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The small molecule TGF- β signaling inhibitor SM16 synergizes with agonistic OX40 antibody to suppress established mammary tumors and reduce spontaneous metastasis

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Abstract

Effective tumor immunotherapy may require not only activation of anti-tumor effector cells, but also abrogation of tumor-mediated immunosuppression. The cytokine, TGF- β , is frequently elevated in the tumor microenvironment and is a potent immunosuppressive agent and promoter of tumor metastasis. OX40 (CD134) is a member of the TNF- α receptor superfamily and ligation by agonistic antibody (anti-OX40) enhances effector function, expansion and survival of activated T cells. In this study, we examined the therapeutic efficacy and anti-tumor immune response induced by the combination of a small molecule TGF- β signaling inhibitor, SM16, plus anti-OX40 in the poorly immunogenic, highly metastatic, TGF- β -secreting 4T1 mammary tumor model. Our data show that SM16 synergizes with anti-OX40 to elicit a potent anti-tumor effect against established primary tumors, with a 79% reduction in tumor size, a 95% reduction in the number of metastatic lung nodules and a cure rate of 38%. This positive treatment outcome was associated with a 3.2-fold increase of tumor-infiltrating, activated CD8⁺ T cells, an overall accumulation of CD4⁺ and CD8⁺ T cells, and an increased tumor-specific effector T cell response. Complete abrogation of the therapeutic effect *in vivo* following depletion of CD4⁺ and CD8⁺ T cells, suggests that the anti-tumor efficacy of SM16 + anti-OX40 therapy is T cell dependent. Mice that were cured of their tumors were able to reject tumor re-challenge and manifested a significant tumor-specific peripheral memory IFN- γ response. Taken together, these data suggest that combining a TGF- β signaling inhibitor with anti-OX40 is a viable approach for treating metastatic breast cancer.

Keywords

Breast cancer; 4T1; OX40; TGF- β ; TGF β RI/ALK5; small molecule kinase inhibitor; SM16

INTRODUCTION

Transforming growth factor-beta (TGF- β) is a pleiotropic cytokine with multiple functions key among which is the ability to potently suppress the anti-tumor immune response [1]. TGF- β is produced in copious amounts by tumor cells of different histological types and elevated serum levels of TGF- β commonly seen in cancer patients have been associated with tumor progression, metastases formation and disease recurrence [2–8]. These tumor-

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CONFLICT OF INTEREST

The authors report no conflict of interest.

promoting activities of TGF- β have provided a rationale for developing strategies to improve anti-tumor response by targeting TGF- β using antibodies [9–13] and fusion proteins [14,15] to neutralize TGF- β or small molecule TGF- β receptor kinase inhibitors to block TGF- β signaling [16–23]. SM16 is a small molecule, orally bioavailable TGF- β signaling inhibitor that binds to the kinase active site of TGF- β type I receptor (ALK5) and prevents phosphorylation of downstream Smad2 and Smad3 proteins following ligand engagement [19,23,24]. We recently demonstrated that oral SM16 administered in mouse chow, abrogates TGF- β signaling in tumors, inhibits growth of established 4T1 mammary tumors and reduces spontaneous metastasis [23]. The ease of oral delivery of SM16 underscores its translational potential, as it could be administered to cancer patients in pill form.

OX40 (CD134) is a member of the TNF- α receptor (TNFR) superfamily and is up-regulated on activated CD4⁺ and CD8⁺ T cells [25,26]. OX40 ligation by its cognate ligand (OX40L) or agonistic antibody (anti-OX40) enhances effector function, expansion and survival of activated T cells [25,27–30]. In addition to these effects, anti-OX40 has been shown to inhibit regulatory T (Treg) cell development and function [31–34] and decrease the number of tumor-infiltrating Tregs [35]. In several mouse tumor models, ligation of OX40 by anti-OX40 has been shown to significantly improve the frequency of durable tumor regressions and increase survival of treated mice [35–38]. Based on the individual salutary effects of SM16 and anti-OX40 on the tumor microenvironment, we hypothesized that concurrent anti-OX40 and SM16 treatment of established 4T1 mammary tumors will lead to improved anti-tumor responses and increase the frequency of tumor regressions.

In this study, we assessed the anti-tumor activity of SM16 in combination with anti-OX40 antibody against established primary mammary tumors and their metastases as well as changes in the tumor microenvironment (TME) during the combination therapy. The data demonstrate that anti-OX40 and SM16 therapy mutually enhanced each other in suppressing primary tumor growth and metastasis. The combination therapy stimulated regression of established tumors and induced a memory response capable of rejecting tumor re-challenge. Furthermore, the anti-tumor response was characterized by recruitment of activated CD4⁺ and CD8⁺ T cells to the tumor microenvironment. In summary, the results suggest that blockade of TGF- β signaling in combination with OX40 co-stimulation is a viable strategy for treating metastatic breast cancer.

MATERIALS AND METHODS

Tumor cells and cell culture

The 4T1 tumor cell line is a poorly immunogenic, highly metastatic variant of 410.4, a tumor sub-line isolated from a spontaneous mammary tumor that developed in a BALB/c/c3H mouse [39]. It was kindly provided by Dr. Fred Miller of the Michigan Cancer Foundation (Detroit, MI). The 4T1 cells were maintained for a limited time *in vitro* by passage in DMEM (Lonza, Walkersville, MD), containing 100 U/mL penicillin, 100 mg/mL streptomycin (Hyclone Laboratories, Logan, UT), 0.025 mg/mL Amphotericin B (Hyclone) and 10% fetal bovine serum (FBS) (Lonza). The Renca cell line was derived from a tumor that arose spontaneously as a renal cortical adenocarcinoma in BALB/cCr mice [40] and was purchased from the American Tissue Culture Collection (ATCC). The Renca cells were cultured in RPMI-1640 (Lonza) containing antibiotics as above with the addition of 0.1 mM non-essential amino acids (Lonza), 1 mM sodium pyruvate (Lonza), 2 mM L-glutamine (Lonza) and 10% fetal bovine serum (FBS) (Lonza).

SM16 and control diets

SM16 was synthesized by Biogen Idec (Cambridge, MA) and was incorporated into standard Purina rodent chow (#5001) by Research Diets (New Brunswick, NJ) at a concentration of 0.3 g SM16 per kg chow (0.03%). A calorie and nutrient-matched diet without SM16 (Purina) was used as the control diet

Reagents

The agonistic anti-OX40 antibody (Rat IgG1 isotype) was kindly provided by Dr. Andrew Weinberg (Earle A. Chiles Cancer Research Institute, Portland, OR) or purchased from BioXCell (West Lebanon, NH). Rat IgG1 isotype control antibody was purchased from BioXCell.

Animals

Six-week-old female BALB/c mice were purchased from the Harlan Laboratory (Indianapolis, IN). All mice were housed at the Providence Cancer Center Vivarium in accordance with the Principles of Animal Care (NIH publication no. 85-23, revised 1985). All studies were reviewed and approved by the institutional animal care and use committee (IACUC) of the Earle A. Chiles Research Institute.

Animal Studies

In vivo tumor establishment and therapy—All mice received a subcutaneous (s.c.) injection of 5×10^4 4T1 cells into the mammary pad. Ten days later, when tumors became palpable ($\sim 22 \text{ mm}^2$), mice were randomized into the following treatment groups: control diet only (+rat IgG1; isotype control for anti-OX40), 0.03% SM16 diet only (+rat IgG1), anti-OX40 only (+control food), SM16 diet + anti-OX40. Mice were placed on standard mouse diet or SM16 diet 5 days prior to the first antibody injection. Mice received 3 injections of OX40 (250 μg /injection/mouse) or isotype control antibody on day 15 (when the average tumor size in all groups was $\sim 40 \text{ mm}^2$), day 18 and day 21 post-tumor implantation. Tumors were measured every 3–4 days for the duration of the study, and all mice were sacrificed when the tumors in the control group reached $>200 \text{ mm}^2$, or animals were moribund (~ 4 weeks post-tumor cell injection). Tumor growth was determined by measuring tumor length (L) and width (W) and tumor size (mm^2) was calculated using the formula $L \times W$. Mice and chow were weighed every 4 days. Tumor-draining lymph nodes (TDLN), and tumors were removed for FACS analysis, and lungs were fixed and stained for enumeration of metastases as previously described [41].

T cell depletion experiments—For T cell depletion, mice were administered intraperitoneal (i.p.) injections of 200 μg of CD4-specific (GK1.5) and/or CD8-specific (2.43) antibodies (BioXCell, West Lebanon, NH) on day 9 post-tumor injection (one day before initiation of SM16 treatment) and weekly thereafter. Control animals received rat-IgG2b (LTF-2) isotype control antibody (BioXCell). Depletion of CD4^+ and CD8^+ T cells was monitored in spleens by flow cytometry and was typically found to be greater than 98%.

Tumor re-challenge experiments—Tumor-free mice in the SM16 + anti-OX40 treatment group were transferred to normal diet on day 28 and observed for an additional 31 days to confirm that tumor regression was permanent. On day 59 post-tumor cell injection, the tumor-free mice were re-challenged with 5×10^4 4T1 cells on one flank, and 2×10^5 Renca cells on the opposite flank. Naïve mice injected with equivalent numbers of 4T1 or Renca tumor cells served as controls. Tumor growth in all groups of mice was monitored over the next 3 weeks.

Isolation of tumor-draining lymph node and tumor infiltrating immune cells

Inguinal tumor draining lymph nodes (TDLN) were resected and pushed through a 70 μ m nylon sieve (BD Biosciences Discovery Labware, Two Oaks, CA) to produce a single cell suspension. After red blood cell lysis, the cells were then washed (300xg, 7 min) and filtered through a 40 μ m nylon sieve (BD Biosciences). To isolate tumor infiltrating cells (TIC), tumors were resected and minced using scissors in triple enzyme digest mix containing 10 mg/mL collagenase type IV (Worthington Biochemical Corp. Lakewood, NJ), 1 mg/mL hyaluronidase (Sigma-Aldrich, St Louis, MO), 200 μ g/mL DNase I (Roche Applied Sciences, Indianapolis, IN) in Hank's balanced salt solution (HBSS, Lonza). The tumors were then incubated with agitation (37°C, 45 min). After addition of ethylenediaminetetraacetic acid (EDTA, 10 mM), the digest was incubated for another 15 min. The digested tissue was pushed sequentially through 70 μ m and 40 μ m nylon sieves, washed (300xg, 7 min), overlaid on Ficoll (FicoLite-LM, Atlanta Biologicals, Lawrenceville, GA) and centrifuged (1500xg, 25 min, slow brake). The interface was collected and washed twice (300xg, 7 min). These tumor infiltrating cells (TIC) were used for flow cytometric analysis and contain tumor infiltrating T cell but also other cell populations including natural killer cells (NK) cells and myeloid derived suppressor cells (MDSC).

In vitro stimulation of lymph node cells

Tumor-draining lymph nodes were pooled from mice in each of the treatment groups, mechanically dissociated into a single cell suspension and incubated at a concentration of 2×10^6 cells/mL with 2×10^5 irradiated (300 Gy) 4T1 or Renca tumor cells for 48 hr in a 24-well tissue culture plate in 1 mL RPMI-1640 containing 100 U/mL penicillin (Hyclone), 100 mg/mL streptomycin (Hyclone), 0.025 mg/mL Amphotericin B (Hyclone), 70 μ M beta-2-mercaptoethanol (Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids, 10 mM HEPES and 10% FBS. The supernatants were collected and assayed for IFN- γ and IL-4 production by ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA).

Flow cytometric Analysis

Tumor infiltrating cells were obtained after tumor disaggregation and ficoll enrichment and were incubated with pre-determined optimal concentrations of fluorophore-conjugated antibodies specific for surface or intracellular antigens and analyzed by 5- or 6-color flow cytometry on a LSR-II flow cytometer (BD Biosciences, San Jose, CA). Non-specific binding of antibody to Fc receptors (FcR) was blocked with purified FcR-blocking agent (anti-mouse CD16/32, eBioscience). Antibodies used for 5-color flow cytometry were: CD3-FITC (eBioscience, San Diego, CA), CD4-APC-H7 (BD-Pharmingen, San Diego, CA), CD8-Pacific Orange (Caltag, Carlsbad, CA), CD25-PE (Caltag) and Foxp3-APC (eBioscience). For intracellular Foxp3 staining, cells were permeabilized and fixed using a Fixation and Permeabilization kit (eBioscience). Antibodies used for 6-color flow cytometry were: CD3-PerCP-Cy5.5 (eBioscience), CD4-APC-H7 (BD-Pharmingen), CD8-Pacific Orange (Caltag), CD25-APC (eBioscience), CD44-FITC (eBioscience) and CD62L-PE-TR (Caltag). Cell viability was assessed using the LIVE/DEAD Fixable Violet stain (Invitrogen, Carlsbad, CA). Data were acquired using DIVA software (BD Biosciences) and analyzed using FlowJo v8.8.4 (Tree Star Inc., Ashland, OR).

Statistical Analysis

Statistical significance of differences among data sets of treatment groups was assessed by Student's *t*-test for pair wise comparisons or for comparisons of multiple groups by one-way analysis of variances (ANOVA) with Tukey's HSD test to adjust for multiple comparisons.

All analyses were performed using Prism software (GraphPad, San Diego, CA). Probability values (P) of ≤ 0.05 were considered indicative of significant differences between data sets.

RESULTS

Combination SM16 + anti-OX40 therapy elicits a potent anti-tumor effect against established primary tumors and spontaneous metastases

First, we evaluated the impact of concurrent anti-OX40 plus SM16 treatment on established tumors. For this purpose, mice with established tumors received SM16 diet for 18 days and 3 separate injections of anti-OX40 beginning on day 15 post-tumor cell injection. The data (Figure 1a) show that the combination treatment of anti-OX40 plus SM16 exerted the strongest anti-tumor effect. The average tumor size in this group on day 28 was 39 mm² compared to 185 mm² in the untreated group, which amounted to a 79% reduction in tumor area. Most importantly, 11 of 31 (38%) mice in the combination group were completely tumor free (Figure 1a, 1b). Singly, SM16 or anti-OX40 modestly suppressed primary tumor growth with average tumor sizes of 136 mm² and 118 mm² respectively. In the mice on SM16 diet, complete tumor regression was observed in only 2 of 32 mice (6%, Figure 1a, 1b). Each mouse consumed ~3 g food per day, which is equivalent to a daily dose of ~0.9 mg SM16/mouse. Animal weights were not significantly different between groups (data not shown) and SM16 and anti-OX40 were well tolerated with no overt signs of toxicity.

As with the control of primary tumor growth, SM16 + anti-OX40 treatment exhibited the most dramatic anti-metastatic effect, reducing the average number of lung metastases 20-fold from 124 visible nodules/mouse in the control mice to 6 visible lung nodules/mouse (Figure 1c). Interestingly, although SM16 alone had a modest effect on primary tumor growth, it significantly decreased the number of lung metastases 5-fold compared to control mice (Figure 1c). This decrease in the number of lung metastases was independent of primary tumor size (Figure 1d) as there was no proportional correlation between the number of metastases and tumor size in the SM16 and SM16 + anti-OX40 groups. This is in contrast to the control and the anti-OX40 alone groups where there was a clear positive correlation of increasing primary tumor size and the number of lung metastases.

SM16 + anti-OX40 treatment increases the frequency of total and activated tumor-infiltrating CD4⁺ and CD8⁺ T cells

To examine the changes occurring in the tumor microenvironment (TME) of mice treated with SM16 + anti-OX40 combination therapy, tumor infiltrating cells (TIC) were enriched from disaggregated tumors using a ficoll gradient. The recovered cell population contains tumor infiltrating T cell but also other cell populations including natural killer cells (NK) cells and myeloid derived suppressor cells (MDSC). TIC were analyzed for CD4⁺ and CD8⁺ content by flow cytometry (Figure 2a). The data (Figure 2b) show a substantially increased accumulation of CD4⁺ and CD8⁺ T cells in the tumors of SM16 + anti-OX40-treated mice. CD4⁺ T cells made up 11% of tumor-infiltrating cells in the combination treatment group compared to 6–8% in the other groups and there were 2–3 times more CD8⁺ T cells in the SM16 + anti-OX40 group (22%) compared to the other groups (range of 7–12%). Following the observation of increased frequency of tumor-infiltrating CD4⁺ and CD8⁺ T cells in the SM16 + anti-OX40 treatment group, we evaluated their activation status by staining with CD44 and CD62L antibodies and performing flow cytometric analysis. The analysis shows that in all groups, the majority (77% to 91%) of the infiltrating T cells had an activated (CD44⁺CD62L⁺) phenotype (Figure 2b). In the SM16 + anti-OX40 group, activated CD8⁺ T cells (CD44⁺CD62L⁺) made up 20% of the total tumor-infiltrating cells (Figure 2b), which amounted to 91% of the total CD8⁺ T cells in the tumor. The percentage of activated CD8⁺ T cells in the combination group was at least twice that of CD8⁺ T cells in the other

treatment groups including the untreated group (range of 5.4% to 11.9%) (Figure 2b). The frequency of activated CD4⁺ T cells was lower than that of activated CD8⁺ T cells and was comparable in all groups (Figure 2b). Compared to the CD8⁺ T cell population, T cells exhibiting a regulatory phenotype (CD4⁺CD25⁺Foxp3⁺), (Figure 2c) made up a small fraction of tumor infiltrating cells ranging from 0.4% to 1.5% of the total tumor-infiltrating cells (Figure 2d). Interestingly, the percentage of Foxp3⁺ regulatory T cells increased significantly in tumors from mice treated with SM16 and anti-OX40 compared to the percentage in tumors from mice treated with control or either agent alone.

Effect of SM16 + anti-OX40 therapy on cytokine production

We wanted to assess if the anti-tumor activity of the combination treatment was associated with increased T cell effector function. To address this question, tumor draining lymph node cells were isolated and re-stimulated *in vitro* for 48 hours with irradiated 4T1 tumor cells or irradiated syngeneic (irrelevant, haplotype-matched) Renca cells. The data (Figure 3a) show high 4T1 tumor-specific IFN- γ production in the anti-OX40, SM16 and SM16 + anti-OX40 groups compared to the control (untreated) group. 4T1 tumor-specific IFN- γ secretion was 3-fold higher in the combination group compared to the anti-OX40 and SM16 alone groups. A reverse correlation in IL-4 production was observed with the highest-tumor-specific IL-4 secretion occurring in the control mice (Figure 3b). These results suggest enhanced tumor-specific T cell priming and a shift towards a more robust T_H1-like anti-tumor immune response in the SM16 + anti-OX40 group compared to the control group.

The anti-tumor activity of SM16 + anti-OX40 therapy is T-cell dependent

We next evaluated the role of CD4⁺ and CD8⁺ T cells in the anti-tumor effect caused by concurrent SM16 and anti-OX40 treatment. For this purpose, only mice in the combination group in which the anti-tumor effect was most obvious were evaluated. Mice in this group bearing 8-day established tumors were depleted of CD4⁺ and/or CD8⁺ T cells by administering 3 weekly i.p. injections of CD4⁺ and/or CD8⁺-specific monoclonal antibodies on days 8, 15 and 22 post-tumor implantation and monitored for tumor growth. The data (Figure 4A) show that simultaneous depletion of CD4⁺ and CD8⁺ T cells significantly enhanced tumor growth ($p < 0.001$) compared to tumor growth in T cell intact mice, suggesting that T cells play a significant role in SM16 + anti-OX40 therapy-mediated tumor suppression. Depletion of either CD4⁺ or CD8⁺ T cells appeared to increase tumor size compared to T cell intact mice but the increase did not reach statistical significance. SM16 + anti-OX40 therapy-mediated suppression of metastatic spread was also dependent on T cells, as T cell depletion abrogated the effect of SM16 + anti-OX40 on metastatic tumor burden (Figure 4b). Similar to tumor burden, depletion of either CD4⁺ or CD8⁺ T cells seemed to increase the number of metastases in the lung compared to T cell intact mice, but the increases did not reach statistical significance.

SM16 + anti-OX40 therapy-mediated tumor rejection elicits a tumor-specific memory response

We examined whether a tumor-specific memory response was induced in SM16 + anti-OX40-treated mice in which complete primary tumor regression had occurred. After thirty-one days of tumor-free survival, a group of mice was re-challenged with 4T1 cells on one flank and Renca cells on the opposite flank and tumor growth was monitored for an additional 21 days (Figure 5a). The data (Table 1) show rejection of challenge 4T1 tumor cells, but not Renca tumor cells in the cured mice indicative of acquisition of a memory anti-tumor immune response. In contrast, 4T1 and Renca tumors grew progressively in naïve mice. To directly assess the presence of a 4T1-specific memory T cell response, draining lymph node cells from mice that were “cured” then re-challenged with 4T1 tumor cells were isolated and stimulated *in vitro* with either irradiated 4T1 or Renca cells or control (media

alone). Stimulation with irradiated 4T1 cells resulted in at least a 6-fold increase in IFN- γ production in these *in vitro* cultures compared to control-stimulated or Renca-stimulated counterparts (Figure 5b). These results indicate that a tumor-specific memory response is induced consequent to tumor rejection and is accompanied by a robust T_H1-type response.

DISCUSSION

In this report we evaluated the effect of global TGF- β blockade on the efficacy of agonist OX40 antibody therapy in a poorly immunogenic, TGF- β -secreting, and highly metastatic model of murine mammary cancer. We demonstrate that the TGF- β signaling inhibitor, SM16, which binds to the ATP pocket of TGF- β R1, synergizes with anti-OX40 to suppress the growth of large established primary tumors, increase the frequency of tumor regressions and decrease the incidence of spontaneous metastasis. Tumor regression was observed in 38% of mice in the SM16 + anti-OX40 combination group compared to 6% and 0% in the SM16 and anti-OX40 treatment groups respectively. Complete tumor regression in the mice receiving the combination treatment was accompanied by the acquisition of a long-term anti-tumor memory response indicated by the ability of tumor-free mice to reject tumor re-challenge in a tumor-specific fashion. Although previous reports using the 4T1 model demonstrated enhanced survival and tumor suppression against incipient tumors that were treated at 3 or 7 days post-tumor injection with anti-OX40 [36] or OX40L fusion protein [42], our study showed a modest effect on tumor growth during anti-OX40 treatment. The lack of a significant anti-tumor effect by anti-OX40 alone in our system could be due to the fact that treatment was begun 15 days post-tumor injection when tumors were larger and had reached an average size of 38 mm².

Our finding that SM16 in combination with anti-OX40 curtails tumor growth extends earlier reports demonstrating the usefulness of SM16 in combination therapy to treat other less invasive tumors [20–22]. Of interest is that our study used a lower dose (0.03%) of SM16 than the 0.045% dose that was shown to inhibit primary tumor growth in previous studies [19–23]. This lower dose of SM16 is likely to be less toxic and therefore could be of greater clinical utility [43]. At 0.03% in feed, equivalent to ~0.9mg/day, SM16 by itself resulted in only 23% tumor growth reduction, however we observed tumor regression in 6% of the mice. This small effect on primary tumors, is in contrast to an earlier report that showed more substantial tumor growth reduction by a daily dose of 0.8mg SM16 when given by intra peritoneal injection [23], suggesting differences in bioavailability of SM16 depending on the route of administration. Although, SM16 by itself had little effect on the primary tumor, SM16 significantly inhibited the incidence of spontaneous lung metastases. This anti-metastatic effect, which corroborates our earlier report using 0.045% SM16 diet [23] could be due to SM16-mediated inhibition of TGF- β -induced epithelial to mesenchymal transition (EMT), a phenotype that has been directly correlated with tumor invasiveness and metastatic spread [44–46]. Alternatively, abrogation of TGF- β signaling by SM16 alleviating the immune suppressive effects of TGF- β on T cells, may result in better control of metastatic tumor cells by the T cell-mediated immune response.

Examination of the tumor microenvironment (TME) in mice receiving the combination treatment revealed changes that were tantamount to re-shaping of the tumor milieu to favor an effective anti-tumor immune response. In particular, there was a significant increase in the percentage of CD4⁺ and CD8⁺ T cells in the tumors of mice that received the combination treatment compared to mice receiving anti-OX40 or SM16 alone. There was a 1.8 to 3-fold increase in CD8⁺ T cells in the combination group (22%) over the SM16 (12%) and anti-OX40 (6.5%) groups respectively. Compared to the untreated (6.9%) and anti-OX40 (6.5%)-treated groups, SM16 treatment caused a modest increase in the percentage of tumor-infiltrating CD8⁺ T cells (12%), which however did not reach statistical significance.

As with the CD8⁺ T cells, there was close to a 2-fold increase in CD4⁺ T cells in the tumors of the combination treatment group compared to the anti-OX40 and SM16 groups. A reasonable explanation for the apparent lack of effect of anti-OX40 treatment on CD4⁺ and CD8⁺ T cell frequency in tumors is that anti-OX40-driven expansion/survival of CD4⁺ and CD8⁺ T cells is hampered by TGF- β in the microenvironment of the established 4T1 tumors, which could be overcome by SM16 inhibition of immunosuppressive TGF- β signaling. This possibility is supported by well-documented evidence that abrogation of TGF- β signaling leads to enhanced CD4⁺ and CD8⁺ T cell proliferation [47] and enhancement of anti-tumor immunity [48]. However, it is unclear why in our study, the increase in TIL appears to favor CD8⁺ over CD4⁺ T cells since both activated populations have been shown to express OX40 and are capable of responding to OX40 co-stimulation [49,50]. Congruent with the increased frequency of CD8⁺ T cells in the TME was our finding that a high percentage (78–91%) of these cells exhibited an activated phenotype (CD44⁺ CD62L⁻). Although activated CD8⁺ T cells predominated in the TME, CD4⁺ and CD8⁺ T cells cooperated to mediate tumor suppression in the combination treatment group since simultaneous depletion of both subsets led to unchecked tumor growth whereas depletion of either CD4⁺ or CD8⁺ T cells did not result in a statistically significant enhancement of tumor growth. In addition, CD4⁺ and CD8⁺ T cells were also important in mediating the restriction of metastatic tumor spread in the combination treatment, as simultaneous depletion of CD4⁺ and CD8⁺ T cells resulted in a significant increase in the number of lung metastases. Although these results clearly show that the T cell response is important in limiting lung metastases, it is not clear at this point if SM16+anti-OX40 treatment together with the T cell response limited metastasis by abrogation of dissociation of metastatic tumor cells from the primary tumor or by inhibition of implantation of circulating metastatic tumor cells in the lung. The superior anti-tumor effect of the combination therapy was also associated with enhanced tumor-specific effector function in the periphery evidenced by increased IFN- γ secretion by tumor-draining lymph node cells.

T cells exhibiting a regulatory T cell (Treg) phenotype made up a minor fraction of T cells in the TME in all the groups and ranged from 0.4% to 1.5% of total tumor-infiltrating cells. A surprising finding was that the frequency of Treg was highest in the SM16 + anti-OX40-treated mice in which tumor suppression was most pronounced. These findings are contrary to previous reports that have demonstrated a decrease in the frequency and effector function (suppression) of Treg in the TME following single or combination anti-OX40 therapy [35,51]. Despite the increased frequency of Treg in the combination group, the higher percentage of cells displaying an activated phenotype in these tumors, suggests an impairment of Treg suppressive activity in the TME. Impairment of Treg functional activity in the TME through inhibition of their suppressive activity has been reported during anti-OX40 therapy [35].

In summary, our finding that blockade of TGF- β signaling using low dose SM16 synergizes with agonistic OX40 antibody to inhibit the growth of well-established primary tumors and reduce the incidence of spontaneous metastasis is novel and suggests that combining SM16 with anti-OX40 could be a potentially useful strategy for treating metastatic breast cancer. The combination of these immune effector-activating and anti-immunosuppressive strategies could potentially result in more significant and durable tumor responses in the clinic.

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The abbreviations used are

TGF-β	transforming growth factor beta
TβRI	transforming growth factor beta-receptor type I
TβRII	transforming growth factor beta-receptor type II
TME	tumor microenvironment
TIC	tumor-infiltrating cells
FBS	fetal bovine serum
ELISA	enzyme-linked immunosorbent assay
IFN-γ	Interferon-gamma
IL-4	Interleukin-4

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Figure 1. SM16 plus anti-OX40 combination therapy suppresses growth of established mammary tumors and spontaneous metastases

Mice were injected in the mammary pad with 5×10^4 4T1 tumor cells. On day 10 when tumors were palpable, (~ 22 mm²) mice were randomized to the control and various treatment groups. Mice received 3 injections of OX40 (250 μ g/injection/mouse) or isotype control antibody at 3-day intervals beginning on day 15 after tumor establishment when the average tumor size in all groups was ~ 40 mm². **(a)** Individual tumor sizes; numbers in parentheses represent mean $\pm$ SEM of tumor areas. (*) represents significant difference between the SM16 + anti-OX40 group and the control, SM16 and anti-OX40 treatment groups, ($P < 0.05$). **(b)** Tumor growth kinetics of individual mice. The number of mice per treatment group ($N = 31-32$) is combined from 4 independent experiments. **(c)** On day 28 post-tumor cell injection, untreated mice and mice treated with SM16, anti-OX40 or both were culled and visible lung metastases were enumerated. Number of visible lung metastases; numbers in parentheses represent mean $\pm$ SEM combined from 3 independent experiments. **(d)** Relationship between tumor size and frequency of visible lung metastases.

Figure 2. SM16 + anti-OX40 treatment increases the number of activated T cells in the tumor microenvironment

Mice with established 10-day tumors received 0.9 mg SM16/mouse/day orally in the diet for 18 days and were each given 3 separate injections of anti-OX40 on days 15, 18 and 21 post-tumor injection. On day 28, tumor-infiltrating immune cells (TIC) were isolated and analyzed by polychromatic flow cytometry. **(a)** Gating strategy of tumor-infiltrating, activated CD4⁺ and CD8⁺ T cells. **(b)** Frequency of total and activated CD8⁺ and CD4⁺ T cells. Activated T cells were identified as CD4⁺CD44⁺CD62L⁻ or CD8⁺CD44⁺CD62L⁻; **(c)** Gating strategy of CD4⁺ CD25⁺ Foxp3⁺ Treg. **(d)** Frequency of CD4⁺ CD25⁺ Foxp3⁺ Treg. TICs were analyzed from 4 individual mice per group in 3 independent experiments. The data represent mean $\pm$ SEM of 12 mice/group. Dead cells were excluded for all analyses by viability stain.

Figure 3. Effect of SM16 + anti-OX40 therapy on cytokine production

Draining inguinal lymph nodes were harvested and pooled from tumor-bearing mice (N = 3/group) on day 24 post-tumor cell injection. The isolated cells were incubated with irradiated 4T1 or Renca tumor cells for 48 h. Supernatants were collected and assessed for IFN- γ (a) and IL-4 (b) secretion by ELISA. Data represent IFN- γ and IL-4 levels after subtracting cytokine levels in Renca-stimulated cultures from 4T1-stimulated cultures.

Figure 4. Depletion of T cells abrogates efficacy of SM16 + anti-OX40 therapy

Mice with 8-day established 4T1 mammary tumors (N = 3/group) were depleted of T cells by weekly intra-peritoneal injections of CD4-and/or CD8-specific antibodies prior to (day 8), and during SM16 + anti-OX40 therapy (day 15 and day 22 post-tumor implantation). A cohort of T cell replete mice received matched isotype antibody and SM16 + anti-OX40 treatment. a) Tumor growth was monitored weekly. b) Mice in all groups were culled when tumor size in the control group reached ~200mm² and number of lung metastases were determined.

Figure 5. SM16 + anti-OX40 treatment generates a tumor-specific memory response

(a) Schematic representation of treatment protocol. Mice bearing established 4T1 tumors were started on SM16 diet on day 10 followed by 3 anti-OX40 injections on days 15, 18 and 21. SM16 diet was discontinued on day 28 and tumor-free mice were challenged with 4×10^4 4T1 and 2×10^5 Renca cells tumor cells in opposite flanks. Tumor growth was monitored for up to 21 days (day 80). **(b)** Draining inguinal lymph nodes were harvested from SM16 + anti-OX40-treated mice 21 days after 4T1 tumor re-challenge and isolated cells were incubated for 48 h with irradiated (300 Gy) 4T1 or Renca tumor cells. Supernatant fluid was collected and assessed for IFN- γ secretion by ELISA.

Table 1

SM16 + anti-OX40-treated, tumor-free mice reject re-challenge of 4T1 tumor cells

	Tumor Challenge	^c Tumor Incidence
^a Mice cured by SM16+anti-OX40 treatment	4T1	0/8
	Renca	4/4
^b Naïve Mice	4T1	8/8
	Renca	4/4

^a Mice treated with SM16 + anti-OX40 that were cured of their tumors were re-challenged with 4T1 or Renca tumor cells and tumor growth was monitored for 21 days. Age of mice 16–17 weeks.

Mice were

^b Age of mice 10–12 weeks.

^c Number of re-challenge mice without tumors by day 21 divided by the total number of challenged mice.