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Different Potentials of $\gamma\delta$ T Cell Subsets in Regulating Airway Responsiveness: Vγ1$^+$ Cells, but Not Vγ4$^+$ Cells, Promote Airway Hyperreactivity, Th2 Cytokines, and Airway Inflammation[1]

Youn-Soo Hahn,*‡ Christian Taube,† Niyun Jin,* Laura Sharp,* J. M. Wands,* M. Kemal Aydintug,* Michael Lahn,* Sally A. Huber,§ Rebecca L. O'Brien,* Erwin W. Gelfand,† and Willi K. Born*[2]

Allergic airway inflammation and hyperreactivity are modulated by $\gamma\delta$ T cells, but different experimental parameters can influence the effects observed. For example, in sensitized C57BL/6 and BALB/c mice, transient depletion of all TCR-δ^+ cells just before airway challenge resulted in airway hyperresponsiveness (AHR), but caused hyporesponsiveness when initiated before i.p. sensitization. Vγ4$^+$ $\gamma\delta$ T cells strongly suppressed AHR; their depletion relieved suppression when initiated before challenge, but not before sensitization, and they suppressed AHR when transferred before challenge into sensitized TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice. In contrast, Vγ1$^+$ $\gamma\delta$ T cells enhanced AHR and airway inflammation. In normal mice (C57BL/6 and BALB/c), enhancement of AHR was abrogated only when these cells were depleted before sensitization, but not before challenge, and with regard to airway inflammation, this effect was limited to C57BL/6 mice. However, Vγ1$^+$ $\gamma\delta$ T cells enhanced AHR when transferred before challenge into sensitized *B6*.TCR-$\delta^{-/-}$ mice. In this study Vγ1$^+$ cells also increased levels of Th2 cytokines in the airways and, to a lesser extent, lung eosinophil numbers. Thus, Vγ4$^+$ cells suppress AHR, and Vγ1$^+$ cells enhance AHR and airway inflammation under defined experimental conditions. These findings show how $\gamma\delta$ T cells can be both inhibitors and enhancers of AHR and airway inflammation, and they provide further support for the hypothesis that TCR expression and function cosegregate in $\gamma\delta$ T cells. ***The Journal of Immunology*, 2004, 172: 2894–2902.**

The mechanisms leading to airway hyperresponsiveness (AHR)[3] and inflammation, pathological conditions associated with asthma and other diseases involving the airways, are not yet fully understood. Even when considering only T lymphocytes, different populations have diverse effects that are often in opposition. In a primary allergic airway response, $\alpha\beta$ T cells are essential for the development of allergen-specific IgE Abs, eosinophilic inflammation, goblet cell hyperplasia, and airway hyper-reactivity to cholinergic agonists (1). It appears that both CD4$^+$ and CD8$^+$ $\alpha\beta$ T cells can mediate airway inflammation and AHR (2). However, allergen-specific $\alpha\beta$ T cells have also been implicated in the regulation of allergic airway inflammation and AHR. CD4$^+$CD25$^+$ $\alpha\beta$ T cells can diminish the allergic airway response (3), but cells of the same phenotype may also be able to promote it (4). Nonallergen-specific NK T cells expressing an invariant $\alpha\beta$ TCR can also be regulatory and may have different effects from those of non-T NK cells (5).

More recently, involvement of $\gamma\delta$ T cells in the regulation of airway inflammation and AHR has been demonstrated. Although available data do not clearly support the idea of allergen-specific $\gamma\delta$ T cells, surprisingly strong effects on eosinophilic inflammation, IgE, and AHR were documented (6–8). Exactly which signals trigger the responses of $\gamma\delta$ T cells and their regulatory effects remains unresolved, although signaling through both the T cell and cytokine receptors may be essential (9, 10).

The regulatory influence of $\gamma\delta$ T cells on the allergic response in the airways appears to be complex. According to differing reports, $\gamma\delta$ T cells promoted allergen-specific IgE responses, eosinophilic airway inflammation, and AHR (7, 8, 11, 12); suppressed the development of specific IgE Abs and AHR (6, 8); or had no effect (13). One possible explanation for some of these discrepancies is that subsets of $\gamma\delta$ T cells affect AHR and airway inflammation differently. Indeed, $\gamma\delta$ T cells fall into discrete subpopulations based on their development and tissue distribution (14, 15). These subpopulations also differ in their expression of TCRs. The observation that TCR-Vγ expression alone can be sufficient to define functionally distinct $\gamma\delta$ T cell subsets (16) led us to propose that in $\gamma\delta$ T cells, TCR expression and function cosegregate (cosegregation hypothesis) (17). With this concept in mind, we examined the role of TCR-Vγ-defined subsets of $\gamma\delta$ T cells in allergic airway inflammation and AHR. Recently, we reported that Vγ4$^+$ $\gamma\delta$ T cells suppress AHR (9, 18). Vγ4$^+$ $\gamma\delta$ T cells are present in several tissues, but also form a resident population in the

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[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; BALF, bronchoalveolar fluid; C_{dyn}, dynamic compliance; MBP, major basic protein; MCh, methacholine; R_L, lung resistance.

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lung (9, 19). Their regulatory effects appear to be local, independent of $\alpha\beta$ T cells and Abs, and largely bypass the inflammatory response (9, 18). In contrast, we show in this study that $\gamma\delta$ T cells defined by the expression of Vγ1 (20) can enhance AHR as well as levels of Th2 cytokines in the airways and eosinophilic infiltrates in the lung. The regulatory effects of V$\gamma1^+$ cells manifest themselves at an earlier stage in the progressive host response, and some are only evident against a defined, genetically $\gamma\delta$ T cell-deficient background (21, 22).

Materials and Methods

Animals

Female C57BL/6, C57BL/10, B6.TCR-$\delta^{-/-}$, B6.TCR-$\beta^{-/-}$, and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B10.TCR-V$\gamma4^{-/-}/6^{-/-}$ (10th backcross generation) were produced in our own laboratory by backcrossing TCR-V$\gamma4^{-/-}/6^{-/-}$ mice (a gift from Dr. K. Ikuta, Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Kyoto, Japan) onto the C57BL/10 genetic background. All mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the institutional animal care and use committee of National Jewish Medical and Research Center. All mice were 8- to 10-wk-old at the time of the experiments.

Sensitization and airway challenge

Groups of mice were sensitized by i.p. injection of 20 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO) emulsified in 2.25 mg of aluminum hydroxide (AlumImuject; Pierce, Rockford, IL) in a total volume of 100 μl on days 0 and 14. Mice were challenged via the airways with OVA (1% in saline) for 20 min on days 28, 29, and 30 by ultrasonic nebulization (particle size, 1–5 μm; De Vilbiss, Somerset, PA). Lung resistance (R_L) and dynamic compliance (C_{dyn}) were assessed 48 h after the last allergen challenge, and the mice were sacrificed to obtain tissues and cells for further assay.

Administration of anti-TCR mAbs

Hamster anti-TCR δ mAbs GL3 (23) and 403.A10 (24), anti-Vγ4 mAb UC3 (25), and anti-Vγ1 mAb 2.11 (20) were purified from hybridoma culture supernatants using a protein G-Sepharose affinity column (Pharmacia Biotech, Uppsala, Sweden). T cell depletion was achieved after injection of 200 μg of hamster anti-TCR-δ mAb (a 1/1 mixture of GL3 and 403.A10) or anti-Vγ4 or -Vγ1 mAbs into the tail veins of mice 3 days before the first and 3 days before the second OVA sensitization. Depletion was monitored as previously described (8, 26). Sham Ab treatments were performed with the same amount of nonspecific hamster IgG (The Jackson Laboratory). The treatments with anti-TCR-δ and anti-Vγ mAbs did not significantly change $\alpha\beta$ T cell numbers in lung and spleen (18).

Note that throughout this paper we use the nomenclature for murine Vγ genes introduced by Tonegawa and colleagues (27).

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after provocation with aerosolized methacholine (MCh) using a method described by Takeda and colleagues (28). MCh aerosol was administered for 10 s (60 breaths/min, 500-μl tidal volume) in increasing concentrations. Maximum values of R_L and minimum values of C_{dyn} were taken and expressed as the percent change from baseline after saline aerosol.

Bronchoalveolar lavage

Immediately after assessment of airway responsiveness, lungs were lavaged via the intratracheal tube with HBSS (1 ml), and total leukocyte numbers were measured with a Coulter counter (Coulter, Hialeah, FL). Differential cell counts were performed on at least 200 cells on cytocentrifuged preparations (Cytospin 2; Shandon, Runcorn, U.K.), stained with Leukostat (Fisher Diagnostics, Fair Lawn, NJ), and differentiated by standard hematologic procedures.

Histochemistry

Lungs were fixed by inflation (1 ml) and immersion in 10% formalin. Cells containing eosinophilic major basic protein (MBP) were identified using rabbit anti-mouse MBP (provided by Dr. J. J. Lee, Mayo Clinic, Scottsdale, AZ) by immunohistochemical staining as previously described (29). The slides were examined in a blinded fashion with a microscope (Nikon, Melville, NY) equipped with a fluorescein filter system. The numbers of eosinophils in the peribronchial tissues were evaluated using IPLab2 software (Signal Analytics, Vienna, VA) for the Macintosh, counting six to eight different fields per animal.

Cell purification and adoptive transfer of V$\gamma1^+$ or V$\gamma4^+$ T lymphocytes

V$\gamma1^+$ or V$\gamma4^+$ cells were purified 2 wk after the second injection of OVA from the spleens of B6.TCR-$\beta^{-/-}$ mice. Briefly, a suspension of splenocytes was prepared by pushing the splenic tissue through a 70-μm pore size mesh (Falcon). Suspended cells were treated with Gey's RBC lysis solution and passed through nylon wool columns to obtain T lymphocyte-enriched cell preparations containing >75% T cells as previously described (30, 31). Total cell counts were determined using a Coulter counter. Nylon wool nonadherent cells (5×10^5) in PBS/5% FBS were incubated with biotinylated anti-Vγ1 mAb 2.11 or anti-Vγ4 mAb UC3 (15 min, 4°C), then washed and incubated with streptavidin-conjugated magnetic beads (streptavidin microbeads; Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at 4°C and passed twice through magnetic columns to purify V$\gamma1^+$ or V$\gamma4^+$ cells. This produced a cell population containing >95% V$\gamma1^+$ or V$\gamma4^+$ viable cells as determined by two-color staining with anti-TCR-δ and anti-Vγ1 or Vγ4 mAbs. These splenic V$\gamma1^+$ or V$\gamma4^+$ cells were washed in PBS and resuspended to 2×10^5 cells/ml PBS, and 1×10^4 cells/mouse was injected in 100 μl of PBS via the tail vein into OVA-sensitized B6.TCR-$\delta^{-/-}$ mice within 1 h before the first airway challenge. For adoptive cell transfers in B10.TCR-V$\gamma4^{-/-}/6^{-/-}$ mice, V$\gamma1^+$ or V$\gamma4^+$ cells were purified 2 days after the last challenge from the lungs of OVA-sensitized and challenged C57BL/10 mice. Briefly, lungs were dissected into small pieces and exposed to an enzymatic digestion mixture containing 0.125% dispase II (Roche, Indianapolis, IN), 0.2% collagenase II (Sigma-Aldrich), and 0.2% collagenase IV (Sigma-Aldrich) for 75 min. After lung digestion, purified V$\gamma1^+$ or V$\gamma4^+$ cells were obtained by nylon wool enrichment and positive selection using magnetic beads as described above, with similar results. Purified cells were adoptively transferred to OVA-sensitized B10.TCR-V$\gamma4/6^{-/-}$ mice within 1 h before the first airway challenge.

Measurement of cytokines in bronchoalveolar fluid (BALF)

IL-5, IL-10, and IL-13 in BALF were detected by ELISA. For IL-5 and IL-10, the OptEIA set was used according to the manufacturer's directions (BD PharMingen, San Diego, CA). For IL-13, a commercial kit was used (R&D Systems, Minneapolis, MN). Cytokine levels were determined by comparison with known standards. The limits of detection were 30 pg/ml for IL-10 and 10 pg/ml for the other two cytokines.

Flow cytometric analysis

For flow cytometric analyses, anti-Vγ4 or anti-Vγ1 mAbs were conjugated with *N*-hydroxysuccinimido-biotin (Sigma-Aldrich), and anti-TCR δ mAbs GL3 were conjugated with FITC-isomer I on Celite (Sigma-Aldrich). Nylon-wool nonadherent cells (2×10^5/well) in 96-well plates (Falcon; BD Biosciences, Franklin Lakes, NJ) were stained by two-color techniques and analyzed on a FACScan flow cytometer (BD Biosciences) counting a minimum of 25,000 events/gated region.

Statistical analysis

Data are presented as the mean ± SEM. The Mann-Whitney test was used for analysis of the effects of mAb treatment on AHR, and ANOVA was used for analysis of differences in cytokine levels. Pairwise comparisons were performed using the Tukey-Kramer honest significant difference test. Statistical significant levels were set at a value of $p < 0.05$.

Results

Depletion of all TCR-δ^+ or only V$\gamma1^+$ $\gamma\delta$ T cells during Ag sensitization decreases AHR in normal mice

We have previously shown that in OVA-sensitized and challenged mice, depletion of all TCR-δ^+ cells, by i.v. injection of anti-TCR mAbs after sensitization, but 3 days before the airway challenge, increased AHR (8). Depletion of V$\gamma4^+$ cells had similar effects as depletion of all TCR-δ^+ cells, whereas depletion of V$\gamma1^+$ cells had no effect (18). Moreover, adoptively transferred $\gamma\delta$ T cells that contained V$\gamma4^+$ cells suppressed AHR, whereas transferred $\gamma\delta$ T cells depleted of V$\gamma4^+$ cells did not (18). Despite the decrease in AHR, no effect on eosinophilic airway inflammation was detected.

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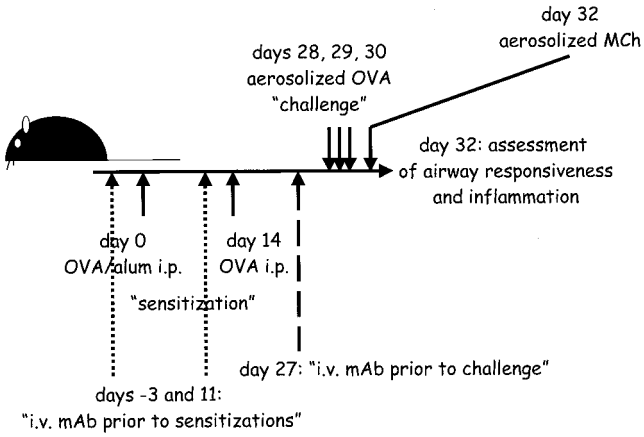

FIGURE 1. Schematic of experimental procedures.

These studies indicated that at least after airway challenge of sensitized mice, $V\gamma4^+$ γδ T cells function to reduce the development of AHR by a mechanism independent of eosinophilic airway inflammation.

In the current study we examined mice sensitized and challenged with OVA and treated with mAbs to deplete all γδ T cells or certain subsets, but altered the time point of Ab treatment. In this study we injected the depleting Abs twice, each 3 days before one of two sensitizing i.p. injections of OVA (Fig. 1). At 3 days after the first mAb injection, splenic expression of the targeted γδ TCRs was substantially reduced (Fig. 2). A reduction in TCR expression levels was still discernible 20 days after the second of two mAb injections, in the spleen and even in the small γδ T cell populations of the lung.

Interestingly, the effect of treatment with anti-TCR-δ mAbs before sensitization was entirely different. AHR was reduced instead of increased when the mice were treated during the sensitization phase (Fig. 3). The effects of treatment during sensitization were similar in C57BL/6 and BALB/c mice. In marked contrast to our previous findings implicating γδ T cells as negative regulators (8, 9, 18), this suggested that at least during the sensitization phase γδ T cells can also function as positive regulators of AHR. To test whether the same or different cells were involved in the two functions, we injected subset-specific mAbs (specific for Vγ1 and Vγ4 instead of TCR-δ) using the same experimental protocol (Fig. 3). Anti-Vγ1 mAb decreased AHR to the same extent as did the anti-TCR-δ mAbs; anti-Vγ4 mAb had no effect. This suggested that the AHR-enhancing effect of γδ T cells during the sensitization phase could be attributed to $V\gamma1^+$ cells. In contrast, at this stage of the response, $V\gamma4^+$ cells had no particular effect (neither inhibitory nor enhancing).

We also examined cytokine levels and cellular accumulation in BALF in Ab-treated mice. In C57BL/6 mice treated with anti-TCR-δ or anti-Vγ1 mAbs during the sensitization phase, IL-13 levels were reduced by ~50%, whereas IL-10 levels were increased (Fig. 4*A*). In BALB/c mice treated in the same way, no reduction in IL-13 levels or increase in IL-10 levels was seen (Fig. 4*C*). However, BALB/c controls (non-Ab treated) showed IL-13 at lower and IL-10 at higher levels in BALF by comparison with C57BL/6 controls. By these criteria, γδ T cells, and $V\gamma1^+$ cells in particular, appear to facilitate Th2 activity in C57BL/6 mice. However, as they did not change Th2 activity in BALB/c mice, this effect may be nonessential to their ability to enhance AHR.

Finally, we examined airway-infiltrating cells in mice treated with Abs during the sensitization phase. C57BL/6 controls (non-Ab treated) showed higher total cell numbers and eosinophils in BALF than did BALB/c controls, confirming previous results. In both strains, treatment with anti-TCR-δ or anti-Vγ1 mAbs had only small effects, if any, on numbers of cells in BALF (Fig. 4, *B* and *D*). Small reductions of eosinophils in anti-Vγ1 mAb-treated

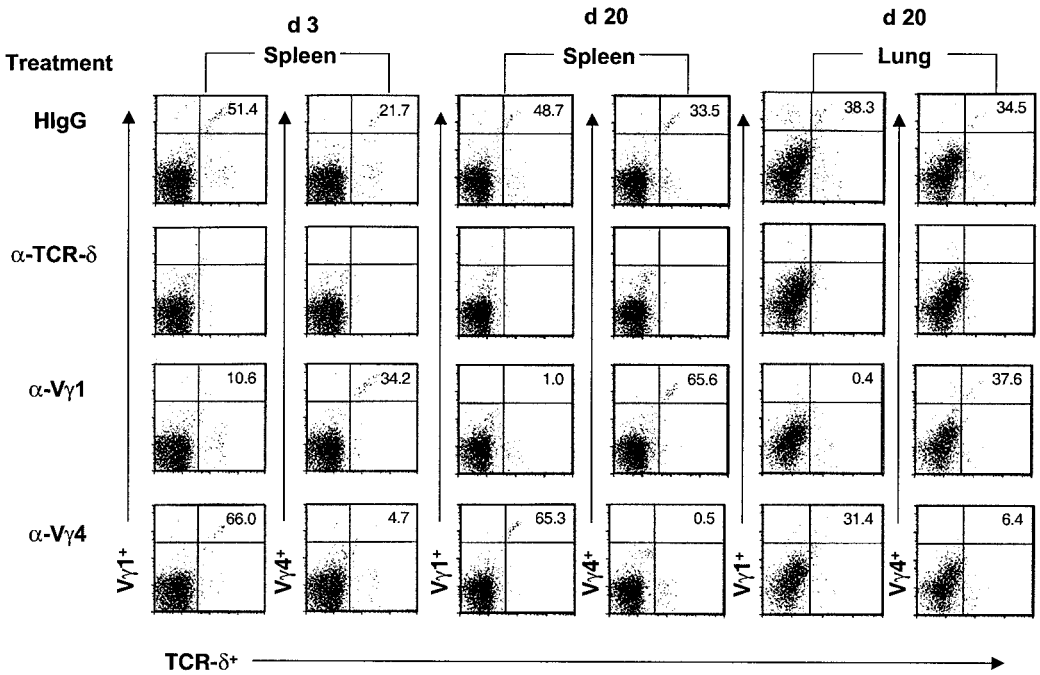

FIGURE 2. Effects of mAb treatments on γδ T cells in spleen and lung. Normal C56BL/6 mice were injected with nonspecific hamster IgG (HIgG), anti-TCR-δ, anti-TCR Vγ1, or anti-TCR Vγ4 mAbs on days −3 and 11 (relative to the regular sensitization and challenge protocol shown in Fig. 1). Flow cytometric analyses of nylon wool-nonadherent splenic or pulmonary $\gamma\delta^+$ T cells were performed 3 days after the first and 20 days after the second injection. A representative experiment is shown. Numbers in the *upper right quadrants* indicate percent frequencies of the respective γδ T cell subsets in relation to total γδ T cells.

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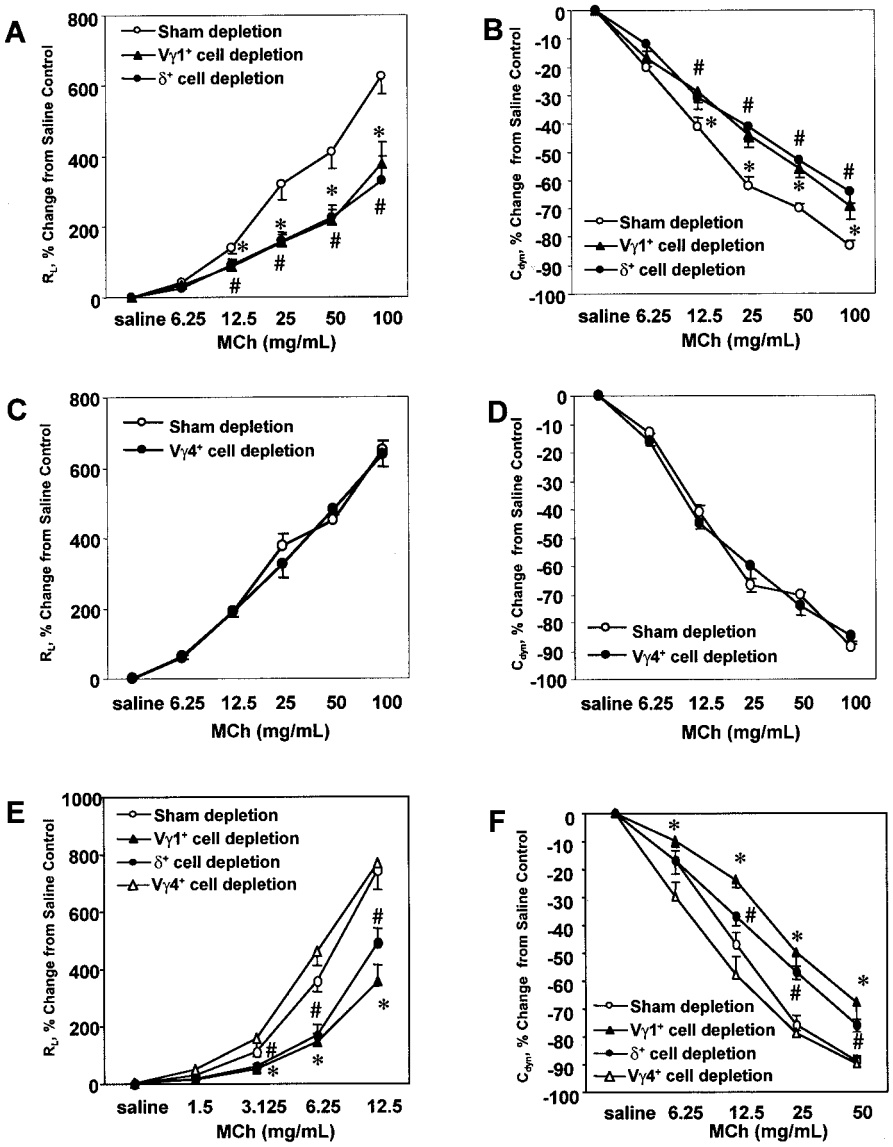

FIGURE 3. Effect of anti-TCR mAbs injected at the time of sensitization on AHR in OVA-sensitized and challenged mice. Airway responses to MCh (*A*, *C*, and *E*,: R_L; *B*, *D*, and *F*: C_{dyn}) were measured 48 h after the last OVA challenge in C57BL/6 (*A–D*) and BALB/C (*E* and *F*) mice and are shown as the percent change from controls that received saline. Mice were sensitized and challenged with OVA (2ip3n protocol). OVA-sensitized and challenged mice were treated twice more with anti-TCR-δ mAb, anti-TCR Vγ1 mAb, anti-TCR Vγ4 mAb, or nonspecific hamster IgG (sham depletion) by i.v. injection 3 days before each OVA/alum injection. Results for each group are expressed as the mean ± SEM ($n = 9$ in *A*, *B*, *E*, and *F*; $n = 6$ in *C* and *D*). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences ($p < 0.05$) are indicated (#, between HIgG- and anti-TCR-δ mAb-treated groups; *, between HIgG- and anti-TCR Vγ1 mAb-treated groups).

C57BL/6 mice (but not in BALB/c mice) and of macrophages in anti Vγ1 mAb-treated BALB/c mice (but not in C57BL/6 mice) were noted. However, as with the cytokines, the effects of the Ab treatment on airway-infiltrating cells varied despite concordant reductions in AHR.

Adoptively transferred Vγ1$^+$, but not Vγ4$^+$, cells increase AHR and airway inflammation in TCR-δ$^{-/-}$ mice (deficient in all γδ T cells)

Eight- to 10-wk-old TCR-δ$^{-/-}$ mice ("young," C57BL/6 genetic background; B6.TCR-δ$^{-/-}$), sensitized and challenged with OVA, exhibited decreased AHR compared with wild-type (C57BL/6) controls (Fig. 5, *A* and *B*). In these B6.TCR-δ$^{-/-}$ mice, IL-13 and IL-5 levels in BALF were also substantially decreased, whereas IL-10 levels were increased (Fig. 5*C*). Moreover, total cells and eosinophils in BALF were significantly reduced (Fig. 5D).

Because the mAb treatments had implicated Vγ1$^+$ cells as positive regulators of AHR, we reconstituted OVA-sensitized and challenged young B6.TCR-δ$^{-/-}$ mice with Vγ1$^+$ cells (10^4 purified cells i.v., derived from the spleen of OVA-sensitized B6.TCR-β$^{-/-}$ donors and positively selected on magnetic beads) or with Vγ4$^+$ cells from the same source as a control (Fig. 6). Vγ4$^+$ cells had no effect on AHR (Fig. 6, *A* and *B*). In contrast, Vγ1$^+$ cells restored AHR to levels matching those of wild-type C57BL/6 mice (Fig. 6, *A* and *B*; compare with Fig. 3, *A* and *B*, and Fig. 5, *A* and *B*). The Vγ1$^+$ cells also substantially increased BALF levels of IL-13 and IL-5 and decreased levels of IL-10 (Fig. 6*C*). Vγ4$^+$ cells, in contrast, did not alter IL-13/IL-5 levels, but reduced IL-10 levels to some extent (Fig. 6*C*). Finally, Vγ1$^+$ cells supported a small, but significant, increase in eosinophils in both airways (BALF; Fig. 6*D*) and lung parenchyma (Fig. 7), whereas Vγ4$^+$ cells did not.

Adoptively transferred Vγ4$^+$, but not Vγ1$^+$, cells decrease AHR in TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice (deficient in Vγ4$^+$ and Vγ6$^+$ γδ T cells only)

Adult sensitized and challenged C57BL/10 mice genetically deficient in both Vγ4$^+$ and Vγ6$^+$ γδ T cells (B10.TCR-Vγ4$^{-/-}$/6$^{-/-}$) showed normal or slightly increased AHR by comparison with wild-type mice (Fig. 8, *A* and *B*). Into these sensitized mice we transferred Vγ4$^+$ or Vγ1$^+$ cells purified from the lungs of adult OVA-sensitized and challenged wild-type C57BL/10 donors (10^4 cells i.v., positively selected on magnetic beads) just before the first of three challenges. The transferred Vγ4$^+$ cells decreased

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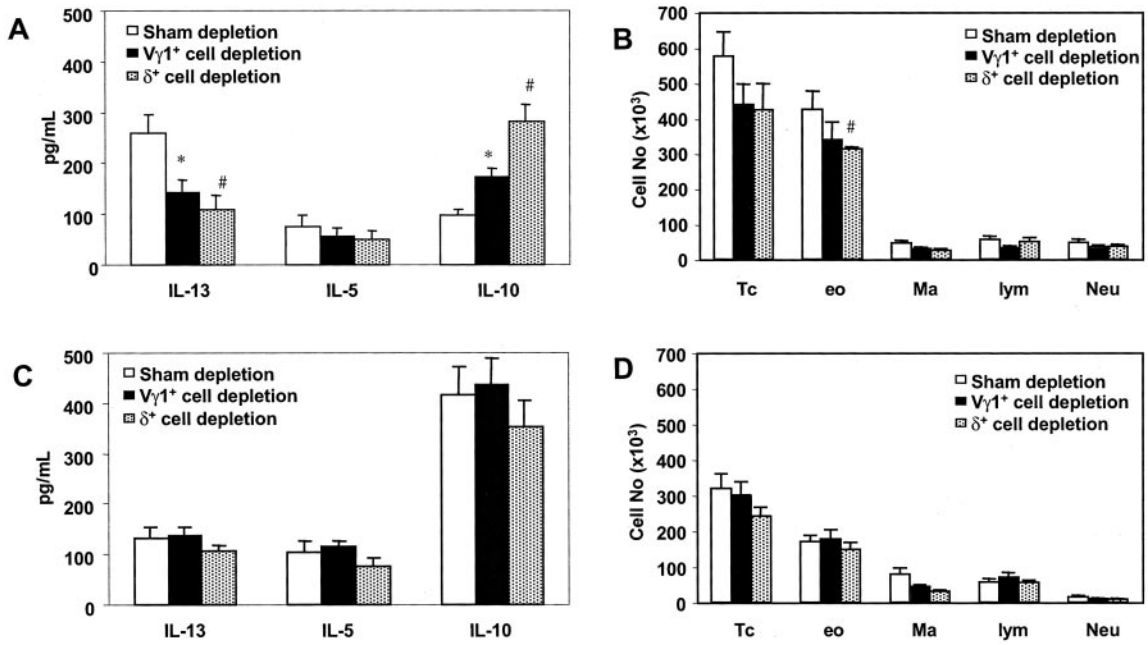

FIGURE 4. Effect of anti-TCR mAbs injected at the time of sensitization on airway-infiltrating cells and cytokines in OVA-sensitized and challenged mice. Cytokines (*A* and *C*) and infiltrating cells (*B* and *D*) in BALF, 48 h after the last OVA challenge, are shown. C57BL/6 (*A* and *B*) and BALB/C mice (*C* and *D*) were treated as described in Fig. 3. TC, total cells; Eo, eosinophils; Ma, macrophages; Lym, lymphocytes, Neu: neutrophils. Results for each group are expressed as the mean ± SEM ($n = 9$ in each group). Significant differences ($p < 0.05$) are indicated (#, between HIgG-treated and anti-TCR-δ mAb-treated groups; ∗, between HIgG-treated and anti-TCR Vγ1 mAb-treated groups).

AHR, whereas $V\gamma1^+$ cells had no effect (Fig. 8, *C* and *D*). This result with positively selected cells confirmed that $V\gamma4^+$ cells, but not $V\gamma1^+$ cells, are sufficient to suppress AHR, and it complemented our previous study comparing γδ T cells depleted or not depleted of $V\gamma4^+$ cells that led us to conclude that $V\gamma4^+$ cells are necessary for suppression.

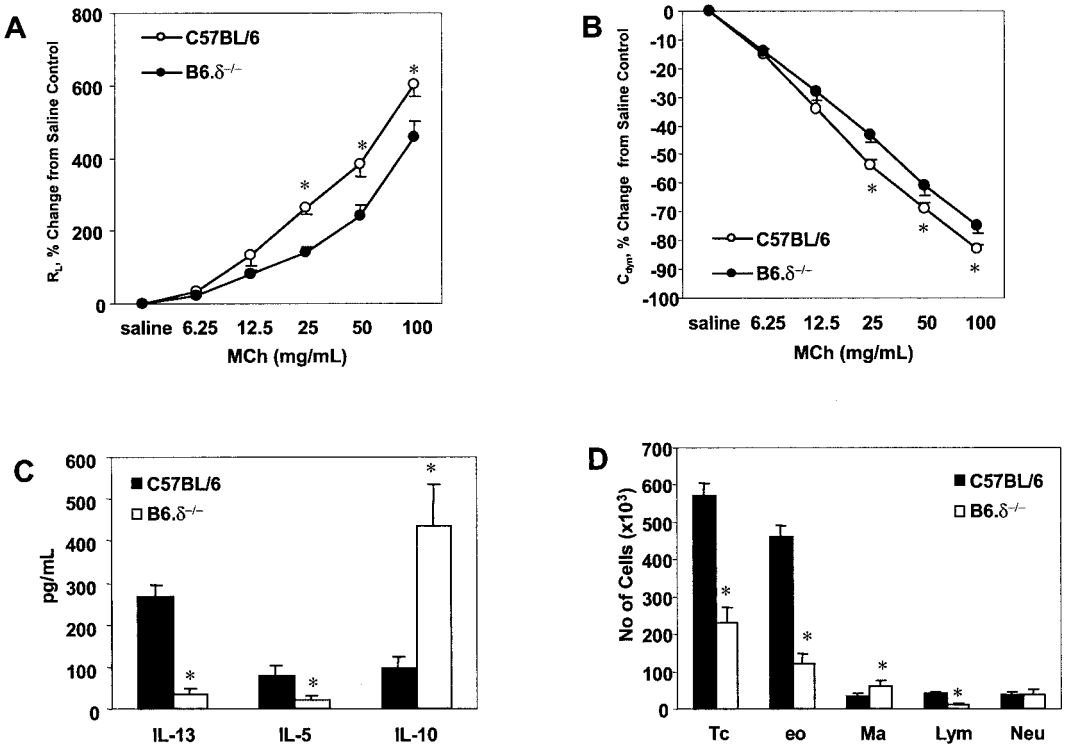

FIGURE 5. Diminished AHR and airway inflammation in OVA-sensitized and challenged young B6.TCR-$\delta^{-/-}$ mice. Airway responses to MCh (*A*, R_L; *B*, C_{dyn}) were measured 48 h after the last OVA challenge in 2ip3n-treated C57BL/6 and B6.TCR-$\delta^{-/-}$ mice and are shown as the percent change from controls that received saline. Cytokine assays (C) and counts of airway-infiltrating cells (*D*) were performed as described in Fig. 4. Results for each group are expressed as the mean ± SEM ($n = 8$ in each group). No significant differences in baseline responses to saline were observed in any of these groups. ∗, Significant difference ($p < 0.05$) between C57BL/6 and B6.TCR-$\delta^{-/-}$ mice.

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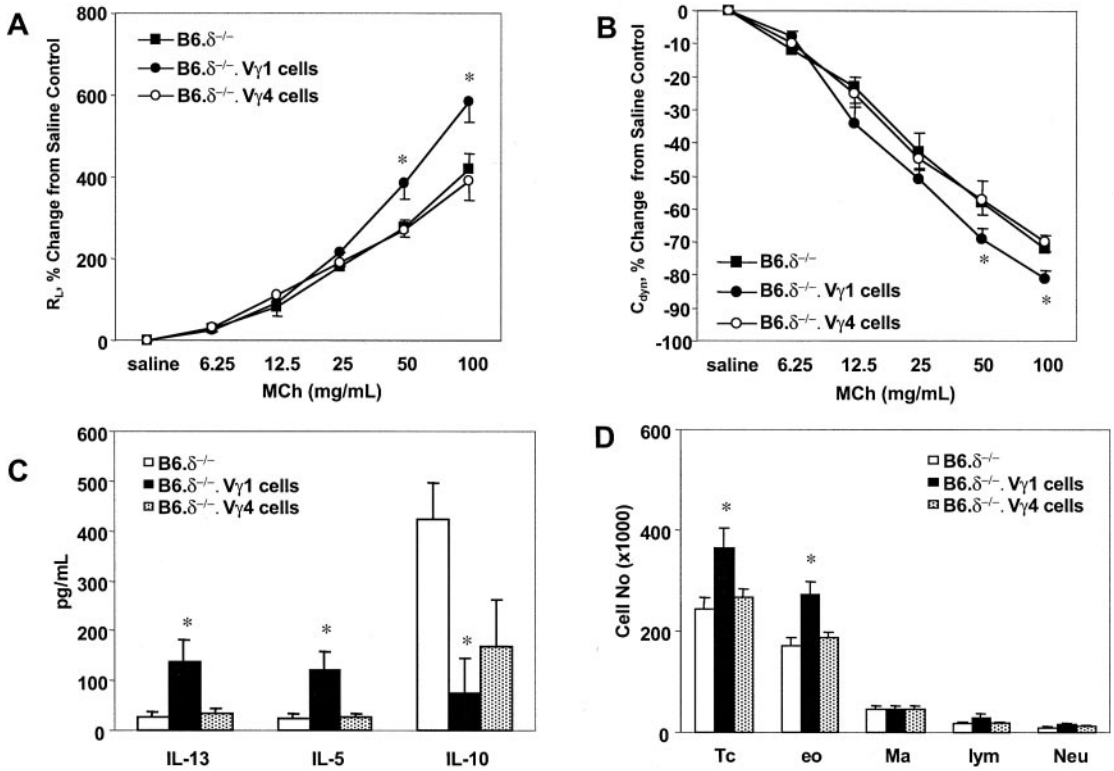

FIGURE 6. Increased AHR in OVA-sensitized and challenged B6.TCR-$\delta^{-/-}$ mice after adoptive transfer of V$\gamma1^+$ cells. Airway responsiveness (*A*, R_L; *B*, C_{dyn}) was measured in 2ip3n-treated B6.TCR-$\delta^{-/-}$ mice that were untreated or received purified splenic V$\gamma1^+$ or V$\gamma4^+$ cells just before the first OVA challenge. Donor cells were derived from OVA-sensitized (2ip) B6.TCR-$\beta^{-/-}$ mice. Airway responses are expressed as the percent change from saline controls in relation to increasing concentrations of MCh. Cytokine assays (*C*) and counts of airway infiltrating cells (*D*) were performed as described in Fig. 4. Results for each group are expressed as the mean ± SEM ($n = 4$ in each group). *, Significant differences ($p < 0.05$) between B6.TCR-$\delta^{-/-}$ mice and B6.TCR-$\delta^{-/-}$ mice reconstituted with V$\gamma1^+$ cells.

Discussion

Although recent studies clearly indicated an involvement of $\gamma\delta$ T cells in airway inflammation and AHR (6–8, 11, 12), the nature of this involvement has remained unclear due to an array of contradictory observations. Some of these contradictions may be explained by the findings of this study, which show that both the

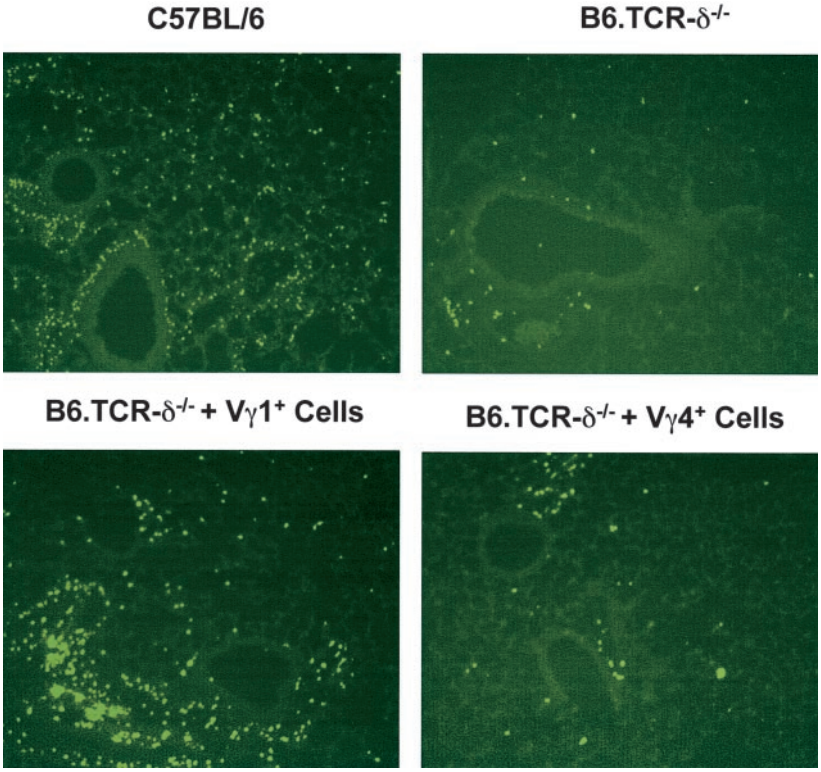

FIGURE 7. Increase in MBP$^+$ eosinophils in the lung of OVA-sensitized and challenged young B6.TCR-$\delta^{-/-}$ mice after adoptive transfer of V$\gamma1^+$ cells. Lung tissues from 2ip3n-treated C57BL/6 and B6.TCR-$\delta^{-/-}$ mice were stained with anti-MBP mAb. B6.TCR-$\delta^{-/-}$ mice that were untreated or reconstituted with splenic V$\gamma1^+$ or V$\gamma4^+$ cells (as described in Fig. 6) are compared.

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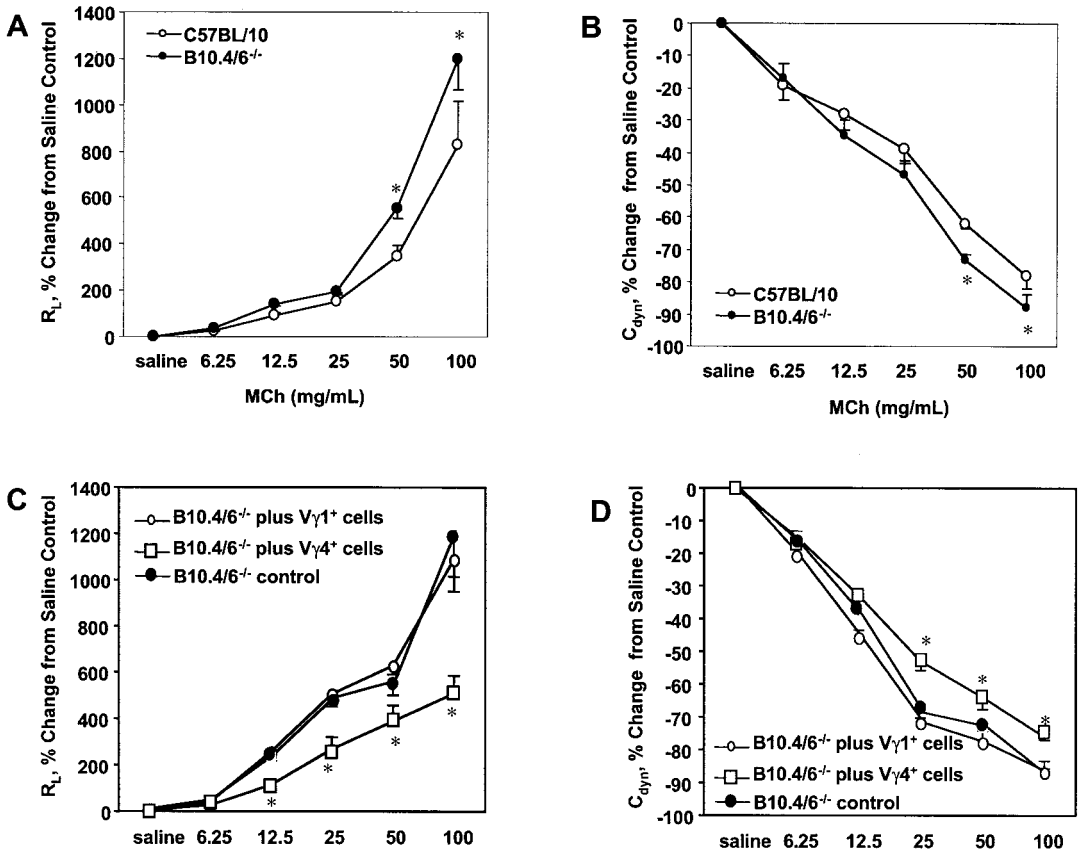

FIGURE 8. Decrease in AHR in OVA-sensitized and challenged B10.TCR-V$\gamma4^{-/-}/6^{-/-}$ mice after adoptive transfer of V$\gamma4^+$ cells. Airway responsiveness (R_L and C_{dyn}) was measured in 2ip3n-treated C57BL/10 and B10.TCR-V$\gamma4/6^{-/-}$ mice (*A* and *B*). Airway responsiveness was also measured in 2ip3n-treated B10.TCR-V$\gamma4/6^{-/-}$ mice reconstituted with adoptively transferred V$\gamma1^+$ or V$\gamma4^+$ cells, purified from the lungs of 2ip3n-treated C57BL/10 mice. Airway responses are expressed as the percent change from saline controls in relation to increasing concentrations of MCh. Results for each group are expressed as the mean ± SEM ($n = 4$ in each group). *, Significant differences ($p < 0.05$) between the congenic mice (*A* and *B*) and recipients of V$\gamma1^+$ or V$\gamma4^+$ cells (*C* and *D*).

timing of experimental intervention and a focus on functionally distinguishable γδ T cell subsets can be critical in dissecting the effects of these cells on airway inflammation and AHR. In addition, immune competence of the host can determine whether a particular γδ T cell function is detected.

We have previously reported that treatment with Abs specific for TCR-δ that transiently deplete all γδ T cells can increase AHR (8), suggesting that γδ T cell depletion relieves negative regulation of AHR. However, the experiments in this study show that the timing of the Ab treatment is critical in demonstrating this effect. The same anti-TCR-δ mAbs that increased AHR when injected before airway challenge decreased AHR when injected during the sensitization phase. If one assumes that the anti-TCR-δ mAbs deplete or at least functionally inactivate the targeted T cells, a mechanism supported by cytofluorometric analysis of the targeted cells (Fig. 2) (16, 26, 32) and by the inverse effect of adoptive cell transfers (16, 18) (see below), these results imply that total γδ T cells not only can inhibit, but also can enhance, AHR, and that the net effect observed depends on the timing of their depletion. Moreover, their inhibitory effect predominates during challenge, as the later, but not the earlier, Ab treatments reveal it, whereas the enhancing effect may be more spread out, although dominant during sensitization (see also below).

What might be the basis for the different regulatory influences of γδ T cells in the course of an allergic response? In principle, the same cells may have different effects depending on external circumstances or their own functional maturation, or different cell populations may become involved sequentially and then exert different effects. Our data support the latter alternative. Not only did depletion treatments with two subset-specific mAbs have different effects on AHR, but again the timing of these treatments was critical as well. Anti-Vγ1 mAbs were as effective as anti-TCR-δ mAbs in reducing AHR as long as they were injected during the sensitization phase. The same mAbs had no effect when injected into sensitized mice before the airway challenges. Conversely, anti-Vγ4 mAbs had no effect during the sensitization phase, but increased AHR to the same degree as anti-TCR-δ mAbs when injected before the challenges. Therefore, the opposite effects of early- and late-injected anti TCR-δ mAbs seem to reflect an involvement of AHR-enhancing V$\gamma1^+$ γδ T cells and AHR-inhibiting V$\gamma4^+$ γδ T cells, respectively. This apparent functional difference between the two subsets was confirmed in transfer experiments with purified γδ T cells (see below). V$\gamma1^+$ cells are the larger subset in the spleen (33), a circumstance that might explain their predominant effect during sensitization. V$\gamma4^+$ cells are the largest subset in the adult lung; unlike other subsets, they further increase in the lung during airway challenges, and local treatment with aerosolized Abs abrogates their inhibitory effect (9, 18). Taken together, these findings may explain their functional dominance during the challenge, but not the sensitization, phase.

Opposite effects on AHR and different timing of their functional engagement or activation are not the only differences demonstrated between V$\gamma1^+$ and V$\gamma4^+$ γδ T cells. In recent studies we have shown that the inhibitory V$\gamma4^+$ γδ T cells also regulate AHR

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independently of $\alpha\beta$ T cells (9), and that their regulatory effect largely bypasses the inflammatory response, insofar as effects on eosinophilic infiltration and goblet cell differentiation are concerned (18). The current study reveals that, unlike Vγ4$^+$ $\gamma\delta$ T cells, Vγ1$^+$ $\gamma\delta$ T cells have substantial effects on cytokine levels and on eosinophilic infiltration in the airways. This difference was most prominent using adoptive transfer of small numbers of purified cells into sensitized young B6.TCR-$\delta^{-/-}$ recipients. In this study transferred Vγ1$^+$ cells increased not only challenge-induced AHR, but also IL-13 and IL-5 in BALF. Small, but significant, increases in BALF and parenchymal eosinophil infiltrations were also detected. These findings are consistent with earlier studies showing proinflammatory effects of $\gamma\delta$ T cells (7, 11, 12) and also with a previously reported bias of Vγ1$^+$ $\gamma\delta$ T cells for IL-4 production (34). In contrast, transferred Vγ4$^+$ cells had none of these effects. Most likely, the effects of the transferred cells were so prominent due to the very weak allergic response of the young B6.TCR-$\delta^{-/-}$ mice to OVA sensitization and challenge (see discussion below), as shown by reduced AHR, and the very low levels of IL-13, IL-5, and airway eosinophils by comparison with wild-type C57BL/6 mice. In the Ab-treated, wild-type mice, the predicted inverse effects were detectable, but were much smaller. However, our study does not resolve whether the enhancing effect of Vγ1$^+$ cells on IL-13 and IL-5 levels is critical for their AHR-enhancing role. In BALB/c mice, depletion of Vγ1$^+$ cells did not affect these cytokines even though AHR was reduced.

Transferred Vγ1$^+$ cells decreased IL-10 in BALF of sensitized and challenged TCR-$\delta^{-/-}$ mice, but transferred Vγ4$^+$ cells had a similar effect. Consistently, depletion of all TCR-δ^+ cells in wild-type C57BL/6 mice increased BALF IL-10 more than did depletion of Vγ1$^+$ cells alone, but there was no obvious connection between the inhibitory effect of either subset on IL-10 and the differential effects of the two subsets on AHR.

Although we and others have previously noted that TCR-$\delta^{-/-}$ mice develop a reduced eosinophilic response to OVA sensitization and challenge by comparison with wild-type mice (7, 8), their reduced AHR was unexpected in light of our earlier finding that these mice exhibit increased AHR (8). However, the TCR-$\delta^{-/-}$ mice used in the current study were younger, and we have recently found that young and old B6.TCR-$\delta^{-/-}$ mice differ far more in airway responsiveness than do their wild-type counterparts (L. Sharp and C. Taube, unpublished observations). We are presently investigating whether age-related differences in the dependence of the allergic response on the regulatory effects of $\gamma\delta$ T cells (both enhancing and inhibitory) can explain this change.

Differences between genetically $\gamma\delta$ T cell-deficient (TCR-$\delta^{-/-}$) mice and transiently $\gamma\delta$ T cell-depleted mice (with Abs) are also noteworthy. Others (35) have reported developmental changes in the epithelia of TCR-$\delta^{-/-}$ mice, and we (8) have found reduced numbers of macrophages in BALF of TCR-$\delta^{-/-}$, but not in transiently depleted mice. The differential effect of Vγ1 depletion in C57BL/6 mice and BALB/c mice (decreased BALF eosinophils in C57BL/6, but not BALB/c, mice) suggests that enhancement of eosinophilia by Vγ1$^+$ cells is not critical for their AHR-enhancing effect in normal mice. The inverse effect after reconstitution of Vγ1$^+$ cells in the TCR-$\delta^{-/-}$ recipient was stronger, but still could be unrelated to AHR enhancement, given that it was only shown on the C57BL/6 genetic background.

In addition to the timing during the allergic response and the functional differences among $\gamma\delta$ T cells subsets, the recipient has a critical influence on the functional effects of the transferred cells. In contrast to TCR-$\delta^{-/-}$ recipients, in which AHR was increased after transfer of Vγ1$^+$ $\gamma\delta$ T cells, no effect was seen in Vγ4$^{-/-}$/6$^{-/-}$ recipients. These recipient mice contain Vγ1$^+$ cells and exhibit normal or slightly increased AHR. (It remains to be determined whether their AHR is also subject to increased age-dependent changes.) Nevertheless, transferred Vγ4$^+$ cells strongly suppressed AHR, as predicted by our earlier studies in similar mice (18). In this particular set of experiments, donor cells were derived from the lung, but we have found that spleen-derived Vγ4$^+$ cells also suppress AHR in Vγ4$^{-/-}$/6$^{-/-}$ recipients (N. Jin, unpublished observations). Therefore, this experiment emphasizes the importance of the host environment in determining which of the functional effects of $\gamma\delta$ T cells emerges.

The results of this study support the broader idea that TCR-Vγ expression and function cosegregate in $\gamma\delta$ T cells, at least with respect to the two subsets examined in this study. The findings are reminiscent of our earlier study in a murine model of virus-induced myocarditis involving the same two subsets where Vγ4$^+$ cells were found to promote cardiac inflammation, whereas Vγ1$^+$ cells inhibited it (16). Cosegregation of TCR expression and function is not typical with $\alpha\beta$ T cells and thus may be a distinctive property of $\gamma\delta$ T cells (17).

Our study also reveals some of the complexity of $\gamma\delta$ T cell involvement in airway inflammation and AHR. To date, we have only examined the functional properties of two Vγ-defined subsets of $\gamma\delta$ T cells. However, other Vγ-defined subsets are likely to become involved, especially Vγ6$^+$ cells, which are known to colonize the lung early during development (36). In addition, heterogeneity within the Vγ-defined subsets (e.g., with regard to Vδ and CD8 expression) probably has functional significance and may determine the precise roles of distinct subpopulations (9). Given the small size of such groups of cells, their potential to exert such a large influence on AHR and airway inflammation is indeed remarkable. It seems unlikely that they could control AHR directly. Rather, they might control a cellular intermediary capable of mediating AHR. We consider the far more frequent pulmonary myeloid cells (dendritic cells and alveolar macrophages) likely candidates for such a role, especially as we have shown that $\alpha\beta$ T cells are not required for $\gamma\delta$ T cell-regulated AHR (8, 9).

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