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Signaling through OX40 Enhances Anti-tumor Immunity

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Abstract

The existence of tumor-specific T cells, as well as their ability to be primed in cancer patients confirms that the immune response can be deployed to combat cancer. However, there are obstacles that must be overcome to convert the ineffective immune response commonly found in the tumor environment to one that leads to sustained destruction of tumor. Members of the tumor necrosis factor (TNF) superfamily direct diverse immune functions. OX40 and its ligand, OX40L, are key TNF members that augment T-cell expansion, cytokine production, and survival. OX40 signaling also controls regulatory T cell differentiation and suppressive function. Studies over the past decade have demonstrated that OX40 agonists enhance anti-tumor immunity in preclinical models using immunogenic tumors; however, treatment of poorly immunogenic tumors has been less successful. Combining strategies that prime tumor-specific T cells together with OX40 signaling could generate and maintain a therapeutic anti-tumor immune response.

Introduction

Advances in the field of tumor immunotherapy have been borne out of our increased understanding of the mechanisms of the immune system. The ability to elicit immune responses against tumor antigens demonstrates that harnessing the immune system to combat tumors is feasible: however, strategies must be devised that augment and maintain tumor-directed immune responses. Activation of the T cell arm of the immune system

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Disclosure Statement

ADW is a founder of Agonox, a company that has licensed OX40 agonists for cancer treatment. H-MH and BAF are founders of UbiVac, a company developing autophagosome vaccines for treatment of cancer.

requires recognition of cognate antigen by the T-cell receptor (TCR) in conjunction with costimulation provided by antigen-presenting cells (APC). These initial interactions are important for the successful priming of T-cells; however, the tumor microenvironment in which these primed T cells reside will ultimately determine their ability to produce a clinically relevant anti-tumor immune response and establish immunological memory (1). Soon after T-cell priming negative regulatory molecules, such as CTLA-4, are induced on T cells leading to downregulation of the T-cell response (1,2). Successful tumor immunotherapy will require adequate numbers of tumor-specific T cells that avoid downregulation and survive over an extended period of time. Members of the tumor necrosis factor (TNF) superfamily contribute to this post-activation environment that influences the fate of recently primed T cells. These interactions, exemplified by OX40, 4-1BB, CD27, GITR, and DR3, provide proliferative and survival signals as well as facilitate T-cell differentiation. The goal of this review is to describe our current understanding of OX40/OX40L interactions as they relate to their role in tumor immunotherapy.

OX40 and OX40L Expression

An antibody (MRC OX-40) that bound to activated rat CD4 T cells and augmented the proliferation of those T cells led to the discovery of OX40 (CD134, TNFRSF4) in 1987 (3). OX40 is a 50 kDa type I transmembrane receptor containing 277 amino acids (a 49 amino acid cytoplasmic tail and a 186 amino acid extracellular region) (4). Early studies of OX40 expression on T cells demonstrated that T-cell activation was required for its expression (3). Neither naïve T cells nor resting memory T cells express OX40 (3,5). Potent signaling through the TCR can induce OX40; however optimal expression of OX40 on the surface of T cells requires additional costimulation through CD28 and/or other cytokine receptors (6). Initial work suggested that OX40 expression was limited to CD4⁺ T cells, but subsequent studies demonstrated that OX40 was also expressed on activated CD8⁺ T cells (7). Expression on activated CD8⁺ T cells was more transient. The induction of OX40 occurs within 24 hours and peaks 48–72 hours following initial TCR stimulation. The duration of OX40 expression depends on the potency of TCR signaling and costimulation, but typically lasts 3–4 days. The induction of OX40 on memory T cells following antigen rechallenge occurs much more rapidly than naïve T cells (8). Other cell types e.g. (natural killer T cells, neutrophils, and natural killer cells) also express OX40 (9–11).

OX40 is constitutively expressed on murine FoxP3⁺CD4⁺ naturally-occurring T_{reg} cells (nT_{reg}), and is inducible on human T_{reg} cells (12). Thymic development of nT_{reg} cells does not require OX40 as OX40^{-/-} have nT_{reg} cells, which are present at a reduced frequency suggesting a role for OX40 in nT_{reg} cell homeostasis (12). In addition to nT_{reg} cells, naïve T cells can become induced T_{reg} cells (iT_{reg}) when activated in the presence of TGF-β (13). The induction of iT_{reg} cells is affected by OX40, although the results have been controversial. The addition of an agonist anti-OX40 antibody to *in vitro* conditions that induce iT_{reg} cells inhibited the generation of iT_{reg} cells (14,15). However, Ruby et al., demonstrated that anti-OX40 agonist treatment increased the polarizing cytokines IFN-γ and IL-4 and diverted naïve T cell differentiation away from the iT_{reg} pathway toward Th1 and Th2 phenotypes. If IL-4 and IFN-γ were blocked during the Treg induction cultures then the agonist anti-OX40 antibody enhanced iT_{reg} generation (16).

The ligand for OX40 (OX40L, CD252, TNFSF4) was initially discovered on HTLV-1 transformed T cells, and termed gp34 (17,18). Subsequently, gp34 was identified as the binding partner of OX40 (19,20). OX40L is a type II transmembrane receptor containing 183 amino acids (a 23 amino acid cytoplasmic tail and a 133 amino acid extracellular domain). It is expressed on the surface as a trimer allowing it to bind to three OX40 molecules (21). OX40L is expressed on activated APCs, including dendritic cells, B cells

and macrophages (22,23). Signaling through CD40 as well as inflammatory stimuli that signal through Toll-like receptors (TLR2, TLR4, TLR9) induce OX40L expression on APCs (23). Inducible OX40L expression on APCs ensures that activated OX40⁺ T cells only receive signaling through OX40 locally when they are in an inflammatory environment. OX40L is also found on NK cells, mast cells, activated T cells, human airway smooth muscle cells, and vascular endothelial cells (24–26).

OX40 and OX40L Signaling

Antigenic stimulation of T cells proceeds through four phases: activation, expansion, contraction, and development of long-lived memory (27). Naïve Ag-specific T cells proliferate upon antigen stimulation and form a pool of effector T cells (Figure 1A). As the antigenic challenge is cleared, the pool of antigen-specific T cells contracts leaving a smaller pool of memory T cells. The temporal expression of OX40 after the priming event suggests its importance in late proliferation and survival of effector T cells. In the absence of OX40, antigen-primed T cells proliferate normally for 2–3 days after antigen stimulation and differentiate into effector T cells, however, there is a significant reduction in their survival by day 12 (Figure 1B)(28,29). The interaction of OX40 with OX40L results in the recruitment of TNFR-associated (TRAFs) molecules to the intracellular domain of OX40. TRAF2 and 3 activate both the canonical NF- κ B1 pathway as well as the noncanonical NF- κ B2 pathway, which are known to be important for cell survival. The activation of the anti-apoptotic molecules BCL-2, BCL-xL, and survivin (29,30) in OX40-stimulated T cells may be responsible for the increased clonal expansion and a larger pool of memory T cells (31), although others have not observed up-regulation of these genes (32,33).

OX40 signaling also augments cytokine secretion (IL-2, IL-4, IL-5, IFN- γ) by activated CD4 T cells (8,34,35). Although early reports suggested that OX40 signaling supported Th2 differentiation (22,34), it has become clear that OX40 signaling can enhance the development of both Th1 and Th2. However, T-cell differentiation is more directly determined by the strength of TCR stimulation, antigen dose, and local cytokine milieu rather than OX40 signaling (36,37). OX40-stimulated T cells also increase expression of IL-2R α (CD25), IL-7R α (CD127), and IL-12R β 2 (35,38–40), receptors associated with T-cell survival. For example IL-2R α signaling activates PKB/AKT which induces the anti-apoptotic molecules, BCL-2 and BCL-xL (41). Enhanced IL-12R β 2 expression is required for OX40-mediated survival through a STAT4-dependent mechanism on OX40-treated CD4 T cells (35). Since the initial OX40/OX40L work focused on CD4⁺ T cells, the understanding of OX40 on CD8⁺ T cells has lagged behind that of CD4⁺ T cells. Several studies using agonist anti-OX40 antibody have shown enhanced expansion and survival of CD8⁺ T cells (7,42). Clearly though, there is both a direct and indirect effect of OX40 on CD8⁺ T cells since OX40^{-/-} CD8⁺ T cells can expand after OX40 treatment although expansion was less than OX40^{+/+} CD8⁺ T cells (39,43,44).

Preclinical Tumor Models

The potent immunological effects mediated by the OX40/OX40L interaction make it an attractive target for improving tumor immunotherapy, especially to increase the frequency and survival of tumor-specific T cells. As stated earlier, TLR agonists and CD40/CD40L are key for the upregulation of OX40L on APCs. However, administration OX40 agonist most likely bypasses the need for DC maturation, at least at the level of OX40 signaling (Figure 1B). Studies have demonstrated therapeutic efficacy when OX40 agonists were delivered three days after subcutaneous tumor challenge with melanoma, glioma, breast and colon carcinoma, sarcoma, renal carcinoma, and prostate cancer (45–49). In each tumor model a percentage of mice (20–55%) remained tumor free. Mice that survived from tumor challenge

through OX40 treatment had tumor-specific memory T cells that could protect naïve mice against a tumor challenge after adoptive transfer (45). The ability of OX40-agonists to protect appears to correlate with the immunogenicity of the tumor. Tumor protection was achieved against immunogenic tumors, however poorly immunogenic tumors did not respond to OX40 agonist treatment (50). This is not surprising since OX40 expression on tumor-specific T cells would require sufficient priming likely not provided by poorly immunogenic tumors.

Strategies employing OX40 agonist treatment in conjunction with other agonist antibodies or factors that stimulate T-cell function have been examined. As stated earlier, OX40 engagement upregulates IL-12R β 2 and the addition of IL-12 to OX40-treated CD4⁺ T cells resulted in greater IFN- γ secretion (38). The addition of IL-12 and an anti-4-1BB antibody to OX40 treatment resulted in a significantly higher cytotoxic T cell activity against tumor cells, and better survival rates in treated mice compared to controls (51). Combining an agonist OX40 antibody, a blocking CTLA-4 antibody and intratumoral injections of CpG oligonucleotides induced regression of systemic lymphoma tumors and provided long lasting protection against tumor rechallenge (52).

T_{reg} cells and OX40-based tumor immunotherapies

It has become increasingly clear that tumors establish immunosuppressive environments that are an obstacle for successful tumor immunotherapy. One such obstacle is the presence of T_{reg} cells that suppress anti-tumor immune responses (53). As discussed above, recent studies have demonstrated a role for OX40 in the homeostasis of T_{reg} cells (12,14–16). Specifically, an agonist anti-OX40 antibody reduced the frequency of TGF- β -induced iTregs *in vitro* (14,15). Our lab has investigated a model where tumor recurrence correlates with increased frequencies of T_{reg} cells. Briefly, lymphopenic mice with large (150–200 mm²) B16BL6-D5 tumors receive an adoptive transfer of tumor-specific CD4⁺ (Trp1 TCR transgenic) and CD8⁺ (pmel TCR transgenic) T cells. Following tumor regression approximately 30% of mice experience tumor recurrence. In an initial experiment mice treated with agonist anti-OX40 antibody had a delay in tumor recurrence (Figure 2A) which correlated with lower frequencies of FoxP3⁺ CD4⁺ T_{reg} cells in the peripheral blood (Figure 2B). However, in three subsequent experiments anti-OX40-treated mice had frequencies of FoxP3⁺ CD4⁺ T_{reg} cells comparable to rat-Ig treated controls and there was no reduction in tumor recurrence. While there is good correlation with low frequencies of FoxP3⁺ T_{reg} cells and prevention of tumor growth, we are puzzled by the failure of anti-OX40 treatment to keep T_{reg} low in subsequent experiments. One possible explanation for these conflicting data builds on the observation that OX40 signaling accentuates T-cell differentiation (Th1, Th2, T_{reg}) driven by the local cytokine milieu (16, 54). Possibly, the cytokine milieu was optimal for reducing T_{reg} when anti-OX40 was administered in the first experiment, but not in subsequent studies. These findings underscore the complexity of the anti-tumor immune response and emphasize the importance of further studying the conditions that favor anti-tumor immune responses in recipients of anti-OX40.

It has also been shown that agonistic anti-OX40 antibody can inhibit the suppressive activity of T_{reg} cells. T_{reg}-mediated suppression of GVHD was abrogated by intraperitoneal injection of anti-OX40 antibody, or by *in vitro* pretreatment of T_{reg} cells with anti-OX40 antibody before transfer (55). Piconese et al., demonstrated in a colon carcinoma model that OX40 stimulation enhanced effector T-cell function and inhibited suppression mediated by T_{reg} cells. Using OX40^{-/-} T_{reg} cells and effector T cells it was shown that OX40 signaling in both T_{reg} cells and effector T cells must occur for the tumor to be rejected (56). Thus, OX40 treatment can affect both T_{reg}-mediated suppression as well as render effector T cells resistant to the inhibition mediated by T_{reg} cells.

Age and OX40-based tumor immunotherapies

It is well established that age-related changes occur in both innate and adaptive immune cells, which may impact tumor immunity and immunotherapy efficacy. Indeed, the systemic administration of anti-OX40 that greatly improved the tumor-free survival of young (2–6 months old) tumor-bearing mice, failed to improve the tumor-free survival in older tumor-bearing mice (12–20 months old) (57). Surprisingly upon further investigation, it was found that older T cells and younger T cells respond comparably following OX40 stimulation, and that the age of the cells of the local innate environment, which promote and support ongoing T cell responses, appear to be responsible for the age-related deficiency in OX40-mediated tumor immunity (57). Further studies are needed to identify and understand the primary age-related defect in order to design optimal OX40-based strategies for the treatment of elderly cancer patients. A potential candidate is IL-12, as administration of this cytokine partially restored OX40-mediated tumor regression in older tumor-bearing mice (57).

Cancer Vaccines

Single agent treatment with OX40 agonist has provided protection against a variety of immunogenic tumors. However, these studies relied on the presence of sufficient endogenous antigen priming to induce the OX40 on tumor-specific T cells, by which the OX40 agonists could exert their effects (Figure 1C). Cancer vaccines prime tumor-specific immune responses, and can be enhanced with the addition of granulocyte macrophage colony-stimulating factor (GM-CSF) (58). Murata et al., showed that the combination of a GM-CSF secreting tumor cell vaccine with anti-OX40 antibody induced a potent CD8⁺ T-cell response that eliminated established breast cancer in mice (42). Importantly, these authors showed that treatment of OX40 costimulation combined with a GM-CSF-secreting tumor vaccine enhanced the expansion and prolonged the survival of tumor-specific T cells compared to vaccine alone (42). In other studies Hu and colleagues identified that anti-OX40 given in combination with a vaccine generated from tumor autophagosomes was significantly more therapeutic than either agent administered alone (figure 3). These data further support the concept that coordinating immunotherapeutic interventions can improve therapeutic efficacy. Based on these studies our group is preparing to initiate a combination immunotherapy trial of breast cancer autophagosomes from an allogeneic breast cancer cell line together with anti-OX40 antibody.

Radiation and Chemotherapy

While it is likely tumors that shed small quantities of antigen as cancer cells turn over in the process of tumor growth and invasion (59), engineering large-scale death of cancer cells *in vivo* is a potential source of antigen to prime new immune responses. Thus, over the course of radiation therapy, patients can develop new tumor antigen-specific immune responses (60). In immunocompetent animal models, CD8 T cells significantly contribute to the efficacy of radiation therapy (61,62). The combination of agonistic anti-OX40 antibodies and radiation therapy has been shown to be significantly more effective than either therapy alone (62,63). Importantly, OX40 therapy was shown to increase tumor antigen-specific cytotoxicity (63) and result in increased infiltration of activated CD8 T cells into the tumor (62).

While many chemotherapy drugs are toxic to lymphocytes, many chemotherapeutic agents have also been shown to engender an immune response that is responsible for some portion of their efficacy (64). With careful timing to take advantage of lymphocyte repopulation following lymphodepleting chemotherapy, agonistic anti-OX40 antibodies have been shown to enhance control of tumors in animal models (54). In this system the cytotoxic therapy is posited to provide tumor antigen to tumor-draining lymph nodes, priming T cells and inducing OX40 expression. Based on these data our group has just received funding to

initiate a clinical investigation to evaluate anti-OX40 in combination with chemotherapy and radiation therapy in patients with metastatic prostate cancer.

Surgery

During cancer surgery, particular care is to avoid disruption of the tumor and minimize release of cancer cells; therefore, tumor antigen release during the operation may be minimal. However, it has been proposed that surgical removal of the primary tumor has a net positive effect on anti-tumor immunity by removal of a source of immune suppression (65). In animal models, CD8 T cells have been shown to play a significant role in controlling residual disease following surgical removal of the primary tumor (62). In an animal model of surgical therapy, treatment with agonistic anti-OX40 antibodies resulted in increased tumor antigen specific T cell cytotoxicity and significantly enhanced control of residual disease (62).

Adoptive Immunotherapy

Vaccination strategies rely on the ability to prime the endogenous immune system sufficiently to eradicate tumor. The ability to expand tumor-specific cells *ex vivo* enables adoptive immunotherapy to achieve potent antitumor effects above what is often achievable by vaccination alone. The role of OX40 in augmenting proliferation and survival makes it a logical candidate for combination with adoptive immunotherapy. Intracranial tumors that had been established for 10 days could be cured with the adoptive transfer of 3-fold fewer T cells if the adoptive transfer was combined with anti-OX40 treatment (66). Using OX40^{-/-} CD8 T cells it was shown that OX40 signaling was important for the survival and accumulation of CD8 T cells at the tumor site (67). These data show that agonistic anti-OX40 antibodies may combine well with adoptive immunotherapy to increase the activity and survival of tumor antigenspecific T cells.

Our group has also investigated whether adding soluble anti-OX40 to cultures of tumor-infiltrating lymphocytes (TIL) augmented the production of tumor-specific T cells. Analysis of 128 independent and parallel cultures of TIL cloids (64 with anti-OX40 and 64 without) from melanoma surgical specimens from 10 different patients failed to show any striking difference in either the frequency or number of tumor-specific T cells recovered. It is still an open question whether immobilized anti-OX40, which typically provides T cells with a stronger proliferative signal, may increase TIL recovery. Another option is to employ anti-OX40 treatment during the TIL rapid expansion cycle or REP. The REP is the massive expansion phase where TIL are cultured with excess feeders and anti-CD3 alone or together with anti-CD28. Given the importance of OX40 signaling in providing a survival signal to T cells we anticipate that applying anti-OX40 during the REP may increase recovery of TIL.

Clinical Studies

A mouse monoclonal agonistic antibody to OX40 was developed by our group under the direction of Dr. Andrew Weinberg and produced under GMP conditions for administration to patients with cancer. A first-in-man phase I trial was performed to establish a safe dose with biologic activity. Three doses of anti-OX40 were administered on days 1, 3 and 5 of a treatment cycle at 0.1, 0.4 and 2 mg/kg. The dose levels were chosen based on pre-clinical primate models showing immunological effect and good tolerability (68). The schedule was chosen because multiple doses of anti-OX40 induced tumor regression in animal models. Furthermore, it is known that OX-40 cycles over a 24–48 hour period in activated T cells as reviewed above. Finally, the murine anti-OX40 that we are using induces HAMA, although not during the first week of anti-OX40 exposure. Thus, repetitive weekly dosing was not feasible with this particular antibody. KLH and tetanus were injected at the same time as the

OX40 agonist and used as reporter antigens. Thirty patients received anti-OX40. Ten patients were treated at each dose level to learn more about the immunological effects of the OX40 pathway engagement in humans. Toxicities were mild with fatigue and transient lymphopenia as the most commonly observed side effects (69). The clinical results and assessment of immunological effects are currently being prepared for publication.

As noted above future plans include clinical trials combining anti-OX40 with chemotherapy, radiation or vaccines. Other studies are planned to examine tumor-specific T-cell immune responses induced by OX40 in patients with metastatic melanoma. A human OX40L-Ig fusion protein (70) and a humanized antibody to OX40 are in preparation and will be used to study the effects of repetitive dosing of OX40 agonists in patients with cancer.

Conclusions

Combining OX40 treatment with other anticancer approaches, including immunotherapy, that are consistent with the functional role of OX40 in augmenting expansion and survival of tumor-specific T cells makes OX40 a promising partner in combinatorial therapy.

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Figure 1. OX40/OX40L interactions impact the four phases of the T cell lifespan

A) Activation of T cells begins with the T cell interaction with the antigen-presenting cell. Cognate antigen presented in MHC molecules by the antigen-presenting cell are recognized by the T cell receptor (TCR) on the T cell. Costimulation through CD28 in conjunction with TCR signaling activates the T cell and leads to upregulation of OX40. Similarly, stimulation of antigen-presenting cells through CD40/CD40L (not shown) or TLR agonists induces expression of OX40L. Binding of OX40 to the OX40L enhances proliferation and survival of the T cells leading to a larger expansion of effector T cells and a larger pool of memory T cells. B) In the absence of OX40 on the T cell or OX40L on the antigen-presenting cell, T cell expansion is reduced and the pool of memory T cells is fewer. C) The administration of OX40 agonists bypasses the need for OX40L supplied by mature antigen-presenting cells.

Figure 2. OX40 treatment delays tumor recurrence

A) Lymphopenic ($Rag1^{-/-}$) mice were challenged with the B16BL6-D5 melanoma cell line. On day 15, Trp1-specific TCR transgenic CD4 T cells together with gp100-specific TCR transgenic CD8 T cells were adoptively transferred into the tumor-bearing mice followed by a vaccination with an irradiated tumor cell vaccine that secretes GM-CSF. Mice received anti-OX40 antibody or Rat IgG on days 15, 20, 24, and 37. B) The percent FoxP3⁺ cells of the CD4⁺ T cell population is determined from peripheral blood samples of mice on the indicated days.

Figure 3. OX40 treatment significantly improves therapeutic response to vaccination with tumor-derived antophagosomes

Mice received 2×10^5 3LL tumor cells i.v. and on day 3 were treated with 3LL DRibble vaccine alone (antophagosomes), 3LL DRibble vaccine combined with anti-OX40, or anti-OX40 alone. Untreated mice were included as a control. 3LL DRibble vaccine (20 μ g) was injected into both inguinal lymph nodes. Anti-OX40 (75 μ g/mouse) was administered ip. All mice were sacrificed on day 28, the lungs were collected and metastases were enumerated. Typically five mice were included in each group. Results are representative of three independent experiments.