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AWARD NUMBER: DAMD17-03-1-0093

TITLE: Enhancing Anti-Prostate Cancer Immunity Through OX40 Engagement

PRINCIPAL INVESTIGATOR: Andrew D. Weinberg, Ph.D.

CONTRACTING ORGANIZATION: Providence Health Center
Portland, Oregon 97213-2933

REPORT DATE: February 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE (DD-MM-YYYY) February 2006		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 Feb 03 – 31 Jan 06	
Enhancing Anti-Prostate Cancer Immunity Through OX40 Engagement				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0093	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Andrew D. Weinberg, Ph.D. E-mail: andrew.weinberg@providence.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Providence Health Center Portland, Oregon 97213-2933				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of the proposed studies is to extend our OX40-specific anti-tumor responses to prostate tumor models by using a protein found on the surface of the T helper subset of leukocytes (OX40). Anti-OX40 delivered into animals with ongoing immune responses are able to clear the tumors and pathogens quicker following the acute immune response and also are left with a greater amount of immunologic "memory". The greater number of memory T cells patients have that recognize these tumors, increases their chance to fight off subsequent metastatic disease. We have found that prostate cancer patients treated with androgen ablation have a large influx of leukocytes into their prostate gland. These cells enter the prostate gland to recognize and destroy tumor cells. Leukocytes that invade the prostate gland after androgen ablation are OX40+. Therefore, we hypothesize that androgen ablation followed by anti-OX40 treatment will enhance anti-tumor immunity in these patients and we propose to exploit our discovery in prostate cancer mouse model. Fine-tuning our approach to gain preclinical data will help us to understand the most efficient way to augment anti-prostate cancer immunity in prostate cancer patients for future clinical trials.					
15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award) tumor immunology; T cells; co-stimulation; adjuvants					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION: OX40 is a cell surface TNF-receptor family member that is primarily found on activated CD4⁺ T cells. Our lab has shown that when this receptor is engaged through its ligand or an anti-OX40 Ab it induces proinflammatory signals that are clinically advantageous when delivered in combination with tumor vaccines or in mice with existing tumors. We also have found expression of OX40 on T cells infiltrating human primary tumors for head and neck cancer, breast cancer, melanoma, and colon cancer. Recently, in collaboration with Dr. Eugene Kwon we have found a large increase of OX40⁺ T cells in prostate tumors of patients that had androgen ablation twenty days prior to surgery. We now feel there is compelling evidence that OX40 engagement in patients with prostate cancer could have beneficial effects leading to improved clinical results. Therefore, we hypothesize that OX40 engagement in vivo will enhance anti-prostate cancer immunity leading enhanced tumor-free survival. The objective of this study is to obtain enough preclinical data to warrant the design of an OX40-specific clinical trial in patients with prostate cancer. The specific aims of the study are as follows; 1) To determine whether OX40 engagement in vivo will enhance anti-prostate cancer specific immunity, 2) Can OX40 engagement in vivo enhance adoptive immunotherapy against prostate cancer, and 3) To investigate whether the combination of androgen ablation and anti-OX40 treatment are synergistic in the treatment of primary prostate cancer in TRAMP mice. The study design will determine whether OX40-specific tumor immunotherapy treatment regimens will be therapeutic in the TRAMP mouse tumor model. We will look at the efficacy of anti-OX40 therapy in both a tumor transplant setting and in transgenic TRAMP mice destined to succumb to prostate cancer through endogenous tumor formation. The molecular mechanism of anti-OX40 enhanced tumor immunity will also be assessed by gene array analysis of T cells stimulated through OX40. We feel that using anti-OX40 in a prostate cancer setting will ultimately benefit an ongoing anti-prostate cancer immune response leading to enhanced immunity against disease that recurs. This is a therapy that is relatively non-toxic and takes advantage of the body's own defense against malignant cells. The experiments proposed will provide the preclinical data necessary to fine-tune our observations so that we will have a favorable chance to succeed in a prostate cancer clinical in the future.

BODY:

Task #1: To perform dose titration experiments in the s.c. TRAMP tumor model.

Previous literature has shown that the TRAMP-C1 cell line needs to be administered at a dose of 0.5×10^6 cells per s.c. injection to get the tumors to grow in male Black/6 (Bl/6) mice (1). Therefore, we performed a tumor titration in male Bl/6 mice starting at 0.5×10^6 and continuing with 1 and 2 million cells. We found that greater than 70% of mice receiving 0.5×10^6 cells formed lethal tumors and 100% of the mice receiving either 1 or 2 million cells formed lethal tumors. Subsequently, we found that inoculation of 0.75 million TRAMP-C1 cells was sufficient for a 100% lethal tumor dose. Therefore, we performed all of the subsequent "therapy" experiments with between 0.75 and 2 million tumor cells in order to have 100% of the control mice develop tumors. We noted that the tumors grew quicker in the mice that received 2 million tumor cells compared to mice that had received 0.75-1 million tumor cells. The initial anti-OX40-specific therapy experiment inoculated mice with either 1 or 2 million tumor cells and on days 3 and 7 the mice were injected with 250 μ g of anti-OX40 or control mice were injected with rat Ig. Tumor growth was slightly delayed in the anti-OX40-treated groups at both doses of tumor compared to rat Ig treated mice, but eventually all the anti-OX40 treated mice

succumbed to tumor growth. We also tested different doses of anti-OX40 and found that 250 µg gave the best efficacy as far as delayed tumor growth in the TRAMP-C1 model. We were unable to obtain the TRAMP-C2 cell line, therefore all the experiments performed within the parameters of this grant used the TRAMP-C1 cell line as well as TRAMP mice.

Task #2: To inhibit metastatic disease via anti-OX40 engagement.

We had originally proposed to accomplish this task with the TRAMP-C2 cell line, but as discussed above were unable to obtain this cell line. Therefore, we attempted to ascertain whether anti-OX40 would inhibit spontaneous metastatic prostate cancer in TRAMP mice. These were experiments performed as part of Task #6. We found that anti-OX40 did not inhibit metastatic disease in the TRAMP model, nor did it delay growth of primary disease in these mice. The TRAMP model may not be a fair assessment of the ability of anti-OX40 to enhance immunity in prostate cancer patients, because every single prostate epithelial cell in the TRAMP mice is destined to become a tumor cell. As will be discussed in Task #6 we did notice a significant difference in T cell infiltration in the prostates of TRAMP mice treated with anti-OX40.

Task #3: To test whole prostate tumor vaccines in conjunction with anti-OX40 treatment.

Previously, researchers have shown that whole tumor vaccines secreting GM-CSF stimulated potent anti-tumor immunity (2). Therefore, we tested whether the combination of anti-OX40 and a GM-CSF secreting prostate-specific tumor cell line would show therapeutic synergy in the TRAMP tumor setting. We performed these experiments in the treatment setting, where the mice were given s.c. TRAMP-C1 and then treated with anti-OX40 and irradiated TRAMP-C1 secreting GM-CSF. Mice injected with s.c. tumor were given anti-OX40 on days 3 and 7 after tumor inoculation and irradiated GM-CSF tumor on days 3, 7 and 10. We found no synergistic or additive therapeutic effects when whole cell vaccine was used in combination with anti-OX40. While this is negative data, it is of interest because a similar combination of TRAMP cell lines secreting GM-CSF in combination with anti-CTLA-4 (another immune enhancing Ab) showed enhanced anti-tumor immunity (1). The data suggest that the mechanism of tumor immune enhancement may be different between these two Ab. Because of the negative results obtained with anti-OX40 alone or in combination with whole tumor vaccines, we tested whether other cytokines (IL-15, IL-2, or IL-12) administered in vivo would augment anti-OX40 therapy in the TRAMP-C1 s.c. model. In particular, we obtained a large amount of purified IL-15 from Amgen Inc. and tested combination therapy with anti-OX40. Anti-OX40 was administered on days 3 and 7 after tumor inoculation and IL-15 was given daily from days 3-10. Again, the combination therapy was no better than anti-OX40 alone. We also administered IL-2 in combination with anti-OX40 in mice harboring the TRAMP-C1 tumor and found no additive or synergistic therapeutic effect (see Figure 1).

Task #4: To test adoptive immunotherapy treatment in mice harboring TRAMP lung metastases.

We were not able to perform the experiments within this task due to a lack of time. Hopefully future grant support will allow this aim to be pursued.

Task #5: Gene array analysis of anti-OX40 stimulated T cells.

Our initial attempts to treat the TRAMP-C1 cell line with anti-OX40 as a solo reagent were not successful. At best, we observed a delay in tumor growth, but eventually all of the mice would succumb to the TRAMP-C1 tumor. Therefore, we searched for ways to enhance the anti-OX40 therapy, by first identifying mechanisms that drive the OX40-enhanced response. This was accomplished by performing gene array experiments, comparing RNA from Ag-specific T cells that received anti-OX40 versus control Ab (rat Ig) in two different CD4 T cell adoptive transfer models. In particular, we were looking for cytokine receptor genes that might be upregulated via OX40 engagement on the Ag-specific T cells that could be potentially used to enhance OX40 stimulation by combination therapy (OX40/cytokine). In both models anti-OX40 greatly upregulated the IL-2 receptor (CD25) and these results

were confirmed at the protein level by FACS analysis (3, 4). However, as shown in Figure 1A we did not see therapeutic synergy between anti-OX40 and IL-2 in the TRAMP-C1 model. We also discovered through gene array analysis that the signaling

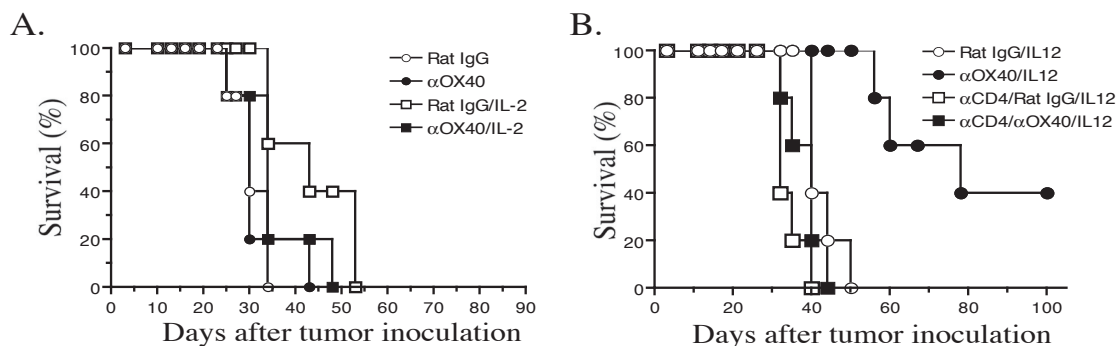


Figure 1. Anti-OX40 and IL-12, but not IL-2, synergize to improve survival of prostate tumor-bearing mice. **A.** Mice were injected s.c. in their right flanks with 7.5×10^5 TC1 tumor cells. Three days later mice were injected i.p. with 250 ug of anti-OX40 and 90,000 U rIL-2, or 250 ug rat IgG and 90,000 U rIL-2, or 250 ug anti-OX40 and PBS control, or 250 ug rat IgG and PBS control. Seven days after tumor challenge mice were given a second injection of 250 ug anti-OX40 or rat IgG. **B.** Half the mice were injected i.p. with 200 ug of anti-CD4 to deplete CD4 cells. One day later mice were injected s.c. in their right flanks with 7.5×10^5 TC1 tumor cells (day 0). On days 3 and 7 mice were injected i.p. with 250 ug anti-OX40 or rat IgG. Recombinant IL-12 (100 ng) was injected i.p. on days 4 through 9. The survival data shown represents 5 mice/group.

subunit of the IL-12 receptor (IL-12R β 2 subunit) was upregulated in both CD4 T cell models and we recently confirmed the IL-12R gene array results at the protein level by FACS analysis of Ag-specific T cells isolated from the draining lymph nodes (see Figure 2A). The expression of the IL-12R was tightly regulated on Ag-specific CD4 T cells,

peaking 4 days after Ag stimulation and completely downregulated 4 days later (Figure 2B). We also found that signaling through IL-12 on anti-OX40 activated Ag-specific CD4 T cells had a profound effect on the ability of these T cells to survive and become memory

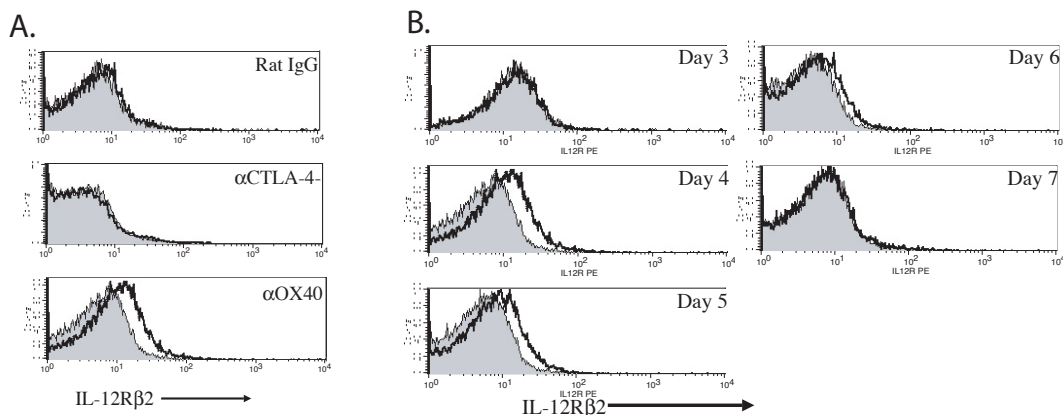


Figure 2: Anti-OX40 induced expression of IL-12R β 2 on Ag specific CD4 T cells. Ova-specific CD4 T cells were transferred into Balb/c recipients (day -1). One day later mice were injected with soluble ova and 50 μ g of anti-OX40, rat Ig, or 100 μ g anti-CTLA-4. Ag-specific T cells (gated on the anti-idiotypic TCR marker KJ-126) obtained from the draining LNs were examined for IL-12R β 2 expression. A) Compares rat Ig, anti-CTLA-4, and anti-OX40 on day 4 after immunization and B) Examines a time course of expression after anti-OX40 administration in the draining LNs. The shaded histogram represents isotype control while the dark open histogram displays IL-12R β 2 staining.

cells. This is depicted in Figure 3, which shows that CD4 T cells transferred into IL-12 knockout hosts and stimulated with Ag and anti-OX40 proliferate just as well as in WT hosts (4 days after activation), but the cells start to disappear between day 4 and 12 after activation in the IL-12 deficient environment. This is the first description of the mechanism of how anti-OX40 enhances memory T cell survival (manuscript in prep). The IL-12R β 2 gene array data and the T cell expression data suggested that the combination of anti-OX40 and IL-12 may have synergistic effects in tumor models. Therefore we tested whether this combination would

enhance therapeutic efficacy in TRAMP-C1 model. As shown in Figure 1B, when anti-OX40 was combined with IL-12 a significant delay in tumor growth was observed in 100% of the dual treated mice and 40% of the mice remained tumor-free for 100 days post-tumor inoculation. Because we previously showed expression of the IL-12R β 2 protein on anti-OX40 CD4 T cells, we determined whether CD4 T cells were essential for the anti-OX40/IL-12 treatment scheme. Figure 1B shows that depletion of CD4 T cells prior to anti-OX40/IL-12 treatment completely negated the efficacy of the combined treatment. These results are a significant step forward in our attempts to augment anti-prostate tumor immunity and future anti-OX40-based clinical trials might attempt this combination therapy for enhanced therapeutic efficacy.

Task #6: To investigate androgen withdrawal and anti-OX40 in TRAMP mice.

Our initial observation in prostate cancer patients showed that androgen ablation prior to prostate cancer surgery greatly increased the numbers of OX40⁺ T cells within the tumor of these patients. Therefore we wanted to test whether the combination of androgen withdrawal and anti-OX40 would show enhanced anti-tumor effects in mice with TRAMP tumors. As shown in Figure 4, we have tested anti-OX40 in combination with androgen ablation (castration) versus androgen ablation alone in the subcutaneous TC1 model and saw no increase in therapy with the combined

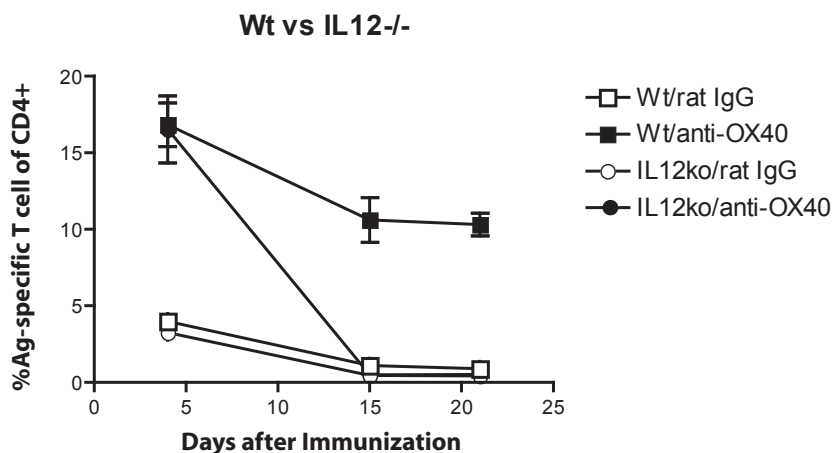


Figure 3: Anti-OX40 mediated survival of CD4 T cells is IL-12 dependent. DO11.10 T cells were transferred into WT or IL-12 knockout naive recipients. The mice were then immunized with ova and injected with anti-OX40 or rat Ig. The mice were bled, lymphocytes were purified on ficoll, and stained with the KJ-126 Ab and CD4 (Ag-specific cells). Each time point represents the mean of 5 mice assessed per group. The LNs and spleens were also evaluated at the later time point with similar results (data not shown).

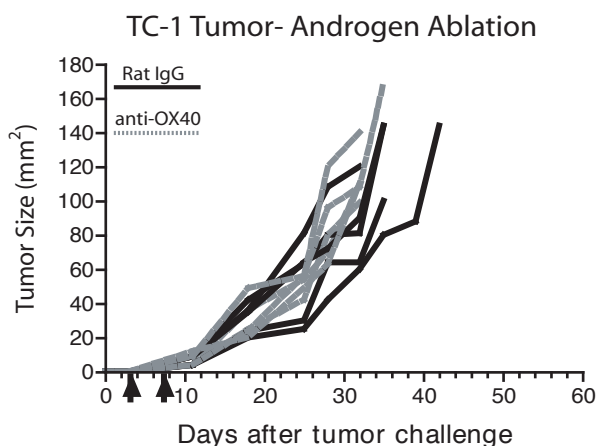


Fig 4. Anti-OX40 treatment had no effect of TC-1 tumor growth in androgen ablated animals. Six-Eight week old male C57BL/6 male mice were castrated and allowed to recover for seven days. Mice were then challenged with 7.5×10^5 TC-1 tumors injected s.c. on the right flank. Anti-OX40 (250 mg) or rat IgG control were injected i.p. three and seven days following tumor challenge (arrows), and monitored for tumor growth. Mice were sacrificed if tumors were ulcerated or if tumor growth reached 150mm². (n=5 per group)

treatment.

We next attempted to treat TRAMP mice that develop spontaneous prostate cancer with an irradiated TRAMP-C1/GMCSF cell line alone or in combination with anti-OX40, IL-12, or the combination of anti-OX40 and IL-12. The TRAMP mice were crossed onto the FVBN background and these mice typically develop tumors by 20 weeks of age. The mice were treated twice with anti-OX40 or IL-12 and irradiated TRAMP-C1 at weeks 10 and 15 and all groups were sacrificed at 20 weeks of age. The sacrificed mice were examined for primary tumor growth, metastatic tumor growth, and the prostates were removed for pathologic examination. There were 5 mice/group and the extent of primary tumor growth and metastatic disease between the groups was indistinguishable. Upon pathologic examination all the groups treated with anti-OX40 (w/wo IL-12) showed somewhat unusual pathology in that stromal thickening and hypercellularity accompanied the areas of transformation/hyperplasia. It was also noted for all the mice treated with anti-OX40, that the dorsal lateral prostate had loose connective tissue associated with inflammation. We stained these prostate tissue sections with anti-CD3 to look for T cell infiltrates and found increased numbers of CD3⁺ associated with areas of hyperplasia in the anti-OX40 treated groups (Figure 5). These results suggest that anti-OX40 altered the pathology of the prostate in TRAMP mice leading to increased numbers of T cell infiltrates.

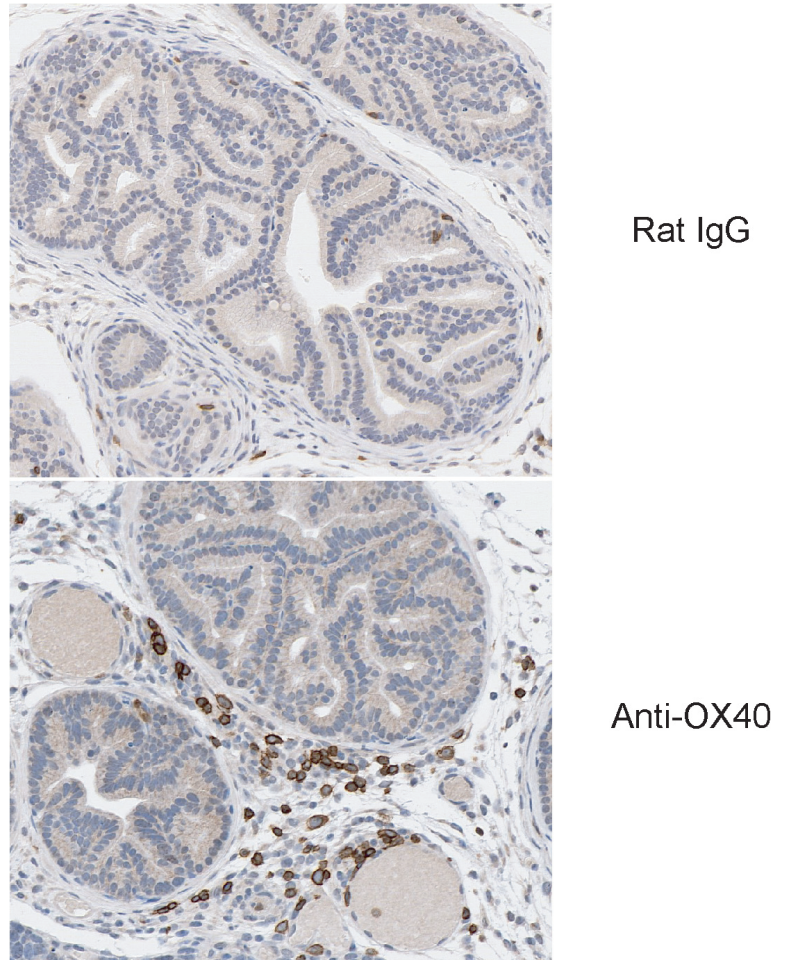


Figure 5. Anti-OX40 treatment of TRAMP mice. Ten-week old male TRAMP mice were injected with 250 ug anti-OX40 or rat Ig i.p. at 10 and 15 weeks of age along with an irradiated TRAMP-C1 cell line that produces GM-CSF. At 20 weeks of age, mice were sacrificed and prostate tissues were harvested and formalin-fixed. Paraffin-embedded sections processed and stained for CD3⁺ lymphocyte infiltrates by immunohistochemistry (reddish-brown stain). Note the increased presence of CD3⁺ infiltrates in the anti-OX40 treated TRAMP mice prostate section. Images shown at 20X magnification.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Discovering that both the IL-2 receptor and IL-12 receptor are upregulated on Ag-stimulated CD4 T cells following anti-OX40 engagement in vivo.
- 2) IL-12 receptor upregulation is an essential component for generating OX40-mediated CD4 T cell memory.
- 3) The combination of anti-OX40 and IL-12 showed therapeutic synergy in the TRAMP-C1 tumor model.
- 4) The anti-OX40/IL-12 therapy was dependent on CD4 T cells in the TRAMP-C1 tumor model.
- 5) Anti-OX40 therapy in TRAMP mice led to increased inflammation within the prostate, which was associated with increased numbers of CD3⁺ T cells.

REPORTABLE OUTCOMES:

Prell, R.A., Evans, D.E., Thalhoffer, C., Shi, T., Funatake, C., and **Weinberg, A.D.** (2003) OX40-mediated memory T cell generation is TRAF dependent. *Journal of Immunology*, 171:5997.

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CONCLUSIONS:

During the 3 years of funding we have explored the TRAMP prostate tumor model and its sensitivity to anti-OX40-mediated tumor therapy. Previously, we had found that anti-OX40 as a solo agent was able to enhance anti-tumor immunity leading to a therapeutic outcome in several tumor models including sarcoma, melanoma, mammary carcinoma, colon cancer, and glioma. Therefore we hypothesized that the TRAMP prostate cancer model would show a similar response to anti-OX40. However, we found that anti-OX40 alone showed little anti-tumor efficacy in the TRAMP model. Therefore, our effort during this grant tenure was refocused to understand how to make this therapy work in prostate tumor models via a combination strategy with other immune enhancing agents. In order to accomplish this task we analyzed several gene array experiments from T cells isolated from mice stimulated with or without anti-OX40 stimulation. Two cytokine receptors, IL-2 and IL-12, were upregulated upon anti-OX40 stimulation of T cells, therefore we tested whether the combination of either cytokine with anti-OX40 in prostate tumor-bearing hosts would have treatment value. IL-2 with anti-OX40 showed no enhancement of therapeutic efficacy; however, the combination of IL-12 with anti-OX40 did show synergy delaying tumor growth in 100% of prostate tumor-bearing mice with 40% long-term survival. We further showed that this synergy was dependent on CD4 T cells, which confirmed our gene array results that initially showed an increase of IL-12 receptor expression on Ag-activated CD4 T cells stimulated with anti-OX40. We have also found that anti-OX40 greatly increases the survival of memory CD4 T cells and that IL-12 is a key component of this survival loop. The IL-12/OX40 findings are currently being compiled for submission to Nature Medicine and we have recently written an NIH R01 submission based on these findings to further study this area of research.

Our group has pioneered the effort to bring anti-OX40 therapy to cancer patients. After years of human Ab preparation, the FDA approved our phase I clinical trial and the first cancer patient was treated in March of 2006. Our hope is to use the knowledge that we have gained through the DOD prostate cancer support to treat prostate cancer patients in the near future.

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