Combined Targeting of Costimulatory (OX40) and Coinhibitory (CTLA-4) Pathways Elicits Potent Effector T Cells Capable of Driving Robust Antitumor Immunity

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Abstract
Ligation of the TNF receptor family costimulatory molecule OX40 (CD134) with an agonist anti-OX40 monoclonal antibody (mAb) enhances antitumor immunity by augmenting T-cell differentiation as well as turning off the suppressive activity of the FoxP3⁺CD4⁺ regulatory T cells (Treg). In addition, antibody-mediated blockade of the checkpoint inhibitor CTLA-4 releases the "brakes" on T cells to augment tumor immunotherapy. However, monotherapy with these agents has limited therapeutic benefit particularly against poorly immunogenic murine tumors. Therefore, we examined whether the administration of agonist anti-OX40 therapy in the presence of CTLA-4 blockade would enhance tumor immunotherapy. Combined anti-OX40/anti-CTLA-4 immunotherapy significantly enhanced tumor regression and the survival of tumor-bearing hosts in a CD4 and CD8 T cell–dependent manner. Mechanistic studies revealed that the combination immunotherapy directed the expansion of effector T-beh/high/Eomeshigh granzyme B⁺ CD8 T cells. Dual immunotherapy also induced distinct populations of Th1 [interleukin (IL)-2, IFN-γ], and, surprisingly, Th2 (IL-4, IL-5, and IL-13) CD4 T cells exhibiting increased T-bet and Gata-3 expression. Furthermore, IL-4 blockade inhibited the Th2 response, while maintaining the Th1 CD4 and effector CD8 T cells that enhanced tumor-free survival. These data demonstrate that refining the global T-cell response during combination immunotherapy can further enhance the therapeutic efficacy of these agents. Cancer Immunol Res; 2(2); 142–53. ©2013 AACR.

Introduction
The generation of potent cytotoxic CD8 T cells capable of destroying tumors requires T cell receptor (TCR) stimulation along with the provision of costimulatory signals (1). Previous studies have shown that ligation of the TNF receptor (TNFR) family costimulatory receptor OX40 (CD134) on T cells can significantly enhance antitumor immunity against various tumor types, including melanoma, breast, and prostate cancer (2). Mechanistic studies have revealed that OX40 ligation with an agonist anti-OX40 monoclonal antibody (mAb) boosts cytokine production and the expression of prosurvival molecules associated with enhanced T-cell expansion, differentiation, and the generation of long-lived memory cells (3–5). OX40 ligation has also been shown to augment or inhibit the expansion and suppressive activity of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Treg) depending on several factors, including the cytokine milieu present during stimulation (6–9).

CTLA-4 is a negative regulatory protein that serves as a checkpoint inhibitor to limit T-cell responses by attenuating T-cell proliferation and cytokine production. In tumor-bearing hosts, inhibition of CTLA-4 improves antitumor immunity by releasing the "brakes" on T cells (10). CTLA-4 blockade was shown to improve the overall survival in patients with metastatic (stage IV) melanoma highlighting the efficacy of this cancer immunotherapy (11). Current studies are investigating whether CTLA-4 blockade has similar benefits for patients with breast, lung, or prostate cancer (12–15).

OX40 and CTLA-4 are both upregulated on CD4 and CD8 T cells shortly after TCR stimulation and are constitutively expressed on Tregs (16–18). Treatment with an agonist anti-OX40 mAb or CTLA-4 blockade has distinct and overlapping functional effects on different T-cell compartments. For example, both anti-OX40 therapy and CTLA-4 blockade enhance the expansion and production of cytokines by naïve CD4 T cells (4, 10). However, anti-OX40 therapy drives significantly greater formation of long-lived memory CD4 T cells (19). OX40 ligation has been shown to augment CD8 T-cell expansion and effector differentiation through a combination of CD8 T-cell direct and indirect pathways. Studies have revealed that during priming CTLA-4 blockade...
indirectly enhances CD8 T-cell effector function through cell-extrinsic effects on the responding CD8 T cells (20–24). OX40 and CTLA-4 are constitutively expressed on Tregs and anti-OX40 therapy or CTLA-4 blockade has been shown to alleviate Treg suppression, although there are reports that under certain conditions OX40 ligation can drive Treg expansion (6–9, 18, 25, 26).

Preclinical data have shown that monotherapy with anti-OX40 or anti-CTLA-4 has limited therapeutic efficacy against many tumor types, suggesting that combination immunotherapy likely will be required to generate optimal therapeutic responses. We hypothesized that OX40 ligation in the presence of CTLA-4 blockade would augment tumor immunotherapy by simultaneously increasing the number and function of effector CD4 and CD8 T cells, while relieving the inhibitory effects of Tregs. Indeed, recent work demonstrated that combined anti-OX40/anti-CTLA-4 therapy in the presence of repeated intra-tumoral vaccination with the Toll-like receptor (TLR) ligand CpG improved antitumor immunity in a murine lymphoma model (27). Therapeutic efficacy of this regimen was associated with increased IFN-γ expression by T cells and a concomitant reduction in the frequency of Tregs in the tumor. However, in this study, the authors only examined the effects of combination therapy versus CpG alone; there was no comparison of the effects of single versus dual therapy or analysis of the effects on CD8 T-cell differentiation and the impact on the cytokine milieu. In the current study, we demonstrate that in tumor-bearing mice the combined anti-OX40/anti-CTLA-4 regimen improves the limited therapeutic efficacy of monotherapy by augmenting the Th1/Th2 CD4 and effector CD8 T-cell responses. In addition, limiting Th2 CD4 T cells via interleukin (IL)-4 blockade further enhances tumor immunotherapy. These data provide insight into the mechanisms by which the combined anti-OX40/anti-CTLA-4 immunotherapy drives potent polyclonal effector T-cell (Teff) responses and suggest that the clinical combination of these agents may offer a novel means of boosting tumor immunotherapy in patients with cancer.

Materials and Methods

Mice

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. OT-I Thy1.1 TCR transgenic and POET-1 transgenic mice (28) were bred in our facility. All mice were bred and maintained under specific pathogen-free conditions in the Providence Portland Medical Center (Portland, OR) animal facility. Experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals.

Lymphocyte isolation and analysis

Peripheral (axillary, brachial, and inguinal) lymph nodes were harvested and processed to obtain single-cell suspensions. Ammonium chloride potassium (ACK) lysing buffer (Lonza) was added for 5 minutes at room temperature to lyse red blood cells. Cells were rinsed with RPMI-1640 medium containing 10% FBS (10% cRPMI), 1 mol/L HEPES, nonessential amino acids, sodium pyruvate (Lonza), and penicillin-streptomycin glutamine (Invitrogen). For flow cytometry analysis, cells were incubated for 30 minutes at 4°C with Ki-67 fluorescein isothiocyanate (FITC), FoxP3 eFluor 450, granzyme B phycoerythrin (PE), CD3 eFluor 710, CD4 V500, CD4 FITC, CD8 eFluor 605, CD8 PE-Cy7, Thy1.1 eFluor 450, KLRG-1 APC, CD25 Alexa Fluor 700, IL-2 PE, IL-2 Alexa Fluor 700, IL-4 FITC, IL-5 PE, IL-13 eFluor 710, TNF-α PE-Cy7, IFN-γ APC, Rb-6 PE, Eomes eFluor 710, T-bet PE-Cy7, Gata-3 eFluor 660, and Fixable Viability Dye eFluor 780. All antibodies were obtained from eBioscience, BD Biosciences, BioLegend, or Invitrogen. Flow cytometry plots were gated on viable CD3+ T cells excluding doublets. For intracellular staining, cells were fixed and permeabilized with the FoxP3 Staining Buffer Set (eBioscience). For intracellular cytokine production, lymphocytes were stimulated with 5 µg/mL plate-bound anti-CD3 (clone 2C11) or SPAS-1 (SNC9-H8; STHVNLHC) peptide in 10% cRPMI with 1 µL/mL of brefeldin A containing Golgi-Plug solution (BD Biosciences) for 4 hours at 37°C before intracellular staining. Cells were analyzed with an LSR II flow cytometer using FACSDiva software (BD Biosciences).

Cytokine production by multiplex ELISA

CD4 or CD8 T cells were purified by cell sorting (B220/CD11b/MHC II-) and 2 × 10^6 cells per well were stimulated with media or plate-bound anti-CD3 (2 µg/mL) in 24-well plates. Supernatants were collected after 24 hours and cytokine expression was determined using a FlowCytomix Th1/Th2/Th17 kit (eBioscience). Cytokine expression levels were visualized with a heatmap using the Genesis software package (29). Cytokine expression levels were log_2 transformed and shown in a yellow/red (low/high) scale.

Tumor challenge and TIL isolation

A total of 7.5 × 10^5 (for monotherapy or combination therapy) or 1 × 10^6 TRAMP-C1 (for tumor harvest and IL-4 blockade) murine prostate tumor cells or 1 × 10^5 MCA-205 murine sarcoma cells were injected (day 0; s.c.) into male C57BL/6 mice. TRAMP-C1 and MCA-205 cells were kindly provided by Dr. Andy Weinberg [Earle A. Chiles Research Institute (EACRI), Portland, OR]. TRAMP-C1-mOVA tumor cells were generated in our laboratory and described previously (20). A total of 2 × 10^6 TRAMP-C1-mOVA cells were injected (day 0; s.c.) into male POET-1 transgenic mice. All cell lines were tested and confirmed to be Mycoplasma-free using the MycoAlert Detection Kit (Lonza). TRAMP-C1 tumor-bearing mice received rat immunoglobulin G (IgG; 200 µg; Sigma), anti-OX40 (250 µg; clone OX86; BioXCell), and/or anti-CTLA-4 (200 µg; clone 9D9; BioXCell) mAb at the indicated time points. All mAbs were verified to be endotoxin-free and injected intraperitoneally (i.p.) into recipient mice. T cells were depleted with anti-CD4 (clone GK1.5) and/or anti-CD8 (clone 53-6.72) mAb on day 3, 10, and 17 (200 µg) after tumor inoculation. For IL-4 blockade, anti-IL-4 mAb (clone 11B.11) was given every 2 to 3 days (200 µg; i.p.) for 3 weeks [National Cancer Institute (NCI) Biological Resources Branch Preclinical Repository, Frederick, MD]. Tumor growth (area) was assessed every 2 to 3 days with microcalipers and mice were sacrificed when tumors reached more than 150 mm^2. Tumor-infiltrating lymphocytes (TIL) were harvested by dissection of tissue into small fragments followed by digestion in 1 mg/mL collagenase.
Adoptive transfer and activation of OT-1 T cells

Single-cell suspensions were prepared from the spleens of OT-1 Thy1.1 TCR transgenic mice. OT-1 T cells were purified by negative selection using the Dynal Mouse CD8 Cell Isolation Kit (Invitrogen) and injected (i.v.; day 0) in 200 μL of PBS into TRAMP-C1-mOVA tumor-bearing POET-1 transgenic mice. Mice were then treated with 100 μg anti-OX40 (clone OX86) or control rat IgG antibody (days 0 and 1), and/or 200 μg anti-CTLA-4 mAb (clone 9D9; days 0, 2, and 4).

Treg functional assay

TRAMP-C1 tumor-bearing mice received anti-OX40 mAb or rat IgG (days 4 and 8) ± anti-CTLA-4 mAb (days 4, 6, and 8). Eight or 9 days later (day 12 or 13 after tumor implantation), lymph nodes were harvested and CD4+CD25+ Tregs (CD8/ B220/CD11b/MHC II−) were isolated by cell sorting (>99% purity). Tregs were seeded in triplicate at 5 × 10^5 cells per well in 96-well round-bottomed plates previously coated with plate-bound anti-CD3/anti-CD28 (2 μg/mL; overnight). Naïve responder CD8 cells (Teff) were prepared from wild-type mice using the Dynal CD8 T Cell Negative Selection Kit (Invitrogen), carboxyfluorescein succinimidyl ester (CFSE)-labeled, and 5 × 10^4 cells per well were added to triplicate wells containing media (positive control) or Tregs. A total of 2 × 10^5 irradiated (3,000 rads) T cell–depleted (Dynal beads; Invitrogen) accessory cells were added to all wells. Cells were harvested after 72 hours, stained for CD8, and the extent of CFSE dilution in the CD8 responder cells was determined.

Statistical analysis

Statistical significance was determined using one-way ANOVA (for comparisons among three or more groups; with Tukey correction for multiple comparisons), Student t test (for comparisons between two groups: two-tailed), or Kaplan–Meier survival and log-rank (Mantel–Cox) test (for tumor survival studies) using GraphPad Prism software (GraphPad); P value less than 0.05 was considered significant: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Agonist anti-OX40 therapy in the presence of CTLA-4 blockade augments the antitumor response

TRAMP-C1 prostate or MCA-205 sarcoma tumors were implanted into cohorts of wild-type mice. Four (TRAMP-C1) or 8 (MCA-205) days later, tumor-bearing mice were treated with control antibody (rat IgG), agonist anti-OX40 mAb, blocking anti-CTLA-4 mAb, or combined anti-OX40/anti-CTLA-4 therapy. Monotherapy with anti-OX40 or anti-CTLA-4 had limited effects on tumor growth or survival, but the combined immunotherapy significantly enhanced tumor regression and survival (Fig. 1A–C). The therapeutic efficacy was T cell dependent as CD4 and/or CD8 T cell depletion before the initiation of therapy abrogated the survival benefit of the combined anti-OX40/anti-CTLA-4 immunotherapy (Fig. 1D).

Combined anti-OX40/anti-CTLA-4 therapy boosts CD4 and CD8 T-cell expansion and differentiation but does not alter the ratio of Teff to Treg or the Treg suppressor function

We characterized the phenotypic and functional response of the CD8 Teffs, CD4 Teffs, and CD4 Tregs isolated from the lymph nodes and tumors following combination immunotherapy. Dual anti-OX40/anti-CTLA-4 therapy enhanced significantly the proliferation and effector function of the CD8 T cells as characterized by the expression of Ki-67 and granzyme B, respectively (Fig. 2A and B). We observed in the lymph nodes low levels of expression of KLRG-1, a marker of terminal differentiation and senescence in activated T cells (30, 31), regardless of treatments (<2% KLRG-1+/total CD8 T cells; Fig. 2B). KLRG-1 expression was markedly increased on tumor-infiltrating CD8 T cells (~20%–30% KLRG-1+/total CD8 T cells; Fig. 2B); however, there was a trend toward lower KLRG-1 expression on CD8 T cells present in the tumors of anti-OX40/anti-CTLA-4–treated mice, which corresponded with a higher proliferative index (Fig. 2B; %Ki-67+/CD8 T cells in the tumor). These data suggest that anti-OX40/anti-CTLA-4 therapy may help retain the proliferative capacity of tumor-reactive T cells by limiting their terminal differentiation into KLRG-1+ CD8 T cells. We evaluated the effects of combined anti-OX40/anti-CTLA-4 immunotherapy on effector and regulatory CD4 T cells. Analogous to its effects on CD8 T cells, dual therapy increased the proliferation (Ki-67) of effector FoxP3+ CD4+ T cells as compared with the other groups (Fig. 3A and B). Although the frequency of FoxP3+ CD4 T cells in the lymph nodes increased, the frequency of Tregs within the tumor did not change following combination immunotherapy (Fig. 3C and D). Similarly, the total cell number was relatively unchanged among the treatment groups (Supplementary Fig. S1). Other studies have demonstrated that CTLA-4 blockade augments tumor immunotherapy in part by increasing the ratio of Teffs to Tregs (25, 32). We analyzed the effect of anti-OX40/anti-CTLA-4 therapy on the Teff to Treg ratio in tumor-bearing mice. Monor or dual-therapy did not increase the ratio of effector CD8 or CD4 T cells to Treg; instead, there was a slight decrease in the Teff/Treg ratio in the lymph nodes (Fig. 4A and B). To investigate whether the combination immunotherapy abrogated the suppressive activity of Tregs, CD4+CD25+ T cells were isolated by cell sorting from tumor-bearing hosts treated with either the control antibody (rat IgG), anti-OX40, anti-CTLA-4, or combined anti-OX40/anti-CTLA-4. Titrated numbers of Tregs were cocultured with CFSE-labeled CD8 T cells and the extent of proliferation was determined after 72 hours. Tregs isolated from mice receiving either monotherapy or combination immunotherapy exhibited comparable suppressor function (Fig. 4C), indicating that the therapeutic efficacy of dual immunotherapy was not due to an abrogation of suppressor Treg function.

Studies have revealed an integral role for the costimulatory molecule, inducible T-cell costimulator (ICOS), in mediating the antitumor efficacy of anti-CTLA-4 in mice. ICOS expression
was associated with increased effector function between CD4 and CD8 T cells (33). Anti-CTLA-4 therapy led to a transient increase in ICOS-expressing T cells, which may serve as a prognostic factor for increased long-term survival (34–36). We investigated ICOS expression following anti-OX40/anti-CTLA-4 therapy in TRAMP-C1 tumor-bearing mice, and found a significant increase in ICOS-expressing CD4 Teff and Treg with a higher ratio of ICOS+ Teff:Treg following combination immunotherapy (Fig. 4D). No changes in ICOS expression were detected in the CD8 T-cell compartment (data not shown). These data suggest that dual therapy augments ICOS expression, although further studies are needed to determine whether ICOS directly affects the function of CD4 Teff and Treg following anti-OX40/anti-CTLA-4 therapy.

**Agonist anti-OX40 therapy plus CTLA-4 blockade enhances CD4 and CD8 T-cell cytokine production and generates distinct subsets of Th1 and Th2 CD4 T cells**

TRAMP-C1 tumor-bearing mice were treated with the combined immunotherapy and the CD4 or CD8 T cells from the lymph nodes were purified by cell sorting and cytokine secretion was determined by multiplex ELISA following restimulation (24 hours) with plate-bound anti-CD3 mAb. We observed a striking increase in the magnitude and breadth of cytokine production in the CD4 T-cell compartment following combined immunotherapy including a significant increase in Th1 (IFN-γ, IL-2) and, surprisingly, Th2 (IL-4, IL-5, IL-13) cytokines (Fig. 5A and C). Dual therapy also led to a significant increase in IL-2 and IFN-γ production by the effector CD8 T cells (Fig. 5B and D).

We investigated whether the combined therapy generated distinct Th1/Th2 subsets, or created multifunctional CD4 T cells as was shown in mice receiving anti-OX40 plus chemotherapy-induced lymphopenia (37). Cytokine expression was determined by intracellular cytokine staining (ICS) and flow cytometry and revealed distinct populations of CD4 T cells within the effector (FoxP3+) compartment: a Th1 subset containing IFN-γ+ cells and a Th2 subset with IL-4+IFN-γ- cells that included the IL-5+ and IL-13+ subset (Fig. 6A). No cytokine production was detected among the FoxP3+CD4+ T cells (data not shown). Combination immunotherapy also boosted the frequency and total number of cytokine-producing CD4 and CD8 T cells (Fig. 6A–D). Although NKT cells can also produce copious amounts of IL-4 (38, 39), anti-OX40/anti-CTLA-4 therapy did not increase the number of NKT cells nor did it elicit NKT cell-specific IL-4 production (data not shown).

To determine whether the combined anti-OX40/anti-CTLA-4 therapy could augment the expansion of antigen-specific CD8 T cells, OVA-specific OT-I T cells were transferred into TRAMP-C1-mOVA tumor-bearing mice along with rat IgG,
anti-CTLA-4, and/or anti-OX40 therapy. Seven days later, the antigen-specific CD8 T-cell response was determined. The combination therapy increased significantly the expansion (% OT-1+/CD8) and differentiation (% grzB+/OT-1) of antigen-specific OT-1 T cells in tumor-bearing mice (Supplementary Fig. S2A). SPAS-1 is an endogenous tumor-antigen expressed in TRAMP-C1 prostate tumor cells (40). We determined the frequency and function of prostate tumor (SPAS-1)–specific CD8 T cells in the TRAMP-C1 tumor-bearing mice. The combined anti-OX40/anti-CTLA-4 therapy boosted the frequency and total number of endogenous effector (IFN-γ+) SPAS-1–specific CD8 T cells (Supplementary Fig. S2B).

T-cell differentiation is regulated by the master transcription factors Bcl-6, T-bet, Eomes, and Gata-3. IFN-γ–induced T-bet promotes Th1 differentiation; IL-4–induced Gata-3 favors Th2 development. Similarly, reciprocal expression of Bcl-6 and T-bet controls CD8 T-cell differentiation; the less differentiated cells are Bcl-6high/T-betlow, whereas the terminal effector cells are Bcl-6low/T-bethigh (41, 42). Our data revealed that the dual anti-OX40/anti-CTLA-4 therapy led to increased T-bet and Gata-3–expressing CD8 T cells (Fig. 6E), which reflected increased Th1/Th2 cytokine production (Fig. 6C). We examined the coexpression of T-bet and Gata-3 among the different treatment groups and found that the combination therapy increased Gata-3 expression primarily in the T-bethigh population (Supplementary Fig. S3). T-bethigh and Eomeshigh effector CD8 T cells were generated following combination immunotherapy (Fig. 6F).

Given the striking increase in Th2 cytokines, particularly IL-4, following anti-OX40/anti-CTLA-4 therapy, we asked whether the IL-4–producing Th2 CD4 T cells were beneficial or detrimental to the generation of antitumor immunity. Pilot studies demonstrated that the efficacy of combination therapy was reduced at higher tumor doses (data not shown). Therefore, we increased the initial dose of TRAMP-C1 tumors (to 1 × 106) and then treated tumor-bearing mice with rat IgG, anti-CTLA-4, anti-OX40, and/or anti-IL-4 mAb. IL-4 blockade was sufficient to abrogate the induction of the Th2 CD4 T-cell response in tumor-bearing mice, while maintaining Th1 and effector CD8 T cells (Fig. 7A and B). More importantly, the inclusion of IL-4 blockade led to a striking increase in survival (Fig. 7D), while anti-IL-4 alone, or in combination with either anti-CTLA-4 or anti-OX40 monotherapy, had no effect (Fig. 7C).

Discussion

Studies have shown that anti-CTLA-4 or anti-OX40 mAb monotherapy can improve antitumor immunity (2, 10). CTLA-4 blockade significantly improved survival in patients with metastatic melanoma, highlighting the therapeutic potential of cancer immunotherapy (11). Despite these successes, only a fraction of patients benefited, suggesting that combination immunotherapy may help generate a more potent and broadly effective response. For example, preclinical studies have shown that repeated intratumoral vaccination with CpG in the presence of anti-OX40/anti-CTLA-4 therapy enhanced antitumor immunity in a murine lymphoma model (27). These data revealed an increase in IFN-γ+ T cells in the tumors of mice receiving combination therapy.

In the current study, we demonstrated that combined anti-OX40/anti-CTLA-4 immunotherapy enhanced survival of mice with TRAMP-C1 (prostate) or MCA-205 (sarcoma) tumors (Fig. 1A–C) through a T cell–dependent mechanism.
Dual anti-OX40/anti-CTLA-4 therapy resulted in a robust increase in effector CD4 and CD8 T cell expansion and differentiation (Figs. 2 and 3), including a striking increase in cytokine production, including Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5, and IL-13) cytokines (Fig. 5). OX40 ligation has been shown to promote either Th1 or Th2 responses, depending upon the cytokine milieu present during the priming of naïve CD4 T cells and the strength of TCR stimulation (43–45).

Recent data from Hirschhorn-Cymerman and colleagues also identified strong Th1 and Th2-cytokine secretion following agonist anti-OX40 therapy in conjunction with lymphodepletion (with cyclophosphamide) and adoptive transfer of melanoma-specific (Trp1 TCR transgenic) CD4 T cells (37), suggesting that combination therapy may direct the development of a novel bifunctional Th1/Th2-like population of CD4 T cells. Work from Gallo and colleagues highlighted the development of IL-13–producing Th1 and Th17 CD4 T-cell subsets, even in the absence of IL-4Rα signaling (46). In contrast, our data

Figure 3. Effects of anti-OX40/anti-CTLA-4 therapy on effector and regulatory CD4 T cells. Cohorts of TRAMP-C1 tumor-bearing mice were treated with anti-OX40 and/or anti-CTLA-4 (as in Fig. 2). Eight days later (day 25 after tumor implantation), lymph nodes and tumors were harvested and the phenotype of (A) FoxP3− effector CD4 T cells and (C) FoxP3+ regulatory CD4 T cells was determined by flow cytometry. A and C, graphs depict representative data from the lymph nodes of individual mice. A and B, the extent of proliferation (Ki-67) of FoxP3− CD4+ T cells was assessed. C and D, the frequency of FoxP3+ Treg/total CD4 T cells and proliferation (Ki-67) of FoxP3+ CD4+ T cells was determined. Graphs are representative of the results obtained from (A) and (C) individual mice or (B) and (D) cumulative data (n = 8/group) from two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
showed that the combined anti-OX40/anti-CTLA-4 therapy led to the generation of distinct Th1 and Th2 CD4 T-cell subsets (Fig. 6A), not bifunctional Th1/Th2 cells. The explanations for these disparate results are unclear, but may reflect differences in the complexity of boosting the endogenous polyclonal CD4 T-cell compartment (the current study) versus the use of adoptively transferred TCR transgenic CD4 T cells with a higher affinity for cognate antigen (37). Indeed, recent work showed that low-affinity TCR stimulation preferentially leads to increased IL-4 production among CD4 T cells (47).

Anti-OX40 or anti-CTLA-4 monotherapy has also been shown to increase the formation of IL-17–producing CD4 T cells in murine models and in peripheral blood mononuclear cells (PBMC) of patients with metastatic melanoma (48). However, we did not detect significant levels of IL-17 among any of the treatment groups (Fig. 5A), suggesting that increased IL-17 production was not responsible for the therapeutic efficacy we observed in the current study. Other groups have shown that OX40 ligation in the presence of IL-4 and TGF-β can induce CD4 differentiation into IL-9–producing cells (49).
were treated with anti-OX40 (days 4 and 8) and/or CTLA-4 blockade (days 4, 6, and 8). Eight days later (day 12 after tumor implantation), CD4 and CD8 T cells were purified from the lymph nodes by cell sorting. Following stimulation for 24 hours (with plate-bound anti-CD3), supernatants were collected and cytokine levels from (A) CD4 or (B) CD8 T cells were determined using a multiplex ELISA. Total cytokine levels (pg/mL) were log2 transformed and visualized with a heatmap (white squares indicate cytokines were not detected). C and D, graphs depict the mean ± SEM (n = 3/group) from one of two independent experiments. *, P < 0.05; ***, P < 0.001.

producing Th9 cells (49). Although we detected high levels of IL-4 following dual immunotherapy (Fig. 5C), TGF-β and IL-9 were undetectable in any of the treatment groups (data not shown).

Along with the potent effects of combination immunotherapy on effector CD4 and CD8 T-cell proliferation and differentiation (Figs. 2 and 3), we also observed an expansion of Treg posttreatment (Fig. 3C and D). Reports have shown that OX40 or CTLA-4–specific monotherapy can shift Teff to Treg ratios in favor of effector cell expansion, relieve the inhibitory effects of Tregs, reduce FoxP3 expression on Tregs, and in certain circumstances, induce Treg apoptosis (7, 25, 50). However, our studies showed that single or dual anti-OX40/anti-CTLA-4 immunotherapy did not alter the effector CD8 or CD4 T cell to Treg ratios in the tumor (Fig. 4A and B), inhibit the suppressor function of Treg (Fig. 4C), reduce FoxP3 expression, or induce Treg apoptosis (data not shown; ref. 51). Thus, it seems that the main effects of the therapy are through increasing the extent of cytokine-producing Teffs, rather than inhibiting Treg function. The explanation for these differences remains unclear, but may reflect the different model systems used for these studies (e.g., cell lines, timing of tissue harvest). Another possibility is that in our study, immune therapy was given in the absence of vaccination, whereas other groups administered anti-CTLA-4 mAb with a granulocyte macrophage colony-stimulating factor (GM-CSF) or FLT3-ligand–secreting vaccine (32, 52). When anti-CTLA-4 was used as a monotherapy in the absence of vaccination, no changes were seen in the Teff:Treg ratio (53). Notably, we did detect a significant increase in ICOS expression among Teff and Treg CD4 T cells following the combination therapy (Fig. 4D), although the functional consequence of this remains to be determined.

Recent work showed that combination immunotherapy with agonist anti-OX40 and anti-4-1BB mAbs favored the formation of T-bethigh/Eomeshigh CD4 T cells with enhanced effector function (54). Although we observed an increase in T-bethigh CD4 T cells, no differences were observed in Eomes...
expression following anti-CTLA-4/anti-OX40 therapy (Fig. 6E), suggesting that T-bet, rather than Eomes, upregulation, was likely the main driver of effector CD4 T cells differentiation in this model system. Given that the IL-4–dependent induction of Th2 cells reduce, rather than promote, the therapeutic efficacy of the dual anti-OX40/anti-CTLA-4 therapy (Fig. 7D), it seems that directing the extent of Th1/Th2 responses may be an important consideration when translating these therapies into the clinic. We also found that anti-OX40/anti-CTLA-4 therapy induced the formation of T-bet\textsuperscript{high}/Eomes\textsuperscript{high} CD8 T cells (Fig. 6F). Because a high T-bet:Eomes ratio is associated with increased effector CD8 T-cell differentiation (42), it remains unclear whether the coexpression of these two transcription factors represents a transient or a long-lived stable population.

Figure 6. Altered transcriptional programming of T cell following combination immunotherapy. A–F, cohorts of mice received $1 \times 10^5$ TRAMP-C1 tumor cells (day 0) and then were treated with rat IgG/anti-OX40 (days 4 and 8) and/or anti-CTLA-4 (days 4, 6, and 8). Eight days later, lymph nodes were harvested and (A–D) cells were restimulated with plate-bound anti-CD3 (4 hours) and the expression of the indicated cytokines was determined by flow cytometry. E and F, cells were harvested as described above and expression (% and MFI) of the indicated transcription factors (Bcl-6, T-bet, Eomes, and Gata-3) was determined directly \textit{ex vivo} by flow cytometry. Dot plots depict the data from individual mice and bar graphs depict the mean ± SD ($n=3$–5/group) from one of two independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. MFI, mean fluorescence intensity.
of effector/effector-memory CD8 T cells or a more central memory-like subset. Additional studies will be needed to clarify the role of transcriptional regulation of the CD8 T-cell compartment in generating optimal tumor immunotherapy.

Th2-derived IL-4 can promote macrophage polarization toward an M2 phenotype, which in turn enhances tumor progression (55, 56). Similarly, tumor-induced myeloid-derived suppressor cells (MDSC) can also suppress T-cell function to limit antitumor immunity (57, 58). Currently, we are examining whether IL-4 affects the expansion and/or suppressive activity of M2 macrophages or MDSC in TRAMP-C1 tumor-bearing mice receiving dual therapy. However, recent work has shown that MDSC accumulate slowly over a period of several weeks in TRAMP-C1 tumor-bearing mice (59). Thus, it is unlikely that potential changes in MDSC function significantly affected the ability of anti-OX40/anti-CTLA-4 therapy to enhance tumor regression as therapeutic efficacy was observed by 10 to 14 days posttreatment (Fig. 1A).

Despite the ability of combination immunotherapy to augment tumor regression, treatment of large well-established tumors remains challenging due to the induction of tumor-specific tolerance and immune-suppression. Given that the combined anti-OX40/anti-CTLA-4 therapy was able to boost antigen-specific CD8 T-cell responses (Supplementary Fig. S2), it will be of great interest to investigate whether the inclusion of tumor-specific vaccination will facilitate the expansion of polyclonal tumor-reactive Teffs capable of further enhancing tumor regression, particularly at later stages of disease. In summary, these data demonstrate that agonist anti-OX40 therapy in conjunction with CTLA-4 blockade improves the limited therapeutic efficacy of monotherapy to elicit potent antitumor immunity and provide the rationale to study the activity of combination regimens such as anti-OX40/anti-CTLA-4 therapy in clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: W.L. Redmond, S.N. Linch
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