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

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CD8 tissue-resident T cells ($T_{RM}$) provide efficient local control of viral infection, but the role of CD4 $T_{RM}$ is less clear. Here, by using parabiotic mice, we show that a preexisting pool of CD4 $T_{RM}$ cells in the genital mucosa was required for full protection from a lethal herpes simplex virus 2 (HSV-2) infection. Chemokines secreted by a local network of macrophages maintained vaginal CD4 $T_{RM}$ in memory lymphocyte clusters (MLCs), independently of circulating memory T cells. CD4 $T_{RM}$ within the MLCs were enriched in clones that expanded in response to HSV-2. Our results highlight the need for vaccine strategies that enable establishment of $T_{RM}$ for protection from a sexually transmitted virus and provide insights as to how such pool might be established.

Effector memory T cells circulate throughout the peripheral tissues, and although they are sufficient to provide protection against systemic infections (1–4), they cannot control infection localized to peripheral tissues (4–6). After viral skin infection, CD8 tissue-resident memory T ($T_{RM}$) cells control infection more efficiently than circulating CD8 T cells (7–10) and recruit circulating T cells to the sites of infection (11). In contrast to CD8 $T_{RM}$ cells, little is known regarding the existence or importance of CD4 $T_{RM}$ cells. Unlike CD8 T cells, memory CD4 T cells readily traffic through circulation to provide protection at distal sites (12). Respiratory infection with influenza virus induces protective CD4 T cells capable of migrating and establishing residency in the lung (13). However, to what extent resident versus circulating memory CD4 T cells are required for protection of the host and the mechanism by which such a memory pool is maintained are unclear.

To address these questions, we used a mouse model of genital herpes. Intravaginal immunization with an attenuated herpes simplex virus 2 (HSV-2) strain [thymidine kinase–negative $(TK)^-$ HSV-2] results in complete protection from secondary challenge with wild-type (WT) HSV-2 (14). $TK^-$ HSV-2 replicates in the vaginal keratinocytes and establishes latent infection in the dorsal root ganglia but does not reactivate (15). The protective memory response to HSV-2 vaginal challenge requires CD4 T cells that secrete the cytokine interferon-γ (IFN-γ) but does not require either CD8 T cells or B cells (16). Although all

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SUPPLEMENTARY MATERIALS
www.sciencemag.org/content/vol/issue/page/suppl/DC1
Materials and Methods
Figs. S1 to S20
References (26–39)

routes of immunization induced systemic CD4 T cell responses, only intravaginal (ivag) immunization with $TK^-$ HSV-2 recruited substantial numbers of viral-specific CD4 T cells to the vagina (17, 18) and conferred protection against lethal disease (fig. S1), which indicated that the local immunization is required to mount protective immunity.

To examine the selective ability of local immunization to confer protective immunity, we used parabiotic mice. Full chimerism was achieved in systemic circulation within 2 weeks of parabiosis (fig. S2) (19). The vaginal memory pool, analyzed 6 weeks after parabiosis of ivag Iijima, page 2 $TK^-$ HSV-2 immune pairs, was largely disconnected from the rest of the circulation (Fig. 1A). Host-derived CD4 tissue-resident memory T cells ($T_{RM}$ cells) were mainly distributed within memory lymphocyte clusters (MLCs), whereas the rare donor-derived circulating CD4 T cells were found at the periphery of the MLCs (Fig. 1B). In contrast, CD8 $T_{RM}$ cells resided within the epithelium and within the MLCs, and circulating CD8 T cells were rarely found (Fig. 1B). Within the MLCs, $CD11c^+$ and major histocompatibility complex (MHC) class $II^+$ cells were present (fig. S3, A and B). Unlike the tertiary lymphoid organs that contain peripheral node address in-positive ($PNAd^+$) high endothelial venules and a developed lymphatic endothelial network (20), $PNAd^+$ blood vessels and lymphatic vessel endothelial hyaluronic acid receptor 1–positive ($LYVE\text{-}1^+$) lymphatic endothelial vessels were not found within the MLCs (fig. S3). Other features typical of tertiary lymphoid tissues were also absent, such as germinal center B cells (fig. S3D) and naive lymphocytes (fig. S4C)

HSV-2–specific $CD4^+$ $T_{RM}$ cells were T cell receptor αβ–positive ($TCR\alpha\beta^+$) (fig. S4A) and secreted IFN-γ, tumor necrosis factor–α (TNF-α), and interleukin 2 (IL-2) (fig. S4B) in response to viral antigen stimulation. The cell surface phenotype of $CD4^+$ $T_{RM}$ cells resembled that of CD8 $T_{RM}$ cells ($CD62L^-$ $CD44^{hi}$ $CD11a^+$ $CD69^+$ $CD49d^+$ CCR7) (7, 21) (fig. S4, B and C). Expression of KLF2, a zinc-finger transcription factor whose suppression promotes retention of CD8 $T_{RM}$ cells in tissues (22)—and its downstream genes *S1pr1* and *Sell* (CD62L)—were also suppressed in CD4 $T_{RM}$ cells residing within the MLCs (Fig. 1C). Only a small subset of CD4 $T_{RM}$ cells expressed CD103 (fig. S4, B and C), a molecule important for CD8 $T_{RM}$ cell survival within the epithelia (23).

Next, to investigate whether a continuous supply of circulating lymphocytes is required to maintain the vaginal memory pool, mice immunized intravaginally were treated with FTY720, an antagonist of sphingosine-1-phosphate receptor 1 (S1PR1) that blocks lymphocyte egress from lymph nodes. Although circulating lymphocytes were depleted from peripheral blood and accumulated in the lymph nodes after 2 weeks of FTY720 treatment (fig. S5, A and B) (24), the number of HSV-specific IFN-$\gamma^+$ CD4 T cells and $K^b$ glycoprotein B (gB) $tetramer^+$ CD8 T cells (fig. S5) in the vagina remained unchanged. Thus, MLCs, the main site for CD4 $T_{RM}$ cell residency, are distinct from tertiary lymphoid organs and do not require circulating cells for maintenance.

Next, we compared the ability of tissue-resident versus circulating memory lymphocytes to mediate protection against a lethal HSV-2 challenge using various parabiotic combinations. Mice relying on only the circulating memory cells (group 4) were impaired in their ability to suppress viral replication, and about half of this group succumbed to viral disease, in

contrast to the fully protected mice in group 3 that harbor $T_{RM}$ cells (Fig. 2, A to C, and fig. S6). Transient virus control seen in group 4 (first 2 days of challenge) is likely mediated by HSV-2–specific immunoglobulin G (IgG) as previously reported (25, 26). Protection conferred by the circulating memory cells depended on $CD4^+$ T cells of the immune donor mice (group 5) but not $CD8^+$ T cells (group 6). In addition, IFN-γ responsiveness by the cells within the challenged animal was required for protection by WT circulating memory T cells (group 7) (Fig. 2, A to C, and fig. S6). Thus, circulating CD4 memory T cells are able to provide some level of protection against HSV-2 infection and disease, by secreting IFN-γ and inhibiting viral replication in the vaginal epithelium, an observation consistent with skin HSV-1 infection (12). However, for complete protection from disease and mortality, local CD4 $T_{RM}$ cells are crucial in providing antiviral defense within the genital mucosa.

Why are circulating memory CD4 T cells inferior to $T_{RM}$ cells? After vaginal infection of the naive partner with WT HSV-2, circulating T cells from the immunized partner entered the tissue within 2 days (Fig. 2D, filled bars). However, the newly arriving circulating HSV-2–specific memory CD4 T cells did not secrete IFN-γ until 5 days after challenge with HSV-2 (Fig. 2E) but not an irrelevant virus, influenza (figs. S7 and S8). Because rapid delivery of IFN-γ to the vaginal epithelium is crucial in HSV-2 control (27), this may be why circulating HSV specific memory CD4 T cells provided poor protection. In contrast, in the presence of MLCs, circulating HSV-2–specific memory T cells were barely recruited upon intravaginal secondary challenge with viruses (fig. S8). These data indicated that the alarming function of $T_{RM}$ cells (11) might be trumped by the abundance and efficiency of preexisting $T_{RM}$ cells within the MLCs in clearing the secondary infection.

To examine the antigen-specificity of $T_{RM}$ cells, we analyzed the sequence of the CDR3 region of TCRβ gene of CD4 T cells isolated from MLCs by laser capture microdissection. Six TCRβ CDR3 sequences were shared by $CD4^+$ T cells in MLCs and those in the draining lymph nodes (DLNs) at the peak of primary immune response in $TK^-$ HSV-2 immunized mice but not in naive mice (Fig. 3A). One of these sequences, CTCSAAGGDAEQFF (TCRVβ01-01*01 and TCRJβ02-01*01) [with variable (V) and joining (J) regions as described], represented a dominant clone (33%) in the CD4 T cells in the MLCs (Fig. 3, B and C). This dominant clone was also detected in the DLNs and in a portion (11%) of CD4 T cells residing outside of MLCs (Fig. 3, B and C). Furthermore, we found that HSV-2–specific CD4 $T_{RM}$ cells expressed TCRVβ01-01*01 (Fig. 3D and fig. S9). These results indicated that MLCs are highly enriched for HSV-2–specific CD4 $T_{RM}$ cells.

We next addressed the mechanism by which $T_{RM}$ cells are maintained in the vagina. Treatment of $TK^-$ HSV-2 intravaginally immunized mice with pertussis toxin (PTX), which inhibits $G\alpha_i$ signaling downstream of many chemokine receptors, resulted in a considerable reduction in HSV-specific memory T cell numbers in the vagina and CD4 $T_{RM}$ cells expressing TCR Vβ CDR3 sequence (CTCSAAGGDAEQFF) in MLCs (figs. S9B and S10A), the disappearance of the MLCs (fig. S10B), and lack of protection against ivag HSV-2 challenge (fig. S10C). The disappearance of $T_{RM}$ cells was not associated with cell death (fig. S11A) but was due to the expulsion of CD4 T cells into the vaginal lumen (fig. S11, B and C). These data suggested that chemokine receptor signaling is required for the retention of $T_{RM}$ cells within the MLCs. Quantitative reverse transcription polymerase chain

reaction (qRT-PCR) revealed selective up-regulation of CCL5 and CXCL9 in vaginal tissue at 5 and 12 weeks after $TK^-$ HSV- 2 immunization, and this expression was largely dependent on IFN-γ (Fig. 4A). Expression of CCR1, CCR5, and CXCR3 was detected in the CD4 $T_{RM}$ and CD8 $T_{RM}$ cells by flow cytometry (fig. S12A). To determine whether these chemokines are required for the retention of $T_{RM}$ cells within the vagina, we treated HSV-2–immunized mice with neutralizing antibodies against CXCL9 and CCL5. The dose of antibody used was deemed saturating (fig. S13). Blockade of CCL5 diminished the number of HSV-2–specific CD4 $T_{RM}$ and CD8 $T_{RM}$ cells in the vagina (Fig. 4, B and C, and fig. S12B) and led to a partial collapse of MLCs (fig. S14A). CXCL9 blockade reduced the number of CD8 $T_{RM}$ cells. It is noteworthy that neutralizing CCL5 resulted in diminished protective immune responses to secondary challenge with HSV-2 ivag infection to the level similar to CD4 T cell depletion (Fig. 4C). Thus, these data indicate that CD4 $T_{RM}$ cells are maintained through continuous interaction with the chemokine CCL5 and that this interaction is required for protection from secondary challenge.

Next, we determined the localization and cellular source of CCL5 within the MLC. Five weeks after ivag immunization, $CCL5^+$ cells were mainly localized within MLCs and were maintained for at least 13 weeks (fig. S14B). Furthermore, the CCL5 producers within the MLCs were hematopoietic ($CD45^+$), and were $CD11b^+$ $CD11c^{lo}$ (Fig. 4D). By flow cytometry, CCL5 expression was detected in mainly macrophages ($Autofluoresence^{hi}$, $CD11c^{lo}$, $CD11b^+$, $MerTK^+$, $CD64^+$) (28), with variable expression of MHC II (fig. S15) but not T cells (fig. S15, C and F), and the number of $CCL5^+$ cells increased after immunization (fig. S15E). Staining for CCL5 was specific (fig. S15, B and D). Depletion of $CD11b^+$ cells in diphtheria toxin receptor–inducible CD11bDTR→WT bone marrow chimera led to a reduction of $CCL5^+$ cells in the vagina (fig. S16, C and D), complete elimination of MLC structure, and diminished CD4 $T_{RM}$ cells in the vaginal tissue within 5 days (Fig. 4E and fig. S16, A and B). Collectively, our results suggest a model in which CD4 $T_{RM}$ cells are maintained through a positive-feedback loop, in which CD4 $T_{RM}$ cell stimulation by local macrophages results in low IFN-γ secretion, which in turn induces chemokines, such as CCL5, to retain the T cells within the MLCs. Although there was no detectable viral genomic DNA or mRNA expression in the vagina (fig. S17), it remains possible that small amount of viral peptide presentation by MHC II is required for the feedback loop. In support of this hypothesis, single cells were isolated from the vagina in the absence of ex vivo stimulation, and secreted IFN-γ was captured onto the cell surface and stained. This uncovered two types of cell populations—those that constitutively express intermediate levels of IFN-γ and those that secrete high levels of IFN-γ after HSV-2 secondary challenge but not after an irrelevant virus infection (influenza) (fig. S18). The majority of the $IFN\text{-}\gamma^{int}$ cells (where superscript int indicates intermediate) were CD4 $T_{RM}$ cells (fig. S19, A and B) and required the presence of $CD11b^+$ cells for their IFN-γ production (fig. S16F). In contrast, after WT HSV-2 secondary challenge, antigen-presenting cells that had captured HSV-2 antigen induced the secretion of large amounts of IFN-γ by $CD4^+$ $T_{RM}$ cells in a antigen-dependent manner (figs. S18 and S19C). Therefore, these results support a model in which CD4 $T_{RM}$ cells are maintained by chemokines secreted from local macrophages, which are in turn induced by basal levels of IFN-γ continuously secreted by $T_{RM}$ cells (fig. S20). Upon infection with HSV-2, the CD4 $T_{RM}$

cells are stimulated by cognate antigen to secrete high levels of IFN-γ, which results in viral clearance.

Our study highlights the importance of local memory T cells in antiviral defense against genital HSV-2 infection and supports the hypothesis that a local T cell presence correlates with viral control on the basis of human studies (29–31). Circulating memory T cells failed to access the genital mucosa at steady state. After infection, whereas circulating memory CD4 T cells do enter the genital tissue and provide protection, viral control is both delayed and incomplete. In contrast, preexisting CD4 $T_{RM}$ cells in the MLCs were able to respond rapidly to secondary challenge and suppressed replication before the virus spread to the nervous system. MLCs are maintained through interaction between CD4 $T_{RM}$ cells and macrophages, which results in T cell secretion of IFN-γ and induction and expression of CCL5 by the local macrophages. Similar MLC-like structures have been reported in humans infected with HSV-2 (32) and *Chlamydia trachomatis* (33), which suggests that this may be a common mechanism of adaptive immune defense in the genital mucosa. Our data strongly support the need for designing vaccines for sexually transmitted infections that establish local memory in the T cell pool.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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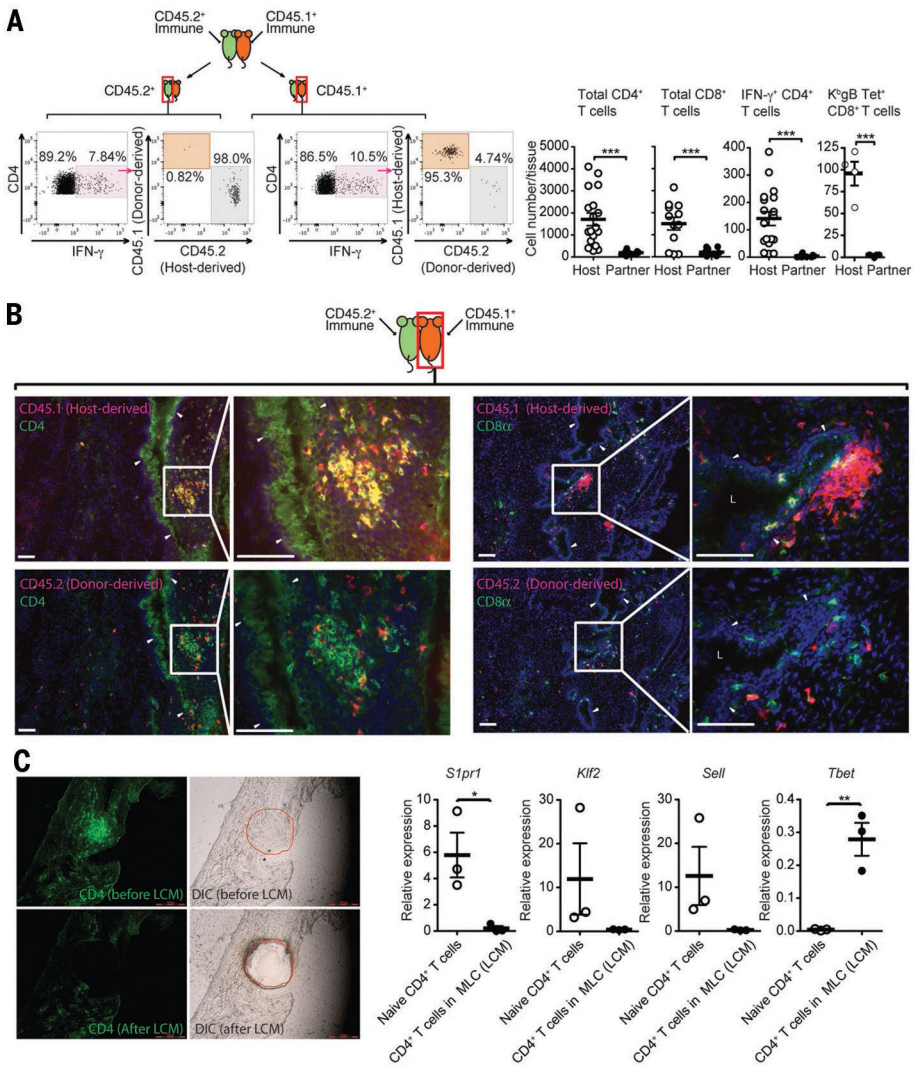
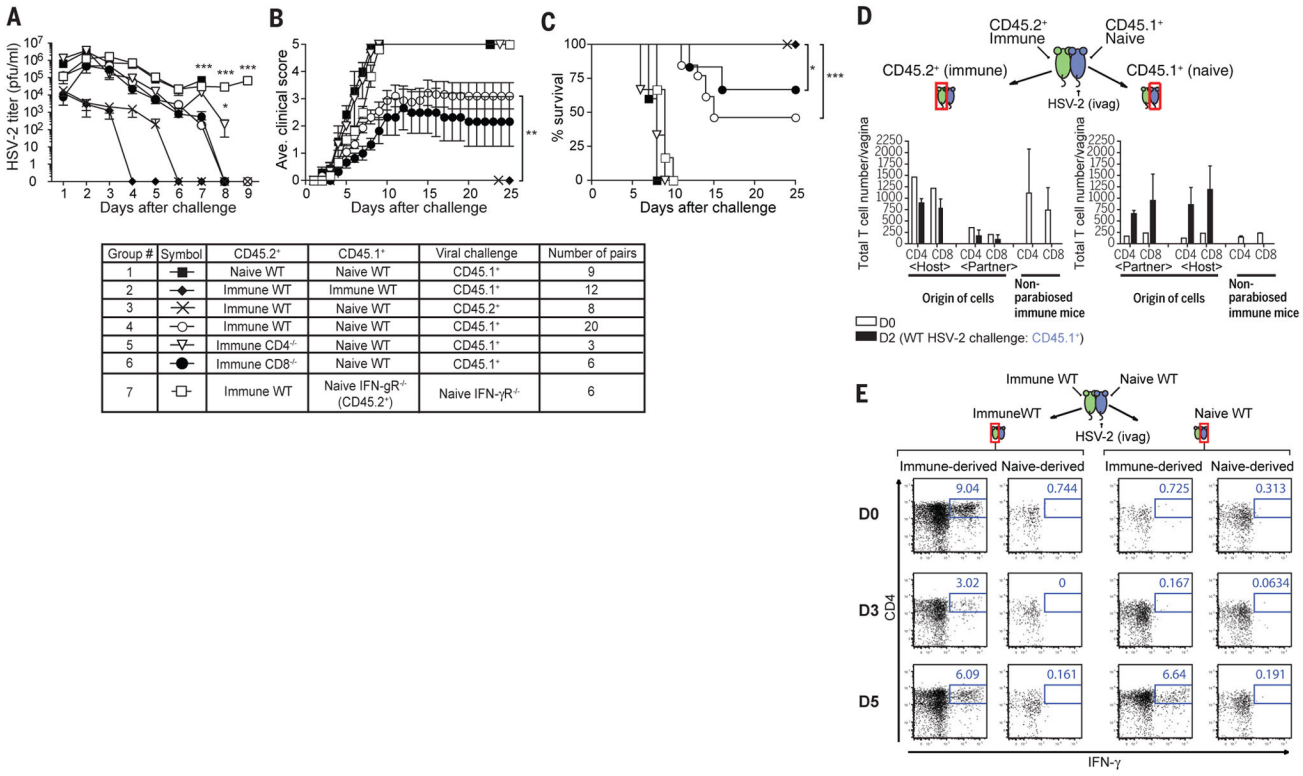



**Fig. 1. Vagina-resident memory T cells are found in MLCs**

$CD45.2^+$ and $CD45.1^+$ mice immunized with $TK^-$ HSV-2 5 weeks before were surgically joined. (**A**) Six weeks later, the presence of total and HSV-specific host and donor CD4 and CD8 T cells in vaginal tissues of parabiotic mice was analyzed by flow cytometry. Host-derived and partner-derived cell numbers of total $CD4^+$ and $CD8^+$ T cells ($n$ = 6 to 9 pairs), HSV-2–specific IFN-$\gamma^+$ $CD4^+$ T cells ($n$ = 9 pairs), and $K^b$ gB tetramer$^+$ $CD8^+$ T cells ($n$ = 2 pairs) in vaginal tissues of parabiotic mice were analyzed by flow cytometry. These data were combined from multiple experiments and analyzed by two-tailed unpaired $t$ test. (**B**) Three weeks after parabiosis, frozen sections of vaginal tissue were stained with antibodies against CD4 or CD8 (green) and CD45.1 or CD45.2 (red). The images were captured by using a 10× or 40× objective lens. Arrows indicate the basement membrane. Scale bars indicate 100 μm. These are representative of two pairs of parabiosed mice. (**C**) Frozen section of vaginal tissues of C67BL/6 mice immunized with $TK^-$ HSV-2 (5 weeks) was stained with antibody against CD4. $CD4^+$ cells in MLCs were collected by laser capture microdissection (LCM). LCM samples were pooled from three vaginal tissues, four or five

slides per mouse. The images were captured using a 20× objective lens. Scale bars indicate 100 μm. DIC, differential interference contrast. qRT-PCR analysis of indicated mRNA was performed in CD4 T cells in MLCs and naive CD4 T cells that were sorted from spleen ($n$ = 3). Their gene expression was normalized to that of *Cd4* for each sample. Data are means ± SEM. *$P$ < 0.05; **$P$ < 0.001; ***$P$ < 0.001 (two-tailed unpaired $t$ test).




| Group # | Symbol | CD45.2+ | CD45.1+ | Viral challenge | Number of pairs |
|---|---|---|---|---|---|
| 1 | ■ | Naive WT | Naive WT | CD45.1+ | 9 |
| 2 | ◆ | Immune WT | Immune WT | CD45.1+ | 12 |
| 3 | ✕ | Immune WT | Naive WT | CD45.2+ | 8 |
| 4 | ○ | Immune WT | Naive WT | CD45.1+ | 20 |
| 5 | ▽ | Immune CD4-/- | Naive WT | CD45.1+ | 3 |
| 6 | ● | Immune CD8-/- | Naive WT | CD45.1+ | 6 |
| 7 | □ | Immune WT | Naive IFN-gR-/- (CD45.2+) | Naive IFN-γR-/- | 6 |

**Fig. 2. CD4 $T_{RM}$ cells are superior to circulating memory T cells in mediating protection against lethal HSV-2 challenge**

(**A** to **C**) CD45.2$^+$ mice were surgically joined with CD45.1$^+$ mice of the indicated genotypes. Mice were either naive or immunized with TK$^-$ HSV-2 5 weeks before parabiosis as indicated in the table. Two to 3 weeks after parabiosis, the indicated partner was challenged with a lethal dose of WT HSV-2 intravaginally. Virus titer in the vaginal secretion (A), clinical score (B), and mortality (C) after viral challenge are depicted. (**D** and **E**) CD45.2$^+$ mice immunized with TK$^-$ HSV-2 5 weeks before were surgically joined with naive CD45.1$^+$ mice for 2 to 3 weeks. On the indicated days (e.g., D0, D2)after WT HSV-2 challenge of the CD45.1$^+$ partner, the number of total CD4$^+$ and CD8$^+$ T cells (D) and the percentage of HSV-2–specific IFN-γ–producing CD4 T cells among the CD90.2$^+$ population (E) of host and donor origins in the vaginal tissue of both parabiotic partners were analyzed by flow cytometry. Data are means ± SEM. *$P$ < 0.05; **$P$ < 0.001; ***$P$ < 0.001.

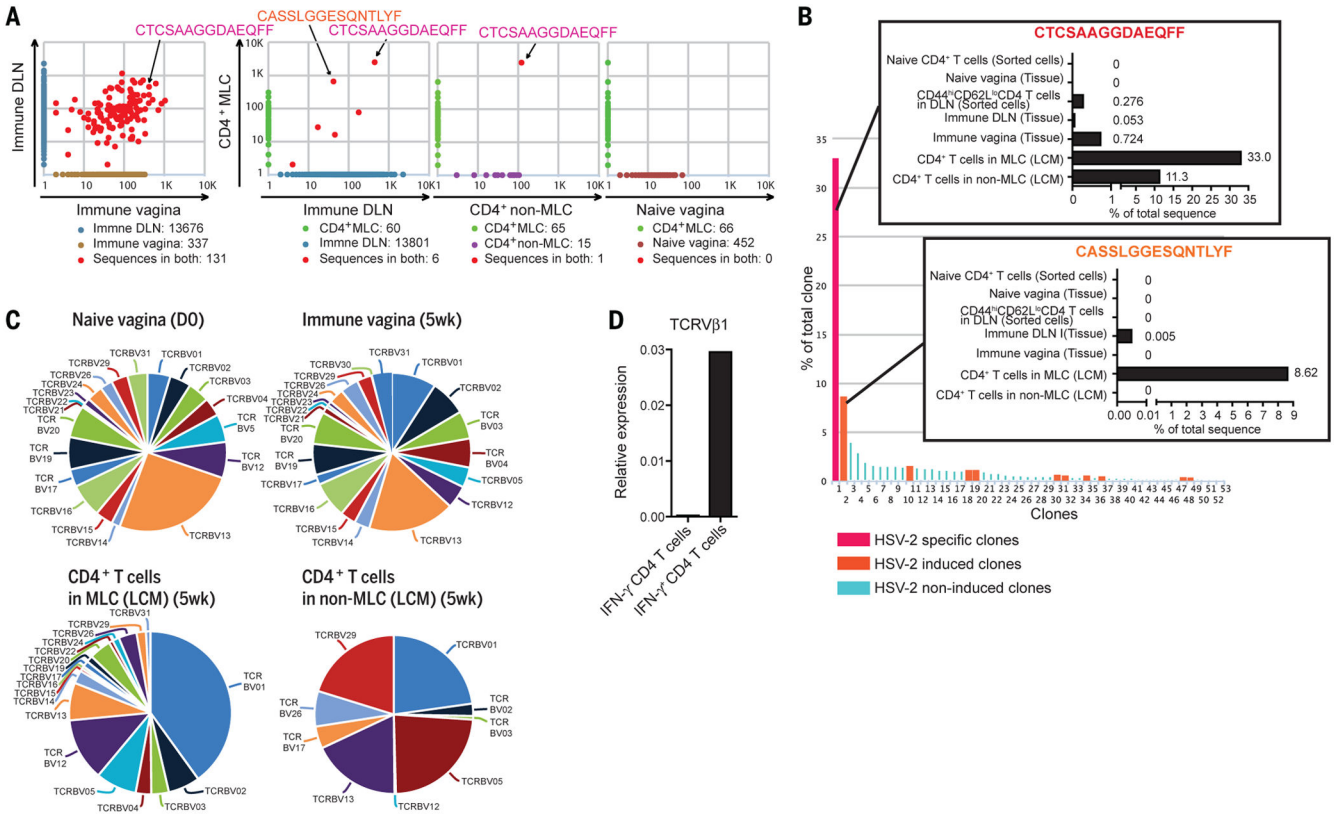



**Fig. 3. Analysis of CD4$^+$ TCR β-chain repertoires in MLCs**

Frozen sections of vaginal tissues of C57BL/6 mice immunized with TK$^-$ HSV-2 (for 5 weeks) was stained with antibody against CD4. CD4$^+$ cells within the MLCs and non-MLC tissue were collected by LCM (pooled from vaginal tissues from 10 mice). DNA from LCM samples, total vaginal tissues of immunized mice, sorted naive CD44$^{lo}$CD62L$^{hi}$ CD4$^+$ T cells and sorted CD44$^{hi}$CD62L$^{lo}$ CD4$^+$ T cells in DLNs 7 days after TK$^-$ HSV-2 immunization (pooled from at least three mice) were subjected to TCR β-chain sequencing. (**A**) Common CDR3 sequences of TCR β chains between the indicated samples are depicted. (**B**) In MLCs, 53 clones of TCR β-chain sequences were detected. Percentage of total clones for HSV-2–specific clones (pink), HSV-2–induced clones (orange), and HSV-2 noninduced clones (blue) are depicted. (**C**) The pie charts show the frequency of Iijima, page 10 TCR Vβ families in vaginal tissue (naive and immune), CD4$^+$ T cells in MLCs and non-MLC tissue. (**D**) Single-cell suspensions from vaginal tissues of mice vaginally immunized with TK$^-$ HSV-2 5 weeks before were restimulated with splenocytes loaded with HSV-2 antigen. IFN-γ$^+$ CD4$^+$ and IFN-γ$^-$ CD4$^+$ cells as shown in fig. S9C were sorted for qPCR analysis of TCR Vβ1. Gene expression of these was normalized to that of *GAPDH* for each sample.

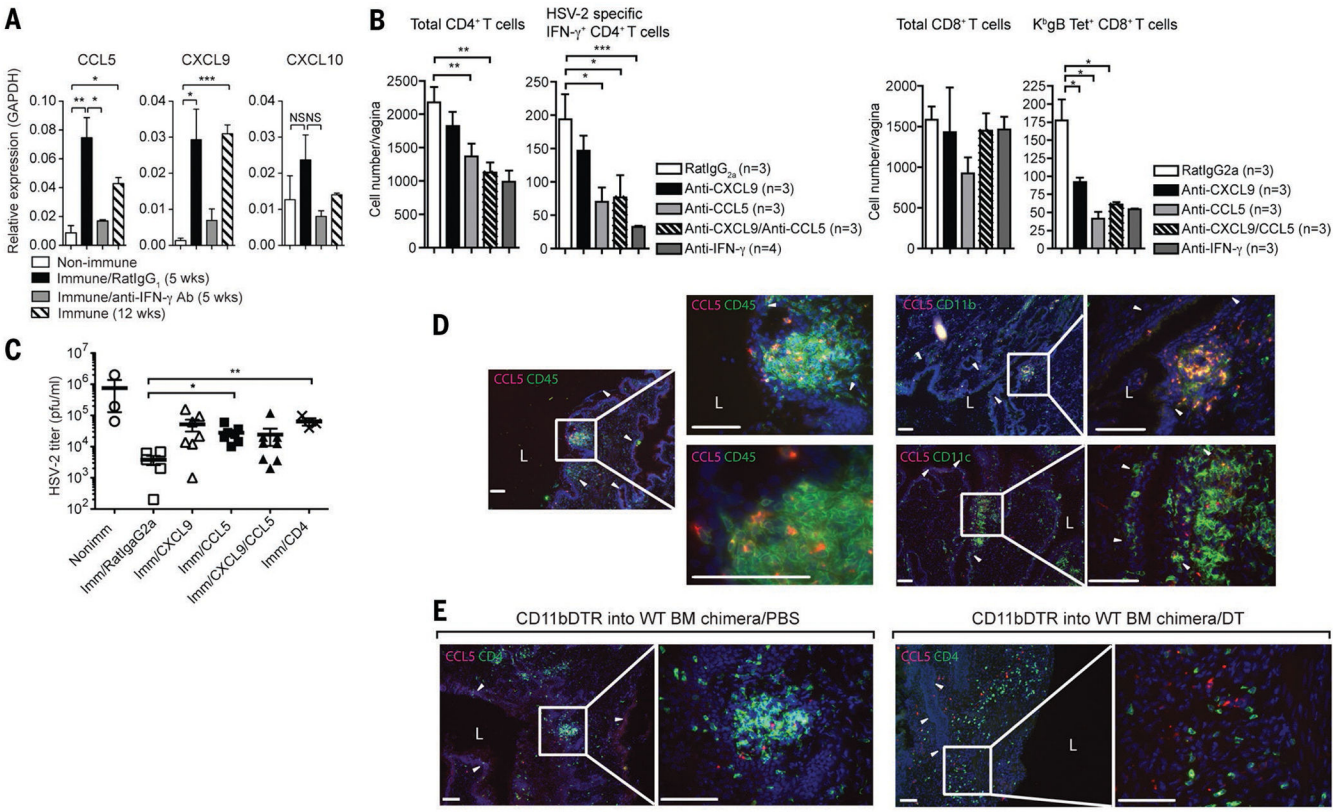



**Fig. 4. The chemokine CCL5 is required for the retention of $T_{RM}$ in vaginal tissues after TK⁻ HSV-2 immunization**

(**A**) Expression levels of CCL5, CXCL9, and CXCL10 in vaginal tissues of C57BL6 mice immunized with TK⁻ HSV-2 5 or 12 weeks before were analyzed by qRT-PCR. A group of mice immunized for 5 weeks was treated with rat $IgG_1$ ($n$ = 5) or antibody against IFN-γ (R4-6A2) ($n$ = 3) intravaginally for four consecutive days. mRNA expression of the indicated genes was normalized to that of *GAPDH* for each sample. (**B**) C57/BL6 mice intravaginally immunized with TK⁻ HSV-2 5 weeks before were treated with isotype control IgG and CXCL9- and/or CCL5-specific antibody intravaginally for four consecutive days. On day 5, HSV-2–specific IFN-$\gamma^+$ $CD4^+$ T cells and tetramer$^+$ $CD8^+$ T cells in the vagina were analyzed by flow cytometry. (**C**) Mice immunized and treated as in (B) were challenged with a lethal dose of WT HSV-2 intravaginally 1 day after the antibody treatment regimen. Virus titers in the vaginal wash were measured on day 1 after challenge. (**D**) Frozen sections of vaginal tissue from mice immunized with ivag TK⁻ HSV-2 5 weeks before were stained with antibodies against CCL5 (red) and CD45, CD11b, or CD11c (green). (**E**) CD11bDTR into WT bone marrow chimera immunized with TK⁻ HSV-2 5 weeks before were treated with DT, and frozen sections of vaginal tissue were stained with antibodies against CCL5 (red) and CD4 (green). Nuclei are depicted by 4′,6′-diamidino-2-phenylindole (DAPI) stain (blue). Images were captured using a 10× or 40× objective lens. Scale bars indicate 100 μm. These data are representative of three similar experiments. Data are means ± SEM. *$P$ < 0.05; **$P$ < 0.001; ***$P$ < 0.001.