

# Persistence of mucosal T-cell responses to herpes simplex virus type 2 in the female genital tract

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Relatively little is known about the human T-cell response to herpes simplex virus type 2 (HSV-2) in the female genital tract, a major site of heterosexual HSV-2 acquisition, transmission, and reactivation. In order to understand the role of local mucosal immunity in HSV-2 infection, T-cell lines were expanded from serial cervical cytobrush samples from 30 HSV-2-infected women and examined for reactivity to HSV-2. Approximately 3% of the CD3+ T cells isolated from the cervix were HSV-2 specific and of these, a median of 91.3% were CD4+, whereas a median of 3.9% were CD8+. HSV-2-specific CD4+ T cells expanded from the cervix were not only more frequent than CD8+ T cells but also exhibited greater breadth in terms of antigenic reactivity. T cells directed at the same HSV-2 protein were often detected in serial cervical cytobrush samples and in blood. Thus, broad and persistent mucosal T-cell responses to HSV-2 were detected in the female genital tract of HSV-2+ women suggesting that these cells are resident at the site of HSV-2 infection. Understanding the role of these T cells at this biologically relevant site will be central to the elucidation of adaptive immune mechanisms involved in controlling HSV-2 disease.

## INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is the major cause of genital herpes, one of the most common sexually transmitted infections worldwide and a significant risk factor of HIV acquisition.<sup>1</sup> In the United States, 17% of people are seropositive for HSV-2 and 58% are seropositive for HSV-1.<sup>2</sup> Elsewhere, the seroprevalence rate is much higher, reaching 95% in some populations of HIV-infected persons and female sex workers (reviewed in Gupta *et al.*<sup>3</sup>). The lack of success of HSV-2 vaccines<sup>4–6</sup> and the observation that HSV-2 reactivation can occur in persons taking standard or high doses of antiviral therapy<sup>7</sup> underscore the urgency of defining mechanisms of resistance in order to design strategies to prevent primary HSV-2 infection and reduce or eliminate reactivation of latent HSV-2.

Clinical and experimental data indicate an important role of the adaptive immune response, especially T-cell responses, in controlling genital herpes infections. Persons with defects in cellular immunity have more severe HSV-2 infections.<sup>8–10</sup> The observations that HSV-2 clearance from genital herpes lesions correlates temporally with the infiltration of CD8 T cells,<sup>11</sup>

functional HSV-2-specific CD4+ and CD8+ T cells populate the site of HSV-2 reactivation,<sup>12–14</sup> and that functional CD8+ T cells persist in genital skin contiguous to sensory neuronal nerve endings,<sup>15–17</sup> suggest a role of the peripheral mucosal-adaptive immune response in controlling HSV reactivation. Mathematical modeling predicts prolonged duration and increased severity of HSV-2-shedding episodes is strongly associated with a low density of CD8+ T cells within genital skin.<sup>18</sup> That the virus has devised numerous strategies to evade host innate and adaptive mechanisms of resistance to primary and recurrent HSV infection further emphasizes the importance of host immune mechanisms.

Although numerous human studies characterize the T-cell response to HSV-2 in blood, skin, and eye (reviewed in Ouwendijk *et al.*<sup>19</sup>), few studies address the local mucosal cellular immune response directed at HSV-2 in the female genital tract.<sup>20</sup> In order to more comprehensively analyze the cervical resident T-cell response to HSV-2, T cells isolated and expanded from serial cytobrush samples were obtained from 30 HSV-2+ women and analyzed with respect to phenotype, antigenic reactivity, and persistence. Cervical cytobrush

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sampling is a relatively non-invasive procedure for obtaining mucosal T cells from the female genital tract and short-term polyclonal expansion of these cells allowed a more detailed characterization of cervically derived T cells to HSV-2.

**RESULTS**

**Clinical and demographic characteristics of study participants**

The study group consisted of 32 female participants in total including 2 who were seronegative to HSV-1 and HSV-2 (HSVneg) and 30 who were seropositive to HSV-2 (HSV-2+): of these 30 HSV-2+ participants, 9 were coinfecting with HSV-1 and HSV-2 (HSV-1+/2+), whereas 21 were seropositive to HSV-2 only (HSV-1-/2+) (Table 1). The average age of HSV+ participants was 35 years (range 19–73 years) and the HSVneg participants were 26 and 51 years old. Most HSV-2+ participants were white (84%) and had been infected with HSV-2 a median of 8.3 years (range 0.2–37.9 years) (Table 1).

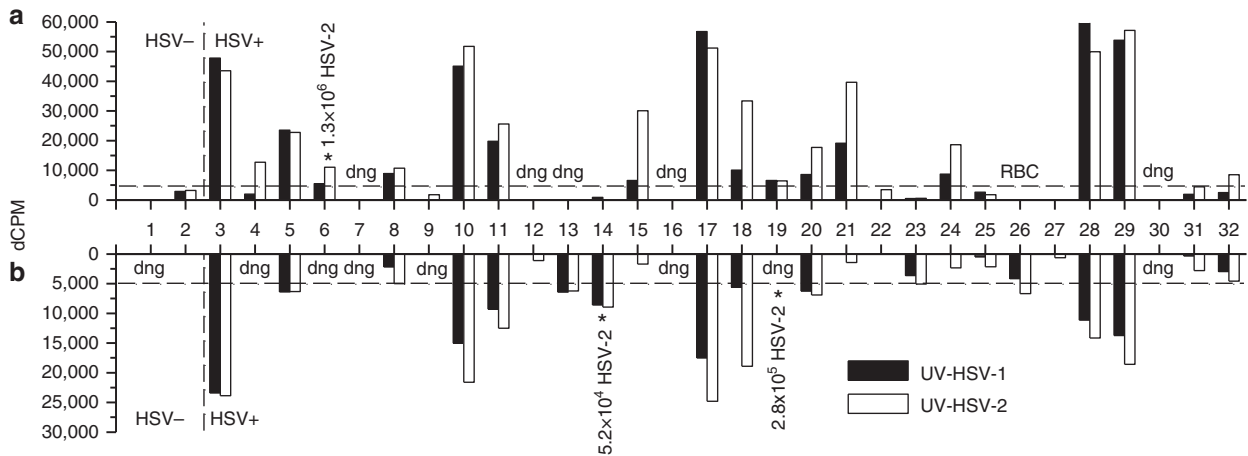
**Table 1 Demographic and clinical characteristics of study participants**

	HSV-2+	HSVneg
No. of participants	30	2
Median age: years (range)	35 (19–73)	39 (26, 51)
<i>HSV serostatus: n (%)</i>		
HSV-1-/2+	21 (70%)	NA
HSV-1+/2+	9 (30%)	NA
Total HSV-2+	30 (100%)	NA
Race	25 White, 5 Black	2 White
Years infected with HSV-2: median (range)	8.3 (0.2–37.9)	NA

Abbreviations: HSV-2, herpes simplex virus type 2; NA, not available.

**HSV-specific lymphoproliferative (LP) responses in cervical lymphocytes**

All study participants (2 HSVneg, 30 HSV-2+) provided two cervical cytobrush samples spaced approximately 1 month apart. A median of  $0.2 \times 10^6$  mononuclear cells (range  $0.08 \times 10^6$ – $1.8 \times 10^6$  cells) per cytobrush were obtained *ex vivo*. Of the 64 cytobrush samples, 1 was discarded owing to red blood cell contamination and T cells could not be expanded from 13 of the samples; cells could not be expanded from either of the two cytobrushes from three HSV-2+ participants (participants 7, 16, and 30) (Figure 1). The remaining 51 cervical cytobrush samples (48 from HSV-2+ participants, 3 from HSVneg participants), where cell growth was evident after 18–24 days in culture, were tested for HSV-specific T-cell activity by LP assays. Of the 48 samples from the HSV-2+ participants, where cell growth was evident, 31 or 65% proliferated in response to HSV-2; 26 of these also proliferated in response to HSV-1 (Figure 1). Considering only those subjects with samples containing HSV-2-specific LP responses, of the subjects who were seropositive for HSV-1 and HSV-2 ( $n = 5$ ), 1 (20%) had no LP responses to HSV-1, whereas 4 (80%) had LP responses to both HSV-1 and HSV-2. Similarly, of the subjects who were seropositive for HSV-2 only ( $n = 16$ ), 4 (25%) had no LP response to HSV-1, whereas 12 (75%) had LP responses to both HSV-1 and HSV-2; these results suggest that HSV serostatus did not influence the ability to detect HSV-1-specific LP responses in the cervical T-cell lines. From 10 of the 30 HSV-2+ participants, cervical T-cell lines were positive for LP responses to HSV-2 from both cytobrush samples, whereas cervical T-cell lines from 17 HSV-2+ participants had positive LP responses in only 1 cytobrush sample (Figure 1). Although T cells expanded from 3 of the 4 cytobrush samples obtained from the 2 HSVneg participants, none were positive for LP responses to HSV-1 or HSV-2 (Figure 1), although LP responses to PHA were positive in the 3 samples (data not



**Figure 1** Herpes simplex virus (HSV)-specific LP responses in cervical cytobrush samples. Cervical cytobrush samples were obtained from 2 HSVneg (HSV-) participants and 30 HSV-2+ participants at study entry (a) and at 1 month (b). Cervical T-cell lines expanded from these samples were tested for HSV-specific LP responses using UV-HSV-1 or UV-HSV-2 as the stimulatory antigen. Dashed line represents threshold for positivity (> 5,000 dCPM). HSV-2 DNA was detected in three samples (\*) and the total amount of virus detected in the sample is displayed. dng, did not grow; RBC, red blood cell contamination.

shown). LP responses to HSV-1 ranged from 1– to 61,827 dCPM (median 5,491 dCPM), responses to HSV-2 ranged from 1 to 57,088 dCPM (median 6,696 dCPM), and PHA responses ranged from 0 to 180,774 dCPM (median 28,890 dCPM): cells expanded from one sample (participant 15, second cytobrush sample) did not proliferate in response to PHA. An additional cytobrush sample was obtained from 5 HSV-2+ participants ~6 months after the first sample; T cells expanded in all five samples and all had positive LP responses to HSV-1, HSV-2, and PHA (data not shown).

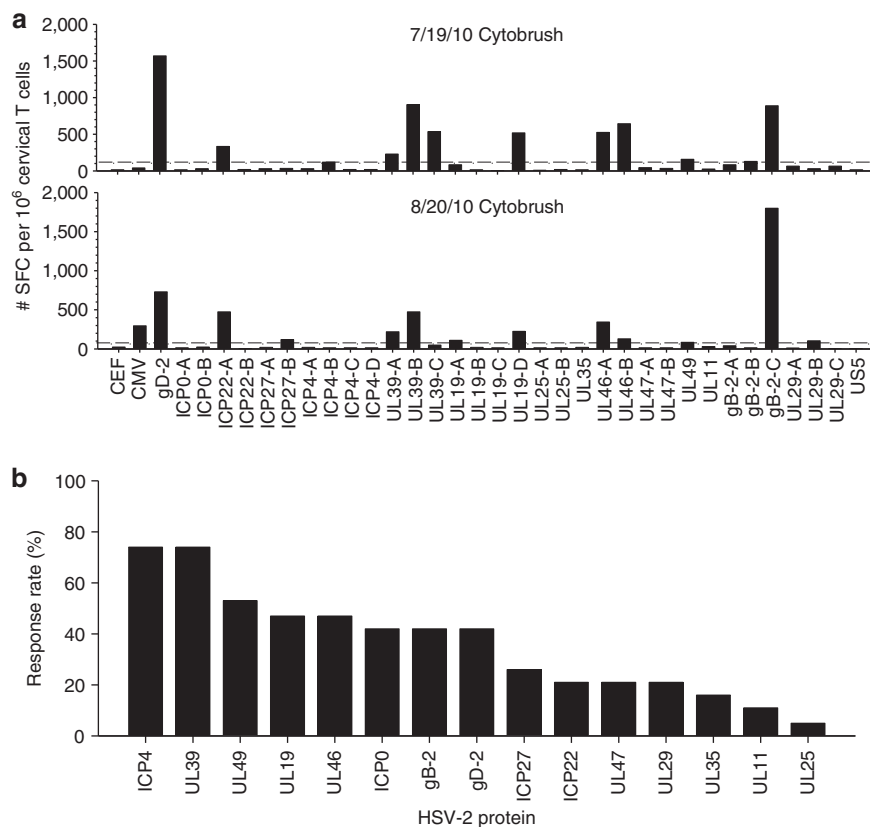
### HSV-2 DNA in cervical samples

Cervicovaginal lavage (CVL) fluid was collected from both clinic visits from each participant and tested for the presence of HSV-2 DNA by quantitative real-time PCR. Of the 60 CVL collected from the 30 HSV-2+ participants, 3 samples (5%) from 3 different participants tested positive for HSV-2 DNA (**Figure 1**). From the three visits where CVL were positive, low levels of HSV-2-specific LP were detected in the corresponding cytobrush samples from two of the participants (participant 6, visit 1; participant 14, visit 2), whereas T cells did not grow from the corresponding cytobrush sample from the third participant

(participant 19, visit 2) (**Figure 1**). It was interesting to note that HSV-2 DNA was not detected in any CVL sample when a corresponding cytobrush sample possessed a high level of HSV-2-specific LP. As expected, no HSV-2 DNA was detected from the four CVL collected from the HSVneg participants (**Figure 1**).

### HSV-2 proteins recognized by cervical T-cell lines

Cervical T-cell lines that tested positive for HSV-specific LP responses were subsequently tested by interferon (IFN)- $\gamma$  enzyme-linked immunospot assay (ELISPOT) for reactivity to a panel of 34 HSV-peptide pools representing 16 HSV-2 proteins. **Figure 2a** displays two representative IFN- $\gamma$  ELISPOT assays from two cytobrush samples obtained 1 month apart from HSV-2 participant 17. Multiple HSV-2 peptide pools were recognized by the cervical T-cell lines from each date (11 pools from 19 July 2010 and 12 pools from 20 August 2010) and multiple sub-pools from an individual HSV-2 protein were also recognized (UL39, UL19, and UL46). In addition, IFN- $\gamma$  ELISPOT responses to eight of the HSV-2 peptide pools were detected in both cytobrush samples (**Figure 2a**). In total, 33 cytobrush samples from 19 HSV-2 participants were analyzed by IFN- $\gamma$  ELISPOT: 5 of these



**Figure 2** Antigenic diversity of herpes simplex virus type 2 (HSV-2)-specific T cells in cervical T-cell lines. **(a)** Cervical T-cell lines that were positive by HSV-LP from HSV-2+ Participant 17 obtained on 19 July 2010 (top) and 20 August 2010 (bottom) were stimulated with HSV-2, a peptide pool containing epitopes from cytomegalovirus, Epstein–Barr virus and influenza (CEF), or a cytomegalovirus pp65 peptide pool and tested for reactivity by interferon (IFN)- $\gamma$  enzyme-linked immunospot assay (ELISPOT); Y-axis is net spot-forming cells (SFC) per million cervical T cells (SFC in dimethyl sulfoxide (DMSO) control wells were subtracted). Dashed lines represent the threshold for positivity ( $4 \times$  median of DMSO control wells and  $\geq 55$  SFCs per million cervical T cells). **(b)** Cervical T-cell lines from HSV-2+ participants that were positive by HSV-LP were screened for reactivity using the 34 peptide pools representing 16 HSV-2 proteins by IFN- $\gamma$  ELISPOT. The frequency of detecting HSV-2 protein-specific T-cell responses in one or both cervical cytobrush samples from 19 HSV-2+ participants is displayed: HSV-2 proteins are stratified based on their hierarchy in % response rate.

participants had 3 cytobrush samples screened, 4 of these participants had 2 cytobrush samples screened, whereas 10 had 1 cytobrush sample screened (**Table 2**). Positive IFN- $\gamma$  ELISPOT responses to >1 sub-pool of an individual HSV-2 protein are grouped together. The median number of HSV-2 proteins recognized in individual cytobrush samples was 4 (range 1–11) (**Table 2**). The HSV-2 proteins most frequently recognized by cervical T-cell lines (positive in one, two, or three cytobrush samples) were ICP4 (74%), UL39 (74%), UL49 (53%), UL46 (47%) and UL19 (47%), ICP0 (42%), gD-2 (42%), and gB-2 (42%), (**Table 2**; **Figure 2b**). This underestimates the

total number of HSV-2 epitopes recognized because in many cases, >1 sub-pool from an individual HSV-2 protein was positive for a given cervical T-cell line: 11 cervical T-cell lines recognized >1 sub-pool of UL39 (5 recognized 2 sub-pools and 6 recognized 3 sub-pools), whereas 5 cervical T-cell lines recognized both sub-pools of UL46 (**Figure 2a** and data not shown). Interestingly, T cells directed at the same HSV-2 protein were often detected in serial cytobrush samples: 8 of the 9 participants who had 2–3 cytobrush samples positive for HSV-LP responses had T-cell responses directed at the same HSV-2 protein in >1 cytobrush sample;  $\sim 1/2$  of the IFN- $\gamma$

**Table 2** Antigenic reactivity of cervical T-cell lines

ID	Study day	HSV-2 protein															Proteins + per cytobrush
		Glycoprotein		Immediate-early				Capsid			Tegument				Other		
		gD-2	gB-2	ICP0	ICP22	ICP27	ICP4	UL19	UL25	UL35	UL46	UL47	UL49	UL11	UL39	UL29	
3	4/5/2010						+					+				+	3
	6/11/2010	+		+			+				+					+	5
	2/8/2011	+	+				+	+			+		+			+	7
4	4/20/2010										+		+			+	3
5	4/21/2010						+	+									2
	7/12/2010			+						+		+				+	4
6	4/26/2010						+	+									2
8	5/17/2010						+				+		+				3
10	6/1/2010		+							+			+				3
	7/1/2010			+			+			+			+		+		5
	5/10/2011		+									+					2
11	6/7/2010										+				+		2
	7/14/2010			+			+								+		4
13	8/4/2010						+	+			+					+	4
14	8/9/2010	+	+				+	+	+		+		+		+	+	9
15	7/1/2010		+							+			+				3
17	7/19/2010	+	+				+	+			+		+		+		8
	8/20/2010	+	+				+	+			+		+		+	+	9
	2/8/2011	+	+							+					+		5
18	7/20/2010	+				+	+	+				+		+		+	7
	8/19/2010	+		+									+	+	+		5
19	7/22/2010						+	+			+		+				4
20	7/2/2010		+	+			+								+		4
	8/23/2010			+			+								+		3
	2/15/2011					+									+		2
21	7/27/2010						+								+		2
23	9/2/2010	+													+		2
26	9/9/2010	+					+					+			+		4
28	8/12/2010		+														1
	9/14/2010	+	+	+			+			+					+		6
29	8/19/2010	+									+	+	+		+		5
	9/20/2010	+	+	+			+	+			+	+	+	+	+	+	10
	3/22/2011	+	+	+	+	+	+	+			+		+	+	+	+	11
#pos/subject (n = 19)		8	8	8	4	5	14	9	1	3	9	4	10	2	14	4	4

Abbreviations: HSV-2, herpes simplex virus type 2; IFN- $\gamma$  ELISPOT, interferon- $\gamma$  enzyme-linked immunospot assay.

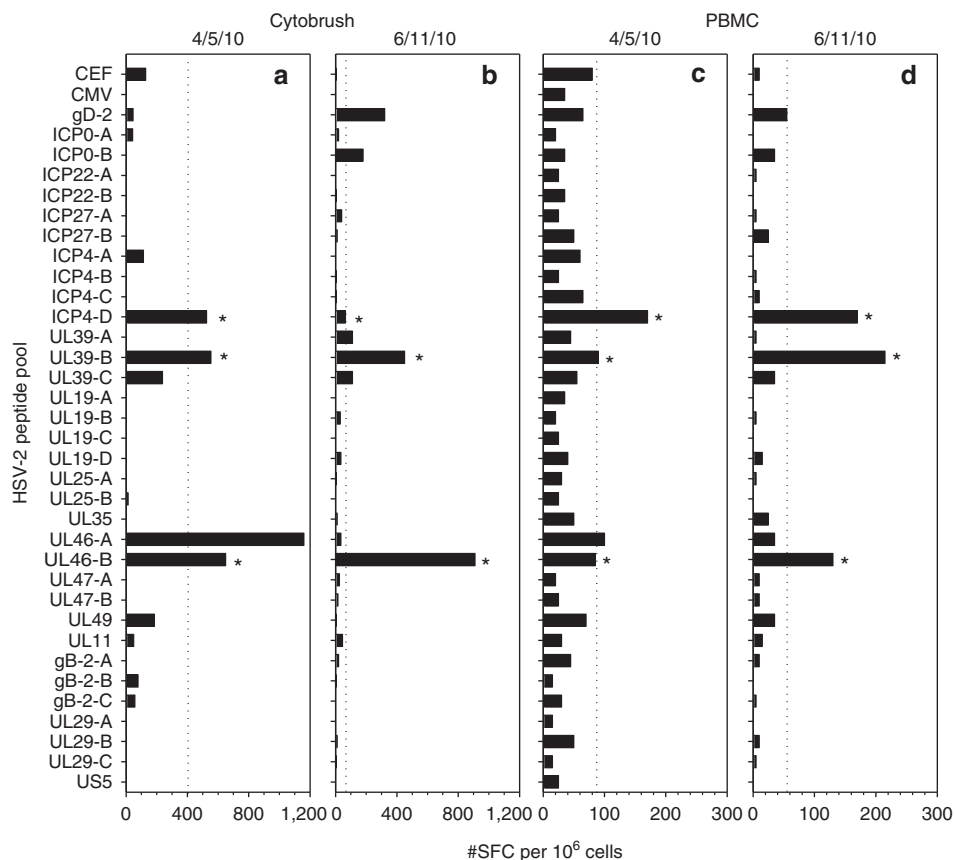
Cervical T-cell lines from HSV-2+ participants with positive HSV-2-specific lymphoproliferative responses were screened by IFN- $\gamma$  ELISPOT using 34 pools of overlapping peptides representing 16 HSV-2 proteins that are classified as glycoproteins, immediate-early, capsid, tegument, or other proteins. Positive IFN- $\gamma$  ELISPOT responses to sub-pools are grouped together. +, positive IFN- $\gamma$  ELISPOT response; shaded cells, T cells directed at the same HSV-2 protein detected in >1 cytobrush sample from the same participant.

ELISPOT responses to a given HSV-2 protein were detected in >1 cytobrush sample from the same participant (**Table 2**; **Figure 2a**).

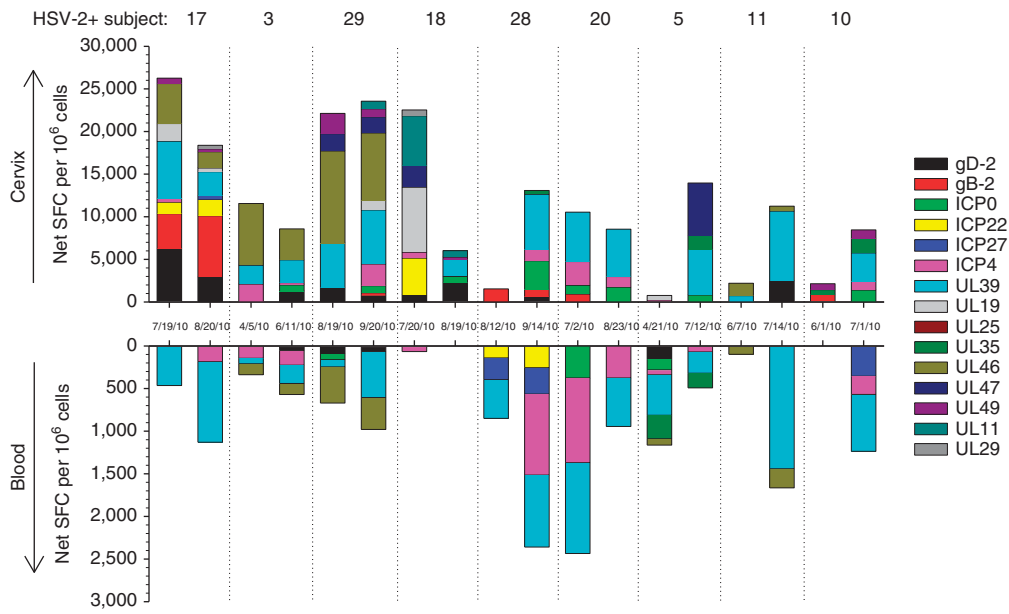
### HSV antigenic specificity of cervical T cells vs. blood-derived T cells

To compare the breadth of the HSV-2-specific T-cell response between the cervix and blood, serial cytobrush samples and corresponding peripheral blood mononucleated cells (PBMC) were tested for reactivity to the HSV-2 peptide pools using IFN- $\gamma$  ELISPOT. **Figure 3** displays data from a representative HSV-2+ participant: positive IFN- $\gamma$  ELISPOT responses were measured to multiple HSV-2 peptide pools and positive responses to the ICP4-D, UL39-B, and UL46-A sub-pools were present in all four samples (**Figure 3**). In addition, positive IFN- $\gamma$  ELISPOT responses to UL46-A were measured in both compartments on 5 April 2010 (**Figure 3a,c**), whereas positive IFN- $\gamma$  ELISPOT responses to gD-2 were detected in both compartments on 11 June 2010 (**Figure 3b,d**). **Figure 4** displays the cumulative IFN- $\gamma$  ELISPOT response to HSV-2 in cervically derived (top) and blood-derived (bottom) T cells from nine HSV-2+ participants who had two cytobrush samples positive for HSV-specific T-cell activity; only those proteins with

positive IFN- $\gamma$  ELISPOT responses are included and positive responses to sub-pools are grouped together. In general, cervically derived T-cell responses were broader in terms of antigenic reactivity compared with those measured in the corresponding PBMC samples. In all but three cases (5 April 2010 sample from participant 3, 12 August 2010 sample from participant 28, and 21 April 2010 sample from participant 5), cervically derived T cells were reactive to more HSV-2 proteins than the corresponding PBMC-derived T cells (**Figure 4**): a median of five HSV-2 proteins (range 1–10) were recognized by these cervically derived T-cell lines compared with a median of three HSV-2 proteins (range 0–6) recognized by PBMC-derived T cells, although this was not statistically significant ( $P > 0.05$ ). Similar antigenic reactivity patterns were observed between serial cytobrush samples from the same participant and often IFN- $\gamma$  ELISPOT responses were positive in both compartments. UL39 was the most commonly recognized HSV-2 protein: T cells directed at UL39 were detected in all cytobrush samples from these nine participants and in at least one PBMC sample from eight of the participants (**Figure 4**). The frequency of the cumulative T-cell response was at least an order of magnitude greater in the cervix compared with the blood suggesting that T cells are enriched in this mucosal compartment.



**Figure 3** Interferon (IFN)- $\gamma$  T-cell response to herpes simplex virus type 2 (HSV-2) peptide pools in blood and genital tract of HSV-2+ women. Cervical T-cell lines (**a** and **b**) and PBMC (**c** and **d**) from HSV-2+ participant 3 obtained on 5 April 2010 (**a** and **c**) or 11 June 2010 (**b** and **d**) were screened by IFN- $\gamma$  enzyme-linked immunospot assay for reactivity to the HSV-2 peptide pools. X-axis is net spot-forming cells (SFC) per million cervical T cells (**a** and **b**) or peripheral blood mononucleated cells (PBMC) (**c** and **d**) (SFC in dimethyl sulfoxide control wells were subtracted). Dashed lines represent the threshold for positivity as described in **Figure 2a**. \*, individual HSV-2 peptide pool responses detected in all four samples from the two different compartments. CEF, cytomegalovirus, Epstein–Barr virus and influenza.



**Figure 4** Cumulative interferon (IFN)- $\gamma$  T-cell response to herpes simplex virus type 2 (HSV-2) in blood and genital tract of HSV-2+ women. IFN- $\gamma$  enzyme-linked immunospot assay responses to HSV-2 peptide pools were measured in serial cervical T-cell lines and peripheral blood mononucleated cells from nine HSV-2+ women. Each bar represents an individual woman's response in the cervix or blood on the date displayed; the SFC from background wells (dimethyl sulfoxide) is subtracted from each HSV-2 peptide pool that is considered positive and only data from those pools that are positive are displayed.

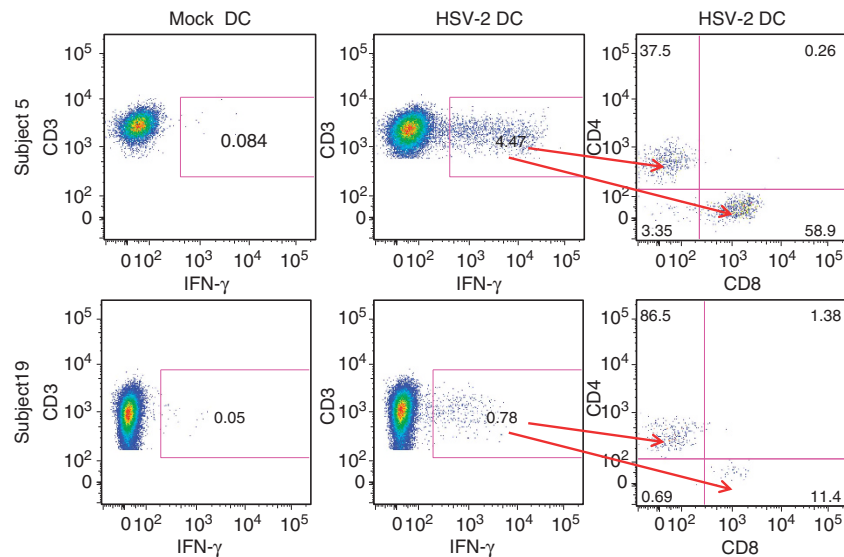
### Phenotype of HSV-2-specific T cells derived from the cervix

Cervical T-cell lines that tested positive for HSV-2-specific LP responses were subsequently stimulated with autologous dendritic cells (DCs) that were mock infected or infected with HSV-2, and the expression of CD3, CD4, CD8, and IFN- $\gamma$  were measured by intracellular cytokine staining (ICS) and flow cytometry. **Figure 5** displays data from two representative HSV-2+ participants, participants 5 and 19. Low background staining ( $<0.10\%$ ) was observed to mock DC in both participants (left panels); in contrast, 4.47% of the CD3+ cells from participant 5 and 0.78% of CD3+ cells from participant 19 co-expressed IFN- $\gamma$  in response to exposure to HSV-2 (**Figure 5**, center panels). In participant 5, 37.5% of the CD3+ /IFN- $\gamma$ + cells were CD4+ and 58.9% were CD8+; in participant 19, 86.5% of the CD3+ /IFN- $\gamma$ + cells were CD4+ and 11.4% were CD8+ (**Figure 5**, right panels). In total, HSV-2-specific CD3+ /IFN- $\gamma$ + cells were detectable in 25 of the cervical T-cell lines that possessed HSV-2-specific LP responses (**Figure 6**); the frequency of HSV-2-specific CD3+ /IFN- $\gamma$ + from 11 of the cervical T-cell lines that possessed HSV-2-specific LP responses were below the level of detection in our ICS/flow cytometry assay and are not included in **Figure 6**. The median frequency of HSV-2-specific CD3+ /IFN- $\gamma$ + T cells detected in the cervical T-cell lines was 2.95% (range 0.45–31.39%; background subtracted) (**Figure 6a**). The HSV-2-specific CD3+ /IFN- $\gamma$ + population was predominantly comprised of CD4+ T cells: a median of 91.30% (range 30–99.40%) of this population was CD4+, whereas a median of 3.90% was CD8+ (range 0–67.70%) ( $P=0.0012$ ) (**Figure 6b**). Not only was the viral-specific CD3+ T-cell

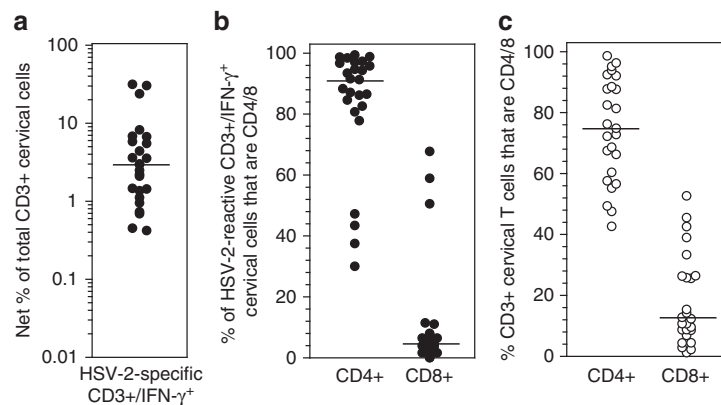
response comprised primarily of CD4+ T cells, but the composition of the total T-cell population was also predominantly CD4+: a median of 74.8% of the total T cells expanded from the cervical cytobrushes was CD4+ (range 42.7–98.6%), whereas a median of 12.8% was CD8+ (range 1.2–52.6%) ( $P=0.00044$ ) (**Figure 6c**). The proportion of total CD4+ T cells that were HSV-2 specific was 2.63% (median; range 0.24–30.14%), whereas the proportion of total CD8+ T cells that were HSV-2 specific was 0.79% (median; range 0–8.79%) (data not shown).

### Phenotype of cervical T cells reactive to HSV-2 peptide pools

To determine whether cervical T cells responding to the HSV-2 peptide pools were CD4+ or CD8+, cervical T-cell lines were incubated with the HSV-2 peptide pools that tested positive by IFN- $\gamma$  ELISPOT, and phenotypes of the responding cells were measured by ICS and flow cytometry. As displayed in representative histograms in **Figure 7a**, seven of the HSV-2 peptide pools that were positive in the 19 July 2010 cytobrush from HSV-2 participant 17 were recognized by CD4+ T cells (gD-2, UL39-B, UL39-C, UL19-D, UL46-A, UL46-B, and gB-2-C), whereas two pools were recognized by CD8+ T cells (ICP22-A and gB-2-C). In total, 28 cervical T-cell lines from 17 participants were analyzed for the phenotype of the responding T cells: 4 participants had 3 cervical T-cell lines, 3 participants had 2 cervical T-cell lines, and 10 participants had 1 cervical T cell analyzed. There were a total of 133 positive responses to individual HSV-2 peptide pools that could be measured by ICS and flow cytometry from all 28 cervical T-cell lines; 105 of these



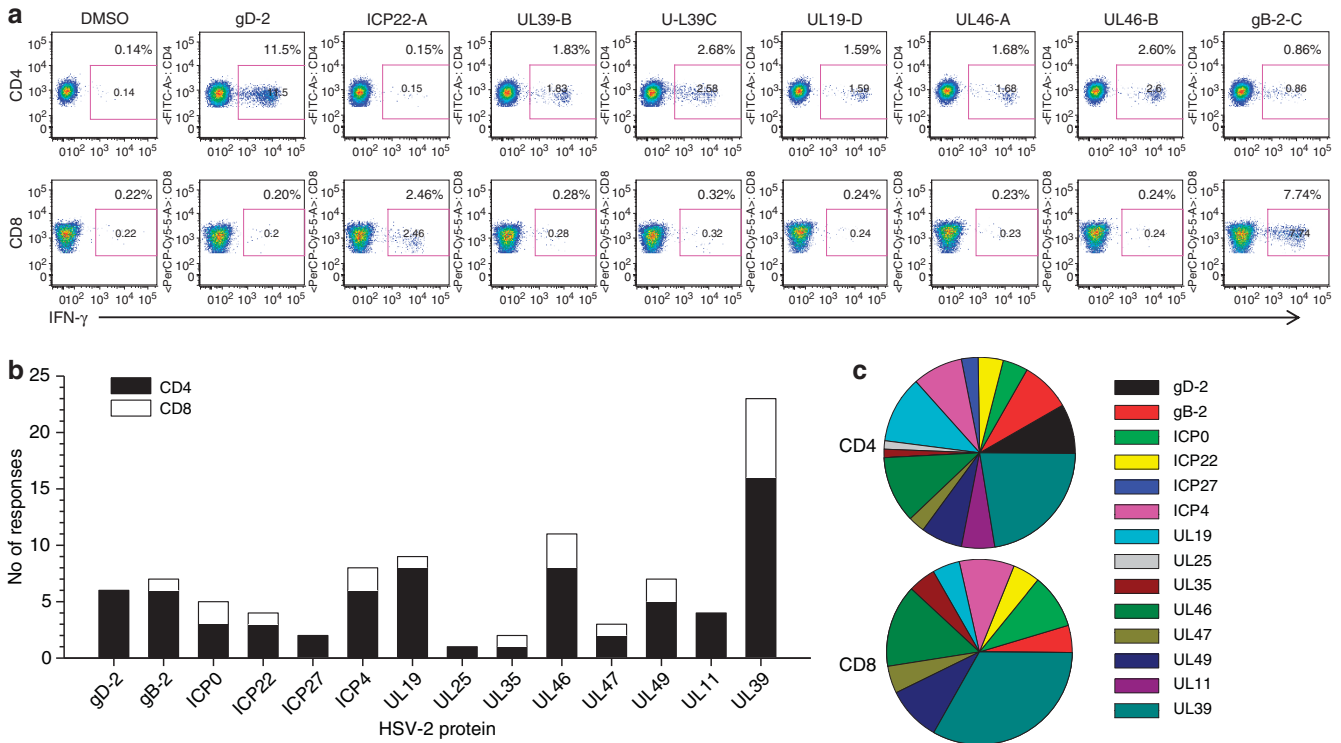
**Figure 5** Phenotype of cervically derived T cells reactive to herpes simplex virus type 2 (HSV-2). Cervical T-cell lines expanded from two HSV-2+ participants (participant 5 and participant 19) were stimulated with a mock dendritic cell (DC) or HSV-2 DC and the production of interferon (IFN)- $\gamma$  from CD3<sup>+</sup> cells was determined. Far right graphs display the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the HSV-2-reactive CD3<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> population.



**Figure 6** Frequency of CD3, CD4, and CD8 T-cell responses to herpes simplex virus type 2 (HSV-2) in T cells derived from the cervix. Cervical T-cell lines were incubated with a mock dendritic cell (DC) or HSV-2 DC and the expression of CD3, CD4, CD8, and interferon (IFN)- $\gamma$  was determined by intracellular cytokine staining and flow cytometry. (a) The proportion of cervically derived CD3<sup>+</sup> T cells that were HSV-2 specific was calculated by subtracting %CD3<sup>+</sup> + IFN- $\gamma$ <sup>+</sup> cells with mock DC from %CD3<sup>+</sup> + IFN- $\gamma$ <sup>+</sup> cells with HSV-2 DC. (b) The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the HSV-2-reactive CD3<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> fraction from (a) is displayed. (c) The proportion of total cervically derived CD3<sup>+</sup> T cells that were CD4<sup>+</sup> or CD8<sup>+</sup> is displayed. In each graph, median frequencies are displayed by the dashed line.

were CD4<sup>+</sup> and 28 were CD8<sup>+</sup>. On an individual basis, CD4 T cells derived from a cervical T-cell line were reactive to an average of three different HSV-2 peptide pools (range 0–13) compared with CD8 T cells that were reactive to an average of one HSV-2 peptide pool (range 0–4) ( $P=0.00044$ ). In **Figure 7b**, all CD4 and CD8 responses to individual HSV-2 proteins from all 17 participants were grouped together to assess the CD4/CD8 phenotype of T cells responding to the HSV-2 proteins; when a CD4 or CD8 response to an individual HSV-2 protein was detected in >1 cytobrush sample from the same individual, we only included this response once. On a population level, CD4 responses were directed at a broader

range of HSV-2 proteins compared with CD8 responses (**Figure 7b**). **Figure 7c** displays pie graphs depicting the percentage of the total number of CD4 (top) or CD8 (bottom) responses directed at individual HSV-2 proteins. UL39 was the most common HSV-2 protein recognized by both CD4 and CD8 T cells derived from the cervix (**Figure 7b,c**). CD8 responses were also commonly directed at ICP0, ICP4, UL46, and UL49 (**Figure 7b,c**), whereas CD4 responses were commonly directed at gB-2, gD-2, UL19, UL46, and ICP4 (**Figure 7b,c**). Taken together, these data suggest a greater antigenic diversity in the cervically derived CD4 T-cell response compared with the cervically derived CD8 T-cell response to



**Figure 7** Antigenic diversity of herpes simplex virus type 2 (HSV-2)-specific CD4 and CD8 T-cell responses in cervical T-cell lines. **(a)** The expanded cervical T-cell line from HSV-2 + participant 17 from the cytobrush sample obtained on 19 July 2010 (**Figure 3a**, top graph) was stimulated with dimethyl sulfoxide (DMSO) only (negative control) or one of the HSV-2 peptide pools that were positive in interferon (IFN)- $\gamma$  enzyme-linked immunospot assays and the phenotype of reactive cells was determined using intracellular cytokine staining (ICS)/flow cytometry. Top row of the graphs are CD4 vs. IFN- $\gamma$  and the bottom row of graphs are CD8 vs. IFN- $\gamma$ ; the percentage of CD4 + /IFN- $\gamma$  + or CD8 + /IFN- $\gamma$  + cells is displayed. **(b)** Positive CD4 and CD8 responses to individual HSV-2 proteins measured in cervical T-cell lines from all HSV-2 + participants determined by ICS/flow cytometry were collated. Black bars, CD4 + /IFN- $\gamma$  + responses, open bar, CD8 + /IFN- $\gamma$  + responses. **(c)** The percentage of CD4 + /IFN- $\gamma$  + responses (top pie graph) and CD8 + /IFN- $\gamma$  + responses (bottom pie graph) directed at individual HSV-2 proteins present in cervical T-cell lines from HSV-2 + participants is displayed.

HSV-2 on both an individual and population basis. Thus, not only do CD4 + T cells comprise a greater percentage of the HSV-2-specific CD3 + /IFN- $\gamma$  + population within the cervical T-cell lines (**Figure 6b**), but they are also directed at a broader range of HSV-2 proteins as compared with the CD8 + T cells derived from the cervix (**Figure 7b,c**).

**DISCUSSION**

This study markedly extends the knowledge of HSV-2-specific T cells populating the human female genital tract. HSV-specific T cells were frequently detected in the cervix and ~3% of all T cells expanded from the cervical cytobrush samples were directed at HSV-2. The HSV-2-specific T-cell response was predominantly comprised of CD4 T cells, which were directed at a broader range of HSV-2 proteins compared with CD8 T cells. HSV-2-specific T cells appeared to be enriched in the cervix compared with the blood compartment, and T-cell responses directed at the same HSV-2 protein and peptide pool were often detected in serial cytobrush samples as well as in PBMC.

Total CD4 + T cells and HSV-2-specific CD4 + T cells were consistently detected in the cervical T-cell lines and at high frequencies compared with CD8 + T cells, which were present

at significantly lower frequencies and sometimes undetectable. This observation is consistent with a comprehensive analysis of memory T-cell subsets within various peripheral tissues, which demonstrated that memory CD4 + T cells represent the majority subset in mucosal tissue including the lung and intestinal tract, although the female genital tract was not analyzed.<sup>21</sup> CD4 + T cells were shown to be present in high numbers in the endocervix in a distinct band underneath the epithelium<sup>22</sup> and a study of 30 healthy Caucasian women showed that CD3 + T cells made up ~1% of the total cells isolated from the endocervix and of those, 61% were CD4 +.<sup>23</sup> A recent study by McKinnon *et al.*<sup>24</sup> showed a 2:1 CD4:CD8 ratio in T cells isolated from healthy women in Seattle, Chicago, and Nairobi. Our study demonstrates that ~75% of the total CD3 + population within the cervical T-cell lines were CD4 + (**Figure 6c**). These data suggest that the total T-cell population obtained upon cytobushing is skewed toward CD4 + T cells. This dominance in CD4 + T cells is more pronounced in the HSV-2-specific CD3 + population, where a median of 91% of HSV-2-specific CD3 + T cells were CD4 + (**Figure 6b**). The presence of sexually transmitted infections has been shown to influence the T-cell composition in the cervix: higher numbers of CD4 + T cells were measured in the endocervix in subjects



with non-ulcerative sexually transmitted infections including chlamydia, gonorrhea, and trichomoniasis.<sup>25</sup> However, because the non-specific stimulation of T cells isolated from the cervix may preferentially expand T cells with higher proliferative capacity, the most accurate assessment of the T-cell composition of both total T cells and HSV-2-specific T cells would be performed *ex vivo*. It is likely that a significant fraction of CD8 + T cells are resident memory T cells that have limited proliferative capacity and thus unlikely to expand in our protocol requiring cells to undergo multiple rounds of cell division. The impact of expansion methods on the maturational state of cervical T cells in HIV + women suggested that expansion using anti-CD3, similar to the current study, led to the accumulation of effector memory T cells (CD45RO + CCR7 – CD27 –) after 7 days;<sup>26</sup> however, these studies did not measure virus-specific responses but instead total CD4 and CD8 T cells. Our limited data analyzing cytobrush-derived T cells *ex vivo* for HSV-2-specific CD4 + and CD8 + T cells suggest that CD8 + T cells were at lower frequencies than CD4 + T cells or undetectable, similar to the phenotype of cervical T-cell lines generated upon *in vitro* expansion (unpublished data). Interestingly, higher numbers of CD8 + T cells were present in ectocervical biopsy specimens compared with endocervical cytobrush specimens obtained from healthy women,<sup>24</sup> suggesting that CD8 + T cells may reside at tissue locations not sampled during cytobushing and perhaps providing another possibility as to why low frequencies of HSV-2-specific CD8 + T cells were measured. In any event, although the presence of high frequencies of HSV-2-specific CD4 + T cells in the cervix may suggest an important role in the local control of genital HSV-2 infection, it may also have significant implications for HIV acquisition as HSV-2 increases the risk of HIV acquisition, possibly owing in part to increased CD4 + T-cell activation in the cervix and an increased expression of HIV susceptibility markers, CCR5 and  $\alpha 4\beta 7$ .<sup>27–29</sup>

HSV-2 disease is characterized by frequent clinical and subclinical shedding. The frequent detection and high frequency of HSV-specific T cells in the cervix suggests the ongoing exposure to an antigen, although cervical shedding of HSV-2 tends to occur at lower rates than from other areas of the lower genital tract.<sup>30</sup> The current study detected HSV-2 DNA in only three of the cytobrush samples (5% of samples); this is similar to what was observed in a cross-sectional study of 509 HSV-2 seropositive women where 7% of all CVL samples were positive for HSV-2 DNA.<sup>31</sup> The antimicrobial activity of CVL, which increases at the time of clinical HSV-2 outbreaks, has been proposed as a mechanism to prevent the spread of HSV-2 from external genital sites to the upper genital tract.<sup>32</sup> The high frequency of HSV-2-specific cervical T cells detailed in the current study may contribute to the control of HSV-2 spread in the female genital tract; anecdotally, HSV-2 DNA was not detected in any CVL with a correspondingly high level of HSV-2-specific LP responses in the cytobrush samples. A more intense study of mucosal sampling, including multiple external and internal genital sites, and local T cells is warranted to assess the relationship between local mucosal HSV-specific

T-cell immunity and viral shedding in order to determine the mechanism of viral control at the site of infection and reactivation.

Short-term polyclonal expansion of the T cells obtained from cytobushing provided sufficient cells to analyze the antigenic repertoire of cervical T-cell lines. In general, T-cell recovery was too low to perform *ex vivo* functional and other phenotypic T-cell studies. We have recently obtained cervical biopsies that may provide a larger source of cells that can be tested *ex vivo* to determine the memory/effector phenotype, cytokine profile, and lytic function of the cervical resident T cells; such studies are best done *ex vivo* to prevent changes in biologically relevant mechanisms that may be altered upon short-term and long-term cell culture.<sup>26,33</sup> These *ex vivo* studies will aid in the determination of the mechanisms utilized by local T cells to limit or prevent HSV reactivation and spread in HSV-2-infected participants or protection from infection in HSV-resistant populations. Recently, our group demonstrated that CD8 $\alpha\alpha$  + T cells are the dominant resident population of dermal–epidermal junction CD8 + T cells that persist at the site of previous reactivation in skin near the genital region.<sup>17</sup> Importantly, these cells (1) lacked the expression of CCR7 and S1PR1, suggesting that they may be tissue-resident T cells, and (2) possessed gene signatures of T-cell activation and antiviral activity suggesting a role in immune surveillance and in the containment of HSV-2 reactivation in human peripheral tissue.<sup>17</sup> It will be important to determine whether these CD8 $\alpha\alpha$  + cells also persist in the human female genital tract as a means to control local HSV-2 reactivation; presumably, these cells lack significant proliferative potential and may not be expanded using the techniques employed in this study but instead may only be detected if assessed *in situ* or *ex vivo*.

UL39 and ICP4 were the most common HSV-2 proteins recognized by cervical T-cell lines, including both CD4 + and CD8 + T cells, from HSV-2 + participants. This is similar to what we have previously reported in blood-derived HSV-2-specific CD8 + T cells from HSV-2 + participants<sup>34,35</sup> and immune seronegative participants.<sup>36</sup> Studying the antigenic repertoire of local T-cell responses will be important for the rational design of prophylactic and therapeutic vaccines against HSV-2. Induction of local T-cell immunity will be an essential feature of any HSV-2 vaccine; frequent cervical cytobushing was well tolerated by our study participants and may represent a viable strategy for assessing whether candidate HSV-2 vaccines elicit local adaptive immune responses. Although the design of therapeutic vaccines may be informed by the study of HSV-infected participants with the lowest rates of mucosal HSV reactivation (“virus controllers”), strategies for the prevention of HSV infection require the elucidation of protective mechanisms employed by participants who resist infection, especially those who remain seronegative even after frequent exposure to HSV from infected sexual partners.<sup>36,37</sup> We are currently exploring whether local T-cell responses are present in these HSVneg participants in order to assess whether these responses may be linked to protection from HSV infection at the genital mucosa.

The current study has utilized HSV-2 peptide pools representing 16 HSV-2 proteins commonly recognized by systemic and skin-derived T cells from HSV-2+ participants. HSV-2 encodes 80 open-reading frames (ORFs) and thus, the current study screens T cells directed at only 20% of the HSV-2 proteome. Dr David Koelle has developed an elegant method to efficiently generate a genome-wide map of the responsiveness to HSV-1-specific T cells<sup>38</sup> and more recently, to HSV-2-specific T cells (unpublished data). This technology will provide a global analysis of both the CD4 and CD8 T-cell response to HSV-2. It is likely to reveal novel T-cell epitopes recognized by cervically derived T cells and allow a precise comparison of the antigenic reactivity between T cells derived from the mucosa and blood.

In summary, this study assessed the frequency and antigenic profile of mucosal T-cell responses to HSV-2 in the human female genital tract using non-specific *in vitro* polyclonally expanded cervical cytobrush-derived T cells from HSV-2-infected women. Local immunity at sites of HSV-2 exposure is likely to have an important role in preventing or controlling HSV reactivation or HSV infection and may inform the design of future HSV-2 vaccine strategies. Efficacious HSV-2 vaccines will require the elicitation of local T-cell immunity, and sampling the cervix for the detection of vaccine-induced T cells would be a feasible strategy and one of high priority to employ in future vaccine trials. Although the study of polyclonally expanded T cells has provided valuable insight into the phenotype and antigenic profile of local T-cell immunity to HSV-2, the study of local T cells *ex vivo* should provide mechanistic data that may lead to correlations of immune protection and novel strategies to suppress HSV-2 reactivation or prevent HSV-2 infection.

## METHODS

**Study population.** Healthy HSV-1 and HSV-2 seronegative (HSV neg), HSV-1-seronegative/HSV-2 seropositive (HSV-1 -/2 +), and HSV-1-seropositive/HSV-2-seropositive (HSV-1 +/2 +) female participants were enrolled into Institutional Review Board-approved protocols at the University of Washington Virology Research Clinic, Seattle, WA. All participants provided written informed consent. HSV western blot to detect antibodies to HSV-1 and HSV-2 was performed as previously described.<sup>39,40</sup>

**PBMC, DC, lymphoblastoid cell lines and viruses.** Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque and cryopreserved within 8 h of venipuncture as previously described.<sup>41</sup> DCs were generated from PBMC using granulocyte-macrophage colony-stimulating factor and interleukin-4 as previously described.<sup>34</sup> Lymphoblastoid cell lines were generated as previously described.<sup>42</sup> HSV-1 strain E115 and HSV-2 strain 333 were used at a multiplicity of infection of 10 or were ultraviolet (UV)-inactivated to use as viral antigen where indicated.

**Cervical mucosal sample collection, processing, and T-cell expansion.** Cervical cells were collected during speculum examination using a Cytobrush Plus Cell Collector (Medscand, Malmo, Sweden) inserted into the cervical os, rotated through 360° once, and immediately placed in 5 ml of cold transport medium RPMI-CVX (RPMI 1640 medium supplemented with 5 mM l-glutamine, 50 U ml<sup>-1</sup> penicillin, 50 µg ml<sup>-1</sup> streptomycin, and 10% human serum, 10 µl ml<sup>-1</sup> amphotericin B, and 0.5 µl ml<sup>-1</sup> ciprofloxacin). Each participant provided two cervical cytobrush samples spaced ~1 month apart; five

HSV-2+ participants provided an additional cytobrush sample approximately 6–10 months post study entry. Cervical samples with visible red blood cell contamination were discarded. Under sterile conditions, cervical cells were isolated within 2 h of collection by gently vortexing the tube containing the cytobrush followed by rotating the cytobrush against the sides of the tube to dislodge cells. The transport medium was flushed through the cytobrush bristles and the cell suspension was passed through a 40-µm filter into a clean 50-ml centrifuge tube. Cells were pelleted at 250 × g for 10 min, resuspended in RPMI-CVX, and counted. Cervical cells were cultured in 24-well plates (up to 2 × 10<sup>6</sup> per well) along with 5 × 10<sup>6</sup> autologous irradiated PBMC, OKT3 (1 µl in 25 ml) and CD28 (0.5 µl ml<sup>-1</sup>) (BD Biosciences, San Jose, CA) in a final volume of 2 ml per well. Interleukin-2 (50 U ml<sup>-1</sup>) was added every 2–3 days in fresh RPMI-CVX.

CVL fluid was obtained from each participant in order to measure HSV DNA by washing the cervical os and posterior vaginal wall with 10 ml of phosphate-buffered saline (pH 7.0) as previously described.<sup>43</sup> Samples were transported on ice and centrifuged at 1000 × g for 20 min at 4 °C, aliquoted, and stored at -80 °C.

**HSV-specific LP responses.** Cervical T-cell lines were washed twice and 1 × 10<sup>5</sup> cells were incubated in triplicate with 1 × 10<sup>5</sup> irradiated autologous PBMC as antigen-presenting cells in 96-well round-bottomed plates. Cervical T-cell lines were stimulated with 1:500 dilution of UV-inactivated (UV) HSV-1, UV-HSV-2, mock antigen (Ag), or purified PHA (0.4 µg ml<sup>-1</sup>) (Murex, distributed by Remel, Lenexa, KS) as previously described.<sup>9</sup> After 3 days, 1 µCi [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA) was added to each well for 18 h and its incorporation into DNA was measured. Δcpm were calculated by subtracting cpm from mock Ag wells from cpm from UV-HSV wells. An LP response was considered positive if the Δcpm were > 5,000 cpm.

**Synthetic peptides and peptide pools.** We selected 16 HSV-2 ORFs for peptide synthesis as previously described.<sup>36</sup> ORFs were mostly virion proteins (capsid, tegument, and glycoprotein) or immediate-early proteins. Amino-acid sequences were derived from the HSV-2 strain HG52 genome (GenBank accession no. NC-001798). Peptides were 15 amino acids long and overlapping by 11 amino acids and synthesized in crude form by either CBI/Mimotopes (San Diego, CA) (gD-2, ICP0, ICP4, ICP22, ICP27, and UL39) or New England Peptide LLC (Gardner, MA) (UL19, UL25, UL35, UL46, UL47, UL49, UL11, UL27, UL29, and US5) and lyophilized. Each peptide was dissolved in 10 mg ml<sup>-1</sup> in sterile endotoxin-free dimethyl sulfoxide (Sigma, St Louis, MO) and stored at 4 °C. The mass of each peptide was approximately 4–5 mg. Control peptide pools included a pool comprising immunodominant CD8+ T-cell epitopes within cytomegalovirus (CMV), Epstein-Barr, and influenza<sup>36,41,44</sup> (CBI/Mimotopes) and the CMV pp65 peptide mix containing 138 15-mers overlapping by 11 amino acids (Becton Dickinson, San Jose, CA).

Peptide pools (library and array) were prepared for each ORF by grouping peptides linearly across an ORF and were used to screen PBMC, cervical T-cell lines, or T-cell clones. A total of 2,633 peptides were synthesized and grouped into pools composed of peptides ranging from 21 to 100 peptides per pool (median 85 peptides per pool) as previously described.<sup>36</sup> Array pools, used to deconvolute library pools that were positive in the T-cell screen, were prepared by arranging the peptides within a single library pool in a row-and-column format and pooling the peptides in each column or row.

**IFN-γ ELISPOT.** The production of IFN-γ by HSV-specific T cells obtained from PBMC or cervical T-cell lines was analyzed by ELISPOT as previously described<sup>36</sup> with modifications. PBMC were thawed in R10 (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, 50 µg ml<sup>-1</sup> streptomycin, 50 U ml<sup>-1</sup> penicillin containing 50 U ml<sup>-1</sup> benzonase (Novagen, Madison, WI)), and then washed and rested overnight in R10 at 37 °C, 5% CO<sub>2</sub> before assay. Cervical T-cell lines were washed twice and counted. Using the

IFN- $\gamma$  ELISPOT kit (Mabtech, Cincinnati, OH) according to the manufacturer's instructions, PBMC were plated at  $2 \times 10^5$  cells per well (no additional antigen-presenting cells added) and cervical T-cell lines were plated at  $5 \times 10^4$  cells per well along with  $5 \times 10^4$  irradiated autologous PBMC as antigen-presenting cells. Peptides were added to the wells at a final concentration of  $1 \mu\text{g ml}^{-1}$  for overnight stimulation. Wells containing R10 alone or R10 and dimethyl sulfoxide served as negative controls, and those containing  $1 \mu\text{g ml}^{-1}$  PHA served as positive controls. Negative controls were tested in four replicate wells, whereas HSV-2, CMV and cytomegalovirus, Epstein-Barr virus and influenza peptide pools, and PHA were tested in duplicate wells. Spots were counted and analyzed using the automated Bioreader (Biosys GmbH, Karben, Germany). Responses were considered positive if (1) the spot-forming cells (SFC) per well were four times greater than the mean SFC in the four dimethyl sulfoxide wells and (2) the SFC per well was  $\geq 11$  SFC per well.<sup>36,45</sup> Results are expressed as SFC per  $10^6$  cells.

**ICS and flow cytometry.** To determine the phenotype of HSV-2 peptide-specific cervically derived T cells, ICS and flow cytometry was performed using a six-color ICS panel in a 96-well plate format modified from<sup>46</sup> as previously described.<sup>36</sup> Briefly, in order to assess T-cell responses to whole HSV-2, cervical T-cell lines ( $0.5\text{--}1 \times 10^6$  per well) were co-incubated with irradiated autologous lymphoblastoid cell lines that were mock infected (negative control) or infected overnight with HSV-2 at a cell ratio of 10:1; in order to assess HSV-2 peptide-specific T-cell responses, irradiated autologous lymphoblastoid cell lines were co-incubated with HSV-2 peptide pools ( $1 \mu\text{g ml}^{-1}$ ) or individual HSV-2 peptides ( $1 \mu\text{g ml}^{-1}$ ). Controls included dimethyl sulfoxide (negative control), Staphylococcus enterotoxin B (positive control), cytomegalovirus, Epstein-Barr virus and influenza, or CMV peptide pools. During the 6-h incubation at  $37^\circ\text{C}$ , brefeldin A ( $10 \mu\text{g ml}^{-1}$ , Sigma) and the co-stimulatory antibodies CD28 and CD49d (each at  $1 \mu\text{g ml}^{-1}$ , BD Biosciences) were included. Antibodies CD4-FITC, CD8 PerCP-Cy5.5, IFN- $\gamma$  APC, and IL-2 PE were purchased from BD Biosciences, CD3 ECD was purchased from Beckman Coulter (Marseille, France), and the LIVE/DEAD Fixable Violet Dead Cell Stain was purchased from Invitrogen/Molecular Probes (Eugene, OR). Samples were collected from 96-well plates using a high-throughput sample (BD) device for analysis by the LSRII and all FACS analyses were performed using FlowJo software (TreeStar, Ashland, OR). To be considered positive, IFN- $\gamma$  or interleukin-2 production needed to exceed the background by a factor of at least two.<sup>47</sup>

**Measurement of HSV DNA in cervical secretions.** CVL fluid collected at both clinic visits from HSVneg and HSV-2+ women was evaluated for HSV DNA by quantitative, real-time, fluorescence-based PCR as described.<sup>48</sup>

**Statistical analysis.** The Wilcoxon matched-pair signed ranks test was employed to determine the differences between compartments. Statistical significance was defined as  $P < 0.05$  with a two-tailed  $\alpha$ -test.

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#### DISCLOSURE

C.M.P. and L.C. are coinventors on several patents involving potential HSV vaccine candidates. L.C. is on the scientific advisory board for and holds

stock (<1% of company) in Immune Design Corp. A.W. has received research funding from Gilead, Agenus, Genocoea, and Vical and has been a consultant for Aicuris. The remaining authors declared no conflict of interest.

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