Supplemental Materials for

A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells

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Materials and Methods

Mice

Six to eight-week old female C57BL/6 ($CD45.2^+$) and congenic C57BL6 B6.SJL-PtprcaPep3b/BoyJ (B6.Ly5.1) ($CD45.1^+$) mice were purchased from the National Cancer Institute and Jackson Laboratory. B6.129S2-*cd4*^{tm1mak}/J ($CD4^{-/-}$) and B6.129S2-*Cd8a*^{tm1Mak}/J ($CD8^{-/-}$), B6.129S7-Ifngr^{tm1Agt}/J (IFN-$\gamma R^{-/-}$), B6.129P2-Ccl5tm1Hso/J ($CCL5^{-/-}$) and B6.FVB-Tg [ITGAM-DTR/EGFP] 34Lan/J (CD11bDTR) mice were obtained from the Jackson Laboratory. All procedures used in this study complied with federal guidelines and institutional policies by the Yale animal care and use committee.

Viruses
HSV-2 strains $186syn^-$ TK^- and $186syn^+$ were generous gifts of Dr. David Knipe (Harvard Medical School, Boston, MA). These viruses were propagated and titered on Vero cells as previously described *(27)*. Influenza virus A/Puerto Rico/3/334 (A/PR8; H1N1) was propagated as previously described *(27)*.

Virus infection
Six- to eight-week-old female mice injected s.c. with progesterone (Depo Provera, Pharmacia Upjohn, 2 mg per mouse) were immunized intravaginally with 10^5 PFU of HSV-2 ($186syn^-$ TK^-) as previously described *(27, 34)*. For secondary challenge, TK^- immunized mice were challenged vaginally with 10^4 pfu of WT HSV-2 ($186syn^+$). The severity of disease was scored as *(35)*; 0, no sign; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions; 4, hind-limb paralysis; 5, pre-moribund. Due to humane concerns, the animals were euthanized prior to reaching moribund state.

Antibodies
Anti-CD3ε (145-2C11 and 17A2), anti-CD90.2 (30-H12), anti-CD45 (30-F11), anti-CD45.2 (104), anti-CD45.1 (A20), anti-CD4 (GK1.5, RM4-5 and RM4-4), anti-CD11b (M1/70), anti-CD8α (53-6.7 and KT15), anti-CD19 (6D5), anti-CD45R/B220 (RA3-6B2), anti-MHC class II (I-A/I-E, M5/114.15.2), anti-CD69 (H1.2F3), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD11a (M17/4), anti-CD103 (2E7), anti-CCR7 (4B12), anti-CD49d (R1-2), anti-NKp46 (29A1.4), anti-TCRγδ (GL3) anti-FoxP3 (FJK-16s), anti-IFN-γ (XMG1.2 and R4-6A2), anti-mouse IL-2 (JES6-5H4), anti-human/mouse T-bet (eBio4B10), and anti-CD62L (MEL14) were purchased from e-Bioscience or Biolegend. Anti-CD4 (H129.19), anti-Ly6C (AL-21), anti-mouse Lyve-1 (ALY7), anti-mouse CD31 (390) was obtained from BD Bioscience. Mouse IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE) was obtained from Miltenyi Biotec. H-$2K^b$-$gB_{498\text{-}505}$ tetramer was obtained from the NIH tetramer core facility.

Isolation of leukocytes from vaginal tissues.
The genital tracts of vaginal tissues treated with Depo-Provera were dissected from the urethra and cervix. The tissues were then incubated with 0.5 mg/mL Dispase II (Roche) for 15 min at 37 °C. Thereafter, vaginal tissues were digested with 1 mg/mL collagenase D (Roche) and 30 μg/mL DNase I (Sigma-Aldrich) at 37 °C for 25 min. To stop enzymatic reaction, 5 mM EDTA

at the final concentration was added and incubated for an additional 5 min. The resulting cells were filtered through a 70- μm filter *(27)*.

Flow cytometry

Preparation of single cell suspensions from spleen, draining LNs (inguinal LN and iliac LNs) and vagina were described previously *(27)*. Multiparameter analyses were carried out on the LSR II flow cytometer (Becton Dickinson) and were analyzed using the FlowJo software (Tree Star, Ashland, OR). To detect HSV-2-speific $CD4^+$ T cells ($CD45.1^+$ or $CD45.2^+$), single cell suspensions from vaginal tissues of TK^- HSV-2 immunized mice were stimulated in the presence of 5 μg/ml Brefeldin A with naïve splenocytes ($CD45.1^+$ $CD45.2^+$) loaded with heat-inactivated HSV-2 antigen (0.5 pfu equivalent/cells) for 10-12 hr. To stain intracellular CCL5, the vaginal tissues were digested with the mentioned above medium containing dispase II and then collagenase D/DNase I in the presence of 10 μg/ml Brefeldin A (Sigma) to prevent chemokine secretion. Thereafter, these cells were stained with cell surface markers in FACS buffer (5% BSA, 2 mM EDTA in PBS) in the presence of 10 μg/ml Brefeldin A. After washing, these cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit. These cells were stained with Rabbit Anti-CCL5/RANTES Polyclonal Antibody (BIOSS Inc. or Cedarlane). To detect extracellular IFN-γ derived from IFN-γ-producing cells (IFN-γ^{int} and IFN-γ^{hi}), single cell suspensions were incubated with the reagent, which is included in Mouse IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE) (Miltenyi Biotec).

DNA isolation from tissues.

C57/BL6 mice were immunized with TK^- HSV-2 virus. Five weeks later, vaginal tissues of these mice were lysed in 10 mg/ml Proteinase K (Roche) to isolate DNA at 55°C overnight on a gently rocking platform. After removing these tubes, phenol equilibrated with Tris pH8.0 was added. Thereafter, upper aqueous phase was added to phenol/chloroform (1:1). The upper aqueous phase was re-suspended with sodium acetate, pH6.0 and 100% ethanol at RT. After shaking and centrifuging, the concentration of isolated DNA pellet was measured. Level of HSV-2 genomic DNA in vaginal tissue was analyzed by qPCR using purified viral DNA genome as standard.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

C57/BL6 mice were immunized with TK^- HSV-2 virus. Five or twelve week later, vaginal tissues of these mice were lysed and homogenized in TRIzol Plus RNA Purification System (Invitrogen) to isolate RNA. Contaminating DNA was removed using recombinant DNaseI (Roche), and cDNA was generated with AMV Reverse Transcriptase (Promega), each according to the manufacturer'sinstructions. Quantitative PCR was performed on a Stratagene MX3000P unit using SYBRgreen-based quantification (Qiagen). Expression of mRNA of various host or HSV-2 genes in vaginal tissues was quantitated by RT-qPCR using the following set of primers. CXCL9 (F, cgagaccatttactgcaacag; R, taattgggccaacagtagcc), CCL5 (F, gctgcaactgcatccatatc; R, gtggcaatgatctcaacacg), CXCL10 (F, gctgcaactgcatccatatc; R, gtggcaatgatctcaacacg), HSV-2 gD (F, taacctgggattcctgatgc; R, cggtgctccaggataaattg), HSV-2 gB (F, ctttatgtccaacccccttcg; R, ttgagttccttggtggtgag), HSV-2 gG (F, tcagcccatcctccttcggcagta; R, cgcgcggtcccagatcggca), HSV-2 gE (F, aaatggatccggctttgag; R, atttacgcccctcgctatg), HSV-2 ICP0 (F, ttacgtcaacaagactatcacggg; R, tccatgtccaggatgggc), HSV-2 ICP4 (F, ggcctgcttccggatctc; R, ggtgatgaaggagctgctgtt), KLF2 (*Klf2*) (F, cattgcaactgggaaggatg; R, aaagggtctgtgacctgtgtg), CD62L (*Sell*) (F, tgtggagcatctggaaactg; R, tgaaagcacacttggactgg), T-bet (*Tbet*) (F, ttcccattcctgtccttcac; R,

ccacatccacaaacatcctg), CD4 (F, tccttcccactcaactttgc; R, agggctggaagaaagaatcc), S1PR1 (*S1pr1*) (F, accttccgcaagaacatctc; R, ttgcagcccacatctaacag), TCRVβ1 (F, ccagggcagaaccttgtact; R, gcagggtagtctgtgtgtgg) *(36),* TCRVβ1 (for nested PCR) (External F, taccacgtggtcaagctg; R, ccagaaggtagcagagaccc; Internal F, gtatccctggatctg; R, gggtagccttttgtttgtttg) *(37).*

***In vivo* treatment with toxins and neutralizing/depleting antibodies.**
C57/BL6 mice were immunized with TK^- HSV-2 virus. Five weeks later, these mice were treated with PTX (0.5 μg/vagina) intravaginally for five consecutive days. In the case of antibody neutralization/depletion, C57/BL6 mice immunized with TK^- HSV-2 five weeks prior were treated with anti-mouse CXCL9 Ab (R &D systems, 1μg/ vagina), anti-mouse CCL5 Ab (R &D systems, 1μg/ vagina), LEAF™ Purified anti-mouse IFN-γ Ab (R4-6A2 (Biolegend), 1μg/vagina) or LEAF™ Purified anti-mouse CD4 Ab (GK1.5; 10 μg/vagina) intravaginally for four consecutive days. The dose of Ab needed to cover all accessible antigens in the vagina was determined by injecting between 0.5 – 5 μg/vagina of RM4-5 Ab and analysing single cell suspension by flow cytometry. Mice were analyzed the following day for the presence and function of memory T cells, or were challenged with a lethal dose of WT HSV-2 intravaginally and viral titers from vaginal wash were measured one day later. To deplete $CD11b^+$ cells in CD11bDTR mice, BM from CD11bDTR mice were transplanted into irradiated WT mice. After full reconstitution, these mice are treated with 10 ng/g body weight from intraperioneally and intravenously on days -4, -1 and 50 ng DT intravaginally on days -4, -3, -2, -1. On day 0, these mice were sacrificed for immunohistochemical analysis.

Immunofluorescence staining.
Frozen sections 8 μm in thickness were cut, fixed, left to dry at ambient temperature. These tissues were stained with the Abs (anti-CD4 [H129.19], anti–CD8-α [53–6.7], anti–MHC class II [M5/114.15.2], anti-CD11b [M1/70], anti-mouse CD11c [HL3], anti-mouse CD45 [30-F11], anti-mouse CD3 [17A2], anti-mouse CD45.2 [104], anti-mouse CD45.1 [A20], anti-PNAd [MECA-79], anti-mouse Lyve-1 [ALY7], anti-mouse CD31 [390] and rabbit anti-mouse RANTES (Cedarlane or Bioss) as previously described *(27).* These slides were washed and incubated with DAPI and mounted with Fluoromount-G (SouthernBiotech). These slides were analyzed by fluorescence microscopy (BX51; Olympus).

Parabiosis
Parabiosis was performed as previously described *(38)* with slight modifications. Mice were anesthetized with a mixture of Ketamine/Xylazine (100 mg/kg and 10 mg/kg respectively). After shaving the corresponding lateral aspects of each mouse, matching skin incisions were made from behind the ear to hip and sutured together with Chromic Gut (4-0, HENRY SCHEIN, UK) absorbable suture, then these areas were clipped with 7-mm stainless-steel wound clips (ROBOZ).

Laser capture microdissection
Frozen vaginal tissues in OCT compounds were cut on Actrurus PEN Membrane Glass Slides (10 μm in thickness, Invitrogen). After fixation, these tissues were stained with anti-CD4 Ab [H129.19] as previously shown *(31).* $CD4^+$ T cells in vaginal tissues were acquired by laser capture microdissection (Zeiss). For TCRβ-chain sequencing, $CD4^+$T cells in MLC and non-MLC ($n > 5$), vaginal tissues from naïve ($n = 3$), immunized mice (5 wk) with PBS ($n = 3$) or

PTX (n= 3), $CD62L^{hi}CD44^{lo}CD4^{+}$ T cells from naïve mice (n = 3) and $CD62L^{lo}CD44^{hi}CD4^{+}$ T cells in DLN of mice immunized with TK^{-}HSV-2 for 7 days (n = 3) were pooled.

TCRβ sequencing

DNA extraction and amplification was followed by previous report *(31)*. TCRβ-chain sequencing was conducted by Adaptive Biotechnologies based on the ImmunoSEQ platform *(39)*.

Statistical Analysis

Survival curve was analyzed using the log-rank test. For other data, normally distributed continuous variable comparisons were performed using two-tailed unpaired *t*-test using Prism software. For comparison of two nonparametric datasets, the Mann-Whitney U-test was used. Binomial confidence interval for CDR3 sequence was calculated using the Wilson interval method.

Any Additional Author notes: Experiments were conceived and designed by N. I. and A.I.. Experiments were performed by N. I.. Data were analyzed by N.I. and A. I. Paper was written by N.I. and A.I..

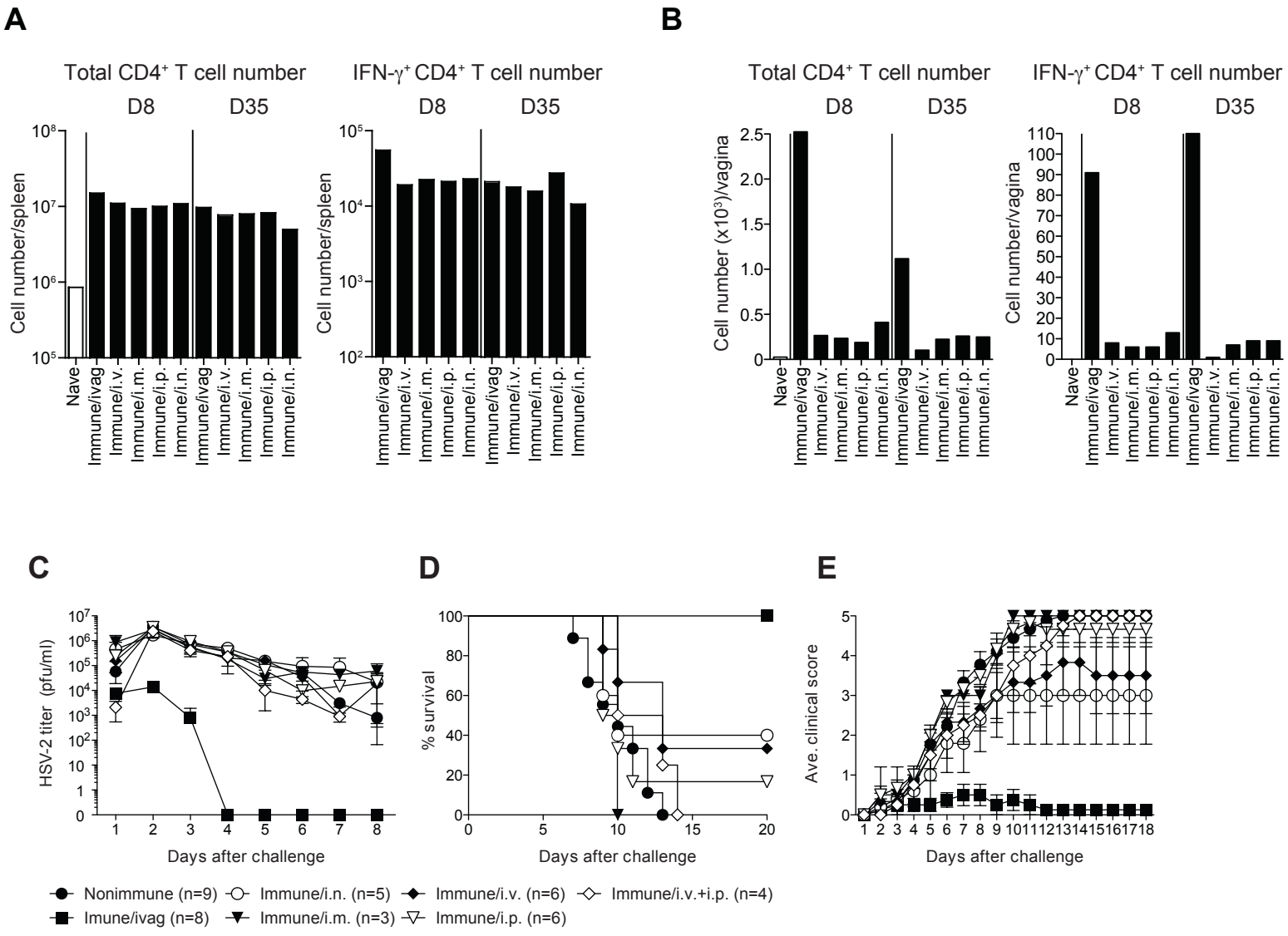


Fig. S1. Intravaginal immunization with attenuated HSV-2 generates local memory CD4 T cells in vaginal tissues and protects the host against HSV-2 secondary challenge.
(**A&B)** C57BL6 mice were immunized with TK$^-$ HSV-2 (10^5 pfu/mouse) via the intravaginal (ivag), intranasal (i.n.), intraperitoneal (i.p.), or intramuscular (i.m.) route. Eight days or thirty-five days later, the number of total CD4$^+$T cells and HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells in spleen (**A**) and vaginal tissues (**B**) were analyzed by flow cytometry. Virus titer in vaginal wash (**C**), mortality (**D**) and clinical score (**E**) were measured at the indicated time points. These data are representative of at least two similar experiments.

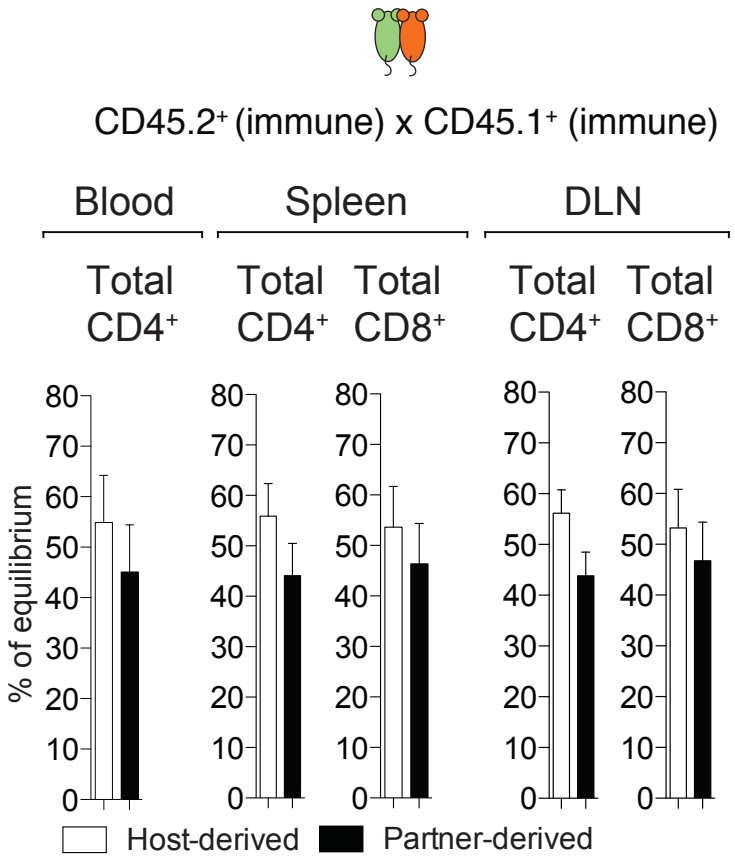


Fig. S2. Equilibrium between parabiotic mice within the circulating compartment is reached within two weeks. CD45.2$^+$ C57BL6 mice immunized with TK$^-$ HSV-2 five weeks prior were surgically joined with similarly immunized CD45.1$^+$ mice (CD45.2$^+$ (immune) x CD45.1$^+$ (immune), n=6 pairs). Two weeks later, the percentage of circulating T cells derived from the host and partner mice in the peripheral blood, spleen and DLN was analyzed by flow cytometry.

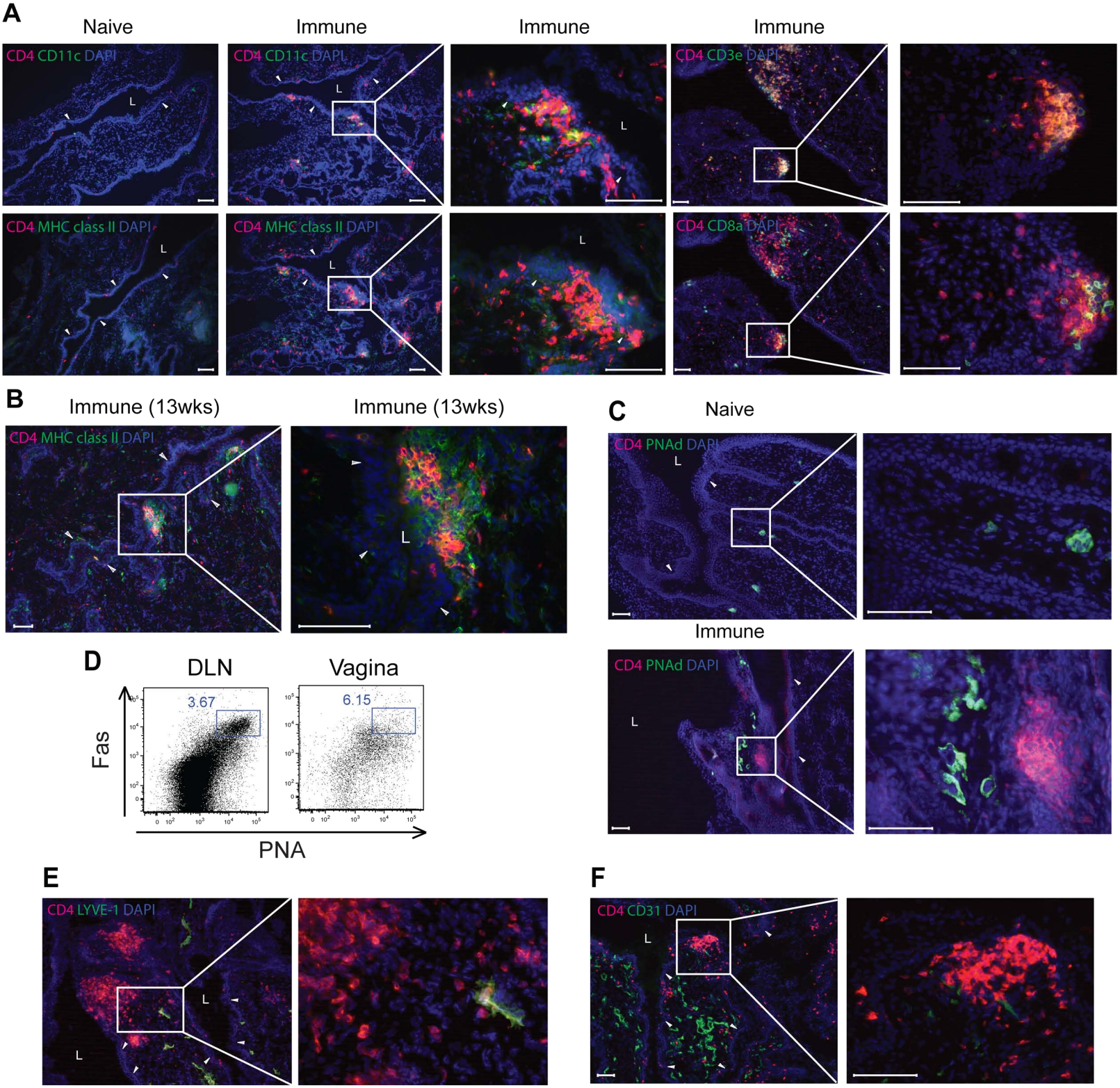


Fig. S3. Formation of memory T cell clusters in the vaginal submucosa following HSV-2 infection. C57BL6 mice were infected vaginally with TK$^-$ HSV-2. Five weeks (**A**, **C-F**) or thirteen weeks (**B**) later, frozen sections of vagina were stained with antibodies against CD4 (red) and CD11c, MHC class II, CD3, CD8, PNAd, LYVE-1 and CD31 (green). Nuclei are depicted by DAPI stain (blue). Images were captured using a 10x or 40x objective lens. Arrows indicate the basement membrane. Scale bars indicate 100 μm. L = lumen. (**D**) Phenotypic markers of germinal center B cells were analyzed by flow cytometry on cells isolated from the draining lymph node (DLN) or the vagina . These data are representative of six similar experiments.

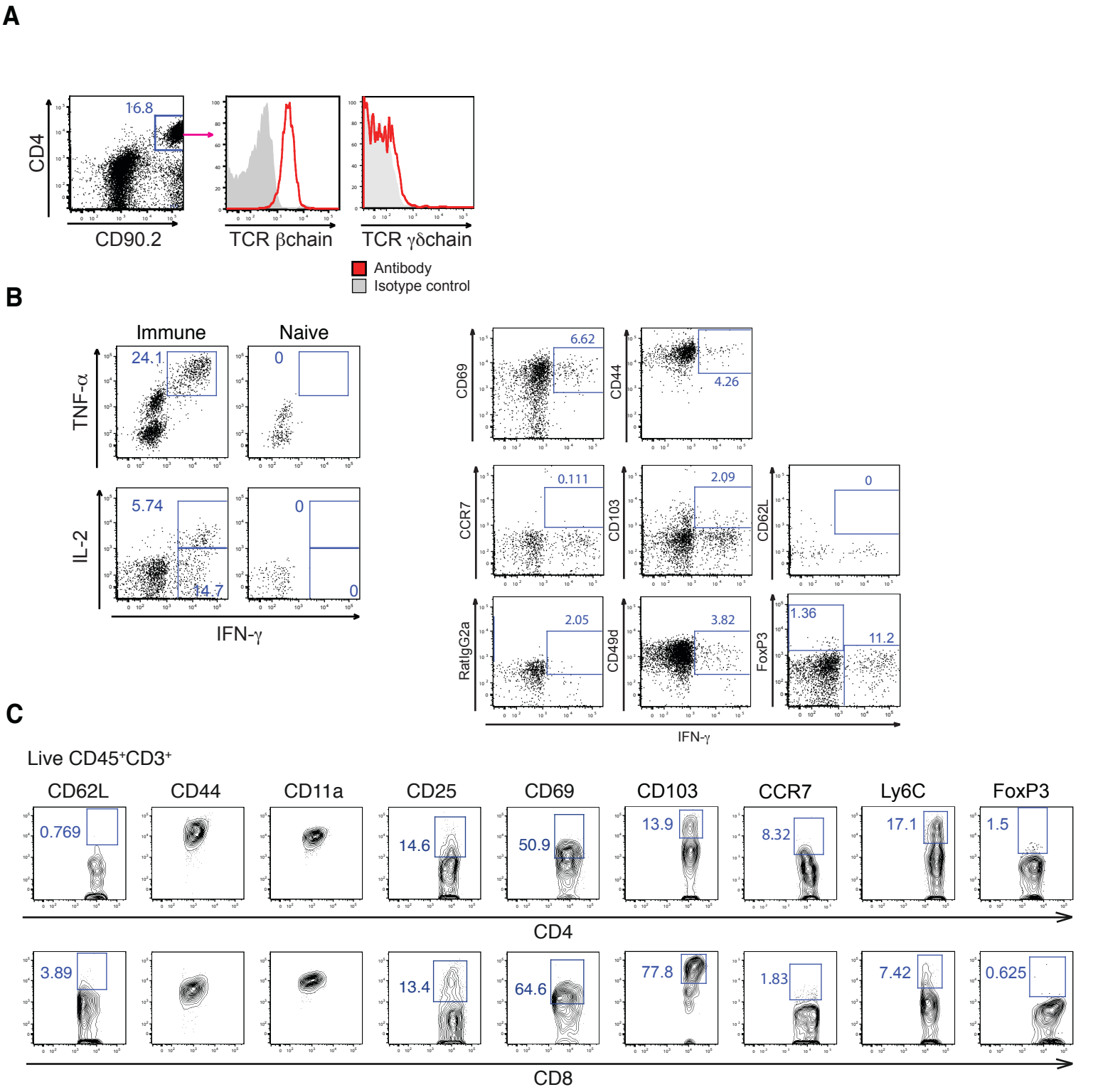


Fig. S4. Phenotypic and functional characteristics of T_{RM} localized in vaginal tissues following ivag HSV-2TK$^-$ immunization.

C57BL6 mice were infected vaginally with TK$^-$ HSV-2 five weeks prior. Vaginal cells were analyzed for expression of TCRαβ chain and γδ chain (GL3) within the CD90.2$^+$ CD4$^+$ population (**A**). (**B**) Detection of surface markers and intracellular cytokines by CD4$^+$ T cells upon restimulation of vaginal cells with HSV-2 antigens. (**C**) Phenotypic markers on the vagina-resident T cells were analyzed by flow cytometry.
These data are representative of at least three similar experiments.

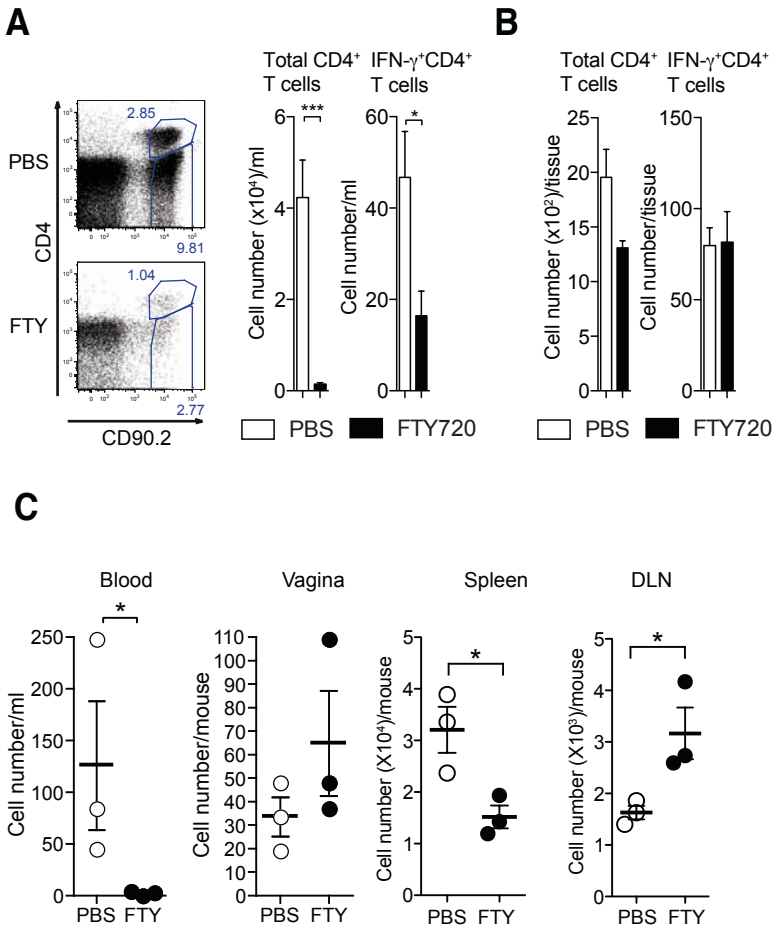
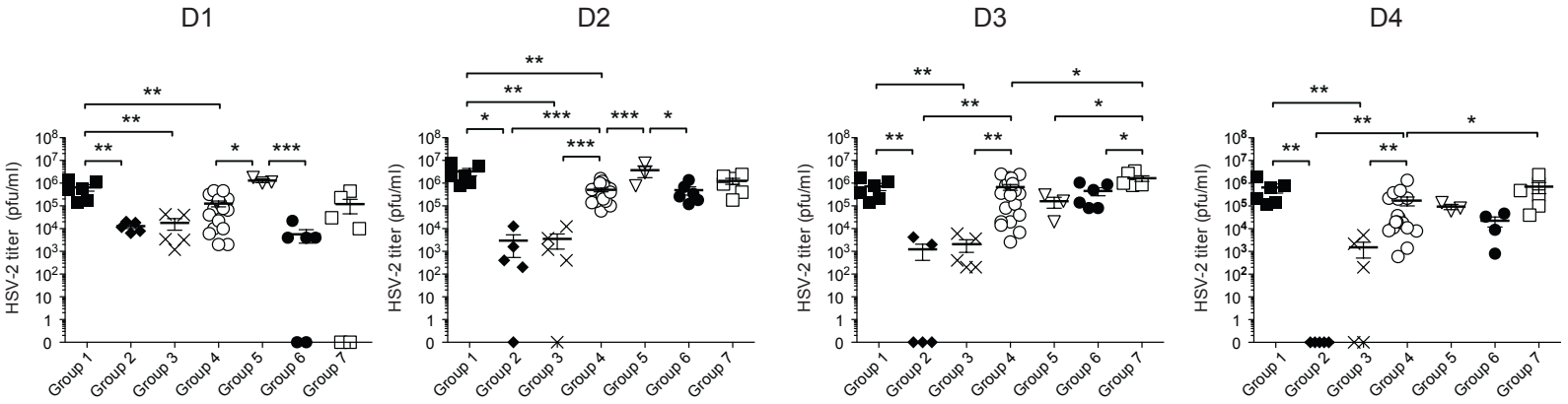


Fig. S5. Tissue-resident T cell pool in the vagina receives little input from circulation.
(**A**) C57BL6 mice were immunized with TK$^-$ HSV-2. Five weeks later, mice were given drinking water containing 4 μg/ml of FTY720 for two weeks. Thereafter, the percentage and number of total CD4$^+$ T cells (n=6) and HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells (n=6) in peripheral blood (**A**), the vagina (**B**), or tetramer$^+$ CD8$^+$ T cells in the indicated organs (n=3) (**C**) were analyzed by flow cytometry.
Data are means ± SE. *, $p<0.05$; ***, $p<0.001$.



Group #	Symbol	CD45.2^{+}	CD45.1^{+}	Viral challenge
1	■	Naive WT	Naive WT	CD45.1^{+}
2	◆	Immune WT	Immune WT	CD45.1^{+}
3	×	Immune WT	Naive WT	CD45.2^{+}
4	○	Immune WT	Naive WT	CD45.1^{+}
5	▽	Immune CD4$^{-/-}$	Naive WT	CD45.1^{+}
6	●	Immune CD8$^{-/-}$	Naive WT	CD45.1^{+}
7	□	Immune WT	Naive IFN-γR$^{-/-}$ (CD45.2^{+})	Naive IFN-γR$^{-/-}$

Fig. S6. Virus titers at indicated time point in challenged parabiotic mice.
CD45.2^{+} mice were surgically joined with CD45.1^{+} mice of the indicated genotypes as in Fig.2 **A-C**. Two to three weeks after parabiosis, the indicated partner was challenged with a lethal dose of WT HSV-2 intravaginally. Virus titer in the vaginal secretion following viral challenge are depicted.
Data are means ± SE. *, p<0.05; **, p<0.001; ***, p<0.001 (The Mann-Whitney U-test).

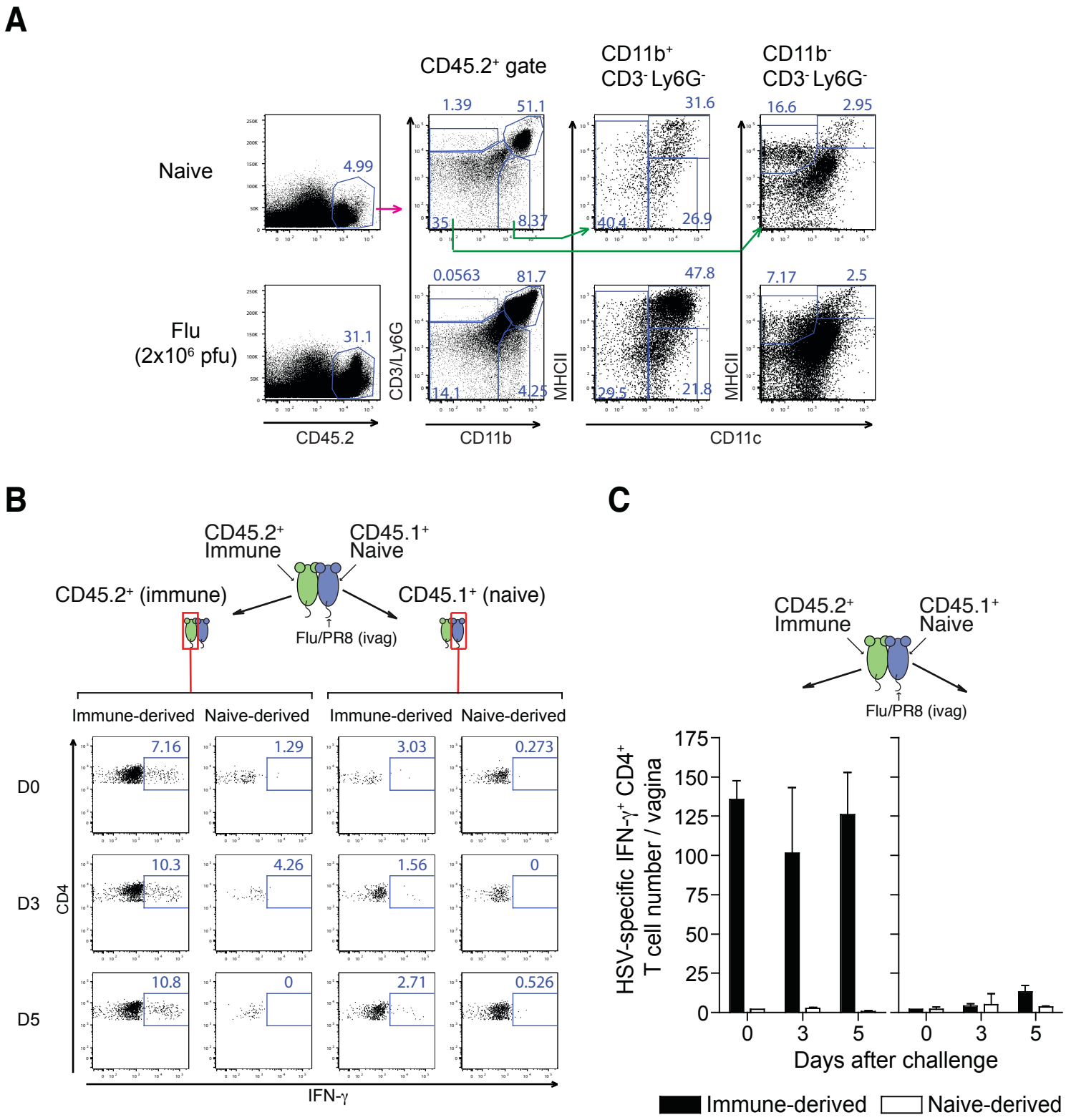


Fig. S7. An irrelevant infection fails to recruit circulating HSV-2-specific Th1 memory T cells into vaginal tissues.

(**A**) Naive C57BL6 mice were infected with Flu (A/PR8; 2x10^6 pfu) intravaginally. Two days later, single cells from vaginal tissues were analyzed by flow cytometry. (**B & C**) CD45.2$^+$ mice immunized with TK$^-$ HSV-2 five weeks prior were surgically joined with naive CD45.1$^+$ mice. Three weeks later, the CD45.1$^+$ partner mice were infected with Flu (A/PR8; 2x10^6 pfu) ivag. On the indicated days following Flu/PR8 challenge, the number of HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells of partner and host origins in the vaginal tissue of both parabiotic partners were analyzed by flow cytometry. These data are representative of two similar experiments.

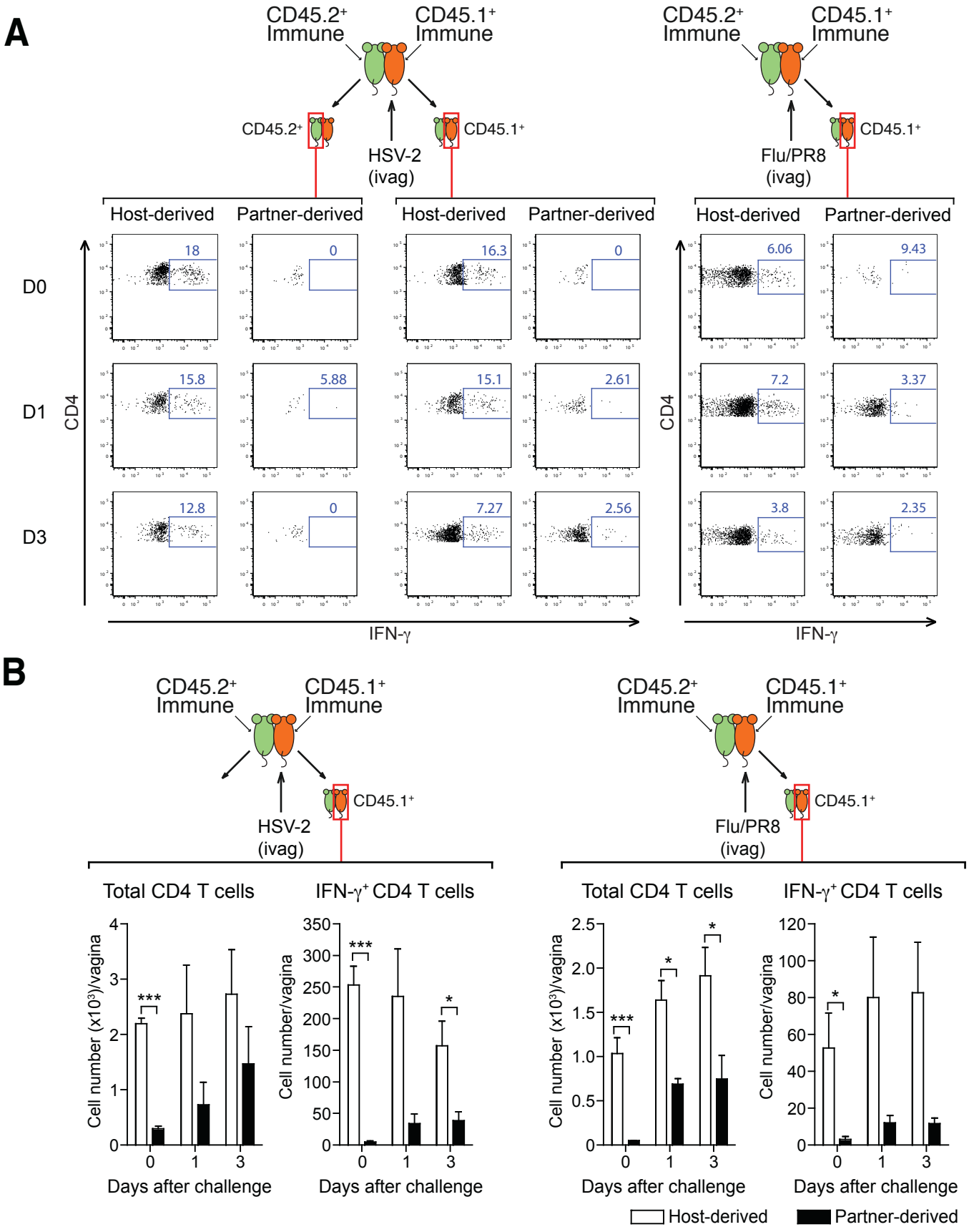


Fig. S8. In the presence of MLC, secondary challenge with cognate or irrelevant virus induces little recruitment of circulating memory cells.

CD45.2+ and CD45.1+ mice immunized ivag with TK- HSV-2 five weeks prior were surgically joined. On the indicated days following WT HSV-2 or Flu/PR8 challenge of the CD45.1+ partner, the number of total CD4+ T cells and HSV-2-specific IFN-γ+ CD4+ T cells of immune (CD45.2+) and donor (CD45.1+) origins in the vaginal tissue of both parabiotic partners were analyzed by flow cytometry. Data are means ± SE. *, p<0.05, ***, p<0.001.

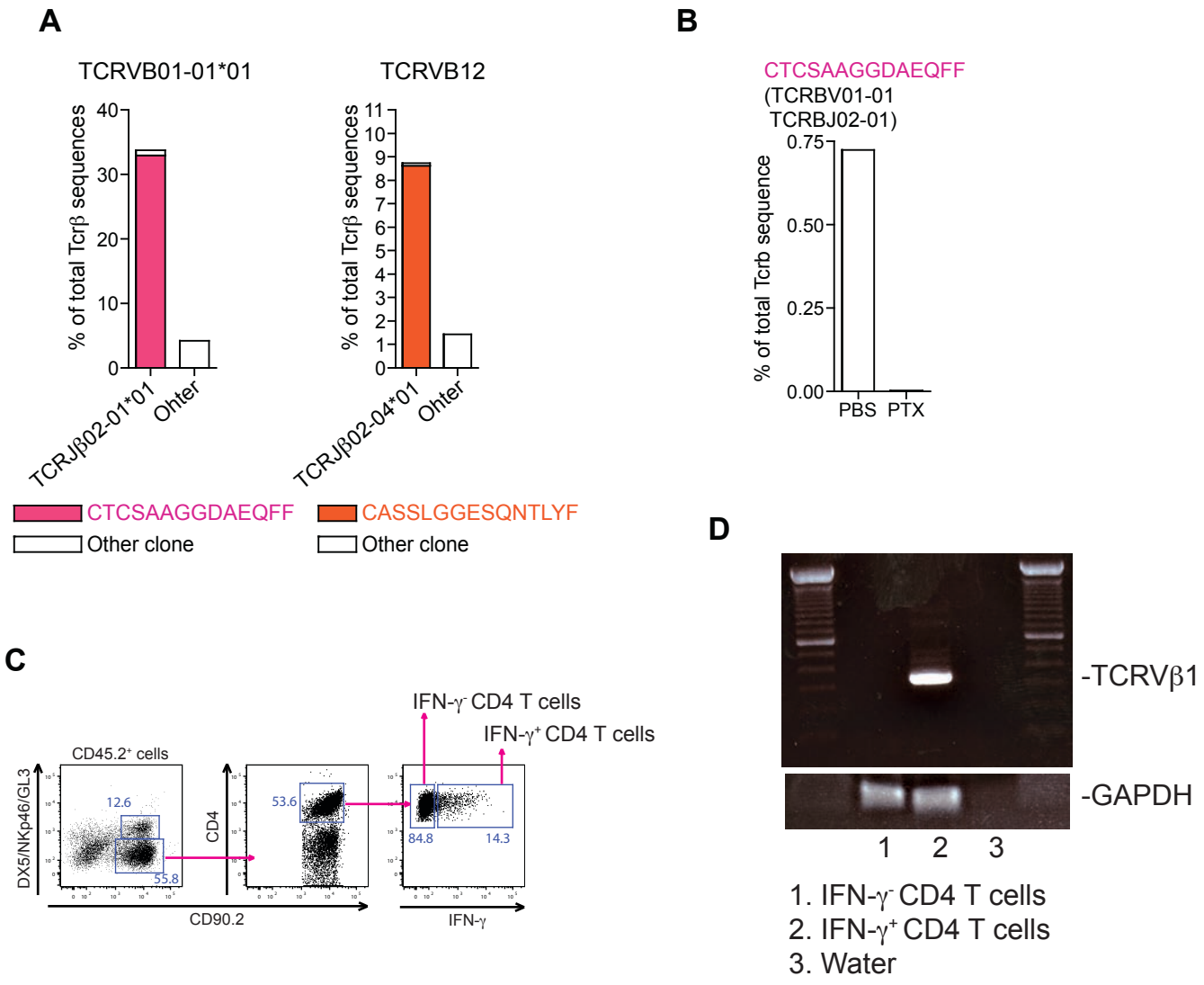


Fig. S9. HSV-2-specific Th1 cells in MLC following ivag TK$^-$HSV-2 immunization are enriched in TCRVβ01-01 and TCRJβ02-01.

C57/BL6 mice were immunized ivag with TK$^-$ HSV-2. Five weeks later, frozen section of vaginal tissues was prepared for laser capture microdissection (LCM) to collect CD4$^+$ T cells in MLC and non-MLC. Using DNA from LCM samples, TCR β-chain sequencing was performed. (**A**) The percentages of TCRBV01-01*01 and TCRVβ12 in MLC, and the occupancy of CTCSAAGGDAEQFF (TCRBV01-01*01/TCRBJ02-01*01) and CASSLGGESQNTLYF (TCRVβ12/TCRJβ02-04*01) among the MLC CD4$^+$ cells (TCRBJ02-01*01 or TCRJβ02-04*01$^+$) are depicted. (**B**) The percentage of total TCRβ sequences of CTCSAAGGDAEQFF (TCRVβ01-01*01) among TCR β-chain CDR3 sequences in vaginal tissues of TK$^-$ HSV-2 immunized mice (5 wk) treated with PBS or PTX. (**C&D**) Single cell suspension from vaginal tissues of mice vaginally immunized with TK$^-$HSV-2 5 week prior were restimulated with splenocytes loaded with HSV-2 antigen.Thereafter, IFN-γ$^+$CD4$^+$ or IFN-γ$^-$CD4$^+$ cells among CD45.2$^+$CD90.2$^+$DX5$^-$NKp46$^-$GL3$^-$ cells following IFN-γ secretion assay were sorted for PCR or qPCR analysis (**C**). (**D**) TCRVβ1 expression level was analyzed by nested PCR.

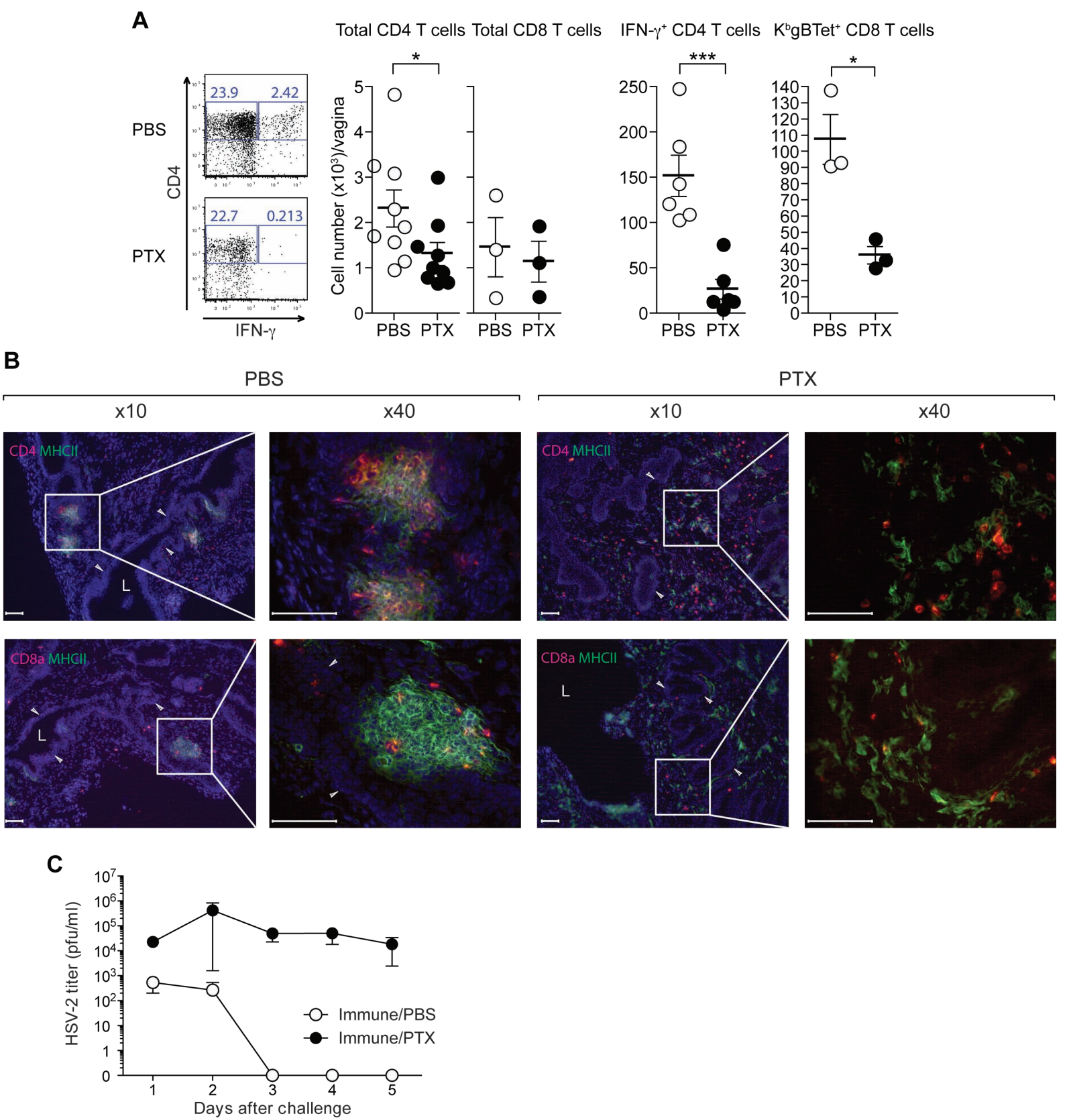


Fig. S10. Vaginal memory lymphocyte clusters are maintained by PTX-sensitive signaling pathway. C57/BL6 mice were immunized ivag with TK$^-$ HSV-2. Five weeks later, these mice were treated with PTX intravaginally for five consecutive days. On the sixth day, total CD4$^+$ T cells, total CD8$^+$ T cells, HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells and K^{b}gB tetramer$^+$ CD8$^+$ T cells in the vagina were analyzed by flow cytometry (**A**). Frozen sections of vagina were stained with antibodies against CD4 or CD8 (red) and MHC class II (green) (**B**). Nuclei are depicted by DAPI stain (blue). Images were captured using a 10x or 40x objective lens. Scale bars indicate 100 μm. (**C**) Average virus titers from vaginal wash at the indicated time points after challenge with WT HSV-2 ivag are depicted. These data are representative of three similar experiments. Data are means ± SE. *, $p<0.05$; **, $p<0.001$; ***, $p<0.001$.

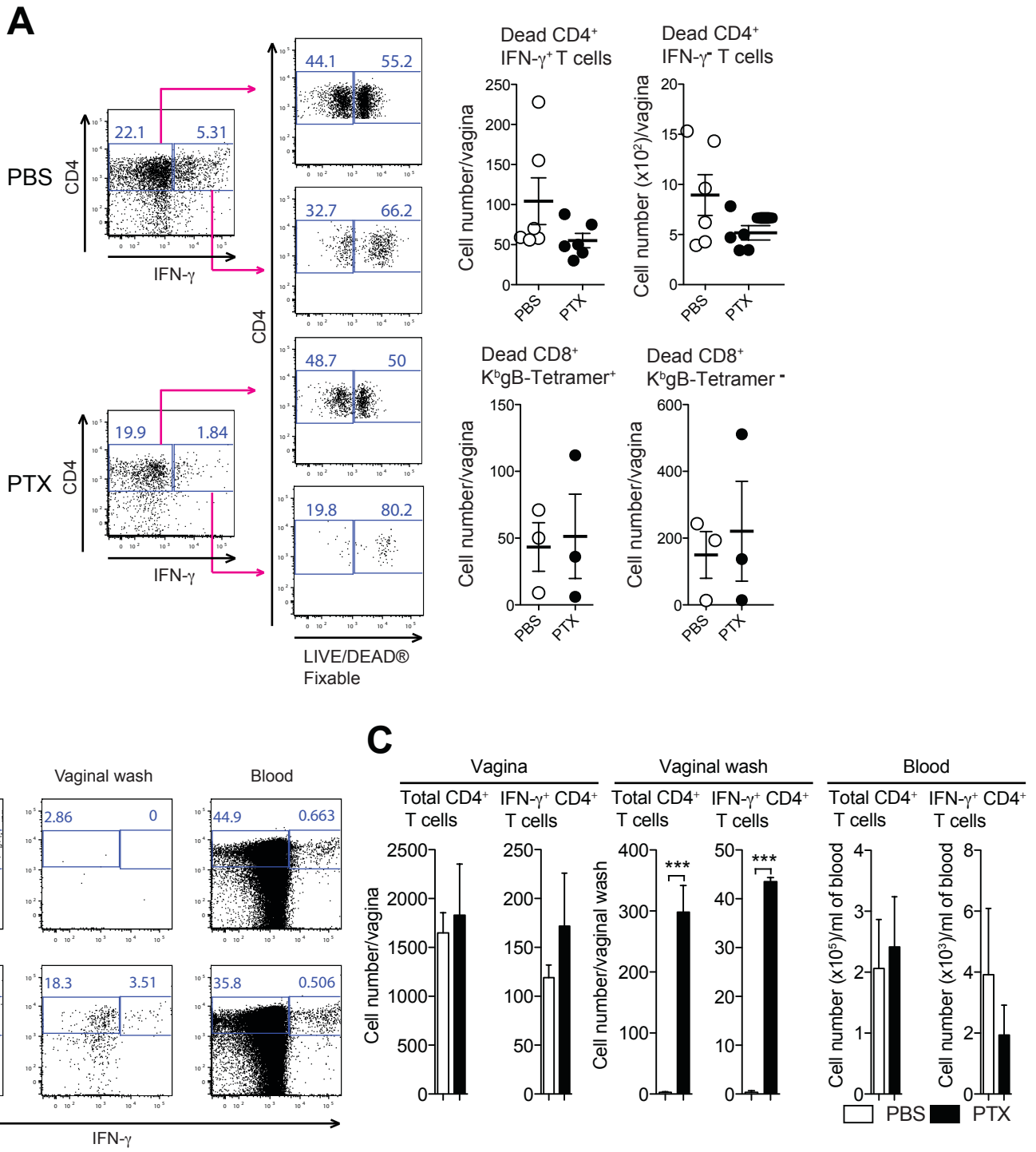


Fig. S11. PTX treatment results in expulsion of CD4 T cells into the vaginal lumen but not cell death in vaginal lymphocytes after PTX treatment.

(**A**) C57/BL6 mice were immunized ivag with TK$^-$ HSV-2. Five weeks later, these mice were treated with PTX intravaginally for five consecutive days. On the sixth day, dead cells (LIVE/DEAD® Fixable$^+$) among HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells and K^{b}gB tetramer$^+$CD8$^+$ T cells in vaginal tissues were analyzed by flow cytometry. (**B&C**) C57/BL6 mice were immunized ivag with TK$^-$ HSV-2. Five weeks later, these mice were treated with PTX intravaginally. Five hours later, total CD4$^+$ T cells and HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells in the vaginal tissues, vaginal wash and peripheral blood were analyzed by flow cytometry (n=3). These data are representative of three similar experiments. Data are means ± SE. ***, $p<0.001$.

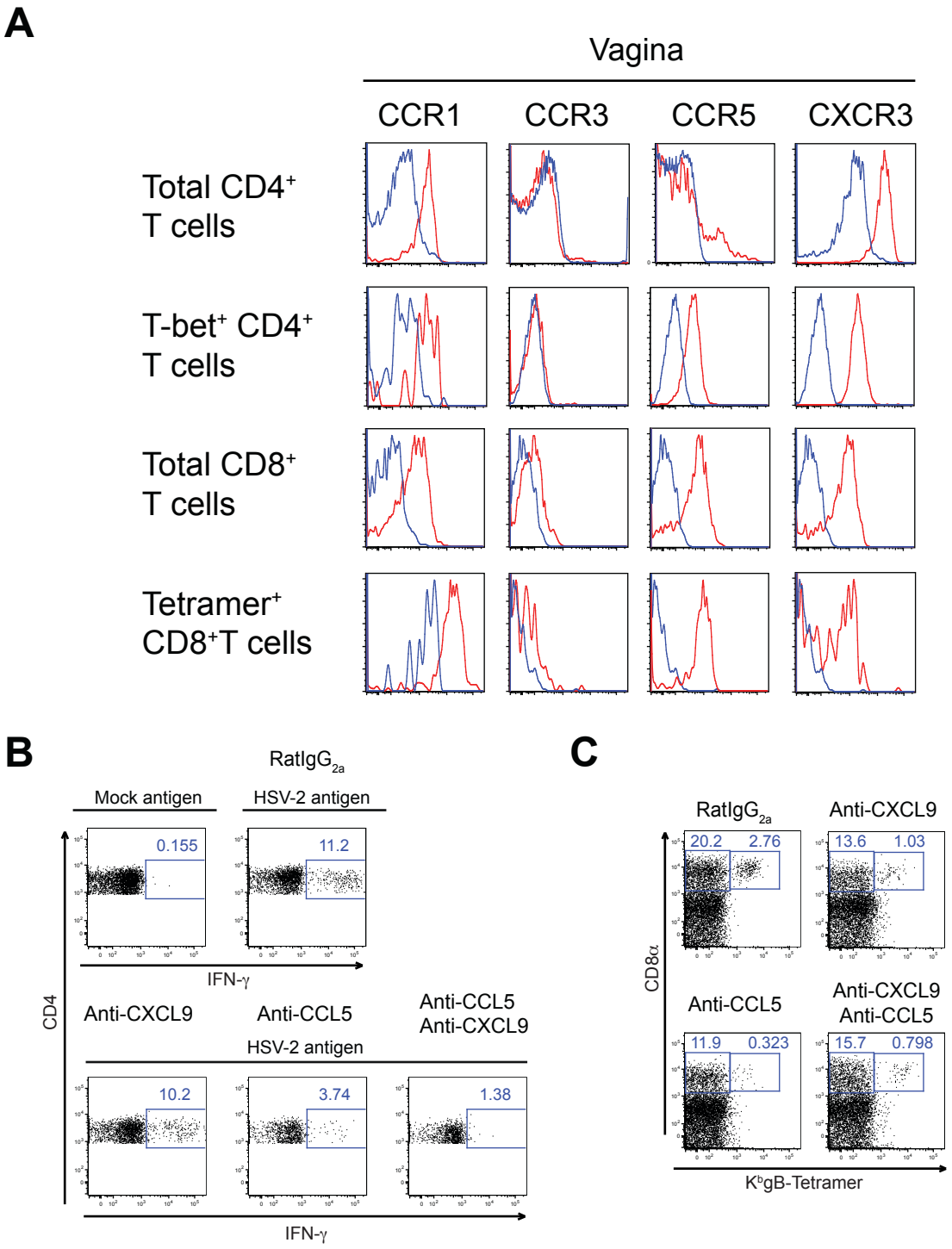


Fig. S12. CCL5 and CXCL9 control retention of CD4 T_{RM} and CD8 T_{RM}.
(**A**) C57/BL6 mice were immunized ivag with TK$^-$ HSV-2. Chemokine receptor expression on total CD4$^+$ T cells, or K^{b}gB-Tetramer$^+$ CD8 T cells in the vaginal tissues five weeks post TK$^-$ HSV-2 immunization was analyzed. Red lines indicate staining with specific antibodies to chemokine receptors, and blue lines indicate staining with isotype-matched control Ab. These data are representative of three similar experiments. (**B&C**) C57/BL6 mice were immunized ivag with TK$^-$ HSV-2. Five weeks later, these mice were treated with isotype control IgG, anti-CXCL9 and/or anti-CCL5 Ab intravaginally for four consecutive days. The following day, HSV-2-specific IFN-γ$^+$ CD4$^+$ T cells (**B**) and K^{b}gB-tetramer$^+$ CD8$^+$ T cells (**C**) in the vagina were analyzed by flow cytometry. These data are representative of three similar experiments.

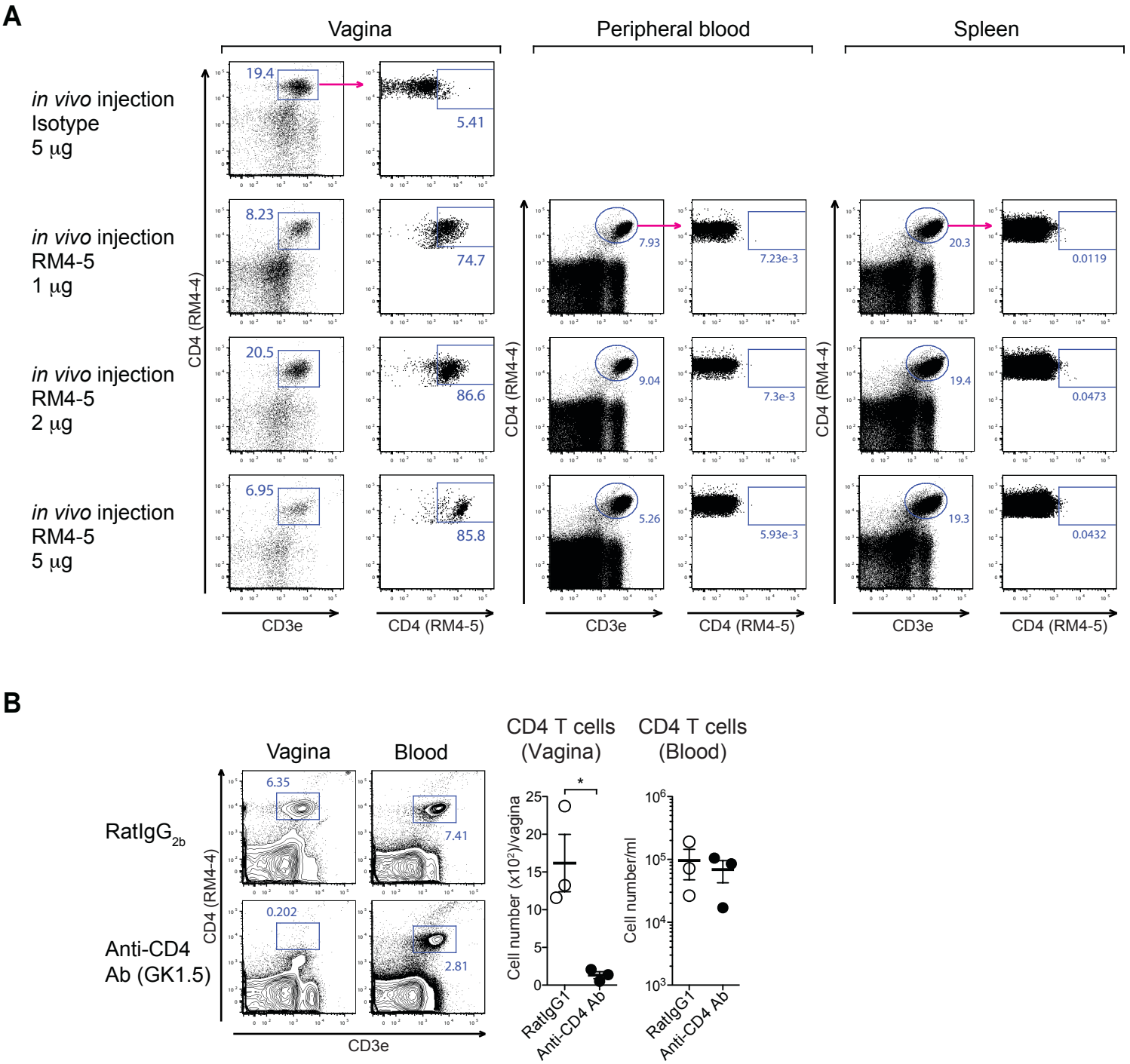


Fig. S13. Intravaginal antibody injection targets the vaginal lymphocytes for staining and depletion. (**A**) C57/BL6 mice were immunized ivag with TK⁻ HSV-2. Five weeks later, these mice were treated with dialyzed FITC-conjugated anti-CD4 Ab (RM4-5, 1μg, 2 μg or 5 μg per vagina) intravaginally for four consecutive days. The following day, FITC-RM4-5-bound cells in the vagina, peripheral blood and spleen were detected by flow cytometry. Labeling specificity was confirmed with a non-cross-reactive anti-mouse CD4 Ab (RM4-4). Notice that ivag injection of Ab selectively stains cells within the vagina but not other organs. These data are representative of three similar experiments.
(**B**) C57/BL6 mice were immunized ivag with TK⁻ HSV-2. Five weeks later, these mice were treated with LEAF™ anti-mouse CD4 Ab (GK1.5, 10 μg per vagina) intravaginally for four consecutive days. The following day, CD4⁺ T cells in the vagina and peripheral blood were detected by flow cytometry.

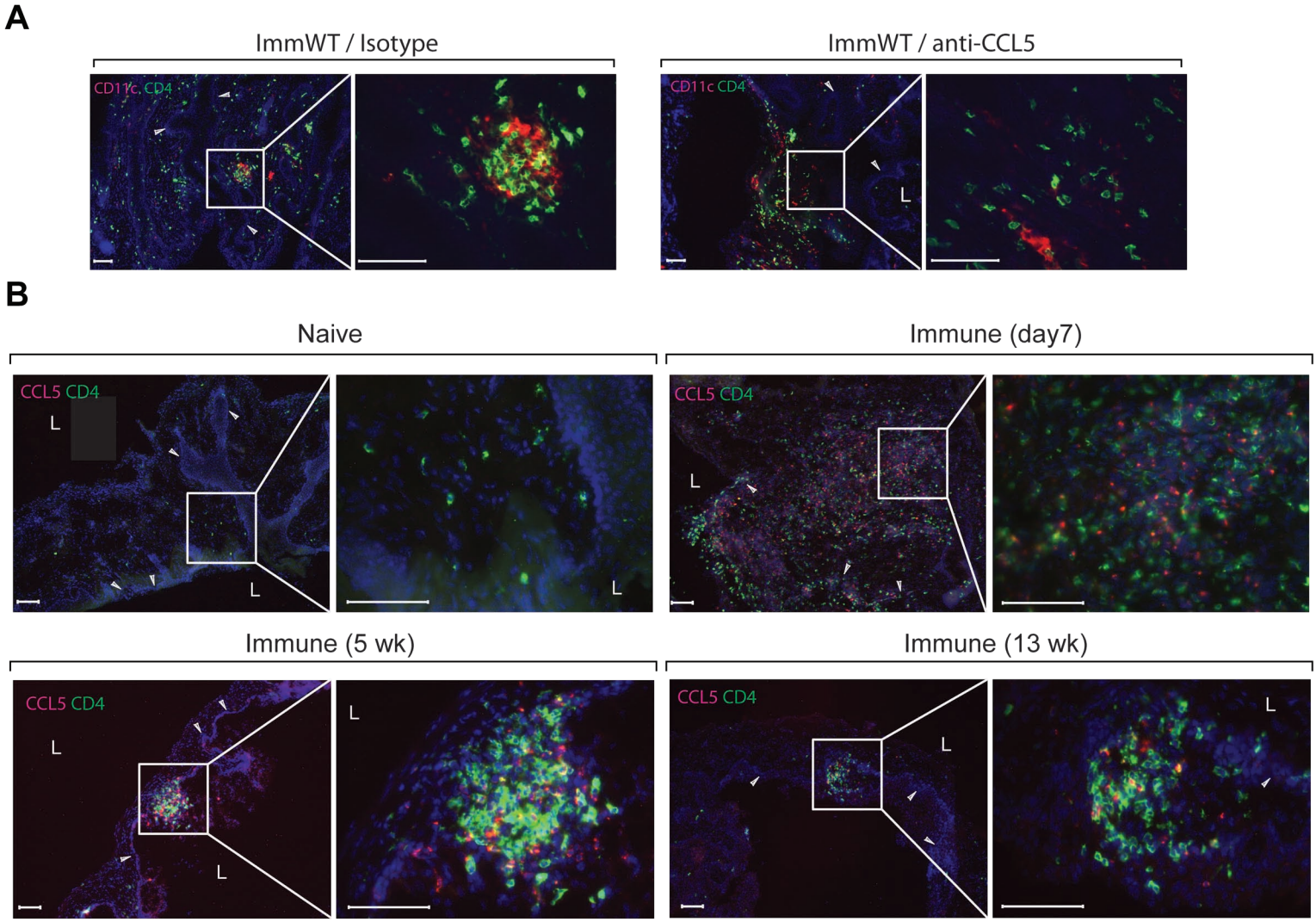
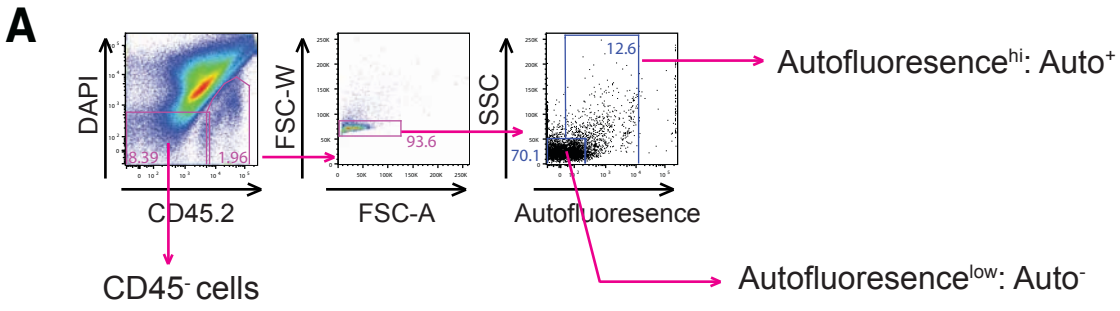
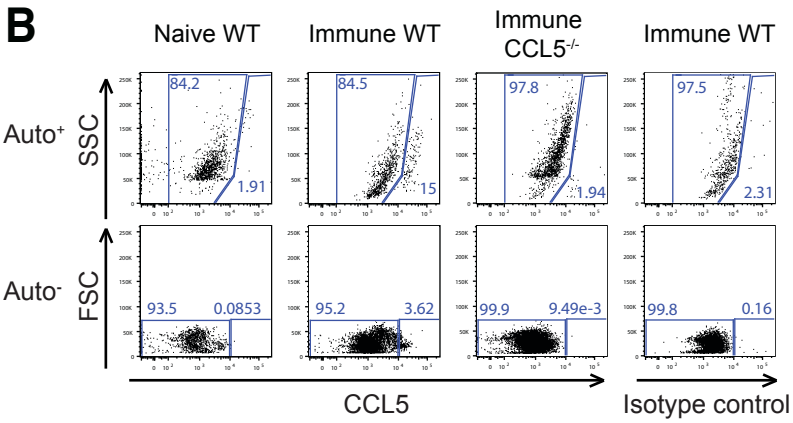
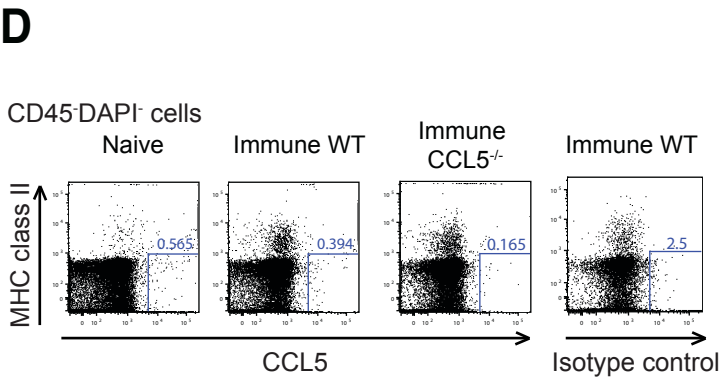
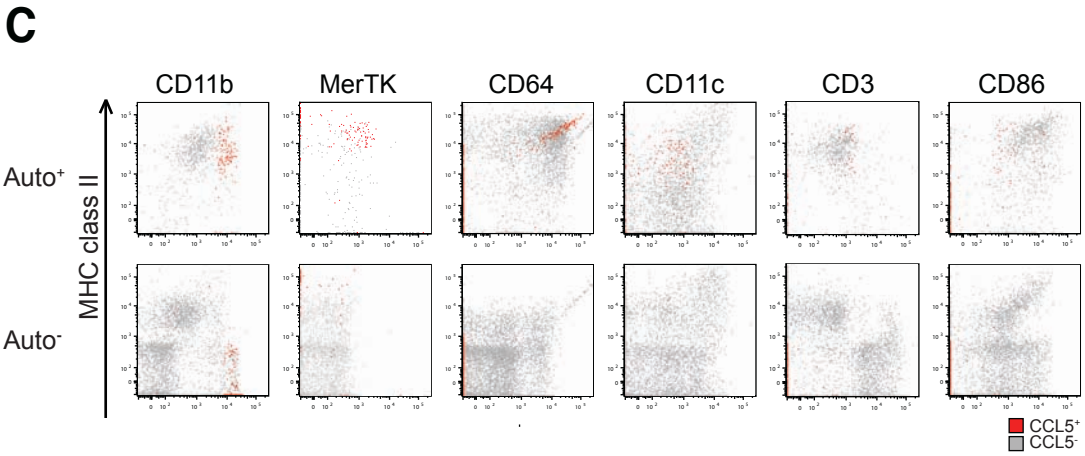


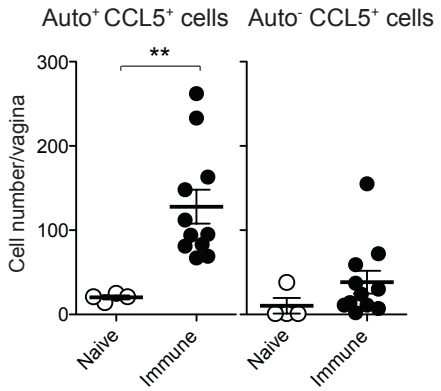
Fig. S14. CCL5 expression and consequences of CCL5 blockade by Ab.
(**A**) C57BL6 mice infected vaginally with TK$^-$ HSV-2 five weeks prior were injected with isotype control IgG or with anti-CCL5 Ab intravaginally for four consecutive days. On the fifth day, frozen sections of the vagina were stained with anti-CD4 (green) and anti-CD11c (red) Abs. (**B**) C57BL6 mice infected vaginally with TK$^-$ HSV-2 and at the indicated days post infection, vaginal tissues were stained with anti-CD4 (green) and anti-CCL5 (red) Abs. Nuclei are depicted by DAPI stain (blue). Images were captured using a 10x or 40x objective lens. Arrows indicate the basement membrane. Scale bars indicate 100 μm. L = lumen. These data are representative of at least five similar experiments.











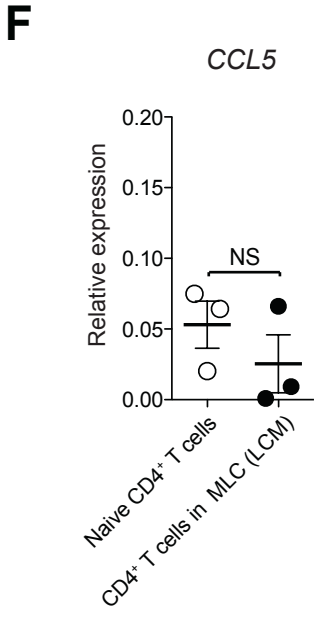


Fig. S15. CD11b$^+$ macrophages produce CCL5 in the vaginal tissues of TK$^-$ HSV-2-immunized mice.

Single cell suspension of vagina from C57BL6 mice (**A-D**) or CCL5$^{-/-}$ mice (**B&D**) infected vaginally with TK$^-$ HSV-2 five weeks prior or naive C57BL6 mice (**B&D**) were stained with the indicated myeloid markers as shown, (**C**) and stained intracellularly with Ab against CCL5. (**E**) The numbers of CCL5$^+$ cells among Auto$^+$ and Auto$^-$ population are indicated for naïve and 5 wk-immune mice. (**F**) Frozen section of vaginal tissues of C67BL/6 mice immunized with TK$^-$ HSV-2 (5 wk) was stained with anti-CD4 Ab. CD4$^+$ cells in MLC were collected by laser capture microdissection (LCM). qRT-PCR analysis of *CCL5* mRNA in CD4$^+$ T cells in MLC was performed. Gene expression was normalized to that of *CD4* for each sample. Data are means ± SE. **, $p<0.001$

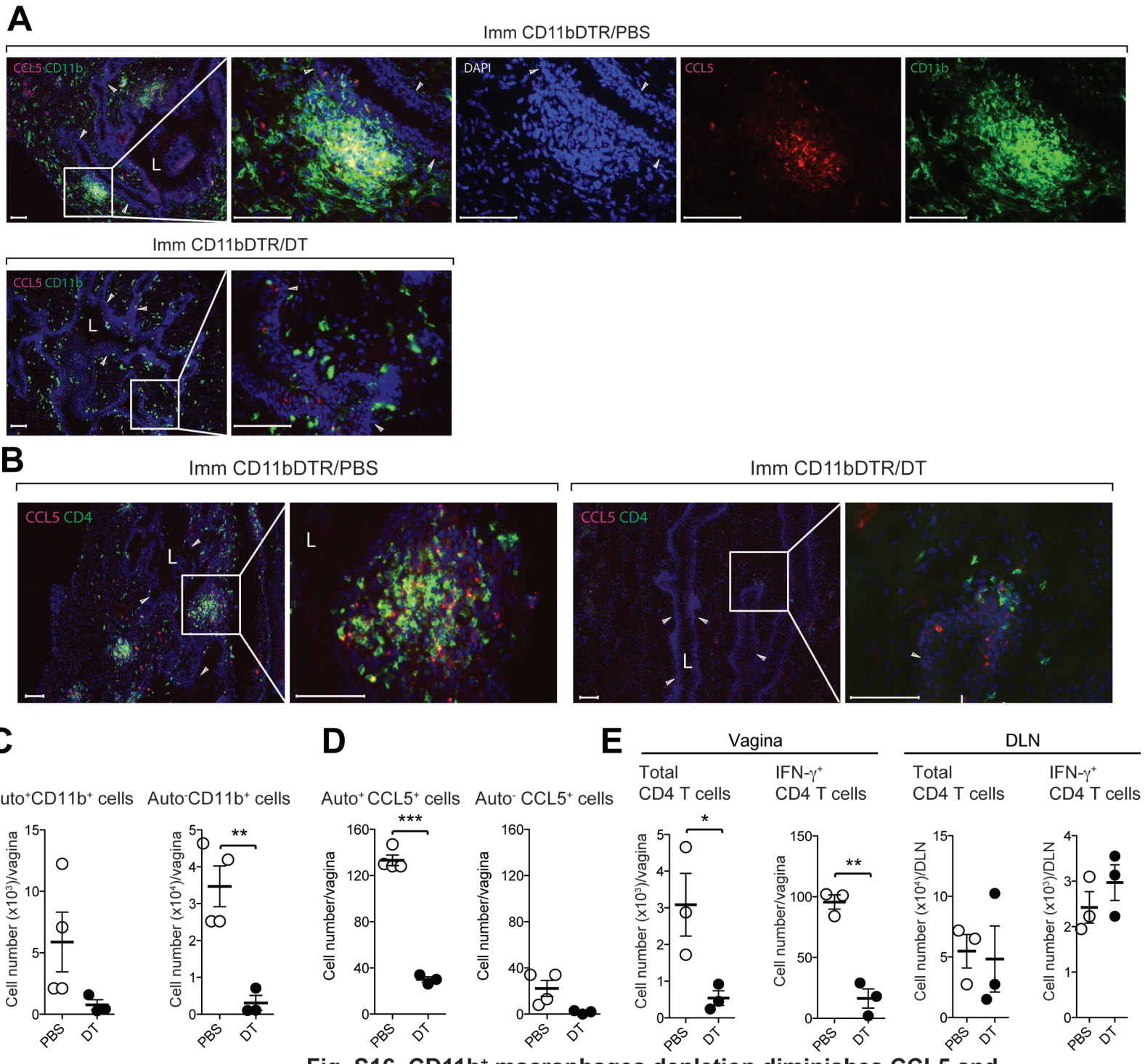


Fig. S16. CD11b⁺ macrophages depletion diminishes CCL5 and HSV-2-specific memory Th1 cells in vaginal tissues.

CD11bDTR (**A&B**) or CD11bDTR into WT BM chimera (**C-F**) were immunized ivag with TK⁻ HSV-2. Five weeks later, these mice were treated with DT to deplete CD11b⁺ macrophages. After depletion, vaginal tissues were stained with anti-CD11b (**A**) or anti-CD4 (**B**)(green) and anti-CCL5 (red)(**A&B**). Nuclei are depicted by DAPI stain (blue). Images were captured using a 10x or 40x objective lens. Arrows indicate the basement membrane. Scale bars indicate 100 μm. L = lumen. (**C**) The numbers of Auto⁺CD11b⁺ cells and Auto⁻CD11b⁺ in vagina tissues of BM chimera with or without DT treatment are depicted. (**D**) The numbers of CCL5⁺ cells among Auto⁺ and Auto⁻ cells in the vagina of such mice are depicted.(**E**) After DT treatment, HSV-2-specific Th1 cells in CD11bDTR BM chimera in vaginal tissue and DLN was detected by flow cytometry. (**F**) After DT treatment, IFN-γ-producing CD69⁺CD4⁺ T cells (IFN-γ^{int}) were analyzed by flow cytometry. Data are means ± SE. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. These data are representative of at least two similar experiments.

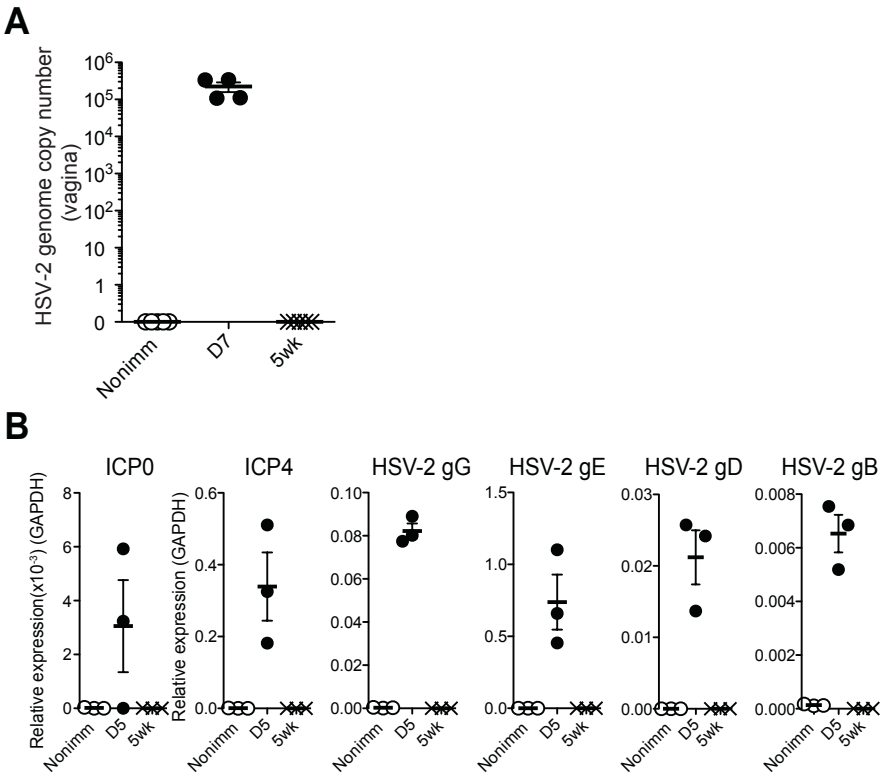


Fig. S17. Detection of HSV-2 viral genome and transcripts in vaginal tissues.
C57BL6 mice were immunized with TK$^-$ HSV-2 virus intravaginally. At the indicate time points, total viral genomic DNA (shown as pfu equivalent) (**A**) and indicated viral mRNA (**B**) in the vaginal tissue was measured by quantitative PCR.

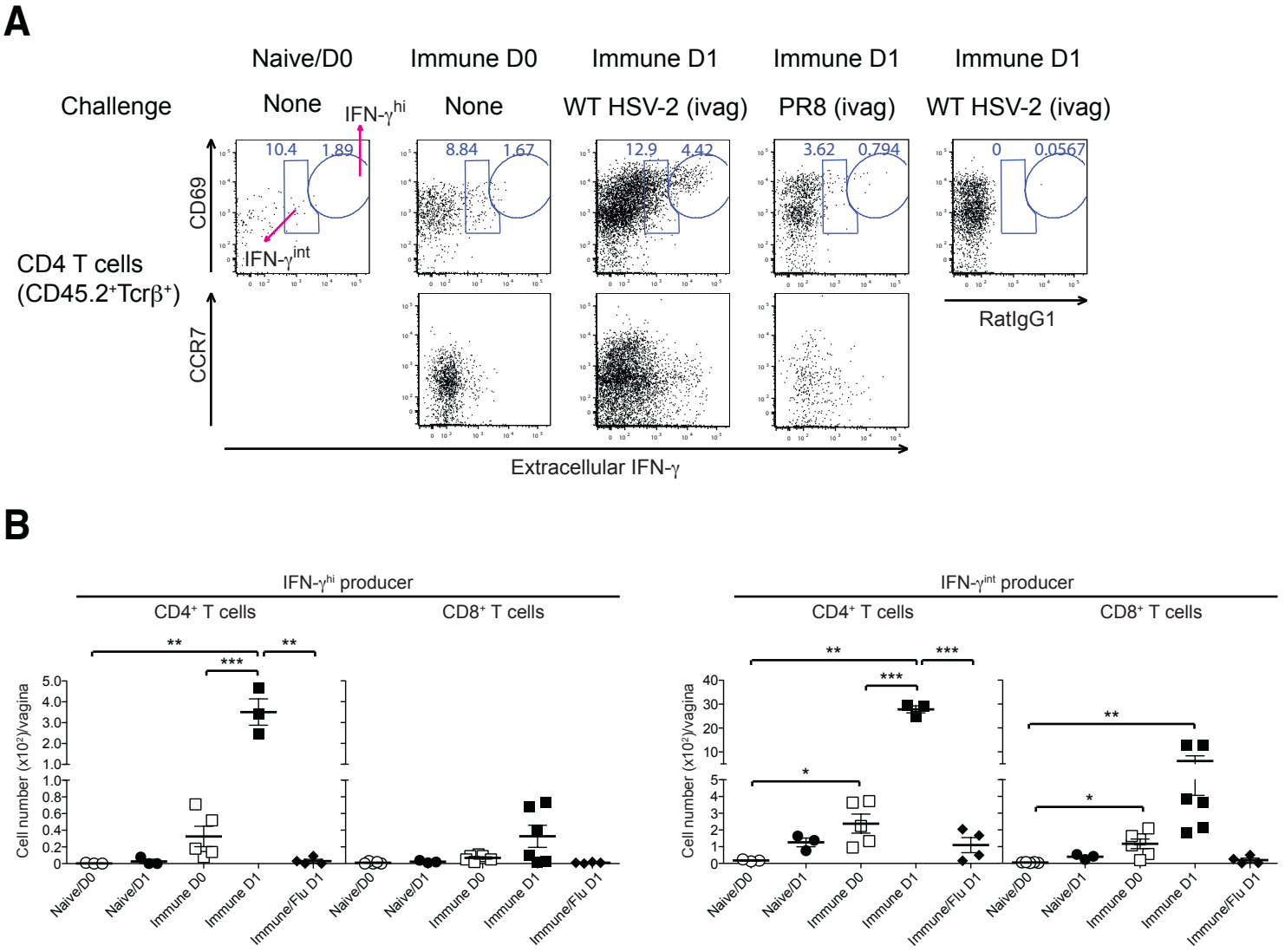


Fig. S18. WT HSV-2 challenge rapidly triggered high levels of IFN-γ from memory CD4⁺ T cells in vaginal tissues.

(**A&B**) C57/BL6 mice were immunized ivag with TK⁻ HSV-2. Five weeks later, these mice were challenged with WT HSV-2 or Influenza/PR8 intravaginally. One day after challnge, extracellular IFN-γ-producing cells (IFN-γ^{hi} and IFN-γ^{int} producer) in vaginal tissues were analyzed by flow cytometry.

Data are means ± SE. *, p<0.05; **, p<0.001; ***, p<0.001

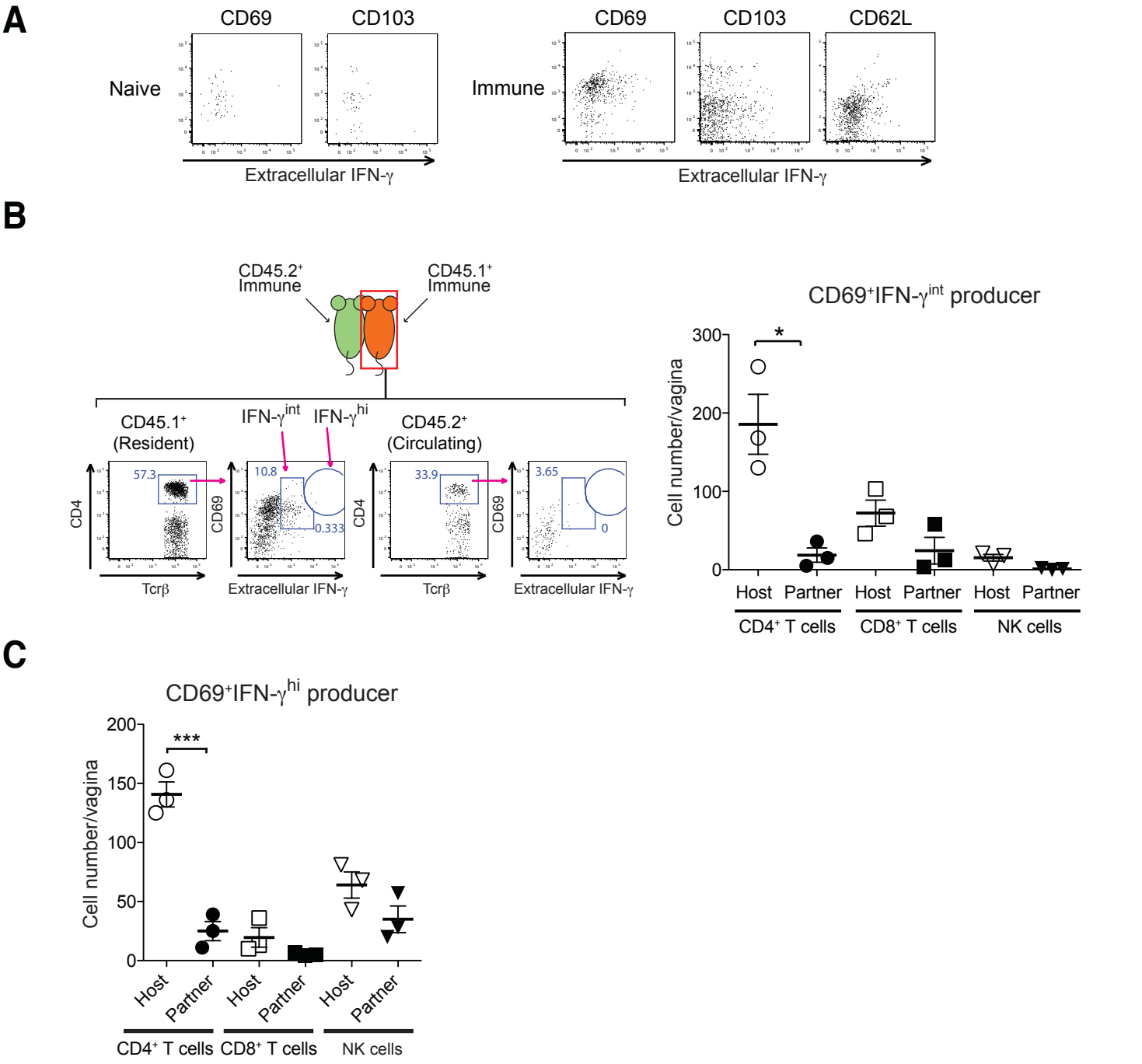


Fig. S19. Tissue-resident CD4⁺ T cells produce constitutive basal level of IFN-γ and rapidly triggered high levels of IFN-γ follwoing WT HSV-2 challenge.
(**A**) C57/BL6 mice were immunized ivag with TK⁻ HSV-2. Five weeks later, CD69, CD103 and CD62L expression on CD45.2⁺Tcrβ⁺CD4⁺ T cells following extracellular IFN-γ staining method. (**B&C**) CD45.2⁺ and CD45.1⁺ mice immunized with TK⁻ HSV-2 five weeks prior were surgically joined. Three weeks after surgery, constitutive IFN-γ-producing cells (CD69⁺IFN-γ^{int}) were analyzed by flow cytometry (**B**). FACS profile of Tcrβ⁺CD4⁺ T cells and the number of IFN-γ^{int} producer of CD4⁺ T cells, CD8⁺ T cells and NK cells were depicted. (**C**) CD45.1⁺ mice of these parabionts were challenged with WT HSV-2 intravaginally. One day after challenge, extracellular IFN-γ⁻-producing cells (CD69⁺IFN-γ^{hi} producer) in vaginal tissues are analyzed by flow cytometry. Data are means ± SE. *, p<0.05; **, p<0.001; ***, p<0.001

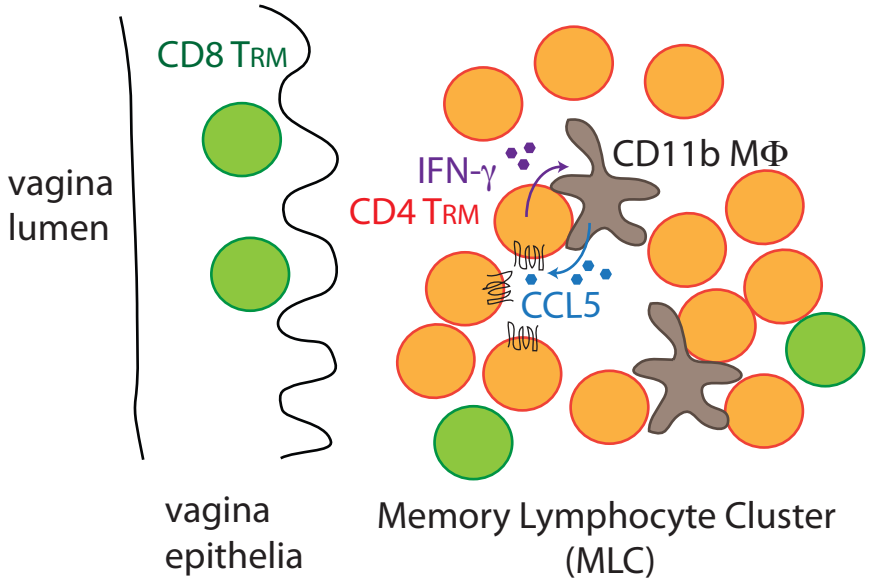


Fig. S20. Proposed mechanism of CD4 T_{RM} maintenance within the MLC.
Local CD4 T_{RM} cells are retained through CCL5 secreted by CD11b^{+} macrophages in the MLCs. CD4 T_{RM} and local macrophages may establish a feedback loop consisting of T cell secreted IFN-γ, inducing CCL5 expression from macrophages, which in turn help retain such T cells within the MLCs.