Therapeutic and Prophylactic Antitumor Activity of an Oral Inhibitor of Fucosylation in Spontaneous Mammary Cancers

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ABSTRACT

2-fluorofucose (2FF) inhibits protein and cellular fucosylation. Afucosylation of IgG antibodies enhances antibody-dependent cell-mediated cytotoxicity by modulating antibody affinity for FcγRIIIα, which can impact secondary T-cell activation. Immune responses toward most common solid tumors are dominated by a humoral immune response rather than the presence of tumor-infiltrating cytotoxic T cells. IgG antibodies directed against numerous tumor-associated proteins are found in the sera of both patients with breast cancer and transgenic mice bearing mammary cancer. We questioned whether 2FF would have antitumor activity in two genetically distinct transgenic models; TgMMTV-neu (luminal B) and C3(1)-Tag (basal) mammary cancer. 2FF treatment significantly improved overall survival. The TgMMTV-neu doubled survival time compared with controls (P < 0.0001; HR, 7.04; 95% confidence interval (CI), 3.31–15.0), and survival was significantly improved in C3(1)-Tag (P = 0.0013; HR, 3.36; 95% CI, 1.58–7.14). 2FF treated mice, not controls, developed delayed-type hypersensitivity and T-cell responses specific for syngeneic tumor lysates (P < 0.0001). Serum IgG from 2FF-treated mice enhanced tumor lysis more efficiently than control sera (P = 0.004). Administration of 2FF for prophylaxis, at two different doses, significantly delayed tumor onset in both TgMMTV-neu; 20 mmol/L (P = 0.0004; HR, 3.55; 95% CI, 1.60–7.88) and 50 mmol/L (P = 0.0002; HR: 3.89; 95% CI, 1.71–8.86) and C3(1)-Tag; 20 mmol/L (P = 0.0020; HR, 2.51; 95% CI, 1.22–5.18), and 50 mmol/L (P = 0.0012; HR, 3.36; 95% CI, 1.57–7.18). Mammary cancer was prevented in 33% of TgMMTV-neu and 26% of C3(1)-Tag. 2FF has potent antitumor effects in mammary cancer models. The agent shows preclinical efficacy for both cancer treatment and prevention.

Introduction

Patients with breast cancer have robust type II tumor-directed immune responses with little evidence of type I immunity (1, 2). The dominance of a type II immune microenvironment is established early in breast tumorigenesis (3). As a result, breast cancer is associated with abundant autoantibodies directed against tumor-associated antigens, high levels of tumor-infiltrating B cells, and few infiltrating CD8+ T cells in the majority of patients (2, 4, 5). Clinical studies of immune checkpoint inhibitor mAbs have shown that strategies which augment endogenous T-cell immunity can have an impact on tumor growth and overall survival in a variety of cancers where tumor-infiltrating T cells predominate (6). Agents designed to “unleash” the numerous tumor-specific endogenous antibodies and improve their antitumor activity could have a similar clinical impact in malignancies dominated by a humoral antibody response, such as breast cancer.

Depleting core fucose residues from oligosaccharides present on the Fc portion of human IgG antibodies can markedly enhance antibody-dependent cellular cytotoxicity (ADCC; ref. 7). Afucosylation increases the binding avidity of the Fc region of IgG to FcγRIIIα receptors on the surface of effector cells (8–10). The ability of afucosylated antibodies to more efficiently mediate ADCC results in direct lysis of cancer cells and activation of natural killer (NK) cells and antigen-presenting cells, which then can subsequently process and provide shed antigens to T cells. 2-fluorofucose (2FF, compound 1 as described in Okeley and colleagues; ref. 10, custom synthesized by SynChem) is an orally available agent that can inhibit protein and cellular fucosylation. This process occurs following entry of 2FF into cells and the metabolic conversion of 2FF to GDP-2FF. GDP-2FF inhibits the de novo synthesis of GDP-fucose from GDP-mannose in addition to the activity of various fucosyltransferases, such as FUT8 (10).

Transgenic mouse models of breast cancer develop spontaneous mammary tumors with low to moderate tumor-infiltrating lymphocytes, but significant levels of IgG antibodies directed against tumor-associated antigens (4, 11–13). The TgMMTV-neu, a transgenic mouse model representing luminal B breast cancer displays an antibody repertoire similar to that of postmenopausal women with breast cancer including multiple antibodies targeting proteins involving glycolysis and spliceosome (e.g., ALDOA, ENO1, and PKM2; ref. 11). The C3(1)-Tag mouse, a model of basal mammary cancer develops tumor-associated autoantibodies that mirror those found in patients with triple-negative breast cancer including numerous keratins, ubiquitins, S100A11, and vimentin to name but a few (4). In both models, dozens of tumor-associated proteins could be identified bound to naturally derived tumors. We questioned whether treatment with 2FF could inhibit the growth of established tumors or even prevent the development of mammary cancers in mice.
Materials and Methods

Mouse models and associated cell lines

TgMMTV-neu mice [FVB/N-Tg(MMTVneu)202Mul/J, strain #002236; Jackson Laboratory] and C3(1)-Tag mice [FVB-Tg(C3-1-TAg)cEgg/Tgg; Dr. J Green, NIH, Bethesda, MD] were established and maintained as described previously (13). Animals were housed in a specific pathogen-free facility at the University of Washington (Seattle, WA), were fed ad libitum with irradiated Picolab Rodent Diet 20 #5053 (PMI Nutrition International), and provided nestlet enrichment. Work described was performed in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines. The mouse mammary tumor cell lines MMC and M6 were derived from spontaneous mammary tumors from TgMMTV-neu (14) and C3(1)-Tag mice (15), respectively. Both cell lines were authenticated before use. The MMC cell line was verified to express rat neu by flow cytometry and the M6 cell line was verified to express the SV40 antigen by Western blot analysis.

2FF treatment of established mammary tumors

2FF (20 mmol/L or 50 mmol/L) was prepared in autoclaved sterile drinking water by agitating gently until thoroughly dissolved. Doses were chosen based on previous experiments which showed that afucosylation of circulating IgG decreased dose dependently, approaching 100% by 100 mmol/L and that both 20 and 50 mmol/L doses provided tumor growth delay in xenografts (10). The resulting solution was sterile filtered and stored at room temperature. Water bottles were filled with 2FF containing solutions or water and refilled 1–2x per week. Control (water only), 2FF-treated TgMMTV-neu mice, or 2FF-treated C3(1)-Tag mice were randomized into treatment groups at 8 weeks of age and observed weekly for spontaneous tumor development. When the first palpable tumor reached a volume of 75 mm³, mice were provided 20 mmol/L 2FF or plain water ad libitum until the end of the experiment (Supplementary Fig. S1). Tumors were measured 2–3 times per week, as previously described, until tumor volume exceeded 1,000 mm³, the animal became sick, or developed tumor ulcers (13).

Prevention of mammary tumor formation

TgMMTV-neu mice were randomized into treatment groups at a mean (SEM) of 6.8 (1.5) weeks while C3(1)-Tag mice were randomized at 5 (1.5) weeks of age due to the earlier onset of tumors in this strain (13). Either plain autoclaved water, 20 mmol/L 2FF water, or 50 mmol/L 2FF water was provided ad libitum to mice upon randomization as described previously (Supplementary Fig. S1). For TgMMTV-neu mice, 2FF was discontinued at an average of 22–28 weeks of age due to insufficient weight gain. For the C3(1)-Tag mice, both doses of 2FF were discontinued at an average of 28 weeks of age to ensure similar treatment times between strains. Animals were observed twice weekly for tumors then lesions were measured 2–3 times per week until sacrifice. Age of tumor onset was calculated as the [(date of first palpable tumor observation) – (mouse date of birth)], accurate to 4 days. We have previously shown that TgMMTV-neu mice develop tumors at a mean and SD of 35.1 (5.9) weeks of age (13). Therefore, we excluded tumor-free animals from tumor-free survival curves if they died prior to 23.3 weeks of age [n = 1, 20 mmol/L (death during blood draw) and n = 2, 50 mmol/L (death due to unspecified reasons)].

Estimation of tumor growth rate

Tumor growth rates were calculated as described previously, with the exception that the average growth rate was used in place of the median growth rate per animal (13). Mice with tumor growth lasting ≤7 days or with ≤3 measurements were excluded from all tumor rate analysis due to insufficient data (TgMMTV-neu mice: n = 2, 20 mmol/L; n = 1, 50 mmol/L, and C3(1)-Tag: n = 1, untreated; n = 3, 50 mmol/L). Nontumor-bearing TgMMTV-neu mice >35 weeks old and C3(1)-Tag mice >19 weeks old were assigned a growth rate of 0.0 mm³/m. TgMMTV-neu mice: n = 1, untreated; n = 3, 20 mmol/L; and n = 7, 50 mmol/L and C3(1)-Tag: n = 2, untreated; n = 6, 20 mmol/L; n = 4, and 50 mmol/L. Growth rate was reported as change in first primary tumor volume (mm³/day).

Cellular immune response

For delayed type hypersensitivity (DTH) response, MMC (TgMMTV-neu) and M6 (C3(1)-Tag), syngeneic mammary tumor cell lines, were lysed over three consecutive freeze thaw cycles. Cell lines were authenticated before use as described previously (16). Protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s directions. Twenty to 30 days after treatment with 2FF was initiated, 30 μg of MMC or M6 lysate was applied topically to the right ear of the mice in both the therapeutic and preventative studies. The lysate was administered in a 1:1 mixture in DMSO in a total volume of 20 μL. The right ear was measured before challenge and again 24 hours after challenge in three replicate measurements per timepoint by the same operator. The response was calculated as (average 24 hours measurements) – (average prechallenge measurements) to determine the change in ear thickness (in mm). In the case where a prechallenge measurement was not taken, raw 24-hour measurements (mm) were compared.

Spleens were processed as described previously (16). IFNy ELISPOT was then performed on 3.5 × 10⁷ splenocytes per well, as described previously (16) with the exception that Cyclin B1 (1 μg/mL) and MMC lysate (1 μg/mL) were included as antigens. Data are reported as the corrected spots per well which is the mean number of spots for each experimental antigen minus the mean number of spots detected in no antigen control wells ± SD.

Immune cell depletion

Mice were randomized into treatment groups at 6–8 weeks of age and provided either with 20 mmol/L 2FF in autoclaved water or normal autoclaved water. Two weeks later, mice were injected intraperitoneally daily for 3 consecutive days with antibodies; 100 μg IgG2b as an isotype control [LTF-2 (University of California San Francisco, UCSF), San Francisco, CA)] or RG7/11.1 (BioXCell), 300 μg anti-CD4 [GK1.5 (UCSF, San Francisco, CA)], 100 μg anti-CD22 [Cy34.1 (BioXCell); ref. 17], 100 μg anti-CD8 [2.43 (UCSF, San Francisco, CA)], 100 μg anti-NK [PK136 (UCSF, San Francisco, CA)], or no treatment. Four days after antibody priming, mice were injected subcutaneously with 5 × 10⁶ MMC. The cell-depleting antibodies were then continued twice weekly for the duration of the study. The percent depletion of each antibody for its target was 99.8%, CD4; 97% CD22 (peripheral blood), 94.7% CD8, and 82% NK. Example depletions are shown in Supplementary Fig. S2.

Evaluation of IgG fucosylation and in vitro cell growth

IgG was isolated from 2FF treated and untreated mouse sera from the therapeutic MMC study (collected at day 88), as described previously and tested for fucosylation using a lectin-binding assay (10). The amount of IgG recovered was quantitated using UV and an
extinction coefficient $E_{280}$ (1%) of 1.4 for murine IgG. A standard curve was generated using a titration of pooled untreated IgG. Test samples were coated in 96-well ELISA plates, washed with PBST, blocked with 1% BSA/PBST, and washed again followed by incubation with fucose-binding *Aleuria aurantia* lectin-biotin (AAL-biotin, 0.5 μg/mL in 1% BSA/PBST, Vector Laboratories). After incubation, plates were washed and treated with Poly-horseradish peroxidase streptavidin in 1% BSA/PBST. After a final wash, signal was generated with TMB solution (Invitrogen), quenched with sulfuric acid, and read at 450 nm in a 96-well plate reader (Molecular Devices SpectraMax Plus). Sample signals were converted to concentration of fucosylated IgG using the untreated IgG standard curve. Percent fucosylated IgG of untreated was calculated according to the formula: \(\frac{\text{mean concentration of fucosylated IgG in untreated pooled IgG}}{\text{mean concentration of fucosylated IgG in untreated IgG}}\) × 100%. Growth of MMC and M6, either untreated or pretreated with or without 100 μmol/L 2FF for >7 days was evaluated in the presence or absence of 100 μmol/L 2FF using the CellTiter-Glo Cell Viability Assay (Promega) according to the manufacturer’s instructions. Plates were plated in 96-well plates and growth was monitored by luminescence over 7 days.

**ADCC**

IgG was purified from murine serum using HitTrap Protein G HP Columns (Sigma) according to the manufacturer’s instructions. Evaluation of ADCC was performed using Calcein-Acetoxymethyl Release Assay (Calcein-AM; Life Technologies). MMC were labeled with Calcein-AM for 45 minutes at 37°C. After washing, the cells were plated at 1 × 10⁴ cells/well. IgG was purified from serum from mice either untreated or treated with 2FF and added at a concentration of 25 μg/mL. Lymphocytes were added as effector cells at an effector to target ratio (E:T) of 10:1, 25:1, 50:1, and 100:1. For maximal release, the 25 was generated using a titration of pooled untreated IgG standard curve. Percent fucosylated IgG of untreated was calculated according to the formula: \(\frac{\text{mean concentration of fucosylated IgG in untreated pooled IgG}}{\text{mean concentration of fucosylated IgG in untreated IgG}}\) × 100%. Growth of MMC and M6, either untreated or pretreated with or without 100 μmol/L 2FF for >7 days was evaluated in the presence or absence of 100 μmol/L 2FF using the CellTiter-Glo Cell Viability Assay (Promega) according to the manufacturer’s instructions. Plates were plated in 96-well plates and growth was monitored by luminescence over 7 days.

**Cell surface fucose analysis**

MMC and M6 cells were cultured with or without 100 μmol/L 2FF for >7 days. Cells were harvested and washed with FACS buffer (PBS, 2% FBS, 0.02% sodium azide) and stained with biotinylated-LCA, -AAL (Vector Laboratories) or -AOL (TCI America) on ice. Cells were washed and resuspended in FACS buffer, followed by incubation with FITC-conjugated avidin D on ice (Vector Laboratories, Burlington, CA), washing, and then analysis by FACS (Attune NXT).

**Assessment of tumor cell–binding antibodies**

Evaluation of tumor-binding antibody using both the sera and purified IgG was performed using FACS. MMC cells were harvested and washed with FACS buffer (PBS, 2% FBS, 0.02% sodium azide) and incubated with 10 μg/mL purified IgG or 1:100 dilution of sera in FACS buffer on ice. Cells were washed and resuspended in FACS buffer followed by incubation with AF488 anti-mouse IgG/M on ice (Jackson ImmunoResearch Labs), washed, and analyzed by FACS.

**Cytokine analysis**

Frozen sera were thawed overnight at 4°C and processed for cytokine production using a Luminex Multiplex Kit (Millipore) as per the manufacturer’s instructions. Briefly, assay plates were washed with 200 μL of wash buffer per well, followed by addition of 25 μL standard or buffer, 25 μL matrix or sample, and 25 μL of multiplexed analyte beads to each well. Samples were incubated overnight with vigorous shaking at 4°C. Plates were washed twice with wash buffer.

**Results**

All graphing and statistical analysis was performed with GraphPad Prism Software v7.01. Comparing one variable between two treatment groups was done using a Student t test. Comparing one variable between three or more treatment groups was performed using a one-way ANOVA with Tukey multiple comparison post-test. Survival was compared using a Gehan–Breslow–Wilcoxon test and the Mantel–Haenszel hazard ratio (HR). Linear regression comparisons were performed to determine a correlation coefficient and whether the slope of the linear regression fit was significantly nonzero. Comparing two variables between two or more treatment groups was performed using a two-way ANOVA. Analysis was all two-sided. Significance was considered at $P < 0.05$, except in survival analysis of three groups where significance was considered at $P = 0.05/3 = 0.017$ to correct for multiple comparisons.

**Statistical analysis**

All graphing and statistical analysis was performed with GraphPad Prism Software v7.01. Comparing one variable between two treatment groups was done using a Student t test. Comparing one variable between three or more treatment groups was performed using a one-way ANOVA with Tukey multiple comparison post-test. Survival was compared using a Gehan–Breslow–Wilcoxon test and the Mantel–Haenszel hazard ratio (HR). Linear regression comparisons were performed to determine a correlation coefficient and whether the slope of the linear regression fit was significantly nonzero. Comparing two variables between two or more treatment groups was performed using a two-way ANOVA. Analysis was all two-sided. Significance was considered at $P < 0.05$, except in survival analysis of three groups where significance was considered at $P = 0.05/3 = 0.017$ to correct for multiple comparisons.

**Treatment with 2FF significantly improves survival, inhibits tumor growth, and induces systemic tumor-specific immunity in mice with established spontaneous mammary tumors**

We sought to evaluate drug activity in two transgenic mouse models that were genetically and pathologically distinct (13). Mammary tumors in the TgMMTV-neu mouse display a gene expression profile similar to HER2+ luminal B breast cancer (18). Oral administration of 20 mmol/L 2FF, when mice had established tumors, resulted in improved overall survival compared with untreated animals ($P < 0.0001$; Fig. 2A). Animals in the untreated group survived a median of 46 days beyond tumor onset. Mice treated with 2FF doubled survival time to a median of 94 days after treatment was initiated (HR, 7.04; 95% CI, 3.31–15.0). Mammary tumors in the C3(1)-Tag mouse are of a basal phenotype and do not express HER2 or estrogen receptor (18). Treatment with 20 mmol/L 2FF at the time of established tumors also resulted in improved overall survival compared with untreated animals ($P = 0.0013$; Fig. 2A). Animals in the untreated group survived a median of 28 days beyond tumor onset. Mice treated with 2FF survived a median of 43 days after treatment was initiated (HR, 3.36; 95% CI, 1.58–7.14).

To assess the development of a systemic tumor-specific immune response, mice were tested by DTH. A significantly greater local inflammatory response to MMC was induced in TgMMTV-neu 2FF-treated mice as compared with untreated mice ($P < 0.0001$; Fig. 2B). The change in ear thickness (mean ± SEM) of control animals was 0.004 ± 0.003 mm, compared with 0.187 ± 0.024 mm in treated animals. Similarly, DTH responses to M6 were significant in the C3(1)-Tag 2FF-treated mice as compared with controls ($P < 0.0001$; Fig. 2B). The change in ear thickness of control animals was 0.005 ± 0.003 mm, compared with 0.152 ± 0.025 mm in treated animals.

2FF treatment also significantly inhibited the rate of tumor growth in both models. We found that the average tumor growth rate of
TgMMTV-neu controls was significantly higher than that of 2FF-treated animals ($P < 0.0001$; Fig. 1C). Controls demonstrated a mean (SEM) tumor growth rate of 17.3 (2.2) mm$^3$/day, whereas the tumors of animals receiving 2FF treatment progressed at less than half of this rate, 7.30 (0.57) mm$^3$/day. The average rate of tumor growth of control C3(1)-Tag animals was significantly higher than that of 2FF-treated animals ($P = 0.0008$); 27.1 (3.0) mm$^3$/day as compared with 14.0 (2.0) mm$^3$/day in treated mice (Fig. 2C). We observed TgMMTV-neu mice with the most robust DTH responses had the slowest growing tumors; correlation coefficient 0.2034 ($r = 0.451$; $P = 0.0017$; Fig. 1C). No

![Figure 1.](image1)

Treatment with 2FF significantly improves survival, inhibits tumor growth, and induces systemic tumor-specific immunity in mice with established luminal B mammary tumors. A, Kaplan–Meier curve (solid line) and SEM (dashed line) with percent TgMMTV-neu mice alive at indicated time since tumor onset is shown for untreated (gray, $n = 24$) and 20 mmol/L treated mice (black, $n = 23$). B, DTH (mean change in ear thickness ± SEM) is shown for control ($n = 23$) and 20 mmol/L treated mice ($n = 25$). C, Growth rate (mm$^3$/day) of tumors is compared with DTH response ($n = 46$; ****, $P < 0.0001$).

![Figure 2.](image2)

Treatment with 2FF significantly improves survival, inhibits tumor growth, and induces systemic tumor-specific immunity in mice with established basal mammary tumors. A, Kaplan–Meier curve (solid line) and SEM (dashed line) with percent C3(1)-Tag mice alive at indicated time since tumor onset is shown for untreated (gray, $n = 20$) and 20 mmol/L treated mice (black, $n = 20$). B, DTH response (mean change in ear thickness ± SEM) is shown for control ($n = 18$) and 20 mmol/L treated groups ($n = 20$). C, Growth rate (mm$^3$/day) of tumors is compared with DTH response ($n = 38$; ****, $P < 0.0001$).
association was evident between the DTH response and rate of tumor growth in the C3(1)-Tag mouse, correlation coefficient 0.065 ($r = 0.255; P = 0.123$; Fig. 2C).

**Treatment with 2FF initiates ADCC and stimulates functional tumor-specific T cells**

2FF augments ADCC by inhibiting fucosylation of antibodies (10). In this study, fucosylation of IgG from 2FF-treated mice was shown to be reduced to 2% of the untreated IgG (Supplementary Fig. S3B). There was no difference in the total amount of IgG isolated from sera of untreated versus treated mice (Supplementary Fig. S3A). Pooled Ig derived from mice treated with 2FF mediated significantly enhanced lysis of MMC target cells at each E:T ratio as compared with pooled Ig collected from untreated controls ($P = 0.0040$; Fig. 3A). Evaluation of the ability of antibodies in the sera to bind MMC cells using FACS demonstrated that there was no increase in the amount of tumor-binding antibody in 2FF-treated sera compared with untreated (Supplementary Fig. S4A).

In addition, splenocytes from 2FF-treated TgMMTV-neu mice responded to stimulation with MMC lysate, in an IFN-$\gamma$ ELISPOT, with a mean corrected spots per well of 35.8 ± 6.08 as compared with 3.78 ± 2.61 spots in untreated animals ($P < 0.0001$; Fig. 3B). No significant difference was observed between control and 2FF-treated animals when splenocytes were stimulated with a nonspecific foreign peptide derived from the human immunodeficiency virus protein sequence ($P = 0.967$) or human Cyclin B1 protein ($P = 0.927$) demonstrating specificity of the cellular immune response to the tumor in treated mice. In *in vivo* tumor inhibition was partially mediated by CD4 T cells. When mice treated with 2FF were depleted of CD4 T cells, the antitumor effects of 2FF were significantly reduced compared with the 2FF + IgG-treated mice ($P < 0.0001$; Fig. 3C). Depletion of CD22-expressing cells had no impact on tumor growth when compared with 2FF + IgG–treated mice ($P = 0.998$; Fig. 3D). Similarly, 2FF-treated mice depleted of CD8 (Supplementary Fig. S5A) and NK (Supplementary Fig. S5B) T cells demonstrated no change in tumor growth when compared with 2FF + IgG–treated mice ($P = 0.978$ and $P = 0.144$, respectively). Furthermore, *in vitro* fucosylation of the syngeneic MMC cell line was assessed by lectin binding. The 2FF-treated cells showed reduction in binding of LCA, AOL, and AAL (UEA did not bind the cells). The reduced fucosylation of cells is consistent with results observed previously (19). This suggests that fucosylation is reduced following 2FF treatment, but treatment did not affect the rate of *in vitro* cell growth, which was identical to the control (Supplementary Fig. S4C).

**Prophylactic administration of 2FF significantly delays or prevents the development of HER2$^{+}$ luminal B and basal mammary tumors**

Daily oral administration of 2FF was reported to be well tolerated in mice (10), so we questioned whether the agent could be used chronically for mammary cancer prevention. We evaluated two different

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**Figure 3.** Treatment with 2FF initiates ADCC and stimulates functional tumor-specific T cells. A, Percent-specific lysis of MMC from 2FF treated ($n = 5$; ○) or untreated ($n = 5$; □) murine splenocytes by E:T ratio. B, IFN-$\gamma$ corrected spots per well (CSPW) for specific antigens or controls for TgMMTV-neu mice treated with water (□), $n = 8$ or 20 mmol/L 2FF (○, $n = 8$). C, Tumor volume over days since implant for mice treated with IgG2b (□), anti-CD4 (○), 20 mmol/L 2FF + IgG2b (●), or 20 mmol/L 2FF + anti-CD4 (▲), $n = 5$ per group. D, Tumor volume over days since implant for mice treated with IgG2b (□), anti-CD22 (○), 20 mmol/L 2FF + IgG2b (●), or 20 mmol/L 2FF + anti-CD22 (▲), $n = 5$ per group (*$; P < 0.01$; **$; P < 0.0001$; ns, not significant).
Inhibiting Fucosylation to Treat and Prevent Breast Cancer

In the TgMMTV-neu, oral delivery of either dose resulted in a significantly delayed tumor onset compared with untreated animals (20 mmol/L: P = 0.0004; 50 mmol/L: P = 0.0002; Fig. 4A). The improvement was not dose dependent (P = 0.743). 2FF treatments were stopped because of insufficient weight gain when compared with untreated TgMMTV-neu animals (Supplementary Fig. S6), an effect that was not dose dependent (P = 0.5717). This weight loss was not observed in the treatment experiments and the mice did not have any other sequelae which would indicate toxicity. 2FF treatment was concluded at an average of 22.8 weeks of age for the 50 mmol/L treatment group, at which time animals had gained a mean (SEM) of 3.11 (0.62) compared with 5.36 (0.45) grams in untreated animals (P = 0.0039). Subsequently, 2FF treatment was concluded at an average of 27.7 weeks of age for the 20 mmol/L group, at which time animals had gained 3.78 (0.26) compared with 5.09 (0.41) grams in untreated animals (P = 0.0092).

Six of 16 (38%) mice at the 20 mmol/L and 4 of 14 (29%) mice at the 50 mmol/L dose never developed a mammary mass. Animals in the control group developed tumors at a median of 28 weeks and 2FF administration delayed tumor onset compared with controls was 20 mmol/L: 42.3 weeks, P = 0.021; 50 mmol/L: 42.3 weeks, P = 0.015; and untreated: 35.5 weeks. After correcting for multiple comparisons, the overall survival for 2FF-treated TgMMTV-neu compared with controls was 20 mmol/L: 42.3 weeks, P = 0.021; 50 mmol/L: 42.3 weeks, P = 0.015; and untreated: 35.5 weeks and for 2FF treated C3(1)-Tag compared with controls was 20 mmol/L: 28.7 weeks, P = 0.095; 50 mmol/L: 30.1 weeks, P = 0.019; and untreated: 25.7 weeks.
The kinetics of tumor growth was significantly slower in treated TgMMTV-neu mice than that observed in control mice. For control animals, the mean (SEM) of tumor growth rate was 16.0 (1.92) mm^3/day versus 8.84 (2.32) mm^3/day (20 mmol/L, \( P = 0.047 \)) and 7.32 (2.37) mm^3/day (50 mmol/L, \( P = 0.033 \)). When tumors arose in C3(1)-Tag, there was no significant difference between the rate of tumor growth in control versus 2FF groups. The mean (SEM) of growth rate was 25.9 (3.73) mm^3/day (untreated) versus 22.0 (7.32) mm^3/day (20 mmol/L, \( P = 0.888 \)) and 17.7 (6.95) mm^3/day (50 mmol/L, \( P = 0.592 \)).

Both strains developed an inflammatory response to syngeneic tumor cell lysates as compared with controls as measured by ear swelling. In the TgMMTV-neu, MMC lysate induced a significantly greater local inflammatory response in 2FF-treated mice compared with untreated mice (20 mmol/L, \( P = 0.0036 \) and 50 mmol/L, \( P < 0.0001 \); Fig. 4B). For the C3(1)-Tag, there was also a significantly greater local inflammatory response in 2FF-treated mice compared with untreated mice (20 mmol/L, \( P = 0.0087 \) and 50 mmol/L, \( P < 0.0001 \); Fig. 5B).

Significant levels of tumor-specific IFNγ-secreting T cells were documented by ELISPOT in the TgMMTV-neu at both the 20 (\( P < 0.0001 \)) and 50 mmol/L (\( P < 0.0001 \)) 2FF doses compared with controls demonstrating a T-cell response could also be generated in the prophylactic setting (Supplementary Fig. S7).

**Discussion**

2FF is a novel immune modulator that can enhance ADCC of endogenous IgG. The ability to manipulate the large tumor-associated antibody repertoire present in most patients with cancer offers an immunotherapeutic approach particularly suited to common solid tumors. These studies were designed to assess the antitumor activity of 2FF in relevant mouse models of breast cancer, and data presented here demonstrates that afucosylation of endogenous antibodies in addition to T-cell modulation and possible direct effects of afucosylation on the tumor may well contribute to growth inhibition of established spontaneous mammary tumors. Serum derived from 2FF-treated mice contains afucosylated antibodies that more potently mediate lysis of tumor cells compared with control sera. Moreover, the 2FF-treated mice develop tumor-specific type I T cells in the peripheral blood that contribute to the antitumor activity as evidenced by the DTH response and the negative effect of depleting CD4^+ T cells on the activity of 2FF. Finally, oral administration of 2FF can inhibit or prevent the development of cancer in mice indicating a potential use in cancer prevention or adjuvant therapy.

Antibodies directed against tumor-associated antigens are found in patients with all stages of breast cancer. Although most patients with breast cancer have a vigorous humoral immune response directed against their tumor, few studies have been able to show that antibodies directed against specific tumor antigens have prognostic importance (20, 21). One potential reason for tumor specificity but lack of antitumor activity is dysfunction of Fc binding to Fc gamma receptors (22). Matrix metalloproteinases present in cancer can cleave the hinge region of IgG which results in sIgGs with reduced FcγR binding and effector function. Removal of the core fucose residue from the Fc of IgG will result in increased affinity of Fc for the FcγIIa receptor and enhanced ADCC (9) via stabilization of the FcγIIa receptor–ligand interaction. Removal of fucose may also increase antibody-dependent cellular phagocytosis as a result of increased binding of human IgG to hFcyRIIb, and in mice mlgG to mFcyV (10, 23–25). We show, in this study, that 2FF treatment leads to a 98% reduction in fucosylation of endogenous antibody. 2FF treatment significantly augmented the ADCC effect of endogenous antibody as assessed by tumor cell lysis. These data are in-line with increased ADCC of resident antibodies possibly playing a role in the antitumor response. The generation of new systemic tumor-specific type I T cells, supported by the DTH response and the negative effect of depleting CD4^+ T cells on the activity of 2FF, suggests that afucosylated endogenous antibodies may elicit activation of NK cells by this enhanced interaction and secretion of IFNγ in turn activating antigen-presenting cells in the tumor microenvironment to initiate a cellular immune response. This possibility is in-line with amplified systemic cytokines observed following 2FF treatment; IL5, GCSF, IL6, IL12p70, IL15, and IP10 were elevated in the 2FF-treated groups (Supplementary Fig. S8). Alternatively, 2FF may have direct impact on the activity of T cells. Through either mechanism, the impact of CD4^+ T-cell depletion on efficacy implies that an amplification of the T-cell response resulting from 2FF treatment may also play a role in the antitumor activity observed (26).

Antibody immunity directed against tumor-associated proteins, in many cases, predates the diagnosis of breast cancer. Proteomic analysis of plasma samples derived from women enrolled in the Women’s Health Initiative Observational Study, and collected a year prior to the diagnosis of triple-negative breast cancer, demonstrated robust networks of antibodies directed against proteins involved in the pathogenesis of the disease (e.g., p53 and cytokeratin networks) as compared with case-matched controls (4). Autoantibody signatures specific for numerous glycolysis and spliceosome proteins precede the detection of breast cancer in postmenopausal women demonstrating the development of tumor-directed humoral immunity prior to a breast cancer diagnosis occurs in all breast cancer subtypes (11). Similar to human breast cancer, transgenic mice developing spontaneous mammary tumors also develop prediagnostic antibody immunity to evolving breast lesions (12). The autoantibody repertoire predating mouse mammary cancers is highly similar to those identified in humans allowing a model to assess the manipulation of antibody function for breast cancer prophylaxis. Administration of 2FF to mice prior to the development of breast cancer significantly delayed tumor development and completely inhibited the development of breast cancer in 33% of TgMMTV-neu and 26% of C3(1)-Tag mice. Systemic tumor-specific immunity was also generated suggesting similar mechanisms of action in stimulating effective antitumor immunity in both the prophylactic and treatment setting. The generation of long-lived antibody and cellular immunity by 2FF therapy may also represent an opportunity to evaluate prevention of disease recurrence in the adjuvant setting.

There are some limitations to our study. First, our work is constrained to two transgenic mammary mouse models so we do not know the potential applicability to other cancers. Second, the C3(1)-Tag mouse develops a highly aggressive tumor with many lesions present in the animals at birth (13). The early lesions and rate of tumor growth may have limited the ability to develop a robust immune response rapidly enough to have an impact on tumor progression. Furthermore, additional mechanisms may contribute to the antitumor activity we observe, such as the direct effects on the tumor or on other components of the tumor microenvironment. Finally, MMTV infection can impact cells of the immune system and there may be a role for MMTV in contributing to the immune responses observed in that mouse model.

T-cell–based immune therapy is showing clinical benefit in the treatment of selected cancer patients. Benefit is predicted by evidence of type 1 immunity and significant levels of T cells infiltrating the cancer (6). For most patients with solid tumor, tumor-infiltrating lymphocytes are present in modest numbers but systemic naturally
occuring tumor-associated antibodies are abundant. 2FF offers a novel approach to harnessing the endogenous antibody response present in patients with cancer for therapeutic purpose.

Disclosure of Potential Conflicts of Interest
M.L. Disis reports receiving a commercial research grant from Seattle Genetics, Pfizer, Celgene, EMD Sorono, Precigen, and Janssen and has ownership interest (including patents) in Epithany. L.R. Corulli has ownership interest (including patents) in Seattle Genetics (stockholder). M.R. Koehnlein has ownership interest (including patents) in Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

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