Supporting Information

Keck et al. 10.1073/pnas.1403271111

SI Materials and Methods

Mice. Six- to 8-wk-old C57BL/6, B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ (CD45.1), B3K508 T-cell receptor (TCR) transgenic (1), and CD3ε$^{-/-}$ mice (2) were bred in our colony. Mb1-Cre mice used to generate Igα$^{-/-}$ mice were kindly provided by Dr. Michael Reth (Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany) (3). IL-2 GFP Ki mice (4) were crossed with B3K508 TCR transgenic mice to examine IL-2 production after 24 h of in vitro activation.

Infections. Recombinant ActA-deficient *Listeria monocytogenes* (Lm) strains expressing the FEAQKAKANKAVD (3K) epitope or the altered peptide ligands FEAQKARANKAVD (P5R), FEAAKAKANKAVD (P2A) or FAAQKAKANKAVD (P-1A) were generated using previously described expression constructs (5–7), which were manipulated by site-directed mutation to introduce the altered sequence. Virulent Lm and ActA-deficient Lm stably expressing chicken ovalbumin have been previously described (8). Frozen stocks of these strains were grown in brain–heart infusion broth to mid log phase. Bacterial numbers were determined by measuring the OD at 600 nm. Mice were injected i.v. with 10^7 total colony forming units (cfu) ActA deficient. In the case of low dose 3K infections, mice received 10^5 cfu of Lm expressing 3K and 9.9×10^6 cfu of wild-type Lm. For recall infections, mice were injected with 10^6 cfu of virulent Lm.3K, diluted in PBS. Vaccinia virus strains expressing 3K or 3K.APLs were previously described (9). Frozen stocks were diluted in PBS and 10^7 pfu were injected intraperitoneally.

Immunofluorescent Microscopy. Spleens were isolated on day 6 after *Listeria* infection, embedded in OCT matrix (CellPath) and frozen on dry ice. Seven-micrometer sections were cut with a cryostat, air dried, and fixed with cold acetone. Sections were stained with 1 μg/mL mAb against B220, F4/80, and Ly5.2. Some sections were additionally stained with CD3 to confirm T-cell and B-cell boundaries. Images were captured on a Nikon A1R-A1 microscope. Random and nonconsecutive sections were used for quantitative analysis to avoid recounting duplicate T-cell and B-cell zones.

Purification of T Cells, Adoptive Cell Transfer, and Cell Labeling. Spleen, thymus, and lymph node suspensions were obtained by mashing total organs through a 100-μm nylon cell strainer (BD Falcon). Red blood cells were lysed with a hypotonic ACK lysis buffer. Unless otherwise indicated, 1×10^4 B3K508 TCR transgenic T cells were injected into the tail vein of congenic CD45.1 mice. For analysis of donor T cells after infection, spleen and lymph node cells were prepared and stained with allophycocyanin-conjugated CD45.2 antibody (BD Pharmingen). Samples were enriched for bead-bound cells on magnetized columns and an aliquot was removed for counting as described (10).

Surface and Intracellular Antibody Staining and Flow Cytometry. To detect surface phenotype, purified donor cells were stained with a mixture of antibodies against CXCR5 (2G8), B220 (RA3-6B2), CD11b (MI-70), CD11c (N418), PD-1 (J43), CD4 (RM4-5), CD3ε (145-2C11), CD25 (PC61.5), CD44 (IM7), CD45.2 (104), and Ly6c (AL-21) each conjugated with a different fluorochrome. In experiments to detect T-bet and Bcl6 expression, surface-stained cells were treated with Fixation/Permeabilization Concentrate and Diluent (eBioscience) and stained for 1 h on ice with antibodies against T-bet (4B10; Biolegend) or Bcl6 (K112-91; BD). In experiments to detect intracellular cytokine production, splenocytes and lymph node cells from infected mice were cultured in vitro with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (200 ng/mL), and Golgi-stop for 4 h before donor cell enrichment. Enriched cells were surface stained, fixed and permeabilized as described above, and then stained with antibodies against IL-2 (JES6-5H4) or IFNγ (XMG1.2).

Analysis of Follicular Helper T Function. The 1×10^5 B3K508 TCR transgenic T cells were transferred into congenic mice followed by infection with either Lm.3K or Lm.P2A. Flow cytometric sorting was used to purify follicular helper T (T_{FH}) (CXCR5^{+}PD-1^{hi}) and T helper (Th)1 (CXCR5^{-}Ly6c^{high}) cells from infected mice before adoptive transfer of $2–5 \times 10^3$ cells into CD3ε$^{-/-}$ recipients that had been immunized 1 d earlier with 100 μg of 3K-OVA (Eurogentec), emulsified in alum (Pierce). Splenocytes were analyzed by FACS after 6 d.

1. Govern CC, Paczosa MK, Chakraborty AK, Huseby ES (2010) Fast on-rates allow short dwell time ligands to activate T cells. *Proc Natl Acad Sci USA* 107(19):8724–8729.
2. Malissen M, et al. (1995) Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *EMBO J* 14(19):4641–4653.
3. Hobeika E, et al. (2006) Testing gene function early in the B cell lineage in mb1-cre mice. *Proc Natl Acad Sci USA* 103(37):13789–13794.
4. Naramura M, Hu RJ, Gu H (1998) Mice with a fluorescent marker for interleukin 2 gene activation. *Immunity* 9(2):209–216.
5. Zehn D, Lee SY, Bevan MJ (2009) Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458(7235):211–214.
6. Pope C, et al. (2001) Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. *J Immunol* 166(5):3402–3409.
7. Lauer P, Chow MY, Loessner MJ, Portnoy DA, Calendar R (2002) Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors. *J Bacteriol* 184(15):4177–4186.
8. Tvinnereim AR, Hamilton SE, Harty JT (2002) CD8(+)-T-cell response to secreted and nonsecreted antigens delivered by recombinant Listeria monocytogenes during secondary infection. *Infect Immun* 70(1):153–162.
9. Vanguri V, Govern CC, Smith R, Huseby ES (2013) Viral antigen density and confinement time regulate the reactivity pattern of CD4 T-cell responses to vaccinia virus infection. *Proc Natl Acad Sci USA* 110(1):288–293.
10. Moon JJ, et al. (2009) Tracking epitope-specific T cells. *Nat Protoc* 4(4):565–581.

A

Peptide	Sequence	K_D	$t_{1/2}$	t_a	Thymic selection
3K	FEAQKAKANKAVD	29	2.2	2.8	above threshold
P5R	FEAQKARANKAVD	92	0.7	0.9	above threshold
P2A	FEAAKAKANKAVD	175	0.2	0.3	above threshold
P-1A	FAAQKAKANKAVD	263	ND	ND	threshold
P3A	FEAQAAKANKAVD	>550	ND	ND	below threshold

B

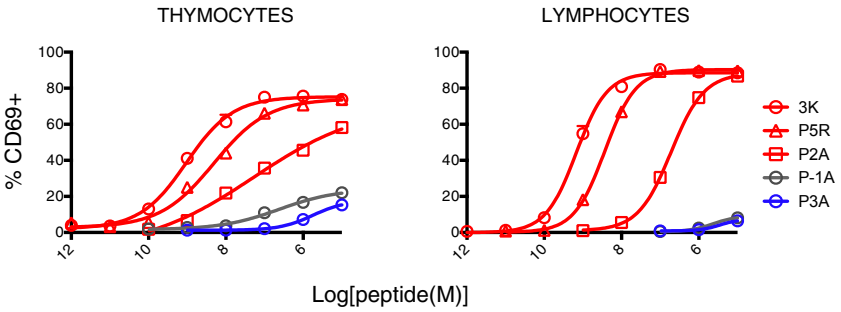

Fig. S1. Establishing the TCR ligand affinity hierarchy. (*A*) Table of peptides and thymic selection outcome in B3K508 fetal thymic organ culture. K_D, $t_{1/2}$ and t_a values were previously reported (1). (*B*) B3K508 thymocytes (*Left*) or lymphocytes (*Right*) were activated with peptide pulsed splenocytes. CD69 expression was assessed by flow cytometry after overnight culture. The corresponding peptide dose–response curves are shown. Data are representative of three independent experiments.

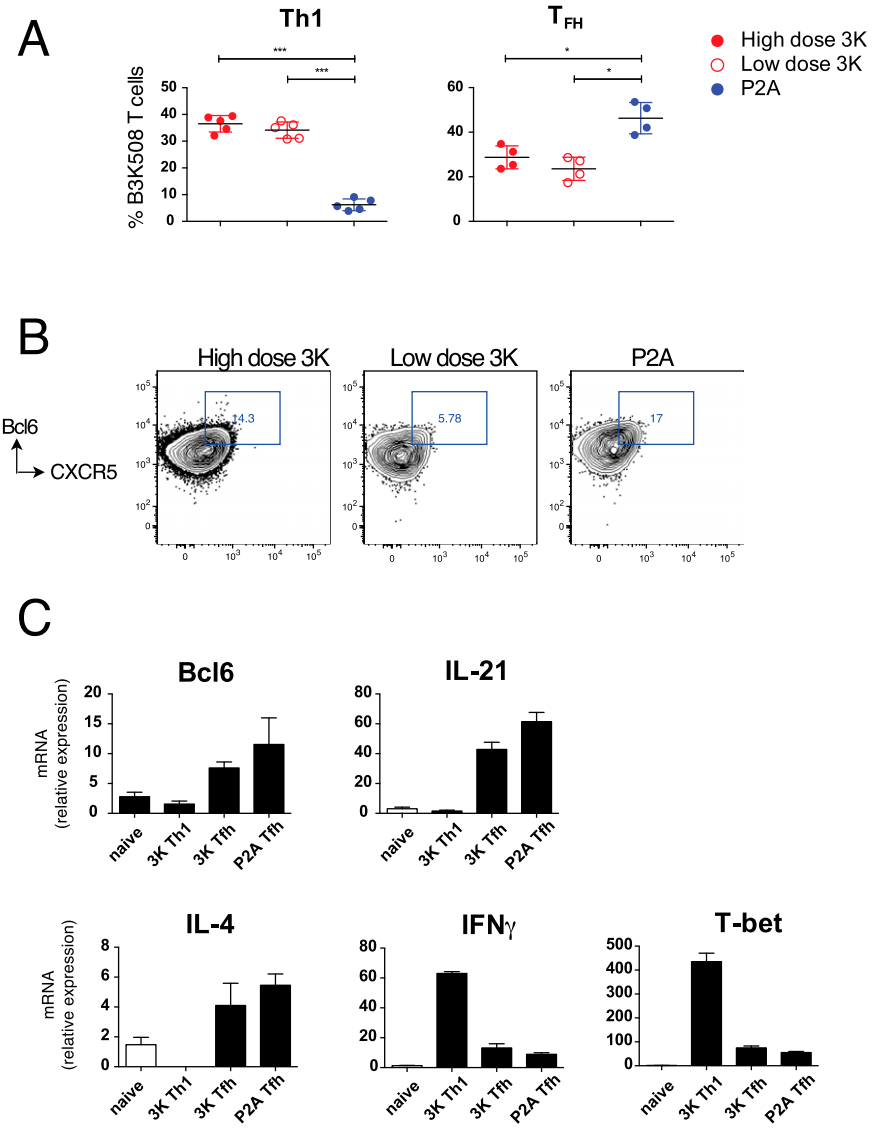

Fig. S2. Antigen affinity and effector T-cell differentiation. (*A*) Percentage of T-bet^{high}CXCR5^{-} Th1 cells and T-bet^{low}CXCR5^{+} T_{FH} cells. Statistical analysis was performed with an unpaired two-tailed Student *t* test. Error bars denote SEM. (*$P < 0.05$, ***$P < 0.0001$). (*B*) Bcl6 expression in B3K508 T cells at day 8 after infection. Representative data are from two independent experiments, $n = 5$ mice per group. (*C*) B3K508 Th1 cells (CXCR5^{-}Ly6c^{+}) generated after Lm.3K infection or T_{FH} cells (CXCR5^{+}PD-1^{+}) generated after Lm.3K or Lm.P2A infection were sorted and pooled for mRNA expression analysis by quantitative RT-PCR. The results are presented as relative expression ± SD between triplicate wells. Data represent $n \geq 3$ (Lm.3K) and $n \geq 8$ (Lm.P2A) pooled mice per sample and two individual experiments.

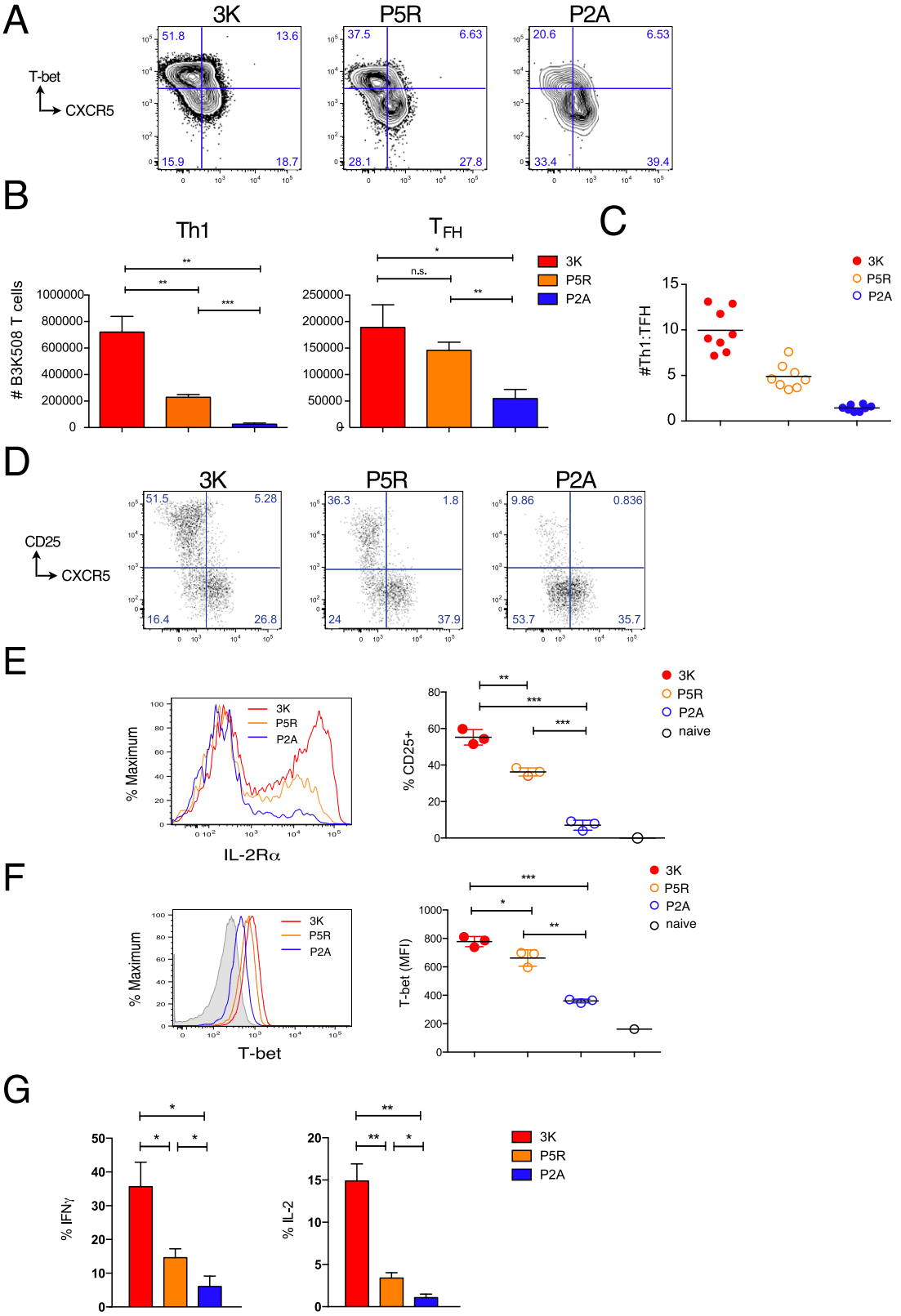

Fig. S3. Phenotype of B3K508 T cells responding to intermediate affinity ligand P5R. Ten thousand B3K508 T cells were transferred into congenic mice followed by infection with Lm.3K, Lm.P5R, or Lm.P2A. Representative flow cytometry plots (*A*) and absolute cell numbers (*B*) at day 6 after infection. (*C*) Ratio of the number of Th1:T_{FH} effectors is shown using combined data from two independent experiments. (*D–F*) IL-2Rα, CXCR5, and T-bet expression at day 3 after infection. Data represent $n = 4$ and are representative of two independent experiments. (*$P < 0.05$, ***$P < 0.0001$). (*G*) Intracellular cytokine production after 4 h of stimulation with PMA and ionomycin in vitro. Data represent $n = 3$ mice from two independent experiments.

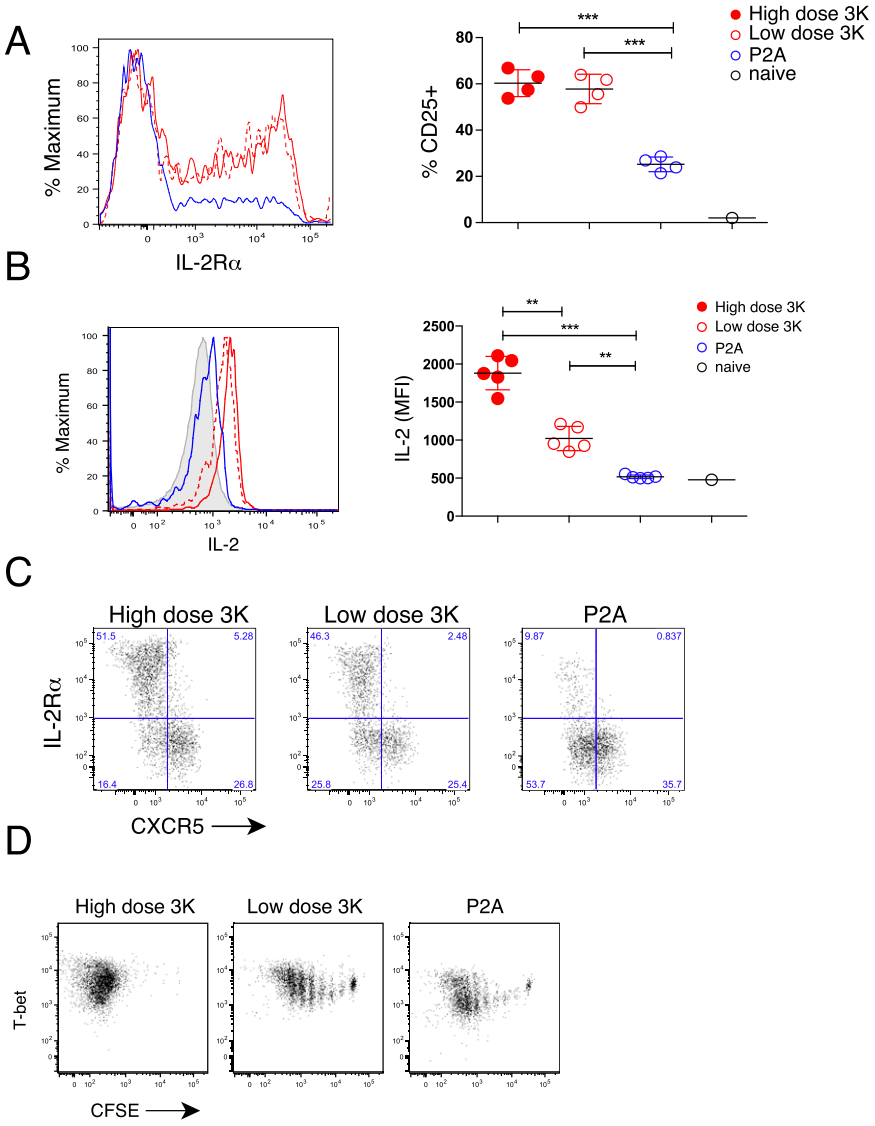

Fig. S4. Antigen affinity and early IL-2 induction. Two hundred thousand carboxyfluorescein succinimidyl ester-labeled B3K508 T cells were transferred into congenic mice followed by infection with Lm.3K or Lm.P2A. B3K508 T cells from spleen and lymph node were analyzed for (*A*) IL-2Rα expression and (*B*) intracellular IL-2 at day 2 after infection. (*C*) IL-2Rα and CXCR5 expression and (*D*) T-bet expression after 3 d of infection. Data represent $n \geq 3$ for each data point and are representative of two independent experiments (**$P < 0.001$, ***$P < 0.0001$).

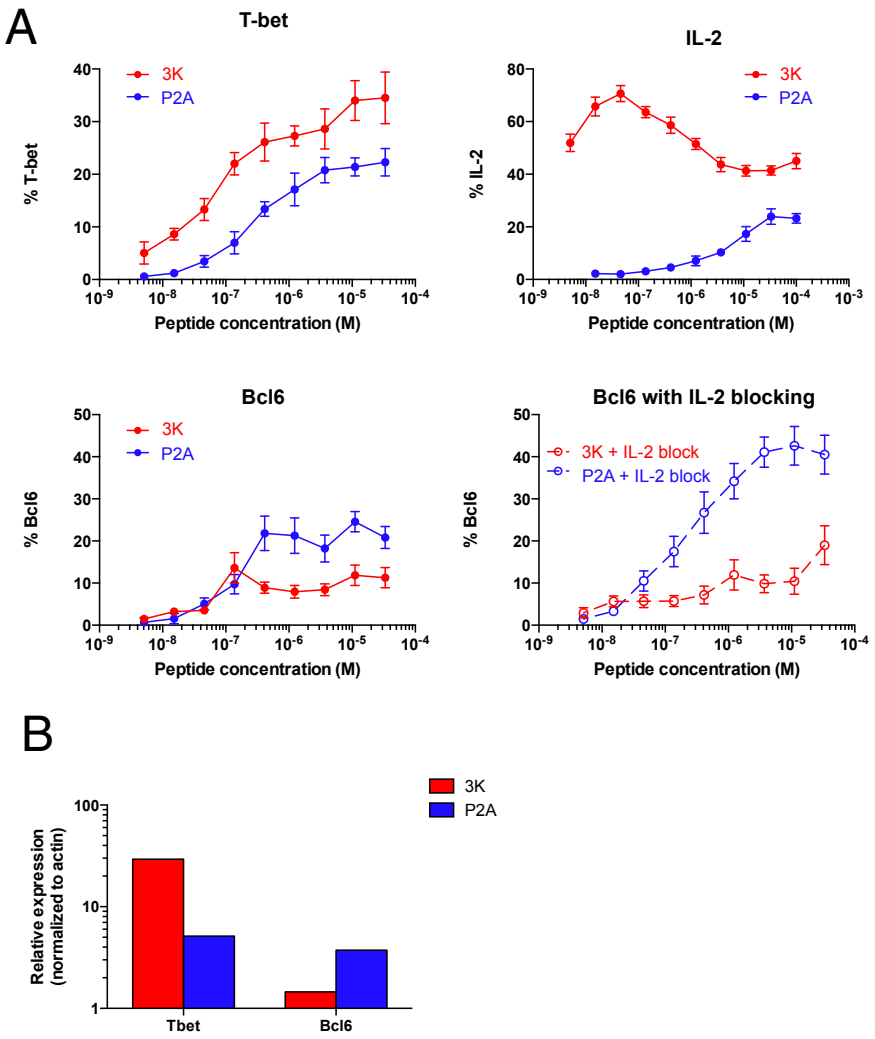

Fig. S5. Lymph node T cells from B3K508 RAG$^{-/-}$ or B3K508 x IL-2 GFP K_i mice were stimulated for 24 h with antigen pulsed dendritic cells in the presence or absence of IL-2 blocking antibodies (10 μg each of anti-CD25 and anti–IL-2). (*A*) T-bet, IL-2, and Bcl6 expression were measured by flow cytometry. (*B*) mRNA expression of T-bet and Bcl6 in B3K508 T cells stimulated with 10 μM 3K or P2A peptide.

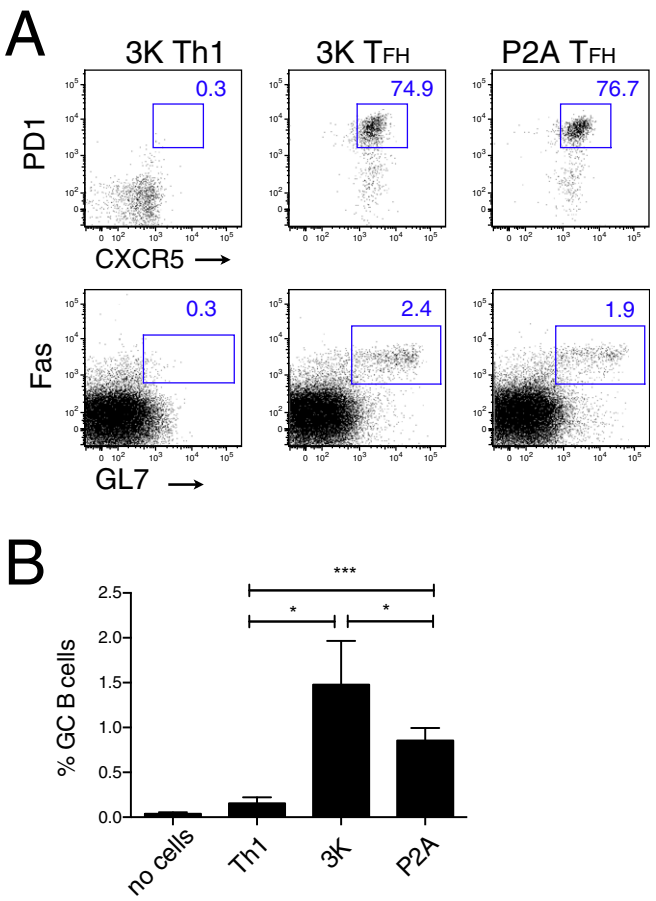

Fig. S6. Function of low affinity activated T cells. The 1×10^5 B3K508 T cells were transferred into congenic mice followed by infection with Lm.3K or Lm.P2A. At day 6, B3K508 Th1 cells from Lm.3K-infected mice, and T_{FH} cells from Lm.3K- or Lm.P2A-infected mice were sorted, pooled, and transferred into CD3$\varepsilon^{-/-}$ mice immunized 1 d earlier with 3K-OVA and alum. (*A*) Representative flow cytometry plots of B3K508 T cells and germinal center B cells (Fas^+GL7^+). (*B*) Percentage of GC B cells among total B cells.

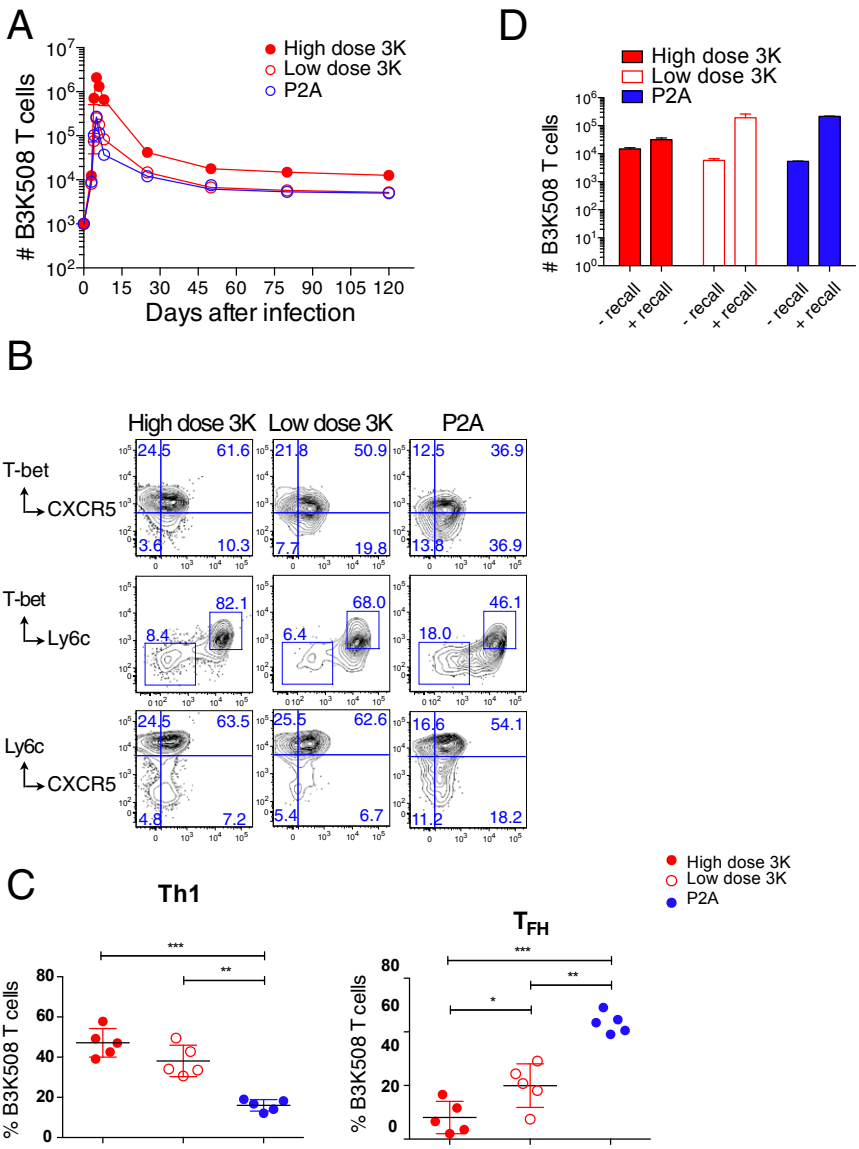

Fig. S7. Low affinity antigen supports memory T-cell differentiation. (*A*) Mean number of B3K508 T cells ($n \geq 3$ mice for each data point) recovered from congenic mice up to 120 d after transfer and infection with either Lm.3K or Lm.P2A. (*B*) Flow cytometry plots of B3K508 T cells recovered from spleen and lymph nodes ≥ 45 d after infection. Data representative of two independent experiments, $n \geq 3$ mice per experiment. (*C*) Percentage of T-bet^{high}CXCR5^{-} Th1 cells and T-bet^{low}CXCR5^{+} T_{FH} cells B3K508 T cells recovered from spleen and lymph nodes at day 4 after recall infection with virulent Lm.3K. (*D*) Number of B3K508 T cells recovered at day 4 after recall infection with virulent Lm.3K. Statistical analysis was performed with an unpaired two-tailed Student *t* test. Error bars denote SEM. (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

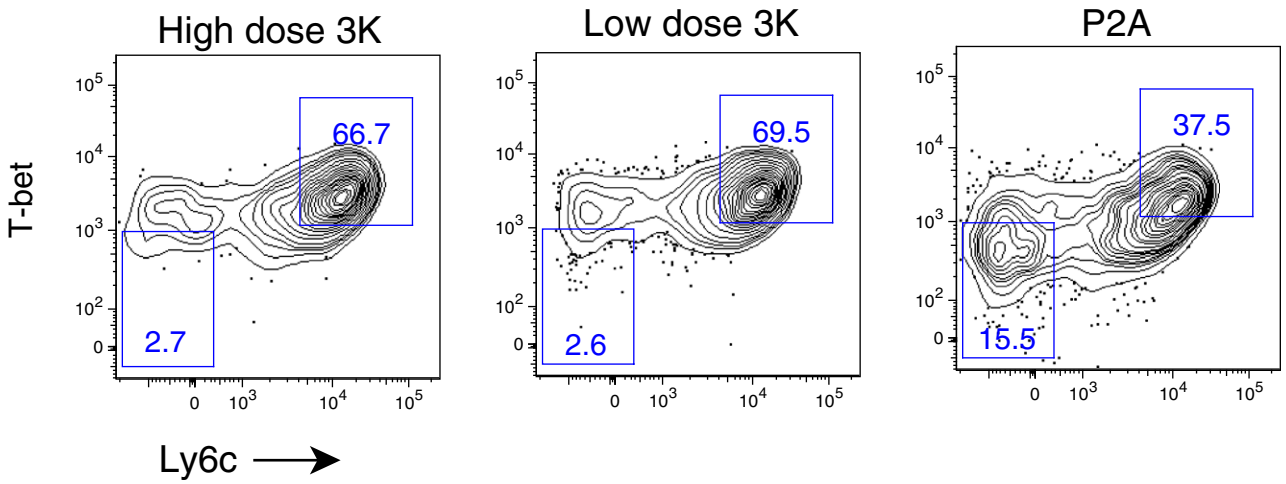

Fig. S8. B3K508 T-cell recall response following secondary infection with vaccinia virus. Ten thousand B3K508 T cells were transferred into congenic mice, followed by infection with Lm.3K or Lm.P2A. After 30–45 d, mice were given a secondary infection with vaccinia expressing 3K. The phenotype of B3K508 T cells was analyzed 3 d later. Data represent two independent experiments, $n = 3$ mice per group.

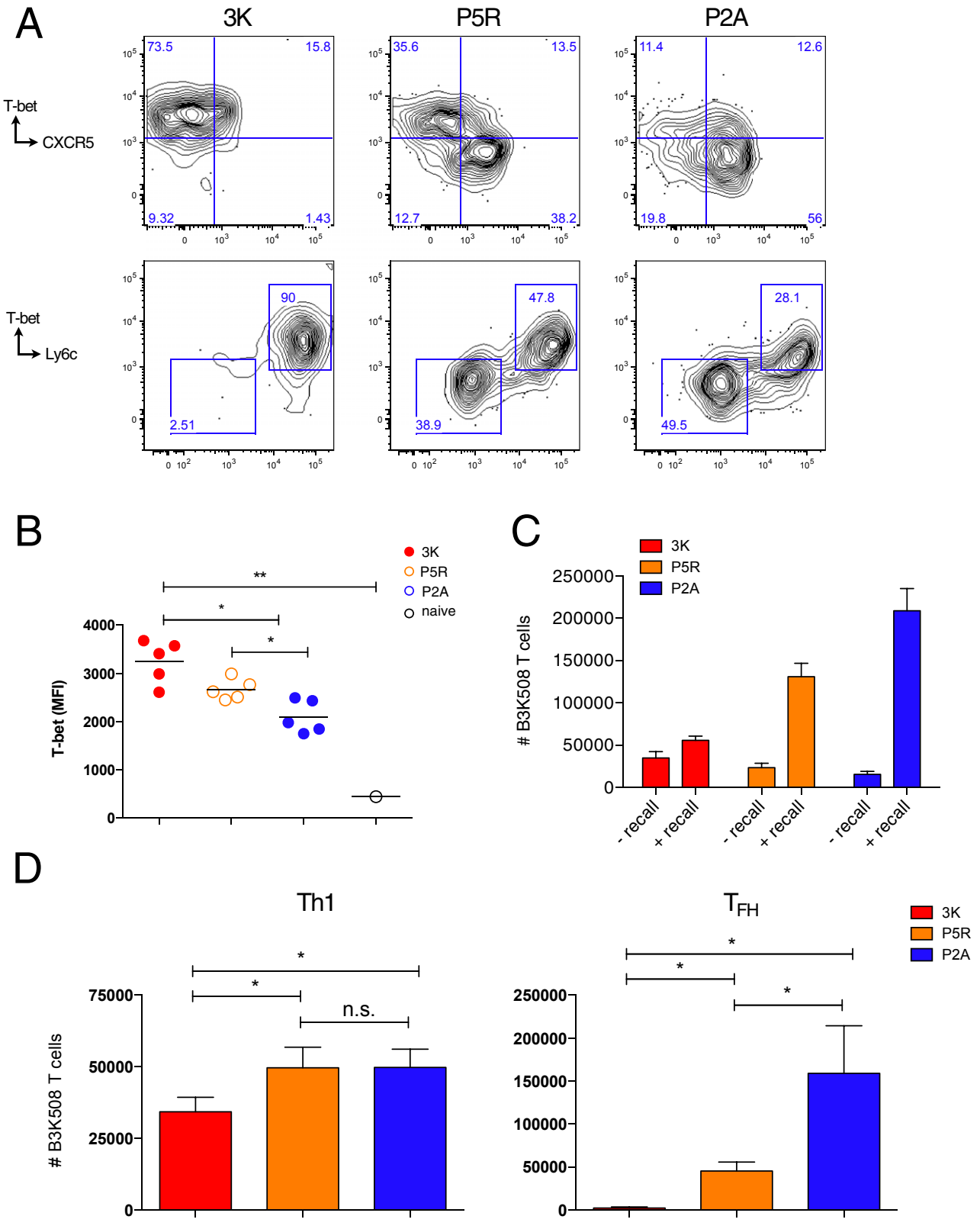

Fig. S9. Recall responses of memory T cells generated after P5R priming. Phenotype (*A* and *B*) and absolute cell numbers (*C* and *D*) of B3K508 T cells 4 d after recall infection with virulent Lm.3K.

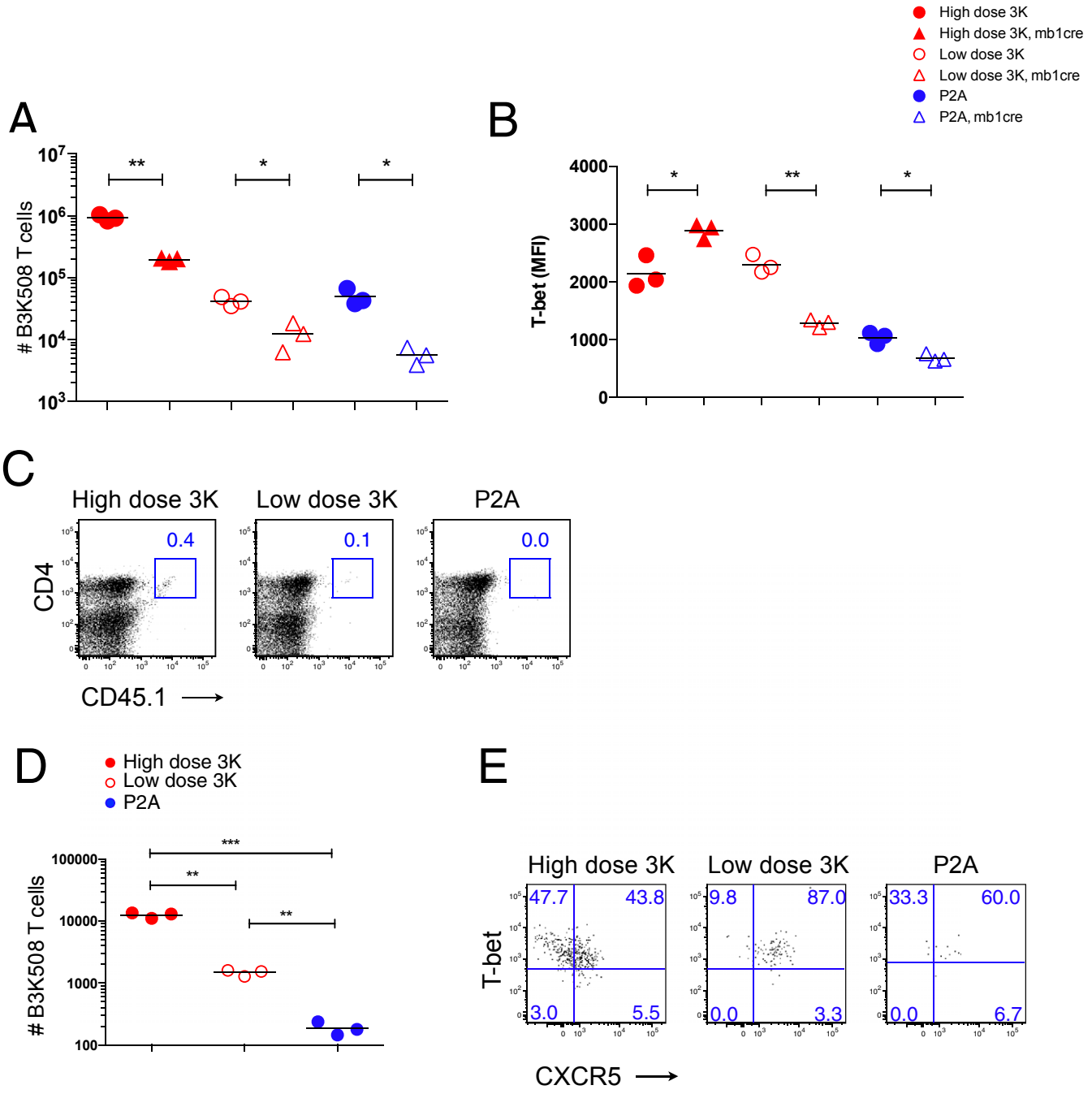

Fig. S10. Effector and memory T-cell differentiation in B-cell–deficient mice. Ten thousand naïve B3K508 Ly5.1 T cells were transferred into B6 or mb1-cre mice followed by infection with Lm.3K or Lm.P2A. The mean number (*A*) and T-bet mean fluorescence intensity (MFI) (*B*) of B3K508 T cells recovered from spleen and lymph nodes at day 6 after infection. (*C*) Decreased proportion of B3K508 T cells recovered from mb1-cre mice at >30 d after infection. Number (*D*) and phenotype (*E*) of B3K508 T cells at day 4 after recall infection with virulent Lm.3K. Data represent $n \geq 3$ for each data point and are representative of two independent experiments. (*$P < 0.005$, **$P < 0.001$).