

# STAT3 Signaling Is Required for Optimal Regression of Large Established Tumors in Mice Treated with Anti-OX40 and TGF $\beta$ Receptor Blockade

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## Abstract

In preclinical tumor models,  $\alpha$ OX40 therapy is often successful at treating small tumors, but is less effective once the tumors become large. For a tumor immunotherapy to be successful to cure large tumors, it will most likely require not only an agonist to boost effector T-cell function but also inhibitors of T-cell suppression. In this study, we show that combining  $\alpha$ OX40 antibodies with an inhibitor of the TGF $\beta$  receptor (SM16) synergizes to elicit complete regression of large established MCA205 and CT26 tumors. Evaluation of tumor-infiltrating T cells showed that SM16/ $\alpha$ OX40 dual therapy resulted in an increase in proliferating granzyme B<sup>+</sup> CD8 T cells, which produced higher levels of IFN $\gamma$ ,

compared with treatment with either agent alone. We also found that the dual treatment increased pSTAT3 expression in both CD4 and CD8 T cells isolated from tumors. Because others have published that STAT3 signaling is detrimental to T-cell function within the tumor microenvironment, we explored whether deletion of STAT3 in OX40-expressing cells would affect this potent combination therapy. Surprisingly, we found that deletion of STAT3 in OX40-expressing cells decreased the efficacy of this combination therapy, showing that the full therapeutic potential of this treatment depends on STAT3 signaling, most likely in the T cells of tumor-bearing mice. *Cancer Immunol Res*; 3(5): 526–35. ©2015 AACR.

## Introduction

OX40 (CD134, TNFRSF4) is a member of the TNF receptor (TNFR) superfamily expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (1–3). Engagement of OX40 with cognate ligand or soluble agonist enhances T-cell proliferation, survival, and cytokine production by activated T cells (4). In addition to OX40 being an activation antigen on T cells, regulatory T cells (Treg) constitutively express OX40 in mice. Under certain conditions, treatment with OX40 agonist antibodies ( $\alpha$ OX40) mitigates Treg development and function while at the same time enhancing effector T-cell function (4, 5). Importantly, a murine anti-human OX40 antibody has recently completed a phase I clinical trial for the treatment of patients with advanced cancer with encouraging results (6). Currently, humanized OX40 agonists are being developed to conduct future trials in patients with cancer.

Although  $\alpha$ OX40 therapy has been effective at controlling small tumors (0–50 mm<sup>2</sup>) in a variety of mouse tumor models (3, 7, 8), it has been less effective at eradicating large established tumors (>50 mm<sup>2</sup>; refs. 8–11). A potential explanation for this deficiency is that the microenvironment of large tumors becomes immune suppressive, which most likely reflects tumors observed in patients with late-stage cancer (10, 12–14). Therefore, it is conceivable that a strategy that blocks immunosuppression will improve the therapeutic efficacy of anti-OX40 immunotherapy, especially in mice with large established tumors.

TGF $\beta$  is an immunosuppressive cytokine that is produced by a variety of tumor types (15–17) as well as by immune cells found within the tumor microenvironment (TME; refs. 18–20), and it has been shown that elevated levels of TGF $\beta$  are associated with tumor progression (15, 16). TGF $\beta$  protumor functions are mediated through direct action on tumor cells (20) as well as inhibition of immune cells, including monocytes, dendritic cells, natural killer cells, and T cells (19, 20). These include inhibition of antigen presentation, T-cell differentiation, effector/cytotoxic activity of CD8<sup>+</sup> T cells, and Th1 cytokine production by CD4<sup>+</sup> T cells (19–22). In addition to suppression of effector T cells, TGF $\beta$  signaling in peripheral Tregs has also been shown to be important for their expansion, survival, and suppressive function *in vivo* (23, 24).

Collectively, the previously discussed studies provide a rationale for blocking TGF $\beta$  to enhance an antitumor immunity. This can be achieved using neutralizing antibodies (25), fusion proteins (26), or small-molecule TGF $\beta$  receptor signaling inhibitors that have demonstrated some efficacy in suppressing primary tumor growth and metastasis in several mouse tumor models (27–30). Unlike antibodies and fusion proteins, whose ability to effectively restore immune function depends on complete

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neutralization of circulating TGF $\beta$ , small-molecule TGF $\beta$  receptor inhibitors, which interfere with TGF $\beta$  receptor signaling, allow immune cells to retain normal function even in a TGF $\beta$ -rich environment. SM16, which is used in this study, is an orally bioavailable small molecule that has demonstrated antitumor efficacy when administered in the diet (27). SM16 binds to the kinase active site of TGF $\beta$  type I receptor (ALK5), thus inhibiting phosphorylation of SMAD proteins and downstream signal transduction (28). We hypothesized that using an agonist ( $\alpha$ OX40) to boost effector T-cell function in conjunction with an inhibitor of T-cell suppression (SM16) would endow the immune system with the ability to eradicate large established tumors. Indeed, this combination has recently been shown to be effective in eradicating smaller tumors via an immune-dependent mechanism in the poorly immunogenic 4T1 breast carcinoma model (31). Therefore, we wanted to determine if this combination would be effective in curing mice of large established tumors.

Our data demonstrate that SM16 plus  $\alpha$ OX40 stimulates a vigorous antitumor response that results in complete and durable tumor regression in mice bearing large (50–120 mm<sup>2</sup>) well-established MCA205 and CT26 tumors that resulted in tumor-specific immunity. Examination of the tumors in mice receiving  $\alpha$ OX40 + SM16 therapy revealed an increase in tumor-infiltrating T cells (TIL) that were phenotypically and functionally distinct from T cells found in tumors from mice treated with the single agents. The combination therapy increased the frequency of OX40-expressing, Ki67<sup>+</sup>/granzyme B<sup>+</sup> CD8<sup>+</sup> TILs, pSTAT3-expressing CD4<sup>+</sup> and CD8<sup>+</sup> TILs, and IFN $\gamma$ -producing TILs. Although the increased effector function in T cells was to be expected, the increase in pSTAT3 was somewhat unexpected as others have shown that STAT3 signaling in T cells is detrimental to their function, especially in regard to tumor eradication (32). Surprisingly, we found that when STAT3 was deleted in OX40-expressing T cells, the efficacy of this potent combination therapy was significantly reduced. Collectively, these results demonstrate that combining OX40 agonists with an orally bioavailable inhibitor of TGF $\beta$  signaling can overcome the immunosuppressive environment in large established tumors, resulting in tumor regression. We also show that this potent immunotherapy combination is mediated in part by STAT3 signaling in OX40-expressing cells.

## Materials and Methods

### Mice

Six- to eight-week-old C57BL/6 female mice were purchased from The Jackson Laboratory. C57BL/6 OX40-Cre mice were kindly provided by Dr. Nigel Killeen (UCSF, San Francisco, CA) and were crossed with mice carrying the Rosa26-loxP-STOP-loxP-YFP allele (33) obtained from The Jackson Laboratory (JAX). In the double transgenic OX40-Cre/Rosa-YFP mice, cells that express OX40-driven cre protein will knock out a floxed stop cassette that is juxtaposed between a promoter that is constitutively active in lymphocytes and a gene encoding YFP (34). STAT3<sup>fllox/fllox</sup> mice were supplied by Dr. Hua Yu (City of Hope Research Institute, Duarte, CA), and the OX40<sup>cre/ut</sup> mice were supplied by Dr. Nigel Killeen (UCSF). STAT3-deficient mice were generated by crossing OX40<sup>cre/wt</sup>  $\times$  STAT3<sup>fllox/fllox</sup> (STAT3<sup>-/-</sup>) with OX40<sup>cre/wt</sup>  $\times$  STAT3<sup>wt/wt</sup> (STAT3<sup>+/+</sup>) mice. It should be noted that the OX40<sup>cre</sup> gene is knocked into the OX40 locus, which only allows for hemizygous expression of OX40 in the crossed progeny

described above. We have found that the OX40 agonist therapy is less effective in mice that express hemizygous levels of OX40 (data not shown). All mice were maintained under specific pathogen-free conditions in the Providence Portland Medical Center animal facility 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 of the Earle A. Chiles Research Institute.

### Tumor cell lines

Murine MCA205 sarcoma cells, 3LL Lewis lung carcinoma cells, and CT26 colon carcinoma cells were propagated *in vitro* using (complete media) RPMI (Lonza) containing 0.292 ng/mL glutamine (Hyclone Laboratories), 100 U/mL streptomycin (Hyclone), 100 U/mL penicillin (Hyclone), 1  $\times$  nonessential amino acids (Lonza BioWhittaker), 1 mmol/L sodium pyruvate (Lonza BioWhittaker), and 10 mmol/L HEPES (Sigma). Both tumor cell lines were routinely tested and shown to be free of *Mycoplasma* contamination.

### Reagents

The following antibodies were used for analysis by flow cytometry: CD4 Pacific Blue or PerCP-Cy5.5 (eBioscience; GK1.5), CD8 PerCP-Cy5.5 (ebioscience; 53-6.7), OX40 PE (Biolegend; OX-86), CD25 APC (eBioscience; PC61.5), Ki67 FITC (BD; 35), Foxp3 Pacific Blue (eBioscience; FJK-16s), Granzyme B PE (Invitrogen; GB12), and IFN $\gamma$  APC (ebioscience; 4S.B3). Live cells were identified using the eFluor 780 viability dye (eBioscience). The OX40 agonist is a rat anti-mouse OX40 antibody (clone OX86). Rat Ig (Sigma) was used as a control for OX86. SM16 was synthesized by Biogen Idec and was incorporated into standard Purina rodent chow (#5001) by Research Diets at a concentration of 0.3 g SM16 per kg of chow (0.03%). A calorie and nutrient-matched diet without SM16 (Purina) was used as the control diet.

### Flow cytometry

Cells (1–5 million) in 100  $\mu$ L of flow wash buffer (FWB, PBS with 0.5% FBS) were incubated with the appropriate antibodies at 4°C for 30 minutes. For intracellular staining, cells were fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Intracellular staining was performed at 4°C for 30 minutes with the appropriate antibodies to detect specific intracellular proteins. Cells were analyzed using an LSR II flow cytometer (Becton Dickinson) and FlowJo4 software.

### *In vivo* tumor growth and survival experiments

For primary tumor challenge, mice were injected s.c. in the right flank with 4  $\times$  10<sup>5</sup> MCA205 or CT26 tumor cells. Eleven days (~50–120 mm<sup>2</sup>) after tumor inoculation, mice were randomized into the following treatment groups: control diet + rat IgG1 (isotype control for  $\alpha$ OX40), control diet +  $\alpha$ OX40 (OX86), SM16 diet (0.03% SM16) + rat IgG1, and SM16 +  $\alpha$ OX40. Mice were then given i.p. injections of 250  $\mu$ g of either  $\alpha$ OX40 or rat IgG1 on days 2 and 6 after starting the diet. Mice were taken off the control and SM16 diets after 2 weeks and monitored for tumor growth two to three times a week by measuring tumor length (*L*) and width (*W*) to determine tumor size [*L*  $\times$  *W* (mm<sup>2</sup>)]. All mice were sacrificed when the tumors either ulcerated or reached >150 mm<sup>2</sup>. For rechallenge experiments, mice were injected s.c. with 4

$\times 10^5$  MCA205 tumor cells in the right flank and  $4 \times 10^5$  3LL cells in the left flank and were examined for tumor growth in the absence of any additional therapy.

### Phenotyping of TILs and splenocytes

Mice were injected s.c. with  $4 \times 10^5$  MCA205 cells. Once tumors were established for 11 days ( $\sim 50$ – $120$  mm<sup>2</sup>), mice were put on SM16 or control diet and administered 250  $\mu$ g of either  $\alpha$ OX40 or Rat IgG1 on days 2 and 6 after starting diet. Seven days after starting feed, tumors and spleens were harvested. Resected tumors were minced with scissors and disaggregated using a triple-enzyme digest cocktail containing 10 mg/mL of collagenase type IV (Worthington Biochemical Corp.), 1 mg/mL hyaluronidase (Sigma-Aldrich), and 200  $\mu$ g/mL DNase I (Roche Applied Sciences) in complete media for 30 minutes with agitation at 37°C using a shaker. The tumor digest was then passed through a 70- $\mu$ m nylon filter, subjected to red blood cell lysis using ACK lysing buffer (Lonza), and then washed and used for analysis. Spleen cells were minced between slides; subjected to red blood cell lysis; and passed through 70- $\mu$ m nylon filter, washed, and used for analysis. Cells were then incubated with fluorescently labeled antibodies and analyzed by flow cytometry.

### In vitro stimulation of isolated immune cells

Half of tumor digest or  $1 \times 10^7$  spleen cells were resuspended in 1 mL of complete media and stimulated for 1 hour at 37°C with  $1 \times$  phorbol myristate acetate (PMA)/ionomycin cocktail (eBioscience) in 48-well plates. Brefeldin A (BioLegend) was

added to a final concentration of 5  $\mu$ g/mL and incubated with the cells for an additional 4 hours at 37°C. Cells were then stained for surface markers, fixed and permeabilized, and then evaluated for intracellular IFN $\gamma$  production as described above.

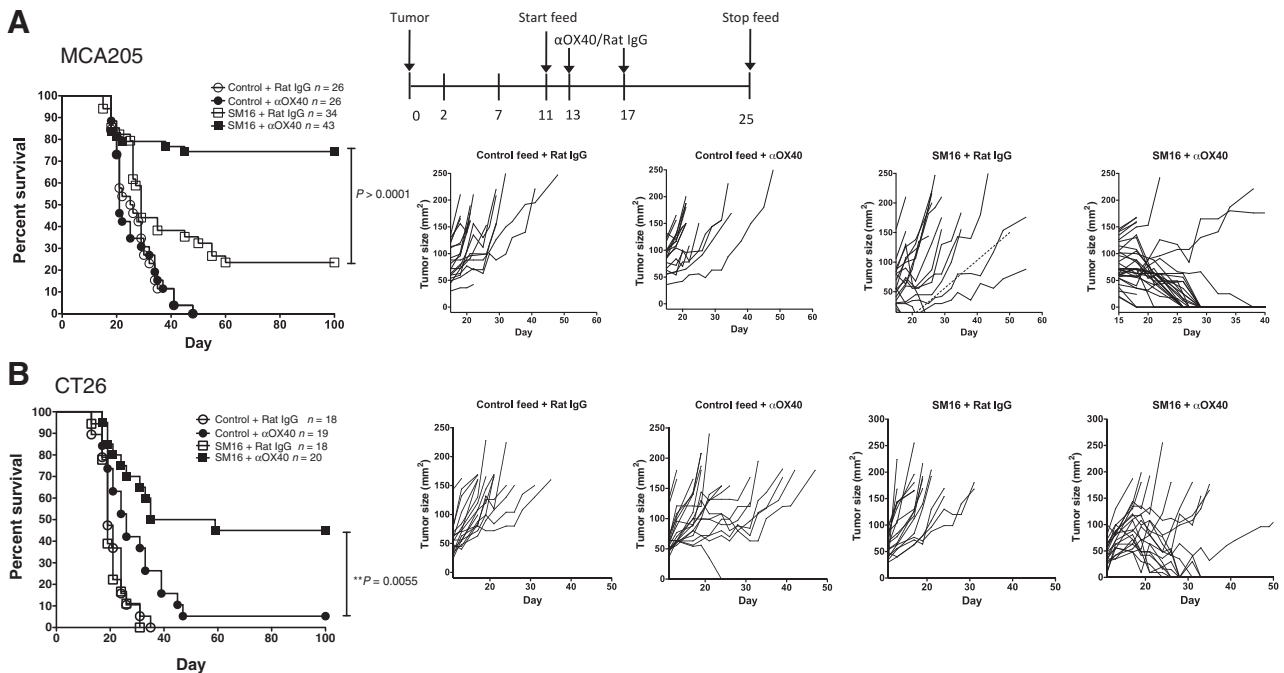
### Statistical analysis

Statistical significance was determined by one-way ANOVA (for comparison among three or more groups), unpaired Student *t* tests (for comparison between two groups), or Kaplan–Meier survival and log-rank (Mantel–Cox) test (for tumor survival studies) using GraphPad Prism software (GraphPad); *P* value is either stated in the figure or indicated by \*, *P* < 0.05.

## Results

### SM16 plus anti-OX40 therapy stimulates regression of large established tumors and prolongs overall survival

$\alpha$ OX40 therapy has been shown to cure mice in several different mouse tumor models if administered shortly after tumor challenge when tumors are either not yet palpable or small (0–50 mm<sup>2</sup>; refs. 3, 7, 8). However,  $\alpha$ OX40 has been less effective at curing large established tumors (>50 mm<sup>2</sup>), either having no impact or only delaying tumor growth, with very little extension of long-term survival (Fig. 1A and B; refs. 8–11). Examination of the TME of large tumors after OX40 agonist treatment revealed that the  $\alpha$ OX40 antibody was able to bind to tumor-infiltrating T cells (data not shown). Yet, despite being able to penetrate large tumors and bind to infiltrating T lymphocytes, anti-OX40 was unable to initiate a therapeutic immune response. The inability of



**Figure 1.**

Therapeutic benefit in large established tumors of combined agonistic antibody to OX40 and a small-molecule blockade of the TGF $\beta$  receptor. **A**, MCA205 sarcoma cells were injected s.c. on the flank of 6- to 8-week-old C57Bl/6J female mice and allowed to grow for 11 days. Eleven days after tumor inoculation, SM16 feed (0.3 g SM16/kg of feed) or control feed was administered and then stopped 14 days later. On days 13 and 17 after tumor inoculation, 250  $\mu$ g of anti-OX40 (clone OX86) or isotype control antibody was injected i.p. Tumor growth and survival were monitored for 100 days. **B**, CT26 colon cancer cells were injected s.c. into 6- to 8-week-old Balb/C mice that were treated as described in **A**. For both tumor types, survival and tumor growth curves are shown, and statistics were performed on the Kaplan–Meier survival analyses.

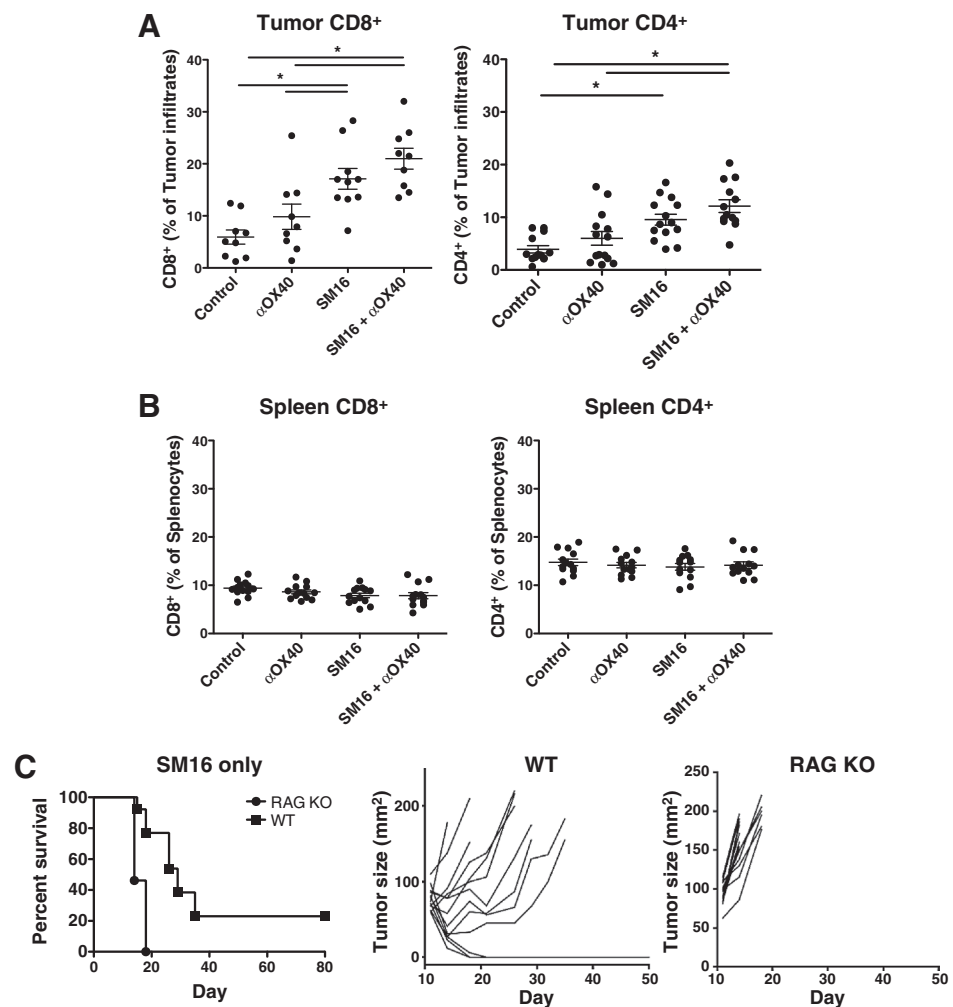
$\alpha$ OX40 to activate an effective antitumor T-cell response may be due to immunosuppressive signals present within the tumor as it grows in size (19, 35–38). Therefore, we hypothesized that using an OX40 agonist antibody in conjunction with a small-molecule inhibitor of the TGF $\beta$  type 1 receptor will initiate a potent antitumor immune response with the potential to cure mice harboring large tumors. To test this hypothesis, MCA205 sarcoma cells and CT26 colon carcinoma cells were implanted into mice and allowed to grow until the tumors were approximately 40 to 120 mm<sup>2</sup> in size (11 days after implant). Tumor-bearing mice were fed SM16 or control feed for 14 days, during which they were treated with either rat IgG or an  $\alpha$ OX40 antibody on days 13 and 17 as depicted in Fig. 1A. The SM16 + anti-OX40 combination therapy caused complete tumor regression in 75% and 45% of mice harboring large preestablished MCA205 and CT26 tumors, respectively (Fig. 1). Tumor-free survival was sustained over 100 days and was significantly lower with single-agent treatment of anti-OX40 or SM16. Tumor regression was not observed in mice that received control IgG. In summary, treatment with an OX40 agonist antibody in conjunction with a TGF $\beta$  signaling inhibitor (SM16) provided a therapeutic immune-boosting combination that caused regression of large established tumors in two separate tumor models tested in two different mouse strains.

### SM16/ $\alpha$ OX40 therapy increases the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in tumors

To understand how SM16/ $\alpha$ OX40 mediates tumor regression, TILs were evaluated and compared with peripheral lymphocytes. Lymphocytes from the TME were analyzed from large tumors in mice that were put on the SM16 or control diet, administered two injections of  $\alpha$ OX40 or control antibodies (as in the scheme in Fig 1A), and then sacrificed 1 day after the second antibody injection. Excised tumors were subjected to triple-enzyme digest without density gradient centrifugation so as not to enrich for immune cells and thus evaluate the proportion of total cells within the tumor digest that were CD4<sup>+</sup> or CD8<sup>+</sup> T cells. This analysis revealed an increase in the proportion of cells that were CD4<sup>+</sup> and CD8<sup>+</sup> in the SM16- and SM16/ $\alpha$ OX40-treated mice compared with that of the control and  $\alpha$ OX40-treated mice (Fig. 2A and B). No increase in proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleens was found with any of the treatments. The increase of T cells within the tumors of SM16- and SM16/ $\alpha$ OX40-treated mice suggested that the mechanism of tumor regression was immune mediated. However, TGF $\beta$  is also known to directly affect tumor growth, independent of immune suppression (20). Therefore, to confirm a role for lymphocytes in SM16-mediated antitumor responses, we performed the SM16 therapy

**Figure 2.**

Increased T-cell infiltration in tumor-bearing mice treated with combination therapy. A and B, MCA205 sarcoma cells were injected s.c. on the flank of 6- to 8-week-old C57Bl/6J female mice and allowed to grow for 11 days. Eleven days after tumor inoculation, SM16 feed or control feed was initiated. On days 13 and 17, 250  $\mu$ g anti-OX40 or isotype control antibody was administered. Tumors and spleens were harvested on day 18, and T cells were assessed for (A) proportion of singlet gate that was CD4<sup>+</sup> or CD8<sup>+</sup> and (B) proportion of splenocytes that were CD4<sup>+</sup> or CD8<sup>+</sup>. Each dot on all graphs represents results from individual mice from at least three independent experiments. C, RAGKO mice or C57Bl/6 female mice were inoculated with MCA205. On day 11, mice were fed SM16 and taken off the SM16 feed 14 days later ( $n = 13$ /group). Both tumor growth and survival curves are shown, and these mice were monitored for 80 days after tumor inoculation. Statistical significance for all graphs was determined by one-way ANOVA analysis. \*,  $P < 0.05$ .



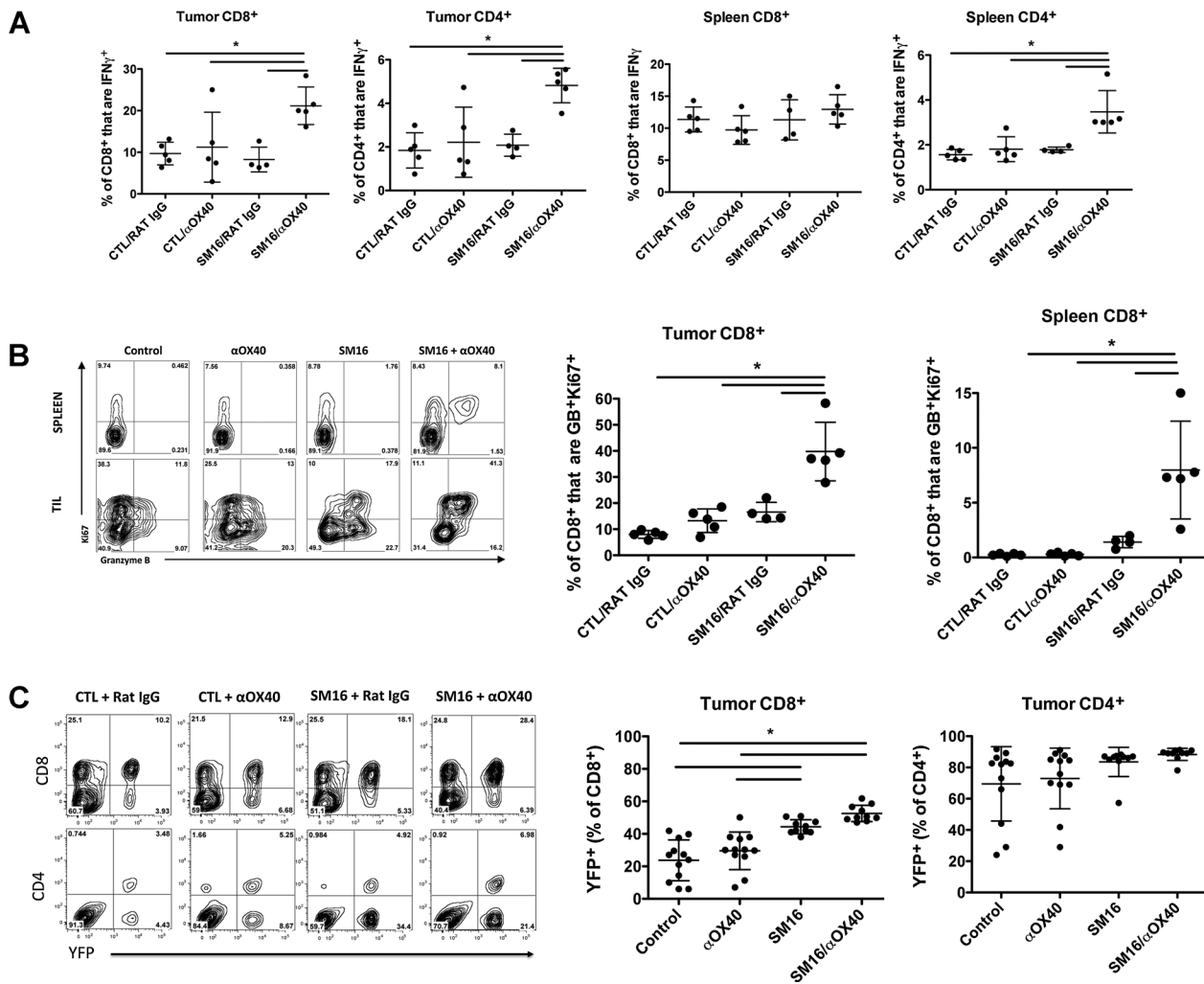
experiment using RAG<sup>-/-</sup> mice. As shown in Fig. 2C, SM16 was unable to alter the outgrowth of large tumors in mice devoid of lymphocytes in the MCA205 model (log-rank test:  $P < 0.0001$ ).

**SM16/ $\alpha$ OX40 combination therapy affects the phenotype and effector function of TILs**

We further evaluated the TILs to determine if there was simply an increase of T cells in the tumor or if these T cells were phenotypically and functionally distinct between treatment groups. Because IFN $\gamma$  is a known beneficial antitumor cytokine (39–43), we evaluated the ability of TILs from the different treatment groups to produce IFN $\gamma$ , directly *ex vivo*. When the cells were stimulated with PMA/ionomycin, we found an increase in the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> TILs capable of producing IFN $\gamma$

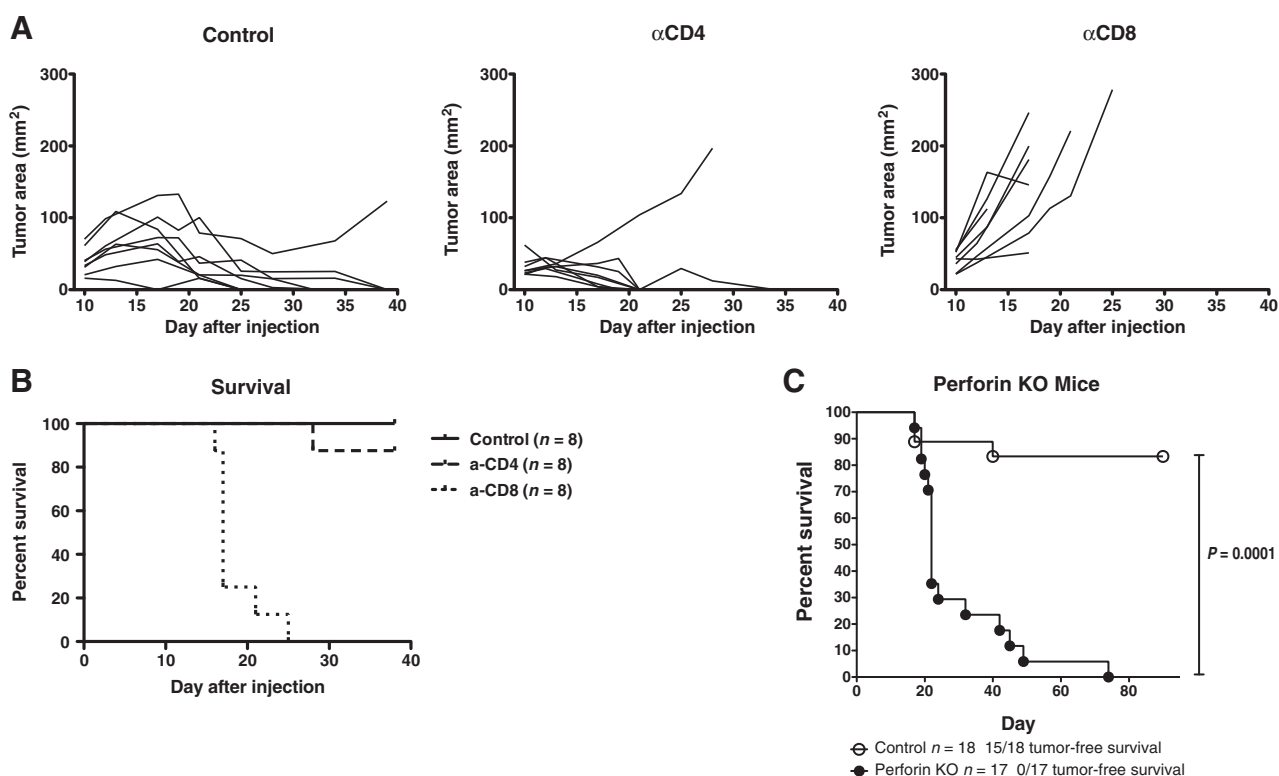
from mice treated with SM16/ $\alpha$ OX40 compared with that from mice in the other treatment groups (Fig. 3A). A significant increase in IFN $\gamma$  production was also observed in splenic CD4<sup>+</sup> T cells from the SM16/ $\alpha$ OX40 treatment group (Fig. 3A).

To further characterize these lymphocytes, we determined the proportion of CD8<sup>+</sup> cells that were actively undergoing proliferation as assessed by Ki67 expression. Although we found no difference in the proportion of CD8<sup>+</sup> cells proliferating in tumors of SM16/ $\alpha$ OX40-treated mice, there was a significant increase in proliferating (Ki67<sup>+</sup>)/granzyme B<sup>+</sup> CD8<sup>+</sup> T cells in both the tumors and the spleens (Fig. 3B). The increase in proliferating granzyme B<sup>+</sup> CD8<sup>+</sup> T cells suggested that there might be an increase in CD8<sup>+</sup> T-cell-mediated killing of tumors in mice injected with the combination therapy. To determine whether



**Figure 3.** Effector function and OX40 expression are increased with SM16/anti-OX40 combination therapy. Tumor-bearing mice were treated as in Fig. 2. A, TILs were stimulated with PMA/ionomycin, after which the cells were treated with brefeldin A and evaluated for intracellular IFN $\gamma$ . Graphs show proportion of CD4<sup>+</sup> and CD8<sup>+</sup> cells that were IFN $\gamma$ <sup>+</sup>. B, representative dot plots of CD8<sup>+</sup> cells isolated from spleens and tumors for expression of Ki67 and granzyme B and Ki67/granzyme B double-positive CD8<sup>+</sup> T cells (right) for an individual experiment (each dot represents an individual mouse). C, OX40cre-YFP reporter tumor-bearing mice were treated as in A and B, and representative flow cytometry plots of TILs for expression of YFP in CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations are shown in the left plots. A summary of the proportion of CD8<sup>+</sup> and CD4<sup>+</sup> cells from the tumor that were YFP<sup>+</sup> is shown in the right plots, which represent a compilation of three independent experiments; the wide horizontal bar indicates mean  $\pm$  SD. Each dot on all graphs represents results from an individual mouse. Statistical significance for all graphs was determined by one-way ANOVA analysis. \*,  $P < 0.05$ .

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**Figure 4.**

Tumor regression observed via the combination therapy is dependent on CD8<sup>+</sup> T cells. MCA205 tumor-bearing mice were treated as in Fig. 1 and were administered CD8- or CD4-depleting antibodies or an isotype antibody on days -1, +4, +7, +14, and 1 week after SM16 and anti-OX40 administration. Tumor growth (A) and survival (B) were monitored for 40 days. C, perforin KO C57/Bl/6 or C57Bl/6 WT mice were treated as in Fig. 1, and tumor growth and survival were monitored for 80 days.

the SM16/ $\alpha$ OX40 dual treatment was reliant on granule-mediated killing of tumors, we tested the dual treatment in tumor-bearing perforin knockout (KO) mice. We found that although 15 of 18 wild-type (WT) tumor-bearing mice receiving the dual treatment survived, none of the perforin KO tumor-bearing mice treated with SM16/ $\alpha$ OX40 survived (0/17; Fig. 4C). We next explored the contribution of CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells to the therapeutic efficacy of the anti-OX40/SM16 combination therapy. Previous studies have shown that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are required for the therapeutic efficacy of OX40 agonists when delivered in mice harboring smaller tumors (5). However, Fig. 4A and B show that only the CD8<sup>+</sup> T cells are necessary for the dual therapy to be effective. Depletion of CD4<sup>+</sup> T cells had no effect on the therapeutic efficacy of the combination therapy; however, there were some autoimmune symptoms associated with depleting the CD4<sup>+</sup> T cells (data not shown), which was most likely due to depletion of Tregs in mice receiving this potent combination immune therapy. Together, these data suggest that the dual therapy is highly dependent on CD8<sup>+</sup> T cells and that increased IFN $\gamma$  production and cytolytic granule activity in the CD8<sup>+</sup> T cells likely contribute to the potent therapeutic effect.

Previous studies have shown that TGF $\beta$  signaling can promote Treg proliferation and survival (23, 24). Therefore, one would expect to observe a decrease in the frequency of Tregs when TGF $\beta$  signaling is blocked. We found that in mice from the SM16-treated groups, there was an increase in the proportion of CD4<sup>+</sup> cells that were FOXP3<sup>+</sup> in the spleens; however, no statistical difference was

observed in the tumor (data not shown). We also found an increase in the proportion of the Tregs undergoing proliferation in the spleens of SM16/ $\alpha$ OX40-treated mice. The expansion of peripheral Tregs in the absence of TGF $\beta$  signaling has also been observed in other tumor models (18, 31), and in our model, the increase in peripheral Tregs may help quell off-target inflammation while the mice are receiving this potent dual therapy.

#### SM16/ $\alpha$ OX40 combination therapy increases the proportion of OX40-expressing TILs

OX40-expressing tumor-infiltrating T cells are the most likely target for  $\alpha$ OX40 therapy. Therefore, we wanted to determine if there was an increase in the proportion of T cells that express or previously expressed OX40 in the SM16- and SM16/ $\alpha$ OX40-treated mice. Because our anti-OX40 detection antibody is the same as the therapeutic antibody and OX40 expression on CD8<sup>+</sup> TILs is low and hard to detect (data not shown), we took advantage of the OX40<sup>Cre</sup>/ROSA<sup>YFP</sup> reporter mouse system that produces the YFP protein in cells that express or had previously expressed OX40 (33). Using these mice, we found a significant increase in the proportion of CD8<sup>+</sup> TILs that were YFP<sup>+</sup> in both the SM16 and SM16/ $\alpha$ OX40 groups (Fig. 3C). There was no statistically significant difference in the proportion of tumor-infiltrating CD4<sup>+</sup> that were YFP<sup>+</sup> because the majority (~90%) of them were YFP<sup>+</sup> in all groups. This increase in CD8<sup>+</sup> T cells that express OX40 in the tumor is most likely relevant to the mechanism of tumor destruction because previous work has shown

that direct engagement of OX40 by CD8<sup>+</sup> T cells is necessary to help drive a potent antitumor immune response by OX40 agonists (10, 11, 44). We also found that the YFP<sup>+</sup> T cells within the tumor were significantly increased for both IFN $\gamma$  and granzyme B, and this observation was consistent for all treatment groups, although the trend showed a greater increase in mice treated with the SM16/ $\alpha$ OX40 dual therapy.

#### SM16/ $\alpha$ OX40-cured mice develop long-term antitumor immunity

We wanted to determine if mice cured of their tumors following SM16 or SM16/ $\alpha$ OX40 therapy developed long-term immunity. To achieve this goal, tumor-free mice in both treatment groups were challenged >60 days after primary tumor injection with viable MCA205 tumor cells. None of the 7 mice from the SM16/ $\alpha$ OX40 treatment group that were cured developed tumors, whereas a fraction (2/7) of the SM16-treated mice developed tumors and, as expected, all (7/7) of the naïve mice developed tumors (data not shown). In contrast, neither the SM16- nor SM16/ $\alpha$ OX40-cured mice were protected from challenge with 3LL tumor cells (data not shown). These results indicate that SM16/ $\alpha$ OX40-mediated tumor regression resulted in a tumor-specific immunologic memory that protects against tumor rechallenge.

#### Conditional ablation of STAT3 in OX40-expressing cells reduces overall survival of mice receiving SM16/anti-OX40 combination therapy

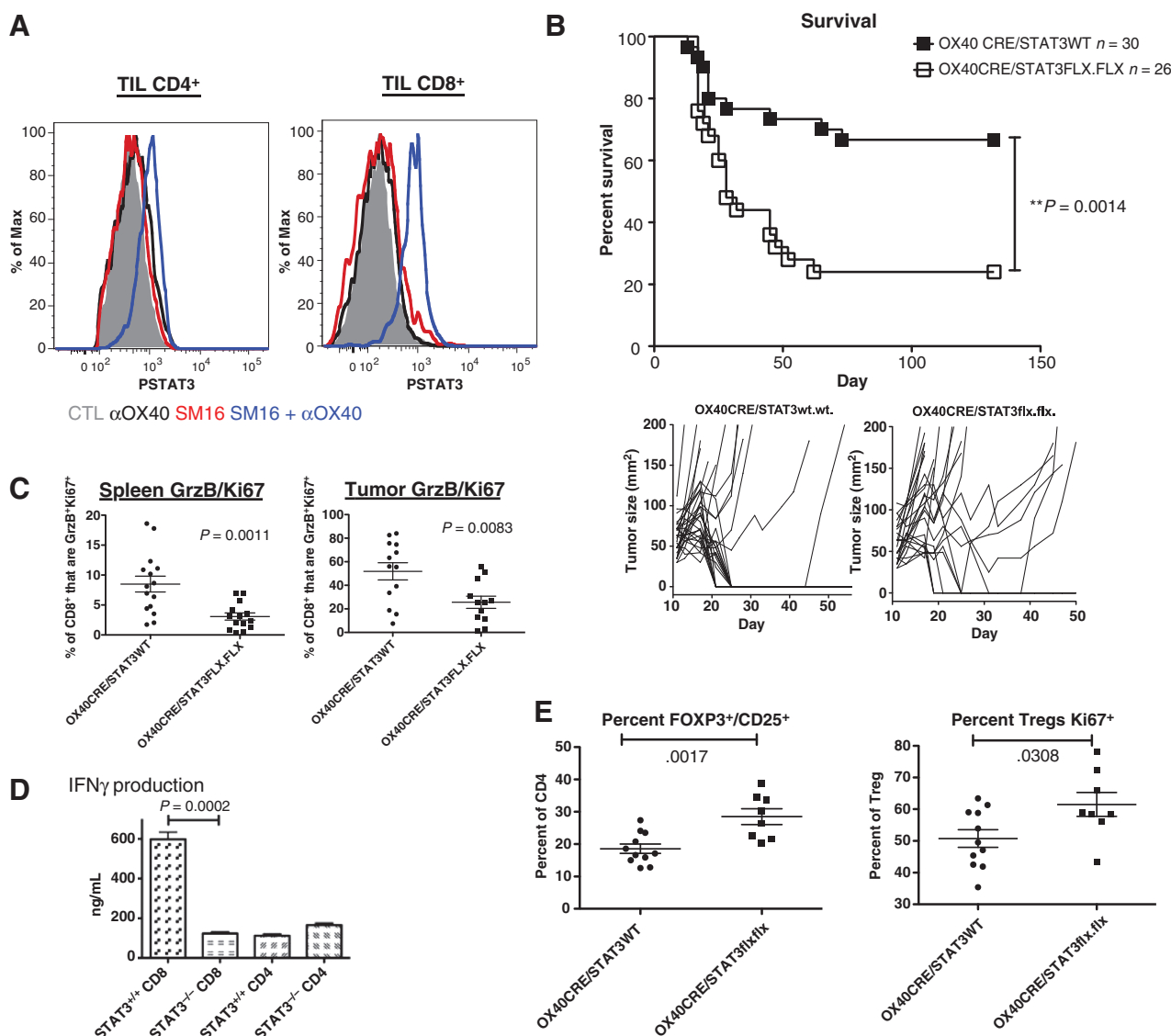
In trying to understand the mechanism(s) underlying the efficient therapeutic activity of the SM16/anti-OX40 therapy, we unexpectedly found that STAT3 phosphorylation was increased in tumor-infiltrating CD8<sup>+</sup> and CD4<sup>+</sup> T cells in SM16/anti-OX40-treated animals compared with that in the other treatment groups (Fig. 5A). Previously, it had been published that pSTAT3-expressing tumor-specific T cells were associated with a dysfunctional T-cell response leading to decreased antitumor efficacy (32). Hence, to better understand the role that STAT3 plays in this combination therapy, we crossed STAT3<sup>lox/lox</sup> mice with OX40<sup>cre</sup> mice and assessed the antitumor efficacy of the combination treatment. We found that conditional deletion of the STAT3 gene reduced the efficacy of the combination therapy as we observed a significant decrease in overall survival and tumor shrinkage in the STAT3 KO mice (Fig. 5B). We also observed that the STAT3-deficient, tumor-bearing mice receiving the dual treatment had decreased frequencies of proliferating, granzyme B<sup>+</sup> CD8<sup>+</sup> T cells in the tumors as well as decreased IFN $\gamma$  production by CD8<sup>+</sup> TILs (Fig. 5C and D). We also found a significant increase in the percentage and proliferation of Tregs in the tumors of the STAT3-deficient mice. These data show for the first time that STAT3 function in T cells plays a positive role in T-cell-mediated rejection of tumors, a concept that opposes the current paradigm that STAT3 function is detrimental to T-cell function in tumor-bearing mice. This observation is most likely context dependent, and in this study, in which mice were treated with a potent immune-stimulatory therapy, STAT3 function plays a positive role in immune-mediated clearance of tumors.

## Discussion

It has been reported that the microenvironment of large tumors is immunosuppressive due to the presence of inhibitory cytokines and other cellular and soluble factors (19, 35–38). Therefore, a successful tumor immunotherapy may require

both an agent to boost effector T-cell function and an inhibitor of tumor-mediated T-cell suppression (8–11). In this study, we found that  $\alpha$ OX40 therapy alone was ineffective at treating large established tumors, but was capable of causing complete regression of large tumors if given in conjunction with a TGF $\beta$  receptor inhibitor (SM16). The dual treatment resulted in long-term survival in approximately 85% of mice compared with approximately 20% of mice treated with SM16 alone in the MCA-205 model (Fig. 1) and 45% survival of the  $\alpha$ OX40/SM16-treated tumor-bearing mice compared with 5% of mice treated with  $\alpha$ OX40 alone in the CT26 model. Furthermore, complete tumor regression was accompanied by the host's ability to resist tumor rechallenge in a tumor-specific fashion in mice cured by the dual therapy. Examination of the TME showed that the T-cell composition was significantly different in tumors obtained from the  $\alpha$ OX40/SM16-treated mice compared with that with either treatment alone. Cytokine analyses revealed that a greater percentage of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from  $\alpha$ OX40/SM16-treated mice were capable of producing IFN $\gamma$  *ex vivo* (Fig. 3A). The finding that the efficacy of the anti-OX40/SM16 combination therapy is reliant on CD8<sup>+</sup> T cells and not CD4<sup>+</sup> T cells led us to focus our efforts in understanding the differences in CD8<sup>+</sup> T cells in mice treated with the combination versus the monotherapies. Phenotypic analyses revealed a significant increase in the proportion of CD8<sup>+</sup> T cells expressing granzyme B (GrzB) in the tumors of  $\alpha$ OX40/SM16-treated mice (~60%) when compared with the SM16- (~30%) and  $\alpha$ OX40-treated mice (~30%; data not shown). Interestingly, a large proportion of these CD8<sup>+</sup>GrzB<sup>+</sup> cells coexpressed the proliferation marker Ki67 in the  $\alpha$ OX40/SM16-treated mice (Fig. 3B). To our knowledge, these CD8<sup>+</sup>GrzB<sup>+</sup> Ki67<sup>+</sup> T cells represent a novel subset of terminally differentiated proliferating T cells within the tumors. Others have described granzyme B<sup>+</sup> CD8 T cells as end-stage nonproliferating cells involved with tumor-cell killing (45); however, the CD8<sup>+</sup> T cells in the dual therapy-treated group seem to have a novel phenotype potentially capable of multiple functions. Previous studies have shown that OX40 signaling increases CD8<sup>+</sup> T-cell proliferation, IFN $\gamma$  production, and granzyme B expression (46). Conversely, TGF $\beta$  signaling in CD8<sup>+</sup> T cells can inhibit all of these functions (22). However, it is clear that single treatment with either SM16 or  $\alpha$ OX40 was unable to significantly increase the proportion of proliferating/effector CD8<sup>+</sup> T cells within the tumor. These data show that promoting OX40 signaling while simultaneously blocking TGF $\beta$  signaling has an additive/synergistic effect on tumor-infiltrating CD8<sup>+</sup> T-cell differentiation and proliferation, which most likely led to the enhancement of tumor-cell killing. The enhanced efficacy of the dual therapy may in part be explained by the ability of SM16 to increase the proportion of CD8<sup>+</sup> T cells that express(ed) OX40, hence increasing the likelihood of increased OX40 signaling within the TME (Fig. 4C).

Because OX40 and TGF $\beta$  signaling have been shown to modulate Treg proliferation, survival, and function (4, 5, 23, 24), we expected to find differences in the frequency of tumor-infiltrating Tregs. Surprisingly, we found no statistical change in the proportion of CD4<sup>+</sup> T cells that expressed FOXP3<sup>+</sup> within tumors of the different treatment groups in WT mice. However, we did observe a statistically significant increase in the percentage of tumor-isolated Tregs (FoxP3<sup>+</sup>CD25<sup>+</sup> CD4 cells) in the STAT3 KO mice compared



**Figure 5.**

STAT3 signaling is essential for full therapeutic potency of the anti-OX40/SM16 combination immunotherapy. A, mice with established tumors were treated with SM16 and anti-OX40, after which tumors were resected and tumor-infiltrating cells were assessed *ex vivo* for phosphorylated STAT3 (pSTAT3) expression by intracellular staining as in Fig. 2. Mean fluorescence intensity of pSTAT3 was quantified in CD8<sup>+</sup> and CD4<sup>+</sup> TILs. B, OX40cre/wt  $\times$  STAT3flx/flx (STAT3<sup>-/-</sup>) and OX40cre/wt  $\times$  STAT3wt/wt mice were inoculated with MCA205 tumor cells and treated as in Fig. 1 and monitored for tumor growth and overall survival. C, frequency of Ki67<sup>+</sup>GrzB<sup>+</sup>CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells. On day 18, splenocytes and TILs were isolated and assessed for Ki67 and GrzB by flow cytometry. D, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were sorted from TILs of SM16- and anti-OX40-treated OX40cre/wt  $\times$  STAT3flx.flx and OX40cre/wt  $\times$  STAT3wt.wt and restimulated for 5 hours with PMA/ionomycin, from which the supernatant was collected and analyzed by ELISA for IFN $\gamma$  production. E, TILs from treated mice were analyzed for FOXP3<sup>+</sup>/CD25<sup>+</sup>CD4<sup>+</sup> T-regulatory cell percentages and percent proliferating based on anti-Ki67 staining. All statistics were performed by the Student *t* test analysis.

with that in WT mice treated with the dual therapy. There was also an increase in Treg proliferation in the STAT3 KO mice compared with WT mice. Hence, there was most likely a STAT3-dependent cytokine signaling event that was increased during dual therapy, which can influence Treg percentages within the TME. It is interesting that despite the high frequency of CD4<sup>+</sup> cells that are FoxP3<sup>+</sup> (sometimes >30% of CD4 cells) within the tumors of  $\alpha$ OX40/SM16-treated WT mice, CD8<sup>+</sup> T cells appear capable of proliferating and differentiating into effector CD8<sup>+</sup> GrzB<sup>+</sup> cells. It currently remains unknown if  $\alpha$ OX40/SM16

treatment tempers Treg function and/or alters CD8<sup>+</sup> T-cell susceptibility to Treg suppression, but these types of questions will be addressed in future studies.

We found that combining  $\alpha$ OX40 antibodies with a TGF $\beta$  receptor signaling inhibitor (SM16) resulted in complete and durable regression of large established tumors. These responses were accompanied by changes in the T-cell frequencies and phenotype within these tumors and the ability to resist tumor rechallenge. Interestingly, we observed an increase in STAT3 phosphorylation in both CD8<sup>+</sup> and CD4<sup>+</sup> T cells infiltrating



tumors of mice receiving the combination therapy compared with tumor-bearing mice receiving either agent alone. Genetic ablation of the STAT3 gene in OX40-expressing T cells significantly reduced tumor cures in the dual therapy-treated mice. Characterization of TILs revealed an increase of proliferating effector CD8<sup>+</sup> T cells expressing granzyme B in tumors of WT mice that received combination treatment, and these populations were significantly reduced in STAT3 KO mice. These findings are opposite to the prevailing paradigm that STAT3 activation in T cells is deleterious to their function within tumor-bearing hosts (32). Instead, STAT3 activates a beneficial pathway(s) in T cells leading to tumor regression during anti-OX40 immunotherapy when the TGF $\beta$  signaling pathway is blocked. These findings justify rethinking the current dogma that STAT3 expressed in T cells decreases their function leading to increased tumor progression and suggest that blocking STAT3 signaling could have detrimental effects, especially when potent immune-based cancer therapies are delivered to cancer-bearing hosts.

Recent articles about STAT3 signaling in T cells have shown a critical/positive role in regulating survival of CD8 and CD4 T cells (47, 48). Hence, recent evidence suggests that STAT3 is important for proinflammatory functions of T cells, especially in the setting of viral infections (48). One report showed that STAT3-deficient T cells had an increased proliferative capacity, but were more prone to activation-induced cell death (AICD). This increase in proliferation and sensitivity to AICD in STAT3 KO T cells correlated with a decrease in antiapoptotic proteins, including OX40, and an increase in proapoptotic proteins (48). In the context of Herpes simplex virus infection, STAT3 deficiency led to a decrease in the percentage of CD8 T cells producing viral-specific IFN $\gamma$ . In humans, a dominant-negative mutation in STAT3 leads to a significant decrease in central memory CD4 and CD8 T cells, and patients carrying this defect are more susceptible to bacterial and fungal infections (47). Therefore, STAT3 is important for the

survival of T cells, and the survival could be mediated through gamma-chain cytokines, which are known to signal through STAT3 and STAT5. We hypothesize that in the context of increased inflammation, such as viral Toll-like receptor signaling or SM16/ $\alpha$ OX40 stimulation, STAT3 signaling increases T-cell survival and enhances antitumor immunity, and these results are quite opposite to previously published reports (32). Based on these differences, we favor a model in which STAT3 signaling could have opposing roles in T-cell function, depending on the environment the T cell encounters upon T-cell receptor engagement.

### Disclosure of Potential Conflicts of Interest

A.D. Weinberg is president/CSO at Agonox, Inc., reports receiving a commercial research grant from MedImmune, and has ownership interest in OX40 patents. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

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