OX40 Is a Potent Immune-Stimulating Target in Late-Stage Cancer Patients

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Abstract

OX40 is a potent costimulatory receptor that can potentiate T-cell receptor signaling on the surface of T lymphocytes, leading to their activation by a specifically recognized antigen. In particular, OX40 engagement by ligands present on dendritic cells dramatically increases the proliferation, effector function, and survival of T cells. Preclinical studies have shown that OX40 agonists increase antitumor immunity and improve tumor-free survival. In this study, we performed a phase I clinical trial using a mouse monoclonal antibody (mAb) that agonizes human OX40 signaling in patients with advanced cancer. Patients treated with one course of the anti-OX40 mAb showed an acceptable toxicity profile and regression of at least one metastatic lesion in 12 of 30 patients. Mechanistically, this treatment increased T and B cell responses to reporter antigen immunizations, led to preferential upregulation of OX40 on CD4+ FoxP3+ regulatory T cells in tumor-infiltrating lymphocytes, and increased the antitumor reactivity of T and B cells in patients with melanoma. Our findings clinically validate OX40 as a potent immune-stimulating target for treatment in patients with cancer, providing a generalizable tool to favorably influence the antitumor properties of circulating T cells, B cells, and intratumoral regulatory T cells. Cancer Res; 73(24); 7189–98. ©2013 AACR.

Introduction

Antibodies, which target T-cell surface proteins, have been shown to restore and enhance the function of tumor-reactive T cells in vivo in tumor-bearing hosts (1–5). The antagonists, anti-CTLA-4 and anti-PD-1, block negative signals to the T cells, whereas the agonists, anti-4-1BB and anti-OX40, enhance T-cell function by increasing costimulation (6). A phase III clinical trial in patients with metastatic melanoma demonstrated enhanced survival in patients receiving anti-CTLA-4 and these results led to the recent U.S. Food and Drug Administration (FDA) approval of this antibody (7). Antibodies directed to PD-1 or PD-1 ligand have produced complete and partial responses as well as durable stable disease in patients with cancer (8, 9). The strategy of blocking inhibitory T-cell pathways has shown clinical activity and there is ample preclinical evidence that T-cell costimulation via 4-1BB (10, 11) or OX40 can induce antitumor effects (12–15).

We completed a translational research study to determine the potential value of immunostimulatory antibody against OX40. OX40 is a TNF-receptor family member that is expressed primarily on activated CD4+ and CD8+ T cells (16–18). Preclinical cancer models have shown that anti-OX40 has potent antitumor activity against multiple tumor types, which is dependent on both CD4+ and CD8+ T cells (12–15). Immunization models have shown that anti-OX40 increased T-cell proliferation, effector cytokine production, cytotoxicity, and decreased activation-induced cell death leading to an increase in memory T cells (19–23). This report describes the clinical, immunological, and antitumor effects of an agonist antibody to OX40 in patients with advanced cancer.

Materials and Methods

Clinical trial ID: NCT01644968

Clinical trial was designed and performed as described in Supplementary Materials and Methods.

Anti-OX40 mAb (9B12)

9B12 is a murine IgG1, anti-OX40 monoclonal antibody (mAb) directed against the extracellular domain of human OX40 (CD134). The mAb was selected as described in Supplementary Material and Methods.
ELISA assays for tetanus and KLH
Recombinant tetanus toxoid C fragment (Roche), at 2 μg/mL, or keyhole limpet hemocyanin (KLH; Biosyn Corp.), at 10 μg/mL, was absorbed on the surface of 96-well plates (Fisher). Serum samples were then incubated for 1 hour, followed by peroxidase-conjugated goat anti-human immunoglobulin G (IgG; Jackson Immunoresearch Lab). TMB substrate solution (SureBlue TMB, KPL Inc.) was added, followed by a stopping solution (85% O-Phosphoric Acid; Fisher). Spectrophotometry measurements were made at 450 nm (Wallac Victor2 spectrophotometer; PerkinElmer).

ELISA assay for measurement of anti-OX40 (CD134) in human serum
Titer in human serum were measured as indicated in Supplementary Material and Methods.

Flow cytometry
Peripheral blood mononuclear cells (PBMC) were obtained from patients and cryopreserved samples were used for flow cytometry studies. The fluorochrome-labeled antibodies to CD3, CD4, CD8, CD95, HLA-DR, CD45RA, CCR7, and Ki-67 were purchased from BD Pharmingen, Foxp3 and CXCR5 from eBioscience, CD28 from Beckman Coulter, CD25 from Miltenyi Biotech, CD38 and OX40 from BioLegend, and Streptavidin AF-700 from Invitrogen. Intracellular staining was performed using the Fix/Perm kit from eBioscience according to the manufacturer’s instructions. To prevent the interference of human anti-mouse antibody (HAMA) with staining, cells were preincubated with the mAb anti-OX40 (9B12). Detection of anti-OX40 binding was performed on fresh PBMCs using an anti-mouse IgG and fluorescein isothiocyanate-labeled anti-rat IgG (Invitrogen). Stained cells were analyzed on an LSRII or the fluorescence-activated cell sorting (FACS) Aria (BD Biosciences). Data analysis was performed using either Winlist (Verity Software House) or FACSdiva (Becton Dickinson) software.

Tumor-specific T-cell assays
Tumor-specific reactivity before and after anti-OX40 administration was assessed in PBMCs from 3 patients with melanoma. Autologous or HLA-matched melanoma cells were cocultured with PBMCs at a PBMC:tumor ratio of 8:1 for 5 days. IFN-γ in the supernatant was quantified using an IFN-γ ELISA kit (BD Biosciences). For Western blot analysis, FEMX cell lysates were heated to 100°C in gel sample buffer with SDS for 5 minutes and 15 μg of protein were separated by electrophoresis on a 10% SDS–PAGE gel. Proteins were transferred to nitrocellulose. The blocked membrane was incubated with patient sera, then with a peroxidase-conjugated secondary antibody, and exposed with enhanced chemiluminescence (ECL) Western Blotting substrate (Pierce).

T-cell proliferation assay
Cryopreserved PBMCs (12 arm A samples and 11 arm B samples), 1 × 10⁵ PBMCs per well, were stimulated with 0.5 μg/mL of anti-CD28/anti-CD95 (BD Biosciences) and with tetanus toxoid antigen (EMD Calbiochem) at 1 μg/mL for 4 days. Cells were then labeled with [³²P]Thymidine (MP Biomedicals) for 18 hours, then harvested and counted on a Wallac TriLux Microbeta scintillation counter. Tetanus-specific proliferation was measured by subtracting proliferation in the absence of antigen from the tetanus toxoid-stimulated cultures. Fold increases were calculated by dividing the proliferation measured on day 43 (arm A) or day 15 (arm B) from the proliferation observed before anti-OX40 administration.

Statistical analyses
Ki-67 analyses. For each cell population, the mean difference between treatment cohorts at study days 8 and 15 were compared using one-way ANOVA on the log₁₀ fold change from baseline. The log₁₀ transformation was performed to satisfy statistical model assumptions. Comparisons between cohorts were not adjusted for multiple comparisons due to the exploratory nature of the analyses. Mean differences in fold change between responders and nonresponders at each study day were analyzed using one-way ANOVA. Analyses were performed using JMP version 9 (SAS Institute).

Tetanus and KLH-specific ELISA. For all statistical tests of serum antibody response to KLH and tetanus, a cutoff optical density value was established that would determine the dilution at which the sera would be considered negative (endpoint dilution). A best-fit curve generated by a 1-phase decay model was used to determine the optical density values for the bottom plateau for each ELISA plate. The mean for all of the plateaus on the plate +/− 2SDs was used as the cutoff optical density. The dilution at which each curve intersected the cutoff is the endpoint dilution (titer) for that curve. The two arms from all three cohorts were compared using a two-tailed Mann–Whitney (Wilcoxon rank-sum test) analysis to determine whether the mean fold change increased significantly from baseline between the two arms.

T-cell proliferation assay. A two-tailed Mann–Whitney (Wilcoxon rank-sum test) was used to determine whether the mean thymidine incorporation was significantly increased when comparing the pretreatment levels with either day 43 for arm A or day 15 for arm B.

Tumor-specificity assay. IFN-γ secretion from supernatants containing either pretreatment or posttreatment PBMCs cocultured with either autologous, HLA-matched cell line, or flu, were compared using a one-tailed unpaired t test to determine statistical significance.

Results
Clinical results
Patients with metastatic solid malignancies refractory to conventional therapy were enrolled in a phase I dosescalation study using the 9B12 murine agonistic anti-human OX40 mAb, Trial ID#NCT01644968 (see Supplementary Fig. S1 and Supplementary Materials and Methods). Consenting participants received a single cycle of anti-OX40 given intravenously on days 1, 3, and 5. The study was designed with three cohorts of 10 patients each. Patients in cohort 1 received one cycle of 0.1 mg/kg, cohort 2, 0.4 mg/kg, and cohort 3, 2 mg/kg of the mAb. The main objectives of the clinical trial were to determine the...
maximum tolerated dose, toxicity, immunologic activity, and potential clinical activity following one cycle of anti-OX40.

**Demographics.** Supplementary Table S1 provides the clinical characteristics and details of prior treatments. Prior therapy included surgery, radiation, chemotherapy, targeted therapy, and immunotherapy. The median number of prior cancer treatments was 2 (range, 0–8) and all had progression of their cancer at the time of enrollment. All patients had performance status Eastern Cooperative Oncology Group (ECOG) 0 or 1.

**Toxicity assessment.** Twenty-eight of 30 patients received all three planned anti-OX40 doses at the assigned dose level. Table 1 summarizes the toxicities related to anti-OX40. Most toxicity was grade 1 or 2, with the exception of lymphopenia in which grade 3 and 4 events were observed. Lymphopenia was transient (Supplementary Fig. S2), and generally resolved 72 hours later. Lymphopenia was well tolerated and the maximum tolerated dose was not reached.

**Pharmacokinetics and HAMA production.** There was a dose-dependent increase in peak serum anti-OX40 levels after the first and third doses (serum was not assessed following the second dose), and a decrease thereafter (Supplementary Fig. S3). Peak anti-OX40 levels were observed 2 hours after the third dose. Anti-OX40 antibody was detected on the surface of CD8 T lymphocytes. To determine the effects of anti-OX40 on T cells, we performed multicolor flow cytometry with antibodies that recognized CD3, CD4, CD8, CD95, CD25, FoxP3, CD28, and Ki-67. Changes in T-cell subpopulation frequencies were determined using FCOM analysis tool in Winlist (24) and statistically significant changes were assessed using exhaustive expansion analysis (25). Ki-67 expression, which is present only on T cells, we performed multicolor flow cytometry with an anti-mouse–specific antibody. Between 9.9% and 26.9% of CD4 T cells and 0.6% and 7.8% of CD8 T cells were positive using this technique (Supplementary Fig. S4). All but one patient tested positive for Ki-67 by flow cytometry, using an antibody that was associated with significant changes in the percentage of Ki-67–positive CD8 T cells on day 29; however, the majority of Ki-67–positive CD8 T cells later (day 15) after anti-OX40 administration and from a normal individual immunized with tetanus were analyzed (Fig. 2A and B and Supplementary Fig. S6A and S6B). PBMCs were gated on CD3, CD95, and CD4; these markers are reported to identify antigen-experienced/memory T cells (26), and further analyzed for FoxP3 [regulatory T cell (Treg)] and Ki-67 (Fig. 2A). PBMCs from the same patients were also gated on CD3, CD95, and CD8 and analyzed for CD28 and Ki-67 (Fig. 2B). At baseline, the percentage of proliferating/Ki-67–positive CD4+ and CD8+ T cells ranged between 0.5% and 6.0% in patients and normal controls (Fig. 2 and data not shown). The percentage of Ki-67–positive CD4+ T cells started to increase early (day 8) and the Ki-67–positive CD8+ T cells later (day 15) after anti-OX40 (summarized in Fig. 2C–F); the percentage of proliferating cells in both populations usually returned to pretreatment percentages by day 57. Typically, proliferating CD8+ T cells were observed in both the CD28-positive and -negative populations on day 15; however, the majority of proliferating CD8+ T cells on day 29 were CD28+. Significant changes in the percentage of Ki-67–positive peripheral blood lymphocytes (PBL) were detected among the normal donors that were immunized with tetanus toxoid for any of these populations at any time points (Fig. 2C–F). There was a significant increase in Ki-67–positive lymphocytes in anti-OX40–treated patients compared with controls. Significant increases

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<th>Table 1. Summary of maximum toxicities related to anti-OX40 at all dose levels</th>
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<td><strong>Adverse events</strong></td>
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<td>Lymphopenia</td>
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<td>Fatigue</td>
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<td>Rash/skin changes</td>
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<td>Arthritis/myalgias</td>
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<td>Nausea/vomiting</td>
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<td>Increased AST, ALT, or alkaline phosphatase</td>
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<td>Anemia</td>
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**NOTE:** Patients with multiple instances of the same toxicity were tabulated once with the highest grade for that toxicity.

**Tumor regression.** Figure 1A shows a waterfall plot for best response by Response Evaluation Criteria in Solid Tumors (RECIST) after anti-OX40. No patient achieved a partial response by RECIST (>30% overall tumor shrinkage). However, at least one tumor nodule regressed in 12 patients and no change in the measurement of target lesions was observed in 6 additional individuals during the 57-day observation period. Regression and SD were observed in patients with melanoma, renal cancer, squamous cell carcinoma of the urethra, prostate cancer, and cholangiocarcinoma. The longest interval of stable disease lasted 470 days in a patient with renal cancer, who received no other therapy during that time. Some individuals had a decrease in measurable lesions at day 29, but had progression by day 57. Figure 1B–E and Supplementary Fig. S5A–S5H show examples of tumor regression in 3 patients following anti-OX40. Mixed responses (e.g., simultaneous regression of at least one tumor deposit and progression at other sites) were observed in 2 patients with melanoma and 2 patients with renal cancer.

**Immunologic results**

Anti-OX40 increases proliferation of CD4 FoxP3neg and CD8 T lymphocytes. To determine the effects of anti-OX40 on T cells, we performed multicolor flow cytometry with antibodies that recognized CD3, CD4, CD8, CD95, CD25, FoxP3, CD28, and Ki-67. Changes in T-cell subpopulation frequencies were determined using FCOM analysis tool in Winlist (24) and statistically significant changes were assessed using exhaustive expansion analysis (25). Ki-67 expression, which is present only on T cells, we performed multicolor flow cytometry with an anti-mouse–specific antibody. Between 9.9% and 26.9% of CD4 T cells and 0.6% and 7.8% of CD8 T cells were positive using this technique (Supplementary Fig. S4). All but one patient tested positive for Ki-67 by flow cytometry, using an antibody that was associated with significant changes in the percentage of Ki-67–positive CD8 T cells on day 29; however, the majority of Ki-67–positive CD8 T cells later (day 15) after anti-OX40 administration and from a normal individual immunized with tetanus were analyzed (Fig. 2A and B and Supplementary Fig. S6A and S6B). PBMCs were gated on CD3, CD95, and CD4; these markers are reported to identify antigen-experienced/memory T cells (26), and further analyzed for FoxP3 [regulatory T cell (Treg)] and Ki-67 (Fig. 2A). PBMCs from the same patients were also gated on CD3, CD95, and CD8 and analyzed for CD28 and Ki-67 (Fig. 2B). At baseline, the percentage of proliferating/Ki-67–positive CD4+ and CD8+ T cells ranged between 0.5% and 6.0% in patients and normal controls (Fig. 2 and data not shown). The percentage of Ki-67–positive CD4+ T cells started to increase early (day 8) and the Ki-67–positive CD8+ T cells later (day 15) after anti-OX40 (summarized in Fig. 2C–F); the percentage of proliferating cells in both populations usually returned to pretreatment percentages by day 57. Typically, proliferating CD8+ T cells were observed in both the CD28-positive and -negative populations on day 15; however, the majority of proliferating CD8+ T cells on day 29 were CD28+. Significant changes in the percentage of Ki-67–positive peripheral blood lymphocytes (PBL) were detected among the normal donors that were immunized with tetanus toxoid for any of these populations at any time points (Fig. 2C–F). There was a significant increase in Ki-67–positive lymphocytes in anti-OX40–treated patients compared with controls. Significant increases
were observed for CD4$^+$ FoxP3$^+$ T cells, CD8$^+$ T cells, and CD3$^+$/NK cells (Fig. 2C, E, and F). The proliferation of CD4$^+$ FoxP3$^+$ Treg was unaffected in anti-OX40–treated patients at any dose when compared with controls (Fig. 2D). At day 15, the proliferation of CD4$^+$ FoxP3$^+$ cells showed a statistically significant increase in cohorts 1 and 2 compared with the control group ($P < 0.02$). The augmentation of CD4$^+$ FoxP3$^+$ T-cell proliferation in patients within the third cohort peaked on day 8 and declined thereafter, which was a different pattern of expression than the first two cohorts. The increase in proliferation of CD8$^+$ T cells peaked on day 15 for all three cohorts; however, a significant increase in proliferation was only observed in the second cohort when compared with the control group ($P = 0.001$). A sustained increase in Ki-67$^+$ expression was found predominantly in patients from the second cohort (through day 29). The average increase in proliferation of CD8$^+$ T cells in cohort 3 was less than that observed in cohort 2, despite the fact that patients received a 5-fold higher dose of anti-OX40. The increase in Ki-67 expression within the CD3$^+$/CD56$^+$ cells was statistically significant in all cohorts compared with normal donors and peaked 15 days after mAb treatment. The largest average fold increase in the percentage of cycling cells for all three cell populations (CD4$^+$/FoxP3$^+$, CD8$^+$, and NK cells) was observed in cohort 2 (Fig. 2C, E, and F). In a post hoc analysis, patients were designated as "nonprogressors" (initial decrease or stabilization of the disease) and "progressors" (tumor progression). Ki-67$^+$ T cells among the CD4$^+$/FoxP3$^+$ population in nonprogressors on day 8 and among CD8$^+$ T lymphocytes on days 5, 8, and 15 were significantly greater than progressors (Supplementary Fig. S7A and S7B). No significant differences were observed in Ki-67 expression in CD4$^+$ FoxP3$^+$ T cells or CD3$^+$ lymphocytes (Supplementary Fig. S7C and S7D).

The generation of HAMA in all patients led us to consider the possibility that the increase in Ki-67$^+$ cells could be attributed to an anti-mouse–specific immune response or tetanus immunization rather than enhancing the endogenous T-cell repertoire. Because this could not be directly tested in humans, we performed a study in non-human primates to determine whether the infusion of a control mouse IgG with tetanus vaccination would elicit a similar increase in Ki-67$^+$ expression by CD4$^+$ and CD8$^+$ T cells as was observed in the clinical trial. Two groups of monkeys received tetanus on day 1 and an intravenous injection of either 1 mg/kg of anti-OX40 mAb or mouse IgG on days 1, 3, and 5. PBMCs were analyzed by flow cytometry with a panel similar to the one described for human PBL. The mouse anti-human OX40 antibody increased Ki-67 expression in both
CD4\(^+\) and CD8\(^+\) T cells (Supplementary Fig. S8A and S8B). However, no significant increases in Ki-67 expression were observed in either CD4\(^+\) or CD8\(^+\) lymphocytes isolated from monkeys that had received tetanus and mouse IgG. These data show that increased Ki-67 expression was induced by anti-OX40 and not by a de novo immune response elicited to a foreign mouse protein or tetanus immunization. To definitively establish that the increased proliferation of CD4\(^+\) and CD8\(^+\) T cells was induced by the engagement of OX40, we injected 4 monkeys with an agonistic monkey OX40L-Ig fusion protein, which increased the mean percentage of Ki-67\(^+\) CD8\(^+\) T cells (Supplementary Fig. S8A and S8B).

**CD4\(^+\) T-cell analysis.** We sought to explain why proliferation of Treg in PBL was less than other T-cell subsets. Therefore, we examined OX40 expression on Treg and CD4\(^+\)/FoxP3\(^+\) T cells in tumors (Fig. 3A). We also compared surface OX40 expression on Treg and CD4\(^+\)/FoxP3\(^+\) T cells in PBL and tumor-infiltrating lymphocytes (TIL) in 3 patients with melanoma enrolled in this study and 2 patients with untreated ovarian cancer. OX40 surface expression on non-Treg and Treg in the PBL was less than 20%, whereas more than 50% of Treg from TIL expressed OX40 (Fig. 3B and C). Moreover, the mean fluorescence intensity for OX40 was up to 16-fold higher in TIL compared with PBL Treg, suggesting that the anti-OX40 may modulate Treg function in the tumor.

**Anti-OX40 induces a transient decrease of follicular helper CD4\(^+\) T cells in the PBL.** Anti-OX40 increased anti-TT and anti-KLH antibodies, suggesting enhanced activity in T follicular helper cells. We analyzed the percentage of CD4\(^+\) CD45RA\(^-\) CCR7\(^+\) CXCR5\(^+\) follicular helper T cells (27) in PBL from patients and controls before and after anti-OX40 antibody. Figure 3D shows that there is significant decrease of this cell subset at day 8 in study patients compared with controls, who have received the TT vaccine on day 1, but have not received anti-OX40 antibody (Fig. 3E).

**Anti-OX40 administration induces qualitative changes in cycling CD8\(^+\) T cells.** The effect of anti-OX40 on the activation phenotype of cycling CD8\(^+\) T cells was examined by assessing coexpression of CD38 and HLA-DR, which is a hallmark of proliferating, viral-specific human CD8\(^+\) T cells following vaccination (28). The expression of CD38 and HLA-DR on CD8\(^+\)Ki-67\(^+\) T cells after anti-OX40 was analyzed. Figure 4A shows an individual patient, in whom 13% of the CD8\(^+\) T cells were Ki-67\(^+\) and 64.2% of them coexpressed CD38 and HLA-DR before treatment. The
percentage of Ki-67+ CD8+ T cells increased to 16.3% and 20.3% on days 15 and 29, respectively. On day 29, more than 95% of cycling cells expressed both CD38 and HLA-DR (Fig. 4A). A higher percentage of cycling CD8+ T cells expressed both activation markers 56 days after anti-OX40 even though the percentage of CD8+/Ki-67+ cells had returned to baseline. The mean percentage of CD38+/HLA-DR+/Ki-67+ CD8+ T cells in 11 anti-OX40-treated patients was compared with nine normal donors immunized with tetanus (Fig. 4B). At days 29 and 57, there was a statistically significant increase in the percentage of HLA-DR+CD38+Ki-67+ CD8+ T cells compared with controls, suggesting that engagement of OX40 increased proliferation and activation of CD8+ T cells.

**Antibody and T-cell responses to reporter antigens.**

Patients at all dose levels were randomized into two groups: arm A patients received KLH on day 1 (the same day anti-OX40 started) followed by a tetanus vaccine on day 29, and arm B patients received tetanus on day 1 and KLH on day 29. Peak antibody levels to KLH and tetanus were assessed 15 days after immunization, which was either on day 15 or 43 (arm A vs. arm B, respectively) after anti-OX40. Tetanus antibody titers were calculated both pre- and post–anti-OX40 and fold increases were calculated for all patients. A significant fold increase in antibody response was found in patients who were immunized with tetanus or KLH on the same day as anti-OX40 compared with patients immunized 28 days later (Fig. 5A–C and Supplementary Fig. 9). Tetanus-specific T-cell proliferation was assessed from PBMCs obtained before and 15 days after immunization. There was a significant fold increase in proliferation when the post–anti-OX40 samples from arm B were compared with arm A, $P = 0.0127$ (Fig. 5B). Patients in arm A who had increases in their anti-KLH antibody response also had a significantly higher increase of Ki-67+ CD8+ T cells on day 15 as compared with low antibody responders ($P = 0.017$, the Satterthwaite method).

**In vitro assessment of antitumor reactivity.** Autologous tumor cell lines from three patients with melanoma enrolled in the clinical trial were available for testing. PBMCs collected before and 57 days after anti-OX40 were cocultured with autologous tumor. HLA-matched or HLA-mismatched melanoma cell lines for up to five days. Significant increases in IFN-γ were found in the PBMCs/tumor supernatants after anti-OX40 in two out of 3 patients (Fig. 6A and B). IFN-γ levels were...
increased in response to autologous tumor in 2 patients, but not to HLA-mismatched tumor lines. The increase in tumor-specific IFN-γ, following anti-OX40 was primarily elicited by CD8⁺ T cells, as the increase in IFN-γ production was blocked by anti-HLA-ABC antibody, but not by anti-HLA-DR antibody and it was selectively detected in CD8⁺ T cells by flow cytometry (data not shown). There were no significant differences in influenza-induced IFN-γ production by PBMCs collected before or after anti-OX40 (Fig. 6A and B).

Serum from 1 patient with melanoma was used to probe protein lysates from a melanoma cell line (FMEX) and an embryonic kidney cell line (HEK293) by Western blot analysis before and days 0, 8, 15, 29, 36 after anti-OX40 (Fig. 6C). There is a 27-kd band present in the melanoma lysate lanes that increases in intensity over time after anti-OX40 (peaking at day 15), but is absent in kidney lysate lanes. The 27-kd band was also detected in two other melanoma cell lines with the day 15 sera from this patient (data not shown). It seems that anti-OX40 increased a tumor-associated antibody response in this patient.

Discussion
Antibodies that enhance T-cell proliferation, survival, and effector function are currently being assessed in clinical trials for patients with cancer. Ipilimumab, an anti-CTLA-4 mAb, was recently approved by the FDA for the treatment of advanced melanoma (7). Preclinical studies indicate that anti-OX40 and anti-CTLA-4 have similar abilities to
stimulate T cells in both basic and tumor immunology models (4, 13, 29, 30): anti-OX40 by costimulation and anti-CTLA-4 by blocking inhibition. Here, we report the first clinical and immunologic assessment of anti-OX40 in patients with cancer. Anti-OX40 was well tolerated: mild to moderate side effects included a brief period of lymphopenia, fatigue, fever/chills, and mild rashes. The MTD was not reached within the dose levels tested. There was tumor shrinkage in 12 of 30 patients following just one cycle, although there were no responses using RECIST. The major limitation of this mouse mAb was the induction of HAMA, which precluded the administration of multiple cycles. Due to the differences in OX40 expression between PBL and TIL, Ki-67 expression by T cells has also recently been assessed in prostate cancer patients treated with anti-OX40. We do not know the biologic reason for the difference observed at the highest anti-OX40 dose. Anti-OX40 did not seem to increase CD4+ /FoxP3+ Treg proliferation possibly due to the differences in OX40 expression between PBL and TIL. Ki-67 expression by T cells has also recently been assessed in prostate cancer patients treated with anti-CTLA-4 (33). Anti-CTLA-4 increased proliferation of both FoxP3+ and FoxP3− CD4+ T cells in a dose-dependent manner (33). The pattern of immunologic response seen with anti-OX40 could be of benefit to patients with cancer, because Treg are known to dampen immunity to tumor (34). Our observation that TIL Treg express more OX40 than PBL Treg suggests that tumor biopsy studies will be important in understanding OX40 biology and antitumor effects.

Anti-OX40 induced proliferation of CD8+ T cells, including CD8+ T cells expressing the activation markers CD38 and HLA-DR, increasing the immunologic potency of these cells. We speculate that a percentage of the Ki-67+ T cells in these patients with cancer are tumor-specific. Although the antigens recognized by the proliferating T cells have not been identified, we have evidence that anti-OX40 increased CD8+ T cell IFN-γ production in 2 of 3 patients with melanoma, in which autologous tumor lines were used to assess tumor reactivity. Similar enhanced antitumor effects

Figure 6. Anti-OX40 infusion increases tumor-specific immune response. A and B, PBMCs from two patients with melanoma, before and after anti-OX40, were cocultured with either autologous, HLA-mismatched melanoma cell lines or flu. IFN-γ in the supernatant was measured by ELISA. Antitumor-specific antibodies were measured by Western blot analysis. C, serum from a patient with melanoma was used to probe lysates (15 μg of protein/lane) from a melanoma cell line (FEMX) or a human embryonic kidney cell line (HEK 293) at different times after anti-OX40.
have been recently described in patients with prostate cancer receiving anti-CTLA-4 (35).

Anti-OX40 significantly increased antibody titers and T-cell recall responses to tetanus immunization. Although similar effects have been observed in mouse models with anti-OX40 and other immune stimulating antibodies (36), to our knowledge this is the first verification of these activities in humans. This effect might be mediated by follicular helper T cells that were transiently decreased in the PBL in OX40-treated patients compared with normal controls. This decrease might suggest that these cells trafficked to lymph nodes, in which they influenced B-cell–mediated antibody production. This observation provides a rationale for testing anti-OX40 in conjunction with vaccination to increase T- and B-cell responses to immunizing Ags in future clinical studies. Anti-OX40 could also serve as adjuvant after disruption of a tumor by radio- or chemotherapy to release tumor Ags.

In summary, administration of anti-OX40 mAb was well tolerated and enhanced both humoral and cellular immunity in patients with cancer. Anti-OX40 increased proliferation of peripheral blood CD4+ and CD8+ T cells, increased responses to recall and naive reporter Ags, and increased endogenous tumor-specific immune responses. We anticipate that multiple doses of a humanized anti-OX40 agonist will lead to stronger immunologic activation and greater tumor regression (37).

Disclosure of Potential Conflicts of Interest

N. Morris is employed as Research Advisor in Agonox and has ownership interest (including patents) in the same. E. Walker is a consultant/advisory board member of Advanced Cytometry Solutions. W.J. Urba has honoraria from Speakers Bureau of BMS and is a consultant/advisory board member of the same. A.D. Weinberg is employed as President of Agonox, has commercial research grant from MedImmune, and has ownership interest (including patents) in Agonox/OX40 Agonists. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Julie Cramer, Katrina Herz, and Scott Lary who assisted in many clinical research activities, Iliana Gonzales and Owen Kramer for the cryopreservation of PBMCs, and Jonna Vercellini for technical assistance. The authors also thank Drs. Nora Doss, John Smith, Jr., and John Thompson for their help in safety monitoring during the clinical trial.

Grant Support

This work was supported by R-01 CA109563-06 and the Providence Portland Medical Foundation.

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Received November 13, 2012; revised September 16, 2013; accepted September 16, 2013; published OnlineFirst October 31, 2013.

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Brendan D. Curti, Magdalena Kovacsovics-Bankowski, Nicholas Morris, et al.


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