[CANCER RESEARCH 59, 301–306, January 15, 1999]

Advances in Brief

A Superagonist Variant of Peptide MART1/Melan $A_{27–35}$ Elicits Anti-Melanoma $CD8^+$ T Cells with Enhanced Functional Characteristics: Implication for More Effective Immunotherapy[1]

Licia Rivoltini,[2] Paola Squarcina, Douglas J. Loftus, Chiara Castelli, Paolo Tarsini, Arabella Mazzocchi, Francesca Rini, Vincenzo Viggiano, Filiberto Belli, and Giorgio Parmiani

Divisions of Experimental Oncology D [L. R., P. S., C. C., P. T., A. M., F. R., G. P.], Medical Oncology A [V. V.], and Surgical Oncology B [F. B.], Istituto Nazionale Tumori, 20133 Milan, Italy, and Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892 [D. J. L.]

Abstract

In the present study, we show that a singly substituted peptide derived from the epitope $MART1_{27–35}$ and containing a Leu in position 1 (LAGIGILTV; 1L) behaves as a superagonist by *in vitro* inducing specific T cells with enhanced immunological functions. 1L-specific CTLs can be raised from peripheral blood of HLA-$A2^+$ melanoma patients more efficiently than T cells specific for the cognate peptide. These T cells show a greater sensitivity to native $MART1_{27–35}$ when compared with CTL variable raised to parental peptide from the same patients. More importantly, anti-1L but not anti-native T cells display high levels of interferon γ production at early time points, and readily secreted interleukin-2 in response to native epitope endogenously presented by melanoma cells. Additionally, anti-1L T cells are insensitive to the inhibitory effects of $MART1_{27–35}$ natural analogues that antagonize the lytic response of CTLs raised to the cognate peptide. Analysis of T-cell receptor variable β usage suggests that the native and 1L peptides stimulate different components of the $MART1_{27–35}$-reactive T cell population. These data provide rationale to the use of superagonist analogues of tumor antigens for inducing *in vivo* immunization potentially able to overcome tumor immune escape and mediate a more significant control of tumor growth.

Introduction

It is now well established that $CD8^+$ tumor infiltrating lymphocytes recovered from melanoma patients recognize a variety of unmodified self peptides that are derived from lineage-specific proteins (1, 2), and are bound to MHC class I molecules. Among such epitopes are those derived from the melanosomal proteins tyrosinase, gp100/Pmel17, and MART1. Although robust CTL activity may be observed *ex vivo*, often in the presence of excess cytokines and antigen, a clear understanding of the functional status of these cells *in vivo*, and their impact on tumor growth, has been elusive. In contrast, several mechanisms have been described that potentially contribute to tumor cell evasion of the immune response (3), suggesting that any antitumor efficacy achieved by immune effectors may be offset by countermeasures that result ultimately in tumor progression.

However, the prevailing dynamic *in vivo* between immune effector and tumor cells may not reflect a "fair fight." Little evidence is available to indicate that CTLs directed to tumor-expressed self epitopes are indeed engaged in a full-fledged autoaggressive response, stemming from a true breach of tolerance. In recent years, studies focused on the influences of costimulation and cytokines on immune responses in general, and autoimmunity in particular, have led to a better appreciation of the factors that contribute to a sustained and effective response. Several such studies also indicate that qualities of the peptide ligand itself can significantly impact the quality of initiated or existing responses (4–7), by providing either optimal or suboptimal stimuli that ultimately affect T cell functions. These studies also extend the relevance of peptides acting in the capacity of APLs[3] (8), suggesting that the activities described for APLs *in vitro* (ranging from superagonists to antagonists) might be harnessed to gain control over immune responses *in vivo*. Although the majority of such studies has involved $CD4^+$ T cells, recent studies indicate that parallels exist for $CD8^+$ cells as well (5, 9). These latter reports in particular highlight the critical differences that may exist among CTL responses obtained with different, but related, immunogenic agonists.

Such studies suggest the potential for finding highly optimized peptides that might positively modulate CTL autoreactivity as it occurs in melanoma patients, thereby better exploiting these responses for therapeutic purposes. In a previous study, we described a singly substituted analogue of the $MART1_{27–35}$ peptide (AAGIGILTV, native peptide; LAGIGILTV, variant "1L") that showed heteroclitic activity toward the CTL raised to the native $MART1_{27–35}$ peptide (10). We view heteroclitic activity, as do others (5), as applying to peptides of which the dose-response curves in functional assays are dramatically left-shifted relative to those of the native peptide, without a commensurate gain in binding affinity for HLA class I. Here, we report that the 1L superagonist can generate CTLs with quantitatively and qualitatively enhanced recognition of melanoma cells expressing the native peptide. These results suggest that a superagonist peptide might be used clinically to exploit a latent capacity of the T cell repertoire to respond more aggressively to the $MART1_{27–35}$ self epitope.

Materials and Methods

Lymphocytes and Tumor Cells. Anti-MART1 and 1L T cells were generated from peripheral blood of HLA-$A2.1^+$ melanoma patients, as previously described in detail (11). Briefly, lymphocytes were stimulated weekly with autologous irradiated PBMCs pulsed with 1 μM peptide. Exogenous IL-2 (30–60 IU/ml) was added 1 day after restimulation. Before being used as effectors in functional assays, bulk cultures were depleted of $CD4^+$ cells by incubation with Mab-coated magnetic beads (Dynal Dynabeads; Oxoid, Oslo, Norway). A42 is a $MART1_{27–35}$-reactive CTL clone isolated from tumor infiltrating lymphocytes, as described previously (12); it was maintained in culture with 120 IU/ml IL-2. The HLA-$A2.1^+$ melanoma line 501 mel is autologous to clone A42. T2 is a peptide transport-deficient cell line that

Received 10/8/98; accepted 12/1/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by grants from the Italian Association for Cancer Research (AIRC Milano) and from the Italian Ministry of Health.

[2] To whom requests for reprints should be addressed, at the Division of Experimental Oncology D, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-2390-631; Fax: 39-02-2362-692.

[3] The abbreviations used are: APL, altered peptide ligand; TCR, T-cell receptor; PBMC, peripheral blood mononuclear cell; IL-2, interleukin 2; Flu, influenza; IFNγ, interferon γ; Mab, monoclonal antibody; TCRBV, TCR β variable region.

effectively presents exogenously supplied peptides in the context of HLA-A2.1.

Peptides. Peptides were synthesized as described previously (13). $MART1_{27–35}$, also referred to in the text as native peptide, has the sequence AAGIGILTV and derives from the melanosomal protein MART1. Mono-substituted variants of $MART1_{27–35}$ used in this study carry Leu or Glu at position 1 (P1) of the peptide (designated 1L and 1E, respectively), Val at P6 (6V), and Ser at P8 (8S). The sequences and origins (shown in parentheses) of additional human protein-derived peptides used are as follows: (*a*) LIGLGVISI (renal sodium/phosphate transporter); (*b*) LAGLGLLVI (signal sequence receptor, a-subunit); (*c*) LLVAGVLVL (CD8b); (*d*) LLGIILLVL (IFN-induced monokine); (*e*) VMGLGVLLL (angiogenin); (*f*) AAGLSLLTL (E-selectin); (*g*) SLGLGLLPV (G protein coupled receptor); (*h*) AVGIGIAVV (CD9); (*i*) IGGIGTPVP (translation elongation factor); (*j*) LVVLGLLAV (glutamyl transferase); (*k*) ALGLGLLPV (G protein-coupled receptor); (*l*) AIVIGILIA (glucose transporter); (*m*) AVVIGIIIV (glutamate transporter); (*n*) LLIGIGLVL (bone marrow stromal antigen-2). Also used as a negative control was the Flu A matrix protein M1 peptide (designated "M1Flu" in figures; residues 58–66, GILGFVFTL).

Cytotoxicity, Cytokine Release, and Antagonist Assays. Cytolytic activity was measured in a standard 4-h ^{51}Cr release assay using target cells treated as described in each figure. Specific lysis was calculated from [(experimental cpm-nonspecific cpm)/(total cpm-nonspecific cpm)] $\times$ 100%, where total cpm was determined from detergent-releasable counts, and nonspecific cpm were the counts released in the absence of added CTLs. HLA-A2 blocking of T-cell lysis was performed by preincubating target cells with the anti-HLA-A2 Mab CR11.351. For cytokine release immunoassay, 1×10^5 target cells were incubated in the absence or presence of different peptides in round-bottomed microtiter plates for 2 h at 37°C. Lymphocytes (1×10^5) were added in 150 μl/well (total volume, 250 μl/well) and incubated overnight (20 h). Plates were then centrifuged, and 200-μl aliquots of supernatant were removed from each well and stored at −80°C until assayed; for cytokine detection, 50 μl (for IFNγ) or 100 μl (for IL-2) were used. Immunoassays were done using ELISA kits for IFNγ (MABTECH, Nacka, Sweden) and IL-2 (Quantikine; R&D Systems, Minneapolis, MN). In the antagonist assays, T2 cells were incubated for 2 h at 37°C with suboptimal concentrations of the $MART1_{27–35}$ peptide (*i.e.*, 30 ng/ml for T cells raised with $MART1_{27–35}$ peptide and 30 pg/ml for T cells generated with 1L peptide), using for both lymphocyte cultures an E:T ratio of 2.5:1. T2 cells were then washed and plated into microwells containing different concentrations of the peptides being tested for antagonism. After incubation for 1 h at 37°C, CTLs were added, and the standard cytolysis assay was carried out as described above. Baseline cytolysis under these conditions typically resulted in a 30–40% specific ^{51}Cr release. Data are reported as percentages of lysis inhibition, calculated as follows: [100 - (Lysis in the absence of antagonist peptide): (% lysis in the presence of the antagonist peptide) $\times$ 100].

Quantification of cDNA and TCRBV Analysis. Total RNA from T lymphocytes was extracted by using the RNAzolB (Cinna/Biotecs, Friendswood, TX) Single-stranded cDNA synthesis was carried out on 2 μg of total RNA with oligo-dT and Moloney murine leukemia virus-derived reverse transcriptase without RNase H activity (MMLV RT RNaseH-, Superscript; Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. cDNA was suspended in 40 μl of water. Quantitative assessment of the *TCRBV* gene usage among peptide-specific CTL lines was carried out with PCR, as previously described (13). To verify that under our PCR conditions the amplified product was proportional to the amount of the target template in the original sample, each cDNA was serially diluted (2-fold dilution up to 1:128), and each dilution was amplified by PCR with TCRBC-specific primers. The amplification was performed in 25 μl of reaction mixture in the presence of 1 μl of the diluted cDNA, 200 μM of each dNTPs, 1 μM of each primer, and 0.625 units of Taq polymerase (Ampli Taq). By a DNA thermal cycler (Perkin-Elmer Corp., Emeryville, CA), 25 cycles of amplification were performed under the following conditions: 95°C, 30-s denaturation; 60°C, 30-s annealing; and 72°C, 1-min extension. Negative controls were included with no cDNA in the mixture. PCR reactions (10 μl) were analyzed by Southern blot using a C β oligonucleotide probe. Autoradiographs were scanned by a computer-assisted imaging system (Molecular Dynamics, Sunnyvale, CA), and the existing linearity between PCR-amplified products and cDNA dilution was verified for each sample used in this study. The same amount of β-specific cDNA template was then used for all of the samples used in the present study. PCR analysis of the TCRBV repertoire was conducted using a panel of described oligonucleotide primers (14). The level of TCRBV-specific amplification was measured by a densitometric scanning (Eagle-eye; Stratagene) and computer-assisted analysis (Eagle Sight; Stratagene) of ethidium bromide-stained gel loaded with 10 μl of each amplification product. Each TCRBV was expressed as a percentage of the sum of all TCRBV signals detected. Repertoire determination was performed at least three times for each sample.

Results

Recognition of Heteroclitic MART1 Analogues by Specific CTL. We previously identified a synthetic $MART1_{27–35}$ analogue, containing a Leu at P1 (LAGIGILTV; "1L"), that shows heteroclitic behavior with a variety of anti-$MART1_{27–35}$ effectors (10). To confirm this previous observation, we tested recognition (assessed as cytotoxic activity) by the $MART1_{27–35}$-specific clone A42 (12) of different singly substituted $MART1_{27–35}$ analogues. As shown in Fig. 1*A*, 1L peptide is the most efficiently recognized among several variant peptides of which the dose-response curves are left-shifted relative to that of the native peptide. The increased responsiveness to the 1L peptide cannot be attributed to enhanced class I binding, because it binds to HLA-A2.1 with an affinity similar to that of the native peptide (10).

Generation of Anti-MART1 T Cells from HLA-$A2^+$ Melanoma Patients by *in Vitro* Sensitization with 1L or MART1/Melan $A_{27–35}$ Peptides. Given the strong recognition of the 1L analogue by anti-MART1 CTL, we evaluated whether this heteroclitic epitope could be more efficient in the generation of specific T cells able to cross-react with the native peptide $MART1_{27–35}$. Peripheral blood lymphocytes from HLA-$A2^+$ melanoma patients were stimulated *in vitro* either with the 1L analogue or with the native peptide, according to a protocol previously described (11). After 2 weeks of culture, T cells were tested for cytotoxic activity against peptides endogenously presented by HLA-$A2^+$ melanoma cells (501mel) or exogenously pulsed onto T2 cells. As reported in Fig. 1*B*, 1L resulted in the generation of cytotoxic T cells more effective in mediating lysis of $MART1_{27–35}$-expressing targets (either peptide-pulsed T2 or 501mel) than the T cells raised with the native peptide. In two out of five patients (1 and 4), specific cytotoxic T cells were generated only with 1L, and no significant activity was obtained using the native epitope. Background cytolysis of T2 pulsed with the irrelevant peptide from Flu matrix M1Flu was, in all cases, tested below 5%, supporting the fine antigen specificity of 1L-generated T cells. Lysis by the anti-MART1/Melan A clone A42 is also reported in Fig. 1*B* as a positive control. Cytolytic activity of anti-1L T cells on 501mel was mediated by a specific recognition of HLA-A2/MART1 complex because it could be blocked by an anti-HLA-A2 Mab and no significant killing of the 501 lymphoblastoid cell line could ever be detected (data not shown). No lysis of autologous lymphoblastoid cell lines was observed either, thus confirming the absence of detectable autoreactivity toward antigens other than MART1 (data not shown).

Peptide titrations were done to assess how efficiently anti-1L CTLs recognized the native peptide. As shown in Fig. 2*A*, 1L-specific T cells responded to significantly lower concentrations of the $MART1_{27–35}$ peptide than those detected by anti-native T cells. Anti-1L T cells were also able to mediate effective melanoma lysis at lower E:T ratios compared with CTLs raised to cognate peptide (Fig. 2*B*).

Specific CTLs exert several immunological functions in addition to cytotoxic activity against antigen-bearing targets. For a more extensive characterization of anti-1L T cell functions, IFNγ release in response to the parental MART1 peptide (exogenously presented by T2 cells or endogenously expressed by the melanoma 501mel) was evaluated at different weeks of *in vitro* culture. As reported in Fig. 2,

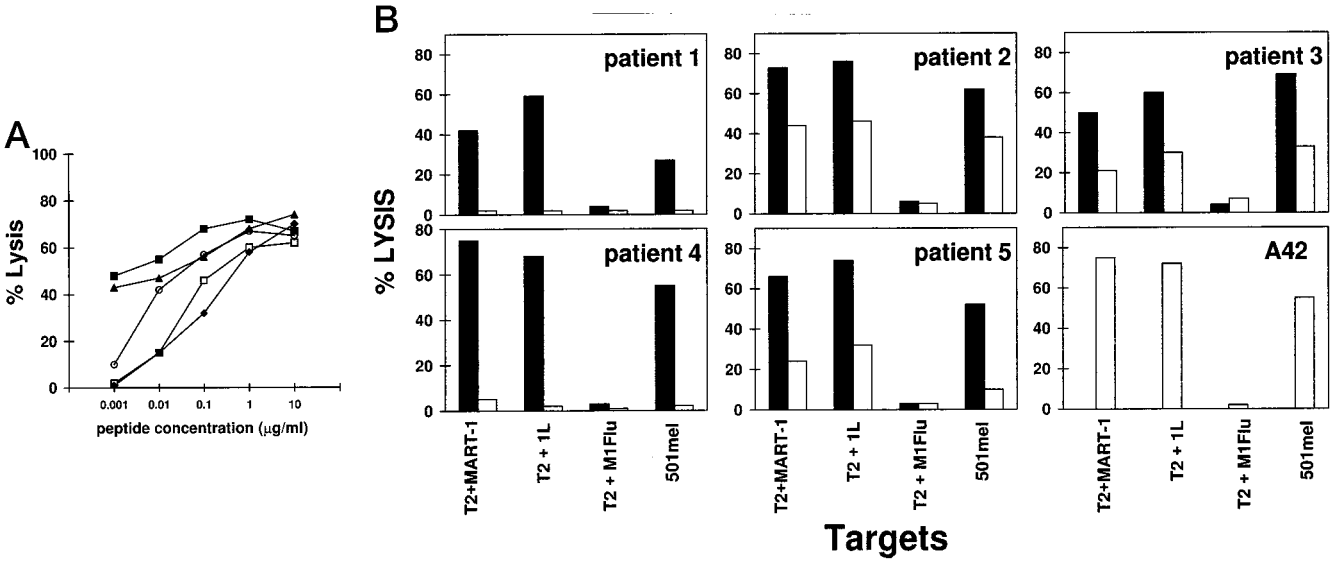

Fig. 1. Immunogenicity of the variant 1L as compared with the native peptide MART1/Melan $A_{27–35}$. *A*, recognition of heteroclitic analogues of MART1/Melan $A_{27–35}$ by the anti-native clone A42. T2 cells were pulsed with different peptide concentrations and tested as target in a 4-h ^{51}Cr-release assay. Data refer to an E:T ratio of 10:1. □, $MART1_{27–35}$; ♦, 1E; ■, 1L; ○, 6V; ▲, 8S. *B*, cytotoxic activity of T cells generated either with 1L or native peptide. Specific T cells were generated from PBMCs of HLA-$A2^+$ melanoma patients, following a protocol described in details elsewhere (19). T cells were tested after 2 weeks of *in vitro* culture, and data refer to a 4-h ^{51}Cr-release assay (E:T ratio = 5:1). Peptide concentration for T2 cell pulsing was 1 μM. M1Flu (an immunodominant peptide from the Flu matrix protein) was used as negative control. ■, anti-1L T cells; □, anti-native T cells.

C and *D*, when effectors were stimulated by the T2 plus native peptide and 501mel, respectively, all of the anti-1L T cells tested showed a higher and earlier production of IFNγ than T cells raised with the cognate peptide. Significant recognition of the HLA-$A2^+$/$MART1^+$ melanoma cells (501mel) could also be detected in all cases tested.

IL-2 Production by Anti-1L T *versus* Anti- $MART1_{27–35}$ Cells in Response to the Cognate Peptide. IL-2 production has been shown recently to be a feature not only of $CD4^+$ helper T cells, but also of fully activated $CD8^+$ cells (15). Impaired IL-2 secretion also has been associated with decreased functional efficacy of $CD8^+$ T cells (5, 16). In our system, only anti-1L T cells were able to secrete IL-2 in response to the $MART1_{27–35}$ cognate peptide, either exogenously presented on T2 cells or endogenously expressed by HLA-$A2^+$ melanoma cells (501mel; Fig. 3*A*). No significant IL-2 secretion in response to melanoma cells could be observed with anti-native T cells, which were found in one case (patient 5) to release barely detectable levels of IL-2, only in response to peptide-pulsed T2 cells.

Resistance of Anti-1L T Cells to Peptide Antagonism. We previously have shown that several natural analogues (peptide sequences contained within endogenous human proteins) of $MART1_{27–35}$ act as partial agonists or antagonists for anti-$MART1_{27–35}$ T cells (10, 13). Under conditions typically used to assay antagonist activity, we noted that several of these analogues effectively inhibited CTL lysis of target cells pulsed with a suboptimal concentration of native MART1/Melan A peptide. However, we also observed that these same antagonist peptides, tested over a broad concentration range, could not inhibit lysis of target cells when suboptimally pulsed with the 1L variant peptide (10). To evaluate whether CTLs raised with 1L analogue could show a comparable insensitivity to antagonist peptides, anti-1L T cells were tested in an antagonist assay with target cells suboptimally pulsed with the MART1/Melan $A_{27–35}$ peptide, using a panel of natural analogues, which included peptides previously observed to be antagonists with other anti-MART1/Melan $A_{27–35}$ CTLs (10). As shown in Fig. 3, *B* and *C*, 6 of 14 tested peptides inhibited lysis of MART1/Melan $A_{27–35}$-pulsed targets by CTLs raised against the native peptide. The anti-native peptide effector populations derived from patients 2 and 3 were each antagonized by different, though partially overlapping, subsets of peptides, most likely reflecting differences in the composition of their responding repertoires, as further suggested by the TCRVβ analysis presented below. On the contrary, no significant antagonism was observed when anti-1L T cells were used (Fig. 3, *B* and *C*), thus confirming the improved immune functions of T cells generated with a superagonist variant.

TCRBV Repertoire of Anti-1L and Anti-MART1/Melan $A_{27–35}$ T Cells. To evaluate whether the different functional behavior of T cells generated with the 1L analogue or with the native peptide could be correlated to an *in vitro* selection of a different TCR repertoire, the TCRBV families expressed by the two specific T cell populations were analyzed by a semiquantitative reverse transcription-PCR. Each repertoire was compared with the TCRBV expressed by unstimulated $CD8^+$ fresh lymphocytes. Fig. 4 reports data obtained for patients 2 and 5 for whom unstimulated PBMCs were available. Comparing the TCRBV repertoire of fresh $CD8^+$ T cells with the corresponding cell line obtained after specific *in vitro* stimulation with 1L or native peptide, a different distribution of TCRBV was observed, suggesting that an antigen-driven selection occurred during the *in vitro* culture. In addition, inside each patient, the TCRBV repertoire was differently affected by stimulation with 1L or with the native peptide, indicating that the final effector T cells greatly differs for their general TCR composition. Comparable data were obtained in patient 3 (data not shown). Additionally, in both patients reported here (Fig. 4, *A* and *B*), a preferential expansion of TCRBV13 and 14 could be observed in 1L-stimulated T lymphocytes, whereas no common usage was detectable for T cells generated with the cognate peptide.

Discussion

T lymphocytes have been shown to have no single ligand specificity, but to recognize a large array of peptides, termed APLs (8). These APLs can mediate a number of different outcomes in interacting T cells, ranging from inducing selective immunological functions (*partial agonist*) to completely turning off their functional capacity (*antagonist*). In addition to suboptimal ligands, TCR can also be triggered by superagonists, which are analogues that enhance T-cell stimulation by inducing immunological functions not detected with the cognate ligand (4–7).

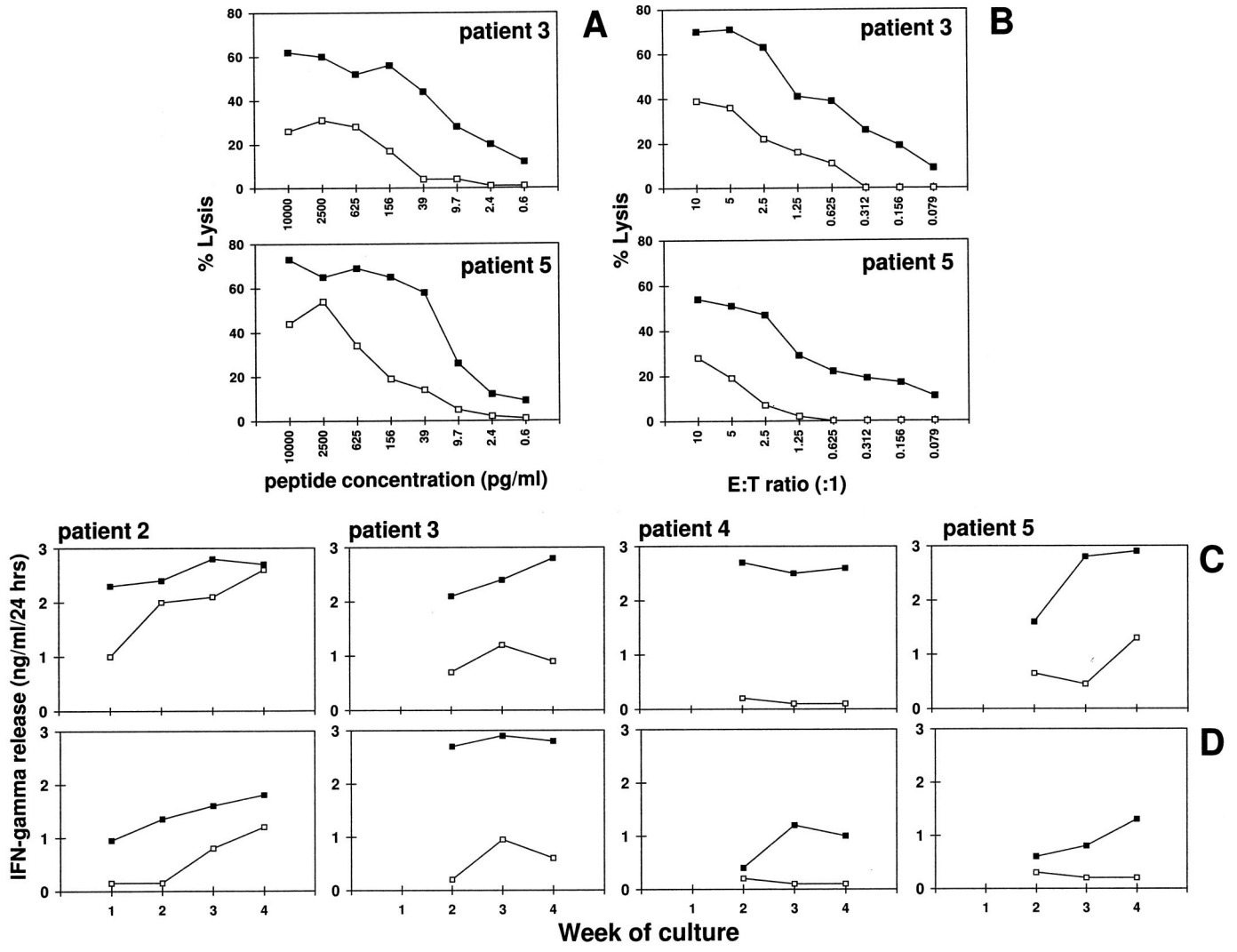

Fig. 2. Functional characterization of anti-1L *versus* anti-native peptide T cells in response to the MART1/Melan $A_{27–35}$. *A*, $MART1_{27–35}$ peptide titration, as tested in a 4-h ^{51}Cr-release assay (E:T ratio = 10:1), with anti-1L (■) or anti-native (□) T cells cultured *in vitro* for 3 weeks. *B*, titration of E:T ratio. T cells (■, anti-1L; □, anti-native) were tested against the melanoma cell line 501mel. *C* and *D*, IFNγ release by anti-1L (■) or anti-native (□) in response to cognate MART1/Melan $A_{27–35}$ peptide exogenously pulsed on T2 cells (*C*) or endogenously expressed by the HLA-$A2^+$ melanoma line 501mel (*D*). T cells were tested at different weeks of *in vitro* culture.

We recently showed that a heteroclitic variant of the melanoma-melanocyte-derived epitope MART1/Melan $A_{27–35}$, containing a Leu instead of an Ala at P1 (LAGIGILTV; 1L), could hyperstimulate MART1/Melan A-specific T cells (10). Here, we reported that this analogue, which binds to MHC with affinity comparable with the native peptide, displays superagonist activity by promoting the generation, in HLA-$A2^+$ melanoma patients, of MART1/Melan A-specific T cells with enhanced immunological functions as compared with cells raised with the native epitope. The superagonist activity of 1L can be detected as an increase in the magnitude of T cell responses to the original epitope (either exogenously pulsed on T2 cells or endogenously expressed by melanoma cells); 1L-raised T cells indeed lyse with higher efficiency MART1/Melan $A_{27–35}$-expressing targets and release in their presence cytokines such as IFNγ earlier and to a higher extent than T cells generated with the original epitope. Furthermore, these T cells exert half-maximal effector responses at concentrations of the MART1/Melan $A_{27–35}$ peptide significantly lower than that required to sensitize lymphocytes raised with the cognate epitope.

In addition to these quantitative modifications, 1L superagonist can induce qualitatively new responses, driving T cells to further secrete IL-2 in response to tumor cells (not detected in T cells generated with the native peptide), and to undergo a faster and different clonal expansion. TCR repertoire analysis shows, in fact, that 1L expands a different and more restricted panel of Vβ families than that obtained when the native peptide is used.

The magnitude of IL-2 production has been shown to be critical for determining the overall extent of the immune responses in both $CD4^+$ and $CD8^+$ T cell subpopulations (4, 15), whereas its lack is considered a key factor in mediating T cell anergy (4, 15–17). The evidence that T cells generated with the 1L superagonist produce IL-2 in response to the MART1/Melan $A_{27–35}$ peptide expressed on tumor cells, a feature undetectable with lymphocytes raised in the presence of the native epitope, represents in our opinion an important observation that may significantly change the therapeutic potential of the $MART1_{27–35}$ peptide. Anti-melanoma T lymphocytes have been, in fact, described as defective in IL-2 production when interacting with tumor cells, and this defect associated with the lack of efficiency to control tumor growth *in vivo* (18). Moreover, data from our laboratory have recently shown that IL-2 production is one of the first functions that T cells lose when interacting with partial agonists or antagonists (10, 13). The possibility of stimulating, by a superagonist variant, a T

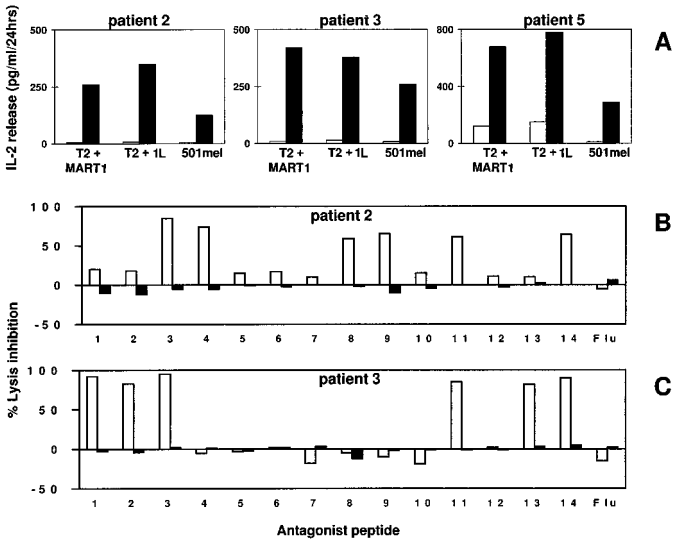

Fig. 3. *A*, IL-2 secretion by anti-1L (■) or anti-native (□) T cells in response to peptide-pulsed T2 cells or to the melanoma line 501mel. T cells were tested after 3 weeks of *in vitro* culture. *B* and *C*, peptide antagonism by MART1/Melan $A_{27–35}$ natural analogues. Antagonist assay was performed as described in "Materials and Methods." T2 cells were prepulsed with 30 ng/ml or 30 pg/ml (for anti-native and anti-1L T cells, respectively) MART1/Melan $A_{27–35}$ peptide for obtaining suboptimal lysis (30–40%) at a 2.5:1 E:T ratio. T2 cells were then washed and incubated with 1 μg/ml antagonist peptide. Each number in the *x* axis corresponds to a different antagonist peptide, as listed in the "Peptide" section of "Materials and Methods." T cells were tested after 3 weeks of *in vitro* culture, in a 4-h ^{51}Cr-release assay at 2.5:1 E:T ratio. Data are reported as percentages of lysis inhibition. □, anti-native; ■, anti-1L. M1Flu peptide was also used as negative control.

cell population potentially able to substain its own growth, once interacting with relevant antigen, and contribute in activating bystander cells should create a more favorable milieu for T-cell activity.

A further factor supporting the ability of T cells generated with 1L to better interact with their HLA-A2/$MART1_{27–35}$ ligand is their refractoriness, at least *in vitro*, to the antagonist effect mediated by MART1/Melan $A_{27–35}$ natural analogues. These analogues have been shown on the contrary to induce a strong inhibitory effect on T cells raised with the native peptide (10), therefore, supporting the hypothesis that anti-MART1/Melan A-reactive T cells may be negatively regulated *in vivo* by encountering antagonist epitopes (3).

Given the lack of evidence that tumor-specific T cells can significantly impact tumor growth *in vivo* and the limited results obtained with the first clinical protocols (19), new strategies for increasing the immunogenicity of tumor-derived peptides and to potentially improve their therapeutic efficacy have to be found. Thus far, the most commonly attempted approach has been to increase HLA binding affinity of native peptides by introducing conservative substitutions at HLA-binding anchor positions, given the correlation observed with viral antigens between immunogenicity and MHC peptide-binding affinity (20). Melanoma-derived modified peptides, the HLA binding affinity/stability of which is higher than that displayed by the native epitopes, were found to be significantly more immunogenic *i.e.*, more efficient than the cognate peptide in inducing cytotoxic T cells to gp100 (21) and MART1-derived HLA-A2.1 binding epitopes (22). The strategy of modifying natural tumor epitope to improve their immunogenicity has been also recently tested in clinics with promising results. In fact, Rosenberg *et al.* (23) have shown that vaccination with a modified analogue of an HLA-A2-binding peptide of the melanoma antigen gp100 can mediate tumor regression in 42% of patients with metastatic melanoma, a success rate not achieved in the previous studies with natural gp100 epitopes (23). Of note is the fact that clinical responses could only be observed when IL-2 was administered along with the vaccine. According to the authors, this observation suggests that exogenous IL-2 may help overcoming the state of anergy possibly induced in T cells by the interaction with the tumor antigen in the absence of costimulation, evidence that has been also proved in murine models (24). These latter *in vivo* data confirm the central role of IL-2 in T cell-mediated antitumor responses and further highlight the clinical potential of a vaccine based on the use of superagonist peptides, which could mediate *in situ* IL-2 production by specific T cells once interacting with the relevant tumor antigen.

Tumor immunotherapy is, perhaps, the one clinical setting in which the induction of an autoaggressive cellular response seems to be desirable. The superagonist variant of $MART1_{27–35}$ that we describe

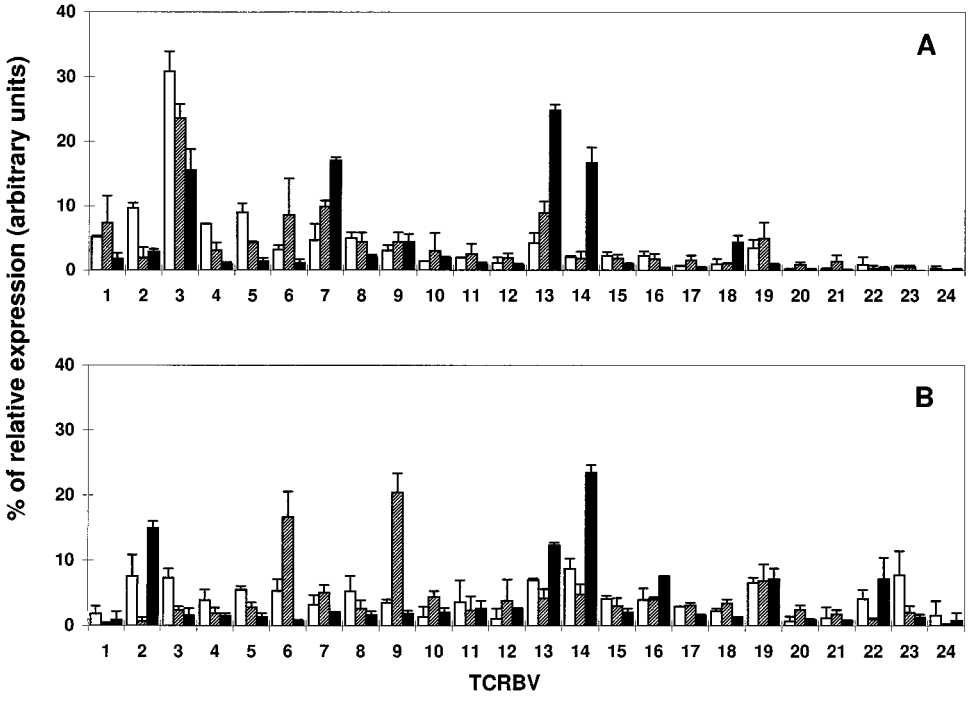

Fig. 4. TCRBV repertoire in anti-1L and anti-MART1/Melan A T cells. TCRBV usage of 1L-specific (■) and native-specific (▨) T cell lines, analyzed by semiquantitative reverse transcription-PCR, as described in "Materials and Methods," was compared with the TCRBV repertoire in fresh $CD8^+$ T lymphocytes of the same patient (□). TCRBV is expressed as a mean percentage of the sum of all TCRBV signals obtained in the same experiments. Each determination was repeated at least three times, and standard deviations are indicated. *A* and *B*, TCRBV repertoire in peptide-specific CTL lines and $CD8^+$ fresh lymphocytes of patients 2 and 5, respectively.

extends the concept of using modified self peptide immunogens to achieve this goal. Although modifications that improve peptide binding and complex stability offer demonstrable advantages, a key issue is the overall quality of the CTL response induced. The data presented here suggest that the 1L variant is capable of optimally inducing CTL *in vitro* that subsequently are capable of responding to low levels $MART1_{27–35}$ with enhanced production of IFN-γ and IL-2, in addition to cytolysis, and reduced susceptibility to negative regulatory mechanisms. The strategy of identifying self-peptide analogues with bona fide superagonist activity offers the potential of improving *in vivo*, peptide-based tumor immunotherapy.

Acknowledgments

We are grateful to Drs. Yongsheng Song and Ettore Appella (National Cancer Institute/NIH, Bethesda, MD) for peptide sythesis. We also thank Drs. Claudia Lombardo and Flavio Arienti of our Institute for HLA typing and buffy coats of melanoma patients.

References

1. Rosenberg, S. A. Cancer vaccines based on the identification of genes encoding cancer regression antigens. Immunol. Today, *18:* 175–182, 1997.
2. Boon, T., Coulie, P. G., and Van den Eynde, B. Tumor antigens recognized by T cells. Immunol. Today, *18:* 267–268, 1997.
3. Rivoltini, L., Loftus, D. J., Squarcina, P., Castelli, C., Rini, F., Arienti, F., Belli, F., Marincola, F. M., Geisler, C., Borsatti, A., Appella, E., and Parmiani, G. Recognition of melanoma-derived antigens by CTL: possible mechanisms involved in down-regulating anti-tumor T cell reactivity. Crit. Rev. Immunol., *18:* 55–64, 1998.
4. Rogers, P. R., Grey, H. M., and Croft, M. Modulation of naive CD4 T cell activation with altered peptide ligands: the nature of the peptide and presentation in the context of costimulation are critical for a sustained response. J. Immunol., *160:* .3698–3704, 1998.
5. Dressel, A., Chin, J. L., Sette, A., Gausling, R., Hollsberg, P., and Hafler, D. A. Autoantigen recognition by human CD8 T cell clones: enhanced agonist response induced by altered peptide ligands. J. Immunol., *159:* 4943–4951, 1997.
6. Vergelli,M., Hemmer, B., Kalbus, M., Vogt, A. B., Ling, N., Conlon, P., Coligan, J. E., McFarland, H., and Martin, R. Modifications of peptide ligands enhancing T cell responsiveness imply large numbers of stimulatory ligands for autoreactive T cells. J. Immunol., *158:* 3746–3752, 1997.
7. Nicholson, L. B., Waldner, H., Carrizosa, A. M., Sette, A., Collins, M., and Kuchroo, V. K. Heteroclitic proliferative responses and changes in cytokine profile induced by altered peptides: implications for autoimmunity. Proc. Natl. Acad. Sci. USA, *95:* 264–269, 1998.
8. Kersh, G. J., and Allen, P. M. Essential flexibility in the T-cell recognition of antigen. Nature (Lond.), *380:* 495–498, 1996.
9. Höllsberg, P., Weber, B., Dangond, F., Batra, V., Sette, A., and Hafler, D. Differential activation of proliferation and cytotoxicity in human T-cell lymphotropic virus type I Tax-specific CD8 T cells by altered peptide ligand. Proc. Natl. Acad. Sci. USA, *92:* 4036–4044, 1995.
10. Loftus, D. J, Squarcina, P., Nielsen, M. B, Geisler, C., Castelli, C., Ødum, N., Appella, E., Parmiani, G., and Rivoltini, L. Peptides derived from self proteins as partial agonists and antagonists of human $CD8^+$ T cell clones reactive to the melanoma-melanocyte epitope $MART1_{27–35}$. Cancer Res., *58:* 2433–2439, 1998.
11. Rivoltini, L., Kawakami, Y., Sakaguchi, K., Southwood, S., Sette, A., Robbins, P. F., Marincola, F. M., Salgaller, M. L., Yannelli, J. R., Appella, E., and Rosenberg, S. A. 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 MART1. J. Immunol., *154:* 2257–2265, 1995.
12. Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P. F., Rivoltini, L., Yannelli, J. R., Appella, E., and Rosenberg, S. A. Identification of the immunodominant peptides of MART1 human melanoma antigen recognized by the majority of the HLA-A2 restricted tumor-infiltrating lymphocytes. J. Exp. Med., *180:* 347–352, 1994.
13. Loftus, D. J., Castelli, C., Clay, T. M., Squarcina, P., Marincola, F. M., Nishimura, M. I., Parmiani, G., Appella, E., and Rivoltini, L. Identification of epitope mimics recognized by CTL reactive to the melanoma/melanocyte-derived peptide $MART1_{27–35}$. J. Exp. Med., *184:* 647–657, 1996.
14. Genevee, C., Diu, A., Nierat, J., Caignard, A., Dietrich, P., Ferradini, L., Roman-Roman, S., Triebel, F., and Hercend, T. An experimentally validated panel of subfamily specific oligonucleotide primers (Va1–w29/Vb1–w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. Eur. J. Immunol., *22:* 1261–1269, 1992.
15. Dutton, R. W. The regulation of the development of CD8 effector T cells. J. Immunol., *157:* 4287–4292, 1996.
16. Sad, S., and Mosmann, T. R. Interleukin (IL) 4, in the absence of antigen stimulation, induces an anergy like state in the differentiated $CD8^+$ TC1 cells: loss of IL-2 synthesis and autonomous proliferation but retention of cytotoxicity and synthesis of other cytokines. J. Exp. Med., *182:* 1505–1515, 1995.
17. Madrenas, J., Schwartz, R. H., and Germain, R. N. Interleukin 2 production, not the pattern of early T-cell antigen receptor-dependent tyrosine phosphorylation, controls anergy induction by both agonists and partial agonists. Proc. Natl. Acad. Sci. USA, *93:* 9736–9741, 1996.
18. Gervois, N., Guilloux, Y., Diez, E., and Jotereau, F. Suboptimal activation of melanoma infiltrating lymphocytes (TIL) due to low avidity of TCR/MHC-tumor peptide interactions. J. Exp. Med., *183:* 2403–2407, 1996.
19. Cormier, J. N., Salgaller, M. L., Prevette, T., Barracchini, K. C., Rivoltini, L., Restifo, N. P., Rosenberg, S. A., and Marincola, F. M. Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART1/Melan A. Cancer J. Sci. Am., *3:* 37–44, 1997.
20. Sette, A., Vitiello, A., Reherman, B., Fowler, P., Nayersina, R., Kast, W. M., Melief, C. J., Oseroff, C., Yuan, L., Ruppert, J., Sidney, J., del Guercio, M., Southwood, S., Kubo, R., Chestnut, R., Grey, H., and Chisari, F. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J. Immunol., *153:* 5586–5592, 1994.
21. Parkhurst, M. R., Salgaller, M. L., Southwood, S., Robbins, P. F., Sette, A., Rosenberg, S. A., and Kawakami, Y. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J. Immunol., *157:* 2539–2548, 1996.
22. Valmori, D., Fonteneau, J. F., Lizana, C. M., Gervois, N., Lienard, D., Rimoldi, D., Jongeneel, V., Jotereau, F,. Cerottini, J. C., and Romero, P. Enhanced generation of specific tumor-reactive CTL *in vitro* by selected Melan-A/MART1 immunodominant peptide analogues. J. Immunol., *160:* 1750–1758, 1998.
23. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.
24. Bronte, V., Tsung, K., Rao, J. B., Chen, P. W., Wang, M., Rosenberg, S. A., and Restifo, N. P. IL-2 enhances the function of recombinant poxvirus-based vaccines in the treatment of established pulmonary metastases. J. Immunol., *154:* 5282–5292, 1995.

A Superagonist Variant of Peptide MART1/Melan $A_{27–35}$ Elicits Anti-Melanoma $CD8^+$ T Cells with Enhanced Functional Characteristics: Implication for More Effective Immunotherapy

Licia Rivoltini, Paola Squarcina, Douglas J. Loftus, et al.

Cancer Res 1999;59:301-306.