JEM



# Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies

Yannick Bulliard,[1] Rose Jolicoeur,[2] Maurice Windman,[2] Sarah M. Rue,[3] Seth Ettenberg,[1] Deborah A. Knee,[3] Nicholas S. Wilson,[1] Glenn Dranoff,[4,5] and Jennifer L. Brogdon[1]

[1]Department of Oncology and [2]Laboratory Animal Services, Novartis Institute for Biomedical Research, Cambridge, MA 02139
[3]Genomic Institute of the Novartis Research Foundation, San Diego, CA 92121
[4]Department of Medical Oncology and Cancer Vaccine Center, Dana Farber Cancer Institute and [5]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02215



The Journal of Experimental Medicine

**Fc γ receptor (FcγR) coengagement can facilitate antibody-mediated receptor activation in target cells. In particular, agonistic antibodies that target tumor necrosis factor receptor (TNFR) family members have shown dependence on expression of the inhibitory FcγR, FcγRIIB. It remains unclear if engagement of FcγRIIB also extends to the activities of antibodies targeting immunoregulatory TNFRs expressed by T cells. We have explored the requirement for activating and inhibitory FcγRs for the antitumor effects of antibodies targeting the TNFR glucocorticoid-induced TNFR-related protein (GITR; TNFRSF18; CD357) expressed on activated and regulatory T cells (T reg cells). We found that although FcγRIIB was dispensable for the in vivo efficacy of anti-GITR antibodies, in contrast, activating FcγRs were essential. Surprisingly, the dependence on activating FcγRs extended to an antibody targeting the non-TNFR receptor CTLA-4 (CD152) that acts as a negative regulator of T cell immunity. We define a common mechanism that correlated with tumor efficacy, whereby antibodies that coengaged activating FcγRs expressed by tumor-associated leukocytes facilitated the selective elimination of intratumoral T cell populations, particularly T reg cells. These findings may have broad implications for antibody engineering efforts aimed at enhancing the therapeutic activity of immunomodulatory antibodies.**

CORRESPONDENCE
Jennifer Brogdon:
jennifer.brogdon@novartis.com

Abbreviations used: ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, ADCC and phagocytosis; FcγR, Fc γ receptor; GITR, glucocorticoid-induced TNFR-related protein; TNFR, TNF receptor.

Activating Fc γ receptors (FcγRs) stimulate immune cell effector mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP), which combine to facilitate antibody-mediated tumor cell killing (Nimmerjahn and Ravetch, 2008; Hogarth and Pietersz, 2012). The importance of FcγR-mediated immune effector cell function has been demonstrated in preclinical efficacy studies for antibodies targeting a range of tumor cell–expressed receptors, including trastuzumab (HER2) and rituximab (CD20; Clynes et al., 2000; Nimmerjahn and Ravetch, 2012). The inhibitory FcγR, FcγRIIB, functions to modulate activating FcγR-mediated effector mechanisms in immune cells that coexpress both FcγR classes, such as macrophages and dendritic cells. FcγRIIB has recently been implicated in augmenting antibody-mediated receptor forward signaling through a mechanism of cross-linking in target cells expressing the TNF receptor (TNFR) family members TNFRSF10, TNFRSF10B (DR4 and DR5, respectively), and TNFRSF5 (CD40; Wilson et al., 2011; Li and Ravetch, 2012). It remains unclear what contribution FcγR biology has in the modality of antibody therapeutics that target other cell surface receptors. In particular, the emerging clinical benefit of agonistic antibodies targeting the T cell–APC interface raises the possibility that FcγR coengagement may contribute to their in vivo mechanism of action (Mellman et al., 2011).

N.S. Wilson's present address is ImmunoOncology, EMD Serono Research and Development Institute, Inc., Billerica, MA 01821.



The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 9 1685-1693
www.jem.org/cgi/doi/10.1084/jem.20130573

Preclinical studies in mice using agonistic antibodies targeted to glucocorticoid-induced TNFR-related protein (GITR)—a costimulatory TNFR expressed by regulatory and activated T cells—have shown compelling antitumor activity in syngeneic mouse tumor models (Turk et al., 2004; Ko et al., 2005). In vitro, stimulation of GITR with agonist antibodies can induce forward signaling into T cells, which promotes proliferation and cytokine production (Kanamaru et al., 2004; Ronchetti et al., 2007). In vivo, several mechanisms have been proposed to contribute to the antitumor activity of antibodies targeting GITR; however, the current paradigm stipulates that agonist properties of these antibodies promotes cytotoxic effector T cell generation, while dampening the immunosuppressive effects by FoxP3$^{+}$ CD4$^{+}$ T reg cells (Ronchetti et al., 2012; Schaer et al., 2012). The recent findings that antibodies targeted to TNFR family members require FcγRIIB interaction for their in vivo activities led us to explore a common mechanism for antibodies targeting TNFRs expressed by T cells, using GITR to test this paradigm.

## RESULTS AND DISCUSSION

### Activating, but not inhibitory, FcγRs are necessary for antitumor activity of a GITR-targeting antibody

To evaluate the contribution of activating or inhibitory FcγRs in the mechanism of tumoricidal activity of an agonist antibody targeting GITR (clone DTA-1, rat IgG2b), Colon26 colorectal cancer cells were implanted subcutaneously in wild-type, FcγRIIB-, or Fc common γ chain–deficient mice. The common γ chain cofactor is required for assembly and membrane expression of the activating FcγRs I, III, and IV (Nimmerjahn and Ravetch, 2008). Mice with preformed tumors (~70 mm$^3$) were treated with a single dose of the anti-GITR antibody (clone DTA-1) or a rat IgG2b isotype control. As previously shown for this tumor model, DTA-1–mediated single dose regressions in 100% of wild-type mice (Fig. 1 A; Zhou et al., 2007). In contrast to recent reports studying anti-TNFR antibodies targeting DR4, DR5, or CD40, the antitumor efficacy of DTA-1 was independent of FcγRIIB expression (Fig. 1 B; Wilson et al., 2011; Li and Ravetch, 2012). Instead, activating FcγRs were required for the tumoricidal activity of a GITR-targeting antibody (Fig. 1 C).

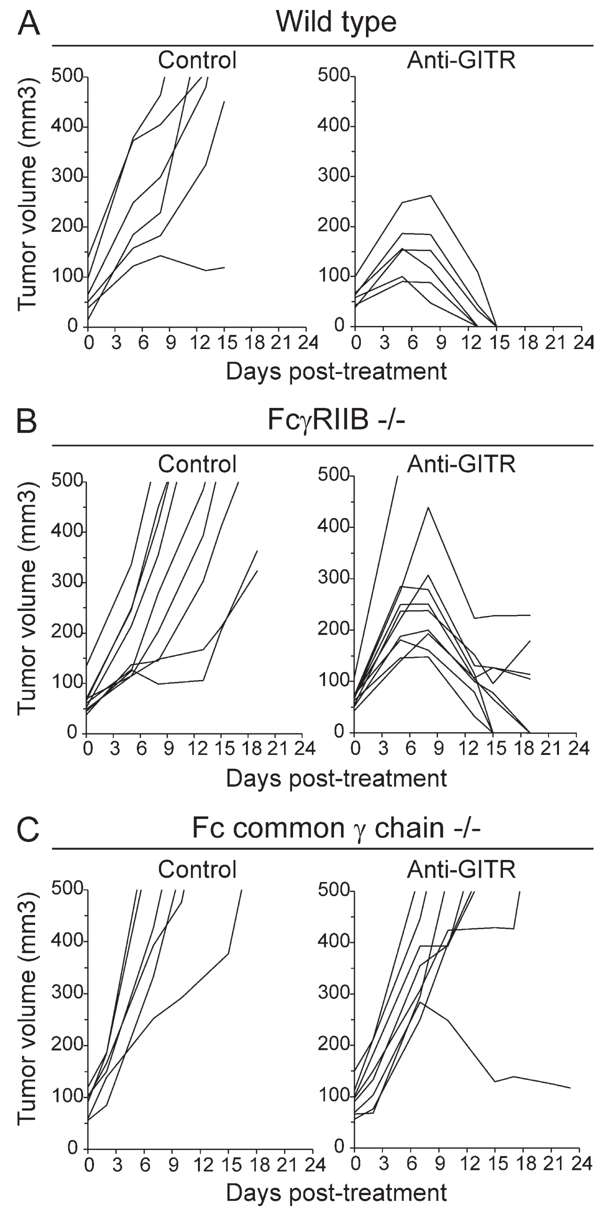


**Figure 1. Activating, rather than inhibitory, FcγRs are necessary for the antitumor activity of an agonistic antibody to GITR.** Efficacy study of anti-GITR antibody (DTA-1 rIgG2b; 5 mg/kg i.p.) in wild type (A), FcγRIIB$^{-/-}$ (B), and Fc common γ chain$^{-/-}$ (C) BALB/c mice bearing Colon26 tumors (*n* = 6–10 mice per treatment group). Day 0 refers to treatment day, 6–8 d after tumor inoculation. Data is a representative of two or more independent experiments.

### Co-engagement of FcγRs by DTA-1 is required for optimal antitumor activity

To further examine the contribution of activating FcγRs for the tumoricidal activity of antibodies to GITR, we generated two chimeric antibodies from the parental DTA-1 rat IgG2b: a murine IgG2a (mIgG2a), and mIgG2a with a N297A mutation that eliminates binding to all murine FcγRs (not depicted; Shields et al., 2001; Chao et al., 2009; Wilson et al., 2011). Binding to in vitro–stimulated splenic T cells was conserved for the murine IgG2a DTA-1 variants and was comparable to parental DTA-1 rat IgG2b (Fig. 2, A and B). All versions of DTA-1 showed a similar ability to induce NF-κB signaling in a reporter cell assay (Fig. 2 C). Moreover, the three DTA-1 variants similarly enhanced anti-CD3–mediated T cell proliferation and cytokine production in vitro (Fig. 2 D and not depicted). Both DTA-1 rIgG2b and mIgG2a showed potent antitumor efficacy, which was abrogated in absence of activating FcγRs but was not impacted by the absence of FcγRIIB expression (Fig. 1 and Fig. 2 E). Eliminating FcγR interaction through the N297A mutation abrogated the antitumor activity of DTA-1 (Fig. 2 F). The three versions of DTA-1 showed similar GITR saturation properties in the tumor and draining lymph, supporting common pharmacokinetic/pharmacodynamic properties

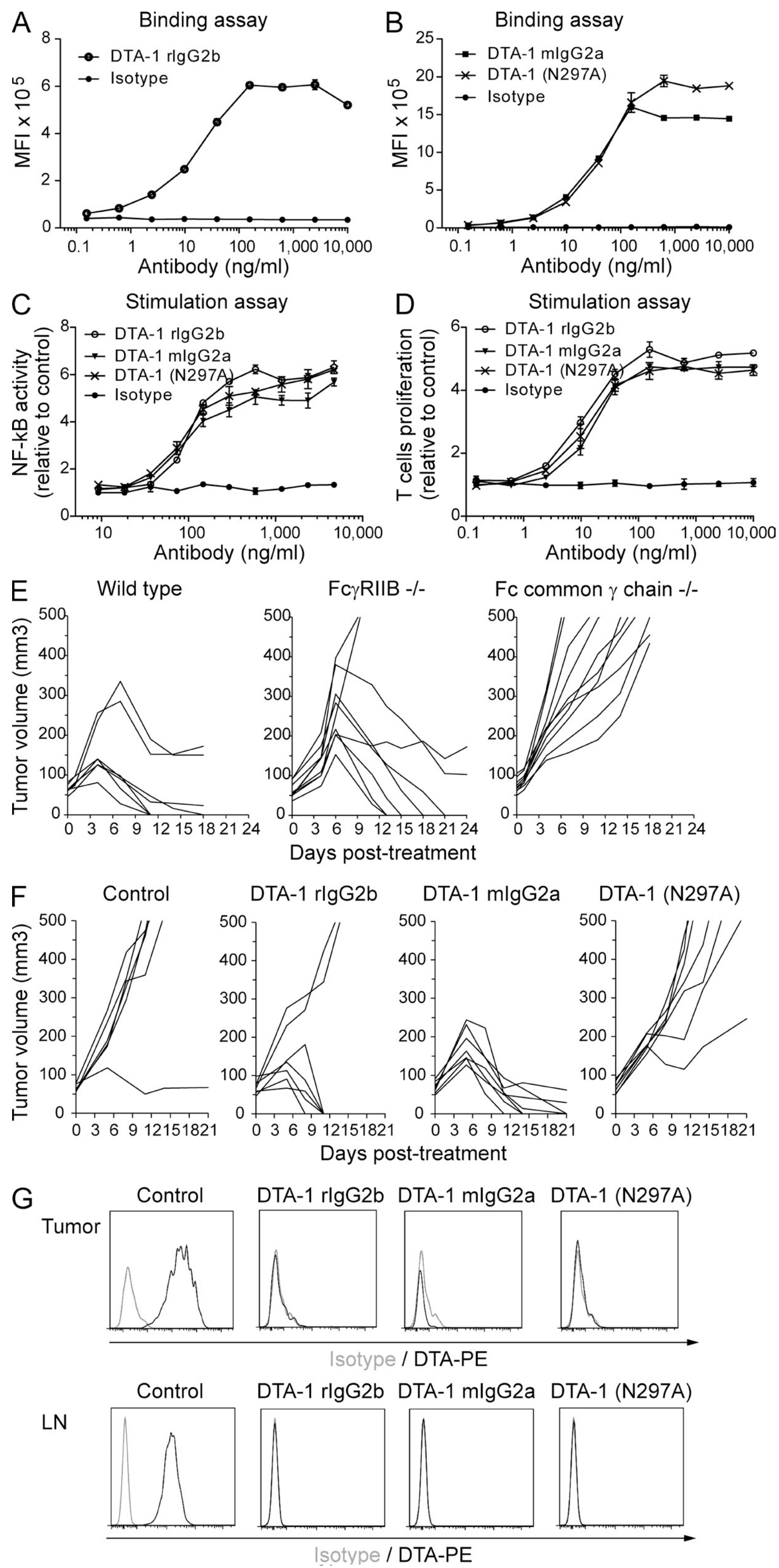


**Figure 2. Engagement of FcγRs by DTA-1 is required for antitumor activity.** (A and B) GITR-binding assay. Primary splenocytes stimulated with CD3- and CD28-specific antibodies served as targets. DTA-1 variants were detected using rat IgG2b-specific (A) or murine IgG2a-specific (B) PE-conjugated antibodies. (C and D) In vitro activity of GITR-specific antibodies at various concentrations tested on GITR-expressing NF-kB 293 reporter cell line (C) and splenocytes incubated with suboptimal doses of anti-CD3 and anti-CD28 antibodies (D). The in vitro data are derived from triplicates and are a

of these variants (Fig. 2 G). Collectively, our results support that agonist antibodies targeting GITR require co-engagement with activating FcγRs for their tumoricidal activities in this model.

### Co-engagement of FcγRs by DTA-1 results in intratumoral loss of T cells

To further understand the underlying FcγR-dependent mechanism of DTA-1, we analyzed the immune cell populations in tumor and draining lymph node after administration of the DTA-1 variant antibodies, focusing on early time points before tumors typically begin to regress. First, we confirmed that GITR was expressed on T cells in the draining lymph node and tumor (Fig. 3 A; Shimizu et al., 2002). The highest expression of GITR was by T cells in the tumor compared with the draining lymph node, with T reg cells in the tumor showing approximately fourfold stronger signal than the equivalent lymph node population. Next, we profiled myeloid and NK cells for expression of FcγRIII and IV (Fig. 3, B and C). Tumor-associated myeloid and NK cell populations were abundant in Colon26 tumors (>50% of all $CD45^+$ leukocytes) and expressed activating FcγRIII/FcγRIV and FcγRIII, respectively. FcγRIII was distinguished from FcγRIIB using mice deficient for FcγRIIB in combination with the 2.4G2 antibody, which cross-reacts with FcγRIIB and III. In contrast, the same innate immune cell populations were underrepresented in the tumor-draining lymph node (<0.5% of all leucocytes; Fig. 3 B). Treatment with the parental or mIgG2a DTA-1 variant resulted in a strong reduction in the percentage of intratumoral $FoxP3^+$ T reg cells (Fig. 3 D). This effect was specific to the tumor, with no significant change in the T reg cell population in draining lymph nodes. In contrast, treatment with the DTA-1-N297A variant did not alter intratumoral T cell populations, supporting dependence on FcγR interactions for this effect. To quantify the change in T cell populations after DTA-1 variant treatment, we monitored the density of T cells in the tumor over 5 d. By 24 h, the parental rat IgG2b or DTA-1 mIgG2a mediated a dramatic reduction in the density of $FoxP3^+$ T reg cells in the tumor, which did not occur in the DTA-1-N297A–treated cohort (Fig. 3 E). Although the loss of intratumoral T cells was most dramatic at early time points in the T reg cell population, $CD4^+$ T cells were also significantly reduced on days 3 and 5 after antibody treatment (Fig. 3 F). A slight reduction in the density of $CD8^+$ T cells was also observed, particularly in the DTA-1 mIgG2a cohort. The depletion of T reg cells was most pronounced, which resulted in a significant shift in the ratio of $CD8^+$ T cells to T reg cells in the tumor early after treatment (Fig. 3 G). This finding correlated with previous reports showing an obvious shift in the ratio of $CD8^+$ T cells to T reg cells in the tumor, although in the previous reports, tumors were extracted at later time points after the onset of regression (Ko et al., 2005; Sharma et al., 2008; Cohen et al., 2010). Importantly, DTA-1 treatment did not affect the overall density of tumor-associated leukocytes, supporting that T cell elimination was a specific event (Fig. 3 H). Again, the cellularity of T cells in the tumor-draining lymph node, as well as the ratio of $CD8^+$ T cells to T reg cells, remained mostly unchanged (Fig. 3 I and not depicted). Together, our data reveal that DTA-1–mediated an FcγR-dependent loss of T cells in the tumor, with the magnitude of depletion correlating with cell surface expression of GITR (T reg→FoxP3-$CD4^+$→$CD8^+$). Furthermore, we demonstrate that the loss of intratumoral T cells precedes tumor regression, supporting that this depletion mechanism may serve as an initial event that culminates in tumor rejection.

### Activating, but not inhibitory, FcγRs are required for intratumoral T reg cell depletion

To evaluate if the depletion of intratumoral T cells required activating FcγR expression, we monitored T cell populations in wild-type, FcγRIIB-, and Fc common γ chain–deficient mice after treatment with DTA-1-mIgG2a. Murine IgG2a DTA-1 mediated potent T reg and non–T reg cell elimination in both wild-type and FcγRIIB-deficient animals, which resulted in a shift in the ratio of $CD8^+$ T cells to T reg cells in the tumor, whereas the ratio remained unchanged in the draining lymph node (Fig. 4, A and B). In contrast, no significant depletion or shift in T cell ratio was observed in mice devoid of activating FcγR expression (Fig. 4 C). GITR expression on T cell populations isolated from tumors and lymph nodes was conserved between the three strains and was highest among intratumoral T reg cells (Fig. 4, D and F). These data support the notion that treatment with antibodies targeting GITR-expressing tumor-infiltrating T cells leads to activatory FcγR-dependent elimination, the degree of which correlates with levels of GITR expression.

### Activating FcγRs are also required for the antitumor activities of an antibody targeting the non-TNFR antigen CTLA-4

With the unexpected dependence on activatory FcγRs for DTA-1–mediated antitumor activity, we next sought to understand if this finding was restricted to a T cell–expressed immunostimulatory TNFRs. To evaluate this, we chose an antagonist antibody (clone 9D9, murine IgG2b) targeting the T cell antigen CTLA-4, which functions to negatively regulate T cell proliferation upon signaling (Leach et al., 1996; Curran et al., 2010). Consistent with earlier reports, a single

representative of two or more independent experiments. (E) Efficacy study of 5 mg/kg DTA-1 mIgG2a in wild-type, $Fc\gamma RIIB^{-/-}$, and Fc common γ $chain^{-/-}$ mice bearing Colon26 tumors (*n* = 7–10 mice per treatment group). Mean and standard errors are based on triplicates, and the data is a representative of two or more independent experiments. (F) In vivo efficacy study after treatment with the DTA-1 variant antibodies (*n* = 7). The efficacy data are a representative of two or more independent experiments. (G) Saturation of GITR on T reg cells in the tumor and draining lymph node by the three versions of DTA-1.

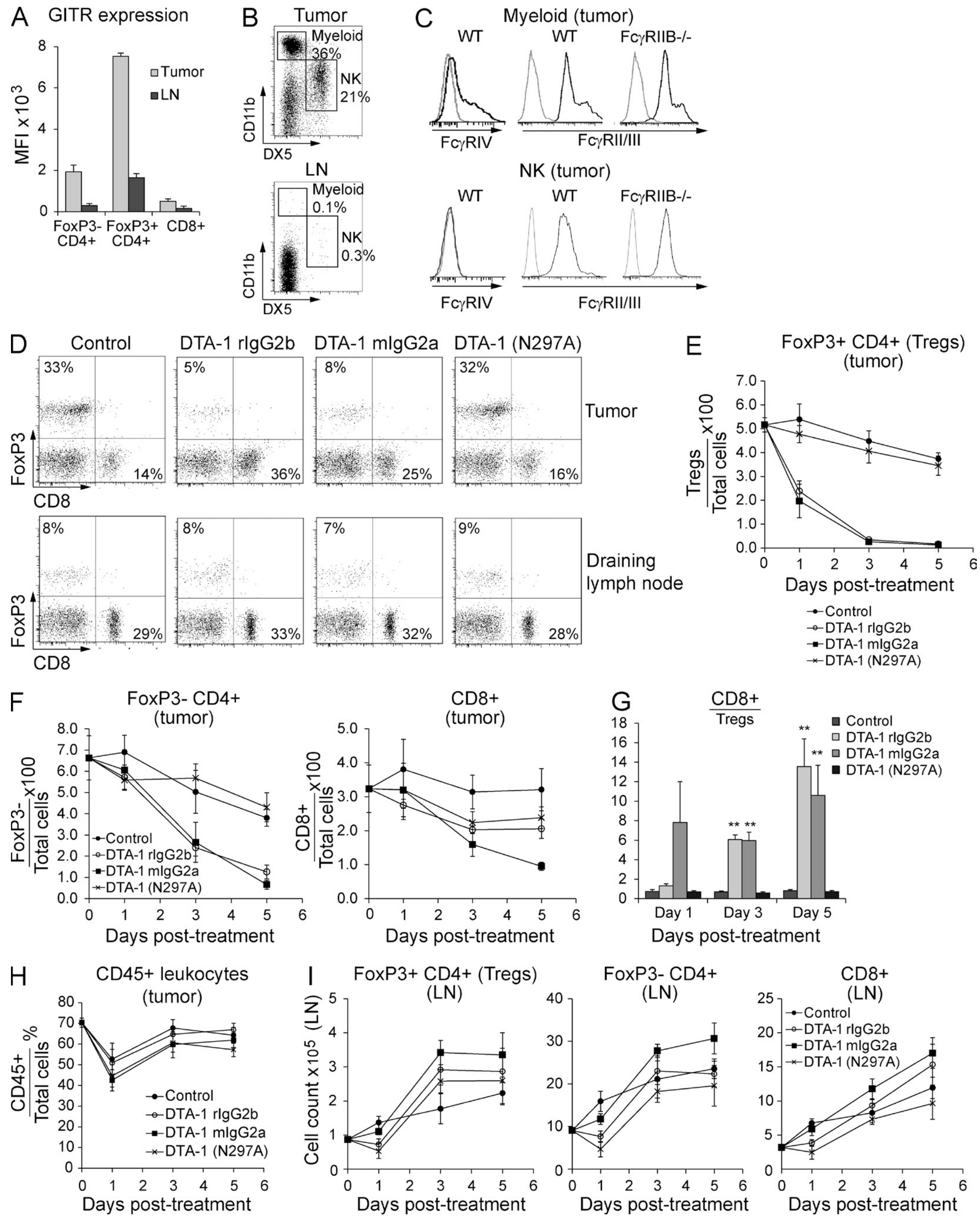


**Figure 3. Engagement of FcγRs by DTA-1 induces loss of intratumoral T reg cells early after treatment.** (A) Cell surface expression of GITR on day 0 ($n = 3$). (B and C) Dot plots of CD11b+ myeloid and DX5+ NK cells, gated on live CD45+ leukocytes (B) and expression of FcγRII/III and IV by the two cell populations in the tumor (C). (D) Dot plots of T cells 3 d after treatment with the DTA-1 variant antibodies. Percentages of live CD45+ CD3+ T cells are indicated. The data are a representative of two or more independent experiments. (E and F) Density of T reg cells (E) and FoxP3-CD4+ T cells and CD8+ T cells (F) in the tumor after treatment with the 5 mg/kg DTA-1 variants. (G) Ratio between CD8+ T cells and T reg cells in the tumor. (H) Percentage of intratumoral CD45+ leukocytes. (I) T cell number in the draining lymph nodes after treatment with 5 mg/kg of the DTA-1 variants. Mean and standard errors are based on triplicates from two independent experiments. P-values were calculated using an unpaired Student's *t* test (**, $P < 0.001$).

intraperitoneal injection of 9D9 mediated tumor regressions in 100% of animals (Fig. 5 A; Leach et al., 1996; Curran et al., 2010). To explore the involvement of FcγR in anti–CTLA-4–mediated antitumor efficacy, we evaluated tumoricidal activity of anti-CTLA4 in mice devoid of activating FcγR expression (Fig. 5 A). Similar to what we observed in DTA-1–treated animals, 9D9 efficacy was strictly dependent on activating FcγRs. Similar to the expression profile of GITR, CTLA-4 was expressed by intratumoral CD4+ T cells, most notable the T reg cell population (Fig. 5 B). 9D9 administration resulted in the

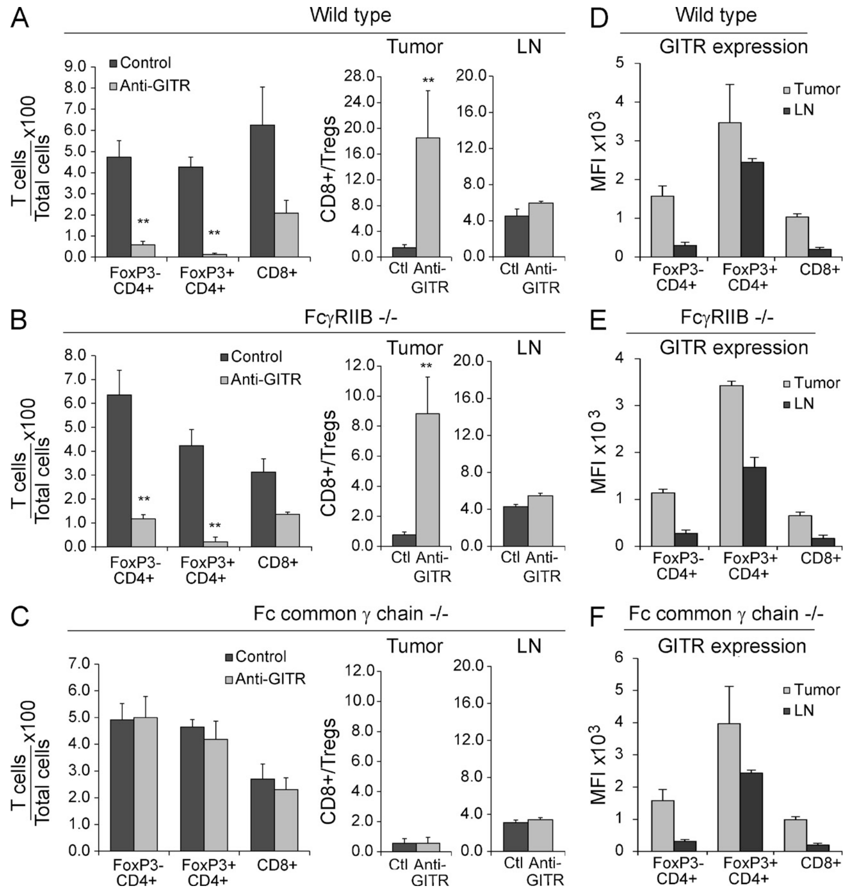


**Figure 4. Activating, but not inhibitory, FcγRs are required for intratumoral T reg cell depletion by antibodies targeting GITR.** Intratumoral T cell density and CD8⁺ T cells to T reg cells ratios 5 d after treatment with 5 mg/kg DTA-1-mIgG2a using wild-type (A), FcγRIIB$^{-/-}$ (B), or Fc common γ chain$^{-/-}$ (C) mice bearing Colon26 tumors. (D–F) Cell surface expression of GITR on T cells. Mean and standard errors are based on triplicates from two independent experiments. P-values were calculated using an unpaired Student's *t* test (**, $P < 0.001$).

specific depletion of tumor-associated T cells and a shift in the ratio of CD8$^+$ to T reg cells, which was dependent on activating FcγR expression (Fig. 5 C). Together, these data support a common antitumor mechanism shared between antibodies targeting receptors highly expressed at the surface of intratumoral CD4$^+$ T cells, which requires the function of activating FcγRs.

Here, we have shown that antibodies targeting two functionally distinct immunoregulatory receptors on T cells require the coengagement of activatory FcγRs to mediate their antitumor effect. A single dose of the GITR targeting antibody DTA-1 mediated the rapid and selective elimination of T cells within the tumor microenvironment, particularly those of the T reg cell lineage, as defined by intracellular FoxP3 expression. The dependence on the expression of activating FcγRs for T cell depletion appears consistent with involvement of the immune effector cell mechanisms ADCC or ADCP. Indeed, tumor-associated immune effector cells expressing activating FcγRIII and IV were abundant in Colon26 tumors and are known to mediate ADCC or ADCP effects in other model systems (Nimmerjahn and Ravetch, 2005; Albanesi et al., 2012). In general, the depletion of T cell populations correlated with the overall level of target antigen expression, although with anti–CTLA-4 treatment this observation was less clear with an overall reduction of intratumoral CD4$^+$ T cells on day 5. We reconcile these observations with the ability of activatory FcγR-expressing immune effector cells to elicit ADCC, based on a receptor threshold model (Lewis et al., 1993; Niwa et al., 2005). In contrast, few myeloid or NK cells were present in the draining lymph nodes, and the expression of GITR and CTLA-4 on FoxP3$^+$ T reg cells in this compartment was lower than in the tumor. An alternative hypothesis is that agonist GITR signaling mediated by DTA-1 could alter the stability of the transcription factor FoxP3 expressed by intratumoral T reg cells (Cohen et al., 2010). However, the loss of FoxP3-negative CD4$^+$ T cells from DTA-1–treated and anti–CTLA-4–treated tumors indicates that this FcγR-dependent depletion mechanism may rather correlate with target antigen expression. Collectively, our data support a common mechanism shared between antibodies targeting receptors highly expressed on intratumoral T reg cells and CD4$^+$ T cells, at least in the Colon26 colorectal cancer model. Additional studies are required, however, to address whether these findings may be translatable to other less immunogenic tumor models. In addition, further studies will be required to establish the

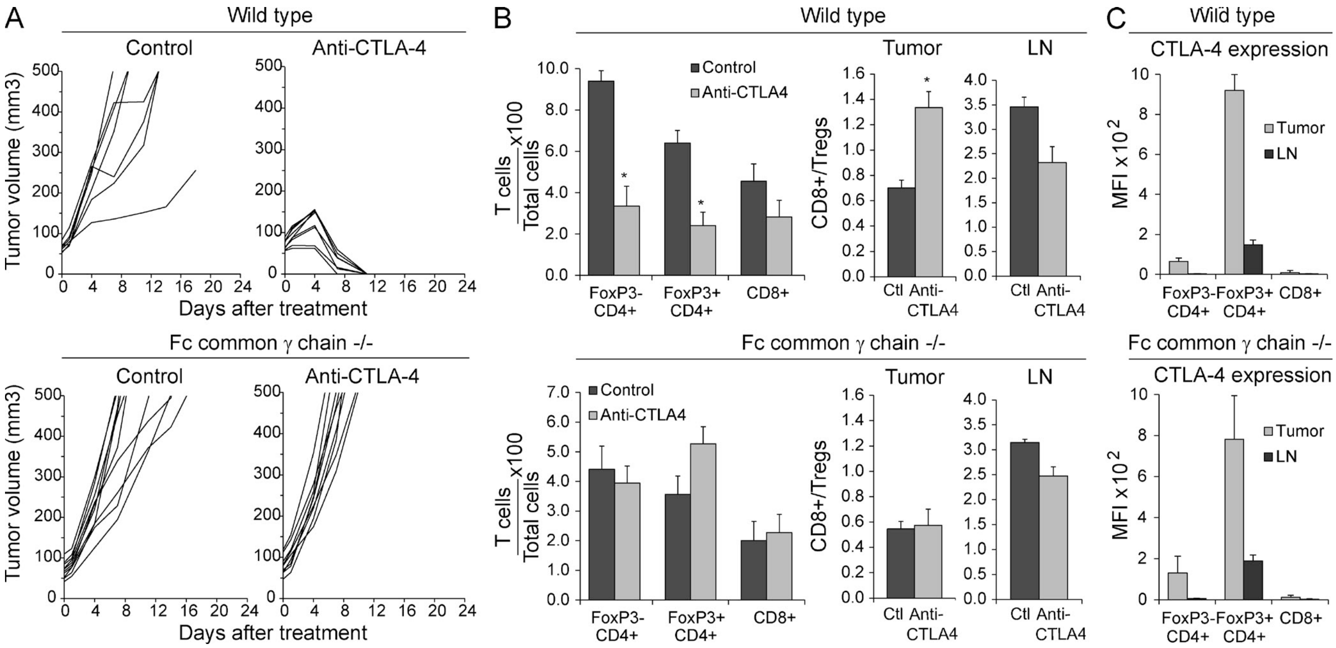


**Figure 5. Engagement of activating FcγRs is also required for the antitumor activity of an antibody targeting the non-TNFR CTLA-4.** (A) Efficacy study using 15 mg/kg CTLA-4–specific antibody (9D9) in wild-type (top) or Fc common γ chain$^{-/-}$ (middle) mice bearing Colon26 tumors (*n* = 8–10 mice per treatment group). (B) Intratumoral T cell density and CD8$^{+}$ to T reg cells ratios 5 d after treatment. (C) Cell surface expression of CTLA-4 on T cells. The efficacy data are a representative of two or more independent experiments. Mean and standard errors are based on triplicates from two independent experiments. P-values were calculated using an unpaired Student's *t* test (*, P < 0.01).

exact mechanism of T cell depletion, to understand how depletion of T reg cell and non–T reg cell populations augment subsequent adaptive antitumor immune response, and, finally, to further explore how antibody-mediated GITR forward signaling contributes to antitumor immunity.

The requirement for activating FcγR coengagement for antibodies targeting costimulatory and inhibitory cell surface targets within the T cell–APC interface supports the possibility of using antibody engineering to enhance their therapeutic activity. This conclusion draws parallels with recent efforts to augment activating FcγR coengagement of antibodies targeting tumor associated targets, such as HER2 or CD317 (Junttila et al., 2010; Nordstrom et al., 2011; Tai et al., 2012). Interestingly, ipilimumab, an antagonist antibody targeting CTLA-4 with human IgG1 framework, has shown efficacy in metastatic melanoma patients. Human IgG1 antibodies have an intrinsically higher activating to inhibitory (A/I) FcγR ratio, which is proposed to favor ADCC effector mechanisms (Hodi et al., 2010; Robert et al., 2011; Hogarth and Pietersz, 2012). In contrast, another antagonist antibody targeting CTLA-4, tremelimumab, is a human IgG2 antibody and has a lower A/I ratio. This antibody failed to reach its endpoints in a clinical trial (Chung et al., 2010). Although these two antibodies remain to be compared directly in patients, it is tempting to speculate that the varying ability of IgG1 versus IgG2 to bind human activating FcγRs may explain, at least in part, the apparent difference in clinical response. Accordingly, Fc-optimized variants of ipilimumab, such as mutations designed to enhance ADCC or ADCP, could also be considered for clinical evaluation. In parallel, a prospective or retrospective study to analyze patients carrying the allelic variants of the activating FcγRIIA and III could be valuable, particularly given the striking correlation with progression-free survival in patients treated with Rituximab (Weng and Levy, 2003). In these settings, a challenge will be to design relevant preclinical models to evaluate the efficacy of immunomodulatory antibodies that better correlate clinical response, and our findings provide a framework to test this with future immunomodulatory antibodies. For instance, mice engineered to express the human FcγR system might be of great utility (Smith et al., 2012).

## MATERIALS AND METHODS

**Mice and tumor models.** 6–10-wk-old wild-type BALB/c female mice were obtained from Charles River Laboratory or Taconic and aged-matched BALB/c FcγRIIB$^{-/-}$ and Fc common γ chain$^{-/-}$ female mice from Taconic. The BALB/c-derived colorectal carcinoma cell line Colon26 was obtained from the DCTD Tumor Repository, National Cancer Institute, Frederick, MA. Colon26 cells were maintained at subconfluent density and under low number of passages before being inoculated, typically by subcutaneous injection of $5 \times 10^5$ cells onto the right flank of BALB/c mice. Tumor diameter was measured by electronic caliper every 2–3 d, and tumor volume was determined by length × width$^2$/2. For all experiments, mice were housed at the Novartis Cambridge Laboratory Animal Services. All procedures were performed in accordance with the standards of the US Department of Health and Human Services and were approved by the Novartis Animal Welfare Committee.

**Antibody production and treatment.** The parental rat IgG2b DTA-1–producing hybridoma was provided by S. Sakaguchi (Immunology Frontier Research Center, Osaka University, Osaka, Japan). The variable region sequences of the parental antibody were cloned from the hybridoma and inserted into a pRS5a derivative vector containing publicly available sequences for the constant domain region of murine IgG2a to create the chimeric antibody DTA-1-mIgG2a. The N297A mutation was introduced into the DTA-1-mIgG2a–encoding vector by site-directed mutagenesis to generate DTA-1-N297A. DTA-1 antibodies were produced from HEK 293 FreeStyle cells (Invitrogen) transfected with appropriate expression vectors and purified using Fast Flow rProtein A Sepharose (GE Healthcare), followed by size exclusion chromatography. The integrity of the antibodies was verified by SDS-PAGE and analytical SEC. The LTF-2 (control; rat IgG2b), C1.18.4 (control; mouse IgG2a), DTA-1 (GITR; rat IgG2b), and 9D9 (CTLA-4; mouse IgG2b) antibodies used for in vivo studies were purchased from BioXCell. Before injection in vivo, antibodies were cleared from precipitates by centrifugation and confirmed to contain endotoxin levels below 1 EU/mg of antibody (LAL assay). For in vivo studies, tumor-bearing mice were typically treated 8–9 d after tumor inoculation (~70 $mm^3$) by intraperitoneal injection of antibodies at indicated doses.

**Tumor dissociation and analyses by flow cytometry.** CD45-, CD25-, CD4-, CD8-, and FcγRII/III (clone 2.4G2)-specific and matching isotype control antibodies were purchased from BD. GITR-, CD11b-, DX5-, CTLA-4–, and FoxP3-specific antibodies were purchased from eBioscience, CD3-specific antibody from BioLegend, and FcγRIV-specific antibody from Sino Biologicals. To obtain single cell suspensions from excised tumors, the tumors were first minced, dissociated with collagenase (Liberase; Roche) and DNase I (Life Technologies), filtered on a 70-µM sieve, and then treated with RBC lysis buffer (eBioscience) before washes with PBS + 2% FBS. For most stainings, cells were incubated with saturating doses of anti-CD16/32 FcγR block (BD) before incubation with fluorochrome-conjugated antibodies. For FcγRII/III staining, the directly conjugated 2.4G2 antibody was incubated before FcγR block. For staining of intracellular markers, extracellular markers were stained first, before fixation/permeabilization of the cells (eBioscience), followed by staining of intracellular proteins using primary-conjugated antibodies. During staining procedure, cells were maintained on ice. Acquisition was performed on an LSR-II flow cytometer (BD). The machine performances were verified daily using Cytometer Setup and Tracking beads (BD), and weekly using Sphero Rainbow Fluorescent 1 Peak particles (BD) and AccuCount Blank beads (Spherotech, Inc). For each analysis, the population of interest was gated on live leukocytes using a combination of morphological parameters, CD45-specific labeling and dead cells exclusion using 7AAD (BD Biosciences), or Live/Dead yellow (Life Technologies). The cell density in the tumor was calculated by dividing the number of cells of interest to the total number of cells extracted. Before each run, a compensation matrix was generated from primary cells stained separately with individual fluorochrome-conjugated antibodies. P-values were calculated using an unpaired Student's *t* test.

**NF-kB activation reporter assay.** The 293-NFkB-luc cell line was generated by stable transfection of HEK293 cells with pNF-κB-Luc plasmid (Takara Bio Inc.), which had been modified to express the Zeocin resistance gene. GITR sequence was cloned from cDNA (Life Technologies), introduced into a pcDNA3 expression plasmid (Invitrogen), and transfected into 293-NFκB-luc cell line to generate the 293-GITR-NFκB-luc reporter cell line. $10^6$ cells/ml cells were incubated with indicated concentrations of GITR-specific antibodies and incubated at 37°C for 24 h. NF-κB activation levels were indirectly determined by measuring NF-κB–induced luciferase activity using CellBright-Glo (Promega).

**In vitro T cell stimulation assays.** CD3- and CD28-specific antibodies were purchased from R&D Systems. $5 \times 10^4$ splenocytes were stimulated with 0.1 µg/ml CD3- and 0.2 µg/ml of CD28-specific antibodies together with indicated concentrations of GITR-specific antibodies in round-bottom culture-treated 96-well plates. After 72 h of incubation, cell proliferation was indirectly determined using CellTiter-Glo (Promega).

We would like to thank Dr. Shimon Sakaguchi for kindly providing the hybridoma for parental DTA-1 rat IgG2b. Particular thanks to Pu Zhang for expert assistance with flow cytometry and Seguinde Arora for critical technical support.

Glenn Dranoff is a consultant and receives sponsored research support from Novartis, Inc. The authors have no additional financial interests.

**Submitted: 18 March 2013**
**Accepted: 1 July 2013**

## REFERENCES

Albanesi, M., D.A. Mancardi, L.E. Macdonald, B. Iannascoli, L. Zitvogel, A.J. Murphy, M. Daëron, J.H. Leusen, and P. Bruhns. 2012. Cutting edge: FcγRIII (CD16) and FcγRI (CD64) are responsible for anti-glycoprotein 75 monoclonal antibody TA99 therapy for experimental metastatic B16 melanoma. *J. Immunol.* 189:5513–5517. http://dx.doi.org/10.4049/jimmunol.1201511

Chao, D.T., X. Ma, O. Li, H. Park, and D. Law. 2009. Functional characterization of N297A, a murine surrogate for low-Fc binding anti-human CD3 antibodies. *Immunol. Invest.* 38:76–92. http://dx.doi.org/10.1080/08820130802608238

Chung, K.Y., I. Gore, L. Fong, A. Venook, S.B. Beck, P. Dorazio, P.J. Criscitiello, D.I. Healey, B. Huang, J. Gomez-Navarro, and L.B. Saltz. 2010. Phase II study of the anti-cytotoxic T-lymphocyte-associated antigen 4 monoclonal antibody, tremelimumab, in patients with refractory metastatic colorectal cancer. *J. Clin. Oncol.* 28:3485–3490. http://dx.doi.org/10.1200/JCO.2010.28.3994

Clynes, R.A., T.L. Towers, L.G. Presta, and J.V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.* 6:443–446. http://dx.doi.org/10.1038/74704

Cohen, A.D., D.A. Schaer, C. Liu, Y. Li, D. Hirschhorn-Cymmerman, S.C. Kim, A. Diab, G. Rizzuto, F. Duan, M.A. Perales, et al. 2010. Agonist anti-GITR monoclonal antibody induces melanoma tumor immunity in mice by altering regulatory T cell stability and intra-tumor accumulation. *PLoS ONE.* 5:e10436. http://dx.doi.org/10.1371/journal.pone.0010436

Curran, M.A., W. Montalvo, H. Yagita, and J.P. Allison. 2010. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc. Natl. Acad. Sci. USA.* 107:4275–4280. http://dx.doi.org/10.1073/pnas.0915174107

Hodi, F.S., S.J. O'Day, D.F. McDermott, R.W. Weber, J.A. Sosman, J.B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J.C. Hassel, et al. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* 363:711–723. http://dx.doi.org/10.1056/NEJMoa1003466

Hogarth, P.M., and G.A. Pietersz. 2012. Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. *Nat. Rev. Drug Discov.* 11:311–331. http://dx.doi.org/10.1038/nrd2909

Junttila, T.T., K. Parsons, C. Olsson, Y. Lu, Y. Xin, J. Theriault, L. Crocker, O. Pabonan, T. Baginski, G. Meng, et al. 2010. Superior in vivo efficacy of afucosylated trastuzumab in the treatment of HER2-amplified breast cancer. *Cancer Res.* 70:4481–4489. http://dx.doi.org/10.1158/0008-5472.CAN-09-3704

Kanamaru, F., P. Youngnak, M. Hashiguchi, T. Nishioka, T. Takahashi, S. Sakaguchi, I. Ishikawa, and M. Azuma. 2004. Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells. *J. Immunol.* 172:7306–7314.

Ko, K., S. Yamazaki, K. Nakamura, T. Nishioka, K. Hirota, T. Yamaguchi, J. Shimizu, T. Nomura, T. Chiba, and S. Sakaguchi. 2005. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating $Foxp3^+CD25^+CD4^+$ regulatory T cells. *J. Exp. Med.* 202:885–891. http://dx.doi.org/10.1084/jem.20050940

Leach, D.R., M.F. Krummel, and J.P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* 271:1734–1736. http://dx.doi.org/10.1126/science.271.5256.1734

Lewis, G.D., I. Figari, B. Fendly, W.L. Wong, P. Carter, C. Gorman, and H.M. Shepard. 1993. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol. Immunother.* 37:255–263. http://dx.doi.org/10.1007/BF01518520

Li, F., and J.V. Ravetch. 2012. A general requirement for FcγRIIB co-engagement of agonistic anti-TNFR antibodies. *Cell Cycle*. 11:3343–3344. http://dx.doi.org/10.4161/cc.21842

Mellman, I., G. Coukos, and G. Dranoff. 2011. Cancer immunotherapy comes of age. *Nature*. 480:480–489. http://dx.doi.org/10.1038/nature10673

Nimmerjahn, F., and J.V. Ravetch. 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*. 310:1510–1512. http://dx.doi.org/10.1126/science.1118948

Nimmerjahn, F., and J.V. Ravetch. 2008. Fcgamma receptors as regulators of immune responses. *Nat. Rev. Immunol.* 8:34–47. http://dx.doi.org/10.1038/nri2206

Nimmerjahn, F., and J.V. Ravetch. 2012. Translating basic mechanisms of IgG effector activity into next generation cancer therapies. *Cancer Immun.* 12:13.

Niwa, R., M. Sakurada, Y. Kobayashi, A. Uehara, K. Matsushima, R. Ueda, K. Nakamura, and K. Shitara. 2005. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. *Clin. Cancer Res.* 11:2327–2336. http://dx.doi.org/10.1158/1078-0432.CCR-04-2263

Nordstrom, J.L., S. Gorlatov, W. Zhang, Y. Yang, L. Huang, S. Burke, H. Li, V. Ciccarone, T. Zhang, J. Stavenhagen, et al. 2011. Anti-tumor activity and toxicokinetics analysis of MGAH22, an anti-HER2 monoclonal antibody with enhanced Fcγ receptor binding properties. *Breast Cancer Res.* 13:R123. http://dx.doi.org/10.1186/bcr3069

Robert, C., L. Thomas, I. Bondarenko, S. O'Day, J.W. M D, C. Garbe, C. Lebbe, J.F. Baurain, A. Testori, J.J. Grob, et al. 2011. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N. Engl. J. Med.* 364:2517–2526. http://dx.doi.org/10.1056/NEJMoa1104621

Ronchetti, S., G. Nocentini, R. Bianchini, L.T. Krausz, G. Migliorati, and C. Riccardi. 2007. Glucocorticoid-induced TNFR-related protein lowers the threshold of CD28 costimulation in CD8+ T cells. *J. Immunol.* 179:5916–5926.

Ronchetti, S., G. Nocentini, M.G. Petrillo, and C. Riccardi. 2012. CD8+ T cells: GITR matters. *ScientificWorldJournal*. 2012:308265. http://dx.doi.org/10.1100/2012/308265

Schaer, D.A., J.T. Murphy, and J.D. Wolchok. 2012. Modulation of GITR for cancer immunotherapy. *Curr. Opin. Immunol.* 24:217–224. http://dx.doi.org/10.1016/j.coi.2011.12.011

Sharma, S., A.L. Dominguez, S.Z. Manrique, F. Cavallo, S. Sakaguchi, and J. Lustgarten. 2008. Systemic targeting of CpG-ODN to the tumor microenvironment with anti-neu-CpG hybrid molecule and T regulatory cell depletion induces memory responses in BALB-neuT tolerant mice. *Cancer Res.* 68:7530–7540. http://dx.doi.org/10.1158/0008-5472.CAN-08-1635

Shields, R.L., A.K. Namenuk, K. Hong, Y.G. Meng, J. Rae, J. Briggs, D. Xie, J. Lai, A. Stadlen, B. Li, et al. 2001. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J. Biol. Chem.* 276:6591–6604. http://dx.doi.org/10.1074/jbc.M009483200

Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3:135–142. http://dx.doi.org/10.1038/ni759

Smith, P., D.J. DiLillo, S. Bournazos, F. Li, and J.V. Ravetch. 2012. Mouse model recapitulating human Fcγ receptor structural and functional diversity. *Proc. Natl. Acad. Sci. USA*. 109:6181–6186. http://dx.doi.org/10.1073/pnas.1203954109

Tai, Y.T., H.M. Horton, S.Y. Kong, E. Pong, H. Chen, S. Cemerski, M.J. Bernett, D.H. Nguyen, S. Karki, S.Y. Chu, et al. 2012. Potent in vitro and in vivo activity of an Fc-engineered humanized anti-HM1.24 antibody against multiple myeloma via augmented effector function. *Blood*. 119:2074–2082. http://dx.doi.org/10.1182/blood-2011-06-364521

Turk, M.J., J.A. Guevara-Patiño, G.A. Rizzuto, M.E. Engelhorn, S. Sakaguchi, and A.N. Houghton. 2004. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J. Exp. Med.* 200:771–782. http://dx.doi.org/10.1084/jem.20041130

Weng, W.K., and R. Levy. 2003. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J. Clin. Oncol.* 21:3940–3947. http://dx.doi.org/10.1200/JCO.2003.05.013

Wilson, N.S., B. Yang, A. Yang, S. Loeser, S. Marsters, D. Lawrence, Y. Li, R. Pitti, K. Totpal, S. Yee, et al. 2011. An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell*. 19:101–113. http://dx.doi.org/10.1016/j.ccr.2010.11.012

Zhou, P., L. L'italien, D. Hodges, and X.M. Schebye. 2007. Pivotal roles of CD4+ effector T cells in mediating agonistic anti-GITR mAb-induced-immune activation and tumor immunity in CT26 tumors. *J. Immunol.* 179:7365–7375.