Supplemental Figure legends

Figure S1. Schematic of LoxP-targeted *Sap* allele and tamoxifen-induced SAP deletion. (A) Germline transmission of a novel *Sap* allele in which exon 1 of *Sap* is flanked by LoxP sites were obtained. Cre-mediated excision of exon 1, which contains the ATG start codon, leads to loss of functional SAP protein. The neomycin resistance cassette is flanked by FRT sites. To eliminate the neomycin cassette, $Sap^{fl/fl}$ mice were bred to transgenic animals expressing the FLP1 recombinase in all cells. (B) $Sap^{fl/fl}$ mice were bred to animals harboring an ubiquitin promoter-driven Cre selectively inducible by tamoxifen (ubiquitin-CRE-ERT2 from Gary Koretzky, University of Pennsylvania). Following tamoxifen administration, SAP expression is eliminated in multiple lineages, and further NKT cell development is impaired. After several days of rest to allow for degradation of residual SAP protein, SAP protein loss is evaluated by flow cytometry. In this system, treatment with tamoxifen leads to reproducible induction of YFP in 50-80 % of lymphoid cells and almost complete elimination of SAP.

Figure S2. SAP-deficient iNKTs produce cytokines, activate bystander cells and induce NK cells to produce IFNγ. (A-D) B6 and tamoxifen-treated $Sap^{fl/fl}$ Cre– or $Sap^{fl/fl}$ Cre+ mice were injected i.p. with 4μg of PBS57 or left untreated. After 2h, the percentages of splenic and hepatic iNKT cells producing IFNγ (A) and IL-4 (B) directly *ex vivo* were analyzed by intracellular cytokine staining and serum IFNγ (C) and IL-4 (D) levels measured by ELISA. Data represent the mean ± SEM from 4 experiments. (E-G) Mice were injected with PBS57 or left untreated. After 4 h, splenocytes were analyzed for the percentage of CD4+, CD8+, NK1.1+, and B220+ cells expressing CD69 (E). The percentage of NK1.1+TCRβ− cells (as gated in the *contour plots*) in the spleen (F) and liver (G) producing IFNγ directly *ex vivo* was determined by intracellular cytokine staining. This experiment was repeated 3 times with a total of 6 mice of each genotype analyzed per condition.

Figure S3. Basal expression of death receptors and lytic molecules are not altered in SAP-deficient iNKT cells. (A) Thymocytes and (B) intrahepatic lymphocytes from *Sap*$^{fl/fl}$Cre– and *Sap*$^{fl/fl}$Cre+ mice (gated on PBS57-CD1d tetramer+TCRβ+ iNKT cells) were analyzed for surface expression of Fas ligand (FasL) and TRAIL. Cells were also fixed, permeabilized and stained for intracellular levels of perforin and granzyme. Representative flow cytometric data from 3 independent experiments are shown. (C) B6 splenocytes were left untreated or cultured overnight on plate-bound anti-CD3 antibody (5μg/ml). After 18h, cells were stained with TCRβ and FasL and analyzed by flow cytometry. Representative histograms (gated on TCRβ+ cells) from 2 independent experiments are shown.

Figure S4. iNKT cells do not express the inhibitory SAP family protein EAT-2 and ERT. (A, B) Total RNA isolated from flow-sorted iNKTs was analyzed for *Eat-2* (A) and *Ert* (B) expression by real-time quantitative PCR. The samples were normalized to GAPDH. The relative expression of *Eat-2* and *Ert* was compared to the expression of these genes in thymic iNKT cells from *Sap*$^{fl/fl}$ Cre− mice. IL-2 activated purified NK cells served as positive control (+ve C). (C) Expression of Eat-2/ErT, SAP or Fyn in purified iNKTs was determined by immunoblotting. β-Actin served as positive control. Data are from 1 of 2 experiments.

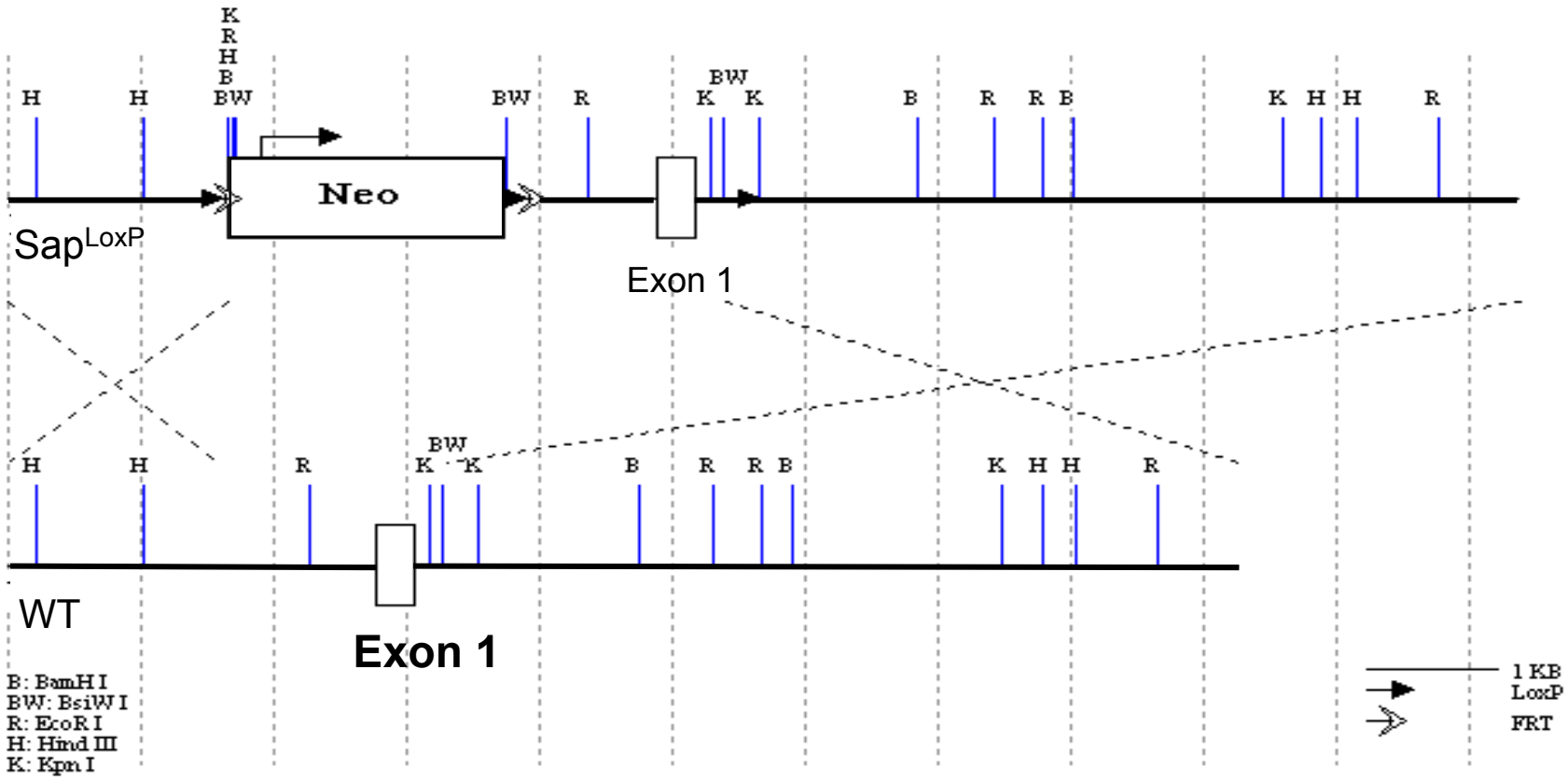
Figure S5. Fyn and SAP-Fyn interaction are dispensable for iNKT cell cytotoxicity. EL4 or A20CD1d cells were pulsed with 100 ng/ml of PBS44 or left untreated. (A, B) Cytolysis of EL4s (A) or A20-CD1d (B) cells by hepatic iNKTs from B6 or Fyn −/− mice. Representative data from 1 of 5 experiments is shown. (C, D) Cytolysis of EL4s (C) or A20-CD1d (D) cells by hepatic iNKTs from B6 or *Sap*^{R78A} mice. Representative data from 1 of 2 experiments is shown.

Figure S6. Human iNKTs isolated from αGC-expanded PBMCs are CD1d-tetramer reactive cells. (A) Representative flow cytometric plots showing percentage of NKT cells in freshly isolated PBMCs of a healthy donor. (B, C) PBMCs were cultured in the presence of αGC and cytokines as described. After 8 days, expansion of iNKT cells was determined by flow cytometry using monoclonal antibodies against human Vα24, Vβ11, and CD3 as well as PBS57 loaded-hCD1d tetramer (B). iNKT cells were MACS-sorted using FITC conjugated Vα24 antibody and anti-FITC magnetic beads (C). Purity of iNKT cells was confirmed by flow cytometry. Data are representative of at least 12 donors.

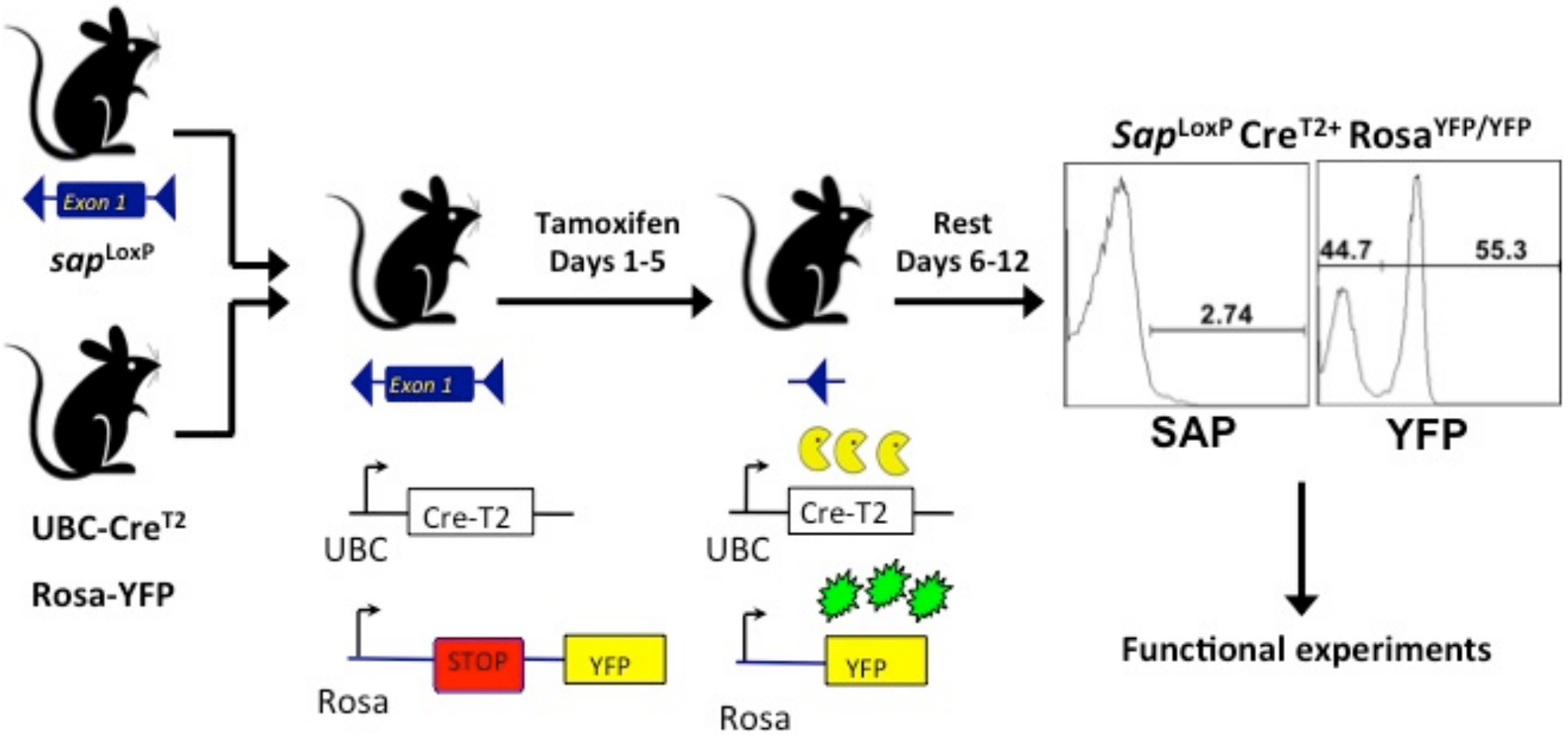
Figure S7. Murine and human iNKTs form conjugates with antigen-pulsed targets. Conjugates of BRSE-labeled B6 (A) or human iNKTs (B) and CFSE-labeled PBS44-loaded or unloaded EL4 cells at different time points. The number in the upper right quadrant represents the percentage of iNKTs in conjugate in relation to the total cell population (iNKT cells plus EL4 cells). Representative flow cytometry plots from 1 of 3 experiments are shown.

Figure S8. SAP-deficient iNKT cells produce less cytokines in response to EL4 targets *in vitro*. iNKT cells from tamoxifen-treated $SAP^{fl/fl}$ Cre– or $SAP^{fl/fl}$ Cre+ mice were co-cultured with PBS44-loaded or unloaded targets (EL4 cells or purified CD11c+ splenic dendritic cells [DC]). iNKTs and targets were mixed at a 40:1 ratio and incubated overnight at 37°C. After 16h, culture supernatants were harvested and analyzed for IFN-γ (A) and IL4 (B) by ELISA. Data represent the mean ± SEM from 3 independent experiments. Statistical significance was determined by unpaired two-tail t-test. * $p<0.05$ ns: not significant.

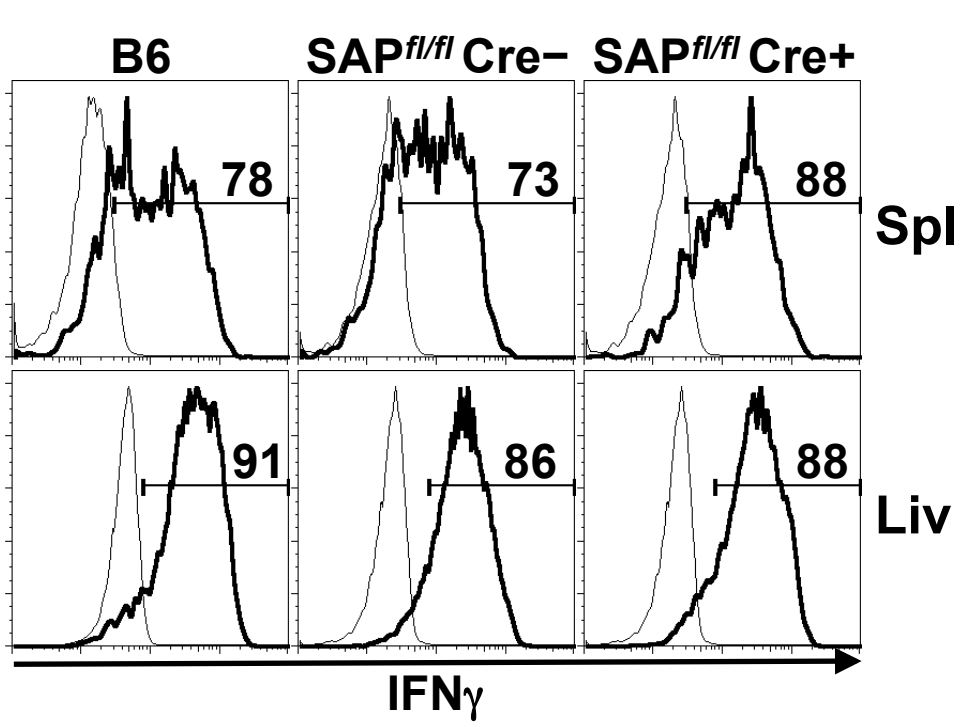
A


K
R
H
B
BW
H
H
BW
BW
R
BW
K
K
B
R
R
B
K
H
H
R
Neo
Sap^LoxP
Exon 1
H
H
R
BW
K
K
B
R
R
B
K
H
H
R
WT
Exon 1
B: BamH I
BW: BsiW I
R: EcoR I
H: Hind III
K: Kpn I
1 KB
LoxP
FRT

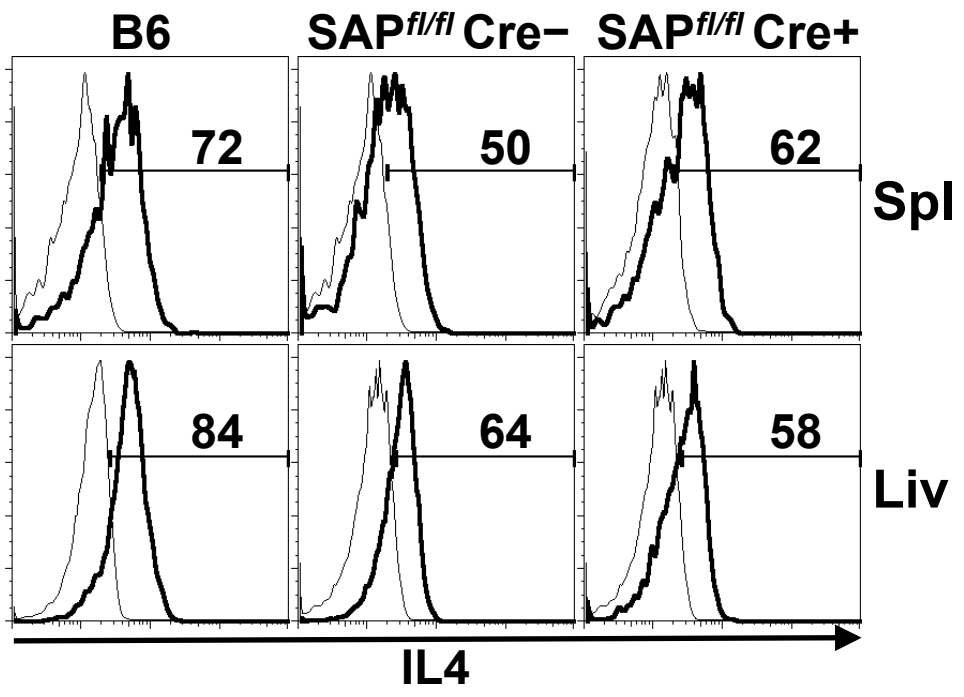
B


Exon 1
sap^LoxP
UBC-Cre^T2
Rosa-YFP
Tamoxifen
Days 1-5
Exon 1
Cre-T2
UBC
STOP
YFP
Rosa
Rest
Days 6-12
Cre-T2
UBC
YFP
Rosa
Sap^LoxP Cre^T2+ Rosa^YFP/YFP
2.74
44.7
55.3
SAP
YFP
Functional experiments

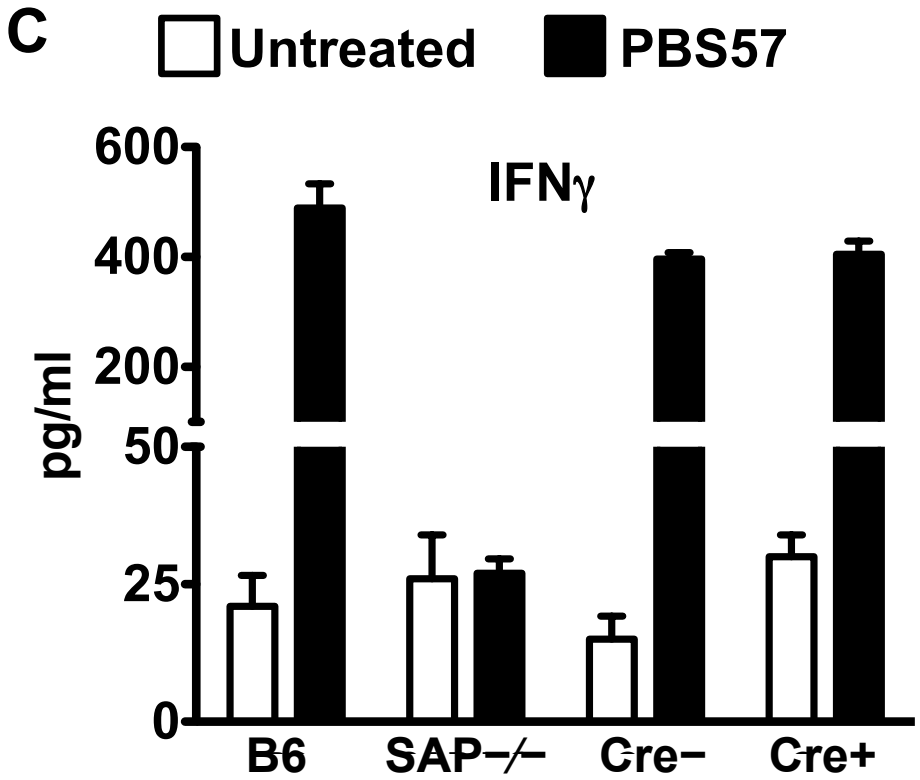
A


Untreated
PBS57
B6
SAP^fl/fl Cre−
SAP^fl/fl Cre+
78
73
88
Spl
91
86
88
Liv
IFNγ

B


B6
SAP^fl/fl Cre−
SAP^fl/fl Cre+
72
50
62
Spl
84
64
58
Liv
IL4

C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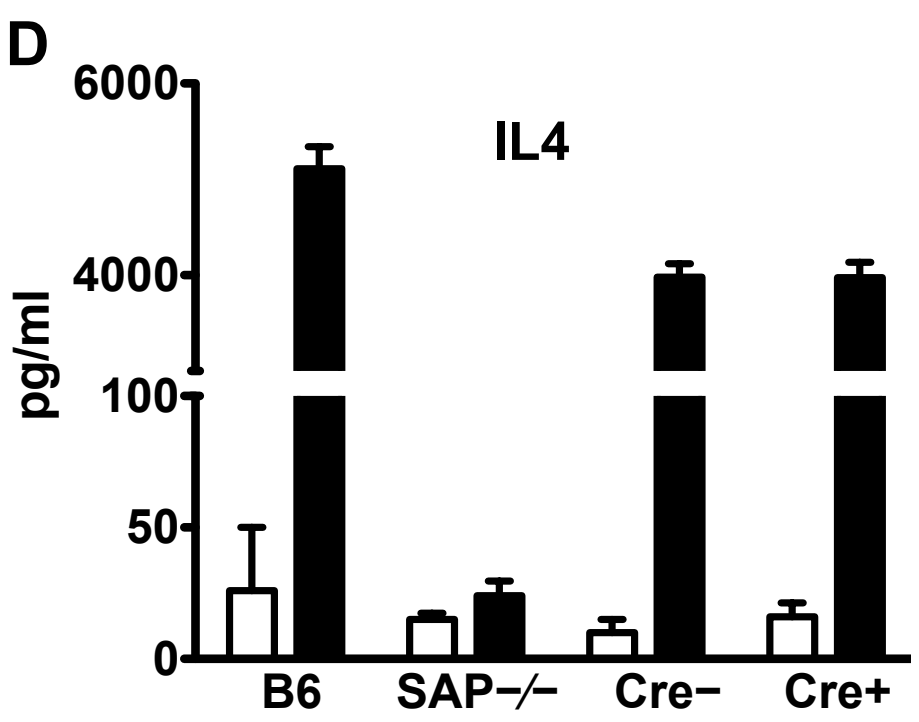
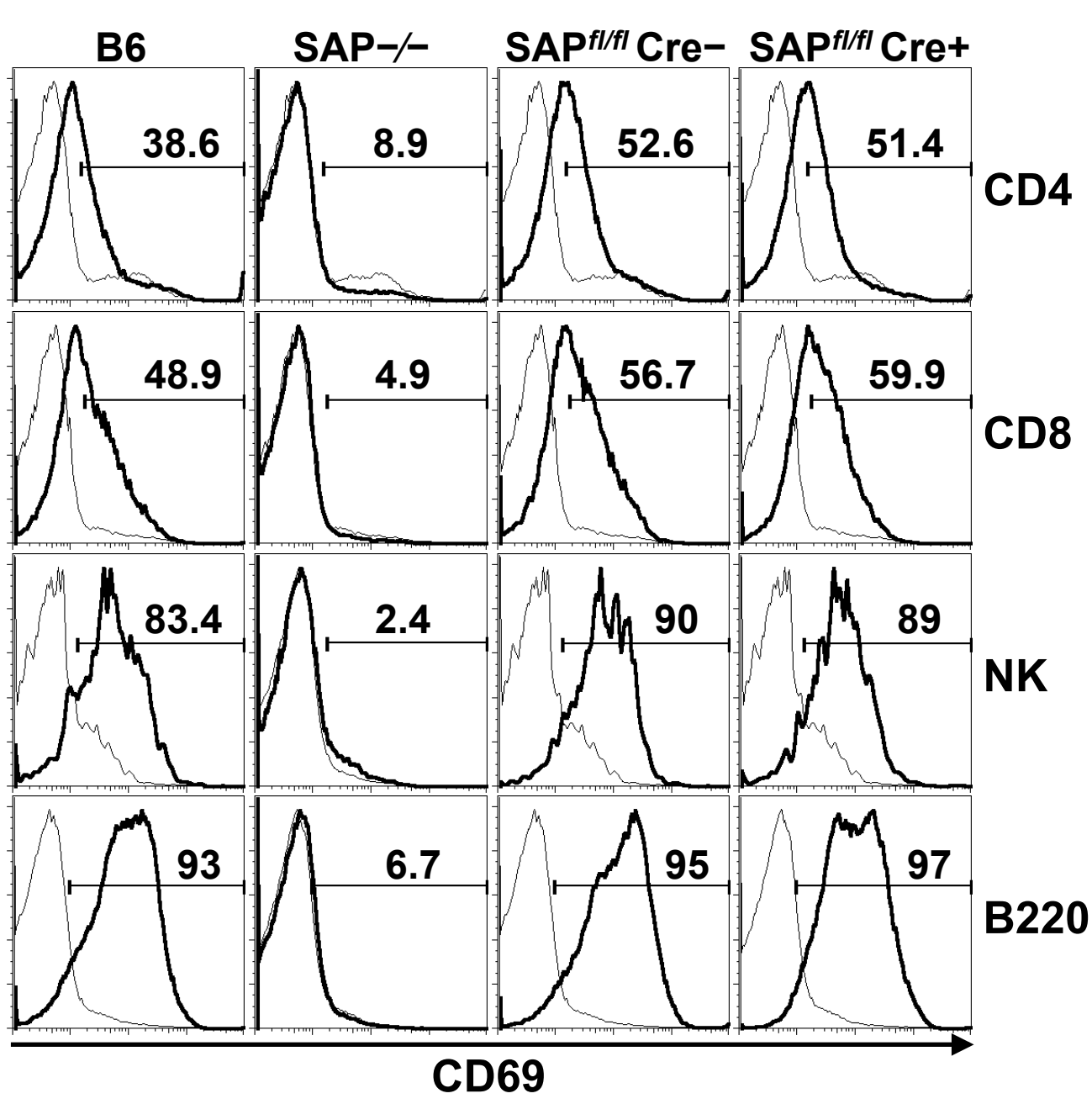
PBS57
IFNγ
pg/ml
600
400
200
50
25
0
B6
SAP−/−
Cre−
Cre+

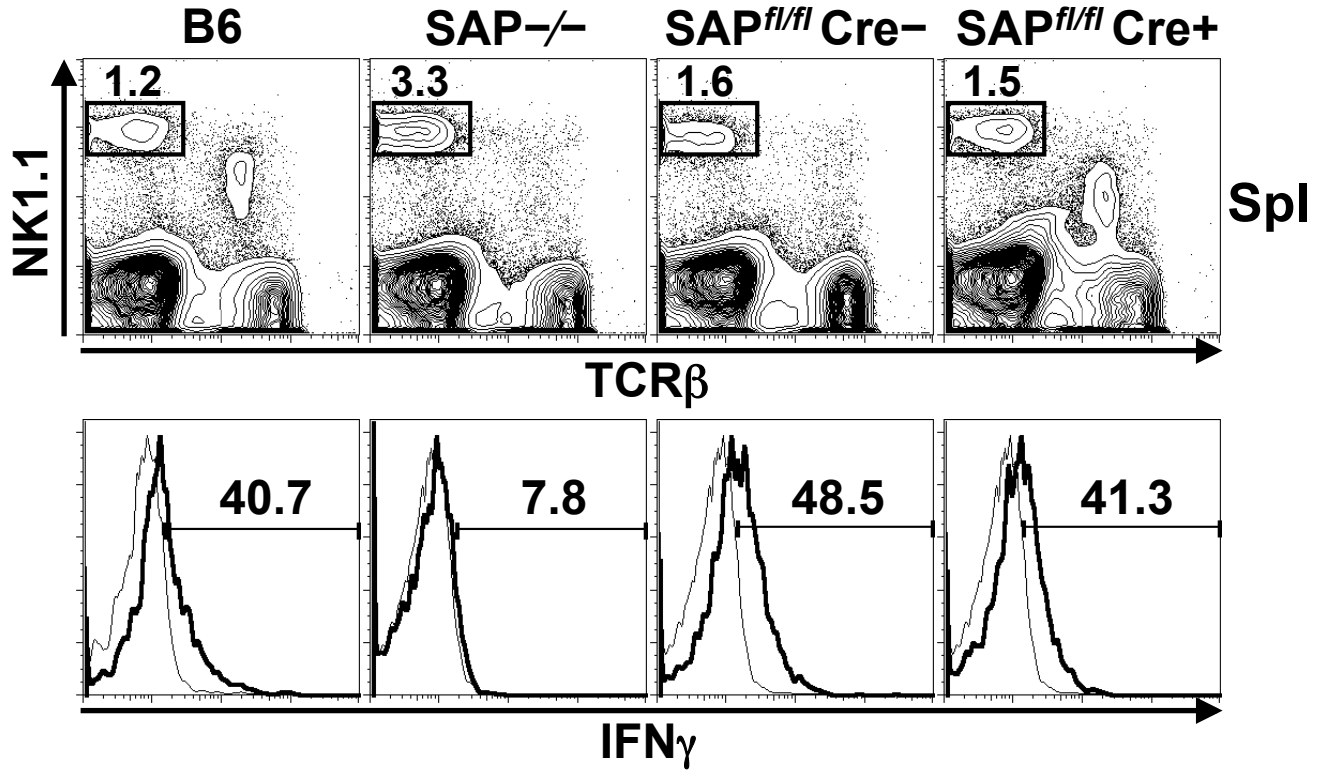
D


IL4
pg/ml
6000
4000
100
50
0
B6
SAP−/−
Cre−
Cre+

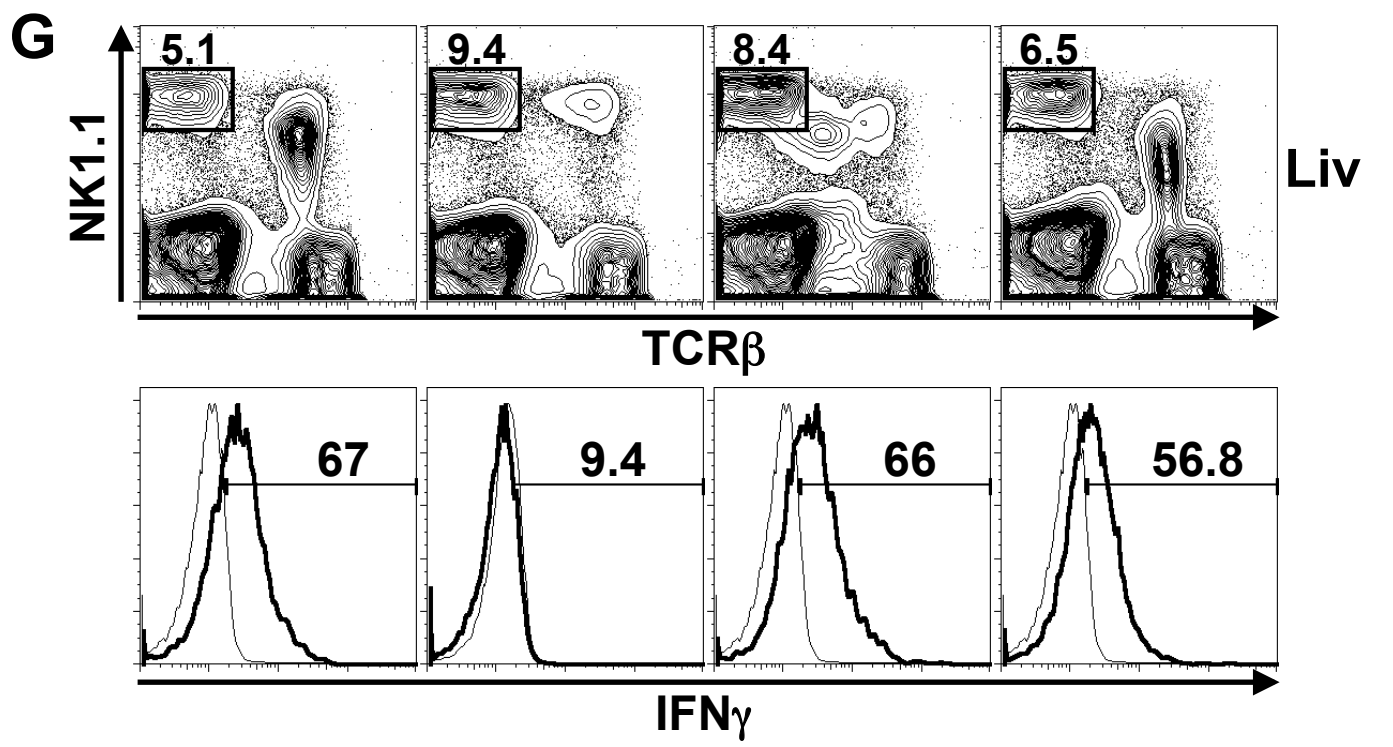
E


Untreated
PBS57
B6
SAP−/−
SAP^fl/fl Cre−
SAP^fl/fl Cre+
38.6
8.9
52.6
51.4
CD4
48.9
4.9
56.7
59.9
CD8
83.4
2.4
90
89
NK
93
6.7
95
97
B220
CD69

F


Untreated
PBS57
B6
SAP−/−
SAP^fl/fl Cre−
SAP^fl/fl Cre+
NK1.1
1.2
3.3
1.6
1.5
Spl
TCRβ
40.7
7.8
48.5
41.3
IFNγ

G


NK1.1
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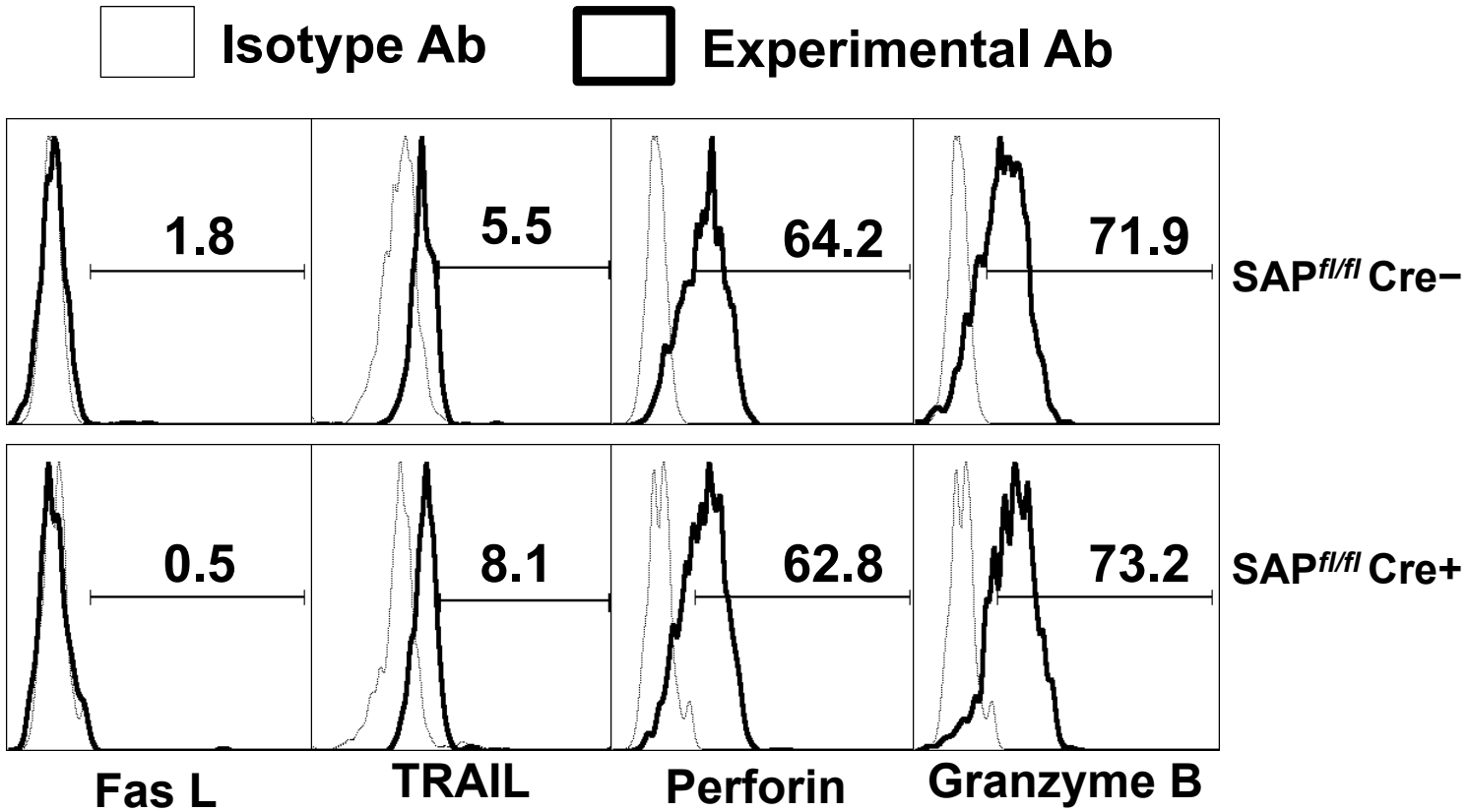
9.4
8.4
6.5
Liv
TCRβ
67
9.4
66
56.8
IFNγ

Supp. Fig 3

A



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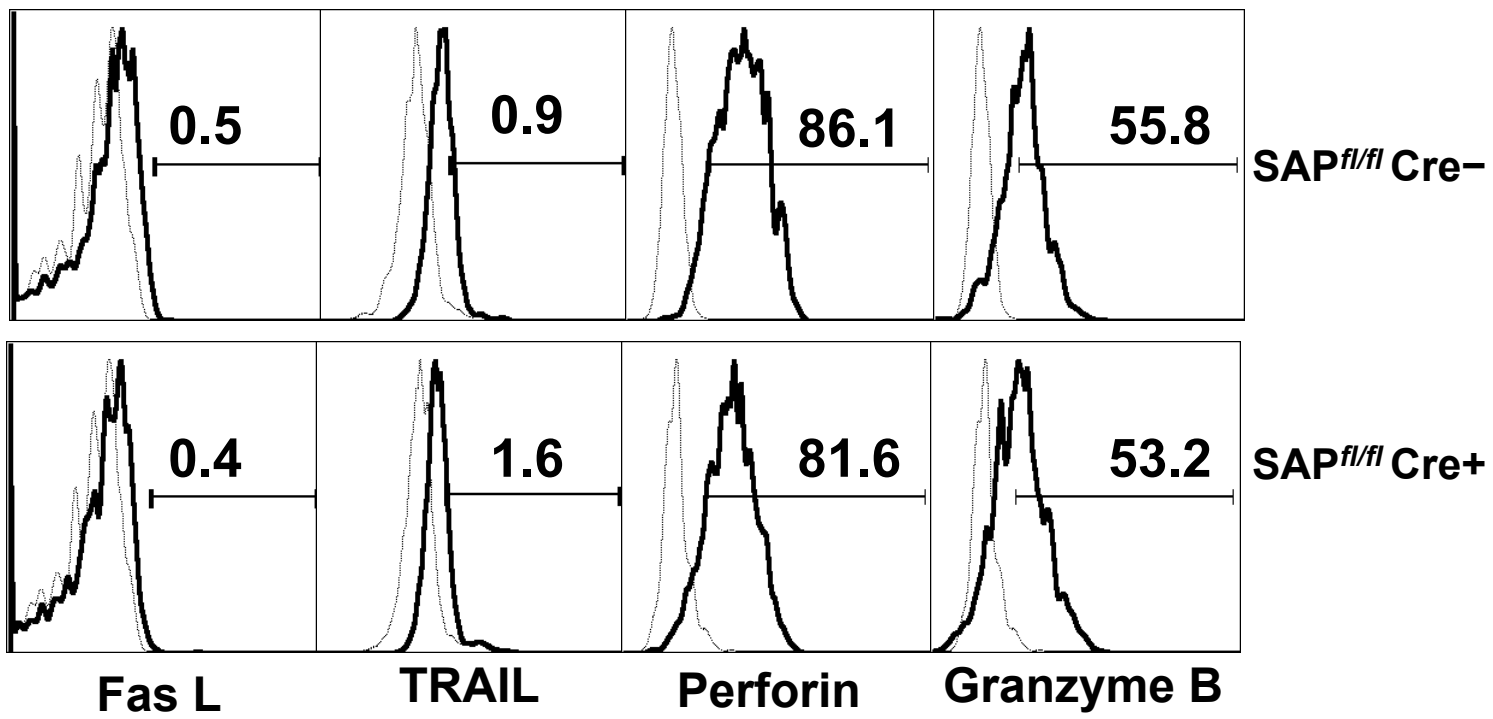
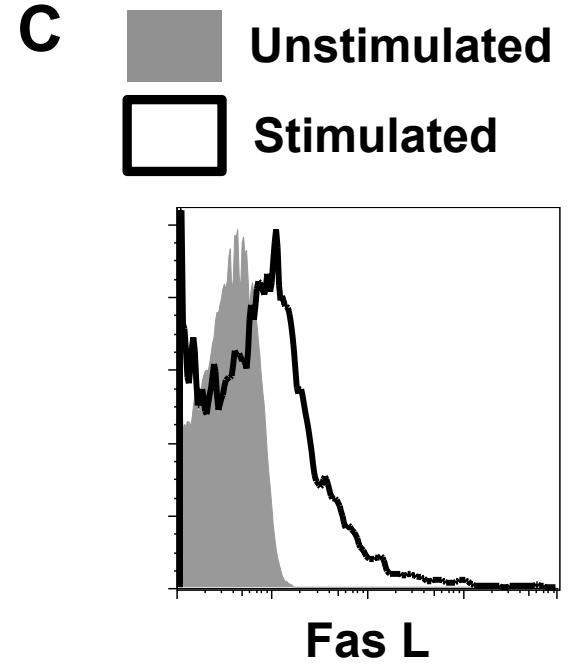
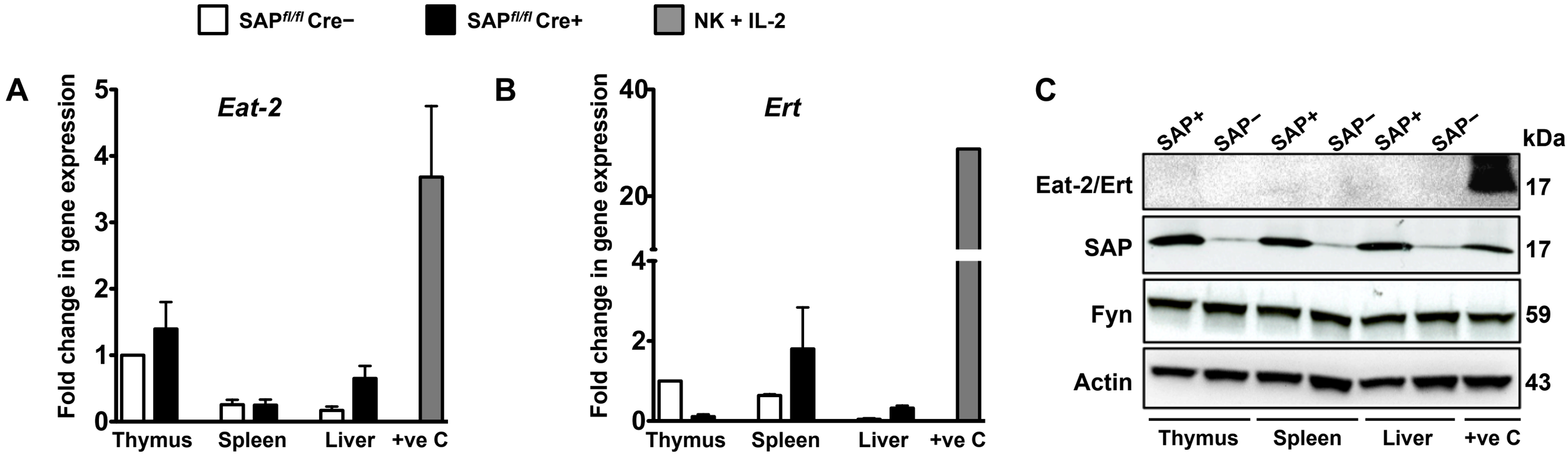


C




SAP fl/fl Cre−
SAP fl/fl Cre+
NK + IL-2
A
Eat-2
Fold change in gene expression
0
1
2
3
4
5
Thymus
Spleen
Liver
+ve C
B
Ert
Fold change in gene expression
0
2
4
20
40
Thymus
Spleen
Liver
+ve C
C
SAP+
SAP−
SAP+
SAP−
SAP+
SAP−
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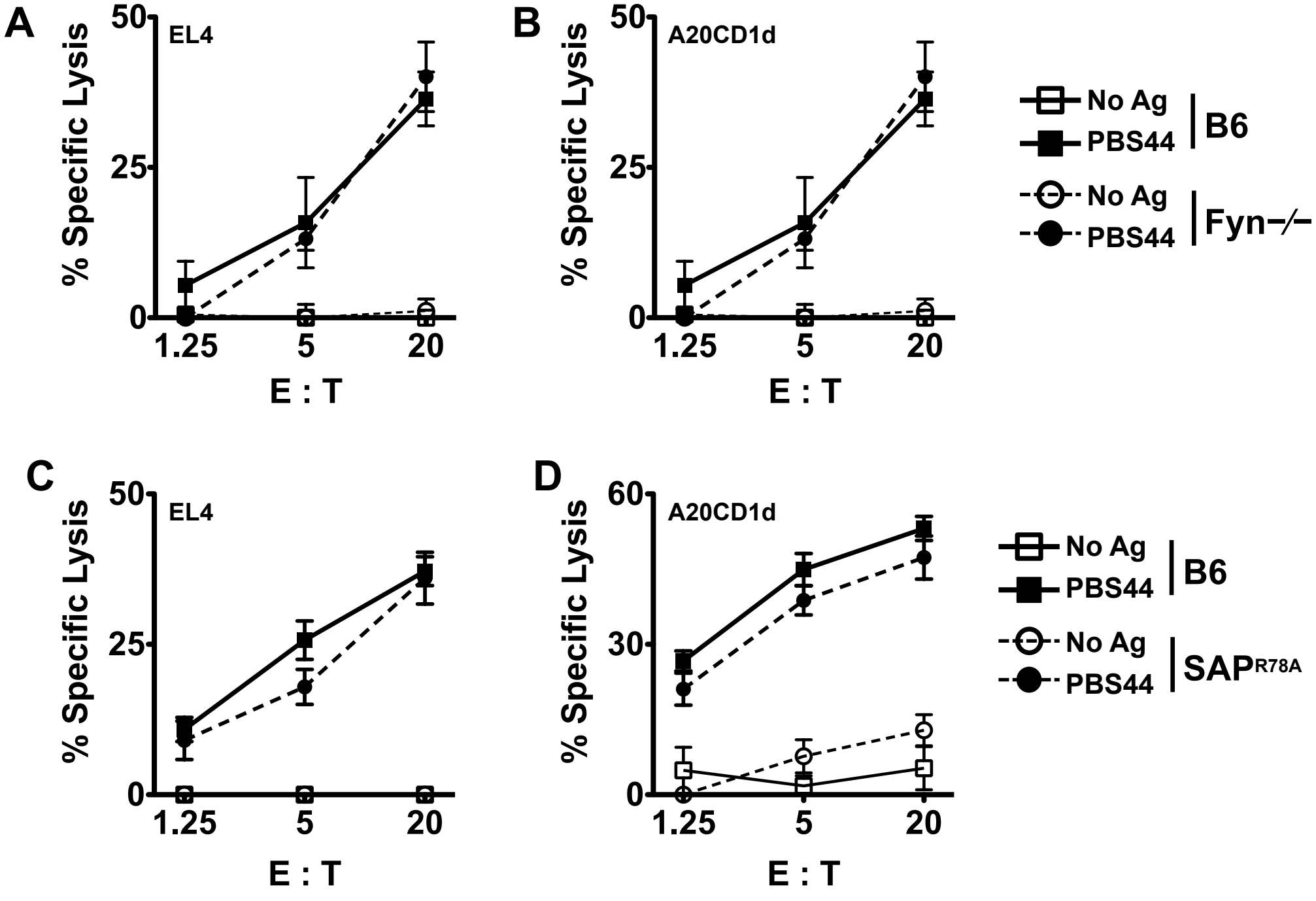
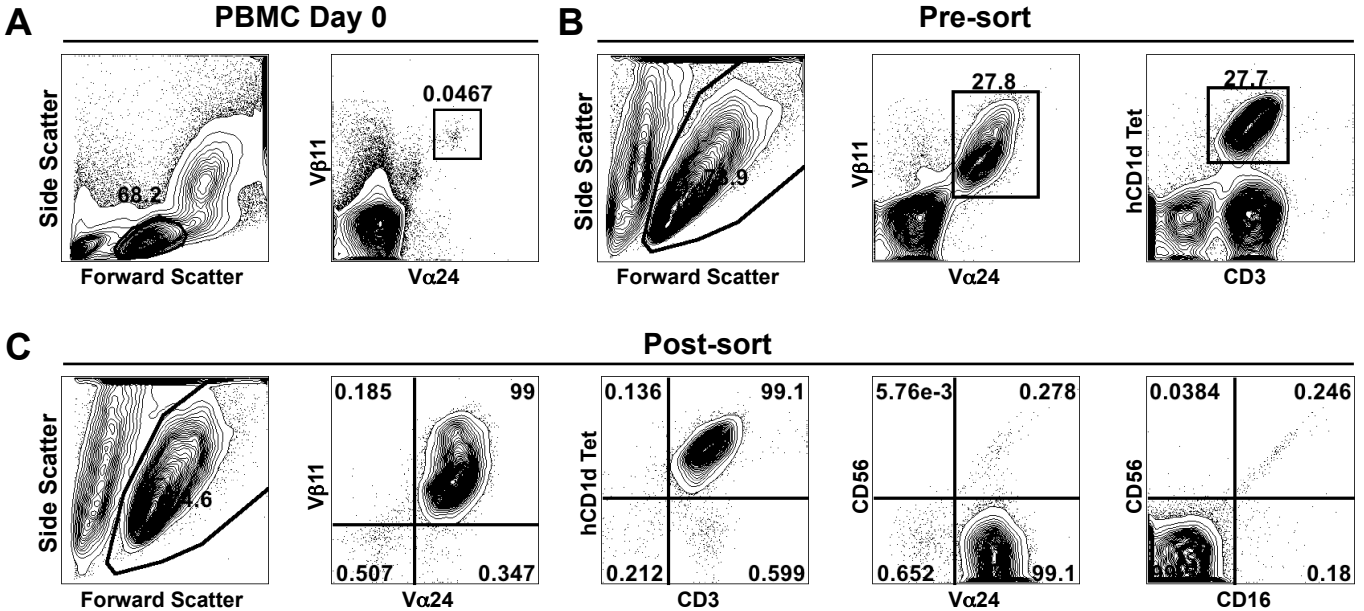
59
Actin
43
Thymus
Spleen
Liver
+ve C


A
EL4
% Specific Lysis
50
25
0
1.25
5
20
E : T
B
A20CD1d
% Specific Lysis
50
25
0
1.25
5
20
E : T
No Ag
PBS44
B6
No Ag
PBS44
Fyn−/−
C
EL4
% Specific Lysis
50
25
0
1.25
5
20
E : T
D
A20CD1d
% Specific Lysis
60
30
0
1.25
5
20
E : T
No Ag
PBS44
B6
No Ag
PBS44
SAPR78A

Supp. Fig 6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PBMC Day 0
Side Scatter
68.2
Forward Scatter
Vβ11
0.0467
Vα24
B
Pre-sort
Side Scatter
Forward Scatter
Vβ11
27.8
Vα24
hCD1d Tet
27.7
CD3
C
Post-sort
Side Scatter
Forward Scatter
Vβ11
0.185
99
0.507
0.347
Vα24
hCD1d Tet
0.136
99.1
0.212
0.599
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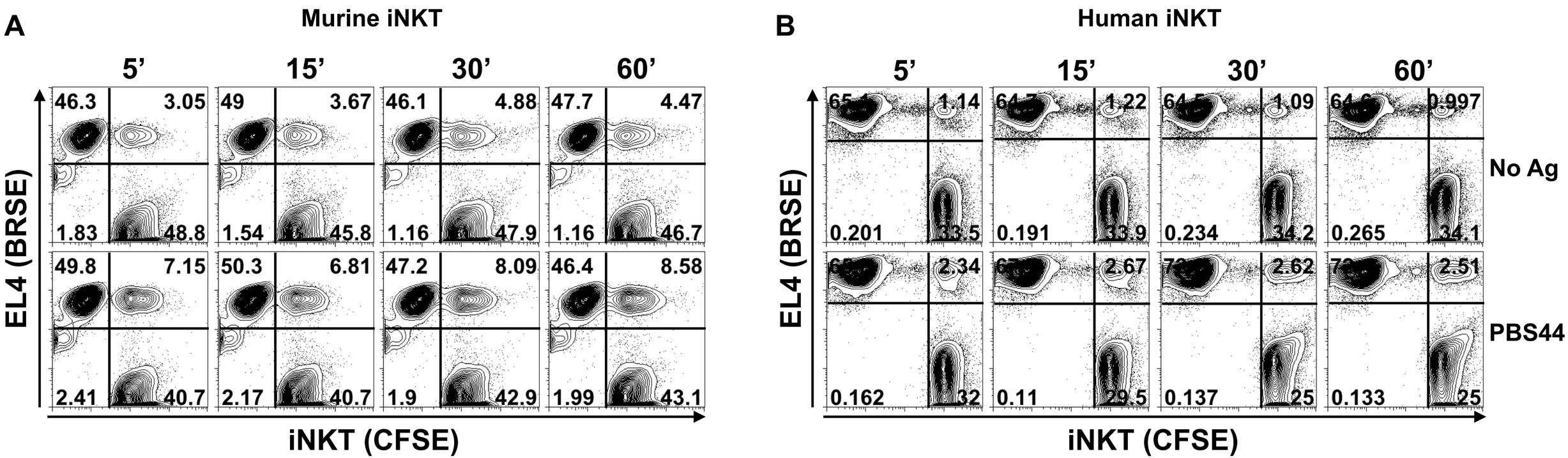
CD16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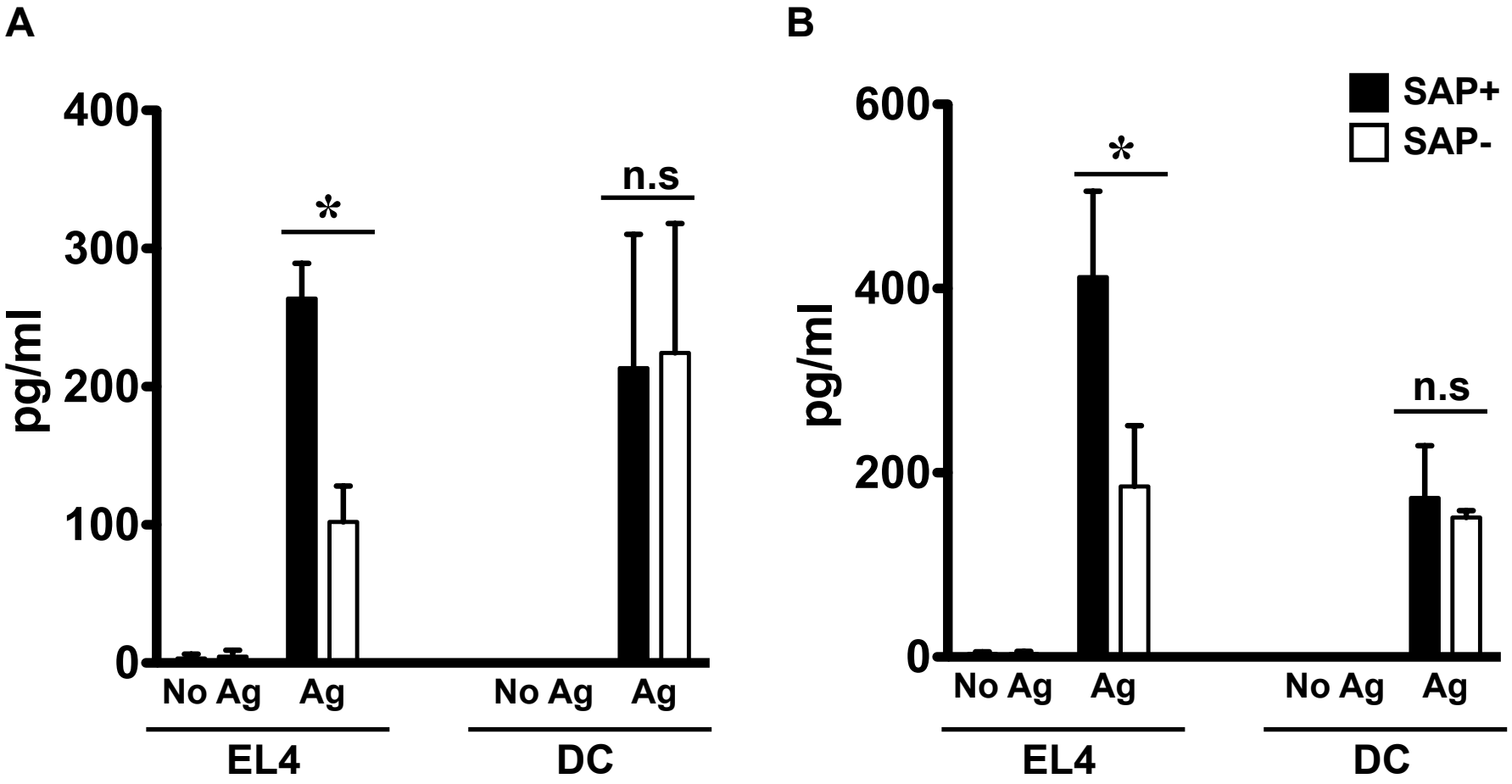
Murine iNKT
5’
15’
30’
60’
46.3
3.05
1.83
48.8
49
3.67
1.54
45.8
46.1
4.88
1.16
47.9
47.7
4.47
1.16
46.7
49.8
7.15
2.41
40.7
50.3
6.81
2.17
40.7
47.2
8.09
1.9
42.9
46.4
8.58
1.99
43.1
EL4 (BRSE)
iNKT (CFSE)
B
Human iNKT
5’
15’
30’
60’
1.14
0.201
1.22
0.191
1.09
0.234
0.997
0.265
2.34
0.162
2.67
0.11
2.62
0.137
2.51
0.133
No Ag
PBS44
EL4 (BRSE)
iNKT (CFSE)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pg/ml
*
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Ag
No Ag
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EL4
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B
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200
0
pg/ml
SAP+
SAP-
*
n.s
No Ag
Ag
No Ag
Ag
EL4
DC