GITR Agonism Enhances Cellular Metabolism to Support CD8\(^+\) T-cell Proliferation and Effector Cytokine Production in a Mouse Tumor Model

Simran S. Sabharwal\(^1\), David B. Rosen\(^1\), Jeff Grein\(^1\), Dana Tedesco\(^1\), Barbara Joyce-Shaikh\(^1\), Roanna Ueda\(^1\), Marie Semana\(^2\), Michele Bauer\(^2\), Kathy Bang\(^2\), Christopher Stevenson\(^2\), Daniel J. Cua\(^1\), and Luis A. Zúñiga\(^1\)

Abstract

GITR is a costimulatory receptor currently undergoing phase I clinical trials. Efficacy of anti-GITR therapy in syngeneic mouse models requires regulatory T-cell depletion and CD8\(^+\) T-cell costimulation. It is increasingly appreciated that immune cell proliferation and function are dependent on cellular metabolism. Enhancement of diverse metabolic pathways leads to different immune cell fates. Little is known about the metabolic effects of GITR agonism; thus, we investigated costimulation via GITR altered CD8\(^+\) T-cell metabolism. We found activated, GITR-treated CD8\(^+\) T cells upregulated nutrient uptake, lipid stores, glycolysis, and oxygen consumption rate (OCR) in vitro. Using MEK, PI3K, and metabolic inhibitors, we show increased metabolism is required, but not sufficient, for GITR antibody (DTA-1)-induced cellular proliferation and IFN\(\gamma\) production. In an in vitro model of PD-L1-induced CD8\(^+\) T-cell suppression, GITR agonism alone rescued cellular metabolism and proliferation, but not IFN\(\gamma\) production; however, DTA-1 in combination with anti–PD-1 treatment increased IFN\(\gamma\) production. In the MC38 mouse tumor model, GITR agonism significantly increased OCR and IFN\(\gamma\) and granzyme gene expression in both tumor and draining lymph node (DLN) CD8\(^+\) T cells ex vivo, as well as basal glycolysis in DLN and spare glycolytic capacity in tumor CD8\(^+\) T cells. DLN in GITR-treated mice showed significant upregulation of proliferative gene expression compared with controls. These data show that GITR agonism increases metabolism to support CD8\(^+\) T-cell proliferation and effector function in vivo, and that understanding the mechanism of action of agonistic GITR antibodies is crucial to devising effective combination therapies. Cancer Immunol Res; 6(10); 1199–211. ©2018 AACR.

Introduction

Immunotherapies have revolutionized the treatment of various cancers (1, 2). Current methods involve checkpoint receptor blockade on cytotoxic effector T cells, attenuating immune inhibitory signals and leading to tumor eradication. Despite remarkable clinical success, the majority of patients still do not respond to these drugs (3). For this reason, the next generation of immunotherapies aims to activate costimulatory receptors to help initiate antitumor responses.

The tumor necrosis factor (TNF) superfamily is a group of related costimulatory receptors that have received much interest as potential cancer immunotherapies (4). These include 4-1BB (CD137), CD27, OX40 (CD134), and glucocorticoid-induced TNFR family-related protein (GITR, CD357). All of these targets are associated with the rapid expansion of stimulated T cells, with increased proliferative gene expression; however, DTA-1 in combination with anti–PD-1 treatment increased IFN\(\gamma\) production. In the MC38 mouse tumor model, GITR agonism significantly increased OCR and IFN\(\gamma\) and granzyme gene expression in both tumor and draining lymph node (DLN) CD8\(^+\) T cells ex vivo, as well as basal glycolysis in DLN and spare glycolytic capacity in tumor CD8\(^+\) T cells. DLN in GITR-treated mice showed significant upregulation of proliferative gene expression compared with controls. These data show that GITR agonism increases metabolism to support CD8\(^+\) T-cell proliferation and effector function in vivo, and that understanding the mechanism of action of agonistic GITR antibodies is crucial to devising effective combination therapies. Cancer Immunol Res; 6(10); 1199–211. ©2018 AACR.

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Current address for S.S. Sabharwal: Pfizer, South San Francisco, California.

Corresponding Author: Luis A. Zúñiga, Merck & Company, Inc., 901 California Avenue, Palo Alto, CA 94304. Phone: 650–496–1181; Fax: 650–496–1200; E-mail: luis.zuniga@merck.com
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glycolysis and mitochondrial priming via enhanced fatty acid oxidation (FAO; refs. 15, 16). 4-1BB was also shown to enhance glycolysis and FAO to support increased T-cell proliferation (17). Conversely, signaling along the PD-L1/PD-1 inhibitory axis prevents T-cell upregulation of glycolysis while promoting lipolysis and FAO, whereas CTLA-4 signaling prevents upregulation of glycolysis and FAO, keeping T cells in a naïve-like, quiescent state (18).

We hypothesized that anti-GITR agonist therapy augments cellular metabolism in CD8+ T cells. In the current study, we demonstrated that GITR antibody therapy enhances CD8+ T-cell activation and metabolism under both suboptimal and supraoptimal stimulation conditions. Using small-molecule and checkpoint inhibitors, we demonstrated that GITR agonist-induced metabolism is required, but not sufficient by itself, for rescuing T-cell activation, depending on what other signaling pathways are being perturbed. In vivo, anti-GITR treatment also enhanced CD8+ T-cell metabolism and upregulated proliferative gene expression. These data show GITR agonism increases metabolism to support CD8+ T-cell effector function and proliferation in vivo, and understanding the mechanism of action of anti-GITR antibodies is crucial to devising effective combination therapies.

Materials and Methods

Mice and reagents

Wild-type C57BL/6J and Foxp3-GDL (C57BL/6J background) mice were obtained from The Jackson Laboratory and housed and bred under specific pathogen-free conditions in the Merck & Co., Inc. animal facility. MC38 mouse colon carcinoma cell line was obtained from the Developmental Therapeutics Program Tumor Repository (Frederick National Laboratory, Frederick, MD) and authenticated using genomic profiling (IDEXX RADIL Cell Check) and tested to be mycoplasma free (IMPACT I PCR Pro). Cells were frozen down at passage five. For each experiment, cells were thawed and placed in T75 flasks, and two days later were expanded into several T175 flasks. Three days later, cells were counted and resuspended at the appropriate concentration prior to injection into mice. Rat anti-mouse DTA-1 GITR antibody (S. Sakaguchi, Kyoto University, Kyoto, Japan) was murinized as previously described (19) for in vivo studies. A proprietary mouse anti-PD-1 (DX400) was made in-house at Merck & Co., Inc. (20). All animal studies were performed in accordance to protocols approved by Merck Research Laboratories’ Ethics board.

In vivo tumor models

For syngeneic tumor experiments, 8- to 12-week old mice were subcutaneously injected with 106 MC38 cells on the right flank. Tumor diameter was measured by electronic calipers and checkpoint inhibitors, we demonstrated that GITR agonist antibody therapy enhances CD8+ T cells were isolated using a positive selection kit (Miltenyi Biotec; cat #130-049-401).

In vitro T-cell isolation and activation

Lymphocytes were isolated from lymph nodes and spleens of naïve C57BL/6J mice. Tissue was mechanically disrupted and passed through a 70-µm filter, and red blood cells were removed using ACK lysis buffer (Gibco; cat #A1049201). CD8+ T cells were isolated using a negative selection kit (Miltenyi Biotec; cat #130-104-075) per manufacturer’s instructions (typical purity ~92-95% of live cells). Cells were plated in 6-well tissue culture plates with plate-bound antibodies. Suboptimal conditions consisted of low-dose plate-bound anti-CD3 (0.1 µg/mL). Supraoptimal conditions consisted of plate-bound anti-CD3 (10 µg/mL), anti-CD28 (2 µg/mL), and IgG1Fc (10 µg/mL). For PD-L1 inhibited cells, PD-L1 (10 µg/mL) was used instead of IgG1Fc. Cells were treated with either IgG2a (10 µg/mL; eBioscience; cat #16-4724-85 or in-house), or DTA-1 (10 µg/mL; ebioscience; cat #16-5874-83 or in-house). For small-molecule inhibitor studies, T cells were activated for 16 hours prior to addition of the inhibitors. Thirty minutes later, antibodies were added, and experiments were performed after an additional 48 hours. Etomoxir (#E1905), PD98059 (#P2125), SW30 (#S26559), and oligomycin A (#75351) were purchased from Sigma-Aldrich. SB203580 (#SYN-1074) was purchased from AdipoGen.

Western blotting

Cells were lysed in M-PER buffer (Thermo Fisher Scientific; cat #78501) with Pierce Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific; cat #88668). Lysates were separated on SDS-polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes that were blotted with primary antibodies. Blots were further incubated with secondary horseradish peroxidase-conjugated antibodies (Cell Signaling Technology) and stained with ECL reagent (Amersham). Chemiluminescence was detected on film. All antibodies were purchased from Cell Signaling Technology. Primary antibodies used were p105/p50 (Cat#3035), phospho-p105 (#4806), p100/p52 (#4882), phospho-p100 (#4810), p65 (#8242), phospho-p65 (3033), Erk1/2 (#4695), phospho-Erk1/2 (#4370), Jnk (#9252), phospho-Jnk (#9255), p38 (#5212), phospho-p38 (9211), p70S6k (#2708), and phospho-p70S6k (#9234).

Flow cytometry

Isolated cells were stained for 30 minutes in PBS, washed, and analyzed on an LSRII or LSRFortessa flow cytometer (BD Biosciences). All flow antibodies were purchased from BD Biosciences as follows: CD44 (#559250), CD62L (#564108), IL7Ra (#SYN-1074) was purchased from AdipoGen.

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Seahorse extracellular flux analysis
Seahorse tissue culture plates were coated with Cell-Tak (Corning, 22.4 µg/mL) per manufacturer’s instructions. Cells were counted on a ViCell Analyzer, and 200,000 viable CD8⁺ T cells were plated per well per manufacturer’s instructions. Seahorse media used consisted of glucose (10 mM), glutamine (2 mM), and sodium pyruvate (1 mM). For in vitro assays, basal metabolic measurements were taken followed by sequential injection of etomoxir (100 µM); Sigma-Aldrich #E1905), oligomycin (1 µM), and rotenone/antimycin A (0.5 µM/mL). For ex vivo assays, basal metabolic measurements were taken followed by sequential injections of oligomycin (1 µM/mL), FCCP (2 µM), and rotenone/antimycin A (0.5 µM/mL).

Cell viability and size
Cell viability and size were assessed using a ViCell Analyzer (Beckman Coulter) per manufacturer’s instructions.

ELISA assays
Cell culture supernatants were collected and interferon γ levels were assessed using a mouse IFNγ DuoSet ELISA kit (R&D Systems; #DY485) per manufacturer’s instructions and read on a SpectraMax microplate reader (Molecular Devices).

RNA expression analysis
For real-time PCR analysis, total RNA was isolated from cells using Arcturus PicoPure RNA Isolation method, according to manufacturer’s protocol (Thermo Fisher Scientific).

Real-time quantitative PCR for gene expression
DNase-treated total RNA was reverse transcribed using Quantitect Reverse Transcription (Qiagen) according to the manufacturer’s instructions. Primers were obtained commercially from Thermo Fisher Scientific. Primer assay ID’s were as follows: Ebi3 = Mm00469294_m1; Cxcl10/IP-10 = Mm00445235_m1; Il2 = Mm00434256_m1; Icam1 = mm00516023_m1; Nt5e – CD73 = Mm00501917_m1; Tbxi2 – Tbet = Mm00450960_m1; Socs1 = Mm00782550_s1; Bcl2l1 – Tnfrsf18 = Mm00438064_m1; Gzmb – Tnfrsf4 = Mm00439191_m1; Gzmk – Mm00438064_m1; Gzma – Mm00438064_m1; Ccnb2 – Mm00484340_m1; Slc3a2 – Mm00434256_m1; Pdk1 – Mm00434256_m1; Plscr1 – Mm00558028_m1; Birc5 – Mm00468875_m1. Gene expression was assessed using a mouse IFNγ DuoSet ELISA kit (R&D Systems) per manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed using GraphPad Prism software. Unless otherwise noted, two samples were compared using Student’s t test and multiple samples were compared using two-way ANOVA followed by the Tukey multiple comparisons test.
Here, DTA-1 treatment increased cell size relative to IgG2a isotype-treated controls (Fig. 2A). Viability of control cells declined by day 3, whereas DTA-1 attenuated this decrease (Fig. 2B). These data are consistent with reports that GITR and other TNFRs increase cell survival through regulation of antiapoptotic proteins such as Bcl-xL (23).

Surface activation markers were assessed to ascertain the extent that DTA-1 treatment augments CD8$^+$ T-cell stimulation under these conditions. IL7Ra and CD25 expression were upregulated by DTA-1 treatment under these optimal activation conditions. DTA-1 treatment also decreased expression of CD62L (Fig. 2C). Despite the supraoptimal conditions used in this study, DTA-1 still upregulated IFN\(\gamma\) expression (Fig. 2D).

TNFRs are defined by their ability to upregulate NF-\(\kappa\)B signaling. Two NF-\(\kappa\)B signaling pathways are known: the canonical pathway (NF-\(\kappa\)B1) and the noncanonical pathway (NF-\(\kappa\)B2). The relative extent by which the various TNFR members can potentiate these two distinct pathways is unclear (24). Here, we show DTA-1 treatment leads to elevated phosphorylation, and therefore activation of p105 (NF-\(\kappa\)B1), p100 (NF-\(\kappa\)B2), and p65 (RelA; Fig. 2E). Although both NF-\(\kappa\)B pathways are activated, DTA-1 enhanced the amount of total NF-\(\kappa\)B2 protein, matching gene-expression data demonstrating significant DTA-1–induced upregulation of \(N\)f\(\kappa\)b2 message without upregulation of \(N\)f\(\kappa\)b1 message (Fig. 2F).

Ikba and \(Gadd45b\), two target genes of NF-\(\kappa\)B, are also upregulated. These data demonstrate increased NF-\(\kappa\)B activity downstream of anti-GITR agonism.

Despite the lack of increased proliferation with DTA-1 under optimal stimulation conditions, we observed significant increases in both the ECAR and OCR (Fig. 2G). Two days after stimulation, the DTA-1–induced increase in OCR is entirely due to increased FAO, as indicated by the etomoxir-sensitive portion of basal OCR (Fig. 2G, panel 3). Etomoxir inhibits carnitine palmitoyl transferase 1a (CPT1a), the rate-limiting step of FAO. However, 3 days after activation, there is virtually no etomoxir effect in either control or DTA-1–treated cells, suggesting a shift in substrate utilization by the mitochondria (Fig. 2G, panel 4).

Figure 1.
Costimulation with the mouse GITR agonist antibody, DTA-1, enhances activation and metabolism in CD8$^+$ T cells stimulated with low-dose anti-CD3. A, Representative CellTrace Violet FACS plots of IgG2a control versus DTA-1–treated CD8$^+$ T cells 3 days after activation. B, Proliferation results of 4 independent experiments. Oxygen consumption rate (OCR; C) and glycolytic rate [extracellular acidification rate (ECAR); D]. N = 3. E, Uptake of the fluorescent glucose analogue 2-NBDG at 72 Hours; N = 5. F, ELISA results for interferon \(\gamma\) (IFN\(\gamma\)) levels; N = 3. Data are shown as mean ± SEM. *\(P\) ≤ 0.05 using Student t test.
DTA-1 treatment significantly increased 2-NBDG uptake. Cells can increase uptake of other nutrients to feed their energy demands, and DTA-1 treatment also increased medium-chain and long-chain fatty acid uptake and increased intracellular lipid stores, assessed by BODIPY staining (Fig. 2H). Lipid stores can be mobilized for ATP production via mitochondrial oxidative phosphorylation (OXPHOS). These data suggest that anti-GITR treatment increases CD8⁺ T-cell fitness in vitro by improving nutrient uptake and allowing cells to have increased flexibility in altering the carbon sources they use to meet their energy and biosynthetic needs.

A panel of genes was associated with increased CD8⁺ T-cell proliferation, activation, or function (Fig. 2I). These include upregulation of IL2 message and its receptor, CD25, downregulation of inhibitory receptor CD73, and confirmation of Bcl-xl upregulation. DTA-1 treatment also upregulated Tbet transcripts, which is important for induction of a type I cytotoxic T-cell (Tc1) phenotype critical for CD8⁺ T-cell–mediated tumor killing (25).

Transcripts for several metabolic targets were also upregulated with DTA-1 treatment (Fig. 2J). These include upregulation of the master metabolic transcription factor c-myc, as well as several other metabolic enzymes and solute transporters (26–28). These data cumulatively suggest that DTA-1 treatment increases global cellular metabolism, even when proliferation is not enhanced.

DTA-1–induced cellular proliferation requires increased glycolytic and mitochondrial metabolism

We next performed experiments following stimulation conditions described above and using 2-deoxyglucose (2-DG), a competitive inhibitor of glycolysis, at a dose sufficient to highly attenuate glycolysis but not completely abolish it. Under these conditions, we demonstrate that DTA-1 is unable to rescue OCR (Fig. 3A), ECAR (Fig. 3B), 2-NBDG uptake (Fig. 3C), proliferation (Fig. 3D), and gene expression (Fig. 3E). These data collectively support the hypothesis that DTA-1 treatment increases global cellular metabolism, even when proliferation is not enhanced.
or IFNγ production (Fig. 3E) by CD8⁺ T cells. Although the metabolic and IFNγ 2-DG isotype controls trend downward versus vehicle controls, there is no significant difference between these groups. Proliferation, however, is significantly blunted in control 2-DG cells, and being unable to rescue metabolic function, DTA-1 is incapable of rescuing cellular proliferation.

Incubating cells with etomoxir to inhibit FAO did not significantly decrease metabolic function, though there is a slight trend downward when comparing DTA-1–treated groups (Fig. 3A–C). Although 100 μmol/L etomoxir is sufficient to fully inhibit FAO upon acute administration during Seahorse experiments, that concentration only partially inhibits FAO after 2 days of incubation. This is supported by the fact that the DTA-1–induced increase in OCR of etomoxir-incubated cells (Fig. 3A) is completely FAO dependent and etomoxir sensitive (Supplementary Fig. S1B). With only partial inhibition of FAO, there is still a significant decrease in cellular proliferation (Fig. 3D; Supplementary Fig. S1A) that DTA-1 is unable to rescue. DTA-1’s inability to rescue proliferation in etomoxir-incubated cells may be dependent on increasing OCR, which indicates that increased FAO following DTA-1 treatment supports increased proliferation.

IFNγ levels trend down in isotype controls with etomoxir treatment, though there is a significant increase with DTA-1 (Fig. 3E). This increase, however, is still significantly lower than DTA-1–treated vehicle controls. These data suggest that FAO may play a role in IFNγ production, though this effect may be due to the proliferative advantage seen in control cells or confounded by only partially inhibiting FAO with etomoxir incubation.

We next used oligomycin to inhibit ATP synthase and block mitochondrial ATP synthesis. OCR was severely attenuated and DTA-1 could not rescue it (Fig. 3F). ECAR was significantly upregulated in isotype control cells with oligomycin, whereas DTA-1 treatment further increased ECAR (Fig. 3G). DTA-1 treatment significantly upregulated 2-NBDG uptake compared with isotype and oligomycin-treated cells (Fig. 3H). Without mitochondrial ATP production, there was a proliferative disadvantage in oligomycin-treated cells that DTA-1 administration was unable to rescue.
to rescue, which highlights the importance of mitochondrial respiration for basal and DTA-1–induced cellular proliferation (Fig. 3I; Supplementary Fig. 5IC). There was a comparable amount of IFNγ production versus vehicle controls (Fig. 3J), despite the reduced proliferation in oligomycin-treated isotype controls (93.7% vs. 47.6%; Fig. 3K), further demonstrating the importance of increased glycolytic function on IFNγ production. DTA-1 significantly increased IFNγ production in cells treated with oligomycin. Although these DTA-1–induced levels were significantly lower than DTA-1 control levels, this is likely due to the lower number of proliferating cells in the oligomycin group (95.6% vs. 45.6%). Collectively, these data underscore the central role of metabolism in cellular proliferation and IFNγ production.

**DTA-1 upregulates MAPK signaling and can rescue CD8+ T cells from MEK inhibition**

TNFRs also signal through the p38, JNK, and ERK MAPK pathways. There are conflicting reports as to which pathways are activated in specific T-cell subsets, depending on which TNFR is involved (12, 29, 30). Here, we demonstrate that phosphorylation and activation of all three MAPK pathways are enhanced by DTA-1 (Fig. 4A). Activation of these pathways in control conditions appears to decrease between 48 and 72 hours, whereas DTA-1–treated cells display enhanced signaling during the same time interval.

To dissect which MAPK pathways are involved in regulating the observed DTA-1–induced changes, we used the p38 inhibitor SB203580 and the MEK inhibitor PD98059. We found that p38 inhibition of isotype-treated cells had no effect on cellular metabolism (Fig. 4B and C) or 2-NBDG uptake (Fig. 4D), whereas MEK inhibition significantly decreased all metabolic readouts. DTA-1 increased metabolic parameters of all treatment groups, including rescue of MEK–inhibited cells to vehicle/lg2A control levels.

Many receptor signals activate both the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways, which both play a role in cell growth and proliferation (31). We hypothesized that GTR agonism by DTA-1 may rescue MEK inhibition, in part, by upregulating the PI3K signaling axis. p70S6k is a kinase downstream of mTOR that is specifically activated by the Akt pathway (32). p70S6k levels decreased with MEK inhibition (Fig. 3E), likely due to cross-talk between the two pathways (31), but DTA-1 treatment rescued the amount of phosphorylated, activated enzyme. Phospho-Akt and phospho-4EBP1, another mTOR-regulated protein, were increased (Supplementary Fig. S2A).

We next used the PI3K inhibitor SB30, together with MEK inhibition (33). Both PD98059 and SW30 attenuated 2-NBDG uptake (47.3% and 40.5%, respectively), and DTA-1 rescued both to vehicle/lg2a control levels (Fig. 4F). The combination of the two drugs reduced 2-NBDG uptake further (59.7% reduction). Although DTA-1 increased 2-NBDG levels when administered with the inhibitor combination, the levels were significantly lower than DTA-1–rescued levels of either small molecule alone. OCR (Fig. 4G) and ECAR (Fig. 4H) were comparably affected. As described earlier (Fig. 2G), the DTA-1–induced increase in OCR 2 days after dosing can be wholly attributed to an increase in FAO, as the increase is etomoxir sensitive. With small-molecule inhibitors, however, OCR is still significantly higher in DTA-1–treated cells after etomoxir administration, compared with isotype controls. This may be due to additional impairment in access to or utilization of other carbon sources for fuel in isotype-treated groups, though further studies are needed to explore this.

Increased pathway signaling and metabolic rescue via DTA-1 treatment were sufficient to rescue cellular proliferation (Fig. 4I; Supplementary Fig. S2B–S2D). There was significant rescue of IFNγ levels by DTA-1 treatment when assessed using multiple different t tests (Fig. 4J, panel 1, untransformed data). The large IFNγ concentration in DTA-1–treated cell controls required a log transformation of the data to compare values between treatment groups. ANOVA of the transformed data showed that DTA-1 did rescue MEK inhibition IFNγ levels to isotype vehicle control levels (Fig. 4J, panel 2, transformed data). DTA-1 also significantly increased IFNγ levels in PI3K inhibiting-treated cells, though this was significantly lower than the MEK–inhibited and vehicle control cells treated with DTA-1. DTA-1 did not rescue the IFNγ levels in the combination treatment group. These data show that, although metabolic rescue may be sufficient for increased proliferation, increased metabolism alone is not sufficient to rescue effector function, as indicated by IFNγ levels.

**Combined checkpoint blockade and anti-GITR therapy overcome PD-L1–induced T-cell inhibition**

As current clinical immunotherapy strategies involve combination treatment with immune-checkpoint inhibitors, we sought to test if anti-GITR treatment would beneficially combine with anti–PD-L1 administration in an in vitro system. In addition to stimulating CD8+ T cells with anti–CD3/anti–CD28, we used plate-bound PD-L1 to inhibit activation and simulate PD-L1–associated immunosuppression that T cells may experience in certain TMEs.

PD-L1 inhibition decreased cell viability (Fig. 5A). Monotherapy with either anti-GITR agonism or PD-1 blockade partially rescued viability, whereas combination therapy significantly restored cell viability to uninhibited levels. A similar pattern was seen with basal OCR (Fig. 5B), basal ECAR (Fig. 5C), and 2-NBDG uptake (Fig. 5D). Cellular proliferation showed a similar response (Fig. 5E). PD-L1 attenuated the percentage of cells undergoing four or five cell divisions (gate 5 and 6, respectively), while increasing the percentage of cells that underwent only one or no cell divisions (gates 2 and 1, respectively). Monotherapy partially rescued PD-L1–associated inhibition of proliferation, whereas combination therapy rescues it further.

Although PD-L1 signaling abrogated IFNγ production, DTA-1 treatment alone did not significantly rescue production of this cytokine, whereas anti–PD-1 monotherapy displayed partial rescue (Fig. 5F). Combination therapy combined to fully restore IFNγ production to non-PD-L1–treated levels. Although enhanced glycolytic flux can lead directly to enhanced IFNγ production, here, DTA-1 monotherapy rescues ECAR and 2-NBDG uptake (Fig. 5C and D, respectively), but not IFNγ production. These data suggest that metabolic enhancement alone is not sufficient to rescue IFNγ production, indicating that other signals through the PD-L1/PD-1 axis inhibit IFNγ production.

**DTA-1 treatment in a mouse model enhances CD8+ T-cell activation and proliferation in vivo**

After demonstrating that anti-GITR agonism alters CD8+ T-cell activation, PI3K/MEK/mTOR signaling, and metabolism in vitro, we wanted to test whether similar changes are observed in vivo. To
this end, we challenged mice with syngeneic MC38 colon cancer cells, which are known to respond well to anti-GITR therapy. Tumors were harvested 8 days after treatment, at a time where tumor regression is just beginning to occur (Fig. 6A). DTA-1–treated TIL and DLN CD8<sup>+</sup>T cells also had enhanced PI3K/MEK/mTOR signaling (Supplementary Fig. S3A and S3B). DTA-1–treated DLN were significantly larger than IgG2a-treated DLN, suggesting substantially more cellular proliferation was occurring in the DLN from DTA-1–treated mice (Fig. 6B). This increase in cellular proliferation is further verified by upregulation of a panel of proproliferative genes in DLN CD8<sup>+</sup>T cells (Fig. 6C) and Ki67 staining (Supplementary Fig. S3C).

Gene transcripts for cytotoxic effector molecules were significantly upregulated in both TIL and DLN CD8<sup>+</sup>T-cell populations (Fig. 6D). Ifng, Gzma, Gzmb, and Gzmk transcripts were significantly elevated in response to DTA-1 treatment. These data suggest that DTA-1 promotes a Tc1 phenotype that enhances antitumor immunity.

GITR and other TNFRs are also associated with enhanced memory cell formation (34). As expected, DTA-1 treatment increased the CD44<sup>+</sup>CD62L<sup>−</sup>effector memory and CD44<sup>+</sup>CD62L<sup>+</sup>central memory pools relative to IgG2a controls (Fig. 6E). Enhanced memory formation by DTA-1 was supported by increased gene transcript of the memory marker Klrg1 (35) in both TIL and DLN (Fig. 6F). This indicated that the T-cell population within the DLN was a complex mixture of naive, newly activated, and memory cells. The shift from naive cells toward effector and memory cells, along with our previous in vitro data, implies GITR agonism may participate in the priming phase of CD8<sup>+</sup>T cells (36).

Because DTA-1 treatment depletes TIL, not DLN Tregs (19), the increased CD8<sup>+</sup>T-cell proliferation in the DLN, and the increased...
effector molecule transcript levels can be attributed to GITR agonist effects of the DTA-1 antibody. This suggests that GITR agonism contributes to CD8\(^+\) T-cell expansion and priming in the DLN to enhance antitumor immunity.

GITR agonism increases CD8\(^+\) T-cell metabolism in the DLN and tumor of MC38-bearing mice

DTA-1 treatment in MC38-bearing mice significantly increased both OCR and ECAR in DLN CD8\(^+\) T cells (Fig. 7A and B, respectively). TIL CD8\(^+\) T cells also had significantly increased OCR (Fig. 7C). Although reports indicate improved effector function is generally accompanied by increased glycolysis (13, 14), the increase we saw in TIL CD8\(^+\) T-cell ECAR with DTA-1 treatment was not significant, although 2 of 3 experiments showed increases (Fig. 7D). Several reports have highlighted the importance of mitochondrial function on proper effector T-cell performance (37–40), although no clear mechanism of action has yet been described. It is possible that the increased OCR seen with DTA-1 allows effector CD8\(^+\) T cells to function properly in the TME. TIL CD8\(^+\) T cells from DTA-1–treated mice had significantly increased spare glycolytic capacity (Fig. 7E), which indicates a cell’s ability to respond to cellular stress and increased energetic demands.

TIL CD8\(^+\) T cells did not display the same proliferative gene signature seen in DLN CD8\(^+\) T cells but did regulate several metabolic gene transcripts affected by DTA-1 treatment (Fig. 7F). DTA-1 also significantly increased BODIPY staining of internal lipid stores in DLN CD8\(^+\) T cells, whereas TIL CD8\(^+\) T cells trended upward (Fig. 7G).

These data suggest that anti-GITR agonism significantly increases CD8\(^+\) T-cell metabolism during the priming phase in the DLN, as well as during the effector phase inside the TME, thereby increasing T-cell fitness and enhancing the CD8\(^+\) T-cell–mediated antitumor response.

Discussion

In this study, we aimed to better understand the mechanism of action of anti-GITR agonism, as opposed to the contribution of Treg depletion, on antitumor efficacy. Here, we show that DTA-1 treatment upregulated OCR and ECAR in CD8\(^+\) T cells both in vitro and in vivo. In vitro, we demonstrated that GITR agonism...
increases cellular proliferation and IFNγ production, and that metabolic changes elicited by DTA-1 treatment were required, but not sufficient by themselves, for those changes. In vivo, enhanced metabolism was accompanied by increased proliferation in the DLN and increased effector molecule transcription in the DLN and TIL populations.

Understanding the mechanism of action of DTA-1–mediated signaling will better inform upon how GITR agonist therapy will combine with other immuno- and chemotherapies.

Costimulatory signals are reported to increase FAO, which supports multiple CD8⁺ T-cell functions (16, 17). CD8⁺ T cells in hypoxic and hypoglycemic TMEs enhance fatty acid catabolism to maintain effector function, mainly by utilizing endogenous fatty acids (41). Our in vitro data show that DTA-1 increased FAO and internal lipid stores. Our in vivo data also demonstrate increased lipid droplet formation in DLN CD8⁺ T cells. Whether DTA-1–induced increases in lipid stores during activation in the DLN are then mobilized upon entry into the TME to help fuel effector function is not yet known.

Tumor cells outcompeting T cells for nutrients in the TME is one mechanism of action of tumor immunosuppression (22). One study shows that enhancing tumor cell metabolism converts a regressive murine cancer line into a progressive cancer (21). In cases of nutrient competition, boosting T-cell metabolism with a GITR agonist antibody may prove beneficial. However, repression of T-cell metabolism can also result from direct interaction between T cells and tumor cells or suppressive immune cells, or indirect interaction, via metabolites such as adenosine, or tryptophan depletion via IDO overexpression (42, 43). In these cases, GITR agonism may prove insufficient in rescuing an antitumor immune response. It is vital to understand which immune inhibitory pathways can or cannot be overcome by GITR agonism in order to devise the most effective combination therapy strategies.

Our finding that GITR agonism can potentiate T-cell activation and function has potential therapeutic relevance. MEK inhibitors are FDA-approved against melanomas with certain mutations, and ongoing clinical trials are testing these inhibitors with checkpoint blockade therapy. Previous work showed that MEK
inhibition reduces T-cell-receptor–induced apoptosis that typically occurs in exhausted T cells in the TME, leading to increased efficacy when paired with anti–PD-1 blockade (44). The study, however, notes that T-cell priming in the DLN is suppressed by MEK treatment. Our data show that GITR agonism can rescue the MEK inhibitor-associated decreases in metabolism, proliferation, and IFNγ production, which suggests that adding anti-GITR treatment to the MEK/anti–PD-1 combination may boost antitumor clearance further by enhancing activation in the DLN. Indeed, other murine studies show that triple combination therapy of TNFR agonist antibodies with MEK inhibitor/anti–PD-1 therapy improves tumor clearance significantly compared with MEK/anti–PD-1 combination alone (45, 46). The TNFR antibodies in these studies target 4-1BB and OX40, but a GITR agonist antibody is likely to act similarly. Although these studies did not identify a molecular mechanism of action, it is probable that the enhanced PI3Kα/Ark/mTOR signaling and augmented metabolic function that we have ascribed to GITR agonism plays a role in rescuing MEK inhibition of T-cell activation in the DLN; however, further studies are required to confirm this hypothesis.

Although much of the focus has remained on the Treg depletion effects of DTA-1, it is still unclear to what extent Treg depletion contributes to efficacy. Several reports demonstrate that Treg depletion alone does not account for the antitumor effects of GITR treatment. Our study substantiates that GITR agonist effects of DTA-1 are necessary for proper tumor clearance (19, 47, 48). Immune cell metabolism is increasingly appreciated for its role in influencing immune cell function. Here, we elucidated some of the metabolic effects of anti-GITR agonism to better understand the mechanism of action of GITR agonism-induced tumor clearance. Current cancer treatment strategies are increasingly focused on combination therapies, with anti–PD-1 therapy as the foundation (49). Understanding the mechanism by which anti-GITR treatment increases metabolic function, and circumstances by which this increased metabolism can rescue T-cell proliferation and effector function can provide improved insight into the effects of combining small molecules and immunotherapies to modulate immune cell metabolism. These insights may lead to enhanced therapeutic strategies that will improve patient outcomes.
Disclosure of Potential Conflicts of Interest

D.B. Rosen reports receiving commercial research support from and has ownership interest (including stock, patents, etc.) in Merck Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S.S. Sabharwal, D.B. Rosen, B. Joyce-Shaikh, L.A. Zúñiga

Writing, review, and/or revision of the manuscript: S.S. Sabharwal, D.B. Rosen, J. Grein, D. Tedesco, K. Bang, L.A. Zúñiga

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.S. Sabharwal, D.B. Rosen, R. Ueda, L.A. Zúñiga

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.S. Sabharwal, D.B. Rosen, J. Grein, D. Tedesco, K. Bang, L.A. Zúñiga

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Bang, C. Stevenson, L.A. Zúñiga

Study supervision: S.S. Sabharwal, R. Ueda, L.A. Zúñiga

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References

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Simran S. Sabharwal, David B. Rosen, Jeff Grein, et al.


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