

# Engagement of the ICOS pathway markedly enhances efficacy of CTLA-4 blockade in cancer immunotherapy

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**Cytotoxic T lymphocyte antigen-4 (CTLA-4) blockade with a monoclonal antibody yields durable responses in a subset of cancer patients and has been approved by the FDA as a standard therapy for late-stage melanoma. We recently identified inducible co-stimulator (ICOS) as a crucial player in the antitumor effects of CTLA-4 blockade. We now show that concomitant CTLA-4 blockade and ICOS engagement by tumor cell vaccines engineered to express ICOS ligand enhanced antitumor immune responses in both quantity and quality and significantly improved rejection of established melanoma and prostate cancer in mice. This study provides strong support for the development of combinatorial therapies incorporating anti-CTLA-4 and ICOS engagement.**

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Abbreviations used: ICOS, inducible co-stimulator; ICOSL, ICOS ligand; i.d., intradermal(iy).

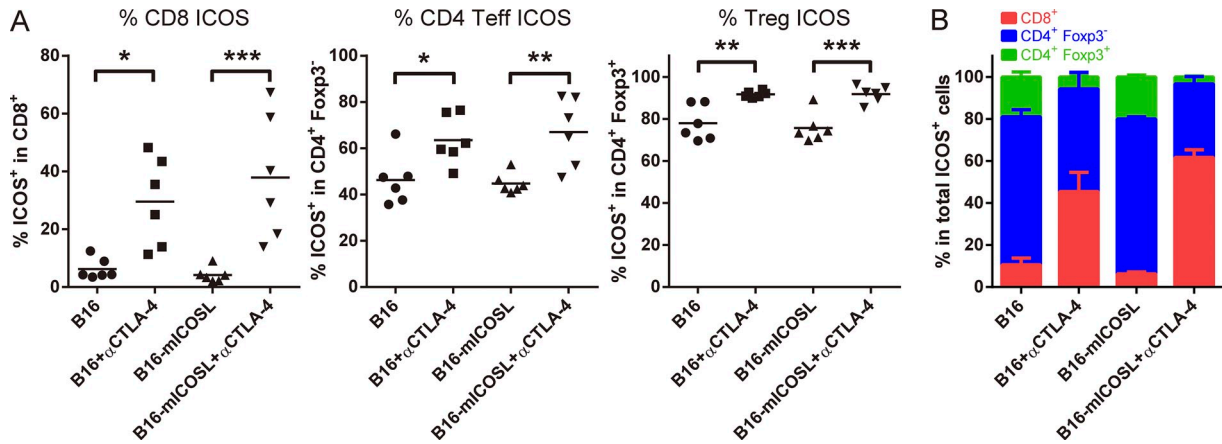
Harnessing T cell responses to eradicate tumors has been difficult in part because of the complexity of regulation of T cell responses. Early T cell activation requires an antigen-specific signal mediated by the TCR plus additional co-stimulatory signals generated by engagement of molecules such as CD28 with their ligands (Harding et al., 1992). CD28 co-stimulation is subject to down-regulation by inhibitory molecules such as cytotoxic T lymphocyte antigen-4 (CTLA-4; Walunas et al., 1994; Krummel and Allison, 1995). Beginning in 1996, we showed that inhibitory signals mediated by CTLA-4 were responsible for limiting antitumor responses in a series of mouse models because administration of antibodies blocking the interaction of CTLA-4 with its ligands could result in tumor rejection and long-lived immunity (Leach et al., 1996).

These preclinical studies led to the generation of antibodies to human CTLA-4, ipilimumab and tremelimumab (Sharma et al., 2011). To date, over 20,000 patients have been treated with these antibodies, the majority receiving ipilimumab. Objective responses have been observed

in patients with melanoma, ovarian, prostate, renal cell, and lung cancers. A randomized phase III clinical trial with ipilimumab was reported in 2010, showing a significant increase in survival for patients with advanced melanoma who received ipilimumab therapy (Hodi et al., 2010). Treatment with ipilimumab improved median overall survival by 3.7 mo and ~23% of treated patients were alive with durable clinic benefit for the 4.5 yr of follow up. Ipilimumab was the first therapy of any kind to show a survival benefit in phase III trials (Hodi et al., 2010; Robert et al., 2011) for patients with advanced melanoma and was approved in March 2011 by the Food and Drug Administration (FDA) as both first and second line therapy for the treatment of patients with advanced melanoma. A recent retrospective study of 177 metastatic melanoma patients from the earliest clinical trials of ipilimumab showed an 88-mo median duration of objective responses (Prieto et al., 2012). And a recent trial of ipilimumab in combination with an antibody to PD-1

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**Figure 1.** Treatment of B16/F10 tumors with anti-CTLA-4 led to increased frequency of ICOS expression on tumor-infiltrating CD8 and CD4 T<sub>eff</sub> cells. (A) Frequency of ICOS expression on CD8, CD4 Foxp3<sup>-</sup>, and CD4 Foxp3<sup>+</sup> T cells in the tumor. Horizontal bars represent means. (B) Breakdown of total intratumoral ICOS<sup>+</sup> T cells in terms of CD8, CD4 Foxp3<sup>-</sup>, and CD4 Foxp3<sup>+</sup> subsets. Data are pooled from two independent experiments ( $n = 3$  mice per group). Error bars represent means  $\pm$  SEM. Data were analyzed with one-way ANOVA and Bonferroni's multiple comparisons test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

(nivolumab) in metastatic melanoma showed an objective response rate of  $\sim 50\%$  (Wolchok et al., 2013).

Together these data demonstrate that blockade of inhibitory signals mediated by CTLA-4 can be quite effective against large bulky tumors and metastatic disease. However, there is clearly a need to extend the therapeutic benefit of this treatment to more patients. We have uncovered a novel immune-based strategy that can significantly enhance the efficacy of CTLA-4 blockade.

In a presurgical clinical trial in which patients with localized bladder cancer were treated with ipilimumab, the frequency of T cells expressing inducible co-stimulator (ICOS) was significantly increased both in tumor tissues and peripheral blood of patients (Liakou et al., 2008). ICOS is a T cell-specific molecule that belongs to the CD28/CTLA-4 family (Hutloff et al., 1999; Sharpe and Freeman, 2002). ICOS expression is up-regulated upon T cell activation, which is enhanced in the setting of CTLA-4 blockade, thereby leading to a higher frequency of ICOS<sup>+</sup> T cells detected in cancer patients receiving anti-CTLA-4 therapy, with the ICOS<sup>+</sup> population containing the bulk of tumor-specific, IFN- $\gamma$ -producing CD4 T cells (Liakou et al., 2008; Carthon et al., 2010; Vonderheide et al., 2010). In a retrospective study of advanced melanoma patients, we also found a significant correlation between sustained elevation of ICOS<sup>+</sup> CD4 T cells in the peripheral blood after ipilimumab treatment and increased survival (Carthon et al., 2010). These clinical studies suggested that ICOS might play an important role in the therapeutic effect of anti-CTLA-4. Our finding that mice deficient in ICOS or ICOS ligand (ICOSL) had impaired antitumor responses after treatment with anti-CTLA-4, as compared with wild-type mice, further supported the notion that the ICOS/ICOSL pathway is critical for the therapeutic effect of anti-CTLA-4 (Fu et al., 2011). These data prompted us to investigate the potential benefit of providing additional signal to the ICOS pathway in the setting of CTLA-4 blockade as a strategy to further improve antitumor responses.

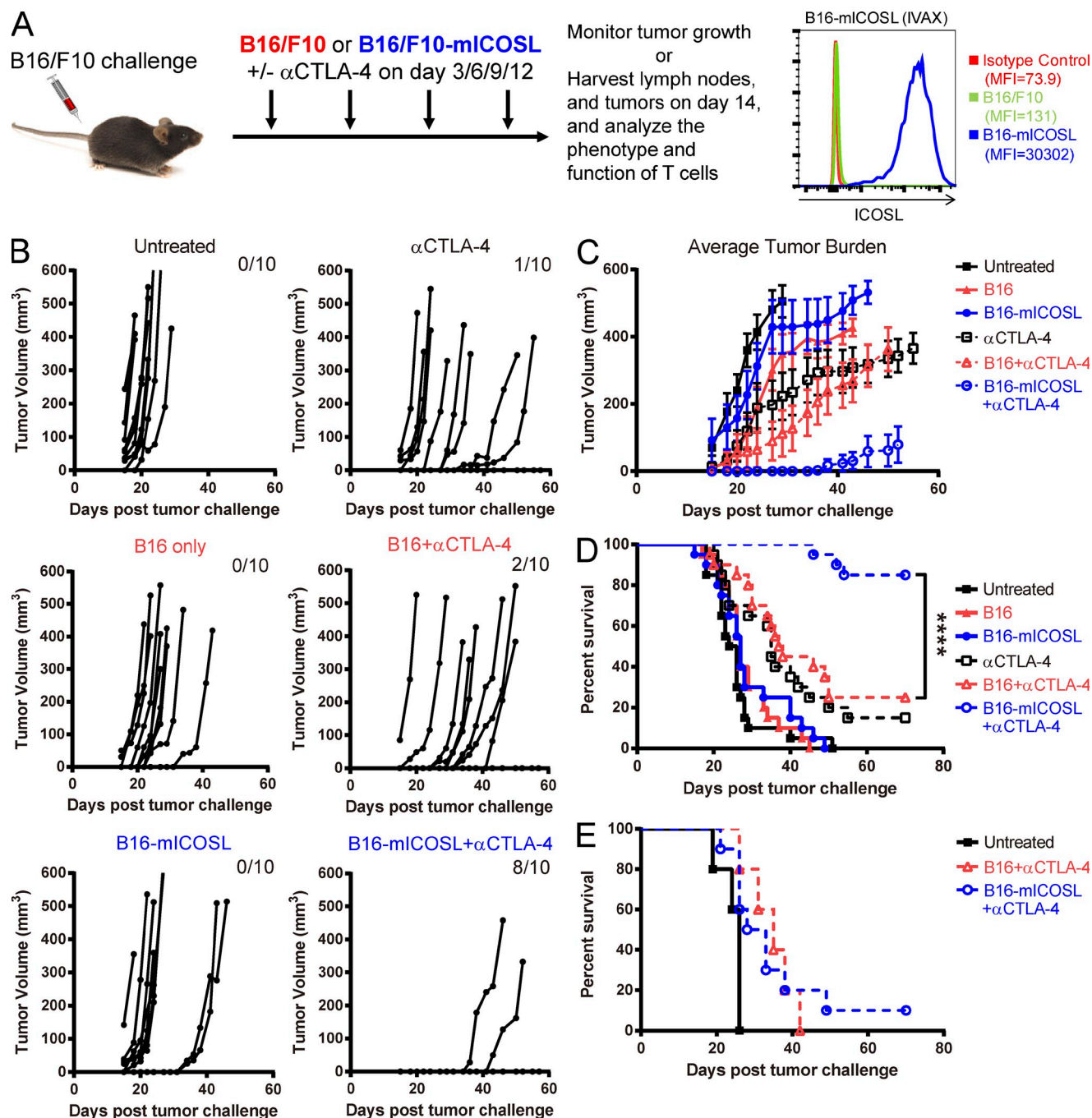
## RESULTS

### ICOS is selectively up-regulated on intratumoral CD8 and CD4 effector T cells (T<sub>eff</sub> cells)

Similar to what we previously observed in cancer patients but even more dramatically, ICOS was up-regulated on CD8 and CD4 Foxp3<sup>-</sup> T<sub>eff</sub> cells in mouse B16/F10 melanoma after treatment with CTLA-4 blockade. We used irradiated parental B16 tumor cells as a control vaccination approach, which did not affect ICOS expression on any T cell compartment (unpublished data). In this situation, a very small fraction of CD8 T cells in the tumor expressed ICOS, whereas about half of CD4 T<sub>eff</sub> cells and the majority of CD4 Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub> cells) were ICOS positive (Fig. 1 A). Blockade of CTLA-4 in addition to the vaccination released the limit on T cell activation and generally increased ICOS expression in all of the T cell compartments, but the most significant change was observed on CD8 T cells, with a six- to eightfold increase in frequency. This trend led to a much greater presence of CD8 T cells, but much less presence of T<sub>reg</sub> cells in the total ICOS-positive pool inside the tumor (Fig. 1 B). These data further support the rationale of activating the ICOS pathway as immunotherapy, as it would be more likely to benefit the anti-tumor CD8 T cells rather than immunosuppressive T<sub>reg</sub> cells.

### Synergistic tumor protection by CTLA-4 blockade and ICOS engagement

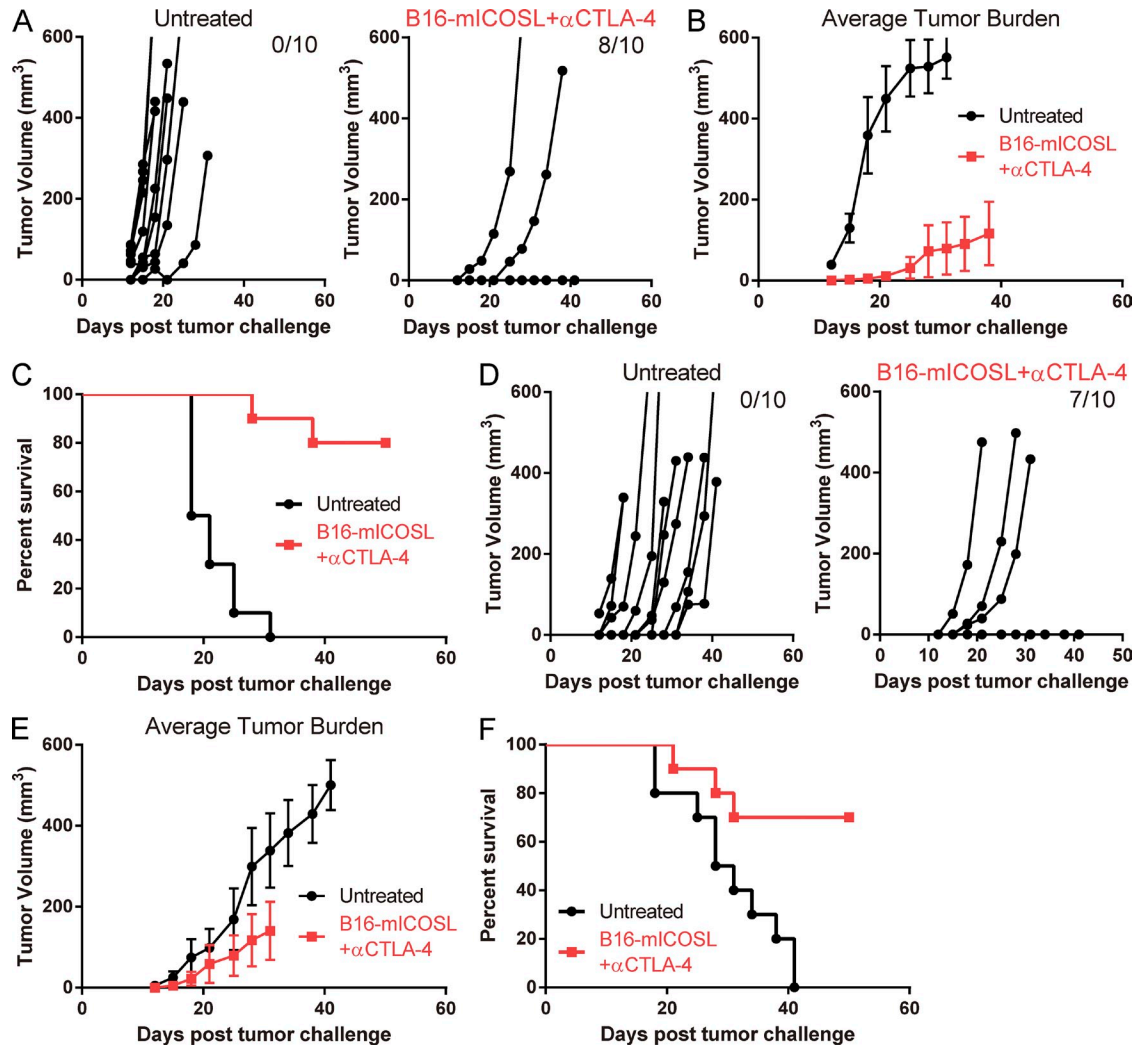
In light of the significant up-regulation of ICOS on intratumoral CD8 T cells, we developed a strategy to activate the ICOS pathway by transducing tumor cells with the cognate ligand, ICOSL (Yoshinaga et al., 1999), and using the irradiated ICOSL-positive tumor cells as a vaccine (IVAX) to treat tumor-bearing mice. B16/F10 melanoma cells were engineered to express ICOSL on the cell surface and tested for stable expression throughout the treatment process both in vitro and ex vivo (Fig. 2 A). ELISA of tissue culture supernatant from these cells failed to show the presence of soluble/



**Figure 2. Cellular vaccine with ICOSL-expressing tumor cells (IVAX) synergized with CTLA-4 blockade to provide protection against B16/F10 tumors.** (A) Treatment schedule of vaccination and CTLA-4 blockade and the verification of ICOSL expression on IVAX. Expression of ICOS on B16 and IVAX were followed in vivo up to day 14 after tumor challenge. (B) Individual tumor growth curves after B16/F10 challenge. Numbers on the top right side represent tumor-free mice. Data are representative of three independent experiments ( $n = 10$  mice per group). (C) Tumor growth curves depict average tumor volume in each group. Error bars represent means  $\pm$  SEM. Data are representative of three independent experiments ( $n = 10$  mice per group). (D) Cumulative survival curves from two independent experiments ( $n = 10$  mice per group). Survival curves were analyzed with Log-rank test. \*\*\*\*,  $P < 0.0001$ . (E) Cumulative survival curves of ICOS-deficient hosts from two independent experiments ( $n = 5$  mice per group).

shed ICOS (detection range  $>0.1$  ng/ml). Mice were given an intradermal (i.d.) tumor challenge with parental (ICOSL negative) B16/F10 cells and subsequently treated with anti-CTLA-4 plus a vaccine comprised of irradiated ICOSL-negative

B16/F10 cells or ICOSL-positive B16/F10 cells (IVAX). Neither IVAX nor the control vaccine of irradiated untransduced B16/F10 cells had any protective effect against tumor growth in the absence of anti-CTLA-4, probably because of

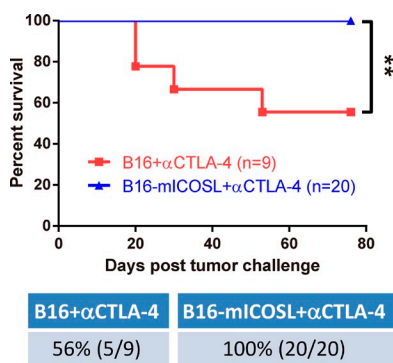


**Figure 3. Combination of IVAX and CTLA-4 blockade led to rejection of higher doses of injected tumor cells and increased eradication of established tumors.** (A–F) Mice were challenged with 100K B16/F10 cells and treated from day 3 (A–C) or challenged with 50K B16/F10 but treated from day 6 (D–F). (A) Individual tumor growth curves after challenge with 100K B16/F10 cells. (B) Tumor growth curves depict average tumor volume in each group. (C) Overall survival curves representative of two independent experiments ( $n = 10$  mice per group). (D) Individual tumor growth curves after challenge with 50K B16/F10 cells. (A and D) Numbers on the top right side represent tumor-free mice. Data are representative of two independent experiments ( $n = 10$  mice per group). (E) Tumor growth curves depict average tumor volume in each group. (B and E) Error bars represent means  $\pm$  SEM. Data are representative of two independent experiments ( $n = 10$  mice per group). (F) Overall survival curves representative of two independent experiments ( $n = 10$  mice per group).

poor activation status and low frequency of ICOS expression on CD8 and CD4  $T_{eff}$  cells (Fig. 1 A). In this model, anti-CTLA-4 treatment alone also failed to elicit tumor rejection. The combination of anti-CTLA-4 with control vaccine resulted in tumor rejection in a minority of mice, whereas anti-CTLA-4 in combination with IVAX led to tumor rejection in >80% of mice, a fourfold increase in efficacy (Fig. 2, B–D). The increase in efficacy was specific to ICOS engagement, as the effect was completely lost in ICOS-deficient hosts (Fig. 2 E). To further test the potency of this combination therapy in a more stringent and clinically relevant situation, we doubled the initial dose of tumor challenge or delayed the onset of therapy. The combination of IVAX and CTLA-4 blockade still generated robust protection against a high dose

of B16/F10 challenge (Fig. 3, A–C) or a more established tumor (Fig. 3, D–F).

The combination of IVAX and CTLA-4 blockade also improved memory response against secondary challenge in the tumor survivors (Fig. 4). We pooled the mice surviving from either the combination of control vaccine and CTLA-4 blockade or the IVAX and anti-CTLA-4 combination. At least 4 mo after their initial tumor rejection, they were challenged with a very high dose (four times higher than regular) of the same B16/F10 tumor without any further intervention. About half the mice from the control combination group obtained enough memory response against the secondary challenge, but all of the mice that had undergone IVAX and anti-CTLA-4 treatment rejected the second tumor without



**Figure 4. Stimulation of the ICOS pathway also improved memory response against B16/F10 rechallenge.** Mice that had been treated with the indicated combination therapies and survived the primary B16/F10 tumor were rechallenged with 200K B16/F10 cells but with no further treatment. Data are representative of two independent experiments. Survival curves were analyzed with Log-rank test. \*\*,  $P < 0.01$ .

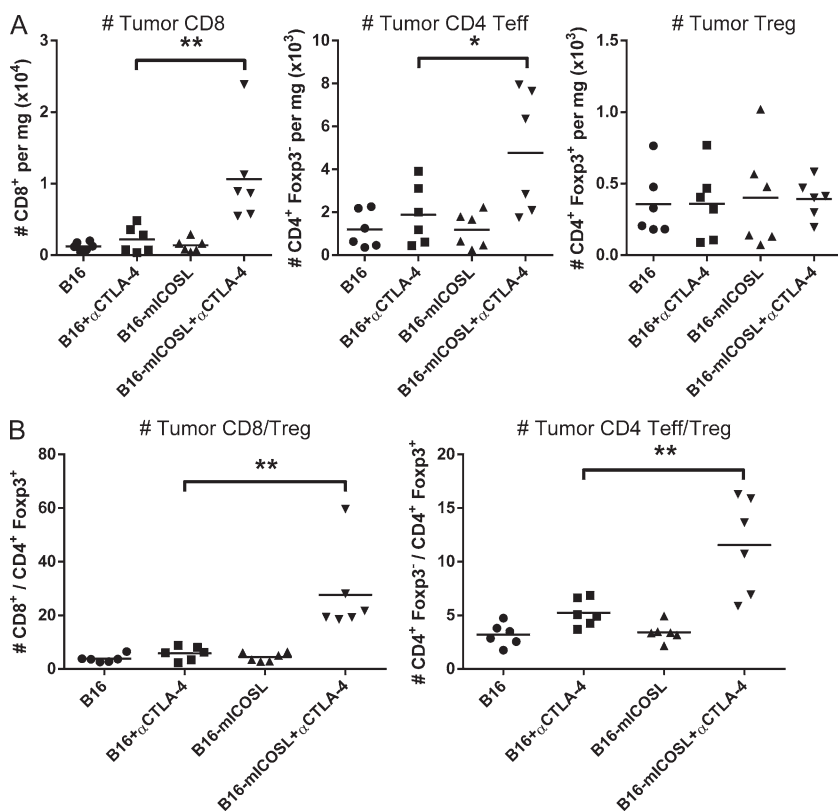
need of additional therapy. This is especially promising because one of the major advantages of tumor immunotherapy is immune memory.

These results suggest that the elevated expression of ICOS on T cells in anti-CTLA-4-treated tumor-bearing hosts is not just a marker for T cell activation, but ICOS can actively participate in further enhancing immune responses against tumors. Thus, in the context of CTLA-4 blockade, which leads to significant up-regulation of ICOS on CD8 and CD4

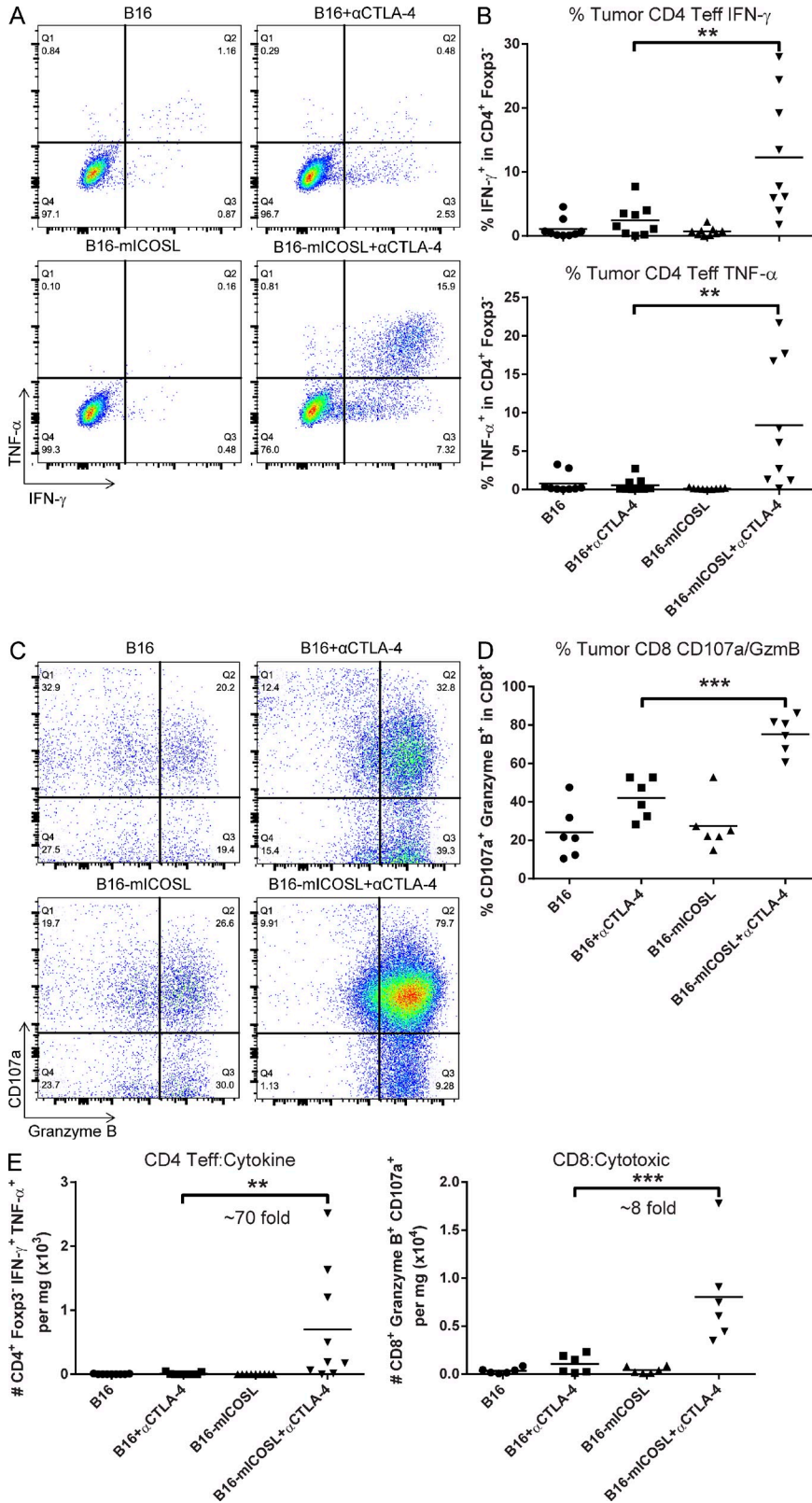
$T_{eff}$  cells, a cellular vaccine expressing ICOSL can trigger the ICOS pathway to synergize with CTLA-4 blockade to provide potent tumor protection.

**Changed balance of antitumor  $T_{eff}$  cells and immunosuppressive  $T_{reg}$  cells**

We next sought to dissect the basis for the enhanced efficacy of anti-CTLA-4/IVAX combination. We have previously shown that anti-CTLA-4, when combined with GM-CSF-secreting cellular vaccine (GVAX), increases the ratio of CD8 T cells to  $T_{reg}$  cells in the tumor (Quezada et al., 2006). To evaluate the impact of IVAX and CTLA-4 blockade on the intratumoral cell composition, we counted the absolute numbers of CD8, CD4 Foxp3<sup>-</sup>, and CD4 Foxp3<sup>+</sup> T cells in B16/F10 tumors on day 14 and normalized these numbers by the tumor weight. IVAX alone did not change the composition of T cells in the tumor but, when administered together with anti-CTLA-4, dramatically increased the density of CD8 (approximately fivefold) and CD4  $T_{eff}$  cells (approximately threefold) compared with the combination of control vaccine and anti-CTLA-4 (Fig. 5 A, left and middle). It is notable that the density of CD4 Foxp3<sup>+</sup>  $T_{reg}$  cells was unaffected (Fig. 5 A, right). The enrichment of CD4 and CD8  $T_{eff}$  cells was primarily observed at the tumor site but not in draining lymph nodes or spleen (unpublished data). Because the density of  $T_{reg}$  cells remained unchanged and CD8 or CD4  $T_{eff}$  cells increased several fold, the combination of IVAX and anti-CTLA-4 blockade raised the intratumoral CD8/ $T_{reg}$  cell ratio almost



**Figure 5. Combination of IVAX and CTLA-4 blockade enriched CD8 and CD4 Foxp3<sup>-</sup> T cells in the tumor and raised the intratumoral CD8/ $T_{reg}$  and CD4  $T_{eff}$ / $T_{reg}$  cell ratios.** (A) Density of CD8, CD4 Foxp3<sup>-</sup>, and CD4 Foxp3<sup>+</sup> T cells depicted as absolute number of cells per milligram of tumor on day 14 after tumor challenge. Numbers of T cells in tumors were calculated as described in Materials and methods. Data are pooled from two independent experiments ( $n = 3$  mice per group). (B) Cumulative quantification of CD8/ $T_{reg}$  and CD4  $T_{eff}$ / $T_{reg}$  cell ratios in day 14 B16/F10 tumors from two independent experiments ( $n = 3$  mice per group). Horizontal bars represent means. Data were analyzed with one-way ANOVA and Bonferroni's multiple comparisons test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 6. Combination of IVAX and CTLA-4 blockade enhanced proinflammatory cytokine production by CD4 Foxp3<sup>-</sup> T cells and cytotoxicity of CD8 T cells.** (A) Dot plots of IFN- $\gamma$  and TNF staining in tumor-infiltrating CD4 Foxp3<sup>-</sup> T cells. Numbers in the quadrants are relative frequency. Data are representative of three independent experiments ( $n = 3$  mice per group). (B) Cumulative quantification of the frequency of IFN- $\gamma$  and TNF production in tumor-infiltrating CD4 Foxp3<sup>-</sup> T cells from three independent experiments ( $n = 3$  mice per group). (C) Dot plots of granzyme B and CD107a staining in tumor-infiltrating CD8 T cells. Numbers in the quadrants are relative frequency. Data are representative of two independent experiments ( $n = 3$  mice per group). (D) Cumulative quantification of the frequency of granzyme B<sup>+</sup> CD107a<sup>+</sup> in tumor-infiltrating CD8 T cells from two independent experiments ( $n = 3$  mice per group). (E) Density of IFN- $\gamma$ <sup>+</sup> TNF<sup>+</sup> CD4 Foxp3<sup>-</sup> T cells (left) and granzyme B<sup>+</sup> CD107a<sup>+</sup> CD8 T cells (right) depicted as absolute numbers of these cells per milligram of tumor. Numbers of T cells in tumors were calculated as described in Materials and methods. Data are pooled from two or three independent experiments ( $n = 3$  mice per group). Horizontal bars represent means. Data were analyzed with one-way ANOVA and Bonferroni's multiple comparisons test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

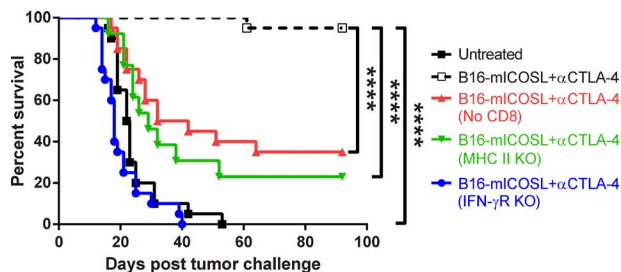
sixfold and doubled the CD4 T<sub>eff</sub>/T<sub>reg</sub> cell ratio as compared with values in mice treated with control vaccine and anti-CTLA-4 (Fig. 5 B). The enhanced ratio of effector to

regulatory T cells marked the shift from an immunosuppressive to immunostimulatory tumor microenvironment and provides one possible explanation for the potent antitumor

efficacy observed with the combination therapy of anti-CTLA-4 and IVAX.

### IVAX combination therapy greatly enhances the function of antitumor $T_{\text{eff}}$ cells

The higher intratumoral CD8/ $T_{\text{reg}}$  and CD4  $T_{\text{eff}}$ / $T_{\text{reg}}$  cell ratios could quantitatively contribute to the tumor protection effect of IVAX and CTLA-4 blockade. Furthermore, there were also profound qualitative changes in the immune functions of these enriched CD8 and CD4  $T_{\text{eff}}$  cells. We isolated tumor-infiltrating lymphocytes from day 14 tumors, briefly restimulated them *ex vivo* with DCs loaded with B16 lysate and assayed their cytokine production and cytotoxic activity. It has been reported that ICOS signaling in human T cells induced Th17 polarization (Paulos et al., 2010). However, in our models, we were unable to detect IL-17A expression in CD4  $T_{\text{eff}}$  cells isolated from the tumors or the vaccination sites (unpublished data). Although mice treated with cellular vaccine alone (either control vaccine or IVAX) or a combination of control vaccine and anti-CTLA-4 had quite few CD4  $T_{\text{eff}}$  cells producing IFN- $\gamma$ , there was a distinct population of polyfunctional CD4  $T_{\text{eff}}$  cells secreting both IFN- $\gamma$  and TNF, with an increase of  $\sim 25$ -fold in the frequency of IFN- $\gamma^+$  TNF $^+$  cells after treatment with anti-CTLA-4 plus IVAX (Fig. 6 A). This cytokine profile indicated that these cells were potent tumor antigen-specific Th1 cells. We also observed that the frequency of either IFN- $\gamma^+$  or TNF $^+$  CD4  $T_{\text{eff}}$  cells was significantly higher in tumors treated with IVAX and anti-CTLA-4 blockade as compared with the combination of control vaccine plus anti-CTLA-4 (Fig. 6 B). These data suggested that the synergy between IVAX and anti-CTLA-4 was capable of inducing Th1 polarization *in vivo* and thus providing strong help to the antitumor cytotoxic CD8 T cells. The CD8 T cells in the treated tumors did not produce as much pro-inflammatory cytokines (IFN- $\gamma$  or TNF) as CD4  $T_{\text{eff}}$  cells, although the combination of IVAX and CTLA-4 blockade still induced higher IFN- $\gamma$  and TNF production than the combination of control vaccine and anti-CTLA-4 (unpublished data). However, the cytolytic activity of tumor-infiltrating CD8 T cells was considerably enhanced by IVAX and anti-CTLA-4, as measured by coexpression of granzyme B and CD107a (LAMP-1; Fig. 6, C and D), which is one of the most striking features of this novel combinatorial approach. Thus the combination of IVAX and anti-CTLA-4 not only enriched CD8 and CD4  $T_{\text{eff}}$  cells in the tumors, but also enhanced their antitumor functions, including secretion of pro-inflammatory cytokines and cytolytic activity at the tumor site. Calculating the effect of these changes altogether, the numbers of CD4  $T_{\text{eff}}$  cells producing IFN- $\gamma$  and TNF and degranulating cytotoxic CD8 T cells were increased by 70- and 8-fold, respectively (Fig. 6 E). Overall, the combination of anti-CTLA-4 and IVAX could increase the density of tumor-reactive helper and killer T cells in the tumor to significantly higher levels, which might be the leading cause for tumor rejection.



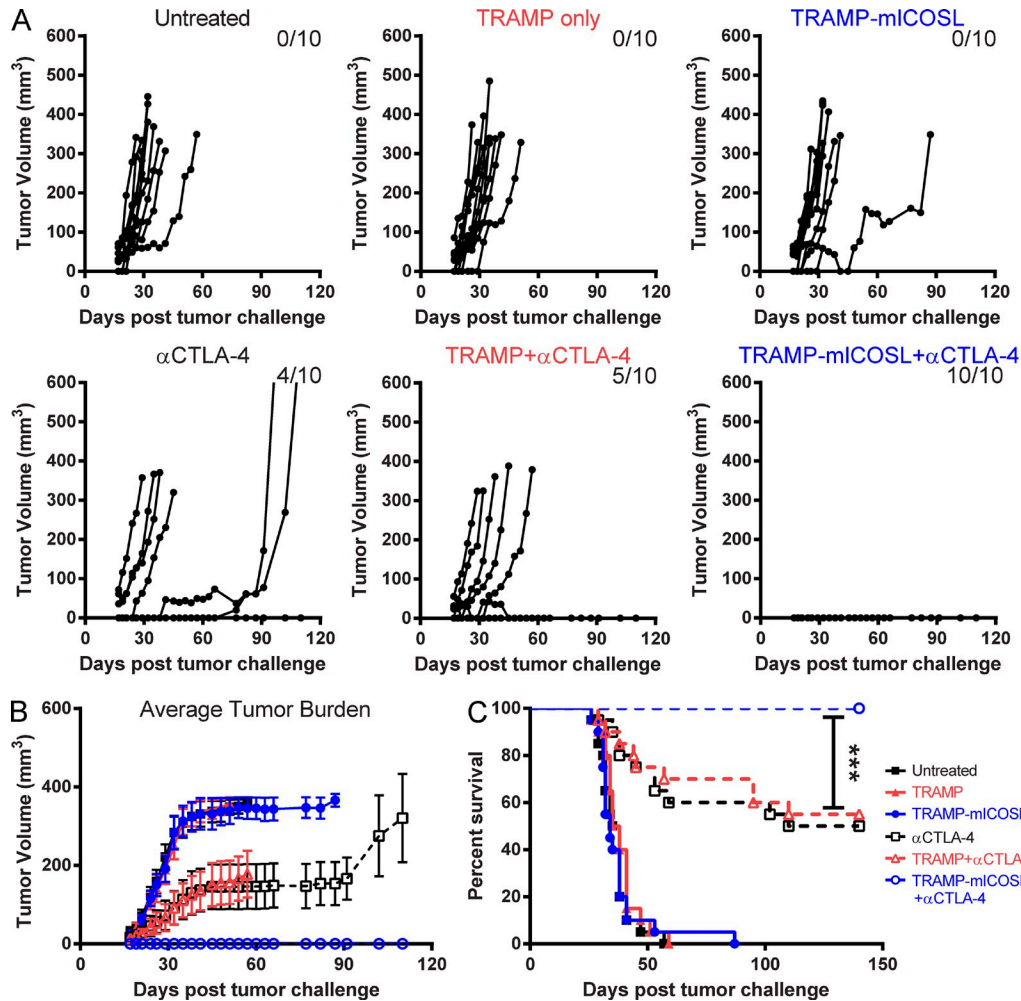
**Figure 7. CD8, CD4 T cells, and IFN- $\gamma$  were indispensable for therapeutic efficacy.** B16/F10 tumor-bearing mice were treated with IVAX and CTLA-4 blockade as described in Fig. 2 A and were depleted of CD8 cells with anti-CD8 (clone 2.43;  $n = 20$ ). The tumor protection rate was also measured in MHC class II KO ( $n = 13$ ) or IFN- $\gamma$ R KO ( $n = 20$ ) hosts. Data were pooled from two independent experiments. Survival curves were analyzed with Log-rank test. \*\*\*\*,  $P < 0.0001$ .

### CD8, CD4 $T_{\text{eff}}$ cells, and IFN- $\gamma$ are required for the tumor protection

The expanded pool of CD8 and CD4  $T_{\text{eff}}$  cells were critical in the IVAX-driven tumor rejection. We repeated our tumor protection experiments with additional treatment with depleting antibody or with genetically deficient hosts. Mice receiving CD8 depleting antibody or lacking MHC class II molecules suffered greatly diminished therapeutic efficacy (Fig. 7). Of note, although both the CD8 and CD4 population were required for the maximum protection, other cell populations also play a role in the absence of either one, suggesting that the tumor rejection caused by this treatment regimen involves multiple helper and effector cell populations. However, the Th1 cytokine IFN- $\gamma$  was indispensable for any tumor protection as IFN- $\gamma$ R KO mice completely lost the survival benefit (Fig. 7).

### IVAX combination therapy has robust protection efficacy in multiple tumor models

To show that the protection efficacy of IVAX combination therapy is not tumor model specific, we also used the same protocol to generate ICOSL-expressing tumor cell vaccines from another mouse melanoma cell line, B16/BL6, as well as the mouse prostate cancer cell line TRAMP C2. As we observed with the B16/F10 model, the combination of IVAX and anti-CTLA-4 can also cure TRAMP prostate cancer. All of the mice rejected TRAMP C2 tumors after treatment with IVAX plus anti-CTLA-4, as compared with the 50% protection rate in the group of control vaccine plus anti-CTLA-4 (Fig. 8 A). The control TRAMP C2 vaccine did not synergize with anti-CTLA-4, as the tumor growth kinetics is very similar between the group of anti-CTLA-4 alone and the one of control vaccine plus anti-CTLA-4 (Fig. 8 B). In contrast, there was statistically significant survival benefit with the combination of IVAX plus anti-CTLA-4 (Fig. 8 C). These data were also reproducible in a third tumor model, B16/BL6 (unpublished data). Thus, the synergy between IVAX and CTLA-4 blockade was observed across different tumor models, and in each model, the combination therapy significantly improved tumor rejection and overall survival. These results



**Figure 8. Combination therapy of IVAX and CTLA-4 blockade was also therapeutic against mouse prostate tumors.** (A) Individual tumor growth curves after challenge with TRAMP C2 cells. Numbers on the top right side represent tumor-free mice. Data are representative of two independent experiments ( $n = 10$  mice per group). (B) Tumor growth curves depict average tumor volume in each group. Error bars represent means  $\pm$  SEM. Data are representative of two independent experiments ( $n = 10$  mice per group). (C) Cumulative survival curves from two independent experiments ( $n = 10$  mice per group). Survival curves were analyzed with Log-rank test. \*\*\*,  $P < 0.001$ .

suggest that the underlying principle of this combination therapy could potentially be applied to multiple types of cancer in clinical application.

#### Tumor protection by IVAX requires presentation in cis

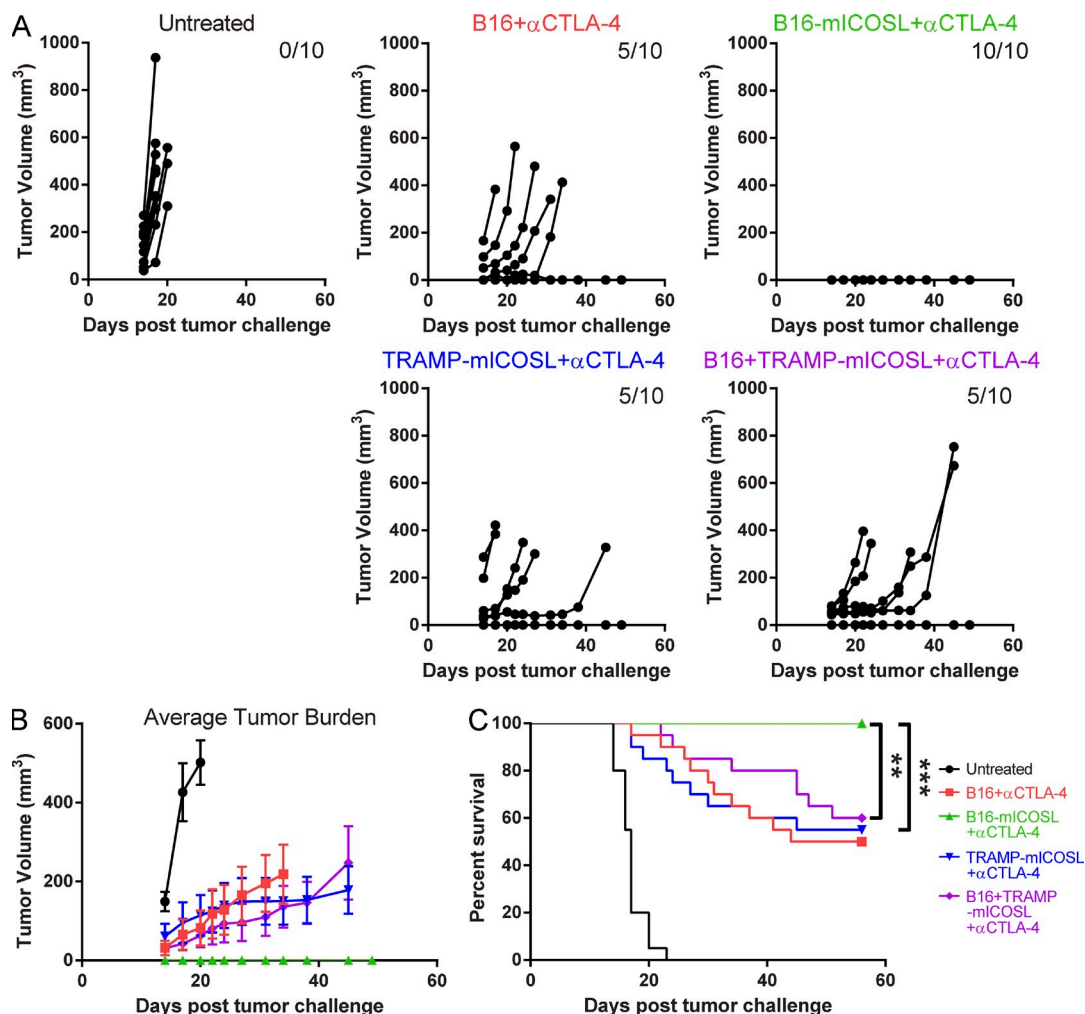
One aspect that could potentially expand the clinical application of IVAX therapy is whether the ICOS signal can be delivered independently of the cognate tumor antigen. This, if true, would lead to easier development of off-the-shelf ICOS agonist that can enhance T cell immunity against any target antigen. We tested this hypothesis by treating mice with B16 tumors with TRAMP-based IVAX or a 1:1 mixture of irradiated wild-type ICOSL-negative B16 and TRAMP-based IVAX. In this setting, the primary TCR signal (B16 tumor antigen) and the secondary ICOS signal were presented in trans. This strategy was unable to generate the same degree of tumor protection as with B16-based IVAX where the two

signals were presented in cis (Fig. 9). The cognate TCR signal is required in order for ICOS signal to take effect, as TRAMP-based IVAX alone was no more effective than irradiated wild-type B16. The ICOS signal in trans did provide some additional survival benefit when compared with mice treated with irradiated B16 control vaccine alone, but the difference was not significant. This result suggests that cognate TCR signal and ICOS stimulation should be incorporated on the same vehicle for optimal therapeutic effect.

#### DISCUSSION

With the FDA approval of PROVENGE and more recently ipilimumab, the effectiveness of immunotherapy in the treatment of cancer is firmly established. Ipilimumab has quickly become a standard-of-care agent for the treatment of late-stage melanoma, and its application will possibly expand as results are reported from ongoing phase III trials in prostate





**Figure 9. Tumor protection by IVAX requires presentation in cis.** B16/F10 tumor-bearing mice were treated with TRAMP-based IVAX or a 1:1 mixture of irradiated wild-type ICOSL-negative B16 and TRAMP-based IVAX. Irradiated wild-type B16 and B16-based IVAX were included as control. CTLA-4 blockade was given in all the treatment groups. (A) Individual tumor growth curves after B16/F10 challenge. Numbers on the top right side represent tumor-free mice. Data are representative of two independent experiments ( $n = 10$  mice per group). (B) Tumor growth curves depict average tumor volume in each group. Error bars represent means  $\pm$  SEM. Data are representative of two independent experiments ( $n = 10$  mice per group). (C) Cumulative survival curves from two independent experiments ( $n = 10$  mice per group). Survival curves were analyzed with Log-rank test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

and other tumor types. As with previous standard-of-care therapies, it will be necessary to develop combination strategies to improve clinical benefit. Here, we demonstrate that the efficacy of anti-CTLA-4 therapy is greatly enhanced by targeting the ICOS/ICOSL pathway with a cellular vaccine (IVAX).

The synergy of IVAX with anti-CTLA-4 results in a dramatic enhancement of tumor rejection. However, in the absence of ICOS up-regulation in CD8 and CD4  $T_{\text{eff}}$  cells as a result of CTLA-4 blockade, IVAX monotherapy has minimal effects. This finding is consistent with previous reports of minimal efficacy when ICOS was targeted as monotherapy. For example, it has been shown that ectopic expression of ICOSL could elicit tumor-specific T cell response, but antitumor responses could only be generated in a prophylactic and not a therapeutic setting (Liu et al., 2001; Wallin et al., 2001; Zuberek et al., 2003). Similarly, engagement of the ICOS pathway with

an ICOSL-Ig fusion protein alone failed to induce rejection of poorly immunogenic tumors such as B16 melanoma (Ara et al., 2003; Zuberek et al., 2003). This was probably caused by the fact that in the absence of CTLA-4 blockade, ICOS was expressed by only a few CD8 and/or CD4  $T_{\text{eff}}$  cells but expressed highly by the majority of  $T_{\text{reg}}$  cells (Fig. 1), so that engagement of the ICOS pathway primarily stimulated the regulatory rather than the effector population. This hypothesis was supported by the fact that pretreatment of mice with cyclophosphamide, which preferentially depleted  $T_{\text{reg}}$  cells, could help improve the efficacy of ICOSL-Ig against poorly immunogenic tumors (Ara et al., 2003). A recent study showed that human melanoma cells expressing high levels of ICOSL facilitated expansion and IL-10 production in the  $T_{\text{reg}}$  cell population in the setting of high-dose IL-2 treatment (Martin-Orozco et al., 2010). In our study, however, there was no expansion of

$T_{reg}$  cells in mice treated with IVAX alone or with anti-CTLA-4, but the balance between effector and regulatory T cells in the tumor was enhanced considerably by the combination of IVAX and CTLA-4 blockade as the result of selective accumulation of effector CD4 and CD8 cells.

CTLA-4 blockade has been reported to selectively reduce the frequency and number of  $T_{reg}$  cells in B16 tumors but not in the peripheral lymphoid organs (Quezada et al., 2006; Simpson et al., 2013). The depletion is strongest in the combination of GVAX and anti-CTLA-4 on adoptively transferred TCR-transgenic Trp1 CD4 cells when compared with GVAX monotherapy. This effect is potentially driven by heavy infiltration of macrophages in the tumor (Simpson et al., 2013), which is a hallmark of GVAX therapy but not observed in the IVAX model. We did verify that CTLA-4 blockade reduced the frequency of  $T_{reg}$  cells in the tumor, but that was primarily because of relatively greater expansion of CD8 and CD4  $T_{eff}$  cells rather than a decrease in the number of  $T_{reg}$  cells per milligram of tumor. In contrast, this also opens up new avenues of combination therapy by incorporating  $T_{reg}$  cell depletion regimen into IVAX therapy to further improve the efficacy.

The underlying mechanism of IVAX therapy is likely to be distinct from GVAX and Flt3L-secreting vaccine (FVAX; Curran and Allison, 2009). An optimal cancer immunotherapy entails ample tumor antigen presentation, co-stimulation, and/or removal of co-inhibition on T cells. Both GVAX and FVAX enhance the differentiation, maturation, and chemoattraction of DCs, which synergizes very well with CTLA-4 blockade. Although IVAX and anti-CTLA-4 combination strategy focuses more on the two facets of T cell activation, adding positive signals and blocking negative ones, using irradiated tumor cell vaccine as the vehicle to carry the positive ICOS signal still provides some help to tumor antigen presentation, but we reason that it would provide further benefit to combine the strength of IVAX and other strategies like GVAX or FVAX.

Our working hypothesis is that CTLA-4 blockade leads to enhanced activation of tumor-reactive T cells with concomitant up-regulation of ICOS, thereby enabling their responses to be enhanced by ICOS engagement. Thus, in the context of CTLA-4 blockade, IVAX triggers the ICOS pathway to enhance the proliferation, survival, and/or migration of effector cells into the tumor, which led to a higher density of  $T_{eff}$  cells inside the tumor, as indicated by an increase in the  $T_{eff}/T_{reg}$  cell ratio and marked increases in both Th1 CD4  $T_{eff}$  and cytolytic CD8 T cells. These results clearly demonstrate a proof-of-concept that antitumor responses enhanced by CTLA-4 blockade can be greatly improved by targeting the ICOS/ICOSL pathway with ICOSL-expressing tumor cell vaccines. We are currently exploring other strategies that might be more suitable for clinical application for providing agonistic signals through ICOS locally and systemically in combination with CTLA-4 blockade. We anticipate that these combination strategies will translate to the clinic to increase the number of patients who derive benefit from anti-CTLA-4 therapy.

## MATERIALS AND METHODS

**Mice.** 6-wk-old C57BL/6 and ICOS<sup>-/-</sup> mice were purchased from the Jackson Laboratory. Mice were housed in specific pathogen-free conditions in accordance with institutional guidelines. All animal experiments were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

**Cell lines.** The poorly immunogenic mouse melanoma cell lines B16/F10 and B16/BL6 were obtained from I. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX) and described previously (van Elsas et al., 1999). The prostate cancer cell line TRAMP C2 was maintained as described previously (Foster et al., 1997). B16/F10, B16/BL6, and TRAMP C2 were transduced with retrovirus to express full-length mouse ICOSL on the cell membrane and tested for stable expression throughout the treatment process.

**Development of IVAX.** HEK 293T cells were transfected with vectors encoding full-length mouse ICOSL (provided by W. Sha, University of California, Berkeley, Berkeley, CA), envelope glycoprotein from the vesicular stomatitis virus (VSV-G), and Gag-Pol using FuGENE HD (Roche). Supernatant containing packaged virus was collected 48 and 72 h later, filtered with 0.45- $\mu$ m microfilters, and applied to cultured B16/F10, B16/BL6, or TRAMP C2 cells. 5  $\mu$ g/ml polybrene was also added to the virus solution. Target cells were spun at 2,600 rpm and 32°C for 2 h before being transferred into 37°C incubators. Tumor cells stably expressing ICOSL were selected by surface staining with anti-ICOSL.

**Antibodies.** Anti-CTLA-4 (9H10) was purchased from Bio X Cell and administered i.p. Antibodies for flow cytometry were purchased from eBioscience and BD.

**Tumor challenge and treatments.** Mice were challenged i.d. on the right flank with  $5 \times 10^4$  B16/F10,  $2 \times 10^4$  B16/BL6, or  $7.5 \times 10^5$  TRAMP C2 tumor cells on day 0. In experiments in which mice would be sacrificed on day 14, initial B16/F10 challenge was  $2 \times 10^5$ . Mice were then treated with i.p. injection of 100  $\mu$ g anti-CTLA-4 (clone 9H10) and i.d. vaccination on the left flank with  $10^6$  irradiated (150 Gy) ICOSL-expressing tumor vaccine (IVAX) on days 3, 6, 9, and 12. The dose of anti-CTLA-4 was doubled on day 3. The mice were then followed for tumor growth or sacrificed on day 14 for dissection of lymphoid organs and tumors.

**Phenotypic and functional analyses of tumor-infiltrating lymphocytes.** Mice used for functional experiments were sacrificed on day 14 after tumor challenge, and tumor draining lymph nodes, vaccine draining lymph nodes, and tumors were isolated. Tumors were digested in Liberase TL (Roche) and DNase I (Roche) at 37°C for 30 min, filtered, and centrifuged over Histopaque-1119 (Sigma-Aldrich). Tumor-infiltrating T cells were restimulated for 4 h at 37°C with  $5 \times 10^4$  DCs loaded with B16 lysate, in the presence of Golgi-Plug (BD). When cytolytic activity was measured, tumor infiltrates was incubated with anti-CD107a at 37°C for 2 h before staining with other antibodies, in the presence of monensin (BD).

**Flow cytometry and quantification.** Samples were stained with anti-CD4-APC-eFluor 780 (L3T4), anti-CD8-PerCP-Cy5.5 (53-6.7), and anti-ICOS-PE (17G9), fixed and permeabilized (eBioscience) according to the manufacturer's instructions, and stained with anti-Foxp3-Alexa Fluor 700 (FJK-16s), anti-IFN- $\gamma$ -Alexa Fluor 488 (XMG1.2), anti-TNF-Pacific Blue (MP6-XT22), anti-granzyme B-allophycocyanin (GB11), and anti-CD107a-PE (1D4B). Flow cytometry reference beads (PeakFlow blue; Invitrogen) were added to the samples before analysis for quantification of T cells in each tumor. The absolute number of a subset of T cells per milligram of tumor was calculated as the following example shows: density of CD8 cells = (number of beads added to each sample  $\times$  count of CD8 cells/count of beads)/tumor weight.

**Statistical analyses.** Data were analyzed with Prism 5.0 (GraphPad Software). Experiments were repeated two to three times. Statistical significance

was determined by one-way ANOVA and Bonferroni's multiple comparisons test. Tumor survival data were analyzed with the Kaplan-Meier method. The log-rank test was used to compare survival curves for different groups on univariate analyses.  $P < 0.05$  was considered statistically significant.

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