Surrogate Tumor Antigen Vaccination Induces Tumor-Specific Immunity and the Rejection of Spontaneous Metastases

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Abstract

The nonimmunogenic 4T1 murine mammary carcinoma model and a model surrogate tumor antigen (sTA) were employed to explore the possibility of inducing tumor-specific immunity through active immunization in the absence of defined tumor-associated antigens. Immunization of naive mice with protein-based sTA resulted in protection from s.c. challenge, with 4T1 modified to express the sTA (4TL.sTA), or from a sTA-expressing unrelated tumor cell line (mKSA). Immunization had no effect on parental 4T1 tumor growth or the formation of parental 4T1 spontaneous lung metastases. Mice that were sTA immunized and successfully rejected 4T1.sTA challenge also rejected a subsequent challenge in the contralateral flank with parental 4T1 and strikingly prevented the formation of spontaneous parental 4T1 lung metastases. The rejection of parental 4T1 seemed to be specific for and associated with unknown 4T1 tumor-associated antigens, because rejection of mKSA did not induce cross-protection against a challenge with parental 4T1. To evaluate the effect of this vaccine approach on established disease, mice were simultaneously challenged on day 0 with 4T1.sTA and parental 4T1 in contralateral flanks and then immunized on days 3, 10, 17, and 24 with sTA protein. Tumor growth and metastasis were delayed in four of five animals, and 20% (2 of 5) of the animals were tumor free at the completion of the experiment. Together, these data suggest that prior vaccination with a sTA followed by inoculation with poorly immunogenic tumor cells modified to express the sTA activates determinant spreading and the induction of systemic tumor immunity resulting in indigenous tumor rejection. (Cancer Res 2005; 65(7): 2938-46)

Introduction

The field of cancer vaccine development is growing rapidly and has become the focus of much intense investigation. Cancer vaccines have evolved from crude tumor cell lysates given with an adjuvant to the sophisticated application of ex vivo engineered dendritic cells. Indeed, proteins, peptides, DNA, viral vectors, heat shock proteins, and dendritic cells as well as various combinations and permutations of these modalities have been and are being explored and refined as vaccines against cancer (1, 2). In the midst of this intense study, one thing is becoming increasingly clear: the most effective vaccines may be those composed of a defined tumor-associated antigen(s) (TAA). Thus, the need to discover, define, and validate additional TAA for cancers to include prostate, breast, and lung cancers remains an obstacle for effective vaccine development (3). To this end, new vaccine methods that circumvent the need for in hand, defined TAA could be extremely valuable.

The BALB/c-derived 4T1 mammary carcinoma model is a unique nontransgenic model of metastatic breast cancer based on explantable, syngeneic, clonal tumor cells (4). The growth characteristics, lack of immunogenicity, and aggressive, spontaneous metastatic properties of this model closely resemble those characteristics observed for human breast cancer (5). 4T1 tumor cells are relatively easy to culture and form tumors in healthy, immunocompetent BALB/c mice following s.c. inoculation with as few as 5,000 to 7,000 cells (6, 7), making this a useful in vivo model to study the effects of active immunization against aggressive, nonimmunogenic cancers for which no TAs have been defined.

Epitope or determinant spreading as it relates to specific immune responses against a given antigen was first described as the dispersion of immunologic specificities from a single dominant epitope to subdominant epitopes (8–10) within the original antigenic target (intramolecular determinant spreading) or to epitopes carried by distinct antigens (intermolecular determinant spreading; ref. 11). Early studies showed that clones of specific T cells were responsible for driving determinant spreading in models of autoimmunity (12). Driver T-cell clones as the mechanism of determinant spreading initiation in autoimmunity were later supported by murine models of tumor vaccination. A vaccine composed of a single peptide epitope from chicken ovalbumin delivered on irradiated RMAS cells initiated a CTL response that resulted in the rejection of ovalbumin-expressing tumors and the generation of CTL with specificities for additional ovalbumin epitopes (intramolecular determinant spreading) as well as the generation of CTL with specificities for epitopes on distinct antigens (intermolecular determinant spreading; ref. 13). In a separate study, a single peptide, P1A, induced CTL that rejected P1A+ tumor challenge and subsequently yielded CTL activity and tumor protection against challenge with a P1A− variant, which resulted from determinant spreading to a second defined epitope (14). These studies showed that peptide vaccines directed at the induction of CTL responses are capable of inducing tumor immunity and that determinant spreading occurs, resulting in the expansion of the immune response to additional epitopes in murine tumor models of tumor protection.

In the present study, we employed the aggressive, tumorigenic, and nonimmunogenic 4T1 murine mammary carcinoma model and SV40 large tumor antigen (Tag) as a model surrogate tumor antigen (sTA) to explore the possibility of inducing tumor-specific immunity through active immunization in the absence of a defined TAA and to address whether vaccination with a soluble whole protein would activate determinant spreading and tumor rejection in vivo. Mice immunized with sTA rejected challenge with 4T1...
Materials and Methods

**Surrogate tumor antigen.** Recombinant SV40 Tag served as a model sTA and was generated in the Sf9 insect cell line using the baculovirus AcNPV expression system. Recombinant Tag was extracted from insect cells and immunoaffinity purified by methods described previously (15). Purity was assessed by SDS-PAGE and silver staining (16) and the concentration was determined using an absorption coefficient at 280 nm for a 1% solution.

**Mice and cell lines.** Female, 6- to 8-week-old female BALB/c mice were purchased from the NIH (Frederick, MD). All animals were cared for and treated according to Institutional Animal Use and Care Committee guidelines at Texas Tech University Health Sciences Center (Lubbock, TX). The tumorigenic murine mammary carcinoma cell line 4T1 was a kind gift from Fred Miller (Karmanos Cancer Institute, Detroit, MI). The tumorigenic SV40-transformed BALB/c mouse kidney cell line designated mKSA (17) was used as a control for tumor growth and immunity following sTA immunization (18). Cells were cultured in RPMI 1640 (Fisher Scientific, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 250 ng/mL fungizone, 50 IU/mL penicillin, 50 µg/mL streptomycin, 30 µg/mL gentamicin sulfate, and 10 mM HEPES. The 4T1.sTA cell line was generated by transfecting parental 4T1 tumor cells with a plasmid encoding SV40 Tag as a model sTA using LipofectAMINE (Invitrogen Corp., Carlsbad, CA) and methods described previously (19).

**RNA isolation and reverse transcription-PCR.** Briefly, total RNA was isolated from mKSA (sTA positive), 4T1.sTA (sTA transfected), P815 (sTA negative), and 4T1 (sTA negative) using Trizol reagent (BioWhittaker, Walkersville, MD) and 1 µg was reverse transcribed to obtain cDNA. The primers and PCR conditions were described previously (19). PCR results were visualized on a 2% agarose gel containing ethidium bromide; assessment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression served as an internal reference control.

**Western analysis.** The cell lines mKSA, P815, 4T1.sTA, and 4T1 were grown to confluency in RPMI supplemented as described above. Cells were harvested and counted and whole cell protein lysates were prepared for 4T1, 4T1.sTA, P815, and mKSA at a concentration of 1 × 10⁶ cells/mL using methods described previously (20). Briefly, samples were loaded on a standard reducing SDS-PAGE (4% stacking, 7% resolving) and run at 200 V for 1 hour. Next, the gel-separated samples were electrophoretically transferred to nitrocellulose and the membrane was blocked in PBS containing 1% normal goat serum, 1% milk, and 0.02% Tween for 2 hours at room temperature. To visualize the expression of the model sTA (SV40 Tag), the membrane was probed with the anti-SV40 Tag monoclonal antibody (mAb) Pab 405 overnight at 4°C (19) and then washed thrice with PBS - 0.02% Tween, following which goat anti-mouse IgG-specific horseradish peroxidase reagent was added at a 1:2,000 dilution for 2 hours at room temperature. Finally, the membrane was washed and developed with 3,3'-diaminobenzidine reagent as a substrate (Sigma, St. Louis, MO).

**Immunohistochemistry.** Formalin-fixed, paraaffin-embedded tumor sections were deparaffinized and subjected to antigen retrieval using a pressure cooker or microwaves in a citrate buffer (pH 6.0). After blocking with normal horse serum, the sections were incubated with primary antibody overnight at 4°C. Following incubation, cells were pelleted, the supernatant was aspirated, and 1× permeabilization/wash solution (1 mL) was added for 20 minutes at 37°C in the dark. Permeabilization/wash buffer (1×) was used in all subsequent washes and dilutions to maintain permeabilization of cells. Following permeabilization, cells were washed once and pelleted, the supernatant was aspirated, and FITC-conjugated antibody (IgG1) was added with incubation for 30 minutes at 4°C in the dark. Finally, cells were washed, resuspended in 1× permeabilization/wash buffer (1 mL), and stored in the dark. All assays were analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ) within 2 hours of staining.

**Immunization and tumor protection.** Mice were immunized four times i.p. with sTA in alum at 1-week intervals at a concentration of 5 µg/animal for the first three injections and a concentration of 15 µg/animal for the final injection. Control groups of mice were immunized with either PBS or alum alone using identical methods and schedules (18). Mice in all groups were bled from the dorsal tail vein before immunization and 7 days following each immunization. Three tumor cell lines were used for s.c. tumor challenge at the following doses: mKSA (1 × 10⁶), 4T1.Tag (1 × 10⁴), and 4T1 (1 × 10⁶). Following harvest and counting, tumor cells were resuspended in versene (PBS-EDTA, Fisher Scientific) to prevent aggregation and injected s.c. in 100 µL. Primary challenge was given in the right flank with either mKSA (sTA positive) or 4T1.sTA followed 2 weeks later by a secondary challenge in the left flank of all animals with parental 4T1 tumor cells. Tumor size was recorded by measuring perpendicular angles with calipers every 2 to 3 days. Tumor volume (mm³) was calculated using the equation: (a × b²) / 2, where b is the smaller of the two measurements. To evaluate metastasis, lungs were harvested and injected with an India ink solution and tumor nodules were enumerated as described below.

**Immunization and tumor treatment.** Parental 4T1 and sTA-modified 4T1 (4T1.sTA) tumor cells at the following doses were used in the therapeutic immunization model: 4T1.sTA tumor cells (1 × 10⁶) and 4T1 tumor cells (1 × 10⁶). Tumor cells were harvested, counted, and processed as described above. Mice were challenged s.c. simultaneously in contralateral flanks on day 0. On days 3, 10, 17, and 24, mice were immunized i.p. with sTA in alum using doses of 5, 5, 10, and 15 µg, respectively. Immunization with alum alone or PBS served as controls. Tumor size was recorded by measuring perpendicular angles with calipers every 2 to 3 days. Tumor volume (mm³) was calculated using the equation: (a × b²) / 2, where b is the smaller of the two measurements. To evaluate metastasis, lungs were harvested and injected with an India ink solution and tumor nodules were enumerated as described below.

**Enumeration of 4T1 spontaneous lung metastases.** To analyze tumor metastasis to the lungs, mice were sacrificed and their lungs were removed and injected with India ink to visualize individual tumor nodules. Briefly, an India ink solution was injected through the trachea and allowed to fill the lungs. The lungs were removed and placed in Fekete's solution for destaining. Tumor nodules do not absorb India ink, which results in the normal lung tissue staining black and the tumor nodules remaining white. Tumor nodules were counted blindly and size was noted by three independent investigators.

**Immunofluorescence for detection of surrogate tumor antigen–specific antibodies.** An ELISA was employed to detect the presence of sTA (Tag)–specific antibodies in the sera of mice following immunization using methods described previously (21, 22). Briefly, 96-well microtiter plates (Nunc Maxisorp, Fisher Scientific) were coated overnight at 4°C with 50 µL of the model sTA, recombinant SV40 Tag, in PBS at a final concentration of 200 ng/mL. Plates were blocked with 10% normal goat serum/PBS for 1 hour at 37°C to prevent nonspecific binding. Next, 2-fold dilutions (beginning dilution,
I50; 50 μL) of mouse sera were added to individual wells in duplicate and plates were incubated overnight at 4 °C. Antibody-antigen reactivity was detected using 50 μL goat anti-mouse IgG-Fc-specific antibody conjugated to horseradish peroxidase (Sigma). Plates were washed between steps with 0.02% Tween-PBS. Reactions were developed with 100 μL peroxidase substrate [2,2′-azino-di(3-ethylbenzthiazolone sulfonic acid) containing 0.01% hydrogen peroxide. Enzyme substrate reactions were terminated following the addition of 5% w/v SDS (100 μL). Absorbance was measured at 405 nm using the Victor3 plate reader (Wallac, Perkin-Elmer, Boston, MA).

T-cell culture and assay for IFN-γ production. T cells were isolated by culturing Ficoll-separated spleen cells with irradiated 4T1 tumor cells in the presence of interleukin (IL)-2 (10 ng/mL), IL-7 (5 ng/mL), and IL-12 (5 ng/mL; Peprotech, Rocky Hill, NJ) at 37 °C for 5 to 7 days. Assessment of cytokine secretion by antigen-specific T-cell cultures (generated as described above) was accomplished by applying culture supernatants to commercially available sandwich ELISA kits for IFN-γ detection (R&D Systems, Minneapolis, MN). Supernatants were harvested from 24-hour cultures of T cells (1 x 10^5 cells/mL in 200 μL medium in 96-well plates) in medium alone compared with T cells cultured with 4T1 tumor cells (1:1 ratio). To confirm MHC-I-restricted tumor recognition, CTL blocking assays were done by incubating 4T1 tumor cells with anti-H-2d (Kd, Ld, and Dd) or anti-H-2b (Kb; control) mAb before incubation with CTLs. Briefly, cultures of T cells (1 x 10^6 cells/mL in 200 μL medium in 96-well plates) in medium alone compared with T cells cultured with 4T1 tumor cells (1:1 ratio). To confirm MHC-I-restricted tumor recognition, CTL blocking assays were done by incubating 4T1 tumor cells with anti-H-2d (Kd, Ld, and Dd) or anti-H-2b (Kb; control) mAb before incubation with CTLs. Briefly, mAb (10 μL) in PBS (final concentration, 30 μg/mL) was added to individual wells of 96-well round-bottomed plates in triplicate. Next, 4T1 tumor targets (100 μL) were added to each well and incubated for 30 minutes at room temperature. Finally, effectors (100 μL) were added to the appropriate wells and the plates were incubated for 24 hours at 37 °C. Assays were analyzed using the Victor3 plate reader. Graphical data in Fig. 3D represent the mean of triplicate values for CTL incubated with 4T1 tumor cells after subtraction of the values for CTLs alone.

Results

Generation of surrogate tumor antigen-positive murine tumor cell lines. To generate a tumor cell line expressing the model sTA, parental 4T1 tumor cells were transfectioned with a plasmid encoding SV40 Tag using LipofectAMINE Plus reagent; the resulting cell line was designated 4T1.sTA. Expression of mRNA encoding the model sTA by 4T1.sTA was confirmed by reverse transcription-PCR (RT-PCR) using methods published previously by our laboratory (19). sTA expression was not detected in the untransfected parental 4T1 cell line or the sTA-negative P815 plasmacytoma cell line (Fig. 1A). The SV40-transformed BALB/c, kidney-derived tumor cell line mKSA, served as a positive control for expression of the model sTA (SV40 Tag; Fig. 1A).

Protein expression of the sTA was confirmed by Western blot analysis. A band of ~90 kDa (23) was observed for the transfected 4T1.sTA tumor cell line as well as for the positive control mKSA tumor cell line (Fig. 1B). Examination of the parental 4T1 cell line before sTA transfection revealed no detectable levels of sTA protein expression by Western blot (Fig. 1B). To further evaluate the expression of sTA protein by 4T1.sTA cells, intracellular staining and flow cytometry were done using a FITC-labeled mAb (Pab 405) with specificity for the model sTA (Fig. 1C). Consistent with RT-PCR and Western analysis, 4T1.sTA expressed the sTA at detectable levels (III) compared with lack of expression by the parental 4T1 tumor cell line (II), albeit much less than the positive control tumor cell line mKSA (I). The disparity in expression between 4T1.sTA and mKSA is likely because mKSA was generated by transformation with SV40 resulting in multiple copies of the gene for Teg being integrated into the cell genome.

Tumor protection following immunization with surrogate tumor antigen. Next, we evaluated whether expression of the model sTA affected the growth kinetics of 4T1 tumor cells in vivo and whether immunization with sTA protein in alum would result in rejection of 4T1.sTA tumor cells compared with parental 4T1 tumor cells. In brief, groups of five mice were immunized i.p. with 5 μg purified recombinant sTA in alum in a total volume of 100 μL once every 7 days for 3 weeks followed by 15 μg sTA in alum on the fourth week. Following immunization with sTA, mice were monitored for the induction of sTA-specific immunity by collecting serum samples and measuring anti-sTA antibody titers using methods and an ELISA generated in and reported by our laboratory (22). All three groups of mice (total of 15 mice) immunized with sTA in alum generated detectable IgG antibody titers specific for the sTA 2 weeks following the final immunization with near-identical endpoint titers of 1:2,000. In contrast, mice immunized with PBS or alum alone showed no detectable IgG antibody titers to the sTA when sera were analyzed by ELISA with the sTA on the solid phase.

Following immunization with sTA in alum, alum alone, or PBS, mice were challenged with either mKSA, parental 4T1, or 4T1 sTA at an inoculation dose of 5 x 10^5, 1 x 10^6, or 1 x 10^7, respectively, and tumor growth was measured using calipers as described in Materials and Methods. Mice challenged with mKSA tumor cells following immunization with sTA in alum did not develop tumors (Fig. 2A), whereas mice that were immunized with alum alone or PBS developed tumors at approximately the same rate (Fig. 2A-C). All the mice challenged with parental 4T1 tumor cells following immunization with either sTA in alum, alum alone, or PBS developed tumors at similar rates regardless of the immunization they were given (Fig. 2B). Importantly, mice challenged with 4T1.sTA tumor cells following immunization with sTA in alum did not develop tumors compared with mice immunized with alum alone or PBS, which developed tumors at similar rates (Fig. 2C). These data establish that immunization with a soluble, whole protein sTA induces immune responses capable of rejecting lethal challenge with sTA-positive tumor cells as well as show that transfection of parental 4T1 tumor cells with the sTA had no effect on in vivo tumor growth of the 4T1 cell in unimmunized mice.

4T1 tumor cells spontaneously metastasize to the lungs forming lethal tumor burdens. To evaluate metastasis, lungs were harvested and stained with an India ink solution and tumor nodules were enumerated. S.c. 4T1.sTA tumor cells following immunization with sTA in alum not only resulted in rejection of the s.c. tumor but also prevented the development of spontaneous lung metastases (Table 1). In contrast, if mice were immunized with sTA in alum and challenged with parental 4T1 tumor cells, lung tumor nodules were present ranging from 10 to 18 large visible tumor nodules (Table 1; mice had to be sacrificed by day 42 following primary challenge with parental 4T1 tumor cells due to morbidity associated with the tumor burden). Therefore, immunization with sTA in alum induced sTA-specific immunity that was capable of preventing tumor formation and metastasis by nonimmunogenic 4T1 tumor cells modified to express the model sTA.

Tumor protection as a result of surrogate tumor antigen-induced determinant spreading. To determine whether a sTA-based vaccine could be used to induce determinant spreading and rejection of indigenous tumors in the absence of known, defined TAA, groups of mice were immunized with sTA in alum and challenged with either mKSA tumor cells or 4T1.sTA tumor cells (as described for Fig. 2). Mice that were immunized with sTA in alum and rejected a primary s.c. challenge with the control sTA-positive, kidney-derived mKSA tumor cell line failed to reject a subsequent s.c. challenge with parental 4T1 tumor cells given 2 weeks later in
the opposite flank (Fig. 3A). However, for the group of mice immunized with sTA in alum that rejected a primary s.c. challenge with 4T1.sTA tumor cells, subsequent rejection of a secondary s.c. challenge with parental 4T1 tumor cells given 2 weeks later in the opposite flank was observed (Fig. 3B). These data suggest that mice were protected from mKSA and 4T1.sTA tumor growth in the primary challenge due to the specific immune response that was generated following immunization with sTA in alum as seen in Fig. 3A and C and that protection from secondary challenge with parental 4T1 mammary tumor cells (Fig. 3B) was due to cross-presentation of unknown TAA shared by 4T1.sTA and parental 4T1 tumor cells resulting in determinant spreading and rejection of parental 4T1 tumor cells. This was supported by the observation that rejection of primary challenge with the unrelated kidney-derived mKSA tumor cells, although sTA-based vaccine driven, had no effect on 4T1 mammary-derived tumor cell growth (Fig. 3A). Thus, the initial immune response induced by immunization with sTA in alum was specific for the model sTA and enabled mice to reject 4T1.sTA tumor challenge resulting in the initiation of determinant spreading and cross-priming to unknown TAA in 4T1 tumor cells, providing protection against secondary s.c. challenge with parental 4T1 tumor cells.

In addition to sTA-based, vaccine-induced protection against s.c. challenge with parental 4T1 tumor cells as a result of apparent determinant spreading, immunization with sTA in alum prevented the formation of spontaneous 4T1 lung metastases in mice that rejected a primary s.c. challenge with 4T1.sTA tumor cells (Table 2; Fig. 3C, c). However, when mice were immunized with sTA in alum and rejected a primary s.c. challenge with 4T1.sTA, a secondary s.c. challenge with parental 4T1 tumor cells was rejected and the formation of spontaneous

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**Figure 1.** Expression of the model sTA in murine tumor cells. A, RT-PCR demonstrating expression of the model sTA (SV40 Tag) in 4T1 murine tumor cells following transfection with a plasmid encoding the full-length cDNA for SV40 Tag. mKSA is a SV40-transformed, SV40 Tag-positive murine kidney-derived tumor of BALB/c origin and served as a positive control. P815 is a Tag-negative murine tumor cell line. GAPDH served as an internal reference control. B, Western blot analysis of murine tumor cell lysates generated from 1 x 10⁶ cells demonstrating protein expression of sTA using a mAb (Pab 405) with specificity for the model sTA (SV40 Tag). The model sTA typically blots as two bands ~ 90 kDa. mKSA is a sTA-positive murine kidney-derived tumor of BALB/c origin and served as a positive control. The sTA-negative tumor cell line P815 served as a negative control. GAPDH served as a control for protein loading. C, expression of sTA protein by flow cytometry. Intracellular sTA detection was accomplished using a Cytofix/Cytoperm reagent and a mAb (Pab 405) with specificity for SV40 Tag. Left, staining of mKSA tumor cells as a positive control; center, lack of staining of parental 4T1 tumor cells; right, staining of 4T1 tumor cells modified to express the model sTA (SV40 Tag). Solid, fluorescence of cells alone; solid line, staining with an isotype-matched control antibody (IgG); dashed line, staining with the FITC-conjugated sTA (SV40 Tag)-specific mAb (Pab 405).
tumor cells as they failed to secrete detectable levels of IFN-γ. Splenocytes cultured with H-2d-matched, syngeneic tumor cells (mKSA) and parental 4T1 lung metastases was prevented (Table 2; Fig. 3C (B) challenged s.c. with either ((1) sTA-positive mKSA kidney-derived tumor cells, (2) sTA-negative 4T1 mammary tumor cells, or (3) sTA-positive 4T1.sTA mammary tumor cells. Tumor growth was monitored thrice weekly (n = 5). Representative of three experiments.

Determination of sTA-induced cross-protection, splenocytes from mice that rejected parental 4T1 tumor cells (Fig. 3C, d). In contrast, parental 4T1 lung tumor nodules ranged from 2 to 17 in mice that rejected a primary s.c. challenge with parental 4T1 tumor cells (Table 2). These results suggest that unknown TAA present in sTA-modified 4T1 tumor cells. Treatment of established 4T1 tumors as a result of surrogate tumor antigen–induced determinant spreading. Finally, we evaluated whether vaccinating with a model sTA would have an effect on established tumors in a treatment scenario. Mice were inoculated s.c. simultaneously with 4T1 and 4T1.sTA tumor cells in contralateral flanks. Three days after tumor inoculation, animals in each group were immunized i.p. with sTA in alum or alum alone on days 3, 10, 17, and 24. Growth of the tumors on each flank was monitored and measurements were taken with calipers as described in Materials and Methods. Given that it

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NOTE: Mice were immunized i.p. with sTA in alum four times at 1-week intervals and challenged s.c. with tumor cells 14 days following the last immunization. All values represent s.c. tumor growth (mm³) and number of lung metastases for individual mice.

*S.c. tumor size was determined with calipers, measuring perpendicular angles.

Lungs were harvested when s.c. tumor size reached ∼1×1 cm or by the day indicated. Tissue was stained with India ink solution and destained with Fekete's solution and tumor nodules were counted and recorded.
takes ~10 to 14 days to induce immunity following immunization, this represents a 13- to 17-day established tumor, albeit minimal disease. Immunization with sTA in alum resulted in the cure of 4T1.sTA tumors in five of five mice (Fig. 4C), whereas alum had no significant effect on 4T1.sTA s.c. tumor growth (data not shown). Mice that were immunized with alum alone also failed to regress 4T1 parental tumors in the opposite flank (Fig. 4D). Strikingly, two of five mice were cured and two additional mice had a significant reduction in tumor growth with delayed onset of their parental 4T1 tumors when immunized with the sTA and simultaneously inoculated with 4T1.sTA and 4T1 tumor cells (Fig. 4B). All five mice in this group were cured of their right flank sTA-expressing 4T1 tumors (Fig. 4C) and 80% (4 of 5) had a complete or partial response against their parental 4T1.sTA-negative left flank tumors (Fig. 4B), demonstrating that it may be possible to treat established disease with sTA-based vaccination to induce determinant spreading to unknown TAA expressed by indigenous tumors, represented herein by parental 4T1 tumor cells.

Discussion

No other form of medical intervention has had a greater impact on world health than vaccination. The identification of tumor rejection antigens and the genes that encode them, with the development of relevant animal tumor models involving the very same antigens, should provide the information and opportunity necessary to bring strategies for active immunization against many
forms of human cancer into the clinic. In the past 5 to 7 years alone, great strides have been made toward the definition of relevant molecular targets on human tumor cells that may serve as TAAs for immunotherapy and vaccine development. Active vaccination against cancer is a treatment approach that has proven effective in preclinical murine models. However, vaccines have been difficult to exploit for highly tumorigenic and poorly immunogenic tumors, such as the 4T1 mammary carcinoma model for which no TAAs have been reported. We hypothesized that in the absence of known TAA it is possible to induce tumor-specific immunity using a vaccine that is composed of a sTA to induce cross-presentation of unknown TAA and tumor-specific immunity as a result of intermolecular determinant spreading. To address this, we used the 4T1 murine mammary carcinoma model and SV40 Tag as a sTA to induce tumor-specific immunity and subsequent cross-presentation of unknown mammary carcinoma-associated antigens.

The observation that T cells could be primed against antigens carried by cells lacking the proper MHC necessary to be recognized by T cells was first reported by Bevan (24). Since the first reports, the phenomenon has been extensively studied in models of autoimmunity, infectious disease, and, to a lesser degree, cancer (25). It has been shown that cross-presentation is mediated by professional, bone marrow–derived antigen-presenting cells and most potently by dendritic cells (26). For example, as tumor cells die or are actively destroyed, they release their complement of antigens into the microenvironment. These antigens are captured and processed by dendritic cells, which then migrate to regional lymph nodes to present the processed antigens to naive T cells. Once primed and activated, these T cells are capable of finding intact tumor cells that share the antigen and the appropriate MHC, recognize the tumor cells, and destroy them. The proper maturation and activation of the dendritic cells is required before they are capable of priming naive T cells. The microenvironment and cytokines necessary and capable of dendritic cell activation are best elicited by infectious agents or danger signals induced by infectious agents. Our model sTA is a viral protein and as such

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NOTE: Mice were immunized i.p. with sTA in alum four times at 1-week intervals and challenged s.c. with tumor cells following the last immunization. All values represent the number of lung metastases for individual mice.

* Mice challenged with mKSA following sTA immunization had no effect on subsequent 4T1 tumor growth or the formation of spontaneous 4T1 lung metastases.

† Mice challenged with 4T1.sTA following immunization rejected parental 4T1 tumor growth and prevented the formation of spontaneous 4T1 lung.

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**Figure 4.** sTA-induced rejection of established 4T1 tumors. Mice were simultaneously challenged s.c. in contralateral flanks with 4T1.sTA and 4T1 tumor cells and immunized on days 3, 10, 17, and 24 with either sTA in alum or alum alone. Tumor development was monitored and recorded. A, treatment of 4T1 tumor cells as a result of immunization with alum alone ([n = 4]). B, treatment of 4T1 tumor cells as a result of immunization with sTA in alum ([n = 5]). C, treatment of 4T1.sTA tumor cells as a result of immunization with sTA in alum ([n = 5]). Representative of two separate experiments.

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Table 2. Effect of 4T1.sTA and mKSA rejection following sTA immunization on the formation of spontaneous 4T1 lung metastases

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possesses the immunogenicity required to induce an immunologic environment that would be optimal for antigen cross-presentation and subsequent determinant spreading.

Previously, we reported that SV40 Tag was capable of eliciting protective tumor immunity in BALB/c mice (27) and that the primary mechanism of immunity was antibody dependent and CD8+ T cell independent (28). In addition, we showed that CD4+ T cells are important for the induction of Tag-specific tumor protective immune responses (29). Based on these data, we used SV40 Tag as a model sTA to transfect the 4T1 mammary tumor cell line enabling us to immunize against this model sTA, subsequently challenge with the sTA-expressing 4T1 tumor cell line and the parental 4T1 tumor cell line, and assess the induction of protective immunity against parental 4T1 tumor challenge. The subline 4T1.sTA was generated by transfecting the parental cell line 4T1 with a plasmid containing the full-length cDNA for SV40 Tag, the model sTA. The presence of sTA in 4T1.sTA cells had no observable effect on the rate of s.c. tumor formation or spontaneous lung metastasis in naïve mice compared with parental 4T1 tumor cells.

In the present studies, we immunized naive mice with recombinant SV40 Tag protein as a model sTA, which resulted in the induction of sTA-specific immunity that had no effect on parental 4T1 tumor formation but completely protected against challenge with sTA-transfected 4T1 tumor cells as would be expected. However, 4T1 tumor growth and spontaneous lung metastasis was prevented when mice were challenged with 4T1 tumor cells following rejection of 4T1.sTA tumor cell challenge. This effect was not seen when unrelated sTA-positive tumor cells were rejected before parental 4T1 tumor cell challenge, suggesting the cross-presentation of shared murine mammary carcinoma antigens and the induction of systemic immunity via intermolecular determinant spreading. We went a step further to evaluate the effect of this vaccine approach on established disease and showed that we were able to dramatically slow the growth of both 4T1.sTA and parental 4T1 established tumors following immunization with the sTA and to cure 40% (2 of 5) of the mice of their parental non-sTA-expressing 4T1 tumors. Together, these data suggest that prior vaccination with a sTA expressed as a transgene in poorly immunogenic tumor cells activates cross-presentation of unknown tumor-specific antigens and the induction of systemic tumor immunity via the targeted immunologic destruction of the sTA-expressing tumor cells.

Previous studies by other investigators employing the 4T1 tumor model showed reduction in metastases of 4T1 (30–32), prolong survival (30, 32, 33), and a slowing in growth of primary tumors (6, 34); however, few reported cure of the primary tumor or complete protection from spontaneous lung metastasis. Using the sTA-based vaccine, we were able to elicit a potent immune response that resulted in complete protection from parental 4T1 tumor challenge and rejection of the formation of lethal, spontaneous lung metastases. Moreover, we were able to cure 40% of the animals in a treatment model of established disease. Our initial studies indicate that CD8+ T cells may play a role in the rejection of the non-sTA-expressing parental 4T1 tumors. However, additional studies, such as in vivo depletion of T-cell subsets (29), will need to be done to further elucidate the mechanism of rejection. Previously, we reported that antibody-mediated rejection of SV40 Tag–expressing tumors is a primary mechanism following Tag immunization (28); this is possible due to the surface expression of SV40 Tag on SV40-transformed or Tag-transfected cells. Additional studies will be done to confirm that antibodies mediate the immune destruction of the sTA-expressing tumor, which lead to cross-presentation of unknown TAA and determinant spreading that results in CTL activation and rejection of non-sTA-expressing parental tumors. Evidence to this effect is our recent unpublished observation that 4T1 cells expressing ovalbumin following transfection with ovalbumin plasmid (kind gift from Dr. M.J. Bevan, University of Washington, Seattle, WA) as a model sTA are not rejected in vivo following ovalbumin in alum immunization and the induction of ovalbumin-specific IgG antibodies. This is in contrast to rejection of 4T1 transfected with SV40 Tag as the model sTA as described herein and may reflect the simple lack of surface expression of ovalbumin on 4T1 tumor cells following transfection, thus supporting a role for antibodies in the afferent phase of this vaccination regimen. We have cloned the ovalbumin cDNA into a construct that was engineered to directly surface expression of the transgene and will use this to transfect 4T1 tumor cells and further evaluate the role of antibody as a mechanism in rejecting sTA-expressing tumors following sTA protein immunization.

Others have approached the idea of vaccinating against cancer by using hapten-coated tumor cells or allo-MHC molecules as the foreign antigen (35, 36) called xenogenization (37, 38). Haptens are not as immunogenic as whole foreign proteins and the mechanisms of immunity may be different for allo-MHC than for foreign protein antigens. These approaches largely centered on administration of the modified or xenogenized tumor cells as the vaccine and were minimally effective. Whole cell xenogenized tumor vaccines may not elicit the proper immune response necessary to induce potent tumor immunity via cross-presentation and determinant spreading, which is likely why these approaches have largely been abandoned. A variation on this approach was to mutagenize the nonimmunogenic tumor cells, immunize with the mutant cells, and challenge with parental tumor cells. Van Pel and Boon showed that rejection of a nonimmunogenic thymic leukemia cell line could be accomplished if the challenge followed immunization with the same cell line that was treated with the mutagen N-methyl-N-nitro-N-nitrosoguanidine (39); this approach led to the discovery of the first human melanoma-associated antigen.

Cross-presentation in human cancer vaccine studies has been reported and is believed to be important in rejection of cancer in patients following immunotherapy. Thomas et al. showed the first direct evidence that T-cell responses can be generated via cross-presentation in pancreatic cancer patients vaccinated with pancreatic cancer cell lines engineered to secrete granulocyte macrophage colony-stimulating factor. Three patients generated MHC-I-restricted T-cell responses against epitopes in the TAA mesothelin carried by the vaccine cancer cell lines, although none of the patients possessed the MHC-I of the vaccine cancer cell line (40). In addition, human monocyte-derived dendritic cells were shown to be more effective at cross-presentation in association with patients with head and neck cancer when loaded with apoptotic tumor cells if proinflammatory cytokines or CD40L was included in the preparation (41).

Our approach is unique in that it employs the use of a defined, foreign protein as a sTA-based vaccine to drive determinant spreading to unknown TAA. By vaccinating with a nonsel sTA, an immune response is induced that focuses the proper immunologic mechanism(s) and environment on the target tumor cells, enabling the potent cross-priming of immune cells for the subsequent rejection of the indigenous/parental tumor.
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References


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