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Article Immunology

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Expression of cutaneous lymphocyte-associated antigen by CD8⁺ T cells specific for a skin-tropic virus

See the related Commentary beginning on page 441.

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Virus-specific CD8⁺ T cells traffic to infected tissues to promote clearance of infection. We used herpes simplex virus type 2 (HSV-2) as a model system to investigate CD8⁺ T cell trafficking to the skin in humans. Using human leukocyte antigen (HLA) class I tetramers, we observed that HSV-specific CD8⁺ T cells in the peripheral blood expressed high levels of cutaneous lymphocyte-associated antigen (CLA). In contrast, CD8⁺ T cells specific for non-skin-tropic herpesviruses lacked CLA expression. CLA-positive HSV-2-specific CD8⁺ T cells had the characteristics of central memory cells, expressing CCR7, CD62L, and CD28, and they proliferated briskly in response to antigen. CLA is related to a functional E-selectin ligand, and both E-selectin and CLA-positive cells were detected in HSV-2-infected skin. HSV-2-specific T cells adhered to cells transfected with E-selectin. A higher proportion of HSV-specific CD8⁺ T cells recovered from herpes lesions express CLA compared with blood, consistent with a role for CLA in skin homing. To our knowledge, this is the first report of expression of tissue-specific adhesion-associated molecules by virus-specific CD8⁺ T cells. The evaluation of vaccines for skin and mucosal pathogens should include study of the induction of appropriate tissue-specific homing molecules.

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Introduction

Models of homing of memory T cells to sites of antigen challenge incorporate an initial step of rolling adherence to activated vascular endothelium (1). The cutaneous lymphocyte-associated antigen (CLA) is a fucose-containing carbohydrate that can decorate P-selectin glycoprotein ligand-1 on T cells (2). CLA is expressed

on the surface of most T cells recovered from skin, and on about 5–10% of circulating CD8⁺ T cells (3). E-selectin (also known as ELAM and CD62E) is expressed by venular endothelial cells in inflamed skin, oral mucosa, and the female genital tract (4–6). An epitope related, although not identical (7), to CLA can interact with E-selectin, leading to the tethering and rolling of CLA-expressing cells in vitro (2). This interaction has been hypothesized to direct lymphocyte trafficking to skin in vivo. However, the role of CLA-associated E-selectin ligands in the trafficking of virus-specific CD8⁺ T cells to cutaneous sites of infection has not previously been investigated.

Herpes simplex virus type 2 (HSV-2) causes intermittent lytic infection of skin and genital mucosa, with or without lesion formation and symptoms. Replicating virus is mostly limited to the epidermis or mucosal squamous keratinocytes (8, 9). During symptomatic recurrent infection, antigen-specific CD4⁺ T cells and NK cells infiltrate the subjacent dermis by day 2 of lesion formation, while CD8⁺ T cell infiltration, local virus-specific cytotoxic activity, and viral clearance typically occur a few days later (10). HSV-specific CD8⁺

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Conflict of interest: D.M. Koelle and L. Corey are co-inventors on patents covering the use of HSV-2 antigens as vaccines. The patents have been licensed to Corixa Inc. (Seattle, Washington, USA) by the University of Washington.

Nonstandard abbreviations used: cutaneous lymphocyte-associated antigen (CLA); herpes simplex virus type 2 (HSV-2); human leukocyte antigen (HLA); HSV-2 virion protein (VP); HSV-2-infected cell protein 0 (ICP0); cytomegalovirus (CMV); Epstein-Barr virus (EBV); EBV-transformed B cell (EBV-LCL); Chinese hamster ovary (CHO); phytohemagglutinin (PHA); amino acids (a.a.); phycoerythrin (PE); transporter associated with antigen processing (TAP).

CTL clones can be obtained at high frequency from lesion biopsies (10, 11). Thus, we investigated whether interactions between the CLA-associated E-selectin ligand and E-selectin might promote migration of HSV-2-specific CTLs to local cutaneous sites of infection.

Fluorescent human leukocyte antigen (HLA) tetramers were used to detect CD8⁺ cells specific for the HSV-2 virion proteins 22 (VP22) and 13/14 (VP13/14) (11) and HSV-2-infected cell protein 0 (ICP0). As controls, T cells specific for cytomegalovirus (CMV) and the Epstein-Barr virus (EBV) were also studied. The majority of memory HSV-2-specific T cells in the blood expressed CLA, while CMV- and EBV-specific T cells lacked CLA expression. Recurrent herpetic skin biopsies showed upregulated E-selectin expression and contained a CLA-expressing dermal infiltrate locally enriched in HSV-specific CD8⁺ T cells. These data suggest that homing receptor expression on memory T cells may be programmed by the site of original antigen encounter, promoting migration for immune surveillance or responses to reactivation of infection. Additional studies indicated that circulating CLA⁺ HSV-2-specific CD8⁺ T cells have a preserved capacity for self-renewal and the characteristics of central memory T cells.

Methods

Subjects and specimens. Subjects were HLA typed (12). Subjects used for HSV-2 analyses were HSV-2-seropositive (13), HIV-1-seronegative, generally healthy, and not taking immune-suppressive medication. For subjects with a clinical history of genital herpes, the first clinical episode had occurred at least 6 months prior to phlebotomy. No subject was experiencing a symptomatic recurrence of genital herpes or receiving antiviral therapy at the time of phlebotomy. HSV-2-seropositive subjects filled out a questionnaire concerning their history of genital herpes. Some subjects had HSV cultures of multiple genital and perirectal sites on a daily basis for more than 50 consecutive days to determine their rates of HSV shedding, as described (14). PBMCs were cryopreserved after Ficoll-Hypaque centrifugation. For CD62L, flow cytometry used freshly isolated PBMCs (15). For two subjects, biopsies of perianal HSV-2 culture-positive lesions and normal forearm skin were performed (10). Portions were frozen in OCT (Sakura Finetek, Torrance, California, USA). Subjects used for EBV and CMV analyses were healthy lab personnel known to be seropositive for these agents and to have appropriate HLA types. Protocols were approved by the institutional review board of the University of Washington and were conducted according to Declaration of Helsinki principles.

Cells and viruses. EBV-transformed B cells (EBV-LCLs) were cultured as described (11). Chinese hamster ovary (CHO) and CHO-E cells, stably transfected with human E-selectin cDNA, were maintained as described (16). PBMCs were restimulated with peptide, IL-2, and IL-7 in T cell medium (11) exactly as described (11, 17),

or alternatively in T cell medium with 1.6 µg/ml phytohemagglutinin (PHA-P; Remel Inc., Lenexa, Kansas, USA) and 64 units/ml human natural IL-2 (Hemagen Diagnostics Inc., Columbia, Maryland, USA) beginning on day 3. To test tetramer B7-RPR, peptide-stimulated cells were stained 12 days after stimulation with tetramer and FITC-conjugated anti-CD8α clone MHCD0801 (Caltag Laboratories Inc., Burlingame, California, USA) and sorted using the FACSVantage system (Becton, Dickinson and Co., San Jose, California, USA). Cells were then rested overnight in T cell medium with 50 U/ml IL-2 (Chiron Corp., Emeryville, California, USA), cloned at 1 cell/well, expanded as described (11), and tested for cytotoxicity (see below). Skin-derived lymphocytes were expanded from biopsies in T cell medium with PHA-P and human natural IL-2 beginning on day 3 as described (10) for 10–14 days. HSV-2 strain 333 (18) and HSV-1 strain E115 (19) were grown and titrated in Vero cells (20).

HSV-2 CD8⁺ T cell epitopes. Methodology and definition of the HLA B7-restricted epitope VP22 amino acids (a.a.) 49–57 have been described (11). Identical methods (11) were used to define the epitope recognized by genital herpes lesion-derived CD8⁺ T cell clone 5491.2000.81. HLA restriction was assigned by transfection/infection of Cos-7 cells with HLA B7 cDNA/HSV-2, coculture with 5491.2000.81, and measurement of IFN-γ secretion by ELISA (11). To define HSV-2 antigens, a library of *Sau3a* I-digested HSV-2 strain HG52 (21) DNA was cotransfected with HLA B7 cDNA into our previously described Cos-7 expression-cloning system (11). This method uses IFN-γ secretion to detect T cell activation. Positive library pools were decoded to yield single antigenic HSV-2 fragments (11). The positive library “hit” encoded a.a. 306–825 of ICP0 (21). Epitope localization was done by C-terminal truncation analysis with nested PCR-generated fragments originating at a.a. 306. Transfection/IFN-γ readout was used to narrow the epitope to a.a. 708–778. Algorithms (22, 23) predicted HLA B7 binding by a.a. 743–751. Peptides were synthesized as described (24).

Lymphocyte assays. Four-hour, triplicate cytotoxicity assays used ⁵¹Cr release and an effector/target ratio of 20:1 (11) unless otherwise indicated. Target infection used a multiplicity of 10 for 18 hours; peptides were loaded for 90 minutes at 37°C. After infection or loading, targets were washed. Spontaneous release was less than 25%. To measure adhesion, CHO or CHO-E cells were plated at 2 × 10⁶ per 60-mm-diameter dish. The next day, PBMCs (2 × 10⁶ unstimulated or 1 × 10⁶ after stimulation with peptide for 8 days) were added. Dishes were rotated at 50 rpm for 1 hour at 37°C. Unbound cells and cells from a PBS wash were pooled to form the unbound fraction. Cells bound to the CHO or CHO-E cells were collected with chilled PBS, 4 mM EDTA, and vigorous pipetting. Microscopy confirmed the removal of lymphocytes. Fractions were washed and processed for flow cytometry. Results are expressed as the proportion of

CD8-high cells that stain with tetramer in the bound fraction divided by the proportion of similar cells in the unbound fraction.

Tetramers. Phycoerythrin-labeled (PE-labeled) tetramers from the National Institutes of Allergy and Infectious Diseases core facility (Emory University, Atlanta, Georgia, USA) were B7-RPR-PE (HLA B7 with HSV-2 VP22 a.a. 49–57), B7-APA-PE (HLA B7 with HSV-2 ICP0 a.a. 743–751), and A2-GLA-PE (HLA A2-GLA, with HSV-2 VP13/14 a.a. 551–559), which was previously described (11). Tetramer A2-NLV-PE (HLA A2 with CMV pp65 a.a. 595–603) was produced according to published methods (25). Briefly, HLA A2 heavy chain and β_2 -microglobulin were produced separately in *E. coli*. The heavy chain was truncated to contain the extracellular domain. A substrate sequence for BirA biotinylation was added at the C terminus. HLA

complexes were folded using 30 mg of heavy chain, 25 mg of β_2 -microglobulin, and 10 mg of peptide. Biotinylation used BirA at 5 mg/ml, 0.5 mM biotin, and 5 mM ATP for 16 hours at room temperature. Biotinylated complexes were purified by HPLC, and tetramers were assembled by mixing biotinylated complexes with streptavidin-phycoerythrin at a 4:1 molar ratio. Tetramers A2-CLG-PE (HLA A201 with EBV LMP2 a.a. 426–434 (26), and A2-VLE-PE (HLA with CMV IE-1 a.a. 316–324) (27), were produced similarly by ProImmune Ltd. (Oxford, United Kingdom). Tetramer A2-GLC-PE (HLA A201 with EBV BMLF-1 a.a. 280–288) has been described (26).

Flow cytometry. For detection of HSV- or EBV-specific T cells, 1×10^6 to 5×10^6 cryopreserved, thawed PBMCs, or approximately 2×10^5 cultured PBMCs, were stained with $1 \mu\text{g}$ phycoerythrin-labeled tetramer in $50 \mu\text{l}$ T cell

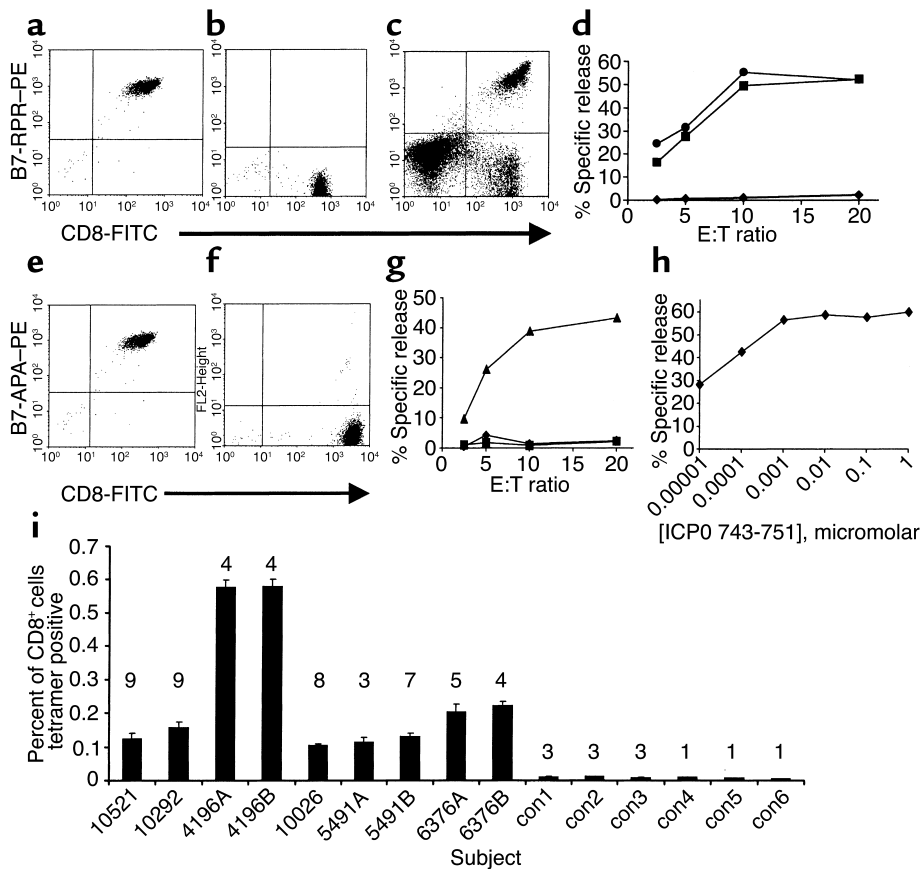


Figure 1

Specificity of tetramer staining and detection of HSV-specific cells in PBMCs. (a) Clone 5491.2000.48, specific for HSV-2 VP22 a.a. 49–57, stained with phycoerythrin-conjugated tetramer B7-RPR (B7-RPR-PE) and anti-CD8 α . (b) Similar analysis of control clone 5491.2000.48, specific for ICP0 a.a. 743–751. (c) Similar analysis of PBMCs from subject 7282 stimulated for 12 days with VP22 a.a. 49–57. (d) Cytotoxicity of a typical clone, 7282.12, derived after sorting the cells in c for high expression of CD8 α and tetramer binding. Targets were autologous EBV-LCLs either untreated (diamonds), infected with HSV-2 (squares), or pulsed with peptide VP22 a.a. 49–57 (circles). (e) Clone 5491.2000.81 stains with tetramer B7-APA-PE. (f) Control clone 5491.2000.48 does not stain with tetramer B7-APA-PE. (g) Clone 5491.2000.81 kills autologous EBV-LCLs infected with HSV-2 (triangles) but not HSV-1-infected (diamonds) or -uninfected EBV-LCLs (squares). (h) Lysis of autologous EBV-LCLs pulsed with peptide ICP0 a.a. 743–751 by clone 5941.2000.81. (i) CD8 α -high cells in whole PBMCs of HLA B7-expressing subjects analyzed for binding of tetramer B7-RPR-PE. Integers above bars show number of replicate aliquots of PBMCs stained. Bar heights are means, and error bars represent standard deviations. For some subjects, staining used PBMCs thawed on two separate days (A and B) but obtained at a single phlebotomy. Controls (con) 1–3 are HSV-2-infected but HLA B7-negative; controls 4–6 are HSV-2-uninfected and HLA B7-positive.

Table 1

Characteristics of the HSV-2-infected subjects

Subject	Duration of HSV-2 infection ^A	Recurrences/year ^B	Shedding rate ^C
10521	11	Not avail.	10.5
10292	0.5	Not avail.	Not avail.
4196	17	Not avail.	26.1
10026	29	0	Not avail.
5491	26	10	Not avail.
6376	Unknown ^D	0	0
10433	16	10	1.4
5101	17	12	31.7
7282	8	2.5	18.5

^AYears between the first clinical episode of a syndrome consistent with genital herpes and phlebotomy for this study, rounded off to nearest whole year except for subject 10292. ^BDerived from subject self-report about the number of episodes of genital ulceration in the 6 months prior to enrollment. ^CPercentage of days during which any anogenital anatomic site was positive for HSV-2 by culture during more than 50 consecutive days of sampling. ^DSubject is HSV-2-seropositive but has no history consistent with genital herpes.

medium at 20°C for 1 hour. Twenty microliters of anti-CD8 α -Cychrome or 20 μ l anti-CD8 α -PerCP and 20 μ l FITC-labeled anti-CLA monoclonal antibody HECA-452 or FITC-labeled anti-CD62L or FITC-labeled anti-CD28 (Pharmingen, San Diego, California, USA) were added for a 30-minute incubation at 4°C, followed by washes and fixation. For CCR7, tetramer was followed by 2 μ g anti-CCR7 clone 2H4 (Pharmingen), washes, and then FITC-labeled goat anti-mouse (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA). For CMV-specific T cells, 5 \times 10⁵ PBMCs were incubated with 10 μ g/ml tetramer in 20 μ l PBS with 20% FCS for 20 minutes at 37°C. Cells were washed, incubated at 4°C for 30 minutes with 20 μ l anti-CD8-PerCP (Becton, Dickinson and Co.) and anti-CLA-FITC, washed, and fixed. Cells were analyzed with a FACScan or FACSCalibur (Becton, Dickinson and Co.) and WinMDI 2.8 software (<http://facs.scripps.edu/software.html>). CD8⁺ T cells were lym-

phocytes (forward/side scatter) staining intensely with anti-CD8 α . Tetramer binding was expressed as the percentage of CD8 α -high cells with bright (usually greater than 100 fluorescence units) tetramer staining. CLA positivity was defined from the FL1/cell number histogram for all lymphocytes at the junction between negative cells and a "tail" of FL1-brighter events, typically at 10^{1.0} to 10^{1.1} fluorescence units. Two-color analyses used FITC-conjugated anti-CD8 α (Caltag Laboratories Inc.) after the tetramer. To document expression of E-selectin, CHO and CHO-E cells were stained with 10 μ g/ml anti-CD62E (Becton, Dickinson and Co.) or isotype control at 4°C for 30 minutes, washed, stained with 2 μ l phycoerythrin-labeled goat anti-mouse (Biomedex, Hayward, California, USA) for 30 minutes at 4°C, washed, and fixed.

Immunohistochemistry. Frozen 4- μ m sections were acetone-fixed, quenched in 4:1 methanol/hydrogen peroxide, and stained as described (28). Briefly, E-selectin was detected with anti-CD62E (see above) followed by isotype-specific peroxidase-conjugated secondary antibody and an ABC peroxidase kit with 3,3'-diaminobenzidine substrate (Vector Laboratories Inc., Burlingame, California, USA). CLA was visualized using biotin-conjugated monoclonal antibody HECA-452 at 1:200 (Pharmingen), followed by anti-biotin monoclonal antibody MB-9100 at 1:200 (Vector Laboratories Inc.), and detection as above. To control for nonspecific binding, staining was performed with isotype-matched primary antibodies specific for irrelevant antigens. Sections were counterstained with Mayer's hematoxylin.

Statistics. Expression of surface antigens was compared between tetramer-staining and -nonstaining CD8⁺ lymphocytes by the two-tailed Mann-Whitney test.

Results

Detection of HSV-2-specific CD8⁺ T cells in PBMCs. We hypothesized that HSV-2-specific CD8⁺ T cells in the blood would express a characteristic pattern of cell-surface molecules involved in cell trafficking. To examine

Table 2CLA expression by CMV- and EBV-specific CD8⁺ T cells in PBMCs

Subject	Virus	Tetramer	Tetramer ^A	CLA expression by CD8 ⁺ cells	
				Tetramer ⁺	Tetramer ⁻
1	CMV	A2-NVP	0.70	4.1	12.9
2	CMV	A2-NVP	6.6	5.1	6.0
3	CMV	A2-NVP	0.51	5.6	2.7
4	CMV	A2-NVP	0.33	2.9	3.4
5	CMV	A2-VLE	3.7	2.1	2.5
6	CMV	A2-VLE	0.47	17.5	11.1
7	CMV	A2-VLE	2.3	11.1	9.5
8	CMV	A2-VLE	0.98	10.4	7.8
9	EBV	A2-GLC	0.11	1.2	2.7
10	EBV	A2-GLC	0.15	4.4	1.6
11	EBV	A2-CLG	0.57	7.2	6.2
12	EBV	A2-CLG	0.74	10.5	15.8

^APercentage of CD8 α -high cells that stain with the indicated tetramer.

this, we first developed and validated tools to identify HSV-specific T cells. An HLA B7 tetramer, B7-RPR, was folded with peptide VP22 a.a. 49–57 from the HSV-2 UL49 open reading frame (21). This tetramer specifically stained the HLA B7-restricted T cell clone 5491.2000.48 (11), isolated from a cutaneous HSV-2 lesion (Figure 1, a and b). To confirm that this tetramer bound HSV-2-specific CTLs, PBMCs from an HSV-2-infected, B7 subject were stimulated with VP22 a.a. 49–57, sorted on the basis of tetramer binding and CD8⁺ expression, and cloned by limiting dilution. Resultant clones had specific cytotoxicity (Figure 1, c and d).

To obtain an additional marker of the HSV-2-specific CD8⁺ response, we determined the fine specificity

of CD8⁺ clone 5491.2000.81, also recovered from an HSV-2 skin lesion. The epitope was found to be a.a. 743–751 of the immediate early viral protein ICP0 (Figure 1, g and h). An HLA B7 tetramer, B7-APA, was constructed and specifically bound clone 5491.2000.81 (Figure 1, e and f).

We then examined the frequency of CD8⁺ T cells for these HSV-2 epitopes in PBMCs from HSV-2-seropositive, HLA B7-expressing adults with symptomatic genital herpes of 0.5–29 years' duration (Table 1). Six of 11 subjects had VP22 a.a. 49–57 specific CD8⁺ cells in their PBMCs. From 0.11% to 0.60% of CD8⁺ high lymphocytes stained with tetramer B7-RPR (Figure 1i). Control PBMCs from control HSV-2-infected,

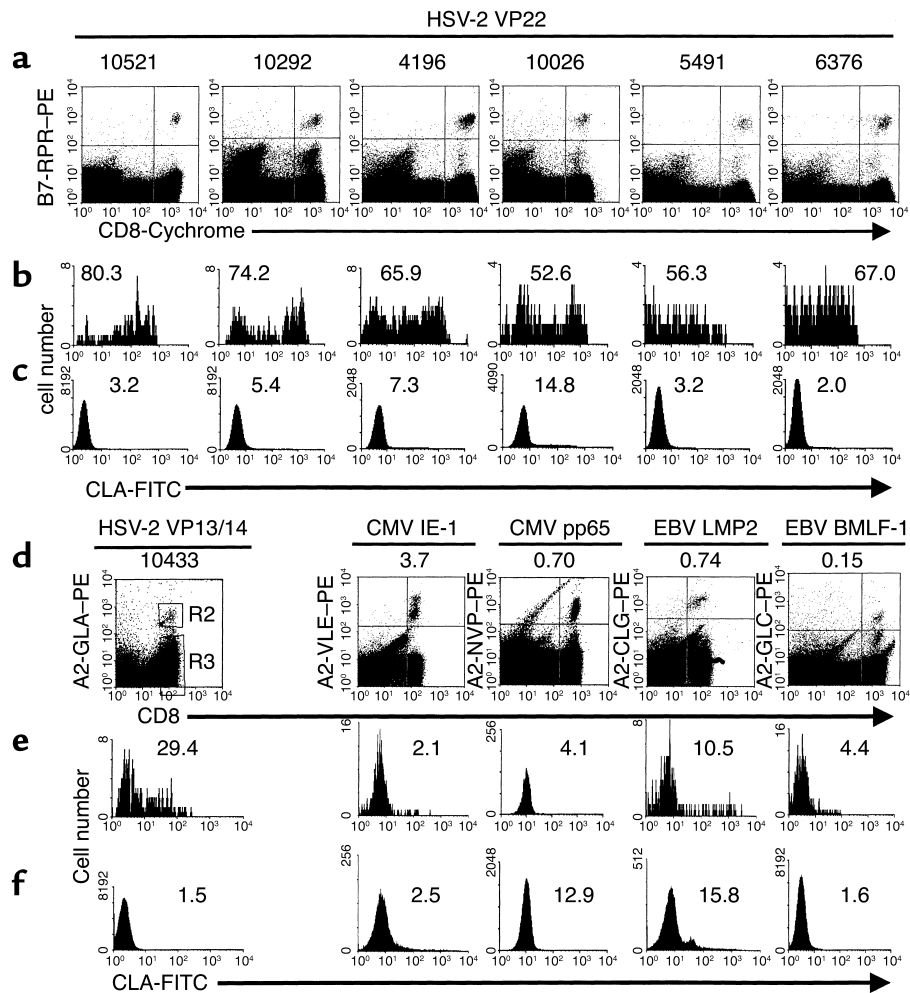


Figure 2

CLA expression by circulating CD8⁺ lymphocytes specific for human herpesviruses. Subject ID numbers (Table 1) are included for HSV-2 analyses. (a) Tetramer B7-RPR-PE and anti-CD8 α staining of lymphocytes in PBMCs from HSV-2-infected, B7 subjects. The percentages of CD8 α -high lymphocytes staining with the tetramers are given in Figure 1i. Quadrants show criteria for CD8 α -high cells and tetramer binding. (b) Expression of CLA by CD8 α -high lymphocytes staining with tetramer B7-RPR-PE. The percentage of CLA-positive cells is indicated. (c) Expression of CLA by CD8 α -high lymphocytes that do not stain with tetramer B7-RPR. (d) Staining of PBMCs from an HLA A2 subjects with anti-CD8 α and tetramers A2-GLA specific for HSV-2 (panel 1), A2-VLE-PE or A2-NVP-PE specific for CMV (panels 2 and 3), or A2-CLG-PE or A2-GLC-PE specific for EBV (panels 4 and 5). For subject 10433, the gates for tetramer-high and tetramer-low CD8⁺ cells are shown. The proportion of CD8 α -high cells staining with tetramer and the quadrant criteria for CD8 α -high cells and tetramer binding are shown. The CMV and EBV data correspond to lines 5, 1, 10, and 12 of Table 2. (e) Expression of CLA by CD8 α -high lymphocytes that stain with herpesvirus tetramers. (f) Expression of CLA by CD8 α -high lymphocytes not staining with the indicated tetramers. Percentages of CLA-positive cells are indicated.

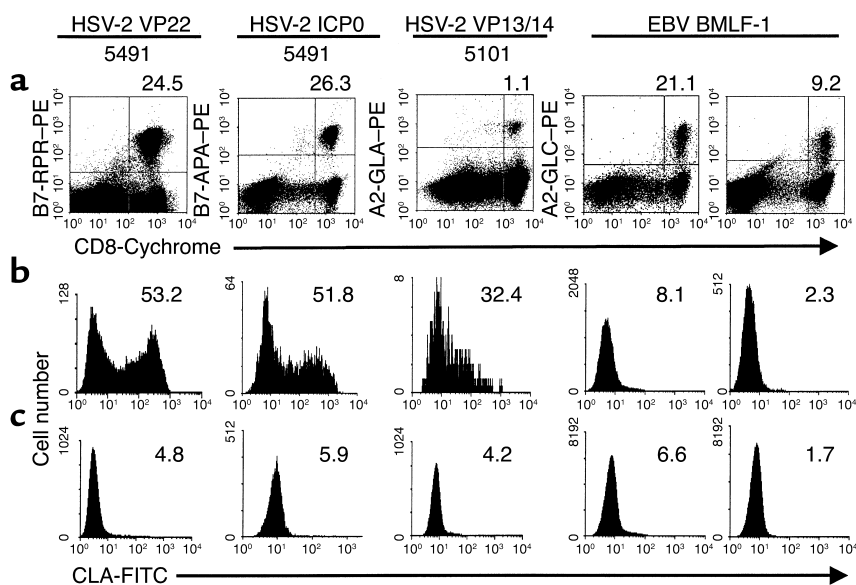


Figure 3
 Expression of CLA by in vitro restimulated herpesvirus-specific CD8⁺ T cells. For HSV-2-specific T cells, unique subject ID numbers are indicated below the HSV-2 antigens. (a) PBMCs from an HLA B7-bearing, HSV-2-infected (panel 1) or two different HLA A2-bearing subjects (panels 2 and 3) stimulated for 13 days with HSV-2 VP22 a.a. 49–57, HSV-2 ICP0 a.a. 743–751, or HSV-2 VP 13/14 a.a. 551–559, respectively. Subjects are described in Table 1. PBMC from two HLA A2-bearing subjects were stimulated with EBV BMLF-1 a.a. 280–288 (panels 4 and 5). Dot plots display binding of the relevant tetramers and anti-CD8 α . Quadrant lines are cutoffs for CD8 α -high cells and tetramer binding. The percentages of CD8 α -high cells that are tetramer-positive are indicated. (b) Expression of CLA by CD8 α -high lymphocytes that stain with HSV-2 or EBV tetramers. (c) Expression of CLA by CD8 α -high lymphocytes that do not stain with tetramer. Percentages of CLA-positive cells are indicated.

HLA B7-negative persons and HSV-uninfected, HLA B7-positive persons had less than 0.01% tetramer-positive CD8⁺ cells.

CLA expression by circulating virus-specific memory CD8⁺ cells. It is not known whether cells destined to traffic to the skin express CLA in the circulation prior to extravasation. It has previously been hypothesized that dermal T cells could acquire CLA after entering the skin (29). We compared the expression of CLA by circulating cells specific for the skin-tropic virus HSV-2 and the non-skin-tropic viruses EBV and CMV.

CLA was expressed by 52.6–80.3% of circulating CD8 α -high cells that stained with tetramer B7-RPR

(66.0 \pm 10.4, mean \pm SD) (Figure 2, a–c). Only 2.0–14.8% of tetramer-negative CD8⁺ cells from these same subjects expressed CLA (6.0 \pm 4.3, mean \pm SD). CLA was expressed by 29.4% of circulating CD8 α -high cells specific for an epitope in HSV-2 protein VP13/14, using tetramer A2-GLA-PE (11), compared with 1.5% of tetramer-negative CD8⁺ cells (Figure 2, d–f). Overall, for HSV-2, expression of CLA by tetramer-positive CD8⁺ cells was higher than expression of CLA by tetramer-negative CD8⁺ cells ($P = 0.006$). In this small study, we did not observe any association between the proportion of VP22-specific CD8⁺ T cells that expressed CLA (Figure 2, d–f) and the severity of HSV-2 infection (Table 1).

We examined CLA expression by HLA A2-restricted CD8⁺ cells specific for either CMV or EBV. For each virus, we studied two independent epitopes. Expression of CLA by EBV- and CMV-specific CD8⁺ cells was low and generally similar to that of tetramer-negative CD8⁺ cells

(examples in Figure 2). A similar pattern was noted for each subject and each epitope (Table 2 summarizes the entire data set). For CMV, the mean \pm SD values for the expression of CLA by virus-specific and bystander CD8⁺ cells were 7.5% \pm 5.1% and 7.7% \pm 3.6%, respectively. For EBV, CLA was expressed by 5.2% \pm 3.1% of EBV-specific cells and 5.4% \pm 4.2% of other CD8⁺ cells, respectively. For CMV and EBV analyses combined, there was no difference in CLA expression between tetramer-positive and tetramer-negative CD8⁺ cells ($P > 0.99$).

Proliferative capacity and phenotype of circulating HSV-2-specific CD8⁺ cells. The above data indicate that HSV-2-specific memory CD8⁺ T cells express the skin-associated

Table 3
 Phenotype of CD8 α -high cells in PBMCs analyzed by binding of tetramer B7-RPR, which identifies cells specific for HSV-2 VP22 a.a. 49–57

Subject	CD28		CD62L		CCR7	
	B7-RPR ⁺	B7-RPR ⁻	B7-RPR ⁺	B7-RPR ⁻	B7-RPR ⁺	B7-RPR ⁻
10521	92.6	55.2	82.6	49.7	75.9	31.2
4196	84.6	43	74.2	28.5	46.3	22.9
10026	95	80.4	35.5	49.7	88.7	64
5491	95.7	30.6	79.5	44.6	53.4	32.7
6376	98.6	46.7	53	47	50.1	43.2
10292	80.9	76.4	ND	ND	ND	ND
Mean \pm SD	91.2 \pm 6.9	55.4 \pm 19.5	65.0 \pm 20.1	43.9 \pm 8.9	62.9 \pm 18.5	38.8 \pm 15.8

ND, not done.

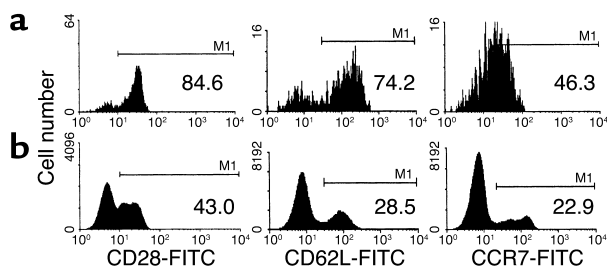


Figure 4
Expression of cell-surface antigens by circulating HSV-2-specific CD8⁺ T cells. (a) PBMCs from donor 4196 were gated for lymphocyte size and scatter, high CD8 α expression, and binding of tetramer B7-RPR, which is specific for VP22 a.a. 49–57. Expression of CD28, CD62L, and CCR7 is displayed in the indicated histograms. (b) Similar data for CD8 α -high lymphocytes, which did not bind tetramer B7-RPR, from the same donor.

adhesion molecule CLA while still in the circulation. We next investigated whether these cells had the property of self-renewal in response to antigen. Circulating cells specific for VP22 a.a. 49–57, VP13/14 a.a. 551–559, or ICP0 a.a. 743–751 were able to expand briskly in vitro in response to one restimulation with specific HSV-2 peptide (Figure 3a). The proportions of VP22- and VP13/14-specific cells that expressed CLA were similar before and after their peptide-driven expansion (Figure 2, b and e, and Figure 3b). We again noted that the proportion of VP13/14-specific CD8⁺ T cells expressing CLA was somewhat lower than the proportion of VP22-specific T cells. This comparison could not be made for ICP0, as the cells were not abundant enough to identify in unmanipulated PBMCs. The same peptide restimulation protocol did not induce CLA expression by EBV-specific cells. These results are consistent with a model in which lineages of CLA-expressing and CLA-negative HSV-2-specific cells can proliferate in vitro, although shifts in phenotype during the expansion of initially CLA-expressing and CLA-negative cells, or shorter-term fluctuations during progression through the cell cycle, cannot be ruled out.

It has been reported that circulating CD8⁺ cells can be divided into central memory cells expressing CD62L and CCR7 that can traffic to lymph nodes, and effector memory cells lacking CD62L and CCR7

but expressing cytolytic molecules. Effector memory cells may have reduced replicative potential (30–33). Circulating VP22-specific cells were greater than 50% CD62L⁺ in four of the five subjects studied (Table 3 and Figure 4). CCR7 expression varied from 46% to 89%. VP22-specific cells were also greater than 80% CD28⁺ from each donor, correlating with their ability to expand in vitro (Table 3). Each of these markers was more highly expressed by VP22-specific cells than by CD8 α -high lymphocytes with other specificities (Table 3). Comparison between tetramer-staining and nontetramer-staining CD8⁺ groups reached statistical significance ($P = 0.009$) for CD28 expression, but not for CCR7 or CD62L for these small groups. These results are consistent with most HSV-2 VP22 a.a. 49–57-specific CD8⁺ T cells having the central memory phenotype. The concept of central memory can be extended to include CD8⁺ T cells that have acquired the ability to selectively home to sites of antigenic challenge.

CLA and CLA ligand expression by T cells infiltrating genital HSV-2 lesions. To explore the possible role of CLA-associated E-selectin ligand in the migration of HSV-specific CD8⁺ T cells to herpetic lesions, we obtained skin biopsy tissue from an HLA B7-expressing person. Because too few cells were available from skin biopsies for direct analysis, we expanded skin-infiltrating cells with PHA-P and IL-2, treatment that provides a fairly uniform replication stimulus to most T cells and NK cells. HSV-2-specific T cells were locally enriched among cells expanded from an HSV-2 culture-positive lesion obtained on the third day of symptoms compared with cells expanded from normal skin and cells in unmanipulated PBMCs. The percentage of CD8 α -high cells from an HSV-2 biopsy specific for VP22 a.a. 49–57 (Figure 5g) was 6.4%, compared with 0.1% for normal skin and 0.21% from blood obtained the day of biopsy (not shown), representing a 60-fold local enrichment at the site of infection. The percentage of lesion-infiltrating CD8 α -high cells specific for HSV-2 ICP0 a.a. 743–751 was 2.3%, while the level in normal skin was 0.06% and the level in blood was below the limit of detection (not shown). Circulating cells with this specificity were detectable after peptide restimulation (Fig-

Table 4
Binding of virus-specific CD8⁺ lymphocytes to E-selectin

Subject	Stimulation	Virus	Tetramer	Input ^A	CHO-E enrichment ^B	CHO enrichment ^B
5491	None ^C	HSV-2	B7-RPR	0.14	10.1	1.1
4196	None ^C	HSV-2	B7-RPR	0.26	5.6	0.7
6376	None ^C	HSV-2	B7-RPR	0.19	13.2	0.8
5491	VP22 a.a. 49–57	HSV-2	B7-RPR	23.6	9.5	1.1
5491	ICP0 a.a. 743–751	HSV-2	B7-APA	12.4	8.3	1.3

Cells were analyzed by flow cytometry before or after 1 hour of incubation with either CHO cells expressing E-selectin or control CHO cells. ^APercentage of CD8 α -high cells that stain with tetramer in PBMC. ^BThe percentages of CD8 α -high cells that stain with the indicated tetramers were measured separately in the fractions of cells that either bound to, or did not bind to, CHO-E or CHO cell monolayers. Each number listed is the quotient of the tetramer-positive percentage in the bound fraction divided by the tetramer-positive percentage in the unbound fraction. ^CUnmanipulated PBMCs were thawed, washed, and used for binding experiments. Phlebotomy for subject 4196 was performed on a different date from specimens used for Figure 1 and Figure 2.

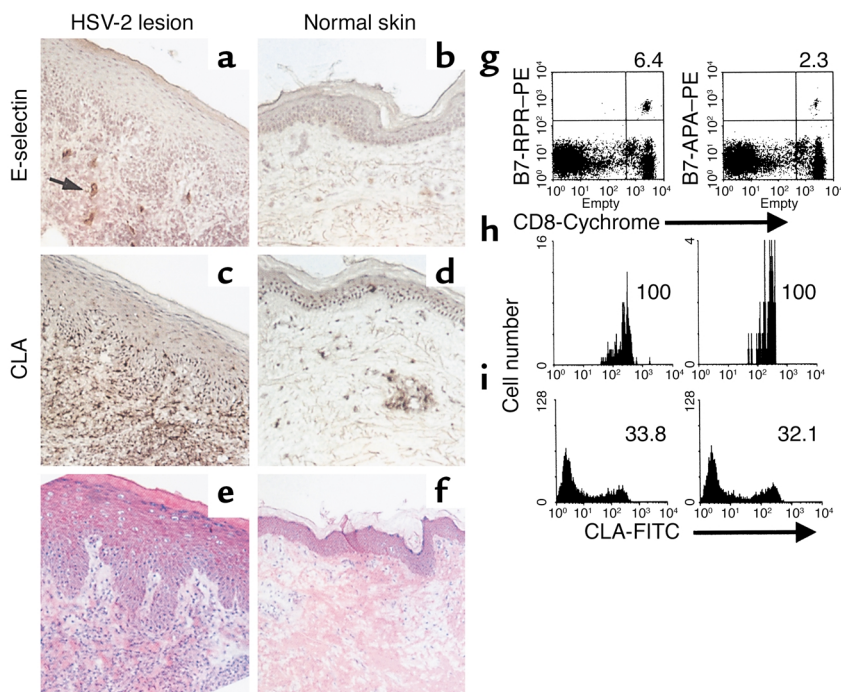


Figure 5 CLA and CLA-ligand expression in skin and by lesion-derived cells. (a) Frozen section of HSV-2 lesion from subject 5491 stained with anti-E-selectin and hematoxylin. Epidermis oriented to the top and original magnification $\times 200$ for all panels. (b) Normal skin from subject 5491 stained with anti-E-selectin. (c) HSV-2 lesion stained with anti-CLA. (d) Normal skin stained with anti-CLA. (e) HSV-2 lesion stained with hematoxylin and eosin. (f) Normal skin stained with hematoxylin and eosin. (g) Lymphocytes expanded for 11 days from the biopsy stained with HSV-2-specific tetramers B7-RPR-PE (left) or B7-APA-PE (right) and anti-CD8 α . Quadrant lines are cutoffs for CD8 α -high cells and tetramer binding. The percentages of CD8 α -high cells that are tetramer-positive are indicated. (h) Expression of CLA by CD8 α -high, tetramer-binding cells for tetramer B7-RPR-PE (left) or B7-APA-PE (right). (i) Expression of CLA by CD8 α -high, tetramer nonbinding cells. Percentages of CLA-positive cells are indicated.

ure 3a). Similar results for both T cell specificities were obtained from a biopsy of a recurrent HSV-2 lesion obtained 2 months after the first; again, both local enrichment and almost universal (>90%) CLA expression was noted. To rule out nonspecific induction of CLA expression during replication of skin-derived T cells in vitro, PBMCs from four donors were expanded for 11 days with the culture conditions used for skin biopsies. CLA was expressed by $4.5\% \pm 2.3\%$ of CD8 α -high cells, similar to fresh PBMCs.

We compared CLA expression by HSV-2-specific T cells derived from different sites. HSV-2-specific cells in PBMCs displayed a broad distribution of CLA expression, including some CLA⁻ cells (Figure 2, b and e). HSV-specific cells from the herpetic lesion were uniformly CLA⁺ (Figure 5h). The tetramer-negative CD8⁺ lymphocytes from these cultures also displayed a higher level (~30% total expression) of CLA expression than did similar cells from PBMCs (Figure 2, c and f).

Immunohistologic examination of an HSV-2 culture-positive buttock lesion from subject 5491, obtained on day 3 of symptoms of recurrent genital herpes, showed that about 30% of small dermal

mononuclear cells stained with anti-CLA antibody. E-selectin was strongly expressed in a dermal venular pattern (Figure 5). In normal skin, E-selectin staining showed a less intense venular pattern, while CLA⁺ cells were rarely observed. The presence of CLA and E-selectin in HSV-2-infected skin suggests that a CLA-associated E-selectin ligand and E-selectin may participate in leukocyte trafficking to recurrent HSV-2 lesions.

Binding of CLA-expressing HSV-specific cells to E-selectin. We determined whether CLA expression by circulating HSV-2-specific CD8⁺ T cells was associated with functional binding to E-selectin. PBMCs from three HSV-2-infected, HLA B7 subjects were incubated with E-selectin-expressing CHO-E cells, which uniformly expressed E-selectin, or control CHO cells, which lacked expression (not shown). Measurement of the proportion of CD8 α -high cells that were tetramer B7-RPR⁺ in the bound and unbound fractions indicated that T cells specific for HSV-2 VP22 a.a. 49–57 were enriched about tenfold by adherence to E-selectin (Table 4). HSV-specific CD8⁺ T cell lines generated in vitro by restimulation with the HSV-2 peptide (Figure 4) were

also tested. Again, HSV-2-specific cells detected with fluorescent HLA tetramers were selectively bound by CHO-E cells but not by control CHO cells.

Discussion

This is the first description of the selective expression of a putative tissue-specific homing molecule by circulating microbe-specific CD8⁺ T cells. The cell-surface expression and functional data in this report are consistent with a role for a CLA-associated E-selectin ligand in the trafficking of circulating HSV-2-specific memory CD8⁺ T cells to skin during recurrent genital herpes. Because many patients with genital herpes have lesions on keratinized epithelial surfaces of the external genitalia, perineum, back, or legs (34), CLA expression by HSV-2-specific T cells is anatomically appropriate.

In common with HSV-2, EBV and CMV undergo intermittent reactivations and are episodically shed in infectious form by most immunocompetent, infected individuals (35, 36). In contrast to HSV-2, neither primary nor recurrent infection with EBV or CMV is associated with cutaneous infection. The most common

site of EBV shedding is the oropharynx, while the most common sites of CMV shedding are the uterine cervix, the urinary tract, and the oropharynx. Reactivations of EBV and CMV in immunocompetent persons are usually asymptomatic (35, 36). We did not study the presence or absence of EBV or CMV reactivation at mucosal or skin sites in our subjects. Reactivations of EBV and CMV are intermittent, brief, and anatomically unpredictable, complicating the assessment of the possible influence of reactivation status on homing receptor expression at the time of phlebotomy.

We found that expression by CD8⁺ T cells specific for EBV and CMV was similar to the background level of 5–10% (37) observed for circulating CD8⁺ lymphocytes. The possibility that expression levels of CLA might change during reactivations could be addressed by combining intensive virologic monitoring with flow cytometry. Study of additional subjects and epitopes, and T cells specific for a variety of skin-tropic and non-skin-tropic pathogens, will help to determine to what extent CLA expression is tightly associated with infection in the skin. Given the anatomic loci of EBV and CMV infection, measurement of the expression of CD103 ($\alpha_E\beta_7$ integrin), a putative homing molecule for mucosal epithelium (4), by T cells specific for these viruses would also be of interest.

HSV-specific CD8⁺ T cells are functionally important in containing HSV-2 infection. Levels of CD8⁺ CTLs correlate inversely with the severity of HSV-2 in men coinfecting with HIV and HSV-2 (38), and correlate temporally with the local clearance of HSV-2 in lesions (8, 10). CD8⁺ CTLs are also important in the control of ganglionic infection, maintenance of neuronal latency, and in protection against infectious challenge in murine models (39–42). HSV evades CD8⁺ T cells by inhibiting the transporter associated with antigen processing (TAP) and degrading host mRNA (43). The tetramer-based measurements in this report reveal higher levels of circulating HSV-2-specific CD8⁺ T cells than were previously observed with limiting dilution CTL assays (38). In particular, high levels of VP22-specific CD8⁺ T cells were detected. VP22 may be recognized efficiently due to its delivery into the class I antigen processing pathway before TAP inhibition can occur. Our previous data show that virion input VP22 can be processed and that endogenous synthesis is not required (11). VP22 also has efficient intercellular spread and has CTL adjuvant activity (44, 45) although it is not known if this is related to its antigenicity.

In the present report, we studied HSV-2-specific T cells before and after trafficking from the circulation to HSV-2-infected skin, extending previous research concerning the role of CLA in pathogenesis of autoimmune and atopic disorders. MelanA-specific CD8⁺ T cells in PBMCs from subjects with vitiligo express higher levels of CLA than do similar cells from normal subjects (46). In atopic subjects, proliferative responses to allergy-associated antigens are enriched among CLA⁺ CD4⁺ T cells (47). Few reports have examined homing

receptor expression by circulating virus-specific T cells. Circulating memory rotavirus-specific CD4⁺ cells preferentially express the adhesion molecule $\alpha_4\beta_7$ integrin (48). Our data indicate that memory CD8⁺ T cells specific for the skin-tropic herpesvirus HSV-2 express CLA prior to leaving the circulation.

E-selectin is expressed at low basal levels in noninflamed skin, and is increased in diverse inflammatory skin conditions. We observed apparent upregulation of E-selectin in HSV-2-infected tissue. This is not surprising, since IFN- γ , IL-1 β , and TNF- α , which are upregulated in HSV lesions (49–51), cooperate to increase E-selectin expression by endothelial cells (52). Additional work is required to document the magnitude and time course of upregulation. Lymphocytes infiltrating the dermis commonly express CLA (35, 53–55). The influx of HSV-2-specific CD4⁺ cells and NK cells into recurrent HSV-2 lesions precedes the inflow of HSV-2-specific CD8⁺ T cells (10), and additional work is required to define the molecules involved in the trafficking of early responder cells. The proportion of HSV-2-specific CD8⁺ T cells that express CLA appears to be higher in the skin than in the blood (Figure 5). The approximately 50–80% of circulating HSV-2-specific cells that express CLA (Figure 2) may preferentially migrate to skin, or the local microenvironment may further promote CLA expression.

Our finding that circulating HSV-2-specific memory cells express CLA implies that expression of this antigen is upregulated during the priming of naive HSV-2-specific CD8⁺ T cells or at a subsequent stage of conditioning. The control of CLA expression is incompletely understood. Expression of $\alpha(1,3)$ -fucosyltransferase VII is a probable key regulator of CLA expression, although control over other glycosyltransferases (56) and the primary polypeptide backbone, P-selectin glycoprotein ligand-1 (2), may also be important. In vivo, CLA is expressed by cells coexpressing CD45RA and CD45RO in skin-draining lymph nodes, consistent with upregulation during the priming of naive T cells (57). In a murine model, molecules associated with skin-homing are upregulated during cutaneous priming (58). It is rational to hypothesize that inflammatory cytokines and local antigen-presenting cells could influence CLA expression during priming.

In this small cross-sectional study, the proportion of HSV-2 VP22-specific CD8⁺ T cells that expressed CLA was relatively constant in our set of six subjects (Figure 2). We recently studied two more HLA B7-bearing subjects (not included in Table 1) who are HSV-2 seropositive but have no clinical history of genital herpes. Staining of unmanipulated PBMCs with tetramer B7-RPR showed that 54.5% and 62.1% of tetramer-high, CD8 α -high cells expressed CLA, similar to the six subjects shown in Figure 2. We do not know whether CLA expression is influenced by recurrences of HSV-2 infection. While the number of subjects that we studied with frequently recurrent versus asymptomatic HSV-2 infection were too small for statistical comparisons, we

observed no obvious segregation of CLA expression by HSV-2-specific CD8⁺ T cells by the clinical or virologic (shedding) severity of HSV-2 infection (Table 1 and Figure 2). Similarly, we do not yet know whether CLA expression by HSV-2-specific cells in the periphery fluctuates temporally in association with symptomatic or asymptomatic recurrences of HSV-2.

Epitope-specific heterogeneity in CLA expression by HSV-2-specific CD8⁺ T cells cannot be excluded with the available data. CLA expression by VP13/14-specific cells was somewhat lower than for VP22-specific cells, both before and after peptide restimulation, but was still clearly above background levels for tetramer-negative cells. In sorting experiments using unmanipulated PBMCs, based on CLA and CD8 expression, we have successfully enriched HSV-2-specific CTLs from several subjects at the bulk and clonal levels. Expression cloning to determine fine specificity, using the methods described in this report, has yielded five additional novel epitopes recognized by HLA class I-restricted CD8⁺ CTLs (Koelle et al., unpublished observations). CLA does appear to be expressed above background levels by CD8⁺ CTLs specific for a diverse set of HSV-2 epitopes.

TGF- β and IL-12 (57, 59, 60) upregulate CLA expression in vitro, while IL-4 may downregulate CLA (56). Each of these cytokines is upregulated at the protein and/or mRNA levels in HSV-associated lesions (50, 61). Secretion of TGF- β and IL-12 in response to HSV infection has been demonstrated in vitro (51, 62–64). Draining lymph nodes in animals display expression of IL-12, TGF- β , IL-4, and other cytokines (65–67). While a cytokine milieu appropriate for CLA induction is associated with HSV infection, the key factor or factors controlling CLA expression by CD8⁺ T cells responsive to HSV-2 remain to be determined.

Memory cells that acquire tissue-specific homing molecules may be specialized for extravasation to sites of antigen challenge. They may also retain a capacity for self-renewal and recirculation through lymphoid tissue. We found that most circulating VP22-specific CD8⁺ T cells expressed CD28 (Figure 4), an important molecule for the delivery of costimulatory signals to memory T cells. HSV-2-reactive T cells specific for each of three different viral epitopes were able to proliferate in vitro in response to peptide, sometimes to very high levels (Figure 3). We also found that the majority, but not all, circulating VP22-specific CD8⁺ T cells expressed CD62L and CCR7, adhesion and chemokine receptor molecules, respectively, associated with recirculation to lymphoid tissue and a substantial capacity for self-renewal (68). As CD62L ligands are expressed in skin (69) as well as lymph nodes, it is possible that CD62L on HSV-specific T cells may participate in homing to this site. There are conflicting data on the expression of CCR7 by CLA⁺ cells in the circulation, with published findings concentrating on CD4⁺ cells (33, 70). Our findings for CCR7 are similar to those noted for

CD8⁺ T cells specific for some EBV epitopes, but contrast with findings for CMV- and HIV-specific CD8⁺ T cells (71–73). Taken together, our data indicate that virus-specific CD8⁺ T cells with the central memory phenotype may also be capable of trafficking to sites of antigenic challenge.

Rolling adhesion to the vessel wall is only the initial stage of lymphocyte trafficking into tissue. Both integrin maturation and chemotaxis have been associated with chemokines and their receptors. Several candidate chemokine receptors have been implicated in homing to skin, including CCR4 and CCR10 (74–76). Most of the published data have concerned CD4⁺ T cells. Future flow cytometric and functional studies will examine the expression of chemokine receptors by HSV-2-specific CD8⁺ T cells.

In summary, subjects with recurrent, symptomatic HSV-2 infection have readily detectable circulating VP22-specific CD8⁺ T cells that express CLA. CLA is tightly associated with functional E-selectin binding activity, which is an anatomically appropriate property for HSV-2-specific T cells. HSV-specific CD8⁺ T cells in PBMCs expressed functional E-selectin binding activity. Neither CLA expression nor E-selectin binding was noted among the responses to CMV or EBV, two non-skin-tropic herpesviruses. We propose that vaccines and immunotherapies for HSV (77–79) should not only elicit specific T cells but also guide these T cells to express appropriate homing molecules. More broadly, preventative and therapeutic T cell-based treatments may be optimized if the identity, mechanisms of action, and control mechanisms for homing molecules can be understood and manipulated.

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