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Phospholipase C γ 1 is required for pre-TCR signal transduction and pre-T cell development

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Abstract

Pre-T cell receptor (TCR) signaling is required for pre-T cell survival, proliferation, and differentiation from the CD4 and CD8 double negative (DN) to the double positive (DP) stage. However, the pre-TCR signal transduction pathway is not fully understood and the signaling molecules involved have not been completely identified. Phospholipase C γ (PLC γ) 1 is an important signaling molecule that generates two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, that are important to mediate PKC activation and intracellular Ca²⁺ flux in many signaling pathways. Previously, we have shown that PLC γ 1 is important for TCR-mediated signaling, development and T-cell activation, but, the role of PLC γ 1 in pre-TCR signal transduction and pre-T cell development is not known. In the current study, we demonstrated that PLC γ 1 expression level in pre-T cells was comparable to that in mature T cells. Deletion of PLC γ 1 prior to the pre-TCR signaling stage partially blocked the DN3 to DN4 transition and reduced thymic cellularity. We also demonstrated that deletion of PLC γ 1 impaired pre-T cell proliferation without affecting cell survival. Further study showed that deficiency of PLC γ 1 impaired pre-TCR mediated Ca²⁺ flux and Erk activation. Thus our studies demonstrate that PLC γ 1 is important for pre-TCR mediated signal transduction and pre-T cell development.

Keywords

Phospholipase C γ 1; pre-T cell; pre-TCR; T-cell development; signal transduction

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Conflict of interest

The authors have no financial conflict of interest.

Introduction

T-cell developmental stages can be defined by the expression pattern of the surface molecules. CD4^{lo} T-cell precursors enter the thymus and are first CD4 and CD8 double negative (DN) thymocytes that can further be separated into four developmental stages depending on CD44 and CD25 expression: DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁺), and DN4 (CD44⁻CD25⁻) [1]. T-cell receptor (TCR) β -chain rearrangement, the first check point during T-cell development, is detected in DN3 cells [2]. The cells with in-frame rearrangement of the TCR β -chain gene will express the β -chain, which then associates with the pre-T α chain and the CD3 molecules to form the pre-TCR complex [3, 4]. Signals emanating from the pre-TCR are essential for pre-T cell survival and allow the cells to undergo a prolific expansion and further differentiation [2, 5].

Similar to TCR signaling, pre-TCR signaling mediates thymocyte survival, proliferation and differentiation through activation of the Src family tyrosine kinases p56Lck and Fyn, which recruit and phosphorylate Syk family tyrosine kinases Zap-70 and Syk. Zap-70 and Syk in turn phosphorylate downstream adaptor molecules LAT and SLP-76 to recruit and activate downstream effector molecules, including PLC γ 1 [6]. Consistent with this model, deletion of Lck partially arrests thymocytes at the DN3 stage [7], and deletion of both Lck and Fyn kinases completely blocks T-cell development at the DN3 stage [8, 9]. Likewise, deletion of both Zap-70 and Syk completely abrogates T-cell development at the DN3 stage [10]. Deletion of either of two essential adaptor molecules downstream of Zap-70 and Syk, SLP-76 and LAT, completely arrests T-cell development at the DN3 stage [11–13]. Downstream of Syk family kinases, Tec family tyrosin kinases, Itk and Rlk, are important for TCR signaling, thymic T-cell selection, and mature T-cell differentiation, and one of the essential functions of Tec family kinases in TCR signaling is to activate PLC γ 1 [14–17]. However, Deficiency of Itk and Rlk either alone or together does not affect pre-TCR mediated DN3 to DN4 transition [17, 18]. In fact, deficiency of Itk alone or deficiency of both Itk and Rlk renders a competitive repopulation advantage for DN thymocytes over those from the wild-type (WT) mice [18]. These studies indicate that Itk and Rlk either alone or together are not essential for pre-T cell development. LAT, SLP-76 and Itk and Rlk are all important for PLC γ 1 activation in TCR signal transduction [14–17, 19–22], and PLC γ 1 is essential for TCR-mediated T-cell development and activation [23]. It is not known whether PLC γ 1 behaves like LAT and SLP-76 and is thus essential in pre-TCR signaling and pre-T cell development, or, acts instead like Itk and Rlk and is not essential for pre-TCR signaling and pre-T cell development.

PLC γ 1 activation is mediated by membrane recruitment to receptor signaling complexes, tyrosine phosphorylation either by kinase activities intrinsic to the receptor or by kinases associated with the receptor, and its own protein conformational changes [24]. Activated PLC γ 1 hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), which function as second messengers to activate protein kinase C (PKC) and induce intracellular Ca²⁺ mobilization, respectively [24]. DAG-activated PKC leads to the activation of NF- κ B via the CARMA1/Bcl10/Malt1 ternary complex in T cells [25]. DAG also regulates the activation of AP-1 through the RasGRP/Ras/Erk signaling pathway in T cells [26, 27]. Elevation of Ca²⁺

is essential for the activation of NFAT through the calmodulin/calcineurin pathway [28]. The ability of PLC γ 1 to regulate multiple signaling pathways and transcription factors raises considerable interest in the biological role of PLC γ 1. Whereas it is known that deficiency of PLC γ 1 impairs TCR-mediated T-cell development and activation [23], the role of PLC γ 1 in pre-TCR signaling and pre-T cell development remains unknown.

In the current study, we used LckCre-driven PLC γ 1 deletion, which deletes PLC γ 1 just prior to pre-TCR expression, to examine the role of PLC γ 1 in pre-TCR signaling and pre-T cell development [29]. We observed that PLC γ 1 deficiency impaired pre-TCR mediated signaling, partially blocked the DN3 to DN4 transition, reduced the proliferation of DN3 and DN4 thymocytes, and resulted in reduction of thymic cellularity. Our study provides conclusive evidences that PLC γ 1 is critical for pre-TCR signaling and pre-T cell development.

Results

PLC γ 1 expression pattern

The pre-TCR signal transduction pathway, which is important for pre-T cell development, is not fully understood and the signaling molecules important for the pathway have not been completely identified. PLC γ 1 is important for TCR signal transduction and TCR-mediated T-cell development and T-cell activation [23]. It is not known whether it also participates in pre-TCR signaling and pre-T cell development. To address this question, we first examined PLC γ 1 expression levels in T cells at different developmental stages by western blotting analysis. The expression level of PLC γ 1 is relatively constant throughout the T-cell development, including the CD4 and CD8 double negative (DN), double positive (DP), CD4 single positive (SP) and CD8 SP thymocytes, and peripheral splenic CD4⁺ and CD8⁺ T-cell populations (Fig. 1A) [30]. The expression level of PLC γ 1 is also constant in the four DN subsets: DN1, DN2, DN3, and DN4 (Fig. 1A). Given that PLC γ 1 plays an important role in TCR signaling [23] and that pre-TCR signaling and TCR signaling bear similarities [2, 31], marked expression of PLC γ 1 indicates a potential role of this molecule in pre-TCR signaling and pre-T cell development.

PLC γ 1 deficiency impairs pre-TCR mediated T-cell development

To determine the role of PLC γ 1 in pre-T cell development, we bred mice bearing the LckCre transgene, which induces deletion of floxed genes prior to the pre-TCR signaling stage [29, 32], with PLC γ 1^{fl/+} and PLC γ 1^{+/-} mice to generate LckCre/PLC γ 1^{fl/-} (PLC γ 1-deficient) and LckCre/PLC γ 1^{fl/+} (WT) mice. PLC γ 1 protein level was reduced in DN3 thymocytes and largely absent in DN4 thymocytes in the LckCre/PLC γ 1^{fl/-} mice (Fig. 1B). We also crossed mice bearing the ROSA-26YFP reporter gene with LckCre/PLC γ 1^{fl/+} and LckCre/PLC γ 1^{fl/-} mice to generate LckCre/YFP/PLC γ 1^{fl/+} and LckCre/YFP/PLC γ 1^{fl/-} mice to report the deletion of PLC γ 1 gene. Consistent with our previous finding in mature T cells, YFP positivity closely correlated with the absence of PLC γ 1 protein expression in DN thymocytes from the LckCre/YFP/PLC γ 1^{fl/-} mice (Fig. 1C) [23]. The effect of PLC γ 1 deficiency on pre-TCR mediated T-cell development could be observed in both total and YFP⁺ thymocyte populations. PLC γ 1 deficiency significantly reduced total thymic

cellularity in PLC γ 1-deficient mice compared with the control mice (Table 1). The percentage of CD4 and CD8 DN thymocytes was significantly increased in PLC γ 1-deficient mice compared with that in the WT control mice in both total (Table 1) and in YFP⁺ thymocytes (Fig. 2A and Table 1). The percentage of DN3 thymocytes was significantly increased and the percentage of DN4 thymocytes was significantly reduced in both total DN thymocyte (Table 1) and YFP⁺ DN thymocyte populations (Fig. 2B and Table 1), resulting in a significant reduction of DN4 thymocyte cellularity in both total and YFP⁺ populations in the PLC γ 1-deficient mice relative to those in the WT control mice (Table 1). These data indicate an impairment of DN3 to DN4 transition in the absence of PLC γ 1. As expected, development of early T cell progenitors, which is at the stage before PLC γ 1 deletion was not affected (data not shown). PLC γ 1-deficiency did not affect the development of $\gamma\delta$ T cells as neither the percentage nor the absolute number of the $\gamma\delta$ T cells in the DN4 thymocytes were affected (data not shown). Further analysis showed that both the percentage and absolute number of TCR β ⁺ DN4 thymocytes was substantially reduced (Fig. 2C and Table 2). The absolute number of the immature single positive thymocytes (ISP), a population after DN4 but before DP thymocytes, defined as CD4⁻CD8⁺CD24⁺TCR β ^{low} was also reduced, to an extent similar to that observed in the DN4 thymocytes [33, 34] (Fig. 2D, and Table 2). However, the percentage of the ISP cells was significantly increased in PLC γ 1-deficient mice. Taken together, these data demonstrate that PLC γ 1 plays an important role in pre-T cell development, and deletion of PLC γ 1 prior to pre-TCR signaling impairs the DN3 to DN4 transition, resulting in reduction of total thymocyte cellularity.

The effect of PLC γ 1 deficiency on thymocyte proliferation and survival

Signals emanating from the pre-TCR control cell proliferation and survival at the DN3 to DN4 transition [5]. We first examined cell proliferation *in vivo* by BrdU incorporation assay. Based on cell sizes, DN3 thymocytes can be separated into early DN3 (DN3-E), corresponding to the smaller DN3 cells and late DN3 (DN3-L), corresponding to the larger DN3 cells. BrdU assay revealed a consistent reduction of BrdU incorporation *in vivo* in PLC γ 1-deficient DN3-E and DN3-L cells, and to a less extent in DN4 thymocytes compared with their WT counterparts (Fig. 3A and 3B). Cell proliferation is preceded by cell size increase and accompanied by CD71 upregulation [35, 36]. We thus examined the percentage of thymocytes with increased cell size and CD71 upregulation on DN3-E, DN3-L, and DN4 thymocytes. Increase in cell size and upregulation of CD71 molecule appear to be concurrent in both DN3 and DN4 thymocytes (Fig. 3C and 3D). In WT mice, whereas large majority of DN4 thymocytes displayed CD71 upregulation and enlarged cell sizes, only close to 30% of DN3 thymocytes showed the same phenotype (Fig. 3C and 3D). PLC γ 1 deficiency significantly increased the percentage of CD71⁻ small DN3 thymocytes and reduced the percentage of CD71⁺ large DN3 thymocytes (Fig. 3C and 3D), further supporting a role of PLC γ 1 in proliferation at the pre-TCR signaling stage. TUNEL analysis and Annexin V staining did not reveal significant differences in cell survival between the WT and PLC γ 1-deficient thymocytes (Figs. 4A to 4C). Thus, PLC γ 1 is important for thymocyte proliferation but dispensable for cell survival at the DN3 to DN4 transition.

PLC γ 1 deficiency impairs pre-TCR mediated Ca²⁺ flux and Erk activation

To directly demonstrate that PLC γ 1 deficiency impairs pre-TCR signaling, we examined pre-TCR induced Ca²⁺ mobilization and Erk phosphorylation in TCR β ⁺ thymocytes derived from LckCre/YFP/PLC γ 1^{f/-} and LckCre/YFP/PLC γ 1^{f/+} mice. Due to the limited number of TCR β ⁺ thymocytes that we could recover, we expanded thymocytes by culturing lineage negative bone marrow cells from these mice on OP9-DL1 cells, and harvested the expanded thymocytes to perform the analysis. We examined Ca²⁺ flux in the YFP⁺TCR β ⁺ DN3 and DN4 thymocytes. Crosslinking of CD3 on the WT YFP⁺TCR β ⁺ DN3 and DN4 thymocytes elicited a quick increase of intracellular Ca²⁺ that reached peak in about 90 seconds in both DN3 and DN4 thymocytes (Fig. 5A). In contrast, CD3 crosslinking-induced Ca²⁺ flux in the PLC γ 1-deficient YFP⁺TCR β ⁺ DN3 and DN4 thymocytes was markedly reduced, indicating an impairment of pre-TCR mediated Ca²⁺ flux in the absence of PLC γ 1 (Fig. 5A). Due to low percentage of TCR β ⁺ thymocytes in the DN3 population and reduction of TCR β staining signal during fixation step in the p-Erk staining procedure, it was technically impossible to examine p-Erk in YFP⁺TCR β ⁺ DN3 thymocytes. We thus only compared pre-TCR mediated Erk phosphorylation level in TCR β ⁺ DN4 thymocytes from WT and PLC γ 1-deficient cells. CD3 crosslinking induced Erk phosphorylation in WT YFP⁺TCR β ⁺ DN4 thymocytes (Fig. 5B), whereas CD3 crosslinking-induced Erk phosphorylation was significantly reduced in the PLC γ 1-deficient YFP⁺TCR β ⁺ DN4 thymocytes (Fig. 5B and 5C). Taken together, we conclude that PLC γ 1 deficiency impaired pre-TCR signaling and pre-TCR mediated thymocyte proliferation, thus impeding thymocyte development at the DN3 to DN4 transition.

Discussion

In the current study, we have demonstrated that PLC γ 1 is an important signal transducer for the pre-TCR signal transduction pathway. We observed that PLC γ 1 deficiency impaired pre-TCR-induced Ca²⁺ mobilization and ERK activation, reduced the proliferation of DN3 and DN4 thymocytes, resulted in reduction of thymic cellularity, and partially blocked DN3 to DN4 transition in thymocyte development. PLC γ 1 deficiency impaired BrdU incorporation in both DN3 and DN4 thymocytes, suggesting impairment of cell proliferation (Figs. 5A and 5B). Although the difference in the BrdU incorporation between WT and PLC γ 1-deficient DN3 and DN4 appear to be subtle, it is consistent and statistically significant. The BrdU was injected into the mouse peritoneal cavity for only 4 hours before we sacrificed the mice and performed the analysis. We believe that the 10–20% reduction in BrdU incorporation in the PLC γ 1-deficient thymocytes at DN3 and DN4 developmental stage this 4 hours could result in a significant difference in final cell numbers during two to three days of mean time of lymphoid residence at these developmental stages [37]. Consistent with the impaired cell proliferation, PLC γ 1-deficient DN3 thymocytes also displayed impaired cell size enlargement and upregulation of CD71 (Figs. 3C and 3D), phenotypic changes precede lymphocyte proliferation [35, 36, 38]. In addition, PLC γ 1-deficient DN4 thymocytes also displayed a slight reduction of cell size increase and CD71 expression. We did not observe a defect in cell survival in the PLC γ 1-deficient pre-T cells by either Annexin V or TUNEL assays (Fig. 4). Thus, impaired pre-TCR mediated T-cell development in the PLC γ 1-deficient mice is largely due to reduced cell proliferation.

Pre-TCR and TCR signal transduction pathways share many signaling molecules, and deficiency of these signaling molecules can block both pre-TCR and TCR signaling and functions. For example, both pre-TCR and TCR signal transduction pathways employ CD3 complexes [39], Src [40–42] and Syk [10, 43–45] family tyrosin kinases, and the adaptor molecules LAT [11, 46] and SLP-76 [12, 13, 47]. However, TCR and pre-TCR signal transduction pathways also differ. For example, whereas RasGRP is essential for TCR signal transduction and T-cell positive selection, it has a minimal role in pre-TCR signaling [48, 49]. Similarly, whereas Itk and Rlk are important for TCR signal transduction, they are dispensable for pre-T cell development [14–18]. PLC γ 1 is essential for TCR signal transduction and TCR-mediated T-cell development [23, 30]. Previous studies have shown that stimulation of DN4 thymocytes with anti-CD3 could induce Ca²⁺ mobilization, and NFAT and NF- κ B activation [50]. However, mice deficient for single or two members of NFAT family of transcription families did not display developmental defect at the pre-TCR signaling stage [51, 52].

One of the mechanisms underlying TCR-induced PLC γ 1 activation is through phosphorylation of PLC γ 1 by tyrosine kinase Itk and Rlk [14]; however, deficiency of Itk and Rlk does not block pre-TCR-regulated DN3 to DN4 transition [18]. Thus, it is possible that a tyrosine kinase other than Itk or Rlk is involved in PLC γ 1 phosphorylation at the pre-TCR signaling stage, or that phosphorylation of PLC γ 1 on tyrosine residues is not important for its role in pre-TCR signal transduction and in regulating the DN3 to DN4 transition. In the former case, a possible candidate is Tec kinase, which is also expressed in pre-T cells and can be activated by TCR stimulation [18, 53]. However, a role of Tec kinase alone in pre-T cell signaling and pre-T cell development have not been demonstrated. The distinct dependence of Tec family tyrosine kinase for PLC γ 1 activation of pre-TCR and TCR highlights one of the many intrinsic differences between the two receptor signaling systems.

The DN3 to DN4 developmental block in the PLC γ 1-deficient mice appeared to be mild. This phenotype is not as severe as that observed in mice homozygous for a LAT mutant with a mutation of a tyrosine that is important for PLC γ 1 activation (LATY136Fm/m), which also exhibit a block at DN3 to DN4 transition [54]. We have demonstrated previously that TCR stimulation induces the association of the other PLC γ isoform, PLC γ 2, with LAT and SLP-76, and that both PLC γ 1 and PLC γ 2 participate in TCR signal transduction and TCR-mediated T-cell development and activation [30]. In addition, we observed that PLC γ 2 is also expressed in DN thymocytes (our unpublished result). It is thus likely that PLC γ 2 is also recruited to the phosphorylated Y136 on LAT and participates in pre-TCR signaling and in pre-TCR mediated T-cell development. Therefore, PLC γ 2 may partially compensate for the absence of PLC γ 1 and support pre-T cell development in PLC γ 1-deficient mice. Our preliminary study in mice deficient for both PLC γ 1 and PLC γ 2 at the early T cell developmental stage also support a role of PLC γ 2 in pre-TCR signal transduction (our unpublished result).

PLC γ 1-deficient mice displayed a significant reduction of ISP thymocyte cell numbers at a level similar to that observed in the DN4 thymocytes (Fig. 2D and Table 2). However, in contrast to the significant reduction of the percentage of the DN4 thymocytes, the percentage of the ISP thymocytes was significantly increased in PLC γ 1-deficient mice compared with

that in the control mice. The reduction of the absolute number of ISP thymocytes is likely due to impaired pre-TCR signaling as the reduction is at a level similar to or only slightly more severe than that observed in the DN4 thymocytes. The increase in the percentage of the ISP is likely due to the compromised development of mature CD8 SP thymocytes as positive selection of CD8 thymocyte is impaired in PLC γ 1-deficient mice [30]. In support of this reasoning, deletion of LAT at thymic DP stage showed an increase of the ISP percentage without affecting the pre-TCR signaling and the absolute ISP cell number [55].

Materials and Methods

Mice

LckCre transgenic mice were purchased from Taconic Laboratory. Rosa-26-YFP mice were provided by F. Costantini (Columbia University, New York, NY) [56]. PLC γ 1 floxed (PLC γ 1^{f/+}) and PLC γ 1^{+/-} mice have been described previously [23, 57]. LckCre/PLC γ 1^{f/+} and PLC γ 1^{+/-} mice have been backcrossed to B6 background for five generations before intercrossing. Rosa-26-YFP mice were crossed with LckCre/PLC γ 1^{f/+} and LckCre/PLC γ 1^{f/-} mice to generate LckCre/YFP/PLC γ 1^{f/+} and LckCre/YFP/PLC γ 1^{f/-} mice. All the experiments were performed using mice between 8 to 12 week old. All the mice were bred and maintained in the Biological Research Center of the Medical College of Wisconsin. The animal protocol (AUA2664) has been approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Western blotting analysis

Single thymocyte and splenocyte were obtained by pressing thymi or spleens through a cell strainer using a plunger of a 3 ml syringe. Cells were counted using a hemocytometer. Thymic CD4 and CD8 double negative (DN), double positive (DP), CD4 single positive (SP) and CD8 SP cells, and splenic CD4⁺ and CD8⁺ T cells were sorted by FACSaria (BD Biosciences) following FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 staining. To obtain DN1 to DN4 thymic subsets, DN thymocytes were first purified through negative sorting using anti-CD4 and anti-CD8 conjugated magnetic beads and MACS columns (Miltenyi Biotech), then the DN thymocytes were sorted into DN1 to DN4 subpopulations by FACSaria after being stained with PE-conjugated antibodies against lineage markers B220, CD4, CD8, Gr-1, Mac-1, NK1.1, and $\gamma\delta$ TCR, FITC-conjugated anti-CD44, and PECy7-conjugated anti-CD25. Cell lysate from 2 $\times$ 10⁵ cells was subjected to direct western blotting analysis with antibodies against PLC γ 1 (sc-81; Santa Cruz Biotechnology). All antibodies used in FACS analysis or cell sorting were purchased from eBioscience or BD Bioscience.

BrdU incorporation assay

Each mouse was injected intraperitoneally with 2 mg BrdU in 200 μ l PBS and euthanized four hours later. Single cell suspension of thymocytes was then prepared and stained with PE-conjugated antibodies against lineage markers B220, CD4, CD8, Gr-1, Mac-1, NK1.1, and $\gamma\delta$ TCR, PECy7-conjugated anti-CD44, and APCCy7-conjugated anti-CD25. Then BrdU staining using APC-conjugated anti-BrdU antibody was performed according to the

manufacturer's instructions (BD Biosciences). Samples were examined on LSR II Flow Cytometer and data analyzed using FACSDiva software (BD Biosciences).

TUNEL assay and Annexin V staining

Single cell suspension of thymocytes was prepared from the mice and stained with biotinylated antibodies against lineage markers B220, CD4, CD8, Gr-1, Mac-1, NK1.1, and $\gamma\delta$ TCR, followed by PerCPCy5.5-conjugated streptavidin, APC-conjugated anti-CD44, and PECy7-conjugated anti-CD25 (all from BD Biosciences or eBioscience). Then TUNEL assay was performed using *In Situ* Cell Death Detection Kit, TMR red (Roche Life Science) following the manufacturer's instructions. For Annexin V staining, thymocytes were stained with PE-conjugated antibodies against the same lineage markers, APCCy7-conjugated anti-CD44, PECy7-conjugated anti-CD25, and APC-conjugated Annexin V. DAPI was added to the samples just before the cells were examined on LSR II Flow Cytometer. Data was analyzed using FACSDiva software (BD Biosciences).

In vitro culture of thymocytes on OP9-DL1 cells

To obtain more thymocytes for in vitro experiments, we cultured lineage marker negative (Lin^-) bone marrow (BM) cells on OP9-DL1 to expand thymocytes as previously described with modification [58]. Lin^- BM cells were purified by using biotinylated antibodies against B220, CD4, CD8, Gr-1, Ter119, and Mac-1 at $2 \times 10^7/\text{ml}$, streptavidin-conjugated magnetic beads and magnetic columns according to the manufacturer's instructions (BD Biosciences). The Lin^- BM cells were collected and cultured in a mixture of 50% T-cell medium (RPMI1640 complete medium containing 100 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, 10 mM HEPES, pH 7.0, 10 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μM β -mercaptoethanol, 10% fetal bovine serum) and 50% OP9 cell medium (alpha MEM containing 20% FBS, 100 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM L-glutamine) at $5 \times 10^4/\text{mL}$ in the presence of 10 ng/ml of IL-7 and 5 ng/ml of Flt-3L and were passed every 4–5 days. At the 3rd passage, IL7 was reduced to 2 ng/ml. After being in culture for total 22–24 days, the cells were used for analysis.

Pre-TCR induced calcium flux analysis

Ca^{2+} analysis was performed with thymocytes cultured on OP9-DL1 cells as previously described [23]. Briefly, the cultured thymocytes were collected and stained with APCCy7-conjugated anti-CD44, PECy7-conjugated anti-CD25, APC-conjugated anti-TCR β , and PE-conjugated antibodies against lineage markers B220, CD4, CD8, Gr-1, Mac-1, NK1.1, and $\gamma\delta$ TCR, and incubated with 30 $\mu\text{g}/\text{ml}$ of biotinylated anti-CD3. After being washed the cells were loaded with Indo-1 AM (Invitrogen) at 10 μg per $4 \times 10^6/\text{ml}$ at room temperature for 30 mins, followed by washing and re-suspension in 1 ml of medium and run on a LSR II Flow Cytometer (BD Biosciences). Baseline data were collected for 1 min, streptavidin (Pierce) was added to a final concentration of 8 $\mu\text{g}/\text{ml}$ to cross-link the pre-TCR, and data were then collected for another 9 mins. Ionomycin was finally added at 500 ng/ml to all samples to verify the Indo-1 AM loading.

Pre-TCR induced Erk phosphorylation

Pre-TCR-induced Erk phosphorylation was performed with thymocytes cultured on OP9-DL1 cells as previously described with modification [59]. The cultured thymocytes were collected and stimulated with 10 µg/ml of biotinylated anti-CD3 and 10 µg/ml of biotinylated anti-CD28 for 20 secs followed by crosslinking with 20 µg/ml of streptavidin for 2.5 mins. The cells were fixed with paraformaldehyde (Electron Microscopy Science), permeabilized with ice-cold methanol, and then stained with Alexa Fluor® 647 mouse anti-Erk1/2 (pT202/pY204) or Alexa Fluor® 647 mouse IgG1 κ isotype control (all from BD Biosciences) as previously described [59]. Then PE-conjugated antibodies against CD4, CD8, Gr-1, Mac-1, NK1.1, B220, and γδTCR, APCCy7-conjugated anti-CD44, PECy7-conjugated anti-CD25, and PECy5.5-conjugated anti-TCRβ were directly added to the samples, incubated at RT for 30 mins. The cells were washed and re-suspended in PBS, and subjected to FACS analysis immediately.

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Abbreviations used

PLCγ1	phospholipase Cγ1
pre-TCR	pre-T cell receptor
DN	double negative
DP	double positive
SP	single positive.

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Figure 1.

PLC γ 1 expression levels in different T-cell subsets from WT mice. (A) PLC γ 1 expression levels in T-cell subsets at different developmental stages. Different T-cell subsets, including DN1 to DN4 thymocytes, DN, DP, CD4SP and CD8SP thymocytes, and splenic CD4⁺ and CD8⁺ T cells were purified by FACS and direct western blotting analysis was performed on 2×10^5 cells of each T-cell subset. (B) Western blotting analysis of PLC γ 1 protein levels in different DN thymocyte subsets derived from LckCre/PLC γ 1^{f/+} (WT) and LckCre/PLC γ 1^{f/-} (KO) mice. (C) Western blotting analysis of PLC γ 1 levels in YFP⁺DN thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} (WT) and LckCre/YFP/PLC γ 1^{f/-} (KO) mice. Blots are representative of two (A and C) or three (B) independent experiments. Actin blot was used as a loading control in all the figures.

Figure 2.

PLC γ 1 deficiency impaired pre-T cell development. (A) CD4 and CD8 expression profile in YFP⁺ thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} and LckCre/YFP/PLC γ 1^{f/-} mice.

(B) CD25 and CD44 expression profile in YFP⁺ DN thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} and LckCre/YFP/PLC γ 1^{f/-} mice. (C) Surface TCR β expression on YFP⁺ DN4 thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} and LckCre/YFP/PLC γ 1^{f/-} mice. (D)

CD24 and TCR β expression level in YFP⁺ CD8SP thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} and LckCre/YFP/PLC γ 1^{f/-} mice. The plots in (A) to (C) are representative of data derived from seven WT and ten PLC γ 1-deficient mice in five independent experiments. The plot in (D) is representative of data derived from four WT and seven PLC γ 1-deficient mice in three independent experiments.

Figure 3.

PLC γ 1 deficiency impaired pre-T cell proliferation. (A) BrdU incorporation assay to determine in vivo cell proliferation of YFP⁺ DN3-E, DN3-L, and DN4 thymocytes. (B) Statistical analysis of BrdU incorporation in YFP⁺ thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} (n=3) and LckCre/YFP/PLC γ 1^{f/-} (n=3) mice. (C) Flowcytometry analysis of CD71 expression and cell size on YFP⁺ DN3 and DN4 thymocytes derived from LckCre/YFP/PLC γ 1^{f/+} and LckCre/YFP/PLC γ 1^{f/-} mice. (D) Statistical analysis of the percentages of the CD71⁺ large and CD71⁻ small cells in DN3 and DN4 thymocytes in LckCre/YFP/PLC γ 1^{f/+} (n=3) and LckCre/YFP/PLC γ 1^{f/-} (n=3) mice. (A and C) Data are representative of three independent experiments. (B and D) Data are shown as mean $\pm$ SD and representative of three independent experiments. *: $P < 0.05$. **: $P < 0.01$ in Student's t -test.

Figure 4.

PLCγ1 deficiency did not affect pre-T cell survival. (A) TUNEL assay to determine apoptosis of YFP⁺ DN3-E, DN3-L and DN4 thymocytes. Data are representative of four independent experiments. (B) Statistical analysis of TUNEL assay of YFP⁺ DN3-E, DN3-L, and DN4 thymocytes derived from LckCre/YFP/PLCγ1^{f/+} (n=4) and LckCre/YFP/PLCγ1^{f/-} (n=4) mice. (C) Statistical analysis of Annexin V⁺ cells in YFP⁺ DN3-E, DN3-L and DN4 thymocytes derived from LckCre/YFP/PLCγ1^{f/+} (n=5) and LckCre/YFP/PLCγ1^{f/-} (n=4) mice. (B and C) Data are shown as mean ± SD and representative of four independent experiments.

Figure 5.

PLCγ1 deficiency impaired pre-TCR mediated Ca²⁺ flux and Erk activation. (A) Ca²⁺ flux following pre-TCR stimulation is impaired in PLCγ1-deficient pre-T cells. The analysis was performed on gated YFP⁺TCRβ⁺ DN3 (upper panel) and DN4 (lower panel) thymocytes.

The arrows indicated the time when streptavidin was added to crosslink the biotin-anti-CD3 or when ionomycin was added. Data is a representative six independent experiments. (B)

Erk phosphorylation following pre-TCR stimulation is impaired in PLCγ1-deficient pre-T cells. pErk analysis was gated on YFP⁺TCRβ⁺ DN4 thymocytes. The upper panel represents the isotype control staining of anti-CD3 + anti-CD28 stimulated LckCre/YFP/PLCγ1^{f/+}

(solid line) and LckCre/YFP/PLCγ1^{f/-} (dashed line) YFP⁺TCRβ⁺ DN4 thymocytes. The lower panel represents anti-pErk staining of the LckCre/YFP/PLCγ1^{f/+} (solid line) and LckCre/YFP/PLCγ1^{f/-} (dashed line) YFP⁺TCRβ⁺ DN4 thymocytes treated with medium

(grey line) or stimulated with anti-CD3 + anti-CD28 (black line). Data is a representative of 3 independent experiments. (C) Statistics analysis of the ratio of pErk mean fluorescent intensity (MFI) between LckCre/YFP/PLCγ1^{f/-} (n=3) and LckCre/YFP/PLCγ1^{f/+}

thymocytes (n=3) when cells are treated with medium or stimulated with anti-CD3 + anti-

CD28. Data are shown as mean +SD and are representative of three independent experiments. $P=0.03$ in Student's t -test.

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Table 1

PLCγ1 plays an important role in pre-TCR mediated pre-T cell development

Genotype	Total thymocytes				YFP ⁺ thymocytes			
	Total thymocytes	DN	DN3	DN4	DN	DN3	DN4	
Cre/YFP/γI ^{fl/+} (n=7)	%	3.8±0.7	58±5.3	34±7.4	2.3±0.7	42±7.2	53±8.2	
	# (×10 ⁶)	79.5±15.1	3.0±0.2	1.7±0.2	1.7±0.2	0.7±0.1	0.9±0.2	
Cre/YFP/γI ^{fl/-} (n=10)	%	6.9±1.0 ^{***}	68±4.6 ^{***}	27±5.7 [*]	4.4±0.9 ^{***}	60±9.1 ^{***}	39±9.5 ^{***}	
	# (×10 ⁶)	41.3±17.7 ^{***}	2.7±1.1	1.8±0.8	1.6±0.7	1.0±0.5	0.6±0.3 [*]	

%; percentage of cells; #: absolute cell numbers; n: the number of mouse used in the experiments. The DN population was analyzed in total or YFP⁺ thymocytes. The DN3 and DN4 populations were analysed in total DN or YFP⁺ DN thymocytes.

* : *P* 0.05;

*** : *P*<0.0005.

Table II
PLC γ 1 deficiency reduced TCR β^+ DN4 thymocytes and immature single positive cells

Genotype	TCR β^+ DN4 cells in		ISP cells	
	total DN4	YFP $^+$ DN4	total cells	YFP $^+$ cells
Cre/YFP/ γ T $^{fl/+}$	Mouse #	n=7	n=4	
	%	11.9 $\pm$ 7.0	12.3 $\pm$ 7.2	40 $\pm$ 6.0
	# ($\times 10^4$)	12.4 $\pm$ 8.7	11.7 $\pm$ 8.3	96 $\pm$ 15
Cre/YFP/ γ T $^{fl/-}$	Mouse #	n=10	n=7	
	%	3.0 $\pm$ 1.4 ^{**}	2.5 $\pm$ 1.0 ^{**}	74 $\pm$ 17 ^{**}
	# ($\times 10^4$)	2.0 $\pm$ 1.3 ^{**}	1.4 $\pm$ 0.8 ^{**}	52 $\pm$ 21 ^{**}

%; percentage of cells; #; absolute cell numbers; n: the number of mouse used in the experiments. ISP cells: CD4 $^-$ CD8 $^+$ CD24 $^+$ TCR β^{low} ; immature single positive thymocytes.

* : P 0.05;

** : P <0.005