Combining the Antigen Processing Components TAP and Tapasin Elicits Enhanced Tumor-Free Survival

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Abstract Purpose: Tpn is a member of the MHC class I loading complex and functions to bridge the TAP peptide transporter to MHC class I molecules. Metastatic human carcinomas often express low levels of the antigen-processing components Tapasin and TAP and display few functional surface MHC class I molecules. As a result, carcinomas are unrecognized by effector CTLs. The aim of this study is to examine if Tapasin (Tpn) plays a critical role in the escape of tumors from immunologic recognition.

Experimental Design: To test our hypothesis, a nonreplicating adenovirus vector encoding human Tpn (AdhTpn) was constructed to restore Tpn expression in vitro and in vivo in a murine lung carcinoma cell line (CMT.64) that is characterized by down-regulation of surface MHC class I due to deficiency in antigen-processing components.

Results: Ex vivo, Tpn expression increased surface MHC class I and restored susceptibility of tumor cells to antigen-specific CTL killing, and AdhTpn infection of dendritic cells also significantly increased cross-presentation and cross-priming. Furthermore, tumor-bearing animals inoculated with AdhTpn demonstrated a significant increase in CD8+ and CD4+ T cells and CD11c+ dendritic cells infiltrating the tumors. Provocatively, whereas syngeneic mice bearing tumors that were inoculated with AdhTpn a significant reduction in tumor growth and increased survival compared with vector controls, combining AdhTpn inoculation with AdhTAP1 resulted in a significant augmentation of protection from tumor-induced death than either component alone.

Conclusions: This is the first demonstration that Tpn alone can enhance survival and immunity against tumors but additionally suggests that Tpn and TAP should be used together as components of immunotherapeutic vaccine protocols to eradicate tumors.

The MHC class I antigen presentation pathway is important both in the initiation of antitumor immune responses through cross-presentation of tumor antigens to CD8+ T cells and in the recognition and killing of tumor cells by tumor-specific CTLs. An important component in both these processes is the chaperone Tapasin (Tpn), a 48-kDa type I membrane glycoprotein whose only known function is assisting in the loading of antigenic peptides onto class I molecules in the ER (1). The mechanisms by which Tpn mediates this function include retaining empty MHC class I molecules in the ER until loaded with peptides, stabilizing TAP and bridging MHC class I to TAP (2), and supporting the binding of high-affinity peptides to MHC class I in conjunction with ERP57 (3, 4). In the presence of Tpn, surface MHC class I molecules are more stable and thus more efficient at presenting antigens to CTLs or their precursors (5, 6). Defects in Tpn expression lead to destabilization of the MHC class I loading complex including TAP1 and TAP2 and a reduction in the expression of MHC molecules at the cell surface (1, 7).

It is an intriguing and consistent finding that Tpn is down-regulated in many human carcinomas, such as breast cancer (8, 9), melanoma (10), colorectal carcinoma (11), and both small cell and non–small cell lung carcinoma (12), as well as mouse cancers, such as mouse fibrosarcoma (13) and mouse melanoma (14). Remarkably, in human colorectal cancers, Tpn is more frequently lost than TAP1, LMP2, and LMP7 (11), suggesting that the loss of Tpn could be a key event in overcoming immune surveillance in these tumors. Moreover, down-regulation or deficiency of components, including Tpn in the MHC class I antigen presentation pathway, results in reduced immunogenicity of tumors (12) and is associated with disease progression and disease outcome in a variety of human carcinomas (10). The mouse lung carcinoma cell line CMT.64, derived from a spontaneous lung carcinoma in a C57BL/6 mouse (15), is characterized by the down-regulation of many components of the antigen presentation pathway, including...
MHC class I heavy chain, β2-microglobulin, LMP2 and LMP7, TAP1 and TAP2 (16, 17), and Tpn. 6 A number of studies have shown that the restoration of TAP1 expression in CMT.64 and other tumor cells using replicating vaccinia virus or nonreplicating adenovirus increases the tumor antigen-specific immune responses and prolongs animal survival (12, 18–22). With these observations in mind, we tested the hypothesis that providing human Tpn (hTpn) either alone or in combination with human TAP1 (hTAP1) expressed from nonreplicating adenoviruses could restore antigen presentation, increase tumor antigen-specific immune responses, and prolong the survival of tumor-bearing mice.

**Materials and Methods**

**Cells, viruses, and mice.** HEK 293 cells (American Type Culture Collection), C8E8 cells (23), CMT.64 cells (12, 15), and CMT.64 transfected with vesicular stomatitis virus–nucleoplasid protein (CMT/ VSV-NP) minigene containing the immunodominant epitope from amino acids 52 to 59 presented on H-2Kb and T1 (American Type Culture Collection; CRL-1991, a hTpn-positive cell line) were cultured in DMEM supplemented with 10% fetal bovine serum. CMT.64 cells have a β-actin–based expression cassette driving a Cere recombinase gene with an N-terminal nuclear localization signal stably integrated into HEK 293 cells (23). The virus was an E1 and E3 deleted version of Ad5 containing loxP sites flanking the packaging site (23). The recombinant adenovirus was propagated and titrated in HEK 293 cells. Primary mouse splenocytes and 721.220 cells (Tpn-deficient myeloma cells provided by Dr. Peter Cresswell, Yale University School of Medicine) were cultured in complete culture medium consisting of RPMI 1640 + 10% fetal bovine serum. Six-week-old to eight-week-old C57BL/6 (H-2b) female mice were obtained from The Jackson Laboratory and housed at the Biotechnology Breeding Facility, University of British Columbia, under Canadian Council on Animal Care guidelines.

**Construction of nonreplicating adenovirus vector encoding human Tpn.** FirstChoice total RNA from human spleen was obtained from Ambion, Inc. cDNA was synthesized using RETROscript first strand synthesis kit for reverse transcription–PCR (Ambion, Inc.) using Oligo(dT) primers as per the manufacturer’s instructions. Tpn cDNA was amplified using primers designed based on the sequence of hTpn transcript variant 1 (NM_003190) with hTpn DNA polymerase (Invitrogen Life Technologies), and both strands were sequenced to ensure no mutations were present. The PCR product was digested from TOPO/hTpn with PstI and BamHI and then cloned into a PstI-digested and BamHI-digested shuttle vector, pLPC plasmid (23). The resulting vector Pad/hTpn was isolated and sequenced to ensure the flank sequence fidelity. The adenovirus vector encoding human Tpn (AdhTpn) was generated as previously described (23). Briefly, the pad/hTpn, linearized with SfiI, was cotransfected along with hTpn DNA into C8E8 cells using LipofectAMINE Plus reagent (Invitrogen Life Technologies) to generate AdhTpn. AdhTpn recombinant viral clones were identified by immunofluorescence assay and plaque purified thrice in HEK 293 cells. The recombinant virus was amplified in large-scale stock in HEK 293 cells, purified by CsCl density gradient centrifugation, and titrated in HEK 293 cells. The identity of AdhTpn was confirmed by PCR and DNA sequencing of purified viral DNA using primers specific for Tpn and adenovirus DNA flanking either side of the Tpn gene. The primer sequences were as follows: forward primer 5′-AAG AGC ATG CAT GAA GTC CTC TCT G-3′ and reverse primer 5′-AAT AAG TCG AGC AGT GAG TCG CCT AAC TCT CCT GCT GCT TTC-3′ for amplification of Tpn and forward primer 5′-GTC TTA TCT ATA GCG CGT AA-3′ and reverse primer 5′-CCA TCA AAC GAG GTG TGT CTC-3′ for amplification of adenoviral flanking sequence.

**TAP and Tpn expression after AdhTpn infection of CMT cells.** To examine Tpn and TAP expression in response to increasing doses of AdhTpn, CMT.64 cells were infected with AdhTpn at 1, 5, 25, 50, and 100 pfu/cell or W5 (negative control) at 100 pfu/cell. T1 cells and 721.220 cells were respectively used as hTpn-positive and hTpn-negative controls. CMT.64 cells treated with IFN-γ were a positive control for mouse TAP1 (mTAP1), mTAP2, and mouse Tpn expression. Two days after infection, cells were lysed and subjected to SDS-PAGE and electrophoresed to Hybond polyvinylidene difluoride membrane (Amersham Biosciences). The blots were treated with rabbit anti-hTpn antibodies (StressGen Biotechnologies Corp.), rabbit anti–mouse Tpn antibodies (a gift from Dr. David Williams, University of Toronto), rabbit anti-mTAP1 and rabbit anti-mTAP2 [made by our laboratory by immunizing rabbits with synthetic peptides generated from the mTAP1 (RGCGYRAMVLEAAPAD-C) or mTAP2 (DGQDYYAHLVQQRLEA, a peptide corresponding to the last 16 amino acids at C-terminal end of mTAP2) sequences conjugated to keyhole limpet hemocyanin; refs. 19, 20] , and mouse monoclonal antibody (mAb) against human β-actin (Sigma-Aldrich). Goat anti-rabbit IgG (H+L)–horseradish peroxidase and goat anti–mouse IgG (H+L)–horseradish peroxidase (Jackson ImmunoResearch Lab) were used as secondary antibodies. The bands were visualized by enhanced chemiluminescence and exposure to Hyperfilm (Amersham Biosciences). Line densitometry was done using the AlphaEaseFC software, version 6.0.0 (Alpha Innotech).

**Effect of AdhTpn on surface expression of MHC class I.** CMT.64 cells were infected with AdhTpn or W5 at 50 pfu/cell. Two days after infection, the cells were incubated with anti-mHc class I mAbs, y3 (H-2Kb specific), and 28.14.8S (H-2Dβ specific), at 4°C for 30 min (17). Bound antibodies were detected by goat anti–mouse IgG-FITC (Jackson ImmunoResearch Lab). The fluorescence-activated cell sorting (FACS) analysis was done using FACS Calibur (Becton Dickinson).

**CTL assay.** Cytotoxicity was measured in a standard 4-h 51Cr release assay. In brief, CMT/VSV-NP, which contains an immunodominant viral peptide consisting of amino acids 52 to 59, were infected with AdhTpn or W5 at 50 pfu/cell for 1 day. These cells were labeled with anti-mHc class I mAbs, y3 (H-2Kb specific), and 28.14.8S (H-2Dβ specific), at 4°C for 30 min (17). Bound antibodies were detected by goat anti–mouse IgG-FITC (Jackson ImmunoResearch Lab). The fluorescence-activated cell sorting (FACS) analysis was done using FACS Calibur (Becton Dickinson).

**In vitro cross-presentation of ovalbumin by dendritic cells.** Spleens were obtained from C57BL/6 mice as described (and disrupted by injection of 1 mL RPMI 1640 containing 5% FCS and 1 mg collagenase D; Roche Applied Science) and incubated for 30 min at 37°C. Subsequently, dendritic cell (DC)–enriched cell populations were obtained by centrifugation of cell suspension on Ficoll-Paque (Amersham Biosciences) gradients. DCs were then purified by positive selection with anti-CD11c MACS beads (Miltenyi Biotech) with the resulting population being >98% CD11c+ (26). DCs were then infected with adenovirus containing hTpn and hAd5 at a multiplicity of infection of 5 × 104 pfu of VSV into mouse spleen cells. Spleocytes were collected 5 days after infection and cultured in RPMI 1640 complete medium plus 1 μmol/L VSV-NP (52-59) peptide for 5 days.

**TAP and Tpn Increase Immune Response to Tumors**


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In vivo cross-presentation of OVA and generation of specific immune responses. On day 0, mice were infected i.p. with $1 \times 10^7$ pfu AdhTpn, $\Psi^5$, or PBS. Soluble OVA (30 mg in 100 $\mu$L) was injected s.c. 16 h later, and the animals were boosted with the same dose of virus and OVA at day 7. To study the cross-priming activity of DCs, splenic DCs were isolated from mouse spleens 24 h later, fixed in 0.005% glutaraldehyde, and cultured at 37°C for 7 days. To study the cross-priming activity of DCs, splenic DCs were cultured at 37°C for 7 days. To study the cross-priming activity of DCs, splenic DCs were cultured at 37°C for 7 days.

Tumor-infiltrating lymphocytes and DCs. Tumor-infiltrating lymphocytes (TIL) and tumor-infiltrating DCs were analyzed using both FACS and immunohistochemistry staining. Tumors were disaggregated in ice-cold PBS, and tumor-infiltrating lymphocytes were purified by centrifugation and enriched lymphocyte populations were obtained by centrifugation of blood on Ficoll-Paque gradient. Spleens were also harvested and digested as described above, and splenocyte-enriched populations were generated in the same fashion. Lymphocytes and splenocytes were double stained with iTAg H-2Kb/SLNFEKL isotope control for anti-CD8 and anti-CD4 antibodies, whereas hamster IgGc was the control for the antibody detecting CD11c+ cells. Antibody binding was detected with biotinylated polyclonal antirat IgG and biotinylated antihamster IgG secondary antibodies and streptavidin–horseradish peroxidase and a 3,3′-diaminobenzidine detection system (all the reagents were purchased from BD Biosciences PharMingen).

Statistical analysis. For the cross-presentation assays, the $\chi^2$ test (multivariate comparison, FlowJo 3.7.1) was used to analyze FACS histograms for differences in total H-2Kb or H-2Db/OVA57–267 complexes expressed on DCs infected with AdhTpn or $\Psi^5$ (control vector) after incubation with OVA. Results were considered significant if $P < 0.01$ (99% confidence) and $T(X) > 10$ was empirically determined as a cutoff value. Student’s $t$ test was used to compare $T(B3Z)$ activation after incubation with CD11c+ cells obtained from mice receiving AdhTpn or $\Psi^5$ followed by OVA. The difference was significant if $P < 0.05$ (two-tailed test). Survivorship data was analyzed using the "comparison of survival distributions." The data were considered statistically different if $P < 0.05$.

Results

AdhTpn increases MHC class I surface expression and immunogenicity in CMT.64 cells. CMT.64 cells infected with AdhTpn expressed H-2Kb and H-2Db proteins in a dose-dependent manner (Fig. 1A). However, no increase in endogenous mouse Tpn, mTAP1, or mTAP2 protein expression was detected in AdhTpn-infected CMT.64 cells by Western blot. Nevertheless, flow cytometry analysis showed that cell surface expression of H-2Kb and H-2Db was increased in CMT.64 cells infected with AdhTpn (Fig. 1B).

http://biostat.hitchcock.org/BSR/Analytics/CompareTwoSurvivalDistributions.asp
whereas cells infected with $\Psi 5$ showed no such increase. CMT.64 cells treated with IFN-$\gamma$ were used as a positive control and showed much larger increases in H-2K$^b$ and H-2D$^b$ surface expression (Fig. 1B), as well as increases in endogenous mouse Tpn, mTAP1, and mTAP2 protein levels in Western blot analysis (Fig. 1A). AdhTpn also enhanced the ability of CMT/VSV-NP minigene to present the immunodominant VSV-NP$_{52-59}$ peptide to CTLs. CMT/VSV-NP cells infected with

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**Fig. 2.** AdhTpn increases DC cross-priming of OVA antigen. A. AdhTpn increases DC cross-presentation of OVA antigen in vitro. Splenic DCs were infected with AdhTpn or $\Psi 5$ for 2 h followed by incubation with OVA for 16 h and then stained with 25.D1.16 and measured by FACS analysis. Histograms representative of four repeated experiments have been shown. The difference in H-2K$^b$/SIINFEKL was considered significant if $P < 0.01$ (99% confidence) as assessed by $\chi^2$ test. B. AdhTpn infection promotes cross-priming of CD8$^+$ T cells after immunization with soluble OVA. C57BL/6 mice were i.p. injected with AdhTpn, $\Psi 5$, or PBS; 16 h later, mice were injected s.c. with OVA and boosted with the same virus and OVA at day 7. After 8 d, splenic DCs were cultured at different ratios with B3Z T cells. After 24 h of coculture, B3Z activation, assessed by $\beta$-galactosidase production, was measured by ELISA plate reader. A graph representative of two repeated experiments done in triplicate has been shown. The difference was considered significant if $P < 0.05$ (two-tailed test) as assessed using Student’s $t$ test. C and D, percentage of CD8$^+$ T cells that recognize the OVA-derived immunodominant peptide SIINFEKL on MHC class I molecules of spleen and blood antigen-processing components were quantified by H-2K$^b$/SIINFEKL tetramer staining. Graph and dot plot representative of two repeated experiments.
AdhTpn and AdhTAP1 prolong the survival of tumor-bearing mice. A, C57BL/6 mice were injected i.p. with CMT.64 cells (4 x 10^6 cells per mouse) and were treated on days 1, 3, 5, and 8 with either AdhTAP1 at 1.25, 2.5, 5.0, 10 x 10^7 pfu, or AdhTpn at 1 x 10^8 pfu in 500 µL PBS, or PBS, and survival was followed for 90 d. The lowest dose showing a protective effect (2.5 x 10^7 pfu) was chosen for complementation studies with AdhTpn. B, treatment with AdhTpn, AdhTAP1, AdhTAP1 and AdhTpn, PBS (5 x 10^7 pfu/500 µL PBS) or PBS was done as above, and survival was followed for 90 d (n = 10 mice per group). To ensure all groups received the same number of Ad particles, mice treated with AdhTAP1 alone or AdhTpn alone were complemented with an equal amount of i5 vector to maintain a total Ad dose of 5 x 10^7 pfu. At the same dose, AdhTAP1 and AdhTpn together resulted in maximal protection that was statistically more significant than AdhTAP1 alone and PBS and controls, but not AdhTpn alone (P < 0.0061 for AdhTAP1 + AdhTpn versus AdhTAP1 alone). Survival of mice treated with AdhTAP1 + AdhTpn (2.5 x 10^7 pfu of each virus) was similar to that of mice treated with the highest dose (1 x 10^8 pfu) of AdhTAP1 alone.

AdhTpn were sensitive to the cytolytic activity of VSV-specific effector T lymphocytes, whereas CMT/VSV-NP cells alone or infected with ψ5 were resistant to killing (Fig. 1C), presumably due to the lack of H-2Kb/VSV peptide on the cell surface of the latter cells. These results show that hTpn expression and activity after AdhTpn infection can restore sufficient MHC class I–restricted antigen presentation of a specific epitope (VSV-NP52-59) to make these cells susceptible to specific CTL activity.

AdhTpn increases DC cross-presentation and cross-priming. The model antigen OVA was used to assess the ability of DCs infected with AdhTpn to cross-present the immunodominant peptide SIINFEKL in the context of H-2Kb. Flow cytometry provides a semiquantitative readout of the number of cell surface H-2Kb/SIINFEKL complexes, allowing assessment of cross-presentation efficiency. Splenic CD11c+ DCs infected in vitro with AdhTpn showed significantly increased cross-presentation of SIINFEKL on H-2Kb compared with DCs infected with ψ5 (P < 0.01; Fig. 2A). The total surface H-2Kb levels were also slightly increased in AdhTpn-infected DCs compared with ψ5-infected DCs. To examine this effect in vivo, we administered ψ5, PBS, or AdhTpn i.p. and injected OVA s.c. to test the effect of AdhTpn in the generation of H-2Kb/SIINFEKL-specific CD8+ T cells. Spleen-derived DCs taken ex vivo from mice infected with AdhTpn and immunized with OVA had a greater capacity to activate the H-2Kb/SIINFEKL-specific T-cell hybridoma B3Z than DCs from mice infected with vector alone (Fig. 2B). AdhTpn-infected mice immunized with OVA showed a greater general immune response detected by an increased number of total CD8+ T cells (data not shown) and a significantly increased OVA-specific response, as shown by a greater number CD8+ T cells specific for H-2Kb/SIINFEKL (measured with tetramer staining) in the spleen compared with vector control (ψ5) or PBS control. This increase in OVA-specific CD8+ T cells was even more prominent in peripheral blood from AdhTpn-infected mice compared with ψ5 and PBS controls (Fig. 2C and D). This indicates that infection of splenic DCs with AdhTpn, but not ψ5 alone, accounted for the increase in both general and antigen-specific CD8+ T-cell responses, which in turn is likely due to increased cross-presentation of exogenous antigen in vivo.

AdhTpn treatment increases survival of tumor-bearing mice: maximal protection is achieved by combining both AdhTpn and AdhTAP1. Previously, we showed that treatment of CMT.64 tumor-bearing mice with recombinant adenovirus expressing human TAP1 (AdhTAP1) resulted in increased survival compared with mice treated with ψ5 or PBS alone (12). Because AdhTpn increases MHC-I antigen surface expression and restores susceptibility to CTL killing in a manner similar to AdhTAP1 treatment, we examined if AdhTpn in combination with AdhTAP1 could enhance the inhibition of CMT.64 tumor formation. To avoid cytotoxicity associated with high adenoviral loads, a suboptimal dose of 2.5 x 10^7 pfu of AdhTAP1 determined by titration (Fig. 3A) that was shown to have a protective effect was used in combination with an equal dose of AdhTpn. To balance the viral load, AdhTAP1 and AdhTpn alone treatments were mixed with an equal number of ψ5 viruses. Dual treatment with AdhTpn and AdhTAP1 resulted in even greater mouse survival than either virus with ψ5 alone, with 50% long-term survival without visible tumors (>100 days) compared with 30% with AdhTpn and 10% with the low dose of AdhTAP1 (Fig. 3B). The dual treatment was statistically more effective than ψ5 or AdhTAP1 treatment alone at the same viral dose (P < 0.01), but not statistically different from AdhTpn treatment alone at the same dose. AdhTpn and AdhTAP1 at 2.5 x 10^7 pfu of each virus (5 x 10^7 pfu total virus) was equivalent to a much higher dose (1 x 10^8 pfu) of AdhTAP1 alone, demonstrating that dual treatment is more efficacious at a given dose (Fig. 3B).

AdhTpn treatment increases TILs and tumor-infiltrating DCs in tumors. Between four to eight mice from the AdhTpn treatment group, as well as ψ5 and PBS control groups, were examined for patterns in tumor growth 20 days after the last treatment injection. The peritoneal cavities of mice treated with AdhTpn were tumor-free or had only a few small tumors <1 or 2 mm in diameter. Both the liver and intestine seemed normal upon visual inspection. This was in sharp contrast to mice treated with PBS or ψ5. These mice had large volumes of bloody ascites fluid (2-5 mL), and many tumors were distributed throughout the peritoneal cavity. Tumors were
observed growing on the liver and intestine and were associated with large fibrotic adhesions. Tumors harvested from the mice were examined for TILs and DCs infiltrates by FACS and immunohistochemistry staining. Immunohistochemistry staining showed that mice treated with AdhTpn had significantly greater numbers of CD8+ and CD4+ T cells and CD11c+ DCs in the tumor mass in tumors taken from mice treated with C5 or PBS (Fig. 4). FACS analysis also confirmed that mice treated with AdhTpn had significantly greater CD8+ and CD4+ TILs (P = 0.011 and P = 0.042, respectively) than in tumors taken

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**Fig. 4.** TILs and DCs were increased in CMT.64 tumors treated with AdhTpn in vivo. Immunohistochemistry staining for CD4+ (A), CD8+ (B), or CD11c+ (C) cells in CMT.64 tumors treated with AdhTpn, C5 (Ad vector control), or PBS. Tumors were analyzed 19 d after CMT.64 cells were introduced into mice. C57BL/6 mice were injected i.p. with CMT.64 cells (4 × 10⁵ cells per mouse) and were treated on days 1, 3, 5, and 8 with either 2.5 × 10⁷ pfu/mouse of AdhTpn, C5, or PBS only. A positive stain is indicated by the intense brown labeling of cell surface membranes (magnification, 200×).
The restoration of surface MHC class I expression and increased immunogenicity of the tumor cells occur despite multiple antigen-processing component defects in CMT.64 cells, which include the down-regulation of MHC class I heavy chain, β2-microglobulin, TAP1, TAP2, LMP2, and LMP7 (16, 17). Residual transport of the peptides into the ER may be due to low levels of TAP expression (undetectable by Western blot) providing sufficient MHC class I peptide complexes in the presence of Tpn-mediated chaperone activity for a significant increase in susceptibility to killing by specific T-cell effectors. Steady-state levels of other components of the antigen presentation pathway, including TAP, have been shown to be stabilized by Tpn (30–32). Therefore, Tpn expression in tumor cells may stabilize the low level of TAP present in these cells and therefore significantly increase the H-2Kb and H-2Dβ surface expression and immunogenicity of CMT.64 cells in this manner. Combining AdhTpn1 and AdhTpn in treating this carcinoma (which is deficient in both these components) resulted in enhanced protection and survival in tumor-bearing animals.

Adding to the novelty of these findings is the observation that Tpn expression increases antigen-specific immune responses to exogenously acquired antigens (OVA). These findings likely also extend to enhanced cross-presentation of tumor-associated antigens in vivo; however, as there are no reagents to measure this specifically, we can only hypothesize that this is the case. Components of the peptide loading complex that are essential for "direct" antigen presentation by virus-infected cells or tumor cells to circulating CD8+ T cells are also required for "indirect" presentation by professional antigen-presenting cells to precursor CD8+ T cells during the initiation of tumor antigen-specific immune responses (33, 34). Augmenting Tpn expression that increases cross-presentation activity of DCs in vitro could be a combination of Tpn and a vector effect, suggesting an interaction between the antigen presentation pathway and innate mechanisms. The ability of DCs from animals infected with AdhTpn in combination with OVA to activate SIINFEKL-specific B3Z cells shows a physiologically relevant in vivo correlate of the effects seen in vitro. The increase in cross-priming activity in vivo due to AdhTpn infection was further shown by increases in the number of SIINFEKL-specific CD8+ T cells in both peripheral blood and spleen as measured by tetramer staining. In our study, the mechanism of increased cross-priming seems to coincide with the significant increase of CD4+ TILs within tumor masses of mice treated with AdhTpn. This is likely related to immunogenicity of the adenovirus vector itself (35, 36). Large numbers of CD4+ T cells favor the CD4+ T-cell–dependent pathway of CD8+ T-cell activation, whereby the CD4+ T cells may stimulate DCs through CD40 ligand and/or present alternative signals that can license DCs for cross-priming (37) or directly stimulate CD8+ T cells by cytokines, such as interleukin-2 (38).

In conclusion, adenoviral vectors containing the antigen-processing component genes encoding Tpn and TAP1 could play an important role in future cancer immunotherapies. The restoration of Tpn together with TAP have several advantages over other existing approaches and provide a general method for increasing immune responses against tumors regardless of the antigenic composition of the tumor or the MHC haplotypes of the host.

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