Characterization of MK-4166, a Clinical Agonistic Antibody That Targets Human GITR and Inhibits the Generation and Suppressive Effects of T Regulatory Cells

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Abstract

GITR is a T-cell costimulatory receptor that enhances cellular and humoral immunity. The agonist anti-mouse GITR antibody DTA-1 has demonstrated efficacy in murine models of cancer primarily by attenuation of Treg-mediated immune suppression, but the translatability to human GITR biology has not been fully explored. Here, we report the potential utility of MK-4166, a humanized GITR mAb selected to bind to an epitope analogous to the DTA-1 epitope, which enhances the proliferation of both naive and tumor-infiltrating T lymphocytes (TIL). We also investigated the role of GITR agonism in human antitumor immune responses and report here the preclinical characterization and toxicity assessment of MK-4166, which is currently being evaluated in a phase I clinical study. Expression of human GITR was comparable with that of mouse GITR in tumor-infiltrating Tregs despite being drastically lower in other human TILs and in many human peripheral blood populations. MK-4166 decreased induction and suppressive effects of Treg, in vitro. In human TIL cultures, MK-4166 induced phosphorylation of NFkB and increased expression of dual specificity phosphatase 6 (DUSP6), indicating that MK-4166 activated downstream NFkB and Erk signaling pathways. Furthermore, MK-4166 downregulated FOXP3 mRNA in human tumor infiltrating Tregs, suggesting that, in addition to enhancing the activation of TILs, MK-4166 may attenuate the Treg-mediated suppressive tumor microenvironment. Cancer Res; 77(16); 1–11. ©2017 AACR.

Introduction

The significant clinical benefit obtained from the use of immune checkpoint inhibitors in the treatment of cancer and the durability of such responses has generated tremendous interest in immunotherapy of cancers (1–4). Therapeutic antibodies designed to block the inhibitory feedback mechanisms of CTLA-4 or PD-1 have been approved for the treatment of cancer. In addition, several agonist antibodies that target costimulatory molecules on T cells like GITR, CD27, 4-1BB, CD40, and OX40 are in various stages of pre-clinical or clinical development (5, 6).

Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), also referred to as TNFRSF18, is a type I transmembrane protein of the tumor necrosis factor receptor superfamily that is expressed on human T lymphocytes (preferentially on Tregs; refs. 7–10) and natural killer (NK) cells (11–13). Expression of GITR is significantly increased upon T-cell activation, and ligation of GITR provides a costimulatory signal that positively modulates antigen-specific T-cell responses, leading to enhanced cellular and humoral immunity (11, 12, 14). The counter structure, GITR-Ligand (GITR-L), is expressed on antigen-presenting cells (dendritic cells, B cells, and macrophages; refs. 15–18).

Engagement of murine GITR by either an agonistic anti-mouse GITR antibody (clone DTA-1) or GITR-L, in the presence of a primary TCR signal, results in enhanced T-cell proliferation and cytokine production (11, 12). In mice, DTA-1 abrogates Treg-mediated suppression either by eliminating GITR-expressing tumor-infiltrating Tregs (19, 20) or by causing them to become unstable thereby attenuating their suppressive activity (21, 22). Agonist anti-GITR antibodies can prevent tumor growth or cure established tumors in murine tumor models (21, 23–28), but the question of whether or not human GITR biology parallels that of the mouse remains largely unanswered (29). Several human GITR

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agonists are currently in early clinical development for the treatment of solid tumors (NCT01239134, NCT01216436, NCT02583165, NCT02628574, NCT02697591, NCT02132754, NCT02437916).

On the basis of the potent antitumor efficacy of DTA-1 in murine models (21, 23–26), we developed the humanized IgG1 agonist anti-GITR mAb MK-4166, focusing our selection on binding to an epitope on human GITR analogous to the epitope that DTA-1 binds on mouse GITR. We show that engagement of GITR by MK-4166 is a high-affinity interaction that enhances TCR-driven in vitro proliferation of human and cynomolgus monkey naïve CD4+ T cells similar to the effect of DTA-1 on mouse T cells. In addition, MK-4166 enhanced the proliferation of human tumor-infiltrating T cells. To address the translatability of murine efficacy data to human cancers, we evaluated GITR expression in mouse and human. The expression of GITR on tumor-infiltrating Tcells was comparable between mouse and human despite differences in GITR expression on many peripheral blood populations and tumor-infiltrating CD4+ and CD8+ T cells. Because Tc cells have been shown to play a critical role in antitumor efficacy of DTA-1, we focused on characterization of MK-4166 activity on human Tc cells. MK-4166 significantly inhibited the generation of Tc cells in mixed lymphocyte reactions (MLR) and decreased the suppressive effects of Tc cells. Moreover, MK-4166 induced phosphorylation of NFkB, upregulated ERK pathway genes, and decreased expression of FOXP3 in tumor infiltrating T cells in ex vivo human tumor cultures. MK-4166 did not induce any adverse events when administered intravenously to cynomolgus macaques at a broad range of doses. The safety and tolerability of MK-4166 in patients with advanced solid tumors is currently being evaluated in a phase I study (NCT02132754).

Materials and Methods

Antibodies

Humanized IgG1, anti-human GITR (MK-4166) and humanized IgG4, anti-human GITR (MK-1248, which has the same CDR regions but significantly reduced Fc effector function) were developed at Merck Research Laboratories, and for certain uses labeled with Dylight650 (Thermo Fisher). Generation of MK-4166 is described in the Supplementary Material. A murinized IgG2a version of rat anti-mouse GITR antibody DTA-1 was generated on the basis of published sequences (30). Antibodies used for flow cytometry are listed in the Supplementary Material.

Mice and cell lines

C57BL/6, BALB/cAnN, DBA/2, or C3H/HeN mice (4 weeks old) were purchased from The Jackson Laboratory or Taconic and maintained in specific pathogen-free facilities according to approved IACUC protocols. The cell lines MC38 (NCI, 2015), MB49 (University of Iowa, 2015), B16F10 (ATCC, 2015), LI/2 (ATCC, 2015), TC-1 (Johns Hopkins University, 2015), 4T1 (ATCC, 2015), CT26 (ATCC, 2015), RECA (ATCC, 2011), EMT6 (ATCC, 2015), CM3 (ATCC, 2016) were all authenticated by IDEXX CellCheck authentication service. The source of each cell line and year of authentication, including IDEXX CellCheck authentication service. The source of each cell line and year of authentication, including Mycoplasma testing, are indicated in parentheses. Tumor cell lines were implanted at passages 4–6 after thawing.

Murine and human tissues

Approximately 200 μL of blood from healthy female C57BL/6 mice or C57BL/6 mice bearing MC38 syngeneic tumors of approximately 100 mm3 was collected by cardiac puncture. Blood from cynomolgus monkeys (Bioreclamation IVT) and patients with cancer (MT Group) was collected into K3-EDTA tubes. Blood from healthy human subjects was collected as part of the voluntary blood donor program at Merck Research Laboratories. Human buffy coats from healthy volunteers were obtained from the Stanford Blood Center. All blood samples were collected from voluntary donors in accordance with Institutional Review Board (IRB) protocols after obtaining an informed consent. The IRBs for MT Group, Merck volunteer blood donor program, and Stanford Blood Center are Sterling IRB, Merck Research Laboratories Palo Alto IRB, and Panel on Medical Human Subjects, respectively. All human blood and tumor samples were collected in accordance with the Declaration of Helsinki ethical guidelines.

Mouse tumor cell lines were injected subcutaneously into the hind flanks of syngeneic female mice and tumors were harvested when they reached a volume of 100 mm3. Single-cell suspensions were obtained by fine mincing with a scalpel, followed by a 60-minute incubation at 37°C in 2.5 mL of digestion media containing DMEM, 0.208 WU/mL liberase TM (Roche), and 400 U/mL DNase I (Worthington). Human tumor specimens were obtained from commercial sources (MT Group, Cureline) or University of Rochester in accordance with IRB protocols after obtaining informed consent. The IRBs for MT Group, Cureline, and University of Rochester are Sterling IRB, Western IRB, and Research Subjects Review Board, respectively. Single-cell suspensions from tumors were obtained by fine mincing with a scalpel, followed by a 30-minute incubation at 37°C in digestion medium containing 8 mL of RPMI1640 medium, 40 μL of 100 mg/mL Collagenase I (Thermo Fisher Scientific), and 320 μL of 10,000 U/mL DNase I.

ELISA

GITR variants with E-tag were serially diluted and added to ELISA plates coated with the parental mouse clone of MK-4166. HRP-conjugated anti-E-tag mAb was used as the detection reagent. The reaction was developed with 2, 2’-Azino-di(3-ethyl-benzthiazoline-6-sulfonate; ABTS) and read at 405 nm.

T-cell proliferation assays

Naïve CD4+ T cells were isolated from blood by negative selection using EasySep Human Naïve CD4+ T-cell Enrichment kits (Stemcell Technologies) per kit instructions. Cells expressing CD32, CD58, and CD80 on their surface were irradiated with 7000 rads, plated at 2.5 × 104 cells per well, and then cocultured with 2 × 106 naïve CD4+ T cells in Yssel’s medium with 1% human AB serum. The T cells were stimulated with 0.3 ng/mL of anti-human CD3 mAb (clone UCHT1) in the presence or absence of MK-4166. T-cell proliferation was assessed after 4 or 5 days by incorporation of tritiated thymidine (3H-thymidine) added 18 to 24 hours before harvesting. T-cell proliferation in cynomolgus monkeys was assayed similarly, except that a NHP naïve CD4+ T-cell kit (Miltenyi Biotec) was used to enrich naïve CD4+ T cells and clone FN-18 at 0.2 ng/mL was used to stimulate T cells. The degree of response to MK-4166 costimulation was variable; 6 of 12 human donors and 5 of 10 cynomolgus monkeys had dose-response curves sufficient to calculate an EC50 value.

Mouse naïve CD4+ T cells were isolated from spleen by negative selection using an EasySep mouse naïve CD4+ T-cell kit and 2 × 104 cells were stimulated with 100 ng/mL of anti-mouse CD3 mAb (clone 145-2C11) and cultured with or without DTA-1 in complete RPMI-1640. Irradiated mouse splenocytes (2,000 rads,
1 × 10^5), depleted of T cells using CD90.2 microbeads, were used to provide costimulation and T-cell proliferation was quantified on day 3 using ^3^H-thymidine incorporation.

Single-cell suspensions of human tumors were layered on 25 mL of Histopaque and centrifuged at 2,000 RPM to remove dead cells and to enrich for TILs. A total of 0.5 × 10^6 enriched TILs were stimulated with 5 ng/mL of soluble anti-CD3 mAb (clone OKT3) for 7 days in the presence of 10 μg/mL of MK-4166, MK-1248 or corresponding isotype matched control antibodies in complete DMEM. Samples were stimulated in triplicates and proliferation was quantified on day 7 using ^3^H-thymidine incorporation.

Generation of iTregs in MLR cultures
Monocytes enriched from human PBMCs (RosetteSep human monocyte enrichment kit) were cultured in complete DMEM with 10% FBS (SAFC Biosciences), 1,000 U/mL GM-CSF (PeproTech) and 400 U/mL IL4 (R&D Systems) for 7 days to generate monocyte enrichment kit (Stemcell Technologies), CD4^+^ T cells, Tregs and HLA-DR^+^ cells were isolated from the culture supernatant media were aspirated from each well and cells were centrifuged. Supernatant media were aspirated from each well and cells were centrifuged at a concentration of 5 × 10^6 cells/mL in the presence of IL2 (100 U/mL) and IL15 (5 ng/mL). MK-4166, MK-1248, or isotype control mAb was added to the cultures and the relative abundance of CD4^+^FoxP3^bright^iTregs was evaluated at day 7 using flow cytometry.

nTreg suppression assay
Total CD3^+^ T cells, Tregs, and HLA-DR^+^ cells were isolated from PBMCs of the same donor with EasySep Human T-cell isolation kit (Stemcell Technologies), CD4^CD25^+^CD127dim/^ regulatory T-Cell Isolation Kit II, human (Miltenyi Biotec) and human anti–HLA-DR MicroBeads (Miltenyi Biotec), respectively. T_{effs} (Total CD3^+^) were labeled with 1.5 μmol/L CFSE (CellTrace Cell Proliferation Kits, CFSE, Invitrogen) and T_{reg} were labeled with 1.5 μmol/L CellTrace Violet (CellTrace Cell Proliferation Kits, Violet, Invitrogen). After labeling, cells were counted and cultured at indicated T_{eff}:T_{reg} ratios without the addition of exogenous IL2. T_{effs} were stimulated with autologous HLA-DR^+^ cells (HLA-DR^+^:T{eff} ratio of 2:1) and 2 μg/mL of anti-CD3 (clone OKT3) in the presence of indicated amounts of MK-4166 or control Ab for 4 days. Cells were labeled with anti-CD3, anti-CD4, and anti-CD8, and proliferation of specific T-cell subsets was measured as a decrease in CFSE-labeling intensity by flow cytometry.

Results
Selection of MK-4166 and epitope matching with DTA-1
On the basis of several reports that indicate that GITR signaling enhances antitumor immunity in mouse models, a humanized IgG1 agonist mAb against human GITR, MK-4166, was developed to treat patients with advanced malignancies. Because the anti-mouse GITR mAb DTA-1 has shown impressive antitumor efficacy in rodent models, the drug candidate was selected to bind to an epitope that is analogous to that of DTA-1. To this end, the amino acid residues important for DTA-1 and MK-4166 binding to murine GITR and human GITR, respectively, were determined by domain swapping and site-directed mutagenesis experiments as detailed in the Supplementary Material. An alignment of the region containing the amino acids identified as important for antibody binding is shown in Fig. 1A. Six out of the 7 key residues were different in mice compared with humans, and when these residues on mouse GITR were replaced with corresponding residues from human GITR, MK-4166 was able to bind the modified mouse GITR, albeit with lower affinity (Fig. 1B). The average affinities of MK-4166 for human and cynomolgus monkey GITR at 25°C as determined by cell-based KinExA were comparable at 5.5 and 7.6 pmol/L, respectively (Supplementary Table S1). MK-4166 did not bind to mouse GITR (Fig. 1B) as determined by ELISA. The affinity of DTA-1 for mouse GITR was determined to be 26 pmol/L (Supplementary Table S1) by cell-based KinExA, and was comparable with that of MK-4166 for human and cynomolgus monkey GITR.

MK-4166 and DTA-1 costimulate naive T cells, resulting in enhanced proliferation
A dose-dependent augmentation of T-cell proliferation was observed when human or cynomolgus monkey naive CD4^+^ T cells were costimulated with anti-CD3 and L-cells in the presence of MK-4166 (Fig. 2A and B). A dose-dependent enhancement of murine T-cell proliferation was observed in an analogous in vitro proliferation assay using splenic naive CD4^+^ T cells costimulated with T-cell–depleted, irradiated splenocytes and anti-CD3 in the presence of DTA-1 (Fig. 2C). The bioactivity of MK-4166 was comparable in human and cynomolgus monkey T cells, with median EC_{50} values of 7.7 and 7.9 pmol/L, respectively (Supplementary Table S2). The median EC_{50} value for DTA-1 in mouse T cells was 99 pmol/L (Supplementary Table S2). Considering the relative high potency (picomolar range) of both MK-4166 and DTA-1, and the variability attributable to the differences between rodent and primate assay formats, the bioactivities of both mAbs were deemed fairly comparable.

The ability of MK-4166 or MK-1248 (which has the same CDRs as MK-4166, but is an IgG4 with a different FcγR binding profile
and minimal Fc effector function) to costimulate human TILs was determined in single-cell suspensions obtained from NSCLC tumor tissues. In the presence of either MK-4166 or MK-1248, increased proliferation of anti–CD3-stimulated TILs was observed (Fig. 2D).

Expression of GITR in peripheral blood cells is significantly different between mice and humans or non-human primates

Translational approaches in pursuing GITR as a target for immunotherapy require knowledge of GITR expression in healthy and tumor-bearing mice, non-human primates (NHP), healthy human donors, and cancer patients. GITR expression was observed on blood CD4⁺ T cells, NK cells, and NKT cells in healthy human donors and cancer patients, but was very low to undetectable on CD8⁺ T cells, B cells, monocytes, and granulocytes (Fig. 3A and B). Among the CD4⁺ T-cell subsets evaluated, Tregs, T H17 and effector memory T cells (TEM) had the highest frequencies of GITR⁺ cells (Fig. 3C; gating scheme depicted in Supplementary Fig. S1). In addition, we observed that the upregulation of GITR upon activation of sorted CD4⁺ T-cell subsets was highest on TEM, followed by central memory (TCM) and then naïve T cells (Fig. 3C and D). In cynomolgus monkeys, GITR expression was similar with the exception of NK cells, which did not express GITR (Fig. 3A). Similar to the profile in peripheral blood, a significant difference in the frequency of GITR⁺ TILs was observed between human and mouse tumors (Fig. 4A and B). In the 3 human tumor types analyzed, GITR was expressed on 22% to 42% of CD4⁺ TILs, whereas it was typically expressed on less than 11% of the CD8⁺ TILs (Fig. 4A). In contrast, GITR was expressed on nearly 100% of CD4⁺ and CD8⁺ TILs in all analyzed syngeneic mouse tumor models (Fig. 4B). Both, frequency (Fig. 4C; Supplementary Fig. S3A and S3B) and intensity (Fig. 4D) of GITR expression were considerably higher on Tregs compared with non-Treg CD4⁺ TILs from NSCLCs and were similar to GITR expression on Tregs in infiltrating mouse tumors (Fig. 4E and F).

Expression of GITR on tumor-infiltrating lymphocytes is significantly different between mice and humans

Expression of GITR on CD4⁺ and CD8⁺ TILs obtained from non-small cell lung carcinoma (NSCLC), melanoma, and renal cell carcinoma (RCC) tumor tissues was evaluated by flow cytometry and compared with that on mouse TILs from 10 syngeneic mouse tumor models (MC38, MB49, LL/2, B16F10, TC1, RENCA, 4T1, CT26, EMT6, and CM3). Similar to the profile in peripheral blood, a significant difference in the frequency of GITR⁺ TILs was observed between human and mouse tumors (Fig. 4A and B). In the 3 human tumor types analyzed, GITR was expressed on 22% to 42% of CD4⁺ TILs, whereas it was typically expressed on less than 11% of the CD8⁺ TILs (Fig. 4A). In contrast, GITR was expressed on nearly 100% of CD4⁺ and CD8⁺ TILs in all analyzed syngeneic mouse tumor models (Fig. 4B). Both, frequency (Fig. 4C; Supplementary Fig. S3A and S3B) and intensity (Fig. 4D) of GITR expression were considerably higher on Tregs compared with non-Treg CD4⁺ TILs from NSCLCs and were similar to GITR expression on Tregs infiltrating mouse tumors (Fig. 4E and F). Furthermore, almost all of the tumor-infiltrating CD4⁺CD25⁺ cells expressed high levels of FoxP3 protein (Supplementary Fig. S4A), high mRNA levels of Treg markers such as Helios and Eos (Supplementary Fig. S4B) and were able to suppress proliferation of CD8⁺ TILs isolated from NSCLC tissues by cell sorting (FACS; Supplementary Fig. S4C), thus confirming their identity as Tregs.

MK-4166 decreases both the induction and suppressive effects of Tregs

Since Treg have been shown to play a central role in antitumor efficacy of DTA-1 and the expression of GITR was the highest on human intratumoral Tregs as compared with the other TIL
populations, we focused on characterization of the ability of MK-4166 to affect the induction of human T\textsubscript{regs}, and their suppressive effects in vitro.

The effect of MK-4166 on the induction of T\textsubscript{regs} (iT\textsubscript{regs}) in MLR cultures was assessed. The iT\textsubscript{regs} were identified as CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{high} and the expression of GITR in this population was measured by flow cytometry (Supplementary Fig. S5A and S5B). Because activated T cells can express low levels of FoxP3, we confirmed that the iT\textsubscript{regs} were functionally suppressive (Supplementary Fig. S5C and S5D). The addition of MK-4166 to MLR cultures on day 0 resulted in a dose-dependent decrease in abundance of iT\textsubscript{regs} after 7 days, measured either as relative abundance (Fig. 5A) or as absolute numbers (Supplementary Fig. S6A). This decrease in iT\textsubscript{regs} was observed only when MK-4166 was present from the start of culture. When it was added on day 7 after iT\textsubscript{regs} were already established, the effect was not observed, indicating that the decrease in the number of iT\textsubscript{regs} is due to lack of induction as opposed to loss of established iT\textsubscript{regs} (Supplementary Fig. S6B and S6C). A similar dose-dependent decrease in the induction of iT\textsubscript{regs} was observed with MK-1248 (Fig. 5B), indicating that Fc effector functions do not likely play a role in this assay, even though MK-4166 does demonstrate the potential to induce ADCC in human MLR-derived iT\textsubscript{regs} using a Jurkat reporter cell line (Supplementary Fig. S7A and S7B).

To determine whether MK-4166 could reduce the suppressive effects of human natural T\textsubscript{regs} (nT\textsubscript{regs}), a nT\textsubscript{reg} suppression assay was used in which donor-matched CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{low} nT\textsubscript{regs} and CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{low} nT\textsubscript{regs} isolated from blood were stimulated with anti-CD3 and autologous HLA-DR\textsuperscript{+} cells and proliferation measured as dilution of CFSE (Fig. 5C and D; Supplementary Fig. S8A and S8B). This was further confirmed in independent experiments using a MLR suppression assay where T cells were stimulated with allogeneic-DCs and T-cell proliferation was tracked using \textsuperscript{3}H-thymidine incorporation (Supplementary Fig. S8C). The purity of nT\textsubscript{regs} (CD4\textsuperscript{+}FoxP3\textsuperscript{+}CD127\textsuperscript{low}) used in the nT\textsubscript{reg} suppression assay was $>$85\% (Supplementary Fig. S9A) and in the MLR suppression assay was 40 to 70\% (Supplementary Fig. S9B). Addition of MK-4166 increased T-cell proliferation compared with isotype-matched controls at several Teff:nT\textsubscript{reg} ratios tested (Fig. 5C and D), indicating that MK-4166 partially attenuates the suppressive effects of T\textsubscript{regs}. MK-4166 did not enhance proliferation of T\textsubscript{eff} alone (T\textsubscript{eff}:nT\textsubscript{reg} ratio1:0; Fig. 5C and D; Supplementary Fig. S8A–S8C), indicating that there was no costimulatory effect of GITR agonism in this assay. In addition, MK-4166 did not enhance the proliferation of nT\textsubscript{regs} in an independent experiment where nT\textsubscript{regs} were stimulated by anti-CD3/anti-CD28–coated beads (Supplementary Fig. S10A and S10B).

MK-4166 engagement triggers NFkB phosphorylation in T\textsubscript{regs} and T\textsubscript{eff}

Published reports indicate that GITR signaling is mediated by NFkB and MAP kinases p38, JNK, and ERK in mice (31–35). To determine whether binding of the agonist mAb, MK-4166, to GITR elicits similar early signaling events, phosphorylation of MAPK/Erk and NFkB was evaluated by flow cytometry. MK-4166 and MK-1248 bound at similar levels to T\textsubscript{regs} (CD4\textsuperscript{+}FoxP3\textsuperscript{+}) or to T\textsubscript{effs} (CD4\textsuperscript{+}FoxP3\textsuperscript{−}) as determined by flow cytometry (Fig. 6A). Following stimulation with MK-4166, a rapid induction of phospho-NFkB p65 was observed in MLR-derived GITR\textsuperscript{+}CD4\textsuperscript{+} T cells but not in GITR\textsuperscript{−}CD4\textsuperscript{+} T cells (Fig. 6B). Maximal phosphorylation of NFkB p65 was observed at 5 minutes following engagement of GITR by MK-4166, and this time point was chosen for further analysis. Increases in phosphorylation of NFkB p65 were observed in both MLR-derived and intratumoral T\textsubscript{regs} and T\textsubscript{effs} (Fig. 6C) following stimulation with MK-4166 but not with isotype control mAb. DyLight650-labeled MK-4166 was used in
these experiments to identify T cells that bound to MK-4166 (GITR expressing) and T cells that did not (GITR non-expressing). Within each sample stimulated with DyLight650-labeled MK-4166, T cells that expressed GITR had significantly higher phospho-NFkB levels compared with T cells that did not (Fig. 6D). Furthermore, all of these results were reproduced with MK-1248, indicating that GITR signaling is independent of isotype in this assay (Fig. 6C and D). Phosphorylation of Erk1/2 in CD4+ T cells was not detected in this assay upon addition of MK-4166 (data not shown).

Gene-expression changes in human TILs upon engagement of GITR by MK-4166

To determine the downstream effects of GITR signaling, human tumor single-cell suspensions were cultured either with DyLight650-labeled MK-4166 or DyLight650-labeled isotype matched control mAb. CD4+ T cells were sorted after 24 hours or 7 days, and gene expression was analyzed by RTqPCR. Even though phospho-Erk1/2 protein was not detected in TILs from NSCLCs, an increase in the expression of DUSP6, a gene induced by the Erk signaling pathway (36), was observed in CD4+ T cells in RCC and colorectal carcinoma after 24 hours of incubation with DyLight650-labeled MK-4166 (Fig. 7A). Cells from NSCLC were not available for this experiment. Some reports indicate that GITR signaling among murine intratumoral Tregs decreases expression of FoxP3 and causes instability of Treg lineage commitment (21, 22). We also observed a decrease in FOXP3 mRNA in CD4+ T cells sorted from human RCC and NSCLC tumor cultures treated with MK-4166 for 7 days (Fig. 7B).

Safety and toxicity assessments in cynomolgus monkeys

Potential toxicity of MK-4166 was characterized in a 1-month study in cynomolgus monkeys with a 2-month post-dosing monitoring period, where a broad range of doses (0.03, 1, 30, or 200 mg/kg/dose) was administered once weekly by intravenous infusion. MK-4166 was well tolerated at all doses and no treatment-related toxicity was detected. Details can be found in the Supplementary Materials.

Discussion

Increased understanding of the immunosuppressive mechanisms in cancers has identified several molecular pathways, including members of the TNF/TNFR family, as potential targets for anticancer therapies (6). In particular, the agonist anti-GITR antibody DTA-1 has impressive antitumor efficacy in murine syngeneic tumor models (21, 23–26). On the basis of these observations, we developed MK-4166, a humanized agonist mAb against human
Figure 4.
Expression of GITR on human and mouse tumor-infiltrating lymphocytes. A, The frequency of GITR⁺ human CD4⁺ and CD8⁺ TILs in NSCLC (n = 21), melanoma (n = 7), and renal cell carcinoma (n = 9) was determined by flow cytometry. B, The frequency of GITR⁺ CD4⁺ and CD8⁺ TILs from 10 different mouse syngeneic tumors (6 mice/model) was determined by flow cytometry. The frequency (C) and the geometric mean fluorescence intensity (D) of GITR expression in Treg (CD4⁺ CD25⁺) and T eff (CD4⁺ CD25⁻) in tumor tissues obtained from patients with NSCLC. *** P < 0.001 calculated by paired Student t test. The trend lines in D show donor-matched populations. Representative histograms of expression of GITR on human (E) or mouse (F) CD4⁺, CD4⁺CD25⁺, and CD8⁺ TILs are also shown. Error bars, where shown, indicate SD.
GITR for the treatment of solid tumors. To take advantage of the robust efficacy seen with DTA-1, we ensured that MK-4166 and DTA-1 bound to highly analogous epitopes in the respective species. This innovative approach of ensuring that the clinical candidate binds to an analogous epitope as that of its mouse surrogate enabled us to make translational comparisons between the observations in the two species.

Although the role of GITR is studied extensively in murine models, there is a dearth of information on the role GITR plays in humans (29), and the experiments reported here not only provide insights on the translatability of observations made in murine models, but extend our understanding of GITR biology in humans. DTA-1 has been shown to deplete intratumoral Tregs in vivo (20) and to decrease the induction of Tregs in murine spleenocytes treated in vitro with TGFβ (21). In contrast, GITR agonism (with DTA-1 or mouse GITRL) has been reported to enhance proliferation of Treg^in vitro and in vivo (37, 38). This suggests that the effects of GITR agonism can be context dependent (12). It is unclear whether our observation that MK-4166 did not enhance nTreg proliferation in vitro is due to conditions of testing or to species-specific differences. We found that the expression of human GITR is comparable to that of mouse GITR in tumor infiltrating Tregs, despite being drastically lower in other TIL populations and in peripheral blood. MK-4166 decreased the number of Tregs induced in a MLR culture. This is likely not due to ADCC because the decrease in the number of Tregs was also seen with MK-1248 (an IgG4, which has the same CDRs but possesses minimal Fc effector functions) and only when MK-4166 was added at the beginning of the coculture. Furthermore, the NK cell-to-target cell ratio in MLR cultures is insufficient for significant ADCC. However, in an assay optimized to detect ADCC potential (Supplementary Fig. S7A and B), we show that Treg-bound MK-4166 has the potential to induce ADCC of human Tregs. Taken together these data suggest that MK-4166 may deplete intratumoral Treg, as well as potentially inhibiting their de novo generation in the tumor.

Figure 5.

Effect of MK-4166 on iTreg generation and nTreg-mediated suppression of Teff. A and B, Human PBMCs were stimulated with irradiated allogeneic DCs in the presence of MK-4166, MK-1248, or isotype-matched control mAb for 7 days, and the relative abundance of iTregs (CD4^+ CD25^+ FoxP3^High) generated in these allo-MLR cultures was measured by flow cytometry and is plotted as a fraction of the total CD4^+ population. Aggregate data from 7 individual donors are shown and one-way ANOVA/Kruskal-Wallis test was used to determine statistical significance. Experiments with MK-4166 and MK-1248 were conducted using blood from the same 7 donors. C and D, CFSE-labeled Teffs were stimulated with anti-CD3 in cultures containing lethally irradiated autologous HLA-DR^+ feeder cells. The ability of responder matched nTregs to inhibit proliferation of CD4^+ (C) or CD8^+ (D) Teffs at indicated Teff:nTreg ratios was measured as dilution of CFSE-labeled intensity in the presence of MK-4166 or isotype-matched control mAb. Data shown are representative of three donors. *, P < 0.05; **, P < 0.01; and ***, P < 0.0001, respectively.
Furthermore, using an in vitro nTreg suppression assay, we show for the first time that an agonist anti-human GITR antibody (MK-4166) attenuates the suppressive effects of human nTregs on Teff proliferation. Unlike in assays where TCR signaling is suboptimal (Fig. 2A–D), a direct proliferative effect of MK-4166 on Teff was not observed in these assays where a very strong primary TCR signal is present (Teff/nTreg ratio 1:0; Fig. 5C and D; Supplementary Fig. S8A–C). However, we cannot rule out the possibility that MK-4166 might act directly on Teff in these cultures to increase their refractoriness to Treg suppression as has been suggested for mouse GITR agonism (21, 23, 39, 40). In either case, MK-4166 decreases the suppressive effects of nTregs.

Analysis of proximal signaling events showed that NFκB p65 is phosphorylated immediately upon engagement of GITR on intratumoral Tregs and Teffs (Fig. 6B–D) by MK-4166, suggesting that direct GITR signaling may contribute to the effects of MK-4166 on both populations. Some studies indicate that DTA-1 causes lineage instability and dedifferentiation of Tregs, and this instability is mechanistically...
tied to its anti-tumor and pro-inflammatory activity (21, 22), though other studies do not (19, 20, 41). We observed a MK-4166-mediated decrease in the expression of Foxp3, a transcription factor linked to Treg function, suggesting that MK-4166 may function by attenuating the suppressive activity of Tregs.

MK-4166 has been shown to inhibit growth of established SK-MEL-5 tumors in humanized mice (19). In this model, MK-4166 reduced the number of Treg in the spleen and to a lesser extent in tumor. In both tissues, a decrease in the activation marker ICOS was observed on Treg, indicating that MK-4166 can attenuate human Treg function in vivo.

The data presented here elucidate the effects of GITR agonism on human T cells and provide a strong scientific and translational rationale for the clinical development of MK-4166 to treat advanced malignancies. These are very early insights into the effects of agonist mAbs on human GITR using ex vivo models and suggest that MK-4166 offers an alternative immune-modulatory mechanism to the reversal of checkpoint inhibition in the treatment of cancer. TNFR-agonist antibodies elicit a higher concern for safety, however MK-4166 was well tolerated in non-human primates at all doses administered and no treatment-related toxicity was detected. The safety and tolerability of MK-4166 antibody either as a monotherapy or in combination with pembrolizumab (anti–PD-1 mAb) in patients with advanced solid tumors is currently being evaluated in a phase I study (NCT02132754).

Disclosure of Potential Conflicts of Interest
S. Sukumar has ownership interest (including patents) in Merck & Co. G. Ermakov has ownership interest (including patents) in WO 2011/028683 A1 and 14/261,152. No potential conflicts of interest were disclosed by the other authors.

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