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## Pivotal Roles of CD4<sup>+</sup> Effector T cells in Mediating Agonistic Anti-GITR mAb-Induced-Immune Activation and Tumor Immunity in CT26 Tumors

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Pengfei Zhou, Lawrence L'italien, Douglas Hodges and Xiao  
Min Schebye

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# Pivotal Roles of CD4<sup>+</sup> Effector T cells in Mediating Agonistic Anti-GITR mAb-Induced-Immune Activation and Tumor Immunity in CT26 Tumors

Pengfei Zhou, Lawrence L'italien, Douglas Hodges, and Xiao Min Schebye<sup>1</sup>

Glucocorticoid-induced TNF receptor family related protein (GITR) is a member of the TNFR superfamily. Previous studies have shown that in vivo administration of a GITR agonistic Ab (DTA-1) is able to overcome tolerance and induce tumor rejection in several murine syngeneic tumor models. However, little is known about the in vivo targets and the mechanisms of how this tolerance is overcome in a tumor-bearing host, nor is much known about how the immune network is regulated to achieve this antitumor response. In this study, we demonstrate that the in vivo ligation of GITR on CD4<sup>+</sup> effector T cells renders them refractory to suppression by regulatory T (T<sub>reg</sub>) cells in the CT26 tumor-bearing mouse. GITR engagement on T<sub>reg</sub> cells does not appear to directly abrogate their suppressive function; rather, it increases the expansion of T<sub>reg</sub> cells and promotes IL-10 production, a cytokine important for their suppressive function. Moreover, CD4<sup>+</sup> effector T cells play a crucial role in mediating DTA-1-induced immune activation and expansion of CD8<sup>+</sup>, NK, and B cells in the tumor-draining lymph nodes. This includes increased CD69 expression on all of these subsets. In addition, NK and tumor-specific CD8<sup>+</sup> T cells are generated that are cytolytic, which show increased intracellular IFN- $\gamma$  production and CD107a mobilization, the latter a hallmark of cytolytic activities that lead to tumor killing. *The Journal of Immunology*, 2007, 179: 7365–7375.

Emerging evidence supports the existence of elevated numbers of regulatory T (T<sub>reg</sub>)<sup>2</sup> cells in human tumors, as well as in tumor-bearing mice (1–5). T<sub>reg</sub> cells not only engage in the maintenance of immunologic self-tolerance in the periphery but also play a predominant role in impeding immunosurveillance against autologous tumor cells (6, 7). Therefore, overcoming the tolerance to tumor Ags maintained by T<sub>reg</sub> cells in vivo, either by depletion, reducing their differentiation and suppressive mechanism, or increasing the resistance of effector cells to suppression by T<sub>reg</sub> cells, represents new strategies for tumor immunotherapy.

Glucocorticoid-induced TNF receptor family related protein (GITR) is a member of the TNF receptor superfamily that is constitutively expressed at high levels on CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells and presented ubiquitously at lower levels on various immune subsets including effector T cells, as well as NK and B cells (8, 9). Upon activation, the expression is up-regulated on CD4<sup>+</sup>, CD8<sup>+</sup>, and NK cells (8, 10). Administration of an anti-mGITR agonistic Ab (clone DTA-1) induces rejection of several murine syngeneic tumors with no obvious autoimmune manifestations (11–13). The results illustrate that administration of DTA-1 promotes activation of effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and alters the intratumor balance of T<sub>reg</sub>/T effectors. It has also been shown that IFN- $\gamma$  is required for the antitumor response induced by DTA-1 (12, 13).

Nevertheless, the exact in vivo targets of DTA-1 and how the immune network is regulated to achieve the antitumor response still remain to be studied.

Tumor rejection upon DTA-1 treatment points to the fact that GITR-GITR-L ligation in vivo can break immune tolerance to tumor Ags. However, investigation into how suppression of T<sub>reg</sub> cells in vitro is abrogated has yielded contradicting results as to whether GITR stimulation on T<sub>reg</sub> cells directly abrogates their suppressive function (14–16). One study of coculture of T<sub>reg</sub> cells and responder CD4<sup>+</sup> T cells from GITR<sup>-/-</sup> and GITR<sup>+/+</sup> mice shows that ligation of GITR on the responder T cells, but not on the T<sub>reg</sub> cells, was required to abrogate suppression (16). This conclusion was supported by experiments using rat CD4<sup>+</sup> responder T cells and mouse T<sub>reg</sub> cells in coculture (16). However, a separate study using GITR<sup>-/-</sup> mice shows that GITR ligation acted on T<sub>reg</sub> cells, as well as on the CD4<sup>+</sup> T responders, to abrogate the suppression (15).

In a tumor-bearing mouse, it has been shown that T<sub>reg</sub> depletion has no influence on DTA-1-induced B16 melanoma rejection (11, 13), indicating that the effect of DTA-1 on effector T cells is sufficient to induce tumor immunity when T<sub>reg</sub> cells are absent. Because the T<sub>reg</sub> cell is a negative regulator, one cannot rule out that DTA-1 has an effect on T<sub>reg</sub> cells when they are naturally present in vivo or that GITR engagement on T<sub>reg</sub> cells can have a functional role in GITR-induced tumor immunity.

Fundamental to understanding how GITR ligation overcomes immune tolerance to tumor Ags is knowing whether the abrogation of suppression actually exists in a tumor-bearing host and which cell subset, T<sub>reg</sub> cells or T effectors, is functionally responsible for this break in immune tolerance. Equally important to developing an effective immunotherapy for cancer is to identify in vivo targets of GITR ligation and to elucidate how the immune regulation is achieved. To this end, we studied the immune activation of T, B, NK, and T<sub>reg</sub> cells upon administration of DTA-1 into a CT26 tumor-bearing mouse, and mechanistically dissected the functional relationship between CD4<sup>+</sup> effector T cells and T<sub>reg</sub> cells. We

Discovery Research, Schering-Plough Biopharma (Formerly DNAX Research Institute), Palo Alto, CA 94304-1104

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<sup>1</sup> Address correspondence and reprint requests to Dr. Xiao Min Schebye, Schering-Plough Biopharma, 901 California Avenue, Palo Alto, CA 94304. E-mail address: xiaomin.schebye@spcorp.com

<sup>2</sup> Abbreviations used in this paper: T<sub>reg</sub>, regulatory T cell; GITR, glucocorticoid-induced TNF receptor family related gene; TDLN, tumor draining lymph node.

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further identified the essential target of DTA-1 *in vivo* and its contribution to immune activation and tumor immunity.

## Materials and Methods

### *Mice and tumor models*

Female BALB/c mice 6–8 wk of age were purchased from Taconic Farms. All animal procedures were approved by the DNAX Institutional Animal Care and Use Committee. The murine tumor cell lines, CT26 (BALB/c derived colorectal carcinoma) and 4T1 (murine mammary tumor line) were purchased from American Type Culture Collection. BALB/c mice were inoculated by intradermal injection of CT26 cells ( $3 \times 10^5$  per mouse) on the right flank. Tumor diameter was measured by electronic caliper every 2–3 days, and tumor volume was determined by length  $\times$  width<sup>2</sup>/2.

### *Anti-GITR mAb treatment and Ab depletions*

Mice received 500  $\mu$ g/mouse of affinity-purified anti-GITR mAb (DTA-1; BioExpress) or control Ab rat-anti-mouse IgG2b subcutaneously in the neck region, at the specified time points, as indicated in the text. For T cell depletion, mice were depleted of CD4<sup>+</sup> (GK1.5; BioExpress) and CD8<sup>+</sup> (53.6.72, BioExpress) by i.p. injection of 500  $\mu$ g of the mAbs at day 5, 6, 7, 12, and 17 following tumor inoculation. For depletion of NK cells, 50  $\mu$ l/mouse of anti-asialo GM1 Ab (Cedarlane) was injected i.p. at day 5, 7, 12, and 17 after tumor challenge. Rabbit IgG (Sigma-Aldrich) was used as control Ab. Depletion efficacy was confirmed in spleen; >95% depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and >80% depletion of NK cells were achieved, respectively.

### *FACS analysis*

Anti-mouse CD3, CD4, CD8, CD25, CD19, DX5, CD69, Ly6A/E, CD16/CD32 FcR, CD122, IFN- $\gamma$ , and isotype controls rat IgG2a and rat IgG2b were all purchased from BD Pharmingen. allophycocyanin-conjugated anti-GITR (DTA-1) was purchased from eBiosciences. Biotin-conjugated rat-anti-mouse GITR mAb (clone GITR-MAb1), a staining Ab for GITR raised by Schering-Plough Biopharma (formerly DNAX Research Institute), does not interfere with the surface staining of DTA-1 (data not shown). Lymphocytes were washed in PBS with 2% FBS and incubated for 15 min at 4°C with anti-CD16/CD32 FcR block. Subsequently, cells were incubated with staining Abs for 30 min at 4°C and washed twice. Stained cells were analyzed on a LSR-II flow cytometer with BD FACSDiva software (BD Bioscience). For detection of Foxp3<sup>+</sup> T cells, the APC-anti-mouse FoxP3 staining set (eBioscience) was used. Cells were incubated with surface molecules, such as CD4, CD3, and CD25 first, and then fixed and permeabilized in the fixation/permeabilization working solution (according to the manufacture manual) for 30 min, washed two times with permeabilization buffer and then incubated with anti-mouse Foxp3 (FJK-16s) at 4°C for 30 min in the dark. Cells were then washed and resuspended in PBS with 2% FBS and analyzed by flow cytometry.

### *CD107a mobilization assay and intracellular cytokine staining*

Degranulation of intracellular vesicles by lymphocytes can be measured using CD107a, as described recently for CD8<sup>+</sup> T cells (17). A similar procedure can also be used to determine the frequency of NK degranulation following stimulation (18). Mononuclear cells ( $1 \times 10^6$ /well) were suspended in RPMI 1640 (Sigma-Aldrich) containing 10% FBS (Atlanta Biologicals), 2 mM L-glutamine (Mediatech), and 50 IU/ml penicillin (Mediatech), and incubated with PMA (5 ng/ml) and ionomycin (500 ng/ml), or CT26 tumor cells ( $0.2 \times 10^6$ /well), and 1.25  $\mu$ g/ml FITC-conjugated anti-mouse CD107a Ab. Following 1 h of incubation at 37°C in 5% CO<sub>2</sub>, GolgiPlug (BD Biosciences) was added for a final concentration of 5  $\mu$ g/ml and incubated for an additional 4 h at 37°C in 5% CO<sub>2</sub>. Samples were then surface-stained using NK markers and T cell markers, and then fixed, permeabilized, and stained for intracellular cytokines such as IL-10, IL-2, and IFN- $\gamma$ . To identify CD107a expression and IFN- $\gamma$  on CD8<sup>+</sup> T cells, total lymphocytes were incubated with CT26 cells at the ratio of 25:1 for 4 days. Then the cells in suspension were collected and restimulated with CT26 cells or 4T1 cells at the indicated ratio for additional 4–6 h in the presence of anti-CD107a Ab. GolgiPlug was added after 1 h of incubation. Cells were then surface-stained with CD3, CD8, and CD107a markers, and then fixed, permeabilized, and stained for intracellular cytokine IFN- $\gamma$  using a Cytofix/Cytoperm kit.

### *In vitro proliferation assay*

T cell subsets were purified from spleen or inguinal lymph nodes of mice. Mononuclear cells were stained with a mixture of depleting mAbs including B220, CD8, DX5, and MAC-1 to enrich CD4<sup>+</sup> T cells. The enriched

cells were then incubated with anti-CD25 PE and subsequently labeled with anti-PE magnetic microbeads and purified on an autoMACS (Miltenyi Biotec), according to the manufacturer's protocol. Purity of the CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells was typically above 95%. CD4<sup>+</sup>CD25<sup>+</sup> T cell suppressive assay was performed as described (19). In brief, CD4<sup>+</sup>CD25<sup>-</sup> T cells ( $4 \times 10^4$ /well) were cocultured with CD4<sup>+</sup>CD25<sup>+</sup> T cells in the presence of  $\gamma$ -irradiated (2000R)-CD4<sup>+</sup>-depleted splenocytes ( $4 \times 10^4$ /well) and anti-CD3 mAb (2C11) at 65 ng/ml in 96-well round-bottom plates. Titrated numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells were added to the culture as indicated. The culture was pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine for the final 8 h of a 72-h culture, and the readouts were performed in triplicate.

### *Enrichment of tumor-specific T cells and flow cytometry-based cytotoxic T-lymphocyte assay*

To prepare effector CD4 and CD8<sup>+</sup> T cells for CTL assay, splenocytes and lymph node cells from CT26-bearing mice (DTA-1 or IgG2b-treated) were harvested and incubated with  $\gamma$ -irradiated (2000 rad) CT26 cells at a ratio of 20:1 (lymphocytes:CT26 cells). The cultures were incubated for 4 days, and viable lymphocytes were collected using centrifugation over Lympholyte-M (Cedarlane Laboratories) at  $100 \times g$  for 20 min. To purify CD8 and CD4<sup>+</sup> T cells, the live lymphocytes were washed and resuspended in PBS/BSA staining buffer at  $100 \times 10^6$ /ml. Subsequently the anti-CD8 microbeads (Miltenyi Biotec) were added to the cell suspension at a final concentration according to manufacturer's instruction, and incubated for 15 min at 4°C. CD8<sup>+</sup> cells were positive selected using MACS MiDi separator with LS columns. The cells in the flow-through fraction were then incubated with anti-CD4 microbeads to positive select CD4<sup>+</sup> T cells. The purity of both CD8 and CD4<sup>+</sup> T cells after positive selection was confirmed to be >97%.

To quantify the target-cell killing activities mediated by tumor-specific T lymphocytes, we used the flow cytometry-based CTL assay to detect the specific cleaved caspase-3 in the target cells following the published method (20, 21). In brief, target cells (CT26 or 4T1) were harvested and labeled with DDAO-SE (Molecular Probes) at 0.6  $\mu$ M for 15 min at room temperature. The cells were then washed and resuspended in culture medium at  $2 \times 10^6$ /ml. Target cells ( $2 \times 10^5$ /well) were mixed with titrated effector CD4 or CD8<sup>+</sup> T cells at different E:T ratios in 96-well plates. The cell mixture was centrifuged at  $50 \times g$  for 1 min, and then incubated for 4 h. The cells were fixed and permeabilized with Fix/Perm solution and then stained with PE-anti-activated caspase-3 mAb (BD Pharmingen). The cells were washed twice in staining buffer and analyzed on LSR II flow cytometer.

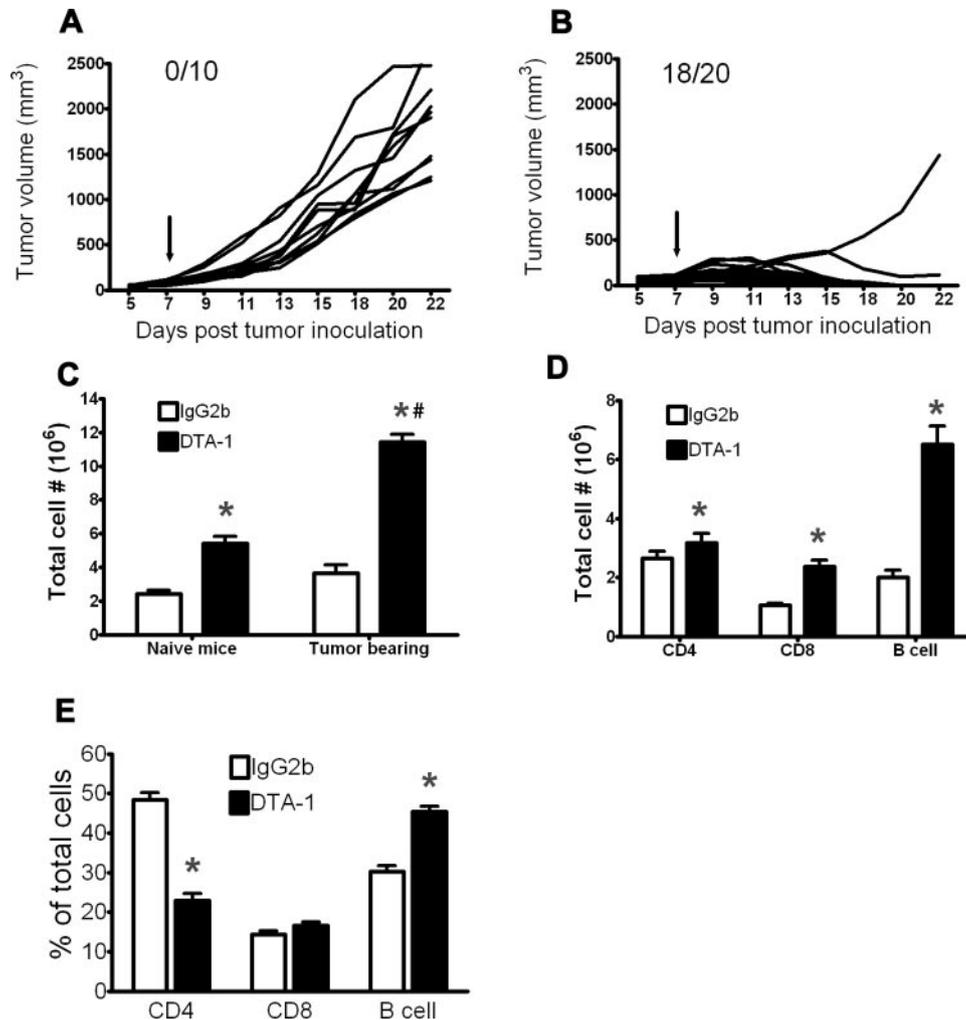
### *Statistical analysis*

Data were expressed as mean  $\pm$  SEM. Statistical analysis was performed using the two-tailed Student's *t* test for independent samples. One-way ANOVA was used for time course data. The differences between the mean of the two groups were considered significant when  $p < 0.05$ .

## Results

### *GITR stimulation induced expansion and activation of T and B cells in the tumor draining lymph nodes*

We confirmed the previous finding (12) that injection of the GITR agonistic Ab, DTA-1, was able to eliminate an established CT26 tumor, a GITR-nonexpressing BALB/c-derived colorectal carcinoma cell line (Fig. 1, A and B). Previous studies have shown that treating tumor-bearing mice with DTA-1 induces activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen and significantly alters the intratumor balance of effector and regulatory T cells (12, 13). The activation status of GITR expressing immune subsets in the tumor-draining lymph nodes (TDLN), which are the anatomic sites of initial tumor Ag presentation and naive T cell priming, has not been examined in detail. To study this, CT26 cells were intradermally inoculated into the right flank of a BALB/c mouse. Seven days after tumor inoculation, 500  $\mu$ g of either DTA-1 or control Ab, rat anti-mouse IgG2b, was injected subcutaneously in the neck region. On various days after Ab injection, the inguinal and axillary TDLNs were collected for analysis. In some cases, we injected DTA-1 to naive mice and collected the inguinal and axillary lymph nodes for comparison. We observed that starting 3 days after DTA-1 treatment, the TDLNs were enlarged, and reaching peak



**FIGURE 1.** Anti-GITR mAb induces tumor rejection and lymphocyte expansion. The right flanks of 6–8 wk BALB/c mice were inoculated with CT26 tumor cells ( $3 \times 10^5$  per mouse) on day 0; rat IgG2b control Ab (A) or DTA-1 (500  $\mu\text{g}/\text{mouse}$ ) (B) was injected subcutaneously on day 7. Tumor size was measured every 2–3 days. C, TDLNs were dissected 4 days after mAb injections and average cell counts from each mouse were determined. For naive mice, the right inguinal LNs were used. D, The absolute cell number of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells obtained from TDLNs of each mouse. E, The percentage of individual lymphocyte subsets observed in the total lymphocyte gate. \*,  $p < 0.05$ , DTA-1 compared with IgG2b control treatment; #,  $p < 0.05$ ; DTA-1 treated tumor-bearing mice compared with naive mice. Each bar represents the average of three independent experiments ( $n = 12\text{--}15$  mice per group).

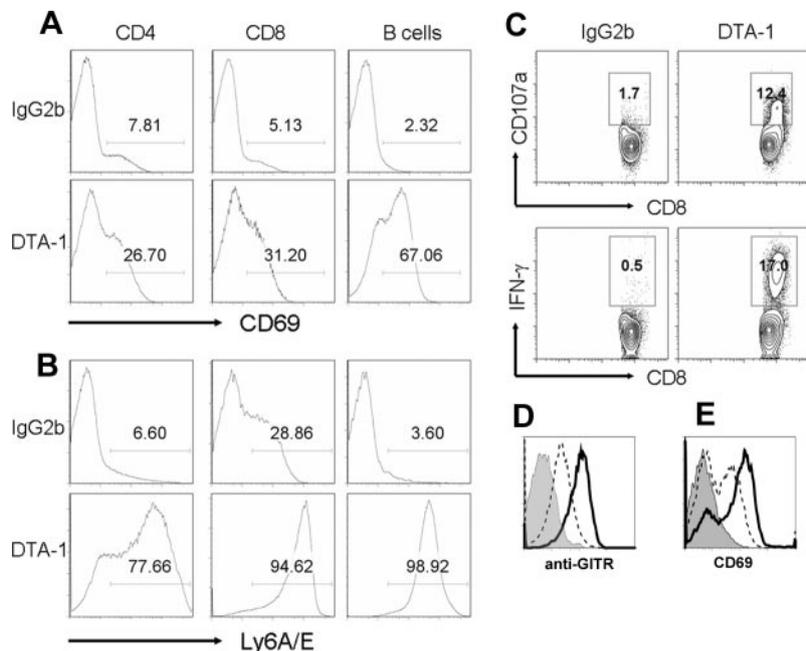
size in 4 to 6 days (data not shown). If no additional DTA-1 was given, then the DLN sizes gradually returned to normal (data not shown). The transient enlargement of TDLN after a single injection of DTA-1 suggests that the immune activation induced through GITR ligation was reversible.

Four days after treatment, DTA-1 induced an increase in the total cell number of TDLNs, compared with IgG2b, in both naive and tumor-bearing mice, with a much larger population increase observed in the CT26 tumor-bearing mice (Fig. 1C). By flow cytometry analysis, we found that DTA-1 treatment induced a 3-fold increase of CD8<sup>+</sup> T cells, a 4.5-fold increase of B cells, and a 1.5-fold increase of CD4<sup>+</sup> T cells in TDLNs compared with that of the control treated mice (Fig. 1D). CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively, remained the same or significantly decreased as a percentage of TDLN cells, which reflects the expansion of other immune cell subsets, especially B cells in the TDLNs (Fig. 1E). DTA-1 treatment clearly induces the expansion of CD4<sup>+</sup>, CD8<sup>+</sup>, and B cells.

CD69 and Ly6A/E, the former an early activation marker and the latter a marker for IFN- $\gamma$ -induced cell activation (22, 23), were used to assess cell activation status. As shown in Fig. 2, A and B,

the expression of both markers showed significant increases on CD4<sup>+</sup>, CD8<sup>+</sup>, and B cells after DTA-1 administration. To examine whether CD8<sup>+</sup> T cells were actually expressing IFN- $\gamma$  and becoming cytolytic, IFN- $\gamma$  intracellular staining and a CD107a mobilization assay (a surrogate for lytic degranulation) were conducted by restimulating lymphocytes with CT26 tumor cells *ex vivo*. CD107a mobilization and IFN- $\gamma$  production increased in DTA-1 treated mice compared with that of IgG2b-treated mice (Fig. 2C), whereas CD8<sup>+</sup> T cells incubated with 4T1 remained at the basal levels for IFN- $\gamma$  production or CD107a mobilization (data not shown). Taken together, these findings indicate that both T cells and B cells were expanded and activated in TDLNs after DTA-1 treatment, where tumor-specific CD8<sup>+</sup> T cells were generated *in vivo* and were capable of producing high levels of IFN- $\gamma$  and releasing significant amounts of cytolytic granules.

Previous studies have shown that GITR is expressed on murine resting B cells, and that the expression level is slightly enhanced by *in vitro* LPS treatment (9). We further examined GITR expression on B cells upon anti-CD40 mAb stimulation and then studied whether DTA-1 could enhance the effect of anti-CD40 to stimulate B cells *in vitro*. Purified B cells from spleens of naive BALB/c



**FIGURE 2.** GITR stimulation increases CD4<sup>+</sup> T cell, CD8<sup>+</sup> T cell, and B cell activation. Lymphocytes from TDLNs collected 4 days after DTA-1 or IgG2b treatment were analyzed by flow cytometry. Percentage of CD69 expression (A) or Ly6A/E (B) was shown on gated CD3<sup>+</sup>CD4<sup>+</sup> (CD4), CD3<sup>+</sup>CD8<sup>+</sup> (CD8) T cells and CD19<sup>+</sup> (B) cells. Data are representative of three independent experiments ( $n = 10$  mice per group). C, CD107a mobilization and intracellular IFN- $\gamma$  expression by CD8<sup>+</sup> T cells recovered from TDLNs of control IgG2b or DTA-1-treated tumor-bearing mice. The mononuclear cells from TDLNs were restimulated in vitro for 72 h with CT26 cells. The restimulated leukocytes were then cultured for an additional 4–6 h with CT26 (shown) or 4T1 (not shown) in a CD107a mobilization assay or stained afterward for intracellular IFN- $\gamma$ . The percentages of CD107a expressing and IFN- $\gamma$  producing CD8<sup>+</sup> T cells are indicated. One representative experiment of three is shown. D, GITR expression by B cells following anti-CD40 treatment. Purified B cells by magnetic beads from naive BALB/c spleen were treated with anti-CD40 (1  $\mu$ g/ml, solid line) or isotype control (dotted line) for 24 h, GITR expression on B cells was identified by staining the cells with anti-B220 and anti-GITR. Shaded area indicates isotype control for anti-GITR. E, CD69 expression on purified B cells treated with anti-CD40 and/or DTA-1 in vitro for 24 h. DTA-1 (1  $\mu$ g/ml, shaded) treatment alone does not induce CD69 expression. Combined treatment of anti-CD40 (1  $\mu$ g/ml) and DTA-1 (solid line) significantly enhances CD69 expression compared with anti-CD40 (1  $\mu$ g/ml) alone (dotted).

mice were treated with anti-CD40 mAb (FGK4.5, BioExpress), or isotype control for 24 h and then GITR expression was measured by flow cytometry analysis. To examine the effects of DTA-1 on anti-CD40-induced B cell activation, purified resting B cells were treated with 1  $\mu$ g/ml anti-CD40, with or without adding DTA-1 for 24 h. As shown in Fig. 2D, anti-CD40 significantly enhanced GITR expression on B cells. Consistent with the observation that DTA-1 is a costimulator for T-effectors, treatment of resting B cells by DTA-1 alone in vitro did not induce B cell activation. Addition of DTA-1 significantly enhanced anti-CD40 mAb-induced CD69 expression on B cells (Fig. 2E). Thus, we speculate that in vivo, B cell activation is not attributed to the direct effect of GITR ligation on B cells, but to the synergistic effect between anti-GITR and other stimuli, such as CD40-CD40L interaction.

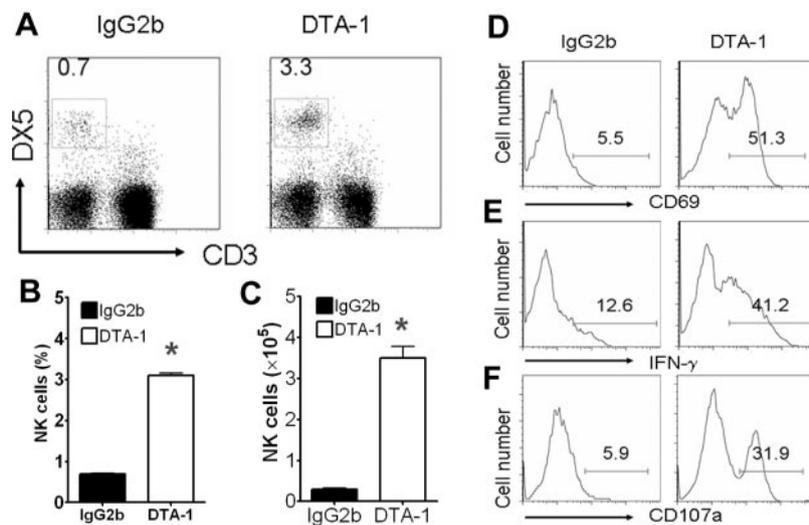
#### NK cells were recruited and activated in tumor-draining lymph nodes

It has been reported that GITR is expressed on murine resting NK cells at the intermediate level (13). A study of human NK cells conducted in vitro has indicated that recombinant GITR ligand costimulated NK cytotoxicity and IFN- $\gamma$  production (10). Exactly how GITR ligation impacts NK function in vivo has not been determined. Under physiological conditions, NK cells circulate in the blood and spleen but are rarely found in the lymph nodes. A single injection of DTA-1 into tumor-bearing mice significantly increased NK cells observed in the TDLNs, percentage-wise and in absolute numbers, compared with IgG2b-injected mice ( $3.1\% \pm 0.30\%$  vs  $0.69\% \pm 0.16\%$ ;  $3.5 \pm 0.32 \times 10^5$  vs  $0.30 \pm 0.04 \times 10^5$ ), as illustrated in Fig. 3, A–C.

Although only a minor population of NK cells from TDLNs in IgG2b-treated mice expressed CD69 and produced IFN- $\gamma$ , >50% of the NK cells expressed CD69 in DTA-1-treated mice (Fig. 3D), and around 40% of NK cells produced IFN- $\gamma$ , as demonstrated by intracellular cytokine staining (Fig. 3E). To examine whether the NK activation induced by DTA-1 treatment was associated with cytolytic activity, we used CD107a as a marker to identify NK cell degranulation following stimulation. Lymphocytes were incubated with PMA/ionomycin and CD107a-specific Abs for 5 h and then stained with DX5 and CD3 mAb to identify NK cells. Surface expression of CD107a was low in unstimulated NK cells (2.5%). Following stimulation with PMA/ionomycin, we observed >30% of NK cells mobilized CD107a to the cell surface in DTA-1 treated mice compared with ~6% in IgG2b treatment (Fig. 3F). Taken together, these data demonstrate that NK cells were recruited to TDLNs after DTA-1 treatment, where they became activated and were capable of producing IFN- $\gamma$  and of releasing cytolytic molecules.

#### DTA-1 treatment in tumor-bearing mice promoted expansion of Treg cells and their IL-10 production

As described earlier, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells express a high level of GITR; however, the impacts of GITR ligation on T<sub>reg</sub> cells in vivo have not been fully examined. We found that a single injection of DTA-1 to tumor-bearing mice induced a significant increase of the absolute number of T<sub>reg</sub> cells in the TDLNs at day 4 after the treatment (Fig. 4B, right), which is in agreement with previous findings in the B16 melanoma model (13). As shown in Fig. 4, A and B (left), the reduction of the percentage of



**FIGURE 3.** NK cells are recruited and activated in TDLNs. BALB/c mice were given a single treatment of 500  $\mu\text{g}/\text{mouse}$  of either DTA-1 or IgG2b on day 7 post CT26 tumor inoculation; at day 11, TDLNs were dissected. The percentage of NK cell and their activation status were examined. *A*, Representative FACS plots indicating the percentages of  $\text{DX5}^+\text{CD3}^-$  NK cells in TDLNs after IgG2b or DTA-1 treatment. *B*, The average percentage of NK cells in TDLNs after IgG2b or DTA-1 treatment. *C*, The total NK cell numbers in TDLNs calculated by multiplying the percentage of NK cells and the total cell count in the TDLNs. Data are the mean  $\pm$  SEM of three separate experiments with  $n = 8-10$  mice in each group for both *B* and *C*. *D*, Expression of CD69 on NK cells from control or DTA-1 treated TDLNs. *E*, Intracellular IFN- $\gamma$  expression by  $\text{DX5}^+\text{CD3}^-$  NK cells recovered from TDLNs of control or DTA-1-treated tumor-bearing mice, after in vitro stimulation with PMA/ionomycin for 5 h. The percentages of IFN- $\gamma$ -producing NK cells are indicated. *F*, Mobilization of CD107a captured on NK cells after in vitro stimulation with PMA/ionomycin for 5 h. The percentages of CD107a positive cells in total NK cells are indicated (gated on  $\text{DX5}^+\text{CD3}^-$  NK cells). Data are representative of three independent experiments.

$\text{Foxp3}^+\text{CD4}^+\text{CD25}^+$  likely reflects the relative expansion of other subsets.

Staining of the cells with rabbit-anti-rat IgG2b Abs to track injected DTA-1 indicated that DTA-1 bound with  $\text{T}_{\text{reg}}$  cells in vivo (data not shown). Moreover, there appeared to be GITR receptor reduction on  $\text{T}_{\text{reg}}$  cells as measured with GITR-MAB1, an in-house raised anti-GITR mAb that is not blocked by DTA-1 binding, between 24 and 120 h after DTA-1 in vivo treatment (Fig. 4C). FACS analysis indicated that the  $\text{Foxp3}^+\text{CD4}^+\text{CD25}^+$   $\text{T}_{\text{reg}}$  cells from DTA-1 treated mice expressed higher levels of CD69 and CD44, and a lower level of CD62L, compared with IgG2b treated mice (Fig. 4D), all of which are associated with activated  $\text{T}_{\text{reg}}$  cells (24). Intracellular cytokine staining of  $\text{Foxp3}^+\text{CD4}^+\text{CD25}^+$   $\text{T}_{\text{reg}}$  cells indicated that production of both IL-2 and IL-10 was increased (Fig. 4E). The increased IL-10 expression suggests that these  $\text{T}_{\text{reg}}$  cells were likely more suppressive (25, 26).

#### *DTA-1 treatment in vivo rendered CD4<sup>+</sup> effectors from CT26-bearing mice resistant to the suppression of T<sub>reg</sub> cells*

To investigate whether DTA-1 treatment functionally altered the suppressive activities of  $\text{T}_{\text{reg}}$  cells and/or resistance of T effectors to suppression, we conducted ex vivo coculture experiments of  $\text{CD4}^+\text{CD25}^+$  T cells and  $\text{CD4}^+\text{CD25}^-$  T responders from the TDLNs of DTA-1 or IgG2b-treated tumor-bearing mice. The coculture assay was divided into two parts: first,  $\text{CD4}^+\text{CD25}^+$  T cells from DTA-1 treated mice were mixed with control, IgG2b-treated  $\text{CD4}^+\text{CD25}^-$  T responders in the presence of anti-CD3 and  $\gamma$ -irradiated-CD4-depleted mononuclear cells. The second part was the reciprocal coculture between DTA-1 treated  $\text{CD4}^+\text{CD25}^-$  T responders and control  $\text{CD4}^+\text{CD25}^+$   $\text{T}_{\text{reg}}$  cells. As shown in Fig. 5A,  $\text{CD4}^+\text{CD25}^+$  T cells from both DTA-1 and IgG2b-treated mice were equally suppressive of control IgG2b-treated  $\text{CD4}^+\text{CD25}^-$  T responders. Therefore, in vivo GITR ligation on the  $\text{T}_{\text{reg}}$  cells did not result in a loss of suppressive capability. When the limitation of this assay is taken into account, there are

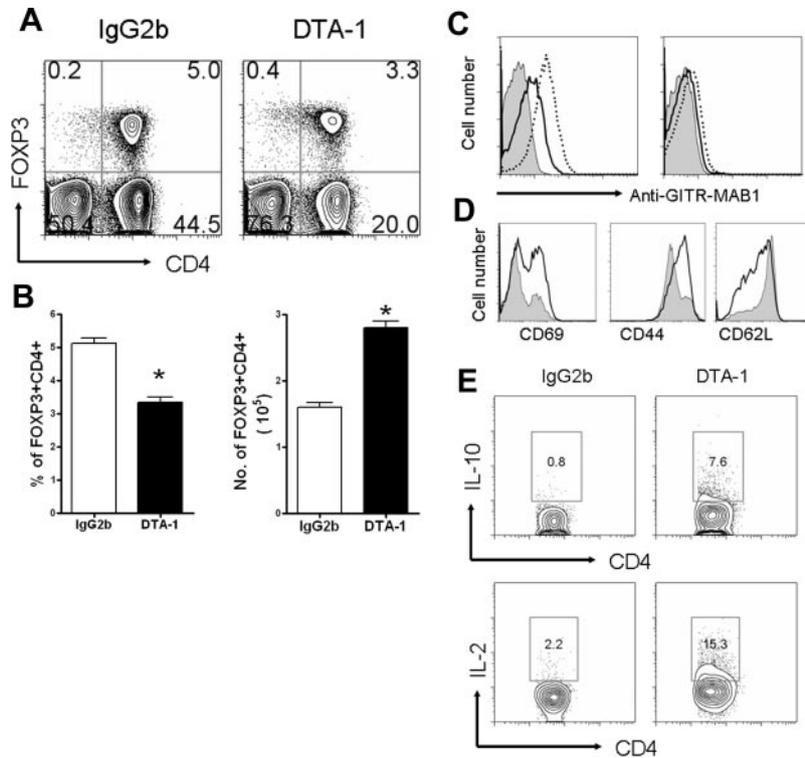
more  $\text{Foxp3}^-\text{CD4}^+\text{CD25}^+$  T cells in the purified  $\text{CD4}^+\text{CD25}^+$  T cells from DTA-1 treated mice than that of IgG2b treated mice (19% vs 6% respectively, Fig. 5C), which likely represent newly activated  $\text{CD4}^+$  effectors. The equal suppression in fact suggests that the  $\text{T}_{\text{reg}}$  cells from DTA-1-treated may be more suppressive than the control  $\text{T}_{\text{reg}}$  cells.

Conversely,  $\text{CD4}^+\text{CD25}^-$  T responders from tumor-bearing mice treated with DTA-1 were not only more proliferative than control IgG2b T responders, when no  $\text{T}_{\text{reg}}$  cells were included in the coculture, but were also more resistant to suppression by IgG2b-treated  $\text{T}_{\text{reg}}$  cells across most  $\text{T}_{\text{reg}}/\text{T}$  responder ratios tested (Fig. 5B). The costimulatory effect of GITR ligation on  $\text{CD4}^+\text{CD25}^-$  T responders, seems to be sufficient to render  $\text{CD4}^+$  T responders resistant to  $\text{T}_{\text{reg}}$  cell suppression. Similar results were obtained in the splenocytes with the same experimental protocol (data not shown).

#### *CD4<sup>+</sup> effector T cells played a pivotal role in mediating DTA-1 induced immune activation and tumor immunity*

To dissect possible roles of  $\text{CD8}^+$  T cells,  $\text{CD4}^+$  T cells, and NK cells in DTA-1 induced antitumor immunity, we conducted depletion experiments, treating mice with purified rat anti-mouse CD8, rat anti-mouse CD4, and rabbit anti-asialo GM1, respectively. We started depletions 5 days after tumor inoculation and just before giving DTA-1, as described in *Materials and Methods*. We reasoned that depleting the cell subsets after tumor inoculation but before initial treatment would ensure that their potential involvement in tumor initiation would not be perturbed and that the results were obtained from a host with an already established tumor. The depletion schedule resulted in greater than 95% of the  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells being depleted, while NK cells were depleted to slightly  $>80\%$ .

As shown in Fig. 6A, both CD8 and NK cells were important effectors in natural antitumor immunity, tumors grew much faster in CD8 or NK depleted mice than nondepleted mice. However,



**FIGURE 4.** The effects of in vivo GITR ligation on T<sub>reg</sub> cells. Mice were treated with 500  $\mu$ g DTA-1 or IgG2b on day 7 post CT26 tumor cell inoculation. Cells from TDLNs were harvested and stained for CD4, CD3, CD25, and Foxp3 as well as markers CD69, CD44, and CD62L. Data are presented as cytometric dot plots (A) and graphically as percentages and absolute numbers (B) of CD4<sup>+</sup>FoxP3<sup>+</sup> cells in an average of three independent experiments. C, Reduced expression of GITR after DTA-1 treatment on gated CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (left) and CD4<sup>+</sup>CD25<sup>-</sup>FoxP3<sup>-</sup> T cells (right). TDLNs were isolated 5 days after DTA-1 (heavy line), IgG2b treatment (dot line), or isotype control stained (tinted area), and GITR expression was measured by staining with biotin-conjugated anti-mouse GITR mAb (GITR-MAB1) and other markers such as CD3, CD4, CD25, and Foxp3, followed by staining with streptavidin-APC. D, CD69, CD44, and CD62L expression on gated CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells derived from IgG2b treated mice (tinted area) and DTA-1-treated mice (thick line). E, Intracellular IL-10 and IL-2 expression by CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells after DTA-1 or IgG2b treatment. Total lymph node cells were cultured with PMA/ionomycin for 5 h and cells were stained with anti-CD3, CD4, CD25, FoxP3, IL-10, and IL-2 Abs. The numbers represent the percentage of cells that produced the indicated cytokines. Gated on CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>. \*,  $p < 0.05$ . Data are representative of three independent experiments.

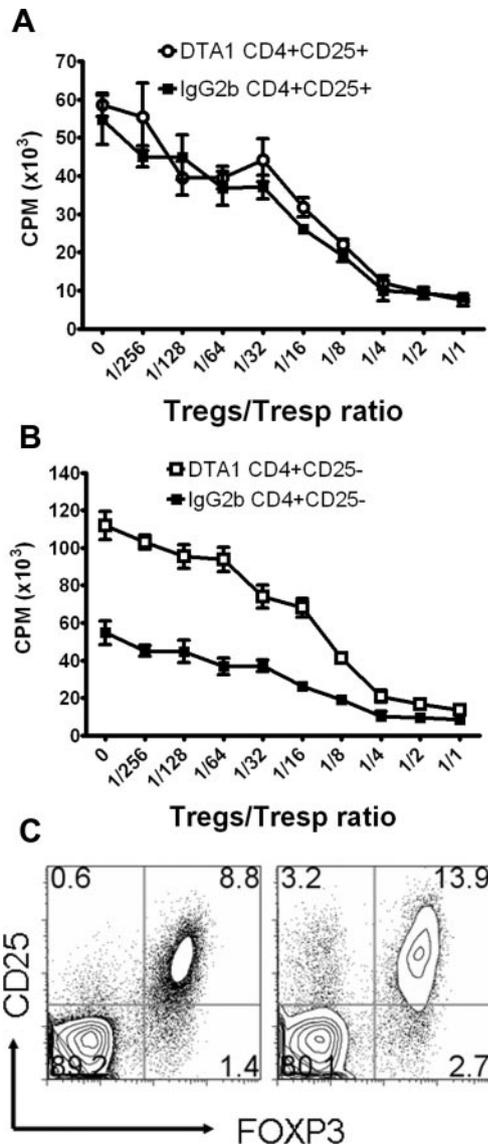
neither CD8<sup>+</sup> nor NK appeared to be absolutely required for DTA-1 induced antitumor immunity because DTA-1 was able to induce significant tumor rejection after CD8 depletion (Fig. 6C) or NK cell depletion (Fig. 6D). Combined depletion of CD8<sup>+</sup> and NK yielded a significantly compromised effect of DTA-1 (Fig. 6B), suggesting that there may be a redundant mechanism in killing tumors between CD8<sup>+</sup> and NK. The fact that double depletion of NK and CD8<sup>+</sup> could not completely block DTA-1-induced effects may simply be a result of incomplete depletion. Alternatively, there could be another or more effectors.

Depleting of CD4<sup>+</sup> cells, however, resulted in the complete abrogation of DTA-1- induced tumor immunity, comparing the tumor growth in the CD4-depleted mice treated with DTA-1 or IgG2b in Fig. 6E. DTA-1-induced tumor immunity was also absent in DTA-1-treated CT26-bearing nude mice, which further supports the primary involvement of CD4<sup>+</sup> T cells in DTA-1-induced immunity (Fig. 6F). Worth noting is that the scale of the overall tumor volume in this experiment is significantly smaller than that of the isotype depletion control (data not shown). The explanation may be that this protocol also depletes CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells; it has been shown that depletion of T<sub>reg</sub> cells inhibits tumor growth (27).

The requirement for CD4<sup>+</sup> T cells in DTA-1-induced tumor immunity raises the possibility of CD4<sup>+</sup> functioning as helper T cells to augment downstream effector mechanisms. If CD4<sup>+</sup> T cells, after DTA-1 treatment, are primarily functioning as helpers,

then when CD4<sup>+</sup> T cells are depleted before DTA-1 treatment, downstream CD8<sup>+</sup> and NK cell subsets should not be activated or induced into a cytolytic killing state. We decided to test this hypothesis using the same experimental procedure as above, where CD4<sup>+</sup> T cells were depleted after tumor implantation but before the first DTA-1 treatment. FACS analysis showed that CD69 expression on NK cells, B cells, and CD8<sup>+</sup> T cells from CD4<sup>+</sup> depleted mice remained at baseline level after DTA-1 treatment. This was in stark contrast to the fact that CD69 was still highly expressed on immune cells after CD8<sup>+</sup> T cell, NK cell, or control depletion (Fig. 7A).

Similarly, the expression of CD107a on NK cells or CD8<sup>+</sup> T cells, which were increased upon DTA-1 treatment, (Fig. 2C and Fig. 3F), remained at background levels upon CD4 depletion (Fig. 7, B and C). Interestingly, CD8<sup>+</sup> T cell depletion caused ~50% less NK CD107a mobilization in the DTA-1-treated mice (Fig. 7B), whereas NK depletion did not affect CD107a mobilization in CD8<sup>+</sup> T cells (Fig. 7B). It is possible that CD8<sup>+</sup> may have positive feedback on NK cells. In nude mice, DTA-1 treatment failed to induce any significant level of CD107a mobilization in NK cells (Fig. 7B), supporting the notion that T cells are required for DTA-1-induced NK activation. Intracellular IFN- $\gamma$  staining showed similar trends, i.e., low expression in CD8<sup>+</sup> T cells upon CD4 depletion (Fig. 7D). The results presented in this study clearly establish that CD4<sup>+</sup> T cells are indispensable for DTA-1-induced tumor



**FIGURE 5.** GITR stimulation increases CD4<sup>+</sup>CD25<sup>-</sup> T effector responses without blocking the suppressive capacity of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells ex vivo. **A**, TDLN-derived CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from DTA-1 or IgG2b-treated tumor-bearing mice were mixed at varying numbers with a constant number of CD4<sup>+</sup>CD25<sup>-</sup> T responder (Tresp) cells (40,000/well) isolated from the TDLNs of IgG2b-treated tumor-bearing mice. Cells were cultured with irradiated CD4<sup>+</sup> T cell-depleted splenocytes in the presence of 62.5 ng/ml anti-CD3 mAb. [<sup>3</sup>H]thymidine was added for the last 8 h of a 72-h culture. **B**, CD4<sup>+</sup>CD25<sup>-</sup> Tresp cells were isolated from DTA-1 or IgG2b-treated tumor-bearing mice mixed with varying numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from TDLNs of control IgG2b-treated tumor-bearing mice, and stimulated in vitro for 72 h as in **A**. Data are representative of results obtained from both TDLNs and splenic T cells. **C**, CD4<sup>+</sup> T cells isolated from the TDLNs of DTA-1 or IgG2b treated mice were stained with CD25 and Foxp3. The numbers in the dot plot indicate the percentage of total CD4<sup>+</sup> T cells.

immunity and play an essential role in mediating immune activation and cytolytic activities of CD8<sup>+</sup> and NK cells in vivo.

#### *DTA-1-treated CD4 T cells are not sufficient for tumor clearance in CT26 tumors*

Recent work has suggested that CD4<sup>+</sup> T cells can be more effective at tumor rejection than CD8 T cells in a TCR transgenic system (28). The indispensable role of CD4<sup>+</sup> T cells in DTA-1-in-

duced tumor immunity also prompted us to investigate whether CD4<sup>+</sup> effectors contributed to direct killing in this context. CD4<sup>+</sup> or CD8<sup>+</sup> T cells purified from DTA-1 or IgG2b-treated CT26-bearing mice were adoptively transferred into SCID-recipients. One day after cell transfers, the SCID recipients were inoculated with CT26 cells. As expected, transfer of CD4<sup>+</sup> and CD8<sup>+</sup> T cells derived from IgG2b-treated mice did not affect tumor growth in the recipient mice (Fig. 8, *A* and *B*). The transferring of CD4<sup>+</sup> T cells derived from DTA-1-treated mice delayed tumor growth, but did not appear to be sufficient to induce tumor rejection. However, of SCID recipients transferred with CD8<sup>+</sup> T cells derived from DTA-1-treated mice, 80% completely rejected the CT26 tumor challenge (Fig. 8, *A* and *B*). These results were supported by in vitro flow cytometry-based CTL assay, that CD4<sup>+</sup> T cells derived from DTA-1-treated tumor bearing mice were weaker CTLs than CD8<sup>+</sup> T cells (Fig. 8*C*). Hence, even though CD4<sup>+</sup> effector T cells were required for DTA-1-induced antitumor immunity, they were not sufficient to induce completed clearance of the tumor burden in the CT26 tumors.

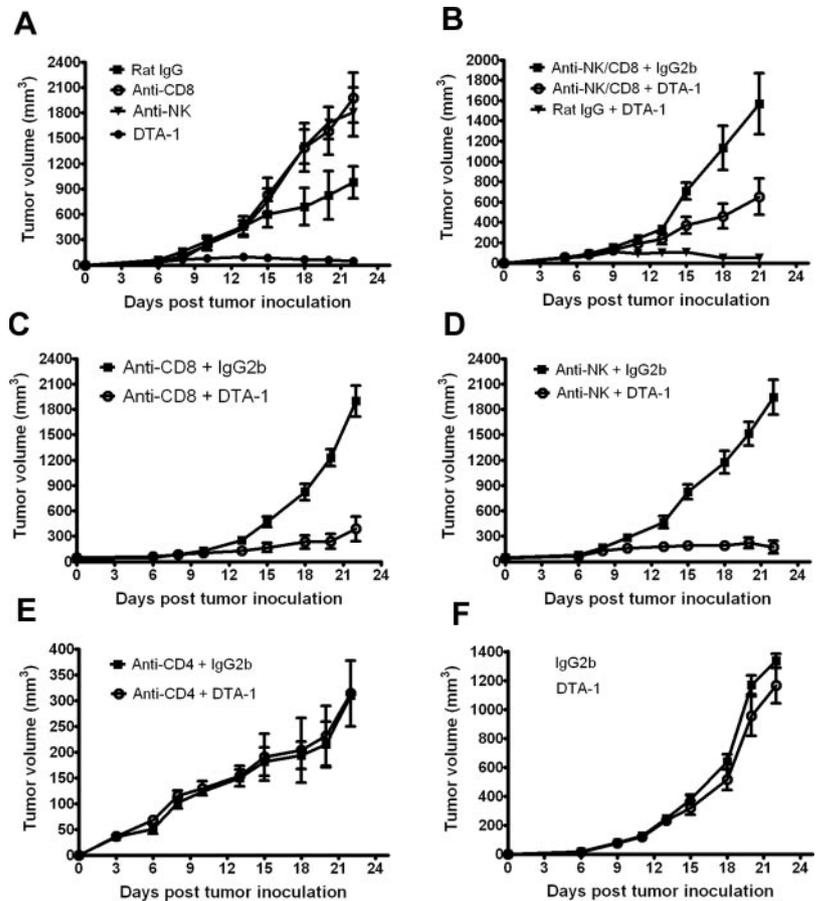
#### **Discussion**

We have used a CT26 mouse colorectal tumor model to characterize the mechanisms of anti-GITR mAb induced antitumor immunity. We have identified CD4<sup>+</sup> effector T cells as the essential targets of GITR ligation in vivo, mediating further activation of CD8<sup>+</sup>, NK, and B cells as well as antitumor response. GITR ligation in vivo does not induce the abrogation of T<sub>reg</sub> cell function directly; rather, it does so indirectly, through the costimulation of the CD4<sup>+</sup> effector T cells. Therefore, the costimulation of CD4<sup>+</sup> effectors, and their subsequent activation of the CD8<sup>+</sup> and NK cells, contribute to breakdown of the immune tolerance.

DTA-1-induced CD8<sup>+</sup> T cell, B cell, and NK cell activation, as well as antitumor immunity were completely diminished when CD4<sup>+</sup> T cells were depleted. This indicates that CD4<sup>+</sup> T cells play a central role in helping generate the activities of CD8<sup>+</sup>, B cell, and NK cell activities. However, the in vivo mechanism of how anti-GITR-stimulated CD4<sup>+</sup> T cells activate B cells, NK cells, and CD8<sup>+</sup> T cells is still not clear. Given that many immune cell populations such as CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, B cells, and NK cells express GITR at substantial levels, it is likely that a GITR agonist costimulates all of these populations. We have found that in vitro, DTA-1 treatment alone does not activate resting B cells, but it seems to synergize with anti-CD40 to enhance CD69 surface expression on B cells (Fig. 2*E*). This implies that CD40L expressed on activated CD4 T cells might promote anti-GITR stimulation on B cells in vivo. We further speculate that factors/cytokines secreted by activated CD4<sup>+</sup> may synergize with GITR agonist to activate downstream subsets, such as NK or CD8<sup>+</sup> T cells. Indeed, it has been shown that the GITR agonist costimulates CD8<sup>+</sup> T cells to proliferate and costimulates NK cells to become cytolytic in vitro (9, 10). Future studies aiming to identify factors/cytokines expressed or released by CD4<sup>+</sup> T cells, which synergize with GITR ligation to promote the activation of other GITR expressing cells may provide insight into the design of effective combination antitumor immunotherapy.

The indispensable role of CD4<sup>+</sup> T cells in DTA-1-induced antitumor immunity presents another possibility that CD4<sup>+</sup> effectors may be sufficient for direct killing of CT26 tumors in addition to their regulatory role. The results from adoptive transfer and the ex vivo killing assay indicate that DTA-treated CD4<sup>+</sup> effectors are not sufficient for tumor clearance, nor do they generate as high activities of CTLs as CD8<sup>+</sup>, suggesting that CD4<sup>+</sup> T cells have a weak direct antitumor effect. In contrast, DTA-1-treated CD8<sup>+</sup> T cells are much more effective at rejecting tumor challenge. Hence,

**FIGURE 6.** CD4<sup>+</sup> T cells are essential for DTA-1 induced tumor rejection. BALB/c or nude mice received 500  $\mu\text{g}/\text{mouse}$  of DTA-1 or rat IgG2b on day 7 after CT26 inoculation. Depletion of individual immune cells was done by i.p. injection of anti-CD4 (GK1.5, 500  $\mu\text{g}$  Ab/mouse), anti-CD8 (53.6.72, 500  $\mu\text{g}$  Ab/mouse), or anti-NK (anti-mouse Asialo GM1, 50  $\mu\text{l}/\text{mouse}$ ) at days 5, 6, 7, 12, and 17 relative to tumor inoculation. Rat-IgG and rabbit IgG were used as control Abs for depletion. *A*, Tumor growth as measured by volume ( $\text{mm}^3$ ) was indicated in anti-CD8 and anti-asialo GM1 treated mice compared with control depletion Ab rat-IgG treatment. DTA-1 treatment to non-cell-depleted mice showed a completed tumor rejection. Tumor growth after DTA-1 or IgG2b treatment in CD8<sup>+</sup> plus NK depletion (*B*), CD8<sup>+</sup> depletion alone (*C*), NK depletion alone (*D*). *E*, Tumor growth in CD4<sup>+</sup> cell depleted mice after DTA-1 or IgG2b treatment. *F*, Tumor growth in nude mice after DTA-1 or control treatment. All data are representative of at least three independent experiments ( $n = 8\text{--}15$  per group).



the effect of CD4<sup>+</sup> effector T cells in DTA-1 treated-CT26-bearing mice depends on their contribution to the activations and activities of CD8<sup>+</sup> T and other host immune cells.

It has been demonstrated that although the primary cytotoxic T lymphocyte (CTL) response can be independent of CD4<sup>+</sup> T cell help, all secondary responses require CD4<sup>+</sup> T cell help, irrespective of whether the stimulus is a cellular Ag or viral infection (29, 30). Therefore, the strong costimulation of effector CD4<sup>+</sup> T cells may allow for long-term memory to be generated, maintaining long-lived and functional tumor-specific memory T cells. The initial evidence to support this is that the Ag-specific CD8<sup>+</sup> memory responses toward a xenogeneic vaccine were enhanced upon DTA-1 treatment in the B16 model (11). In addition, it has been shown that mice treated with DTA-1 rapidly rejected a subsequent challenge at a later time point by the same tumors with a 10-fold larger dose (12), suggesting that DTA-1 treatment induces a long-lasting antitumor immunity. We have obtained similar results in the CT26 model, i.e., at 2 months after the rejection of CT26 tumors, those DTA-1-treated mice rejected a subsequent challenge (data not shown). Such studies suggest that treatment by a GITR agonist may prevent tumor recurrence.

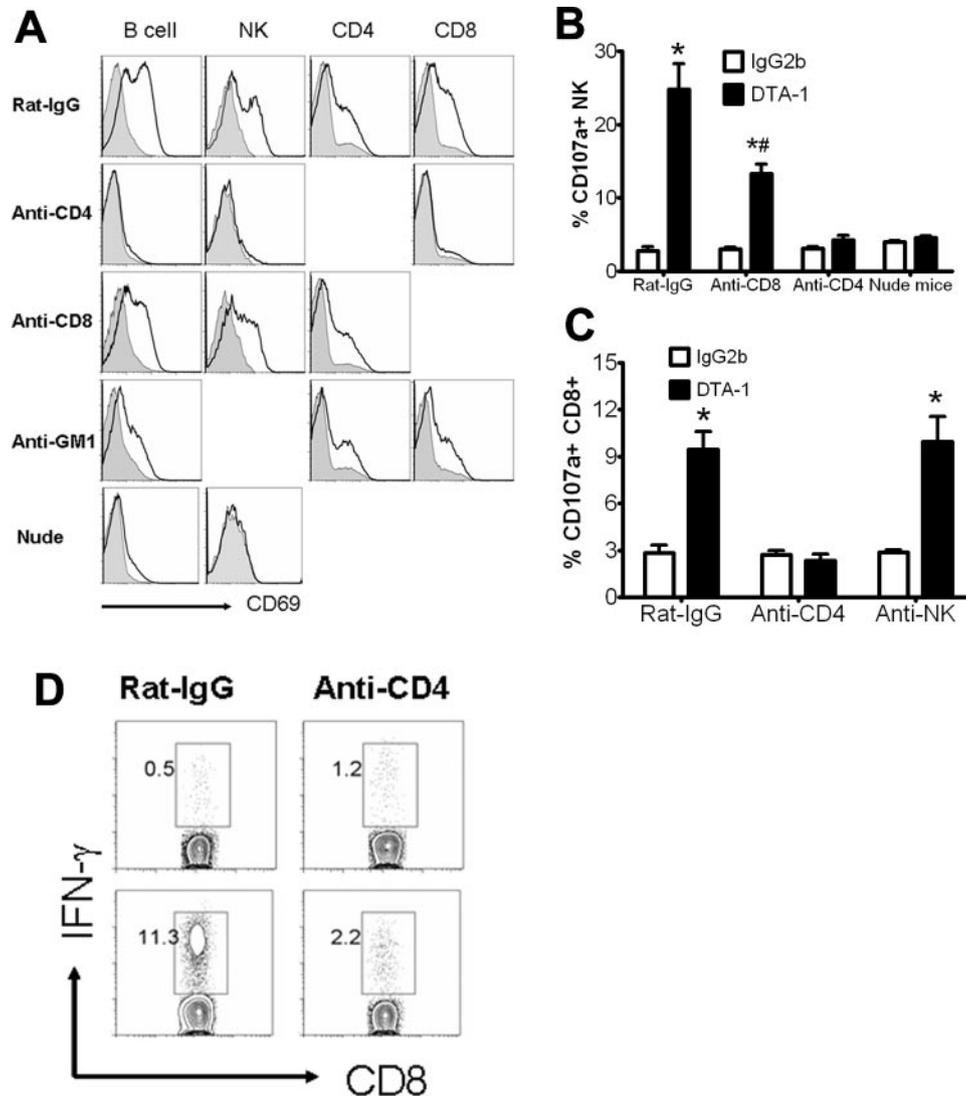
We found significant NK cell recruitment and activation in tumor-draining lymph nodes after DTA-1 treatment. The failure of DTA-1 to induce activation in TDLNs in both nude mice and CD4<sup>+</sup> T cell depleted BALB/c mice suggests that DTA-1 cannot directly activate NK activities without help from CD4<sup>+</sup> T cells. The finding that DTA-1's effect is not significantly compromised after NK depletion does not rule out NK contribution, given the limited depletion efficacy (80%) and the redundancy of the host immunity. The latter possibility was illustrated by the increased average tumor volume in the combined NK and CD8 depletion

compared with each individual depletion upon DTA-1 treatment (Fig. 6, *B–D*). Additional support evidence is that the cytolytic CD8 and NK cells are generated in the TDLNs (Figs. 2*C* and 3*F*).

Because GITR is widely expressed on many types of immune cells, GITR ligation potentially has broader regulatory effects on other immune cells in addition to CD4<sup>+</sup>, CD8<sup>+</sup> T cells, and NK cells. GITR ligation on NKT cells enhanced IL-4 and IFN- $\gamma$  production and contributed to the NKT-mediated hypersensitivity pneumonitis (31). We have shown that DTA-1 dramatically induced activation and expansion of B cells in tumor bearing mice. How B cell activation contributes to DTA-1-induced tumor immunity, and/or autoimmunity, is currently under investigation.

T<sub>reg</sub> cells maintain tolerance through multiple mechanisms, such as inhibiting T cell priming in tumor-draining lymph nodes (32, 33), blocking tumor-specific CD8<sup>+</sup> T cell activation, or abrogating their cytotoxicity (34–36). T<sub>reg</sub> cells also suppress the generation of tumor-killing NK cells through inhibiting IL-2 production or NKG2D-mediated cytotoxicity (37, 38). We have shown in the CT26-bearing mice that DTA-1 treatment does not directly abrogate the suppressive capacity of T<sub>reg</sub> cells, but rather that effector CD4<sup>+</sup> from mice treated with DTA-1 are able to overcome the suppression. This agrees with previous conclusions drawn from in vitro studies that the effect on CD4<sup>+</sup> T responders is responsible for the abrogation (16). To date, this is the first evidence demonstrated in a tumor-bearing host after DTA-1 treatment that the CD4<sup>+</sup>CD25<sup>+</sup> cells are still suppressive, whereas the strong costimulation of DTA-1 on CD4<sup>+</sup>CD25<sup>-</sup> population abrogates the suppressive activity of T<sub>reg</sub> cells.

What is of great interest is what direct role GITR ligation has on T<sub>reg</sub> cells, especially in a tumor-bearing host. We have discovered



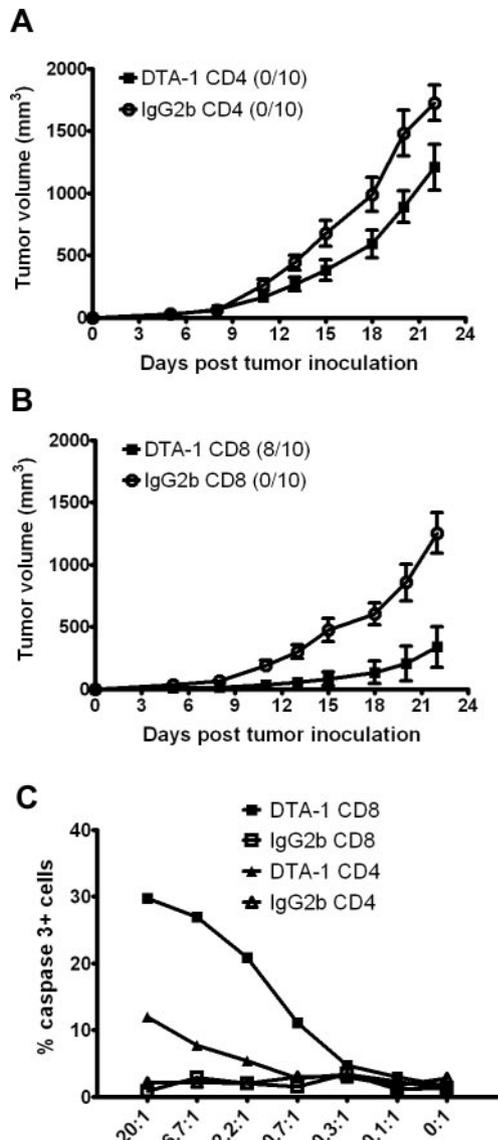
**FIGURE 7.** CD4<sup>+</sup> T cells are required for DTA-1 induced immune activation of CD8<sup>+</sup>, NK, and B cells in the TDLNs. BALB/c mice depleted of individual CD4, CD8 T cells, or NK cells, or nude mice were treated with DTA-1 or IgG2b at day 7 after CT26 tumor inoculation. Four days post DTA-1 or IgG2b treatment, cells derived from TDLNs were collected and analyzed by flow cytometry to determine the activation status of immune cell subsets. **A**, CD69 expression on CD4<sup>+</sup>, CD8<sup>+</sup>, B cells after IgG2b (tinted) and DTA-1 treatment (heavy line). **B**, CD107a mobilization on NK cells. Cells isolated from TDLNs of tumor-bearing nude mice, CD4<sup>+</sup>, CD8<sup>+</sup> or control depleted mice, were stimulated with PMA/ionomycin for 5 h in vitro. CD107a mobilization was determined by flow cytometry analysis on gated DX5<sup>+</sup>CD3<sup>-</sup> NK cells. **C** and **D**, CD107a and IFN- $\gamma$  expression on CD8<sup>+</sup> T cells after in vitro restimulation with CT26 cells for 5 h. Data are representative of three independent experiments with  $n = 5$  mice per group.

that GITR engagement on T<sub>reg</sub> cells in vivo results in an up-regulation of activation markers such as CD69 and CD44, and down-regulation of CD62L, all of which are associated with T cell activation. We have also demonstrated that T<sub>reg</sub> cells from DTA-1-injected TDLNs produce more IL-10 and IL-2 compared with isotype-injected mice. Both cytokines are critical in developing and maintaining the suppressive function of T<sub>reg</sub> cells (39–41). We also noted in the ex vivo coculture assay there was 19% of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> in the CD4<sup>+</sup>CD25<sup>+</sup> population purified from DTA-1-treated mice vs 6% in the IgG2b-treated mice. This population most likely accounts for the recently activated CD4<sup>+</sup> effector T cells. Considering the potential impact of the contaminated effector T cells, the equal suppression shown in Fig. 5A suggests that T<sub>reg</sub> cells from DTA-1 treated mice are slightly more suppressive.

Substantial evidence supports the role of Foxp3<sup>+</sup> T<sub>reg</sub> cells in controlling autoreactivity during and subsequent to the development of the peripheral immune system (7, 42). New evidence

shows that that in vivo ablation of T<sub>reg</sub> cells in adult mice elicits rapid onset of an autoimmune disease, emphasizing the essential role of these T<sub>reg</sub> cells as a dominant force in controlling lymphomyeloid cell proliferation and activation (43, 44). Our in vivo data support the recent model raised by Shevach and Stephens, in which GITR ligation costimulates both effector T cell functions and the suppressive function of T<sub>reg</sub> cells; the costimulation of effector T cells overrides the suppression of T<sub>reg</sub> cells (45). From the standpoint of developing the GITR agonist to treat cancer, because the suppressive function of T<sub>reg</sub> cells is not compromised, we hypothesize that such perturbation may have less severe autoimmune side effects than ablation of T<sub>reg</sub> cells.

The TNF receptor superfamily includes several members besides GITR that deliver costimulatory signals to T cells. The agonists to both human 4-1BB and OX40 are currently in clinical trials for treating cancers (46). GITR, 4-1BB, and OX40 appear to be closely related in terms of their inducible expression patterns on T cells and their apparent signaling pathways (47). However, their



**FIGURE 8.** DTA-1-treated CD4<sup>+</sup> T cells are not sufficient to induce tumor rejection compared with CD8<sup>+</sup> T cells. On day 21 after tumor inoculation (one week after second dose of DTA-1 or IgG2b treatment), CD4<sup>+</sup> ( $10 \times 10^6$ /mouse) and CD8<sup>+</sup> T cells ( $10 \times 10^6$ /mouse) purified from mixed splenocytes and lymph nodes were adoptively transferred into SCID mice. One day after cell transfer, SCID mice were inoculated intradermally with  $3 \times 10^5$  CT26 cells. **A**, Tumor growth in SCID mice received DTA-1-CD4 or IgG2b-CD4 ( $n = 10$  in each group). **B**, Tumor growth in SCID mice received DTA-1-CD8 or IgG2b-CD8 ( $n = 10$  in each group). Numbers in parentheses refer to tumor rejection ratios. **C**, Ex vivo tumor killing activity. Flow cytometry-based CTL assay to measure killing activity of DTA-1 or IgG2b treated effector CD4<sup>+</sup> or CD8<sup>+</sup> T cells against CT26 tumor cells. X-axis indicates the E:T ratios. The percentages of cleaved caspase-3 in total DDAO-SE labeled-target cells are shown on Y-axis. One of two independent experiments is shown.

expression and function may not be totally overlapping, e.g., in mouse, GITR is substantially expressed by naive T cells, whereas the expressions of OX40 and 4-1BB are more restricted to activated T cells (47). Functionally, OX40 positively regulates effector function and late accumulation/survival, whereas the evidence as regards 4-1BB is intriguing. It has been shown that 4-1BB initially operates in a negative manner to limit primary CD8 responses, but enhances CD8 T cell response only at a late stage when memory has formed or is beginning to form (48). It has also been shown

that an agonistic 4-1BB mAb induces proliferation of CD8<sup>+</sup> and leads to amplification of CTL responses (46). Relating to abrogation of suppressive function of T<sub>reg</sub> cells, GITR ligation was found to reverse suppression of naive T<sub>reg</sub> cells and in vitro activated T<sub>reg</sub> cells (49), whereas anti-OX40 only abrogated the suppression of naive T<sub>reg</sub> cells (49). In addition, OX-40 ligation can inhibit the generation of IL-10-producing Tr1 cells, but 4-1BB and GITR do not appear to have this capability (50). Therefore, these TNF receptor superfamily members may have preferential effects on special types of T cells at different stages of activation. Further studies to differentiate these costimulators' roles in tumor immunity of mouse models and their clinical implications will be beneficial to cancer immunotherapy.

In conclusion, CD4<sup>+</sup> T cells play a central role in orchestrating multiple effector cells of anti-GITR mAb-induced tumor rejection. GITR agonist-mediated breakdown of immune tolerance to tumor Ags, without direct abrogation of T<sub>reg</sub> cell function, provides a promising means to enhance anti-tumor immunity with potentially less severe autoimmunity than perturbing T regulatory cells themselves.

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## Disclosures

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