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Antigen-Specific Regulation of IgE Antibodies by Non-Antigen-Specific $\gamma\delta$ T Cells

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We re-examined the observation that $\gamma\delta$ T cells, when transferred from mice tolerized to an inhaled conventional Ag, suppress the allergic IgE response to this Ag specifically. Using OVA and hen egg lysozyme in crisscross fashion, we confirmed the Ag-specific IgE-regulatory effect of the $\gamma\delta$ T cells. Although only $V\gamma 4^+$ $\gamma\delta$ T cells are regulators, the Ag specificity does not stem from specificity of their $\gamma\delta$ TCRs. Instead, the $V\gamma 4^+$ $\gamma\delta$ T cells failed to respond to either Ag, but rapidly acquired Ag-specific regulatory function in vivo following i.v. injection of non-T cells derived from the spleen of Ag-tolerized mice. This correlated with their in vivo Ag acquisition from i.v. injected Ag-loaded splenic non-T cells, and in vivo transfer of membrane label provided evidence for direct contact between the injected splenic non-T cells and the $V\gamma 4^+$ $\gamma\delta$ T cells. Together, our data suggest that Ag itself, when acquired by $\gamma\delta$ T cells, directs the specificity of their IgE suppression. *The Journal of Immunology*, 2013, 190: 000–000.

Mucosal allergen exposure can induce a state of tolerance, in which subsequent immunization with the same allergen fails to elicit an allergic response (1, 2). This tolerance is important because it prevents potentially harmful immunity, and can be used in the desensitization of individuals suffering from atopy (3, 4). However, direct allergen exposure during the process of tolerization carries the risk of accidental hypersensitization, emphasizing the need for a better understanding of underlying mechanisms. Nonresponsiveness induced through mucosal allergen exposure may be mediated by conventional T cell tolerance (5), engagement of regulatory $\alpha\beta$ T cells (6), or inducible inhibitory $\gamma\delta$ T cells (7–9).

The $\gamma\delta$ T cells are increasingly appreciated as critical players in the immune responses (10). Like B cells and $\alpha\beta$ T cells, they express selectable (adaptive) Ag receptors, although the specific modalities of their Ag recognition remain to be established. There is no evidence that $\gamma\delta$ T cells recognize protein Ags such as OVA or hen egg lysozyme (HEL) when they are processed and presented, although their ability to recognize native proteins has been described (11). However, studies in vitro indicated that $\gamma\delta$ T cells can also process and present protein Ags, without having to recognize these Ags via their TCR (12, 13). In this regard, $\gamma\delta$ T cells resemble professional APCs.

The $\gamma\delta$ T cells have been shown to regulate allergic responses (7, 14, 15). Moreover, subsets of $\gamma\delta$ T cells can play different regulatory roles, either inhibiting or enhancing disease pathology (16), and, in mouse models of allergic disease, $V\gamma 4^+$ $\gamma\delta$ T cells suppressed and $V\gamma 1^+$ $\gamma\delta$ T cells promoted allergic airway hyperresponsiveness (17). The $\gamma\delta$ T cells have been shown in particular to regulate IgE Abs (7, 15, 18).

Selective tolerance to OVA can be induced by exposing mice to inhaled aerosolized OVA. McMenamin et al. (7) reported that IgE-suppressive $\gamma\delta$ T cells derived from the spleen of mice tolerized by repeated inhalation of OVA were responsible for this tolerance. When such $\gamma\delta$ T cells were adoptively transferred, very small numbers mediated the suppressive effect, which seemed to depend on IFN- γ . In a prior study, we confirmed these results and determined that the IgE suppressors are $V\gamma 4^+CD8^+$ $\gamma\delta$ T cells. These cells had to be derived from OVA inhalation-treated mice to develop their IFN- γ -dependent IgE-regulatory function (8). In contrast, $V\gamma 4^+CD8^-$ $\gamma\delta$ T cells had no inducible regulatory function, whereas $V\gamma 1^+$ $\gamma\delta$ T cells from nontreated (NT) mice increased the IgE response, but lost this capability following the inhalation treatment (8, 19).

McMenamin et al. (7) also claimed that the OVA inhalation-induced IgE-suppressive $\gamma\delta$ T cells were Ag specific, because they failed to suppress an IgE response to the house dust mite allergen DerP1. However, whether DerP1, if used as a tolerogen, could elicit specific $\gamma\delta$ T cell-mediated suppression of a DerP1 IgE response was not shown. Because of this lack of information, and because $\gamma\delta$ TCRs specific for conventional Ags such as OVA have not been described in general, we decided to investigate the proposed Ag specificity of $\gamma\delta$ T cell-mediated IgE suppression. Instead of OVA and DerP1, which due to their different origins and structure might elicit immune responses by different mechanisms (20), we used the more closely matched Ags OVA and HEL, both of which are avian proteins. This allowed a better focus on specificity alone. We compared the development of regulatory $\gamma\delta$ T cells induced by repeated inhalation of these two Ags in the cell transfer model introduced by McMenamin et al. (7), with minor modifications, and examined the effect of $\gamma\delta$ T cells induced by each Ag on the IgE responses induced by the same or the other

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Abbreviations used in this article: DC, dendritic cell; DQ-OVA, high-density FITC-labeled self-quenched OVA; HEL, hen egg lysozyme; 10N, tolerogenic treatment with 10 aerosol inhalations; NT, nontreated.

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Ag. We thus confirmed the Ag specificity of inhalation-induced $\gamma\delta$ T cells, and this led us to ask the question of how the IgE-regulatory $\gamma\delta$ T cells acquire their specificity. Inhaled Ags and allergens can cross the pulmonary epithelial barrier intact and enter blood circulation (21, 22). Blood-borne Ags are filtered and processed in the spleen (23). Therefore, we then examined the possibility that the regulatory $\gamma\delta$ T cells are not induced by direct exposure to the inhaled Ags but rather by interaction with a splenocyte involved in this filter/processing function. Our data support this notion, and indicate that such intermediate cells induce Ag-specific IgE-regulatory $\gamma\delta$ T cells with rapid kinetics. Furthermore, evidence of Ag and cell membrane transfer suggests that the Ag specificity of the inducible regulatory $\gamma\delta$ T cells depends on their ability to acquire the Ag from their inducers rather than being determined by the specificity of the $\gamma\delta$ TCR.

Materials and Methods

Animals

Female C57BL/6 mice and several mutant strains with the same genetic background (B6.TCR- $\beta^{-/-}$, B6.TCR- $\delta^{-/-}$, B6.TCR- $\beta^{-/-}\delta^{-/-}$, B6.MyD88 $^{-/-}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.MHCII $^{-/-}$ was provided by P. Marrack (National Jewish Health). All mice were 8–12 wk old at the time of the experiments. Mice were maintained on an OVA-free diet, and were cared for at National Jewish Health (Denver, CO), following guidelines for immune-deficient animals. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Ag exposure and immunization

The animals were exposed to 1% OVA (w/v) ($5\times$ crystalline; EMD Biosciences, La Jolla, CA) or 1% HEL (w/v) (L6876; Sigma-Aldrich) or 1% BSA (w/v) (A7906; Sigma-Aldrich) in saline by aerosol inhalation for 30 min daily, 5 d per week for 2 wk, and subsequently once per week (designated 10N), following a method described by others (7). To induce Ag-specific IgE, mice were immunized by i.p. injection of 10 μ g OVA or HEL in aluminum hydroxide (AlumImject; Pierce, Rockford, IL) (7).

T cell purification from spleen and adoptive T cell transfer

Spleens were harvested from NT or tolerogenic treatment with 10 aerosol inhalations (10N) 10N-treated mice at the time of the experiments. A suspension of splenocytes was prepared by mechanical dispersion. Cell suspensions were treated with Gey's red cell lysis solution and passed through nylon wool columns to obtain T lymphocyte-enriched cell preparations containing $>75\%$ T cells, as previously described (17). Total cell counts were determined using a Coulter counter. Splenic nylon wool-nonadherent cells from NT and 10N mice (C57BL/6, B6.TCR- $\beta^{-/-}$, B6.MHCII $^{-/-}$) were incubated with biotinylated anti-V γ 4 mAb (clone UC3) for 15 min at 4°C, washed and incubated with streptavidin-conjugated magnetic beads (Streptavidin Microbeads; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C, and passed through magnetic columns to purify V γ 4 $^{+}$ cells, as previously described in detail (24). This produced a cell population containing $>85\%$ V γ 4 $^{+}$ viable cells, as determined by dye exclusion and two-color staining with anti-TCR- δ and anti-V γ 4 mAbs. These splenic V γ 4 $^{+}$ cells were washed in PBS and resuspended to a concentration of 1.5×10^5 cells/ml in PBS, and 3×10^4 cells/mouse were injected in 200 μ l PBS via the tail vein into B6.TCR- $\delta^{-/-}$ mice directly before the OVA immunization. The optimal number of transferred $\gamma\delta$ T cells was determined in the preceding study (8).

Throughout this article, we use the nomenclature for murine TCR-V γ genes introduced by Heilig and Tonegawa (25).

Transfer of splenocytes and non-T cells

Whole spleens from NT or aerosol-treated (10N) mice (B6.TCR- $\delta^{-/-}$, B6.TCR- $\beta^{-/-}\delta^{-/-}$, B6.MyD88 $^{-/-}$) were harvested at the time of the experiments and mechanically dispersed to generate single-cell suspensions. Cell suspensions were treated with Gey's red cell lysis solution. Total cell counts were determined using a Coulter counter. Splenic cells either were used directly for cell transfers or further purified prior to cell transfer. For further purification, splenocytes were incubated with FITC-conjugated anti-CD11c mAb (clone HL3) and PE-conjugated anti-CD8 α (clone 53-6.7; BD Pharmingen) (20 min at 4°C) and then washed. Cells were then sorted based on their expression of CD11c and CD8 α using

a MoFlo XDP cell sorter (DakoCytomation). Purified cells were washed in PBS and resuspended in PBS, and 2×10^5 to 1×10^6 cells/mouse were injected in 100 μ l PBS via the tail vein into B6.TCR- $\delta^{-/-}$ mice.

Flow cytometric analysis

For flow cytometric analyses, whole spleen or nylon wool-nonadherent cells (2×10^5 /well) were stained in 96-well plates (Falcon; BD Biosciences, Franklin Lakes, NJ) for TCR, CD8, and MHC class II (MHCII) expression using an allophycocyanin-labeled pan anti-TCR δ mAb (clone GL3) and FITC-conjugated anti-V γ 4 mAb (clone UC3) or anti-V γ 1 mAb (clone 2.11), followed by PE-conjugated anti-MHCII mAb (M5/114) and biotinylated anti-CD8 α mAb (clone 53-6.7) plus streptavidin conjugated with allophycocyanin-Cy7. The early activation Ag CD69 was detected with mAb H1.2F3, L-selectin (CD62L) with mAb MEL-14, and CD44 (Pgp-1) with mAb (clone IM7). In the experiments of Fig. 3, whole spleen cells were gated based on forward and side scatter (lymphocyte gate), forward scatter height and amplitude, and side scatter width and amplitude (doublets), as well as expression of various non-T cell markers, including CD11c (mAb clone HL3), CD11b (mAb clone (M1/70), Gr1 (mAb clone RB6-8C5), F4/80 (mAb clone BM8), and CD19 (mAb clone ID3), as indicated. All samples were analyzed on FACScan (BD Biosciences) or LSRII flow cytometers, counting a minimum of 25,000 events per gated region, and the data were processed using FlowJo 6.4.1 software (Tree Star).

Determination of serum IgE levels

Sera were harvested on day 14 after the i.p. immunization with OVA/alum. For OVA-specific serum IgE determinations, plates were coated overnight at 4°C with 2 μ g/ml rat anti-mouse IgE Ab (clone R35-72; BD Biosciences). The serum samples were then added, and biotin-labeled OVA was subsequently added to the wells. Prior to biotinylation, OVA was first dialyzed at 4°C overnight against 0.1 M borate buffer (pH 8.4). Biotinylated OVA was then prepared by reacting 1 ml OVA in PBS (1 mg/ml) with 150 μ l N-hydroxy-succinimido-biotin in DMSO (1 mg/ml) for 4 h at room temperature, followed by overnight dialysis against PBS at 4°C. The bound OVA-biotin was detected with streptavidin-conjugated HRP (BD Biosciences), followed by 100 μ l/well tetramethylbenzidine substrate solution. OVA-specific IgE levels in the samples were compared with an internal standard obtained from pooled sera of hyperimmunized BALB/c mice, which was arbitrarily assigned to equal 1000 ELISA units. Total IgE levels were measured by a sandwich ELISA using rat anti-mouse IgE at 2 μ g/ml (clone R35-72; BD Biosciences) as a capture Ab, followed by biotinylated rat anti-mouse IgE H chain mAb (clone R35-118; BD Biosciences) at 2 μ g/ml, and detected, as described above. All samples were read using a VERSAmax tunable microplate reader and processed using SoftMax Pro 4.7.1 software.

In vitro cell proliferation assay

Whole splenocytes were prepared from either NT or 10N-treated B6.TCR- $\beta^{-/-}$ mice. CFSE vital labeling (26) was performed by incubating these cells at room temperature for 5 min with 0.2 μ M CFSE. Labeling was stopped by adding 1/5 vol FBS, followed by extensive washing to remove excess CFSE. Cells were then distributed to 24-well plates at a concentration of 5×10^6 /ml. After 72 h of incubation in the presence of plate-bound anti-CD3 ϵ mAb (clone 145-2C11), or rIL-2 alone (15 U/ml) or together with OVA or HEL (200 μ g/ml), cells were collected, and the proliferation of $\gamma\delta$ T cells was determined cytofluorimetrically, based on CFSE dilution.

In vivo assay for synaptic transfer using the membrane dye PKH67

Whole splenocytes from T cell-deficient mice (B6.TCR- $\beta^{-/-}\delta^{-/-}$) were prepared by mechanical dispersion. Cells were labeled with lipophilic green dye PKH67 (Sigma-Aldrich), according to the manufacturer's instructions. Excess dye was removed by adding an equal volume of FBS, followed by three repeated washes with PBS. Labeled cells were then resuspended in PBS, and 5×10^7 cells/mouse were injected in 200 μ l PBS via the lateral tail vein into B6.TCR- $\beta^{-/-}$ mice. After 24 h of incubation in vivo, splenocytes from the recipient B6.TCR- $\beta^{-/-}$ mice were prepared, and membrane transfer to $\gamma\delta$ T cells was assessed by flow cytometry, based on the presence of PKH67 dye associated with $\gamma\delta$ T cells.

Live cell microscopic analysis of individual Ag-carrying $\gamma\delta$ T cells

Whole splenocytes from T cell-deficient mice (B6.TCR- $\beta^{-/-}\delta^{-/-}$) were prepared by mechanical dispersion. Cells were loaded with a high-density

FITC-labeled self-quenched OVA (DQ-OVA; Molecular Probes, Eugene, OR) during 1 h of *in vitro* culture (37°C, 1 mg DQ-OVA/ml balanced salt solution, 1×10^7 cells), followed by washing three times with PBS. Ag-loaded cells were then resuspended in PBS, and 5×10^7 cells/mouse were injected in 200 μ l PBS via the lateral tail vein into B6.TCR- $\beta^{-/-}$ mice. After 24 h of incubation *in vivo*, splenocytes from the recipient B6.TCR- $\beta^{-/-}$ mice were sorted for TCR-V γ 4 expression and DQ-OVA fluorescence (FITC green fluorescence, indicative of processed unquenched OVA fragments). Sorted cells were transferred to imaging medium (complete RPMI 1640 without phenol red, with the addition of 0.2% low melting point agarose to keep the live cells in place during imaging), and were then examined on a spinning disc confocal microscope system (3i Intelligent Imaging Innovations, Denver CO) equipped for live cell analysis. For quantitative analysis, cells sorted as V γ 4⁺/DQ-OVA⁺ were compared with an equal number of negative control cells sorted as V γ 4⁺/DQ-OVA⁻, using the programs Slidebook 5.0 (Intelligent Imaging Innovations) and Fiji (27). To calculate the mean fluorescence intensity of the cells, the background fluorescence was subtracted and the average fluorescence of at least 20 DQ-OVA⁺ and negative control cells was determined.

Statistical analysis

Data are presented as means $\pm$ SEM. The unpaired *t* test was used for two group comparisons, and ANOVA was used for analysis of differences in three or more groups. Statistically significant levels are indicated as follows: $p < 0.05$, $p < 0.01$, $p < 0.001$.

Results

Ag-specific IgE suppression by inhalation-induced $\gamma\delta$ T cells

We previously characterized the OVA-induced IgE-suppressive $\gamma\delta$ T cells in the spleen as IFN- γ -dependent, V γ 4⁺CD8 α ⁺ $\gamma\delta$ T cells, confirmed their remarkable efficiency, and showed that they suppress both OVA-induced total and OVA-specific IgE (8). To assess the Ag specificity of these cells, we used the experimental design shown in Fig. 1A. In a preliminary experiment, repeated inhalation of BSA instead of OVA (the matched Ag) failed to induce these cells (Fig. 1B), consistent with the claim that the regulatory $\gamma\delta$ T cells are Ag specific (7). Then, to rigorously address the question of Ag specificity, we examined the effect of two Ags in a crisscross design such that $\gamma\delta$ T cells exposed to one Ag during tolerance induction would be tested for their IgE-suppressive function in mice sensitized to the same or the other Ag. Because BSA by itself does not elicit strong IgE Abs, we instead paired OVA with HEL, which as a second chicken protein is a well-matched Ag. We used the two proteins both as inhaled tolerogen and as *i.p.* injected Ag (Fig. 1C, 1D). Of note, HEL is a weaker IgE inducer than OVA in the C57BL/6 mouse strain, albeit better than BSA (28). Following a single *i.p.* Ag injection of B6.TCR- $\delta^{-/-}$ (recipient) mice in the presence of alum, both Ags elicited primary IgE Abs (Fig. 1C, 1D). Yet, treatment of cell donor B6.TCR- $\beta^{-/-}$ mice with aerosolized HEL (10N) induced regulatory V γ 4⁺ $\gamma\delta$ T cells in the spleen, which when transferred specifically suppressed the IgE Abs elicited by HEL (Fig. 1D), but not by OVA immunization (Fig. 1C). Conversely, aerosolized OVA (10N) induced V γ 4⁺ $\gamma\delta$ T cells to specifically suppress the IgE Abs elicited by OVA (Fig. 1C), but not by HEL immunization (Fig. 1D), clearly demonstrating the Ag specificity of the IgE regulation. We have previously shown in this setting that OVA-induced $\gamma\delta$ T cells suppress OVA-specific IgE Abs (8).

To test whether inhaled OVA and HEL stimulate Ag-specific responses of $\gamma\delta$ T cells, we examined responses of V γ 4⁺ $\gamma\delta$ T cells from NT or 10N-treated B6.TCR- $\beta^{-/-}$ donor mice to either Ag *in vitro*. However, the cells were not activated based on lack of expression of markers of activation (CD69^{high}, CD62L^{low}, and CD44^{high}) (Fig. 1E), nor did they proliferate in response to these Ags (Fig. 1F), regardless of whether derived from NT or Ag-treated mice. Thus, neither selected nor endogenous TCR-

dependent specificities of the $\gamma\delta$ T cells for OVA and HEL were evident.

Rapid induction of the Ag-specific IgE regulators by CD11c⁺ CD8⁻, MyD88-dependent non-T splenocytes

Because there is little, if any, lymphocyte traffic between lung and spleen (29, 30), functional induction or licensing of the splenic regulatory $\gamma\delta$ T lymphocytes might occur inside the spleen. This process might involve another splenic cell type such as the non-T splenocytes involved in the filtering/processing of blood-borne Ags (23). To investigate these possibilities, and to determine the kinetics of $\gamma\delta$ T cell licensing, which remained obscure in the original model of transferred inhalation tolerance (7), we established a new expanded cell transfer model designed to reveal aspects of the induction/licensing process (Fig. 2A). In this model, a first mouse devoid of $\gamma\delta$ T cells (inhaler) is exposed to aerosolized allergen. Spleen cells from this mouse are transferred to a second mouse (incubator), which is at no time directly exposed to the inhaled allergen. This second mouse contains $\gamma\delta$ T cells and eventually donates $\gamma\delta$ T cells to a third mouse, which again lacks endogenous $\gamma\delta$ T cells (indicator). This third mouse is then immunized to elicit primary IgE Abs. This final mouse serves to indicate possible regulatory effects mediated by the transferred $\gamma\delta$ T cells, in a manner like the second mouse in the original model shown in Fig. 1A.

Using this expanded model, we treated B6.TCR- $\delta^{-/-}$ inhaler mice with aerosolized OVA as before (10N protocol), and then transferred splenocytes from these mice or from NT controls via *i.v.* injection to B6.TCR- $\beta^{-/-}$ incubator mice. One to 3 d later, we purified V γ 4⁺ splenic $\gamma\delta$ T cells from the incubator mice and injected them *i.v.* into B6.TCR- $\delta^{-/-}$ indicator mice, which were at the same time also *i.p.* injected with OVA/alum to elicit IgE Abs. The Abs were measured as total serum IgE (Fig. 2B–E, 2G) or as OVA-specific IgE (Fig. 2F) on day 14 following the immunization. Under this regimen, endogenous V γ 4⁺ $\gamma\delta$ T cells capable of suppressing OVA/alum-induced IgE Abs developed in the spleen of the incubator mice within 24 h following spleen cell transfer (Fig. 2B). As previously shown in the original model (8), $\gamma\delta$ T cells induced in the expanded model also suppressed OVA-specific IgE (Fig. 2F). The V γ 4⁺ $\gamma\delta$ T cells were only induced by splenocytes from 10N OVA-treated, but not NT inhalers (Fig. 2C). This experiment revealed that induction of IgE-regulatory $\gamma\delta$ T cells in the spleen does not require their direct exposure to the inhaled allergen. Instead, they can be induced by another activated splenocyte. It also showed this induction occurs rapidly, within 24 h, because $\gamma\delta$ T cells transferred from the incubator mice only 1 d after receiving inducer cells were IgE suppressive (Fig. 2B). Finally, we again found that the induced $\gamma\delta$ T cells suppressed the elicited IgE Abs only when the immunizing Ag matched the inhaled Ag (Fig. 2C, 2D), indicative of their Ag specificity.

In the same and subsequent experiments, we also aimed at better defining the inducer cells. Spleen cells from B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$ inhalers still induced V γ 4⁺ IgE-suppressive $\gamma\delta$ T cells (Fig. 2C), demonstrating that T cells are not required as inducers. Moreover, purified CD11c⁺CD8 α ⁻ spleen cells from these mice (Fig. 2E) induced the IgE-regulatory $\gamma\delta$ T cells more efficiently than whole splenocytes. Thus, the inducers might be CD11c⁺CD8 α ⁻ dendritic cells (DCs), or other types of CD11c⁺ splenocytes that become enabled as a consequence of the Ag inhalations (31, 32). Because protein preparations from hen eggs can contain LPS, we considered that TLR signaling might play a role in the responses to OVA and HEL. The adaptor protein MyD88 mediates signals through the LPS receptor TLR-4, as well as the IL-1R and others (33, 34). We found that treatment of B6.MyD88-deficient mice

A Adoptive transfer of induced regulatory $\gamma\delta$ T cells

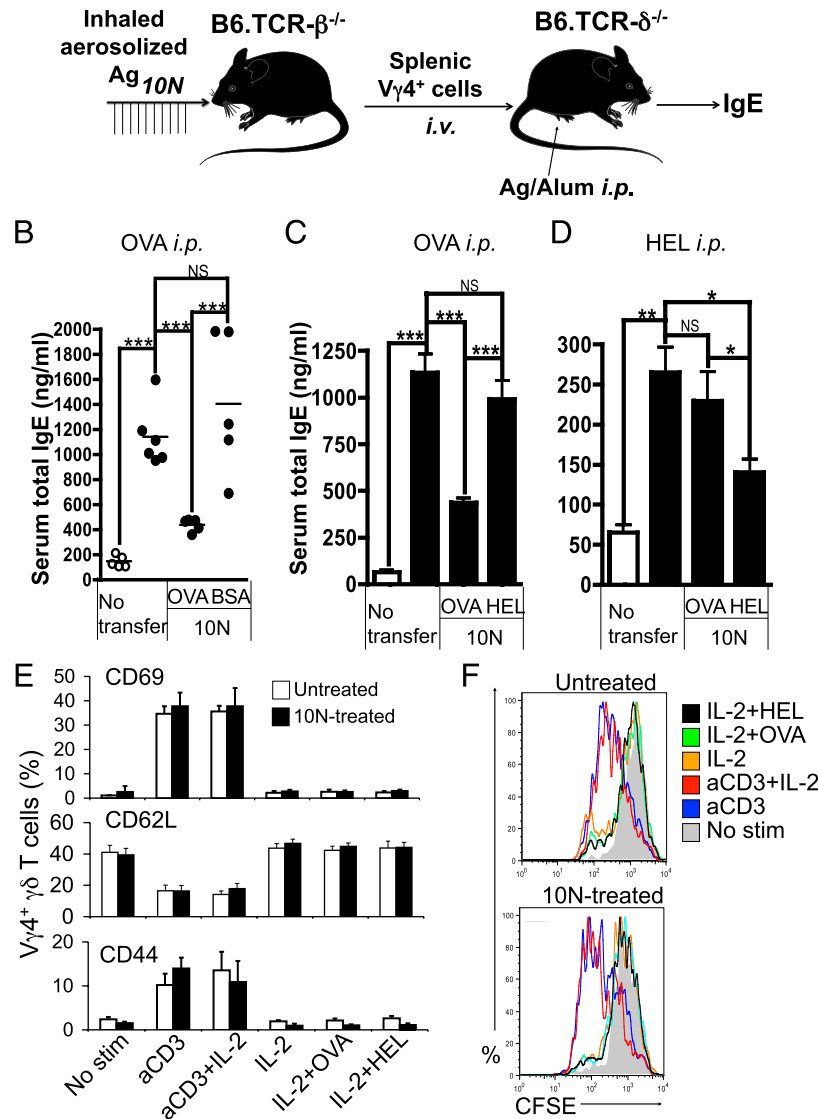


FIGURE 1. Ag-specific IgE suppression by inhalant allergen-induced $\gamma\delta$ T cells. **(A)** In our modification of the tolerance model published by McMenamin et al. (7), mice deficient in all $\alpha\beta$ T cells (B6.TCR- $\beta^{-/-}$) were tolerized to Ag via 10 or more inhalations of aerosolized Ag, and used as cell donors. Purified splenic $\gamma\delta$ T cells from these mice were transferred to recipients deficient in all $\gamma\delta$ T cells (B6.TCR- $\delta^{-/-}$), which were at the same time also immunized with a single i.p. injection of Ag/alum. Serum IgE levels in the recipients were measured 14 d after the immunization. **(B)** Experimental design as described in (A); donor mice were tolerized with OVA or BSA. Recipients remained nonimmunized (open circles) or were immunized with OVA/alum i.p. (filled circles). Immunized recipients either received no cells (No transfer) or 3×10^4 purified V $\gamma 4^+$ $\gamma\delta$ T cells from OVA-tolerized (10N OVA) or BSA-tolerized (10N BSA) donors. In (B), each circle/dot represents one mouse, and mean values are indicated as horizontal bars. **(C and D)** Crisscross experimental design, tolerance induction, cell transfers, and immunization, as described in (A) and (B); donor mice were tolerized with OVA or HEL. Recipients remained nonimmunized (open columns) or were immunized with Ag/alum i.p. (filled columns). Immunized recipients either received no cells (No transfer) or 3×10^4 purified V $\gamma 4^+$ $\gamma\delta$ T cells from OVA-tolerized (10N OVA) or HEL-tolerized (10N HEL) donors. In (C), recipients were immunized with OVA/alum i.p., and in (D) with HEL/alum i.p. Note that V $\gamma 4^+$ cells from nontolerized donors are not IgE suppressive (see Fig. 2). (C and D) Show mean values as columns. Results for each group in (C) and (D) are calculated as the mean, and show also $\pm$ SEM ($n \geq 4$ in each group). Significant differences between groups are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(E)** Spleen cells from B6.TCR- $\beta^{-/-}$ mice, either NT (open columns) or previously 10N OVA inhalation-tolerized (filled columns), were incubated 1 h in vitro with OVA or HEL, or stimulated by plate-bound anti-CD3e mAbs, in the presence of IL-2. At the end of the incubation period, harvested cells were stained with Abs specific for V $\gamma 4$ and the activation markers CD69, CD62L, and CD44. Results for each group in (E) are calculated as the mean $\pm$ SEM ($n = 3$ independent determinations). **(F)** CFSE-labeled spleen cells from B6.TCR- $\beta^{-/-}$ mice, either NT or previously 10N OVA inhalation-tolerized (as indicated), were stimulated in vitro with plate-bound anti-CD3e mAbs or with Ag, in the presence of IL-2. Anti-CD3e-stimulated V $\gamma 4^+$ cells uniformly responded with several divisions to the plate-bound Abs (culture period 72 h), but no divisions over background were detected in the presence of the Ags. Data of one experiment representative of four similar experiments are shown.

with 10N OVA failed to produce CD11c⁺CD8⁻ splenocytes capable of inducing IgE-suppressive $\gamma\delta$ T cells (Fig. 2G), suggesting that the OVA-inducible licensing activity of the CD11c⁺CD8 α^{-} non-T splenocytes depends on MyD88-mediated signaling.

In vivo Ag transfer from non-T splenocytes to V $\gamma 4^+$ $\gamma\delta$ T cells

To determine whether the in vivo interaction between the splenic inducer cells and IgE-regulatory V $\gamma 4^+$ $\gamma\delta$ T cells involves the transfer of Ag, we prepared non-T splenocytes from NT or 10N

A Separation of allergen exposure and $\gamma\delta$ T cell induction

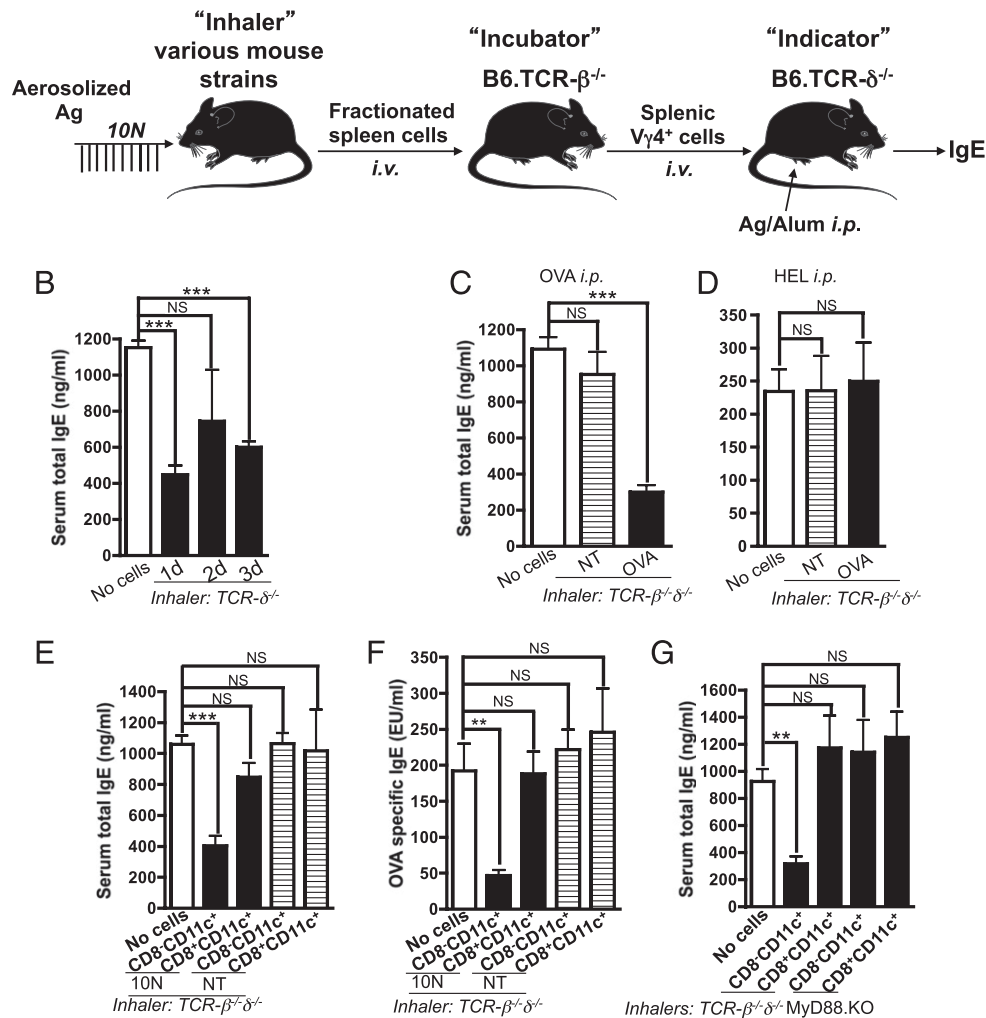


FIGURE 2. Induction of Ag-specific V γ 4 $^{+}$ IgE-regulatory cells by CD11 $^{+}$ CD8 α^{-} cells. **(A)** Shows the extended model of inhalation tolerance used in this study. Inhaler mice (various mouse strains, usually deficient in $\gamma\delta$ T cells or in all T cells) were treated with nebulized OVA (10N). Whole or fractionated splenocytes from these mice were then injected i.v. into incubator mice containing $\gamma\delta$ T, but no $\alpha\beta$ T cells (B6.TCR- $\beta^{-/-}$). After an in vivo incubation period [24 h, except for the time course in **(B)**], V γ 4 $^{+}$ $\gamma\delta$ T cells were purified from the spleen of the incubator mice, and purified cells were i.v. injected into indicator mice (B6.TCR- $\delta^{-/-}$), which were also immunized with Ag/alum, as described in Fig. 1. Serum IgE levels in these mice were measured 14 d after the immunization. **(B)** Whole splenocytes from OVA-treated B6.TCR- $\delta^{-/-}$ inhaler mice were injected into incubator mice 24–72 h prior to the transfer of purified V γ 4 $^{+}$ $\gamma\delta$ T cells into indicator mice, which were also i.p. immunized with OVA/alum. Open column, No transfer of $\gamma\delta$ T cells; filled columns, 3×10^4 purified V γ 4 $^{+}$ $\gamma\delta$ T cells transferred. Levels of whole serum IgE are shown. **(C and D)** Whole splenocytes from B6.TCR- $\beta^{-/-}$ inhaler mice that either remained NT or received the 10N OVA treatment (OVA) were injected into incubator mice 24 h prior to the transfer of purified V γ 4 $^{+}$ $\gamma\delta$ T cells into indicator mice. The indicator mice were immunized with OVA/alum **(C)** or with HEL/alum **(D)**. Open columns, No transfer of $\gamma\delta$ T cells; hatched columns, 3×10^4 purified V γ 4 $^{+}$ $\gamma\delta$ T cells were transferred from incubator mice that received splenocytes from 10N OVA-treated inhalers. **(E–G)** Fractionated splenocytes from B6.TCR- $\beta^{-/-}$ or MyD88-deficient inhaler mice that either remained NT or received the 10N OVA treatment (OVA) were injected into incubator mice 24 h prior to the transfer of purified V γ 4 $^{+}$ $\gamma\delta$ T cells into the indicator mice. The indicator mice were immunized with OVA/alum, as in **(B)** and **(C)**. **(E)** Shows a comparison of CD8 α^{+} and CD8 α^{-} CD11c $^{+}$ non-T cells as inducers of IgE-regulatory $\gamma\delta$ T cells. Open columns, No transfer of $\gamma\delta$ T cells; hatched columns, 3×10^4 purified V γ 4 $^{+}$ $\gamma\delta$ T cells were transferred from incubator mice that received fractionated purified splenocytes from NT inhalers; filled columns, 3×10^4 purified V γ 4 $^{+}$ $\gamma\delta$ T cells were transferred from incubator mice that received fractionated purified splenocytes from 10N OVA-treated inhalers. **(F)** Shows the same comparison of inducer cells as in **(E)**, but, instead of total IgE, OVA-specific IgE is measured in the serum of the immunized indicator mice. **(G)** Shows a comparison of CD8 α^{+} and CD8 α^{-} CD11c $^{+}$ non-T cells [prepared as in **(E)**] from T cell-deficient (B6.TCR- $\beta^{-/-}$) and MyD88-deficient (B6.MyD88 $^{-/-}$) mice as inducers of IgE-regulatory $\gamma\delta$ T cells. Open columns, No transfer of $\gamma\delta$ T cells; filled columns, 3×10^4 purified V γ 4 $^{+}$ $\gamma\delta$ T cells were transferred from incubator mice that received fractionated purified splenocytes from the indicated 10N OVA-treated inhalers. **(B–G)** Show mean values as columns. Results for each group are calculated as the mean $\pm$ SEM ($n \geq 4$ in each group). Significant differences between groups are indicated. *** $p < 0.001$, ** $p < 0.01$.

OVA-treated B6.TCR- $\beta^{-/-}$ inhaler mice, loaded them in vitro with high-density FITC-labeled self-quenched OVA (DQ-OVA) (35), and transferred them by i.v. injection into the incubator mice. Twenty-four hours later, we analyzed V γ 4 $^{+}$ cells for

FITC fluorescence, which is indicative of processed DQ-OVA. We found that at this time point, a small fraction of V γ 4 $^{+}$ $\gamma\delta$ T cells from the incubator mice had acquired fluorescent signal (Fig. 3A). To ascertain that the DQ-OVA FITC fluorescence was contained

within these cells and specific to DQ-OVA, we purified $V\gamma 4^+$ $\gamma\delta$ T cells by cell sorting (Supplemental Fig. 1), separately collected $V\gamma 4^+/DQ\text{-OVA}^+$ and $V\gamma 4^+/DQ\text{-OVA}^-$ cells, and analyzed the collected cells individually by spinning disc confocal microscopy. This analysis confirmed that fluorescent $V\gamma 4^+$ $\gamma\delta$ T cells in the spleen contained processed or partially processed DQ-OVA (Fig. 3B–D), indicative of Ag transfer from splenic non-T cells to the $\gamma\delta$ T cells. The fact that only a small fraction of the $V\gamma 4^+$ $\gamma\delta$ T cells carried DQ-OVA fluorescence raised new questions about the kinetics and efficiency of *in vivo* Ag transfer, Ag processing (required for unquenched fluorescence), and possible heterogeneity among the Ag recipient $\gamma\delta$ T cells.

Evidence for *in vivo* contact involving a subset of $V\gamma 4^+$ $\gamma\delta$ T cells

To determine whether the *in vivo* interaction between the splenic inducer cells and IgE-regulatory $V\gamma 4^+$ $\gamma\delta$ T cells involves direct cell contact, we prepared non-T splenocytes from NT or 10N OVA-treated B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$ inhaler mice, labeled them *in vitro* with the fluorescent membrane dye PKH67, and transferred them into

the incubator mice. Twenty-four hours later, we examined $V\gamma 4^+$ $\gamma\delta$ T cells from the incubator spleens cytofluorimetrically. After gating out doublets (Supplemental Fig. 2), a small fraction of $V\gamma 4^+$ $\gamma\delta$ T cells (1–2%) still exhibited PKH67 fluorescence (Fig. 4A), and treatment of the $\gamma\delta$ T cells with EDTA to break up cellular aggregates did not substantially alter this fluorescent signal (data not shown). Non-T splenocytes from NT mice also transferred dye to the splenic $\gamma\delta$ T cells (Supplemental Fig. 3A), indicative of close cellular contacts that might occur constitutively, independent of any specific prior Ag exposure. Again, only a small fraction of the $V\gamma 4^+$ $\gamma\delta$ T cells was PKH67 positive.

Strong activation has been found to induce MHCII expression in mouse $\gamma\delta$ T cells (13). Thus, we examined MHCII expression in splenic $\gamma\delta$ T cells of inhalation-treated C57BL/6 mice. After rigorous gating to eliminate doublets and non-T cells (Supplemental Fig. 2), a small MHCII⁺ subpopulation of $V\gamma 4^+$ $\gamma\delta$ T cells remained (Fig. 4B). However, among PKH67-labeled $V\gamma 4^+$ cells in the dye transfer experiment, gated to exclude doublets and non-T cells (Supplemental Fig. 2), most cells (>80%) were also MHCII⁺ (Fig. 4C), suggesting that MHCII-expressing $V\gamma 4^+$ $\gamma\delta$ T cells are primary dye transfer recipients and thus might be preferentially involved in interactions with non-T splenic inducer cells, or up-regulate MHCII following the interaction.

In a separate experiment, we found that inhalation-treated B6 mice lacking MHCII, despite having normal numbers of $V\gamma 4^+$ $\gamma\delta$ T cells expressing CD8 and IFN- γ (Supplemental Fig. 3B, 3C), failed to produce $\gamma\delta$ T cells that can regulate IgE (Fig. 4D). Thus, MHCII appears to have a role connected with IgE-regulatory $V\gamma 4^+$ $\gamma\delta$ T cells, perhaps because these cells must be able to express it.

Discussion

This study was undertaken to further examine the claimed Ag specificity of inducible IgE-regulatory $\gamma\delta$ T cells (7), and to investigate its possible underlying mechanisms. In support of the claim, we found that OVA-induced $V\gamma 4^+$ $\gamma\delta$ T cells suppressed the IgE response to OVA, but not HEL, whereas HEL-induced $V\gamma 4^+$ $\gamma\delta$ T cells suppressed the IgE response to HEL, but not OVA, clearly indicating Ag specificity of the regulatory effect. However, $V\gamma 4^+$ $\gamma\delta$ T cells did not respond to stimulation with OVA or HEL. Moreover, we found that OVA-specific IgE-suppressive $V\gamma 4^+$ $\gamma\delta$ T cells could be induced in nonimmunized mice within 24 h of *in vivo* coinoculation with CD11c⁺CD8[−] non-T splenocytes transferred from OVA inhalation-treated donors (expanded inhaler/incubator/indicator model). Together, these findings argue against the possibility of $\gamma\delta$ TCRs specific for these Ags, whether intrinsically Ag specific or selected for Ag specificity. If $\gamma\delta$ TCR specificities for OVA and HEL were intrinsic (germline encoded and oligoclonal), akin to superantigen specificities of $\alpha\beta$ T cells (36), they should have been detectable in the stimulation assays. In contrast, if they were merely selected, as are conventional Ag specificities of $\alpha\beta$ T cells, 24 h of *in vivo* coinoculation with APCs should not have provided sufficient time to select them. Instead, these findings provide indirect evidence for the alternative possibility that the Ag itself determines specificity. In the inhaler/incubator/indicator model, the inducible IgE-regulatory $\gamma\delta$ T cells are never directly exposed to the inhaled Ag, but they could encounter Ag-carrying inducer cells and perhaps acquire Ag from them. After the purified $V\gamma 4^+$ $\gamma\delta$ T cells are transferred into the third (indicator) mouse, they could then suppress the IgE response in an Ag-specific manner, because they themselves now carry the Ag. Assuming that the Ag is transferred from non-T splenocytes, we *i.v.* injected such cells, which were previously loaded with self-quenched fluorescent OVA (DQ-OVA), and examined $V\gamma 4^+$ splenic $\gamma\delta$ T cells in the recipients. Twenty-four

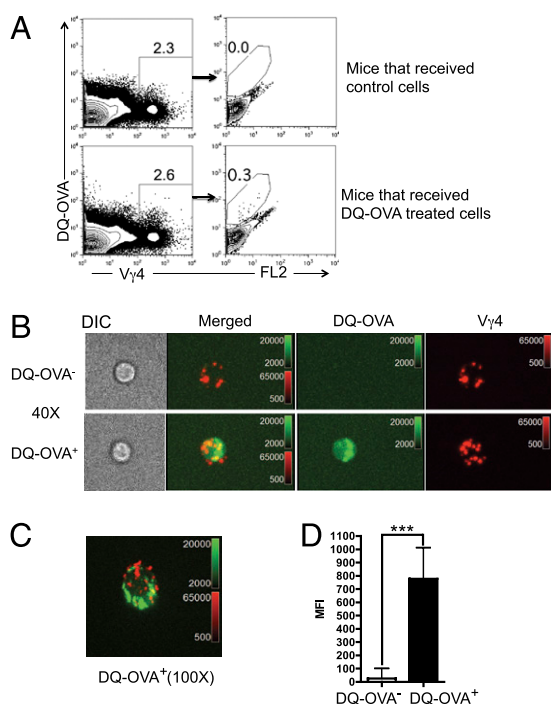


FIGURE 3. Evidence of *in vivo* Ag transfer onto splenic $V\gamma 4^+$ $\gamma\delta$ T cells. **(A)** Splenocytes from 10N OVA-treated B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$ inhaler mice were loaded *in vitro* with self-quenched FITC-labeled OVA (DQ-OVA), and injected *i.v.* into NT B6.TCR- $\beta^{-/-}$ incubator mice. After 24 h of *in vivo* incubation, $V\gamma 4^+$ $\gamma\delta$ T cells in the spleen of the cell transfer recipients were examined cytofluorimetrically for FITC fluorescence indicative of processed OVA. Cells from mice that received non-DQ-OVA-loaded splenocytes are used for comparison. Doublet exclusion and further gating were as in Supplemental Fig. 1. The experiment shown is representative of several similar experiments. **(B and C)** Splenic $V\gamma 4^+$ $\gamma\delta$ T cells from mice that received DQ-OVA-loaded cells as in (A) were sorted into OVA fluorescence-positive and -negative fractions and analyzed by confocal microscopy for the localization of processed OVA. OVA fluorescence was present inside individual $\gamma\delta$ T cells. Original magnifications as indicated in (B) and (C). **(D)** Comparison of OVA fluorescence-positive and -negative $V\gamma 4^+$ $\gamma\delta$ T cells for their mean fluorescence intensity. Same microscopic analysis as in (B) and (C). Results for each group are calculated as the mean cell fluorescence $\pm$ SEM, after background subtraction ($n = 20$ in each group). Significant differences between groups are indicated. *** $p < 0.001$.

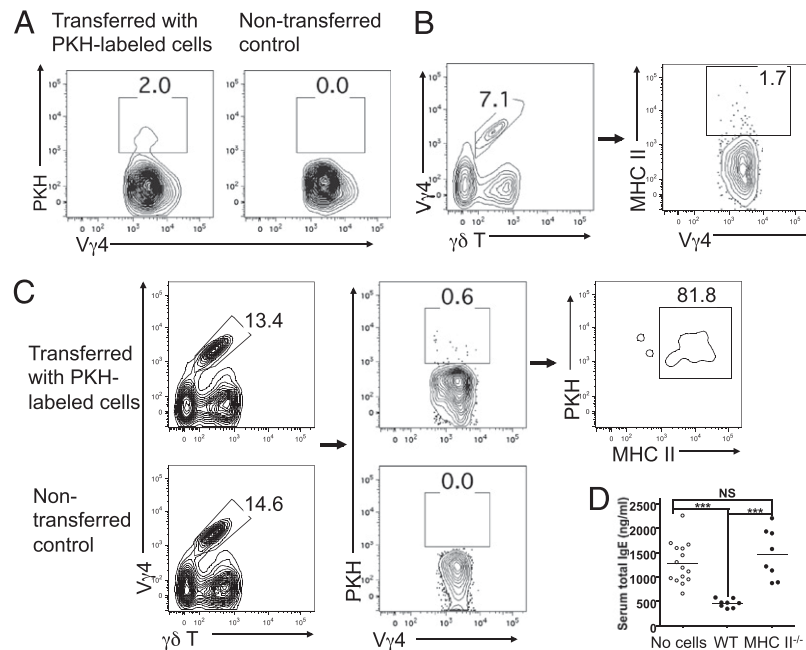


FIGURE 4. Evidence for direct contact with inducer cells and requirement of MHCII by inducible IgE-regulatory Vγ4⁺ γδ T cells. **(A)** Splenocytes from 10N OVA-treated B6.TCR-β^{-/-}γδ^{-/-} inhaler mice were labeled in vitro with the lipophilic dye PKH67 and injected i.v. into NT B6.TCR-β^{-/-} incubator mice. After 24 h of in vivo incubation, γδ T cells from the spleen of the cell transfer recipients were examined cytofluorimetrically for acquisition of the PKH67 label. Cells from mice that did not receive PKH67-labeled splenocytes were used for comparison. Doublet exclusion and further gating were as in Supplemental Fig. 2. The experiment shown is representative of several similar experiments. **(B)** Splenocytes from 10N OVA-treated B6.TCR-β^{-/-} inhaler mice were examined cytofluorimetrically for MHCII expression. Doublet exclusion and further gating are shown in Supplemental Fig. 1. The experiment shown is representative of several similar experiments. **(C)** Same experimental protocol as in (A), but using a more rigorous gating strategy (Supplemental Fig. 1) to eliminate all cellular aggregates as well as single non-γδ T cells. In addition, cells were also stained for MHCII to detect MHCII⁺ cells among Vγ4⁺ γδ T cells with acquired PKH67 fluorescence. **(D)** Comparison of wild-type C57BL/6 mice and B6 mice deficient in MHCII expression for their ability to generate IgE-regulatory Vγ4⁺ γδ T cells. Using the same experimental protocol as in Fig. 1, indicator mice deficient in γδ T cells (B6.TCR-δ^{-/-}) were immunized by i.p. injection with OVA/alum to induce a primary IgE response, or were immunized and received in addition i.v. transferred purified splenic Vγ4⁺ γδ T cells (3 × 10⁴ cells/recipient) derived from wild-type or MHC II^{-/-} 10N OVA-treated donors, just prior to being injected with the Ag. Open dots, No γδ T cells transferred; filled dots, γδ T cells transferred from C57BL/6 mice (WT) or B6 mice deficient in MHCII expression (MHC II^{-/-}). Serum total IgE levels in immunized mice were measured 14 d after the OVA/alum injection. Results for individual mice are shown (each dot represents one mouse), and mean values are indicated by horizontal bars (n ≥ 8 in each group). Significant differences between groups are indicated. ***p < 0.001.

hours after the injection, we observed OVA fluorescence within the γδ T cells. This shows that splenic γδ T cells can acquire Ag in vivo from a non-T splenocyte, consistent with the idea that they might be functionally connected with those cells in the spleen that are involved in the filtering/processing of blood-borne Ags (23). It seems likely that our data do not reflect the full extent of Ag transfer because we only examined one time point and because, in the case of DQ-OVA, Ag processing is a requirement for fluorescence (37). However, due to the low frequency, it also seems likely that only a subpopulation of the Vγ4⁺ γδ T cells is capable of acquiring such Ags. We already reported that the IgE-regulatory function coincides with the Vδ5⁺ CD8⁺ fraction of the Vγ4⁺ cells (~10% of splenic Vγ4⁺ cells in B6.TCR-β^{-/-} mice) (8), and suspect that the inducible IgE-regulatory function of Vγ4⁺ γδ T cells runs parallel with their capability to acquire Ag. In the current study, we further observed that splenic non-T cells in vivo transfer the lipophilic dye PKH67 to a fraction of splenic Vγ4⁺ γδ T cells. Such transfer is indicative of direct cell contact and might occur during active membrane exchange between two cells, or following uptake of one (dead) cell by another (38). We found such transfer to splenic Vγ4⁺ γδ T cells with non-T splenocytes from both OVA inhalation-treated and NT mice, suggesting that it occurs constitutively and does not require any particular exposure. Such a constitutive mechanism would be consistent with the idea of splenic γδ T cells that linked to the Ag-filtering/processing cells of the spleen. However, as with DQ-OVA, only a small

fraction of the γδ T cells acquired pKH67. Because there is no processing requirement with PKH67 fluorescence, inefficiency due to lack of Ag processing can be excluded, emphasizing other possible mechanisms. Indeed, we found that >80% of the PKH67-fluorescent Vγ4⁺ splenic γδ T cells also expressed MHCII. MHCII can be expressed by activated γδ T cells (13), but it can also be transferred from cell to cell during cell contact (39). Although <2% of total splenic Vγ4⁺ γδ T cells were MHCII⁺, this >40-fold enrichment among PKH67-fluorescent Vγ4⁺ γδ T cells suggests that MHCII expression, whether pre-existing or provoked, identifies a subset more likely to acquire transferred membrane and hence presumed to make contact with inducer cells. Conceivably, only the MHCII⁺ fraction of the γδ T cells might end up carrying Ag. Finally, our observation in this study that MHCII-deficient mice fail to produce IgE-regulatory γδ T cells (despite having a normal composition of splenic γδ T cells) also emphasizes the role of MHCII in γδ T cell-mediated inhalation tolerance.

Our current findings are consistent with an earlier study in which we found adoptively transferred splenic DCs in close proximity with endogenous γδ T cells in the spleen (40). They are also consistent with a study showing γδ T cells in trogocytosis with other cells in vitro, a process dependent on cellular activation (38).

It remains to be determined how inhaled tolerogenic Ag reaches the spleen. Based on current information (22, 41, 42), we favor a pathway in which inhaled Ag crosses the lung epithelial barrier

intact, enters the circulation soluble or in vesicles, and becomes engulfed by non-T cells for the first time when entering the splenic filter. However, it has also been demonstrated that migratory DCs from peripheral tissues, including lung and skin, can transfer Ag to resident DCs in lymph nodes and spleen (43, 44), and splenic $\gamma\delta$ T cells might receive the Ag similarly to resident splenic DCs, or even downstream to these DCs in the chain of Ag transfers (40). Further studies are needed to reveal which of these pathways might be operative, and whether different pathways are used with different Ags (22).

How IgE-regulatory $\gamma\delta$ T cells exert their function also remains unclear. Our data suggesting that the regulatory population is small are consistent with an earlier estimate of the size of this population (7). Because such $\gamma\delta$ T cells suppress Ag-specific IgE, which is dependent on T cell help, they could function in the manner of APCs (13) and regulate Th cell differentiation. Such a mechanism would not require many cells and could explain the previously observed requirement for IFN- γ (7, 8). However, at this point, we cannot rule out direct interactions with developing B cells.

In sum, we show that repeated Ag inhalation enables distinct $\gamma\delta$ T cells in the spleen to become Ag-specific IgE regulators. These $\gamma\delta$ T cells do not rely on their TCRs for Ag specificity, but rather on non-T inducer cells in the spleen. Additional data suggest that such inducer cells can transfer Ag onto them. We therefore suggest that tolerance through Ag inhalation mediated by $\gamma\delta$ T cells is Ag specific not through Ag recognition via the $\gamma\delta$ TCR, but through the Ag itself, which is acquired, and then perhaps presented, by the IgE-regulatory $\gamma\delta$ T cells.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figures

Supplemental Figure 1 Cytofluorimetric gating for the detection of $V\gamma 4^+$ $\gamma\delta$ T cells containing acquired processed Ag (DQ-OVA) based on FITC-fluorescence

Gating for Fig. 3

Splenocytes from 10N OVA-treated B6.TCR- $\beta^{-/-}/\delta^{-/-}$ mice were loaded *in vitro* with DQ-OVA and subsequently injected i.v. into B6.TCR- $\beta^{-/-}$ mice. 24 hrs later, spleen cells of the cell transfer recipients were stained with antibodies as indicated and analyzed cytofluorimetrically. Cells were gated based on forward and side scatter (lymphocyte gate) and signal height and widths (doublet exclusion). After this, 0.6% of $V\gamma 4^+$ $\gamma\delta$ T cells still exhibited DQ-OVA fluorescence. The experiment and gating strategy shown are representative of several similar experiments in which the percentage of dye-labeled $\gamma\delta$ T cells varied between 0.2 and 0.6%.

Sorting strategy

Singlet lymphocytes were determined based on the area of forward scatter and side scatter (FSC-A versus BSC-A), followed by two extra gates (FSC-A versus FSC-L and BSC-A versus BSC-L) to eliminate cell doublets. $V\gamma 4^+$ $\gamma\delta$ T cells were selected according to the fluorescence intensity of APC-labeled anti- $V\gamma 4$ antibody (UC3), and were further divided into DQ-OVA⁺ and DQ-OVA⁻ populations based on the DQ-OVA signal (FL1 channel). Unused FL2 channel was displayed to eliminate autofluorescence (FL1⁺FL2⁺). Post-sorting analyses are shown below.

Supplemental Figure 2 Cytofluorimetric gating for the detection of $V\gamma 4^+$ $\gamma\delta$ T cells with acquired PKH67 fluorescence and expressing MHCII

Gating for Fig. 4A

Splenocytes from 10N OVA-treated B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$ mice were labeled *in vitro* with the lipophilic dye PKH67 and subsequently injected i.v. into B6.TCR- $\beta^{-/-}$ mice. 24 hrs later, spleen cells of the cell transfer recipients were stained with antibodies as indicated and analyzed cytofluorimetrically. Cells were gated based on forward and side scatter (lymphocyte gate) and signal height and widths (doublet exclusion). After this, 1.96% of V γ 4⁺ $\gamma\delta$ T cells still exhibited PKH67 fluorescence. The experiment and gating strategy shown are representative of several similar experiments in which the percentage of dye-labeled $\gamma\delta$ T cells varied between 2.0 and 0.5%.

Gating for Fig. 4B

Splenocytes from 10N OVA-treated B6.TCR- $\beta^{-/-}$ mice were stained with antibodies as indicated and analyzed cytofluorimetrically. Cells were gated based on forward and side scatter (lymphocyte gate), signal height and widths (doublet exclusion) and based on the presence of marker antigens not expressed by $\gamma\delta$ T cells (dump gate). After this, 1.7% of V γ 4⁺ $\gamma\delta$ T cells still exhibited MHCII staining. The experiment and gating strategy shown are representative of several similar experiments.

Gating for Fig. 4C

Splenocytes from 10N OVA-treated B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$ mice were labeled *in vitro* with the lipophilic dye PKH67 and subsequently injected i.v. into B6.TCR- $\beta^{-/-}$ mice. 24 hrs later, spleen cells of the transfer recipients were stained with antibodies as indicated and analyzed cytofluorimetrically. Cells were gated based on forward and side scatter (lymphocyte gate), signal height and widths (doublet exclusion) and based on the presence of marker antigens expressed by B cells (CD19) and myeloid cells (CD11b). After this, the 0.5% of V γ 4⁺ $\gamma\delta$ T cells that exhibited PKH67 fluorescence were analyzed for MHCII expression as shown in Fig 4C. The experiment and gating strategy shown are representative of several similar experiments.

Supplemental Figure 3

***Panel A* Direct contact *in vivo* between transferred non-T cells and splenic V γ 4⁺ $\gamma\delta$ T cells**

We compared non-T splenocytes from 10N-treated and untreated donors for their ability to transfer the lipophilic dye PKH67 onto splenic V γ 4⁺ $\gamma\delta$ T cells. Either type of donor cells was able to transfer the dye indicative of direct membrane contact with the $\gamma\delta$ T cells.

Splenocytes from 10N OVA-treated B6.TCR- $\beta^{-/-}$ / $\delta^{-/-}$ or untreated mice were labeled *in vitro* with the lipophilic dye PKH67, and injected i.v. into untreated B6.TCR- $\beta^{-/-}$ incubator mice. After 24 hrs of *in vivo* incubation, $\gamma\delta$ T cells in the spleen of the cell transfer recipients were examined cytofluorimetrically for acquisition of the PKH67 label. Cells from mice that did not receive PKH67-labeled splenocytes were used as controls. Doublet-exclusion and further gating were as in Supplemental Figure 2. The experiment shown is representative of several similar experiments.

***Panels B, C* V γ 4⁺ $\gamma\delta$ T cells develop normally in MHCII-deficient mice**

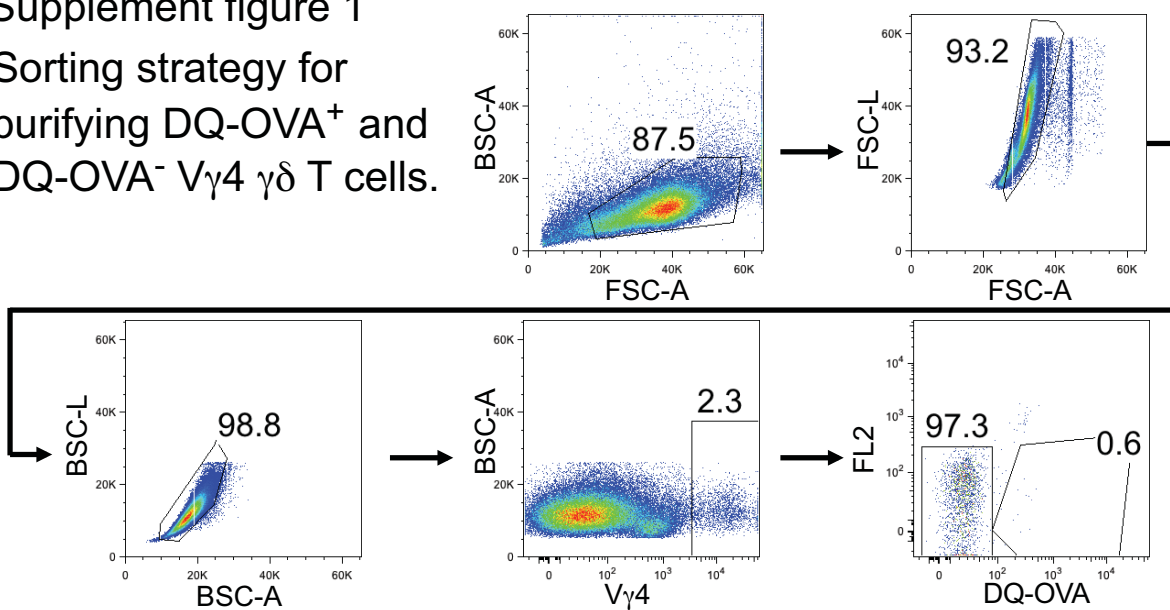
We compared splenic V γ 4⁺ $\gamma\delta$ T cells in C57BL/6 mice and congenic mice deficient in MHCII expression (B6.MHCIIKO) in order to detect abnormalities in the mutant strain.

Panels A show comparisons for total cell numbers and relative frequencies of V γ 4⁺CD8 α ⁺ $\gamma\delta$ T cells.

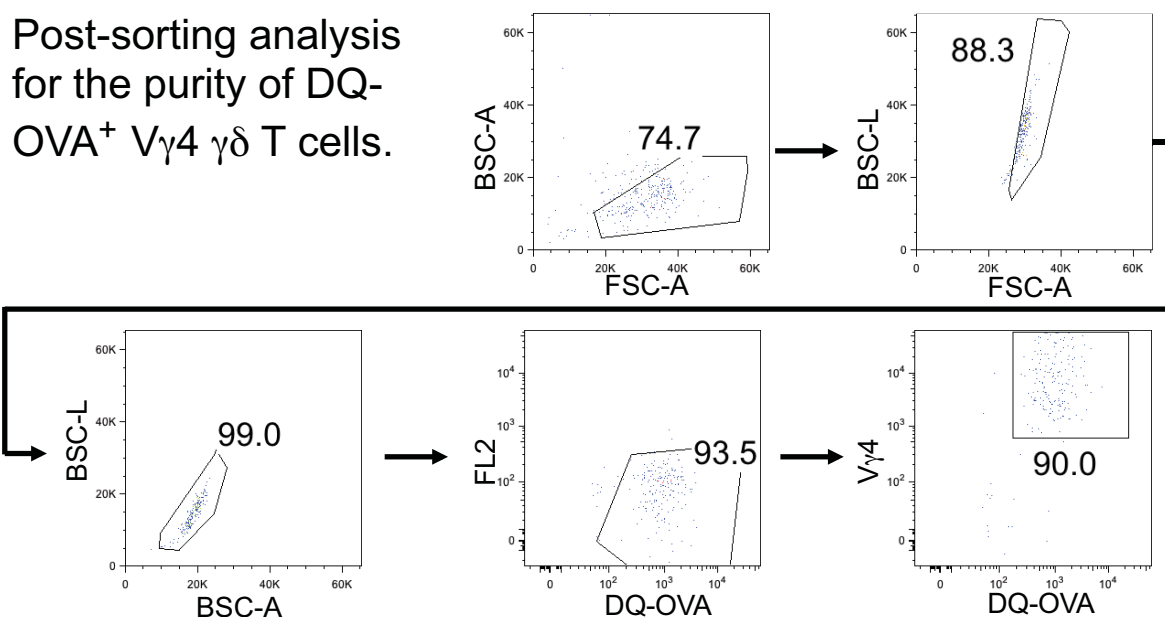
Panels B show comparisons for relative frequencies of cells capable of expressing the IgE-regulatory cytokine IFN- γ , among total splenic V γ 4⁺ cells and among splenic V γ 4⁺CD8 α ⁺ cells.

No significant differences between the two strains were detected. Results for individual mice are shown, and mean values indicated by horizontal bars (n = 3 in each group). Significant differences between groups are indicated. NS, not significant.

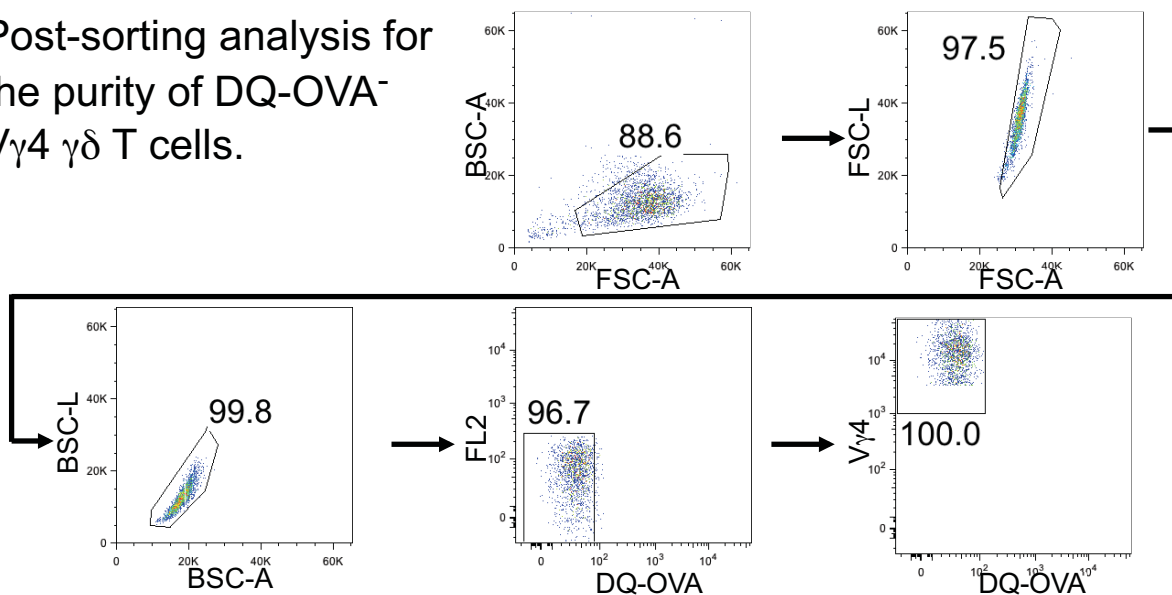
Supplement figure 1
Sorting strategy for
purifying DQ-OVA⁺ and
DQ-OVA⁻ V γ 4 $\gamma\delta$ T cells.



Post-sorting analysis
for the purity of DQ-
OVA⁺ V γ 4 $\gamma\delta$ T cells.

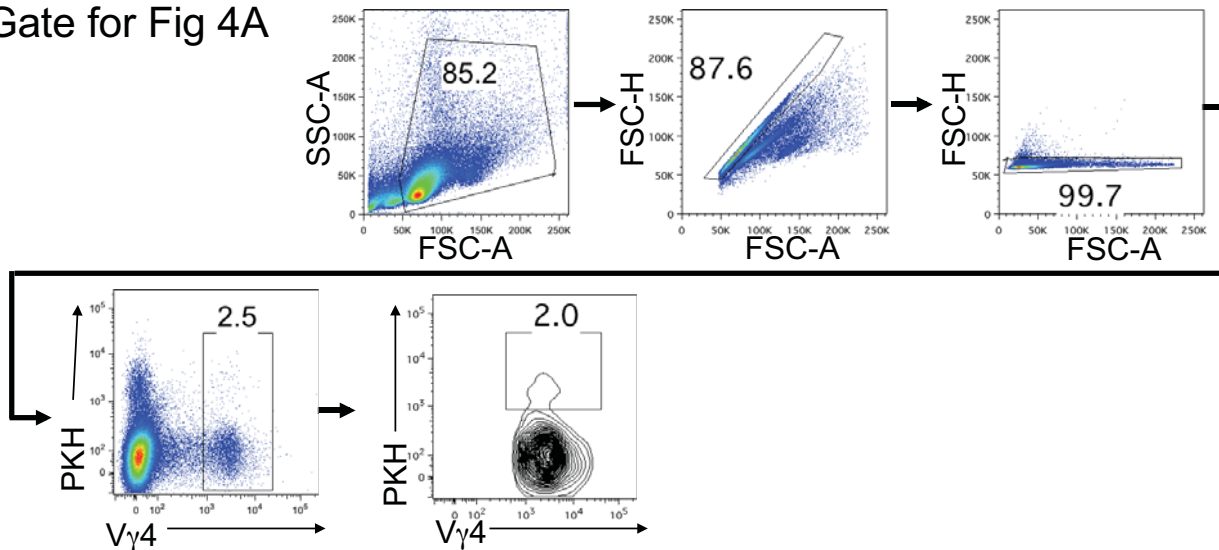


Post-sorting analysis
for the purity of DQ-OVA⁻
V γ 4 $\gamma\delta$ T cells.

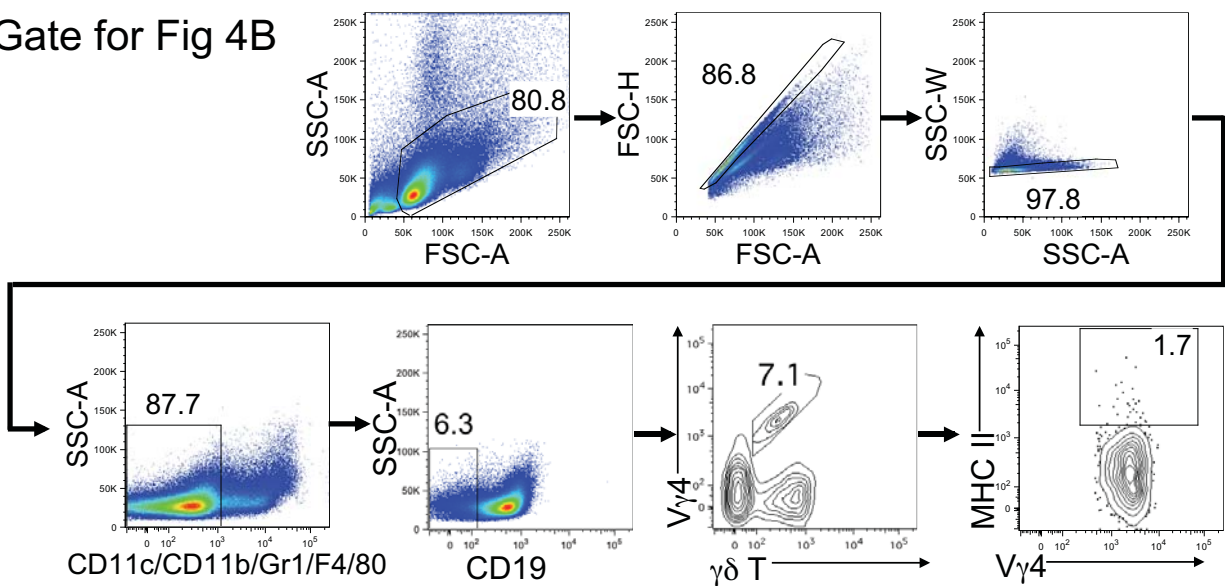


Supplement figure 2

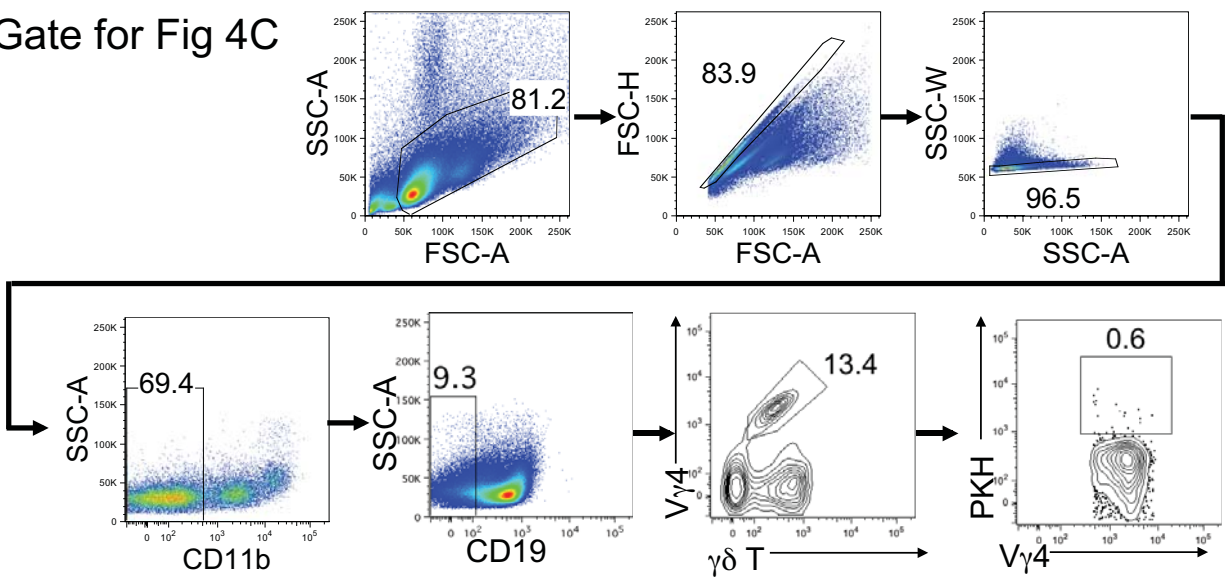
Gate for Fig 4A



Gate for Fig 4B



Gate for Fig 4C



Supplement figure 3

