

Diversity in CD8⁺ T Cell Function and Epitope Breadth Among Persons with Genital Herpes

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Abstract CD8⁺ T cells are known to be important in clearing herpes simplex virus (HSV) infections. However, investigating the specific antiviral mechanisms employed by HSV-2-specific T cell populations is limited by a lack of reagents such as CD8⁺ T cell epitopes and specific tetramers. Using a combination of intracellular cytokine staining flow cytometry and ELISpot methods, we functionally characterized peripheral HSV-2-specific CD8⁺ T cells from peripheral blood mononuclear cell (PBMC) that recognize 14 selected HSV-2 open-reading frames (ORFs) from 55 HSV-2 seropositive persons; within these ORFs, we subsequently identified more than 20 unique CD8⁺ T cell epitopes. CD8⁺ T cells to HSV-2 exhibited significant heterogeneity in their functional

characteristics, proliferation, production of inflammatory cytokines, and potential to degranulate *ex vivo*. The diversity in T cell response in these *ex vivo* assessments offers the potential of defining immune correlates of HSV-2 reactivation in humans.

Keywords HSV-2 · T cells · antigens/peptides/epitopes · virus

Abbreviations

ICS Intracellular cytokine staining
ORF Open-reading frame
CEF CMV, EBV and flu
MFI Median fluorescence intensity

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Introduction

Genital herpes is a pandemic sexually transmitted infection that causes substantial morbidity and increases the risk of HIV acquisition two- to threefold [1, 2]. While antiviral therapy is available, treating infection has no effect on the epidemiology of the disease. As such, development of novel therapeutic approaches is needed. At most, there is limited information regarding the effector mechanisms of the adaptive immune system that control herpes simplex virus-2 (HSV-2) and the functional epitopes encoded by the virus that may be recognized by lymphocytes. Increased knowledge of adaptive immunity against HSV-2 is thus essential to the development of preventative and therapeutic vaccines for HSV-2.

Several lines of evidence suggest that T lymphocytes are essential to reducing the magnitude and duration of active herpetic disease and in controlling viral latency [3, 4]. In particular, cytotoxic (CD8⁺) T cells infiltrate and clear HSV-2 from genital herpes lesions and remain concentrated around the sensory neurons at the skin after the lesion has healed [5]. These cells also accumulate around the sensory neurons during subclinical reactivation [5]. Both cytolytic activity and interferon gamma (IFN- γ) secretion by T cells are required in rodents for complete clearance of HSV-2 from genital epithelium [6]. However, the mechanisms of lymphocyte-based immune control of HSV-2 reactivation and clearance in humans are largely unknown.

Few surveys of adaptive host responses of persons with HSV-2 have been performed. However, the diversity of T cell responses to HSV-2 proteins or epitopes has been determined; the numbers of CD8⁺ T cells that produce IFN- γ or exhibit cytolytic activity when presented with an HSV-2-derived antigen have been estimated in HSV-2 seropositive individuals. Recent studies have identified several proteins encoded by HSV-2 that activate CD8⁺ T cells in a high proportion of HSV-2 seropositive subjects [7–9]. Due to the low level of circulating HSV-2-specific CD8⁺ T cells, previous approaches used for epitope identification have required enrichment of these T cells, either by positive selection or clonal expansion of CD8⁺ cells from peripheral blood mononuclear cell (PBMC). Since the enrichment steps may alter the behavior of the cell, their inclusion in functional assays is not ideal when investigating the immune function of HSV-2-specific T cells. Recently, new assays have been developed that allow the detection of low numbers of activated T cells within whole PBMC populations without enrichment. For example, an eight-color intracellular cytokine staining (ICS) assay, developed as a quantitative and qualitative high-throughput approach to evaluate T cell activity against HIV-1 [10], can detect and quantify co-expression of different cytokines in distinct cell populations, even when the cell of interest is infrequent.

The current study expands on our initial survey [7] to identify immunocompetent responses against HSV-2 in humans and reports specific CD8⁺ T cell responses to HSV-2-derived peptides in HSV-2 seropositive individuals using a modification of this validated ICS method. Detection of CD8⁺ T cell responses directly from PBMC was obtained without prior enrichment, and the polyfunctionality of these responses was determined. We observed monofunctional and polyfunctional cells in populations of HSV-2-specific CD8⁺ T cells that differed proportionately within individuals responding to multiple epitopes and also between different individuals. HSV-2-specific CD8⁺ T cells proliferate and mobilize CD107a and express lytic proteins such as perforin and granzyme. However, intra- and inter-individual variation was observed for these activities. Exploring this diversity in functional activity of CD8⁺ T cells in relation to clinical variability of mucosal HSV-2 infection between individuals is likely to provide novel insights on the immune control of HSV-2. Studies interrogating the epitope-specific T cell responses as they relate to disease expression may offer new insights into host–viral interaction and their interplay in containment of the virus.

Methods

Selection of Viral Open-Reading Frames and Peptides

Fourteen open-reading frames (ORFs) from the HSV-2 genome, which were previously found to induce IFN- γ production from CD8⁺ lymphocytes in a high frequency of HSV-2 seropositive subjects [7], were targeted during this study to assess T cell function. These are listed in Table I with their size and ORF class. The peptides were designed against the HG52 strain of HSV-2 (Genbank accession number: Z86099) with modifications to incorporate common local sequence variations, which are also listed (Table I). The proteins encoded by each ORF were synthesized as peptide arrays (Tier two; New England Peptide, Gardner, MA) of 15-mer peptides of ~70% purity that overlapped by 11 amino acids. Sequences from low-passage clinical isolates were obtained for UL46 and UL47 as previously reported [11] and similarly for ICP0 and UL25 (primer sequences available on request) and deposited in GenBank (accession numbers: EU029137–EU029151 or pending). The peptides were reconstituted in DMSO to give a concentrated stock of 20 mg/mL, and combined in pools containing up to 99 peptides at 200 μ g/mL. The number of pools for the 14 ORFs was thus 29 in total. These pools were stored at –80°C until used. Singular peptides identified as CD8⁺ T cell-inducing peptides during this investigation were synthesized with at least 90% purity (New England Peptide) and reconstituted in DMSO at 20 mg/mL and stored as described above.

Table I Open-Reading Frames of HSV-2 Used During This Study

ORF	Expression kinetics	Number of amino acids	Modifications from HG52	Pool	Number of peptides in pool
UL19	Leaky-late	1,374		1	85
				2	85
				3	85
				4	86
UL25	Leaky-late	585	P540L, T555A	5	72
				6	72
UL35	Late	112		7	26
UL46	Leaky-late	721	T364R, C471F, E594K, G613S, P634L, R637Q, L638D, L644P, R672P, R673G, 587-590 delA	8	89
				9	89
UL47	Late	696	V38G, N156S, P172A, S177P	10	86
				11	86
UL49	Early	300		12	74
UL11	Early	96		13	22
UL27	Late	904		14	74
				15	87
				16	63
				17	68
ICP0	Immediate-early	826	A204S, V385F, D560G, 587-589 insA	18	68
				19	68
				20	63
ICP27	Immediate-early	512		21	63
				22	99
UL29	Early	1,196		23	99
				24	99
				25	94
UL39	Early	1,142		26	94
				27	95
				28	21
US5	Late	92		29	96
US6	Late	393			

Positive control pools containing CEF and CMV peptides were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEF control peptide pool (Cat # 9808) from DAIDS, NIAID; CMV pp65 peptide mix (Cat # 551969) from BD Biosciences. These were reconstituted, stored, and used according to the manufacturer's recommendations.

Research Subjects

Patients were recruited into a study approved by the University of Washington (UW) and Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA. All participants provided written informed consent. Participants were healthy, HIV seronegative adults. Fifty-five HSV-2 seropositive participants, with a history of genital HSV-2 infection for more than 1 year, were enrolled between

November 2006 and November 2007. A control group, comprising 18 HSV-1 and HSV-2 seronegative persons, was also enrolled to establish baseline responses for each antigen. HSV-2 serostatus was established by the UW Western blot [12]. All of the subjects were recruited specifically for the study and were not part of our previously published cohort [7]. The demographic and clinical characteristics for the study groups are shown in Table II. Low-resolution HLA typing was performed for all seropositive subjects at the Puget Sound Blood Center, Seattle, WA.

Collection, Preservation, and Thawing of PBMC

Blood (200 mL) was collected from all study subjects using heparinized syringes. PBMC isolation was performed less than 8 h from the time of blood draw. Whole blood was

diluted 2:1 with Hank's balanced salt solution (HBSS; Invitrogen-Gibco, USA) and loaded onto 50 mL Accuspin tubes (Sigma, USA) containing 15 mL Ficoll 1.077 (Sigma). PBMC were isolated from buffy coats, following centrifugation at $800\times g$ for 15 min and washed in 40 mL HBSS at $1,000\times g$ for 10 min. Cell pellets were resuspended to 2×10^7 PBMCs per milliliter in fetal calf serum (FCS; Gemini Bioproducts, CA, USA) containing 10% DMSO, and slowly frozen at -80°C in an insulated container for 1–2 days, prior to transfer to liquid nitrogen (-140°C) for long-term storage. Frozen PBMC were quickly thawed in a 37°C waterbath and washed in RPMI-1640 (Invitrogen-Gibco). They were rested at $37^\circ\text{C}/5\%$ CO_2 overnight in RPMI-1640 containing L-glutamine (2×10^6 cells per milliliter) and supplemented with 10% FCS and penicillin (100 U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$)—referred to as R10 medium—prior to use in stimulation experiments.

Intracellular Cytokine Staining Assays

We adapted the validated ICS assays developed at the FHCRC HIV Vaccine Trials Network laboratories for these studies [10]. Rested PBMC were washed, resuspended to 10×10^6 cells per milliliter in RPMI-1640 containing human serum (10%; Gemini Bioproducts), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$), and added to 96-well plates at 1×10^6 PBMC/well. CD107a-PeCy5 antibody was incubated, at a dilution 7:100, for 10 min at room temperature, and culture volumes made up to 200 μL . Stimulations with HSV-2 peptide pools (10 $\mu\text{g}/\text{mL}$ each peptide) or single peptides (2 $\mu\text{g}/\text{mL}$) were performed at $37^\circ\text{C}/5\%$ CO_2 for 6 h in the presence of co-stimulation using antibodies

against CD28 (1 $\mu\text{g}/\text{mL}$, BD Biosciences, San Jose, CA) and CD49d (2 $\mu\text{g}/\text{mL}$, BD Biosciences) adding, after the initial hour of incubation, Brefeldin A (10 $\mu\text{g}/\text{mL}$, Sigma, St. Louis, MO) and GolgiStop (0.25%; BD Biosciences). As positive controls for T cell activation, individual responses to both CEF and CMV peptide pools, and to the superantigen staphylococcal enterotoxin B (SEB; 2.5 $\mu\text{g}/\text{mL}$), were also measured. Peptide solvent (1% DMSO) or media alone (non-stimulated) served as negative controls. Cultures were transferred to 4°C overnight at the end of the incubation period.

Following 10 min incubation at room temperature with EDTA (20 mM), cells were pelleted and stained with LiveDead Violet stain (1:1,000; Invitrogen) for 20 min. The cells were subsequently washed, lysed (FACS lyse, BD Biosciences), permeabilized (FACS perm II; BD Biosciences), and stained with a cocktail of conjugated antibodies: CD3-ECD (Beckman Coulter), CD4-FITC (BD Biosciences), CD8-PerCP/Cy5.5 (BD Biosciences), IFN- γ -APC (BD Biosciences); IL-2-PE (BD Biosciences), TNF- α -Alexa700 (BD Biosciences), and CD14-APC/Cy7 (BD Biosciences), CD19-APC/Cy7 (BD Biosciences). Stained cells were resuspended and fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry as described below.

Proliferation Assay

PBMC were thawed and rested and washed as described above. Cell pellets were resuspended in 2 mL PBS containing 1.4 μg CFSE (Vybrant CFDA SE cell tracer kit, Invitrogen) for 8 min. The staining processes was

Table II Demographics of Study Subjects

	HSV-2 seropositive subjects ($N=55$) <u>N (%)</u>	HSV-2 seronegative subjects ($N=18$) <u>N (%)</u>
Gender		
Female	26 (47)	8 (44)
Male	29 (53)	10 (56)
Race		
Caucasian	45 (82)	14 (78)
Non-Caucasian	10 (18)	4 (22)
Age		
Min	27.8	23.1
Max	69.1	60.2
Median	51.9	29.8
<30	2 (4)	10 (56)
31-50	24 (44)	6 (33)
>50	29 (53)	2 (11)
HSV-1 status		
Positive	21 (38)	0 (0)
Negative	34 (62)	18 (100)

stopped by addition of cold human serum and cells washed and resuspended to 2×10^6 cells per milliliter in RPMI containing 10% human serum, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). For each stimulation, 4×10^6 CFSE-labeled cells were added per well of a 24-well plate, in the presence of 2 $\mu\text{g}/\text{mL}$ HSV-2-specific peptide or CMV peptide pool. An equivalent volume of DMSO was added to negative control wells. As a positive control and to define number of proliferation events, PBMC were incubated with anti-CD3 (OKT3; 0.1 ng/mL) and anti-CD28 (2 $\mu\text{g}/\text{mL}$; BD Biosciences) antibodies. Following incubation at 37°C for 6 days, cells were harvested, washed, and dispensed at 1×10^6 cells/well of a 96-well plate. Cells were stained with LiveDead stain and CD3-ECD, CD8-PerCP/Cy5.5, and CD4-APC/Cy7 (BD Biosciences) antibodies, washed, fixed, and analyzed by flow cytometry as described below.

Detection of Perforin and Granzyme B in T Cells

PBMC were prepared and stimulated with HSV-2-specific peptides or control reagents as described as for ICS assays. Cells were pelleted and stained using LiveDead Violet stain. CD3-ECD, CD8-PerCP/Cy5.5, IFN- γ -APC, Perforin-PE (BD Biosciences), and Granzyme B-FITC (BD Biosciences) antibodies were used to stain the cells following treatment with FACS lyse (10 min) and FACS perm II (10 min). After washing and fixing steps, the stained cells were analyzed by flow cytometry as described below.

Flow Cytometric Analysis

Stained cells were analyzed using an LSRII (BD Biosciences). The flow cytometer was calibrated at the beginning of each run using SPHERO™ UltraRainbow Fluorescent Particles (Spherotech, Inc.), ensuring the positions of bead histograms, for each appropriate color, did not vary more than 1% (with a CV of less than 10) between different days. Single antibody-stained cells were used for compensation under the same conditions. All events were collected without compensation.

Flow cytometry data were analyzed and compensated using FlowJo Software (Version 8; TreeStar, Inc.). The gating strategy allowed for selection of single lymphocytes (using FSC-A versus FSC-H parameters) and removal of dead cells from all analyses. Differences in gating strategies for distinct analyses were as follows:

ICS Assay Cells positive for CD14 (monocytes) and CD19 (B cells) were excluded from viable cells. The remaining CD3 positive (T) cells were separated into CD4⁺ and CD8⁺ cells and gated for cytokine (IFN- γ , IL-2, or TNF- α) or CD107a expression as described previously [10]; CD4⁺ T

cells were excluded from the analyses. Multiple functional marker expression events were observed using the Boolean gate feature in FlowJo.

Proliferation Assay CD3⁺CD8⁺ cells were selected from live and single cells as described above. Gate locations of proliferated cells were established using CD3/CD28 stimulated cells; proliferated (CFSE^{lo}) cells were defined as those with half or less CFSE staining as parental cells.

Granzyme and Perforin Assay Selection of CD3⁺CD8⁺ T cells from live and single cells was performed as described above. Granzyme B⁺, Perforin⁺, or IFN- γ ⁺ events were independently gated from CD8⁺ T cells. Multiple functional marker expression events were observed using Boolean gates.

Scoring and Comparison of Antigen-Specific Responses

ICS Assay—Single Cytokine Positivity

Positive or negative responses were evaluated separately for each cytokine and for each antigen within each subject. Individual cytokine responses were scored on total cell frequencies expressing a given cytokine regardless of whether or not other cytokines were co-expressed. The Fisher's exact test (one-sided) was used to determine whether the proportion of cells expressing a given cytokine was higher in the antigen-exposed wells versus the appropriate negative control. Each SEB-treated positive control was determined to be valid when it contained a significantly greater proportion of cells expressing cytokine than non-stimulated control wells. All other experimental assays (cells exposed to HSV-2 pools 1–29, CEF pools, or CMV pools) were determined to be positive when the experimental well had a significantly greater proportion of cells expressing cytokine than solvent-exposed wells (DMSO). A p value <0.02 was used as a cutoff for all positive subject responses; this was determined to provide positivity in 5% of seronegative subjects through trial and error experimentation during validation, thus allowed for a standard 5% type I (false positive) error rate. SEB well positivity was used to confirm assay validity, and all were found valid.

To evaluate immunodominance of HSV-2 ORFs, the frequency of subjects with positive responses to a given ORF were compared for each cytokine. To assess if timing of ORF expression impacted response frequencies, ORFs were classified into groups depending on their expression kinetics (Table I) and the response rate per ORF group determined for each cytokine. Statistically significant differences in response rate were tested using Poisson

regression for correlated outcomes (generalized estimating equations) [13]. Similar analyses were performed to examine length-adjusted response rates, in which the response rate was divided by the number of amino acids in the region and offset by the log of that length.

ICS Assay—Multifunctional Analysis

Activated T cells were further assessed for co-expression patterns of IFN- γ , IL-2, TNF- α , and/or CD107a to determine if HSV-2-specific T cells produce monofunctional or polyfunctional responses; this was performed using an approach described previously [14]. Positive CD8⁺ T cell responses in subjects were divided according to HSV-2 ORF, and proportions of all responding cells displaying each possible functional phenotype were calculated; there were 15 possible combinations of the four functional markers, and the number of cells within each combination was observed as the percent of total (summed) responding cells for all 15 combinations. Responses detected in cells exposed to DMSO (background) were subtracted from responses in antigen-stimulated samples for each functional combination; all negative values were set to zero.

Distinct monofunctional or polyfunctional populations were also assessed for levels of each cytokine or CD107a, estimated from differences in the median fluorescence intensity (Δ MFI) relative to cells negative for a given protein. Differences in Δ MFI within the antigen-specific T cells of the tested subjects were compared using a non-parametric Friedman's (Conover pairs) test and the Δ MFI of the unique cell populations were considered significantly different if $p < 0.05$.

Proliferation Assay

Cell division indices (CDI) were calculated by dividing the percent of CD8⁺ cells that were CFSE^{lo} in antigen-exposed cells by the percent of CD8⁺ cells that were CFSE^{lo} following exposure to DMSO; proliferation was considered positive if CDI ≥ 2 [15].

Granzyme and Perforin Assay

The number of HSV-2-specific cells expressing lytic granules was assessed in two ways. First, CD8⁺ T cells expressing IFN- γ , following antigen exposure, were selected and the proportions of IFN- γ ⁺ cells co-expressing granzyme B and/or perforin determined. Second, granzyme B or perforin positive cells were selected and IFN- γ positive cells, relative to each of these lytic granules, were visualized and counted; differences in the numbers of IFN- γ ⁺ T cells, in HSV-2 peptide exposed versus control

(DMSO-treated) cultures in cells, expressing or lacking granzyme B or perforin, were compared using the Wilcoxon test for matched pairs and considered significantly different if $p < 0.05$.

Using Boolean gates, cell populations positive for different combinations of granzyme B, perforin, and/or IFN- γ were identified; levels of each granule protein or cytokine were estimated in each distinct population by observing the Δ MFI relative to cells negative for a given protein. Differences in Δ MFI within the antigen-specific T cells of the individuals tested, were compared using a non-parametric Friedman's (Conover pairs) test and the Δ MFI of the unique cell populations were considered significantly different if $p < 0.05$.

Epitope Identification and Confirmation

Peptide pools that were determined to give positive responses in CD3⁺CD8⁺ lymphocytes were subjected to further analysis in order to identify the responsible peptide. Subpools of no more than ten peptides were generated such that each peptide was represented once in two subpools. PBMC were thawed and rested as described above and resuspended at 2×10^6 cells per milliliter in R10 medium. R10 medium (100 μ L) was added to each well of an ELISpot plate precoated with anti-IFN- γ (Human IFN- γ ELISpot PLUS kit (ALP); Mabtech, Ohio, USA). Peptide pools were added to give a final concentration 0.2 μ g/mL and 2×10^5 cells added per well. Following overnight incubation of cultures at 37°C/5% CO₂, plates were washed with PBS and incubated with detection antibody (1:200) in PBS, containing 1% FCS, for 2 h. Bound antibody was detected using filtered NBT substrate following the manufacturer's instructions, the plates dried overnight, and spots counted using Bioreader[®] 3000 ELISpot reader (BioSys) within the Immune Monitoring Laboratory at the Fred Hutchinson Cancer Research Center. Responses with ≥ 11 spots per well and $\geq 3 \times$ the number of spots in control (DMSO) wells were considered positive.

Single 15-mers predicted to give responses in CD8⁺ T cells, using this method, were verified by further ELISpot analysis. T cell (9-mer) epitopes embedded within a given 15-mer were predicted with the assistance of epitope prediction software at the Immune Epitope Database (beta.immuneepitope.org). A common 9 amino acid epitope (FLWEDQTLL) predicted within UL25 was synthesized at >90–95% purity (New England Peptide) and verified by serial (log) dilution using ELISpot, and sent to John Sidney at La Jolla Institute for Allergy and Immunology (La Jolla, California), to perform peptide-binding assays to MHC class I, as described previously [16]. HLA-A*0201 tetramers binding FLWEDQTLL or a human CMV pp65 HLA-A*02 specific epitope (NVLPMVATV) were synthe-

sized at the Immune Monitoring Laboratory at the Fred Hutchinson Cancer Research Center, conjugated with Phycoerythrin (PE), and used to recognize HSV-2-specific CD3⁺CD8⁺ cells in responding individuals by flow cytometry.

Results

HSV-2 ORF-Specific Responses by Cytotoxic T Cells

Before assessing the function of HSV-2-specific T cells, we were first required to identify the antigen-specific responses unique to each study subject. CD3⁺CD8⁺ T cell cytokine responses to the HSV-2 peptide pools were detected in 43 (78%) of the 55 HSV-2 seropositive persons. The most frequent responses in HSV-2 infected subjects were to ICP0, UL39, UL49, and UL19 (29%, 29%, 27%, and 26%, respectively) (Fig. 1a). UL25 and UL46 peptide pools each produced responses in 11 (20%) subjects, and 9 (16%) subjects responded to UL27 (gB) derived peptides. Seven subjects (13%) each gave responses to UL47 and UL29, 4 (7%) subjects each responded to ICP27 and UL11, while 2 (4%) each responded to UL35 and US6 (gD). No seropositive subjects had detectable responses to US5 (gJ). We did detect responses in 3 of the HSV-1/HSV-2 seronegative persons but, as shown in Fig. 1d, all of these were barely above baseline responses; none of the T cell responses in these HSV seronegative subjects exceeded 0.05% (ranges; 0.01–0.05% IFN- γ ⁺, 0.02% IL-2⁺, 0.02–0.04% TNF- α ⁺ CD8⁺ T cells) of CD8⁺ T cells above background levels for any cytokine. In contrast, the magnitude of IFN- γ ⁺, IL-2⁺, or TNF- α ⁺ CD8⁺ T cells of HSV2 seropositive subjects was much greater (Fig. 1c vs. Fig. 1d).

Since the most frequent CD8⁺ T cell responses were identified to immediate-early and early ORFs, and few responses were detected to late ORFs, we wanted to know if response rate varied depending on the expression kinetics of the ORFs. ORFs were categorized into groups according to their expression kinetics (Table I) and compared with and without adjustment for the number of amino acids contained per ORF (Fig. 2). To determine whether the number of ORFs to which individuals responded differed significantly, we performed a Poisson regression analysis that allowed within-participant correlation. For IFN- γ , both the immediate-early and late responses were less frequent than the early (RR=0.56, 95% CI 0.34–0.90, p =0.016, and RR=0.33, 95% CI 0.18–0.63, p <0.001, respectively). For IL-2, the late response was less frequent than the early (RR=0.38, 95% CI 0.15–0.92, p =0.032), but no differences were found between the early and the IE or leaky-late. For TNF- α , the IE response was less frequent than the early response (RR=0.47, 95% CI 0.23–0.98, p =0.044).

Since this was performed separately for each cytokine, p values \leq 0.0167 were considered statistically significant, following Bonferroni adjustment for multiple comparisons. Thus, the apparent differences in IL-2 and TNF-expression by CD8⁺ T cells, according to the viral replication cycle, may be due to chance.

The number and length of proteins within each ORF class varies. To exclude the effect of ORF size on response rate, we compared response rates by classification of ORFs after adjustment for number of amino acids. Responses to each cytokine were compared using a similar analysis to that described above. For IFN- γ , the amino acid-adjusted late response remained less common than the early response (RR=0.53, 95% CI 0.28–0.99, p =0.049), thus ORF length appears not to be responsible for the observed difference in response frequency, although this is not significant with Bonferroni correction. No differences were observed for ORF length-adjusted responses for IL-2 or TNF- α . Thus IFN- γ responses to HSV proteins appeared to be more common to the early proteins, which included UL11, UL29, UL39, and UL49.

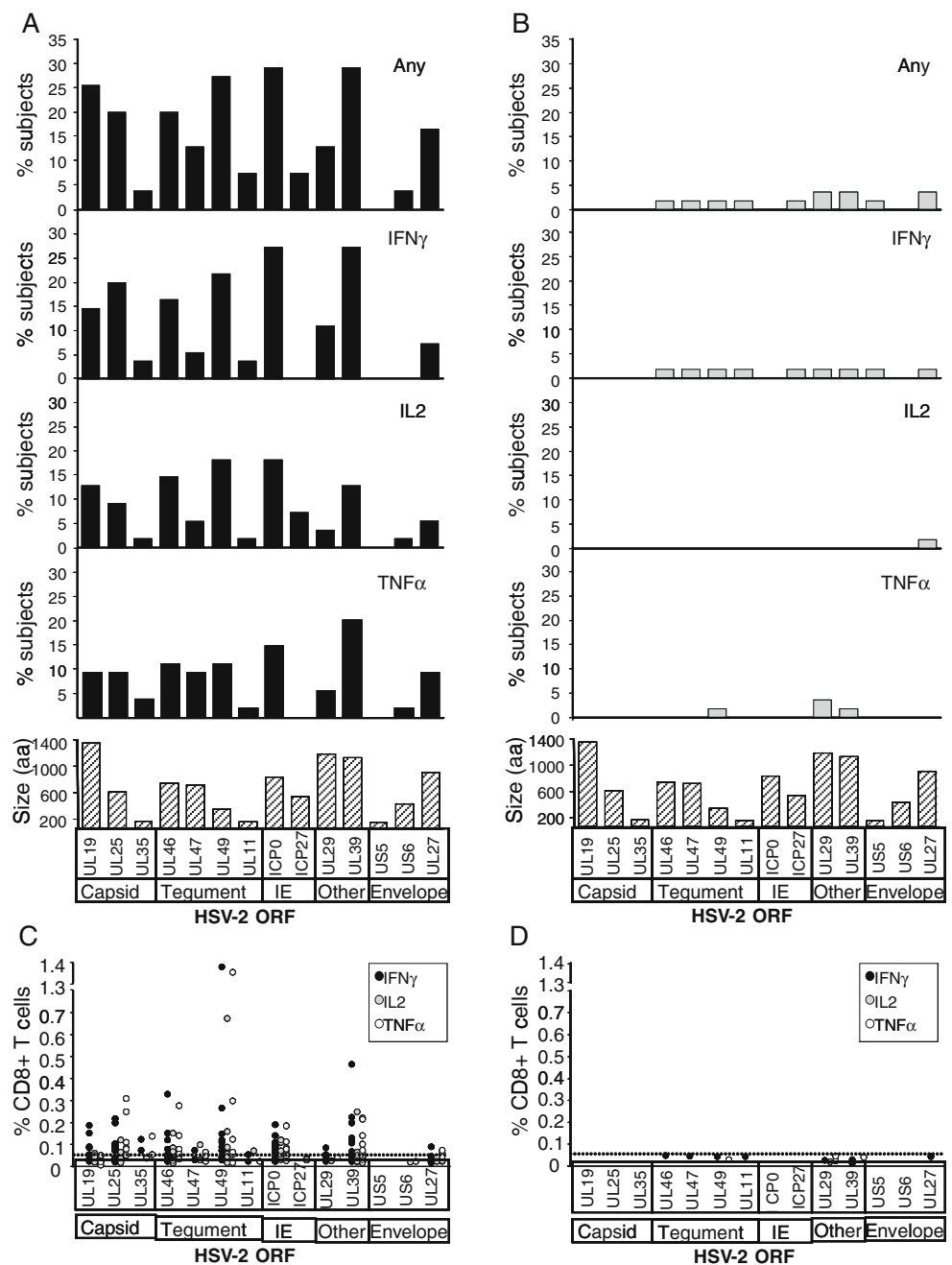
Cytotoxic T Cells Responding to HSV-2 Peptide Pools Make IFN- γ and are Often Monofunctional

Analysis of T Cell Populations

HSV-2-specific CD8⁺ T cells produced multiple cytokines, but were overall, more likely to express IFN- γ than IL-2 or TNF- α following exposure to HSV-2 peptide pools. Poisson regression for correlated data confirmed that the differences in ORF-specific response rates between IFN- γ and both IL-2 (p =0.009) and TNF- α (p =0.010) were statistically significant. Considering all positive responses identified in CD8⁺ T cells of seropositive subjects, regardless of ORF, 80% were positive for IFN- γ (median value; range 0–100%), 50% were positive for IL-2 (median value; range 25–100%), and 50% were positive for TNF- α (median value; range 0–100%) (Supplementary Table S1). Relative to all seropositive subjects, the highest proportion of subjects with IFN- γ ⁺CD8⁺ T cell responses was identified for both UL39 and ICP0 (27% each), followed by UL49 (22%), UL25 (20%), and UL46 (16%) (Fig. 1). UL19-specific IFN- γ ⁺CD8⁺ cells were observed in only 14% subjects, while CD8⁺ T cells of 11% subjects produced IFN- γ in response to UL29 peptides. IFN- γ ⁺ responses against other ORFs were identified in <10% subjects each.

Although the frequency of subjects with detectable responses for other cytokines was lower than for IFN- γ , the same general ORF immunodominance was reflected. IL-2⁺CD8⁺ T cells were most frequently identified in response to ICP0 and UL49 peptides (18% of subjects

Fig. 1 Percentage of HSV-2 seropositive (**a**; $N=55$) or seronegative (**b**; $N=18$) individuals, detected by high-throughput ICS analysis, who display specific immune responses against a given HSV-2 ORF in their CD8⁺ T cell population. Responses are displayed as the percent of individuals with CD8⁺ T cells positive for **any** of the three observed cytokines (IFN- γ , IL-2, or TNF- α ; *top panels*), as well as the percentage with detectable responses observed separately for each cytokine; the individual cytokine responses were determined by observing total cell frequencies expressing a given cytokine regardless of whether or not other cytokines are co-expressed. The amino acid sizes of each ORF are shown in the *bottom panels*. The percentages of CD8⁺ T cells expressing each cytokine in positively scored responses to HSV-2 peptide pools are shown for seropositive (**c**) and seronegative (**d**) subjects; the values presented represent percentage of cytokine-positive CD8⁺ T cells exposed to HSV-2 peptides minus the percentage of cytokine-positive CD8⁺ T cells exposed to DMSO. The *dashed line* represents 0.05% CD8⁺ T cells



each), followed by UL46 (15%), UL19, and UL39 (12% each). Each of the other ORFs stimulated IL-2 production in <10% subjects. The most frequent TNF- α ⁺CD8⁺ T cell responses were observed following stimulation with UL39 (20%) and ICP0 (14%), followed by UL49 and UL46 (11% each) similar to the pattern observed for IFN- γ , although at lower frequencies. Peptides to other ORFs each produced TNF- α ⁺CD8⁺ T cells in <10% subjects. Only two ORFs, with responses to HSV-2 peptides, gave no detectable IFN- γ ⁺CD8⁺ T cells; four responses to ICP27 were IL-2⁺ only and one response each to US6 was singly positive for IL-2 and TNF- α . All responses to both ORFs comprise $\leq 0.05\%$

CD8⁺ T cells above background levels. No significant differences in the type of cytokine produced by HSV-2-specific CD8⁺ T cells were observed when comparing responses between different ORFs.

Analysis of Single Cells

After confirming that exposure to HSV-2 antigens induces T cell populations to produce several cytokines, we examined, by ORF, the polyfunctionality of the CD8⁺ T cells at the single cell level; we aimed to know, within each responsive group of T cells, if an individual T cell produced

multiple cytokines or single cytokines, with or without degranulating. First, the proportion of responding HSV-2-specific cells (mean percent of total response) positive for one, two, three, or four functional markers were determined for all subjects with confirmed positive responses to a given HSV-2 ORF (Fig. 3). Monofunctional cells ranged from 35–98% (mean 70%) of responsive cells, whereas expression of two functional markers was found in 16% (mean; range 0–35%) of responsive cells, expression of three functional markers was found in 12% of responsive cells

(mean; range 0–31%), and expression of all four functional markers was found in 1% of responsive cells (mean; range 0–3%).

Next, we assessed the frequency of responding cells with distinct phenotypes (15 were possible) based upon different combinations of IFN- γ , IL-2, TNF- α , and CD107a expression. Most HSV-2 ORFs induced polyfunctional cells that were largely IFN- γ ⁺; polyfunctional CD8⁺ T cells predominantly co-expressed IFN- γ , IL-2, and TNF- α (highest mean response 22% of total; UL49), or IFN- γ and TNF- α

Fig. 2 Comparison of responses to ORFs with different expression kinetics. ORFs are grouped according to their expression kinetics (immediate-early [IE], early, leaky-late, or late) as listed in Table I, and the frequency of persons with positive responses was determined for each category. **a** The percentage of subjects with positive cytokine (IFN- γ , IL-2, or TNF- α) responses to HSV-2 peptides. **b** The percentage of subjects divided by the number of amino acids (AA) represented per ORF class. **c** The number of amino acids contained per ORF class

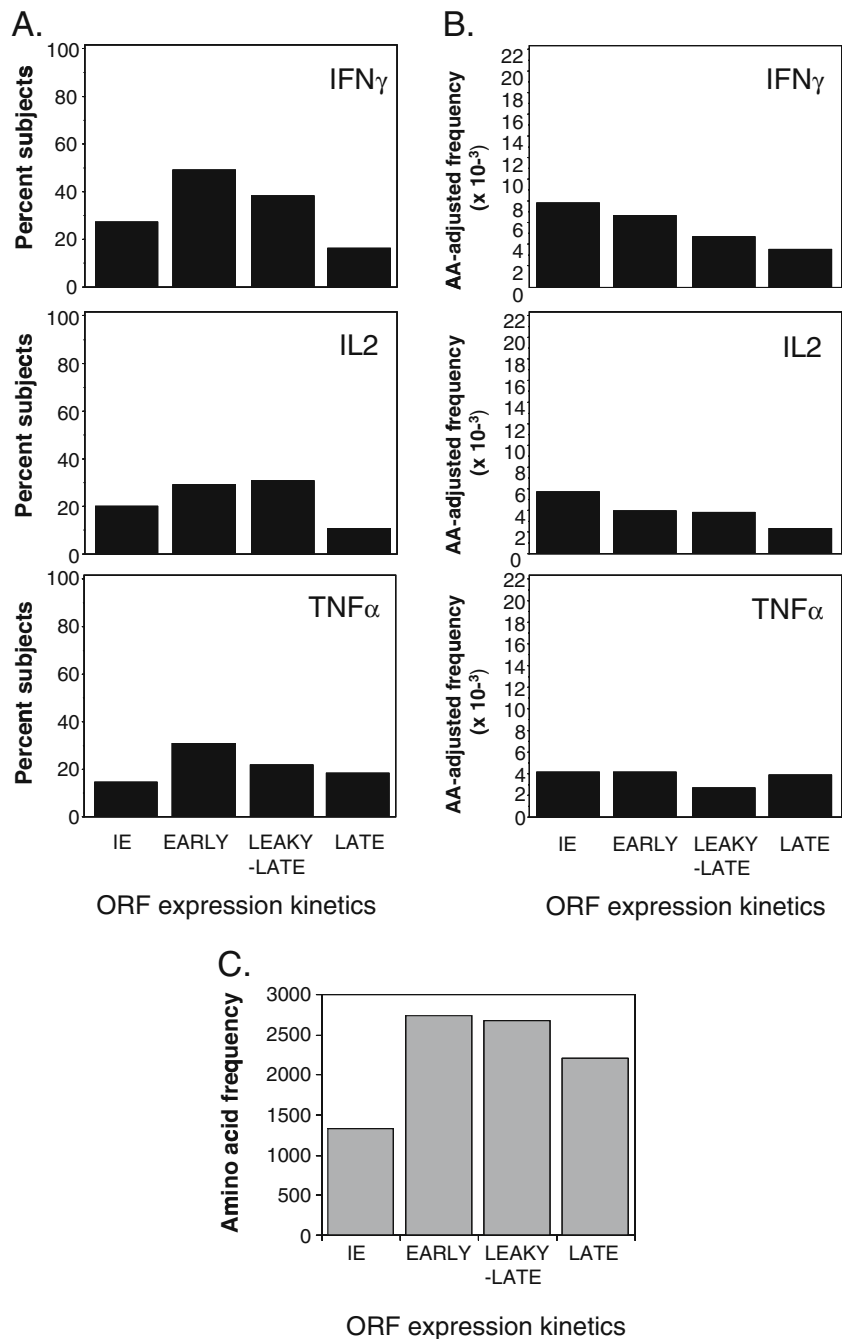


Fig. 3 HSV-2-specific T cell populations are polyfunctional. High-throughput ICS assays were used to detect expression of the functional markers IFN- γ , IL-2, TNF- α , and CD107a in T cells that react to HSV-2-derived peptide pools. The proportion of HSV-2-specific CD8⁺ T cells that expressed one, two, three, or all four of these markers was determined—these data are summarized as pie charts, where one pie represents the proportional average of responding T cells for all individuals with responses to a given ORF. Each section of the pie represents cells that express one (*yellow*), two (*blue*), three (*red*), or four (*black*) of the functional markers. Qualitative representation of the 15 distinct combinations of functional markers on responsive HSV-2 ORF-specific CD8⁺ T cells are shown as *dot plots*, where each *graph* represents a distinct ORF and each *dot per column* represents a unique individual. The number of responding individuals (*n*) per ORF is given. Values shown on the *y*-axes of each graph represent the percent of the total CD8⁺ T cells that express functional markers

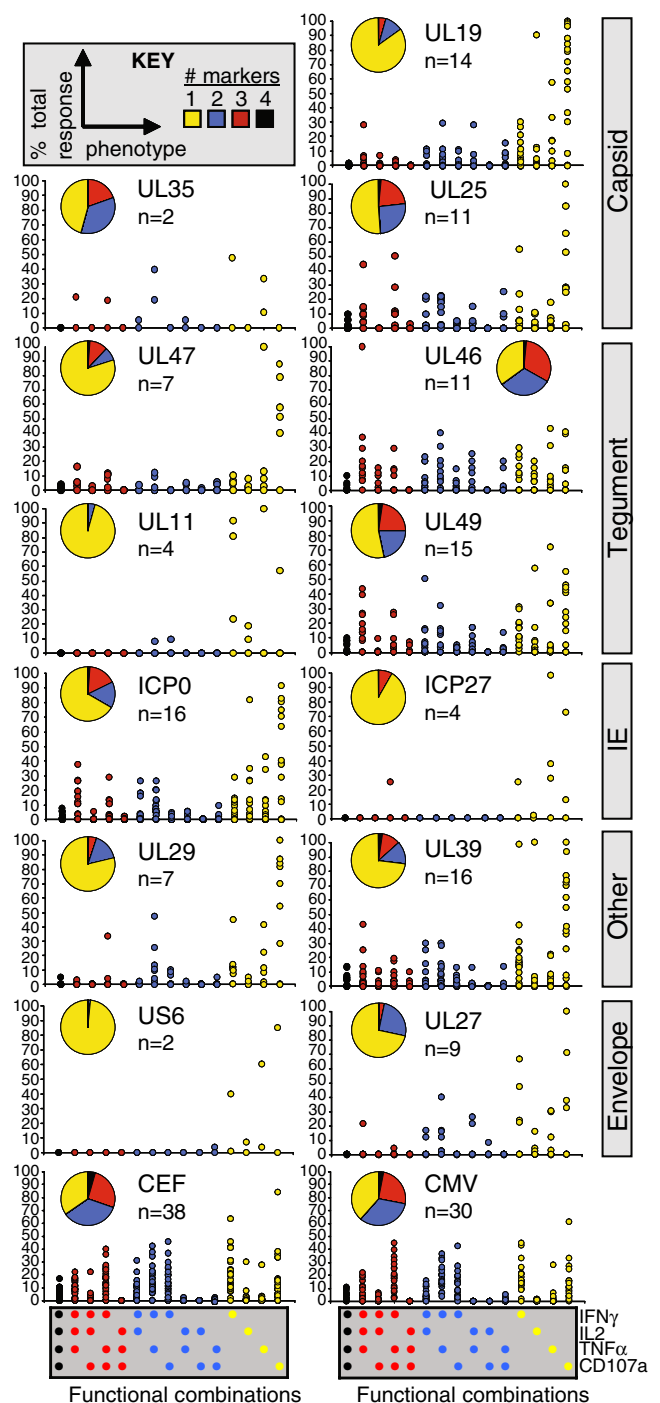
(highest mean response 29% of total; UL35). Cytokine-secreting cells did degranulate; there were CD107a⁺ polyfunctional cells. The co-expression of IL-2 and CD107a, particularly in the absence of IFN- γ expression, was infrequent (mean 0%; range 0–8%); typically, of total responding CD8⁺ T cells, 1–3% of co-expressed all four markers, 1–4% co-expressed IFN- γ , IL-2 and CD107a, 1% co-expressed IL-2, TNF- α , and CD107a, and 1% co-expressed IL-2 and CD107a (UL27 only).

Monofunctional T cells were more likely IFN- γ ⁺ (highest mean response 49%; UL11) or CD107a⁺ (highest mean response 63%; UL19) than IL-2⁺ (highest mean response 11%; ICP0) or TNF- α ⁺ (highest mean response 32%; US6). Their proportions were highest (92–98% of responsive CD8⁺ T cells) during reactions to ICP27 (IL-2 only), UL11, and US6 (IFN- γ only) and lowest (35–53% of responsive CD8⁺ T cells) during reactions to UL25, UL35, UL46, and UL49. ORFs inducing the highest proportions of monofunctional cells also induced the lowest frequency of cytokine-positive CD8⁺ T cells, suggesting that monofunctional phenotype and low frequency of activated cells are related (Fig. 1). Only 2 (UL35 and UL46) of the 14 ORFs studied produced responses in which >50% of the cells were polyfunctional. In contrast, <50% of the CD8⁺ T cells activated by CMV or CEF control peptide pools were monofunctional.

In summary, most CD8⁺ T cells to HSV-2 were monofunctional, with IFN- γ being the most commonly expressed cytokine and many HSV-2-specific T cells able to degranulate. No differences in the functional profiles were noted between the prevalent ORFs of HSV-2.

Identification of Specific Epitopes Within HSV-2 ORFs

ELISpot assays measuring IFN- γ production were used to deconvolute peptide pools that induced responses in CD3⁺CD8⁺ lymphocytes and to identify the responsible 15-mer peptides. The specific epitopes identified, by HSV-2 protein, are listed in Table III.



Of particular interest, we identified a previously uncharacterized peptide embedded in the HSV-2 capsid-associated protein UL25 that is antigenic in multiple HLA-A02 positive persons. This peptide (UL25_{369–383}) elicited T cell responses in at least 5 (17%) of the 29 (53%) subjects that possessed an HLA-A02 allele. A listing of the HLA alleles in our population is shown in Supplementary Table S2. Analysis of the peptide sequence by epitope prediction programs identified a high putative

Table III Peptides that Induce Responses in CD8+ T Cells of HSV-2 Seropositive Subjects

ORF	Peptide ^c	Location in ORF	Subject	HLA-A and -B types ^b	Epitope IC50 (μM) ^d
UL19	AF <u>EDRSYPAVFYLLQ</u>	617–633	Pt 04	A01, A03, B08 , B63	358
UL25	HNL <u>FLWEDQTLLRAT</u>	369–383	Pt 11	A02 , A11, B08, B15	2–32
	“	“	Pt 02	A02 , A34, B35, B62	
	“	“	Pt 09	A01, A02 , B08, B44	
	“	“	Pt 10	A02 , A24, B35, B44	
	“	“	Pt 07	A02 , A03, B07, B44	
	DRLDNRLQLGMLIPG	405–419	Pt 12	A02 , A32, B14, B44	79–102
UL46	RLGPADRRFVALSGS	249–263	Pt 13	A03, B07 , B47	112
	“	“	Pt 01	A03, B07	
	“	“	Pt 08	A01, A03, B07 , B08	
	AQREAAAGVYDAVRTW	533–547	Pt 14	A03, A68 , B35, B53	7 (EV9)
	“	“	Pt 03 ^a	A01, B57	216 (GW9)
UL49	PMRAR <u>PRGEVRFLHY</u>	45–59	Pt 01	A03, B07	23
	“	“	Pt 15	A02, A30, B07 , B35	
	“	“	Pt 16 ^a	A01, A31, B07 , B60	
	“	“	Pt 17 ^a	A02, A74, B07 , B14	
	“	“	Pt 08 ^a	A01, A03, B07 , B08	
	“	“	Pt 05	A24, A31, B07 , B62	
	<u>ARPRRSASVAGSHGPG</u>	81–96	Pt 01	A03, B07	7
	“	“	Pt 18	A02, A68, B07 , B14	
	HGPGPARAPPPGGPV	93–108	Pt 01	A03, B07	47
	PKASATPATDPARGR	129–143	Pt 01	A03, B07	170
	KNLLQRANELVNPDA	249–263	Pt 08	A01, A03, B07, B08	35
ICP0	EAGLMDAATPPARPPA	77–92	Pt 19	A24, A30 , B18, B40	54
	LHPFCIPCMTWIPL	145–159	Pt 20	A01, A03 , B07, B08	119
	DFIWTGNPRTAPRSL	209–223	Pt 13 ^a	A03, B07 , B47	5
	“	“	Pt 07	A02, A03, B07 , B44	
	LPIAGVSSVVALAPY	689–703	Pt 21	A33, A74, B18, B35	169
	“	“	Pt 10	A02, A24, B35 , B44	
	DMETGHIGAYVVLVD	717–731	Pt 06 ^a	A03, A11, B39 , B51	B39 allele not in IEDB
	GHIGAYVVLVDQTGN	721–735	Pt 22	A29, A68 , B71, B37	250. B37/71 not in IEDB
	RAAAPAWSRRTLLPE	741–755	Pt 01	A03, B07	7
	“	“	Pt 07	A02, A03, B07 , B44	
	“	“	Pt 05	A24, A31, B07 , B62	
	PVGNMLFDQGTLVGA	779–793	Pt 23 ^a	A02 , B50, B62	2–36
UL39	LMLEYFCRCAREESK	346–359	Pt 24	A03 , A11, B07, B51	710
	GVLVHLRIRTREASF	433–447	Pt 25 ^a	A02, A03, B49, B62	94
	“	“	Pt 04	A01, A03, B08 , B63	32
	FGGHYMESVFQMYTR	515–529	Pt 03	A01 , B57	183
	SMSLADFHGEEFEKL	725–739	Pt 08 ^a	A01, A03, B07 , B08	184
	KTSNALCVRGARPFS	911–925	Pt 16	A01, A31 , B07, B60	17
UL29	CPLLIFDRTRKFLVA	1,013–1,027	Pt 26 ^a	A24, A34, B14, B50	Alleles not in IEDB

^a Responses to the adjacent downstream 15-mer were also detected, ^b HLA types in bold text represent those most likely restricting the ^c epitope response, predicted by commonly shared types, supertype motif conservation and/or ^d lowest IC50 (smm method) scores using HLA binding prediction programs at IEDB

affinity HLA-A02 binding epitope (FLWEDQTLL) embedded in the middle of this 15-mer. Likely, proteasomal processing of this epitope was also predicted using NETCHOP (not shown).

The predicted 9-mer was subsequently synthesized and tested for its ability to induce IFN-γ production by serial dilution analysis using ELISpot (Fig. 4a). This 9-mer could stimulate IFN-γ production in PBMC of all responding

individuals at concentrations as low as 10^{-11} M, with peak stimulation occurring at 10^{-8} M; this exceeded the ability of the corresponding 15-mer to generate T cell responses at low concentrations. By peptide-binding studies; low IC_{50} values (<10 nM) confirmed that FLWEDQTLL could bind HLA-A*0201, HLA-A*0202, HLA-A*0203, and HLA-A*0206 with high affinity, but not HLA-A*6802, which belongs to a different serogroup (Fig. 4b). To further confirm the HLA-A02 restriction of this epitope, PE-labeled HLA-A*0201 tetramers binding FLWEDQTLL were synthesized. $CD3^+CD8^+$ cells from PBMC of subjects with known responses to this peptide were able to bind this tetramer (Fig. 4c); the HLA-A*0201 tetramers did not bind to T cells of individuals that lacked HLA-A02. Thus, we identified a common high affinity novel HLA-A2-restricted epitope to one of the major capsid proteins of HSV-2.

Qualitative Analysis of $CD8^+$ T Cells to Single Epitopes

We randomly selected eight individuals from our cohort for evaluation of their T cell function using a single epitope/

peptide. This allowed us to evaluate responses across several ORFs and in subjects with different HLA types. ICS analysis revealed that a greater percentage of responding T cells were polyfunctional when optimal peptide/epitope stimulation was performed; single peptide stimulation generated less monofunctional cells than peptide pools. Initial comparison to T cells exposed to peptide pools indicated single peptides induced an equivalent or higher frequency of $CD8^+$ T cells producing IFN- γ for six individuals and a lower frequency for two individuals (Fig. 5a); for all subjects, median ratio of single peptide(s)/peptide pool is 1.1 (range 0.5–10.5).

In other viral infections, polyfunctional T cells have higher functional capacity than monofunctional T cells [17–19]. To determine whether this is the case in HSV-2-specific T cells, we assessed the relative levels of each functional marker in responding cells expressing different cytokine and degranulation markers. To determine per-cell differences in cytokine or degranulation levels in polyfunctional and monofunctional HSV-2-specific T cells, the relative intensities of cytokine and degranulation markers

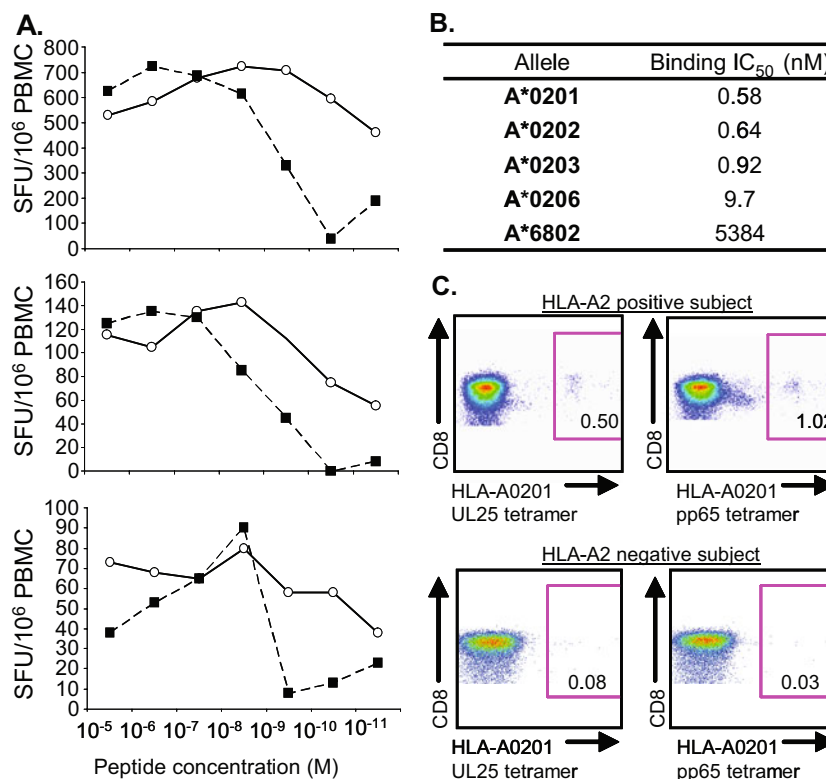


Fig. 4 Confirmation of a novel 9-mer epitope embedded in UL25 that is antigenic in HLA-A2 possessing individuals. **a** Decreasing concentrations of the UL25_{369–383} 15-mer (dashed lines and black boxes) and UL25_{372–380} 9-mer (solid line and white circles) were assessed by ELISpot for their ability to induce secretion of IFN- γ from $CD8^+$ T cells within PBMC of known responders to the 15-mers. IFN- γ release was detected at low doses ($<10^{-10}$ M) of peptide. All responding individuals possessed an HLA-A2 allele. **b** Binding

analyses comparing the affinity (IC_{50} ; nM) of UL25_{372–380} for different HLA-A*02 proteins, or the unrelated HLA-A*6802 molecule. **c** Representative surface staining of $CD3^+CD8^+$ lymphocytes from a responsive HSV-2 seropositive individual with HLA-A*0201 tetramers binding FLWEDQTLL (HSV-2 UL25 epitope) or NVLPMVATV (CMV pp65 epitope). Lack of staining of cells from an HLA-A*0201 negative individual is shown for comparison. Plots represent an average of duplicate analyses

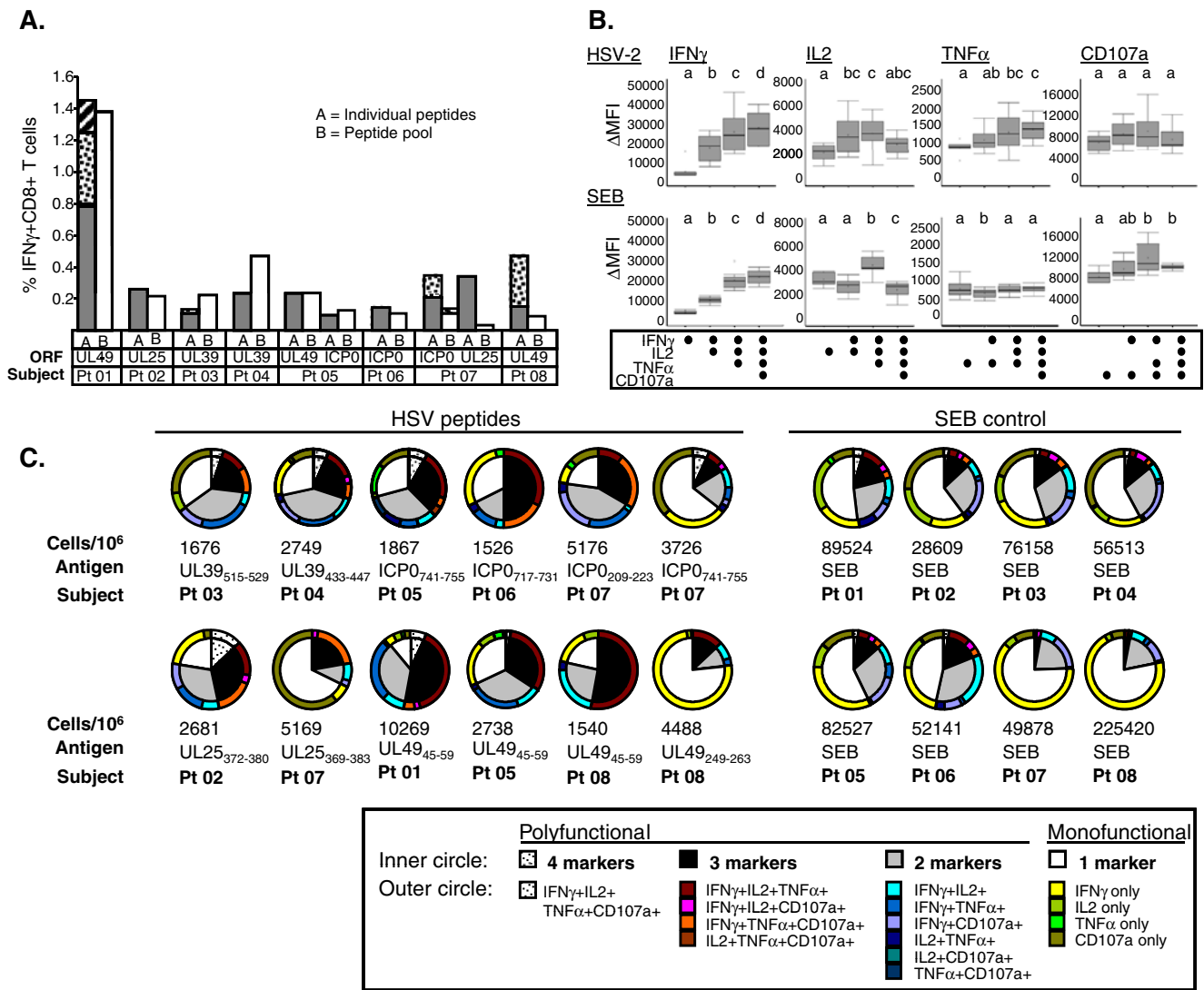


Fig. 5 **a** Comparing the frequency of IFN- γ ⁺CD8⁺ T cells recalled by single HSV-2 peptides (*shaded bars*) and peptide pools (*white bars*) in selected HSV-2 seropositive subjects with mapped responses. Each distinct shading pattern in bars for single peptide responses represents a unique peptide within a given ORF. **b** Changes in median fluorescence intensity (Δ MFI) of IFN- γ , IL-2, TNF- α , and CD107a in monofunctional and polyfunctional CD8⁺ T cells following exposure to HSV-2-specific peptides or to SEB. Average responses of all individual peptide responses within an individual were calculated to reduce bias. Distinct combinations of functional markers are indicated below box-plots. For box-plots, *gray boxes* represent the 25–75% range, and *bars* represent the 5–95% range, while the *horizontal line within the bar* represents the median. Significantly

different groups ($n=8$; Friedman's non-parametric test followed by Conover; $p<0.05$) are indicated with distinct letters (*a, b, c, or d*), thus groups not found different will share the same letter. **c** Individual peptides from HSV-2 ORFs activate polyfunctional cells, most of which are IFN- γ positive. Single 15-mers derived from HSV-2 ORFs UL39, ICP0, UL25, or UL49 were used to specifically stimulate PBMC in responsive individuals *in vitro*. Cytokine production and CD107a expression were detected in CD8⁺ T cells by ICS; the relative proportions of responsive cells stained with one, two, three, or four functional markers are presented as pie charts. For each antigen, the total number of responsive cells detected by ICS per million CD8⁺ T cells is shown below the pies. Mono- and polyfunctional cell proportions are shown, as per color key, in the *outer circles*

were compared between cells expressing one or more markers. IFN- γ and TNF- α levels were higher in polyfunctional cells than in monofunctional cells (Fig. 5b). Significant increases in median fluorescence intensity were observed for IFN- γ (8-, 11-, and 13-fold) in polyfunctional HSV-2-specific cells with two, three, and four detectable markers, confirming that polyfunctional cells are making

large amounts of this cytokine; this is consistent with increases in IFN- γ observed following SEB stimulation. Increases in TNF- α levels were also observed (1.4 and 1.6-fold) in polyfunctional cells with three and four detectable markers, relative to monofunctional cells following exposure to HSV-2 peptides, but not to SEB. IL-2 levels were increased in cells with two or three functions (1.5- and 1.6-

fold, respectively), but not in those with four functions, relative to monofunctional cells; cells that mobilized CD107a did not have a higher Δ MFI for IL-2 (not shown), suggesting that IL-2 production is less likely to occur in cells that degranulate. The quantity of CD107a mobilization was not significantly different between monofunctional or polyfunctional HSV-2-specific T cells, although increases were observed for SEB-challenged cells.

Single peptides were able to recall polyfunctional cells regardless of their specific ORF or subject variability. However, while cytokine and degranulation marker expression patterns for SEB-stimulated cells between individuals were similar, responses to HSV-2-specific peptides and ORFs varied by individual. Consistent with studies using peptide pools, IFN- γ was the most abundant cytokine produced by HSV-2-specific CD8⁺ T cells; the majority of HSV-2-specific CD8⁺ T cells were positive for IFN- γ , regardless of whether these cells were mono- or polyfunctional, reflecting rises in IFN- γ production with higher functional complexity, as well as the high frequency of IFN- γ ⁺ monofunctional cells (Fig. 5c).

The most variable functional markers, in our study, were CD107a and IL-2. Although the polyfunctional cells in two individuals (Pt 03 and Pt 07) were still IFN- γ positive, these subjects had a larger percentage of monofunctional cells that, respectively, stained for IL-2 (51% of monofunctional T cells) or CD107a (58–61% of monofunctional T cells). Higher levels of CD107a⁺ CD8⁺ T cells, in general, were detected in Pt 07 than in the other subjects. For all subjects, very few HSV-2-specific T cells were observed that both produced IL-2 and exposed CD107a, although Pt 07 showed the highest cellular frequency (~30% of trifunctional T cells) with detectable expression of both IL-2 and CD107a; these were observed following stimulation with peptide ICP0_{209–755}. Of particular interest, all three individuals tested with UL49-derived peptides had an unusual pattern in their “triple positive” T cells relative to responses to the other ORFs; most (~80–100%) CD8⁺ T cells, responding to UL49 peptides and positive for three activation markers, produced IFN- γ , IL-2, and TNF- α but were negative for CD107a staining. In fact, for these persons, only a small percentage of T cells with recalled responses were CD107a positive, suggesting the UL49-specific T cells of these individuals degranulate less than cells that recognize the other ORFs.

Thus, although HSV-2-specific cells predominantly expressed IFN- γ in all subjects, there were qualitative differences in the proportions of cells expressing IL-2 and CD107a, potentially reflecting differential proliferation or cytotoxic capability of epitope-specific cells in distinct subjects. This is interpreted with a caveat; since T cells were activated with 15-mer peptides in these studies rather than exact epitopes (8–10-mer), it is plausible that

suboptimal T cell activation generated some of these differences.

Epitope-Specific T Cell Responses Vary Within an Individual

To evaluate functional differences between CD8⁺ T cells within an individual person, we assessed in detail one subject (Pt 01) in whom we detected four activating peptide sequences from the same ORF that were likely restricted by the same HLA (Table III). The four 15-mers from UL49 were tested by ICS for differential cytokine expression and degranulation marker exposure, as described above. We first confirmed that the single peptides stimulate IFN- γ production in PBMC using ELISpot (Fig. 6a). Differences in the magnitude as well as the functional profiles of the T cells to individual epitopes were evident. The ELISpot values were 1,570 SFU/10⁶ PBMCs for UL49_{45–59}, 510 and 610 SFU/10⁶ PBMC for UL49_{93–108} and UL49_{129–143}, respectively, and 144 SFU/10⁶ PBMC for UL49_{81–96}. The differences in relative abundance of each peptide-specific T cell population were confirmed when observing activated T cells using ICS (Fig. 6b).

Variations in functional phenotypes were observed between T cells activated by different peptides (Fig. 6c). Peptide UL49_{45–59} produced the largest proportion (46%) of CD8⁺ T cells that were positive for three functional markers; of these, >80% produced IFN- γ , IL-2, and TNF- α , but did not show detectable degranulation (CD107a negative). Cells specific for this peptide also showed the fewest monofunctional cells (11%) and gave bifunctional cells that were predominantly IFN- γ ⁺TNF- α ⁺. Although T cell responses to peptides UL49_{93–108} and UL49_{129–143} showed similarities in their polyfunctional profiles to UL49_{45–59} specific T cells, a larger percentage of IFN- γ ⁺TNF- α ⁺CD107a⁺IL-2⁻ cells were observed for these stimuli, and greater variation in cytokine combinations were observed for bifunctional cells. Both peptides generated monofunctional responses in a significant proportion of CD8⁺ T cells; 42% and 54% of responding cells were monofunctional following exposure to UL49_{93–108} and UL49_{129–143}, respectively. Polyfunctional T cells for these latter peptides produced mainly IFN- γ , while contrasting UL49_{45–59} monofunctional cells were frequently positive for CD107a. The relative levels of IFN- γ also appeared higher for UL49_{45–59} than the other peptides (Fig. 6d); this was not observed for other functional markers.

Thus, both immunodominance and functional differences between CD8⁺ T cells to the same ORF within one subject were apparent. These cannot be related to genetic differences or HLA restriction, and are presumably influenced in some way by the biochemical properties of different peptide epitopes and/or the T cell receptors that bind them.

Detection of Granule Proteins in HSV-2-Specific CD8⁺ T Cells

During ICS analyses, we used mobilization of CD107a as an indicator of degranulation following T cell exposure to antigen; this protein is exposed on the inner membrane of a granule as it opens to release its contents, but provides no precise information on the stored constituents.

To address whether the granules of HSV-2-specific T cells store lytic proteins, we examined the expression of both granzyme B (GrzB) and perforin (Perf) in T cells of six HSV-2 seropositive individuals (Fig. 7). In all six, GrzB expression was detected more frequently than Perf in HSV-2-specific T cells (Fig. 7a, b); HSV-2-specific T cells were detected by their ability to produce IFN- γ following exposure to peptides. In fact, few activated HSV-2-specific T cells contained detectable levels of Perf, 2.2% (median; range 0–9.1%) of responding T cells were

GrzB⁺Perf⁺IFN- γ ⁺, and 6.5% (median; range 1.6–13.3%) were GrzB⁺Perf⁻IFN- γ ⁺. GrzB⁺ cells were more frequent, with 42.3% (median; range 34.1–50.8%) of responding T cells having a GrzB⁺Perf⁻IFN- γ ⁺ phenotype, although similar levels of IFN- γ ⁺ T cells possessed neither GrzB nor Perf (median 47.6%; range 40.0–53.2%). Similar data were obtained for SEB-stimulated CD8⁺ T cells, although higher numbers of cells lacking granule proteins were observed (median 58%; range 44.5–70.9%).

Likewise, increases in IFN- γ ⁺ T cells were detected in GrzB⁺, GrzB⁻, and Perf⁻ T cell populations, but not Perf⁺ cells, following activation with HSV-2 peptides (Fig. 7c, d). Comparing averaged HSV-2 responses within subjects, a 0.14% (median; range 0.04–0.28%) increase in IFN- γ ⁺GrzB⁺ and 0.18% (median; range 0.11–0.41%) increase in IFN- γ ⁺GrzB⁻ cells above baseline (DMSO control) values was observed. The frequency of IFN- γ ⁺ T cells increased by 0.17% (median; range 0.09–0.24%)

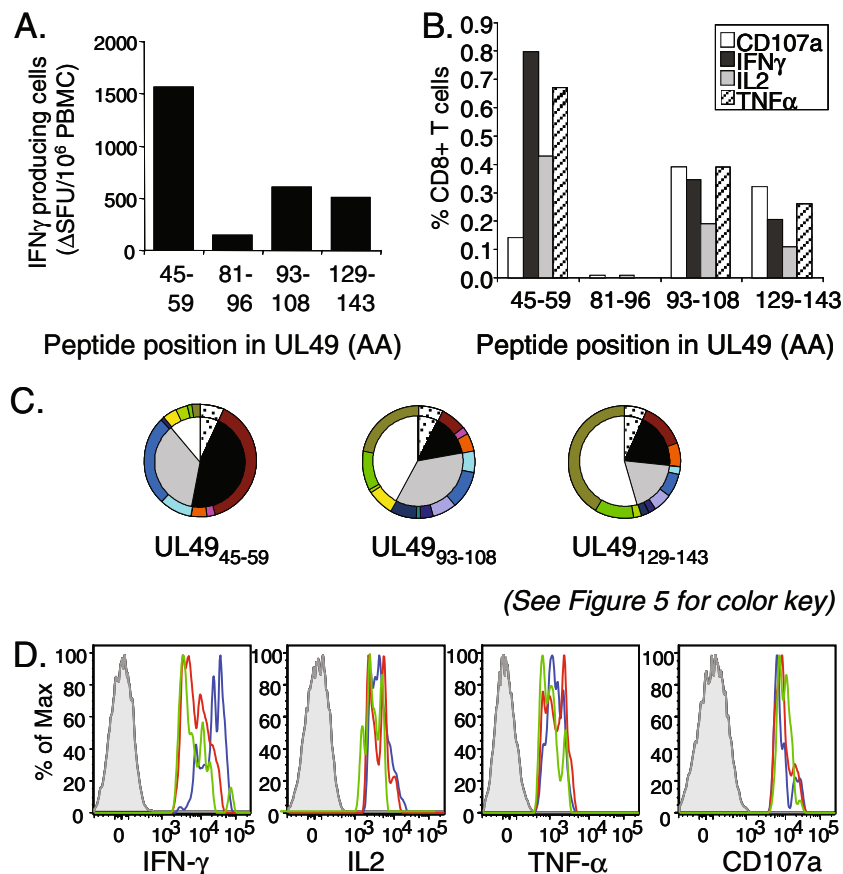
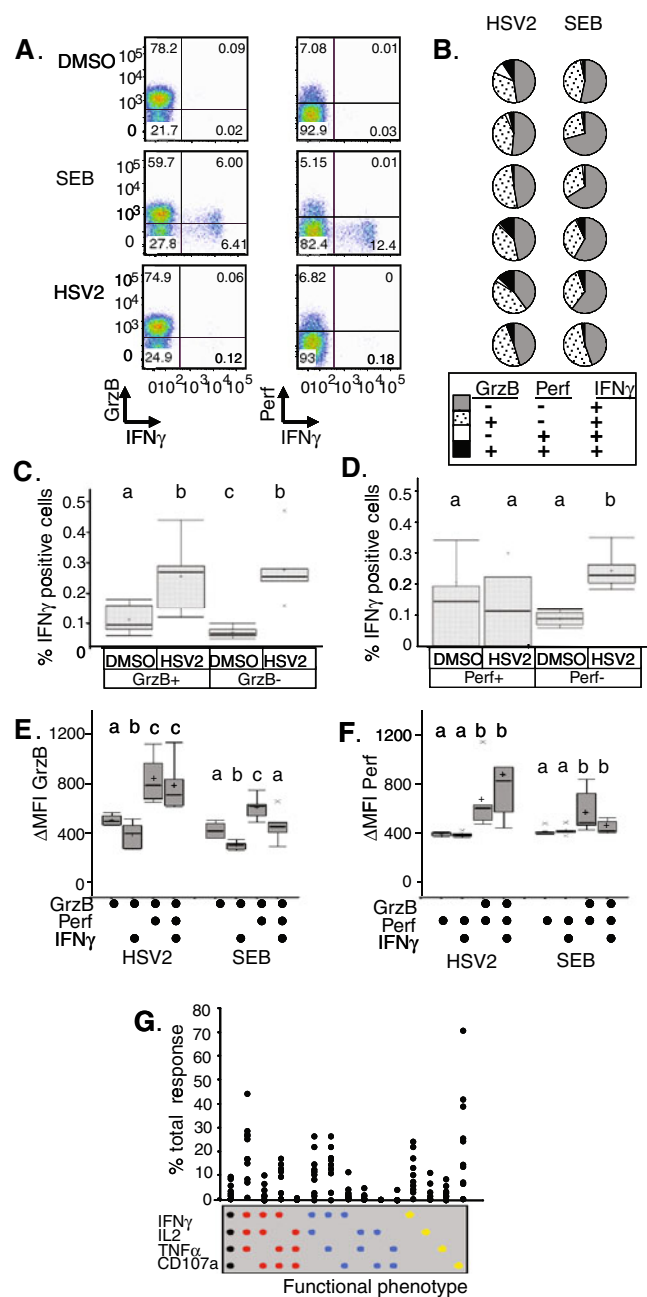


Fig. 6 Multiple HSV-2 epitope-specific T cells can be detected within a single individual. **a** ELISpot detection of IFN- γ production in PBMC incubated in the presence of HSV-2 UL49 specific peptides in a single HSV-2 seropositive subject. **b** ICS detection of IFN- γ , IL-2, TNF- α , and CD107a in CD8⁺ T cells of a single HSV-2 seropositive individual following incubation of PBMC with UL49 specific peptides. *Bars* show percent of CD8⁺ T cells that express each marker. **c** Proportions of CD8⁺ T cells that express one, two, three, or

four functional markers for three UL49-derived peptides are shown in the *central pies*; *outer circles* show representative phenotypes. The same color key was used as shown in Fig. 5. **d** Relative levels of each of the four functional proteins, estimated by median fluorescence intensity levels. *Gray shaded histogram* represents cells negative for a given protein; *blue, red, and green histograms* represent CD8⁺ T cells specific for peptides UL49_{45–59}, UL49_{93–108}, or UL49_{129–143}, respectively

Fig. 7 Detection of granzyme B and perforin in HSV-2-specific CD8⁺ T cells. **a** Representative plot of IFN- γ ⁺ cells in CD8⁺ T cells versus either granzyme B (GrzB) or perforin (Perf) following challenge with DMSO (negative control), SEB, or HSV-2 peptide. **b** The proportion of CD8⁺IFN- γ ⁺ T cells that express, co-express or lack Perf or GrzB following stimulation with HSV-2 peptides (averaged response of multiple peptides within an individual) or SEB. Each row represents a different subject. **c** CD8⁺ T cells were separated into GrzB⁺ and GrzB⁻ populations, and the percent IFN- γ ⁺ events quantified following stimulation with HSV-2 peptides or DMSO (negative control). Box-plots compare the average response per subject for all HSV-2 peptides, or DMSO control, in GrzB⁺ or GrzB⁻ CD8⁺ T cells. **d** As in panel c with Perforin⁺ and Perforin⁻ populations. **e** Box-plots comparing the change in median fluorescence intensity (Δ MFI) of GrzB in CD8⁺ T cells co-expressing perforin and/or IFN- γ . **f** Box-plots comparing the change in median fluorescence intensity (Δ MFI) of perforin in CD8⁺ T cells co-expressing GrzB and/or IFN- γ . For all box plots (e–f), gray boxes represent 25–75% range, and bars represent the 5–95% range, while the horizontal line within the bar represents the median ($n=6$). Letters (a, b, or c) displayed above the bars indicate differences between groups; if groups are significantly different (Friedman non-parametric test with Conover posthoc test for pairs; $p < 0.05$) within a graph, they are assigned a distinct letter. **g** Functional phenotypes (as per Fig. 3) of activated CD8⁺ T cells observed during ICS assays in cells stimulated with the same epitopes used to detect granzyme and perforin in HSV-2-specific T cells



relative to controls in Perf negative cells from cultures exposed to HSV-2 peptides. Although, this increase was not observed in Perf⁺ CD8⁺ T cells (median increase 0%; range 0–0.45%), the analysis was limited by low numbers of cells with this phenotype.

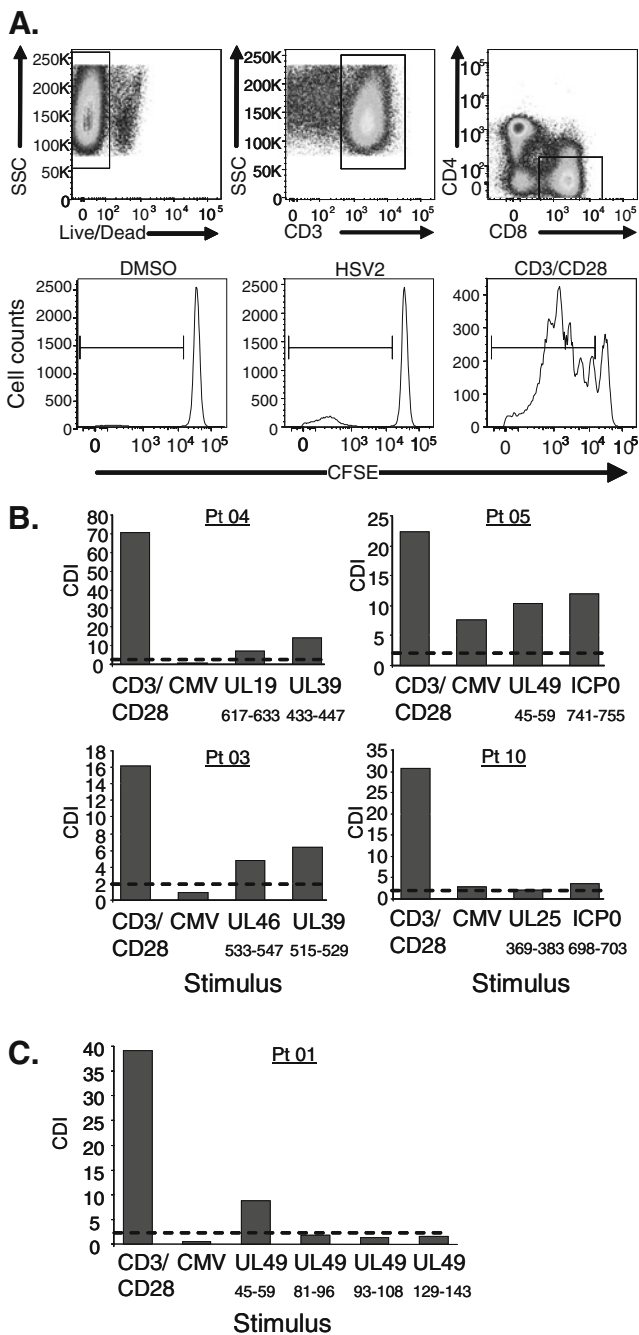
HSV-2-specific T cells lacking lytic proteins may have released their granule contents in response to peptide activation prior to intracellular staining. To assess this, we investigated whether GrzB or Perf levels decreased in IFN- γ ⁺ (activated) T cells. Mean fluorescent intensities (Δ MFI; see “Methods”) were used for estimating the relative levels of GrzB or Perf in cells co-expressing either granule protein and/or IFN- γ (Fig. 7e, f). Δ MFI for GrzB were approximately twofold higher in HSV-2 peptide exposed CD8⁺ T cells that co-expressed Perf (median Δ MFI=782; $p < 0.001$) or Perf and IFN- γ (median Δ MFI=707; $p < 0.001$) than in cells that expressed IFN- γ but lacked Perf (median Δ MFI=398), similar to the relative Δ MFI of GrzB in SEB-challenged T cells. The GrzB⁺IFN- γ ⁺ cells that lack Perf have likely released Perf in response to T cell activation by their specific antigen; reduced GrzB levels in these cells is consistent with co-release of GrzB during degranulation. Similarly, Perf levels were higher in CD8⁺ T cells that co-expressed GrzB (median Δ MFI=600; $p < 0.001$) or GrzB and IFN- γ (median Δ MFI=820; $p < 0.001$) than in GrzB⁻IFN- γ ⁺ CD8⁺ T cells (median Δ MFI=384), consistent with release of Perf and GrzB following activation of the antigen-specific T cells.

The same HSV-2 peptides were able to increase CD107a mobilization, as well as release cytokines, in all but one of the subjects tested (Fig. 7g), relating evidence that HSV-2-

specific T cells can degranulate with observed decreases in lytic proteins.

Proliferation of HSV-2-Specific CD8⁺ T Cells

To evaluate the proliferative capacity of HSV-2-specific CD8⁺ T cells, we utilized CFSE to stain PBMC prior to epitope-specific stimulation (Fig. 8a). Four subjects were each tested with two distinct CD8⁺ T cell peptides; a CDI index > 2 was seen with all HSV-2 epitopes from all four subjects (Fig. 8b). Variability was seen in the CDI index (range=2.1–14, median=7). This variability was also seen



with the CDI index to the four distinct epitopes with UL49 of Pt 01 (Fig. 8c). CD8⁺ T cells that recognized UL49_{45–49} did proliferate (CDI=8.7), but CDI values were below threshold for peptides UL49_{81–96}, UL49_{93–108}, and UL49_{129–143} (respective CDI=1.8, 1.4, and 1.6). Since T cells specific for UL49_{45–49} are more abundant than those specific for the other epitopes, the ability of these cells to proliferate likely reflects the epitope dominance observed within UL49. Of interest, this epitope also generated the lowest frequency of CD107a⁺ CD8⁺ T cells relative to the other UL49 derived epitopes during polyfunctional analysis

Fig. 8 The proliferative capacity of HSV-2-specific CTL was estimated by measuring the frequency of CFSE^{lo} CD8⁺ T cells following incubation with HSV-2 derived peptides. PBMC of HSV-2 seropositive individuals were incubated with previously determined stimulatory peptides, negative control (DMSO), or positive control stimuli. CD3⁺CD8⁺ cells were isolated by flow cytometry, and cells with ≥2× reduction in CFSE staining were counted (a); these are considered proliferated cells. CFSE^{lo} frequency of antigen-challenged cells was divided by the CFSE^{lo} frequency in negative controls to provide the cell division index (CDI); these values are shown in *bar graphs* for subjects tested with two peptides from distinct HSV-2 ORFs (b) and one individual tested with four distinct peptide epitopes from the same ORF, UL49 (c). Proliferation was considered positive when CDI ≥2; this value is shown with *dashed lines* on each graph

(Fig. 6), reflecting the inverse relationship of proliferation and degranulation abilities of these T cell populations.

Discussion

Our studies provide several new insights about the CD8⁺ T cell response to HSV-2 in immunocompetent persons with genital herpes. This investigation is the first to use a newly optimized high-throughput flow cytometry technique to detect HSV-2-specific T cell responses. Given the low level of HSV-2-specific CD8⁺ T cells relative to T cells that respond to other viruses, our ability to detect these cells and monitor differential cytokine production represents a significant methodological advance in HSV immunology. Using this novel method, we confirm that HSV-2-specific CD8⁺ T cells, although varied between individuals, are often directed at the immediate-early, capsid and tegument proteins and can be detected without prior enrichment from PBMC. Interestingly, at the single cell level, many of the CD8⁺ T cells appear to be of the effector phenotype and secrete IFN-γ alone. Polyfunctional responses correlate with a high magnitude of T cell response and are often found at higher levels when induced with single peptides than in assays induced with peptide pools. The latter phenomenon likely reflects methodological issues, related to higher levels of DMSO added to cultured cells when using peptide pools (0.5% v/v) than single peptides (0.005%), since DMSO has inhibitory effects on T cells [20]. HSV-2-specific CD8⁺ T cells store lytic proteins and are able to degranulate and proliferate but do show considerable inter- and intra-individual variation. Even within an individual and with responses directed at a single ORF, there is considerable variation in the magnitude and function of T cell responses.

Proteins encoded by immediate-early or early genes were the predominant targets for HSV-2-specific CD8⁺ T cells and induced IFN-γ, IL-2, and TNF-α production. IFN-γ responses were identified for a higher proportion of

subjects, consistent with HSV-2-specific T cells having a predominantly effector phenotype with lower levels of IL-2 and TNF- α production. The highest frequency of CD8⁺ T cell responses were detected for the immediate-early protein, ICP0, whose gene is one of the first expressed following HSV reactivation [21] and UL39 (ICP10), which is transcribed with early kinetics [22]. In contrast, late genes, such as the glycoproteins gB, gD, and gJ, were rarely recognized by CD8⁺ T cells; IFN- γ producing CD8⁺ T cells specific for these ORFs were found in significantly fewer persons than those recognizing early ORFs. Although not thought to occur during HSV infections [23], a similar distinction was previously observed for virus-specific T cell responses, with up to threefold more responses observed against immediate-early proteins than late proteins from CMV [24, 25]. This may occur because viral gene products synthesized with earlier kinetics become processed and presented by the host cell presentation machinery, to control viral activity, before late gene products are produced. Alternatively, the virus may employ a mechanism such as inhibition of TAP activity by HSV ICP47 [26, 27], encoded by immediate-early gene US12, to reduce the likelihood of peptides from proteins synthesized later during the viral lytic phase being presented to T cells of the host. As we were primarily concerned with investigating the functional characteristics of HSV-2-specific T cells, the present investigation concentrated on screening prevalent HSV-2 ORF responses in a generalizable population of HSV-2 infected patients. Study subjects were, hence, distinct to our original studies, were newly enrolled and assessed, and we were required to identify HSV-2-specific responses in our cohort de novo. Whether investigation of less prevalent ORFs would provide different insights is of course unknown.

For many HSV-2-specific CD8⁺ T cell responses, we succeeded at identifying the single peptide antigen and could predict the probable HLA-restricted epitopes embedded within. Epitopes derived from ORFs expressed after immediate-early stages lacked diversity and variety in HLA restriction, contrasting the high diversity of identified epitopes, and associated HLA restrictions were observed for ICP0. This is consistent with the hypothesis that ICP47 inhibits TAP and, therefore, TAP-dependent HLAs during later stages of viral activity. Although we should bear in mind that our methods may favor activation of cells restricted by HLAs that bind exogenous peptide from endosomal compartments rather than T cells that are specific for TAP-dependent epitopes, TAP-independent epitope presentation pathways or proteins made early during the lytic phase of viral activity should be considered during the design of therapeutic or preventative interventions that invoke T cell responses against HSV. TAP-independent presentation of HSV-derived peptides in mice

has been suggested previously [28]. Further confirmation of TAP-dependency on HSV-2 epitope processing and loading in humans is required.

While multiple epitopes were evident across several HSV-2 ORFs, common epitopes were identified and constituted the majority of responses for UL49—the most abundant tegument protein, VP22, that is expressed early [29]—and UL25—an abundant C-capsid-associated protein with leaky-late expression that assists in maturation, packaging, and retention of viral DNA, and increases capsid stability [30–34]. A high frequency of subjects responded to UL49, confirming the findings of previous investigations [7, 35]. In UL49, a previously confirmed common and potent HLA-B07 restricted epitope (RPRGEVRFL) [35, 36] was responsible for CD8⁺ T cell activation in most responding subjects. However, we also identified four novel UL49 epitopes; three were predicted to be restricted by HLA-B07 with one (UL49_{249–263}) restricted by HLA-B08. The common epitope (FLWEDQTLL) found in UL25 is the first common CD8⁺ T cell epitope identified for HSV-2 that is restricted by HLA-A*0201; the same epitope may also be presented by HLA-A*0202, *0203, and *0206. Accordingly, all subjects identified with CD8⁺ T cell responses to UL25 possessed an HLA-A2 allele. FLWEDQTLL is conserved in UL25 of HSV-1 and is thus potentially type common; responses were detected in HSV-1⁺/HSV-2⁺ and HSV-1⁺/HSV-2⁺ individuals. Since the epitopes identified in UL49 and UL25 were recognized by T cells of multiple individuals and utilize HLA-types that are common in the population, development of T cell-inducing HSV vaccines with a wide population coverage may benefit from inclusion of these targets [37].

Screening for HSV-2-specific CD8⁺ T cells facilitated investigating our major interest—to determine if functional effector CD8⁺ T cells could be identified in subjects with chronic HSV-2 infection. Chronic viral infections often cause T cell exhaustion that culminates in loss of cytotoxic function, proliferation, and cytokine production [38], and we predicted this would be the case for HSV-2 infection. However, HSV-2-specific CD8⁺ T cells did not appear dysfunctional. Polyfunctionality within HSV-2 epitope-specific CD8⁺ T cell populations was confirmed for the first time in our study. Since polyfunctionality is associated with better viral control [17–19], HSV-2-specific T cells appear to have retained some high level effector function to control HSV-2 reactivations even after long-term infection. CD8⁺ T cell populations that were activated by HSV-2 peptides comprised a mixture of monofunctional and polyfunctional cells with differing ability to produce IFN- γ , IL-2, and TNF- α or to degranulate. IFN- γ levels were higher in polyfunctional cells than in monofunctional T cells, similar to vaccinia virus-specific T cells identified in a

previous study [14]. Also consistent with other studies, IL-2 was typically lower in cells that mobilize CD107a, suggesting that cells that degranulate have less potential to induce proliferation, or vice versa. IL-2 was also the least abundant cytokine—in terms of detectable cellular frequency—in our assay; higher frequencies of HSV-2-specific CD8⁺ T cells made TNF- α and IFN- γ . CD8⁺ T cells are known to produce less IL-2 than CD4⁺ T cells [39], thus observing that lower frequency of IL-2⁺ than IFN- γ ⁺ CD8⁺ T cells is typical and not an indicator of dysfunction [40, 41], thus HSV-2-specific T cells do not appear to be visibly impaired.

In addition to producing multiple cytokines, a high frequency of CD8⁺ T cells mobilized CD107a, an indicator of degranulation, and had decreased granzyme B and perforin levels following exposure to HSV-2-derived peptides; this implies that HSV-2-specific CD8⁺ T cells can kill infected cells by releasing cytotoxic mediators from granules and supports the hypothesis that these T cells can control virally infected cells by means of degranulation. In a recent study in mice, granzyme B has been shown to control HSV reactivation by directly damaging ICP4, an essential HSV protein with a role in maintaining viral latency [42]. It would be of use to know if viral control of HSV-2 by granzyme B also occurs in humans.

We also confirmed that HSV-2-specific CD8⁺ T cells retain the ability to proliferate, although there are clear differences in the magnitude and proliferative ability of distinct T cell clonotypes that appear related to the level of activation achieved by those cells and opposed to a preponderance to degranulate. Our observation was verified during a recent study that compared proliferation and cytotoxicity of CD8⁺ T cells specific for several different viruses; markers associated with proliferation were identified on distinct T cell populations from markers associated with degranulation and cytotoxicity, whereby central memory T cells had high proliferative capacity but low cytotoxicity, and both effector memory and terminal effector memory subsets displayed high cytotoxic activity but low potential to proliferate [43].

Conclusions

In summary, our study demonstrates the use of ICS for confirming polyfunctionality of HSV-2-specific CD8⁺ T cells, and for identifying numerous novel epitopes responsible for activating these T cells from selected HSV-2 ORFs. Ex vivo analysis of the HSV-2-specific T cells revealed a variety of effector functions, raising the question whether CD8⁺ T cell function is associated with the frequency and severity of HSV-2 reactivation in the genital mucosa. The relationship between disease severity and the

diversity of cytokine proteins, kinetics of proliferation or degranulation are questions raised by our study, such that a future investigation into the ability of cells with different functional profiles to control viral replication is on our agenda. The epitopes discovered during this investigation and the flow cytometric techniques we have optimized will be highly beneficial tools that can be utilized for answering such questions.

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