

Antigen-specific antitumor responses induced by OX40 agonist are enhanced by IDO inhibitor indoximod

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## **ABSTRACT**

Although an immune response to tumors may be generated using vaccines, so far, this approach has only shown minimal clinical success. This is attributed to the tendency of cancer to escape immune surveillance via multiple immune suppressive mechanisms. Successful cancer immunotherapy requires targeting these inhibitory mechanisms along with enhancement of antigen-specific immune responses to promote sustained tumor-specific immunity.

Here we evaluated the effect of indoximod, an inhibitor of the immunosuppressive indoleamine-(2,3)-dioxygenase (IDO) pathway, on antitumor efficacy of anti-OX40 agonist in the context of vaccine in the IDO<sup>-</sup> TC-1 tumor model. We demonstrate that although the addition of anti-OX40 to the vaccine moderately enhances therapeutic efficacy, incorporation of indoximod into this treatment leads to enhanced tumor regression and cure of established tumors in 60% of treated mice. We show that the mechanisms by which the IDO inhibitor leads to this therapeutic potency include (i) an increment of vaccine-induced tumor-infiltrating effector T cells that is facilitated by anti-OX40, and (ii) a decrease of IDO enzyme activity produced by non-tumor cells within the tumor microenvironment that results in enhancement of the specificity and the functionality of vaccine-induced effector T cells.

Our findings suggest a translatable strategy to enhance the overall efficacy of cancer immunotherapy.

## **Introduction**

Improved cancer immunotherapy strategies would both evoke an immune response and resist tumor-driven immunosuppression. Successful immunotherapy

depends on breaking tumor-mediated immune tolerance such that the immune system can be modulated to deliver T cells that could recognize and eradicate tumors. Induction of immune response against tumors is an efficient method to educate T cells with tumor antigens; however, suppressive mechanisms within the tumor microenvironment weaken the effector immune response. Thus, mitigating the immunosuppression while concurrently augmenting vaccine-induced antigen-specific T cells could improve the overall potency of cancer immunotherapy.

Favorable outcomes follow increased number of tumor-specific effector T cells within the tumor environment [1-3]. To generate strong and long-lasting anti-cancer immune response upon antigen recognition, an engagement of secondary costimulatory receptors is often employed in combinational immune therapeutic studies [4-8]. A member of the tumor necrosis factor receptor (TNFR) superfamily, OX40 (CD134) is a costimulatory molecule [9] that is currently in clinical trials [10]. The OX40 receptor is inducible on activated T cells [11-15] and its engagement with cognate ligand or agonistic antibody enhances T-cell proliferation, cytokine production, survival and memory development [16, 17]. In animal models, anti-OX40 has shown favorable antitumor activities and in autoimmune models increases the severity of disease [18-23]. In addition to augmenting the effector arm of the immune system, extensive studies have been devoted to immune tolerance and tumor-mediated immunosuppressive mechanisms, which render effector T cells ineffective in clearing malignant cells [24-26]. Tumor-mediated immune suppression includes inhibitory mechanisms elicited by tumor cells directly (co-inhibitory receptor/ligand interactions, secretion of inhibitory cytokines and other molecules) or indirectly (mechanisms based on tumor-recruited suppressive immune cells). We believe that targeting the indirect mechanisms would have wide application and that such a strategy could be used for immune therapy of a broad spectrum of tumors, regardless of their expression of specific molecules.

One of the protective mechanisms is production of indoleamine-(2,3)-dioxygenase (IDO). IDO is produced not only by some tumors but also by inhibitory immune cells such as suppressive dendritic cells and monocyte-derived macrophages [27, 28]. In fact, IDO expression within the tumor microenvironment and tumor draining lymph nodes correlates with poor cancer prognosis [29-32]. IDO enzymatic activity potentiates tryptophan degradation, causing cell-cycle arrest and induction of anergy in effector T cells. Toxic tryptophan catabolites may lead to apoptosis of T cells and induction of regulatory T cells (Tregs) [33-38]. Accordingly, inhibition of the IDO pathway increases antitumor responses in murine models [27, 33, 38-40]. One of the relatively well characterized IDO pathway inhibitors is indoximod (1-methyl-D-tryptophan) [27, 39, 40].

We and others have previously demonstrated that successful cancer immunotherapy requires simultaneous targeting of both effector and suppressor arms of the immune system [41-45]. Since OX40 leads to increased  $\text{INF}\gamma$  expression in the periphery and within the tumor microenvironment [16, 17] and  $\text{INF}\gamma$  promotes the induction of IDO, we hypothesized that OX40 based antitumor immunotherapy could be improved by reducing immune suppression through inhibition of IDO. We therefore evaluated the therapeutic efficacy and immune mechanisms of treatment combining anti-OX40Ab with indoximod in a vaccine setting in mice with established tumors. To test the effect of IDO inhibition on the immune component of the tumor microenvironment, we tested the combination in an IDO<sup>+</sup> tumor model, TC-1. We demonstrate that this treatment is therapeutically potent and discuss immune mechanisms that explain the role of each component within the combination. Here, we provide evidence that simultaneous targeting of the effector arm with anti-OX40 and the suppressor arm of immune system with an IDO inhibitor, indoximod, produces a synergistic effect on the tumor immune response resulting in the enhancement of vaccine potency and in tumor

eradication. Considering that, on average, more than half of human tumors are IDO<sup>-</sup> [46], we believe that this treatment strategy is promising, can be applied to a wide range of tumors (including IDO<sup>-</sup> tumors), and can improve the efficacy of cancer treatment.

## **Material and Methods**

### **Mice and cell lines**

C57BL/6 female mice (6-8 weeks old, The Jackson Laboratory, Bar Harbor, ME) were housed in pathogen free environment. All procedures were carried out with approved Augusta University institutional animal protocols and NIH guidelines.

TC-1 cell line, derived by co-transfection of human papilloma-virus strain 16 (HPV16) early proteins 6 and 7 (E6 and E7) and activated h-ras oncogene into primary lung epithelial cells, was obtained from American Type Culture Collection (Manassas, VA) and propagated in RPMI1640 supplemented with 10% FBS, penicillin and streptomycin (100 U/ml each), L-glutamine (2 mM) at 37°C with 5% CO<sub>2</sub>. Cells were maintained at a confluence of 70% to 80% and were always used from a passage number less than 10. These cells were tested routinely for absence of mycoplasma by using PCR at Georgia Cancer Center, Augusta University (Augusta, GA). All tests were negative. During the last one year, these cells were not re-authenticated.

This cell line was deliberately selected, as these tumor cells do not express IDO, allowing the study of the effect of the IDO inhibitor on the tumor microenvironment without interference from the direct effect on tumor cells.

### **Vaccine and other reagents**

The CTL epitope from HPV16 E7<sub>49-57</sub> (9aa peptide, RAHYNIVTF, 100µg/mouse) mixed with 13 amino acid synthetic nonnatural pan DR CD4<sup>+</sup> T helper epitope (PADRE) [47] (13aa peptide, aK-Cha-VAAWTLKAAa, where a is D-alanine, and Cha is L-cyclohexylalanine, 20µg/mouse) (both from Celtek Bioscience, Franklin, TN) and QuilA adjuvant (20µg/mouse) (Brenntag, Westbury, NY) was used as the model vaccine (that contains both CD8 and CD4 epitopes) in all studies [subcutaneous (s.c.) injections].

indoximod (1-MT, Sigma-Aldrich) was provided in drinking water (freshly prepared every three days) at 2mg/mL concentration. Water was supplemented with low-fat sweetener to improve the water intake by mice. Once all the material was dissolved, the pH was adjusted to a range of 7-7.5 with 1N HCl and filtered through a 75mm filter unit.

indoximod drinking solution was provided to mice in amber-colored or aluminum foil-wrapped drinking bottle to protect from exposure to light.

Agonist rat anti-mouse OX40 (clone OX86, rat IgG1) was provided by Medimmune (Cambridge, UK), and delivered by intraperitoneal (i.p.) injections at a dose of 1 mg/kg.

### **Tumor implantation and treatment**

For both therapeutic and immunology experiments  $7 \times 10^4$  TC-1 cells were inoculated s.c. into the right flank of the mice on day 0. Vaccine was given weekly s.c. starting on day 10-12 after tumor implantation when tumor diameter reached 4-5 mm. For therapeutic experiments, vaccine was given weekly throughout the experiment. Anti-OX40 and indoximod treatments started on the same day with vaccination. Anti-OX40 was given twice a week for the entire duration of treatment. Similarly, indoximod was provided in drinking water until the end of the study. A total of eight groups of mice ( $n = 5$ ) were utilized in these experiments, including non-treated, single and double agent-treated and vaccine/anti-OX40/indoximod treated groups. Tumor growth and survival was monitored. Tumors were measured every 3–4 days using digital calipers, and tumor volume was calculated using the formula  $V = (W^2 * L) / 2$ , whereby V is the volume, L is the length (longer diameter) and W is width (shorter diameter). Mice were sacrificed when moribund or if tumor volume reached  $1.5 \text{ cm}^3$ . For immunology experiments mice were treated similarly and were sacrificed six days after the second immunization. Tumors were harvested for analysis of tumor infiltrating cells and IDO activity.

### **Profiling tumor-infiltrating immune cells**

Resected tumors were homogenized using GentleMACS Dissociator (Miltenyi Biotec, San Diego, CA), and strained through a 70  $\mu$ m nylon filters (BD Biosciences, San Jose, CA). Red blood cells were lysed with ACK buffer according to manufacturer's suggestions (Life Technologies, Grand Island, NY). Samples were fixed and permeabilized using BD Pharmingen Mouse FoxP3 buffer set (BD Biosciences, San Jose, CA). LSRII SORP flow cytometer (BD Biosciences, San Jose, CA) was used to analyze the following tumor-infiltrating cell subsets: CD8<sup>+</sup>, CD8<sup>+</sup>E7<sup>+</sup>, CD8<sup>+</sup>E7<sup>+</sup>GrzB<sup>+</sup>, CD4<sup>+</sup>Foxp3<sup>-</sup> (non-Treg), and CD4<sup>+</sup>Foxp3<sup>+</sup> (Treg) cells within live CD3<sup>+</sup>CD45<sup>+</sup> population.

The following fluorophore conjugated antibodies purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA) were used for flow cytometry assay: CD45-PE, CD3-PE-CF594 or V450, CD8-V450 or APC, CD4-FITC, FoxP3-APC or PE-CF594, Granzyme B-APC. The FITC-conjugated dextramers specific to E7<sub>49-57</sub> peptide was purchased from Immudex (Fairfax, VA). Live cells were identified by fixable near infra-red Live/Dead viability staining as suggested by manufacturer (Life Technologies, Grand Island, NY). Acquired data were analyzed using FlowJo software, version 10 (TreeStar, Ashland, OR).

The absolute numbers of tumor-infiltrating cells were standardized per 10<sup>6</sup> of total tumor cells. The fold change of tumor infiltrating immune cells was presented as fold change over the non-treated group.

### **Evaluation of IDO activity**

The IDO enzymatic activity in tumor homogenates was assessed using high-performance liquid chromatography (HPLC) techniques as described earlier [48]. Briefly, a concentration of kynurenine (tryptophan catabolite) was measured before and after 2 hrs of addition of exogenous L-tryptophan substrate solution into tumor homogenates.



Tumors were harvested from control and treated mice. The treatment schedule and tumor harvest were the same as for the assessment of immunology response.

### **Statistical analysis**

All statistical parameters (average values, SD, significant differences between groups) were calculated using GraphPad Prism software. Statistical significance between groups was determined by One-way ANOVA with a Tukey multiple comparison post-test ( $P < 0.05$  was considered statistically significant).

### **Results**

#### **Indoximod enhances agonistic effect of anti-OX40 to eradicate tumors**

It is known that engagement of OX40 on T cells with its ligand or agonist antibody leads to increased  $\text{INF}\gamma$  expression in the periphery and within the tumor microenvironment [16, 17].  $\text{INF}\gamma$  induces IDO, which in turn produces a range of immune inhibitory effects within the tumor microenvironment [49, 50]. Accordingly, we tested whether inhibiting the IDO pathway would enhance the therapeutic effect of anti-OX40. We tested this hypothesis in the TC-1 tumor model that lacks IDO expression to identify the effects of the combination specifically on the immune component of the tumor microenvironment rather than on the tumor cells. We first evaluated tumor growth and survival of animals after treatment of TC-1 tumor-bearing mice with vaccine, anti-OX40 agonist Ab and indoximod. TC-1 tumor cells were implanted on day 0 and treatment was initiated when tumors reached 4-5mm in diameter. Tumor-bearing mice were treated weekly with an E7 peptide vaccine to generate an effector T-cell response against the tumor. Anti-OX40 agonist was administered twice a week and indoximod was provided in drinking water

throughout the experiment as depicted in Fig. 1A, and tumor growth and survival were monitored.

We found that although none of the single-agent treatments affects the tumor growth, vaccine/anti-OX40 leads to slower tumor progression and complete tumor regression in 20% of the mice when added to the vaccine (Fig. 1B). The addition of indoximod to this treatment significantly enhanced therapeutic efficacy resulting in a complete eradication of established tumors in 60% of the mice. In addition, enhanced therapeutic efficacy after treatment with vaccine and anti-OX40 was seen early in the treatment (Fig. 1C; day 26 representing the last day when all mice are surviving in all control groups). However, incorporation of indoximod into this treatment improved the long term therapeutic outcome as shown at day 40 (Fig. 1D). Moreover, compared to the single treatments, vaccine/anti-OX40 led to prolonged survival and addition of indoximod to this treatment resulted in a sustained response beyond 65 days (Fig. 1B, E). These data demonstrate that IDO inhibition with indoximod significantly enhances the effect of OX40 agonist antibody.

### **Combination of anti-OX40 with indoximod results in significant increase of CD8<sup>+</sup> T cell to Treg ratio**

To dissect the immune mechanisms leading to the enhanced therapeutic efficacy of vaccine/anti-OX40/indoximod treatment, we tested the effect of the combination on the tumor-infiltrating T cells. Animals were treated as mentioned above; six days after the second vaccination, mice were sacrificed and tumors were harvested for evaluation of tumor-infiltrating T-cell subsets by flow cytometry.

Neither the single agent treatments nor the vaccine/anti-OX40 combination significantly affected CD4<sup>+</sup>FoxP3<sup>-</sup> T-cell tumor infiltration compared with either agent alone. However, we found that the addition of indoximod to vaccine/anti-OX40 led to a significant

increase in tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup> (non-Treg CD4<sup>+</sup> T cells) (Fig. 2A). None of the treatments significantly affected the numbers of tumor-infiltrating Tregs (Fig. 2B).

Although the percentage of tumor-infiltrating Treg cells within the total CD4<sup>+</sup> T-cell population was decreased in the vaccine/anti-OX40 group (due to increase of CD4<sup>+</sup>FoxP3<sup>-</sup> T cells), this reduction was more significant when indoximod was added to the treatment (Fig. 2C).

Next, we explored the effect of the addition of indoximod to anti-OX40 on tumor-infiltrating CD8<sup>+</sup> T cells. We found that treatment with vaccine/anti-OX40 significantly increased the number of CD8<sup>+</sup> T cells infiltrating the tumor compared to mice from control groups (Fig. 3A). Although addition of indoximod to vaccine/anti-OX40 resulted in better antitumor response, no significant differences were found in the numbers of tumor-infiltrating CD8<sup>+</sup> T cells over the vaccine/anti-OX40 group (Fig. 3A). Both groups showed a significant increase in CD8<sup>+</sup>/Treg ratio when compared to control groups (Fig. 3B).

These data demonstrate that the enhanced antitumor efficacy caused by inhibition of IDO is not facilitated by the increase in CD8<sup>+</sup> T cell number, suggesting the existence of other mechanisms driving the therapeutic efficacy of the combination treatment.

### **Addition of indoximod to anti-OX40 treatment increases antigen-specific functional CD8<sup>+</sup> T cells**

We have demonstrated that the addition of indoximod to vaccine/anti-OX40 immunotherapy did not affect the total number of tumor infiltrating CD8<sup>+</sup> T cells or the CD8<sup>+</sup>/Treg ratio, despite the fact that addition of indoximod improved therapeutic efficacy of anti-OX40.

Therefore, to understand the reasons for improved therapeutic outcome when indoximod is added to vaccine/anti-OX40, we first confirmed the efficacy of indoximod for the IDO

enzymatic activity within tumor homogenates by measuring the levels of kynurenine catabolites. As expected, we detected no differences of kynurenine catabolites produced by tumor homogenates after addition of exogenous tryptophan in untreated mice and animals treated with vaccine alone or vaccine/anti-OX40 compared to non-treated mice (Fig. 4A). Moreover, there was no difference in IDO activity in mice treated with anti-OX40/indoximod and vaccine/indoximod. However, mice treated with vaccine/anti-OX40/indoximod had significantly decreased IDO activity ( $P < 0.05$ ) compared to non-treated (Fig. 4A).

Next we tested the antigen-specificity and functionality of tumor-infiltrating CD8<sup>+</sup> T cells, which could also be responsible for the differences in therapeutic efficacy between vaccine/anti-OX40 and vaccine/anti-OX40/indoximod treatments.

The specificity and functionality of tumor-infiltrating CD8<sup>+</sup> T cells were evaluated from mice treated with vaccine, vaccine/anti-OX40, vaccine/indoximod, or vaccine/anti-OX40/indoximod as described above. Although the addition of indoximod to anti-OX40/vaccine was unable to increase the total number of CD8<sup>+</sup> T cells in the tumor microenvironment compared to vaccine/OX40 group (Fig. 3A), incorporation of indoximod into the treatment resulted in a significantly higher number of tumor-infiltrating antigen (E7)-specific CD8<sup>+</sup> T cells compared to all other groups (Fig. 4B). Granzyme B (GrzB) expression was used to characterize the functionality of these CD8<sup>+</sup> T cells. We demonstrated that the addition of indoximod to vaccine/anti-OX40 also led to a significant increase in the number of tumor-infiltrating CD8<sup>+</sup>GrzB<sup>+</sup> antigen-specific T cells compared to all other groups (Fig. 4C).

These data demonstrate that providing indoximod simultaneously with vaccine/anti-OX40 not only leads to decreased IDO activity in tumor but also increases numbers of functional antigen-specific CD8<sup>+</sup> T cells, which might explain the differences in therapeutic efficacy observed between the treatment groups.

Thus, we show that although the addition of anti-OX40 to vaccine treatment enhances the numbers of CD8<sup>+</sup> T cells in tumors (“quantity”), addition of indoximod improves the “quality” of these CD8<sup>+</sup> T cells (enhanced antigen-specificity and functionality).

## **Discussion**

Here we tested the agonist anti-OX40 in combination with the IDO pathway inhibitor, indoximod in the context of cancer vaccine. We showed that anti-OX40 agonist can enhance the effect of vaccine treatment resulting in slower tumor progression and complete regression of established tumors in 20% of treated mice. We hypothesized that the addition of indoximod to anti-OX40 treatment can improve the overall therapeutic efficacy of vaccine treatment. We showed that combinational treatment with vaccine/anti-OX40/indoximod significantly enhanced antitumor efficacy and resulted in complete tumor eradication in 60% of mice. While evaluating immunologic mechanisms responsible for this potent therapeutic outcome, we found that an increase of tumor-infiltrating CD8<sup>+</sup> T cells for the combinational treatments is mediated by anti-OX40, since both vaccine/anti-OX40 and vaccine/anti-OX40/indoximod treatments had similar numbers of CD8<sup>+</sup> T cells. Likewise, anti-OX40 was responsible for the increase in non-Treg CD4<sup>+</sup> T-cell infiltration into the tumor, thus resulting in decreased percentage of Treg cells within the tumor-infiltrating CD4<sup>+</sup> T-cell population.

We found no significant effect of anti-OX40 on the number of tumor infiltrating Tregs. This contrasts with results in the CT26 tumor model, where the same anti-OX40 clone resulted in Treg depletion within the tumor and led to enhanced therapeutic efficacy [51].

This could be attributed to the different immune profiles of the two tumor models.

We found no differences in numbers of tumor-infiltrating CD8<sup>+</sup> T cells or in CD8<sup>+</sup>/Treg ratio, a well-established criteria that correlates with cancer prognosis [45, 52], between vaccine/anti-OX40 and vaccine/anti-OX40/indoximod treatments, even though the

therapeutic efficacy for these treatments was significantly different. To understand this phenomenon, we evaluated the specificity and functionality of tumor-infiltrating CD8<sup>+</sup> T cells. We found that numbers of both E7-specific and Granzyme B<sup>+</sup> functional antigen-specific CD8<sup>+</sup> T cells were elevated within the tumors of mice treated with vaccine/anti-OX40/indoximod compared to other groups. This at least partially explains the differences in antitumor therapeutic efficacies between these two treatments.

Considering that more than half of the human tumors are IDO<sup>-</sup> [46], we selected the TC-1 tumor model for evaluation of indoximod-based immunotherapy. As these tumor cells (in culture and *in vivo*) do not express IDO, the observed effect elicited by indoximod would be due not to its direct effect on tumor cells but rather to its effect on immune suppressive cells within the tumor microenvironment. Under normal physiological conditions, IDO contributes to maintenance of immune tolerance [27]. The catabolic effect of IDO on tryptophan depletion and kynurenine accumulation leads to T-cell arrest or apoptosis [34-37]. In the tumor setting this phenomenon enhances tumor growth by hindering immune effector cell function [28, 33, 38]. We observed no differences in IDO activity between animals treated with vaccine or with vaccine/anti-OX40, whereas the addition of indoximod to vaccine/anti-OX40 decreased IDO activity compared to other groups. Thus, one possibility for increased specificity and functionality of tumor-infiltrating CD8<sup>+</sup> T cells from mice treated with indoximod in addition to vaccine/anti-OX40 is the decrease in IDO enzymatic activity within the tumors of these mice.

In conclusion, here we demonstrate the synergistic antitumor therapeutic effect of vaccine/anti-OX40/indoximod combination that leads to tumor regression, and explore the immune mechanisms responsible for that synergy. Dissecting the mechanisms for each compound within the combination shows that anti-OX40 is responsible for the overall increase in numbers, and indoximod for specificity and functionality of vaccine-induced CD8<sup>+</sup> T cells. We provide evidence that simultaneous targeting of the effector

and suppressor arms of the immune system synergistically promotes tumor eradication.

This translatable strategy may enhance the overall efficacy of cancer treatment.

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## Figure legends

**Figure 1. Combination of vaccine, anti-OX40 Ab and indoximod provides potent antitumor therapeutic efficacy.** **A.** C57BL/6 mice ( $n = 5/\text{group}$ ) were injected s.c. in the right flank with  $7 \times 10^4$  TC-1 cells. Mice from appropriate groups were injected weekly with vaccine (s.c.) or PBS as a control into the right flank. Anti-OX40 was injected i.p. twice a week starting on Day 10 after tumor implantation throughout the experiment. Indoximod was provided in drinking water starting Day 10 throughout the experiment. Tumor sizes were measured every 3-4 days. **B.** Plots represent tumor volumes of individual mice for each treatment. Ratio on the plots shows number of mice with completely regressed tumors over total number of mice in the group. Average tumor volumes  $\pm$  SD on days 26 (**C**) and 40 (**D**). **E.** Kaplan–Meier plot of the overall survival. Similar results were obtained from two independent experiments. \* $P < 0.05$ , \*\*  $P < 0.01$ . Experiment was repeated three times with similar results.

**Figure 2. Vaccine/anti-OX40/indoximod treatments lead to an increase of non-Treg CD4<sup>+</sup> T-cell infiltration into the tumor without affecting Treg cell number.** C57BL/6 mice ( $n = 4-5/\text{group}$ ) were injected with  $7 \times 10^4$  TC-1 cells and treated as for therapeutic studies, except on Day 23 mice were sacrificed, and tumor-infiltrating CD4<sup>+</sup> T cells were analyzed in tumor homogenates by flow cytometry. The absolute numbers of tumor-infiltrating cells were standardized per  $10^6$  of total tumor cells and presented as fold change over non-treated (NT) group for **A.** non-Treg CD4<sup>+</sup> T cells (CD4<sup>+</sup> FoxP3<sup>-</sup>), **B.** Treg cells (CD4<sup>+</sup> FoxP3<sup>+</sup> - no statistically significant differences were observed between any of the groups), or as a percentage of Tregs within CD4<sup>+</sup> population (**C**). \*  $P < 0.05$ , \*\*  $P < 0.01$ . Combined data from three independent experiments are shown.

**Figure 3. Both vaccine/anti-OX40 and vaccine/anti-OX40/indoximod treatments lead to an increase of CD8<sup>+</sup> T-cell infiltration into the tumor and CD8<sup>+</sup>/Treg ratio.** C57BL/6 mice ( $n = 4-5/\text{group}$ ) were injected with  $7 \times 10^4$  TC-1 cells and treated as for Fig. 2, and tumor-infiltrating CD8<sup>+</sup> T cells were analyzed in tumor homogenates by flow cytometry. The absolute numbers of tumor-infiltrating CD8<sup>+</sup> T cells were standardized per  $10^6$  of total tumor cells and presented as fold change over non-treated (NT) group (**A**). **B.** The CD8<sup>+</sup>/Treg ratio was calculated from normalized numbers of CD8<sup>+</sup> T cells and Tregs per  $10^6$  of total tumor cells. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . Combined data from three independent experiments are shown.

**Figure 4. Addition of indoximod to vaccine/anti-OX40 treatment leads to decrease in kynurenine and an increase of tumor-infiltrating E7-specific and E7<sup>+</sup>GrzB<sup>+</sup> CD8<sup>+</sup> T cells.** **A.** Enzymatic activity of IDO was evaluated in tumor homogenates from control and treated mice. IDO activity was calculated as pmol kynurenine/hr/mg tissue. **B and C.**

Tumor-bearing C57BL/6 mice ( $n = 5/\text{group}$ ) were treated with vaccine alone, vaccine/anti-OX40, vaccine/indoximod or vaccine/anti-OX40/indoximod as described above. The absolute numbers of tumor-infiltrating E7 dextramer<sup>+</sup> CD8<sup>+</sup> T cells (**B**) and E7<sup>+</sup>GrzB<sup>+</sup> CD8<sup>+</sup> T cells (**C**) were standardized per  $10^6$  of total tumor cells and presented as mean values  $\pm$ SD. \* $P < 0.05$  compared to all other groups. Similar results were obtained from two independent experiments.

**Figure 1.**

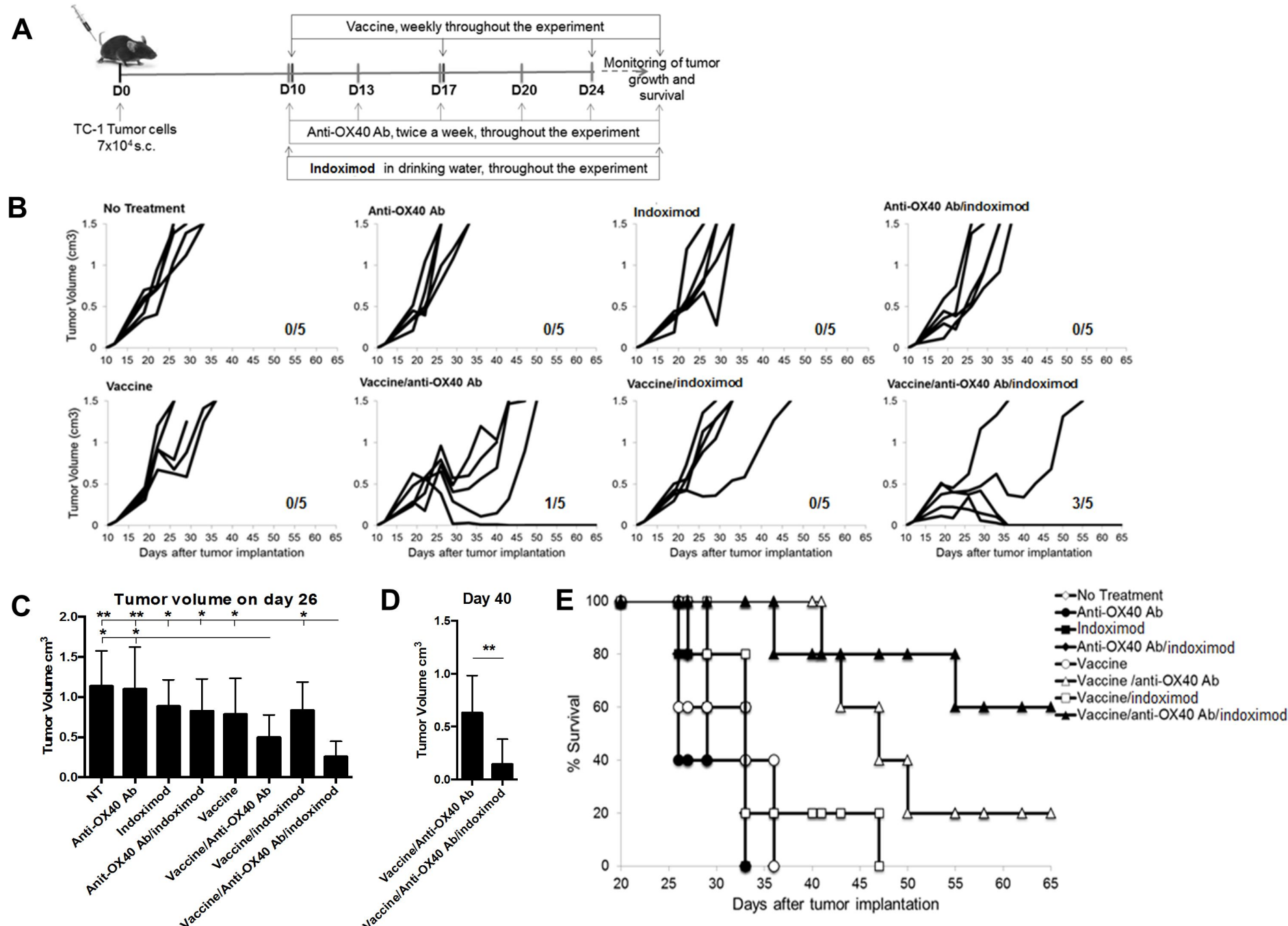
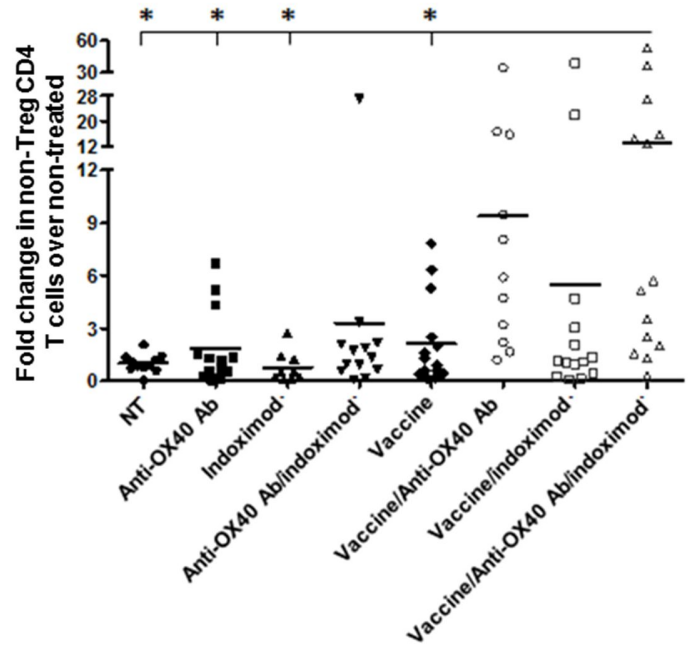
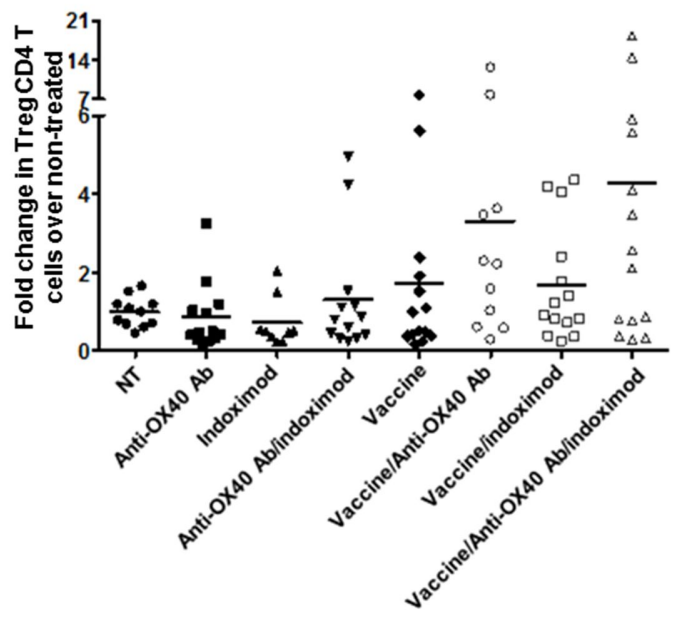


Figure 2.

A



B



C

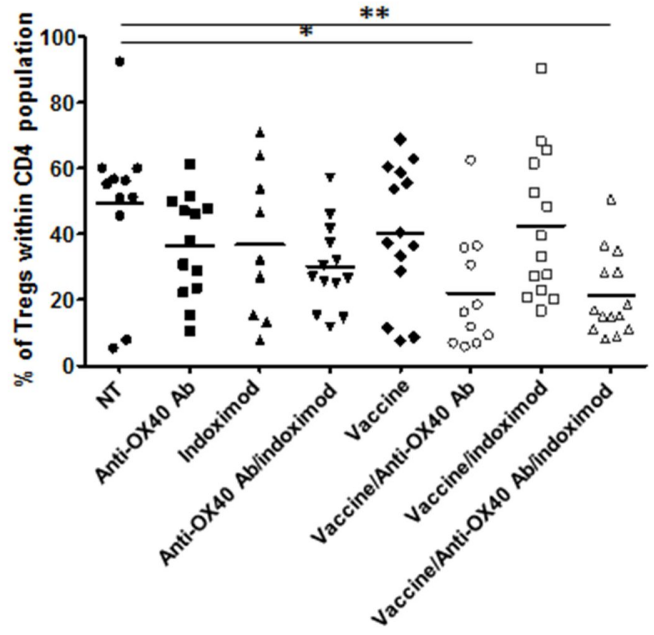
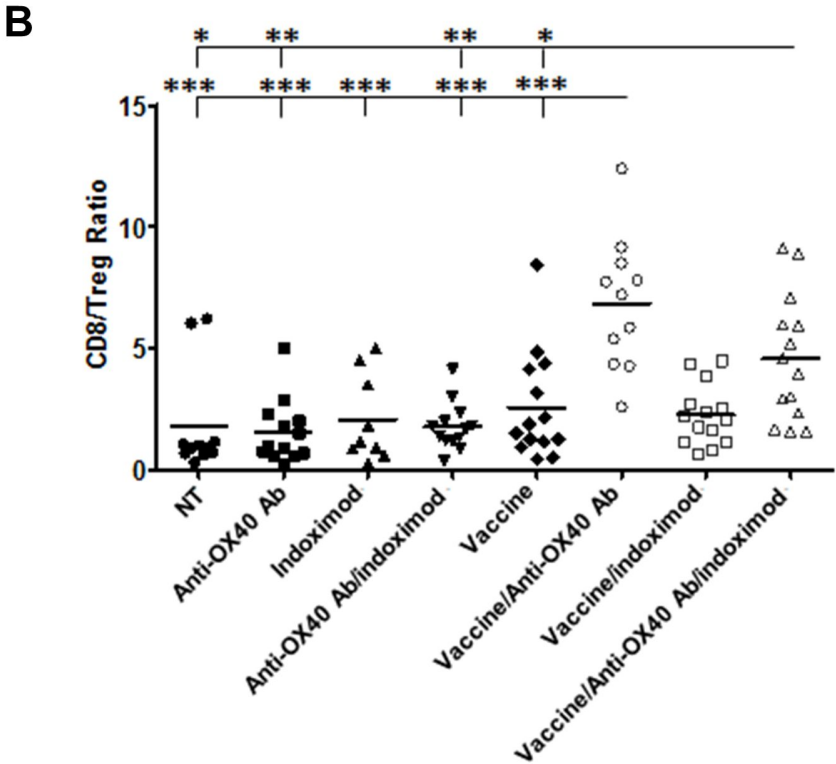
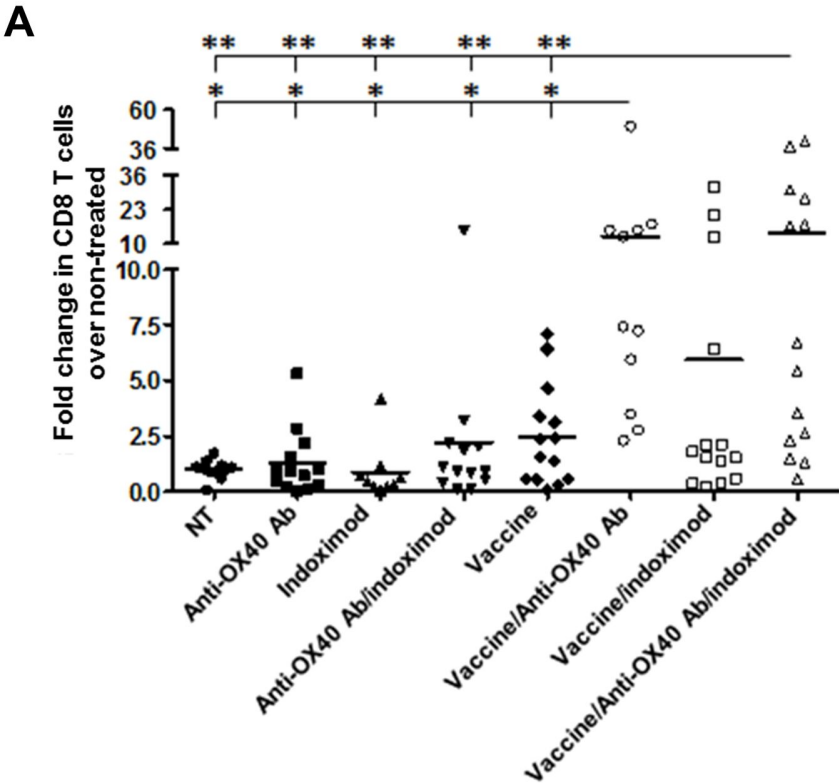
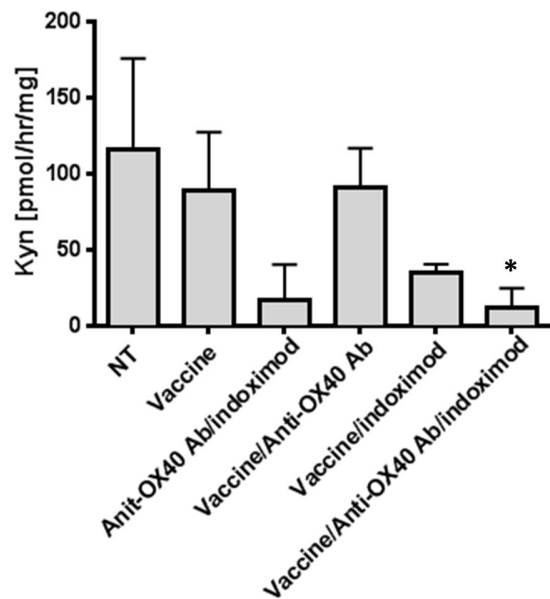


Figure 3.

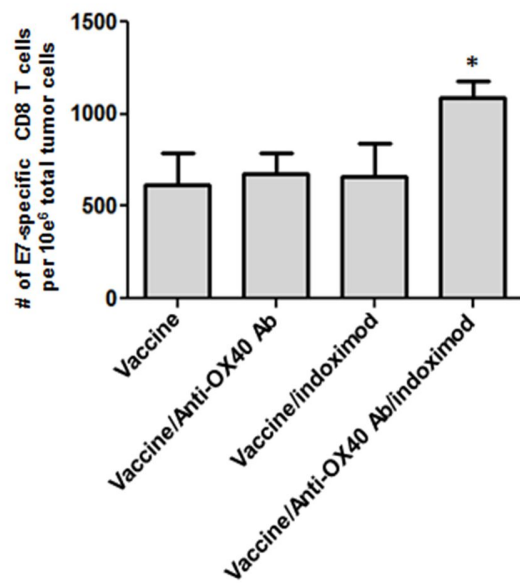


**Figure 4.**

**A**



**B**



**C**

