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Murinization and H Chain Isotype Matching of the Anti-GITR Antibody DTA-1 Reduces Immunogenicity-Mediated Anaphylaxis in C57BL/6 Mice

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Recent advances in immuno-oncology have shown that the immune system can be activated to induce long-term, durable antitumor responses. For immuno-oncology drug development, immune activation is often explored using rat Abs in immunocompetent mouse models. Although these models can be used to show efficacy, antidrug immune responses to experimental protein-based therapeutics can arise. Immunogenicity of surrogate Abs may therefore represent an important obstacle to the evaluation of the antitumor efficacy of immunomodulator Abs in syngeneic models. A recent publication has shown that anti-glucocorticoid-induced TNFR family-related protein agonistic Ab DTA-1 (rat or murinized IgG2a) can induce the development of anaphylaxis in C57BL/6 mice upon repeated i.p. dosing because of an anti-idiotypic anti-drug Ab immune response. This study was undertaken to address the impact of the immunogenicity derived from the Fc and variable domains. To this end, chimerized (rat V domains/mouse constant regions) and murinized (95% mouse sequence) DTA-1-based surrogate Abs with a murine IgG2c H chain isotype were created. Chimerization and murinization of DTA-1 did not affect receptor binding and glucocorticoid-induced TNFR family-related protein-induced T cell agonistic properties. Similar in vivo antitumor efficacy and intratumoral CD8⁺/regulatory T cells were also observed. Finally, treatment of C57BL/6 mice with the chimerized and murinized DTA-1 Abs on a C57BL/6-matched IgG2c isotype resulted in reduced development and severity of anaphylaxis as measured by decline of body temperature, behavioral effects, serum IL-4, IgE, and anti-drug Ab levels. These results suggest that careful murinization and selection of a strain-matched H chain isotype are critical to generate ideal surrogate Abs for testing immuno-oncology mechanisms in vivo. *The Journal of Immunology*, 2017, 198: 000–000.

Cancer immunotherapy harnesses the cytotoxic capabilities of the immune system and directs it toward the destruction of tumor cells (1, 2). Therapies directed against immune checkpoint receptors including programmed death cell protein 1 (PD-1) and CTLA-4 have proven benefits to some cancer patients in clinical trials (3–8). Recently, agonistic mAbs against the costimulatory TNFR superfamily (i.e., CD40, 4-1BB, OX40, glucocorticoid-induced TNFR family-related protein [GITR], ICOS, and CD27) have entered clinical trials (9–19). Some of these costimulatory biologics are used not only to directly induce T lymphocytes activation, but also to concomitantly inhibit regulatory T cell (T_{reg}) suppression (20, 21). GITR is a type I transmembrane protein with three extracellular TNFR domains. This TNFR is primarily expressed

on activated CD4⁺ and CD8⁺ T cells, constitutively expressed on T_{regs}, and minimally expressed on B, NK, NKT cells, and macrophages (22, 23). It has been shown in vitro that GITR engagement increases T cell activation, proliferation, and survival, and modulates T_{reg} suppressive activity (24–27). A role of GITR/GITR ligand triggering in endothelial cells (ECs) has also been suggested to regulate leukocyte adhesion and extravasation (28). Multiple studies in mice have demonstrated that GITR ligation by an agonistic GITR Ab (clone DTA-1) or an Fc–GITR ligand induces an immunological response and increases resistance to tumors and viral infections in various in vivo models (29–34). Anti-GITR (DTA-1) Ab treatment was shown to decrease Foxp3 expression and alter Foxp3 intracellular localization (30). It has also been suggested that this Ab led to a modest accumulation of CD8⁺ T cells and a decrease of T_{regs} (↑ CD8⁺/T_{regs}) in tumor, which highlights that engagement of activating FcγRs is crucial for DTA-1 antitumor efficacy (31). Other studies demonstrated that CD4⁺ T cells and B cells play a central role to support DTA-1-mediated increase of CD8⁺ T and NK activity, cytotoxicity, and degranulation (32, 33). Moreover, a separate study demonstrated that GITR triggering does not prolong the survival of mice when NK and NKT cells are depleted (34). Preclinical evidence has also demonstrated that CTLA-4 or PD-1 blockade and agonistic anti-GITR Ab are synergistic in Meth-A sarcoma, CT-26 colon, and ID8 ovarian murine cancer models (35–37).

The therapeutic effect of agonist Abs against immune costimulatory pathways has been demonstrated in several preclinical studies. However, severe toxicities occurred because of immunogenicity,

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Abbreviations used in this article: ADA, anti-drug Ab; ADCC, Ab-dependent cellular cytotoxicity; EC, endothelial cell; GITR, glucocorticoid-induced TNFR family-related protein; HEK293, human embryonic kidney 293; IC, immune complex; PD-1, programmed death cell protein 1; TIL, tumor-infiltrating lymphocyte; T_{reg}, regulatory T cell.

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autoimmunity, and cytokine release syndrome upon administration of therapeutics such as anti-CD3 and anti-CD28 mAbs (38, 39). These serious immune-related adverse events emphasize the power of the immune system and the need for a conservative trial design and extreme care when testing immune activator molecules. In this regard, several preclinical strategies have been proposed to minimize the potential risk for immunogenicity and toxicity of immunomodulatory biologics, while maintaining their therapeutic functions by approaches such as humanization, engineering of agents with fast clearance, developing better in vitro T cell activation assays, studies in transgenic animal models, and so on (40, 41). A thorough understanding of the immunopharmacology of agonistic Abs in animal models is required to predict the clinical risk for adverse immunotoxicological events and to determine a safe starting dose for first-in-human clinical studies. There is very limited information concerning the in vivo immunogenic response to mouse surrogate Abs against the costimulatory TNFR superfamily. A recent publication has shown that repetitive i.p. doses of a rat IgG2b or murinized IgG2a DTA-1 agonistic Ab in C57BL/6 mice induce anaphylaxis because of an anti-idiotypic anti-drug immune response (42). However, it has been previously reported that inbred mouse strains (C57BL/6, C57BL/10, SJL, and NOD) with the *Igh-1^b* allele do not carry the gene for IgG2a and instead express the IgG2c isotype (43–47). To gain a better understanding of the role of a strain-matched H chain isotype on anti-drug Ab (ADA) formation, we have assessed the development of an anaphylaxis response with a murinized IgG2c anti-GITR Ab in C57BL/6 mice. Our results demonstrate that the murinization of the DTA-1 Ab and inclusion of a strain-matched IgG2c H chain isotype Ab inhibits immunogenicity-mediated anaphylaxis in C57BL/6 mice. In this article, we describe the profound effects that murinization and selection of a strain-matched H chain isotype have on immunogenicity in rodents and highlight the importance of understanding the immunopharmacology of immunomodulator Abs in syngeneic models.

Materials and Methods

Abs and murinization

The parental rat IgG2b DTA-1–producing hybridoma was provided by Dr. E. Shevach (National Institutes of Health, Bethesda, MD) with authorization of Dr. S. Sakaguchi (Immunology Frontier Research Center, Osaka University, Osaka, Japan). The V region sequences of the parental Ab were cloned from the hybridoma and inserted into a pHyBE derivative vector containing publicly available sequences for the constant domain region of murine IgG2a and IgG2c to create the chimeric Abs. Murinization of chimeric forms of DTA-1 was performed as described in the *Results*. DTA-1 Abs were produced and purified using a previously reported methodology (48). The integrity of the Abs was verified by SDS-PAGE and analytical size-exclusion chromatography (% monomer >95%). The isotype control rat IgG2b (clone LTF-2) Ab used for in vivo studies was purchased from BioXCell (West Lebanon, NH). Before in vitro T cell assays and in vivo injections, endotoxin levels in Abs were confirmed to be <1 endotoxin unit/mg (Endosafe; Charles River Laboratory, Wilmington, MA).

Binding

Human embryonic kidney 293 (HEK293) or CHO-K1 cells were transfected with murine GITR or FcγR (I, IIB, III, and IV) expression constructs using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) to generate corresponding transfectants. These cell lines were FACS sorted using a MoFlo XDP cell sorter (Beckman Coulter, Brea, CA). Cells were incubated at 4°C with indicated concentrations of anti-mouse GITR Abs, and binding was detected using an AffiniPure PE-labeled F(ab')₂ fragment goat anti-mouse/rat IgG Fc fragment-specific and F(ab')₂ fragment-specific secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). The analysis was performed using FlowJo software (Tree Star, Ashland, OR). All cells were maintained from passages 3–6 in the appropriate media containing FBS (10%) at 37°C, 5% CO₂. All cell culture media and serum were purchased from Hyclone (Thermo Fisher

Scientific, Waltham, MA). In vitro data are derived from triplicates and are representative of two independent experiments.

In vitro T cell stimulation assays

CD3⁺ T cells were isolated from mouse splenocytes and lymph nodes by negative selection (STEMCELL, Vancouver, BC). T cell proliferation and viability were evaluated using a plate-bound system in the presence of accessory cells. Anti-muCD3 (1.0 μg/ml, clone 5000A2; eBioscience, San Diego, CA) in PBS was coated on a nontissue culture-treated 96-well flat-bottom plate for 2 h. After plates were washed twice with PBS, a combination of 2 × 10⁴ irradiated splenocytes and 2 × 10⁴ mouse CD3⁺ T cells was plated and treated with indicated concentrations of anti-muGITR Abs at 37°C in a CO₂ incubator. After 72 h, 1.0 μCi/well methyl-[³H]thymidine was added, and proliferation was assessed by thymidine incorporation 12 h later. Cell survival was evaluated after 120 h using CellTiter-Glo (Promega, Madison, WI). Luminescence was measured using an EnSpire Alpha fluorescence plate reader (PerkinElmer, Waltham, MA). In vitro data are derived from triplicates and are a representative of two independent experiments.

NF-κB activation reporter assay

The 293–NF-κB–reporter luciferase cell line was generated by transduction of HEK293 cells with an NF-κB lentiviral vector (Qiagen, Valencia, CA) at a multiplicity of infection of ~10:1. The 293–NF-κB–luciferase cells were transfected with a muGITR expression construct using Lipofectamine 2000 (Life Technologies) to generate a 293–NF-κB–luciferase–muGITR cell line. This cell line was functionally assayed for the stable presence of the NF-κB reporter construct using 200 ng/ml PMA (InvivoGen, San Diego, CA) and went through two rounds of FACS by surface staining with anti-muGITR Ab (BD Biosciences, San Jose, CA). Cells were incubated overnight at 37°C with indicated concentrations of anti-mouse GITR Abs. NF-κB activation levels were indirectly determined by measuring NF-κB–induced luciferase activity using Britelite Plus (PerkinElmer). Luminescence was measured using an EnSpire Alpha fluorescence plate reader (PerkinElmer). Effect of cross-linking was evaluated using a F(ab')₂ goat anti-mouse IgG, Fc fragment-specific Ab at a 4:1 cross-linker/Ab ratio (Jackson ImmunoResearch Laboratories). In vitro data are derived from triplicates and are representative of two independent experiments.

Ab-dependent cellular cytotoxicity activation reporter assay

A total of 1 × 10⁴ adherent HEK293–muGITR transfected target cells and 1.25 × 10⁴ mFcyRIV transfected Jurkat/NFAT-luciferase effector cells (Promega) were cocultured for 6 h at 37°C with indicated concentrations of anti-mouse GITR Abs. Ab-dependent cellular cytotoxicity (ADCC) was indirectly determined by measuring NFAT-mediated luciferase activity using a Bio-Glo Luciferase assay (Promega). Luminescence was measured as previously described. In vitro data are derived from triplicates and are a representative of two independent experiments.

Mice, tumor models, and treatment

Five- to six-week-old wild type C57BL/6 female mice were obtained from Taconic (Oxnard, CA) or Charles River Laboratory. C57BL/6-derived colorectal adenocarcinoma MC-38 cell line was a generous gift from Dr. Y.-X. Fu (The University of Texas Southwestern Medical Center, Dallas, TX). MC-38 cells were cultured at subconfluent density under low number of passages, and 1 × 10⁶ cells were injected s.c. onto the right flank of C57BL/6 mice. Tumor diameters were measured by electronic caliper every 3–5 d, and tumor volume was determined by length × width × height/2. For in vivo studies, tumor-bearing mice were randomized into size-matched groups 7–8 d after tumor inoculation (~100 mm³ on average) and treated by i.p. injection of Abs at indicated doses on days 7, 9, and 11 after tumor challenge. For all experiments, mice were housed at AbbVie Biotherapeutics Vivarium. All studies were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of the American Association for Assessment and Accreditation of Laboratory Animal Care (Frederick, MD).

Tumor dissociation and analyses by flow cytometry

At day 12 after treatment, mice were sacrificed and the tumors were removed. To obtain single-cell suspensions, we dissected the tumors into ~2-mm fragments followed by dissociation using a gentleMACS Dissociator system (Miltenyi Biotec, San Diego, CA) in a mixture of 2.0 μg/ml collagenase A and 0.1 μg/ml DNase I (Roche Life Science, Indianapolis, IN)



FIGURE 1. Computational modeling shows the importance of back mutations for conformational stabilization of DTA-1 after murinization using the IgG2c framework. **(A and B)** Phenylalanine (F2) within the DTA-1 V_L domain CDRs forms a tight pocket with L2/L3 [(A) ribbon diagram and (B) space-filling model]. **(C)** The original mouse V_L germline sequence contains a leucine amino acid residue at position 2. However, computational modeling reveals that a F2L mutation causes a steric clash of the DTA-1 conformation. Images were generated using PyMOL Molecular Graphics System, Version 1.8 (Schrödinger, New York, NY).

at 37°C. The digest was mashed and filtered through a 40- μ m cell strainer to remove macroscopic debris, and the final cell preparation was washed with PBS plus 1% FBS. Cells were counted and stained for flow cytometry. Fc γ receptor binding was blocked by preincubating cells with a mouse Fc γ R blocking reagent (BD Biosciences). Phenotyping of tumor-infiltrating cells was performed using fluorochrome-conjugated Abs (BD Biosciences, eBioscience) against CD45 (allophycocyanin-Cy7, 30-F11), CD4 (PerCp Cy5.5, RM4-5), CD8 (FITC, CT-CD8b/H35-17.2), CD25 (PE, PC61), and Foxp3 (allophycocyanin, FJK-16s)-specific and matching isotype control Abs. For Foxp3 intracellular staining, cells were surface stained, fixed, and permeabilized using a staining kit (eBioscience). During staining procedures, cells were kept on ice. Acquisition was performed on a BD FACS Canto II flow cytometer (BD Biosciences). At least 5×10^5 cells were acquired per sample. The machine performance was verified daily using Cytometer Setup and tracking beads (BD Biosciences). For analysis, the population of interest was gated on CD45 $^+$ lymphocytes. The analysis was performed using FACSDiva software (BD Biosciences). Before each run, a compensation matrix was generated using primary cells stained separately with individual fluorochrome-conjugated Abs.

Temperature measurement

The temperature of each animal was measured at baseline and every 15 min after induction of anaphylaxis after either the third dose (three doses, i.p., 3.0 or 50 mg/kg on days 0, 4, and 11) or the fourth dose (four doses, i.p., 3.0 mg/kg on days 0, 4, 11, and 18) by measuring the tail temperature using an infrared thermometer (Thermo Fisher Scientific). The range of body temperature loss is slightly different between results presented in Figs. 4 and 5 but is within the expected error because of different operators.

ELISA

Serum drug level determined by ELISA was used to indirectly detect sera (collected at day 21 after the fourth dose) ADAs levels of mice treated with parental, chimeric, and murinized variants of DTA-1. Plates were coated with a muG1TR-Fc fusion protein (in $1 \times$ PBS), and a goat anti-rat IgG2b-HRP (BD Biosciences) or a goat anti-mouse IgG2c-HRP (Jackson ImmunoResearch Laboratories) were used as secondary Abs. The lower limits of quantification were 50 ng/ml for parental DTA-1 and 10 ng/ml for chimeric and murinized DTA-1. Total serum IgEs (collected at day 21 after the fourth dose) were detected using an IgE ELISA assay (Bethyl Laboratories, Montgomery, TX). In brief, plates were coated with a goat anti-mouse IgE, and a HRP-conjugated IgE detection Ab was used as a secondary Ab. A standard calibration curve was generated using a mouse IgE calibrator. Also, total sera ADA from mice treated with chimeric or murinized variants of DTA-1 was detected using an MSD platform assay (Meso Scale Discovery, Rockville, MD). Combinations of ruthenylated and biotinylated mouse IgG2c and chimeric and murinized

DTA-1 IgG2c were incubated with diluted serum samples (collected 7 d after the third i.p. dose of three) for 3 h at room temperature on a shaker at 350 rpm. The mixture was then added to an MSD streptavidin-coated electrode plate to immobilize the formed complexes bridged by ADAs. After incubation and wash, read buffer containing tripropylamine was added for detection using an MSD SI2400 sector imager reader (Meso Scale Discovery). Sera from untreated mice were used as a negative control. A bridging ELISA was used for the detection of anti-idiotypic ADAs in parental DTA-1-treated mice sera (collected at day 12 after the third dose). Plates were coated with either DTA-1 or LTF-2 (in $1 \times$ PBS), and biotinylated LTF-2/DTA-1 with streptavidin-HRP was used as a secondary Ab (Jackson ImmunoResearch Laboratories). Nonneutralizing ADAs were detected by ELISA in mice sera (collected at day 12 after the third dose) after parental DTA-1 treatment. Plates coated with either DTA-1/LTF-2 (in $1 \times$ PBS) and biotinylated rat anti-mouse IgG1 or IgG2b (BD Biosciences) with streptavidin-HRP were used as secondary Abs (Jackson ImmunoResearch Laboratories). All ELISA plates were read with a SpectraMax 190 (Molecular Devices, Sunnyvale, CA) at 650 nm.

Multiplexed cytokine assays

Serum was analyzed for the concentrations of IFN- γ (at day 12 after the third dose) and IL-4 (at day 21 after the fourth dose) using luminescence bead-based (Milliplex Map) multiplex assays (Millipore, Billerica, MA) and a Bio-Plex system (BioRad, Hercules, CA).

Statistical analysis

Statistical differences between experimental groups were calculated by unpaired Student *t* test ($*p < 0.05$) using Prism software (GraphPad Software, La Jolla, CA).

Results

Chimerization and murinization of G1TR Ab

DTA-1 was murinized to an IgG2c/ λ framework to address immunogenicity-related anaphylaxis. The rat DTA-1 V_H domain CDRs were grafted into a consensus sequence of the mouse IGHV-5 V_H germline family, and two back mutations were made at positions L2 and T74 (Kabat numbering scheme). J-region IGHJ4-01 was used to connect to a mouse IgG2c Fc region. The DTA-1 L chain variable domain (λ) CDRs were grafted into a mouse V_L germline sequence (GenBank ID: AAH02129.1, derived from cDNA library), as previously described in the literature, and two back mutations were inserted at positions F2 and V48 (Kabat numbering scheme) (42). This phenylalanine (F2) may be critical for conformational stabilization of DTA-1 because it forms a tight pocket with L2/L3. A F2L mutation causes a steric clash of the mAb conformation based on computational modeling as shown in Fig. 1.

Chimerization and murinization of G1TR Ab does not affect *in vitro* properties and *in vivo* antitumor efficacy

In vitro binding of the chimerized ($EC_{50} = 0.20 \mu\text{g/ml}$) and murinized ($EC_{50} = 0.15 \mu\text{g/ml}$) IgG2c DTA-1 variants to a muG1TR-HEK293 transfectant cell line was conserved in comparison with the parental rIgG2b DTA-1 ($EC_{50} = 0.14 \mu\text{g/ml}$) (Table I). The slight differences in the binding titration curve were due to the different secondary Ab used for detection (Fig. 2A). The three DTA-1 versions showed a similar ability to augment TCR-triggered (anti-CD3-mediated) T cell proliferation ($EC_{50} = 0.01\text{--}0.02 \mu\text{g/ml}$) and viability ($EC_{50} = 0.01\text{--}0.02 \mu\text{g/ml}$) using preactivated splenocytes

Table I. Binding affinity, T cell proliferation, viability, and NF- κ B signaling of DTA-1 Ab variants

DTA-1 Abs	Binding	T Cell Proliferation	T Cell Viability	NF- κ B Signaling	NF- κ B Signaling + Linker
Rat IgG2b	0.14	0.02	0.02	0.08	0.08
Chimeric IgG2c	0.20	0.01	0.01	0.05	0.02
Murinized IgG2c	0.15	0.02	0.02	0.06	0.04

All values are EC_{50} ($\mu\text{g/ml}$).

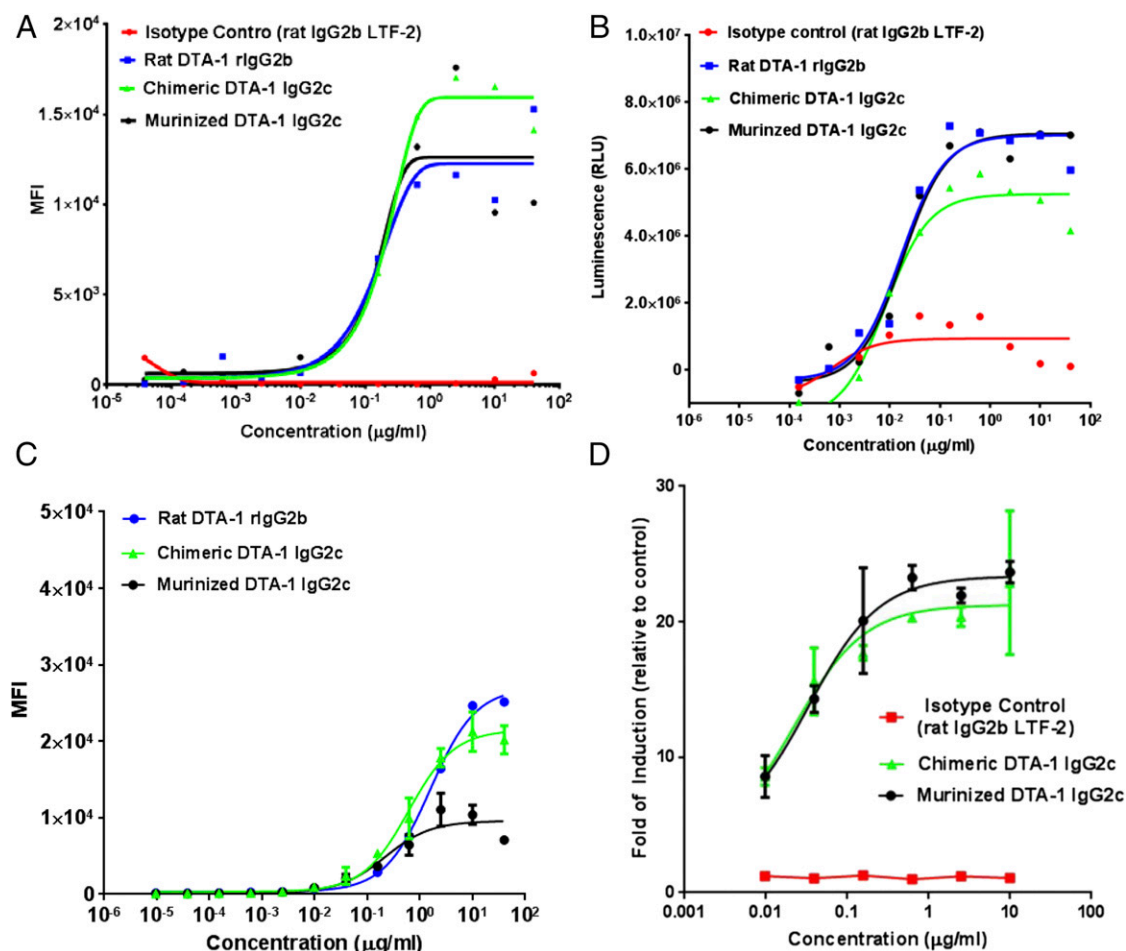


FIGURE 2. Surrogate anti-GITR Ab variants show similar in vitro activity before and after murinization. **(A)** GITR-binding assay. muGITR-HEK transfectant served as targets. DTA-1 variants were detected using rat or murine-specific PE-conjugated Abs. **(B)** T cell viability assay. Mouse CD3⁺ T cells and irradiated splenocytes were incubated with anti-muCD3 Ab at indicated concentrations of anti-muGITR Abs. **(C)** mFcγRIV-binding assay. Titration binding curves of DTA-1 variants to mFcγRIV-CHO-K1 transfectants cells. Binding was detected using a PE-labeled goat anti-mouse F(ab')₂, F(ab')₂ fragment-specific secondary Ab. **(D)** mFcγRIV-ADCC assay. DTA-1-mediated ADCC was detected using HEK293-muGITR target cells, mFcγRIV transfected Jurkat/NFAT-luciferase effector cells, and indicated concentrations of anti-GITR Abs. The in vitro data are derived from triplicates and are representative of two independent experiments.

(Fig. 2B, Supplemental Fig. 2C, Table I). NF-κB signaling induced by the three forms of DTA-1 were similar ($EC_{50} = 0.05$ – 0.08 μg/ml) by a luminescence-reporter assay (Supplemental Fig. 2A). NF-κB signaling was not enhanced using an artificial cross-linking agent ($EC_{50} = 0.02$ – 0.08 μg/ml) (Supplemental Fig. 2B, Table I). The function of the Fc portion of each DTA-1 mAb isotype forms was assessed by comparing their binding with each of the mouse FcγR (I, IIB, III, and IV) stable transfectants. Consistent with previous reports in the literature, mIgG2c showed a similar FcγR binding titration profile to mIgG2a mAbs: high-affinity binding to FcγRI ($EC_{50} = 0.28$ – 1.51 μg/ml) and FcγRIV ($EC_{50} = 0.22$ – 0.61 μg/ml), and weak binding to FcγRIIB ($EC_{50} = 4.15$ – 8.75 μg/ml) and FcγRIII ($EC_{50} = 15.80$ – 18.14 μg/ml) (Table II) (49). In comparison, the parental DTA-1 showed similar FcR binding affinity (FcγRI, $EC_{50} = 4.31$ μg/ml; FcγRIIB, $EC_{50} = 7.54$ μg/ml; FcγRIII, $EC_{50} = 21.62$ μg/ml; and FcγRIV, $EC_{50} = 1.57$ μg/ml) (Table II). The slight differences in the binding titration curve to mFcγRIV were due to the different secondary Ab used for detection (Fig. 2C). We also examined the ability of these DTA-1 variants to induce ADCC in vitro using an mFcγRIV ADCC activation reporter assay. For this assay, we used a cell line overexpressing muGITR as target cells to maximize the ability to detect ADCC. As expected from their FcγR binding profiles, both mIgG2c mAbs had similar ADCC activities (chimeric

DTA-1, $EC_{50} = 0.022$ μg/ml; murinized DTA-1, $EC_{50} = 0.033$ μg/ml) (Fig. 2D). An isotype control rat IgG2b Ab (clone LTF-2) did not show any ADCC activity (Fig. 2D). Consistent with published results, we found that i.p. injection of parental and murinized IgG2a forms of DTA-1 at 3.0 mg/kg on days 7, 9, and 11 resulted in significant regression of established MC-38 colorectal adenocarcinoma tumors in syngeneic BALB/c mice (50). The murinized IgG2c DTA-1 variant showed a comparable antitumor efficacy in vivo (Fig. 3A). Systemic administration of all these DTA-1 variants resulted in a significant reduction of tumor-infiltrating T_{regs} (% of CD4⁺) and CD4⁺Foxp3⁺ T cells (% of CD45⁺), but not CD8⁺ T cells (% of CD45⁺), which led to an increase in the ratio of intratumoral CD8⁺ T cells to T_{regs} (Fig. 3B). A similar skewed CD8⁺/T_{reg} ratio has recently been reported for Abs targeting other TNFR and non-TNFR immunoregulatory molecules, such as OX40 and CTLA-4 (51, 52). Detectable levels of IFN-γ (<200 pg/ml) were observed in the sera of mice 24 h after the third dose of all DTA-1 mAb variant treatments. Intracellular IFN-γ-producing tumor-infiltrating lymphocyte (TILs) has been previously proposed as a pharmacodynamics indicator of target engagement (T cell activation) upon treatment with anti-TNFRSF mAbs (37, 53). In conclusion, these data suggest that the murinization of the parental rat GITR Ab alters neither the in vitro physicochemical/agonistic properties nor the mechanism of action/in vivo antitumor potency.

Table II. Murinization of rat anti-GITR does not affect murine FcγR binding

DTA-1 Abs	mFcγRI	mFcγRIIB	mFcγRIII	mFcγRIV
Rat IgG2b	4.31	7.54	21.62	1.57
Chimeric IgG2c	1.51	8.75	15.80	0.61
Murinized IgG2c	0.28	4.15	18.14	0.22

All values are EC50 (μg/ml).

Anaphylaxis does not occur after repetitive doses of GITR Ab in isotype strain-matched C57BL/6 mice

It was reported previously that repetitive doses of a surrogate rat anti-GITR IgG2b Ab (clone DTA-1) induced an anaphylaxis reaction in C57BL/6 mice, which was confirmed by a fast and severe decline in body temperature after the third i.p. dose (42). Similar anaphylactic symptoms were observed using a murinized IgG2a form of this Ab (42). These observations were reproduced after three i.p. doses of 3.0 mg/kg on days 0, 4, and 11 (Fig. 4A). A similar anaphylactic reaction was observed between 3.0 and 50 mg/kg (Supplemental Fig. 1), so a dose of 3.0 mg/kg was used for all anaphylaxis in vivo studies. An isotype control rat IgG2b Ab (clone LTF-2) did not cause symptoms of anaphylaxis (Fig. 4A, Supplemental Fig. 1). Furthermore, this rat DTA-1-induced anaphylaxis is titratable, and it occurs not only in naive C57BL/6 but also in an MC-38 tumor-bearing mouse syngeneic model (Fig. 5). Based on the literature, BALB/c (Igh-1^a) and C57BL/6 (Igh-1^b) γ2 Igh genes are not derived from a common ancestral gene; they are derived from two distinct haplotypes, γ2b-γ2a and γ2b-γ2c, respectively (43, 44, 46). These allele variants give rise to different IgG2 Ab isotypes—BALB/c: IgG2b, IgG2a; and C57BL/6:

IgG2b, IgG2c. Murine IgG2a (IgG2a^a) and IgG2c (IgG2a^b) Abs share a total percentage of homology of 85%, with main differences at the hinge (29%) and C_H3 (28%) domains (45). Chimerized and murinized DTA-1 Abs on a C57BL/6-matched IgG2c isotype resulted in reduced development and severity of anaphylaxis as measured by decline of body temperature and physical body signs (i.e., scruffy hair, weakness/dizziness) (Fig. 4A, Table III). A significant difference (**p* < 0.05) in body temperature drop has been observed between the rat DTA-1 IgG2b and the murinized IgG2c form after a dosing schedule of three doses (days 0, 4, and 11) (Fig. 4C). A reduced anaphylaxis was also observed with a chimerized anti-OX40 mIgG2c mAb (clone OX86) (data not shown). Equivalent data were obtained after four i.p. doses of 3.0 mg/kg on days 0, 4, 11, and 18 (Fig. 4B). These observations demonstrate that the murinization of an anti-GITR IgG2c Ab inhibits immunogenicity-mediated anaphylaxis in C57BL/6 mice.

Murinized anti-GITR IgG2c Ab does not induce an ADA response, elevated total serum IgEs, or IL-4 in C57BL/6 mice

To indirectly assess the relative levels of total mouse ADAs, we analyzed serum samples from DTA-1-treated (parental, chimeric, and murinized) C57BL/6 mice (collected 3 d after the fourth i.p. dose of 3 mg/kg) for serum drug levels by ELISA. We found high DTA-1 levels in serum from mice treated with chimeric and murinized DTA-1 IgG2c (Fig. 6A) and low DTA-1 levels in rodents treated with parental DTA-1. These results suggest that rat IgG2b DTA-1 Ab was cleared more quickly than chimeric and murinized IgG2c DTA-1 variants, which correlates with the generation of a robust ADA response. Similarly, we detected high levels of IL-4 and IgE in serum of mice treated with the rat DTA-1, indicating a type I hypersensitivity allergic

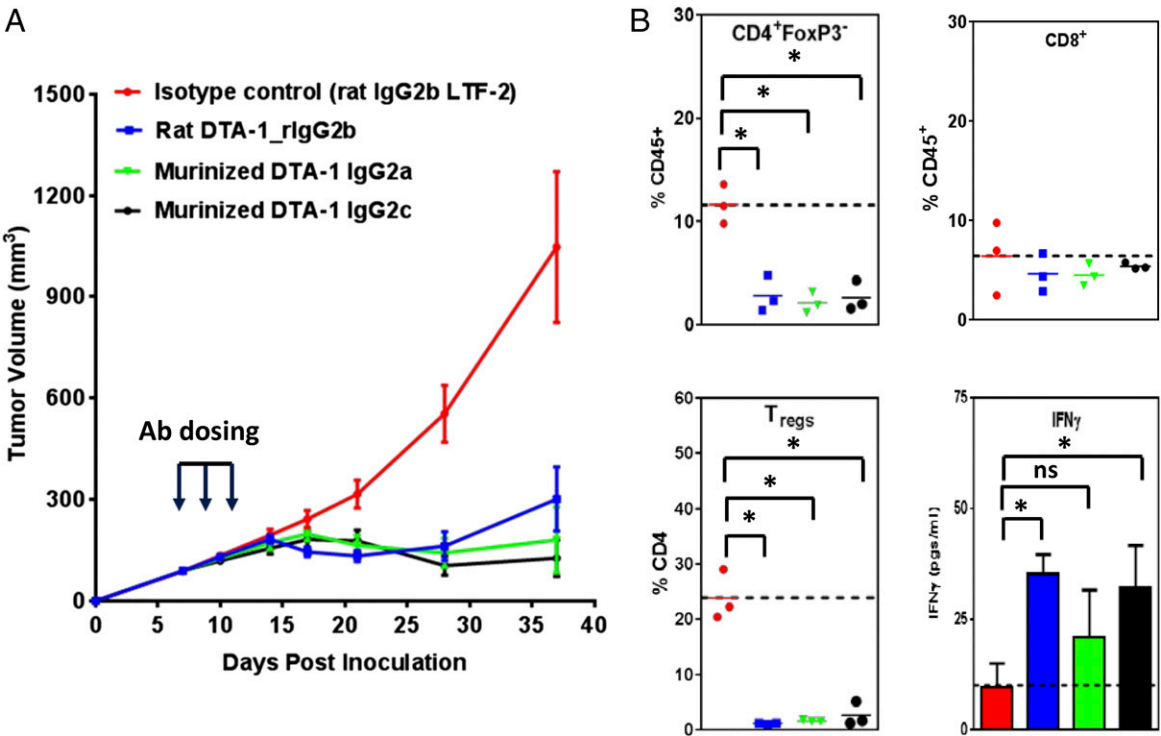


FIGURE 3. DTA-1 H chain isotype Ab variants induce comparable in vivo antitumor efficacy through the engagement of GITR leading to the increase in the ratio of intratumoral CD8⁺ T cells to T_{reg}. **(A)** Efficacy study of anti-GITR Abs in MC-38 syngeneic tumor model. C57BL/6 mice were challenged s.c. with 1×10^6 colorectal adenocarcinoma MC-38 cells and treated with 3.0 mg/kg of rat IgG2b, murinized IgG2a and IgG2c DTA-1, or rat IgG2b isotype control (clone LTF-2) on days 7, 9, and 11 after tumor challenge. Tumor growth was measured every 2–3 d, and mean tumor volume + SEM over time is depicted in the graph. Mice were euthanized when tumor volume reached 2500 mm³. Data are representative of two independent experiments (*n* = 10 per group). **(B)** Frequency of CD4⁺Foxp3⁺, CD8⁺, and T_{reg} TILs, and serum IFN-γ levels after treatment (day 12) with 3 mg/kg DTA-1 isotype variants. Representative FACS plots with mean for frequency of CD4⁺Foxp3⁺ and CD8⁺ within live CD45⁺ TIL gate and CD4⁺CD25⁺Foxp3⁺ within live CD4⁺ TIL gate. Serum IFN-γ concentration is shown in picograms per milliliter. Data are representative of two independent experiments (*n* = 3 per group). The *p* values were calculated using an unpaired two-tailed Student *t* test (**p* < 0.05).

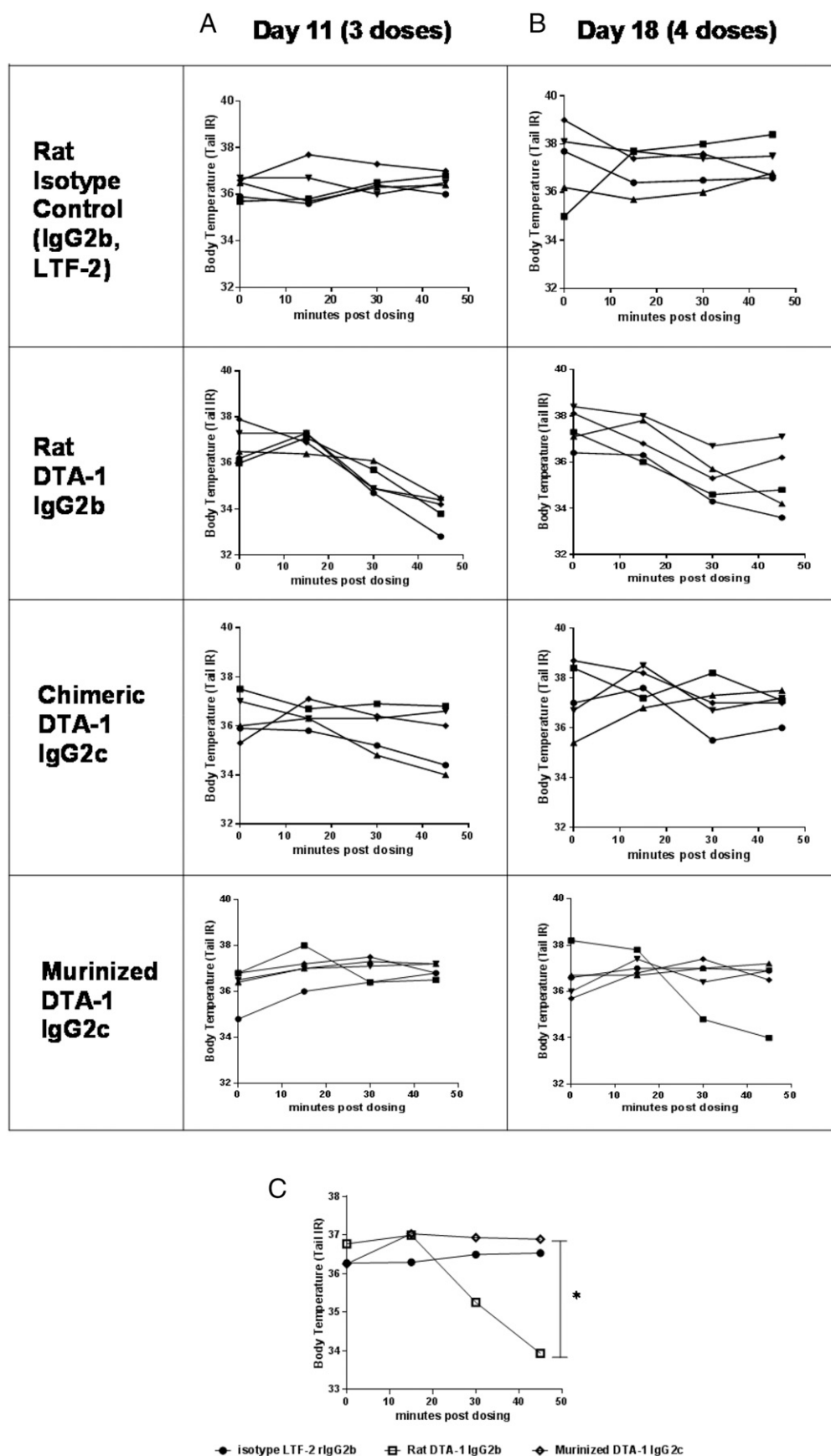


FIGURE 4. Murinization of a rat anti-GITR IgG2b DTA-1 Ab into an IgG2c isotype inhibits immunogenicity-mediated anaphylaxis in C57BL/6 mice. Mice were treated with three or four i.p. doses of 3.0 mg/kg on days 0, 4, and 11 (**A**) or days 0, 4, 11, and 18 (**B**), respectively. Temperature ($^{\circ}\text{C}$) of each animal was measured at baseline and every 15 min after the final dose injection using an infrared thermometer. Temperature of each animal is represented by individual lines in the graphs. (**C**) Mean comparison graph between rat IgG2b and mouse IgG2c (*Figure legend continues*)

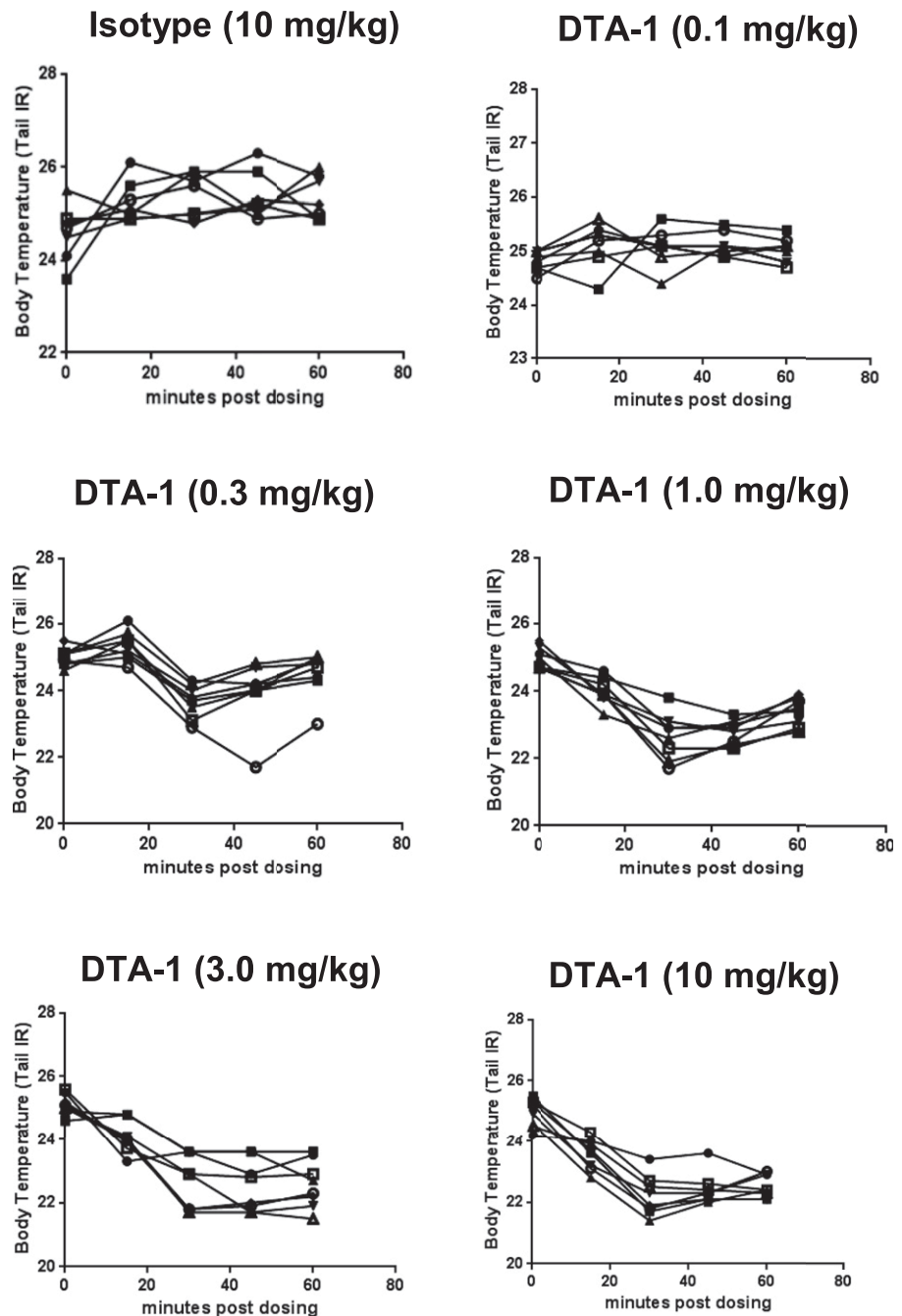


FIGURE 5. Immunogenicity-mediated anaphylaxis is titratable and tumor Ag independent. MC-38 tumor-bearing C57BL/6 mice were treated with three i.p. doses of 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg rat IgG2b DTA-1 Ab on days 0, 4, and 11. Temperature ($^{\circ}\text{C}$) of each animal was measured at baseline and every 15 min after the final dose injection by using an infrared thermometer. Data are representative of two independent experiments ($n = 8$ per group).

response (Fig. 6B). These results were confirmed by a total ADA assay (Fig. 6C), where higher ADA levels were found in the sera of mice treated with the chimeric than with the murinized DTA-1 IgG2c Ab (collected 7 d after the third i.p. dose of 3 mg/kg). To further assess the involvement of ADAs in rat DTA-1-induced anaphylaxis, mouse serum samples were tested for neutralizing (anti-idiotypic) or non-neutralizing activities. Low levels of DTA-1-specific anti-idiotypic ADAs were detected in the serum of rat DTA-1-treated mice (Fig. 7A), whereas in comparison, high levels of DTA-1- and isotype-specific IgG1 and IgG2b nonneutralizing ADAs were found in the same serum samples (Fig. 7B). These results suggest that the rat DTA-1

Ab induces an ADA response mainly directed to its rat sequence-derived variable and Fc domains, rather than the idiotype region of the variable domains, demonstrating that immunogenicity-mediated anaphylaxis can be caused by the inappropriate selection of the Ab isotype, which can be monitored by measuring body temperature, behavioral changes, nonneutralizing ADAs, IL-4 levels, and IgE levels.

Discussion

Although prior studies have demonstrated that repetitive i.p. bolus doses of DTA-1 cause anaphylaxis in rodents through a type I hypersensitivity pathway, the exact mechanism involved remains

Table III. Physical signs after dosing of different isotypes of anti-GITR Abs in C57BL/6 mice

DTA-1 Abs	Day 11 (Three Doses)		Day 18 (Four Doses)	
	Scruffy Hair	Weakness/Dizziness	Scruffy Hair	Weakness/Dizziness
Rat IgG2b isotype control	0/5	0/5	0/5	0/5
Rat IgG2b	5/5	4/5	5/5	5/5
Chimeric IgG2c	0/5	4/5	0/5	3/5
Murinized IgG2c	0/5	0/5	1/5	1/5

elusive. This study sought to determine whether this antidrug immune response is primarily mediated by GITR ligation and stimulation of CD4⁺ T and B cells or is due to an immune response induced by the Fc and variable domains of DTA-1. The Igh-1^b

allele in mouse strains such as C57BL/6, C57BL/10, SJL, and NOD is not an allele but is derived from a separate gene whose product is designated as IgG2c isotype (43–47). In the C57BL/6 mouse strain, the IgG2a gene is deleted, whereas in inbred strains

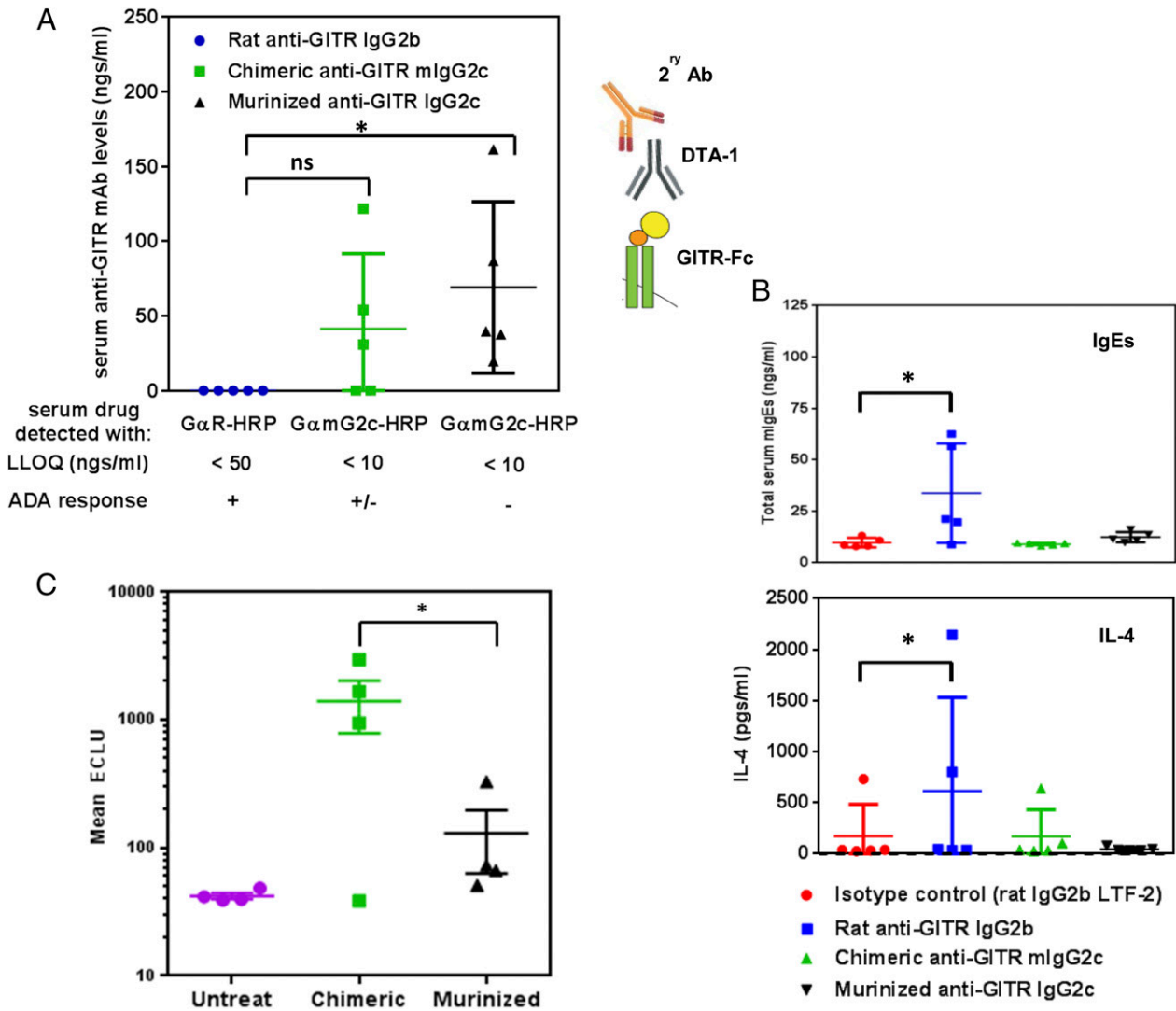


FIGURE 6. ADA response does not occur after repetitive doses of GITR Ab in isotype strain-matched C57BL/6 mice. **(A)** Serum drug level ELISA. C57BL/6 mice were treated with DTA-1 isotype Ab variants on days 0, 4, 11, and 18. On day 21, sera were collected from individual mice and assayed by ELISA as described in *Materials and Methods*. DTA-1 serum concentration levels were measured by anti-rat IgG2b or anti-mouse IgG2c Abs. High ADAs levels impair detection of DTA-1 levels in the serum. The lower limits of quantification were <50 ng/ml for parental DTA-1 and <10 ng/ml for chimeric and murinized DTA-1. Diagram for ELISA assay format has been included on the right. Mean drug level + SD is depicted in the graph. Data are representative of two independent experiments ($n = 5$ per group). **(B)** Total serum IgEs and IL-4 concentration levels. Mice treatment and sera collection follow the same schedule as described in (A). IgEs were detected using an ELISA assay, and IL-4 was measured using a luminescence bead-based multiplex assay. Mean IgE or IL-4 level + SD is depicted in the graph. Data are representative of two independent experiments ($n = 5$ per group). **(C)** Total serum ADA MSD assay. C57BL/6 mice were treated with chimeric and murinized DTA-1 IgG2c variants on days 0, 4, and 11. On day 18, sera were collected from individual mice and assayed by MSD as described in the *Materials and Methods*. Sera from untreated mice were used as a negative control. Mean ADA level + SD is depicted in the graph. Data are representative of two independent experiments ($n = 4$ per group). The p values were calculated using an unpaired two-tailed Student t test (* $p < 0.05$).

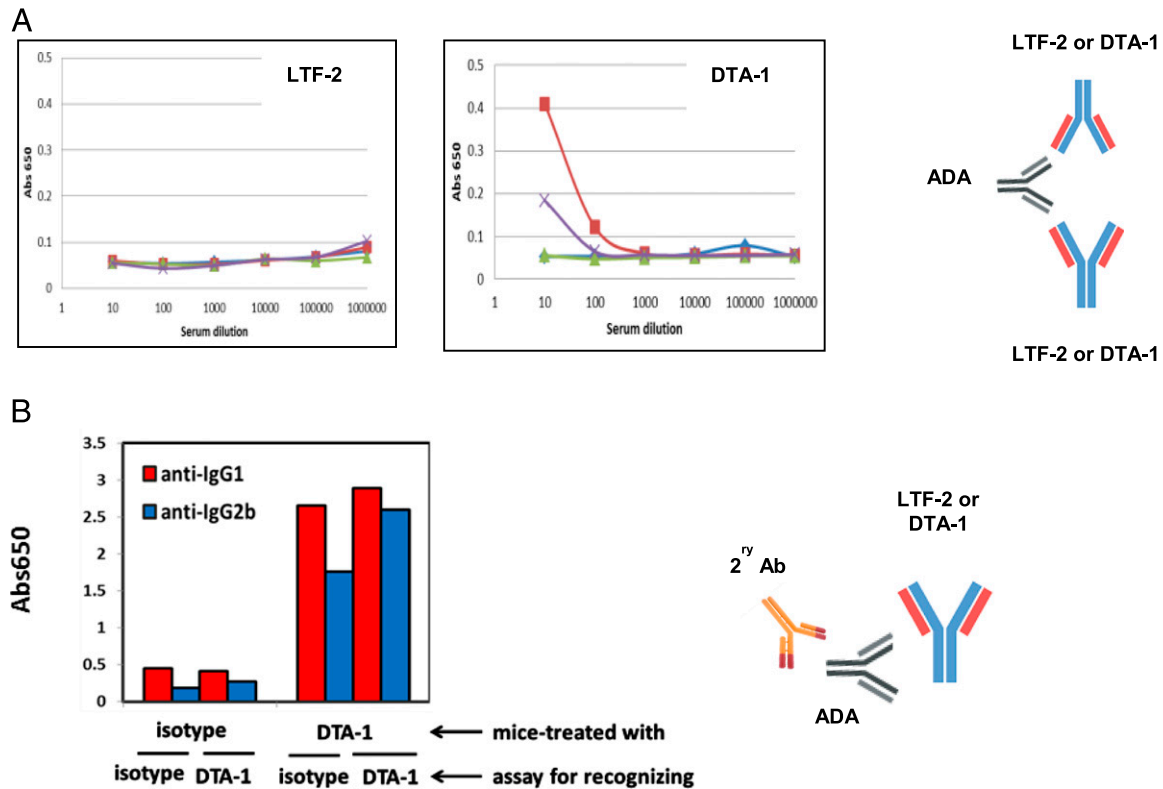


FIGURE 7. Rat DTA-1 Ab induces an ADA response mainly directed to its rat sequence-derived variable and Fc domains, rather than the idiotype region of the variable domain. **(A)** Anti-idiotypic ADA bridging ELISA. C57BL/6 mice were treated with rat DTA-1 or isotype control on days 7, 9, and 11. Day 12 sera were analyzed by ELISA for anti-DTA-1 Abs. ADA level of each animal is depicted in the graph. **(B)** Nonneutralizing ADA ELISA. Mice sera [from the same group of mice as indicated in (A)] were assayed by ELISA for anti-IgG1 and -IgG2b Abs. Diagrams for ELISA assay formats have been included on the right. Mean ADA level is depicted in the graph. Data are representative of two independent experiments ($n = 3$ per group).

with the *Igh-1^a* allele, such as BALB/c, the *IgG2c* gene is deleted (43–47). We hypothesized that the immunogenicity-mediated anaphylaxis observed by Murphy et al. (42) was in part due to the murinization of the anti-GITR agonist Ab DTA-1 into the *IgG2a* murine H chain isotype, an *IgH* that is nonendogenous in C57BL/6-mice. Murinization of DTA-1 using an *IgG2c* isotype framework did not affect in vitro agonistic activity (T cell proliferation, viability, and NF- κ B signaling) or antitumor immunity in a murine adenocarcinoma model (MC-38). The ability of engaging stimulatory mFc γ Rs (predominately mFc γ RIV) and depleting intratumoral T_{regs} was identical between the rat and murinized versions of DTA-1. We demonstrate that following the same i.p. dosing schedule, a murinized DTA-1 with a murine *IgG2c* H chain isotype did not induce an immunogenicity-mediated anaphylaxis, contrary to what was observed with the rat or murinized *IgG2a* variants in C57BL/6 mice. A chimerized *IgG2c* DTA-1 variant has been shown to slightly induce an anaphylaxis effect, which suggests that the rat variable domains of this Ab may still play a role in the immunogenicity. It has also been observed that the rat *IgG2b* isotype control (LTF-2) did not induce a decline in body temperature on the animals, which suggest that the anaphylaxis observed with the parental DTA-1 variant is not only derived from rat Fc or variable domains, but in part may be a sign of the side effects of a humoral GITR-mediated immune response, because DTA-1 has been proposed to increase tumor Ag-specific serum *IgG1* Abs in CT-26 tumor-bearing mice (33).

DTA-1 Ab drug levels detected in the serum of mice treated with the chimerized and murinized *IgG2c* DTA-1 variants was higher

than those in mice treated with the rat Ab. Loss of bioavailability is likely due to the development of ADA directed at the rat Ab. These observations were confirmed by the detection of total ADA only in the sera of the mice treated with the chimeric DTA-1 *IgG2c* and not in sera from the murinized DTA-1 *IgG2c*-treated mice. Exacerbated immune-mediated clearance of Ab only in mice treated with parental DTA-1 is consistent with the high IL-4 and IgE serum levels detected in these mice. As previously discussed, these results suggest that this atypical anaphylactic sensitization may result from a combination of two different pathways: an IL-4-induced B cell activation via immune complex (IC)–Fc γ R cross-linking on neutrophils leading to the release of platelet activation factor and an IgE–IC–Fc ϵ R cross-linking on basophils and mast cells leading to the release of histamine (42). The specificity of the anti-drug Abs induced by the anti-GITR mAbs is consistent with the evidence of a Th2-type allergic response. Our results suggest that the rat *IgG2b* isotype is still highly efficacious in the MC-38 syngeneic tumor model because it induces mostly a nonneutralizing anti-DTA-1 Ab response. In vivo efficacy of the rat anti-GITR Ab may also be preserved because of the transient levels and low affinity exhibited by the anti-idiotypic IC, or the high concentration of active drug at the site of action (tumor draining lymph node and tumor). In contrast with the robust antitumor efficacy, the fast clearance of parental DTA-1 (data not shown) may be explained by a rapid hepatic catabolism of high m.w. IC, where the drug is in complex with nonneutralizing ADAs. The uptake and degradation of DTA-1–induced IC through Fc γ R recognition and internalization by Kupffer cells (reticuloendothelial system) have been recently

reviewed in detail by Brunn et al. (54). The experimental assessment that we used to characterize ADAs shows typical analytical challenges, especially clear limitations to differentiate between bivalent and multivalent ADAs.

Although the manifestations of immunogenicity in preclinical models are generally not considered predictive in humans, our preclinical observations highlighted the importance of a well-designed strategy for murinization of immunoregulatory mAbs and selection of a strain-matched H chain isotype to generate ideal “surrogate” Abs that can serve as a model to predict immunopathological pathways in humans. A similar hypersensitivity/ADA reaction has been recently described after a repetitive i.p. dosing with murine Ab treatments against checkpoint inhibitors (hamster anti-PD-1 and rat IgG2b anti-PD-L1) in a 4T1 tumor syngeneic model because of the xenogeneic nature of the Ab treatments (55). Strategies to mitigate risk factors from the clinical use of human protein therapeutics (i.e., humanization, CDR modification, formulation, biophysical characteristics, routes of delivery, etc.) should also be applied to anti-mouse Abs (56–61). Currently, no serious immune-related adverse events have been reported in phase I clinical trials of humanized anti-GITR agonistic Abs (62, 63).

In conclusion, murinization and selection of a strain-matched isotype play a critical role in the immune-mediated anaphylaxis in rodents for murine anti-GITR and OX40 mAbs. Our findings should be applied to the evaluation of any protein-based tumor immunology agents in mouse syngeneic models. Taken together, these results highlight the importance of understanding the interplay between the antitumor efficacy of surrogate agonistic immunoncology biologics and the pathophysiology of immunogenicity that may arise after treatment.

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Supplemental Materials.

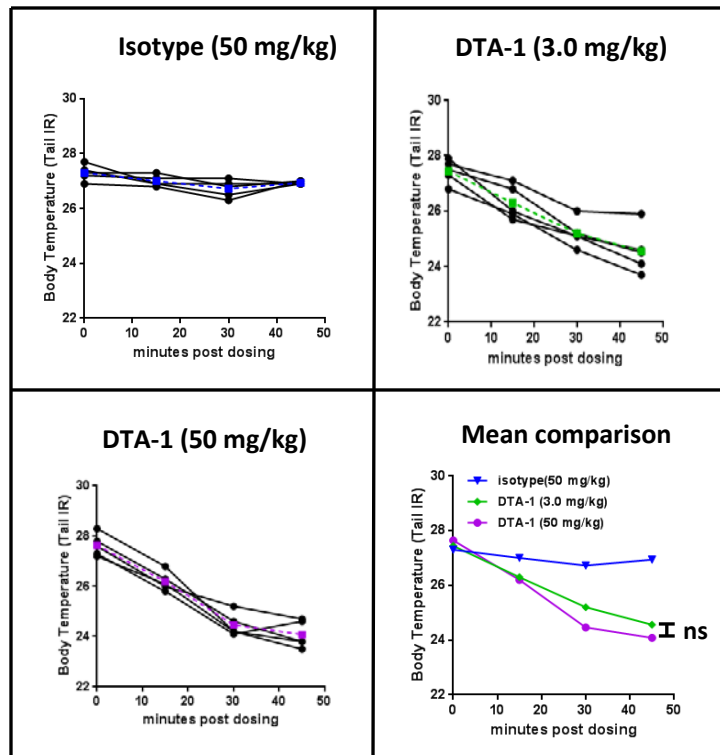
Murinization and heavy chain isotype matching of the anti-GITR antibody DTA-1 reduces immunogenicity-mediated anaphylaxis in C57BL/6 mice

Heavy chain IgG2c isotype of DTA-1 reduces anaphylaxis

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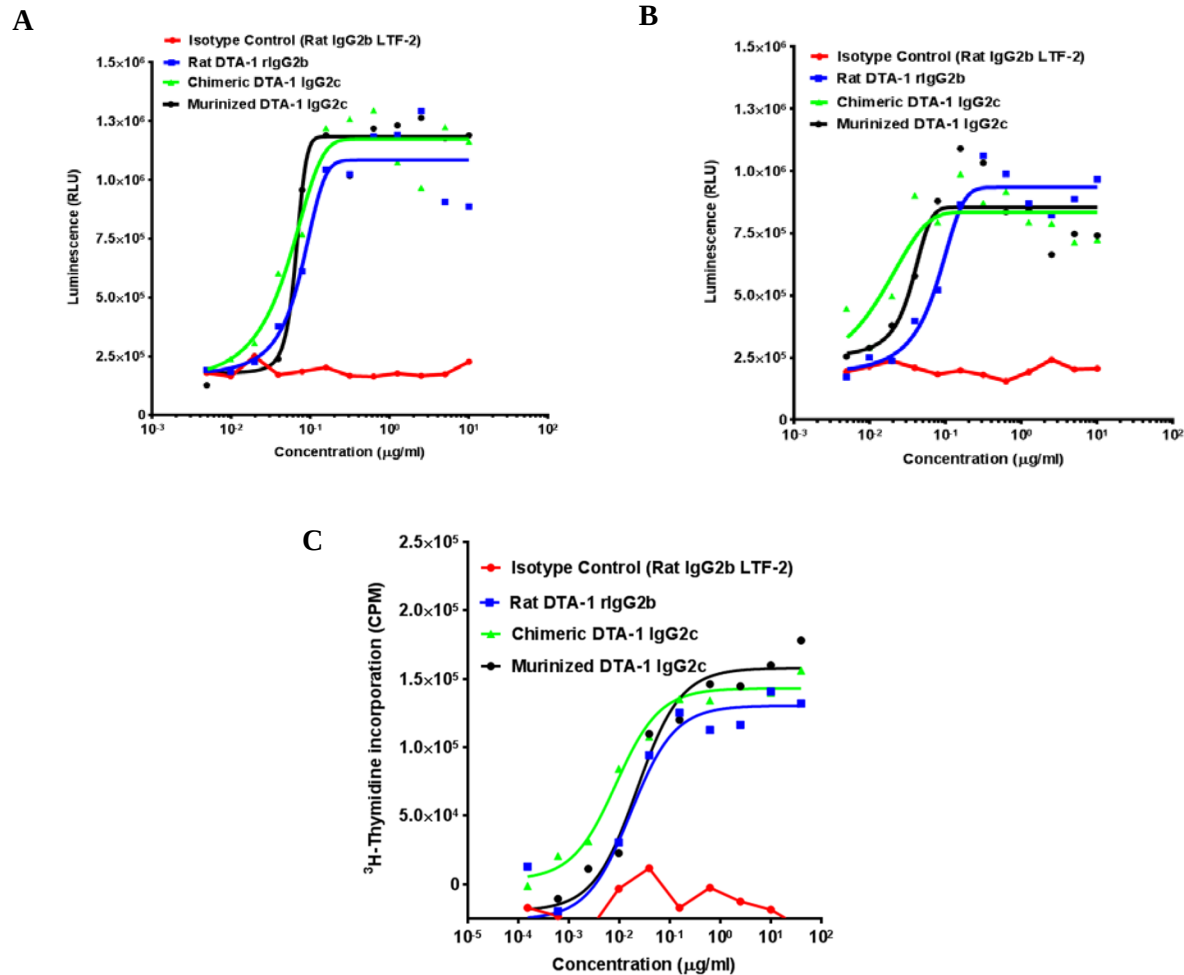
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Supplemental Figure 1.



Supplemental Figure 1. Rat anti-GITR IgG2b DTA-1 antibody induces similar levels of immunogenicity-mediated anaphylaxis in C57BL/6 mice at low and high doses. Mice were treated with three intraperitoneal doses of 3.0 and 50 mg/kg on days 0, 4 and 11. Temperature of each animal was measured at baseline and every 15 minutes following the final dose injection using an infrared thermometer. Temperature of each animal is represented by individual lines in the graphs. Color lines in the graphs represent the mean of each group of mice. Data are representative of two independent experiments (n = 5/group). P-values were calculated using an unpaired 2-tailed Student's *t* test (*, $P < 0.05$).

Supplemental Figure 2.



Supplemental Figure 2. Surrogate anti-GITR antibody variants show similar in vitro activity before and after murinization. (A) NF κ B activation reporter assay. A 293-NF κ B-reporter luciferase cell line was generated for this assay as described in Materials and Methods. Cells were incubated overnight at 37°C with indicated concentrations of anti-mouse GITR antibodies. (B) NF κ B activation reporter assay with cross-linking effect. Effect of cross-linking was evaluated using a F(ab')₂ goat anti-mouse IgG, Fc fragment-specific antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). (C) T cell proliferation assay. Mouse CD3⁺ T cells and irradiated splenocytes were incubated with anti-muCD3 antibody at indicated concentrations of anti-muGITR antibodies. The in-vitro data are derived from triplicates and are a representative of two independent experiments.