Published in final edited form as:

J Immunother. 2002 ; 25(3): 252–263.

Frequency of MART-1/MelanA and gp100/PMel17-Specific T Cells in Tumor Metastases and Cultured Tumor-Infiltrating Lymphocytes

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Summary

Melanoma differentiation antigens, such as MART-1/MelanA and gp100/PMel17, frequently are observed as targets of tumor infiltrating lymphocytes (TIL) originated from HLA-A*0201-expressing patients with melanoma. Furthermore, particular clinical relevance was attributed to gp100/pMel17 based on the impression that the adoptive transfer of gp100-recognizing TIL was associated with clinical responses in a small group of patients. However, the actual frequency of specific T cells for these melanoma differentiation antigens has never been directly enumerated in ex vivo or in vitro expanded TIL cultures. Here, we enumerated melanoma differentiation antigen-specific T-cell precursor frequency in TIL using tetrameric HLA/epitope complexes, functionally characterizing their responsiveness to cognate epitope by cytokine release assay. T-cell precursor frequencies were enumerated in 11 fresh-tumor preparations and 17 TIL adoptively transferred into patients bearing HLA-A*0201. MART-1 or gp100-specific T cells could be detected respectively in 5 and 2 of the 11 fresh preparations and in 5 and 2 of the 17 adoptively transferred TIL. With one exception, melanoma differentiation antigen-specific T-cell precursor frequency in fresh material and TIL ranged between 5,000 to 21,000/10⁶ CD8⁺ T cells. T-cell precursor frequency was not significantly higher in TIL whose administration was associated with clinical response. These data provide direct enumeration of MART-1/MelanA and gp100/pMel17 reactivity ex vivo and in vitro in the context of HLA-A*0201.

Keywords

Melanoma antigens; Immunotherapy; Tumor-specific antigens

Melanoma differentiation antigens (MDA), such as MART-1/MelanA (1,2) and gp100/Mel17 (3), have been originally identified in the context of HLA-A*0201 and recognized by most long-term tumor infiltrating lymphocyte (TIL) cultures (4). Clinical observations have associated the adoptive transfer of TIL in combination with systemic interleukin (IL)-2 with treatment-induced regressions (5) particularly when gp100-reactive TIL was given (3). MART-1 has received particular attention, because immune competence toward this MDA

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appears to be enhanced in patients with HLA-A*0201-expressing melanoma compared with healthy individuals (6,7), and it is comparable with reactivity toward common pathogens like the Flu Matrix protein HLA-A*0201-associated epitope (FluM1:58–66) (6). Furthermore, in vitro expansion of peripheral blood mononuclear cells with HLA-A*0201-matched tumor-cell lines has been reported to frequently induce cytotoxic T lymphocyte (CTL) cultures that recognize MART-1. Although other hypotheses have been raised to explain the relative ease of identifying MART-1-specific circulating T cells (8), it was reasonable to postulate that this phenomenon relates to the immunodominant role that this MDA might play in the tumor microenvironment (9). Finally, significant attention has been devoted in the clinical application of antigen-specific vaccination toward these MDA compared with other tumor antigens (TA) (10). However, a recent analysis by our group revealed that the frequency of MDA-specific shorter-term TIL cultures might be lower than previously suspected (11). Because of the interest that these two MDA have generated in the past on either research or clinical grounds, we considered it important to directly enumerate the occurrence of T cells specific for these MDA in tumors ex vivo as well as after in vitro expansion.

The T-cell precursor frequency (*Tc-pf*) in TIL populations that recognize these MDA has never been directly documented. Until recently, the techniques used to assess the frequency of T cells that recognize these TA have been limited to indirect methods that depend on in vitro manipulation of tumor tissues before quantitative assessment is performed. These techniques included in vitro expansion of TIL by limiting dilution analysis (12). The enumeration of epitope-specific interferon (IFN)- γ -producing colonies by enzyme-linked immunospot assays has provided measurements without necessity for in vitro expansion. (13,14). However, enzyme-linked immunospot-based assessment is not a direct enumeration of antigen-specific T cells, because of the dependency on cytokine secretion that may underestimate the frequency of CTL precursors by missing T cells with a threshold for activation above the stimulus applied by the assay (15). Moreover, naïve T cells, less responsive to epitope-specific stimulation, might be missed by these functional assays (16).

Recently, the use of HLA/epitope tetrameric complexes (tHLA) (17) has offered a tool to directly measure the frequency of CTL precursors. Measurements with this assay demonstrated CTL precursor frequencies considerably higher than those suggested by enzyme-linked immunospot assays, limiting dilution assays, and intra-cellular assessment of cytokine production (15,18). Studies on tumor reactivity have been primarily limited to the analysis of MART-1, gp100, and tyrosinase-reactive T cells in the peripheral circulation and in lymph nodes of patients with metastatic melanoma. In patients with melanoma with vitiligo, MART-1-specific CTL have been identified at a frequency of up to 0.67% of CD8⁺ T cells (19). Furthermore, MART-1-specific T cells could be identified in melanoma-infiltrated lymph nodes with a frequency ranging from 0.22% to 1.8% of CD8⁺ T cells and correlated with MART-1/MelanA expression (20). Characterization of circulating lymphocytes demonstrated identifiable MDA-specific T cells in approximately half of the patients affected with metastatic melanoma (21). However, in our experience, the frequency of circulating MDA-specific T cells was surprisingly lower than we would have expected based on the ease with which reactivity toward these MDA can be raised with in vitro sensitization methods (22).

Here, we directly tested the *Tc-pf* of MART-1/gp100-reactive TIL in fresh-tumor preparations from HLA-A*0201-expressing patients with melanoma. In addition, the kinetics of the ex vivo expansion was characterized. Furthermore, we tested whether a quantitative correlation existed between clinical effectiveness and MDA-specific *Tc-pf* in adoptively transferred TIL populations.

MATERIALS AND METHODS

Cell Lines

The melanoma cell clones 624.38 MEL and 624.28 MEL were established and cultured as previously described (23). These clones were derived from the same bulk culture and are identical in expression of melanoma antigens and HLA-alleles with the exception of HLA-A*0201 whose expression is lost in 624.28 MEL as a result of aberrant splicing of HLA-A*0201 heavy chain mRNA (24). T2 cells (American Type Culture Collection [ATCC], Rockville, MD, U.S.A.) were used to test TIL specificity toward HLA-A*0201-restricted epitopes (25). The g209/209-2M-reacting T-cell clone 1520 and the MART-1-reacting T-cell clone 1235 have been previously described (26). All tumor-cell lines were maintained in complete medium consisting of RPMI 1640 (Biofluids, Rockville, MD, U.S.A.) with 10 mmol/L HEPES buffer, 100 U/mL penicillin-streptomycin (Biofluids), 10 µg/mL Ciprofloxacin (Bayer West Haven, CT, U.S.A.), 0.03% L-glutamine (Biofluids), 0.5 mg/mL amphotericin B (Biofluids), and 10% heat-inactivated fetal bovine serum (Biofluids). T-cell clones were cultured in complete medium consisting of Iscoves (Biofluids) with 10 mmol/L HEPES buffer, 100 U/mL penicillin-streptomycin (Biofluids), 10 µg/mL Ciprofloxacin (Bayer West Haven), 0.03% L-glutamine (Biofluids), 0.5 mg/mL amphotericin B (Biofluids), and 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA, U.S.A.) plus IL-2 (6,000 IU/mL) (Chiron, Emeryville, CA, U.S.A.).

Peptides

The following peptides were used for in vitro sensitization and in vitro testing: gp100:209–217 (IT-DQVPFSV, g209), gp100:209–217 (210M) (IM-DQVPFSV, g209–2M), gp100:154–162 (KTWGQY-WQV, g154), gp100:280–288 (YLEPGPVTV, g280), MART-1:27–35 (AAGIGILTV, MART-1), MART-1:26–35 (27L) (ELAGIGILTV, MART-1–2L), and Flu-M1:58–66 (GILGFVFTL, FluM1). These peptides were produced according to Good Manufacturing Practice (Princeton Biomolecules, Langhorne, PA, U.S.A.) by solid phase synthesis techniques and solubilized in sterile water or dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, U.S.A.) according to their biochemic characteristics. Peptide identity was confirmed by mass spectral analysis.

Patient Selection

The patient population included in this study was selected from patients with metastatic malignant melanoma referred to the Surgery Branch of the National Cancer Institute for immunotherapy between 1988 and 2000. The HLA class I phenotype of patients was determined on peripheral blood mononuclear cells using sequence-specific primer polymerase chain reaction (27). Polymerase chain reaction was also used for molecular subtyping (28).

Epitope-Specific T-Cell Staining Using HLA-A2 Tetramer

Tetrameric peptide-HLA-A*0201 complexes were produced as described previously (17,22). Recombinant HLA-A*0201 heavy chain containing a biotinylation site and recombinant c₂-microglobulin were synthesized and used for refolding of soluble HLA molecules in the presence of a HLA-A*0201-binding peptide. Soluble HLA molecules were prepared for the following epitopes: gp100:209–217, gp100:209–217 (210M), MART-1:26–35 (27L), and Flu-M1:58–66. All peptides were commercially synthesized and purified by gel filtration (Princeton Biomolecules, Columbus, OH, U.S.A.). The refolding reaction was dialyzed and concentrated for purification of correctly refolded soluble HLA on gel filtration. Monomeric HLA/peptide complexes were biotinylated with BirA (Avidity, Denver, CO, U.S.A.) at the heavy chain and separated from free biotin by gel filtration. Biotinylated HLA complexes were tetramerized by adding avidin-PE (Pierce, Rockford, IL, U.S.A.) at a 4:1 molar ratio. The final

concentration of tHLA was adjusted to 2 mg/mL. As examined by gel filtration, all tHLA were without detectable free avidin-PE. Tumor infiltrating lymphocytes at 1×10^6 cells/50 μ L ice-cold FACS buffer (phosphate buffer plus 5% inactivated fetal calf serum [Biofluids]) were incubated on ice with 1 μ g tHLA for 15 minutes, then continued for 30 minutes with 10 μ L anti-CD8 monoclonal antibody (100 μ g/mL; Becton Dickinson, San Jose, CA, U.S.A.). Cells were washed twice in 2 mL cold buffer before analysis by FACS (Becton Dickinson). Fifty thousand events were acquired for analysis of TIL.

T-Cell Precursor Frequency Analysis

T-cell precursor frequency was calculated as previously described (22). The frequency of tHLA⁺ T cells per million CD8⁺ T cells (*Tc-pf*) was calculated according to the formula: $f_{\text{tHLA}} = \text{URQ}/(\text{URQ} + \text{LRQ}) \times 10^6$ with the upper-right quadrant (URQ) containing the tHLA⁺, CD8⁺ T cells and the lower-right quadrant (LRQ) containing the tHLA⁻, CD8⁺ T cells. T-cell precursor frequency values superior to any observed staining with tHLA of non-HLA-A*0201 TIL were considered positive (typed as bold in tables). This cut-off point corresponded to *Tc-pf* > 5,000/10⁶ CD8⁺ T cells for all epitopes.

Preparation of Single-Cell Suspension From Tumor Tissue and Establishment of Tumor Infiltrating Lymphocytes Cultures

Tumor tissue was obtained after surgical excision from patients with histologically confirmed metastatic malignant melanoma. The diagnosis was reconfirmed on a representative sample of the tissue used for culture. Tumor tissue was incubated overnight with an enzyme cocktail to obtain single-cell suspension of the tumor tissue as previously published (29). After overnight treatment, cells were counted for tumor cell/lymphocyte ratio and viability. Cells from tumor digests were plated in 24 well plates at 2×10^6 cells per well in complete medium with the addition of 6,000 IU IL-2/mL. Interleukin-2 was supplemented every second. For TIL expansion in response to epitope-specific in vitro sensitization, 1 μ L of each peptide at a concentration of 1 mg/mL was added to each mL of media on day 0 after 24 hours of IL-2 administration.

Assessment of Ag Recognition by T Cells

Ag recognition by T TIL was assessed by IFN- γ release assay. Effector cells (5×10^5) were plated with 5×10^5 stimulator cells (tumor or T2 cells pulsed with 1 μ mol/L peptide) in 96 well round-bottom plates in 200 μ L complete medium. After a 24-hour incubation at 37°C, the plates were centrifuged, and the supernatant was harvested for enzyme-linked immunosorbent assay (ENDOGEN, Cambridge, MA, U.S.A.). Interferon- γ was reported as pg/mL in the coculture supernatant. Values double the background, and > 100 pg/mL were considered positive.

Selection of Tumor Infiltrating Lymphocytes Cultures Used in Adoptive Therapy

Twenty-three frozen samples of TIL cultures established at the Surgery Branch of the National Cancer Institute between 1988 and 1995 and used for adoptive transfer therapy were randomly selected. HLA-typing and sub-typing for HLA class I and II had been performed on all patients; 17 patients included in this study were HLA-A*0201. To obtain representative samples, we selected TIL vials derived as closely as possible from the TIL cultures used for adoptive transfer just before a clinical response.

Immunohistochemic Analysis of Tumor-Antigen Expression

For immunohistochemic studies, mouse anti-MART-1/MelanA (C10), monoclonal antibody (30), and the anti-gp-100/Pmel17 HMB45 monoclonal antibody (Biogenex Laboratories, San Ramon, CA, U.S.A.) were used to stain tumors from which T cultures were expanded. Purified

myeloma protein, mouse IgG1k (Organon Teknika Corporation, West Chester, PA, U.S.A.) was used as negative control. The secondary antibody consisted of affinity purified, biotinylated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.). The immunohistochemical procedure has been described in detail elsewhere (30).

RESULTS

g209, MART-1, and FluM1-Specific T-Cell Precursor Frequency in Fresh-Tumor Preparations and After Short-Term Culture

Excised melanoma metastases from 13 patients operated on at the Surgery Branch of the National Institutes of Health were prepared overnight to obtain single-cell suspensions (29). HLA-A*0201 expression by the patients, previous treatment received, levels of expression of MART-1 and gp100 in the lesions, and the frequency of CD8⁺ T cells among the whole tumor-cell population are shown in Table 1. MART-1 and gp100-specific *Tc-pf* were enumerated on day 0, using tHLA for MART-1–2L and g209. Tumor infiltrating lymphocytes 61, 67, 42, 44, and 46 demonstrated *Tc-pf* for MART-1–2L higher than 7,000 tHLA⁺ T cells/10⁶ CD8⁺ T cells. Furthermore, TIL 62 and 42 demonstrated identifiable *Tc-pf* for g209 (Table 2A). To support the findings suggested by tHLA enumeration of fresh-tumor preparations, *Tc-pf* were recalculated 10 days after in vitro expansion of tumor preparations plated in 24 well plates at a concentration of 1×10^6 /mL and cultured in the presence of IL-2 (6,000 IU/mL) at 37°C. After in vitro expansion, TIL 61, 67, 42, 44, and 46 demonstrated detectable levels of MART-1–2L-specific CD8⁺ T cells whereas, no expansion of MART-1-specific T cells could be identified in cultures of fresh preparations that had undetectable MART-1-specific T cells by tHLA staining (Table 2B). This finding suggests a strong correlation between ex vivo analysis of tHLA staining T cells and their proliferative activity as previously noted for circulating lymphocytes (22). T-cell precursor frequency had almost tripled in TIL 61 (Fig. 1), remained unchanged for TIL 67, 42, and 46, and decreased but remained detectable in TIL 44. T-cell precursor frequency for g209 became detectable in TIL 44 and 63, remained detectable in TIL 42, and became undetectable in TIL 62. Thus, the good correlation noted between ex vivo enumeration and that of expanded cultures for MART-1 was not as clear for gp100. Five of 11 (45%) TIL cultures were positive for MART-1 and 3 of 11 (27%) for gp100. This is in accordance with previous observations by Kawakami et al. (11) about the reactivity of TIL cultures in HLA-A*0201-expressing patients. In that study, 22 of 57 (39%) TIL cultures examined were MART-1 reactive, and 3 of 11 (23%) were gp100 reactive. Thus, reactivity toward MART-1/MelanA and gp100 is detectable in less than 50% of lesions from patients carrying the HLA-A*0201 phenotype whether this is tested directly in fresh-tumor preparations or after in vitro expansion with IL-2.

T-Cell Precursor Frequency of g209, MART-1, and FluM1-Specific T Cells in Tumor Infiltrating Lymphocytes Cultures Expanded In Vitro From Surgical Specimens

Fresh-tumor preparation from four HLA-A*0201-expressing patients (TIL 61, 62, 63, and 64) and two HLA-A*0201-negative patients (TIL 65 and 66) were also expanded in vitro in the presence of IL-2 (6,000 IU/mL) after stimulation with different epitopes. The tumor from one of the HLA-A*0201-negative patients (patient 65) was noted by immunohistochemistry not to express either gp100 or MART-1 (Table 1). Tumor infiltrating lymphocytes 65 and 66 served as controls for the specificity of HLA-A*0201-associated induction in vitro of tHLA-staining T cells. The other four cultures were induced in HLA-A*0201-expressing patients who had or had not undergone TA-specific vaccination. In addition, parallel cultures were separately stimulated at day 0 with g209, g209–2M, g154, g280, MART-1, MART-1–2L, or FluM1. After 20 days, the TIL were cocultured with T2 cells alone (background) or pulsed with 1 μ mol/L peptide or by coculture with HLA-A*0201-expressing tumor targets. Supernatants were tested for IFN- γ release by enzyme-linked immunosorbent assay. Cultures from HLA-A*0201-

negative patients did not develop HLA-A*0201-associated reactivities even when exposed to epitope-specific stimulation. Amongst HLA-A*0201-expressing patients, it was possible to raise MDA-specific reactivity in TIL 61, 62, and 63 (Table 3). Tumor infiltrating lymphocyte 61 developed MART-1-specific T cells in concordance to the tHLA-based phenotyping of fresh-tumor preparation. In addition, epitope stimulation raised antigen reactivity toward alternate epitopes of gp100 (gp100:154–162 and gp100:280–288) (31) but not g209. The reactivities identified might have been caused by previous vaccination against either TA. In TIL 61, MART-1 *Tc-pf* remained elevated for as long as 71 days in culture. Similarly, g209 specific *Tc-pf* remained sustained for 40 to 70 days (data not shown). In TIL 62 and 63, we could raise g209-specific T cells in accordance to tHLA phenotyping performed in fresh tumors or after in vitro expansion with IL-2 alone. Interestingly, stimulation with native or heteroclytic g209 epitope demonstrated differential response of TIL 62 that recognized only the modified g209–2M epitope at the peptide concentration used in the study. This can be most likely explained by the higher affinity of g209–2M for the HLA-A*0201 allele that allows higher epitope density on the stimulating cells. Tumor infiltrating lymphocyte 62 did not appear to sustain the *Tc-pf* in culture and only TIL 61 and 63 maintained the *Tc-pf* for at least 20 days in culture in the presence of IL-2 (Fig. 2). Tumor infiltrating lymphocyte 63 demonstrated reactivity toward g209, and also, in this case, the patient had previously received a gp100-based vaccine. Finally, patient 64, who had previously received vaccination with MART-1 and gp100, did not develop reactive T cells against either TA. Overall, no correlation was noted between previous treatment and identifiable reactivity toward individual TA. In no cases, FluM1 reactive T cells could be elicited by stimulation with the Flu Matrix epitope (Flu M1:58–66), a finding dramatically in contrast with the ease of in vitro induction of Flu-reactive T cells in circulating lymphocytes (6,22). In summary, these data suggested that the frequency of epitope-specific CD8⁺ T cells is not dramatically altered by in vitro culture conditions whether expansion is performed in the presence or absence of antigen-specific stimulation.

T-Cell Precursor Frequency of g209, MART-1, and FluM1-Specific T Cells in Tumor Infiltrating Lymphocyte Cultures Used for Adoptive Transfer

We enumerated the *Tc-pf* of MDA-specific T-cells in 23 TIL populations expanded in vitro with IL-2 (6,000 IU/mL) at the Surgery Branch of the National Cancer Institute for the adoptive therapy of patients with meta-static melanoma (from 1988 to 1996). This was done to test whether the frequency of MART-1 and/or gp100-reactive TIL had any predictive value toward clinical outcome. Samples were randomly selected from leftover TIL that had been frozen coincidentally to the adoptive transfer (Table 4). Eleven TIL cultures were from patients who experienced either complete (three patients) or partial response (eight patients) in association with adoptive transfer (Table 5). The other 12 TIL cultures were from patients who did not respond. The majority of TIL analyzed were from HLA-A*0201-expressing patients. However, two of the TIL from patients who responded (TIL 31 and 33) and four from the nonresponders (TIL 11, 17, 18, and 22) did not express this phenotype and were studied as controls. CD8⁺ T cells predominantly composed the majority of TIL. In all cases, *Tc-pf* was assessed with tHLA for g209, g209–2M, MART-1–2L, and FluM1.

T-cell precursor frequency against these epitopes were low in the majority of the cultures studied. One culture (TIL 8) whose adoptive transfer had induced a partial response demonstrated *Tc-pf* of 10,532 g209 tHLA⁺/10⁶ CD8⁺ T cells (Fig. 3 [middle panel]). Furthermore, TIL 55 and 56 were from patients who had achieved a partial remission after adoptive therapy and showed a MART-1–2L-specific *Tc-pf* of 5,224 and 8,153 tHLA⁺/10⁶ CD8⁺ T cells, respectively (Fig. 4 [middle panel]). Tumor infiltrating lymphocyte cultures from patients who experience a complete response did not demonstrate tHLA staining for either MART-1 or gp100. A culture from a patient who did not respond to adoptive transfer (TIL 19) had g209-specific *Tc-pf* of 927,772 tHLA⁺/10⁶ CD8⁺ T cells (Fig. 3 [lower panel]).

Furthermore, cultures from three patients (TIL 51, 52, and 54) who also did not respond clinically had MART-1–2L-specific *Tc-pf* ranging between 5,481 to 21,614/10⁶ CD8⁺ T cells, respectively (Fig. 4 [lower panel]). To verify results obtained by T-cell phenotyping with tHLA, we further tested antigen recognition measuring IFN- γ release by enzyme-linked immunosorbent assay upon antigen-specific stimulation. Cocultures of TIL and peptide-pulsed (1 μ g/mL) T2 cells were incubated for 24 hours at 37°C at 1:1 ratio (10⁶ cell/mL) in U bottom 96 well plates (200 μ L total per well). Results (Table 6) support the data obtained by tHLA phenotyping. The majority of the TIL cultures tested did not recognize the antigen presented on T2 cells. Amongst clinical responders, only TIL 8 that had demonstrated higher *Tc-pf* with g209 released detectable amounts of IFN. Furthermore, TIL 55 and 56 that demonstrated detectable MART-1–2L-specific *Tc-pf* released IFN- γ in response to MART-1-specific stimulation. Among the cultures from TIL that were not associated with clinical response, TIL 19 and 21 show IFN- γ release after coculture with g209. Tumor infiltrating lymphocytes 51, 52, and 54 that had detectable *Tc-pf* for MART-1 also showed IFN- γ production after coculture with MART-1. Overall, in this study, neither the frequency of detection of MDA-specific TIL nor their enumeration in individual cultures correlated with clinical effectiveness.

DISCUSSION

This study enumerated the frequency of MDA-specific T cells in TIL cultures and fresh tumor preparations derived from patients with melanoma. In addition, the frequency of MDA-reactive T cells was analyzed in TIL used for adoptive transfer in clinical settings. No correlation was noted between MDA reactivity and clinical outcome, suggesting that these antigens have no specific bearing in clinical settings. Therefore, these findings may have practical implications for how TIL are expanded for immunotherapy and what the end points of such expansions should be.

Besides the obvious clinical implications suggested by this study, these findings raise some more fundamental questions regarding the significance of MDA in tumor immunology. Contrary to most vaccinations, which are aimed at priming the immune system against future pathogens, immunizations against cancer are given in a therapeutic setting (32,33). Epitope-based vaccinations demonstrate that acquired T-cell responses can be elicited against cancer (34). Interestingly, these pivotal studies underlined a puzzling discrepancy between the effectiveness of vaccines in inducing systemic T-cell responses and their limited clinical success. Thus, it remains important to identify the roots of natural immune competence toward cancer and its innate and/or acquired potential in response to different endogenous and/or exogenous factors.

The peptides that function as antigenic determinants for a given protein are generally fewer than the potential binders to relevant HLA alleles and are dominant (35,36). In practical terms, the identification within the context of a specific HLA restriction element of immune dominant epitopes may suggest a more susceptible target for antigen-specific vaccination (9). We have been particularly interested in two molecules (MART-1 and gp100) whose frequent expression in melanoma tissues (37) seemed to be mirrored by a high frequency of TIL capable of recognizing them in the context of HLA-A*0201. In particular, MART-1 appeared to demonstrate several immune-relevant characteristics, because it is frequently expressed in melanoma metastases (37), and MART-1-specific T cells have been detected frequently in TIL (31). Furthermore, MART-1 immunodominance at the tumor site appears to correlate with an enhanced immune competence toward a unique epitope (MART-1:27–35) in HLA-A*0201-expressing individuals. Because repeated exposure of peripheral blood mononuclear cells from HLA-A*0201 individuals to minimal epitopic sequence from MART-1/MelanA (6,38), HLA-matched melanoma cell lines (39), and antigen-presenting cells infected with pox viruses, encoding for this protein (40) seems to consistently induce reactivity toward this

immunodominant epitope, and MART-1:27–35 has sparked interest in its utilization as a vaccine against cancer.

Opposing theories have been evoked to explain the predominant role that this TA seems to play. We noted that MART-1 reactivity is more readily elicited in patients with melanoma compared with healthy individuals (6). This finding suggested that tumors could prime the host immune competence. Others confirmed this finding and suggested that differences in immune competence between healthy and melanoma-bearing individuals correlate with a respective predominance of naïve and memory CTL in the two populations (7,41). Loftus et al. (8) suggested that MART-1:27–35 immune dominance detected in circulating lymphocytes is not driven by tumor immunogenicity, but is explainable by biochemic commonalities coincidentally shared by MART-1 and analog endogenous or exogenous epitopes to which individuals might become repeatedly exposed to during their lifetime. Others have noted that MART-1:27–35 has low affinity for HLA-A*0201 and, therefore, negative T-cell receptor selection toward this self epitope might be less strictly enforced during embryogenesis thus, leaving a robust T-cell receptor repertoire against this epitope (38).

It is now clear that immune competence toward cancer can be influenced by peripheral stimuli artificially provided in the form of naturally occurring or modified minimal epitopic determinants (34,42). However, evidence that human tumors can naturally shape the host immune competence has remained indirect (7,9,41,43). The surprisingly low frequency of MART-1 and/or gp100-reactive T cells in TIL populations detected in this study raises the question of why the systemic induction of MART-1 and (to a lesser degree) gp100-specific T cells can be elicited from circulating T cells. Possibly, immune competence toward these TA has a nondetermined environmental origin unrelated to the tumor-bearing status.

The conclusion of this study is that at the tumor site, the frequency of MART-1 and/or gp100-reactive TIL in individual patients is not as high as originally predicted. These results are not totally surprising, because Kawakami et al. (31) have recently observed that the frequency of MART-1 and gp100-reactive T cells in bulk TIL cultures from 123 patients was comparable to the estimates found in this study (11). Therefore, this study confirms Kawakami's results and provides a more accurate measurement of the *Tc-pf* expected in random TIL populations from HLA-A*0201 patients. Furthermore, this study provides additional information about *Tc-pf* in TIL populations that contain MART-1 and/or gp100-reactive T cells. With few exceptions, *Tc-pf* are relatively low suggesting that the majority of CD8⁺ TIL may recognize other specificities, indirectly suggesting that the immune competence of the host toward melanoma might be broader than generally suspected. This analysis obviously questions what other epitopes, if any, are recognized by TIL that may or may not be related to other TA.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SS).

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FIG. 1.

Kinetics of the development of short-term tumor infiltrating lymphocyte (TIL) cultures from TIL 61 expanded in vitro from fresh-tumor preparations. Single-cell suspension from tumor digest was cultured under standard conditions with interleukin-2 (6,000 IU/mL) for 20 days. On days 0, 3, 10, and 20, staining with g209, MART-1, and Flu HLA/epitope tetrameric complexes-PE (y-axis) and CD8-FITC (x-axis) was performed. The first row shows the time course of MART-1-specific T-cell precursor frequency from day 0 to 20. The second and third rows depict the kinetics for g209 and FluM1, respectively.

FIG. 2.

Kinetics of the development of short-term tumor infiltrating lymphocyte (TIL) cultures expanded in vitro from fresh-tumor preparations with 6,000 IU/mL interleukin-2. Data are presented for the four HLA-A*0201-expressing patients TIL 61, 62, 63, and 64 over a period of 20 days. T-cell precursor frequency (*Tc-pf*) was calculated according to the formula: $f_{\text{tHLA}} = \text{URQ} / (\text{URQ} + \text{LRQ}) \times 1,000,000$ with the upper-right quadrant containing the HLA/epitope tetrameric complexes (tHLA)⁺, CD8⁺ T cells and the lower right quadrant containing the tHLA⁻, CD8⁺ T cells (see “Statistical” section of this article). The time course of *Tc-pf* specific for g209, g209-2M, MART-1-2L, and FluM1 is shown in separate graphs for each patient. The x-axis shows days in culture, and the y-axis shows the *Tc-pf*.

FIG. 3.

Phenotypic characterization of HLA/epitope tetrameric complexes staining T cells in tumor infiltrating lymphocyte (TIL) populations adoptively transferred for treatment of patients with metastatic melanoma. The long-term TIL culture 1520, previously described as g209 reactive, is used as a positive control (upper panel) (26). Staining with g209, g209-2M, and MART-1-2L is shown for TIL 8 (middle panel) and 19 (lower panel).

FIG. 4. Phenotypic characterization of HLA/epitope tetrameric complexes staining T cells in tumor infiltrating lymphocyte (TIL) populations adoptively transferred for the treatment of patients with metastatic melanoma. The long-term TIL cultures 1235, previously described as MART-1 reactive, is used as a positive control (38). Staining with g209, g209-2M, and MART-1-2L is shown for TIL 55 (middle panel) and 51 (lower panel).

TABLE 1
Characteristics of fresh-tumor preparations in which TA-specific *Tc-pf* was assessed

TIL	Previous treatment			TA expression		TIL phenotype (% CD8)
	HLA-A*0201	MART-1	gp100	MART-1	gp100	
61	+	yes	yes	25-50	50-75	9.7
62	+	no	no	25-50	50-75	10.8
63	+	no	yes	NA	NA	10.7
64	+	yes	yes	> 75	25-50	38.8
65	-	no	no	negative	negative	41.6
66	-	no	no	> 75	> 75	20.9
67	+	yes	yes	(> 75)	(> 75)	33.5
41	+	no	yes	negative	50-75	0
42	+	no	no	50-75	50-75	3.9
43	+	no	yes	> 75	negative	2.6
44	+	no	no	> 75	> 75	26.2
45	+	yes	yes	50-75	negative	23.4
46	+	no	no	> 75	> 75	32.2

Immunohistochemistry (IHC) data is presented as percent of cells expressing the given TA. Negative are called sample with < 25% cells expressing the given TA. In parenthesis is TA expression in lesions different from the one analyzed and obtained closest to it: patient 67 IHC from lesions obtained 2 months before. CD8⁺ cells frequency is in relation to all tumor-cell populations.

HLA, human leukocyte antigen; NA, not available; TA, tumor antigens; *Tc-pf*, T-cell precursor frequency; TIL, tumor infiltrating lymphocytes.

TABLE 2

TABLE 2A. <i>Tc-pf</i> of tHLA ⁺ /10 ⁶ CD8 ⁺ T cells in fresh-tumor preparations			
TIL	MART-1-2L	g209	g209-2M
61	15032 *	3990	2400
62	3817	7143 *	3422
63	0	3797	1597
64	1277	1500	1349
65	443	1923	1534
66	52	268	1173
67	12470 *	1381	1319
41	0	0	0
42	7737 *	5599 *	5362 *
43	0	0	0
44	10603 *	3051	0
45	1388	1730	2563
46	9996 *	3470	5620 *

TABLE 2B. <i>Tc-pf</i> of tHLA ⁺ /10 ⁶ CD8 ⁺ T cells expanded from TIL for 10 days in the presence of interleukin-2 (6,000 IU/mL)			
TIL	MART-1-2L	g209	g209-2M
61	41332 *	2803	3222
62	3992	2080	2750
63	0	5496 *	2515
64	125	0	822
65	0	255	0
66	113	1320	2500
67	13696 *	1973	630
41	0	0	0
42	6459 *	6736 *	9617 *
43	0	0	0
44	5376 *	7880 *	0
45	6	539	5
46	9829 *	2582	7840 *

Values above 5,000 considered to represent *Tc-pf* truly above background (see “Materials and Methods”).

* Values above 5,000 considered to represent *Tc-pf* truly above background (see “Materials and Methods”).

HLA, human leukocyte antigen; *Tc-pf*, T-cell precursor frequency; +HLA, HLA/epitope tetrameric complexes; TIL, tumor infiltrating lymphocytes.

Analysis of TIL cocultured with T2 cells loaded with relevant and irrelevant peptide as well as HLA-A*0201 expressing (624.38) and not expressing (624.28) tumor-cell targets (data are presented as levels of IFN- γ , pg/mL)

TIL culture	T2 cells ± peptide							624.38	624.28
	None	+g209	+g209-2M	+MART-1	+MART-12L	+g100-154	+g100-280		
TIL 61									
No peptide	—	—	—	1225	1290	506	—	—	—
g209	—	—	—	1225	1367	—	—	—	829
g209-2M	—	—	—	374	1174	—	—	—	1321
MART-1	—	—	—	315	984	—	—	—	1159
g100-154	—	—	—	623	1292	293	—	—	1291
g100-280	—	—	—	1302	1339	—	486	—	939
FLU-M1	—	—	—	—	—	—	—	—	1277
TIL 62									1211
g209-2M	—	—	270	—	—	—	—	—	—
g100-280	—	—	—	—	—	—	135	—	—
TIL 63									
g209	—	172	237	—	—	—	—	—	234
g209-2M	—	—	167	—	—	—	—	—	140

TIL 61–66 were cultured in vitro with IL-2 (6,000 IU/mL) in all the experiments presented in this Table. In addition, cultures were separately stimulated at day 0 with g209, g209-2M, g154, g280, MART-1, MART-1-2L, or FluM1 (as indicated in the first column). After 20 days, the TIL were cocultured with T2 cells alone (background) or pulsed with 1 μmol/L peptide. In addition, TIL were cocultured with the HLA-A*0201-expressing melanoma cell line 624.38 as well as the HLA-A*0201-negative sister clone 624.28. Supernatants were tested for IFN-γ protein production by ELISA. Only TIL cultures that showed IFN-γ data above > 100 pg/mL and at least twice background are shown. Data represent the average of at least two consecutive experiments. All TIL cultures were induced in vitro with all the peptides as for TIL 61. For simplicity, however, data are presented only when at least one positive value was observed for each culture condition. Similarly, results from patients 64, 65, and 66 are not presented for simplicity, because they were completely negative in two separate experiments.

ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; TIL, tumor infiltrating lymphocytes.

TABLE 4

Characterization of TIL cultures used for adoptive transfer

TIL	Date of treatment	Date TIL frozen	Age of culture (days)	Expansion ($\times 1,000$)
1	9/10/92	9/9/92	66	1261
2	11/15/91	11/15/91	71	460
5	11/11/93	11/11/93	NA	NA
6	6/23/92	6/23/92	46	574
7	4/7/94	4/7/94	NA	NA
8	11/11/88	11/10/88	NA	NA
55	11/7/91	11/7/91	48	1173
56	2/8/88	2/5/88*	NA	NA
31	1/3/95	1/3/95	42	17
32	9/4/91	9/4/91	NA	NA
33	5/16/94	5/16/94	NA	NA
3	4/23/92	4/23/92	74	78
11	4/14/92	4/20/92	59	60
12	11/19/92	11/16/92	50	349
13	2/6/91	2/6/91	NA	NA
54	1/28/94	3/7/94*	NA	NA
17	12/27/88	11/20/89*	NA	NA
18	4/20/92	4/20/92	38	21
19	10/29/96	8/28/96*	40	11
21	1/5/93	6/18/93*	55	6
22	12/3/92	12/3/92	42	1
51	12/20/94	12/19/94	NA	NA
52	8/25/95	8/25/95	45	490

Cases in which TIL were obtained from bags of TIL and frozen. After thawing the TIL cultures were re-expanded in IL-2 (6,000 IU/mL) for a period ranging 10–20 days before being refrozen at the date shown in the Table. In these cases, there is lower confidence that the material used for this study is representative of the TIL population used for adoptive transfer. Age of culture refers to the time interval between TIL harvest and adoptive transfer. Fold expansion refers to the number of TIL available after expansion compared with the starting number after TIL harvested $\times 1,000$ fold.

IL, interleukin; Na, not available; TIL, tumor infiltrating lymphocytes.

TABLE 5
Characteristics of TIL cultures used for adoptive transfer in combination with systemic IL-2

TIL	HLA-A* A0201	Tc-pf of tHLA+/10 ⁶ CD8+ T cells						TIL phenotype (% CD8)	Outcome
		g209	g209-2M	MART-1-2L	FluM1	Number of TIL infused (1 × 10 ¹¹)			
1	+	2057	1113	1509	1	2.2		96.9	PR
2	+	1468	3389	204	202	0.8		98.0	PR
5	+	2437	2458	2905	303	5.0		98.4	PR
6	+	502	2389	0	402	1.6		97.8	PR
7	+	501	3902	100	0	6.3		96.7	PR
8	+	10532	15175	0	0	2.4		84.1	PR
55	+	2890	2890	5224	0	1.5		93.1	PR
56	+	602	1312	8153	112	7.5		99.3	PR
31	-	403	1325	3814	403	6.8		98.0	CR
32	+	331	304	2515	267	3.8		90.4	CR
33	-	1845	4721	4966	312	6.2		95.0	CR
3	+	0	0	0	0	0.8		1.1	NR
11	-	2332	3467	803	101	1.5		99.4	NR
12	+	1014	3890	0	0	6.9		86.6	NR
13	+	4921	8131	99	0	0.5		98.2	NR
54†	+	745	213	5481	0	1.1		98.1	NR
17†	-	402	1089	0	190	2.6		39.3	NR
18	-	711	2834	0	203	4.4		98.4	NR
19	+	927772	925673	1023	534	2.5		99.2	NR
21	+	637	1987	670	185	2.9		99.8	NR
22	-	527	2861	211	425	5.6		98.6	NR
51	+	308	308	21614	442	6.6		96.0	NR
52	+	213	107	10198	0	2.1		93.3	NR
1235 TIL		89	1220	376820	345				
1520 TIL		853400	873450	102	780				

Numbers of specific T-cell precursor frequencies in melanoma-reactive TIL used in adoptive therapy by HLA-A*0201/peptide tetramers.

* Vials of TIL cultures from samples which had been taken from the infusion bags used for treatment of the patients, expanded in culture and refrozen with a week of use for adoptive with the exception of.†

HLA, human leukocyte antigen; IL, interleukin; Tc-pf, T-cell precursor frequency; TIL, tumor infiltrating lymphocytes.

TABLE 6
Cocultures of TIL and peptide-pulsed T2 cells

	No peptide	Stimulator T2 cells loaded with						FLU
		g209	g209-2M	MART-1	MART-1-2L	g154	g280	
IFN- γ (pg/mL)								
Long-term TIL cultures								
1235 TIL	—	—	—	1367	—	—	—	—
1520 TIL	—	1890	1759	—	—	—	—	—
Responders								
TIL 1	—	—	—	—	—	—	—	—
TIL 2	—	—	—	—	—	—	—	—
TIL 5	—	—	—	—	—	—	—	—
TIL 6	—	—	—	—	—	—	—	—
TIL 7	—	—	—	—	—	—	—	—
TIL 8	—	498	521	178	—	—	—	—
TIL 55	—	—	—	625	1233	—	1426	—
TIL 56	—	—	—	312	757	—	736	—
TIL 31	—	—	—	—	—	—	712	—
TIL 32	—	—	—	—	—	—	—	—
TIL 33	—	—	—	—	—	—	—	—
Nonresponders								
TIL 3	—	—	—	—	—	—	—	—
TIL 11	—	—	—	—	—	—	—	—
TIL 12	—	—	—	—	—	—	—	—
TIL 13	—	—	—	—	—	—	—	—
TIL 54	—	—	—	240	705	—	—	—
TIL 17	—	—	—	—	—	—	—	—
TIL 18	—	—	—	—	—	—	—	—
TIL 19	—	1270	1338	—	—	—	—	—
TIL 21	—	895	1189	—	—	—	—	—
TIL 22	—	—	—	—	—	—	—	—
TIL 51	—	—	—	1459	1284	—	—	—
TIL 52	—	—	—	632	982	—	—	—

IFN- γ (pg/mL) release by TIL cocultured with peptide-pulsed T2 cells. Only values deemed to represent specific cytokine release upon stimulation are reported. Cytokine release was considered specific when above 100 mg/mL and at least two-fold the background release in cocultures with T2 cells without peptide. Therefore, the negative signs refer to cultures that did not meet those requirements in at least two separate experiments. Data presented as IFN- γ levels, pg/mL.

IFN, interferon; TIL, tumor infiltrating lymphocyte.