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Reduction of Established Spontaneous Mammary Carcinoma Metastases following Immunotherapy with Major Histocompatibility Complex Class II and B7.1 Cell-based Tumor Vaccines[1]

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ABSTRACT

For many cancer patients, removal of primary tumor is curative; however, if metastatic lesions exist and are not responsive to treatment, survival is limited. Although immunotherapy is actively being tested in animal models against primary tumors and experimental metastases (i.v. induced), very few studies have examined immunotherapy of spontaneous, established metastatic disease. The shortage of such studies can be attributed to the paucity of adequate animal models and to the concern that multiple metastatic lesions may be more resistant to immunotherapy than a localized primary tumor. Here, we use the BALB/c-derived mouse mammary carcinoma, 4T1, and show that this tumor very closely models human breast cancer in its immunogenicity, metastatic properties, and growth characteristics. Therapy studies demonstrate that treatment of mice with established primary and metastatic disease with MHC class II and B7.1-transfected tumor cells reduces or eliminates established spontaneous metastases but has no impact on primary tumor growth. These studies indicate that cell-based vaccines targeting the activation of $CD4^+$ and $CD8^+$ T cells may be effective agents for the treatment of malignancies, such as breast cancer, where the primary tumor is curable by conventional methods, but metastatic lesions remain refractile to current treatment modalities.

INTRODUCTION

In human breast cancer, if metastases are not present, surgical removal of the primary tumor can lead to full recovery of the patient. However, if the primary tumor has metastasized, then other therapies such as hormone therapy (1), chemotherapy (2, 3), and/or radiation therapy (4) are used to eliminate metastatic cells. In many cases, these conventional treatments only lead to temporary control of the disease and provide only an average 3-year survival rate postdiagnosis (5). More effective therapies are clearly necessary for treating metastatic disease. Immunologists have recently proposed and tested a variety of novel strategies for generating cell-based tumor vaccines, and these approaches hold promise for additional treatment modalities. These approaches have focused on the stimulation of $CD8^+$ CTLs because these effector cells are capable of specifically and directly destroying malignant tumor cells. For example, various cytokine genes and/or surface molecules have been transfected into tumors, and the modified tumor cells have been used as cell-based vaccines to enhance antitumor immune responses (reviewed in Refs. 6 and 7). Although some of these studies were designed to circumvent the need for $CD4^+$ T_h[3] lymphocytes by allowing the tumor cells to directly supply cytokines to CTLs (6), other studies were directly aimed at increasing T_h cell generation (8, 9). Both approaches demonstrated that optimal immunity required both $CD4^+$ and $CD8^+$ T cells (8–12). Most of these studies have focused on the treatment of primary tumors, and only a limited number have addressed experimental metastases (*e.g.*, Refs. 13–16). Although even fewer groups focused on established spontaneous metastatic disease, those studies used either severe combined immunodeficient mice or anatomically incorrect tumor challenges in the footpad (17–19). Effective therapies for distant metastatic cells, therefore, have not been extensively studied and remain elusive.

T cells recognize antigen (peptide)/MHCs through their T-cell antigen receptor (20). However, to achieve maximum activation of $CD4^+$ or $CD8^+$ T-cells, a second T-cell antigen receptor-independent signal (costimulation) is required (21). Numerous studies have demonstrated the role of B7.1 and B7.2 in costimulation (22). Other molecules, such as intercellular adhesion molecule-1, VCAM-1, heat-stable antigen, and 4-1BB ligand have also been shown to function in a costimulatory role (23–27). Previously, we demonstrated that the transfection of MHC class II genes into mouse sarcoma and melanoma cells enhanced primary tumor rejection and reduced experimental (i.v.) metastases, respectively (8). Furthermore, expression of either B7.1 or B7.2 in addition to MHC class II increased these effects (8, 9). Not surprisingly, these responses were dependent on both $CD4^+$ and $CD8^+$ T cells. We now propose that by designing tumor cells as vaccination vehicles for stimulating both $CD4^+$ and $CD8^+$ T-cells, it should be possible to induce tumor-specific immunity to treat spontaneous metastatic disease.

To test this hypothesis, we have used the poorly immunogenic BALB/c mouse-derived 4T1 mammary carcinoma (28–30). This tumor shares many characteristics with human mammary cancers, making it an excellent animal model, and it expresses adequate levels of MHC class I molecules, making it a suitable target for $CD8^+$ T cells. Because 4T1 is 6-thioguanine resistant, micrometastatic cells can readily be detected at very early stages of growth, allowing us to quantitatively monitor the effects of the immunotherapy approach on spontaneous metastasis development.

MATERIALS AND METHODS

Animals and Reagents. Female BALB/c and BALB/c *nu/nu* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the University of Maryland Baltimore County animal facility and were used at 8 weeks of age. Reagents were purchased as indicated: Lipofectin and G-418 sulfate (Geneticin; Life Technologies, Inc., Gaithersburg, MD); collagenase types 1 and 4 (Worthington Biochemical Corp., Freehold, NJ); elastase (ICN, Costa Mesa, CA); hyaluronidase, BSA, 6-thioguanine (2-amino-6-mercaptopurine), and methylene blue, Sigma Chemical Co. (St. Louis, MO).

cDNA Expression Vectors. The expression vector pHβ-Apr-1-neo has been described previously (31). Using PCR, cDNAs encoding the A_α^d and A_β^d class II MHC genes were amplified from RNA isolated from A20 B-lymphoma cell line. Primers for the A_α^d chain (sense, 5′-CTCCGCGAGTCGACGATGCCGTGCAGCAGA-3′; and antisense, 5′-ACAGCGGATCCTCATAAAGGCCCTG-3′) and A_β^d chain (sense, 5′-CCTGTGCAGTCGACATGGCTCTGCAGAT-3′; and antisense, 5′-GACACGGATCCTCACTGCAG GAGCC-3′) incorporated a *Sal*I site at their 5′ end and a *Bam*HI site at their 3′ end for

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[3] The abbreviations used are: T_h, T-helper; TD, mean tumor diameter; LN, lymph node; CC, correlation coefficient; NK, natural killer; APC, antigen-presenting cell; mAb, monoclonal antibody.

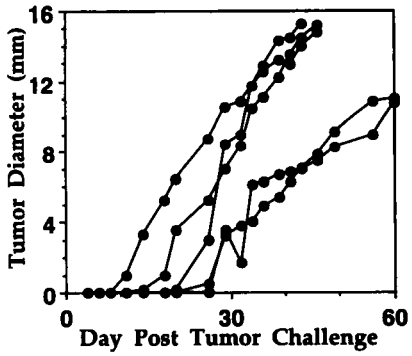

Fig. 1. 4T1 cells are highly tumorigenic. Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells. Primary tumors were measured every 3–4 days, and the mean TD was calculated as described in "Materials and Methods." *Lines*, individual mice.

subcloning into the parental vector. The expression vector containing the B7.1 cDNA was also generated using PCR and was described previously (32). The final constructs contained only the sequence within the coding region for each cDNA and conferred resistance to G-418.

Cell Lines and Transfectants. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma, was kindly supplied by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI; Ref. 30). Unmodified tumor cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine product (Hyclone, Logan, UT) and 1× antibiotic-antimycotic (Life Technologies, Inc.). Transfectants were made to express either MHC class II or B7.1 by using Lipofectin according to the manufacturer's instructions. Cells were selected with 400 μg/ml G-418, cloned by limiting dilution 48 h after transfection, stained for surface antigen expression, and analyzed by flow cytometry as described previously (8, 9). The following antibodies were used: 34-5-8, mouse anti-H-2D^d (33); 16.3.1, mouse anti-H-2K^k (34); MKD6, mouse anti-I-A^d (35); 3JP, mouse anti-I-A^{b,k} (36); and 1G10, rat anti-B7.1 (37).

***In Vivo* Tumor Growth.** Mice were challenged s.c. in the abdominal mammary gland with either parental or transfected 4T1 tumor cells. Primary tumors were measured every 3 or 4 days following tumor challenge using vernier calipers. Mean TD was calculated as the square root of the product of two perpendicular diameters. Animals were sacrificed when the TD reached 14–16 mm or when the mice became moribund, according to University of Maryland Baltimore County Institutional Animal Care and Use Committee guidelines.

Spontaneous Metastases Assay. Spontaneous metastases were measured by adapting methods described previously by Aslakson and Miller (30). Mice were challenged s.c. in the abdominal mammary gland with 5×10^3 parental or transfected 4T1 tumor cells and sacrificed at the times indicated. Several organs were removed from each mouse, uniquely identified, and further prepared as follows: Blood and draining LNs were prepared as described previously (30). Liver samples were finely minced and digested in 5 ml of enzyme cocktail containing 1× PBS, 0.01% BSA, 1 mg/ml hyaluronidase, and 1 mg/ml collagenase type 1 for 20 min at 37°C on a platform rocker. Lung samples were finely minced and digested in 5 ml of enzyme cocktail containing 1× PBS, 1 mg/ml collagenase type 4 and 6 units/ml elastase for 1 h at 4°C on a rotating wheel. Brain samples were finely minced and digested for 2 h at 37°C on a platform rocker with 5 ml of the same enzyme cocktail used for lung samples. After incubation, all samples were filtered through 70-μm nylon cell strainers and washed two to three times with 1× HBSS. Resulting cells were resuspended and plated neat or serially diluted in 10-cm tissue culture dishes in medium containing 60 μM thioguanine for clonogenic growth. 6-Thioguanine-resistant tumor cells formed foci within 10–14 days, at which time they were fixed with methanol and stained with 0.03% methylene blue for counting. Clonogenic metastases were calculated on a per-organ basis.

Statistical Analyses. A Student's *t* test for unequal variances was performed using Microsoft Excel Version 5.0 to determine the statistical significance of indicated data.

RESULTS

Inoculation of Small Quantities of 4T1 Mammary Carcinoma Induces Primary Tumor Formation and Spontaneous Metastatic Disease in Syngeneic BALB/c Mice. Previous studies by Miller and colleagues (29, 30) and others (28) established that the 4T1 mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice. Because we are developing immunotherapy strategies for the treatment of metastatic malignancies, we have confirmed these results and assessed metastatic disease in additional target organs as a prelude to our therapeutic studies. As shown in Fig. 1 and Table 1, primary tumors form in 100% of BALB/c mice when as few as 5×10^3 cells are injected s.c. in the abdominal mammary gland. These tumors are palpable within 11–26 days after injection and reach 14–16 mm in TD within 40–69 days. At higher doses ($>10^4$), primary tumors develop more rapidly, as reflected in a shortened tumor onset and decreased survival time. Although inoculation of lower doses of 4T1 (10^3) also induces primary tumor formation, the tumor incidence decreases to 60% of inoculated mice. The 4T1 tumor, therefore, is highly tumorigenic, even at relatively low doses of inoculating cells.

To confirm the metastatic potential of the 4T1 mammary carcinoma, female BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 4T1 cells, and metastasis formation was assessed. Mice were sacrificed at varying times after inoculation and the kinetics of spontaneous metastasis formation were assessed in the draining LN, lung, liver, blood, and brain by plating out dissociated organs in medium supplemented with 6-thioguanine. Because 4T1 cells are 6-thioguanine resistant, individual tumor cells form foci in culture, each focus representing an individual clonogenic tumor cell. The number of foci, therefore, is a direct measure of the number of metastatic tumor cells per organ, and the *in vitro* amplification allows for the quantitation of micrometastatic tumor cells, which would otherwise not be detectable.

Table 2 shows the distribution and subsequent spread of metastatic tumor cells in the various organs at progressive times after inoculation. For example, at day 14 or 18 after primary s.c. inoculation, distant spontaneous metastases were measurable in the LN of 11 of 12 mice and the lungs of 13 of 13 mice. By day 22, the livers of three of five mice had clonogenic metastases, whereas the blood of only one of eight mice contained tumor cells. Because only a portion of the blood was recovered, this value may be an underestimate. By week 4, the blood, liver, and lungs of 75–100% of mice contained tumor cells. Some of the organs with clonogenic tumor cells showed visible metastatic lesions; however, many of the organs appeared phenotypically normal and showed no visible signs of tumor. Also by week 4, the draining LN of five of eight mice had been engulfed by the primary tumor and, thus, could not be tested. Metastatic cells in the brain were first detected at week 5 (27% of mice) and the frequency of mice with metastatic cells in the brain increased (67%) as time progressed. Metastases in the blood, LN, liver, and/or brain of indi-

Table 1 *Tumor growth analysis of 4T1 mammary carcinoma in syngeneic BALB/c mice*

BALB/c mice (five mice/group) were challenged s.c. in the abdominal mammary gland with the indicated number of parental 4T1 tumor cells. The tumor incidence is the number of animals that developed progressive tumors. As described in "Materials and Methods," animals that developed tumors were sacrificed when the TD reached 14–16 mm or when the mice became moribund.

Challenge dose	Tumor incidence	Tumor onset (days)	Time to sacrifice (days)
1×10^3	3/5	15–20	45–61
5×10^3	5/5	11–26	40–69
1×10^4	5/5	8–10	35–46
1×10^5	5/5	6–8	35
1×10^6	5/5	4–7	30

Table 2 *4T1 mammary carcinoma cells spontaneously metastasize in BALB/c mice*

BALB/c mice were challenged s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 tumor cells. Mice were sacrificed at various times after tumor challenge, and the draining lymph node, lung, liver, blood, and brain tissues were removed. Each organ was individually prepared as described in "Materials and Methods" and plated for metastatic cell outgrowth. Data indicate the number of animals positive for spontaneous metastases of the total number tested for each organ. The numbers in parentheses show the range of clonogenic metastases found in the positive organs.

	Spontaneous metastases				
Harvest day	LN	Lung	Liver	Blood	Brain
14–18	11/12 (2–57)	13/13 (1–43)	0/11	0/13	ND[a]
22	7/9 (5–35)	6/11 (32–338)	3/5 (1)	1/8 (1)	ND
30–32	2/3 (15–83)	10/10 (6–116,500)	7/8 (7–3,700)	3/4 (6–82)	ND
34–37	ND	10/12 (315–267,000)	11/14 (32–7,800)	5/11 (1–24)	3/11 (1–116)
>42	ND	14/14 (1,109–200,000)	6/8 (1,100–12,200)	6/8 (25–490)	4/6 (5–613)

[a] ND, not done.

vidual mice were only present when the individual contained lung metastases and not *vice versa*. The pathway of metastasis for the 4T1 tumor, therefore, appears to be from the primary tumor to the lungs and the draining LN and, subsequently, to the liver, blood, and brain.

There is frequently a correlation in human disease between the size of primary tumor and extent of metastatic disease. To determine whether this observation is modeled by the 4T1 tumor, the number of clonogenic tumor cells in the lung, liver, blood, LN, and brain has been plotted as a function of the TD at the time of harvest. As shown in Fig. 2*A*, there is a positive correlation (CC = 0.684) between size of primary tumor at time of sacrifice and the number of clonogenic lung metastases. Similar correlations between TD at the time of harvest and clonogenic metastases were also seen for liver (Fig. 2*B*, CC = 0.520), blood (Fig. 2*C*, CC = 0.396), and brain (Fig. 2*D*, CC = 0.426). No correlation was seen between the number of clonogenic metastases in LN and the size of primary tumor (Fig. 2*E*, CC = 0.134) because the number of samples were limiting. The 4T1 tumor, therefore, shows a pattern of metastatic spread comparable to human mammary carcinoma, and assessment of lung metastases best approximates the extent of metastatic disease in tumor-bearing mice.

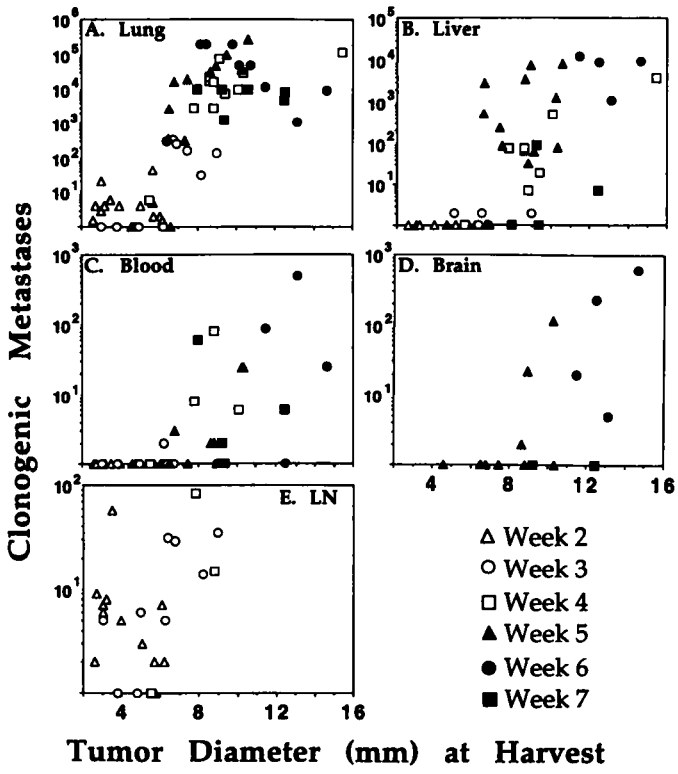

Fig. 2. 4T1 tumor cells spontaneously metastasize to the lungs (*A*), liver (*B*), blood (*C*), brain (*D*), and LN (*E*). Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells. Mice were sacrificed at varying times after inoculation (weeks 2–7), and the number of metastatic tumor cells was determined as described in "Materials and Methods." *Data points*, individual mice.

Expression of MHC Class II or B7.1 by 4T1 Transfectants Reduces Tumorigenicity and Metastatic Potential. In previous studies, we demonstrated that sarcoma cells transfected with syngeneic MHC class II plus B7.1 genes are an effective cell-based vaccine for the treatment of established, primary, solid tumors (9). That strategy was based on the hypothesis that such vaccines could activate both $CD4^+$ and $CD8^+$ tumor-specific T-cells and that optimal activation of $CD8^+$ T-cells requires "help" from $CD4^+$ T-cells. Because such vaccines might be very desirable agents for the treatment of disseminated metastatic disease, we have now extended our studies to the spontaneously metastatic 4T1 breast carcinoma.

4T1 tumor cells were transfected with plasmids containing MHC class II, B7.1, and/or the selectable neomycin resistance genes. Following limiting dilution cloning, several clones were chosen based on their surface expression of MHC class I, class II, and B7.1, as detected by indirect immunofluorescence staining (Fig. 3). All transfectants express similar levels of MHC class I as compared to parental 4T1 cells (Fig. 3, *a–h*). Two of the MHC class II transfectant clones (4T1/A^d-12 and 4T1/A^d-30) express similar levels of MHC class II, whereas the third class II transfectant (4T1/A^d-1) expresses higher levels (Fig. 3, *j–l*). Of the four B7.1 transfectants, two clones (4T1/B7.1-1 and 4T1/B7.1-6) express similar levels of B7.1, which are slightly higher than the levels expressed by the two other transfectants (4T1/B7.1-15 and 4T1/B7.1-23 (Fig. 3, *u–x*). 4T1 cells transfected with the empty parental vector (4T1/neo) do not express either MHC class II or B7.1 (data not shown), as observed with untransfected 4T1 cells (Fig. 3, *i* and *q*).

To test the immunogenicity and tumorigenicity of the class II and B7.1 transfectants, syngeneic female BALB/c mice were challenged in the abdominal mammary gland with 5×10^3 tumor cells, and the challenged mice were followed for primary tumor growth and metastasis formation. Fig. 4 shows the number of clonogenic tumor cells in the lungs *versus* TD at time of sacrifice (*A–H*), and the growth rate of the primary tumor (*A–H, insets*) for the various transfectants. With the exception of 4T1/A^d-30 (Fig. 4*D*, *inset*), all of the transfectants show some reduction in primary tumor growth rate and/or lack of tumorigenicity, although only the 4T1/A^d-12 transfectant does not form primary tumors in any of the inoculated mice (Fig. 4*C*). In contrast, the metastatic potential of both the class II^+ and $B7.1^+$ transfectants is markedly reduced relative to 4T1 cells. For example, 17 of 21 mice inoculated with class II^+ transfectants contained <5,000 metastatic cells in the lung (Fig. 4, *B–D*), whereas 15 of 15 mice inoculated with wild-type 4T1 cells have 5,000–120,000 metastatic cells in the lung (Fig. 4*A*). For the $B7.1^+$ transfectants, 19 of 20 inoculated mice contained 0–432 metastatic cells, with only one mouse displaying >10,000 tumor cells in the lungs (Fig. 4, *E–H*). Primary tumor growth in immunocompetent syngeneic mice, therefore, is inconsistently re-

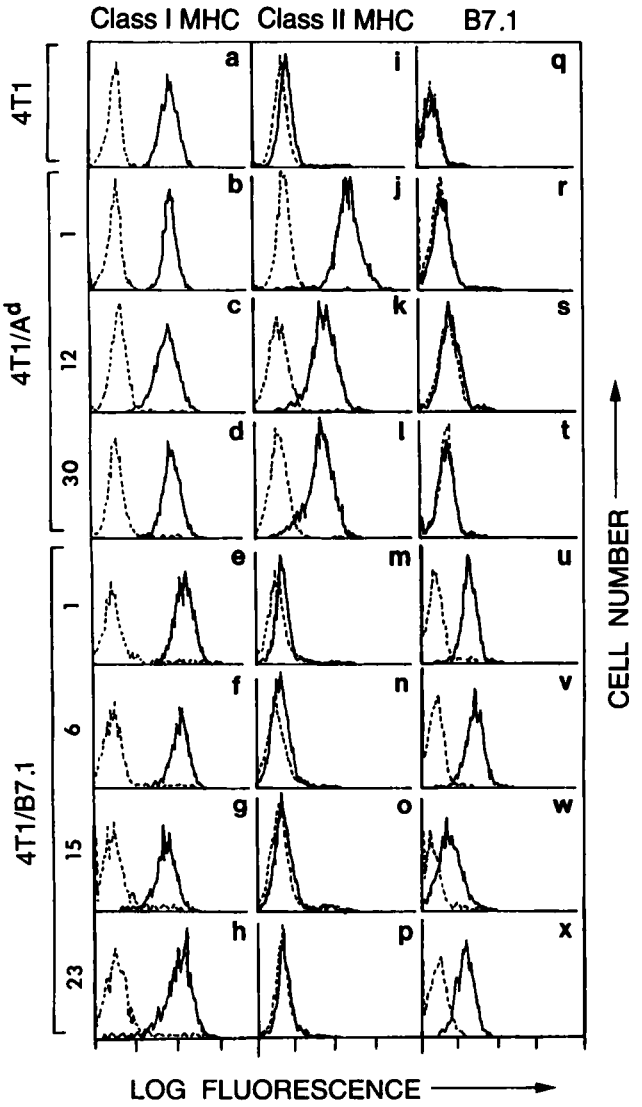

Fig. 3. 4T1 mammary carcinoma transfectants express either I-A^d class II MHC or B7.1 molecules. Parental 4T1 cells and transfectants were stained by indirect immunofluorescence as described in "Materials and Methods." Class I MHC expression (*a–h*) was measured using the mouse anti-H-$2D^d$ mAb 34-5-8 (——) and irrelevant control mouse anti-H-$2K^k$ mAb 16.3.1 (·····). Class II MHC expression (*i–p*) was measured using the mouse anti-A^d mAb MKD6 (——) and the isotype-matched irrelevant control mouse anti-$A^{b,k}$ mAb 3JP (·····). B7.1 expression (*q–x*) was measured using the rat anti-B7.1 mAb 1G10 (——) with the conjugate alone (·····) as control. The *X* axis shows four logarithmic cycles of fluorescence intensity.

duced by expression of MHC class II or B7.1 genes; however, metastatic potential is reproducibly decreased.

Primary Tumor Growth and Metastasis Formation Are Regulated by T Lymphocytes. To determine whether T cell-mediated immunity is involved in the reduced tumorigenicity and metastatic spread of the class II^+ and $B7.1^+$ transfectants, T cell-deficient *nu/nu* mice were tumor-challenged (5×10^3 cells) and followed for primary tumor growth and metastasis formation. Two MHC class II transfectants and two B7.1 transfectants were used. As shown in Fig. 5, one of the class II^+ transfectants (4T1/A^d-1; Fig. 5*B*) and one of the $B7.1^+$ transfectants (4T1/B7.1-6; Fig. 5*D*) formed tumors and metastases in nude mice similar to unmodified wild-type 4T1 tumor cells (Fig. 5*A*). In contrast, 4T1/A^d-12 (Fig. 5*C*) and 4T1/B7.1-23 (Fig. 5*E*) lines formed primary tumor comparable to 4T1; however, their metastatic potential was much reduced relative to wild-type 4T1 tumor cells. To analyze the effects of T cells in immunocompetent *versus* T cell-deficient mice, primary tumor incidence in BALB/c and BALB/c *nu/nu* mice were compared. As summarized in Table 3, 87% of the BALB/c *nu/nu versus* 20% of the BALB/c mice developed progressive primary tumor following s.c. challenge. The class II^+ and $B7.1^+$ transfectants, therefore, have different primary growth kinetics and metastasis formation in T cell-deficient nude mice *versus* immunocompetent BALB/c mice, suggesting that T lymphocytes are important effector cells for regulating tumor growth *in vivo*.

Immunization of Naive Mice with 4T1 Transfectants Expressing MHC Class II or B7.1 Protects against Metastatic Disease but not Primary Tumor Growth following Wild-type 4T1 Challenge. The experiments of Figs. 1–5 suggest that the reduced primary tumor and metastasis formation of the class II^+ and $B7.1^+$ transfectants *versus* 4T1 cells is due to increased tumor cell immunogenicity. We, therefore, have tested the transfectants as immunotherapeutic agents. In the first regimen, naive, tumor-free syngeneic BALB/c mice were immunized i.p. with 10^6 irradiated transfectants and challenged s.c. 4 weeks later with 5×10^3 live 4T1 parental cells. Mice were sacrificed 5 weeks after the 4T1 challenge and clonogenic tumor cells measured in the lungs. As shown in Fig. 6, all of the transfectants provided some protection against 4T1 metastasis, with 4T1/A^d-12 and the mixture of 4T1/A^d-12 plus 4T1/B7.1-23 providing the maximum protection (<1400 clonogenic cells in each individual lung), and immunization with wild-type 4T1 providing minimal protection. Clonogenic metastatic cells in the liver and blood were also similarly reduced in the transfectant-treated animals (data not shown). Other organs were not monitored for metastatic cells. However, none of the transfectants significantly reduced the growth of the primary tumor (data not shown). Immunization of naive mice with the class II^+ and/or $B7.1^+$ transfectants significantly protects against spontaneous metastatic disease but does not affect primary tumor growth of wild-type 4T1 tumor.

Treatment of Tumor-bearing Mice with Transfectants Expressing MHC Class II or B7.1 Reduces Established Wild-type Metastatic Disease but Does Not Affect Primary Tumor Growth. To model a more realistic clinical situation and to test the transfectants more rigorously, the therapeutic efficacy of two transfectant clones was further tested in mice against established metastases. BALB/c mice were challenged s.c. with 5×10^3 wild-type 4T1 tumor cells and, starting at either day 9 or 14 after 4T1 challenge, they were given injections of irradiated transfectants (4T1/A^d-12 and/or 4T1/B7.1-6) twice a week until the day of sacrifice, approximately 4 weeks later. At the time of sacrifice, primary TDs of control-treated mice (*i.e.*, mice given irradiated 4T1 cells), 6.8–12.5 mm, were comparable to TDs in transfectant-treated animals, 6.3–13.6 mm. The two-tailed *P* was 0.29 when tumor sizes of mice treated with control cells were compared with those of transfectant-treated mice combined. Lungs were subsequently removed, and the number of clonogenic tumor cells was determined. Because this therapy will be used to treat patients with established tumor, the results of this experiment have been plotted as number of clonogenic cells in the lungs *versus* TD at the start of treatment. As shown in Fig. 7, administration of 4T1/A^d-12, 4T1/B7.1-6, or a mixture of cells significantly reduces the number of lung metastases (Fig. 7, *B–D*) relative to treatment with wild-type 4T1 cells (Fig. 7*A*) when primary TDs at the start of treatment were <4 mm. After transforming the number of clonogenic metastases to logarithmic values and analyzing as described in "Materials and Methods," the two-tailed *P* was 0.008 when control-treated mice were compared with transfectant-treated mice combined. When TDs, however, were >4 mm on the initial treatment day, no significant reduction in primary tumor growth or metastatic cells was seen (data not shown). Metastatic spread, therefore, can be significantly reduced by im-

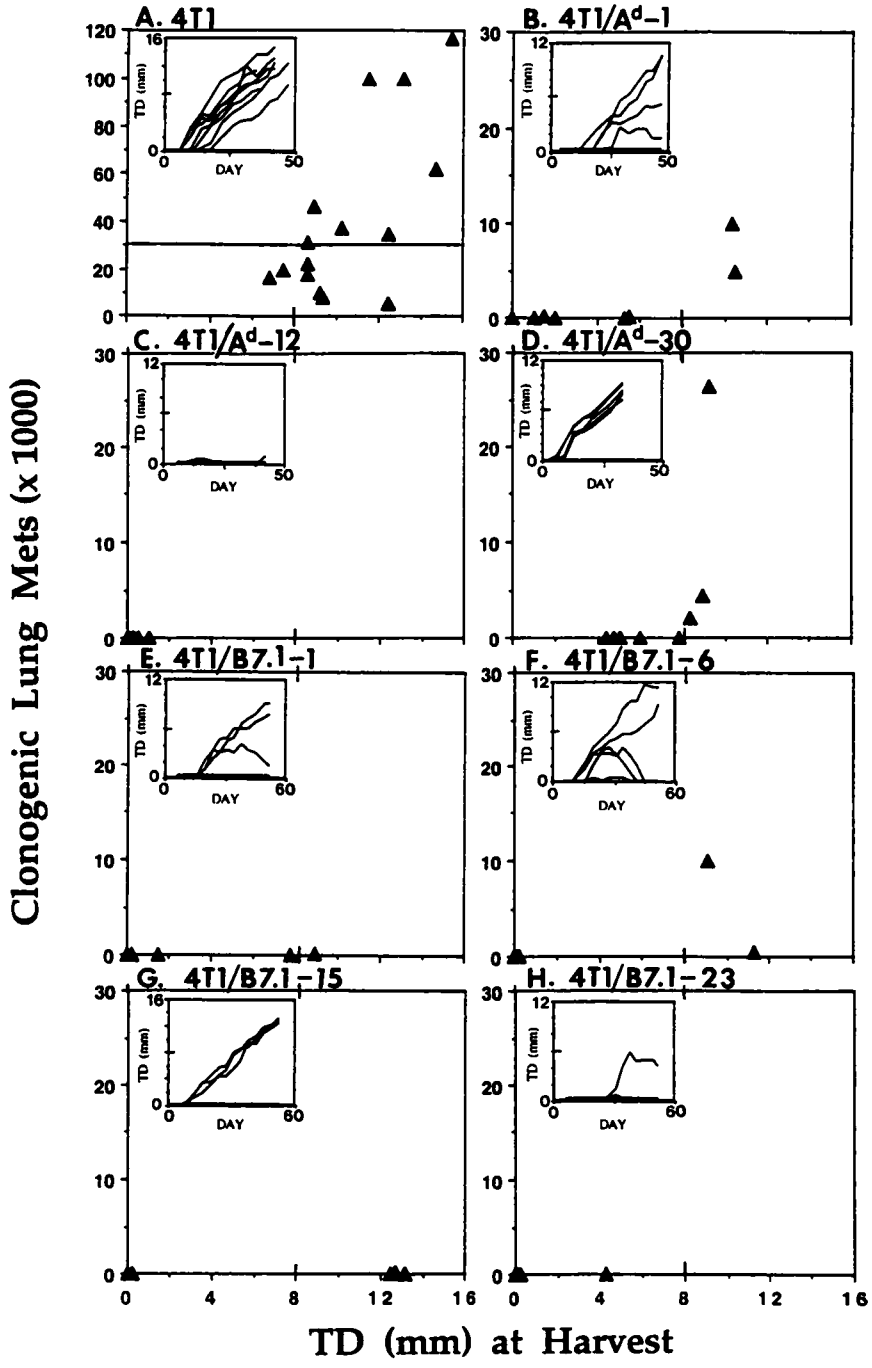

Fig. 4. Expression of either class II MHC or B7.1 reduces metastatic potential and tumorigenicity of the 4T1 transfectants. Female BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells (15 mice; *A*), 4T1/A^d-1 (9 mice; *B*), 4T1/A^d-12 (10 mice; *C*), 4T1/A^d-30 (8 mice; *D*), 4T1/B7.1-1 (5 mice; *E*), 4T1/B7.1-6 (5 mice; *F*), 4T1/B7.1-15 (5 mice; *G*), or 4T1/B7.1-23 (5 mice; *H*) and sacrificed 32–55 days later, and the number of metastatic cells in the lungs was determined as described in "Materials and Methods." Primary tumors were measured every 3–4 days. *A–H*, numbers of clonogenic lung metastases (× 1000) *versus* the TD at the time the mice were sacrificed. ▲, individual mice. *Insets*, mean TD (*Y* axis) *versus* days postinoculation (*X* axis). *Lines*, individual mice. Note that the number of clonogenic lung metastases shown on the *Y* axis ranges from 0 to 120 in *A*, as opposed to a range of 0–30 for *B–H*.

munotherapy in mice carrying spontaneously metastatic established tumors, provided treatment originates when the primary tumor is <4 mm in diameter.

DISCUSSION

Many studies during the past 5–10 years have focused on developing immunotherapy strategies for the treatment of solid tumors and have used animal systems to model human disease and to test the efficacy of immunotherapy. Most of these studies have used transplanted primary solid tumors (6, 7) or short-term established experimental (i.v. induced) metastatic cancers, in which therapy was performed very early during metastatic disease (13–16). A small number of studies focused on spontaneous metastases; however, these models used severe combined immunodeficient mice or anatomically incorrect tumor challenge sites (17–19). In many cases, the growth characteristics and kinetics of the model tumors used did not closely follow the natural history of their corresponding human tumor and, hence, were not optimal model systems. In contrast to many mouse tumors, the BALB/c-derived 4T1 mammary tumor, originally derived by Miller and colleagues (29, 30) and others (28), shares many characteristics with its human counterpart mammary carcinoma. For example, 4T1 spontaneously metastasizes while the primary tumor is in place, analogous to human mammary tumors. Sites of metastasis are common between the mouse and human malignancies: spreading first to the lungs and liver in 24–77% and 22–62% of women, respectively, *versus* >95% and >75%, respectively, of BALB/c mice (Table 2; Refs. 38–41). Metastasis to the central nervous system is characteristically less frequent than metastasis to other sites in both

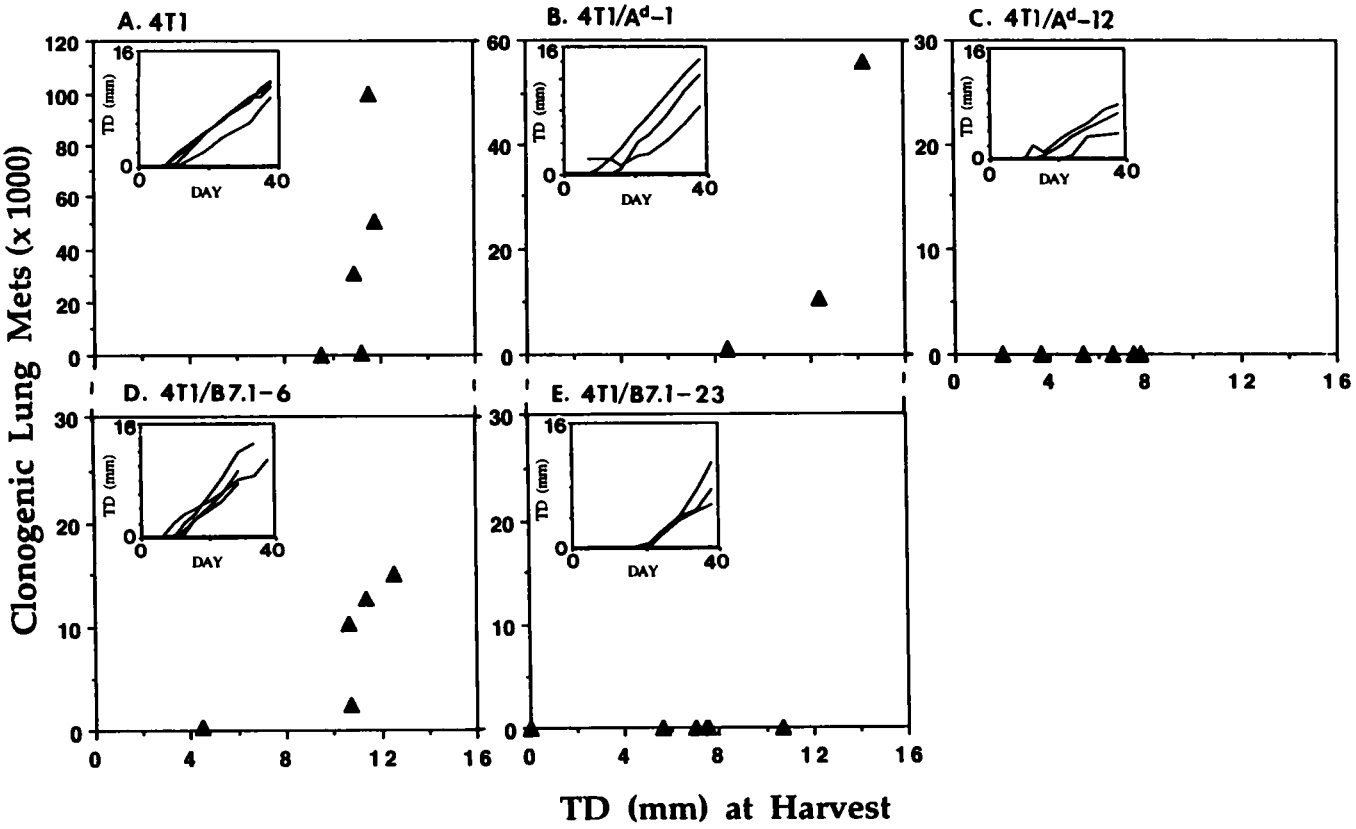

Fig. 5. Different immune effector cells alter primary tumor growth *versus* spontaneous metastasis formation. BALB/c *nu/nu* mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells (5 mice; *A*), 4T1/A^d-1 (3 mice; *B*), 4T1/A^d-12 (6 mice; *C*), 4T1/B7.1-6 (5 mice; *D*), or 4T1/B7.1-23 (6 mice; *E*), and tumor growth was measured every 3–4 days. Data are plotted as in Fig. 4. Note that the number of clonogenic lung metastases shown on the *Y* axis ranges from 0 to 120 in *A*, as opposed to ranges of 0–60 for *B* and 0–30 for *C–E*.

Table 3 *Tumor incidence of 4T1 transfectants in syngeneic BALB/c versus BALB/c nu/nu mice*

Mice were challenged s.c. in the abdominal mammary gland with 5×10^3 transfected 4T1 tumor cells. The tumor incidence is the number of animals that developed progressive tumors. As described in "Materials and Methods," animals that developed tumors were sacrificed when the TD reached 14–16 mm or when the mice became moribund.

Tumor challenge	Tumor incidence	
	BALB/c	BALB/c *nu/nu*
4T1/A^d-1	3/10	3/3
4T1/A^d-12	1/10	5/6
4T1/B7.1-6	2/5	7/8
4T1/B7.1-23	1/5	5/6

humans and mice (30% and 40%, respectively) and, statistically, occurs later in the disease process (Table 2; Refs. 41 and 42).

In addition to its growth characteristics, the 4T1 tumor has several experimental characteristics that make it an ideal model for testing immunotherapy strategies. A major asset is its stable resistance to 6-thioguanine, enabling the precise quantitation of very small numbers of tumor cells, long before they could be detected visually or accurately quantitated by other methods. Because metastasis to the lungs precedes and always accompanies metastasis to other organs (Table 2), quantitation of lung metastases accurately assesses metastatic disease. The similarity in growth between the 4T1 tumor and human mammary cancer plus the ease of assessing metastatic disease, therefore, make the mouse 4T1 tumor an excellent model for testing potential immunotherapy strategies.

Previous immunotherapy studies using MHC class II and/or B7.1-expressing tumor cells as cell-based vaccines have dealt predominantly with solid, primary tumors (7–9). Here, these vaccines are used for the treatment of metastatic disease. The current studies are also distinct from earlier studies using a variety of cell-based vaccines, including cytokine-transduced/transfected tumor cells, in that spontaneous, established metastases are being treated, rather than short-term experimental (i.v.) metastases. These disease conditions much more closely mimic those of human breast cancer patients, and hence, the

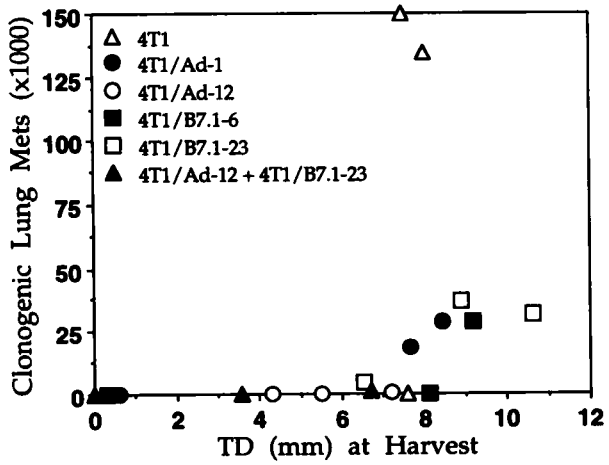

Fig. 6. Immunization with MHC class II$^+$ or B7.1$^+$ transfectants protects naive mice against metastatic disease from parental 4T1 tumor challenge. Syngeneic BALB/c mice (three mice/group) were vaccinated i.p. with 1×10^6 irradiated parental 4T1 cells (△), 4T1/A^d-1 (●), 4T1/A^d-12 (○), 4T1/B7.1-6 (■), 4T1/B7.1-23 (□), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-23 (▲). Four weeks later, mice were challenged s.c. in the abdominal mammary gland with 5×10^3 live parental 4T1 cells. Five weeks postparental tumor challenge, the TD and the number of clonogenic lung metastases were measured.

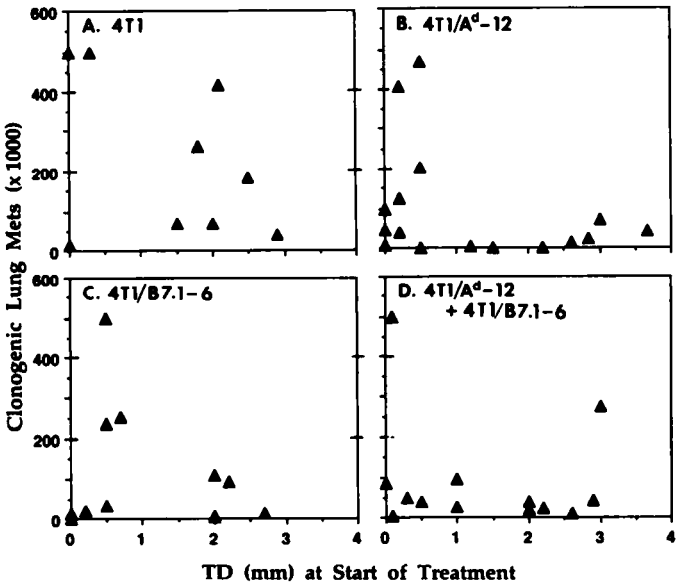

Fig. 7. Immunotherapy of established 4T1 tumors with MHC class II$^+$ and/or B7.1$^+$ transfectants reduces metastatic disease. Syngeneic BALB/c mice were challenged s.c. in the abdominal mammary gland with 5×10^3 live parental 4T1 cells. At day 9 or 14 postparental tumor challenge, the TD was measured, and the therapeutic injections began. Mice were treated i.p. twice a week until the time of sacrifice with 1×10^6 irradiated parental 4T1 (*A*), 4T1/A^d-12 (*B*), 4T1/B7.1-6 (*C*), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-6 (*D*) cells. Mice were sacrificed 6 weeks after initial 4T1 tumor challenge, and the number of clonogenic lung metastases was determined. The data are plotted as the TD at the time the therapeutic treatment began *versus* the number of clonogenic lung metastases (×1000) at the time of sacrifice. ▲, individual mice. Statistical analysis was performed using a Student's *t* test for unequal variances as described in the text (two-tailed $P = 0.008$).

observed results may be useful in projecting experimental animal results to human clinical situations.

Treatment of mice carrying 9–14-day established 4T1 tumors with MHC class II and/or B7.1-transfected tumor cells results in a dramatic reduction in the number of metastatic tumor cells relative to mice treated with wild-type 4T1 (Fig. 7), suggesting that such cell-based vaccines may be useful immunotherapeutic agents for the treatment of metastases. The finding that metastatic growth is greatly reduced or eliminated, whereas primary tumor growth is not significantly impacted, is surprising and suggests that immunotherapy may be more useful against metastatic disease than against primary tumor. Because many primary tumors can be successfully surgically resected whereas many metastatic lesions are refractile to current therapy, immunotherapy may have a unique role in cancer treatment.

Because mice with primary tumors with TDs of >2 mm contain LN and lung metastatic cells (Fig. 2), the immunotherapy is limiting proliferation of pre-established metastases. Likewise, because treatment of naive mice produces some animals with no metastases, the immunotherapy is also preventing establishment of new metastases. Therefore, although not routinely curative, this immunotherapy may slow progression of metastatic disease.

Previous therapy studies with B7.1 transfected tumors and primary or experimental metastases indicated that costimulatory molecule expression was effective in vaccines containing "moderately" immunogenic tumor cells but not in vaccines containing "poorly" immunogenic tumor cells (7). By definition, 4T1 cells are poorly immunogenic because immunization of tumor-free mice with irradiated wild-type cells does not provide protective immunity against subsequent challenge with wild-type tumor cells (Figs. 6 and 7). Because immunization with B7.1 transfected tumor cells does not result in reduced primary tumor growth in the immunotherapy protocol, our results agree with these earlier studies (7). However, the finding that B7.1-transfected tumor cells promote significantly reduced metastatic growth in the therapy protocol (Fig. 7) revives B7.1 as a potential candidate for immunotherapy.

The mechanism by which the class II$^+$ and B7.1$^+$ transfectants are providing their protection is not clear. Because these transfectants displayed varying *in vivo* phenotypes, different types of effector cells may be activated. In most cases, T cells were important in regulating primary tumor growth (Fig. 5); however, their role in outgrowth of metastases is less clear cut. This could easily be explained by an enhancement of nonspecific effectors, such as lymphokine-activated killer cells and/or NK cells, as it has been previously shown that B7.1 can induce NK activity against tumors (32, 43). Alternatively, limiting dilution cloning of the transfectants may have cloned out tumor cells that lost their ability to metastasize (44). Regardless of the *in vitro* and *in vivo* phenotypes of the transfectants (*i.e.*, level of expression of class II and/or B7.1, metastatic potential, and tumorigenicity in BALB/c *versus nu/nu* mice), most clones provide some protection against wild-type metastatic disease (Figs. 6 and 7). Thus, these studies suggest that most transfectants will be useful as vaccines and that cell-based vaccines may be more effective than previously thought.

Transfection of tumor cells with MHC class II plus B7.1 genes was originally designed to produce tumor cells that could directly present antigen to CD4$^+$ T_h cells and CD8$^+$ CTL and, thereby, facilitate optimal antitumor immunity (9, 45). Genetic experiments using bone marrow chimeras and sarcoma tumor cells support this hypothesized mechanism of CD4$^+$ T-cell activation and demonstrate that the genetically modified tumor cells function as the APC for tumor-encoded antigen (46, 47). In contrast, class I-restricted tumor-encoded antigen appear to be presented indirectly via host-derived APCs (48–50). Increased antitumor activity following immunization, therefore, is probably the result of enhanced presentation of tumor antigens and the subsequent activation of multiple helper and effector cell populations.

Why the effectiveness of this treatment is limited to mice with starting tumors with TDs of <4 mm is unclear. Factors such as immunosuppression of tumor-bearing individuals, immunogenicity of tumor antigens, the timing of the developing immune response *versus* outgrowth of the tumor, and involvement of nonspecific effector cell types (*i.e.*, lymphokine-activated killer cells, NK cells, and macrophages) have been discussed at length in the context of other immunotherapy approaches (51–53), and some or all of these factors may be implicated here. Optimal T-cell activation is achieved when B7.1 and MHC class II molecules are expressed by the same APC (9, 54). Our cell-based vaccine, therefore, might be more effective if double transfectants were used rather than the mixture of single transfectants tested in this study. Regardless of the limitations, however, the promising therapeutic responses are encouraging for further testing and development of this approach either alone or in combination with other immunotherapeutic and/or conventional modalities.

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