Cancer Immunol Immunother (2008) 57:1067–1077
DOI 10.1007/s00262-007-0438-5

ORIGINAL ARTICLE

In vivo responses to vaccination with Mage-b, GM-CSF and thioglycollate in a highly metastatic mouse breast tumor model, 4T1

Claudia Gravekamp · Belinda Leal · Ashley Denny · Rumana Bahar · Shellye Lampkin · Francisco Castro · Sun Hee Kim · Dan Moore · Robert Reddick

Received: 1 October 2007 / Accepted: 5 December 2007 / Published online: 20 December 2007
© Springer-Verlag 2007

Abstract Metastatic breast cancer is an important contributor to morbidity and mortality. Hence, new therapies are needed that target breast cancer metastases. Here, we focus on Mage-b as a possible vaccine target to prevent the development of breast cancer metastases, through activation of Mage-b-specific cytotoxic T lymphocytes (CTL). The syngeneic cell line 4T1, highly expressing Mage-b, was used as a pre-clinical metastatic mouse breast tumor model. BALB/c mice received three preventive intraperitoneal immunizations with Mage-b DNA vaccine mixed with plasmid DNA, secreting granulocyte–macrophage colony stimulating factor (GM-CSF). In addition, antigen-presenting cells were more efficiently recruited to the peritoneal cavity by the injection of thioglycollate broth (TGB), prior to each immunization. Immunization with Mage-b/GM-CSF/TGB significantly reduced the number of metastases by 67% compared to the saline/GM-CSF/TGB and by 69% compared to the vector control/GM-CSF/TGB. Also, tumor growth was significantly reduced by 45% in mice vaccinated with Mage-b/GM-CSF/TGB compared to the saline/GM-CSF/TGB and by 47% compared to the control vector/GM-CSF/TGB group. In vivo, the number of CD8 T cells significantly increased in the primary tumors and metastases of mice vaccinated with Mage-b/GM-CSF/TGB compared to the saline/GM-CSF/TGB and the control vector/GM-CSF/TGB group, while the number of CD4 T cells significantly decreased. The combination of Mage-b, GM-CSF and TGB did not only induce significantly higher levels of IFNγ in the lymph nodes of vaccinated compared to control mice, but also induced significantly higher expression levels of Fas-ligand (FasL) in the primary tumors (expressing Fas protein constitutively), compared to the control mice. Whether the interaction between Fas and FasL may have contributed to the smaller tumors needs to be further analyzed.

Keywords Metastases · Mage-b DNA vaccine · Breast tumor model · 4T1 · GM-CSF · Thioglycollate

C. Gravekamp (✉) · F. Castro · S. H. Kim · D. Moore
California Pacific Medical Center Research Institute,
475 Brannan Street, San Francisco, CA 94107, USA
e-mail: gravekc@cpmcri.org

B. Leal · A. Denny
Department of Cellular and Structural Biology,
University of Texas Health Science Center San Antonio,
15355 Lambda Drive, San Antonio, TX 78245, USA

R. Bahar
Buck Institute for Age Research,
8001 Redwood Blvd, Novato, CA 94945, USA

S. Lampkin · R. Reddick
Department of Pathology,
University of Texas Health Science Center San Antonio,
7703 Floyd Curl Drive, San Antonio, TX 78229, USA

Introduction

Breast cancer is the most common cancer among women around the world [1], and 40% of the women diagnosed with breast cancer will progress to metastatic disease [2]. Current treatment options for localized breast cancer include surgical resection of the primary tumor, assessment of the regional lymph nodes, and removal if found positive, followed by adjuvant chemotherapy or hormonal therapy [3]. Although the first-line endocrine therapy with tamoxifen or the newer third generation aromatases is promising [4], the cure rate of metastatic breast cancer is low [5]. Despite aggressive treatment, for most patients the elimination of metastases or residual tumor cells after surgery is

incomplete, due to chemoresistance [6]. Thus, metastases and not the primary tumor is the most important contributor to breast cancer morbidity and mortality. Treatments that specifically reduce or eliminate distant metastases or residual tumor cells should therefore be the focus of our efforts, and will offer the greatest promise in improving the outcome for patients with metastatic breast cancer. Enhancement of specific helper and cytotoxic T lymphocyte (CTL) responses to breast tumors through vaccination with tumor-associated antigens (TAA) could potentially lead to the specific elimination of micro-metastases and/or residual tumor cells.

So far, many TAA have been identified in human tumors of various histological origins. The MAGE antigens are particularly interesting for the development of breast cancer vaccines, because their expression (MAGE-A and/or MAGE-B) has been frequently detected in human breast tumor biopsies (92%) [7], but not in normal tissues.

Human clinical trials show the potential of MAGE vaccination against metastases but also the need to further optimize the efficacy of MAGE-based vaccines in order to improve clinical outcome [8–12]. Such optimizations are ideally done in the mouse. Several preclinical vaccines studies in mice with human and mouse Mage-a have been reported, showing Mage-a-specific immune responses and protective immunity against Mage-a-expressing tumors [13–17]. We found evidence that DNA vaccination with mouse Mage-b in a metastatic mouse breast tumor model 4TO7cg, expressing Mage-b, eliminated almost all metastases, albeit not completely [18].

In the study presented here we used a much more aggressive, highly metastatic breast cancer model, 4T1, expressing Mage-b [19], in an attempt to further optimize the mouse Mage-b vaccine for its eventual use in humans based on its homologous MAGE-B equivalent. In this more aggressive model, the Mage-b vaccine alone is only weakly protective (unpublished). Therefore we tested it in combination with plasmid DNA secreting granulocyte–macrophage colony-stimulating factor (GM-CSF). GM-CSF has been identified as an adjuvant for vaccines and promotes the differentiation of DC and activates macrophages [20, 21]. To increase the frequency of antigen-presenting cells (APC), required for DNA uptake, we recruited macrophages to the peritoneal cavity by the injection of thioglycollate broth (TGB) [22], prior to each intraperitoneal (i.p.) vaccination. To determine the efficacy of the vaccine, we measured the direct effect of Mage-b DNA vaccination on the development of metastases, and on the growth of primary tumors. Vaccine-induced immune responses were analyzed in the draining lymph nodes (LNs), primary tumors and metastases. The results of this study indicate a robust effect of Mage-b/GM-CSF/TGB on the metastases and primary tumors.

Materials and methods

Mice

Normal female Balb/c mice (3-month-old) were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the animal husbandry facility of the University of Texas Health Science Center, San Antonio (UTHSCSA), according to the Association and Accreditation of Laboratory Animal Care (AACAC) guidelines.

Plasmids

Mouse Mage-b DNA plasmid (pcDNA3.1-Mage-b/V5) and the control vector (pcDNA3.1/V5) were developed in a previous study [18]. Mouse GM-CSF plasmid (CMV1-GM-CSF) was kindly provided by Dr. Stephen Johnston (The Center for Innovations in Medicine, The Biodesign Institute at Arizona State University) [23].

Cells and cell culture

The 4T1 cell line was derived from a spontaneous mammary carcinoma in a BALB/c mouse [24]. Various 4T1 sub lines have been generated with different patterns of metastases [25]. The 4T1 cell line used in this study is highly aggressive, metastasizing to the mesenteric lymph nodes (MLN), and diaphragm and less frequently to the surface of the liver, kidney and spleen [19]. The 64pT cell line is a spontaneous fusion between mammary cell lines 4TO7 and 68H and is nonmetastatic [26]. Both 4T1 and 64pT were kindly provided by Dr. Fred Miller of the Karmanos Cancer Institute, Detroit, MI. All mammary cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM mixed nonessential amino acids, 2 mM L-glutamine, insulin (0.5 HSP units/ml) penicillin (100 units/ml) and streptomycin (100 μg/ml).

Breast tumors and metastases

Breast tumors and metastases were generated in BALB/c mice by the injection of 10^5 cells of the mouse mammary tumor cell line 4T1, into a mammary fat pad as described previously [19]. Primary tumors were detected by palpation within 1–2 weeks in live mice. To determine the tumor size in situ, the perpendicular largest diameters of the tumor were measured with a caliper. Twenty-two days after injection of the tumor cell line, the mice were euthanized, weighted, and necropsied to evaluate the presence and frequency of metastases and to determine the weight and size of primary tumors. Primary tumors extended to the chest cavity lining, and subsequently metastasized to the mesenteric

lymph nodes (MLN) and diaphragm, and less frequently to distant organs at the surface of the liver, spleen, and kidneys. Metastases were visible to the naked eye as nodules. The total number of metastases per mouse (MLN, diaphragm, liver, kidney, and spleen) was determined. Normal and tumor tissues were collected aseptically and kept at −80°C, or fixed in 10% Zinc solution for 48 h and kept in 70% ethanol, until use. The primary tumors and metastases were confirmed by histology in the laboratory of Dr. Robert Reddick. In some cases the metastases were too small for histological analysis, and only RT-PCR for the detection of Mage-b expression was performed. Mage-b is highly expressed in 4T1 metastases and primary tumors, but not in normal cells [19].

Immunization and tumor challenge

Mice were immunized as described previously [19]. Briefly, Balb/C mice were injected with 1 ml of 2.9% (w/v) TGB (Brewer thioglycollate broth B2551, Sigma-Aldrich, St Louis, MO) into the peritoneal cavity in order to recruit professional antigen-presenting cells, i.e., macrophages, to the peritoneal cavity [22]. After 4 days, the mice were immunized intraperitoneally with 100 μg of the Mage-b (pcDNA3.1-Mage-b), or with 100 μg of the control vector (pcDNA3.1), or saline. In order to improve the processing and presentation of the vaccine antigen, the vaccine and control vector were mixed with 100 μg of a plasmid-secreting GM-CSF (kindly provided by Dr. Johnston, Director of Center for Innovations in Medicine Biodesign Institute, Arizona State University). GM-CSF plasmid DNA (100 μg) was also added to the saline group. After 3 and 6 weeks, an identical second immunization was given. After 2 weeks from the second immunization, primary tumors and metastases were induced as described above. Twenty-two days after tumor challenge, the mice were euthanized and analyzed for tumor size, frequency and location of metastases.

RT-PCR and Southern blotting

RNA was isolated using Trizol according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Conversion of 1 μg of mRNA into cDNA was performed with Superscript Preamplification system (Life Technologies). Subsequently, 10 μl of the cDNA was amplified by hot start PCR (Platinum PCR SuperMix, Life Technologies) (40 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 2 min) in a thermocycler from Perkin–Elmer (Norwalk, CT). The primers for Mage-b, Fas, FasL, TGFβ, and β-actin used in this study are listed in Table 1. Mage-b RT-PCR products were hybridized with a chemiluminescence-labelled 993-bp Mage probe (AY196960) according to the manufacturer's instructions (enhanced chemiluminescence; Amersham). For comparison of expression levels between groups, intensity of RT-PCR bands in an ethidium bromide-stained agarose gel was measured with a densitometer (Fluorchem 8900 from Alpha InnoTech, San Leandro, CA). For each individual gene (Fas, FasL, TGFβ, or β-actin), all samples of vaccinated and control mice were subjected to RT-PCR in the same experiment, and analyzed in the same gel. Lymph nodes of tumor-bearing mice were used as an internal positive control in each experiment.

Analysis of active caspase-3 and caspase-8 in primary tumors

From each tissue, 3 mm^3 was homogenized in an Eppendorf tube with glass beads and 1 ml of PBS, containing protease inhibitors, by a Wig-L-Bug (Dentsply C020200, Elgin, IL) for 2–5 × 30 s. The protein concentration from each sample was determined by bicinchoninic acid (BCA) [27]. Active caspase-3 was analyzed by western blotting following the manufacturer's instructions (Zymed, Invitrogen, Carlsbad, CA), using anti-active caspase-3 antibodies (Cell Signaling, Danvers MA), and intensity of protein bands were measured with a densitometer, averaged per

Table 1 Primers used in this study

Gene	Primer sequence 5′–3′	Size of RT-PCR product (bp)	Reference
Mage-b	F: GAG CTT GAT CCA CGA GTT C R: AGG AGA CCT GTC CTA GGC	632	U19032
FasL	F: CGG TGG TAT TTT TCA TGG TTC TGG R: CTT GTG GTT TAG GGG CTG GTT GTT	380	DQ846747
Fas	F: GCT GCA GAC ATG CTG TGG ATC R: TCA CAG CCA GGA GAA TCG CAG	408	NM_007987
TGFβ	F: AGA CGG AAT ACA GGG CTT TCG ATT CA R: CTT GGG CTT GCG ACC CAC GTA GTA	489	M13177
β-actin	F: TCA TGA AGT GTG ACG TTG ACA TCC GT R: CCT AGA AGC ATT TGC GGT GCA CGA TG	285	Life Technologies

group, and subjected to statistical analysis. Active caspase-8 was measured by an enzymatic reaction using acetyl (Ac)-Ile-Glu-Thr-Asp coupled to compound 7-amino-4-trifluoromethyl coumarin (AFC), which is a substrate for active caspase-8, according manufacturer's instructions (MP Biomedicals, Solon, OH).

In vivo analysis of primary tumors and metastases by immunohistochemistry

Tumors and metastases were dissected from the mice in two independent experiments. Briefly, Zinc-fixed (10% v/v) tissues were embedded in paraffin and 5-μm thick sections were cut from each block. The sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope. All tumors were confirmed by histological analysis. Metastases were confirmed either by histology or by RT-PCR, due to the small amount of tissues available for examination. For immunohistochemistry, the paraffin-embedded sections were stained with primary antibody to CD4, CD8, CD25 (IL-2 receptor), and subsequently incubated with a biotin-labeled secondary antibody, followed by incubation with streptavidin conjugated with horseradish peroxidase (HRP), and finally incubated with diamino benzidine (DAB) substrate. All antibodies were purchased from Pharmingen.

In vitro analysis of cells from LNs after re-stimulation

Cells from inguinal draining lymph nodes (LNs) were isolated from vaccinated and control mice according to standard protocols [28]. Within each group (10 mice per group), the cells of the draining LNs were pooled, and re-stimulated with syngeneic 64pT tumor cells, expressing highly Mage-b, as described previously [18]. Briefly, cells from draining LN of vaccinated and control mice were re-stimulated with γ-irradiated (40 Gy) 64pT tumor cells (0.5×10^6 LN cells/ml and 2×10^5 or 5×10^4 64pT tumor cells/ml). All re-stimulation assays were performed in DMEM, and 10% FBS. Levels of IL-2 and IFNγ were determined by quantitative ELISA according standard protocols (Pharmingen, San Diego, CA).

Analysis of IL-6 and IL-10 in primary tumors and metastases by ELISA

From each tissue, 3 mm^3 was homogenized in an Eppendorf tube with glass beads and 1 ml of PBS containing 0.1% Triton-X100, 2 mM EDTA, and protease inhibitors by a Wig-L-Bug for 2–5 $\times$ 30 s, and serial dilutions were made of the supernatant after homogenization (undiluted, 10$\times$, 100$\times$). The ELISA was performed as described above. The lymphokine levels were normalized for protein concentration. Three tumors and six metastases were analyzed from each group (vaccinated or control mice), as well as the 4T1 cell line.

Results

Preventive effect of vaccination with Mage-b/GM-CSF/TGB

To determine the effect of Mage-b/GM-CSF/TGB, the total number of metastases in each mouse was measured, then averaged per mouse, and compared with the average number of metastases per mouse in the control groups. Immunization with Mage-b/GM-CSF/TGB significantly reduced the number of metastases by 67% compared to the saline/GM-CSF and by 69% compared to the vector control/GM-CSF/TGB. The average number of metastases per mouse as determined for each group was 28 (Mage-b/GM-CSF/TGB), 83 (saline/GM-CSF/TGB), and 93 (control vector/GM-CSF/TGB) (Fig. 1a). Also, tumor growth was significantly reduced by 45% in mice vaccinated with Mage-b/GM-CSF/TGB compared to the saline/GM-CSF/TGB and by 47% compared to the control vector/GM-CSF/TGB group. The average tumor size (mm^2) per mouse as determined for each group was 83 (Mage-b/GM-CSF/TGB), 152 (saline/GM-SCF/TGB), and 158 (control vector/GM-CSF/TGB) (Fig. 1b). These results indicate a robust protective effect of the combination of Mage-b, GM-CSF and TGB. To exclude that the protective effect was delivered just by GM-CSF and/or TGB, we have tested all combinations, Mage-b + GM-CSF/no TGB, Mage-b + TGB/no GM-CSF, and TGB alone in the 4T1 model. None of them showed a significant effect on the metastases or primary tumors (Fig. 2a, b).

Expression of Fas, FasL, and active caspase-3 and -8 in 4T1 primary tumors

In previous vaccine studies with Mage-b, but without GM-CSF and TGB, no effect was observed on the primary tumors. In the study presented here, a significant reduction in growth of primary tumors was observed when vaccinated with Mage-b and GM-CSF and TGB. To determine whether apoptosis may have played a role here, we analyzed the primary tumors of all TGB combination for the expression of Fas and FasL, known to be involved in apoptosis of breast tumor cells [29]. The expression of FasL was significantly higher in those combinations that contained TGB compared to the tumors of mice that received no treatment, while Fas expression was constitutively high in all primary tumors (Fig. 2c, d). The 4T1 cell line did not express FasL, but Fas was highly expressed (Fig. 2c, d).

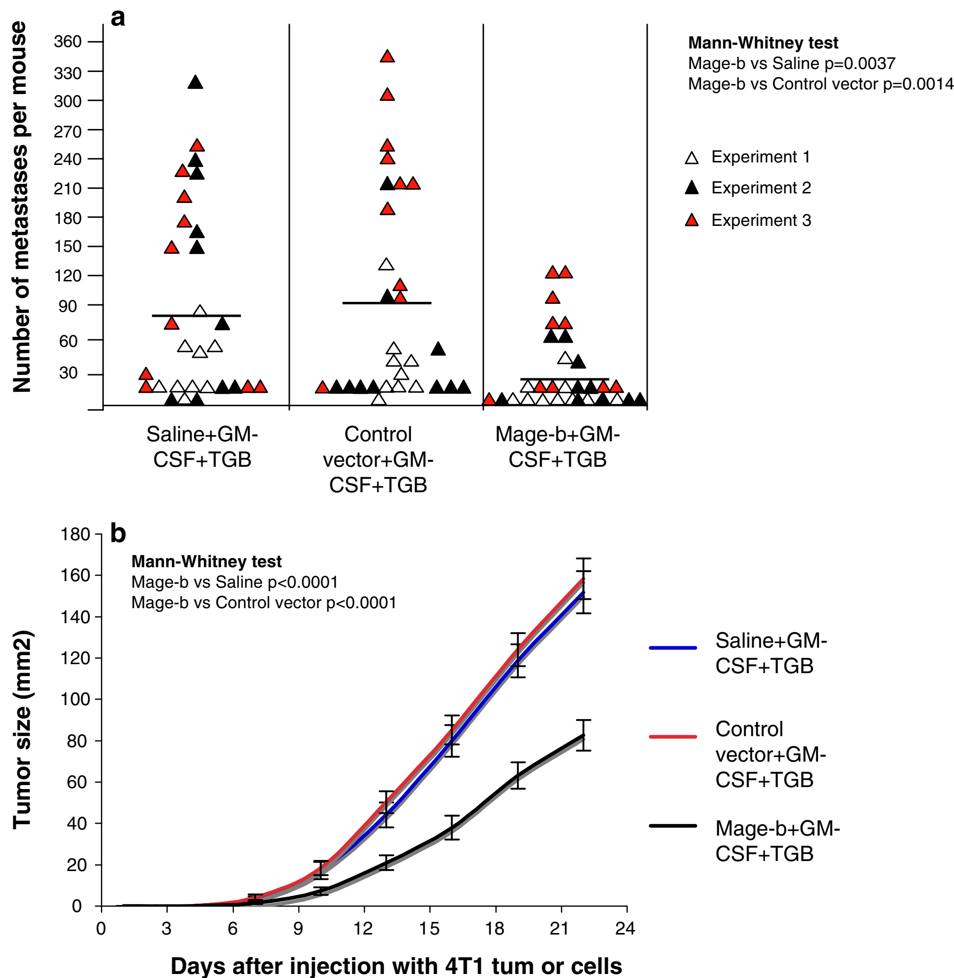

Fig. 1 Effect of Mage-b + GM-CSF + TGB on 4T1 metastases (**a**), and primary tumors (**b**). Mice were immunized preventively three times i.p. with saline/pCMV-GM-CSF DNA, pcDNA3.1/GM-CSF DNA or pcDNA3.1-Mage-b/GM-CSF DNA. TGB was injected 4 days prior to each immunization. 4T1 tumor cells were injected into a mammary fat pad, 2 weeks after the second immunization. Twenty-two days after injection of 4T1 tumor cells, the mice were euthanized and analyzed for the frequency of metastases (MLN, diaphragm, liver, kidneys, spleen). The results from three vaccine studies are presented in this figure. In each vaccine study, ten mice per group were used. In **a**, each *triangle* represents the number of metastases per individual mouse. The number of metastases per mouse of the three vaccine studies was averaged in each group, and subjected to statistical analysis using the Mann–Whitney test (2-tailed). The *vertical bar* in each group represents the average number of metastases per mouse. In **b**, tumor sizes were measured every 3 days, and averaged within each group. Statistical difference between tumor sizes at day 22 only (after dissection) was calculated using the Mann–Whitney test (2-tailed). Tumor growth was also compared across the six measurement days using Hotelling's T statistic, a multivariate method that takes into account correlation of measurements on the same tumor over time ($P = 0.0003$ for Mage-b/GM-CSF/TGB vs. saline /GM/CSF/TGB and $P < 0.0001$ for Mage-b/GM-CSF/TGB vs. control vector/GM-CSF/TGB). *Error bars* represent the standard error of the mean

We then analyzed the primary tumors of the vaccinated and control mice for the expression levels of Fas and FasL. Significantly higher levels of FasL was observed in the primary tumors of mice vaccinated with Mage-b/GM-CSF/TGB compared to the control groups, and this correlated with smaller tumor weight (Fig. 3a, b). The expression of Fas was high in the primary tumors of both vaccinated and control mice (Fig. 3c). We also tested the levels of Fas and FasL expression in metastases. The effect of Mage-b/GM-CSF/TGB on FasL expression in the metastases (not shown) was less robust than in the primary tumors, and therefore was not further analyzed.

The interaction between Fas and FasL may activate the death receptor pathway, via activation of caspase-8 and subsequent caspase-3. Therefore, we analyzed the tumor samples for the presence of active caspase-8 and -3. Active caspase-3 was strongly expressed in the 4T1 primary tumors (Fig. 3d) but not in the untreated 4T1 cell line (data not shown). However, no significant difference in the levels of active caspase-3 was observed between vaccinated and control mice. The expression levels of active caspase-8 in the 4T1 primary tumors did not exceed the expression levels of active caspase-8 in the 4T1 cell line (data not shown).

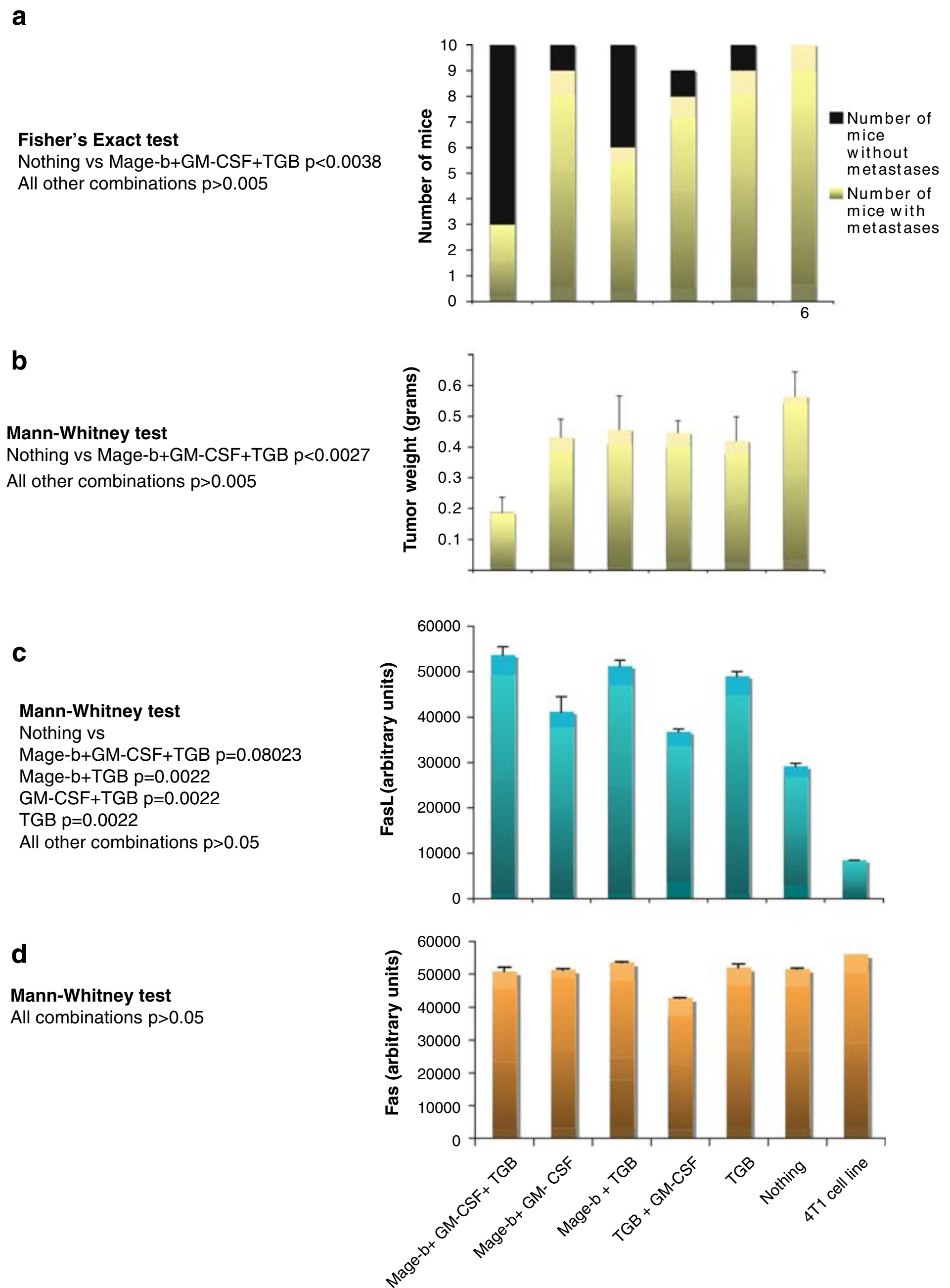

Fig. 2 The effect of Mage-b with TGB and/or GM-CSF on 4T1 primary tumors and metastases, and on FasL/Fas expression. Mice were immunized, challenged with 4T1 tumor cells and euthanized as described in Fig. 1. The number of mice with and without metastases (**a**) as well as the tumor weight of each mouse (**b**) was determined, and subjected to statistical analysis. For these in vivo studies, ten mice were used in each group. The primary tumors were then analyzed for the expression of FasL (**c**) and Fas (**d**) by RT-PCR. From each group four tumors and four metastases of different mice were analyzed and measured three times. The results were averaged per group and subjected to statistical analysis. The *error bars* represent standard error of the mean

Immune responses in vivo, at the site of 4T1 primary tumor and metastases

In order to evaluate vaccine-induced immune responses in vivo, we analyzed sections of primary tumors and metastases for the presence of CD8 T cells, for CD4 T cells, and for CD25 cells. CD25 is expressed by activated CD4, CD8 T cells, or NK cells, and by activated macrophages or B cells. A significant higher number of CD8 T cells were found in the tumors and metastases of Mage-b/GM-CSF/TGB-vaccinated compared to the saline/GM-CSF/TGB and/or vector control group (Fig. 4a, b). The average number of CD8 T cells/field in the primary tumors was ten (Mage-b/GM-CSF/TGB), four (saline/GM-CSF/TGB), and four (control vector/GM-CSF/TGB), and in the metastases nine (Mage-b/GM-CSF/TGB), four (saline/GM-CSF/TGB), and two (control vector/GM-CSF/TGB). In contrast to CD8 T cells, a significantly lower number of CD4 T cells were found in the tumors and metastases of Mage-b/GM-CSF/TGB-vaccinated compared to the saline/GM-CSF/TGB and/or vector control group (Fig. 4c, d). The average number of CD4 T cells in the primary tumors was 3 (Mage-b/GM-CSF/TGB), 19 (saline/GM-CSF/TGB), and 9 (control vector/GM-CSF/TGB), and in the metastases 1 (Mage-b/GM-

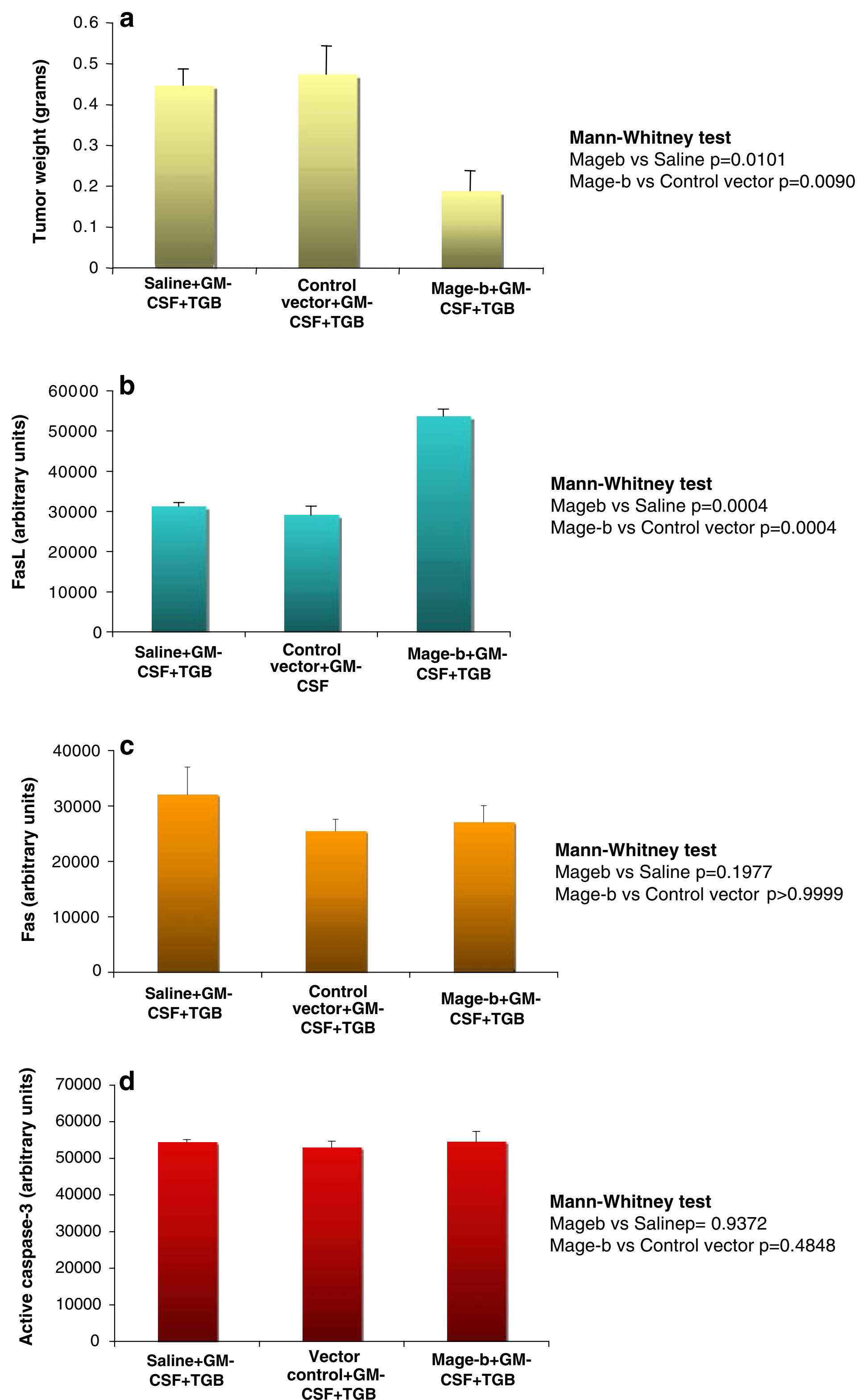

Fig. 3 Expression of Fas/FasL/active caspase-3 in relation to growth of 4T1 primary tumors. Mice were immunized, challenged with 4T1 tumor cells and euthanized as described in Fig. 1. 4T1 primary tumors were analyzed for weight (**a**), expression of FasL (**b**), Fas (**c**), and active caspase-3 (**c**). Expression of Fas and FasL was determined by RT-PCR. Active caspase-3 was determined by western blotting, and expression levels were measured by densitometer. From each group 4–6 tumors of different mice were analyzed and measured three times. The results were averaged per group and subjected to statistical analysis. The *error bars* represent standard error of the mean

CSF/TGB), 8 (saline/GM-CSF/TGB), and 9 (control vector/GM-CSF/TGB). The number of CD25-positive cells in the tumors and metastases did not significantly differ between vaccinated and control groups, in the primary tumors and metastases (Fig. 4e, f). Finally, we analyzed the samples for activated macrophages using Mac3 antibodies. All tumors and metastases from vaccinated and control mice yielded a dramatically high number of macrophages,

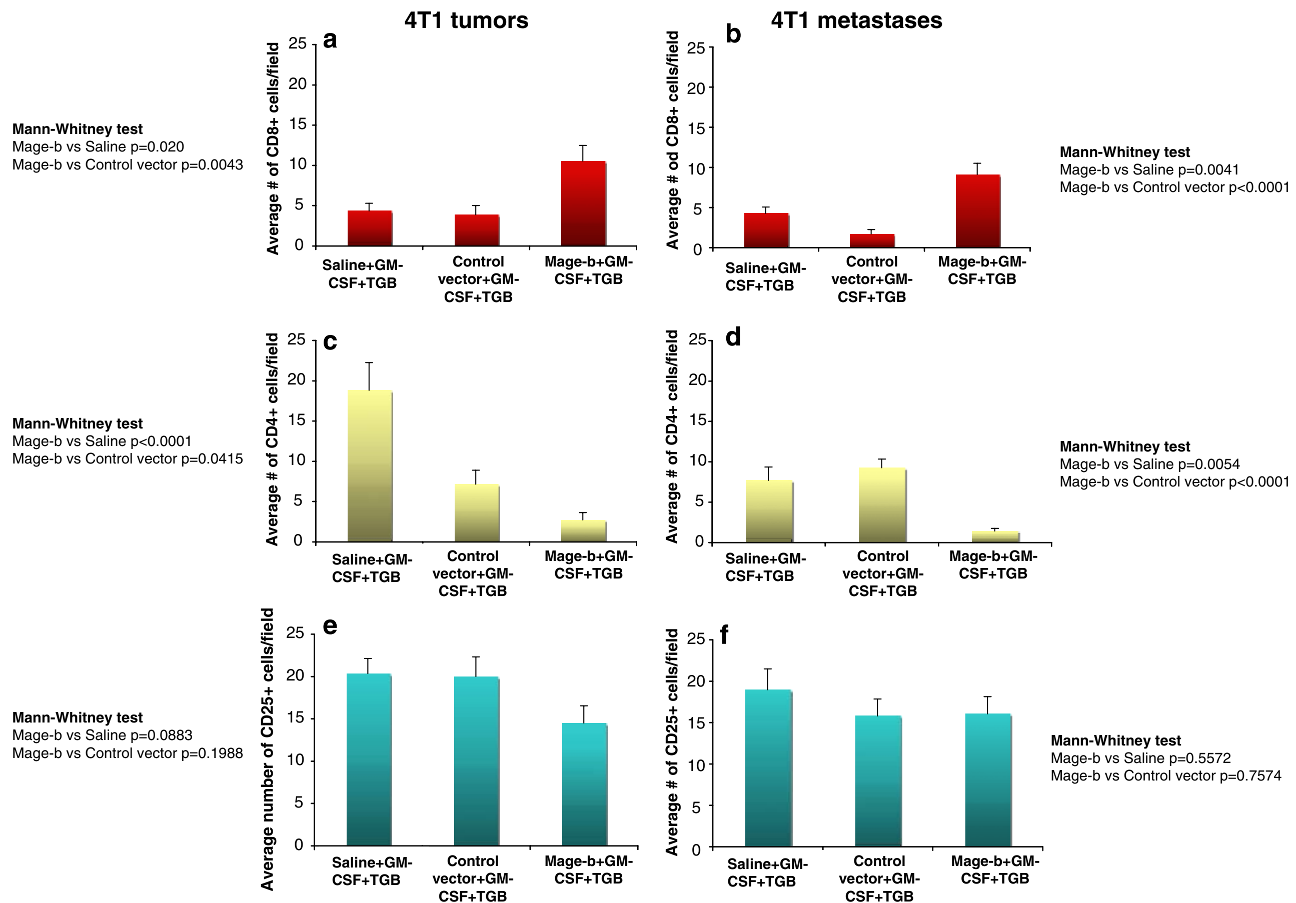

Fig. 4 Immunohistochemical analysis of tissue sections from 4T1 tumors and metastases of vaccinated and control mice. Mice were immunized, challenged with 4T1 tumor cells and euthanized as described in Fig. 1. The results from two vaccine studies were averaged and presented in this figure. Sections of 4T1 tumor and metastases were stained with anti-CD8 (**a**, **b**), -CD4 (**c**, **d**), and -CD25 (**e**, **f**) antibodies. From each group four tumors and four metastases were blinded-analyzed. From each tumor or metastasis, two sections were cut and the total number of positive cells in ten fields were averaged per group and subjected to statistical analysis, using the Mann–Whitney test (2-tailed). The *error bars* represent standard error of the mean

i.e., more than 60% of the tumors and metastases consisted of macrophages (data not shown).

In vitro analysis of cells from LNs after re-stimulation

The in vivo analysis suggests that Mage-b vaccination recruited Mage-b specific T cells at the site of the primary tumors and metastases. However, we have not proven yet whether the CD8 and CD4 T cells in the primary tumors and metastases were functionally active. Therefore, we analyzed cells in the draining LNs of Mage-b-vaccinated and control mice, bearing 4T1 tumors, for the production of IL-2 and IFNγ by quantitative ELISA. LNs were re-stimulated with 64pT, a syngeneic mouse breast tumor cell line, highly expressing Mage-b. We found a significant increase in the level of IFNγ in the LN of Mage-b/GM-CSF/TGB-vaccinated mice compared to the control mice (Fig. 5a), but the responses were not very strong. Moreover, IL-2 levels in the draining LN of these mice with tumors were very weak (Fig. 5b).

Production of IL-6, IL-10, and TGFβ in 4T1 primary tumors and metastases

An important problem in cancer vaccination is the negative effect that primary tumors may have on vaccine-induced immune responses. To evaluate this possibility in our model, we analyzed the primary tumors and metastases by ELISA or RT-PCR for the production of lymphokines that potentially could inhibit vaccine-induced immune responses lymphokines such as IL-6, TGFβ, and IL-10. It appeared that all primary tumors and metastases produced high levels of IL-6 (Fig. 6a), and expressed high levels of TGFβ (Fig. 6b), while IL-10 was not produced (data not shown). IL-6 and TGFβ were also expressed in the 4T1 cell line. No significant differences in the expression levels of IL-6 or TGFβ between tumor and metastases were observed, and vaccination with Mage-b/GM-CSF/TGB did not modulate the expression levels of IL-6 and TGFβ. Each sample that has been analyzed contained the same amount of protein or mRNA.

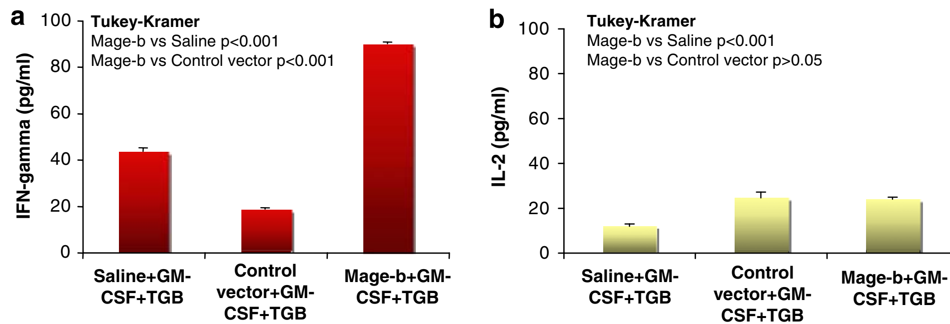

Fig. 5 Generation of Mage-b-specific immune responses in spleens and LNs of BALB/c mice with 4T1 tumors and metastases. Mice were immunized, challenged with 4T1 tumor cells and euthanized as described in Fig. 1. Two weeks after the last immunization mice were killed and draining (inguinal) LN of mice with tumors were isolated, pooled, and subsequently restimulated with syngeneic breast 64pT tumor cells, highly expressing Mage-b. Levels of IFNγ (**a**) and IL-2 (**b**) were measured by quantitative ELISA. Experiments were performed in triplicates, averaged and subjected to statistical analysis, using ANOVA. *Error bars* represent standard deviations. The results from one vaccine study are presented in this figure. In each group 5–7 mice were used

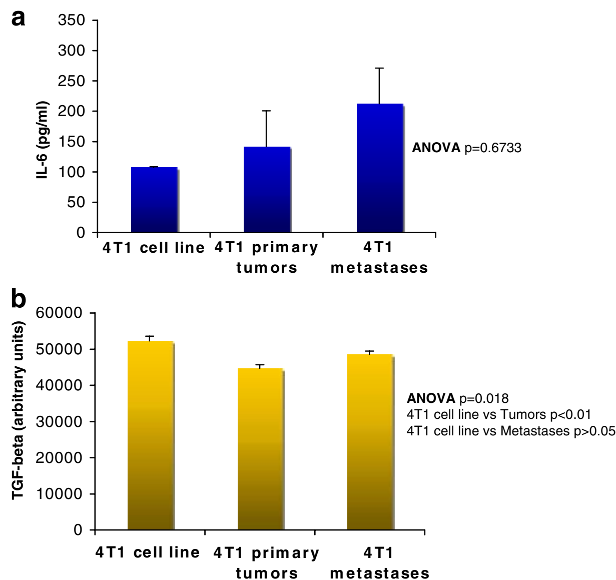

Fig. 6 High levels of the expression of TGFβ and IL-6 in 4T1 primary tumors and metastases. 4T1 tumors and metastases were analyzed for the expression of TGFβ by RT-PCR (**a**), and for the production of IL-6 by ELISA (**b**). Three tumors and six metastases were analyzed as well as the 4T1 cell line. This experiment was performed in triplicate, averaged and analyzed for statistical difference by ANOVA. *Error bars* represent the standard error of the mean. The tumors and metastases analyzed in this experiment were from different mice

Discussion

Results from clinical trials underscore the need for therapies that are effective against metastatic breast cancer. In a previous study [18], we demonstrated that Mage-b vaccination had a preventive effect on the development of metastases in a moderately metastatic breast tumor model 4TO7cg (2–20 metastases per mouse). This is in keeping with the positive effects of human clinical trials with MAGE-based vaccines. However, to greatly increase the effectiveness of such vaccines more extensive preclinical studies in the mouse are opportune. In the study presented here, we used a much more aggressive metastatic breast tumor model 4T1 (5–300 metastases per mouse). In this aggressive 4T1 model the same Mage-b vaccine used previously, was only weakly protective (unpublished results). However, when Mage-b was combined with plasmid DNA secreting GM-CSF, and when APC were recruited to the pc with TGB prior to each vaccination, a robust protective effect was observed on the 4T1 metastases. To exclude that the protective effect was delivered just by GM-CSF and/or TGB, we have tested all combinations Mage-b + GM-CSF/no TGB, Mage-b + TGB/no GM-CSF, as well as TGB alone in the 4T1 model. None of them had a significant effect on the metastases. Interestingly, the combination of Mage-b and GM-CSF and TGB not only protected against metastases, but also inhibited the growth of 4T1 primary tumors. This is in contrast to previous studies with the metastatic 4TO7cg and nonmetastatic 64pT models, in which Mage-b alone had a significant effect on the metastases but not on the primary tumors [18]. These results raised several questions. The first question was whether the smaller tumors were the result of immune responses induced by vaccination with Mage-b/GM-CSF/TGB, and the second question was, whether other mechanism(s) contributed to the smaller tumors as well.

To answer the first question, we analyzed the primary tumors for the presence of CD8, CD4 and CD25 cells. We found a significant higher number of CD8 T cells and simultaneously a significant lower number of CD4 T cells in tumors and metastases of mice vaccinated with Mage-b/GM-CSF/TGB compared to both control groups. To identify whether regulatory T cells are responsible for this decrease in CD4 T cells more detailed analyses with antibodies against CD4, CD25, and Foxp3 will be needed. The significant increase in CD8 T cells in the group vaccinated

with Mage-b/GM-CSF/TGB are promising and suggest that Mage-b vaccination may have recruited antigen-specific CD8 T cells at the site of the primary tumors. However, we have not proven that they are functionally active. In vitro analysis of the draining LN showed significantly higher levels of IFNγ in the group of vaccinated compared to the control mice. However, the responses were not very strong. Moreover, IL-2 production was almost undetectable. These results suggest that immune responses may have been down regulated at the site of the 4T1 primary tumors. In addition, we cannot exclude that NK cells and macrophages may have played a role here as well.

Many primary tumors produce lymphokines that reduce vaccine-induced T cell responses [30]. We found high levels of TGFβ and IL-6, in the primary tumors and metastases of the 4T1 model. TGFβ and IL-6 are both known for their ability to prevent maturation of DC [31–33] that subsequently leads to inhibition of T cell function. We found evidence that IL-6-neutralizing antibodies could restore IFNγ levels in LNs of 4T1 tumor-bearing mice, while purified IL-6 completely abrogated IFNγ levels in the spleen of 4T1 tumor-bearing vaccinated mice, after re-stimulation with bone marrow cells expressing Mage-b (manuscript in preparation). A significant difference in expression levels of IL-6 or TGFβ was not observed between tumors and metastases in vitro. However, in vivo the primary tumors are 100–100,000 times larger than the metastases. Therefore, IL-6 and TGF-beta levels are expected to be 100–100,000 times higher in the environment of the tumor compared to the environment of the metastases, and consequently the inhibition of T cell functions are expected to be much stronger at the site of the primary tumors than at the site of the metastases.

To answer the second question, i.e., whether other mechanism were activated by the combination of Mage-b, GM-CSF and TGB and contributed to smaller tumors, we analyzed the primary tumors for the expression of various genes that could possibly be involved in apoptosis of the breast tumor cells. Evidence for the interaction of Fas with FasL in breast tumors activating the death receptor apoptotic pathway [29], exists. Fas expression was constitutively high in all primary tumors and in the 4T1 cell line. However, FasL was significantly higher in those combinations that contained TGB, compared to tumors of mice that received no treatment. This was quite surprising since TGB was originally used for its improvement of DNA uptake in macrophages. FasL was not expressed in the 4T1 tumor cell line, suggesting that immune cells infiltrated in the primary tumors expressed FasL. Correlation between high expression level of FasL and smaller tumors was only observed in the group of Mage-b/GM-CSF/TGB, but not in the other groups that contained TGB. In addition, significantly higher levels of FasL were observed in the primary tumors of mice vaccinated with Mage-b/GM-CSF/TGB compared to the control groups (vector/GM-CSF/TGB and saline/GM-CSF/TGB), and this correlated with the smaller tumor weight. These results suggest that FasL may be expressed in different immune cells, but only the FasL-expressing cells in the group of Mage-b/GM-CSF/TGB may have contributed to smaller tumors. We found significantly higher number of CD8 T cells in the primary tumors of mice vaccinated with Mage-b/GM-CSF/TGB compared to the control mice. Mage-b (and GM-CSF) may have recruited CD8 T cells to the primary tumors, while TGB may have induced FasL expression in the CD8 T cells. Also macrophages, abundantly present in all 4T1 primary tumors (and metastases), may express FasL. It is possible that FasL-expressing CD8 T cells are more effective in killing Fas-expressing tumor cells than FasL-expressing macrophages. Interaction of Fas–FasL can activate caspase-8, and subsequently caspase-3 [32]. Caspase-3 was strongly activated in both vaccinated and control mice, but significant differences between the groups could not be observed. The 4T1 cell line did not express active caspase-3. In addition, levels of active caspase-8 in the 4T1 primary tumors did not exceed those levels in the 4T1 cell line. Our results suggest that we have not looked at the correct time point for the detection of active caspase-8 and -3, since the tumor cells in which apoptosis was induced were already dead. However, CD8 T cells (expressing FasL) are not expected to die, and therefore the higher expression levels of FasL in the 4T1 primary tumors of vaccinated compared to the control mice are still detectable at a later time point. The similar levels of active caspase-3 observed in the 4T1 primary tumors of vaccinated and control mice, might be the result of interaction between FasL on macrophages, which were abundantly present in all primary tumors of vaccinated and control mice (60% of all 4T1 primary tumors consist of macrophages), and the Fas-expressing tumor cells. We have analyzed other genes involved in apoptotic pathways such as active caspase-9 and -12, TRAIL and DR5, DcR1, DcR2, as well as TNFα. None of them were positive or significantly higher expressed in the 4T1 primary tumors of vaccinated compared to the control mice (data not shown).

In conclusion, the most important result here is that the combination of Mage-b, GM-CSF and TGB provided a robust protective effect against 4T1 metastases. Our results suggest that immune responses are down regulated at the site of the primary tumor but not at the site of the metastases. At the site of the primary tumors, expression of FasL was induced in immune cells by the combination of Mage-b, GM-CSF and TGB. Whether this may have contributed to smaller tumors needs to be further analyzed. If so, then the vaccination of Mage-b and GM-CSF DNA combined with drug treatment that induce the expression of FasL (and could replace TGB) may lead to an improved therapy against metastatic breast cancer expressing Fas protein.

Acknowledgments Authors gratefully acknowledge Rita Busuttil, Ana-Maria Garcia, Shanique Leonard, and Karl Rodriguez, for their excellent technical assistance in the mouse studies. This work was supported by The American Federation for Aging Research (AFAR) A000106 grant, and by NIA grant 1RO1 AG023096-01.

References

1. Althuis MD, Dozier JM, Anderson WF, Devesa SS, Brinton LA (2005) Global trends in breast cancer incidence and mortality 1973–1997. Int J Epidemiol 34:405–412
2. Berkowitz N, Gupta S, Silberman G (2000) Estimates of the lifetime direct costs of treatment for metastatic breast cancer. Value Health 3:23–30
3. Scart H, Cantin J, Levin M (2002) Clinical practice guidelines for the care and treatment of breast cancer: mastectomy or lumpectomy? The choice of operation for clinical stages I and II breast cancer (summary of the 2002 update). CMAJ 167:145–155
4. Kurtz JE, Dufour P (2002) Strategies for improving quality of life in older patients with metastatic breast cancer. Drugs Aging 19:605–622
5. Alberg AJ, Singh S (2001) Epidemiology of breast cancer in older women: implications for future health care. Drugs Aging 18:761–762
6. Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895–902
7. Park JW, Kwon TK, Kim IH, Sohn SS, Kim CI, Bae OS, Lee KS, Lee KD, Lee CS, Chang HK, Choe BK, Ahn SY, Jeon CH (2002) A new strategy for the diagnosis of MAGE-expressing cancers. J Immunol 79:79–86
8. Van Baren N, Bonnet MC, Dreno B et al (2005) Tumoral and immunological response after vaccination of melanoma patients with ALVAC virus encoding MAGE antigens recognized by T cells. J Clin Oncol 23:9008–9021
9. Marchand M, Punt CJ, Aamdal S, Escudier B, Kruit WH, Keilholz U, Hakansson L, van Baren N, Humblet Y, Mulders P, Avril MF, Eggermont AM, Scheibenbogen C, Uiters J, Wanders J, Delire M, Boon T, Stoter G (2003) Immunization of metastatic cancer patients with MAGE-3 protein combined with adjuvant SBAS-2: a clinical report. Eur J Cancer 39:70–77
10. Lurquin C, Lethe B, De Plaen E, Corbiere PG, Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. JEM 201:249–257
11. Kruit HJ, van Ojik HH, Brichard VG et al (2005) Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma. Int J Cancer 117:596–604
12. Thurner B, Haendle I, Roder C et al (1999) Vaccination with Mage-3A1 peptide-pulsed mature monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced Stage IV melanoma. J Exp Med 190:1169–1178
13. Sun X, Hodge LM, Jones HP, Tabor L, Semecka JW (2002) Co-expression of granulocyte–macrophage colony-stimulating factor with antigen enhances humoral and tumor immunity after DNA vaccination. Vaccine 20:1466–1474
14. Okamoto T, Kaneda Y, Yuzuki D, Huang SK, Chi DD, Hoon DS (1997) Induction of antibody response to human antigens by gene therapy using a fusigenic viral liposome vaccine. Gene Ther 4:969–976
15. Ma JH, Sui YF, Huang YY, Li ZS, Chen GS, Ou P, Song HP, Zhang XM (2005) Heat shock protein 70/MAGE-3 fusion protein vaccine can enhance cellular and humoral immune responses to MAGE-3 in vitro. Cancer Immunol Immunother 54:907–914
16. Van Pel A, De Plaen E, Duffour MT, Warnier G, Uytttenhove C, Perricaudet M, Boon T (2001) Induction of cytolytic T lymphocytes by immunization of mice with an adenovirus containing a mouse homologue of the human MAGE-A genes. Cancer Immunol Immunother 49:593–602
17. Eggert AO, Andersen MH, Voigt H, Schrama D, Kampgen E, thor Straten P, Becker JC (2004) Characterization of mouse MAGE-derived H-2K^{b}-restricted CTL epitopes. Eur J Immunol 34:3285–3290
18. Sypniewska RK, Hoflack L, Tarango M, Gauntt S, Leal BZ, Reddick RL, Gravekamp C (2005) Prevention of metastases with a Mage-b DNA vaccine in a mouse breast tumor model: potential for breast cancer therapy. Breast Cancer Res Treat 91:19–28
19. Gravekamp C (2007) Cancer vaccines in old age. Exp Gerontol 42:441–450
20. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Mulligan RC (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte–macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci USA 15:35–39
21. Shi Y, Roberts AI, Das J, Xu G, Ren G, Zhang Y, Zhang L, Yuan ZR, Tan HS, Das G, Devadas S (2006) Granulocyte–macrophage-colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. Cell Res 16:126–33
22. Yasuda K, Kawano H, Yamane I, Ogawa Y, Yoshinaga T, Nishikawa M, Takakura Y (2004) Restricted cytokine production from mouse peritoneal macrophages in culture in spite of extensive uptake of plasmid DNA. Immunol 111:282–290
23. Chambers RS, Johnston SA (2003) High-level generation of polyclonal antibodies by genetic immunization. Nat Biotechnol 21:1088–1092
24. Aslakson CJ, Miller FR (1992) Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 52:1399
25. Lelekakis M, Moseley JM, Martin TJ et al (1999) A novel orthotopic model of breast cancer metastasis to bone. Clin Exp Metastasis 17:163–170
26. Rak JW, McEachern D, Miller FR (1992) Sequential alteration of peanut agglutinin binding-glycoprotein expression during progression in murine mammary neoplasia. Br J Cancer 65:641–648
27. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 163:279
28. Reeves FR, Reeves PA (2003) Current protocols in immunology, vol 1. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (eds) Care and handling of laboratory animals. City of University of New York Medical School, Wiley, New York, P1.9.1
29. Simstein R, Burow M, Parker A, Weldon C, Beckman B (2003. Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system. Exp Biol Med 228:995–1003
30. Gajewski TF, Meng Y, Harlin H (2006) Immune suppression in tumor microenvironment. J Immunother 29:233–240
31. Chen ML, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, Khazaie K (2005) Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo. PNAS 102:419–424
32. Park SJ, Nakgawa T, Kitamura H, Atsumi T, Kamon H, Sawa SI, Kamimura D, Ueda N, Iwakura Y, Ishihara K, Murakami M, Hirano T (2004) IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. J Immunol 173:3844–3854
33. Chen W, Jin W, Hardegen N, Lei KJ, Li L et al (2003) Conversion of peripheral CD4+CD25+ naïve T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 198:1875–1886