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Generation of human-melanoma-specific T lymphocyte clones defining novel cytolytic targets with panels of newly established melanoma cell lines

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Abstract Melanoma is a cancer where the immune system is believed to play an important role in the control of malignant cell growth. To study the variability of the immune response in melanoma patients, we derived melanoma cell lines from several HLA-A2$^+$ and HLA-A2$^-$ patients. The melanoma cell lines studied were designated FM3, FM6, FM9, FM28, FM37, FM45, FM55$_P$, FM55$_{M1}$ and FM55$_{M2}$ and were established from eight metastatic tumors as well as from one primary tumor from a total of seven different patients. On the basis of the ability of tumor cells to induce specific cytotoxic T lymphocytes (CTL) from peripheral blood lymphocytes (PBL) in mixed lymphocyte/tumor culture with HLA-A2$^+$ melanoma cells, the FM3 cell line was characterized as highly immunogenic. To investigate the expression of different melanoma-associated antigens recognized by CTL on different melanoma cell lines, we selected the cell line FM3 for restimulation and further T cell cloning experiments. The lytic activity of CTL clones with good proliferative activity was examined using a panel of HLA-A2$^+$ and HLA-A2$^-$ melanoma cell lines. None of the tested HLA-A2$^-$ melanoma cell lines were susceptible to lysis by the CTL clones, whereas allogeneic HLA-A2$^+$ melanoma cell lines were lysed only by a few CTL clones. On the basis of their reactivity with different melanoma cell lines, it was possible to divide the present CTL clones into at least four groups suggesting the recognition of at least four different antigens. Three of these target structures probably are different from already-described HLA-A2-restricted melanoma-associated antigens, because their expression in the different melanoma cell lines do not correlate with the recognition of melanoma cells by these CTL. The results first indicate that poorly immunogenic melanoma cells may express melanoma-associated antigens, and also suggest that, by using CTL clones obtained against different HLA-class-I-matched melanoma cells, it is possible to define such antigens.

Key words Melanoma · Melanoma-associated antigens · Cytolytic T cell clones · HLA-A2 Immunogenicity

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Introduction

Melanoma is a cancer where the immune system is important in controlling malignant cell growth. Autologous melanoma-specific cytolytic T lymphocytes (CTL) can be generated from peripheral blood lymphocytes (PBL), lymph nodes, and tumor-infiltrating lymphocytes (TIL) from melanoma patients [2, 21, 26, 30, 32, 37, 45]. The existence of melanoma-associated antigens (MAA) recognized by CTL was first proposed from data on the lytic activity of HLA-class-I-restricted CTL clones on a large panel of melanoma cell clones obtained after mutagenesis and immunoselection [30]. Lately seven of these antigens have been identified: MAGE-1 [40], MAGE-3 [22], tyrosinase [11, 46], gp100/Pmel17 [5, 29], Melan-A/MART-1 [18, 28], gp75 [44] and BAGE [10]. It was observed that not all melanoma TIL cultures, usually only one-third, manifest in vitro cytolytic specificity for autologous melanoma targets [37]. HLA-class-I-antigen-matched allogeneic melanoma cells could also be lysed by such CTL lines, pointing to the presence of shared MAA [19–21, 34, 38, 43, 45]. However, not all melanoma cell lines were lysed by such effector cells, even when they shared dominant restriction elements, e.g. the HLA-A2 antigen [43]. This raises the question whether some melanomas lack MAA or whether the absence of a specific immune response is due to other factors, e.g.

low expression of accessory adhesion molecules [1, 4, 35, 39] or local induction of tolerance [6]. In this study, we show that several poorly immunogenic HLA-A2$^+$ melanoma cell lines that were incapable of stimulating antigen-specific syngeneic CTL were sensitive to some allogeneic melanoma-specific CTL clones that were obtained against a highly immunogenic HLA-A2$^+$ melanoma cell line. These findings suggest that poorly immunogenic melanoma cells still may express MAA, which can be recognized by HLA-matched allogeneic CTL clones.

Materials and methods

Patients and cell lines

We have established melanoma cell lines from a total of seven different patients with malignant melanoma: patients 3, 6, 9, 28, 37, 45 and 55. The corresponding melanoma cell lines designated FM3, FM6, FM9, FM28, FM37, FM45, FM55$_P$, FM55$_{M1}$ and FM55$_{M2}$ were established from biopsy materials from these seven patients. The three different melanoma cell lines from patient 55 were derived from a primary tumor (FM55$_P$) and from two different subcutaneous metastases (FM55$_{M1}$ and FM55$_{M2}$). The FM3 cell line has been described previously [33]. We established these cell lines in culture according to the protocol described by Vessière-Louveaux et al. [42]. In brief, cell suspensions were prepared from biopsy material within 2 h of surgical removal. Cells were seeded into T25 or T75 tissue-culture flasks (Nunc, Denmark) in culture medium (Dulbecco's modified Eagle's medium containing 20% fetal calf serum (FCS), 10 mM HEPES, 3.5 g/l glucose and 216 mg/l L-glutamine). Irradiated (100 Gy) NIH 3T3 mouse fibroblasts were added weekly as feeder cells. Cultured cells were detached using 0.5 g/l trypsin and 0.2 g/l EDTA. The culture medium was later changed to RPMI-1640 supplemented with 10% FCS. In addition, the melanoma cell lines FM2, FM57, FM58, FM59, FM60, FM66, FM74 and FM76 obtained in our laboratory were used in some experiments for further characterization of the class I restriction of CTL clones.

HLA typing of all melanoma patients was carried out at the Tissue Typing Laboratory, Copenhagen University Hospital, using peripheral blood lymphocytes (PBL) from all these patients. The HLA-A2 subtypes of HLA-A2$^+$ melanoma cell lines were investigated by sequence analysis of RT-PCR products using primers specific for HLA-A2 (see below). All HLA-A2$^+$ were found to be HLA-A*0201. The B-lymphoblastoid cell line CALOGERO (HLA-A2, B61, C2, DR16, DQ5, DP4 from the ninth and tenth Histocompatibility Antigen Workshops) was obtained from the Tissue Typing Laboratory, Copenhagen University Hospital. An autologous Epstein-Barr-virus(EBV)-transformed lymphoblastoid B cell line FB3 was obtained from lymphocytes of patient 3. The HLA-A2$^+$ melanoma cell line BLM and its gp-100-transfected counterpart [5] were kindly provided by Dr. G. J. Adema, University Hospital, Nijmegen, The Netherlands. Normal cultured melanocytes (NHEM2486) were purchased from Clonetics (San Diego, Calif., USA) and determined as HLA-A2$^+$ by FACS analysis with HLA-A2-specific monoclonal antibodies (see below). Melanocytes were cultured in melanocyte growth medium (MGM, Clonetics).

Isolation of the HLA-A2 loss variant FM55p.R22

The HLA-A2 loss variant of the FM55$_P$ melanoma cell line, designated FM55$_P$.R22, was isolated as follows: 6×10^6 FM55p melanoma cells and 20×10^6 CTL clone 22 cells were mixed in 5 ml medium, centrifuged mildly for 5 min at 150 *g* in a round-bottomed 10-ml centrifuge tube, and then incubated for 6 h at 37 °C in 5% CO_2. After this immunoselection, the remaining melanoma cells were allowed to adhere in medium in a culture flask, and the remaining CTL were washed away the following day. This procedure was repeated once again 1 month later, after which the cells were expanded again and characterized. They were found to be completely resistant to CTL clone 22 and in fluorescence-activated cell sorting (FACS) analysis positive for expression of HLA class I antigens with the polymorphic monoclonal antibody W6/32, but negative for HLA-A2 with both of the HLA-A2-specific antibodies MA2.1 and PA2.1. Its loss of expression of the HLA-A2 allele was further confirmed by the reverse transcriptase/polymerase chain reaction (RT-PCR) with HLA-A2-specific primers (see below).

Mixed lymphocyte/tumor cell culture

Mixed lymphocyte/tumor cell cultures (MLTC) were carried out mainly as described by Hérin et al. [23]. PBL were separated from heparinized blood by centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Aliquots were frozen in liquid N_2 and thawed immediately before setting up MLTC. Samples containing 10^6 PBL were distributed into the wells of a 24-well cluster tray and 10^5 irradiated (100 Gy) melanoma cells were added in a final volume of 2 ml complete medium [RPMI-1640 supplemented with 20% AIM-V (Gibco, Grand Island, N.Y., USA), 10% FCS, 10 mM HEPES buffer, 116 mg/l L-arginine, 36 mg/l L-asparagine, 216 mg/l L-glutamine]. On day 3, 25 U/ml interleukin-2 (IL-2) and 5 U/ml IL-4 (Genzyme, Boston, Mass., USA) were added. On day 7, the lymphocytes were collected, spun at 300 *g* for 7 min, resuspended in fresh medium and counted. Samples containing 5×10^5 lymphocytes were restimulated with 5×10^4 irradiated melanoma cells in 2 ml complete medium containing 25 U/ml IL-2 and 5 U/ml IL-4. Restimulation was repeated at weekly intervals.

Cytolytic T lymphocyte clones

MTLC responder lymphocytes from patient 3 were cloned by limiting dilution in 96-well U-bottomed microtiter plates. Aliquots of 10, 3 and 1 responder cells were seeded in 200 µl complete medium containing 25 U/ml IL-2, 5 U/ml IL-4; 3×10^3 irradiated (50 Gy) melanoma cells were added as stimulator cells and 10^4 irradiated (50 Gy) CALOGERO cells were used as feeder cells. One selected batch of FCS (0970-47903 CSL Diagnostics, Australia), supporting high cloning efficiency, was used for all experiments involving the cloning of CTL. On day 3, 100 µl supernatant was exchanged with 100 µl complete medium containing 50 U/ml IL-2 and 10 U/ml IL-4. The T lymphocyte clones were restimulated on day 7 and on day 14.

On day 21, 50 µl cell suspension from wells with growing clones was mixed with ^{51}Cr-labelled target cells for the assessment of cytolytic activity. Lymphocyte clones with lytic activity were restimulated in 96-well flat-bottomed microtiter plates with a mixture of stimulator melanoma cells and feeder cells in complete medium containing 25 U/ml IL-2 and 5 U/ml IL-4. The clones were expanded in 24-well plates with 2 ml complete medium containing 25 U/ml IL-2, 5 U/ml IL-4, 5×10^4 irradiated stimulator cells and 2×10^5 irradiated feeder cells. The CTL clones were tested for their lytic activity against melanoma cells on day 28 and specific clones were selected. Long-term culture of CTL clones was achieved by restimulation every 7 days.

Cytotoxicity assays

Samples containing 10^6 target cells were labelled for 90 min at 37 °C with 100 µCi Na_2CrO_4 (Amersham, UK). After being washed twice

with cold RPMI-1640 medium, the targets were adjusted to a concentration of 5×10^4 cells/ml in RPMI-1640 medium with 10% FCS. The effector cells were resuspended at various concentrations. Effector and target cells were seeded in 100-µl aliquots in 96-well U-bottomed microtiter plates, spun at 60 *g* for 5 min and incubated at 37 °C in 5% CO_2. After 4 h 100 µl supernatant was aspirated and the radioactivity was determined (Cobra 5005, Packard Instruments, Meriden, Conn., USA). The specific lysis was calculated according to the following equation:

$$\text{Specific lysis (\%)} = \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

where the spontaneous release was assessed by incubating target cells in the absence of effectors and the maximum release was determined in the presence of 1% SDS.

Inhibition of lysis by the monoclonal antibody W6/32 (anti-HLA class I), MA2.1 and PA2.1 (both anti-HLA-A2) was performed by pre-incubating target cells with 10–50 µg/ml antibody in 100 µl medium at 37 °C for 45 min prior to addition of effector cells. In these experiments 10% pooled human serum was used instead of 10% FCS. Natural killer (NK) and lymphokine-activated killer (LAK) cell cytotoxicity was assessed using the NK-sensitive erythroleukemia cell line K562, and the LAK-sensitive lymphoma line Daudi. LAK cells were induced from PBL from normal donors with high concentrations of IL-2 as described previously [24].

Antibodies and flow-cytometric analysis

Unconjugated, fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated monoclonal antibodies against CD3, CD4, CD8, CD56, ICAM-1, LFA-3, BB1/B7 and HLA-DR were purchased from Becton Dickinson (Mountain View, Calif., USA). The monoclonal antibodies W6/32, MA2.1 and PA2.1 were purified from hybridoma supernatants using a protein-A–Sepharose 4B column (Pharmacia, Uppsala, Sweden). For indirect immunofluorescence staining, a goat anti-(mouse Ig) FITC-labelled-$F(ab')_2$ antibody was used as the secondary antibody. Flow-cytometric analyses were carried out using a FACScan flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, Calif., USA). Antibodies against the melanocyte lineage expressed antigen gp100 (HMB-45, Enzo Diagnostics, New York, USA) were used for immunohistological staining.

Reverse-transcriptase-coupled polymerase chain reaction

Expression of tyrosinase, Melan-A/MART-1 and MAGE-3 was determined by the reverse-transcriptase-coupled polymerase chain reaction (RT-PCR). RNA was extracted from various melanoma cell lines using the method of Chomczynski and Sacchi [16] with minor modifications. Briefly, $(1.5–4 \times 10^6$ cells were resuspended in 180 µl lysis buffer (0.14 M NaCl, 10 mM TRIS/HCl, 1.5 mM $MgCl_2$, pH 7.5) and 20 µl 10% Triton X-100 and incubated on ice for 10 min. The nuclear fraction was removed by spinning at 10 000 *g* for 10 min. The supernatant was mixed with 400 µl 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 400 µl phenol, 40 µl 3 M sodium acetate and 100 µl chloroform/isoamyl alcohol (49 : 1), left on ice for 15 min and spun at 10 000 *g* for 10 min at 4 °C. Supernatants were supplemented with one volume of chloroform/isoamylalcohol, mixed and centrifuged at 10 000 *g* for 10 min at 4 °C. RNA was precipitated with two volumes of 99% ethanol for 1 h at − 20 °C and pelleted by centrifugation at 20 000 rpm for 20 min, after which the pellet was washed with 70% ethanol.

Synthesis of cDNA was performed using 5–10 µg RNA, 1 µM oligo-dT primer (DNA Technology, Aarhus, Denmark), 800 U murine moloney leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburgh, Md., USA), 0.6 mM dNTP (Pharmacia LKB, Uppsala, Sweden), 3 U RNaseBlock II (Stratagene, La Jolla, Calif., USA), 0.01 M dithiothreitol, in a total of 100 µl 1 × buffer (Gibco-BRL) incubated at 42 °C for 1 h and at 99 °C for 5 min and placed on ice. Polymerase chain reactions (PCR) were performed with 0.5- to 5-µl aliquots of cDNA in a total volume of 50 µl 1 × PCR buffer (500 mM KCl, 200 mM TRIS pH 8.4, 25 mM $MgCl_2$, 0.05% bovine serum albumin) containing 20 pmol each primer, 0.1 mM dNTP (Pharmacia LKB, Uppsala, Sweden) and 0.625 U Super Taq Polymerase (HT Biotechnology Ltd., Cambridge, UK). For the detection of tyrosinase transcripts, the primer sequences were HTYRA: 5′-CCCACAAATCCTAACTTACTCAGCCCAGC-3′ (upstream) and HTYRB: 5′-ATCGTTGGCAGATCCTGTACCTGGG-3′ (downstream), based on the cDNA sequence of the human tyrosinase gene [31], giving rise to a RT-PCR product of 377 base pairs (bp). The parameters used for amplification were 94 °C 30 s, 67 °C 30 s, 72 °C 60 s for 40 cycles and finally 5 min at 72 °C. For the detection of Melan-A/MART-1 transcripts, the primers used were (upstream) 5′-AA**GGTGTC**CTGTGCCCTGACCC-3′, the *Bam*H1 site is in bold type, and (downstream) 5′-**GGCTTG**CATTTTTCCTACAC-CATTCC-3′, the *Xba*I site is in bold type, which are based on the previously published cDNA sequence of Melan-A/MART-1[28] giving rise to a RT-PCR product of 498 bp. The parameters for the amplification of Melan-A/MART-1 were as follows: 94 °C 30 s, 66 °C 30 s, 72 °C 60 s for 5 cycles; 94 °C 30 s, 64 °C 30 s, 72 °C 60 s for 5 cycles; 94 °C 30 s, 62 °C 30 s, 72 °C 60 s and 94 °C 30 s, 60 °C 30 s, 72 °C 60 s for 25 cycles. For the detection of MAGE-3 transcripts, the primers used were (upstream) 5′-ACCAGAGGCCCCCGGAG-GAG-3′ and (downstream) 5′-CTGCCAATTTCCGACGACA-CTCC-3′, based on the published sequence of MAGE-3 [22] giving rise to a RT-PCR product of 633 bp. The parameters for amplification of MAGE-3 were as for Melan-A/MART-1 above.

For all amplifications, a "hot-start" procedure was used in which the Taq polymerase and dNTP were added to the reaction at the 80 °C step between the denaturation and annealing steps of the first cycle. Amplifications were carried out on a Hybaid Omnigene Thermal Cycler (Hybaid, Teddington, Middlesex, UK) using standard microtubes (Multi Technology Inc., Salt Lake City, Utah, USA). The PCR products were separated by agarose gel electrophoresis (NuSieve 3 : 1; FMC BioProducts, Vallensbaek Strand, Denmark) flanked by 1 µg 100-bp DNA ladder (Pharmacia, Uppsala, Sweden).

For the cloning and sequencing of HLA-A2 from tumor cells, cDNA was amplified with HLA-A2-specific primers: 5′-GCTCTCAC-TCCATGAGGTATTTC-3′ (upstream) and 5′-AGC**GGATCC**AG-TCATATGCGTTTTGGGGGC-3′ (downstream), based on published DNA sequences [12]. The downstream primer includes a *Bam*HI site (in bold type) for directional ligation. The parameters for the amplification were as described above for Melan-A. The PCR products were treated with Klenow enzyme, cut with *Bam*-HI, ligated to Bluescript KS + and transformed into *E. coli* XL1-Blue. Positive colonies were screened with the insert specific primers and isolation of plasmids was carried out with QIAprep-spin Plasmid kit (Qiagen Inc., Chatsworth, Calif., USA). Sequencing was performed by the dideoxy-DNA chain-termination procedure using Sequenase 2.0 (United States Biochemical, Cleveland, Ohio, USA) following the manufacturer's instructions. After electrophoresis in 6% acrylamide, the gel was vacuum-dried and exposed to PhosphorImager storage screens (Molecular Dynamics, Sunnyvale, Calif., USA). After a 48-to 72-h exposure, screens were scanned using a PhosphorImager (Molecular Dynamics) and saved as computer files [27].

Results

Characterization of melanoma cell lines

All melanoma cell lines used in these studies were obtained in our laboratory from fresh biopsy materials.

Table 1 Properties of melanoma cell lines

Patients	HLA class I phenotype of patient PBL	Designation of cell line	HLA-A2 expression on melanoma cells[a]	HMB-45 staining	Tyrosinase expression	Melan-A/MART-1 expression	MAGE-3 expression
3	A2,3; B7,44	FM3	+	+ +	+ +	+	+
6	A1,2; B7,8	FM6	+	+/−	+	+	+
9	A1,3; B8,35; Cw4	FM9	−	−	−	+	+
28	A1,2; B8,15	FM28	+	+	−	−	−
37	A2,3; B7,40; Cw3	FM37	−	+ +	+	+	+
45	A23,28; B17,35; Cw4	FM45	−	+	+ +	+	+
55	A1,2; B15,28; Cw3	$FM55_P$	+	+	+	+	+
		$FM55_{M1}$	+	+	ND[b]	+	+
		$FM55_{M2}$	+	+	ND	+	+

[a] All HLA-A2$^+$ melanoma cell lines were determined to HLA-A*0201$^+$ by sequencing of reverse transcriptase/polymerase chain reaction products (see Materials and methods); [b] **ND**: not done

Melanoma cell cultures were established either with or without the use of mouse NIH-3T3 fibroblasts as feeder cells according to the protocol of Vessière-Louveaux et al. [42]. We observed that, in some of the cultures, addition of feeder cells was not necessary and, for some melanoma cell lines, such as $FM55_P$ and $FM55_{M1}$, addition of mouse fibroblasts even appeared to confer a suppressive effect. This is in agreement with the previously reported effect of NIH-3T3 mouse fibroblasts on the growth of human melanoma cells in vitro [8]. The HLA phenotypes of patients and melanocyte-lineage-specific antigens in melanoma cells are presented in Table 1. The classification of these cell lines as melanomas was confirmed by immunohistological staining with antibodies against the HMB-45 antigen [5] and by the analysis of tyrosinase [11], Melan-A/MART-1 [18, 28] and MAGE-3 [22] gene expression using RT-PCR (Table 1). The cell line FM28, while expressing the HMB-45 antigen, did not express tyrosinase, which is in accordance with previous published results describing the absence of tyrosinase gene expression in some cultured melanoma cells [11]. Interestingly, this cell line also lacked expression of Melan-A/MART-1 and MAGE-3. The HLA-A2$^-$ cell line FM9 did not express HMB-45 or tyrosinase, but expressed both Melan-A/MART-1 and MAGE-3 and therefore was considered as a melanoma line that served as a HLA-A2$^-$ control in some of the experiments. All melanoma cell lines showed high levels of expression of HLA class I antigens, except FM37, which showed low expression of total HLA class I antigens in FACS analysis, but lacked expression of HLA-A2 antigens in FACS analysis as well as in RT-PCR assays for HLA-A2. The FM37 cell line served as an additional control for tests of HLA-A2 restriction by CTL clones. The expression of HLA-A2 by the remaining cell lines from the HLA-A2$^+$ patients was confirmed by FACS analysis. The HLA-A2 subtype of all HLA-A2$^+$ melanoma cell lines was determined by sequencing of the HLA-A2 RT-PCR products and, in all cases, was found to be HLA-A2.1 (HLA-A*0201).

Comparison of immunogenicity of melanoma cells in mixed lymphocyte/tumor cell cultures

To investigate the in vitro immunogenicity of melanoma cells we set up autologous mixed lymphocyte/tumor cultures (MLTC) using PBL as responder cells. MLTC was performed only with HLA-A2$^+$ melanoma cells. Table 2 presents the data on cytotoxicity of lymphocytes 21 days after initiation of the MLTC. The development of significant cytotoxicity under these conditions was seen only with lymphocytes from donor 3, and this cytotoxicity was specific for HLA-A2$^+$ FM3 cells, because the level of lysis with HLA-A2$^-$ melanoma cells was at background level. The lack of development of cytotoxicity with the melanoma lines FM6, FM28 and $FM55_P$ correlated with the much lower extent of proliferation of PBL in these cultures in comparison to MLTC with FM3 cells ([33] and data not shown).

The low development of cytotoxicity in MLTC with some targets might not only be the result of low expression of antigenic determinants, but could also be explained by low expression of adhesion and/or costimulatory molecules. To test this possibility, we investigated the expression of known co-stimulatory and adhesion molecules, which are thought to be important for interaction of T-lymphocytes with antigen-presenting cells, namely BB1/B7 [15, 25, 35, 39], ICAM-1 and LFA-3 [1, 4], using FACS analysis. In some cell lines such as FM3 [33], the expression of B7 was high but it was low in others, in agreement with observations made elsewhere [25]. The levels of expression of ICAM-1 and LFA-3 were high in some cell lines and intermediate in others, and generally did not correlate with the immunogenic properties of cells in the syngeneic systems. To exclude further a major contribution of costimulatory and adhesion molecules to the immunogenicity of melanoma cells, we also investigated the ability of all these melanoma cell lines to induce cytotoxicity in allogeneic MLTC. The results presented in Table 3 clearly indicate that all melanoma

Table 2 Lytic activity of lymphocytes derived from syngeneic mixed lymphocyte/tumor cell culture (*MLTC*) Peripheral blood lymphocytes (*PBL*) were stimulated three times at weekly intervals with syngeneic melanoma cells. Cytotoxic T lymphocyte (CTL):TC ratio = 4

MTLC lymphocytes	Lytic activity (specific ^{51}Cr release, %); syngeneic melanoma cells	FM45
PBL3-anti-FM3	30	9
PBL6-anti-FM6	3	2
PBL28-anti-FM28	6	3
PBL55-anti-FM55$_P$	4	2

Table 3 The ability of melanoma cells to induce allogeneic MLTC. PBL were stimulated three times at weekly intervals with the indicated allogeneic melanoma cells. CTL:TC ratio = 4. (*ND* not done)

	Lytic activity (specific ^{51}Cr release,%) with:			
MLTC lymphocytes	FM3	FM6	FM28	FM55$_P$
PBL28-anti-FM3	51	ND[a]	4	ND
PBL3-anti-FM6	16	37	ND	ND
PBL3-anti-FM28	9	ND	35	ND
PBL3-anti-FM55$_P$	18	ND	ND	42

[a] **ND**: not done

cell lines are able to induce significant cytotoxicity in allogeneic MLTC, indicating that all melanoma lines fulfill the basic requirements to elicit a cytotoxic response with appropriately primed PBL.

These data suggest that the weak induction of CTL against FM6, FM28 and FM55$_P$ cells in the syngeneic systems (Table 3) could be due to the low immunogenicity of these cells which, in turn could correspond to a low frequency of melanoma-specific CTL$_p$ with PBL from patients with low immunogenic tumors. Prolonged MLTC usually led to the development of a higher level of cytotoxicity even with these weakly immunogenic melanoma cells (data not shown). However, comparison of the expression of known HLA-A2-restricted MAA by the melanoma cell lines investigated (Table 1) shows that all the cell lines, with the exception of FM28, expressed some of these antigens. This suggests that these antigens may not be major contributors to the high immunogenicity of FM3 cells in the syngeneic system. For this reason, we decided to produce CTL clones specific for the highly immunogenic melanoma cell line FM3, and to use these CTL clones to detect MAA present on FM3 cells and the remaining less immunogenic melanoma cell lines.

Identification of four clusters of melanoma antigens using CTL clones

As a source of lymphocytes for in vitro immunization in MLTC, we used PBL from patient 3 and applied the protocols of Hérin et al. [23] and of Coulie et al. [17]. After 3 weeks in culture, the proportion of CD8$^+$ cells in the cultures was very high (above 80%). However, the specificity of the resultant T cell line (FT3) was not restricted to FM3 cells, since allogeneic melanoma cells (FM9 and FM45) were also killed, albeit with a lower efficiency. Further, the FT3 CTL line was capable of lysing both NK-sensitive and LAK-sensitive target cells (data not shown). Prolonged cultivation (more than 2 months) led to an increasing specificity of the FT3 line, but the proportion of CD8$^+$ cells decreased with a corresponding increase in CD4$^+$ cells (up to 60%). Cloning was then performed using a 3-week-old MLTC culture. After screening of 400 T cell clones, more than 100 of these clones showed lytic activity against FM3 cells. The specificity of these CTL clones was subsequently investigated using syngeneic HLA-A2$^+$ melanoma cells (FM3) as well as allogeneic HLA-A2$^-$ melanoma cells (FM9 and FM45) as targets for cytotoxicity assays. The majority of the CTL clones only lysed FM3 target cells and not FM9 or FM45 cells.

We further tested the lytic activity of several CTL clones against a panel of different HLA-A2$^+$ melanomas as target cells (Table 4). This table includes one additional HLA-A2$^-$ melanoma cell line, FM37, included as an additional control. The investigated CTL clones were divided into four different groups according to their pattern of reactivity with different HLA-A2$^+$ melanoma cells: group A CTL clones lysed FM3 cells only; group B CTL clones lysed FM3 and FM28 cells (FM28 to a lesser degree than FM3 cells) (CTL clones 21, 39, 49, 80); group C CTL clones lysed FM3, FM28 as well as FM55$_P$ cells (FM3 cells to a lesser degree than FM28 and FM55$_P$ cells) (CTL clone 47); group D CTL clones lysed FM28, FM6, FM55$_P$ and FM3 with increased activity (CTL clones 22 and 32). The selected CTL clones did not kill the syngeneic EBV-transformed lymphoblastoid B cell line FB3, Daudi or K562 cells (data not shown). The clustering of these CTL specificities reflects the expression of different MAA epitopes by the different target cells, and suggests the definition by the CTL clones of at least four different putative antigens: A, B, C and D. The distribution of these putative antigens is illustrated schematically in Fig. 1. Our results also indicate that the CTL clones belonging to groups B–D recognize shared antigens.

HLA restriction of selected CTL clones

To investigate the HLA restriction of the shared antigens mentioned, CTL clones 21, 22 and 47 were used for further experiments. It was noticed that restimulation of clone 47 with FM55$_P$ melanoma cells instead of autologous FM3 cells resulted in better growth of this CTL clone, and therefore FM55$_P$ cells were

Table 4 Lytic activity of CTL clones on different melanoma target cells. Specific ^{51}Cr release (%); CTL:TC ratio = 5; [a] **ND**: not done

CTL clone group/number	Cytotoxicity on melanoma target cells (%)						
	FM3	FM6	FM9	FM28	FM37	FM45	$FM55_P$
Group A							
29	38	3	1	5	0	0	0
35	24	0	0	0	ND	0	0
36	22	ND[a]	1	0	ND	ND	0
54	35	0	0	1	0	1	0
65	16	1	1	1	ND	0	0
67	10	0	0	2	0	0	0
70	43	0	0	0	0	0	0
73	18	ND	0	0	ND	0	0
82	26	1	1	1	ND	0	0
83	21	0	0	1	0	0	0
Group B							
21	39	1	1	13	0	0	0
39	59	0	0	21	0	1	0
49	40	0	3	16	0	ND	1
80	27	0	0	16	0	1	0
Group C							
47	26	2	1	51	0	0	50
Group D							
22	39	21	0	8	ND	2	25
32	30	7	0	4	ND	1	19

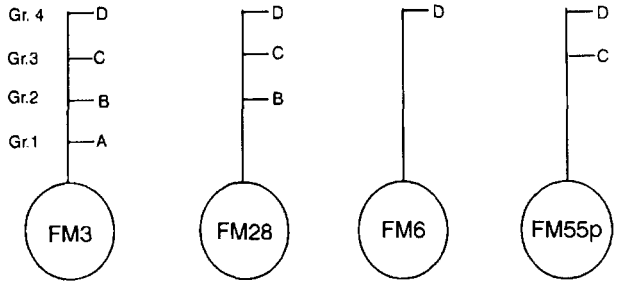

Fig. 1 Schematic representation of the expression of antigenic epitopes by different melanoma cell lines recognized by cytotoxic T lymphocyte (CTL) clones belonging to different groups

subsequently used for restimulation of this clone. The interaction of CTL clones 21, 22 and 47 with different HLA-$A2^+$ and HLA-$A2^-$ melanoma cells, including some additional new melanoma cell lines, is presented in Table 5. CTL clone 21 did not lyse any of the HLA-$A2^-$ melanoma cell lines. CTL clone 22 lysed all HLA-$A2^+$ melanoma cells, and no lysis of HLA-A^- melanoma cells was observed. CTL clone 47 showed high reactivity with some of the HLA-$A2^+$ melanoma cell lines, but also exhibited low reactivity with one additional HLA-$A2^-$ cell line (FM66). These data therefore suggest that the restriction element for clones 22 and 47 is HLA-A2. None of the HLA-$A2^-$ melanoma cell line were lysed by CTL clones 21, 22 and 47 whereas HLA-$A2^+$ poorly immunogenic melanoma cells were lysed by some of the CTL clones (Table 5).

Table 5 Interaction of CTL clones 21, 22 and 47 with different melanoma cells

Target cells	HLA-A2 expression on melanoma cells	Cytotoxicity (%) of CTL clones		
		Clone 21	Clone 22	Clone 47
FM2[a]	–	0	0	1
FM3	+	25	38	15
FM6	+	0	22	2
FM28	+	14	15	66
$FM55_P$	+	1	25	38
FM57[a]	+	0	56	3
FM58[a]	+	0	15	38
FM59[a]	+	ND[b]	ND	42
FM60[a]	+	1	22	2
FM66[a]	–	0	0	10
FM74[a]	–	0	1	3
FM76[a]	+	3	22	37

[a] Additional melanoma cell lines (unpublished data)
[b] **ND**: not done

The lytic activity of CTL clones 22 and 47 was blocked by a monoclonal antibody against HLA class I antigens (W6/32) (Fig. 2). Direct proof of the HLA-A2 restriction of CTL clones 22 and 47 was obtained from experiments with $FM55_P$.R22 cells, a variant of $FM55_P$ cells which had lost expression of HLA-A2 antigens during immunoselection experiments with the CTL clone 22 (see description in Materials and methods). These cells were not lysed by CTL clone 22, and their sensitivity to CTL clone 47 was also significantly decreased (Fig. 3). In separate experiments, it was demon-

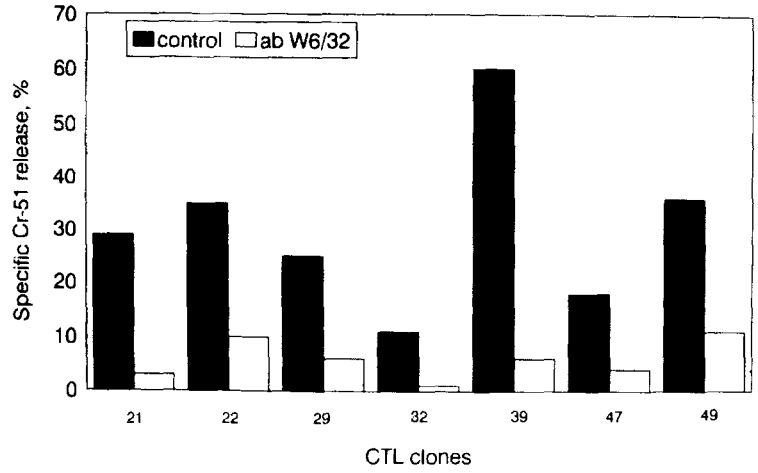

Fig. 2 Inhibition of lytic activity of CTL clones by an anti-HLA class I monoclonal antibody (W6/32). Cytolytic activity was detected in a standard 4-h ^{51}Cr-release assay using an effector-to-target cell ratio of 5. The target cells were incubated with antibody for 45 min before the addition of CTL. The final concentration of the monoclonal antibody was 50 μg/ml

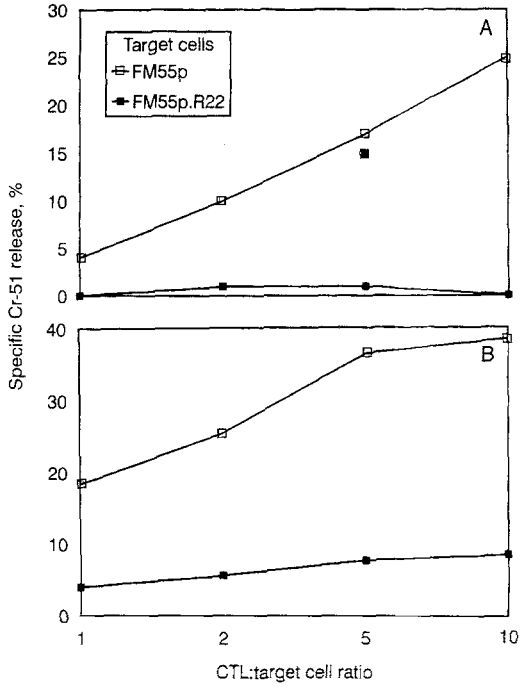

Fig. 3A, B Comparison of sensitivity of FM55$_P$ and FM55$_P$R22 melanoma cells to lysis by (**A**) CTL clone 22 and (**B**) CTL clone 47. The lytic activity was determined in a 4-h ^{51}Cr-release assay using an effector-to-target cell ratio of 5

strated that both the parental FM55p cells and the HLA-A2 loss variant FM55$_P$.R22 were sensitive to MHC-unrestricted lysis mediated by LAK cells (data not shown). Together, these data indicate that the HLA-A2 allele is the restriction element for CTL clones 22 and 47. The CTL clone 21 is probably also restricted by HLA-A2, since the melanoma lines FM3 and FM28, which were sensitive to this CTL clone only, shared the

Table 6 Effect of HLA-A2-specific monoclonal antibodies on the activity of CTL clones 21, 22 and 47. CTL : TC ratio = 5. Concentration of antibodies during pretreatment = 50 μg/ml

	Inhibition of lytic activity by antibodies (%)					
	Clone 21		Clone 22		Clone 47	
Target melanoma cells	MA2.1	PA2.1	MA2.1	PA2.1	MA2.1	PA2.1
FM3	44	16	97	22	− 20	0
FM28	28	0	100	53	0	0
FM55$_P$	ND	ND	ND	ND	0	0

HLA-A2 allele. We also tried to use HLA-A2-specific monoclonal antibodies (MA2.1 and PA2.1) for blocking the interaction of the CTL clones with sensitive target cells, but the results from these experiments were rather conflicting. The results of one representive experiment are summarized in Table 6 and show that the activity of CTL clone 22 was completely blocked by the MA2.1 antibody, but only partially by the PA2.1 antibody. The activity of CTL clone 21 was also blocked by the MA2.1 antibody but to a lower degree whereas the activity of CTL clone 47 could not be blocked completely by either of the anti-HLA-A2 antibodies (MA2.1 or PA2.1).

The interaction of the CTL clones with HLA-A2$^+$ normal melanocytes is shown in Fig. 4. Only CTL clone 22 induced significant lysis of normal melanocytes, indicating that the relevant target structure recognized by this CTL clone is present on normal melanocytes and therefore probably corresponds to a melanocyte-lineage-specific differentiation antigen.

As can be seen from Table 4, a large number of CTL clones exclusively kill FM3 melanoma cells (antigen A). The activity of CTL clones belonging to this group

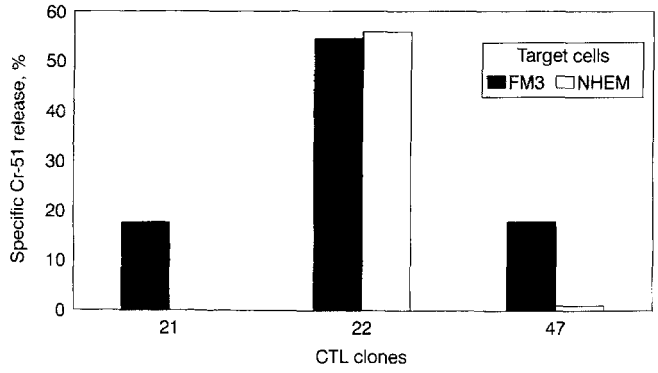

Fig. 4 Interaction of CTL clones 21, 22 and 47 with FM3 cells and normal HLA-A2$^+$ melanocytes. The lytic activity was determined in a 5-h ^{51}Cr-release assay using an effector-to-target cell ratio of 10. The lysis of normal HLA-A2$^+$ melanocytes by CTL clone 21 was 0

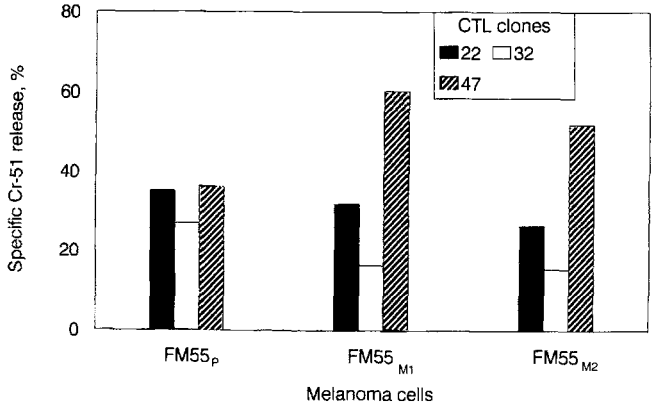

Fig. 5 The recognition of three CTL clones (clones 22, 32 and 47) of primary (FM55$_P$) and metastatic (FM55$_{M1}$ and FM55$_{M2}$) melanoma cell lines from patient 55. The lytic activity of these CTL clones was determined in a standard 4-h ^{51}Cr-release assay using an effector-to-target cell ratio of 5

were not blocked by HLA-A2-specific antibodies (data not shown), which by itself does not exclude the participation of HLA-A2 in the restriction of these CTL clones, but the present data do not allow any definite conclusions concerning their HLA restriction elements.

It has recently been demonstrated [7] that the reactivity of T cell lines derived from primary and metastatic melanomas exhibit significant differences when tested against primary and metastatic melanoma cells. We therefore investigated the lytic activity of CTL clones 22, 32 and 47 towards the primary melanoma cell line (FM55$_P$) and the two different metastatic melanoma cell lines, (FM55$_{M1}$ and FM55$_{M2}$) from patient 55. Both the primary and metastatic melanoma cell lines were lysed by CTL clone 22 (Fig. 5) strongly suggesting the possibility of shared antigenic determinants (antigen D) on primary and metastatic melanoma cells.

Discussion

The purpose of this study was to compare the immunogenicity of several HLA-A2$^+$ melanoma cell lines, and to show that poorly immunogenic tumors may express certain melanoma-associated antigens (MAA), which can function as target structures for already established CTL. In the course of this study, we unexpectedly found that the target structures involved differed from previously described HLA-A2-restricted MAA.

As a general approach to evaluate the immunogenicity of melanoma cells in vitro, we selected MLTC using PBL as responder cells. Comparison of the ability to induce the development of cytotoxicity of several HLA-A2$^+$ melanoma cell lines in a 3-week MLTC indicated that only FM3 cells could induce the appearance of significant specific cytotoxicity. The immunogenicity of tumor cells is determined by several factors: the presence of specific antigens and the expression of MHC class I molecules [3, 14, 18–21, 32, 34, 37, 38, 41, 43–46] as well by the presence of costimulatory [15, 25, 35, 39] and adhesion molecules [1, 4]. All the investigated HLA-A2$^+$ melanoma cell lines showed high expression of HLA-A2 and total levels of HLA class I molecules. The expression of the B7 costimulatory molecule, which is thought to be crucial for induction of a primary immune response [15, 25, 35, 39] varied among the investigated melanoma cells, as also found by others [25], and may therefore not be crucial for the induction of cytotoxicity by highly immunogenic tumor cells. The expression of the adhesion molecules ICAM-1 and LFA-3 was high or intermediate on all tested melanoma cell lines, and their expression level did not correlate with the immunogenicity of these cells (data not shown). Naturally, other costimulatory or adhesion molecules could be important for the level of immunogenicity, but the ability of all the present HLA-A2$^+$ melanoma cell lines to induce specific cytotoxicity in allogeneic MLTC probably excludes these factors as the main determinants of immunogenicity in our experiments. It is noteworthy, that the poorly immunogenic melanoma cell lines FM6 and FM55$_P$ have the same distribution of known MAA, which are recognized in an HLA-A2-restricted manner (Table 1), as the highly immunogenic FM3 cell line. This probably indicates that the known MAA are not the major determinants of the high immunogenicity of FM3 cells, and additional antigens may be present which could contribute to their immunogenic properties. The melanoma line FM3 was therefore considered as the best candidate as a target cell for the production of CTL clones. Consequently, PBL from this patient were selected for in vitro immunization experiments. In these experiments, we obtained a large number of CD8$^+$ CTL clones against FM3 cells, and some of these clones were found also to recognize allogeneic, HLA-A2$^+$

melanoma cells. None of the investigated CTL clones recognized HLA-A2$^-$ melanoma cells. Several CTL clones with strong proliferative activity were tested on different HLA-A2$^+$ melanoma cells. On the basis of their reactivity with the different melanoma cell lines, we were able to classify the CTL clones into at least four different groups, which suggests the presence of at least four antigens (or antigenic epitopes): A, B, C and D, three of which are shared by several melanoma cell lines (Fig. 1). The restriction element for the shared antigens is probably HLA-A2. This conclusion, based on the reactivity of the different CTL clones with different HLA-A2$^+$ and HLA-A2$^-$ melanoma cell lines, is supported in the case of CTL clone 22 by blocking of its cytotoxic activity against HLA-A2$^+$ melanoma targets by anti-HLA-A2 monoclonal antibodies. For unknown reasons, the same antibodies did not block the activity of CTL clone 47, and these antibodies only showed low blocking activity with CTL clone 21 (Table 5). The poor blocking of lytic activity of CTL with melanoma target cells by polymorphic HLA class I antibodies has also been noted by other investigators, both in the case of HLA-A2-restricted CTL [21] and for HLA-A3 restricted CTL [14], while in other cases these antibodies were able to block cytolytic activity [3]. It is of interest to note that inhibition of melanoma-specific CTL by HLA-A2-specific monoclonal antibodies has mainly been shown with CTL that are specific for differentiation antigens present also on normal melanocytes [3]. For a typical melanocyte-lineage-associated differentiation antigen such as tyrosinase [46], the results may be explained by the presence of CTL with low-affinity T-cell receptors, since CTL with high-affinity T-cell receptors for self antigens will be expected to be eliminated during thymic selection [36]. We also observed strong inhibition by HLA-A2-specific antibodies for the CTL clone 22, which similarly recognizes normal melanocytes and therefore probably a melanocyte-lineage-associated differentiation antigen (Fig. 4). It is therefore a possibility that only the interaction of CTL carrying low-affinity T cell receptors can be blocked by HLA-A2-specific antibodies, whereas CTL with high-affinity T cell receptors may not be significantly influenced by the binding of HLA-A2 antibodies. This interpretation implies that CTL clones not influenced by binding of HLA-A2-specific antibodies (such as CTL clones 21 and 47) might not be directed against differentiation antigens, but rather against progression antigens, which could be analogous to the MAGE [40, 41] and BAGE [10] antigens. On the other hand, the inhibitory effect of MA2.1-specific antibodies on the interaction of CTL with relevant target cells could be indirect. It is noteworthy here that the MA2.1 antibody is commonly used in order to facilitate peptide binding by HLA-A2 molecules [9, 13, 46], which may suggest that this particular antibody could, in fact, facilitate the interaction of CTL with peptide-HLA-A2 complexes.

The clustering of the above CTL clones may reflect the expression of different MAA epitopes by the different target cells and suggests the definition of at least four different antigens: A, B, C and D respectively. This is illustrated schematically in Fig. 1.

The antigens B, C and D are all shared antigens, as detected by their presence on several HLA-A2$^+$ melanoma cell lines. One of these antigens, antigen D, was apparently present on all HLA-A2$^+$ melanoma cell lines. It appears to be different from other previously described HLA-A2-restricted antigens, i.e. tyrosinase [11], gp100/Pmel17 [5, 29], Melan-A/MART-1 [18, 28] and MAGE-3 [22, 41]. The CTL clone 22, defining antigen D, recognizes FM28 melanoma cells, and although its cytolytic activity was low with these cells, it was blocked by W6/32, MA2.1 and PA2.1 antibodies (data not shown) indicating the HLA-A2 restriction of this interaction. The sensitive FM28 target cells did not express tyrosinase, Melan-A/MART-1 or MAGE-3 (Table 1), which exludes these antigens as possible targets for CTL clone 22. The sensitive FM28 target cells expressed gp100/Pmel17 [5, 29], as detected by staining with the HMB-45 antibody, but the CTL clone 22 was tested in cytotoxicity with gp100-transfected HLA-A2$^+$ BLM melanoma cells [5] and did not show any cytolytic activity (data not shown). Our data further indicate that the target structure D is HLA-A2-restricted, since its activity was blocked by HLA-A2-specific antibodies. The D antigen was expressed by HLA-A2$^+$ melanoma cell lines, established both from a primary tumor and from metastases as well as by normal HLA-A2$^+$ melanocytes. On the basis of these results, we suggest that antigen D may be a new stably expressed melanoma-associated differentiation antigen restricted by HLA-A2.

The antigens B and C, recognized by CTL clones 21 and 47 respectively, were not expressed by normal HLA-A2$^+$ melanocytes. The only reported MAA that is absent on normal melanocytes [22] and can be recognized by CTL in an HLA-A2-restricted manner [41] is MAGE-3 [22]. MAGE-3 was expressed similarly to Melan-A/MART-1 and was detected in all four HLA-A2$^+$ melanoma cell lines used to group our CTL clones (Table 1). However, some of these cell lines were resistant either to CTL clone 21 (FM6 and FM55$_P$) or to clone 47 (FM6). These results appear to exclude MAGE-3 as a target for CTL clones 21 and 47, and suggest the identification of antigens B and C as two new MAA that may be classified as progression antigens, since neither of these antigens was expressed by normal melanocytes. Work is now in progress to study the interaction of the described CTL clones with the already known MAA-derived HLA-A2-binding nonapeptides and peptide-loaded T2 cells [32]. The peptides tested so far (derived from tyrosinase [45] and Melan-A/MART-1 [18] respectively) did not induce the sensitization of T2 cells to lysis by CTL clones 22 and 47 (data not shown).

An additional conclusion from this study is that a number of MAA are also present on poorly immunogenic melanoma cells, exemplified here by FM6 and FM55p, and may be recognized by specific CTL raised against highly immunogenic melanoma cells, such as the FM3 melanoma line [33]. Our finding that such MAA are also expressed by poorly immunogenic tumors and can serve as targets for cytotoxicity by appropriately stimulated CTL is in support of future novel immunization protocols or adoptive immunotherapy focusing on these target antigens in HLA-A2$^+$ patients. Our continuing studies will concentrate on the cloning and characterization of the putative antigenic molecules B, C and D. Among these antigens, the antigen provisionally designated D (defined by CTL clone 22) would appear to be the most interesting candidate for further work, since it was found to be stably expressed by all the HLA-A2$^+$ melanoma cell lines studied, including cell lines from both primary and metastatic tumors. Its stable expression should be an obvious advantage as a target antigen for the development of vaccination or other immunotherapeutic protocols.

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