

Recognition of a Shared Human Prostate Cancer-Associated Antigen by Nonclassical MHC-Restricted CD8⁺ T Cells

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To identify prostate cancer-associated Ags, tumor-reactive T lymphocytes were generated using iterative stimulations of PBMC from a prostate cancer patient with an autologous IFN- γ -treated carcinoma cell line in the presence of IL-2. A CD8⁺ T cell line and TCR $\alpha\beta$ ⁺ T cell clone were isolated that secreted IFN- γ and TNF- α in response to autologous prostate cancer cells but not to autologous fibroblasts or lymphoblastoid cells. However, these T cells recognized several normal and malignant prostate epithelial cell lines without evidence of shared classical HLA molecules. The T cell line and clone also recognized colon cancers, but not melanomas, sarcomas, or lymphomas, suggesting recognition of a shared epithelium-associated Ag presented by nonclassical MHC or MHC-like molecules. Although Ag recognition by T cells was inhibited by mAb against CD8 and the TCR complex (anti-TCR $\alpha\beta$, CD3, V β 12), it was not inhibited by mAb directed against MHC class Ia or MHC class II molecules. Neither target expression of CD1 molecules nor HLA-G correlated with T cell recognition, but β_2 -microglobulin expression was essential. Ag expression was diminished by brefeldin A, lactacystin, and cycloheximide, but not by chloroquine, consistent with an endogenous/cytosolic Ag processed through the classical class I pathway. These results suggest that prostate cancer and colon cancer cells can process and present a shared peptidic Ag to TCR $\alpha\beta$ ⁺ T cells via a nonclassical MHC I-like molecule yet to be defined. *The Journal of Immunology*, 1999, 163: 6330–6337.

The antitumor immune response, similar to immunity against microbial agents, alloantigens, and autoantigens, is a complex and finely orchestrated series of events involving different cell types. In murine models and cancer patients, the existence of CD8⁺ CTL that are capable of direct tumor killing upon recognition of MHC class I-bound, tumor-associated peptide Ags is well documented (1, 2). The emphasis in the current literature on MHC class I-restricted antitumor responses has been further reinforced by the results of clinical immunization protocols with MHC class I-restricted peptides from melanoma-associated Ags demonstrating tumor regressions (3–5). However, searching for a better understanding and improvement of cytotoxic antitumor responses, recent works have pointed to the central role of MHC class II-restricted CD4⁺ T cells in regulating Ag-specific immune responses (6, 7). Experimental studies in mouse models have established that long lasting antitumor immunity requires specific signals emanating from so-called CD4⁺ Th cells, which then activate professional APC through CD40 and by secreting costimulatory cytokines (7). A few studies have implicated nonclassical T cells in antitumor immunity, although their general role is not well delineated (8).

Most studies of human antitumor immunity have been conducted in melanoma. The isolation of immune effectors from tumors of other histologic origins remains only sparsely described in the literature, in part due to the apparently weaker inherent immu-

nogenicity of these tumors, but importantly also due to the difficulty of obtaining sufficient biological materials to conduct in-depth immune studies. This is the case for prostate carcinoma, in which primary operative specimens are commonly small and, until recently, cultured tumor cell lines were scarce. However, prostate cancer is the most commonly diagnosed cancer in men in the United States, with 184,500 new cases and 39,200 deaths anticipated this year (9). Disseminated prostate cancer remains virtually incurable using standard treatment modalities. Immunotherapy offers an alternative form of treatment, which has already been successful for some patients with metastatic melanoma or kidney cancer. To better characterize the human immune response to prostate cancer, we initially focused our efforts on banking lymphocytes from prostate cancer patients derived from PBMC or surgically resected draining lymph nodes and on generating autologous immortal cell lines from fresh primary prostate cancer specimens (10). This unique set of reagents has now been used to test the ability of T lymphocytes to recognize autologous prostate cancer cells using repetitive in vitro stimulation under specialized culture conditions.

In the present work we describe the generation of prostate-reactive CD8⁺ T cells from the PBMC of a patient with localized prostate carcinoma, using iterative stimulations with an IFN- γ -treated autologous carcinoma cell line. Through the TCR- $\alpha\beta$, these CD8⁺ T cells recognize an Ag that is shared by several prostate and colon cancer cell lines and is processed through the classical MHC class I pathway but is presented by a nonclassical MHC-like molecule.

Materials and Methods

Cell lines

Prostate epithelial cell lines derived from cancerous (CPTX)³ or normal (NPTX) tissue were generated in our laboratory from fresh prostatectomy specimens and immortalized by transduction with a recombinant retrovirus

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³ Abbreviations used in this paper: CPTX, immortalized prostate carcinoma cell line; β_2m , β_2 -microglobulin; FTX, immortalized fibroblast cell line; KIR, killer inhibitory receptor; NPTX, immortalized normal prostate cell line; TIL, tumor-infiltrating lymphocytes; TPI, triosephosphate isomerase.

encoding the HPV16 E6 and E7 transforming proteins. Cell cultures were characterized genetically as previously described (10). These cell lines were maintained in keratinocyte serum-free medium (Life Technologies, Grand Island, NY) containing 25 $\mu\text{g}/\text{ml}$ bovine pituitary extract, 5 ng/ml epidermal growth factor, 2 mM L-glutamine, 10 mM HEPES buffer, antibiotics, and 5% heat-inactivated FBS (Biofluids, Rockville, MD). Immortalized fibroblast cultures and EBV-transformed B cell lines were generated and maintained in our laboratory as previously described (10).

HLA typing

HLA serotypes and DNA genotypes of fresh PBMC and cell lines were determined by the National Institutes of Health HLA Laboratory as previously described (11). The HLA type of patient 1542 is HLA-A1, A23; B50, B70; Cw2; DR β 1*1101/0301; DQ β 1*0201/0301; DR β 3*0202.

PBMC cultures and T cell clones

PBMC from prostate cancer patient 1542 were separated from a fresh leukapheresis specimen by centrifugation over Ficoll (Nycomed, Oslo, Norway). PBMC were cultured in 24-well plates at 2×10^6 cells/well in the presence of irradiated (12,000 rad) IFN- γ -treated autologous cultured prostate carcinoma cells, 1542-CP $_3$ TX (3×10^5 cells/well). Exposure of 1542-CP $_3$ TX and other prostate epithelial cell lines to IFN- γ (Biogen, Cambridge, MA; 500 U/ml for 72 h) was performed to enhance expression of cell surface MHC class I and II molecules and ICAM-1, as previously shown (10). Individual wells from 24-well plate PBMC cultures were maintained as separate cultures (macrocultures). In some instances, CD4 $^+$ T cells were depleted from cultures using immunomagnetic beads according to the manufacturer's instructions (Dyna, Oslo, Norway). Medium for macrocultures consisted of RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 mM L-glutamine, 10 mM HEPES buffer, and antibiotics. After 7 days of incubation at 37°C and 5% CO $_2$, IL-2 was added to the cultures at 150 IU/ml (Chiron, Emeryville, CA). Cultures were restimulated every 14 days with IFN- γ -treated 1542-CP $_3$ TX, in the presence of IL-2 (150 IU/ml). In addition, IL-2 was replenished every 7 days. A CD8 $^+$ T cell clone, designated W3.9, was generated from a macroculture, designated W3, by limiting dilution in microtiter plates in the presence of irradiated allogeneic PBMC (5×10^4 /well), irradiated allogeneic EBV-B cells (1×10^4 /well), anti-CD3 (OKT3, American Type Culture Collection, Manassas, VA; 30 ng/ml), and rIL-2 (100 IU/ml). The gp100-specific clone, B1F11, is HLA-A2 restricted and reacts toward the epitope gp100 $_{209-217}$ as well as the 1088-mel melanoma cell line (HLA-A2 $^+$, gp100 $^+$). The CD4 $^+$ tumor-infiltrating lymphocyte (TIL) line designated TIL 1558 is HLA-DR1 restricted, specific for a mutated triosephosphate isomerase (TPI) (12), and recognizes the 1558-mel melanoma cell line (DR1 $^+$, TPI $^+$).

Assessment of T cell reactivity

To evaluate T cell recognition of malignant or benign cells, T cells (1×10^5 cells/well) were cocultured overnight with irradiated target cells (1×10^5 cells/well) in flat-bottom 96-well plates as previously described (13). For mAb blocking experiments, targets or effectors were preincubated for 30 min at room temperature with preservative-free mAb, after which the assay was completed. T cell reactivity was measured by the specific secretion of cytokines using commercially available ELISA kits for IFN- γ , TNF- α , IL-4, and IL-10 (R&D Systems, Minneapolis, MN). The mAbs directed against HLA determinants included W6/32 (against HLA-A, -B, -C), IVA12 (against HLA-DR, -DP, -DQ), and L243 (against HLA-DR) and were used at a final concentration of 20 $\mu\text{g}/\text{ml}$. Abs against TCR $\alpha\beta$ (WT31), CD8, and CD4 were purchased from Becton Dickinson (San Jose, CA) and were used at 1–10 $\mu\text{g}/\text{ml}$. The mAb specific for CD1d (CD1d 27.2, 42, and 51) were provided by Dr. Steven Porcelli (Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Boston, MA) and used at a concentration of 50 $\mu\text{g}/\text{ml}$. The Ab 1H1 against CD1d was purchased from PharMingen (San Diego, CA).

Flow cytometry

T lymphocytes were phenotyped using anti-CD4, anti-CD8, anti-CD56, anti-CD16, anti-CD57, and anti-TCR mAb (all from Becton Dickinson). Furthermore, we used anti-p58 (killer inhibitory receptor; Immunotech, Seattle, WA), anti-CD94 (NK inhibitory receptor; Immunotech), and anti-V β 12 mAb (Endogen, Woburn, MA) to characterize T cells. When necessary, FITC-conjugated goat anti-mouse IgG F(ab) $'_2$ (Roche Molecular Biochemicals, Indianapolis, IN) was used for counterstaining.

T cell receptor analysis

RNA was extracted from 5×10^6 T cells using Trizol reagent according to the manufacturer's procedure (Life Technologies). First-strand cDNA was synthesized (Superscript Preamplification System, Life Technologies) followed by PCR amplification using V β subfamily-specific forward primers combined with a C β reverse primer from the constant region of the TCR as previously described (14). The positive control used the constant region primers C β F and C β R. Negative controls used water instead of the forward primer in the PCR reaction. RT-PCR products were resolved on a 1% agarose gel. DNA sequencing of the CDR3 region of the TCR β -chain was performed as previously described (12). Briefly, PCR products were ligated into the plasmid pCRII (Invitrogen, San Diego, CA), and recombinant clones were sequenced using a V β 12S2 subfamily-specific primer (CA GACTGAGAACCACCGC) by cycle sequencing with the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer/ABI, Foster City, CA).

HLA-G PCR

Total RNA was prepared from cultured cell lines using Trizol reagent. First-strand cDNA was synthesized (Superscript Preamplification System, Life Technologies) followed by PCR amplification of HLA-G using the primers G.257 (+) and G.1225 (–) as previously described (15). As a positive control for amplification, we simultaneously amplified MHC class I cDNA using the primers class I (+) and class I (–) as previously described (15).

Inhibition of Ag processing

In some experiments, tumor cells in suspension were treated with chloroquine (Sigma Aldrich, Milwaukee, WI), cycloheximide (Sigma Aldrich), brefeldin A (Calbiochem, San Diego, CA), or lactacystin (Calbiochem) before use as targets in cytokine secretion assays to inhibit specific events in MHC class I or class II processing pathways. Cells were preincubated for 1 h with chloroquine (100 μM), brefeldin A (10 $\mu\text{g}/\text{ml}$), or lactacystin (25 $\mu\text{g}/\text{ml}$) or for 4 h with cycloheximide (5 $\mu\text{g}/\text{ml}$) in PBS and then cultured with reduced concentrations of inhibitors overnight. After treatment, cells were washed and fixed with 0.5% paraformaldehyde (Sigma Aldrich). Tumor cells were subsequently washed three times in PBS and incubated with T cells in the functional assay.

β_2 -Microglobulin (β_2m) gene replacement study

A recombinant vaccinia virus encoding β_2m (16) was used to infect target cells at a multiplicity of infection of 10. After a 3-h infection, cells were washed and cultured overnight in complete medium at 37°C, then harvested the following day for use in assays.

Results

Establishment and characterization of a prostate cancer-reactive T cell line

Eight separate macrocultures of PBMC from patient 1542, a patient with localized high grade prostate cancer, were generated and maintained by stimulation with the IFN- γ -treated autologous carcinoma cell line (1542-CP $_3$ TX) in the presence of IL-2. One macroculture manifested tumor-specific reactivity, as measured by cytokine secretion upon cocultivation with IFN- γ -treated 1542-CP $_3$ TX, but not with autologous transformed fibroblasts (1542-FTX), which was sustained after long term cultivation. Cytokine secretion in response to 1542-CP $_3$ TX by 1542-W3 was 7-fold greater than background; when 1542-CP $_3$ TX was pretreated with IFN- γ , recognition increased to 37-fold above background (data not shown). No reactivity was observed against 1542-FTX regardless of whether it was pretreated with IFN- γ . Irrelevant reactivity against autologous 1542-EBV B cells was eliminated by depleting CD4 $^+$ T cells from the culture, which at this point was a 50/50 mixture of CD4/CD8 by flow cytometric analysis. The purified (>95%) CD8 $^+$ W3 T cell line demonstrated specific cytokine secretion in response to the autologous carcinoma, which was enhanced by pretreatment of target cells with IFN- γ , but failed to recognize IFN- γ -treated autologous fibroblasts or EBV-B cells (Fig. 1). The W3 T cell line was not cytotoxic and failed to secrete IL-4 or IL-10, while it secreted TNF- α and IFN- γ consistently when stimulated with specific Ag.

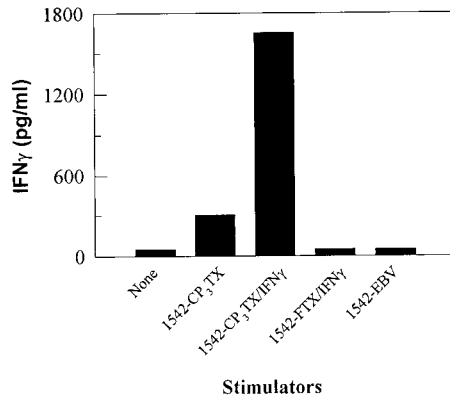


FIGURE 1. CD8⁺ T cells from macroculture 1542-W3 specifically recognize autologous prostate carcinoma cells. CD8⁺ T cells were incubated in the presence of various autologous cell lines. Target recognition by T cells was measured by IFN-γ secretion. Background IFN-γ secretion from target cells alone was <15 pg/ml. 1542-CP₃TX, prostate carcinoma cell line; 1542-FTX, fibroblast cell line; 1542-EBV, EBV-transformed B cell line. 1542-CP₃TX/IFN-γ and 1542-FTX/IFN-γ were pretreated with IFN-γ for 72 h before this assay.

To assess the mechanism by which CD8⁺ W3 T cells recognized autologous prostate cancer cells, mAb directed against MHC- or TCR-associated molecules were used to inhibit the interaction (Fig. 2). Unexpectedly, neither blocking of MHC class I nor class II molecules inhibited W3 recognition of 1542-CP₃TX. However, target recognition was almost completely abrogated by an mAb against the CD8 molecule, suggesting involvement of an MHC/TCR complex. Because recognition of autologous tumor cells by 1542-W3 could not be inhibited by mAb directed against

classical MHC class I or II molecules, we proceeded to test recognition of allogeneic prostate epithelial cell lines and tumors of other histologic types (Table I). We observed that the 1542-W3 T cell line reacted against all eight allogeneic prostate epithelial cell lines tested, including normal (NPTX) and malignant (CPTX) cell lines, to various degrees and regardless of HLA type. Autologous benign prostate epithelium (1542-NPTX) and seminal vesicle epithelium (1542-SVTX) were well recognized, while autologous fibroblasts and EBV-B cells were not. Furthermore, no IFN-γ secretion from T cells was detected in the presence of melanoma cells or the K562 erythroleukemia cell line. These results suggested that the Ag recognized by 1542-W3 T cells might be commonly expressed by normal and malignant prostate epithelial cells.

Characterization of a CD8⁺ T cell clone reactive with prostate and colon cancers

To determine more precisely the nature of the shared Ag recognized by the CD8⁺ 1542-W3 T cell line, clone 1542-W3.9 was obtained by limiting dilution. This CD8⁺, TCR αβ⁺ clone, similar to the parent line, was found to be highly reactive against 1542-CP₃TX without any recognition of the autologous 1542-EBV or 1542-FTX cell lines. It was characterized by type I cytokine secretion (IFN-γ, TNF-α), but failed to secrete IL-4 or IL-10 and was not lytic. A clonal analysis of 1542-W3.9 was conducted by performing RT-PCR with primers specific for 27 different TCR Vβ gene segments and 32 TCR Vα segments. We found a single TCR that consisted of Vα21 and Vβ12. DNA sequencing of the Vβ gene demonstrated a unique CDR3 sequence (only one combination, Vβ12S2/Jβ1S1, with the unique sequence CCCACTAGGGG). This molecular characterization confirmed the monoclonality of W3.9,

FIGURE 2. Tumor-reactive 1542-W3 T cells are not restricted by classical MHC molecules. *A*, 1542-W3 T cells were cocultured with IFN-γ-treated 1542-CP₃TX for 20 h in the absence or the presence of mAb directed against HLA-A, -B, -C (W6/32); HLA-DR (L243); CD4; or CD8. Tumor recognition was measured by IFN-γ secretion. IFN-γ background secretion for each target alone was <15 pg/ml. *B*, Autologous lymphokine-activated killer (LAK) cells were used to assess nonspecific blocking activity of mAb. *C*, As a control for mAb blocking of MHC class I-restricted T cells, the gp100-specific, HLA-A2-restricted CD8⁺ B1F11 clone was cocultured with 1088-mel. *D*, As a control for mAb blocking of MHC class II-restricted T cells, HLA-DR1-restricted CD4⁺ TIL 1558 specific for a mutated TPI were cocultured with 1558-mel. W6/32 blocked the B1F11 T cell clone, but not 1542-W3 or CD4⁺ TIL 1558. L243 and anti-CD4 blocked TIL 1558, but not 1542-W3 or the CD8⁺ B1F11 T cell clone. Anti-CD8 blocked the CD8⁺ 1542-W3 T cell line and the B1F11 T cell clone, but not TIL 1558. LAK cells were not inhibited by any mAb. This experiment was repeated twice with similar results.

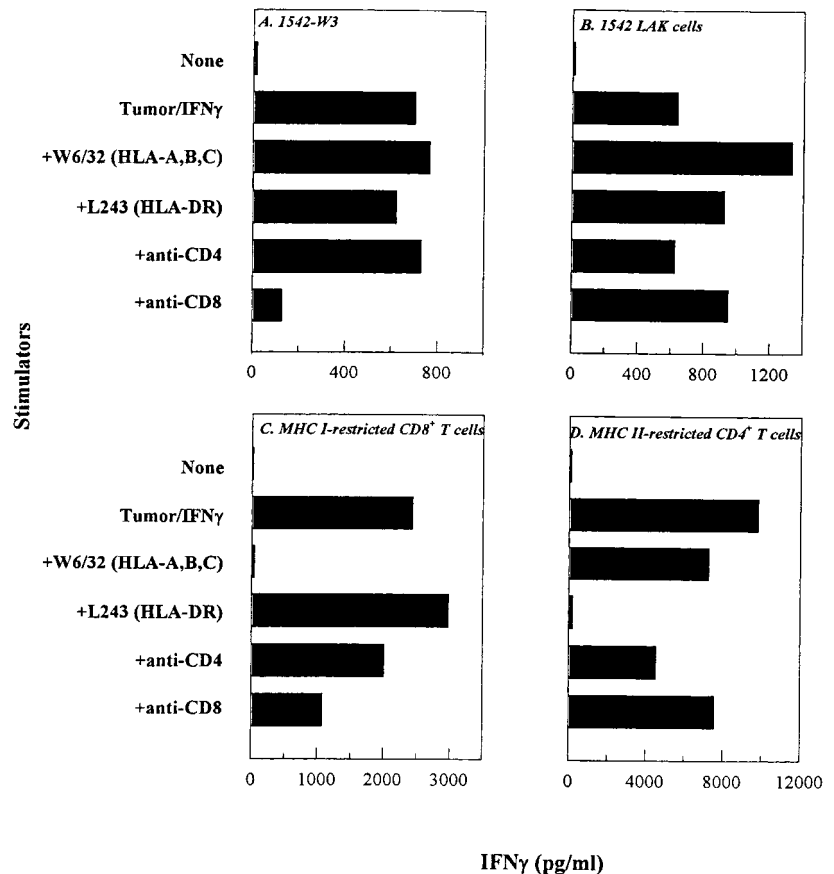


Table I. 1542-W3 T cells recognize a shared prostate epithelial Ag without evidence of classical HLA restriction

Targets ^a	IFN- γ (pg/ml) ^b	HLA Molecules Shared Between T Cells and Targets ^c	
		Class I	Class II
Autologous targets			
CP ₃ TX	703	All	All
NPTX	166	All	All
SVTX	510	All	All
Allogeneic prostate epithelial cell lines			
1510-CPTX	94	None	DQB1*0201
1512-CPTX	141	None	None
1519-CPTX	53	None	DQB1*0201
1532-CPTX	169	A1	DR β 1*0301 DQB1*0201, DQB1*0301
1535-CPTX	221	A1	DQB1*0201
1550-CPTX	586	A1, Cw2	None
1560-NPTX	51	A1	None
1654-CPTX	512	A23	None
Melanoma cell lines			
697-mel	<15	None	None
1011-mel	<15	Cw2	None
1088-mel	<15	A1	DQB1*0201
1359-mel	<15	A1	DQB1*0201, DQB1*0301
Miscellaneous			
K562	<15	None	None

^a Pretreated with IFN- γ 500 U/ml for 72 h.

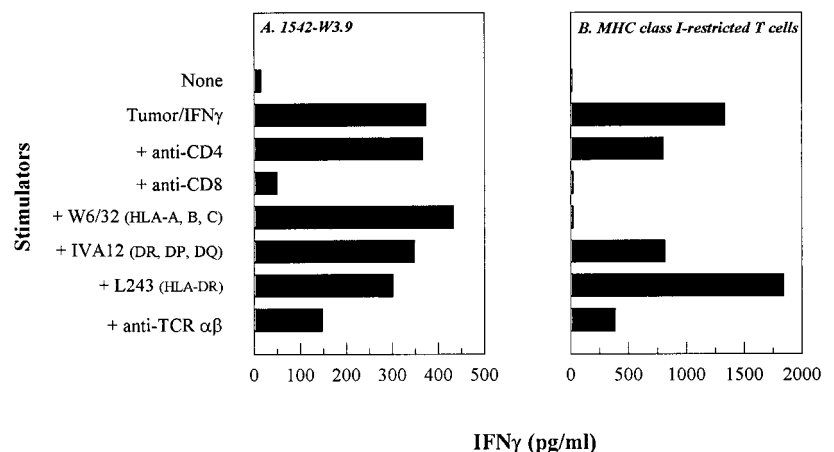
^b Measured by ELISA.

^c Determined by HLA serotyping for class I, genotyping for class II.

but did not demonstrate expression of particular V α or V β rearrangements, which have been associated with human T cells recognizing Ag presented by nonclassical restriction elements (17). Although flow cytometric analysis revealed monomorphic expression of CD8 and V β 12 by W3.9, 32% of these cells also expressed CD56, usually associated with NK cells. However, following purification, CD8⁺/CD56⁺ and CD8⁺/CD56⁻ T cells displayed similar profiles of reactivity (data not shown), and no other NK markers were detected (CD16, p58, CD94). Similar to the parental W3 T cell line, recognition of autologous tumor by clone W3.9 was not inhibited by mAb against classical MHC class I and II molecules, but was almost completely abrogated by anti-CD8 (Fig. 3). The requirement for TCR engagement was suggested by the inhibitory effects of mAb against TCR $\alpha\beta$ (Fig. 3), CD3, and V β 12 (data not shown).

Also similar to the W3 T cell line, clone W3.9 recognized autologous benign prostate epithelium (1542-NPTX) as well as a series of allogeneic tumor cell lines. The primary prostate cancer cell lines 1510-CPTX and 1519-CPTX were recognized, whereas the metastatic PC3 and DU145 lines were not (not shown). Importantly, W3.9 also recognized several nonimmortalized colon cancer cell lines (Fig. 4), but did not recognize other epithelial tumors such as breast cancer cell lines (MCF-7 and SK-BR-3) or the transformed renal epithelial cell line 293 (data not shown). There was no recognition of fibroblasts (1542-FTX, 1510-FTX, and 1532-FTX), sarcomas (RD-ES, TC-71, or 6647), or leukemias (MOLT-4, THP-1). These experiments were repeated with similar results. Thus, clone W3.9 seemed to recognize a shared Ag expressed by benign and malignant prostate epithelium as well as colon cancer in the context of a nonclassical restriction element.

FIGURE 3. Autologous tumor recognition by the 1542-W3.9 T cell clone is CD8 and TCR dependent. A, W3.9 cells were coincubated with IFN- γ -treated 1542-CP₃TX for 20 h in the absence or the presence of anti-CD4, anti-CD8, anti-MHC I (W6/32), anti-MHC II (IVA12), anti-HLA-DR (L243), or anti-TCR $\alpha\beta$ (WT31) mAb. Tumor recognition was measured by IFN- γ secretion. IFN- γ background for each target alone was <15 pg/ml. B, The specificity of the mAb was confirmed using the CD8⁺ gp100-specific, HLA-A2-restricted B1F11 T cell clone coincubated with the gp100⁺, HLA-A2⁺ 1088-mel. Only mAb against CD8 and the TCR blocked tumor recognition by the 1542 W3.9 T cell clone, while the B1F11 anti-gp100 clone was also inhibited by W6/32.



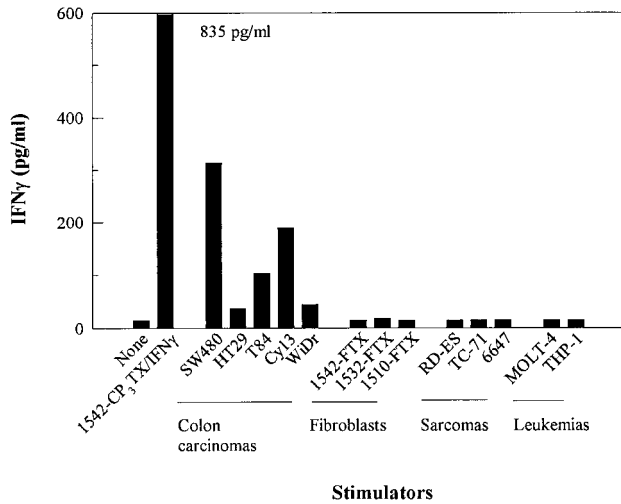


FIGURE 4. The 1542-W3.9 T cell clone cross-reacts with prostate and colon carcinoma cell lines. T cells were coincubated with cell lines of different histologic types, such as colon cancers (SW480, HT29, T84, Cy13, WiDR), fibroblasts (FTX), Ewing's sarcomas (RD-ES, TC-71, 6647), and leukemias (MOLT-4, THP-1). Only the autologous 1542-CP₃TX cell line was pretreated with IFN-γ, because the viability of some of the other cell lines decreased after IFN-γ incubation. Target recognition by T cells was measured by IFN-γ secretion. IFN-γ background secretion from each target alone was <15 pg/ml.

Intracellular processing of the Ag recognized by 1542-W3.9 requires proteasomal degradation and endoplasmic reticulum-Golgi trafficking

The reactivity of clone 1542-W3.9 toward several epithelial cell lines, without apparent shared classical HLA molecules or inhibition by mAb directed against classical MHC molecules, suggested that Ag presentation could be occurring in the context of a non-polymorphic molecule such as MHC class Ib (HLA-E, -F, -G, -H) or CD1. Although some of these molecules are known to bind peptides (MHC class Ib, CD1d), others present glycolipid Ag (CD1a, b, c) (18, 19). We first assessed whether the Ag recognized by W3.9 could be processed through the exogenous/endosomal pathway characteristic of class II-restricted and CD1b-restricted Ag (20). For this purpose, lysates of 1542-CP₃TX tumor were pulsed onto various APC, including autologous EBV-B cells, autologous cultured dendritic cells, or the THP-1 monocytic leukemia cell line (12), but pulsed APC were not recognized by W3.9. To derive some clues about the chemical nature and processing pathway of the Ag recognized by the CD8⁺ W3.9 clone, inhibitors known to interfere with discrete stages of Ag processing were used. The specificity of these inhibitors was assessed using an HLA-A2-restricted CD8⁺ T cell clone (B1F11) specific for gp100 and a CD4⁺ T cell line that was HLA-DR1 restricted and specific for a TPI mutation (TIL 1558). The respective tumor cell targets were preincubated with processing inhibitors, then fixed with paraformaldehyde and used to stimulate specific cytokine release from T cells. Results are depicted in Fig. 5. IFN-γ secretion by 1542-W3.9 in response to stimulation with the autologous prostate carcinoma was completely abrogated by lactacystin (blocks proteasomal degradation), brefeldin A (blocks protein egress from the endoplasmic reticulum), and cycloheximide (blocks protein synthesis). In contrast, 1542-W3.9 still recognized chloroquine-treated prostate carcinoma cells, indicating that the Ag did not localize to the endosomal-lysosomal compartment. As a control, the MHC class I-restricted T cell clone B1F11 failed to recognize 1088-mel cells pretreated with brefeldin A, cycloheximide, or lactacystin,

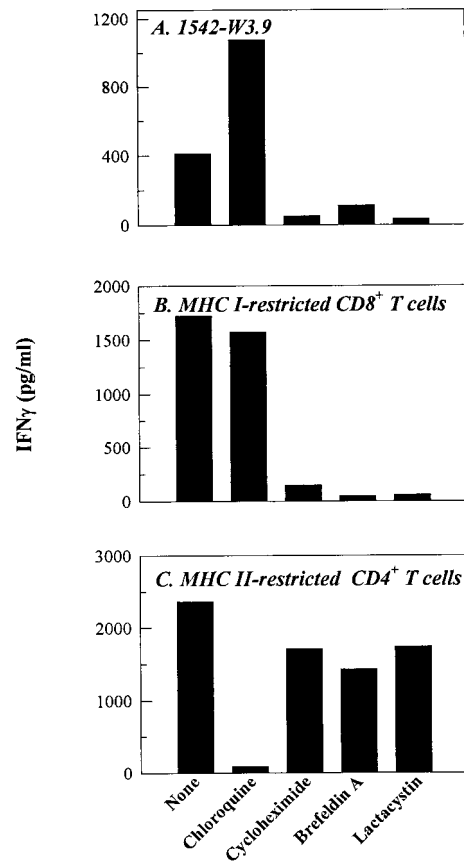


FIGURE 5. Processing requirements for the 1542-CP₃TX Ag indicate utilization of the cytosolic class I pathway. A, The W3.9 CD8⁺ T cell clone was coincubated with autologous 1542-CP₃TX tumor cells or with 1542-CP₃TX previously treated with 100 μM chloroquine, 5 μg/ml cycloheximide, 10 μg/ml brefeldin A, or 25 μg/ml lactacystin. B, As a control for Ag processing through the class I pathway, the gp100-specific, HLA-A2-restricted CD8⁺ B1F11 T cell clone was cocultured with 1088-mel. C, As a control for Ag processing through the class II pathway, HLA-DR1-restricted CD4⁺ TIL 1558 specific for a mutated TPI were cocultured with 1558-mel. Tumor recognition by T cells was measured by IFN-γ secretion. IFN-γ background secretion for each target alone was <15 pg/ml. These results demonstrate a similar recognition pattern for W3.9 and the B1F11 CD8⁺ clone, suggesting that the 1542-CP₃TX Ag is processed through the class I pathway.

but was unaffected by chloroquine. Conversely, MHC class II-restricted TIL 1558 were inhibited from recognizing 1558-mel by chloroquine, but not by the other agents. In parallel, we used flow cytometry to assess the impact of inhibitor treatment on the relative intensity of cell surface MHC class I expression by 1542-CP₃TX and EBV-B cells. Whereas chloroquine and lactacystin treatment of the cells did not affect class I expression within the time period of our assay, expression was somewhat decreased in presence of brefeldin A and cycloheximide. However, EBV-B cells remained competent to present exogenously pulsed peptides to specific class I-restricted T cells (data not shown), suggesting that the reduced HLA expression alone could not account for the complete inhibition of Ag presentation to 1542-W3.9 T cells by cycloheximide, brefeldin A, and lactacystin. These results indicate that the W3.9 T cell clone recognizes a peptidic Ag processed through the classical class I pathway.

To investigate the possibility that the Ag recognized by W3.9 is glycosylated, 1542-CP₃TX cells were grown in the presence of

tunicamycin, an inhibitor of *N*-glycosylation, or phenyl-acetyl-galactosamidase, an inhibitor of *O*-glycosylation. Neither agent diminished tumor recognition by W3.9 T cells. In addition, neuraminidase or *O*-sialoglycoprotein endopeptidase were used to cleave carbohydrates or *O*-glycosylated peptides, respectively, from the surface of 1542-CP₃TX cells. Neither agent affected T cell recognition of the prostate cancer cells (data not shown).

Ag presentation to the 1542-W3.9 T cell clone is β_2m dependent

Because the previous results suggested that the Ag recognized by W3.9 might be processed through the classical MHC I pathway, we questioned whether an MHC class I-related structure could present this Ag. Therefore, the possible role of β_2m in presentation of the prostate/colon cancer-associated Ag was investigated. For this purpose, we used the observation that the β_2m -deficient LoVo colon carcinoma cell line (21) was unable to stimulate the 1542-W3.9 T cell clone (data not shown), while other colon carcinomas could be recognized (Fig. 4). To determine whether the absence of reactivity to LoVo was due to lack of β_2m , the LoVo cell line was infected with Vac- β_2m and then used to stimulate the 1542-W3.9 T cell clone. Fig. 6 shows that Vac- β_2m -infected LoVo stimulated IFN- γ secretion from the CD8⁺ T cell clone, while uninfected LoVo or LoVo infected with an empty vaccinia vector were not recognized. Furthermore, overexpression of β_2m alone was not sufficient to stimulate 1542-W3.9, because Vac- β_2m infection of the β_2m -deficient Daudi lymphoma cell line (22) caused cell surface expression of MHC class I molecules but did not result in T cell recognition. Therefore, while β_2m expression is necessary for recognition of the shared prostate/colon carcinoma Ag, it is not sufficient for recognition. These results further demonstrate the nonubiquitous nature of the Ag recognized by clone W3.9.

Assessment of CD1d and HLA-G as potential presenting molecules for the prostate/colon carcinoma Ag

The class Ib molecules, HLA-E, -F, -G, and -H, as well as CD1d are nonpolymorphic, contain CD8 binding sites, complex to peptides, and associate with β_2m (for review, see Ref. 23). Additional properties of CD1d, including its expression on intestinal epithelium, up-regulation by IFN- γ , and recognition by $\alpha\beta^+$ T cells (24), are consistent with the characteristics of Ag recognition by clone W3.9. However, none of several anti-CD1d mAb (CD1d27.2, CD1d42, CD1d51, 1H1) inhibited recognition of 1542-CP₃TX by clone W3.9. Moreover, flow cytometric analysis of prostate epithelial cell lines failed to consistently detect CD1d at the cell surface, whereas CD1d was detected on the thymus-derived cell line MOLT-4 (data not shown). Thus, it seemed unlikely that CD1d was the presenting molecule for the prostate/colon carcinoma Ag. Among the class Ib molecules, HLA-G appeared to be a reasonable candidate to present the 1542-CP₃TX Ag. Even though only interactions with CD94 or KIR on NK cells have been demonstrated to date (25), HLA-G is reported to bind a large array of peptides from which a specific motif has been defined, making this class MHC class Ib molecule compatible with peptidic Ag presentation to specific T cells (26). However, HLA-G was not detectable by flow cytometry using the Ab 87G (27) at the surface of prostate and colon cancer cells recognized by 1542-W3.9. To investigate further, PCR amplification of HLA-G from tumor cell lines recognized or not by clone W3.9 was performed. No correlation was observed between expression of mRNA for HLA-G and T cell recognition of these targets, suggesting that HLA-G is unlikely to be the Ag-presenting molecule recognized by W3.9.

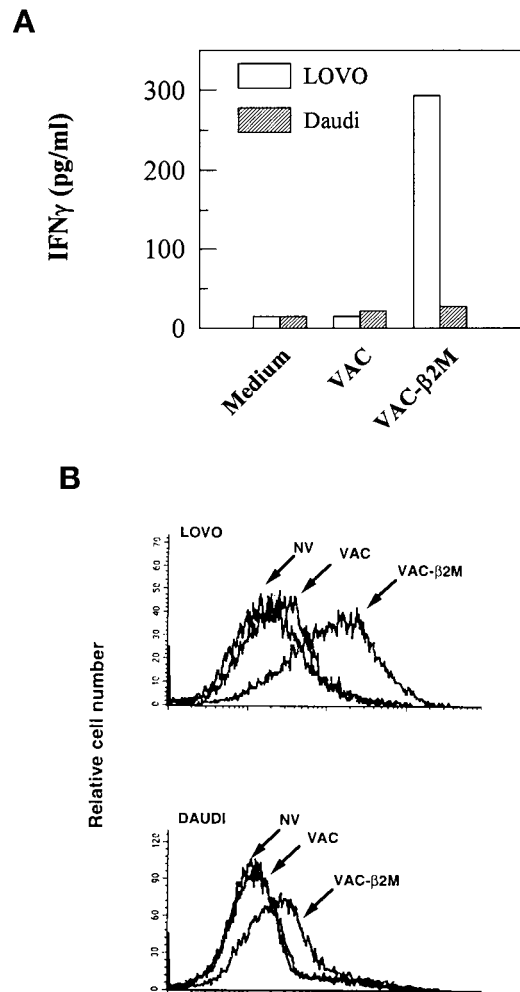


FIGURE 6. Presentation of Ag recognized by the W3.9 T cell clone is β_2m dependent. **A**, Two β_2m -deficient cell lines, LoVo (colon carcinoma) and Daudi (Burkitt's lymphoma), were infected with an empty vaccinia vector (VAC) or a recombinant virus encoding β_2m (VAC- β_2m) and then fixed with 0.5% paraformaldehyde. Uninfected or infected cells were co-cultured with the W3.9 T cell clone, and recognition by T cells was measured by IFN- γ secretion. IFN- γ background secretion for each target was <15 pg/ml. **B**, Flow cytometric analysis of the infected target cells for MHC class I expression (mAb W6/32) was performed simultaneously.

Discussion

In this study we report the derivation and characterization of a CD8⁺ T cell line and clone specific for a shared prostate/colon carcinoma Ag presented on a nonclassical MHC I-like molecule. Successful isolation of such tumor-targeted immune cells from one prostate cancer patient validates our strategy to raise prostate cancer-reactive T cells in vitro using autologous sets of reagents comprising carcinoma cell lines and lymphocytes from PBMC or draining lymph nodes. To our knowledge, this report is the first to describe the isolation of prostate cancer-reactive T cells in a completely autologous system. The immortalized prostate cancer cell line used as a stimulator to raise reactive T cells, 1542-CP₃TX, was previously certified as malignant in origin through a genetic loss of heterozygosity analysis and was clearly distinct from autologous normal prostate epithelium, seminal vesicle epithelium, and fibroblast cell lines established following the same procedures (10). In preliminary unpublished experiments, numerous attempts to raise prostate cancer-reactive CD8⁺ or CD4⁺ T cells from six different prostate cancer patients, including patient 1542, through

different strategies, including repetitive *in vitro* stimulation with whole autologous irradiated tumor cells, with tumor cells expressing a costimulatory B7.1 transgene, or with lysates of tumor cells processed by autologous dendritic cells, were unsuccessful. Because such techniques have proved useful in the melanoma setting, failure here implied either an extremely low precursor frequency of reactive lymphocytes in PBMC or an inherently low immunogenicity of the prostate cancer cells used for *in vitro* stimulation. To overcome these impediments, T cell lines were generated from individual macrocultures, each containing 2 million PBMC, and cultured prostate cancer cells were exposed to IFN- γ to enhance expression of MHC and adhesion molecules before use as stimulators. This last approach was successful in raising CD8⁺, TCR $\alpha\beta$ ⁺ T cells capable of recognizing autologous prostate cancer cells in patient 1542 and a second patient currently under study. Unexpectedly, 1542 T cells did not appear to be restricted by classical MHC molecules, even though mAb blocking studies demonstrated the critical roles of the CD8 and TCR molecules in target recognition. A panel of prostate and colon carcinoma cell lines was recognized without evidence for shared classical MHC molecules, and recognition was not inhibited by mAb against MHC class Ia or II determinants. However, involvement of a restriction element for Ag presentation is supported by the findings that the Ag is subject to proteasomal processing (inhibited by lactacystin) and endoplasmic reticulum-Golgi transport (inhibited by brefeldin A), and requires β_2m for presentation. These results mitigate against the possibility that the recognized Ag is a complex molecule with a repeating subunit structure capable of directly cross-linking the TCR, such as MUC-1 (28). Rather, they imply the existence of a peptidic Ag derived from a cytosolic protein and presented by an MHC class I-like molecule with limited or no polymorphism.

The functional attributes of the T cell-tumor cell interactions described here suggest a possible role for CD1d in Ag presentation. Although CD1a, -b, -c, and -d molecules on bone marrow-derived APC are involved in presenting bacterial derived lipoglycans for immune recognition (19, 20, 29), CD1d has also been found on human intestinal epithelium and is involved in educating thymus-independent CD8⁺ T cells (24). Strikingly, the CD1 mouse homologue has been described to present long hydrophobic peptides to CD8⁺, TCR $\alpha\beta$ ⁺ T cells, whose function could be inhibited by anti-CD8 mAb (30, 31). However, using available Abs to stain prostate cancer cells and to inhibit target recognition by the prostate cancer-reactive T cells, we were unable to demonstrate a definitive role for CD1d in presentation of the shared prostate/colon cancer Ag.

The MHC class Ib molecules (HLA-E, -F, -G, and -H) also appeared to be candidates for presenting the shared prostate/colon cancer Ag. It is well established that HLA-G, selectively expressed by MHC class Ia-negative trophoblast cells, interacts with KIR on maternal NK cells to inhibit their cytotoxic activity (32). It has also been recently postulated that ectopic expression of HLA-G on melanomas might be a mechanism by which these malignant cells could escape NK attack (33). Importantly, elution and sequencing of peptides bound to HLA-G has demonstrated a wide array of peptides compatible with Ag presentation to specific T cells (26). However, in the current study the absence of correlation between expression of HLA-G mRNA by target cells and their recognition by the prostate-reactive T cells allowed us to rule out the role of this MHC class Ib molecule in Ag presentation. Another potential candidate for Ag presentation in the 1542 system is HLA-E, which has been shown to preferentially bind peptides derived from the signal sequences of most HLA-A, -B, -C, and -G molecules (34). Recent studies have revealed that HLA-E molecules function as ligands for NK cell inhibitory receptors and can protect target cells

from lysis by NK cells (34, 35). However, the W3.9 T cell clone expresses none of the receptors described as interacting with HLA-E, such as CD94 or KIR, nor does it express the NK markers CD57 or CD16. Furthermore, it has been shown in an HLA-E transgenic mouse model that CTL recognition of target cells expressing HLA-E molecules can be inhibited by the mAb W6/32 (36), whereas the activity of the W3.9 T cell clone is not inhibited. Finally, because little is currently known about the functions of HLA-F and HLA-H (23), Ag presentation by these molecules remains a possibility, which will be the subject of future studies.

Although some of the nonclassical molecules mentioned above have known Ag-presenting functions, other MHC class I-related molecules are described in the literature with unknown functions, such as MR1 (37), or functions unrelated to Ag presentation, such as MIC-A and MIC-B (38). MIC-A and MIC-B are stress induced, are directly recognized by $\gamma\delta$ T cells independently of Ag processing, and do not seem to bind β_2m . Thus, it seems unlikely that either molecule is involved in recognition of a processed Ag by the TCR $\alpha\beta$ ⁺ clone W3.9.

Altogether, these results demonstrate the existence of prostate cancer-reactive CD8⁺ T cells, which recognize a shared Ag processed and presented by a select group of epithelial cells through a seemingly classical class I pathway, via an unconventional restriction element. Regarding the nature of the Ag recognized, we have few clues. The protein Ag is shared by prostate and colon carcinomas as well as normal prostate epithelium and seems to be epithelium restricted because none of multiple cell lines derived from immune cells or mesenchyme were recognized by these CD8⁺ T cells. Ag expression in nonmalignant prostate epithelial cells may imply a cell lineage-specific Ag analogous to a number of melanoma-melanocyte Ag that have been characterized (39, 40). This Ag is recognized by noncytolytic Th1-type CD8⁺ T cells expressing TCR $\alpha\beta$. Compared with the conventional MHC class I-restricted CD8⁺ CTL widely considered as the dominant effector cells mediating tumor killing, such type I cytokine-secreting CD8⁺ T cells have not received much attention. Although the physiological relevance of noncytolytic cytokine-secreting CD8⁺ T cells for antitumor immunity remains to be elucidated, these cells may exert toxic effects against tumor targets either directly or indirectly, through the cytokines that they secrete and the other antitumor effector cells that they recruit.

The field of tumor immunology has traditionally focussed on identifying CD8-recognized, MHC class Ia-restricted tumor Ag for clinical development as cancer vaccines. More recently, increased attention has been paid to characterizing Ag recognized by MHC class II-restricted CD4⁺ antitumor effectors for the development of multivalent vaccines capable of activating a plurality of immune responses (7). Finally, the results of the current study suggest a role for nonclassical effectors in mediating antitumor immunity. Due to the nonpolymorphic nature of the relevant Ag-presenting molecules, elucidation of tumor Ag recognized by these immune cells holds promise for the development of universally applicable cancer vaccines.

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