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Inhibition of Spontaneous Breast Cancer Metastasis by Anti–Thomsen-Friedenreich Antigen Monoclonal Antibody JAA-F11[1]

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Abstract

Thomsen-Friedenreich antigen (TF-Ag) is expressed in many carcinomas, including those of the breast, colon, bladder, and prostate. TF-Ag is important in adhesion and metastasis and as a potential immunotherapy target. We hypothesized that passive transfer of JAA-F11, an anti–TF-Ag monoclonal antibody, may create a survival advantage for patients with TF-Ag–expressing tumors by cytotoxicity, blocking of tumor cell adhesion, and inhibition of metastasis. This was tested using *in vitro* models of tumor cell growth; cytotoxicity assays; *in vitro*, *ex vivo*, and *in vivo* models of cancer metastasis; and, finally, *in vivo* effects in mice with metastatic breast cancer. Unlike some anti–TF-Ag antibodies, JAA-F11 did not enhance breast carcinoma cell growth. JAA-F11 did not induce the killing of 4T1 tumor cells through complement-dependent cytotoxicity or apoptotic mechanisms. However, JAA-F11 blocked the stages of metastasis that involve the adhesion of human breast carcinoma cells to human endothelial cells (human umbilical vein endothelial cells and human bone marrow endothelial cells 60) in *in vitro* static adhesion models, in a perfused *ex vivo* model, and in murine lung vasculature in an *in vivo* metastatic deposit formation assay. JAA-F11 significantly extended the median survival time of animals bearing metastatic 4T1 breast tumors and caused a > 50% inhibition of lung metastasis.

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Introduction

During carcinogenesis, alterations in the biosynthesis of carbohydrate structures occur, and several different carbohydrate moieties linked to either proteins or lipids have been recognized as tumor-associated glycoantigens. One of them is the Thomsen-Friedenreich antigen (TF-Ag), which was discovered by Thomsen, Friedenreich, and Hueber in the late 1920s [1]. TF-Ag is a disaccharide galactoseβ1–3*N*-acetyl galactosamine attached to proteins by an α *O*-serine or *O*-threonine linkage. It is cryptic on cell membranes of various normal cells, including epithelial cells, red blood cells, and lymphocytes [2], because it is either further glycosylated or covalently masked by highly charged sialic acid [3]. TF-Ag is exposed and immunoreactive, however, to an array of adenocarcinomas, including breast, lung, bladder, prostate, and pancreas adenocarcinomas [1,2,4,5]. The expression of TF-Ag has been proposed as a tool for the detection of tumors and as a criterion for prognosis [6]. TF-Ag densities predict the histopathological grade of carcinomas [7], their invasive potential, and the probability of early recurrence in breast [1,8,9], urinary bladder, and prostatic tumors [10].

TF-Ag is not simply an immunopathological marker. It has been postulated to have a role in adhesion and metastasis [1]. Reversible or irreversible intercellular adhesion is one of the primary steps in metastasis [11], which may occur when TF-Ag recognizes ligands such as galectins or other lectins [5,12]. There is an increased expression of TF-Ag in metastatic tumors, and lectins that bind TF-Ag are in some of the common sites of metastatic tumor growth [13]. Ligands for

Abbreviations: CDC, complement-dependent cytotoxicity; HUVEC, human umbilical vein endothelial cells; HBMEC-60, human bone marrow endothelial cells 60; LDH, lactate dehydrogenase; MOM, Mouse on Mouse; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide: thiazoyl blue; RT, room temperature; TF-Ag, Thomsen-Friedenreich antigen

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TF-Ag adhesion have been found in the vascular endothelium, liver, bone marrow, and lymph nodes [14,15], and this may explain how TF-Ag levels are related to carcinoma aggressiveness [16]. Additional evidence that TF-Ag is involved in tumor metastasis is that adhesion of breast tumor cells to normal breast cells was blocked by polyclonal antibody to TF-Ag [11]. Thus, TF-Ag was chosen for this study as a cancer-associated antigen proposed to be involved in tumor progression and metastasis [3,6,17–21], which could be clinically significant in tumor immunotherapy.

There have been several reports concerning the immunotherapeutic value of an induced immune response to carbohydrate tumor-associated antigens [19,22–26]. In a particularly relevant work by Springer et al. [11], vaccination with TF-Ag–Tn vaccine composed of O MN red blood cell–derived glycoprotein resulted in improved breast cancer patient survival, even though only small amounts of IgM antibody were produced. Interestingly, immunization of breast cancer patients with Globo H (a TF-Ag–containing hexasaccharide) conjugated to keyhole limpet hemocyanin and injected with the adjuvant QS21 also resulted in most patients forming only an IgM response [27]. Although IgM (representing a less mature immune response) is generally of lower affinity and specificity than IgG, many of the studies concerning antibodies to TF-Ag involve IgM responses.

In contrast, by immunization with a bovine serum albumin (BSA) conjugate of Galβ1–3GalNAc, we were able to generate a hybridoma designated as JAA-F11, which produces IgG_3 monoclonal antibody (mAb) against TF-Ag [28]. IgG subclasses are usually superior to those of IgM in terms of higher affinity, specificity, and lower background binding. JAA-F11 characterization using the structurally relevant saccharide GM1 confirmed the ability of JAA-F11 antibody to discriminate between α and β linkages to Gal and to prefer Galβ1–3GalNAcα to GM1 [28]. This is important because it shows that related carbohydrate structures on normal cells would not bind the JAA-F11 antibody. This point also differentiates JAA-F11 from the anti–TF-Ag antibody 170H82, which was reported previously by Dessureault et al. [29]. The 170H82 antibody recognizes both Galβ1–3GalNAcα and Galβ1–3GalNAcβ derivatives and, therefore, could potentially interact with carbohydrate structures expressed in normal tissues such as GM1, whereas JAA-F11 does not. Furthermore, the lack of JAA-F11 binding to NANAα2–3Galβ1–3GalNAcα-*O*-DP-BSA, the sialylated analog of TF-Ag derivatives commonly found in normal cells [28], also suggests that JAA-F11 may recognize tumor tissues with higher specificity in either localization or therapy. In a previous study of six human breast adenocarcinoma tissues, all stained positively for JAA-F11. One sample each of the brain, ovary, lung, kidney, colon, liver, testes, endometrium, heart, and spleen was tested and found to be negative [28]. Some antibodies and lectins that bind to TF-Ag have been found to increase tumor cell growth *in vitro* [12,30–32], but, importantly, our data show that JAA-F11 does not enhance growth.

Based on the above points, we hypothesize that passive transfer of JAA-F11 anti–TF-Ag IgG_3 antibody could create a survival advantage for patients with TF-Ag–expressing tumors either by blockade of tumor cell adhesion to the vascular endothelium or by different mechanisms of cellular cytotoxicity. This was tested in *in vitro* models of cellular cytotoxicity [complement-dependent cytotoxicity (CDC) and apoptosis]; in an *in vitro* model of the direct effect of JAA-F11 on tumor cell growth; in *in vitro*, *ex vivo*, and *in vivo* human models of metastasis involving the adhesion of human breast cancer cells to the vascular endothelium [5,33]; and, finally, in *in vivo* effects in mice with metastatic breast cancer. In our experiments, JAA-F11 did not induce the significant killing of 4T1 tumor cells *in vitro* through CDC or apoptotic mechanisms. However, the addition of the antibody to *in vitro* cultures of tumor cells inhibited their growth by a modest (up to 16%) but significant extent ($P < .01$). In *in vitro* and *ex vivo* models of human breast cancer metastasis, JAA-F11 inhibited tumor cell adhesive interactions with human umbilical vein endothelial cells (HUVEC) and human bone marrow endothelial cells (HBMEC), as well as with well-differentiated porcine microvessels. These *in vitro* effects translated *in vivo* into a significant ($P = .05$) extension of the survival time of animals bearing 4T1 breast cancer tumors and > 50% inhibition of spontaneous lung metastasis ($P = .0155$).

Materials and Methods

Antibody Purification

JAA-F11 mAb was partially purified from a supernatant using ammonium sulfate precipitation followed by dialysis and lyophilization. A stock solution of partially purified antibody was made at 1 mg/ml total protein containing ~160 μg/ml JAA-F11 and used for *in vitro* experiments. For *in vivo* experiments, the antibody was additionally purified and concentrated using size exclusion chromatography on a Sephadex G-200 column (Pharmacia Fine Chemicals, Piscataway, NJ) yielding a stock solution containing ~1.2 mg/ml purified JAA-F11 antibody.

Cell Lines and Cultures

The mouse mammary gland adenocarcinoma cell line 4T1 was purchased from ATCC (Manassas, VA; no. CRL-2539). The 4T1 cell line is a relevant animal model for stage IV human breast cancer [34,35]. When injected into BALB/c mice, 4T1 produces highly metastatic tumors that can spontaneously metastasize to the lung, liver, lymph nodes, and brain, whereas the primary tumor grows *in situ* [34–36]. Mouse myeloma P3-X63-Ag8 (ATCC; no. CRL-1580), which served as the fusion partner for producing JAA-F11 hybridoma [28], was used in this study as a $TF\text{-}Ag^-$ control cell line. The highly metastatic MDA-MB-435 human breast carcinoma cell line was kindly provided by Dr. J. Price (M. D. Anderson Cancer Center, Houston, TX). The tumor cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and adjusted to contain 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. HUVEC were purchased from Cascade Biologics (Portland, OR). Basal Medium 200

(Cascade Biologics) supplemented with low-serum growth supplement containing FBS (final concentration, 2% vol/vol), hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor was used for culturing HUVEC. The cells at population doublings of approximately 8 to 12 were used for adhesion experiments. HBMEC-60 were kindly provided by Dr. C. E. van der Schoot (University of Amsterdam, Amsterdam, The Netherlands). HBMEC-60 were shown to maintain their normal phenotype and adhesive properties, specifically their ability to bind hematopoietic progenitor cells [37]. Basal Medium 200 (Cascade Biologics) supplemented with 20% FBS and low-serum growth supplement containing hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor was used for HBMEC-60. All cells were maintained as monolayer cultures in a humidified incubator in 5% CO_2/95% air at 37°C.

Immunohistochemistry

The vector Mouse on Mouse (MOM) Immunodetection Kit (Vector Laboratories, Burlingame, CA) was used to detect the expression of TF-Ag on paraffin-embedded formalin-fixed tissue samples with JAA-F11 antibody. After blocking endogenous peroxidase with 3% H_2O_2 and endogenous Ig using a mouse Ig block reagent, a stock solution of purified JAA-F11 antibody (1.2 mg/ml) diluted 1:5 (vol/vol) with the MOM diluent was applied for 1 hour as primary antibody. Biotinylated MOM anti-mouse IgG reagent, ABC reagent, and 3c3-diaminobenzidine HCl were used for subsequent color development. Next, slides were counterstained with hematoxylin and examined by light microscopy.

Indirect Cellular Enzyme-Linked Immunosorbent Assay (ELISA)

Two-hundred-microliter aliquots of a single-cell suspension (2×10^5 cells) of 4T1, MDA-MB-435, or P3-X63-Ag8 myeloma cells that had been harvested by scraping without trypsin were placed in tubes, and 200 μl of 4% formaldehyde solution in phosphate-buffered saline (PBS) was added for 20 minutes to fix the cells. The tubes were centrifuged and decanted, and 200 μl of PBS–Tween–1% BSA was added to each to prevent fixed cells from drying. The tubes with fixed cells were stored at 4°C overnight for up to 2 weeks. For peroxidase-linked immunoassay, 200 μl of indicated JAA-F11 mAb dilutions was added to each tube and incubated for 2 hours at 37°C. The tubes were washed thrice with PBS–Tween (no azide) then centrifuged for 10 minutes at 1200 rpm between each wash. Two hundred microliters of anti-mouse IgG (γ-chain–specific) peroxidase conjugate (1:1000, 0.7 mg/ml; Sigma, St. Louis, MO) in PBS–Tween–1% BSA was added to the tubes for 1 hour at room temperature (RT). After three washes, *O*-phenylenediamine dihydrochloride substrate (Sigma) was used for color development, which was terminated after 1 hour with 100 μl of stop solution (1 N H_2SO_4). The tubes were centrifuged at 1200 rpm for 10 minutes, and 200 μl of the supernatant was removed to microtiter plate wells. The plate was read at 490 nm using a well of unreacted substrate as blank. Inhibition was performed by adding 0.4 mg of TF-Ag–BSA conjugate to the 1:5 dilution of F11, incubating for 1 hour at RT, and centrifuging in a microfuge for 3 minutes before 4T1 and MDA-MB-435 reactions. Inhibition was calculated as: 1−(optical density [OD] inhibited/OD uninhibited).

Effects of JAA-F11 on Tumor Cell Growth In Vitro

To determine the direct effect of JAA-F11 on tumor cell growth, *in vitro* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazoyl blue, MTT) proliferation analyses were performed [38,39]. 4T1 mouse mammary adenocarcinoma cells (at 2.5×10^4 cells/well) and MDA-MB-435 human breast tumor cells (at 1.25×10^4 cells/well) were plated in 96-well plates in the presence of JAA-F11 at an indicated concentration or in the presence of a control isotype-matching antibody at an equivalent concentration. When plated at these amounts, the cells are in the linear portion of their growth curves at 72 hours. Cells grown in a culture medium without antibody and a culture medium alone were used as additional negative controls. After 68 hours of cell growth, 10 μl of the tetrazolium salt MTT [38,39] was added to each well, and the plates were returned to the incubator for a total growth time of 72 hours. The resulting formazan product in each well was solubilized by adding 120 μl of 5% formic acid in isopropanol with forceful mixing, and the absorbance of each well was measured at 570 nm.

Apoptosis Assay on JAA-F11–Treated Tumor Cells

The APO-BRDU Apoptosis kit (catalog no. AU1001; Phoenix Flow Systems, San Diego CA) was used with few changes, as listed below. The kit is based on terminal deoxynucleotide transferase dUTP nick end labeling assay (TUNEL) assay technique for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry. Fixation was performed as suggested in the kit's directions, and cells (4T1 and myeloma, untreated or treated with 8 μg/ml JAA-F11) were incubated for 30 minutes with 1% paraformaldehyde before centrifugation, washing, and dropwise addition of ice-cold ethanol during vortexing. Cells were stored overnight at −20°C before staining. Staining was performed as stated in the kit's directions, with the exception that the supernatant was not aspirated off but rather poured off, and the tube was blotted and vortexed. After DNA labeling and staining with fluorescein and propidium iodide, the cells were analyzed on a FACS Calibur machine (Becton Dickinson Biosciences, Franklin Lakes, NJ). Appropriate controls were included in the kit and prepared as stated above.

CDC

The CytoTox 96 Nonradioactive Cytotoxicity Assay (Promega, Madison, WI) based on the release of lactate dehydrogenase (LDH) was performed in this study to analyze the effect of JAA-F11 antibody on 4T1 cells in CDC assays *in vitro*. As a colorimetric alternative to ^{51}Cr release cytotoxicity assays, this assay quantitatively measures LDH, a stable cytosolic enzyme that is released on cell lysis, in much the same way as ^{51}Cr is released in radioactive assays. A mouse anti-mouse H-2D^d mAb (Becton Dickinson), which recognizes an epitope on N-terminal domains (α_1 and α_2 of

H-2D^d) [38,40,41], was used as positive control. JAA-F11 and anti–H-2D^d mAb were both brought to 0.01 mg/ml. The source of the complement was baby rabbit serum, which was reconstituted as directed (CL3441, 10 × 1-ml lyophilized vials; Cedarlane Laboratories, Burlington, NC). The complement was used within 1 hour of reconstitution. The optimal cell concentration for the assay was determined as 1×10^4 cells/50 μl per well.

Fifty microliters of 4T1 cell suspension (or medium), 40 μl of complement dilutions (or medium), and 10 μl of antibody solutions (or PBS) were mixed in each well in quadruplicate sets in a round-bottom 96-well culture plate by centrifuging at 1000 rpm for 4 minutes. The plate was incubated for 2 hours at 37°C with 5% CO_2. Forty-five minutes before harvesting the supernatant, 10 μl of lysis solution (×10) for each 100 μl of target cells was added to the wells containing the target cell maximum LDH release control and the volume correction control. After 2 hours of incubation, the plate was centrifuged at 1000 rpm for 4 minutes, and 50-μl aliquots were transferred from all wells to a fresh 96-well plate. Next, 50 μl of reconstituted substrate mix was added to each well, then the plate was covered with foil to protect from light and was incubated for 30 minutes at RT. The reaction was stopped by adding 50 μl of stop solution to each well, and absorbance was recorded using a microplate reader at 490 nm.

Static Adhesion Assay

Static adhesion experiments were performed exactly as described [5,17]. A single-cell suspension of cancer cells prelabeled with a 3-μg/ml solution of acridine orange (5×10^4 cells/chamber in 2.5 ml of a culture supernatant at approximately 50 μg/ml antibody) was added to the monolayer of endothelial cells grown to confluence directly on microscope slides using four-well chamber slides (Nalge-Nunc, Naperville, IL). The chambers were sealed, and cells were allowed to adhere for 1 hour at 37°C, after which the chambers were inverted and left upside down for 30 minutes to allow for sedimentation of nonadherent cells. Next, the medium was drained, and samples were gently rinsed with PBS, fixed for 30 minutes in 2% formaldehyde solution in PBS, mounted with cover glass, and examined by fluorescent microscopy. Four random fields in each well were photographed at ×250 magnification, and the total number of adhered cells in every field was counted. The assay was performed in quadruplicate for each experimental condition.

Ex Vivo – *Perfused Porcine Dura Mater Model*

Porcine dura mater was used in adhesion experiments, as described elsewhere [33,42]. Briefly, the dura mater corresponding to one hemisphere, collected from mature female Yucatan miniature swine (Charles River Laboratories, Wilmington, MA) within 30 minutes of animal sacrifice (in accordance with the animal care protocol approved by the University of Missouri), was dissected and flattened onto a Sylgard-coated 100-mm dish. A major branch of the median meningeal artery was cannulated, and the dura vasculature was perfused at 15 μl/min initially with Krebs physiological salt supplemented with 1.0 mg/ml porcine serum albumin for 20 minutes, then with vessel-labeling solution (0.3 μg/ml acridine orange in RPMI 1640 supplemented with 10 % FBS and 1.0 mg/ml porcine albumin) for an additional 40 minutes. Before injection, cancer cells were prelabeled for 5 minutes with 3 μg/ml acridine orange solution in RPMI 1640 medium, rinsed thrice, dissociated from plastic, pipetted to produce a single-cell suspension, filtered through a 20-μm nylon mesh to remove cell clumps, and adjusted to 5×10^4 cells/ml. The interactions of cancer cells with the dura microvasculature were monitored and videorecorded at 30 frames/sec using a fluorescence videomicroscopy system based on a Laborlux 8 microscope (Leitz Wetzlar, Wetzlar, Germany) equipped with a 75-W xenon lamp and a high-sensitivity charge-coupled device (CCD) camera (COHU, San Diego, CA). For subsequent frame-by-frame analysis, the recorded analog videoimages were digitized using a media converter DVMC-DA2 (Sony, Tokyo, Japan) and Adobe Premier 6 software (Adobe Systems, San Jose, CA). JAA-F11 was added as a culture supernatant, which contained approximately 50 μg/ml antibody.

In Vivo *Metastatic Deposit Formation Assay*

In vivo metastatic deposit formation assay was performed exactly as previously described [43]. Briefly, 6-week-old female HsdIcr:Ha(ICR)-scid mice (Harlan, Indianapolis, IN) were used in this study in accordance with the animal care protocol approved by the University of Missouri. Before intravenous injection, MDA-MB-435 human breast carcinoma cells were prelabeled for 5 minutes with a 3-μg/ml solution of acridine orange in RPMI 1640 medium, rinsed thrice with serum-free RPMI 1640 medium, and dissociated from plastic with a nonenzymatic cell dissociation reagent (Sigma). Immediately following dissociation, tumor cells were resuspended using one of the following: 1) complete RPMI 1640 medium (control); 2) undiluted conditioned supernatant containing JAA-F11 anti–TF-Ag antibody (approximately 50 μg/ml mAb); or 3) undiluted conditioned supernatant containing control antibody; and pipetted to produce a single-cell suspension. All subsequent manipulations with cancer cells were performed using the same media composition. To remove any remaining cell clumps, the tumor cell suspension was filtered through a 20-μm nylon mesh and adjusted to contain 5×10^6 cells/ml. Next, 200 μl (1×10^6 cells) of a single-cell suspension of fluorescently labeled MDA-MB-435 cells was injected into the lateral tail vein of experimental animals. Three hours postinjection, the animals were euthanized, and the lungs were removed, examined by epifluorescence microscopy, and photographed using a QICAM high-performance digital CCD camera (Quantitative Imaging Corporation, Burnaby, Canada). At least two identical experiments were performed for each experimental setup. In each animal, subpleural metastatic deposits were scored in four random observation fields. The results were calculated and presented as mean ± SD.

In Vivo *4T1 Mouse Breast Carcinoma Model*

To study the effect of JAA-F11 on the *in vivo* growth of primary tumors, 4T1 tumors were induced in 20 eight-week-

old female BALB/c mice by injecting 100 μl of a single-cell suspension (1×10^5 viable cells/ml) subcutaneously into the fat pad area of the right abdominal mammary gland. This dose (1×10^4 viable tumor cells/mouse) ensures 100% tumor incidence [34–36]. Three days after the implantation of 4T1 tumor cells, the mice were divided randomly into two groups and received intraperitoneal administration of either purified JAA-F11 (120 μg/100 μl per mouse) as treatment or PBS as control, twice weekly, throughout the duration of the experiment. Beginning on day 11 after implantation, tumor size was measured every 2 days as follows: two measurements of the tumor, perpendicular to each other and spanning the largest portion of the tumor in each direction, were performed with Vernier calipers, and tumor volume was calculated based on these measurements.

To study the effect of JAA-F11 on the spontaneous metastasis of 4T1, tumors were induced as above in 40 eight-week-old female BALB/c mice. Three days after implantation, mice were randomized into two groups (20 mice/group). One group received intraperitoneal injections of purified JAA-F11 (120 μg/100 μl per mouse) as treatment, whereas another group received 100 μl of PBS as control, twice weekly, throughout the duration of the experiment. To further model the clinical situation, 2 weeks after tumor implantation, when spontaneous metastases were present in at least 88% of control animals, the primary tumors were surgically removed, leaving only metastatic disease to be studied in the animal model; this is comparable to a clinical situation where primary breast tumor is surgically removed and metastatic foci remain intact [34–36].

Measurement of Tumor Outcome

Mouse weights were measured and weight loss rates between treated mice and control mice were compared. Clinical symptoms, such as lack of grooming, rough coat, rapid and labored breathing, and loss of mobility, were monitored, recorded, and used as indicators of morbidity. Mice were sacrificed when weight loss reached 20% or when moribund. Daily observations were made by both investigators and animal caretakers. The survival time was recorded and analyzed by the Kaplan-Meier survival curve (MedCalc, Mariakerke, Belgium). Organs of interest (primary tumor, lung, liver, spleen, lymph node, and brain) were collected and fixed using Z-fixative, and paraffin sections for immunohistochemical staining were prepared.

Statistical Analysis

Statistical significance was calculated using *t*-test, and error bars represent ± 1 SD of the mean for some experiments and ± 1 SEM for some experiments, as noted in figure legends. Grubb's test for outlying observations was used to determine any outliers for rejection from the data set. Chi-square analysis was used to determine the statistical significance of lung metastases, whereas the Kaplan-Meier survival curve was used to determine the survival difference between JAA-F11–treated mice and control mice.

Results

Detection of TF-Ag on Tumor Cells Using JAA-F11 Antibody

The ability of JAA-F11 mAb to recognize TF-Ag expressed in tumor cells *in vivo* and *in vitro* was confirmed by immunohistochemical analysis of primary 4T1 tumors induced in BALB/c mice and by indirect cellular ELISA, respectively. Because JAA-F11 is a mouse mAb and 4T1 is a murine mammary adenocarcinoma, we used the MOM Immunodetection Kit (Vector Laboratories) to detect the expression of TF-Ag with JAA-F11 on paraffin-embedded formalin-fixed tissue samples of primary 4T1 tumors removed from BALB/c mice (Figure 1*A* and *B*). The characteristic brown stain (Figure 1*A*) is indicative of the JAA-F11 recognition of TF-Ag

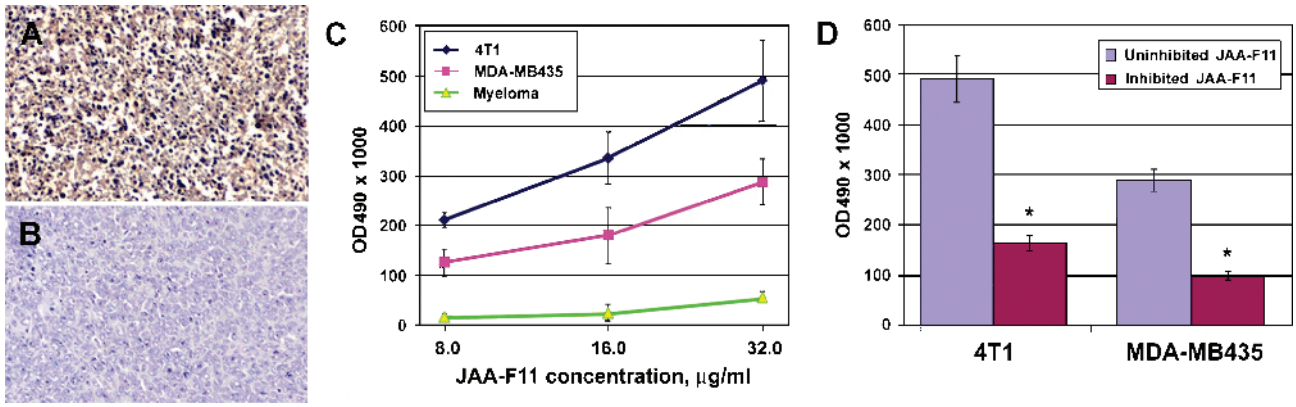

Figure 1. *(A) Immunohistochemical staining of primary 4T1 breast tumor dissected from BALB/c mice using JAA-F11 as primary antibody. Brown color indicates the binding of JAA-F11 antibody to the TF-Ag expressed in tumor cells. (B) Nonimmune (no primary antibody) negative control. (C) Whole-cell indirect ELISA showing 4T1 and MDA-MB-435 dose-dependent reactivity with three JAA-F11 dilutions of 8, 16, and 32 μg/ml. TF-Ag⁻ P3-X63-Ag8 mouse myeloma cells show no significant reactivity with JAA-F11. P < .01, comparing 4T1 to myeloma cells and MDA-MB-435 to myeloma cells at all dilutions, using t-test for significance. Representative of four experiments. Error bars represent ± 1 SEM. (D) Whole-cell indirect ELISA showing the inhibition of JAA-F11 binding to 4T1 and MDA-MB-435 cells by 0.4 mg of TF-Ag–BSA conjugate. Significant inhibition of JAA-F11 at a 1:5 dilution compared to uninhibited JAA-F11 was seen in both cell types. Error bars represent ± 1 SEM and *P < .05.*

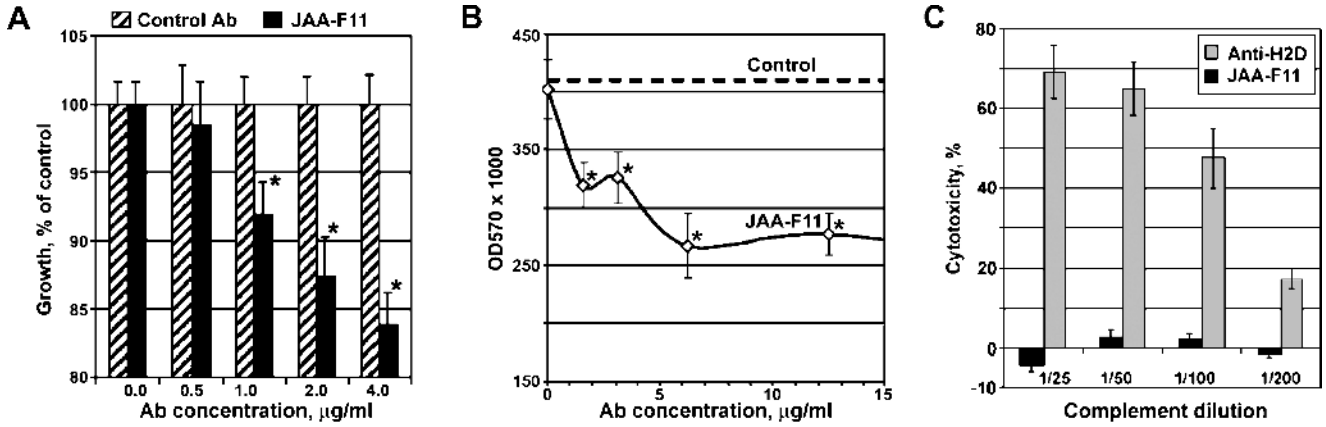

Figure 2. *(A and B) The effect of JAA-F11 antibody on tumor cell growth* in vitro*, as measured by MTT assays. (A) TF-Ag$^+$ 4T1 mouse adenocarcinoma cells show a modest but statistically significant (*) inhibition of growth in response to JAA-F11 antibody compared to no-antibody controls ($P < .001$) and control IgG_3 antibody ($P < .005$). The average of five experiments is graphically shown. Error bars represent ± 1 SEM. (B) Dose-dependent inhibition of TF-Ag$^+$ MDA-MB-435 human breast carcinoma cells in response to JAA-F11 treatment. Data are presented as mean ± 1 SEM. *Statistically significant difference ($P < .05$) compared to controls. (C) CDC: compared to anti–H-2D^d antibody, JAA-F11 lacks the ability to cause complement-mediated tumor cell cytotoxicity* in vitro.

expressed in cancer cells. No staining was observed in nonimmune control samples (Figure 1*B*). In other experiments, radiolocalization of ^{124}I-labeled JAA-F11 showed a lack of reactivity of other mouse tissues and the localization of the antibody to 4T1 cells in mice (data not shown).

To test the ability of JAA-F11 to detect the TF-Ag expressed on surfaces of tumor cells grown *in vitro*, indirect cellular ELISA was performed. Mouse myeloma P3-X63-Ag8 cells, which do not express TF-Ag, were used as negative control in these experiments. Two series of ELISA experiments were performed. In the first series, the binding of various concentrations of JAA-F11 mAb to the same amount of cells was measured. The results of these experiments demonstrated that the binding of JAA-F11 to TF-Ag–expressing human breast tumor MDA-MB-435 and 4T1 tumor cells was linear and increased in agreement with escalating mAb concentrations (Figure 1*C*). In the second series of ELISA experiments, ELISA was performed in microtiter plates, and various amounts of tumor cells were exposed to the fixed concentration of the JAA-F11 antibody. This reaction was also linear with cell number (data not shown). As expected, JAA-F11 did not react with TF-Ag$^-$ mouse myeloma P3-X63-Ag8 cells (Figure 1*C*). The difference in the binding of JAA-F11 to 4T1 *vs* myeloma was statistically significant in all mAb dilutions ($P < .05$). The addition of TF-Ag–BSA to JAA-F11 before incubation with the cell line caused a 67% inhibition of 4T1 binding and a 65% inhibition of MDA-MB-435 binding (Figure 1*D*).

Effects of JAA-F11 on 4T1 Tumor Cells In Vitro

To determine the effects of JAA-F11 mAb on the *in vitro* growth of 4T1 cells, with and without JAA-F11 and with control antibody, quantitation by MTT assays was performed [38,39]. After performing the assay on five separate days in sets of eight on each day, the average OD was obtained for all five experiments. The average of the cells grown with JAA-F11 was statistically compared to cells grown without JAA-F11 and to cells grown with IgG_3 control antibody. A statistically significant ($P < .005$) dose-dependent decrease in cell growth due to JAA-F11 at 4, 2, and 1 μg/ml was observed in TF-Ag–expressing 4T1 cells, compared to 4T1 cells treated with control IgG_3 antibody and without antibody (average of five experiments; Figure 2*A*). Similarly, JAA-F11 significantly inhibited the growth of MDA-MB-435 human breast tumor cells *in vitro* in a dose-dependent manner (Figure 2*B*). Of note, other TF-Ag–targeted antibodies and lectins were reported to stimulate tumor cell growth; therefore, it is important that JAA-F11 did not stimulate tumor cell growth. This supports the hypothesis that JAA-F11 is able to bind tumor cells, to block adhesion, and to negatively affect cell growth *in vitro*.

Because the binding of some galectins and other lectins can cause apoptosis [43,44], an assay was performed to determine if this was the cause of the inhibition of *in vitro* growth by JAA-F11. With apoptosis assay, the level of apoptosis in positive control cells included in the kit was 29% compared to 0.3% in negative controls. In 4T1 cells, the percentage of apoptotic cells after treatment with JAA-F11 was 2.66 ± 0.34% compared to 3.26 ± 0.27% in untreated controls. Similarly, myeloma cells, although possessing a slightly higher background level of apoptosis (5.86 ± 1.48%), did not show an increase in apoptosis level after treatment with JAA-F11 (8.69 ± 0.76%). Therefore, this assay showed that no apoptosis resulted from the incubation of mouse breast cancer cells or control cells with JAA-F11, suggesting that growth inhibition may be due to antiadhesion or cytostatic effects rather than due to a cytotoxic mechanism.

The ability of JAA-F11 to induce 4T1 cell killing through a CDC mechanism was assessed using the CytoTox 96 Nonradioactive Cytotoxicity Assay based on LDH release. A mouse anti-mouse H-2D^d mAb was used as positive control, and baby rabbit serum was used as the source of the complement. The results of these experiments (Figure 2*C*) indicated that, compared to anti–H-2D^d, JAA-F11 antibody lacks the ability to cause complement-mediated anti–tumor cytotoxicity *in vitro*.

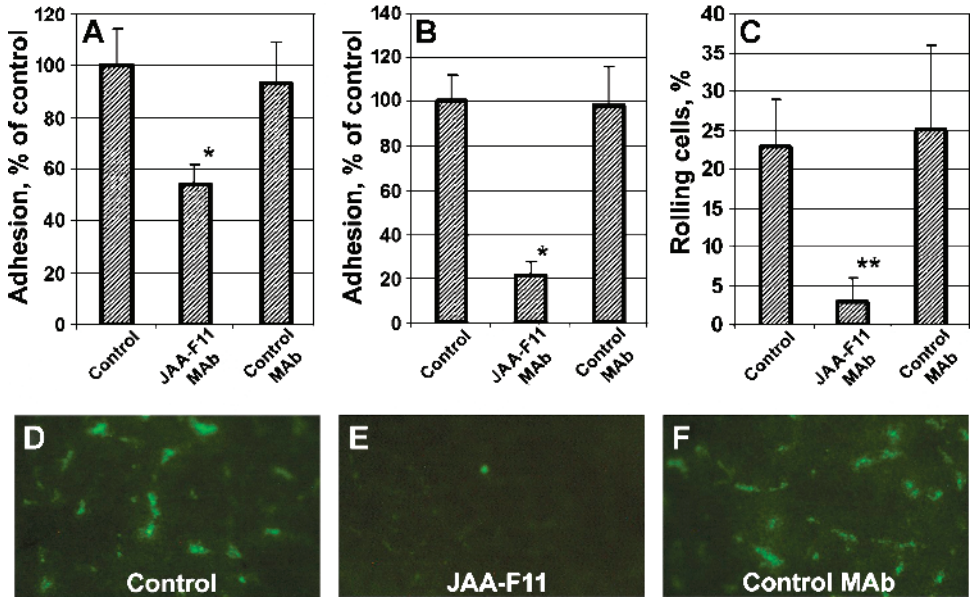

Figure 3. *Inhibitory effect of JAA-F11 antibody on breast cancer cell adhesion to the endothelium. The results of static adhesion experiments (A and B) show the inhibitory effect of JAA-F11 on the MDA-MB-435 metastatic human breast carcinoma cell line's stable adhesion to HUVEC monolayers (A) and HBMEC-60 (B)* in vitro. *(C) The effect of JAA-F11 on MDA-MB-435 rolling in well-differentiated microvessels of perfused porcine dura mater* ex vivo. *(D–F) Inhibition of the formation of MDA-MB-435 human breast carcinoma subpleural metastatic deposits in the mouse lung 3 hours after intravenous inoculation* in vivo *by JAA-F11 antibody (E) compared to untreated controls (D) and control antibodies (F) viewed by fluorescence videomicroscopy. (A–C) *P < .05 and **P < .01. Error bars represent ± 1 SD.*

Human Models of Metastasis

TF-Ag plays an important role in the primary steps of breast and prostate cancer metastases by mediating adhesive interactions between metastatic cells and the endothelium [5,17,45,46]. Here, we show that blocking TF-Ag with JAA-F11 antibody inhibits the stable adhesion of human breast carcinoma cells to the endothelium in two static adhesion models. *In vitro*, cancer cell binding to HUVEC monolayers (Figure 3*A*) and HBMEC-60 (Figure 3*B*) was inhibited by 54% ($P < .05$) and 78% ($P < .05$), respectively. In the *ex vivo*–perfused porcine dura mater model, JAA-F11 inhibited human breast tumor cell adhesive interactions with well-differentiated microvessels by 86% ($P < .01$) (Figure 3*C*). Finally, *in vivo*, JAA-F11 almost completely blocked the formation of MDA-MB-435 human breast carcinoma cells' metastatic deposits in mouse lungs after intravenous inoculation (Figure 3*D–F*).

In Vivo *Immunotherapy*

In the first series of *in vivo* immunotherapy experiments using passive transfer of JAA-F11 antibody, we studied the effect of JAA-F11 on the growth of primary 4T1 tumors induced in mouse mammary fat pads. Due to a potential decrease in active antibody concentration over time, the dose of JAA-F11 was increased in these experiments to 120 μg/injection compared to the 50-μg/ml dose in MDA-MB-435 *in vivo* experiments. The results of these experiments (Figure 4*A*) show that JAA-F11 did not significantly affect the growth kinetics of primary 4T1 tumors *in vivo*. Thus, the *in vitro* inhibitory effects of JAA-F11 on 4T1 cell growth did not translate into a significant inhibition of 4T1 primary tumors *in vivo*.

In the second series of *in vivo* immunotherapy experiments, we investigated JAA-F11 effects on 4T1 spontaneous metastasis and animal survival. Kaplan-Meier analysis (Figure 4*B*) demonstrated that immunotherapy with JAA-F11 antibody provided experimental animals bearing TF-Ag–expressing 4T1 tumors with a significant survival advantage. The median survival time of animals in the PBS-treated group and in the JAA-F11–treated group was 57 and 72 days, respectively, and the difference was statistically significant ($P = .05$). Furthermore, JAA-F11 immunotherapy resulted in a significant reduction in metastatic tumor burden, reflected by both a dramatic decline in the overall incidence of spontaneous lung metastasis (from 88% in the control group to 47% in the JAA-F11–treated group) and a reduced number of macroscopic metastatic lesions (Figure 4*C* and *D*). Chi-square test for trend analysis demonstrated that the difference in the frequencies of lung metastasis between the PBS-treated group and the JAA-F11–treated group was significant ($P = .0155$). Interestingly, although the number of lung metastases was lower in the JAA-F11–treated group, there was no noticeable difference in the size of metastatic lesions between the groups. Furthermore, only 2 of 10 animals without macroscopic lung metastasis had microscopic metastatic foci, and the rest were metastasis-free. This suggests that JAA-F11 likely affects the process of metastatic establishment through antiadhesive mechanisms rather than through the growth of metastatic lesions. In our experiments, there was no detectable brain or liver metastasis in animals from either group. Single-bone (sternum) metastatic lesions were found in 3 of 16 (18.8%) animals in the control group, and in 2 of 20 (10%) in the JAA-F11–treated group, suggesting that JAA-F11 may

have an effect on 4T1 metastatic spread to the bone as well. However, additional experimentation is necessary to support this hypothesis.

Discussion

The IgG_3 JAA-F11 mAb recognizes the TF-Ag that is expressed on tumor cell surfaces with high specificity. Here, we demonstrated that JAA-F11 has a modest but statistically significant inhibitory effect on *in vitro* 4T1 tumor cell growth and MDA-MB-435 (Figure 2*A* and *B*), as reflected by MTT assay. This effect was not due to the induction of apoptosis or CDC. A decrease in MTT reduction to formazan after treatment with JAA-F11 antibody reflects changes in mitochondrial function. In the absence of apoptotic response to JAA-F11 treatment, it may suggest that the antibody could have a cytostatic effect on cancer cells *in vitro*, which may account, in part, for the failure of CDC *in vitro*. JAA-F11, however, did not affect the *in vivo* growth rate of primary 4T1 tumors. We speculate that this could be due to the following factors: 1) biologically, the growth of 4T1 cells as a monolayer culture *in vitro* differs dramatically from *in vivo* tumor growth in a mammary fat pad; 2) due to a dilution factor and administration regimens (twice per week), the effective concentrations of the antibody acting on tumor cells *in vivo* could have been much lower than those *in vitro*. Nevertheless, even though an *in vitro* growth-inhibitory effect of JAA-F11 did not translate into the *in vivo* inhibition of primary 4T1 tumor growth (Figure 4*A*), this represents an obvious advantage of JAA-F11 over some other antibodies against TF-Ag, which enhances *in vitro* tumor growth [12,30–32]. Glinsky et al. [46] suggest that the binding of TF-Ag on tumor cells to galectins on other tumor cells is important in homotypic cancer cell aggregation, which in turn is important in clonogenic survival and growth; this mechanism is likely involved in *in vitro* proliferation assays and *in vivo* experiments.

In the series of *in vitro*, *ex vivo*, and *in vivo* experiments, JAA-F11 dramatically inhibited the adhesion of human breast carcinoma cells to monolayers of human microvascular endothelial cells, well-differentiated porcine microvessels, and mouse lung microvasculature (Figure 3). These *in vitro* and *ex vivo* JAA-F11 antimetastatic effects translated into: 1) a significant ($P = .05$) extension of the survival time of animals bearing metastatic 4T1 breast cancer tumors from 57 days in the PBS-treated control group to 72 days in the JAA-F11–treated group; 2) considerable reduction in metastatic tumor burden; and 3) > 50% inhibition of the incidence of spontaneous lung metastasis ($P = .0155$) (Figure 4*A*–*D*). These results support the theory that JAA-F11 has effective antiadhesion properties that could be clinically significant in blocking important steps in the metastasis of tumor cells. We believe that JAA-F11 antibody disrupts TF-Ag interactions with its binding partner galectin-3 [5,45,46] or possibly other galectins. TF-Ag–galectin-3 interactions are involved in metastatic cell adhesion to the microvascular endothelium, as well as in the homotypic aggregation of cancer cells with each other. Most likely, these carbohydrate–lectin interactions, which are rather weak and transient in nature,

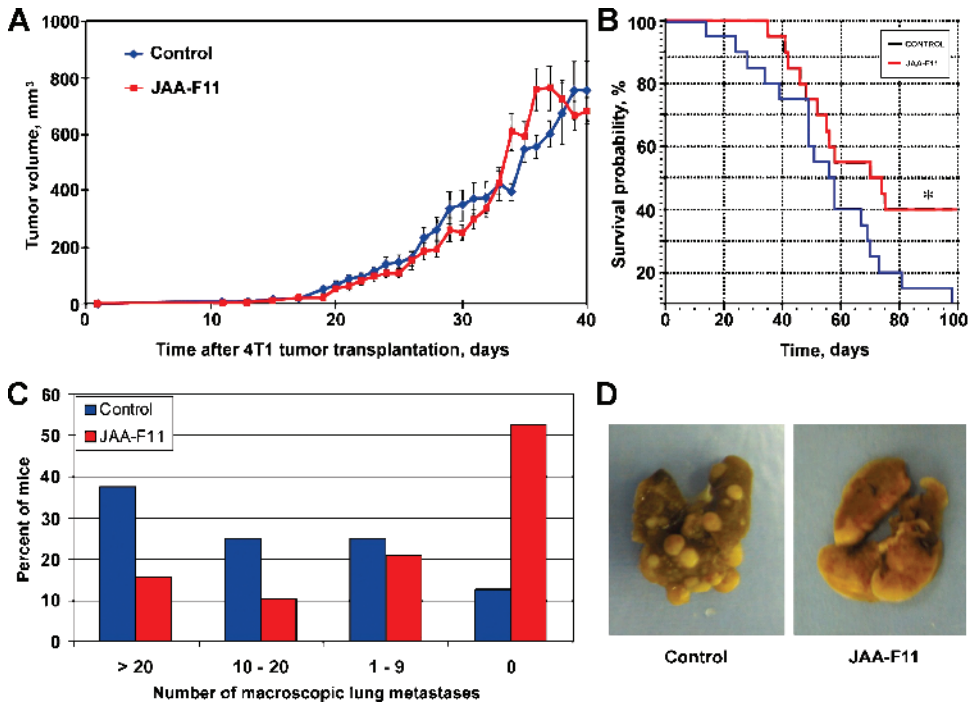

Figure 4. *(A) JAA-F11 causes no effect on the growth of primary 4T1 tumors* in vivo. *Error bars represent ±1 SEM. (B) Kaplan-Meier analysis of* in vivo *spontaneous metastasis immunotherapy experiments shows that JAA-F11–treated animals (red line) have a significant (*P = .05) survival advantage compared to control (blue line) mice. (C) Distribution of lung metastasis in control (blue bars) and JAA-F11–treated (red bars) animals. Chi-square analysis shows that JAA-F11 treatment significantly (P = .0155) reduces the amount of spontaneous lung metastasis in the 4T1 model. (D) Representative lungs from control and JAA-F11–treated mice. Note a decrease in the number of visible metastatic foci on the lungs of JAA-F11–treated mice compared to controls.*

represent some of the earliest events in a multistep cell-to-cell adhesion process [45,46]. We see a role for TF-Ag–galectin-3–mediated adhesion in initiating tumor cell binding to endothelial cells and in mediating subsequent steps in the metastatic cascade, including invasion and extravasation, which further determine the fate of metastatic deposits and the organ specificity of hematogenous cancer metastasis. It appears that therapeutic inhibition of these early metastasis-associated adhesive events may have a significant effect on the outcome of the entire metastatic process and may represent a new paradigm for therapeutic interventions aiming at preventing and controlling metastatic cancer spread.

In addition, the data indicate that the passive transfer of antibody to TF-Ag could be potentially beneficial in patients with TF-Ag–expressing metastatic breast cancer or other TF-Ag–expressing cancers. To date, limited clinical success has been achieved with antibodies or antibody conjugates. For example, trastuzumab, a mAb targeting Her2/neu, has received Food and Drug Administration approval for use in patients with metastatic breast cancer. Similar to JAA-F11, trastuzumab was developed originally as a mouse mAb. To reduce patients' immune reaction to trastuzumab, it was humanized through a recombinant gene technology so that only the antigen-binding region retained a mouse origin whereas the rest of the IgG molecule was made "human" [47]. The same strategy could be used to humanize JAA-F11 antibody.

Trastuzumab is often used in conjunction with paclitaxel, which shows a definite synergistic effect in terms of the number of complete responses and time to disease progression, such that trastuzumab and paclitaxel, when used together, produce a much greater response than the sum of the responses achieved with either agent used independently [47]. Thus, it would be of interest to investigate whether JAA-F11 could synergize cytotoxic drugs. Furthermore, the trastuzumab target Her2/neu is upregulated in about 25% of breast tumors, whereas JAA-F11 would bind to tumor cells from a much higher percentage of breast cancer patients [47]. Given the chemical specificity of JAA-F11 compared to other anti–TF-Ag antibodies, yet another potential avenue could be the use of JAA-F11 as a vehicle for the targeted delivery of cytotoxic drugs and/or radiopharmaceuticals for therapeutic and diagnostic purposes. These studies are currently underway to determine the broad-spectrum clinical utility of JAA-F11 mAb.

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