The Journal of Immunology

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J Immunol 2005; 174:2671-2679; ;
doi: 10.4049/jimmunol.174.5.2671
http://www.jimmunol.org/content/174/5/2671

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Mismatched Antigen Prepares γδ T Cells for Suppression of Airway Hyperresponsiveness[1]

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γδ T cells suppress airway hyperresponsiveness (AHR) induced in allergen-challenged mice but it is not clear whether the suppression is allergen specific. The AHR-suppressive cells express TCR-Vγ4. To test whether the suppressive function must be induced, we adoptively transferred purified Vγ4$^+$ cells into γδ T cell-deficient and OVA-sensitized and -challenged recipients (B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$) and measured the effect on AHR. Vγ4$^+$ γδ T cells isolated from naive donors were not AHR-suppressive, but Vγ4$^+$ cells from OVA-stimulated donors suppressed AHR. Suppressive Vγ4$^+$ cells could be isolated from lung and spleen. Their induction in the spleen required sensitization and challenge. In the lung, their function was induced by airway challenge alone. Induction of the suppressors was associated with their activation but it did not alter their ability to accumulate in the lung. Vγ4$^+$ γδ T cells preferentially express Vδ4 and -5 but their AHR-suppressive function was not dependent on these Vδs. Donor sensitization and challenge not only with OVA but also with two unrelated allergens (ragweed and BSA) induced Vγ4$^+$ cells capable of suppressing AHR in the OVA-hyperresponsive recipients, but the process of sensitization and challenge alone (adjuvant and saline only) was not sufficient to induce suppressor function, and LPS as a component of the allergen was not essential. We conclude that AHR-suppressive Vγ4$^+$ γδ T cells require induction. They are induced by allergen stimulation, but AHR suppression by these cells does not require their restimulation with the same allergen. ***The Journal of Immunology,*** **2005, 174: 2671–2679.**

Functional engagement of γδ T cells in the immune response has been demonstrated in animal models of injury or diseases but the role of the γδ TCR remains unresolved (1). In mice sensitized and challenged with OVA that exhibit airway hyperresponsiveness (AHR),[4] we found that γδ T cells regulated AHR (2, 3) and that subsets of γδ T cells had different regulatory influences. In particular, cells expressing Vγ1 (GV5S1) promoted eosinophilic airway inflammation, increased levels of IL-5 and IL-13 in bronchoalveolar lavage fluids, and exacerbated AHR to methacholine (MCh) (4). In contrast, cells expressing Vγ4 (GV3S1) suppressed AHR (3, 5), without detectable effects on the inflammatory response. In this regard, Vγ4$^+$ γδ T cells seem to complement certain CD4$^+$CD25$^+$ αβ T cells, which have been shown to suppress Th 2 immune responses and inflammation in the lung without effects on AHR (6). Despite the enhancing effect of Vγ1$^+$ cells, the net regulatory effect of total γδ T cells was protection of normal airway function in hypersensitized mice (2, 7, 8). In nonsensitized nonchallenged mice, γδ T cells did not alter baseline airway responsiveness (3).

The regulatory functions of γδ T cells in OVA-sensitized and -challenged mice can be demonstrated by depletion or reconstitution of the appropriate subpopulations. What triggers the functional involvement of the γδ T cells in AHR regulation is not clear. Based on several studies with γδ T cells, conventional Ag recognition is not a predicted mechanism (9). However, the fact that only Vγ4$^+$ γδ T cells suppress AHR suggests that specific TCR-ligand interactions play a role. Associations of TCR-Vγ expression and distinctive functions have been found in other settings as well (4, 10, 11). Furthermore, both of the γδ T cell populations implicated in AHR regulation express diverse TCRs (12, 13), and AHR suppression by the TCR-Vγ4$^+$ cells requires the peptide transporter TAP-1 (5), known to be involved in the presentation of MHC class I-associated peptide Ags of both endogenous and exogenous origins (14–17). Finally, as previously reported by others, γδ T cells that suppress IgE responses to OVA and insulin appeared to recognize these Ags specifically (18–20). Because of this contradictory evidence, we have examined AHR-suppressive γδ T cells by injecting the regulatory cells, isolated from separate donors, into recipients that exhibit AHR (3, 4). We now show that the AHR-suppressive γδ T cells require functional induction and Ag (allergen) challenge of the donor can fulfill this requirement. However, upon transfer into a recipient, the induced γδ T cells do not require restimulation with the same Ag to suppress AHR.

Materials and Methods

Animals

C57BL/6, B6.TCR-β$^{-/-}$ (C57BL/6 mice deficient in TCR-β expression, which lack all αβ T cells), and C.C3-Tlr4^{Lps-d} mice (deficient in TLR4 expression due to a mutation in the *Tlr4* gene of C3H/HeJ mice, backcrossed onto the BALB/cJ genetic background) were obtained from The Jackson Laboratory. B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ (C57BL/6 mice deficient in TCR-Vγ4 and TCR-Vγ6 expression; 10th backcross generation) and B6.TCR-β$^{-/-}$/GFP-tg mice (expressing a ubiquitin promoter-driven GFP

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Received for publication July 2, 2004. Accepted for publication December 13, 2004.

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[1] This work was supported by National Institutes of Health Grants RO1HL65410 and AI40611 (to W.K.B.), HL36557 and HL61005 (to E.W.G.), and AI44920 (to R.L.O.) and by Environmental Protection Agency Grant R825702. C.T. was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG 275/2-1).

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[4] Abbreviations used in this paper: AHR, airway hyper responsiveness; MCh, methacholine; RW, ragweed; EU, endotoxin unit; R_L, lung resistance; C_{dyn}, dynamic compliance.

0022-1767/05/$02.00

transgene) were produced in our own laboratory by the appropriate crosses. The original TCR-V$\gamma4^{-/-}/6^{-/-}$ mice (21) were a gift from Dr. K. Ikuta (Department of Medical Chemistry, Kyoto University, Kyoto, Japan) and B6-UBI-GFP-tg mice (22) were a gift from Dr. B. Schaefer (National Jewish Medical and Research center, Denver, CO). All mice were maintained on OVA-free diets. All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center. The mice were 8–14 wk old at the time of the experiments.

Allergens and LPS-depletion of OVA

The following allergen preparations were used: OVA (OVA grade V; Sigma-Aldrich), BSA (fraction V; Sigma-Aldrich), ragweed (RW, "short ragweed" (*Ambrosia artemisiifolia*), lot XP56-D18-1638.30; Greer Laboratories), and LPS-depleted OVA. LPS was removed from the above-described OVA using a protocol adapted from others (23). Briefly, OVA was dissolved in saline at 30 mg/ml and mixed with Triton X-114 (Sigma-Aldrich) at a ratio of 100:1 (v/v), and the mixture was cooled on ice. Phase separation was accomplished at 37°C and after high-speed centrifugation the upper aqueous layer was collected. Bio-Beads SM-2 (Bio-Rad) were then used to remove residual detergent from the solution (1g beads per 2 ml of aqueous layer at 4°C for 1 h). The detergent-depleted OVA solution was then sterilized with a 0.22-μm filter.

For the detection and measurement of LPS, we used the Charles River Endosafe *Limulus* Amebocyte Lysate Endochrome Assay as directed by the manufacturer. Endotoxin units (EU) per milligram were determined as: OVA (55.5 EU), LPS-depleted OVA (<1.5 EU), RW (2.8 EU), and BSA (2.4 EU).

Sensitization and airway challenge

Groups of mice were sensitized by i.p. injection with 20 μg allergen emulsified in 2.25 mg aluminum hydroxide (AlumImuject; Pierce) in a total volume of 100 μl on days 0 and 14. In the text, this treatment is referred to as "2ip." Mice were challenged via the airways with allergen (10 mg/ml in saline) for 20 min on days 28, 29, and 30 by ultrasonic nebulization (particle size 1–5 μm; De Vilbiss). In the text, this treatment is referred to as "3N" and the combined sensitization and challenge treatment as "2ip3N." In some experiments, mice were challenged via the airways on 10 consecutive days ("10N"). Lung resistance (R_L) and dynamic compliance (C_{dyn}) were assessed 48 h after the last allergen challenge. Although R_L and C_{dyn} tend to be (inversely) correlated in our model, R_L is thought to reflect primarily changes in the large airways, whereas C_{dyn} is thought to be influenced more by changes in the small airways (24). The mice were sacrificed to obtain tissues and cells for further assay.

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

V$\gamma4^+$ cells were purified from the lungs or spleens of B6.TCR-$\beta^{-/-}$ or B6.TCR-$\beta^{-/-}$/GFP-tg mice via positive selection on streptavidin-conjugated magnetic beads (Streptavidin Microbeads; Miltenyi Biotec) as previously described in detail (4). This produced a cell population containing >95% viable V$\gamma4^+$ cells as determined by two-color staining with anti-TCR-δ and Vγ4 mAbs. The purified cells were washed in PBS and resuspended at 1×10^5 cells/ml in PBS, and 1×10^4 cells/mouse were injected via the tail vein into OVA-sensitized B6.TCR-V$\gamma4^{-/-}/6^{-/-}$ mice <1 h before the first airway challenge.

Administration of anti-TCR mAbs

Hamster anti-Vγ4 mAb UC3 (25) was purified from hybridoma culture supernatant using a Protein G-Sepharose affinity column (Pharmacia). T cell depletion was achieved after injection of 200 μg of purified anti-Vγ4 mAb into the tail veins of mice 3 days before the first OVA challenge. Depletion was monitored as previously described (2, 4, 26). Sham Ab treatments were performed with the same amount of nonspecific hamster IgG (The Jackson Laboratory). Treatment with the anti-Vγ4 mAb did not significantly change αβ T cell numbers in lung and spleen (3).

Throughout this article we use the nomenclature for murine TCR-Vγ genes introduced by Tonegawa and Heilig (27).

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after provocation with aerosolized MCh using a method described by Takeda et al. (28). MCh aerosol was administered for 12 s (40 breaths/min, 500-μl tidal volume) in increasing concentrations. Maximum values of R_L and minimum values of C_{dyn} were recorded and expressed as a percentage change from baseline after saline aerosol.

Flow cytometric analysis

For flow cytometric analyses, anti-Vδ mAbs were conjugated with *N*-hydroxysuccinimido-biotin (Sigma-Aldrich) and detected with streptavidin-CyChrome3, anti-TCR-δ mAb GL3 was conjugated with PE, and anti-Vγ4 mAb UC3 was conjugated with FITC isomer I on Celite (Sigma-Aldrich). In brief, 2×10^5 nylon wool nonadherent cells/well in 96-well plates (Falcon; BD Biosciences) were stained as described previously (4) and analyzed on a FACScan flow cytometer (BD Biosciences) counting a minimum of 25,000 events per gated region. Additional Abs used include anti-CD25-PE (PC61), anti-CD44-PE (IM7), anti-CD45RB-PE (23G2), anti-CD62L-PE (MEL-14), and anti-CD69-PE (HI.2F), all from BD Pharmingen. Abs specific for Vδ4 (DV104S1; mAb GL2 (29)), Vδ5 (DV105S1; mAb F45.152 (30)), Vδ6.3 (ADV7S1; mAb 17C (31)), Vδ6λ12 (DV7S3/4/5; mAb F4.22 (30)), and Vδ8 (DV2S8; mAb B20.1.1 (32)) were used as biotin derivatives, either purchased from BD Biosciences or prepared in our laboratory.

Statistical analysis

Data are presented as means ± SEM. The unpaired *t* test was used for two group comparisons and ANOVA for analysis of differences in three or more groups. Pairwise comparisons were performed using the Tukey-Kramer honest significant difference test. Statistical significant levels were set at $p < 0.05$.

Results

Ag stimulation induces AHR-suppressive γδ T cells

We used a previously established cell transfer model in which donors were wild-type or TCR-$\beta^{-/-}$mice, and transferred cells were prepared by positive or negative selection. In this model, adoptively transferred V$\gamma4^+$ γδ T cells derived from OVA-sensitized and -challenged (2ip3N) donors diminished AHR in OVA-sensitized, γδ T cell-deficient recipients (B6.TCR-V$\gamma4^{-/-}/6^{-/-}$) (3, 4). This result, along with the finding that selective depletion of the same type of cells increased AHR (5, 8), indicated that V$\gamma4^+$ γδ T cells can function as suppressors of AHR. However, it remained unclear whether the suppressors require functional induction. To address this question, we used the same cell transfer model to investigate the influence of donor sensitization and/or challenge with OVA. We compared purified V$\gamma4^+$ γδ T cells from the lungs and spleen of B6.TCR-$\beta^{-/-}$ mice in terms of their ability to suppress AHR in OVA-sensitized and -challenged recipients (B6.TCR-V$\gamma4^{-/-}/6^{-/-}$), using as donors mice that were either untreated (naive), challenged with aerosolized OVA on 3 or 10 consecutive days (3N or 10N), sensitized with two i.p. injections of OVA/alum (2ip), or both sensitized and challenged (2ip3N). Fig. 1 compares the AHR response of recipient mice that did not received transferred cells (*A* and *B*) with those that received the type of cells indicated above each panel (*C–F*). V$\gamma4^+$ γδ T cells from naive donors had no effect, regardless of whether they were prepared from lung or spleen. In contrast, V$\gamma4^+$ γδ T cells from Ag-stimulated donors were AHR suppressive. Those from the lung strongly suppressed AHR even when the donors were only challenged (3N or 10N), and i.p. sensitization did not significantly increase their effect. Those from the spleen only suppressed AHR when the donors were both sensitized and challenged (2ip3N), but under this condition, they became as potent as the pulmonary cells. Finally, V$\gamma4^+$ cells from the spleens of mice that had been sensitized only (2ip), collected 17 days after the second i.p. OVA/alum injection to match the time point of collection in the sensitized and challenged mice, were not suppressive (Fig. 1, *E* and *F*). Thus, AHR regulatory V$\gamma4^+$ γδ T cells required functional induction and using OVA stimulation they could be induced in either lung or spleen.

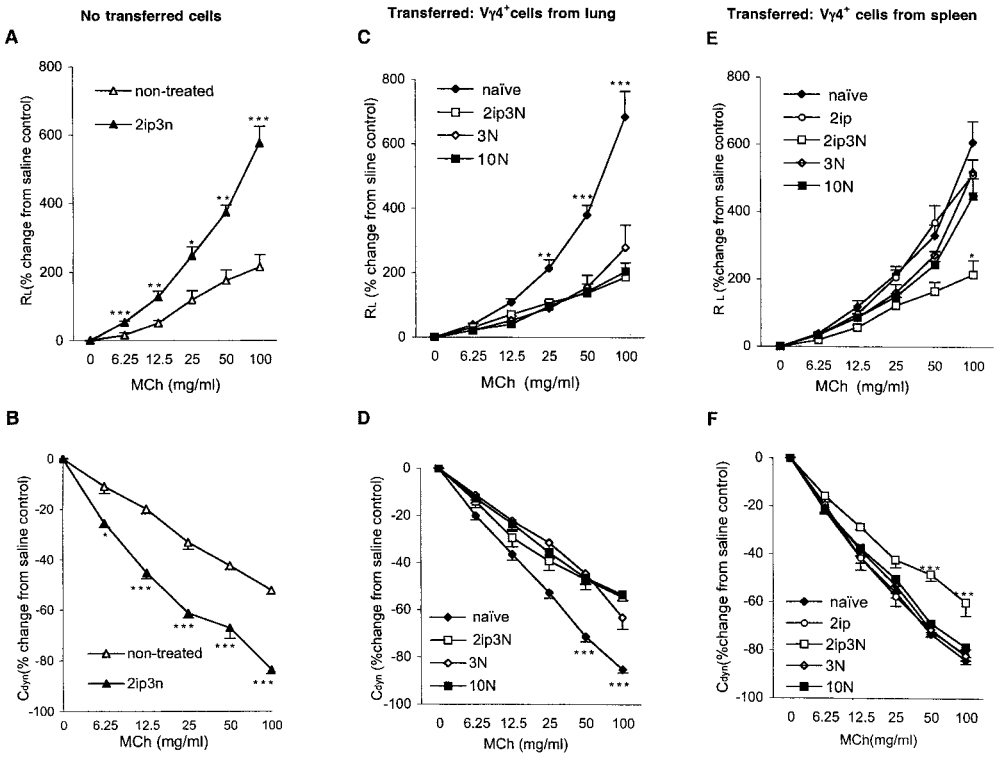

FIGURE 1. AHR-suppressive V$\gamma4^+$ $\gamma\delta$ T cells in lung and spleen require functional induction. Purified V$\gamma4^+$ $\gamma\delta$ T cells derived from the lungs or spleen of naive and OVA-sensitized and -challenged donors (B6.TCR-$\beta^{-/-}$) were compared for their ability to suppress AHR in adoptive cell transfer recipients. In all cell transfers, 10^4 purified cells were injected i.v. just before airway challenges of the recipient. Airway responses to MCh (R_L and C_{dyn}) were measured 48 h after the last OVA challenge and are shown as percent change from the responses to saline. *A* and *B,* AHR of the recipient mice (B6.TCR-V$\gamma4^{-/-}$/$6^{-/-}$) after OVA sensitization and challenge, without cell transfer. In the cell transfer experiments (*C–F*), cell donors were either left naive, challenged 3 times (3N), or 10 times (10N), only sensitized (2ip) or sensitized and challenged (2ip3N) with OVA. AHR of the OVA-sensitized and -challenged recipient mice (B6.TCR-V$\gamma4^{-/-}$/$6^{-/-}$) after transfer of cells purified from the donor lung (*C* and *D*) and spleen (*E* and *F*) is shown. Results of each group are expressed as the mean ± SEM (n = 5–10). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences are indicated by symbols (∗ comparison between nontreated and 2ip3N-treated mice (*A* and *B*), between 2ip3N-treated mice that received naive cells and any of the other stimulated cells (*C* and *D*), and between mice that received 2ip3N-prepared cells and any of the other types of cells (*E* and *F*), and the number of symbols indicates the levels of significance (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

Activation plays a role in the induction of AHR-suppressive $\gamma\delta$ *T cells*

We compared V$\gamma4^+$ $\gamma\delta$ T cells from naive and OVA-stimulated donors (2ip3N) for their expression of CD25, -44, -45RB, -62L, and -69, at the normal time point of donor cell collection, but found that expression levels had changed little. For CD45RB, an increase in the lung contrasted with a small decrease in the spleen. CD25 expression was increased in the spleen but not in the lung and only CD69 was increased in both lung and spleen (Fig. 2*A*). We then FACS-sorted V$\gamma4^+$ $\gamma\delta$ T cells derived from the spleen of OVA-sensitized and -challenged mice into CD69^{high} and CD69^{low} fractions (dividing the entire V$\gamma4^+$ population according to the median of CD69 expression into two equal portions) and compared the two fractions for their ability to suppress AHR. V$\gamma4^+$CD69^{high} cells suppressed AHR more strongly than V$\gamma4^+$CD69^{low} cells (Fig. 2, *B* and *C*), indicating that activation is involved in the induction of the AHR suppressors.

One possible consequence of functional induction and activation of the AHR-suppressive $\gamma\delta$ T cells might be an increased ability to migrate to the lung. This could be critical because the population of $\gamma\delta$ T cells inside the lung appears to regulate AHR (3, 5, 33). We wondered whether the adoptively transferred $\gamma\delta$ T cells accumulate in the lung and whether the Ag stimulation of the donor mouse can enhance the accumulation. To address this question, we purified V$\gamma4^+$ $\gamma\delta$ T cells derived from B6.TCR-$\beta^{-/-}$GFP-tg mice and confirmed that they suppressed AHR (Fig. 3, *A* and *B*). We then determined that 2×10^5 cells is the lower limit of transferred cells for reliable detection and retrieval from the recipient lung (data not shown). To compare induced and noninduced cells within the same recipient, we next mixed cells from nontransgenic B6.TCR-$\beta^{-/-}$ and B6.TCR-$\beta^{-/-}$ GFP-tg donors, leaving one donor untreated while OVA-stimulating the other (2ip3N). To avoid error due to an inherent difference between the two mouse strains, we then repeated the experiment, switching the type of donor that was OVA stimulated. Donor cells were mixed so as to establish a 1:1 ratio of V$\gamma4^+$ cells from the two sources and injected i.v. into OVA-sensitized, TCR-Vγ4-deficient recipients (B6.TCR-V$\gamma4^{-/-}$/$6^{-/-}$). A total of 4×10^5 V$\gamma4^+$ cells (2×10^5 of each type) was thus transferred into each recipient, just before the challenges as in our previous functional studies. One day after the first challenge, we collected cells from the recipient lungs (eight mice) and examined the ratio of transferred V$\gamma4^+$GFP$^+$ to V$\gamma4^+$GFP$^-$ cells. We found that only a fraction of the transferred cells lodged in the lung while others remained in the spleen (Fig. 3, *C* and *D*). Per recipient lung, we retrieved 1016 ± 166 V$\gamma4^+$ cells from naive donors (0.51 ± 0.08% of input) and 1228 ± 330 from OVA-stimulated donors (0.61 ± 0.17% of input) and per spleen, 8380 ± 1259 from naive (4.2 ± 0.63% of input) and 8620 ± 1360 from OVA-stimulated donors (4.3 ± 0.68% of input). Thus, only small fractions of the injected cells were retrieved from lung and spleen,

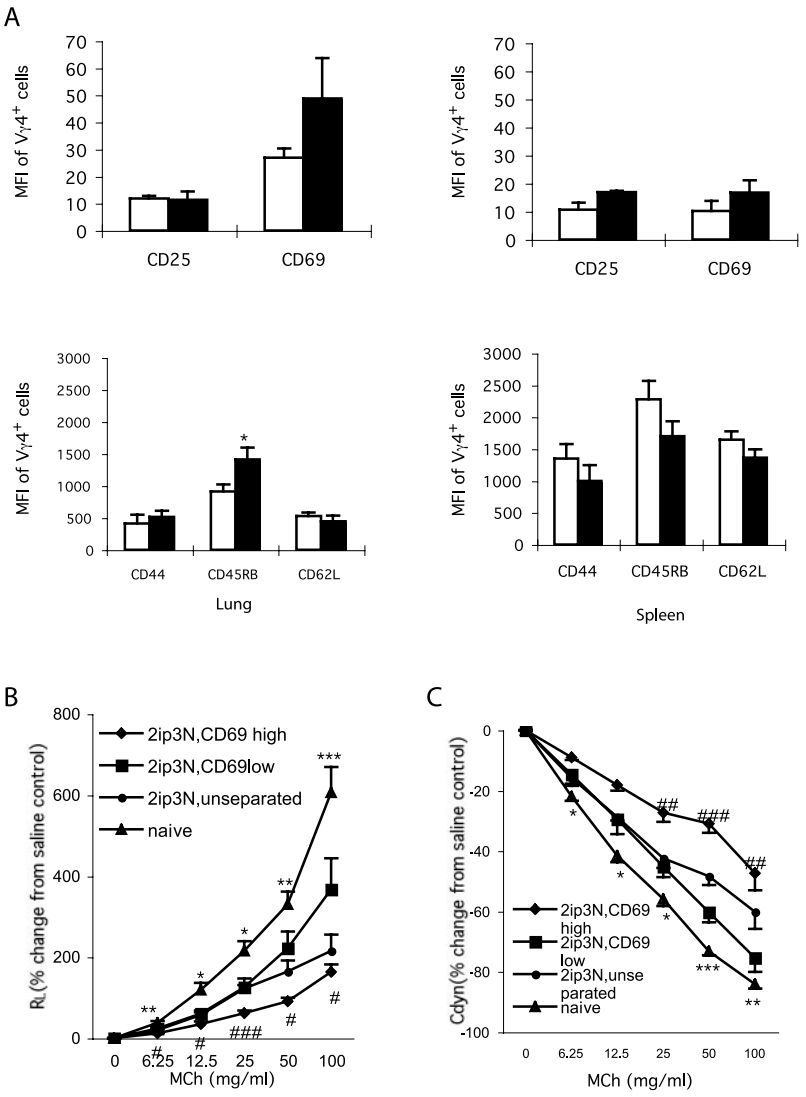

FIGURE 2. Functional induction of AHR-suppressive Vγ4$^+$ γδ T cells is associated with their activation. Vγ4$^+$ γδ T cells from OVA-sensitized and -challenged, and from naive B6.TCR-$\beta^{-/-}$ mice were compared for their expression of activation markers (*A*). □, naive and ■, OVA-stimulated mice. Mean fluorescence intensities (MFI) ± SEM from three independent determinations are shown (n = 4–6). Levels of CD69 expression, in particular, were also correlated with AHR-suppressive function (*B* and *C*). Here, purified Vγ4$^+$ γδ T cells from OVA-sensitized and -challenged donors (B6.TCR-$\beta^{-/-}$) were equally divided into CD69^{high} and CD69^{low} fractions, and the two fractions were compared for their ability to suppress AHR in OVA-stimulated recipients (B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$). Experimental conditions were as described in Fig. 1. Suppression of AHR after transfer of either total TCR-Vγ4$^+$ γδ T cells, naive or 2ip3N-stimulated, or of 2ip3N-stimulated CD69^{high} or CD69^{low} Vγ4$^+$ γδ T cells is shown. Results of each group are expressed as the mean ± SEM (n = 4–9 in *B* and *C*). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences are indicated by symbols (∗ comparison between mice that received naive or 2ip3N-stimulated, unseparated cells; # comparison between mice that received 2ip3N-treated CD69^{high} or CD69 ^{low} cells).

but the ratios of the cells from OVA-stimulated and naive donors (1.1 ± 0.2 in the lung and 1.0 ± 0.1 in the spleen) did not significantly differ from the ratio at the time of inoculation.

Induction of AHR-suppressive Vγ4$^+$ γδ T cells does not change their Vδ expression

Vγ4$^+$ γδ T cells display a strong bias for Vδ4 and Vδ5 expression. To test whether this bias plays a role in their function as AHR suppressors, we divided Vγ4$^+$ cells from OVA-stimulated donors (2ip3N) into Vδ4/5/8$^+$ and Vδ4/5/8$^-$ fractions (approximately equal in relative frequencies). Both types of cells suppressed AHR in the recipients, without significant difference (Fig. 4, *A* and *B*). Also, we compared the occurrence of expressed Vδ's in Vγ4$^+$ γδ T cells of naive and OVA-stimulated (2ip3N) B6.TCR-$\beta^{-/-}$ mice, in lung and spleen, using a collection of Vδ-specific mAbs (Fig. 4, *C* and *D*). Relative frequencies of Vγ4/Vδ pairs in lung and spleen were very similar, and this expression pattern remained essentially unchanged following OVA stimulation. Together, these experiments suggested that the distinctive Vδ bias of Vγ4$^+$ γδ T cells has no particular significance with regard to their function as AHR suppressors.

The AHR-inducing allergen is not required in inducing Vγ4$^+$ γδ T cells to AHR regulatory function

To test whether matched Ags are required in cell donors and recipients, we compared donors sensitized and challenged (2ip3N) with three different and unrelated Ags, OVA, BSA, and RW for their ability to induce Vγ4$^+$ γδ T cells capable of suppressing AHR in OVA-stimulated recipients (Fig. 5, *A–D*). The use of B6.TCR-$\beta^{-/-}$ donors eliminated any influence of Ag-specific αβ T cells. Donors treated with any of the three Ag preparations produced Vγ4$^+$ γδ T cells, in lung and spleen, that suppressed AHR in OVA-sensitized and -challenged recipients.

This indicated that matched Ags are not required. However, the spleen of donors subjected to the process of sensitization and challenge without any Ag (adjuvant plus saline; the lung was not examined) did not yield Vγ4$^+$ cells that suppressed AHR (Fig. 5, *C* and *D*). This suggested that Ag is required or, alternatively, that common components contaminating the various Ag preparations might induce the suppressive Vγ4$^+$ γδ T cells. We examined the role of LPS because of its known effects on AHR and on γδ T cells (34–37). However, only the OVA preparation used in our experiments contained LPS in substantial quantities (>50 EU/mg), whereas BSA and RW contained only small amounts (2.4 and 2.8

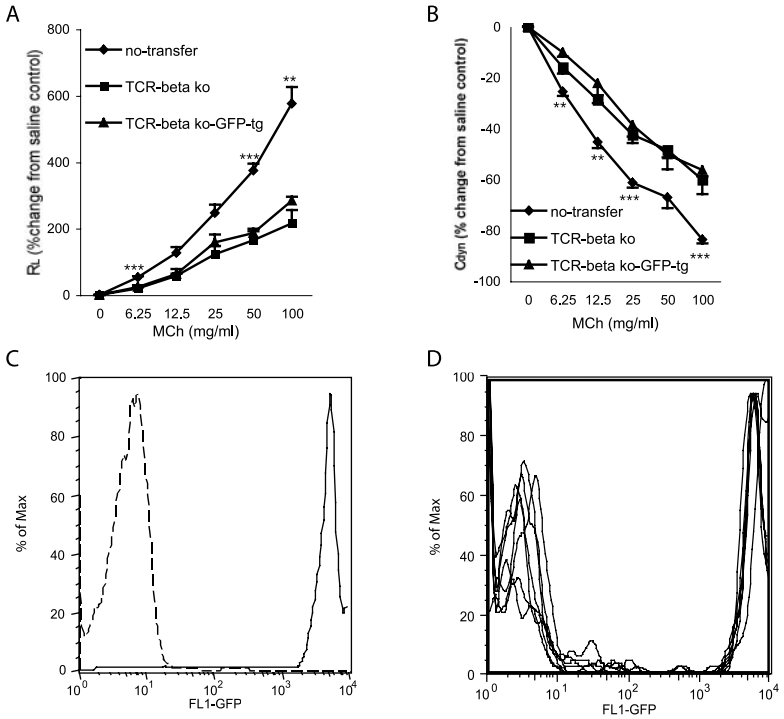

FIGURE 3. Functional induction of AHR-suppressive V$\gamma4^+$ $\gamma\delta$ T cells does not increase their accumulation in the lung. *A* and *B,* GFP-tg V$\gamma4^+$ $\gamma\delta$ T cells have retained their AHR-suppressive potential. Donor mice (B6.TCR-$\beta^{-/-}$ and B6.TCR-$\beta^{-/-}$/GFP-tg) were sensitized and challenged with OVA. and 10^4 purified V$\gamma4^+$ $\gamma\delta$ T cells were transferred into sensitized and challenged recipients, and recipient AHR was measured as in Fig. 1. AHR to MCh (R_L and C_{dyn}) is shown. Results of each group are expressed as the mean ± SEM (n = 4–9). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences are indicated as in Fig. 1 (∗ comparison between mice that received no transferred cells and those that received either B6.TCR-$\beta^{-/-}$ or B6.TCR-$\beta^{-/-}$/GFP-tg-derived cells). *C* and *D,* Donor mice (B6.TCR-$\beta^{-/-}$ and B6.TCR-$\beta^{-/-}$/GFP-tg) were either left untreated or sensitized and challenged with OVA. Nylon wool nonadherent spleen cells from the two types of donors (one sensitized and challenged, the other naive) were mixed to generate an inoculum containing 2×10^5 V$\gamma4^+$ cells of each donor type or a total of 4×10^5 V$\gamma4^+$ cells, of which one-half express GFP. *C,* Superimposed fluorescence profiles of gated V$\gamma4^+$ cells of the two types of donor. The mixed cells were injected i.v. into OVA-sensitized recipients (B6.TCR-V$\gamma4^{-/-}$/$6^{-/-}$) just before the first challenge, and the composition of V$\gamma4^+$ $\gamma\delta$ T cells in the recipient lung was analyzed cytofluorometrically 1 day after the cell transfer. *D,* Fluorescence profiles of V$\gamma4^+$ cells retrieved from the recipient lungs 1 day after the cell transfer (profiles of seven individual recipients superimposed); two experiments (experiment 1: B6.TCR-$\beta^{-/-}$ mice were 2ip3N treated and B6.TCR-$\beta^{-/-}$/GFP-tg mice were left untreated; experiment 2: B6.TCR-$\beta^{-/-}$/GFP-tg were 2ip3N treated and B6.TCR-$\beta^{-/-}$ mice were left untreated) yielded essentially the same result as detailed in the text.

EU/mg, respectively). Because LPS might be critical in the treatment of the recipients (all of which received OVA), we next prepared LPS-depleted OVA (<1.5 EU/mg) and tested it for the induction of AHR, as well as for the AHR regulatory function of V$\gamma4^+$ $\gamma\delta$ T cells (Fig. 5, *E* and *F*). In this experiment, AHR regulatory $\gamma\delta$ T cells were assessed indirectly by treating the OVA-stimulated mice with anti V$\gamma4$ mAbs as described previously (5). The LPS-depleted OVA induced AHR in a manner similar to non-depleted OVA preparations (3). Depletion of V$\gamma4^+$ $\gamma\delta$ T cells further increased the AHR response, indicating that the regulatory function of the V$\gamma4^+$ $\gamma\delta$ T cells was not affected by the absence of LPS (3). Because these experiments do not exclude that very small amounts of LPS are needed, we also examined mice deficient in the LPS receptor Tlr4 (Fig. 5, *G* and *H*). These mice (C.C3-Tlr4$^{Lps\text{-}d}$) had a different genetic background (BALB/cJ), but we have shown previously that V$\gamma4^+$ $\gamma\delta$ T cells regulate AHR in mice of this background also (3). OVA induced AHR in the Tlr4-deficient mice in a manner similar to the wild-type mice, and treatment with anti-V$\gamma4$ mAb further increased the AHR response in the absence of any LPS-driven signal through Tlr4 (Fig. 5, *G* and *H*). The absence of a requirement for matched Ags in the induction of AHR-suppressive $\gamma\delta$ T cells sets the induction process apart from conventional Ag priming and the difference does not seem to be merely due to non-Ag-specific LPS stimulation.

Discussion

Previously, we have reported that $\gamma\delta$ T cells capable of suppressing AHR belong to the TCR-V$\gamma4^+$ subset (3–5, 8, 33). We now demonstrate that these cells require functional induction and that Ag sensitization and/or challenge can induce these cells to become AHR suppressors. However, in an adoptive cell transfer system, we found that these cells did not need to encounter within the recipient animal the same Ag that was present at their induction in the donor animal to function as AHR suppressors. This absence of an Ag-specific recall requirement sets the process of functional induction of TCR-V$\gamma4^+$ AHR suppressors apart from conventional Ag priming and challenge. To make the distinction, we refer to it as "preparation."

In this study, we used an adoptive cell transfer system in which purified $\gamma\delta$ T cells were injected i.v. into OVA-sensitized, $\gamma\delta$ T cell-deficient recipients (B6.TCR-V$\gamma4^{-/-}$/$6^{-/-}$), just before three airway challenges with nebulized OVA (3). These recipients lack TCR-V$\gamma4^+$ and TCR-V$\gamma6^+$ $\gamma\delta$ T cells (21, 38) and exhibit strong AHR in response to OVA sensitization and challenge (3). Their AHR is suppressed upon transfer of functionally competent TCR-V$\gamma4^+$ $\gamma\delta$ T cells derived from OVA-sensitized and challenged donors (C57BL/6 wild-type or B6.TCR-$\beta^{-/-}$). The suppressive cells can be enriched by positive or negative selection, indicating

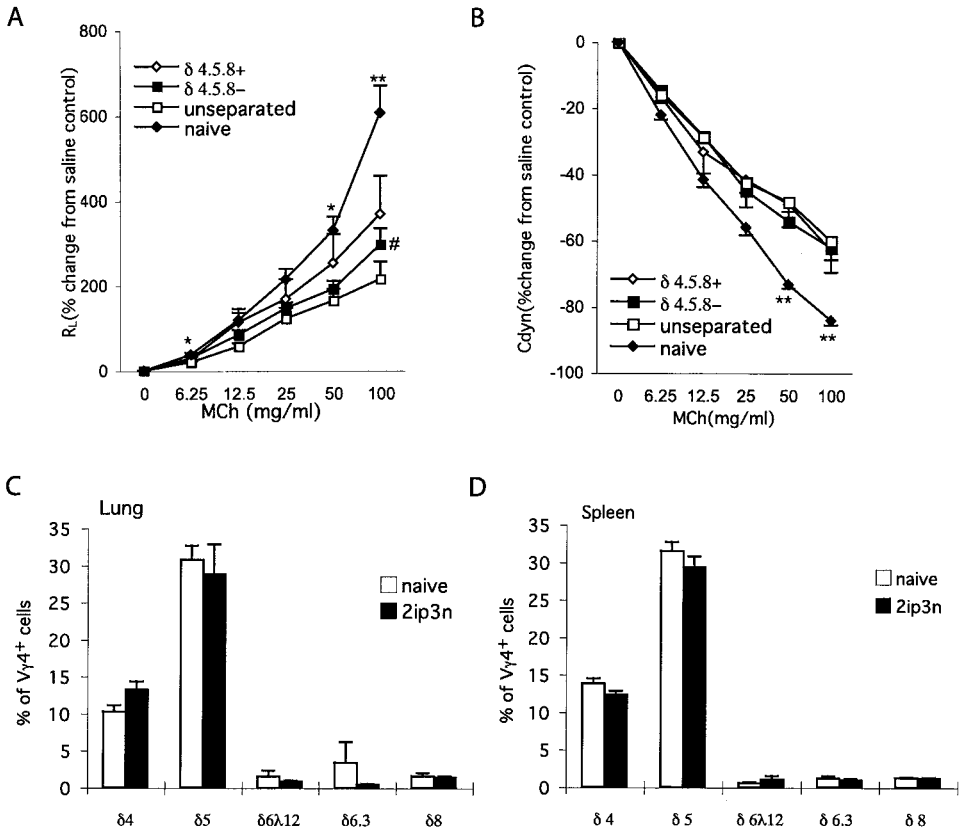

FIGURE 4. The biased TCR-Vδ expression in Vγ4$^+$ γδ T cells is not related to their AHR-suppressive function. *A* and *B,* Purified Vγ4$^+$ γδ T cells from OVA-sensitized and -challenged donors (B6.TCR-$\beta^{-/-}$) were further divided into Vδ4,5,8$^+$ and Vδ4,5,8$^-$ fractions, and the two fractions were compared for their ability to suppress AHR in OVA-stimulated recipients (B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$). Experimental conditions were as described in Fig. 1. Results of each group are expressed as the mean ± SEM (n = 4–6 in *A* and *B*). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences are indicated by symbols (∗ comparison between mice that received naive or 2ip3N-stimulated, unseparated cells; # comparison between mice that received unseparated naive or 2ip3N-treated cells fractionated based on their Vδ expression. There was no significant difference between the two Vδ -defined groups.) *C,* Nylon wool nonadherent cells, isolated from lung and spleen of OVA-sensitized and -challenged B6.TCR-$\beta^{-/-}$ mice, harvested 72 h after the last OVA challenge, or of naive B6.TCR-$\beta^{-/-}$ mice, were stained with mAbs specific for TCR-δ, TCR-Vγ4, and TCR-Vδs as indicated. Stained cells were analyzed by flow cytometry. Results of each group are expressed as the mean ± SEM (n = 3–8). No significant differences were found.

that the AHR-suppressive function is not a result of the Abs/magnetic beads used in the selection. As few as 1×10^4 transferred cells can reduce AHR to background levels (3). The number of actual AHR suppressors is probably smaller still (discussed below).

In this model, we have tested whether Ag treatment of the donors is a prerequisite for the development of the AHR-suppressive γδ T cells. Interestingly, in a study also relying on airway challenge, McMenamin et al. (18) concluded that small numbers of allergen-specific γδ T cells suppressed IgE responses to the challenge Ag (OVA). Our earlier finding that the suppressor cells distinctively expressed TCR-Vγ4 is consistent in principle with allergen specificity (3, 5). Vγ4 is expressed by a subset of γδ T cells in mice that arise late in development and express diverse TCRs (Refs. 30, 39, and 40 and this study), and peripheral selection of TCRs within the Vγ4$^+$ cell subset has been observed by others (41). However, our data now show that although functional induction of the AHR regulatory Vγ4$^+$ γδ T cells is needed, cell donor priming with the Ag used in the recipient is not required. This is inconsistent with conventional Ag-specific T cell responses that rely on the expansion of Ag-specific clones. Although there could exist an intrinsic bias within the TCR-Vγ4$^+$ subset for OVA recognition, it appears more likely that these cells are not allergen specific. The Ags used for donor sensitization and challenge in our study, OVA, BSA, and RW, are entirely unrelated. They also varied greatly in their LPS contents and yet all induced Vγ4$^+$ γδ T cells capable of suppressing AHR in the OVA-sensitized and -challenged recipients. Our model may be comparably insensitive to LPS stimulation because an adjuvant is used during the sensitization (42). The occurrence of Vγ4 γδ T cell-regulated AHR in the absence of the LPS receptor Tlr4, or induced with LPS-depleted OVA, further supports the notion that LPS is not essential. Importantly, the possibility that functional induction occurs in the recipient can be excluded because cells derived from naive donors, or from donors sensitized and challenged with adjuvant/saline only, were unable to regulate AHR in the OVA-challenged recipients.

Consistent with our earlier studies on γδ T cell-depleted mice (2, 5), 3N OVA challenge was sufficient to induce transferable AHR-suppressive cells in the donor lung. In contrast, induction of AHR-suppressive cells in the donor spleen required both sensitization and challenge. This difference might indicate a dependence of the splenic population on alum-inducible Gr1$^+$ accessory cells, as recently described for B lymphocytes (43).

3N OVA challenge was not sufficient to induce AHR suppression in recipients of cells transferred from naive donors, indicating that the adoptively transferred cells are not functionally equivalent to the endogenous cells resident in the lung. What might explain

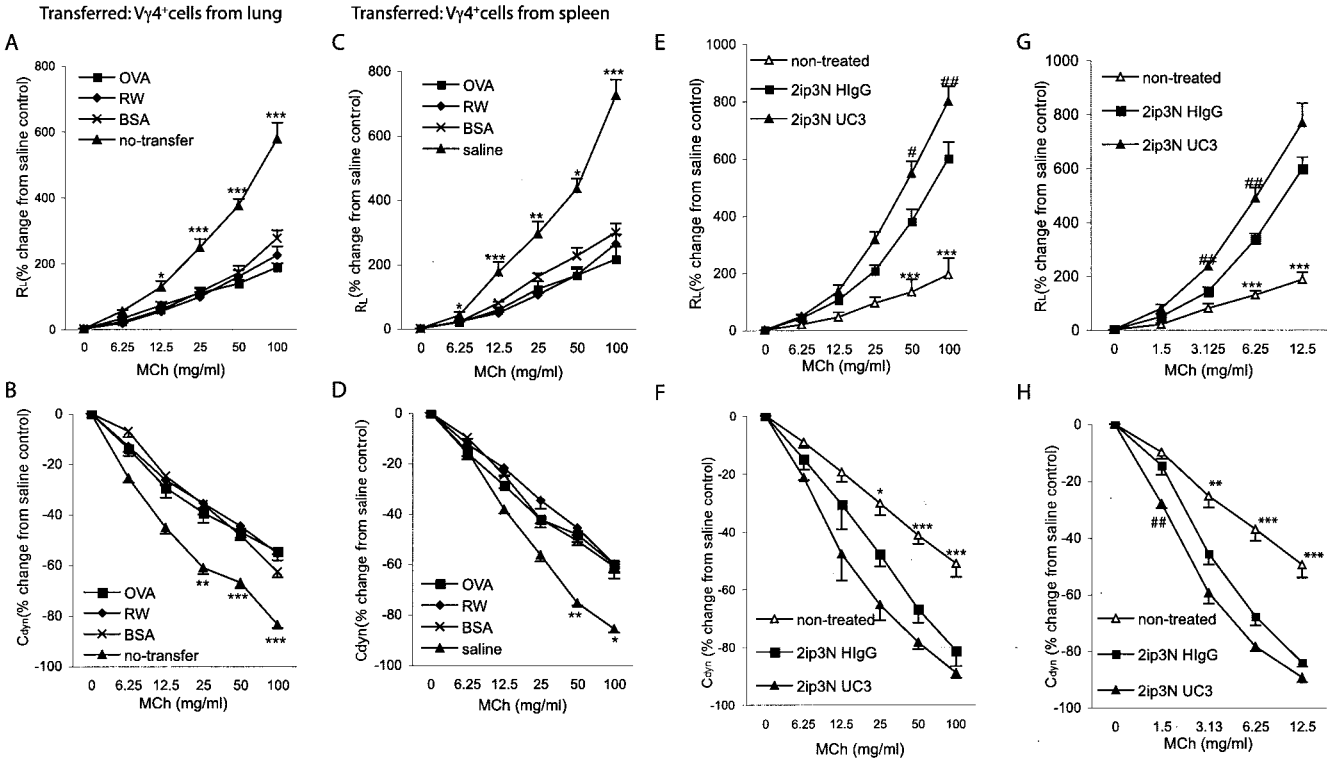

FIGURE 5. Mismatched allergens "prepare" V$\gamma4^+$ $\gamma\delta$ T cells for AHR suppression. Purified V$\gamma4^+$ $\gamma\delta$ T cells derived from the lungs or spleen of donors (B6.TCR-$\beta^{-/-}$) sensitized and challenged with OVA, RW, or BSA or saline alone (spleen only) were compared for their ability to suppress AHR in adoptive cell transfer recipients. Experimental conditions were as described in Fig. 1. AHR of the OVA-sensitized and -challenged recipient mice (B6.TCR-V$\gamma4^{-/-}$/$6^{-/-}$) after transfer of cells purified from the donor lung (*A* and *B*) and spleen (*C* and *D*) is shown. Results of each group are expressed as the mean ± SEM ($n = 6–8$ in *A* and *B* and $n = 4–8$ in *C* and *D*). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences are indicated by symbols as in Fig. 1 (∗ comparison between 2ip3N-treated mice that received no cells and any of the other groups (*A* and *B*), and between 2ip3N-treated mice that received cells from adjuvant/saline-treated donors and any of the other groups (*C* and *D*). The number of symbols indicates the levels of significance (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$). For further reference, compare *C* and *D* also with Fig. 1, *A* and *B*. *E–H* address the role of LPS. Mice were OVA stimulated and depleted of V$\gamma4^+$ $\gamma\delta$ T cells using Ab treatments. Airway responses to MCh (R_L and C_{dyn}) were measured 48 h after the last OVA challenge and are shown as percent change from the responses to saline. *E* and *F,* AHR of C57BL/6 mice after sensitization and challenge (2ip3N) with LPS-depleted OVA and additional treatments with hamster mAb UC3 for depletion of V$\gamma4^+$ $\gamma\delta$ T cells or hamster IgG as a control. *G* and *H,* AHR of Tlr4-deficient mice (BALB/cJ genetic background) after OVA sensitization and challenge and the same Ab treatments. Results of each group are expressed as the mean ± SEM ($n = 4$ in *E* and *F* and $n = 5$ in *G* and *H*). No significant differences in baseline responses to saline were observed in any of these groups. Significant differences are indicated as in Fig. 1 (∗ comparison between nontreated and 2ip3N/HIgG or/mAb UC3-treated mice; # comparison between 2ip3N/HIgG and 2ip3N/mAb UC3-treated mice).

this difference? In this study, we found that only a small percentage of i.v. transferred V$\gamma4^+$ cells arrive in the lung of the OVA-sensitized and -challenged recipients. In an earlier study, we showed that only V$\gamma4^+$ cells within the lung actually mediate AHR suppression (5). The endogenous pulmonary population of V$\gamma4^+$ cells in normal adult C57BL/6 mice consists of $2–4 \times 10^4$ cells, based on cell retrieval (3, 5). Therefore, transferred AHR suppression may rely on a still smaller set of suppressors than endogenous AHR suppression, and functional demands on the suppressors may be greater. The transferred cells also may not be optimally localized or may even be altered during the purification. These differences are of some concern but at the same time, they were essential to using the transfer model for studying the effect of Ag stimulation in the donor.

What might be the significance of the induced regulatory function? Our data indicate that "prepared" V$\gamma4^+$ T cells do not need to encounter the same priming Ag in the adoptive recipient to cause suppression. Interestingly, it has been shown recently that chronic inhaled OVA exposure induces "inhalational" tolerance that is Ag nonspecific yet Ag dependent (44), and we have previously reported that extensive inhaled OVA exposure results in $\gamma\delta$ T cell-dependent suppression of AHR (8). Thus, AHR-suppressive $\gamma\delta$ T cells could be mediators of this Ag-nonspecific inhalational tolerance. In the cell transfer model studied here, it is even questionable whether the $\gamma\delta$ TCR is still required during the process of functional preparation, because preparation was not associated with marked changes in the TCR-δ repertoire. This is somewhat reminiscent of the ability of memory CD8$^+$ T cells to provide inducible protection in the absence of cognate Ag (45). Our observation thus might support the concept that peripheral $\gamma\delta$ T cells resemble $\alpha\beta$ T memory cells (46). However, in the process of inducing AHR-suppressive function, during preparation, the allergens themselves or some component within them do seem to play a role because sham sensitization and challenge (with saline and adjuvant only, no allergen) were not sufficient to induce function. For example, the process of allergen uptake by APCs could have a nonspecific activating effect that results in stimulatory signals for the $\gamma\delta$ T cells. LPS is a contaminant in the OVA preparations, with documented effects on AHR (34, 37, 47) and on $\gamma\delta$ T cells (35, 36, 48). However, we did not find that LPS plays a role in the induction of the suppressors. Whether other contaminants are important remains to be investigated. Clearly, the mere absence of a requirement for matched Ags in donors and recipients does not rule out a role for the $\gamma\delta$ TCR. Even if the TCR is not involved in allergen

recognition, it might still be required during preparation of the AHR-suppressive γδ T cells or for their development before Ag stimulation. Conceivably, autologous ligands for the γδ TCR could play a role. Our earlier study showing that AHR regulatory $V\gamma4^+$ γδ T cells are nonfunctional in β_2-microglobulin-negative or peptide transporter TAP-1-negative mice is consistent with such a possibility (5). Moreover, other studies have implicated $V\gamma4^+$ γδ T cells in particular in the recognition of MHC class I and related molecules (49, 50). MHC class I recognition via the TCR probably would involve both TCR-Vγ and -Vδ. However, based on the cell transfers, more than one Vδ must be compatible with the AHR-suppressive function of the $V\gamma4^+$ subset. Since we did not find evidence for TCR-Vδ selection within $V\gamma4^+$ cells during Ag stimulation of the donors or the phase of functional preparation of the AHR suppressors, it seems possible that the TCR only matters before preparation, e.g., during the ontogenetic establishment of the $V\gamma4^+$ subset.

We have now demonstrated that a process of preparation is required in the generation of the AHR-suppressive γδ T cells. This process involves the activation of these cells (51–53) but apparently not the selective priming of Ag-specific clones. The γδ TCR requirement might come into play at earlier developmental stages, before the encounters with allergens.

Acknowledgments

We acknowledge the help of William Townend and Shirley Sobus with cell sorting and of M. Kemal Aydintug with the preparation of purified Abs. We are also grateful to Anthony Joetham for help with the allergen preparations. Philippa Marrack suggested the term "preparation" in contrast to "priming," in an attempt to distinguish the requirements of γδ T cells from those of conventional Ag-specific αβ T cells.

Disclosures

The authors have no financial conflict of interest.

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