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HLA-A*0201-Restricted CD8+ Cytotoxic T Lymphocyte Epitopes Identified from Herpes Simplex Virus Glycoprotein D1

Aziz Alami Chentoufi,2* Xiuli Zhang,2* Kasper Lamberth,† Gargi Dasgupta,* Ilham Bettahi,* Alex Nguyen,* Michelle Wu,* Xiaoming Zhu,* Amir Mohebbi,* Søren Buus,‡ Steven L. Wechsler,* Anthony B. Nesburn,* and Lbachir BenMohamed3*‡

Evidence obtained from both animal models and humans suggests that T cells specific for HSV-1 and HSV-2 glycoprotein D (gD) contribute to protective immunity against herpes infection. However, knowledge of gD-specific human T cell responses is limited to CD4+ T cell epitopes, with no CD8+ T cell epitopes identified to date. In this study, we screened the HSV-1 gD amino acid sequence for HLA-A*0201-restricted epitopes using several predictive computational algorithms and identified 10 high probability CD8+ T cell epitopes. Synthetic peptides corresponding to four of these epitopes, each nine to 10 amino acids in length, exhibited high-affinity binding in vitro to purified human HLA-A*0201 molecules. Three of these four peptide epitopes, gD53–61, gD70–78, and gD278–286, significantly stabilized HLA-A*0201 molecules on T2 cell lines and are highly conserved among and between HSV-1 and HSV-2 strains. Consistent with this, in 33 sequentially studied HLA-A*0201-positive, HSV-1-seropositive, and/or HSV-2-seropositive healthy individuals, the most frequent and robust CD8+ T cell responses, assessed by IFN-γ ELISPOT, CD107a/b cytoplasmic degranulation, and tetramer assays, were directed mainly against gD53–61, gD70–78, and gD278–286 epitopes. In addition, CD8+ T cell lines generated by gD53–61, gD70–78, and gD278–286, peptides recognized infected target cells expressing native gD. Lastly, CD8+ T cell responses specific to gD53–61, gD70–78, and gD278–286 epitopes were induced in HLA-A*0201 transgenic mice following ocular or genital infection with either HSV-1 or HSV-2. The functional gD CD8+ T cell epitopes described herein are potentially important components of clinical immunotherapeutic and immunoprophylactic herpes vaccines. The Journal of Immunology, 2008, 180: 426–437.

Herpes simplex types 1 and type 2, HSV-1 and HSV-2, are infectious pathogens that cause serious diseases at every stage of life, including fatal disseminated disease in newborns, cold sores, genital ulcers, eye disease, and fetal encephalitis in adults (1–6). Recurrent ocular herpes disease (primarily HSV-1) causes herpetic stromal keratitis, an immunoinflammatory and immunopathological lesion of the cornea that can lead to blindness (1, 2, 7, 8). More than 80% of adults shed reactivated HSV in their tears many times per year, and >450,000 people in the U.S. have a history of recurrent ocular herpes disease (1, 9). Recurrent genital herpes disease (primarily HSV-2), termed herpes genitalis, also has an immunoinflammatory and immunopathological course that leads to the development of genital lesions, ulcers, and scarring (7, 8). Genital herpes affects up to 33% of adults in the United States and the European Union (1, 7, 10). Together, ocular and genital herpes represent an enormous reservoir for horizontal and vertical transmission (1, 11). The rapid spread of herpes infection, which occurs mostly during unrecognized asymptomatic shedding, is reflected in more than 1 million new cases per year in the United States alone (1, 11, 12).

Despite the availability of many interventional strategies such as sexual behavior education, barrier methods, and costly guanidine nucleoside antiviral drug therapies (e.g., Acyclovir and derivatives), controlling the spread of ocular and genital herpesvirus remains a challenge as clinical manifestation rates have continued to rise over the last decade (8, 9, 13). An effective immunoprophylactic and/or immunotherapeutic vaccine would be the most cost-effective approach that could be helpful not only in developed nations but also in underdeveloped regions such as some sub-Saharan African areas, where 70% of high-risk, HIV-negative individuals and 85% of HIV-infected individuals are seropositive for herpes (12, 14, 15). To date, however, no clinical herpes vaccine is yet available. Several challenges face its development, including the identification of target Ags and derived human epitopes and uncertainty about the effector mechanisms mediating protective immunity.

Among some 11 herpes glycoproteins (2, 7, 8), the glycoprotein D (gD)4 has been successful in eliciting T cell-mediated immunity

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4 Abbreviations used in this paper: gD, glycoprotein D; LCL, lymphoblastoid cell line; MFI, mean fluorescence intensity; rVV, recombinant vaccinia virus; SFU, spot-forming unit; Tg, transgenic; VVGd, vaccinia virus expressing gD.
in mouse, rabbit, and guinea pig herpes models when delivered emulsified in experimental Freund’s or derivative adjuvants (18, 16).

However, in the two most recent phase II/III clinical trials, the delivery of gD with the clinical adjuvants MF59 and alum-mono-

phosphoryl lipid A only induced moderate and transient protection or only protected previously uninfected women while failing to protect men or HSV-1-seropositive women (4, 10, 17, 18). In both trials, the HSV-specific Ab responses were similar to those of nat-

ural HSV infection. However, in vitro studies of T cell responses induced by the gD vaccines were limited to IFN-γ production, as no functional human CD8+ T cell gD epitopes have been previ-

ously identified (4, 10). The mapping of human minimal CD8 functional IFN-γ has been a major concern in the develop-

ment of effective immunotherapeutic and immunoprophylactic vaccine strategies. The sera collected from 61 patients were tested for HSV-1 and HSV-2 type specific serotyping.

Materials and Methods

BioInformatics analyses

HSV-1 gD open reading frames used in this study were from strain 17 (GenBank/GenPept accession no. Q69091). The 394-aa sequence of the immature gD protein, including the 25-aa leader sequence, was screened for HSV-A*0201 binding regions. The HLA-A*0201 supertype family is represented in >50% of the world’s population irrespective of gender and ethnicity (19). Using several immunological parameters, including peptide binding affinity to purified HLA-A*0201 molecules, stabilization of HLA-A*0201 molecules on T2 cells by high-affinity gD peptides, ex vivo tetramer staining, and in vitro functional IFN-γ ELISPOT and cytotoxic CD8+ T cell assays, we found three CD8+ T cell epitopes: gD53–61, gD70–76, and gD278–286. These minimal T cell epitopes are highly conserved among and between HSV-1 and HSV-2 strains. They recalled functional HSV-specific CD8+ T cells in HLA-A*0201-positive individuals that were seroresistant for HSV-1, HSV-2, or both and in humanized HLA-A*0201 transgenic (Tg) mice ocularly or intravaginally in-

fected with either HSV-1 or HSV-2.

Peptide synthesis

To determine potential vaccine candidate, 10 HLA-A*0201 binding pep-

tides of herpes simplex virus type 1 (strain 17) glycoprotein D (GenBank/ GenPept accession no. Q69091) have been selected based on predictive computational algorithms using a computer-based program accessed through the National Institutes of Health BioInformatics and Molecular Analysis Section (BIMAS) software (National Institutes of Health, Bethesda, MD; http://

bimas.dct.nih.gov/gha/bind/). The SYFPEITHI algorithm (http://www.syfpeithi.de/) (20). Potent cleavage sites for human protea-

some were identified using NetChop 3.0 (http://www.cbs.dtu.dk/services/ NetChop/) (21) and MHC Pathway (http://www.mhc-pathway.net) and were also used in this screening (22).

MHC-peptide binding assays

gD peptide epitope binding with purified HLA-A*0201 molecules was per-

formed as previously described (30–32). Briefly, denatured and purified recombinant HLA-A*0201 heavy chains were diluted into a renaturation buffer containing β2-microglobulin and graded concentrations of the test peptide and incubated at 18°C for 48 h, allowing equilibrium to be reached. The concentration of peptide-HLA complexes generated was measured in a quantitative ELISA and plotted against the concentration of peptide of-

tered (30). Because the effective concentration of HLA-A*0201 (2 nM) used in this assay is below the equilibrium dissociation constant (Kd) of most high-affinity peptide-HLA interactions, the peptide concentration leading to half-saturation of the HLA is a reasonable approximation of the affinity of the interaction. The concentration of peptide-HLA complexes generated was measured in a quantitative ELISA and plotted against the concentration of peptide of-

tered (30). Because the effective concentration of HLA-A*0201 (2 nM) used in this assay is below the equilibrium dissociation constant (Kd) of most high-affinity peptide-HLA interactions, the peptide concentration leading to half-saturation of the HLA is a reasonable approximation of the affinity of the interaction.

Stabilization of HLA-A*0201 on class I-HLA-transfected B × T hybrid cell lines (T2 lines)

to determine whether synthetic peptides could stabilize HLA-A*0201 molecule expression on the T2 cell surface, peptide-inducing HLA-A*0201 up-regulation on T2 cells was examined according to a protocol described previously (33, 34). T2 cells (3 × 10^5/well) were incubated with different concentration of individual gD peptide (as indicated in Fig. 1) in 48-well plates for 18 h at 26°C. Cells were then incubated at 37°C for 3 h in the presence of 0.7 µl/ml Golgi Stop (BD Pharamingen) to block cell expression of newly synthesized HLA-A*0201 molecules and human β-2 microglobulin (1 µg/ml). The cells were washed with FACS buffer (1% BSA and 0.1% sodium azide in PBS) and stained with anti-HLA-A21-specific mAb BB7.2 (BD Pharmingen) at 4°C for 30 min. After incubation, the cells were washed with FACS buffer, fixed with 1% paraformaldehyde in PBS, and analyzed by flow cytometry and analyzed by CellQuest software (BD Biosciences). The acquired data were analyzed with CellQuest software (BD Biosciences). Expression was measured by a FACSCalibur cytometer (BD Biosciences) and mean fluorescence intensity (MFI) was recorded. The percentage of MFI increase was calculated as follows: percentage MFI increase = (MFI with the given peptide − MFI without peptide)/(MFI without peptide × 100. Each experiment was performed three times and means ± SD values were calculated.

HLA-A*0201 Tg mice

HLA-A*0201/Kb (referred to throughout as HLA-A*0201) Tg mice provided by Dr. F. Lemmonier (Pasteur Institute, Paris, France) and supplied by Dr. D. Diamond (City of Hope, Duarte, CA) were bred at the University of California, Irvine, CA. These mice represent the F1 generation resulting from a cross between HLA-A*0201/Kb Tg mice (expressing a chimeric gene consisting of the α1 and α2 domains of HLA-A*0201 and the α1, β1, and β2 domains of H-2Kd) created on the C57BL/6 background with BALB/c mice (The Jackson Laboratory). All studies were conducted in facilities appo-

proved by the Association for Assessment and Accreditation of Laboratory Animal Care and according to Institutional Animal Care and Use Commit-

tee-approved animal protocols.

Cells

JAU2.1 cells are human Jurkat cells that express the HLA-A*0201/Kb chi-

meric gene (25, 26), and LPS blasts were prepared as previously described (27–29, 35). T2 and RS cells (both from American Type Culture Collection) were grown as directed by the supplier.

PBMC isolation

Healthy individuals (negative for HIV and hepatitis B virus and without any HSV infection history) were recruited at University of California Ir-

vine General Clinical Research Center. Approximately 175 ml of an indi-

vidual’s blood was drawn into yellow-top Vacutainer tubes (BD Bio-

sciences). The serum was isolated and underwent centrifugation for 10 min at 800 × g. The PBMCs were isolated by gradient centrifugation using leucocyte separation medium (Cellgro). The cells were washed in PBS and resuspended in complete culture medium consisting of RPMI 1640 me-

edium containing 10% FBS (Bio-Products) supplemented with 1× penicillin/ 

l-glutamine/streptomycin, 1× sodium pyruvate, 1× nonessential amino acids, and 50 µM 2-ME (Invitrogen Life Technologies). Aliquots of freshly isolated PBMCs were also cryopreserved in 90% FCS and 10% DMSO in liquid nitrogen for future testing.

HSV-1 and HSV-2 type specific serotyping

The sera collected from 61 patients were tested for HSV-1 and HSV-2 status using HerpeSelect IgG1 and HerpeSelect IgG2 HSV ELISA kits (Focus Diagnostics) as previously described (36). Although in general this
In vivo generation of gD-peptide specific CD8 T cell line

CD8 T cell lines were isolated from PBMCs using a CD8 T cell isolation kit (BD Pharmingen) as previously described (37). Briefly, LCLs were derived from patients with PBMCs stimulated with PHA (4 μg/ml) in RPMI 1640 plus 10% FCS for 3 days. Half of the medium was then replaced by fresh medium containing IL-2 (10 ng/ml) and IL-7 (20 ng/ml). On day 6, the LCLs were infected overnight with an empty vaccinia virus (control), a vaccinia virus expressing gD (VVgD), HSV-1, or HSV-2, each at a multiplicity of infection of 10. The next day, infected cells were washed three times and treated with mitomycin C (50 μg/ml). The LCLs were then washed three times before a 6-h incubation with effector CD8 T cells at the indicated E:T ratio. The cytotoxic activity was detected in a CD107a/b degranulation assay as previously described (40).

In vitro generation of gD-peptide specific CD8 T cell line

CD8 T cells were isolated from PBMCs using a CD8 T cell isolation kit II (MACS; Miltenyi Biotec). A CTL line was established by stimulating CD8 T cells (2 x 10^6/ml) with mitomycin C-treated (50 μg/ml) autologous PBMCs (10^6/ml) incubated 3 h with individual peptides (20 μM). IL-2 (5 ng/ml; R & D Systems) was added to the culture every other day starting on day 3. On day 7, cells were restimulated with mitomycin C-treated autologous individual peptide-pulsed PBMCs (10^6/ml) and IL-2 (20 ng/ml) added every other day. On day 14, cultures were expanded and restimulated as described above for another week and used as effector cells for CD107 assay.

IFN-γ ELISPOT assays

T cell stimulation was measured by IFN-γ production in peptide-stimulated PBMCs using a BD IFN-γ ELISPOT kit (BD Pharmingen) as previously described (41). Briefly, 5 x 10^6 PBMCs were stimulated with individual gD peptides (20 μM) or with heat-inactivated HSV-1 (strain McKrae) (multiplicity of infection of 5) for 6 days. Then, activated PBMCs were harvested, washed, and stimulated with the gD peptides for 24 h in IFN-γ ELISPOT plates (Millipore) that have been previously coated with anti-human IFN-γ capture Abs in a humidified incubator at 37°C with 5% CO2. The spot-forming cells were developed as described by the manufacturer (BD Pharmingen) and counted under a stereo microscope.

CD107 cytotoxicity assay

To detect cytolytic CD8 T cells recognizing gD peptides in freshly activated and in vitro activated PBMCs, we used the CD107a/b cytotoxicity assay. Betts and colleagues (40, 42) recently described the CD107 assay as an alternative cytotoxicity assay that is able to address some of the shortcomings of the 51Cr release assay. The CD107 assay was performed as described by Betts and colleagues (40, 42) with a few modifications. On the day of the assay, unstimulated or in vitro gD peptide-stimulated PBMCs (7, 14, or 21 days) were incubated at 37°C for 5 to 6 h with BD Golgi Stop (BD Biosciences), costimulatory anti-CD28 and anti-CD49d Abs (1 μg/ml), and 10 μl of CD107a-FITC and CD107b-PE. At the end of the incubation period the cells were harvested into separate tubes and washed once with FACS buffer then stained with PE-conjugated anti-human CD8 Ab for 30 min. The cells were then washed again and analyzed using a FACScan (BD Biosciences).

Statistical analyses

To evaluate whether a CD8 T cell response to peptide-pulsed or recombinant vaccinia virus (rVV)-HSV-infected target cells was statistically significant, we used the Student’s t test to compare two sets of variables: 1) mean IFN-γ spots generated by CD8 T cells (from HSV-seropositive humans or infected HLA-A*0201 mice) in response to JA2.1 cells pulsed with peptide (relevant vs irrelevant) or infected with an rVV or HSV construct (relevant vs irrelevant); or 2) mean IFN-γ spots generated in response to peptide-pulsed or rVV- or HSV-infected JA2.1 cells by CD8 T cells from infected vs noninfected humans or mice. We used 2-by-2 contingency tables to determine whether an association existed between peptides with binding affinities of <100 nM or >100 nM and peptides that were immunogenic in HSV seropositive human or infected HLA-A*0201 mice. The p values from the two-tailed Fisher exact test are reported.

Results

In silico prediction and in vitro binding of potential HSV-1 gD

The HLA-A*0201 allele, the prototypic member of the HLA-A2 supertype family, is predominant in the human population (26–29). In addition, mice transgenic for this molecule are available (26–28). We therefore decided to search the deduced HSV-1 (strain 17) gD amino acid sequence (including the signal sequence indicated below by negative numbers) for potential HLA-A*0201 binding regions using BIMAS, SYFPEITHI, MAPPP, and MHC Pred predictive computational algorithms. Based on these analyses, 10 potential peptide epitopes with high predicted affinity to HLA-A*0201 molecules were selected (Table I). Nine of the 10
gD peptide epitopes shared the HLA-A*0201-binding motifs: leucine, isoleucine, or methionine at the second position and a valine, alanine, leucine, methionine, or threonine at the ninth position. Based on the above computational algorithms, these gD peptides, bearing putative antigenic and immunogenic HLA-A*0201-binding epitope(s) are probably less constrained than other parts of the gD molecule and are therefore more readily accessible to proteolysis, an event that precedes T cell epitope presentation in association with HLA molecules (26–29, 43). Three of the 10 predicted epitopes are located within the gD signal sequence (gD13 to 11, gD11 to 12, and gD18 to 10). Four belong to the external N-terminal portion of gD (gD53–61, gD70–78, gD95–103, and gD153–161). Three are adjacent to the hydrophobic membrane anchor domain (gD224–232, gD253–262, and gD278–286). Interestingly, none of the predicted peptides are within the hydrophilic C-terminal cytoplasmic portion of gD, and all 10 peptides are in nonglycosylated regions.

The 10 highest probability gD epitope peptides were synthesized and screened for binding affinity to purified HLA-A*0201 molecules (Table I). The gD18 to 10, gD36–61, gD70–78, and gD278–286 peptides had the highest affinity binding to HLA-A*0201 molecules (Kd affinity values of 19–62 nM). Of the remaining six peptides, gD53–61, gD224–232, and gD253–262 showed medium affinity to HLA-A*0201 molecules (Kd affinity values of 2002–4891 nM), whereas gD13 to 11, gD11 to 12, and gD95–103 had low affinity (Kd > 50,000 nM). Overall, these results suggest that seven of the 10 gD peptides bind to HLA-A*0201, with gD53–61, gD70–78, and gD278–286 being the highest binders.

**High-affinity gD peptides stabilized HLA-A*0201 molecules on T2 cells**

We next performed a stabilization assay of HLA-A*0201 molecules on class I HLA-transfected B × T hybrid cell lines (T2 lines). This assay measures the increase of HLA-A*0201 molecules on the surface of T2 cells, which is normally at a low level (44, 45). Each gD peptide was tested at a concentration of 60 μM (not shown) and then titrated in serial 2-fold dilution steps (Fig. 1). Of the four peptides previously showing the highest affinity binding to purified HLA-A*0201 molecules, gD53–61, gD70–78, and gD278–286 peptides significantly increased levels of HLA-A*0201 molecules on the surface of the T2 cells (p < 0.005) in a dose-dependent manner (Fig. 1). In contrast, the remaining seven peptides produced no significant stabilization of HLA-A*0201 molecules detectable by FACS on the surface of T2 cells (Fig. 1).

**gD epitope-specific, IFN-γ-producing CD8+ T cells detected in healthy HLA-A*0201-positive, HSV-seropositive individuals**

We next assessed the ability of high and low affinity peptides to recall HSV-specific CD8+ T cells in a total of 61 HLA-A*0201-positive and HLA-A*0201-negative healthy individuals (Table II). All individuals had well-defined clinical histories with one or no episodes of recurrent disease per year (i.e., “protected asymptomatic” or “low-recurrent” disease), were healthy, hepatitis seronegative, and HIV seronegative. At the time of the study, none of them had experienced any clinical ocular or genital herpes symptoms for at least 1 year. In addition, the PBMCs from all 61 individuals produced a positive T cell response to the mitogens LeuA and PHA.

We used fresh peripheral blood-derived CD8+ T cells isolated directly ex vivo to minimize artifacts that could arise from in vitro restimulation of CD8+ T cells. The number of gD epitope-specific IFN-γ-producing CD8+ T cells was determined by ELISPOT assay. Analysis revealed that 95 or more spots per 10^5 CD8+ T cells produced no significant stabilization of HLA-A*0201 molecules detectable by FACS on the surface of T2 cells (Fig. 1).

**Table II. Cohort of individuals used in this study**

<table>
<thead>
<tr>
<th>Subject Level Characteristic</th>
<th>Results for All Subjects (n = 61)</th>
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</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male 28 (46%)</td>
</tr>
<tr>
<td></td>
<td>Female 33 (54%)</td>
</tr>
<tr>
<td>Race</td>
<td>Nonwhite 24 (39%)</td>
</tr>
<tr>
<td></td>
<td>White 37 (61%)</td>
</tr>
<tr>
<td>Age</td>
<td>31 (18–63 years)</td>
</tr>
<tr>
<td>HSV status</td>
<td>HSV-1-positive 19 (31%)</td>
</tr>
<tr>
<td></td>
<td>HSV-2-positive 8 (13%)</td>
</tr>
<tr>
<td></td>
<td>HSV-1- and HSV-2 positive 6 (10%)</td>
</tr>
<tr>
<td></td>
<td>HSV-negative 28 (46%)</td>
</tr>
<tr>
<td>HLA</td>
<td>A*0201-positive 34 (56%)</td>
</tr>
<tr>
<td></td>
<td>A*0201-negative 27 (44%)</td>
</tr>
</tbody>
</table>
gave a 99.5% probability for defining a positive response, and 95 spot-forming units (SFU) per 10^5 CD8\(^+\) T cells was therefore used as the cutoff point for subsequent analyses. Of the 560 separate ELISPOT wells used to screen the 27 HSV-seronegative individuals, only 11 wells (2%) exceeded 95 SFU per 10^5 CD8\(^+\) T cells.

Of the 1386 separate ELISPOT assays used to screen the seropositive individuals, 478 (17%) had readings of 95 SFU per 10^5 CD8\(^+\) T cells. Overall, 32 of 33 HSV-seropositive individuals responded to at least one of the gD peptides (i.e., had 95 or more spots per 10^5 CD8\(^+\) T cells).

Next, we compared the IFN-γ-producing CD8\(^+\) T cells from HLA-A*0201-positive vs HLA-A*0201-negative seropositive individuals (HSV-1 and/or HSV-2) (Fig. 2A). Positive IFN-γ-producing CD8\(^+\) T cell responses were assessed by comparison with control wells without Ag. For all but one peptide (gD_{224-232}), significantly more CD8\(^+\) T cells producing IFN-γ were seen in HLA-A*0201-positive compared with HLA-A*0201-negative individuals. The highest IFN-γ response appeared to be recalled by the gD_{53–61} epitope peptide (p < 0.0001; Fig. 2, A and B).

For each of the high-affinity peptides (gD_{53–61}, gD_{70–78}, and gD_{278–286}), significant IFN-γ-producing CD8\(^+\) T cell responses were detected in 45–95% of HLA-A*0201-positive, HSV-seropositive individuals. The highest frequency of IFN-γ-producing CD8\(^+\) T cell responses was detected against gD_{53–61} peptide epitope (95%). In comparison, only 14–24% of individuals showed positive T cell responses to the remaining low affinity peptides (gD_{13–19}, gD_{11–20}, gD_{18–24}, gD_{70–78}, gD_{95–103}, gD_{153–161}, and gD_{224–232}). There was no difference in T cell responses detected in individuals seropositive for HSV-1, HSV-2, and HSV-1/HSV-2 (data not shown). We then stimulated CD8\(^+\) T cells with high-affinity peptides (gD_{53–61}, gD_{70–78}, and gD_{278–286}) for 6 days and analyzed the T cell response. Fig. 2B shows IFN-γ producing CD8\(^+\) T cells specific to three immunodominant peptides detected in HLA-A*0201-positive but not in HLA-A*0201-negative individuals.
whether gD epitope-specific CD8\(^{+}\) T cells display lytic activity, fresh PBMC-derived CD8\(^{+}\) T cells from HLA-A*0201-positive and HLA-A*0201-negative individuals were stimulated in vitro with individual gD peptides and CD107a/b expression was examined by flow cytometry on gated CD8\(^{+}\) T cells from healthy HLA-A*0201-positive, HSV-seropositive individuals. Although, gD\(_{18-10}\), gD\(_{13-11}\), and gD\(_{224-232}\) peptides induced ex vivo significant CD107a/b expression on CD8\(^{+}\) T cells from HLA-A*0201-negative individuals. This suggests that the presentation of this particular epitope may be affected by factors in HSV-infected target cells. As a control, no cytolytic activity was detected when gD\(_{53-61}\)-, gD\(_{70-78}\)-, or gD\(_{278-286}\)-specific CD8\(^{+}\) T cell lines were incubated with HLA-A*0201-positive target cells infected with empty vaccinia virus (designated VVC (control) in Fig. 4). Therefore, these gD\(_{53-61}\), gD\(_{70-78}\), and gD\(_{278-286}\)-specific CTLs

Because the CD8\(^{+}\) CTLs were generated by in vitro stimulation with peptides to ascertain whether they recognized the processed products of endogenously synthesized HSV-derived protein and not just peptides, we analyzed the cytolytic activity of CD8\(^{+}\) T cell lines generated by each gD peptide against HLA-A*0201-positive target cells infected with HSV-1, HSV-2, VVgD, or an empty vaccinia virus (control; not expressing gD). Fig. 4 shows that CD8\(^{+}\) T cell lines specific to the gD\(_{53-61}\) and gD\(_{278-286}\) peptides undergo significant CD107a/b degranulation when incubated with HLA-A*0201-positive target cells infected with VVgD, HSV-1 (strain McKrae), or HSV-2 (strain 333) (\(p < 0.005\)). In contrast, the gD\(_{70-78}\)-specific T cell line was able to undergo significant CD107a/b degranulation only when incubated with HLA-A*0201-positive target cells infected with VVgD, HSV-1 (strain McKrae), or HSV-2 (strain 333) (\(p < 0.005\)). This suggests that the presentation of this particular epitope may be affected by factors in HSV-infected target cells. As a control, no cytolytic activity was detected when gD\(_{53-61}\), gD\(_{70-78}\), or gD\(_{278-286}\)-specific CD8\(^{+}\) T cell lines were incubated with HLA-A*0201-positive target cells infected with empty vaccinia virus (designated VVC (control) in Fig. 4). Therefore, these gD\(_{53-61}\), gD\(_{70-78}\), and gD\(_{278-286}\)-specific CTLs
were specific for naturally processed products of endogenously synthesized gD protein, not just exogenous peptides.

High frequency of gD53–61, gD70–78, and gD278–286 epitope-specific CD8<sup>+</sup> T cells detected in HLA-A*0201-positive, HSV-seropositive individuals

To obtain an objective enumeration of gD epitope-specific CD8<sup>+</sup> T cells, we constructed HLA-A*0201 tetramers incorporating all but one gD peptide (i.e., gD<sub>13 to 25</sub>). For technical reasons we were unable to produce a tetramer specific to the gD<sub>13 to 25</sub> peptide epitope. Using peptide/PE-labeled HLA-A*0201 tetramers together with an FITC-conjugated mAb specific to human CD8<sup>+</sup> T cells, we directly visualized the number of circulating CD8<sup>+</sup> T cells specific to each epitope ex vivo (i.e., without in vitro stimulation) and after in vitro stimulation. For each of the high-affinity peptides gD<sub>53–61</sub>, gD<sub>70–78</sub>, and gD<sub>278–286</sub>, a significantly higher percentage of tetramer<sup>+</sup> CD8<sup>+</sup> T cells was detected after in vitro stimulation of the PBMCs of HLA-A*0201-positive, HSV-seropositive individuals (Fig. 5) compared with HLA-A*0201-negative, HSV-seropositive individuals. Similar to the IFN-γ-producing CD8<sup>+</sup> T cell and CD8<sup>+</sup>/CD107a/b responses described in Figs. 2A and 3, the highest frequency of tetramer<sup>+</sup> CD8<sup>+</sup> T cells was detected against the gD<sub>53–61</sub> peptide epitope (6.2%). Despite repeated attempts, the remaining six gD peptide/HLA-A*0201 tetramers consistently detected a low percentage of CD8<sup>+</sup> T cells. To enumerate the frequency of gD epitope-specific CD8<sup>+</sup> T cells in unstimulated PBMCs, freshly ex vivo sorted CD8<sup>+</sup> T cells were analyzed for the percentage of tetramer<sup>+</sup> CD8<sup>+</sup> T cells. A high frequency of gD<sub>53–61</sub>- (7.2%) but not gD<sub>70–78</sub>- and gD<sub>278–286</sub>-specific CD8<sup>+</sup> T cells was detected ex vivo in HLA-A*0201-positive HSV-seropositive individuals. Collectively, these results show a high frequency of gD<sub>53–61</sub>-, gD<sub>70–78</sub>- and gD<sub>278–286</sub>-specific CD8<sup>+</sup> T cells from healthy HLA-A*0201-positive HSV-seropositive individuals, suggesting that these peptides contain functional HLA-A*0201-restricted CD8<sup>+</sup> T cell epitopes generated during HSV infection.

**FIGURE 4.** CD8<sup>+</sup> T cell lines generated by immunodominant CD8<sup>+</sup> T cell epitope peptides recognized infected target cells expressing native gD. gD<sub>3–61</sub>, gD<sub>70–78</sub> and gD<sub>278–286</sub> peptide-specific CD8<sup>+</sup> T cell lines were generated from HLA-A*0201-positive individuals and their cytotoxic activity was tested (i.e., capacity to degranulate) in the presence or absence of target cells infected with HSV-1, HSV-2, or with VVgD. Target cells infected with an empty vaccinia (VVC) were used as negative controls. Results are representative of three independent experiments. * p < 0.05.

**FIGURE 5.** High frequency of gD53–61, gD70–78, and gD278–286 epitope-specific CD8<sup>+</sup> T cells detected in HLA-A*0201-positive HSV-seropositive individuals. In vitro activated PBMC and freshly purified CD8<sup>+</sup> T cells were analyzed for the frequency of CD8<sup>+</sup> T cells recognizing gD peptide/tetramer complexes. Tetramer analysis of PBMCs from HLA-A*0201-positive HSV-1-seropositive and HLA-A*0201-negative HSV-1-seropositive individuals that have been stimulated in vitro with heat inactivated HSV-1 (PFU = 5). Results shown are representative of seven experiments.
Induction of gD peptide-specific CD8⁺ T cells in HSV-1- and HSV-2 infected HLA-A*0201 Tg mice

To ascertain the lack of cross-reactivity of gD-specific CD8⁺ T cell responses with self-derived or other pathogen-derived epitopes as has been reported for some heterologous infections (47–49), we assessed the percentage of tetramer CD8⁺ T cells (Fig. 6A) as well as the frequency of IFN-γ-producing CD8⁺ T cells (Fig. 6B) specific to each gD peptide in HLA-A*0201 Tg mice infected with HSV-1 (strain McKrae). HLA-A*0201 Tg mice infected with HSV-1 developed high frequencies of CD8⁺ T cells specific to gD₁₈ to ₁₁₀, gD₅₃–₆₁, gD₇₀–₇₈, gD₉₅–₁₀₃, gD₁₅₃–₁₆₁, gD₂₂₄–₂₃₂, and gD₂₇₈–₂₈₆, detected by either tetramer (Fig. 6A) or IFN-γ-ELISPOT (Fig. 6B). Unlike HLA-A*0201-positive, HSV seropositive humans, HLA-A*0201 Tg mice infected with HSV-1 did mount CD8⁺ T cell responses against cryptic gD₁₈ to ₁₁₀, gD₉₅–₁₀₃, gD₁₅₃–₁₆₁, and gD₂₂₄–₂₃₂ epitopes, which could reflect a higher level of infection in HLA-A*0201 Tg mice compared with HLA-A*0201-positive humans. However, in the HLA-A*0201 Tg mice

<table>
<thead>
<tr>
<th>Virus and Strain (GenBank Accession No.)</th>
<th>gD₅₃–₆₁</th>
<th>gD₇₀–₇₈</th>
<th>gD₂₂₄–₂₃₂</th>
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<tr>
<td>HSV-1</td>
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<td>Strain 17 (Q69091)</td>
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<td>ALLEDPTVT</td>
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<tr>
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<td>VLNAPSEA</td>
<td>ALLEDPTVT</td>
</tr>
<tr>
<td>Strain HZT (P06476)</td>
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<tr>
<td>HSV-2</td>
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</tr>
<tr>
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<td>VLNAPSEA</td>
<td>ALLEDPAQT</td>
</tr>
<tr>
<td>gD precursor (P03172)</td>
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<td>VLNAPSEA</td>
<td>ALLEDPAQT</td>
</tr>
<tr>
<td>Virion gD (Q69467)</td>
<td>SLPITVYYA</td>
<td>VLNAPSEA</td>
<td>ALLEDPAQT</td>
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</tbody>
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* The nonidentical amino acids between HSV-1 and HSV-2 are bolded and underlined.

* The nonidentical amino acids between HSV-1 strains are bolded, underlined, and italicized.
infected with HSV-1, the highest frequency of CD8+ T cells appeared to be directed against the gD53–61, gD70–78, gD153–161, gD224–232, and gD278–286 epitopes. In addition, CD8+ T cell responses specific to the immunodominant gD53–61, gD70–78, and gD278–286 peptides were detected in HLA-A*0201 mice following infection with either HSV-1 (strain McKrae) or HSV-2 (strain 333) (Fig. 6C). This is consistent with the sequences of these three epitopes being highly conserved among and between HSV-1 and HSV-2 strains (Table III).

**Discussion**

This study identified three human CD8+ CTL epitopes from HSV-1 gD, a glycoprotein that produces protective immunity in both animal models and humans (3, 4, 7, 8, 10, 50). These new gD epitopes displayed high-affinity binding to purified HLA-A*0201 molecules, stabilized HLA-A*0201 molecules on target cells, recalled CD8+ CTLs in HSV-1 and HSV-2 seropositive individuals as well as in HLA-A*0201 Tg mice infected with either HSV-1 or HSV-2, and were naturally processed from native HSV-1/2 gD in HLA-A*0201-positive target cells.

In humans, HSV-specific CD4+ and CD8+ T cells play a crucial role in controlling both primary and recurrent infections (51, 52). Results from a number of studies have documented HSV gD as being a target for both CD4+ and CD8+ CTLs. In the late 1980s and early 1990s, Zarling and coworkers (53, 54) generated several human CD4+ CTL clones from HSV-1-seropositive individuals by stimulating PBLs with a rVV expressing HSV-1 gD. Five of these CD4+ CTL clones lysed autologous HSV-1-infected LCLs and their cytotoxicity was restricted to HLA class II molecules. Later, it was demonstrated that some of these HSV-1-specific CTL clones were directed against both gD1 and gD2 by using target cells infected with wild-type HSV strains, a glycoprotein C deletion mutant of HSV-1, and a recombinant HSV-1 × HSV-2 virus (55, 56). In another study, purified HSV-1 gD expressed in mammalian cells stimulated the proliferation of and the IL-2 production by the CD4+ T cell clone 53-61. HSV-specific memory T cells (57). In addition, T cell clones generated by the stimulation of PBL with HSV-1 were found to proliferate in response to gD1 in the absence of exogenous IL 2 and to lyse HSV-1 or both HSV-1/2-infected autologous target cells (53). HSV-specific cytotoxic CD4+ and CD8+ T cell clones have been recovered ex vivo from the HSV-2 lesions of five patients by Koelle and coworkers (58, 59). By isolating HSV-specific CD8+ CTL clones from a patient with recurrent genital herpes, Burke and coworkers (60) also reported one CD8+ T cell clone that lysed vaccinia virus/gD2-infected target cells. Cunningham and coworkers (61, 62) have also reported that HSV gD is a major target for both CD4+ and CD8+ CTLs using human epidermal keratinocytes as targets. Although, the above findings demonstrated gD as a major target for human HSV-specific CD4+ and CD8+ CTLs, no specific gD minimal epitopes were defined.

Therefore, to our knowledge the present study reports the first demonstration that lysed vaccinia virus/gD2-infected target cells. Cunningham and coworkers (61, 62) have also reported that HSV gD is a major target for both CD4+ and CD8+ CTLs using human epidermal keratinocytes as targets. Although, the above findings demonstrated gD as a major target for human HSV-specific CD4+ and CD8+ CTLs, no specific gD minimal epitopes were defined. Therefore, to our knowledge the present study reports the first demonstration that HSV-1-specific T cells respond to gD53–61, gD70–78, and gD278–286 epitopes as recognized by human CD8+ T cells. Although, the profile of responding peptides appears different between HLA-A*0201-positive humans and HLA-A*0201 Tg mice.
mice (Fig. 6), all three immunodominant peptides (gD53–61, gD70–78, and gD278–286) detected in HLA-A*0201-positive humans were also positive in HLA-A*0201 Tg mice. The Tg mice also responded to additional epitopes including gD18–26, gD95–103, gD153–161, and gD224–232. This nonsurprising discrepancy might be due to the following: 1) the class I molecule in HLA-A*0201 Tg mice is composed of human α1 and α2 domains and the mouse α3 domain to allow mouse CD8 binding, which could affect the presentation of some epitope peptides; 2) the amount of HSV used to infect the mice may greatly exceed the amount for a typical primary human infection, which might differentially affect CD8+ T cell stimulation for some epitopes; and 3) the processing/presentation machinery of the HSV virus in mice and humans is different.

The ability of CD8+ T cells generated by gD53–61, gD70–78, and gD278–286 epitopes to kill target cells endogenously expressing the gD protein (i.e., infected with HSV or VVgD) is a critical parameter, especially given the observation that CTLs induced by peptide in vitro may not kill targets expressing the endogenous protein. However, a difference in the level of gD expression by LCL target cells might occur following VVgD, HSV-1, and HSV-2 infection. This could affect the functional presentation of some gD epitopes by infected target LCLs as detected by CD107a/b degranulation and IFN-γ ELISPOT assays. Indeed, although the gD53–61-specific CD8+ T cell line efficiently recognized HSV-1- and HSV-2-infected target cells, its level of cytotoxicity was slightly lower compared with gD53–61 and gD278–286-specific T cell lines. In contrast, the same gD53–61-specific CD8+ T cell line failed to recognize the same LCL target cells when infected with VVgD. Thus, although the gD53–61 epitope appeared to be efficiently presented by HSV-1- and HSV-2-infected LCL target cells, this did not appear to be the case in VVgD-infected LCL target cells. One possibility to account for this difference might be that the gD expression level in VVgD-infected LCLs, although sufficient for efficient presentation of other gD epitopes, was for as yet undetermined reasons insufficient for efficient presentation of the gD53–61 epitope. It is also possible that the correct presentation of this epitope requires one or several factors produced in HSV-infected cells, because this epitope was efficiently presented in HSV-1- and HSV-2-infected LCLs. Although some gD-expressing cell lines are available, HLA-A2.1-positive target cell lines constitutively expressing gD are not currently available. In the future, we hope to construct such a cell line. Thus, it is very likely that the gD53–61, gD70–78, and gD278–286 epitopes detected in humans were not cross-reactive but were induced following HSV-1/HSV-2 infection. This does not imply excluding the involvement of other infectious agents in shaping HSV T cell epitope-specific responses.

The vast majority of the world’s human population is infected with HSV-1 and/or HSV-2 and might potentially benefit from therapeutic vaccination approaches designed to boost HSV-specific cellular immunity. Although the primary goal of this study was to identify human CD8+ T cell epitope peptides on HSV gD, a secondary but important goal is to develop the knowledge of herpes T cell epitopes required for the development of a totally synthetic, self-adjuvant lipopeptide human vaccine. We now plan first to construct lipopeptide vaccine candidates incorporating the CD8+ T cell epitope identified in the present study together with the human CD4+ T helper epitopes that we (Ref. 1 and X. Zhang, F. A. Castelli, M. Y. Wu, I. Bettahi, D. Carpenter, A. Mohebbi, A. B. Nesburn, S. L. Wechsler, B. Maillère, and L. BenMohamed, submitted for publication) and others (A. L. Cunningham, unpublished observations) have recently identified. Such a combination would produce CD4+CD8+ chimeric lipopeptide vaccine candidates similar to those we have recently described in mice against ocular and genital herpes infection. Secondly, we plan to study the safety, immunogenicity, and protective efficacy in humans of a CD4+CD8+ chimeric epitope lipopeptide candidate vaccines.

Recently, we reported four immunodominant H2d-restricted CD4+ T cell epitopes on HSV-1 gD, among which is the peptide gD49–52, a naturally processed epitope that protects H2d mice against lethal ocular HSV-1 challenge (1). We have also recently found that the gD49–52 epitope represents an immunodominant HLA-DR*0101 and HLA-DR*0104-restricted human CD4+ T cell epitope (X. Zhang et al. submitted for publication). Interestingly, the gD53–61 sequence, one of the three immunodominant human CD8+ T cell epitopes identified in this study, is completely contained within the gD49–52 sequence. Therefore, gD49–52 harbors at least two human epitopes, the gD53–61 CD8+ T cell epitope and a CD4+ T cell epitope and, as such, this gD region represents a good candidate for inclusion in a CD4+ and CD8+ T cell epitope-based human vaccine.

A question of practical importance is the translation of the current immunological findings into the development of an epitope-based vaccine for a genetically heterogeneous human population. Although the high degree of HLA polymorphism is often pointed to as a major hindrance to the use of epitope-based vaccines, this constraint can be dealt with through the inclusion of multiple supertype-restricted epitopes recognized in the context of diverse related HLA alleles and by designing mixtures of peptide-based vaccines with higher epitope densities (2, 3, 7, 8, 11, 24, 25). Broad population coverage can be established providing that epitopes corresponding to multiple HLA supertype families are incorporated into the vaccine. Sette and Sidney (73) defined nine HLA class I supertypes that provide an almost perfect coverage (>99%) of the entire repertoire of HLA class I molecules. Because the HLA-A2 supertype family is present in ~50% of the general population (regardless of ethnicity), the CD8+ T cell epitopes identified in this study should prove to be useful in vaccine trials. To maximize efficacy, a multipitope-based herpes vaccine should probably include several T cell epitopes from several different structural glycoproteins and regulatory proteins, each chosen to represent at least the HLA supertypes known to provide recognition in a large proportion of the global population regardless of race and ethnicity. Hence, our laboratory is trying to identify such HLA-promiscuous T cell epitopes.

CD8+ T cell infiltrates appear to correlate with HSV clearance from mucocutaneous lesions (52). However, pathogens such as HSV that establish a latent infection have evolved a variety of immune evasion tactics to avoid being detected by the host’s immune system. Among the central issues in the immunobiology of HSV is its ability to evade the cellular immune system control and periodically reactivate from latent infected sensory neurons (74–78). Unlike HIV and other pathogens, antigenic variation is not used by HSV as a mechanism to escape cell-mediated immune surveillance. Rather, HSV has developed alternative mechanisms in which specific viral genes inhibit antigenic processing and presentation to T cells, including CD8+ T cells (78–80). The HSV immediate-early protein ICP47 blocks the peptide transporters (TAP1/TAP2) associated with Ag processing (74, 76, 81). This decreases the access of antigenic peptides into the MHC pathway, preventing the transport of HLA molecules to the surface of APCs (74, 80, 82). The product of the US1 gene (76, 80) and the virion host shutoff (vhs) or UL41 gene rapidly degrade host cell mRNA, contributing to decreased synthesis of new HLA class I molecules (83). All of these mechanisms might render HSV-infected neurons, fibroblasts, keratinocytes, LCL/dendritic cells, and other mucocutaneous target cells insensitive to lysis by infiltrating CD8+ CTLs. Therefore, these powerful immune evasion mechanisms must be
taken into account in future vaccine strategies against herpes. Their effect on epitope processing/presentation might be counteracted by IFN-γ or other treatments (e.g., acyclovir), thus providing a better role for CD8+ T cells in the cellular immunity to HSV (83, 84).

In conclusion, in this article we report three immunodominant HLA-A*0201-restricted epitopes derived from the HSV-1 gD that recall CD8+ CTL responses in HLA-A*0201-positive, HSV-seronegative individuals and in HSV infected HLA-A*0201 Tg mice. These functional CD8+ T cell epitopes shared high sequence homology between and within HSV-1 and HSV-2 strains. Hence, they should be considered in the design of effective immunotherapeutic and immunoprophylactic strategies against HSV-1 and HSV-2 in humans.

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Disclosures
The authors have no financial conflict of interest.

References


