Gp96-Ig/Costimulator (OX40L, ICOSL, or 4-1BBL) Combination Vaccine Improves T-cell Priming and Enhances Immunity, Memory, and Tumor Elimination

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Abstract

T-cell costimulation typically occurs in a defined microenvironment that is not recapitulated by agonistic antibody therapy. To deliver such stimulation under more favorable conditions, we investigated whether an allogeneic cell-based vaccine that secreted Fc-OX40L, Fc-ICOSL, or Fc-4-1BBL would activate and expand T cells comparably with systemically administered agonist antibodies. Among these costimulators, locally secreted Fc-OX40L provided superior priming of antigen-specific CD8+ T cells, compared with combinations with OX40 antibodies or vaccine alone. Vaccine-expressed Fc-OX40L also stimulated IFNγ, TNFα, granzyme B, and IL2 by antigen-specific CD8+ T cells similarly to OX40 antibodies, without off-target consequences such as proinflammatory cytokine induction. Vaccine-secreted Fc-OX40L increased CD127+ KLRG-1+ memory precursor cells during the contraction phase, resulting in improved proliferation upon secondary antigen challenge, as compared with OX40 antibody. A cell-based vaccine cosecreting gp96-Ig and Fc-OX40L led to even more pronounced tumor control, complete tumor rejection, and increased tumor antigen–specific T-cell proliferation, including in tumor-infiltrating lymphocytes, as compared with combinations of gp96-Ig vaccine and OX40 antibodies, in mice with established melanoma or colorectal carcinoma. These data suggest that local modulation of the vaccine microenvironment has unexpected advantages over systemic costimulation with agonistic antibodies, which may simplify the clinical translation of such combination immunotherapies into humans.

Introduction

The dramatic clinical success of cancer immunotherapies in a small percentage of patients has highlighted the need to identify combination approaches that increase the frequency of responders. Synergistic benefits in preclinical models generally result from combinations of individual modalities that rely on distinct mechanisms of action. For this reason, combinations of (i) vaccines that provide signal 1, which primes T cells to new antigens (mechanism of action for gp96-Ig vaccine in Supplementary Fig. S1A to S1D; refs. 1, 2); (ii) T-cell costimulatory agents that provide signal 2, by directly activating T cells (mechanism of action in Supplementary Fig. S1E; refs. 3); and (iii) blockade of immune inhibitory molecules (i.e., checkpoint inhibition) are being tested and developed in ongoing clinical trials.

Agonist antibodies targeting T-cell costimulatory molecules such as 4-1BB, OX40, TNFRSF25, GITR, and ICOS are in clinical development based on their antitumor effects in a variety of preclinical model systems (8–15). These agonist monoclonal antibodies (mAb) are typically delivered i.v. at target-saturating doses and achieve systemic biodistribution within minutes, often resulting in global pathway activation (16). The normal context in which the ligands for these T-cell proteins meet their receptors, however, is localized to antigen-presenting cell plasma membranes in specific tissue microenvironments (17–19). The kinetics of ligand expression include rapid induction and subsequent downregulation within a 2- to 5-day window (17, 18), a pattern that is not reproduced by systemic costimulation with mAbs. Microinjecting agonist mAbs directly into tumor lesions increases antitumor benefits over systemic antibody administration (20), again underscoring the advantage of localized costimulation.

We developed an allogeneic, cell-based, therapeutic vaccine that selectively activates CD8+ T cells toward cell-derived antigens. The vaccine cells secrete a heat-shock protein, gp96-Ig (gp96 linked to an Fc region of immunoglobulin), a chaperone specialized for antigen cross-presentation (refs. 1, 21; mechanism of action in Supplementary Fig. S1). To date, over 160 patients with non–small cell lung or bladder cancer have been treated with gp96-Ig–based vaccines. Patients in single-armed phase I trials specifically activated their CD8+ T cells, which more readily infiltrated tumors, correlating with recurrence-free survival in bladder cancer (ref. 22 and unpublished data). The vaccine is constructed from individual cancer cell lines selected because they shared expression of differentiation and embryonal antigens identified from patients analyzed as part of The Cancer Genome Atlas project. Selected cancer cells were stably
transfected with an episomal-resident, bicistronic plasmid encoding gp96-Ig in the first expression cassette and a cell surface marker in the second cassette (ImPACT—immune pan-antigen cytotoxic therapy; Fig. 1A). We hypothesized that coexpression of a T-cell costimulatory ligand fusion protein together with gp96-Ig could provide an efficacious combination immunotherapy within a single product.

We developed coexpression vectors incorporating gp96-Ig along with either Fc-OX40L, Fc-ICOSL, or Fc-4-1BBL, resulting in new cellular vaccine products that we refer to as ComPACT (combination pan-antigen cytotoxic therapy; ComPACT/OX40L, ComPACT/ICOSL, or ComPACT/4-1BBL). Our data show that local OX40L costimulation (at the site of vaccination) provides the most improved antigen-specific CD4+ /CD8+ T-cell proliferation and tumor rejection compared with OX40 agonist mAbs. The success of this strategy suggests that combination immunotherapy that recapitulates the microenvironment in which such pathways are typically engaged may have distinct advantages over systemic therapy.

Materials and Methods

Cell culture and vaccine cell line generation

All cell lines were cultured under standard conditions. The 3T3-Ovalbumin-Hygro parental cell line was established using a chicken ovalbumin (ova) encoding pcDNA3.1-hygro plasmid through nucleofection with the 4D-Nucleofector and Cell Line Nucleofector Kit SE (Lonza) according to the manufacturer’s directions. Single-cell clones secreting a high concentration of ova were screened by ELISA and used to generate 3T3-ova-gp96-Ig (ImPACT, fused to murine IgG1 subclass Fc), 3T3-ova-gp96-Ig/Fc-OX40L (ComPACT/OX40L, fused to murine IgG4 subclass Fc), 3T3-ova-gp96-Ig/Fc-ICOSL (ComPACT/ICOSL), and 3T3-ova-gp96-Ig/Fc-4-1BBL (ComPACT/4-1BBL) through nucleofection of B45-neo plasmids encoding either murine gp96-Ig or gp96-Ig and the extracellular domains of Fc-OX40L, Fc-ICOSL, and Fc-4-1BBL, respectively. Single-cell 3T3 clones of both ImPACT and ComPACT versions were generated through G418 antibiotic selection and individual clones secreting comparable levels of mouse gp96-Ig were selected by qRT-PCR and secreted levels of OX40L-Fc were determined by quantitative RT-PCR (qRT-PCR) and protein levels by Western blot (see Supplementary Fig. S2).

Murine melanoma (B16.F10) cell lines were first established by generating an ova parental clone (B16.F10-ova: as described above for 3T3 cells). Then, B16.F10-ova versions of ImPACT (B16.F10-ova-gp96-Ig) and ComPACT/OX40L (B16.F10-ova-gp96-Ig/Fc-OX40L) were again transfected with the identical plasmids as described above, and selected for high gp96-Ig secretion. Vector-specific expression of Fc-OX40L mRNA was confirmed by qRT-PCR and secreted levels of OX40L-Fc were determined by ELISA (see Supplementary Fig. S2). CT26 versions of ImPACT (CT26-gp96-Ig) and ComPACT/OX40L (CT26-gp96-Ig/Fc-OX40L) were generated using similar procedures.

Mouse models, OT-I/OT-II adoptive transfer, and analysis

Ova-specific CD8+ T cells (OT-I) and CD4+ T cells (OT-II) were isolated and adoptively transferred as previously described (13). For Ova/OT-I experiments performed using 3T3 cell lines, mice on days 0 and 35 (in the case of boosted mice) were either untreated, vaccinated with the 3T3-ova parental clone as a control, vaccinated with ImPACT (alone or in combination with 100 µg of agonist antibodies to ICOS, BioLegend #313512), 4-1BB (3H3 antibody; BioXCell), or OX40 (OX86 antibody; BioXCell Supplimentary Fig. S1F), or vaccinated with all ComPACT versions (ComPACT/OX40L, ComPACT/ICOSL, or ComPACT/4-1BBL). Vaccinations consisted of 1 x 106 cells and were administered by intraperitoneal injection (i.p.). Lymphocytes harvested from peripheral blood were analyzed by flow cytometry throughout the time course.

All mouse vaccinations were i.p. Parenteral injections such as this produce high molecular bioavailability by avoiding the first-pass effect of hepatic metabolism and therefore serve as comparable modes of delivery as those used for human vaccination (intradermal injection; ref. 20). Similar magnitudes of gp96-Ig–induced activation of an MHC/CD8 response have been previously characterized following different modes of vaccine delivery, including intraperitoneal, intradermal, intrarectal, and intravaginal (22, 23–25).

CT26 and B16.F10-ova tumor model and analysis

For CT26 tumor studies, BALB/C mice were inoculated with 5 x 105 tumor cells via s.c. injection into the rear flank, indicating day 0. For B16.F10-ova studies, C57BL/6 mice were inoculated with 5 x 105 tumor cells into the rear flank, indicating day 0. Mice were vaccinated and used in experiments only if they had an established tumor that was 3 to 5 mm in diameter by day 4 vaccination. On vaccination days (days 4, 7, and 10), tumor-bearing mice were either untreated or vaccinated with 1 x 106 mitomycin-C (Sigma) treated ImPACT, ImPACT + 100 µg anti-OX86 [referred to as OX40(ab) throughout], or ComPACT cells. Tumor area (mm2) and overall survival were assessed throughout the time course. The 30-day survival criteria included total tumor area less than 175 mm2 with no sign of tumor ulceration. When a mouse reached tumor burden, it was recorded as a death, and this final tumor area was used for the remaining tumor size time course. "Tumor rejection" was counted if a tumor was established and subsequently disappeared completely because of treatment.

A cohort of B16.F10 experimental mice (without OT-I adoptive transfer) was euthanized on day 13 for SIINFEKL-tetramer analysis in peritoneal cells and tumor-infiltrating lymphocytes (TIL). FOXP3-RFP reporter mice were inoculated and vaccinated as described above. On day 13, peritoneal cells were harvested and analyzed by flow cytometry. From these same mice, tumors were excised and dissociated with a tumor dissociation kit (Miltenyi #130-096-730), then enriched for total mouse T cells using a cocktail of antibodies described below, and finally, analyzed by flow cytometry.

A cohort of CT26 experimental mice was euthanized on day 12 for AH1-tetramer analysis in splenocytes and genetic analysis of tumor tissue. Tumors were excised from these mice, trypsinized at 37°C for 10 to 15 minutes, dissociated and homogenized through a 100-µm strainer. Cells were pelleted and processed for RNA isolation (see below).

Flow cytometry

Flow cytometry and cell sorting were performed using standard procedures on a Sony SH800, and the data were analyzed using Sony SH800 software and Flowjo version v10.1r5.

www.aacrjournals.org Cancer Immunol Res; 4(9) September 2016 767

Published OnlineFirst June 30, 2016; DOI: 10.1158/2326-6066.CIR-15-0228

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For B16.F10-tetramer analysis, total peritoneal cells and T-cell-enriched TILs were incubated with aliphophycocyanin (APC)-labeled tetramer for B16.F10-ova antigen SiINFELK (MBL International) for 30 minutes on ice in the dark. Immediately, a cocktail of the following antibodies was added and incubated for a further 30 minutes on ice in the dark: CD8a-FITC, CD4-PE/Cy7, NK1.1-BV421, CD11b-BV421, CD11c-BV421, and Gr-1-BV421. Following this incubation period, stained cells were washed and resuspended in FACS buffer. For the analysis, cells positive for BV421 (Nk1.1, CD11b, CD11c, or Gr-1 positive) were gated out. From the remaining cells, the SiINFELK+ fraction was calculated from cells also staining positive for CD8a.

For CT26-tetramer analysis, CD8+ -enriched splenocytes (using magnetic isolation as described above) were incubated with APC-labeled tetramer for CT26 antigen AH1 (SPSYVYHQF-APC; MBL International) for 30 minutes on ice in the dark. Immediately, a cocktail of the following antibodies was added and incubated for a further 30 minutes on ice in the dark: CD8a-FITC, CD3-APC, NK1.1-BV421, CD11b-BV421, CD11c-BV421, and Gr-1-BV421. Following this incubation period, stained cells were washed and resuspended in FACS buffer. For the analysis, cells positive for BV421 (Nk1.1, CD11b, CD11c, or Gr-1 positive) were gated out. From the remaining cells, the AH1+ fraction was calculated from cells also staining positive for both CD3 and CD8a.

ELISAs
Standard mouse IgG ELISA conditions were set such that 1 × 10^6 cells were plated in 1 mL of culture media and analyzed after 24 hours. High-binding ELISA plates were coated overnight at 4°C with 10 μg/mL mouse IgG (Jackson Laboratories; #115-005-062) in sodium bicarbonate buffer. The following morning, plates were blocked with casein buffer for 1 hour at room temperature (RT) and then probed with 50 μL of cell supernatants along with an 11-point mouse IgG standard set (Jackson Laboratories) for 1 hour at RT. Plates were washed, and then detection antibody (Jackson Laboratories; #115-035-071) was added for 1 hour at RT in the dark. For mouse OX40L ELISAs, plates were coated overnight at 4°C with 5 μg/mL mouse OX40 (Bio-Rad) and 20 μg/mL mouse OX40L (MBL International) for 1 hour at RT. Plates were washed, and then detection antibody (Jackson Laboratories) for 1 hour at RT. Plates were washed and then the second detection antibody (Jackson Laboratories; #705-036-147) was added for 1 hour at RT in the dark. For development, all plates were washed again and SureBlue TMB Microwell Peroxidase Substrate (KPL) was added to each well and allowed to incubate at RT for 20 minutes in the dark. To stop the reaction, 100 μL of 1N sulfuric acid was added to each well, and absorbance at 450 nm was read immediately on a BioTek plate reader. Samples were run at a minimum in triplicate and at multiple dilutions.

RNA isolation and qRT-PCR
Total RNA from cells and tissue was prepared using RNaseq and RNeasy Micro kits (Qiagen), according to the manufacturer's recommendations. An on-column DNAse I (Qiagen) treatment was performed during RNA isolation. A total of 1 μg (using RNeasy) or 100 ng (using RNeasy Micro) of RNA was used to synthesize cDNA with the First-strand cDNA synthesis kit (OnGene). qPCR detection of selected gene targets was performed using SYBR green master mix (Kapa Biosystems) and then analyzed on a Roche Light Cycler 96. Values were normalized to 18S mRNA and represent the average ± standard error of the mean (SEM) from a minimum of 3 biological replicates, all run in triplicate. Primer sequences are available upon request.

Western blot analysis
ImPACT and ComPACT cells were treated for 16 hours ± Brefeldin-A to inhibit protein transport and secretion and in the case of ComPACT/OX40L, ± the reducing agent beta-mercaptoethanol. Cells were then lysed in RIPA buffer with 1X complete protease inhibitor cocktail (Roche) for 10 minutes on ice. Protein concentration was determined using the DC Protein Assay kit (Bio-Rad) and 20 μg of protein was probed. Antibodies used for probing were CD252 (OX40L, Abcam #ab156285, 1:1,000 dilution), B7-H2 (ICOSL, R&D Systems #AF158, 1:1,000 dilution), TNFSF9 (4-1BBL, Abcam #ab86575, 1:1,000 dilution), histone H3 (Active Motif #61278, 1:10,000 dilution), and β-actin (Abcam #ab8226, 1:10,000 dilution).

LEGENDplex cytokine analysis
Experimental mice were euthanized through CO2 asphyxiation and cervical dislocation and whole blood was collected via cardiac puncture. Serum was then transferred to a new 1.5 mL eppendorf tube. Cytokine analysis was performed using the LEGENDplex Cytokine Analysis kit (BioLegend) according to the manufacturer's recommendations and analyzed on the Sony SH800. Flow cytometry results were converted to pg/mL secretion using BioLegend LEGENDplex software.

Experimental animal guidelines
All mouse protocols were designed based on IACUC guidelines and approved by a licensed veterinarian. Experimental mice were monitored daily and euthanized by CO2 asphyxiation and cervical dislocation prior to any signs of distress.

Statistical analysis
Experimental replicates (n) are shown in figures and figure legends. Unless noted otherwise, values plotted represent the mean from a minimum of three distinct experiments and error is SEM. Statistical significance (P value) was determined using one-way ANOVA with multiple comparisons. Significant P values are labeled with asterisks: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Mantel–Cox statistical tests were used to determine the significance between survival curves.

In the B16.F10 model, survival curve P values were as follows when compared with untreated mice: B16.F10 only control (P = 0.3666), OX40(ab) (P = 0.0004), ImPACT (P < 0.0001), ImPACT + OX40(ab) (P < 0.0001), and ComPACT/OX40L (P < 0.0001); and when comparing ImPACT + OX40(ab) versus ComPACT/OX40L (P = 0.3770) and when comparing ImPACT versus ComPACT/OX40L (P = 0.1636).

In the CT26 model, survival curve P values were as follows when compared with untreated mice: CT26 only control (P = 0.6430), OX40(ab) (P = 0.0087), ImPACT (P = 0.0006),
Results

Activity of OX40, ICOS, and 4-1BB agonist mAbs in the presence of gp96-Ig vaccine

We performed a series of head-to-head studies in preclinical mouse models to identify potential synergy between a gp96-Ig–based vaccine (ImPACT) and T-cell costimulatory molecules targeting OX40, ICOS, or 4-1BB to further costimulate antigen-specific T cells (Fig. 1A–C; ref. 1). C57BL/6 mice adoptively transferred with ovalbumin-specific CD8\(^+\) T cells expressing EGFP (OT-I) were either untreated or immunized with a 3T3-ova-gp96-Ig vaccine alone or in combination with agonist mAbs and monitored for OT-I cell proliferation over an 11-day time course. Vaccination with 3T3-ImPACT cells led to proliferation of OT-I cells (prime response) between 4 and 7 days after vaccination, which corresponded to ~10% of the total peripheral blood CD8\(^+\) T cells. This OT-I response was doubled (~20%) by coadministration of an OX40 agonist mAb, but not with ICOS or 4-1BB costimulatory mAbs (Fig. 1B). Interestingly, the addition of a 4-1BB agonist mAb resulted in a significant increase in FOXP3\(^+\) regulatory T cells (Treg), which is consistent with previous findings (26). This increase was not observed with 3T3-ImPACT vaccination alone, OX40, or ICOS agonist Abs (Fig. 1C).

gp96-Ig combination vaccine: ComPACT/OX40L, ComPACT/ICOSL, and ComPACT/4-1BBL

Because vaccines are typically administered locally, we asked whether OX40L, ICOSL, or 4-1BBL proteins fused at the N-terminus to the Fc region of mouse IgG (Fc-OX40L, Fc-ICOSL, and Fc-4-1BBL, respectively) could be coexpressed with gp96-Ig as a strategy to enable combination immunotherapy within a single vaccine product (Fig. 2A). As proof of concept, 3T3 single-cell clonal lines coexpressing soluble ovalbumin and
either gp96-Ig alone (ImPACT) or gp96-Ig together with Fc-OX40L (ComPACT/OX40L), Fc-ICOSL (ComPACT/ICOSL), or Fc-4-1BBL (ComPACT/4-1BBL) were generated. These cell lines stably secrete comparable levels of both ova and gp96-Ig as confirmed by ELISA (all cell lines are characterized in Supplementary Fig. S2A–S2J). Expression of OX40L, ICOSL, and 4-1BBL from the 3T3-ComPACT vaccine cells was confirmed at the mRNA and protein levels by qRT-PCR and Western blotting, respectively (Supplementary Fig. S2C and S2D). The in vivo activity of ImPACT, either alone or in combination with OX40 agonist mAbs, was compared with that of all ComPACT cell lines using the OT-I model described in Fig. 1.

Figure 2.
The combination of Gp96-Ig with T-cell costimulator Fc-OX40L, Fc-ICOSL, or Fc-4-1BBL in a single vaccine vector termed ComPACT produces superior antigen-specific CD8+ T-cell expansion compared with agonist antibodies. A, as shown in Fig. 1B, FOXP3-reporter mice adoptively transferred with OT-I cells on day −1, were tracked for antigen-specific CD8+ expansion in the peripheral blood on the indicated days for each treatment group. This time, mice were treated with new ComPACT vaccines, shown in comparison with ImPACT. Top, ComPACT/OX40L (red line) and ImPACT + OX40(ab) (blue dotted line); middle, ComPACT/ICOSL (orange line); bottom, ComPACT/4-1BBL (green line). Treatment days are noted with a syringe. B, a cohort of experimental mice was sacrificed on day 8 following vaccination. Total RNA was harvested from peritoneal cells and splenocytes for gene expression analysis. Values were normalized to 18S mRNA, ACTB serves as an unchanged control, and the first "untreated" only replicate was set at 1. These data are inclusive of five (A, top), three (A, middle and bottom), and one (B) independent experiments. Experimental replicates are shown; plotted values represent the mean ± SEM, and P values are denoted by asterisks.
Immunization with all versions of ComPACT significantly improved proliferation of OT-I cells, with differing kinetics following initial immunization or boost. At the peak of initial T-cell expansion, ComPACT/OX40L reached the highest frequency, compared with ImPACT, ImPACT + OX40L agonist mAb, ComPACT/ICOSL, and ComPACT/4-1BBL (Fig. 2A; Supplementary Fig. S3A and S3B). Not only did ComPACT/OX40L increase OT-I cells between days 4 and 6 of peak expansion, but the contraction phase was also prolonged, critical for programming effective memory T-cell responses (Fig. 2A; days 6–29; refs. 27, 28). ComPACT/ICOSL expanded more antigen-specific OT-I cells compared with ImPACT alone on days 8 and 28 following the initial vaccination (Fig. 2A, middle). ComPACT/4-1BBL provided minimal addition to the peak OT-I response, with a significant increase observed only on day 8 (Fig. 2A, bottom). The absolute value of OT-I expansion varied slightly between Figs. 1 and 2, likely due to the different 3T3-ova vaccine parental cell line clones that needed to be established to generate ImPACT and ComPACT versions.

Mice boosted with OX40 agonist have relatively weak expansion within the antigen-specific CD8+ compartment (13). To determine how well antigen-specific CD8+ cells in ComPACT-treated mice expanded, we analyzed the memory response following re-vaccination on day 35 after primary immunization (Fig. 2A; Supplementary Fig. S3A). Consistent with previous results, gp96-Ig/OX40 agonist combinations provided a weak boost in OT-I response (13). However, ComPACT/OX40L–treated mice elicited a boost response that nearly matched the magnitude of the primary response of all other treatment groups. ComPACT/ICOSL showed no response after challenge, but ComPACT/4-1BBL elicited a memory response after boost, with OT-I levels significantly higher than those for ImPACT alone on days 39, 42, 43, and 46, albeit lower than what was generated using ComPACT/OX40L. The improved antigen-specific OT-I activation observed with all ComPACT vaccines was achieved without Treg activation (Supplementary Fig. S3C). Even ComPACT/4-1BBL did not increase the percentage of FOXP3+ CD4+ T cells, as was observed with its agonist antibody counterpart (Fig. 1C; Supplementary Fig. S3C). Together, these data indicate that vaccine-secreted antigen-specific T-cell costimulatory fusion proteins in the context of gp96-Ig vaccine (especially Fe-OX40L), significantly increases both primary and boost responses in antigen-specific CD8+ T cells, corresponding to an increase in memory precursor cells and a prolonged contraction phase following priming.

Because day 8 after initial vaccination was a common time point when all ComPACT versions enhanced antigen-specific CD8+ expansion over that of ImPACT alone, we analyzed other characteristics associated with T-cell activation by gene expression analysis (qRT-PCR) of cells isolated from the peritoneal cavity and spleen (Fig. 2B). Peritoneal cells isolated on day 8 from ImPACT only and all ComPACT treated mice, had elevated IFNG (interferon-γ), IL2, and GZMB (granzyme-B) consistent with the site of vaccination serving as the primary location of T-cell activation. It is unclear whether the apparent reduction in IFNG following ComPACT/ICOSL or ComPACT/4-1BBL is specific, a result of unique kinetics or other factors, and will be further studied. Only ComPACT-treated mice showed elevated IFNG, IL2, and GZMB in splenocytes, indicating the presence of a systemic T-cell response beyond the site of vaccination, as the peritoneal-activated T cells enter the blood stream and circulate. More specifically, ComPACT/OX40L and ComPACT/4-1BBL expressed significantly more of all three T-cell activation markers tested compared with ImPACT alone, whereas ComPACT/ICOSL only showed higher IL2 and GZMB (Fig. 2B). Thus, ComPACT/OX40L and ComPACT/4-1BBL led to enhanced activation of T cells with cytolytic potential.

**Fc-OX40L–secreting ComPACT vaccines increased memory T cells**

Given the significant increase in antigen-specific CD8+ OT-I cells with ComPACT after a boost vaccination, we questioned whether such a phenomenon could be attributable to an increase in memory precursor T cells (CD127+ KLRG1−) and more mature memory cells (CD127+ KLRG1−). Flow cytometric analysis of peritoneal cells and splenocytes from mice receiving ComPACT/OX40L revealed a marked increase in CD127+ KLRG1− cells in both the peritoneal cavity and the spleen compared with the other groups, indicating an increase in memory precursor cells (Fig. 3, left). ComPACT/OX40L did not induce an increase in short-lived effector cells (CD127+ KLRG1+, Fig. 3, middle), although this was the primary effect observed in OX40 agonist mAb–treated mice. ComPACT/OX40L groups also showed an increase in mature memory T cells (CD127+ KLRG1−) within the spleen (Fig. 3, right), consistent with OT-I boost response shown in Fig. 2A. ComPACT/ICOSL and ComPACT/4-1BBL yielded modestly greater numbers of CD127+ KLRG1− mature memory cells than ImPACT alone; however, they did not reach statistical significance.

Together, these experiments revealed a novel and unexpected mechanism of action for ComPACT-treated mice in comparison to those treated with ImPACT. Due to the superior activation and expansion of antigen-specific CD8+ and memory T cells with ComPACT/OX40L, we did a more detailed mechanistic analysis of its function in addition to assessing its role in antitumor immunity.

**Vaccine cell–secreted Fe-OX40L and specificity of immune response**

We speculated that the increased primary and boost responses following ComPACT/OX40L treatment were likely due to decreased off-target (antigen nonspecific) immune activation compared with systemic administration of OX40 agonist antibodies. To confirm this hypothesis, peritoneal cells, splenocytes, and inguinal lymph node cells were isolated on day 8 from mice that were immunized with ImPACT ± OX40L agonist mAb or ComPACT/OX40L and analyzed by flow cytometry and qRT-PCR to distinguish between off-target immune activation and an antigen-specific response. Increased levels of total CD4+ and FOXP3+ Tregs were detected in the peritoneal cavity, spleen, and inguinal lymph nodes in mice treated with OX40 agonist mAbs (Fig. 4A and B; Supplementary Fig. S4A and S4B). In contrast, ComPACT/OX40L–treated mice specifically amplified antigen-specific OT-I (CD8+) and OT-II (CD4+) cells with no apparent stimulation of Tregs (Fig. 4A and B; Supplementary Fig. S4A and S4B). Serum cytokine analysis revealed a systemic increase in IFNγ, TNFα, IL5, and IL6 concentrations in mice treated with OX40 agonist mAbs, but not with ComPACT/OX40L (Fig. 4C). To investigate the cellular source of this systemic cytokine increase, qRT-PCR was performed on FACS-sorted OT-I and OT-I+ CD8+ cells on day 8 following immunization (to compare antigen
Fromm et al. Superior therapeutic tumor immunity with ComPACT/OX40L

ImPACT vaccines elicit an antigen-specific CD8⁺ T-cell response that leads to antitumor activity in preclinical mouse tumor models (21, 23, 29). Given the superior CD8⁺ T-cell priming and boost response observed with the combination therapy ComPACT/OX40L vaccine in the 3T3-ova model system, we asked whether such an activity would translate to a more potent antitumor effect in preclinical mouse tumor models. The B16.F10–ImPACT and –ComPACT cell lines were homogenized and the supernatants were harvested and concentrated using Centricon-30 columns. The concentration was determined by a protein assay and the samples were used in the ELISA assays described below. The supernatants were adjusted to pH 7.4 and were heat-inactivated at 56°C for 30 min before use.

To assess the efficacy of ComPACT/OX40L treatment in vivo, we used the B16.F10–ImPACT and –ComPACT cell lines. We injected 10⁶ cells s.c. into the right flank of 8-week-old male BALB/c mice. The mice were then treated with ComPACT/OX40L vaccine in the peritoneal cavity (Supplementary Fig. S5A). Mice were sacrificed at day 14 after tumor inoculation for analysis of the CD8⁺ T-cell response to the H2-Kb–restricted ovalbumin antigen, SIINFEKL. Mice treated with ComPACT/OX40L generated significantly greater numbers of CD4⁺, CD8α⁺, and SIINFEKL(CD8) cells at the site of vaccination in the peritoneal cavity (Supplementary Fig. S5A). ImPACT and ImPACT + OX40mAb–treated mice showed increased CD4⁺ cells and ImPACT–treated mice had increased numbers of CD8⁺ cells (Supplementary Fig. S5A). These antigen-specific CD8⁺ cells (SIINFEKL(CD8)) increased only in frequency significantly in TILs of ComPACT/OX40L–treated mice, providing evidence that ComPACT/OX40L vaccines were effective at initiating a specific immune response. The ability of ComPACT/OX40L vaccines to induce antitumor immunity in vivo was demonstrated by the increased numbers of CD8⁺ T cells in the peritoneal cavity of mice treated with ComPACT/OX40L vaccine (Supplementary Fig. S5A). The increased numbers of CD8⁺ T cells in the peritoneal cavity of mice treated with ComPACT/OX40L vaccine correlated with the increased numbers of CD8⁺ T cells in the draining lymph nodes of the tumor-bearing mice (Supplementary Fig. S5A). The increased numbers of CD8⁺ T cells in the peritoneal cavity of mice treated with ComPACT/OX40L vaccine were also associated with increased levels of IFN-γ and TNF-α in the peritoneal cavity (Supplementary Fig. S5A). Our results show that ComPACT/OX40L vaccines are effective at generating an antitumor immune response in vivo.

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B16.F10-ova-ComPACT/OX40L (Fig. 5C). Consistent with the data obtained in the 3T3-ova model system (Fig. 2), OT-I cells in B16.F10-ova-ComPACT/OX40L–treated mice robustly expanded between days 10 and 19 (which corresponded to days 6–15 following the initial vaccination), more than OT-I cells in ImPACT/C6 OX40 agonist mAb–treated mice, with durable kinetics similar to the contraction phase previously observed in tumor-free mice. The B16.F10-ova-ComPACT/OX40L–vaccinated mice displayed a more potent antitumor effect than ImPACT–only vaccinated mice and ImPACTþOX40 agonist mAb–treated mice did not completely reject their tumors and their overall survival rates were 50% and 62.5%, respectively, 30 days following tumor implantation (Fig. 5E). The addition of an OX40 agonist mAb to ImPACT did not significantly improve tumor progression or overall survival more than ImPACT alone (Fig. 5D and E).

We expanded the antitumor analysis of ComPACT/OX40L into a colorectal carcinoma tumor model system that is more receptive to immunotherapy with OX40 agonist antibodies (33). CT26-ImPACT and CT26-ComPACT/OX40L cell lines were generated as described for 3T3 and B16.F10 model systems. CT26 vaccines lack Ova and therefore allow for the analysis of a native tumor antigen (AH1; SPSYVYHQF). Secretion of IgG and OX40L from CT26 vaccine cell lines was measured by ELISA (Supplementary Fig. S2H and I, respectively), and mRNA expression of OX40L by qRT-PCR (Supplementary Fig. S2J). The experimental time line used to assess CT26-ComPACT/OX40L vaccine antitumor effects in syngeneic BALB/C mice is shown in Fig. 6A. Mice were
inoculated with CT26 cells on day 0, and then immunized with mitomycin C–treated CT26 cells. CT26-ImPACT, CT26-ImPACT combined with OX40 agonist mAb, or with CT26-Com-PACT/OX40L cells on days 4, 7, and 10 after tumor inoculation, if the tumor had a diameter of 3 to 5 mm by day 4. qRT-PCR on tumor tissue isolated on day 12 after tumor inoculation revealed increased expression of CD8a, IL2, and IFNγ in OX40 agonist mAb, ImPACT, ComPACT/OX40L, and ImPACT + OX40 agonist mAb combination–treated groups, indicating immune cell activation and tumor infiltration. Only mice receiving OX40 agonist mAbs (either alone or together with ImPACT) showed increased CD4 and FOXP3 expression within the tumor (Fig. 6B). CT26 antigen–specific CD8+ expansion, as detected by AH1-tetramer staining, was significantly elevated ~4-fold in ImPACT + OX40 mAb and ~5-fold in ComPACT/OX40L-treated mice compared with the untreated group (Fig. 6C). Tumor progression was significantly delayed in mice receiving either ImPACT + OX40 agonist or ComPACT/OX40L treatments as compared with the control or monotherapy arms (Fig. 6D). This finding led to a significant increase in long-term survival and a higher rate of complete tumor rejection in ComPACT/OX40L-treated mice (Fig. 6E; 80% and approximately 47%, respectively) compared with what we observed with the B16.F10 tumor model. Moreover, four ComPACT/OX40L-treated mice that rejected tumors were rechallenged with a secondary tumor consisting of 5 x 10^5 tumor cells on day 30 after initial tumor inoculation. These mice were monitored for an additional 30 days (60 days in total) and their primary and secondary tumors remained undetectable (data not shown). Thus, in two tumor models, ComPACT/OX40L generated potent antigen-specific T-cell expansion and tumor infiltration and, delays in tumor growth and resulted in a significant increase in the frequency of complete tumor rejection, while concomitantly providing a potent memory response capable of preventing relapse.

**Discussion**

To prevent development of lethal autoimmunity, mammalian immunity is tilted toward tolerance in the absence of specific innate stimuli. The phenotypic distinction between an antigen-presenting cell that has been activated by innate stimuli and one

Figure 5.

ComPACT/OX40L generates antigen-specific CD8+ expansion, delayed tumor growth, increased overall survival, and tumor rejection in aggressive melanoma model (B16.F10-ova). A, mice were either untreated or adoptively transferred with 5 x 10^5 OT-I cells on day −1 and then inoculated on day 0 with 5 x 10^5 B16.F10-ova tumor cells. Untreated mice were sacrificed on day 13 for the analysis of the SIINFEKL tetramer, and adoptively transferred mice were monitored for 30 days to assess OT-I expansion, tumor growth, and survival. B, mice that were not adoptively transferred with OT-I cells were inoculated with tumor and vaccinated on days 4, 7, and 10. On day 13, mice were sacrificed, and total T cells were enriched from the TILs of treated mice and analyzed by flow cytometry for populations of CD4+, CD8a+, and SIINFEKL + CD8a+ cells. C, peripheral blood lymphocytes from mice that were adoptively transferred with OT-I cells were assessed for OT-I expansion over a time course of 25 days following treatment (indicated by images of a syringe). Experimental values represent the mean ± SEM, and P values are denoted by asterisks. D, tumor area was measured throughout a 25-day time course following initial tumor inoculation, as described in A. Experimental replicates are listed; error is SEM, and significant P values when comparing ImPACT versus ComPACT/OX40L are shown. E, overall survival was determined throughout a 50-day time course. Complete tumor rejection was observed only in the ComPACT/OX40L treatment group, at ~11%. A Mantel–Cox test was used to determine the significance (P value) of differences between untreated and treated groups. Additional P-value comparisons are listed in Materials and Methods. These data are inclusive of two (B) or three (C–E) independent experiments and represented as mean ± SEM.
that has not is determined mostly by the expression of B7 and TNF receptor superfamily costimulatory molecules (which include OX40L, ICOSL, and 4-1BBL), and not by the ability of an antigen-presenting cell to continually display antigens on MHC I and MHC II. Thus, it should not be unexpected that systemic provision of exogenous costimulatory molecules (using agonist mAbs) could lead to activation of T cells that engage with antigen-presenting cells that are displaying antigen in the absence of costimulatory molecules. Indeed, this was very likely a factor in 4-1BB agonist mAbs causing lethal toxicity in a phase II clinical trial (34).

To investigate the contribution of local vaccine-secreted versus systemic T-cell costimulation, the current study directly investigated the relative immune and antitumor activity of vaccine cell-secreted costimulatory molecules, Fc-OX40L, Fc-ICOSL, and Fc-4-1BBL, compared with systemically administered agonist antibodies. OX40L stimulation was prioritized over ICOSL and 4-1BBL as a result of superior antigen-specific T-cell activation and expansion, as well as its effective generation of memory precursor and mature memory cells. These studies showed a significant combinatorial benefit in mice treated with ImPACT and OX40 mAbs, but not 4-1BB or ICOS agonist mAbs. An explanation for the lack of costimulation seen with 4-1BB or ICOS agonists was not the aim of this study, but may be related to a variety of different factors, including the lack of CD4+ T-cell stimulation by ImPACT vaccines, possible activation-induced cell death by 4-1BB agonist antibodies, concurrent stimulation of Treg and Teff responses by ICOS agonists in the absence of CTLA-4 blockade, or other factors (35–37).

The initial feasibility of a ComPACT vaccine incorporating Fc-OX40L depended on whether a physiologically active concentration of Fc-OX40L could be secreted by a transfected vaccine cell to exert a local costimulatory signal. This was not a trivial feasibility question, as systemic administration of OX40 agonist antibodies is typically achieved with receptor saturating

Figure 6. ComPACT/OX40L treatment resulted in CD8+-specific tumor infiltration, hindered tumor growth, increased overall survival, and led to significant tumor rejection in colorectal carcinoma model (CT26). A, mice were inoculated on day 0 with 5 x 10^5 CT26 tumor cells and either untreated or treated according to the groups listed on days 4, 7, and 10. A cohort of mice was sacrificed on day 12 for tumor gene expression analysis. Remaining mice were monitored for 30 days to measure tumor area and overall survival. B, day 12 tumor gene expression analysis. Total RNA was isolated from dissociated tumors, reverse transcribed, and analyzed by qPCR. Transcript levels were assessed at CD4, FOXP3, CD8a, IL2, and IFNG. Values were normalized to 18S mRNA and the first “untreated”-only replicate was set at 1. C, total splenocytes were harvested from experimental mice on day 12 after treatment and enriched for CD8+ cells. AH1-tetramer (representing CT26 antigen-specific CD8+) cells were determined from cells also staining double positive for CD8a and CD3e. The gating strategy is described in Materials and Methods. D, similar to Fig. 5D, tumor area was measured daily for 21 days following initial tumor inoculation. Significant P values when comparing ImPACT versus ComPACT/OX40L are shown. E, overall survival was determined throughout a 30-day time course. Complete tumor rejection was observed in ComPACT/OX40L-treated mice (~47%), ImPACT + OX40(ab)-treated mice (~17%), and ImPACT-only-treated mice (~17%). A Mantel-Cox test was used to determine the significance (P value) of differences between untreated and treated groups. Additional P value comparisons are listed in Materials and Methods. These data are inclusive of two (B, C) or three (D, E) independent experiments. Plotted values represent the mean ± SEM, and P values are denoted by asterisks.
or supra-saturating doses of antibody in the range of hundreds of micrograms. In contrast, ComPACT/OX40L was defined to secrete nanogram range quantities of Fc-OX40L (Supplementary Fig. 52F and S2I). Surprisingly, the initial feasibility studies in tumor-naïve mice demonstrated that not only did this relatively small quantity of Fc-OX40L provide costimulation, but that the use of ComPACT/OX40L also led to superior antigen-specific immune responses at priming, during contraction, and again at the time of boosting as compared with ComPACT/ICOSL, ComPACT/4-1BBL, ImPACT, OX40 antibodies alone, or the combination of the two.

We hypothesized that the benefit of vaccine-secreted Fc-OX40L may be due to increased specificity to antigen-specific T cells as compared with systemically administered OX40 agonist mAbs. Indeed, subsequent experiments demonstrated that OX40 agonist mAbs led to cytokine release in the peripheral blood (with increased TNF, IFN, IL4, IL5, IL6, and IL10) as compared with ComPACT/OX40L. Further, OX40 mAbs stimulated proliferation of non–antigen-specific CD4 + and FOXP3 + T cells in the peripheral blood, spleen, and peripheral lymph nodes, which was not observed with ComPACT/OX40L vaccination. However, ComPACT/OX40L could stimulate greater antigen-specific CD8 + T-cell responses and similar antigen-specific CD4 + T-cell responses as observed with ImPACT combined with OX40 agonist mAbs. Perhaps most importantly for cancer therapy, relevant to the length of time many patients have disease prior to diagnosis, the use of ComPACT/OX40L led to improvements in the frequency of CD127 + KLRG1 + memory precursor cells, CD127 + KLRG1 + mature memory cells, and improved boost responses at the time of antigen rechallenge.

These findings in tumor-naïve mice suggested that ComPACT/OX40L may provide superior antitumor immunity in preclinical models. To test this, the activity of ComPACT/OX40L was compared with ImPACT and OX40 agonist mAbs in both a stringent preclinical melanoma model (B16.F10) and in a less stringent model of mouse colon cancer (CT26). In both cases, high doses of tumor cells were administered, and treatment was initiated following the formation of palpable subcutaneous tumors in the range of 3 to 5 mm in diameter. Although no mouse tumor model accurately reflects the long latency with which human tumors are typically established prior to treatment, this represents a therapeutic tumor model commonly used to determine the activity of cancer immunotherapy in the preclinical setting.

Because the CT26 tumor expresses the native antigen AH1, and B16.F10-ova tumors express the peptide, SIINFEKL, antigen-specific CD8 + T-cell responses were measured against “authentic” tumor antigens, which demonstrated higher frequencies of these cells in ComPACT/OX40L-treated animals, in keeping with all data collected using OT-I cells as the readout.

The data from both tumor models tested were consistent in showing that ComPACT/OX40L outperformed any of the monotherapy regimens or the combination of ImPACT together with OX40 agonist mAbs. In established B16-F10 tumors, ComPACT/OX40L was the only group that included complete tumor rejection (~11% of mice) and in the CT26 model nearly 50% of the ComPACT/OX40L-treated mice completely rejected established tumors as compared with ~16% with the combination of ImPACT and OX40 agonist antibodies. ComPACT/OX40L-treated mice that rejected tumors by day 30 also rejected secondary tumor challenge when monitored until day 60, providing evidence that the new ComPACT vaccine could provide a durable memory response inhibiting the recurrence of disease.

Unexpectedly, in the CT26 tumor model, OX40 agonist mAbs led to increased CD4 and FoxP3 mRNA expression within the tumor microenvironment. These data are in contrast with what was observed in the B16.F10 tumor model, where the percentage of FoxP3 + CD4 + cells (as measured by flow cytometry) decreased in the peritoneal cavity under all treatment conditions and in total T-cell–enriched TILs in the ImPACT + OX40(ab) and ComPACT/OX40L treatment groups. The latter observation is more in line with previous characterizations of the OX40 agonist mAb clone OX86 (28, 38). This could be explained in a number of ways, including but not limited to, the analysis of RNA from total lysed tumors versus T-cell–enriched TIL populations analyzed by flow cytometry (for example, FoxP3 message could be expressed at a high level from a small number of cells or originating from non-T cells altogether), unique variations between the microenvironmental context between tumor models, and/or by differences in the mode of therapy delivery (IP in our experiments versus intratumoral in the aforementioned references).

Interest in the compartmental function of T-cell costimulators and checkpoint inhibitors is growing rapidly, and it will therefore be interesting to characterize the differences we observed in the context of ComPACT/OX40L vaccination and Tregs in the near future.

Together, these data demonstrate that locally provided T-cell costimulation secreted from vaccine cells may have dramatically different outcomes to systemically administered, antibody-mediated, costimulation. These findings extend the work of Marabelle and colleagues in providing evidence that microdoses of locally administered agonist mAbs lead to improved therapeutic tumor immunity as compared with larger doses of antibody administrated systemically (28). Although no obvious clinical signs of distress or toxicity were observed in these mice, the observation that OX40 agonist mAbs leads to off-target T-cell responses and systemic cytokine release should be carefully monitored in clinical trials combining these agents with anti-PD-1/L1 or anti-CTLA-4 antibodies. Many costimulatory antibodies have now entered clinical development for use in oncology (4-1BB, OX40, CD27, CD40, GITR, ICOS, and TNFRSF25; ref. 8). These agents are likely to be used in combination with either traditional cancer therapeutics or other immuno-oncology compounds, and are expected to have limited efficacy as monotherapies (30–32). In preclinical studies, antitumor efficacy is generally dose dependent and requires saturating or supra-saturating doses (33, 39, 40). Such dosing regimens do not recapitulate the natural biology of costimulatory molecule expression, which occurs in a spatiotemporally restricted context. The current study provides an alternative strategy for combination immunotherapy, by incorporating vaccination and T-cell costimulation within a single cell–based compound, leading to initial immune stimulation at the site of vaccination. Intriguingly, locally secreted Fc-OX40L provided superior antigen-specific T-cell activation, increased CD8 + memory response, and decreased off-target T-cell activation (in comparison to a widely used murine OX40 agonist antibody), ultimately leading to improved antitumor immunity in ComPACT/OX40L-treated mice. These data may translate into...
the clinical development of future immunotherapeutics that merge several modalities into single compounds, enhancing antigen-specific immunity, simplifying development, and providing improved clinical outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G. Fromm, S. de Silva, T.H. Schreiber

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Writing, review, and/or revision of the manuscript: G. Fromm, S. de Silva, L. Giffin, T.H. Schreiber

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Received September 4, 2015; revised May 18, 2016; accepted June 6, 2016; published OnlineFirst June 30, 2016.

www.aacrjournals.org Cancer Immunol Res; 4(9) September 2016 777

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Gp96-Ig/Costimulator (OX40L, ICOSL, or 4-1BBL) Combination Vaccine Improves T-cell Priming and Enhances Immunity, Memory, and Tumor Elimination

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doi:10.1158/2326-6066.CIR-15-0228

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