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*J Immunol* published online 6 September 2013
http://www.jimmunol.org/content/early/2013/09/06/jimmunol.1202752

Supplementary Material  
http://www.jimmunol.org/content/suppl/2013/09/06/jimmunol.1202752.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Antibodies Targeting Human OX40 Expand Effector T Cells and Block Inducible and Natural Regulatory T Cell Function

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Current cancer vaccines induce tumor-specific T cell responses without sustained tumor regression because immunosuppressive elements within the tumor induce exhaustion of effector T cells and infiltration of immune-suppressive regulatory T cells (Tregs). Therefore, much effort has been made to generate agonistic Abs targeting members of the TNFR superfamily, such as OX40, 4-1BB, and GITR, expressed on effector T cells and Tregs, to reinvigorate T cell effector function and block Treg-suppressive function. In this article, we describe the development of a panel of anti-human OX40 agonistic mouse mAbs that could promote effector CD4+ and CD8+ T cell proliferation, inhibit the induction of CD4+ IL-10-producing type 1 regulatory T cells, inhibit the expansion of ICOS+IL-10+ Tregs, inhibit TGF-β-induced FOXP3 expression on naive CD4+ T cells, and block natural Treg-suppressive function. We humanized two anti-human OX40 mAb clones, and they retained the potency of their parental clones. These Abs should provide broad opportunities for potential combination therapy to treat a wide realm of cancers and preventative vaccines against infectious diseases. The Journal of Immunology, 2013, 191: 000–000.

Numerous therapeutic cancer vaccines have been developed that induce tumor-specific T cell responses in patients (1–4); however, patient clinical response rates following vaccination have been low. This low response rate has been attributed largely to the presence of immunosuppressive elements at the tumor sites that induce exhaustion of tumor-infiltrating lymphocytes (TILs), influx of immune-suppressive CD4+ regulatory T cells (Tregs), and secretion of the anti-inflammatory cytokines TGF-β and IL-10 that induce the generation of regulatory dendritic cells and maintain CD4+ naturally occurring FOXP3+ Tregs (nTregs) or convert CD4+ T cells into inducible IL-10+/TGF-β+ Tregs (iTregs) (5–11). Indeed, recent reports showed that tumor-specific CD8+ T cells from melanoma patients were functionally impaired and expressed high levels of the inhibitory receptors PD-1, TIM-3, CTLA4, and LAG3 (5, 12). In addition to impaired CD8+ T cells, a large number of CD25+CD4+ Tregs were found in the tumors and draining lymph nodes of many cancer patients (13). The accumulation of Tregs at tumor sites has been attributed to the secretion of the chemokine CCL22 by cancer cells and macrophages that actively recruit Tregs expressing CCR4 (14, 15). Furthermore, TGF-β and IL-10 secreted by cancer cells can induce TGF-β–producing Tregs or IL-10–producing Tregs, which actively suppress effector T cell (Teff) function and expansion (10, 16), either directly or indirectly through the induction of regulatory dendritic cells (10). These two cytokines not only can induce iTregs, they also were shown to maintain the expression of the transcription factor FOXP3 and the suppressive function of Tregs (17, 18). As a consequence of these negative factors in the tumor environment, the majority of TILs are either functionally impaired CD4+/CD8+ T cells (5, 12) or have been converted into IL-10–producing (19, 20) or TGF-β–producing (21) Tregs that prevent antitumor immune responses.

Evidence from the literature suggests that these negative elements within the tumor microenvironment can be modulated by triggering members of the TNFR superfamily, such as OX40, 4-1BB, and GITR, which are highly expressed on Teffs and Tregs, to reinvigorate T cell effector function and block Treg-suppressive function (13, 22–27). Therefore, intense research over the last decade focused on generating reagents that trigger these molecules. We recently showed that triggering of human OX40 (hOX40) with OX40 ligand shuts down the generation and function of IL-10–producing type 1 Tregs (Tr1), whereas agonists of 4-1BB and GITR were ineffective (28). Recent studies further showed that triggering of OX40 turns off FOXP3+ Tregs and inhibits TGF-β– and Ag-driven conversion of naive CD4+ T cells into CD25+FOXP3+ T cells (29, 30). Studies from mice demonstrated that targeting OX40 using agonistic mAbs (31, 32) could promote Teff function and memory by promoting T cell survival and clonal expansion (33, 34), inhibit the function or survival of normal and tumor-derived FOXP3+ nTregs (23, 31), and induce changes in the tumor stroma, including a decrease in the number of macrophages and myeloid-derived suppressor cells (35). Abs against hOX40, generated using phage Abs (U.S. Pat. No. 7,550,140) or mice immunized with either hOX40 DNA (36) or OX40-transfected L929 cells (37), are capable of promoting Teff function. In particular, the Abs generated by Weinberg et al. (36)
have in vivo efficacy in prolonging T cell survival in nonhuman primates.

In this article, we describe the successful generation of a new panel of agonistic anti-hOX40 mAbs that can potently enhance CD4+ and CD8+ T cell function, inhibit induction of Tr1 cells and FOXP3+ Tregs, and completely block the suppressive function of nTregs. We also show that these Abs can block the function of Tregs derived from follicular lymphoma (FL) tumors. Further characterization involving mechanistic studies suggest that our anti–hOX40 mAbs block Treg suppression directly by inhibiting the function of Tregs and indirectly by making Tregs resistant to suppression by Tregs. In summary, our Abs can simultaneously promote human T eff function and block human T-reg suppressive function, both key targets for developing effective immunotherapy strategies to cure cancers.

## Materials and Methods

### Reagents and cell lines

The following reagents were purchased from the indicated manufacturers: TLR ligands PamCysK4 and flagellin (FLA-STA ultrapure; InvivoGen), IL-2 and IL-10 ELISA kits (R&D Systems), FCS and human serum (Gemiini), CFSE (Invitrogen), and PHA (Sigma-Aldrich). Mouse fibroblast L cells expressing hTGFα (hTGFα-L cells), CD32 (CD32-L cells), or CD32 plus the ICOS ligand or OX40 ligand were generated in our laboratory and maintained with RPMI 1640 culture medium containing 10% FCS, 1% glutamine, and 1% penicillin-streptomycin.

### Generation and screening of anti–hOX40 mAbs

Anti–hOX40 mAbs were generated using BALB/c female mice immunized with hTGFα-L cells at the M.D. Anderson Monoclonal Antibody Core Facility following established protocols. Hybridsomas secreting mAbs recognizing hOX40 were identified by ELISA and flow cytometry, and agonistic function was identified by screening clones for the ability to shut down IL-10+ Tr1 cell induction and block the suppressive function of nTregs. For functional assays, Abs were purified using fast protein liquid chromatography–protein A FF HITrap (GE Healthcare) and eluted with Gentle Ag/Ab elution buffer (Pierce). The generation of humanized anti–hOX40 mAbs was performed by JNJ Biosciences.

### Abs, FACS analysis, and cell sorting

The following Abs were used for flow cytometry analysis: IL-2–PE, CD4–allophycocyanin–Cy7, CD71–DE-PE, CD25–PE–Cy7, CD14–FITC, CD16–FITC, CD20–FITC, CD36–FITC, CD11c–FITC, and TCY6–FITC (BD Pharmingen). FoxP3–allophycocyanin was from BioLegend (clone 259D). Functional-grade Abs were anti–CD3 (OKT3; Centocor Ortho Biotech), rhesus monkey cross-reactive anti–CD3 (clone SP34; BD Biosciences), and anti–ICOS and anti–CD28 (eBioscience). Fl(αβγ)2 goat anti-human IgG, Fcγ fragment–specific secondary Ab and ChromPure goat IgG, Fl(αβγγ)2 fragment control Ab were from Jackson ImmunoResearch Laboratories. Human IgG1 isotype control was purchased from Sigma. Anti-human CD134 (clone ACT35) was purchased from BD Biosciences. Flow cytometry was performed on a flow cytometer (FACSCalibur; Becton Dickinson). FACS sorting was conducted on a cell sorter (FACSRia IU; Becton Dickinson). All flow cytometry analyses were gated on live T cells.

### Isolation of CD4+ T cell subsets from healthy donors and FL patient

Buffy coat samples prepared from the peripheral blood of healthy, adult donors were obtained from the Gulf Coast Regional Blood Center (Houston, TX) (IRB LAB03-0390 “Isolation of human dendritic cells, T cells and hematopoietic progenitor cells from human blood and tissue samples”). CD4+ CD45RA+ Teffs, and Tregs (purity 99% for all) were isolated from PBMCs using a CD4+ T cell enrichment mixture (STEMCELL Technologies), followed by gating out lineage-negative markers (CD14, CD16, CD20, CD56, CD11c, TCY6) and cell sorting the CD4+ CD127low CD25high (top 4–6%) fraction as nTregs, the CD4+ CD127low CD25low CD45RA+ CD45RO+ fraction as Tregs, and the CD4+ CD127low CD25low CD45RA+ CD45RO high fraction of naive T cells. To isolate T cells from FL patients, single-cell suspensions were prepared from FL patient tumor tissues obtained from the National Cancer Institute (IRB LAB04-0717).

### Generation of Tr1 cells from CD4+ T cells and IL-10 assays

A total of 2 × 10^5 freshly isolated CD4+ T cells was cultured with irradiated (60 Gy) ICOS ligand–expressing CD32-L cells (8 × 10^5), which were precoated with anti–CD3 (0.2 μg/ml) in the presence of dexamethasone (5 × 10^-6 M; Life Technologies) and 1α,25-dihydroxyvitamin D3 (1 × 10^-7 M; Life Technologies) in T cell culture medium containing 10% FCS, RPMI, Glutamax (Life Technologies), 1% penicillin-streptomycin, IL-2 (50 IU/ml), and soluble anti–CD28 (0.2 μg/ml), for 7 d in a 48-well tissue culture plate. Expanded T cells were restimulated with 50 ng/ml PMA and 2 μg/ml ionomycin (ION) for 6 h; 10 μg/ml brefeldin A (from Sigma) was added during the last 4 h. The cells were stained with Alexa Fluor 647–IL-10 Ab (clone JES5-9D7; eBioscience) using a Caltag FIX and PERM Kit. To evaluate the effects of OX40 signaling on IL-10+ Tr1 cell frequency, sorted CD4+CD127lowCD25highICOS+ Tregs were cultured for 14 d with anti–CD3 (0.2 μg/ml) and anti–CD28 (1 μg/ml) in the presence of IL-2 (300 IU IL-2/ml) and mouse fibroblast L cells expressing the OX40 and CD32 ligands or in the presence of CD32-L cells plus anti–hOX40 mAbs or control Ab for 5 d. Cells were then restimulated with plate-bound anti–CD3 (2 μg/ml) plus soluble anti–CD28 (1 μg/ml) for 24 h, and supernatants were assayed for IL-10 by ELISA or stimulated with PMA/ION and stained with IL-10 Ab, as described above.

### Proliferation assays

Two systems were used to evaluate T cell proliferation in the presence or absence of Tregs.

- **Plate-bound proliferation assay**
  - In the presence of accessory cells: anti–CD3 (3 μg/ml) and anti–hOX40 (2 μg/ml) in PBS were co-administered on a nontissue culture–treated 96-well flat-bottom plate for 1 h at 37°C in a CO2 incubator. To determine the proliferation of naive T cells, 10^5 naive T cells were added per well containing T cell culture medium containing 10% FCS (RPML Glutamax), 10% human AB serum, 1% penicillin-streptomycin.
  - On the third day, 1 μCi/well methyl-[3H]thymidine was added, and proliferation was assessed by thymidine incorporation 15 h later. To determine the proliferation of Teffs in the presence of Tregs, CFSE (4 μM)-labeled T effs and nTregs, each at 8 × 10^4, were added per well.
  - CD14+ monococyte–based proliferation system: CFSE-labeled Teffs (8 × 10^4) and Tregs, at a 1:1 or 2:1 ratio, were cultured in T cell medium in the presence of irradiated (60 Gy) monocytes at a 1:1 lympocyte/Teff ratio for 3.5 d in the presence of 0.3 μg/ml anti–CD3 Ab (1, 2). T eff proliferation in the presence of Tregs was determined after 3.5 d of stimulation by CFSE dilution assessed by flow cytometry.

### Direct stimulation of Teffs and nTregs with anti–hOX40 mAbs

To directly treat T effs, they were pre-stimulated with plate-bound anti–CD3 (2 μg/ml) in T cell culture medium plus IL-2 (300 IU/ml) for 12 h, to upregulate OX40 on all T effs, in a 24-well plate. Activated T effs were then pulsed with the anti–hOX40 Abs (20 μg/0.5 × 10^5 cells) in T cell culture medium for 4 h at 37°C in a CO2 incubator. Cells were then washed and cultured with CFSE–CFSE-labeled Teffs in the presence of monocytes and soluble anti–CD3 (0.3 μg/ml). To directly treat Teffs, they were stimulated with plate-bound anti–CD3 (0.8 μg/ml) in T cell culture medium containing 10% FCS for 12 h to upregulate OX40, pulsed with anti–hOX40 Abs (20 μg/0.5 × 10^5 cells) in T cell culture medium for 4 h, as above; washed three times; and labeled with CFSE, as described. Next, 8 × 10^4 CFSE–CFSE-labeled T effs were cultured with nTregs in the presence of monocytes and soluble anti–CD3.

### Induction of FOXP3 expression from naive T cells

CFSE-labeled freshly sorted naive CD4+ T cells (10^5) were stimulated with plate-bound anti–CD3 (3 μg/ml) and an anti–hOX40 mAb clone (2 μg/ml), for 119-122, 106-222, or 120-270 in the presence of soluble anti–CD28 (1 μg/ml) and increasing concentrations of TGF-β in serum-free AIM V lymphocyte culture medium (Life Technologies). Three days after stimulation, expanded cells were stained with FOXP3 Ab using a FOXP3 stain buffer system (eBioscience).

### Generation of activated human PBMCs and rhesus T cells for OX40 mAb-binding assays

Human PBMCs were stimulated with soluble PHA (10 μg/ml) for 2 d in 10% FCS/RPMI 1640. Staining of CD3OX40+ T cells was performed using primary anti–hOX40 mAbs followed by a secondary Ab against PE-conjugated mouse Ig. CD3+ T cells were detected using anti–CD3-Pacific.
Blue–conjugated Ab. Ag-binding competition assays were performed by preincubating 0.5 μg/ml allophycocyanin-conjugated 106-222 Ab (Ab labeling kit; A20186; Life Technologies) with increasing concentrations of recombinant hOX40 protein (TP311253; OriGene) in FACS buffer (1% FCS/2 mM EDTA) for 20 min. The Ab–Ag complex formed was added to 5 × 10⁶ unstimulated or activated human T cells and incubated for another 20 min. The binding was then analyzed on the CD4⁺ cell subpopulation. The indicated mean fluorescence was plotted for 0, 0.078, 0.625, 2.5, and 10 μg/ml the recombinant protein. Rhesus CD4⁺ T cells were isolated from rhesus PBMCs using anti-CD4 MicroBeads (clone M-T466; Miltenyi Biotec). Enriched CD4⁺ T cells were stimulated with 8 μg/ml plate-bound anti-CD3 (clone SP34) for 72 h in the presence of IL-2 (50 IU/ml). Staining of OX40-expressing T cells was performed with allophycocyanin-conjugated 106-222 and compared with isotype control mouse IgG1-allophycocyanin (BD Biosciences).

**Statistical analysis**

Statistical differences between experimental groups were determined by either paired or unpaired t tests or two-way ANOVA using Prism software (GraphPad).

**Results**

**Generation and identification of potent agonistic anti-hOX40 mAbs**

Although OX40 signaling can break immune tolerance, the commercially available anti-hOX40 mAb ACT35 is ineffective in blocking the function of nTregs. Therefore, we decided to immunize mice with mouse fibroblast L cells stably expressing recombinant...
hOX40 on the cell surface. We performed three fusions and screened the generated hybridoma clones by ELISA for binding to plate-bound hOX40-L cells. We found that >500 hybridoma clones bound to hOX40-L cells but not the control L cells (data not shown). We performed flow cytometry analysis to demonstrate that these clones bound specifically to hOX40 expressed on the cell surface. Supernatants from hybridomas were screened by staining a mixture of hOX40-L cells and parental L cells at a 1:1 ratio. We found that only 20 of 500 of the anti-hOX40 mAbs stainedOX40-expressing L cells but not the parental L cells (Fig. 1A), indicating that they bind specifically to surface hOX40.

OX40 signaling was shown to block the induction of Tr1 cells, TGF-β–mediated conversion of naïve CD4+ T cells to FOXP3+ Tregs, and suppressive function of nTregs, but it augmented the proliferation of CD4+ and CD8+ T cells (28, 29, 34, 38). To evaluate the agonistic activity of the 20 anti-hOX40 Abs, we purified the Abs by protein A/G–mediated affinity chromatography and then tested them for their ability to inhibit the induction of Tr1 cells. We found that 9 of the 20 hOX40-specific Abs could block vitamin D3/dexamethasone-mediated generation of Tr1 cells from total CD4+ T cells (Fig. 1B). We next titrated the Abs to determine their potency. As shown in Fig. 1C, five of the nine Abs potently suppressed the generation of Tr1 cells at a concentration as low as 4 ng/ml. Fig. 1D further shows that, although the induction of Tr1 cells was inhibited by anti-hOX40 mAbs, the percentages of TNF-α+ and IFN-γ+ cells were increased. To evaluate the ability of our anti-hOX40 mAbs to also inhibit the expansion of IL-10+FOXP3+ Tregs, we stimulated freshly sorted CD4+CD127lowCD25highICOS+ Tregs with anti-CD3 Ab-coated human CD32-L cells in the presence of anti-CD28 and an anti-hOX40 mAb or control IgG1 isotype for 5 d. Then cells were either restimulated with anti-CD3/CD28 Abs for IL-10 cytokine secretion or PMA/Ionomycin for staining of IL-10+IL-2+ T cells. Fig. 1E shows that all nine anti-hOX40 mAbs tested blocked the secretion of IL-10 from expanded IL-10+FOXP3+ Tregs. This reduction in IL-10 release was reflected by the 80% decrease in the number of IL-10+IL-2+ Tregs (Fig. 1EII). To further evaluate the ability of our anti-hOX40 mAbs to inhibit TGF-β–mediated conversion of naïve CD4+ T cells to FOXP3+ Tregs, we stimulated naïve CD4+ T cells with plate-bound anti-CD3 and an anti-hOX40 mAb with increasing concentrations of TGF-β in serum-free medium. Fig. 1F shows that an anti-hOX40 mAb (three represented in figure) potently inhibited the induction of FOXP3+ T cells. Together, these results suggest that our anti-hOX40 mAbs inhibit the induction and expansion of IL-10+ Tregs and FOXP3+ Tregs.

We further determined which of the 20 originally selected Abs could augment TCR-triggered naïve CD4+ or CD8+ T cell pro-

FIGURE 2. Anti-hOX40 Abs enhance CD4+ and CD8+ T cell proliferation. (A) A total of 1 × 10^5 CFSE-labeled freshly sorted CD4+CD25−CD45RO−CD45RA+ naïve T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and the indicated anti-hOX40 mAb (2 μg/ml). T cell proliferation was evaluated 4 d after stimulation. Mouse IgG1 and 119-42 served as negative controls. Results from the commercially available anti-hOX40 mAb ACT35 are shown to the far right. Fifteen anti-hOX40 mAbs (under the bracket; 119-122 to 119-220C) significantly enhanced T cell proliferation compared with control mouse IgG1 (*p < 0.05). Error bars represent mean ± SD. The p values were calculated by paired t tests. (B) CD3+CD45RA+CD27+CD8+ T cells were stimulated as in (A); [3H]thymidine was added on the third day of culture, and cells were harvested after another 15 h of incubation (left panel). Proliferation of T cells was evaluated by thymidine incorporation. CFSE-labeled CD8+ T cells were stimulated with plate-bound anti-CD3 (1.5 μg/ml) and an anti-hOX40 mAb (0.5 μg/ml) (right panel). T cell proliferation was evaluated 3.5 d after stimulation. Mouse IgG1 and anti-CD28 served as negative and positive controls, respectively. Data are representative of two donors. Error bars represent means ± SD. The p values were calculated by unpaired t tests.
liferation. Plate-bound anti-hOX40 and anti-CD3 mAbs were used to stimulate CFSE-labeled T cells. We found that 11 of the anti-hOX40 mAbs potently enhanced (5–9-fold) the proliferation of naive CD4+ T cells, whereas the only commercially available anti-hOX40 mAb, ACT35, had no such effect (Fig. 2A). In addition to this stimulation of CD4+ T cells, one of the clones tested, 119-122, also stimulated CD8+ T cell proliferation (Fig. 2B). We next investigated whether these anti-hOX40 mAbs could inhibit nTreg-suppressive function. We performed similar proliferation assays at a 1:0 or 1:2 ratio of CFSE-labeled CD4+CD25+CD45RA+CD45RO+Teffs to CD4+CD127lowCD25high nTregs (nTregs). We found that the 11 Abs that were strong activators of naive CD4+ T cell proliferation were also strong activators of memory Teff proliferation (Fig. 3A) and could also completely block the suppression of nTregs (Fig. 3B). We note that the commercial anti-hOX40 mAb ACT35 is a relatively weak activator of Teff proliferation and a weak blocker of nTreg-suppressive function. Together, these results indicate that several of our anti-hOX40 mAbs are potent agonists that can potently inhibit the generation and suppressive function of iTregs and nTregs.

Anti-hOX40 mAbs block normal donor- and cancer patient–derived Treg function in the presence of accessory cells

Because Abs are likely to interact with accessory cells in vivo to block Treg function, we evaluated the ability of our anti-hOX40 mAbs to block Treg suppression in the presence of CD14+ monocytes, CFSE-labeled Teffs, nTregs, and soluble anti-CD3 Abs. We found that 20 μg/ml of clone 106-222, 119-122, 119-43, or 120-270 blocked the suppressive function of nTregs (Fig. 3C). Surprisingly, clones 119-173B and 119-8B, which blocked nTreg function in the plate-bound proliferation system, could not block nTreg function in this system. We next wondered whether the anti-hOX40 mAbs could also block the function of allogeneic CD4+CD127lowCD25high Tregs isolated from a FL tumor sample in the presence of monocytes.

**FIGURE 3.** Anti-hOX40 mAbs block the activity of CD4+CD25high nTregs. (A and B) Blocking of nTreg function in the absence of accessory cells. Syngeneic CFSE-labeled CD4+CD25highCD127+CD45RA-CD45RO+Teffs and CD4+CD25lowCD4+ Tregs were cultured at a 1:2 ratio with soluble anti-CD28 (0.4 μg/ml), plate-bound anti-CD3 (3 μg/ml), and the indicated anti-hOX40 mAb or isotype control (2 μg/ml). Representative FACS data showing the proliferation of Teffs in the presence of naive CD4+ T cells, nTregs, or nTregs plus the anti-hOX40 mAb 119-33A (left panels). Percentage of Teff proliferation from six to nine donors in the absence (A) or presence (B) of nTregs after treatment with the indicated anti-hOX40 mAb. Error bars represent means ± SD. Anti-hOX40 mAbs under the bracket significantly enhanced T cell proliferation and blocked nTreg-suppressive function. (C) Anti-hOX40 mAbs inhibit normal donor–derived nTreg function in the presence of accessory cells. Freshly sorted healthy donor–derived nTregs (3.5–5 × 10⁵) were cultured for 3–4 d with CFSE-Teff (7 × 10⁵), irradiated CD14+ monocytes (7 × 10⁶; 60 Gy), anti-CD3 (0.3 μg/ml), and increasing concentrations of anti-hOX40 mAb. (D) Inhibition of FL-derived Treg function. CD4+CD127lowCD25highTregs were sorted from FL-infiltrating lymphocytes. CFSE-labeled CD4+CD127−CD25+Teffs and monocytes were derived from a healthy donor. In (A–C), representative FACS analyses are shown in left panels, unlabeled CD4+ T cells replace nTregs as control, and cell proliferation was assessed by flow cytometry for CFSE dilution. Percentages of Teff proliferation after treatment with the indicated Abs are shown in the right panels. All experiments were performed in duplicate. Data are representative from two or three donors. *p < 0.05, compared with control mouse IgG1, paired t test.
We found that 119-43 and 119-122 Abs completely restored the proliferation of Teffs at the three Treg:Teff ratios tested, whereas the control Abs IgG1 and 106-118 (does not bind to hOX40) did not (Fig. 3D). These results demonstrate that some of the anti-hOX40 mAbs are capable of blocking Tregs isolated from healthy donors, as well as cancer patients.

Anti-hOX40 mAbs act directly on T cells to block Treg suppression

Evidence from the literature suggests that a strong OX40-triggering signal can block Treg suppression either directly or indirectly (23, 39). To test these possibilities, we first determined the sensitivity of a T cell subset to strong OX40 triggering. Each T cell subset, including naive, memory, and nTregs, was stimulated with soluble anti-CD3 Abs in the presence of CD14+ monocytes and increasing concentrations of the anti-hOX40 mAb 106-222, which blocks nTreg-suppression function. We found that the anti-hOX40 mAb stimulated the proliferation of naive T cells and nTregs in a dose-dependent manner (data not shown). However, when the Ab concentration reached 20 μg/ml, the percentage of viable nTregs evaluated by gating out live cells on a forward light scatter and side scatter plot decreased significantly (Fig. 4A, 4B). With an Ab concentration of 30 μg/ml, the viability of nTregs was reduced from 60% to <20%, whereas the viability of memory T cells was only reduced 10–15% and was unaffected in naive T cells. To further monitor the precise apoptotic effect of OX40 triggering, we stained nTregs with annexin V and propidium iodide (PI). Consistently, we found that, at high doses of anti-hOX40 mAb, a significant number of nTregs stained positive for annexin V or annexin V and PI (Fig. 4C). These results suggest that a stronger OX40 triggering can preferentially induce apoptosis on nTregs, leading to the loss of suppressive function. We next asked whether a brief pulse of preactivated nTregs with anti-hOX40 mAb was sufficient to block suppression activity. We pulsed preactivated nTregs with anti-hOX40 mAbs for 4 h and then cocultured them with CFSE-labeled Teffs in the presence of monocytes. We found that the brief pulse was sufficient to completely block nTreg suppression, whereas control Abs had no such effect (Fig. 4D). Because Teffs are resistant to killing by OX40 triggering, we next determined whether OX40 triggering could instead render Teffs

**FIGURE 4.** High concentrations of anti-hOX40 mAb kill nTregs. (A and B) Naive T cell subsets (CD4+CD25lowCD127+CD45RO+CD45RA+), Teffs (CD4+CD25highCD127+CD45RA+), or nTregs (CD4+CD25highCD127low) were cultured with CD14+ monocytes (1:1) plus soluble anti-CD3 (0.3 μg/ml) and increasing concentrations of the 106-222 anti-hOX40 mAb. Cell viability was determined after 3 d of culture by flow cytometry by gating out live cells on a forward light scatter and side scatter plot (two donors). (C) nTregs were stimulated as in (A), except that, after 3 d of culture, the nTregs were stained with annexin V and PI. (D) Anti-hOX40-treated Tregs are unable to suppress Teff proliferation. Cell viability was determined after 3 d of culture by flow cytometry by gating out live cells on a forward light scatter and side scatter plot (two donors). (E) Anti-hOX40 mAb confers Teff resistance to suppression by nTregs.
resistant to suppression by nTregs. We found that preactivated Teffs became resistant to suppression by nTregs when they were subjected to a brief pulse with the anti-hOX40 mAb clone 119-122, whereas a control Ab had no such effect (Fig. 4E). Together, these results suggest that several of our anti-hOX40 mAbs act on both Teffs and nTregs to block nTreg function.

Anti-hOX40 mAbs bind specifically to human and rhesus OX40

For the purpose of future preclinical testing of the efficacy and toxicity of our anti-hOX40 mAbs, we asked whether anti-hOX40 mAbs could bind specifically to activated CD3+ T cells in human PBMCs and rhesus OX40 expressed on rhesus-activated CD4+ cells. We obtained fresh human PBMCs from healthy donors and performed Ab-binding assays. We found that two of our best Abs (119-122 and 106-222) specifically bound to 10–25% of activated CD3+ T cells but not CD32 cells, whereas the same Abs bound to only 2–5% of unstimulated CD3+ T cells (Fig. 5A). In contrast, no such binding was observed with control Abs, mouse IgG1 and 319. Surprisingly, we found that the commercially available anti-hOX40 mAb ACT35 bound to both CD3+ and CD32 T cells in unstimulated PBMCs (Fig. 5A), suggesting that the Ab is less specific for OX40 binding. To further assess the specificity of OX40 mAbs, we performed a competition assay in which a fixed amount of fluorochrome-conjugated 106-222 OX40 Ab was preincubated with increasing concentrations of recombinant hOX40 protein. The Ab–Ag complex formed was added to unstimulated or activated human T cells, and the binding was analyzed on the CD4+ cell subpopulation. We found that the binding of 106-222 to activated CD4+ T cells could be blocked by recombinant hOX40 protein in a dose-dependent manner, whereas control protein had no such effect (Fig. 5B, 5C). Mean fluorescence was plotted for only four of the concentrations used of the recombinant protein (0.078, 0.625, 2.5, and 10 μg/ml), showing that, even if the percentage of CD4+ proliferating cells at lower concentrations is similar (>40%), the mean fluorescence was reduced (Fig. 5C). Taken together, these results demonstrate that our anti-hOX40 mAbs could bind specifically to rhesus OX40, we performed similar binding assays using fresh and anti-CD3 polyclonally stimulated CD4+ T cells. We found that the allophycocyanin-conjugated anti-hOX40 mAb 106-222 bound only to activated CD4+ T cells (Fig. 5D), whereas no such binding was observed using mouse IgG1 isotype control, suggesting that our OX40 mAb also binds specifically to rhesus OX40. We next extended our study to all of the anti-hOX40 mAbs. We purified CD4+ T cells from rhesus PBMCs and stimulated them with PHA for 2 d. Then, anti-hOX40 mAbs were tested for binding to the activated CD4+ T cells. We found that 10 Abs (107, 108, 148, 8B, 58, 69A, 122, 140A, 222, and 270) could bind to rhesus CD69+CD4+ T cells (Fig. 5E), whereas control Ab IgG1 did not bind, suggesting that these Abs recognize rhesus OX40 on activated T cells.

FIGURE 5. Anti-hOX40 mAbs bind specifically to human and rhesus OX40. (A) Anti-hOX40 mAbs bind specifically to activated human CD3+ cells. Fresh and PHA-activated PBMCs were tested with two agonistic anti-hOX40 mAbs (1 μg/106 cells) and negative controls (mouse IgG1 and mAb 106-319, which do not bind to hOX40). Bound cells were determined by flow cytometry. Data are representative from three donors. (B and C) Ag-binding competition assay. Allophycocyanin-conjugated 106-222 Ab (0.5 μg/ml) was preincubated with increasing concentrations of recombinant hOX40 protein. The Ab–Ag complex formed was added to unstimulated or activated human T cells, and the binding was analyzed on the CD4+ cell subpopulation. Bound cells were expressed as the percentage of CD4+OX40+ cells (B) or mean fluorescence intensity (MFI) (C). (D) 106-222 mAb binds specifically to activated rhesus CD4+ T cells. CD4+ T cells were activated using plate-bound anti-CD3, and binding was detected using increasing concentrations of allophycocyanin–106-222 or allophycocyanin-IgG1. Bound cells were determined by flow cytometry and shown as the percentage of CD4+OX40+ cells. (E) Ten anti-hOX40 mAbs bind to activated rhesus CD4+ cells. CD4+ T cells were stimulated with 10 μg/ml PHA. After 2 d, cells were stained with the indicated anti-hOX40 mAb, followed by goat anti-mouse Ig-allophycocyanin and CD69-PE. Mouse IgG1 and 106-317 served as negative controls. Bound cells were determined by flow cytometry. Data are representative from two donors.
In view of a future clinical trial, we selected the anti-hOX40 mAbs 119-122 and 106-222 for humanization based on their ability to inhibit the induction of Tr1 cells and FOXP3+ Tregs, enhance the proliferation of naive CD4+ T cells, block the suppressive function of nTregs in the absence or presence of accessory cells, and bind to rhesus OX40. The humanized anti-hOX40 mAbs Hu106-222 and Hu119-122 bound to OX40 with similar binding affinity (Supplemental Fig. 1). We next tested the ability of the humanized Abs to enhance naive CD4+ T cell proliferation and block nTreg function. As shown in Fig. 6A, plate-bound Hu106-222 and Hu119-122 mAbs have similar potency in enhancing CD4+ T cell proliferation. They both stimulated naive CD4+ T cell proliferation to a level similar to that induced in the presence of anti-CD28 Ab (Fig. 6A). However, when the humanized or mouse anti-hOX40 mAbs were tested as soluble Abs, they failed to stimulate T cell proliferation (Fig. 6A), suggesting that cross-linking is required for Ab activity. To demonstrate that cross-linking was required for Ab activity, naive CD4+ T cells were stimulated with plate-bound anti-CD3 Abs plus soluble mouse anti-hOX40 mAb 119-122 in the presence or absence of a secondary Ab against Fcey. We found that the addition of a secondary Ab against Fcγ to the cell culture restored the Ab’s ability to enhance T cell proliferation, whereas control Abs were ineffective (Fig. 6B), thus demonstrating that cross-linking is required for anti-hOX40 mAb function. We further determined whether Hu119-122 and Hu106-222 could block nTreg function. Fig. 6C shows that both plate-bound Abs potently stimulated Teff proliferation and blocked nTreg-suppressive function. Interestingly, in the presence of Hu106-222 and nTregs, the percentage of Teff proliferation exceeded that in the absence of nTregs, suggesting that, in these conditions, OX40 signaling blocks nTreg function by the combined action of reversing nTreg function and inducing Teff resistance to suppression. These results suggest that the Hu106-222 and Hu119-122 mAbs retained the functional activities of their parental mAbs.

**Discussion**

A key question in the development of effective immunotherapy against cancer is how to reinvigorate Teff function and eliminate or block the suppressive function of Tregs. Recent studies focused on targeting OX40 because it is highly expressed on the surface of Tregs and activated CD4+ and CD8+ T cells, and OX40 activation on effectors CD4+ and CD8+ T cells was shown to promote their survival and expansion (33). Importantly, triggering of mouse OX40 on Tregs using agonistic anti-mouse OX40 Abs was shown to block Treg-suppressive function and induce Treg-specific apoptosis and tumor rejection in mice previously subjected to chemotherapy (23, 24, 27, 30, 33). Therefore, OX40 represents an attractive target molecule to develop immunotherapy for a wide realm of cancers. In this study, we performed an exhaustive immunization strategy using hOX40-L cells and innovative multitask screening procedures to identify a set of robust agonistic anti-hOX40 mAbs that enhance naive CD4+ and CD8+ T cell proliferation, inhibit expansion of IL-10+FOXP3+ nTregs, shut down the induction of Tr1 cells and FOXP3+ Tregs from CD4+ T cells, and act directly on Tregs to inhibit their function. We found that 11 anti-hOX40 mAbs (132, 222, 8B, 33A, 43, 58, 122, 173B, 157A, 140A, 270) enhanced the levels of naive CD4+ T cell proliferation, each >5–10-fold, compared with the control Ab. Although less robust, the anti-hOX40 mAb 119-122 also enhanced CD3+CD8+ T cell proliferation. This effect might explain why some of our anti-hOX40 mAbs could completely inhibit nTreg suppression of Teff proliferation. Importantly, we found that activated nTregs were highly susceptible to anti-hOX40 mAb– or OX40 ligand (OX40L)-triggered cell death, whereas other T cell subtypes, such as naive and memory CD4+ T cells, were less susceptible. These results are in agreement with two previous reports showing that stimulation with OX40L or OX40L plus TNF induced apoptosis of an OX40-expressing CD4+ T cell line and that pretreatment of mice with the alkylation agent cyclophosphamide, which upregulates the expression of OX40 on CD4+ FOXP3+ Tregs, combined with OX40 triggering could induce apoptosis in vivo.

**FIGURE 6.** Anti-hOX40 mAbs require cross-linking to enhance T cell proliferation and block Treg-suppressive function. (A) Only plate-bound anti-hOX40 mAbs enhance CD4+ T cell proliferation. Freshly sorted naive CD4+ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) plus the indicated humanized or mouse anti-hOX40 mAb (2 μg/ml), plate-bound or soluble, in the absence of accessory cells. After 3 d, [3H]thymidine was added to the culture, and cell proliferation was assessed after 15 h by thymidine incorporation. Human and mouse IgG1 and anti-CD28 served as negative and positive controls, respectively. (B) Cross-linked soluble anti-hOX40 mAbs augment CD4+ T cell proliferation. Naive CD4+ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml). Soluble anti-hOX40 mAb clone 119-122 (2 μg/ml) was added alone or in combination with an equal amount of a secondary Ab against Fcγ. Mouse IgG1 and ChromPure goat IgG, F(ab')2 fragment, served as negative controls. Cell proliferation was evaluated as described in (A). Each treatment in (A) and (B) was performed in triplicate, and data are representative from two donors. (C) Plate-bound humanized anti-hOX40 mAbs Hu106-222 and Hu119-122 block nTreg-suppressive function. Teffs and nTregs cells were prepared and cultured as described in Fig. 3A and 3B. The percentage of Teff proliferation taken from seven donors in the absence (●) or presence (○) of nTregs after treatment with Hu106-222 or Hu119-122. Human IgG1 served as a negative control. Error bars represent mean ± SD. Statistical significance between treatment groups (p < 0.05 compared with control Hu IgG1, paired t test) is indicated by a top bracket.
that the majority of our Abs could bind specifically to CD3+ T cells showed that 10 of the anti-hOX40 mAbs could bind to rhesus-from human PBMCs, demonstrating that there is no off-target reagents are a suitable option for developing robust immunotherapy cancer patient–derived Treg-suppressive function. Thus, these novel and survival of Teffs, as well as blocking normal donor– and CD4+ T cell proliferation and blocking nTreg-suppressive function. We found that strong OX40 signaling preferentially kills Tregs is novel and significant. It opens up the possibility of preferentially expanding Tefs at tumor sites to induce potent immune responses against cancer cells. These results suggest a new biological role for OX40 signaling in controlling the fate of Tregs in the tumor microenvironment. These results demonstrate that we identified a set of anti-hOX40 mAbs with potent activity to stimulate T cell proliferation and block the induction or suppressive function of Tregs.

With the purpose of generating reagents suitable for preclinical testing, we further examined the ability of our Abs in terms of their cell-binding specificity, their ability to block normal donor– and cancer patient–derived Treg-suppressive function in the presence of accessory cells, and their binding to rhesus CD4+ T cells. We found that the majority of our Abs could bind specifically to CD3+ T cells from human PBMCs, demonstrating that there is no off-target binding molecules for our Ab in the human system. We further showed that 10 of the anti-hOX40 mAbs could bind to rhesus-activated CD4+ T cells, suggesting that they will most likely bind to rhesus OX40 and activate OX40 signaling, allowing us to test their toxicity in monkeys. Although our Abs require cross-linking for activity, we found that they are able to restore T cell proliferation in the presence of Tregs and monocytes, suggesting that other accessory cells in the peripheral blood might be responsible for the cross-linking and triggering of OX40 signaling. Based on these results, two mouse anti-hOX40 mAbs, 119-122 and 106-222, were chosen for humanization. We found that, in comparison with their parental mouse Abs, the humanized anti-hOX40 mAbs Hu119-122 and Hu106-222 retained the same potency in enhancing naive CD4+ T cell proliferation and blocking nTreg-suppressive function. Collectively, these results indicate that we generated potent anti-hOX40 mAbs that are suitable for promoting clonal expansion and survival of Tefs, as well as blocking normal donor– and cancer patient–derived Treg-suppressive function. Thus, these novel reagents are a suitable option for developing robust immunotherapy strategies to cure human cancers.

Acknowledgments
We thank Drs. Iagamandha K. Sastry and Sattva S. Neelapu for the gifts of rehues PBMCs and IL-TILs, Karen Ramirez and Zhiiwei He for cell sorting and support, Janis Johnson (M.D. Anderson Monoclonal Antibody Core Facility) for Ab purification, and Melissa Wentz for careful reading of the manuscript.

Disclosures
K.V., L.B., and Y.J.L developed the mAb described in this article and are named inventors on a patent application from which they received royalty payments, exclusively licensed to GlaxoSmithKline. The other authors have no financial conflicts of interest.

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