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TAP expression reduces IL-10 expressing tumor infiltrating lymphocytes and restores immunosurveillance against melanoma

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Many immune therapeutic strategies are under development for melanoma to treat metastatic disease and prevent disease reoccurrence. However, human melanoma cells are often deficient in antigen processing and this appears to play a role in their expansion and escape from immunosurveillance. For example, expression of the transporters associated with antigen processing (TAP1 and TAP2) is down-regulated in the mouse melanoma cell line B16F10. This results in a lack of tumor-associated antigen processing, low surface expression of MHC Class I molecules and low immunogenicity. We observe that restoration of TAP1 expression by transfection resurrects the processing and presentation of viral antigens, and the melanoma-associated antigen, TRP-2. Immunization with irradiated B16F10/rTAP1 transfected cells generates CTLs that are capable of killing B16F10/rTAP1 transfected targets and B16F10 targets deficient in TAP1. Furthermore, B16F10/rTAP1 transfectants grow at a significantly slower rate in mice than B16F10 cells. In an experimental model that closely recapitulates the clinical situation, treatment of B16F10 tumors in mice with a vaccinia virus vector expressing TAP1 also significantly decreases tumor growth *in vivo*. Furthermore, tumors treated with vaccinia TAP1 had significantly reduced numbers of immunosuppressive, $CD3^+$/IL-10 positive, tumor infiltrating lymphocytes. Therefore, TAP1 expression restores both antigen presentation and immunogenicity in B16F10 melanoma cells and concomitantly reduces immunosuppressive IL-10 production at the local tumor site, thereby increasing immunosurveillance mechanisms against tumors.

Key words: TAP1; MHC class I; melanoma; B16F10; vaccinia; mouse

Melanomas (MAAs) are neoplasms that arise from pigmented melanocytes differentiated from the neural crest. Cutaneous MAA represents only 4% of all skin cancers but accounts for almost all of the deaths associated with the disease. The incidence of MAA over the last 30 years has more than doubled in the United States with 54,000 new cases and 7,600 deaths in 2003. Intermittent acute sun exposure and lifetime sun exposure, age, skin pigment type, immune suppression, atypical nevi and family history are risk factors for MAA. The early stage disease is curable but reoccurrence is common for later stage disease and the prognosis is poor if the disease has spread to the lymph nodes and other tissues. Standard treatment is biopsy and 2-cm wide excision after histological determination that the lesion is malignant. In some cases, regional and sentinel lymph nodes are also removed and, if positive, adjuvant therapy consisting of interferon-α or IL-2 may be administered but response rates low and long term benefit are not significant.[1–3] However, cases of spontaneous remission and reoccurrence of the disease many years after remission or resection, and correlation of remission with immune cell infiltrates all suggest that there is an immune surveillance process that could be leveraged to develop treatments for late stage disease.[4–8]

The discovery of tumor antigens associated with MAA[9] has led to research and development of therapeutic vaccines designed to generate antitumor responses with the aim of controlling metastasis. A wide variety of vaccination approaches and protocols are currently being tested.[10] The results show that even though specific cellular immune responses are generated against the antigens, the response of the disease to the treatment is low. The reasons for the low response rates are thought to be low immunogenicity of the antigens, which are usually self-antigens, and the natural selection of tumor variants that have immune-suppressive phenotypes arising through a process called immuno-editing.[11] The nature of the immune suppression may include secretion of immunosuppressive cytokines,[12] the expression of ligands (FAS-L) that initiate apoptosis in cytotoxic T-cells[13] and tumor variants that are deficient in antigen processing and presentation.[14] Variants that are deficient in the MHC Class I antigen processing pathway do not express MHC Class I antigens on the cell surface. As a consequence, specific cytotoxic T-cells generated by the vaccine protocol are unable to recognize and kill these tumor variants due to defective presentation of tumor associated antigen-derived peptides recognized by the CTLs.

The MHC Class I restricted antigen presentation pathway consists of a number of genes encoded in the MHC Class I locus of human chromosome 6. The pathway generates peptides from endogenous proteins by the degradative action of proteolytic enzymes LMP2 and LMP7, located in a proteolytic complex called the proteasome. The peptides are transported from the cytoplasm into the endoplasmic reticulum (ER) by the ABC transporter, transporters associated with antigen processing (TAP), a heterodimer that is composed of 2 subunits, TAP1 and TAP2. Within the ER, the peptide may be trimmed further by resident ER proteases and subsequently loaded onto an MHC Class I molecule, which consists of MHC heavy chain and beta-2-microglobulin (β2M). The assembly of this complex is aided by a number of chaperone proteins; these include calreticulin and calnexin, which are responsible for the folding of MHC Class I heavy chains and stabilization of their association with β2M, and tapasin, which is responsible for anchoring the MHC molecules to TAP and loading the peptides onto the MHC Class I molecules. The properly assembled complex is then transported to the cell surface by the secretory pathway. On the cell surface, the functional MHC Class I molecules offer a ligand to the TCR of $CD8^+$ T-cells. If the TCR binds with high enough affinity, activation of the T-cell occurs and the target cell can be destroyed.

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Any alterations or deficiencies in the antigen presentation pathway can lead to variants, which give rise to nonimmunogenic tumors. These deficiencies can be due to chromosomal lesions leading to loss of heterozygosity or mutations in the genes of the pathway (such as β2M) and can be referred to as hard lesions. In most cases however, deficiencies in MHC Class I antigen expression on the cell surface are due to soft lesions characterized by the down-regulation of components of the antigen presentation pathway.[14,15] Down-regulation of TAP1 is a critical factor in MHC Class I antigen deficiencies and has been associated with disease progression and death in cutaneous and orbital MAA.[16–18] Conversely TAP1 expression has been associated with tumor infiltrating lymphocytes (TILs), a characteristic of good clinical outcome[8,16,19] and spontaneous regression of MAAs.[4]

In our study, we examine the effect of the restoration of TAP1 expression on MHC Class I antigen surface expression in the murine MAA cell line, B16F10. B16F10 cells are a subclone of the mouse B16 MAA cell line that are weakly immunogenic and have been widely used as a tumor model for tumor–host immune interactions. This tumor, like most metastatic carcinomas, has deficiency in components of MHC Class I antigen-processing pathway, including TAP, MHC Class I antigen surface expression, proteasome subunits LMP2, LMP7 and LMP10, PA28α and β, and the chaperone tapasin.[20,21] This down-regulation of the antigen presentation pathway can be reversed by IFN-γ treatment. We test the hypothesis that restoration of TAP1 expression in B16F10 cells increases MHC Class I antigen surface expression and immunogenicity, making these cells visible to immune surveillance mechanisms.

Material and methods

Animals

The mouse strain C57BL/6 (H-2^b) was obtained from The Jackson Laboratory (Bar Harbor, ME), and maintained at the Biotechnology Breeding Facility (University of British Columbia, Vancouver, BC, Canada). The mice were maintained according to the guidelines of the Canadian Council on Animal Care. Mice were kept on a standard diet with water *ad libitum*. The colony was routinely screened for *Mycoplasma pulmonis* and *Mycoplasma arthritidis,* rodent coronaviruses (including hepatitis) and Sendai virus, using the Murine ImmunoComb Test (Charles River Laboratories, Wilmington, MA). The mice used in the experiments were between 6 and 12 weeks of age.

Viruses

Vesicular Stomatitis Virus, Indiana Strain (VSV), a gift from Frank Tufaro (University of British Columbia), was cultured on Vero cells [American Type Tissue Culture (ATCC), Rockville, MD]. Recombinant vaccinia virus (VV) either carrying rat-TAP1 genes (VV-rTAP1) or the empty plasmid PJS-5 (VV-PJS-5, vector negative control) is described previously.[22] All VV strains were grown in CV-1 cells (ATCC). VSV and VV titres were determined by tissue culture infective dose ($TCID_{50}$) assay or standard plaque assay (PFU), using Vero cells and CV-1 cells, respectively.

Cell lines

RMA, RMA-S, Vero and B16F10 cells (murine MAA) were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT), L-glutamine (2 mM), penicillin(100 IU/ml), streptomycin (100 μg/ml) and HEPES (20 mM). CV-1, CMT.64 (murine lung carcinoma)[22] and murine tapasin$^{-/-}$ fibroblasts (a kind gift from Dr. Luc Van Kaer, Vanderbilt University School of Medicine, Nashville, TN) cell lines were cultured in DMEM with the same supplements. The clones of rat TAP1 (rTAP1) and vector-only transfectants of B16F10 cells were created by transfecting the cells with the rTAP1 cDNA in mammalian expression vector pH (Apr-1neo) and maintained in Geneticin (Invitrogen, Burlington, ON, Canada) selecting RPMI-1640 medium.[22] Two rTAP1-transfected clones were designated as B16/rTAP1 3–3 and B16/rTAP1 3–8 and a clone to control for the transfection vector was designated as B16/PHβ 1–1.

Detection of TAP protein expression in B16F10 cells transfectants by immunoblotting

rTAP1 and mouse TAP2 expression in B16/rTAP1 3–3 cells and B16/rTAP1 3–8 cells was examined by immunoblotting. Total extracts from 5×10^5 cells were separated on 10% polyacrylamide-SDS gels and blotted onto nitrocellulose filters. The blots were probed for mouse or rTAP1 and TAP2 with relevant specific rabbit antiserum at a 1:2,000 dilution. The blots were then incubated with horseradish peroxidase-labelled anti-rabbit IgG antibodies at a 1:20,000 dilution. The immune complexes were visualized by enhanced chemiluminescence (ECL) according to the instructions of the manufacturer (GE Healthcare, Chalfont St. Giles, UK). The rabbit antiserum against mouse and rTAP1 protein was created by immunizing rabbits with a common TAP1 peptide sequence, RGGCYRAMVEALAAPAD-C with a cysteine at the C-terminal, linked to keyhole limpet hemocyanin (Pierce Biotechnology, Rockford, IL). The specificity of the antiserum was confirmed by the detection of a band of ~70 kDa in size in lysates from TAP-expressing cells (RMA) that was absent in lysates of fibroblasts derived from TAP1$^{-/-}$ mice. The rabbit serum against mouse and rat TAP2 (116/4) was kindly provided by Dr. Geoff Butcher (University of Cambridge, Cambridge, UK). For mouse tapasin expression, 1.2×10^6 cells were lysed in 1% NP-40 lysis buffer 30 min on ice, spun at 10,000 g for 15 min at 4°C and precleared with Protein G-sepharose (50 μl) (GE Healthcare). Tapasin was immunoprecipitated with a rabbit antitapasin antiserum[23] (number 2668, courtesy of Dr. Ted Hansen, Washington University School of Medicine, St. Louis, MO) and Protein G sepharose, followed by separation by 12% SDS-PAGE, transferred to PDVF membranes (GE Healthcare) and probed with the same antiserum followed by ECL as described earlier.

Detection of surface H-2K^b, H-2D^b and I-A^b antigen expression

B16F10 or B16/rTAP1 clone 3–8 cells were infected with VV-PJS-5 or VV-rTAP1 [multiplicity of infection (MOI) of 10] and incubated for 3 days (37°C, 5% CO_2), followed by fixation and preparation for FACS analysis. Indirect immunofluorescence staining with conformational specific monoclonal antibodies (Abs) for H-2K^b (Y-3, ATCC) and H-2D^b (28.14.8.S, ATCC) detected MHC Class I surface expression. Aliquots of 10^6 cells were incubated (30 min at 4°C) with the primary Ab (50 μl) for H-2K^b or H-2D^b antigens. After washing twice with PBS, the cells were resuspended and incubated (30 min at 4°C) in FITC-conjugated rabbit anti-mouse IgG secondary Ab (50 μl) (Dakopatts, Glostrup, Denmark). For I-A^b antigen, PE-conjugated anti-mouse I-A^b AF6-120.1 (BD Biosciences, Mississauga, ON, Canada) was used at a 1:100 dilution for staining 10^6 cells 30 min at 4°C, then washed 3 times with PBS. A FACScan analyzer (Becton Dickinson, Mountain View, CA) measured the mean logarithmic fluorescence intensity associated with the staining of surface antigens.

Generation of VSV, TRP-2 and B16F10 tumor-specific effector CTLs

All splenocytes were cultured in RPMI-1640 complete medium containing 10% heat-inactivated HyClone FBS, L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, 0.1 mM nonessential amino acids, 1 mM Na-pyruvate and 50 μM 2-ME at 37°C, 5% CO_2. H-2K^b antigen-restricted, VSV-specific CTLs were generated by infection of mice (i.p.) with VSV (10^7 $TCID_{50}$). Splenocytes were harvested 5 days after infection and cultured for an additional 5 days in media (10^6 cells/ml) containing VSV-$Np_{52–59}$ peptide (RGYVYQGL) (1 μg/ml) at 37°C, 5% CO_2. To generate H-2K^b antigen-restricted tyrosinase related protein-2 (TRP-2)-specific CTLs, TRP-2 peptide (VYDFFVWL) (100 μg) was mixed with 50-μl TiterMax adjuvant (Cedarlane Laboratories, Hornby, ON, Canada) and 50-μl PBS and injected

subcutaneously into mice. This procedure was repeated after 7 days. Fourteen days after the initial injection, mice received an additional injection (i.p.) with γ-irradiated RMA-S cells (5×10^6 cells in 300 μl). The irradiated RMA-S cells were prepared by incubating 5×10^6 cells with TRP-2 peptide (10 μg/ml peptide in 2 ml media) overnight at room temperature followed by γ-irradiation (10,000 rads). Cells were washed and resuspended in PBS (300 μl). Seventeen days after the initial injection, the immunized spleen was removed, and the splenocytes (10^8 cells) were cultured for 5 days with γ-irradiated naÿve splenocytes (5×10^7 cells) pulsed with VYDFFVWL peptide (10 μg/ml).

To generate B16F10 tumor-specific CTLs, C57BL/6 mice were injected (i.p.) with γ-irradiated (10,000 rads) B16F10 cells, B16/PHβ 1–1 or B16/rTAP1 3–3 cells (3×10^6 cells/mouse). About 5 days after immunization, splenocytes were removed and cultured with stimulators at a 1:20 (stimulator/splenocyte) ratio for another 5 days at 37°C, 5% CO_2. The stimulators were prepared by incubating B16F10 cells, B16/rTAP1 3–3, or B16/PHβ 1–1 cells (1 hr at 37°C) with mitomycin C (30 μg/ml) followed by γ-irradiated (10,000 rads) and washed 3 times before addition to the splenocyte culture.

Cytotoxicity assay for VSV, TRP-2 specific and B16F10 tumor-specific effector CTLs

The cytotoxic activities were measured in standard 4 hr ^{51}Cr release assays. Overnight VSV (MOI of 10) infection of B16F10, B16/rTAP1 3–3 or B16/PHβ 1–1 cells provided targets for VSV-specific CTL assays. For TRP-2-specific and tumor-specific killing, B16F10 cells, B16/rTAP1 3–3 or B16/PHβ 1–1 target cells were untreated. All targets were labeled with $Na^{51}CrO4$ (70 μCi/10^6 cells) (GE Healthcare) for 1 hr at 37°C and washed extensively.

Animal studies

Mice ($n = 49$) were injected subcutaneously into the hindquarter with 1.5×10^5 cells of either B16F10 cells or B16/rTAP1 3–8 cells. About 1 and 6 days after introduction of tumor cells, mice were treated with injections localized to the tumor site of VV-rTAP1 (2×10^6 PFU/injection), VV-PJS-5 (2×10^6 PFU/injection), or PBS. About 21 days after the introduction of tumor cells, mice were killed and the tumor masses measured.

Detection of IL-10 in TILs

To detect IL-10-producing, CD3-positive cells, the tumor masses were homogenized, passed through a nylon cell strainer (40 μm), and the TILs were isolated by centrifugation on Ficoll-Paque. The TILs were then stimulated with phorbol 12-myristate 13-acetate (50 ng/ml) (Sigma-Aldrich Canada, Oakville, ON, Canada), calcium ionophore A23187 (1 μg/ml) (Sigma-Aldrich) and GolgiPlug (Becton Dickinson) at 37°C for 6 hr. Afterwards, the Fc-receptors of the stimulated TILs were blocked by anti-mouse CD16/CD32 (BD Biosciences) for 15 min at 4°C. The cells were then fixed, permeabilized and double stained with PE-conjugated anti-mouse CD3 and FITC-conjugated anti-mouse IL-10 (BD Biosciences) according to the protocol provided in the Cytofix/Cytoperm Plus Kit (BD Biosciences). Finally, the mean logarithmic fluorescence intensity was measured, using a FACScan analyzer (Becton Dickinson).

Statistical analysis

The effect of VV-rTAP1 infection on surface MHC Class I antigen expression was analyzed, using the Probability Binning Chi (*T*) Test (FlowJo, Ashland, OR). Results were considered statistically different if the T(X) value was greater than 4, implying that the distributions are different with a $p < 0.01$ (99% confidence). The effect of rTAP transfection and treatment with vaccinia vectors on the growth of B16F10 tumors was analyzed by 2-way ANOVA. The Tukey HSD Test was used for multiple comparisons to determine the differential effects of the treatments on tumor growth. The effect of vaccinia vectors on the percentage of $CD3^+$ TILs producing IL-10 was analyzed by ANOVA. The *p* values less than 0.05, after corrections for multiple comparisons, were considered significant.

Results

B16F10 cells transfected with rTAP1 up-regulate H-2K^b and H-2D^b antigen surface expression, but not MHC Class II (I-A^b) antigen expression

rTAP1 cDNA was used for transfection to allow for distinction, by polymerase chain reaction, between endogenous mouse TAP1 and transfected rTAP1 during the determination of transfection efficiency and clone stability (data not shown). rTAP1, mouse TAP2 and mouse tapasin (Tpn) protein expression was examined in B16F10 cells transfected with rTAP1 by immunoblotting. RMA cells were used as a positive control for TAP1, TAP2 and tapasin expression, and CMT.64 cells were used as a negative control for TAP1 and TAP2 expression.[22,24] Fibroblasts derived from tapasin$^{-/-}$ mice were used as a negative control for mouse tapa-

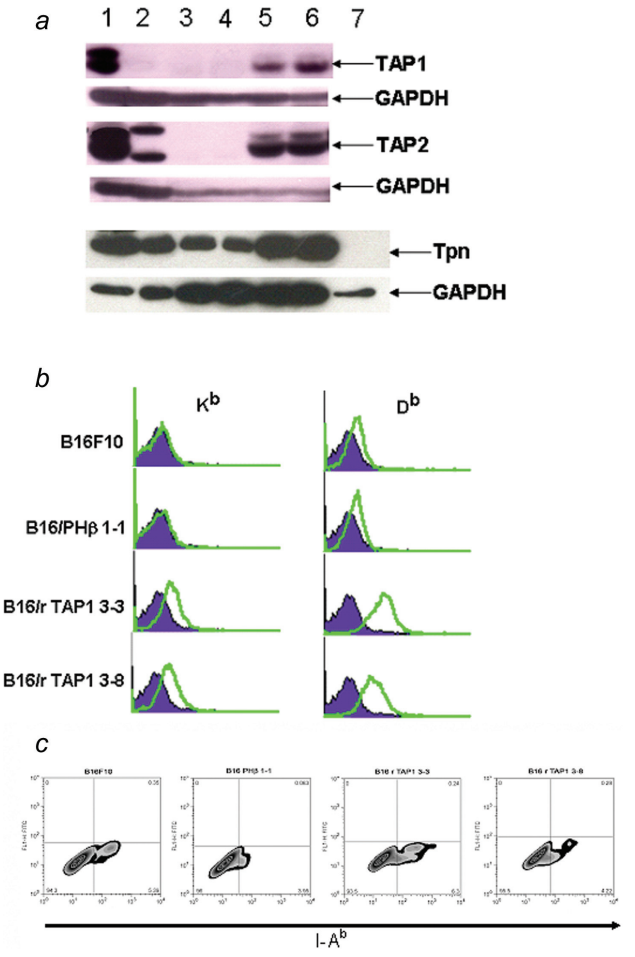

Figure 1 – The transfection of B16F10 cells with rTAP1 increases endogenous mouse TAP2 and mouse tapasin, and induces MHC Class I, but not MHC Class II antigen surface expression. (*a*) TAP1, TAP2 and tapasin expression was detected by Western blot: Lane 1-RMA cells, Lane 2-CMT.64 cells, Lane 3-B16F10 cells, Lane 4-B16/PHβ 1–1 cells, Lane 5-B16/rTAP1 3–3 cells, Lane 6-B16/rTAP1 3–8 cells, Lane 7â Tapasin$^{-/-}$ murine fibroblasts (tapasin blot only). (*b*) Surface MHC Class I antigen H-2K^b and H-2D^b expression is detected by flow cytometry. Purple filled areas represent negative controls, and green lines represent H-2K^b or H-2D^b stained cells. (*c*) Surface MHC Class II I-A^b antigen expression is detected by flow cytometry.

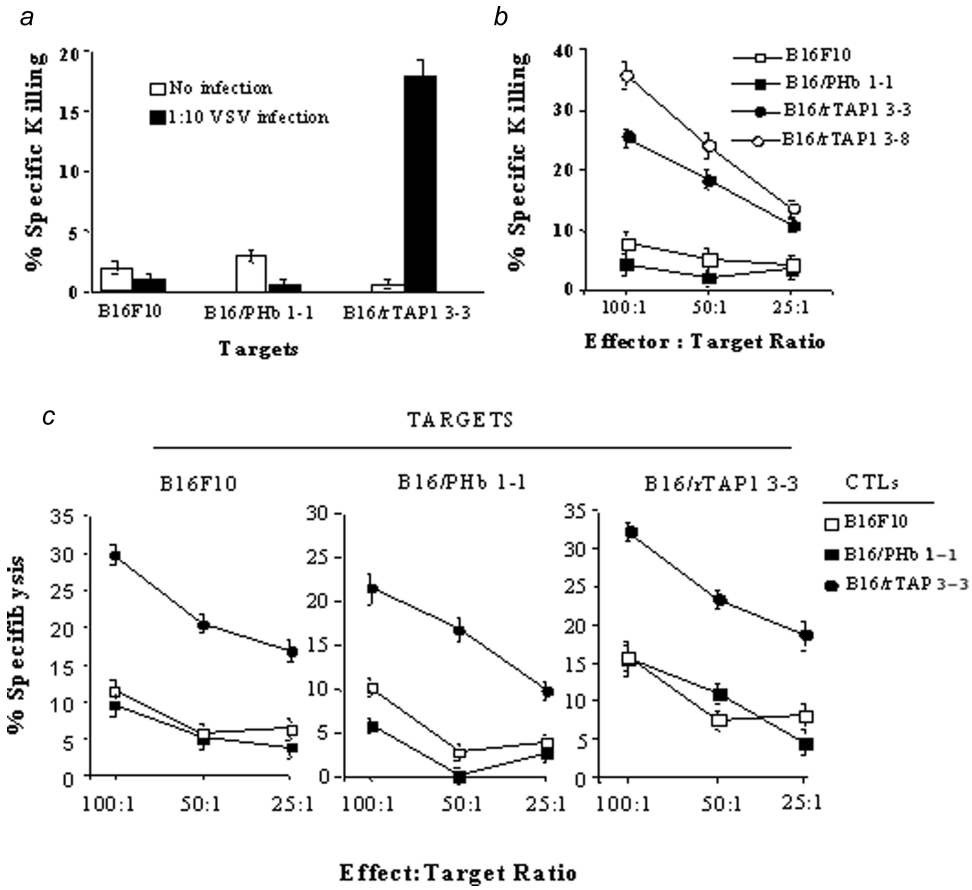

FIGURE 2 – TAP1 transfected B16F10 cells present both viral antigens (*a*) and tumor associated antigens (*b*), and are more immunogenic (*c*). A standard cytotoxicity assay was performed to detect antigen presentation capacity and capacity to induce tumor-specific T-cells in TAP1 transfectants of B16F10 cells. (*a*) Splenocytes from VSV-immunized mice were used as effectors and VSV-infected B16F10 cells, B16/PHβ 1–1 cells and B16/rTAP1 3–3 cells were used as targets. (*b*) Effectors were splenocytes from mice immunized with the tumor associated antigen peptide, TRP-2, followed by irradiated RMA cells pulsed with TRP-2 peptide. B16F10 cells, B16/PHβ 1–1 cells, B16/rTAP1 3–3 cells and B16/rTAP1 3–8 cells were used as targets. (*c*) Splenocytes from mice immunized with irradiated B16F10 cells, B16/PHβ 1–1 cells and B16/rTAP1 3–3 cells were used as effectors against the indicated targets.

sin. B16/rTAP1 3–3, B16/rTAP1 3–8 and B16/PHβ 1–1 cells were tested for rTAP1 and mouse TAP2 and tapasin expression (Fig. 1*a*). Normal B16F10 cells and B16/PHβ 1–1 cells were negative for both TAP1 and TAP2, but expressed some mouse tapasin. Both B16/rTAP1 3–3 and B16/rTAP1 3–8 cells were positive for rTAP1 expression. The expression of rTAP1 also induced and/or stabilized the expression of endogenous mouse TAP2 in B16/rTAP1 3–3 and B16/rTAP1 3–8 cells. rTAP1 expression also greatly increased endogenous levels of mouse tapasin.

MHC Class I and Class II antigen surface expression on B16F10 cells was compared to surface expression on B16/rTAP1 3–3, B16/rTAP1 3–8 and B16/PHβ 1–1 cells, using FACS analysis. The antibodies used for the FACS analysis are conformation-specific and only bind to H-2K^b, H-2D^b and I-A^b antigens properly folded and loaded with antigen peptide. Both B16/rTAP1 3–3 and B16/rTAP1 3–8 cells exhibited significant expression of conformation specific, mature H-2K^b and H-2D^b antigens on the cell surface (Fig. 1*b*). This was in contrast to B16F10 cells and B16/PHβ 1–1 cells, which had undetectable levels of H-2K^b antigen on the cell surface and only a small amount of H-2D^b antigen. Both rTAP1 and vector-alone transfected B16F10 cells exhibited a small population of cells positive for surface I-A^b antigen expression, between 4 and 6% of the total population. However, this population was neither increased nor decreased in cells expressing TAP1 compared to untransfected or vector-alone transfected cells.

TAP1 expression restores antigen presentation and immunogenicity in B16F10 cells

We tested whether TAP1 expression and the subsequent increase in H-2 antigen surface expression restores immunogenicity and T-cell recognition of B16F10 cells by measuring the ability of B16/rTAP1 3–3, B16/PHβ 1–1 and B16F10 cells to process and present the H-2K^b antigen-specific, immuno-dominant VSV antigen: VSV-Np$_{52–59}$. VSV-Np$_{52–59}$-specific cytotoxic splenocytes were able to kill VSV-infected B16/rTAP1 3–3 cells but not VSV-infected B16/PHβ 1–1 or B16F10 cells in a ^{51}Cr release assay (Fig. 2*a*). B16/rTAP1 3–3 cells were able to correctly process and present viral antigens but B16/PHβ 1–1 or B16F10 cells were not. We also examined if rTAP1 expression was able to restore the surface presentation of the H-2K^b antigen-restricted tumor-associated antigen, TRP-2. The ^{51}Cr release assay showed that both B16/rTAP1 3–3 and B16/rTAP1 3–8 cells were sensitive to killing by TRP-2-specific splenocytes compared to B16F10 cells or B16/PHβ 1–1 cells, which were resistant to killing (Fig. 2*b*). Both B16/rTAP1 3–3 and B16/rTAP1 3–8 cells were therefore able to present tumor-associated antigens, in the context of H-2K^b antigen, making the cells sensitive to killing by TRP-2 specific splenocytes.

A CTL assay measured the immunogenicity of B16F10 cells expressing TAP1. Splenocytes from mice immunized with B16/rTAP1 3–3 cells were able to kill all 3 target cell lines in contrast with

TABLE I – VV-rTAP1 INFECTION OF B16F10 AND B16/r TAP1 3–8 CELLS RESULTS IN INCREASED SURFACE MHC CLASS I ANTIGEN EXPRESSION COMPARED TO VV-PJS-5

Cell line	Fold increase H-2K^b	$T(X)$ value, p value	Fold increase H-2D^b	$T(X)$ value, p value
B16F10	0.52	4.4711, $p < 0.01$	1.8	24.052, $p < 0.01$
B16/r TAP1 3–8	0.16	10.462, $p < 0.01$	0.81	2.1617, $0.01 > p > 0.17$

Data represent the fold increase in mean fluorescence intensity of VV-rTAP1-infected cells with the fold increase in VV-PJS-5-infected cells subtracted, as detected by flow cytometry, using antibodies to H-2K^b and H-2D^b antigens.

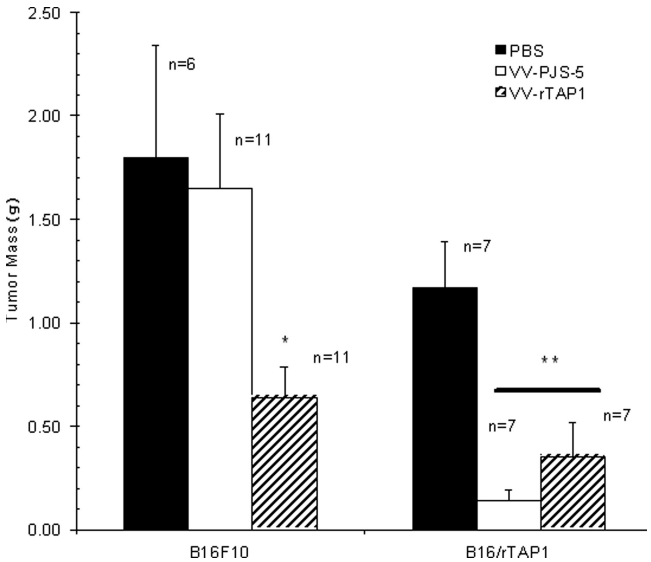

FIGURE 3 – The effect of TAP1 expression on tumor growth was determined in subcutaneous B16F10 and B16/rTAP1 3–8 tumors. Mice were injected subcutaneously with 1.5×10^5 cells in the hindquarter. About 1 and 7 days later, mice were injected at the site of the tumor with 2×10^6 PFU of VV-rTAP1, VV-PJS-5 (vector control) or PBS. Tumor mass (mean + SE) was measured after 21 days. The 2-way ANOVA showed that both TAP1 transfection and infection with VV-TAP1 retarded tumor growth. (*) Significant reduction in B16F10 cells tumor growth when treated with VV-rTAP1. (**) Significant reduction in B16/rTAP1 3–8 tumor growth when treated with VV-rTAP1 or VV-PJS-5.

splenocytes from mice immunized with B16/PHβ 1–1 or B16F10 cells, which possessed diminished cytotoxic activity (Fig. 2*c*).

These experiments demonstrate that rTAP1 expression restores antigen processing sufficiently to make B16F10 cells sensitive to killing by antigen-specific cytotoxic cells. In addition, rTAP1 expression by B16F10 cells stimulates immune responses that can generate cytotoxic cells capable of killing not only cells that express surface H-2 antigens but also those that have very low levels of H-2 antigen expression.

B16F10 cells infected with VV-rTAP1 up-regulate H-2K^b and H-2D^b antigen surface expression

We infected B16F10 or B16/rTAP1 3–8 cells with either VV-PJS-5 or VV-rTAP1 and examined the effect after 3 days on MHC Class I antigen surface expression. FACS analysis demonstrated that H-2K^b antigen is upregulated by 0.52-fold [$p < 0.01$; $T(X) = 4.4711$] in VV-rTAP1-infected B16F10 cells relative to VV-PJS-5-infected cells, H-2D^b antigen is upregulated 1.8-fold [$p < 0.01$; $T(X) = 10.462$]. Interestingly, in B16/rTAP1 3–8 cells, which already express TAP1 as a result of transfection, infection with VV-rTAP1 resulted in only 0.16-fold increase relative to VV-PJS-5 infection for H-2K^b antigen [$p < 0.01$; $T(X) = 24.052$] and 0.81-fold for H-2D^b antigen [$p < 0.17$ but >0.01; $T(X) = 2.1617$] (Table I).

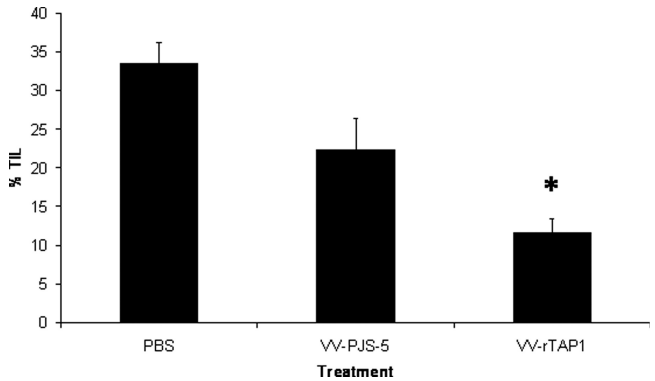

FIGURE 4 – IL-10-producing TILs are reduced in VV-rTAP-1 treated animals. The percentage of CD3$^+$/IL-10 positive TILs (mean i ± SE, $n = 5$/group) in B16F10 cell tumors treated with VV-rTAP1, VV-PJS-5, or PBS. *One-way ANOVA shows a significant reduction in IL-10 positive TILs in tumors after treatment with VV-rTAP1 ($p < 0.001$).

TAP1 expression reduces B16F10 cell growth in vivo

The effect of TAP1 expression on tumor growth rate was examined in a syngeneic mouse model. B16/r TAP 3–8 and B16F10 tumors were grown in the hindquarters of mice and treated with either a vaccinia rTAP1 expression vector (VV-rTAP1), vaccinia vector control (VV-PJS-5) or PBS. Tumor growth rates were analyzed by 2-way ANOVA, which revealed significant reductions in tumor mass due to the effects of both rTAP transfection ($p < 0.001$) and the vaccinia treatments ($p < 0.001$). There was a significant interaction ($p < 0.01$), however, between the main effects. The nature of the interaction was further characterized by comparisons between treatment groups. Multiple comparisons showed that the growth rates of B16F10 tumors were significantly slowed by treatment with VV-rTAP1 ($p < 0.001$) but not by treatment with VV-PJS-5. On the other hand B16/r TAP 3–8 tumor growth rates were significantly retarded by both VV-rTAP1 and VV-PJS-5 ($p < 0.001$) but there was no difference between the 2 vaccinia vectors ($p > 0.05$) (Fig. 3).

IL-10 expression by tumor infiltrating lymphocytes is decreased in tumors treated with VV-rTAP1

The expression of IL-10, a Th2 cytokine capable of inhibiting cytotoxic T-cell responses, was measured in T lymphocytes infiltrating B16F10 cells tumors treated with VV-rTAP1, VV-PJS-5 or PBS. The percentage of TILs expressing IL-10 in tumors was determined by FACS analysis, and the treatments compared using 1-way ANOVA (Fig. 4). Tumors treated with VV-rTAP1 had significantly reduced CD3$^+$ lymphocytes expressing IL-10 compared to VV-PJS-5 ($p < 0.05$) and PBS ($p < 0.001$) treatments. The treatment of tumors with VV-PJS-5 also significantly reduced the number of IL-10 expressing CD3$^+$ lymphocytes compared to tumors treated with PBS ($p < 0.05$).

Discussion

The restoration of antigen processing, MHC Class I antigen expression, and immunogenicity by transfection or infection of TAP1 alone into B16F10 MAA cells occurs despite numerous other deficiencies in the antigen presentation pathway. This has also been demonstrated in other cell lines with similar antigen presentation-deficient phenotypes, such as the murine NSCLC cell line CMT.64, human MAA, human small cell lung carcinoma, human squamous cell carcinoma of the head and neck and human renal cell carcinomas.[20,22,25–29] In B16F10 cells, TAP1 expression stabilized the expression of TAP2 and increased the expression of endogenous tapasin. This indicates that the re-expression of TAP1 may lead to a general reconstitution of several other components of the MHC Class I antigen-processing pathway, and may therefore increase the amount of antigenic peptide available for assembly onto MHC Class I molecules in the ER. Restoration of TAP expression in TAP-deficient cancer cells should make a wide variety of peptides derived from tumor-specific and -associated antigens available and perhaps this may compensate for any unpredictable deficiencies in the cell's MHC Class I antigen allele repertoire.

In the case of B16F10 cells, rTAP1 gene transfer was able to resurrect the presentation of the appropriate TRP-2 peptide on H-2K^b antigens to allow for TRP-2-specific CTL killing. B16/rTAP1 cells present H-2D^b antigen-specific peptides derived from gp100, making B16/rTAP1 cells susceptible to specific CTL lysis both *in vitro* and *in vivo*[30]. Vaccination by irradiated cells expressing TAP1 enhances greatly the CTL activity not only towards B16/rTAP1 target cells but also to untransfected B16F10 target cells. This indicates that it is likely not necessary for every cell to re-express TAP1 for immune tolerance to the tumor to be broken, allowing CD8$^+$ cytotoxic T-cell responses to occur. This is further supported by our previous *in vivo* study with CMT.64 lung carcinoma, in which mice initially immunized with CMT.64 expressing TAP1 were better able to reject a challenge with untransfected CMT.64, unlike mice initially immunized with untransfected CMT.64.[22] Perhaps encouraging for applications to metastatic disease, the lysis of B16F10 cells by splenocytes generated by vaccination with irradiated B16/rTAP1 cells demonstrates that there is sufficient H-2 antigen on the surface of B16F10 cells to facilitate cytolytic activity. TAP1 activity or the products of TAP1 activity in B16/rTAP1 cells must be transferred in some way to the dendritic cells involved in the cross-presentation of MHC Class I tumor antigens, a crucial step in generating specific CD8$^+$ cytotoxic T-cell responses. TAP1 expression in B16/rTAP1 cells results in a source of antigen that may be bound to MHC Class I antigens on the surface of B16/rTAP1 cells and these MHC Class I antigen-restricted antigens are transferred to the dendritic cell MHC Class I antigens. Alternatively, dendritic cells may access processed tumor antigens from the ER compartment of B16/rTAP1 cells during internalization and antigen cross-presentation.

The enhanced immunogenicity of B16/rTAP1 cells has a significant effect on tumor growth in mice. Vaccination of mice with MAA antigens specific for H-2D^b antigen are protected from B16/rTAP1 tumor challenge but not by challenge with B16F10 cells by CD8$^+$ T-cells.[30] In our study, B16/rTAP1 tumor growth was retarded without prior vaccination with tumor differentiation antigens, though the protection was not as complete as with prior vaccination. The effect also appears when TAP1 is transferred *in vivo* by vaccinia expression vectors. In this case, not only does TAP1 expression have an effect on tumor growth, but also the vector provides an adjuvant effect. In addition, we found that *in vitro* infection of B16F10 cells transfected with TAP1 (B16/ r TAP1 3–8 cells) with VV-rTAP1 did not greatly enhance H-2K^b and H-2D^b antigen surface expression relative to VV-PJS-5 infected cells. This may indicate that TAP activity is already at a maximum as a result of transfection, and that additional expression of TAP1 from the VV construct does not further upregulate MHC Class I antigen expression to a great extent. We propose that in addition to tumor antigens, upon infection the tumor cells also present viral antigens in the presence of TAP that provide further epitopes for CTL recognition. The presence of the VV during the processing tumor antigens by DCs may also assist in breaking the tolerance of the immune system towards the tumor cells by acting as a ''danger signal'' that contributes to the priming of tumor antigen-specific immune responses.[31,32] Furthermore, B16F10 tumors are known to harbor regulatory T-cells that secrete cytokines, such as IL-10, which energize CD8$^+$ T-cells in tumors.[33] TAP1 gene transfer by vaccinia vectors reduced the number of CD3$^+$/IL-10 secreting lymphocytes in the tumors, one of the modulating factors that have been implicated in the resistance of MAAs to antitumor immune responses. TAP1 expression, in conjunction with viral gene transfer vector, promotes a Th1 type response that can function to retard tumor growth. Thus, immunotherapeutic approaches that utilize TAP have the potential to restore the priming and expansion of specific T-cells and the subsequent ability of the tumors to process and present tumor antigens.

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References

1. Hauschild A, Weichenthal M, Balda BR, Becker JC, Wolff HH, Tilgen W, Schulte KW, Ring J, Schadendorf D, Lischner S, Burg G, Dummer R. Prospective randomized trial of interferon α-2b and interleukin-2 as adjuvant treatment for resected intermediate- and high-risk primary melanoma without clinically detectable node metastasis. J Clin Oncol 2003;21:2883–88.
2. Hancock BW, Wheatley K, Harris S, Ives N, Harrison G, Horsman JM, Middleton MR, Thatcher N, Lorigan PC, Marsden JR, Burrows L, Gore M. Adjuvant interferon in high-risk melanoma: the AIM HIGH Study–United Kingdom Coordinating Committee on Cancer Research randomized study of adjuvant low-dose extended-duration interferon α-2a in high-risk resected malignant melanoma. J Clin Oncol 2004;22:53–61.
3. Kleeberg UR, Suciu S, Brocker EB, Ruiter DJ, Chartier C, Lienard D, Marsden J, Schadendorf D, Eggermont AM; EORTC Melanoma Group in cooperation with the German Cancer Society (DKG). Final results of the EORTC 18871/DKG 80-1 randomised phase III trial. rIFN-α2b versus rIFN-γ versus ISCADOR M versus observation after surgery in melanoma patients with either high-risk primary (thickness > 3 mm) or regional lymph node metastasis. Eur J Cancer 2004;40:390–402.
4. Panagopoulos E, Murray D. Metastatic malignant melanoma of unknown primary origin: a study of 30 cases. J Surg Oncol 1983;23:8–10.
5. Barnetson RS, Halliday GM. Regression in skin tumours: a common phenomenon. Australas J Dermatol 1997;38:S63–S65.
6. Menzies SW, McCarthy WH. Complete regression of primary cutaneous malignant melanoma. Arch Surg 1997;132:553–6.
7. Wagner SN, Schultewolter T, Wagner C, Briedigkeit L, Becker JC, Kwasnicka HM, Goos M. Immune response against human primary malignant melanoma: a distinct cytokine mRNA profile associated with spontaneous regression. Lab Invest 1998;78:541–50.
8. Dissemond J, Gotte P, Mors J, Lindeke A, Goos M, Ferrone S, Wagner SN. Association of TAP1 downregulation in human primary melanoma lesions with lack of spontaneous regression. Melanoma Res 2003;13:253–8.
9. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643–7.
10. Smith C, Cerundolo V. Immunotherapy of melanoma. Immunology 2001;104:1–7.
11. Khong HT, Wang QJ, Rosenberg SA. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother 2004;27:184–90.

12. Vicari AP, Caux C, Trinchieri G. Tumour escape from immune surveillance through dendritic cell inactivation. Semin Cancer Biol 2002;12:33–42.
13. Whiteside TL. Tumor-induced death of immune cells: its mechanisms and consequences. Semin Cancer Biol 2002;12:43–50.
14. Ruiz-Cabello F, Cabrera T, Lopez-Nevot M, Garrido F. Impaired surface antigen presentation in tumors: implications for T cell-based immunotherapy. Semin Cancer Biol 2002;12:403–5.
15. Seliger B, Cabrera T, Garrido F, Ferrone S. HLA class I antigen abnormalities and immune escape by malignant cells. Semin Cancer Biol 2002;12:3–13.
16. Kageshita T, Hirai S, Ono T, Hicklin DJ, Ferrone S. Down-regulation of HLA class I antigen-processing molecules in malignant melanoma: association with disease progression. Am J Pathol 1999;154: 745–54.
17. Kamarashev J, Ferrone S, Seifert B, Boni R, Nestle FO, Burg G, Dummer R. TAP1 down-regulation in primary melanoma lesions: an independent marker of poor prognosis. Int J Cancer 2001;95:23–8.
18. Cresswell AC, Sisley K, Laws D, Parsons MA, Rennie IG, Murray AK. Reduced expression of TAP-1 and TAP-2 in posterior uveal melanoma is associated with progression to metastatic disease. Melanoma Res 2001;11:275–81.
19. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence and survival in epithelial ovarian cancer. N Engl J Med 2003;348:203–13.
20. Seliger B, Ritz U, Abele R, Bock M, Tampe R, Sutter G, Drexler I, Huber C, Ferrone S. Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. Cancer Res 2001;61:8647–50.
21. Seliger B, Wollscheid U, Momburg F, Blankenstein T, Huber C. Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. Cancer Res 2001;61:1095–9.
22. Alimonti J, Zhang QJ, Gabathuler R, Reid G, Chen SS, Jefferies WA. TAP expression provides a general method for improving the recognition of malignant cells in vivo. Nat Biotechnol 2000;18:515–20.
23. Harris MR, Lybarger L, Yu YY, Myers NB, Hansen TH. Association of ERp57 with mouse MHC class I molecules is tapasin dependent and mimics that of calreticulin and not calnexin. J Immunol 2001; 166:6686–92.
24. Layton MG, Franks LM. Heterogeneity in a spontaneous mouse lung carcinoma: selection and characterisation of stable metastatic variants. Br J Cancer 1984;49:415–21.
25. Kallfelz M, Jung D, Hilmes C, Knuth A, Jaeger E, Huber C, Seliger B. Induction of immunogenicity of a human renal-cell carcinoma cell line by TAP1-gene transfer. Int J Cancer 1999;81:125–33.
26. Singal DP, Ye M, Bienzle D. Transfection of TAP 1 gene restores HLA class I expression in human small-cell lung carcinoma. Int J Cancer 1998;75:112–6.
27. Gabathuler R, Reid G, Kolaitis G, Driscoll J, Jefferies WA. Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. J Exp Med 1994;180: 1415–25.
28. Lopez-Albaitero A, Nayak JV, Ogino T, Machandia A, Gooding W, DeLeo AB, Ferrone S, Ferris RL. Role of antigen-processing machinery in the in vitro resistance of squamous cell carcinoma of the head and neck cells to recognition by CTL. J Immunol 2006;176:3402–9.
29. White CA, Thomson SA, Cooper L, van Endert PM, Tampe R, Coupar B, Qiu L, Parsons PG, Moss DJ, Khanna R. Constitutive transduction of peptide transporter and HLA genes restores antigen processing function and cytotoxic T cell-mediated immune recognition of human melanoma cells. Int J Cancer 1998;75:590–5.
30. Leitch J, Fraser K, Lane C, Putzu K, Adema GJ, Zhang QJ, Jefferies WA, Bramson JL, Wan Y. CTL-dependent and -independent antitumor immunity is determined by the tumor not the vaccine. J Immunol 2004;172:5200–5.
31. Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology 1996;219:339–49.
32. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 2001;413:732–8.
33. Seo N, Hayakawa S, Takigawa M, Tokura Y. Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. Immunology 2001;103:449–57.