



Co-expression of P1A₃₅₋₄₃/β2m fusion protein and co-stimulatory molecule CD80 elicits effective anti-tumor immunity in the P815 mouse mastocytoma tumor model

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Abstract. A strong CTL response is dependent upon a high level of expression of specific class I major histocompatibility complex (MHC)/peptide complexes at the cell surface. An epitope-linked β2-microglobulin (β2m) molecule could provide a simple and more efficient means to enhance the formation of defined MHC/peptide complexes. However, the ability of an epitope-linked β2m molecule to elicit primary CTL responses *in vivo* is still unknown. In this study, we modified the P1A tumor cell vaccine by addition of the tumor-associated epitope (TAE)-linked β2m molecule and co-stimulatory molecule CD80 to improve the efficiency in the application of the vaccine. A eukaryotic co-expression vector consisting of the P1A₃₅₋₄₃-linked β2m molecule and the murine *CD80* gene was constructed. P815 cell lines stably expressing P1A₃₅₋₄₃-linked β2m molecule and/or CD80 were established after transfection, by selection under G418. Administration of these inactivated tumor cell vaccines allowed the TAE-specific CD8⁺ T cell responses to be examined *in vivo*. Our results indicate that immunization with P815 cells expressing both the P1A₃₅₋₄₃-linked β2m molecule and the murine *CD80* gene elicited a significantly stronger antitumor immune response than the single-modified tumor cell vaccines (expressing either P1A₃₅₋₄₃-linked β2m or CD80 alone). These findings support the feasibility and effectiveness of developing a dual-modified tumor cell vaccine consisting of the epitope-linked β2m molecule and a co-stimulatory molecule.

Introduction

Major histocompatibility complex (MHC) class I molecules are heterotrimers consisting of a transmembrane (type I) heavy chain, a soluble light chain [β2-microglobulin (β2m)], and a peptide ligand of typically 8-10 amino acids. Complete assembly of the heterotrimer is necessary for ER egress and stable surface expression of class I molecules (1,2). Since the density of specific MHC/peptide complexes on the cell surface can influence T cell responsiveness (3-5), the ability to express high levels of a particular class I complex could be potentially useful for eliciting strong CTL responses in the development of vaccinations or immunotherapy.

One potential approach to augmenting the surface display and immunogenicity of an epitope is to physically couple it to its presenting MHC molecule. The resulting fusion proteins have been shown to elicit CTL responses when expressed in transfected cells (6-8). More recently, it has been shown that generating an epitope-linked β2m molecule could provide a simple and more efficient means of enhancing the formation of defined MHC/peptide complexes (9-11). Previously, we have reported that the expression of the OVA₂₅₇₋₂₆₄-linked β2m fusion molecule increases the formation of H2-K^b-OVA molecules on the cell surface of EL-4 cells (12). However, the ability of peptide-β2m molecules to elicit primary CTL responses *in vivo* is still unknown. In the present study, we examined the antitumor effects of genetically engineered P815 cells expressing the epitope-linked β2m molecule in the mouse mastocytoma tumor model. Induction of a T-cell response to an antigen usually requires two signals (13,14). Signal 1 is delivered to the TCR via the peptide-MHC complex of the APC. Signal 2 involves the interaction of a costimulatory molecule of the APC with its ligand on the APC. Data are emerging that the level of signal 1 required for effective T-cell activation is influenced by the level of expression of signal 2 (15). CD80 plays an important role in the activation of T cells, exogenous costimulatory molecules in tumor cells can lead to the strengthening of the effects of T cells, including cell proliferation, secretion of cytokine and CTL activity (16,17). We have successfully constructed a co-expression vector of CD80 and GFP-tagged K^b molecules to enhance the response of specific T cells and thus increase the

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uptake of the antigen peptide-MHC class I-GFP complex (18). Therefore, we also examined the role of CD80, a member of the B7 family, in the T-cell response induced by genetically engineered P815 cells.

Materials and methods

Animals and cells. Female DBA/2 mice (H-2^d), 6- to 8-weeks-old, were purchased from the Experimental Animal Center of Shanghai (China) and bred in our specific pathogen-free animal facility. P815, a mastocytoma cell line of DBA/2 origin, was cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM HEPES, 5 μ M 2-ME, 100 IU/ml penicillin and 100 μ g/ml streptomycin [all from Gibco (Carlsbad, CA, USA)] at 37°C in a humidified atmosphere consisting of 5% CO₂.

Construction of eukaryotic expression vectors. The plasmids, pcDNA3/P1A and pcDNA3/P198, are the eukaryotic expression vectors pcDNA3 (Invitrogen, CA, USA) containing the human β 2m leader followed by either the P1A₃₅₋₄₃ or P198₁₄₋₂₂ peptide epitope tethered to the β 2m through an 8-amino acid (8aa) linker [(GGGS)₂]. The constructs were generated using a template from a similar construct, the human β 2m leader-OVA₂₅₈₋₂₆₅-8aa linker-h β 2m, inserted as a *HindIII*/*XbaI* fragment in pcDNA3 (12). The OVA₂₅₈₋₂₆₅ peptide sequence was replaced with either the P1A₃₅₋₄₃ or P198₁₄₋₂₂ sequence using PCR. Using the *BamHI* site in the linker sequence, a PCR product covering the leader sequence, the peptide and the first 12 bases of the linker was generated using the following reverse primers: 5'CGGGATCCTCCTCCGAAGACCAGCCACCCTAGATAAGGCAGAGCCTCCAGGCCA GAAAG3' for pcDNA3/P1A; and 5'CGGGATCCTCCTCCAGAGTGGTTGTCACTGCCTGGTACTTAGCCTCCAGGCCAGAAAG3' for pcDNA3/P198. The forward primer, 5'CCCAAGCTTATGTCTCGCTCCGTG3' (containing a *HindIII* site) was used for both constructs. PCR products were subsequently cloned, as a *HindIII*/*BamHI* fragment, into the template vector.

Full-length mouse CD80 cDNA was amplified by RT-PCR from Con A-activated C57BL/6 splenocytes. The former primer is 5'TCCCAAGCTTCAAAGCATCTGAAACCA TGGCT3' (containing a *HindIII* site), the reverse primer is 5'TGCTCTAGACTAAAGGAAGACGGTC3' (containing an *XbaI* site). Then CD80 cDNA was cloned into the pcDNA3 and pGL3/promoter plasmids (Promega, Madison, WI, USA). The resulting plasmids were named pcDNA3/CD80 and pGL3/CD80, respectively. To construct the co-expression vector consisting of the peptide-linked β 2m molecule and CD80, a *BglIII*/*SmaI* fragment (covering the CMV promoter, the leader, the peptide, the linker, h β 2m and the SV40 polyA signal) of pcDNA3/P1A or pcDNA3/P198 was digested and cloned into pGL3/CD80, and were named pGL3/CD80-P1A or pGL3/CD80-P198, respectively. All of these constructs were confirmed by sequence analysis.

Stable transfection of P815 cells. P815 cells were transfected with pGL3/CD80-P1A, pGL3/CD80-P198, pcDNA3/P1A, pcDNA3/P198, pcDNA3/CD80 and pcDNA3 by electro-

poration using a Bio-Rad gene pulser II (Hercules, CA, USA). The electroporation was performed on cells diluted in serum-free RPMI-1640 media. Cells were pulsed at 960 μ F and 0.25 kV/cm. The co-transfection of pSV40/neo (which carries a eukaryotic resistance marker) and pGL3/CD80-P1A or pGL3/CD80-P198 (1:50) was selected using G418. Forty-eight hours after transfection, the transfected cells (designated as P815/CD80-P1A, P815/CD80-P198, P815/P1A, P815/P198, P815/CD80 and P815/neo, respectively) were selected in complete media containing 500 μ g/ml G418 (Invitrogen) and were subsequently cloned by limiting dilution. The expression of the peptide-linked β 2m fusion molecule was confirmed by PCR and RT-PCR. Mouse CD80⁺ P815 cells were identified by FACS analysis, using an anti-CD80 antibody (eBioscience, San Diego, CA, USA). A representative clone of each transfectant was selected for further studies.

Tumorigenicity of gene-modified tumor cells. The gene-modified P815 cells were tested for tumorigenicity by subcutaneous (s.c.) transplantation of syngeneic DBA/2 (H-2^d) mice. DBA/2 mice (six mice per group) were injected s.c. in the right flank with 1x10⁵ P815 cells stably transfected with the backbone vector, peptide-linked β 2m molecule and/or CD80 vector pcDNA3, pGL3/CD80-P1A, pGL3/CD80-P198, pcDNA3/P1A, pcDNA3/P198 or pcDNA3/CD80. The tumor size was measured by vernier calipers every 4 days. Animal experiments were performed in accordance with institutional guidelines for animal care by Jiao Tong University (19).

Immunization with gene-modified tumor cells. DBA/2 (H-2^d) mice (three mice per group) were injected s.c. in the right flank with 1x10⁶ P815/P1A-CD80, P815/P1A, P815/CD80 or P815/Neo cells (irradiated with Cs¹³⁷). The injections were repeated one week later and the mice were sacrificed 14 days after the second immunization for the collection of lymphocytes. Spleen mononuclear cells (SMC) were isolated by density gradient centrifugation (Nycoprep, Axis-Shield PoC AS, Norway) and used in H-2L^d/LPYLGWLVF pentamer staining and IFN- γ ELISPOT assays.

Ex vivo enumeration of tumor associated epitope (TAE)-specific CD8⁺ T cells with MHC pentamers. Pentamer staining assays were performed with an R-PE labeled Pro5 MHC Pentamer H-2L^d/LPYLGWLVF kit (Proimmune, Oxford, UK) according to the manufacturer's instructions. Briefly, 5x10⁵ SMC were incubated with one test (10 μ l) R-PE labeled H-2L^d/LPYLGWLVF Pentamer for 10 min at room temperature (22°C). After washing, cells were incubated with one test anti-CD8⁺ FITC (clone KT15) for 20 min at 4°C. Following a further two washes, cells were fixed in formaldehyde and analyzed using a FACSCalibur with CellQuest (BD Biosciences, San Jose, CA, USA).

ELISPOT assays. The number of IFN- γ -secreting, peptide specific T cells in the fresh splenocyte preparations was determined by the ELISPOT method. ELISPOT assays were performed with a Mouse IFN- γ ELISPOT kit (U-CyTech Biosciences, Utrecht, The Netherlands) according to the manufacturer's instructions. Briefly, 96-well plates were



SPANDIDOS PUBLICATIONS with 50 μ l of diluted coating antibodies overnight at 4°C. After washing five times with washing buffer [PBS containing 0.05% Tween-20 (PBST)], the plates were blocked with 200 μ l blocking solution B at 37°C for 1 h. After removal of the blocking media, SMC were incubated at 2×10^5 per well in six separate wells in a total volume of 100 μ l RPMI-1640 containing 10% FCS, in the presence or absence of 8 μ g/ml P1A non-peptide (LPYLGWLVF, Proimmune) for 24 h. Plates were washed three times with PBST, followed by incubation with biotinylated detection antibodies (100 μ l/well) at room temperature for 2 h. Plates were washed three times with PBST and PBS alone, then 50 μ l of GABA solution was added and samples were incubated at room temperature for 2 h. After washing five times with PBST, 30 μ l of freshly prepared Activator I/II solution was added. The plates were incubated at room temperature in the dark. The colorimetric reaction was stopped by washing the plates under running water. Plates were allowed to dry, and the number of spots was read on an ELISPOT reader (Bioreader® 4000 PRO-X, Germany). Data are presented as the mean number of spot forming units per 2×10^5 cells \pm standard error of the mean (SEM).

Challenge experiments following immunization with gene-modified tumor cells. DBA/2 mice were injected i.p. with either 1×10^6 P815/CD80-P1A cells (irradiated by Cs¹³⁷), P815/CD80 cells (irradiated by Cs¹³⁷) or PBS (20). Before injection, tumor cells were washed twice in PBS, re-suspended in 1 ml PBS, then injected i.p. using a 21-gauge needle. Three weeks later, 4×10^5 wild-type P815 cells were injected i.p. and the incidence of death was monitored daily to determine the survival rate of the mice.

Statistical analysis. The data were expressed as mean \pm SEM. Statistical analyses were performed using an unpaired Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

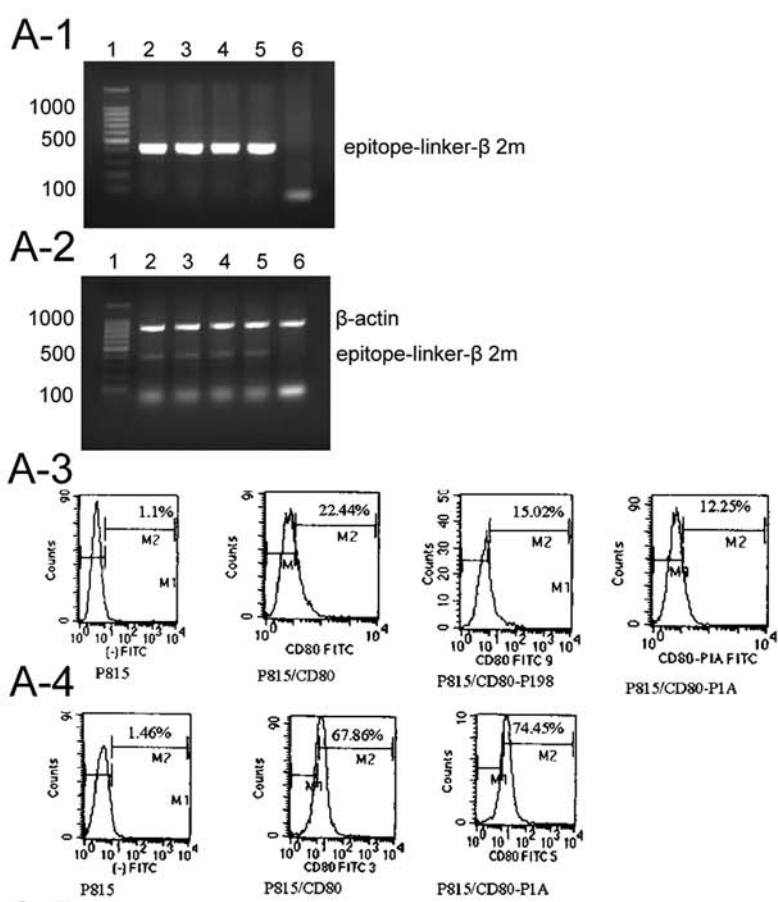
In vivo tumor growth of gene-modified P815 cells. The expression of fusion protein and CD80 in G418 selected P815 cells which had been transfected with different plasmids is shown in Fig. 1A. DBA/2 mice were injected with different G418 screened 1×10^5 P815 cells and tumor development was monitored. As shown in Fig. 1B, the *in vivo* growth of P815 cells co-expressing the peptide-linked β 2m molecule and CD80 (P815/CD80-P1A, P815/CD80-P198) was significantly slower than P815 cells transfected with other genes, including the peptide-linked β 2m molecule alone or CD80 alone gene modified P815 cells (P815/P1A, P815/P198 and P815/CD80) ($P < 0.01$). In contrast, the growth of the P815/CD80-P1A transfected cells was slower than that of the P815/CD80-P198 transfected cells ($P < 0.05$). The level of *in vitro* proliferation of the gene-modified P815 cells was assessed by tetrazolium bromide (MTT) colorimetry. No differences were observed between the gene-modified and wild-type P815 cells (data not shown), suggesting that decreased tumorigenicity was not due to impaired proliferation of the gene-modified cells.

In order to eliminate the influence of transfection efficiency on the tumorigenicity of gene-modified P815 cells, monoclonal transgene expressing P815 subline cells were selected from transfected cells by limiting dilution and G418 selection. The tumorigenicity of these sublines was assessed (Fig. 1C). DBA/2 mice (six mice per group) were injected s.c. with 1×10^5 wild-type P815 cells or stably transfected P815 sublines. Tumors were monitored every 2-4 days once the tumor became palpable. We found that tumors derived from the CD80-expressing P815 sublines grew significantly slower than those from the non-CD80-expressing P815 sublines and wild-type P815 cells ($P < 0.01$). However, no significant difference between the P815 cells transfected with the peptide-linked β 2m molecule alone and the wild-type P815 cells was observed (Fig. 1C).

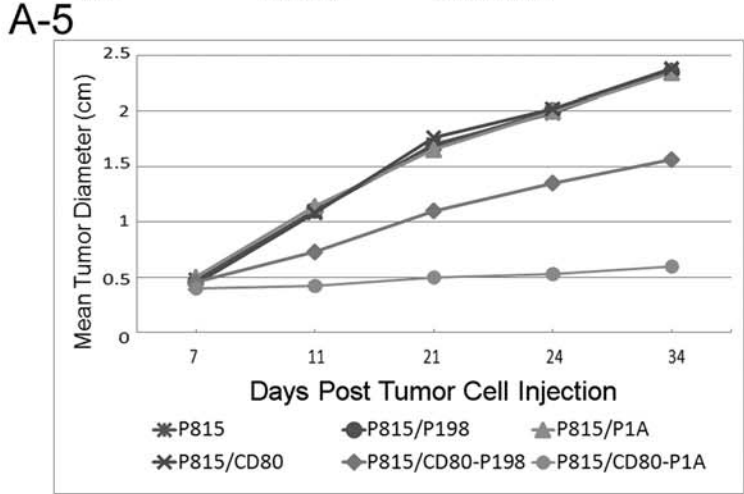
Analysis of antigen-specific CD8⁺ T cells from immunized mice. To investigate the potential mechanism underlying the differences in tumorigenicity of the gene-modified P815 cells, we immunized mice with P815/neo, P815/P1A, P815/CD80 and P815/CD80-P1A and analyzed the frequency of P1A-specific T cells. Splenocytes from the immunized mice were isolated and stained with H-2L^d/LPYLGWLVF pentamers to assess the frequency of antigen-specific CTL precursors (CTLp). The co-staining of the H-2L^d/LPYLGWLVF pentamer with anti-CD8⁺, indicates that immunization with P815/P1A, P815/CD80 and P815/CD80-P1A results in a significant induction of pentamer-positive CD8⁺ T cells (Fig. 2). We found that P815/CD80-P1A-immunized mice had the highest number of pentamer positive CD8⁺ T cells (Fig. 2D). Surprisingly, the overall frequency of LPYLGWLVF-specific CD8⁺ T cells after the P815/P1A and P815/CD80 immunizations were very similar (Fig. 2B and C). These results show that the expression of peptide-linked β 2m molecule in P815 cells elicits a TAE-specific CD8⁺ T cell response that is enhanced by the co-expression of CD80.

IFN- γ -producing cell frequency of splenic lymphocytes from immunized mice. To determine whether the co-expression of the peptide-linked β 2m molecule and CD80 enhanced tumor-specific immune responses in mice, the number of cells expressing IFN- γ , a cytokine secreted by CTL, was evaluated using the IFN- γ enzyme-linked immune spot (ELISPOT) assay. Fourteen days after the last immunization, splenocytes were isolated from the immunized mice, co-cultured in the presence or absence of the P1A nonapeptide (LPYLGWLVF) for 24 h and the IFN- γ -producing cell frequency was determined. As shown in Fig. 3, minimal detectable reactivity was observed in splenocytes from P815/neo or P815/P1A-immunized mice, whereas significant reactivity was observed in P815/CD80 or P815/CD80-P1A-treated mice ($P < 0.05$). In particular, no difference in the IFN- γ -producing cell frequency of lymphocytes was observed in P815-immunized mice after the spleen cells were re-stimulated with the minimal P1A CD8⁺ T cell epitope, LPYLGWLVF compared to non-stimulated spleen cells. However, a significant difference was observed in P815/P1A or P815/CD80-P1A-immunized mice ($P < 0.05$), indicating that the CD8⁺ T cell epitope was correctly processed and that the P1A-specific CD8⁺ T cell response was effectively induced. Furthermore, these data

A



B



C

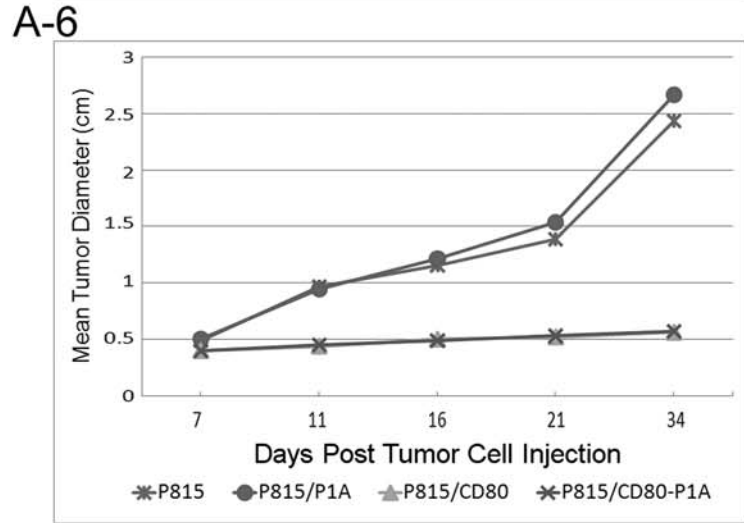


Figure 1. Tumor growth of gene-modified P815 cells. Legend on page 5.

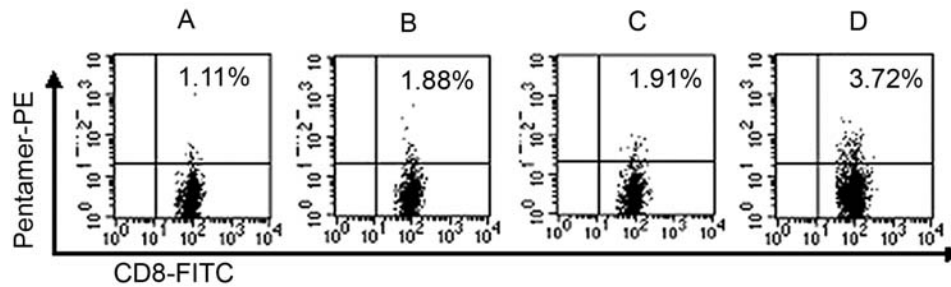


Figure 2. Tumor associated epitope (TAE)-specific CD8⁺ T cell responses induced by gene-modified P815 cells. Two weeks after the final immunization of mice with irradiated P815 cells (A) P815/neo; (B) P815/P1A; (C) P815/CD80; (D) P815/CD80-P1A), spleen cells were co-stained with a FITC-conjugated anti-CD8⁺ mAb and a PE-conjugated H2-L^d-P1A₃₅₋₄₃-pentamer. Samples were analyzed by FACSCalibur. The data presented here are a representative experiment of three independent experiments that gave similar results.

suggest that the expression of CD80 in P815 cells increased the IFN- γ -producing cell frequency in a non-peptide-specific manner.

Immunization with inactivated P815/CD80-P1A cells induces protection against P815 cells. To determine whether immunization of DBA/2 mice with irradiated gene-modified tumor cells could result in protective immunity, we examined the survival of mice injected with a lethal dose of P815 tumor cells after injection with the irradiated gene-modified P815 cells. Mice (ten animals in each group) were immunized with irradiated P815/CD80-P1A cells, P815/CD80 cells or PBS. Three weeks after immunization, mice were challenged i.p. with wild-type P815 cells and monitored for survival. As shown in Fig. 4, animals immunized with P815/CD80 or PBS showed no evidence of protection from the tumor challenge. However, a strong protective effect was observed in animals immunized with P815/CD80-P1A cells. Sixty days after the tumor challenge, the survival rate of P815/CD80-P1A-immunized mice was approximately 50%, indicating that the epitope-specific immune response was critical for this protection.

Discussion

MHC class I molecules bind intracellular peptide antigens, both foreign and self, and display them at the cell surface for scrutiny by CD8⁺ CTLs. A single cell may display thousands of different class I bound peptides, the vast majority of which are present in extremely low amounts (<0.1% of total) (21). Previous studies have shown that an epitope-linked β 2m

molecule could provide a simple and more efficient means of enhancing the formation of defined MHC/peptide complexes (9-12). Here, we show that immunization with P815 cells expressing both the P1A₃₅₋₄₃-linked β 2m molecule and the murine CD80 gene elicited an effective antitumor immune response. Furthermore, our data indicate that the immune response to P1A was a major component which induces the tumor rejection.

Among the different murine tumors used in antitumor immunity studies, the mastocytoma P815 mouse model is the most thoroughly characterized. Five different antigens are recognized by CTLs on this tumor (22,23). Among these antigens, the major target of the rejection response was shown to consist of a non-america peptide encoded by the P1A gene, which is silent in normal tissue and expressed in several types of mouse tumor (24). Antigen P198 is a different antigen that is expressed by an immunogenic variant obtained after mutagenesis of P815 tumor cells, and which consists of a peptide corresponding to a point mutation in a gene expressed ubiquitously (25,26). Different modes of immunization with these two antigens have been tested for their efficiency to induce antitumor CTL responses or antitumor protection. In this study, we show that the co-expression of the epitope-linked β 2m molecule (P1A₃₅₋₄₃-linked β 2m molecule or P198₁₄₋₂₂-linked β 2m molecule) and the co-stimulatory molecule CD80 in P815 tumor cells could significantly decrease tumorigenicity in syngeneic DBA/2 mice. In contrast, the *in vivo* growth of P815 cells expressing both the P1A₃₅₋₄₃-linked β 2m molecule and CD80 is slower than that of P815 cells expressing both the P198₁₄₋₂₂-linked β 2m molecule and CD80. In agreement with other reports (27), our data show that the

Figure 1. Tumor growth of gene-modified P815 cells. (A) Expression of fusion protein and CD80 in transfected P815 cells. The expression of the peptide-linked β 2m fusion molecule was confirmed by PCR and RT-PCR. Mouse CD80⁺ P815 cells were identified by FACS analysis, using an anti-CD80 antibody. (A-1) PCR amplification of epitope-linker- β 2m fusion gene from the genomic DNA of transfected P815 cells; (A-2) RT-PCR analysis of epitope-linker- β 2m fusion gene in transfected P815 cells. (1, 100 bp DNA Ladder Marker; 2, RT-PCR result from P815/P1A; 3, RT-PCR result from P815/P1198; 4, RT-PCR result from P815/CD80-P1A; 5, RT-PCR result from P815/CD80-P198; 6, RT-PCR result from P815 cell). (A-3) The expression of CD80 molecule on the surface of G418 selected transfectants. (A-4) The expression of CD80 molecule on the surface of monoclonal cell lines. (B, A-5) Decreased tumor growth of peptide-linked β 2m molecule and CD80 gene-modified P815 cells. DBA/2 mice (six mice per group) were injected s.c. with 1×10^5 G418-screened transfectants. Tumors were monitored every 3-5 days once the tumor became palpable. Values are given as mean tumor diameter (cm) \pm SEM, where n=6 independent experiments. (C, A-6) Decreased tumor growth of CD80 gene-modified P815 clones. DBA/2 mice (six mice per group) were injected s.c. with 1×10^5 different monoclonal cell lines. Tumors were monitored every 3-5 days once the tumor became palpable. Values are given as mean tumor diameter (cm) \pm SEM, each experiment was repeated with similar results.

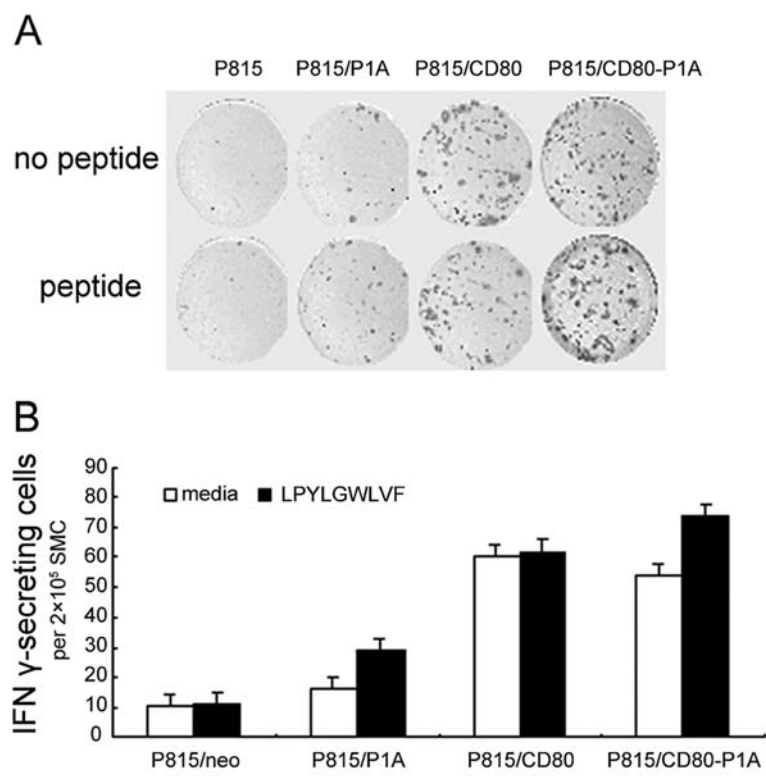


Figure 3. Functional analysis of peptide-specific CD8⁺ T cells responses by ELISPOT. P1A₃₅₋₄₃-specific CD8 T cell responses were measured using the IFN- γ -ELISPOT as described in Materials and methods. Mice were immunized twice, one week apart and assayed 14 days after the last immunization. Mice were tested for IFN- γ responses to LPYLGWLVF peptide (black bars) compared with media alone (white bars) measured by ELISPOT assay (n=3/group). (A) Examples of the spot-forming cell response. (B) The number of spots detected in the IFN- γ -ELISPOT assays. Each bar represents the mean spot number \pm SEM per 2×10^5 splenocytes. Similar results were seen with repeat experiments.

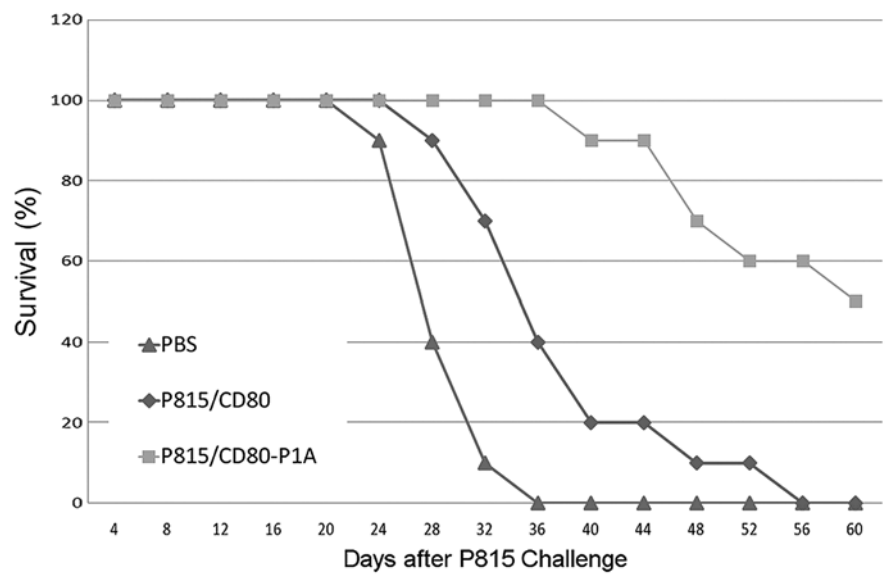


Figure 4. Tumor protection induced by P1A-specific immunity. DBA/2 mice were immunized i.p. with either 10^6 irradiated P815/CD80-P1A cells (n=10), 10^6 irradiated P815/CD80 cells (n=10) or PBS (n=10). Three weeks later, 4×10^5 wild-type P815 cells were injected i.p. and survival was monitored daily for 60 days. Each experiment was repeated with similar results.

tumor specific peptide, P1A, is more effective than P198 in terms of inducing antitumor immunity.

Peptide/MHC tetramer analysis and the ELISPOT assay have been widely used to assess antigen-specific T cell responses (28,29). To investigate the potential mechanism

involved in the differences in tumorigenicity of gene-modified P815 cells, we analyzed the P1A-specific CD8⁺ T cell responses using the H-2L^d/LPYLGWLVF pentamer analysis and mouse IFN- γ ELISPOT assay. We found that although immunization with P815 cells expressing P1A₃₅₋₄₃-linked β 2m



SPANDIDOS alone increased the frequency of TAE-specific cells, the total number of IFN- γ -producing splenocytes was still small. These findings may explain why epitope-linked β 2m molecule expression in P815 cells does not affect its tumorigenicity. Bartholdy *et al* (30) have reported that vaccinations using DNA vaccines encoding the immunodominant MHC class I-restricted epitopes of lymphocytic choriomeningitis virus covalently linked to β 2m only protected against systemic infection, whereas protection against peripheral challenge was limited.

In addition to antigen presentation, successful cancer immunotherapy requires the engagement of the T-cell receptor with an antigen/MHC and a costimulatory molecule such as CD80 or CD86 (31,32). Most tumor cells lack the B7 family (33,34), and thus exhibit T-cell tolerance. Several studies (35-37) have shown that tumor cell vaccines expressing the co-stimulatory molecule, CD80, can induce much stronger antitumor immunity than the wild-type tumor vaccine. Therefore, in this study, the co-expression vector of epitope-linked β 2m molecule and CD80 was constructed and transfected into P815 cells. We show that the expression of the co-stimulatory molecule CD80 in P815 cells significantly increases the number of IFN- γ -producing splenocytes, which is non-specific to the non-peptide LPYLGLVF. This may explain the difference in tumorigenicity between CD80-modified tumor cells and other tumor cells.

In order to evaluate the protective capacity of different modified P815 tumor vaccines, we have further examined the ability of the peptide-linked β 2m molecule to induce protective immunity against wild-type P815 challenge. Compared with P815 cells expressing the co-stimulatory molecule CD80 alone, immunization with irradiated P815 cells expressing both the epitope-linked β 2m molecule and CD80 significantly elicited protective immunity. We found that approximately 50% of the mice immunized with P815 cells expressing both the epitope-linked β 2m molecule and CD80 were protected against a challenge with a lethal dose of P815 tumor cells injected into the peritoneal cavity, consistent with previous studies (19,20). Therefore, our results indicate that the co-expression of both the peptide-linked β 2m molecule and co-stimulatory molecule CD80 in P815 cells elicits antigen-specific T cell responses. Our data suggest that the immune response to P1A was the major component of the tumor rejection.

In summary, our findings show that tumor vaccines modified with peptide-linked β 2m molecule and CD80 can generate a more effective antitumor immunity than a single-modified tumor vaccine *in vivo*, indicating that the peptide-linked β 2m molecule and CD80 were able to stimulate anti-tumor immune responses synergistically. These results provide experimental evidence that supports the feasibility and effectiveness of this novel approach in cancer immunotherapy.

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