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Combinational immunotherapy of anti-OX40 antibody and IDO inhibitor synergistically enhances anti-tumor immune T cell-mediated response

(Under the direction of Dr. SAMIR KHLEIF)

One of the major goals of cancer immunotherapy is to disrupt the immunosuppressive environment that allows tumors to thrive in and to generate potent and enduring anti-tumor specific-immune responses. Cancer vaccines may elicit antigen-specific immune responses; however, this is, in many tumor models and in human cancer, insufficient for positive outcomes due to existence of multiple immune-inhibitory mechanisms in tumors. A relatively recently introduced strategy to increase the therapeutic efficacy of tumor vaccination is to combine different immunological approaches that target different immunosuppressive pathways and to enhance the efficacy of vaccines by T cell agonists. OX40 is a co-stimulatory receptor expressed on T cells that can lead to proliferation and enhancement of T cell effector function when bound by its ligand or targeted with agonist antibody. Here, we show that different doses of anti-OX40 antibody (Ab) elicit differential impacts on the T cell immune response resulting in either efficacious or detrimental therapeutic effect in immunized tumor-bearing mice. We demonstrate that treating tumor-bearing mice with an optimal dose of 1 mg/kg anti-OX40 Ab leads to a potent therapeutic and immune anti-tumor effect when combined with vaccine, whereas higher dose at 2.5 mg/kg of anti-OX40 Ab with vaccine increases the accumulation of regulatory T cells in the tumor and diminishes the therapeutic effect. Furthermore, we proposed that OX40 downstream molecular signaling through AKT activation in T cells may elucidate the differential T cell response when stimulated with anti-OX40 Ab.

After optimizing the dose of agonist anti-OX40 Ab to stimulate the immune

system toward maximal anti-tumor response when combined with vaccine, we strategized to improve the combinational therapy by targeting the so far untouched immunosuppressive environment. One of the immune suppressive molecules correlating with cancer progression is indoleamine-(2,3)-dioxygenase (IDO) enzyme. The catalytic activity of IDO hinders effector T cells from properly eliciting an anti-tumor effect. Here, we further evaluated the therapeutic outcome and immune mechanisms of the vaccine-induced immune response enhancement by agonist anti-OX40 antibody, while inhibiting the immunosuppressive IDO enzyme. We demonstrate that therapeutic efficacy of this combinational treatment leads to a profound inhibition of tumor growth and complete regression of established tumors in 60% of treated mice. We show that the mechanisms responsible for this therapeutic potency are: i) an increase in vaccine-induced tumor-infiltrating effector T cells that is facilitated by anti-OX40 antibody, and ii) a decrease of IDO enzyme activity within the tumor and the enhancement in the functionality of effector T cells that are facilitated by 1-methyl tryptophan (1-MT, IDO inhibitor).

Our findings provide a promising and translatable strategy that can enhance the overall efficacy of cancer immunotherapy.

KEY WORDS: (OX40, Immunotherapy, Cancer, IDO, AKT)

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This thesis/dissertation is submitted by Zuzana Jirina Berrong and has been examined and approved by an appointed committee of the faculty of the Graduate School of Augusta University.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis/dissertation has received final approval with reference to content, form and accuracy of presentation.

This thesis/dissertation is therefore in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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COMBINATIONAL IMMUNOTHERAPY OF ANTI-OX40 ANTIBODY AND IDO INHIBITOR SYNERGISTICALLY ENHANCES ANTI-TUMOR IMMUNE T CELL-MEDIATED RESPONSE

By

Zuzana Jirina Berrong

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Table of Contents

Page

List of Figures

Page

I. INTRODUCTION

As of 2016, there are seven billion people in the world, and about fourteen million humans will learn they have cancer. Less than half of them will survive it. In some countries, cancer surpasses heart disease in mortality, becoming the leading cause of death.

Cancer is a collection of diseases that have the common trait of uncontrollable cell growth. Cancer is an intricate disease because, at a developmental stage, cancer cells very closely resemble features of a healthy cell, which makes it difficult for the immune system to eradicate them.

A. STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

Tumors use multiple strategies to escape immune surveillance and thus hamper cancer immunotherapies. Even when immune responses are generated with tumor vaccines, the anti-tumor therapeutic outcome is often not feasible due to tumor-mediated immune suppression. These inhibitory mechanisms involve co-inhibitory receptor-ligand interactions, such as PD-1/PD-L1, secretion of inhibitory molecules, such as TGFβ, IL-

10, indoleamine-(2,3)-dioxygenase (IDO), and recruitment of suppressive cells, such as regulatory T cells (Tregs), and myeloid derived suppressor cells (MDSCs).

We and others have previously demonstrated that successful immunotherapy requires simultaneous targeting of both effector and suppressor arms of the immune system (Curran, Montalvo, Yagita, & Allison, 2010; M. Mkrtichyan et al., 2011; M. Mkrtichyan et al., 2012; N. C. Mkrtichyan, Rasha Abu Eid, Anu Wallecha, Reshma Singh, John Rothman and Samir N Khleif 2013) to overcome immune inhibition by tumors.

Thus, we hypothesized that an effective cancer immunotherapy will modulate the patient's immune system to attack cancer by recognizing the tumor, enhancing immunity, and neutralizing tumor immune-suppressive mechanisms. Accordingly, in this project we evaluated the therapeutic and immune potency of anti-tumor immunotherapy based on anti-OX40 antibody (to enhance the effector arm) and 1-methyl-tryptophan (1-MT), IDO inhibitor, (to inhibit the suppressive arm), and vaccine.

- OX40 (CD134) is a tumor necrosis factor receptor (TNFR) superfamily co-stimulatory molecule (Mallett & Barclay, 1991), inducible on activated $CD4^+$ and $CD8^+$ T cells (Croft, 2010; Gramaglia et al., 2000; Gramaglia, Weinberg, Lemon, & Croft, 1998; Mallett, Fossum, & Barclay, 1990; Rogers, Song, Gramaglia, Killeen, & Croft, 2001; Sleckman et al., 1987). Targeting OX40 with agonistic antibody leads to T cell expansion/proliferation, and an increase in effector function, survival and memory development (Biagi et al., 2005; Curti et al., 2013;

Gough et al., 2008; Humphreys et al., 2007; Piconese, Valzasina, & Colombo, 2008; Sugamura, Ishii, & Weinberg, 2004; Weinberg et al., 2000).

- IDO, which is produced mainly by the tumor cells, MDSCs, macrophages and dendritic cells (DCs) (Munn et al., 2004; Uyttenhove et al., 2003), is shown to mediate suppressive effects directly on effector T cells and activate Tregs (Munn & Mellor, 2004, 2007; Munn et al., 2002). Inhibition of IDO with 1MT has been shown to result in an increase of T cell-dependent antitumor responses in murine models (Friberg et al., 2002; Muller, Malachowski, & Prendergast, 2005; Uyttenhove et al., 2003).

Before approaching combinational immunotherapy, it was imperative to optimize the dose for vaccine and anti-OX40 antibody therapy that would provide an efficacious anti-tumor immune response resulting in a positive therapeutic and immune outcome. Therefore to address this, we present the following aim:

Specific aim 1: ***To determine the optimum dose for anti-OX40 antibody (Ab) treatment with vaccine to enhance the effector immune response, resulting in slower tumor progression in an animal model.***

Surprisingly, we found that the therapeutic efficacy for this combination is not linearly dose-dependent. Given these findings, we hypothesized that different doses of anti-OX40 Ab differently affects T cell subsets, which impacts anti-tumor therapeutic

efficacy. Dissection of mechanisms responsible for this differential effect is crucial for future generation of anti-OX40-based combinational strategies and for successful translation of the treatment into clinical studies.

Specific aim 2: ***To determine the immune response mechanisms responsible for differential effect of various doses of anti-OX40 Ab on therapeutic efficacy.***

Therapy involving anti-OX40 Ab and vaccine effectively stimulates the effector arm of the immunity. However, to combat the immunosuppressive existence in the tumor environment, we hypothesized that targeting the suppressive arm of immunity by IDO inhibitor, while stimulating the immune system with anti-OX40 Ab, would lead to synergistic anti-tumor immune response outcome.

Specific aim 3: ***To determine if inhibiting IDO in the tumor micro-environment would enhance the therapeutic efficacy when added to the vaccine with anti-OX40 Ab, and to determine the immune mechanism of such enhanced effect.***

B. IMMUNOEDITING

In 2004, Shreiber, Old, and Smyth presented a novel idea, immunoediting, which proposes that the immune system interacts with cancer in ways that can both eliminate and promote its growth or survival (Schreiber, Old, & Smyth, 2011).

Cancer immunoediting is the capacity of the immune system to eradicate transformed cells expressing antigens that strongly differentiate from healthy cells. However, this process of *elimination* leads to a survival of the fittest, where sporadic transformed cells that have not been eliminated by the immune system, enter a dormancy phase, *equilibrium*, where their rate of proliferation is equaled by their rate of eradication by the immune system. However, under the editing influence of the immune system, the cells undergo additional mutational changes or adaptations that facilitate an *escape* from the immune system. In this escape phase, the growth of immunologically unrecognized tumors promote an immunosuppressive environment, which further protects the cancer from immunological assault (de Visser, Eichten, & Coussens, 2006; Schreiber et al., 2011; Smyth, Godfrey, & Trapani, 2001).

Elimination

In the elimination phase, the host's immune cells recognize and destroy premalignant cells before they become tumor cells; it keeps the host at a normal healthy stage. This stage is the hallmark of immunosurveillance (Vesely, Kershaw, Schreiber, & Smyth, 2011). When premalignant cells occur, first, the immune cells of the innate lineage are recruited to the tissue site in response to pro-inflammatory cytokines (IL-12, IFN-γ) that are generated by the premalignant cells, macrophages, and stromal cells (Gasser, Orsulic, Brown, & Raulet, 2005; Kang et al., 2011). Tumor-associated antigens

(TAAs) are engulfed, processed and presented by DCs. Activated DCs migrate from the periphery to a local draining lymph node for TAA presentation to the adaptive immune cells, through the expression of MHC-I and T cell receptor on CD8 T cells (cytotoxic T cells, CTLs, also known as killer Tcells)(Shalapour & Karin, 2015). This leads to a stimulation of antigen-specific CD8 T cells and their clonal expansion for cancerous cell recognition and a direct attack on tumor cells. CD8 T cells perfom their cytotoxic function on tumor cells by secreting cytolytic factors, such as Perforins and Granzyme B, to eventually initiate apoptosis in the malignant cells (Jr, P, & M, 2001). The elimination is a set of processes, where the innate and adaptive immune systems orchestrate together to reject emerging cancer before it becomes clinically relevant. However, cancerous cells not only exhibit TAAs but non-mutated self antigens as well, which creates challenges for the host's immune system to detect them for eradication or retention (Mittal, Gubin, Schreiber, & Smyth, 2014).

Equilibrium

At this stage, the immune and tumor cells have entered a dynamic equilibrium, when the tumor growth is controlled, but not eliminated. Cancer is held in a dormant state, where the rate of proliferation of the tumor is equaled by the immunologic elimination of the tumor. In the equilibrium state, the tumor cells are more immunogenic

but as a result of immunoediting, they become less so and their rate of proliferation exceeds that of immune elimination (Teng, Swann, Koebel, Schreiber, & Smyth, 2008).

During this period, tumor cells gain genetic modifications and epigenetic alterations that heighten the oncogenic transcripts and anti-apoptotic factors, which promote proliferation and prolong the survival of the fittest. This resilient transformation against the immune stakeout generates a robust cancer cell that escapes the phase of equilibrium dormancy (Schreiber et al., 2011; Teng et al., 2008).

Escape

For cancer to progress and stay in this phase, it endures several intracellular distortions that shape the tumor mass into a phenotypically unrecognizable form and is indestructible by the host's immune system without intervention (Hanahan & Weinberg, 2000, 2011). This phase is the ultimate admission to malignancy. As a consequence, a cell that develops in a hostile immune environment gains the privilege to evolve immunosuppressive dynamics that will further support tumor oncogenesis. There are many mechanisms of immune suppression imposed by the tumor itself, called *tumor intrinsic immune mechanisms.* Also, the tumor environment can generate a vast number of immunosuppressive elements, such as the expression of *co-inhibitory molecules* on tumor or immune cells, or secretion of *soluble inhibitory factors*, which further results in a recruitment of *suppressive immune cells (Schreiber et al., 2011)*.

Tumor Immune Escape Mechanisms

i) Tumor Intrinsic Immunosuppression

One well-characterized mechanism is down-regulation of MHC class I molecule expression on malignant cells, which impairs the proper presentation of tumor antigens and allows the tumor to escape destruction by cytotoxic T cells (Bubenik, 2004). For example, mutations in the beta 2-microglobulin gene cause an absence of MHC-I expression, which prevents recognition by CTLs (Bicknell, Rowan, & Bodmer, 1994). Furthermore, many cancers display defects in their antigen processing machinery, decreasing their effectiveness in antigen presentation. A transporter-associated antigen processing (TAP) protein, either polypeptide TAP1 or TAP2, are necessary for proper peptide loading on newly synthesized MHC-I (DeMars et al., 1985). Defects in the TAP proteins decrease the ability to induce tumor-specific immunity in fibrosarcoma (Kuroda et al., 1995). These tumor intrinsic immunosuppressive mechanisms crafted by the tumor are just one part of a strategy that protects it from immune recognition.

ii) Immune Co-inhibitory Molecules

Immune cells express a repertoire of cell surface molecules, which can either stimulate or suppress the immune system when engaged. The tumor-mediated immunosuppressive mechanisms involve co-inhibitory receptor-ligand interactions expressed predominantly on T cells that have the capacity to put a “break” on the immune response. One of the best characterized immunological checkpoints is cytotoxic

T lymphocyte antigen receptor (CTLA-4), which is constitutively expressed on T lymphocytes (Leach, Krummel, & Allison, 1996). When B7 (B7-1 and B7-2) membrane proteins found on activated antigen presenting cells (APCs) are bound to cluster of differentiation 28 (CD28) on T cells, it results in a co-stimulatory immune effect; however, this is negated by CTLA-4 signaling due to its higher binding affinity for B7 (Alegre, Frauwirth, & Thompson, 2001). Numerous pre-clinical studies showed combinatorial anti-cancer therapies, where the incorporation of blocking CTLA-4 led to a partial or complete tumor regression. In 2011, ipilimumab (anti-CTLA-4 Ab) was approved to treat late-stage melanoma (Callahan, Flaherty, & Postow, 2016). Another co-inhibitory receptor on T cells, which reached the forefronts in clinical trials, is program cell death protein-1 (PD-1) (Freeman et al., 2000; Pardoll, 2012; Topalian, Drake, & Pardoll, 2012). Similar to CTLA-4 receptor, engagement of PD-1 receptor with its cognate ligands (PD-L1 and PD-L2), that are predominantly expressed on APCs, results in most cases in inhibition of T cell activation, and with persistent signaling can eventually lead to T cell apoptotic death (Freeman et al., 2000). Blocking the axis of PD-1 and PD-L1 interaction has therapeutic benefits in many tumor models (Voena & Chiarle, 2016). Molecules that block this pathway, nivolumab and pembrolizumab, are approved by the FDA for the treatment of melanoma, lung and kidney cancer, with many more approvals likely to come in other disease entities in the coming months and years.

iii) Soluble Factors

In addition to suppressive cell-to-cell contact through co-inhibitory receptors and ligands in the tumor microenvironment, immune soluble molecules are exerting suppressive mechanisms on effector immune cells preventing them from attacking tumor.

Transforming growth factor beta (TGF Beta) and IL-10 are among the best known cytokines important in regulation of the immune system, and their secretion is mostly associated, but not restricted to, suppressive regulatory Tcells (Tregs) (Morisaki et al., 1996; Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009; Thomas & Massague, 2005). Other suppressive soluble molecules, such as the inflammatory cytokine, granulocyte macrophage colony-stimulating factors (GM-CSF), angiogenic chemokine vascular endothelial growth factor (VEGF), and indoleamine 2,3-dioxygenase (IDO) enzyme are elevated in cancers and secreted by various tumors and immune cells. Expression of GM-CSF in the tumor microenvironment can lead to differentiation of immature DCs (iDCs) into MDSCs)(Morales, Kmieciak, Knutson, Bear, & Manjili, 2010). IDO is shown to mediate suppressive effects directly on effector T- cells by tryptophan degradation and increased levels of toxic kynurenine catabolite, which inhibits T cell activation (Munn & Mellor, 2004, 2007; Munn et al., 2002). Targeting suppressive cytokines in immunotherapy has been shown to result in an increase of T cell-dependent antitumor responses in murine models (Friberg et al., 2002; Muller et al., 2005; Uyttenhove et al., 2003). In general, there are many types of cancers that show the expression of immunosuppressive cytokines, which further facilitates the recruitment of

suppressive immune cells that promote tumor progression (Ikubo et al., 1995; Morisaki et al., 1996).

iv) Suppressive Immune Cells

Expansion, recruitment and activation of regulatory Tregs are highly correlated with tumor invasiveness due to an induction of immunologic tolerance by preventing exuberant immune system activation. Tregs are generally phenotypically $CD4^+CD25^+$ positive and express forkhead/winged helix transcription factor protein 3 (FoxP3) that specifies the Treg cell lineage and is required for their sustained suppressive function (Sakaguchi et al., 2009). To revert the effect of Tregs on tumor growth, blocking the interleukin-2 receptor alpha (CD25) needed for Treg survival with anti-CD25 antibody showed an anti-tumor effect that in some instances led to tumor rejection in leukemia, myeloma, and sarcoma (Onizuka et al., 1999). Tregs exhibit their suppressive function both by secreting suppressive cytokines, such as IL-10 and TGF-beta, or by a direct cell-to-cell contact via CTLA-4 receptor (Mills, 2004). A specific subset of human $CD14^+CTLA4^+$ regulatory DCs in patients with hepatocellular carcinoma secrete high levels of suppressive IL-10 and IDO molecules, which are responsible for the tolerogenic activity of these regulatory DCs (Han et al., 2014). DCs develop in the bone marrow from hematopoietic progenitor cells (HPCs). DCs play an important role in antigen presentation, and their proper effector function is required for an effective anti-tumor response. However, in patients with cancer, DCs express low levels of the co-stimulatory

molecules CD80 and CD86 necessary for immune activation, and in some cases, such as in metastatic melanoma in humans, DCs do not express CD86 at all (W. Zou et al., 2001). These DCs, which induce T cell anergy or tolerance by the lack of co-stimulatory signals, are not fully developed and are known as immature dendritic cells (iDCs). Almost all patients and animal models with cancer reveal high levels of circulating MDSCs in the blood or residing in the tumor environment (Morales et al., 2010). Two subtypes of MDSCs, granulocytic and monocytic, produce reactive oxygen species (ROS), nitric oxide (NO), and arginase-1 (ARG-1) molecules, which impair T cells from demonstrating proper anti-tumor function (Gabrilovich & Nagaraj, 2009).

In summary, all these tumor intrinsic, co-inhibitory, soluble, and cellular immunosuppressive factors coordinate together to prevent tumors from immune elimination and, at the same time, facilitating tumor progression. Once a tumor is left in an immune hostile environment, eventually at a later cancer progression, immunosuppressive factors outnumber the effector immune response. Under immunosuppressive stress, the effector anti-cancer immune cells, such as cytotoxic CD8 T cells, lose their ability to perform effective anti-tumor lytic function, or become anergic or undergo apoptosis due to improper activation or imposed suppression by regulatory immune factors (Rabinovich, Gabrilovich, & Sotomayor, 2007; Schreiber et al., 2011). Similarly, helper T conventional CD4 cells (CD4 Tconvs, $CD4^{+}FoxP3^{-}$) may secrete effector cytokines for CD8 T cell expansion; however, in the tumor environment, they make become defective or outgrown by regulatory T cells ($CD4^{+}FoxP3^{+}$). The

immunological relevant ratio of effector CD8 Tcells to Tregs predicts the clinical outcome in cancer development in many tumor models and is under investigation in patients with cancer. Not only a decreased ratio of CD8/Treg cells, but also lower functionality of CD8 T cells, is associated with cancer progression (Hadrup, Donia, & Thor Straten, 2013).

In this study, we strategized to harness an effective anti-tumor specific immune response to abrogate the immunosuppressive barriers in cancer in order to deliver positive therapeutic outcome.

C. STRATEGIES TO HARNESS ANTI-TUMOR IMMUNE RESPONSE

An extensive number of studies have provided evidence showing that tumors prosper in an immunologically suppressive environment, which overpowers the effective anti-tumor immune response (Rabinovich et al., 2007).

Cancer immunotherapy is a treatment that acts by harnessing and modulating the immune system to recognize and fight cancer. However, as described above, the tumor imposes numerous immunosuppressive mechanisms, for which cancer immunotherapy with a single agent is put to a challenge. The types of immunotherapy that can utilize anti-tumor immune responses can be achieved by i) stimulating the effector arm of immunity, such as antigen-specific therapy (e.g., vaccines, adoptive cell transfer), stimulatory cytokines (e.g., IL2, IL-12, IL-15), and immune co-stimulatory receptors

(e.g., OX40, GITR, 4-1BB), and by ii) targeting the suppressive arm of immunity, such as blocking immune inhibitory soluble (e.g., IDO, TGFbeta) and cellular (e.g., Tregs, MDSCs) factors, and co-inhibitory receptors (e.g., PD-1, CTLA-4).

The broad goal of my research was to find an immune-based anti-cancer combinational therapy strategy to cultivate an effective anti-tumor immune response that will provide a strong enough stimulus to override the immunosuppressive environment in the tumor microenvironment, resulting in a tumor free outcome.

We hypothesized that the strategy for a successful anti-cancer immunotherapy consists of three components that will i) educate effector cells with the tumor antigen, ii) overcome the suppressive environment, and iii) become functionally strong enough to eradicate the tormented malignancy. In this research, the above strategies were achieved by combining an anti-cancer therapeutic vaccine with the immune-stimulating agonist anti-OX40 Ab, and an IDO inhibitor to partially reverse the immune inhibitory environment of the tumor. Here, I show a novel anti-cancer combinational therapy approach that resulted in an increase of functional effector CD8 T cells infiltrating a tumor that led to prolonged survival, slower tumor progression, and, in some animals, led to a complete eradication of established tumors in mice.

Vaccine therapy is an efficient method to educate T cells with tumor antigens; however, multiple suppressive mechanisms within the tumor microenvironment cause deterioration of the effector immune response (Drake, 2012). Thus, mitigating the

immunosuppressive environment and at the same time augmenting vaccine-induced antigen-specific T cells could improve the overall potency of cancer immunotherapy.

Enhancing the number of tumor-specific effector T cells within the tumor environment is an important factor to achieve a more favorable clinical outcome (Carretero et al., 2015; Naito et al., 1998; Romano et al., 2015). Therefore to generate a strong and long-lasting anti-cancer immune response upon antigen recognition, an engagement of secondary co-stimulatory receptors are often implemented in combinational immune therapeutic studies (Almasbak et al., 2015; Burns et al., 2010; Chacon et al., 2013; Hombach & Abken, 2013; Hombach, Heiders, Foppe, Chmielewski, & Abken, 2012).

D. IMMUNE CO-STIMULATORY OX40 RECEPTOR

Introduction

OX40 (also known as CD134, ACT35, TNFRSF4) is a member of the tumor necrosis factor receptor (TNFR) superfamily and is relatively well characterized among co-stimulatory molecules (Mallett & Barclay, 1991). The OX40 receptor is inducible on activated T cells (Croft, 2010; Gramaglia et al., 2000; Gramaglia et al., 1998; Mallett et al., 1990; Sleckman et al., 1987), and its engagement with cognate ligand or agonistic antibody enhances T cell proliferation, cytokine production, survival, and memory development (Rogers et al., 2001; Sugamura et al., 2004). In animal models, treatment with anti-OX40 Ab has shown favorable anti-tumor activities (Biagi et al., 2005; Curti et

al., 2013; Gough et al., 2008; Humphreys et al., 2007; Piconese et al., 2008; Weinberg et al., 2000). This eventually led to the initiation of the OX40 co-stimulatory molecule to be tested in multiple ongoing clinical trials in cancer ("www.clinicaltrials.gov," 2015).

OX40 Structure

In 1987, Alan Williams generated several monoclonal antibodies that bound to rat CD4 T lymphocytes and one of them (MRC OX40) detected cell surface protein, which led to the discovery of OX40 receptor (Paterson et al., 1987). The 50-kD glycoprotein OX40 is a type 1 transmembrane protein, extending 277 amino acids, with a 186 amino acid extracellular region and 49 amino acid cytoplasmic tail comprising of three complete and one truncated cysteine-rich domains, which are characteristics of the TNFR family (Mallett et al., 1990). In humans, the OX40 gene is mapped to chromosome band 1p36, and in mice, it is located on chromosome 4. The cognate OX40 ligand (OX40L, CD252) is clustered on both human and mouse chromosome 1 with other TNF family members, such as FasL and GITRL (Latza et al., 1994). The crystal structure of OX40 receptor depicts three monomers that bind into grooves of its associated ligand OX40 homotrimer (Compaan & Hymowitz, 2006; Croft, 2010). The complex of OX40 receptor and OX40 ligand has a similar size of other interacting immune co-stimulatory molecules, such as CD28 receptor binding to B7 proteins that mediate T cell receptor (TCR) and APC activity, resembling the similarity of OX40 cellular functions (Gramaglia et al., 1998).

OX40 Expression

OX40 is predominantly expressed on activated T cells (CD8, CD4, Th2, Th1, Th17 and $CD4^{+}FoxP3^{+}$ cells) and was detected on polymorphonuclear (PMN), natural killer (NK), natural killer T cells (NKT) and DCs (34,35). It is not expressed on naïve Tcells, nor on central or effector memory T cells, but can be expressed promptly after antigen re-challenge (Zaini et al., 2007). The expression on T cells can be visualized after 12 hours of antigen encounter, peaking at 24-48 hours, and lasting as long as 6 days (Jensen et al., 2010). Stimulating TCR/CD3 complex is enough for OX40 expression, which can be amplified or prolonged with second stimulatory signals, such as CD28, or the cytokines IL-1, IL-2, IL-4, and TNF (Jensen et al., 2010). In general, OX40 expression is sustained on immune cells at sites of inflammation, such as cancer, and is barely detected in the periphery, which resembles the OX40 receptor expression induction by antigen (Ag)-stimulated T cells (Croft, 2010). OX40 receptor signaling can be triggered only with OX40 ligand (OX40L). The expression of OX40L is induced on activated or matured APCs (including B cells, DCs, and macrophages). OX40L has also been detected on NK cells, mast cells, activated T cells, human airway smooth muscle, and vascular endothelial cells (Jensen et al., 2010). Because OX40 receptor and its ligand are expressed on activated immune cells and not naïve cells, it ensures that OX40-dependent stimulation occurs at sites of inflammation, which limits the potential to develop treatment side effects.

Additionally, it was suggested that OX40 expression could be used as a marker for self-antigen-specific T cells in autoimmune encephalomyelitis (EAE) because sequencing analysis of TCRs belonging to auto-reactive T cells induced with myelin-basic protein (MBP)-specific Lewis rat EAE revealed that the receptor of $OX40^+$ T cells was predominantly specific for the MBP compared to OX40- T cells (K. Murata et al., 2002). This further implies that OX40 expression is associated with antigen-specific T cells.

OX40 Prolongs T cell Survival by Suppressing Apoptosis

When T cells switch from the naïve to active state, a cascade of intracellular signaling is initiated upon TCR priming by an antigen bound to MHC complex and along with the help of a secondary co-stimulatory signal between CD28 and B7 molecules. The well characterized, CD28 co-stimulatory signal promotes clonal expansion and enhances survival by increasing the expression of anti-apoptotic proteins, such as Bcl-xL (Wells, Gudmundsdottir, & Turka, 1997). However, apoptosis of T cells follows shortly after antigen recognition (Rogers et al., 2001; Wells et al., 1997).

In comparison to constitutively expressed co-stimulatory molecule CD28 on T cells, the co-stimulatory OX40 receptor is inducible right after antigen priming, and is considered to extend the survival of CD4 T cells (Gramaglia et al., 1998).Triggering the OX40 receptor of IL-4 primed (Th1-like) and IFN-γ primed (Th2-like) naïve effector T cells significantly promotes sustained proliferation over an extended time of at least 90

hrs, whereas stimulating T cells with the co-stimulatory molecule B7 showed a dramatic drop in proliferation at 66 hrs, which was comparable to T cells lacking OX40 co-stimulatory molecule (Gramaglia et al., 1998).

The molecular mechanism responsible for the increased CD4 T cell survival is due to an induction of the anti-apoptotic molecules, Bcl-2 and Bcl-xL, which are essential for long-lasting immunity. OX40 or OX40L knockout mice show decreased T cell division and survival (Gramaglia et al., 2000) It is reported that OX40-/- T cells fail to retain high levels of Bcl-xL and Bcl-2 proteins, which leads to apoptotic fate observed by increased levels of annexin V when compared to wild type. Retroviral transduction of Bcl-xL or Bcl-2 or inhibition of caspase reverted the defective survival effect of OX40-/- T cells (Stuber, Neurath, Calderhead, Fell, & Strober, 1995). Tumor necrosis factor receptor-associated factors (TRAFs) are essential OX40 receptor adaptor proteins required for downstream NF-κB signaling pathway activation, which is necessary for survival. Also, the PI3K/AKT signaling pathway plays an important role in OX40-mediated survival, where OX40 stimulation recruits and activates AKT leading to expression of anti-apoptotic proteins in CD4 T cells (Kawamata, Hori, Imura, Takaori-Kondo, & Uchiyama, 1998). Sustained protein kinase B (PKB) activation maintains survivin expression. The lack of OX40 in CD4 T cells results in decreased survivin expression, which is important for *in vivo* antigen-driven clonal expansion (J. Song, So, Cheng, Tang, & Croft, 2005). Early studies assumed that the OX40/OX40L interaction did not have any impact on active CD8 T cells because OX40L transgenic animals

showed CD4 T cell accumulation (Brocker et al., 1999; K. Murata et al., 2002). However, other more probing studies show that the interaction between OX40 and its ligand is crucial for CD8 T cell immunity as well. Investigators demonstrated that OX40-deficient CD8 T cells lack the ability to expand in number. Furthermore, CD8 T cells stained positive for annexin V after blocking the OX40/OX40 L interaction, implying that the involvement of OX40 triggering is important for CD8 T cell survival as well as CD4 T cells (Pakala, Bansal-Pakala, Halteman, & Croft, 2004). Moreover, CD4 T cell-depleted mice with established tumors accumulated high levels of CD8 T cells after agonist anti-OX40 antibody treatment, demonstrating that OX40 stimulation on CD8 T cells can be CD4-independent (Pakala et al., 2004). Just as in CD4 T cells, adoptively transferred OX40-/- CD8 effector T cells experienced defective survival due to decreased expression of the Bcl-xL gene and positive staining for annexin V in lymph node, spleen, and peritoneal cavity. Moreover, Bcl-xL restores the impaired survival of OX40-deficient CD8 T cells, resulting in decreased tumor burden and reduced mortality of tumor-bearing mice after adoptive T cell therapy with transduced Bcl-xL OX40-/- CD8 T cells (Pakala et al., 2004).

OX40 Generates Long-Lived Memory T cells

As mentioned earlier, the ligation of co-stimulatory molecule CD28 on T cells during TCR priming *in vivo* leads to significant clonal expansion; however, apoptosis of T cells shortly follows. It was hypothesized that another signaling trigger is necessary to

promote long-lasting memory T cells. It is now emerging that the interaction between OX40 and OX40L is a necessity for T cell memory. As mentioned earlier, anti-OX40 antibody prolongs the survival of effector T cells after Ag-specific stimulation. It was shown that OX40-mediated signaling inhibited activation-induced cell death (AICD) of Ag-induced T cells and led to the accumulation of long-lived CD4 T cells, which attained memory cell fate *in vivo* (Valzasina et al., 2005). Injection of a superantigen followed by administration of agonist anti-OX40 antibody produced a 10-fold increase in cell survival, with an accumulation of memory T cells (Maxwell, Weinberg, Prell, & Vella, 2000). Furthermore, delivery of anti-OX40 antibody together with vaccinia virus (VACV) peptides strongly promoted immune protection by increasing the memory CD8 T cell pool residing in lung mucosal tissue for an immediate response against virus infection (Salek-Ardakani, Moutaftsi, Sette, & Croft, 2011). One of the vital molecules for CD8 memory is c-Myc, which expression is dramatically reduced in OX40-/- and CD28-/- memory CD8 T cells when compared to their wild-type counterpart. Overexpression of c-Myc in these knockout T cells reversed the developmental impairment of memory CD8 T cells. It is implied that the increase of c-Myc-mediated memory T cells is facilitated by the increase of cell number via CD28 stimulation and by the inhibition of cell death by OX40 trigger (Haque et al., 2016). The maintenance of long-lived Ag-specific T cells driven by OX40 stimulation proves that OX40 has the potential to cultivate the properties for the enhancement of vaccine efficacy.

OX40 Mediates T cell Differentiation and Function

In addition to OX40-mediated enhancement on T cell survival and memory, an engagement of OX40 receptor augments cytokines that promote an effector function or differentiation of T cells. It was demonstrated that anti-OX40 Ab has the ability to enhance IL-2, IL-4, IL-5, IL-13, and IFN-γ secretion by effector-secreting Th1 and Th2 cells in an Ag dose-dependent manner. While a small dose of 0.01uM of T102S peptide in the presence of anti-OX40 Ab accumulated IL-5 and IL-13 cytokines (Th2 phenotype), a higher dose of 1-100uM gradually increased IFN-γ cytokine (Th1 phenotype). Interestingly, IL-2 secretion by anti-OX40 Ab was increased at all doses (Rogers & Croft, 2000). From this study it was suggested that OX40 signaling augments the already existing T cell response in the environment rather than shifting their differentiation lineage (Rogers & Croft, 2000). Furthermore, it was supported that stimulating naïve human CD4 T cells with anti-OX40 Ab or OX40L expressing cells significantly increased the production of IL-4 at priming, which caused differentiation of naïve T cells into high IL-4-secreting Th2 T cells (Ohshima et al., 1998).

The production of IL-4 and IL-13 was abrogated in muMT model mice (B cell-deficient mice). The defective Th2 development of adoptively transferred OT-II Thy 1.1 cell can be restored by B cell injections into the mice at time of priming; however, IL-4 and IL-13 secretion was not restored if adoptively transferred B cells were OX40 ligand deficient, thus, suggesting that the impairment of OX40 ligand in B cells is sufficient to limit Th2 development *in vivo* (Linton et al., 2003). Moreover, CDCR5, a chemokine that

promotes maturation of B cells in the germinal center, is up-regulated upon the interaction of OX40 and its ligand of CD4 T cells at time of priming (Chevalier et al., 2011). This shows an imperative role for T cell and B cell contact, where OX40 ligation may assist a proper recruitment of activated T cells to sites of inflammation.

Stimulating OX40 also has an impact on effector CD8 T cells. Treatment with anti-OX40 Ab resulted in a significant up-regulation of IL-2 receptor (CD25) and GranzymeB (GrzB) in adoptively transferred OT-I CD8 T cells on a per cell basis. This enhancement was evident for either OX40-positive or deficient transferred cells, suggesting that engaging OX40 receptor imposes a direct and indirect effect on CD8 T cells. Furthermore, the investigators evaluated the CD8 T cells' cytolytic effect on peptide-pulsed target cells, and found that anti-OX40 antibody remarkably improved the cytotoxic lytic CD8 T cell function (Redmond, Gough, Charbonneau, Ratliff, & Weinberg, 2007). An interesting study demonstrated that OX40 agonist can reverse anergic CD8 T cells into responsive cells *in vivo*. Investigators revealed that anergic CD8 T cells could undergo expansion and gain cytolytic effector function upon anti-OX40 treatment, which led to a prolonged survival of tumor-bearing mice (Redmond, Gough, & Weinberg, 2009). It is evident that engaging co-stimulatory OX40 molecules can deliver effector properties that are imperative for anti-cancer treatment.

The Effect of OX40 Stimulation on Regulatory T cells

It is well established that OX40 ligation on antigen-engaged effector CD8 and CD4 T cells enhances their survival, memory, differentiation, and function, which are necessary traits to effectively eradicate a tumor. One of the major impediments in immunotherapy is the increased numbers of suppressive regulatory T cells (Tregs) in the cancer environment. OX40 stimulation also has an effect on Treg proliferation and function; however, the results are somewhat contradictory. Tregs are suppressive immune T cells that maintain tolerance to self-antigens by dampening over-stimulated immune responses that may cause tissue damage (Sakaguchi et al., 2009). Tregs are phenotypically defined by the expression of transcription factor FoxP3, which is needed for their stability (Sojka, Huang, & Fowell, 2008). There are two types of Tregs; naturally occurring Tregs that develop in the thymus and induced Tregs (iTregs) that are converted from effector Tconv cells in the periphery under the influence of TGF-β cytokine (Sakaguchi et al., 2009). OX40 is dispensable in natural Treg (nTreg) development because OX40-deficient mice have the same number of Tregs as do wild-type mice *in vivo,* and the suppressive ability of OX40 -/- and wild-type Tregs on effector T cells is similar *in vitro (Jensen et al., 2010)*. However, it was reported that OX40 costimulation in the presence of TGF-β prevents induction of Tregs from effector T cells by down-regulating FoxP3 expression, which ameliorates their suppressive function on Tconv cells (So & Croft, 2007; Vu et al., 2007). On the other hand, genetically modified APCs expressing OX40L increased the human cord blood Treg population without changing

their suppressive ability. Additionally, these expanded Tregs protected from graft-versus-host disease (GVHD) *in vitro* and *in vivo* (Hippen et al., 2008). Studies suggest that the correlation between OX40 receptor and Treg populations may be regulated by their environmental cytokine milieu. Treg differentiation was inhibited in response to antigen stimulation by the Th1/Th2-polarizing cytokines, IL-12, IFN-γ, and IL-4, while blockade of IFN-γ and IL-4 induced the Treg population from Tconv cells, in the presence of low levels of TGF-β *in vitro* and *in vivo* (Wei et al., 2007). Further it was demonstrated that stimulating OX40 on CD4 Tconv cells led to an increase of IFN-γ and IL-4 secretion and that inhibition of Treg expansion was dependent either on IFN-γ and IL-4 presence or OX40 blockade by antagonist OX40L Ab or by lack of OX40 on APCs (Duan, So, & Croft, 2008). Investigators who studied OX40 importance in an experimental autoimmune encephalomyelitis (EAE) model demonstrated that when agonist anti-OX40 treatment was provided at the time of antigen priming, it resulted in Treg expansion while Tregs contracted when anti-OX40 was given at disease progression. Thus EAE was exacerbated when anti-OX40 Ab was administered at time of Ag priming, while ameliorated when treated at time of disease onset. It was evident that anti-OX40 Ab has an impact on Tregs, but the degree of the impact is possibly based on the local cytokine milieu at the time of anti-OX40 stimulation (Ruby et al., 2009). It was further supported that OX40 directly acts on the expansion of Tregs by using wild-type or OX40 -/- Treg adoptive cell transfer into an OX40-depleted mouse model. However, this resulted in poorly suppressive Treg expansion due to the limited amount of essential IL-2 (Xiao et

al., 2012). Furthermore, the discrepancy of OX40 triggering on Tregs may be due to the affinity differences between soluble antibodies or cell-based OX40 ligand-transfected cells, or due to the timing of anti-OX40 Ab administration or the anti-OX40 Ab doses used. In light of these differences, more exploration into the mechanism of OX40 on CD4 T cells needs to be investigated.

OX40 Downstream Signaling

OX40 receptor is one of the many members of the TNFR superfamily. The TNFR family can be subdivided into three groups based on their receptor intracellular sequence. The first group of TNF receptors, such as death receptor (DR3), Fas and TRAIL, contain a death domain (DD) in their cytoplasmic tail. Upon receptor activation, adaptors such as FAS-associated death domain and TNFR-associated domain are recruited to the DD, which initiates apoptosis through caspase signaling. The second group of TNF receptors includes OX40, CD30, CD40, and 4-1BB, which lack the DD but contain TRAF-interacting motifs (TIMs). Upon ligation of these receptors, TRAF adaptor-associated proteins are recruited to bind TIMs on the TNF receptor intracellular domain, which leads to an activation of downstream signal transduction important for T cell survival or death. The third, lesser-known group of receptors, such as TRAIL-R3 or decoy receptor 3 (DcR3), are considered to compete with the two other TNF receptors in order to protect cells from Fas ligand or TRAIL-induced apoptosis (Croft, 2003; Hehlgans & Pfeffer, 2005).

It was demonstrated that ligating OX40 causes an aggregation of multiple OX40 receptor trimers and migration into cholesterol- and sphingolipid-rich detergent-soluble membrane lipid microdomains (DIMs), sometimes called lipid rafts, which contain a signaling complex of TRAF2, PKB (also known as AKT), and the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) subunit p85. Additionally, it was shown that the recruitment of TRAF2 occurred before the OX40 lipid raft formation, and that TRAF2 was crucial for downstream OX40 signaling. Furthermore, TRAF3 and TRAF5 were shown to interact with OX40 receptor, although their correlation to OX40 is not well established (So, Choi, & Croft, 2011). Transgenic OX40-/- CD4 T cells initially divide normally and produce IL2, but ultimately do not survive (Rogers et al., 2001). It was demonstrated that OX40 -/- T cells have an impaired ability to recruit AKT, which is essential for T cell survival, proliferation, function, and memory generation (J. Song et al., 2004). Without AKT, T cells undergo apoptosis. It was further supported that OX40 -/- T cells had a lower expression of the anti-apoptotic proteins Bcl-xL, Bcl-2, and survivin. The absence of OX40 in T cells causing T cell impairment was reversed by constitutive expression of AKT (J. Song et al., 2004). Another study looking at the effect of OX40L transduced APCs on pAKT level in Tregs with and without IL-2 stimulation showed that OX4O ligation alone remarkably enhanced the pAKT level in Tregs when compared to IL-2 stimulation alone. Consequently IL-2 addition to OX40L transgenic APCs further increased pAKT level (Xiao et al., 2012). This suggests that OX40 signaling is AKT pathway dependent. Furthermore, the OX40-mediated recruitment of

PI3K and AKT was T cell receptor (TCR) or Ag recognition independent. Once OX40 was stimulated together with TCR trigger, the phosphorylation of AKT was increased when compared to Ag/TCR engagement only (So et al., 2011). Moreover, OX40 signaling involves activation of the NFkB pathway, which is also important for T cell survival, expansion, and functionality (Rowe et al., 2013).

OX40-Mediated Immunotherapy

Since OX40 signaling has the capacity to modulate the immune system by predominantly enhancing effector immune cells and cytokines, and under some conditions reduces suppressive Treg number or functionality, then OX40 may be a feasible target for anti-cancer immunotherapy. The anti-tumor activity of stimulating OX40 receptor was studied in a variety of murine tumor models such as breast, sarcoma, colon, glioma, melanoma, prostate, ovarian, hepatoma, leukemia, and lung tumor (Bulliard et al., 2014; Gray et al., 2008; Kjaergaard et al., 2000; S. Murata et al., 2006; Murphy et al., 2012; Redmond, Linch, & Kasiewicz, 2014). The effective anti-tumor activity of OX40 led to tumor eradication and prolonged survival. The molecules used to trigger OX40 signaling to treat cancer include agonist anti-OX40 antibodies, or OX40L-Fc proteins involving the Fc portion of immunoglobulin linked to two murine OX40L molecules.

i) Agonist Anti-OX40 Antibody as Anti-Cancer Monotherapy

The administration of anti-OX40 antibody (clone OX86) in sarcoma has led to 50% tumor regression, if the tumor burden was not too high, and if the anti-OX40 Ab dose (300ug) was high but administered at less frequency (2 doses) or was at a lower dose (150ug) but provided more frequently (4 doses). The therapy generated tumor-specific immune memory, as evident by tumor re-challenge. Depletion of CD4 or CD8 T cells abrogated the anti-cancer therapeutic efficacy (Kjaergaard et al., 2000). Another study suggests that antibody dependent cell cytotoxicity (ADCC) may be needed to deplete intratumoral Tregs by activating Fc gamma receptors (FcγRs). In this study, the Fc portion of anti-OX40 antibody, when bound to OX40 receptor on Tregs, was recognized by FcγR expressed on natural killer (NK) or myeloid cells, which targeted Tregs for destruction. This ADCC effect eliminated colon (CT26) tumors in mice when treated with one dose of 15mg/kg anti-OX40 Ab (Bulliard et al., 2014). Treg depletion by anti-OX40 Ab in the same tumor colon model was also observed by another group of investigators who demonstrated that OX40-induced tumor regression is dependent on the migration of DCs into the tumor microenvironment, which was shown by CCR7 knockout mice restricting DCs from entering lymph nodes upon CCL21 stimulus (Ruby & Weinberg, 2009b). (81). An interesting study exhibited that the anti-OX40 Ab effect is age-related. Tumor (sarcoma MCA205)-bearing mice that were young (2 and 6 months old) benefited from anti-OX40 Ab treatment, showing 80% survival, while older mice (12 and 20 months old) completely lost the therapeutic efficacy. This effect was reversed

by the addition of proinflammatory IL-12 cytokine (Ruby & Weinberg, 2009a, 2009b). Additionally anti-OX40 antibody monotherapy increased the number of effector CD8 T cells infiltrating the tumor (Ruby & Weinberg, 2009b).

Monotherapies may provide an anti-cancer response, but often times, developing tumors gain resistance against the therapy, causing tumor outgrowth. Pre-clinical studies involving monotherapies may provide the unique mechanism of action for each agent, which are an excellent source to develop strategies to combine them with complimentary therapeutic molecules. Adequately chosen combinational immunotherapy will deliver a synergistic anti-cancer response that will strengthen the scope to overcome tumor burden.

ii) Anti-OX40 in Anti-Cancer Combinational Immunotherapy

Numerous therapeutic approaches have involved targeting OX40 receptor combined with therapeutic vaccine, adoptive cell transfer, immune based soluble factors, other immune checkpoint receptors, and current conventional treatments (radiation, chemotherapy, surgery, chemotherapy). Patients treated with therapeutic vaccine have shown an immunologic response but it was not enough to provide a curative anti-cancer outcome. A combination of therapeutic vaccine with targeting OX40 receptor led to more potent tumor specific immunotherapy. It was shown that a GM-CSF-based vaccine provided synergy by increasing the OX40 expression on T cells. Thus, the co-existing synergy between the vaccine and anti-OX40 Ab led to an increase of antigen-specific CD8 T cells in the NT2 mammary tumor model (S. Murata et al., 2006). Also, a

concomitant administration of tumor lysate vaccine with OX40L-Fc in glioma increased the proliferative ability of CD4 and CD8 T cells depicted by Ki67 and CD8 anti-tumor lytic function (Murphy et al., 2012). The strong co-stimulatory ability of OX40 receptor to expand effector T cells has been put to test, when anti-OX40 Ab treatment expanded adoptively transferred T cells in tumor-bearing mice, resulting in 40% complete tumor regression (A. Song, Song, Tang, & Croft, 2007). Soluble factors, such as providing IL12 cytokines, have improved OX40-based immunotherapy in older mice having CT26 established tumors (Ruby & Weinberg, 2009a, 2009b). As mentioned above, IL-4 was one of the cytokines that helped to eliminate the generation of induced Tregs in OX40-treated T cells. Surprisingly, blocking IL-4 cytokine together with agonist anti-OX40 Ab and antagonist anti-CTLA-4 Ab significantly enhanced survival of mice having TRAMP-prostate large tumors (Redmond et al., 2014). Moreover a triple combination therapy involving peptide-based vaccine together with two immune co-stimulatory molecules, anti-OX40 and anti-41BB Abs, significantly enhanced antigen-specific CD8 T cells, secreting IFN-γ when compared to control groups, which resulted in prolonged survival in 40% of mice injected with B16-F10 melanoma (Gray et al., 2008). In general, studies involving OX40-mediated immunotherapy have significant implications on enhancing T cell activity that result in a dramatic anti-tumor immunity, which initiated multiple currently ongoing clinical trials.

E. IDO

Introduction

It is now evident that tumors generate immunosuppressive factors in the tumor microenvironment that prevent them from immune recognition and elimination from the system. It is conceivable that disrupting the suppressive environment may provide an effective anti-tumor response, where the immune system properly identifies the tumor-associated antigens for rejection. It has been proven that tumors can escape immunosurveillance by acquiring immune tolerance. This was evident by a study demonstrating a transgenic tumor with strong antigens that should normally be eradicated but instead progressed, due to an immune hostile environment (Sotomayor et al., 2001). Moreover, transgenic human papillomavirus oncogene E7-specific CD8 T cells were rendered tolerant by an APC-dependent mechanism (Doan et al., 2000). The acquired tumor tolerance can defeat the purpose of immunizations targeting E7 oncoprotein when used as part of immunotherapy.

One of the immune molecules responsible for the tissue antigen to be accepted as self by the immune system is indoleamine 2,3-dioxygenase (IDO). The initial and well characterized mechanism mediated by IDO was demonstrated by Mellor, Munn and their colleagues, who showed that IDO is a catabolizing enzyme involved in the first and rate-limiting step of tryptophan degradation, resulting in the generation of kynurenine product (Mellor & Munn, 1999). An abundance of the amino acid tryptophan is essential for proper proliferation of T cells; therefore, the expression of IDO in the local environment

of T cells results in T cell arrest in the G1 phase of the cell cycle (Munn et al., 1999). It was argued that the purpose of IDO synthesis is to protect the mammalian fetus from T cell rejection (Mellor & Munn, 1999). This was supported by a study showing that tryptophan catabolism is indeed one of the mechanisms accountable for avoiding mouse allogeneic fetus rejection, which was reverted by pharmacological IDO inhibitor, 1-methyl-D-tryptophan (1-MT) (Munn et al., 1998). Due to IDO's ability to suppress the immune response of T cells, an interest was sparked to evaluate IDO in the tumor environment. Evidence of the presence of immunosuppressive IDO in progressing tumors initiated pre-clinical studies showing the benefits of IDO inhibition in tumor-bearing mice, which eventually resulted in the development of IDO inhibitors that are currently being tested in clinical trials.

Tryptophan Catalyzing Enzymes

Historically, before IDO was acknowledged to be responsible for tumor suppression, a significant increase of tryptophan (trp) catabolites was found to be present in the urine in patients affected by cancer (Ivanova, 1959; Rose, 1967; Wolf, Madsen, & Price, 1968).(Gailani, Murphy, Kenny, Nussbaum, & Silvernail, 1973) Four trp catabolic enzymes were recognized: tryptophan hydroxylase 1 (TPH1), which is associated with indoleamine neurotransmitter synthesis, such as serotonin and melatonin, mediating neuropsychiatric disorders; tryptophan 2,3-dioxygenase (TDO), and indoleamine 2,3-dioxygenases (IDO1 and IDO2). TDO is known to be the most prevalent tryptophan-

degrading enzyme. TDO expression is limited to liver, and while elevated trp levels in the tumor environment were interpreted as cancer progression, there were occasions that TDO presence in the tumor was not detected when the trp level was high (Gailani et al., 1973). This implies the existence of another tryptophan catabolic constituent in the tumor environment. IDO1 and IDO2 have a highly similar sequence homology (Ball et al., 2007; Metz et al., 2007). IDO2 was not strongly found to be expressed in many tumors, and its suppressive activity in the tumor environment has yet to be further established (van Baren & Van den Eynde, 2015). IDO1 is well established to be expressed by numerous tumors *in vitro* and *in vivo*. The overexpression of IDO1 in the tumor environment fosters malignant cell tolerance toward the immune system (Munn & Mellor, 2004). At the same time, it was verified that the inhibition of IDO1 by a pharmacological compound, 1-MT, can exert an anti-tumor effect (Hou et al., 2007; Koblish et al., 2010).

The Immunosuppressive Influence of IDO Activity in a Disease

The overexpression of IDO in a lung transplant prevented allograft rejection, while 1-MT promoted inflammatory tissue damage, depicted by an infiltration of lymphocytes into the lungs *in vivo* (Swanson, Zheng, Heidler, Mizobuchi, & Wilkes, 2004). IDO is involved in mitigating EAE, while pharmacological inhibition of IDO exacerbated the developed disease (Kwidzinski et al., 2005). However, it is important to mention that 1-MT in healthy mice does not induce inflammatory diseases nor do IDO-/-

mice develop symptoms of autoimmunity (Mellor et al., 2003; Uyttenhove et al., 2003). This proves that IDO inhibition is expected to have a limited amount of toxicity exhibiting autoimmune manifestation. Many studies have been devoted to IDO expression in tumors as was demonstrated by Uyttenhove, who identified many human IDO-positive tumors by immunohistochemistry staining. Additionally, an administration of 1-MT decreased the IDO-expressing tumor in mice, whereas concomitant depletion of CD4 and CD8 T cells eliminated the effect of 1-MT, leading to an interesting observation that tumors expressing IDO resist T cell-based immune rejection (Uyttenhove et al., 2003).

As mentioned above, IDO enzyme catabolizes tryptophan along the kynurenine pathway (Mellor & Munn, 1999). An insufficient profusion of tryptophan promotes T cell anergy due to their elevated level of the stress response kinase GCN2 in response to amino acid deficiency through a high abundance of uncharged tRNA (Munn & Mellor, 2007). It was further shown that toxic metabolites in the kynurenine pathway can lead to anergy or apoptosis of T cells and natural killer cells, and can cause an induction of Tregs (Fallarino et al., 2002; Frumento et al., 2002; Mellor et al., 2003).

IDO-Mediated Tolerance Inducing Mechanisms

There has been evidence of IDO expression in a specific subset of dendritic cells (DCs, a class of professional APCs). Upon antigen presentation, IDO-expressing DCs lead to T cell anergy or may cause T cell tolerance, rendering them to be unresponsive to

tumor antigens (Munn et al., 2004; Munn et al., 2002). It was demonstrated that ligation of CD80/86 on DCs with co-inhibitory receptor CTLA-4, which is constitutively expressed on Tregs, led to nuclear localization of transcription factor Foxo3 in DCs, and caused IDO expression up-regulation and enzymatic activity in mouse CD11c+ DCs, generating immunosuppressive DCs (Bronte, 2011; Dejean et al., 2009; Fallarino et al., 2003; Grohmann et al., 2002). IDO can be induced by a variety of stimuli; other cell surface proteins, such as CD40 and GITR have been shown to up-regulate IDO-induced trp catabolism and Treg expansion (Grohmann et al., 2007; Jenabian et al., 2014). Additionally, generation of trp metabolites bound to aryl hydrocarbon receptor (AhR) on Tregs led to Treg expansion (Mezrich et al., 2010). Furthermore, it is also well established that soluble factors can enhance the expression of IDO. One of the major known IDO enhancers is a T cell stimulatory cytokine, interferon gamma (IFN-γ). Administration of both type I or type II interferons (IFNs) to patients with cancer induced IDO enzyme, reduced the level of trp in sera and increased urinary metabolites of the kynurenine pathway (Brown et al., 1991; Taylor & Feng, 1991). IDO is expressed by many tumors, and one of the molecular mechanisms is a loss of the bridging integrator 1 (Bin1) gene, a tumor suppressor gene (Jia et al., 2015). IDO is induced by inflammatory stimuli, and its expression is not only restricted to some tumors and DCs but can also be identified in macrophages, eosinophils, epithelial and endothelial cells, and fibroblasts (Munn & Mellor, 2007).

Overall, while IDO exhibits immunosuppressive properties, inhibition of IDO with 1-MT as monotherapy in tumor-bearing mice has not been able to produce a strong enough immune response to eliminate cancer. However, combinational immunotherapies involving IDO have exhibited profound anti-cancer results that initiated studies of 1-MT in clinical trials (Holmgaard, Zamarin, Munn, Wolchok, & Allison, 2013; Moon, Hajjar, Hwu, & Naing, 2015; Spranger et al., 2014).

II. MATERIALS AND METHODS

Mice and cell lines

C57BL/6(H-2b) female mice (6-8 wk old, The Jackson Laboratory, Bar Harbor, ME) were housed in a pathogen-free environment. All procedures were carried out with approved GRU institutional animal protocols and NIH guidelines.

The 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 filtered RPMI1640 supplemented with 10% FBS, penicillin and streptomycin (100 U/ml each), L-glutamine (2 mM) at 37°C with 5% CO_2.

Treatment reagents

A preparation of all treatment reagents was prepared in an aseptic environment. BD 1mL TB syringes (25G x 5/8) were used for tumor and treatment injections.

Vaccine

The CTL epitope from HPV16 $E7_{49–57}$ (9aa peptide, RAHYNIVTF, 100μg/mouse) mixed with synthetic T helper epitope PADRE (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 in all studies (subcutaneous (s.c.) injections of 100uL in

PBS). Peptides HPV16 $E7_{49–57}$ and PADRE were stored in -20ºC in DMSO aliquots, and QuilA adjuvant (Brenntag) was stored in -4ºC in PBS.

Anti-OX40 antibody

Agonist rat anti-mouse OX40 antibody (clone OX86) was provided by Medimmune (Cambridge, UK), and used as intraperitoneal (i.p.) injections at a dose of 0.5, 1, and 2.5 mg/kg (or 10, 20, 50 ug/mouse respectively in 100uL PBS injections). Anti-OX40 Ab was stored in -80ºC.

1-methyl-D-tryptophan (1-MT, IDO inhibitor)

The 1-D-MT was purchased from Sigma Aldrich (St. Louis, MO) and provided in drinking water (freshly prepared every three days) at 2mg/mL concentration. 1-MT was dissolved in basic tap water. Water was supplemented with 3 grams of low fat sweetener per 1L of 2mg/mL 1-MT solution to improve the water intake by mice. Once all was dissolved, the pH was adjusted to a range of 7-7.5 with 1N HCl and filtered through a 75mm filter unit (Nalgene Rapid Flow, Thermo Scientific). 1-MT drinking solution was provided to mice in amber-colored or aluminum foil-wrapped drinking bottle to protect from exposure to light.

Tumor implantation and treatment

For both therapeutic and immunology experiments, 100uL of $7x10^4$ TC-1 cells in PBS

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 the tumor diameter reached 4-5 mm. For therapeutic experiments, vaccine was given weekly throughout the experiment. Anti-OX40 Ab and 1-MT treatments were provided on the same day with vaccination. Anti-OX40 Ab was given twice a week, 2-3 days apart, for the entire duration of a treatment. Similarly, the 1-MT was provided on a daily basis in drinking water until the end of the study. A treatment was stopped if a mouse regressed tumor. 5 mice per group were utilized in these experiments, including non-treated, treated with agonist anti-OX40 Ab (testing doses of 0.5 mg/kg, 1mg/kg, or 2.5 mg/kg) with or without vaccine for an optimal dose determination in tumor-bearing mice therapeutic studies. For vaccine/anti-OX40/1-MT combinational immunotherapy studies, all control non-treated, single and double agent-treated tumor-bearing mice were included with the vaccine/anti-OX40/1-MT-treated group to evaluate therapeutic efficacy. Tumor growth and survival were 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 cm^3. For immunology experiments, mice were treated similarly and were sacrificed four days after the second immunization. Tumors were harvested for analysis of tumor infiltrating cells and IDO activity, while spleen was evaluated for antigen-specific immune response.

Profiling tumor-infiltrating immune cells

Processing of tissue was performed in an aseptic environment and on ice at all times.

Tumor and spleen tissue processing

Resected tumors were homogenized individually in PBS using GentleMACS Dissociator (Miltenyi Biotec, San Diego, CA) and then filtered through Falcon 70 μm nylon filters (BD Biosciences, San Jose, CA), while each spleen either from tumor-bearing or naïve mice was processed separately to a single-cell suspension through 70 μm nylon filters in PBS. Cell suspensions were brought up to 10mL of PBS and centrifuged at 500xg for 5-7 minutes, followed by discarding of supernatant. 1mL of ACK buffer (Life Technologies, Grand Island, NY) per one sample was added to the spleen or tumor pellet for 5 minutes to lyse erythrocytes. Immediately, PBS was added up to 10mL and strained through 70 μm nylon filter once more and centrifuged at 500xg for 5-7 minutes. One additional wash with PBS and centrifugation were performed before fixation for tumor cells for immune response evaluation by flow cytometry, or two more washes and centrifugation were performed for spleen before proceeding to culture the cells for ELISPOT assay or for *in vitro* protein analysis by Western blot.

Tumor cell fixation and permeabilization

A Cellometer Auto T4 Cell Viability Counter was used to count processed tumor cells. One million (1 mL of $2.5x10^6$ cells/mL) re-suspended tumor cells in PBS were distributed into pre-labeled flow tubes (tumor samples were pooled only if the tumor size was too small to accommodate 1 million tumor cells, which was sometimes the case in mice responding to vaccine/anti-OX40 Ab or vaccine/anti-OX40 Ab/1-MT treatments). PBS was decanted after cells were centrifuged at 500xg for 5 min and stained for viability by fixable Live/Dead cell stain. (Live/Dead staining is described below in staining tumor cells and acquisition by flow cytometry**).** After live/dead staining, the pellet in the flow tube was carefully vortexed and fixed with 2mL of cold 1x fixation buffer (BD Pharmigen Mouse FoxP3 buffer set, BD Biosciences, San Jose, CA, Cat.No.566409) in PBS and incubated for 30 minutes in the dark at 4ºC. Fixation buffer was decanted from the samples following 500xg centrifugation for 5 mins. Samples were carefully vortexed, washed with 2 mL of 1x staining buffer (1x staining buffer: 10%PBS, 0.6% Azide, 20% FBS in milique H_2O), and pelleted by 500xg centrifugation for 5 min. If staining for intracellular FoxP3 or GrzB proteins was required, the permeabilization step was necessary; otherwise, it was omitted if extracellular staining with fluorophore-conjugated antibodies against CD45, CD3, CD8, CD4, E7, or OX40 cellular surface markers was performed.

Tumor sample permeabilization was achieved by adding 2mL of 1x permeabilization buffer (BD Pharmigen Mouse FoxP3 buffer set, BD Biosciences, San Jose, CA, Cat.No.

566409) in PBS to the sample and incubating for 30 min at 37ºC in an incubator in the dark. Then samples were washed with 2mL PBS, pelleted with 500xg centrifugation for 5 min and preceded to staining for flow cytometry.

Staining tumor cells and acquisition by flow cytometry

Live/Dead staining for cell viability

Sample staining with infra-red Live/Dead (Molecular Probes, Life Technologies) was performed before re-suspending the sample in a fixation buffer. After tumor cells were processed and washed as described above in the tumor cell fixation and permeabilization methodology section, the samples were stained and gently mixed with 1 uL of re-constituted Live/Dead fluorescent dye (one vial was re-constituted in 50uL DMSO) in 1mL PBS for 30 minutes at room temperature and protected from light. Next, samples were washed with 2mL of PBS and pelleted for fixation/permeabilization.

Staining for extracellular and intracellular markers

Before staining with fluorophore-conjugated antibodies to detect tumor intracellular or extracellular markers, Fc receptor was blocked by 1ug of a purified rat anti-mouse CD16/CD32 antibody (Mouse BD Fc Block) to eliminate non-specific antibody binding and then incubated for 10mins at 4ºC. Fc block was washed with 2mL of 1x staining buffer, and sample was pelleted and decanted for staining cellular markers.

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 (clone:30-F11) or Alexa Fluor 700 (clone:30-F11), CD3-PE-CF594 (clone:145-2C11) or V450 (clone:500A2), CD8-V450 (53-6.7) or APC (53-6.7), CD4-FITC (GK1.5), FoxP3-APC (MF23), and GranzymeB-APC (GB11). The FITC-conjugated dextramer specific to $E7_{49–57}$ peptide (MHC Dextramec, H-2 Db/RAHYNIVTF) was purchased from Immudex (Fairfax, VA). Two staining panels were established; to determine tumor T cell infiltration, samples were stained with CD45, CD3, CD8, CD4, and FoxP3, and to evaluate antigen-specific and functional CD8 T cells in the tumor environment, samples were stained with CD45, CD3, CD8, E7, and GrzB. Samples were stained with 0.5 grams of each antibody for 30 minutes, in the dark, at 4ºC and then washed twice with 2mL of PBS. At the same time, single stained compensation controls for every fluorophore-conjugated antibody was prepared in addition to a non-stained control sample following the same incubation and washing procedure as for tested samples (for compensation, 0.5 grams of each specific fluorophore-conjugated antibody was used for strongly expressing antigen to get a clear optical signal).

LSRII SORP flow cytometer (BD Biosciences, San Jose, CA) was used to acquire the following tumor-infiltrating cell subsets: $CD8^+$, $CD8^+E7^+$, $CD8^+E7^+GrzB^+$, $CD4^+Foxp3^-$ (non-Treg), and $CD4^+Foxp3^+$ (Treg) cells within live $CD3^+CD45^+$ population.

Acquired data were analyzed using FlowJo software, version 10 (TreeStar, Ashland, OR).

Evaluation of IDO enzyme activity

Tumor tissue was harvested from tumor-bearing mice treated with vaccine, vaccine/anti-OX40, vaccine/1-MT, and vaccine/anti-OX40 Ab/1-MT therapy (five tumor-bearing mice per group) following the same schedule and treatment as for the immune response assessment. Each tumor was homogenized by grinding in a pinch of sand using a mortar and pestle and brought up to 25 to 50mg/mL in PBS (no less than 25mg/mL). 100uL of homogenate samples were transferred into a 96-well plate and mixed with an equal amount of 100uL of substrate solution (reagents for substrate solution were purchased from Sigma and prepared by combining 100 mM potassium phosphate buffer pH 6.5, Cat.No. P9791 or P222; 50 μM methylene blue, Cat.No. M9140; 50mM ascorbate, Cat.No. 11140; 0.4 mM L-Tryptophan, Cat.No. 936659; 20 μg catalase, Cat.No. C3515). Immediately, 100 uL of tumor homogenate with substrate was transferred into a new 96-well plate, and mixed with Trichloroacetic acid (TCA, see below) as "time 0". The remaining 100 uL of tumor solution with homogenate in the plate were incubated for two hours at 37ºC and 550 rpm on a thermomixer for IDO activity reaction mixture as "time 2hrs" and then processed with TCA (see below). Additionally, one well of PBS blank was used for time 0 and time 2hrs.

TCA: 20 μL of 30% trichloroacetic acid was added to 100uL of IDO activity reaction mixture (time 0, and time 2hrs) in the plate, mixed with pipette and allowed to sit on ice for at least 5 minutes, following spin at 2000g for 10 minutes at 4ºC. The supernatant was carefully pipetted out (limiting the disturbance of bottom residue) into MultiScreen

Solvinert filter plate (Millipore, Cat.No. MSRLN0410) and centrifuged at 2000g for 10 minutes at 4C. The filtered supernatants were analyzed by high-performance liquid chromatography (HPLC, using LC-20 instrument) for kynurenine level. IDO enzyme activity was expressed in pmol/hour/mg tissue using the following formula:

Activity = ([Kyn] at 2 hours (in mM) - [Kyn] at 0 hour) × 2400 / (2hours × tissue concentration)

Analysis of antigen-specific cellular immune responses by ELISPOT

The detection of antigen-specific immune response in immunized and non-treated tumor-bearing mice was evaluated by secretion of IFN-γ from cultured spleens that were re-stimulated with vaccine-peptide components, and assessed by Enzyme-Linked ImmunoSpot (ELISPOT). Spleens were harvested from tumor-bearing mice four days after the second vaccination. The termination, treatment schedule, and dosing were the same as described above for the immunology response evaluations, with the exception that only two groups (non-treated and vaccinated) were analyzed. Spleens were processed in PBS to single cell suspension through 70 μm nylon filters (BD Biosciences, San Jose, CA). Red blood cells were lysed with ACK buffer (Life Technologies, Grand Island, NY). A detailed spleen preparation was described in the tumor and spleen tissue processing methodology section. 24 hours before seeding processed splenocytes into the ELISPOT plate (Immunospot TM M200, BD ELISPOT Millipore), the plate was coated

with 100uL per well of 1mg/mL purified anti-IFNγ Ab (BD ELISPOT) in PBS as a capture antibody in 4ºC. Also right before culturing splenocytes, the IFNγ-coated plate was washed with 200uL per well of complete RPMI media once, following blocking the plate with 200uL per well of complete RPMI media for 2 hours in a 37ºC incubator. Each splenocyte culture sample was re-stimulated with individual vaccine peptide separately (E7 peptide and PADRE peptide) as follows. The blocking media was discarded from the plate, and 100uL of 40ug/mL of E7 peptide in RPMI complete media was added into a well for each spleen sample. The same was done for PADRE peptide (100uL of 40ug/mL of PADRE peptide in complete RPMI media was distributed into a new pre-blocked well per sample), and as a negative control, 100uL of blank complete RPMI media was included. Then 250,000 spleen cells in 100uL complete RPMI media were added into each peptide-containing well and blank media well (making a final concentration of 20ug/mL of peptide in each well). The Millipore plate with splenocyte cultures comprising peptide or blank media was covered and sealed for overnight incubation in 37°C with 5% CO_2. The next day, the supernatant together with splenocyte cultures were decanted from the plate. Further leftover cells in the wells were lysed and washed with 200uL of tap H_2O twice, having 3 minutes lysing incubation time at room temperature. Then three washes with 200uL of 0.05% Tween PBS were performed, and 100uL of 0.5mg/mL of biotinylated anti-mouse anti-IFNγ Ab (BD ELISPOT) detection antibody in blocking solution (0.5% BSA in 0.05% Tween PBS) was added into a well and let rest for 1 hour at room temperature. Next, four washes with 200uL of 0.05% Tween PBS

followed to remove any residual detection antibody before adding 100uL of 100ug anti-mouse streptavidin-HRP (BD ELISPOT) in blocking solution for another 1 hour in room temperature. After, the wells were washed twice with 200uL of 0.05% Tween PBS buffer, followed with two extra washes of 200uL of PBS to remove any Tween traces. The development of spots was achieved by adding 100uL of BD-streptavidin mix (10mL BD Elispot AEC Substrate with 7 drops of substrate AEC chromogen) into the wells. Immediately after spots were resolved (3 - 10 minutes), the reaction was stopped by addition of 100uL of tap water to eliminate yellowing background. Finally, the wells were decanted and washed with 200uL of tap water, and decanted once more and air-dried overnight to count the spots. An automated plate reader, CTL Immunospot Analyzer (Cellular Technology, Shaker Heights, OH), was used to estimate the number of IFN-γ secreting cells in the form of spots. The analysis was performed in Microsoft Excel; the number of spots was calculated by subtracting negative control wells from experimental E7- or PADRE- containing wells.

Spleen CD4 T cell isolation

$CD4^+CD25^-$ T cells (Tconvs) were purified from spleens of naïve C57BL/6 female mice and further used for *in vitro* assays to determine the effect of: i) 1-MT treatment on OX40 receptor expression on Tconvs, ii) anti-OX40 Ab on IL-2 cytokine secretion from Tconvs, and iii) anti-OX40 Ab on the activation of AKT protein. Spleens were processed and erythrocytes were lysed according to the description in tumor and spleen tissue

processing methodology section. $CD4^+CD25^-$ T cells were enriched using mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec MACS, Cat.No. 130-091-041). First, splenocytes were depleted of non-$CD4^+$ T cells by resuspending 10^7 total mouse processed splenocytes in 40 µL of cold MACS buffer (0.5% FBS with 2mM EDTA in PBS) and labeled with 10 µL of $CD4^+CD25^+$ Regulatory T cell biotin-antibody cocktail. After the cell suspension was well mixed and incubated for 10 minutes at 4ºC, an additional 30 µL of cold MACS buffer, 20 µL of anti-biotin microbeads, and 10 µL of CD25-PE antibody were mixed in and incubated for an extra 15 minutes at 4ºC (following the manufacturer's protocol using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, which does not provide the amount of antibodies, thus the concentration is not known). Then cells were washed with 3 mL of MACS buffer and pelleted by 500xg centrifugation for 5 minutes at 4C. Next, the labeled cell pellet was resuspended in 500 µL of cold MACS buffer and applied onto the LD column prewashed with 2 mL of MACS buffer (set in a magnetic field of MACS separator) for non-CD4 T cell depletion. Unlabeled cells in the flow-through were collected, centrifuged at 500xg for 5 minutes, and then supernatant was aspirated out. Next followed magnetic labeling of $CD25^+$ cells by resuspending the pellet (10^7 total cells) in 90 µL of buffer and mixing it with 10 µL of anti-PE microbeads. Labeled cells were incubated for 15 mins in 4ºC before magnetic separation. A magnetic bead MS column was rinsed with 500 µL of buffer and the labeled cell suspension was applied for flow-through. The column was washed twice with additional 500 uL of MACS buffer. Finally, the unlabeled fraction of cells ($CD4^+CD25^-$

T cell, Tconvs) that passed through was collected and used for further experimental studies.

Analysis of IL-2 cytokine by Cytometric Bead Array (CBA) assay

The following assay was to evaluate the effect of anti-OX40 Ab on T conv cell secretion of IL-2 cytokine. The acquisition of IL-2 cytokine secretion in the supernatant of Tconv cultures was assessed by BD^{TM} Cytometric Bead Array (CBA) kit (BD Biosciences, mouse Th1/Th2/th17 cytokine kit). Spleens of naïve C57BL/6 female mice were isolated and processed as described in tumor and spleen tissue processing methodology section, and the enrichment of CD4+CD25- Tconvs followed the same procedure defined in the spleen CD4 T cell isolation segment. Tconv cells were activated by seeding 200 uL of 0.3 million of Tconv cells in complete RPMI media in a 10ug/mL of anti-CD3 Ab (BD Pharmingen, clone 145-2C11) pre-coated 96-well culture plate. Half of the culture was treated with 1ug/mL of anti-OX40 Ab (clone OX86) and half was left untreated as a control. Cell cultures were incubated at 37°C with 5% CO_2 for 72 hours for cytokine secretion. Then supernatant was collected and further centrifuged at 500xg for 5 minutes to remove any remaining cells. The next step was to proceed to evaluate IL-2 cytokine level in the supernatant by CBA assay using BD CBA Mouse Th1/Th2/Th17 cytokine kit. A bead master mix was made by adding 10 µL of each cytokine bead per determined numbers of supernatant samples and standards. A standard vial was reconstituted with 2 mL of dilution buffer resulting in 5000 pg/mL. Standard concentrations were as follows:

1000, 500, 250, 125, 62.5, 31.25, and 0 pg/mL. Flow cytometry tubes were labeled with standards and tested samples. 50 µL of supernatant sample and standards were added into pre-labeled flow cytometry tubes accordingly. Next, 50 µL of bead master mix, together with 50 µL of a detection reagent was distributed to all flow tubes, vortexed, and incubated for 2 hours at room temperature protected from light. Next, samples and standards were washed with 1 mL of wash buffer, pelleted by 500xg spin for 5 minutes, and finally reconstituted in 300 µL of wash buffer. Data acquisition was performed on LSRII SORP flow cytometer (BD Biosciences, San Jose, CA) and FCAP Array™ software was used to analyze cytokine level in samples. The concentration of IL-2 in the experimental culture supernatants was determined by an interpolation from the standard curve generated from IL-2 cytokine bead standards. Data were normalized by dividing the anti-OX40 Ab-treated sample concentration value by the average concentration value of the untreated sample, and the final result was expressed in a percentage having set untreated as 100%.

Evaluation of OX40 receptor expression on T cells

The expression of OX40 receptor was assessed for CD8, $CD4^{+}FoxP3^{-}$ (Tconvs), and $CD4^{+}FoxP3^{+}$ (Tregs). Harvested spleen from naïve C57BL/6 mice was processed and lysed for erythrocytes as explained in tumor and spleen tissue processing methods section. Half a million cells of whole spleen single cell suspension in complete RPMI was seeded per well and activated with soluble 10ug/mL of anti-CD3 Ab and 20U of IL-2

in a 48-well plate. Splenocyte culture was incubated at 37°C with 5% CO_2 and then collected at 24, 48, and 78 hours time intervals for OX40 expression detection by flow cytometry. Collected cells were washed with PBS and pelleted out at 500xg for 5 minutes. After, cells were stained with Live/Dead staining and permeabilized as described in Live/Dead staining for cell viability and tumor cell fixation and permeabilization methodology section. Then cells were labeled with fluorophore-conjugated antibody, CD8-V450 (53-6.7), CD4-Fitc (GK1.5), FoxP3-APC (MF23), OX40-PerCP (OX-86) and let stand for 30 minutes at 4ºC protected from light. Finally, cells underwent two washes with 2 mL of PBS, which was followed with data acquisition on LSRII SORP flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software, version 10 (TreeStar, Ashland, OR).

Assessment of 1-MT on OX40 expression

The effect of 1-MT on OX40 expression on $CD4^{+}FoxP3^{-}$ T cells was determined by flow cytometry assay. Spleens of naïve C57BL/6 female mice were isolated and processed as described in tumor and spleen tissue processing methodology section, and the enrichment of CD4+CD25- Tconvs followed the same procedure defined in spleen CD4 T cells isolation segment. Naïve CD4+CD25- enriched Tconvs in complete RPMI media were activated by plating in a 48-well plate pre-coated the day before with 10ug/mL of anti-CD3 Ab in 4ºC at a ratio of 0.5 million of cells per well, with additional 2.5 ug/mL of soluble anti-CD28 Ab (BD Pharmingen) and 20U/mL IL-2 cytokine (Peprotech). Cell

culture was incubated at 37°C with 5% CO_2 for 24 hours before IDO inhibitor (1-MT) was added into the culture at a titration of 10, 100, and 1000 ug/mL of 1-MT. The activated and treated cell culture with 1-MT was incubated for an extra 48 hours at 37°C with 5% CO_2. Alongside, non-stimulated CD4+CD25- enriched Tconvs were incubated at the same time and at 37°C with 5% CO_2 as a control. After a total of 72 hours of incubation, cell cultures were collected, washed twice with PBS, and pelleted out for staining with fluorophore-conjugated antibodies OX40-PerCP (OX86), CD4-Fitc (GK1.5), FoxP3-APC (MF23) including DAPI to detect the effect of 1-MT on OX40 expression on CD4 Tconvs. The staining procedure, data acquisition, and analysis followed the same steps as described in evaluation of OX40 receptor expression on T cells methodology section.

Assessment of IDO RNA expression in TC-1 cells by qRT-PCR

Culture and treatment of TC-1 tumor model

TC-1 cell line passages 8 at ~ 20% confluence was treated with recombinant mouse 20ng/mL of IFN-gamma (Invitrogen, CA) for 48 hours in complete RPMI media at 37°C with 5% CO_2, to evaluate the induction of IDO level in cells.

RNA extraction and quantification

Trypsinized cells were washed in ice cold PBS and centrifuged (500xg for 5 minutes) twice to avoid contamination with the culture media. At least 10^6 cells for extraction were

transferred into 1.5 mL Eppendorf tubes with the addition of 1 mL TRIzol and were let stand for 5 minutes at room temperature. Then 250 uL of chloroform was added and the tube was shaked vigorously for 15 seconds and incubated for extra 5 minutes at room temperature. Next layers were separated by 10,000 rpm spin for 5 minutes (top layer: clear aqueous, interphase: white precipitated DNA, bottom layer: pink organic phase). The top aqueous layer was carefully extracted and placed into a new 1.5 mL Eppendorf tube. Then 550 uL of isopropanol was added to the aqueous layer, mixed gently and left alone for 5 minutes at room temperature. Next, the sample was centrifuged at maximum speed (14,000 rpm) for 30 min. Working on ice, the isopropanol/aqueous solution was removed, while leaving the pellet in the tube. The pellet was precipitated by mixing with 1 mL of 75% ethanol in DEPC treated water and centrifuged for 9,500 rpm for 5 minutes. Then ethanol was poured out and pellet was let air-dry for approximately 5 minutes. After making sure that the pellet does not get over dried, while all ethanol was removed, 15-25 uL (depending on yield) of DEPC treated water was added. Finally absorbance was measured at 260 nm. The 260/280 ratio should be greater than 1.8. Next followed DNase treatment by adding 2ug/8uL of RNA to 9 uL of DNase cocktail (1uL of RQ1 RNase free DNAse, 2uL of DNase 10x reaction buffer, 6uL DEPC-treated H_2O, and 0.5 RNase Out). The sample was incubated in thermal cycler for 37ºC for 15 mins followed by 65C for 20 mins.

IDO RNA quantification by RT-PCR

Complementary DNA (cDNA) transcript from total RNA was performed by reverse transcription using 10uL of Bio-rad SsoAdvance Universal SYBR Green Supermix, 250nM of forward and reverse random primers, 1ug of RNA template, and nuclease-free H_2O to bring up the volume to 20uL. Samples were added to the PCR tubes, mixed well and vortexed for 5 seconds. cDNA reverse transcription was performed by incubating the samples at 37ºC for 1hour, then 95ºC for 5 mins on iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to measure the probe fluorophore amplification of cDNA in the sample. IDO primers were purchased from Qiagen, CA. The threshold for CT (threshold cycle) was used to calculate relevant expression as $2^{Ct(\beta\text{-actin})-Ct(\text{target gene})}$.

Detection of pAKT by Western blot

Culturing and treatment of $CD4^+CD25^-$ Tconvs

Enriched $CD4^+CD25^-$ T convs were seeded (.25 million per well) in anti-CD3 Ab (10ug/mL) pre-coated 48-well-plate for 48 hours at 37°C with 5% CO_2. On day 2, activated Tconvs were pooled and treated with 50ug/mL of anti-OX40 Ab for 20mins in 37ºC. (The detailed procedure was as follows: 250 uL of 100ug/mL of anti-OX40 Ab in PBS and 250 uL of blank PBS (as a control) was put into wells of a 48-well plate. While plate was put into 37ºC incubator to heat up the treated and non-treated PBS filled wells, the activated T convs were washed with PBS and centrifuged at 500xg for 5 minutes

twice. Then 250 uL of washed activated cells re-suspended (2milion cells/mL) in PBS were added to the wells having either blank PBS or anti-OX40 Ab treated PBS, and incubated for 20 min at 37ºC. Thus a well contained half a million of activated Tconvs in 400 uL total volume, with or without anti-OX40 Ab; having 50ug/mL of anti-OX40 Ab final concentration). Protein was extracted from cell cultures as described below.

Protein purification

Cells were lysed with 10%Phospatase (Sigma Aldrich) and 10% of ProteinaseK (Thermo Scientific) in RIPA buffer (Thermo Scientific) onto a shaker for 1 hour in 4ºC. More specifically, plate with treated and non-treated cells were centrifuged at 500xg for 15 seconds. A supernatant was carefully decanted by tipping the plate over and put onto a tissue to remove all PBS traces. 80uL of prepared RIPA buffer containing 10%Phospatase and 10% of ProteinaseK was added into each well and incubated on a shaker (level 7 for tilt and speed) for 1 hour in 4ºC. Cells were collected into 1.5mL Eppendorf tubes and centrifuged at the highest speed for 90 mins in order to collect cell lysate in the supernatant.

Protein quantification

Protein concentrations were quantified using Coomassie Blue (Thermo Scientific, IL) at 570 Absorbance. Sample concentration was calculated using standards (50, 25, 12.5,

6.25, 0 ug/mL of BSA in Coomassie Blue). If protein was used for Western blot analysis, then aliquots of protein cell lysates in 1x BME were prepared and stored in -80C.

pAKT detection by Western blot assay

To reveal pAKT by Western blot assay, 20 ug of total protein lysate from treated and non-treated stimulated Tconvs were denatured at 95ºC for 10 minutes and loaded into wells of a gel (Bolt 4-12% Bis-Tris Plus 10 well, Invitrogen) together with a dye (Precision Plus Protein Standards, BioRad). After gel electrophoresis ran at 150V for approximately 1 ½ hours in 1x running buffer (NuPAGE SDS Running Buffer, Life Technologies), the protein transfer to nitrocellulose membrane was performed by dry blot process using the iBlot® 7-Minute Blotting System (ThermoFisher, Scientific), which involves assembling of iBlot™ Gel Transfer Stacks (Nitrocellulose-Mini, Invitrogen). Then the nitrocellulose membrane was blocked for 1 hour with 3% milk in 0.1% tPBS at room temperature on low speed rocker. After blocking solution was decanted and rabbit pAKT (D7F10) at 1:1000 dilution in 0.1% tPBS containing 1% milk was added at 4ºC for overnight incubation on low speed rocker. Next day, three washes with 0.1% tPBS for 10 mins at room temperature on maximum speed of rocker were performed to remove any free antibody, followed with the addition of secondary detection Ab anti-rabbit HRP (Cell Signaling) at 1:2500 in 0.1% tPBS containing 1% milk for 1 ½ hours at room temperature on gentle speed rocker. Finally, three washes with 0.1% tPBS for 10mins at

room temperature on maximum speed rocker were performed before developing pAKT in dark box (LAS-300).

Next, a blot was restripped (Restore Western Blot Stripping Buffer, Thermo Scientific) for 20 minutes at room temperature on medium speed rocker, followed with two 0.1% tPBS washes for 20 minutes, and blocked for 1 hour with 3%milk in 0.1%tPBS before adding goat β-actin (Sigma) at 1:2000 ratio in 0.1% tPBS containing 1% milk for overnight incubation at 4ºC on low speed rocker. The next day, β-actin was detected by labeling it with secondary anti-goat HRP (Cell Signaling) at 1:2500 ratio in 0.1% tPBS containing 1% milk for 1 ½ hours at room temperature on gentle speed rocker, followed with three washes of 0.1% tPBS at 10 minutes intervals, on maximum speed rocker at room temperature.

Additionally, an enhancer (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) was used to augment the chemiluminescent detection sensitivity, which was developed in dark box (LAS-3000) in .tif image format. The density of resolved bands on the digital image was analyzed by ImageJ software and normalized to β-actin and fold over non-treated sample.

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).

III. RESULTS

Vaccine enhances tumor-specific CD8 T cells

Priming T cells with tumor antigen is one of the strategies to effectively educate T cells for tumor recognition to elicit anti-tumor function while limiting the side effects on peripheral cell toxicity. One of the methods to train T cells to identify tumors for rejection is an immunization with therapeutic vaccine. Therefore in our immunotherapy study, we have decided to implement vaccine composed of a CTL epitope from HPV16 $E7_{49–57}$ (9aa peptide), synthetic T helper PADRE (13aa peptide) and QuilA adjuvant for tumor recognition. The tumor model we used is a lung epithelial TC-1 cell line expressing human papillomavirus strain 16 (HPV16) early proteins 6 and 7 (E6 and E7).

Here, we evaluated the effectiveness of the vaccine to induce antigen-specific cytotoxic CD8 T cells infiltrating the tumor environment by flow cytometry. Mice were implanted with 70,000 TC-1 cells into the right flanks at Day 0. The subcutaneous vaccine immunization started when the tumor reached 4-5 mm in diameter (approximately day 10). Mice were immunized on days 10 and 17. Tumor was harvested on day 21 for antigen-specific immune response evaluation by staining cells with live/dead, CD45, CD3, CD8, and E7-dextramer flourophore-conjugated antibodies.

We show that the immunization significantly increased the number of CD8 T cells and the percentage of antigen-specific CD8 T cells within the CD8 population (Figure 1A, B) when compared to control mice. A representative gating strategy for non-treated and vaccine immunized mice is shown in Figure 1C. Here, we show that vaccine alone is

effective in priming T cells against tumor antigen, which is a necessary immunotherapy application to direct T cells for tumor recognition.

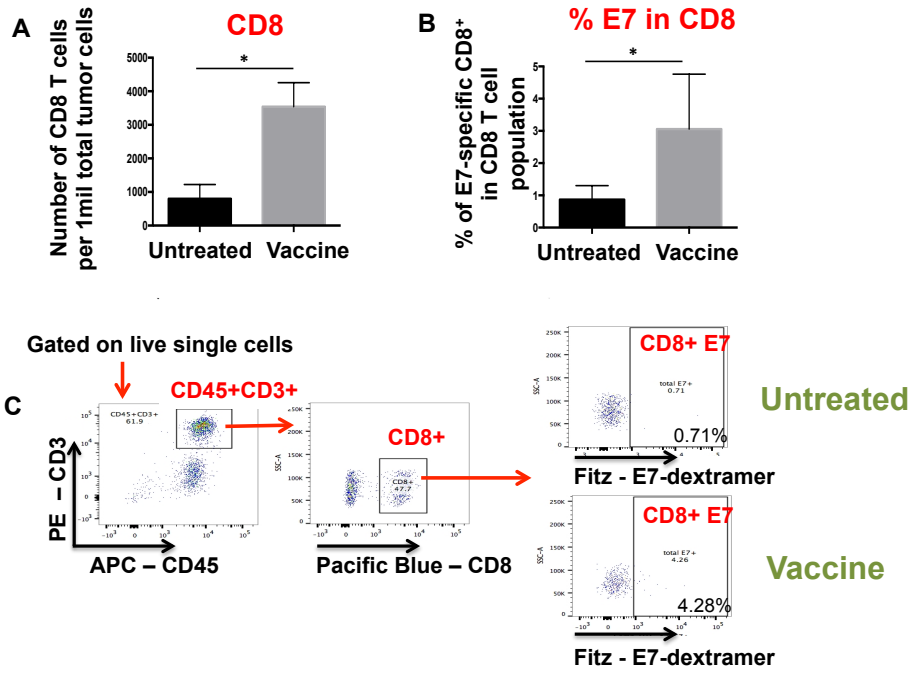

Figure 1. Vaccine promotes antigen specificity in CD8 T cells. Tumor-bearing mice were immunized with HPV16 E7 peptide specific for CTL epitope and T helper PADRE peptide. **A.** Tumor tissue was characterized for number of CD8 T cells, and **(B)** percent of antigen-specific CD8 T cells within the CD8 population in the tumor. **C.** A representative gating strategy for vaccinated and non-treated mice. *$p<0.05$. Data represent one out of three independent experiments.

Anti-OX40 Exhibits a Bell-Shaped Dose-Response Curve of Immunomodulatory Activity.

Due to OX40 receptor immune stimulatory properties on T cells, we hypothesized that vaccine efficacy may be enhanced when combined with anti-OX40 antibody. Mice bearing TC-1 tumors were subjected to vaccine with anti-OX40 Ab treatment. Different doses of anti-OX40 Ab (0.5, 1, and 2.5 mg/kg) were evaluated. The tumor implantation and vaccine treatment followed the same schedule as for the $CD8^+$ E7-antigen-specific T cell immune response evaluation in Figure 1, with the addition that on days 10, 13, 17, and 20, intraperitoneal injections with different doses of anti-OX40 Ab were provided, as depicted in Figure 2A. A day after the last treatment, on day 21, spleens were harvested and restimulated with each vaccine peptide component in culture. After 24 hours, splenocyte cultures were evaluated for IFN-γ cytokine in the media by ELISPOT. In Figures 2B and C, spleens from vaccinated mice showed an increased number of IFN-γ secreting cells for each peptide (either E7 peptide that is specific for cytotoxic T cell lymphocytes or T helper peptide PADRE) restimulation *in vitro* when compared to non-treated animals. Interestingly, the addition of anti-OX40 Ab at dose 1mg/kg to vaccine significantly enhanced IFN-γ secreting cells in splenocytes re-stimulated with E7 peptide when compared to all other groups. It was surprising that the same peptide restimulation for a high dose of 2.5 mg/kg of anti-OX40 Ab with vaccine did not have any effect on the secretion of IFN-γ cytokine; the outcome of a high dose of anti-OX40 Ab with vaccine was comparable to vaccine alone and to 0.5 mg/kg of anti-OX40 Ab with vaccine

treatments (Figure B). While the re-stimulation with T helper peptide PADRE significantly enhanced IFN-γ secreting splenocytes for all doses of anti-OX40 Ab with vaccine (Figure 2C), the dose of 1mg/kg of anti-OX40 Ab with vaccine radically enhanced IFN-γ secreting cells among all other groups (Figure 2C). These data show that anti-OX40 Ab doses exhibit a non-linear correlation for antigen-specific immune response, where 1mg/kg of anti-OX40 Ab is the most promising dose to enhance vaccine-specific efficacy.

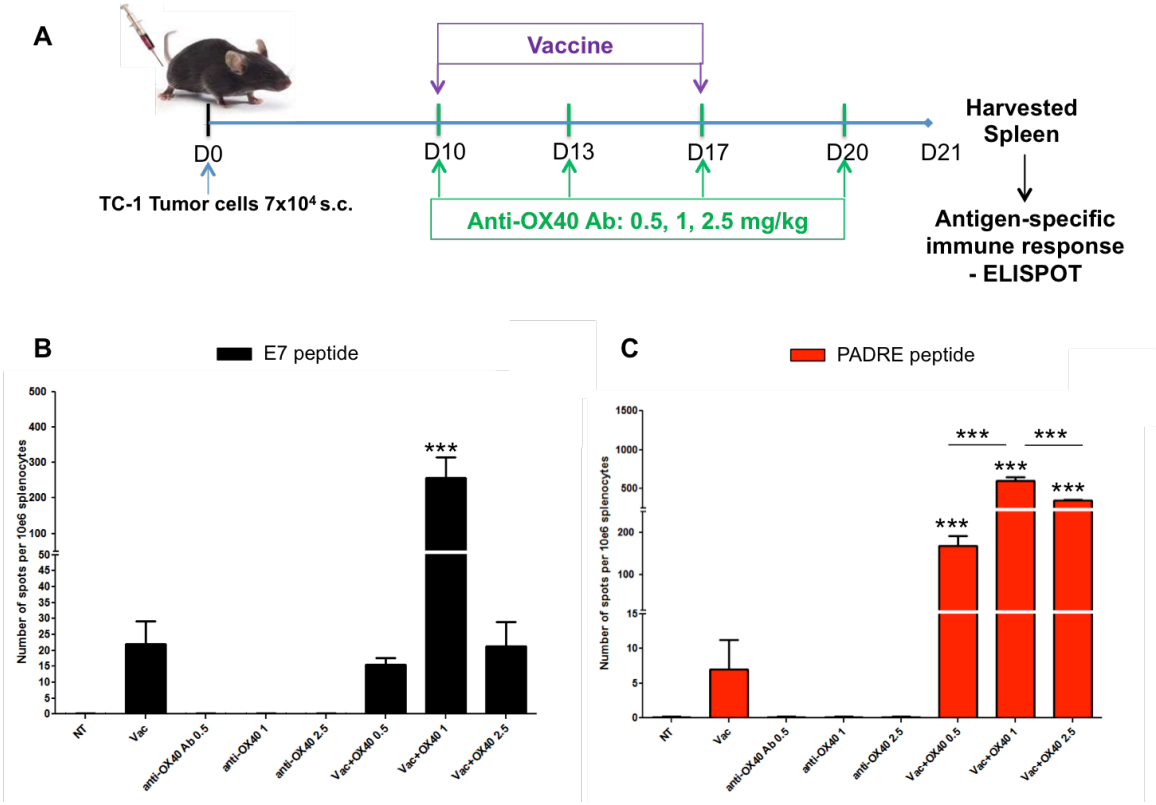

Figure 2. Dose of 1mg/kg of anti-OX40 Ab enhances vaccine efficacy. A. Spleens were harvested from tumor-bearing mice that were immunized and treated with different doses of anti-OX40 as depicted above. **B.** Restimulated splenocyte cultures with E7-peptide and **C.** PADRE peptide were analyzed for secreted IFNγ in media by ELISPOT. *** $p<0.001$.

Anti-OX40 Ab Demonstrates Anti-Tumor Activity Pattern Consistent With Optimal Biologic Dosing

To evaluate the optimal dose of anti-OX40 Ab that would provide promising therapeutic effect in tumor-bearing mice, we have tested three doses of anti-OX40 Ab (0.5, 1, 2.5 mg/kg) with and without vaccine, in a subcutaneous murine tumor model. TC-1 tumor cells were implanted on day 0, and treatment was initiated when the tumor reached 4-5 mm in diameter. Tumor-bearing mice were treated weekly with a peptide vaccine, and different doses of anti-OX40 Ab were administered twice a week throughout the whole experiment, as depicted in Figure 3A. Tumor growth and survival were monitored.

Tumor-bearing mice treated with vaccine did not benefit in survival compared with untreated controls;, similarly, anti-OX40 Ab at a dose of 0.5 mg/kg with vaccine did not significantly improve the anti-tumor outcome (Figure 3A). A dose of 1mg/kg of anti-OX40 Ab with vaccine significantly prolonged the survival, led to slower tumor progression, and a complete regression of tumors in 20% of mice (Figures 3C and 4). Unexpectedly, a further vaccine dose increase to 2.5 mg/kg resulted in loss of therapeutic efficacy (Figures 3D and 4). Altogether, the optimal dose of anti-OX40 Ab (1mg/kg) was determined for vaccine-based therapy, and the effect of anti-OX40 Ab treatment was not dose-dependent. Thus, further assessment of tumor infiltrating immune cells was elucidated to understand the differential therapeutic effect between 1 and 2.5 mg/kg anti-OX40 Ab doses.

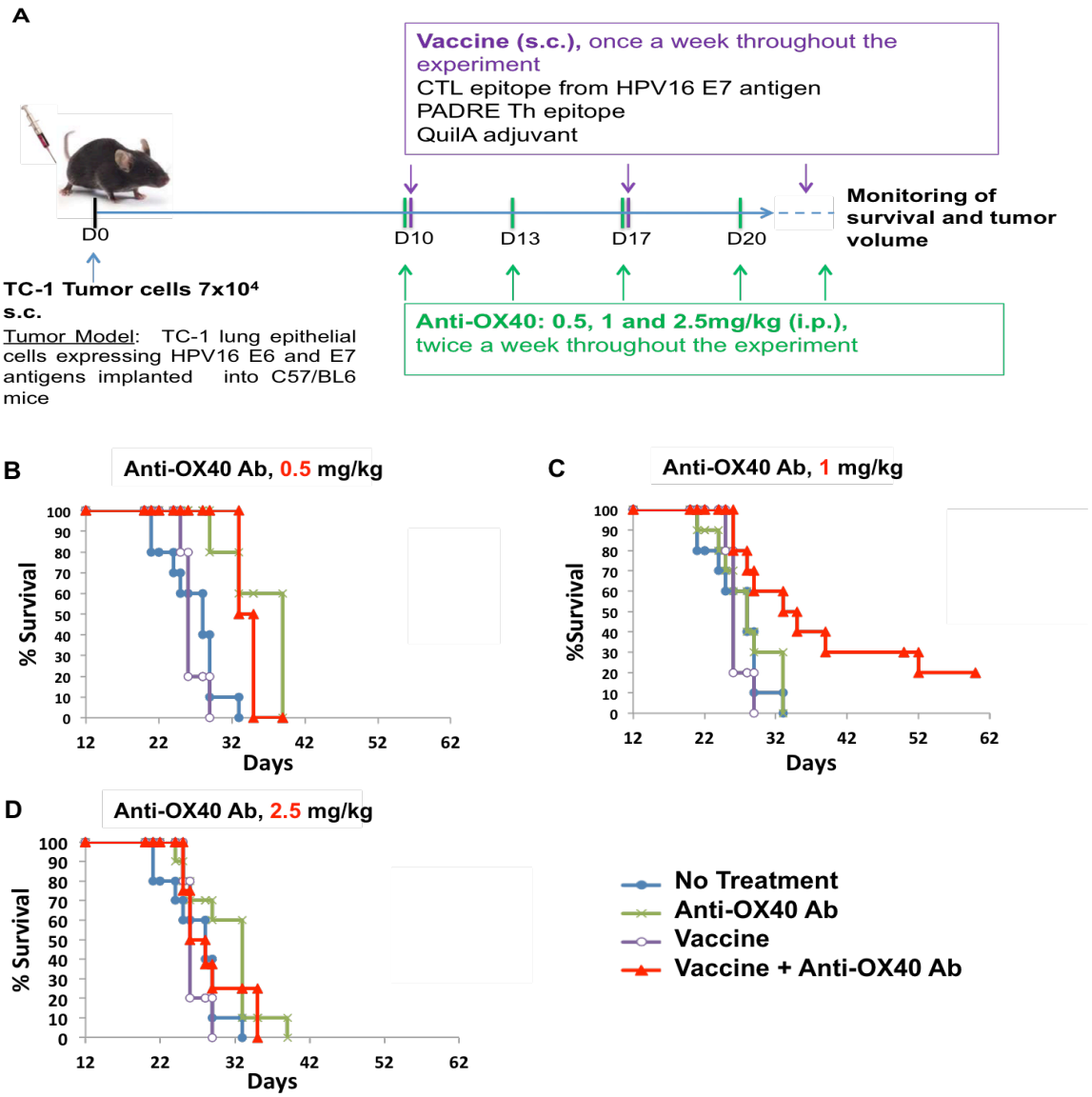

Figure 3. Survival evaluation for anti-OX40 Ab with vaccine. Tumor-bearing mice were immunized and treated with different doses of anti-OX40 as presented **(A)**. Survival of mice treated with 0.5 mg/kg **(B)**, 1 mg/kg **(C)**, and 2.5 mg/kg of anti-OX40 Ab with vaccine was monitored **(D)**. Data represent two combined independent experiments. Five mice were evaluated for each group and experiment.

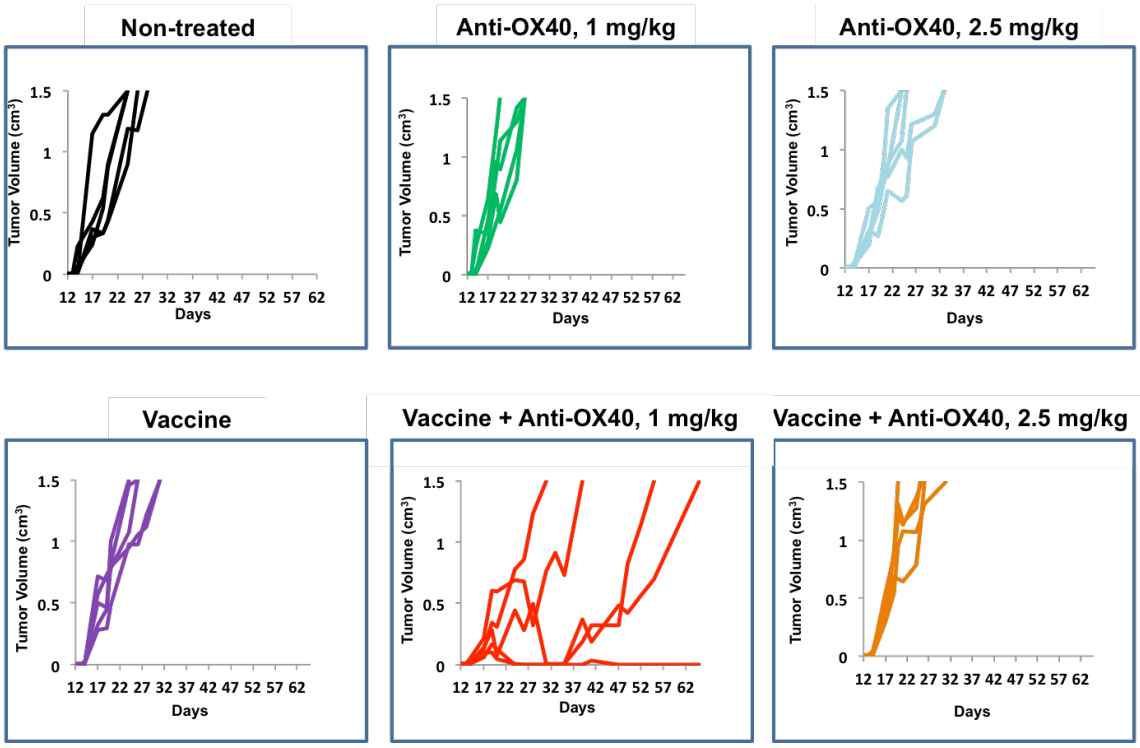

Figure 4. Tumor volume assessment for anti-OX40 Ab with vaccine. Tumor-bearing mice were immunized and treated with different doses of anti-OX40 as presented in Figure 3A. Tumor volume of mice treated with 0.5 mg/kg, 1 mg/kg, and 2.5 mg/kg of anti-OX40 Ab with vaccine was monitored every 3-4 days. Data represent one of two independent experiments. Plots represent lines for each individual mouse. Five mice in each group were tested for each experiment.

1 mg/kg of Anti-OX40 With Vaccine Enhances Effector CD8/Treg Ratio Whereas 2.5 mg/kg Diminishes Efficacy.

To understand the loss of activity of higher anti-OX40 Ab doses in mice, the effect of triggering OX40 receptor on tumor infiltrating immune cells was evaluated. Tumor-bearing mice were treated in the same manner as for the therapeutic efficacy experiments with the exception that tumor was harvested four days after the second immunization with vaccine, as illustrated in Figure 5. We show that either immunized or single agent-treated animals did not significantly show an increase in the number of tumor infiltrating CD8 T cells when compared to untreated tumor-bearing mice. However, combining vaccine therapy with anti-OX40 Ab at 1mg/kg resulted in a significant increment of CD8 T cells when compared to their single agent control (Figure 6A). Remarkably, CD8 T cells did not undergo further enhancement when the dose of anti-OX40 Ab was boosted to 2.5 mg/kg with vaccine (Figure 6A). In fact, to our surprise, the high dose of anti-OX40 (2.5mg/kg) treatment significantly increased the number of tumor infiltrating regulatory T cells (CD4+FoxP3+ T cells), either provided with or without vaccine, when compared to non-treated or any single agent-treated animals (Figure 6B). More importantly, the positive therapeutic outcome for anti-OX40 Ab dose at 1 mg/kg when added to vaccine may partially be due to significant enhancement of the CD8/Treg ratio when compared to all treated and non-treated groups, while the high dose (2.5 mg/kg) of agonist anti-OX40 Ab with vaccine significantly

reduced the effector T cell ratio when compared to 1 mg/kg of anti-OX40 Ab with vaccine therapy as shown in Figure 6C. Due to significant variability in the Tregs and CD8 T cells infiltrating the tumor, close attention was put on three groups involving anti-OX40 Ab (2.5 mg/kg), vaccine with anti-OX40 Ab (1 mg/kg), and vaccine with anti-OX40 Ab (2.5 mg/kg). Representative dot plots from these three groups, including the no treatment group (Figure 6D), showed that the addition of anti-OX40 Ab at a dose of 1 mg/kg to vaccine increased the percentage of CD8 T cells, while a high dose of anti-OX40 Ab (2.5 mg/kg) treatment, either alone or with vaccine, enhanced the percentage of Tregs in the tumor environment when compared to other groups.

In summary, these data suggest that there is an optimal dose of anti-OX40 Ab (1 mg/kg) that increases the number of effector CD8 T cells without significantly increasing the number of suppressive Tregs, whereas a high dose of anti-OX40 Ab (2.5 mg/kg) increases the number of Tregs, which may partially explain the difference in therapeutic efficacy portrayed in Figures 3 and 4.

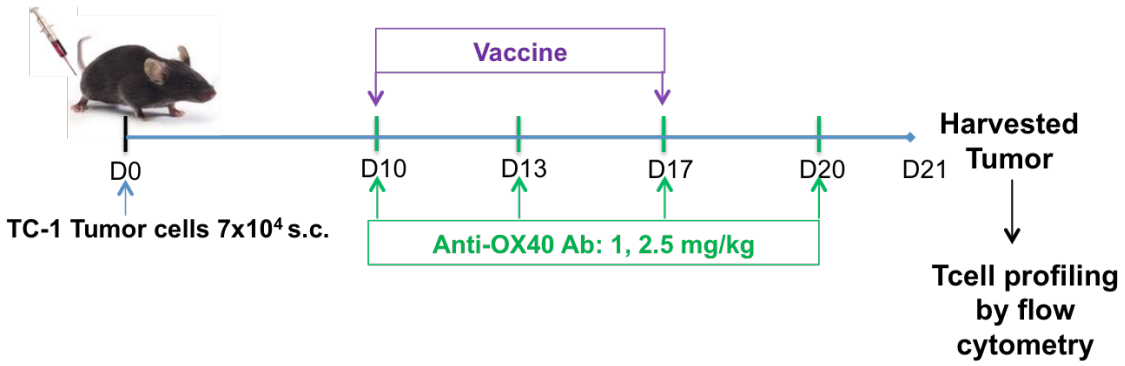

Figure 5. Treatment regimen for vaccine and anti-OX40 Ab. Mice were inoculated with $70x10^4$ TC-1 cells subcutaneously. When tumors reached 3-4 mm in diameter (approximately on day 10), the treatment with vaccine and anti-OX40 Ab was initiated. Mice were immunized subcutaneously twice on days 10 and 17. Intraperitoneal injections of anti-OX40 Ab were provided on days 10, 13, 17, and 20. Two doses of 1 and 2.5 mg/kg of anti-OX40 Ab were tested. Tumor tissue was harvested from treated and non-treated mice four days after immunization. Tumor infiltrating regulatory and CD8 T cells were analyzed by flow cytometry. The results are depicted in Figure 6.

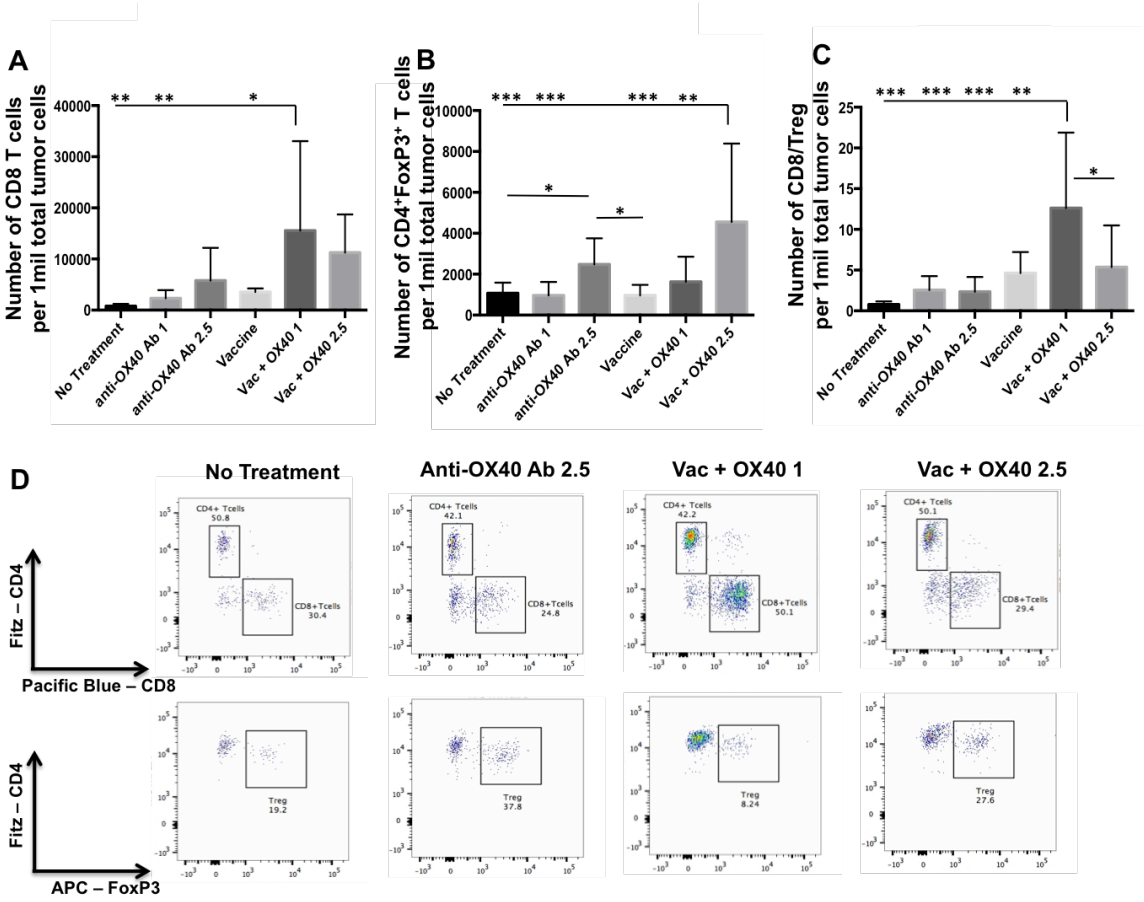

Figure 6. Dose of 1 mg/kg anti-OX40 with Vaccine Enhances Effector CD8/Treg Immune Response Whereas the Higher 2.5 mg/kg Dose Diminishes the Ratio. Tumor-bearing mice were treated as illustrated in Figure 5. **(A)** Number of CD8 T cells **(B)** CD4+FoxP3^{+} (Tregs), and **(C)** CD8/Treg ratio in tumor were analyzed by flow cytometry. **(D)** Representative gating strategy. *p<0.05, **p<0.01, *** p<0.001. Data shown are from two combined independent experiments.

Anti-OX40 Ab Mediates Dose-dependent Increase of Treg Tumor Infiltration

Due to the variable immune response in CD8 T cells and Tregs when treated with 1 or 2.5 mg/kg of anti-OX40 Ab, we decided to further evaluate the immune and molecular mechanisms *in vitro* and *vivo*.

Since OX40 receptor is expressed on activated T cells, we assessed the effect of anti-OX40 Ab treatment on three T cell subsets in tumor tissue: $CD4^+FoxP3^-$ (non-Tregs), $CD4^+FoxP3^+$ (Tregs). Tumor-bearing mice were treated with anti-OX40 Ab only (Figure 7A). Strikingly, we found that while all the anti-OX40 Ab doses significantly increased the number of tumor infiltrating non-Treg $CD4^+$ cells (Figure 7B), only the high dose (2.5 mg/kg) of anti-OX40 Ab significantly increased the number of suppressive Treg cells when compared to no treatment (Figure 7C). This finding demonstrates the 1 mg/kg of agonist anti-OX40 Ab to be the optimal dose that enhances effector T cells without increasing suppressive regulatory T cells.

We show that various doses of anti-OX40 Ab have a differential effect on T cell subsets; therefore we have evaluated the molecular mechanism responsible for the outcome discrepancy. Before assessing OX40-dependent downstream signaling in T cells, we evaluated the expression of OX40 receptor on T cell subsets (by flow cytometry), which may have determined one of the possible explanations for anti-OX40 Ab doses variability on T cells. To simulate the *in vivo* effect, whole splenocytes from naïve mice were stimulated with anti-CD3 antibody and IL-2 cytokine. The OX40 receptor was analyzed on CD8, $CD4^+FoxP3^-$ (CD4 non-Tregs), and $CD4^+FoxP3^+$ (Tregs)

at three different time points: 24, 48, 72 hours of cell culture collection. We demonstrate that while there is a slight increase of OX40 receptor on Tregs at 24 hours when compared to non-Treg and CD8 T cells, the expression of OX40 receptor is closely similar among all the T cell subsets at 48 and 72 hours (Figure 8). In summary, despite the slight OX40 receptor increase on Tregs at initial activation, there is no dramatic difference in OX40 receptor expression between the T cell subsets analyzed.

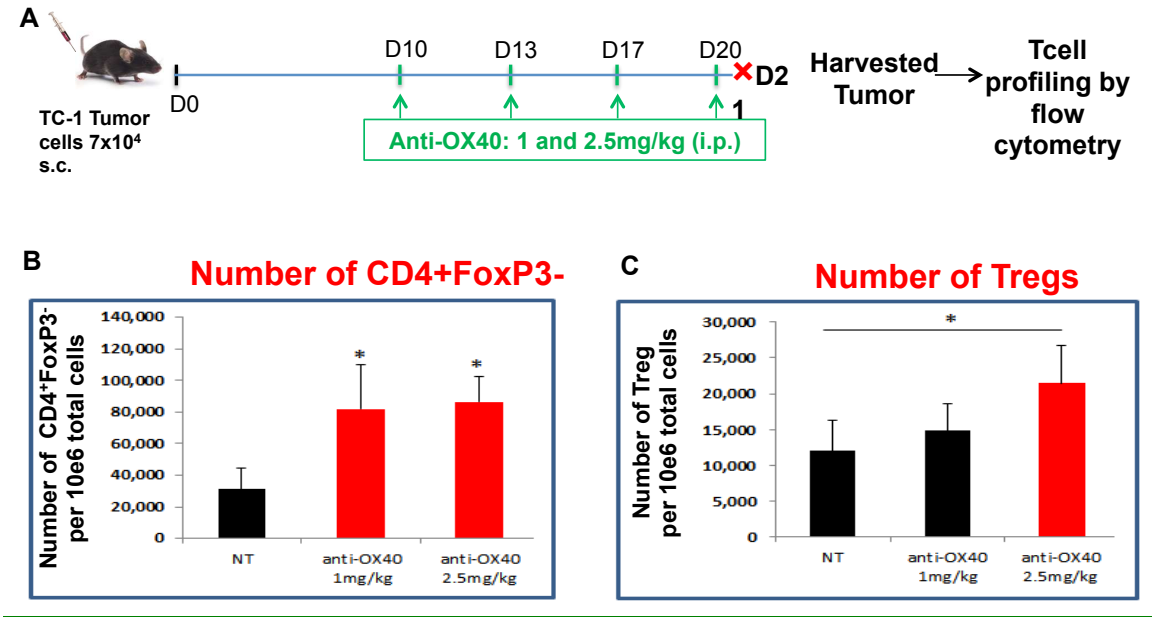

Figure 7. Evaluating the impact of anti-OX40 dose on T cell immune response in tumor. (A) Tumor-bearing mice were treated with anti-OX40 Ab only. Two doses (1, and 2.5 mg/kg) were analysed. **(B)** Number of CD4+FoxP3$^-$ (CD4 Tconvs), and **(C)** CD4+FoxP3$^+$ (Tregs) in tumors were analyzed by flow cytometry. *p<0.05 Data shown are from three combined independent experiments. Gated within CD45$^+$CD3$^+$ population.

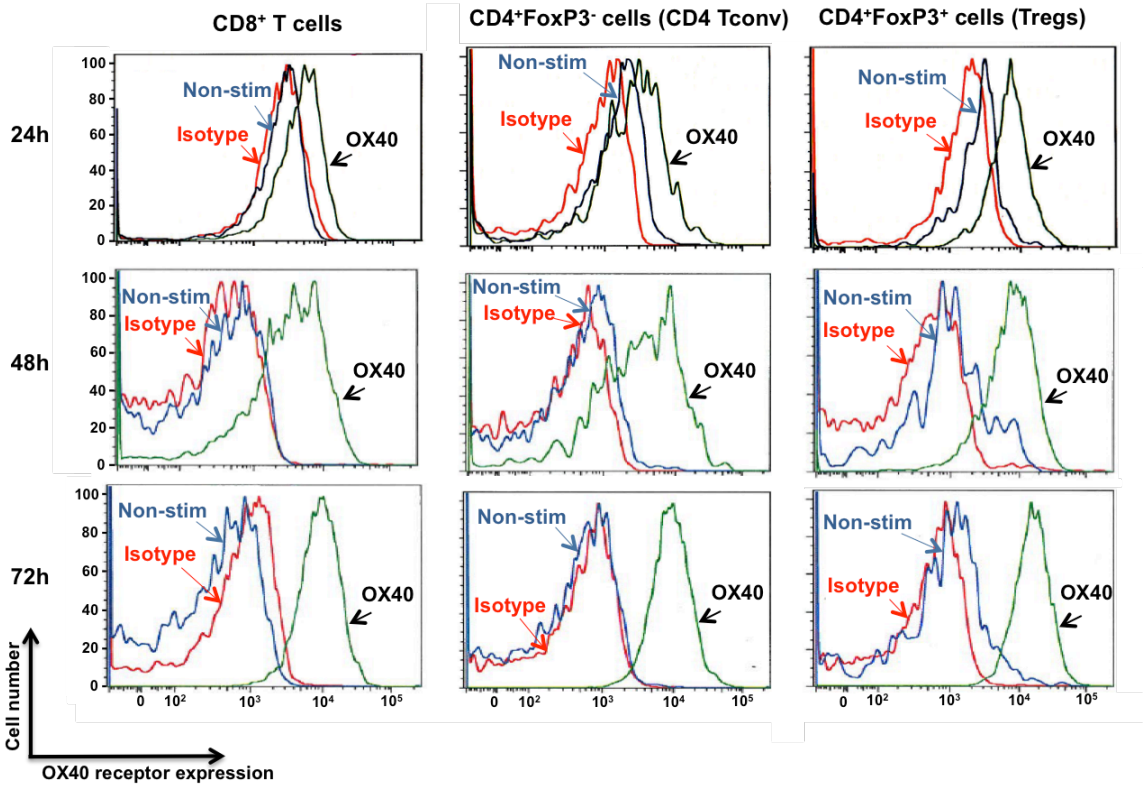

Figure 8. Evaluation of OX40 receptor expression on T cells. Single cell suspension from naïve mouse spleens was stimulated with anti-CD3 Ab and IL-2 and assessed for OX40 expression on CD8 T cells, CD4+FoxP3$^-$ (CD4 Tconvs), and CD4+FoxP3$^+$ (Tregs) at different time points (24, 48, 72 hours) by flow cytomtery. Two negative controls, non-stimulated and isotype for anti-OX40 Ab were included.

Engaging OX40 Receptor on T cells Increases pAKT Protein and IL-2 Secretion

To understand the differential effect on the CD4 T cell subset immune response triggered by 1 and 2.5 mg/kg of anti-OX40 Ab, the molecular mechanism in T cells mediated by OX40 receptor was assessed *in vitro*. Purified $CD4^+CD25^-$ T cells from mouse spleen were stimulated with anti-CD3 Ab. After 48-hour incubation, cultures were treated with anti-OX40 Ab for an additional 20 minutes. The level of phosphorylated AKT from cell lysates was visualized by Western blot assay, as illustrated in Figure 9A. To assure OX40 receptor presence on activated T cells for anti-OX40 antibody binding, the induced OX40 receptor was detected before anti-OX40 Ab treatment by flow cytometry (Figure 9B). Since AKT protein plays an important role in T cell survival, proliferation, and function, and OX40 downstream signaling goes through the PI3K/AKT pathway, we decided to evaluate the co-stimulatory OX40 effect on AKT activity in T cells. We show that anti-OX40 Ab treatment notably increased the active form of AKT protein when compared to the control group (Figures 9C and D).

Next, the impact of engaging OX40 receptor on T cell activity was analyzed for IL-2 cytokine secretion. Purified $CD4^+CD25^-$ T cells from spleen of naïve mice were cultured with plate-bound anti-CD3 Ab (10 ug/mL) and anti-OX40 at 1 mg/kg for 48 hours at 37C as shown in Figure 10A. Next, the IL-2 concentration in media was measured by CBA. We observed that anti-OX40 Ab significantly enhanced IL-2 secretion in T cell cultures. These data prove the role of OX40 receptor as a stimulant for

AKT activity, which is an indication of functional T cells as further confirmed by enhanced IL-2 secretion.

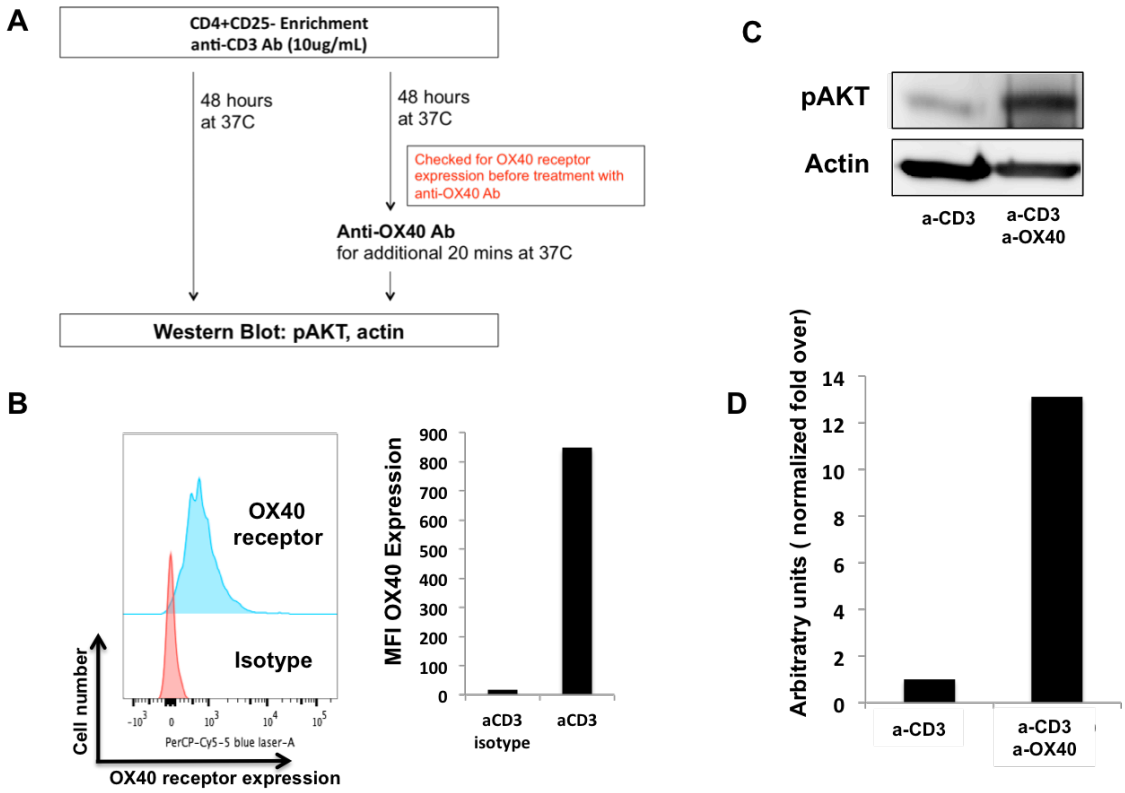

Figure 9. OX40-mediated stimulation increases pAKT in T convs. Purified CD4 Tconvs ($CD4^{+}CD25^{-}$) from naïve BL/6 mice spleen were activated with 10 ug/mL anti-CD3 Ab for 48 hours at 37C. The cell culture was then treated with anti-OX40 Ab (50ug/mL) for an extra 20 minutes, alongside non-treated control. Cells were lysed and protein homogenate was extracted and quantified for detection of active AKT level by Western blot assay (**A**). **B.** OX40 receptor was determined by flow cytometry before anti-OX40 Ab treatment. **C.** Level of pAKT was assessed by Western blot. Samples were normalized to actin, and the experimental group was expressed as fold change over the control group.

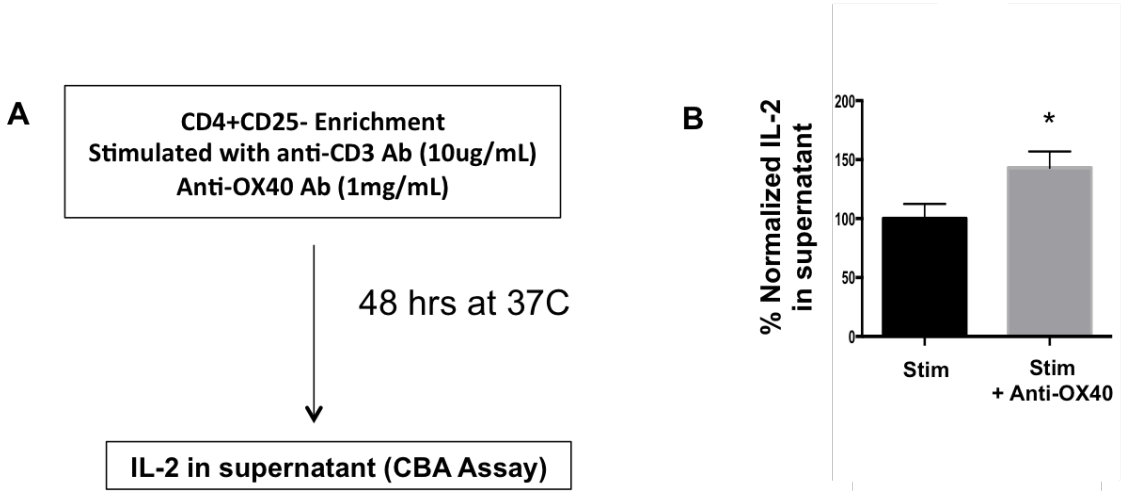

Figure 10. OX40 receptor engagement enhanced IL-2 secretion in T convs. Purified $CD4^{+}CD25^{-}$ T cells from spleens of naïve mice were cultured with plate-bound anti-CD3 Ab (10 ug/mL) and anti-OX40 at 1 mg/kg for 48 hours at 37C (**A**). Media was collected and measured for secreted IL-2 cytokine by CBA **(B).** The concentration of IL-2 was normalized to control stimulated sample and expressed in percentage. *p<0.05. Data shown are combined from two independent experiments.

Non-IDO Secreting TC-1 Tumor-Bearing Mice Exhibit Abundance of Suppressive IDO Enzymatic Activity in Tumor Microenvironment.

Many tumors, immune cells, epithelial cells, and fibroblasts may secrete the suppressive IDO enzyme. In order to apply the combinational immunotherapy of vaccine with anti-OX40 antibody while inhibiting the suppressive IDO enzyme to a variety of tumors, we challenged this immunotherapy to stringent conditions by choosing a tumor model that does not secrete IDO enzyme. In this tumor model, IDO is produced by the host cells only. The TC-1 cell line was subjected to IFN-γ cytokine for 48 hours, and the IDO RNA level was evaluated by qRT-PCR (Figure 11A). We show that IDO RNA was not found in TC-1 tumor *in vitro*, detecting the same level as in IDO-knockout (KO) mouse spleen tissue as a negative control, while the IDO level was significantly reduced when compared to the positive control (depicting spleen tissue from a mouse that was infected with murine leukemia virus, which is known to induce IDO enzyme), represented in Figure 11B. Additionally, the 4T1 breast cancer model was used as a control in IDO induction through the presence of IFN-γ in culture. To exhibit IDO expression by host cells *in vivo,* while demonstrating that pharmacological IDO inhibitor (1-MT) decreased the IDO enzymatic activity in the tumor environment, tumor cells were implanted on day 0 and treatment was initiated when the tumor reached 4-5mm in diameter. Tumor-bearing mice were treated with a peptide-based vaccine on days 10 and 17, and anti-OX40 Ab at an optimal dose of 1mg/kg was administered on days 10, 13, 17, and 20 while 1-MT was provided in drinking water throughout the whole experiment as

depicted in Figure 12A. Tumor was harvested on day 21, and the IDO enzymatic activity was assessed by detecting the kynerunine level (kynerunine is an IDO enzyme catabolite) by HPLC. Here, we not only demonstrated that the enzymatic activity of IDO is present in the tumor environment of non-IDO secreting TC-1 tumor cells for non-treated and vaccine with anti-OX40 Ab-treated mice, but the addition of 1-MT to the vaccine and anti-OX40 Ab significantly neutralized the suppressive IDO catalytic activity (Figure 12B).

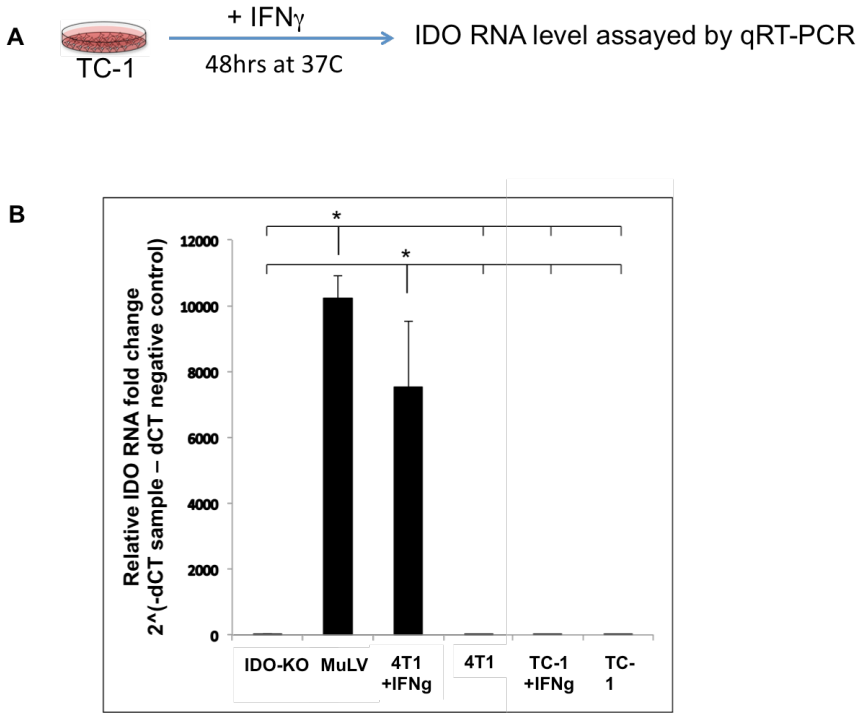

Figure 11. TC-1 tumor model does not secrete IDO. A. The TC-1 cell line was treated with IFNγ. After 48 hours incubation at 37C, **B.** the culture was assayed for IDO RNA by PCR. Three controls were included: tissue from IDO knockout mice (IDO KO), tissue from murine leukemia virus-infected mice, known for high IDO expression (MuLv), and IDO-inducible breast tumor model (4T1). *p<0.001.

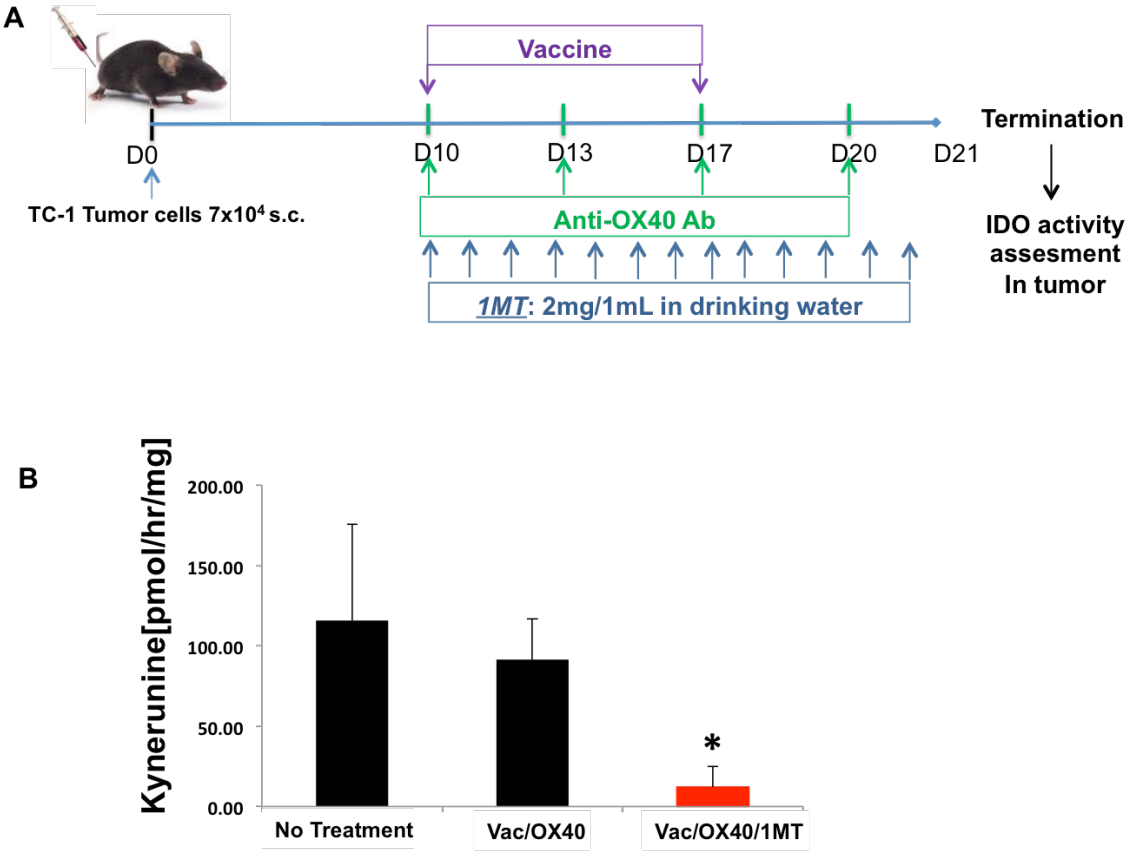

Figure 12. IDO secreted by host cells in tumor tissue. (A) Tumor-bearing mice were immunized on days 10 and 17 and treated with anti-OX40 Ab (1mg/kg) on days 10, 13, 17, and 20. IDO inhibitor (1-MT) was provided every day. **(B)** On day 21, tumor tissue was harvested and assessed for IDO activity by detecting the IDO catabolite, kynerunine. *p<0.001. Data representation is from combined three independent experiments.

Agonist anti-OX40 Ab Synergizes with IDO Inhibition and Vaccine in Promoting Tumor Eradication

To test the hypothesis that vaccine-mediated anti-tumor efficacy could be improved by combining it with IDO inhibition and agonist anti-OX40 Ab, we first evaluated tumor growth and survival of animals after combinational treatment in the TC-1 mouse tumor model. Tumor cells were implanted on day 0 and treatment was initiated when the tumor reached 4-5mm in diameter. Tumor-bearing mice were treated weekly with a peptide vaccine, anti-OX40 Ab was administered twice a week, 1-MT was provided in drinking water throughout the whole experiment as depicted in Figure 13A, and tumor growth and survival were monitored.

None of the single agent treatments significantly affects tumor growth, but the addition of 1-MT to vaccine and agonist anti-OX40 Ab treatment markedly enhanced the therapeutic efficacy from 20 % of mice having regressed tumors to a complete eradication of established TC-1 tumors in 60% of the mice that was sustained beyond 65 days (Figures 13B and 14). Re-challenging the cured mice with double the number of TC-1 tumor cells ($1.4x10^5$ cells) on day 70 resulted in no tumor growth in all animals tested (data not shown). These data demonstrate that the combination of anti-OX40 Ab, 1-MT and vaccine is a therapeutically potent strategy, which results in complete regression of established tumors and generates a protective immunity against tumor re-challenge.

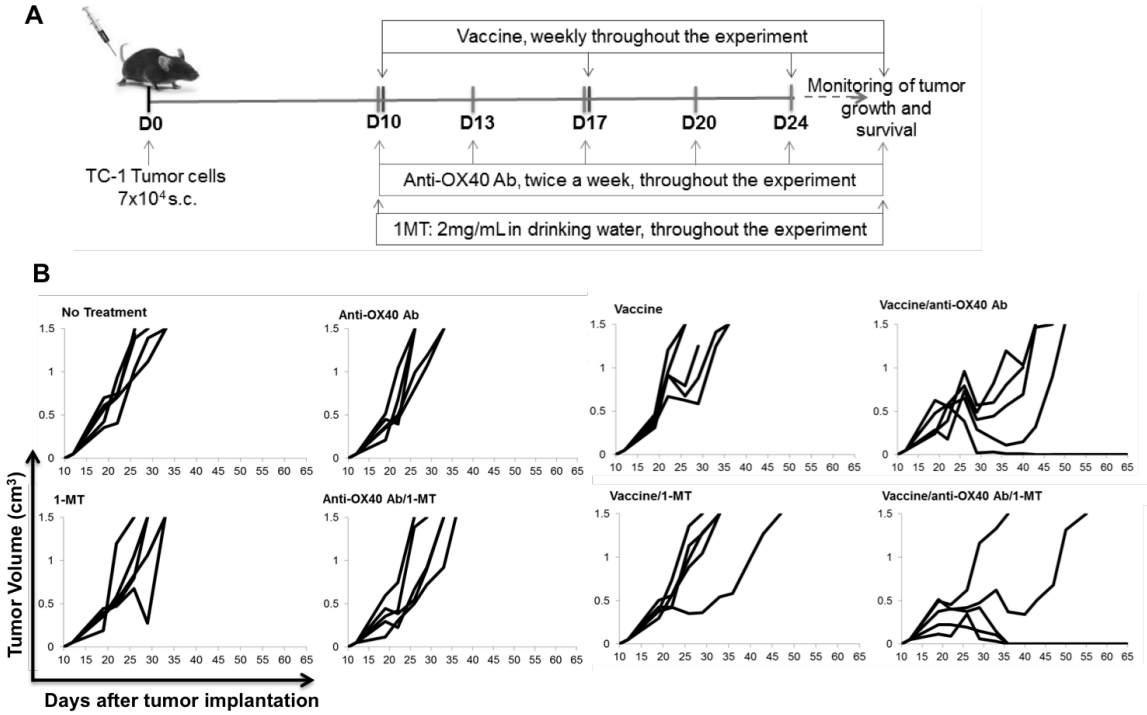

Figure 13. Combination of vaccine, anti-OX40 Ab and 1-MT provides potent anti-tumor therapeutic efficacy. A. C57BL/6 mice (n=5/group) were injected s.c. in the right flank with $7x10^4$ TC-1 cells. Mice from the appropriate groups were injected weekly with vaccine (s.c.) or PBS as a control. Anti-OX40 Ab was injected i.p. twice a week starting on Day 10 after tumor implantation throughout the experiment. 1-MT was provided in drinking water starting on Day 10 throughout the experiment. Tumor sizes were measured every 3-4 days. **B.** Plots represent tumor volumes of individual mice for each treatment. Similar results were obtained from two independent experiments.

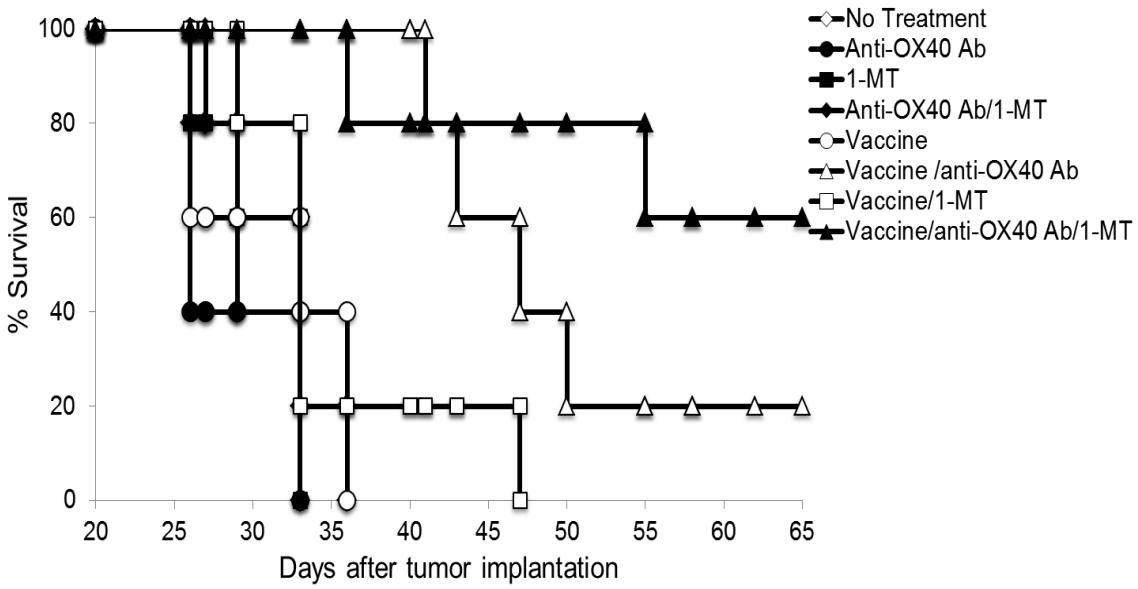

Figure 14. Combination of vaccine, anti-OX40 Ab and 1-MT prolongs survival of tumor-bearing mice. Inoculation of tumor, treatment dosing and schedule followed the same illustration as depicted in Figure 12 A. Kaplan–Meier plot of the overall survival for treated and non-treated mice. Similar results were obtained from two independent experiments.

IDO Inhibition Does Not Alter OX40 Expression

To understand the mechanism for the improved therapeutic outcome when vaccine with anti-OX40 Ab therapy was combined with 1-MT, we elucidated its impact on OX40 expression *in vitro*. Purified CD4 non-Tregs from naive murine spleen were cultured and activated with anti-CD3 and anti-CD28 Abs in the presence of IL-2 cytokine for 24 hours, followed with 1-MT treatment for an additional 48 hours (Figure 15A). The effect of three concentrations (10, 100, 1000 ug/mL) of 1-MT on OX40 receptor was evaluated by flow cytometry. While the OX40 receptor is not expressed on naïve T cells (Figure 15B), and is inducible upon the activation of T cells (Figure 15C), the mean fluorescence intensity (MFI) of OX40 receptor expression is not altered by 1-MT at any concentration, either in naïve or activated T cells (Figure 15D).

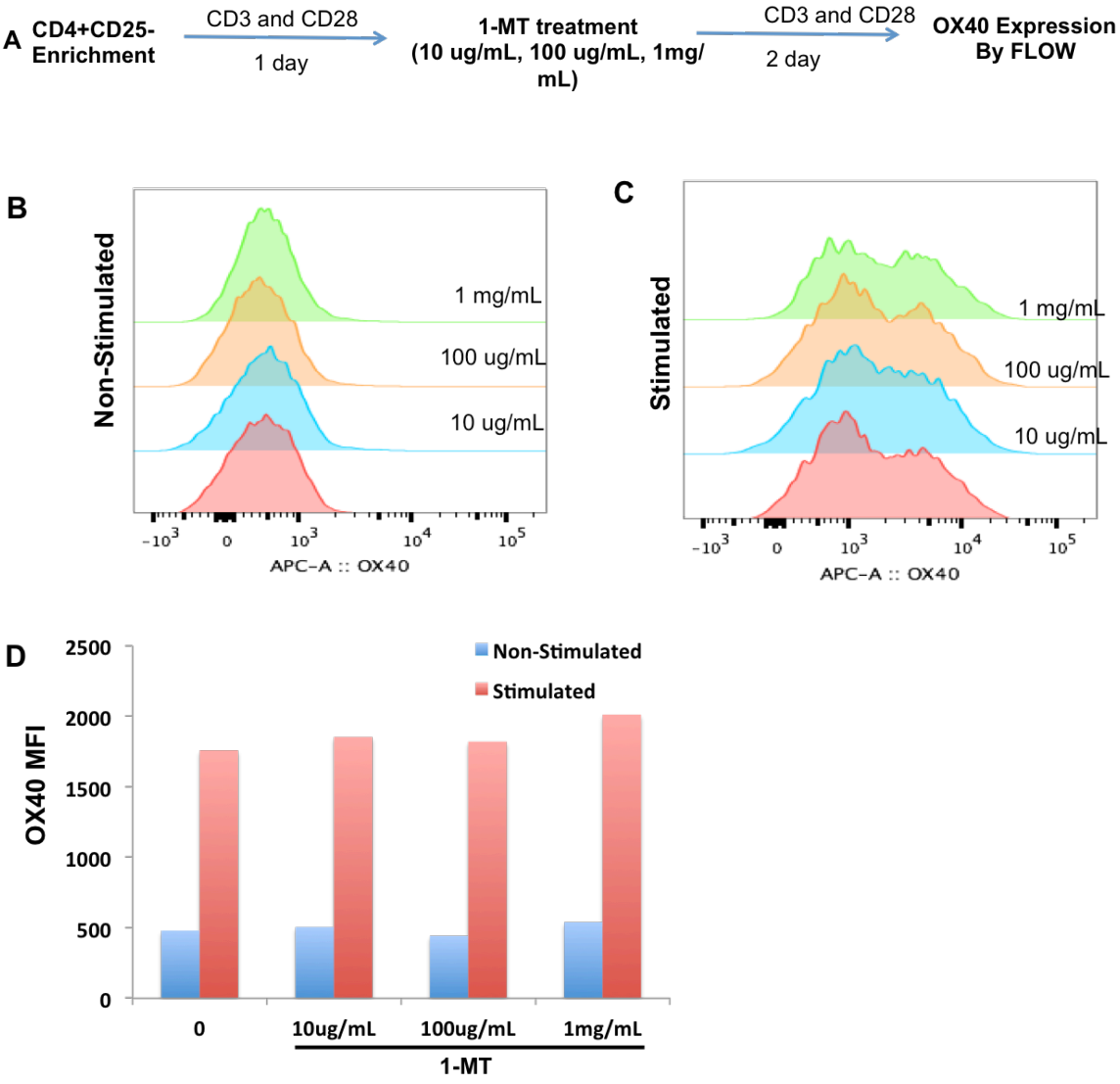

Figure 15. IDO inhibition does not alter OX40 receptor expression. (A) Purified $CD4^{+}CD25^{-}$ T cells (CD4 Tconvs) were activated with anti-CD3 and CD28 antibodies for 24 hours and then treated with IDO inhibitor 1-MT for an additional 48 hours at 0.01, 0.1, and 1 mg/mL. OX40 expression was assessed for (B) non-stimulated, and (C) stimulated cell cultures. (D) Data represents MFI for non-stimulated and stimulated, treated cells.

Combinational Treatment with Vaccine/anti-OX40 Ab/1-MT Results in Significant Increase of CD8/Treg Ratio within Tumor Environment

To understand the immune mechanisms leading to the therapeutic efficacy of vaccine/anti-OX40 Ab/1-MT treatment, we next profiled the tumor-infiltrating T cells. Animals were treated as described above in Figure 13A; however, four days after the second vaccination, mice were sacrificed and tumors were harvested for the evaluation of tumor-infiltrating T cell subsets by flow cytometry. A representative gating strategy for T cells is illustrated in Figure 16, where a single cell population of live cells was gated on $CD45^{+}CD3^{+}$ lymphocytes and further separated into CD4 and CD8 T cells. CD4 T cells were divided into FoxP3-expressing Tregs or $CD4^{+}Foxp3^{-}$ non-Tregs.

We observed a significant enhancement of non-Treg CD4 T ($CD4^{+}FoxP3^{-}$) cells when the vaccine was combined with anti-OX40 Ab (Figure 17A). This non-Treg CD4 T cell enhancement resulted in a significant decrease in the percentage of tumor-infiltrating Treg cells in the total CD4 population, for both vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT groups (Figure 17B).

Next, we explored the effect of combinational treatment on tumor-infiltrating CD8 T cells. We found that treatment with the vaccine in combination with anti-OX40 Ab significantly increased the number of CD8 T cells when compared to mice from control groups (Figure 18A). The addition of 1-MT treatment to vaccine/anti-OX40 Ab non-significantly increased the number of tumor-infiltrating CD8 T cells over the vaccine/anti-OX40 Ab group (Figure 18A). As a result of the CD8 T cell increase in

tumors, both vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT treatments showed a significant increase in the CD8/Treg ratio when compared to control groups (Figure 18B).

These data demonstrate that vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT treatments exert an effective anti-tumor immune response through an increase of tumor-infiltrating non-Treg CD4 and CD8 T cells, suggesting that this effect is predominantly facilitated by addition of anti-OX40 antibody to vaccine treatment.

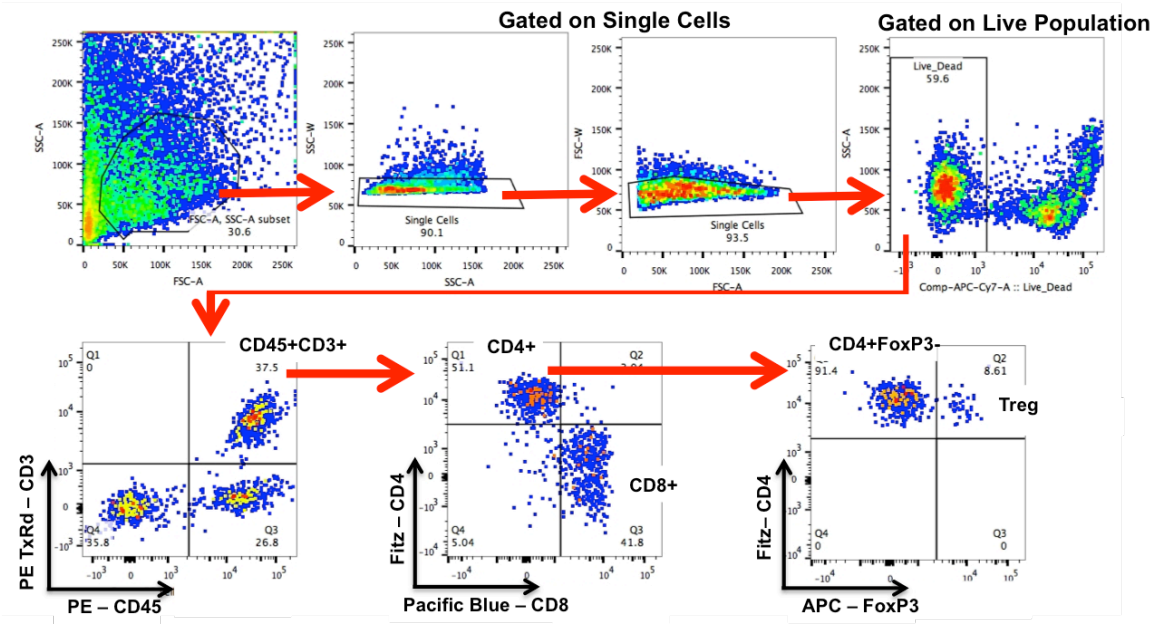

Figure 16. A representative gating strategy by flow cytometry. C57BL/6 mice (n=4-5/group) were injected with 7x10^4 TC-1 cells and treated as for therapeutic studies as illustrated in Figure 13A, except on Day 21 mice were sacrificed, and tumor-infiltrating lymphocytes were analyzed in tumor homogenates by flow cytometry. The live single cell population was gated on CD45$^+$CD3$^+$ lymphocytes, which were separated into CD8$^+$ and CD4$^+$ T cells. CD4$^+$ T cells were further divided into CD4$^+$FoxP3$^+$ (Tregs) and CD4$^+$FoxP3$^-$ (CD4 Tconv) subsets.

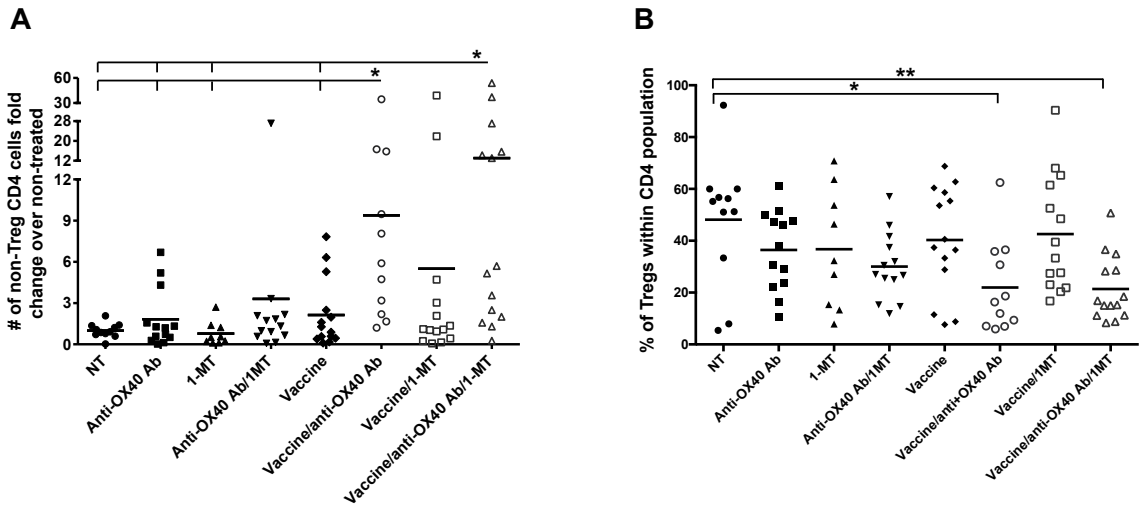

Figure 17. Both vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT treatments led to an increase of non-Treg CD4 T cell infiltration into the tumor. Inoculation of TC-1 tumor treatment protocol is illustrated in Figure 13A with the exception that tumors were harvested on day 21 and analyzed for CD4 T cell infiltration into the tumor 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 the non-treated group for **(A)** non-Treg CD4 T cells ($CD4^+$ $FoxP3^-$), or as **(B)** a percentage of Tregs within the $CD4^+$ population. *p<0.05, **p<0.01. Combined data from three independent experiments are shown. Gated within $CD45^+CD3^+$ population.

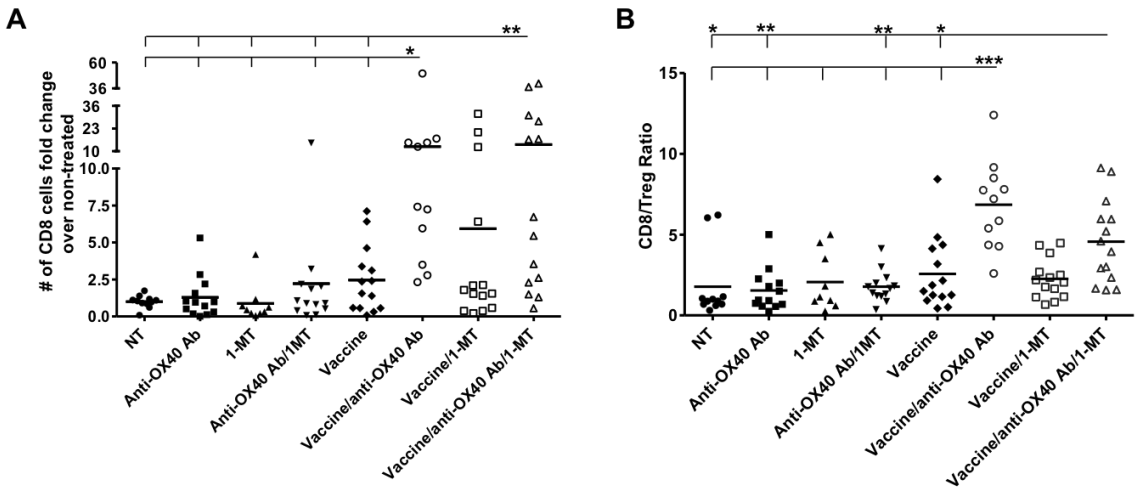

Fig. 18. Both vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT treatments led to an increase of CD8 T cell infiltration into the tumor and in the CD8/Treg ratio. C57BL/6 mice (n=4-5/group) were injected with $7x10^4$ TC-1 cells and treated as for Figure 17, and tumor-infiltrating CD8 T cells were analyzed in tumor homogenates by flow cytometry. **A.** The absolute numbers of tumor-infiltrating CD8 T cells were standardized per 10^6 of total tumor cells and presented as fold change over non-treated group **B.** The CD8/Treg ratio was calculated from normalized numbers of CD8 T cells

and Tregs per 10^6 of total tumor cells. *p<0.05, **p<0.01, ***p<0.001. Combined data are from three independent experiments.

Addition of 1-MT to vaccine/anti-OX40 Ab Treatment Leads to Increase in Functional CD8 T cell

We have demonstrated that the addition of 1-MT to vaccine/anti-OX40 Ab immunotherapy did not increase the number of tumor infiltrating CD8 T cells or the CD8/Treg ratio compared with vaccine and anti-OX40 (Figure 18A and B), although we observed significant differences in the therapeutic efficacy between these treatments (Figures 13 and 14). Therefore in addition to evaluation of the total numbers of cells, we tested the specificity and functionality of tumor-infiltrating CD8 T cells, which could be responsible for the differences in therapeutic efficacy between vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT treatments.

The functionality of tumor-infiltrating CD8 T cells were evaluated from mice treated with vaccine, vaccine/anti-OX40 Ab, vaccine/1-MT, or vaccine/anti-OX40 Ab/1-MT as described above. Despite the fact that the addition of anti-OX40 Ab to vaccine was able to significantly increase the number of CD8 T cells compared to vaccine alone (Figure 18A), we further evaluated the functionality of these CD8 T cells. Granzyme B (GrzB) expression was used to characterize the functionality of the CD8 T cells. We demonstrated that addition of 1-MT to vaccine/anti-OX40 Ab also led to a significant increase in the number of tumor-infiltrating $CD8^{+}GrB^{+}$ T cells compared to all other groups (Figure 19A). Furthermore, the addition of 1-MT to vaccine significantly increased the percentage of GrzB+ CD8 T cells (Figure 19B) within the CD8 population. A representative gating strategy is portrayed in Figure 19C.

These data demonstrate that addition of 1-MT to vaccine/anti-OX40 Ab leads to increased numbers of functional CD8 T cells and might at least partially explain the differences in therapeutic efficacy observed between the treatment groups.

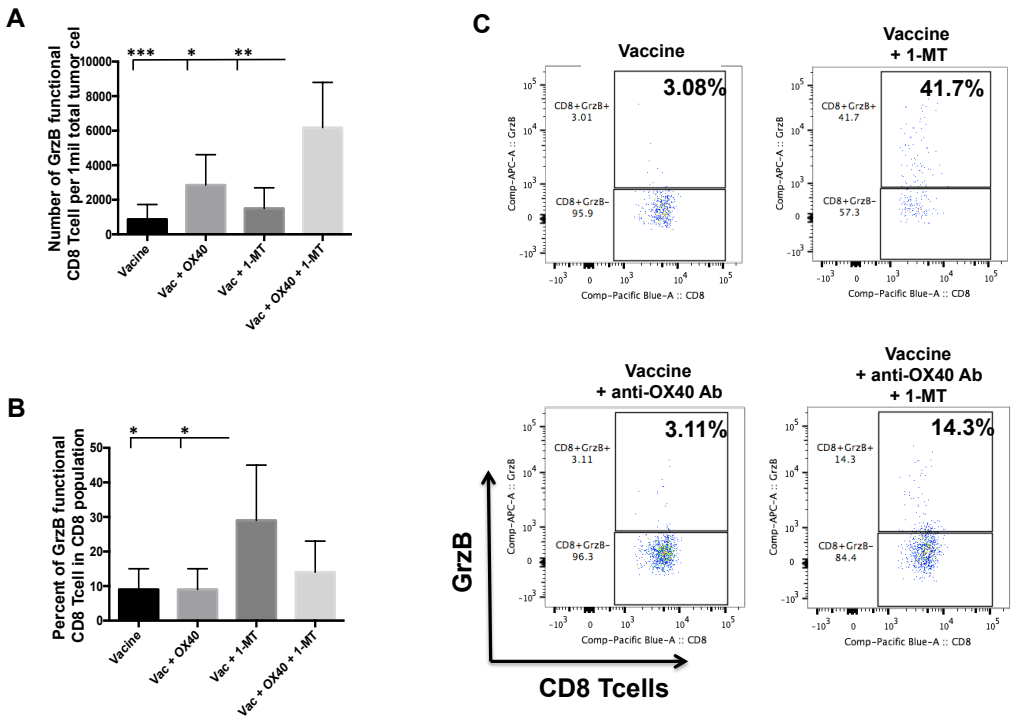

Figure 19. Addition of 1-MT to vaccine/anti-OX40 Ab treatment leads to an increase of GrzB$^+$ CD8 T cells in tumor. Tumor-bearing C57BL/6 mice (n=5/group) were treated with vaccine alone, vaccine/anti-OX40 Ab, vaccine/1-MT or vaccine/anti-OX40 Ab/1-MT as described above. The absolute numbers of tumor-infiltrating GrzB$^+$ CD8 T cells (**A**) were standardized per 10^6 of total tumor cells. And percent of GrzB$^+$ CD8 T cells (**B**) within the CD8 population were evaluated by flow cytometry. Data are presented as mean values ±SD. *p<0.05 compared to all other groups. Data represent combined three independent experiments. Gating is within CD45$^+$CD3$^+$ population.

IV. DISCUSSION

Immunosurveillance is an important strategy used by the immune system to protect our body from any foreign invaders and to recognize cells that provide strong enough immunogenic signaling for elimination. However, if malignant cells are not eliminated, the interactions between these cells and the host immune system in the tumor microenvironment create an immunosuppressive network, which can have a negative impact in the long term. The elimination and equilibrium phases of immunoediting provide a selection against immunogenic malignant cells, while progressively leaving the less immunogenic behind, which eventually become immune-resistant cancerous cells. Cancerous cells strive in this tumor immunosuppressive environment not only by gaining resistance due to tumor intrinsic genetic changes, which equip the malignant cells with an overexpression of anti-apoptotic molecules and by the down-regulation of immunogenic antigens that are no longer accessible for immune system recognition, but more importantly, the malignant cells impose negative extrinsic immunosuppressive aspects that protect the tumor from immune assault. The synchronization of immunosuppressive soluble molecules, inhibitory checkpoint receptors, and suppressive immune cells attenuates the efficacy of immunotherapy approaches. Thus, it is essential to boost immunity by harnessing anti-tumor-specific immune cells that must endure this immunosuppressive network in the tumor microenvironment and as well impose an immune cytotoxic force for tumor elimination. To combat this strong tumor-promoting

environment, a T cell must first be able to recognize the tumor and be directed to it. Therefore, we incorporated a therapeutic vaccine into our immunotherapy that would generate a tumor antigen-specific immune response. While peptide-based vaccines integrate CD8 cytotoxic peptides, some studies report an abrogation of vaccine response when CD4 T cells are depleted (Riddell & Greenberg, 1995). Thus, the vaccine composition in this study involved the incorporation of T helper peptide within the CTL-specific epitope. Here, we demonstrate that E7-transfected tumor-bearing mice immunized with peptide-based vaccine against human papilloma virus 16 (HPV16) E7 cytotoxic T lymphocyte and PADRE T helper epitopes generated tumor E7-antigen-specific CD8 T cell infiltration into the tumor environment and triggered an antigen-specific immune response of splenocytes from immunized mice when re-stimulated with both vaccine peptide components (Figure 1 and 2). It was reported that an antigen-specific immune response can be induced in almost all peptide-vaccinated patients (Slingluff et al., 2007), and CTLs induced by peptide vaccine can recognize and lyse melanoma cells when co-cultured (Yamshchikov et al., 2001). Although vaccine-based immune responses are promising and effective to direct T cells for tumor recognition, the immunogenic response is often times poor to effectively impose killing of the tumor, especially for advanced established tumors (Rosenberg, Yang, & Restifo, 2004). Additionally, adoptive transfer of T cells primed with a single tumor peptide effectively eliminated tumor cells expressing that antigen, but the malignancy did not regress due to antigen-loss variants (Yee et al., 2002). To partially overcome the drawbacks associated with the selection for a tumor-specific antigen, a vaccination comprising multiple peptides against a diverse set of tumor antigens may be implemented, or an immunization

with a whole tumor lysate may provide targeting against a repertoire of antigens expressed by the tumor; however, the low immunogenic response delivered by vaccination is still not resolved (Chiang, Coukos, & Kandalaft, 2015; Slingluff et al., 2007; Walter et al., 2012). In our study, we strengthened the antigen-specific T cell immune response by targeting the immune co-stimulatory receptor, OX40, expressed on T cells and combining it with vaccine therapy. Triggering OX40 receptor with its associated ligand, or agonist anti-OX40 antibody, or OX40L-Fc fusion protein led to prolonged T cell persistence and to enhanced T cell function, proliferation, and memory. Two properties make OX40 an appealing target for anti-cancer therapies; the OX40 receptor is predominantly expressed on activated T cells and it is restricted to sites of inflammation, such as cancer. Therefore, direct stimulation of immune cells of non-T cell lineage and peripheral side effects are limited. Altogether, therapies targeting immune co-stimulatory OX40 receptor promote anti-tumor immune responses, which lead to promising anti-cancer outcomes.

Here, we demonstrate that anti-OX40 antibody enhances vaccine efficacy for both CD4 and CD8 T cell antigen-specific immune responses, which correlates with other studies showing that anti-OX40 Ab has a positive effect on expansion of CD8 T cells to exogenous antigens (S. Murata et al., 2006). However, some report that anti-OX40 Ab has no effect on OT-1 CD8 T cell expansion in response to OVA with anti-OX40 Ab (Lee et al., 2004), and moreover, the OX40/OX40L interaction is not required for the CD8 and CD4 T cell response to SEA superantigen *in vivo* (Dawicki, Bertram, Sharpe, & Watts, 2004). The discrepancy may be due to the antigen immune stimulatory strength already inducing a strong enough antigen-specific immune response; thus, a further

OX40/OX40L interaction would be redundant. More importantly, we show that 1mg/kg of anti-OX40 Ab with vaccine enhanced the antigen-specific immune response, while increasing the dose to 2.5 mg/kg abolished the effect and is comparable to the level of vaccine stimulation alone (Figure 2). Therefore, in the study where anti-OX40 Ab treatment did not have any effect on OT-1-specific CD8 T cell stimulation (Lee et al., 2004), the dose they used was above 50ug/mL, which is 20 times above the high dose (2.5mg/kg) in our study, which did not enhance the antigen-specific immune response as well. Therefore, these data emphasize that the dose optimization of anti-OX40 Ab is necessary when considering tumor-reactive T cells.

Interestingly, we detected that a high dose of anti-OX40 Ab with vaccine triggered an antigen-specific immune response for the CD4 T helper epitope (PADRE) but not for CD8 T cell-specific E7-peptide. This observation closely relates with the cellular immune response of T cells, which we found in the tumor microenvironment. Here, we demonstrate that a therapy of a high dose (2.5 mg/kg) of anti-OX40 Ab with vaccine significantly accumulated suppressive T regulatory cells in the tumor. It is well established that Tregs have the ability to suppress other T cells to limit the stimulatory immune response (Sakaguchi et al., 2009). Therefore, it is conclusive to suggest that this high Treg buildup in tumor that we observed, suppressed the number of effector CD8 T cells as we observed in the decreased CD8 T cell infiltration into tumor at a high dose of anti-OX40 Ab when compared to the optimal dose of 1mg/kg of anti-OX40 Ab with vaccine (Figure 5). The ability of OX40 agonist to promote CD8 T cell expansion is well known (Pakala et al., 2004); however, there are conflicting reports regarding Tregs. To date, there are several reports speculating about OX40 suppressing Treg function and

development (So & Croft, 2007; Takeda et al., 2004; Valzasina et al., 2005; Vu et al., 2007). Some studies have found that ligation of OX40 receptor suppresses Treg function by turning off transcription factor FoxP3 (Vu et al., 2007) and preventing the development of Tregs (So & Croft, 2007), while other studies report OX40 having no effect on Tregs (Curti et al., 2013; Redmond et al., 2014) or showing that Treg cells expanded upon OX40 receptor trigger (Takeda et al., 2004). An interesting study revealed that OX40-dependent Treg mediation is based on the cytokine milieu in which the Tregs reside (Ruby et al., 2009). While Ruby and his colleagues showed that triggering OX40 receptor increased IL-4 and IFN-γ cytokine production, which led to TGF-beta-mediated Treg suppression, we also believe that this inconsistency in preclinical studies may be due to differences in the treatment schedules and doses used. In fact, Ruby showed that anti-OX40 Ab given at disease onset in the EAE murine model exacerbated the disease, whereas if given at antigen priming, the disease progression and severity was reduced (Ruby et al., 2009). Another study focused on schedule optimization targeting OX40 receptor in cancer and found that Fc-OX40L provided at the same time with vaccine led to a greater therapeutic index than if Fc-OX40L was given alone or at a later time after immunization (Murphy et al., 2012).

Furthermore, one of the mechanisms for OX40-mediated Treg depletion was shown to be dependent on the expression of activating Fc gamma receptors (FcγRs) (16). The expression of FcγRs on macrophages, monocytes, eosinophils and NK cells can recognize the Fc region of an antibody bound to a cell and kill it via the induction of antibody dependent cell cytotoxicity (ADCC) or antibody dependent cell phagocytosis (ADCP). Although these ADCC or ADCP phenomena are an interesting tactic to

eliminate Tregs, and manufactured antibodies inducing cytotoxicity have been tested in anti-cancer preclinical studies with successful outcomes (Bulliard et al., 2014; Chester, Ambulkar, & Kohrt, 2016; Smyth, Ngiow, & Teng, 2014), the capacity to achieve the ADCC or ADCP effect is dependent on a few factors. The isotype of antibody classes or the antibody glycosylation structure may affect the level of ADCC effector function, and the host environmental immune factor plays an important role. Anti-cancer immune responses would not be ADCC-mediated if there are no activating FcγR-expressing cells present. While it has been reported that anti-OX40 Ab at a higher dose than we used has eliminated Tregs in an ADCC manner (Bulliard et al., 2014)(18), it is unlikely that in our study, the ADCC function has occurred due to the Treg enhancement at the high dose of anti-OX40 Ab; however, although it is beyond the scope of this study, it would be interesting to evaluate if it may be due to the lack of meeting the above factors to accommodate effective ADCC function.

Numerous studies show that there is an abundance of Tregs in tumor tissue, suppressing the induction and activation of effector T cells, which creates a suppressive immune obstruction that prevents the tumor from immune elimination (Nishikawa & Sakaguchi, 2014). Therefore, many immunotherapies specifically target Tregs to achieve an anti-cancer immune response. Thus, it is appealing to conclude that the diverse effect of the different doses that we see on the immune response in our data is the reason for the distinct outcome in anti-cancer therapy in tumor-bearing mice. We demonstrate that 1mg/kg of anti-OX40 Ab with vaccine has led to slower tumor progression, complete tumor regression in 20% of mice having established tumor, and prolonged survival when compared to all other experimental groups (Figure 3). Strikingly we show that treatment

with a higher dose (2.5 mg/kg) of anti-OX40 Ab with vaccine completely abrogated the anti-cancer therapeutic properties, resembling the outcomes of animals either untreated or treated with a monotherapy (Figure 3). We have shown that the T cell response correlates with the survival outcome.

In summary, we demonstrate that there is an optimal dose of anti-OX40 Ab (1mg/kg) with vaccine that leads to effector immune stimulation without accumulating Tregs in the tumor environment, which is responsible for the positive therapeutic outcome. In contrast, a dose increase to 2.5mg/kg of anti-OX40 Ab with vaccine enhances the suppressive regulatory T cells, leading to no significant effector increase in the clinically relevant CD8/Treg ratio, with attendant loss in anti-cancer therapeutic efficacy (Figure 3,4).

Further, we have been intrigued by the surprising results, where different doses of anti-OX40 Ab has differential effect on CD4 T cells. While all the doses of agonist OX40 Ab significantly increased the number of effector CD4 Tconvs, only the high dose of 2.5 mg/kg increased the number of Tregs in tumor when compared to non-treated tumor-bearing mice (Figure 7). Here, for the first time to our knowledge, we have titered OX40 doses *in vivo* and demonstrated the doses' differential impact on T cell subsets. The dosage of anti-OX40 Ab may be one of the possible explanations in studies showing the inconsistency of OX40-mediated effect on Tregs (Hippen et al., 2008; Ruby et al., 2009; So & Croft, 2007). Taken together, this emphasizes that the strength of anti-OX40 Ab dosage mediates the ratio between effector and suppressive immune response, which reflects on the therapeutic outcome in cancer. In addition, one must be cautious to generalize the OX40-mediated effect on T cell subsets *in vivo* due to the different factors

each tumor model carries.

We further tried to understand the above results among the CD4 T cell subsets mediated by the different doses of anti-OX40 Ab. We showed that it is not due to variability of the OX40 receptor among the T cell subsets (Figure 8). Further, we studied the molecular mechanism. T cell activation is initiated upon the TCR recognition of peptide-MHC complex expressed on APCs and upon the binding between co-stimulatory molecules, which initiates a downstream signaling cascade (Wells et al., 1997). One of the essential molecules for T cell growth, survival, and differentiation is the activation of AKT (Okkenhaug, Turner, & Gold, 2014). It was also reported that stimulation of OX40 receptor plays a major role in AKT recruitment and activation (J. Song et al., 2004). In our laboratory, we have previously demonstrated that different pharmacological AKT inhibitors can selectively inhibit the activation and proliferation of Tregs with a minimum effect on CD4 Tconvs (Abu-Eid et al., 2014). We thought that OX40 downstream PI3K/AKT pathway may be closely related to the differential effect on T cell subsets for the various doses of anti-OX40 Ab we present here (Figure 7). To evaluate the molecular mechanisms responsible for differential outcome of the various doses of anti-OX40 Ab on CD4 T cells *in vivo*, we assessed the OX40-mediated direct effect on the activation of AKT in $CD4^{+}CD25^{-}$ Tconvs isolated from naïve mouse spleen treated with anti-OX40 Ab *in vitro*. It is important to mention that to demonstrate the pure OX40 influence on T cells, we stimulated T cells only with anti-CD3 Ab (in the absence of CD28 stimulation and IL-2 cytokine addition to culture) to induce OX40 receptor expression (Figure 9B). We found that the engagement of OX40 receptor with anti-CD3 Ab stimulation significantly increased the level of active AKT in splenocyte cultures when compared to

CD3-stimulated only sample (Figure 9C). This closely relates to a study reporting that the OX40/OX40L interaction can mediate AKT activation in CD4 T cells when MHC-specific TCR-transfected T cells with OX40 receptor are co-cultured with OX40L-transfected DCEK cells expressing endogenous CD80 (as a source of APCs) in the presence of MCC peptide (So et al., 2011). One key difference between our study and theirs is that their assay involves the interaction between co-stimulatory molecules CD28 and CD80. Binding of CD28 on T cells to CD80 on APCs led to phosphorylation of AKT (Okkenhaug et al., 2014). Thus, in our assay, we omitted CD28/CD80 co-stimulation in order to see the pure anti-OX40 Ab effect on the activation of AKT.

Here, for the first time to our knowledge, we show that OX40 engagement alone with anti-CD3 Ab (anti-CD3 Ab stimulation of T cells was the minimum requirement for OX40 receptor expression *in vitro*) significantly engages in the activation of AKT in the CD4 T cell subset (Tconv cells) (Figure 9C). Furthermore, OX40 receptor stimulation significantly increased cytokine IL-2 secretion by Tconv cells (Figure 10B), which promotes growth and survival. The augmented IL-2 secretion upon OX40 triggering signifies the enhanced activity and functionality of T cells. Another study reports that IL-2 is important for Treg proliferation (T. Zou et al., 2012). These data provide the evidence that OX40 alone plays an important role in AKT activation in conventional CD4 T cells. And while the AKT inhibition differentially affects the CD4 T cell subpopulation resulting in selective Treg reduction while leaving CD4 Tconvs intact, it prompts the intriguing assumption that the possible explanation for the CD4 T cell subset's distinct response upon OX40 engagement *in vivo* that we show here may be due to variable stimulatory OX40 strength on downstream AKT signaling. Another additional

theory may be that OX40 stimulation leads to a high number of CD4 Tconvs causing a prominent amount of IL-2 cytokine secretion, which is consumed by Tregs, resulting in their proliferation (Moran et al., 2015). This highlights and may correlate with our finding for Treg increase *in vivo* facilitated by a high dose stimulation of agonist anti-OX40 Ab. In our study, the stronger dose of anti-OX40 Ab may gradually intensify IL-2 secretion from the high accumulation of CD4 T convs *in vivo*, which indirectly impacts the expansion of Tregs. It would be noteworthy to investigate the direct (AKT signaling) and indirect (IL2 cytokine) effect on CD4 T cell subsets mediated by OX40 receptor stimulation in order to elucidate the potential mechanism of action that will help in effective planning for future combinational immunotherapies.

As mentioned in the introduction, the major hurdle of immunotherapy is overcoming the immune suppressive environment imposed by the tumor. We showed that agonist anti-OX40 antibody with vaccine is robust in promoting an immune stimulatory response mediated by a significant increment of effector T cells in the tumor environment. However, while the anti-OX40 Ab with vaccine therapy led to significant inhibition of tumor growth, only 20% of animals showed complete regression of established tumors (Figure 13). This could be attributed to the existence of multiple tumor-mediated inhibitory mechanisms preventing it from immune elimination. One of the main suppressive factors that prevent T cells from properly exhibiting killing function against tumor is the high abundance of inhibitory IDO enzyme in cancer (Munn & Mellor, 2007). We hypothesized that the power of targeting both stimulatory and inhibitory arms of immunity will lead to anti-cancer immune restructuring in the tumor environment, providing an effective anti-tumor response. Thus, we tested if addition of

stimulating the immunity by anti-OX40 Ab, while targeting the suppressive immunity by IDO inhibitor, 1-MT, would lead to increased therapeutic outcome due to the interaction of these compounds generating novel synergistic mechanisms.

First, we deliberately selected the TC-1 tumor model for the evaluation of IDO inhibitor-based immunotherapy, since these tumor cells (in culture and *in vivo*) do not express IDO (Figure 11B, data not shown for *in vivo*), so the observed effect elicited by 1-MT is not due to its direct effect on tumor cells but rather by the host cells within the tumor microenvironment. Furthermore, under these stringent conditions, where IDO secretion is limited, the application may be provided to a broader spectrum of cancers, assuming that 1-MT treatment would deliver a greater benefit for IDO-secreting tumors.

Secondly, we show by HPLC that the suppressive IDO enzymatic activity in the tumor tissue is in abundance in animals either untreated or treated with vaccine/anti-OX40 Ab, which manifests the immune suppressive environment. To target the inhibitory IDO molecule and to confirm the efficacy of 1-MT, we tested for IDO enzymatic activity within tumor homogenates from vaccine/anti-OX40 Ab/1-MT and its controls by HPLC. No difference in IDO activity between vaccine and vaccine/anti-OX40 Ab-treated mice was observed whereas the addition of 1-MT to vaccine/anti-OX40 Ab significantly decreased IDO activity compared to the other groups (Figure 12B).

We observed that the addition of IDO inhibitor to vaccine/anti-OX40 Ab treatment led to substantial enhancement of anti-tumor efficacy and resulted in complete tumor eradication in 60% of mice (Figure 13B). Moreover, the combinational vaccine/anti-OX40 Ab/1-MT therapy generated long-lasting immunity; when mice with entirely regressed tumors were re-challenged with double the amount (140,000 tumor

cells) of TC-1 tumor than injected initially, they were protected against developing new cancer when compared to the no treatment group (data not shown).

After showing that 1-MT does not alter the expression of OX40 receptor on conventional CD4 T cells *in vitro* (Figure 15), we continued to analyze the cellular immune mechanism responsible for this potent therapeutic outcome. We found that a significant increase of tumor-infiltrating CD8 T cells after combinational treatments is mediated by anti-OX40 antibody, since both vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT -treated mice had similar numbers of CD8 T cells (Figure 18A). Likewise, the anti-OX40 Ab was shown to be responsible for the increase in non-Treg CD4 T cell infiltration into the tumor, thus resulting in decreased percentage of Treg cells (Figure 17). Additionally, one must be careful to consider the number of mice responding to a treatment when evaluating the immune response. Here, we show that adding 1-MT to vaccine/anti-OX40 Ab further increased the number of CD8 and non-Treg cells, when compared to other groups, except for the vaccine/anti-OX40 Ab group, but the enhancement of effector T cells may be due to the higher number of responders in vaccine/anti-OX40/1-MT-treated animals.

Finding no differences in the numbers of tumor-infiltrating CD8 T cells as well as in the CD8/Treg ratio, which are well-established criteria that correlate with cancer prognosis (Curran et al., 2010; Shen et al., 2010), between the vaccine/anti-OX40 Ab and vaccine/anti-OX40 Ab/1-MT treatments was somewhat surprising, since the therapeutic efficacy for these treatments was significantly different (Figures 14 and 18). To understand this phenomenon, we evaluated the functionality of tumor-infiltrating CD8 T cells. Notably, we found that the addition of 1-MT to vaccine significantly increased the

percentage of GranzymeB^{+} functional CD8 T cells within the tumor infiltrating CD8 population (Figures 19B). Additionally, the number of GranzymeB^{+} functional CD8 T cells were significantly elevated within the tumors of mice when 1-MT was concomitantly provided with vaccine/anti-OX40 Ab in comparison to other groups, including vaccine/anti-OX40 Ab-treated mice (Figures 19A). This might at least partially explain the differences that we observe in anti-tumor therapeutic efficacies between these two treatments.

Under normal physiological conditions, it is well established that IDO contributes to the maintenance of immune tolerance (Uyttenhove et al., 2003), and the suppressive phenomenon in the tumor can drive effector T cells into dysfunctional or unresponsive to tumor derived antigens (Makkouk & Weiner, 2015). Thus, one possibility for increased functionality of vaccine-induced effector CD8 T cells within the tumors of these mice (Figure 19) is the decrease in IDO enzymatic activity by 1-MT.

The soluble IDO expression imposes immunosuppressive activity by its catalytic ability to degrade tryptophan in the tissue. The essential amino acid deficiency of tryptophan leads to accumulation of uncharged tRNA recognized by the stress sensing kinase GCN2 in T cells (Platten, von Knebel Doeberitz, Oezen, Wick, & Ochs, 2014). Increased GCN2 causes proliferative arrest and anergy in T cells (Munn et al., 2005). Therefore, restoring sufficient amounts of tryptophan in the tumor environment by 1-MT for T cell proper function may be one of the reasons to observe the increase in cytotoxic CD8 T cells (Figure 19).

Another reason for CD8 T cell mediated functionality may be due to direct priming by dendritic cells. One of the immune cells that are well studied and known to

express IDO are DCs, more specifically plasmacytoid (pDCs (Munn et al., 2004). The generation of IDO enzyme in DCs can be in response to immune factors, such as the induction of cytokines IFN-γ and TGF-β (Chen, 2011), the interaction between co-stimulatory receptor 4-1BB on T cells with its ligand on DCs (Seo et al., 2004), and the binding of co-inhibitory CTLA-4 receptor on Tregs to the CD80 molecule on DCs (Finger & Bluestone, 2002). IDO-positive DCs display increased PD-L1 and PD-L2 level compared to IDO-negative DCs (Von Bubnoff, Scheler, Wilms, Fimmers, & Bieber, 2011). Binding of PD-L1/2 ligand to PD-1 receptor on T cells leads to their exhaustion (Pauken & Wherry, 2015). Therefore it is probable that in this study, the inhibition of IDO activity in DCs may lower the expression of the PD-L1 checkpoint, hence preventing T cell tumor-specific anergy and preserving their full activity in eliciting cytotoxic function against tumor.

Furthermore, CD80 preferentially binds to co-inhibitory CTLA-4 receptor than to co-stimulatory CD28 receptor on T cells. Thus, the high binding affinity of CTLA-4 receptor to CD80 on DCs limits the available CD80 for binding to co-stimulatory CD28 receptor, which is necessary for full T cell activity. It is possible that OX40 molecule, being the second co-stimulatory receptor may partially substitute for the defective CD28 T cell stimulation, which may resemble the effective therapeutic potency when combined with vaccine (Figures 3 and 4). On the contrary, since 4-1BBL and OX40L are both immune stimulatory molecules expressed on DCs, it may well be probable that stimulation of OX40L resembles 4-1BBL triggering IDO expression in DCs, which would explain the synergistic therapeutic effect when 1-MT is added to vaccine/anti-OX40 Ab therapy. It is important to mention that we have not seen increased IDO

activity in tumors for anti-OX40 Ab with vaccine when compared to vaccine therapy alone; therefore three factors may need to be considered. First, the small amount of IDO-secreting DCs in the tumor may not result in a significant change in IDO activity in the whole tumor tissue. Second, the comparative infiltration of immune cells may be different between vaccine and vaccine/anti-OX40 Ab therapies; therefore, normalization for specific cell number would be required. Third, priming of tumor-specific T cells is predominantly in tumor draining lymph nodes (TDLs); hence, the IDO enzymatic activity may show different results in TDLs when compared to the tumor environment.

Another immune suppressive mechanism mediated by IDO is through the induction and activation of Tregs (Munn, 2011), and many studies show a 1-MT-mediated drop of Tregs in the tumor environment (Mezrich et al., 2010). It is rather surprising that in our study, tumor-bearing mice that were provided with 1-MT did not show reduced Treg infiltration into tumors when compared to mice with no treatment (Figure not shown), but perhaps the suppressive functionality of Tregs may have been affected. It is established that Treg abundance restricts vaccine efficacy in tumors (Ndure & Flanagan, 2014). Here, 1-MT therapy may have altered the suppressive Treg activity, which may have boosted vaccine effectiveness in producing antigen-specific CD8 T cells. Furthermore, studies show that Tregs may undergo reprogramming from suppressive to inflammatory Th17-like cells (Kimura & Kishimoto, 2010). Treg reprogramming was prevented by the presence of IDO in tumors (Baban et al., 2009); however, the inhibition of IDO with 1-MT converted Tregs into cells expressing pro-inflammatory cytokines, which facilitated a less suppressive environment, generating improved vaccine efficacy (Sharma et al., 2010). Mechanistically, IL-6 can exert reprogramming of Tregs into pro-

inflammatory IL-17 cells, but IDO is known to suppress IL-6 (Baban et al., 2009; Sharma et al., 2009). These data closely relate to our study and may provide another mechanism for CD8-induced functionality when 1-MT is combined with vaccine. In our case, 1-MT may not be decreasing the number of Tregs, but may have reprogrammed Tregs into less suppressive cells that may preserved the functionality for CD8 T cells.

In general, the significantly increased functional CD8 T cells elicited by the addition of 1-MT to vaccine (Figure 19) may be due to explicit inhibition of IDO by providing a sufficient amount of tryptophan for the necessary effector T cell functionality and by increasing priming of CD8 T cells via the reduction of suppressive activity of DCs and Tregs.

Furthermore, this study is focused on the T cell immune response; however, it is highly plausible that the scope of vaccine/anti-OX40 Ab/1-MT affects other immune factors that are responsible for the anti-tumor therapeutic outcome.

Gathering the data together for the combinational immunotherapy, here for the first time to our knowledge, we demonstrate a therapeutic outcome for a novel combinational vaccine/anti-OX40 Ab/1-MT therapy and elucidate the synergistic immune mechanism for it. We show that vaccine/anti-OX40 Ab/1-MT treatment elicits potent anti-tumor efficacy by increasing the number (anti-OX40 Ab-mediated) and functionality (1-MT-mediated) of vaccine-induced CD8 T cells.

Accordingly, the question of how to target and improve the anti-cancer environment so that mice that are non-responders to the treatment can become responders in future combinational immune therapy yet remains. Identifying the tumor genetic

signature for possible predictive biomarkers may provide the therapeutic strategies needed to develop maximal anti-tumor efficacy.

V. SUMMARY

To enhance the effector immune response resulting in slower tumor progression in an animal model, we first optimized the dose for anti-OX40 Ab with vaccine combination. We found that the therapeutic efficacy for this combination is not linearly dose-dependent but rather shows an optimal biologic dose for tumor efficacy. We demonstrate that 1 mg/kg of anti-OX40 antibody with vaccine enhances CD8 T cell number and the clinically relevant CD8/Treg ratio. The enhanced effector immune response resulted in slower tumor progression and in complete tumor eradication in 20% of mice. However, increasing the anti-OX40 antibody dose to 2.5 mg/kg with vaccine diminished the therapeutic outcome. One of the possible immune cellular mechanisms responsible for the loss of therapeutic efficacy is due to enhanced accumulation of tumor infiltrating Tregs induced by higher dose of anti-OX40 antibody. Altogether, we show that while a low dose of anti-OX40 significantly increased the number of CD4 Tconvs, only the high dose led to a prominent increase in the number of in Tregs *in vivo*.

Here, we propose a potential molecular mechanism mediated by OX40 receptor stimulation that results in differential effect on CD4 T cell subsets. Previously, we have shown that AKT inhibition in T cells differently affect CD4 T cell activation and proliferation. Here we demonstrate that triggering OX40 downstream signaling mediates AKT activation in CD4 Tconvs. It will be noteworthy to elucidate the potential variation in phosphorylated AKT protein in CD4 T cell subsets when stimulated with different

doses of anti-OX40 antibody. Dissection of the OX40/AKT mechanistic correlation may explain the observed difference on CD4 Tconvs and Tregs *in vivo*, which is crucial for generating future anti-OX40-based combinational studies.

Next, we demonstrate the novel, synergistic, anti-tumor therapeutic effect of vaccine/anti-OX40 Ab/1-MT combination that leads to profound tumor regression, and we elucidate the immune mechanisms responsible for that synergy. Dissecting the mechanisms for each compound within the combination shows that anti-OX40 antibody is responsible for the overall increase in cell numbers, and 1-MT is responsible for the functionality of CD8 T cells. We provide evidence that simultaneous targeting of effector arm and suppressor arm of the immune system has a synergistic effect for tumor eradication and is a promising and translatable strategy that can enhance the overall efficacy of cancer treatment.

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