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CUTTING EDGE

# Cutting Edge: Ligation of the Glucocorticoid-Induced TNF Receptor Enhances Autoreactive CD4$^+$ T Cell Activation and Experimental Autoimmune Encephalomyelitis[1]

*Adam P. Kohm, Julie S. Williams, and Stephen D. Miller*[2]

***The glucocorticoid-induced TNFR (GITR) is expressed at high levels on resting CD4$^+$CD25$^+$ T regulatory ($T_R$) cells and regulates their suppressive phenotype. Accordingly, we show that anti-GITR mAb treatment of SJL mice with proteolipid protein 139–151-induced experimental autoimmune encephalomyelitis significantly exacerbated clinical disease severity and CNS inflammation, and induced elevated levels of Ag-specific T cell proliferation and cytokine production. Interestingly, prior depletion of $T_R$ cells failed to result in exacerbated experimental autoimmune encephalomyelitis suggesting alternative targets for the anti-GITR mAb treatment. Importantly, naive CD4$^+$CD25$^-$ T cells up-regulated GITR expression in an activation-dependent manner and anti-GITR mAb treatment enhanced the level of CD4$^+$ T cell activation, proliferation, and cytokine production in the absence of $T_R$ cells both in vivo and in vitro. Taken together, these findings suggest a dual functional role for GITR as GITR cross-linking both inactivates $T_R$ cells and increases CD4$^+$CD25$^-$ T cell effector function, thus enhancing T cell immunity. The Journal of Immunology,* 2004, 172: 4686–4690.***

One population of T regulatory ($T_R$)[3] cells display a mixed phenotype of naive and activated cell surface marker (1), e.g., CD4$^+$CD25$^+$L-selectin (CD62L)$^{high}$, and inhibit T cell effector function in a TCR-dependent manner. Although the exact mechanism by which $T_R$ cells exert their suppressive influence is still unknown, IL-10 production, surface CTLA-4 expression, IL-2 sequestration, costimulatory molecule blockade, and surface/secreted TGF-$\beta$ expression are all proposed mechanisms by which $T_R$ cells may down-regulate effector CD4$^+$ and CD8$^+$ T cell responses (1).

The glucocorticoid-induced TNFR family gene (GITR or TNFRSF18) is a member of the TNF growth factor receptor family that includes CD40, CD27, 4-1BB, and OX40 (2, 3). GITR is predominantly expressed on resting CD4$^+$ CD25$^+$ $T_R$ cells (4) and functions to regulate $T_R$ cell function such that anti-GITR Ab treatment of normal mice exacerbates several models of spontaneous autoimmune disease (4–6).

In the current study, anti-GITR mAb treatment during the induction phase of experimental autoimmune encephalomyelitis (EAE) significantly enhanced the level of clinical disease severity, CNS inflammation, and autoreactive T cell responses. Importantly, $T_R$ cell-depleted CD4$^+$ T cell populations expressed high levels of GITR in an activation-dependent manner and cross-linking of GITR expressed on CD4$^+$CD25$^-$ T cells during activation enhanced their level of activation, proliferation, and cytokine production in a concentration-dependent manner suggesting a positive immunoregulatory role for GITR expression on CD4$^+$CD25$^-$ T cells.

## Materials and Methods

### *Mice and materials*

SJL female mice, 5- to 6-wk-old, were purchased from Harlan Sprague Dawley (Indianapolis, IN). DO11.10 ($OVA_{323–339}$/I-A$^d$-specific) and 5B6 (proteolipid protein $(PLP)_{139–151}$/I-A$^s$-specific) (7) TCR transgenic mice were maintained and bred as previously described (8). The DTA-1 anti-GITR clone was a generous gift of Dr. S. Sakaguchi (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan).

### *$T_R$ cell depletion*

For in vitro depletion, single cell lymph node (LN) suspensions were depleted of $T_R$ cell populations by positive selection of CD25$^+$ $T_R$ cells using anti-CD25 mAb (7D4), anti-rat $\kappa$ microbeads (Miltenyi Biotec, Auburn, CA) and an AutoMACs (Miltenyi Biotec). For in vivo depletion, mice received two injections of anti-CD25 mAb (7D4; 500 $\mu$g/injection i.p.) on days $-4$ and $-2$ before disease initiation. The efficiency of $T_R$ cell depletion was determined to be 96–98% effective at the time of disease initiation by immunofluorescence.

### *Induction and clinical evaluation of $PLP_{139–151}$-induced EAE*

Six- to 7-wk-old female mice were immunized s.c. with 200 $\mu$l of an emulsion containing 800 $\mu$g of *Mycobacterium tuberculosis* H37Ra (Difco, Kansas City, MO) and 100 $\mu$g of $PLP_{139–151}$ distributed over three spots on the flank. Individual animals were observed daily and clinical scores were assessed in a blinded fashion on a 0–5 scale as follows: 0 = no abnormality, 1 = limp tail, 2 = limp tail and hind limb weakness, 3 = hind limb paralysis, 4 = hind limb paralysis and forelimb weakness, and 5 = moribund. The data are reported as

Department of Microbiology-Immunology and Interdepartmental Immunobiology Center, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

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[2] Address correspondence and reprint requests to Dr. Stephen D. Miller, Department of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, IL 60611. E-mail address: s-d-miller@northwestern.edu

[3] Abbreviations used in this paper: $T_R$, T regulatory; GITR, glucocorticoid-induced TNFR; EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; Tg, transgenic; LN, lymph node; CD62L, L-selectin.



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the mean daily clinical score. Mice were age- and sex-matched for all experiments. In vitro proliferation and ELISPOT assays were performed as previously described (9)

*Immunohistochemistry and immunofluorescence*

CNS immunohistochemistry was performed as previously described (9). For immunofluorescence, single cell suspensions were washed and incubated with fluorescently tagged Abs directly against a panel of cell surface markers (BD PharMingen, San Diego, CA). Fluorescent staining was analyzed using a LSRII and CellQuest Pro Analysis Software (BD Biosciences, San Jose, CA).

*Statistical analysis*

Comparisons of clinical scores and ELISPOT frequencies between the various treatment groups were analyzed by unpaired Student's *t* test. Values of $p < 0.01$ were considered significant.

## Results

We previously reported a functional role for $CD4^+CD25^+$ $T_R$ cells in regulating the onset and progression of EAE such that $T_R$ cell supplementation ameliorated clinical disease severity and CNS inflammation in both active and adoptive EAE (8). To further investigate the protective role of $T_R$ cells during EAE, mice were treated with five 1-mg doses of anti-GITR mAb (DTA-1) to inactivate the $T_R$ cell population (4, 5) at times spanning the induction and preacute disease phases. Anti-GITR-treated mice displayed a 0% survival rate upon reaching the "onset" phase of clinical disease (Fig. 1*A*). This was an unexpected result, because the typical survival rate in this EAE system is 100%, suggesting that anti-GITR mAb treatment lethally enhanced the inflammatory response that normally precedes clinical disease onset. However, it was equally possible that the treatment regimen of repeated injections of a high dose of anti-GITR mAb produced a cumulative toxic effect. To test this possibility, similar studies were performed in which mice were immunized with a peptide known to lack inflammatory potential in SJL mice, $OVA_{323–339}$, in combination with anti-GITR mAb treatments. There was a 100% survival rate in $OVA_{323–339}$-primed mice receiving the identical anti-GITR mAb treatment regimen (Fig. 1*B*) suggesting a role for the autoreactive inflammatory response in anti-GITR-treated mice primed with $PLP_{139–151}$.

In light of the detrimental effect on survival following high dose anti-GITR treatment, similar experiments were performed with a lower dose of anti-GITR mAb. Mice treated with 500 $\mu$g of anti-GITR mAb displayed a significantly exacerbated clinical disease severity in the absence of any observed mortality (Fig. 1*C*). In agreement with clinical disease severity, anti-GITR mAb treatment enhanced the level of $PLP_{139–151}$-specific T cell proliferation in both the LNs (Fig. 1*D*) and spleen (data not shown). Anti-GITR mAb treatment also significantly increased the frequency of both $PLP_{139–151}$-specific Th1- (IFN-$\gamma$; Fig. 1*E*) and Th2- (IL-4; Fig. 1*F*) cytokine-producing cells, suggesting that anti-GITR mAb treatment functioned to generally enhance the autoantigen-specific immune response during EAE.

It is believed that the clinical symptoms of both EAE and multiple sclerosis manifest as a direct result of CNS inflammation and subsequent myelin damage apparently from the direct and indirect effects of chemokines (10) and proinflammatory cytokines such as IFN-$\gamma$ and lymphotoxin/TNF$\beta$ (11, 12). We have previously shown that supplementation of the $T_R$ cell population prevents CNS inflammation and ameliorates clinical disease severity potentially via effects on both autoreactive T cell activation and cell trafficking (8). Therefore, we next determined the effect of anti-GITR mAb treatment on the level of CNS inflammation at times corresponding to the peak of the

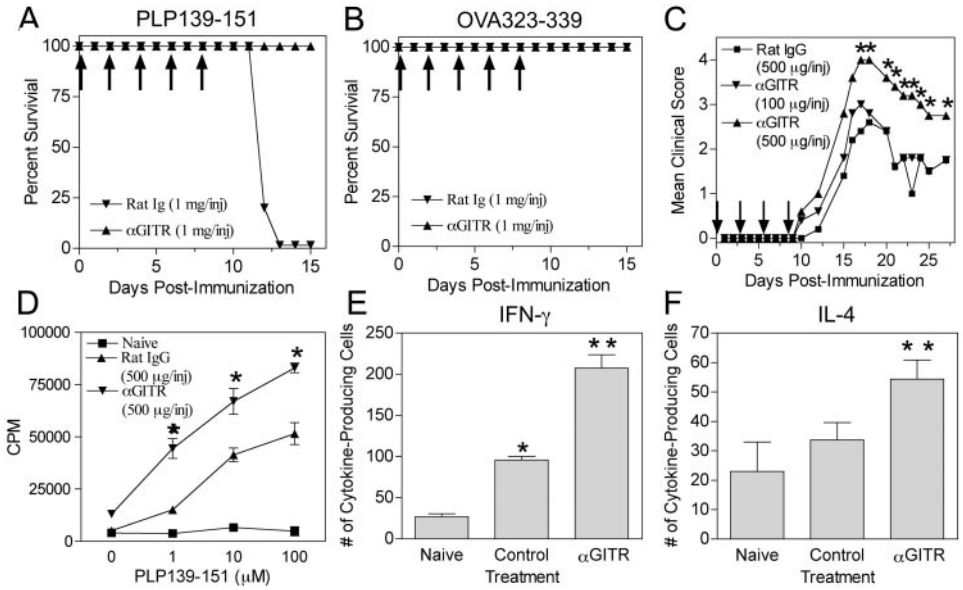


**FIGURE 1.** Anti-GITR Ab treatment exacerbates EAE clinical disease progression and autoreactive T cell effector function. *A* and *B*, Effect of high-dose anti-GITR mAb treatment on EAE progression. Mice received five i.p. injections (arrows) of either anti-GITR or rat IgG control Ab (1 mg/injection) on alternating days either (*A*) beginning with the day of active EAE induction by s.c. injection of $PLP_{139–151}$/CFA or (*B*) the day of s.c. injection of $OVA_{323–339}$/CFA. Data are presented as the daily percent survival and are representative of three separate experiments. *C–F*, Effect of moderate-low dose anti-GITR mAb treatment on EAE progression. Beginning with the day of active EAE induction by s.c. injection of $PLP_{139–151}$/CFA, mice received four injections of anti-GITR mAb (100 or 500 $\mu$g/injection) on alternating days and were followed for clinical disease (*C*). *D*, Effect of anti-GITR mAb treatment on $PLP_{139–151}$-specific T cell proliferation during EAE. Data are presented as the mean cpm and are representative of three separate experiments. *E* and *F*, Effect of anti-GITR mAb on the frequency of $PLP_{139–151}$-specific Th1 (IFN-$\gamma$) and Th2 (IL-4) cells during EAE as measured by ELISPOT. Data are presented the number of $PLP_{139–151}$-specific IFN-$\gamma$ (*E*) and IL-4 (*F*)-secreting cells per $10 \times 10^5$ LN cells. Data presented are representative of three separate experiments. Significant differences from naive (*) or control (**), $p < 0.01$.

acute clinical disease. In agreement with our previous findings, the level of CNS inflammation correlated with the degree of clinical disease severity such that anti-GITR mAb specifically increased the extent of $CD4^+$ T cell infiltration within the CNS (Fig. 2), without any discernable effects on either the $CD8^+$ or $B220^+$ cell populations which are usually not detected at high levels within the CNS of SJL mice with EAE. Taken together, these findings suggest that anti-GITR mAb treatment enhances autoreactive T cell activation and homing to the CNS resulting in an exacerbated level of clinical disease possibly via the functional inactivation of $T_R$ cell populations.

As previously discussed, anti-GITR mAb treatment is believed to specifically target and block the suppressive function of $T_R$ cells (4, 5). Therefore, we reasoned that $T_R$ cell depletion before EAE induction should result in enhanced clinical disease severity similar to that seen in mice treated with anti-GITR mAb. To our surprise, $T_R$ cell depletion failed to influence the severity of acute clinical disease in our model system (Fig. 3*A*). In addition, we found that SJL mice depleted of $T_R$ cells and subsequently treated with anti-GTIR mAb surrounding $PLP_{139-151}$/CFA priming displayed enhanced clinical disease (Fig. 3*B*) suggesting that anti-GITR has direct effects on cells other than $CD4^+CD25^+$ $T_R$ cells. To further investigate the possibility that the anti-GITR mAb may target additional cell types, we examined the level of GITR expression on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. In agreement with previous findings (4), GITR was predominantly expressed on resting $CD4^+CD25^+$ T cells with only a small percentage of resting $CD4^+CD25^-$ T cells expressing detectable levels of the receptor (Fig. 4*A*). However, GITR expression was rapidly up-regulated on the surface of $CD4^+CD25^-$ T cells within 24 h of activation by anti-CD3 mAb (Fig. 4*B*) or following stimulation of naive $PLP_{139-151}$ TCR transgenic $(Tg)^+$ T cells by Ag (data not shown). Interestingly, the observed up-regulation of GITR expression on $CD4^+$ T cells correlated with their level of CD69 and CD25, both markers of T cell activation, and these experiments have also been repeated using $T_R$ cell-depleted cultures (data not shown). To confirm the activation state dependency of GITR expression on $CD4^+CD25^-$ effector T cells,

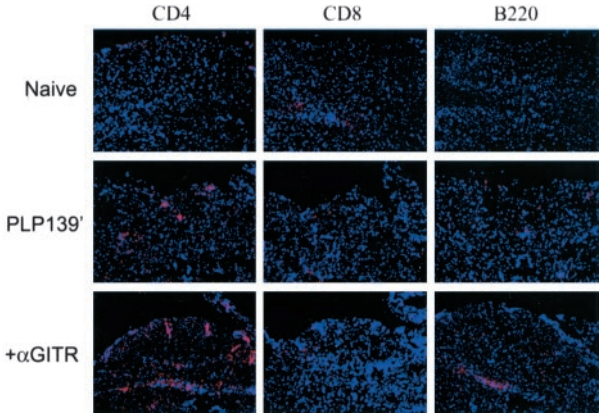


**FIGURE 2.** Anti-GITR mAb treatment increases CNS inflammation during EAE. Spinal cord tissues from mice, receiving either saline, $PLP_{139-151}$, or $PLP_{139-151}$ + anti-GITR mAb (500 $\mu$g/injection on days 0, 2, 4, and 6 postimmunization), were examined on days 15–20 post-disease induction for the presence of $CD4^+$, $CD8^+$, and $B220^+$ cells (red). Tissues were also counterstained with 4′,6′-diamidino-2-phenylindole (blue). No positive staining was observed in isotype-matched control samples (data not shown); ×100 magnification.

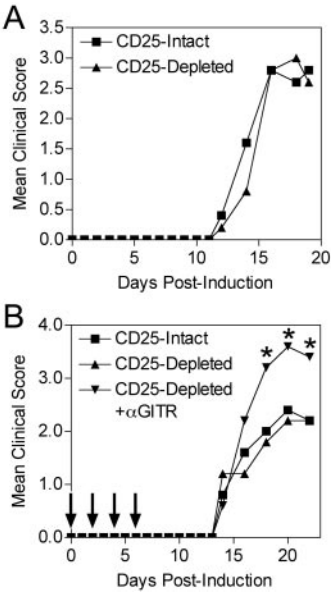


**FIGURE 3.** $T_R$ cell depletion fails to influence EAE disease progression. *A*, SJL mice were depleted of $T_R$ cells by anti-CD25 mAb injection (500 $\mu$g/injection i.p.) on days −4 and −2 before EAE induction by s.c. injection of $PLP_{139-151}$/CFA. Data are presented as the mean clinical disease score and are representative of three separate experiments. *B*, SJL mice were depleted of $T_R$ cells by anti-CD25 mAb injection (500 $\mu$g/injection i.p.) on days −4 and −2 before EAE induction by s.c. injection of $PLP_{139-151}$/CFA. Mice received four injections of anti-GITR mAb (500 $\mu$g/injection) on alternating days and were followed for clinical disease.

$CD4^+CD25^-$ T cells were also stimulated in the presence of $F(ab')_2$ of the anti-CD3 Ab (CD3-$F(ab')_2$) which blocks TCR-induced activation of $CD4^+$ T cells.[4] As expected, CD3-$F(ab')_2$ blocked $CD4^+$ T cell activation as determined by CD25, CD54, CD62L, and CD69 expression (data not shown), as well as activation-induced GITR expression (Fig. 4*C*), indicating that GITR is a phenotypic marker of $CD4^+$ T cell activation.

Because both activated $CD4^+$ T cells and $T_R$ cells expressed GITR, the possibility existed that anti-GITR mAb treatment may also directly influence $CD4^+$ effector T cell function. To test this, we examined the effects of Ab-mediated cross-linking of GITR on $CD4^+$ T cell proliferation in the absence of $T_R$ cells. Activation of $T_R$ cell-depleted LN (Fig. 4*D*) or spleen (data not shown) cells in the presence of anti-GITR mAb increased the level of anti-CD3-induced T cell proliferation in a concentration-dependent manner. Interestingly, anti-GITR mAb treatment produced similar findings when added to $T_R$ cell-depleted cultures of naive $OVA_{323-339}$-specific Tg T cells (DO11.10) activated with suboptimal (0.1 $\mu$g/ml), but not optimal, levels of Ag (1 $\mu$g/ml) (Fig. 4*E*). Of direct relevance to the observed effects in our EAE model system, anti-GITR mAb treatment also enhanced the proliferation of $T_R$ cell-depleted naive $PLP_{139-151}$-specific TCR Tg $CD4^+$ T cells in a concentration-dependent manner (Fig. 4*F*) suggesting that anti-GITR mAb treatment can enhance the effector function of both naive and previously activated $CD4^+$ T cells (Fig. 1*D*) in the absence of $CD4^+CD25^+$ $T_R$ cells. Lastly, anti-GITR mAb treatment of

[4] A. Kohm, J. Williams, A. Bickford, L. Chatenoud, J.-F. Bach, and S. Miller. Treatment with non-mitogenic anti-CD3 mAb induces $CD4^+$ T cell tolerance and functional reversal of established autoimmune disease. *Submitted for publication.*

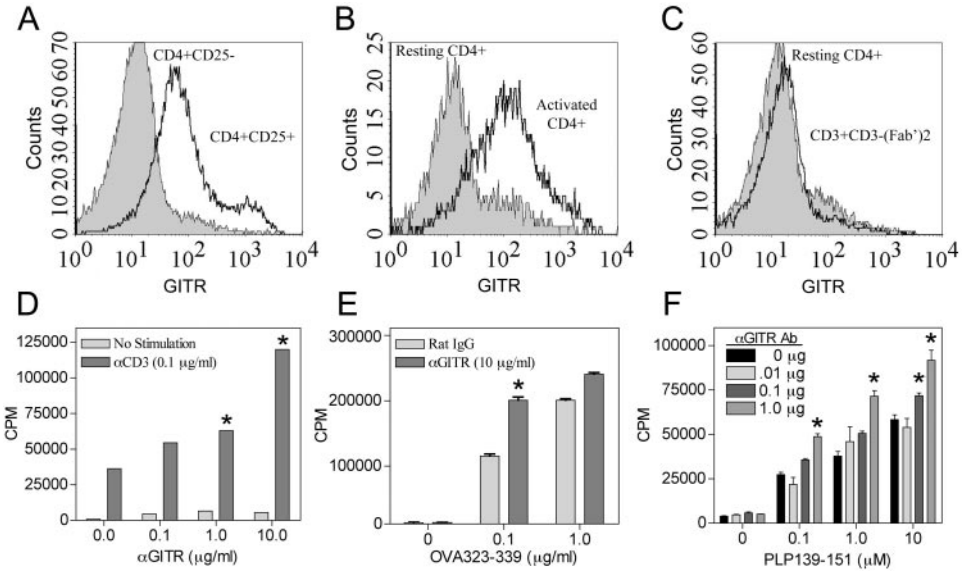


**FIGURE 4.** Expression and functional role for GITR in $CD4^+CD25^-$ effector T cells. *A–C*, GITR Expression on $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. *A–C*, LN cells isolated from naive mice were stained with Abs specific for CD4, CD25, CD62L, and GITR. Some cells were either activated with (*B*) anti-CD3 Ab (1 $\mu$g/$10^6$ cells) or (*C*) anti-CD3 Ab + CD3-F(ab′)$_2$ (1 $\mu$g/$10^6$ cells) for 24 h before immunofluorescence staining. Data are presented as the relative fluorescence intensity of GITR cells. All data are representative of three separate experiments. *D–F*, Effect of anti-GITR mAb ligation on $CD4^+CD25^-$ T cell proliferation. *D*, Naive SJL LN cells were depleted of $CD4^+CD25^+$ $T_R$ cells before activation with anti-CD3 mAb (0.1 $\mu$g/$10^6$ cells) in the presence of an increasing concentration of anti-GITR mAb. *E*, Naive SJL LN cells were depleted of $CD4^+CD25^+$ $T_R$ cells before activation with increasing concentrations of anti-CD3 mAb in the presence of anti-GITR mAb (1 $\mu$g/$10^6$ cells). *F*, LN cells isolated from naive 5B6 $PLP_{139-151}$ TCR transgenic mice were depleted of $CD4^+CD25^+$ $T_R$ cells and activated with an increasing concentration of $PLP_{139-151}$ in the presence of an increasing concentration of anti-GITR mAb. All data are presented as the mean cpm and are representative of three separate experiments.

$T_R$ cell-depleted $OVA_{323-339}$-specific T cells also enhanced the level of cellular activation as determined by CD54, CD62L, and CD69 expression in a concentration-dependent manner (Fig. 5), but failed to influence CD25 expression.

## Discussion

Consistent with previous reports showing that GITR is predominantly expressed on $CD4^+CD25^+$ $T_R$ cells and that anti-GITR inactivates $T_R$ cell functionality (4, 5), we found that treatment of SJL mice with anti-GITR mAb significantly exacerbated $PLP_{139-151}$-induced clinical EAE, CNS inflammation, and peptide-specific T cell responses (Figs. 1 and 2). However, our present findings also demonstrate that GITR is rapidly up-regulated on naive $CD4^+CD25^-$ T cells following either mitogen- or Ag-induced T cell activation and that anti-GITR enhanced the level of $CD4^+$ T cell activation, proliferation, and cytokine production in the absence of $T_R$ cells in vitro (Figs. 4 and 5). The expression pattern of GITR is thus similar to that of CD25 which is also preferentially expressed at higher levels on resting $T_R$ cells, but quickly up-regulated on naive $CD4^+$ T cells upon TCR activation, raising the possibility that CD25 and GITR expression may be regulated by similar mechanisms. Thus, it may be more appropriate to consider GITR expression as a marker of T cell activation rather than defining the $T_R$ population.

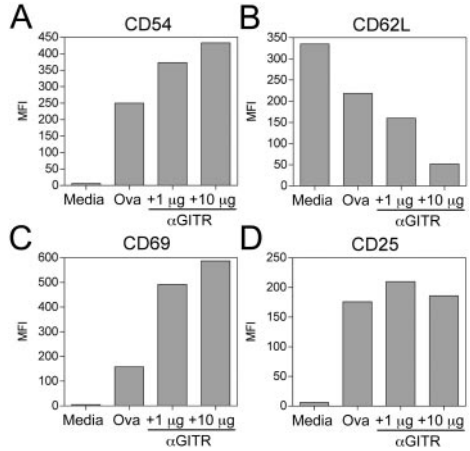


**FIGURE 5.** Anti-GITR mAb treatment enhances $CD4^+CD25^-$ T cell activation. LN cells were isolated from naive DO11.10 TCR transgenic mice and depleted of $CD4^+CD25^+$ $T_R$ cells before activation in the presence of $OVA_{323-339}$ (10 $\mu$M) and an increasing concentration of anti-GITR mAb for 24 h before determination of the level of CD54, CD62L, CD69, and CD25 surface expression by flow cytometry. Data are presented as the relative fluorescence intensity of each activation marker on $CD4^+$ T cells; MFI, mean fluorescence intensity.

We previously showed that $T_R$ cell supplementation blocked the activation of both naive and previously activated autoreactive $CD4^+$ T cells and protected against the initiation and progression of EAE (8). However, the question remains as to why anti-GITR-mediated $T_R$ inactivation (Fig. 1), but not anti-CD25-mediated $T_R$ cell depletion, led to enhanced acute clinical EAE (Fig. 3*A*). One possible explanation may be that the anti-CD25 depletion protocol used in the current studies simply down-regulated the level of CD25 expression on $T_R$ cells rather than deplete this population. However, in vitro studies using the same depleting and detecting Ab clones did not result in significant down-regulation of CD25 expression on $CD4^+$ T cells (data not shown). Regardless, the possibility exists that distinct mechanisms may function in vivo and current experiments are closely examining the fate of $CD4^+CD25^+$ $T_R$ cells

following anti-CD25 mAb injection. An alternative explanation for the failure of $T_R$ cell depletion to influence the clinical disease progression in EAE may be the existence of redundant compensatory regulatory mechanisms in vivo including other regulatory cell populations. However, further phenotypic and functional characterization of these alternative regulatory populations is necessary to determine their contribution to immune homeostasis during EAE.

Of particular interest is our finding that ligation of GITR by anti-GITR mAb enhanced $CD4^+$ T cell activation, proliferation, and cytokine production in the absence of $T_R$ cells both in vivo (Fig. 3*B*) and in vitro (Fig. 4). Moreover, ligation of GITR appears to optimally augment $CD4^+$ T cell activation in conditions of suboptimal TCR ligation. Therefore, in addition to previously reported effects of anti-GITR on inactivating $T_R$ cell activity (4, 5), GITR ligation appears to directly enhance $CD4^+$ T cell effector function by delivering a costimulatory signal thus lowering the signaling threshold of the TCR complex. Collectively, our findings suggest a dual functional role for GITR wherein GITR ligation both inactivates $T_R$ cells and, at the same time, increases the activation and effector functions of $CD4^+CD25^-$ T cells, thus resulting in enhanced T cell-mediated immunity.

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