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\(_{(27-35)}\)...
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_3s>. 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 turn in 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<258_666>, 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 lyzes 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 usage analysis as described previously (27). An anti-influenza CTL line specific for the matrix peptide M1<258_666> 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 51Cr-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, 51Cr-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 51Cr-labeled T2 cells were incubated with the indicated peptides and then washed extensively before use. CTL were added to microtiter wells at a fixed effector: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 (10⁵ cells/ml) were incubated overnight at 37°C with 10⁵ PFU/ml of recombinant virus. After the incubation, cells were washed extensively and labeled with 51Cr for use in lysis assays.

TcRV Analysis. Quantitative assessment of Vα 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 Jurkat cDNA were amplified by PCR with TcRV-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 Cα 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 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 TcRVβ transcripts and preliminary experiments showed that
TcRV₃ 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]xG 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, TcRV₃ 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.](image)
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 ²⁹Cr release values are normalized to values obtained with MART-1 (27,35), set at 100%.

| Peptide Position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------|---|---|---|---|---|---|---|---|---|
| MART-1 Input pattern | A | A | G | I | G | I | L | T | V |
| Flu M1 Input pattern | G | I | L | G | F | V | F | T | L |

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 alphabetic) 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. Shown with each pattern is the probability of finding 9-mer derived from a healthy donor. This pattern represents 864,000 different positions P2 and P9 of HLA-A*0201-binding peptides 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 ²⁹Cr release values are normalized to values obtained with MART-1 (27,35), set at 100%.

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 ²⁹Cr release values are normalized to values obtained with MART-1 (27,35), set at 100%.

MART-1 pattern, 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
| Sequence     | Species            | Protein                                           |
|--------------|--------------------|---------------------------------------------------|
| AAGIGILTV    | Human              | MART-1/melan A (27-35)                            |
| AVGIGIAVV    | Human              | CD9                                               |
| IGGIGTVPV    | Human              | Glutamyl transferase                             |
| LVLGLLAV     | Human              | G protein-coupled receptor                        |
| ALGLGLLPV    | Human              |                                                   |
| AIVIGILIA    | Human              |                                                   |
| AVVGIHV     | Human              |                                                   |
| LGVLGLVAL   | Human              |                                                   |
| LLGLGVLET   | Measles virus      |                                                   |
| AMAPATIAA   | Human              |                                                   |
| GIGIGVLAA   | Herpes simplex virus-1 |                                                |
| GAGIGVAVL   | Herpes simplex virus-2 |                                                |
| IAGIGILAI   | Pseudorabies virus |                                                |
| GAVPGIASV   | Adenovirus 2       |                                                |
| LIVIGILIL   | Adenovirus 3,7     |                                                   |
| VDGIGILTI   | Saccharomyces cerevisiae |                                               |
| ALVIGIVTL   | Saccharomyces cerevisiae |                                         |
| IGAIGLIFT   | Candida albicans   |                                                   |
| LAGIGLIAA   | Streptomyces lincolnensis |                                                |
| LGGLGLFFA   | Mycobacterium tuberculosis |                                         |
| IAGPGTITL   | Mycobacterium tuberculosis |                                         |
| LAGVALLAT   | Streptococcus gordonii |                                              |
| EIVGIIIA    | Streptococcus mutans |                                              |
| LGIGLIL    | Bondetella pertussis |                                              |
| IIIVGLIL    | Pneumocystis carinii |                                              |
| ALGLGVFAA   | Pseudomonas putida  |                                              |
| FIGVALVAL   | Pseudomonas aeruginosa |                                    |
| LIAIAIFAL   | Pseudomonas aeruginosa |                                    |
| LIGLAVLST   | Pseudomonas aeruginosa |                                    |
| LYGIVVATA   | Pseudomonas aeruginosa |                                    |
| GAGIGVLTA   | Bacillus polymyxa   | β-endoxylanase                                   |
| GVGLGVLSL   | Yersinia enterocolitica |                          |
| LIALGVIII   | E. coli            |                                                   |
| AGGIGIFTL   | E. coli            |                                                   |
| FMGIGLIAT   | E. coli            |                                                   |
| AAGIGIIQI   | E. coli            | Methionine synthase                              |
| AIGIGILGG   | E. coli            |                                                   |
| QGIGILTV    | E. coli            |                                                   |
| PLGIGVLT    | 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.
necessary but not sufficient for recognition by anti-lular responses in swine and murine models (39). Likewise, productive TcR engagement.

pulsed targets were lysed significantly by T cell lines CLW yield the motif described in the text.

also common human pathogen, though not as prevalent as has been described as an immunodominant antigen for cel-

thetic peptides gl[1(4ss_463) (Pseudorabies virus), g0(480 488) clone 620.62. The three viral glycoproteins from which syn-

are common and highly prevalent human pathogens. The species represented in Table 1, adenovirus and HSV-1

force these peptides to adopt conformations that do not fa-

sequence indicates that conformity to this P3-P7 motif is

ment at P3-P7 comprised by [G,V]-[I,L]-[G]-[I,L,V]-

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.

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 A42 (3/40 positive), however the other two 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.

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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 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),
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 gC480-488 by T cell lines is mediated by the same receptor(s). Unlabeled T2 cells pulsed with peptides as indicated in the key above. CTL were used at an effector/target ratio of 10:1.

cold target inhibition experiments were done to confirm that recognition of MART-1(27-35) and gC480-488 by T cell lines is mediated by the same receptor(s). Unlabeled T2 cells pulsed with peptides as indicated in the key above. CTL were used at an effector/target ratio of 10:1.

Figure 8. Autoradiogram showing TcR Vβ 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 TcR Vβ segments and Southern blotting, as described in Materials and Methods. TcR Vβ segments are designated at the top of the upper panel. Following the nomenclature adopted by Wei et al. (35), Vβ segments 17, 18, 19, and 20 were amplified by primers Vβ-17, 18, 20, and 18, respectively. Numbers below lanes indicate the level of Vβ expression as a percentage of the sum of 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 TcRV3 repertoire characterized by the predominant expression of two to three Vβ transcripts. Prior analyses have revealed common usage of Vβ14 and Vβ7 segments among TIL- and PBL-derived anti-MART-1 clones from different melanoma patients (reviewed in reference 41), and anti-MART-1 clones using Vβ3 (27) and Vβ4 (42) have also been described. These previous analyses have also revealed that anti-MART-1 TcR from melanoma patients are generally characterized by restricted Vβ 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 close conformity to the MART-1(27_35) 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 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(481_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(481_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. Prevête, 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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References

1. Boon, T., T.F. Gajewski, and P.G. Coulie. 1995. From defined tumor antigens to effective immunization? Immunol. Today. 16:334–336.
2. Coulie, P.G., F. Lehmann, B. Lethe, J. Herman, C. Lurquin, M. Andrawiss, and T. Boon. 1995. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. Proc. Natl. Acad. Sci. USA. 92:7976–7980.
3. Wolfgél, T., M. Hauer, J. Schneider, M. Serrano, C. Wolfgél, H.E. Klehmán, E. De Plaen, T. Hankeln, K. H. Meyer zum Buschenfelde, and D. Beach. 1995. A p16INK4a–insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science (Wash. DC). 269:1281–1284.
4. Robbins, P.F., M. El-Gamil, Y.F. Li, Y. Kawakami, D. Loftus, E. Appella, and S.A. Rosenberg. 1996. A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J. Exp. Med. 183:1185–1192.
5. van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash. DC). 254:1643–1647.
6. Boel, P., C. Wildmann, M.L. Sensi, R. Brasseur, J.C. Renaud, P. Coulie, T. Boon, and P. van der Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. Immunity. 2:167–175.
7. Van den Eynde, B., O. Peeters, O. De Backer, B. Gaugdler, S. Lucas, and T. Boon. 1995. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J. Exp. Med. 182:689–698.
8. Brichard, V., A. Van Pel, T. Wolfgél, C. Wolfgél, E. De Plaen, B. Lethe, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med. 178:489–495.
9. Coulie, P.G., V. Brichard, A. Van Pel, T. Wolfgél, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, J.P. Szikora et al., 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med. 180:35–42.
10. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, L. Rivoltini, S.L. Topalian, T. Miki, and S.A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl. Acad. Sci. USA. 91:3515–3519.
11. Cox, A.L., J. Skipper, Y. Chen, R.A. Henderson, T.L. Darrow, J. Shabanowitz, V.H. Engelhard, D.F. Hunt, and C.J. Slingluff. 1994. Identification of a peptide recognized by five melanoma–specific human cytotoxic T cell lines. Science (Wash. DC). 264:716–719.
12. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, K. Sakaguchi, E. Appella, J.R. Yannelli, G.J. Adema, T. Miki, and S.A. Rosenberg. 1994. Identification of a human melanoma antigen recognized by tumor infiltrating lymphocytes associated with in vivo tumor rejection. Proc. Natl. Acad. Sci. USA. 91:6458–6562.
13. Wang, R.F., P.F. Robbins, Y. Kawakami, X.Q. Kang, and S.A. Rosenberg. 1995. Identification of a gene encoding a melanoma tumor antigen recognized by HLA-A31–restricted tumor infiltrating lymphocytes [published erratum appears in J. Exp. Med. 181:1261]. J. Exp. Med. 181:799–804.
14. Marincola, F.M., D. Venzon, D. White, J.T. Rubin, M.T. Lotze, T.B. Simonis, J. Balkissoon, S.A. Rosenberg, and D.R. Parkinson. 1992. HLA association with response and toxicity in melanoma patients treated with interleukin 2–based immunotherapy [published errata appear in Cancer Res. 1993 53:3846 and 1993 53:6079]. Cancer Res. 52:6561–6566.
15. Kawakami, Y., S. Eliyahu, K. Sakaguchi, P.F. Robbins, L. Rivoltini, J.R. Yannelli, E. Appella, and S.A. Rosenberg. 1994. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2–restricted tumor infiltrating lymphocytes. J. Exp. Med. 180:347–352.
16. Spagnoli, G.C., C. Schaefer, T.E. Willimann, T. Kocher, A. Amoroso, A. Juretic, M. Zuber, U. Luschker, F. Harder, and M. Heberer. 1995. Peptide-specific CTL in tumor infiltrating lymphocytes from metastatic melanomas expressing MART-1/Melan-A, gp100 and Tyrosinase genes: a study in an unselected group of HLA-A2.1-positive patients. Int. J. Cancer. 64:309–315.

17. Rivoltini, L., Y. Kawakami, K. Sakaguchi, S. Southwood, A. Sette, P.F. Robbins, F.M. Marincola, M.L. Salgaller, J.R. Yannelli, E. Appella, and S.A. Rosenberg. 1995. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. J. Immunol. 154:2257–2265.

18. Salgaller, M.L., A. Afsar, F.M. Marincola, L. Rivoltini, Y. Kawakami, and S.A. Rosenberg. 1995. Recognition of multiple epitopes in the human melanoma antigen gp100 by peripheral blood lymphocytes stimulated in vitro with synthetic peptides. Cancer Res. 55:4972–4979.

19. Stevens, E.J., L. Jacknin, P.F. Robbins, Y. Kawakami, M. El-Gamil, S.A. Rosenberg, and J.R. Yannelli. 1995. Generation of tumor-specific CTLs from melanoma patients by using peripheral blood stimulated with allogeneic melanoma tumor cell lines. Fine specificity and MART-1 melanoma antigen recognition. J. Immunol. 154:762–771.

20. Bhardwaj, V., V. Kumar, H.M. Geyser, and E.E. Sercarz. 1993. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells. J. Immunol. 151: 5000–5010.

21. Garza, K.M., and K.S.K. Tung. 1995. Frequency of molecular mimicry among T cell peptides as the basis for autoimmune disease and autoantibody induction. J. Immunol. 155: 5444–5448.

22. Shimoda, S., M. Nakamura, H. Ishibashi, K. Hayashida, and Y. Niho. 1995. HLA DRB4 0101-restricted immunodominant T cell autoepitope of pyruvate dehydrogenase complex in primary biliary cirrhosis: evidence of molecular mimicry in human autoimmune diseases. J. Exp. Med. 181:1835–1845.

23. Quaranta, S., C.J. Thorpe, P.J. Travers, and M. Londei. 1995. Similar antigenic surfaces, rather than sequence homology, dictate T-cell epitope molecular mimicry. Proc. Natl. Acad. Sci. USA. 92:10398–10402.

24. Wucherpfennig, K.W., and J.L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell. 80:695–705.

25. Ohashi, P.S., S. Oechen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner. 1991. Ablation of tolerance and induction of diabetes by virus infection in viral antigen transgenic mice. Cell. 65: 305–317.

26. Oldstone, M.B.A. 1990. Molecular mimicry and autoimmune disease. Cell. 50:819–820.

27. Cole, D.J., D.P. Weil, P. Shamamian, L. Rivoltini, Y. Kawakami, S. Topalian, C. Jennings, S. Eliyahu, S.A. Rosenberg, and M.I. Nishimura. 1994. Identification of MART-1-specific T-cell receptors: T cells utilizing distinct T-cell receptor variable and joining regions recognize the same tumor epitope [published erratum appears in Cancer Res. 1994 54: 6014]. Cancer Res. 54:5265–5268.

28. Ljunggren, H.G., N.J. Stan, C. Ohlen, J.J. Neefjes, P. Hoglund, M.T. Heemels, J. Bastin, T.N. Schumacher, A. Townsend, K. Karre, and H. Ploegh. 1990. Empty MHC class I molecules come out in the cold. Nature (Lond.). 346: 476–480.

29. Milford, E.L., L.J. Kennedy, S.Y. Yang, B. Dupont, J.-M. Lalouel, and E.J. Yunis. 1987. Serological characterization of the reference panel of B-lymphoblastoid cell lines for factors of the HLA system. In Immunobiology of HLA, Vol. 1 Histocompatibility Testing. B. Dupont, editor. Springer, New York. 19.

30. Weir, J.P., M. Bennet, E.M. Allen, K.L. Elkins, S. Martin, and B.T. Rouse. 1989. Recombinant vaccinia virus expressing the herpes simplex virus type 1 glycoprotein C protects mice against herpex simplex virus challenge. J. Gen. Virol. 70: 2587–2594.

31. Rivoltini, L., D.J. Luftas, K. Barracchini, F. Arienti, A. Mazzocchi, W.E. Biddison, M. Salgaller, E. Appella, G. Parmiani, and F. Marincola. 1996. Binding and presentation of peptides derived from melanoma antigens MART-1 and gp100 by HLA-A2 subtypes: implications for peptide-based immunotherapy. J. Immunol. In press.

32. Smith, G.L., J.Z. Levin, P. Palese, and B. Moss. 1987. Synthesis and cellular location of the ten influenza polyptides individually expressed by recombinant vaccinia viruses. Virol. 160:336–345.

33. Salvi, S., F. Segalla, S. Rao, F. Arienti, M. Sartori, G. Brantina, E. Caronno, A. Anichini, C. Clemente, G. Parmiani, and M. Sensi. 1995. Overexpression of the T-Cell Receptor beta-chain variable region TCRBV14 in HLA-A2-matched primary human melanomas. Cancer Res. 55:3374–3379.

34. Genevee, C., A. Diu, J. Nierat, A. Caignard, P. Dietrich, L. Ferradini, S. Roman-Roman, F. Triebl, and T. Hercend. 1992. An experimentally validated panel of subfamily-specific oligonucleotide primers (Vα1-w29/Vβ1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. Eur. J. Immunol. 22:1261–1269.

35. Wei, S., P. Charmlay, M.A. Robinson, and P. Concannon. The extent of the human germline T-cell receptor V beta gene segment repertoire. 1994. Immunogenetics. 40:27–36.

36. Kast, W.M., R.M. Brandt, J. Sidney, J.W. Drijfhout, R.T. Kubo, H.M. Grey, C.J. Melief, and A. Sette. 1994. Role of HLA-A motif in identification of potential CTL epitopes in human papillomavirus type 16 E6 and E7 proteins. J. Immunol. 153:3904–3912.

37. Pearson, W.R., and D.J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. 85:2444–2448.

38. Data made available and distributed electronically by Protein Information Resource (PIR), National Biomedical Research Foundation, Washington, D.C. 20007, USA.

39. Zuckerma, F.A., L. Zsak, T.C. Mettenleiter, and T. Ben-Porter. 1990. Pseudorabies virus glycoprotein gill is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. J. Virol. 64:802–812.

40. Martin, S., C.M. Mercadal, J.P. Weir, and B.T. Rouse. 1993. The proportion of herpes simplex virus-specific cytotoxic T lymphocytes (Tc) that recognize glycoprotein C varies between individual mice and is dependent on the form of immunization. Viral Immunol. 6:21–33.

41. Sensi, M., and G. Parmiani. 1995. Analysis of TCR usage in human tumors: a new tool for assessing tumor-specific immune responses. Immunol. Today. 16:588–595.

42. Sensi, M., C. Traversi, M. Radizophreni, S. Salvi, C. Macalli, R. Mortarini, L. Rivoltini, C. Farina, G. Nicolini, T. Woflel et al. 1995. Cytotoxic T-lymphocyte clones from different
patients display limited T-cell-receptor variable-region gene usage in HLA-A2-restricted recognition of the melanoma antigen Melan-A/MART-1.

43. Madden, D.R., D.N. Garboczi, and D.C. Wiley. 1993. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2 [published erratum appears in Cell. 1994 76:410]. Cell. 75:693–708.

44. Stern, L.J., J.H. Brown, T.S. Jardetzky, J.C. Gorga, R.G. Urban, J.L. Strominger, and D.C. Wiley. 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature (Lond.). 368:215–221.

45. Whitely, R.J. 1994. Herpes simplex virus infections. In Herpesvirus infections. R. Glaser and J. F. Jones, editors. Marcel Dekker, New York. 1–57.

46. Borysiewicz, L.K., and J.G. Sissons. 1994. Cytotoxic T cells and human herpes virus infections. Curr. Top. Microbiol. Immunol. 189:123–150.

47. Marrack, P., J. McCormack, and J. Kappler. 1989. Presentation of antigen, foreign major histocompatibility complex proteins and self by thymus cortical epithelium. Nature (Lond.). 338:503–505.

48. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1994. Specificity and flexibility in thymic selection. Nature (Lond.). 369:750–752.

49. Dunkley, M., R. Pabst, and A. Cripps. 1995. An important role for intestinally derived T cells in respiratory defence. Immunol. Today. 16:231–236.

50. Sztein, M.B., M.K. Tanner, Y. Polotsky, J.M. Orenstein, and M.M. Levine. 1995. Cytotoxic T lymphocytes after oral immunization with attenuated vaccine strains of Salmonella typhi in humans. J. Immunol. 155:3987–3993.

51. Dellacona, P., G. Casorati, B. Friedli, L. Angman, F. Sallusto, A. Tunnahlciff, E. Roosneck, and A. Lanzavecchia. 1993. In vivo persistence of expanded clones specific for bacterial antigens within the human T cell receptor α/β CD4–8– subset. J. Exp. Med. 177:1763–1771.

52. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. Science (Wash. DC). 248:1349–1356.

53. Ferrone, S., and F.M. Marincola. 1995. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. Immunol. Today. 16:487–494.

54. Lehmann, F., M. Marchand, P. Hainaut, P. Pouillart, X. Sastre, H. Ikeda, T. Boon, and P.G. Coulie. 1995. Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. Eur. J. Immunol. 25:340–347.

55. Sette, A., J. Alexander, J. Ruppert, K. Snoke, A. Franco, G. Ishioka, and H.M. Grey. 1994. Antigen analogs/MHC complexes as specific T cell receptor antagonists. Annu. Rev. Immunol. 12:413–431.

56. Cao, W., S.S. Tykodi, M.T. Esser, V.L. Braciale, and T.J. Braciale. 1995. Partial activation of CD8+ T cells by a self-derived peptide. Nature (Lond.). 378:295–298.