Brief Definitive Report

Identification of the Immunodominant Peptides of the MART-1 Human Melanoma Antigen Recognized by the Majority of HLA-A2–restricted Tumor Infiltrating Lymphocytes

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Summary

Four melanoma proteins, MART-1, gp100, tyrosinase, and tyrosinase-related protein-1 (gp75) were evaluated for recognition by HLA-A2–restricted melanoma-specific cytotoxic T lymphocytes (CTLs) derived from the tumor-infiltrating lymphocytes (TIL) of 10 different patients. 9 of 10 TIL recognized MART-1, 4 recognized gp100 (including 3 that also recognized MART-1), but none of the TIL recognized tyrosinase or gp75. Based on the known HLA-A2.1 peptide binding motifs, 23 peptides from MART-1 were synthesized in an attempt to identify the epitopes recognized by TIL. Three peptides were recognized by TIL when pulsed on T2 target cells. One of the 9-mer peptides, AAGIGILTV, was most effective in sensitizing the T2 cells for TIL lysis. This peptide was recognized by 9 of 10 HLA-A2–restricted melanoma-specific CTLs. Therefore, this peptide appears to be a very common immunogenic epitope for HLA-A2–restricted melanoma-specific TIL and may be useful for the development of immunotherapeutic strategies.

The adoptive transfer of cultured tumor-infiltrating lymphocytes (TIL) with IL-2 can mediate tumor regression in mice with lung or liver metastases (1) and in 30–40% of patients with metastatic melanoma (2, 3). Many TIL recognize, in vitro, autologous melanoma cells. Allogeneic melanoma cells and cultured melanocytes that share appropriate antigen-presenting HLA molecules are also recognized (4–7) suggesting that some CTLs recognize common antigens expressed on melanoma and melanocytes (8, 9). Identification of these common antigens recognized by TIL can lead to increased understanding of the immune responses to tumors as well as to improvement of immunotherapeutic strategies for patients with melanoma.

The existence of multiple human melanoma antigens recognized by T cells has been suggested (10, 11) and five antigens have previously been identified and isolated. MZ2-E encoded by the MAGE-1 gene and MZ2-D encoded by the MAGE-3 gene are expressed in melanoma, other types of cancers including head and neck cancer, lung cancer, colorectal cancer, breast cancer, sarcoma, and normal testis, and were recognized by HLA-A1–restricted CTL (12–14). MART-1, gp100, and tyrosinase are specifically expressed in melanoma and melanocytes and are recognized by HLA-A2–restricted CTL (15–18). The epitopes of MART-1 recognized by T cells have not previously been identified.

The importance of these antigens in the recognition of melanoma by T cells, in vivo, in patients with melanoma has not yet been defined. The immunogenicity of these antigens in vivo may be related to the level of antigen expression on the tumor surface and the repertoire of T cell receptors expressed on patient's T cells as well as the degree of tolerance towards these epitopes.

In this study, we have identified highly immunogenic common epitopes of MART-1 that were recognized by the majority of HLA-A2–restricted melanoma-specific TIL isolated from HLA-A2 melanoma patients. These common antigenic peptides may be useful for the development of immunotherapeutic strategies against melanoma and in studies of the mechanism of T cell recognition and immunological tolerance to tumor antigens.

Materials and Methods

Generation of Melanoma-specific CTL Lines and a Clone from TIL. Melanoma-specific CTL lines were generated by culturing a single cell suspension made from metastatic melanoma with 6000 U/ml of IL-2 (Cetus-Oncology Division, Chiron Corp., Emeryville, CA) as previously reported (19). A T cell clone, A42, was established by limiting dilution methods from patient 501.

Assessment of Antigen Recognition by CTLs. ^{51}Cr release cytotoxicity assays and cytokine release assays using ELISA to measure

IFN-γ, GM-CSF, and TNF-α were performed to analyze the reactivity of TIL as previously described (17, 19). Melanoma cell lines were established in our laboratory. For analysis of the recognition of known antigens by TIL, the COS7 cell line transfected with cDNAs encoding either, MART-1, gp100, tyrosinase, or tyrosinase-related protein (gp75) along with HLA-A2.1 cDNA were incubated with TIL for 20 h and the amount of IFN-γ secreted into the supernatant was measured by ELISA (17). The cDNA encoding MART-1 or gp100 in plasmid pcDNA3 (Invitrogen, San Diego, CA) was previously cloned from a 501mel melanoma cDNA library by screening with TIL1235 or TIL1200, respectively (15, 17). The cDNA encoding tyrosinase in pcDNA3 was cloned from an 888mel melanoma cDNA library by screening with TIL888 (20). The cDNA encoding tyrosinase related protein (gp75) in pCEV27 plasmid was isolated from 501mel melanoma cDNA library using a probe generated by PCR based on the reported sequence of gp75 (21).

Peptide Synthesis and Identification of Antigenic Peptides. Peptides were synthesized by a solid phase method using a multiple peptide synthesizer (model AMS 422; Gilson Co. Inc., Worthington, OH). The peptides were purified by HPLC on a C-4 column (VYDAC, Hesperia, CA) with 0.05% TFA/water-acetonitrile. To identify the antigenic peptides, TIL lysis of T2 cell lines preincubated for 2 h with each peptide was measured using a ^{51}Cr release cytotoxicity assay.

Results

HLA-A2–restricted Melanoma-specific TIL. HLA-A2–restricted melanoma-specific CTL lines and a clone, A42, were established from lymphocytes infiltrating into tumors of 10 melanoma patients. These TIL represented all available cell lines with HLA-A2–restricted antimelanoma reactivity grown in our laboratory and thus should be representative of other TIL with similar in vitro reactivity. These TIL recognized autologous and most allogeneic fresh or cultured melanoma cells expressing HLA-A2, but did not recognize HLA-A2$^-$ melanomas or HLA-A2$^+$ nonmelanoma cell lines (5). They also recognized HLA-A2$^+$ normal cultured melanocytes derived from neonatal skin (9, 15, 17). Thus, these TIL recognized nonmutated self-peptides derived from proteins expressed in melanoma and melanocytes in association with HLA-A2.

Recognition of the Previously Identified Melanoma Proteins by TIL. To evaluate the frequency of recognition of four previously isolated melanoma proteins including MART-1, gp100, tyrosinase, and tyrosinase-related protein (gp75), we tested the reactivity of TIL to COS7 cell lines transfected with cDNAs encoding these four proteins with or without the cDNA encoding HLA-A2.1. One of several experiments with nine TIL is shown in Table 1. Eight of the nine HLA-A2–restricted melanoma-specific TIL secreted IFN-γ when incubated with COS7 cotransfected with MART-1 and HLA-A2.1. Only TIL1200, which is a relatively oligoclonal CTL line (22), did not respond to this COS transfectant. Four TIL (620, 660, 1143, 1200) recognized gp100 when transfected along with HLA-A2.1. TIL1200 secreted large amounts of IFN-γ compared with TIL620, 660, and 1143, suggesting

Table 1. *Recognition of Melanoma Antigens by HLA-A2–restricted Melanoma-specific TIL*

Stimulator cell line	Transfected cDNA	HLA-A2	IFN-γ Secretion								
			TIL 501	TIL 620	TIL 660	TIL 1074	TIL 1088	TIL 1128	TIL 1143	TIL 1200	TIL 1235
			pg/ml								
501mel	none	+	93	720	530	670	491	272	354	736	750
397mel	none	–	0	0	0	0	0	0	0	0	0
COS7	none	–	0	0	0	0	0	0	14	0	0
COS7	HLA-A2.1	+	0	0	0	0	0	0	0	0	0
COS7	MART-1	–	0	0	0	0	0	0	12	0	0
COS7	gp100	–	0	0	0	0	0	0	14	0	0
COS7	tyrosinase	–	0	0	0	0	0	0	0	0	0
COS7	gp75	–	0	0	0	0	0	0	0	0	0
COS7	HLA-A2.1 + MART-1	+	270	196	131	625	328	52	184	0	743
COS7	HLA-A2.1 + gp100	+	0	89	17	0	0	0	41	391	8
COS7	HLA-A2.1 + tyrosinase	+	0	0	0	0	0	0	0	3	0
COS7	HLA-A2.1 + gp75	+	0	0	0	11	0	0	7	0	0

IFN-γ was measured in the supernatants after HLA-A2–restricted melanoma-specific TIL were coincubated with COS7 cells cotransfected with cDNAs encoding proteins expressed in melanoma with or without HLA-A2.1 cDNA. All TIL except TIL1200 secreted IFN-γ when cultured with COS7 cotransfected with cDNAs encoding MART-1 and HLA-A2.1. TIL620, 660, 1143, and 1200 secreted IFN-γ when cultured with COS7 cotransfected with the cDNAs encoding gp100 and HLA-A2.1.

that only a small subset of T cells in these latter three TIL lines recognized gp100. None of these TIL recognized tyrosinase or gp75 using this assay. Thus, MART-1 is a common melanoma antigen recognized by most HLA-A2–restricted TIL derived from melanoma patients.

Identification of MART-1 Epitopes for TIL. To identify the MART-1 epitopes for these TIL, 23 peptides were selected based on the known peptide binding motifs to HLA-A2.1 (23–25), synthesized (>90% purity), and screened by testing lysis of the HLA-A2.1$^+$ T2 cell line by TIL after incubation of the T2 line with each peptide (Table 2). The T2 cell line was lysed well by all four HLA-A2–restricted melanoma-specific TIL tested when preincubated with either peptides M9-2, M10-3, or M10-4. Both 10-amino acid peptides, M10-3 and M10-4, contain the M9-2 sequence, with M10-3 having an additional glutamic acid at its NH_2 terminus and M10-4 having an extra isoleucine at its COOH-terminus. These peptides are located in a hydrophobic putative transmembrane domain in MART-1 (17). The same lysis was observed when other HLA-A2$^+$ cells incubated with these peptides were used as targets including the K4B and 501EBVB Epstein-Barr virus–transformed B cells or HMY-C1R B cells transfected with the HLA-A2.1 gene (data not shown).

The peptides, M9-1, M9-2, M9-3, M10-2, M10-3, M10-4, and M10-5 were further purified and titrated in order to evaluate their relative ability to sensitize T2 cells to lysis by

Table 2. *Lysis of T2 Cells Preincubated with Synthetic MART-1 Peptides*

Target		Peptide	Percent specific lysis			
			A42	TIL 1235	TIL 660	TIL 1074
501mel		none	47	30	31	41
397mel		none	1	0	1	2
T2		none	−2	−3	−1	1
T2	M9-1	TTAEEAAGI	−10	−5	−5	−4
T2	M9-2	AAGIGILTV	64	80	40	56
T2	M9-3	GIGILTVIL	18	20	0	10
T2	M9-4	GILTVILGV	1	−1	−3	2
T2	M9-5	ILTVILGVL	−2	−1	−5	−1
T2	M9-6	LTVILGVLL	1	0	1	0
T2	M9-7	TVILGVLLL	−2	−3	−2	1
T2	M9-8	VILGVLLLI	1	5	−2	−2
T2	M9-9	ALMDKSLHV	−1	−4	−8	0
T2	M9-10	SLHVGTQCA	−1	1	−8	4
T2	M9-11	PVVPNAPPA	−2	0	4	−1
T2	M9-12	NAPPAYEKL	1	−2	0	6
T2	M10-1	YTTAEEAAGI	−4	−2	−3	3
T2	M10-2	TAEEAAGIGI	7	11	12	15
T2	M10-3	EAAGIGILTV	55	66	31	51
T2	M10-4	AAGIGILTVI	34	68	29	21
T2	M10-5	GILTVILGVL	−1	2	7	10
T2	M10-6	ILTVILGVLL	1	6	6	7
T2	M10-7	LTVILGVLLL	−2	−1	−1	2
T2	M10-8	TVILGVLLLI	−6	−1	−1	11
T2	M10-9	RALMDKSLHV	3	5	8	11
T2	M10-10	SLHVGTQCAL	−2	−8	2	9
T2	M10-11	SLQEKNCEPV	3	2	2	9

23 peptides (twelve 9-mers and eleven 10-mers) (>90% purity) were synthesized and the lysability by TIL clone A42, TIL lines TIL1235, TIL660, and TIL1074 derived from different patients was tested against HLA-A2$^+$ T2 cells preincubated with each peptide (10 μg/ml) in a 4-h ^{51}Cr release cytotoxicity assay at E/T ratio of 20:1 for A42 and 40:1 for other TIL lines. T2 cells were lysed well when incubated with M9-2, M10-3, and M10-4. M10-3 and M10-4 contain the entire M9-2 sequence (underlined).

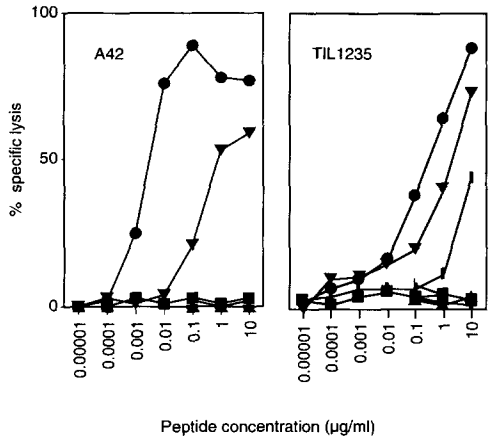

Figure 1. Titration of MART-1 peptides for recognition by TIL. T2 cells were incubated with varied concentrations of the purified MART-1 peptides, M9-1, M9-2, M9-3, M10-2, M10-3, M10-4, and M10-5, and lysis by TIL clone A42 or TIL line TIL1235 was measured by 4-h ^{51}Cr release cytotoxicity assay at an E/T ratio of 20:1 for A42 and 40:1 for TIL1235. Peptide M9-2 sensitized T2 cells at a concentration of 1 ng/ml. The purified peptide M10-4 was recognized by TIL1235, but not by A42. (M9-1 |---|, M9-2 ●---●, M9-3 ■---■, M10-2 ▲---▲, M10-3 ▼---▼, M10-4 ▌---▐, M10-5 +---+).

MART-1 reactive TIL1235 or T cell clone A42 (Fig. 1). The purified peptides M9-2, M10-3, and M10-4 were required in minimum concentrations of 1 ng/ml, 100 ng/ml, and 1,000 ng/ml, respectively. The purified M10-4 was not recognized by TIL clone A42 even at 10 μg/ml as shown in Fig. 1. M9-1, M9-3, M10-2, and M10-5 peptides were not recognized by either A42 or TIL1235.

Recognition of MART-1 Peptides by HLA-A2–restricted TIL Established from Different Patients. To evaluate whether a variety of HLA-A2–restricted MART-1–specific TIL recognized the same or different epitopes in the MART-1 antigen, lysis of T2 cells preincubated with each peptide was tested with TIL derived from 10 melanoma patients. A representative experiment with 10 TIL is shown in Table 3. M9-2 and M10-3 were recognized by 9 of 10 TIL (only TIL1200 were negative) as well as the A42 clone with the same pattern of lysis as COS7 cells cotransfected with cDNAs encoding MART-1 and HLA-A2.1. Only TIL620 and TIL1088 demonstrated low level of nonspecific lysis of T2 cells without peptides or after the addition of irrelevant peptides, but showed significant increase of lysis of T2 cells preincubated with M9-2, M10-3, and M10-4 peptides. The recognition of M10-4 differed

Table 3. *Recognition of MART-1 Peptides by HLA-A2–restricted Melanoma-specific TIL*

		Percent specific lysis										
Target	Peptide	TIL 501	TIL 620	TIL 660	TIL 1074	TIL 1088	TIL 1128	TIL 1143	TIL 1200	TIL 1235	TIL 1363	clone A42
	μg/ml											
501mel	none	42	49	35	32	31	19	24	41	32	43	41
397mel	none	3	16	6	1	1	4	3	3	3	4	1
T2	none	0	7	−3	−6	7	−6	−7	−6	−7	−7	−6
T2	M9-1 (1)	4	15	−4	1	31	1	−5	−1	1	4	3
T2	M9-2 (1)	86	75	73	79	98	30	36	2	92	82	91
T2	M9-2 (0.001)	52	49	23	32	81	9	6	1	10	41	63
T2	M9-3 (1)	5	25	0	1	19	0	1	−2	0	−2	−4
T2	M10-2 (1)	10	22	5	8	21	8	3	7	7	7	6
T2	M10-3 (1)	84	68	68	73	79	24	27	1	42	67	62
T2	M10-3 (0.001)	91	50	33	25	86	13	14	0	14	39	1
T2	M10-4 (1)	83	47	16	35	80	6	3	1	14	53	0
T2	M10-4 (0.001)	0	11	3	0	14	4	−1	−1	2	−3	−3
T2	M10-5 (1)	4	14	1	4	13	2	3	0	3	0	2

Lysability by TIL clone A42 and TIL lines derived from 10 patients of T2 cells preincubated with the purified peptides M9-1, M9-2, M9-3, M10-2, M10-3, M10-4, and M10-5 was tested in a 4-h ^{51}Cr release assay at an E/T ratio of 20:1 for A42 and 40:1 for other TIL lines. 9 of 10 TIL lysed T2 cells incubated with peptides M9-2 or M10-3. 7 of 10 TIL lysed T2 incubated with peptide M10-4 at a concentration of 1 μg/ml.

among the TIL, but was similar to the different reactivity to M10-4 by the T cell clone A42 or the T cell line TIL1235 (Fig. 1). Higher concentrations (1 μg/ml) of M10-4 were required for lysis than were required for M9-2 or M10-3. These 10 TIL and clone A42 also secreted cytokines including IFN-γ, GM-CSF, and TNF-α when incubated with T2 cells preincubated with M9-2 or M10-3 (data not shown). Therefore, M9-2 or M10-3 are common epitopes recognized by a majority of HLA-A2–restricted melanoma-specific TIL.

Discussion

In this study, we have analyzed the relative frequency of recognition of known melanoma proteins by T cells derived from the TIL of 10 melanoma patients. We have also identified the common epitopes, M9-2 and M10-3, in the MART-1 antigen that were dominantly recognized by nine of these TIL. The cDNA encoding MART-1 was previously isolated by cDNA expression cloning using TIL1235 in screening assays. MART-1 is a 118-amino acid protein containing a single transmembrane domain and is expressed in most melanoma cells as well as cultured melanocytes and retina (17) similar to the expression pattern of gp100 (15) and tyrosinase. gp100 is recognized by 4 of 10 TIL, but recognition of tyrosinase or tyrosinase related protein was not detected by any of the 10 TIL in these assays.

Based on dose response analysis, peptide M9-2 most effectively sensitized T2 cells for lysis (Fig. 1), suggesting that this peptide may be naturally processed and presented on tumor cells. The T cells recognizing M9-2 may react with peptide M10-3 or M10-4 because the latter 10-mer peptides contain the 9-amino acid sequence of peptide M9-2. There is some difference in recognition of these three peptides by different TIL. For example, M10-4 was poorly recognized by the T cell clone A42, but was well recognized by some TIL lines, although a higher concentration of M10-4 was necessary to observe the lysis. This may be due to the variation of TCR affinity for the M9-2 and M10-4 peptides in the context of HLA-A2, or alternatively, TIL lines may contain different T cell clones that only recognize either M9-2 or M10-4. Peptides M10-3 and M10-4 may also be naturally processed and presented by tumor cells. The existence of multiple melanoma antigens presented by HLA-A2 has previously been suggested by analyzing the recognition of melanoma cell clones by a variety of T cell clones (10, 11) or by analyzing HPLC peptide fractions that were isolated from HLA-A2 melanoma cells (26, 27). The M9-2, M10-3, and M10-4 peptides analyzed in this paper may correspond to peptides present in HPLC fractions found by others to stimulate T cells. If these peptides are present on tumor cells, it should be possible to identify them in extracts from melanoma cells.

The observation that most HLA-A2–restricted TIL from melanoma patients recognize common MART-1 peptides but not tyrosinase or gp75 suggests that the M9-2 or M10-3 MART-1 peptides may be more immunogenic in inducing T cell responses in vivo than other known melanoma antigens. However, it is not clear whether these proteins are tumor rejection antigens. Some of the TIL used in this study were injected along with IL-2 into autologous patients, and interestingly, all four TIL (620, 660, 1143, 1200) that recognize gp100 effectively induced tumor regression (>50% reduction of tumor). All but TIL1200 also recognized MART-1. Since many factors are likely to be involved in the induction of tumor regression by adoptive therapy with TIL, it is difficult at this time to conclude which of these melanoma antigens, if any, are responsible for rejection. It would be interesting to conduct clinical trials using active immunization with these identified melanoma antigens or with CTL populations specifically sensitized in vitro to individual antigenic epitopes such as the MART-1 peptides described in this report.

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