Cutting Edge: Spontaneous Development of IL-17–Producing \( \gamma\delta \) T Cells in the Thymus Occurs via a TGF-\( \beta1 \)–Dependent Mechanism

Jeong-su Do, Pamela J. Fink, Lily Li, Rosanne Spolski, Janet Robinson, Warren J. Leonard, John J. Letterio and Booki Min

*J Immunol* published online 8 January 2010
http://www.jimmunol.org/content/early/2010/01/08/jimmunol.0903539

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/01/08/jimmunol.0903539.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
In naive animals, γδ T cells are innate sources of IL-17, a potent proinflammatory cytokine mediating bacterial clearance as well as autoimmunity. However, mechanisms underlying the generation of these cells in vivo remain unclear. In this study, we show that TGF-β1 plays a key role in the generation of IL-17+ γδ T cells and that it mainly occurs in the thymus particularly during the postnatal period. Interestingly, IL-17+ γδ TCR+ thymocytes were mainly CD44highCD25low cells, which seem to derive from double-negative 4 γδ TCR+ cells that acquired CD44 and IL-17 expression. Our findings identify a novel developmental pathway during which IL-17–competent γδ T cells arise in the thymus by a TGF-β1–dependent mechanism.

The Journal of Immunology, 2010, 184: 000–000.

Although they are widely distributed throughout the epithelial cell-rich tissues (1) and are known to be an important source of IL-17 in response to a number of pathogens, recruiting neutrophils to the site of inflammation (2, 3), γδ T cells constitute a small proportion (<5%) of total peripheral T lymphocytes. γδ T cells are also the major IL-17–producing cells in naive animals (4–6). It was reported that Ag–naïve CD122+ γδ T cells preferentially produce IL-17, whereas Ag–experience CD122+ γδ T cells produce IFN-γ (5). It was recently shown that the expression of the TNF family member b1 and IFN-γ (5) T cells acquire IL-17 expression in vivo remains unclear. In this study, we report that developing γδ T cells acquire IL-17–producing capacity within the thymus via a TGF-β1–dependent mechanism. Interestingly, peripheral IL-17+ γδ T cells primarily accumulate in the peripheral but not in the mesenteric LN (mLN). An ontogeny study revealed that the highest frequency of IL-17+ γδ T cells was found in the postnatal thymus. IL-17+ γδ TCR+ thymocytes were CD44highCD25low (double-negative [DN] 1) phenotype cells, which are in fact the DN4 cells that upregulated CD44 and acquired IL-17 expression. The generation of IL-17+ γδ T cells was dramatically abolished by the absence of TGF-β1 but not of other Th17-inducing cytokines. Consistent with this, γδ T cells in the thymus expressed the highest levels of TGF-β receptors. Taken together, the current study highlights a unique pathway of thymic γδ T cell development during which the differentiation of natural IL-17+ γδ T cells takes place, revealing an irreplaceable role for TGF-β1 to promote this process.

Materials and Methods

Mice

C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lymphoid cells from IL-6−/− and IL-23 p19−/− mice were provided by Drs. Robert Fairchild and Steve Stohlman (Cleveland Clinic Foundation, Cleveland, OH). IL-21−/− mice were purchased from the Mutant Mouse Regional Resource Centers (Columbia, MO; www.mmrrc.org). Rag2pGFP and Tgfb1−/− mice were previously described (8, 9). All experimental procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation, Case Western Reserve University, and the University of Washington.

Ex vivo stimulation

Spleen, axillary and cervical LN (pLN), and mLN cells were separately harvested and ex vivo stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 4 h in the presence of 2 μM monensin (Calbiochem, San Diego, CA) during the last 2 h. Cells were immediately fixed with 4% paraformaldehyde, permeabilized, and stained with fluorescence conjugated Abs (see below).

Flow cytometry

The following Abs were used: biotinylated anti-γδ TCR (GL3), PE–anti-γδ TCR (GL3), PE–anti-CD25 (PC61), PE–anti-CD44 (IM7), PE–anti-CD82 (MEL-14), PE–anti–IFN-γ (XMG1.2), streptavidin-PE, PE-Cy5–

"Department of Immunology, Lerner Research Institute, and †Cleveland Clinic Lerner College of Medicine, Cleveland Clinic Foundation, Cleveland, OH 44195; ‡Department of Immunology, University of Washington, Seattle, WA 98195; §Laboratory of Molecular Immunology and Immunology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and †Division of Pediatric Hematology/Oncology, Department of Pediatrics, Case Western Reserve University, Cleveland, OH 44106.

Received for publication November 2, 2009. Accepted for publication December 18, 2009.

Supported by National Institutes of Health Grant R01-AI074932 (to B.M.) and R01-AI064318 (to P.J.F.), and by the Intramural Research Program, National Heart, Lung, and Blood Institute, National Institutes of Health (to R.S. and W.J.L.).
anti-CD44 (IM7), APC-anti-CD4 (RM4-5), APC-anti-CD8 (53-6.7), APC-anti-IL-17 (dhol1B7), FITC-anti-CD4 (RM4-5), FITC-anti-CD8 (53-6.7), FITC-anti-NK1.1 (PK136), FITC-anti-B220 (RA3-6B2), and FITC-anti-IFN-γ (XMG1.2) Abs. All Abs were purchased from eBioscience (San Diego, CA) or BD Pharmingen (San Jose, CA). Cells were acquired using a FACSCalibur (Becton Dickinson) RNA was extracted using RNeasy reagent (Qiagen, Valencia, CA), and cDNA was subsequently generated by Superscript III RTase (Invitrogen, Carlsbad, CA). Taqman primers/probes specific for Tgfbr1 (Mm03024015_m1) and Tgfbr2 (Mm03024091_m1) were purchased from Applied Biosystems (Foster City, CA), and their expression was determined using an Applied Biosystems 7500 PCR system. Expression level was normalized based on the 18S rRNA (VIC-TAMRA, purchased from Applied Biosystems) expression.

Data analysis
Statistical significance was determined by the Student t test using the SigmaPlot 9.0 (SPSS, Chicago, IL). p < 0.05 was considered to indicate a significant difference.

Results and Discussion
γδ T cells are the major IL-17–producing cells in native animals
In naive animals, very few (∼0.1%) LN CD4 T cells expressed intracellular IL-17 following PMA/ionomycin stimulation, but ∼0.5% non-CD4 T cells expressed IL-17 under the same conditions (Fig. 1A). IL-17+ non-CD4 T cells were not CD8, B, or NK1.1+ cells (Fig. 1A); instead, ∼75% of the IL-17+ cells expressed γδ TCR (Fig. 1B). A significant portion of γδ T cells expressed an activated phenotype compared with αβ T cells: CD44high and CD62Llow (Fig. 1C). IL-17 production was noticed only from CD44high γδ T cells (Fig. 1D), which differs from a previous study showing that IL-17 is preferentially produced from naive CD122low γδ T cells after TCR cross-linking (5). Our finding agrees with a recent report that CD27low γδ T cells that mainly produce IL-17 are CD44highCD62Llow cells (7). Indeed, IL-17+ γδ T cells in the pLN displayed the same CD27low phenotype (Fig. 1E, top panel).

Interestingly, the proportion of IL-17+ γδ T cells in regional lymphoid tissues displayed a substantial heterogeneity; the highest frequency of IL-17+ γδ T cells was found in pLN, whereas γδ T cells from mLNs failed to express IL-17 (Fig. 1F). IL-17+ γδ T cells in the spleen were also present at a low frequency (∼3%). Notably, <3% of CD27low mLN γδ T cells expressed IL-17, suggesting that CD27low phenotype does not necessarily define IL-17–producing γδ T cells (Fig. 1E). By contrast, IFN-γ+ γδ T cells were found in all lymphoid tissues (Fig. 1F). Consistent with previous reports (5, 7), γδ T cells producing IL-17 and IFN-γ did not overlap (data not shown). Unlike lymphoid γδ T cells, skin-resident γδ T cells, γδ T cells from Peyer’s patches, or intraepithelial γδ lymphocytes from the small intestine and the colon expressed very little IL-17 (Fig. 1G). Why peripheral IL-17+ γδ

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** γδ T cells preferentially express IL-17 following activation. A, LN cells were ex vivo stimulated with PMA/ionomycin and subsequently stained for CD4, NK1.1, CD8, B220, and IL-17. B, LN cells stimulated as above were stained for B220, CD4, CD8, IL-17, and γδ TCR. Shown is γδ T cell IL-17 expression by non-B/non-T cells. γδ, CD4, and CD8 T cells from the indicated tissues were examined for the surface expression of CD44 and CD62L. D, IL-17 expression of CD44low and CD44high γδ T cells (spleen and pLN) was examined by intracellular staining. E, γδ T cells from the indicated tissues were stimulated and stained for IL-17 and CD27. F, Proportions of IFN-γ+ and of IL-17–producing γδ T cells (and CD4 T cells) in the indicated tissues were examined. Shown is the mean ± SD (n = 4). G, Cells isolated from the indicated tissues were subsequently stained with PMA/ionomycin, and cytokine production was determined by flow cytometric analysis. Shown are cytokine profiles of γδ TCR+ gated cells. All the experiments were repeated more than twice and similar results were observed. **p < 0.01; ***p < 0.001. DETC, dendritic epidermal γδ T cell; IEL, intraepithelial lymphocyte; PP, Peyer’s patches; SI, small intestine.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** IL-17 expression of γδ T cells in mice of different ages. A–C, Spleen, pLN, mLN, and thymic cells from mice at the indicated ages were stimulated as described in Fig. 1 were stained for IL-17, IFN-γ, and γδ TCR. Proportions of IL-17– (A) and of IFN-γ–expressing (C) γδ T cells were examined. B, Total numbers of IL-17–expressing γδ T cells were determined by flow analysis. Shown are the mean ± SD (n = 3–4).
T cells display a lymphoid tissue-specific accumulation is unclear. IL-17+ γδ T cells may express a chemokine receptor (s) that allows them to preferentially migrate to and accumulate in the pLN. Indeed, Martin et al. (10) recently reported that IL-17+ γδ T cells uniformly express CCR6. Whether CCR6 is necessary for IL-17+ γδ T cell accumulation in the pLN remains to be determined. Alternatively, a microenvironment within the mLN may suppress IL-17 expression by γδ T cells. These results demonstrate that CD44high γδ T cells display IL-17+—producing capacity and that these IL-17+ γδ T cells are primarily enriched in the pLN but not in the mLN or in other epithelial cell-rich tissues.

**Age-dependent generation of IL-17+ γδ T cells in the thymus**

It was recently demonstrated that the IL-17+ phenotype of γδ T cells is established during thymic development (5, 7). Analysis of γδ TCR+ thymocytes from mice of different ages revealed a striking pattern in IL-17+ production. Thymus from newborn mice contained γδ TCR+ thymocytes, 30–40% of which expressed IL-17+ following stimulation (Fig. 2A). The proportions of IL-17+ γδ TCR+ thymocytes reached a peak around 5 d of age and declined thereafter (Fig. 2A). Interestingly, IL-17+ γδ T cells in the pLN increased as thymic IL-17+ γδ TCR+ thymocytes declined (between 7 and 14 d of age), suggesting that the IL-17+ cells differentiated within the thymus appear to populate the periphery. Particularly striking is that the total numbers of IL-17+ γδ T cells were constant regardless of age (Fig. 2B), strongly suggesting a tight homeostatic mechanism that controls the generation of IL-17+ γδ T cells in the thymus. Notably, thymic γδ TCR+ thymocytes expressed little IFN-γ, whereas peripheral γδ T cells uniformly expressed IFN-γ in all tested lymphoid tissues (Fig. 2C), suggesting that IFN-γ production, unlike IL-17+, may be acquired in the periphery at least at the protein level.

**DN stage γδ T cells become CD44+ and express IL-17**

To characterize a developmental pathway leading to the generation of IL-17+ γδ thymocytes, adult thymocytes were stimulated ex vivo and the phenotypes of IL-17+ γδ TCR+ cells were examined. IL-17+ γδ TCR+ thymocytes were mainly found within CD4negCD8negCD44highCD25low cells, the phenotype of DN1 thymocytes (Fig. 3A). Notably, developing DN thymocytes initiate γδ TCR rearrangement following the DN1 stage and start to express surface γδ TCR after the DN2/3 stage (11). Therefore, IL-17+ γδ T cells found in the DN1 stage might be peripheral γδ T cells that have recirculated from the periphery (12). Alternatively, it is possible that the DN1-stage γδ T cells are activated DN4 cells that upregulated CD44 and acquired IL-17+ expression. To test this, we used Rag2p-GFP transgenic (Tg) mice (13). It was previously shown that Rag2 promoter-driven GFP expression is induced during the late DN2 stage, reaches peak expression in DP thymocytes, and gradually diminishes during the transition from the DP to the SP thymocytes (14). In the periphery, GFP expression is primarily found in recent thymic emigrants, yet the expression is lower than that in DP thymocytes (14). The distribution of γδ TCR+ thymocytes in Rag2p-GFP Tg adult mice among the DN subsets was comparable to that of non-Tg mice (data not shown). Similarly, most IL-17+ γδ T cells were...
found within the DN1 subsets (data not shown). DN1 γδ TCR+ thymocytes showed two populations based on GFP expression (Fig. 3B), and IL-17–producing γδ TCR+ thymocytes were mostly GFP<sup>-</sup> cells (Fig. 3C). As expected, only GFP<sup>+</sup> γδ T cells were found in the pLN (Fig. 3C).

To further examine if GFP<sup>+</sup> IL-17–producing γδ T cells are mature cells recirculated from the periphery, we examined the thymus from 5-d-old Rag2-GFP Tg mice, an age before IL-17–producing γδ T cells are seen in the periphery (Fig. 2A). Even at this age, the majority of CD4<sup>+</sup> γδ TCR+ thymocytes did not express GFP (data not shown). Moreover, IL-17–producing γδ TCR+ thymocytes from these mice were mostly GFP<sup>+</sup> cells (Fig. 3D), whereas all DN4 γδ TCR+ thymocytes expressed GFP (data not shown). Therefore, it is likely that the GFP<sup>+</sup> IL-17–producing γδ TCR+ thymocytes in 5-d-old mice have undergone extensive proliferation and have lost GFP expression (15). A similar pattern was observed in 14-d-old mice (data not shown). Therefore, our data strongly suggest that CD4<sup>+</sup> IL-17–producing γδ T cells in the thymus are DN4 cells that acquire CD4<sup>+</sup> expression. To test this possibility, sorted DN4 (CD4<sup>+</sup>lowCD25<sup>+</sup>) γδ TCR+ thymocytes were cocultured with the stromal cell line OP9-DL<sub>4</sub> (16). OP9-DL<sub>4</sub> cells transduced with the Notch ligands (Delta-like 4) efficiently promoted T lineage development (17). DL4 (16). OP9-DL4 cells transduced with the Notch ligands supported the transition from DN4 to DN1-like cells.

**TGF-β is required for the generation of IL-17–producing γδ T cells**

In case of CD4 T cells, multiple factors including IL-23, IL-21, IL-6, and TGF-β play roles in the differentiation of naive CD4 T cells into Th17 cells (18–20). We thus explored whether these cytokines are required for the endogenous generation of IL-17–producing γδ T cells. Lymphoid cells from the indicated gene-deficient mice were stimulated and cytokine production was examined. As shown in Supplemental Fig. 2, the lack of an IL-23 p19 subunit and IL-6 did not alter IL-17 production by γδ T cells. IL-17 production of γδ T cells in the spleen and mLN of IL-21–deficient mice was slightly reduced; yet, pLN γδ T cell IL-17 production in these mice was not altered (Supplemental Fig. 2). Notably, the total numbers of γδ T cells in the lymphoid tissues of these mice were equivalent, indicating that the generation of γδ T cells is independent of these cytokines (data not shown). Endogenous CD4 T cell IL-17 expression was partially reduced by IL-23 p19 deficiency and completely abolished by the lack of IL-6 (data not shown). Therefore, these cytokines, while playing an important role in the generation of endogenous Th17 cells (21), play little or no role in γδ T cell acquisition of IL-17 expression. Similarly, Lochner et al. (6) also reported that IL-17–producing γδ T cells were unaffected by the absence of IL-6. Whether TGF-β1 plays a role in the generation of IL-17–producing γδ T cells was next examined. As all Tgfβ1<sup>/−</sup> mice develop severe lymphoproliferative disease early in life, LN and spleen cells from 2- to 3-wk-old mice were used. TGF-β1 deficiency completely abolished IL-17 expression by γδ T cells (Fig. 4A). Of note, γδ T cell generation was not impaired in Tgfβ1<sup>/−</sup> mice (Supplemental Fig. 3). Likewise, γδ T cells deficient in Smad3, a TGF-β–signaling adaptor molecule (22), expressed significantly lower levels of IL-17 compared with littermate controls (Fig. 4B). In contrast, IFN-γ production of γδ T cells was not different in Tgfβ1<sup>/−</sup> and in Smad3<sup>/−</sup> mice (Supplemental Fig. 4).

We then examined if TGF-β1 is needed for the development of IL-17–producing γδ T cells in the thymus. γδ TCR+ thymocytes from Tgfβ1<sup>/−</sup> and littermate control mice were analyzed for IL-17 expression. The DN distribution of developing γδ TCR+ thymocytes was not different (Fig. 4C). However, IL-17–producing capacity of the developing γδ TCR+ thymocytes was greatly impaired in Tgfβ1<sup>/−</sup> mice (Fig. 4D). In support of this finding, γδ TCR+ thymocytes expressed the highest levels of type I and type II TGF-β receptors (Fig. 4E). Notably, some thymic γδ T cells still acquire IL-17 expression in Tgfβ1<sup>/−</sup> mice (Fig. 4D), and this finding might be supported by the expression of either TGF-β2 or TGF-β3 by thymic epithelia. However, these cells disappeared in the periphery (Fig. 4A), suggesting that TGF-β1 may play an important role in maintaining IL-17–producing γδ T cells in the periphery. It was previously reported that thymic TGF-β is expressed on subcapsular and cortical thymic epithelium, which interacts with developing thymocytes (23). Identifying the source of TGF-β in the thymus as well as in the periphery will be an important subject for future study. Taken together, these results strongly suggest that TGF-β1, although dispensable for the phenotypic maturation (i.e., CD4<sup>+</sup> upregulation during DN4 to DN1-like transition), plays an irreplaceable role in the acquisition of IL-17–producing capacity in the thymus.

What are the immunologic roles of IL-17–producing γδ T cells in vivo? Following *Escherichia coli* infection, γδ T cell–derived IL-17 was shown to play critical roles in recruiting neutrophils and in neutrophil-mediated bacterial clearance (2). IL-17 production by γδ T cells is also associated with lethal pulmonary aspergillosis in mice with chronic granulomatous disease (24). Moreover, γδ T cell IL-17 production is undoubtedly involved in exacerbating collagen-induced arthritis or autoimmunity (25, 26). The current study provides an important basis to define the mechanism(s) of how γδ T cells acquire IL-17 expression and of how endogenous γδ T cell–derived IL-17 influences immunity in vivo.

**Acknowledgments**

We thank Drs. Rob Fairchild and Steve Stohlman (Cleveland Clinic) for providing IL-6<sup>+</sup> and IL-23 p19<sup>+</sup> cells, respectively, Dr. Lan Zhou (Case Western Reserve University) for providing OP9 and OP9-DL4 cell lines, and Ms. Jennifer Powers for cell sorting.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

1. Caude, S. R., and P. J. Egan. 2002. Gammadelta T cells: functional plasticity and heterogeneity. *Nat. Rev. Immunol.* 2: 336–345.
2. Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007. Resident Vdelta1+ γδ T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J. Immunol.* 178: 4466–4472.
3. Roark, C. L., P. L. Simonian, A. P. Fontenot, W. K. Born, and R. L. O’Brien. 2008. Gammadeltal T cells: functional plasticity and heterogeneity. *Nat. Rev. Immunol.* 2: 336–345.
4. Stark, M. A., Y. Huo, T. L. Burcin, M. A. Morris, T. S. Olson, and K. Ley. 2005. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 22: 285–294.
5. Jensen, K. D., X. Su, S. Shin, L. Li, S. Yousef, S. Yamasaki, L. Steinman, T. Saito, R. M. Lockey, M. M. Davis, et al. 2008. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity* 29: 90–100.
