Immunization with tumor-associated epitopes fused to an endoplasmic reticulum translocation signal sequence affords protection against tumors with down-regulated expression of MHC and peptide transporters

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Abstract

Treatment of human cancers with an inherent antigen-processing defect due to a loss of peptide transporters (TAP-1 and TAP-2) and/or MHC class I antigen expression remains a considerable challenge. There is now an increasing realization that tumor cells with down-regulated expression of TAP and/or MHC class I antigens display strong resistance to cytotoxic T lymphocyte (CTL)-mediated immune control, and often fail to respond to the conventional immunotherapeutic protocols based on active immunization with tumor-associated epitopes (TAE) or adoptive transfer of tumor-specific T cells. In the present study, we describe a novel approach based on immunization with either genetically modified tumor cells or naked DNA vectors encoding TAE fused to an endoplasmic reticulum (ER) signal sequence (ER-TAE) which affords protection against challenge by melanoma cells with down-regulated expression of TAP-1/2 and MHC class I antigens. In contrast, animals immunized with a vaccine based on TAE alone showed no protection against tumor challenge. Although MHC–peptide tetramer analysis showed a similar frequency of antigen-specific CTL in both ER-TAE- and TAE-immunized mice, functional analysis revealed that CTL activated following immunization with ER-TAE displayed significantly higher avidity for TAE when compared to animals immunized with the TAE alone. These observations provide a new strategy in anti-cancer vaccine design that allows activation of a highly effective and well-defined CTL response against tumors with down-regulated expression of TAP and MHC class I antigens.

Introduction

There is now considerable evidence to support the notion that CD8+ T cells play an important role in the control of human cancers (1–3). This control is directed through expression of tumor-associated antigens which are processed intracellularly and presented on the surface of the tumor cell in a manner similar to that seen in virus-infected normal cells (3). Nevertheless, it is clear that in many instances CD8+ T cells fail to recognize tumor cells as efficient targets for lysis (reviewed in 4). Malignant melanoma is a classic example of a human tumor that evades cytotoxic T lymphocyte (CTL) surveillance (5–7). This resistance to immune surveillance is not due to an obvious CTL dysfunction in these patients but has been attributed, in many cases, to the down-regulation of essential components of antigen processing in these tumor
cells, such as peptide transporters (TAP-1 and TAP-2) and/or surface MHC class I antigen expression (5,7). More recent studies by Kageshita and colleagues have shown that TAP down-regulation plays an important role in the clinical course of malignant melanoma, probably by providing melanoma cells with a mechanism to escape from CTL recognition during disease progression (8). A similar antigen-processing defect has also been documented in other human cancers (9–12). Most of these studies have shown that loss of TAP and MHC class I antigen expression is more frequently seen in metastases than in primary lesions (7,8,12).

Recently, immunotherapeutic protocols based on vaccination with autologous dendritic cells (DC) sensitized with tumor-associated epitopes (TAE) have been used for the treatment of metastatic human melanoma (13,14). Although these clinical trials demonstrated limited evidence of clinical regression of tumors, a large proportion of the patients showed a detectable immune response following DC-based vaccination. The lack of tumor regression in these patients was not due to an underlying immune dysfunction, but correlates with the possibility of immune escape by the tumor cells (15). Indeed, in vitro analyses of malignant melanoma cell lines and metastatic melanoma biopsies frequently show loss of TAP and/or HLA expression (5,6,8,15). Alternative therapeutic approaches based on in vivo use of proinflammatory cytokines, such as IFN, or transfection of wild-type TAP genes in tumor cells are limited in their practical applications. Previous studies from our laboratory have shown that direct translocation of CTL epitopes into the endoplasmic reticulum (ER) lumen with the help of an ER translocation signal sequence can completely repair the antigen-processing defect in TAP-down-regulated Burkitt’s lymphoma cells in vitro (9). If such an approach can restore the antigen presentation in vitro, it is possible that activation of the immune system following immunization with CTL epitopes fused to an ER signal sequence will result in destruction of tumor cells and thus prevent tumor outgrowth in vivo? To examine this possibility, we have tested a novel approach based on immunization with genetically modified tumor cells or naked DNA vectors encoding TAE fused to an ER translocation signal sequence to determine whether such an approach can afford protection against challenge by antigen-processing-defective tumor cells.

Methods

Establishment and maintenance of cell lines
B16-F10, a mouse melanoma cell line of H-2b haplotype (ATCC, Rockville, MD) of C57BL/6J origin, was used in the present study. In addition, EL4 (H-2b), a mouse thymoma cell line, and E.G7OVA cells which is a clone of EL4 stably transfected with chicken ovalbumin (OVA) cDNA (16) (a kind gift from Dr F. Carbone), were also used in this study. A CTL clone (B3) specific for the H-2Kb antigen-restricted CTL epitope SIINFEKL from OVA (17) (a kind gift from Dr F. Carbone) was used to assess the antigen-processing function in tumor cells. All tumor cell lines were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS and with or without 5×10⁻⁵ M 2-mercaptopethanol (referred to as growth medium). The B3 CTL clone was maintained in growth medium supplemented with recombinant IL-2 (20 U/ml; Chiron, Emeryville, CA) and stimulated weekly with irradiated E.G7OVA cells.

Analysis of surface MHC class I antigen expression on B16 and E.G7OVA cells
MHC class I antigen expression on B16 and E.G7OVA cells was analyzed by surface immunofluorescence labeling followed by flow cytometry (FACS). Cells were initially incubated with H-2Kb antigen-specific mAb (20.8.4, 28.8.6 and Y3), washed and further incubated with polyclonal affinity-purified FITC-conjugated goat anti-mouse Ig (Silenus, Melbourne, Australia). Labeled cells were analyzed using the FACScan (Becton Dickinson, Mountain View, CA) and results were expressed as mean fluorescence intensity. In some experiments, surface MHC expression on B16 cells was also analyzed following treatment with IFN-γ. For this treatment B16 cells were incubated with recombinant IFN-γ (200 U/ml) for 48 h at 37°C. Following this incubation, cells were washed in growth medium and incubated with H-2Kb antigen-specific mAb, and analyzed using FACS as described above.

Analysis of antigen-processing genes in B16 cells
Expression of peptide transporters (TAP-1 and TAP-2) and H-2Kb genes in B16 and E.G7OVA cells was analyzed by RT-PCR. Total RNA from B16 and E.G7OVA cells was isolated by using a single-step RNA isolation reagent (Advanced Biotechnologies, London, UK) and mRNA was reverse transcribed with oligo(dT) primer. Semiquantitative PCR analysis of relative amounts of TAP-1, TAP-2 and H-2Kb cDNA was performed using the sequence specific primers as follows: 5’-TCTCTACCTGTGTTCTGTTC-3’ and 5’-GCACTGTTCACTAATGGACTC-3’ for TAP-1, 5’-CGGGTCTGTATATTGACATC-3’ and 5’-GCAGCAGGATGCCCATTG-3’ for TAP-2, and 5’-GCAAAGAGACAAGTTCGCCAG-3’ for H-2Kb. Amplified products were separated on 2% agarose gels and scanned densitometrically using ImageQuant software version 3.3 (Molecular Dynamics, Sunnyvale, CA).

Construction and transfection of minigene DNA expression vectors
Five distinct expression plasmids were used in this study. One of these constructs encoded the full-length OVA gene, while the other two constructs were designed to encode a minigene that included H-2Kb antigen-restricted CTL epitopes from either OVA, SIINFEKL (referred to as pSIIN), or tyrosinase-related protein (TRP)-2, VYDFFVWL (referred to as pTRP) (18). In addition, another two constructs expressed either SIINFEKL or VYDFFVWL fused to an ER translocation signal sequence (referred to as pSIINER and pTRPER). This signal sequence is derived from the adenovirus E3/19 kDa protein (19). These expression plasmids were constructed by annealing the specific synthetic oligonucleotides followed by extension with Klenow fragment, DNA polymerase I and dNTPs, and cloned into specific restriction sites of expression vectors as described earlier (9).

The B16 cell line was electroporated at 280 mV, 960 μF with 10 μg of the plasmid DNA (pSIIN and pSIINER) as
described elsewhere (9). After 48 h, cells were resuspended in 20 ml of growth medium containing hygromycin B (400 µg/ml; Sigma, St Louis, MO). Following 3 weeks of hygromycin selection, transfected cells were cloned by limiting dilution in a 96-well plate. These clones were expanded and maintained in growth medium containing hygromycin B (400 µg/ml).

Immunization with genetically modified tumor cells or naked plasmid DNA
C57BL/6J mice were immunized s.c. with either 1×10⁵ irradiated (8000 rad) B16 cells, B16 cells transfected with pSIIN expression vector or B16 cells transfected with pSIINER expression vector. This immunization procedure was repeated 7 days later. Four days after the final immunization, animals from all three groups were challenged s.c. with 10⁴ live B16.SIIN cells.

For DNA immunizations, C57BL/6J mice were immunized with 80 µg of plasmid DNA (pSIIN, pSIINER, pTRP and pTRPER) intramuscularly into the quadriceps muscle. These injections were repeated after 2 and 6 weeks. Two weeks after the final immunization, animals were challenged s.c. with 10⁶ live B16 cells (for pTRP- and pTRPER-immunized mice) or 10⁴ live B16.SIIN cells (for pSIIN- and pSIINER-immunized mice). Following challenge, these animals were monitored for 21 days and the tumor load was measured in each group after completion of the follow up period.

Ex vivo enumeration of TAE-specific memory CD8 T cells with MHC tetramers
Splenocytes form mice immunized with B16, B16.SIIN, B16.SIINER or OVA TCR transgenic mice (OT-1; generously provided by W. R. Heath, WEHI, Parkville, Australia) were isolated, and OVA-specific CD8 T cells were detected using H-2Kb tetramers containing the OVA protein-derived peptide SIINFEKL. MHC tetramers were produced as previously described (20,21). Briefly, H-2Kb-containing the biotin–protein ligase-dependent biotinylation substrate sequence was encoded either in the full-length OVA (referred to as B16.OVA) or a minigene sequence encoding the H-2Kb antigen-processing genes in B16 melanoma cells (5,7,8,15). To confirm and extend the results obtained by RT-PCR, surface H-2Kb protein expression in these tumor cells was also analyzed by FACS analysis using H-2Kb-specific mAb. This analysis also showed complete loss of H-2Kb antigen expression on B16 cells, while pretreatment of B16 with IFN-γ restored surface H-2Kb antigen expression (Fig. 1B). These results strongly suggest that B16 cells might be defective in processing MHC class I antigen-restricted CTL epitopes endogenously.

In the next set of experiments we analyzed the implication of loss of TAP and MHC class I gene expression on the endogenous processing function of CTL epitopes in B16 cells. B16 cells were transfected with expression vectors encoding either the full-length OVA (referred to as B16.OVA) or a minigene sequence encoding the H-2Kb antigen-restricted CTL epitope (SIINFEKL) from OVA (referred to as B16.SIIN). These transfectants were then exposed to the OVA-specific CTL clone B3. Data presented in Fig. 1(C) shows that although TAP+E.G7.OVA cells were efficiently recognized by the B3 clone, none of the B16 transfectants (B16.OVA or B16.SIIN) were recognized by this CTL clone. The lack of CTL lysis was not due to a defect in peptide binding by H-2Kb molecules expressed by B16 cells, since exogenous sensitization with synthetic SIINFEKL peptide epitope induced strong lysis by the B3 clone (data not shown). More importantly, endogenous presentation of the SIINFEKL epitope by the B16 transfectants was completely restored following treatment with IFN-γ (Fig. 1C). Taken together, these observations indicated that down-regulated expression of antigen-processing genes in B16 cells significantly blocked endogenous presentation of MHC class I-restricted CTL epitopes and thus interferes with immune recognition by specific T cells.

To determine whether the peptide transport defect can be rectified in B16, the cells were transfected with an expression vector encoding the SIINFEKL epitope fused to an ER translo-
Vaccine strategy for antigen-processing-defective tumors

Consequently B16 transfectants were tested for susceptibility to the B3 clone in a standard CTL assay. B16 cells transfected with pSIINER (referred to as B16.SIINER) showed significant lysis by B3 clone (Fig. 1C) and this lysis was not dependent on IFN-γ treatment as seen for B16.SIIN cells. Thus it seems that the TAP-down-regulated B16 cells are unable to transport sufficient levels of peptides into the ER unless TAP-independent mechanisms operate to directly translocate peptide epitopes into the ER compartment thereby restoring the presentation of MHC–peptide complexes at the surface of the cell.

Immunization with TAE fused to an ER signal sequence affords protection against B16 cells

Recent studies on human melanoma patients strongly argue that a functional MHC class I antigen-processing and -presentation defect is one mechanism by which malignant melanoma escapes immune recognition, and thus must be accounted for by future rational immunotherapies for human melanoma (5,8,15). Since B16 cells displayed an antigen-processing defect similar to that seen in human melanoma, we next explored the possibility that immunization with B16.SIINER cells might afford protection against B16 tumor cells. Three groups of mice (seven animals in each group) were immunized with irradiated B16, B16.SIIN or B16.SIINER cells respectively. Following immunization, mice were challenged s.c. with B16.SIIN cells and monitored for tumor load. Animals immunized with B16 or B16.SIIN rapidly developed aggressive tumors and showed no evidence of protection from tumor challenge. Surprisingly, on the other hand, animals immunized with B16.SIINER cells showed strong protection and the tumors grew much less aggressively. By day 21, the average tumor load in B16.SIINER-immunized mice was 13- or 21-fold lower when compared to B16- or B16.SIIN-immunized mice respectively (Fig. 2A–C).

Although immunization with B16.SIINER tumor cells afforded a high level of protection against challenge by an antigen-processing defective tumor, one of the limitations of applying this approach for the treatment of human melanoma is that it would require a custom-designed vaccine based on autologous tumor cells. To overcome this limitation, we designed a set of expression vectors based on the CTLa minigene encoding the H-2K b antigen-restricted CTL epitope epitope (VYDFFVWL) from the B16 melanoma-specific anti-(SIINFEKL) from OVA (referred to as B16.SIIN) or a minigene encoding SIINFEKL epitope fused to an ER translocation signal sequence (B16.SIINER).

In addition, these cells were also used as targets in a CTL assay following treatment with IFN-γ. E.G7OVA cells expressing full-length OVA were used as a positive control in this assay. An E:T ratio of 2:1 was used in this assay. Representative data from one of three experiments is shown here.

![Fig. 1.](image)

(A) Semiquantitative RT-PCR analysis of MHC class I antigen-processing gene expression in B16 cells. Relative levels of TAP-1, TAP-2 and H-2K b mRNA were measured by using semiquantitative RT-PCR analysis. Each of the PCR products was amplified using primers specific for TAP-1, TAP-2 and H-2K, and the housekeeping gene HPRT. E.G7OVA cells were used as a positive control in this analysis. (B) Analysis of surface H-2K b antigen expression on E.G7OVA cells, B16 and IFN-γ-treated B16 cells. Cells were initially incubated with H-2K b antigen-specific mAb (20.8.4), washed and further incubated with affinity-purified FITC-conjugated goat anti-mouse Ig (Silenus). Labeled cells were analyzed using the FACScan (Becton Dickinson). (C) Analysis of endogenous processing of a MHC class I antigen-restricted CTL epitope in B16 cells. B16 cells or tumor cells transfected with full-length OVA (referred to as B16.OVA), a minigene encoding the H-2K b antigen-restricted CTL epitope (SIINFEKL) from OVA (referred to as B16.SIIN) or a minigene encoding SIINFEKL epitope fused to an ER translocation signal sequence (B16.SIINER) were exposed to the OVA-specific CTL clone B3. In mice (seven animals in each group) were immunized s.c. with either plasmid alone or plasmid encoding the TRP-2 epitope (VYDFFVWL) from the B16 melanoma-specific antigen, tyrosinase-related protein-2 (TRP-2). Three groups of mice (seven animals in each group) were immunized with plasmid DNA encoding SIINER, tyrosinase-related protein-2 (TRP-2) or plasmid encoding the TRP-2 epitope fused to an ER translocation signal sequence (referred to as pTRPER). Following immunization, these animals were challenged with TAP-down-regulated B16 cells. Data presented in Fig. 2(D) clearly demonstrate that prior immunization with pTRPER afforded strong protection against B16 challenge, while animals immunized with plasmid alone or pTRP showed a significantly higher tumor load following challenge with live B16 cells. These results were also confirmed by immunization with another plasmid DNA encoding SIINER.
Analysis of antigen-specific CTL from immunized mice

To investigate the potential mechanism of protection afforded by ER signal sequence-based immunization, we analyzed antigen-specific T cells from the different groups of immunized mice using two different methods. Firstly, splenocytes from B16-, B16.SIIN- and B16.SIINER-immunized mice were isolated and stained with H-2K\(^b\)/SIINFEKL tetramers to assess the frequency of antigen-specific CTL precursors (CTLp). Representative data based on this tetramer analysis is shown in Fig. 3. Splenocytes from OT-1 mice, used as a positive control, showed strong staining and >76% of CD8\(^+\) T cells were positive for SIINFEKL tetramer (Fig. 3A). Although immunization with B16.SIIN and B16.SIINER resulted in a significant induction of tetramer-positive CD8 T cells, surprisingly, the overall numbers of SIINFEKL-specific CTL in these two groups were very similar (Fig. 3C–D). Indeed, tetramer analysis of splenocytes indicated that B16.SIIN-immunized mice had 8.52 ± 0.20% CD8\(^+\) T cells specific for the SIINFEKL peptide, while 8.35 ± 0.91% CD8\(^+\) T cells were specific for the SIINFEKL epitope in B16.SIINER-immunized mice. Furthermore, limiting dilution analysis based on \(^{51}\)Cr-release assay of CTL recovered from B16.SIIN- and B16.SIINER-immunized mice also showed comparable numbers of CTLp in splenocytes (data not shown).

In the second set of experiments splenocytes from these mice were stimulated with irradiated autologous LPS blasts coated with saturating concentrations of the SIINFEKL peptide (10 \(\mu\)g/ml). Following a single round of stimulation, the lytic activity of these T cells against H2-K\(^b\)-positive EL4 cells sensitized with varying concentrations of the synthetic peptide epitope SIINFEKL was determined. Representative data from one such experiment is shown in Fig. 4. This analysis clearly showed that although CTL from both B16.SIIN- and B16.SIINER-immunized mice showed comparable lytic activity against the target cells sensitized at high concentrations of peptide when compared to T cells from B16.SIIN-immunized mice. Taken together, these results suggest that although immunization with a minigene CTL epitope and signal sequence-tagged CTL epitope induce comparable numbers of epitope-specific CTLp, antigen-specific T cells recovered from signal sequence-based immunization display significantly high levels of avidity for the peptide epitope. A similar difference in the CTL avidity was also seen in mice immunized with pTRP and pTRPER (data not shown).

Data presented in this study demonstrate that loss of antigen-processing function, due to down-regulated expression of TAP and MHC genes, is an important immune-resistance mechanism operative in B16 cells and this phenotype very closely resembles that seen in a large number of human malignant melanomas (5,8,15). These observations provided a unique opportunity to explore a vaccination strategy in a mouse model, which might be capable of controlling the outgrowth of TAP-down-regulated tumor cells. In the last few years, there has been an increasing trend towards the use of gene-modified melanoma cells or synthetic peptides from melanoma-associated antigens for anti-tumor immunotherapy. Although it is a sound conceptual rationale that these...
two approaches might induce very strong CTL responses in vivo, their efficacy in the control of rapidly proliferating TAP-negative tumor cells may be significantly reduced. In fact, such vaccination strategies might also contribute towards the selection of TAP-down-regulated tumors. Indeed, a recent study by Nestle and colleagues showed that defective loading of peptides in the ER and loss of MHC class I antigen expression were consistently detected in situ in metastases from non-responders to a DC vaccination protocol (15). These observations are further supported by an analysis of in vitro established human malignant melanoma cell lines which showed that >60% of these lines expressed low or undetectable levels of TAP-1 and/or TAP-2 and surface expression of MHC class I molecules (5,8).

In the present study we describe a novel vaccine strategy which is capable of restricting the outgrowth of not only the TAP but also TAP-down-regulated cancer cells in vivo. Data presented here clearly demonstrate that immunization with tumor-associated CTL epitope(s) fused to an ER translocation signal sequence is an extremely efficient method of inducing a strong immune response which affords protection from a challenge by TAP-down-regulated melanoma cells. Although the precise mechanism of protection is still unclear, in vitro analyses strongly suggest that T cells from animals immunized with epitope(s) fused to an ER translocation signal sequence display stronger avidity for the CTL epitopes compared to the CTL induced by epitope alone. It is therefore possible that T cells from the signal-sequence-based immunization protocol are capable of recognizing suboptimal peptide levels presented by TAP-down-regulated tumor cells in vivo, while T cells from animals immunized with epitope alone fail to recognize TAP-down-regulated tumor cells in vivo. It is important to mention here that this hypothesis is also supported by earlier studies of Restifo and colleagues who also showed that CTL responses in mice immunized with CTL epitopes fused to an ER signal sequence are consistently enhanced and persist long term (22,23). We strongly believe that the current anti-tumor vaccines which are designed to induce a quantitatively enhanced tumor-specific CTL
response might be inadequate in the control of TAP-downregulated human cancers in vivo. However, based on the data presented in this study we propose that if vaccine strategies can be specifically designed to enhance the TAE-specific avidity of CTL, strong protection against TAP-downregulated tumors can be achieved.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>CTLp</td>
<td>precursor cytotoxic T lymphocytes</td>
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<td>DC</td>
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<td>TAE</td>
<td>tumor-associated epitope</td>
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<td>TRP</td>
<td>tyrosinase-related protein</td>
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**References**