Supplementary Methods

Immunofluorescence Staining

Samples were quenched and blocked with Casein and BSA solution, then incubated with primary antibody for 90 minutes at room temperature, followed by incubation with HRP conjugated secondary antibody that was further incubated with green fluorescent Alexa Fluor Tyramide substrate following the manufacturer's protocol (Molecular Probes). The slides were washed with PBS and incubated with a second monoclonal antibody conjugated with Alexa-Fluor 647 at 4° C overnight and then counterstained with DAPI (Fluka) and mounted in Mowiol 40-88 containing 2.5% DABCO (Sigma). For the quantification of T cells with various markers, multiple 445 by 333 μm fields covering the entire biopsy section were captured using the Mosaix acquisition function and then stitched to make one large image file. To calculate cell density, the number of cells was divided by the area of the section (in μm^2, and multiplied by a million to convert to cells/mm^2).

Droplet Digital PCR (ddPCR)

cDNA samples were diluted 12 fold, and 10 μl of diluted cDNA was then used for each ddPCR reaction. Due to the high copy number of actin B in the samples, an additional 10 fold dilution of the cDNA was required for actin B ddPCR. The PCR thermo-cycling conditions for all these 4 genes are as following: 95° C for 10 minutes, 40 cycles of 94° C for 30 seconds and 60° C for 1 minute, 86 followed by 98° C for 10 minutes and ending at 4° C.

Statistics

To investigate the association between shedding status and biopsy results, a linear mixed-effects model was used to estimate the difference in biopsies (CD4+ T-cell density, CD8+ T-cell density, Foxp3+ T-cell density, the ratio of Foxp3+ and CD4+Foxp3+ T-cell density, and the ratio of Foxp3+ and CD8+ T-cell

density) across groups defined by whether the HSV shedding status was detected on the biopsy date. The model accounted for correlation between biopsy results from the same persons over time. At the time of lesion, linear regression was used to assess association of biopsy quantification and HSV DNA quantity. The linear regression model was used to evaluate the association between biopsy HSV quantity and CD4+, CD8+ and Foxp3+ T-cell densities. To calculate the density, we measured the area of the biopsy (in μm^2), divided the total number of the cells by the area and converted to cells/mm^2.

To compare the proportion of Foxp3 at vaginal tissue and PBMCs at lesion time and to determine whether the percentage of CLA positive Foxp3 cells is higher in post heal sections than in acute lesion stage, we used non-parametric method, the Wilcox signed rank test, which tests the median of the difference between the two matched samples, since the observations are paired for each subject and the sample size is small for parametric methods.

Laser Capture Microdissection (LCM)

Six to eight μm sections of frozen biopsies were adhered on a polyethylene naphthalate membrane slide (Carl Zeiss). Tissue sections were fixed in 100% acetone, dried at room temperature, rinsed in PBS and incubated with Foxp3 –AF647 or CD4–AF647 on ice for 10 min. Slides were then washed in PBS and dried with ethanol (75%, 95% and 100%) and xylene.

Supplementary Data Legends

Supplementary Table 1. Demographic and clinical characteristics of the subjects.

Supplementary Table 2. Study procedures.

Supplementary Table 3. Antibodies utilized.

Supplementary Table 4. Primer/probe sets used for digital droplet PCR assays.

Supplementary Figure 1. Characteristics of CD4+ T cells in genital biopsies. (A-C) Lesional biopsies were stained for (A) CD3 (green) and CD4 (pink); (B) Foxp3 (green) and CD127 (pink); and (C) Foxp3 (red) and Ki67 (green). Samples were counter stained with DAPI (blue). Scale bar = 50 μm.

Supplementary Figure 2. Phenotypic characterization of Tregs in peripheral blood. PBMCs were isolated from peripheral blood at time of Lesion (top) and at 8 wph (bottom) and analyzed by flow cytometry. Percentage of positive cells are provided. This is a representative experiment of one patient's PBMCs. PBMCs from the same patient were stained with the Treg cocktail with the anti-Foxp3 antibody omitted. The isolated cells were used in the experiments depicted in Figure 3D. See Material and Methods for experimental detail.

Supplementary Figure 3. CD8+ T-cell kinetics during HSV-2 reactivation. The density of CD8+ T cells (cells/mm^2) in tissue sections of serial biopsies. X-axis depicts the time points acute lesion (Lesion), recently healed (Healed), and 2, 4, and 8 wph; as well as control biopsy cell density (Control). Each data point represents one patient (10 total). These data are similar in both number and pattern as previously published [5, 7].

Supplementary Table 1. Demographic and clinical characteristics of the subjects.

Patient	Gender	Age at outbreak (yrs)	Race	Years infected (yrs)	Anti-viral during lesion episode	Lesion location
1	F	61	White	22	No	Buttocks
2	F	62	White	33	No	Buttocks
3	F	57	White	27	No	Perineum
4	M	60	Black	1	No	Perineum
5	F	64	White	44	No	Buttocks
6	F	66	White	22	No	Buttocks
7	F	71	White	46	No	Buttocks
8	F	21	Mixed	1	No	Pubis
9	M	26	White	10	No	Pubis
10	F	48	White	0.5	No	Buttocks

Supplementary Table 2. Study procedures.

Visit	Time	Genital lesion biopsy	Control skin biopsy	Blood draw
1	Lesion	X	X	X
2	Healed	X		
3	2 wph	X		
4	4 wph	X		
5	8 wph	X	X	X

Supplementary Table 3. Antibodies utilized.

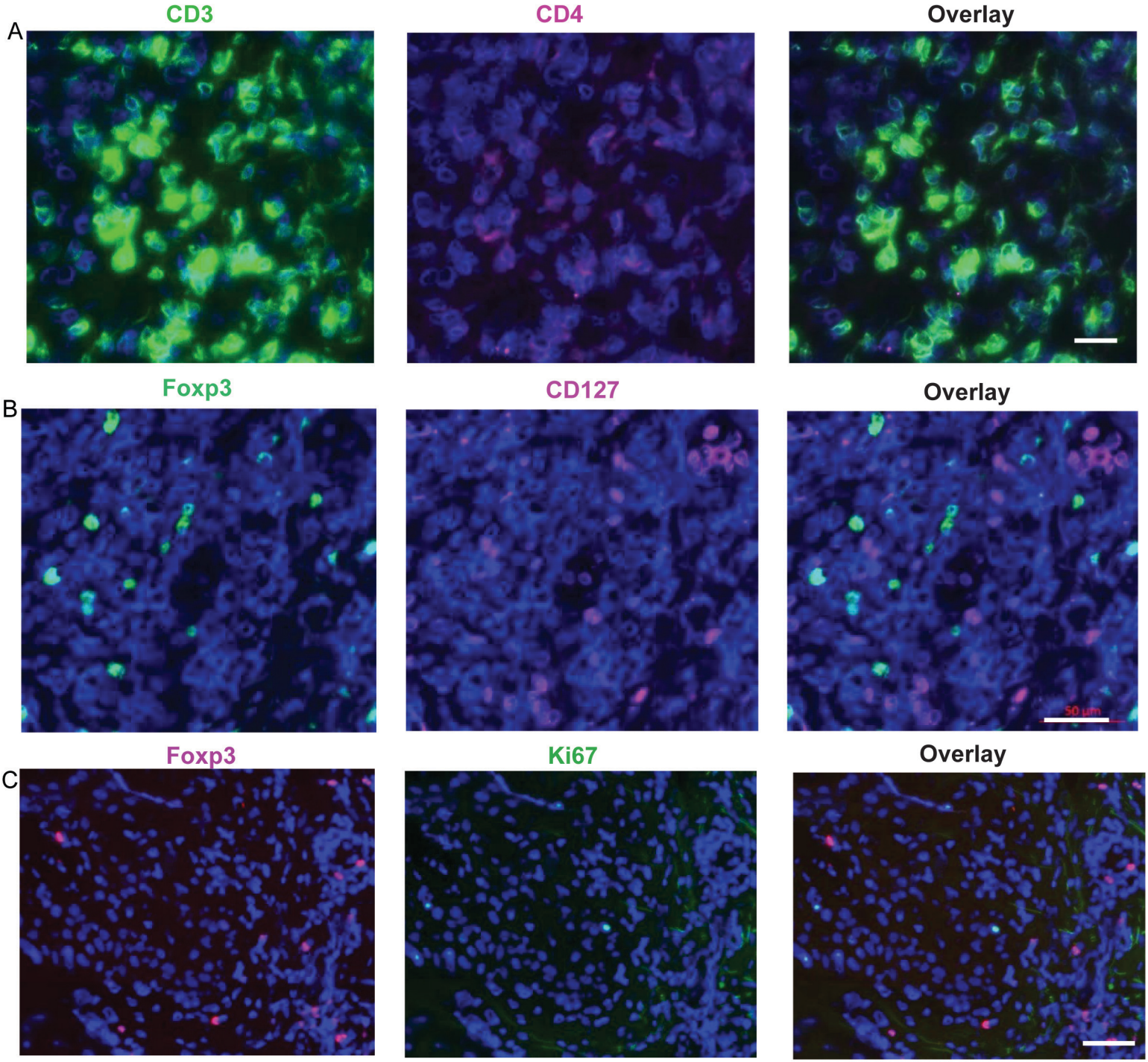
Application	Antibody	Manufacturer
Immuno-fluorescence	Mouse anti human Foxp3	eBioscience
microscopy	Mouse anti human Foxp3 Alexa Fluor® 647	BD Pharmingen
	Mouse anti human CD4	Biologend
	Mouse anti human CD4Alexa Fluor® 647	Biolegend
	Rat anti human CD4	abcam
	Mouse anti human CD8Alexa Fluor ®647	Biolegend
	Rabbit anti mouse IL7R	LS Bio
	Mouse anti human antiCd25	Leica
	Rat anti human/mouse Cutaneous Lymphocyte	eBioscience
	Antigen (CLA) eFluor®	
	Mouse anti human CD3ε	Santa Cruz
	Mouse anti human CD45 RO	BD pharmingen
	Mouse anti Ki67	DAKO
	Goat ant Mouse HRP	Life technologies
	Donkey anti mouse Alexa Fluor®647	Molecular probes
	Donkey anti rat Alexa Fluor® 594	Molecular probes
	Donkey anti rabbit Alexa Fluor® 594	Molecular probes
	Donkey anti rabbit Alexa Fluor® 647	Life technologies
Flow cytometry	Mouse anti human CD3	BD pharmingen
	Mouse anti human CD4	Biolegend
	Mouse anti human CD25	Biolegend

Mouse anti human CD127	Biolegend
Mouse anti human Foxp3	eBiosciences
Mouse anti human CD39	BD biosciences

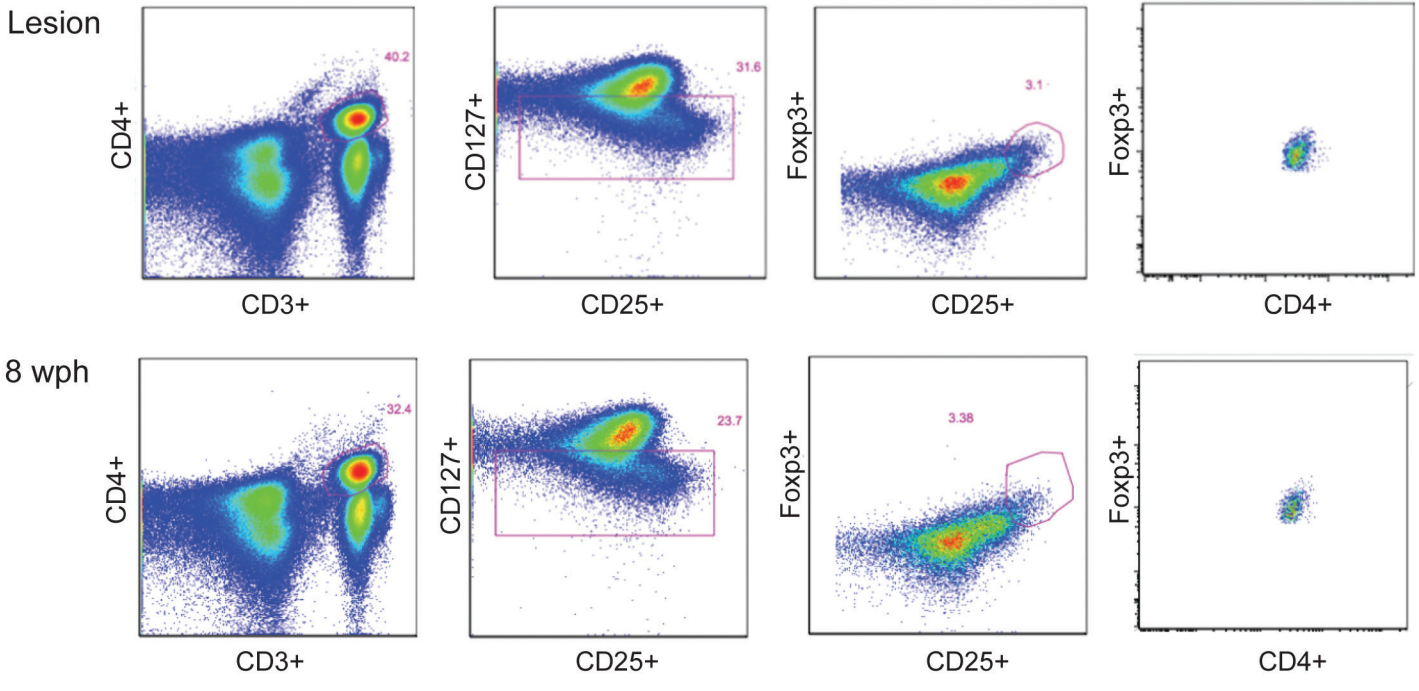
Supplementary Table 4. Primer/probe sets used for digital droplet PCR assays.

Gene	Gene Assay ID
ACT B	Hs01060665_g1
Foxp3	Hs01085833_g1
CTLA -4	Hs03044418_m1
IL-2RA (CD25)	Hs00907779_m1

Supplementary
Figure 1



Supplementary Figure 2



Supplementary Figure 3