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Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene

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ABSTRACT **Although commonly expressed human melanoma-associated antigens recognized by CD8+ cytolytic T cells have been described, little is known about CD4+ T-cell recognition of melanoma-associated antigens. Epstein–Barr virus-transformed B cells were used to present antigens derived from whole cell lysates of autologous and allogeneic melanomas for recognition by melanoma-specific CD4+ T-cell lines and clones cultured from tumor-infiltrating lymphocytes. HLA-DR-restricted antigens were detected in the lysates on the basis of specific release of cytokines from the responding T cells. Antigen sharing was demonstrated in the majority of melanomas tested, as well as in cultured normal melanocytes, but not in other normal tissues or nonmelanoma tumors. T-cell clones manifested a single recognition pattern, suggesting the presence of an immunodominant epitope. This epitope was identified as a product of the tyrosinase gene, which has also been shown to encode class I-restricted epitopes recognized by CD8+ T cells from melanoma patients. Identification of commonly expressed tumor-associated protein molecules containing epitopes presented by both class I and class II major histocompatibility molecules may provide optimal reagents for cancer immunization strategies.**

Commonly expressed melanoma-associated antigens can be recognized by CD8+ cytotoxic T lymphocytes (CTLs) derived from melanoma patients. In short-term lysis assays, CTLs grown from *in vitro* sensitized peripheral blood lymphocytes (PBLs) or lymph node lymphocytes or from lymphocytes infiltrating metastatic melanoma lesions have been shown to recognize autologous and major histocompatibility complex (MHC) class I-compatible allogeneic melanomas but not HLA-matched nonmelanoma tumors, lymphoblasts, or cultured fibroblasts (1, 2). Similar recognition patterns have been observed by measuring cytokine secretion from tumor infiltrating lymphocytes (TILs) cocultivated with autologous or HLA-matched allogeneic tumor stimulators (3). Expression of shared melanoma antigens even in tumors of discrepant HLA phenotypes has been shown by genetically modifying these tumors to express the HLA-A2.1 molecule: HLA-A2-restricted melanoma-specific CTLs lysed the majority of gene-modified melanomas tested, but not HLA-A2+ nonmelanoma tumors or normal tissues (4, 5). Recently, melanoma-specific HLA-A2-restricted CTL clones have been shown to recognize cultured normal melanocytes as well as their malignant counterparts, suggesting that shared melanoma antigens may be lineage specific (6). The multiplicity of melanoma-associated antigens present within isolated melanoma lesions, and even within individual melanoma cells, has been suggested on the cellular level (7, 8) and confirmed on the peptide level (9, 10). Five MHC class I-restricted melanoma-associated antigens have been molecularly defined (11–16), encoding only a subset of these commonly expressed antigens. Therapeutic vaccination strategies are evolving based on these CD8-recognized epitopes.

While animal models of malignant and viral diseases have shown the importance of CD8+ T cells in the effector phase of the immune response, the CD4+ helper arm has been shown to mediate critical priming and effector functions as well. We therefore addressed the issue of whether shared melanoma antigens are recognized by melanoma-specific CD4+ T cells. To facilitate screening procedures for shared CD4-recognized melanoma determinants, we devised an assay system which uses an autologous or MHC class II-matched allogeneic Epstein–Barr virus (EBV)-transformed B-cell line to present antigens derived from tumor cell lysates; reactivity in CD4+ T cells was measured by cytokine secretion (17). By using a single professional antigen-presenting cell (APC) to present putative tumor-specific antigens from various sources for T-cell recognition, rather than measuring direct T-cell–tumor cell interactions, we circumvented issues of hidden HLA genotype discrepancies (18–20) as well as potential antigen-processing defects which have been documented in some human tumors (21). We report here the identification of a commonly expressed MHC class II-restricted melanoma-associated antigen recognized by CD4+ T cells from TILs.

MATERIALS AND METHODS

Lymphocyte Cultures and Clones. TILs were cultured from enzymatically digested single-cell suspensions of solid metastatic melanoma lesions as described (22), in the presence of recombinant interleukin 2 (IL-2, 6000 units/ml; Chiron), and were used in bioassays after 45–70 days. CD4+ and CD8+ TIL subsets were purified from bulk cultures by positive panning (23). CD4+ T cells were cloned by limiting dilution in microtiter plates, in the presence of IL-2 (600 units/ml), pooled allogeneic PBLs from three donors (total of 3×10^4 cells per well, 3000 rads; 1 rad = 0.01 Gy), and autologous EBV-B cells (1×10^4 cells per well, 10,000 rads) pulsed with a freeze/thaw lysate of autologous tumor. Clones were restimulated weekly. The clones used for bioassays were grown from 0.3- or 1-cell-per-well dilutions and tested after 58–155 culture days.

EBV-transformed B-cell lines were established from PBLs of melanoma patients by standard techniques and were maintained as suspension cultures in RPMI 1640 with 10% fetal bovine serum.

Tumors and Normal Melanocytes. Melanoma cultures were established from single-cell suspensions of metastatic lesions

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Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; GM-CSF, granulocyte/macrophage-colony-stimulating factor; MHC, major histocompatibility complex; IL, interleukin; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; TIL, tumor-infiltrating lymphocyte.

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and maintained as adherent monolayers in RPMI 1640 with 10% fetal bovine serum (24). The normal melanocyte cultures FM 707, FM 708, FM 902, FM 906 and FM 907, generated from neonatal foreskin, were a gift of Meenhard Herlyn (Wistar Institute, Philadelphia) (25) and were maintained in melanocyte growth medium (MGM; Clonetics, San Diego). The cultured colon carcinoma CY13 was a gift of J. Yannelli (National Institutes of Health, Bethesda, MD). Colon carcinomas WiDr, LoVo, and SW480; breast carcinomas ZR-75-1 and MCF7; and Ewing sarcomas 6647, RD-ES, and TC-71 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 with 10% fetal bovine serum. All cultures were mycoplasma free.

Fresh tumor specimens prepared from enzymatically dispersed single-cell suspensions of solid tumors were immediately cryopreserved and were rapidly thawed for use on the day of bioassay.

Antigen Presentation to T Cells by EBV-Transformed B Cells. Optimization of a bioassay for tumor-reactive $CD4^+$ T cells, using EBV-B cells as antigen-presenting cells for lysates of whole tumor cells, has been described (17). To prepare putative tumor antigens, whole tumor cells were subjected to three rapid freeze/thaw cycles, and cell fragments were cultured with EBV-B cells. These "tumor-pulsed" B cells were maintained at 37°C for 20–24 hr, and then TILs were added to these cultures. As a positive control, TILs were also cultured in plates coated with anti-CD3 monoclonal antibody (mAb) OKT3 (Ortho Pharmaceuticals). After an additional 20–24 hr, culture supernatants were harvested and assayed with ELISA kits (R & D Systems) for granulocyte/macrophage-colony-stimulating factor (GM-CSF, detectable at 8–500 pg/ml), tumor necrosis factor α (15–1000 pg/ml), IL-4 (31–2000 pg/ml), and IL-6 (3–300 pg/ml). GM-CSF assays were calibrated with international reference standard 88/646 (National Cancer Institute, Frederick, MD). Interferon γ was measured with an ELISA developed with reagents from BioSource International (Camarillo, CA) (20–10,000 pg/ml). The response of TILs to tumor stimulation was considered to be significant when cytokine secretion in response to tumor-pulsed EBV-B cells exceeded the response to EBV-B cells alone by $\geq$3.0-fold.

Antibodies were used to inhibit TIL recognition of tumor-pulsed EBV-B cells (17). Antibodies directed against HLA determinants included W6/32 (against HLA-A, -B, -C; IgG2a; Sera-Lab, Crawley Down, Sussex, U.K.), IVA12 (HLA-DR, -DP, -DQ; IgG1), L243 (HLA-DR; IgG2a), Genox 3.53 and G2b.2 (HLA-DQw1; IgG1 and IgG2a, respectively), and IVD12 (HLA-DQw3; IgG1) (all purified from ATCC hybridoma supernatants).

Transfection of COS-7 Cells. Genes cloned into the expression vectors pcDNA3 (Invitrogen) or pCEV27 (26) were transiently transfected into the monkey kidney COS-7 cell line (gift of W. Leonard, National Institutes of Health) by the DEAE-dextran method (27). EBV-B cells were pulsed with lysates of transfected COS-7 cells for recognition by T cells. The tyrosinase gene was isolated from a cDNA library from the cultured melanoma line 1290A-mel, and its identity was confirmed by partial DNA sequencing, which gave a sequence identical to that published by Bouchard *et al.* (28). The tyrosinase-(1–3) gene, also isolated from the 1290A-mel library, lacks the exons 4 and 5 and encodes a truncated product. The gene encoding tyrosinase-related protein (gp75) was isolated by screening a cDNA library from 501-mel with a probe constructed on the basis of the published gene sequence (29).

RESULTS AND DISCUSSION

$CD4^+$ T cells were purified by positive selection from a heterogeneous population of TILs cultured from a metastatic melanoma lesion from patient 1088. Selected cultures were >95% $CD4^+$. Preliminary experiments indicated that these $CD4^+$ T cells secreted cytokines specifically when cocultivated with autologous EBV-B cells (1088-EBV) which had been pulsed with lysates of autologous melanoma cells (1088-mel). TILs secreted large quantities of GM-CSF and much smaller quantities of tumor necrosis factor α, IL-4, and interferon γ in response to autologous tumor; specific IL-6 secretion was not observed. Thus, GM-CSF secretion was monitored as a measure of T-cell recognition in subsequent assays. In 11 separate experiments, $CD4^+$ TILs stimulated with tumor-pulsed EBV-B cells secreted 8- to 138-fold more GM-CSF (median, 46-fold) than TILs stimulated with EBV-B cells in the absence of tumor. GM-CSF secretion was inhibited 96% by blocking with the anti-HLA-DR mAb L243 (Fig. 1), suggesting that TIL reactivity was HLA-DR restricted. Significant blocking (71%) of cytokine secretion was also observed with the anti-class II framework mAb IVA12 but not with isotype-matched mAb directed against a monomorphic MHC class I determinant or against two HLA-DQ determinants (HLA type of patient 1088: HLA-DR 4,17; HLA-DQw2,3; HLA-DRw52,53). Conversely, in the same experiment, cytokine secretion by purified $CD8^+$ TIL 1088 in response to whole autologous melanoma cells (MHC class I^+, class II^-) was inhibited by anti-class I mAb W6/32 but not by L243 or IVA12 (data not shown). As expected, $CD8^+$ TIL 1088 failed to react to 1088-EBV cells pulsed with tumor lysate. Thus, $CD4^+$ TIL 1088 recognized autologous tumor

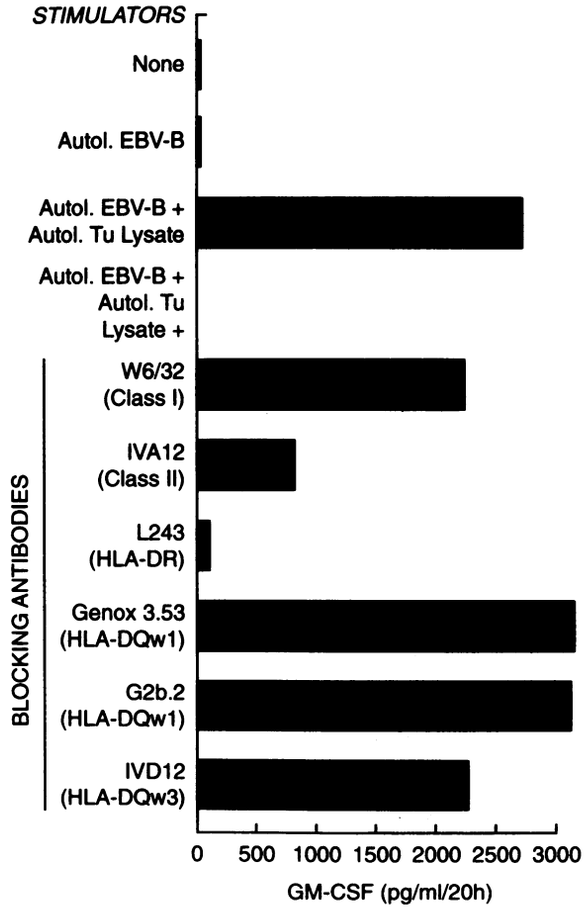

FIG. 1. Response of $CD4^+$ TIL 1088 to autologous tumor is HLA-DR restricted. TIL (10^6 per ml) were cultured in the presence of autologous EBV-B cells (10^6 per ml) alone or B cells pulsed with a lysate of autologous cultured melanoma cells (7×10^5 cell equivalents per ml). Secretion of GM-CSF following autologous tumor stimulation was significantly inhibited by mAb L243 (anti-HLA-DR) and IVA12 (anti-HLA-DR, -DP, -DQ).

antigen presented by EBV-B cells in a specific, MHC class II-restricted manner.

A variety of allogeneic tumors and normal tissues were screened for the presence of antigens recognized by CD4+ TIL 1088. In a representative experiment (Fig. 2) lysates of the autologous cultured melanoma line and all seven allogeneic melanoma cultures tested were stimulatory. Stimulation indices [SI = GM-CSF secretion by TILs with EBV-B plus cell lysate)/secretion by TILs with EBV-B)] ranged from 4.2 for 938-mel to 104.8 for 553-mel. In separate experiments, among 18 fresh or cultured melanomas that were screened for recognition, 12 were positive (67%), 4 were negative, and 2 were equivocal on repeat experiments. Taken together, these results suggest that the antigens recognized by CD4+ TIL 1088 are broadly but not universally expressed in melanoma lesions; alternatively, recognition may reflect relative degrees of antigen expression and the sensitivity of our detection system. Although TILs recognized a number of allogeneic melanomas, they consistently failed to react with normal cells of nonmelanocytic lineage derived from the same patients (data not shown). TILs also recognized autologous fresh melanoma cells (SI = 19.0 and 13.1 in two experiments) as well as tumor cultured from the same lesion (1088-mel), from passages 6 through 48. Thus, the recognized antigens were present *in vivo* and were not a function of culture artifact; their expression was conserved through almost 1 year of continuous *in vitro* culture.

CD4+ TIL 1088 cells were tested for recognition of a variety of nonmelanoma tumors, both fresh and cultured. These TILs failed to recognize lysates from 14 tumors of various histologic types, including colon carcinomas, breast carcinomas, lymphomas, and sarcomas. However, CD4+ TIL 1088 did recognize all four normal melanocyte lines assayed, on repeated occasions. Measured levels of GM-CSF secretion approached those observed in response to 1088-mel. An example is shown in Fig. 3. These results suggested that the antigens recognized by CD4+ TIL 1088 might be specific for the melanocytic lineage.

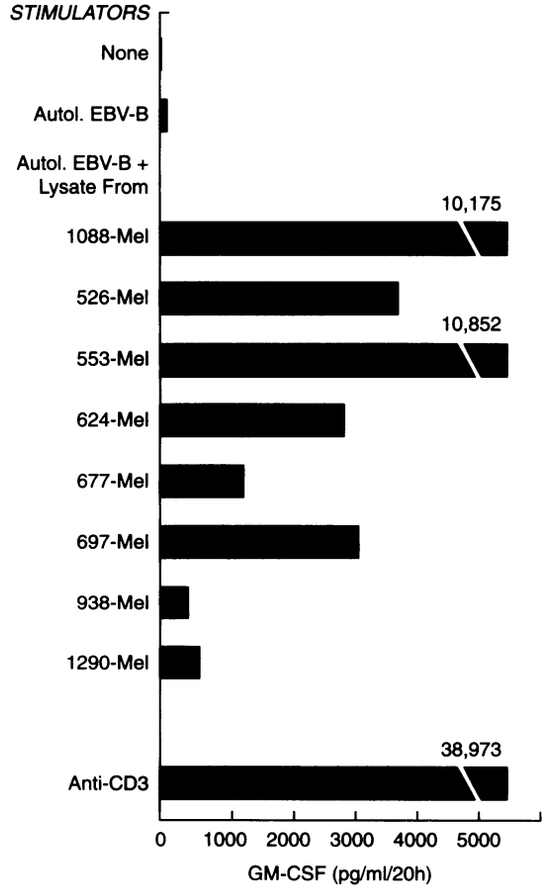

FIG. 2. CD4+ TIL 1088 cells recognize lysates of autologous and allogeneic cultured melanoma lines presented by autologous EBV-B cells. TILs cultured 58 days without tumor restimulation were incubated for 20 hr with tumor-pulsed 1088-EBV. GM-CSF secretion was measured by ELISA. All cells were at 10^6 per ml.

We previously identified two other melanoma patients whose CD4+ TILs recognized lysates of autologous melanoma cells presented by autologous or HLA-matched EBV-B cells (17). These TILs appeared to be MHC class II-restricted and to recognize antigens unique to autologous melanoma cells, since they failed to react with 15 allogeneic melanomas including 1088-mel or with normal cells including cultured melanocytes. These results suggest the existence of multiple class II-restricted melanoma determinants which are differentially and specifically recognized by CD4+ T cells from these three patients.

TILs cultured in bulk under the conditions described have been shown to be oligoclonal, but not monoclonal, cell populations (30–32). To determine whether multiple shared antigens were being recognized by CD4+ TIL 1088, CD4+ T-cell clones were raised from these TILs and assayed for target recognition. Four clones recognized the autologous melanoma as well as multiple allogeneic melanomas and all three normal melanocyte lines tested (FM 902, 906, and 907) (Table 1). The target recognition profiles of all four clones were remarkably similar, and antibody blocking studies suggested that all were HLA-DR restricted. For two clones, HLA-DR restriction was confirmed by using allogeneic EBV-B cell lines or macrophages as APCs for tumor antigens; only APCs sharing the HLA-DR4 molecule were stimulatory. Thus, a single antigenic protein seemed to be present in all of the melanomas recognized by these CD4+ T-cell clones and was shared by normal melanocytes. Further experiments with these and seven additional CD4+ TIL 1088 clones revealed a homogeneous recognition profile.

CD4+ TIL 1088 clones were assessed for recognition of melanoma-associated gene products expressed by 1088-mel as shown by Northern blotting and which are known to contain commonly expressed CD8 epitopes recognized by CTLs from melanoma patients. The genes encoding the tyrosinase, MART-1, and gp100 proteins (12–16) were cloned into plasmid vectors and transiently expressed in COS-7 cells. Lysates of transfected COS-7 cells were incubated with 1088-EBV and used to stimulate cytokine secretion from CD4+ T-cell clones. All six T-cell clones tested secreted significant amounts of GM-CSF in response to lysates of tyrosinase-transfected COS-7 cells as well as to lysates from 1088-mel cells (Table 2). Transfection of COS-7 cells with a truncated tyrosinase gene or with genes encoding the gp75 tyrosinase-related protein, β-galactosidase, or HLA-A2.1 did not confer recognition. As a control, CD8+ T cells from patient 1088 failed to react with any of these stimulator cells. Uncloned CD4+ T cells did not react as strongly as the clones with the tyrosinase gene product. This may reflect the presence of CD4+ TIL 1088 subpopulations not represented by these clones, which can recognize additional antigens, as also suggested by some discrepancies in the recognition of allogeneic melanomas by the bulk-cultured TILs compared with the T-cell clones (compare Fig. 2 and Table 1). Alternatively, this may reflect the decreased sensitivity of the uncloned TILs compared with the tumor-restimulated clones. In three additional experiments, CD4+ T-cell clones specifically recognized the products of tyrosinase genes isolated from two different patients' melanomas and expressed in two different plasmids, while failing to react with MART-1 or gp100 (data not shown). Together with demonstrations of CD8+ T-cell reactivity in melanoma patients

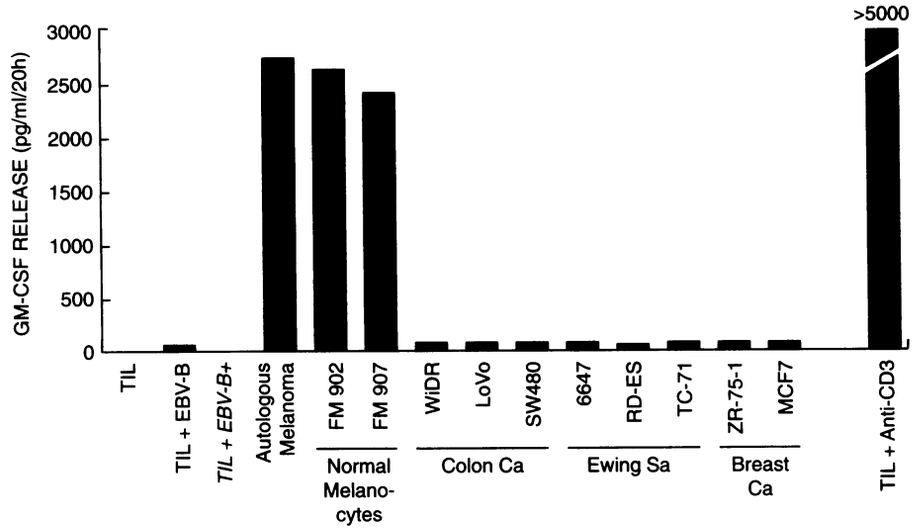

FIG. 3. $CD4^+$ T cells secrete GM-CSF specifically in response to lysates of autologous melanoma cells and allogeneic cultured normal melanocytes, but not to a variety of cultured tumors of other histologic origins. All cells were at 10^6 per ml. Ca, cancer; Sa, sarcoma.

against HLA-A2- and HLA-A24-restricted epitopes encoded by the tyrosinase gene (12, 33), our findings suggest that a single gene product, tyrosinase, contains epitopes recognized by both $CD4^+$ and $CD8^+$ T lymphocytes. Further, that our $CD4^+$ TILs react to lysates of melanomas as well as normal melanocytes suggests that the recognized epitope is nonmutated. Some melanoma lines which are not recognized by $CD4^+$ TIL 1088 clones nevertheless express tyrosinase mRNA detectable by Northern blotting. This apparent lack of T-cell recognition may reflect posttranscriptional events (34), allelic or mutational diversity of the tyrosinase gene affecting peptide structure, or the sensitivity threshold of our assay system. However, we cannot exclude the possibility that a by-product of a chemical reaction catalyzed by tyrosinase, rather than the tyrosinase molecule itself, may be the source of specific antigen for $CD4^+$ TIL 1088. Though formally possible, this latter mechanism is unlikely, since none of the known reaction products of tyrosinase are peptidic in nature.

Tyrosinase regulates the conversion of tyrosine to the complex melanin pigment polymer. Tyrosinase gene expression is tissue specific, normally restricted to cells of melanocytic lineage. The recognition of its protein product by T cells infiltrating a metastatic melanoma lesion suggests a breach of immunologic tolerance. This breach has presumably occurred within the tumor milieu and perhaps has been facilitated by *ex vivo* culture conditions. Such immunoreactivity, while potentially toxic to normal tissues, might be manipulated to therapeutic advantage in the setting of cancer treatment. Proteins such as tyrosinase, containing epitopes recognized by both $CD4^+$ and $CD8^+$ T cells, may prove to be

Table 1. $CD4^+$ T-cell clones recognize a shared antigen expressed on autologous and allogeneic melanomas and normal melanocytes

	GM-CSF secretion,† pg/ml per 24 hr (SI‡)			
Stimulator*	NT9	1D6	1B7	1E7
1088-mel	14,581 (504)	27,992 (384)	21,497 (169)	13,767 (101)
1011-mel	13,626 (471)	18,937 (260)	15,882 (125)	9,292 (68)
553-mel	2,856 (99)	11,067 (153)	9,357 (74)	4,777 (36)
697-mel	2,451 (86)	8,887 (123)	6,497 (52)	3,382 (26)
526-mel	168 (7)	2,117 (30)	1,400 (12)	196 (2)
624-mel	44 (3)	1,537 (22)	1,187 (10)	98 (2)
1087-mel	37 (2)	806 (12)	675 (6)	53 (1)
1290B-mel	<8 (1)	168 (3)	120 (2)	33 (1)
938-mel	<8 (1)	50 (2)	32 (1)	<8 (1)
1102-mel	<8 (1)	<8 (1)	<8 (1)	<8 (1)
586-mel	<8 (1)	55 (2)	18 (1)	<8 (1)
537-mel	<8 (1)	34 (1)	<8 (1)	<8 (1)
501A-mel	<8 (1)	24 (1)	<8 (1)	<8 (1)
677-mel	<8 (1)	22 (1)	<8 (1)	<8 (1)
CY13	<8 (1)	<8 (1)	<8 (1)	<8 (1)
1088-EBV	<8 (1)	<8 (1)	<8 (1)	<8 (1)
FM 902	5,831 (202)	12,667 (175)	10,517 (83)	6,557 (49)
FM 906	11,376 (393)	17,767 (244)	16,307 (128)	NT
FM 907	9,401 (325)	17,382 (239)	15,202 (120)	NT

NT, not tested.

*Cell lysates of cultured lines listed were incubated with 1088-EBV cells for 20 hr. In the same experiment, $CD8^+$ TIL 1088 failed to react with these stimulators (data not shown).

†Net secretion = (secretion by TILs with EBV-B plus cell lysate) − (secretion by TILs with EBV-B). All cells were at 4×10^5 per ml in microtiter plates.

‡Stimulation index. GM-CSF secretion by TIL with EBV-B in the absence of cell lysate ranged from 29 to 138 pg/ml.

Table 2. $CD4^+$ T-cell clones recognize a product of the tyrosinase gene

Stimulator*	Transfected gene†	GM-CSF secretion,‡ pg/ml per 24 hr							
		$CD4^+$ bulk TILs	$CD8^+$ bulk TILs	NT9	1D6	1B7	1E7	2G2	2F9
1088-mel	None	6169	56	32,904	58,515	43,550	17,563	11,702	38,295
COS-7	Tyrosinase	123	<8	2,804	7,015	9,150	2,163	12,202	39,995
COS-7	Tyrosinase-(1–3)	12	<8	<8	14	<8	<8	<8	<8
COS-7	Tyrosinase-related gp75	<8	<8	<8	<8	46	<8	<8	37
COS-7	β-Galactosidase	8	<8	<8	16	36	16	<8	<8
COS-7	HLA-A2.1	8	<8	<8	22	<8	<8	<8	12

*Cell lysates were cocultivated with 1088-EBV for 20 hr.
†Genes were expressed in the plasmid vector pcDNA3 except for the tyrosinase-related gp75 gene, which was in pCEV27.
‡Net secretion is defined in footnote † to Table 1. Values for TIL with EBV-B ranged from 10 to 237 pg/ml.

optimal immunogens in vaccination strategies which will simultaneously recruit the helper and cytotoxic arms of the anti-tumor immune response.

The importance of $CD4^+$ T cells in the priming and effector phases of the anti-tumor immune response has been shown in animal models (35–38). Although experimental immunization strategies for patients with melanoma and other cancers currently emphasize shared class I-restricted tumor antigens recognized by $CD8^+$ T cells, immunization against both class I- and class II-restricted epitopes may increase the effectiveness of these approaches. Previous reports suggesting the presence of shared MHC class II-restricted melanoma antigens have measured direct T-cell–tumor cell interactions and were thus limited by the extensive polymorphism of human class II molecules and reagent availability (39–42). However, as demonstrated here, a bioassay which utilizes one APC for multiple sources of antigen will permit rapid screening and detection of shared CD4-recognized tumor antigens. This detection system, coupled with chromatographic protein separation techniques to isolate different components of antigen-containing whole cell lysates, may be ideal for cloning additional relevant genes.

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