Supplemental Experimental Procedures

Mice

C57BL/6 mice, 6-8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in filtered cages. IFNγ-deficient mice and OT-II mice (purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms, respectively), gDT-II mice (Bedoui et al., 2009; Gebhardt et al., 2011) (kindly provided by Drs. Andrew Brooks (The University of Melbourne) and David Leib (Dartmouth College)), and *Foxp3*^{DTR} (Kim et al., 2007) and CTLA-4$^{flox/flox}$ x *Foxp3*^{Cre} mice (kindly provided by Dr. Alexander Rudensky, Memorial Sloan-Kettering Cancer Center) were bred onsite at the animal facility at Fred Hutchinson Cancer Research Center (FHCRC). All animal experiments were approved by the FHCRC Institutional Animal Care and Use Committee. The Office of Laboratory Animal Welfare of the National Institutes of Health (NIH) has approved the FHCRC's Animal Welfare Assurance (#A3226-01), and this study was carried out in strict compliance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals.

Bone marrow chimeras

Foxp3^{DTR} Ly 5.1 mice were irradiated with 900 Rads 1 day before reconstitution with bone marrow (BM) cells collected from the femurs and tibiae of Foxp3^{DTR} Ly 5.1 mice mixed with an equal number of cells from either CTLA-4$^{flox/flox}$ x Foxp3^{Cre} or CTLA-4$^{WT/WT}$ x Foxp3^{Cre} Ly 5.2 mice. BM cells were flushed from the bones with a needle, red blood cells were lysed, and a single cell suspension was prepared by forcing cells through a 100 □M filter. After counting, equal numbers of the appropriate cells were mixed together, and then 5 million total cells

suspended in HBSS were injected intravenously into each recipient mouse. BM chimeras were allowed to rest for at least three months prior to being used.

Infections

Mice were injected subcutaneously with 2 mg of Depo Provera (Greenstone, Peapack, NJ) 5 to 7 days prior to being infected with HSV-2. At the time of infection, mouse vaginal tracts were swabbed with calcium alginate tipped swabs before 1.88×10^5 - 1.0×10^6 PFU of virus was applied to the vaginal tract in a 10 □L volume. HSV-2 was propagated on and titered by plaque assay on Vero cells. All infections used HSV-2 186 □Kpn (Jones et al., 2000) (kindly provided by Dr. David Knipe, Harvard Medical School) or TK- HSV-2 expressing OVA (Dobbs et al., 2005) (kindly provided by Dr. Gregg Milligan, University of Texas Medical Branch at Galveston).

Vaginal Washes

Vaginal washes were collected from mice by swabbing vaginal canals with titration buffer-soaked calcium-alginate tipped swabs, followed by washing the vaginal canals with 50 □L of titration buffer, followed by a final swab with a dry calcium-alginate tipped swab. The tips of both swabs as well as the 50 □L used for washing were stored in 1 mL titration buffer. Samples were collected on ice and either assayed immediately or stored at -80C. Viral titer of vaginal washes was determined by plaque assay on Vero cells. IFN□ concentration in vaginal washes was determined using the Ready-Set-Go ELISA kit (eBioscience, San Diego, CA) according to manufacturer's instructions.

Antibodies

The following antibodies were used in this study: anti CD16/32 (clone 93) anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-Ly 5.1 (clone A20), anti-Ly 5.2 (clone 104), anti-Nur77 (clone 12.14), anti-CD69 (clone H1.2F3), anti-CD11c (clone N418), anti-MHC II (clone M5/114.15.2), anti-CD11b (clone M1/70), anti-CTLA-4 (clone UC10-4B9), anti-Foxp3 (clone FJK-16s) (all from eBioscience, San Diego, CA) and anti-CD3 (clone 145-2C11) (from eBioscience, San Diego, CA and BD Biosciences, San Jose, CA).

Tissue Collection and Processing

When lymph node or spleen lymphocytes were being isolated, a single cell suspension was prepared by forcing the tissue through a 100 □M filter into PBS. When DCs from the lymph node or vaginal tract cells of any type were being isolated, tissues were chopped into small pieces in the presence of DMEM medium supplemented with Collagenase D and DNase I (Roche, Basel, Switzerland) then incubated in the Collagenase D/DNase I solution for 30 minutes at 37 degrees C. Tissue was then re-suspended in HBSS supplemented with EDTA and FCS and incubated for 5 minutes at 37 degrees C. Tissue was then forced through a 100 □M filter to prepare a single cell suspension. Blood was collected from the orbital sinus using a capillary tube and collected into PBS supplemented with EDTA. In spleen and blood samples, red blood cells were lysed using ACK lysis buffer.

Cell sorting

For *ex vivo* co-culture experiments, DCs were sorted based on expression of CD11c and MHC II. After dissection of the draining iliac lymph nodes (dLN), cells were isolated as described above. After counting and normalizing the number, cells were plated onto a 96 well plate. Cells were blocked for Fc binding through incubation with anti CD16/32 antibody (clone 93) (eBioscience, San Diego, CA) for 10 minutes at 4 degrees C. Cells were then stained with anti-MHC II antibody and anti-CD11c antibody (eBioscience, San Diego, CA) by incubating cells for 15 minutes at 4 degrees C in antibody cocktail. Cells were re-suspended in PBS supplemented with EDTA and forced through a Falcon filter-tipped FACS tube. Cells from dLN were sorted into two groups; an MHC II high/CD11c mid population of migratory DCs and MHC II mid/ CD11c + population of non-migratory DCs. All sorting was done on a FACSAria (BD Biosciences, San Jose, CA). CD4 T-cells that were already enriched using the CD4+ T-cell negative selection kit (Stem Cell Technologies) were also stained using anti-CD4 (clone GK1.5) and anti-MHC II antibodies (eBioscience, San Diego, CA) and sorted into a population of CD4+ and MHC II – cells to ensure that the CD4 T-cells were not contaminated with antigen presenting cells.

Flow Cytometry

Single cell suspensions from various tissues were prepared as described above. LN cells were counted and 2 x 10^6 cells were transferred to a 96 well plate for staining. All blood and vaginal tract cells were transferred to a 96 well plate for staining. Cells were first incubated in eFlour 780 fixable viability dye (eBioscience, San Diego, CA) diluted in PBS for 30 minutes then were blocked for Fc staining by incubating in anti-CD16/32 antibody (clone 93) (eBioscience, San Diego, CA) diluted in .5% FCS in PBS for 10 minutes at 4 degrees C. After washing, cells were stained using various combinations of antibodies listed above in 0.5% FCS in PBS for 15

minutes at 4 degrees C. Cells with no intracellular staining needed were then fixed by incubating in 1% paraformaldehyde for 10 minutes at room temperature before being suspended in PBS. Cells with intracellular staining targets were then incubated in fix/perm solution (eBioscience, San Diego, CA) for 30 minutes at 4 degrees C. After washing with permeabilization buffer (eBioscience, San Diego, CA), cells were re-suspended in a solution of various combinations of antibodies listed above suspended in permeabalization buffer (eBioscience, San Diego, CA). Cells were suspended in PBS for analysis on an LSR II. For vaginal tract samples, cells were forced through a Falcon filter topped FACS tube and combined with CountBright beads (Thermo Fisher Scientific, Waltham, MA) for enumeration. Data were analyzed using FlowJo software (Treestar, Ashland, OR). Cell counts from LN samples were determined by multiplying appropriate cell populations fractions by the cell count determined by hemacytometer. Blood cell fractions were determined by multiplying appropriate cell population fractions together.