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Expression of a "Self-"Antigen by Human Tumor Cells Enhances Tumor Antigen-specific CD4+ T-Cell Function

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Abstract

$CD4^+$ T cells can recognize "self" tumor antigens, but the impact of tumor cell expression of self-antigens on $CD4^+$ T-cell function in humans is unknown. Here, we identify a new epitope (ISPNSVFSQWRVVCDSLEDYD) derived from tyrosinase-related protein-1 (TRP-1) using a predictive algorithm and mice transgenic for a chimeric HLA-DRB1*0401 molecule. We then compared the functions of TRP-1-epitope-specific, $CD4^+$ T-cell responses in normal healthy individuals to those found in patients with metastatic malignant melanoma. Surprisingly, we found that tumor-bearing patients had significantly higher levels of TRP-1-specific, $CD4^+$ T-cell function than healthy volunteers as measured *ex vivo*. Thus, the net effect of "self" antigen expression by tumor cells was the enhancement of tumor antigen-specific $CD4^+$ T-cell function, rather than immunosuppression. These findings indicate that antigens expressed by malignant melanoma cells can partially activate $CD4^+$ T lymphocytes.

Introduction

Tumor-specific T cells frequently recognize nonmutated "self" antigens that are expressed by both normal cells and by growing tumor cells. Although much research in T-cell-based immunotherapy has focused on direct targeting of tumor cells using $CD8^+$ T cells, accumulating evidence suggests that $CD4^+$ T cells also play an important role in orchestrating the host immune response against cancer (1). $CD4^+$ T cells function as "helper" cells (*i.e.,* cells that are capable of augmenting the functions of other immune cells, such as dendritic cells, B cells and $CD8^+$ T cells; Refs. 2, 3). They also can directly recognize tumor cells (4). Tumor cells may, for a variety of reasons, fail to display the target antigens, thereby leading to their "escape" (5, 6). Those T cells that are specific for self antigens expressed on normal tissues are potentially susceptible to the mechanisms of self-tolerance that can alter, suppress, or abrogate their functions. These mechanisms can result in T-cell anergy or deletion (7). Interactions of T cells with tumor cells expressing cognate antigens may also influence their activities. Many workers have asserted that tumor cells tolerize host T cells through deletional or nondeletional mechanisms (8–11), although this reigning paradigm has recently been called into question (12). The relative impact of antigen expression by normal and tumor cells on T-cell functionality remains unelucidated. In this report, we studied how tumor cell expression of a self antigen affects $CD4^+$ T-cell function *ex vivo*. We analyzed $CD4^+$ T cells from melanoma patients specific for the melanocyte differentiation antigen TRP-1[2] and compared them to a matched group of normal donors using a newly identified DR4-restricted epitope. Unexpectedly, we found that expression of a self antigen by a growing tumor deposit does not suppress or tolerize self-reactive T cells, but rather enhances the function, as assessed *ex vivo*, of these autoreactive cells.

Materials and Methods

Animals, Peptides, and Recombinant Protein. Six to 10-week-old murine class II-deficient, DR4-IE transgenic mice have been described previously (13). Four DR4-IE transgenic mice were vaccinated three times at 2-week intervals using the Accell gene gun (Agracetus, Middleton, WI) with plasmid DNA encoding the full-length *hTRP-1* sequence within pcDNA3.1 (Invitrogen, Carlsbad, CA). Mouse cells specific for HLA-DR4-associated TRP-1 were grown with a previously published method (13). Peptides were synthesized using a solid-phase method based on fluorenylmethoxycarbonyl (Fmoc) chemistry on a multiple peptide synthesizer (Model AMS 422; Gilson Co., Inc., Worthington, OH). The molecular masses of peptides were verified by laser desorption mass spectrometry (Bio-Synthesis, Inc., Lewisville, TX). A truncated fragment corresponding to amino acids 25–477 from full-length (537 amino acids) TRP-1 (Gene Bank, accession no. AF001295) was amplified by PCR using specific primers (forward: AGCATATGACCATGGGAAGATCTGCTCCTAAACTCCT; reverse: AAGCTAGCTCATATTCTTTCTTCAGC). *hTRP-1*-transfected BL21(DE3) *Escherichia coli* (Novagen) were grown to A_{600} = 0.6, then induced with 1 μg/ml isopropyl-thiogalactoside for 3 h. Inclusion bodies were isolated and lysed in 6 M urea. Preparative scale SDS-PAGE was performed using a Prep cell (Bio-Rad, Hercules, CA) electrophoresis chamber, and fractions were dialyzed against PBS. Purity was >70% based on SDS-PAGE with Colloidal Blue (Novex, San Diego, CA) staining. Recombinant full-length ovalbumin protein (Sigma, St. Louis, MO) was used as a control protein.

HLA Typing of PBMCs and Testing of Human T-Cell Lines. All of the studies were done in accordance with National Cancer Institute-Institutional Review Board-approved protocols. The NIH-HLA Laboratory performed all of the genotyping. HLA-DRB1*0401+ PBMCs were obtained from patients before any form of immunotherapy. Seven patients with established metastatic malignant melanoma and four healthy individuals who did not have any history of melanoma were studied. Human T cells were grown as described previously (13). To evaluate human T-cell lines or clones, EBV-B cells or activated B-cells were incubated for 5–18 h with peptide, protein or freeze-thaw lysates of tumor cells (10^5 cell equivalents per well). Whole tumor cells or pulsed antigen presenting cell (10^5 cells/well) were cocultured with 10^5 T cells/well in U-bottomed 96-well plates for 24 h. Culture supernatants were assayed for cytokine, using commercially available ELISA kits (Endogen, Woburn, MA). In blocking experiments, mAbs, used at 50 μg/ml, included HB55 (anti-HLA-DR; IgG2a) and W6/32 (anti-HLA-A, B, C; IgG2a). PBMCs from patients with melanoma or healthy volunteers were cultured at 2×10^5 cells per well in flat-bottomed 96-well plates in 200 μl of CM containing hTRP-$1_{277-297}$ at 50 μM (48 wells per sample) or $HA_{306-318}$ at 50 μM (12 wells per sample) and plates were angled at 10 degrees. On day 7, T cells were restimulated with

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[2] The abbreviations used are: TRP-1, tyrosinase-related protein-1; CIITA, MHC class II transactivator; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; CM, complete medium; IL, interleukin.

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autologous, peptide-pulsed (50 μM for 5 h), irradiated (3000 rads) PBMCs at 2×10^5 cells per well in CM. On day 8, 100 μl of CM were removed from each well, and 100 μl of CM containing IL-2 at 100 CU/ml (final concentration of 50 CU/ml) were then added and plates were laid flat. On day 21, all wells from the original parental 96-well plate were tested for specific peptide reactivity using DR4$^+$ EBV-B cells (1088 EBV-B) pulsed with either $HA_{306-318}$ or hTRP-$1_{277-297}$, depending on the type of initial stimulation. To generate CD40-activated B cells, PBMCs were stimulated with 500 ng/ml soluble CD40 ligand (Immunex Corp, Seattle, WA) and 200 units/ml IL-4 (Peprotech, Rocky Hill, NJ) in supplemented Iscove's medium. After 8–12 days, 60–95% of the cells expressed the B-cell marker CD19. When designated, tumor cell line 526 Mel was induced to express MHC class II molecules using 500 units/ml of IFN-γ for 48 h (13). Other tumor cell lines used to test direct recognition were treated with CIITA retroviral transduction (construct kindly provided by Jeremy Boss, Emory University, Atlanta, GA).

Results and Discussion

Identification of an HLA-DRB1*0401-Restricted Epitope from *hTRP-1*. We chose to study *TRP-1* because of its demonstrable expression in normal melanocytes and its high and consistent level of expression in metastatic melanoma lesions. In addition, mouse data using a recombinant virus encoding TRP-1 has linked a CD4$^+$ T cell-mediated induction of vitiligo and tumor regression (14), although the phenomena can be uncoupled (15). Using expressed sequence tag (EST) and serial analysis of gene expression (SAGE) databases, we compared *TRP-1* gene expression in 6 total libraries of metastatic melanoma cells containing 33,623 sequences with 93 total libraries containing 402,493 sequences derived from normal tissues (excluding dermis). This analysis revealed a highly statistically significant difference in expression of *TRP-1* in melanoma cells ($P = 0.00173$).

To enable experiments exploring the frequency and functionality of human TRP-1-specific CD4$^+$ T cells, we first focused our efforts on identifying an epitope restricted by HLA-DRB1*0401, the most commonly expressed MHC class II allele in our patient population (13). We used a computer-based algorithm for performing HLA-DRB1*0401-allele-specific epitope forecasting (http://surgery.nci.nih.gov), which we have previously developed to predict the peptides capable of forming stable complexes with HLA-DRB1*0401 molecules (13). Twenty-four candidate peptide epitopes forecasted by the prediction algorithm (Table 1) were used to stimulate splenocyte cultures from mice transgenic for a chimeric HLA-DRB1*0401 molecule (called *DR4-IE* mice) that had been immunized by gene gun vaccination with a plasmid encoding the full-length *hTRP-1* gene. Individual mouse *ex vivo* splenocyte cultures were evaluated for specific IFN-γ secretion using human HLA-DRB1*0401$^+$ EBV-B cells (697 EBV-B) pulsed with control peptide $HA_{306-318}$ (previously shown to bind to DR-4; Ref. 13) or the specific peptide in question. Screening of peptides predicted to bind best revealed that only one peptide (ranked 2 of 57 overall, core amino acid positions 283–291) induced significant reactivity (Table 1). T cells from that culture were verified to be predominantly CD4$^+$ (86%) by cytoflourometric analysis (not shown).

Table 1 *Identification of an HLA-DRB1*0401-restricted epitope from hTRP-1*

Rank	AV[a]	Position	xx (Core sequence) xx	697 EBV-B + HA[b]	697 EBV-B + Peptide[b]
1	4.9	410	DA (VFDEWLRRY) NA	3.0	2.8
2	12.3	283	SV (FSQWRVVCD) SL	4.4	84.3
3	18.2	190	FV (WTHYYSVKK) TF	3.1	5.1
4	23.4	226	HR (YHLLRLEKD) MQ	3.7	2.6
5	35.8	36	EA (LRSGMCCPD) LS	2.5	2.3
6	36.0	223	LT (WHRYHLLRL) EK	1.4	1.6
7	43.5	319	GN (VARPMVQRL) PE	6.9	2.9
8	53.2	464	YT (YEIQWPSRE) FS	2.4	2.3
9	63.8	293	DS (LEDYDTLGT) LC	1.3	1.4
10	89.8	213	VD (FSHEGPAFL) TW	1.9	1.8
11	111.8	257	KN (VCDICTDDL) MG	1.9	1.9
12	125.1	19	LL (FQQARAQFP) RQ	3.3	2.7
13	127.6	117	PG (WRGAACDQR) VL	2.7	2.6
14	150.6	33	AT (VEALRSGMC) CP	2.9	2.3
15	173.4	288	WR (VVCDSLEDY) DT	1.9	1.5
16	184.3	196	YS (VKKTFLGVG) QE	3.0	2.3
17	198.9	17	PL (LLFQQARAQ) FP	4.2	2.7
18	200.6	402	FV (LLHTFTDAV) FD	6.8	5.9
19	227.8	26	AQ (FPRQCATVE) AL	0.6	0.5
20	234.9	145	HF (VRALDMAKR) TT	2.0	1.9
21	259.1	48	SP (VSGPGTDRC) GS	1.5	1.4
22	263.5	379	HN (LAHLFLNGT) GG	1.2	1.1
23	271.0	179	PQ (FENISIYNY) FV	1.6	1.8
24	281.7	481	IA (IAVVGALLL) VA	4.1	3.9

[a] AV, algorithm value (binding prediction).
[b] INF-γ [ng/ml].

Table 2 *Identification of an HLA-DRB1*0401-restricted epitope from hTRP-1*

Position	Sequence	IFN-γ [pg/ml]
277–297	ISPNSVFSQWRVVCDSLEDYD	40,069
277–296	ISPNSVFSQWRVVCDSLEDY	1,363
278–297	SPNSVFSQWRVVCDSLEDYD	37,483
278–296	SPNSVFSQWRVVCDSLEDY	1,590
278–295	SPNSVFSQWRVVCDSLED	12
279–296	PNSVFSQWRVVCDSLEDY	2,652
279–295	PNSVFSQWRVVCDSLED	16
m277–297	ISPNSVFSQWRVVCESLEEYD[a]	1,903

[a] Amino acids that are different between the human and murine peptide are underlined.

The initial screening of candidate peptides had resulted in the tentative identification of a new DR4-restricted epitope (TRP-$1_{283-291}$). Previous work (13) had indicated that peptides identified with this method could be optimized for length. A representative optimization experiment using one line, TD 6/11, is shown in Table 2, which revealed a 21-residue peptide (hTRP-$1_{277-297}$; ISPNSVFSQWRVVCDSLEDYD) that conferred significantly enhanced recognition when pulsed onto target cells. Although the NH_2 terminus of the minimal peptide determinant was not definitively characterized, truncation of the 21-mer from the COOH terminus resulted in a significant reduction of T-cell recognition yielding a minimal determinant of 17 amino acids between positions 279 and 296. We observed some cross-reactivity to the murine version of the peptide, mTRP-$1_{277-297}$ (ISPNSVFSQWRVVCESLEEYD), which differs from its human counterpart at positions 291 and 295, both D to E (conservative amino acid substitutions). Identification of this HLA-DR4-restricted, TRP-1 epitope enabled us to further probe the functionality of human CD4$^+$ T cells.

Human T Cells Specific for TRP-$1_{277-297}$ Recognize Antigen-Pulsed Targets and Human Melanoma Cells. The cloning of cells from long-term cultures stimulated with TRP-$1_{277-297}$ peptide enabled the generation of TRP-1-reactive CD4$^+$ T cell-clones. Cloning by limiting dilution of one line designated T/11 resulted in a clone called TD Clone-2, characterized by flow cytometry and Vβ analysis to be a monoclonal homogeneous population of CD4$^+$Vβ5$^+$ cells (not shown). TD Clone-2 was capable of recognizing intact TRP-1 protein pulsed onto DR4$^+$ EBV-B cells, or TRP-1-containing tumor cell lysates pulsed onto DR4$^+$ CD40-activated B cells (Fig. 1*A*). In the same assay, peptide recognition by TD Clone-2 could be distinguished from background at nanomolar concentrations when pulsed onto DR4$^+$ EBV B cells (Fig. 1*B*). These data indicated that high-avidity human CD4$^+$ T cells with specificity for TRP-1 could be grown from the available repertoire.

To determine whether the TRP-1 epitope that we identified could be processed and presented by human melanoma cells in the context of HLA class II antigens, we tested the CD4$^+$ T-cell TD Clone-2 for recognition of a panel of melanoma cells (Fig. 2). CD4$^+$ T cells are shown to recognize DR4$^+$ human melanoma cells (624 and 1088 Mel,

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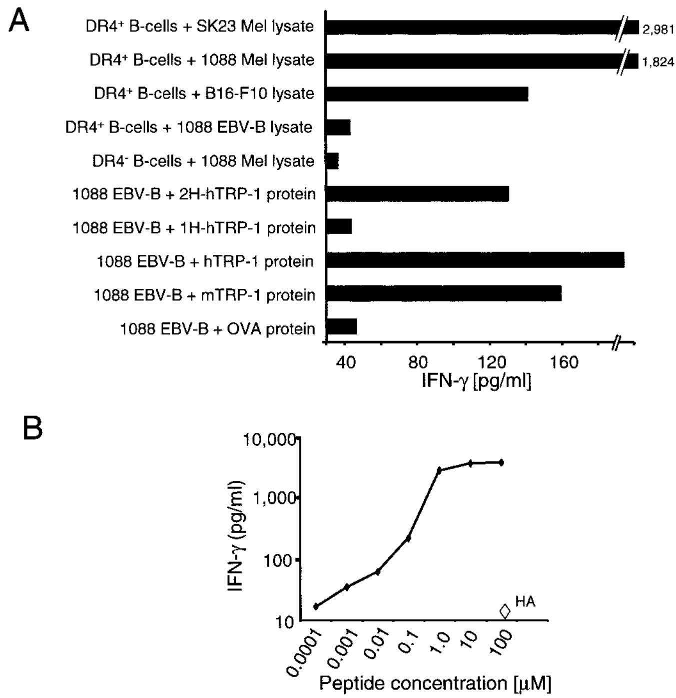

Fig. 1. Tumor-associated TRP-1 is processed and presented for MHC class II-restricted recognition by CD4^{+} T cells. *A*, tumor lysates and recombinant TRP-1 protein are processed and presented for recognition by an hTRP-$1_{277–297}$-specific T-cell clone. Limiting dilution was used to generate the TRP-1-specific TD Clone-2 and clonality was verified by β chain analysis (not shown). TD Clone-2 specifically recognized recombinant hTRP-1 protein, mTRP-1 protein, and a distal fragment of hTRP-1 protein (called 2H) corresponding to amino acids 266–477 pulsed onto DR4^{+} (1088 EBV-B) EBV-B cells as well as melanoma cell lysates 1088 and SK23 Mel and murine melanoma cell lysate B16-F10 (containing mTRP-$1_{277–297}$) pulsed onto DR4^{+} CD40-activated B-cells. No reactivity was observed to 1088 Mel lysate pulsed on DR4^{-} CD40-B-cells and 1088 EBV-B lysate pulsed onto CD40-DR4^{+} B-cells and there was no significant recognition of the control peptide ($HA_{306–318}$), control protein (OVA), and 1H-hTRP-1 protein (amino acids 25–265). *B*, TD Clone-2 recognition of titrated doses of hTRP-$1_{277–297}$ pulsed onto DR4^{+} (1088 EBV-B) EBV-B cells.

induced to express class II antigen with CIITA and 526 Mel with IFN-γ), but not a DR4^{-} and A2^{-} melanoma cell (888 Mel CIITA). In addition, both peptide and tumor reactivity are shown to be completely and specifically blocked by a mAb directed against HLA-DR (HB55). A human CD8^{+} T-cell line (M18) recognizing MART-$1_{27–35}$ in the context of *HLA-A*0201*, was used to test for nonspecific or deleterious effects of the mAbs used on target cell interaction and was specifically blocked by a mAb directed against MHC class I (W6/32). These results demonstrated that the hTRP-$1_{277–297}$ epitope was indeed processed and presented by human melanoma cells.

We were able to generate TRP-$1_{277–297}$ peptide-specific human CD4^{+} T-cell lines and clones from multiple non-tumor-bearing individuals. However, unlike growing CD4^{+} T cells from patients with metastatic malignant melanoma, the process was laborious and required long-term cultures and multiple (three or more) *ex vivo* restimulations. On the basis of these initial, albeit anecdotal observations, we set out to study more systematically whether human CD4^{+} T-cell cultures could more easily be obtained from patients with established metastatic malignant melanoma than from healthy individuals who did not have melanoma. This study promised to shed light on the impact of the tumor-bearing state on the functionality, as defined by the release of IFN-γ, of human CD4^{+} T cells specific for this epitope.

Impact of the Tumor-bearing State on Tumor-specific T Cells. To explore whether tumor antigen-specific T-cell responses were altered by the expression of the HLA-DRB1*0401-restricted hTRP-$1_{277–297}$ epitope by tumor cells, we analyzed seven DR4-matched patients who had measurable, established malignant melanoma and four healthy individuals who had undergone an identical leukapheresis procedure at the National Cancer Institute and who did not have any medical history of malignant melanoma (Fig. 3). As a positive control, we used the HLA-DRB1*0401-restricted epitope from influenza A hemagglutinin ($HA_{306–318}$), to which most individuals have been exposed. To overcome problems associated with the objective quantification of low-frequency CD4^{+} T cells from human peripheral

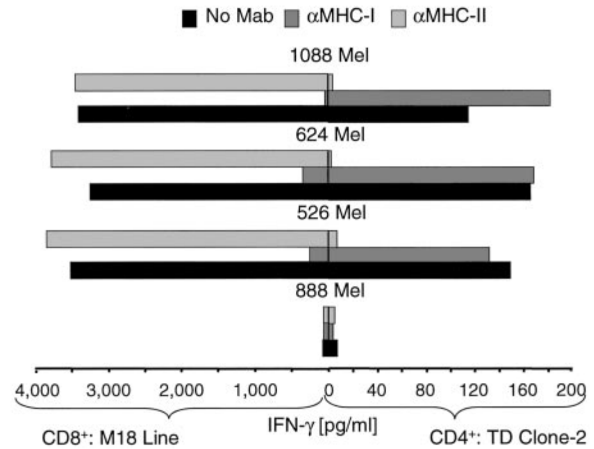

Fig. 2. TRP-1-specific human CD4^{+} T cells recognize TRP-1 in an MHC-class II-restricted fashion on human melanoma cells. *Left panel*, MHC class I-restricted recognition of tumor targets by a control human MART-$1_{27–35}$-specific, *HLA-A*0201*-restricted CD8 $^{+}$ T-cell line (M18) of CIITA-transfected 1088 Mel, 624 Mel and IFN-γ-treated 526 Mel (all DR4^{+} and A2^{+}) but not CIITA-transfected 888 Mel DR4^{-} and A2^{-} melanoma cells (888 Mel). *Right panel*, MHC class II-restricted recognition of DR4^{+} but not the DR^{-} melanoma line in the same assay by TRP-1-specific TD Clone-2. MHC-restriction of the recognition was determined by blocking with anti-MHC-I or anti-MHC-II blocking antibodies as described in "Materials and Methods."

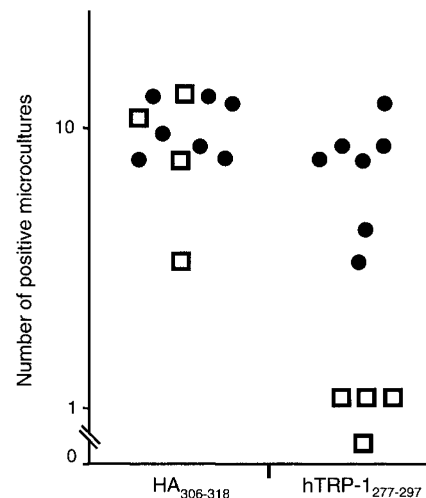

Fig. 3. Antigen expressed by tumor primes low-avidity T cells. hTRP-$1_{277–297}$-specific T cells are induced from melanoma patients after short-term (two *in vitro* stimulations) culture. A comparative *in vitro* sensitization assay was performed with PBMCs from four DR4^{+} healthy individuals (□) and seven DR4^{+} melanoma patients (●) to $HA_{306–318}$ and hTRP-$1_{277–297}$. Individual wells were tested for specific IFN-γ release measured by ELISA using peptide pulsed (50 μM) DR4^{+} EBV-B cells (1088 EBV-B) as stimulators for 24 h. The results are shown as the number of specifically reactive wells (12 microwells for the $HA_{306–318}$ stimulation and 48 microwells for the hTRP-$1_{277–297}$ stimulation). The median number of positively reactive wells (defined as IFN-γ secretion that was more than twice that of the control peptide) was statistically analyzed using an exact rank Wilcoxon rank sum test as follows: $P = 0.63$, not significant for melanoma patients *versus* healthy individuals for $HA_{306–318}$; $P = 0.003$, highly significant for melanoma patients *versus* healthy individuals for hTRP-$1_{277–297}$. The experiment was independently repeated with similar results.

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blood, we used a microculture technique previously developed in our laboratory (13).

Of the 12 microcultures tested on each subject, healthy individuals were found to have a similar number of growth-positive, antigen-specific wells as melanoma patients to $HA_{306-318}$ (Fig. 3, median 8.5 *versus* 9, respectively, with a nonsignificant P of 0.63). In sharp contrast, T-cell reactivity that was specific for the "self" epitope, hTRP-$1_{277-297}$ was poor or absent in short-term cultures derived from healthy individuals. All seven of the melanoma patients that were tested responded to this epitope. Statistical analysis revealed that the difference observed between patients with and without metastatic malignant melanoma were highly significant (Fig. 3, $P = 0.003$) and were independently reproduced with similar results. These findings indicated that patients with metastatic melanoma had elevated $CD4^+$ T-cell functionality that was specific for the hTRP-$1_{277-297}$ epitope.

Thus, we report here that the expression of a normal, nonmutated self-antigen by tumor tissue increases antigen-specific $CD4^+$ T-cell functionality *ex vivo*. Many workers have asserted that the tumor-bearing state only exacerbates the immunological unresponsiveness that was a result of the expression of "self"-antigens in normal tissues (8–11). In contrast, others claim that tumors, including melanoma cells, release immunosuppressive cytokines or express death receptor ligands such as FasL, although aspects of these reports have been disputed (16).

Our results are not necessarily at odds with findings that tumors express soluble or membrane-bound factors that negatively impact on T cells. Our results indicate, however, that the net effect of the expression of a class II-restricted self-antigen can be an increase in tumor antigen-specific $CD4^+$ T-cell function. The generalizability of these findings is unknown. Antigen-specific induction of $CD4^+$ T-cell function may be limited expression of this particular antigen in cases of metastatic malignant melanoma. These findings are consistent with previously reported elevations in $CD8^+$ T-cell function that were specific for MART-1, another nonmutated melanoma/melanocyte differentiation antigen (17). Tumor priming and expansion of T cells specific for some antigens may be the mechanism whereby a nonspecific immunotherapy such as IL-2 can have antitumor effects in humans.

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