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### Imbalanced immunity in multiple sclerosis

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# ***Balanced immunity by GITR-GITRL***

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GITR triggering induces expansion of both effector and regulatory CD4<sup>+</sup> T cells *in vivo*

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*Manuscript submitted*



## **Abstract**

*Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is expressed on activated and regulatory T cells, but its role on these functionally opposing cell types is not fully understood. Here we describe that transgenic expression of GITR's unique ligand (GITRL) induces a prominent increase of both effector and regulatory CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells. Regulatory T cells from GITRL-transgenic mice are phenotypically activated and retain their suppressive capacity. The accumulation of effector and regulatory T cells is not due to enhanced differentiation of naïve T cells, but a direct result of increased proliferation. Functional consequences of increased numbers of both regulatory and effector T cells were tested in an autoimmune model and show that GITR stimulation is protective, as it significantly delays disease induction. These data indicate that GITR regulates the balance between regulatory and effector CD4<sup>+</sup> T cells by enhancing proliferation of both populations in parallel.*

## Introduction

Members of the TNF receptor (TNFR) superfamily are able to directly and indirectly affect the course of an immune response.<sup>194</sup> The glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a member of this family and has been implicated in regulating both innate and adaptive immune responses.<sup>195,196</sup> Regulatory T cells are well-known for their expression of GITR, though this receptor is also expressed on activated non-regulatory T cells, as well as B cells, monocytes and macrophages, dendritic cells and mast cells.<sup>196-200</sup> Its ligand (GITRL) can be found on a variety of cells, including dendritic cells, macrophages and B cells.<sup>200-202</sup> GITRL is transiently up-regulated on these antigen presenting cells upon stimulation via the transcription factor NF- $\kappa$ B<sup>203</sup> and it most likely exerts its main function during inflammatory responses. Indeed, agonistic antibodies and (cells expressing) recombinant GITRL enhance T cell proliferation *in vitro* upon TCR triggering,<sup>198,204-207</sup> which suggests that GITR acts as a costimulatory factor for T cells. GITR stimulation on regulatory T cells in coculture with effector T cells has been suggested to neutralize the suppressive capacity of regulatory T cells.<sup>198,208,209</sup> However, it was later shown that GITR ligation on regulatory T cells does not directly affect their suppressive capacity, but that GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells.<sup>210</sup> GITR is not essential for regulatory T cell function, as regulatory T cells from GITR<sup>-/-</sup> mice display a normal capacity to suppress T cell proliferation *in vitro*.<sup>211,212</sup> This leaves unanswered what the function of GITR is on regulatory T cells.

Studies using GITR<sup>-/-</sup> mice showed that the absence of GITR was protective in several disease models, which was attributed to an impaired effector function of T cells.<sup>213-215</sup> Correspondingly, studies that have directly addressed the function of GITR on T cells *in vivo* by deliberate stimulation of the receptor with agonistic antibodies conclude that GITR has a pro-inflammatory role within the immune system through its costimulatory effects on T cells.<sup>200,216-218</sup> However, these antibodies have their limitations when studying the impact of GITR stimulation in complex disease models, in particular since anti-GITR antibodies have been reported to cause depletion of regulatory T cells.<sup>200</sup> Moreover, recent studies on the crystal structure of GITRL have revealed that this ligand can exist in multiple oligomerization states that depend on binding to the receptor,<sup>219,220</sup> and it therefore remains to be addressed whether crosslinking GITR with agonistic antibodies exerts the same downstream effects as signaling induced by membrane-bound GITRL. Thus, in order to properly address the consequence of direct GITR stimulation on T cell function *in vivo*, we generated transgenic (TG) mice in which GITRL is constitutively expressed on

B cells. Our findings demonstrate that GITR stimulation *in vivo* very effectively increases the absolute number of both effector and regulatory CD4<sup>+</sup> T cells through enhanced proliferation of both cell types. In agreement with increased regulatory T cell numbers, GITRL TG mice showed a marked delay in disease onset upon induction of experimental autoimmune encephalomyelitis (EAE), an experimental model for multiple sclerosis. We propose that GITR plays an important role in the regulation of both regulatory and effector CD4<sup>+</sup> T cell numbers *in vivo* by enhancing their turnover.

## Methods

### **Generation of GITRL TG mice**

cDNA encoding murine GITRL was obtained via PCR on total splenic cDNA and cloned into the pGEM-T plasmid (PROMEGA). This construct was digested with NotI and XhoI to obtain a 600 bp fragment containing the mGITRL cDNA, which was cloned into the NotI – XhoI site of a CD19-pC3 plasmid (kindly provided by Patrick Derksen, Academic Medical Center, The Netherlands), resulting in the GITRL expression construct under control of the human CD19 promoter. This construct was linearized via AatII digestion (see Fig. 1A) and microinjected into pronuclei of C57BL/6 fertilized oocytes and implanted into pseudopregnant female C57BL/6 mice. Transgenic (TG) founders were identified by PCR analysis of tail or ear DNA, using the following PCR primers: pC3s1 (5'-GCAGTGACTCTCTTAAGGTAGCC-3') and mGITRL4a (5'CTTGAGTGAAGTATAGATCAGTGTA-3'). Three GITRL TG founder lines (RW14, RW18, RW20) were propagated by mating with wild type C57BL/6 (WT) mice and offspring was tested for the presence of the transgene by PCR analysis of tail or ear DNA with the same primers.

### **Mice**

GITRL TG mice were maintained on a C57BL/6 background and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions. Mice were used at 6-24 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines. For measurement of *in vivo* T cell proliferation, mice were injected i.p. with 1 mg (+)-5-Bromo-2'-deoxyuridine, (BrdU, Aldrich) and sacrificed for analysis 16 hours later.

### **Cell staining and flow cytometry**

Single-cell suspensions were obtained by mincing the specified organs through 40  $\mu\text{m}$  cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe System). Cells ( $5 \times 10^5$  -  $5 \times 10^6$ ) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 minutes at 4°C with antibodies in the presence of anti-CD16-CD32 (clone 2.4G2). The following fluorescently or biotin-labeled monoclonal antibodies (and clone names) were obtained from Pharmingen: anti-B220 (RA3-6B2), anti-CD3 $\epsilon$  (145-2C11), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-CD62L (clone MEL-14), anti-CD69 (H1.2F3) or from eBioscience: anti-GITRL (ebioYGL386), anti-FoxP3 (NRRF-30), anti-GITR (DTA-1), anti-CD44 (IM7), anti-CTLA4 (4C10-4B9), anti-PD1 (RMP1-30), anti-CD134 (OX86), anti-CD27 (LG.7F9), anti-CD103 (M290), anti-CD45.1 (104 or A20). PE-conjugated anti-CD25 was obtained from Miltenyi Biotec. For the detection of biotinylated antibodies, streptavidin-PE (Caltag Laboratories, CA), streptavidin-APC (Pharmingen) or streptavidin-conjugated PerCP-Cy5.5 (Pharmingen) was used. Intracellular stainings for FoxP3 and/or BrdU were performed using FoxP3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufacturer's protocol. For BrdU staining, FITC-conjugated anti-BrdU/Dnase (Becton Dickinson) was added during the FoxP3 staining step and stained for 25 min at RT. Data were collected on a FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed using FlowJo software (Treestar).

### **Intracellular cytokine staining**

To determine direct *ex vivo* cytokine production, splenocytes were plated at  $1 \times 10^6$  cells/well in a 96-well round-bottom plate and stimulated with 1 ng/ml PMA and 1  $\mu\text{M}$  ionomycin. After 2 hours incubation at 37°C, 1  $\mu\text{g/ml}$  of the protein-secretion inhibitor Brefeldin A was added (Sigma) and cells were cultured for another 4 hours. Hereafter, cells were washed and stained for CD4 and CD8, followed by fixation and permeabilization (Becton Dickinson). Cells were then incubated for 30 min with fluorescently labelled antibodies against IFN- $\gamma$  or IL-2 (eBioscience), thoroughly washed and analyzed by flow cytometry.

### **T cell proliferation assay**

To analyze the effect of GITR engagement on the proliferative capacity of WT responder cells, T cells were enriched from spleens of WT mice by negative selection using CD19<sup>+</sup> beads (Miltenyi Biotec). T cell enriched splenocytes were labeled with 0.25  $\mu\text{M}$  carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37°C for 10 minutes

and stimulated with 100 ng/ml anti-CD3 (clone 145-2C11) for 3 days in the presence of irradiated (10 Gray) WT or GITRL TG B cells with or without 200 U/ml IL-2. B cells were isolated by positive selection using CD19<sup>+</sup> beads (Miltenyi Biotec). For the analysis of the expression of CD25 and CD69, non-CFSE-labeled enriched WT T cells were used, stimulated similarly and analyzed after 1 day. To determine the effects of GITR ligation on IL-2 production *in vitro*, T cell enriched splenocytes were stimulated as described above, in the presence of 10 µg/ml blocking anti-CD25 antibody (clone PC61) to prevent IL-2 consumption. Culture supernatant was harvested after 1 day of stimulation and frozen at -20°C. The IL-2 ELISA (Becton Dickinson) was performed according to instructions from the manufacturer.

### **Regulatory T cell assay**

Splenic CD4<sup>+</sup>CD25<sup>-</sup> (responder cells) and CD4<sup>+</sup>CD25<sup>hi</sup> (regulatory cells) T cells were isolated by cell sorting using a FACSAria (Becton Dickinson) and purity of sorted populations was consistently >96%. Responder cells were mixed with regulatory T cells at different ratios in 96-well tissue culture plates. The cells were stimulated with 10 µg/ml soluble anti-CD3 (clone 145-2C11) plus irradiated (10 Gray) WT splenocytes (APCs) at 37°C for 72 hours. Hereafter, cells were pulsed for 16 hours with 1 µCi 3H-TdR ([Methyl-<sup>3</sup>H]Thymidine, Amersham Pharmacia)/well, and incorporation of 3H-TdR was determined using a Beta Plate scintillation counter (Wallac, 1450 microbeta Plus Liquid Scintillation counter). Data are presented as percentage proliferation compared to maximum responder cell proliferation of triplicate assays.

### **MLPA**

RNA isolation and MLPA analysis was performed as previously described with the murine apoptosis kit (RM002, MRC-HOLLAND) in collaboration with MRC-HOLLAND.<sup>221</sup>

### **Adoptive transfer of naïve T cells into WT and GITRL TG mice**

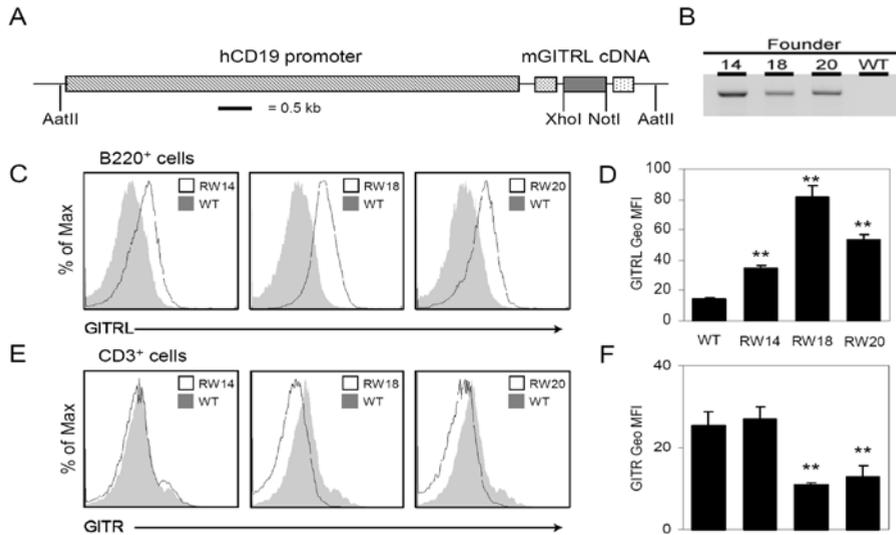
For adoptive transfers, naïve (CD25<sup>-</sup>) CD4<sup>+</sup> T cells were purified from spleens and peripheral lymph nodes of Ly5.1 mice by negative selection using the CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell isolation kit (Miltenyi Biotec). Purified CD4<sup>+</sup>CD25<sup>-</sup> cells (purity >90%) were labeled with 0.25 µM carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37°C for 10 min and injected after washing ( $\pm 1 \times 10^6$  in 200 µl PBS) i.v. into WT and GITRL TG recipient mice. Distribution and phenotype of transferred cells was analyzed 3 days later by flow cytometry.

**EAE induction**

EAE was induced by s.c. immunization of mice in the hind flanks using 50 µg of MOG<sub>35-55</sub> peptide in CFA containing 1 mg/ml heat-inactivated Mycobacterium tuberculosis (Difco) on day 0. Mice also received 200 ng of pertussis toxin (Sigma) i.v. on days 0 and 2. Disease severity was assessed according to the following scale: 0, no disease; 1, flaccid tail; 2, loss of hind leg spreading reflex; 3, hind limb weakness; 4, unilateral hind limb paralysis; 5, bilateral hind limb paralysis; 6, abdominal paralysis; 7, moribund; 8, dead. All mice were sacrificed 14 days following EAE induction after which brain and spinal cord was frozen in Tissue-Tek (Sakura Finetek, The Netherlands) at -80°C for immunohistochemical analysis.

**Immunohistochemistry**

Cryostat sections (8 µm) of spinal cord and brain of 4 WT and 4 TG mice were fixed in acetone, containing 1% H<sub>2</sub>O<sub>2</sub> for 10 minutes. Then, sections were incubated with monoclonal rat anti-mouse antibodies to CD68 (a kind gift from Siamon Gordon, Oxford, UK, clone FE-11), CD4 and FoxP3 (eBioscience), diluted in PBS with 8% bovine serum albumine (BSA), 10% normal mouse serum (NMS) and 0.05% NaN<sub>3</sub> for 1



**Fig. 1** Generation of B cell specific GITRL TG mice. (A) Schematic representation of the hCD19-mGITRL DNA construct. The human CD19 promoter (hatched box) is followed by a chimeric intron (white box), mGITRL cDNA (dotted box) and a poly A tail (black box). (B) PCR analysis of genomic tail DNA from WT or GITRL TG mice (founder lines RW14, 18 and 20). (C) Representative staining for GITRL on splenic B220<sup>+</sup> cells from WT, RW14, RW18 and RW20 mice and (D) expression as the average geometric mean fluorescence intensity (geoMFI) ± SD for 3 mice per group. (E) Representative staining for GITR expression on splenic CD3<sup>+</sup> cells from WT, RW14, RW18 and RW20 mice and (F) expression as the average geoMFI ± SD. Asterisks denote significant differences (\*\* p < 0.005).

hour at 4°C. After washing in PBS, the sections were incubated with anti-rat HRP diluted in PBS/8% BSA/10% NMS/0.05% NaN<sub>3</sub>/350 mM NaCl. Staining was visualized with DAB (Sigma Laboratories, St. Louis, MO, USA) applied for 10 minutes. Sections were counterstained with haematoxylin for 30 seconds, dehydrated and mounted in entellan (Merck, Darmstadt, Germany). As negative controls, primary antibodies were either omitted or substituted with an isotype control antibody. No immunoreactivity was seen for all negative controls.

### **Statistical analysis**

Statistical analysis of the data was performed using the unpaired Student's t-test or Wilcoxon rank-sum test where mentioned. For the EAE experiments, effect on mean clinical score was assessed by calculating the area under the curve using the trapezoidal rule, followed by the Wilcoxon rank-sum test. Differences in cumulative incidence were analyzed on a per day basis, using a  $\chi^2$  test of a contingency table.

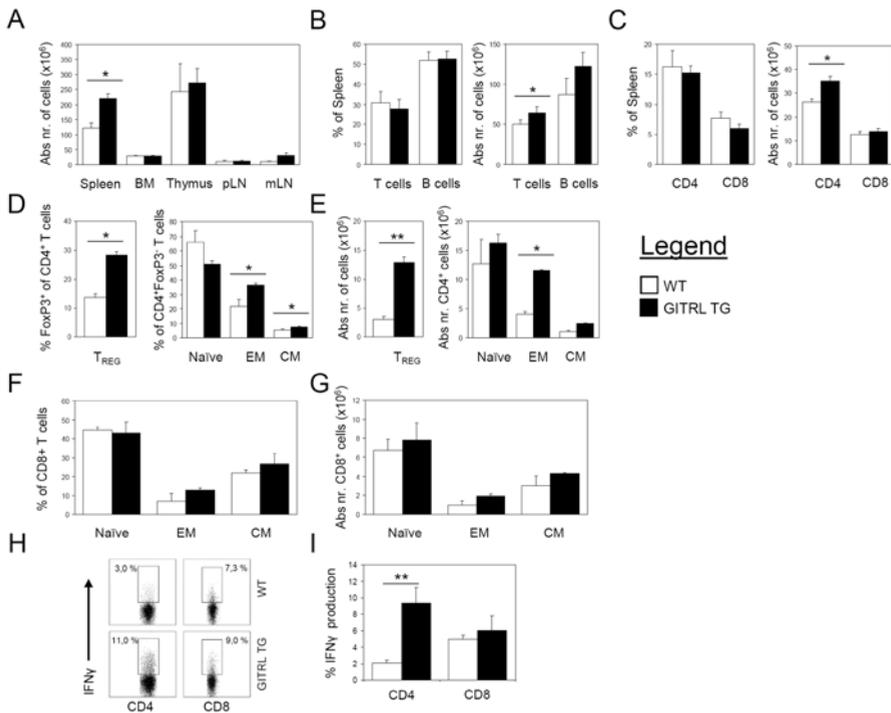
## **Results**

### **Generation of B cell specific GITRL TG mice**

To study the function of GITR on T cells *in vivo*, we generated B cell specific GITRL TG mice by expressing GITRL cDNA under control of the human CD19 promoter (Fig. 1A). Through microinjection of fertilized oocytes, we acquired three founder lines (RW14, RW18 and RW20), which were identified by genomic PCR analysis (Fig. 1B). GITRL TG mice were fertile, born at expected Mendelian frequencies and appeared as healthy as their littermate controls. Flow cytometry showed that GITRL was indeed significantly expressed on B cells in all founder lines, with highest expression on the RW18 line (Fig. 1C-D). As receptor downregulation upon ligand stimulation is a hallmark for TNFR-superfamily members, we determined the expression of GITR on T cells. T cells from GITRL TG mice showed decreased GITR expression compared to WT mice, which correlated with the level of GITRL expression (Fig. 1E-F). This indicates that GITR is indeed functionally engaged by its ligand in these mice. The data shown below are obtained from experiments with the GITRL TG RW18 line, though a similar, but less pronounced phenotype was also found in the other founder lines (data not shown).

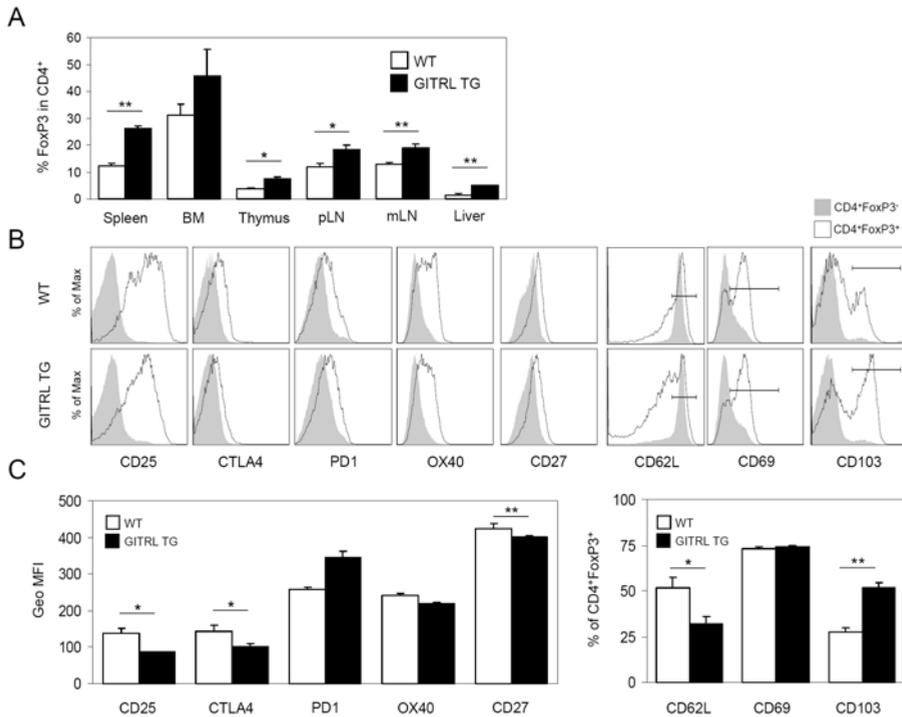
### **GITRL TG mice have more CD4<sup>+</sup> effector memory-like and regulatory type T cells**

To establish the effects of GITR triggering *in vivo*, we analyzed the primary and secondary lymphoid organs of GITRL TG mice. T cell differentiation in the thymus



**Fig. 2** GITRL TG mice have more effector and regulatory type CD4<sup>+</sup> T cells. (A) Absolute number of cells in spleen, bone marrow (BM), thymus, peripheral (pLN) and mesenteric (mLN) lymph nodes in 4-8 weeks old WT (white bar) and GITRL TG (black bar) mice. Percentage and absolute number of (B) T and B cells or (C) CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen of WT and GITRL TG mice. (D, E) Percentage and absolute number of splenic regulatory (FoxP3<sup>+</sup>) and non-regulatory (FoxP3<sup>-</sup>) CD4<sup>+</sup> T cells in WT and GITRL TG mice. Non-regulatory CD4<sup>+</sup> T cells were subdivided in naïve (CD44<sup>+</sup>CD62L<sup>+</sup>), effector memory (EM) (CD44<sup>+</sup>CD62L<sup>-</sup>) and central memory (CM) (CD44<sup>+</sup>CD62L<sup>+</sup>) cells. (F, G) Percentage and absolute number of naïve, EM and CM cells of splenic CD8<sup>+</sup> T cells. Production of IL-2 (H) and IFN- $\gamma$  (I) by CD4<sup>+</sup> or CD8<sup>+</sup> T cells in WT and GITRL TG mice after stimulation with PMA/ionomycin. Asterisks denote significant differences (\*  $p < 0.05$ ; \*\*  $p < 0.005$ ). Data represent the average value  $\pm$  SD of 3-5 mice and are representative for 2-4 independent experiments.

of these mice was comparable to WT littermates (data not shown), as was cellularity of bone marrow, thymus, peripheral and mesenteric lymph nodes (Fig. 2A). However, spleens of GITRL TG mice contained significantly more leukocytes than WT mice (Fig. 2A). This increase was due to elevated numbers of CD4<sup>+</sup> T cells (Fig. 2B-C), whereas numbers of B cells and CD8<sup>+</sup> T cells were not significantly altered (Fig. 2B-C). Analysis of FoxP3 expression indicated that a substantial part of this increase in CD4<sup>+</sup> T cells could be attributed to an enlarged regulatory T cell compartment, as up to three times more CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells were present in the spleens of GITRL TG mice (Fig. 2D-E). Phenotypic analysis of FoxP3<sup>-</sup> CD4<sup>+</sup> T cells indicated that the non-regulatory fraction was also affected in GITRL TG mice, as



**Fig. 3** Systemic increase of regulatory T cells via GITR signaling. (A) The percentage of FoxP3<sup>+</sup> cells within the CD4<sup>+</sup> T cell compartment in spleen, bone marrow (BM), thymus, peripheral (pLN) and mesenteric (mLN) lymph nodes and liver in WT and GITRL TG mice. (B) Phenotypic analysis of splenic FoxP3<sup>+</sup> (filled graph) and FoxP3<sup>-</sup> (open graph) CD4<sup>+</sup> T cells for surface expression of CD25, CTLA4, PD1, OX40, CD27, CD62L, CD69 and CD103 in WT and GITRL TG mice. (C) Average intensity (geoMFI  $\pm$  SD) or percentage positive cells (%  $\pm$  SD) of these molecules on FoxP3<sup>+</sup>CD4<sup>+</sup> T cells. Asterisks denote significant differences (\*  $p < 0.05$ , \*\*  $p < 0.005$ ). Data are representative for 2 independent experiments with each at least 3 mice per group.

significantly more CD4<sup>+</sup> T cells with an effector memory-like (CD44<sup>+</sup>CD62L<sup>+</sup>) and central memory-like (CD44<sup>+</sup>CD62L<sup>+</sup>) phenotype were identified (Fig. 2D-E). This increase of CD4<sup>+</sup> T cells with either a regulatory or a memory-like phenotype in GITRL TG mice apparently did not develop at the cost of the naïve CD4<sup>+</sup> population, since absolute numbers of naïve CD4<sup>+</sup> T cells were comparable with WT littermates (Fig. 2E). No differences were found for CD8<sup>+</sup> T cells with respect to their naïve, effector memory-like and central memory phenotype (Fig. 2F-G). Corroborating the specific increase in CD4<sup>+</sup> effector T cells in GITRL TG mice, splenocyte stimulation with PMA-ionomycin showed increased production of the effector cytokines IL-2 (Fig. 2H) and IFN $\gamma$  (Fig. 2 I-J) by CD4<sup>+</sup>, but not CD8<sup>+</sup> T cells. These data thus indicate that GITR triggering *in vivo* enhanced the number of both regulatory and effector CD4<sup>+</sup> T cells.

**Distribution and activation status of regulatory T cells in GITRL TG mice**

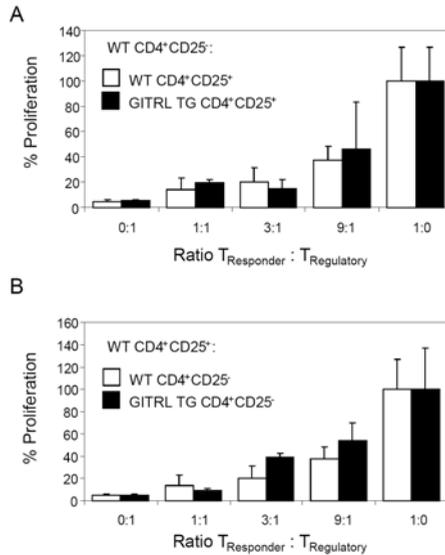
To determine whether the strong increase of regulatory T cells in GITRL TG mice was restricted to the spleen, we analyzed the presence of these cells in bone marrow, thymus, peripheral and mesenteric lymph nodes and liver in these mice. We found that GITRL TG mice have a systemic increase in regulatory T cell numbers, as all analyzed compartments, except for the bone marrow, showed a significantly higher fraction of FoxP3<sup>+</sup> CD4<sup>+</sup> cells compared to WT mice (Fig 3A).

Next, we analyzed the activation status of splenic regulatory and non-regulatory T cells. Apart from the described changes in CD44 and CD62L expression (see Fig. 2D-E), non-regulatory CD4<sup>+</sup> T cells in GITRL TG mice were comparable with their WT counterparts on the basis of several other costimulatory and activation molecules (Fig 3B). On the other hand, we found that CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells from GITRL TG mice were quite distinct from their counterparts in WT mice, as they expressed lower levels of CD25, CD62L and CTLA-4 (Fig 3B-C). In addition, the expression of PD-1 was increased in GITRL TG mice, while a large fraction of regulatory T cells from GITRL TG mice expressed the adhesion molecule CD103 (αE integrin) on their surface (Fig 3B-C). The expression of OX40, CD27 and CD69 was comparable to WT mice (Fig 3B-C). Based on these characteristics, in particular the decreased levels of CD62L and the high percentage of CD103<sup>+</sup> cells, we conclude that constitutive GITR stimulation not only leads to more regulatory T cells, but also stimulates their differentiation towards an effector phenotype.<sup>222</sup>

**GITR engagement *in vivo* does not affect the suppressive capacity of regulatory T cells**

To determine if the altered phenotype of regulatory T cells in GITRL TG mice mirrored a change in their function, we performed *in vitro* proliferation assays, in which WT responder T cells (CD4<sup>+</sup>CD25<sup>-</sup>) were stimulated with anti-CD3/CD28 in the presence of increasing numbers of regulatory T cells (CD4<sup>+</sup>CD25<sup>+</sup>) from WT or GITRL TG mice. From these experiments it can be concluded that regulatory T cells from GITRL TG are fully capable to suppress responder T cell proliferation and were equally anergic as regulatory T cells from WT mice (Fig. 4A). This conclusion challenges previous reports, which have suggested that GITR stimulation on regulatory T cells is sufficient to abrogate their suppressive capacity.<sup>198,223</sup>

We also analyzed the susceptibility of GITRL TG vs WT derived responder T cells to the suppressive capacity of WT regulatory T cells, as it has been reported that GITR stimulation allows T cells to escape suppression by regulatory T cells.<sup>210</sup> These experiments revealed that responder T cells from GITRL TG mice could still be adequately suppressed by regulatory T cells (Fig. 4B), thereby indicating that chronic GITR stimulation *in vivo* is not sufficient to induce an enduring state of insensitivity

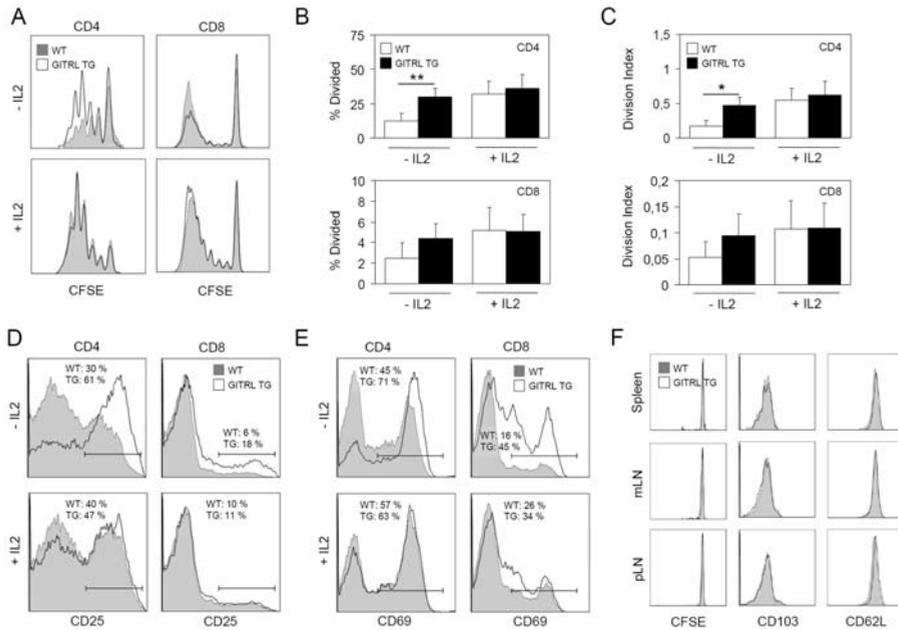


**Fig. 4** Regulatory T cell function of WT and GITRL TG mice. (A) Ability of purified WT and GITRL TG regulatory ( $CD4^+CD25^+$ ) T cells to suppress WT responder ( $CD4^+CD25^-$ ) T cells. (B) Ability of WT regulatory T cells to suppress proliferation of WT and GITRL TG responder T cells. Cells were cultured at different ratios for 4 days with  $10 \mu\text{g/ml}$  soluble anti-CD3 mAb in the presence of irradiated WT splenocytes as antigen presenting cells; for the final 16 hours  $[^3\text{H}]$ thymidine was added and incorporation was measured. Data are depicted as the percentage proliferation compared to responder T cells alone (average of triplicate wells  $\pm$  SD) and are representative of 2 independent experiments.

to regulatory T cell activity. Instead, it rather suggests that this previously described capacity of GITR is only effective when given together with TCR stimulation.<sup>210</sup>

### ***GITR engagement does not enhance the survival profile of T cells***

To investigate whether the increase in effector memory-like and regulatory type T cells could be explained by an increased survival potential mediated through GITR signaling, we examined the expression profile of approximately 40 pro- and anti-apoptotic proteins using an advanced PCR approach called multiplex ligation-dependent amplification (MLPA).<sup>221</sup> Naïve ( $CD25^-CD62L^+CD44^-$ ), effector ( $CD25^-CD62L^-CD44^+$ ) and regulatory ( $CD25^{\text{hi}}$ ) type  $CD4^+$  T cells from WT and GITRL TG mice were purified by cell sorting and their mRNA was isolated and subjected to MLPA analysis. These data revealed no obvious differences in the apoptotic gene expression profile of naïve, effector and regulatory type T cells for WT and GITRL TG mice (Supplemental data, Table 1). The only exception was the expression of granzyme B, which was significantly increased in effector  $CD4^+$  T cells of GITRL TG compared to WT mice. This finding is indicative for enhanced effector T cell formation in GITRL



**Fig. 5** GTR costimulation *in vitro* enhances CD4<sup>+</sup> T cell proliferation and IL-2 production. (A) Anti-CD3-induced proliferation of CFSE-labeled WT T cells cultured for 3 days in the presence of WT (filled graph) or GITRL TG (open graph) B cells with or without IL-2. Triplicate wells analyzed for (B) the average precursor frequency and (C) the average division index (i.e. the number of divisions that the dividing population underwent). (D) IL-2 concentration in supernatants (average of triplicate wells  $\pm$  SD) after stimulating WT T cells as in (A) for 24 hours in the presence of a blocking antibody against CD25 to prevent IL-2 consumption. Expression of CD25 (E) and CD69 (F) on CD4<sup>+</sup> T cells stimulated as in (A) after 24 hours. (G) Naïve WT CD4<sup>+</sup>CD25<sup>-</sup> T cells from Ly5.1 mice were CFSE-labeled and injected intravenously in WT (filled graph) or GITRL TG (open graph) mice. Donor cells, gated on CD45.1<sup>+</sup>CD4<sup>+</sup> T cells, were analyzed 3 days after transfer for expression of CFSE, CD103 and CD62L expression. A representative staining from 3 mice per group is shown. Asterisks denote significant differences (\*  $p < 0.05$ , \*\*  $p < 0.005$ ).

TG mice and corresponds to the increased number of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells in these mice (Fig. 2I). Based on these data it is highly unlikely that basic changes in cell survival underlie the increase of effector and regulatory T cells in GITRL TG mice.

### ***GTR costimulation enhances CD4<sup>+</sup> T cell proliferation and IL-2 production***

Next, we set out to determine the effects of GTR triggering on T cell proliferation. CFSE-labeled T cells from WT mice were stimulated with suboptimal concentrations anti-CD3 in a 1:1 ratio with WT or GITRL TG irradiated B cells for a period of 3 days. We found that increased GITRL availability enhanced CD4<sup>+</sup> T cell proliferation, but did not affect CD8<sup>+</sup> T cell proliferation (Fig. 5A). The enhanced proliferation of CD4<sup>+</sup>

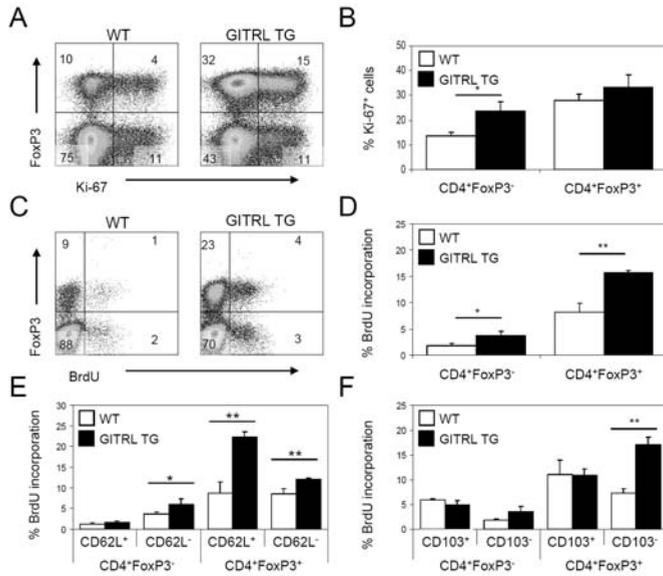
T cells via GITR engagement was no longer apparent when extra IL-2 was added to these cultures, indicating that GITR engagement affects the early proliferative capacity of CD4<sup>+</sup> T cells. We found that increased GITR ligation raised the percentage of CD4<sup>+</sup> T cells entering cell division (Fig. 5B), as well as the number of divisions that these cells underwent (Fig. 5C). Since the addition of IL-2 enhanced T cell proliferation to a similar extent as the addition of GITRL TG B cells, we questioned whether GITR stimulation induced IL-2 production. Indeed, when WT T cells were stimulated with anti-CD3, the addition of GITRL TG B cells induced almost 3-fold more IL-2 than WT B cells (Fig. 5D). Moreover, GITR ligation increased the expression of CD25 and CD69 on CD4<sup>+</sup> T cells, confirming the enhanced IL-2 production and increased activation induced by GITR stimulation (Fig. 5E-F).

To determine if constitutive GITR triggering alone was sufficient to induce activation and/or proliferation of naïve T cells *in vivo*, we isolated naïve non-regulatory CD4<sup>+</sup>CD25<sup>-</sup> T cells from Ly5.1<sup>+</sup> WT donor mice, labeled them with CFSE and transferred them into WT or GITRL TG (Ly5.2<sup>+</sup>) recipients. Three days after transfer, naïve T cells transferred to both WT and GITRL TG mice showed no CFSE dilution and did not alter their expression levels of CD62L or CD103 (Fig. 5G). Thus, despite the fact that GITRL TG mice contained more T cells with an effector memory-like phenotype, these data imply that stimulation through GITR alone is not sufficient to induce activation or proliferation of naïve T cells. Yet, when TCR-triggering is provided, GITR stimulation enhanced the production of IL-2 and increased proliferation of CD4<sup>+</sup> T cells *in vitro*.

### ***GITR engagement in vivo increases the proliferation of effector and regulatory CD4<sup>+</sup> T cells***

As GITR engagement could directly and specifically enhance CD4<sup>+</sup> T cell proliferation, we investigated the proliferative capacity of CD4<sup>+</sup> T cells in WT and GITRL TG mice *in vivo*. As measured by Ki-67 expression, GITRL TG mice had more non-regulatory T cells (CD4<sup>+</sup>FoxP3<sup>-</sup>) in cell cycle in the spleen than WT mice (24% ± 2.2 in GITRL TG mice vs 14% ± 1.2 in WT mice) (Fig 6A-B). The fraction of regulatory T cells (CD4<sup>+</sup>FoxP3<sup>+</sup>) that stained positive for Ki-67 was not significantly different between WT and GITRL TG mice, but the fraction of Ki-67<sup>+</sup> cells within the regulatory T cell compartment is already high (~30%) in WT mice (Fig. 6A-B).

To directly assess T cell proliferation *in vivo*, WT and GITRL TG mice were injected i.p. with BrdU and incorporation of this compound was analyzed 16 hours later (Fig. 6C-D). Within the non-regulatory CD4<sup>+</sup> T cell compartment, we found that GITRL TG mice contained more BrdU<sup>+</sup> cells than WT mice and this increase was most pronounced in the CD62L<sup>-</sup> effector fraction (Fig. 6C-E). A GITR-mediated increase in

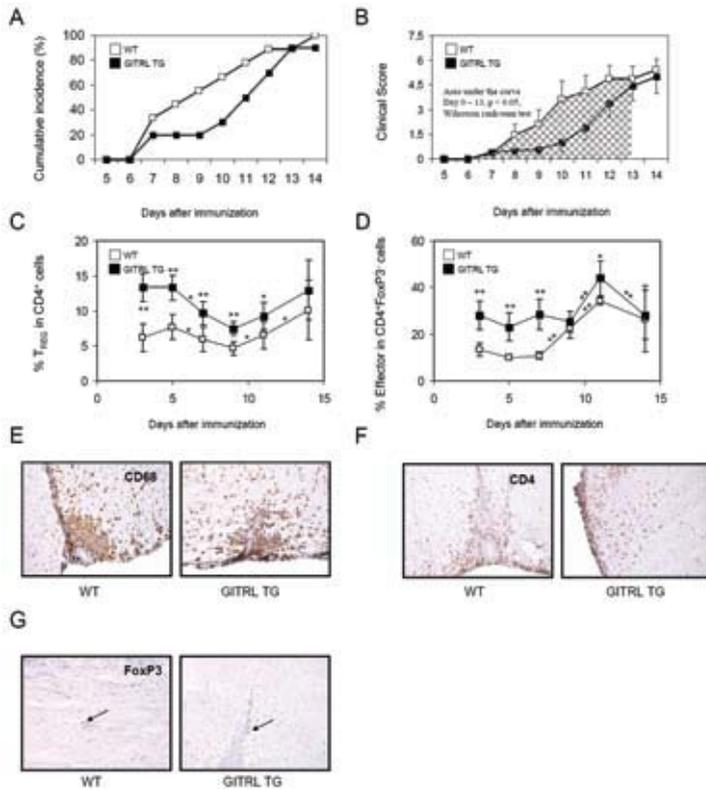


**Fig. 6** GITR triggering enhances proliferation of effector and regulatory T cells *in vivo*. (A) Representative intracellular staining for FoxP3 and Ki-67 on splenic CD4<sup>+</sup> T cells from WT and GITRL TG mice and (B) the percentage Ki-67<sup>+</sup> cells of FoxP3<sup>-</sup> and FoxP3<sup>+</sup> CD4<sup>+</sup> T cells (average  $\pm$  SD). (C) Representative intracellular staining for BrdU and FoxP3 on splenic CD4<sup>+</sup> T cells from WT and GITRL TG mice, 16 hours after i.p. injection of 1 mg BrdU. (D) The percentage BrdU<sup>+</sup> cells of FoxP3<sup>-</sup> and FoxP3<sup>+</sup> CD4<sup>+</sup> T cells (average  $\pm$  SD). Characterization of proliferating FoxP3<sup>-</sup> and FoxP3<sup>+</sup> CD4<sup>+</sup> T cells based on CD62L (E) or CD103 (F) expression. The percentage of BrdU<sup>+</sup> cells in each fraction is depicted for WT (white bar) and GITRL TG (black bar) mice (average  $\pm$  SD). Data are representative for 2 independent experiments with each at least 3 mice per group. Asterisks denote significant differences (\*  $p < 0.05$ , \*\*  $p < 0.005$ ).

proliferation was even more profound for regulatory T cells, as the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells that had incorporated BrdU had more than doubled compared with WT littermates (Fig 6C-D). In this case, it was the CD62L<sup>+</sup>CD103<sup>-</sup> fraction of regulatory T cells that showed the most profound increase in BrdU incorporation (Fig. 6E-F). No effect of GITRL overexpression on BrdU incorporation was found in CD8<sup>+</sup> T cells (data not shown). Overall, these data indicate that GITR affects the numbers of regulatory T cells as well as the memory/effector pool of non-regulatory CD4<sup>+</sup> T cells *in vivo* by regulating their proliferation.

### ***Enhanced GITR ligation delays experimental autoimmune encephalomyelitis***

To examine the significance of the expansion of both regulatory and effector CD4<sup>+</sup> T cells on a complex immune response *in vivo*, GITRL TG mice were subjected to an experimental model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). We selected this model as it induces autoimmunity by selective



**Fig. 7** Delayed EAE induction through GITR ligation. (A) The cumulative incidence and (B) the average clinical score following EAE induction in WT and GITRL TG mice. Experiment depicted contains 10 animals per group and is representative of 2 independent experiments. Significant differences ( $p < 0.05$ ) of the area under the curve were determined by Wilcoxon rank-sum test (highlighted area). (C) Percentage regulatory (FoxP3<sup>+</sup>) and (D) non-regulatory effector (FoxP3<sup>-</sup> CD62L<sup>+</sup>) T cells within the CD4<sup>+</sup> compartment was determined in peripheral blood following immunization (day 0) and pertussis toxin injection (day 0 and 2) of WT and GITRL TG mice. Data represent the average  $\pm$  SD of 4 mice. Asterisks denote significant differences between WT and GITRL TG mice on a particular day (\*  $p < 0.05$ , \*\*  $p < 0.005$ ). Black dots denote significant differences between consecutive days for either group ( $\cdot$   $p < 0.05$ ,  $\bullet$   $p < 0.005$ ). (E) Cellular infiltrates in brain and spinal cord were analyzed for mice with a clinical score of five and in which disease onset occurred four to five days prior to experimental endpoint. Representative immunohistochemical staining of the spinal cord of WT and GITRL TG mice shows the presence of (F) CD68<sup>+</sup> macrophages (G) CD4<sup>+</sup> T cells and (H) FoxP3<sup>+</sup> regulatory T cells on a haematoxylin background staining (20x magnification). Arrow indicates a single FoxP3<sup>+</sup> cell.

depletion and loss of function of regulatory T cells through treatment of immunized mice with pertussis toxin, which probably facilitates autoreactive T cells to develop and cause nerve damage in the central nervous system (CNS).<sup>224,225</sup> It is therefore conceivable that GITRL TG mice would be protected from disease (i.e. limb paralysis), because they have more regulatory T cells, but it could also be that

they would display enhanced susceptibility to EAE than WT mice because of their increased effector T cell formation. We found that regulatory mechanisms dominated the EAE response in GITRL TG mice, as they had a significant delay in disease onset compared to WT mice, which correlated with a lower clinical score (Fig. 7A-B). Although disease was delayed in GITRL TG mice, it was not inhibited, as the cumulative incidence and clinical score were similar between both groups at the experimental endpoint (Fig. 7A-B), suggesting that autoreactive cells did develop in these mice.

To gain insight in the relative contribution of regulatory and effector CD4<sup>+</sup> T cells in disease development, we examined the levels of both cell types in circulation following EAE induction. During the first 9 days, the fraction of regulatory CD4<sup>+</sup> T cells decreased in both groups of mice, but GITRL TG mice continuously displayed more regulatory T cells in circulation than WT mice (Fig. 7C). Both GITRL TG and WT mice showed a disease-related increase of effector type CD4<sup>+</sup> T cells in peripheral blood, but this occurred later in GITRL TG than in WT mice, which correlates with the observed delay in disease development (Fig. 7D). Finally, to investigate whether the observed differences in EAE signified merely a difference in time or also in quality of the immune response, we analyzed the cellular infiltrates in the central nervous system of mice with a similar clinical score. GITRL TG and WT mice showed a comparable influx of macrophages (Fig. 7E) and CD4<sup>+</sup> T cells (Fig. 7F) in the central nervous system and the influx of FoxP3<sup>+</sup> cells was equally low in both groups (Fig. 7G). Therefore, these data suggest that GITR engagement does not change the quality of this autoimmune response, but rather delays disease development by delaying the generation of disease-related effector type CD4<sup>+</sup> T cells.

## Discussion

Since its discovery in 1997, GITR has been the focus of many studies that address its biological function in cellular immunology.<sup>198,226-229</sup> These studies have indicated that GITR has costimulatory effects during T cell activation, but it is still not fully understood at what level GITR triggering affects both T cell activation and regulatory T cell function and how this influences immune responses *in vivo*. Here we describe that *in vivo* GITR stimulation through its natural ligand increased absolute numbers of both effector and regulatory type CD4<sup>+</sup> T cells, which was not at the expense of the naïve CD4<sup>+</sup> T cell pool. Detailed analysis revealed that this increase was a direct consequence of enhanced proliferation of both effector and regulatory type CD4<sup>+</sup> T cells in GITRL TG mice. GITR ligation *in vivo* did not affect the anergic state of

regulatory T cells *in vitro*, nor did it influence the suppressive function of regulatory T cells. These data are congruent with the phenotype of GITR<sup>-/-</sup> mice, which have normally functioning regulatory T cells, but slightly fewer absolute numbers.<sup>230</sup> *In vitro* studies have shown that agonistic anti-GITR antibodies can induce proliferation of regulatory T cells in an IL-2 dependent manner, also without affecting their suppressive activity.<sup>198,210</sup> We found that GITRL expression on B cells increased IL-2 production by CD4<sup>+</sup> T cells *in vivo* (Fig. 2H) and *in vitro* (Fig. 5A), which is most likely a direct effect, as GITR crosslinking with antibodies can induce IL-2 production through TRAF-5 mediated NF-κB activation.<sup>231</sup> These results have two important implications for our understanding of the biological function of GITR on T cells. First, since regulatory T cells depend on exogenous IL-2 for their proliferation,<sup>232</sup> these findings indicate that GITR drives proliferation of both regulatory and effector T cells through the induction of IL-2 from the latter. Second, it explains why GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells,<sup>210</sup> since it was recently shown that regulatory CD4<sup>+</sup> T cells exert their suppressive function through consumption of IL-2 produced by activated T cells, leading to apoptosis of the latter.<sup>233</sup> Since GITR triggering increases the production of IL-2, it allows them to escape or delay this cytokine deprivation-induced apoptosis. These implications fit in a previously postulated model for GITR function,<sup>200</sup> in which it was also suggested that when GITRL expression decreases at the end of an immune response, this would render effector T cells susceptible to suppression by an expanded, activated regulatory T cell pool. Transgenic GITRL expression does not allow us to test this hypothesis in our system, but it is worth following up this idea, as it implies that GITR is indirectly involved in termination of a T cell response.

Detailed analysis revealed that GITR ligation *in vivo* modified the expression of several key proteins expressed by regulatory T cells (Fig. 3). We found that the IL-2 receptor is down-regulated on regulatory T cells of GITRL TG mice, which is most likely a direct consequence of increased IL-2 consumption driving enhanced proliferation.<sup>232</sup> This is in agreement with recent findings that homeostatically proliferating regulatory CD4<sup>+</sup> T cells *in vivo* express lower levels of the IL-2 receptor than non-proliferating cells.<sup>234</sup> Furthermore, GITRL TG mice contained more regulatory T cells with an activated phenotype, expressing low levels of CD62L and high levels of CD103 (Fig. 3).<sup>222</sup> This is interesting, because we found that BrdU predominantly incorporated in the CD62L<sup>+</sup> and CD103<sup>-</sup> population of regulatory T cells in GITRL TG mice (Fig. 6). This would thus indicate that GITR ligation induces proliferation of CD62L<sup>+</sup>CD103<sup>-</sup> regulatory T cells and that during this proliferation they become activated and accumulate as CD62L<sup>-</sup>CD103<sup>+</sup> regulatory T cells. This would be in agree-

ment with an earlier study, which described that regulatory T cells with a high turnover downmodulate CD62L after several cell divisions.<sup>235</sup> Since CD62L is required for high endothelial venule dependent lymphocyte entry into lymph nodes and CD103 is an integrin necessary for the homing and retention of cells at inflammatory sites, these data suggest that regulatory T cells in GITRL TG mice are more prone to enter (inflamed) peripheral tissues than secondary lymphoid organs compared to their WT counterparts. Indeed, we found that liver and bone marrow of GITRL TG mice accumulate more CD62L<sup>+</sup>CD103<sup>+</sup> regulatory T cells than WT mice (data not shown), but since the supply of regulatory T cells is also increased in these mice, it requires more specific migration experiments to adequately address this issue.

An intriguing finding from our analysis of GITRL TG mice is that the functional consequences of GITR engagement were restricted to CD4<sup>+</sup> T cells, as no effects on the proliferation or effector cell formation of CD8<sup>+</sup> T cells could be detected, neither *in vitro* nor *in vivo* (Fig. 2, 5A-C and data not shown). This is in contrast with other studies in which a role for GITR on CD8<sup>+</sup> T cell responses was demonstrated, using agonistic GITR antibodies or GITR<sup>-/-</sup> mice.<sup>236-238</sup> We found that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in GITRL TG mice had down-modulated surface expression of GITR compared to WT mice (Fig. 1 and data not shown), which indicates that GITR was functionally engaged by its ligand on both cell types. In WT mice, GITR expression is higher on CD4<sup>+</sup> non-regulatory T cells than on CD8<sup>+</sup> T cells<sup>239</sup> (and data not shown), which could be the reason why GITRL expression has a stronger effect on CD4<sup>+</sup> T cells than CD8<sup>+</sup> T cells. This might also relate to the finding that the costimulatory effect of GITR crosslinking with an anti-GITR antibody is apparent at a lower anti-CD3 concentration in CD4<sup>+</sup> T cells than in CD8<sup>+</sup> T cells.<sup>210</sup> Moreover, GITR upregulation following T cell activation is dependent on CD28 engagement in CD4<sup>+</sup>, but not CD8<sup>+</sup> T cells.<sup>210,238,240</sup> Thus, although GITR functions on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, it is differently regulated in these subsets. In our hands, deliberate triggering of GITR on CD8<sup>+</sup> T cells *in vivo* by its natural ligand clearly does not translate into functional consequences, or at least not as strong as the effects found on CD4<sup>+</sup> T cells.

The synchronized expansion of regulatory and effector CD4<sup>+</sup> T cells that is induced upon GITR stimulation might seem contradictory for protective immunity, as these cell types obviously have opposite functions. However, recent *in vivo* studies have shown that regulatory T cells expand with similar kinetics as effector CD4<sup>+</sup> T cells upon HSV-2 infection<sup>241</sup> or immunization with Freund's complete adjuvants,<sup>242</sup> so that their ratio remains relatively constant. Coincident expansion of regulatory and effector T cells could be a direct consequence of responsiveness of regulatory T cells to IL-2 produced by effector T cells<sup>243</sup> and our data suggest that GITR could play a role in this process. The clinical consequence of an immune response might

even depend on this ratio, as the experimental induction of both adjuvant arthritis and type 1 diabetes correlates with an increased ratio of effector versus regulatory T cells.<sup>244-246</sup> The same might apply for the EAE model, as depletion of regulatory T cells resulted in enhanced disease progression and severity.<sup>247</sup> We found that the delay in disease induction observed in GITRL TG mice correlated with a delay in the increase of effector CD4<sup>+</sup> T cells in circulation (Fig. 7C-D). As no differences were observed in final disease severity nor cellular infiltrates in the brain parenchyma, these observations suggest that GITR stimulation sets a threshold for T cell activation and can delay autoimmunity by regulating the numbers of both regulatory and effector T cells.

In conclusion, we have shown that GITR serves as a costimulatory molecule in that it induces proliferation of regulatory as well as effector CD4<sup>+</sup> T cells *in vivo*. We suggest that upregulation of GITRL on antigen presenting cells during the initiation of an immune response, through the increase of pro-inflammatory stimuli, enhances IL-2 production and thereby the proliferation of cognate CD4<sup>+</sup> T cells, which also makes them less susceptible to suppression by regulatory T cells. At the same time, GITRL expression during this early phase induces the expansion of regulatory T cells, aided by the presence of exogenous IL-2 from proliferating non-regulatory T cells. These regulatory T cells might be important to re-establish the status quo of the immune system at later stages of the response.

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## Supplemental data

Table 1. MLPA analysis on CD4<sup>+</sup> T cells from WT and GITRL TG mice

Family	MLPA probe	Naïve T cells			Effector T cells			Regulatory T cells			
		WT mice	GITRL TG mice	Differential expression (Log <sub>2</sub> )	WT mice	GITRL TG mice	Differential expression (Log <sub>2</sub> )	WT mice	GITRL TG mice	Differential expression (Log <sub>2</sub> )	
Anti-apoptotic	Bel-1	0.00 ± 0.00	0.05 ± 0.05	0	0.08 ± 0.07	0.19 ± 0.03	1.272	0.10 ± 0.01	0.22 ± 0.07	1.136	
	Bel-X1	1.34 ± 0.03	1.13 ± 0.05	-0.246	2.82 ± 0.21	2.07 ± 0.17	-0.446	3.21 ± 0.13	2.50 ± 0.29	-0.361	
	Bel-2	5.10 ± 0.40	5.81 ± 0.43	0.187	2.68 ± 0.30	2.34 ± 0.20	-0.195	7.05 ± 0.66	3.81 ± 0.95	-0.888	
	A1a, A1b & A1d	4.44 ± 0.32	5.06 ± 0.53	0.189	15.04 ± 0.80	15.80 ± 1.22	0.071	9.34 ± 1.43	14.45 ± 0.72	0.629	
	Mcl-1	5.13 ± 0.34	5.64 ± 0.26	0.137	4.03 ± 0.70	4.13 ± 0.24	0.034	5.44 ± 0.57	4.48 ± 0.74	-0.281	
Pro-apoptotic	BH3-only	Bid	1.46 ± 0.05	1.48 ± 0.06	0.022	1.75 ± 0.32	2.01 ± 0.13	0.200	1.77 ± 0.20	1.77 ± 0.21	-0.007
		Bik	0.16 ± 0.02	0.12 ± 0.01	-0.369	0.79 ± 0.34	0.25 ± 0.01	-1.647	0.10 ± 0.01	0.08 ± 0.08	-0.310
		Bim	2.60 ± 0.21	2.49 ± 0.16	-0.063	1.78 ± 0.59	1.38 ± 0.11	-0.372	2.27 ± 0.42	1.03 ± 0.23	-1.137
		BMF	0.16 ± 0.03	0.11 ± 0.10	-0.534	0.27 ± 0.10	0.10 ± 0.10	-1.424	0.23 ± 0.12	0.05 ± 0.08	-2.274
		PUMA	0.86 ± 0.13	0.83 ± 0.05	-0.045	0.53 ± 0.07	0.66 ± 0.04	0.322	1.04 ± 0.20	1.03 ± 0.14	-0.006
	Bax/ Bak like	Noxa	0.00 ± 0.00	0.23 ± 0.05	0	0.44 ± 0.11	1.12 ± 0.04	1.337	0.35 ± 0.05	1.03 ± 0.20	1.572
		Bax	0.94 ± 0.08	0.83 ± 0.08	-0.180	0.99 ± 0.11	0.96 ± 0.10	-0.040	0.91 ± 0.16	0.95 ± 0.14	0.064
		Harakiri	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0
		Bax	3.06 ± 0.13	2.95 ± 0.13	-0.056	2.90 ± 0.85	2.06 ± 0.05	-0.492	2.12 ± 0.30	2.29 ± 0.52	0.115
		Bak	1.98 ± 0.11	2.07 ± 0.13	0.065	1.93 ± 0.55	1.50 ± 0.10	-0.358	1.24 ± 0.20	1.27 ± 0.26	0.029
	Others	Bok	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0
		Bcl-G	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0
		Bcl-Rambo	1.99 ± 0.09	1.89 ± 0.04	-0.075	1.58 ± 0.20	1.41 ± 0.14	-0.157	1.59 ± 0.23	1.69 ± 0.26	0.090
		MiAP1	0.48 ± 0.05	0.36 ± 0.06	-0.444	0.49 ± 0.14	0.31 ± 0.04	-0.651	0.41 ± 0.06	0.30 ± 0.07	-0.448
		Boo/Divs	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0
		XIAP	0.29 ± 0.02	0.22 ± 0.02	0.146	0.30 ± 0.07	0.22 ± 0.03	-0.420	0.29 ± 0.09	0.20 ± 0.03	-0.563
		NAIP	0.00 ± 0.00	0.16 ± 0.01	0	0.98 ± 0.10	1.72 ± 0.13	0.819	2.24 ± 0.26	2.31 ± 0.44	0.042
IAP Family	IAP1	6.04 ± 0.30	7.17 ± 0.13	0.246	4.91 ± 0.78	5.23 ± 0.60	0.089	6.06 ± 0.74	5.59 ± 1.06	-0.119	
	IAP2	1.05 ± 0.04	1.32 ± 0.16	0.335	1.39 ± 0.14	1.64 ± 0.13	0.233	1.68 ± 0.23	3.01 ± 0.41	0.840	
	LIVIN	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	
	Apolon	13.34 ± 0.47	13.44 ± 0.56	0.032	11.15 ± 0.70	8.82 ± 0.80	-2.337	9.71 ± 1.65	8.48 ± 1.76	-0.196	
	PI9	0.46 ± 0.02	0.65 ± 0.06	0.481	0.74 ± 0.30	0.91 ± 0.02	0.322	0.70 ± 0.08	0.77 ± 0.06	0.119	
Miscellaneous	CFLAR	6.76 ± 0.12	6.94 ± 0.39	0.036	5.32 ± 0.25	5.19 ± 0.38	-0.035	4.83 ± 0.47	4.56 ± 0.71	-0.082	
	Gzmb	0.00 ± 0.00	0.00 ± 0.00	0	0.22 ± 0.20	0.93 ± 0.16	2.065	0.26 ± 0.03	0.23 ± 0.04	-0.160	
	Preferin1	2.91 ± 0.10	2.64 ± 0.30	-0.139	0.75 ± 0.21	1.18 ± 0.22	0.650	0.48 ± 0.08	0.26 ± 0.05	-0.911	
	Apaf1	2.21 ± 0.15	2.46 ± 0.06	0.141	2.54 ± 0.25	3.95 ± 0.34	0.635	1.79 ± 0.34	2.39 ± 0.34	0.415	
	Smac/Diablo	1.89 ± 0.20	1.50 ± 0.10	-0.332	1.23 ± 0.40	1.19 ± 0.09	-0.046	1.40 ± 0.10	1.11 ± 0.15	-0.333	
	AIF	0.94 ± 0.05	0.93 ± 0.07	-0.020	0.96 ± 0.05	0.79 ± 0.05	-0.281	0.79 ± 0.16	0.67 ± 0.10	-0.248	
	HtrA2	0.82 ± 0.16	0.82 ± 0.15	-0.002	0.85 ± 0.04	0.87 ± 0.05	0.022	0.95 ± 0.16	1.53 ± 0.25	0.681	
	P21	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00	0	
	HomoKeeping	B2M	11.52 ± 0.14	11.37 ± 0.76	-0.018	12.89 ± 3.47	15.11 ± 5.99	0.229	15.11 ± 9.89	18.05 ± 12.05	0.256
		GUS	1.11 ± 0.02	1.04 ± 0.03	-0.093	1.08 ± 0.14	0.83 ± 0.13	-0.379	0.97 ± 0.21	0.77 ± 0.16	-0.343
TBP		2.40 ± 0.13	2.05 ± 0.06	-0.225	1.49 ± 0.01	1.32 ± 0.05	-0.176	1.38 ± 0.15	1.04 ± 0.15	-0.410	
PAK2		10.33 ± 0.09	8.56 ± 0.14	-0.271	6.96 ± 1.15	5.92 ± 0.76	-0.233	7.19 ± 1.53	5.26 ± 0.92	-0.451	