Characterization of MK-4166, a clinical agonistic antibody that targets human GITR and inhibits the generation and suppressive effects of T regulatory cells

Selvakumar Sukumar1,2*, Douglas C. Wilson1*, Ying Yu1, Jerelyn Wong1, Saraswathi Naravula1, Grigori Ermakov1, Romina Riener1, Bhagyaushree Bhagwat1, Antoaneta S. Necheva3, Jeff Grein1, Tatyana Churakova1, Ruban Mangadu1, Peter Georgiev3, Denise Manfra3, Elaine M. Pinheiro3, Venkataraman Sriram1,4, Wendy J. Bailey5, Danuta Herzyk5, Terrill K. McClanahan1, Aarron Willingham1, Amy M. Beebe1, Svetlana Sadekova1

1Merck Research Laboratories, Palo Alto, CA, 3Merck Research Laboratories, Boston, MA, 5Merck Research Laboratories, West Point, PA

2Current address: CSL Behring, King of Prussia, PA

4Current address: Pionyr Immunotherapeutics, San Francisco, CA

* These authors contributed equally to the work presented in this manuscript

Conflicts of Interest: All authors are current or former employees of Merck & Co.

Running Title: Characterization of MK-4166, an agonistic anti-GITR mAb

Key words: Antibody immunotherapy, Antireceptors, Immunomodulation, Tumor Immunobiology

Corresponding Author: Selvakumar Sukumar, CSL Behring, 1020 First Ave., King of Prussia, PA, 19406. Phone: 610-878-4271; Email: selvasukumar@yahoo.com
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

Abstract

GITR is a T cell co-stimulatory 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 T<sub>reg</sub>-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 monoclonal antibody selected to bind to an epitope analogous to the DTA-1 epitope, which enhances the proliferation of both naïve and tumor-infiltrating T lymphocytes (TILs). We also investigated the role of GITR agonism in human anti-tumor 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 to that of mouse GITR in tumor-infiltrating T<sub>reg</sub>s despite being drastically lower in other human TILs and in many human peripheral blood populations. MK-4166 decreased induction and suppressive effects of T<sub>reg</sub>s <i>in vitro</i>. In human TIL cultures, MK-4166 induced phosphorylation of NFκB and increased expression of dual specificity phosphatase 6 (<i>DUSP6</i>), indicating that MK-4166 activated downstream NFκB and Erk signaling pathways. Furthermore, MK-4166 downregulated <i>FOXP3</i> mRNA in human tumor infiltrating T<sub>reg</sub>s, suggesting that, in addition to enhancing the activation of TILs, MK-4166 may attenuate the T<sub>reg</sub>-mediated suppressive tumor microenvironment.
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 co-stimulatory 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 T_{reg}) (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 co-stimulatory 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) (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 T_{reg}-mediated suppression either by eliminating GITR expressing tumor-infiltrating T_{reg} (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).
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

Based on the potent anti-tumor 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 Tregs was comparable between mouse and human despite differences in GITR expression on many peripheral blood populations and tumor-infiltrating CD4+ and CD8+ T cells. Since Tregs have been shown to play a critical role in anti-tumor efficacy of DTA-1, we focused on characterization of MK-4166 activity on human Tregs. MK-4166 significantly inhibited the generation of Tregs in mixed lymphocyte reactions (MLR) and decreased the suppressive effects of Tregs. Moreover, MK-4166 induced phosphorylation of NFκB, up-regulated 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, Palo Alto, CA and for certain uses labeled with DyLight650 (Thermofisher). Generation of MK-4166 is described in the supplementary material. A murinized IgG2a version of rat
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

anti-mouse GITR antibody DTA-1 was generated based on published sequences (30). Antibodies used for flow cytometry are listed in the supplementary material.

**Mice and cell lines**

C57BL/6J, BALB/cAnN, DBA/2J or C3H/HeN mice (4 weeks old) were purchased from Jackson Laboratories 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), 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, Palo Alto. Human buffy coats from healthy volunteers were obtained from the Stanford Blood Center. All blood samples were collected from voluntary donors in accordance with 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 RPMI 1640 medium, 40 µL of 100 mg/mL Collagenase I (Thermo Fisher), 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 monoclonal antibody was used as the detection reagent. The reaction was developed with 2, 2'-Azino-di(3-ethylbenzthiazoline-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. L-cells expressing CD32, CD58, and CD80 on their surface were irradiated with 7000 rads, plated at 2.5×10^4 cells per well, and then co-cultured with 2×10^4 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) was used to enrich naïve CD4+ T
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

cells and clone FN-18 at 0.2 ng/mL was used to stimulate T cells. The degree of response to MK-4166 co-stimulation was variable; 6 of 12 human donors and 5 of 10 cynomolgus monkeys had dose response curves sufficient to calculate an EC50.

Mouse naïve CD4+ T cells were isolated from spleen by negative selection using an EasySep mouse naïve CD4+ T cell kit and 2×10^4 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 (2000 rads, 1×10^5), depleted of T cells using CD90.2 microbeads, were used to provide co-stimulation and T cell proliferation was quantified on day 3 using ^3^H-thymidine incorporation.

Single cell suspensions of human tumors were layered on 25mL of Histopaque and centrifuged at 2000 RPM to remove dead cells and to enrich for TILs. A total of 0.5×10^5 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), 1000 U/mL GM-CSF (PeproTech) and 400 U/mL IL-4 (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 (2×10^6 cells/mL) with γ-irradiated (30 Gy) allogeneic mo-DCs (0.2×10^6 cells/mL) in the presence of IL-2 (100 U/mL) and IL-15 (5 ng/mL). MK-4166, MK-1248, or isotype control mAb was added to the cultures and the relative abundance of CD4+FoxP3^{high} T_{regs} was evaluated at day 7 using flow cytometry.
**nT_{reg} suppression assay**

Total CD3+ T cells, T_{regs} 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) and human anti-HLA-DR MicroBeads (Miltenyi), respectively. T_{effs} (Total CD3+) were labeled with 1.5 μM CFSE (CellTrace™ Cell Proliferation Kits, CFSE, Invitrogen) and T_{regs} were labeled with 1.5 μM CellTrace Violet (CellTrace™ Cell Proliferation Kits, Violet, Invitrogen). After labeling, cells were counted and cultured at indicated T_{effs}:nT_{reg} ratios without the addition of exogenous IL-2. T_{effs} were stimulated with autologous HLA-DR+ cells (HLA-DR+:T_{effs} ratio of 2:1) and 2 ug/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.

**Analysis of early signaling events triggered by MK-4166**

Nine day old MLR cultures were frozen for future analysis. Upon thawing cells were rested for 24 hours to allow for stabilization of basal phosphoprotein levels. T_{regs} were then stimulated with either DyLight650-labeled MK-4166, DyLight650-labeled MK-1248 or DyLight650-labeled isotype matched control mAb at a concentration of 5 μg/mL for 5, 10, 15, or 30 minutes. After stimulation, cells were immediately chilled on ice and centrifuged. Supernatant media was 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 NFkB p65 (clone 93H1) and phosphorylated Erk1/2 (clone D13.14.4E).
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

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 IL-2 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 RTqPCR as detailed in the supplementary material.

Results

Selection of MK-4166 and epitope matching with DTA-1

Based on several reports which indicate that GITR signaling enhances anti-tumor immunity in mouse models, a humanized IgG1 agonist mAb against human GITR, MK-4166, was developed to treat patients with advanced malignancies. Since the anti-mouse GITR mAb DTA-1 has shown impressive anti-tumor 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 Figure 1A. Six out of the 7 key residues were different in mice compared to 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 pM and 7.6 pM, 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 pM (Supplementary Table
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

S1) by cell-based KinExA®, and was comparable to that of MK-4166 for human and cynomolgus monkey GITR.

**MK-4166 and DTA-1 co-stimulate naïve T cells resulting in enhanced proliferation**

A dose-dependent augmentation of T cell proliferation was observed when human or cynomolgus monkey naïve CD4+ T cells were co-stimulated 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 co-stimulated 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 pM, respectively (Supplementary Table S2). The median EC50 value for DTA-1 in mouse T cells was 99 pM (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 co-stimulate 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, TH17 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 up-regulation 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). 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 B). 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 to that on mouse TILs from ten 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 22-42% of CD4+ TILs, while 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 B) and intensity (Fig. 4D) of GITR expression were considerably higher on Tregs compared to 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...
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

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 Tregs have been shown to play a central role in anti-tumor efficacy of DTA-1 and the expression of GITR was the highest on human intratumoral Tregs as compared to 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+ FoxP3<sup>high</sup> and the expression of GITR in this population was confirmed by flow cytometry (Supplementary Fig. S5A and B). Since activated T cells can express low levels of FoxP3, we confirmed that the iTregs were functionally suppressive (Supplementary Fig. S5C and D). The addition of MK-4166 to MLR cultures on day 0 resulted in a dose dependent decrease in abundance of iTregs after 7 days, measured either as relative 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 C). 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-4166 does demonstrate the potential to induce ADCC in human MLR-derived iTregs using a Jurkat reporter cell line (Supplementary Fig. S7A and B).

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+CD127<sup>low</sup> 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 B). 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 $^3$H-thymidine incorporation (Supplementary Fig. S8C). The purity of nT$\text{regs}$ (CD4+FoxP3+CD127$^{low}$) used in the nT$\text{reg}$ 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 to isotype matched controls at several Teff:nT$\text{reg}$ ratios tested (Fig. 5C and D), indicating that MK-4166 partially attenuates the suppressive effects of T$\text{regs}$. MK-4166 did not enhance proliferation of Teffs alone (Teff:nT$\text{reg}$ ratio 1:0; Fig. 5C and D; Supplementary Fig. S8A-C) indicating that there was no co-stimulatory effect of GITR agonism in this assay. Additionally, MK-4166 did not enhance the proliferation of nT$\text{regs}$ in an independent experiment where nT$\text{regs}$ were stimulated by anti-CD3/anti-CD28 coated beads (Supplementary Fig. S10A and B).

**MK-4166 engagement triggers NFκB phosphorylation in T$\text{regs}$ and Teff**

Published reports indicate that GITR signaling is mediated by NFκB and MAP kinases p38, JNK, and ERK in mice (31-35). To determine if 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 T$\text{regs}$ (CD4+FoxP3+) or to Teffs (CD4+FoxP3-) 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 T$\text{regs}$ 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-NF\(\kappa\)B levels compared to 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 (CRC) 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 T\(_{\text{regs}}\) decreases expression of FoxP3 and causes instability of T\(_{\text{reg}}\) 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 anti-cancer
therapies (6). In particular, the agonist anti-GITR antibody DTA-1 has impressive anti-tumor efficacy in murine syngeneic tumor models (21,23-26). Based on these observations, we developed MK-4166, a humanized agonist mAb against human 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 \textit{in vivo} (20) and to decrease the induction of Tregs in murine splenocytes treated \textit{in vitro} with TGFβ (21). In contrast, GITR agonism (with DTA-1 or mouse GITRL) has been reported to enhance proliferation of Treg \textit{in vitro} and \textit{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 \textit{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 since the decrease in the number of Tregs was also seen with MK-1248 (an IgG4 which has the same CDRs but possess minimal Fc effector functions) and only when MK-4166 was added at the beginning of the co-culture. 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 \textit{de novo} generation in the tumor.
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 Tregs 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 Tregs in the spleen and to a lesser extent in tumor. In both tissues a decrease in the activation marker ICOS was observed on Tregs, 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).

Acknowledgements

The cell lines MB49 and TC-1 were a generous gift from Dr. Michael O'Donnell (University of Iowa) and Dr. Tzyy-Chou Wu (Johns Hopkins University). We thank Gary Starling for a critical review of the manuscript.

References

Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb


Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb


Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb


33. Esparza EM, Arch RH. Glucocorticoid-induced TNF receptor functions as a costimulatory receptor that promotes survival in early phases of T cell activation. J Immunol 2005;174:7869-74


Figure Legends

Figure 1. Epitopes of DTA-1 and MK-4166. (A) Partial sequence alignment of mouse and human GITR. Residues critical for DTA-1 binding to mouse GITR and MK-4166 binding to human GITR are boxed. The connecting lines represent disulphide connectivity predicted using homology modeling. Residue numbering is according to Nocentini et al (42) (B) Binding of the parental mouse clone of MK-4166 to GTR80 (modified mouse GITR with six residues mutated to match human GITR - K59R, Y61H, D74K, I75F, V76S, R80Q), human GITR-His and mouse GITR-His as determined by ELISA. The mean and S.D. of three independent experiments are shown.

Figure 2. Co-stimulation of naïve peripheral T cells from human, cynomolgus monkey or mouse blood and from human TILs by agonist GITR antibodies. Naïve 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) Naïve 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 4 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.

Figure 3. Expression of GITR in blood of humans, cynomolgus monkeys and mice. (A & B) The frequency of GITR+ cells on the indicated cell types from healthy human donors (n=3-15), cancer patients (n=7-22), cynomolgus monkey (n=15-30), healthy mice (n=5-10) and tumor bearing mice (n=5) was evaluated by flow cytometry. (C) The frequency of GITR+ cells on the indicated T cell subtypes
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb

from healthy human donors (n=8). The gating scheme used to identify these cells is shown in Supplementary Figure S1. (D) The frequency of GITR+ cells on naïve CD4+ T cells (CD45RA+CCR7+), CD4+ T_{CM} (CD45RA-CCR7+), and CD4+ T_{EM} (CD45RA-CCR7-). The dark histograms show labeling with control Ab while light gray histograms show labeling with MK-4166. Naïve, T_{CM} and T_{EM} were FACS-sorted based on the gating scheme shown, stimulated with anti-CD3 and anti-CD28 microbeads (at a bead-to-cell ratio of 1:2) in complete RPMI-1640 and expression of GITR on their surface was determined on day 2 post-activation by flow cytometry. One of two representative donors is shown.

Figure 4. Expression of GITR on human and mouse tumor-infiltrating lymphocytes. (A) The frequency of GITR+ human CD4+ and CD8+ TILs in non-small cell lung carcinoma (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 per model) was determined by flow cytometry. The frequency (C) and the geometric mean fluorescence intensity (D) of GITR expression in T_{effs} (CD4+CD25-) and T_{regs} (CD4+CD25+) in tumor tissues obtained from patients with non-small cell lung carcinoma (NSCLC). *** indicates a p-value < 0.001 calculated by paired student t-test. The trend lines in panel 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 standard deviation.

Figure 5: Effect of MK-4166 on iT_{reg} generation and nT_{reg}-mediated suppression of T_{eff}. (A & 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 iT_{regs} (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 is shown and 1-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&D) CFSE-labeled T_{effs} were stimulated with anti-CD3 in cultures containing lethally irradiated autologous HLA-DR+ feeder cells. The ability of responder
matched nT\textsubscript{reg} to inhibit proliferation of CD4+ (C) or CD8+ (D) T\textsubscript{eff}\nT\textsubscript{reg} ratios was measured as dilution of CFSE-label intensity in the presence of MK-4166 or isotype matched control mAb. Data shown are representative of three donors. * indicates a p value < 0.05 calculated by paired student t-test. Symbols *, ** and *** indicate statistical significance at p < 0.05, p < 0.01 and p < 0.0001 respectively.

**Figure 6. Effects of MK-4166 engagement of GITR on CD4+ T cells derived from human MLR cultures and tumors.** (A) The fraction of T\textsubscript{reg} or T\textsubscript{eff} expressing GITR and able to bind DyLight650-labeled MK-4166 or MK-1248 was evaluated using flow cytometry. (B) DyLight650-labeled MK-4166 or DyLight650-labeled isotype matched control antibody was added to MLR cultures and the level of phosphorylated NF\kappa B p65 in CD3+CD4+ T cells was measured using flow cytometry after 5, 10, 15, or 30 minutes. The level of NF\kappa B phosphorylation in GITR+ CD4+ T cells was compared against GITR- CD4+ T cells. (C) Induction of phosphorylated NF\kappa B, 5 minutes after treatment with MK-4166, MK-1248 or isotype matched control antibody is shown as fold change over media control. (D) In the samples treated with MK-4166 or MK-1248 the geometric mean fluorescence intensity of phosphorylated NF\kappa B labeling at 5 minutes is compared between T cells expressing GITR (DyLight650-positive) and T cells not expressing GITR (DyLight650-negative). Aggregate data from 4 (MLR) or 5 (NSCLC) donors is shown in panels A, C and D. Symbols * and ** indicate statistical significance at p < 0.05 and 0.01, using the paired, two-tailed t-test.

**Figure 7. MK-4166 affects gene expression in ex vivo treated human TILs.** Single cell suspensions from human RCC (n=6), NSCLC (n=3) or 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 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.
Sukumar, et al. Characterization of MK-4166, an agonistic anti-GITR mAb
Figure 1

A

mGITR  |  CSLYAPGKED  |  CPKERCIVTPEYHGD|  DPQCKIC|  KHPPQGQRVESQGDIVFGFRC

hGITR  |  CRVHTTRCRCRDYPGEECSEWD|  CMCVQPFHGDPCCTC|  RHPCPPGQVQSQKFSFGFRC

B

ABTS OD 405nm

GTR80-His
murine GITR-His
human GITR-His

Downloaded from cancerres.aacrjournals.org on June 22, 2017. © 2017 American Association for Cancer Research.
Figure 2

A

B

C

D

Downloaded from cancerres.aacrjournals.org on June 22, 2017. © 2017 American Association for Cancer Research.
Figure 3

A

B

C

D

Downloaded from cancerres.aacrjournals.org on June 22, 2017. © 2017 American Association for Cancer Research.
Figure 4

A

% GITR Positive

CD8+ TILs

CD4+ TILs

NSCLC  Melanoma  RCC

B

% GITR Positive

CD4+ TILs

CD8+ TILs

MC38  MB49  LL2  B16F10  TC1  Renca  4T1  CT26  EMT6  CM5

C

% GITR Positive

CD4+ CD25-  CD4+ CD25+

D

GITR MFI

CD4+ CD25-  CD4+ CD25+

E

CD4+

GITR+ 48.5%

CD4+ CD25+

GITR+ 81.3%

CD8+

GITR+ 8.87%

F

CD4+

GITR+ 85.9%

CD4+ CD25+

GITR+ 100%

CD8+

GITR+ 99.2%
Figure 5

A

% CD25+ FoxP3^high of total CD4+ T cells

B

% CD25+ FoxP3^high of total CD4+ T cells

C

CD4+ Teff

D

CD8+ Teff

Downloaded from cancerres.aacrjournals.org on June 22, 2017. © 2017 American Association for Cancer Research.
Figure 7

(A) DUSP6

(B) FOXP3
Characterization of MK-4166, a clinical agonistic antibody that targets human GITR and inhibits the generation and suppressive effects of T regulatory cells

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

Cancer Res Published OnlineFirst June 13, 2017.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-16-1439

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2017/06/13/0008-5472.CAN-16-1439.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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

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

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.