

## Anti-CTLA-4 Antibodies of IgG2a Isotype Enhance Antitumor Activity through Reduction of Intratumoral Regulatory T Cells

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### Abstract

Antitumor activity of CTLA-4 antibody blockade is thought to be mediated by interfering with the negative regulation of T-effector cell (Teff) function resulting from CTLA-4 engagement by B7-ligands. In addition, a role for CTLA-4 on regulatory T cells (Treg), wherein CTLA-4 loss or inhibition results in reduced Treg function, may also contribute to antitumor responses by anti-CTLA-4 treatment. We have examined the role of the immunoglobulin constant region on the antitumor activity of anti-CTLA-4 to analyze in greater detail the mechanism of action of anti-CTLA-4 antibodies. Anti-CTLA-4 antibody containing the murine immunoglobulin G (IgG)2a constant region exhibits enhanced antitumor activity in subcutaneous established MC38 and CT26 colon adenocarcinoma tumor models compared with anti-CTLA-4 containing the IgG2b constant region. Interestingly, anti-CTLA-4 antibodies containing mouse IgG1 or a mutated mouse IgG1-D265A, which eliminates binding to all Fc $\gamma$  receptors (Fc $\gamma$ R), do not show antitumor activity in these models. Assessment of Teff and Treg populations at the tumor and in the periphery showed that anti-CTLA-4-IgG2a mediated a rapid and dramatic reduction of Tregs at the tumor site, whereas treatment with each of the isotypes expanded Tregs in the periphery. Expansion of CD8<sup>+</sup> Teffs is observed with both the IgG2a and IgG2b anti-CTLA-4 isotypes, resulting in a superior Teff to Treg ratio for the IgG2a isotype. These data suggest that anti-CTLA-4 promotes antitumor activity by a selective reduction of intratumoral Tregs along with concomitant activation of Teffs. *Cancer Immunol Res*; 1(1): 32–42. ©2013 AACR.

### Introduction

The immune system is capable of controlling tumor development and mediating tumor regression. This requires the generation and activation of tumor-antigen-specific T cells. Multiple T-cell costimulatory receptors and T-cell negative regulators, or coinhibitory receptors, act in concert to control T-cell activation, proliferation, and gain or loss of effector function. Among the earliest and best-characterized T-cell costimulatory and coinhibitory molecules are CD28 and CTLA-4 (1). CD28 provides costimulatory signals to T-cell receptor engagement by binding to B7-1 and B7-2 ligands on antigen-presenting cells, whereas CTLA-4 provides a negative signal downregulating T-cell proliferation and function. CTLA-4, which also binds the B7-1 and B7-2 ligands but with higher affinity than CD28, acts as a negative regulator of T-cell function through both cell autonomous (or intrinsic) and cell

nonautonomous (or extrinsic) pathways. Intrinsic control of CD8 and CD4 T-effector (Teff) function is mediated by the inducible surface expression of CTLA-4 as a result of T-cell activation, and inhibition of T-cell proliferation and cytokine proliferation by multivalent engagement of B7 ligands on opposing cells (2). Anti-CTLA-4 antibodies, when cross-linked, suppress T-cell function *in vitro* (3–6).

Regulatory T cells (Treg), which express CTLA-4 constitutively, control Teff function in a non-cell autonomous fashion. Tregs that are deficient for CTLA-4 have impaired suppressive ability (7), and antibodies that block CTLA-4 interaction with B7 can inhibit Treg function (8, 9). More recently, Teffs have also been shown to control T-cell function through extrinsic pathways (10, 11). Extrinsic control of T-cell function by Tregs and Teffs occurs through the ability of CTLA-4-positive cells to remove B7 ligands on antigen-presenting cells, thereby limiting their costimulatory potential (12, 13).

Antibody blockade of CTLA-4/B7 interactions is thought to promote Teff activation by interfering with negative signals transmitted by CTLA-4 engagement; this intrinsic control of T-cell activation and proliferation can promote both Teff and Treg proliferation (3, 9). In early studies with animal models, antibody blockade of CTLA-4 was shown to exacerbate autoimmunity (14, 15). By extension to tumor immunity, the ability of anti-CTLA-4 to cause regression of established tumors provided a dramatic example of the therapeutic potential of CTLA-4 blockade (16).

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Human antibodies to human CTLA-4, ipilimumab and tremelimumab, were selected to inhibit CTLA-4-B7 interactions (17, 18) and have been tested in a variety of clinical trials for multiple malignancies (19, 20). Tumor regressions and disease stabilization were frequently observed, and treatment with these antibodies has been accompanied by adverse events with inflammatory infiltrates capable of affecting a variety of organ systems. In 2011, ipilimumab, which has an immunoglobulin G (IgG)1 constant region, was approved in the United States and European Union for the treatment of unresectable or metastatic melanoma based on an improvement in overall survival in a phase III trial of previously treated patients with advanced melanoma (21).

Several different antibodies have been used to show activity of anti-CTLA-4 blockade in mouse models, including hamster anti-CTLA-4 antibodies, 9H10 [Syrian hamster IgG2b (3)] and 4F10 [Armenian hamster IgG1 (4)], and mouse anti-mouse CTLA-4 antibody (9D9-murine IgG2b) generated in a human CTLA-4 transgenic mouse (9, 22). Anti-CTLA-4 9D9-IgG2b has been tested in a variety of mouse subcutaneous tumor models, such as Sa1N fibrosarcoma, MC38 and CT26 colon adenocarcinomas, and B16 melanoma. Except for Sa1N, anti-CTLA-4 monotherapy has shown modest antitumor activity (refs. 9, 23; see later and unpublished data). Murine IgG2b can bind to immunoglobulin Fc $\gamma$  receptors (Fc $\gamma$ R), including Fc $\gamma$ RIIB, Fc $\gamma$ RIII, and Fc $\gamma$ RIV receptors (24). Consequently, it is possible that multivalent engagement of CTLA-4 by 9D9-IgG2b bound to T cells and Fc $\gamma$ R-positive cells could result in an agonistic negative signal and render this antibody less effective in CTLA-4 blockade than a blocking antibody with no Fc $\gamma$ R-binding properties.

To determine the relative potency of mouse anti-CTLA-4 antibodies in antitumor activity, we generated a series of 9D9 isotype variants that differ in their affinity for Fc $\gamma$ Rs. Unexpectedly, we found enhanced antitumor activity of anti-CTLA-4 containing the IgG2a constant region and an absence of antitumor activity with the use of an IgG1 constant region. These results directed us to examine the effects of anti-CTLA-4 on intratumoral and peripheral T-cell subpopulations which express CTLA-4. We found that anti-CTLA-4 IgG2a isotype antibody, and to a lesser extent IgG2b, resulted in loss of intratumoral Tregs along with expansion of CD8 T<sub>H</sub>1, resulting in an enhanced T<sub>H</sub>1 to Treg ratio for this isotype. These results show an additional mechanism of action of anti-CTLA-4, tumor-specific elimination of Tregs, which is independent of CTLA-4-B7 interactions.

## Materials and Methods

### Antibody generation, purification, and characterization

The 9D9 hybridoma (kindly supplied by J. Allison, University of Texas, MD Anderson, Houston, TX) is a mouse anti-mouse CTLA-4 antibody derived by immunization of human CTLA-4 transgenic mice (22). 9D9 blocks the binding of murine CTLA-4-Ig to B7-1-positive cells (data not shown). To generate 9D9 isotypes, total RNA was prepared from 9D9 hybridoma cells using the RNeasy Mini Kit (Qiagen). cDNA was prepared by the 5'-RACE protocol using the SMARTer RACE cDNA Amplification and Advantage 2 PCR Kits (Clontech Laboratories, Inc.).

Variable regions were amplified using a 3'-murine-specific constant region primer, paired with the 5'-RACE universal primer mix. PCR products containing the V-region were cloned into the pCR4-TOPO vector (Invitrogen) and transformed into *Escherichia coli* strain TOP10 (Invitrogen). Templiphi (GE Healthcare Biosciences) samples were prepared and subjected to DNA sequencing (Sequetech).

For expression of recombinant antibodies (mouse IgG2a, mouse IgG1, and mouse IgG1-D265A isotypes), the 9D9 variable regions were amplified by PCR to introduce cloning sites and cloned into UCOE expression vectors (EMD Millipore) that contain the osteonectin signal sequence and the desired constant region. Heavy and light chain vectors were linearized and cotransfected into CHO-S cells (Invitrogen). Stable pools and/or clones were selected. For mouse IgG2a, the BALB/c allotype, IgG2a<sup>a</sup> (haplotype Igh-1<sup>a</sup>) sequence was used.

Culture supernatants from 9D9 hybridoma or CHO cell transfectants were harvested for antibody production. Antibodies were purified using Protein A or Protein G by standard methods and dialyzed into PBS. All antibodies were free of endotoxin (<0.05 EU/mg) and shown to have less than 5% aggregates as determined by size exclusion chromatography/high-performance liquid chromatography (HPLC). Each of the CTLA-4 isotypes was assessed for binding to cells constitutively expressing CTLA-4 (58 $\alpha$ - $\beta$ -CTLA-4/CD3 $\zeta$ ) by flow cytometry. 58 $\alpha$ - $\beta$ -CTLA-4/CD3 $\zeta$  is a murine T-cell hybridoma that expresses murine CTLA-4 fused to CD3 $\zeta$  and is analogous to a similar construct for human CTLA-4 (17). A total of  $1 \times 10^5$  cells per well in fluorescence-activated cell sorting (FACS) buffer [ $1 \times$  Dulbecco's PBS (DPBS; CellGro), 0.02% sodium azide, 2% FBS (Hyclone), and 1 mmol/L EDTA] were stained with serial dilutions of antibodies starting at 20  $\mu$ g/mL and incubated for 30 minutes at 4°C. Cells were washed and secondary antibody [R-PE Donkey anti-mouse IgG (Jackson ImmunoLabs)] was added and incubated for 30 minutes at 4°C. Cells were then washed and resuspended in FACS buffer and analyzed on a BD FACS Canto flow cytometer.

### Murine tumor models and antibody pharmacokinetics

C57BL/6 or BALB/c mice were subcutaneously injected with 2 million MC38 or 1 million CT26 tumor cells, respectively. After 7 days, tumor volumes were determined and mice were randomized into treatment groups so as to have comparable mean tumor volumes (45–50 mm<sup>3</sup>/2). Antibodies formulated in PBS were administered intraperitoneally (i.p.) on days 7, 10, and 14 at 200  $\mu$ g per dose in a volume of 200  $\mu$ L for MC38 and on days 7, 10, 14, and 17 for CT26. Tumor volumes were recorded 3 times weekly. The control antibody used for the studies is a recombinant human anti-diphtheria toxin antibody with a mouse IgG1 isotype.

For characterization of pharmacokinetics of the anti-CTLA-4 antibodies, 9 female C57BL/6 mice were injected intraperitoneally with 10 mg/kg of each isotype of anti-CTLA-4 (IgG1, IgG1-D265A, IgG2a, or IgG2b). Blood samples were taken at 1, 6, 24, 48, 72, 120, 168, 336, and 504 hours and the sera were analyzed by ELISA. Chemiluminescent ELISA was used to measure serum levels of anti-CTLA-4 monoclonal antibodies. Recombinant mouse CTLA-4-Ig was used as a capture in

combination with a horseradish peroxidase conjugate of goat anti-mouse IgG (light chain-specific) polyclonal antibody. Standards, controls, and samples were diluted 100-fold with 1% bovine serum albumin/PBS/0.05% Tween 20. Concentrations of anti-CTLA-4 antibodies in mouse serum samples were calculated from luminescence intensity as measured by M5 plate reader (Molecular Devices) using a 5-parameter logistic (5-PL) calibration curve generated from corresponding anti-CTLA-4 antibody calibrators.

### Lymphocyte staining analysis

All mice were sacrificed and tumor and draining lymph node were harvested for analysis on day 15 after tumor implantation. Single cell suspensions were prepared by dissociating tumor and lymph node with the back of a syringe in a 24-well plate. Cell suspensions were passed through 70- $\mu$ m filters, pelleted, resuspended, and counted. Cells were then plated in 96-well plates with  $1 \times 10^6$  cells per well for staining. Cells were treated with 24G.2 (BioXcell), which blocks Fc binding to Fc $\gamma$ RIIB and Fc $\gamma$ RIII, and subsequently stained with antibodies against CD8 (clone 53-6.7; Biolegend), CD4 (clone GK1.5; Biolegend), and CD45 (clone 30-F11; Biolegend) or antibodies to CD11c (clone N418; eBioscience), CD45, CD8, CD11b (clone M1/70; Biolegend), and Gr-1 (clone Rb6-8C5; eBioscience). For intracellular staining, samples were fixed, permeabilized, and stained with antibodies to Foxp3 (clone FJK-16s; eBioscience), Ki-67 (clone SolA15; eBioscience), and CTLA-4 (clone 4F10; BD Pharmingen). Samples were then analyzed on a FACS Canto flow cytometer (BD).

### Intratumoral cytokine analysis

Tumors were harvested into 1 mL of complete T-cell media (RPMI-1640 supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, and  $\beta$ -mercaptoethanol; Life Technologies) in 24-well plates and manually dissociated into single-cell suspensions. Cells and debris were spun down and supernatant was harvested and frozen to allow for batch processing of samples. Upon thawing, 25  $\mu$ L of supernatant from each sample was assessed for concentrations of intratumoral interleukin (IL)-1 $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-21, IL-22, IL-27, IP-10, granulocyte macrophage colony-stimulating factor (GM-CSF), TNF- $\alpha$ , and IFN- $\gamma$  in duplicate using a bead-based cytokine array according to the manufacturer's instructions (FlowCytomix; eBioscience).

### Antibody binding to Fc $\gamma$ R by surface plasmon resonance

Fc $\gamma$ Rs were procured from R&D systems with the exception of Fc $\gamma$ RI. For expression of Fc $\gamma$ RI, the extracellular domain was amplified by PCR and cloned into a UCOE expression vector (EMD Millipore) in-frame with an osteonectin signal sequence and a C-terminal 6XHis tag and stop codon. CHO-S cells (Invitrogen) were transfected using Amaxa Nucleofector II (Lonza Group, AG), and stable pools and clones were selected and expanded and the subsequent supernatants collected for purification. The soluble recombinant proteins were purified using standard techniques through immobilized metal nickel affinity chromatography (IMAC Life Technologies Corporation) nickel-charged resin columns.

Fc $\gamma$ R interactions were determined by coating the antibodies directly on a CM5 chip to a density of about 1,500 response units (RU) and flowing 8 concentrations of mFcRs over the immobilized antibodies until equilibrium was attained. The equilibrium response unit was plotted as a function of FcR concentration using GraphPad Prism and equilibrium  $K_D$  was obtained. Alternatively, the 6X-His tagged FcRs were captured (to about 200 RUs) on an anti-His antibody-coated CM5 surface and flowing 8 concentrations of the antibody over the Fc $\gamma$ R captured surface. The equilibrium  $K_D$  obtained by this approach was lower by about 4-fold due to the absence of multivalent binding present when antibodies are directly coated on surface. FcRn interaction was characterized by coating 500 RUs of mFcRn on a CM5 chip and flowing 8 concentrations of antibodies over the FcRn-coated surface at pH 6.0, in 50 mmol/L 2-(N-morpholino)ethanesulfonic acid, 150 mmol/L NaCl running buffer. The antibody bound surface was regenerated using pH 8.0 Tris solution.

### Statistical analysis

Statistical analyses were conducted using GraphPad Prism. Error bars represent the SEM calculated using Prism. Specific statistical tests used were unpaired *t* tests and one-way ANOVA, *P* values less than 0.05, 0.01, and 0.001 were noted as \*, \*\*, and \*\*\*, respectively, in each figure.

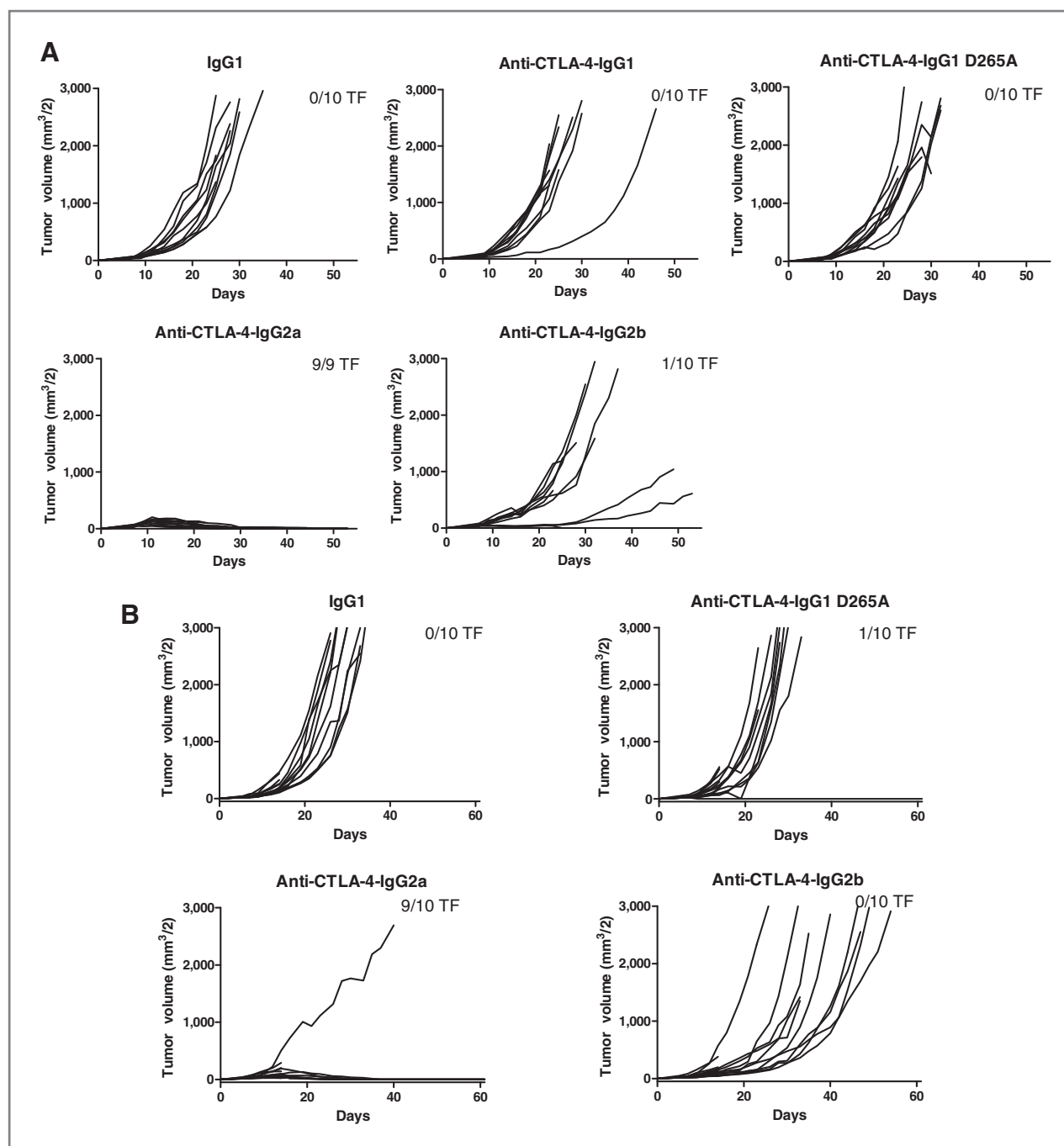
## Results

### Activity of isotype variants of anti-CTLA-4 in subcutaneous tumor models

To determine the relative potency of different isotypes of anti-CTLA-4 in antitumor activity, 4 versions of the 9D9 antibody with different immunoglobulin heavy chain constant regions were generated and purified from CHO transfectants or parental hybridoma. These included IgG1 containing a D265A mutation (IgG1-D265A), which is a non-Fc $\gamma$ R-binding mutant (25), IgG1, IgG2b, and IgG2a. Each of these isotype variants bind equivalently to cells constitutively expressing mouse CTLA-4 (Supplementary Fig. S1). In addition, each of the isotypes was characterized for binding to soluble forms of Fc $\gamma$ RI, Fc $\gamma$ RIIB, Fc $\gamma$ RIII, Fc $\gamma$ RIV, and FcRn by surface plasmon resonance. Affinities of the different anti-CTLA-4 isotypes for Fc $\gamma$ Rs were determined to be as previously described (refs. 24, 26; Supplementary Table S1).

Each of the CTLA-4 antibodies was tested for antitumor activity in therapeutic MC38 (Fig. 1A) and CT26 colon adenocarcinoma (Fig. 1B) tumor models, with treatment initiated 7 days after implantation. Anti-CTLA-4 9D9-IgG2a treatment resulted in nearly complete tumor rejection, whereas 9D9-IgG2b showed moderate tumor growth inhibition in both models. Unexpectedly, anti-CTLA-4 containing IgG1 or IgG1-D265A showed little activity; the growth of tumors in mice treated with these antibodies was comparable with those in the control IgG1 treatment group.

Pharmacokinetic analysis of each of these antibodies was evaluated in normal nontumor-bearing C57BL/6 mice (Supplementary Table S2 and Supplementary Fig. S2). Systemic

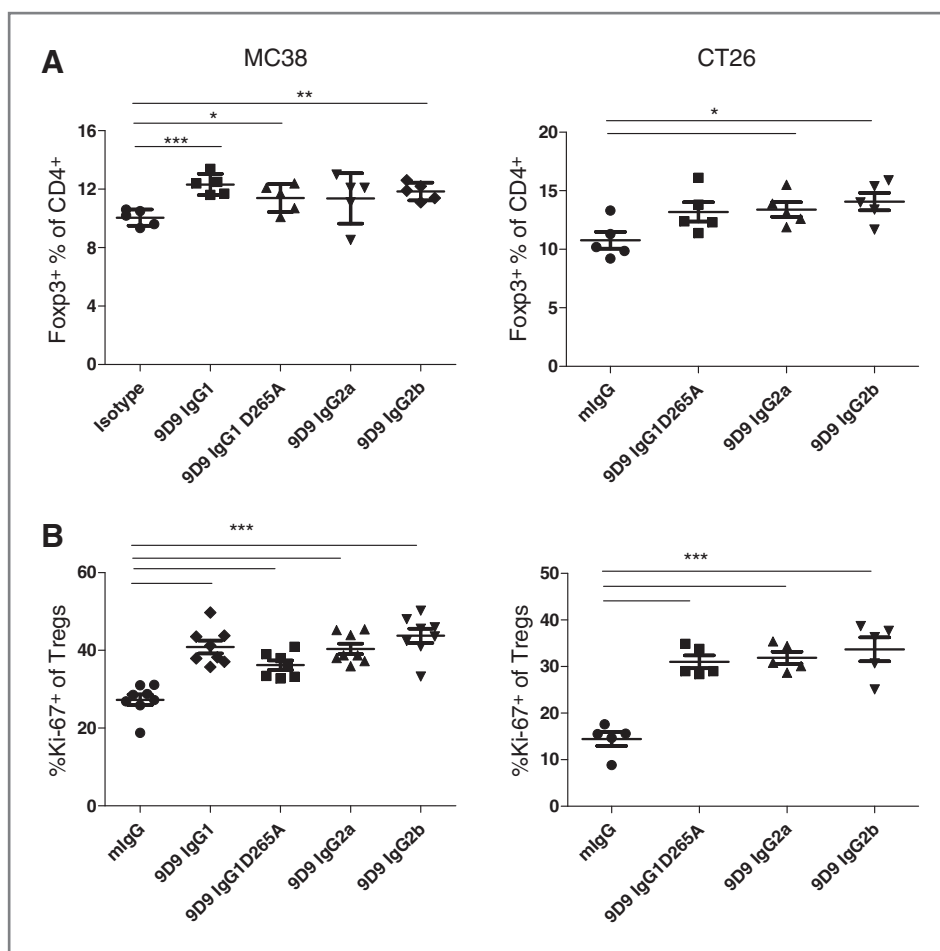


**Figure 1.** Isotype-dependent activity of CTLA-4 antibodies in subcutaneous tumor models. **A**, activity of anti-CTLA-4 isotypes in MC38 adenocarcinoma model in C57BL/6 mice. **B**, activity of anti-CTLA-4 isotypes in CT26 adenocarcinoma model in BALB/c mice. C57BL/6 or BALB/c mice were injected subcutaneously with  $2 \times 10^6$  MC38 or  $1 \times 10^6$  CT26 cells on day 0. On day 7, tumors were measured, randomized, and then treated with the designated antibody (200  $\mu$ g/dose i.p.) and again on days 10 and 14 for MC38 and days 10, 14, and 17 for CT26. Tumor volumes were measured 3 times weekly. The number of tumor-free (TF) mice per group is shown for each group. The experiment shown is representative of at least 3 different experiments using 10 mice per group. For CT26, tumor growth with anti-CTLA-4-IgG1 and anti-CTLA-4-IgG1-D265A were equivalent (not shown).

exposure of the 4 isotypes was largely similar, although the area under the concentration versus time curve (AUC) of IgG1-D265A (185  $\mu$ mol/L·h) and IgG2b (170  $\mu$ mol/L·h) were slightly higher than IgG1 (119  $\mu$ mol/L·h) and IgG2a (125  $\mu$ mol/L·h). The terminal half-lives of the antibodies were also similar (156–

174 hours), although there was an accelerated terminal decay observed only for IgG2a from week 2 to week 3; this was presumably due to the formation of antidrug antibody as a consequence of allotypic differences between the Balb/c IgG2a<sup>a</sup> constant region and that of C57BL/6 mice tested here





**Figure 2.** CTLA-4 blockade expands peripheral Tregs. A, a total of  $2 \times 10^6$  MC38 colon tumor cells (left) or  $1 \times 10^6$  CT26 (right) were implanted subcutaneously into C57BL/6 or BALB/c mice, respectively. At day 7 postimplantation, tumor-bearing mice were randomized and dosed with 10 mg/kg of antibody by intraperitoneal injection, every 3 days  $\times$  3 for MC38 and every 3 days  $\times$  4 for CT26. On day 15 postimplantation, spleen (MC38) or tumor draining lymph nodes (CT26) were harvested, manually dissociated into single-cell suspensions, and stained for flow cytometry. Percentage of CD4<sup>+</sup> cells that are FoxP3<sup>+</sup> is displayed. B, splenic Tregs from anti-CTLA-4 isotype-treated MC38 or lymph node Tregs from CT26 tumor-bearing mice were analyzed for their levels of Ki-67. Percentage of Tregs that are Ki-67<sup>+</sup> is displayed. Data are representative of 2 (CT26) or 3 (MC38) independent experiments with  $\geq 5$  mice/group/experiment.

(27). Thus, the differences in antitumor efficacy of the anti-CTLA-4 isotypes cannot be explained by differences in drug exposure.

#### Analysis of intratumoral and peripheral T cells

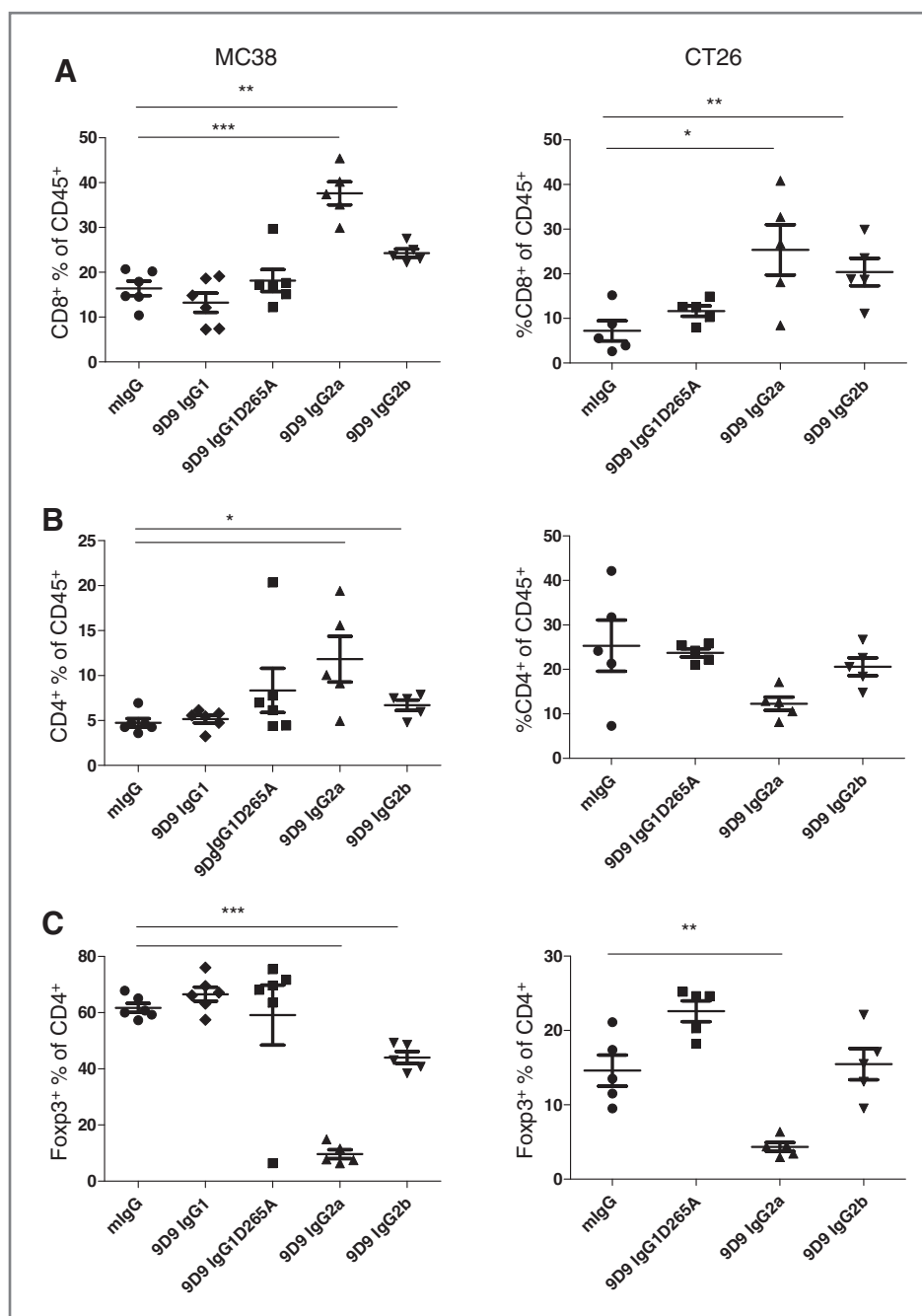
As CTLA-4 can be expressed both by Tregs and Teffs, we monitored multiple cell populations from different locations. Previous data showed that anti-CTLA-4 antibody blockade results in expansion of Tregs in the lymph nodes of treated mice (9). The effect of CTLA-4 antibody isotype on peripheral Treg expansion was tested in MC38 and CT26 tumor-bearing mice. All antibodies enhanced the numbers of Tregs in the spleen or at other sites in the periphery, such as lymph nodes or blood (Fig. 2A and Supplementary Fig. S3 for representative FACS plots). In addition, Tregs in animals treated with anti-CTLA-4 also have higher expression of Ki-67 (Fig. 2B), a marker for proliferation, suggesting that CTLA-4 blockade is removing an inhibitory signal, regardless of the antibody isotype. Similar results were obtained in an analysis of lymph nodes from nontumor-bearing mice treated with each of the anti-CTLA-4 isotypes (data not shown).

We next interrogated the effect of anti-CTLA-4 isotypes on cells at the tumor site. T-cell subsets were analyzed in tumors of MC38 and CT26 tumor-bearing mice at day 15 after tumor

implantation. Treatment with anti-CTLA-4 antibodies resulted in an increase in the percentage of CD8<sup>+</sup> CD45<sup>+</sup> cells at the tumor site, with the greatest increases in the IgG2a and IgG2b isotype-treated groups (Fig. 3A). Analysis of the percentage of intratumoral CD4 effector cells revealed that anti-CTLA-4 with an IgG2a or IgG2b isotype resulted in a slight increase of CD4<sup>+</sup> T cells in the MC38 but not in CT26 model (Fig. 3B). When intratumoral CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs were analyzed, profound differences in each of the treatment groups were observed. Treatment with anti-CTLA-4 of the IgG2a isotype resulted in dramatic decreases in Tregs at the tumor, whereas IgG2b showed a modest reduction. Isotypes IgG1 and IgG1-D265A resulted in slight increases in Treg prevalence (Fig. 3C; representative FACS plots in Supplementary Fig. S4A). Reduction of intratumoral Tregs is observed after a single anti-CTLA-4-IgG2a treatment within 24 hours ( $\sim 25\%$  reduction) and is reduced by approximately 75% by day 5 after treatment (Supplementary Fig. S4B).

The changes in Teff and Treg numbers mediated by the IgG2b and IgG2a anti-CTLA-4 antibodies result in dramatic differences in the intratumoral CD8 to Treg ratio as well as the CD4 effector to Treg ratio (Fig. 4A and 4B). Anti-CTLA-4-IgG2a isotype promoted the highest Teff to Treg ratio. An increased ratio of CD8 Teff to Tregs, originally described by Quezada and

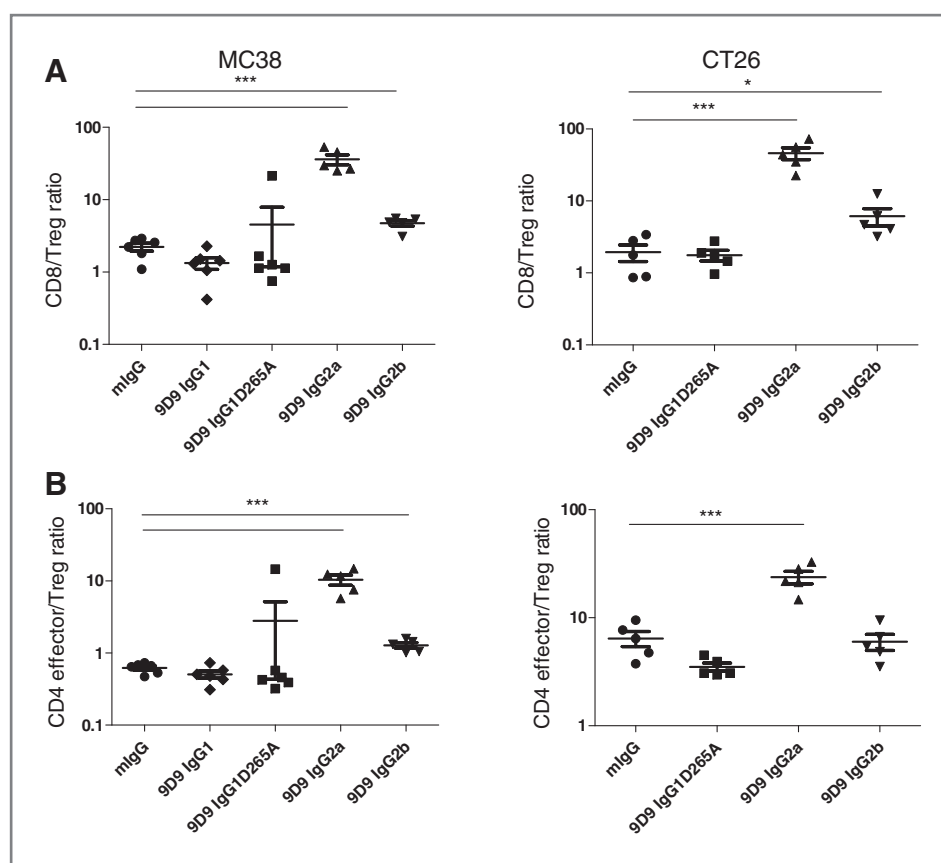
**Figure 3.** Isotype-dependent expansion of Tregs and reduction in Tregs. A total of  $2 \times 10^6$  MC38 colon tumor cells (left) or  $1 \times 10^6$  CT26 (right) were implanted subcutaneously into C57BL/6 or BALB/c mice, respectively. At day 7 postimplantation, tumor-bearing mice were randomized and dosed with 10 mg/kg of antibody by intraperitoneal injection, every 3 days  $\times$  3 for MC38 and every 3 days  $\times$  4 for CT26. On day 15 postimplantation, tumors were harvested, manually dissociated into single-cell suspensions, and stained for flow cytometry. A, percentage of CD45<sup>+</sup> cells that are CD8<sup>+</sup>. B, percentage of CD45<sup>+</sup> cells that are CD4<sup>+</sup>. C, percentage of CD4<sup>+</sup> cells that are foxp3<sup>+</sup>. Data are representative of 2 (CT26) or 3 (MC38) independent experiments with  $\geq 5$  mice/group/experiment.



colleagues (9), correlated with antitumor activity in this study. Because we observed similar results with respect to changes in T-cell subsets in both MC38 and CT26 tumor models, as well as in Sa1N fibrosarcoma (data not shown), it seems that the effects of anti-CTLA-4-IgG2a isotype represent a general observation across multiple tumor models and mouse MHC haplotypes.

The differing outcomes of anti-CTLA-4 IgG2a on intratumoral Treg numbers compared with peripheral Tregs or activated effectors could be attributed to differences in the expression levels of CTLA-4. To address this possibility,

CTLA-4 expression levels (both total and cell surface) on Tregs from tumor and periphery, as well as intratumoral CD4 and CD8 T cells, were analyzed by FACS from control IgG-treated animals. The expression of total CTLA-4 on intratumoral Tregs was increased 2- to 3-fold relative to peripheral Tregs from the spleen (Fig. 5A, left, and Fig. 5B) and significantly higher than CD4 and CD8 tumor-infiltrating Tregs. When CTLA-4 cell surface expression was examined, only tumor Tregs showed detectable levels at the cell surface (Fig. 5A, right). This difference in expression may account for the selective loss of tumor Tregs by an Fc $\gamma$ R-dependent



**Figure 4.** Isotype-dependent increase in the Teff to Treg ratio after treatment with anti-CTLA-4. Tumors from CT26 and MC38 mice were treated and harvested as described in Fig. 3, and the ratios of Teffs to Tregs at the tumor were calculated. A, ratio of CD8 effector cells to Treg cells. B, ratio of CD4 effector cells to Treg cells. Data are representative of 2 (CT26) or 3 (MC38) independent experiments with  $\geq 5$  mice/group/experiment.

mechanism such as antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP).

In addition to T cells, myeloid-derived suppressor cells (MDSC), defined by expression of the surface markers CD11b and Gr-1, were analyzed in tumors and spleens of anti-CTLA-4 antibody-treated MC38 tumor-bearing mice. No changes were observed in splenic MDSC for any of the anti-CTLA-4 isotypes (data not shown). Interestingly, MDSC numbers were substantially increased in tumors of mice treated with the IgG2a isotype (Supplementary Fig. S5A).

#### Intratumoral cytokine expression in response to anti-CTLA-4 treatment

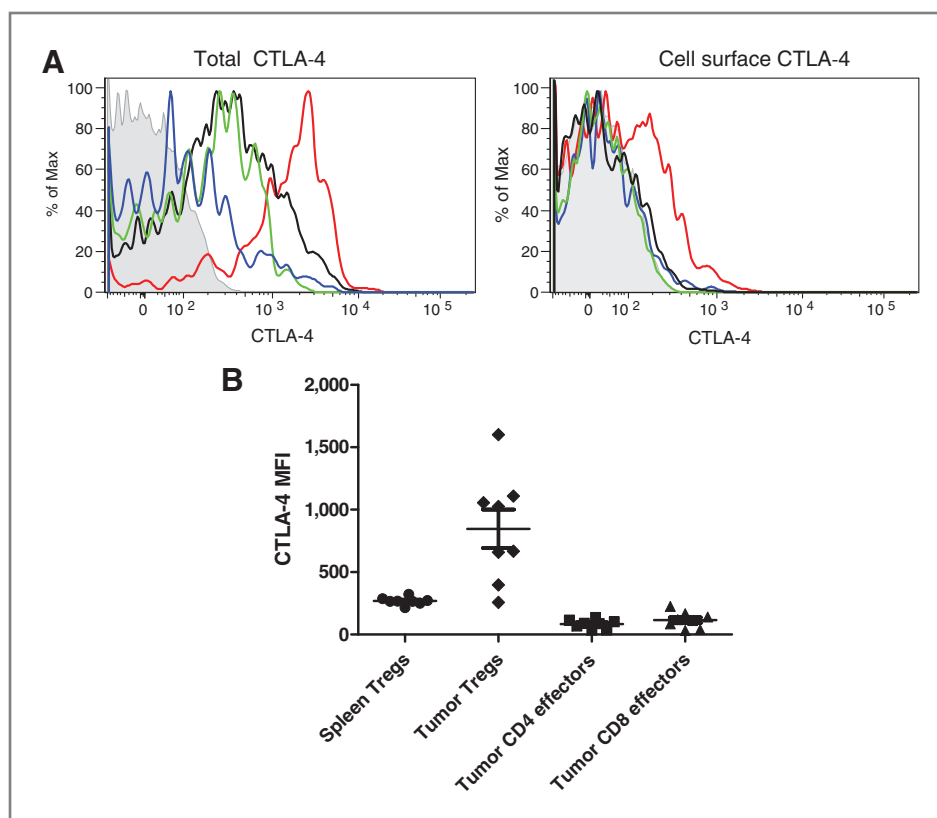
To determine whether the decrease in tumor-infiltrating Tregs and concomitant increase in effector CD8 numbers was associated with changes in T-cell function, we measured cytokine levels present within the tumor microenvironment in each of the treatment groups in MC38 tumor-bearing mice (Fig. 6). Anti-CTLA-4-IgG2a treatment resulted in the most pronounced increases in intratumoral levels of both T-helper ( $T_H$ )1 and  $T_H$ 2 cytokines, with significant enhancement of IFN- $\gamma$ , TNF- $\alpha$ , IL-13, and IL-10 compared with each of the other isotype variants (Fig. 6). Interestingly, the levels of IL-1 $\alpha$  were also both specifically and significantly higher in tumors of mice that had been treated with CTLA-4-IgG2a (Supplementary Fig. S5B) compared with all other isotypes. This upregulation was

unique to treatment with the IgG2a isotype and not simply associated with tumor destruction and regression as this increase in IL-1 $\alpha$  was not observed with MC38 tumor-bearing mice treated with the combination of anti-PD-1 and anti-CTLA-4-IgG2b antibodies, which leads to similar antitumor efficacy and expansion of Teffs (data not shown).

#### Discussion

The data presented here show profound differences among different isotypes on the antitumor potency of anti-CTLA-4 antibodies in subcutaneous mouse tumor models. Remarkably, anti-CTLA-4 with the IgG2a isotype was most effective, showing nearly complete monotherapy activity in both MC38 and CT26 tumor models, whereas IgG1 and IgG1-D265A displayed no antitumor activity. Isotype differences in antibodies have been shown to have markedly different effects on their biologic activities (28). The antitumor activity of antibodies directed against a melanoma differentiation antigen, antibody TA99, which targets gp75/TRP-1 on B16 melanoma cells, was shown to require binding to activating receptors (24, 29). This work defined the A/I ratio [the ratio of the binding of immunoglobulin Fc regions to activating Fc $\gamma$ R (Fc $\gamma$ RIV or Fc $\gamma$ RIII) to inhibitory Fc receptors (Fc $\gamma$ RIIB)], which correlated with the relative potency for antibodies mediating ADCC and/or ADCP function. The specific activating Fc $\gamma$  receptors that are required for maximal tumor clearance using targeted antibody is actively studied, and the results vary according to the model

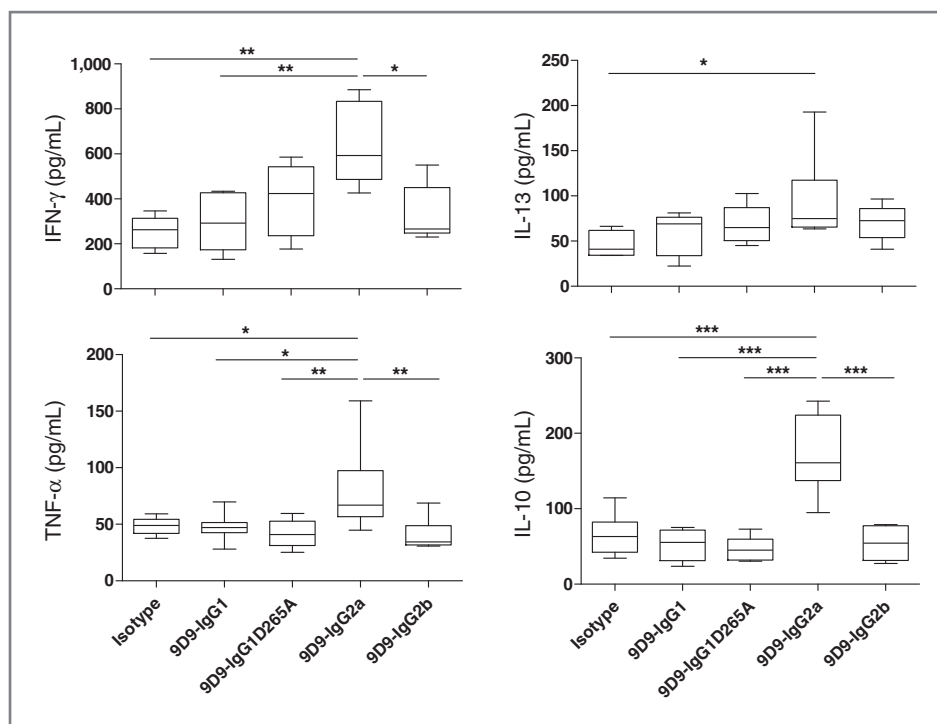
**Figure 5.** Expression of CTLA-4 on intratumoral and peripheral Tregs and Teffs. A, comparison of total CTLA-4 levels (left) or cell surface CTLA-4 levels (right) from either tumor CD4 effectors (blue histogram), tumor CD8s (green histogram), splenic Tregs (black histogram), and tumor Tregs (red histogram). Shaded gray histogram is isotype control staining. B, mean fluorescence intensity (MFI) from T-cell subsets of 8 control-IgG1 tumor-bearing mice.



and the tissue locations studied (24, 30). Interestingly, the antitumor activity of anti-CTLA-4 follows the hierarchy defined by the A/I ratio: IgG2a > IgG2b > IgG1, and suggests that maximal antitumor activity of anti-CTLA-4 requires bind-

ing to activating Fc $\gamma$ R. Anti-CTLA-4 IgG1 and IgG1-D265A were equally inactive in antitumor activity, suggesting that Fc $\gamma$ R1IB and Fc $\gamma$ R1II do not appreciably contribute to the antitumor effect of anti-CTLA-4-IgG2a and IgG2b isotypes.

**Figure 6.** Isotype-dependent enhancement of intratumoral T<sub>H</sub>1/2 cytokine secretion. A total of  $2 \times 10^6$  MC38 colon tumor cells were implanted subcutaneously into C57BL/6 mice. At day 7 postimplantation, tumor-bearing mice were randomized and received 3 doses of antibody by intraperitoneal injection (10 mg/kg) every 3 days. On day 15 postimplantation, tumors were harvested, manually dissociated into single-cell suspensions, and levels of intratumoral cytokines were assessed via bead-based cytokine arrays (FlowCytomix; eBioscience). Data are representative of 3 independent experiments with  $\geq 5$  mice/group/experiment.





Anti-CTLA-4-IgG2a, and to a lesser extent anti-CTLA-4-IgG2b, results in the elimination or depletion of Tregs from the tumor site consistent with their ability to bind to activating Fc $\gamma$ R<sub>s</sub>. This occurs with the concomitant activation and expansion of CD8 T<sub>eff</sub>s (and CD4 T cells), which is likely mediated by inhibiting CTLA-4-B7 interactions. However, our experiments do not rule out that T<sub>eff</sub> activation is solely a consequence of Treg depletion. Thus, when compared with the other isotypes, the murine IgG2a isotype of anti-CTLA-4 is able to potentially reduce Treg numbers, while sparing activated T<sub>eff</sub>s that mediate the antitumor response. Indeed, the finding of augmented effector cytokine secretion [IFN- $\gamma$ , TNF- $\alpha$ , and IL-13, and perhaps IL-10, (31, 32)] at the tumor site is consistent with a loss of Treg suppression and an increase in activated CD8 effectors.

The absence of antitumor activity of the IgG1 and IgG1-D265A isotypes in the therapeutic treatment of MC38 and CT26 models is also noteworthy. Inhibiting CTLA-4-B7 interactions with anti-CTLA-4 IgG1 or anti-CTLA-4 IgG1-D265A leads to activation and expansion of Tregs in the periphery, whereas blockade of T<sub>eff</sub> cells alone (i.e., in the absence of Treg elimination) is insufficient to promote a detectable antitumor response. In addition, blocking CTLA-4 on Tregs, while shown to diminish Treg function (9, 12, 13) also does not appreciably enhance antitumor activity. In contrast, in a B16 melanoma model, using anti-CTLA-4 (hamster anti-CTLA-4 9H10), GVAX therapy, and reconstitution of irradiated recipient mice with T-cell subsets expressing either human or mouse CTLA-4, mouse CTLA-4 expression was required on both T<sub>eff</sub> and Tregs for full antitumor activity (22). However, unlike the studies described here, anti-CTLA-4 blockade targeted to T<sub>eff</sub> only resulted in partial antitumor effects in this model.

Although anti-CTLA-4 IgG2a mediates its effect through elimination of intratumoral Tregs, intratumoral CD4 and CD8 T<sub>eff</sub>s are spared, as well as peripheral Tregs. The differential sensitivity of intratumoral Tregs to elimination may be due to higher levels of CTLA-4 expressed by intratumoral Tregs compared with intratumoral T<sub>eff</sub>s. Although we cannot formally exclude the elimination of some T<sub>eff</sub>s expressing high levels of CTLA-4, any loss of these cells was not apparent in either their overall percentage or absolute numbers within the tumor. Elimination of T<sub>eff</sub>s would be expected to limit the antitumor response. Interestingly, high levels of CTLA-4 cell surface expression were not a prerequisite for Fc $\gamma$ R-mediated effector activity. Similarly, the melanosomal antigen gp75/TRP-1, which is an intracellular melanosome protein, is a target of antibody TA99-mediated tumor rejection, despite its low level of cell surface expression (33).

The cellular composition of Fc $\gamma$ R-bearing cells at the tumor site may account for the differential effects of anti-CTLA-4 on intratumoral Tregs as opposed to peripheral Tregs. In this regard, it is interesting that there is an increased prevalence of CD11b<sup>+</sup>Gr-1<sup>hi</sup> myeloid cells specifically in tumors from mice treated with anti-CTLA-4-IgG2a. Although cells with this phenotype are normally ascribed to a suppressive subset, they are likely to express activating Fc $\gamma$ R and may be capable of mediating Treg reduction. These cells may be recruited by

or responsible for the high levels of IL-1 $\alpha$  induced by anti-CTLA-4-IgG2a treatment (34). Further experiments are required to determine the cell types, specific Fc $\gamma$ R, and the cellular mechanisms involved in anti-CTLA-4-mediated intratumoral Treg reduction. Preliminary experiments suggest that natural killer (NK) cell depletion has no appreciable effect on the antitumor activity of anti-CTLA-4-IgG2a (data not shown).

The results described here also have implications for the activity of anti-CTLA-4 antibodies in man. The antihuman CTLA-4 antibody, ipilimumab, has been approved for the treatment of metastatic melanoma and is in clinical testing in other cancers (19, 21). The isotype of ipilimumab is human IgG1, which binds best to most human Fc receptors (35). Ipilimumab has been shown to increase the absolute lymphocyte counts (36) and numbers of activated T cells in the blood of treated patients (as detected by increases in the frequency of HLA-DR<sup>+</sup> or ICOS<sup>+</sup> T cells; refs. 37, 38), indicating that depletion of T cells does not occur in the periphery in humans. Minor changes in peripheral Treg frequency in the blood of patients treated with ipilimumab have been observed (37), but little information of the effect of ipilimumab on intratumoral Tregs is available. However, a positive correlation between a high CD8 to Treg ratio and tumor necrosis in biopsies from metastatic melanoma lesions from patients treated with ipilimumab has been described (39). In addition, tumor tissue from ipilimumab-treated patients with bladder cancer had lower percentages of CD4<sup>+</sup> Foxp3<sup>+</sup> T cells than tumors from untreated patients with bladder cancer (38). These data support the possibility that ipilimumab mediates Treg reduction at the tumor site.

In contrast, tremelimumab, another CTLA-4 antibody that has been tested in human clinical trials, is an IgG2 isotype that binds poorly to human Fc receptors, except for the Fc $\gamma$ RIIa variant H131 (35). Tremelimumab, which—like ipilimumab— inhibits CTLA-4-B7 interactions, has demonstrable antitumor activity in metastatic melanoma (40, 41). However, it is possible that tremelimumab may be limited in mediating Treg reduction at the tumor. Indeed, studies on the mechanism of action of tremelimumab show, in small number of samples analyzed by immunohistochemistry, that increases in tumor-infiltrating CD8 T cells occur as a result of therapy, along with an increase or no change in the number of Foxp3<sup>+</sup> cells in the tumor (42).

Potential biomarkers of ipilimumab activity are also suggested by the results presented here. Anti-CTLA-4 may function best in those patients with high intratumoral Tregs. The presence of Tregs is likely to be the consequence of an ongoing immune response to tumor antigens. Indeed, response to ipilimumab has been associated with the presence of tumor-infiltrating lymphocytes (43, 44). Ipilimumab therapy is also more effective in patients with preexisting responses to tumor antigen, such as for NY-ESO-1 (45). Moreover, Treg elimination may depend on the presence of specific cell types in the tumor microenvironment. Consequently those cells present in the tumor, such as monocytes, macrophages, or NK cells, which bear the relevant Fc $\gamma$  receptors, may be required for the antitumor effect of anti-CTLA-4.

### Disclosure of Potential Conflicts of Interest

M.J. Selby is employed (other than primary affiliation; e.g., consulting) as a Director and has ownership interest (including patents) in Bristol-Myers Squibb. J.J. Engelhardt has ownership interest (including patents) in Bristol-Myers Squibb. M. Srinivasan is employed (other than primary affiliation; e.g., consulting) as a Director in Bristol-Myers Squibb. A.J. Korman has ownership interest (including patents) in Bristol-Myers Squibb stock. No potential conflicts of interest were disclosed by the other authors.

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