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2 **Supplementary Information for**

3 Protein phosphatase 2A has an essential role in promoting 4 thymocyte survival during selection

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Supporting information-Materials and Methods

Antibodies. The biotin anti-mouse CD3 (145-2C11), biotin anti-mouse CD4 (GK1.5), anti-mouse CD28 (37.51), antibodies were from Biolegend (San Diego, CA, USA). The anti-PLC-γ phospho-Tyr783 (2821), anti-ERK1-ERK2 (9102), anti-ERK1-ERK2 phospho-Thr202 and Tyr204 (9101), anti-Jnk1-Jnk2 (56G8), antibody to Jnk1-Jnk2 phosphorylated at Thr183 and Tyr185 (9251), anti-Akt (C67E7), antibody to Akt phosphorylated at Ser473 (D9E), anti-Bcl2, anti-Bcl-xl, anti-Bim, anti-Bax, anti-Bad, anti-GAPDH were from Cell Signaling Technology (Danvers, MA, USA). The anti-PP2Ac was from Millipore.

Fluorescein isothiocyanate–conjugated antibody to mouse CD4 (L3T4), CD8(Ly-2), CD69 (H1.2F3), CD5 (Ly-1), CD3 (145-2C11) or BrdU (PRB-1); allophycocyanin-conjugated antibody to mouse CD62L (MEL-14) and TCRβ (H57-597) were from BD Biosciences.

Immunoblot analysis. Cells were lysed in SDS sample buffer by the addition of 1/4 volume of 5×SDS sample buffer directly into cell suspension. The samples were then boiled for 5 min and separated by 10% SDS-PAGE.

Ca^{2+} flux. 1×10^6 thymocytes in suspension were first labeled with 4 μg/ml Fluo4 (Invitrogen) for 1 h at 37℃, washed with ice-cold PBS and re-suspended in PBS. Cells were surface labeled with phycoerythrin-indocarbocyanine-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD8 for 30 min on ice and then incubated with biotinylated anti-CD3 (5 μg/ml) and anti-CD4 (5 μg/ml). The labeled cells were warmed for 20 min at room temperature and then crosslinked with streptomycin (25 μg/ml) or stimulated with PMA (phorbol 12-myristate 13-acetate) and ionomycin immediately before flow cytometry analysis. Mean fluorescence ratios were plotted after analysis with FlowJo software (TreeStar).

Proliferation *in vivo* (Brdu). Intraperitoneal injection was used to administer BrdU (1mg/mouse), 18 hours later, mice were sacrificed to obtain the thymocytes and splenocytes. 2×10^6 cells were suspended in 100μl PBS with surface marker antibodies for CD4 or CD8 (eBioscience) in 4℃ for 30 min. These were centrifuged, and IC fixation buffer was added at 4℃ overnight. Cells were treated with1×permeabilization buffer for 2min on ice, and then treated with DNase I buffer(30μl 10×buffer, 220μl ddH2O, 50U DNase I) for 37℃ for 30 min, then

51 resuspended with 3 µl FITC anti-BrdU (eBioscience) in 300 µl 1 × BrdU staining buffer
52 at 4 ℃ for 30 min before analysis with flow cytometry.

53 **Cell Cycle analysis.** 2×10^6 cells were stained for CD4 or CD8 (eBioscience) at 4 ℃
54 for 30 min and fixed in buffer IC prior to permeabilization. Propium Iodide (PI) and
55 RNase A buffer was then added at 4 ℃ for 40 min before analysis with flow
56 cytometry.

57 **Glycolytic and mitochondrial respiration rate measurement.** For metabolic
58 experiments, a Seahorse XF24 instrument was used. Total thymocytes were seeded at
59 a density of 5×10^5 per well. The extracellular acidification rate (ECAR) and the
60 oxygen consumption rate (OCR) for each well were calculated, while the cells were
61 subjected to the XF Cell Mito or the XF Glycolytic stress test protocols. The XF Cell
62 Mito and the XF Glycolytic stress test kit were purchased from Seahorse Biosciences.

63 Statistical significance was defined as $P < 0.05$.

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Supplementary Figures

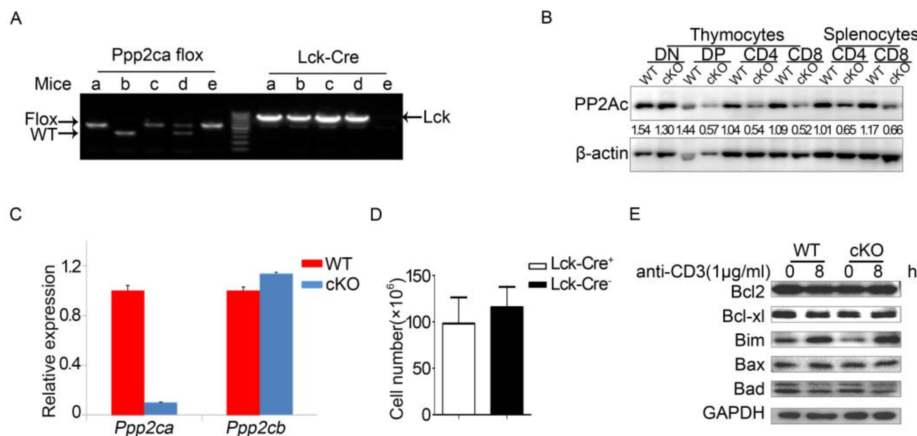


Fig. S1 Generation of PP2A conditional knockout mice

(A) Genotyping: *Ppp2ca* Floxed allele (593 bp) was detected by the primer set F/R. Flox forward primer: 5'-TAGCCCATGCCTTTAATCTCAGAGC-3'. Flox reverse primer: 5'-CACTCGTCGTAGAACCCATAAACC-3'. Lck-Cre transgene was detected by Lck-Cre forward primer: 5'-GGAAAATGCTTCTGTCCGTTTG-3'. Lck-Cre reverse primer： 5'-TTGGTCCAGCCACCAGCTTG-3'. Resulted in the amplification of a 800 bp band. Mice a and c were Lck-Cre^{+}*Ppp2ca*$^{flox/flox}$ cKO mice. **(B)** Western Blot analysis of PP2A catalytic α subunit prepared from populations sorted from central and peripheral lymphoid organs in WT and cKO mice. The values for densities of PP2Ac/β-actin band from the same lane were determined by using the Image J software. **(C)** RT- PCR reaction was performed to detect the expression of *Ppp2ca* and *Ppp2cb* in cKO thymocytes. *Ppp2cb* serves as a negative control. **(D)** Total thymocyte number of Lck-Cre^{+} mice and its litter mate Lck-Cre^{-} mice. Data are representative of three experiments. Error bars show mean ± SEM. **(E)** Immunoblot analysis of pro- (Bim, Bax, Bad) and anti- (Bcl2, Bcl-xl) apoptosis protein levels. GAPDH serves as a loading control.

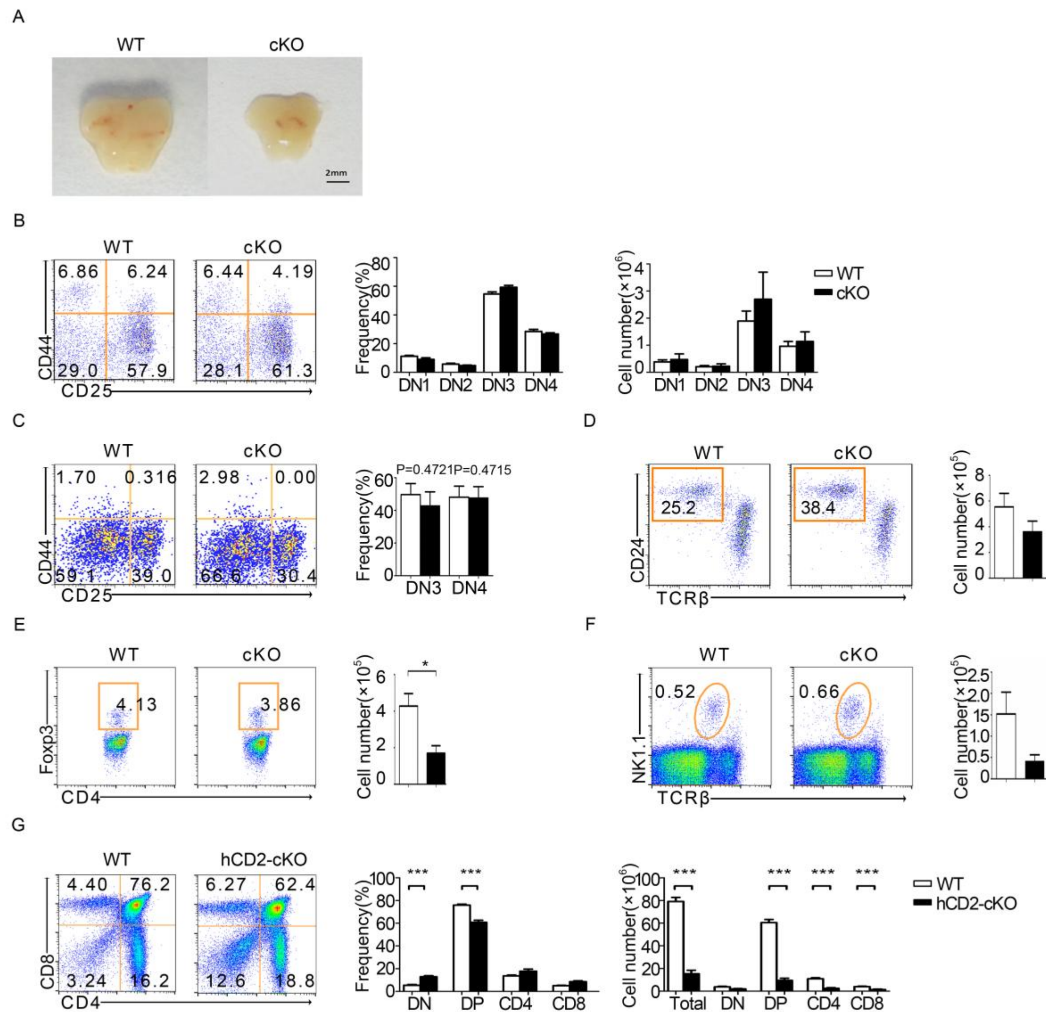
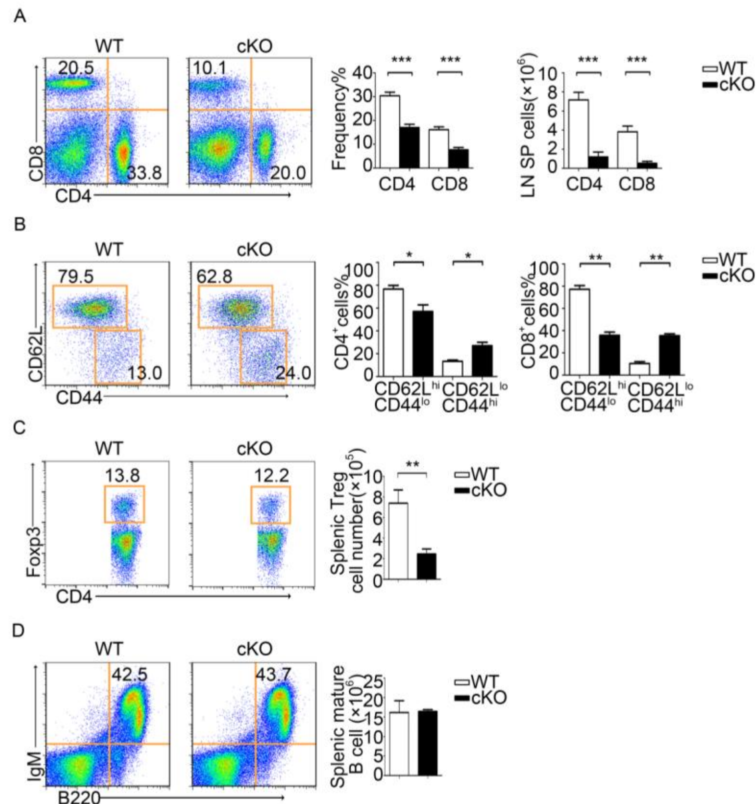


Fig. S2 PP2A conditional deletion in T cell lineage

(A) Thymus size in WT and cKO mice (Scale bar 2mm). **(B)** Two parameter dot plots show CD44 versus CD25 surface staining of $CD4^-CD8^-$ thymocytes. Right, the frequency and cell number of DN1-DN4 thymocyte subpopulations. **(C)** Flow cytometry analysis of CD44 versus CD25 gated on DN thymocytes co-cultured with OP9-DL1 cells at day 5. Right, the frequency DN3, DN4 subpopulations. **(D)** Surface staining of CD24 and TCRβ to analyze cell number of ISP. **(E, F)** Analysis of nTreg (E) and NKT cells (F) in thymus. **(G)** Cell surface staining of CD4 and CD8 on WT and *Ppp2ca*$^{flox/flox}$ hCD2-Cre cKO thymocytes. Numbers in or adjacent to outlined areas (or in quadrants) indicate percentage. Bar charts indicate the cell frequencies and numbers for total thymocytes DN, DP, $CD4^+$ or $CD8^+$ SP thymocyte subpopulations. Data are representative of five experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.



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104 **Fig. S3 Phenotype of lymph node (LN) T cells in cKO mice**
105 **(A)** Surface staining of CD4 and CD8 on WT and cKO LN (left), and frequency
106 (middle) and cell number (right) of LN subpopulations. **(B)** Expression of CD62L and
107 CD44 on $CD4^+$ LN from WT and cKO mice (left), and frequency of $CD62L^{hi}CD44^{lo}$
108 $CD4^+$ and $CD62L^{lo}CD44^{hi}$ $CD4^+$ subpopulations. **(C, D)** Analysis of pTreg (C) and B
109 cell (D) in the peripheral. Data are representative of three experiments. Error bars
110 show mean ± SEM.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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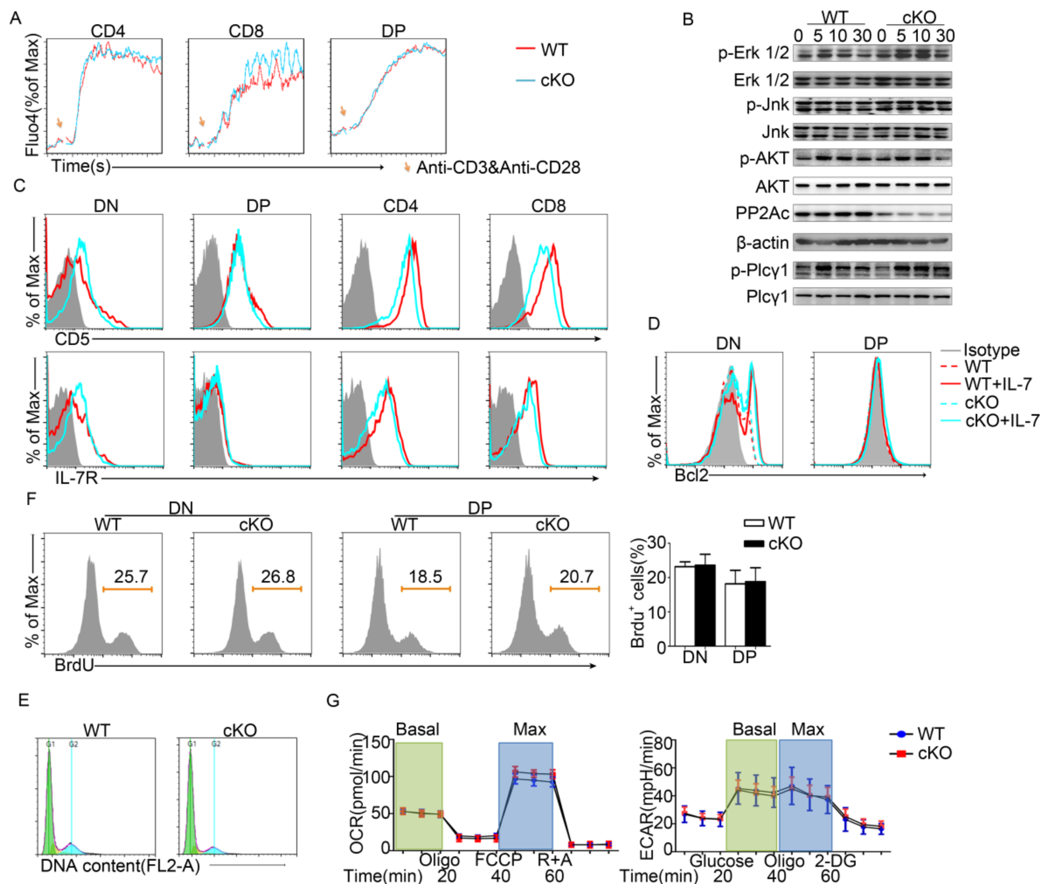

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118 **Fig. S4 Intact TCR Signaling in PP2Ac cKO thymocytes**

119 **(A)** Calcium flux in CD4, CD8 or DP thymocytes from WT and cKO mice after
120 stimulation (downward arrows) with anti-CD3 and anti-CD28 (1). **(B)** Immunoblot
121 analysis of total and phosphorylated proteins of sorted WT and cKO DP thymocytes
122 stimulated for 0, 5, 10 or 30 min with anti-CD3 and anti-CD28. β-actin serves as a
123 loading control. **(C)** Surface staining of CD5 and IL-7R on DN and DP thymocytes in
124 WT and cKO thymocytes. **(D)** The expression of Bcl2 in DN and DP cells with or
125 without 10 ng/ml IL-7 overnight. **(E)** Cell cycle of DP cells by Propidium Iodide (PI)
126 staining (1). **(F)** BrdU uptake by DN or DP cells, numbers above bracketed lines
127 indicate percent of $BrdU^+$ cells (1). Data are representative of four independent
128 experiments. **(G)** The oxygen-consumption rate (OCR) (left) and extracellular
129 acidification rate (ECAR) (right) of WT and cKO total thymocytes in basal conditions
130 (Basal) or at maximum (Max), with the addition (vertical dashed lines) of oligomycin,
131 the mitochondrial uncoupler FCCP and rotenone plus antimycin A (R+A) (left) or
132 glucose, oligomycin (Oligo) and 2-deoxy-d-glucose (2-DG) (right), assessed by a
133 mitochondrial stress test (left) or glycolysis stress test (right)(2).

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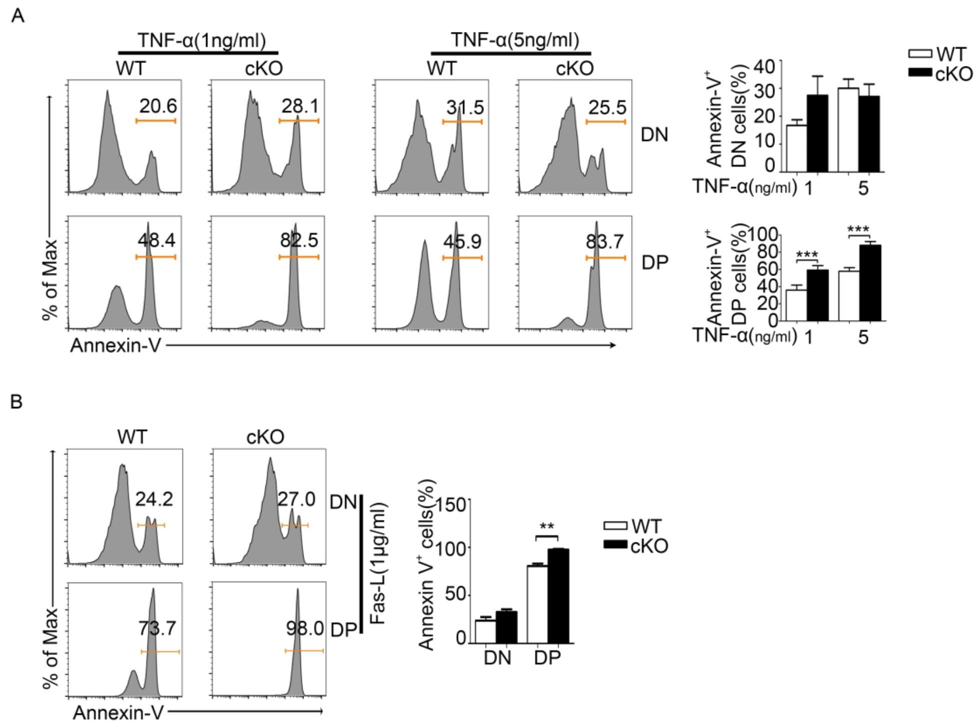

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138 **Fig. S5 Cell apoptosis is increased after stimulation of Fas-L and TNF-α**

139 **(A)** Annexin V staining of DN and DP cells left stimulated with different dose of
140 TNF-α for 20 h in vitro, and quantification of Annexin V^+ cells (right). $^{**}P < 0.01$ and
141 $^{***}P < 0.001$. **(B)** Annexin-V staining of DN and DP cells left stimulated with
142 Fas-L(1μg/ml) for 20h in vitro, and quantification of Annexin V^+ cells (right). Data
143 are representative of three experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

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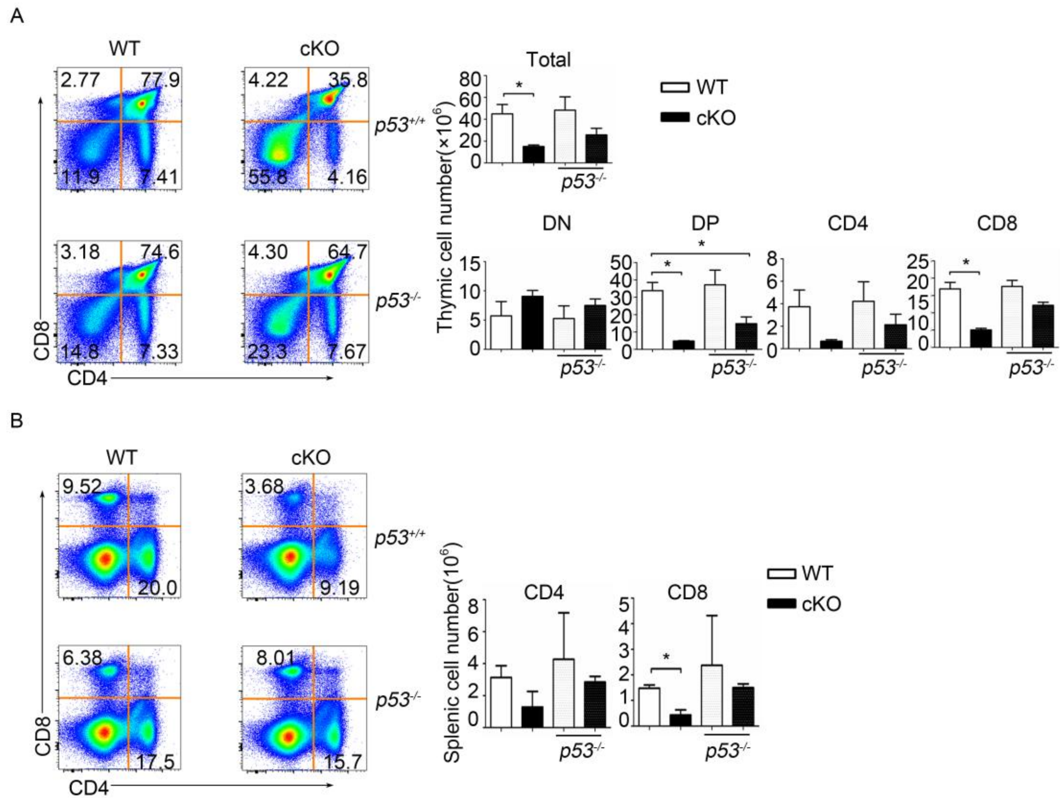

Fig. S6 Knockout of *p53* can partly rescue cell number

(A) Surface staining of CD4 and CD8 on WT and cKO on *p53*$^{-/-}$ background thymocytes (left). Numbers in or adjacent to outlined areas (or in quadrants) indicate percent cells in each throughout. Right, the frequency and cell number of total, DN, DP thymocyte subpopulations. **(B)** Surface staining of CD4 and CD8 on WT and cKO on *p53*$^{-/-}$ background splenocytes (left), and cell number of splenocyte subpopulations (right). Data are representative of three experiments. *$P < 0.05$.

166 **References:**

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