Identification of Epitope Mimics Recognized by CTL Reactive to the Melanoma/Melanocyte-derived Peptide MART-$1_{(27-35)}$

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Summary

CTL reactivity to the epitope MART-$1_{(27-35)}$, of the melanoma (self) antigen MART-1/melan A is frequently observed in tumor-infiltrating lymphocytes and may be readily elicited from the peripheral blood of melanoma patients that express HLA-A*0201. Available data suggest that these observations contrast with those made for other HLA-A*0201-presented melanoma self antigens regarding the regularity of observed CTL responses. Based on preliminary findings, we hypothesized that the CTL response to MART-1 might be augmented in part by T cell encounters with peptides derived from sources other than MART-1, which show sequence similarity to MART-$1_{(27-35)}$. To test this idea, a protein database search for potential MART-1 epitope mimics was done using criteria developed from analyses of effector recognition of singly-substituted peptide analogues of MART-$1_{(27-35)}$. Synthetic peptides were made for a portion of the sequences retrieved; 12/40 peptides tested were able to sensitize target cells for lysis by one or more anti-MART-1 effectors. The peptides recognized correspond to sequences occurring in a variety of proteins of viral, bacterial, and human (self) origin. One peptide derives from glycoprotein C of the common pathogen HSV-1; cells infected with recombinant vaccinia virus encoding native glycoprotein C were lysed by anti-MART-1 effectors. Our results overall indicate that sequences conforming to the A2.1 binding motif and possessing features essential to recognition by anti-MART-1 CTL occur frequently in proteins. These findings further suggest that T cells might encounter a variety of such sequences in vivo, and that epitope mimicry may play a role in modulating the CTL response to MART-$1_{(27-35)}$.

Several melanoma-associated antigens have been identified in recent years that are recognized by $CD8^+$ CTL present in tumor-infiltrating lymphocytes (TIL)[1] and peripheral blood of melanoma patients (1). Antigens recognized by these CTL fall into three main classes, comprised of mutated cellular proteins (2–4), ectopically expressed proteins (5–7), and lineage-specific (non-mutated) self proteins (8–13). To date, $CD8^+$ T cell responses to HLA-A*0201-presented epitopes have been most extensively studied, mainly due to the high prevalence of this class I allele in the melanoma patient population (14). Among HLA-$A*0201^+$ patients, CTL responses directed to epitopes derived from the melanocyte-specific self proteins tyrosinase, Pmel17/gp100, and MART-1/melan A are frequently observed. In particular, recognition of the MART-$1_{(27-35)}$ (AAGIGILTV) epitope is observed with high regularity among numerous independently-derived TIL lines (15, 16). CTL reactive to this epitope can be elicited readily from TIL and PBMC of melanoma patients by in vitro stimulation with MART-$1_{(27-35)}$ peptide (17, 18) or with HLA-$A*0201^+$ allogeneic melanoma cells (19). Where comparisons have been made, responses to epitopes derived from tyrosinase or gp100 were less frequently observed in TIL (15, 16), or were less readily induced from peripheral blood of HLA-$A*0201^+$ patients (18, 19). Though systematic, comparative studies of responses to epitopes from tyrosinase, gp100 and MART-1 are limited, a view emerges from these reports suggesting that the MART-$1_{(27-35)}$ epitope may be "immunodominant" among melanoma/melanocyte autoantigens (16).

To gain a better understanding of the factors underlying the CTL response to MART-$1_{(27-35)}$, we have focused attention in this study on features intrinsic to the MART-

[1] *Abbreviations used in this paper:* NP, nucleoprotein; TIL, tumor-infiltrating lymphocytes.

$1_{(27-35)}$ sequence, AAGIGILTV, which might influence responsiveness. We questioned whether a postulated mechanism for autoreactive T cell induction, involving epitope mimicry (20–24), might play a role in the self-directed CTL response to MART-$1_{(27-35)}$. In particular, we sought to determine whether CTL in vivo might encounter mimics of the MART-$1_{(27-35)}$ epitope derived from non-tumor sources, a circumstance which might result in an augmented population of CTL nominally specific for MART-$1_{(27-35)}$.

The central premise of epitope mimicry as an etiological mechanism is that T cells triggered by a foreign (pathogen-derived) antigen might in turn react with a previously ignored (25) self antigen, providing that sufficient similarity exists between the foreign and self epitopes in question (26). This premise is supported by accumulating reports which suggest that TcR specificities may not be as tightly restricted as previously imagined. Detailed studies of T cell epitope mimicry have thus far been confined mainly to $CD4^+$ T cells and have indicated that synonymous epitopes may arise when two or more peptides simply share select features important for TcR recognition (20–24).

In the present study, variously derived MART-$1_{(27-35)}$-specific effector populations were analyzed to define criteria important for epitope recognition, and to effectively search for potential mimicry peptides. Our findings indicate that MART-$1_{(27-35)}$-like sequences, potentially capable of being endogenously presented by HLA-A*0201, occur with high frequency among a variety of self and non-self proteins, and moreover, that MART-$1_{(27-35)}$-specific effectors are capable of recognizing many such sequences. While we deem it unlikely that a single mimetic agent underlies the anti-MART-1 CTL response in melanoma patients, one of the mimicry peptides we identified derives from the common virus HSV-1, and is endogenously processed and presented by HLA-A*0201-expressing cells. Our combined observations suggest that CTL might encounter a variety of MART-$1_{(27-35)}$-like sequences in vivo, with various possible functional consequences for the anti-tumor response to MART-$1_{(27-35)}$.

Materials and Methods

Peptides. MART-$1_{(27-35)}$ derives from the melanoma/melanocyte protein MART1/Melan A, and has the sequence AAGIGILTV, in one-letter amino acid code. Flu $M1_{(58-66)}$, used as a control in these studies, derives from the Influenza A matrix protein M1 and has the sequence GILGFVFTL. The sequences and origins of other synthetic peptides are described in the text and figures. All peptides were synthesized by standard F-moc, solid phase chemistry using a multiple peptide synthesizer (AMS Model 422; Gilson, Middleton, WI). Peptide quality after cleavage from the resin was assessed by HPLC and peptides generally were used without further purification. The purity and mass of peptides shown in Fig. 3 were further analyzed by mass spectrometry. Peptide stock solutions were made at 1–5 mg/ml in pure DMSO.

Cells. Anti-MART-1 T cell lines CLW and TIL-GDN are independently derived HLA-A*0201-restricted cytolytic effectors obtained from separate patients by either in vitro stimulation of patient PBL using MART-$1_{(27-35)}$ peptide-pulsed cells (CLW) (17), or by high-dose IL-2, in vitro expansion of tumor-infiltrating lymphocytes (TIL-GDN) (15). Both T cell lines were determined to be >90% $CD8^+/CD3^+$. Clone A42 is an HLA-A*0201-restricted, $CD8^+$ clone which specifically lyses HLA-matched melanoma cell lines and MART-$1_{(27-35)}$-pulsed target cells, and was initially isolated from TIL (10). Details regarding the generation, characterization, and maintenance of these effector cells are described in the sources cited above. Anti-MART-1 clones 620.41 and 620.62 were isolated by limiting dilution cloning of TIL 620 (15); clonality was verified by $TcRV_\alpha$ sequence analysis as described previously (27). An anti-influenza CTL line specific for the matrix peptide $M1_{(58-66)}$ was generated from an HLA-A*0201 individual by in vitro stimulation with peptide-pulsed PBMC. T2 cells (28) are HLA-A*0201^+ human lymphoid cells that are defective in antigen processing, but effectively present exogenously supplied peptides. LCL 721 is an HLA-A*0201^+, Epstein-Barr virus (EBV)-transformed human B cell line (29). Both lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Vaccinia Constructs. VV-gC5 (30), kindly provided by Dr. Barry Rouse (University of Tennessee), encodes native glycoprotein C of herpes simplex virus-1 (HSV-1), under the control of an early viral promoter. Vac-MART1 (31) and vac-NP (32), provided by Dr. N. Restifo (Surgery Branch, NCI), are vaccinia constructs that encode native MART-1 protein and influenza nucleoprotein, respectively.

Cytotoxicity Assays. Standard ^{51}Cr-release assays were used to assess T cell recognition of peptide-loaded target cells and cells infected with recombinant vaccinia virus. In peptide loading experiments, ^{51}Cr-labeled T2 cells were added to microtiter wells containing peptide and incubated at 37°C for 1 h before the addition of CTL at the effector:target ratios indicated in the figures. For cold target inhibition experiments, unlabeled or ^{51}Cr-labeled T2 cells were incubated with the indicated peptides and then washed extensively before use. CTL were added to microwells at a fixed effector/labeled target ratio; wells contained unlabeled and labeled T2 cells at the cold/hot target ratios shown. Experiments using vaccinia virus-infected target cells were done as previously described (31). Briefly, LCL 721 cells (10^6 cells/ml) were incubated overnight at 37°C with 10^7 PFU/ml of recombinant virus. After the incubation, cells were washed extensively and labeled with ^{51}Cr for use in lysis assays.

$TCRV_\beta$ Analysis. Quantitative assessment of V_β gene usage among CTL populations was carried out by PCR. To verify that the PCR-amplified product was proportional to the amount of the target template in the original sample, dilutions of T cell cDNA were amplified by PCR with $TcRC_\beta$-specific primers (33). Amplification was done on a DNA thermal cycler (Perkin-Elmer Cetus) under the following conditions: 30 s denaturation at 95°C, 30 s annealing at 60°C, followed by a 1-min extension at 72°C, for 25 cycles. Negative controls were included with no cDNA in the mixture. 10-μl aliquots of each PCR reaction were analyzed by Southern blot using a C_β oligonucleotide probe. Autoradiographs were scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the existing linearity between PCR-amplified products and cDNA dilution was verified for each sample. The same amount of β-specific cDNA template was then used for all of the samples in this study.

PCR analysis of $TcRV_\beta$ usage was conducted using a panel of previously described oligonucleotide primers (34, 35), and carried out as described above except that 30 amplification cycles were used. These conditions allowed the detection of low levels of $TcRV_\beta$ transcripts and preliminary experiments showed that

$TcRV_\beta$ PCR products amplified from all the cDNA used in this study accumulate exponentially.

Results

Recognition of MART-$1_{(27-35)}$ Analogues. To determine criteria important for recognition of the MART-1 epitope, three HLA-A*0201-restricted, anti-MART-1 effector populations were assessed for their ability to recognize analogues of MART-$1_{(27-35)}$ (Fig. 1). A total of 46 analogues, singly-substituted at peptide positions P1-P9, were tested for their ability to sensitize T2 target cells for lysis by A42, CLW, and TIL-GDN cells. The substitutions employed were biased toward residues having aliphatic/hydrophobic side chains, in keeping with the overall character of the parent peptide. These peptides were tested at a concentration of 10 μg/ml to minimize differences in recognition related to a given analogue's affinity for the A*0201 molecule. Effectors were tested at ratios of 20:1 and 5:1 (effectors/targets) to obtain an indication of the efficiency of analogue recognition.

Recognition by clone A42 was least tolerant of substitutions, especially at central positions (P3-P7) of the peptide. While at least one substitution at most peptide positions was permissive for recognition, none of the substitutions at P5 resulted in lysis of peptide-loaded T2 cells by A42 CTL. In contrast, CLW and TIL-GDN effectors recognized the majority of analogues tested. As observed for A42 CTL, recognition by CLW and TIL-GDN was most sensitive to substitutions for GlyP5 of MART-$1_{(27-35)}$, though CLW, and to a lesser extent, TIL-GDN, were both capable of recognizing the analogue GlyP5 >Ala. Additionally, all three lines showed a similar preference for an aliphatic side chain at P6, each recognizing the isomeric replacement IleP6 >Leu, as well as the analogue P6Val; P6Thr was also recognized by CLW and TIL-GDN. While residue requirements at P3 were less stringent for CLW relative to TIL-GDN and A42, a common preference is observed among the three effector groups for a central [G,A,V]xGx motif at P3-P6, where x is a residue with an aliphatic side chain (Leu, Ileu, or Val).

One implication of these results is that GlyP5 plays a crucial role in maintaining the epitope recognized by the majority of anti-MART-$1_{(27-35)}$ TcR within these effector populations. Possibly, TcR contacts might be formed with peptide main chain atoms at this position, or the introduction of a side chain at P5 might alter peptide conformation in a manner that disturbs TcR-peptide interaction at other positions. Another immediate implication of these results concerns the TcR profiles of the CLW and TIL-GDN cell lines. While examination of Fig. 1 reveals specificity differences between these two T cell populations, the overall similarity of their recognition patterns is highly apparent, suggesting that different methods of CTL isolation (see Materials and Methods under Cells) have given rise to functionally similar T cell populations. To better characterize these effector populations and to obtain a comparison to previously characterized anti-MART-1 CTL, $TcRV_\beta$ gene usage was analyzed in both CLW and TIL-GDN cell lines, as described below.

To obtain an indication of whether the reactivities observed for CLW and TIL-GDN might be typical for CTL lines, similar experiments were conducted using an anti Flu $M1_{(58-66)}$ CTL line and singly-substituted analogues of the matrix peptide. Similar results (data not shown) were obtained, with respect to tolerance for conservative substitu-

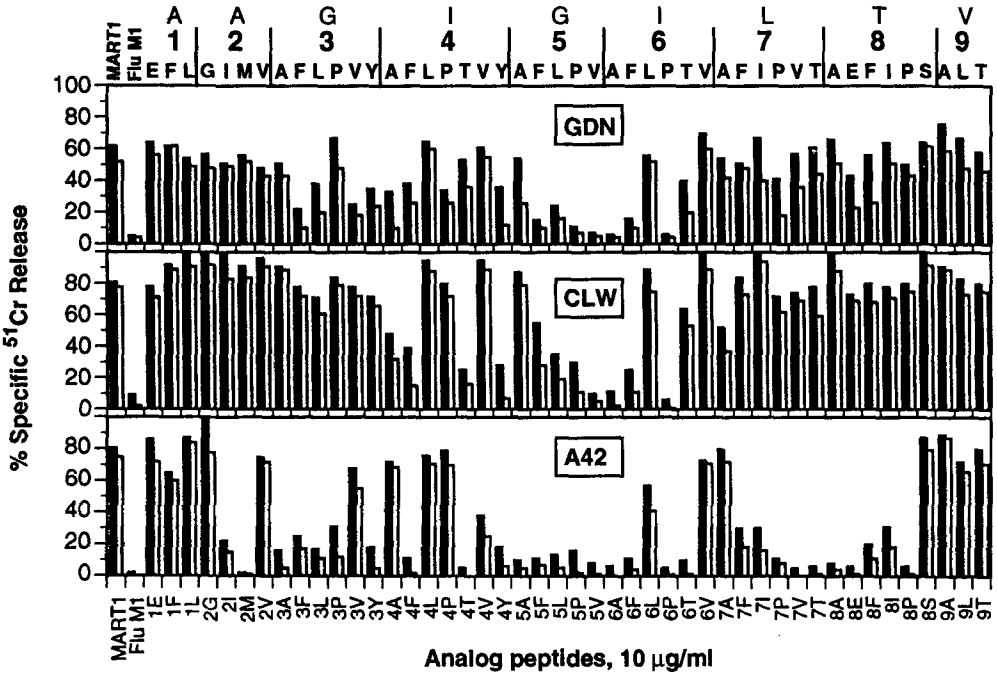

Figure 1. Synthetic, singly-substituted analogs of the MART-$1_{(27-35)}$ peptide tested for their ability to sensitize T2 cells for lysis by TIL-GDN, CLW and A42 CTL. Residue substitutions are shown at the bottom and top of the figure, along with residues of the parent peptide at positions P1-P9. The Flu $M1_{(58-66)}$ peptide is used as a control. Solid and open bars indicate lysis obtained at effector/target ratios of 20:1 and 5:1, respectively. Results shown are representative of those obtained in two or more experiments with each effector.

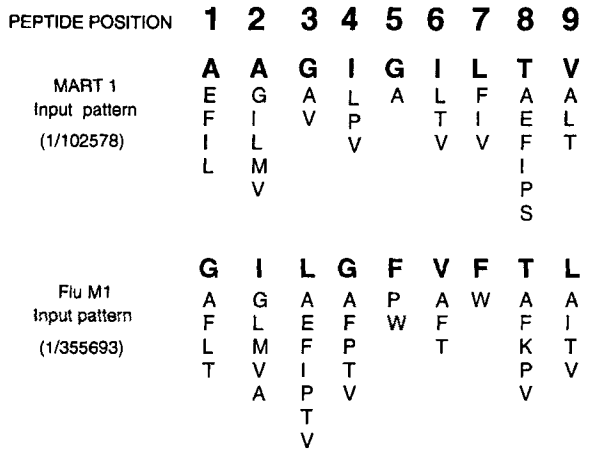

Figure 2. Input patterns used to initiate searches of the PIR protein database using the program *findpatterns*. (*Top*) MART-1 pattern, showing residues allowed at each position in addition to those of the parent peptide, shown in bold. The general features of this pattern reflect the inclusion of residues permissive for HLA-A*201 binding (36) at anchor positions P2 and P9, and the requirements imposed by T cell recognition for a limited variety of (mainly aliphatic) residues at positions P3-P7. Allowed residues were chosen based on evaluation of analogue recognition datasets, and represent a compromise between the stringency observed with clone A42 and the broader tolerance for substitutions observed with CTL lines, as shown in Fig. 1. The pattern combinatorially represents 322,560 different peptide sequences. (*Bottom*) Flu M1 pattern, obtained following analogue recognition assays using a single anti-Flu effector line derived from a healthy donor. This pattern represents 864,000 different sequences. Shown with each pattern is the probability of finding 9-mer peptides meeting the specified requirements, calculated as described in the text.

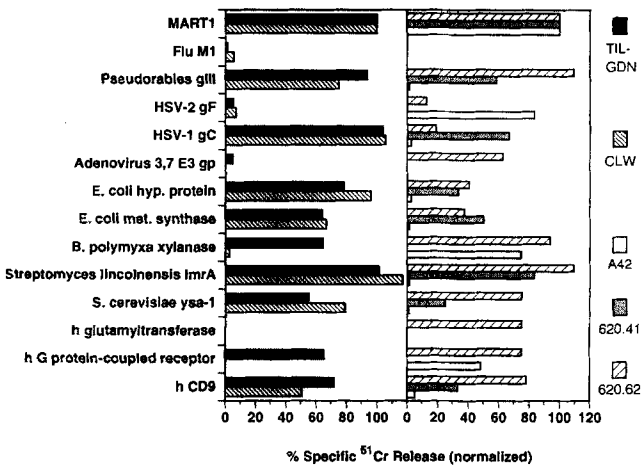

Figure 3. Sensitization of T2 cells for lysis by anti-MART-1 CTL lines and clones by synthetic peptides derived from the proteins listed on the vertical axis. The sequences of the peptides shown appear in bold type in Table 1. Each peptide was tested at 10 μg/ml, using an effector/target ratio of 10:1. To permit clearer comparisons of peptide recognition by different effectors, percent ^{51}Cr release values are normalized to values obtained with MART-$1_{(27-35)}$, set at 100%.

tions at most positions, indicating that CLW and TIL-GDN are not unique in their ability to recognize singly substituted analogues of their cognate peptide.

Database Search for Potential MART-$1_{(27-35)}$ Mimics. Guided by the analogue recognition results shown in Fig. 1, and by previously established residue preferences at anchor positions P2 and P9 of HLA-A*0201–binding peptides (36), we used the pattern shown in Fig. 2 to initiate a search of the PIR database using the program *findpatterns* in the Genetics Computer Group software. This strategy was employed previously by Wucherpfennig and Strominger (24) to identify class II–presented epitope mimics. A direct alignment search (37) of the database was also done for the MART-$1_{(27-35)}$ sequence. For the purpose of comparison, data gathered from anti-Flu M1 effector recognition of matrix peptide analogues were similarly used to compose an input pattern (also shown in Fig. 2) for the *findpatterns* program. This search served as a control to determine the degree to which sequence similarities might be generally observed using a highly degenerate test sequence.

The *findpatterns* search for "natural analogues" of MART-$1_{(27-35)}$ retrieved 348 non-identical peptide sequences, while the search using the Flu M1 matrix peptide pattern retrieved only 69 non-identical sequences. This disparity in the number of sequences retrieved may be largely accounted for by amino acid occurrence rates. Residues Gly, Ala, Val, and Leu, featured prominently in the MART-1 pattern, occur with high relative frequencies in proteins. Overall probabilities for each pattern, calculated based on the percent occurrence of amino acids in a pooled database of prokaryotic and mammalian proteins (38), suggest that sequences conforming to the MART-1 input pattern are approximately three times more likely to occur at random than sequences specified by the Flu matrix peptide pattern, despite the difference in pattern degeneracy noted in Fig. 2. The MART-1 pattern search retrieved 120 sequences more than predicted by chance (n = 228), while the number retrieved by the Flu M1 pattern search was roughly equal to that suggested by probability alone (n = 66).

CTL Recognition of Naturally Occurring Sequences. Sequences retrieved by direct alignment were examined for HLA-A*0201 binding motifs, and together with those retrieved by *findpatterns*, nearly 400 sequences variously similar to MART-$1_{(27-35)}$ were gathered for consideration. Inspection of these sequences led us to synthesize 40 candidate mimicry peptides. Sequences were chosen based primarily on how closely they conformed to the central [G,A,V]xGx motif at P3-P6 of MART-$1_{(27-35)}$, with preference given to sequences derived from pathogens and self proteins. Test sequences are shown in Table 1. These peptides were tested for their ability to sensitize T2 cells for lysis by A42, CLW, and TIL-GDN cells, and by two additional anti-MART 1 CTL clones, 620.41 and 620.62. The results for those peptides testing positive with at least one of the effector groups are shown in Fig. 3; equivalent profiles were obtained using either 10 μg/ml (shown) or 1 μg/ml peptide. The sequences and proteins of origin of these peptides appear in bold type in Table 1; the remainder tested negative in lysis assays.

As depicted in Fig. 3, CLW and TIL-GDN cells recognized 7 and 9 of the 40 peptides, respectively, in nearly

Table 1. *Sequences Retrieved from PIR Protein Database*

Sequence	Species	Protein
AAGIGILTV	**Human**	**MART-1/melan A (27-35)**
AVGIGIAVV	**Human**	**CD9**
IGGIGTVPV	Human	
LVVLGLLAV	**Human**	**Glutamyl transferase**
ALGLGLLPV	**Human**	**G protein-coupled receptor**
AIVIGILIA	Human	
AVVIGIIIV	Human	
LGVIGLVAL	Human	
LLGLGVLET	Measles virus	
AMAPATIAA	Herpes simplex virus-1	
GIGIGVLAA	**Herpes simplex virus-1**	**Glycoprotein C**
GAGIGVAVL	**Herpes simplex virus-2**	**Glycoprotein F**
IAGIGILAI	**Pseudorabies virus**	**Glycoprotein III**
GAVPGIASV	Adenovirus 2	
LIVIGILIL	**Adenovirus 3,7**	**E3 9K glycoprotein**
VDGIGILTI	***Saccharomyces cerevisiae***	**ysa-1**
ALVIGIVTL	*Saccharomyces cerevisiae*	
IGAIGLIFT	*Candida albicans*	
LAGIGLIAA	***Streptomyces lincolnensis***	**lmr A**
LGGLGLFFA	*Mycobacterium tuberculosis*	
IAGPGTITL	*Mycobacterium tuberculosis*	
LAGVALLAT	*Streptococcus gordonii*	
EIVLGIIIA	*Streptococcus mutans*	
LIGLGLLSA	*Bordetella pertussis*	
IIVLGILIL	*Pneumocystis carinii*	
ALGLGVFAA	*Pseudomonas putida*	
FIGVALVAL	*Pseudomonas aeruginosa*	
LIAIAIFAL	*Pseudomonas aeruginosa*	
LIGLAVLST	*Pseudomonas aeruginosa*	
LVGIGVATA	*Pseudomonas aeruginosa*	
GAGIGVLTA	***Bacillus polymyxa***	**β-endoxylanase**
GVGLGVLSL	*Yersinia enterocolitica*	
LIALGVIIV	*E. coli*	
AGGIGIFTL	*E. coli*	
FMGIGLIAT	*E. coli*	
AAGIGIIQI	***E. coli***	**Methionine synthase**
AIGIGILGG	*E. coli*	
QGGIGLLTV	*E. coli*	
PLGIGVLTT	*E. coli*	
LAVLGVLAL	*E. coli*	
QAGIGILLA	***E. coli***	**Hypothetical protein**

Synthetic peptides screened for MART-1(27-35) mimicry and their origins. The peptides shown represent sequences retrieved by both direct alignment and pattern-based searches of the protein database; those marked by bold type sensitized T2 cells for lysis by at least one anti–MART-1 effector population.

	A A **G I G I L** T V	MART-1
1.	A V **G I G I A** V V	h CD9
2.	L V **V L G L L** A V	h glutamyltranferase
3.	A L **G L G L L** P V	h G protein-coupled receptor
4.	G I **G I G V L** A A	HSV-1 gC
5.	G A **G I G V A** V L	HSV-2 gF
6.	I A **G I G I L** A I	Pseudorabies gIII
7.	L I **V I G I L** I L	Adenovirus 9K glycoprotein
8.	L A **G I G L I** A A	Streptomyces lincolnensis lmrA
9.	V D **G I G I L** T I	Yeast ysa-1
10.	G A **G I G V L** T A	B. polymyxa β-endoxylanase
11.	A A **G I G I I** Q I	E.coli methionine synthase
12.	Q A **G I G I L** L A	E.coli hypothetical protein

Figure 4. Alignment of sequences recognized in lysis assays by one or more anti-MART-1 effectors. Residues at P3-P7 are shown in bold and yield the motif described in the text.

identical fashion. Few of the test peptides were recognized by clone A42 (3/40 positive), however the two other clones tested recognized, to varying degrees, 7 (clone 620.41) and 10 (clone 620.62) of the 40 peptides. Alignment of the 12 sequences showing activity (Fig. 4) reveals a motif at P3-P7 comprised by [**G**,V]-[**I**,L]-[**G**]-[**I,L,V**]-[**L**,I,A]; bold letters indicate the most frequent residues at each position. Comparison of active versus inactive peptide sequences indicates that conformity to this P3-P7 motif is necessary but not sufficient for recognition by anti-MART-$1_{(27-35)}$ effectors. The failure of CTL to recognize some test peptides bearing this motif could reflect poor peptide–class I binding, or residues at P1,2/P8,9 might force these peptides to adopt conformations that do not favor productive TcR engagement.

The test peptides recognized by anti-MART1 CTL derive from both self and non-self protein sequences. Among the species represented in Table 1, adenovirus and HSV-1 are common and highly prevalent human pathogens. The adenovirus 3/7-derived peptide was recognized only by clone 620.62. The three viral glycoproteins from which synthetic peptides $gIII_{(455-463)}$ (Pseudorabies virus), $gC_{(480-488)}$ (HSV-1), and $gF_{(446-454)}$ (HSV-2) are derived are members of the herpesvirus glycoprotein F superfamily. HSV-2 is a also common human pathogen, though not as prevalent as HSV-1. Pseudorabies virus mainly affects swine, and gIII has been described as an immunodominant antigen for cellular responses in swine and murine models (39). Likewise, CTL responses to gC have been noted in murine models of HSV-1 infection (40). Of the HSV-derived peptides, $gF_{(446-454)}$ is recognized only by clone A42, while $gC_{(480-488)}$-pulsed targets were lysed significantly by T cell lines CLW and TIL-GDN, in addition to clone 620.41.

The remainder of the peptides recognized in lysis assays derive from human, bacterial, and yeast proteins. The potential significance of these other reactivities observed for anti-MART-1 CTL is further considered below. However, given the prevalence of HSV-1 as a human pathogen and the observed reactivity of 3/5 effectors to the gC-derived peptide, we focused additional study on $gC_{(480-488)}$. A dose-response curve for the gC peptide (Fig. 5), using CLW effectors, indicates that significant lysis is observed with this peptide at concentrations of 100 ng/ml and

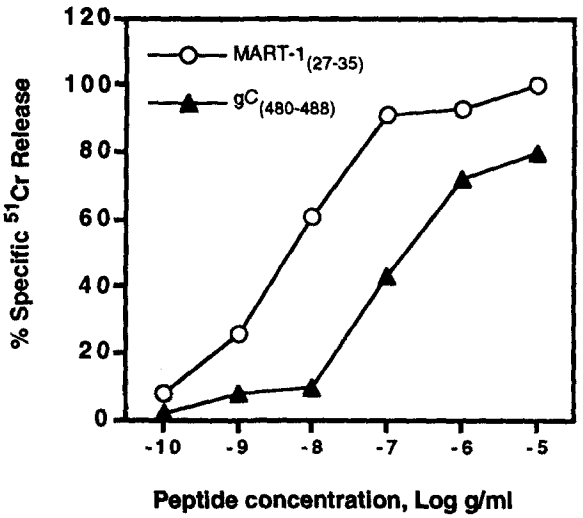

Figure 5. Dose-response plot of lysis obtained with T2 cells loaded with either $MART\text{-}1_{(27-35)}$ or $gC_{(480-488)}$ at the concentrations indicated. CTL line CLW was used at an effector/target ratio of 10:1.

above. The MART-1 peptide is at least 10-fold more potent in its ability to sensitize T2 cells for lysis by CLW, however, judgments of relative potency are complicated by our observation that $gC_{(480-488)}$ is poorly soluble in aqueous media, as were the majority of peptides listed in Fig. 3, likely due to their high content of aliphatic residues.

CTL Recognition of Endogenously Processed HSV-1 gC. To establish whether the reactivity to $gC_{(480-488)}$ might be physiologically meaningful, we tested the ability of CLW and TIL-GDN to lyse cells expressing native gC protein. Using vaccinia constructs separately encoding native MART-1 protein, gC, and influenza nucleoprotein (NP),

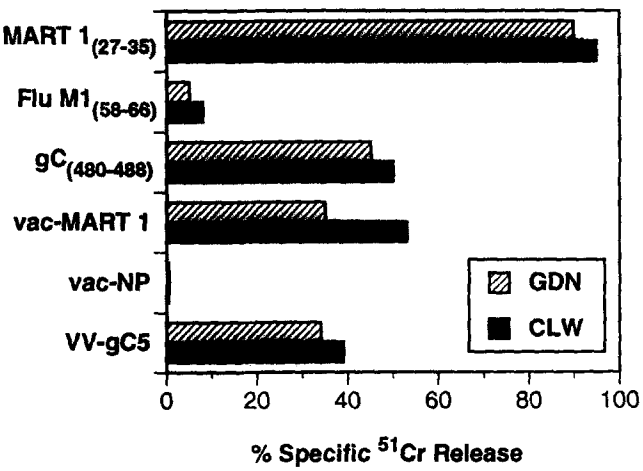

Figure 6. Lysis of LCL 721 cells by anti-MART-1 CTL lines after infection of targets with recombinant vaccinia viruses encoding for either native MART-1 (vac-MART-1) or HSV-1 gC (VV-gC5). Lysis obtained with vac-NP, encoding for the irrelevant antigen influenza nucleoprotein, serves as a control for vaccinia infection. Also shown are results obtained using LCL 721 cells pulsed with the indicated peptides at 1 μg/ml, to compare presentation of endogenously processed versus exogenously supplied antigen.

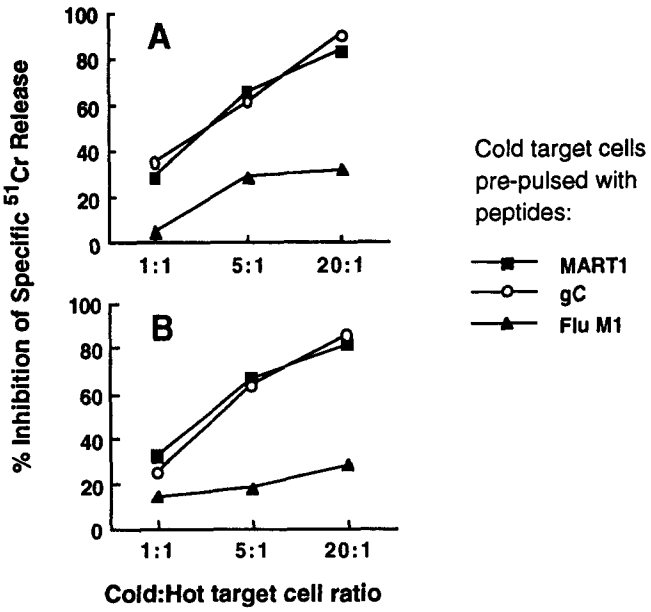

Figure 7. Inhibition of lysis mediated by TIL-GDN using peptide-pulsed cold targets. ^{51}Cr-labeled T2 cells pulsed with MART-$1_{(27-35)}$ (*A*) or $gC_{(480-488)}$ (*B*) were mixed with unlabeled T2 cells pulsed with peptides as indicated in the key above. CTL were used at an effector/target ratio of 10:1.

we observed that LCL 721 cells infected with vac-MART-1 or VV-gC5 recombinants, but not vac-Flu NP, were lysed by both CLW and TIL-GDN effectors (Fig. 6). Thus, both MART-1 and gC-derived peptides are effectively processed and presented through the endogenous pathway. Under these assay conditions, equivalent levels of lysis were obtained for targets infected with vac-MART-1 or VV-gC5, despite the difference in peptide potency noted when using exogenously supplied peptides.

Confirmation of Cross-reactivity. Cold target inhibition experiments were done to confirm that recognition of MART-$1_{(27-35)}$ and $gC_{(480-488)}$ by T cell lines is mediated by the same receptor(s). Unlabeled T2 cells pulsed with peptide $gC_{(480-488)}$ effectively inhibited lysis by TIL-GDN of ^{51}Cr-labeled target cells pulsed with MART-$1_{(27-35)}$ (Fig. 7). Similarly, in the reciprocal experiment, MART-$1_{(27-35)}$-pulsed cold targets inhibited lysis of $gC_{(480-488)}$-pulsed, labeled T2 cells. In addition, equivalent inhibition at different cold/hot target ratios was obtained in each case, whether cold targets were pulsed with gC or MART-1 peptide. Similar results were obtained using CLW effectors (data not shown). These results indicate that the bulk of the lysis observed for target cells sensitized with either MART-$1_{(27-35)}$ or $gC_{(480-488)}$ peptide is mediated by the same TcR.

Analysis of TcR V_β Gene Usage. The expression of TcRV_β family transcripts in effector lines CLW and TIL-GDN (Fig. 8) showed both lines to be oligoclonal, suggesting antigen-driven selection. As a control, the TcRV_β repertoire was also analyzed in fresh, uncultured PBL of patient CLW. In peptide-stimulated effector line CLW, expression of many TcRV_β segments was absent or sharply decreased relative to the uncultured PBL, while the relative expression of V_β14 was significantly increased. As shown in Fig. 8, V_β14, V_β4, and V_β3 in peptide-stimulated PBL together account for more than 70% of the TcRV_β segments expressed. The increased representation of V_β14 in the CLW effector line (21% versus 3% in fresh PBL) suggests that this TcRV_β, as well as V_β4 and V_β3, could be directly involved in recognition of the MART-$1_{(27-35)}$ epitope.

In TIL-GDN, V_β1 and V_β15 segments respectively accounted for 76 and 13% of the TcRV_β transcripts (Fig. 8, *bottom*). Fresh GDN PBL were not available for this study, precluding a comparative analysis and interpretation of this V_β expression pattern. However, the dominance of V_β1 and V_β15 transcripts in the TIL-GDN population is strongly suggestive of a role for these V_β segments in recognition of the MART-$1_{(27-35)}$ epitope.

The TcR of clone A42 previously has been characterized (27) and uses V_β7. TcRV_β sequence analysis of clone 620.41 revealed that the TcR of this clone uses V_β17; data for clone 620.62 were not available for this study. Detailed TcR analyses of the two TIL 620-derived clones will be reported elsewhere.

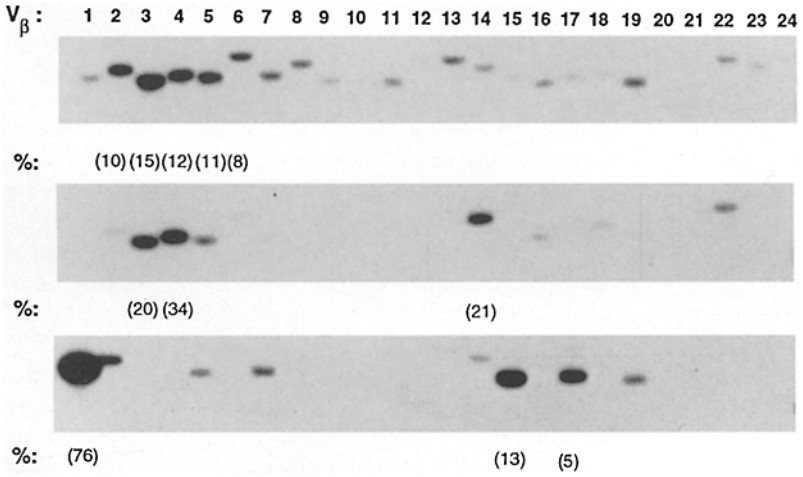

Figure 8. Autoradiogram showing TcRV_β expression in fresh PBL from patient CLW (*top*), anti-MART-1 CTL line CLW (*middle*), and TIL-GDN (*bottom*). Results were obtained following PCR amplification of TcRV_β segments and Southern blotting, as described in Materials and Methods. TcRV_β segments are designated at the top of the upper panel. Following the nomenclature adopted by Wei et al. (35), V_β segments 17, 18, 19, and 20 were amplified by primers V_β-19, 17, 20, and 18, respectively. Numbers below lanes indicate the level of V_β expression as a percentage of the summed pixel values from all lanes after quantitation on a PhosphorImager; values are shown for expression levels greater than 5%.

The two MART-1–specific cell lines analyzed here display different TcRV_β repertoires characterized by the predominant expression of two to three V_β transcripts. Prior analyses have revealed common usage of V_β14 and V_β7 segments among TIL- and PBL-derived anti–MART-1 clones from different melanoma patients (reviewed in reference 41), and anti–MART-1 clones using V_β3 (27) and V_β4 (42) have also been described. These previous analyses have also revealed that anti–MART-1 TcR from melanoma patients are generally characterized by restricted V_β usage and CDR3 regions of diverse length and composition (41). Overall, the CTL lines studied here appear to embody characteristics typical of MART-1–reactive effectors described to date.

Discussion

While numerous factors affect immune responsiveness, especially to self antigens, we sought in the present study to investigate whether sequences closely related to the MART-$1_{(27-35)}$ epitope, AAGIGILTV, derived from sources other than MART-1, might be encountered by the immune system and potentially play a role in shaping the anti–MART-1 CTL response. The criteria for "similarity" to MART-$1_{(27-35)}$ derive from requirements imposed by both T cell recognition and binding to the HLA-A*0201 molecule. These requirements lead to a consensus sequence dominated by aliphatic residues, a feature contributing to the retrieval of a large number of sequences, variously similar to MART-$1_{(27-35)}$, from the protein database.

Alignment of the 12 naturally occurring sequences recognized by anti–MART-1 effectors in this study indicates close conformity to the MART-$1_{(27-35)}$ parent peptide at P3-P7; all of these mimics contain Gly at P5. Previous studies of class II-presented mimicry peptides have indicated a similar requirement for maintenance of identity or close similarity to the parent peptide at two to three critical residues (20, 21, 24), though some of these mimics lacked extensive identity or similarity to the parent peptide overall. Class I and class II molecules are distinguished by the closed (class I) versus open (class II) design of their respective peptide binding grooves (43, 44). Peptides bound to HLA-A2 and related allomorphs are anchored at their amino and carboxy termini, while side chains in the peptide mid-region tend to adopt sequence-specific orientations (43). This feature may impose constraints on the range of sequence possibilities available to class I–presented mimics. In general, however, our results are consistent with the suggestion arising from class II studies that overall, significant sequence identity does not guarantee, nor is it a requirement for, TcR recognition.

Given the currently limited representation of proteins in the database, the number of MART-$1_{(27-35)}$ mimics may not be limited to the sequences described here. The relevance of our observations is predicated on at least a subset of these mimicry peptides being endogenously processed and presented by cell surface HLA-A*0201 molecules. If so, then circulating CTL could encounter an array of epitopes similar to MART-$1_{(27-35)}$ contributed from a variety of self and non-self proteins.

The HSV-1-derived peptide $gC_{(480-488)}$ fulfills the minimal criteria for a pathogen-derived epitope mimic that could play a role in potentiating the anti–MART-1 response. Our data indicate HLA-A*0201–expressing cells endogenously process and present a gC-derived epitope recognized by a subset of anti-MART-$1_{(27-35)}$ CTL, and that these effectors recognize $gC_{(480-488)}$ in a cross-reactive fashion. HSV-1 (non-genital herpes) infection is common among humans, and a majority of adults show evidence of prior exposure to this virus (45). CTL responses to HSV-1 in humans remain poorly characterized (46), and to our knowledge, HLA-A*0201-restricted CTL epitopes have not been described. While it is unlikely that HSV-1 plays a primary causative role in the anti–MART-1 response, it is plausible that exposure to HSV-1, or to multiple pathogens contributing similar epitopes, could result in the generation of CTL which are subsequently recruited in the response to tumor-derived MART-$1_{(27-35)}$ in HLA-A*0201 melanoma patients. It will be of interest to determine whether anti-gC CTL can be raised from melanoma patients or donors which subsequently cross-react with MART-$1_{(27-35)}$.

Our combined observations raise alternative possibilities for mimicry playing a role in shaping the anti-MART-$1_{(27-35)}$ CTL response. The anti–MART-1 effectors we tested also recognized peptides derived from human (self) and *Escherichia coli* (commensal self) proteins. Thymic presentation of a variety of MART-1–like self sequences might play an important role in positive selection of T cells, shaping a TcR repertoire that is, to a degree, biased toward recognizing such sequences (47, 48). There is also evidence indicating that bacterially derived antigens can elicit $CD8^+$ responses (49, 50), and that *E. coli* sequences in particular are visible to the immune system (51). The consequences of potentially constitutive, peripheral presentation of mimicry peptides are difficult to predict; these epitopes might be ignored entirely, or perhaps may drive low level expansion and/or anergy (52) of reactive CTL and contribute to tolerance in vivo.

A puzzling aspect of the anti-melanoma, anti–MART-1 CTL response is the apparent inability of these CTL, in many cases, to effectively eradicate tumors in vivo. A number of mechanisms may contribute to tumor escape, including downregulation of MHC and/or antigen (53), possibly as a result of immunoselection (54). In this case, MART-1–like sequences in the periphery might act to maintain an expanded population of anti-MART-1 CTL whose anti-tumor function is obsolete.

However, it appears that anti-MART-1 CTL in vivo often do not elaborate a full-blown autoimmune response. A recent study examined the effect of immunizing melanoma patients with MART-$1_{(27-35)}$ peptide (Cormier, J., M.L. Salgaller, T. Prevette, L. Rivoltini, K.C. Barracchini, N.P. Restifo, S.A. Rosenberg, and F.M. Marincola, manuscript submitted for publication). Immunization significantly increased anti–MART-$1_{(27-35)}$ CTL reactivity recovered from peripheral blood, though clinical improvement was not ap-

parent. While presently speculative, there may be another role for MART-1–like sequences in shaping CTL activity in vivo. In the context of an anti–MART-1 response to tumor, T cell encounters with MART-$1_{(27-35)}$–like peptides acting as partial agonists or antagonists (55, 56) might result in negative modulation of the CTL response.

We view the utility of the analysis described here as two-fold. A comprehensive search for mimicry peptides can potentially uncover undesirable reactivities to other self peptides, a matter of general importance to self epitope-targeted immunotherapy. In addition, this type of analysis may provide a starting point for asking new questions related to how CTL responses might be shaped in vivo.

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