Cytotoxic T lymphocytes (CTL) recognize and kill virus-infected cells and contribute to immunologic control of viral replication. For many herpesviruses (e.g., Epstein-Barr and cytomegalovirus), virus-specific CTL responses can be readily detected in infected persons, but CTL responses against Kaposi’s sarcoma–associated herpesvirus (KSHV) appear to be weak and remain poorly characterized. Using a human leukocyte antigen (HLA) binding motif–based epitope prediction algorithm, we identified 37 HLA-A*0201 binding peptides from 8 KSHV open-reading frames (ORFs). After in vitro stimulation of peripheral blood mononuclear cells from KSHV-infected persons, CTL responses against 1 peptide in the KSHV kaposin protein (ORF K12) were detected in 2 HLA-A*0201–positive subjects. The optimal CTL epitope was identified by HLA restriction analysis and peptide titration assays. These data describe a latent phase viral gene product targeted by CTL that may be relevant for KSHV immunopathogenesis.

Kaposi’s sarcoma (KS)–associated herpesvirus (KSHV) was first described when Chang et al. [1] detected genome sequences of a new member of the γ-herpesvirus family in tissue from KS lesions [1]. This virus was subsequently sequenced and was termed KSHV or human herpesvirus 8 and is closely related to Herpesvirus saimiri and Epstein-Barr virus (EBV) [1, 2]. It has been closely linked to KS, a subset of non-Hodgkins lymphomas, and multicentric Castleman’s disease [3]. Indirect evidence from studies of KS in transplant recipients and patients with AIDS suggests that immune control may dictate oncoligic disease related to the virus, since patients with AIDS and KS can experience complete regression of the tumor after immunologic improvement during antiretroviral therapy. Furthermore, patients who have undergone transplantation often have regression of KS with a decrease in their immunosuppressive regimens. Although EBV- and cytomegalovirus-specific cytotoxic T lymphocytes (CTL) have been well characterized [4, 5], only limited data exist for CTL responses against KSHV [6, 7]. Consequently, it is largely unknown which proteins are targeted by KSHV-specific CTL, and no minimal HLA class I–restricted CTL epitopes have been defined thus far.

As is typical for DNA viruses, a major obstacle to a detailed analysis of KSHV-specific CTL responses is the large genome with >80 open-reading frames (ORFs) [2]. While CTL responses against RNA viruses can be measured comprehensively by using overlapping peptide sets or a few vaccinia virus constructs, this approach is more complex for the study of large genome DNA viruses. Therefore, we used a peptide prediction algorithm to screen a restricted number of KSHV protein sequences, focusing on potential CTL responses restricted by the most common HLA class I allele in white persons, HLA-A*0201.

Such peptide prediction algorithms are based on the observation that antigenic epitopes presented by HLA class I molecules require certain amino acids in their anchor positions to allow for strong binding to their restricting HLA class I molecule [8]. This has led to the identification of HLA class I allele-specific binding motifs that can be used to screen larger protein sequences for potential HLA class I binding epitopes [9]. A number of different allele-specific binding motifs have been described and have been successfully used to predict class I–restricted CTL epitopes from longer protein sequences [10]. A large number of HLA-A*0201–restricted epitopes have been described, and efficient prediction algorithms for this allele exist [11–13].

In this study, we used the HLA-A*0201 binding motif to screen
structural and nonstructural and lytic and nonlytic KSHV protein sequences for A*0201 binding peptides. In vitro stimulation of peripheral blood mononuclear cells (PBMC) from KSHV-infected subjects gave rise to CTL clones and lines directed against an epitope in ORF K12, which allowed for precise identification of a KSHV-encoded minimal CTL epitope.

Materials and Methods

Subjects. Six HLA-A*0201–positive, KSHV-seropositive, human immunodeficiency virus (HIV) type 1–infected persons were included in this study: 2 are part of the San Francisco City Clinic Cohort (subjects 13010 and 11504), 3 (subjects 012-221L, 010-115, and 145) are from the Boston area, and 1 (subject S405) is enrolled in the San Francisco Men’s Health Study [14–16]. KSHV infection was demonstrated by detecting latency associated nuclear antigen (LANA; KSHV ORF 73)–specific antibody responses in the serum of these subjects [17]. HIV loads and CD4 cell counts were available for subjects 221L (220,000 copies/mL and 894 cells/µL, respectively), 11504 (400 copies/mL and 614 cells/µL, respectively), 13010 (<400 copies/mL and 614 cells/µL, respectively), 11504 (155,000 copies/mL and 601 cells/µL, respectively), and S405 (<50 copies/mL and 294 cells/µL, respectively). Only subject S405 was receiving antiretroviral treatment at the time the samples were obtained, and only this person had a history of a KSHV-associated disease (KS). HIV-negative, HLA-A*0201–expressing control subjects included donors with low risk for KSHV infection, and all tested negative for LANA antibodies.

Cell lines and CTL clones. Autologous and partially HLA-matched EBV-transformed B lymphoblastoid cell lines (B-LCLs) were maintained in RPMI 1640 media containing 20% (vol/vol) heat-inactivated fetal calf serum (FCS), 10 mM Hepes buffer, 50 U/mL of penicillin, 50 µg/mL of streptomycin, and 2 mM L-glutamine [18]. The EBV-transformed B-LCLs were shown to be KSHV negative by polymerase chain reaction (PCR) for KSHV ORF 26 [1]. CTL clones were maintained in the same medium as above, containing 10% FCS (designated R10) supplemented with 50 U/mL of recombinant interleukin (IL)–2 (designated R10-50). CTL clones were restimulated every 2 weeks by using the anti-CD3 specific monoclonal antibody (MAb) 12F6 [19]. Recombinant IL-2 was a gift of M. Gateley (Hoffman-La Roche, Nutley, NJ).

Prediction of HLA-A*0201 binding peptides. KSHV ORF sequences were screened for the presence of the HLA-A*0201–specific binding motif by using a polynomial prediction algorithm, as described elsewhere [12]. The highest scoring sequences from these analyses were synthesized and were tested for HLA-A*0201 binding [20]. Peptides binding with an IC₅₀ <500 nM were considered to be “good binders,” were synthesized in large scale (Chiron), and subsequently used for in vitro stimulations [21].

Peptide stimulation of PBMC. Peptides that demonstrated binding to HLA-A*0201 were pooled in 6 pools containing 5–8 peptides each (with each peptide at 2 µg/mL concentration). The HLA-A*0201–restricted HIV-1 gag-derived epitope SL9 (SL YNTFLKLV H11003), which is recognized by PBMC from HLA-A*0201–positive HIV-1–infected subjects, was added to pool 6 [18, 22]. Frozen PBMC from 6 healthy donors were thawed, and aliquots of 2 × 10⁵ PBMC were stimulated with 15 × 10⁶ irradiated PBMC from a healthy homozygous HLA-A*0201–positive donor and were pulsed with the different peptide pools. This alleloimmune, instead of autologous, stimulation was used because of the limited availability of PBMC from these KSHV-infected persons. Because of potential cross-reactivity between CTL against KSHV-derived peptides and EBV antigens, no EBV-transformed B-LCLs were used as antigen-presenting cells (APCs) in these initial stimulations. All peptide incubations were done with “pulsed” APCs. Thus, no peptides were added directly to the cultures or cytotoxicity assays. The final concentrations in the peptide pulsing steps were generally 200 µg/mL per peptide, except in peptide titration assays (see below). Dimethyl sulfoxide (DMSO) was used to reconstitute peptides; the final concentration of DMSO in all cases was <1%.

Recombinant human IL-2 (50 U/mL) was added to the cultures after 3 days and then twice a week thereafter. After 2 weeks, the cultures were restimulated with peptide-pulsed HLA-A*0201–positive PBMC as above and were tested after 10–14 days for specific lysis of HLA-A*0201–positive B-LCLs, either unpulsed or pulsed with the appropriate peptide pool. Bulk cultures that showed killing against a specific pool subsequently were screened against the single peptides in that pool. CTL clones were generated by limiting dilution from specific cell lines by seeding 1, 3, or 10 cells per well. Limiting dilutions were done with peptide-pulsed HLA-A*0201–positive PBMC as feeder cells. Growing wells were ex-

Table 1. Kaposi’s sarcoma–associated herpesvirus proteins screened for HLA-A*0201 binding motifs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Homology and/or function</th>
<th>Protein size, aa</th>
<th>Peptides fulfilling the HLA-A*0201 binding motif</th>
<th>HLA-A*0201 binders, IC₅₀ &lt;500 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF 72</td>
<td>Cyclin D homologue</td>
<td>257</td>
<td>30</td>
<td>6 4</td>
</tr>
<tr>
<td>ORF 8</td>
<td>Glycoprotein B</td>
<td>845</td>
<td>69</td>
<td>12 3</td>
</tr>
<tr>
<td>ORF K1</td>
<td>Long unique region</td>
<td>289</td>
<td>26</td>
<td>3 3</td>
</tr>
<tr>
<td>ORF K12</td>
<td>Kaposin</td>
<td>60</td>
<td>8</td>
<td>2 1</td>
</tr>
<tr>
<td>T1.1</td>
<td>3 Putative ORFs</td>
<td>143</td>
<td>4</td>
<td>1 1</td>
</tr>
<tr>
<td>K12-DR</td>
<td>Direct repeat region</td>
<td>219</td>
<td>1</td>
<td>1 1</td>
</tr>
</tbody>
</table>

NOTE. ORF, open-reading frame.

* Unclear whether the abundant RNA is translated [24].

* Upstream of ORF K12, 2 direct 23-aa repeats have been described elsewhere [25].

* Size varies depending on the number of direct repeats.

* Peptide was included in the study, despite a low binding affinity to HLA-A*0201 (IC₅₀ <500 nM).
panded by restimulation with an anti-CD3 MAb and feeder cells, as described elsewhere [19, 23].

Peptide titration assays. For the definition of the optimal epitope, peptides were titrated directly in the assays at final concentrations of 100 μg/mL to 10 pg/mL and were incubated with 51Cr-labeled target cells for 45 min before the addition of effector cells [18]. Specific CTL clones were used at indicated effector-to-target ratios, and 51Cr-release assays were done at 37°C.

HLA typing and HLA restriction analysis. PCR-based HLA typing for HLA class I and class II was done by the Massachusetts General Hospital HLA typing laboratory (Boston). The HLA-A2 subtype was determined by PCR, as described elsewhere [18], and only HLA-A*0201-positive subjects were included in these experiments. HLA restriction experiments were done by using allogeneic B-LCLs matching different HLA class I alleles. The cells were pulsed for 90 min with 51Cr and the minimal epitope K12-P3-LL9, were washed 3 times with cold R10, and were used as targets in a standard cytotoxicity assay.

Results

Identification of HLA-A*0201 binding KSHV-derived peptides. To identify potential KSHV-derived HLA-A*0201 binding CTL epitopes, amino acid sequences from 8 KSHV ORFs were screened for the presence of the HLA-A*0201–specific binding motif (table 1). Viral gene encoded products were selected for screening on the basis of expression in latency and similarity to CTL epitope-containing ORFs in other herpesviruses. The selected genes also included structural (ORF 8) and nonstructural (ORF 72) proteins plus 2 proteins unique to KSHV (ORF K1 and ORF K12). In addition, sequences in the T1.1 region of KSHV (containing 3 putative short ORFs) and sequences upstream of ORF K12 were screened [24]. The region upstream of ORF K12 is of special interest, because 2 repeated sequence motifs have been found in this area, which are conserved on the amino acid level in all 3 ORFs and may be expressed during latency [25].

The above protein sequences (1813-aa residues) were screened by using a binding motif search algorithm for HLA-A*0201, as described elsewhere [12], and yielded 138 peptide sequences that fulfilled the binding motif. The algorithm that was used allowed for 9-mer and 10-mer peptides to score. These 138 sequences upstream of ORF K12 was of special interest, because 2 repeated sequence motifs have been found in this area, which are conserved on the amino acid level in all 3 ORFs and may be expressed during latency [25].

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In vitro stimulation of PBMC from KSHV-infected persons. To evaluate whether KSHV-infected persons show CTL activity against ≥1 of these HLA-A*0201 binding peptides, the peptides were grouped into 6 different peptide pools and were used for in vitro stimulation of PBMC from KSHV-infected subjects (table 2). The pools were designed to contain peptides from the same protein, except for pool 5, which contained ORF 72 peptides and the 1 peptide from the direct repeat region upstream of ORF K12 and pool 6, which contained peptides from ORF K12 and T1.1 and the HIV-1 gag p17-derived peptide SL9. Previous studies that used cells from a strong SL9 responder demonstrated that peptide pools can be used for in vitro stimulations of PBMC, even if the targeted epitope is not among the
Six HLA-A*0201–positive subjects were tested in bulk stimulation assays for specific cytotoxic T lymphocyte responses against 6 peptide pools. Peripheral blood mononuclear cells were incubated with autologous (subjects 115i and 221L) or HLA-A*0201 matched B lymphoblastic cell lines (subjects 145, 13010, S405, and 11504), pulsed with different peptide pools (200 μg/mL for 90 min), and tested for specific lysis after 10–14 days of culture (effector-to-target ratio, 100:1). Data are percentage of specific lysis after subtraction of control killing (targets without peptides). Positive bulk cultures (>10% above control lysis) were restimulated with the same peptide pool, as in the initial stimulation, and cultures were retested after 10–14 days (2d stim.).

The strongest HLA-A*0201 binders in the pool (data not shown; table 2), which rules out concerns regarding peptide binding competition among the peptides in the peptide pools.

PBMC from 6 HLA-A*0201–positive, KSHV-seropositive, HIV-1–coinfected persons were used in subsequent in vitro stimulation assays [14–16]. The cultures were set up by using peptide-pulsed HLA-A*0201–expressing PBMC as APCs, as described elsewhere [18]. Cultures were tested after 10–14 days for pool-specific CTL activity by using autologous or HLA-A*0201–positive B-LCLs as target cells, either unpulsed or pulsed, with the peptide pool used in the initial stimulation (figure 1). Only weak responses were observed in the presence of sometimes high background lysis of ≤72%. Three subjects (subjects 115i, 13010, and 221L) showed lysis of ≥10% target cell preparation that exceeded control killing by >10%. These responses were directed against pool 3 in subjects 13010 and 221L and against pool 6 in subjects 115i and 221L. Subject S405 had responses that were directed against pool 2 (8% lysis) and pool 6 (9% lysis above background). PBMC from 3 HLA-A*0201–positive, HIV-negative persons at low risk for KSHV infection also were stimulated with pool 6 peptides and did not show preferential lysis of target cells pulsed with KSHV peptides (data not shown).

From 4 subjects with positive responses after the initial in vitro stimulation (subjects 115i, 13010, 221L, and S405), the most active bulk culture was restimulated with peptide pool–pulsed, HLA-A*0201–matched PBMC and tested again after 2 more weeks in culture (figure 1, “2d stim.”). This second round of stimulation gave rise to 2 cell lines specific for peptide pool 6 (subjects 115i and S405), whereas initial bulk cultures of subjects 221L and 13010 lost their specificity. For the peptide pool 6–stimulated bulk cultures from subjects 115i and S405, it was possible to define the targeted epitope within the peptide pool. The restimulated bulk culture from subject 115i recognized peptide K12-P3 with the same magnitude with which the peptide pool was recognized (figure 2). No reactivity against any other peptide in pool 6 was detectable. Subsequent limiting dilution cloning from this bulk culture yielded 5 peptide K12-P3–specific CTL clones that were used for subsequent studies. The bulk culture from subject S405 was tested only against pool 6–pulsed targets and targets pulsed with the K12-P3 pep-
tide. Both targets were lysed to the same degree, which indicates that the majority of pool 6 reactivity was directed against the K12-P3 peptide (data not shown).

Definition of the optimal CTL epitope within K12-P3. Although the K12-P3 epitope was identified by the prediction algorithm as a 10-aa peptide, longer and shorter versions of the K12-P3 peptide were tested for recognition by CTL clones derived from subject 115i. To identify the optimal CTL epitope, titrations with truncated versions of the epitope were performed [18]. Figure 3A shows the data of a representative experiment, in which 100 μg/mL of the different peptides was used to pulse the autologous target cells. The 6 best recognized and shortest peptides then were used in peptide titration assays, where peptides were titrated directly into the cytotoxicity assays at concentrations ranging from 100 μg/mL to 10 pg/mL (figure 3B). These experiments were performed for 3 clones obtained from subject 115i, and all showed the same results. Unexpectedly, the data revealed that the best recognized epitope is the 9-mer peptide LL9, which is 1 aa shorter than the predicted epitope VL10 and which was not predicted by the algorithm. The 8-mer peptide (LNGWRWRL, position 18–25) also was tested in subsequent studies and was less well recognized than LL9 (data not shown). Thus, these data identify the LL9 peptide as the optimal epitope within the K12-P3 peptide.

HLA restriction analysis. Since a shorter epitope than the predicted 10-mer VL10 was identified as the optimal epitope for CTL clones from subject 115i, additional HLA restriction analyses were performed with the optimal 9-mer LL9 epitope.
by using \(^{51}\)Cr-labeled autologous and partly HLA-matched B-LCL. Only targets that expressed HLA-A*0201 were killed by CTL clone 115i-14 (figure 4). These experiments were repeated by using 2 other clones from the same subject and showed the identical results, which indicates that the LL9 peptide was indeed restricted by HLA-A*0201.

Discussion

Our data identify the first optimal KSHV-derived, HLA class I-restricted CTL epitope. Bulk stimulation of PBMC from KSHV-infected subjects revealed only weak CTL activity after 1 in vitro stimulation, which indicates that the magnitude of these responses is low, compared with other chronic viral infections such as HIV-1 [23, 26]. However, after an additional restimulation and 14 days of culture, efficient killing was observed. Subsequent studies that used ELISPOT techniques and staining for IFN-γ intracellular (ICS) could not detect specific CTL (data not shown), again suggesting that these responses are present at a low frequency only. The lack of responses in the ELISPOT and ICS assays also could be explained by the presence of “silent” CTL, which lack the production of IFN-γ and which have been found in other chronic viral infections (e.g., HCV, HIV, and EBV) [27–30]. Such silent responses could be caused by a lack of appropriate support from T helper cells [31], which also would be in line with the rather weak T helper responses described in KSHV infection [32]. This question could be approached by tetramer studies; however, given the lower sensitivity of the tetramer versus the ELISPOT analysis, no significant responses would be expected from tetramer staining. In addition, the relatively weak T cell responses against KSHV may reflect the well adapted relationship of human host and viral pathogen postulated to have evolved over many thousands of years, resulting in a relatively docile pathogen that is usually controlled by a modest immune response [33–37]. Alternatively, the potentially less efficient cross-presentation of viral antigen by dendritic cells for the induction of the initial immune response may limit the strength of CTL responses against latent protein antigens. This has been shown recently for CTL responses directed against the EBV-encoded latent gene product EBNA1. This also could explain why CTL responses against latent gene products, although weak, are detectable, despite immune modulatory mechanisms employed by KSHV and other herpesviruses during latency [33, 38].

The peptide prediction algorithm that we used yielded 138 peptide sequences with a high probability for binding to HLA-A*0201. By use of binding assays with the 138 peptides, 37 were found to bind efficiently to HLA-A*0201 on the basis of a previously described threshold of IC \(_{50}\) <500 nM that was found to be important for in vitro and in vivo immunogenicity [21]. The proportion of HLA-A*0201 binding 9-mer and 10-mer peptides (24 9-mer vs. 12 10-mer) was similar to the proportion of 9-mer and 10-mer sequences found in the motif search (88 9-mer vs. 50 10-mer), which indicates that the algorithm-based prediction analyses are equally suitable for the identification of 9-mer and 10-mer HLA-A*0201 binding peptides. Furthermore, 106 9-mer and 46 10-mer peptides have been described to be naturally presented by HLA-A*0201 [10], again, a ratio similar to the 9-mer and 10-mer peptides observed for the HLA-A*0201 binding epitopes in this study, further confirming the usefulness of the applied prediction algorithm.

The analyses of the optimal CTL epitopes identified the LL9 truncation of the K12-P3 peptide as the optimal CTL epitope for the 3 CTL clones tested. However, the algorithm used in this study predicted a slightly longer epitope than the LL9 epitope as a potential HLA-A*0201 binding peptide. Therefore, it appears to be important to include truncation and titration studies to identify the optimal epitope in peptide prediction approaches and to incorporate the data obtained from minimal epitope determinations, as provided by the present study, into new designs of epitope prediction algorithms, to further optimize the prediction approach. Also of note, the targeted 10-mer K12-P3 peptide was among the best binders in peptide pool 6 (table 2). However, studies with PBMC that showed strong response to the HIV-1–derived peptide SL9 indicate that binding hierarchy among the peptides in the pool does not prevent the restimulation of CTL recognizing a relatively weaker binding peptide [18].

Peptide prediction approaches do not prove that the identified epitopes recognized by CTL are involved in natural infection. Indeed, de novo generation of CTL responses in vitro or in vitro expansion of cross-reactive memory CTL could complicate interpretation of the results. However, since the CTL responses against peptide pool 6 were partly detectable after a single in vitro stimulation, it is likely that they were based on the in vitro expansion of preexisting KSHV-specific CTL precursors rather than on a de novo generation of these CTL responses restricted by HLA-A*0201.
responses in vitro. De novo generation of CTL responses normally requires multiple rounds of restimulation, “professional” APCs, such as dendritic cells, and additional cytokines, such as IL-7 or IL-12 [39–42]. This and the absence of detectable responses against pool 6 in KSHV-negative persons suggest that the LL9 epitope is processed and presented after natural infection. However, because of a lack of suitable expression systems (e.g., recombinant vaccinia virus expressing the K12 protein) and the fact that KSHV-infected cell lines are weak APCs and thus are difficult to use to address natural processing of antigenic epitopes [33, 35, 36], this remains to be formally proven. Nevertheless, since 3 of 6 subjects had detectable responses against the peptide pool containing the K12-P3 epitope, and 2 subjects showed specific responses to the K12-P3 peptide, this epitope may represent an important CTL target that could prove useful in adoptive immunotherapeutic approaches or in CTL-based KSHV vaccine design.

References

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Addendum. The same K12-P3-LL9 epitope has been found by other investigators and reported in a preliminary manner: Micheletti F, Monini P, Fortini C, et al. Identification of cytotoxic T lymphocyte epitopes within latent and lytic antigens of human herpesvirus 8 [abstract 42]. In: Programs and abstracts of the 5th International AIDS Malignancy Conference (Bestheda, MD). Bethesda, MD: National Cancer Institute, 2001.