

Allergic Airway Hyperresponsiveness-Enhancing $\gamma\delta$ T Cells Develop in Normal Untreated Mice and Fail to Produce IL-4/13, Unlike Th2 and NKT Cells¹

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Allergic airway hyperresponsiveness (AHR) in OVA-sensitized and challenged mice, mediated by allergen-specific Th2 cells and Th2-like invariant NKT (iNKT) cells, develops under the influence of enhancing and inhibitory $\gamma\delta$ T cells. The AHR-enhancing cells belong to the V γ 1⁺ $\gamma\delta$ T cell subset, cells that are capable of increasing IL-5 and IL-13 levels in the airways in a manner like Th2 cells. They also synergize with iNKT cells in mediating AHR. However, unlike Th2 cells, the AHR enhancers arise in untreated mice, and we show here that they exhibit their functional bias already as thymocytes, at an HSA^{high} maturational stage. In further contrast to Th2 cells and also unlike iNKT cells, they could not be stimulated to produce IL-4 and IL-13, consistent with their synergistic dependence on iNKT cells in mediating AHR. Mice deficient in IFN- γ , TNFRp75, or IL-4 did not produce these AHR-enhancing $\gamma\delta$ T cells, but in the absence of IFN- γ , spontaneous development of these cells was restored by adoptive transfer of IFN- γ -competent dendritic cells from untreated donors. The i.p. injection of OVA/aluminum hydroxide restored development of the AHR enhancers in all of the mutant strains, indicating that the enhancers still can be induced when they fail to develop spontaneously, and that they themselves need not express TNFRp75, IFN- γ , or IL-4 to exert their function. We conclude that both the development and the cytokine potential of the AHR-enhancing $\gamma\delta$ T cells differs critically from that of Th2 cells and NKT cells, despite similar influences of these cell populations on AHR. *The Journal of Immunology*, 2009, 182: 2002–2010.

Exposure to pathogens induces protective responses of Ag-specific CD4⁺ T lymphocytes, which are associated with distinct cytokine patterns. Those involving IFN- γ , IL-2, and TNF- β have been classified as Th1 cells, those involving IL-4, IL-5, IL-9, and IL-13 as Th2 cells, and those involving IL-17 as Th17 cells (1). All of these responses depend on Ag priming, and on the induced differentiation of CD4⁺ $\alpha\beta$ T cells into functionally polarized Th cell types.

Polarized Th2 cells in particular promote humoral responses against extracellular parasites such as certain helminths (2), but they are also responsible for the development of hypersensitivity reactions against noninfectious allergens (3). Exactly what leads to the development of Th2 responses in vivo is not clear. According to one theory, weak Ag stimulation favors Th2 reactivity. A Th2 bias also exists after birth when the immune system is still immature (4). However, it is frequently observed that Th2 responses are induced by specific infectious pathogens (5) and by allergens (6). Th2 cytokines such as IL-4 further aid in the development of Th2 immunity (7, 8). Recent studies have implicated the innate immune

system in this process with its ability to recognize non-self motifs of extracellular or intracellular organisms and cell damage in the initiation of induced Th2 reactivity (9–12). For example, it has been proposed that the innate system is able to recognize protease activity, which plays an essential role in the lifecycle of helminths, and that allergens, many of which are proteases also, are recognized by essentially the same mechanism (13, 14). The stimulatory properties of aluminum adjuvants, which are commonly used to induce Th2-type immunity to inert protein Ags and allergens, were shown to be based on the activation of an intracellular innate response system, known as the Nalp3 inflammasome, which then induces the release of specific cytokines capable of polarizing the developing Th2 cells (11).

In mice infected with the Th1-inducing bacterium *Listeria monocytogenes* or the Th2-inducing extracellular parasite *Nippostrongylus brasiliensis* $\gamma\delta$ T cells also produced IFN- γ and IL-4, respectively, suggesting initially that $\gamma\delta$ T cells in general might differentiate in response to Th-polarizing conditions in a manner similar to uncommitted $\alpha\beta$ T cells (15). However, several studies showed that subsets of $\gamma\delta$ T cells, defined by their expression of TCR-V γ , exhibit differential and sometimes opposed Th-like reactivity (16, 17). In particular, cells expressing V γ 1, but not other $\gamma\delta$ T cells, selectively responded to the Th2-inducing parasite *Schistosoma mansoni* (18), inhibited host resistance to the Th1-inducing *L. monocytogenes* (17) and increased Th2 cytokines and allergic airway hyperresponsiveness (AHR)³ in mice sensitized and challenged with OVA (19). To determine whether $\gamma\delta$ T cells

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³ Abbreviations used in this paper: AHR, allergic airway hyperresponsiveness; iNKT, invariant NKT; BAL, bronchoalveolar lavage; DC, dendritic cell; HSA, heat-stable Ag.

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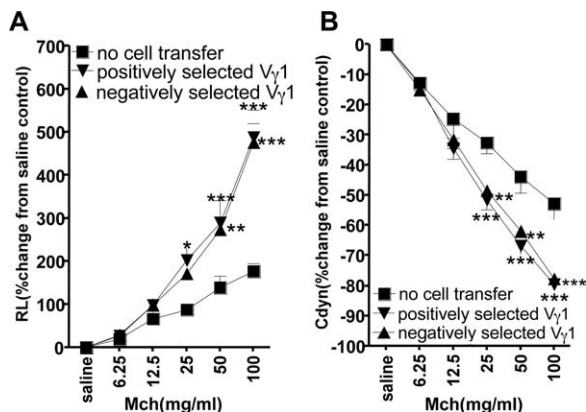


FIGURE 1. $V\gamma 1^+$ $\gamma\delta$ T cells from naive donors mediate AHR regardless of whether they were purified by positive or negative selection. AHR was monitored by measuring lung resistance (A) and dynamic compliance (B). OVA-sensitized B6.TCR- $\delta^{-/-}$ mice received 1×10^4 splenic $V\gamma 1^+$ $\gamma\delta$ T cells from untreated B6.TCR- $\beta^{-/-}$ donors, before airway challenge. The $V\gamma 1^+$ cells were enriched by positive or negative selection, as described in *Materials and Methods*. Recipients that were sensitized and challenged but did not receive cells (no cells transferred) are also shown. Results for each group are mean $\pm$ SEM ($n = 7$ –8 mice). Significant differences between “no cell transfer” and selected “ $V\gamma 1$ cell” transferred groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

must be induced to develop their Th-like functions, we established a model of adoptive cell transfer, in which transferred $\gamma\delta$ T cells can be examined for their ability to modulate OVA-induced AHR (20, 21). In this model, we previously observed that development of AHR-suppressive $V\gamma 4^+$ $\gamma\delta$ T cells had to be induced through repeated airway challenge of the donor mice, although matched Ag was not required (22). In marked contrast, we now demonstrate that development of the AHR-enhancing $V\gamma 1^+$ $\gamma\delta$ T cells does not require any treatment and is already established in the thymus, different from Th2 cells but similar to invariant NKT (iNKT) cells. Moreover, unlike both the induced AHR-mediating Th2 cells and the spontaneously developing AHR-mediating iNKT cells, the spontaneously developing AHR-enhancing $\gamma\delta$ T cells do not exert their effect by producing the Th2 cytokines IL-4 and IL-13.

Materials and Methods

Animals

C57BL/6 mice and several mutant strains with the same genetic background (B6.TCR- $\beta^{-/-}$, B6.TCR- $\delta^{-/-}$, B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$, B6.IL-4 $^{-/-}$, B6.CD8 $\alpha^{-/-}$) were purchased from The Jackson Laboratory. B6.TCR- $\beta^{-/-}$ /IFN- $\gamma^{-/-}$ mice were generated by crossing the single mutants and breeding double mutants identified in the F₂ generation, and B6.TNFRp75 $^{-/-}$ mice were a gift from Dr. D. Lynch (Immunex, Seattle, WA). Mice were 8- to 12-wk-old at the time of the experiments. All mice were maintained on an OVA-free diet and were cared for at the National Jewish Medical and Research Center (Denver, CO), following guidelines for immune-deficient animals. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Sensitization and airway challenge

Groups of mice were sensitized by i.p. injection of 20 μ g of OVA (grade V; Sigma-Aldrich) emulsified in 2.25 mg of aluminum hydroxide (Alum-Imject; Pierce) in a total volume of 100 μ l on days 0 and 14 (2ip). These mice were either subsequently challenged or they served as donors of $V\gamma 1^+$ $\gamma\delta$ T cells. Mice were challenged via the airways with OVA (10 mg/ml in saline) for 20 min each on days 28–30, by ultrasonic nebulization (3N, particle size 1–5 μ m; De Vilbiss). Lung resistance (R_L) and dynamic compliance (C_{dyn}) were assessed 48 h after the last allergen challenge, directly following provocation with aerosolized methacholine. Mice were subsequently sacrificed to obtain tissues and cells for additional analyses.

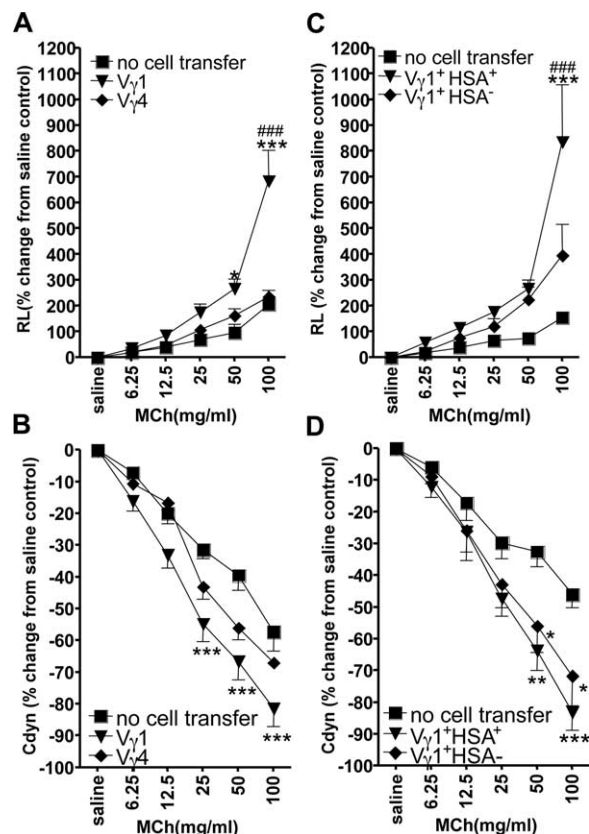


FIGURE 2. $V\gamma 1^+$ thymocytes mediate AHR. AHR was monitored by measuring lung resistance (A and C) and dynamic compliance (B and D). OVA-sensitized B6.TCR- $\delta^{-/-}$ mice received 1×10^4 splenic $V\gamma 1^+$ thymocytes from untreated B6.TCR- $\beta^{-/-}$ donors, before airway challenge. The thymocytes were selected for $V\gamma 1$ or $V\gamma 4$ expression, and $V\gamma 1^+$ thymocytes were further divided into HSA^{high} and HSA^{low} cells, as described in *Materials and Methods*. Recipients that were sensitized and challenged but did not receive cells (no cell transfer) are also shown. Results for each group are presented as mean $\pm$ SEM ($n = 4$ –7 mice). Significant differences between “no cell transfer” and “ $V\gamma 1^+$ cell” transferred groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between $V\gamma 1^+$ cell and $V\gamma 4^+$ cell transferred groups, or $V\gamma 1$ /HSA⁺ cell and $V\gamma 1$ /HSA⁻ cell transferred groups are also indicated. ###, $p < 0.001$.

Bronchoalveolar lavage (BAL)

Immediately following measurements of AHR, lungs were lavaged, and BAL fluid was recovered. Total leukocyte numbers were measured (Coulter Counter). Differential cell counts were performed by light microscopy of cytocentrifuged preparations (Cytospin2, Cytospin; Thermo Shandon), stained with Leukostat (Fisher Scientific). For each sample, at least 200 cells were counted and differentiated by standard hematologic procedures.

Cell purification and adoptive transfer of T cells and dendritic cells (DC)

C57BL/6 or B6.TCR- $\beta^{-/-}$ mice were used as cell donors. Their spleens and thymi were homogenized, treated with Gey's solution for RBC removal, and passed through nylon wool columns for T cell enrichment. Nonadherent cells were collected for further purification and stained with biotinylated anti- $V\gamma$ mAbs. Cells were washed and incubated with streptavidin-conjugated magnetic beads (streptavidin Microbeads; Miltenyi Biotec), and passed through magnetic columns as previously described (22). In particular, repeated positive selection starting with nonadherent spleen cells from B6.TCR- $\beta^{-/-}$ mice produced a cell population containing >90% viable $V\gamma 1^+$ cells. Negative selection starting with nonadherent spleen cells from B6.TCR- $\beta^{-/-}$ mice involved removal of $V\gamma 4^+$ cells and produced a cell population containing >65% viable $V\gamma 1^+$ cells as determined by two-color staining with anti-TCR- δ and anti- $V\gamma 1$ mAbs. To obtain subsets of $V\gamma 1^+$ cells, nonadherent cells were stained with biotinylated subset-specific mAbs (anti-V δ 6.3 mAb 17-C or anti-heat-stable Ag

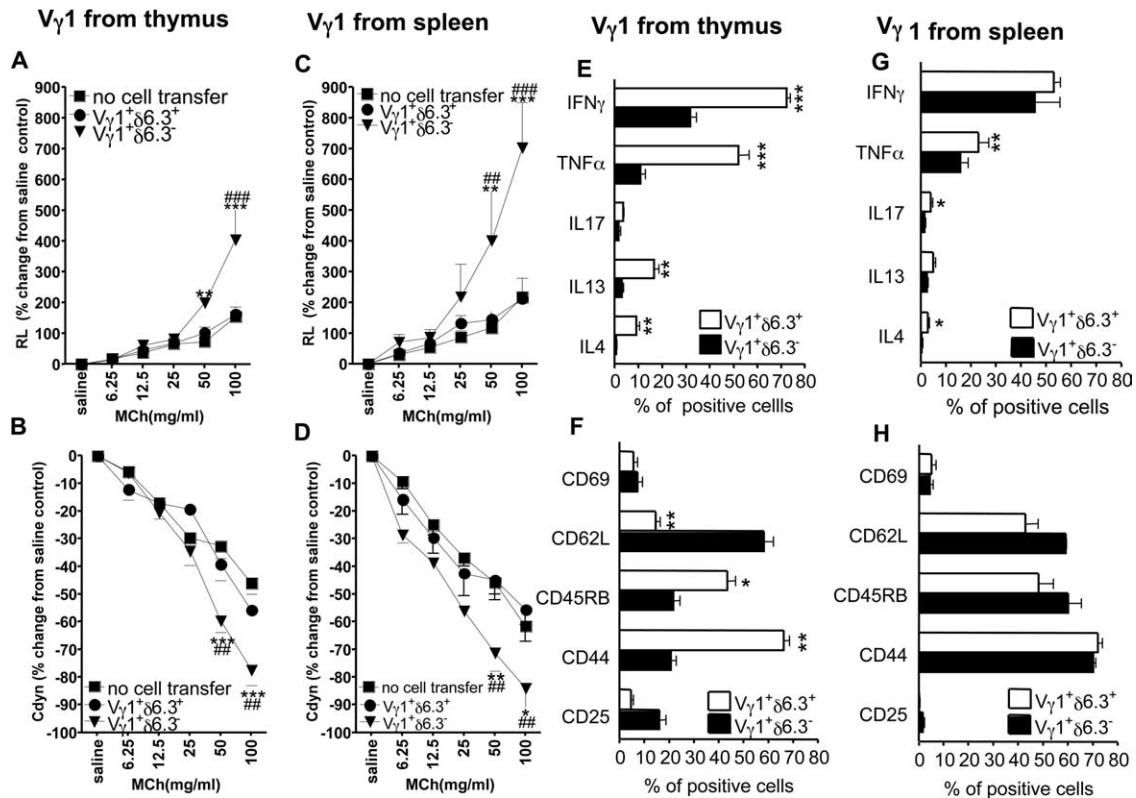


FIGURE 3. $V\gamma 1^+/V\delta 6.3^-$ $\gamma\delta$ T cells mediate AHR but fail to express Th2 cytokines. AHR was monitored by measuring lung resistance (A and C) and dynamic compliance (B and D). Cytokine-producing cells were enumerated by intracellular staining and cytofluorometry (E and G), and activated cells were enumerated by cell surface staining and cytofluorometry (F and H). To determine cellular effects on AHR, OVA-sensitized B6.TCR- $\delta^{-/-}$ mice received 1×10^4 $V\gamma 1^+$ thymocytes or splenic $V\gamma 1^+$ cells from untreated B6.TCR- $\beta^{-/-}$ donors, before airway challenge. The $V\gamma 1^+$ cells were further selected into $V\delta 6.3^+$ and $V\delta 6.3^-$ fractions, as described in *Materials and Methods*. Recipients that were sensitized and challenged but did not receive cells (no cell transfer) are also shown. Results for each group are shown as mean $\pm$ SEM ($n = 4-6$ mice). Significant differences between "no cell transfer" and " $V\gamma 1^+$ cell" transferred groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between $V\gamma 1^+/V\delta 6.3^+$ cell and $V\gamma 1^+/V\delta 6.3^-$ cell transferred groups are also indicated. ###, $p < 0.01$ and ####, $p < 0.001$. To determine the frequency of cells with potential for cytokine production, $V\gamma 1$ cells from B6.TCR- $\beta^{-/-}$ thymus and spleen were activated in vitro with PMA/ionophore in the presence of brefeldin A and stained for TCR expression and intracellular cytokines. The percentage of cytokine-producing cells among $V\gamma 1^+/V\delta 6.3^+$ and $V\gamma 1^+/V\delta 6.3^-$ subsets are shown (E and G). To determine the frequency of cell expression activation/memory markers, freshly isolated cells were stained directly for TCR expression and cell surface markers. The percentage of marker-positive cells among $V\gamma 1^+/V\delta 6.3^+$ and $V\gamma 1^+/V\delta 6.3^-$ subsets are shown (F and H). Results for each group are presented as mean $\pm$ SEM ($n = 8-11$ mice). Significant differences between $V\gamma 1^+/V\delta 6.3^-$ and spleen $V\gamma 1^+/V\delta 6.3^+$ groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(HSA, CD24) mAb M1/69 plus streptavidin-PE and FITC-conjugated anti- $V\gamma 1$ mAb 2.11), then sorted on a MoFlo cell sorter (DakoCytomation) and collected at a purity of $>95\%$. The purified cells were washed and resuspended in balanced salt solution, and injected via the tail vein into OVA-sensitized mice (B6.TCR- $\delta^{-/-}$), less than 1 h before the first airway challenge.

To purify $CD8^+$ DC for reconstitution experiments, a $CD8^+$ DC purification kit (Miltenyi Biotec) was used per the manufacturer's recommendations. Briefly, RBC-depleted splenocytes derived from untreated T cell-deficient mice (B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$) were incubated with biotinylated Ab mixture (anti- $CD90$, anti- $CD45R$, and anti- $CD49b$) in PBS/5% FBS (10 min on ice). Cells were washed and incubated with anti-biotin microbeads (15 min on ice), and washed and passed over an LD magnetic column. These enriched DC were then incubated with anti- $CD8\alpha$ (Ly-2) microbeads (30 min on ice), washed, and passed twice over an MS magnetic column retaining the $CD8^+$ DC. This protocol produced a preparation containing 60–70% viable $CD8\alpha^+$ DC, based on the additional criteria of dual MHC class II and $CD11c$ expression as previously described (23). A total of 1×10^5 wild-type $CD8^+$ DC were transferred i.v. to B6.TCR- $\beta^{-/-}$ /IFN- $\gamma^{-/-}$ recipients, which were used as donors of $V\gamma 1^+$ cells 4 days later.

Of note throughout this study, the nomenclature used for murine $V\gamma$ genes was introduced by Heilig and Tonegawa (24). We use the term "enhancing cells" to refer to purified $V\gamma 1^+$ $\gamma\delta$ T cells capable of enhancing AHR upon adoptive cell transfer into OVA-sensitized and -challenged recipients, and the term "suppressive cells" to refer to purified $V\gamma 4^+$ $\gamma\delta$ T cells derived from OVA-sensitized and -challenged mice, which are capable of suppressing AHR.

Determination of airway responsiveness

Airway responsiveness was assessed as a change in lung function after provocation with aerosolized methacholine using a method previously described (25). Methacholine was administered for 10 s (60 breaths/min, 0.5-ml tidal volume) in increasing concentrations. Maximum values of lung resistance and minimum values of dynamic compliance were recorded and expressed as a percentage of change from the baseline obtained after saline aerosol treatment.

Relative frequency of activated and cytokine-producing cells

Thymus and spleen (B6.TCR- $\beta^{-/-}$) were homogenized and treated with Gey's solution to remove RBC. For analysis of cytokine competence, cells were cultured at $1-2 \times 10^6$ /ml for 4 h at 37°C in the presence of PMA/ionomycin and brefeldin A to activate them while blocking their cytokine release. After culture, cells were stained with anti- $V\gamma 1$ (FITC-conjugated mAb 2.11) and anti- $V\delta 6.3$ (biotinylated mAb 17-C and streptavidin-allophycocyanin mAb), then fixed in 1% paraformaldehyde. After permeabilizing cells in 0.5% saponin buffer, PE-labeled anti-mouse IL-4 (BVD4-1D11), anti-mouse IL-13 (eBio13A), anti-mouse IL-17 (TC11-18H10), anti-mouse IFN- γ (XMG1.2), and anti-TNF- α (MP6-XT22) mAbs (from BD Pharmingen, eBioscience, and BD Biosciences) were then added to detect intracellular cytokines. For analysis of the state of activation, freshly isolated cells were stained immediately with Abs against these makers (anti- $CD25$ (PC-61), anti- $CD44$ (IM7), anti- $CD45RB$ (16A), anti- $CD62L$ (Mel-14), and anti- $CD69$ (H1.2F3), all from BD Pharmingen), in addition to the same

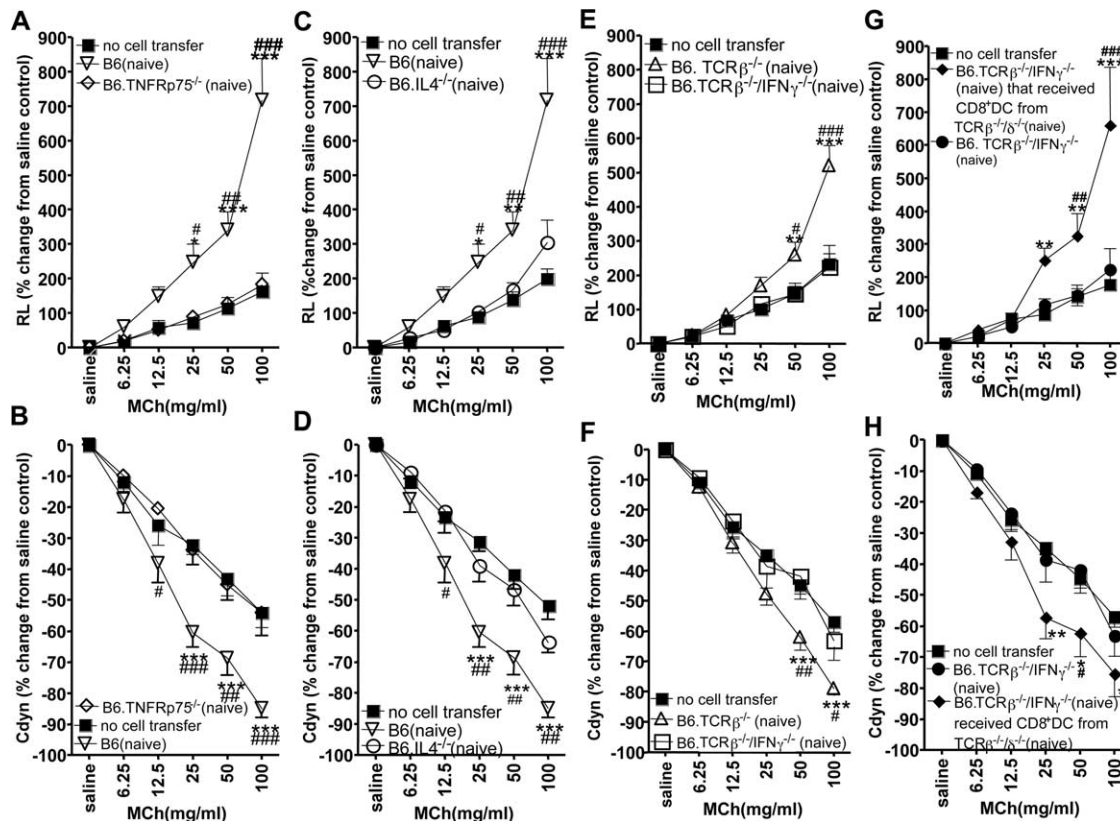


FIGURE 4. $V\gamma 1^+$ $\gamma\delta$ T cells from naive donors deficient in TNFRp75, IL-4, and IFN- γ fail to mediate AHR, and transferred CD8 $^+$ DC restore this ability in $V\gamma 1^+$ cells from IFN- γ -deficient mice. AHR was monitored by measuring lung resistance (A, C, E, and G) and dynamic compliance (B, D, F, and H). OVA-sensitized B6.TCR- $\delta^{-/-}$ mice received 1×10^4 splenic $V\gamma 1^+$ $\gamma\delta$ T cells from untreated donors, or from donors that received 1×10^5 CD8 $^+$ splenic DC (G and H), as described in *Materials and Methods*, before airway challenge. In addition to the cells from the cytokine- or cytokine receptor-deficient mice, cells from matched normal controls (C57BL/6 or B6.TCR- $\beta^{-/-}$ mice) were also transferred. Responses of recipients that were sensitized and challenged but did not receive cells (no cell transfer) are also shown. Results for each group are presented as mean $\pm$ SEM ($n = 6$ mice for A, B, E, and F and $n = 8$ mice for C and D). Significant differences between “no cell transfer” and “ $V\gamma 1^+$ cells” transferred groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between wild-type or mutant-derived $V\gamma 1$ cell transferred group, or between $V\gamma 1^+$ cells derived from IFN- γ -deficient or IFN- γ -deficient DC-reconstituted mice are also indicated. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$.

TCR-specific Abs as in the analysis of cytokine expression. Fluorescent staining profiles were analyzed on a FACscan (DakoCytometry).

Histology

Frozen sections of thymus tissue from adult B6.TCR- $\beta^{-/-}$ mice were dehydrated and stained with Abs as previously described (23, 26). Abs used for histology were anti-TCR- δ (mAb GL3), anti- $V\gamma 1$ (mAb 2.11), anti-CD8 α (mAb 53-6.7) plus secondary reagents as described (26). Stained sections were mounted and slides viewed using a Leica DM-RXMA upright fluorescent confocal microscope using 25X (see Fig. 7A) and 40X (see Fig. 7B) oil immersion objectives. Digital images were captured using a Cooke SensiCam, and processed on an Apple MacIntosh Computer using the SlideBook Imaging program (31).

Statistical analysis

Data are presented as mean $\pm$ SEM. The paired t test was used for two-group comparisons, and two-way ANOVA for analysis of differences in three or more groups, followed by pairwise Bonferroni post hoc test. Statistically significant levels were set at a value for $p < 0.05$.

Results

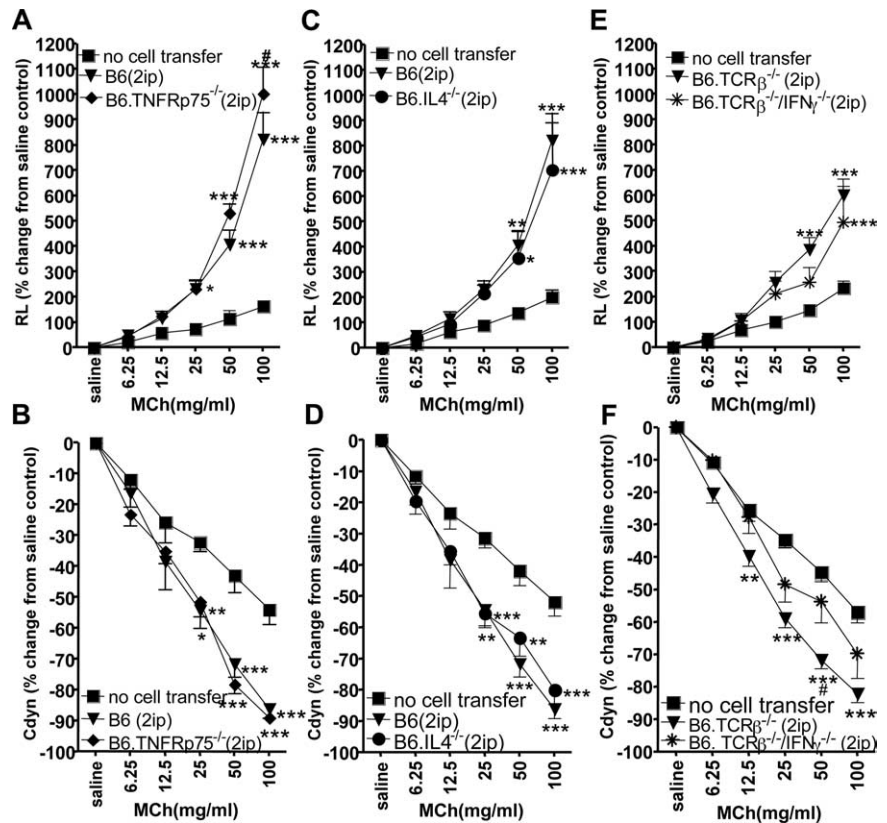
AHR-enhancing $\gamma\delta$ T cells develop in normal untreated mice

We have previously shown that $\gamma\delta$ T cells expressing $V\gamma 1$ enhance AHR and also airway cytokines (IL-5 and IL-13) in C57BL/6 mice sensitized and challenged with OVA, using both TCR depletion (in vivo treatment with anti-TCR mAbs, which inactivates the targeted T cells) and complementary adoptive cell transfer (19, 21). In the

cell transfer model, mice deficient for all $\gamma\delta$ T cells (B6.TCR- $\delta^{-/-}$), which fail to develop AHR following OVA-sensitization and challenge when they are young, develop AHR at levels similar to wild-type mice when they receive small numbers of $V\gamma 1^+$ $\gamma\delta$ T cells before the first challenge. In addition, substantial increases in IL-5 and IL-13, and a decrease in IL-10 are evident in BAL fluid. The Th2-like AHR enhancers can be detected in both OVA-sensitized wild-type C57BL/6 mice and genetically matched mice deficient in all $\alpha\beta$ T cells (B6.TCR- $\beta^{-/-}$), indicating that they arise independently from $\alpha\beta$ T cells (21).

The AHR enhancers are also present in untreated donors, suggesting that they develop without need for any external stimulation (21). However, because the transferred cells in these experiments were purified in vitro using anti-TCR-mAbs and magnetic beads, the possibility remained that these cells became activated and acquired their function through this process of positive selection. To address this possibility, we prepared enriched $V\gamma 1^+$ cells from the spleen of nonsensitized B6.TCR- $\beta^{-/-}$ mice by negative selection, and examined their AHR-enhancing potential in comparison with positively selected cells (Fig. 1). Upon transfer into OVA-sensitized B6.TCR- $\delta^{-/-}$ mice, the negatively selected $V\gamma 1^+$ cells enhanced AHR to the same level as the positively selected cells, indicating that the process of positive selection does not contribute to their

FIGURE 5. $V\gamma 1^+$ $\gamma\delta$ T cells from 2ip-sensitized donors deficient in TNFRp75, IL-4, and IFN- γ mediate AHR. AHR was monitored by measuring lung resistance (A, C, and E) and dynamic compliance (B, D, and F). OVA-sensitized B6.TCR- $\beta^{-/-}$ mice received 1×10^4 splenic $V\gamma 1^+$ $\gamma\delta$ T cells from donors sensitized by i.p. injection of 20 μ g of OVA-V emulsified in 2.25 mg of aluminum hydroxide on days 0 and 14 (2ip), before airway challenge. In addition to the cells from the cytokine- or cytokine receptor-deficient mice, cells from 2ip-sensitized normal controls (C57BL/6 or B6.TCR- $\beta^{-/-}$ mice) were also transferred. Responses of recipients that were sensitized and challenged but did not receive cells (no cell transfer) are also shown. Results for each group are mean $\pm$ SEM ($n = 6$ mice for A and B, $n = 8$ mice for C and D, and $n = 5$ mice for E and F). Significant differences between “no cell transfer” and “ $V\gamma 1^+$ cell” transferred groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between wild-type cell group and mutant-derived $V\gamma 1^+$ cell transferred group (F) are also indicated. #, $p < 0.01$.



enhancing function. In contrast to their potent AHR-inducing effect, neither type of transferred cells substantially altered the inflammatory infiltrate in the airways (data not shown), consistent with our earlier findings (21).

AHR-enhancing $\gamma\delta$ T cells exhibit their functional potential already as thymocytes

Because donors of the splenic AHR enhancers did not require sensitization or any other prior treatment, it seemed possible that $V\gamma 1^+$ $\gamma\delta$ T cells acquire their AHR-enhancing potential already during intrathymic maturation. To investigate this possibility, $V\gamma 1^+$ and $V\gamma 4^+$ thymocytes were purified from the thymi of untreated 8- to 10-wk-old B6.TCR- $\beta^{-/-}$ mice, and transferred into OVA-sensitized B6.TCR- $\delta^{-/-}$ recipients, before airway challenge and the measurement of airway function. Whereas $V\gamma 4^+$ thymocytes had no effect, $V\gamma 1^+$ thymocytes enhanced AHR though not as strongly as $V\gamma 1^+$ splenocytes (Fig. 2, A and B). As was observed with the splenic $\gamma\delta$ T cells, the transferred thymocytes did not change the composition of the cellular infiltrate in the airways (data not shown). Because some peripheral $\gamma\delta$ T cells return to the thymus (27), the possibility remained that the thymic AHR enhancers belong to this returning mature population. Therefore, we fractionated $V\gamma 1^+$ thymocytes based on their expression of HSA (CD24), a marker of thymocyte maturation (28, 29). In general, HSA^{high} thymocytes are less mature than HSA^{low} thymocytes, and peripheral T cells remain HSA^{low}. When we compared the HSA^{high} and HSA^{low} $V\gamma 1^+$ thymocytes, we found that both cell types enhanced AHR (Fig. 2, C and D). Again, the transferred thymocytes did not substantially alter the composition of the cellular infiltrate in the airways (data not shown).

$V\gamma 1^+$ AHR-enhancers fail to produce IL-4/13

We also examined $V\gamma 1^+$ thymocytes coexpressing V $\delta 6.3$, for two reasons. First, these cells have been previously reported to return

to the thymus from peripheral locations (27), raising the possibility that thymic AHR enhancers might consist of this more mature population. Second, these $V\gamma 1^+/V\delta 6.3^+$ cells have been likened to iNKT cells, which do not require Th2 polarization to produce IL-4 and IL-13 and are capable of mediating AHR (30). However, when we compared $V\gamma 1^+/V\delta 6.3^+$ and $V\gamma 1^+/V\delta 6.3^-$ thymocytes, only the latter enhanced AHR (Fig. 3, A and B), and when we extended the comparison to splenocytes, the AHR enhancers again were contained within the $V\gamma 1^+/V\delta 6.3^-$ fraction (Fig. 3, C and D), consistent with our earlier finding that AHR-enhancing $\gamma\delta$ T cells in the spleen express $V\gamma 1/V\delta 5$ (21). Clearly, the AHR-enhancing $\gamma\delta$ T cells in the thymus are not derived from the recirculating NKT-like subset of $\gamma\delta$ T cells (27). Next, we compared the same cell populations ($V\gamma 1^+/V\delta 6.3^+$ and $V\gamma 1^+/V\delta 6.3^-$ cells) in thymus and spleen for their ability to produce cytokines when stimulated with PMA/ionomycin (Fig. 3, E and G). Not surprisingly, large fractions of the NKT-like $V\delta 6.3^+$ cells in the thymus produced cytokines, including IL-4, TNF- α , and IFN- γ . Far fewer cells among the $V\delta 6.3^-$ thymocytes, which contain the AHR enhancers, produced TNF- α and IFN- γ , and IL-4-producing cells were absent. Only the $V\delta 6.3^+$ cells contained IL-13 producers. IL-4/13-producing cells among $V\gamma 1^+$ splenocytes were even less frequent. Only small fractions of $V\gamma 1^+$ cells in thymus and spleen produced IL-17, and there was little if any difference between $V\delta 6.3^+$ and $V\delta 6.3^-$ cells. The two fractions of $V\gamma 1^+$ cells in the thymus also differed in their state of activation (Fig. 3F), with $V\delta 6.3^-$ cells expressing the adhesion molecule CD62L at significantly higher frequency, and more of the $V\delta 6.3^+$ cells expressing CD44 and CD45RB, two markers of activation/memory, but these differences mostly disappeared in the spleen (Fig. 3H). Taken together, these results illustrate the heterogeneity of the $V\gamma 1^+$ $\gamma\delta$ T cells, especially in the thymus, and they provide support for the notion that $V\gamma 1^+$ $\gamma\delta$ T cells capable of mediating AHR become functional in the thymus. In addition, they suggest that the AHR-enhancing

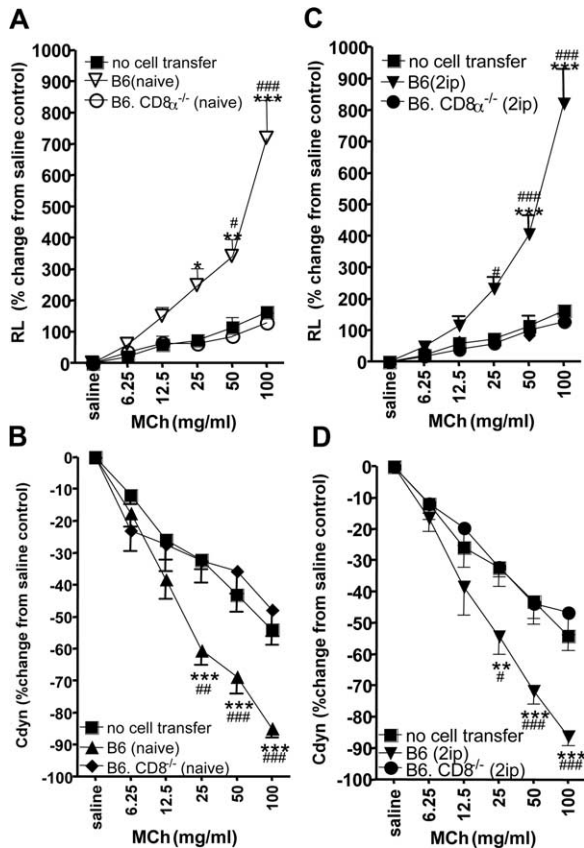


FIGURE 6. $V\gamma 1^+$ $\gamma\delta$ T cells from $CD8\alpha$ -deficient donors fail to mediate AHR even when the donors are 2ip-sensitized. AHR was monitored by measuring lung resistance (A and C) and dynamic compliance (B and D). OVA-sensitized B6.TCR- $\delta^{-/-}$ mice received 1×10^4 splenic $V\gamma 1^+$ $\gamma\delta$ T cells from untreated (A and B) or donors sensitized by i.p. injection of 20 μ g of OVA-V emulsified in 2.25 mg of aluminum hydroxide on days 0 and 14 (2ip), before airway challenge. Recipients that were sensitized and challenged but did not receive cells (no cell transfer) are also shown. Results for each group are presented as mean $\pm$ SEM ($n = 3$ mice for A and B and $n = 5$ mice for C and D). Significant differences between “no cell transfer” and “ $V\gamma 1^+$ cell” transferred groups are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between wild-type and mutant-derived $V\gamma 1$ cells are also indicated. #, $p < 0.05$; ###, $p < 0.001$; ####, $p < 0.0001$.

$\gamma\delta$ T cells exert their effect on the basis of a mechanism that is fundamentally different from that used by AHR-mediating Th2 cells and iNKT cells, both of which produce IL-4 and IL-13.

Requirements for the development/function of AHR-enhancing $\gamma\delta$ T cells

We previously found that $\gamma\delta$ T cells derived from untreated mice express the high affinity TNFR (TNF-Rp75) at higher levels, and are more sensitive to TNF- α compared with $\alpha\beta$ T cells (31). For this reason, we considered the possibility that signaling through TNF-Rp75 might be required in their functional development. Indeed, $V\gamma 1^+$ cells from the spleen of untreated C57BL/6 mice genetically deficient in TNF-Rp75 completely failed to enhance AHR in the cell-transfer model (Fig. 4, A and B). This finding suggested that signaling through TNF-Rp75 is somehow required for the AHR-enhancing function of the $V\gamma 1^+$ cells. Subsequently, we examined strains of mice deficient for additional cytokine genes, and found that $V\gamma 1^+$ cells from untreated C57BL/6 mice genetically deficient in IL-4, and from untreated B6.TCR- $\beta^{-/-}$ mice deficient in IFN- γ , also

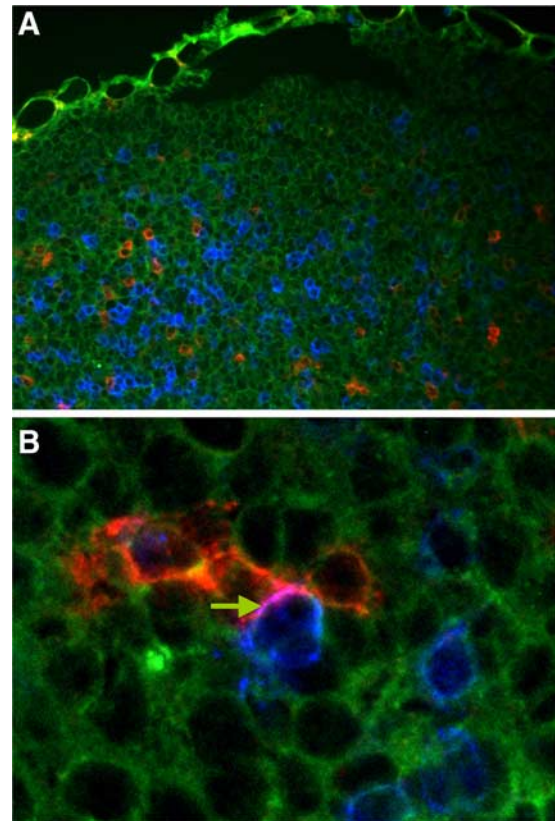


FIGURE 7. $V\gamma 1^+$ thymocytes encounter $CD8\alpha^+$ cells in the thymus. Frozen sections of adult B6.TCR- $\beta^{-/-}$ thymus were stained with Abs specific for TCR- δ (red) and $CD8\alpha$ (blue) (A) or $V\gamma 1$ (red) and $CD8\alpha$ (blue) (B). Autofluorescence (green) shows thymus tissue. The digital image (B) is enlarged $\times 7.4$ relative to A. The arrow (B) points to the area of contact between the $\gamma\delta$ T cell (red) and the $CD8^+$ cell (blue).

have lost their AHR-enhancing potential (Fig. 4, C–F). However, at least for $V\gamma 1^+$ cells in the IFN- γ -deficient mice (B6.TCR- $\beta^{-/-}$ /IFN- $\gamma^{-/-}$), this functional potential was restored following transfer of $CD8^+$ DC, purified from the spleen of untreated mice (Fig. 4, G and H) by a previously described technique (23), confirming that the AHR enhancers themselves need not express IFN- γ . For these experiments, the transferred DC were obtained from donors capable of producing IFN- γ (B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$), and might themselves have provided this cytokine. Taken together, these data revealed a role for several cytokines in connection with the AHR-enhancers, and suggested that IFN- γ and $CD8^+$ DC might be involved in their development.

OVA/aluminum hydroxide injection restores the AHR-enhancing $V\gamma 1^+$ cells in mice that fail to produce them spontaneously

It has been shown that aluminum hydroxide (alum) enables $Gr1^+$, IL-4-secreting innate cells to prime B lymphocytes (32). Moreover, alum together with a second stimulus such as LPS stimulates Nalp3 inflammasome activation and Th2 polarization (11), and a single i.p. injection of OVA/alum is sufficient to induce an Ag-specific IgE response (33). Having identified several genetically altered mouse strains in which AHR enhancers fail to develop “automatically” (mice lacking the expression of IL-4, TNF-Rp75, or IFN- γ), we reexamined cells of these mice after two i.p. injections of OVA/alum. In all cases, the sensitized mice were now able to produce $V\gamma 1^+$ $\gamma\delta$ T cells capable of enhancing AHR (Fig. 5), suggesting that there is also an

inducible pathway for the development of the AHR-enhancing $\gamma\delta$ T cells. Moreover, neither TNF-Rp75 nor IFN- γ or even IL-4 is absolutely required in the functional repertoire of these cells, which develop in the absence of these molecules. Finally, as a way to assess the limit of OVA/alum-mediated AHR enhancer induction, we examined mice lacking CD8 α expression, which also fail to produce AHR enhancers, but can be restored in this regard by adoptive transfer of CD8 α^+ DC (23). In this experiment, in marked contrast to the cytokine or cytokine receptor-deficient mice, sensitization with OVA/alum alone did not restore the AHR enhancers (Fig. 6), suggesting that CD8 $^+$ DC are indispensable for the development of the AHR-enhancing $\gamma\delta$ T cells. We have shown previously that CD8 $^+$ DC and $\gamma\delta$ T cells encounter each other in the spleen, and that the DC are required for the development of the $\gamma\delta$ T cells (23). Because the AHR-enhancing $\gamma\delta$ T cells acquire their functional potential already in the thymus (this study), we examined the thymus histologically (Fig. 7). In this experiment also, as in the spleen, CD8 $^+$ non-T cells could be seen frequently in close proximity or direct contact with $\gamma\delta$ T cells.

Discussion

The development and functions of $\gamma\delta$ T cells remain poorly understood. Previous studies from one group suggested that $\gamma\delta$ T cells in general acquire Th-like functional profiles when they develop under Th-polarizing conditions, so that they might become Th1-like during one type of infection and Th2-like during another (15). In contrast, another group provided evidence to indicate that $\gamma\delta$ T cells develop with a strong bias to produce IFN- γ (34). Our earlier studies and those of others suggested that functional profiles in $\gamma\delta$ T cells actually segregate with TCR-defined subsets, which may be Th-like or NKT-like (16, 19, 27). Investigating mice sensitized and challenged with OVA, which develop allergic airway inflammation and AHR dependent on several types of T cell including Th2 cells, CD8 $^+$ T cells, and iNKT cells, we found that V γ 1 $^+$ $\gamma\delta$ T cells enhanced and V γ 4 $^+$ cells suppressed AHR (19). The V γ 4 $^+$ AHR suppressors produced IFN- γ and depended on IFN- γ for their function (35), in this regard reminiscent of Th1 cells. The V γ 1 $^+$ cells exhibited less of a tendency to produce IFN- γ , and caused IL-5 and IL-13 to increase in the airways in a manner like Th2 cells and iNKT cells (19), suggesting that they might be categorized as Th2-like lymphocytes. However, the findings of the current study modify and refine this picture: they reveal that the AHR-enhancing $\gamma\delta$ T cells develop quite differently from Th2 cells, and that their functional profile in terms of cytokine production differs from those of Th2 cells or Th2-like iNKT cells. The first set of observations in this study suggested that the spontaneously developing V γ 1 $^+$ AHR-enhancing $\gamma\delta$ T cells might resemble AHR-enhancing iNKT cells, which also were found to develop without induction (30). In contrast to conventional Th2 cells, both cell types appear to reach at least some degree of functional competence already in the thymus, in the case of the $\gamma\delta$ T cells demonstrated by their enhancing effect on AHR following adoptive cell transfer (this study), and in the case of the iNKT cells, by their ability to produce Th2-type cytokines in large quantity (36). Although the AHR-enhancing effect of the thymocytes was smaller than that of splenic $\gamma\delta$ T cells, it is not insignificant when one considers that only 10 ^{4} cells were transferred. These $\gamma\delta$ T cells might mature even earlier than the iNKT cells because they exhibited their function already as HSA^{high} thymocytes, whereas the iNKT cells acquire their functions at an HSA^{low} maturational stage (37), assuming that the innate cell types follow a maturational pathway in the

thymus that resembles that of classical T cells. Because of the developmental similarity with iNKT cells, it seemed possible that the AHR-enhancing $\gamma\delta$ T cells actually might belong to a previously identified V γ 1 $^+$ subpopulation with NKT-like properties (38). These cells frequently coexpress V δ 6.3, and like iNKT cells, some express IL-4 already as thymocytes. Phenotypically, they resemble activated/memory-type cells. Our data confirmed these observations, but showed that the AHR-enhancing V γ 1 $^+$ thymocytes do not belong to this NKT-like subset. This observation is consistent with our earlier finding that the AHR-enhancing $\gamma\delta$ T cells are NK1.1 $^-$, unlike the NKT-like population of $\gamma\delta$ T cells (21).

It remains to be addressed whether the thymic precursors of the AHR enhancers must be selected to acquire their functional potential. Thymic selection clearly plays a role in the development of iNKT cells (39, 40), which also transition from HSA^{high} to HSA^{low} maturational stages. Thymic iNKT cells are thought to undergo a Th2-to-Th1 conversion such that the least mature cells only express IL-4, whereas intermediate cells express IL-4/IFN- γ and the most mature cells express IFN- γ (36). A maturational conversion also has been described for T10/T22-reactive $\gamma\delta$ T cells, which were found to express IL-17 in the absence of an expressed ligand in the thymus, and IFN- γ in its presence, suggesting that the ligand experience in the thymus changes their cytokine profile (41). A TCR ligand for the AHR enhancers is not known. However, consistent with a role for the TCR, we found that they exclusively express V γ 1V δ 5 (21), and only very few V γ 1 $^+$ thymocytes in this study could be induced to produce IL-17, whereas many produced IFN- γ .

The AHR-enhancing V γ 1 $^+$ $\gamma\delta$ T cells may develop similarly to NKT cells, but they are not otherwise NKT-like. This difference is further emphasized by the induced cytokine profile of these cells. NKT cells upon induction produce large amounts of cytokine and typically exhibit a mixed profile, including both Th1 and Th2 cytokines (42). Similarly, we found relatively increased frequencies of IFN- γ , TNF- α , and IL-4 producers among the NKT-like V γ 1 $^+$ V δ 6.3 $^+$ thymocytes. However, V γ 1 $^+$ V δ 6.3 $^-$ thymocytes that contain the AHR enhancers did not contain IL-4 producers, and fewer among them produced IFN- γ and TNF- α . These differences are not merely a reflection of different maturational states because the V γ 1 $^+$ cells in the spleen also failed to produce Th2 cytokines. Both V γ 1 $^+$ V δ 6.3 $^+$ and V γ 1 $^+$ V δ 6.3 $^-$ spleen cells hardly contained any IL-13 or IL-17 producers. Therefore, although the effect of the $\gamma\delta$ T cells on airway responsiveness resembles that of Th2-like cells and iNKT cells, the mechanism by which they influence AHR probably is very different. This observation makes sense in view of our earlier findings that the AHR-enhancing $\gamma\delta$ T cells depend on $\alpha\beta$ T cells to exert their enhancing effect (19), and that V γ 1 $^+$ $\gamma\delta$ T cells and iNKT cells synergize to promote AHR (21). When adoptively transferred into mice deficient of all other T cells, the two types of innate T cells depended on each other and either type alone could not induce AHR. It thus appears that the $\gamma\delta$ T cells can exert their AHR-enhancing function only in synergy with a cellular source of essential Th2-type cytokines, especially IL-13, which is not provided by the $\gamma\delta$ T cells. This function remains to be shown, however, and the nature of the $\gamma\delta$ T cell contribution is still unclear.

The AHR-enhancing $\gamma\delta$ T cells that promote Th2 reactivity might arise constitutively as part of a developmental program, or become induced by background levels of immune stimulation. To detect molecular and cellular requirements in their development, we examined the already known requirements for the Th2 response. Some time ago, TNF- α was identified as a

critical component in the induction of protective Th2 responses during helminth infections (43), and it also appears to play a role in the induction of Th2-dependent allergic airway inflammation (10). Our data provide indirect evidence that signals from this cytokine are required for the development of the AHR enhancers in untreated mice. Similarly, IL-4 can play a critical role at the onset of Th2 responses (9), and our data indicate that IL-4 is required for the development of the AHR enhancers in untreated mice. Why IFN- γ might be required also is not obvious given that conventional Th2 responses are inhibited by IFN- γ (44), but it might prevent differentiation of the $\gamma\delta$ T cells along a competing alternative pathway (45), or the requirement might relate to an auxiliary cell rather than the $\gamma\delta$ T cells itself. In fact, we already reported evidence that CD8 α^+ DC are needed for the development of the AHR enhancers in untreated mice (23). Because the AHR-enhancing $\gamma\delta$ T cells do not depend on Ag-priming, the role of the DC remains enigmatic (46). However, as DC also have been implicated in autophagy (47), they might provide autologous ligands for the $\gamma\delta$ T cells. Histological evidence for CD8 α^+ DC encounters with V γ 1 $^+$ cells in the thymus is provided in Fig. 7. Furthermore, we show in the current study that reconstituting IFN- γ -deficient mice with wild-type CD8 $^+$ DC is sufficient to restore the development of the AHR enhancers (Fig. 4).

That i.p. injection of OVA/alum can restore development of the AHR enhancers in the IFN- γ - or IL-4-deficient mice further emphasizes that these cytokines have no essential role in the AHR-enhancing function of these cells. Perhaps, sensitization with OVA/alum overcomes the various deficiencies because it stimulates both TLR-dependent and Nalp3 inflammasome-dependent pathways of innate-activation that help in the induction of Th2 cells (10, 11), and which also might provide inducing signals for $\gamma\delta$ T cell differentiation. In summary, our data suggest that the AHR-enhancing $\gamma\delta$ T cells play an important part in the Th2 response, and that this part is synergistic and complementary rather than additive. In this way, a small number of $\gamma\delta$ T cells might be able to jump-start and regulate the far larger populations of Th2 and Th2-like cells. How this mechanism occurs and what might control cell engagement remain to be determined.

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Disclosures

The authors have no financial conflict of interest.

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