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

Qian-Jin Zhang1,1, Robyn P. Seipp1,2,3, Susan S. Chen1,2,3, Timothy Z. Vitalis1, Xiao-Lin Li1,1, Kyung-Bok Choi1,2, Andrew Jeffries1,2 and Wilfred A. Jeffries1,2,3,4,5*

1Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada
2Department of Zoology, University of British Columbia, Vancouver, BC, Canada
3Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada
4Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada
5Correspondence to: Biomedical Research Center and the Michael Smith Laboratories, 2222 Health Sciences Mall, University of British Columbia, Vancouver, British Columbia, V6T 1Z3 Canada.

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

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 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 tumor cells in mice with a vaccinia virus vector expressing TAP1 also significantly decreases tumor growth in vivo. Furthermore, tumors treated with vaccinia TAPI had significantly reduced numbers of immunosuppressive, CD8+IL-10 positive, tumor infiltrating lymphocytes. Therefore, TAPI 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.

© 2007 Wiley-Liss, Inc.

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 pigmentation, immune suppression, atypical nevi and family history are risk factors for MAA. The early stage disease is curable but recurrence 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 recurrence 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.4,5

The discovery of tumor antigens associated with MAA1 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-cells13 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.

First three authors contributed equally to this work.

© 2007 Wiley-Liss, Inc.

DOI 10.1002/ijc.22371

Published online 2 February 2007 in Wiley InterScience (www.interscience.wiley.com).
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.\textsuperscript{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.\textsuperscript{16-18} Conversely, TAP1 expression has been associated with tumor infiltrating lymphocytes (TILs), a characteristic of good clinical outcome,\textsuperscript{8,16,19} and spontaneous regression of MAA.\textsuperscript{4}

In our study, we examined 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, PA28a and β, and the chaperone tapasin.\textsuperscript{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\textsuperscript{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 \textit{ad libitum}. The colony was routinely screened for \textit{Mycoplasma pulmonis} and \textit{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 MD\textsuperscript{]}. Recombinant vaccinia virus (VV) either carrying rat-TAP1

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 L-glutamine (20 mM). CV-1, CMT.64 (murine lung carcinoma)\textsuperscript{22} and murine 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 (Apri-Neo) and maintained in Geneticin (Invitrogen, Burlington, ON, Canada) selecting RPMI-1640 medium.\textsuperscript{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/PJS-5 1–1.

Detection of TAP protein expression in B16F10 cells transfectants by immunoblotting

rTAP1 and mouse TAP2 expression in B16/rtTAP1 3–3 cells and B16/rTAP1 3–8 cells was examined by immunoblotting. Total extracts from 5 × 10\textsuperscript{6} 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, RGCGYRAMVEALAAPAD-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\textsuperscript{-/-} 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\textsuperscript{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\textsuperscript{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 PVDF membranes (GE Healthcare) and probed with the same antiserum followed by ECL as described earlier.

Detection of surface H-2K\textsuperscript{b}, H-2D\textsuperscript{b} and I-\textsuperscript{a} antigen expression

B16F10 or B16/rtTAP1 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\textsubscript{2}), followed by fixation and preparation for FACS analysis. Indirect immunofluorescence staining with conformational specific monoclonal antibodies (Abs) for H-2K\textsuperscript{b} (Y-3, ATCC) and H-2D\textsuperscript{b} (28.14.8.S, ATCC) was confirmed by the detection of a band of 200–300 amino acids in the immunoprecipitated band 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\textsuperscript{-/-} mice. The rabbit serum against mouse and rTAP2 (116/4) was kindly provided by Dr. Geoff Butcher (University of Cambridge, Cambridge, UK). For mouse tapasin expression, 1.2 × 10\textsuperscript{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\textsuperscript{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 PVDF membranes (GE Healthcare) and probed with the same antiserum followed by ECL as described earlier.

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\textsubscript{2}, H-2K\textsuperscript{b} antigen-restricted, VSV-specific CTLs were generated by infection of mice (i.p.) with VSV (10\textsuperscript{7} TCID\textsubscript{50}). Splenocytes were harvested 5 days after infection and cultured with additional 5 days in media (10\textsuperscript{6} cells/ml) containing VSV-Np52-59 peptide (RGYVYQGL) (1 μg/ml) at 37°C, 5% CO\textsubscript{2}. To generate H-2K\textsuperscript{b} antigen-restricted TAP2-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 medium) 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 (10^6 cells) were cultured for 5 days with γ-irradiated naïve splenocytes (5 × 10^5 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 × 10^5 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% CO₂. 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 ⁵¹Cr 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 Na²⁴CrO₄ (70 μCi/10⁶ 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 × 10⁵ 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 × 10⁶ PFU/injection), VV-PJS-5 (2 × 10⁶ 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 (²) 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 α = 0.05 (95% 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-2Kb and H-2Dd antigen surface expression, but not MHC Class II (I-A^d) 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 (Tp) 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.²²,²³ Fibroblasts derived from tapasin⁻/⁻ mice were used as a negative control for mouse tapa-
sin. B16/rTAP1 3–3, B16/rTAP1 3–8 and B16/PHB b 1–1 cells were tested for rTAP1 and mouse TAP2 and tapasin expression (Fig. 1a). Normal B16F10 cells and B16/PHB b 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/PHB b 1–1 cells, using FACS analysis. The antibodies used for the FACS analysis are conformation-specific and only bind to H-2Kb, H-2Db and I-Ab antigens properly folded and loaded with antigen peptide. 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/PHB b 1–1 cells, using FACS analysis. The antibodies used for the FACS analysis are conformation-specific and only bind to H-2Kb, H-2Db and I-Ab 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-2Kb and H-2Db antigens on the cell surface (Fig. 1b). This was in contrast to B16F10 cells and B16/PHB b 1–1 cells, which had undetectable levels of H-2Kb antigen on the cell surface and only a small amount of H-2Db antigen. Both rTAP1 and vector-alone transfected B16F10 cells exhibited a small population of cells positive for surface I-Ab 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.

**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/PHB b 1–1 cells and B16/rTAP1 3–3 cells and B16/rTAP1 3–8 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/PHB b 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/PHB b 1–1 cells and B16/rTAP1 3–3 cells were used as effectors against the indicated targets.

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/PHB b 1–1 and B16F10 cells to process and present the H-2Kb antigen-specific, immuno-dominant VSV antigen: VSV-Np52–59. VSV-Np52–59-specific cytotoxic splenocytes were able to kill VSV-infected B16/rTAP1 3–3 cells but not VSV-infected B16/PHB b 1–1 or B16F10 cells in a 51Cr release assay (Fig. 2a). B16/rTAP1 3–3 cells were able to correctly process and present viral antigens but B16/PHB b 1–1 or B16F10 cells were not. We also examined if rTAP1 expression was able to restore the surface presentation of the H-2Kb antigen-restricted tumor-associated antigen, TRP-2. The 51Cr 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/PHB b 1–1 cells, which were resistant to killing (Fig. 2b). Both B16/rTAP1 3–3 and B16/rTAP1 3–8 cells were therefore able to present tumor-associated antigens, in the context of H-2Kb 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
TAP1 expression restores immunogenicity

**TABLE I – VV-rTAP1 INFECTION OF B16F10 AND B16/r TAP 1–8 CELLS RESULTS IN INCREASED SURFACE MHC CLASS I ANTIGEN EXPRESSION COMPARED TO VV-PJS-5**

| Cell line          | Fold increase H-2K<sup>b</sup> | T (X) value, p value | Fold increase H-2D<sup>d</sup> | T (X) value, p value |
|--------------------|-------------------------------|---------------------|-------------------------------|---------------------|
| B16F10             | 0.52                          | 4.4711, <i>p</i> < 0.01 | 1.8                           | 24.052, <i>p</i> < 0.01 |
| B16/r TAP 1–8      | 0.16                          | 10.462, <i>p</i> < 0.01 | 0.81                          | 2.1617, 0.01 > <i>p</i> > 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<sup>b</sup> and H-2D<sup>d</sup> antigens.

---

**FIGURE 3** – The effect of TAP1 expression on tumor growth was determined in subcutaneous B16F10 and B16/rTAP 1–8 tumors. Mice were injected subcutaneously with 1.5 × 10<sup>6</sup> cells in the hindquarter. About 1 and 7 days later, mice were injected at the site of the tumor with 2 × 10<sup>6</sup> 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/rTAP 1–8 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. 2c).

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.

**FIGURE 4** – IL-10-producing TILs are reduced in VV-rTAP-1 treated animals. The percentage of CD3<sup>+</sup>/IL-10 positive TILs (mean ± 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 (<i>p</i> < 0.001).

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

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<sup>b</sup> antigen is upregulated by 0.52-fold (<i>p</i> < 0.01; T(X) = 4.4711) in VV-rTAP1-infected B16F10 cells relative to VV-PJS-5-infected cells, H-2D<sup>d</sup> antigen is upregulated 1.8-fold (<i>p</i> < 0.01; T(X) = 10.462). Interestingly, in B16/rTAP 1–8 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<sup>b</sup> antigen (<i>p</i> < 0.01; T(X) = 24.052) and 0.81-fold for H-2D<sup>d</sup> antigen (<i>p</i> < 0.17 but > 0.01; T(X) = 2.1617) (Table I).

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<sup>+</sup> lymphocytes expressing IL-10 compared to VV-PJS-5 (<i>p</i> < 0.05) and PBS (<i>p</i> < 0.001) treatments. The treatment of tumors with VV-PJS-5 also significantly reduced the number of IL-10 expressing CD3<sup>+</sup> lymphocytes compared to tumors treated with PBS (<i>p</i> < 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 carcinoma.

In B16F10 cells, TAP1 expression stabilized the expression of TAP2 and increased the expression of endogenous tapases. 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\(^+\) antigens to allow for TRP-2-specific CTL killing. B16/rTAP1 cells present H-2D\(^+\) antigen-specific peptides derived from gp100, making B16/rTAP1 cells susceptible to specific CTL lysis both in vitro and in vivo.\(^{40}\) 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. 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.\(^{25}\) 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 may therefore increase the 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 antigens to allow for TRP-2-specific CTL killing. B16/rTAP1 cells with VV-TRP1 did not greatly enhance H-2K\(^+\) and H-2D\(^+\) antigen surface expression relative to VV-PIS-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 cell's MHC Class I antigen expression may 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 B16/rTAP1 (1×10\(^{6}\) cells) with VV-rTAP1 did not greatly enhance H-2K\(^+\) and H-2D\(^+\) antigen surface expression relative to VV-PIS-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.

Acknowledgements

The authors acknowledge the assistance of Ms. Eunice Yao, Ms. Kyla Omlulis and Dr. Anna Reinicke in article preparation. The authors also thank Dr. Geoff Butcher for the TAP2 antisera, Dr. Luc Van Kaer for the murine tapasin 1 fibroblasts and Dr. Ted Hansen for the rabbit antisera to mouse tapasin.

References

1. Hauschild A, Weichenthal M, Balda BR, Becker JC, Wolff HH, Tilgen W, Schulte KW, Ring J, Schadendorf D, Lischinger S, Burg G, Dummer R. Prospective randomized trial of interferon-α-2b and interferon-β-2a as adjuvant treatment for resected intermediate- and high-risk primary melanoma without clinically detectable node metastasis. J Clin Oncol 2003;21:2883–8.
2. Hancock BW, Wheatley K, Harris S, Ives N, Harrison G, Horsman 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.
3. Dissemong 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.
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;132:533–6.
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. Van der Bruggen P, Revsland C, Cushner P, Lorquin 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.
9. Smith C, Cerundolo V. Immunotherapy of melanoma. Immunology 2001;104:1–7.
10. Khong HT, Wang QI, 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, Conej-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Ruben SC, Coupar B, Qiu L, Parsons FG, 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.

20. Leitch J, Fraser K, Lane C, Putzu K, Adema GJ, Zhang QJ, Jefferies WA, Branson JL, Wan Y. CTL-dependent and -independent antitumor immunity is determined by the tumor not the vaccine. J Immunol 2004;172:5200–5.

21. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA by Toll-like receptor 3. Nature 2001;413:732–8.

22. 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.