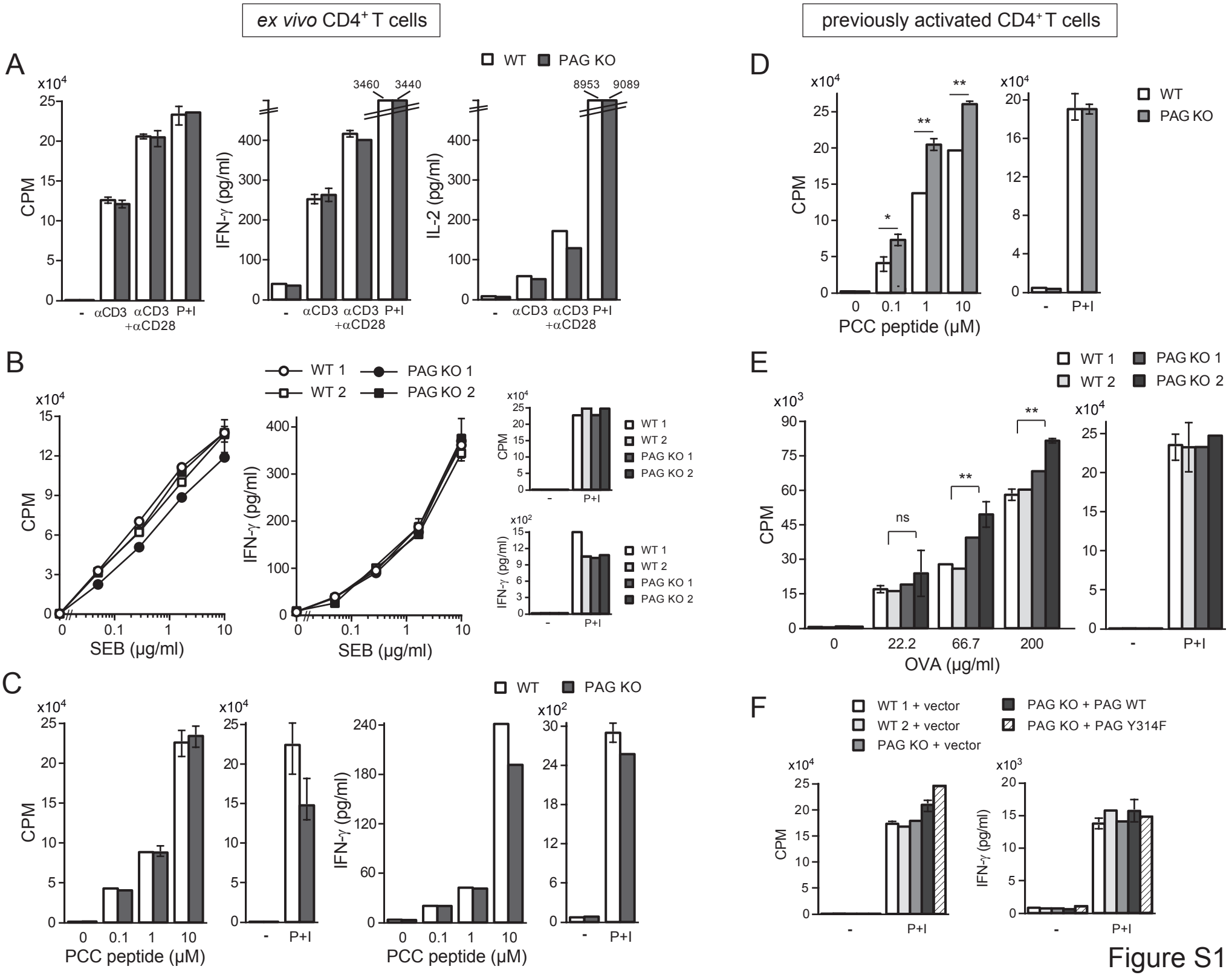

ex vivo CD4+ T cells
previously activated CD4+ T cells
A
WT
PAG KO
CPM
IFN-γ (pg/ml)
IL-2 (pg/ml)
3460
3440
8953
9089
-
αCD3
αCD3 +αCD28
P+I
B
WT 1
WT 2
PAG KO 1
PAG KO 2
SEB (μg/ml)
C
PCC peptide (μM)
D
*
**
E
ns
OVA (μg/ml)
F
WT 1 + vector
WT 2 + vector
PAG KO + vector
PAG KO + PAG WT
PAG KO + PAG Y314F

Figure S1

Supplemental Figure 1. Related to Figure 1. Impact of PAG deficiency in T cells.

(A,B). Freshly isolated ("*ex vivo*") $CD4^+$ T cells from WT or PAG KO mice were stimulated with anti-CD3 (3 μg/ml), anti-CD3 (3 μg/ml) plus anti-CD28 (1 μg/ml) or with P+I (A). Alternatively, they were stimulated with the indicated concentrations of SEB plus WT splenocytes, or P+I (B). Thymidine incorporation and release of IFN-γ and IL-2 were assessed, as detailed for Figure 1B. (C). Purified $CD4^+$ T cells from TCR AND transgenic mice were activated with the indicated concentrations of PCC peptide and WT splenocytes, or with P+I. Proliferation and production of IFN-γ were monitored. (D). As in Figure 1D, except that thymidine incorporation was monitored. (E). As in Figure 1E, except that thymidine incorporation was monitored. (F). Same as Figure 1G, except that cells were simulated with P+I. *$p<0.05$; **$p<0.01$; ns: not significant. Representative of n = 6 (A), n = 4 (B), n = 4 (C), n = 4 (D), n = 3 (E) and n = 2 (F).

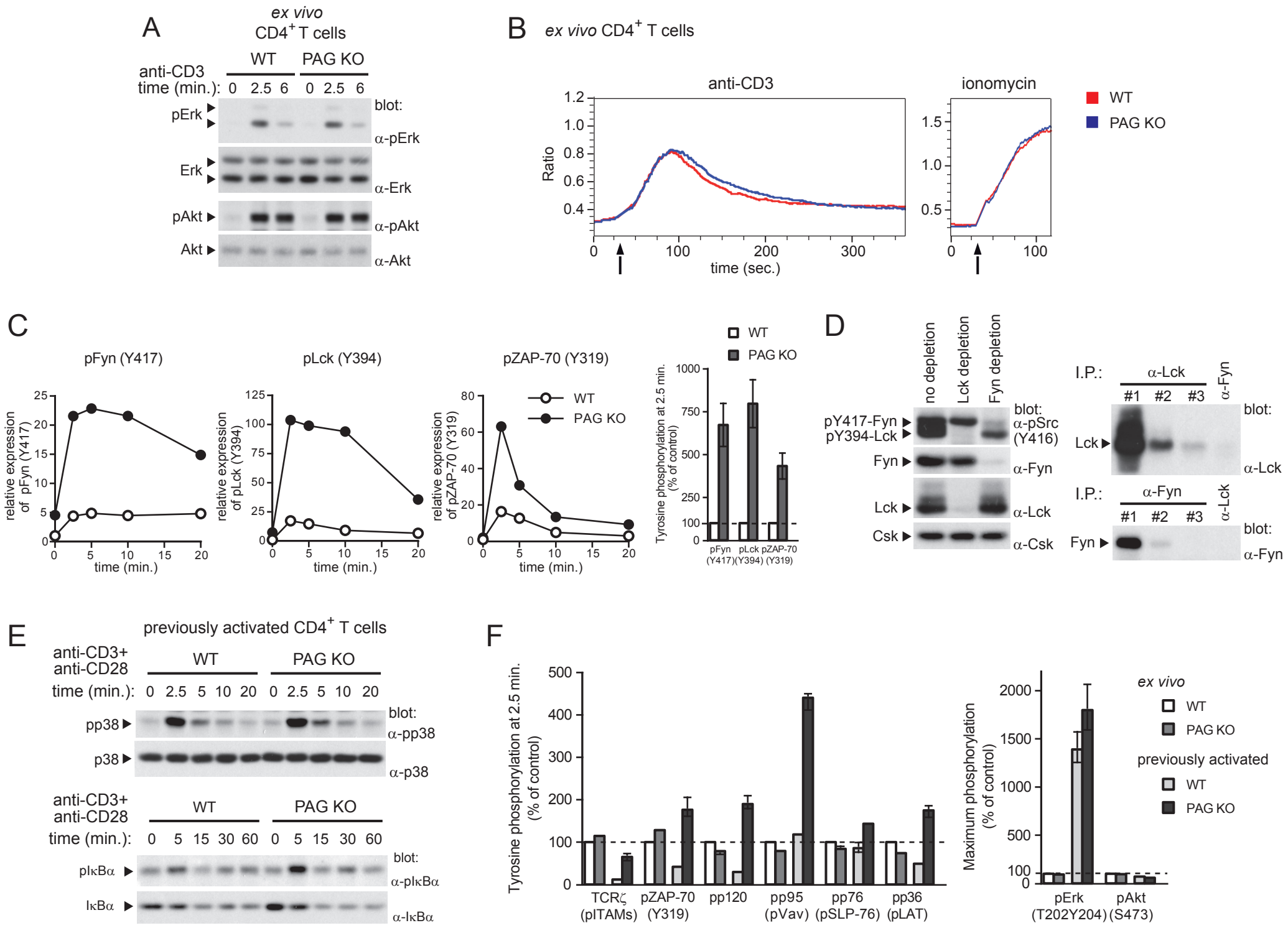

A
ex vivo
CD4+ T cells
anti-CD3
time (min.):
WT
PAG KO
0 2.5 6 0 2.5 6
pErk
Erk
pAkt
Akt
blot:
α-pErk
α-Erk
α-pAkt
α-Akt
B
ex vivo CD4+ T cells
anti-CD3
ionomycin
Ratio
time (sec.)
WT
PAG KO
C
pFyn (Y417)
pLck (Y394)
pZAP-70 (Y319)
relative expression of pFyn (Y417)
relative expression of pLck (Y394)
relative expression of pZAP-70 (Y319)
time (min.)
WT
PAG KO
Tyrosine phosphorylation at 2.5 min. (% of control)
pFyn (Y417)
pLck (Y394)
pZAP-70 (Y319)
D
no depletion
Lck depletion
Fyn depletion
pY417-Fyn
pY394-Lck
Fyn
Lck
Csk
blot:
α-pSrc (Y416)
α-Fyn
α-Lck
α-Csk
I.P.:
α-Lck
α-Fyn
#1 #2 #3
Lck
Fyn
blot:
α-Lck
α-Fyn
E
previously activated CD4+ T cells
anti-CD3+ anti-CD28
WT
PAG KO
time (min.): 0 2.5 5 10 20 0 2.5 5 10 20
pp38
p38
blot:
α-pp38
α-p38
time (min.): 0 5 15 30 60 0 5 15 30 60
pIκBα
IκBα
α-pIκBα
α-IκBα
F
Tyrosine phosphorylation at 2.5 min. (% of control)
TCRζ (pITAMs)
pZAP-70 (Y319)
pp120
pp95 (pVav)
pp76 (pSLP-76)
pp36 (pLAT)
Maximum phosphorylation (% of control)
pErk (T202Y204)
pAkt (S473)
ex vivo
WT
PAG KO
previously activated
WT
PAG KO

Figure S2

Supplemental Figure 2. Related to Figure 2. Influence of PAG deficiency on TCR signaling. (A,B). As in Figure 2D,C, respectively, except that *ex vivo* $CD4^+$ T cells were used. (C). Quantification of data from Figure 2B. Levels of phosphorylation of the indicated proteins in unstimulated previously activated WT T cells were considered as 1.0 (left). Cumulative measurements are shown on the right; extent of phosphorylation 2.5 minutes after stimulation of previously activated WT T cells was taken as 100%. Mean values with SD are shown. (D). To demonstrate that the 59 kDa- and 56 kDa-tyrosine phosphorylated proteins recognized by the anti-pSrc (pY416) antibodies were Fyn and Lck, respectively, PAG KO T cells were stimulated with anti-CD3. After lysis, either Fyn or Lck was depleted from cell lysates by three rounds of sequential immunoprecipitation. Lysates, immunodepleted or not, were then probed by immunoblotting with anti-pSrc (pY416) antibodies (left). The amounts of Fyn and Lck recovered in the sequential immunoprecipitations are shown on the right. (E). Same as Figure 2D, except that cell lysates were probed by immunoblotting with antibodies recognizing phospho-p38 or phospho-IκBα. (F) Quantification of data depicted in Figure 2A,B,D. Extent of phosphorylation 2.5 minutes after stimulation (for protein tyrosine phosphorylation) or at the time of maximal phosphorylation (for pErk and pAkt) of *ex vivo* T cells from WT mice was considered as 100%. Mean values with SD are shown. Representative of n = 3 (A), n = 3 (B), n = 1 (D) and n = 2 (E).

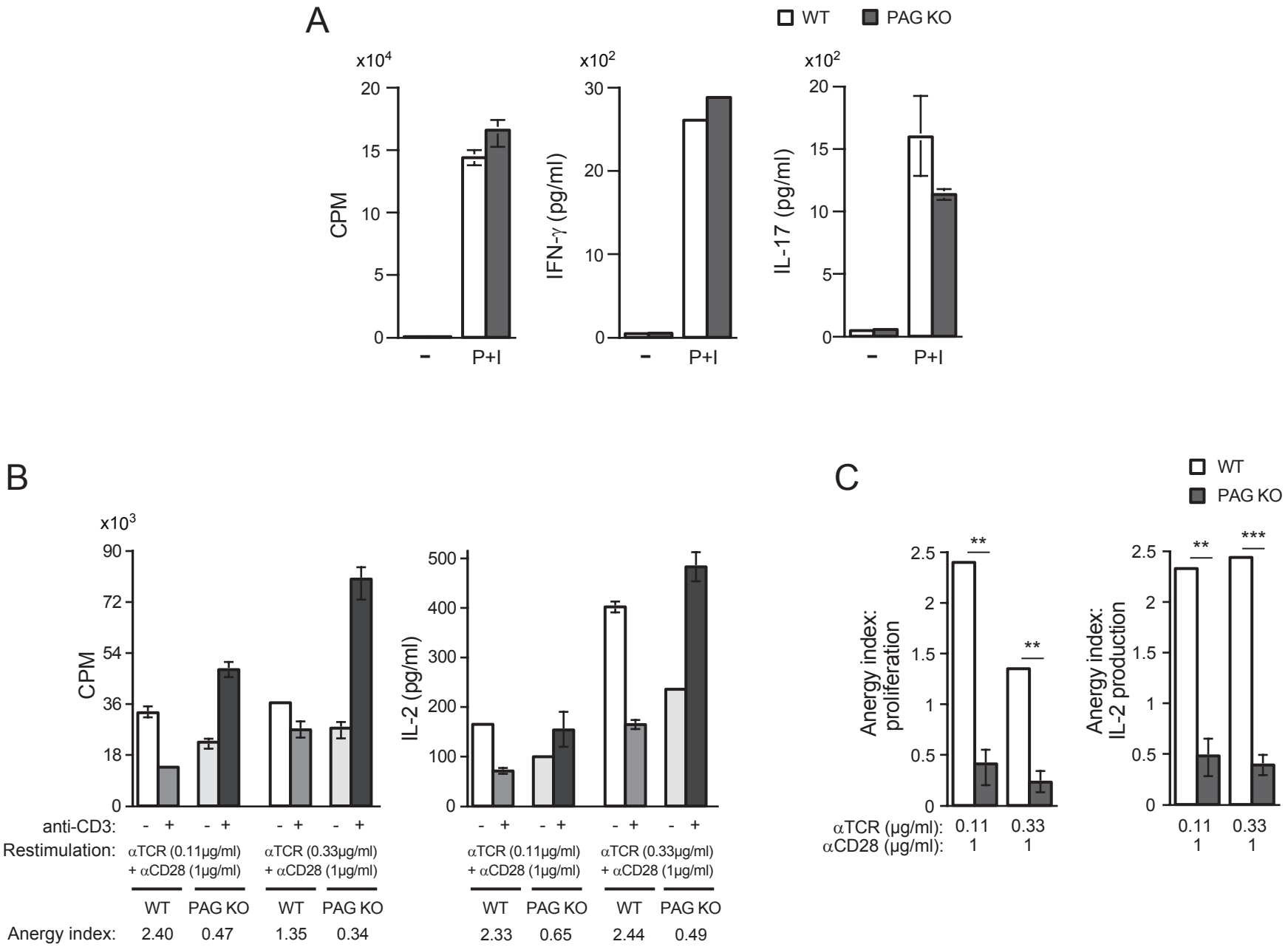
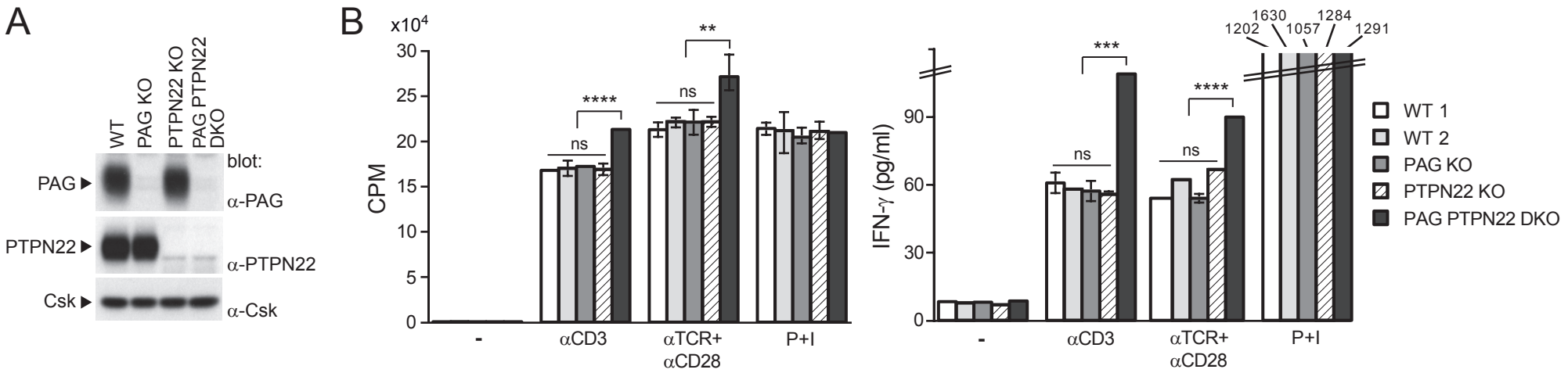
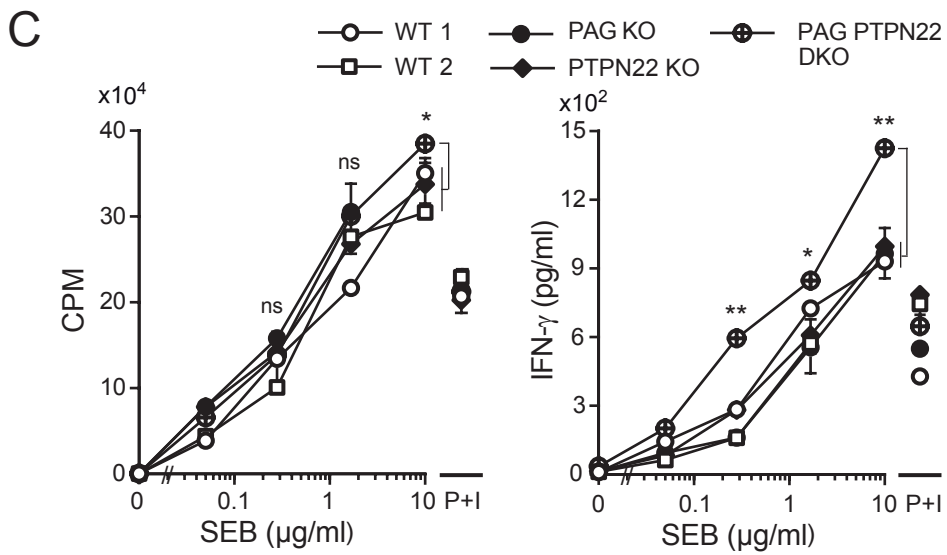

A
WT
PAG KO
x10^4
CPM
20
15
10
5
0
-
P+I
x10^2
IFN-γ (pg/ml)
30
20
10
0
-
P+I
x10^2
IL-17 (pg/ml)
20
15
10
5
0
-
P+I
B
x10^3
CPM
90
72
54
36
18
0
anti-CD3: - + - + - + - +
Restimulation: αTCR (0.11μg/ml) + αCD28 (1μg/ml)
αTCR (0.33μg/ml) + αCD28 (1μg/ml)
WT PAG KO WT PAG KO
Anergy index: 2.40 0.47 1.35 0.34
IL-2 (pg/ml)
500
400
300
200
100
0
- + - + - + - +
αTCR (0.11μg/ml) + αCD28 (1μg/ml)
αTCR (0.33μg/ml) + αCD28 (1μg/ml)
WT PAG KO WT PAG KO
2.33 0.65 2.44 0.49
C
WT
PAG KO
Anergy index: proliferation
2.5
2
1.5
1
0.5
0
**
**
αTCR (μg/ml): 0.11 0.33
αCD28 (μg/ml): 1 1
Anergy index: IL-2 production
2.5
2
1.5
1
0.5
0
**
***
0.11 0.33
1 1

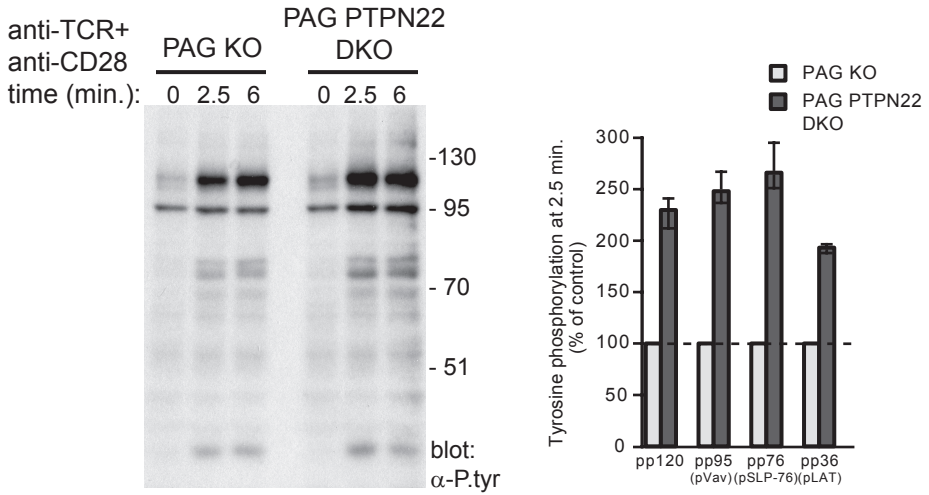
Figure S3

Supplemental Figure 3. Related to Figure 3. Impact of PAG deficiency on EAE and anergy. (A). Same as in Figure 3B, except that cells were re-stimulated with P+I. (B,C). $CD4^+$ T cells were activated with anti-CD3 plus anti-CD28, and subsequently expanded in medium containing IL-2. They were then treated or not with anti-CD3 (1 μg/ml) to trigger anergy, and re-stimulated with anti-TCR and anti-CD28. Thymidine incorporation and IL-2 secretion were assessed. The anergy index is the ratio of thymidine incorporation or IL-2 secretion between untreated and anti-CD3-treated cells. Means with SD of triplicates are shown (B). Anergy indices are represented graphically (C). Representative of n = 2 (A), n = 4 (B,C).

ex vivo CD4$^+$ T cells







previously activated CD4$^+$ T cells

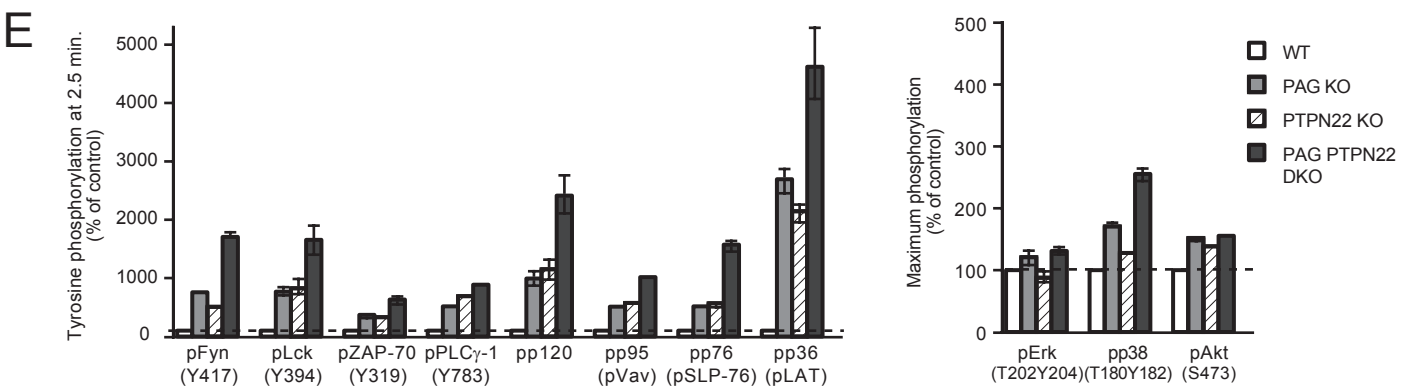


Figure S4

Supplemental Figure 4. Related to Figure 5. Impact of PAG and PTPN22 on T cell responses. (A). Expression of PTPN22 and PAG in purified $CD4^+$ T cells from WT, PAG KO, PTPN22 KO or PAG PTPN22 DKO mice was probed by immunoblotting of total cell lysates. (B-D). Same as Figure 5A-C, respectively, except that purified *ex vivo* $CD4^+$ T cells were used for stimulation either with anti-CD3 (3 μg/ml), anti-TCR (3 μg/ml) plus anti-CD28 (1 μg/ml) (B) or with SEB (C). Quantification of the data depicted in panel D (left) is shown on the right. Extent of phosphorylation 2.5 minutes after stimulation of *ex vivo* PAG KO T cells was considered as 100%. Mean values with SD are shown (D). (E). Quantification of the data depicted in Figure 5C,E. Extent of phosphorylation 2.5 minutes after stimulation (for protein tyrosine phosphorylation) or at the time of maximal phosphorylation (for pErk, pp38 and pAkt) in previously activated T cells from WT mice was considered as 100%. Mean values with SD are shown. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns: not significant. Representative of n > 4 (A), n = 3 (B), n = 2 (C) and n = 2 (D).

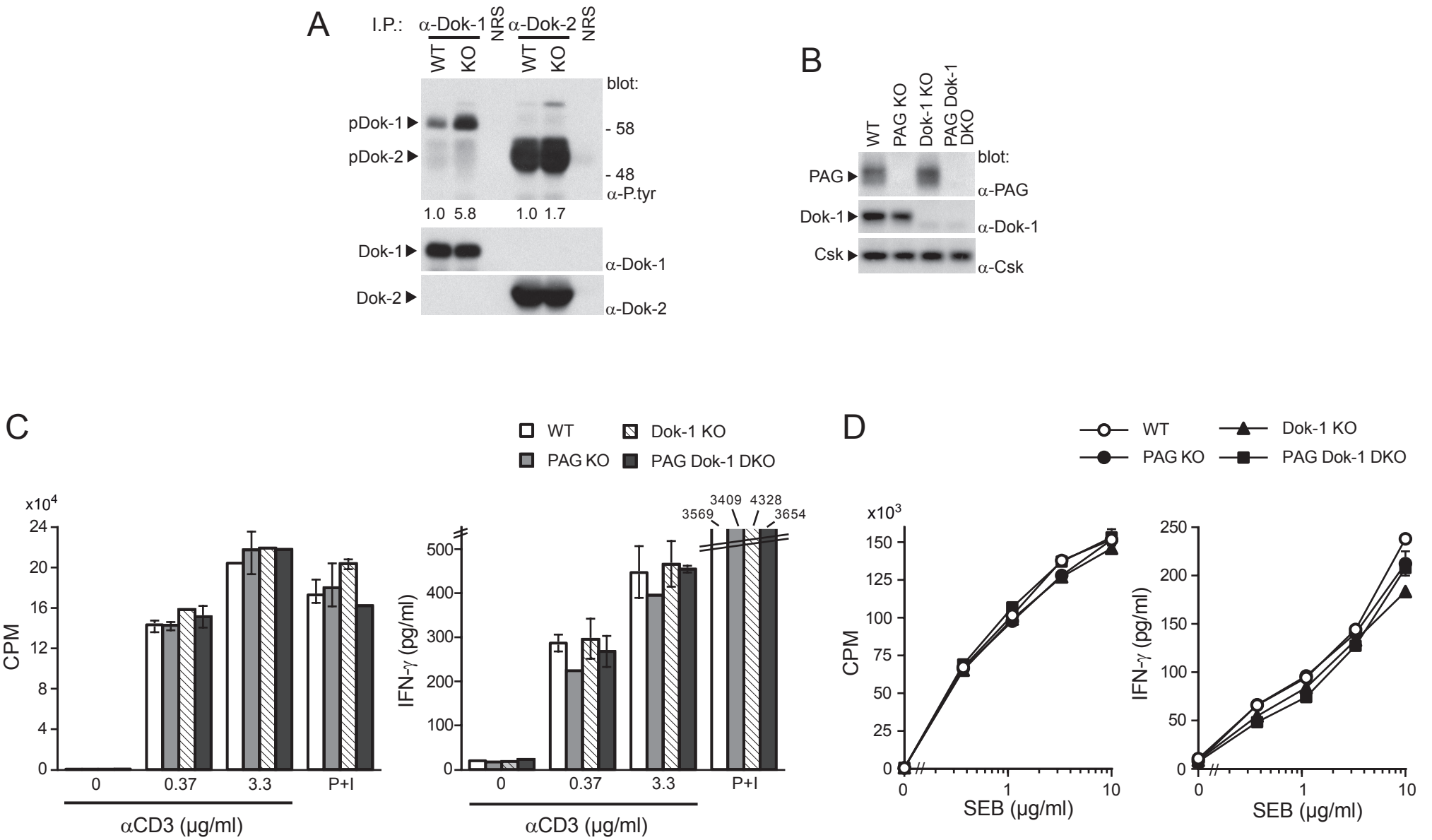

A
I.P.: α-Dok-1 NRS α-Dok-2 NRS
WT KO WT KO
blot:
pDok-1
pDok-2
- 58
- 48
α-P.tyr
1.0 5.8 1.0 1.7
Dok-1
α-Dok-1
Dok-2
α-Dok-2
B
WT PAG KO Dok-1 KO PAG Dok-1 DKO
blot:
PAG
α-PAG
Dok-1
α-Dok-1
Csk
α-Csk
C
WT Dok-1 KO
PAG KO PAG Dok-1 DKO
x10^4
CPM
αCD3 (μg/ml)
IFN-γ (pg/ml)
3569 3409 4328 3654
P+I
D
WT Dok-1 KO
PAG KO PAG Dok-1 DKO
x10^3
CPM
SEB (μg/ml)
IFN-γ (pg/ml)

Figure S5

Supplemental Figure 5. Related to Figure 6. Effect of combined loss of Dok-1 and PAG. (A). Same as Figure 6B, except that immunoprecipitates were probed by anti-P.tyr immunoblotting. Quantification of extent of phosphorylation of Dok-1 and Dok-2 is shown below the top panel. (B). Same as Figure 6D, except that expression of PAG and Dok-1 was analyzed by immunoblotting of total cell lysates. (C,D). Same as Figure 6D, except that freshly isolated T cells were used. Cells were activated with anti-CD3 or P+I (C), or SEB (D). Representative of n = 4 (A), n = 4 (B), n = 4 (C) and n = 2 (D).

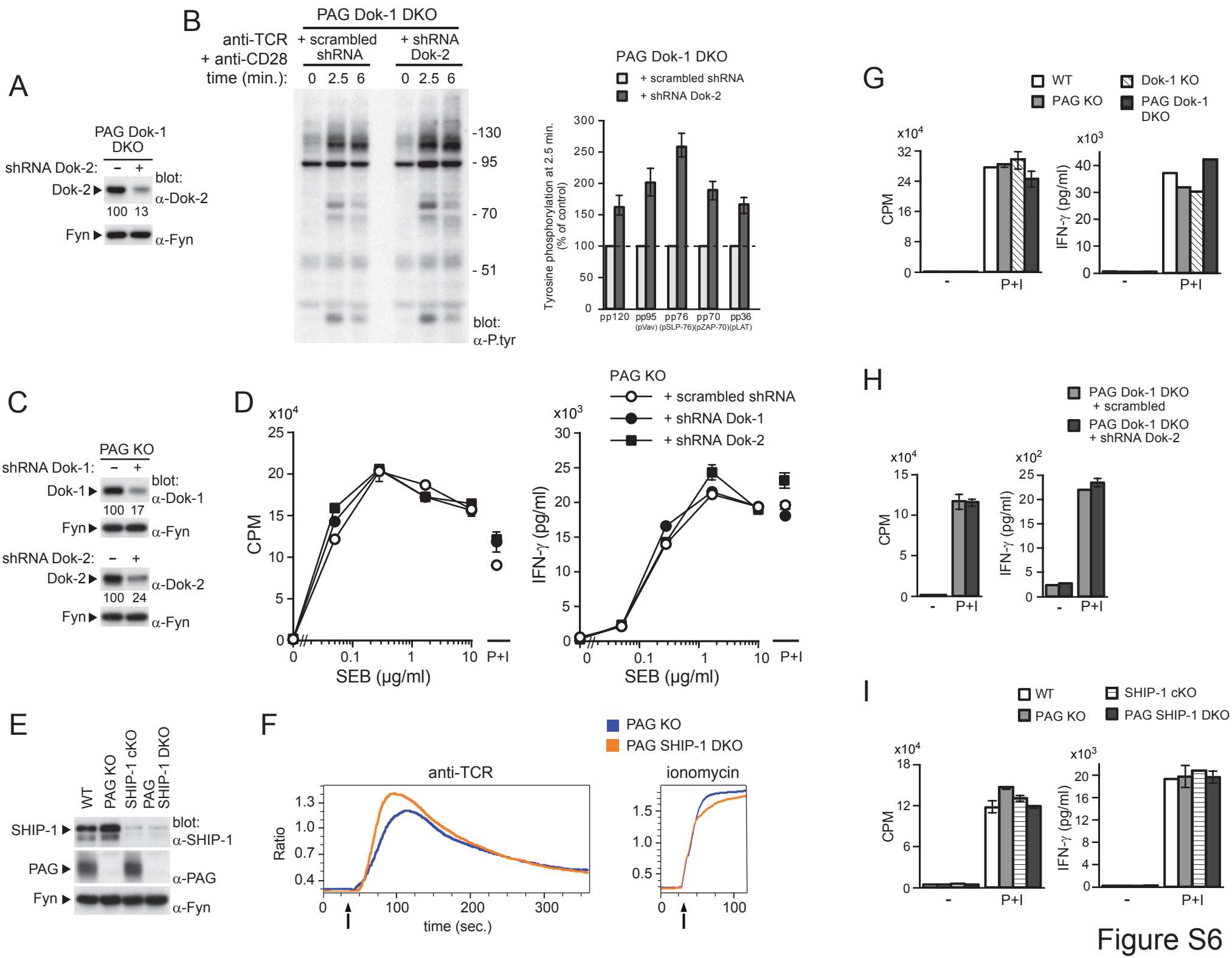

A
PAG Dok-1 DKO
shRNA Dok-2: – +
Dok-2 ▶
100 13
blot:
α-Dok-2
Fyn ▶
α-Fyn
B
PAG Dok-1 DKO
anti-TCR + anti-CD28
time (min.):
+ scrambled shRNA
+ shRNA Dok-2
0 2.5 6 0 2.5 6
-130
- 95
- 70
- 51
blot:
α-P.tyr
PAG Dok-1 DKO
+ scrambled shRNA
+ shRNA Dok-2
Tyrosine phosphorylation at 2.5 min. (% of control)
pp120 pp95 pp76 pp70 pp36
(pVav) (pSLP-76)(pZAP-70)(pLAT)
C
PAG KO
shRNA Dok-1: – +
Dok-1 ▶
100 17
blot:
α-Dok-1
Fyn ▶
α-Fyn
shRNA Dok-2: – +
Dok-2 ▶
100 24
α-Dok-2
Fyn ▶
α-Fyn
D
PAG KO
+ scrambled shRNA
+ shRNA Dok-1
+ shRNA Dok-2
CPM
IFN-γ (pg/ml)
SEB (μg/ml)
P+I
E
WT
PAG KO
SHIP-1 cKO
PAG SHIP-1 DKO
SHIP-1 ▶
PAG ▶
Fyn ▶
blot:
α-SHIP-1
α-PAG
α-Fyn
F
PAG KO
PAG SHIP-1 DKO
anti-TCR
ionomycin
Ratio
time (sec.)
G
WT
PAG KO
Dok-1 KO
PAG Dok-1 DKO
CPM
IFN-γ (pg/ml)
P+I
H
PAG Dok-1 DKO + scrambled
PAG Dok-1 DKO + shRNA Dok-2
CPM
IFN-γ (pg/ml)
P+I
I
WT
PAG KO
SHIP-1 cKO
PAG SHIP-1 DKO
CPM
IFN-γ (pg/ml)
P+I

Figure S6

Supplemental Figure 6. Related to Figure 6. Impact of combined loss of Dok adaptors and PAG, or SHIP-1 and PAG. (A). Expression of Dok-2 in PAG Dok-1 DKO T cells transduced with scrambled (-) or Dok-2-specific (+) shRNAs was analyzed by immunoblotting. Quantification of protein expression is shown below the panel. (B). Same as (A), except that cells were stimulated with anti-TCR plus anti-CD28, and protein tyrosine phosphorylation was analyzed (left). Quantification of the data depicted is shown on the right. Extent of phosphorylation 2.5 minutes after stimulation of previously activated PAG Dok-1 DKO T cells transduced with scrambled shRNAs was considered as 100%. Mean values with SD are shown. (C,D). PAG KO T cells were transduced with scrambled (-), Dok-1- or Dok-2-specific (+) shRNAs. Expression of Dok adaptors was analyzed by immunoblotting. Quantification of protein expression is shown below each panel (C). Cells were also stimulated with SEB and irradiated splenocytes, or P+I, and thymidine incorporation and IFN-γ secretion were analyzed, as detailed for Figure 6D (D). (E). Same as Figure 6F, except that expression of PAG and SHIP-1 was analyzed by immunoblotting of total cell lysates. Since the SHIP-1 KO is the result of breeding of a floxed SHIP-1 allele with *Cd4-Cre*, a small amount of residual SHIP-1 is present, due to incomplete deletion of the allele. (F). Same as Figure 6F, except that cells were stimulated with anti-TCR or ionomycin, and calcium fluxes were analyzed, as detailed for Figure 2C. (G-I). Same as Figure 6D-F, except that cells were stimulated with P+I. Representative of n = 3 (A), n = 2 (B), n = 4 (C), n = 4 (D), n = 2 (E), n = 2 (F) and n = 2-3 (G-I).

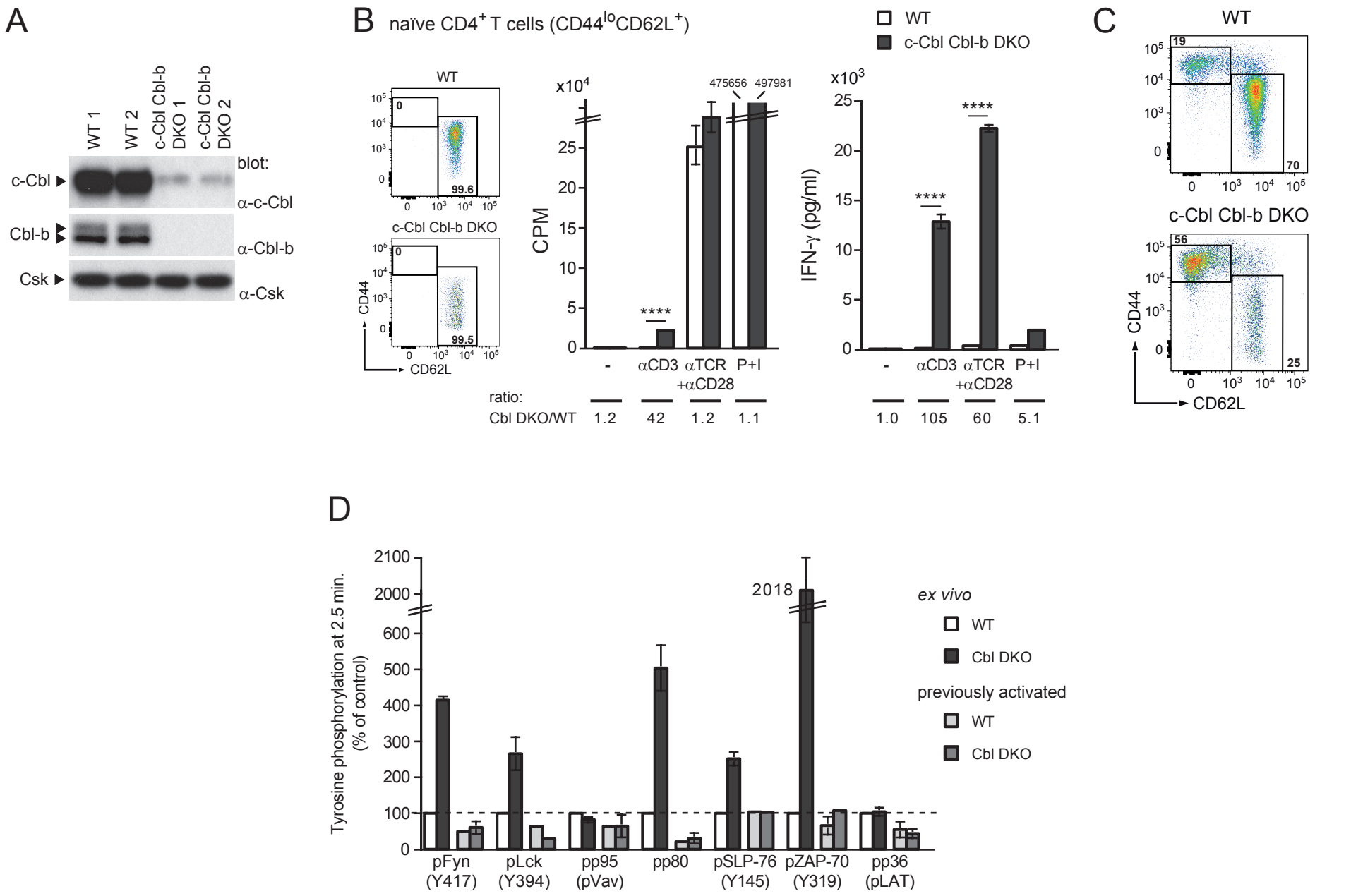

A
WT 1
WT 2
c-Cbl Cbl-b DKO 1
c-Cbl Cbl-b DKO 2
blot:
c-Cbl
α-c-Cbl
Cbl-b
α-Cbl-b
Csk
α-Csk
B naïve CD4+ T cells (CD44loCD62L+)
WT
c-Cbl Cbl-b DKO
CD44
CD62L
99.6
99.5
x10^4
CPM
475656
497981
****
-
αCD3
αTCR +αCD28
P+I
ratio:
Cbl DKO/WT
1.2
42
1.2
1.1
x10^3
IFN-γ (pg/ml)
1.0
105
60
5.1
C
WT
c-Cbl Cbl-b DKO
19
70
56
25
CD44
CD62L
D
Tyrosine phosphorylation at 2.5 min. (% of control)
2018
ex vivo
WT
Cbl DKO
previously activated
WT
Cbl DKO
pFyn (Y417)
pLck (Y394)
pp95 (pVav)
pp80
pSLP-76 (Y145)
pZAP-70 (Y319)
pp36 (pLAT)

Figure S7

Supplemental Figure 7. Related to Figure 7. Impact of Cbl proteins on T cell responses. (A). Same as Figure 7A, except that expression of c-Cbl and Cbl-b was analyzed by immunoblotting of total cell lysates. Since the c-Cbl KO is the result of breeding of a floxed c-Cbl allele with *Cd4-Cre*, a small amount of residual c-Cbl is present, due to incomplete deletion of the allele. Two mice of each genotype were analyzed. (B). Naive $CD4^+$ T cells ($CD44^{lo}CD62L^+$) were purified by cell sorting from WT or c-Cbl Cbl-b DKO mice. Purity was assessed by flow cytometry (left). Cells were then stimulated with anti-CD3 (1µg/ml), anti-TCR (1µg/ml) plus anti-CD28 (1µg/ml), or P+I (right). Thymidine incorporation and IFN-γ secretion were assessed (right). (C). Freshly isolated $CD4^+$ T cells from the indicated mice were stained with anti-CD44 and anti-CD62L antibodies, to identify naive ($CD44^{lo}CD62L^+$) and effector-memory ($CD44^{hi}CD62L^-$) cells. Cells were then analyzed by flow cytometry. (D). Quantification of the data depicted in Figure 7C,D. Extent of phosphorylation 2.5 minutes after stimulation of *ex vivo* T cells from WT mice was considered as 100%. Mean values with SD are shown. ****$p<0.0001$. Representative of n = 2 (A), n = 2 (B) and n = 8 (C).

A Mice aged 1.5 - 3 months:

	WT (n=4)	PAG KO (n=3)	p value	
Thymus				
$CD4^+CD8^-$	7.43 ± 1.50	6.95 ± 1.20	0.6712	ns
$CD4^-CD8^+$	2.69 ± 0.61	3.07 ± 0.62	0.4412	ns
$CD4^+CD8^+$	88.24 ± 2.08	87.15 ± 1.80	0.5006	ns
$CD4^-CD8^-$	2.12 ± 0.19	2.30 ± 0.29	0.3280	ns
Spleen				
$CD4^+CD8^-$	16.87 ± 2.33	17.71 ± 1.98	0.6359	ns
$CD4^-CD8^+$	9.89 ± 0.40	9.88 ± 1.08	0.9823	ns
$CD4^-CD8^-$	73.11 ± 2.17	72.54 ± 3.01	0.7794	ns
$CD4^+CD44^{lo}CD62L^+$	72.60 ± 2.05	74.55 ± 0.66	0.1264	ns
$CD4^+CD44^{hi}CD62L^-$	12.40 ± 1.04	11.30 ± 0.26	0.1503	ns
$CD4^+PD1^+CXCR5^+$	4.93 ± 0.74	3.60 ± 0.38	0.0506	ns
$CD4^+FoxP3^+$	13.57 ± 0.57	12.58 ± 1.79	0.4066	ns
$CD4^+V\beta8.1/8.2$	22.07 ± 0.67	21.25 ± 1.32	0.3774	ns

B Mice aged 9 - 13 months:

	WT (n=6)	PAG KO (n=9)	p value	
Spleen				
$CD4^+CD44^{lo}CD62L^+$	52.77 ± 2.13	59.32 ± 3.12	0.1450	ns
$CD4^+CD44^{hi}CD62L^-$	33.04 ± 2.32	27.85 ± 1.53	0.1051	ns
$CD19^+GL7^+$	1.14 ± 0.056	1.40 ± 0.14	0.0633	ns

Table S1

Supplemental Table 1. Related to Figure 1. Flow cytometry analyses of various immune cell populations in thymus and spleen of WT and PAG KO mice, aged 1.5-3 months (A) or 9-13 months (B). Means (expressed as percentages) with SD and *p* values are indicated. ns: not significant.

A Basal immunoglobulin levels (mice aged 1.5 - 3 months):

	WT (n=12)	PAG KO (n=12)	p VALUE	
IgG1 (µg/ml)	411 ± 40	476 ± 61	0.3711	ns
IgG2b (µg/ml)	366 ± 66	363 ± 49	0.9709	ns
IgG2c (µg/ml)	40 ± 15	53 ± 15	0.5689	ns
IgG3 (µg/ml)	128 ± 31	108 ± 20	0.5913	ns
IgM (µg/ml)	158 ± 22	156 ± 26	0.9417	ns
IgE (ng/ml)	1317 ± 40	1319 ± 86	0.9807	ns

B Basal immunoglobulin levels (mice aged 9 - 13 months):

	WT (n=5)	PAG KO (n=8)	p VALUE	
IgG1 (µg/ml)	894 ± 167	734 ± 145	0.4957	ns
IgG2b (µg/ml)	492 ± 106	480 ± 81	0.9313	ns
IgG2c (µg/ml)	231 ± 36	197 ± 17	0.3471	ns
IgG3 (µg/ml)	368 ± 99	337 ± 67	0.7985	ns
IgM (µg/ml)	560 ± 38	526 ± 83	0.7567	ns
IgE (ng/ml)	2714 ± 410	2160 ± 247	0.2474	ns

Table S2

Supplemental Table 2. Related to Figure 1. Analysis of levels of basal immunoglobulins (Ig) in WT and PAG KO mice, aged 1.5-3 months (A) or 9-13 months (B). Data were obtained by ELISA. Means with SEM and *p* values are indicated. ns: not significant.

Supplemental experimental procedures

DNA constructs and retroviral infection. cDNAs encoding wild-type PAG or PAG Y314F were cloned in the retrovitral vector pMIGR1, which also contains the GFP-encoding gene. To down-regulate Dok-1 or Dok-2 expression using shRNA, we utilized the retroviral vector pSUPER.retro.neo+gfp (OligoEngine, Seattle, WA) containing the sequences 5'GATCCCCGTGCATTCCAAGAAACCTCTTCAAGAGAGAGGTTTCTTGGAATGCACTTTTTAAGCTTAAAAAGTGCATTCCAAGAAACCTCTCTCTTGAAGAGGTTTCTTGGAATGCACGGG 3' (for Dok-1) or 5'GATCCCCAACACGACACGGCAATGAGTTCAAGAGACTCATTGCCGTGTCGTGTTTTTTTAAGCTTAAAAAAACACGACACGGCAATGAGTCTCTTGAACTCATTGCCGTGTCGTGTTGGG 3' (for Dok-2). A vector containing the random sequence 5'GATCCCCGCGCGCTTTGTAGGATTCGTTCAAGAGACGAATCCTACAAAGCGCGCTTTTTAAGCTTAAAAAGCGGCCTTTGTAGGATTCGTCTCTTGAACGAATCCTACAAAGCGCGCGGG 3' was used as non-specific control. In all cases, retroviral infection was performed using previously activated T cells, as outlined elsewhere (Davidson et al., 2010).

Antibodies. For flow cytometry, the following antibodies were used: anti-CD4 MAb GK1.5, anti-CD4 MAb RM4-5, anti-CD8 MAb 53-6.7, anti-CD3 MAb 145-2C11, anti-TCRβ H57.197, anti-TCRVβ8.1/8.2 MAb MR5-2, anti-TCRVα11.1/11.2 MAb RR8-1, anti-TCRVα2 MAb B20.1, anti-CD44 MAb 1M7, anti-CD62L MAb MEL-14, anti-FoxP3 FJK-16a, anti-CXCR5 2G8, anti-CD279 PD-1 J43, anti-CD19 1D3, anti-B220 MAb RA3-6B2, anti-T and B cell activation antigen MAb GL7, anti-CD45 LCA-Ly5 30F11, anti-CD45RA 14.8 and anti-CD45RO PTPRC. They were purchased from BD Biosciences (Mississauga, Ontario, Canada), eBioscience/Affymetrix (San Diego, CA) or Thermo Fisher Scientific. For immunoprecipitation

and immunoblot, we used rabbit antibodies against PAG, Csk, PTPN22, ZAP-70, Fyn, Lck, Dok-1, Dok-2, c-Cbl, SLP-76, CD45, SHIP-1, SHP-1 and phosphotyrosine generated in our laboratory (Davidson et al., 2003; Davidson et al., 2007; Davidson et al., 2010). Antibodies recognizing Erk (catalog no. 9102), Akt (catalog no. 9272), p38 (catalog no. 9212), JNK (catalog no. 9252), PKC-θ (catalog no. sc-212), IκBα (catalog no. sc-371), Cbl-b (MAb D3C12), PLC-γ1 (catalog no. sc-81), Ras-GAP (catalog no. sc-63), β-actin (catalog no. sc-47778) as well as phospho-specific antibodies recognizing Src (pY416; also recognizing Lck Y394 and Fyn Y417; catalog no. 2101), pLck (pY505; catalog no. 2751), ZAP-70 (pY319; catalog no. 2701), Erk (pT202pY204; MAb E10), Akt (pS473; MAb 193H12), p38 (pT180pY182; catalog no. 9211), JNK (pT183pY185; catalog no. 9251), PKC-θ (pT538; catalog no. 9277), IκBα (pS32pS36; MAb 5A5), PLC-γ1 (pY783; catalog no. 2821) and SLP-76 (Y145; catalog no. 14770) were obtained from Cell Signaling Technology (Danvers, MA) or Santa-Cruz Biotechnology Inc. (Santa Cruz, CA); phospho-specific anti-Dok-1/2 (pY361/351; catalog no. DP2241) was obtained from ECM Biosciences.

Calcium fluxes. T cells were loaded with Indo-1 (10 μM; Molecular Probes, Eugene, OR), as described (Davidson et al., 2007). They were then stimulated at 37^{o}C with biotinylated anti-TCR or anti-CD3 and avidin. Intracellular calcium fluxes were monitored using a BD LSR or Fortessa cell analyzer (BD Biosciences). As control, cells were stimulated with ionomycin (100 ng/ml).

Anergy. For anergy induced *in vitro* by anti-CD3, CD4^{+} T cells were activated for 48 hrs with anti-CD3 and anti-CD28. They were then propagated in IL-2 for 3 days. Anergy was subsequently induced by treating cells with anti-CD3 alone (1 μg/ml) for 16 hrs. Controls were with no addition. After resting the cells in growth medium for 30 hrs, they were re-stimulated with anti-TCR and anti-CD28 for 48 hrs. Thymidine incorporation and IL-2 secretion were

measured. To induce anergy *in vivo*, OT-II mice were injected I.V. with OVA peptide [500 μg in phosphate-buffered saline (PBS)] or PBS alone on day 0 and day 3. On day 10, $CD4^+$ $CD44^{hi}$ $V\alpha2^+$ T cells were isolated by cell sorting, and anergy was measured by stimulating cells with OVA peptide and irradiated WT splenocytes. Proliferation and IL-2 secretion were measured. Anergy index was calculated as described elsewhere (Davidson et al., 2007).

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