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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,*,‡‡ Yuan Liu,* Chenyu Li,* Kejing Deng,* 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 VδVγ 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 eβ 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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Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; DN, double-negative; DP, double-positive; DUSP, dual-specificity phosphatase; ES, embryonic stem; FRT, Fop recombinase target; FK, 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 IκBε 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 development, and fetal-derived, IL-17–producing Vγ1d+ T cells by generating and analyzing T cell–specific ablation of conventional deletion of PP6.

### 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 En2 gene. A pair of Loxp sites was also inserted at Spel 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 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+/flx mice were crossed with Act-flox transgenic mice (stock no. 003800) to delete the β-gal and neo cassette to generate PP6flox/mice. PP6flox/mice were backcrossed with C57BL/6 for six generations before crossing with Lck-Cre (17) or Lck-Cre mice. PP6flox/mice were used for genotyping of PP6+/flox; Lck-Cre mice. Flow cytometry data were obtained (23).

#### X-gal staining

PP6+/flox mice were used for X-gal staining to determine the expression pattern of PP6. Tissue processing, freezing, section, X-gal staining, and immunofluorescent microscopic analysis were performed as described previously (24).

#### 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 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 × 10^6 thymocytes were resuspended in 500 μl Fluoro-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-PE-Cy7 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

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plus anti-Armenian hamster IgG (eBioscience) as cross-linking Ab were added. Events were collected for a total time of 10 min.

**Statistics analysis**

Statistical analysis was conducted using an unpaired *t* test by GraphPad Prism. The *p* values <0.05 were considered significant.

**Results**

**Essential role of PP6 in embryonic development**

We first generated a PP6 mutant allele, PP6<sup>galeo</sup>, in mouse ES cells (Supplemental Fig. 1A, 1B) using a “conventional first, conditional ready” gene-targeting strategy (25). RT-PCR analysis indicated that PP6<sup>galeo</sup> allele is a hypomorphic allele (Supplemental Fig. 1C). In PP6<sup>galeo</sup> 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<sup>+/galeo</sup> mice were intercrossed, PP6<sup>galeo/galeo</sup> 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. 1D and data not shown), and rarely found after birth (at postnatal day 10 WT:HE:TETO = 103:159:3). The PP6<sup>f</sup> conditional allele was generated after removing the β-gal reporter gene and neomycin expression cassettes with Flp/FRT-mediated recombination (Supplemental Fig. 1A). PP6<sup>f/f</sup> mice were phenotypically indistinguishable from WT littermates and used as controls in the studies described later. Germline deletion of the PP6<sup>f</sup> allele with P<sup>gk-Cre</sup> resulted in lethality at early embryonic stages (Supplemental Fig. 1H), confirming the critical function of PP6 in embryonic development.

**Abnormal thymocyte development in PP6<sup>f/f</sup>;Lck-Cre mice**

To study the function of PP6 in T cells, we crossed the PP6<sup>f</sup> allele into Lck-Cre mice to produce PP6<sup>f/f</sup>;Lck-Cre mice. Genomic PCR and Western blot analyses showed that PP6 was deleted efficiently in the thymocytes of PP6<sup>f/f</sup>;Lck-Cre mice (Supplemental Fig. 1E, 1F). T cell–specific ablation of PP6 resulted in abnormal thymocyte development. Both proportion and numbers of CD4<sup>-</sup>CD8<sup>-</sup> single-positive (SP) cells, CD8 SP cells, and CD4<sup>+</sup>CD8<sup>-</sup> (double-positive [DP]) cells, as well as numbers of total thymocytes, were significantly reduced in PP6<sup>f/f</sup>;Lck-Cre mice (Fig. 1A). In contrast, the proportion and number of CD4<sup>+</sup>CD8<sup>-</sup> (double-negative [DN]) cells were increased in the mice. However, further analyses revealed that DN subpopulations of PP6<sup>f/f</sup>;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<sup>f/f</sup>;Lck-Cre mice (see Supplemental Fig. 1H), confirming the critical function of PP6 in embryonic 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). CD4<sup>-</sup> expression in SP cells decreases upon their maturation (28). We found that the proportion of CD24<sup>hi</sup> SP thymocytes was increased in the PP6<sup>f/f</sup>;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<sup>f/f</sup>;Lck-Cre mice to test MHC class II and class I–mediated positive selection, respectively. Thymocytes...
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 TCRVb5+ 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 TCRVo2hi and CD8 SP cells (Fig. 2C, middle panels, and Supplemental Fig. 2E) and a higher ratio between post selected OT-I–specific TCRVo2hi 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-κB. Enhancement of OT-I signals by constitutive activation of NF-κB 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/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/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 Vβ5+ and Vβ11+ TCR clonotypic thymocytes in the presence of the class II MHC molecule I-E^d (33). MHC I-E^d is absent in C57BL/6 mice (H-2^b) but present in BALB/c mice (H-2^d). Enhanced deletion of Vβ5+ and Vβ11+ clones was observed for both CD4 and CD8 SP thymocytes in PP6/F;Lck-Cre;H-2^d mice, whereas Vβ8+ 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 gd T cells in PP6/F;Lck-Cre mice

In contrast with the severe reduction of αβ T cells, γδ T cells were dramatically increased in the thymus (Fig. 3A) and the periphery

FIGURE 3. Increased IL-17–producing Vγ6Vδ1+ T cells in PP6/F;Lck-Cre mice. (A) Flow cytometry and cellularity of αβ and γδ T cells in the thymus with indicated genotype. n = 5 for each genotype. (B) Cellularity of γδ T cells in spleen and lymph node (LN) from PP6/F and PP6/F;Lck-Cre mice. n = 5 for each genotype. (C) Cellularity of Vγ1+, Vγ4+, Vγ5+, and Vγ6+ γδ T cells from thymus in PP6/F and PP6/F;Lck-Cre mice. n = 4 for each genotype. (D) FACS analysis of intracellular expression of IL-17 and surface expression of IL-7R of γδ T thymocytes, with indicated genotypes. (E) The cellularity of γδ T cells in the thymus from fetal or postnatal PP6/F and PP6/F;Lck-Cre mice. n ≥ 3 for each genotype at indicated ages. (F) Flow cytometry analysis of thymus of B6.SJL recipient mice transplanted with a mixture of equal number of bone marrow cells from PP6/F; CD45.1.2+ or PP6/F;Lck-Cre;CD45.2.2+ mice. Numbers adjacent to outlined areas indicate the γδ T cell percentage of the gated CD45.1.2+ or CD45.2.2+ thymocytes. n = 4 for each genotype. (G) Percentage of BrdU+ γδ T cells from PP6/F and PP6/F;Lck-Cre mice at P2, which were injected s.c. with BrdU 24 h before sacrificed for flow cytometry. n = 4 for each genotype. (H) Proliferation and apoptosis of CD24hi and CD24lo γδ T cells from adult mice (6–8 wk) with indicated genotypes were evaluated flow cytometrically by measuring BrdU incorporation (H) or Annexin V staining (J), respectively. n = 3 for each genotype. (I) Number of BrdU+ CD24hi and BrdU+ CD24lo γδ T cells in thymus from adult mice (6–8 wk) with indicated genotypes. n = 4 for each genotype. Data shown are representative from two (C and F) or three (A and D) independent experiments. (A–C, E, and G–J) Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
of PP6\(^{-}\);Lck-Cre mice (Fig. 3B and Supplemental 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\(^{-}\);Lck-Cre thymus first by quantitative real-time RT-PCR analysis of seven individual \(\gamma\delta\) genes. We found that the expression of \(\gamma5\) and \(\gamma\delta\) fragments increased ∼30 and ∼50 times, respectively, in the \(\gamma\delta\) T cells purified from PP6\(^{-}\);Lck-Cre thymus in comparison with those of the control mice (Supplemental Fig. 3B). V\(\delta1\) expression was also found to be increased ∼40-fold (Supplemental Fig. 3B). FACS analysis with \(\gamma\delta\)-specific Abs revealed that the dramatic increase of γδ T cell numbers in PP6\(^{-}\);Lck-Cre thymus is primarily due to an increase of V\(\delta6\) cells, even though numbers of V\(\gamma1\) and V\(\gamma5\) cells also increased (Fig. 3C and Supplemental Fig. 3C, 3D). γδ T cells expressing V\(\gamma6\), which are normally pairs with V\(\delta1\), 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 γδ T cells) (34). Indeed, we found that most PP6\(^{-}\);Lck-Cre γδ T cells were IL-7R\(^{+}\), CCR6\(^{+}\), and CD27\(^{+}\) IL-17 producers (Fig. 3D and Supplemental Fig. 3E). V\(\gamma6\) T cells are typically generated in fetal thymus in WT mice (34, 35). However, this population appeared to be continuously increased in PP6\(^{-}\);Lck-Cre mice only after birth (Fig. 3E and Supplemental Fig. 3F). To determine whether the increase of V\(\gamma6\) 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\(^{-}\)-deficient mice (Supplemental Fig. 3H), they failed to recapitulate the mutant increase of V\(\gamma6\)-specific Abs revealed that the majority of CD4\(^+\) and CD8\(^+\) T cells in ex vitro culture (Fig. 5A). Adoptive transfer test further demonstrated an intrinsic survival defect of PP6\(^{-}\)-deficient CD4\(^+\) and CD8\(^+\) 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\(^{-}\);Lck-Cre thymocytes. The surface expressions of TCR\(\beta\) and CD3 were reduced in PP6\(^{-}\)-deficient thymocytes (Fig. 6A). Upon TCR stimulation, the phosphorylations of Zap70, LAT, and LCK proteins were reduced in PP6\(^{-}\)-deficient cells (Fig. 6B). Activation of PLC-\(\gamma\)-1, which is responsible for the production of the second messenger diacylglycerol and the initiation of Ca\(^{2+}\) 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\(^{-}\);CD4\(^{+}\) mice (Supplemental 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\(^{-}\);Lck-Cre mice by intracellular staining. The analysis showed that regardless of PMA stimulation, enhanced phosphorylated ERK was detected in both γδ and εβ thymocytes from PP6\(^{-}\)-deficient mice (Supplemental 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 (Supplemental 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 CD62L and CD44 expression and percentage of CD44hiCD62Llo and CD44loCD62Lhi splenic CD4+ and CD8+ T cells from PP6F/F and PP6F/F;CD4-Cre mice. n = 4 for each genotype. (C) CD69 expression on splenic CD4+ and CD8+ T cells from PP6F/F and PP6F/F;CD4-Cre mice. (D–F) Flow cytometry and percentage of IFN-γ+CD4+ or IFN-γ+CD8+ (D), IL-4+CD4+ (E), and IL-17+CD4+ (F) splenocytes after PMA/ionomycin stimulation for 6 h. (G) Intracellular staining of Ki67 in CD4+ and CD8+ splenocytes with indicated genotypes. n = 3 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.
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 in 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 as observed in our analysis of PP6-deficient thymocytes, an expansion of CD4+ 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. These findings place PP6 at a major control point of distal TCR signaling.

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.

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**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. These findings place PP6 at a major control point of distal TCR signaling.
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 γT 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 γT cells in PP6+/−, Lck-Cre mice, expressed a lower level of CD5 (Supplemental Fig. 3 I). 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. 3 C). Thus, we postulate that PP6 deficiency–mediated hyperactivation of distal TCR signaling (e.g., ERK) may have greater impact on IL-17–producing Vγ6 T cells than other γT cells that possess different affinity of γTCR. Our postulation is consistent with the previous report that stronger TCR signal is consistent with the previous report that stronger TCR signal

References


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Disclosures

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