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Overlapping Synthetic Peptides Encoding TPD52 as Breast Cancer Vaccine in Mice: Prolonged Survival¹

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Summary

Peptide-based vaccines, one of several anti-tumor immunization strategies currently under investigation, can elicit both MHC Class I-restricted (CD8⁺) and Class II-restricted (CD4⁺) responses. However, the need to identify specific T-cell epitopes in the context of MHC alleles has hampered the application of this approach. We have tested overlapping synthetic peptides (OSP) representing a tumor antigen as a novel approach that bypasses the need for epitope mapping, since OSP contain all possible epitopes for both CD8⁺ and CD4⁺ T cells. Here we report that vaccination of inbred and outbred mice with OSP representing tumor protein D52 (TPD52-OSP), a potential tumor antigen target for immunotherapy against breast, prostate, and ovarian cancer, was safe and induced specific CD8⁺ and CD4⁺ T-cell responses, as demonstrated by development of specific cytotoxic T cell (CTL) activity, proliferative responses, interferon (IFN)- γ production and CD107a/b expression in all mice tested. In addition, TPD52-OSP-vaccinated BALB/c mice were challenged with TS/A breast carcinoma cells expressing endogenous TPD52; significant survival benefits were noted in vaccine recipients compared to unvaccinated controls ($P < 0.001$). Our proof-of-concept data demonstrate the safety and efficacy of peptide library-based cancer vaccines that obviates the need to identify epitopes or MHC backgrounds of the vaccinees. We

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show that an OSP vaccination approach can assist in the disruption of self-tolerance and conclude that our approach may hold promise for immunoprevention of early-stage cancers in a general population.

Keywords

Overlapping Synthetic Peptides (OSP); Vaccine; Cancer; Tumor protein 52

1. Introduction

The goal of therapeutic vaccines against cancer is to induce or enhance immune responses directed against tumor antigens. Peptides derived from tumor-specific (mutated) or tumor-associated antigens are presented in the grooves of major histocompatibility complex (MHC) class I or class II molecules on cancer cells, where they activate either CD8⁺ T cells with cytotoxic T-lymphocyte (CTL) activity or CD4⁺ T lymphocytes (T helper) that secrete cytokines capable of inhibiting the growth of tumor cells. An optimal cancer vaccine must therefore contain at least two antigenic epitopes: one that will induce specific CTL responses and a T-helper epitope [1-3].

Currently, several peptide-based vaccines targeting specific tumor antigens are under development [1, 2]. Peptide-based vaccine studies have focused on identifying epitopes that are capable of eliciting either MHC Class I-restricted (CD8⁺) and/or Class II-restricted (CD4⁺) responses. Peptide vaccines may have advantages over other tumor vaccine approaches that rely on intact proteins or tumor cells [4, 5]. Several advantages in terms of relative ease of peptide synthesis, chemical stability, storage, delivery, and the lack of oncogenic or infectious potential has made the peptides attractive vaccine candidates for clinical use. However, a major limitation of the widespread usefulness of peptide vaccines is the need to identify specific T-cell epitopes in the context of MHC alleles [6].

In a previous report, we described a novel strategy that bypasses the need for epitope mapping for an outbred population: vaccination with overlapping synthetic peptides (hereafter referred to simply as OSP) [7]. We demonstrated that OSP derived from viral structural proteins induced strong cell-mediated immunity in outbred and different inbred mice. Potential advantages of OSP vaccines are: 1) OSP are promiscuous, as they contain all the peptide sequences comprising their cognate protein, 2) OSP covering short amino acid sequences of a toxic protein are very likely nontoxic, 3) OSP contain all possible epitopes for both CD8⁺ and CD4⁺ T cells of a genetically diverse population and can bind to different MHC alleles, 4) OSP contain all possible CTL epitopes, thereby minimizing target sequence variations that would result in loss of, or diminished, recognition by CTL, 5) OSP vaccines can be designed rapidly, because only the amino acid sequence of the target protein is required, and 6) OSP may bind directly by MHC molecules expressed on the cell surfaces or ingested and processed by antigen presenting cells. Therefore, neither epitopes nor MHC alleles need to be identified.

In this study, we evaluated the use of an OSP vaccine corresponding to tumor protein D52 (TPD52) for safety, immunogenicity and anti-tumor efficacy in mice. TPD52 was chosen

because 1) it is a small protein (21 kDa, 180 amino acids (aa)) and the entire sequence can be covered with 34 peptides of 15 aa in length, with 10 aa overlaps between sequential peptides and 2) murine orthologue of TPD52 (mD52) represents a “self,” non-mutated tumor-associated antigen (TAA). The mD52 has been cloned and, based on the sequence information, is predicted to be 86% identical to human TPD52 [8]. The human TPD52 gene family is comprised of four genes, *hD52*, *hD53*, *hD54* and *hD55* [8-11]. TPD52 proteins are involved in several cell functions, such as exocytotic secretion, vesicle trafficking, calcium-mediated signal transduction, cell proliferation and apoptosis [10]. The human D52 locus has been mapped to chromosome 8q21, a chromosome band that is frequently overrepresented in human cancer. Several reports have shown that *hD52* is overexpressed in cancers of the breast, lung, prostate, colon, and ovary, as well as in B-cell malignancies [12-15]. Also, expression of TPD52 has been reported in some normal tissues in inbred and outbred mice [16-18]. It has been shown that *hD52* and *hD53* genes encode markers or regulators of cancer-cell proliferation [8], suggesting that TPD52 may be important for initiating and perhaps maintaining a tumorigenic and metastatic phenotype. Recent data also showed that mD52 expression initiated cellular transformation, tumorigenesis, and progression to metastasis [17].

Briefly, our results conclude that TPD52-OSP vaccination was safe and effective in inducing CTL responses with anti-tumor activity in mice. In a breast-cancer cell challenge model, TPD52-vaccinated mice had a significantly prolonged survival compared to control mice. In addition, activation of T cells directed against the tumor-associated self-antigen was without noticeable development of autoimmunity. The information provided by this study may be useful for the development of peptide-based vaccines without identifying either epitopes or MHC backgrounds of the vaccinees.

2. Materials and methods

2.1. Peptides

Tumor Protein D52 (TPD52)-OSP consisted of a group of 34 peptides of 15 aa in length, with 10 aa overlaps between sequential peptides representing the murine form of TPD52; these peptides were custom-made by Millipore/Chemicon (Temecula, CA). Human immunodeficiency virus (HIV) clade B Tat (Tat-OSP, also termed Control-OSP) consisted of 23 peptides of 15 aa in length, with 5 aa overlaps between sequential peptides; the peptides were obtained from the AIDS Research Reagent Repository Program (ARRRP; NIAID, NIH).

2.2. Cell lines

TS/A cells, a spontaneous murine mammary adenocarcinoma cell line of BALB/c origin, were cultured in DMEM medium supplemented with L-glutamine, antibiotics and 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). P815 cells (mastocytoma cells; American Type Culture Collection, Manassas, VA) were cultured in DMEM medium supplemented with L-glutamine, antibiotics and 10% FBS.

2.3. TPD52 expression analysis

To determine expression of mD52 in parental TS/A cells and different tissues (breast, liver, thymus, kidney, heart and spleen tissue), total RNA was extracted by TriZol reagent (Invitrogen, Carlsbad, CA), treated with RNase-free DNase and used for amplification in an RT-PCR reaction using the SuperScript One-Step RT-PCR with Platinum *Taq* (Invitrogen) following the manufacturer's protocol. Two μ l of total RNA were processed in a 50 μ l final volume, the reverse transcription cycles consisted of 1 cycle of 30 min at 50°C; 2 min at 94°C, followed by PCR amplification using primer pairs for mD52: forward: 5'-AAGGTCTGCTGAAGACAGAGC-3'; and mD52 reverse: 5'-TGTGGAATTTCAGGACTTCTC-3', and the housekeeping gene product; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Invitrogen) as internal control. The final amplification consisted of 35 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. The PCR was terminated by elongation at 72°C for 10 min. The results were visualized on 2% agarose gels containing ethidium bromide.

2.4. Mice

Eight to ten-week old outbred NMRI (Harlan, IN) and inbred BALB/c (H-2^d) (Taconic Farms, Germantown, NY) mice were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

2.5. Immunization protocol

Mice were immunized subcutaneously with TPD52-OSP at 5 μ g of each individual peptide in 100 μ l phosphate-buffered saline (PBS) per mouse together with MPL+TDM Adjuvant System (Sigma) or CpG oligodeoxynucleotides (CpG ODNs) (Coley Pharmaceutical, Wellesley, MA) (50 μ g) plus Incomplete Freund's Adjuvant (IFA) (for some of the tumor challenge studies). Control mice were immunized subcutaneously with either with Control-OSP (Tat-OSP), PBS or adjuvant only. Immunizations were given at weeks 0, 3, and 6. Mice were euthanized 10 days after the last boost and their spleens were collected to evaluate cellular immune responses.

2.6. Cell proliferation assay

Splenocytes (5×10^5) from vaccinated and control mice (NMRI, BALB/c) were cultured with either TPD52-OSP, Tat-OSP (1 μ M) or no OSP for 4 days in 96-well U-bottomed plates, then pulsed with 1 μ Ci/well [³H]thymidine overnight. After cells were harvested, [³H]thymidine incorporation was assessed using a β -counter (Beckman, Fullerton, CA). The data were recorded by using the stimulation index (SI), calculated as the ratio of cpm of stimulated cells to cpm of cells grown in medium only.

2.7. IFN- γ ELISPOT assays

Assays were performed using mouse ELISPOT kits from BD PharMingen (San Diego, CA) as described by the supplier. Splenocytes from NMRI and BALB/c mice were restimulated overnight with 1 μ M OSP in the IFN- γ pre-coated plates.

2.8. Intracellular IFN- γ assay

Splenocytes were harvested from immunized and control mice and incubated for 18 h at 37°C in 96 U-well plates at 5×10^5 cells/well together with 1 μ M OSP. During the last 4 hours, 1 μ l/well GolgiStop (BD Pharmingen) was added to the culture. Cells were stained with 1 μ g/well FITC-labeled anti-mouse CD8 and CD4 monoclonal antibody (mAb; BD Pharmingen). Following surface labeling, the cells were fixed with 1% formaldehyde (20 min, 4°C) and then permeabilized with 0.5% saponin (10 min, 4°C) before intracellular labeling with 0.5 μ g/well PE-labeled rat anti-mouse IFN- γ (BD Pharmingen) for 20 min at 4°C. After a final wash, the cells were resuspended in FACS buffer and analyzed immediately by FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

2.9. In vitro CTL activity assay

Ten days after final vaccination, BALB/c mouse splenocytes were harvested. Splenocytes from vaccinated and control mice were cultured with OSP (5 μ M) and recombinant interleukin-2 (rIL-2) (20 U/ml) for 5 days in 24-well plates. Cytolytic activity of the T-cell cultures was assessed by standard 4- to 6-h 51 Cr release assays, using different ratios of effector (E) to target cells (T). As targets, either P815 (H-2^d) cells or TS/A cells were labeled with 51 Cr for 90 min at 37°C, washed, and 10^4 P815 cells were incubated with TPD52-OSP or Control-OSP overnight. All experiments were repeated at least two times, and representative results are shown. The mean percentage of specific lysis in triplicate wells was calculated by the standard formula $[(\text{release by CTL} - \text{release by targets alone}) / (\text{release by 5\% Triton-X100} - \text{release by targets alone})] \times 100\%$. Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X100. Spontaneous release was determined from the target cells without addition of effector cells.

2.10. CD107a/b (lysosome-associated membrane protein, LAMP-1/2) expression assay

Splenocytes from individual groups of BALB/c mice were cultured for 5 days at 37°C in 24 well plates at 5×10^6 cells/well together with 20 U/well rIL-2 and 5 μ M TPD52-OSP or Tat-OSP in accordance with the prior immunization. Splenocytes from control mice given adjuvant only were cultured with rIL-2 only. Splenocytes were washed and resuspended in supplemented RPMI 1640 (Sigma-Aldrich) and stimulated with TS/A cells (at an E:T ratio of 5:1); medium alone served as the negative control. FITC-labeled anti-mouse-CD107a & b (10 μ l/ml) (BD Pharmingen) were added directly to the wells to detect degranulation. Following 1 h of incubation at 37°C in 5% CO₂, 1 μ l of monensin (Golgi-stop; BD Biosciences) was added for a final concentration of 6 μ g/ml and incubated for an additional 5 h at 37°C in 5% CO₂. Samples were then surface-stained using PE-labeled anti-mouse CD8. After washing, cells were resuspended in 1% paraformaldehyde (Sigma-Aldrich) until multicolor flow cytometric analysis was performed on a FACSCalibur.

2.11. Tumor challenge

BALB/c mice (10/group) were immunized subcutaneously according the immunization protocol. To induce lung metastases, mice were challenged intravenously with 10^3 or 5×10^3 TS/A tumor cells in 100 μ l of Hanks solution (Invitrogen) 10 days after the final immunization and followed prospectively. TPD52-OSP vaccine recipients and control

groups were monitored regularly for any distress and weight loss. Mortality rate was tracked daily, and mice were euthanized for humane reasons when they lost > 15% of body weight.

2.12. Immunohistochemistry and histopathology on tissue sections

Analysis of formalin-fixed, paraffin-embedded lung tissue was performed on 5 µm-thick sections that were deparaffinized through xylene and 100% ethanol and blocked for endogenous peroxidase activity by incubation in 0.3% H₂O₂-absolute methanol for 10 min. Sections were fixed with acetone before staining with rabbit polyclonal anti-CD3 antibody (Cell Marque, Rocklin, CA). Matched sections were deparaffinized and stained with hematoxylin and eosin (H&E). Slides were analyzed using an Olympus BX51 microscope. Photomicrographs were taken with a Q Imaging micropublisher 3.3 RTV digital camera.

2.13. Statistical analysis

Survival data were analyzed using the method of Kaplan and Meier [19]. Differences were tested using the log rank test. Two-sided p-values are presented. $P < 0.05$ was considered significant.

3. Results

3.1. TPD52 expression in the TS/A cell line and in normal murine tissues

Murine D52 naturally mirrors human TPD52 with respect to known function and expression in tumor cells compared to normal cells and tissues, and thus represents a “self,” non-mutated TAA with direct relevance to several human cancers, including prostate, breast, lung, ovarian and colon carcinomas. We tested the expression of mD52 in cultured TS/A breast carcinoma cells and normal mouse tissues, such as thymus, kidney, liver, heart, mammary tissue, and spleen. Total RNA was isolated from the TS/A cell line and tissues as described in Materials and Methods. PCR amplification demonstrated expression of mD52 in the TS/A cell line, mammary tissue, and several mouse tissues (Fig. 1); the highest level of expression was in TS/A cells and the kidney.

3.2. OSP induced cell-mediated immunity (CMI) in mice

To determine the safety and immunogenicity of TPD52-OSP vaccination in genetically diverse hosts, groups of inbred mice ($n = 7$ per group) and outbred NMRI mice ($n = 5$ per group) were used. The latter were derived from outbred Swiss mice by random breeding; these mice do not tolerate skin grafts from each other and express MHC molecules that are similar to H-2^d but differ between individuals of the same strain. Mice were immunized either with; TPD52-OSP, Tat-OSP, or PBS and adjuvant only. All animals tolerated the immunization series without untoward effects. Ten days after the last boost, splenocytes from these mice were cultured with the two corresponding OSP or in medium only *in vitro*. Proliferative responses were examined by [³H]thymidine incorporation. Pooled splenocytes from TPD52-OSP-immunized BALB/c (Fig. 2A and B) and splenocytes from individual NMRI mice (Fig. 2, C and D) only showed significant proliferative responses when restimulated with TPD52-OSP but not with Tat-OSP; the converse was true for Tat-OSP-immunized animals.

Next, we examined the production of IFN- γ in mice by ELISPOT analysis and intracellular cytokine staining (ICS) in the cohorts of TPD52-OSP or Tat-OSP-vaccinated or control mice. As shown in Fig. 3A and D, the number of spot-forming units (SFU) was higher in splenocytes in the TPD52-OSP or Tat-OSP immunized mice when the cells were restimulated *in vitro* with the corresponding OSP compared to control mice. Restimulation of splenocytes with corresponding OSP yielded between 714 and 998 SFU/10⁶ for BALB/c mice and 305 and 567 SFU/10⁶ for outbred NMRI mice. Measured directly without *in vitro* restimulation, SFU ranged between 72 and 116 SFU/10⁶ splenocytes in BALB/c mice and 11 and 56 SFU/10⁶ in NMRI mice (TPD52-OSP and Tat-OSP vaccinated mice), respectively; the corresponding numbers for the adjuvant controls were 11-120 SFU/10⁶ splenocytes. In addition, ICS showed that after restimulation with the corresponding peptides, TPD52-OSP and Tat-OSP-immunized BALB/c mice had an increase in the percentage of IFN- γ ⁺CD8⁺ T cells from 0.45% to 1.4% and from 0.39% to 1.19% (Fig. 3B), respectively, and an increase in the percentage of IFN- γ ⁺CD4⁺ T cells from 0.57% to 2.2% and from 0.51% to 1.39% (Fig. 3C), respectively. We observed similar results in outbred NMRI mice (Fig. 3E and F). Of note, every single one of these random-bred mice showed increases in ICS after specific restimulation with peptides corresponding to the OSP immunogens. Together, these results demonstrate that vaccination with OSP was safe and induced specific T-cell responses, involving both CD4⁺ and CD8⁺ T cells, in an inbred population (BALB/c) and within each animal of a genetically diverse population of NMRI mice.

3.3. Assessment of CTL activity *in vitro*

To assess the functionality of the CD8⁺ TPD52-specific cellular immune responses, the ⁵¹Cr-release assay was used to measure antigen-specific CTL activity in OSP-immunized and control BALB/c mice. Splenocytes from immunized mice were expanded *in vitro* in the presence of OSP and rIL-2 for 5 days and used as effector cells. As targets, either P815 cells (H-2^d) were pulsed with relevant OSP and labeled with ⁵¹Cr or TS/A cells were labeled with ⁵¹Cr, and used at different E:T ratios (Materials and Methods). Specific CTL responses were induced by OSP-immunization in all mice, as indicated by specific lysis of MHC-matched P815 cells loaded with corresponding OSP (Fig. 4, A and B) ($p < 0.005$, $p < 0.01$). In addition, to confirm antigen-specific lysis, we used TS/A tumor cells (the same tumor cell line that used for *in vivo* challenge) as targets (Fig. 4C) ($p < 0.01$). These results indicate that by OSP immunization, antigen-specific CTL activity generated and CTL can kill tumor cells.

3.4. Degranulation of TPD52-specific CD8⁺ T cells in response to *in vitro* stimulation with TS/A tumor cells

To confirm specific targeting of syngeneic tumor cells, we assessed the expression of CD107a and b (LAMP-1 and LAMP-2) in CD8⁺ T cells following immunization. Degranulation of intracellular vesicles by lymphocytes can be measured using CD107a and b, as described recently for CD8⁺ T cells [20]. Degranulation is accompanied by the transport of the CD107a and b proteins to the cell surface, followed by immediate reinternalization. Surface expression of the CD107a and b markers on actively degranulating CD8⁺ T cells is inversely correlated with the presence of perforin and directly correlated

with cytotoxic activity [20]. Thus, expression of CD107 is directly correlated with cytotoxic activity.

Splenocytes were harvested from TPD52-OSP and Tat-OSP-immunized BALB/c mice and cultured with the corresponding OSP as described in Materials and Methods; splenocytes from mice given adjuvant only were grown in rIL-2 without peptides. Based upon the expression of CD107a/b by FACS analysis, 8.7% of TPD52-OSP-restimulated splenocytes from TPD52-OSP-immunized mice responded to TS/A tumor cells and degranulated, presumably lysing target cells (Fig. 4D, left panel). In contrast, only 2.6% of Tat-OSP-restimulated splenocytes from Tat-OSP-immunized mice were CD8⁺CD107a/b⁺ when brought in contact with TS/A tumor cells (Fig. 4D, right panel), which was similar to the 2.2% CD8⁺CD107a/b⁺ splenocytes from control mice given adjuvant only. This degranulation assay thus confirmed our standard ⁵¹Cr-release cytotoxicity assay. Together, these data show that OSP immunization generated peptide-specific CTL activity and that activated CTL underwent degranulation, released IFN- γ and effectively killed TS/A tumor cells, which express endogenous mD52 as shown in Fig. 1. Thus, TPD52-OSP immunization effectively broke tolerance and generated specific CTL against tumor cells overexpressing the corresponding TAA.

3.5. Anti-tumor effects of TPD52-OSP vaccination in BALB/c mice

Our next goal was to examine *in vivo* whether vaccination with OSP could protect BALB/c mice against syngeneic tumor cell challenge. Mice were immunized with TPD52-OSP or Control-OSP at 5 μ g of each individual peptide in PBS per mouse together with MPL+TDM adjuvant. As additional control, a group of mice was given adjuvant only. Ten days after the last immunization, all mice were inoculated intravenously with 10³ TS/A tumor cells and monitored prospectively for development of metastatic disease as described (Materials and Methods). TPD52-OSP-vaccinated mice showed a delayed tumor growth and weight loss pattern compared to control mice, all of which died around day 55 post tumor challenge (Fig. 6A). In contrast, TPD52-OSP-immunized mice had a 40 percent long-term survival rate ($p < 0.001$). No significant survival difference was observed between mice given Tat-OSP or MPL+TDM adjuvant only ($p < 0.6$).

Immunized mice undergoing necropsy were also routinely assessed for evidence of autoimmunity. We observed no abnormal lymphocytic infiltrates into organs, such as liver, heart and kidney (data not shown). These observations have been confirmed in another study [18].

Recruitment of innate immunity by optimizing adjuvants may result in improved anti-tumor CTL responses. Enhancing the interaction between T cells and antigen presenting cells (APC) by attracting larger numbers of APC and/or stimulating their function may thus yield greater anti-tumor immune responses. CpG oligodeoxynucleotides (CpG-ODNs) have been shown to activate APC (particularly tissue dendritic cells), leading to up-regulation of costimulatory molecules and the secretion of cytokines necessary for T lymphocyte activation [21-23]. To augment the immunogenicity of our OSP vaccine strategy, we selected CpG-ODN as adjuvant. Mice were immunized with TPD52-OSP plus CpG-ODN in IFA and then challenged with TS/A tumor cells (5 $\times$ 10³ tumor cells/mouse). Fig. 5B shows

that CpG-ODN enhanced the anti-tumor effects of TPD52-OSP vaccination and improved the long-term survival rate (62.5%) ($p < 0.001$); the median survival exceeded 170 days, compared to 65 days in the vaccine trial involving MPL+TDM as adjuvant for the TPD52-OSP vaccine.

3.6. Immunohistochemistry (IHC) on tissue sections

Next, we sought to test whether TPD52-OSP-immunized mice had more tumor-infiltrating lymphocytes in pulmonary tissues compared to control mice. Lung tissue sections were stained by IHC for CD3⁺ cells. Mice vaccinated with TPD52-OSP showed high numbers of CD3⁺ cells in the tumor tissue (Fig. 6, F and H). This pattern of lymphocytic infiltration was in stark contrast to that observed in the control mice (Fig. 6, B and D). High numbers of tumor-infiltrating CD3⁺ cells were significantly associated with small tumor size and better survival rates of the TPD52-OSP vaccinated mice.

In a separate study, macroscopic examination of lungs of TPD52-OSP and Tat-OPS-immunized mice and mice given adjuvant only (MPL+TDM) revealed significantly reduced lung metastases in vaccinees compared to the two controls. In this experiment, higher numbers of tumor cells were inoculated (2×10^5 as opposed 1×10^3 TS/A cells). Consistent with the smaller size of the lung nodules, the mean lung weight of TPD52-OSP-immunized mice was lower than that of control mice (336 mg versus 556 mg for mice given Tat-OPS and 553 mg for mice given adjuvant only; $p = 0.0285$ by Student's T test comparing vaccinees versus the combined controls).

Together, these data show that TPD52-OSP vaccination induced specific cellular immune responses that resulted in the rejection of TPD52⁺ tumors. Of note, our OSP vaccination strategy successfully overcame peripheral T-cell tolerance against the TPD52 self-antigen without inducing autoimmunity.

4. Discussion

Peptide-based vaccines are among one of several approaches that are presently being pursued as cancer vaccines. The relative ease of their production, storage, delivery, their chemical stability, lack of infectious potential without the risk of genetic integration or recombination has made peptides attractive vaccine candidates. The concept of peptide vaccines is based on identification and chemical synthesis of T-cell epitopes, which are immunodominant and can induce specific immune responses. Vaccines based upon several peptides have undergone clinical trials for cancer immunotherapy [1, 2, 4, 24-30].

However, the main obstacle associated with peptide vaccines based upon defined T-cell epitopes is the need to identify the latter in the context of MHC alleles. In patients, this generally restricts a peptide vaccine approach to individuals with HLA A2, unless mixtures of peptides tailored for additional HLA alleles are employed as recently described by Slingluff et al. [27, 31].

We have demonstrated in the past that an OSP vaccine derived from viral structural proteins can elicit robust CMI in multiple strains of outbred and inbred mice [7]. In the present study,

we selected TPD52 as a model because it represents a “self” tumor antigen overexpressed in the cells used as tumor challenge. The human ortholog is overexpressed in various tumors, including breast, prostate, lung and colon carcinomas. Many TAAs have been shown to be expressed during fetal development. Therefore, weak host immune response to such “self-TAAs” would be expected. In addition, the induction of immune responses to a self-antigen raises the concern of development of autoimmunity. Here, we report that our OSP vaccine strategy corresponding to endogenous TPD52 led to strong peptide-specific cell-mediated immunity (CMI) and more importantly, reduced tumor growth and increased survival of mice bearing tumors. We observed a 40 percent long-term survival rate in TPD52-OSP immunized mice in our initial tumor challenge study.

In a separate experiment, we utilized CpG-ODN as adjuvant to enhance the ability of antigen presentation and to induce costimulatory molecules on APC, as well as to secrete cytokines that mediate proliferation and maturation of effector cells [32]. It has been shown that CTL populations that specifically target cancer cells can be expanded by Toll-like receptor (TLR) ligands, which increase the cross talk between the innate and adaptive immune systems [33]. Switching to CpG-ODN as adjuvant increased the survival rate for TPD52-OSP-immunized mice to 62.5%, suggesting that this vaccination induced effective cellular immunity that resulted in the rejection of TPD52⁺ tumors without the development of adverse autoimmunity. A recent study has also shown that immunization with recombinant mD52 protein with CpG/ODN resulted 40-50% in the rejection of tumors over-expressing mD52 protein [18].

Studies have demonstrated that tumor-specific CD4⁺ T helper (Th) cells critically contribute to the development and efficacy of anti-tumor responses [1, 4, 34-38]. The effectiveness of Th cells probably for their capacity to deliver necessary signals to professional APC needed for tumor-specific CTL priming [39-41]. In addition, antigen-specific CD4⁺ T cells may provide CTL with necessary growth stimuli during the effector phase [42]. It would also be valuable to induce CTL responses against a wide range of various epitopes to minimize the generation of tolerized T cells. Since OSP contain all possible epitopes for the CD4⁺ as well as CD8⁺ T cells of a genetically diverse population, OSP can offer pivotal antigen-specific CD4⁺ T-cell help to induce, expand, and sustain an effective CD8⁺ CTL response.

The mechanisms of tolerance that prevent autoimmunity may also obviate the development of sufficient anti-tumor responses, since most tumor antigens are self-antigens. Therefore, cancer vaccines directed against self-antigens must be able to overcome host tolerance in order to elicit a potent cell-mediated immune response capable of completely eliminating the tumor. OSP contain all possible epitopes of an antigen, not only the immunodominant ones, which have been linked to tolerance [43]. Thus, OSP vaccines, unlike vaccines based upon immunodominant epitopes, can break tolerance.

Vaccines designed to generate an immune response against a self-protein raise safety concerns. The lack of an autoimmune response toward normal cells expressing basal levels of TPD52 may imply that a threshold level of TPD52 expression, such as that present on a tumor cell, is required for initiating an autoimmune response. We detected no autoimmune toxicity in our experiments following immunizations. However, CD3⁺ cell infiltration in

lung metastases was pronounced in TPD52-OSP-vaccinated mice and practically absent in tumor tissue of control mice, implying homing of TPD52-specific lymphocytes in the former group of mice. We conclude that the high degree of tumor infiltration by CD3⁺ cells and the small tumor size predicted a favorable outcome for the vaccinated mice.

In summary, we have demonstrated that an OSP vaccine targeting TPD52 broke peripheral T-cell tolerance against this self-antigen and induced immune responses that resulted in rejection of TPD52⁺ tumors in the absence of deleterious autoimmunity. We conclude that our OSP vaccine strategy may be of use for the implementation of peptide-based vaccines without needing to identify either epitopes or MHC backgrounds of the vaccinees. Most importantly, our OSP cancer vaccine strategy could benefit individuals of all genetic backgrounds, not just those with HLA A2. Given that hTPD52 is overexpressed in a variety of human cancers, our novel anti-TPD52 vaccination strategy may hold promise against malignancies other than breast cancer, for which we have provided proof-of-concept in the murine model.

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Figure 1.

TPD52 expression in the TS/A cell line and tissues from normal BALB/c mice (A) and outbred CD-1 mice (B). PCR amplification is shown for mD52 (top panels) and the housekeeping gene GAPDH (bottom panels). Plasmid mTPD52 was used as positive control for the PCR and amplification without template cDNA was used as negative control.

Figure 2.

Antigen-specific proliferative responses induced by OSP immunization in inbred BALB/c and outbred NMRI mice. BALB/c mice were immunized either with TPD52-OSP or Tat-OSP. Control mice received adjuvant only. Ten days after the last boost, pooled BALB/c splenocytes were stimulated overnight with either TPD52-OSP (A) or Tat-OSP (B) and proliferation was measured by [3 H]thymidine incorporation. SI, stimulation index as per Material and Methods. Outbred NMRI mice were immunized with either TPD52-OSP (n = 5) (C) or Tat-OSP (D), and 10 days after the last boost, proliferation of splenocytes from the individual mice was measured by [3 H]thymidine incorporation.

Figure 3.

Antigen-specific IFN- γ production induced by OSP immunization in inbred BALB/c (A-C) and outbred NMRI mice (D-F) as measured by IFN- γ ELISPOT analysis or intracellular cytokine staining (ICS). Animals were immunized as described in the legend to Figure 2. Ten days after the last immunization, splenocytes from BALB/c mice were pooled and stimulated *in vitro* with or without relevant OSP overnight. The number of spot-forming units (SFU) was determined by ELISPOT analysis (A). (B, C) ICS. D, ELISPOT analysis and (E, F) ICS of individual NMRI mice vaccinated as above.

Figure 4.

Quantification of antigen-specific CTL activity and degranulation of TPD52-specific CD8⁺ T cells in OSP-immunized mice. Splenocytes from (A), TPD52-OSP- and (B), Tat-OSP-immunized BALB/c mice were expanded *in vitro* in the presence of OSP and rIL-2 for 5 days and used as effector cells. As target cells, P815 cells (H-2^d) were labeled with ⁵¹Cr and then loaded with OSP. (C), TPD52-OSP- and Tat-OSP-immunized BALB/c mice were used as donors of effector cells and restimulated with TPD52-OSP. TS/A cells were labeled with ⁵¹Cr and used as target cells. Target cells were mixed with various numbers of effector cells. E:T, effector-to-target cell ratio. (D), Splenocytes from BALB/c mice given TPD52-OSP or Tat-OSP were cultured with rIL-2 and the corresponding OSP. Restimulated splenocytes were then mixed with TS/A tumor cells. FITC-labeled anti-mouse-CD107a & b were added directly to the wells to detect degranulation.

Figure 5.

Anti-tumor efficacy of TPD52-OSP vaccination in BALB/c mice. Ten days after the last immunization with (A), MPL+TDM adjuvant, or (B), CpG-ODN adjuvant, BALB/c mice were challenged with TS/A cells (10^3 and 5×10^3 cells, respectively). Control groups consisted of mice given the irrelevant Tat-OSP with adjuvant or adjuvant only. The percent survival is shown by Kaplan-Meier plot; log rank analysis was used to determine statistically significant differences between TPD52-OSP-immunized and control mice ($p < 0.001$).

Figure 6.

CD3⁺ lymphocyte infiltration in lungs of immunized mice. Sections from the lungs were subjected to either H&E staining (A, C, E, F) to reveal tissue topography or immunohistochemistry (IHC) with polyclonal anti-CD3 antibody (B, D, F, H). CD3⁺ cells were seen as brown cells. Magnification at 10x (left column) and 40x (right column).