SUPPLEMENTAL MATERIAL

Iijima et al., http://www.jem.org/cgi/content/full/jem.20082039/DC1

Isolation of lymphocytes and DCs from the epithelium and lamina propria of vagina.
The genital tracts of vaginal tissues treated with Depo-Provera were segregated from urethra and cervix. These vaginal tissues were then incubated in 0.5 mg/ml Dispase II (Roche) for 15 min at 37°C. For lymphocyte analysis, vaginal tissues were cut into small pieces and then digested with 0.425 mg/ml collagenase D (Roche) and 30 μg/ml DNase I (Sigma-Aldrich) at 37°C for 45 min. For vaginal DC analysis, the epithelium of vagina was peeled off from the lamina propria as previously shown (Iijima, N., M. M. Linehan, S. Saeland, and A. Iwasaki. 2007. *Proc Natl Acad Sci USA*. 104:19061–19066.). The epithelium were cut into small pieces and then digested with 0.425 mg/ml collagenase D and 30 μg/ml DNase I at 37°C for 10 min. To analyze the DC population in the lamina propria of vagina, the lamina propria were cut into small pieces and were further digested with 0.425 mg/ml collagenase D and 100 U/ml hyaluronidase (Sigma-Aldrich) and 30 μg/ml DNase I for 45 min. To both of the digested tissues, 5 mM EDTA at the final concentration was added and incubated for additional 5 min. The resulting cells were filtered through a 70-μm filter and used for FACS analyses.

Primary keratinocyte culture and infection.
Primary keratinocyte cell lines were generated from the epidermis of fetal C57BL/6 mice and propagated in the media of 50% EMEM (Ca^{2+} free) and 50% conditioned media from culture supernatant of newborn skin fibroblast containing 2 ng/ml EGF, 0.4 μg/ml of hydrocortisone, 0.75 mM aminoguanidine nitrate, 10^{-10} M of cholera toxin, and 8% Ca^{2+}-free FBS (KC media; Bachmann, M.F., and R.M. Zinkernagel. 1997. *Annu. Rev. Immunol.* 15:235–270). For HSV infection, these cells (2–5 × 10^5 cells/well) were cultured in 24-well plates (BD) and then incubated with 1.56 × 10^{-3} MOI of $186syn^+$ HSV-2 at 37°C. 1 h after infection, the keratinocytes were washed once in PBS and then were incubated in KC media (400 μl/well) for an additional 48 h. To examine the effect of IFN-α or IFN-γ on the virus replication in the keratinocytes, these cytokines at the final concentration of 2.5, 0.625, 0.156, and 0.04 ng/ml were added into the wells 2 h after HSV-2 infection. At the end of the incubation, these cells were incubated with 0.05% trypsin solution (Invitrogen), including 0.53 mM EDTA, for 15 min and then collected for FACS analysis.

ELISPOT assay.
C57BL/6 mice or CD11c-DTR→WT BM chimeras were immunized ivag with 5 × 10^5 PFU TK^-HSV-2. 4 wk later, the vaginal tissues were segregated from urethra and cervix. These vaginal tissues were then cut into small pieces and then incubated in 0.5 mg/ml Dispase II (Roche) for 15 min at 37°C. Thereafter, these cells were digested with 0.425 mg/ml collagenase D (Roche), 30 μg/ml DNase I (Sigma-Aldrich), and 0.054% hyaluronidase IV-S (Sigma-Aldrich) at 37°C for 30 min. The resulting cells were filtered through a 70-μm filter and then passed over Lympholyte M (Cedarlane Laboratories Limited). Lymphocytes were collected from the interface and resuspended with complete DMEM. To remove adherent cells containing macrophages, these cells were incubated in 24-well tissue culture plates (BD) for 1 h at 37°C. The nonadherent lymphocytes were incubated with noninfected splenocytes (1 × 10^5 cells/well) in the presence of mock or HSV-2 antigen (equivalent to 4 × 10^5 PFU/well) in 96-well PVDF Membrane ELISPOT plates (Millipore) that were precoated with anti–mouse IFN-γ Ab (clone, AN-18) for 48 h. The plates were developed with anti–mouse IFN-γ mAb (clone, R4-6A2) according to the manufacturer's instructions (eBioscience).

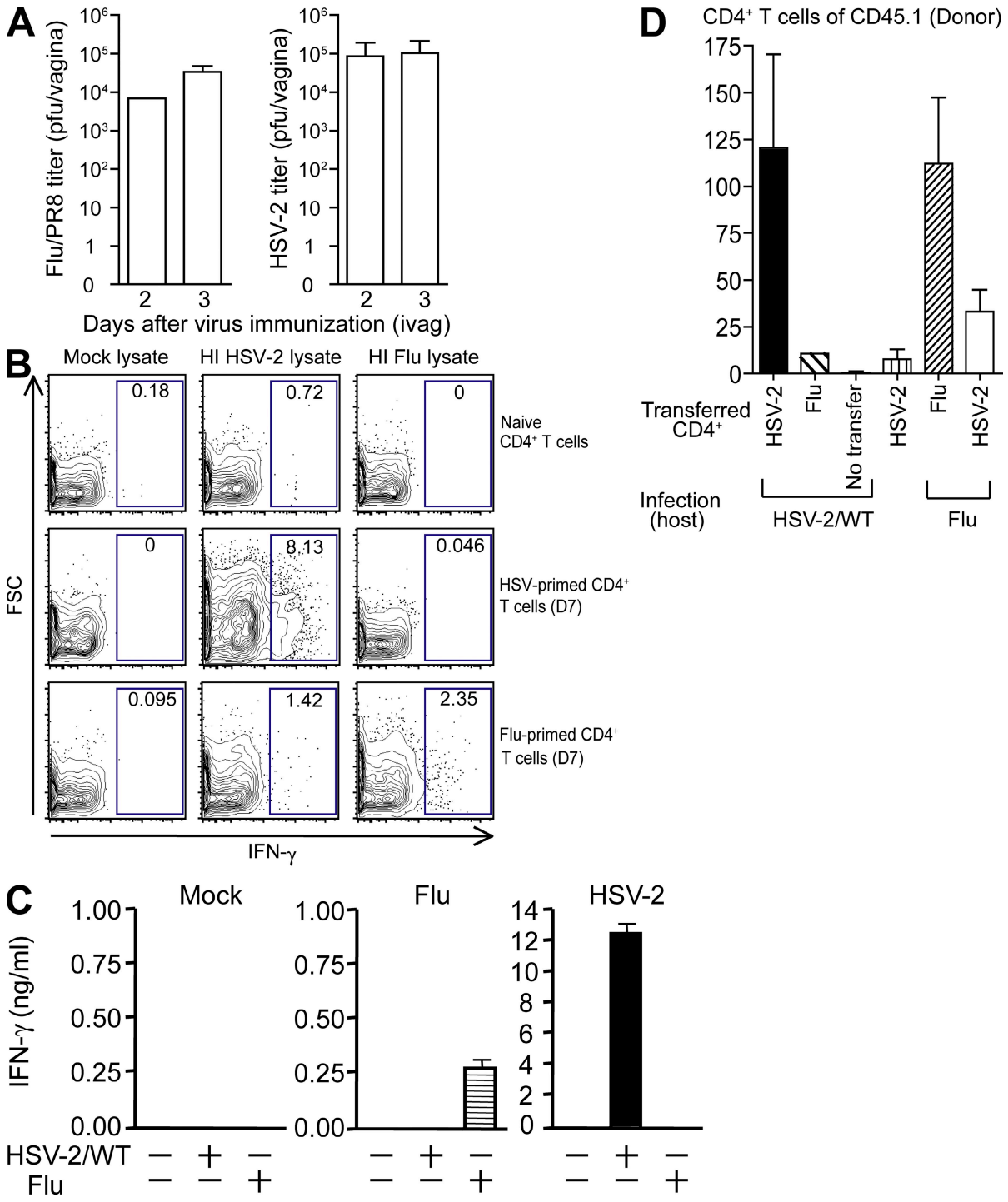

Figure S1. HSV-2–specific IFN-γ production and migration of Th1 cells after lethal WT HSV-2 challenge. (A) C57/BL6 mice were infected ivag with HSV-2 virus (strain TK⁻ mutant, 1 × 10^5 PFU/mouse) or influenza virus (strain A/PR8, 3 × 10^5 PFU/mouse). The virus titer in vaginal wash was measured at days 2 and 3. (B and C) CD4+ T cells were isolated from the draining LNs of mice immunized ivag with TK⁻HSV-2 or A/PR8/Flu for 7 d. These T cells (2 × 10^5 cells) were incubated with splenic APCs (2 × 10^5 cells) in the presence of mock antigen, heat-inactivated HSV-2 antigen (4 × 10^5 PFU equivalent) or heat-inactivated Flu antigen (4 × 10^5 PFU equivalent) for 3 d. Thereafter, these cells were incubated in complete DMEM in the presence of 5 µg/ml Brefeldin A for 24 h, and intracellular IFN-γ staining was performed. (C) CD4+ T cells (mock or HSV/Flu at day 7 after immunization) were incubated with splenic APCs in the presence of mock antigen, heat-inactivated HSV-2 antigen or heat-inactivated flu antigen for 3 d, and then the supernatants were collected for ELISA. (D) CD4+ T cells (10^7 cells/mouse) isolated from draining LNs in mice immunized with TK⁻HSV-2 or A/PR8/Flu for 7 d were adoptively transferred into C57/BL6 mice. 1d later, these recipients were challenged with WT HSV-2 (10^4 PFU) or PR8/Flu (10^6 PFU). 3 d later, single-cell suspensions from infected vaginae were prepared for FACS analysis and the donor CD4+ T cells were enumerated. Error bars represent the mean ± SD of three independent experiments.

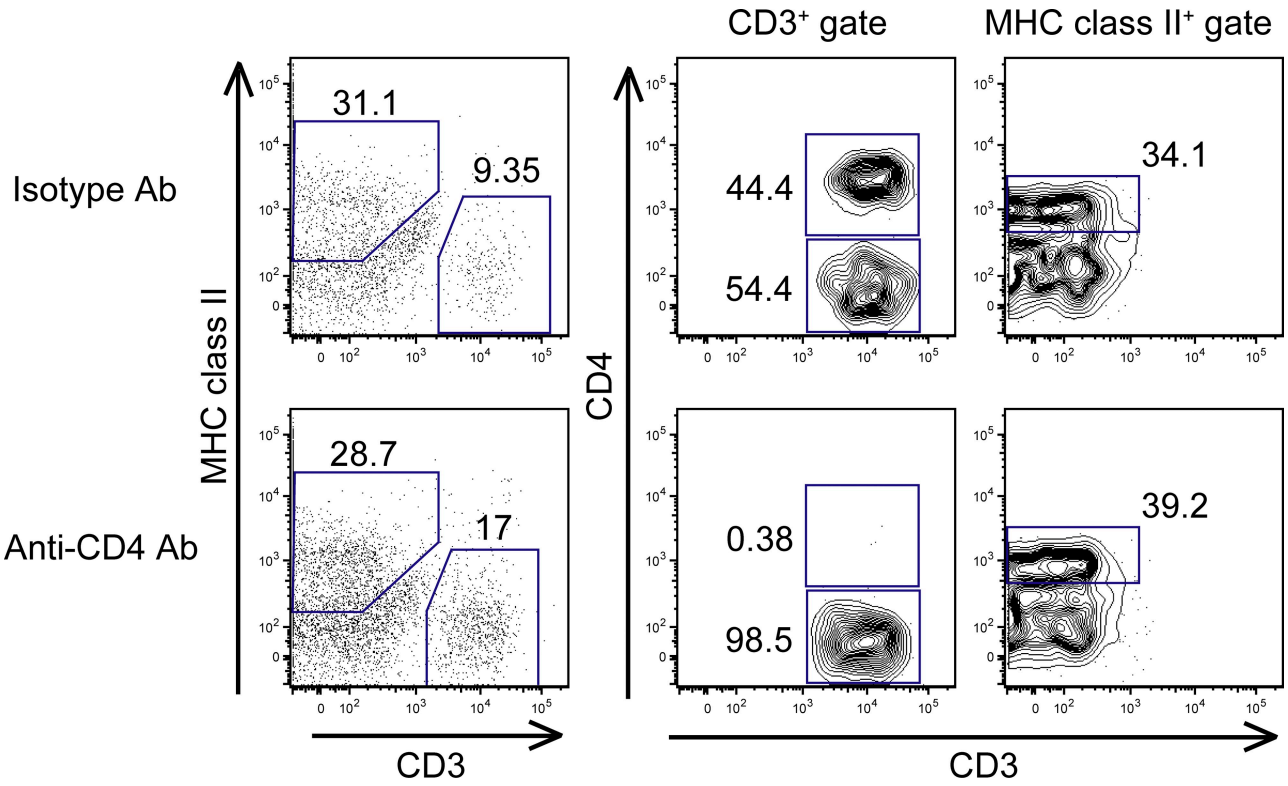

Figure S2. Anti-CD4 Ab depletes CD4+ T cells but not CD4loMHCII+ APCs in vaginae of HSV-2–immunized mice in vivo. C57/BL6 mice were immunized ivag with TKHSV-2 virus (10^6 PFU/mouse). As described in the supplemental Materials and methods, anti-CD4 Ab was injected on days −5, −3, −1, and 1 after secondary viral challenge (2 wk after immunization). 2 d after secondary challenge, the genital tracts of vaginal tissues were segregated from urethra and cervix. These vaginal tissues were then cut into small pieces and digested with 0.425 mg/ml collagenase D and 30 µg/ml DNase I and homogenized using a homogenizer (Power Gen model 1000; Thermo Fisher Scientific). These digested tissues were further incubated at 37°C for 10 min. The resulting cells were filtered through a 70-µm filter and used for FACS analyses. The dot plots depict CD4 expression in CD3+ (middle) and CD3− (right) MHC class II+ cells in the vagina at 2 d after challenge.

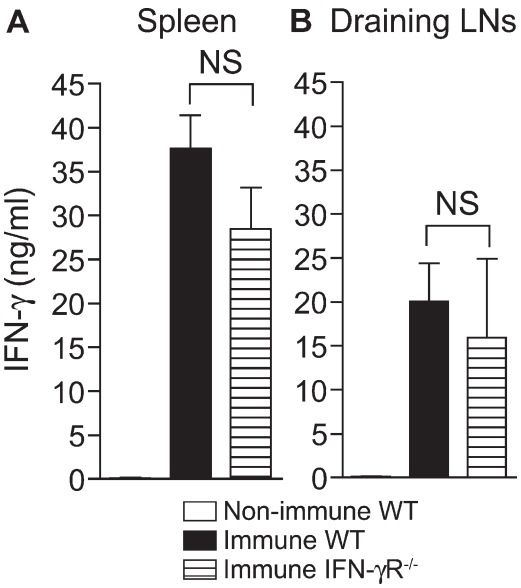

Figure S3. Th1 responses are intact in TK−HSV-2–immunized IFN-γR−/− mice. CD4 T cells isolated from the spleen (A) and draining LNs (B) of 129svj and IFN-gR−/− mice (n = 3), immunized ivag with TK−HSV-2, 3 wk earlier were cocultured with syngeneic splenocytes in the presence of HSV-2 antigens and analyzed for IFN-γ secretion. The bar graph represents the mean of values obtained from the mice in WT and IFN-γR−/− groups.

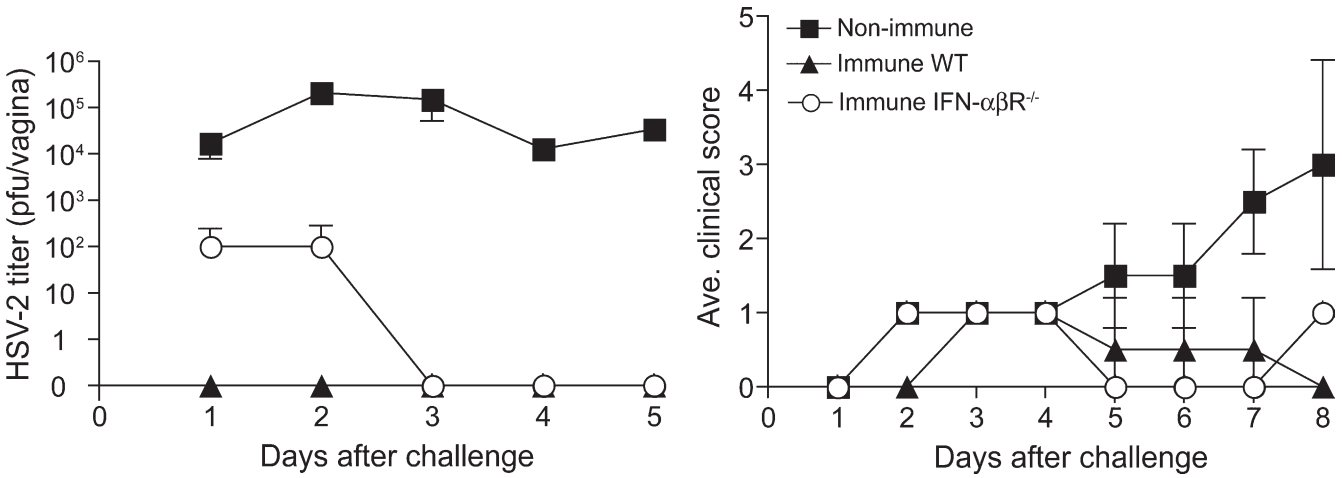

Figure S4. IFN-αβ signaling is not required for protection against secondary HSV-2 infection. 129svj mice and IFN-αβR$^{-/-}$ mice were immunized with TK^{-}HSV-2 virus. 4 wk later, mice were challenged with lethal WT HSV-2 virus. Virus titers and genital mean pathology scores in the vaginal fluids were examined. Error bars represent the mean ± SD of three mice per group.

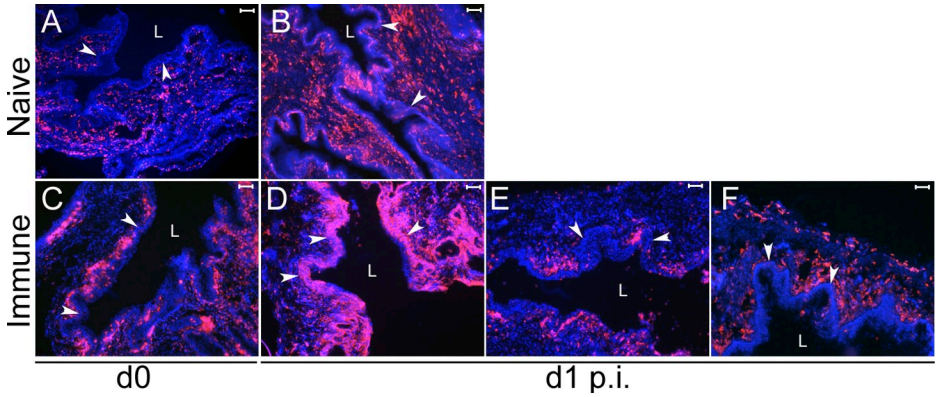

Figure S5. Induction of MHC class II on vaginal cells after secondary HSV-2 challenge. Groups of naive (A and B) or immunized WT (C and D), IFN-γR$^{-/-}$ (E), and WT→IFN-γR$^{-/-}$ (F) mice were challenged with WT HSV-2 ivag and vaginae were harvested 0 (A and C) or 1 (B and D–F) d later. Frozen sections were stained with MHC class II (red) and counterstained with DAPI (blue). Images were captured with a 10× objective. Arrowheads indicate the basement membrane. L, lumen. Bars, 100 µm. These figures are representative of three independent experiments.

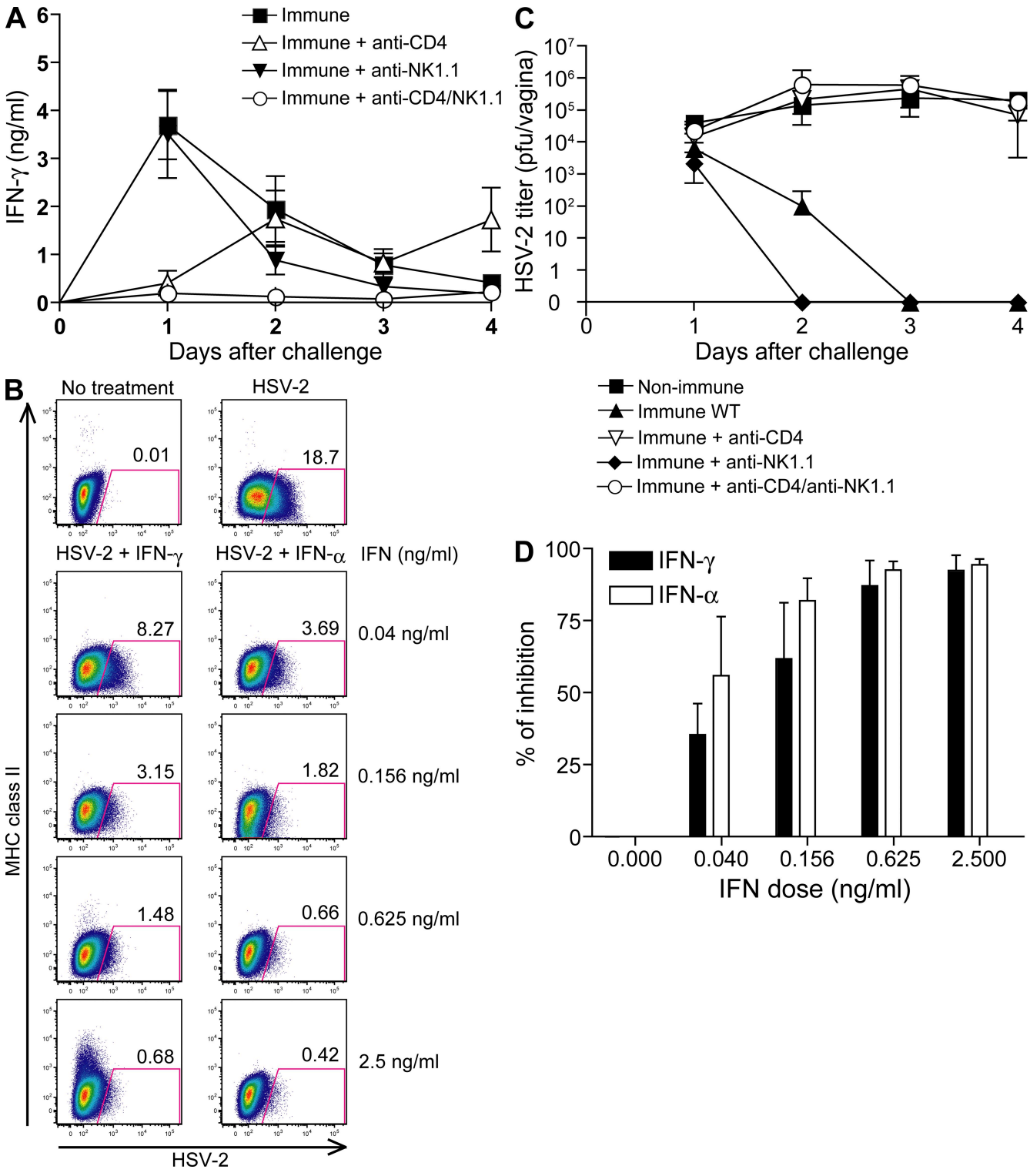

Figure S6. IFN-γ secreted by Th1 cells is sufficient for protection against HSV infection. (A) IFN-γ levels in the vaginal fluids after secondary ivag HSV-2 infection were measured. CD4 T cells and/or NK cells were depleted from TKHSV-2–primed mice and challenged ivag with WT HSV-2 (A). (B) Virus titer was measured in vaginal washes of anti-CD4– and/or anti-NK1.1–treated mice after HSV-2 secondary challenge. Each point represents the mean ± SD of three mice per group. (C and D) Primary keratinocytes were infected with 1.5×10^{-3} MOI of WT HSV-2. 2 h later, these cells were treated with the indicated doses of IFN-γ or IFN-α. 48 h after IFN treatment, HSV-2 infection and MHC class II expression were analyzed by flow cytometry (C). The percentage of inhibition of HSV-2 replication by the treatment of IFN-γ or IFN-α is depicted (D). Three repeated experiments resulted in similar data (mean ± SE).

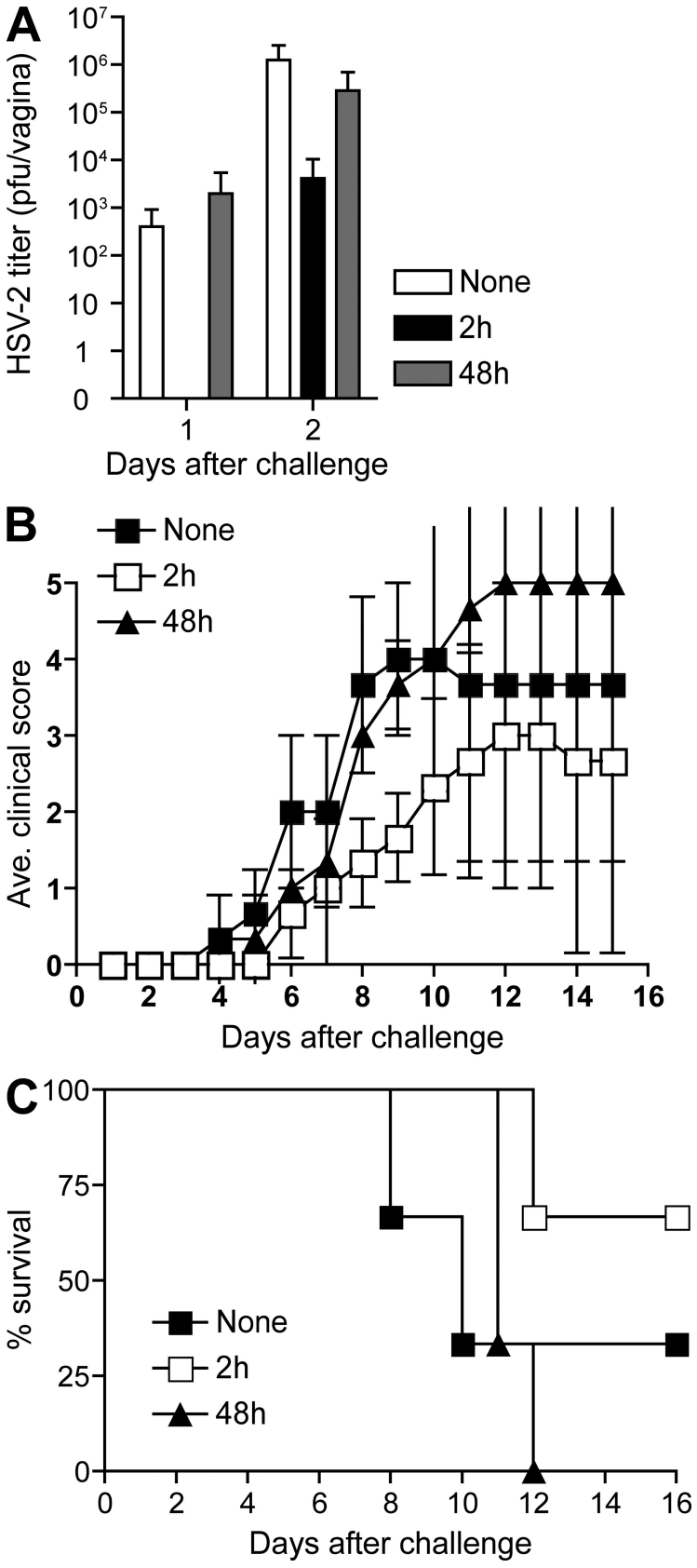

Figure S7. Topical treatment with IFN-γ at an early phase (2 h) of HSV-2 infection confers protection against HSV-2 challenge. (A–C) C57/BL6 mice ($n = 3$ per group) were challenged ivag with WT HSV-2 (5×10^2 PFU/mouse). All mice were given 6 µg of carrier-free recombinant IFN-γ (eBioscience) i.p. 6 h before challenge. Thereafter, 0.8 mg of IFN-γ was inoculated ivag at either 2 or 48 h after viral challenge. Virus titers (A), genital mean pathology scores (B), and survival (C) were examined. Errors represent the mean ± SD of three mice per group.

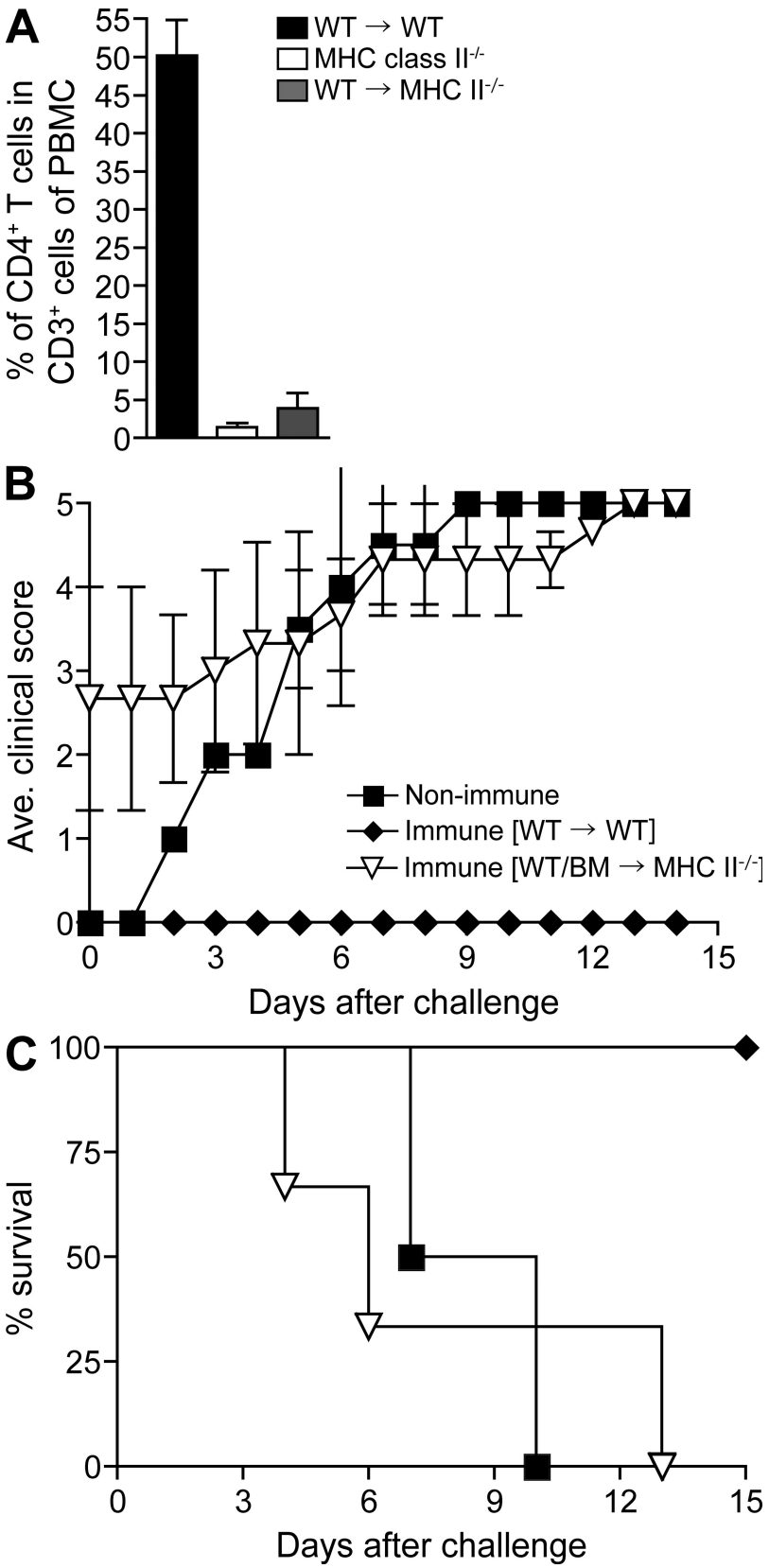

Figure S8. WT→MHCII$^{-/-}$BM chimera succumb to death after HSV-2 challenge. To generate WT/BM into MHC class II$^{-/-}$chimera (WT→MHCII$^{-/-}$), BM from C57/BL6 mice transferred into irradiated MHC class II recipients. (A) 7 wk after BM transfer, the proportion of CD4 T cells in peripheral blood was analyzed by FACS. (B and C) To examine protection against lethal WT HSV-2 challenge, WT→WT and WT→MHCII$^{-/-}$chimera were immunized with TK^{-}HSV-2 (5×10^5 PFU/mouse). 4 wk later, these immunized mice were challenged with lethal WT HSV-2 virus (10^4 PFU). The mean clinical score (B) and mortality (C) are shown. Error bars represent the mean ± SD (A) or SEM (B) of three mice per group.

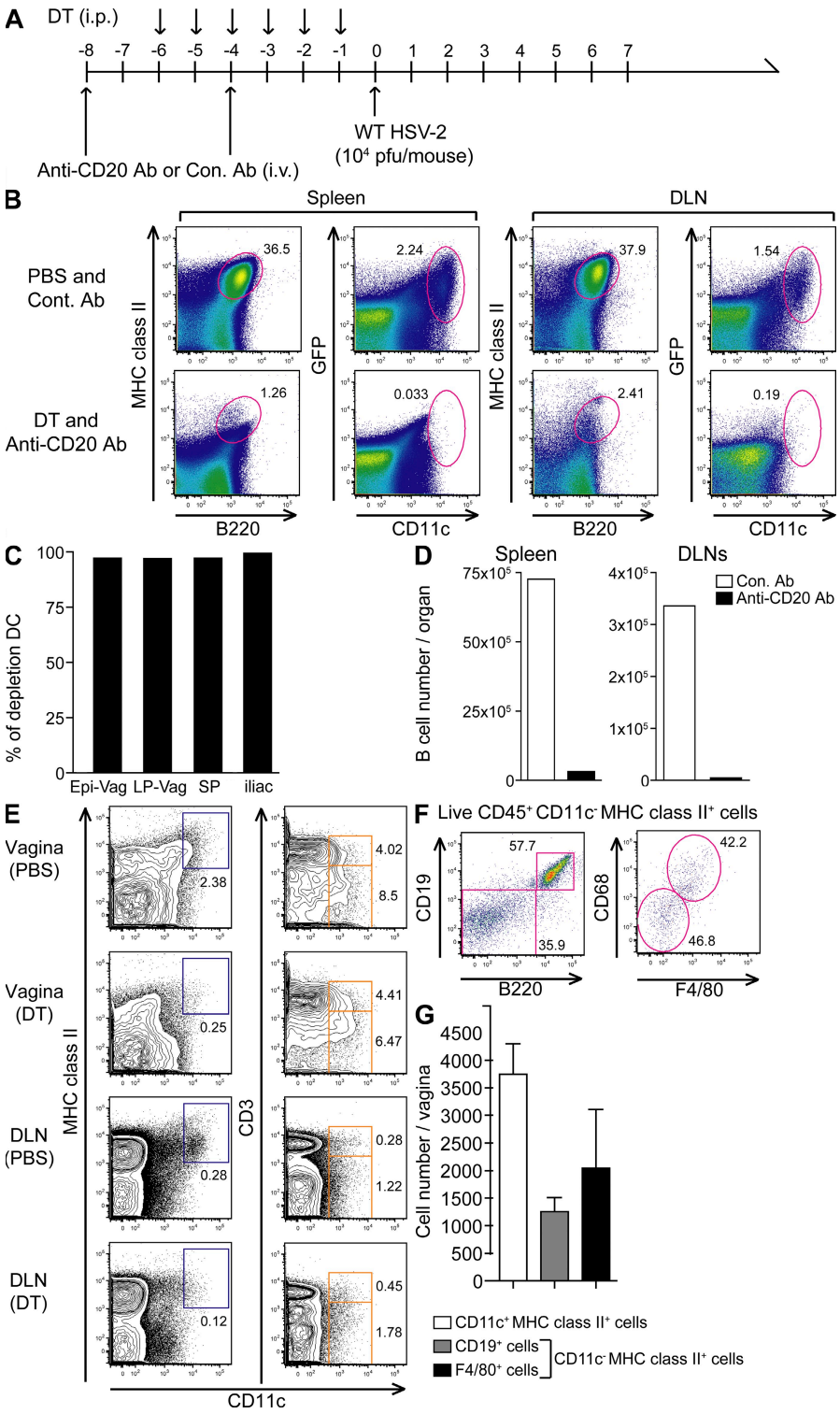

Figure S9. Both B cells and conventional DCs are depleted by DT treatment and anti-CD20 Ab injection. TK⁻HSV-2–immunized CD11c-DTR→WT chimeras were inoculated with DT or PBS i.p. and control Ab (2B8) or anti–mouse CD20 mAb (18B12) i.v. at the indicated time points (A). Depletion of B cells ($CD19^+B220^+$) and conventional DCs ($CD11c^+GFP^+$) in the spleen and draining LNs (iliac) was confirmed by FACS analysis (B). The percentage of depletion of DCs (C) and the cell number of B cells in spleen and draining LNs (iliac) after B cell depletion is depicted (D). $CD11c^+CD4$ T cells in vagina and DLN were not depleted by DT treatment. DT was injected into TK⁻-immunized CD11c-DTR→WT chimeras as shown in Fig. S9 A. 1 d after challenge, $CD11c^+$MHC class II^+ (left) and $CD11c^+$MHC class $II^-CD3^+CD4^+$ (right) populations were detected by FACS. (E and F) B cells ($CD11c^-CD19^+B220^+$MHC class II^+) and macrophage ($CD11c^-F4/80^+CD68^+$MHC class II^+) populations in the vaginae at 3 wk after TK⁻HSV-2 infection were analyzed by FACS. The total numbers of DCs, B cells, and macrophage populations are depicted (F). Error bars represent the mean ± SD of three mice per group.

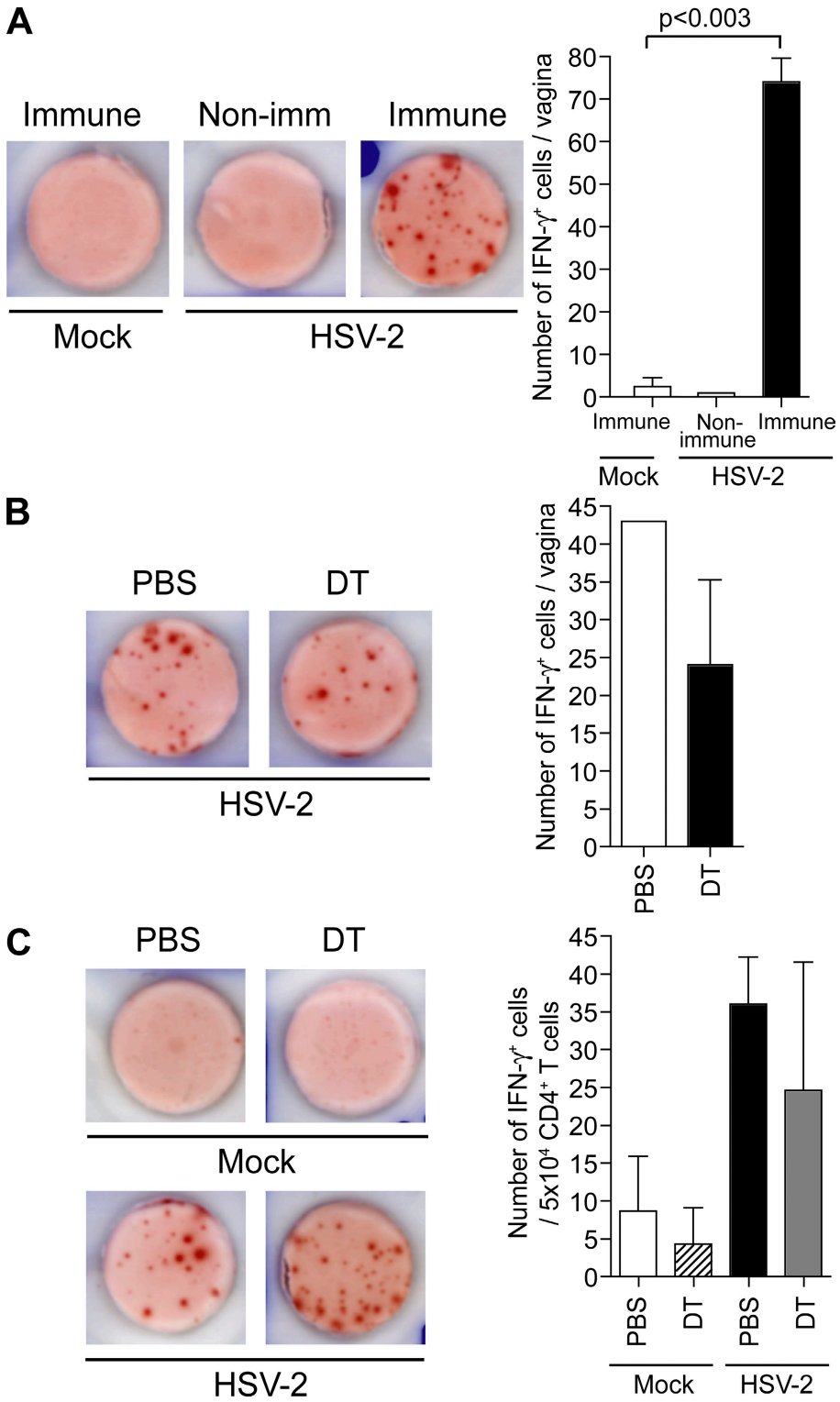

Figure S10. Quantification of HSV-2–specific T cell response in the vagina of TK⁻HSV-2–immunized mice by IFN-γ ELISPOT. C57/BL6 mice (A) or CD11c DTR BM chimeras (B and C) were immunized with TK⁻HSV-2. (A and B) 4 wk later, lymphocyte populations from vaginae were isolated as described in the supplemental Materials and methods. These cells were incubated with splenic APCs (10^5 cells) in the presence of mock or HSV-2 antigen for 48 h. Images show a representative well in each group with HSV-2 antigen. Bar graphs on the right represent the number of IFN-γ–secreting cells per vagina (mean ± SD). (C) $CD4^+$ T cells (5×10^4 cells) purified from spleen of TK⁻-immunized DC depleted or sufficient CD11c DTR BM chimeras were incubated with splenic APCs (1×10^5 cells) in the presence of mock or HSV-2 antigen for 48 h. Bar graphs on the right represent the number of IFN-γ–secreting cells per vagina (mean ± SD). Images show a representative well in each group with Mock or HSV-2 antigen.

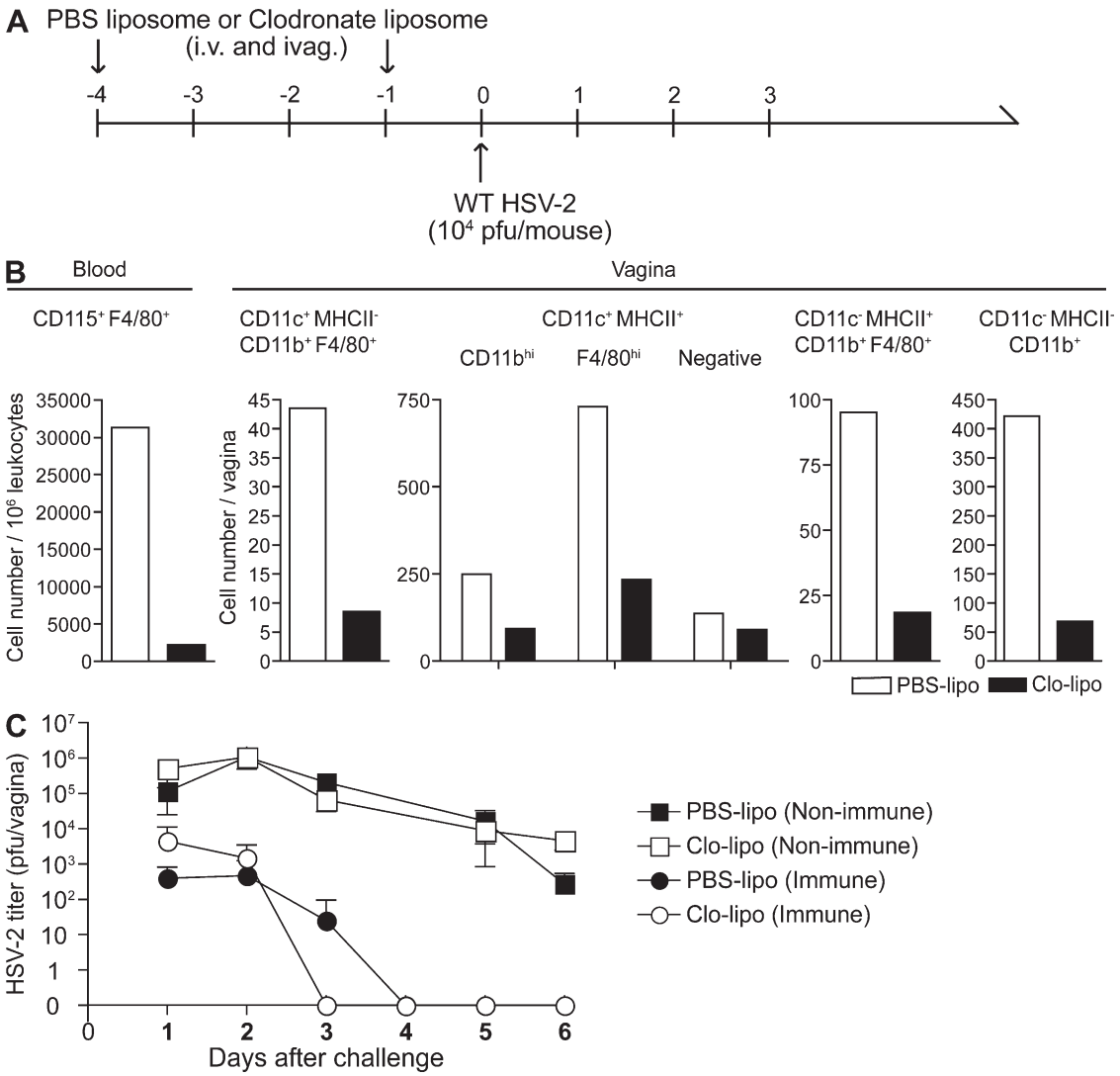

Figure S11. Depletion of DCs and macrophages by Clodronate liposome does not impair protection against HSV-2 secondary challenge. (A) TK⁻HSV-2–immunized C57BL6 mice were inoculated with Clodronate liposome (Clo-lipo) or PBS liposome (PBS-lipo) via the i.v. (250 µl) and ivag (10 µl) routes at the indicated time points. (B) Depletion of monocytes in peripheral blood and phagocytes including DC and macrophages in the vagina at day 0 were analyzed by flow cytometry. (C) Viral titers in the vaginal secretion of C57BL6 mice immunized with or without TK⁻HSV-2 were measured at the indicated days after challenge. Error bars represent the mean ± SD. of four mice per group.

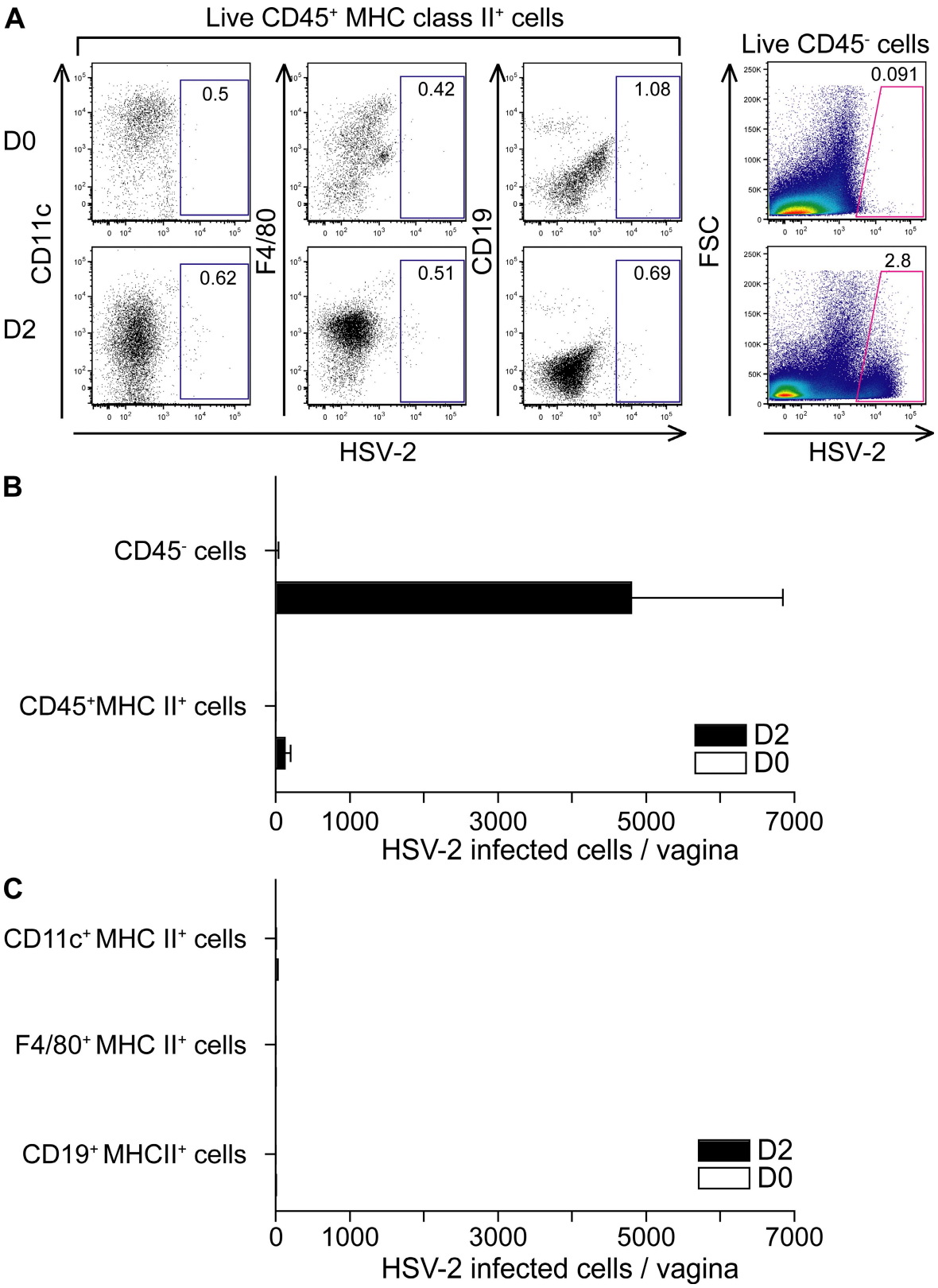

Figure S12. HSV-2 predominantly infects epithelial cells and not APCs. (A–C) C57/BL6 mice were challenged with 10^4 PFU WT HSV-2. Single-cell suspensions of vaginal tissues were analyzed by FACS. (A) The dot plot represents HSV-2 antigen staining of 7-AAD$^-$CD45$^+$MHC class II$^+$ and 7-AAD$^-$CD45$^-$ cells in the vagina 0 or 2 d after challenge. (B and C) Statistical analyses of dot plot data (A) are shown. Error bars represent the mean ± SD of five mice per group.

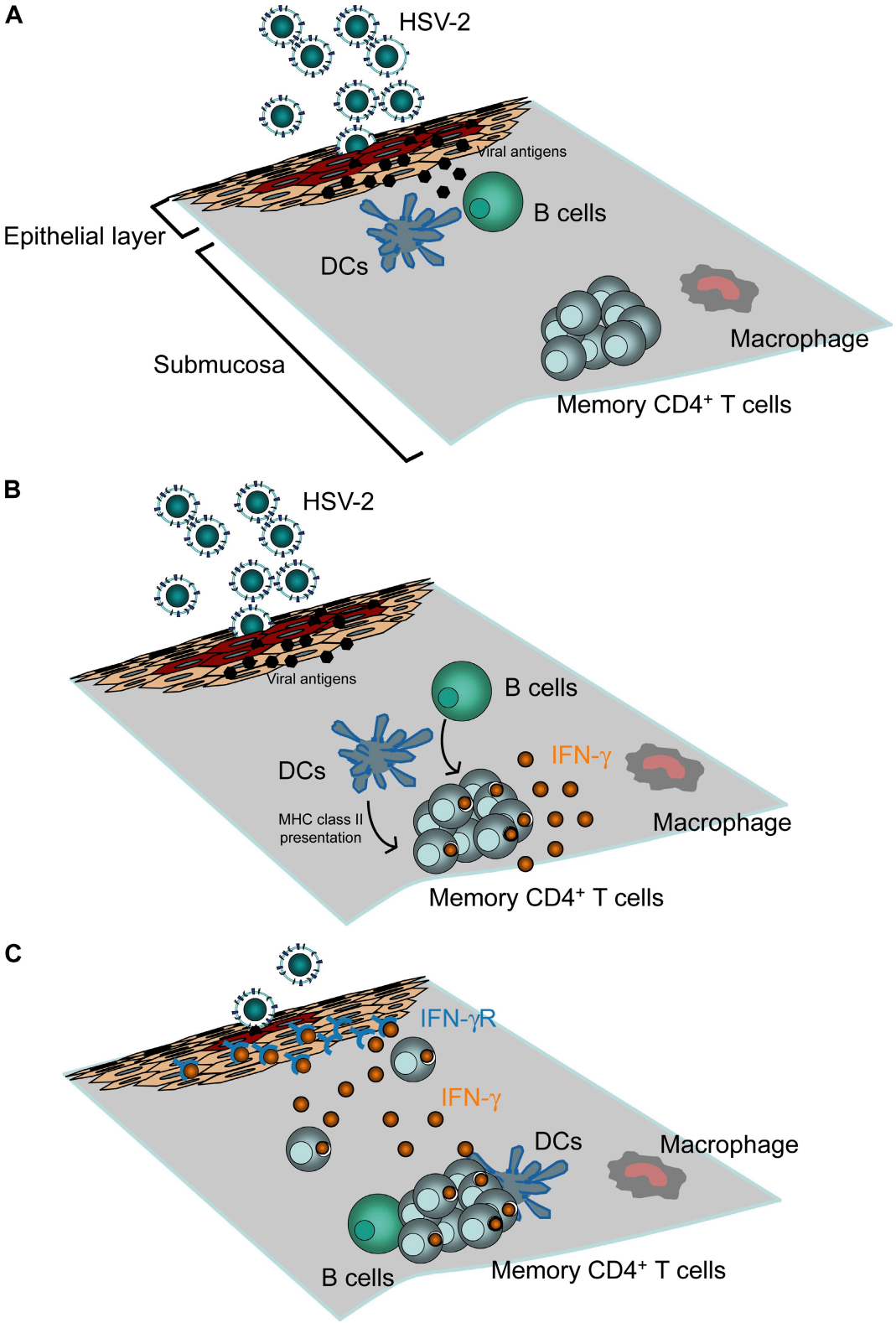

Figure S13. Schematic representation of memory Th1 cell–mediated antiviral immunity in mucosal tissues. (A) The drawing shows resident CD11c$^+$DC and B cells capturing viral antigen from vaginal epithelial cells infected with HSV-2. (B) These cells interact with the memory CD4$^+$ T cells that form clusters in the local tissue (Fig. 1 A) and present viral antigens on MHC II. The recalled CD4 T cells secrete IFN-γ rapidly at the site of infection. (C) Secreted IFN-γ acts on HSV-2–infected vaginal epithelial cells through the IFN-γR and elicits antiviral genes that potently inhibit viral replication at the early stage of secondary challenge.