anti-γδTCR vs. isotype control–treated RV-OVA mice on day 7 (**Figure 3g**; $P < 0.05$), suggesting that γδT cells also suppress humoral immune responses.

In contrast to some pro-inflammatory responses, levels of the regulatory cytokine IL-10 in BAL were significantly

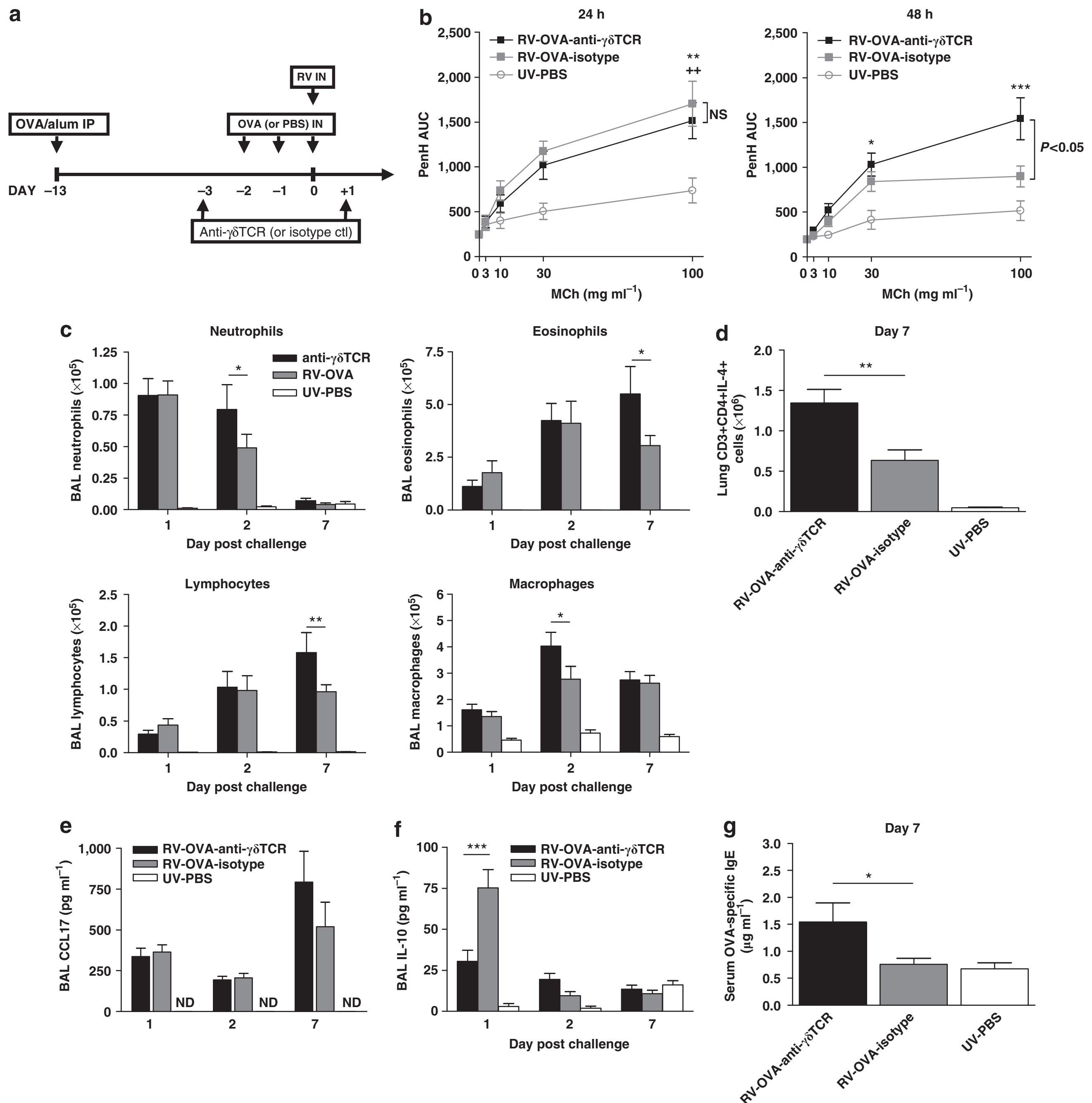

Figure 3 γδT cells suppress airways hyper-reactivity (AHR) and allergic airways inflammation. (**a**) ovalbumin (OVA)-sensitized mice were challenged intranasally (IN) with OVA or phosphate-buffered saline (PBS) and additionally with rhinoviruses (RV) or ultraviolet (UV)-inactivated RV as described. RV plus OVA–challenged mice were also administered anti-γδTCR (anti-γδT-cell receptor) antibody (or isotype control) 1 day before and 1 day after OVA challenges. (**b**) AHR was assessed in response to methacholine (MCh) challenge 24 and 48 h after virus challenge. *RV-OVA-anti-γδTCR vs. UV-PBS, + RV-OVA-isotype vs. UV-PBS. (**c**) Leukocyte populations in bronchoalveolar lavage (BAL) were enumerated by cytospin assay. (**d**) Lung leukocytes harvested at 7 days post infection were stimulated with phorbol myristate acetate and ionomycin and stained for CD3, CD4, and intracellular interleukin (IL)-4. (**e**) C-C motif chemokine ligand 17 (CCL17) and (**f**) IL-10 protein levels in BAL. (**g**) Serum OVA-specific immunoglobulin E (IgE) on day 7 post infection. $n=8$–12 mice/group. */+ $P<0.05$, **/++ $P<0.01$, ***/+++ $P<0.001$, AUC, area under the curve; IP, intraperitoneally; ND, not detected; NS, not significant; Penh, enhanced pause.

lower in anti-γδTCR-treated RV-OVA mice compared with isotype-treated controls (**Figure 3f**; day 1 RV-OVA-anti-γδTCR vs. RV-OVA-isotype $P<0.001$), suggesting that γδT cells may downregulate inflammation during RV-induced asthma exacerbations via production of regulatory cytokines.

γδT cells do not affect anti-viral immune responses

To determine whether the suppressive effect of γδT cells on airways inflammation was via modulation of anti-viral immunity and inhibition of viral replication, we measured viral RNA copy number in lung tissue, observing no differences

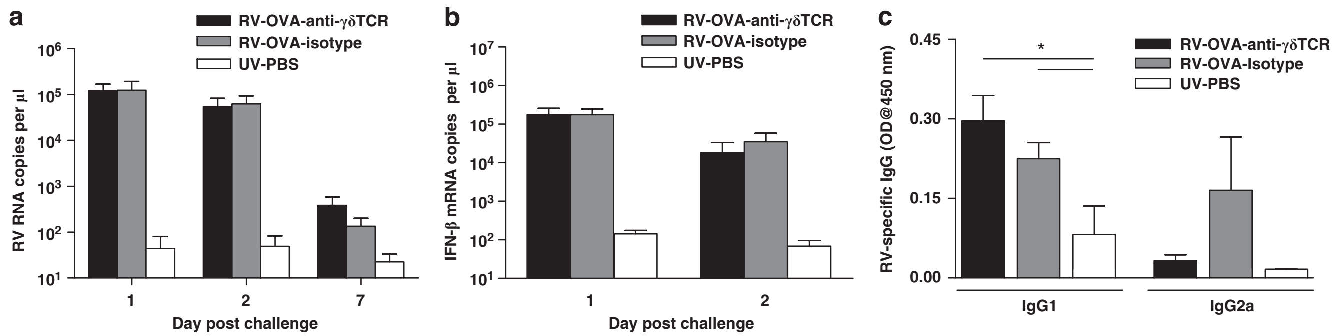

Figure 4 γδT cells do not suppress antiviral immune responses. ovalbumin (OVA)-sensitized mice were challenged intranasally with OVA or phosphate-buffered saline (PBS) and additionally with rhinoviruses (RV) or ultraviolet (UV)-inactivated RV as described. RV plus OVA–challenged mice were also administered anti-γδTCR (anti-γδT-cell receptor) or isotype control antibodies. (**a**) RV RNA and (**b**) interferon (IFN)-β mRNA levels in lung tissue were measured by Taqman quantitative PCR. n = 8 mice/group. (**c**) Serum RV-specific immunoglobulin G1 (IgG1) and IgG2a levels on day 7 post infection measured by enzyme-linked immunosorbent assay. n = 4–6 mice/group. OD, optical density.

between anti-γδTCR and isotype control antibody–treated RV-OVA mice (**Figure 4a**). We also assessed the effect of γδT-cell-mediated suppression of allergic airways inflammation on innate antiviral immune responses and found that anti-γδTCR treatment had no effect on lung interferon (IFN)-β mRNA expression (**Figure 4b**). Similarly, inhibition of γδT-cell responses did not significantly affect RV-specific antibody (IgG1 and IgG2a) responses, although there was a trend for a shift towards increased IgG1 (t-test $P = 0.23$) and reduced IgG2a (t-test $P = 0.21$) (**Figure 4c**). These data suggest that antiviral immune responses were not inhibited by γδT cells during RV-exacerbated allergic airways inflammation.

Lung γδT cells produce IL-17a and IFN-γ in the mouse RV-induced asthma exacerbation model

It has been postulated that γδT cells influence disease in allergic asthma models via the direct production of cytokines, such as IFN-γ[24,31] and IL-17a.[32] Reduced IL-10 in BAL of anti-γδTCR-treated mice indicated that γδT cells might also produce this immune-regulatory cytokine. To determine whether γδT-cell-mediated effects on AHR and airways inflammation might be due to their direct production of cytokines, we assessed the cytokine expression profile of lung γδT cells in the mouse RV-induced asthma exacerbation model.

Less than 5% of lung γδT cells expressed IL-4 or IL-10 in any treatment group, although both the proportion and absolute number of IL-4- and IL-10-producing γδT cells was greater in the OVA- vs. PBS-challenged treatment groups (**Figure 5a,b**). A greater proportion (up to 15%) of γδT cells from OVA-challenged mice expressed IFN-γ, and this was further increased by RV infection such that IFN-γ+ γδT-cell number was significantly greater in the RV-OVA vs. UV-OVA treatment groups on day 2 (**Figure 5a**, $P < 0.05$). In contrast to the modest Th1, Th2, and Treg-associated cytokine production by γδT cells, a high proportion (up to 75%) of γδT cells expressed IL-17a. There were greater total numbers of IL-17a+ γδT cells in the lungs of OVA- vs. PBS-challenged mice, although unlike IFN-γ, RV infection did not further increase γδT-cell IL-17 expression (**Figure 5a**). Notably, 20–40% of all IL-17a+ lung lymphocytes were CD3+γδTCR+.

DISCUSSION

Despite the great disease burden of RV-induced asthma exacerbations, surprisingly little is known about the immune mechanisms involved in disease pathogenesis. T cells are likely to have an important role, but insight to date into T-cell responses has been limited to demonstrations of increased numbers of CD3+ cells in the lower airways of asthmatics during experimental exacerbations[33,34] and more recently associations between CD4+ Th cell responses and disease severity.[4] The specific role of innate lymphocytes such as γδT cells in the pathogenesis of RV-induced asthma exacerbations is unknown.

We have addressed this using an experimental human challenge model. Our observations were consistent with previous studies of stable asthma[16,17] in that there were trends for higher numbers of γδT cells in BAL of asthmatics compared with healthy control subjects at baseline. We extended these observations by showing that γδT-cell numbers were further increased in BAL of asthmatics during RV infection and were significantly greater in asthmatic vs. healthy control subjects by day 4 post infection. Further analyses revealed that BAL γδT-cell number positively correlated with responses associated with airways disease during asthma exacerbations, including lower airway viral load, airway inflammatory cell infiltration, airways obstruction, and AHR. To our knowledge, this is the first study to observe a clear association between γδT cells and disease severity during asthma exacerbations. We also observed concomitant reductions in blood γδT cells after infection in asthmatics, suggesting that increases in BAL γδT cells might be attributed to RV-induced recruitment of γδT cells to the airways from peripheral blood.

The association of γδT cells with clinical and immunological measures of disease is consistent with two possibilities: either γδT cells contribute to disease causing responses or they are recruited to the lung as part of the host response to control airways disease, for example, via limiting viral replication or

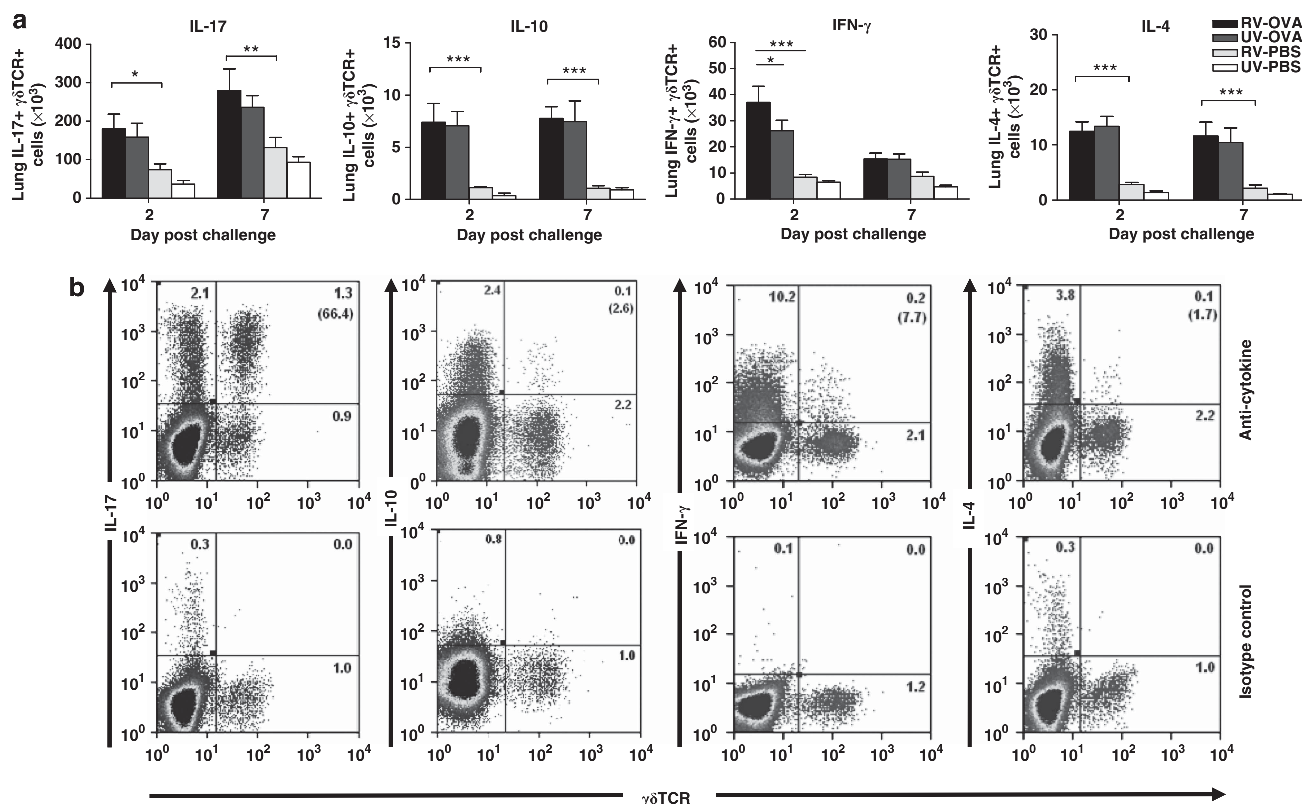

Figure 5 Lung γδT cells produce interleukin (IL)-17a and interferon (IFN)-γ in the mouse model of rhinoviruses (RV)-induced asthma exacerbation. Ovalbumin (OVA)-sensitized mice were challenged with OVA or phosphate-buffered saline (PBS) and infected with RV or ultraviolet (UV)-inactivated RV as described. Harvested lung leukocytes were stimulated with phorbyl myristate acetate and ionomycin and stained for CD3, γδTCR (γδT-cell receptor), and intracellular IL-17a, IL-10, IFN-γ, or IL-4. (**a**) Total numbers of cytokine-positive lung γδT cells in all the treatment groups on days 2 and 7 post infection. (**b**) Representative dot plots of intracellular cytokine staining in lung γδT cells from RV-OVA treated mice on day 2 post infection. Plots are gated on viable forward scatter/side scatter–defined lymphocytes. Numbers indicate the percentage of lymphocyte gate cells in each quadrant and in parenthesis the percentage of cytokine-positive lung γδT cells. Comparisons only shown for RV-OVA vs. UV-OVA and RV-OVA vs. RV-PBS treatments. $n=5$ mice/group. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

regulating host immunity. Previous reports of increased Th2 cytokine-expressing γδT cells in asthmatic vs. healthy airways[16,35] led investigators to postulate that γδT cells promote allergic airways inflammation in asthma. However, in the absence of studies blocking γδT-cell function it was impossible to define their role in human disease. Here we have extended human clinical studies to examine the relationship between γδT cells and airways disease using antibody-mediated transient inhibition of γδT-cell function in a mouse model.

During either severe RV infection or OVA-induced allergic airways inflammation, we observed increased number and activation of γδT cells, suggesting that inflammation induced by diverse stimuli can trigger γδT-cell responses in the lung. When RV infection and allergic airways inflammation were combined, γδT-cell responses were enhanced similarly to infected human asthmatics. The fact that γδT-cell activity was increased as early as 24 h after infection is consistent with a rapidly inducible cell population, which lacks a requirement for conventional antigen processing and presentation.[36–38]

To define the role performed by γδT cells in RV-induced asthma exacerbations, we administered an anti-γδTCR antibody to RV and OVA–treated mice during the allergen and virus challenge phase. This strategy was used because of reports that γδT cells are required for effective allergen sensitization,[18] which makes knockout mice unsuitable for studying exacerbation of pre-existing allergic disease. A recent publication has questioned the efficacy of this strategy for depleting γδT cells, with the authors suggesting that γδT cells remain present in tissues after antibody treatment but with undetectable cell surface TCR expression.[39] Anti-γδTCR treatment has, however, been shown to have significant effects on disease in a variety of mouse models,[24,28,40] indicating that antibody treatment induces at least a major functional impairment of γδT cells. Importantly, some investiagtors have corroborated phenotypes induced by anti-γδTCR antibody treatment using adoptive transfer of γδT cells.[19,32]

Systemic administration of an anti-γδTCR antibody enhanced AHR in RV and OVA–challenged mice, suggesting that γδT cells suppress AHR. Studies in OVA-sensitized and challenged mice genetically deficient in the TCR δ chain have provided conflicting data as to whether γδT cells promote or suppress AHR.[21,22] However, our finding that γδT cells suppress AHR is consistent with a number of studies where pan-γδT-cell "deficiency" is induced by antibody treatment in

the challenge phase of allergic asthma models,[19,20,23,24] suggesting that γδT cells perform a similar function in virus-induced asthma exacerbations as in stable or allergen-induced disease. Other investigators have used adoptive transfer of γδT cells to confirm this capacity for γδT cells to suppress allergen sensitization and challenge-induced AHR.[19,23,32] Some studies have attributed this suppressive function to the TCR Vγ4 expressing subset of γδT cells,[23,24] which we found to be increased in number in the lungs after allergen and virus challenge similarly to total γδT cells.

Whether γδT-cell-mediated effects on AHR are associated with or dependent upon changes in airways inflammation is somewhat unclear. γδT-cell-mediated suppression of AHR has been associated with suppression of allergic inflammation in both a mouse model of more chronic allergen challenge and a rat model of the late asthmatic response.[31,32] Conversely, in an acute mouse allergic airways disease model, a dissociation of effects on AHR and allergic inflammation was proposed.[19,22] We observed significant relationships between γδT-cell responses, worse lung function, increased AHR, and increased inflammation in the human model. In the mouse, anti-γδTCR treatment caused increases in neutrophilic, eosinophilic, and Th2 cell inflammation, and AHR. We have previously reported that in our human study, increased eosinophil and neutrophil number in BAL during infection were associated with increased fall in PEF.[4] Although the specific contribution of each of these cell types to disease requires further investigation, the suppression of these cellular inflammatory responses further supports a disease-suppressing function for γδT cells. Importantly, given that during virus-induced exacerbations there is likely to be a balance between suppression of immunopathology and maintenance of effective anti-viral immune responses, we found that anti-γδTCR treatment had no significant affect on lung viral load, IFN-β gene expression, or RV-specific serum IgG. This suggests that a therapy directed at promoting inflammation-suppressing γδT-cell responses would not compromise innate or adaptive anti-viral immunity.

Attempts to elucidate the mechanisms underlying γδT-cell-mediated regulation of allergic airways disease have to date focused on cytokine production, with γδT-cell-derived IFN-γ[24,31] and IL-17a[32] both having been implicated in murine models. In our model, γδT cells primarily produced IL-17a and, to a lesser degree, IFN-γ after *ex vivo* polyclonal stimulation. The finding that the majority of lung γδT cells produce IL-17a has recently been reported by others in a BALB/c mouse model of allergic asthma.[32] We noted that IL-10 levels in BAL were significantly reduced in anti-γδTCR-treated mice, indicating that γδT cells also affect IL-10 production and identifying a potential mechanism for their immuno-suppressive activity, although it is unlikely that γδT cells were the main source of IL-10 as γδT cells made little IL-10 after *ex vivo* stimulation. The precise role displayed by IFN-γ, IL-10, and, in particular, IL-17a, for which conflicting functions have been described[41–43] in asthma models is yet to be fully elucidated. IFN-γ,[44] IL-17a,[41] and IL-10[45] have, however, each been suggested to be capable of suppressing allergic airways inflammation and/or AHR in mouse asthma models and could therefore have a role in γδT-cell-mediated suppression of RV-induced disease. The ability of γδT cells to promote resolution of allergic airways inflammation in a mouse asthma model has, in fact, been directly attributed to their IL-17a producing capacity.[32]

In summary, we have shown for the first time that γδT-cell responses are associated with RV-induced asthma exacerbations and that severity of disease can be related to the magnitude of this response. We demonstrated that γδT cells exhibit an airways disease suppressing function using a mouse model. These findings add to the growing body of evidence that γδT cells can be potent negative regulators of allergic airways disease and extend this by identifying a role for these cells in regulating RV-induced asthma exacerbations.

METHODS

Human study. The design and subject characteristics of the human RV challenge study have been described in detail previously.[4] Atopic asthmatic (n = 9) or normal healthy adult volunteers (n = 15) were infected intranasally with RV16. No common cold symptoms were reported in any subject for 6 weeks before starting the study, and all subjects were RV16 seronegative at baseline. Asthmatic subjects were not receiving oral or inhaled steroid treatment. Where subject numbers differ from those stated, this was due to availability of matched data for given endpoint analyses.

FEV_1 and PEF data are presented as maximum percentage fall in the 14 days following virus inoculation as compared with baseline. AHR was measured at baseline and on day 6 and is presented as a change from baseline to day 6 in the provocative concentration of histamine required to induce a 10% reduction in FEV_1 (PC_{10}).

γδT cells were enumerated by flow cytometry whereby FC receptors were blocked with human IgG and 1×10^5 viable peripheral blood mononuclear cells, or BAL cells were stained with fluorochrome-conjugated monoclonal antibodies specific for CD3 (clone UCHT1) and γδTCR (clone B1) (both BD Pharmingen, San Diego, CA) to assess the percentage of total lymphocytes in each tissue represented by γδT cells ($CD3^+$, $γδTCR^+$). Analysis was performed on at least 10,000 lymphocyte events. Quantitative PCR for RV RNA in BAL was performed on day 4 post-infection samples as described previously.[4]

Mouse studies. *In vivo protocols:* All studies were performed in 6–8-week old, female BALB/c mice. RV serotype 1B was propagated in Ohio Hela cells as described previously.[29]

The RV infection and RV-induced asthma exacerbation models have been described previously.[29] For the RV infection model, all mice were infected intranasally with 5×10^6 $TCID_{50}$ RV1B. All mice in the asthma exacerbation model were sensitized to OVA and challenged with OVA or PBS control and with 2.5×10^6 $TCID_{50}$ RV1B (RV-OVA, RV-PBS) or UV-inactivated RV control (UV-OVA, UV-PBS). Where indicated, RV-OVA-treated mice were additionally administered 0.5 mg monoclonal hamster anti-mouse γδTCR (Clone GL3) or hamster IgG control antibody (Jackson ImmunoResearch, West Grove, PA) intraperitoneally, 1 day before and 1 day after airway challenges.

Protein assays. Cytokine protein levels in BAL were assayed using commercial "duoset" enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, UK).

For measurement of antibodies, blood was collected from the carotid arteries and serum allergen-specific IgE and virus-specific IgGs were measured by in-house enzyme-linked immunosorbent assay. Detection antibody for the IgE assay was biotinylated rat

anti-mouse IgE (clone 23G3, Southern Biotech, Birmingham, AL), and levels were quantified using a mouse OVA-specific IgE standard (clone 2C6; Genetex, Irvine, CA). IgG detection antibodies were biotinylated rat anti-mouse IgG1 (clone A85-1) and IgG2a (clone R19-15) (both BD Biosciences, San Diego, CA).

Cell assays. BAL was performed and cells were differentially counted by cytospin assay as described previously.[29] For flow cytometry analysis, lung leukocytes were obtained from whole lung tissue by digestion in RPMI medium (PAA laboratories, Pasching, Austria) containing 1 mg ml^{-1} collagenase type XI and 80 units ml^{-1} bovine pancreatic Dnase type IV (both Sigma-Aldrich, St Louis, MO). Red cells were lysed with ACK buffer. For cell surface marker staining, BAL or lung cells were stained with LIVE/DEAD fixable dead cell stain (Life Technologies, Carlsbad, CA), incubated with anti-mouse CD16/CD32 (FC block; BD Biosciences), and subsequently with directly fluorochrome-conjugated monoclonal antibodies specific for: CD3ε (clone 17A2, Biolegend, Cambridge, UK; or clone 500A2, BD Biosciences), γδTCR (clone GL3 or UC7-13D5), CD69 (clone H1.2F3) TCR Vγ4 (clone UC3-10A6), and CD4 (clone RM4-5) (all BD Biosciences). For intracellular staining, leukocytes were stimulated for 5 h at 37 °C with phorbol myristate acetate (50 ng ml^{-1}) and ionomycin (500 ng ml^{-1}) (both Sigma-Aldrich) in the presence of golgi stop (BD Biosciences) protein transport inhibitor, stained for surface markers, and permeablised using 0.5% (w/v) saponin (Sigma-Aldrich) before addition of fluorochrome-conjugated monoclonal antibodies specific for IFN-γ (clone XMG1.2), IL-4 (clone 11B11), IL-17a (clone TC11-18H10) (all BD Biosciences) or IL-10 (clone JES5-16E3; EBioscience, San Diego, CA). Data was acquired with a Cyan ADP 9 color cytometer (Dako, Glostrup, Denmark).

Quantitative PCR. Taqman quantitative PCR was performed on RNA extracted from a left upper lobe of mouse lung. RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using the Omniscript RT kit (Qiagen) with random hexamer primers (Promega, Madison, WI). Primers and probes for 18S ribosomal RNA, RV, and IFN-β have been described previously.[46] Virus and interferon RNA copy number were quantified by comparison to a plasmid DNA standard, normalized to 18S ribosomal RNA levels, and are expressed as copies per μl lung cDNA. Data was acquired using a ABI 7500 Fast Realtime PCR system (Applied Biosystems, Carlsbad, CA).

Assessment of AHR. AHR was measured as enhanced pause (Penh) in response to methacholine challenge using an unrestrained whole-body plethysmography system (Electromedsystems, Bordon, UK). Penh is displayed as area under curve for a 5 min log period post-methacholine challenge.

Statistical analyses. For human experiments, data are presented as median and interquartile range. Differences during infection from baseline were analyzed using Friedman's test and if significant, Wilcoxon's tests were used. Differences between the groups were analyzed using Mann–Whitney's tests. Correlations were examined using Spearman's rank correlation. In mouse experiments, animals were studied in groups of 4–6 and data is presented as mean ± s.e.m, representative of at least two independent experiments. Differences between groups were assessed via analysis of variance and if significant ($P<0.05$), individual differences were identified using Bonferroni's post-tests. Statistical analyses were performed with Graphpad Prism 4.2 software (Graphpad, La jolla, CA).

Ethical approval. The human infection study was approved by St Mary's National Health Service Trust Research Ethics committee. All subjects gave written informed consent. All animal studies were conducted according to the UK home office legislation.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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