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

Selvakumar Sukumar¹, Douglas C. Wilson¹, Ying Yu¹, Jerelyn Wong¹, Saraswathi Naravula¹, Grigori Ermakov¹, Romina Rienert¹, Bhagyashree Bhagwat¹, Antoaneta S. Necheva², Jeff Grein¹, Tatyana Churakova¹, Ruban Mangadu¹, Peter Georgiev², Denise Manfra², Elaine M. Pinheiro², Venkataraman Sriram³, Wendy J. Bailey³, Danuta Herzyk³, Terrill K. McClanahan¹, Aarron Willingham¹, Amy M. Beebe¹, and Svetlana Sadekova¹

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 Tₗ体育彩票-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 naïve 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 Tₐₗ体育彩票 despite being drastically lower in other human TILs and in many human peripheral blood populations. MK-4166 decreased induction and suppressive effects of Tₐₗ体育彩票 in vitro. In human TIL cultures, MK-4166 induced phosphorylation of NFκB and increased expression of dual specificity phosphatase 6 (DUSP6), indicating that MK-4166 activated downstream NFκB and Erk signaling pathways. Furthermore, MK-4166 downregulated FOXP3 mRNA in human tumor infiltrating Tₐₗ体育彩票 suggesting that, in addition to enhancing the activation of TILs, MK-4166 may attenuate the Tₐₗ体育彩票-mediated suppressive tumor microenvironment. Cancer Res; 77(16); 4378–88. ©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 Tₐₗ体育彩票 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 Tₐₗ体育彩票-mediated suppression either by eliminating GITR-expressing tumor-infiltrating Tₐₗ体育彩票 (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
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 translatable of murine efficacy data to human cancers, we evaluated GITR expression in mouse and human. The expression of GITR on tumor-infiltrating Treg 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 Treg have been shown to play a critical role in antitumor efficacy of DTA-1, we focused on characterization of MK-4166 activity on human Treg. MK-4166 significantly inhibited the generation of Treg in mixed lymphocyte reactions (MLR) and decreased the suppressive effects of Treg. Moreover, MK-4166 induced phosphorylation of NFκB, 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 reducedFc 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 (NCL, 2015), MB49 (University of Iowa, 2015), B16F10 (ATCC, 2015), LL/2 (ATCC, 2015), TC-1 (Johns Hopkins University, 2015), 4T1 (ATCC, 2015), CT26 (ATCC, 2015), RENCA (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 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/6J mice or C57BL/6J 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 K2-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 × 104 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 × 106 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 spleenocytes (2,000 rads,
1 × 10⁵), depleted of T cells using CD90.2 microbeads, were used to provide costimulation and T-cell proliferation was quantified on day 3 using ³H-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 × 10⁵ 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 ³H-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-derived DCs (mo-DCs). LPS (0.5 µg/mL) was added to the culture during the last 2 days to mature the mo-DCs. MLR was set up by culturing PBMCs (2 × 10⁵ cells/mL) with γ-irradiated (30 Gy) allogeneic mo-DCs (0.2 × 10⁶ 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⁺FoxP3high Tregs was evaluated at day 7 using flow cytometry.

nTreg suppression assay
Total CD3⁺ T cells, Treg, and HLA-DR⁺ cells were isolated from PBMCs of the same donor with EasySep Human T-cell isolation kit (Stemcell Technologies). CD4⁺CD25⁺CD127dim/C0 T cells were costimulated with anti-CD3 and L-cells in the presence of 2 µg/mL of anti-CD3 (clone OKT3) and Treg at 2:1 ratio. Supernatant media were aspirated from each well and cells were incubated with a viability dye and phenotypic antibodies. All samples were fixed in 100 µL of 1.5% formaldehyde solution for 15 minutes followed by incubation with 200 µL of ice-cold 100% methanol for 30 minutes. Cells were then washed twice with DPBS, blocked 10 minutes with 2% normal mouse serum, and subsequently incubated for 30 minutes on ice with mAbs specific for FoxP3 (clone PCH101), phosphorylated NfκB p65 (clone 93H1), and phosphorylated ErK1/2 (clone D13.14.4E).

Gene expression in CD4⁺ TILs after stimulation of tumor cultures with MK-4166
Dissociated tumor cells were cultured in complete RPMI medium supplemented with 62.5 ng/mL of IL2 and 10 µg/mL of either DyLight650-labeled MK-4166 or DyLight650-labeled isotype control antibody at 37°C with 5% CO₂ in a humidified incubator. After 1 or 7 days in culture, CD4⁺ T cells were sorted using FACSAriaII (BD Biosciences) into individual wells of a 96-well plate containing 5 µL of preamplification buffer with gene-specific primers and probes for RT-PCR as detailed in the Supplementary Material.

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 naïve T cells, resulting in enhanced proliferation
A dose-dependent enhancement of T-cell proliferation was observed when human or cynomolgus monkey naïve 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 naïve 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 EC₅₀ values of 7.7 and 7.9 pmol/L, respectively (Supplementary Table S2). The median EC₅₀ 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 naive 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). The discrepancy in expression of GITR on human and cynomolgus monkey NK cells was further confirmed by gene expression analysis of sorted NK cells (Supplementary Fig. S2A and S2B). In marked contrast, the vast majority of murine CD4⁺ T cells, CD8⁺ T cells, NK cells, and NKT cells expressed GITR (Fig. 3A). Moreover, a large proportion of B cells and a subset of monocytes and granulocytes also expressed GITR in mice (Fig. 3B).

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, Li/2, B16F10, TC1, RENCA, 4T1, CT26, EM6, 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 Treg

Since Tregs 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 Treg, as compared with the other TIL.
populations, we focused on characterization of the ability of MK-4166 to affect the induction of human Tregs and their suppressive effects in vitro.

The effect of MK-4166 on the induction of Tregs (iTregs) in MLR cultures was assessed. The iTregs were identified as CD4+ CD25+ FoxP3high and the expression of GITR in this population was determined by flow cytometry (Supplementary Fig. S5A and S5B). Because activated T cells can express low levels of FoxP3, we confirmed that the iTregs 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 (Fig. 5A) or as absolute numbers (Supplementary Fig. S6A). This decrease in iTregs was observed only when MK-4166 was present from the start of culture. When it was added on day 7 after iTregs were already established, the effect was not observed, indicating that the decrease in the number of iTregs is due to lack of induction as opposed to loss of established iTregs (Supplementary Fig. S6B and S6C). A similar dose-dependent decrease in the induction of iTregs was observed with MK-1248 (Fig. 5B), indicating that Fc effector functions do not likely play a role in this assay, even though MK-1248 does demonstrate the potential to induce ADCC in human MLR-derived iTregs using a Jurkat reporter cell line (Supplementary Fig. S7A and S7B).

To determine whether MK-4166 could reduce the suppressive effects of human natural Tregs (nTregs), a nTreg suppression assay was used in which donor-matched CD4+ CD25+ CD127low nTregs and CD4+ and CD8+ Teffs isolated from blood were stimulated with anti-CD3 and autologous HLA-DR+ 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 [3H]-thymidine incorporation (Supplementary Fig. S8C). The purity of nTregs (CD4+ FoxP3+ CD127low) used in the nTreg 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 Treg:nTreg ratios tested (Fig. 5C and D), indicating that MK-4166 partially attenuates the suppressive effects of Tregs. MK-4166 did not enhance proliferation of Teffs alone (Teff:nTreg ratio 1:0; 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 nTregs in an independent experiment where nTregs were stimulated by anti-CD3/anti-CD28–coated beads (Supplementary Fig. S10A and S10B).

**Figure 2.**

Costimulation of naive peripheral T cells from human, cynomolgus monkey or mouse blood, and from human TILs by agonist GITR antibodies. Naive CD4+ T cells from peripheral blood of human healthy donors (A) or cynomolgus monkeys (B) were incubated with L cells expressing CD32a, CD58, and CD80. MK-4166 or an isotype-matched control antibody was added in a dose-titration manner in the presence of anti-CD3 (0.2 to 0.3 ng/mL of anti-CD3 mAb) and T-cell proliferation was measured after 4 or 5 days. C, Naive CD4+ T cells from mouse spleens were incubated with irradiated splenocytes depleted of T cells in the presence of 100 ng/mL of anti-CD3 mAb. DTA-1 or an isotype-matched control antibody was added in a dose-titration manner and T-cell proliferation was measured. D, Human TILs from four independent donors were stimulated with anti-CD3 antibody (10 ng/mL) in the presence 10 µg/mL of MK-1248 or MK-4166 or an isotype-matched control antibody for 7 days and proliferation was measured.

**MK-4166 engagement triggers NfκB phosphorylation in Treg**

Published reports indicate that GITR signaling is mediated by NfκB and MAP kinases p38, INK, 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 NfκB was evaluated by flow cytometry. MK-4166 and MK-1248 bound at similar levels to Tregs (CD4+ FoxP3high) compared with nTregs (CD4+ FoxP3low) as determined by flow cytometry (Fig. 6A).

Following stimulation with MK-4166, a rapid induction of phospho-NfκB p65 was observed in MLR-derived GITR+ CD4+ T cells but not in GITR− CD4+ T cells (Fig. 6B). Maximal phosphorylation of NfκB 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 NfκB p65 were observed in both MLR-derived and intratumoral Tregs and Teffs (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 GITR, to induce antitumor efficacy in cancer patients. MK-4166 is well tolerated at all doses and provides significant antitumor efficacy in a variety of tumor models (21, 23–26). These results suggest that MK-4166 has the potential to be an effective therapeutic agent for the treatment of cancer.
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. C, The frequency (MFI) and the geometric mean fluorescence intensity (D) of GITR expression in T_em (CD4⁺CD25⁺) and T_reg (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 (21). In contrast, GITR agonism (with DTA-1 or mouse GITRL) has been reported to enhance proliferation of Tregs 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 Tregs 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 iTreg (CD4+CD25+FoxP3High) 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 Treg were stimulated with anti-CD3 in cultures containing lethally irradiated autologous HLA-DR+ feeder cells. The ability of responder matched nTreg to inhibit proliferation of CD4+ (C) or CD8+ (D) Teff at indicated Teff:nTreg ratio 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; *** P < 0.001; **** 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 Teffs 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 Teffs 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 T_{reg} function, suggesting that MK-4166 may function by attenuating the suppressive activity of T_{reg}.

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 T_{reg} in the spleen and to a lesser extent in tumor. In both tissues, a decrease in the activation marker ICOS was noted (CRC; n = 1), specimens were treated either with DyLight650-labeled MK-4166 (10 µg/mL) or DyLight650-labeled isotype control for 24 hours or 7 days and CD4^+ T cells were sorted by FACS. Changes in mRNA expression of DUSP6 and FOXP3 on day 1 (A) and FOXP3 on day 7 (B) in CD4^+ T-cell populations was determined by qPCR and graphed as fold change over isotype-matched control mAb.

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.

Authors’ Contributions
Conception and design: S. Sukumar, D.C. Wilson, Y. Yu, B. Bhagwat, P. Georgiev, V. Sriram, A. Willingham, A.M. Beebe, S. Sadekova
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sukumar, D.C. Wilson, Y. Yu, S. Naravula, G. Ermakov, R. Rieder, B. Bhagwat, A.S. Necheva, J. Grein, T. Churakova, R. Mangadu, P. Georgiev, V. Sriram
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S. Sukumar, D.C. Wilson, Y. Yu, S. Naravula, G. Ermakov, R. Rieder, B. Bhagwat, A.S. Necheva, J. Grein, T. Churakova, R. Mangadu, P. Georgiev, W.J. Bailey, A. Willingham, S. Sadekova
Writing, review, and/or revision of the manuscript: S. Sukumar, D.C. Wilson, Y. Yu, S. Naravula, G. Ermakov, R. Rieder, B. Bhagwat, A.S. Necheva, P. Georgiev, W.J. Bailey, D. Herzyk, A. Willingham, A.M. Beebe, S. Sadekova
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.J. Bailey, T.K. McClanahan, S. Sadekova
Study supervision: D. Mantra, E.M. Pinheiro, W.J. Bailey, T.K. McClanahan, S. Sadekova

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Selvakumar Sukumar, Douglas C. Wilson, Ying Yu, et al.


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