

Timing of PD-1 Blockade Is Critical to Effective Combination Immunotherapy with Anti-OX40

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Abstract

Purpose: Antibodies specific for inhibitory checkpoints PD-1 and CTLA-4 have shown impressive results against solid tumors. This has fueled interest in novel immunotherapy combinations to affect patients who remain refractory to checkpoint blockade monotherapy. However, how to optimally combine checkpoint blockade with agents targeting T-cell costimulatory receptors, such as OX40, remains a critical question.

Experimental Design: We utilized an anti-PD-1-refractory, orthotopically transplanted MMTV-PyMT mammary cancer model to investigate the antitumor effect of an agonist anti-OX40 antibody combined with anti-PD-1. As PD-1 naturally aids in immune contraction after T-cell activation, we treated mice with concurrent combination treatment versus sequentially administering anti-OX40 followed by anti-PD-1.

Results: The concurrent addition of anti-PD-1 significantly attenuated the therapeutic effect of anti-OX40 alone. Combination-treated mice had considerable increases in type I and type II

serum cytokines and significantly augmented expression of inhibitory receptors or exhaustion markers CTLA-4 and TIM-3 on T cells. Combination treatment increased intratumoral CD4⁺ T-cell proliferation at day 13, but at day 19, both CD4⁺ and CD8⁺ T-cell proliferation was significantly reduced compared with untreated mice. In two tumor models, sequential combination of anti-OX40 followed by anti-PD-1 (but not the reverse order) resulted in significant increases in therapeutic efficacy. Against MMTV-PyMT tumors, sequential combination was dependent on both CD4⁺ and CD8⁺ T cells and completely regressed tumors in approximately 30% of treated animals.

Conclusions: These results highlight the importance of timing for optimized therapeutic effect with combination immunotherapies and suggest the testing of sequencing in combination immunotherapy clinical trials. *Clin Cancer Res*; 23(20); 6165–77. ©2017 AACR.

See related commentary by Colombo, p. 5999

Introduction

The potential for immunotherapy to improve outcomes of cancer patients, particularly through the combination of agents targeting immune inhibitory pathways, is becoming increasingly evident (1, 2). Nonetheless, how to optimally combine the myriad of new immunotherapy agents currently being developed remains a major question in cancer research. Antibodies targeting the programmed cell death protein-1 (PD-1, CD279) receptor have made a major therapeutic impact on multiple types of solid tumors (3). Given relative low levels of reported toxicity combined with therapeutic efficacy, PD-1 pathway blockade is currently the building block for testing combinations with other

immunotherapeutics. PD-1 is an inhibitory molecule upregulated after T-cell receptor (TCR) engagement that normally plays a major role in immune contraction, leading T cells to exhaustion and apoptosis (3–5). Cancer, however, can use the PD-1 pathway to its advantage by expressing programmed death-ligand 1 (PD-L1, B7-H1, CD274) on a tumor's surface or inducing it on the surface of other tumor-associated immune cells like macrophages or dendritic cells to suppress an antitumor immune response, making the PD-1 receptor an attractive target for immunotherapeutic intervention (6, 7). By blocking PD-1 or PD-L1, exhausted tumor-specific effector T cells can then be reinvigorated to enhance their function (8).

OX40 (CD134 or TNFRSF4) is a TNF family costimulatory receptor that is also upregulated on T cells after TCR recognition of specific antigen (9, 10). However, when engaged with its ligand, OX40 stimulation results in enhanced proliferation, activation, differentiation, and survival (9, 11, 12). OX40 is expressed on activated, conventional CD4⁺ and CD8⁺ T cells and strongly expressed on CD4⁺FoxP3⁺ regulatory T (Treg) cells, and can also be upregulated shortly after reactivation of primed effector T cells (11, 13). Agonist antibodies specific to OX40 can induce significant antitumor effects in preclinical models (14, 15), and despite OX40 expression occurring mainly on CD4⁺ T cells, antitumor responses have been credited to both CD4⁺ and CD8⁺ cells (13, 16). OX40 costimulation has also demonstrated enhanced preclinical antitumor effects when combined with anticytotoxic T lymphocyte-associated protein-4 (CTLA-4) and either adjuvants, vaccination, or radiation (17–19). Supported by this

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Translational Relevance

In an attempt to improve the percentage of patients who respond to PD-1 blockade, hundreds of studies are combining treatments that block PD-1 signaling with anticancer drugs or other immunologic therapies. Much of the preclinical data supporting these studies were generated from immunogenic tumor models that are responsive to checkpoint blockade. Using tumor models unresponsive to checkpoint blockade, we found that simultaneous administration of a T-cell agonist with checkpoint blockade reduced therapeutic efficacy compared with the agonist alone, but sequencing significantly improved therapy and resulted in apparent cure of some animals. Although no animal model is a perfect surrogate for developing immunotherapy of human cancer, these studies identify that the schedule of drug administration can significantly affect therapeutic efficacy and suggest some parameters that might be evaluated in early combination clinical trials of immunologically active agents.

promising preclinical data, OX40 is currently being evaluated in clinical trials in a variety of solid tumors (20).

Breast cancer is the most commonly diagnosed cancer in women, but conventional therapies, such as radiation, chemotherapy, and targeted therapies like the anti-HER-2 drug trastuzumab (Herceptin), have led to significant improvements in patient survival rates over recent decades. Nevertheless, a substantial portion of patients remain refractory to these conventional treatments, and over the past decade, a multitude of preclinical studies demonstrating immunotherapy-mediated tumor regression, including with anti-OX40 (14), have renewed interest in utilizing immunotherapies in breast cancer and spawned a variety of clinical trials. Adding to this interest, tumor-infiltrating lymphocytes (TIL) have been shown to associate with good clinical outcome (21) and response to therapy (22). PD-1 expression on TIL and PD-L1 expression on breast cancer tumors is associated with worse prognosis (23, 24), and preliminary results of clinical trials with PD-1 blockade have produced objective responses in specific subsets of patients (25). However, overall responses have been modest (5%–19%), leaving a majority of patients refractory to monotherapy. Breast cancer TILs can express OX40 (14, 26), and polymorphisms in OX40L (CD252) have shown to be associated with breast cancer carcinogenesis (27), suggesting that antibodies targeting both OX40 and PD-1 could be effective when given in combination.

To address the potential for antitumor effects by combining immunotherapies that target both receptors, we employed orthotopically transplanted tumors from murine mammary tumor virus polyoma middle T (MMTV-PyMT) mice, a preclinical model of oncogene-driven mammary cancer (28). This model resembles luminal human breast cancer and is infiltrated with myeloid and T cells (29), making it a strong candidate for immunotherapy with T cell–targeting antibodies. Here, we report that this model is refractory to PD-1 blockade, but by stimulating the OX40 receptor with an agonist antibody, we significantly delayed tumor progression. However, concurrent combination of anti-OX40 and PD-1 blockade diminished this effect. Along with weakened antitumor effect, we noted an acute increase in serum cytokines with combination treatment,

and heightened expression of T cell–inhibitory receptors. However, by delaying anti-PD-1 administration, sequential combination treatment greatly enhanced the effects of anti-OX40 monotherapy and provided durable responses dependent on both CD4⁺ and CD8⁺ T cells that eliminated tumors in a substantial portion of animals.

Materials and Methods

Mice and tumor models

FVB/NJ MMTV-PyMT mice were provided by Emmanuel Akporiaye [Earle. A Chile Research Institute (EACRI), Portland, OR]. Female FVB/NJ and BALB/c mice of 6 to 8 weeks were purchased from The Jackson Laboratory. Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 2011), and all animal protocols were approved by the EACRI Animal Care and Use Committee. All mice were maintained in a specific pathogen–free environment. For each independent experiment, three tumors from a MMTV-PyMT mouse were digested with triple enzymes (collagenase, DNAase, hyaluronidase) for 30 minutes and resuspended in Matrigel Matrix (Corning) (19, 30). Cells (10⁶) were orthotopically transplanted into the mammary fat pad of naïve FVB/NJ mice (day 0). Seven days later, mice were randomized and injected intraperitoneally with either 100 µg of anti-OX40 (clone OX86), 250 µg anti-PD-1 (clone G4; ref. 7), both (for concurrent treatment), or nothing on days 7, 9, and 11. Anti-OX40 and anti-PD-1 were a gift from Andrew D. Weinberg (EACRI, Portland, OR). For sequential combination treatment, mice were given anti-OX40 on days 7, 9, and 11, followed by anti-PD-1 on days 13, 15, and 17. For experiments combining anti-OX40 with anti-PD-L1, 200 µg of anti-PD-L1 (clone 10F.962, Bio X Cell) was substituted for anti-PD-1 on the same time course. The 4T1 tumor cell line was a gift from Emmanuel Akporiaye in 2010, from stocks received from Suzanne Ostrand-Rosenberg (UMBC, Baltimore, MD). Their identity was independently confirmed via STR profiling (IDEXX Biorsearch) in 2013, and they were mycoplasma negative. All tumor cells were cultured in complete media (CM) consisting of RPMI1640 (Lonza) with 1% l-glutamine (Lonza), 1% sodium pyruvate (Lonza), 1% nonessential amino acids (Lonza), 0.1% β-mercaptoethanol, 50 mg gentamicin sulfate, and 10% FBS (Atlas Biologicals lot # 1070612), and 4T1 cells were passaged twice before tumor challenge. 4T1 tumor cells (5 × 10³) were transplanted in the mammary fat pad of naïve BALB/c mice. Five days later, mice were randomized and given anti-OX40, anti-PD-1, or both on days 5, 7, and 9 (concurrent) or anti-OX40 on days 5, 7, and 9, followed by delayed anti-PD-1 on days 11, 13, and 15. Tumors were measured three times weekly, and mice were sacrificed when tumors reached 150 mm² (measured width × length).

CD4/CD8 depletion

Mice were injected intraperitoneally with 250 µg anti-CD4 (GK1.5, Bio X Cell), anti-CD8 (53-6.7, Bio X Cell), or rat IgG (Bio X Cell) twice, one week apart (days 6 and 13 after tumor transplant). Mice were analyzed for confirmation of depletion by staining with different clones targeting CD4 or CD8 at 1 µg/mL for whole blood, spleen, and tumor.

Serum cytokines

For serum cytokine analysis, whole blood from treated and nontreated, PyMT tumor-bearing animals was collected on days 7,

9, 11, 13, 14, 17, and 20 after tumor transplant. Serum was isolated by 30 minutes of incubation at 37°C, 16 hours at 4°C, and 15-minute centrifugation at 2,400 RCF. Serum was stained with Th1/Th2/Th17 Cytokine Bead Array Kit (BD Biosciences), and run on an LSRII flow cytometer (BD Biosciences).

Flow cytometry

Surface and intracellular receptors were measured from single-cell suspensions of day 13, 14, or 19 splenocytes and tumor digested from treated and untreated MMTV-PyMT tumor-bearing mice. Cells were stained for CD3, CD8, CD4, CD45.1, ICOS, CD137 (4-1BB), GITR, PD-1, PD-L1, TIM-3, BTLA, and live/dead. All antibodies were obtained from eBioscience, Molecular Probes, or BD Biosciences. Intracellular staining of FoxP3, Ki67, Annexin V, IFN γ , and CTLA-4 was performed with FoxP3 Fixation/Permeabilization Kit (eBioscience). Samples were run on LRSII Fortessa (BD Biosciences) and analyzed using FlowJo software (TreeStar). Cells were gated on lymphocytes > singlets > live cells > CD3⁺ > CD4⁺ or CD8⁺ for splenocytes or lymphocytes > singlets > live cells > CD45.1⁺ > CD3⁺ > CD4⁺ or CD8⁺ for TILs.

Polyoma middle T antigen purification and T-cell assays

Lysates of an MMTV-PyMT-derived tumor cell line, FAT, were run over anti-PyMT (PyMT, Abcam cat # ab15085)–crosslinked magnetic beads using the Pierce Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific). The second elution was used, and PyMT protein was confirmed via SDS-PAGE and Western blot analysis with anti-PyMT. Tumor-bearing treated and untreated day 19 splenocytes were pooled and expanded as previously described by our laboratory (31) for 48 hours at 2×10^6 cells/mL in CM in 24-well plates with 5 μ g/mL of anti-CD3 (clone 2c11), followed by 72 hours at 2×10^5 cells/mL in CM with 60 IU/mL of IL2 (Chiron), all at 37°C. Expanded T cells were cultured with PyMT-loaded antigen-presenting cells (APC). For APC loading, naïve, FVB/NJ splenocytes were cultured for 6 hours in CM with either no (neutralized elution buffer only), 1 μ g/mL, or 10 μ g/mL of PyMT protein and then cocultured 1:1 with 10^6 expanded T cells. After 24 hours, supernatants were assayed by ELISA using purified rat anti-mouse IFN γ antibodies at 2 μ g/mL, rat anti-mouse anti-IFN γ biotin-labeled antibodies at 1 μ g/mL, streptavidin HRP (BD Biosciences), and SureBlue TMB substrate (KPL). Absorbance at 450 nm was read on a Wallac Victor2 plate reader. IFN γ concentration was determined by linear regression using WorkOut 2.5 software (PerkinElmer).

Statistical analysis

All statistical analyses were performed using Gehan–Breslow–Wilcoxon test (survival) or one-way ANOVA with Tukey or Dunnett multiple comparison tests (tumor growth, phenotype comparisons). All statistics were done with Prism 6 (GraphPad Software). In Fig. 4C, one outlier data point was thrown out of the no treatment group due to an error in cell counting.

Results

Concurrent PD-1 blockade diminishes the therapeutic efficacy of OX40 costimulation

Given previous reports of antitumor effects with monotherapy of either anti-OX40 or anti-PD-1 antibodies, we sought to evaluate the therapeutic efficacy of combination therapy in murine

mammary cancer models. We utilized an orthotopically transplanted mammary tumor model where spontaneous tumors generated from FVB/NJ MMTV-PyMT transgenic mice were transplanted into naïve FVB/NJ hosts. Before treatment, 7 days posttransplant, OX40 expression in the tumor was predominantly on CD4⁺FoxP3⁺ Treg cells, although some conventional CD4⁺FoxP3⁻ and CD8⁺ cells also expressed OX40 (Fig. 1A and B). Alternatively, PD-1 expression was only identified on intratumoral CD4⁺FoxP3⁻ and CD8⁺ cells (Fig. 1A and C). We noted very few T cells expressing both receptors. T cells in the draining lymph node and spleen were not PD-1⁺ and only Treg cells expressed OX40, although at a lower frequency than in the tumor (Fig. 1A–C). Beginning on day 7, tumor-bearing mice (average tumor size of ~ 6 – 9 mm²) were treated with three doses of either an agonist antibody targeting OX40 (anti-OX40), a blocking antibody targeting PD-1 (anti-PD-1), or both antibodies on days 7, 9, and 11 (Fig. 1D). Mice that received anti-OX40 treatment exhibited a significant attenuation in tumor growth compared with untreated mice, while anti-PD-1 had no impact on tumor growth (Fig. 1E). However, when anti-OX40 was combined with anti-PD-1, instead of increasing therapeutic efficacy, we noted a significant reduction in tumor control compared with anti-OX40 alone. Survival was also significantly diminished in mice receiving combination therapy compared with anti-OX40 alone (Fig. 1F). These data indicate that anti-PD-1 fails to improve the therapeutic efficacy provided by anti-OX40, and in fact, PD-1 blockade provides an adverse effect on anti-OX40-induced therapy in this model.

Concurrent PD-1 blockade and OX40 costimulation increases serum cytokines and upregulates T-cell–inhibitory receptors

A recent publication reported that OX40 costimulation combined with either PD-1 or PD-L1 blockade leads to excessive IFN γ production and loss of parasite control in the context of plasmodium infection (32). Considering this as a possible mechanism for the reduced efficacy concurrent PD-1 blockade imparted on anti-OX40-treated mice, we measured the levels of both type I and type II cytokines in serum from tumor-bearing animals during and after antibody treatment. A striking increase in IFN γ was detected during treatment that peaked 2 days after the last antibody dose in combination-treated mice compared with mice receiving monotherapy with either agent (Fig. 2A). While anti-PD-1 alone had a substantial effect on cytokine levels compared with untreated mice, combination treatment boosted cytokines 10- to 100-fold higher than in untreated tumor-bearing mice. Serum levels of other cytokines, including IL6 as well as both type I (TNF α) and type II (IL4 and IL10) cytokines were also highly elevated in the combination-treated group compared with anti-OX40 alone (Fig. 2A). Consistent with symptoms of cytokine release syndrome or a cytokine storm-like event, combination-treated mice also exhibited ruffled, unkempt fur and lethargy from approximately days 9 to 14. Untreated mice had minimal or undetectable levels of all cytokines. Intriguingly, we saw consistent patterns in the kinetics of specific types of cytokines over time. Type II cytokines IL4 and IL10 in combination-treated mice peaked early, only 2 days after the first antibody dose, followed by a gradual decline but a late increase 9 days after the last antibody dose. At the same time, type I cytokines TNF α and IFN γ increased during the treatment period, with TNF α peaking first, and then declined after the last antibody dose. Together, these data demonstrate that simultaneously stimulating the OX40

Messenheimer et al.

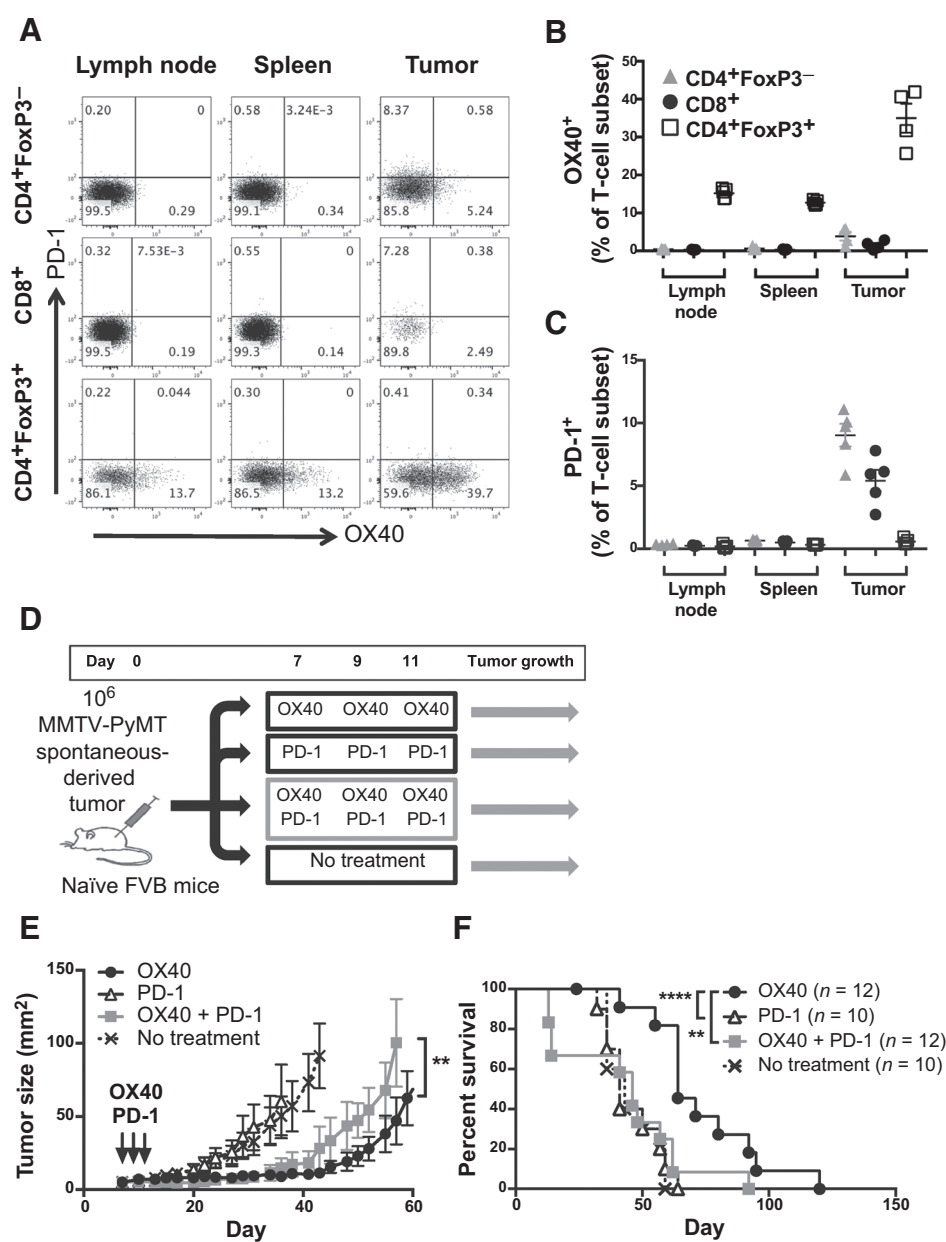


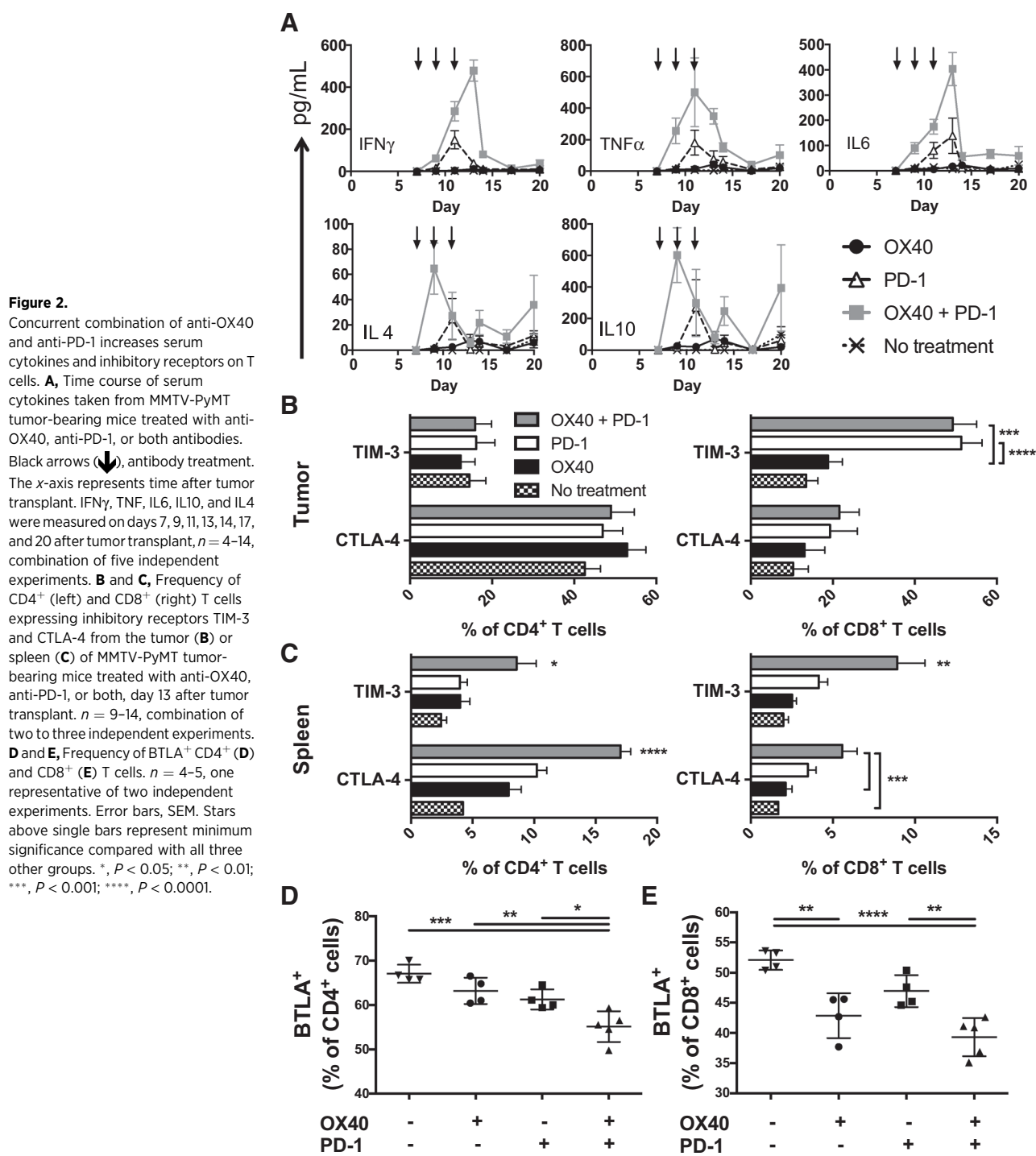
Figure 1. Concurrent administration of anti-PD-1 and anti-OX40 reduces antitumor effect of anti-OX40 alone. **A**, Representative flow cytometric quantification of OX40 and PD-1 on conventional CD4⁺FoxP3⁻, CD8⁺, and CD4⁺FoxP3⁺ T cells in the draining lymph node, spleen, and tumor of pretreatment 7-day MMTV-PyMT tumor-bearing mice. **B**, Frequency of OX40⁺ T cells. *n* = 4–5, one representative of two independent experiments. **C**, Frequency of PD-1⁺ T cells. **D**, Treatment schedule of MMTV-PyMT tumor-bearing mice. Mice were treated on days 7, 9, and 11 with 100 μg anti-OX40, 250 μg anti-PD-1, or both antibodies. **E**, Mean tumor growth of treated tumors, *n* = 6, one representative of two independent experiments. **F**, Kaplan-Meier survival curves of treated mice. *n* = 10–12, combination of two independent experiments. Error bars, SEM. **, *P* < 0.01; ****, *P* < 0.0001.

receptor and blocking the PD-1 receptor can have profound effects on the cytokine milieu, drastically increasing the acute cytokine release stimulated by anti-PD-1 monotherapy.

We noted splenomegaly in tumor-bearing mice treated with the combination of anti-OX40 and anti-PD-1 (Supplementary Fig. S1A). Splenocyte numbers increased after just a single dose of combination antibodies at day 9 and steadily grew over time to significantly outpace untreated or monotherapy-treated mice, peaking at day 17 (Supplementary Fig. S1B). Given the substantial increase in serum cytokines and this splenomegaly, we investigated whether combination treatment associated with an increase in peripheral T-cell activation induced cell death (AICD) or apoptosis. Combination treatment increased the frequency of Annexin V⁺ cells that also stained positive with propidium iodide (PI, Live/Dead), marking them as dead, apoptotic cells, in both

the CD4⁺ and CD8⁺ T-cell splenic compartment (Supplementary Fig. S1C and S1D).

T-cell activation and inhibition is a delicate balance of positive and negative signals that involves a host of transiently and constitutively expressed surface receptors, some of which are compensatory and other independent of each other. With the amplified levels of IFN γ and other cytokines observed in the serum of combination-treated mice, we anticipated substantial changes to T-cell surface receptor expression. Similar to previous reports (33), we found that a majority of untreated splenic T cells expressed PD-L1 on day 13, and treatment further increased the frequency of PD-L1⁺ T cells (Supplementary Fig. S2A and S2B). IFN γ upregulates PD-L1, and after combination treatment, we observed a significant increase in PD-L1 fluorescence intensity on both CD4⁺ and CD8⁺ T cells (Supplementary Fig. S2C and S2D).



The frequency of intratumoral CD8 $^{+}$ T cells expressing inhibitory receptors T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) or CTLA-4 was also increased with anti-PD-1 or combination treatment, with more than double the frequency of TIM-3 $^{+}$ cells compared with anti-OX40 monotherapy (Fig. 2B). The prevalence of CTLA-4 $^{+}$ CD4 $^{+}$ cells also increased in all treatment groups, but there were no changes to TIM-3 $^{+}$ CD4 $^{+}$ cells with treatment (Fig. 2B). In the spleen, however, there were significant increases in the frequencies of both CTLA-4 $^{+}$ and

TIM-3 $^{+}$ cells in both the CD4 $^{+}$ and CD8 $^{+}$ compartments with combination treatment (Fig. 2C). B and T lymphocyte attenuator (BTLA) is another inhibitory marker initially expressed on activated T cells, but subsequent downregulation (BTLA $^{-}$) is associated with terminal T-cell differentiation in the tumor and a decreased capacity to proliferate (34). With combination treatment, BTLA $^{+}$ T cells were significantly decreased in both the CD4 $^{+}$ and CD8 $^{+}$ T-cell populations of the spleen (Fig. 2D and E). The frequency of cells expressing costimulatory molecules,

Messenheimer et al.

inducible T-cell costimulator (ICOS), 4-1BB (CD137), or glucocorticoid-induced TNFR-related protein (GITR) in the tumor was not altered with any treatment (Supplementary Fig. S3A and S3B). However, in the spleen, combination treatment significantly increased the prevalence of ICOS⁺ and 4-1BB⁺ cells in both the CD4⁺ and CD8⁺ compartments (Supplementary Fig. S3C and S3D). Thus, combination treatment appears to primarily impact CD8⁺ T cells in the tumor, but both CD4⁺ and CD8⁺ T cells in the periphery. These data suggest that concurrent anti-OX40 and anti-PD-1 combination treatment impacts peripheral T cells more than either agent alone. But importantly, combination treatment increases the frequency of exhausted T cells in the tumor compared with anti-OX40 monotherapy.

Concurrent combination therapy increases the frequency of proliferating T cells in the tumor

Given the suggestion that combination treatment increased exhaustion in TILs, we next investigated the composition and proliferation of intratumoral T cells. Two days after the completion of treatment (day 13 after tumor transplant), anti-OX40-treated tumors had an increased frequency of CD3⁺ T cells compared with untreated tumors (Fig. 3A). T-cell frequency was further increased with the addition of anti-PD-1. The predominant population of T cells in treated or untreated tumors was CD4⁺ cells, specifically CD4⁺FoxP3⁻ conventional T cells (Fig. 3B). However, only anti-OX40 monotherapy caused a significant increase in the total number of CD4⁺FoxP3⁻ conventional and CD4⁺FoxP3⁺ Treg T cells infiltrating the tumor, while CD8⁺ T cells were unchanged (Fig. 3C). Neither antibody resulted in a significant depletion of Treg cells. Given an attenuated therapeutic effect (Fig. 1E and F), unexpectedly, combination treatment significantly increased the frequency of both Ki67⁺ proliferating CD4⁺FoxP3⁻ and CD8⁺ T cells (Fig. 3D). The prevalence of proliferating CD4⁺FoxP3⁺ Treg cells was significantly increased with combination compared with anti-PD-1 monotherapy or no treatment, but not anti-OX40 treatment. Although combination treatment increases proliferation of TILs at this time point, the increase in TIM-3 expression (Fig. 2B) and loss of BTLA expression (Fig. 2D and E) suggest that this effect may be short lived, and this metric does not correlate with therapeutic response. Thus, we investigated alternative ways to successfully combine OX40 costimulation with PD-1 blockade.

Sequential, delayed PD-1 blockade augments anti-OX40 antitumor effects

Given the diminished antitumor effects generated by the concurrent combination of anti-OX40 and anti-PD-1 and the knowledge that PD-1 plays a critical role in the contraction phase of a normal immune response, we reasoned that combination treatment with anti-PD-1 antibody would be more effective if it was given after the initial T-cell boost generated by anti-OX40. In support of this hypothesis, we noted a significant increase in PD-1 expression on day 13 splenic CD4⁺ and CD8⁺ T cells after anti-OX40 treatment (Fig. 4A). Accordingly, anti-OX40 given on days 7, 9, and 11 after tumor transplant was followed by delayed anti-PD-1 administered on days 13, 15, and 17, providing a sequential treatment (Fig. 4B). This sequential treatment resulted in a significant delay in tumor growth compared with anti-OX40 alone with some tumors reaching complete regression (Fig. 4C). Sequential combination therapy was also far superior to concurrent combination therapy. Delayed anti-PD-1 treatment alone

had no significant impact on tumor growth (Supplementary Fig. S4A). Anti-OX40 plus delayed anti-PD-1 correspondingly resulted in a significant increase in survival, with approximately 30% of the animals experiencing complete regression of their tumors (200+ days; Fig. 4D).

To establish that the specific order of sequential treatment was crucial, we reversed the combination, giving anti-PD-1 first, followed by delayed anti-OX40. This treatment proved to be much less effective than anti-OX40 plus delayed anti-PD-1, demonstrating that providing OX40 costimulation first and then blocking the PD-1 receptor is critical to the effects of sequential combination therapy (Fig. 4E). We confirmed these results in the poorly immunogenic, faster growing 4T1 mammary tumor model. Although therapeutic efficacy was not as profound as in the MMTV-PyMT model, sequential combination treatment provided significant tumor growth delay, but concurrent combination had no effect on tumor growth (Supplementary Fig. S5A). The reversed sequence results were also confirmed in the 4T1 model (Supplementary Fig. S5B).

Despite possible differences in mechanism, targeting the PD-1 receptor or its ligand PD-L1 for immunotherapeutic intervention has shown similar results in the clinic (3, 35). To investigate whether timing was also critical for anti-OX40 combination with PD-L1 blockade, we compared two treatment strategies, concurrent or sequential combination of anti-OX40 and anti-PD-L1. Anti-PD-L1 alone provided a short but significant delay to tumor progression, differentiating it from anti-PD-1 treatment (Supplementary Fig. S4B; Fig. 1D). However, in combination, sequential anti-OX40 and anti-PD-L1 treatment delayed tumor growth for a longer period of time compared with concurrent combination ($P = 0.05$, Fig. 4F). These data establish that sequential combination of anti-OX40 with PD-1 pathway blockade provides superior therapy to concurrent combination in two different preclinical mammary cancer models.

Sequential anti-OX40 and anti-PD-1 combination therapy maintains proliferating T cells and reduces T-cell exhaustion

Unlike 6 days earlier, when concurrent combination treatment increased TIL proliferation (Fig. 3D), on day 19 (2 days after the final sequential anti-PD-1 dose), concurrent combination treatment resulted in a significant reduction in the frequency of Ki67⁺ proliferating CD4⁺FoxP3⁻ and CD8⁺ T cells in the tumor (Fig. 5A and B). However, this drop in proliferation did not occur with sequential combination treatment. In addition, at day 19, we noted significantly more BTLA⁺ CD4⁺ and CD8⁺ T cells in the tumor with sequential combination treatment (Fig. 5C and D). The large spikes in multiple serum cytokines induced by concurrent anti-OX40 and anti-PD-1 led us to investigate cytokine levels following sequential combination therapy. We compared the levels of IFN γ and TNF α after completion of concurrent combination or anti-OX40 alone (day 13) and sequential combination (day 19) and again noted very high levels of both cytokines in concurrent combination animals on day 13 (Supplementary Fig. S6A and S6B). However, sequential combination treatment did not induce high levels of either cytokine at the earlier (anti-OX40 only) or later time points. In addition, the frequency of CD8⁺ T cells expressing TIM-3 in the concurrent combination-treated tumors at day 19 remained at similar frequencies as day 13, but importantly, the sequential addition of anti-PD-1 to anti-OX40 did not increase this population of exhausted cells compared with anti-OX40 monotherapy (Fig. 5E). CTLA-4 expression was the

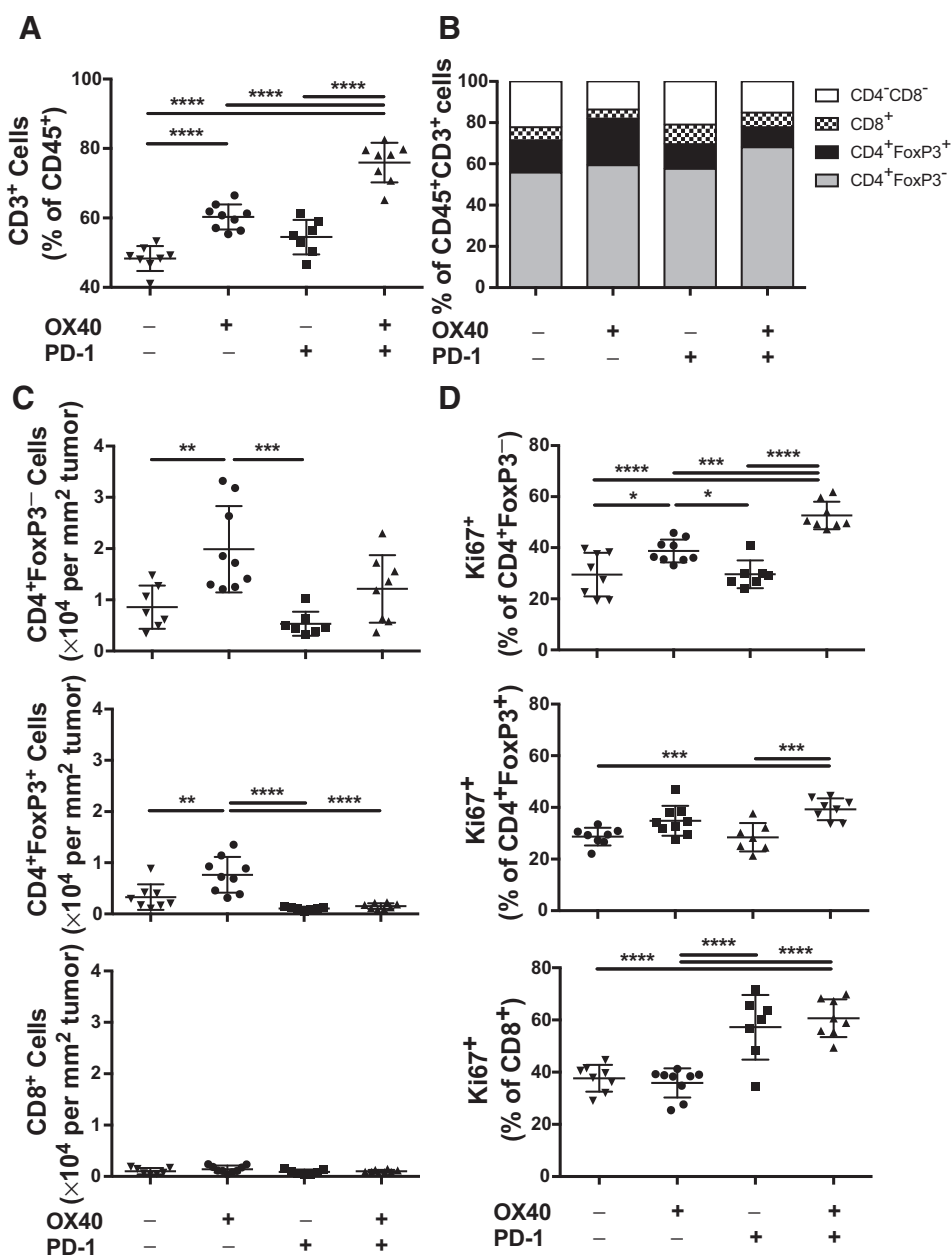
opposite, with increased frequencies of CTLA-4⁺ cells associating with the improved responses of anti-OX40 monotherapy and sequential combination (Fig. 5E). Patterns of expression on CD8⁺ T cells in the spleen were similar, but at much lower frequencies (Fig. 5F). At this later time point, the frequency of T cells expressing costimulatory molecules was not significantly altered by sequential combination treatment in the tumor or spleen (Supplementary Fig. S7). Combined, these data demonstrate that concurrent combination treatment induces a strong, but short-lived burst of intratumoral T-cell proliferation, which coincides with acute cytokine secretion, increased TIM-3⁺CD8⁺ exhausted cells, and attenuated antitumor effect (Fig. 1E and F). But administering anti-OX40 and anti-PD-1 in a sequential fashion maintains less differentiated, BTLA⁺ T cells, sustains proliferation in the

tumor, and avoids the TIL exhaustion induced by concurrent combination treatment.

To evaluate whether combination treatment increased the T-cell response to a specific tumor-associated antigen, we purified whole protein of the viral oncogene polyoma middle T antigen (PyMT) from an MMTV-PyMT-derived cell line previously established in our laboratory (Supplementary Fig. S8A). We loaded whole protein onto APCs and stimulated anti-CD3/IL2 expanded splenocytes from day 19 tumor-bearing mice treated with concurrent or sequential combination therapy. T cells from untreated MMTV-PyMT-bearing mice generated a T-cell response against PyMT, identified by secretion of IFN γ after stimulation with PyMT-loaded APCs (Supplementary Fig. S8B). As expected, sequential combination

Figure 3.

Concurrent combination treatment increases the frequency of proliferating T cells in the tumor, but only anti-OX40 increases total TILs. MMTV-PyMT tumor-bearing mice were treated as in Fig. 1C, and tumors were resected on day 13. **A**, Flow cytometric quantification of the frequency of CD3⁺ T cells of the CD45⁺ cell population in treated tumors. **B**, Average T-cell populations as a frequency of CD45⁺CD3⁺ cells in the tumor. **C**, Total conventional CD4⁺FoxP3⁻ (top), CD4⁺FoxP3⁺ Tregs (middle), and CD8⁺ (bottom) T cells in untreated and treated tumors. Normalized by square millimeters per tumor. **D**, Frequency of Ki67⁺ proliferating conventional CD4⁺FoxP3⁻ (top), CD4⁺FoxP3⁺ Tregs (middle), and CD8⁺ (bottom) T cells in the tumor. *n* = 7-10, combination of two independent experiments. Error bars, SEM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.



Messenheimer et al.

immunotherapy with anti-OX40 followed by anti-PD-1 increased the IFN γ response to PyMT; however, the magnitude of this response was the same as that observed for mice treated with anti-OX40 and anti-PD-1 concurrently.

Antitumor effect of sequential anti-OX40 and anti-PD-1 combination requires both CD4⁺ and CD8⁺ T cells for optimal therapeutic effect

Given the contribution that CD4⁺ T cells can have to T-cell help and the high prevalence of CD4⁺FoxP3⁻ T cells in MMTV-PyMT tumors (Fig. 3B and C), we sought to evaluate the prevalence and necessity of CD4⁺ and CD8⁺ T cells in the antitumor effect provided by sequential combination treatment. Phenotyping tumor-infiltrating T cells in sequential combination-treated tumors on day 19 (2 days after the final anti-PD-1 dose), we noted conventional CD4⁺FoxP3⁻ T cells still made up a majority of the T cells in treated tumors (Fig. 6A). To assess the role of different T-cell populations, either CD4⁺ depleting, CD8⁺ deplet-

ing, or rat IgG nondepleting antibodies were injected on days 6 and 13 into MMTV-PyMT tumor-bearing mice before both anti-OX40 (beginning on day 7) and anti-PD-1 (beginning on day 13) were administered. CD4⁺ T-cell depletion led to a significant increase in tumor-infiltrating CD8⁺ T cells in sequential combination-treated tumors (Fig. 6B), but despite this increase, CD4⁺ T-cell ablation completely eliminated the therapeutic efficacy provided by sequential combination, proving their necessity for the combination treatment (Fig. 6C). CD8⁺ T cells were also necessary for the observed therapeutic efficacy (Fig. 6D). Although initially the therapeutic effect was unaltered, loss of tumor control became apparent at later time points (after day 30). Consequently, depletion of either CD4⁺ or CD8⁺ T cells in combination-treated mice reduced animal survival, although CD8⁺ depleted mice had significantly longer survival than CD4⁺ depleted or untreated animals (Fig. 6E). These data strongly indicate that although conventional CD4⁺ T cells play a major and necessary role in the immediate tumor control provided by sequential

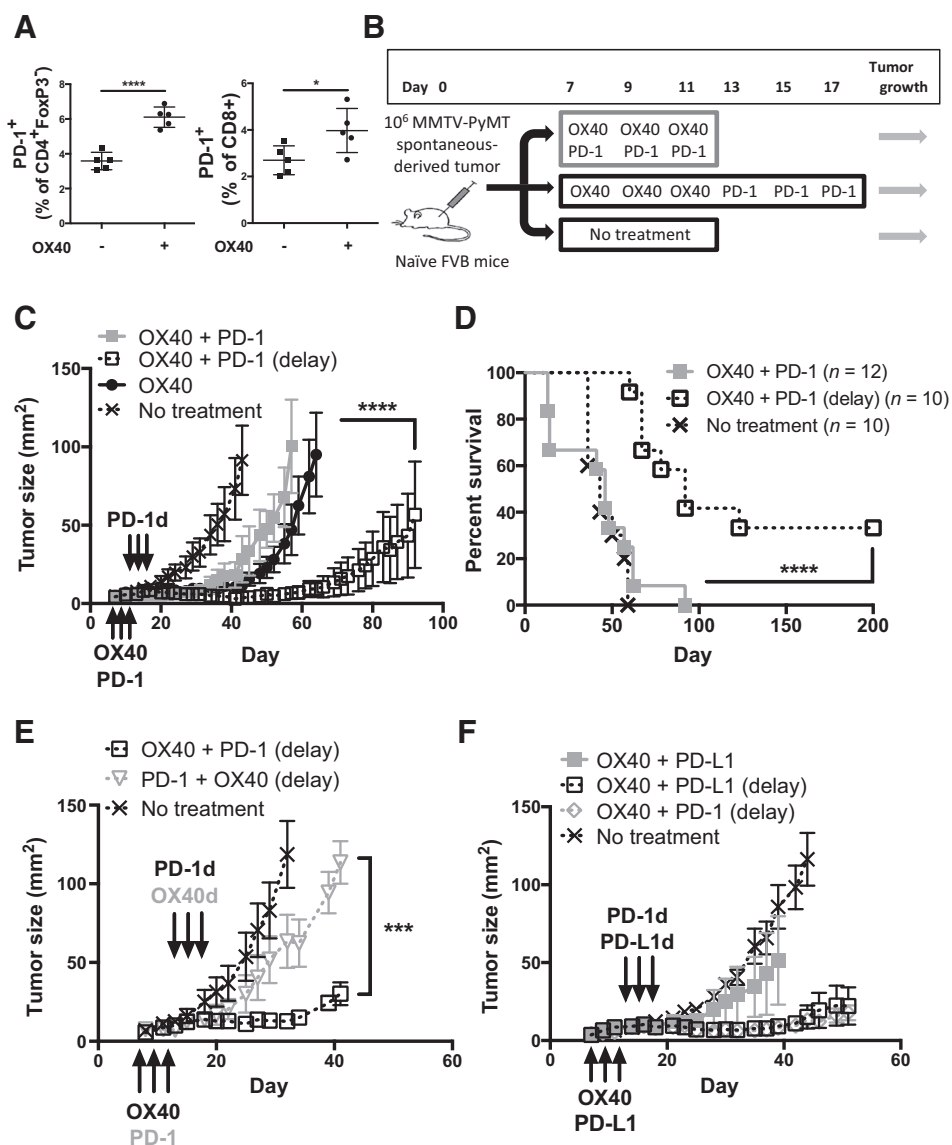


Figure 4.

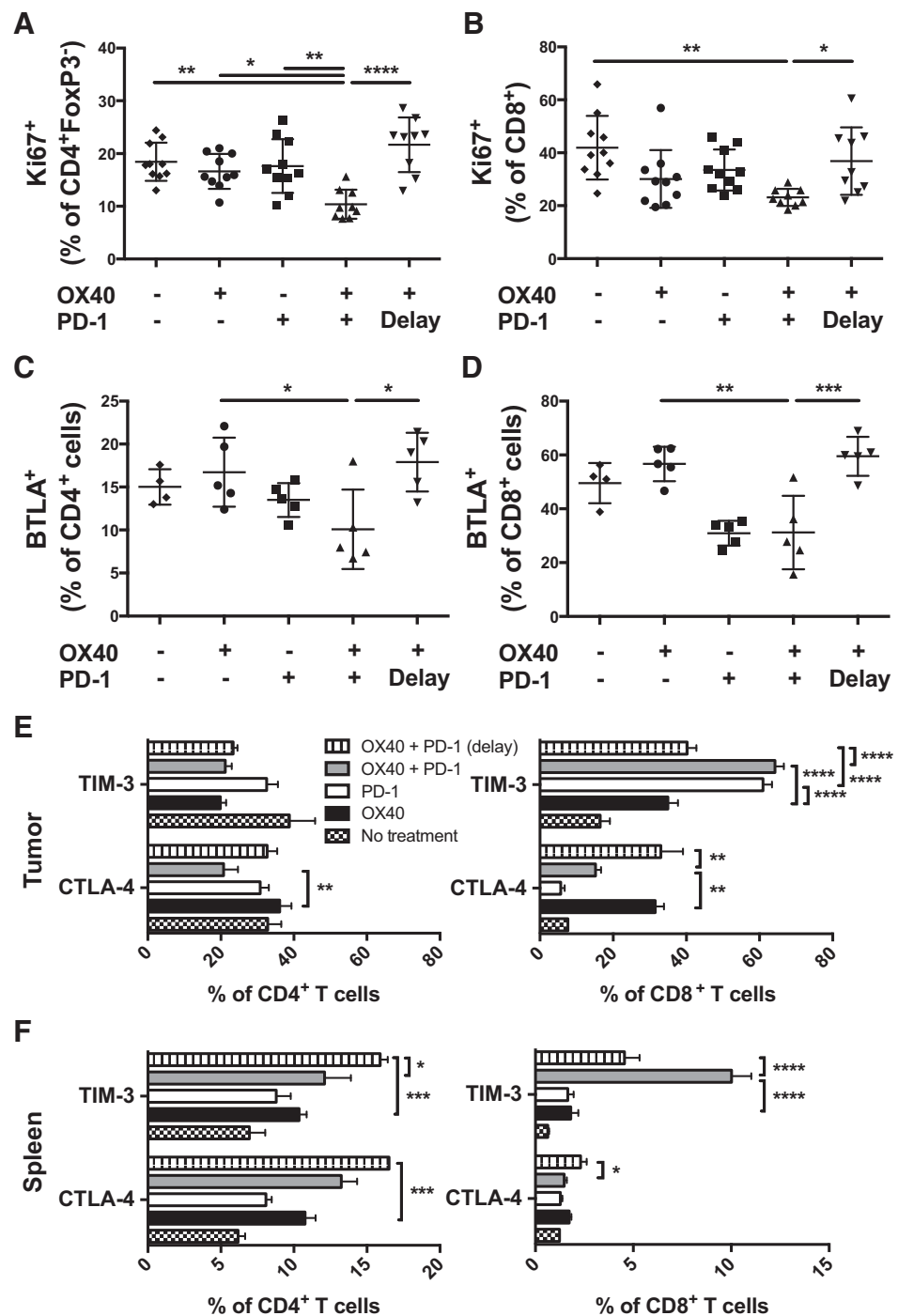
Sequential combination treatment augments anti-OX40 therapeutic efficacy. **A**, Frequency of PD-1 expression on conventional CD4⁺ (left) and CD8⁺ (right) T cells in day 13 MMTV-PyMT tumor-bearing spleens after anti-OX40 therapy. $n = 5$, one representative of four independent experiments. **B**, Treatment schedule of MMTV-PyMT tumor-bearing mice comparing concurrent and delayed (delay, d), sequential therapy. Delayed anti-PD-1 was given on days 13, 15, and 17. **C**, Mean tumor growth of treated tumors, $n = 6$, one representative of two independent experiments is shown. **D**, Survival of combination-treated mice. $n = 10$ –12, combination of two independent experiments. **E**, Mean tumor growth of tumors treated sequentially with anti-OX40 then anti-PD-1 or anti-PD-1 then anti-OX40. $n = 6$, one representative of two independent experiments is shown. **F**, Mean tumor growth of MMTV-PyMT tumor-bearing mice treated with a combination of anti-OX40 plus 200 μ g of either concurrent (days 7, 9, and 11) or delayed (days 13, 15, and 17) anti-PD-L1. $n = 5$ –6, one representative of two independent experiments is shown. Error bars, SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

anti-OX40 plus anti-PD-1, CD8⁺ T cells are also necessary for long-term antitumor effect.

Discussion

Questions of timing and sequence are important aspects of study design when complementing chemotherapy, radiotherapy, or targeted therapies, but as novel immunotherapies continue to demonstrate significant clinical impact, it will be important to

design treatment regimens that recognize and optimize compensatory, regulatory, and homeostatic immune mechanisms. Recent trials have focused on combined checkpoint blockade, but agents that target costimulatory molecules, such as OX40, offer a promising approach to augment the effect of current immunotherapies targeting inhibitory receptors like PD-1. Treatments combining T cell-targeting antibodies with vaccines, adoptive T-cell transfer, and targeted therapies are also being extensively explored preclinically (36–40). However, the designs of these types of studies



Messenheimer et al.

are often empirical and take a straightforward additive approach that may not be optimal considering the unique immunologic mechanisms that influence combination treatments. Although others have demonstrated that administering checkpoint blockade "late" or for a longer duration than costimulatory antibodies is beneficial with preclinical combination immunotherapies (41, 42), to our knowledge, ours is the first report to directly compare concurrent combination treatment with nonoverlapping, sequential treatment with the same agents. Given that a substantial portion of patients does not respond to PD-1 blockade (in monotherapy or in combination; refs. 1, 3), our findings in anti-PD-1-refractory model systems suggest that the use of costimulatory stimulation may impact these patients. PD-1 blockade could then be combined in a sequential manner to boost T-cell activation with anti-OX40 costimulation.

Currently, multiple OX40-targeted antibodies have been developed with humanized IgG1 (increasing the possibility of antibody-dependent cell-mediated cytotoxicity and Treg cell deple-

tion) or IgG2 isotypes and are in phase I clinical trials as monotherapies (NCT01644968, NCT02221960, NCT02318394, NCT01416844, NCT02274155) or in combination with anti-CTLA-4 (NCT01689870), anti-PD-L1 (NCT02205333, NCT02410512, NCT02528357), or anti-4-1BB (NCT02315066). In addition, anti-OX40 is also being investigated in combination with radiotherapy (NCT01303705, NCT01862900). As clinical trials employing antibodies targeting other costimulatory molecules, such as GITR and 4-1BB, are currently being initiated, some in combination with checkpoint blockade, understanding not just which T-cell costimulatory or inhibitory molecules to target but when to stimulate or block them is likely to be a critical component of an optimal therapeutic regimen. Our group highlighted this concept in a recent article demonstrating the importance of timing when anti-CTLA-4 or anti-OX40 is combined with radiation (19).

In a model of B-cell lymphoma, McKee and colleagues recently demonstrated an abrogation of the therapeutic effect of 4-1BB

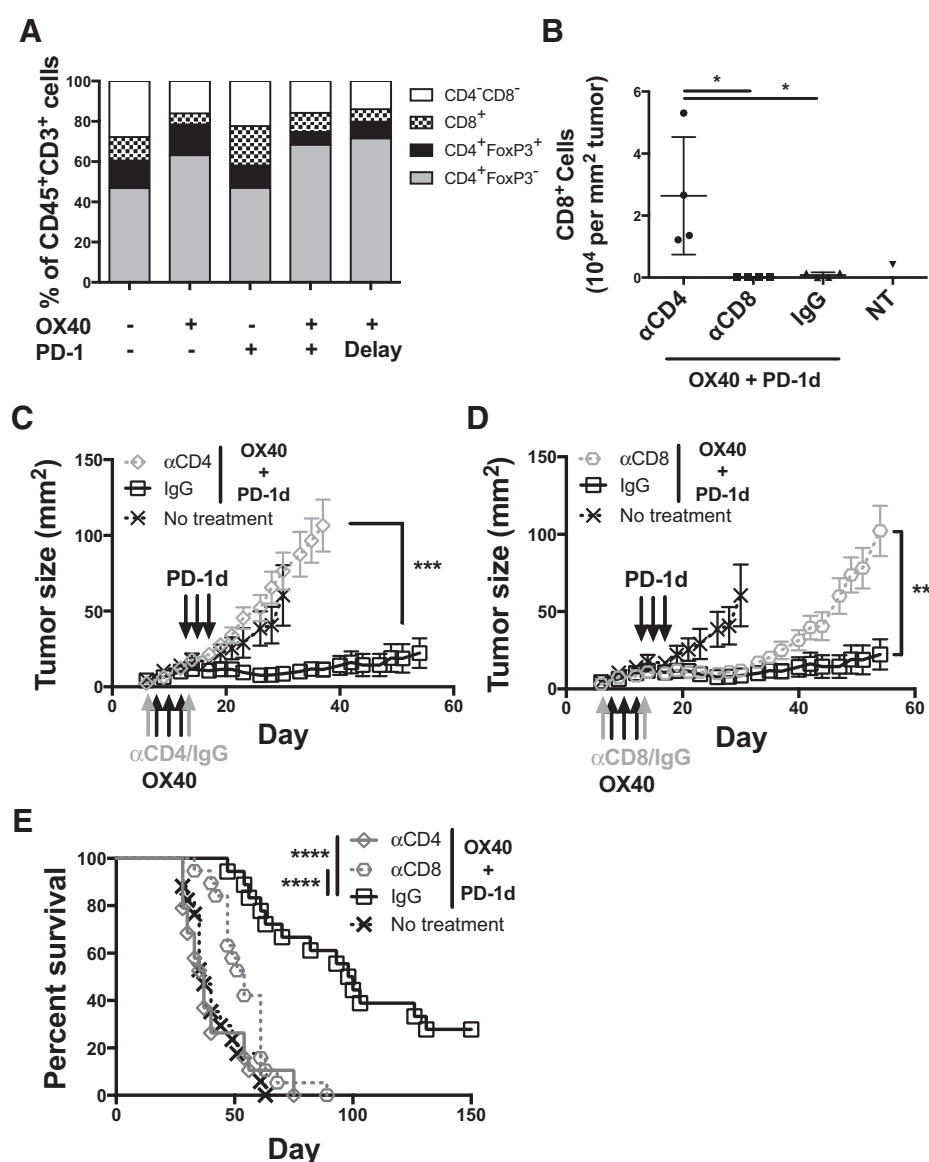


Figure 6.

Sequential combination treatment requires both CD4⁺ and CD8⁺ T cells for optimal therapeutic effect. MMTV-PyMT tumor-bearing mice were treated with anti-OX40 plus delayed anti-PD-1 (PD-1d), combined with 250 μ g anti-CD4, anti-CD8, or rat IgG on days 6 and 13. **A**, Average T-cell populations as a frequency of CD45⁺CD3⁺ cells in nondepleted day 19 treated and untreated tumors. $n = 8-9$, combination of two independent experiments. **B**, Quantification of total CD8⁺ cells per square millimeter of tumor on day 20 or 21 determined via flow cytometry. $n = 4$, combination of two independent experiments. **C-E**, Mean tumor growth of combination-treated CD4-depleted (**C**) or CD8-depleted (**D**) mice. $n = 9-10$, one representative of two independent experiments shown. **E**, Survival of CD4 or CD8-depleted mice. $n = 18-20$, combination of two independent experiments. Error bars, SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

costimulation when anti-PD-1 was combined concurrently (43). In our studies, concurrent anti-OX40 and anti-PD-1 also attenuated anti-OX40-induced therapeutic efficacy, dramatically increased serum cytokine levels, and increased peripheral T-cell apoptosis. A previous report noted reduced gene expression of BTLA in T cells treated with anti-OX40 and anti-PD-L1 (8). Similarly, we noted a significant decrease in the frequency of BTLA⁺ peripheral T cells after concurrent combination treatment. Others have suggested that BTLA⁻ cells are less effective in the tumor, with reduced proliferative capacity and decreased responsiveness to costimulatory molecules (34). We also observed reduced proliferation in concurrent combination-treated T cells in the tumor 7 days after the conclusion of treatment (Fig. 5A and B), despite an increase in proliferation 6 days prior (Fig. 3D). We reason that concurrent combination of anti-OX40 and anti-PD-1 negatively increases T-cell differentiation, indicated by loss of BTLA expression. Despite their initial burst of proliferation, terminally differentiated cells are less proliferative long term, making them less effective at mediating tumor destruction. Differentiation status of T cells is a key determinant of T cell-mediated antitumor responses, and increased success of adoptive T-cell transfer has been linked with less differentiated cells that maintain higher proliferative capacity (44, 45). Notably, we have demonstrated here that by administering combination treatment in a sequential manner, we can reduce terminal differentiation, maintain proliferation, and provide a more substantial antitumor effect compared with concurrent combination treatment.

In addition to a lower frequency of peripheral T cells expressing BTLA, the frequency of intratumoral CD8⁺ T cells expressing the inhibitory receptor TIM-3 was also significantly amplified with concurrent combination treatment. CD8⁺ T cells that express both TIM-3 and PD-1 are commonly recognized as exhausted, with reduced proliferative capacity and effector function (46). Deeply exhausted and terminally differentiated T cells can also more frequently undergo AICD and apoptosis (47, 48). Importantly TIM-3⁺CD8⁺ T cells were less frequent in tumors treated with anti-OX40 monotherapy or sequential combination, suggesting a less exhausted phenotype, and these treatments associated with the best antitumor responses (Fig. 4C and D). With concurrent combination immunotherapies capable of inducing an acute cytokine response, the risk of "whack-a-mole," where one therapeutic target is neutralized but one or more other inhibitory receptor like TIM-3 pops up, is a very real possibility, supported by our data with the concurrent combination of anti-OX40 and anti-PD-1. However, this also presents possible targets for other agents specific to these inhibitory receptors, as well as possible biomarkers to monitor the success of combination treatments in generating an appropriate milieu to support a therapeutic anticancer immune response. In support of this concept, Koyama and colleagues noted "adaptive resistance" with upregulated expression of TIM-3 on TIL in anti-PD-1-resistant tumors and demonstrated improved responses with secondary TIM-3 blockade (49), although some have recently suggested that PD-1⁺TIM-3⁺ deeply exhausted or hyperexhausted T cells are potentially unrecoverable and may not be impacted by checkpoint blockade (50, 51).

A previous report noted a synergistic antitumor effect with concurrent combination of anti-OX40 and anti-PD-1 (52). The ID8 ovarian tumor used was impervious to anti-OX40 or anti-PD-1 monotherapy. It is important to remember that in our transplanted MMTV-PyMT model, the therapeutic effect of concurrent combination, although inferior to sequential

combination and anti-OX40 monotherapy, was not completely abrogated and did have a significant effect on tumor growth (Fig. 1E and F). Other publications using the ID8 model show that anti-PD-L1 monotherapy significantly increases survival, and this effect was not significantly augmented when anti-4-1BB was added in combination (41, 53). Combined, these reports strongly suggest that ID8 is not responsive to costimulation (anti-OX40 or anti-4-1BB), and this may be a key difference between the results in our models and ID8 that have implications in the clinic. Transplanted MMTV-PyMT tumors have a much higher frequency of CD4⁺ T cells compared with CD8⁺ T cells. It is also well documented that OX40 expression is more common on CD4⁺ cells than CD8⁺ cells, which we have confirmed in the MMTV-PyMT tumors (Fig. 1A and B). This provides a possible explanation why OX40 monotherapy generates a therapeutic effect in the MMTV-PyMT model, but not the ID8 model, which was more dependent on CD8⁺ T cells for therapeutic response. Perhaps patients with high frequencies of CD4⁺ TIL may be more likely to respond to anti-OX40. Sequential combination treatments with costimulation and checkpoint blockade may then be most critical to patients who have some initial response to costimulation. Alternatively, perhaps in patients who do not respond to either costimulation or checkpoint blockade, a concurrent combination may be more effective by inducing a strong cytokine response and initial burst of proliferation as we see in our preclinical models.

CD4⁺ T cells can provide cytokine support to "help" CD8⁺ T cells, but have also demonstrated cytotoxic capabilities (54, 55). Conflicting reports over the necessary contribution of CD4⁺ and CD8⁺ T cells for therapeutic benefit in the MMTV-PyMT model have been published, with one reporting CD4⁺ T cells to be dispensable and in fact detrimental (56) and another suggesting that CD8⁺ T cells are dispensable while conventional CD4⁺ T cells are necessary (57). Our findings, showing a variance in the timing of necessary T-cell populations, provide a possible explanation for these discrepancies. Although myeloid-targeted therapies may require CD8⁺ T cells at early time points (30), some T cell-targeted therapies may not. Transient regulatory T-cell ablation does not require CD8⁺ T cells (57), but alone, this therapy only slows tumor growth for a limited period of time before continued progression. Sequential anti-OX40 plus anti-PD-1 provides a much longer period of stable disease, with some tumors reaching full regression. Given that OX40 is expressed almost exclusively on intratumoral CD4⁺ T cells in this model, it should not be surprising that an anti-OX40-generated effect would require CD4⁺ T cells, even when the PD-1 pathway is also targeted. In support of this, when treated tumors were depleted of CD4⁺ cells, even a significant increase in CD8⁺ TILs had no effect on tumor growth. Alternatively, CD8 depletion saw initial tumor growth delay, followed by rapid progression, indicating that the early antitumor effect provided by sequential combination treatment is in fact independent of CD8⁺ T cells, but these cells are necessary for a durable memory response. We hypothesize that as few CD8⁺ TILs express OX40 at the time of anti-OX40 treatment, and the early antitumor effects are dependent only on CD4⁺ T cells, the "help" provided by anti-OX40-stimulated CD4⁺ T cells drives CD8⁺ cells to long-term functionality and makes them necessary for long-term tumor regression. Consistent with this hypothesis, others have demonstrated that anti-OX40 combined with delayed anti-PD-L1 (1 and 3 days after anti-OX40) treatment saw a boost in T-cell function when antigen-specific CD8⁺ T cells were transferred with CD4⁺ help (8). Clearly long-term therapeutic efficacy

targeting OX40 and PD-1 in this model requires both CD4⁺ and CD8⁺ T cells, which corroborates a previous report utilizing anti-OX40 in another tumor model (16).

Our results demonstrate that in some tumors, the sequence and timing of antibody treatment targeting both costimulatory and inhibitory receptors is critical to success of the combined therapy. These data offer a strong rationale for delaying PD-1 or PD-L1 blockade or possibly other inhibitory receptor-targeted therapies, such as CTLA-4, TIM-3, or LAG-3, until after costimulation has boosted the tumor-specific T cells to a state where checkpoints are inhibiting the antitumor response. Sequential treatment has the combined benefit of both optimizing the antitumor immune response as well as potentially minimizing possible toxicity from acute cytokine release. Given the complex nature of T-cell costimulatory and inhibitory signaling, our data underscore the delicate balance that the immune system maintains and suggest a number of factors that ought to be explored in combination immunotherapy clinical trial designs. In our opinion, as these types of combination immunotherapy studies move into clinical trials, the importance of characterizing a patient's baseline immunity and assessing the impact of treatment has never been more clear. Fortunately, the tools to assess immunity are improving. A recent taskforce reviewed the state-of-the-art technologies for performing these analyses (58), and the AACR and FDA have been holding a series of policy forums to discuss a vision for developing effective combination immunotherapy (59). Together, these joint efforts between industry, academia, and regulatory agencies hold promise for the development of a new generation of immunotherapies that take advantage of the latest science and technology to further improve outcomes for a greater percentage of patients.

Disclosure of Potential Conflicts of Interest

W.J. Urba is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, Celldex, eTHERNA, and Oncosec and reports receiv-

ing commercial research support from AstraZeneca and Bristol-Myers Squibb. B.A. Fox is a consultant/advisory board member for AstraZeneca/MedImmune, Bristol-Myers Squibb, Definiens, Janssen/JNJ, and PerkinElmer and reports receiving commercial research support from AstraZeneca/MedImmune. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.J. Messenheimer, S.M. Jensen, Z. Feng, W.J. Urba, B.A. Fox
Writing, review, and/or revision of the manuscript: D.J. Messenheimer, S.M. Jensen, M.E. Afentoulis, M.J. Gough, W.J. Urba, B.A. Fox
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.A. Fox
Study supervision: S.M. Jensen, B.A. Fox

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