

Original Article

$\gamma\delta$ T cell is essential for allergen-induced late asthmatic response in a murine model of asthma

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Background: Gamma-delta ($\gamma\delta$) T cells regulate immune responses at mucosal surfaces. Whether they can modify allergen-induced early (EAR) and late airway responses (LAR) is unknown.

Objective: We have tested the hypothesis that the $\gamma\delta$ T cells enhance allergen-induced airway responses in mice. **Methods:** BALB/c wild-type (WT) mice and $\gamma\delta$ T cell-deficient ($\gamma\delta$ T-cell KO) mice were sensitized by intraperitoneal injection of ovalbumin (OVA) on days 1 and 15, immunized with 1% OVA aerosol on days 29–31, and challenged with 5% OVA or saline on day 33. Enhanced pause (Penh) was measured and BAL fluid was collected after challenge. Serum IgE was measured before challenge. The percentage of interleukin (IL)-4 and interferon (IFN)- γ producing T cells in splenocytes from sensitized animals was determined by flow cytometry. **Results:** Both EAR and LAR were observed in OVA-challenged WT mice. LAR but not EAR was inhibited in OVA-challenged $\gamma\delta$ T-cell KO mice. $\gamma\delta$ T-cell KO mice showed less eosinophilia in BALF and serum OVA-specific IgE. In the sensitization period, the percentage of IFN- γ producing $\alpha\beta$ T cell in $\gamma\delta$ T-cell KO mice was higher than that in

WT mice. **Conclusion:** $\gamma\delta$ T cells enhance LAR and airway inflammation but not EAR in this model of asthma.

Key words: Gammadelta T cell, asthma, murine model, Penh,

Introduction

Inhalation of allergens by allergic asthmatics causes an immediate bronchoconstriction followed by delayed airway narrowing¹. These two responses, the early and late airway responses (EAR and LAR), have several distinctive features based on pathophysiological mechanisms. LAR is the consequence of events including airway inflammation, increased airway secretions, and enhanced microvascular permeability and is strongly associated with the increase of airway responsiveness to inhaled irritants¹. Recent studies have implicated a role of T cells, such as CD4⁺ T cells^{2–6} and CD8⁺ $\alpha\beta$ T cells⁷ in LAR.

In addition to these T cells, immune responses by $\gamma\delta$ T cells may be pivotal in the regulation of allergic airway responses^{8–11}, because $\gamma\delta$ T cells account for 8–20% of pulmonary lymphocytes in adult mice^{12,13}. In sensitized and challenged rats, adoptive transfer of CD8⁺ $\gamma\delta$ T cells from naive donors inhibits LAR and eosinophilic airway inflammation⁸. V γ 4⁺ T cells, a subpopulation of pulmonary $\gamma\delta$ T cells, exert a regulatory effect on airway hyperresponsiveness (AHR) after inhalation of ovalbu-

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min (OVA) in naive mice⁹. In contrast, several studies have indicated that $\gamma\delta$ T cells¹⁰, especially $V\gamma 1^+$ cells in spleen¹¹, enhance AHR in sensitized and challenged mice. Nonetheless, there has been no report of the role of $\gamma\delta$ T cells in allergen-induced airway responses, EAR and LAR, in mice.

Recently, involvement of $\gamma\delta$ T cells in the regulation of allergic airway inflammation, Th2 cytokine responses, and production of IgE has been demonstrated. Though adoptive transfer of $CD8^+\gamma\delta$ T cells from OVA-tolerant mice suppresses IgE antibody production¹⁴, sensitized and challenged $\gamma\delta$ T-cell-deficient ($\gamma\delta$ T-cell KO) mice exhibited diminished allergen-specific IgE production, eosinophilic airway inflammation, and Th2 cytokine responses^{9,10,15,16}. Although administration of interleukin- (IL-)4 to $\gamma\delta$ T-cell KO mice during the sensitization period restored Th2-mediated airway inflammation¹⁵, other studies reported that $\gamma\delta$ T-cell KO mice were not defective in production of IL-4 and IL-5^{16,17}. How $\gamma\delta$ T cells may modulate allergic airway inflammation has not been clearly elucidated.

To study the role of $\gamma\delta$ T cells in allergic airway responses, we first measured EAR and LAR in $\gamma\delta$ T-cell KO mice, using whole-body barometric plethysmography in conscious, unrestrained animals to define EAR and LAR. We also assessed cytokine profile of T-cell-subsets in spleen after sensitization. The results suggested that $\gamma\delta$ T cells promote LAR and skew cytokine profiles away from Th1 response during sensitization.

Material and Methods

Animals

BALB/cCr mice were purchased from Japan SLC, Inc (Shizuoka, Japan). BALB/c-TCR- $\delta^{-/-}$ ($\gamma\delta$ T-cell KO) mice were obtained from Dr. H. Yamamoto at the Osaka University (Osaka, Japan). All mice were maintained in a conventional animal facility of the Tokyo Metropolitan Institute of Medical Science. Experimental procedures were approved by an institutional animal care committee. All mice were 8- to 12-wk old at the time of the experiments.

Sensitization and airway immunization and challenge

WT and $\gamma\delta$ T-cell KO mice were sensitized by intraperitoneal injection of 20 μ g of OVA (Grade V; Sigma-Aldrich, St. Louis, MO) with 2.25mg of aluminum hydroxide (ImjectAlum; Pierce, Rockford, IL) in a total volume of 100 μ l on days 1 and 15. Mice were immu-

nized with aerosolized OVA (1% in saline) for 20min on days 29, 30, and 31. Forty-eight hours after the last OVA immunization, mice were challenged with aerosolized OVA (5% in saline) for 20 minutes (OVA group, n=8). Control mice for measurement of airway responses were challenged with saline (saline group, n=7).

Measurement of airway responses to OVA

We assessed airway responses as changes in enhanced pause (Penh) after challenge with 5% OVA or saline. We placed unrestrained, conscious mice in whole-body plethysmographic chambers (Buxco Electronics, Inc., Wilmington, NC) and then recorded Penh for 20 minutes for baseline. After challenge, recordings were taken at 5, 10, 15, and 30 minutes, and hourly for a total of 9 hours. Each recording was taken and averaged for 5 minutes and each value was expressed as the relative change from the baseline.

Bronchoalveolar lavage (BAL)

BAL were performed in OVA sensitized, OVA immunized, OVA challenged WT and KO mice 24 hours after challenge (n=7 each). Naive WT and KO mice were used as control (n=4 each). Mice were anesthetized with diethyl ether, and the lungs were lavaged through a tracheal tube three times with 3 aliquots of 1ml of saline. Total leukocyte counts were determined with a hemacytometer using trypan blue dye. BAL cytospin slides were prepared using a Cytospin2 (Shandon Ltd., Runcorn, UK), and stained with Diff-Quick (International Reagents Corp., Kobe, Japan). Cellular differentials were assessed by counting 300 or more cells. The supernatants of BAL were kept at -80 °C until use.

Cytokine levels in BAL fluid

To assess the contribution of cytokines to airway inflammation, the levels of IFN- γ , IL-2, IL-4, IL-5 and tumor necrosis factor (TNF)- α , were measured in BAL fluid by Mouse Th1/Th2 Cytokine Cytometric Bead Array kit (BD PharMingen, San Diego, CA) according to the manufacturer's instructions.

Serum OVA-specific IgE measurement by ELISA

The sera of WT mice and $\gamma\delta$ T-cell KO mice were taken for the measurement of OVA-specific IgE levels at day 0 (day0, n=3), at 14 days after the second intraperitoneal sensitization (day29, n=3), and at 2 days after airway immunization (day33, n=4). OVA-specific IgE was measured by ELISA as previously

described¹⁸.

Cytokine expression in splenic T cells

Splenocytes were isolated from sensitized mice 3 days after the second sensitization. Spleens from two mice were dispersed in RPMI-1640 medium supplemented with 10% fetal bovine serum and cell suspensions were passed through nylon mesh. After red blood cell lysis, cells were stimulated with OVA (100 μ g/ml) on 6-well plates for 3 days. To detect intracellular cytokines, cells were restimulated with phorbol 12-myristate 13-acetate (PMA; 10ng/ml, Sigma-Aldrich) and ionomycin (500ng/ml, Sigma-Aldrich) in the presence of Golgi Stop (1.3 μ g/ml, PharMingen) for 3 hours. The cells were double stained for both surface markers using fluorescein isothiocyanate (FITC)-conjugated anti-TCR β , anti-TCR $\gamma\delta$, or isotype control antibody (BD Pharmingen) and intracellular cytokines using allophycocyanin (APC)-conjugated anti IL-4, anti-IFN- γ , or isotype control antibody (BD Pharmingen), according to the company's instruction. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lake, NJ).

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical

comparisons between groups were performed with Kruskal-Wallis test followed by Mann-Whitney test using Statcel (OMS, Tokorozawa, Japan). Cytokine levels in BALF were compared by two-factor factorial ANOVA followed by Scheffé's F test. OVA-specific IgE levels were compared by one-factor ANOVA followed by Scheffé's F test. Statistical significance was set at a value of $P < 0.05$.

Results

$\gamma\delta$ T-cell KO mice show EAR but not LAR

The time course of enhanced pause (Penh) after challenge with OVA or saline is shown in Figure 1. EAR and LAR were determined from the peak in the Penh within 15 minutes and from 4 to 9 hours after challenge respectively. EAR and LAR in WT/OVA challenged mice were shown at 5 minutes and from 8 hours onward after provocation compared to WT/saline challenged mice ($P < 0.05$). EAR was greater in KO/OVA challenged mice than in KO/saline challenged mice ($P < 0.05$), whereas LAR was not different in KO/OVA challenged mice compared to KO/saline challenged mice.

EAR was not different between WT/OVA challenged

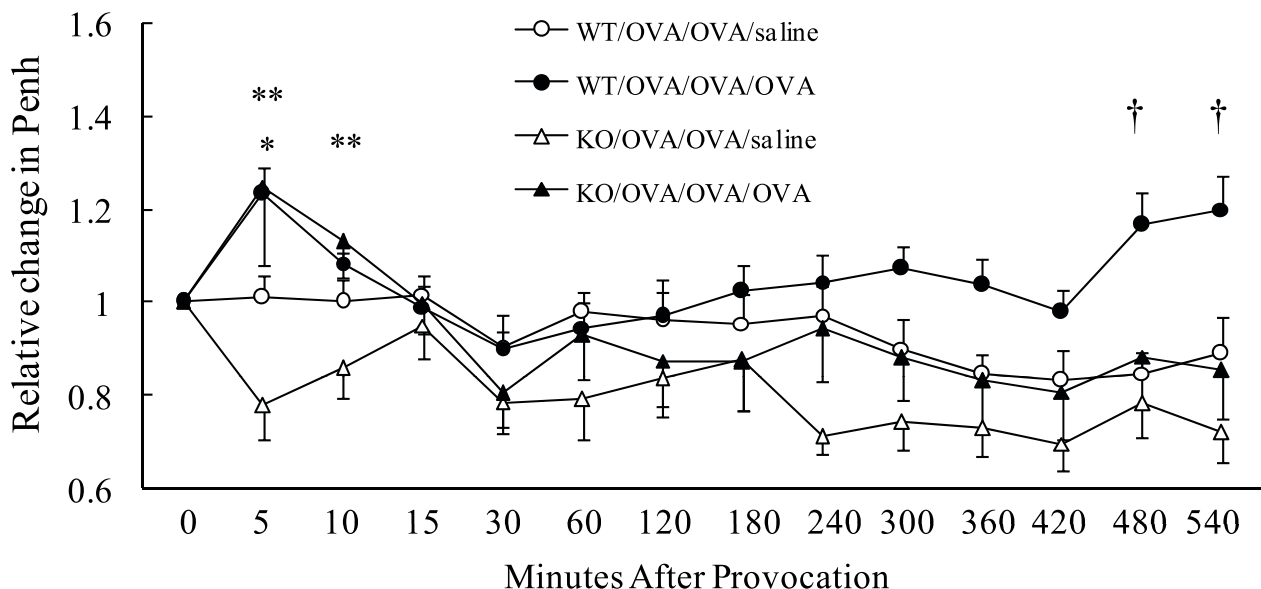


Fig. 1. Measurement of airway responses to OVA challenge in WT and $\gamma\delta$ T-cell KO mice. All animals were sensitized and immunized with OVA. The time course of enhanced pause (Penh) after challenge with OVA ($n=8$) or saline ($n=7$) is shown. Symbols indicate the mean values for each group, and the vertical bars indicate the SEMs. EAR and LAR in WT/OVA challenged mice were shown at 5-10 minutes and from 8 hours onward after provocation compared to WT/saline challenged mice (* $P < 0.05$ WT/OVA/OVA/OVA versus WT/OVA/OVA/saline). EAR was greater in KO/OVA challenged mice than in KO/saline challenged mice (** $P < 0.05$ KO/OVA/OVA/OVA versus, KO/OVA/OVA/saline). The KO/OVA group showed significantly lower LAR than the WT/OVA group († $P < 0.05$ WT/OVA/OVA/OVA versus KO/OVA/OVA/OVA).

mice and KO/OVA challenged mice, whereas the KO/OVA group showed significantly lower LAR than the WT/OVA group ($P<0.05$).

Airway inflammation is diminished in $\gamma\delta$ T-cell KO mice

In naive WT mice ($n=4$), BAL revealed 1 to 3×10^5 total leukocytes per mouse, of which over 98% were alveolar macrophages and <2% were lymphocytes and neutrophils (Table 1). BAL analysis of sensitized, immunized, and challenged WT mice ($n=7$, WT/OVA group) showed a marked increase in total leukocytes and eosinophils as compared with naive control mice ($P<0.01$). In $\gamma\delta$ T-cell KO mice, total leukocytes and eosinophils were also increased after sensitization, airway immunization and challenge ($n=7$, $P<0.05$ and $P<0.01$, respectively), whereas the percentage of eosinophils in challenged $\gamma\delta$ T-cell KO mice was significantly lower than that in challenged WT mice ($P<0.05$).

Cytokine levels in BALF

The concentrations of Th2-type cytokines, IL-4 and IL-5, significantly increased in OVA sensitized, immunized, and challenged WT mice compared with those in WT-naive mice ($P<0.05$, Fig 2A, B). Likewise, the concentrations of IL-4 and IL-5 increased in OVA sensitized

and challenged $\gamma\delta$ T-cell KO mice. IL-4 and IL-5 expression showed a tendency of decrease but not significant change in OVA sensitized and challenged $\gamma\delta$ T-cell KO mice compared to WT mice. Th1-cytokines, IFN- γ , IL-2, and TNF- α were undetectable in BALF (data not shown).

OVA-specific IgE production is decreased in $\gamma\delta$ T-cell KO mice

After sensitization, there was no significant difference between OVA-specific IgE levels in WT mice and those in $\gamma\delta$ T-cell KO mice (Fig 3). Airway immunization with OVA boosted the specific IgE responses in both WT and $\gamma\delta$ T-cell KO mice (Fig 3), but OVA-specific IgE in sensitized and challenged WT mice was higher than that in sensitized and challenged $\gamma\delta$ T-cell KO mice ($*P<0.05$).

IFN- γ production by splenic $\alpha\beta$ T cells is increased in $\gamma\delta$ T-cell KO mice

To evaluate the mechanisms by which $\gamma\delta$ T cells mediate the development of LAR, airway inflammation, and humoral responses, we measured the capacity of OVA-primed splenic T-cell subsets to produce IL-4 and IFN- γ in sensitization period ($n=4$). After sensitization, there was a significant increase in the percentage of IL-4-staining TCR- $\gamma\delta^+$ cells in WT mice ($*P<0.05$),

Table 1. Total cells and cellular profiles in bronchoalveolar lavage fluid (BALF)

animal	sensitization	immunization	challenge	Total cells, $\times 10^5$	Eosinophils, $\times 10^5$ (%)	Macrophages, $\times 10^5$ (%)
WT ($n=4$)	-	-	-	2.45 ± 0.40	0	2.40 ± 0.40 ($98.0 \pm 0.23\%$)
WT ($n=7$)	OVA	OVA	OVA	$8.06 \pm 1.69^{**}$	$4.02 \pm 1.47^{**}$ ($41.5 \pm 6.21\%$) **	2.72 ± 0.17 ($42.6 \pm 6.76\%$) **
KO ($n=4$)	-	-	-	4.28 ± 0.99	0	3.97 ± 1.00 ($91.2 \pm 1.77\%$) *
KO ($n=7$)	OVA	OVA	OVA	$9.40 \pm 1.03^\dagger$	$2.31 \pm 0.55^{\dagger\dagger}$ ($22.7 \pm 5.08\%$) ††,‡	$4.79 \pm 0.51^{\dagger\dagger}$ ($54.4 \pm 6.60\%$)

Data are presented as mean $\pm$ SEM.

$^*P<0.05$, $^{**}P<0.01$ versus WT-naive

$^\dagger P<0.05$, $^{\dagger\dagger}P<0.01$ versus KO-naive

$^\ddagger P<0.05$, $^{\ddagger\ddagger}P<0.01$ versus WT-OVA

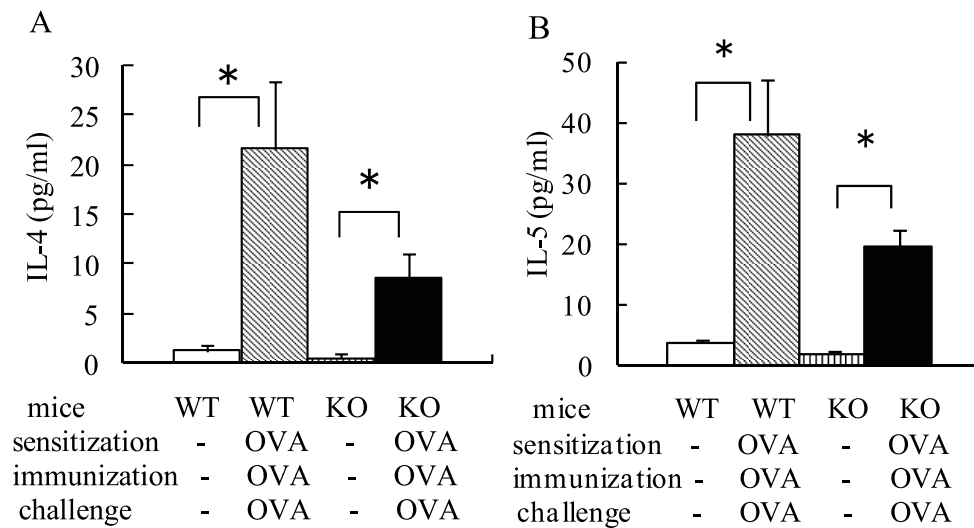


Fig. 2. Levels of IL-4 (A) and IL-5 (B) were measured in sensitized and immunized WT and KO mice after challenge (n=7) by Mouse Th1/Th2 Cytokine Cytometric Bead Array kit. Naive mice were served as controls (n=5). Data are presented as mean $\pm$ SEM. IL-4 and IL-5 expression showed a decreasing tendency but not significant change in OVA sensitized and challenged $\gamma\delta$ T-cell KO mice compared to WT mice. * P <0.05.

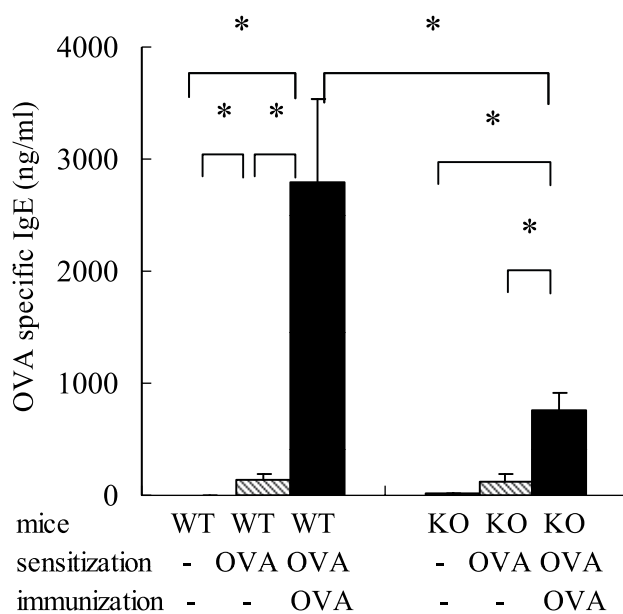


Fig. 3. Serum OVA-specific IgE levels were measured in WT and KO mice after sensitization and immunization (n=4) by ELISA. Sera from naive mice were served as controls (n=3). Airway immunization with OVA boosted the specific IgE responses in both WT and $\gamma\delta$ T-cell KO mice. OVA-specific IgE in sensitized and challenged WT mice was more increased than that in sensitized and challenged $\gamma\delta$ T-cell KO mice (* P <0.05).

whereas there was not a significant change in KO mice (Fig 4A). After sensitization, there was a significant increase in the percentage of IFN- γ -staining TCR- $\alpha\beta$ ⁺ cells in both WT and $\gamma\delta$ T-cell KO mice (* P <0.05), and moreover the percentage of IFN- γ -staining TCR- $\alpha\beta$ ⁺ cells in $\gamma\delta$ T-cell KO mice was higher than that in WT mice (* P <0.05, Fig 4B).

Discussion

In this study, we demonstrated that sensitized and airway-immunized BALB/c mice developed EAR followed by LAR and showed a marked increase in OVA-specific IgE, Th2 cytokines and eosinophils in BAL after allergen airway challenge. $\gamma\delta$ T-cell KO mice lacked LAR and showed significant attenuation of OVA-specific IgE and airway eosinophilia compared to WT mice, suggesting that $\gamma\delta$ T cells promote LAR through Th2-driven airway inflammation. $\gamma\delta$ T cells may promote Th2 responses by shifting the cytokine profile of $\alpha\beta$ T cells to Th2 type during the sensitization period, because we found that $\alpha\beta$ T cells from sensitized WT mice produced lower IFN- γ than those from sensitized $\gamma\delta$ T-cell KO mice.

In our experiments, we found that both WT and $\gamma\delta$ T-cell KO mice developed EAR, meanwhile the level of allergen-specific IgE in $\gamma\delta$ T-cell KO mice was lower

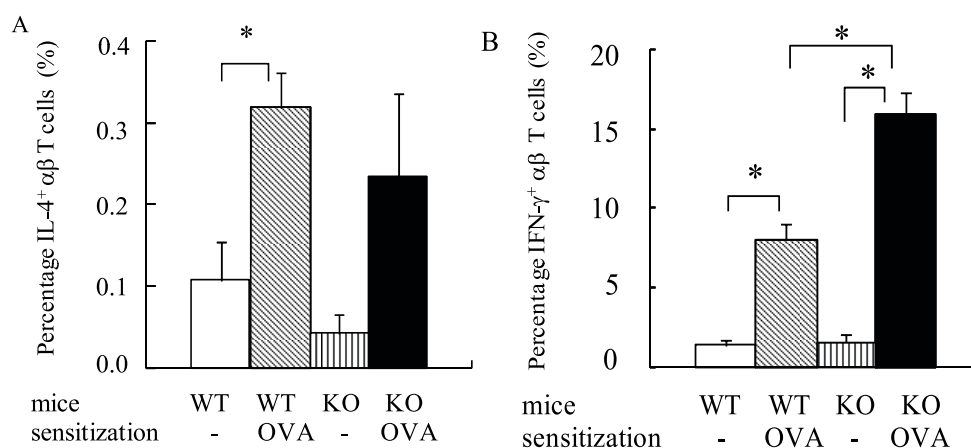


Fig. 4. The percentages of IL-4 (A) and IFN- γ (B) producing cells in splenic $\alpha\beta$ T cells from WT and KO mice after sensitization are shown. Naive mice were served as controls. Splenic cells from two mice were cultured with OVA for 3 days and re-stimulated with mitogen in the presence of GolgiStop for 3 hours. Intracellular cytokine and surface antigen were analyzed by flow cytometry. Each column expresses an average of four experiments. The percentage of IFN- γ -staining TCR- $\alpha\beta$ ⁺ cells in WT mice was lower than that in $\gamma\delta$ T-cell KO mice (B, * P <0.05).

than that in WT mice. Although the cause of this discrepancy is unclear, possible explanation is that IgE may not be indispensable for early allergic responses in mice. Crosby *et al.*¹⁹ reported that EAR was absent in gene knockout mice deficient of either B or T cells, and transfer of allergen-specific IgG, but not IgE, was capable of inducing EAR in both strains of lymphocyte-deficient mice. Some studies showed that sensitized $\gamma\delta$ T-cell KO mice are not defective in the production of allergen-specific IgG^{15,16,20}. Although we have not measured the level of OVA-specific IgG, EAR might be induced through OVA-specific IgG in this murine model.

We found that $\gamma\delta$ T-cell KO mice lacked LAR and showed reduced airway eosinophilia. These results suggest that $\gamma\delta$ T cells enhance airway inflammation and allergen-induced LAR, and appear to conflict with earlier findings indicating a suppressive role of $\gamma\delta$ T cells on allergic airway responses. Isogai *et al.*⁸ found that CD8⁺ $\gamma\delta$ T cells from naive donors significantly decreased LAR in sensitized and challenged Brown Norway rats, whereas eosinophils were decreased by both $\gamma\delta$ T cells from naive and sensitized donors. Lahn *et al.*⁹ demonstrated negative regulation of AHR by a subset of pulmonary $\gamma\delta$ T cells in OVA-challenged mice. These cells expressed V γ 4 and depended for their function on the presence of MHC class I molecules. These studies are similar concerning the CD8⁺ subset of $\gamma\delta$ T cells that produce IFN- γ when they are activated. In contrast, the proinflammatory roles of all $\gamma\delta$

T cells have been suggested by several studies. In these reports, sensitized and challenged $\gamma\delta$ T-cell KO mice showed decreases in airway inflammation^{10,11,15,16}, Th2 cytokines in BALF^{11,15}, and AHR^{10,11}. Transient depletion of all $\gamma\delta$ T cells before sensitization also resulted in airway hyporesponsiveness and/or a diminished airway eosinophilia^{10,11}. One possible explanation for these discrepancies is that subsets of $\gamma\delta$ T cells affect allergic airway responses differently. Hahn *et al.*¹¹ characterized two subsets of $\gamma\delta$ T cells; V γ 4⁺ cells that are the largest subset in the adult lung and V γ 1⁺ cells that are the largest subset in the spleen. In their report, V γ 4⁺ $\gamma\delta$ T cells strongly suppressed AHR when they were transferred into sensitized TCR-V γ ^{4-/-6-/-} mice before airway challenge. In contrast, V γ 1⁺ $\gamma\delta$ T cells enhanced AHR, levels of Th2 cytokines and lung eosinophil numbers when they were transferred before challenge into sensitized $\gamma\delta$ T-cell KO mice. Since mice were sensitized repeatedly with intraperitoneal injection of OVA and Alum before airway challenge in our experiments, the effect of $\gamma\delta$ T cells in spleens as an enhancer of allergic airway responses appeared to exceed the suppressive role of the other subset of $\gamma\delta$ T cells.

$\gamma\delta$ T-cell KO mice demonstrate equivalent Th2 type cytokine expression in the lung or spleen^{16,17} to WT mice. Interestingly, Hahn *et al.*¹¹ reported that in BALB/c mice treated with anti TCR- δ monoclonal antibody during sensitization, no changes in IL-13 levels in BALF was seen, whereas in C57BL/6 mice treated in

the same way IL-13 levels were reduced by ~50%. AHR was reduced in both strains. Because BALB/c controls showed lower IL-13 levels in BALF compared to C57BL/6 controls, they estimated that Th2 activity may be nonessential for $\gamma\delta$ T cells to enhance AHR. Our data showing no significant difference in IL-4 and IL-5 in BALF between BALB/c WT and $\gamma\delta$ T-cell KO mice support these results. However, Zuany *et al.*¹⁵ reported that the administration of IL-4 to BALB/c $\gamma\delta$ T-cell KO mice during sensitization restored the pulmonary airway inflammation, IL-5 levels in BALF, and IgE production. During early phase of infection $\gamma\delta$ T cells may contribute to the development $\alpha\beta$ Th subsets by producing IL-4 or IFN- γ ²¹. Therefore we hypothesized in this study that $\gamma\delta$ T cells regulate the development $\alpha\beta$ Th subsets by producing IL-4 during sensitization.

Contrary to our expectations, our data revealed that the percentage of IL-4 producing $\gamma\delta$ T cells in spleens from WT mice was very low and did not increase after sensitization (data not shown). There were no differences in the frequencies of IL-4 producing $\alpha\beta$ T cells at baseline and after sensitization between WT and $\gamma\delta$ T-cell KO mice. In further experiments, we found that the percentage of IFN- γ producing $\alpha\beta$ T cell in $\gamma\delta$ T-cell KO mice was higher than that in WT mice. Absence of $\gamma\delta$ T cells appeared to result in increase of IFN- γ producing Th1 cells in spleen. In vitro, IFN- γ attenuates the development and expansion of Th2-type lymphocytes and inhibits IgE production²². In murine models, intraperitoneal administration of IFN- γ regulates eosinophil recruitment into airways by inhibiting the infiltration of the CD4⁺ T cell²³. In our model, increased IFN- γ secretion from $\alpha\beta$ T cells in $\gamma\delta$ T-cell KO mice might inhibit IgE production, airway eosinophilia, and LAR after challenge. However, the mechanism how $\gamma\delta$ T cells regulate the development of Th1 $\alpha\beta$ T cells is unclear. Recently, it has been described that $\gamma\delta$ T cells downregulate the activated macrophages during the resolution of bacteria-induced inflammation^{24,25}. Our data showed that the number of macrophages in BALF from OVA-challenged $\gamma\delta$ T-cell KO mice was larger than that in challenged WT mice. Deficiency of $\gamma\delta$ T cells may lead to an increase in the number of activated macrophages. Activated macrophages secrete a range of cytokines including IL-12 which induces the differentiation of CD4⁺ T cells into Th1 cells²⁶. $\gamma\delta$ T cells may modulate the development of $\alpha\beta$ Th subsets through the regulation of activated macrophages.

Our results indicate that, $\gamma\delta$ T cells contribute to the late allergic airway response, airway inflammation and

allergen-specific IgE production, independently of the Th2 cytokine response. The possible mechanism is that $\gamma\delta$ T cells negatively regulate the development of Th1 $\alpha\beta$ T cells during sensitization.

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