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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 γδV6V1+ 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 naïve T cell homeostasis by preventing their premature activation before Ag stimulation. The Journal of Immunology, 2015, 194: 1654–1664.

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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 promotes IkBe 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 Vγ6Vδ1+ T cells are preferentially expanded in PP6−/−Lck-Cre mice. In peripheral lymphoid organs, PP6-deficient mature T cells are no longer able to maintain their naive state and become short-lived effectors. PP6 deficiency leads to a profound hyperactivation of multiple 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 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 injected into C57BL/6J blastocysts to produce chimera mice. PP6+/galeo mice were crossed with Lck-Cre mice. The Journal of Immunology 1655

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 β2m, CD4 (RM4-5), CD8b (H35-17.2), CD3ε (145-2C11), TCRβ (H57-597), TCRγδ (GL3), CD5 (53-73), CD24 (30-1F1), CD69 (H1.2F3), CD25 (PC6.15), CD44 (IM7), CD62L (MEL-14), CD45.1 (A20), CD45.2 (104), TCRβ/J5 (MR9-4), TCRβ/J8 (KJ16), TCRβ/I1 (C21), TCRβ/2a (B2.10), IL-7R (SB/199), TCRγδ (2.11), TCRγδ (UC-10αβ), TCRγδ/5 (536), CCR6 (140706), CD27 (LG79), IFN-γ (XM12.1.2), IL-4 (IL11.01), IL-17 (CT11-18H10), Foxp3, and Ki67 Kit were purchased from BD or eBioscience. Anti-TCR αε was kindly provided by Dr. Xuetao 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 with approval of 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 μg/ml proteinase inhibitor (Roche). Immunoblots were performed as described previously (21) with Abs to p-ERK (4370), ERK (4695, 9107), p-JNK (4668), JNK (9258, p-38 (4511), p86 (8900), p-raf (9427), p-MEK1/2 (9154), p-MEK4 (9156), p-MEK3/6 (9231), MEK1/2 (9126), p-IKKα/β (2697), IKKγ (2682), p-65 (3033), p-IκBα (2859), IκBα (4814), p-IBβ (4924), IκBε (9249), p-Zap70 (2717), Zap70 (3165), p-LAT (3584), LAT (9166), p-LCK (2751), LCK (2787), p-PLCγ1 (2821), and PLCγ1 (5690) purchased from Cell Signaling. Anti-PP6 (07-1224) was from Millipore, and anti–β-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 β2m, CD4 (RM4-5), CD8b (H35-17.2), CD3ε (145-2C11), TCRβ (H57-597), TCRγδ (GL3), CD5 (53-73), CD24 (30-1F1), CD69 (H1.2F3), CD25 (PC6.15), CD44 (IM7), CD62L (MEL-14), CD45.1 (A20), CD45.2 (104), TCRβ/J5 (MR9-4), TCRβ/J8 (KJ16), TCRβ/I1 (C21), TCRβ/2a (B2.10), IL-7R (SB/199), TCRγδ (2.11), TCRγδ (UC-10αβ), TCRγδ/5 (536), CCR6 (140706), CD27 (LG79), IFN-γ (XM12.1.2), IL-4 (IL11.01), IL-17 (CT11-18H10), Foxp3, and Ki67 Kit were purchased from BD or eBioscience. Anti-TCR αε was kindly provided by Dr. Robert E. Tigelaar at Yale University. The staining of Vγ6+ 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 spleenocytes with anti-CD4–coated magnetic microbeads (Miltenyi Biotech). γδ T cells were sorted on FACSARia II (BD Biosciences).

Adoptive transfer

For adoptive transfer, freshly isolated spleenocytes containing an equal amount of wild type (WT) or PP6-deficient CD4+ T cells or CFSE-labeled purified CD4+ T cells were i.v. injected into B6.SJL as described previously (23).

X-gal staining

PP6−/−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 analysis.

Calcium flux

Calcium flux was measured using the Fluor-4 Direct Calcium Assay Kit (Invitrogen). In brief, 2×106 thymocytes were resuspended in 500 μl Fluor-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-PET 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
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-TET:HO = 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 Pgk-Cre 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> single-positive (SP) cells, CD8<sup>+</sup> 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 Increased IL-17–producing γδ T cells in PP6<sup>f/f</sup>;Lck-Cre mice) (Fig. 1D). Although the proportion of peripheral Tregs increased in adult PP6<sup>f/f</sup>;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<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.

![FIGURE 1](http://www.jimmunol.org/)  Defective thymocyte development in PP6<sup>f/f</sup>;Lck-Cre mice. (A) Flow cytometry and cellularity of thymocytes in PP6<sup>f/f</sup> and PP6<sup>f/f</sup>;Lck-Cre mice. Numbers in quadrants (left panel) indicate percent cells in each. n = 8 for each genotype. (B) Cellularity of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from spleen, lymph node (LN), and blood of PP6<sup>f/f</sup> and PP6<sup>f/f</sup>;Lck-Cre mice. n = 8 for each genotype. (C) Flow cytometry of thymocytes gated on DN cells (CD4<sup>+</sup>CD8<sup>+</sup> TCR<sup>+</sup>CD11b<sup>−</sup> NK1.1<sup>−</sup>B220<sup>+</sup>) and frequency of DN thymocyte subpopulations from PP6<sup>f/f</sup> and PP6<sup>f/f</sup>;Lck-Cre mice. Numbers in quadrants (left panel) indicate percent cells in each. n = 4 for each genotype. (D and E) Percentage and cellularity of Tregs gated on CD4 SP thymocytes (D, left panels) or CD4<sup>+</sup> splenocytes (D, right panels) from adult PP6<sup>f/f</sup> and PP6<sup>f/f</sup>;Lck-Cre mice or CD4<sup>+</sup> T cells of LNs from 1-wk-old PP6<sup>f/f</sup> and PP6<sup>f/f</sup>;Lck-Cre mice (E). n = 4 for each genotype. Data shown are representative from at least three (A, B, D, and E) and two (C) independent experiments. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
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 TCRVβ5+ 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 TCRVα2hi and CD24lo CD8 SP cells (Fig. 2C, middle panels, and Supplemental Fig. 2E) and a higher ratio between post selected OT-I–specific TCRVα2hi 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

FIGURE 2. Enhanced positive and negative selection in PP6-deficient mice. (A) Surface expression of CD5, CD24, and CD69 on PP6-deficient and control thymocytes gated as indicated. n ≥ 4 for each genotype. (B) Flow cytometry (top panel) and cellularity (bottom panel) of thymocytes in PP6F/F;OT-II and PP6F/F;Lck-Cre;OT-II mice. Numbers in quadrants (top panel) indicate percent cells in each. (Middle panel) Staining with Ab to the OT-II–specific V region Vβ5. n = 5 for each genotype. (C) Flow cytometry (top panel) and cellularity (bottom panel) of thymocytes in PP6F/F;OT-I and PP6F/F;Lck-Cre;OT-I mice. Numbers in quadrants (top panel) indicate percent cells in each. (Middle panel) Staining with Ab to the OT-I–specific V region Vα2. n = 4 for each genotype. (D) Flow cytometry of thymocytes and percentage of DP thymocytes of P6F/F and PP6F/F;Lck-Cre mice 48 h after administration of 10 μg anti-CD3 mAb or PBS. n = 4 for each genotype. (E and F) Statistical analysis of the percentage of Vβ5+, Vβ11+, and Vβ8+ thymocytes gated on CD4 SP (E) or CD8 SP (F) in PP6F/F (Ct) and PP6F/F;Lck-Cre (KO) mice with (H-2b/d) or without (H-2b/b) expression of MHC molecule I-E. Deletion of Vβ5+ and Vβ11+ cells by superantigen only occurs on H-2b/d, but not H-2b/b backgrounds. Vβ8+ cells are not affected. Each symbol represents a single mouse; small horizontal lines indicate the mean. Data shown are representative from two independent experiments. (B and D) Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
required for clonal deletion, PP6F/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 PP6F/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-Ek (33). MHC I-Ek is absent in C57BL/6 mice (H-2b/b) but present in BALB/c mice (H-2d/d). Enhanced deletion of Vβ5+ and Vβ11+ clones was observed for both CD4 and CD8 SP thymocytes in PP6F/F;Lck-Cre;H-2b/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 PP6F/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...
of PP6<sup>fl/fl</sup>;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<sup>fl/fl</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>fl/fl</sup>;Lck-Cre thymus in comparison with those of the control mice (Supplemental Fig. 3B). Vβ1 expression was also found to be increased ~40-fold (Supplemental Fig. 3B). FACS analysis with Vγ-specific Abs revealed that the dramatic increase of γδ T cell numbers in PP6<sup>fl/fl</sup>;Lck-Cre thymus is primarily due to an increase of Vγ6<sup>+</sup> cells, even though numbers of Vγ<sup>+</sup> and Vγ<sup>-</sup> cells also increased (Fig. 3C and Supplemental Fig. 3C, 3D). γδ T cells expressing Vγ6, which are normally pairs with V81, 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 IFNγ–producing γδ T cells) (34). Indeed, we found that most PP6<sup>fl/fl</sup>;Lck-Cre γδ T cells were IL-7R<sup>+</sup>, CCR6<sup>+</sup>, and CD27<sup>+</sup> IL-17 producers (Fig. 3D and Supplemental 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>fl/fl</sup>;Lck-Cre mice only after birth (Fig. 3E and Supplemental 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>fl/fl</sup>;Lck-Cre mice. Although the mutant donor cells showed defects in αβ lineage development similar to that observed in PP6<sup>fl/fl</sup>;Lck-Cre mice (Supplemental Fig. 3H), they failed to recapitulate the mutant phenotype of PP6<sup>fl/fl</sup>-deficient γδ T cells (Fig. 3F). Therefore, the increase of γδ T cells in PP6<sup>fl/fl</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>fl/fl</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>fl/fl</sup>;Lck-Cre mice (Supplemental 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>+</sup>-deficient mature γδ T cells were less susceptible to apoptosis (Fig. 3I). Therefore, we concluded that PP6 deficiency promotes a continuing expansion of pre-existing Vγγ<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>fl/fl</sup>;CD4<sup>-Cre</sup> mice were ~70 and 42% of the PP6<sup>+</sup> controls, respectively (Fig. 4A). Naive CD4<sup>+</sup> or CD8<sup>+</sup> T cells are significantly reduced in PP6<sup>fl/fl</sup>;CD4<sup>-Cre</sup> 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 IFNγ–producing effector CD4<sup>+</sup> or CD8<sup>+</sup> cells in PP6<sup>+</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 vitro 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>fl/fl</sup>;Lck-Cre thymocytes. The surface expressions of TCRβ 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 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>fl/fl</sup>;CD4<sup>-Cre</sup> 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<sup>fl/fl</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-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-kB 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 PP6+/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 PP6+/F and PP6F/F;CD4-Cre mice. n = 4 for each genotype. (C) CD69 expression on splenic CD4+ and CD8+ T cells from PP6+/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 PP6+/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 (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 IκBα 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 IκBε 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 IκBε in HeLa cells (14). However, we do not think that PP6 deficiency–mediated deregulation of IκBε 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.

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.

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/F;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/F;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...

![FIGURE 5. Reduced viability of PP6-deficient periphery T cells.](http://www.jimmunol.org/)

(A) Cell viability of CD4+ and CD8+ splenocytes from PP6F/F and PP6F/F;CD4-Cre ex vivo cultured for indicated time. Annexin V−/7AAD− cells were considered viable. Four pairs of mice were used for each independent experiment. (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/F;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/F and PP6F/F;CD4-Cre mice, stained with MitoTracker Green (left) or dihydroethidium (right). n = 4 for each genotype. (D) 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) groups. (A, B, and D) Data are presented as mean ± SD. **p < 0.01, ***p < 0.001.
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 PP6Δ/Δ; 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 PP6Δ/Δ and PP6Δ/Δ; Lck-Cre mice. (B) Immunoblot analysis for the activation (phosphorylation) of Zap70, LAT, LCK, and PLCγ1 in thymocytes from PP6Δ/Δ and PP6Δ/Δ; 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 PP6Δ/Δ and PP6Δ/Δ; 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 PP6Δ/Δ and PP6Δ/Δ; 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. 3I). 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 balances physiological and oncogenic nuclear factor-kappa B signaling in T lymphocytes. Immunity 30: 879–886.


Supplemental Figure 1. Generation of PP6 conditional deficient mice.

(A) Schematic illustration of the gene-targeting strategy for PP6. WT: mouse genomic DNA fragment of WT PP6 gene containing exon 3-7. B and N represent the restriction sites of BamH I and Nde I, respectively. Targeting vector: schematic structure of the PP6 targeting vector. β-galPA represents a nuclear β-galactosidase reporter gene with poly A signal. Pgk-Neo and Pgk-DTA represent expression cassettes of positive selection marker neomycin and negative selection marker diphtheria toxin A with Pgk promoter, respectively. FRT and LoxP represent the FRT and LoxP sites, respectively. PP6galeo: genomic structure of mutant PP6galeo allele after homologous recombination. Probe 1 and Probe 2 represent the probes used to identify the recombinant ES clones. PP6F: genomic structure of modified PP6F allele after removing the β-galPA and Pgk-neo cassettes. Arrows p1, p2, and p3 are PCR primers for genotyping of PP6F allele and PP6 null allele. PP6-: genomic structure of PP6 null allele after removing the fragment containing exons 5 and 6.

(B) Genomic Southern blots of WT and targeted ES clones. 11 and 4kb BamH I fragments detected by probe 1 represent the WT and PP6galeo alleles, respectively. 6kb Nde I fragment was only detected by probe 2 (neomycin) in targeted ES cells.

(C) Real time RT-PCR analysis of PP6 expression in live E18 WT and PP6galeo/galeo embryos.

(D) Micrograph of WT, PP6+/galeo or PP6galeo/galeo embryos at E14.5. Scale bar, 2 mm.

(E) Genomic PCR analysis of recombination efficiency of PP6F allele in PP6F/F;Lck-Cre thymocytes. DNAs from toe and thymus with indicated genotypes were used as negative and positive controls, respectively.

(F) Immunoblot analysis of PP6 expression in thymus with indicated genotypes.

(G) Micrograph of X-gal stained cryosections of tissues as indicated from PP6+/galeo mice, revealing PP6 expression patterns.

(H) Micrograph of E6.5 of PP6F- and PP6F/F;Pgk-Cre embryos generated by cross PP6F/F mice with PP6+/F;pgtk-Cre mice. Right, genotyping PCR of the embryos with indicated genotypes. Scale bar, 0.2 mm.

Images in (G) and (D, H) are representative sections (n>3) or representative embryos (n=3 per genotype), respectively. Data shown in (C, E and F) are representative from two independent experiments and presented as mean ± SD. ** p<0.01, *** p<0.001.
Supplemental Figure 2

Supplemental Figure 2. Abnormal thymocyte development in PP6 deficient mice.

(A) Flow cytometry of CD4+ and CD8+ lymphocytes from spleen, lymph node (LN) and blood of PP6+/+ and PP6F/F;Lck-Cre mice. n=8 for each genotype.

(B) Flow cytometry of Tregs gated on CD4 SP thymocytes (left panels) and CD4+ splenocytes (right panels) from PP6+/+ and PP6F/F;Lck-Cre mice. Numbers above boxed areas indicate Treg (Foxp3+) percentage. n=4 for each genotype.

(C) Flow cytometry and cellularity of Tregs in the spleen from 1-week-old PP6+/+ and PP6F/F;Lck-Cre mice. Numbers above boxed areas indicate Treg (Foxp3+) percentage of CD4 T cells. n=4 for each genotype.

(D and E) Cell surface expression of CD69 and CD24 on DP and CD4 SP (D) or CD8 SP (E) thymocytes of the mice with indicated genotypes. B6, C57BL/6 WT mice. n=5 for each genotype.

(F) Flow cytometry of gated TCRα2hi thymocytes from PP6F/F;OT-I and PP6F/F;Lck-Cre;OT-I mice (left) and ratio of post selected CD8 SP and pre-selected DP and CD4 transitional thymocytes (right). Most of CD4 SP cells have been shown to be transitional cells and precursors to CD8 SP thymocytes in the OT-I model. n=4 for each genotype.

Data are representatives of three (A-C) and two (C-F) independent experiments. Data in (C and F) are presented as mean ± SD. * p<0.05, *** p<0.001.
Supplemental Figure 3. Postnatal expansion of IL-17-producing Vγ6Vδ1+ T cells in PP6F/F;Lck-Cre mice.

(A) Flow cytometry of γδ T cells in spleen and LN from PP6F/F and PP6F/F;Lck-Cre mice. Numbers in boxed areas indicate percentage of γδ T cells. n=5 for each genotype.

(B) Real time RT-PCR analysis of expression of individual TCR Vγ and Vδ1 genes γδ T cells purified by FACS from thymus with indicated genotypes. Actinb was used as an internal standard for normalization. The expression levels of each indicated genes in PP6F/F γδ T cells were set as 1. Four pairs of mice were used for each independent experiment.

(C and D) Flow cytometry (C) and percentage (D) of Vγ1+, Vγ4+, Vγ5+ and Vγ6+ γδ T cells from thymus in PP6F/F and PP6F/F;Lck-Cre mice. Numbers in boxed areas indicate the percentage of specific subset Vγ T cells in thymus. n=4 for each genotype.

(E) Surface expression of CCR6 and CD27 on γδ T cells in the thymus of PP6F/F and PP6F/F;Lck-Cre mice.

(F) The percentage of γδ T cells in the thymus from fetal or postnatal PP6F/F and PP6F/F;Lck-Cre mice. n≥3 for each genotype in indicated days.

(G) Flow cytometry and percentage of thymocytes of recipient mice (B6.SJL, CD45.1.1+) 6-8 weeks after transplantation with a mixture of equal numbers of bone marrow cells from PP6+/F;CD45.1.2+ and PP6F/F;Lck-Cre;CD45.2.2+ mice. n=4 for each genotype.

(H) Flow cytometry and frequency of thymocytes subpopulation of gated cell in (G).

(I) The cellularity of CD24hi and CD24lo γδ thymocytes in adult mice (6-8 weeks) with indicated genotypes. n=4 for each genotype.

(J) Surface expression of CD5 on γδ thymocytes from PP6F/F and PP6F/F;Lck-Cre mice.

Data are representatives of at least three (A, E, J) and two (B, C, D, G) independent experiments. Data in (A-B, F-I) are presented as mean ± SD. * p<0.05, ** p<0.01, *** p<0.001.
Supplemental Figure 4

(A) Surface expression of TCRβ and CD3 on the CD4+ and CD8+ splenocytes with indicated genotypes.

(B-C) Immunoblot analysis for the activation (phosphorylation) of Zap70, LAT, LCK, and PLCy1 in purified naïve (CD4+CD44hiCD62Llo) (B) or effector (CD4+CD44hiCD62Llo) (C) splenic T cells from PP6F/F and PP6F/F;CD4-Cre mice. Cells were stimulated with anti-CD3/CD28 mAbs for indicated time. Total Zap70, LAT, LCK, and PLCy1 were served as loading controls.

(E) Flow cytometry for calcium flux in CD4+ and CD8+ splenocytes with indicated genotypes. The cells were stained with calcium indicators Fluo-4 and Fura Red followed by stimulation with anti-CD3/CD28 mAbs.

(F) Intracellular staining and statistical analysis of the mean fluorescent intensity (MFI) for p-ERK in γδ and αβ thymocytes with indicated genotypes w/wo PMA/ionomycin stimulation for 5 minutes. IgG, rabbit IgG staining of thymocytes from PP6F/F mice without stimulation.

(G, H-J) Immunoblot analyses for the activation of MAPKs, AKT (D, G, J), Aurora A (H) and NF-κB signaling pathway (I) using indicated antibodies in PP6F/F and PP6F/F;Lck-Cre thymocytes stimulated with anti-CD3/CD4 (G-I) or in purified naïve (CD4+CD44hiCD62Llo) (D) or effector (CD4+CD44hiCD62Llo) (J) PP6F/F and PP6F/F;CD4-Cre splenocytes stimulated with anti-CD3/CD28 (D and J). Total ERK, JNK, p38, AKT, Aurora A, IKKα and p65 are shown as loading controls, respectively.

Numbers below lanes (B-D and G-J) indicate band intensity relative to the respective total proteins. Data are representative of four (A), three (B-D, F-J), and two (E) independent experiments. ** *p<0.001.