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Engagement of the OX-40 Receptor In Vivo Enhances Antitumor Immunity

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The OX-40 receptor (OX-40R), a member of the TNFR family, is primarily expressed on activated CD4+ T lymphocytes. Engagement of the OX-40R, with either OX-40 ligand (OX-40L) or an Ab agonist, delivers a strong costimulatory signal to effector T cells. OX-40R+ T cells isolated from inflammatory lesions in the CNS of animals with experimental autoimmune encephalomyelitis are the cells that respond to autoantigen (myelin basic protein) in vivo. We identified OX-40R+ T cells within primary tumors and tumor-invaded lymph nodes of patients with cancer and hypothesized that they are the tumor-Ag-specific T cells. Therefore, we investigated whether engagement of the OX-40R in vivo during tumor priming would enhance a tumor-specific T cell response. Injection of OX-40L:Ig or anti-OX-40R in vivo during tumor priming resulted in a significant improvement in the percentage of tumor-free survivors (20–55%) in four different murine tumors derived from four separate tissues. This anti-OX-40R effect was dose dependent and accentuated tumor-specific T cell memory. The data suggest that engagement of the OX-40R in vivo augments tumor-specific priming by stimulating/expanding the natural repertoire of the host’s tumor-specific CD4+ T cells. The identification of OX-40R+ T cells clustered around human tumor cells in vivo suggests that engagement of the OX-40R may be a practical approach for expanding tumor-reactive T cells and thereby a method to improve tumor immunotherapy in patients with cancer. The Journal of Immunology, 2000, 164: 2160–2169.

Two primary vaccine strategies have been used to boost T cell-specific tumor immunity in animal models as well as in human clinical trials. The first involves genetic modification of tumor cells in vitro (transfection of immune-enhancing genes) and immunization with the altered tumor cells in vivo (1–7). The second strategy involves expansion of tumor-reactive T cells either by using Abs to T cell surface markers or with peptides specific for tumor antigens (8–11). Most studies have concluded that the terminal effector cells responsible for T cell-specific tumor killing are CD8+ T cells (11). Therefore, prior strategies have specifically augmented the CD8 arm of the immune system using MHC class 1-specific tumor peptides (8–11). However, it is now apparent in certain animal tumor models that CD4+ T cells can be responsible for recognition and elimination of tumors (7, 12). Ultimately, augmentation of tumor-specific responses to both CD4 and CD8 T cells may be the most beneficial tumor vaccine strategy. In this manuscript, we attempt to stimulate the CD4 T cell arm of immunity by targeting the newly described costimulatory receptor OX-40 (OX-40R), which is preferentially expressed by CD4+ T cells.

The OX-40R (CD134) is a lymphocyte-specific member of a growing family of receptors for membrane-bound and soluble cytokines that has been termed the TNFR superfamily (13). In addition to the TNFR, this family also contains the CD30 Ag, CD40, CD27, FAS (CD95), DR3, and 4-1BB, all of which are expressed predominantly on cells of hematopoietic lineage (13). A common function of the TNFR superfamily seems to be in the regulation of activation/proliferation or apoptosis of lymphocytes. For example, a signal transmitted through CD40 is pivotal for Ig switching and prevents programmed cell death of germinal center B cells (14). In contrast, signaling through FAS induces apoptosis; the absence of a functional FAS protein leads to a generalized autoimmune syndrome that may result from impaired deletion of autoreactive lymphocytes (15, 16). The OX-40R is a membrane-associated glycoprotein with an apparent m.w. of 47,000–51,000, which is found primarily on activated CD4+ T cells and not on normal resting peripheral blood lymphocytes (17). The OX-40 ligand (OX-40L) is a type II membrane protein of ~34,000 m.w. (18), which is expressed on activated B cells (19), activated endothelial cells, dendritic cells, and activated macrophages (20). It is not expressed on normal resting cells. The OX-40L delivers a potent costimulatory signal to OX-40R+ T cells and an OX-40R/OX-40L interaction appears to be directly involved in an adhesion event between endothelial cells and T cells (20).

The presentation of a tumor Ag to T cells by MHC class I or II molecules is insufficient to prime an immune response in vivo. At least two signals are necessary to activate a CD8 or CD4 T cell response (21). The first signal is delivered through the T cell Ag receptor by Ag (peptide) bound to MHC class I or II. If only the}
first signal occurs, the T cell becomes tolerant or can undergoes apoptosis. A second signal involving the ligation of a costimulatory molecule appears to be required for optimal T cell activation. The best-characterized second signal is the one delivered to the T cell CD28 receptor by its ligand B7.1 or B7.2 (now known as CD80 and CD86, respectively). Both B7.1 and B7.2 bind to two determinants on T cells, CD28 and CTLA-4; the former is constitutively expressed on 95% of CD4+ and 50% of CD8+ T cells, and the latter is expressed only on activated T cells (21). The interaction of CD80 or CD86 with CD28 results in increased production of IL-2, which is necessary for the development of a beneficial T cell response. In contrast, the interaction of B7 with CTLA-4 delivers a negative signal (22) to the T cell and could negate the activation signal of CD28. TheOX-40R is expressed on activated CD4+ T cells and when engaged causes a potent costimulatory signal to effector T cells that can enhance long-term survival (23, 24).

TheOX-40R has a very distinct pattern of expression in autoimmune disease, in that it is expressed on CD4+ T cells within the inflammatory compartment but not on peripheral CD4+ T cells (25, 26). Approximately 15–40% of the T cells that invade the inflammatory tissue during an acute episode of autoimmune encephalomyelitis (an animal model for multiple sclerosis) areOX-40R+, whereas 100% of the invading T cells express the IL-2R (25, 27). Spinal cord T cells isolated from the CNS of rats with experimental autoimmune encephalomyelitis (EAE), sorted based onOX-40R expression, had their TCR CDR3 regions (Ag-binding region) sequenced. The sequence analysis revealed that 16 of 17 fractions contained myelin-specific binding motifs, whereas only 5 of 17 in theOX-40R-negative population showed the same motif (28). These data provide strong evidence that theOX-40R+ T cells within the inflammatory site were indeed the cells that recognized autoantigens in vivo. Further support for this hypothesis was derived from experiments that depletedOX-40R+ T cells in animals with EAE. An anti-OX-40 monoclonal antibody was injected into animals with ongoing signs of EAE, and after treatment the disease did not progress and the autoantigen reactive cells were no longer present within the inflammatory site (26). We propose that theOX-40R+ T cells found within the inflammatory compartments in cancer (29) are the tumor-reactive T cells and if they can be specifically expanded it should lead to an increase in tumor-specific immunity.

In this manuscript, we report the antitumor effects ofOX-40R engagement with Ab or OX-40L: Ig in vivo during tumor priming. Injection ofOX-40L: Ig or anti-OX-40R on days 3 and 7 after s.c. tumor inoculation enhanced tumor-free survival of mice compared with untreated controls. The effect, which was dose dependent, was observed in four separate tumor models derived from four different tissues. Anti-OX-40R-treated mice that survived the initial tumor challenge exhibited enhanced tumor-specific CD4+ T cell memory. Examination of surgically removed human breast cancers revealed thatOX-40R+ T cells were found in close proximity to tumor cells. Therefore, theOX-40R-specific antitumor effects observed in the mouse tumor models may also be applicable to the immunotherapy of human cancer.

Materials and Methods

Mice

Both the B16/F10 and MCA 303 were produced on the C57BL/6 (B6) background, whereas the CT26, Renca, and SM1 tumors were produced on the BALB/c background. Therefore, the mice used in this study were either female B6 or BALB/c purchased from The Jackson Laboratory (Bar Harbor, ME) at 4 wk of age. All mice used for injection of tumor were 6–12 wk old, and each individual experiment used mice of the same age. All mice were housed at the Earle A. Chiles Research Institute animal care facility (Providence Portland Medical Center) and were cared for according to National Institutes of Health guidelines, with the exception of the CT26 experiments, which were performed at Cantab Pharmaceuticals (Cambridge, U.K.) according to British animal facility guidelines.

Tumor models

All of the tumor lines used for this study were HMC class II negative and did not express theOX-40L, as assessed by flow cytometry (data not shown). The B16/F10 (F10) melanoma line was originally described by Hart (30) and was selected as an invasive variant of the original B16 line. The F10 variant is considered poorly immunogenic because it does not protect against subsequent challenge to parental tumor when irradiated and used as a vaccine. In all experiments, theF10 tumor was inoculated with in vitro passaged cells s.c. in the flank at a dose of 1 × 106 cells, a dose which usually necessitates the mice to be sacrificed within 25–30 days after inoculation. The D5G6 melanoma is a variant of B16 that was transfected with the GMCSF cDNA (31). The transfection vector was constructed so that the only gene product produced is GMCSF. This line produces 450 ng/106 cells/h of GMCSF and can be cultured and maintained in vitro.

The MCA 303 tumor model was originally described by Huntecker and Fox (32) at the Earle A. Chiles Research Institute and is one of a series of methylcholanthrene-induced sarcomas. It has been shown to be moderately immunogenic because it protects ~50% of mice against subsequent challenge to parental tumor when used as an irradiated vaccine (32). MCA 303 grows slowly in vitro and for this reason we have been using in vivo passaged tumor for the inoculations, except for in Fig. 3A where in vitro passaged tumor was used. We have found with each in vivo passage, the MCA 303 tumor becomes more tumorigenic (data not shown) and the in vitro passaged tumor appeared to be more immunogenic and easier to treat with the anti-OX-40R reagents (see Fig. 3A).

The SM1 breast cancer line was originally developed by Guzman et al. (33), and we have shown it to be weakly immunogenic because it protects >50% of mice against subsequent challenge to parental tumor when used as an irradiated vaccine. The SM1 tumor line grows well in vitro and all mice were inoculated with 5 × 106 in vitro cultured cells and then treated withOX-40R-specific reagents.

CT26 is anN-nitroso-N-methylurethane-induced undifferentiated colon carcinoma (34). All of the mice inoculated with the CT26 cell line in this study were given s.c. (see Fig. 3C) or i.p. (Fig. 4). The renca tumor is a spontaneous arising murine renal cell carcinoma and was isolated and maintained as described previously (35). The mice in Fig. 6B were inoculated s.c. with 1 × 103 Renca cells. All mice were sacrificed when the tumor reached 200 mm3 in the flank, except for the CT26- and Renca-injected mice which were sacrificed when the tumor size reached 100 mm3.

In vivo reagents

Both the murine and human OX-40L:Ig fusion proteins were produced at Cantab Pharmaceuticals by constructing a chimeric cDNA that contained the C-terminal region of theOX-40L (extracellular domain without the membrane spanning region) fused to the constant region of human IgG1 (36). The human DR3 receptor:Ig fusion protein was produced at SmithKline Beecham Pharmaceuticals (Philadelphia, PA) and the extracellular domain (excluding the membrane spanning region) was fused to the constant region of human IgG1. All of these proteins were purified by protein G chromatography and were subjected to SDS-PAGE analysis under reducing conditions and showed a single band of the appropriate m.w. The hydridoma that produces the anti-murine OX-40R-specific mAb was obtained from the European Cell Culture Collection (37) and is referred to asOX-86. The hydridoma was grown in RPMI 1640 with FCS that was stripped of Ig by passing it over a protein G column. The cells were grown to a high density and the supernatant was then poured over a protein G column and purified Ab was eluted. Abs concentrations were quantitated by absorption at 280 nm. All of the injections of fusion proteins or Abs were given i.p. on days 3 and 6 or 7 (except for Fig. 6) after tumor inoculation at amounts ranging from 25 to 250 μg per injection. The dose for each individual experiment is indicated in the figure legends.

CD8 depletion and adoptive transfer

TheOX-40L:Ig-treated mice in Fig. 3 all survived the initial MCA 303 tumor challenge and were rechallenged 60 days later with MCA 303. Fifty-three days after the second challenge, the mice were inoculated with MCA 303 and 10 days later were injected with anti-CD8 (Lyt-2) i.p. to delete CD8+ T cells. These mice were sacrificed 3 days later, and the spleens were analyzed by flow cytometry for both CD4+ and CD8+ cells. Typically, the spleens were <2%CD8+ and the majority of T cells were CD4+. One spleen equivalent of theCD8-depleted cells (1.45 × 105 cells/transfer) were transferred i.p. into one naive recipient for three mice. The recipient mice were then rechallenged with MCA 303 15 days after adoptive transfer.
(3B). All of the mice in this experiment were given tumor doses of $1 \times 10^6$ cells/challenge. The control mice in Fig. 3B were injected with the same volume of saline i.p. that was transferred with the CD8-depleted cells.

**Analysis of OX-40R**

$^{+}$T cells isolated from tumor-draining lymph nodes

B16F10 mice were immunized with the D5G6 tumor (31) described above (1 $\times 10^6$ in four flanks). The cells from the lymph nodes (inguinal, axillary, brachial) were harvested 9–11 days after inoculation. The lymph nodes were processed and put into single-cell suspension as described (31). From five mice our yields were typically 250–300 $\times 10^6$ total cells, of which 2–4% were OX-40R $^{+}$. The cells were then incubated with OX-40L:Ig, washed, and incubated with anti-huIg beads (Miltenyi Biotech, Auburn, CA). The cells were then passed over a VS $^+$ column, and the positive and negative fractions were saved for FACS analysis and cytokine assays. The cells were put in culture for 7 days with 50 U/ml of IL-2 and then stimulated with irradiated tumor-draining lymph nodes. The cells were set up in 96-well round-bottom plates with 20,000 lymph node cells stimulated with 1 $\times 10^6$ irradiated APC. The APC were isolated from D5G6-draining lymph nodes or MCA 205-draining lymph nodes (12), irradiated with 5000 rad, washed, and used as APC in the assay. The cells were stimulated for 72 h, and the supernatants were collected from triplicate wells and analyzed in a standard ELISA assay for IFN-$^\gamma$. The anti-IFN-$^\gamma$ Abs were obtained from PharMingen (La Jolla, CA) and a standard quantitative assay was performed (12). The data represented in Fig. 4, A and B, respectively, were performed as separate experiments, and the MHC class II knockout (ko) mice in Fig. 4B were on the B16F10 background and were able to elicit tumor-specific CD8 $^{+}$ T cells that caused regression of lung (B. Fox, unpublished data).

**Immunohistology of breast cancer**

We obtained paraffin-embedded blocks from two breast cancer patients whom had primary tumor and adjacent lymph nodes removed surgically. The patients signed a standard surgical consent form, and the tissue was removed for the sole purpose of treating and diagnosing the disease. The blocks were sectioned and placed on a histostaining slide for analysis. The sections were hydrated and transferred into containers containing 0.5 M Tris (pH 10.0). The sections were then subjected to a microwave Ag-retrieval technique as described previously (38). Serial sections were then stained with the primary Abs in a humidity chamber overnight. The primary Abs used were anti-human CD4 (Vector Laboratories, Burlingame, CA) used at a dilution of 1/100, and anti-CD45 (PharMingen) used at a dilution of 1/100, and anti-CD45 Ab (ox-1) was used as a negative control for all samples. The tissue was then washed and incubated with a 1/400 dilution of a biotinylated anti-mouse Ig Ab for 25 min (Vector Laboratories). The tissue was then washed and incubated with an avidin-HRP complex (Vector Laboratories) for 30 min, washed, and the color was developed with diaminobenzidine (DAB; Dako, Carpenteria, CA). The Ag-positive cells are visualized as a reddish brown, which is the precipitate caused by the HRP clearing the DAB.

**Statistical analysis**

Survival rates were calculated according to the method of Kaplan and Meier and compared by log rank analysis. The program used for the analysis was S-Plus guide to statistical data analysis (MathSoft, Seattle, WA). The $p$ values are indicated for each analysis in Results.

**Results**

**OX-40R engagement in vivo during tumor priming (sarcoma)**

Engagement of the OX-40R causes a potent costimulatory response leading to T cell proliferation, increased cytokine production, and enhanced survival of effector T cells (23, 24, 39–41). If as we predict, the OX-40R $^{+}$ cells at the tumor site or draining lymph nodes are the tumor-specific T cells in vivo (27–29), then engagement of the OX-40R in vivo should lead to an enhanced antitumor-specific response. Therefore, we investigated whether engagement of the OX-40R in vivo during tumor priming would lead to an increase in tumor-free survival or a delay in tumor development. Fig. 1 depicts the results when mice that were injected with a lethal inoculum of MCA 303 (methylcholanthrene-induced sarcoma (32)) s.c. and were treated 3 and 7 days later with either mOX-40L:lg, DR3:lg, or saline. Saline-treated mice and mice treated with DR3:lg had to be sacrificed at the same time because of progressive tumor growth. In contrast, all of the mice that received mOX-40L:lg experienced delayed tumor growth and 60% were free of tumor for >70 days. The mOX-40L:lg-treated mice were rechallenged with MCA 303 tumor s.c., and the mice failed to develop tumors, as all the control mice did, suggesting they had developed a tumor-specific memory T cell response (data not shown).

Mice injected with MCA 303 s.c. were then subjected to a dose titration of mOX-40L:lg on days 3 and 7 after tumor inoculation. Mice that received 25 or 50 $\mu$g of mOX-40L:lg had to be sacrificed because of progressive tumor growth in a similar time frame as the control “saline” mice. Fifty percent of the mice receiving 100 $\mu$g and all mice receiving 250 $\mu$g experienced delayed tumor growth. Ultimately, 25% of the 100-$\mu$g group and 50% of the 250-$\mu$g group were tumor free for more than 70 days after tumor challenge (Fig. 2). It should be noted that the MCA 303 tumor line becomes more tumorogenic and less immunogenic the more times that it is passaged in vivo. The MCA 303 tumor line in Fig. 2 had been passaged more times in vivo than in Fig. 1, accounting for the observation that the OX-40L:lg treatment was a bit less effective at the 100-$\mu$g dose.

Fig. 3 shows the fate of mice inoculated with in vitro passaged MCA 303 that were then treated with mOX-40L:lg. In all experiments, in vitro passaged MCA 303 was easier to treat than in vivo passaged tumor. All of the mice were inoculated with tumor s.c. and injected with mOX-40L:lg on days 3 and 7 after tumor inoculation survived the initial tumor challenge, whereas all of the mice injected with saline had to be sacrificed due to excessive tumor burden (Fig. 3A). The mOX-40L:lg-treated mice that survived the initial tumor challenge from Fig. 3A were then rechallenged with MCA 303. All of the mice survived the second challenge and were tumor free for 53 days (data not shown). The surviving mice were then reinoculated with MCA 303 s.c. and depleted of CD8 cells by i.p. injection of anti-Lyt-2 Ab 10 days later. Three days later, the mice were sacrificed and shown to be devoid of splenic CD8 cells (<2%). CD8-depleted spleen cells (1.45 $\times 10^5$) from the tumor-immune mice were transferred into naive mice, and 15 days later the mice were challenged with MCA.
The OX-40R was expressed solely on the CD4 and 10 days later the tumor-draining lymph nodes were isolated. Mice were immunized with D5G6 (a B16 melanoma variant (31)), cultured in IL-2 for 7 days and then restimulated with irradiated tumor-specific CD4 T cells and assessed in vivo more times.

303 s.c. Fig. 3B shows that the mice receiving the CD8-depleted spleen cells were resistant to tumor challenge, whereas the control mice had to be sacrificed due to tumor burden. As a control for the adoptive transfer experiments described above, we transferred the same amount of CD8-depleted spleen cells or total splenocytes isolated from naive mice into naive mice and challenged them with MCA 303 15 days after adoptive transfer. As opposed to the adoptive transfer from the OX-40R-treated mice, all of the naive splenocytes conferred no adoptive immunity to the MCA 303 tumor, and all of the mice were sacrificed due to excessive tumor burden (data not shown).

**Tumor-draining lymph node OX-40R T cells have enhanced tumor-specific responses**

To understand the therapeutic effect that the OX-40L:Ig reagent has in tumor-bearing mice, we sorted OX-40R T cells from tumor-draining lymph nodes and assessed their ability to respond in a tumor Ag-specific assay. Our previous data in EAE showed that most of the response (IFN-γ production) is due to a CD4 T cell interacting with an MHC class II molecule.

FIGURE 2. Dose titration of OX-40L:Ig in mice inoculated with MCA 303. Mice were inoculated with MCA 303 s.c. and injected i.p. with escalating doses of OX-40L:Ig (25, 50, 100, and 250 μg) or the same volume of saline on days 3 and 7 after inoculation (four mice per group). The mice were followed up for evidence of tumor and were sacrificed if the tumor reached 200 mm². The mice were followed up for a total of 80 days after tumor inoculation. The MCA 303 tumor used in Fig. 2 was more tumorigenic (aggressive) than the tumor used in Fig. 2 because it had been passaged in vivo more times.

FIGURE 3. Effects of adoptive transfer of CD8-depleted spleen cells from mice immune to tumor treatment following OX-40L:Ig administration. In A, mice were inoculated with in vitro cultured MCA 303 and then treated with 100 μg of OX-40L:Ig or the same volume of saline on days 3 and 7 after inoculation (three mice per group). In B, CD8-depleted spleen cells from the OX-40L:Ig tumor-immune mice were adoptively transferred into naive recipients and challenged with in vivo passaged MCA 303. One spleen equivalent was transferred into each mouse (three mice per group). The three control mice received an equivalent volume of saline before tumor challenge. In both A and B, the mice were followed up for evidence of tumor and were sacrificed once the tumor reached 200 mm². Mice were examined for appearance of tumor for a total of 52 days after tumor inoculation.

B16/Blck/6 background). Fig. 4A presents IFN-γ production from the D5G6 OX-40R T cells and shows a 3-fold enhanced response in the OX-40R T cells, whereas neither group responded to the MCA 205-draining lymph node cells. There were some contaminating cells in our magnetic separation of the OX-40R T cells (mostly B cells and a small percentage of CD8 T cells); therefore, we wanted to assess whether CD4 T cells within the OX-40R T cell fraction were responsible for the cytokine production. The OX-40R T cell fraction was stimulated with either D5G6 tumor-draining lymph node cells from normal mice or D5G6 tumor-draining lymph nodes from MHC class II ko mice. The data confirm that most of the response (IFN-γ production) is due to a CD4 T cell interacting with an MHC class II APC and not from CD8 T cell contamination. It should be noted that the MHC class II ko mice were able to prime D5G6 tumor-specific CD8 T cells that could eradicate 3-day established lung metastases (B. Fox, unpublished data).
OX-40R-specific treatment in a poorly immunogenic tumor model (B16/F10)

The F10 variant of the B16/B16 melanoma line does not elicit a protective immune response when injected as an irradiated vaccine s.c. (data not shown), and has therefore been characterized as a poorly immunogenic tumor. We wanted to determine whether OX-40R engagement during tumor priming would enhance immunity to this aggressive tumor. Fig. 5A shows that treatment with murine OX-40L:Ig on days 3 and 7 also exhibited antitumor effects. Approximately 25% of treated mice survived tumor challenge long term. An Ab to OX-40R (mAb OX-86 (37)) delivered at the same dose and time schedule enhanced tumor-free survival to a level comparable to treatment with mOX-40L:Ig (Fig. 5B). The percentage of tumor-free mice following Ab treatment was very similar to OX-40L:Ig treatment. Both reagents resulted in a statistically significant level of tumor protection as assessed by log rank analysis ($p < 0.007$ (Ab) and $p < 0.05$ (mOX-40L:Ig)). We have now used the m-anti-OX-40R Ab in four separate tumor models, and it has been as therapeutic as the OX-40L:Ig construct in all of the models tested so far.

Enhancement of antitumor immunity in colorectal cancer model (CT26)

A similar protocol was designed to treat mice with colorectal tumors (CT26). The strategy was exactly as described above (mOX-40L:Ig, two-dose regimen). Human OX-40L:Ig was used as a negative control because it does not signal the murine OX-40R (42). The two-dose regimen significantly enhanced tumor-free survival ($p < 0.04$; data not shown). Fig. 6A shows that multiple injections of OX-40L:Ig was beneficial as assayed by tumor-free survival with a $p$ value of higher confidence ($p < 0.01$) than the two-injection dose scheme. Multiple injections (days 2, 7, 14, 21, 27, and 40) appeared to be better (greater numbers of survivors), but no statistical difference was observed when directly compared with the two-dose regimen as assessed by log rank analysis. Seven of the surviving mice from the mOX-40L:Ig-treated group were then rechallenged with CT26. All of the mOX-40L:Ig mice resisted the
tumor challenge and remained tumor free, whereas all of the naive control mice succumbed to the tumor challenge (Fig. 6B). On day 30, the seven tumor-free mice were then rechallenged with a syngeneic tumor of different histology in the opposite flank from the CT26 injections (Renca, renal origin and this tumor does respond to anti-OX-40R-mediated therapy (data not shown)) to test for a tumor-specific T cell memory response. Six of seven of the CT26-resistant mice had to be sacrificed due to tumor burden associated with the Renca tumor, which suggests that the CT26-resistant mice had specificity for tumor Ags associated with colon cancer.

Summary of OX-40R engagement during tumor priming

Table I summarizes the data in four different tumor models in which the antitumor effects of OX-40R engagement during tumor priming have been examined. Although the data suggest that more immunogenic tumors respond better to the therapy, we have therapeutic results even in the poorly immunogenic melanoma model (F10). Data for all of the tumor lines, except for the SM1 breast cancer line, have been presented in the previous figures. The SM1 tumor line is weakly immunogenic (data not shown). Mice that have been injected with the SM1 tumor and treated with OX-40L:Ig on days 3 and 7 after tumor inoculation had enhanced antitumor activity as shown by the increase of tumor-free survival. The SM1 data were subjected to log rank statistical analysis and shown to be significant with a \( p = 0.01 \).

OX-40R expression in human breast cancer

The existence of OX-40R \(^{+} \) T cells within breast cancer samples would suggest the possibility of treating breast cancer patients with anti-OX-40R-specific reagents. To determine the spatial relationship between OX-40R \(^{+} \) T cells and tumor cells, we examined several human breast cancer biopsies by immunohistochemistry. Both primary tumors and tumor-involved lymph nodes were analyzed for CD4 \(^{+} \) and OX-40R \(^{+} \) cells. Fig. 7 is a representative sample from two separate patients (from a study of 44 patients), both with infiltrating duct carcinoma. Fig. 7A depicts the tumor-infiltrating lymphocytes within a primary tumor, whereas Fig. 7B depicts a tumor-infiltrated lymph node. In Fig. 7A, the CD4 \(^{+} \) cells are seen infiltrating the outer edge of the tumor. At higher magnification, one can see that the OX-40R \(^{+} \) T cells represent a subset of the invading lymphocytes and are in close proximity to the tumor cells. A number of the OX-40R \(^{+} \) cells appear to be larger (blasts) and some appear to be undergoing mitosis. Fig. 7B depicts

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Table I. Summary of OX-40R engagement during tumor priming

<table>
<thead>
<tr>
<th>Tumor Origin</th>
<th>Immunogenicity (^{a})</th>
<th>Treatment</th>
<th>Tumor-Free Mice/Mice Injected (^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA 303 (sarcoma)</td>
<td>Moderately</td>
<td>mOX-40L:Ig</td>
<td>9/16</td>
</tr>
<tr>
<td>CT26 (colon carcinoma)</td>
<td>Moderately</td>
<td>mOX-40L:Ig</td>
<td>9/24(^{c})</td>
</tr>
<tr>
<td>SM1 (breast cancer)</td>
<td>Weakly</td>
<td>hOX-40L:Ig</td>
<td>2/22</td>
</tr>
<tr>
<td>B16/F10 (melanoma)</td>
<td>Poorly</td>
<td>mOX-40L:Ig</td>
<td>5/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat Ig</td>
<td>0/20</td>
</tr>
</tbody>
</table>

\(^{a}\) The scale for immunogenicity is based on experiments performed in our laboratory or in other laboratories (55). Irradiated cells were injected s.c. into naive recipients and then challenged with live tumor 1–2 wk after immunization. Poorly immunogenic, 0% of the mice survived the challenge; weakly immunogenic, <30% of the mice survived the challenge; and moderately immunogenic, approximately 30% or more of the mice survived the challenge.

\(^{b}\) Log rank statistical analysis was performed on all groups except for the MCA 303 group and the \( p \) values are reported in Results.

\(^{c}\) Combines all of the experiments where at least 100 \( \mu \)g of mOX-40L:Ig was used.

\(^{d}\) Combines the two CT26 experiments discussed in Results with different time courses of treatment.

\(^{e}\) The human (h) OX-40L does not signal the murine OX-40R (42).
a lymph node where more than half of the node architecture has been invaded by the tumor. There is an abundance of CD4+ cells surrounding the invading tumor. The OX-40R+ cells are found concentrated in areas directly adjacent to the invading tumor cells (lower right panel). OX-40R+ cells are also found in uninvolved areas, but the greatest number was found closest to the site of tumor infiltration. Based on our work in autoimmune disease and the data in Fig. 4, we hypothesize that the OX-40R+ cells within these tissue sections represent the tumor Ag-specific T cells (27, 28).

We have now analyzed 44 patients with breast cancer with both anti-CD4 and anti-OX-40R Abs (our unpublished data). We have found that 18 primary tumors had CD4 infiltrates, 30% of which contained OX-40R+ T cells. Twenty-seven of these patients had lymph nodes that contained infiltrating breast cancer cells. Of the tumor-positive lymph nodes, 48% were highly positive for OX-40R+ T cells, whereas 26% were positive and 26% were negative. In the tumor-negative lymph nodes, there were very few OX-40R+ T cells. In the majority of specimens sampled, the OX-40R+ T cells were found in greatest numbers close to the site of tumor infiltration.
infiltration similar to the lower right panel in Fig. 7. All of the tumors tested in this study were MHC class II negative; therefore, we are not exactly sure why the OX-40R+ T cells would be in close proximity to the tumors. We hypothesize that an APC (such as a dendritic cell) may be near the tumor T cell boader and may present Ag to CD4+ T cells, causing up-regulation of the OX-40R.

Discussion

In vivo engagement of the OX-40R during tumor priming results in a significant therapeutic benefit in four separate tumor models of diverse histology and immunogenicity. The effect was dose dependent and induced long-lasting tumor-specific immunity in the mice that were cured of the initial tumor challenge. Previous data showing that the OX-40R+ cells within the inflammatory lesions in an autoimmune disease model were the T cells that responded to autoantigen suggest that we are targeting tumor Ag-specific cells in vivo with the OX-40R-specific therapy (27, 28). We and others have shown that engagement of the OX-40R in vitro causes a potent costimulatory event that enhances T cell cytokine production, proliferation, and survival (23, 24, 39–41). Therefore, OX-40R engagement during tumor priming in vivo is likely to enhance tumor Ag-specific CD4+ T cell function, expansion, and survival leading to improved antitumor immunity. The appearance of OX-40R+ T cells adjacent to tumor cells in human breast cancer specimen suggests that this strategy could be translated into human clinical trials with similar therapeutic effects.

Engaging the OX-40R in vivo during tumor priming led to 20–60% cure rates for mice challenged with four different solid tumors with varying immunogenicity. The data suggest that OX-40R-based therapy could be a universal enhancer of the immune system, not only for tumor immunity, but also as an immunologic adjuvant for other types of vaccines (e.g., viral, bacterial, etc.). The human OX-40L:Ig protein is a form of a potential immune adjuvant that is available now for use in human clinical trials and has been shown to stimulate human T cells in vitro. Currently, we are in the process of producing a panel of humanized anti-OX-40 mAbs. Although the Ab and the soluble OX-40L fusion protein worked with similar potency in our tumor models (Fig. 5 and data not shown), we feel that the Ab may be less immunogenic and would potentially have a longer half-life in vivo. Both the fusion protein and anti-OX-40R Abs are likely candidates for human vaccine adjuvant therapies in the future. Currently, we do not have a mouse reagent that recognizes the mouse OX-40R; therefore, we cannot make a direct comparison between the reagents we have been using (rat anti-mouse or mouse OX-40L:human Ig) in mice vs a mouse anti-OX-40R reagent. It could be possible that when multiple injections of the OX-40L chimeric fusion protein (mouse/human) are administered (Fig. 6) that Abs may be produced that enhance the response by cross-linking the fusion protein in vivo. Currently, we are synthesizing the mouse/mouse OX-40L:Ig fusion protein to assess multiple injections use of this reagent for long-term experiments in vivo.

Enhancement of tumor immunity with Abs such as anti-4-1BB or anti-CTLA-4 are other examples of T cell activation Ags which when triggered or blocked enhance tumor-specific immunity (8, 43). Like the OX-40R, the 4-1BB receptor, also a member of the TNFR family, was originally described as a T cell activation Ag that has potent costimulatory properties (44). The 4-1BB receptor is expressed on CD8 and CD4 T cells as well as NK cells (44). 4-1BB receptor costimulatory function appears to primarily affect CD8+ T cells (45), and engagement of this receptor during tumor priming led to a 50-fold increase in tumor-specific CD8+ T cell cytolytic function and led to enhanced tumor-free survival (8). The CTLA-4 protein is expressed on both CD8 and CD4 T cells and when engaged by its ligand(s) (B7.1 or B7.2) delivers an inhibitory signal to the T cell. Abs that block CTLA-4/B7 interaction enhance Ag-specific T cell function (22) and can enhance tumor-specific immunity (43). The OX-40R-specific therapy although potent, never led to 100% tumor-free mice. Therefore, combination therapy with anti-CTLA-4 or anti-4-1BB and anti-OX-40R may synergize to accentuate Ag-specific T cell therapy leading to a higher percentage of tumor-free mice. Future experiments will attempt to maximize tumor-specific T cell therapies by combining two or more of these Abs during tumor priming with the hope of enhancing both CD4 and CD8 Ag-specific effector/memory T cell responses.

Recently, we have shown that in vivo engagement of the OX-40R during Ag-specific priming increases the number and life span of Ag-specific CD4+ T cells (see Ref. 24 and data not shown). Most T cells become susceptible to activation-induced cell death after encountering Ag at the effector T cell stage, and only a few go on to become memory T cells (46). We hypothesize that engagement of the OX-40R during tumor priming targets the tumor-reactive CD4+ T cells and rescues them from activation-induced cell death. An increase in the number of Ag-specific T cells may allow the mice to remain tumor-free and fight a secondary tumor challenge. Fig. 3B shows that the adoptive transfer of CD8-depleted spleen cells from OX-40R-treated tumor-immune mice can confer antitumor immunity. These data suggest that there is an increase and/or enhancement of tumor Ag-specific memory CD4+ T cells, and that they are able to transfer adoptive protection. Because the tumor cells in all four models do not express MHC class II, we do not feel that the CD4+ T cells are the ultimate effector cells that interact with the tumor. Instead, we hypothesize that enhanced cytokine production by the tumor Ag-specific CD4+ T cells help to activate CD8+ T cells, NK cells, and/or macrophages, which in turn may directly interact with and destroy tumors. We have some direct evidence for the indirect model of tumor irradiation in the Renca model (Renal cell carcinoma). We found the Renca tumor to respond to anti-OX-40R therapy, but if either the CD4 or CD8 T cells were deleted the therapeutic effect was diminished (data not shown).

We now have direct evidence that engagement of the OX-40R dramatically increases memory T cell development (47). The experiments were performed with T cells that expressed the transgenic TCR for OVA (DO11.10) and could be detected with an anti-idiotypic Ab (48). Soluble Ag was injected into mice seeded with TCR transgenic T cells and given five daily injections of anti-OX-40R in a proinflammatory environment (LPS injection) during the priming phase. The mice were sacrificed 60 days after immunization, and there was a 60-fold increase in Ag-specific cells compared with mice receiving Ag alone or Ag plus LPS. The Ag-specific cells were phenotyped and shown to be small resting T cells, which is the target signal to the T cell. Abs that block CTLA-4/B7 interaction enhance Ag-specific T cell function (22) and can enhance tumor-specific immunity (43). The OX-40R-specific therapy although potent, never led to 100% tumor-free mice. Therefore, combination therapy with anti-CTLA-4 or anti-4-1BB and anti-OX-40R may synergize to accentuate Ag-specific T cell therapy leading to a higher percentage of tumor-free mice. Future experiments will attempt to maximize tumor-specific T cell therapies by combining two or more of these Abs during tumor priming with the hope of enhancing both CD4 and CD8 Ag-specific effector/memory T cell responses.

We have found that the OX-40R is only expressed on CD4+ T cells isolated from the “inflammatory sites” in cancer and autoimmune disease and is turned over quite rapidly (within 24–48 h) (26, 29). However, it has been shown that both CD4 and CD8 T cells can express the OX-40R if stimulated in vitro with Con A or PHA (42). It appears that the only way to up-regulate OX-40R expression on T cells is through TCR engagement. Even in highly inflammatory situations, such as superantigen stimulation, there appears to be no bystander up-regulation of the OX-40R on Ag-nonspecific cells (24, 47). In superantigen-injected mice, the OX-40R is solely expressed on Vβ3/CD4+ T cells, which is the target
TCR for this superantigen (24). Therefore, we believe that engaging the OX-40R during tumor priming in vivo would target the most recently Ag-activated T cells.

It has been shown that the inflammation associated with superantigen stimulation and clinical signs of EAE involves the production of Th1 cytokines (49). We have shown that engaging the OX-40R on Th1 lines accentuates T cell proliferation by up-regulating translation and transcription of IL-2 (39). We have also shown that effector T cells appear to be more sensitive to OX-40R-specific costimulation than naive T cells (23). Effector T cells that have differentiated to produce either Th1 or Th2 cytokines are both sensitive to OX-40R-specific costimulation (23). Engaging the OX-40R on Th2 effector cells increased translation and secretion of IL-4 and IL-5 and enhanced their proliferation. Two reports recently showed that engaging the OX-40R polarizes cells to the Th2 phenotype (40, 41). Our data suggest that T cell polarization is dependent on the cytokine milieu present during T cell differentiation and engagement of the OX-40R will accentuate both a Th1 or Th2 response. It has been shown that a Th2-immune response can be detrimental to tumor eradication in vivo, but a Th1 response is beneficial (50). Therefore, it might be important to enhance Th1 responses during tumor priming (with IL-12, IFN-γ, and/or anti-IL-4) to get an effective antitumor-immune response when administering reagents that engage the OX-40R in vivo. In contrast, other groups have shown that tumor-specific Th2 cells can play a role inactivating eosinophils and might be beneficial to an antitumor immune response (51).

The OX-40L is expressed only on activated APC such as B cells, dendritic cells, endothelial cells, and macrophages (19, 20, 52, 53). In vivo expression of the OX-40L appears to occur in highly inflammatory situations such as infection of mice with mouse mammary tumor virus (draining lymph nodes) or in mice with EAE on macrophages isolated from the inflamed organ (brain) (31, 53). Even in normal primary T cell responses such as immunization with Ag in CFA, OX-40L expression was quite low on spleen macrophages (50). The OX-40R is expressed every time a T cell is triggered through the TCR; therefore, the potent OX-40R costimulatory effects might be regulated by the inexcessability of the OX-40L on APC. The immune system has evolved to generate an immune response to clear foreign entities rapidly and then readily down-regulate itself. Since OX-40L-mediated costimulation is quite potent at the effector T cell stage, it may only be necessary in cases where a massive invasion occurs, which in turn causes a long-lasting inflammatory response. Because aggressive tumors have been shown to down-regulate immune responses through immunosuppressive mechanisms (54), APC near the tumor site would probably not express the OX-40L. We hypothesize that engagement of the OX-40R in vivo enhances a beneficial tumor-specific immune response by adding a signal that is absent in the tumor-bearing mice. This would explain the observation that a percentage of the tumor-challenged mice injected with OX-40R:lg were able to remain tumor free.

In summary, engagement of the OX-40R in vivo during tumor priming significantly delayed and prevented the appearance of tumors compared with control-treated mice. This OX-40R effect was dose dependent and was observed in both immunogenic and non-immunogenic tumor models. OX-40R expression was found on T cells localized at the tumor site in several different human cancers (melanoma, head and neck, and breast cancer) (Ref. 29 and Fig. 7). In this manuscript, we examined the physical relationship of the OX-40R T cells to breast cancer cells in both primary tumors and a tumor-invaded lymph node. The OX-40R T cells were concentrated in areas surrounding the tumor, and we hypothesize (from Fig. 4 and our work in EAE (27, 28)) that they are the tumor-specific T cells. The combination of the OX-40R therapeutic data in the mouse tumor model and the appearance of OX-40R+ in tumor-bearing patients suggests that we will be able to enhance immune tumor reactivity with reagents designed to engage the OX-40R in patients with cancer. The data also suggest that engaging OX-40R may be a useful immune adjuvant to enhance Ag-specific responses in a wide variety of vaccine settings.

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