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Identification of CD25$^+$ γδ T Cells As Fetal Thymus-Derived Naturally Occurring IL-17 Producers[1]

Kensuke Shibata, Hisakata Yamada,[2] Risa Nakamura, Xun Sun, Momoe Itsumi, and Yasunobu Yoshikai

We previously reported that resident γδ T cells in the peritoneal cavity rapidly produced IL-17 in response to *Escherichia coli* infection to mobilize neutrophils. We found in this study that the IL-17-producing γδ T cells did not produce IFN-γ or IL-4, similar to Th17 cells. IL-17-producing γδ T cells specifically express CD25 but not CD122, whereas CD122$^+$ γδ T cells produced IFN-γ. IL-17-producing γδ T cells were decreased but still present in IL-2- or CD25-deficient mice, suggesting a role of IL-2 for their maintenance. IFN-γ-producing CD122$^+$ γδ T cells were selectively decreased in IL-15-deficient mice. Surprisingly, IL-17-producing γδ T cells were already detected in the thymus, although CD25 was not expressed on the intrathymic IL-17-producing γδ T cells. The number of thymic IL-17-producing γδ T cells was peaked at perinatal period and decreased thereafter, coincided with the developmental kinetics of Vγ6$^+$Vδ1$^+$ γδ T cells. The number of IL-17-producing γδ T cells was decreased in fetal thymus of Vδ1-deficient mice, whereas Vγ5$^+$ fetal thymocytes in normal mice did not produce IL-17. Thus, it was revealed that the fetal thymus-derived Vγ6$^+$Vδ1$^+$ T cells functionally differentiate to produce IL-17 within thymus and thereafter express CD25 to be maintained in the periphery. ***The Journal of Immunology,*** **2008, 181: 5940–5947.**

IL-17 (IL-17A) is a proinflammatory cytokine critical for the development of various autoimmune diseases (1–4). IL-17 also plays a role in host defense against various pathogens especially by inducing mobilization of neutrophils (5). Because it was revealed that IL-17 is produced by newly identified CD4 Th cells, Th17, much attention has been paid on the differentiation mechanisms of Th17 from naive CD4 T cells. Differentiation of Th17 depends on TGF-β and several proinflammatory cytokines including IL-6 (6). Thus, naive CD4 T cells that are stimulated with their TCR in such cytokine milieu differentiate to Th17 cells. Similar to the case of Th1 and Th2 cells, whose differentiation requires specific transcription factors, T-bet and GATA-3, respectively, development of Th17 cells depends on a transcription factor, RORγt (7).

In addition to Th17, several subsets of T cells have been reported to produce IL-17. These include CD8 T cells, invariant NKT cells and γδ T cells (8–14). We found that γδ T cells in the peritoneal cavity produced IL-17 immediately after *Escherichia coli* infection, which is involved in the neutrophil-mediated clearance of the pathogen (10). Importance of IL-17 production by γδ T cells was also reported in other models of infection as well as autoimmune disease (11–14). Thus, γδ T cells could be the main source of IL-17 in some aspects of immune responses. In addition to IL-17 production, γδ T cells are known to exert various biological functions by producing different cytokines. Such functional difference of γδ T cells seems to be related with their anatomical location or their TCR repertoire. γδ T cells in the epithelium of skin or intestine are shown to be involved in tissue remodeling (15, 16). Immune regulatory roles of γδ T cells were also reported (17, 18). Notably, γδ T cells in the peritoneal cavity, which produce IL-17 in *E. coli* infection, were also involved in early host defense against *Listeria monocytogenes* by producing IFN-γ (19). However, it has not been clearly shown whether there are functionally different subsets of γδ T cells like CD4$^+$ Th cell subsets. It is also possible that the same γδ T cells produce different cytokines depending on the stimuli or environments provided by infection with different pathogens.

Mechanisms of functional differentiation of γδ T cells are also unclear. Many of γδ T cells in the periphery are already equipped with effector functions and exhibit memory phenotypes in situ. Thus, in contrast to naive αβ T cells, γδ T cells are found in small numbers in lymphoid organs but are more abundant in peripheral tissues or organs. In addition, there are unique features in the development of γδ T cells, especially those expressing Vδ1, which include the IL-17-producing peritoneal γδ T cells (10). Vδ1$^+$ γδ T cells express Vγ5$^+$ or Vγ6$^+$ and are the first lineage of T lymphocytes generated in fetal thymus, (20–22). These Vδ1$^+$ γδ T cells express canonical TCR due to a lack of terminal deoxynucleotidyl transferase activity, thus have limited Ag specificities (23, 24). After the development in the fetal thymus, Vγ5Vδ1$^+$ and Vγ6Vδ1$^+$ γδ T cells are seeded to the periphery, the former into skin and the latter into reproductive tracts as well as peritoneal cavity (21, 22, 25–29). Therefore, it is unclear in which stage of development these γδ T cells acquire effector functions, in contrast to the conventional αβ T cells, which are exported from the thymus as naive T cells, subsequently differentiate into Th cells after encountering with the Ags in the lymph nodes, and then circulate to migrate into peripheral tissues.

In the present study, we first identified and characterized IL-17-producing γδ T cells in the peritoneal cavity. It was revealed that

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IL-17-producing γδ T cells were a functionally independent subset of γδ T cells, which specifically express CD25 but not CD122. Their number but not functional differentiation was regulated by CD25-mediated signaling. Subsequent analysis surprisingly revealed that these γδ T cells already differentiated into IL-17-producing cells in fetal thymus.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Breeding Laboratories. Vδ1-deficient mice were generated as previously described (30). Mice genetically deficient for IL-2, CD25, and IL-15, OT-I mice expressing OVA peptide 257–264 specific TCR in the context of H2-K^b, OT-II mice expressing OVA peptide 323–339 specific TCR in the context of I-A^b, and C57BL/6 Ly5.1 congenic mice were obtained from The Jackson Laboratory. Fetal mice were obtained from timed mating at which the day of finding a vaginal plug was designated as day 0 of embryonic development (ED0). Mice were maintained in specific pathogen-free conditions in our institute. This study was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine (Kyushu University). Experiments were conducted under the control of the Guidelines for Animal Experiment.

Cell preparations from various tissues

Single cell suspensions were prepared from fetal thymi, adult thymus, and spleens by using pairs of slide glasses. Peritoneal exudate cells (PEC)[3] were collected by extensive wash of peritoneal cavity with HBSS. To isolate intestinal intraepithelial T lymphocytes from small bowel, Peyer's patches were removed, and, after being washed with HBSS, the gut was opened and cut into small pieces. The mucosa was dissociated by being stirred in 15 ml of medium 199 (M199; Life Technologies) containing 10% FCS (Sigma-Aldrich) and 1 mM dithioerythritol (Sigma-Aldrich) for 30 min at 37°C. After centrifugation, the pellet was resuspended in RPMI 1640 (Wako, Japan) with 10% of FCS and incubated at 37°C with shaking for 30 min. Intestinal intraepithelial T lymphocytes were purified on a 40%/70% discontinuous Percoll (Amersham Biosciences) density gradients at 600 × *g* for 20 min. To isolate gut lamina propria lymphocytes, Peyer's patches were removed, and the epithelium was eliminated by being stirred, twice in 15 ml of HBSS containing 3 mM EDTA at 37°C for 15 min and then twice in 15 ml of calcium-free RPMI 1640 containing 1% FCS, 1 mM EGTA, and 1.5 mM $MgCl_2$ for 15 min. Gut pieces were collected, cut into 2-mm samples, and stirred at 37°C for 45 min in 15 ml of RPMI 1640 containing 20% FCS, 100 U/ml collagenase (Life Technologies), and 100 μg/ml DNase I (DN25; Sigma-Aldrich). At the middle and at the end of the incubation, the suspension was dissociated by vortexing for 3 min. The pellet was washed, and lamina propria lymphocytes were purified on a 40%/70% discontinuous Percoll gradients at 600 × *g* for 20 min. Epidermal cells were prepared as previously described (31). Briefly, both ventral and dorsal aspects of ear skin were separated from underlying cartilage using fine forceps, followed by flotation, dermal side up, on 1% trypsin/PBS solution for 45 min at 37°C. The epidermis was separated from dermal tissue, and epidermal single cell suspensions were prepared by mechanical agitation. The resulting epidermal cell suspensions were enriched and separated from dead cells by using Lympholyte-M (Cedarlane Laboratories) density gradient centrifugation.

In vitro activation of OT-I and OT-II T cells

Splenocytes from OT-I or OT-II mice were cultured at 2 × 10^5 cells/well in 96-well round-bottom plates. Cells were stimulated with the corresponding peptides (OT-I, SIINFEKL; OT-II, ISQAVHAAHAEINEAGR) for 2 or 3 days in CO_2 incubator at 37°C.

Real-time RT-PCR analysis

Cells were purified by using FACS Aria (BD Biosciences). Total RNA from the purified cells was extracted using TRIzol reagent (Invitrogen Life Technologies). The first-stranded cDNA synthesis was done using Superscript II (Invitrogen) according to the manufacturer's instruction. The following gene-specific primers were used: RORγt, 5′-AGCTTTGTGCAGATCTAAGG-3′, 5′-TGTCCTCCTCAGTAGGGTAG-3′; and GAPDH, 5′-GGCAAATTCAACGGCACA-3′, 5′-GTTAGTGGGGTCTCGCTCTG-3′.

Real-time RT-PCR was performed on an ABI PRISM thermal cycler (Applied Biosystems) using SYBR Premix Ex Taq (Takara, Japan).

[3] Abbreviations used in this paper: PEC, peritoneal exudate cell; Treg, regulatory T cell; ED, embryonic development.

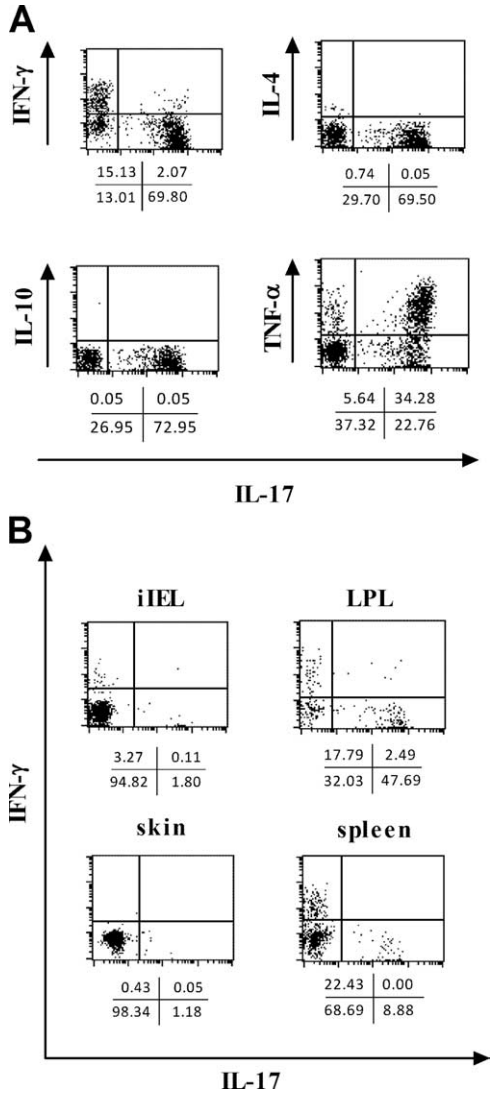

FIGURE 1. Identification of IL-17-producing γδ T cell subset. *A*, Cytokine production by γδ T cells in the peritoneal cavity of naive C57BL/6 mice (7-wk-old) was analyzed after stimulation with PMA and ionomycin. *B*, Expression of IFN-γ and IL-17 in γδ T cells from various tissues was analyzed. Cells were stimulated with PMA and ionomycin and 10 μg/ml brefeldin A was added for the last 4 h of incubation followed by surface staining as described in *Materials and Methods*. Data were shown after gating on γδ T cells (positive for TCRδ and negative for TCRβ, B220, F4/80, and MHC class II). The number indicated under each panel indicates the percentages of positive cells in the respective quadrant. Data are representative of three independent experiments.

Abs and flow cytometric analysis

FITC-conjugated anti-TCRγδ (GL3) and anti-TCRβ (H57-597) mAb, Alexa Fluor 488-conjugated anti-mouse IL-17 (eBio17B7) mAb, PE-conjugated anti-CD25 (PC61.5), anti-CD122 (TMβ1), anti-mouse IFN-γ (XMG1.2), anti-mouse IL-4 (11B11), anti-mouse IL-10 (JES5-16E3), anti-mouse IL-17 (eBio17B7), and anti-mouse TNF-α (MP6-XT22) mAb, allophycocyanin-conjugated anti-CD3ε (145-2C11), anti-TCRγδ (GL3), anti-CD25 (PC61.5), and anti-mouse IFN-γ (XMG1.2) mAb Alexa Fluor 647-conjugated anti-mouse/human T-bet (4B10) mAb, biotin-conjugated anti-MHC class II (M5/114.15.2), anti-F4/80 (BM8), anti-TCRβ (H57-597), anti-B220 (RA3-6B2) mAb were purchased from eBioscience. PE-conjugated CD69 (H1.2F3) mAb and allophycocyanin-conjugated anti-CD25 (PC61.5), anti-CD4 (CT-CD4), and anti-CD8α (CT-CD8α) mAb were purchased from Caltag Laboratories. PerCP-Cy5.5-conjugated anti-CD3ε mAb and PerCP-Cy5.5-conjugated streptavidin were purchased from BD Biosciences. Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using CellQuest software (BD Biosciences).

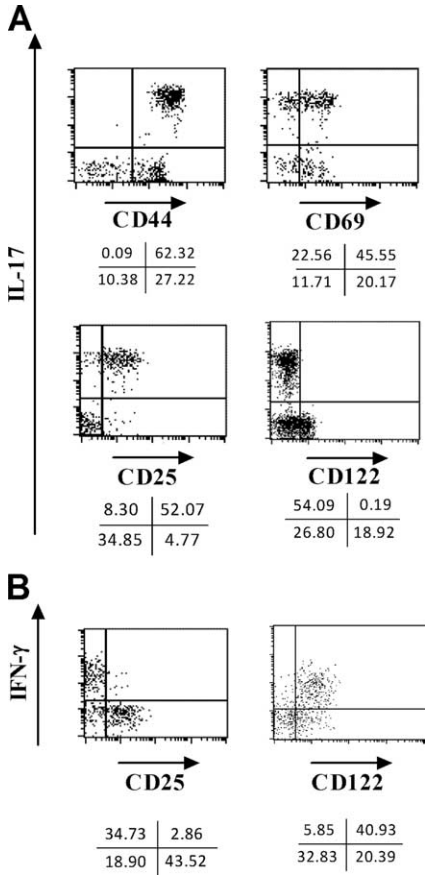

FIGURE 2. Expression of surface markers on IL-17-producing γδ T cells. *A*, Expressions of surface markers on IL-17-producing γδ T cells in the peritoneal cavity were analyzed similar to cells in Fig. 1. *B*, Expression of IFN-γ, CD25, and CD122 in γδ T cells in the peritoneal cavity. Data were shown after gating on γδ T cells. The number shown below each panel indicates the percentage of positive cells in the respective quadrant. Data are representative of three independent experiments.

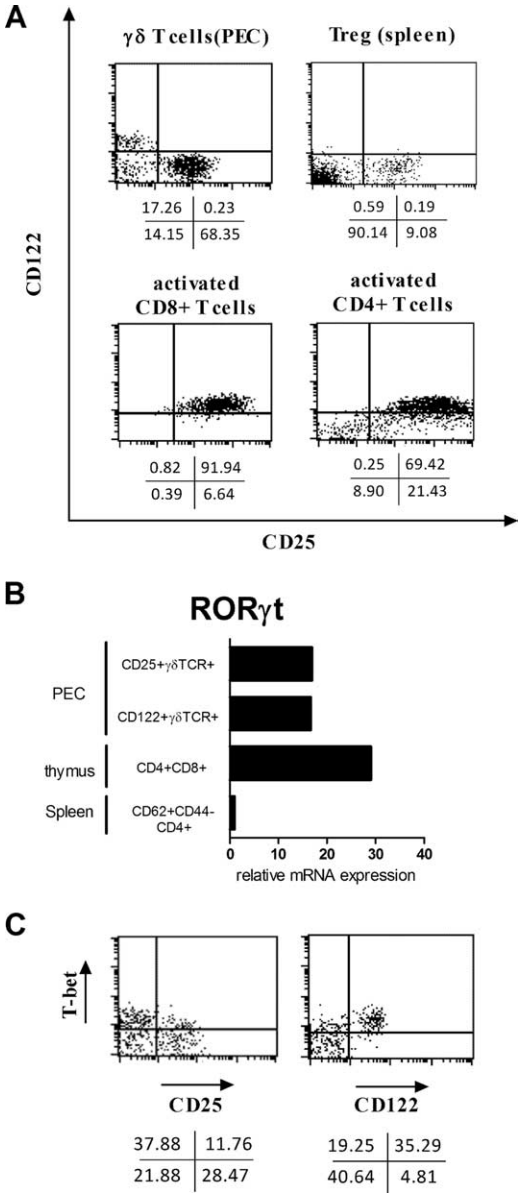

FIGURE 3. Differential expression of CD25 and CD122 discriminates different subsets of γδ T cells. *A*, Expression of CD25 and CD122 on γδ T cells in the peritoneal cavity (*top left*) or $CD4^+$ cells in the spleen (*top right*) in naive C57BL/6 mice, and in vitro-activated $CD8^+$ cells (*bottom left*) or $CD4^+$ cells (*bottom right*) was analyzed. Data were shown after gating on γδ T cells (*top left*), CD4 T cells (*right column*), or $CD8^+$ T cells (*bottom left*). The number shown under each panel indicates the percentage of cells in the respective quadrant. *B*, Expression of *RORγt* mRNA in FACS-sorted $CD25^+$ and $CD122^+$ γδ T cells in the peritoneal cavity, $CD4^+CD8^+$ thymocytes, and $CD62L^+CD44^-$ naive CD4 T cells in the spleen from naive C57BL/6 mice (7-wk-old) was analyzed by real-time RT-PCR. Expression of GAPDH mRNA was used in parallel as the control. Data shown are the relative expression calculated by setting the negative control sample ($CD62L^+CD44^-$ splenic CD4 T cells) to 1. *C*, Expression of T-bet in $CD25^+$ and $CD122^+$ γδT cells in the peritoneal cavity was analyzed by intracellular staining. Data were shown after gating on γδ T cells. The number shown under each panel indicates the percentage of cells in the respective quadrant. Data are representative of three independent experiments.

Intracellular cytokine staining

Cells were stimulated with 25 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) for 5 h at 37°C. Then 10 μg/ml brefeldin A (Sigma-Aldrich) was added for the last 4 h of incubation. After surface staining of the cells with various mAbs for 30 min at 4°C, intracellular staining was performed according to the manufacturer's instruction (BD Biosciences). Briefly, 100 μl of BD Cytofix/Cytoperm solution was added to cell suspension with mild mixing and placed for 20 min at 4°C. Fixed cells were washed with 250 μl of BD Perm/Wash solution twice and were stained intracellularly with PE-conjugated anti-mouse IFN-γ, anti-mouse IL-4, anti-mouse IL-10, anti-mouse IL-17, and anti-mouse TNF-α mAb and Alexa Fluor 488-conjugated anti-mouse IL-17 mAb for 30 min at 4°C.

Statistics

Statistical significance was calculated by the Student's *t* test using Prism software (GraphPad, San Diego, CA). Differences with values for $p < 0.05$ were considered statistically significant.

Results

IL-17-producing γδ T cells are different from IFN-γ-producing γδ T cells and are enriched in the peritoneal cavity

We previously reported that resident γδ T cells in the peritoneal cavity produced IL-17 and contributed to neutrophil-mediated host defense against *E. coli* infection (10). However, because peritoneal γδ T cells were also shown to produce several cytokines including IFN-γ (19), we first examined profiles of cytokine production of γδ T cells in the peritoneal cavity stimulated with PMA and ionomycin (Fig. 1*A*). There was a large number of IL-17-producing γδ T cells, which were different from IFN-γ-producing γδ T cells. Similar to Th17 cells (32), majority of IL-17-producing γδ T cells

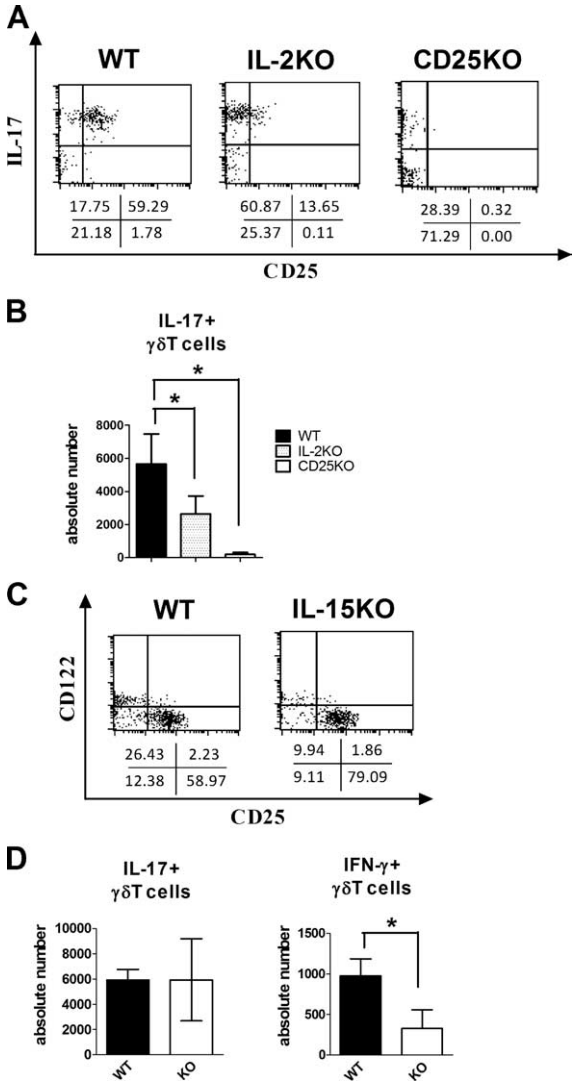

FIGURE 4. Different cytokine-mediated signaling regulates the number of different subsets of γδ T cells. *A*, PEC from wild-type (WT), IL-2-deficient (IL-2KO), and CD25-deficient (CD25KO) mice (all 3-wk-old) were analyzed for the presence of IL-17-producing γδ T cells after stimulation with PMA and ionomycin. Data shown are after gating on γδ T cells. The number shown under each panel indicates the percentage of cells in the respective quadrant. *B*, Absolute cell number of IL-17$^+$ γδ T cells is shown in these mice. Data indicate the mean ± SD of seven mice for each group. *C*, Expression of CD25 and CD122 on γδ T cells in PEC from WT or IL-15-deficient (IL-15 KO) mice (both 7-wk-old) was analyzed. Data are shown after gating on γδ T cells. The number shown under each panel indicates the percentage of cells in the respective quadrant. *D*, The absolute cell number of IFN-γ$^+$ (*left*) or IL-17$^+$ (*right*) γδ T cells were calculated after flow cytometric analysis. Data indicate the mean ± SD of seven mice for each group. *, $p < 0.05$. Data are representative of three independent experiments.

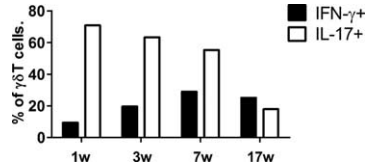

FIGURE 5. Age-related changes in the frequency of IFN-γ- and IL-17-producing γδ T cells. PEC from naive C57BL/6 mice were harvested at the indicated ages. IFN-γ and IL-17 production was examined after stimulation with PMA and ionomycin. Data are shown as a percentage of IFN-γ$^+$ (■) and IL-17$^+$ (□) cells within γδ T cells and are representative of three independent experiments.

also produced TNF-α but not IL-4 or IL-10. Thus, IL-17-producing γδ T cells are independent subsets of γδ T cells resembling Th17 cells in CD4 T cells.

Next we examined tissue distribution of the IL-17-producing γδ T cells in naive mice (Fig. 1*B*). The frequency of IL-17-producing γδ T cells was highest in the peritoneal cavity, and they were relatively abundant in intestinal lamina propria. On the contrary, IL-17-producing γδ T cells were scarcely found in epithelial tissues of both skin and intestine. There were more IFN-γ-producing γδ T cells than IL-17-producing γδ T cells in the spleen. Thus, the frequency of IL-17- or IFN-γ-producing γδ T cells varies greatly among different tissues.

IL-17-producing γδ T cells specifically express CD25 but not CD122

We examined expression of surface molecules on IL-17-producing γδ T cells (Fig. 2*A*). γδ T cells in the peritoneal cavity expressed several activation markers including CD44, CD69, and CD25 (IL-2R α-chain). Among these molecules, CD25 was specifically expressed on IL-17-producing γδ T cells. Importantly, CD25$^+$ γδ T cells did not produce IFN-γ (Fig. 2*B*), indicating that expression of CD25 does not simply reflect their activation state. Unexpectedly, CD122 (IL-2R β-chain) was not expressed on IL-17-producing γδ T cells (Fig. 2*A*), whereas there was a distinct populations of γδ T cells expressing CD122 but not CD25, which produced IFN-γ (Figs. 2*B* and 3*A*). Thus, the functionally different γδ T cell subsets can be discriminated by the expression of CD25 and CD122. As CD25 can be expressed on activated CD4 or CD8 T cells as well as Foxp3$^+$ CD4$^+$ regulatory T cells (Tregs), we examined expression levels of CD25 and CD122 on these T cells. Similar to IL-17-producing γδ T cells, Tregs express CD25 but not CD122, whereas activated CD4 and CD8 T cells expressed both CD25 and CD122 (Fig. 3*A*). However, IL-17-producing γδ T cells were negative for Foxp3 (data not shown).

We next examined expression levels of RORγt, which is a transcription factor for Th17, in the γδ T cell subsets by real-time RT-PCR (Fig. 3*B*). CD4$^+$CD8$^+$ thymocytes were used as positive control (33, 34). It was revealed that *RORγt* mRNA was expressed not only in CD25$^+$ γδ T cells, but also in CD122$^+$ γδ T cells (Fig. 3*B*). However, the level of *RORγt* mRNA expression in both subsets of γδ T cells was lower than that of CD4$^+$CD8$^+$ thymocytes. We also analyzed the expression of T-bet, a transcription factor for Th1 cells, in CD25$^+$ and CD122$^+$ γδ T cells by intracellular staining (Fig. 3*C*). T-bet was highly expressed in CD122$^+$ γδ T cells, which produce IFN-γ, but not in CD25$^+$ γδ T cells.

Differential regulation of the number of CD25$^+$ IL-17-producing γδ T cells and CD122$^+$ IFN-γ-producing γδ T cells by IL-2 and IL-15

To address roles of CD25-mediated signaling in the development and functional maturation of IL-17-producing γδ T cells, we examined PEC of IL-2- and CD25-deficient mice of 3 wk of age, which did not exhibit overt inflammatory disease yet. Although there was a clear population of IL-17-procducing γδ T cells in the peritoneal cavity of both IL-2- and CD25-deficient mice, their expression levels of CD25 were down-regulated in IL-2-deficient mice, nearly comparable to those in CD25-deficient mice. In addition, absolute number of IL-17-producing γδ T cells was decreased in IL-2-deficient mice, which was more severely reduced in CD25-deficient mice (Fig. 4, *A* and *B*). We also analyzed PEC

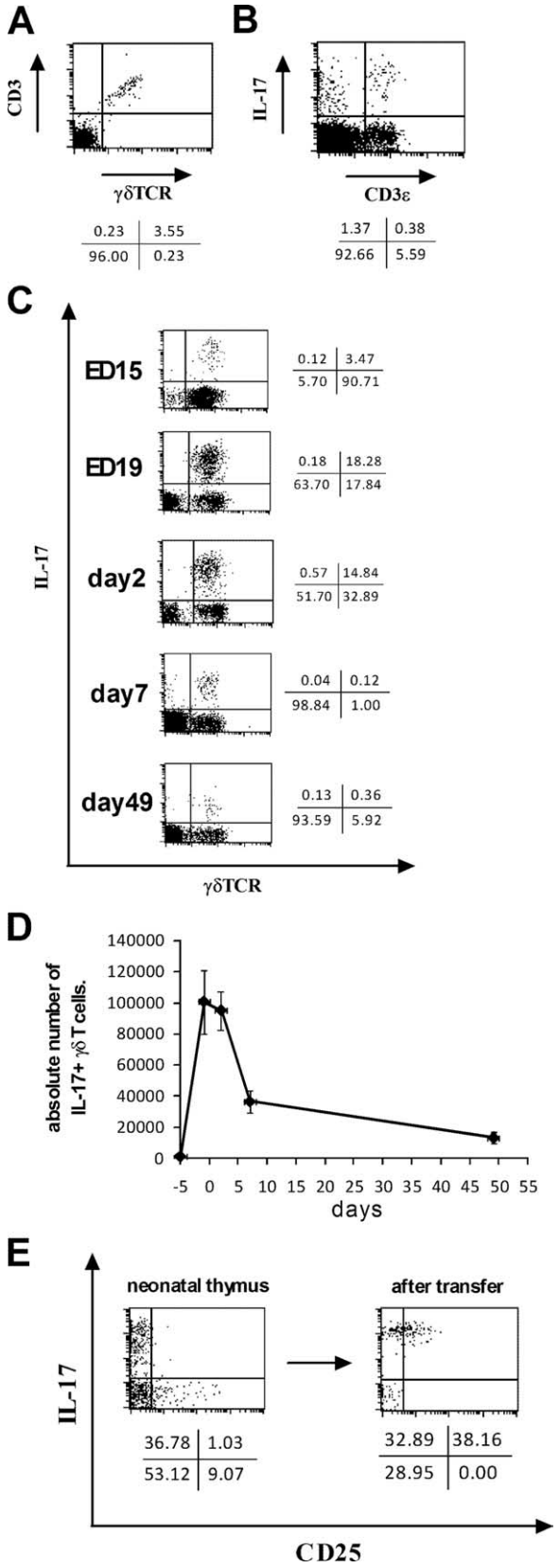

FIGURE 6. Functional maturation of IL-17-producing γδ T cells in fetal thymus. *A*, Expression of γδ TCR and CD3 on fetal thymocytes (ED15) was analyzed. *B*, Fetal thymocytes (ED15) were stimulated with PMA and ionomycin and were analyzed for the expression of CD3 and intracellular IL-17. Data are shown after gating on lymphocytes. The number shown under each panel indicates the percentage of cells in the respective lymphocyte gate. *C*, IL-17 production of thymocytes from various ages was analyzed. Data are shown after gating on $CD3^+$ cells. *D*, The absolute number of IL-17-producing γδ T cells in the thymus of different ages of mice were shown. Data shown indicate the mean ± SD of five to eight mice for each time point. *E*, Expression of CD25 on IL-17-producing γδ T cells in neonatal thymus of C57BL/6 mice ($Ly5.2^+$) was analyzed before and 4 days after transfer into the peritoneal cavity of C57BL/6 Ly5.1 congenic mice. Data are analyzed after gating on $Ly5.1^+$ γδ T cells. Results are representative of three independent experiments. The number shown under each panel indicates the percentage of cells in the respective quadrant.

of IL-15-deficient mice and found that $CD122^+$ γδ T cells were selectively decreased in these mice (Fig. 4*C*). Consistently, the number of IFN-γ-producing γδ T cells was decreased in IL-15-deficient mice, whereas the number of $CD25^+$ or IL-17-producing γδ T cells was not affected (Fig. 4*D*).

Functional maturation of IL-17-producing γδ T cells in fetal thymus before expressing CD25

Because we have found that $V\delta1^+$ γδ T cells in the peritoneal cavity, which are known to develop early in ontogeny, predominantly produce IL-17 in *E. coli* infection (10), we examined age-related changes in the frequency of IL-17-producing γδ T cells in the peritoneal cavity. As shown in Fig. 5, more than 70% of peritoneal γδ T cells produced IL-17 at 1 wk after birth. However, the percentage of IL-17-producing γδ T cells was decreased with age, whereas the percentage of IFN-γ-producing γδ T cells was increased in older mice, supporting perinatal ontogeny of IL-17-producing γδ T cells. These data also indicated that most of γδ T cells in the peritoneal cavity already acquired effector function as early as 1 wk after birth. Therefore, we next examined γδ T cells in fetal thymus, where $V\delta1^+$ γδ T cells predominantly differentiate (20–22). On day 15 of embryonic development (ED15), a few fetal thymocytes were positive for CD3, nearly all of which expressed γδ TCR (Fig. 6*A*). Surprisingly, the thymic γδ T cells on ED15 were already differentiated to produce IL-17 (Fig. 6*B*). IL-17-producing cells were also found in $CD3^-$ population, most of which were $CD44^+CD25^-$, DN1 phenotype (Fig. 6*B* and data not shown). The number of thymic IL-17-producing γδ T cells was peaked at perinatal period, on ED19, and rapidly decreased afterward (Fig. 6, *C* and *D*). In contrast to IL-17-producing γδ T cells in the periphery, those in the thymus were negative for CD25, but they became $CD25^+$ after being transferred into the periphery (Fig. 6*E*). Furthermore, IL-17-producing γδ T cells are equally found in the thymus of IL-2- or CD25-deficient mice (data not shown). These results further suggest that CD25-mediated signaling is not essential for functional differentiation of IL-17-producing γδ T cells.

$V\gamma6^+V\delta1^+$ cells in fetal thymus naturally differentiate to IL-17 producers

The number of IL-17-producing γδ T cells reached the peak on ED19 and was decreased with age, which is synchronized with the development of $V\delta1^+$ γδ T cells (26). $V\delta1^+$ γδ T cells in fetal thymus can be paired with either of the two Vγ chains, Vγ5 or Vγ6. Because mAb specific for Vγ5 was only available, we examined the expression of Vγ5 on IL-17-producing cells in fetal thymus. Although more than half of γδ T cells were positive for Vγ5 on ED15 (Fig. 7*A*), most of IL-17-producing γδ T cells were negative for Vγ5 (Fig. 7*B*) consistent with the absence of IL-17 production by skin γδ T cells expressing Vγ5 (Fig. 1) (21), suggesting an importance of $V\gamma6^+V\delta1^+$ γδ T cells as IL-17-producing cells. We detected *Vγ6* mRNA expression in the fetal thymus by RT-PCR (data not shown). To further confirm the relationship

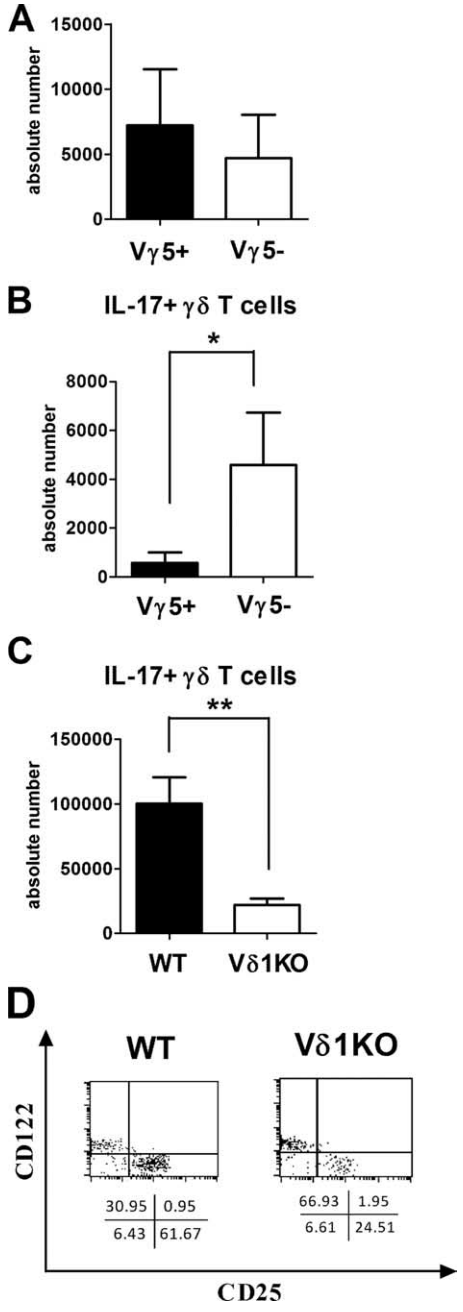

FIGURE 7. TCR repertoire of IL-17-producing γδ T cells. *A*, The absolute number of $V\gamma5^+$ and $V\gamma5^-$ γδ T cells in fetal thymus (ED15) was calculated after flow cytometric analysis. *B*, Fetal thymocytes (ED15) were stimulated with PMA and ionomycin, and the absolute number of IL-17-producing $V\gamma5^+$ and $V\gamma5^-$ γδ T cells was calculated after flow cytometric analysis. Data indicate the mean ± SD of five mice for each group. *, $p < 0.05$. *C*, The absolute number of IL-17-producing γδ T cells in fetal thymus of Vδ1-deficient mice was calculated. Data indicate the mean ± SD of nine mice for each group. **, $p < 0.01$. *D*, Expression of CD25 and CD122 on γδ T cells in the peritoneal cavity of adult (7-wk-old) Vδ1-deficient mice was analyzed. The number shown under each panel indicates the percentage of cells within each respective quadrant after gating on γδ T cells. Data are representative of three independent experiments.

between $V\delta1^+$ cells and the IL-17-producing γδ T cells, we examined Vδ1-deficient mice. The number of IL-17-producing γδ T cells in the fetal thymus of Vδ1-deficient mice were strikingly decreased (Fig. 7*C*), indicating the predominance of $V\gamma6^+V\delta1^+$ repertoire in fetal thymocytes that naturally differentiated to IL-17-producing cells. Consistently, $CD25^+$ γδ T cells were selectively decreased in the peritoneal cavity of adult Vδ1-deficient mice (Fig. 7*D*). This decrease was also consistent with our previous finding of a reduced IL-17 production by γδ T cells in the peritoneal cavity of Vδ1-deficient mice in response to *E. coli* infection (10).

Discussion

We have previously found that γδ T cells in the peritoneal cavity produced IL-17 immediately after infection with *E. coli*, which played an important role in mobilization of neutrophils, although detailed characters of the IL-17-producing γδ T cells have been unclear. In the present study, we first demonstrated that IL-17-producing γδ T cells in the peritoneal cavity did not produce IFN-γ or IL-4, similar to Th17 cells. Furthermore, we found the IL-17- and IFN-γ-producing γδ T cells are distinguishable by the expression of CD25 and CD122. $CD25^+CD122^-$ and $CD25^-CD122^+$ cells represent IL-17- and IFN-γ-producing γδ T cells, respectively.

In $CD4^+$ T cells, differentiation to different subsets of Th cells is dependent on the expression of different transcription factors. T-bet is required for the differentiation of Th1, and Th1 express high levels of T-bet (35). Similarly, we found that the IFN-γ-producing $CD122^+$ γδ T cells expressed high levels of T-bet. RORγt has been identified as differentiation factor for Th17 cells (7). However, we did not detect significant difference in the expression of *RORγt* mRNA between $CD25^+$ and $CD25^-$ γδ T cells, which are IL-17-producing and nonproducing γδ T cells, respectively. It is of note that RORγt is expressed not only in Th17 cells. For example, nearly all double positive thymocytes express RORγt, which is critical for survival of these cells (33, 34, 36). Thus, expression of RORγt does not necessarily mean IL-17 production. Moreover, we found the expression level of *RORγt* mRNA in either of the γδ T cell subsets was lower than that of double positive thymocytes. Additional factors or even other factors than RORγt might be essential for functional differentiation of IL-17-producing γδ T cells. Interestingly, although IL-6 and TGF-β are critical for the differentiation of Th17 cells, we did not detect significant effect of neutralizing IL-6 or TGF-β on the development of IL-17-producing γδ T cells in a fetal thymus culture system (data not shown). However, additional experiments using cytokine gene knockout mice are required to obtain conclusive data. It is of note that Rachitskaya et al. (37) recently reported that IL-17-producing NKT cells developed in the absence of IL-6. Therefore, IL-17-producing γδ T cells and NKT cells might undergo unique differentiation pathway different from that of conventional Th17 cells.

Consistent with the expression of CD25, a subunit of the high-affinity IL-2R complex, on IL-17-producing γδ T cells, their cell number was decreased in IL-2-deficient mice as well as in CD25-deficient mice. However, IL-2-mediated signaling seems dispensable for their development per se because a significant number of IL-17-producing γδ T cells were still found in IL-2- or CD25-deficient mice. IL-17-producing γδ T cells in the thymus were negative for CD25 and were equally found in both IL-2- and CD25-deficient mice. Therefore, it is suggested that IL-2-mediated signaling plays a role on IL-17-producing γδ T cells in the periphery, possibly by promoting their maintenance or expansion rather than by regulating functional differentiation of $V\gamma6^+$ γδ T cells to IL-17-producing cells because we found the total number of γδ T cells in the peritoneal cavity of IL-2- or CD25-deficient mice also reduced. Notably, there was a significant difference in the extent of reduction of IL-17-producing γδ T cells between IL-2- and CD25-deficient mice. The percentage of $IL\text{-}17^+$ cells in

γδ T cells was also reduced in CD25-deficient mice. This suggests an additional role of CD25, but any ligands for CD25 other than IL-2 have not been reported yet. Down-regulation of CD25 on γδ T cells in IL-2-deficient mice indicates that IL-2 is also essential for the expression of CD25. Similarly, down-regulation of CD122 on NK cells and CD8αα intestinal intraepithelial T lymphocytes including γδ T cells in IL-15-deficient mice has been reported (38). The number of these cell subsets was also decreased in the absence of IL-15 signaling (39). We found in this study that the number of IFN-γ-producing γδ T cells, which express CD122, was selectively decreased in IL-15-deficient mice. These results clearly indicate an importance of IL-15 for the maintenance of these CD122-expressing cells.

It is unknown how IL-17-producing γδ T cells in the periphery receive signals from CD25, as cellular sources of IL-2 are unclear. Although activated T cells, especially CD4 T cells, are assumed to be major IL-2 producers in vivo, their IL-2 production is usually dependent on TCR triggering, but is not spontaneous. Thus, continuous exposure to environmental Ag might be responsible. Nevertheless, such T cell responses usually take place in the lymph nodes, where γδ T cells are scarce. In contrast, in the peripheral tissue like peritoneal cavity, there is a predominance of effector memory T cells, which do not produce a large amount of IL-2 (40). Alternatively, IL-2 production by γδ T cells themselves might be involved. In fact, we detected a small part of IL-17$^+$ γδ T cells producing IL-2 after an in vitro stimulation with PMA and ionomycin (data not shown), although in vivo relevance of the γδ T cell-derived IL-2 is still unclear. Additional experiments using cell type-specific IL-2 knockout mice will clarify this issue.

Lack of expression of CD122 on IL-17-producing γδ T cells, which express CD25, was unexpected. We also found that CD25$^+$ Tregs did not express CD122, whereas activated CD4 and CD8 T cells equally expressed both CD25 and CD122 (Fig. 3*A*). Although CD25 is believed to form trimers with CD122 and CD132 (common γ chain), which are critical for the signal transduction, our results suggest the presence of CD25 in another configuration. Importantly, even such a form of CD25 does work as a functional receptor for IL-2 because CD25$^+$ Tregs as well as IL-17-producing γδ T cells are clearly regulated by IL-2 in vivo. We also found that CD25$^+$ γδ T cells proliferate in response to exogenous IL-2 in vitro (data not shown). Although it is still possible that γδ T cells express nearly undetectable but enough levels of CD122 for signal transduction, recent study on crystal structure of IL-2R suggests that CD25 can be expressed without CD122 (41). In addition, although IL-2R on γδ T cells may use CD132 for signal transduction, it is complicated to be analyzed because γδ T cells express IL-7R α-chain, which also forms a heterodimer with CD132. Detailed biochemical investigation on the structure of IL-2R complex on γδ T cells is needed. Notably, it was reported that IL-2 signaling rather has a suppressive effect on Th17 (42). This might also be attributed to the different expression patterns of IL-2R complex on Th17 and IL-17-producing γδ T cells.

We surprisingly found IL-17-producing γδ T cells complete functional differentiation in fetal thymus. Notably, it was also shown that invariant NKT cells also acquire the ability to produce cytokines such as IL-4 and IFN-γ during the developmental stage in the thymus (43). In addition to the IL-17-producing γδ T cells, we detected significant number of IFN-γ-producing γδ T cells in fetal thymus (data not shown). Invariant NKT cells are not deleted but are positively selected upon recognition of CD1d in the thymus, whereas Itohara and Tonegawa (44) reported that γδ T cells in fetal thymus, which might include the IL-17-producing cells, expressed canonical TCR and were not deleted but positively selected by TCR-mediated stimulation. γδ T cells and invariant NKT cells are also similar in their functions as innate lymphocytes that play a role in early protection against infection. Innate lymphocytes also include NK cells as well as memory phenotype CD8 T cells, which rapidly secrete IFN-γ and a part of latter are suggested to be self-specific (45). NKT cells as well as IL-17-producing γδ T cells express memory markers (43). Taken together, it is speculated that such innate T lymphocytes naturally acquire effector functions, similar to NK cells, to rapidly respond to invading pathogens.

Because mAbs specific for Vγ6 are unavailable, it is not yet clear that all the CD25$^+$ IL-17-producing γδ T cells in the peritoneal cavity are identical with Vγ6$^+$ cells or vice versa. Nevertheless, our results strongly suggest that IL-17-producing γδ T cells developed in fetal thymus were V6$^+$Vδ1$^+$ cells, but not Vγ5$^+$Vδ1$^+$ cells. Consistently, Vγ5$^+$ γδ T cells in the skin did not produce IL-17 (Fig. 1*B*). It is interesting how Vγ5$^+$ and Vγ6$^+$ cells, which share same Vδ1 chain, acquire different functions in the thymus. This feature might be due to different TCR signals provided by different ligands. Alternatively, immature thymocytes producing IL-17 are committed to express Vγ6. This finding is supported by the fact that IL-17 production was also detected in CD3$^-$ populations of fetal thymocytes (ED15), which express CD44$^+$CD25$^-$ phenotype of T cell precursors (DN1) (Fig. 6*B*). However, we are unable to exclude other possibilities that the CD3$^-$ IL-17-producing cells differentiate into another cell type or die before being exported from the thymus because we have not found any markers specifically expressed on these cells. Although we have focused on fetal thymus-derived peritoneal IL-17-producing γδ T cells in this study, there seem other subsets of IL-17-prodcuing γδ T cells. Some of Vγ4$^+$ cells in the lung produce IL-17 but are negative for CD25 (our unpublished observation). Vγ4$^+$ γδ T cells express Vδ5 or Vδ6, develop after birth and exist in the lung, thymus, and spleen (46, 47). Despite these differences, these findings also suggest a relationship between certain TCR repertoire and IL-17 production of γδ T cells. Because the specific ligands for these γδ T cells are largely unknown, involvements of TCR-mediated signaling in the development and functions of IL-17-producing γδ T cells are difficult to address. Thus, identification of the natural ligands for γδ T cells remains as a critical issue.

Although biology of Vγ5$^+$ T cells, which uniquely constitute skin epithelial lymphocytes, has been extensively studied, little has been known about Vγ6$^+$ cells, the other subset of fetal thymus-derived Vδ1$^+$ cells. Our results demonstrate that Vγ6$^+$ cells are also unique subset of T cells, which naturally differentiate to IL-17 producers and express CD25, and thus provide a substantial advance in understanding the biology of γδ T cells that comprise immune system from early stage of ontogeny.

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Disclosures

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