Cooperativity of *Staphylococcal aureus* Enterotoxin B Superantigen, Major Histocompatibility Complex Class II, and CD80 for Immunotherapy of Advanced Spontaneous Metastases in a Clinically Relevant Postoperative Mouse Breast Cancer Model

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ABSTRACT

One of the leading causes of death for women is metastatic breast cancer. Because most animal tumors do not accurately model clinical metastatic disease, the development of effective therapies has progressed slowly. In this study, we establish the poorly immunogenic mouse 4T1 mammary carcinoma as a postsurgical animal model. 4T1 growth characteristics parallel highly invasive human metastatic mammary carcinoma and, at the time of surgery, the extent of disease is comparable with human stage IV breast cancer. Progress in understanding the immune response has led to innovative immune-based anticaner therapies. Here, we test in this postsurgical model, a novel cell-based vaccine, combining MHC class II, CD80 (B7.1), and SEB superantigen. Effective treatment of tumor-bearing mice with this immunotherapy requires expression of all three molecules. Mean survival time is extended from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Increased survival is accompanied by a maximum of 100-fold decrease in clonal lung metastases. These therapeutic effects are particularly noteworthy because: (a) the postsurgical model demonstrates that early metastases responsible for morbidity are established by 2 weeks after tumor inoculation with 7 × 10³ parental 4T1 cells into the mammary gland; (b) the immunotherapy is started 4 weeks after tumor inoculation when the mice contain extensive, pre-established, disseminated metastases; and (c) CD4⁺ and CD8⁺ T cells are required for the effect.

INTRODUCTION

As a result of recent discoveries and advances in immunology and molecular cloning, many novel immunotherapeutic strategies for the treatment of cancer are being developed (1, 2). Most of these strategies are focused on eliminating primary tumors, which are frequently successfully treated by conventional methods, such as surgery. In contrast, few immunotherapeutic approaches are targeting disseminated metastatic disease, for which conventional therapies frequently have limited success. Development of therapies for the treatment of metastatic disease is complicated by the shortage of animal models for spontaneously metastatic cancers. We recently described a novel cell-based vaccine for the therapy of metastatic disease and tested it using the mouse 4T1 mammary carcinoma model. The 4T1 tumor shares many characteristics with its human counterpart (3), making it an excellent animal model.

In most clinical situations, primary mammary tumors are cured by surgery, yet approximately 33% of women successfully treated for primary tumors die subsequently from spontaneous metastatic disease (4). To further refine the 4T1 system and to more closely parallel clinical disease, we have now developed a postsurgical model of the 4T1 mammary tumor. In this model, mice receive inoculations s.c. in the abdominal mammary gland and the primary tumor is allowed to grow progressively, become extensively vascularized, and metastasize. The primary tumor is then surgically resected, and therapy with the cell-based vaccines is initiated.

The cell-based vaccines consist of tumor cells transfected with syngeneic MHC class II (I-A^d^) and CD80 (B7.1) costimulatory molecule genes and were designed to enhance activation of tumor-specific CD4⁺ T lymphocytes via improved presentation of tumor-encoded class II–restricted epitopes. Although CD8⁺ T lymphocytes have been traditionally the focus of immunotherapy approaches, accumulating results have demonstrated that CD4⁺ T lymphocytes also play a critical role in effective antitumor immunity (5–9). Whereas our previous vaccines showed significant reduction of established, spontaneous metastatic tumor, the antitumor response was limited to small burdens of metastatic cells and did not completely eliminate metastases (3). In addition, we did not assess the effects of immunotherapy on survival. Furthermore, the vaccine was tested in mice with metastatic disease and carrying intact primary tumor so the model did not mimic the clinical situation in which primary tumor would have been surgically removed before initiation of immunotherapy. We now report a second-generation cell-based vaccine that is significantly more effective than the original vaccine for the treatment of spontaneous 4T1 metastatic mammary cancer and that is tested in a postsurgical model. The new vaccine incorporates a gene encoding the bacterial toxin SEB (3). SEB is a sAg that when complexed with MHC class II molecules on APCs is a potent polyclonal activator of CD4⁺ T lymphocytes (10, 11). Although CD4⁺ T-cell activation by SEB is not antigen specific, we reasoned that the addition of SEB to the MHC class II/CD80 vaccine will provide additional activation signals to the CD4⁺ T cells that have been activated in an antigen-specific fashion by the MHC class II⁺ CD80⁺ vaccinating cells.

MATERIALS AND METHODS

cDNA Expression Vectors. The expression vectors pHβ-Apr-1-neo containing MHC class II (I-A^d^, I-A^B^) and mouse B7.1 have been described previously (3). The SEB gene (12) was subcloned into the SalI/BamHI site of the pHβ-Apr-1-neo expression vector. The final construct, pHβ-SEB-neo, contains the amino acid sequence for the mature SEB protein minus the signal peptide and confers resistance to G-418. The pZeoSV2 plasmid was purchased from Invitrogen (San Diego, CA).

Animals, Cell Lines, and Transfectants. Female BALB/c and BALB/c nude mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the University of Maryland Baltimore County animal facility and used at 8 weeks of age. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma (13), was kindly supplied.
by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI) and grown in culture as described previously (3). Transfectants were made to express MHC class II and CD80, or SEB by using lipofectin (Life Technologies, Inc.) according to manufacturer’s instructions. Cells were selected with 400 μg/ml G-418 (Life Technologies, Inc.) or 200 μg/ml zeocin (Invitrogen), cloned by limiting dilution, stained for surface antigen expression, and analyzed by flow cytometry, as described previously (3).

**SEB Assay.** Naïve BALB/c spleen cells (5–10 × 10⁶) were cultured in serial dilutions of transfectants’ supernatants or purified SEB (Sigma Chemical Co., St. Louis, MO), as indicated. To demonstrate specific SEB activity, a polyclonal rabbit antibody against SEB (Sigma Chemical Co.) was added to cultures, as indicated. After 3 days in culture, spleen cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent, as described previously (14).

**Surgery.** All surgical supplies and surgical equipment were purchased from Henry Schein Veterinary & Medical Supply Catalogue (Melville, NY) and Roboz (Rockville, MD), respectively, unless indicated otherwise. Before surgery, animals were weighed and anesthetized with i.p. injections (0.02 ml/g body weight) of 2.5% recrystalized avertin (2,2,2-tribromomethanol; Sigma Chemical Co.). Once the animals were unconscious, the tumor-bearing abdominal area was prepared and sterilized for surgery by shaving with Oster Finisher Trimmer, followed by 2 washes each with diluted Nolvasan surgical scrub (chlorhexidine) and isopropanol. Tumors were resected with sterilized surgical instruments, removing the smallest amount of skin tissue as possible. Wounds were closed with either Nexaband liquid or stainless steel 9-mm wound clamps with a Mikon autoclamp applier, as necessary. Wound clamps were removed 10 days after surgery with a Mikon autoclamp remover. Mice were monitored for survival, and those that died from surgery (within 1–4 days after surgery, survival rate of 67–80%) were not included in the experiment. All mice were autopsied at the time of death to confirm the presence of lung metastases as well as recurrence of the primary tumor.

**Tumor Challenges, Metastases Assays, and in Vivo Depletions.** Mice were challenged s.c. in the abdominal mammary gland with (7 × 10⁴/50 μl) parental 4T1 tumor cells. Primary tumor growth and spontaneous metastases were measured as described previously (3). Depletions of CD4⁺ and CD8⁺ T cells were performed as described previously (15). Splenocytes of all depleted mice were checked by immunofluorescence for depletion at the conclusion of the experiment. Mice depleted for CD4⁺ or CD8⁺ T cells had <4% or 7% of CD4⁺ T cells or CD8⁺ T cells, respectively.

**Statistical Analyses.** To determine the statistical significance of the data, the Tukey’s Honestly Significant Difference Test was performed at a P set at 0.05. The Tukey’s test is a multicomparison test that determines the statistical significance of data sets of size 3 or greater and allows for unequal sample size (n) and sample variances (16). To determine the statistical significance of the effects of immunotherapy on primary tumor growth, the Student’s t-test for unequal variances (Microsoft Excel, version 5.0) was performed.

**RESULTS**

**Tumor Lethality Is Due to Early Metastases.** Our previous studies have demonstrated that the BALB/c-derived 4T1 mammary carcinoma is a poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes throughout the body in a pattern similar to human breast cancer (3). For example, primary 4T1 tumors that have been established for 2–3 weeks in BALB/c mice typically metastasize to the lymph nodes, lungs, and livers in 86%, 79%, and 20% of mice, respectively, and the numbers of micrometastatic cells found in these organs range between 2–57, 1–338, and 0–1, respectively. In addition, as the primary tumors age (i.e., by 4–5 weeks), the incidence of metastases in the lungs, livers, and now brains increases to 91%, 82%, and 36% of mice, respectively, and the range of metastatic cells for these organs is between 6–250,000, 7–7,800, and 1–116, respectively (3).

As shown in Fig. 1, 4T1 is also similar to human mammary carcinoma in that morbidity is due to outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early (week 2) during primary tumor growth. Groups of female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 × 10⁴ wild-type 4T1 tumor cells. Primary TDs were measured, and tumors were surgically resected at varying times after inoculation (weeks 2–5). Each point represents the survival time in days after primary tumor challenge for an individual mouse.

**METASTASES REDUCTION BY SEB, CLASS II, AND B7.1**

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**Fig. 1.** Early spontaneous metastases are responsible for mortality. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 × 10⁴ live wild-type 4T1 cells. Primary TDs were measured, and tumors were surgically resected at varying times after inoculation (weeks 2–5). Each point represents the survival time in days after primary tumor challenge for an individual mouse.
SEB at a concentration of 2 µg/ml (open circles) or supernatants from parental 4T1 (filled circles), 4T1/SEB-12 (open diamonds), or 4T1/SEB-14 (open triangles) transfectants in the presence (open symbols) or absence (filled symbols) of antibody specific for SEB.

The success of this treatment, however, was limited to small tumor burdens and did not completely eliminate spontaneous metastases (3). A potential problem with this earlier therapy is that the transfectants did not coexpress MHC class II and CD80, and previous data using a mouse sarcoma showed that coexpression of these molecules is synergistic (15). We have, therefore, generated 4T1 transfectants that coexpress MHC class II and CD80 as detected by indirect immunofluorescence staining (data not shown) to test this hypothesis. To further increase the potency of the vaccine, we have combined the MHC class II+/CD80+ double transfectants (4T1/A^/B7.1) with SEB^+ transfectants (4T1/SEB), reasoning that SEB may provide additional proliferation signals to the tumor-specific T cells activated via the MHC class II/CD80 interaction.

SEB expression was tested by coculturing supernatants of transfectants with naive BALB/c spleen cells and monitoring lymphocyte proliferation. To determine a relative amount of SEB secretion, splenocytes were also cultured with soluble SEB. As shown in Fig. 2, supernatants from two independent clones (4T1/SEB-12 and 4T1/SEB-14) stimulated splenocyte proliferation as efficiently as soluble SEB at a concentration of 2 µg/ml. This activity was reduced on the addition of a polyclonal anti-SEB antibody, demonstrating that the spleen cell proliferation was due to SEB expression by the 4T1 transfectants. Supernatants from parental 4T1 cells as well as supernatants from 4T1 cells transfected with empty vector (4T1/neo) did not induce proliferative responses (Fig. 2 and data not shown). Therefore, the 4T1/SEB transfectants secrete SEB, which induces splenocyte proliferation comparable with proliferation induced by soluble exogenously added SEB.

Vaccines such as the 4T1 transfectants are likely to be most useful for the treatment of disseminated spontaneous metastatic disease because primary tumors usually can be eliminated by surgery. Therefore, we have tested the combination vaccine in mice with established, disseminated spontaneous metastases following surgical removal of the primary tumor. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 x 10^3 wild-type 4T1 tumor cells. The tumors were allowed to grow and metastasize for 3 weeks, at which time the primary tumor burden was measured and surgically resected. The experiments in Fig. 1 and our previously published results (3) established that at this stage and size of primary tumor the mice have very extensive disseminated metastases. At the time of surgery (3 weeks after primary tumor challenge), the primary TD in each treatment group ranged between 3.5 mm and 5.7 mm. The extent of spontaneous metastatic cancer at this time is significantly more advanced than that tested in other immunotherapy experiments. Therapeutic injections of irradiated vaccine cells (1 x 10^6 total) were started 1 week after surgery (i.e., 4 weeks after initial tumor challenge) and were administered every 3–5 days for the duration of the experiment. Because we are interested in determining whether primary tumor size affects vaccine efficacy, the results in Fig. 3 are plotted as the survival time versus the size of the primary tumor at the time of surgery. A line denoting the average survival time of the 4T1-treated control group (45 days) is included to demonstrate the effects of the vaccine on survival. The survival time in days for 4T1-treated (Fig. 3A), 4T1/SEB-treated (Fig. 3B), 4T1/A^/B7.1-treated (Fig. 3C), and 4T1/SEB+4T1/A^/B7.1-treated (Fig. 3D) animals was 35–52, 40–59, 47–54, and 41–74 days, respectively. Statistical analyses using the Tukey’s Honestly Significant Difference Test revealed that only the treatment with a 1:1 mixture of 4T1/A^/B7.1+4T1/SEB cells significantly increases the survival time of mice with established wild-type metastatic disease (P < 0.05). Treatment with either 4T1/SEB alone or 4T1/A^/B7.1 alone does not significantly increase survival. Therefore, therapy with this cell-based vaccine requires expression of all three molecules to extend mean survival time from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Although this increase in survival time is relatively small, it is statistically significant and compelling because the immunotherapy was started at week 4 and untreated and/or 4T1-treated mice begin to die as early as 5 weeks after tumor challenge.

Increase in Survival Correlates with Reduction of Metastatic Cancer. To demonstrate that the increase in survival was due to a reduction of spontaneous metastatic cancer, lungs from therapy-treated animals were harvested and the number of clonogenic metastases was quantitated as described previously (3). Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 10^6 total cells of irradiated parental 4T1 (3 mice; A), 4T1/SEB (8 mice; B), 4T1/A^/B7.1 (7 mice; C), or a 1:1 mixture of 4T1/A^/B7.1 plus 4T1/SEB cells (14 mice; D). The 1:1 mixture of 4T1/A^/B7.1 plus 4T1/SEB therapy group is significantly different from the 4T1 control group (P < 0.05, Tukey’s Honestly Significant Difference Test).
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7 \times 10^3 wild-type 4T1 tumor cells. Starting 2 weeks after challenge, they were given i.p. injections of irradiated vaccine cells (1 \times 10^6 total/injection) twice a week until the day of sacrifice. At the time of sacrifice (6 weeks after the initial primary tumor challenge), the number of clonogenic tumor cells. Ten days later, the number of clonogenic metastatic cells could be enumerated because 4T1 cells are resistant to 6-thioguanine, whereas normal cells are not resistant and die. As shown in Fig. 3, we are interested in determining whether primary tumor size versus TD at the start of treatment. A line denoting a level of 10,000 tumor cells in the lungs is also included because >85% of untreated tumor-bearing mice contain >10,000 metastatic cells in their lungs after 42 days of primary tumor growth (3). As shown in Fig. 4, administration of the 4T1 transfectants significantly reduces the number of lung metastases (Fig. 4, B-D) relative to treatment with wild-type 4T1 cells (Fig. 4A). For example, 13 of 16 (81.2%) mice treated with irradiated parental cells (Fig. 4A) contained \(10^6\) clonogenic lung metastases, which contrasts 12 of 23 (52.2%) mice treated with either 4T1/SEB or 4T1/A^B/B7.1 (Fig. 4, B and C) and 6 of 21 (28.6%) mice treated with a mixture of transfectants (Fig. 4D). After transforming the number of clonogenic metastases to logarithmic values and analyzing these data using the Tukey’s Honestly Significant Difference Test, we found that only treatment with a 1:1 mixture of 4T1/SEB+4T1/A^B/B7.1 cells (Fig. 4D) significantly reduced the number of clonogenic lung metastases (5 = 0.05). Treatment with either 4T1/SEB alone (Fig. 4B) or 4T1/A^B/B7.1 alone (Fig. 4C) did not significantly decrease the number of clonogenic lung metastases. Previously, we demonstrated that therapy with MHC class II^+/CD80^+ vaccines significantly reduced clonogenic lung metastases in 50% of mice whose immunotherapy was initiated 9–14 days after tumor challenge but this reduction corresponded to only a 10-fold maximum reduction when compared with the control group (3). In contrast, treatment of mice carrying 14-day established primary and metastatic tumor with the combination therapy of tumor cell transfectants expressing MHC class II, CD80, and SEB genes decreases spontaneous metastases in the lung by a maximum of 100-fold. Therefore, effective immunotherapeutic treatment of tumor-bearing mice with extensively established spontaneous metastases requires expression of all three molecules.

Reduction of Established Wild-Type Metastases with MHC class II, CD80, and SEB Immunotherapy Requires Both CD4^+ and CD8^+ T cells. The concept of combining SEB with MHC class II and CD80 was based on the hypothesis that SEB is a potent polyclonal activator of CD4^+ T lymphocytes (10, 11) and would provide additional activation signals to CD4^+ T cells that have been activated in an antigen-specific fashion by the MHC class II^+ CD80^+ vaccinating cells. Therefore, we tested the immunotherapy described in Fig. 4 in CD4^- or CD8^-depleted animals and BALB/c nu/nu mice. As shown in Fig. 5, C and D, depletion of CD4^+ or CD8^+ T cells (monoclonal antibodies GK1.5 and 2.43, respectively) eliminates the therapeutic effect of the MHC class II^+, CD80^+, SEB^+ vaccine against spontaneous metastases, whereas depletion with control ascites (Fig. 5B) has no effect. In addition, the combination vaccine does not reduce metastatic disease in BALB/c nu/nu mice (Fig. 5F). Collectively, these data demonstrate that the three transfected genes of the cell-based vaccines are working cooperatively to optimally activate both CD4^+ and CD8^+ T lymphocytes and that these lymphocyte populations are essential for the therapeutic effect.

DISCUSSION

sAgs, including SEB, have been previously recognized as potential reagents for up-regulating T lymphocyte responses against tumors. However, their use has been limited and they have not been combined with other factors that might optimize their therapeutic efficacy. For example, several studies describe redirected T-cell activation using sAgs coupled to tumor-specific monoclonal, anti-idiotypic, or bifunctional antibodies (17–19). SEB has also been administered systemically along with tumor cells, and SEB DNA has been inoculated intratumorally along with cytokine DNA to reduce primary tumor growth (20, 21). In addition, sAgs have been used to activate tumor-draining lymph node T cells ex vivo for adoptive transfer into tumor-bearing animals (22, 23). All of these approaches produce some reduction in primary tumor growth and/or decrease in metastatic lesions. However, the test settings have involved relatively small primary tumor and/or very small metastatic tumor burdens, which do not mimic the clinical situation. These results, taken together with the SEB transfected tumor vaccines presented in this study, show that SEB expression alone has only a modest effect on metastatic tumor progression. However, as shown in this study, the antitumor effect of SEB on highly advanced spontaneous metastases is more effective when combined with the cell-based vaccine containing MHC class II and CD80 molecules.

The modified tumor cells may function directly as APCs for the initial activation of tumor-specific CD8^+ and CD4^+ T cells following immunization. Previous studies demonstrate that both CD8^+ and
CD4+ T lymphocytes are involved in immunity induced by MHC class II/CD80 and SEB immunotherapy requires CD4+ and CD8+ T cells. A-D, naïve BALB/c mice received injections s.c. in the abdominal mammary gland of 7 x 10^5 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; A) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (B-D). On days 8, 11, and 13 (i.e., before the start of immunotherapy), mice received injections of either control ascites (10 mice; B), GK1.5 ascites (9 mice; C), or 2.43 ascites (8 mice; D). Antibody injections were continued at least once a week for the duration of the experiment. E and F, BALB/c nu/nu mice received injections s.c. in the abdominal mammary gland of 7 x 10^5 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; E) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (8 mice; F).

Fig. 5. Reduction of established wild-type metastases with MHC class II, CD80, and SEB. Immunotherapy requires CD4+ and CD8+ T cells. A–D, naïve BALB/c mice received injections s.c. in the abdominal mammary gland of 7 x 10^5 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; A) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (B–D). On days 8, 11, and 13 (i.e., before the start of immunotherapy), mice received injections of either control ascites (10 mice; B), GK1.5 ascites (9 mice; C), or 2.43 ascites (8 mice; D). Antibody injections were continued at least once a week for the duration of the experiment. E and F, BALB/c nu/nu mice received injections s.c. in the abdominal mammary gland of 7 x 10^5 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; E) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (8 mice; F).

Fig. 6. Proposed mechanism of action by the MHC class II/CD80 and SEB tumor cell-based vaccine. The SEB modified tumor cell (bottom) secretes sAg/s at the immunization site where the host professional APC (left) and the MHC class II/CD80 modified tumor cell (right) are able to bind the sAg and activate both CD4+ and CD8+ T cells. As a result, immunization occurs by direct and indirect (cross-priming) antigen presentation."
results, and testing in humans, therefore, will always be required. However, the inability to translate therapies developed in experimental animal models to humans may also be because many of the mouse tumor systems used in immunotherapy studies do not closely model human cancers and, hence, the immunotherapies are not being tested in clinically relevant settings. For example, many immunotherapies are tested in so-called “metastatic” settings; however: (a) the extent of metastatic disease is minimal; (b) the metastases have not arisen spontaneously; and/or (c) the metastases have not been established for significant amounts of time. Furthermore, many commonly used mouse models: (a) are not spontaneously metastatic (e.g., CMS-5 fibrosarcoma, RENCA renal cell carcinoma, CT-26 colon adenocarcinoma, SaI sarcoma, and so forth); (b) rapidly lose their metastatic potential when cultured in vitro (e.g., K1735 melanoma); (c) metastasize poorly unless the primary tumor is excised (e.g., B16 melanoma, line 1 carcinoma); or (d) rapidly invade the local environment, such that animals die from primary tumor before metastatic disease is established (e.g., B16 melanoma). In contrast, the 4T1 mammary carcinoma is spontaneously metastatic and metastasizes to many of the organs to which human breast cancer metastasizes (e.g., lung, liver, and brain). Also, similar to human mammary carcinoma, 4T1 metastases spread and progress while primary tumor is in place. In addition, following inoculation of a small number of tumor cells (7 × 10^3) in the mammary gland, lethal metastatic disease develops early (within the first 2–3 weeks) and progresses over several weeks so that immunotherapies can be tested against early or very advanced stage disease. The 4T1 tumor, therefore, is an excellent model for testing experimental immunotherapies. In contrast to our earlier studies with the 4T1 tumor in which relatively early metastases were treated and primary tumor was left in place (3), the studies reported here address very advanced metastatic disease in a postsurgical setting. Although the statistically significant extension of survival time following surgery and administration of immunotherapy was small, we find no comparable studies in the literature in which the efficacy of an immunotherapy is demonstrated in such a clinically relevant model of advanced stage metastatic disease.

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