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**PP6 Controls T Cell Development and Homeostasis by Negatively Regulating Distal TCR Signaling**

Jian Ye,* Hao Shi,* Ye Shen,* Chao Peng,* Yan Liu,* Chenyu Li,* Kejing Deng,* Jianguo Geng,† Tian Xu,**§ Yuan Zhuang,**§ Biao Zheng,‡ and Wufan Tao*

T cell development and homeostasis are both regulated by TCR signals. Protein phosphorylation and dephosphorylation, which are catalyzed by protein kinases and phosphatases, respectively, serve as important switches controlling multiple downstream pathways triggered by TCR recognition of Ags. It has been well documented that protein tyrosine phosphatases are involved in negative regulation of proximal TCR signaling. However, how TCR signals are terminated or attenuated in the distal TCR signaling pathways is largely unknown. We investigated the function of Ser/Thr protein phosphatase (PP) 6 in TCR signaling. T cell lineage-specific ablation of PP6 in mice resulted in enhanced thymic positive and negative selection, and preferential expansion of fetal-derived, IL-17–producing Vγ6Vδ1+ T cells. Both PP6-deficient peripheral CD4+ helper and CD8+ cytolytic cells could not maintain a naive state and became fast-proliferating and short-lived effector cells. PP6 deficiency led to profound hyperactivation of multiple distal TCR signaling molecules, including MAPKs, AKT, and NF-κB. Our studies demonstrate that PP6 acts as a critical negative regulator, not only controlling both αβ and γδ lineage development, but also maintaining naive T cell homeostasis by preventing their premature activation before Ag stimulation. The Journal of Immunology, 2015, 194: 1654–1664.

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J.Y., Y.Z., and W.T. designed the research; J.Y., H.S., Y.S., C.P., Y.L., and C.L. performed research, data collection, and statistical analysis; K.D., T.X., J.G., and J.Y. addressed and interpreted data; and J.Y., Y.Z., B.Z., and W.T. wrote the manuscript.

The online version of this article contains supplemental material.

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; DN, double-negative; DP, double-positive; DUSP, dual-specificity phosphatase; ES, embryonic stem; FRT, Flp recombinase target; PK, protein kinase; PP, protein phosphatase; PTP, protein tyrosine phosphatase; SP, single-positive; Treg, regulatory T cell; WT, wild type.

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activity (11) and facilitating the repair of DNA double-strand breaks by activating DNA-activated protein kinase (DNA-PK) and dephosphorylating γH2AX in HeLa cells (12, 13). Involvement of PP6 in modulating NF-κB activity is suggested by findings that the PP6 protein interacts with and protects IκBe from TNF-α–induced degradation in Cos7 cells (14). PP6 also suppresses IL-1–stimulated TAK1 activation by dephosphorylating TAK1 in 293 cells (15). Recent evidence suggests that PP6 may also regulate Hippo signaling via interaction with MOB1B (16). However, the physiological functions of PP6, especially in T cell biology, are not known.

In this study, we investigated functions of the PP6 gene in T cells by generating and analyzing PP6-deficient mice. Whereas conventional deletion of PP6 led to early embryonic lethality, T cell–specific ablation of PP6 revealed essential regulatory roles for PP6 in both T cell development and activation. Both positive and negative selection events are enhanced during TCR signaling molecules distal to the TCR. Collectively, our study demonstrates that PP6 is a major negative regulator of TCR signals by controlling multiple downstream pathways in the distal end of the TCR signaling cascade.

Materials and Methods

Generation of PP6-deficient mice

To construct the PP6 gene–targeting vector, we isolated a genomic DNA fragment containing exons 3–7 of PP6 from the 129/Sv mouse genomic phage library (Stratagene). In the gene targeting vector, a nuclear β-gal reporter gene with a splicing acceptor and a neomycin expression cassette flanked by Flp recombination target (FRT) sites were inserted after exon 4. Splice acceptor was derived from exon 2 of mouse β2 gene. A pair of LoxP sites was also inserted at SpeI and BamHI sites flanking exons 5 and 6, and a diphtheria toxin A expression cassette was used as a negative selection marker. The linearized vector containing exons 3–7 of PP6, and a diphtheria toxin A expression cassette was used as a negative selection marker. The linearized gene–targeting vector, we isolated a genomic DNA fragment containing exons 3–7 of PP6 from the 129/Sv mouse genomic phage library (Stratagene). In the gene targeting vector, a nuclear β-gal reporter gene with a splicing acceptor and a neomycin expression cassette flanked by Flp recombination target (FRT) sites were inserted after exon 4. Splice acceptor was derived from exon 2 of mouse β2 gene. A pair of LoxP sites was also inserted at SpeI and BamHI sites flanking exons 5 and 6, and a diphtheria toxin A expression cassette was used as a negative selection marker. The linearized PP6 gene–targeting vector was electroporated into mouse embryonic stem (ES) cells (W4/129S6 ES; Taconic Transgenic), and the recombinant ES cell clones were screened by PCR and further confirmed by Southern blot analysis. Two recombinant ES clones were injected into C57BL/6 blastocysts to produce chimera mice. PP6+/flox mice were crossed with Act-Flpe transgenic mice (stock no. 003800) to delete the β-gal and neo cassette to generate PP6+/flox mice. PP6+/flox mice were backcrossed with C57BL/6 for six generations before crossing with Lck-Cre (17) or Cd4-Cre (18) transgenic mice. PP6+/floxed null allele was generated after removal of the fragment containing exons 5 and 6 by introducing a Cre transgene driven by universal Pgk promoter or T cell–specific Lox or Cd4 promoter. Deletion of exons 3 and 6 led to a frame shift and multiple stop codons in exon 7 in truncated PP6 mRNA. Oligos pp6-FRT-F1 (p1: 5′-TGTTGAGATCTGCCTGGTTACAGTCT-3′); PP6-loxP2-R1 (p2: 5′-CACGACCTCCTCCTATTTGTTG-3′); and pp6-FRT-R1 (p3: 5′-GGT-TCTAGTTCAGCTAGCATG-3′) were used for genotyping of PP6+/flox allele and PP6−/floxed null allele by PCR.

Other transgenic mice

Pgk-Cre (19) transgenic mice were described previously. Act-Flo transgenic mice and B6.SJL (stock no. 002014) mice were purchased from the Jackson Laboratory. The OT-II and OT-I TCR transgenic mice were kindly provided by Dr. Xueting Cao. Mice at 6–10 wk old were used for experiments unless otherwise noted. Bone marrow chimeras were generated by i.v. transfer of T cell–depleted bone marrow into sublethally irradiated B6.SJL mice as described previously (20). All mice were kept in specific pathogen-free conditions, and animal-related procedures were performed by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University.

Immunoblot, flow cytometry, and cell sorting

T cells were stimulated with 100 ng/ml PMA plus 1 μM ionomycin or soluble anti-CD3ε plus anti-CD28 or anti-CD4 (10 μg/ml each) for various times before being lysed with radioimmunoprecipitation assay buffer containing 1 mM PMSF and 1× proteinase inhibitor (Roche). Immunoblot were performed as described previously (21) with Abs to p-ERK (4370), ERK (4695, 9107), p-JNK (4696), JNK (9258), p-p8 (4511), p38 (8690), p-p-Caf (9427), p-MEKK1/2 (9154), p-MEEK4 (9156), p-MEKK3/6 (9231), MEKK1/2 (9126), p-IκKβ (2697), IKKα (2682), p65 (3033), p-IκBα (2859), IκBα (4814), p-IκBε (4924), IκBε (9249), p-Zap70 (2717), Zap70 (3165), p-LAT (3584), LAT (9166), p-LCK (2751), Lck (2787), p-PLCy1 (2821), and PLCy1 (5690) purchased from Cell Signaling. Anti-PP6 (07-1224) was from Millipore, and anti–β3-Actin (AC-15) was from Sigma-Aldrich. Images were acquired with Tanon-5200 or Biorad ChemiDoc MP System, and the density of the bands was quantified by ImageJ.

For surface marker analysis, cells were stained with indicated Abs in PBS containing 2% FBS. Intracellular staining was performed according to the manufacturer’s instructions. Abs to B220 (2F11), CD4 (RM4-5), CD8b (H35-17.2), CD3ε (145-2C11), TCRβ (H57-597), TCRγδ (GL3), CD5 (53–73), CD4 (320-F1), CD69 (H1.2F3), CD25 (2D15.1), CD44 (14M7), CD62L (16L-14), CD45.1 (A20), CD45.2 (104), TCRβ/J (MR9-4), TCRβ/J (KJ16), TCRβ/1 (C21), TCRβa (B20.1), IL-7R (SB/199), TCRα (2.11), TCRβα (UC3-10A6), TCRβα (536), CCR6 (140706), CD27 (LG7P9), IFN-γ (XMG1.2), IL-4 (1B11), IL-7 (TC11-18H10), Foxp3, and Ki67 Kit were purchased from BD or eBioscience. Anti-TCRε was a gift from Dr. Robert E. Tigelaar at Yale University. The staining of CD6+ γδ T cells has been described previously (22). Dead cells were excluded during analysis according to their light-scattering characteristics and/or 7-aminocoumarin D (7AAD) staining.

For staining of mitochondria, lymphocytes were incubated with 20 nM MitoTracker Green (Invitrogen) at 37°C for 20 min. Reactive oxygen species were measured by incubation with 10 μM dihydroethidium (Sigma-Aldrich) at 37°C for 30 min. CFSE labeling was performed by incubating sorted cells with 5 μM CFSE (Invitrogen) at 37°C for 20 min followed by adoptive transfer into B6.SJL mice. Flow cytometry data were acquired on FACSCalibur or LSRII (BD) and were analyzed with FlowJo software (Tree Star). CD4+ T cells were isolated from splenocytes with anti–CD4–coated magnetic microbeads (Miltenyi Biotec). Y6 γδ T cells were sorted on FACSaria II (BD Biosciences).

Adoptive transfer

For adoptive transfer, freshly isolated splenocytes containing an equal amount of wild type (WT) or PP6−/floxed CD4+ T cells were i.v. injected into B6.SJL mice. Flow cytometry data were acquired on FACSCalibur or LSRII (BD) and were analyzed with FlowJo software (Tree Star). CD4+ T cells were isolated from splenocytes with anti–CD4–coated magnetic microbeads (Miltenyi Biotec). Y6 γδ T cells were sorted on FACSaria II (BD Biosciences).

Quantitative RT-PCR

Total RNA was isolated from T cells using the RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized using the SensiScript Reverse Transcription Kit (Qiagen). The abundance of mRNA for each gene was determined by real-time quantitative PCR using SYBR Green master mix (Applied Biosystems) in Prism 7500 (Applied Biosystems). A pair of primers specific for PP6 was separately located in exons 5 and 6. Primer sequences will be provided upon request. Expression values were calculated using the 2−ΔΔCt method with Actinb as an endogenous control.

Brdu incorporation assay

Mice were i.p. (for adults) or s.c. (for neonates) injected with 50 mg/kg BrdU two times with a 2-h interval. The thymus and spleen were taken 24 h after the first injection. Cells were first stained with surface markers, then fixed and stained with a BrdU staining kit (BD) followed by FACS analysis.

Calcium flux

Calcium flux was measured using the Fluo-4 Direct Calcium Assay Kit (Invitrogen). In brief, 2 × 10^5 thymocytes were resuspended in 500 μl Fluo-4 Direct calcium reagent with 6 μM Fura Red and incubated at 37°C for 30 min. After washing, the cells were labeled with anti–CD4-Pacific Blue and anti–CD8-allophycocyanin for 15 min on ice followed by a 30-min incubation at room temperature. Calcium influx was analyzed by FACS. After measurement of baseline levels for 30 s, anti-CD3 and anti-CD28
Results

Essential role of PP6 in embryonic development

We first generated a PP6 mutant allele, PP6\textsuperscript{galeo}, in mouse ES cells (Supplemental Fig. 1A, 1B) using a “conventional first, conditional ready” gene-targeting strategy (25). RT-PCR analysis indicated that PP6\textsuperscript{galeo} allele is a hypomorphic allele (Supplemental Fig. 1C). In PP6\textsuperscript{galeo} allele, the expression of β-gal reporter is under the control of the endogenous PP6 promoter, and β-galactosidase is in-frame fused with the first 78 aa of the PP6 protein after splicing. This knocked-in β-galactosidase reporter revealed that PP6 was highly expressed in the brain and testis (Supplemental Fig. 1G). When PP6\textsuperscript{v/galeo} mice were intercrossed, PP6\textsuperscript{galeo/galeo} embryos were recovered with the expected ratio at embryonic day 10, frequently lost at embryonic day 14.5, with remaining ones occasionally exhibiting exencephaly (Supplemental Fig. 1A). PP6\textsuperscript{galeo} mice were phenotypically indistinguishable from WT littermates and used as controls in the studies described later. Germline deletion of the PP6\textsuperscript{b} allele with Pgk-Cre resulted in lethality at early embryonic stages (Supplemental Fig. 1H), confirming the crucial function of PP6 in embryonic development.

Abnormal thymocyte development in PP6\textsuperscript{f/f};Lck-Cre mice

To study the function of PP6 in T cells, we crossed the PP6\textsuperscript{f} allele into Lck-Cre mice to produce PP6\textsuperscript{f/f};Lck-Cre mice. Genomic PCR and Western blot analyses showed that PP6\textsuperscript{f} was deleted efficiently in the thymocytes of PP6\textsuperscript{f/f};Lck-Cre mice (Supplemental Fig. 1E, 1F). T cell-specific ablation of PP6 resulted in abnormal thymocyte development. Both proportion and numbers of CD4\textsuperscript{+}CD8\textsuperscript{−} single-positive cells, CD8 SP cells, and CD4\textsuperscript{+}CD8\textsuperscript{−} (double-positive) (DP) cells, as well as numbers of total thymocytes, were significantly reduced in PP6\textsuperscript{f/f};Lck-Cre mice (Fig. 1A). In contrast, the proportion and number of CD4\textsuperscript{+}CD8\textsuperscript{−} (double-negative) (DN) cells were increased in the mice. However, further analyses revealed that DN subpopulations of PP6\textsuperscript{f/f};Lck-Cre thymocytes appeared normal after removing γδ T cells (Fig. 1C), indicating that the increase of γδ T cells should be the reason for an apparent upsurge of DN thymocytes in PP6\textsuperscript{f/f};Lck-Cre mice (see Increased IL-17-producing γδ T cells in PP6\textsuperscript{f/f};Lck-Cre mice). The PP6\textsuperscript{f/f};Lck-Cre mice had considerably fewer peripheral T cells (7–12% that of the control; Fig. 1B and Supplemental Fig. 2A). Numbers of regulatory T cells (Tregs) were also decreased in the thymus and periphery of PP6\textsuperscript{f/f};Lck-Cre mice (Fig. 1D). Although the proportion of peripheral Tregs increased in adult PP6\textsuperscript{f/f};Lck-Cre mice, it is still significantly lower in the 1-wk-old mice (Fig. 1D, 1E, and Supplemental Fig. 2B, 2C). These data indicate that PP6 plays important roles in T cell development.

Effects of PP6 deficiency on thymic positive and negative selection

The effects of PP6 deficiency on thymocyte development were further examined with the maturation and activation markers: CD5, CD24, and CD69. CD5 expression at the DP stage is proportional to TCR signaling strength during positive selection (26), whereas CD69 upregulation in DP cells is indicative of positive selection in response to TCR signaling (27). CD5 expression was increased in PP6-deficient DP cells, and CD69 expression was elevated in both mutant DP and SP cells (Fig. 2A). CD24 expression in SP cells decreases upon their maturation (28). We found that the proportion of CD24\textsuperscript{hi} SP thymocytes was increased in the PP6\textsuperscript{f/f};Lck-Cre mice (Fig. 2A). Collectively, these observations suggest that PP6 deficiency may promote positive selection and enhance thymocyte maturation.

To further examine the effect of PP6 deficiency on thymocyte positive selection and to overcome the difficulty of evaluating thymocyte selection in mice with a diverse T cell repertoire, we introduced MHC-II–restricted OT-II or MHC-I–restricted OT-I TCR transgenes into PP6\textsuperscript{f/f};Lck-Cre mice to test MHC class II and class I–mediated positive selection, respectively. Thymocytes

![Image](http://www.jimmunol.org/)
expressing the OT-II TCR are positively selected along the CD4 lineage (29). FACS analysis showed that the proportion and numbers of total CD4 SP cells, OT-II–specific TCRV\(\beta\)5+ thymocytes, and CD69hi and CD24lo CD4 SP cells were increased in PP6F/F;Lck-Cre;OT-II mice in comparison with the control mice (Fig. 3B and Supplemental Fig. 2D). These results suggest that PP6 deficiency enhances positive selection of the MHC-II–restricted thymocytes. OT-I TCR–expressing CD8+ thymocytes are favored for positive selection in OT-I transgenic mice (30). In OT-I mice, PP6 deficiency resulted in an increase of proportion of CD69hi DP and CD8 SP cells (Supplemental Fig. 2E), indicating enhancement of positive selection of PP6F/F;Lck-Cre;OT-I thymocytes. In OT-I mice, PP6 ablation also led to a higher proportion of TCR\(\alpha\)2hi and CD24lo CD8 SP cells (Fig. 2C, middle panels, and Supplemental Fig. 2E) and a higher ratio between post selected OT-I–specific TCR\(\alpha\)2hi CD8 SP cells and the preselection stage cells, including DP and the CD4 transitional thymocytes (31) (Supplemental Fig. 2F), implying that more CD8 SP cells become phenotypically mature cells.

However, an overall reduction of total thymocyte, DP, and CD8 SP thymocytes was also observed (Fig. 2C, bottom panels). All these observations are consistent with the previously described behavior of the OT-I thymocytes expressing a constitutive NF-\(\kappa\)B. Enhancement of OT-I signals by constitutive activation of NF-\(\kappa\)B pushed a portion of positive selecting thymocytes into negative selection, a phenomenon called pseudonegative selection (32).

Thymocytes bearing high-affinity TCRs for self-Ags undergo clonal deletion or negative selection. To investigate whether PP6 is
required for clonal deletion, PP6\textsuperscript{F/F};Lck-Cre or control mice were injected with the CD3 Ab to mimic high-affinity TCR signals. Under the experimental condition, DP thymocytes in PP6\textsuperscript{F/F};Lck-Cre mice were almost completely eliminated, whereas 30% of DP thymocytes in control mice survived (Fig. 3D). To further confirm the effect of PP6 deficiency on negative selection, we analyzed PP6-deficient thymocyte development in a superantigen-mediated deletion model. Superantigen Mtv-8 and Mtv-9 specifically lead to deletion of the V\textsubscript{b}5\textsuperscript{+} and V\textsubscript{b}11\textsuperscript{+} TCR clonotypic thymocytes in the presence of the class II MHC molecule I-Ed (33). MHC I-Ed is absent in C57BL/6 mice (H-2\textsuperscript{b/b}) but present in BALB/c mice (H-2\textsuperscript{d/d}). Enhanced deletion of V\textsubscript{b}5\textsuperscript{+} and V\textsubscript{b}11\textsuperscript{+} clones was observed for both CD4 and CD8 SP thymocytes in PP6\textsuperscript{F/F};Lck-Cre;H-2\textsuperscript{b/d} mice, whereas V\textsubscript{b}8\textsuperscript{+} thymocytes that did not recognize the superantigen were not affected (Fig. 2E, 2F). This outcome supports the conclusion that negative selection is enhanced in the absence of PP6, which may account for the overall reduction of mature CD4 and CD8 SP cells in the thymus of the mutant mice.

**Increased IL-17–producing \( \gamma \delta \) T cells in PP6\textsuperscript{F/F};Lck-Cre mice**

In contrast with the severe reduction of \( \alpha \beta \) T cells, \( \gamma \delta \) T cells were dramatically increased in the thymus (Fig. 3A) and the periphery (H-2\textsuperscript{d/d}).
of PP6<sup>FF</sup>;Lck-Cre mice (Fig. 3B and Supplementary Fig. 3A). To determine whether this increase affects all or only subsets of γδ lineages, we examined the repertoire of γδ T cells in the PP6<sup>FF</sup>;Lck-Cre thymus first by quantitative real-time RT-PCR analysis of seven individual Vγ genes. We found that the expression of Vγ5 and Vγ6 fragments increased ~30 and ~50 times, respectively, in the γδ T cells purified from PP6<sup>FF</sup>;Lck-Cre thymus in comparison with those of the control mice (Supplementary Fig. 3B). Vβ1 expression was also found to be increased ~40-fold (Supplementary Fig. 3B). FACS analysis with Vγ-specific Abs revealed that the dramatic increase of γδ T cell numbers in PP6<sup>FF</sup>;Lck-Cre thymus is primarily due to an increase of Vγ6<sup>+</sup> cells, even though numbers of Vγ1<sup>+</sup> and Vγ5<sup>+</sup> cells also increased (Fig. 3C and Supplementary Fig. 3C, 3D). γδ T cells expressing Vγ6, which are normally pairs with Vβ8<sup>1</sup>, represent the major IL-17 producers among γδ lineage T cells and can be identified based on high-level expression of IL-7R and CCR6 (a marker for IL-17-producing T cells) and low-level expression of CD27 (a marker of IFN-γ-producing Vγ T cells) (34). Indeed, we found that most PP6<sup>FF</sup>;Lck-Cre γδ T cells were IL-7R<sup>hi</sup>,CCR6<sup>hi</sup>, and CD27<sup>lo</sup>IL-17<sup>+</sup> producers (Fig. 3D and Supplementary Fig. 3E). Vγ6<sup>+</sup> T cells are typically generated in fetal thymus in WT mice (34, 35). However, this population appeared to be continuously increased in PP6<sup>FF</sup>;Lck-Cre mice only after birth (Fig. 3E and Supplementary Fig. 3F). To determine whether the increase of Vγ6<sup>+</sup> IL-17-producing γδ T cells is a result of enhanced postnatal development or expansion of fetal-derived cells, we performed an adoptive transfer test using bone marrow donors derived from the PP6<sup>FF</sup>;Lck-Cre mice. Although the mutant donor cells showed defects in αβ lineage development similar to that observed in PP6<sup>FF</sup>;Lck-Cre mice (Supplementary Fig. 3H), they failed to recapitulate the mutant phenotype of PP6<sup>FF</sup>-deficient γδ T cells (Fig. 3F). Therefore, the increase of γδ T cells in PP6<sup>FF</sup>;Lck-Cre mice is most likely due to continuing expansion of fetal-derived Vγ6<sup>+</sup> γδ T cells in the postnatal life. To directly assess the proliferation status of these cells, we pulse-labeled the mice with BrdU. Increased BrdU labeling was observed among both immature (CD24<sup>hi</sup>) and mature (CD24<sup>lo</sup>) γδ T cells in PP6<sup>FF</sup>;Lck-Cre mice at the neonatal stage (Fig. 3G). In young adult mice (6–8 wk), there were still a higher percentage of BrdU<sup>+</sup> immature γδ T cells, but the proportion of BrdU<sup>+</sup> mature γδ T cells was reduced (Fig. 3H). However, the net quantity of BrdU<sup>+</sup> mature γδ T cells, which composed the majority of γδ T cells in PP6<sup>FF</sup>;Lck-Cre mice (Supplementary Fig. 3I), was still 35 times more than the controls (Fig. 3I). In addition to the overall increase in cell proliferation, we found that PP6<sup>FF</sup>-deficient mature γδ T cells were less susceptible to apoptosis (Fig. 3J). Therefore, we concluded that PP6 deficiency promotes a continuing expansion of pre-existing Vγ6<sup>+</sup> γδ T cells in the adult life. 

**Chronic T cell activation, hyperproliferation, and enhanced apoptosis of effector T cells in PP6-deficient mice**

To examine functions of PP6 in peripheral T cells, we switched to the CD4<sup>+</sup>-Cre model, in which a significantly higher number of peripheral T cells were found in comparison with the Lck-Cre model. The total numbers of splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells in PP6<sup>FF</sup>;CD4<sup>+</sup>-Cre mice were ~70 and 42% of the PP6<sup>FF</sup> controls, respectively (Fig. 4A). Naive CD4<sup>+</sup> or CD8<sup>+</sup> T cells are significantly reduced in PP6<sup>FF</sup>;CD4<sup>+</sup>-Cre mice (Fig. 4B). Most T cells showed activated phenotype based on increased expression of activation markers including CD69 and CD44 (Fig. 4B, 4C). PMA/ionomycin stimulation of ex vivo culture revealed a significant increase in numbers of IFN-γ–producing effector CD4<sup>+</sup> or CD8<sup>+</sup> cells in PP6<sup>FF</sup>-deficient splenocytes (Fig. 4D). The same analysis also revealed a small increase in IL-4– or IL-17–producing effector CD4<sup>+</sup> cells (Fig. 4E, 4F). Consistent with these effector phenotypes, PP6-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found highly proliferative based on increased fractions of Ki67<sup>+</sup> cells (Fig. 4G). Enhanced proliferation of the effector T cells was further confirmed by CFSE dilution assay of PP6-deficient OT-II T cells. Upon adoptive transfer, PP6-deficient OT-II T cells underwent faster proliferation than PP6-sufficient T cells within 3 d of OVA<sub>323–339</sub> peptide stimulation (Fig. 4H). These results indicate that PP6 deficiency promotes T cell activation and proliferation under normal homeostatic conditions, leading to quick depletion of the naive T cell pool.

To further determine the cause of overall reduction of peripheral T cells in PP6-deficient mice, we examined whether T cell hyperactivation leads to faster turnover of the effector T cells. Annexin V/7AAD FACS analysis showed a decreased viability of PP6-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T cells in ex vivo culture (Fig. 5A). Adoptive transfer test further demonstrated an intrinsic survival defect of PP6-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 5B). The increase in cell death is correlated with an increased loss of mitochondrial membrane potential, increased production of reactive oxygen species, and reduced expression of Bcl-2 (Fig. 5C, 5D). Collectively, these experimental evidences indicate that PP6 deficiency leads to activation, differentiation, proliferation, and apoptosis of the peripheral T cells in the absence of exogenous TCR signals.

**Downregulation of proximal TCR signaling and TCR expression in PP6-deficient T cells**

The overall increase in thymocyte positive and negative selections and T cell activation strongly suggest that PP6 functions as a negative regulator in TCR signaling. To determine how PP6 modulates TCR signaling events, we first assessed the expression and activation status of proximal TCR signaling molecules in PP6<sup>FF</sup>;Lck-Cre thymocytes. The surface expressions of TCRβ and CD3 were reduced in PP6<sup>FF</sup>-deficient thymocytes (Fig. 6A). Upon TCR stimulation, the phosphorylations of Zap70, LAT, and LCK proteins were reduced in PP6<sup>FF</sup>-deficient cells (Fig. 6B). Activation of PLCγ1, which is responsible for the production of the second messenger diacylglycerol and the initiation of Ca<sup>2+</sup> signaling in T cells, was decreased (Fig. 6B). Consequently, TCR-induced calcium flux was also reduced in these thymocytes (Fig. 6C). The similar results were also obtained for the peripheral T cells of PP6<sup>FF</sup>;CD4<sup>+</sup>-Cre mice (Supplementary Fig. 4A–C, 4E). Thus, the hypoactivation of proximal TCR signaling observed in PP6-deficient T cells suggests that PP6 is unlikely involved in negative regulation of the proximal TCR signaling.

**Negative regulation of distal TCR signaling by PP6**

TCR signals consist of both membrane-proximal events and multiple distal pathways. To further understand the molecular mechanism(s) by which PP6 regulates T cell development, we examined the activation of ERK in thymocytes from PP6<sup>FF</sup>;Lck-Cre mice by intracellular staining. The analysis showed that regardless of PMA stimulation, enhanced phosphorylated ERK was detected in both γδ and αβ thymocytes from PP6<sup>FF</sup>-deficient mice (Supplementary Fig. 4F). Then, we further verified ERK hyperactivation and evaluated the activation status of other distal TCR signaling molecules in PP6-deficient thymocytes by Western blot. We found that the phosphorylation levels of ERK, JNK, p38, and AKT were all higher in intensity and retained for a longer duration in PP6-deficient thymocytes after PMA/ionomycin (Fig. 7A) or TCR stimulation (Supplementary Fig. 4G). Moreover, the upstream kinases of MAPKs including c-Raf, MEK1/2, MKK4, and MKK3/
6 were also hyperactivated in these cells (Fig. 7B). Similarly, enhanced activation of MAPKs and AKT was observed in the PP6-deficient periphery T cells from PP6F/F;CD4-Cre mice (Supplemental Fig. 4D, 4J), in which the activation of these signals was critical to promote their proliferation and cytokine production (36, 37). These data suggest that hyper and prolonged upregulation of MAPKs and AKT activities may account for enhanced thymocyte positive/negative selection and hyperactivation of T cells in PP6-deficient mice.

PP6 has been shown to directly regulate TAK1 and Aurora A (11, 15). TAK1 is essential for the activation of JNK, p38, and NF-κB in T cells (23). Aurora A has been shown to activate AKT (38, 39) and ERK (38–41) in cancer cell lines. TAK1 phosphorylation was markedly reduced in PP6-deficient thymocytes after PMA/ionomycin stimulation (Fig. 7C), indicating that hyperactivation of JNK and p38 in PP6-deficient T cells is not due to overactivation of TAK1. In contrast with TAK1, Aurora A in PP6-deficient T cells was observed to be constitutively active with

**FIGURE 4.** Hyperactivation of PP6-deficient T cells in the periphery. (A) Cellularity of CD4+ and CD8+ lymphocytes from spleen and lymph node (LN) of PP6F/F and PP6F/F;CD4-Cre mice. n = 8 for each genotype. (B) Flow cytometry for CD4+ and CD8+ lymphocytes from spleen and lymph node (LN) of PP6F/F and PP6F/F;CD4-Cre mice. n = 8 for each genotype. (C) CD69 expression on splenic CD4+ and CD8+ T cells from PP6F/F and PP6F/F;CD4-Cre mice. (D–G) Flow cytometry for CD4+ and CD8+ lymphocytes from spleen and lymph node (LN) of PP6F/F and PP6F/F;CD4-Cre mice. n = 8 for each genotype. (H) Purified splenic CD4+ T cells from PP6F/F;OT-II and PP6F/F;Lck-Cre;OT-II mice were labeled with CFSE and i.v. transferred into B6.SJL mice. CFSE dilution was analyzed by FACS 2 and 3 d after stimulated with 40 μg OVA323–339 peptide per recipient mice. Data shown are representative from three (B–E, H) or two (G) independent experiments. (A, B, and D–G) Data are presented as mean ± SD. *p < 0.05, ***p < 0.001.
Reduced viability of PP6-deficient periphery T cells. (A) Cell viability of CD4+ and CD8+ splenocytes from PP6F/+ and PP6F/F, CD4-Cre ex vivo cultured for indicated time. (B) Flow cytometry of CD4+ T cells from spleen and lymph node (LN) 3 d after adoptive transfer of splenocytes (with equal number of CD4+ T cells) from PP6F/+;CD45.1.2+ and PP6F/F;CD4-Cre:CD45.2.2+ mice into B6.SJL (CD45.1.1+) recipient mice. Numbers above boxed areas indicate percent cells originated from control and mutant mice. For statistical analysis, viability of PP6-deficient cells was normalized to the control (B, right panels). n = 5 for each genotype. (C) FACS analysis of CD4+ and CD8+ T cells from PP6F/+ and PP6F/F;CD4-Cre mice, stained with MitoTracker Green (left) or dihydroethidium (right). n = 4 for each genotype. (C) FACS analysis of Bcl-2 intracellular staining and statistical analysis of the mean fluorescent intensity (MFI) for Bcl-2 expression in splenic CD4+ and CD8+ T cells with indicated genotypes. Three pairs of mice were used for each independent experiment. Data shown are representative from two (A, B, and D) and three (C). (A, B, and D) Data are presented as mean ± SD. **p < 0.01, ***p < 0.001.

Discussion

In this article, we present genetic and molecular evidences that PP6 is critical for the regulation of T cell development and homeostasis. Our data show that PP6 deficiency results in enhanced positive/negative selection accompanied with a dramatic reduction of αβ thymocytes, an expansion of γδ T cells, and loss of homeostasis of peripheral T cells. Mechanistically, ablation of PP6 leads to hyperactivation of distal TCR signaling involving MAPKs, AKT, and NF-κB pathways, and consequently enhances positive/negative selection and T cell activation.

or without PMA/ionomycin (Fig. 7E) or TCR stimulation (Supplemental Fig. 4H). Thus, PP6 might downregulate distal TCR signals, for example, the ERK and AKT signals, by suppressing Aurora A activity (see Discussion).

The NF-κB pathway also plays critical roles in thymocyte selection and T cell activation (42, 43). Constitutive activation of NF-κB activity in thymocytes pushes the most thymocytes into negative selection (32). The phosphorylation of NF-κB (p65) in PP6-deficient thymocytes was higher than that in the control with or without TCR stimulation (Supplemental Fig. 4I) and can be further enhanced by PMA/ionomycin stimulation (Fig. 7D), whereas its upstream kinase, IKKβ, was hyperactivated in response to TCR (Supplemental Fig. 4I) or PMA/ionomycin stimulations (Fig. 7D). Our further analysis showed that phosphorylation of IkBα in PP6-deficient thymocytes stimulated by PMA/ionomycin was comparable with the control (Fig. 7D), and its degradation in thymocytes could not be efficiently stimulated by TCR engagement (Supplemental Fig. 4I). We also found that IκBα was robustly phosphorylated in the mutant thymocytes after PMA/ionomycin stimulation (Fig. 7D). This result is consistent with the previous report that PP6 negatively regulated phosphorylation and degradation of IκBα in HeLa cells (14). However, we do not think that PP6 deficiency–mediated deregulation of IκBα in T cells may contribute much to the developmental defect of PP6-deficient T cells (see Discussion).

Collectively, these data indicate that PP6 negatively regulates TCR signaling through modulating multiple pathways distal to the initial TCR signal events. Removal of PP6 leads TCR-dependent hyperactivation of MAPKs, AKT, and NF-κB pathways, and consequently enhances positive/negative selection and T cell activation.

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Discussion

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Although distal TCR signals are markedly increased in PP6-deficient cells, the activation of proximal TCR signals are paradoxically decreased and the expressions of TCRβ and CD3 on the surface of PP6-deficient thymocytes and peripheral T cells are lower than the control. One possible explanation for this paradoxical phenomenon could be that the cells expressing higher levels of TCR and CD3 are preferentially eliminated by PP6 deficiency–mediated enhanced negative selection, whereas only those with lower levels of TCR survive. Another explanation could be that hyperactivation of distal TCR signaling in PP6-deficient T cells may increase trafficking of TCR–CD3 complexes to lysosomes for degradation because overexpression of constitutive active LckY505F was reported to reduce TCR surface expression by this mechanism (44). Currently, we also cannot rule out the possibility that PP6 positively regulate the activities of proximal TCR molecules. Further studies are needed to test these possibilities.

Positive and negative selection is defined by the ability of TCR to sense relatively weak and overly strong signals, respectively. However, the regulation, particularly negative regulation, of distal TCR signaling cascade in positive and negative selection of thymocytes is not completely known. The test of positive selection using OT-II transgenic TCR demonstrates that PP6 deficiency enhances positive selection of thymocytes. However, the total number of CD4 SP cells, along with CD8 SP and DP thymocytes, in non-TCR transgenic PP6F/++;Lck-Cre mice is decreased. This discrepancy suggests that enhanced positive selection applies only to a fraction of developing T cells in PP6-deficient thymus, presumably those expressing relatively low-affinity TCR such as OT-II. PP6-deficient thymocytes expressing higher-affinity TCR, such as OT-I, may undergo negative selection instead of increased positive selection as observed in our analysis of PP6F/++;Lck-Cre; OT-I mice. Our finding is reminiscent to that observed in thymocytes expressing a constitutively active NF-κB, which is sufficient to push a portion of positively selecting CD4 and CD8 SP thymocytes into pseudonegative selection (32). Our analysis of distal TCR signaling pathways indicates that PP6 negatively
regulates multiple pathways known to be involved in either positive (e.g., ERK pathway) or negative (e.g., NF-κB and JNK pathways) selection. Thus, the TCR distal signaling pathways responsible for both positive and negative selection are attenuated by the PP6 phosphatase.

Our study has shown that the number of γδ T cells dramatically increased in both thymus and peripheral lymphoid organs of PP6F/F;Lck-Cre mice. However, the expansion of γδ T cells was mainly restricted to the IL-17–producing Vγ6+ γδ T cells, with mild or no increase of other Vγ subpopulations. One possible explanation could...

FIGURE 6. Attenuation of proximal TCR signaling in PP6-deficient thymocytes. (A) Surface expression of TCRβ (top) and CD3 (bottom) on the DP, CD4 SP, and CD8 SP thymocytes from PP6F/F and PP6F/F;Lck-Cre mice. (B) Immunoblot analysis for the activation (phosphorylation) of Zap70, LAT, LCK, and PLCγ1 in thymocytes from PP6F/F and PP6F/F;Lck-Cre mice. Total thymocytes were stimulated with anti-CD3/CD28 mAbs for the indicated time. Total Zap70, LAT, LCK, and PLCγ1 were served as loading controls. Numbers below lanes represent band intensity relative to the respective total proteins. (C) Flow cytometry for calcium flux in DP, CD4 SP, and CD8 SP thymocytes from PP6F/F and PP6F/F;Lck-Cre mice. The cells were stained with calcium indicators Fluo-4 and Fura Red followed by stimulation with anti-CD3/CD28 mAbs. Data are representative of four (A), three (B), and two (C) independent experiments.

FIGURE 7. Hyperactivation of distal TCR signaling in PP6-deficient thymocytes. (A and B) Immunoblot analysis for the activation of MAPKs, AKT (A), and their upstream molecules including c-Raf, and MAP2Ks (MEK1/2, MKK4 and MKK3/6) (B) in PMA/ionomycin-stimulated thymocytes from PP6F/F and PP6F/F;Lck-Cre mice using indicated Abs. Total thymocytes were stimulated with PMA/ionomycin for indicated time. Actin was served as a loading control. (C and E) Immunoblot analysis for the activation of TAK1 (C) and Aurora A (E) with the same lysates in (A) using indicated Abs. Total TAK1 or Aurora A was served as loading controls. (D) Immunoblot analysis for the activation of NF-κB signaling pathway with the same lysates in (A) using indicated Abs. Numbers below lanes represent band intensity relative to the respective total proteins. Data are representative of four (A) and three (B–E) independent experiments.
be that PP6 deficiency might extend the window of using Vγ6 gene fragments beyond embryonic stage. However, our finding that PP6-deficient γδ T cells were not increased in bone marrow recipient mice argues strongly against this possibility. The second possible explanation is that the differential effects of PP6 deficiency on each Vγ subset might depend on their corresponding affinities to selecting ligands. Vγ1Vδ6.3 is thought to be a high-affinity TCR (45), whereas Vγ6Vδ1 may be an intermediate-affinity TCR, because Vγ6 T cells, which composed a majority of γδ T cells in PP6−/−; Lck-Cre mice, expressed a lower level of CD5 (Supplemental Fig. 3J). Correspondingly, the number of Vγ1+ T cells was only slightly (1.5-fold) increased, and Vγ6+ T cells upsurged >100-fold in PP6−/−; Lck-Cre mice (Fig. 3C). Thus, we postulate that PP6 deficiency-mediated hyperactivation of distal TCR signaling (e.g., ERK) may have greater impact on IL-17–producing Vγ6+ γδ T cells than other γδ T cells that possess different affinity of γδ TCR. Our postulation is consistent with the previous report that stronger TCR signal promotes the development of γδ T cells in Vγ6Vδ1 TCR transgenic mice (46). In contrast, the reduced Zap70 activity in the SKG mouse promotes the development of a stable population of naive T cells. We also thank Dr. Xiaohui Wu for scientific discussion and Xiaofeng Tao for English editing.

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