SUPPLEMENTARY FIGURES AND TABLE

NKG2D signaling on CD8+ T cells represses T-bet and rescues CD4-unhelped CD8+ T cell memory recall but not effector responses

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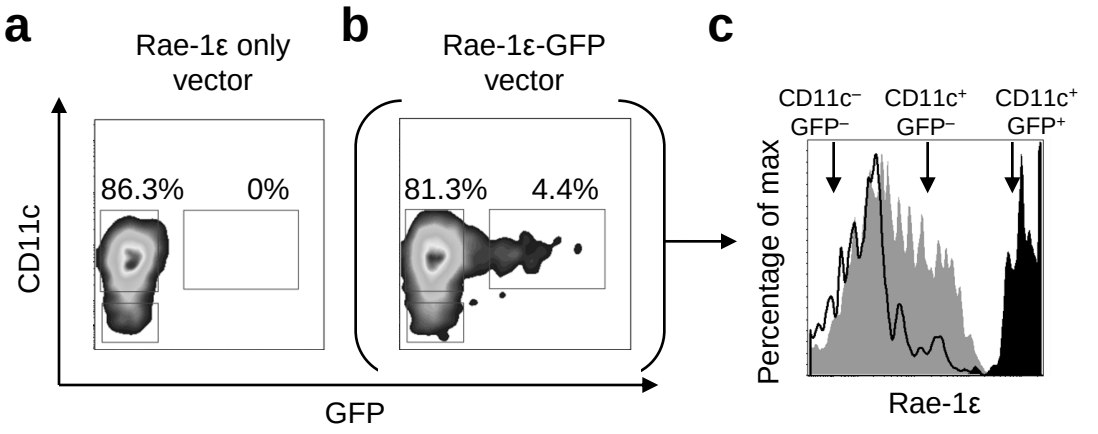
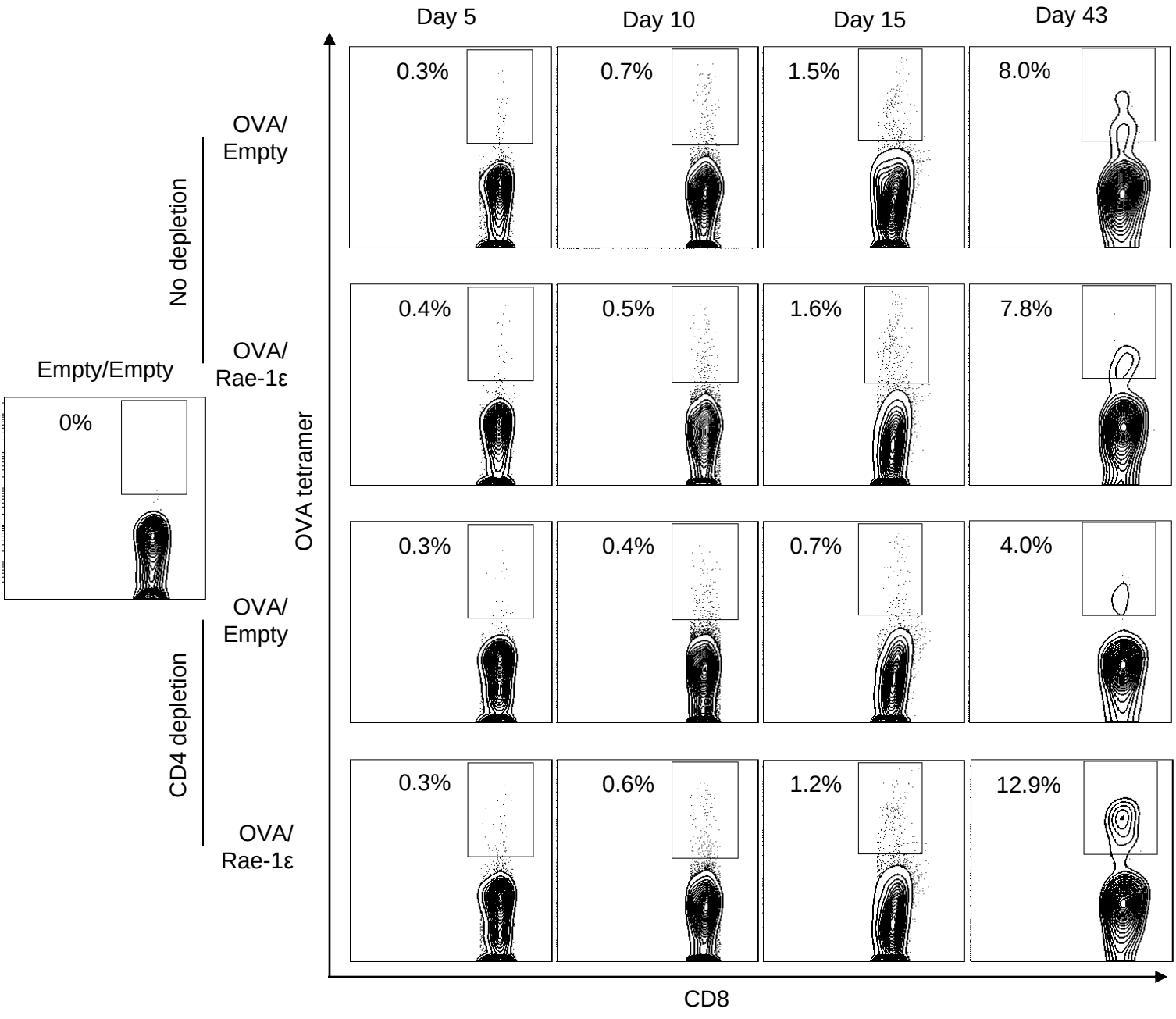
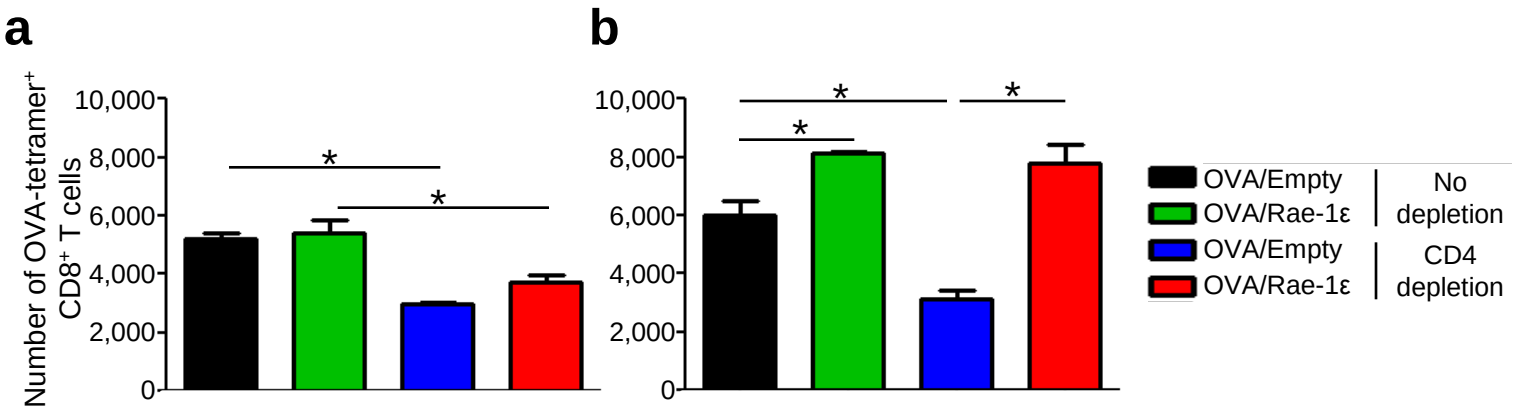
Content



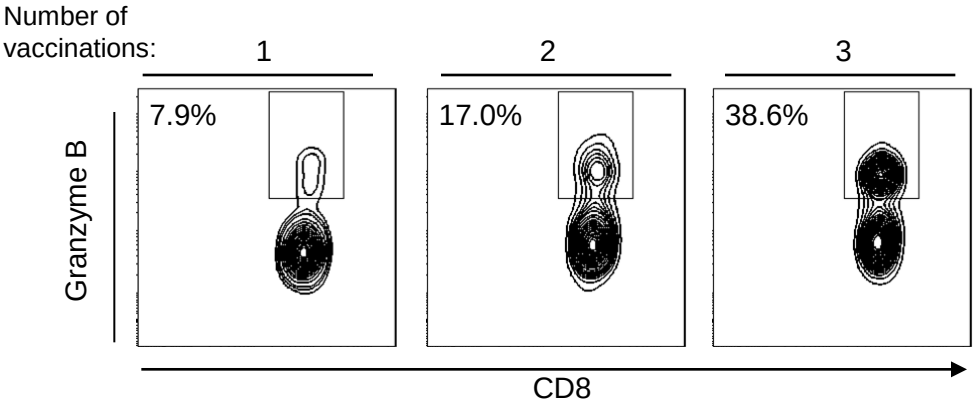
Supplementary Fig. 1 Verification of Rae-1ε expression on draining lymph node APCs upon DNA vaccination with Rae-1ε. (**a,b**) Rae-1ε without the fusion vector (**a**) or Rae-1ε-GFP fusion vector (**b**) were delivered via gene gun vaccination to C57BL/6 mice. Twenty-four hours later, inguinal draining lymph nodes were analyzed by flow cytometry for CD11c and GFP co-expression. (**c**) $CD11c^+$ cells from (**b**) were magnetically enriched and expression of Rae-1ε was determined on $CD11c^-GFP^-$, $CD11c^+GFP^-$, and $CD11c^+GFP^+$ cells. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results.



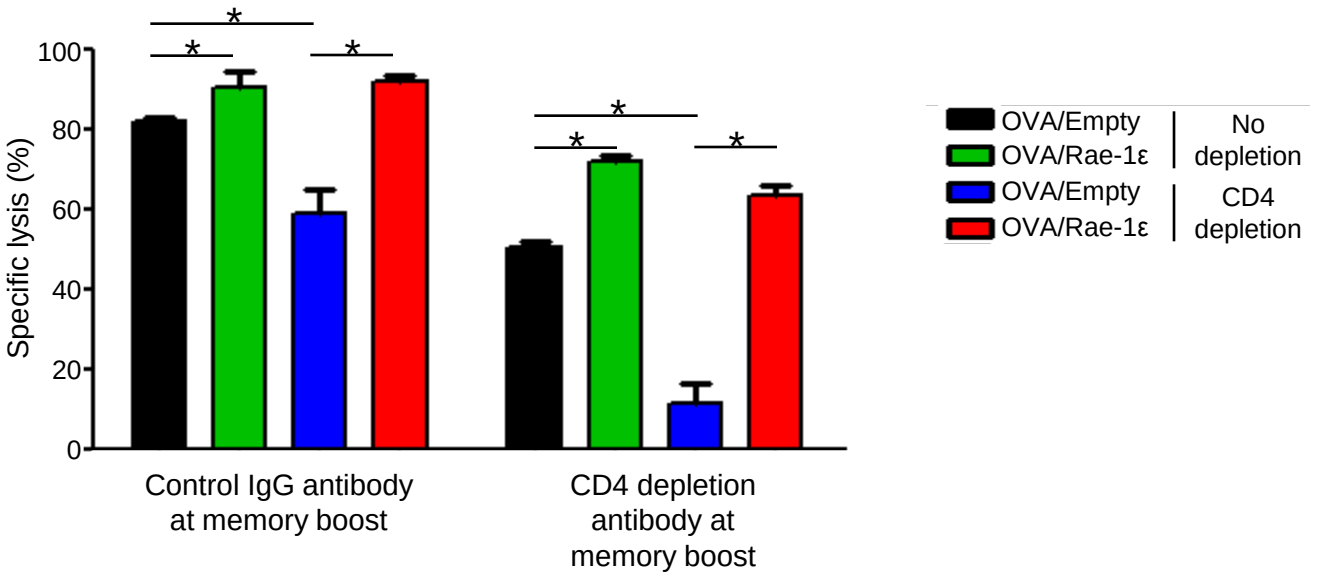
Supplementary Fig. 2 OVA-tetramer staining of effector and memory phase CD8$^+$ T cells. OVA-tetramer ($OVA_{257–264}$: SIINFEKL) flow cytometric analysis of OVA-specific CD8$^+$ T cells detected in spleens of mice vaccinated as described in Fig. 1a for the effector (days 5, 10, and 15) and memory recall (day 43) phases. Data are representative of 3–5 mice analyzed individually per group per experiment from at least three experiments conducted with similar results.



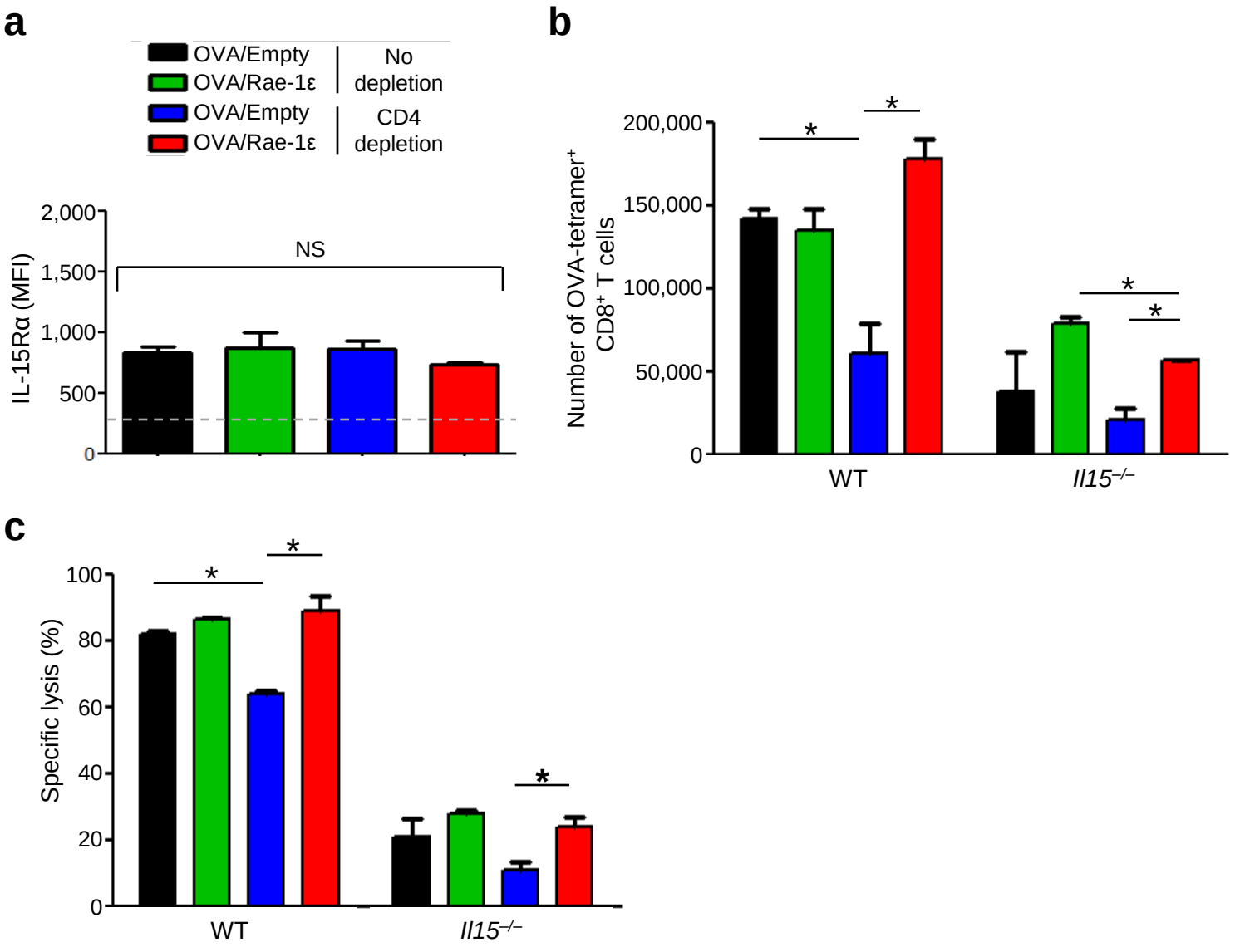
Supplementary Fig. 3 Lymph node OVA-tetramer$^+$ CD8$^+$ T cell numbers at effector and memory recall phases. (**a,b**) Bar graph showing mean number of OVA-tetramer$^+$ CD8$^+$ T cells (+SEM) per inguinal draining lymph node on effector peak day 15 (**a**) and memory recall peak day 43 (**b**) from mice vaccinated as described in Fig. 1a. Data shown are representative of at least two experiments each with 3–5 individually analyzed mice per group per experiment with similar results. *P < 0.05.



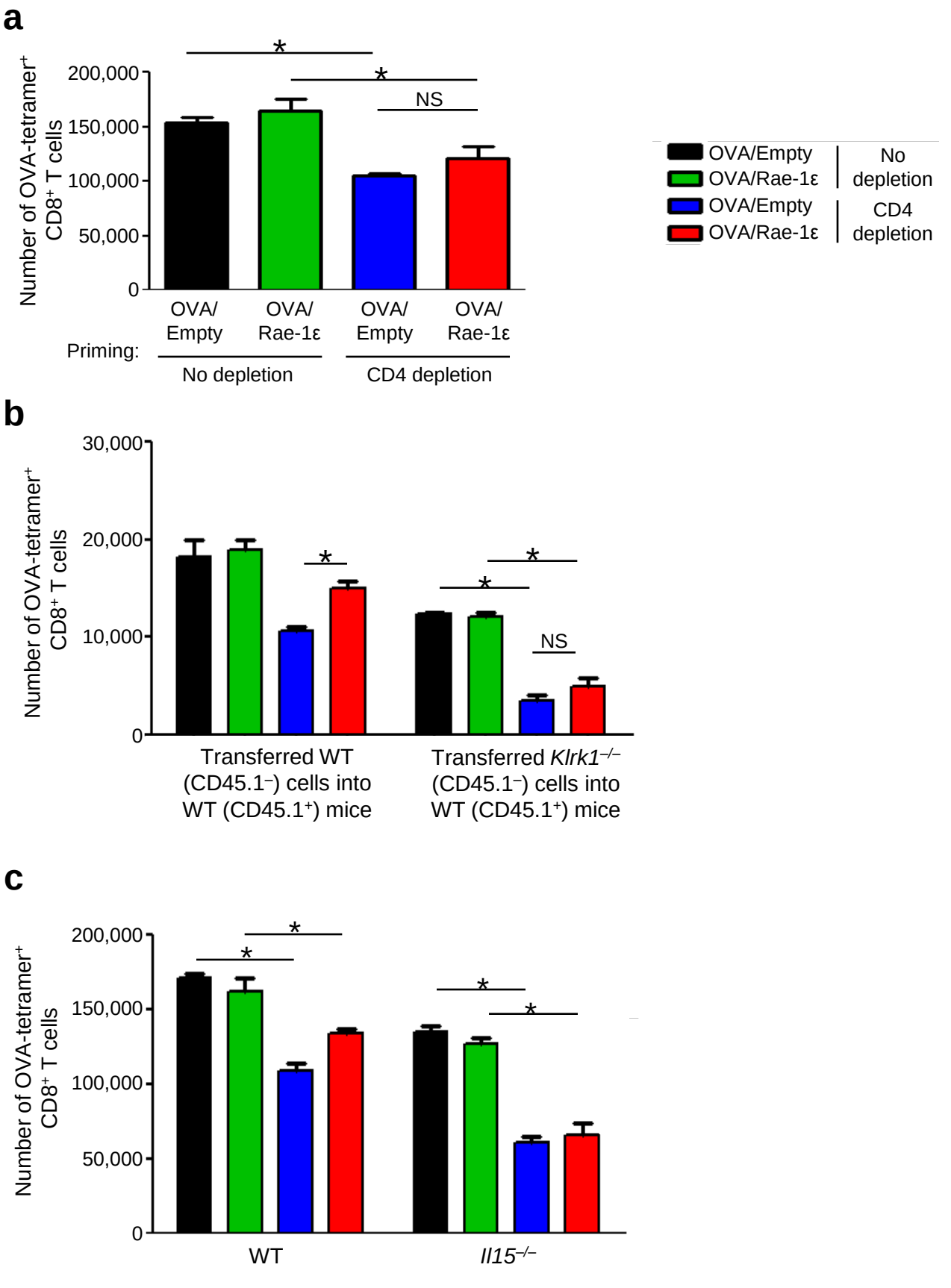
Supplementary Fig. 4 Granzyme B production by OVA-tetramer$^+$ CD8$^+$ T cells after 1, 2, and 3 vaccinations. Representative flow plots of granzyme B production by OVA-tetramer$^+$ CD8$^+$ T cells from C57BL/6 mice vaccinated 1, 2, or 3 times with OVA/Rae-1ε, then restimulated 5 d later *in vitro* for 6 h with $OVA_{257–264}$ peptide (1 μg ml$^{–1}$). Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results.



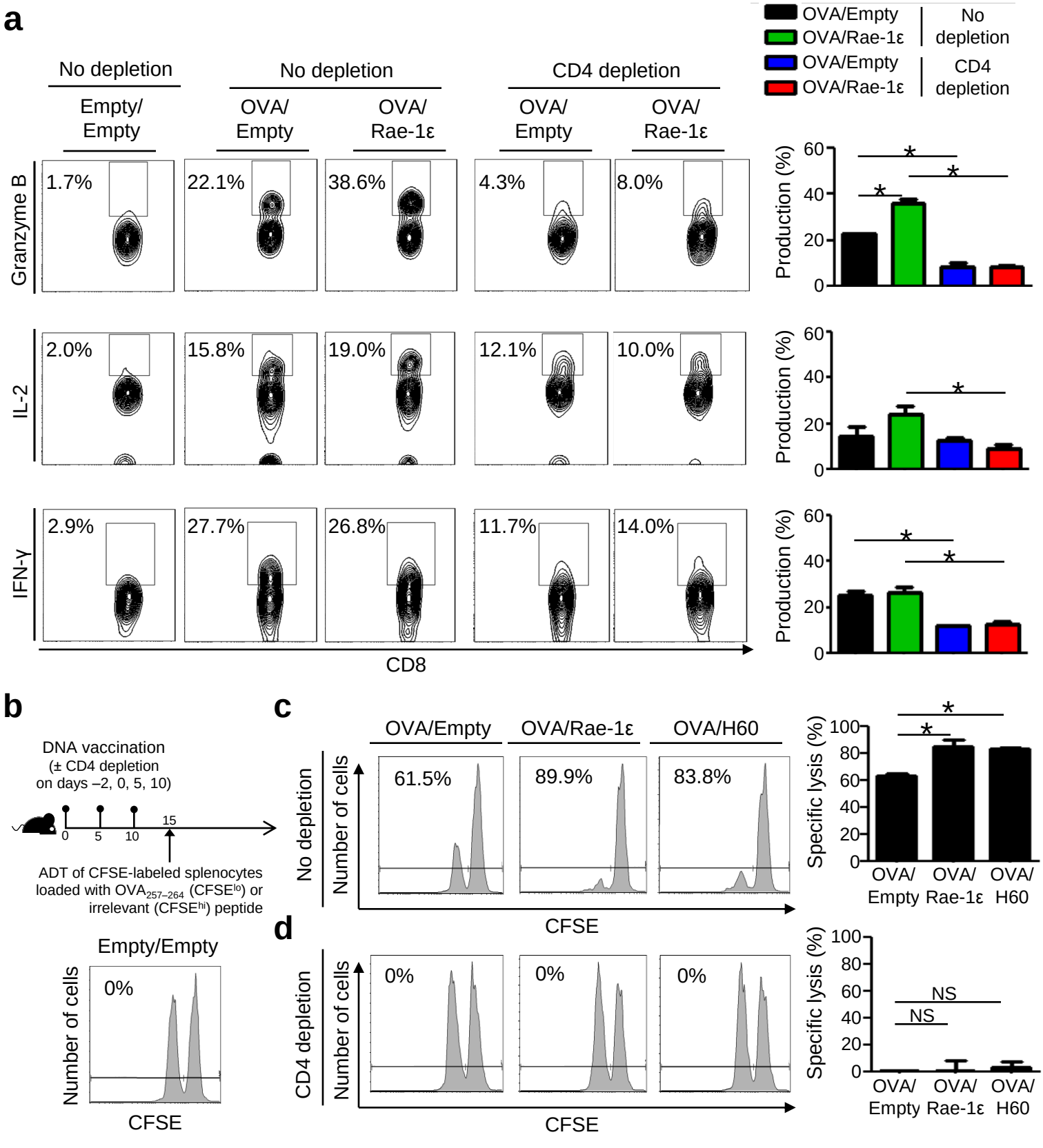
Supplementary Fig. 5 $CD8^+$ T cell memory recall CTL lysis with addition of CD4 depletion at the memory phase. Cumulative figure of mean specific lysis (%) comparing $CD8^+$ T cells after memory boost and target cell transfer into mice vaccinated as described in Fig. 1a versus those mice additionally receiving CD4 depletion (GK1.5; 500 µg per mouse per depletion) on days 36 and 38. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. *$P < 0.05$.



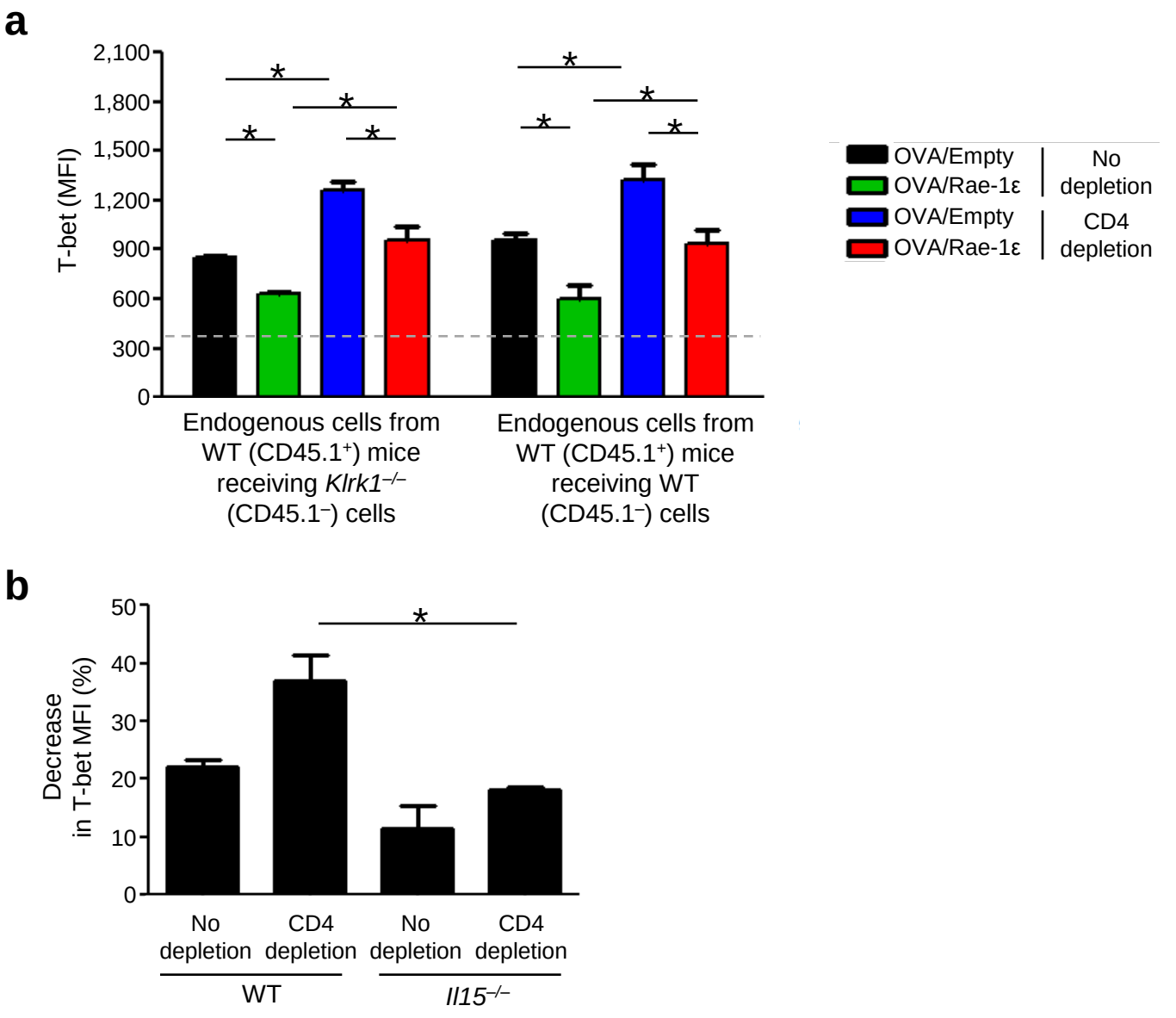
Supplementary Fig. 6 NKG2D-mediated rescue of memory CD8$^+$ T cell response is not independent of IL-15. (**a**) C57BL/6 mice were vaccinated as described in Fig. 1a. Cumulative bar graph shows IL-15Rα MFI (+SEM) determined on dendritic cells at the effector phase (day 15) by flow cytometric analysis. (**b**) IL-15-deficient (*Il15*$^{-/-}$) and wild type C57BL/6 (WT) mice were vaccinated as described in Fig. 1a. Mean number of OVA-tetramer$^+$ CD8$^+$ T cells (+SEM) per spleen was calculated on day 43 (5 d after a memory boost vaccination with OVA only). (**c**) CFSE-labeled C57BL/6 splenocytes loaded with $OVA_{257–264}$ (CFSE^{lo}) and irrelevant peptide ($hgp100_{25-33}$, CFSE^{hi}) were adoptively transferred into mice from (**b**) for 18-h *in vivo* CTL lysis assay. Spleens were then removed and target cell CFSE levels were detected by flow cytometry. Bar graph (**c**) shows mean specific lysis (% + SEM). Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. Dashed lines represent MFI of background flow cytometric staining for IL-15Rα. *$P < 0.05$; NS: $P > 0.05$.


a
Number of OVA-tetramer+ CD8+ T cells
200,000
150,000
100,000
50,000
0
*
*
NS
OVA/ Empty
OVA/ Rae-1ε
OVA/ Empty
OVA/ Rae-1ε
Priming:
No depletion
CD4 depletion
OVA/Empty
OVA/Rae-1ε
OVA/Empty
OVA/Rae-1ε
No depletion
CD4 depletion
b
Number of OVA-tetramer+ CD8+ T cells
30,000
20,000
10,000
0
*
*
*
NS
Transferred WT (CD45.1−) cells into WT (CD45.1+) mice
Transferred Klrk1−/− (CD45.1−) cells into WT (CD45.1+) mice
c
Number of OVA-tetramer+ CD8+ T cells
200,000
150,000
100,000
50,000
0
*
*
*
*
WT
Il15−/−

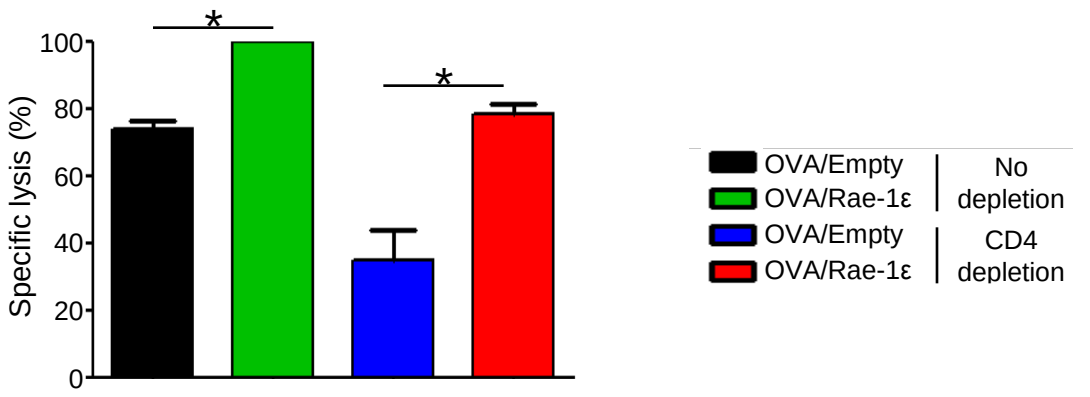
Supplementary Fig. 7 NKG2D co-stimulation regimen does not rescue CD4-unhelped $CD8^+$ T cell effector recall expansion. (**a**) Bar graph showing mean number of OVA-$tetramer^+$ $CD8^+$ T cells (+SEM) per spleen on day 15 in mice vaccinated as described in Fig. 1a. (**b**) $CD8^+$ T cells ($4*10^6$) from NKG2D-deficient (*Klrk1*$^{-/-}$) or wild type C57BL6 (WT) spleens were adoptively transferred into B6.SJL-Ptprca Pepcb/BoyJ ($CD45.1^+$) mice. Mice were vaccinated as described in Fig. 1a. CD45.1 expression was used to distinguish transferred ($CD45.1^-$) from endogenous ($CD45.1^+$) OVA-$tetramer^+$ $CD8^+$ T cells. Effector phase (day 15) mean OVA-$tetramer^+$ $CD8^+$ T cell number (+SEM) was calculated as described in the methods. (**c**) IL-15-deficient (*Il15*$^{-/-}$) or WT mice were vaccinated as described in Fig. 1a. Mean number of OVA-$tetramer^+$ $CD8^+$ T cells (+SEM) per spleen were calculated on day 15. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. *$P < 0.05$; NS: $P > 0.05$.


a
OVA/Empty
OVA/Rae-1ε
No depletion
OVA/Empty
OVA/Rae-1ε
CD4 depletion
No depletion
No depletion
CD4 depletion
Empty/ Empty
OVA/ Empty
OVA/ Rae-1ε
OVA/ Empty
OVA/ Rae-1ε
Granzyme B
1.7%
22.1%
38.6%
4.3%
8.0%
IL-2
2.0%
15.8%
19.0%
12.1%
10.0%
IFN-γ
2.9%
27.7%
26.8%
11.7%
14.0%
CD8
Production (%)
60
40
20
0
*
*
*
b
DNA vaccination
(± CD4 depletion
on days –2, 0, 5, 10)
0
5
10
15
ADT of CFSE-labeled splenocytes
loaded with OVA257–264 (CFSElo) or
irrelevant (CFSEhi) peptide
Empty/Empty
Number of cells
0%
CFSE
c
OVA/Empty
OVA/Rae-1ε
OVA/H60
No depletion
Number of cells
61.5%
89.9%
83.8%
CFSE
Specific lysis (%)
100
80
60
40
20
0
*
*
OVA/ Empty
OVA/ Rae-1ε
OVA/ H60
d
CD4 depletion
Number of cells
0%
0%
0%
CFSE
NS
NS

Supplementary Fig. 8 Effector phase cytokine/effector molecule production and CTL lysis. (**a**) C57BL/6 mice were vaccinated as described in Fig. 1a. On day 15 (5 d after the third vaccination), mouse splenocytes were restimulated *in vitro* for 6 h with $OVA_{257–264}$ peptide (1 μg ml^{-1}). Representative flow plots and cumulative bar graphs show OVA-tetramer$^+$ $CD8^+$ T cell production of granzyme B, IL-2 and IFN-γ as determined by intracellular flow cytometric analysis. Flow plot gate placements were based on non-restimulated cell controls or isotype controls (data not shown) and verified on cells from mice vaccinated with empty vector DNA plasmids (designated in the figures as Empty/Empty). (**b**) Experimental design (upper panel) showing the timeline by which C57BL/6 mice were vaccinated as described in Fig. 1a. Some mice were alternatively vaccinated with NKG2D ligand, H60. On day 15 (5 d after the third vaccination), CFSE-labeled C57BL/6 splenocytes loaded with $OVA_{257–264}$ ($CFSE^{lo}$) and irrelevant peptide ($hgp100_{25–33}$, $CFSE^{hi}$) were adoptively transferred for 18-h *in vivo* CTL lysis assay. Spleens were then removed and target cell CFSE levels were detected by flow cytometry. CFSE profile (lower panel) of target cells from a control mouse vaccinated with Empty/Empty DNA plasmids. (**c**) Representative flow cytometry histograms and bar graphs (+SEM) from (**b**) showing specific lysis (%) calculated as described in the methods section. Data are representative of 3–5 mice analyzed individually per group per experiment from at least three experiments conducted with similar results. *$P < 0.05$; NS: $P > 0.05$.



Supplementary Fig. 9 T-bet expression by endogenous OVA-specific effector phase CD8$^+$ T cells. (**a**) CD8$^+$ T cells (4*10^6) from NKG2D-deficient or wild type C57BL/6 (WT) control spleens were adoptively transferred into B6.SJL-Ptprca Pepcb/BoyJ (CD45.1$^+$) mice. Mice were vaccinated as described in Fig. 1a. CD45.1 expression was used to distinguish transferred (CD45.1$^-$) from endogenous (CD45.1$^+$) OVA-tetramer$^+$ CD8$^+$ T cells. T-bet expression MFI (+SEM) was determined on day 15 in endogenous (CD45.1$^+$) T cells from mice receiving NKG2D-deficient (*Klrk1*$^{-/-}$) and WT CD8$^+$ T cell transfers. (**b**) IL-15-deficient (*Il15*$^{-/-}$) or WT mice were vaccinated as described in Fig. 1a. Mean decrease (%) in T-bet MFI was calculated on day 15 (5 d after the third vaccination) in mice with versus without NKG2D co-stimulation. Data are representative of 3–5 mice analyzed individually per group per experiment from three experiments conducted with similar results. Dashed lines represent MFI of background flow cytometric T-bet staining. *P < 0.05.



Supplementary Fig. 10 Detection of per cell function via an *ex vivo* CTL lysis assay. C57BL/6 mice were vaccinated as described in Fig. 6a. On day 41 (3 d after the memory boost vaccination with OVA only), spleen OVA-tetramer$^+$ CD8$^+$ T cells were detected via flow cytometric analysis. Equalized numbers of untouched OVA-tetramer$^+$ CD8$^+$ T cells from the four vaccination conditions were co-cultured with CFSE-labeled C57BL/6 splenocytes loaded with $OVA_{257–264}$ (CFSE^{hi}) and irrelevant peptide ($hgp100_{25–33}$, CFSE^{low}) at a 10:1 E:T ratio for the 18-h *ex vivo* CTL lysis assay. Lysis of cultured targets was then analyzed by flow cytometry. Mean specific lysis (%) values are representative of 3–5 mice analyzed individually per group per experiment from two experiments conducted with similar results. *$P < 0.05$.

Donor	Age (y)	Race	Sex	LTNP (y)	CD4 (number per mm^3)	Viral load (copies per ml)
LNTP 1	40	AA	F	7	1171	470
LTNP 2	28	AA	M	8	507	<50
LNTP 3	42	H	M	8	635	59
LTNP 4	59	W	F	16	749	<196
LTNP 5	47	W	M	5	845	184
CHR 1	53	AA	M	-	136	>75,000
CHR 2	61	H	M	-	409	1855
CHR 3	39	W	F	-	105	50,400
CHR 4	44	W	F	-	207	>75,000

Supplementary Table 1 Clinical characteristics of HIV-positive donors. AA: African American; CHR: HIV-positive chronically-infected progressor; H: Hispanic; LTNP: HIV-positive long-term non-progressor; W: white.