A Therapeutic OX40 Agonist Dynamically Alters Dendritic, Endothelial, and T Cell Subsets within the Established Tumor Microenvironment

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Abstract

Little preclinical modeling currently exists to support the use of OX40 agonists as therapeutic agents in the setting of advanced cancers, as well as the mechanisms through which therapeutic efficacy is achieved. We show that treatment of mice bearing well-established day 17 sarcomas with a novel OX40 ligand–Fc fusion protein (OX40L-Fc) resulted in tumor regression or dormancy in the majority of treated animals. Unexpectedly, dendritic cells (DC) in the progressive tumor microenvironment (TME) acquire OX40 expression and bind fluorescently labeled OX40L-Fc. Furthermore, longitudinal analyses revealed that DCs become enriched in the tumor-draining lymph node (TDLN) of both wild-type and Rag2−/− mice within 3 days after OX40L-Fc treatment. By day 7 after treatment, a significant expansion of CXCR3+ T effector cells was noted in the TDLN, and by day 10 after treatment, type 1 polarized T cells exhibiting a reactivated memory phenotype had accumulated in the tumors. High levels of CXCL9 (a CXCR3 ligand) and enhanced expression of VCAM-1 by vascular endothelial cells (VEC) were observed in the TME early after treatment with OX40L-Fc. Notably, these vascular alterations were maintained in Rag2−/− mice, indicating that the OX40L-Fc–mediated activation of both DC and VEC occurs in a T-cell–independent manner. Collectively, these findings support a paradigm in which the stimulation of DC, T cells, and the tumor vasculature by an OX40 agonist dynamically orchestrates the activation, expansion, and recruitment of therapeutic T cells into established tumors. Cancer Res; 70(22): 9041–52. ©2010 AACR.

Introduction

As tumors develop and progress, several regulatory mechanisms are responsible for maintaining immune tolerance in the tumor microenvironment (TME). Poor tumor homing and penetration of T effector cells, a consequence of aberrant vasculature and limited chemokine and adhesion molecule expression in the TME (1, 2), is one major barrier to antitumor immunity (3). Furthermore, tumor-specific T cells that effectively infiltrate tumors may be rendered inactive by soluble factors and inhibitory signals associated with tumor cells and through the negative effect of both myeloid- and lymphoid-derived suppressor cells (4, 5). Although systemic immunity may be affected to a variable degree, immune suppression is typically most profound within the TME, with tumor-infiltrating lymphocytes (TIL) exhibiting severe deficiencies in CD8+ T-cell–mediated cytotoxic function (6). In murine tumor models, TIL dysfunction becomes pronounced only at later stages of solid tumor growth, at which point a mature tumor stroma composed of both bone marrow– and non–bone marrow–derived cells has been established (7, 8).

Given the perceived importance of T-cell–mediated immunity underlying effective immunotherapy (9) and better clinical outcome (3), substantial emphasis has recently been placed on the development of treatment modalities that are capable of restoring T-cell function and enhancing tumor penetration in the tumor-bearing host. In particular, immune-stimulating agents that target the costimulatory tumor necrosis factor receptor (TNFR) family member OX40 have shown antitumor efficacy in preclinical models (10). Costimulatory members of the TNFR family are upregulated shortly after T-cell receptor engagement on naive and antigen-experienced cells, where they serve as key modulators of cell activation, survival, and differentiation (11, 12). OX40 is expressed by activated T effector cells, and OX40-mediated signals provided during priming regulate CD4+ and CD8+ T-cell activation and clonal expansion in vitro (13–15). Furthermore, anergic or hyporesponsive OX40+ T cells may be reactivated by OX40 agonists (16). OX40 is also constitutively expressed by CD4+Foxp3+ regulatory T cells (Treg; ref. 17). Indeed, recent studies have shown that agonist signaling through...
OX40 inhibits the suppressor function of natural Foxp3+ Treg (18), prevents the induction of Treg from CD4+ T effector cells (19), and confers resistance to effector cells against Treg-mediated inhibition (13).

To characterize the molecular, cellular, and treatment-associated consequences of OX40 engagement in the setting of well-established tumors, a novel agonistic reagent directed against murine OX40 [OX40 ligand–Fc fusion protein (OX40L-Fc)] was recently constructed and characterized in vitro (20). We observed that the progressive growth of well-established day 17 sarcomas was inhibited by a short course of OX40L-Fc therapy, with complete tumor regression or extended disease stabilization (i.e., tumor dormancy) observed in the majority of treated animals. Comparable findings were obtained in both the MCA205 (H-2d) and CMS4 (H-2b) sarcoma models. We noted that i.p. injection of OX40L-Fc induced significant expansion of T effector cells in the tumor-draining lymph node (TDLN), resulting in the accumulation of activated, type 1 polarized T cells in the TME within 10 days of initiating OX40L-Fc therapy. Moreover, our therapy seemed to dynamically affect denritic cell (DC) and vascular endothelial cells (VEC) in both wild-type and Rag−/− mice bearing well-established tumors. The extensive molecular and cellular alterations observed in this model strongly support the translation of OX40 agonists into human clinical trials, either as single agents or in the context of combinational immunotherapy (21).

Materials and Methods

Mice

Six- to 10-week-old female C57BL/6 (H-2b), B6.129S7-Rag1tm1Mom (Rag−/−; H-2b), and BALB/cj (H-2b) mice were purchased from The Jackson Laboratory and maintained in the pathogen-free animal facility in the Biomedical Sciences Tower at the University of Pittsburgh. All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Tumor establishment

The MCA205 (H-2b) sarcoma cell line was purchased from the American Type Culture Collection. The CMS4 (H-2b) sarcoma has been described in detail previously (22). Cell lines were cultured in complete media [CM; RPMI 1640 supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum (all reagents from Life Technologies)] in a humidified incubator at 37°C and 5% CO2. All cell lines were negative for known mouse pathogens. Tumors were established by injection of 5 × 105 tumor cells s.c. into the right flanks of syngeneic mice, with tumor size assessed every 3 to 4 days and recorded in mm2. Mice were sacrificed when tumors became ulcerated or reached a maximum size of 400 mm2.

Costimulatory therapy

Tumor-bearing mice were injected i.p. with 100 μg of OX40L-Fc or rat IgG isotype control antibody (Sigma-Aldrich) in a total volume of 100 μL PBS on days 17 and 20 after tumor inoculation when tumors were approximately 30 to 50 mm2 in size. The mOX40L-Fc fusion protein has been previously described (20).

Isolation of tumor, lymph node, and spleen cells

Single-cell suspensions were obtained from TDLN as previously described (22). For TILs, tumors were enzymatically digested with 0.1% (w/v) collagenase, 1% (w/v) hyaluronidase, and 0.1% (w/v) DNase (all from Sigma), with lymphocytes isolated as buoyant cells after discontinuous density centrifugation as previously described (23).

In vitro stimulation of T cells

Bulk TILs (n = 1 per group) were restimulated in vitro with irradiated (100 Gy) MCA205 cells for 5 days at a T cell–to-tumor ratio of 10:1 in CM with 20 units/mL of recombinant human interleukin-2 (IL-2). Recovered T cells were then cultured in media alone (to determine background cytokine levels) or with 5 μg/mL anti-CD3 (BioLegend) for 72 hours. Cell-free supernatants were then harvested and assessed for levels of mIFN-γ using a specific OptEIA ELISA set (BD Biosciences) according to the manufacturer's instructions with a lower limit of detection of 32.5 pg/mL. Data are reported as the mean ± SD of duplicate determinations.

Reverse transcription-PCR

Total RNA was isolated from TIL on days 3, 7, and 10 after the initial treatment (day 17 after tumor inoculation), as indicated, using RNeasy Micro kit (Qiagen), according to the manufacturer's instructions. For semiquantitative reverse transcription-PCR (RT-PCR), TILs were cultured with 5 μg/mL anti-CD3 (BioLegend) for 24 hours, followed by RNA isolation and cDNA preparation using random hexamer primers (Applied Biosciences). PCR was performed using the primer pairs listed in Supplementary Table S1. Cycling conditions and temperature were as follows: initial denaturation at 94°C for 2 minutes (1 cycle); denaturation at 94°C for 30 seconds, annealing at 60°C to 65°C for 30 seconds, and elongation at 72°C for 1 minute (35–40 cycles); final extension at 72°C for 5 minutes (1 cycle). Following gel electrophoresis, PCR products were imaged and band density was quantified using LabWorks software (Perkin-Elmer). For quantitative RT-PCR, reverse transcription and PCR amplification were performed by the University of Pittsburgh Genomics and Proteomics Core Laboratories (a shared resource). For quantitative analysis of T-bet, IFN-γ, and control β-actin, previously published primer pairs were used (24), and cDNA was amplified using SYBR Green PCR Master Mix (Applied Biosystems). For quantitative analysis of Foxp3, IL-10, and control β-actin, RT2 qPCR Primer Assays (SABiosciences) were used and cDNA was amplified using RT2 SYBR Green qPCR Master Mix (SABiosciences). For each sample, the cycle threshold (Ct) values for β-actin gene were determined for normalization purposes and the ΔCt values between β-actin and T-bet, Foxp3, IFN-γ, and IL-10 were calculated. ΔΔCt values were calculated to
isotype treatment samples. Relative RNA expression for each gene is depicted as $2^{ΔΔCt}$.

Confocal immunofluorescence staining and imaging

Tumor tissue was processed and sectioned as previously reported (22), followed by immunofluorescence staining and confocal microscopy. The following primary antibodies were used for staining sections: rat anti-mouse CD3 (BD Biosciences), goat anti-mouse OX40 (Santa Cruz Biotechnology), rat anti-mouse OX40 (eBioscience), hamster anti-mouse CD11c (BD Biosciences), rat anti-mouse VCAM-1 (Santa Cruz Biotechnology), and goat anti-mouse CXCL9 (R&D Systems). The following secondary antibodies were used: donkey anti-rat Alexa Fluor 488 (Molecular Probes), donkey anti-goat Alexa Fluor 594 (Molecular Probes), donkey anti-rat Cy3 (Jackson ImmunoResearch), goat anti-rat Fab1 fragment Cy3 (Jackson ImmunoResearch), and goat anti-rat Alexa Fluor 488 (Molecular Probes). All sections were briefly incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) and then mounted. Images were acquired using an Olympus FluoView 1000 confocal microscope (Olympus). Isotype control and specific antibody images were taken using the same level of exposure on the channel settings.

Flow cytometry

Before all stainings, cells were Fc blocked with anti-CD16/CD32 (Becton Dickinson). Single-cell suspensions were stained using the following antibodies: PerCP- and PE-conjugated CD4 and CD8, PE-conjugated Gr-1, FITC-conjugated CD80 and CD25, and PerCP-Cy5.5-conjugated Ki67 (all Becton Dickinson); FITC-conjugated CD27, Class II and F4/80, PE-conjugated CCR7 and OX40, and APC-conjugated CD11c, CD44, CCR3, and CD11b (all eBioscience); or appropriate-matched, fluorochrome-labeled isotype control monoclonal antibody (mAb). For Foxp3 intracellular staining, CD4+ T cells were surface stained as isotype control monoclonal antibody (mAb). For Foxp3 in-eBioscience); or appropriate-matched, fluorochrome-labeled CD11c, CD44, CXCR3, and CD11b (all II and F4/80, PE-conjugated CCR7 and OX40, and APC-Ki67 (all Becton Dickinson); FITC-conjugated CD11c, CD44, CXCR3, and CD11b (all eBioscience); or appropriate-matched, fluorochrome-labeled isotype control monoclonal antibody (mAb). For Foxp3 intracellular staining, CD4+ T cells were surface stained as described above and then further processed using an APC anti-mouse/rat Foxp3 Staining kit (eBioscience) according to the manufacturer’s instructions. FITC conjugation of OX40L-Fc was performed using a FITC protein labeling kit (Molecular Probes). Cells were analyzed using an LSR II flow cytometer (Beckman Coulter), with corollary data assessed using FlowJo software (version 7.6.1; Tree Star, Inc.).

Statistical analysis

All comparisons of intergroup means were performed using a two-tailed Student’s $t$ test, with $P < 0.05$ considered significant.

Results

OX40L-Fc treatment elicits potent antitumor activity against well-established tumors

A novel OX40 agonist, consisting of mOX40L linked to the COOH terminus of the Fc fragment of immunoglobulin, was recently constructed and shown to be curative in early day 5 (H-2b) Colon 26 and RENCA tumor models (20). In contrast, an agonist anti-OX40 (OX86) mAb was only able to extend median survival by ~2 weeks in these models. To determine whether OX40L-Fc would be efficacious in a more established and potentially clinically relevant disease model, we treated H-2b mice bearing day 17 MCA205 sarcomas via i.p. injection of 100 μg of OX40L-Fc or control rat IgG. A second identical dose was provided 3 days later. Tumors in all control-treated mice grew progressively and exhibited a rapid expansion in size around day 30 after inoculation, necessitating euthanasia by day 40 (Fig. 1A). In contrast, OX40L-Fc–treated mice exhibited reduced, stabilized tumor size by day 27 that was durable through day 40. Although OX40L-Fc treatment resulted in the long-term survival of only 13% of treated animals (Fig. 1B), 50% of this cohort exhibited small (~20–40 mm$^3$) lesions that remained “dormant” for >6 weeks, before eventually progressing. Growth of representative tumors exhibiting dormancy in OX40L-Fc–treated mice is shown in Fig. 1C.

Similar therapeutic benefits were observed in the CMS4 (H-2d) sarcoma model, where >80% of animals rejected their tumors after OX40L-Fc treatment on days 17 and 20 (Supplementary Fig. S1). Despite the superior efficacy observed for OX40L-Fc in the CMS4 model, all remaining data were collected in the MCA205 tumor model due to the tendency of progressor CMS4 tumors to ulcerate, necessitating premature euthanasia per Institutional Animal Care and Use Committee regulations.

T-cell and DC expression of OX40 is elevated in the progressor TME

To identify the in situ cellular targets of OX40L-Fc–based therapy, we next assessed OX40 expression on T-cell subsets within the TDLN and the TME of untreated MCA205 tumor-bearing mice between days 17 and 20 after tumor inoculation. Whereas OX40 was barely detectable on CD4$^+$Foxp3$^-$ and CD8$^+$ T cells in the TDLN, ~50% of CD4$^+$Foxp3$^-$ T cells expressed OX40 (Fig. 2A), consistent with previous reports indicating that OX40 expression is restricted to the regulatory subset of resting T cells in peripheral tissues (13). Conversely, OX40 expression was upregulated on all T-cell subsets in the untreated TME, including a median of >20% of CD8$^+$ T cells and >60% of CD4$^+$Foxp3$^-$ T cells.

Because natural killer (NK) and NKT cells can express OX40 under certain conditions (25, 26), we hypothesized that non–T-cell subsets may also represent targets for interaction with OX40L-Fc in the progressor TME. Indeed, OX40 expression was highly upregulated on CD11c$^+$CD11b$^+$ ClassII$^+$ tumor-infiltrating DC (TIDC) when compared with TDLN-localized DC (median of 37.6% versus 2.3% OX40$, respectively; Fig. 2B). OX40 was also detected on TIDC via confocal immunofluorescence microscopy (Fig. 2C). Furthermore, FITC-labeled OX40L-Fc was found to bind to TIDC, but not to TDLN-localized DC (Fig. 2D). These data suggest that although CD4$^+$Foxp3$^-$ T cells may represent the exclusive expressors of OX40 in the periphery, CD4$^+$ Foxp3$^-$ and CD8$^+$ T effector cells, as well as DC, contain
substantial OX40+ populations in the TME, making each of these cell types plausible targets of OX40L-Fc–based therapy.

**T-cell–independent enrichment of mature DC expressing the lymph node–homing receptor CCR7 in the TDLN shortly after treatment with OX40L-Fc**

Given the observed high levels of OX40 expressed by TIDC in untreated tumor-bearing mice, we next evaluated how DC populations were altered in response to OX40L-Fc treatment. By day 3 after the first OX40L-Fc treatment, TIDC expression of the costimulatory molecules CD80 and CD86 was augmented (data not shown), and a concordant increase in CD11c+CD11b+ DC within the TDLN was observed when compared with isotype mAb-treated control mice (Fig. 3A). These TDLN DC populations expressed elevated levels of CD80 and the lymph node–homing chemokine receptor CCR7 when compared with TDLN isolated from isotype mAb-treated control animals (Supplementary Fig. S2). Moreover, enrichment of DC on OX40L-Fc treatment was similarly observed in the TDLN of Rag−/− mice bearing established MCA205 tumors (Fig. 3B). These data are consistent with the T-cell–independent activation/maturation of OX40+ TIDC to become competent for trafficking to the TDLN within the initial 3 days of OX40L-Fc–based therapy. Longitudinal analysis suggests that treatment-induced migration of TIDC to the TDLN persists through day 7 of the therapy period, with a return to control conditions by day 10 (data not shown). Such trafficking of activated DC would be anticipated to sponsor the cross-priming of antitumor T-cell responses in the TDLN. We observed no substantial alterations, however, in DC frequency or phenotype in non-TDLN (data not shown).

**OX40L-Fc treatment promotes the expansion of TDLN T cells expressing the tissue-homing type 1 chemokine receptor CXCR3**

To assess alterations in the TDLN T-cell compartment following OX40L-Fc treatment and TIDC trafficking to the TDLN, we harvested TDLN from mice on days 3, 7, and 10 after initiating treatment and determined absolute numbers of CD4+Foxp3+ and CD8+ T effector cells. Time-dependent increases in both T-cell subpopulations were noted as a consequence of OX40L-Fc treatment, with numbers of TDLN T cells peaking at day 7 after treatment (Fig. 3C). Indeed, 7 days after initiating OX40L-Fc treatment, highly significant upregulation in expression of the proliferation marker Ki67 was
observed for both CD4+ and CD8+ T-cell subsets within the TDLN (P < 0.01; Fig. 3D). Although these T cells did not exhibit any alterations in activation marker expression (CD25 and CD69; data not shown), both CD4+ and CD8+ T cells expanded from OX40L-Fc– versus control mAb–treated mice were enriched in the CD44hi phenotype at day 7 after treatment (Supplementary Fig. S3A), supporting the ability of OX40L-Fc therapy to preferentially stimulate memory T cells.

It has recently been shown that type 1 polarized CD8+ T cells are effectively recruited to tumor sites via CXCR3-mediated chemotaxis in response to the CXCL9-11 chemokines produced within the TME (27). To determine whether TDLN T cells in OX40L-Fc–treated animals are differentially competent to migrate to the TME based on this index, we assessed CD4+ and CD8+ T cells for their expression of CXCR3 7 days after the initiation of therapy. As shown in Supplementary Fig. S3B, we found that the CXCR3+ subpopulation of CD4+ and CD8+ T cells was increased after OX40L-Fc versus control mAb treatment. These data suggest that OX40 agonist therapy not only stimulates expansion of TDLN T cells but also licenses these cells for trafficking to peripheral tissue sites in which CXCR3 ligands are expressed, such as the TME.

**Tumors become enriched in T effector cells by day 10 following OX40L-Fc treatment**

Based on our observation that maximal numbers of CXCR3+ TDLN T cells occurred by day 7 after OX40L-Fc treatment, we hypothesized that these transport-competent T cells might then infiltrate the TME shortly thereafter. Although increased frequencies of CD4+Foxp3− and CD8+ T effector cells were detected in the TME throughout the observation period, a highly significant increase versus control mAb–treated animals was noted for CD4+Foxp3− TIL at days 7 and 10 after treatment and CD8+ TIL at day 10 after treatment (P < 0.01; Fig. 4A). The change in percentages of T effector cells correlated with increases in the number of CD4+Foxp3− and CD8+ TIL per gram of tumor tissue (Supplementary Fig. S4). To distinguish between the recruitment of T effector cells and in situ T-cell expansion within the TME, TILs were analyzed for their expression of Ki67. Although Ki67

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**Figure 2.** T-cell and DC expression of OX40 is elevated in the progressive TME in wild-type mice. A and B, single-cell suspensions (n = 4) were prepared from untreated MCA205 tumors (TME) and TDLNs isolated between days 17 and 20 after tumor inoculation. A, left, percentages of OX40+ T cells among the indicated gated cell populations. Each symbol corresponds to an individual tumor-bearing mouse (bar, median value). Representative histograms are depicted (right), with percentages of OX40+ T cells and mean fluorescence intensity (MFI) of OX40+ T cells indicated. Filled histograms, cells isolated from the TDLN; open histograms, cells isolated from the TME. B, percentages of DC (CD11c+CD11b+ClassIIhi) expressing OX40 in the TDLN and TME are reported (bar, median value). C, untreated MCA205 tumors were isolated between days 17 and 20 after tumor inoculation, and then sectioned, stained, and analyzed by confocal fluorescence microscopy as described in Materials and Methods. Representative staining of DAPI (blue), OX40 (green), and CD11c (red) is shown, with arrows indicating CD11c+OX40+ cells. Inset reflects higher-power (x60 magnification) image. Bars, 10 μm. D, representative staining of gated CD11c+CD11b+ DC with FITC-labeled OX40L-Fc. Filled histogram, cells isolated from the TDLN; open histogram, cells isolated from the TME. Experiments were repeated two times with similar results obtained in each instance.

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**Research.**

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expression was upregulated by T cells in the TDLN of OX40L-Fc–treated mice (Fig. 3D), expression of this marker on TIL was not substantially altered (Fig. 4B), suggesting that T effector cell accumulation in the TME of treated mice is most likely attributed to the enhanced recruitment of these cells, rather than to their expansion within tumor lesions.

Despite an elevated frequency of CD4+Foxp3+ T cells being consistently identified in the TDLN of OX40L-Fc–treated mice (Fig. 3D), expression of this marker on TIL was not substantially altered (Fig. 4B), suggesting that T effector cell accumulation in the TME of treated mice is most likely attributed to the enhanced recruitment of these cells, rather than to their expansion within tumor lesions.

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Tumor-infiltrating T cells exhibit a reactivated memory phenotype and are type 1 polarized

An analysis of the phenotype of TIL on day 10 after OX40L-Fc treatment, at which time T effector cell frequencies in the TME peaked, suggested no differential expression of CD25 or CD69 versus the control mAb–treated cohort (data not shown). However, the frequency of CD4+ and CD8+ TIL expressing high levels of CD44 (CD44hi, antigen experienced) and low levels of CD27 (CD27low, recently activated) was enriched at this time point (Fig. 4C; Supplementary Fig. S7). These phenotypic analyses are consistent with a model in which OX40L-Fc–stimulated T cells are expanded in the TDLN by day 7 after treatment and subsequently infiltrate the TME by day 10 after treatment.

Because effective cancer immunotherapies have been largely associated with a state of type 1 T-cell polarization and an increased T effector cell–to–Treg ratio (29, 30), we...
next examined the polarization status of freshly isolated TIL on days 3 to 10 following OX40L-Fc versus control mAb treatment. Using quantitative RT-PCR, factors associated with type 1 T-cell activity (i.e., T-bet and IFN-γ) and those associated with regulatory T-cell activity (i.e., Foxp3 and IL-10) were assessed for their relative levels of expression. Although minimal alterations were observed in the expression of any of these four transcripts on days 3 and 7 after treatment, by day 10 after treatment, at a time when increased frequencies of TILs were observed, transcript levels for all four gene products were dramatically enhanced in the OX40L-Fc treatment group (Fig. 5A, top). This is consistent with a recent report by Ruby and colleagues (31), who propose that although all T-cell lineages are responsive to OX40 stimulation, the plasticity of the response is dependent on the local cytokine milieu. By considering the “balance” of polarized T-cell responses based on a ratio of the effector-to-regulatory gene transcripts, however, we noted that OX40L-Fc treatment skews the balance in favor of type 1 T-cell immunity at all time points analyzed (Fig. 5A, bottom). Similar alterations in these transcript ratios were observed in anti-CD3–stimulated TIL (Fig. 5B). This suggests that although OX40 signaling may not be a polarizing event per se, a cytokine milieu appropriate for the preferential expansion of type 1 versus regulatory-type immunity exists within the OX40L-Fc–treated TME.

To ensure that alterations in IFN-γ RNA expression correlated with alterations at the protein level, TILs were isolated on days 3 to 10 after treatment and stimulated in vitro before analysis of IFN-γ secretion levels by ELISA. Interestingly, TIL production of IFN-γ was not elevated, and perhaps even slightly reduced on day 3 after treatment with OX40L-Fc versus isotype mAb (Fig. 5C). In contrast, TIL isolated 7 and 10 days after initiating OX40L-Fc–based therapy

![Figure 4. Accumulation of T effector cells in the TME of wild-type mice by day 10 following OX40L-Fc treatment. TME cells were isolated on days 3, 7, and 10 after treatment with isotype control antibody or OX40L-Fc. Graphs show percentages of CD4+Foxp3− and CD8+ T cells (A) and percentages of Ki67+ T cells (B). Each symbol corresponds to one tumor-bearing mouse. The solid line represents the median value. C, TILs were isolated from isotype control mAb–treated mice; filled histograms, cells isolated from OX40L-Fc–treated mice. Percentages of CD44hi of CD4+ and CD44hi of CD8+ cells are indicated on representative histograms. CD27 was evaluated on gated CD4+ and CD8+ cells, as indicated (representative histograms are shown). Open histograms, cells isolated from isotype control mAb–treated mice; filled histograms, cells isolated from OX40L-Fc–treated mice. Similar data were obtained in two independent experiments performed. *, P < 0.05; **, P < 0.01.](https://www.aacrjournals.org/cancerres/doi/abs/10.1158/0008-5472.CAN-10-1369)
produced significantly higher levels of IFN-γ protein versus TIL harvested from control mAb–treated mice at these same time points. Moreover, when comparing the effector-to-regulatory balance at the cellular level within the TME, significant increases were observed in both the CD4+Foxp3− and CD8+ T effector versus Treg ratios at days 7 and 10 after treatment (*, P < 0.05; **, P < 0.01).

**OX40L-Fc treatment renders the TME permissive to type 1 T-cell infiltration via modulation of the tumor vasculature**

In parallel with an increased fraction of CXCRII+ T cells in the TDNL of OX40L–Fc–treated mice, CXCCL9 (a CXCRII ligand) was dramatically upregulated in the TME between days 7 and 10 after treatment (Fig. 6A, left), indicating that OX40L–Fc therapy redundantly promotes TIL trafficking by augmenting chemokine signals in both the periphery and the TME. We have also recently reported that effective recruitment of adoptively transferred type 1 polarized CD8+ T cells into the TME requires tumor-associated VEC expression of VCAM-1 (32). Confocal immunofluorescence microscopy revealed that tumor-associated CD31+ VECs rapidly upregulate (i.e., by day 3) and maintain VCAM-1 expression through day 10 as a consequence of treatment with OX40L–Fc (Fig. 6B, left), suggesting that type 1 T effector cell recruitment into the TME might be further enhanced by OX40L–Fc via additional chemokine-independent mechanisms. Importantly, treatment-associated enhancement of VCAM-1 and CXCCL9 expression by CD31+ VECs was recapitulated in Rag-/- mice (Fig. 6A and B, right), suggesting the T-cell–independent

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**Figure 5.** TILs exhibit a type 1 polarized phenotype. TILs were isolated from wild-type mice on days 3, 7, and 10 after treatment with isotype control antibody or OX40L–Fc. A, top, quantitative RT-PCR was performed on purified RNA (n = 2 per group, pooled) using primers specific for murine T-bet, Foxp3, IFN-γ, and IL-10. ΔΔCt values were calculated to day 3 isotype treatment samples, and relative RNA expression for each gene is depicted as 2^ΔΔCt; bottom, ratios of T-bet/Foxp3 and IFN-γ/IL-10. Similar data were obtained in two independent experiments performed. B, TILs were stimulated with anti-CD3 for 24 h in vitro as outlined in Materials and Methods, with semiquantitative RT-PCR performed on purified RNA (n = 2 per group, pooled) using primers specific for murine T-bet, Foxp3, IFN-γ, and IL-10. Similar data were obtained in two independent experiments performed. C, isolated TILs were restimulated in vitro with irradiated MCA205 cells for 5 d (as described in Materials and Methods). Recovered T cells were then stimulated with anti-CD3 for 72 h, and supernatants were assessed for levels of IFN-γ. Columns, mean of duplicate determinations; bars, SD. Similar data were obtained in two independent experiments performed. D, CD4+Foxp3− and CD8+ T effector-to–CD4+Foxp3+ Treg ratios were determined by flow cytometry (n = 4 per group). Data are representative of three independent experiments performed. *, P < 0.05; **, P < 0.01.
nature of these changes. In support of a direct effect of OX40L-Fc on VEC within the TME, we detected moderate levels of OX40 expression by a subset of CD31⁻CD11b⁻ VEC in untreated, progressor tumors (Supplementary Fig. S8). It is also important to note that Fig. 6A data suggest that additional (non-VEC) stromal cells in the TME produce CXCL9 in wild-type, but not Rag⁻/⁻, mice (most strikingly on day 7 after treatment with OX40L-Fc). Hence, therapeutic production of CXCL9 in the TME seems to result from both T-cell–dependent and T-cell–independent processes.

We also observed a pronounced reorganization of the tumor vasculature after OX40L-Fc treatment, consistent with what has previously been described as a more “normalized” phenotype (33). Whereas CD31⁺ VEC density increased coordinately with disease progression in the tumors of isotype control mAb–treated mice, vessel density in OX40L-Fc–treated tumors was significantly diminished by day 10 after treatment (Supplementary Fig. S9A). These alterations in vascular density at day 10 after treatment additionally correlated with a less tortuous and more organized (i.e., normalized) morphology of OX40L-Fc– versus control mAb–treated tumor vasculature (Supplementary Fig. S9B), consistent with a phenotype favorable for lymphocyte infiltration.

Discussion

In this report, we describe multifaceted antitumor activities associated with a novel mOX40L-Fc fusion protein when applied in the therapeutic setting against well-established tumors.
H-2b and H-2d sarcomas. When administered i.p. on days 17 and 20 after tumor inoculation, OX40L-Fc treatment inhibited tumor growth, resulting in disease stabilization or complete regression in the majority of treated animals.

In the TDNL of mice bearing well-established MCA205 sarcomas, OX40 expression was restricted to CD4+Foxp3+ T cells, whereas CD4+Foxp3− and CD8+ T effector cells, as well as DC and potentially VEC, were observed to be OX40− in the TME. Expression of OX40 has also been reported to be upregulated by T cells in primary tumors, but not in the tumor-free lymph nodes of human cancer patients (34). In alternative disease models, T-cell upregulation of OX40 has been observed at sites of inflammation during the onset of experimental autoimmune encephalomyelitis (35), and within the synovial fluid but not the peripheral blood of patients with rheumatoid arthritis (36). This has been attributed to the influence of inflammatory cytokines, including IL-1 and TNF-α (37, 38). Such cytokines could also play a role in upregulating the expression of OX40 by tumor-localized DC and VEC in our well-established sarcoma models. Furthermore, 4-1BB, an alternate member of the costimulatory TNFR family with structural and functional similarities to OX40, can be expressed by activated DC and atherosclerotic endothelia under the appropriate conditions (39, 40).

Numerous studies have indicated that both CD4+ and CD8+ T effector cells play instrumental roles in antitumor immunity stimulated by OX40 agonists in vivo (41). Consistent with this notion, we observed significant expansion of CXCR3+ TDNL T effector cells on day 7 after initiating OX40L-Fc-based therapy, followed on day 10 after treatment by the accumulation of reactivated, type 1 polarized CD4+Foxp3− and CD8+ T cells in the TME. Our data from Rag2−/− models also now suggest the T-cell–independent nature of OX40L-Fc–mediated events, including (a) the rapid and sustained production of CXCL9 and expression of VCAM-1 on VEC in established tumors and (b) the conversion of CD40+ TIDC into transport-competent APC (deduced from the subsequent enrichment of CD11cCCR7+ myeloid DC in the TDNL by day 3 after treatment). Although a recent study has shown a similar enhancement in DC trafficking to the TDNL on treatment with OX40 agonist mAb in an early-stage tumor model, this finding was attributed to therapy-mediated suppression of Treg function and to a corollary restoration in the migratory capacity of TIDC (42). Instead, based on the constitutive expression of CD40 by a subpopulation of TDNC in the well-established TME and the preservation of DC alterations in Rag2−/− mice following OX40L-Fc treatment, these alterations may occur via the direct engagement of OX40 on these cells. To unequivocally show a direct effect of OX40L-Fc on TDNC, prospective therapeutic models using chimeric mice in which only DCs are genetically deficient in expression of OX40 will be pursued.

The importance of manipulating the tumor vasculature to attract T cells has recently been shown by Quezada and colleagues (43), who report ICAM and VCAM upregulation in the TME on prophylactic Treg depletion as well as therapeutic vaccination (Gvax)/anti-CTLA-4 combination treatment, indicating that vascular activation may be achieved through several distinct mechanisms. Similar to our data, activation of the tumor vasculature in these models correlated with enhanced T-cell infiltration, increased T effector–to–Treg ratios, and improved therapeutic efficacy. Our data further suggest that therapy-associated induction of chemokines and adhesion molecules that render the TME more permissive to immune cell infiltration can be achieved in the absence of T cells. OX40L-Fc could conceivably mediate vascular activation in the TME of Rag2−/− mice via (a) direct stimulation of a subset of CD11c+ VEC, (b) inflammatory cytokine/chemokine production by OX40+ TIDC, or (c) the participation of alternate inflammatory (i.e., NK among others) effector cells present in these mice. The initial effects of nonhematopoietic and/or innate immune cell subsets in therapy-mediated vascular activation, however, are likely to synergize with the effects mediated by T cells in immune-competent hosts. In our model, for example, initial secretion of inflammatory cytokines (i.e., TNF-α) by OX40+ TIDC on OX40L-Fc treatment may induce CXCL9 and VCAM-1 expression in the TME, allowing for the recruitment of type 1 polarized T effector cells that produce IFN-γ (a potent inducer of CXCL9 and other angiostatic chemokines), resulting in further remodeling of the tumor vasculature and enhanced T-cell infiltration. Future studies will investigate the validity of this paradigm.

Based on the tumor growth curves of OX40L-Fc– versus control mAb–treated mice, the effect of therapy only becomes apparent by 7 to 10 days after the initiation of therapy. This is consistent with the infiltration of a highly reactive CD27+CD44hi CD8+ T effector cell population in the TME. As OX40 signaling has been previously shown to enhance recall responses and to preferentially expand CD44hi memory T cells on antigen rechallenge (44, 45), this may suggest that OX40L-Fc treatment leads to the expansion of a tumor antigen–experienced, rather than naive, T effector cell population in the TDNL, and subsequent trafficking of these T cells to the TME. Regardless, these infiltrating T cells seem competent to promote tumor regression or to regulate a state of tumor dormancy (i.e., stable disease) in the majority of OX40L-Fc–treated animals for a period of several weeks before the ultimate “escape” of progressor lesions. We are currently investigating whether this late progression event results from the erosion of protective antitumor T-cell responses, the outgrowth of less-immunogenic tumor cell variants, and/or the reacquisition of aberrant vascular structures in the TME (46). If the former mechanism underlies the ultimate failure of OX40L-Fc to induce complete tumor rejection, we would anticipate the enhanced benefits of extending the number of treatment cycles involving OX40 agonists.

Overall, the therapeutic benefits shown for OX40L-Fc in our well-established sarcoma models strongly support the continued translation of OX40 agonists, particularly those based on a recombinant form of OX40L into human clinical trials. Moving forward, it will be important to better delineate how the various OX40+ target cell populations within the TME (and elsewhere) are affected by OX40-mediated signals to select potential cotherapeutic agents and to define
a strategically rational schedule for the administration of each modality to yield maximal treatment benefit.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

A Therapeutic OX40 Agonist Dynamically Alters Dendritic, Endothelial, and T Cell Subsets within the Established Tumor Microenvironment

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