Supporting Online Material

Supporting materials and methods.

Targeting PEP Exon 8 or Exon 1 to generate *pep*$^{-/-}$ mice. Disruption of the *pep* locus was accomplished through two independent strategies. The first deleted Exon 8 that encompasses the phosphatase domain including the active D195 and C225 residues within the catalytic pocket (fig. S1A). The second strategy altered the translational initiation methionine within Exon 1. *pep*$^{+/-}$ ES cells were generated for both strategies and injected into B6 blastocysts to yield chimeric mice that were crossed with wild type B6 mice to generate *pep*$^{+/-}$ germline mice. Southern blot analysis revealed successful homologous recombination of the targeting construct (*1*) and immunoblot analysis using two independent polyclonal antisera directed to the C-terminus of PEP (aa 588 to 654 and aa 670 to 740, respectively) revealed the lack of wild type or truncated PEP proteins in both *pep(E8)*$^{-/-}$ and *pep(E1)*$^{-/-}$ thymocytes and splenocytes (fig. S1B). Both *pep(E8)*$^{-/-}$ and *pep(E1)*$^{-/-}$ strategies gave rise to developmentally normal viable mice under specific pathogen free (SPF) conditions with normal Mendelian inheritance. No differences were observed between *pep(E8)*$^{-/-}$ *or pep(E1)*$^{-/-}$ mice and hence mice derived by either strategy will hereon be referred to as *pep*$^{-/-}$ mice.

Antibodies.

Anti-PEP polyclonal Abs. Fusion protein for either GST-PEP proline 1 (aa 588-654) or GST-PEP proline 2 (aa 670-740) was produced in bacteria and used for rabbit immunizations. After 4 courses of immunizations, serum was collected and depleted of anti-GST Abs utilizing a GST column.

Anti-PTP-PEST monoclonal Abs. A GST-fusion protein encoding PTP-PEST (aa 600-733) was used to immunize hamsters, followed by selection to generate a panel of hybridomas for

PTP-PEST (clone 8F7. 10, 6G11.3 and 6H7.1). Anti-PTP-PEST polyclonal Ab was kindly provided by Dr. Hidetaka Yakura. All monoclonal and polyclonal Abs utilized demonstrated similar results described in this paper. Anti-Csk and anti-β actin Abs were purchased from Santa Cruz Biotechnologies and Abcam, respectively. Anti-394 phospho-Lck Ab was kindly provided by Dr. Andrey Shaw, anti-505 phospho Lck and anti-319 phospho-ZAP-70 Ab were purchased from Transduction Laboratories. Anti-ZAP-70 Ab was described previously (*2*).

Supporting figures.

Supplemental Fig. 1. Generation of *pep*$^{-/-}$ mice. **(A)** Targeting of PEP exon 8 (upper) or PEP exon1 (lower). The genomic structure surrounding exon 8 of PEP (top), targeting construct (middle) and the targeted allele (bottom) are depicted. Exon 8 includes amino acid 181 through 227 of the PEP coding region, which contains the aspartate (195) as well as cysteine (225) residue essential for the phosphatase activity of PEP. The replacement of this genomic region with a neomycin resistance cassette by homologous recombination converts the 3.5-kb WT into a 10-kb BamHI fragment as detected with the 3' probe. Targeting of PEP exon 1 (lower). The genomic structure surrounding exon 1 of PEP (top), targeting construct (middle) and the targeted allele (bottom) are depicted. The targeting construct contains a puromycin resistance cassette and a mutated Exon1 in which the Exon1 splicing acceptor site is deleted and the translation initiation site is mutated from ATGG to CTGC. The replacement of this genomic region with a puromycin resistance cassette by homologous recombination converts the 5.5-kb WT into a 12-kb NcoI fragment as detected with the 3' probe. **(B)** Absence of PEP protein expression in *pep*$^{-/-}$ mice. Cell lysates of thymus, spleen and bone marrow from *pep*$^{+/+}$, *pep*$^{+/-}$ and *pep*$^{-/-}$ mice of exon 8 (top) or exon 1 (second) targeted mice were immunoblotted with anti-PEP (top two panels),

anti-PTP-PEST (third panel), anti-Csk (fourth panel) and anti-β-actin (bottom) Abs. **(C)** Enhanced positive selection of H-Y TCR^+ T cells. Female WT or *pep*$^{-/-}$ H-Y TCR^+ thymocytes and peripheral T cells were stained for CD4 and CD8. **(D)** Enhanced positive selection of DO11.10 TCR^+ T cells. WT or *pep*$^{-/-}$ DO11.10 TCR^+ thymocytes and peripheral T cells were stained as described in (C). **(E)** Normal negative selection in *pep*$^{-/-}$ H-Y TCR^+ male mice. Thymic, splenic and lymph node H-Y TCR^+ cells from male WT or *pep*$^{-/-}$ H-Y TCR^+ mice were stained for CD4, CD8 and an anti-idiotype H-Y TCR mAb (left) and quantitated (right). Statistical analysis was performed by the two-tailed Student's *t* test (N= 7). **(F)** Normal *in vivo* deletion of $CD4^+CD8^+$ *pep*$^{-/-}$ thymocytes. 4-6 week old WT or *pep*$^{-/-}$ mice were injected with an anti-CD3ε mAb (2C11, 50 μg) or phosphate buffered saline (PBS). 48 h later, thymocytes were stained for CD4 and CD8 (top). Total $CD4^+CD8^+$ thymocyte cell number (x 10^6) was quantitated (bottom) (N=3).

Supplemental Fig. 2. T and B cell subsets in young *pep*$^{-/-}$ mice. Splenocytes **(A)**, lymph node **(B)** and bone marrow **(C)** cells were examined by FACS analysis for each of the indicated cell surface markers. All Abs were purchased from Becton-Dickinson (BD) except for the anti-mouse IgM Ab (Jackson ImmunoResearch Laboratories). **(D)** Increased $CD8^+$ effector and memory T cells in older *pep*$^{-/-}$ mice. The splenic $CD8^+CD44^{hi}CD62L^{lo\text{-}hi}$population was segmented by CD43 (1B11) staining for $CD8^+CD44^{hi}CD62L^{lo\text{-}hi}CD43^{hi}$ effector cells versus $CD8^+CD44^{hi}CD62L^{lo\text{-}hi}CD43^{low}$ memory cells (*3*).

Supplemental Fig. 3. TCR signaling in naïve and effector *pep*$^{-/-}$ T cells. **(A)** Enhanced cycling of $CD44^{lo}CD62L^{hi}$ T cells from either young or old mice at day 4. $CD44^{lo}CD62L^{hi}$ T cells from

either young (2 month) or old mice (12 month) were labeled with CFSE and stimulated with anti-CD3ε and CD28 Abs. 4 days following stimulation, cells were analyzed by FACS analysis. **(B)** Normal activation induced cell death of *pep*$^{-/-}$ T cells. CD44^{lo}CD62L^{hi} T cells from young mice were assessed for cell death using Trypan Blue inclusion. **(C)** CD44^{lo}CD62L^{hi} T cells were stimulated with anti-CD3ε and CD28 Abs for 12 h and supernatants were measured for cytokine production. Cytokine production was quantitated using a Luminex 100 instrument (Austin, TX) and Beadlyte mouse 10-plex kits (Upstate, Lake Placid, NY), allowing for the multiplexed detection of mouse IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ, and TNF in each sample. **(D)** Comparable phosphorylation of cellular proteins in *pep*$^{-/-}$ naïve T cells. CD44^{lo}CD62L^{hi} splenic T cells from WT or *pep*$^{-/-}$ mice were stimulated with anti-CD3ε/CD28 mAbs (2C11 and 37.51 respectively, purchased from BD) for the specified time periods. Cells were lysed and subjected to Western blot analysis as indicated on the right. **(E)** Enhanced phosphorylation of cellular proteins in *pep*$^{-/-}$ effector T cells. *In vitro* generated effector WT (lanes1-4) or *pep*$^{-/-}$ (lanes 5-8) cells from young mice were stimulated with an anti-CD3ε mAb for the indicated time periods. Cells were lysed and analyzed by Western blot analysis as indicated on the right (*2*). **(F)** Normal cycling of effector/memory cells in response to IL-15 or IL-2. CD44^{hi}CD62L^{lo} T cells were labeled with CFSE and cultured with either recombinant IL-15 or IL-2 (100 ng/ml, R&D). Cell division was monitored by FACS analysis at day 4 for either CD4^{+} (top) or CD8^{+} (bottom) cells.

Supplemental Fig. 4. BCR signaling in *pep*$^{-/-}$ B cells. **(A)** Normal BCR-activated tyrosine phosphorylation of *pep*$^{-/-}$ B cells. Splenic B cells from WT or *pep* $^{-/-}$ mice were purified by negative selection using anti-CD43 microbeads, then stimulated with the indicated concentration

of an anti-mouse IgM $F(ab')_2$ for 3 min (left) or for various time periods with an anti-mouse IgM $F(ab')_2$ (20 μg/ml, right). Cells were lysed and analyzed by immunoblotting with an anti-pY (4G10, Upstate), anti-PEP or anti-β-actin (Abcam) Abs. **(B)** Proliferation of *pep*$^{-/-}$ B cells. Splenic B cells isolated from WT or *pep*$^{-/-}$ mice were activated with an anti-mouse IgM $F(ab')_2$ fragment and proliferation was measured by [^{3}H]-thymidine incorporation during last 8 h of a 48-h culture. Stimulation with P (40 ng/ml) and I (0.8 μM) was performed in parallel. **(C)** Normal upregulation of CD86 and CD69 in *pep*$^{-/-}$ B cells. Splenocytes were stimulated *in vitro* with an anti-mouse IgM $F(ab')_2$ for 12 h and analyzed for surface expression of CD86 and CD69 in B220$^+$ cells.

Supporting table.

Supplemental table 1. Complete blood counts, lymphoid subsets and myeloid cell counts in WT or *pep*$^{-/-}$ mice. Complete peripheral blood counts, lymphoid subsets isolated from thymi or spleens of < 6 month (left) or > 6 month (right) old WT or *pep*$^{-/-}$ mice are shown. Statistical analysis was performed by the two-tailed Student's *t* test (N= 14 young and N=9 old littermates).

Supporting references and notes.

1. K. Hasegawa, A. C. Chan, data not shown.
2. A. C. Chan et al., *J Immunol* **152**, 4758 (1994).
3. L. E. Harrington, M. Galvan, L. G. Baum, J. D. Altman, R. Ahmed, *J Exp Med* **191**, 1241 (2000).

fig. S1

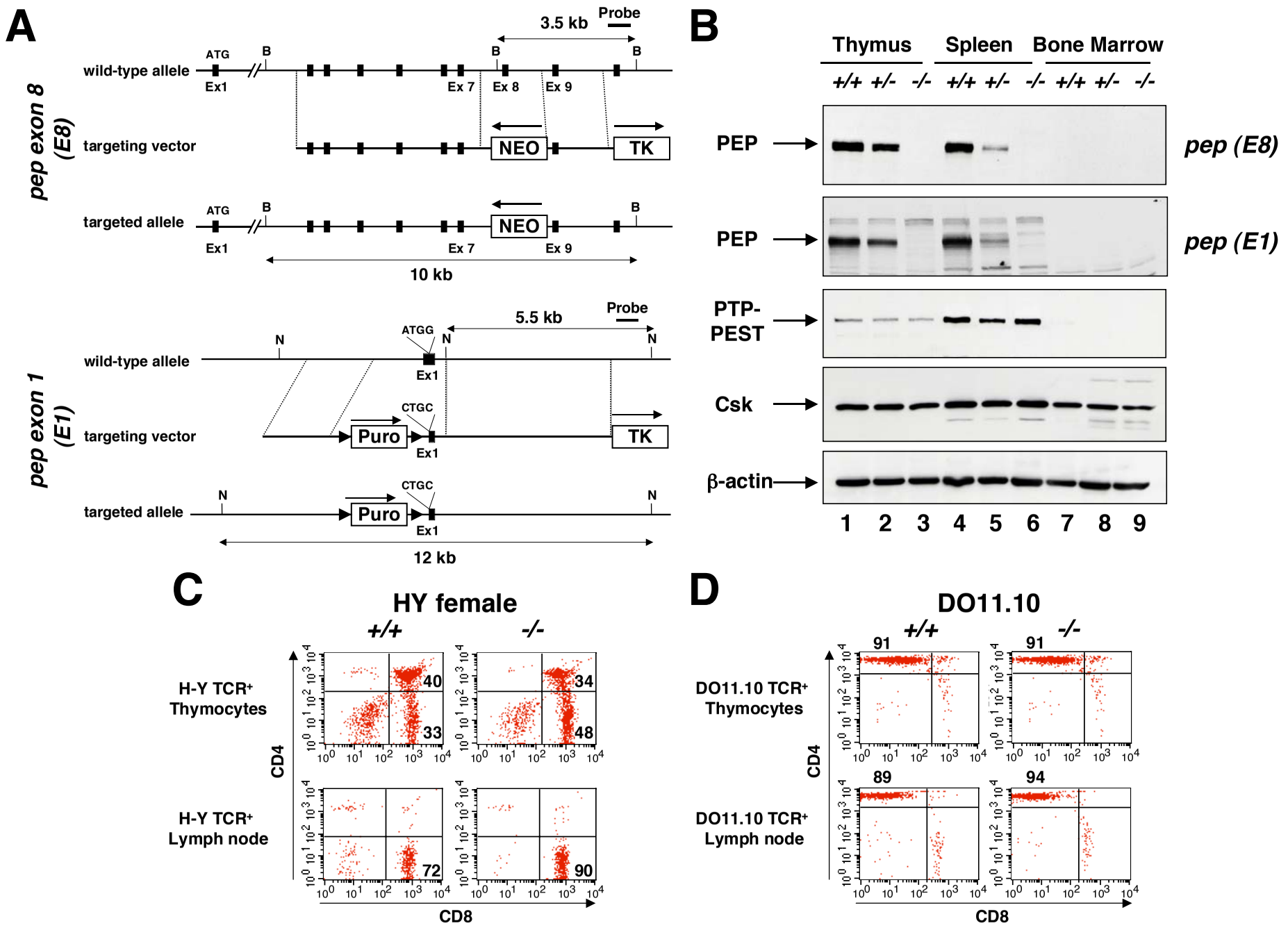
A
pep exon 8 (E8)
wild-type allele
targeting vector
targeted allele
ATG
B
Ex1
Ex 7
Ex 8
Ex 9
NEO
TK
3.5 kb
Probe
10 kb
pep exon 1 (E1)
N
ATGG
5.5 kb
CTGC
Puro
12 kb
B
Thymus
Spleen
Bone Marrow
+/+ +/- -/- +/+ +/- -/- +/+ +/- -/-
PEP
pep (E8)
pep (E1)
PTP-PEST
Csk
β-actin
1 2 3 4 5 6 7 8 9
C
HY female
+/+
-/-
H-Y TCR+ Thymocytes
H-Y TCR+ Lymph node
CD4
CD8
40
33
34
48
72
90
D
DO11.10
DO11.10 TCR+ Thymocytes
DO11.10 TCR+ Lymph node
91
91
89
94

fig. S1

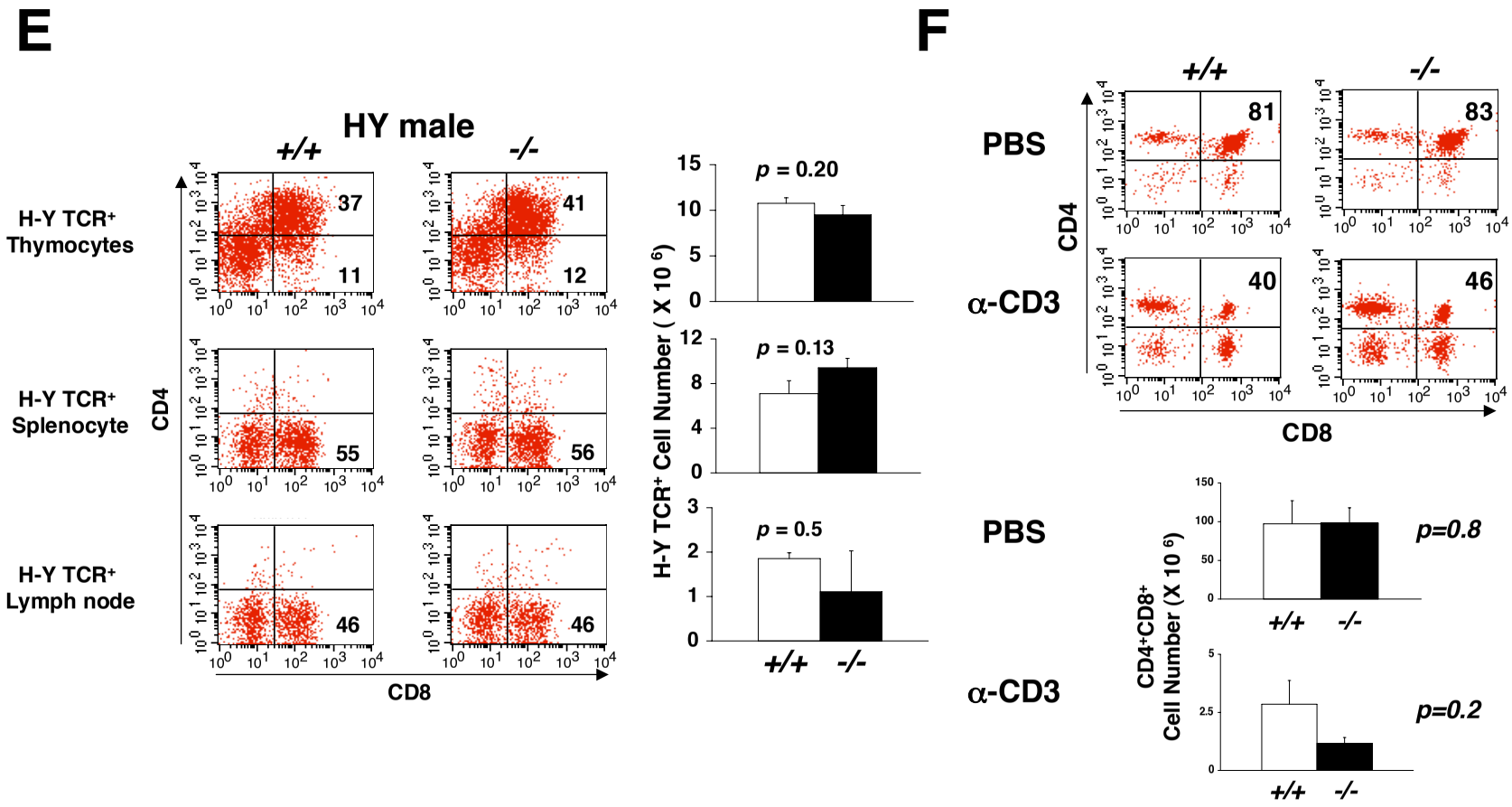
E
HY male
+/+
-/-
H-Y TCR+ Thymocytes
H-Y TCR+ Splenocyte
H-Y TCR+ Lymph node
CD4
CD8
37
11
41
12
55
56
46
46
H-Y TCR+ Cell Number (X 10 6)
p = 0.20
p = 0.13
p = 0.5
+/+
-/-
F
+/+
-/-
PBS
α-CD3
81
83
40
46
CD4
CD8
PBS
α-CD3
CD4+CD8+ Cell Number (X 10 6)
p=0.8
p=0.2
+/+
-/-

fig. S2

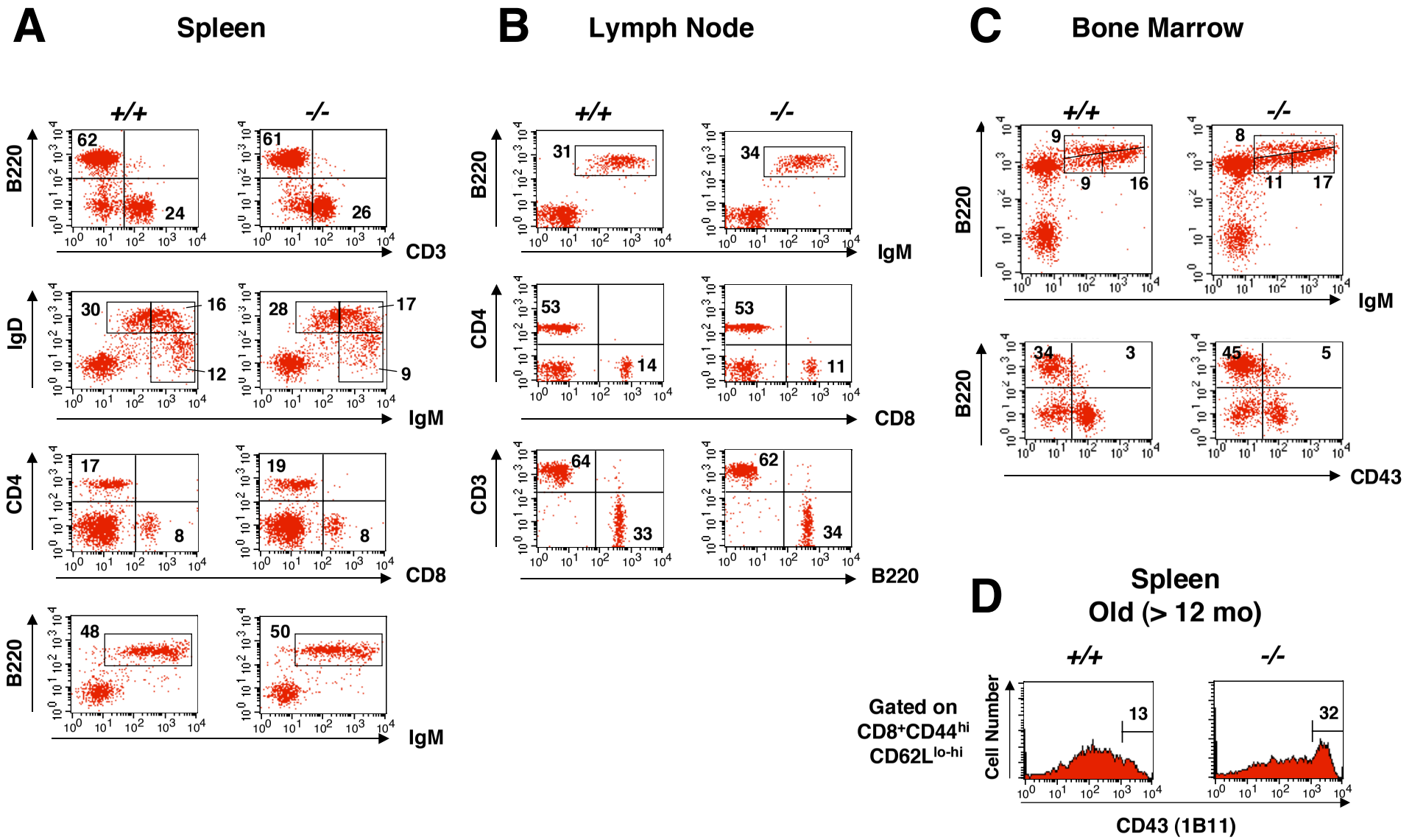
A
Spleen
+/+
-/-
B220
CD3
62
24
61
26
IgD
IgM
30
16
12
28
17
9
CD4
CD8
17
8
19
8
B220
IgM
48
50
B
Lymph Node
+/+
-/-
B220
IgM
31
34
CD4
CD8
53
14
53
11
CD3
B220
64
33
62
34
C
Bone Marrow
+/+
-/-
B220
IgM
9
9
16
8
11
17
B220
CD43
34
3
45
5
D
Spleen
Old (> 12 mo)
+/+
-/-
Gated on
CD8+CD44hi
CD62Llo-hi
Cell Number
13
32
CD43 (1B11)

fig. S3

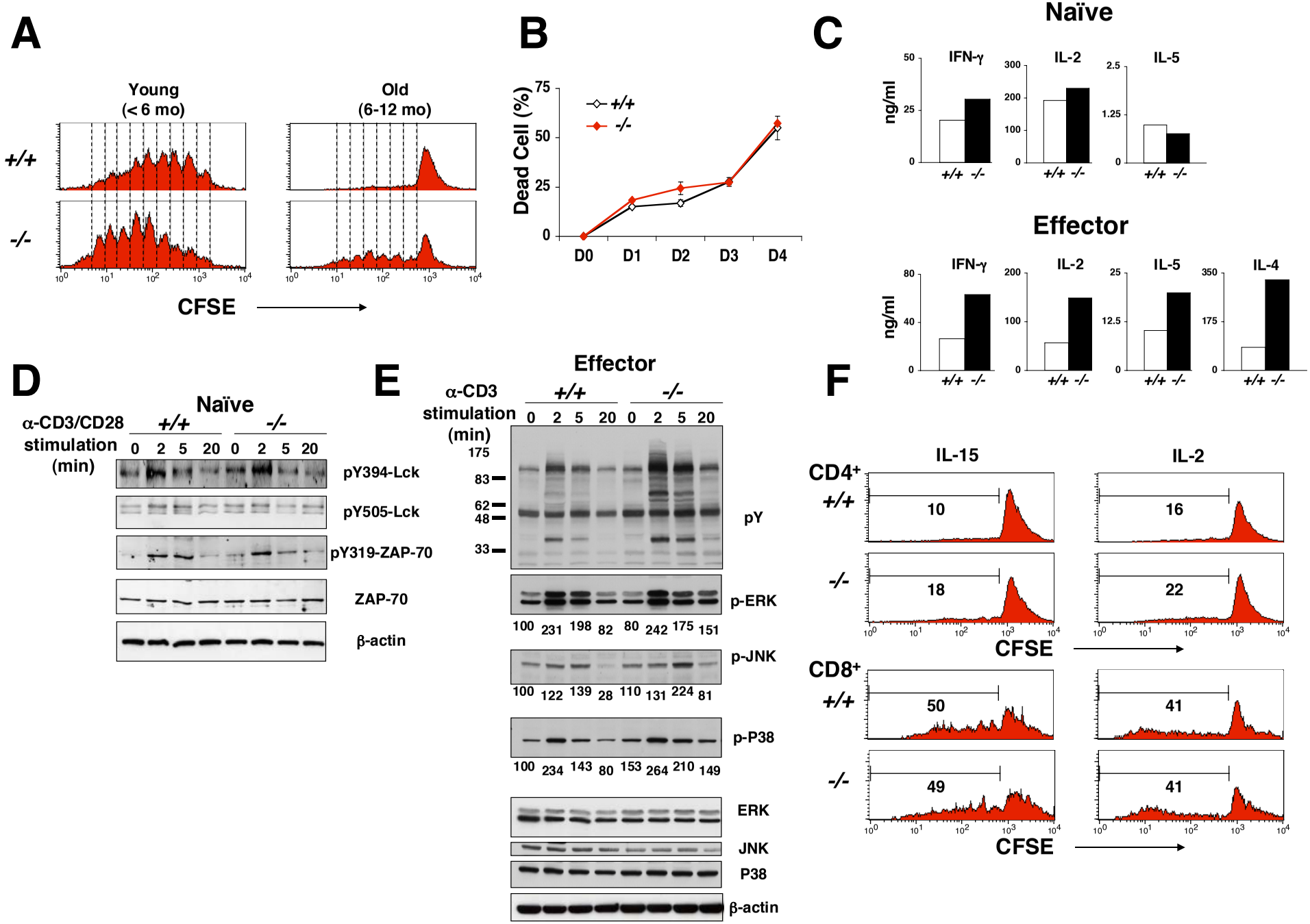
A
Young
(< 6 mo)
Old
(6-12 mo)
+/+
-/-
CFSE
B
Dead Cell (%)
+/+
-/-
D0
D1
D2
D3
D4
C
Naïve
IFN-γ
IL-2
IL-5
ng/ml
Effector
IL-4
D
Naïve
α-CD3/CD28
stimulation
(min)
pY394-Lck
pY505-Lck
pY319-ZAP-70
ZAP-70
β-actin
E
Effector
α-CD3
stimulation
(min)
pY
p-ERK
p-JNK
p-P38
ERK
JNK
P38
β-actin
F
IL-15
IL-2
CD4+
CD8+
CFSE

fig. S4

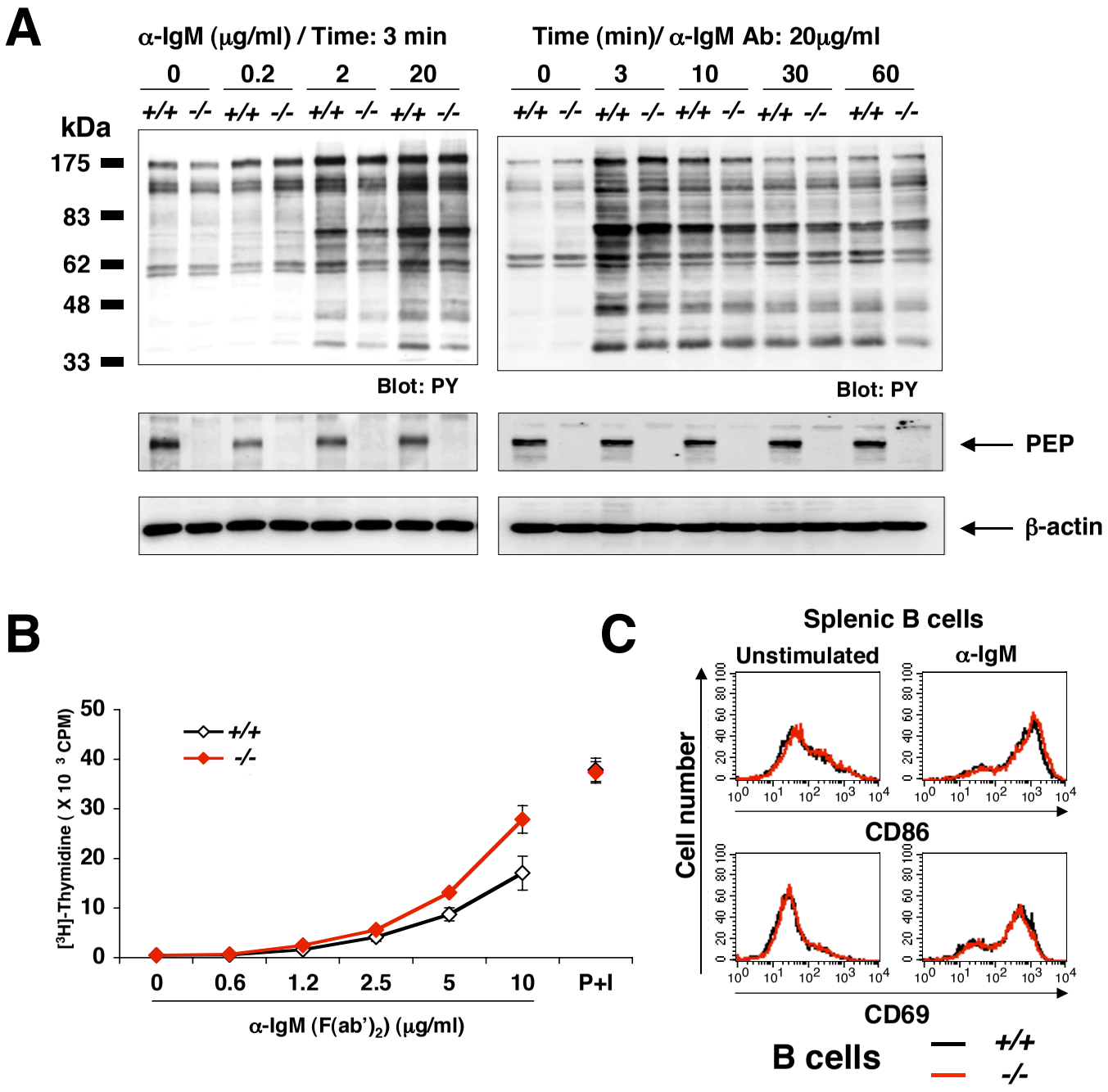
A
α-IgM (μg/ml) / Time: 3 min
0
0.2
2
20
+/+ -/- +/+ -/- +/+ -/- +/+ -/-
Time (min)/ α-IgM Ab: 20μg/ml
0
3
10
30
60
+/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/-
kDa
175
83
62
48
33
Blot: PY
Blot: PY
PEP
β-actin
B
[3H]-Thymidine (X 10 3 CPM)
50
40
30
20
10
0
+/+
-/-
0
0.6
1.2
2.5
5
10
P+I
α-IgM (F(ab')2) (μg/ml)
C
Splenic B cells
Unstimulated
α-IgM
Cell number
CD86
CD69
B cells
+/+
-/-

Table S1.

	Young (< 6 mo)			Old (> 6 mo)		
PBMC	***pep*$^{+/+}$**	***pep*$^{-/-}$**	**p value**	***pep*$^{+/+}$**	***pep*$^{-/-}$**	**p value**
Red blood cells ($10^6/\mu l$)	10.8 ± 0.3	10.4 ± 0.4	0.1180	9.7 ± 0.2	9.6 ± 0.2	0.8720
Platelets ($10^3/\mu l$)	586.4 ± 61	728.0 ± 149	0.3650	977.4 ± 81	932.0 ± 149	0.7060
White blood cells ($10^3/\mu l$)	7.2 ± 0.4	8.0 ± 1.0	0.5980	7.1 ± 1.9	5.3 ± 0.6	0.4020
Lymphocytes (%)	90.8 ± 2.2	90.0 ± 2.9	0.3440	88.6 ± 2.1	88.1 ± 1.3	0.8380
Monocytes (%)	1.2 ± 0.4	0.5 ± 0.5	0.3910	1.5 ± 0.6	1.0 ± 0.3	0.5010
Neutrophils (%)	7.4 ± 7.4	7.7 ± 1.9	0.2280	7.9 ± 1.5	9.1 ± 1.5	0.5880
Eosinophils (%)	0.6 ± 0.2	1.7 ± 1.4	0.5040	1.7 ± 0.3	1.7 ± 0.5	1.0000
Basophils (%)	0 ± 0	0 ± 0	ND	0.1 ± 0.1	0.1 ± 0.1	0.0890
Thymus: Cell no. ($X10^6$)						
Total	143.4 ± 14	167.0 ± 9	0.1424	49.0 ± 16	40.0 ± 8	0.4097
$CD4^-CD8^-$	3.4 ± 0.3	4.8 ± 0.6	0.0610	2.1 ± 0.9	2.1 ± 0.6	0.6543
$CD4^+CD8^+$	118.8 ± 14.2	135.7 ± 11.1	0.1694	43.1 ± 15.6	43.5 ± 16.5	0.2767
$CD4^+CD8^-$	14.9 ± 2.5	19.7 ± 2.7	0.0376*	5.6 ± 1.9	6.2 ± 2.3	0.9292
$CD4^-CD8^+$	5.6 ± 0.5	5.2 ± 1.1	0.5949	1.9 ± 0.7	1.9 ± 0.6	0.6172
Spleen: Cell no. ($X10^6$)						
Total	71.5 ± 5.2	94.9 ± 8.6	0.0003*	122.0 ± 12	189.0 ± 24	0.0001*
$CD4^+$	9.5 ± 0.8	14.1 ± 1.6	0.0260*	17.8 ± 1.2	33.1 ± 2.9	0.0009*
$CD8^+$	5.0 ± 0.4	6.4 ± 0.8	0.2469	11.0 ± 1.2	12.2 ± 2.9	0.0064*
$B220^+$	42.0 ± 3.8	52.8 ± 7.0	0.0541	60.7 ± 4.8	83.0 ± 9.4	0.0029*
$DX5^+$	3.1 ± 0.3	6.3 ± 0.6	0.0707	3.0 ± 0.4	4.2 ± 0.0	0.2375
$CD11b^+$	1.0 ± 0.02	1.9 ± 0.2	0.2092	1.6 ± 0.0	1.5 ± 0.0	0.2839