Brief Definitive Report

Impaired Development of Vγ3 Dendritic Epidermal T Cells in p56^{lck} Protein Tyrosine Kinase–deficient and CD45 Protein Tyrosine Phosphatase–deficient Mice

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Summary

To determine whether p56^{lck} protein tyrosine kinase and CD45 protein tyrosine phosphatase are involved in the signal transduction during intrathymic differentiation of γ/δ T cells, we have examined the development of T cells expressing Vγ3 T cell receptor (TCR) in mice deficient for either protein. The skin from both mice contained significantly reduced numbers of dendritic epidermal T cells expressing decreased levels of Vγ3 TCR at the cell surface. Analysis of the fetal thymus from these mice suggested that maturation of Vγ3 thymocytes was blocked at the immature stage that was characterized by the low level of Vγ3 TCR and the high level of heat stable antigen. These results imply that both p56^{lck} and CD45 are involved in the signal transduction during maturation of Vγ3 T cells in the fetal thymus.

Recent studies have examined the role of several protein tyrosine kinases and protein tyrosine phosphatases in the signal transduction during thymocyte development. p56^{lck}, a *src*-family protein tyrosine kinase, is physically associated with the cytoplasmic domains of CD4 and CD8 and participates in the signal transduction through the TCR in mature α/β T cells (for reviews see references 1, 2). Mice deficient for the p56^{lck} (Lck$^{-/-}$) (3) or carrying a dominant negative mutation of the p56^{lck} gene (4) display an early block in α/β thymocyte development. Interestingly, this developmental block occurs before α/β TCR–regulated positive and negative selection processes commence and appears to be independent of the interaction of p56^{lck} with CD4 and CD8 (3, 4). The cell-surface receptor that interacts with p56^{lck} during early α/β thymocyte development has not been identified, although a putative role for p56^{lck} in the signal transduction through the pre-TCR formed by TCRβ and gp33 was suggested (5).

CD45 protein tyrosine phosphatase specifically dephosphorylates negative regulatory tyrosine residues of *src*-family protein tyrosine kinases and is required for the kinase activity of p56^{lck} (for reviews see references 1, 6). CD45-deficient (CD45−/−) mice also manifest a block in α/β thymocyte development (7). Unlike Lck−/− mice and dominant negative p56^{lck}-transgenic mice, the block in CD45−/− mice occurs at a relatively late stage where α/β thymocytes undergo TCR-regulated selection processes (7). The differential requirements of p56^{lck} and CD45 suggest that protein tyrosine phosphatases other than CD45 may regulate the activity of p56^{lck} in early α/β thymocyte development.

As compared with α/β T cells, little is known about the signal transduction during development of γ/δ T cells. The skin of mice contains dendritic epidermal T cells (DETC) (8) expressing an invariant γ/δ TCR composed of Vγ3 paired with Vδ1-Dδ1-Jδ2 chains, both of which lack junctional diversity (for a review see reference 9). This canonical Vγ3 TCR is identical to the TCR expressed on the first T cells to appear in the fetal thymus and it has been shown that DETC arise from these fetal thymic precursors (9, 10). Recently, maturational steps of Vγ3 T cells in the fetal thymus were defined (11). However, the cell-surface receptor or the signal transduction involved in intrathymic differentiation of Vγ3 T cells remains unknown.

In this report, we demonstrate that development of Vγ3 fetal thymocytes as well as their descendants, DETC, is impaired in both Lck−/− and CD45−/− mice. These results suggest that p56^{lck} and CD45 are involved in the signal transduction required for development of γ/δ T cells in the fetal thymus.

Materials and Methods

Mice. The generation of Lck−/− and CD45−/− mice (H-2^{b}) has been described elsewhere (3, 7). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as a wild-type (+/+) control.

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Immunofluorescence Staining of Epidermal Sheets. EDTA-separated epidermal sheets were prepared from the ear as described (12), fixed in cold acetone for 15 min, and incubated with a FITC-conjugated mAb for 1 h at 37°C. After rinses in PBS, the specimens were mounted in buffered glycerine containing 0.3 M 1,4-diazabicyclo-[2.2.2]octane (Sigma Chemical Co., St. Louis, MO) and examined under a Zeiss microscope (Obercochen, Germany).

Epidermal Cell Preparation. Epidermal cell suspensions were prepared from the ear and the trunk skin by trypsinization as described (13). The cells were cultured overnight in IMDM supplemented with 10% FCS, 5×10^{-5} M 2-ME, penicillin, streptomycin, and 10% supernatant from Con A–stimulated rat spleen cells. Debris and dead cells were removed by Lympholyte-M (Cedarlane Laboratories, Hornby, ON, Canada) density gradient centrifugation. The average yield of viable cells per mouse after overnight culture and density gradient centrifugation did not differ significantly among +/+, Lck−/−, and CD45−/− mice.

Flow Cytometric Analysis. Cells ($2–10 \times 10^5$) were resuspended in PBS supplemented with 2% FCS, 0.1% NaN_3, 25 mM EDTA, and incubated for 1 h at 4°C with saturating amounts of mAbs. The following mAbs were purchased from PharMingen (San Diego, CA): FITC-conjugated anti-Vγ3 TCR, 536; FITC-conjugated anti-α/β TCR, H57-597; FITC-conjugated anti-γ/δ TCR, GL3; FITC-conjugated anti-pan CD45, 30F11.1; PE-conjugated anti-Thy-1.2, 30-H12; PE-conjugated anti-CD3ε, 145-2C11; PE-conjugated anti–heat stable antigen (HSA), M1/69; and PE-conjugated anti-IL-2Rβ, TM-β1. Live gates were set on the basis of forward and side scatters and 10^4 viable cells were analyzed for each sample using a FACScan® flow cytometer with the Lysis II program (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

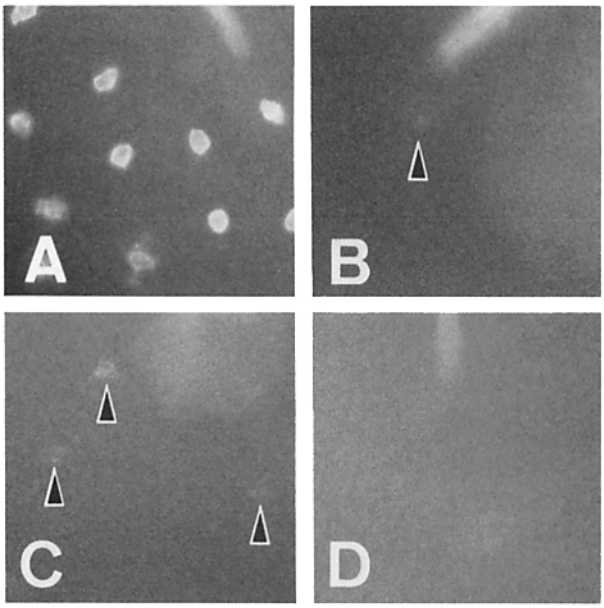

Figure 1. Lck−/− and CD45−/− mice have reduced numbers of Vγ3 DETC. Epidermal sheets from +/+ (*A* and *D*), Lck−/− (*B*), or CD45−/− (*C*) mice were stained with a FITC-conjugated anti-Vγ3 (A-C) or isotype-matched control anti-α/β TCR mAb (*D*). Scattered epidermal cells in Lck−/− and CD45−/− mice are weakly positive for Vγ3 TCR (arrowheads). Similar results were obtained in seven independent experiments. ×400.

Results and Discussion

To determine the role of $p56^{lck}$ and CD45 in development of Vγ3 T cells, epidermal sheets from +/+, Lck−/−, and CD45−/− mice were examined for the presence of Vγ3 DETC by immunofluorescence staining. Whereas $V\gamma3^+$ cells were abundantly found in the epidermis of +/+ mice, only scattered $V\gamma3^{low}$ cells were detected in Lck−/− and CD45−/− mice (Fig. 1). Reduction of Vγ3 DETC in Lck−/− and CD45−/− mice was also demonstrated by flow cytometric analysis of trypsinized epidermal cells (Fig. 2 *A*). Downregulation of Vγ3 TCR on the residual DETC was less apparent in the epidermal cell suspensions than in the in situ staining of epidermal sheets. Increased TCR expression probably occurred during the preparation of epidermal cells as a consequence of the in vitro culture period. Although a small subset of peripheral T cells in CD45−/− mice expressed CD45 (7), the residual Vγ3 DETC in CD45−/− mice were negative for CD45 (data not shown). The presence of small numbers of $V\gamma3^{low}$ DETC in Lck−/− and CD45−/− mice suggests that other protein tyrosine kinases and protein tyrosine phosphatases could partially compensate for the function of $p56^{lck}$ and CD45 in the development of Vγ3 DETC, respectively. Nevertheless, these results indicate that both $p56^{lck}$ and CD45 play critical roles in the development of Vγ3 DETC.

It should be noted that a fraction of $Thy\text{-}1^+$ epidermal cells was found to be negative for Vγ3 TCR (Fig. 2 *A*) in Lck−/− and CD45−/− (as well as +/+) mice. These epidermal cells did not express either α/β or γ/δ TCR (Fig. 2 *B* and data not shown). Since similar $Thy\text{-}1^+$ epidermal cells lacking surface expression of TCR/CD3 were found in athymic nude mice (14) and irradiated thymectomized mice

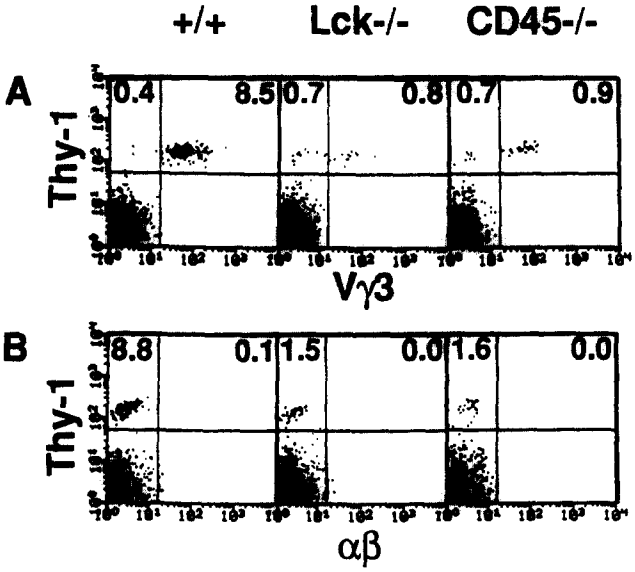

Figure 2. Flow cytometric analysis of epidermal cells. Epidermal cell suspensions from +/+, Lck−/−, or CD45−/− mice were double stained with PE-conjugated anti-Thy-1 and FITC-conjugated anti-Vγ3 (*A*) or anti-α/β (*B*) TCR mAbs. Mean percentages ± SD of $Thy\text{-}1^+/V\gamma3^+$ DETC (n = 7) were 8.1 ± 2.9%, 1.4 ± 1.4%, and 1.1 ± 1.0% in +/+, Lck−/−, and CD45−/− mice, respectively.

Table 1. *Number of FD16 Thymocytes and Vγ3⁺ Cell Subsets*

Mice	Cells/thymus*			
	Total	Total Vγ3⁺	Vγ3^{low}/HSA^{high}	Vγ3^{high}/HSA^{low}
	× 10^{-5}			
+/+	3.9 ± 1.2	10,960 ± 3,100	4,860 ± 670	6,100 ± 2,430
Lck−/−	3.7 ± 0.6	11,000 ± 1,780	9,970 ± 1,480	1,030 ± 870
CD45−/−	3.9 ± 1.2	10,230 ± 1,210	8,090 ± 1,390	2,130 ± 1,170

* Data are expressed as an arithmetic mean ± SEM. For each group, at least 20 fetal thymi were analyzed in three independent experiments.

reconstituted with adult bone marrow cells (15), these cells might represent epidermal lymphocytes of extrathymic origin. This possibility is supported by the observations that extrathymic development of γ/δ T cells in the intestine occurs normally in Lck−/− and CD45−/− mice (16, and Kishihara, K., unpublished data).

Previous studies have demonstrated that the precursors of Vγ3 DETC develop in the fetal thymus (9, 10) and recently, immature Vγ3^{low}/HSA^{high} and mature Vγ3^{high}/HSA^{low} subsets were defined (11). As shown in Table 1, the total thymocyte number was the same in +/+, Lck−/−, and CD45−/− mice at fetal day (FD) 16. Since the thymus is predominantly comprised of CD4⁻/CD8⁻ cells at this stage, the defect in α/β thymocyte maturation observed in adult Lck−/− and CD45−/− mice (3, 7) is not detectable. A similar number of Vγ3 thymocytes was also found in +/+, Lck−/−, and CD45−/− mice at FD16 (Table 1). However, the level of Vγ3/CD3 expression was noticeably reduced in Lck−/− and CD45−/− mice (Fig. 3 *A*). FD16 +/+ thymocytes already contained a significant number of mature Vγ3^{high}/HSA^{low} cells, whereas in Lck−/− and CD45−/− mice the majority of Vγ3 thymocytes remained at the immature Vγ3^{low}/HSA^{high} stage (Fig. 3 *B*). Although CD45−/− thymocytes contained slightly increased numbers of mature Vγ3^{high}/HSA^{low} cells as compared with Lck−/− thymocytes (Table 1), these cells also did not express CD45 (data not shown). It is unlikely that the reduction of mature Vγ3^{high}/HSA^{low} thymocytes in Lck−/− and CD45−/− mice at FD16 is due to delayed differentiation of Vγ3 thymocytes, since the immature Vγ3^{low}/HSA^{high} subset was still dominant after FD17 (data not shown).

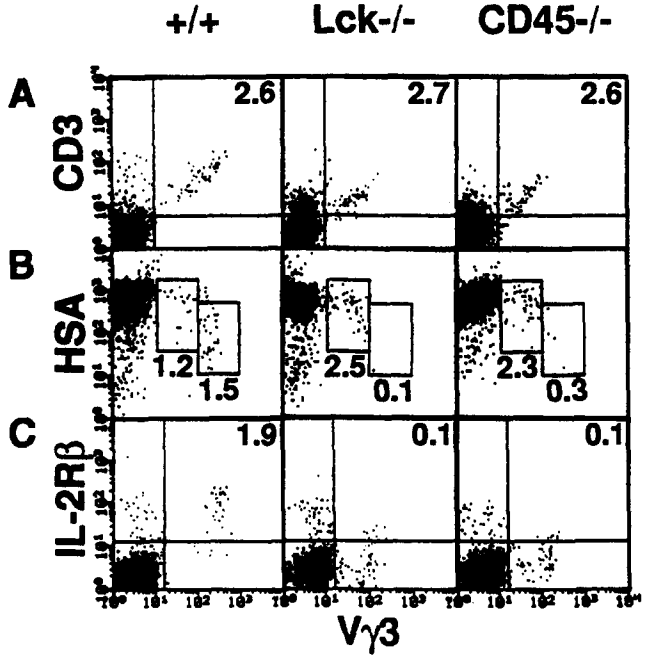

Figure 3. Maturation of Vγ3 thymocytes is blocked at the immature Vγ3^{low}/HSA^{high}/IL-2Rβ⁻ stage. FD16 thymocytes from +/+, Lck−/−, or CD45−/− mice were double stained with FITC-conjugated anti-Vγ3 TCR and PE-conjugated anti-CD3 (*A*), anti-HSA (*B*) ,or anti-IL-2Rβ (*C*) mAbs.

These results suggest that both p56^{lck} and CD45 are involved in intrathymic development of Vγ3 DETC. It is possible that these proteins may participate in intrathymic expansion of mature Vγ3^{high}/HSA^{low} cells. Alternatively, the apparent accumulation of immature Vγ3^{low}/HSA^{high} thymocytes in Lck−/− and CD45−/− mice (Table 1) may be due to the defect in the signal transduction required for Vγ3 thymocyte maturation. In the latter case, CD45 may be crucial for the activation of p56^{lck} during maturation of γ/δ fetal thymocytes. This interdependence does not occur in the α/β lineage since α/β thymocyte development is blocked at different stages in Lck−/− and CD45−/− mice (3, 7).

The requirement of p56^{lck} for intrathymic development of γ/δ T cells was previously reported in Lck−/− mice carrying a γ/δ TCR transgene (16). However, it should be emphasized that this transgenic model used the γ/δ TCR gene typical of T cells present in the adult lymphoid organs (17), and these γ/δ T cells may undergo a different developmental pathway from the epithelial γ/δ T cells arising in the fetal thymus (9).

The cell-surface receptor that interacts with p56^{lck} in immature Vγ3^{low}/HSA^{high} thymocytes is not clear. Since Vγ3 fetal thymocytes generally do not express CD4 or CD8 (11), other receptors that potentially interact with p56^{lck} would transduce the signal required for Vγ3 thymocyte maturation. Recently, it was reported that IL-2Rβ, which is expressed

predominantly on Vγ3$^+$ cells in the fetal thymus, transduces a critical signal for development of Vγ3 DETC (18). The observation that p56^{kk} can interact with IL-2Rβ and is involved in the signal transduction through IL-2Rβ (19) may account for the maturational block of Vγ3 fetal thymocytes in Lck−/− (and CD45−/−) mice. However, this possibility is unlikely because IL-2Rβ was expressed predominantly on mature Vγ3^{high} thymocytes in +/+ mice and was not expressed on the majority of Vγ3^{low} thymocytes in Lck−/− and CD45−/− mice (Fig. 3 *C*).

An alternative candidate for the receptor that interacts with p56^{kk} and transduces the signal required for intrathymic maturation of Vγ3 T cells is the Vγ3 TCR itself. Involvement of TCR-mediated signal transduction in the maturation of Vγ3 thymocytes is supported by the recent observation that addition of cyclosporin A (CsA) to fetal thymic organ cultures blocks the appearance of mature Vγ3^{high}/HSA^{low} cells (11). CsA has been demonstrated to block maturation of α/β thymocytes, presumably by interfering with TCR-mediated signal transduction required for positive and negative selection processes (20–22). Whether Vγ3 fetal thymocytes undergo positive and negative selection processes is not clear (9, 23). It is unlikely that MHC plays a role in the development of Vγ3 thymocytes, since the canonical sequence of Vγ3 TCR is the same in several MHC-disparate mouse strains (9) and the skin of β_2-microglobulin–deficient mice that lack class I MHC expression contains normal numbers of Vγ3 DETC (24). Recognition of self-antigens produced by keratinocytes by the invariant Vγ3 TCR of DETC is also not restricted by classical MHC (25). Taken together, this evidence argues against MHC-dependent selection processes of Vγ3 thymocytes akin to those of α/β T cells. Furthermore, evidence is accumulating that the invariant Vγ3 TCR repertoire in the fetal thymus is mainly shaped intracellularly by recombinase machinery and regulation of gene rearrangement (26, 27). Cellular selection processes may serve to promote survival and maturation of fetal thymocytes with the predetermined invariant Vγ3 TCR, but these selection processes probably involve different mechanisms from those operative in MHC-restricted α/β thymocyte maturation.

Our studies have demonstrated that intrathymic maturation of Vγ3 T cells is mediated by the signal transduction involving both p56^{kk} and CD45. Notably, the block in γ/δ thymocyte maturation in these protein-deficient mice is distinct from the block in α/β lineage (3, 7), suggesting that maturation and "selection" of these two T cell lineages are governed by different pathways.

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