

TAP expression provides a general method for improving the recognition of malignant cells in vivo

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A major class of tumors lack expression of the transporters associated with antigen processing (TAP). These proteins are essential for delivery of antigenic peptides into the lumen of the endoplasmic reticulum (ER) and subsequent assembly with nascent major histocompatibility complex (MHC) class I, which results in cell surface presentation of the trimeric complex to cytolytic T lymphocytes. Cytolytic T lymphocytes are major effector cells in immunosurveillance against tumors. Here we have tested the hypothesis that TAP downregulation in tumors allows immunosubversion of this effector mechanism, by establishing a model system to examine the role of TAP in vivo in restoring antigen presentation, immune recognition, and effects on malignancy of the TAP-deficient small-cell lung carcinoma, CMT.64. To test the potential of providing exogenous TAP in cancer therapies, we constructed a vaccinia virus (VV) containing the TAP1 gene and examined whether VV-TAP1 could reduce tumors in mice. The results demonstrate that TAP should be considered for inclusion in cancer therapies, as it is likely to provide a general method for increasing immune responses against tumors regardless of the antigenic complement of the tumor or the MHC haplotypes of the host.

Keywords: TAP, MHC, CTL, cancer therapy, immunosurveillance, carcinoma

Neoplastic cells arise frequently in the body in response to a variety of external and internal influences, and we depend on the immune system to recognize and destroy these cells before they develop into tumors. However, malignant transformations may be accompanied by phenotypic changes resulting in the ability of the cancer cells to escape the immunosurveillance mechanism. As the phenotypic changes vary with each neoplasm, it has so far been impossible to develop one therapy for all cancer. Fortunately, many tumor phenotypes fall into one of several larger groups.

One such group appears with increased tumorigenicity due to a decrease in major histocompatibility complex (MHC) class I expression¹⁻⁵. A decrease in cell surface expression of MHC class I can be due to a defect in the MHC class I biosynthetic pathway^{4,6}. Among the many cellular proteins that contribute to MHC class I assembly⁷⁻¹⁰, the TAP complex is one of the most important components. It functions to translocate endogenously processed peptides from the cytosol into the endoplasmic reticulum (ER) for binding to relevant MHC class I, resulting in cell surface presentation of these complexes to cytolytic T lymphocytes (CTLs). Loss of the TAP complex is highly correlated with loss of HLA expression in cervical carcinoma¹¹. In addition, a higher frequency of downregulation of this complex has been observed for metastatic lesions than for primary lesions³. The TAP complex has been particularly strongly implicated in tumorigenicity of several cancers such as melanomas, cervical carcinomas, and renal cell carcinomas^{3,12,13}. These findings suggest that TAP downregulation may represent an important mechanism for immune escape of malignant cells in a variety of tumors.

The immune system has evolved a very intricate recognition mechanism to eliminate diseased cells. In order to proliferate, a

tumor must evade the cells involved in tumor recognition. The major antitumor effector mechanisms are the CD8⁺ CTLs and natural killer (NK) cells (reviewed in ref. 4). The functions of both effectors are controlled by MHC class I. CD8⁺ CTLs recognize surface MHC class I-restricted tumor-associated antigens (TAA) and destroy tumor cells¹⁴, whereas NK cells only lyse the targets in the absence or downregulation of surface MHC class I.

Small-cell lung carcinomas (SCLCs) are highly malignant in humans and are generally fatal. In mice, SCLCs have similar characteristics. The CMT.64 cell line is a SCLC that arose spontaneously in a C57BL/6 mouse¹⁵, and is lacking in both MHC class I surface expression and endogenous antigen presentation. Interferon- γ (IFN- γ) treatment corrects these deficiencies, however, the underlying defect remains unknown^{16,17}. We have recently shown that CMT.64 cells are defective in many components of the antigen presentation pathway, such as MHC class I heavy chain, β_2 -m, proteasome subunits (LMP 2 and LMP 7), and TAP-1 and -2¹⁸. These defects can be remedied by IFN- γ treatment¹⁸. Although the CMT.64 cells are very poor at antigen presentation, reconstitution with rat TAP1 alone (rTAP1) or TAP1 and 2 heterodimer (rTAP1,2) restores viral antigen presentation in vitro^{18,19}. These data demonstrated that the blockage of antigen presentation in the SCLC CMT.64 is at the level of transport of peptide from the cytoplasm to the ER.

Many cancers have multiple cellular defects, making treatment difficult. However, the course of treatment would be simplified if restoring one defective component would allow the host's immune system to recognize and destroy the cancer. Many of the tumors lacking surface MHC class I have TAP deficits or dysfunctions³. If CMT.64 transfected with TAP is sufficient to restore antigen presentation in vitro, does it follow that the immune system will recognize

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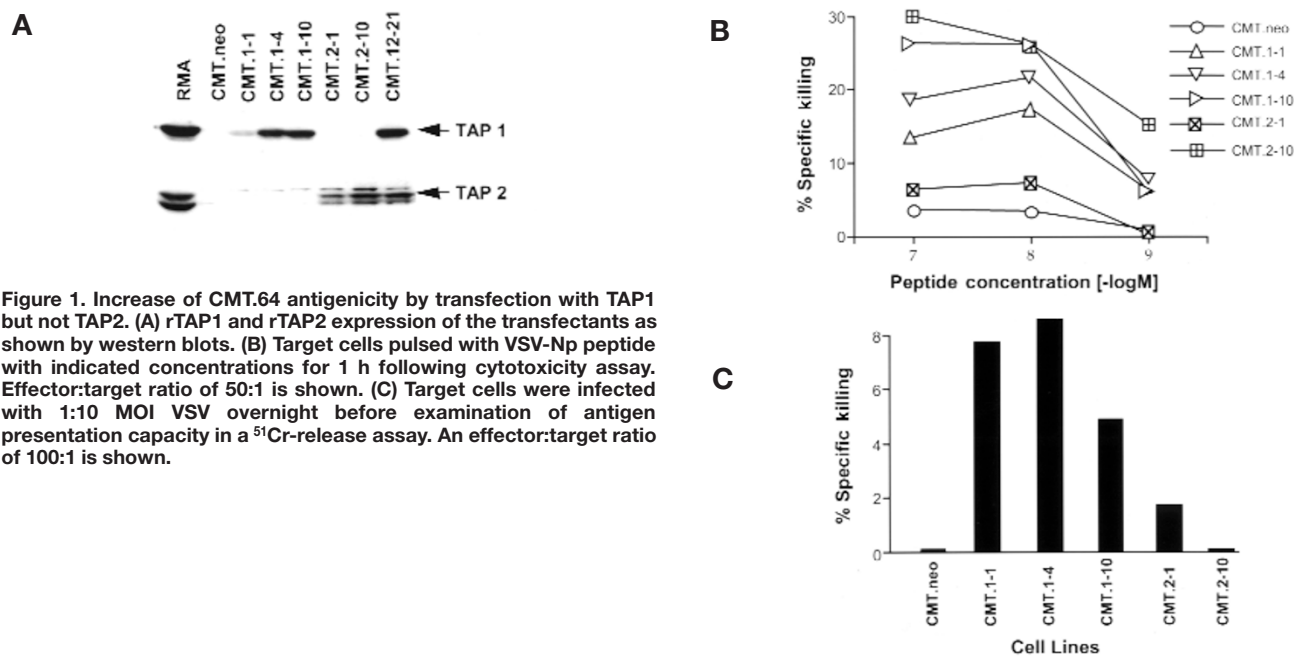


Figure 1. Increase of CMT.64 antigenicity by transfection with TAP1 but not TAP2. (A) rTAP1 and rTAP2 expression of the transfectants as shown by western blots. **(B)** Target cells pulsed with VSV-Np peptide with indicated concentrations for 1 h following cytotoxicity assay. Effector:target ratio of 50:1 is shown. **(C)** Target cells were infected with 1:10 MOI VSV overnight before examination of antigen presentation capacity in a ^{51}Cr -release assay. An effector:target ratio of 100:1 is shown.

the TAP-expressing tumor cells, destroy them, and thus prevent metastasis *in vivo*? To examine this possibility, we tested the TAP-deficient CMT.64, as well as TAP-transfected cell lines derived from CMT.64 *in vivo*, to determine whether TAP could improve the immune response against cancer cells and thus improve survival of animals bearing this tumor.

Results and discussion

Phenotype of TAP transfectants. The phenotype of the CMT.64 cell line has been described¹⁸. Transfection of rat TAPs (rTAP) into this cell line complements the expression of relevant components in the MHC class I-restricted antigen presentation pathway. Figure 1A shows transfectants expressing rTAP proteins. Two rTAP1-transfectant clones, CMT.1-4 and CMT.1-10, and one rTAP1,2-transfectant, CMT.12-21, express higher levels of rTAP1 protein than the CMT.1-1 clone (one of the rTAP1 clones). In comparison with RMA control cell line, these transfectants reveal a similar rTAP1 expression level (except CMT.1-1): CMT.1-1 clones express 10 times less, CMT.1-4 and CMT.1-10 express 2.5 times less, and CMT.12-21 expresses 2 times less. The rTAP2 expression levels in the transfectants were also examined in relation to RMA: CMT.2-1 and CMT.2-10 (two rTAP2-transfectant clones) express four times and two times less, respectively, and CMT.12-21 two times less. Thus, in comparison to RMA, all rTAP transfectants, except CMT.1-1, express similar levels of rTAP proteins.

TAP supplies peptides that bind to MHC class I molecules, resulting in their surface expression. Therefore, we examined MHC class I expression on the surface of transfectants by fluorescence-activated cell-sorting (FACS) analysis. Although TAPs were introduced into the CMT.64 cells, surface expression of MHC class I did not dramatically increase (Table 1), as judged by comparison to IFN- γ -treated CMT.64 cells, which restored high levels of MHC class I expression. This suggests that downregulation of MHC class I in CMT.64 cells likely occurs at the transcriptional level. However, constitutive expression of TAP restores some surface MHC class I expression (Table 1). It is noteworthy that the levels of MHC class I expression on the surface of TAP-transfectant clones quantitatively predict antigenic peptide binding. Cells pulsed with the immunodominant peptide derived from vesicular stomatitis virus nucleoprotein (VSV-Np) were killed equally well in a cytotoxic CTL assay, demonstrating that functional amounts of MHC

class I are expressed on all transfectants, except CMT.neo and CMT.2-1 (Fig. 1B).

It is well known that the presentation of endogenously generated antigenic peptides to the cell surface for CTL recognition requires that peptides have the capacity to be transported by TAP and to bind to relevant MHC class I. An additional requirement is that there be sufficient quantities of peptides generated in the cytosol. Peptide-pulse experiments merely indicate whether surface MHC class I expression for CTL recognition is sufficient but do not confirm the overall antigen presentation capacity. Thus, we infected transfectants with VSV at a multiplicity of infection (MOI) overnight and of 10:1, then performed cytotoxicity assays. As shown in Figure 1C, the results demonstrated that three clones of the rTAP1 transfectant were able to present the immunodominant epitope, VSV-Np, whereas two rTAP2 clones and CMT.neo were unable to present this epitope efficiently. In a separate experiment, a clone transfected with rTAP1,2, CMT.12-21, also presented this epitope efficiently (data not shown). Our results suggest that only rTAP1 or rTAP1 and 2, but not rTAP2-transfected clones increase their antigenicity and acquire the ability to process and present foreign antigens. This difference cannot be attributed to levels of rTAP2 expression, since all TAP transfectants, except CMT.1-1 clone, express similar levels of TAP1 and/or TAP2 compared with RMA-TAPs. Taken together, antigen

Table 1. Comparison of MHC class I expression on surface of the CMT-TAP transfectants^a

Cell line	D ^b	K ^b
CMT.64	18	0
CMT.neo	N.D.	0.1
CMT.1-1	N.D.	62
CMT.1-4	164	64
CMT.1-10	68	36
CMT.2-1	10	3
CMT.2-10	39	48
CMT.12-21	103	22
CMT.64 + IFN- γ	1,100	590

^aSurface expression of D^b and K^b molecules was performed on the CMT.64 and its TAP transfectants by FACS analysis. The monoclonal antibodies, Y-3 (against K^b) and 28.14.8.S (against D^b) were used in this assay. The results are normalized by subtracting immunofluorescence intensity of negative control from each result. N.D., Not determined.

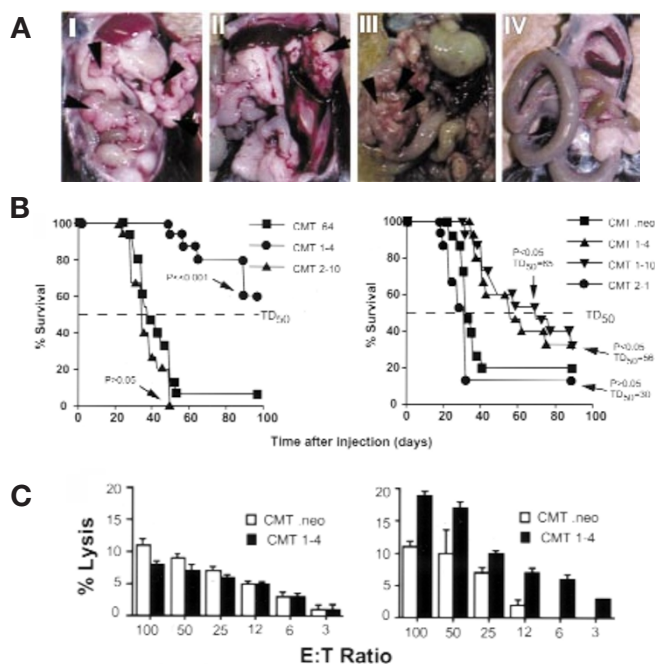


Figure 2. Control of tumor growth in vivo and improvement of mice survival by introducing rTAP heterodimer or rTAP1 but not rTAP2 into CMT.64 tumor cell line. CMT.64 or its transfectants were injected intraperitoneally into syngeneic mice. (A) After one month, one representative mouse from each group was sacrificed and the tumor growth pattern was examined. Panel I, CMT.neo; panel II, CMT.1-10; panel III, CMT.2-10; panel IV, CMT.12-21. The arrows indicate the tumors. (B) The time after injection of morbidity in mice was recorded for each group. Statistical analysis yields p value, comparing survival rates of CMT.64-bearing mice (left panel) or CMT.neo-bearing mice (right panel). (C) The specificity of splenocytes from a mouse injected with CMT.neo (left panel) or CMT.1-4 (right panel) was determined in a CTL assay against the targets CMT.neo and CMT.1-4.

presentation appears to largely depend on TAP1 function or TAP1,2 heterodimer function in CMT.64 transfectants.

TAP1 improves immune recognition of tumors in vivo. Since in vitro experiments provide evidence that rTAP is able to improve specific CTL recognition, these results could be applied in immunosurveillance against tumors in vivo. To test this hypothesis, we first determined whether the host immune system could control the growth of CMT.64 transfectants. Mice were injected with CMT.neo or rTAP-transfected cells. On day 30 after injection, one representative mouse from each group was killed in order to examine the tumor growth pattern. The results are depicted in Figure 2A. Two rTAP2-transfected cell lines, CMT.2-1 and CMT.2-10 (see Fig. 2A, panel III for one example), had a tumor growth pattern identical to the control tumor, CMT.neo (Fig. 2A, panel I). Interestingly, in rTAP1 or rTAP1,2 transfectants, either tumors grew to form one large tumor (CMT.1-1 and CMT.1-10) (Fig. 2A, panel II, one example) or no tumor was present (CMT.1-4 and CMT.12-21) (Fig. 2A, panel IV, one example). Furthermore, on day 60, CMT.1-4 tumors demonstrated the same growth pattern as the other rTAP1-transfected clones (data not shown). These results suggest that tumors with rTAP1 or rTAP1 and 2 have limited tumor foci or are absent, whereas rTAP2-transfected tumors have the same level

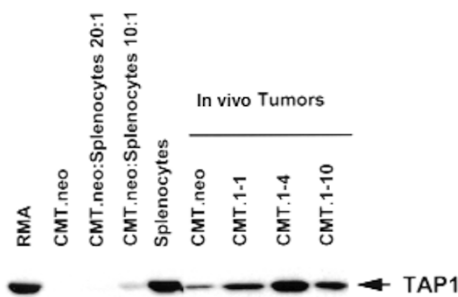


Figure 3. TAP1 expression within growing tumors in mice. TAP1 expression of in vivo tumors or cell lines was detected by western blot using D90 rabbit serum specific for rat and mice TAP1.

of metastasis as the wild-type tumors. This is true for other tumor-bearing mice (data not shown).

In a subsequent set of experiments, we addressed whether or not immune recognition of TAP1-transfected tumors could prolong the survival of tumor-bearing animals. Figure 2B depicts two independent experiments and summarizes the survival rates of mice injected intraperitoneally with CMT.64, or rTAP1- or rTAP2-transfected clones. At day 40–42 postinjection, 50% of CMT.64 and CMT.2-10 tumor-bearing group mice had died (Fig. 2B, left panel), and statistical analysis demonstrated no difference between these two groups ($p \gg 0.05$). In contrast, after 100 days 60% of the CMT.1-4 group mice were still alive ($p < 0.001$) (Fig. 2B, left panel). To confirm that increased survival is not due to variation of rTAP1-transfected clones, in a repeated experiment with other clones, we also confirmed protection in another rTAP1-expressing clone ($p < 0.05$) but not rTAP2 ($p > 0.05$) (Fig. 2B, right panel). This demonstrates that this effect is not specific to a single TAP1-expressing clone. Autopsy examination of all mice represented in Figure 2B revealed the patterns noted Figure 2A. As an experimental control, no difference was observed between CMT.64 and CMT.neo cell lines (data not shown). Our results suggest that improvement of the survival rates of rTAP1 tumor-bearing mice may be due to enhancement of the tumor's immunogenicity, which in turn would trigger the antitumor immune response of the hosts.

The nature of tumor recognition. T cells are critical factors in the defense against the development of most tumors. The presence of lymphocytic infiltrates within many malignant tumors has been suggested to indicate an in vivo antitumor immune response²⁰. Since

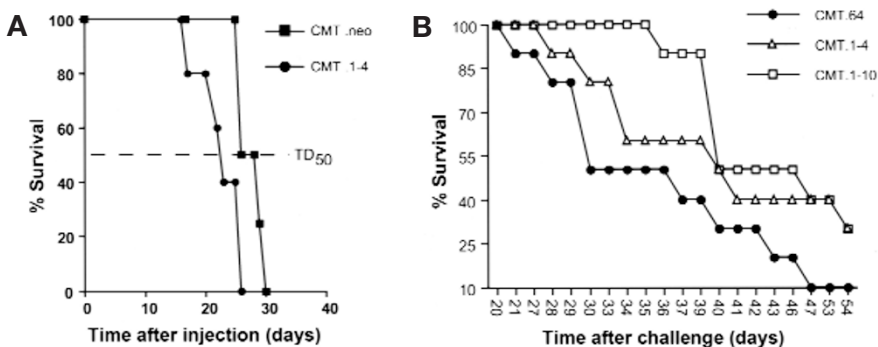


Figure 4. Examination of the survival of mice bearing CMT tumors. (A) rTAP1 does not improve recognition of CMT.64 in nude mice. A total of 5×10^5 cells of either CMT.1-4 (five mice) or CMT.neo (four mice) were injected intraperitoneally into nude mice (H-2^b). The time of morbidity was recorded for each group. (B) Immunization with rTAP-transfected tumors improves the survival of CMT.64-bearing mice. A total of 1×10^7 cells of CMT.neo, CMT.1-4, or CMT.1-10 were treated with mitomycin C (30 mg ml^{-1}) for 2 h and γ -irradiated before intraperitoneal injection into 10 C57BL/6 mice. One month later the mice were challenged intraperitoneally with 5×10^5 CMT.64 cells in PBS. The time of morbidity was recorded.

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Table 2. Percentage of tumor-infiltrating lymphocytes within growing tumors in mice^a

Tumor	One month		Two months		Ratio ^b	
	CD4 %	CD8 %	CD4 %	CD8 %	CD4	CD8
CMT.neo	0.54	0.41				
CMT.1-1	1.02	0.87			1.9	2.1
CMT.1-4			1.07	1.20	2.0	2.9
CMT.1-10	4.19	2.94			7.8	7.2
CMT.2-1	0.24	0.23			0.4	0.6
CMT.2-10	0.12	0.66			0.2	1.6

^aCD4 and CD8 T cells in tumors were detected by FACS analysis using monoclonal antibodies, RM4-5 (against CD4 or CD8 positive cells) and 53-6.7 (against CD8). % = 100% × number total cells (including tumor cells).

^bRatio = CD4 % or CD8 % of TAP transfectants/CD4 % or CD8 % of CMT.neo.

in vivo protection from rTAP1 tumors is controlled by the host immune system, we compared the percentage of tumor-infiltrating lymphocytes (TILs) between different tumors. We examined TILs in animals one month or two months after injection of CMT.64 transfectants using flow cytometry. The results are shown in Table 2. The ratios of CD4⁺ and CD8⁺ T cells were enhanced by two to eight times in rTAP1 tumors compared with control, CMT.neo, and rTAP2 tumors.

The observed increases of TIL in TAP1 tumors suggest the presence of specific cytolytic T cells. This possibility is based on three lines of evidence: (1) TAP1 increases tumor surface MHC class I; (2) TAP1 improves antigen presentation; (3) TAP1 results in tumors being controlled *in vivo* and improves animal survival. If CMT.64 cells contain a TAA, then specific CTLs should be generated by antigen presentation in rTAP1 transfectants *in vivo*. We performed CTL analysis using splenocytes from mice immunized with CMT.neo or CMT.1-4. CMT.neo-stimulated and CMT.1-4-stimulated splenocytes were compared against CMT.neo and CMT.1-4 targets in a standard ⁵¹Cr-release assay. Splenocytes from CMT.1-4-immunized mice were much better than splenocytes from CMT.neo mice at killing target cells (Fig. 2C). Killing of CMT.neo and CMT.1-4 targets by CMT.neo splenocytes was equivalent (Fig. 2C, left panel). In contrast, killing by CMT.1-4-stimulated splenocytes of CMT.1-4 targets was enhanced at both high and low effector:target (E:T) ratios (Fig. 2C, right panel). These results suggest that CMT.1-4 cells contain an antigenic antigen(s), TAA, and that this antigen can be presented by TAP1-expressing tumors, triggering host T-cell recognition.

The importance of T cells in antitumor immunity has been further confirmed by using athymic mice, which are devoid of T lymphocytes²¹. Unlike wild-type animals, the survival rates of athymic mice were not significantly different between CMT.neo- and CMT.1-4-bearing mice groups (Fig. 4A).

Immunization with rTAP1-transfected cells affords protection against wild-type tumor cells. Having confirmed that rTAP1-transfected cells possess antigenicity and immunogenicity, we were interested in testing whether or not immunization with TAP1-positive cells affords protection against TAP-deficient CMT.64 cells. Three groups of mice were immunized intraperitoneally with mitomycin- and irradiation-treated cells: CMT.neo, CMT.1-4, and CMT.1-10, respectively. One month later the mice were challenged intraperitoneally with wild-type CMT.64 cells, and then monitored for survival. The results are shown in Figure 4B. Mice immunized with both immunogenic rTAP1-transfected clones show a successful challenge with TAP-negative CMT.64, compared with CMT.neo immunization. A statistical analysis of regression on logarithmic percentage survival showed this effect to be significant ($p < 0.001$ for CMT.1-10 immunization; $p < 0.05$ for CMT.1-4 immunization).

Successful challenge of wild-type tumors following immunization with rTAP1 but not CMT.neo tumor cells suggests that the

immunization with immunogenic tumors can augment the antitumor response. Obviously, specific T cells participate in this immune response. Although it is not clear how T cells, especially CTLs, directly recognize wild-type CMT.64 cells, *in vivo* they, along with other immune elements, may compose the integral components of the specific antitumor response.

Analysis of rTAP1 expression in *in vivo* growing tumors. At this point we became curious whether the rare large tumors seen in mice receiving the TAP1 transfectants had lost TAP expression, perhaps hastened by selective pressure by the host immune system. A western blot analysis was performed on the rTAP1 tumors that had grown in mice for one month or two months. Tumors, *in vivo*, contain normal mouse cells (such as CD4 and CD8 T cells) that express mouse TAPs. Our TAP1-specific antibody recognizes both mouse and rat TAP1, and it was initially very difficult to judge the expression of rTAP1 in these solid tumors. We, therefore, included controls that consisted of mixing CMT.neo cells with a 20:1 or 10:1 ratio of the wild-type splenocytes from the mice. The results are shown in Figure 3. The CMT.neo tumor contained a stronger TAP1 signal than the CMT.neo-splenocytes mixture at a 10:1 ratio, suggesting that other types of wild-type mouse cells, excluding T cells (CMT.neo tumor contains ~1% T cells; see Table 2), had infiltrated the tumor. As expected, in comparison with rTAP1 tumor signals, the CMT.neo tumor's signal was much less (see Fig. 3). Although we cannot judge whether the amount of mouse wild-type infiltrating cells induced

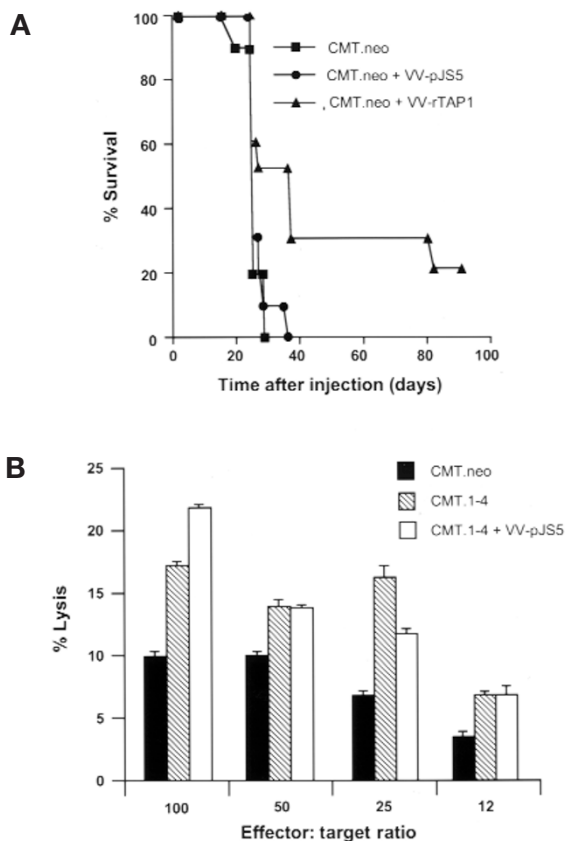


Figure 5. Antitumor immune therapy by VV-rTAP1. (A) Each group of mice was injected intraperitoneally with CMT.neo cells. Two out of three groups subsequently received treatments of either VV-pJS5 or VV-rYAP1 with 10⁶ p.f.u. in PBS containing 2% mouse serum at 24 h and two weeks after cell injection. Control group received only PBS containing 2% mouse serum. Statistical analysis shows that VV-rTAP1 treatment of tumor-bearing mice has a significant p value ($p < 0.05$), comparing with both VV-pJS5 and mimic treatment. **(B)** The specificity of splenocytes from two mice injected with CMT.neo and VV-pJS5 infected CMT.1-4 (10:1 MOI infection for 3.5 h).

the differences in levels of TAP expression between CMT.neo tumor and rTAP1 tumor clones (CMT.1-1 and CMT.1-10), the intensity as judged by gel scanning of TAP1 in CMT.1-4 tumor is identical to that in RMA cells. Since the CMT.1-4 cells express half the amount of rTAP1 protein compared to the RMA cells (see Fig. 1A), and this tumor, *in vivo*, contains fewer T cells than CMT.1-10 tumor (see Table 2) (suggesting less contamination by wild-type infiltrating cells), we conclude that the TAP-transfected tumors maintain the expression of rTAP1 during two months' growth *in vivo*.

Our data suggested that the large tumors are not TAP revertants but also implied a more interesting possibility. The TAP1 levels between the different TAP-expressing clones generally appeared to correspond with the malignancy of the tumor. The more TAP1 expressed, the fewer the number of tumors observed. These data lead us to speculate that the reason we see some large tumors in the TAP1 expressers is that the TAP1 expression levels are too low to provide complete protection. Alternatively, in a naive mouse the initial tumor burden has formed a solid focus, leading to a late-stage metastatic carcinoma that cannot be controlled by a specific antitumor immune response. The latter possibility is supported by our results, which showed that all surviving mice that were initially challenged with live rTAP1 or rTAP1,2 tumor cells (nonirradiated) remained healthy after subsequent challenge with rTAP1 tumor cells (data not shown).

Contribution of TAP1 to cancer therapy. We have shown that TAP1 improves CMT.64 immunogenicity and host survival rates. This has led us to explore whether TAP1 can form the basis of a tumor immunotherapy. An expression vector of recombinant vaccinia virus carrying rTAP1 gene (VV-rTAP1) was generated for these experiments. A faithful model for viral therapy for tumor-burdened individuals entails infection *in vivo* after the tumor load had been established. To examine this scenario, 5×10^5 CMT.neo cells were injected into three mouse groups. After 24 h, mice received either 10^6 p.f.u. VV-rTAP1, VV-pJS5 (control vector), or phosphate-buffered saline (PBS) containing 2% C57BL/6 mouse serum. This procedure was performed again at two weeks. As expected, the vector alone, VV-pJS5, did not increase mouse survival, as judged by comparison to the PBS group ($p \gg 0.05$) (see Fig. 5A). However, the mice receiving VV-rTAP1 treatment had a significantly higher survival rate, as judged by comparison to the PBS group ($p << 0.05$) and the VV-pJS5 group ($p << 0.05$) (see Fig. 5A).

We sought to confirm that the improved survival rate of tumor-bearing mice was due to the host immune system recognizing antigens, including tumor antigens, after VV-rTAP1 infection. We performed CTL analysis by using the splenocytes from mice injected with CMT.neo plus VV-rTAP1. The targets were CMT.neo, CMT.1-4, and CMT.1-4 infected with 10:1 MOI VV-pJS5. If the splenocytes contained TAA-specific CTLs, then they would kill CMT.1-4 targets and, therefore, confirm the presentation *in vivo* of tumor antigens in VV-rTAP1-infected CMT.neo. The results are shown in Figure 5B. In comparison with control CMT.neo, CMT.1-4 targets were killed more efficiently, suggesting that tumor antigens are presented in VV-rTAP1-infected CMT.neo tumors *in vivo*. In addition, these results also show that CMT.1-4 infected with VV-pJS5 achieved levels of killing equal to CMT.1-4, suggesting the splenocytes also contained VV-specific T cells. Thus, viral-infected tumor cells provide additional antigen(s) to the host immune system for recognition. Taken together, we conclude that the improved mouse survival is likely due to the presentation of both tumor and VV antigens after the viral therapy.

Introducing TAP into the tumor-burdened individuals by using a vector provides a possible method for antitumor immune therapy. It has a potential significance for controlling the occurrence of neoplastic metastasis and for treating metastasized neoplasms. This would be particularly true for the tumors with a deficiency in com-

ponents of the antigen-processing machinery. Utilizing VV allows TAP to be produced in any cell of the body, and using TAP in this manner provides a possible treatment for improving survival. The use of VV as gene-delivering system is not the only choice; indeed, many methods of expressing specific proteins in different cancer cells have been developed. Whichever gene-delivering system is used, targeting TAP into cancer cells exclusively is not likely to be necessary, unless evidence is generated suggesting that a risk exists of developing autoimmunity. However, we observed no increased autoimmunity in this system (manuscript in preparation). Tumors that down-regulate their antigen-presenting capabilities successfully evade the immune system. To allow recognition of these tumors, one would have to match the polymorphism of the MHC with the binding and immunogenicity of allele-specific peptide expressed by different tumors. By acting independently of the tumor antigen and MHC polymorphism, TAP vaccines bypass these requirements to increase recognition.

Further investigation is necessary to determine the effects of this treatment on other types of tumors and whether the results seen in this experiment will ultimately translate into a therapy for human disease.

Experimental protocol

Animals. The mouse strain C57BL/6 (H-2^b) was obtained from Jackson Laboratories but housed and bred by Willem Schoorl at the Biotechnology Breeding Facility (University of British Columbia). The H-2^b nude mice (B/6 Nu-M (C57BL/6 NTAC-NuDF)) were obtained from Taconic (Hanover, NY) and kept in specific pathogen-free incubators. The mice were maintained according to the guidelines of the Canadian Council on Animal Care. The mice used in the experiments were between 6 and 12 weeks of age and were killed by CO₂ asphyxiation.

Recombinant vaccinia virus (VV) construction. Recombinant VV was constructed by homologous recombination of the wild-type VV WR strain by infecting CV-1 cells transfected with the plasmids pJS5, pJS5-rTAP1, pJS5-rTAP2, or pJS5-rTAP1,2 according to described protocols²².

Purification of VV stocks. Crude cell stocks were used for the infection of cells in culture, but purified stocks of VV were used when injecting mice. To purify the VV, 3 L batches of VV-infected Hela S3 cultures were used. The VV was released from the cells by homogenization with a Dounce homogenizer before centrifugation at 750 g for 5 min at 4°C. The supernatant was trypsinized with 0.1 volume of 2.5 mg ml⁻¹ trypsin for 30 min at 37°C, then layered onto an equal volume of 36% sucrose in 10 mM Tris-HCl pH 9.0. It was centrifuged for 80 min at 4°C at 25,000 g and the pellet was then resuspended in 1 mM Tris-HCl pH 9.0. The pellet was trypsinized again before being layered onto a 24–40% continuous sucrose gradient and centrifuged for 45 min, at 4°C at 18,750 g. The milky band was collected and saved, whereas the pellet was trypsinized and repurified on another sucrose gradient. All of the bands collected were pelleted by diluting with two volumes of 1 mM Tris-HCl pH 9.0 and centrifuging for 60 min at 4°C at 25,000 g. The viral pellet was resuspended in 1 mM Tris-HCl pH 9.0, and 0.5 ml aliquots were stored at -80°C or -135°C.

Tissue culture. The small-cell lung carcinoma cell line, CMT.64, used in the cancer experiments originated spontaneously from the C57BL/6 mouse strain¹⁵. All of the stable CMT.64 transfectants containing rTAP-1 (CMT.1-1, CMT.1-4, CMT.1-10), rTAP-2 (CMT.2-1, CMT.2-10), rTAP1,2 (CMT.12-21), and the vector-only control (CMT.neo) were created by transfecting CMT.64 cells with the rTAP cDNA in mammalian expression vector pH(Apr-1neo)^{18,19}. All cell lines including the RMA cell line were grown in either Dulbecco's modified Eagle's medium (DMEM) or RPMI containing 10% fetal bovine serum (FBS).

Generation of effector cell populations. Virus-specific CTL populations were generated by infecting mice intraperitoneally with 10^7 tissue culture infection dose (TCID) units of VSV or at the suggested plaque-forming units (p.f.u.) for VV-TAP or VV-pJS5 vector. Cytolytic T lymphocytes were collected on day 5 postimmunization from the cervical lymph nodes (LN) or spleen and cultured in RPMI-1640 medium containing 10% FBS, 20 mM HEPES, 2 mM L-glutamine, 0.1 mM essential amino acids, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol (β-ME), and penicillin/streptomycin (henceforth referred to as RPMI complete medium). The LN cell

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suspensions were cultured at 4×10^6 cells/ml for three to five days in the absence of stimulation before being used in a CTL assay, whereas the splenocyte suspension was cultured for seven days with peptide stimulation. Bulk populations of VSV-specific CTLs were maintained by weekly restimulation with $1 \mu\text{M}$ VSV-Np (amino acids 52–59) plus pulsed irradiated (2,200 rads) stimulator splenocytes. Irradiated stimulator cells and CTLs were incubated together at a ratio of 4:1 in RPMI complete medium containing 20 units ml^{-1} human interleukin 2 (hIL-2). Seven days later, this bulk population was used in a CTL assay.

For antitumor CTL generation, the specificity of splenocytes was generated by injecting mice intraperitoneally with 1×10^7 CMT.neo or CMT.1-4 cells (Fig. 2C). Upon removal the splenocytes were cultured with stimulators at a 3:1 ratio. The stimulators were prepared by incubating CMT1-4 or CMT.neo cells with 30 mg ml^{-1} mitomycin C under hypoxic conditions. After incubation for 2 h the cells were γ -irradiated (10,000 rads) and washed three times before addition to the splenocyte culture. CMT.neo splenocytes received CMT.neo stimulators, whereas CMT1-4 splenocytes received CMT1-4 stimulators. Six days after in vitro stimulation the splenocytes were tested in a standard 4 h ^{51}Cr -release assay.

For antitumor and VV CTL generation, the specificity of splenocytes was generated by injecting mice intraperitoneally with 1×10^7 CMT.neo cells and 1×10^6 p.f.u. VV-rTAP1 (Fig. 5B). The splenocytes were a secondary mass culture that were incubated with stimulator cells, plus γ -irradiated (5,000 rads) naive syngeneic splenocytes, at a 5:1:15 ratio. The stimulator cells were prepared by infecting CMT.neo cells with VV-rTAP1 for 3 h before adding 30 mg ml^{-1} mitomycin C under hypoxic conditions. After incubation for 2 h the cells were γ -irradiated (10,000 rads) and washed three times before addition to the splenocyte culture. After incubation for six days the splenocytes were tested in a standard 4 h ^{51}Cr -release assay.

Cytotoxicity assays. Target cells for the CTL assays were loaded with ^{51}Cr by incubating 10^6 cells with $100 \mu\text{Ci}$ of ^{51}Cr (as sodium chromate; Amersham, Arlington Heights, IL) in $250 \mu\text{l}$ of CTL medium (RPMI-1640 containing 10% (vol/vol) HI FBS, 20 mM HEPES) for 1 h. Following three washes with RPMI, 2% (vol/vol) FBS, the target cells were incubated with the effector cells at the indicated ratios for 4 h. $100 \mu\text{l}$ of supernatant from each well were collected and the ^{51}Cr release was measured by a γ -counter (LKB Instruments, Gaithersburg, MD). The specific ^{51}Cr release was calculated as follows: $((\text{experimental} - \text{media control}) / (\text{total} - \text{media control})) \times 100\%$. The total release was obtained by lysis of the cells with a 5% Triton X-100 (BDH) solution.

FACS assays. Surface expression of the H-2K^b allele was detected by indirect immunofluorescence using the conformational-dependent mouse monoclonal antibodies AF6-88-5.3 (ATCC, Manassas, VA) and 142.23 (a gift from Dr. Sun Kvist), both specific for the complex of K^b- $\beta_2\text{M}$. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Dakopatts, DK) was used as the secondary antibody. The mean logarithmic fluorescence intensity was measured by a FACScan analyzer (Becton Dickinson, Mountain View, CA). For detection of CD4⁺ T cells and CD8⁺ T cells we used a protocol similar to that used for detection of surface MHC class I molecules, with minor modifications. Briefly, tumors were washed extensively and homogenized into single cells. FITC-conjugated rabbit anti-mouse antibodies (BD PharMingen, San Diego, CA) RM4-5 (against CD4) and 53-6.7 (against CD8) were used.

Western blots. The proteins of lysates from 5×10^5 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% resolving gel, and then were transferred to a nitrocellulose membrane. The blots were probed with either the rabbit anti-rat TAP1 (D90) or TAP2 (114/2) polyclonal antibody at a dilution of 1:1,000, and then incubated with horseradish peroxidase-labeled anti-rabbit antibody at a 1:100,000 dilution. The immunocomplexes were visualized by enhanced chemiluminescence (ECL) according to the instructions of the manufacturer (Amersham) and were quantitatively assessed by a densitometry scan.

Inoculation of mice with tumor cell lines. A total of 5×10^5 (unless otherwise indicated in figure legend) cells of CMT.64 or its transfectants in PBS were injected intraperitoneally into C57BL/6 syngeneic mice. For examina-

tion of tumor growth pattern, one representative mouse from each group (four mice for each group) was killed after one month for photographing of in vivo tumors (Fig. 2A). For mice survival experiments, each group contained 15 (Fig. 2B) or 10 (Fig. 4B and 5A) mice.

Statistics. The statistics for the cancer studies were performed using the Kaplan-Meier log rank survival test or regression log percentage survival test before carrying out a paired *t*-test. The computer software program JMP IN version 3.2.1 was used to do the computations²³. The data were considered statistically different if $p < 0.05$.

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