#### Supplemental Figure 1. TCR repertoire of Qa-1–FL9 Tet<sup>+</sup> CD8 T cells.

Single Qa-1–FL9-PE<sup>+</sup> Qa-1–FL9-APC<sup>+</sup> cells were sorted and subjected to sequencing for TCR $\alpha$  and TCR $\beta$ . TCR $\alpha$  and TCR $\beta$  pairs were then analyzed based on their TCR V gene segments. Alignment of TCR $\alpha$  (**A**) and TCR $\beta$  (**B**) sequences obtained from Qa-1–FL9 Tet<sup>+</sup> single cells. TCR affinity to Qa-1–FL9 complex and TCR V $\alpha$  and V $\beta$  gene usage by each Qa-1–FL9 Tet<sup>+</sup> CD8 T cell are shown on the right. TRAV9N3 (V $\alpha$ 3.2) and/or TRBV12.1/2 (V $\beta$ 5.1/2) expressing CD8 T cell clones are highlighted.

Α	тс	Rα cł	nains for FL9	TCR				
		CDR1		CDR2		Affinity	να	Vβ
FL9.15 FL9.8 FL9.34 FL9.41 FL9.40 FL9.23 FL9.30 FL9.2 FL9.35 FL9.35 FL9.35 FL9.38	GDSVTQTEGPVTVSESESLIINCTYS AQSVTQPDARVTVSEGASLQLRCKYS AQSVTQPDARVTYS AVX	ATSIAYP YFGTP YFGTP YFGTP YFGTP YFGTP YFGTP YFGTP YFGTP YFGTP DPNSY DRNFQ	NLFWYVRYPGEGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YLFWYVQYPRQGLQLL: YFFWYRQPPGASLQLL! YFFWYRQPFGASLQLL! YFFWYRQPSGGPKAL! :**CDR3	LEVITAGQ-RG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYPGDPVVG LEYYFGDPVVG LEYYFGDPVVG LEYYFGDPVVG LEYYFGDPVVG LEYYFGDPVVG LEYYFGDPVVG LEYFFGDPVG LEYFFGDPVG LEF	59 58 58 58 58 58 58 58 58 58 58 58 58 58	- +++ + + + + + + + + + + + + + + + + +	TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV9N3 TRAV5-5	TRBV12-1 TRBV-12-2 TRBV-12-2 TRBV-12-2 TRBV12-1 TRBV12-1 TRBV12-1 TRBV12-1 TRBV12-1 TRBV12-1 TRBV-12-2 TRBV12-3 TRBV-13-3
FT.9.15	SSRGFEATYNKETTSFHLOKASVOES	DSAVYY	ALGEA-SSGSWOLLEG	SGTOLTVMP	116			
FL9.8	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFC	AVRANYAQGLT [G]	LGTRVSVFP	113			
FL9.34	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFC	AVRGQGGRALI	IGTTVSVSP	113			
FL9.41	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFC	AVSIWATSSGQKLVFG	QGTILKVYL	116			
FL9.40	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFC	AVTRYG-SSGNKLIFG	IGTLLSVKP	115			
FL9.23	GVNGFEAFFSKSNSSFHLRKASVHWS GVNGFEAFFSKSNSSFHLRKASVHWS	DWAVYFO	AVSNINVLIEG	SGTKLTVEP	114			
FL9.2	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFO	AVRANTGKLT G	DGTVLTVKP	112			
FL9.5	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFC	AVKGGNYKPT G	KGTSLVVHP	112			
FL9.35	GVNGFEAEFSKSNSSFHLRKASVHWS	DWAVYFC	AVSMMGYKLTFG	IGTSLLVDP	112			
FL9.32	-GQGFTVLLNKKDKRLSLNLTAAHPG	DSAAYFC	AVSGAGNTGKLIFG	LGTTLQVQP	113			
FL9.38	-G-RFTAHLNKASLHVSLHIRDSQPS	DSALYFC	ALSESSGSWQLIFG	SGTQLTVMP	112			
В	тс	<b>R</b> β ch CDR1	ains for FL9					
FL9.41	VTLLEQNPRWRLVPRGQAVNLRCIL	NSQYPW	ISWYQQDLQKQLQWLFT	LRSPGEKEVK	5 60			
FL9.32	EAAVTQSPRNKVAVTGGKVTLSCNQT	NN-HNN	IYWYRQDTGHGLRLIHY	SYGAGS TEKGI	59			
FL9.38	EAAVTQSPRSKVAVTGGKVTLSCHQT	NN-HDY	IYWYRQDTGHGLRLIHY	SYVADSTEKGI	59			
FL9.15	DSGVVQSPRHIIKEKGGRSVLTCIPI	SG-HSN	VWYQQTLGKELKFLIQ	HYEKVERDKGI	59			
FL9.35	DSGVVQSPRHITKEKGGRSVLTCIPI	SG-HSNU	VWIQQTLGKELKFLIQ	HYEKVERDKG	- 59 - 59			
FL9.2	DSGVVQSTRIIIKEKGGRSVLTCIPI	SG-HSN	VWYOOTLGKELKFLIO	HYEKVERDKGI	59			
FL9.40	DSGVVQSPRHIIKEKGGRSVLTCIPI	SG-HSN	VWYQQTLGKELKFLIQ	HYEKVERDKGI	59			
FL9.23	NSGVVQSPRYIIKGKGERSILKCIPI	SG-HLS	AWYQQTQGQELKFFIQ	hydk <b>me</b> rdkgi	1 59			
FL9.5	NSGVVQSPRYIIKGKGERSILKCIPI	SG-HLS	VAWYQQTQGQELKFFIQ	HYDKME RDKGI	1 59			
FL9.8	NSGVVQSPRYIIKGKGERSILKCIPI	SG-HLS	AWYQQTQGQELKFFIQ	HYDKMERDKGI	1 59			
FL9.34	NSGVVQSPRYLIKGKGERSILKCIPI	SG-HLS	AWYQQTQGQELKFFIQ	HYDKME RDKGI	N 59			
	· · · · ·	•••••	CDR3					
FL9.41	LPGADYLATRVTDTELRLQVANMS	QGRTLYC	TCSARQGS-GNTLYFG	EGSRLIVV	114			
FL9.32	IPDG-YKASRPSQENFSLILELATPS	QTSVYFC	ASGTGDERLFFG	HGTKLSVL	111			
FL9.38	IPDG-YKASRPSQENFSLILELASLS	QTAVYFO	ASRDRGDQNTLYFG	AGTRLSVL	113			
FL9.15	LPSR-FSVQQFDDYHSEMNMSALELE	DSAMYFO	ASSLGQGN-YAEQFFG	PGTRLTVL	114			
ETA 30	LPSR-FSVQQFDDYHSEMNMSALELE	DSAMYFO	ASSETGGS-AETLYFG	SGTRLTVL	114 114			
FL9.2	LPSR-FSVOOFDDYHSEMNMSALELE	DSAMYFO	ASSPRLAS-AETLIEG	SGTRLTVL SGTRLTVI.	114			
FL9.40	LPSR-FSVQQFDDYHSEMNMSALELE	DSAMYFO	ASSPRLGS-AETLYFG	SGTRLTVL	114			
FL9.23	LPSR-FSVQQFDDYHSEMNMSALELE	DSAVYFO	ASSLRGV-SNERLFFG	HGTKLSVL	114			
FL9.5	LPSR-FSVQQFDDYHSEMNMSALELE	DSAVYFO	ASSLTGAYEQY <mark>F</mark> G	PGTRLTVL	112			
FL9.8	LPSR-FSVQQFDDYHSEMNMSALELE	DSAVYFO	ASSLLGGPSAETLYFG	SGTRLTVL	115			
FL9.34	LPSR-FSVQQFDDYHSEMNMSALELE	USAVYFC	ASSPGPSQNTLYFG	AGTRLSVL	113			
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#### Supplemental Figure 2. TCR repertoire of Qa-1–Hsp60 Tet<sup>+</sup> CD8 T cells.

Single Qa-1–Hsp60-PE<sup>+</sup>Qa-1–Hsp60-APC<sup>+</sup> cells were sorted and subjected to sequencing for TCR $\alpha$  and TCR  $\alpha$  and TCR $\beta$  pairs were analyzed based on their TCR V gene segments. Alignment of TCR $\alpha$  (**A**) and TCR $\beta$  (**B**) sequences obtained from Qa-1–Hsp60 Tet<sup>+</sup> single cells. TCR V $\alpha$  and V $\beta$  gene usage by each Qa-1–Hsp60 Tet<sup>+</sup> CD8 T cell are shown on the right. TRAV9N3 (V $\alpha$ 3.2) and/or TRBV12.1/2 (V $\beta$ 5.1/2) expressing CD8 T cell clones are highlighted.

Α	TCR $\alpha$ chains for Hsp60 TCR			
	CDR1 CDR2		Vα	Vβ
Hsp60.1	GDSVTQTEGPVTVSESESLIINCTYSATSIAYPNLFWYVRYPGEGLQLLLKVITAGQ-K	(G 59	TRAV6D-6	STRBV12-1
Hsp60.15	GDSVTQTEGPVTVSESESLIINCTYSATSIAYPNLFWYVRYPGEGLQLLLKVITAGQ-K	(G 59	TRAV6D-6	STRBV12-1
Hsp60.40	GDSVTQTEGPVTVSESESLIINCTYSATSIAYPNLFWYVRYPGEGLQLLLKVITAGQ-K	G 59	TRAV6D-6	STRBV13-2
Hsp60.43	AQSVTQPDARVTVSEGASLQLRCKYSYFGTPYLFWYVQYPRQGLQLLLKYYPGDPVV	7Q 58	TRAV9N3	TRBV12-2
Hsp60.18	AQSVTQPDARVTVSEGASLQLRCKYSYFGTPYLFWYVQYPRQGLQLLLKYYPGDPVV	7Q 58	TRAV9N3	TRBV12-2
Hsp60.44	AQSVTQPDARVTVSEGASLQLRCKYSYFGTPYLFWYVQYPRQGLQLLLKYYPGDPVV	7Q 58	TRAV9N3	TRBV13-2
Hsp60.29	AQSVTQPDARVTVSEGASLQLRCKYSYFGTPYLFWYVQYPRQGLQLLLKYYPGDPVV	7Q 58	TRAV9N3	TRBV13-2
Hsp60.5	AQSVTQPDARVTVSEGASLQLRCKYS <mark>YFGTPY</mark> LFWYVQYPRQGLQLLLK <mark>Y</mark> YPGDPV <mark>V</mark>	7Q 58	TRAV9N3	TRBV13-2
Hsp60-31	AQSVTQPDARVTVSEGASLQLRCKYS <mark>YFGTPY</mark> LFWYVQYPRQGLQLLLK <mark>YYPGDPV</mark> V	7Q 58	TRAV9N3	TRBV13-2
Hsp60.6	AQSVTQPDARVTVSEGASLQLRCKYS <mark>YFGTPY</mark> LFWYVQYPRQGLQLLLK <mark>YYPGDPV</mark> V	7Q 58	TRAV9N3	TRBV12-1
Hsp60-19	AQSVTQPDARVTVSEGASLQLRCKYSYFGTPYLFWYVQYPRQGLQLLLKYYPGDPVV	7Q 58	TRAV9N3	TRBV12-2
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	<u> </u>			
Hsp60.1	SSRGFEATYNKETTSFHLQKASVQESDSAVYYCALGEASSGSWQLIFGSGTQLTVMP 1	.16		
Hsp60.15	SSRGFEATYNKETTSFHLQKASVQESDSAVYYCALGEASSGSWQLIFGSGTQLTVMP 1	.16		
Hsp60.40	SSRGFEATYNKETTSFHLQKASVQESDSAVYYCALGEASSGSWQLIFGSGTQLTVMP 1	.16		
Hsp60.43	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSI-SNNRIFFGDGTQLVVKP 1	.12		
Hsp60.18	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSSNYNVLYFGSGTKLTVEP 1	.12		
Hsp60.44	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSSNYNVLYFGSGTKLTVEP 1	.12		
Hsp60.29	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSR-A-NTGKLTFGDGTVLTVKP 1	.13		
Hsp60.5	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSK-DSGYNKLTFGKGTVLLVSP 1	.14		
Hsp60-31	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSK-DSGYNKLTFGKGTVLLVSP 1	.14		
Hsp60.6	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSK-STG-SKLSFGKGAKLTVSP 1	.13		
Hsp60-19	GVNGFEAEFSKSNSSFHLRKASVHWSDWAVYFCAVSS-TGGYK-VVFGSGTRLLVSP 1	.13		
	· ·**** : ·* · · :**** : ** *** ********			

TCR $\beta$  chains for Hsp60 TCR

CDR1		
TOM	NNHNN	MYWYRODTCHCLPLT

CDR2

Hsp60.5	EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNNMYWYRQDTGHGLRLIHYSYGAGSTEKGDI	60
Hsp60.29	EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNNMYWYRQDTGHGLRLIHY <mark>S</mark> YGAG <mark>STEKGDI</mark>	60
Hsp60.31	EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNNMYWYRQDTGHGLRLIHY <mark>S</mark> YGAG <mark>STEKGDI</mark>	60
Hsp60.40	EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNN <mark>MYWYRQDTGHGLRLIHY</mark> SYGAG <mark>S</mark> TEKGDI	60
Hsp60.44	EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNNMYWYRQDTGHGLRLIHY <mark>S</mark> YGAG <mark>STEKGDI</mark>	60
Hsp60.18	NSGVVQSPRYIIKGKGERSILKCIPI <mark>SGHLS</mark> VAWYQQTQGQELKFFIQ <mark>HYDKM</mark> ERDKGNL	60
Hsp60.19	NSGVVQSPRYIIKGKGERSILKCIPI <mark>SGHLS</mark> VAWYQQTQGQELKFFIQ <mark>HYDKM</mark> ERDKGNL	60
Hsp60.43	NSGVVQSPRYIIKGKGERSILKCIPI <mark>SGHLS</mark> VAWYQQTQGQELKFFIQ <mark>HYDKM</mark> ERDKGNL	60
Hsp60.1	DSGVVQSPRHIIKEKGGRSVLTCIPI <mark>SGHSN</mark> VVWYQQTLGKELKFLIQ <mark>HYEKV</mark> ERDKGFL	60
Hsp60.15	DSGVVQSPRHIIKEKGGRSVLTCIPI <mark>SGHSN</mark> VVWYQQTLGKELKFLIQ <mark>HYEKV</mark> ERDKGFL	60
Hso60.6	DSGVVQSPRHIIKEKGGRSVLTCIPI <mark>SGHSN</mark> VVWYQQTLGKELKFLIQ <mark>HYEKV</mark> ERDKGFL	60
	::.*.**** : .* : *.** <mark>:</mark> **:* *: *::: * . :** :	
Hsp60.5	PDGYKASRPSQENFSLILELATPSQTSVYF <mark>C</mark> ASGTGDERL <mark>F</mark> FGHGTKLSVL 111	
Hsp60.29	pdgykasrpsqenfslilelatpsqtsvyf <mark>c</mark> asgtgderl <mark>f</mark> fghgtklsvl 111	
Hsp60.31	pdgykasrpsqenfslilelatpsqtsvyf <mark>c</mark> asgtgderl <mark>f</mark> fghgtklsvl 111	
Hsp60.40	pdgykasrpsqenfslilelatpsqtsvyf <mark>c</mark> asgtgderlffghgtklsvl 111	
Hsp60.44	pdgykasrpsqenfslilelatpsqtsvyf <mark>c</mark> asgtgderl <mark>f</mark> fghgtklsvl 111	
Hsp60.18	psrfsvqqfddyhsemnmsaleledsavyf <mark>c</mark> asslvsgs-aeqffgpgtrltvl 113	
Hsp60.19	psrfsvqqfddyhsemnmsaleledsavyf <mark>c</mark> asslag-req <mark>yfgpgtrltvl</mark> 111	
Hsp60.43	psrfsvqqfddyhsemnmsaleledsavyf <mark>c</mark> asslag-req <mark>yfgpgtrltvl</mark> 111	
Hsp60.1	psrfsvqqfddyhsemnmsaleledsamyf <mark>c</mark> asslgqgnyaeqffgpgtrltvl 114	
Hsp60.15	PSRFSVOOFDDYHSEMNMSALELEDSAMYFCASSLGOGNYAEOFFGPGTRLTVL 114	
110000.10		
Hso60.6	PSRFSVQQFDDYHSEMNMSALELEDSAMYFCASSRANYEQYFGPGTRLTVL 111	

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#### Supplementary Figure 3. Control study of TCR chain usage:

technique utilized collect А single-cell sorting was to two types of Т cells: TCRβ<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup>CD122<sup>+</sup>Ly49<sup>+</sup> T cells (45 cells) and TCRβ<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup>CD122<sup>+</sup>Ly49<sup>-</sup> T cells (45 cells). The sorted cells underwent RT-PCR amplification of TCR  $\alpha$  and  $\beta$  chains, followed by electrophoresis. Paired amplification was observed in 37 out of 45 Ly49<sup>+</sup> cells (82%) and 33 out of 45 Ly49<sup>-</sup> cells (73.3%). Sanger sequencing analysis revealed the specific TCR chain usage. Among the Ly49<sup>+</sup> cells, 4 cells exhibited TRAV9N-3 (Vα3.2) gene presence (10.8%), while only 1 cell displayed TRAV9N-3 positivity (3%) in the Ly49<sup>-</sup> population.



#### Supplemental Figure 4. Gating strategy for detection of Ly49<sup>+</sup>V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup> CD8 T cells.

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**A)** Gating strategy for V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup> CD8 cell detection. Frequency of V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup> CD8 cells in the Ly49<sup>+</sup> and Ly49<sup>-</sup> CD8 cells. **B**) Frequency of V $\alpha$ 3.2<sup>+</sup> or V $\beta$ 5<sup>+</sup> cells within Ly49<sup>+</sup> CD8 cells in spleen and LNs of WT B6, Qa-1.D227K KI (DK) and Qa-1 KO (KO) mice at 8 weeks of age.









# Supplemental Figure 5. Generation of FL9 TCR<sup>+</sup> hybridoma and analysis of their responsiveness to FL9 peptide.

**A**)  $58C\alpha^{-\beta^{-}}$  hybridomas were transduced with OT-I TCR or FL9 TCRs that were identified from single cell TCR sequencing from Qa-1–FL9 tet<sup>+</sup> CD8 T cells. Correct folding and assembly of transduced TCRs and their expected specificity were tested by staining with anti-CD3, anti-TCR V $\beta$  and Qa-1–Hsp60 or Qa-1–FL9 tetramers. **B**) Qa-1–FL9 tetramer-based detection of TCRs on 58C hybridomas that were transduced with individual TCR pairs (sequences shown in Suppl. Fig. 1) isolated from Qa-1–FL9-specific single CD8 T cells. **C**) Responsiveness of FL9 TCR<sup>+</sup> hybridoma to increasing concentrations of FL9 peptide depicted by surface CD69 expression.



# Supplemental Figure 6. Qa-1-dependent differentiation of FL9 T cells.

**A)** Tg TCR<sup>+</sup> cells (V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup>) in TCR<sup>+</sup> thymocytes and percent of active-Caspase 3<sup>+</sup>PD1<sup>+</sup> cells in DP (CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes in OT-I  $\rightarrow$  WT B6, FL9.8 Tg  $\rightarrow$  WT B6 BM chimera 8 wks after BM reconstitution. **B**) Ki67 and CD44 expression by OT-I and FL9.8 TCR Tg CD8<sup>+</sup> T cells was measured as an indication of Ag encounter in spleen and liver of OT-I  $\rightarrow$  WT B6 and FL9.8 Tg  $\rightarrow$  WT B6 BM chimeras 8 wks after BM reconstitution. OT-1 is also used as a control in Fig. 1H. **C)** TCR and CD8 expression on polyclonal non-Tg CD8 cells, OT-I, FL9.2 and FL9.8 Tg T cells.



**B** Ki67 and CD44 expression in the periphery establishes recognition of self-antigens by FL9 cells



**C** TCR and CD8 expression on FL9 Tg T cells reflects chronic Ag activation



# Supplemental Figure 7. NKG2D expression by FL9 TCR Tg T cells.

Acquisition of NKG2D expression by FL9.2 (**A**) and FL9.8 (**B**) T cells with age. Percentage of NKG2D<sup>+</sup> cells within CD8 $\alpha\beta^+$  FL9 T cells from spleen, LN and liver in FL9 TCR Tg mice at the ages of 18 days, 9 wks and 4 mos.



Increased expression NKG2D with age by FL9.2 T cells

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Increased expression of NKG2D with age by FL9.8 T cells



#### Supplemental Figure 8. Qa-1 dependent phenotype acquisition by FL9.8 Tg T cells.

**A**) Frequency of V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup> T cells in TCR $\beta$ <sup>+</sup> cells from FL9.8 TCR Tg mice on Qa-1 WT and KO backgrounds. Representative FACS plots for detection of V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup> cells in spleen are shown on the left. **B**) Expression of CD44 and NKG2D by FL9.8 TCR Tg T cells in spleen and LNs of Qa-1 WT and Qa-1 KO mice. Representative FACS plots for NKG2D<sup>+</sup>CD44<sup>+</sup> cells in spleens of FL9.8 TCR Tg mice are shown on the left. **C**) Expression of Ki67 by FL9.8 T cells in the LNs of WT.FL9.8 TCR Tg and Qa-1<sup>-/-</sup>.FL9.8 TCR Tg mice at 8 wks of age.





С



#### Supplemental Figure 9. FL9 Tg CD8 T cells recognize activated CD4 T cells.

**A)** ConA-stimulated CD4 cells from WT B6, Qa-1.D227K KI (DK), K<sup>b</sup>D<sup>b</sup> KO and ERAAP KO mice were co-cultured with FL9.8 T cells isolated from spleen and LNs of FL9.8 TCR Tg mice. After 20 hrs, CD69 expression on FL9 Tg T cells was measured as a readout of TCR stimulation. ConA-stimulated WT CD4 cells loaded with FL9 peptide were used as a positive control. **B)** OT-I or FL9.8 TCR<sup>+</sup> hybridomas were co-cultured with ConA-stimulated CD4 cells from WT or ERAAP KO mice. Stimulation of OT-1 or FL9 TCR was measured according to CD69 expression by hybridoma cells. Mean ± SEM is indicated. \*\*\*\*P < 0.0001, \*\*\*P < 0.001, \*\*P < 0.05.



OT-1 FL9.8

## Supplemental Figure 10. Antibody-dependent depletion of V $\alpha$ 3.2<sup>+</sup> Ly49<sup>+</sup> CD8 T cells.

WT B6 and B6.Qa-1.D227K mice were immunized with Ova/CFA along with injection of rat IgG2b or anti-V $\alpha$ 3.2 Abs at day 0. These mice were boosted with Ova/IFA on day 8 and also injected with Abs. At day 15, the presence of V $\alpha$ 3.2<sup>+</sup>V $\beta$ 5<sup>+</sup> cells was assessed in total T, CD8 T and Ly49<sup>+</sup> CD8 T cells. Shown are plots from analysis of spleen cells from WT mice.



#### Supplemental Figure 11. Failure of native FL9 peptide to stimulate FL9.2 T cells.

CD45.1<sup>+</sup> B6 hosts were adoptively transferred with FL9.2 T cells and immunized i.p. with FL9 in CFA or no peptide (CFA alone) on day 0. Three days later, activation (CD69) and proliferation (Ki67) of FL9.2 T cells were measured.



## Supplemental Figure 12. Identification of superagonist peptides.

Activation of FL9.8 T cells after stimulation with FL9 variants selected from the library screen described in Fig. 4. Dose-dependent activation of FL9.8 T cells was measured by culturing FL9.8 T cells with EL4 (Qa-1<sup>+</sup>) at various concentrations of the indicated peptides (0, 1, 3 and  $10\mu$ g/ml).



# Supplemental Table 1. Primer sequences for the amplification of TCR $\alpha$ and TCR $\beta$ sequences

	Primers for TCRα		Primers for TCRβ
AL-1	AAGGATCCGAATTCCTGCAGGatgctgcagatgtgggggtttg	BL-1	AAGGATCCGAATTCCTGCAGGatgtggcagttttgcattctg
AL-10	AAGGATCCGAATTCCTGCAGGatgaagacatcccttcacac	BL-2	AAGGATCCGAATTCCTGCAGGatgggctccattttcctcag
AL-10-4	AAGGATCCGAATTCCTGCAGGatggataaaacatcccttc	BL-3	AAGGATCCGAATTCCTGCAGGatggatatctggcttctag
AL-10D	AAGGATCCGAATTCCTGCAGGatggattaagacatcccttc	BL-4	AAGGATCCGAATTCCTGCAGGatgggctgtaggctcctaag
AL-11	AAGGATCCGAATTCCTGCAGGatgaaaaagYgcctKagtgcctg	BL-5	AAGGATCCGAATTCCTGCAGGatgagctgcaggcttctc
AL-14-1	AAGGATCCGAATTCCTGCAGGatggacaMgaWcctgacag	BL-12-1	AAGGATCCGAATTCCTGCAGGatgtctaacactgtcctcgctg
AL-14D-1	AAGGATCCGAATTCCTGCAGGatggacaagattctgacag	BL-12-2	AAGGATCCGAATTCCTGCAGGatgtctaacactgccttccctg
AL-15-1	AAGGATCCGAATTCCTGCAGGatgcctcctcacagcctgYtc	BL-13-1	AAGGATCCGAATTCCTGCAGGatgggctccaggctctttc
AL-15-2	AAGGATCCGAATTCCTGCAGGatgcctcctcagagcctgctc	BL-13-2	AAGGATCCGAATTCCTGCAGGatgggctccaggctcttcttc
AL-16	AAGGATCCGAATTCCTGCAGGatgctgattctaagcctgYtg	BL -13-3	
AL-17	AAGGATCCGAATTCCTGCAGGatgttccYagtgaccattctg	BL -14	
AL-19	AAGGATCCGAATTCCTGCAGGatgactggcttcctgaag	BL_15	
AL-2	AAGGATCCGAATTCCTGCAGGatgaagcaggtggcaaaagt	DL-10	
AL-21	AAGGATCCGAATTCCTGCAGGatgggatgtgtgagtggaattg	DL-10	
AL-3-1	AAGGATCCGAATTCCTGCAGGatgaagacRgtgactgga	DL-17	
AL-3-4	AAGGATCCGAATTCCTGCAGGatgaaaacagtgRctgga	BL-19	
AL-4-2	AAGGATCCGAATTCCTGCAGGatggagaggagcccgggaac	BL-20	AAGGATCCGAATTCCTGCAGGatgttactgcttctattacttc
AL-4-3	AAGGATCCGAATTCCTGCAGGatgSagaggaacctggttg	BL-23	AAGGATCCGAATTCCTGCAGGatgggtgcacggctcatttg
AL-4-4	AAGGATCCGAATTCCTGCAGGatgcagaggaacctgggag	BL-24	AAGGATCCGAATTCCTGCAGGatgggtgcaagactgctctg
AL-5-1	AAGGATCCGAATTCCTGCAGGatgaagacagctattcatg	BL-26	AAGGATCCGAATTCCTGCAGGatggctacaaggctcctctg
AL-5D-4	AAGGATCCGAATTCCTGCAGGatgaaaacataYgctcctac	BL-29	AAGGATCCGAATTCCTGCAGGatgagagttaggctcatctctg
AL-6-1	AAGGATCCGAATTCCTGCAGGatgaacWattcYccagcttta	BL-30	AAGGATCCGAATTCCTGCAGGatgtggacattcctgctacttc
AL-6-4	AAGGATCCGAATTCCTGCAGGatgaaYacttctccagYttta	BL-31	AAGGATCCGAATTCCTGCAGGatgctgtactctctccttgc
AL-6-5	AAGGATCCGAATTCCTGCAGGatgaacctttRtcctgaactg		
AL-6-6	AAGGATCCGAATTCCTGCAGGatgRactcttctccaggcttc		
AL-7-1	AAGGATCCGAATTCCTGCAGGatgaagtccttgtgtgtttc		
AL-7-2	AAGGATCCGAATTCCTGCAGGatgaaatccttKagtRtttc		
AL-7-6	AAGGATCCGAATTCCTGCAGGatgcattccttacatgtttc		
AL-7D-4	AAGGATCCGAATTCCTGCAGGatggtacaaacacagatgttc		
AL-7D-4-	AAGGATCCGAATTCCTGCAGGatgaaatccttgagtgtttta		
AL-8-1	AAGGATCCGAATTCCTGCAGGatgcacagcctcctRgggttg		
AL-8-1-3	AAGGATCCGAATTCCTGCAGGatgcacagcctcctggggttg		
AL-8-2	AAGGATCCGAATTCCTGCAGGatgaacagattcctgggaata		
AL-9-1	AAGGATCCGAATTCCTGCAGGatgctcctggtYctcatctc		
AL-9-2	AAGGATCCGAATTCCTGCAGGatgctcctggYgctcctcYcag		
AL-9D-4	AAGGATCCGAATTCCTGCAGGatgctcctggcactcctcccag		
AL-12-1	AAGGATCCGAATTCCTGCAGGatgcgtcctgDcacctgctc		
AL-12D-1	AAGGATCCGAATTCCTGCAGGatgcgtcctgtcacctcctc		
AL-13-1	AAGGATCCGAATTCCTGCAGGatgaacaggctgctgtgctctc		
AL-13-2	AAGGATCCGAATTCCTGCAGGatgaagaggctgctgtgttctc		
AL-13D-1	AAGGATCCGAATTCCTGCAGGatgaagaggctgctgagctctc		