Potent Immune Modulation by MEDI6383, an Engineered Human OX40 Ligand IgG4P Fc Fusion Protein

Michael D. Oberst, Catherine Augé, Chad Morris, Stacy Kentner, Kathy Mulgrew, Kelly McGlinchey, James Hair, Shino Hanabuchi, Qun Du, Melissa Damschroder, Hui Feng, Steven Eck, Nicholas Buss, Lolke de Haan, Andrew J. Pierce, Haesun Park, Andrew Sylwester, Michael K. Axthelm, Louis Picker, Nicholas P. Morris, Andrew Weinberg, and Scott A. Hammond

Abstract

Ligation of OX40 (CD134, TNFRSF4) on activated T cells by its natural ligand (OX40L, CD252, TNFSF4) enhances cellular survival, proliferation, and effector functions such as cytokine release and cellular cytotoxicity. We engineered a recombinant human OX40L IgG4P Fc fusion protein termed MEDI6383 that assembles into a hexameric structure and exerts potent agonist activity following engagement of OX40. MEDI6383 displayed solution-phase agonist activity that was enhanced when the fusion protein was clustered by Fc gamma receptors (FcγRs) on the surface of adjacent cells. The resulting costimulation of OX40 on T cells induced NFκB promoter activity in OX40-expressing T cells and induced Th1-type cytokine production, proliferation, and resistance to regulatory T cell (Treg)-mediated suppression. MEDI6383 enhanced the cytolytic activity of tumor-reactive T cells and reduced tumor growth in the context of an allogeneic human T cell:tumor cell admix model in immunocompromised mice. Consistent with the role of OX40 costimulation in the expansion of memory T cells, MEDI6383 administered to healthy nonhuman primates elicited peripheral blood CD4 and CD8 central and effector memory T-cell proliferation as well as B-cell proliferation. Together, these results suggest that OX40 agonism has the potential to enhance antitumor immunity in human malignancies.

Introduction

The generation of an antitumor immune response as a therapeutic strategy in oncology has been studied for many years. Recently, immuno-oncology drugs have demonstrated significant improvements over standard-of-care therapies in certain malignancies, exemplified by FDA approvals for anti-CTLA-4, anti-PD-1, and anti-PD-L1 mAbs (1). Despite this success, a significant number of cancer patients do not respond to immunotherapies, respond incompletely, or discontinue therapy due to adverse events. Immunosuppressive mechanisms outside of the targeted pathway may prevent an effective antitumor immune response within the tumor microenvironment (TME) despite the presence or recruitment of antitumor T cells (2). Such factors include suppressive immune cells that include regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) capable of suppressing activated T cells. Therefore, additional therapies are needed that expand high affinity, tumor-specific T cells in regional draining lymph nodes or within the TME despite immunosuppression not currently addressed by immunologic checkpoint blockade.

One strategy to promote an antitumor immune response that is different from checkpoint inhibition is to activate the TNF receptor superfamily (TNFRSF) of costimulatory T-cell receptors. Agonist approaches for these receptors currently undergoing clinical trials include antibodies and other technologies targeting CD137 (4-1BB; TNFRSF9), CD40 (TNFRSF5), CD27 (TNFRSF7), GITR (CD357; TNFRSF18), and OX40 (CD134; TNFRSF4; ref. 3). OX40 is a TNFRSF member expressed on activated effector and memory, as well as regulatory, T cells. Development of the mouse mAb 9B12, subsequently termed MEDI6469, was the first anti-human OX40 mAb in clinical development for advanced solid malignancies, and showed encouraging antitumor responses and a tolerable safety profile (4). The mouse origin of the MEDI6469 antibody, however, limits its clinical utility to one cycle of treatment due to the emergence of human anti-mouse...
antibody (HAMA) responses. Subsequently, a humanized version of MEDI6469 termed MEDI0562 was created to avoid the immunogenicity seen with MEDI6469. This and other agonist anti-human OX40 mAbs have entered early-phase clinical testing (5–7).

OX40-specific mAbs, as bivalent OX40 binding moieties, have the potential to induce OX40 signaling when clustered, but have not been shown to be capable of trimerizing OX40 in the absence of clustering. In contrast, the naturally trimeric OX40 ligand (OX40L, CD252, TNFSF4) protein complex expressed by professional antigen-presenting cells (APC) can trimerize OX40 directly. The engagement of OX40 by the OX40L, in concert with other costimulatory signals, promotes T-cell activation, survival, expansion, and the formation of effector and central memory T-cell pools. In contrast to OX40-specific mAbs, engineered fusion proteins containing the OX40L extracellular domain (ECD) have been created to take advantage of the strong agonist properties of the ligand. Previously, a human OX40L ECD linked to a human IgG1 Fc domain via a coiled coil trimerization domain from the yeast GCN4 protein had been expressed and characterized (8). It was found to naturally associate into a hexameric human OX40L fusion protein structure composed of two trimerized molecules covalently bound together through disulfide linkages found in the human IgG1 Fc domains.

To build a hexameric human OX40L fusion protein suitable for clinical use, we designed a fully human OX40L fusion protein termed MEDI6383. This protein contains human OX40L ECDs fused to the trimerization domain of the human TRAF2 protein and to human IgG4 domains to enable the formation of a covalently linked hexamer. Because the human IgG1 Isotype can mediate complement fixation and antibody-dependent cellular cytotoxicity (ADCC), we chose human IgG4 as the human IgG isotype to minimize the possibility of in vivo depletion of OX40-expressing effector T cells. Although human IgG4 was unlikely to mediate ADCC or complement fixation, this isotype can bind with relatively lower affinity to human FcγRs other than CD16 (9), which may be enhanced by avidity effects. Therefore, we were interested to test whether FcγR-mediated clustering may enhance MEDI6383 activity.

On the basis of the structure of the molecule, we further hypothesized that MEDI6383 would lack effector function, induce potent CD4 T-cell activation and expansion both in vitro and in vivo, and mediate antitumor immunity in animal models. Likewise, we hypothesized that MEDI6383 would protect conventional CD4 T cells from Treg-mediated suppression as has been reported for other OX40 agonists. In this manuscript, we report the production and characterization of MEDI6383 and the testing of these hypotheses using human in vitro assays and after the administration of MEDI6383 to immunodeficient mice bearing an admixture of human T cells and tumor cells as well as to healthy rhesus macaques. Results from these studies show that MEDI6383 represents an attractive candidate to activate and expand tumor-reactive T cells in patients with cancer.

Materials and Methods

OX40 binding molecules and control reagents

MEDI6383 was generated by genetically recombining DNA fragments that encode the human IgG4P fragment crystallizable (Fc) domain, the human TNF receptor-associated factor 2 (TRAF2) coiled coil domain (10), and the human OX40 ligand (OX40L) extracellular receptor binding domain (RBD). The control fusion protein lacking binding to OX40, termed F180A OX40L fusion protein control, was generated by mutating phenylalanine at position 180 to alanine which is known to abrogate OX40L binding to OX40 (11). MEDI6469 is a mouse anti-human OX40 mAb previously termed 9B12 and described previously (4).

Analytic size-exclusion chromatography

MEDI6383 protein sample was applied onto a TSK-gelf G3000SWxl column (Tosoh Biosciences), eluted isocratically with an Agilent 1200 HPLC system (Agilent Technologies), and detected using UV absorbance at 280 nm and Daen EOS Multi-Angle Light Scattering (MALS; Wyatt Technology).

Cell lines, tissue culture reagents, and culture conditions

All cell lines were originally purchased from the ATCC and tested at Medimmune for mycoplasma using mycoplasma-specific real-time PCR prior to use. Human A375 melanoma, Raji B, and HEK293 parental cells were authenticated by short tandem repeat profiling (IDEXX BioResearch Laboratories); human OX40-expressing Jurkat NFκB-luciferase reporter cells did not undergo cell line authentication. All cells except those used in regulatory T cell (Treg) suppression assays were cultured in RPMI complete media consisting of RPMI1640 containing supplements (Life Technologies; catalog no. A1049101) plus 10% v/v heat-inactivated FBS and 1% v/v (1:1) penicillin/streptomycin antibiotics (Life Technologies) in a humidified tissue culture incubator at 37°C and 5% CO2. T cells in Treg suppression assays were cultured in RPMI1640 Glutamax-1 (Life Technologies; catalog no. 61870-036) containing 1% v/v penicillin and streptomycin, 5% v/v human AB serum, and 1 μg/mL anti-CD28 antibody, clone 28.8 (BD Biosciences).

Flow cytometry reagents and analysis

BSA and propidium iodide (PI) were purchased from Sigma-Aldrich. OX40 was detected using APC-conjugated anti-human OX40 clone Ber-Act33 (Biologent). Flow cytometry data were collected by a BD LSRII flow cytometer (Becton Dickinson) and flow cytometry standard (FCS) data analyzed using FlowJo software (FlowJo LLC). Absolute CD4 and CD8 cell counts for the rhesus macaque study were generated using TruCount beads (Becton Dickinson) and is described further in Supplementary Methods.

Binding of MEDI6383 and MEDI6469 to activated human CD4 T cells

Primary human CD4+ T cells were obtained through negative selection of PBMCs isolated from sodium heparin anticoagulated whole blood from healthy human or non-human primate (NHP) donors. CD4 T cells were cultured with 2 μg/mL phytohemagglutinin-leucaemoglobin (PHA-L; Roche Life Sciences) and 20 IU/mL recombinant human IL2 (shL2, PeproTech) to activate T cells and upregulate OX40. Activated T cells expressing OX40 were bound to MEDI6383 or MEDI6469 and detected with AlexaFluor 647–conjugated or AlexaFluor 488–conjugated goat anti-human IgG secondary antibody (Life Technologies) on live (PI negative) cells. The apparent (avidity driven) equilibrium binding constants for MEDI6383 or MEDI6469 binding were determined after background subtraction using GraphPad Prism version 5.01 for Windows.
reporter cell line was in some cases used together with an Fc in trans expressing cell line at a 1:1 ratio (100,000 reporter cells plus 100,000 Fc-expressing cells) for clustering of MEDI6383 or MEDI6469 in trans through Fc domain clustering. FcR-expressing CD45+ cells from primary human tumors were isolated using a human CD45 microbead positive selection kit (Miltenyi Biotec) from tumors digested to single-cell suspensions as described in Supplementary Methods. Luciferase production was measured using the SteadyGlo luciferase assay system (Promega) and a Perkin Elmer Envision multi-label micro plate reader (Molecular Devices). FcR-blocking reagents and controls used to demonstrate FcR-dependent activity in 2-cell reporter bioassays included: BSA (Sigma-Aldrich), hexameric GITRL fusion proteins containing human IgG1 (control hexamer IgG1), or human IgG4P (control hexamer IgG4P) Fc domains (12, 13) that do not bind OX40 or activate OX40 reporter cells but can compete with MEDI6383 for FcR binding (generated at MedImmune). LEAF purified murine IgG1 x isotype control antibody clone MOPC-21 (Biologend), LEAF purified mouse anti-human CD64 antibody clone 10.1, sterile PBS-dialyzed purified mouse anti-human CD32 antibody clone FcγRII-2 (Biologend), purified human IgG gamma globulin (Jackson ImmunoResearch), and sterile human AB serum (Thermo Fisher Scientific). These reagents were added to 2-cell bioassays containing parental or FcR-expressing HEK293 cells, as indicated, for 45 minutes prior to addition of 0.5 µg/mL (1.6 nmol/L) MEDI6383 and OX40 Jurkat NKxB-luciferase reporter cells to allow for effective competition for FcγRs.

Primary human T-cell bioactivity assays

Enriched human CD3, CD4, or CD8 T cells were isolated from healthy donor Leuko Paks (AllCells) or whole blood (Medimmune Blood Donor Program) using EasySep human (CD3) T cell, CD4, or CD8 T cell enrichment kits (for leuko paks; StemCell Technologies) or RosetteSep human T cell enrichment cocktails for CD3, RosetteSep CD4, or RosetteSep CD8 T cell enrichment cocktails (for whole blood; StemCell Technologies). Thereafter, T cells were activated using 2 µg/mL PHA-L and 20 IU/mL rhIL2 for 48 hours to upregulate OX40. To test the ability of plate-immobilized MEDI6383 to induce cytokine secretion and T proliferation, activated OX40-expressing T cells were added to nontissue culture-treated round-bottom 96-well assay plates to which MEDI6383 and a suboptimal concentration (2 ng/mL) of UltraLEAF low endpoint clone OKT3 anti-human CD3 mAb (Biologend) had been immobilized. For wells containing solution-phase MEDI6383, antibody-based capture of MEDI6383 was omitted. Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) using the CellTrace CFSE cell proliferation kit (Life Technologies) and cell proliferation at 72 hours measured by CFSE dilution. Cytokine release at 72 hours was measured using Mesoscale Discovery (MSD) Th1/Th2 10-plex tissue culture kit (MesoScale Diagnostics).

To test the activity of MEDI6383 clustered by CD32A-expressing HEK cells, fusion protein was incubated with isolated, activated, and CFSE-labeled primary human CD3, CD4, or CD8 T cells plus CD32A-expressing HEK cells together with 10 µg/mL of the anti-CD3 mAb clone OKT3 to initiate T-cell receptor (TCR) stimulation.

Natural Treg suppression assay

Human CD4 effector T cells (Teff) and regulatory T cells (Treg: CD4+CD25+CD127low) were isolated from the same healthy donor Leukopacks (Hemacare) using an EasySep human CD4+ T cell isolation kit and a EasySep human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (StemCell Technologies), respectively (see Supplementary Fig. S1 for CD25, CD127, and Foxp3 immunophenotypic profile). Teff cells were labeled with CFSE using the CellTrace CFSE cell proliferation kit and Tregs with the CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific). Teff and Treg were cocultured at 1:1 ratio in complete RPMI1640 medium containing 5% v/v human AB serum and 1 µg/mL purified NA/LE anti-CD28 mAb, clone 28.2 (BD Biosciences) in the tissue culture plates coated with anti-human CD3 clone OKT3 (Biologend) alone or with MEDI6383 or F810A OX40L fusion protein control. After 5 days of coculture in a humidified tissue culture incubator at 37°C and 5% CO2, the percentages of divided CD4 Teff cells (CFSE dilution) and divided CD4 Treg cells (CellTrace Violet dilution) were assessed by flow cytometry on an Fortessa flow cytometer (Becton Dickinson) and using FlowJo Software (FlowJo LLC).

Human T cell/tumor cell admixed xenograft model

NOD/SCID mice of 5–9 weeks of age were purchased from Envigo Harlan Laboratories, Inc. Studies with mice were conducted in accordance with and approved by the Institutional Animal Care and Use Committee–approved protocols in the Laboratory Animal Resources facility at MedImmune, an Association for Animal Accreditation of Laboratory Animal Care, and United States Department of Agriculture–licensed facility.

Human CD4 and CD8 T cells were separately isolated from healthy donor peripheral blood using CD4 and CD8 human T cell enrichment kits (StemCell Technologies). To generate A375 melanoma tumor cell line–reactive CD4 and CD8 lines, enriched CD4 and CD8 T cells were cultured separately in RPMI complete media containing rhIL2 with mitomycin C–treated A375 tumor cells for one week. T cells were collected and separately restimulated with mitomycin C–treated A375 tumor cells and rhIL2 again for an additional week. Expanded CD4 and CD8 T-cell lines were combined at a 2:1 ratio and then added to A375 tumor cells to produce a ratio of 6:1 of A375 to total T cells, and a total of 3.5 × 10^6 cells were injected into the flank of NOD/SCID mice (n = 6 female mice per group). MEDI6383 or a control OX40L IgG4P fusion protein containing an F810A point mutation (phenylalanine replaced by alanine at position 180) in the OX40 binding interface that abrogates OX40L:OX40 interactions (11) was injected at the indicated doses levels and times starting at day 3 after admixed tumor cell/T-cell engraftment. Dosing was continued 2 times per week for 2 weeks. Tumor dimensions were measured using a caliper at the indicated time points and tumor volumes calculated using the following formula: V (mm^3) = [length (mm) × width (mm) × width (mm)]/2. Antitumor effects were expressed as percent tumor growth inhibition (% TGI), which was calculated as follows: % TGI = [1 − (mean tumor V of treatment group) − (mean tumor V of control group)] × 100.
**MEDI6383 administration to rhesus macaques**

Indian-origin rhesus macaques (*Macaca mulatta*) were housed at the Oregon National Primate Research Center and experiments with these animals were conducted in accordance with and approved by the Institutional Animal Care and Use Committee for Oregon Health & Science University/Oregon National Primate Research Center and the Guide for the Care and Use of Laboratory Animals. Vehicle control (sterile PBS), MEDI6383, or mouse anti-human OX40 mAb MEDI6469 (previously termed 9B12) were administered via the intravenous (bolus) route (*n* = 5 female macaques per group). Vehicle and MEDI6383 at 1 mg/kg were administered every other day (day 0, 2, and 4) for a total of 3 doses while MEDI6469 was administered as a single 5 mg/kg dose. Following test article administration, anticoagulant citrate dextrose (ACD) anticoagulated peripheral whole blood was collected at the indicated times for immuno-phenotyping by whole blood staining. Antibody panels used to stain rhesus peripheral immune cells for flow cytometry analysis are shown in Supplementary Fig. S2A, with Ki67 antibody included in intracellular Ab cocktails and the remainder added to surface antibody cocktails. Example gating of total, central, and effector memory CD4 or CD8 T cells based on CD95, CD28, CCR5, and CCR7 staining is shown in Supplementary Fig. S2B.

**Statistical analyses**

Statistical differences between proliferation values for MEDI6383-mediated Teff proliferation in the presence or absence of Tregs were determined using unpaired, two-tailed Student *t* test. Differences between experimental conditions in FcγR-expressing HEK293 2-cell bioassays using FcγR blocking reagents and for pharmacodynamic measurements between baseline (day 1 predose) and indicated time points in the rhesus study were analyzed for statistical significance using one-way ANOVA with Dunnett posttest correction for multiple comparisons, reporting multiplicity adjusted *P* values for each comparison using GraphPad Prism version 7.02 (GraphPad Software). Differences between MEDI6383-treated or F180A OX40L FP control–treated conditions in Treg assays were analyzed by two-tailed Student *t* test using GraphPad Prism.

**Results**

**Design and preparation of MEDI6383**

MEDI6383 was designed as a human OX40L IgG4P Fc fusion protein composed of three distinct domains: (i) human OX40 ligand extracellular receptor binding domains (RBD) that form homotrimers and bind OX40; (ii) isoleucine zipper coiled coil domains derived from TRAF2 (10) that stabilize the homotrimeric structure of the OX40L RBDs; and (iii) human IgG4 fragment crystallizable gamma (Fc) domains that facilitate Fcγ receptor clustering of the fusion protein and contain a serine to proline substitution in the hinge regions at position 228 according to EU numbering of the full-length IgG4 heavy chain sequence (IgG4P) to prevent inter-Fab arm exchange (Fig. 1A). The resulting protein has a calculated molecular mass of 306,000 daltons supportive of a glycosylated, hexameric protein. Size exclusion chromatography (SEC) and SEC-MALS suggested a highly purified hexameric form (Fig. 1B) with an apparent molecular mass of 330,000 daltons, consistent with the calculated mass (Fig. 1C).

![Figure 1](https://www.aacrjournals.org/molcanther/article-pdf/17/5/1027/4001664/mct-17-0200.pdf)

Figure 1.

Schematic diagram of the proposed structure of MEDI6383. Figure 1 shows that MEDI6383 is a human OX40 ligand IgG4P Fc fusion protein consisting of three subunits containing an OX40L receptor binding domain (RBD), a TRAF2 coiled coil domain, and an IgG4 Fc domain containing an S→P mutation at position 228 according to EU numbering of the full-length IgG4 heavy chain sequence to prevent inter-Fab arm exchange. A, The proposed structure of the protein product is a hexameric “dimer of trimers”. The OX40L RBDs are oriented from N-terminal amino acids (top) to the C-terminus. B, Analytic size exclusion chromatography (SEC) analysis of intact MEDI6383 showed highly monomeric (>98%) single peak with very low levels of aggregates, no fragments or free chains. C, SEC combined with multi-angle light scattering (SEC-MALS) showed an apparent molecular mass of approximately 330,000 daltons which supports the proposed hexameric form. Light trace spanning entire elution time, MEDI6383 sample; darker trace, molecular mass standards.
MED6383 potently binds OX40 on activated primary human and NHP T cells

After purification, MED6383 was tested for binding to native OX40 on the surface of activated human and NHP T cells. We found that OX40 is highly expressed on activated CD4 T cells as opposed to activated human CD8 T cells (Supplementary Fig. S3A and S3B). Therefore, we used activated primary human and NHP CD4 T cells for binding studies. Results showed potent binding to OX40 on activated human, cynomolgus, and rhesus CD4 T cells, with mean apparent equilibrium binding constant ($K_D$) values of 1.7, 24, and 21 pmol/L, respectively (Supplementary Fig. S3; Supplementary Table S1). The control OX40L fusion protein containing an F180A point mutation (phenylalanine replaced by alanine at position 180) in the OX40L binding interface that abrogates OX40L:OX40 interactions (11) did not demonstrate appreciable binding to human or nonhuman primate OX40. MED6383 binding to OX40 was substantially more potent than that of the mouse anti-human OX40 mAB MED6469. The latter bound to activated human CD4 T cells with a mean apparent $K_D$ value of 669 pmol/L (Supplementary Table S1).

MED6383 induces NFkB signaling in human T cells that is enhanced by Fcy receptor–mediated drug clustering

To demonstrate the ability of MED6383 to induce OX40-mediated signaling in human T cells, we generated T-cell reporter and drug clustering cell lines for use in a series of in vitro bioactivity potency assays. First, we created an OX40-expressing Jurkat human T-cell reporter line infected with a lentivirus directing the NFkB luciferase reporter. Second, we made HEK293 cell lines engineered to express either the human Fcy receptor CD32A (FcγRIIA H131, with histidine at position 131), CD64 (FcγRI), or CD16 (FcγRIII 158F or 158V, with phenylalanine or valine at position 158) to evaluate the drug activity (Supplementary Fig. S4). Upon ligation of OX40 on the FcγB-ligand Jurkat reporter cell line, luciferase enzyme activity is produced in the reporter cells through FcγB promoter activation and can be measured as a bioluminescent readout (Fig. 2A). As was observed for primary human T cells, MED6383 showed potent binding to OX40 expressed exogenously on the FcγB-ligand Jurkat T cell reporter line with an apparent $K_D$ of 4.9 pmol/L (Supplementary Table S1). After the addition of MED6383, moderate FcγB promoter activation was observed in a dose-dependent manner, demonstrating that solution-phase MED6383 is capable of mediating low levels of OX40 clustering on reporter cells (Fig. 2B). No change in this activity was observed after the addition to the assay of parental HEK293 human embryonic kidney cells that lack FcyR expression. However, the activity was significantly enhanced in a dose-dependent manner when the reporter Jurkat cells were coincubated with MED6383 and HEK293 cells expressing CD32A or CD64 (Fig. 2C and D), but not CD16 (Supplementary Fig. S4H and S4I). This demonstrated that OX40 receptor agonism by MED6383 occurred to a limited extent in a solution-phase form that was enhanced when MED6383 was clustered by binding of Fc domains on the fusion protein to FcγRs on the surface of CD32A- or CD64-expressing HEK293 cells.

To further demonstrate the dependency of the clustering on FcγRs, FcγR blocking reagents and appropriate control proteins were used in 2-cell bioassays with FcγR-expressing HEK293 cells, reporter cells and MED6383 at a concentration (0.5 μg/mL, 1.6 nmol/L) to induce optimal activity. With HEK293 parental cells in the 2-cell reporter bioassay, none of the FcγR blocking reagents affected the minimal solution-phase activity of MED6383 except human serum which slightly, but statistically significantly, enhanced bioactivity (Fig. 2E). In contrast, control hexamer fusion proteins of human IgG1 and IgG4P isotypes used in excess to MED6383 or an anti-CD32 mAb significantly inhibited drug activity mediated by HEK CD32A drug clustering, with the strongest inhibition observed for the IgG1 isotype control hexamer fusion protein and the anti-CD32 mAb (Fig. 2F). Human serum at 10% or 20% of assay volume did not significantly affect activity, nor did purified human IgG at 100 μg/mL. Likewise, the control hexamer fusion proteins of human IgG1 and IgG4P isotypes and an anti-CD64 mAb significantly inhibited MED6383-mediated bioactivity when HEK CD64 cells were used, with the IgG1 isotype control hexamer consistently showing the greatest activity (Fig. 2G). In this format, however, 10% or 20% human serum also decreased MED6383 activity, albeit reproducibly less than that of the control hexamer fusion proteins or anti-CD64 mAb. Purified human IgG at 100 μg/mL did not affect activity of MED6383 with HEK CD64 cells. In total, these results demonstrate that MED6383 bioactivity was mediated by FcγR clustering in this bioassay format.

To test whether FcγR-mediated enhancement of MED6383 activity may occur in the context of FcγR clustering by Raji tumor B cells (predominately expressing CD32B; see Supplementary Fig. S4), or by CD45 immune cells isolated from primary tumors (in total expressing CD64, CD32A, CD32B, and CD16; Supplementary Fig. S4), we tested these cell types in 2-cell bioassays. Results showed that both cell types mediated robust MED6383 activity, greater than that observed for MED6383 in the absence of clustering (Fig. 2H–I). The F180A OX40L fusion protein control lacking the ability to bind OX40 did not demonstrate activity in any of the 2-cell assays tested. MED6469 bound with lower apparent affinity than MED6383 to OX40 on the surface of Jurkat reporter cells (apparent $K_D$ of 922 pmol/L; Supplementary Table S1). Likewise, MED6469 had a substantially lower potency than MED6383 for OX40 reporter activity in the 2-cell bioassay format when utilizing either Raji or CD32B-expressing HEK cells to cluster drug (Supplementary Table S2).

Multiple FcγRs were capable of mediating enhanced MED6383 activity except CD16, an FcγR expressed by NK cells that mediates NK cell–mediated cytotoxicity when effectively cross-linked by antibodies with an appropriate isotype (i.e., human IgG1; ref. 14). Therefore, we sought to determine whether MED6383 was capable of inducing NK-cell ADCC of OX40-expressing cells. MED6383 contains six Fc domains of the IgG4 class, and thus the fusion protein would not be expected to mediate ADCC by CD16-expressing NK cells. As predicted, MED6383 did not demonstrate ADCC when added to mixtures of primary human NK cells and CD4 T cells expressing high levels of OX40, whereas an IgG1-formatted version of MED6383 (OX40L fusion protein IgG1) demonstrated potent NK-cell–mediated ADCC activity (Supplementary Fig. S5A). Furthermore, MED6383 did not bind the complement component C1q (Supplementary Fig. S5B). This suggests that it would not mediate complement-dependent cytotoxicity of OX40-expressing cells.
Induction of cytokine release and proliferation in activated primary human T cells by MEDI6383

Results using the OX40-expressing FluskT T cells suggested that MEDI6383 would potentiate primary human T-cell activation. To test this hypothesis, primary human CD3+ T cells were isolated from healthy donors, activated with PHA-L plus IL2 to upregulate cell surface expression of OX40, and then plated onto tissue culture plates coated with a suboptimal T-cell receptor (TCR) stimulus (plate-bound anti-human CD3 mAb clone OKT3) and a range of concentrations of MEDI6383 (Fig. 3A). The F180A OX40L fusion protein control was included to demonstrate OX40-specific effects. The immobilized MEDI6383 induced the release of 5-fold or higher IFNγ and TNFα levels from activated primary human CD3-purified T cells in a concentration-dependent manner (Fig. 3B and C) and at least 2-fold or higher levels of IL10, IL13, IL5, and IL8 (Supplementary Fig. S6), suggesting that MEDI6383 can induce the release of Th1- and Th2-type cytokines under these culture conditions. Likewise, MEDI6383 induced CD4 T-cell proliferation among activated CD3 purified T cells in a concentration-dependent manner (Fig. 3D). The F180A OX40L fusion protein control did not induce cytokine release or proliferation, showing that the effects were specific to OX40 engagement. Interestingly, solution-phase MEDI6383 (i.e., drug not preimmobilized to the assay plate) did not induce cytokine release or proliferation under these experimental conditions. There was also a lack of MEDI6383-induced cytokine release and proliferation in the absence of concomitant TCR stimulation (no anti-CD3 mAb).

Consistent with its enhancement of T-cell cytokine release and proliferation, costimulation with MEDI6383 enhanced the expression levels of the T-cell activation markers CD137, CD30, PD-1, and CD25 on activated CD3 T cells (Supplementary Fig. S7). The increase in cell surface markers occurred on both CD4 and CD8 T-cell subsets, indicating that MEDI6383 engaged low levels of OX40 on CD8 T cells or that cross-talk between OX40-expressing CD4 T cells and CD8 T cells occurred in coculture conditions. Taken together, these results showed that MEDI6383 further enhanced activation of both CD4 and CD8 T cells when present in the same experimental conditions.

To examine the activity of MEDI6383 with activated primary human T cells in the context of FcγR clustered drug together with suboptimal TCR (anti-CD3) stimulation, MEDI6383 was tested in a variation of the 2-cell bioassay using activated primary human T cells. In this assay, drug-clustering HEK cells expressing CD32A were tested with isolated primary human CD3, CD4, or CD8 T cells activated with PHA-L plus IL2 to upregulate cell surface expression of OX40. Consistent with results from bioassays using plate-immobilized drug, MEDI6383 induced a concentration-dependent increase in CD4 T-cell proliferation among purified, activated CD4 T cells (Fig. 3E) or CD3 T cells (Supplementary Fig. S8) when incubated with CD32A-expressing HEK and anti-CD3 mAb. No enhancement of proliferation was observed among CD4 T cells in the absence of HEK CD32A-expressing cells or in the absence of anti-CD3 stimulation, demonstrating the requirement for drug clustering and TCR stimulation to induce a MEDI6383-mediated biological response.

Overall, experiments using activated primary human T cells demonstrated TCR signal–dependent costimulatory activity of MEDI6383 upon drug clustering, but not in solution phase. CD4 cells express higher activation-induced OX40 on cell surfaces than CD8 cells (Supplementary Fig. S3A and S3B), and MEDI6383 demonstrated potent and consistent enhancement of activity among these cells, and less potent and inconsistent enhancement of primary CD8 T-cell activation.

MEDI6383 inhibits nTreg-mediated suppression of conventional T cells

MEDI6383 augmented the activation of conventional CD4 T cells, but in the immunosuppressive tumor microenvironment the activity of T cells can be dramatically suppressed (2). Natural or induced regulatory T cells (nTreg or iTreg, respectively) represent two related but distinct cell types that may mediate inhibition of conventional T-cell activation through cell-to-cell contact-mediated mechanisms as well as by soluble factors (15). To examine the ability of MEDI6383 to overcome nTreg inhibition of conventional T-cell activation, we cocultured TCR-stimulated conventional CD4 T cells with MEDI6383 in the absence and presence of autologous nTreg cells isolated from healthy donor peripheral blood (Fig. 4; Supplementary Fig. S1). Freshly isolated conventional CD4 (Teff) and Treg cells expressed low but detectable OX40 that was upregulated upon Teff plus Treg coculture in the presence of anti-CD3 and anti-CD28 stimulation (Fig. 4A). As had been shown above, MEDI6383 promoted TCR-driven T-cell proliferation in the absence of nTregs. In the presence of nTregs, proliferation was inhibited. The nTreg-mediated inhibition of Teff proliferation was reversed to levels observed in the absence of nTregs when nTreg plus Teff cells were cultured in the presence of MEDI6383, demonstrating the ability of the fusion protein to prevent Teff immunosuppression of T cell proliferation (Fig. 4B and D). Treg cells themselves proliferated poorly when cultured alone. However, Tregs cultured alone were induced to proliferate by MEDI6383 in a concentration-dependent manner, but not by the F180A OX40L FP control (Fig. 4C and E). When cocultured with Teff cells, a greater percentage of Treg cells proliferated at baseline (~ 30%) than when cultured alone (< 10%). As when Tregs were cultured alone, MEDI6383 induced the proliferation of Tregs in a concentration-dependent manner when Tregs were cocultured in the presence of Teff cells. In total, these results show that MEDI6383 induces the proliferation of both Teff and Treg cells cultured alone or in coculture with one another.

MEDI6383-mediated antitumor activity in vivo

Testing the activity of MEDI6383 in immunocompetent mouse syngeneic models is not possible as human OX40L binds to human and primate OX40 but not to mouse OX40. Therefore, to determine whether agonism of OX40 by MEDI6383 could help antitumor T cells overcome the immunosuppressive environment within a tumor, we tested the ability of MEDI6383 to mediate tumor clearance in vivo using a human tumor/T-cell admixed model. In this model, human CD4 cells that express OX40 on their cell surface (Supplementary Fig. S9) and CD8 T cells that are each allogeneic to the human melanoma cell line A375 were mixed with A375 melanoma cells and implanted into the flank of NOD/SCID mice. Thereafter, mice were treated with isotype F180A OX40L fusion protein control or MEDI6383 at various dose levels and antitumor effects were monitored. Administration of MEDI6383 to tumor-bearing mice caused significant tumor growth inhibition when compared with control fusion protein-treated mice (Fig. 5A). This effect was dependent on the activity of T cells as no growth inhibition was observed in their absence, but was restored upon addition of T cells to the admixture (Fig. 5B and C).
Induction of immune cell activation by MEDI6383 administered systemically to NHP.

MEDI6383 at 1 mg/kg was administered by the intravenous route every other day for three injections in five rhesus monkeys (Fig. 6A). Vehicle (PBS) control or a single 5 mg/kg bolus injection of the mouse anti-human OX40 mAb MEDI6469 was administered to separate cohorts of five monkeys each for comparison to MEDI6383 (Fig. 6A). Whole blood was obtained at various time points prior to and after drug administration for immunophenotyping of peripheral blood populations. After MEDI6383 administration, the percentages of total memory CD4 T cells positive for Ki67 (proliferating CD4) increased from a baseline of 7% (day 0 predose) to 52% at day 10 predose (7-fold increase, \( P = 0.003 \); Fig. 6B). Smaller, but statistically significant, increases in the percentages of proliferating total memory CD8 T cells were also seen, rising from 9% at baseline (day 0 predose) to 19% at day 10 predose (2-fold increase, \( P = 0.02 \)). In contrast, no increases in proliferation among vehicle-treated or of treated naive CD4 or CD8 T cells were observed, which remained low throughout the study. Subdividing total memory cells into central (Tcm) and effector memory (Tem) subsets yielded different proliferation profiles among each subset. For CD4 memory T cells, an increase in proliferating CD4 Tcm cells from 5% at day 0 predose to 56% at day 10 (10-fold, \( P = 0.001 \)) was observed, whereas a smaller increase in proliferating CD4 Tem cells was detected with 7% at day 0 predose and 14% at day 14 (~2-fold, \( P = 0.005 \)), reaching a maximum level later than CD4 Tcm cells (day 14 versus day 10). For CD8 memory T cells, the magnitude of the Ki67 increase among CD8 Tcm cells was smaller than that observed for CD4 Tcm, with 6% at day 0 predose and 29% at day 10 (~2-fold, \( P = 0.02 \)). The increase for CD8 Tem was similar to that for CD4 Tem, with 11% at day 0 predose and 21% at day 14 (~2-fold, \( P = 0.02 \)). The kinetics of peak Ki67 induction in the CD8 Tem population (day 14) was delayed relative to that observed in the CD8 Tcm subset (day 10), similar to what was observed for CD4 memory populations. The induction of CD4 memory T-cell proliferation by MEDI6383 was greater than that observed for MEDI6469 among CD4 total memory (11% at day 0 predose to 27% at day 10, ~2.5-fold, \( P = 0.03 \)) CD4 central memory (9% at day 0 predose to 23% at day 10, ~2.5 fold, \( P = 0.02 \)) and CD8 central memory T cells (9% at day 0 predose to 13% at day 7, ~1.5-fold, non-significant at \( P = 0.29 \)). This result was perhaps expected given the higher binding affinity of MEDI6383 for OX40 relative to that of MEDI6469 (Supplementary Table S1) and the relatively greater potency observed for the fusion protein in 2-cell bioactivity assays with FcR-expressing cells (Supplementary Table S2).

We evaluated markers of T-cell activation and found an increase in the percentage of ICOS\(^{+}\) total memory CD4 (2% at day 0 predose to 15% at day 7, ~7-fold, \( P = 0.01 \)) and total memory CD8 T cells (0.7% at day 0 predose to 3% at day 7, ~4-fold, \( P = 0.01 \)), reaching maximum levels around day 7–10 (Fig. 6C). The greatest percentage of the responding cells was higher in CD4 versus CD8 memory cells, but at lower percentages overall when compared with the Ki67 positive fraction of total memory cells at those time points (Fig. 6B). Increases in the percentage of PD-1\(^{+}\) CD4 total memory cells (1.5% at day 0 predose to 3.5% at day 7, ~2-fold, \( P = 0.005 \)) were also observed, but at a lower percentage and fold change than seen for ICOS positivity among CD4 total memory cells. No increases in the percentage of ICOS\(^{+}\) and PD-1\(^{+}\) total memory cells were observed for vehicle-treated animals. Interestingly, the increase in the percentage of ICOS\(^{+}\) total memory cells observed for MEDI6383 was not seen for MEDI6469, while the fold increase in PD-1\(^{+}\) CD4 total memory cells was higher for MEDI6469 (1% at day 0 predose to 5% at day 10, ~5-fold, \( P = 0.04 \)) than for MEDI6383. Enhanced cytokine production may also be an indicator of T-cell co-stimulation following OX40 agonism, as shown in vitro. However, neither intracellular nor plasma levels of cytokine were measured in this study.

Examining B-cell proliferation, MEDI6383 induced an increase in the percentage of circulating Ki67\(^{+}\) B cells (10% at day 0 predose to 24% at day 17, ~2 fold, \( P = 0.01 \)), reaching a maximum proliferation later (day 17) than that observed for proliferating CD4 and CD8 total memory cells (Fig. 6D). MEDI6383 induced a similar fold increase of proliferating B cells as compared with MEDI6469 (8% at day 0 predose to 16% at day 14, ~2-fold, \( P = 0.05 \)).

MEDI6383 and MEDI6469 treatment initially resulted in a decrease in absolute numbers of circulating CD4 T and B cells per mL of blood as measured at day 4 postdose, consistent with driving an exodus of CD4 T and B cells from the circulation. This was followed by increases in CD4, CD8 T-cell, and B-cell absolute numbers over the second week indicating that the observed rise in percent Ki67-positive cells was associated with increases in circulating cell numbers (Supplementary Fig. S10).

**Discussion**

Within the TME, an endogenous antitumor T-cell immune response is shaped by a number of signaling events. First, effective

---

**Figure 2.** OX40 reporter bioactivity of MEDI6383. Figure 2 shows the bioactivity of MEDI6383 using OX40-expressing Jurkat NFκB reporter cells with or without clustering by FcγR expressed on human cells, A. Schematic illustration of the anticipated interactions in a 2-cell bioassay using FcγR-expressing drug “clustering” cells and the NFκB-luciferase reporter Jurkat T cell line that expresses OX40. B, Concentration-dependent response of MEDI6383-mediated activation of an NFκB-luciferase reporter Jurkat T cell line by MEDI6383 in solution phase or in the presence of parental HEK293 cells lacking FcγR expression. F180A OX40L control or no MEDI6383, as indicated, were incubated with HEK293 plus reporter cells as controls. As an additional control, reporter cells were incubated in the absence of HEK293 cells and MEDI6383 (reporter alone), C, Bioactivity of MEDI6383 clustered by CD32A-expressing HEK293 or D, CD64-expressing HEK293 cells compared with MEDI6383 in solution phase or to controls, as indicated. E–G, Effects of various FcγR blocking reagents, or controls, on the bioactivity of 0.5 μg/mL (1.6 nmol/L) of MEDI6383 incubated with parental or FcγR-transduced HEK293 plus reporter cells, as indicated. Upper dotted line indicates mean activity of MEDI6383 without blocking reagent (media) plus HEK293 cells and lower dotted line represents mean activity of reporter cells incubated with MEDI6383 in the absence of HEK293 cells, \( P < 0.001 \) by one-way ANOVA comparing the indicated tests to either media, albumin, or mouse IgG control proteins. H, Bioactivity induced by MEDI6383 or the F180A OX40L control protein clustered by the CD32A-expressing Raj B cell tumor line. I, Bioactivity observed after MEDI6383 clustering by CD45 immune cells* and or J, a resected primary human non-small cell lung cancer. Results are representative of at least three independent experiments with data points and error bars representing the mean and SEM, respectively, of triplicate measurements, with the exception of results from CD45 cells isolated from primary human cancers which represent individual experiments.
presentation of tumor-specific epitopes by tumor cells or antigen-presenting cells provides an essential signal for T-cell activation. Second, costimulatory signals such as that provided by OX40 promote effector T-cell activation and expansion, as well as the generation of effector and central memory T cells required for long-lived antitumor immunity.

As a therapeutic strategy for the treatment of cancer, MEDI6383 was engineered to promote an antitumor immune response through potent stimulation of OX40. On the basis of the biology of OX40 engagement, we hypothesized that MEDI6383 would enhance T-cell costimulatory signaling, resulting in proinflammatory cytokine release and proliferation in the context of a T-cell receptor stimulus. Furthermore, we hypothesized that the drug would expand memory T cells and provide protection of conventional, activated T cells from the inhibitory effects of regulatory T cells. Finally, we hypothesized that OX40 engagement would induce antitumor-specific immunity dependent on T cells.

With regard to T-cell activation, we found that MEDI6383 in solution phase induced the activation of the NFκB promoter in OX40-positive reporter T cells, and that this activity was greatly enhanced by FcγR clustering of the fusion protein. In vivo, cells that express FcγRs would be expected to cluster MEDI6383 and thus drive OX40 costimulation. We found that primary human tumors, including NSCLC and renal cell carcinoma (RCC), contained immune cells expressing FcγRs, and these cells effectively clustered MEDI6383 and mediated potent OX40 reporter activity ex vivo. As expected, reagents that have the potential to strongly block FcγR interactions, such as control hexamer fusion

---

**Figure 3.** Activity of MEDI6383 using primary human T cells. Figure 3 shows the activity of MEDI6383 immobilized to tissue culture plastic or clustered by CD32A-expressing cells with OX40-expressing activated primary human T cells. A, Schematic illustration of the bioactivity assay using plate immobilized M, MEDI6383, IFNγ (B) and TNFα release (C) into cell culture supernatants and proliferation of cells (D) plated under the conditions described next to each graph. Solution-phase MEDI6383 indicates drug added to medium instead of captured on the plate surface, and no anti-CD3 indicates omission of anti-CD3 mAb clone OKT3 stimulation in the wells. E, Bioactivity of MEDI6383 with OX40-expressing activated primary human CD4 T cells cocultured with CD32A-expressing HEK295 cells and a suboptimal amount of anti-CD3 mAb clone OKT3. No drug, no anti-CD3, and no HEK CD32A indicate bioassay conditions containing all components except MEDI6383, anti-CD3 mAb clone OKT3, or CD32A-expressing HEK295 cells, respectively. T cells only indicate the presence of CFSE-labeled CD4 T cells alone in the absence of drug, anti-CD3 mAb clone OKT3, and CD32A expressing HEK295 cells. Results for plate-immobilized or HEK CD32 clustered MEDI6383 are each representative of three independent experiments, with data points and error bars representing the mean and SEM of triplicate measurements, respectively.
proteins of the IgG1 or IgG4P isotypes used in molar excess or anti-FcγR antibodies, interfered with MEDI6383 bioactivity mediated by FcγR-expressing cells. However, the bioactivity was not affected by human IgG at >400 times molar excess of MEDI6383 nor as strongly by human serum that contains high levels of human IgG. The latter demonstrates the ability of MEDI6383 as a hexameric IgG-containing fusion protein to outcompete endogenous IgG for FcγR binding and clustering of drug, a feature that is

Figure 4.
MEDI6383 overcomes the suppressive activity of nTreg cells. Figure 4 demonstrates the ability of MEDI6383 to reverse the immunosuppressive activity of nTreg cocultured with Teff. A, Expression of cell surface OX40 by Treg cells at the time of isolation (day 0) and after 5 days of Teff/Treg coculture in the presence of anti-CD3 and anti-CD28 stimulation. B, Examples of CSFE dilution of Teff cells or C, Treg among untreated, MEDI6383-treated, or F180A OX40L FP control-treated Teff cell, Treg cell, or Teff/Treg cocultures, as indicated. D, Plot of CD4 Teff cell proliferation versus concentration of MEDI6383- or F180A OX40L FP control-treated Teff cell (Teff only) or Teff/Treg cocultures (Teff + Treg), as indicated. E, Plot of CD4 Treg cell proliferation versus concentration of MEDI6383- or F180A OX40L FP control-treated Treg cell (Treg only) or Teff/Treg cocultures (Teff + Treg), as indicated. Data is representative of three independent experiments. Points and error bars represent mean of triplicate measures and SEM, respectively. *P < 0.05 using two-tailed Student t test comparing MEDI6383-treated versus F180A OX40L FP control-treated cells for points 0.15 nmol/L and above for Teff + Treg and 0.62 nmol/L and above for Teff only in D and for points 0.31 and above for Teff + Treg and 0.62 nmol/L and above for Treg only in E.
important to consider when MEDI6383 is administered in vivo where endogenous IgG levels will be high.

In vivo, we used a human tumor/immune cell admixed model in NOD/SCID mice to demonstrate T-cell–dependent tumor eradication by MEDI6383. Although not examined in this study, such tumors would be expected to be infiltrated with FcγR-expressing mouse immune cells, and these potentially could have mediated clustering of MEDI6383. It is anticipated that FcγR-positive immune cells, which may include myeloid cells, dendritic cells, or B cells, will be important in human tumors and tumor draining lymph nodes of patients to potentiate MEDI6383-mediated antitumor activity. However, some activity related to OX40 clustering by the hexameric OX40L fusion protein itself without further immobilization on cell surfaces cannot be ruled out.

The CD64 FcγRI has relatively high affinity for IgG4, whereas the FcγRs CD32 and CD16 have relatively lower affinity (16). We determined the equilibrium binding (K_D) values for MEDI6383 binding to human FcγRs by surface plasmon resonance (Supplementary Table S3) and found expected low, micromolar affinity binding to CD16 and CD32A and CD32B, and higher, nmol/L affinity binding to CD64. CD64 (FcγRI) and CD32A or B (FcγRII isoforms) enhanced drug activity through clustering, but CD16 (FcγRIII) lacked this enhancement. On the basis of the measured

Figure 5.
Antitumor activity of MEDI6383. Figure 5 demonstrates the T-cell–dependent activity of MEDI6383 in a human T cell/tumor cell admixed model in NOD/SCID mice. A, Statistically significant tumor growth inhibition by MEDI6383 administered over a range of dose levels in mice engrafted with human A375 melanoma tumor cells admixed with allogeneic A375-reactive human CD4 and CD8 T cells. B, Lack of MEDI6383 activity in mice engrafted with A375 tumor cells but in the absence of human T cells. C, Activity of MEDI6383 when allogeneic A375-reactive human T cells were engrafted with A375 tumor cells. * P < 0.05 by Mann–Whitney rank sum test of mean tumor size at day 28 for MEDI6383-treated groups compared to isotype control group. Arrows indicate times after tumor injection when MEDI6383 was administered to mice. Statistically significant differences in mean tumor size between MEDI6383 and isotype control group was demonstrated in three independent experiments, with representative data shown.
affinities, it is not clear why CD32A and CD32B would enhance clustering and bioactivity, whereas CD16 would not. It is important to note that the CD32A or CD32B were expressed at high levels in our HEK expressing or Raji 2-cell bioassays, and may explain why these FcyR-presenting cells demonstrated activity despite their relatively low affinity for IgG4. However, the lack of effective clustering by CD16, despite its robust expression in HEK cells, was consistent with its relatively low affinity for IgG4 and our finding that MEDI6383 did not mediate measurable NK-cell–mediated ADCC of OX40-expressing activated T cells. In contrast, a human IgG1 version of the OX40L fusion protein with stronger binding to CD16 mediated potent ADCC of OX40-expressing T cells. Likewise, MEDI6383 did not bind to the complement component C1q as did a human IgG1 control antibody. Thus, MEDI6383 would not be expected to mediate complement-dependent cellular cytotoxicity of OX40-expressing cells. The lack of NK-cell effector function for MEDI6383 was specifically engineered by incorporation of the IgG4 isotype, and was designed to prevent the elimination of activated, tumor-reactive effector T cells in the TME through NK-cell–mediated ADCC or by CDC. Whether MEDI6383 may mediate other effector functions through FcyRs, such as antibody-dependent cellular phagocytosis (ADCP) or ADCC mediated by FcyR-expressing immune cells other than NK cells, remains an area for further investigation.

In line with the observed NF-kB activation in reporter cells, immobilized MEDI6383 induced Th1 and Th2 cytokine release and cellular proliferation in preactivated primary human CD4 T cells. The cytokine release profile induced by OX40 agonism during an antitumor immune response likely depends on the local cellular and cytokine milieu, and may include a mixed Th1/Th2 response (17) as observed here. The activity of MEDI6383 required concomitant TCR signaling, as the fusion protein was inactive in the absence of an anti-CD3 mAb used to

Figure 6. Pharmacodynamic effects of MEDI6383. Figure 6 shows the activity of MEDI6383 on peripheral T and B cells when administered to healthy rhesus macaques. A, Schematic for test article dosing and blood draws for immunophenotyping by flow cytometry. B, Percentage of Ki67+ CD4 (top) and CD8 (bottom) total memory, naive, central and effector memory T cells, as indicated, prior to and after test article dosing on day 0. C, Percentage of ICOS+ (left) and PD-1+ (right) total memory CD4 (top) and CD8 (bottom) T cells. D, Ki67+ CD20 B cells. Error bars were withheld for C and D so that the comparisons between different treatments could be more easily visualized.
stimulate the TCR. Likewise, consistent with the relatively low NKFCR promoter activity observed in reporter cells treated with MEDI6383 in solution phase, activated primary human CD4 T cells only showed evidence of cytokine release and proliferation by clustered, but not solution-phase, MEDI6383. Together, these results suggest that MEDI6383 may only induce T-cell activation in the presence of a TCR stimulus, and will likely have optimal activity when clustered. The enhancement of TNFRSF agonist clustering by FcγRs for drug activity is not unique to MEDI6383 (18), and is likely a common phenomenon for agonists whose target receptors are multimerized by natural ligands as is the case for the OX40:OX40L interaction. As shown by the example in Supplementary Fig. S4, primary human tumors may be infiltrated by FcγR-expressing human myeloid cells, and we anticipate that these cell types will mediate MEDI6383 clustering in the tumor.

In addition to enhancing the activation of conventional CD4 T cells, OX40 agonism of these cells has been reported to protect them from the immunosuppressive effects of Treg cells (19, 20). The expression of OX40 on the surface of Treg cells themselves in human tumors has been found to be high (21). As mentioned above, MEDI6383 was engineered to reduce or eliminate ADCC function, and so would not be expected to mediate cell killing of OX40+ Treg. However, MEDI6383 may modulate Treg formation or function in different ways. For example, OX40 engagement on Tregs has been alternatively reported to promote Treg cell death (22), prevent the formation of FoxP3+ Treg (23, 24), reduce FoxP3 expression and alter the suppressive capacity of Treg (25–27), and even to induce Treg expansion under certain experimental conditions (17, 28). In this study, we showed that MEDI6383 restored the proliferation of activated, conventional CD4 T effector cells in the presence of immunosuppressive natural regulatory T cells to a level observed when conventional CD4 T effector cells were cultured in the absence of Tregs. Whether this effect may occur through engagement of OX40 on nTregs to abrogate their suppressive function or on conventional T cells to protect them from the immunosuppressive effects of regulatory T cells remains to be determined, and is an area of current investigation. Immune cells other than regulatory T cells, such as suppressive myeloid cells or tumor-associated macrophages, may also mediate immunosuppression in the TME. Whether MEDI6383 may overcome some of the immunosuppression mediated by these cell types is also an area for further study. In this study, MEDI6383 also induced the proliferation of Tregs, either alone or in coculture with T effector cells. As mentioned above, this is consistent with other published reports (17, 28). MEDI6469, an agonist murine anti-human OX40 mAb, expanded conventional CD4 T cells but did not demonstrate Treg cell expansion in the peripheral blood of subjects with advanced malignancies treated with the drug; however, whether MEDI6383 may induce Treg expansion in the peripheral blood or tumors of patients remains to be seen. Results reported here, however, indicate that MEDI6383 would likely enhance T effector cell function in the presence of Treg cells in either case.

The antitumor activity of MEDI6383 was demonstrated in an allogeneic human T cell tumor cell admixed model in NOD-SCID immunodeficient mice. In this model, MEDI6383 inhibited the growth of A375 human melanoma tumors in a T-cell–dependent manner. The effectors of this activity are likely cytolytic granzyme and perforin granules released by activated T cells, although cytotoxic or cytostatic activity mediated by cytokines and Fas or TRAIL ligands cannot be ruled out and deserve further study. In addition to enhancing the activation and proliferation of conventional effector T cells, OX40 agonists have been shown to promote the survival and expansion of memory T-cell subsets (29, 30). In particular, administration of OX40 agonists to tumor-bearing mice expands memory T cells and establishes an antitumor memory T-cell response that can provide long-lasting antitumor immunity (31). To test the hypothesis that MEDI6383 is capable of expanding memory T cells, we studied the pharmacodynamic effects of MEDI6383 administered intravenously to healthy untreated rhesus macaques. Immunophenotyping of peripheral blood T cells in a clinical trial of MEDI6469 in patients with advanced malignancies demonstrated activation and proliferation of CD4 and CD8 T cells in the peripheral blood of treated patients (4). In this study, MEDI6469 likewise induced T-cell proliferation and increased absolute CD4 and CD8 T-cell numbers in the peripheral blood of rhesus macaques, justifying the use of this species as a relevant model to study MEDI6383-mediated pharmacodynamic activity.

Results from the rhesus study showed that MEDI6383 induced the proliferation of both CD4 and CD8 Tcm and Tem, but not naïve T cells that have low OX40 expression. The effects were particularly potent with respect to the induction of CD4 Tcm proliferation. This bias toward CD4 was not unexpected as the upregulation of cell surface OX40 on activated CD4 T cells is much higher than that on activated CD8 T cells, where expression levels of OX40 are relatively modest. Consistent with this, we found that isolated CD8 cells activated in vitro respond poorly to OX40 agonists compared with isolated, activated CD4 T cells. However, CD8 T cells among enriched CD3 T cells (CD4 + CD8) have sometimes demonstrated modest proliferative responses to MEDI6383 and both cell populations demonstrated increased cell surface activation marker expression, consistent with a helper cytokine effect from CD4 T cells in the coculture or resulting from low-level OX40 expression on CD8 T cells. The activation of CD4 T cells may have contributed to the proliferation of CD8 T cells in MEDI6383-treated rhesus monkeys, although this is difficult to formally distinguish from direct effects on CD8 T cells expressing low OX40 levels.

Interestingly, we found that Tcm proliferated to a much greater extent than the Tem subset, and the peak proliferation in the latter was delayed relative to the former. This suggests that rhesus Tcm cells may be more responsive to OX40 signals, and/or they may themselves subsequently promote proliferation of Tem cells or convert into a Tem-like phenotype upon encounter with cognate antigen (32). However, the delayed appearance of Tem proliferation could simply reflect a delayed kinetics of proliferation in this T-cell subset relative to that of Tcm cells. The interplay and interconversion of memory T-cell subsets, and their respective proliferation kinetics, in animals treated with OX40 stimulating drugs remains an area of further study. However, given that antitumor Tcm cells may provide more persistent and effective antitumor immunity (33) and that OX40 agonism may potentiate memory phenotypes while preserving cytolytic effector function (34), this will be of particular interest in the context of long-lasting antitumor immunity that may be generated by MEDI6383 in the treatment of cancer.

The initial decrease in absolute numbers of circulating CD4 and CD8 T cells and CD20+ B lymphocytes observed in MEDI6383-treated nonhuman primates is interesting as they are consistent with the possibility that peripheral blood T cells are being mobilized to exit the circulation on initial exposure, although other
hypotheses may need to be considered. A decrease in absolute lymphocyte count within the first week after the administration of checkpoint blocking mAbs has been observed in human clinical trials (35), and so this may be a general phenomenon after pharmacologic activation of peripheral lymphocytes. In this study, reductions of CD4 and CD8 T-cell numbers in the peripheral blood were short-lived. Generally, T-cell counts increased in the second week post treatment, the same timeframe in which increases in T cells entering the cell cycle measured by Ki67 expression were observed. Whether these blood changes had corresponding changes in tissues and lymph nodes is an interesting question but was not addressed in this study.

Consistent with the induction of T-cell proliferation and in vitro enhancement of cell surface T-cell activation markers such as PD-1 and CD25 by MEDI6383, the upregulation of the activation markers ICOS and PD-1 were observed among memory CD4 and CD8 T cells. The peak induction of ICOS was higher than that of PD-1, and higher in CD4 than CD8 total memory T cells. Interestingly, CD25 induction was not observed in vivo, and the induction of PD-1 was surprisingly low considering the levels of T-cell activation as indicated by Ki67 and ICOS levels. In general, ICOS+ or PD-1+ cells were observed in a much smaller fraction of T cells relative to Ki67+ (proliferating) T cells. This may reflect the induction of these markers in a subset of proliferating cells, or a difference in the kinetics of their expression, resulting in lower steady-state percentages of ICOS+ and PD-1+ cells relative to Ki67+ positive cells that reflect cells outside of G0 in the cell cycle. In any case, upregulation of these activation markers further supports the idea that OX40 agonists such as MEDI6383 may combine well in cancer treatment with checkpoint inhibitors such as PD-1/PD-L1 as suggested by mouse syngeneic tumor models (36, 37). Clinical trials with OX40 agonists combined with PD-1/ PD-L1 therapies are ongoing, including MEDI6383 combined with the anti-PD-L1 mAb durvalumab (NCT02221960). MEDI6383 was found to induce B-cell proliferation (Ki67 positivity) in addition to T-cell proliferation. As resting and activated rhesus B cells do not express OX40, this would appear to be an indirect effect of MEDI6383 on B cells. It is possible that MEDI6383 activated CD4 follicular Th (Tfh) cells within lymph node germinal centers, leading to the maturation and proliferation of rhesus B cells. Some evidence suggests that OX40 is expressed by Tfh cells and that engagement of OX40 promotes Tfh cell activity with regard to B-cell expansion (38). Such an effect of MEDI6383 on B-cell expansion may be meaningful in patients with tumors, and in fact increased antibody titers in vaccinated NHP (39) and cancer patients (40) following MEDI6469 treatment have been observed, suggesting B-cell activation in those settings. Potentially, activation and expansion of B cells producing antibodies that recognize tumor neoantigens may induce direct NK-cell–mediated tumor lysis and/or opsonization and phagocytosis (antibody-dependent cellular phagocytosis) by professional phagocytic cells within the TME. Each of these processes may contribute to antitumor activity of OX40 agonists. In contrast, regulatory B cells within a TME or in surrounding tertiary lymphoid structures may act to suppress antitumor-specific T-cell responses, which would serve to oppose antitumor T-cell activity. An expansion of regulatory B cells by OX40 agonists could theoretically oppose antitumor immunity. Given the potential pro- and antitumor roles played by B cells, the effects of MEDI6383 on B-cell form and function remains a subject of particular interest. The effects of MEDI6383 on B-cell activation should also be interpreted with caution, however, as antidrug antibodies are frequently observed in nonhuman primates administered with antibodies or recombinant fusion proteins. Such responses may indicate a nonspecific B-cell activation in response to the drug and are difficult to discern from specific B-cell effects as isotype control proteins would also be expected to induce similar ADA responses. Therefore, the true impact of MEDI6383 on B-cell activation may await results from studies in human subjects where ADA responses may be less common than those observed in nonhuman primates.

In summary, we found that MEDI6383 was a strong activator of T cells in vitro and in vivo, leading to proliferation of effector and memory T cells and protection from Treg-mediated suppression. The activity depended upon concomitant TCR signaling, and was substantially enhanced by FcγR-mediated clustering. As expected, MEDI6383 lacked OX40-directed ADCFc effector function. These properties, coupled with the ability to expand memory T cells, makes MEDI6383 an attractive candidate to induce durable antitumor immunity in patients with cancer. MEDI6383 is currently undergoing testing in patients with advanced solid malignancies, both as monotherapy and in combination with the anti-PD-L1 blocking mAb durvalumab (NCT02221960).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Authors’ Contributions

Conception and design: M.D. Obert, K. Mullgrew, M. Damschroder, H. Feng, S. Eck, N. Buss, A.J. Pierce, L. Picker, N.P. Morris, A. Weinberg, S.A. Hammond
Development of methodology: C. Auge, S. Kentner, K. Mullgrew, J. Hair, Q. Du, H. Feng, S. Eck, A. Sylwester, N.P. Morris, S.A. Hammond
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.D. Obert, C. Auge, S. Kentner, K. Mullgrew, M. Damschroder, J. Hair, S. Hanabuchi, Q. Du, H. Feng, S. Eck, L. de Haan, H. Park, A. Sylwester, M.K. Axthelm, L. Picker
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.D. Obert, C. Auge, C. Morris, S. Kentner, K. Mullgrew, J. Hair, S. Hanabuchi, Q. Du, H. Feng, S. Eck, N. Buss, L. de Haan, A.J. Pierce, H. Park, A. Sylwester, S.A. Hammond
Writing, review, and/or revision of the manuscript: M.D. Obert, C. Auge, S. Kentner, K. Mullgrew, S. Hanabuchi, S. Eck, N. Buss
Study supervision: M.D. Obert, S. Eck, N. Buss, L. de Haan, H. Park, M.K. Axthelm, S.A. Hammond

Acknowledgments

The NHP research conducted at the Oregon National Primate Research Center, Oregon Health and Science University (OHRI) by H. Park, A. Sylwester, M. Axthelm, and L. Picker were supported in part by grants U42OD010426 and P51OD0119255 from the NIH. We thank Alfred Legasse (OHRI) for oversight and planning of the NHP protocol and Shannon Plummer (OHRI) for expert technical assistance related to the NHP study.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 6, 2017; revised August 11, 2017; accepted February 12, 2018; published first March 15, 2018.
References

1. Callahan MK, Postow MA, Wolchok JD. Targeting T cell co-receptors for cancer therapy. Immunity 2016;44:1069–78.
2. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity 2013;39:1–10.
3. Mahoney KJ, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. Nat Rev Drug Discov 2015;14:561–84.
4. Curti BD, Kovacsovics-Bankowski M, Morris N, Walker E, Chisholm L, Floyd K, et al. OX40 is a potent immune-stimulating target in late-stage cancer patients. Cancer Res 2013;73:7189–98.
5. Leidner R, Patel S, Fury M, Ferris R, McDevitt J, Lanasa M, et al. A phase I study to evaluate the safety, tolerability, PK, pharmacodynamics, and preliminary clinical activity of MED0562 in patients with recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN). J Clin Oncol 2015;33:TPS6083.
6. Infante J, Hansen A, Pishvaian M, Chow L, McArthur G, Bauer T, et al. MEDI1873, a fusion protein linked via a coiled-coil trimerization domain. Mol Immunol 2008;415:151–6.
7. Ruby CE, Yates MA, Hirschhorn-Cymerman D, Chlebeck P, Wolchok JD, et al. Anti-OX40 stimulation in vivo enhances CD8+ memory T cell survival and significantly increases recall responses. Eur J Immunol 2007;37:157–66.
8. Hirschhorn-Cymerman D, Budhu S, Kitano S, Liu C, Zhao F, Zhong H, et al. Induction of tumoricidal function in CD4+ T cells confers superior antitumor immunity compared with effector memory CD8+ T cells. J Immunol 2009;183:4853–7.
9. Stewart R, Hammond S, Oberst M, Robert W, Wilkinson R. The role of Fc gamma receptors in the activity of immunomodulatory antibodies for cancer. J Immunother Cancer 2014;2:29–38.
10. Takenaka T, Ito T, Nishiyama MC, Tsurumaki K, Tsuchiya H, et al. Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. J Immunol 2004;172:3580–9.
11. Wang YH, MacRae D, Hanabuchi S, Oba K, et al. OX40 ligand contributes to human lupus pathogenesis by promoting T follicular helper response. Immunity 2015;42:1159–70.
12. Weinberg AD, Thalhofer C, Morris N, Walker JM, Seiss D, Wong S, et al. Anti-OX40 (CD134) administration to nonhuman primates: immunologic correlates. J Clin Oncol 2010;28:3167–70.
13. Montirot R, Bell RB, Thalhofer C, Leidner R, Feng Z, Fox BA, et al. OX40, PD-1 and CTLA-4 are selectively expressed on tumor-infiltrating T cells in head and neck cancer. Clin Transl Immunology 2016;5:e70.
14. Hirschhorn-Cymerman D, Rizzato GA, Mergiouh T, Cohen AD, Avogadri F, Lesokhin AM, et al. OX40 engagement and chemotherapy combination provides potent antitumor activity in preclinical models of concomitantly resistant T cell apoptosis. J Exp Med 2009;206:1103–16.
15. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:415–25.
16. Nimmerjahn F, Ravetch JV. Translating basic mechanisms of IgG effector function to anti-cancer therapy. Immunity 2016;44:1069–78.
17. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:415–25.
18. Nimmerjahn F, Ravetch JV. Analyzing antibody-Fc-receptor interactions. Methods Mol Biol 2008;415:151–6.
19. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:415–25.
20. maize FcγRIIIA polymorphisms in the response to antitumor antibodies. Clin Cancer Res 2013;19:6785–95.
21. Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. J Immunol 2006;177:999–1000.
22. Baeyens A, Saadoun D, Billard F, Roues A, Gregoire S, Zaragoza B, et al. OX40 engagement blocks suppressor regulatory T cells and facilitates tumor rejection. J Exp Med 2005;205:825–39.
23. Valzasina B, Giuducci C, Dislich H, Kilren N, Weinberg AD, Colombo MP. OX40 blocking antibodies concomitantly target regulatory T cells and potentiate antitumor immunity. J Exp Med 2010;209:2113–20.
24. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:415–25.
Molecular Cancer Therapeutics

Potent Immune Modulation by MEDI6383, an Engineered Human OX40 Ligand IgG4P Fc Fusion Protein

Michael D. Oberst, Catherine Augé, Chad Morris, et al.

Mol Cancer Ther 2018;17:1024-1038. Published OnlineFirst March 15, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-17-0200

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2018/03/15/1535-7163.MCT-17-0200.DC1

Cited articles
This article cites 38 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/17/5/1024.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/17/5/1024.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/17/5/1024.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.