Modulating T cell homeostasis via TNF and TNFR superfamily members: characterization and function of effector & regulatory T cells
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Chapter 2

GITR triggering induces expansion of both effector and regulatory CD4$^+$ T cells in vivo.


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GITR Enhances CD4$^+$ T Cell Proliferation in vivo.

Abstract
Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR)$^4$ 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$^+$ T cells, but not CD8$^+$ 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$^+$ 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 \(^1\). 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 \(^2, 3\). 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 \(^4-8\). Its ligand (GITRL) can be found on a variety of cells, including dendritic cells, macrophages and B cells \(^9-11\). GITRL is transiently upregulated on these antigen presenting cells upon stimulation via the transcription factor NF-1 \(^9\) 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 \(^5, 6, 9, 12, 13\), 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 \(^5, 6, 9\). 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 \(^14\). GITR is not essential for regulatory T cell function, as regulatory T cells from GITR\(^{-/-}\) mice display a normal capacity to suppress T cell proliferation in vitro \(^15, 16\). This leaves unanswered what the function of GITR is on regulatory T cells.

Studies using GITR\(^{-/-}\) mice showed that the absence of GITR was protective in several disease models, which was attributed to an impaired effector function of T cells \(^17-19\). 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.
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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 \cite{23}. 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 \cite{24,25}, 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 \textit{in vivo}, we generated transgenic (TG) mice in which GITRL is constitutively expressed on B cells. Our findings demonstrate that GITR stimulation \textit{in vivo} very effectively increases the absolute number of both effector and regulatory CD4$^+$ 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$^+$ T cell numbers \textit{in vivo} by enhancing their turnover.
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).

Figure 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+ cells from WT, RW14, RW18 and RW20 mice and (D) the expression of GITRL on splenic B220+ B cells as the average geometric mean fluorescence intensity (geoMFI) ± SD for 3 mice per group. (E) Representative staining for GITR expression on splenic CD3+ cells from WT, RW14, RW18 and RW20 mice and (F) the expression of GITR on CD3+ T cells as the average geoMFI ± SD. Asterisks denote significant differences (** p<0.005).
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 shedding upon ligand stimulation is a hallmark of various TNFR-superfamily members 26-30, 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⁺ 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 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⁺ T cells (Fig. 2B-C), whereas numbers of B cells and CD8⁺ T cells were not significantly altered (Fig. 2B-C). Analysis of FoxP3 expression indicated that a substantial part of this increase in CD4⁺ T cells could be attributed to an enlarged regulatory T cell compartment, as up to three times more CD4⁺FoxP3⁺ regulatory T cells were present in the spleens of GITRL TG mice (Fig. 2D-E). Phenotypic analysis of FoxP3⁺CD4⁺ T cells indicated that the non-regulatory fraction was also affected in GITRL TG mice, as
Figure 2. GITRL TG mice have more effector and regulatory type CD4+ 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+ and CD8+ T cells in the spleen of WT and GITRL TG mice. (D, E) Percentage and absolute number of splenic regulatory (FoxP3+) and non-regulatory (FoxP3-) CD4+ T cells in WT and GITRL TG mice. Non-regulatory CD4+ T cells were subdivided in naïve (CD44+CD62L-), effector memory (EM) (CD44+CD62L+) and central memory (CM) (CD44+CD62L+) cells. (F, G) Percentage and absolute number of naïve, EM and CM cells of splenic CD8+ T cells. Production of (H) IL2, (I-J) IFNγ and (K-L) IL10 by CD4+ or CD8+ 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 ± SD of 3-5 mice and are representative for 2-4 independent experiments.
significantly more CD4\(^+\) T cells with an effector memory-like (CD44\(^+\)CD62L\(^-\)) and central memory-like (CD44\(^+\)CD62L\(^+\)) phenotype were identified (Fig. 2D-E). This increase of CD4\(^+\) 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\(^+\) population, since absolute numbers of naïve CD4\(^+\) T cells were comparable with WT littermates (Fig. 2E). No differences were found for CD8\(^+\) T cells with respect to their naïve, effector memory-like and central memory phenotype (Fig. 2F-G). Corroborating the specific increase in CD4\(^+\) effector T cells in GITRL TG mice, splenocyte stimulation with PMA-ionomycin showed increased production of the effector cytokines IL2 (Fig. 2H) and IFN\(\gamma\) (Fig. 2I-J) by CD4\(^+\), but not CD8\(^+\) T cells. Consistent with the increase in regulatory T cell numbers, we observed a trend towards more IL10-producing CD4 T cells, but this difference was not significant (Fig. 2K-L). These data thus indicate that GITR triggering in vivo enhanced the number of both regulatory and effector CD4\(^+\) 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\(^+\) CD4\(^+\) 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\(^+\) 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\(^+\)FoxP3\(^+\) regulatory T cells from GITRL TG mice consistently expressed lower
levels of CD25, CD62L and CTLA4 compared to their counterparts in WT mice (Fig 3B-C). In addition, the expression of PD1 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). As FoxP3+ regulatory T cells can be divided in two functionally distinct subsets, namely naïve (CD62L+ CD103-) and effector regulatory T cells (CD62L– CD103+) 31, we conclude that constitutive GITR stimulation not only leads to more regulatory T cells, but specifically stimulates the formation of regulatory T cells with an effector phenotype.

**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+CD25-) were stimulated with anti-CD3/CD28 in the presence of increasing numbers of regulatory T cells (CD4+CD25+) 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 5, 6. 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 14. 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 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 14.

To establish whether regulatory T cells in GITRL TG mice are indeed functionally active in vivo, we examined several organs of 12 months old GITRL TG mice for cellular infiltrates as
Figure 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 μg/ml soluble anti-CD3 mAb in the presence of irradiated WT splenocytes as APCs; for the final 16 hours [3H]thymidine was added and incorporation was measured. Data are depicted as the percentage proliferation compared to responder T cells alone (average of triplicate wells ± SD) and are representative of 2 independent experiments. (C) Wright Giemsa staining of cryo-sections from thyroid gland, kidney, liver, stomach, and small and large intestine of 12 month old WT and GITRL TG mice. Data are representative for 2 mice per group.
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a sign of organ inflammation and autoimmunity. Loss of function of regulatory T cells has been associated with increased numbers of autoreactive T cells, which can induce several forms of autoimmunity, including thyroiditis, glomerulonephritis, gastritis and inflammatory bowel disease.\textsuperscript{32-34} But despite the strong increase of effector CD4 T cells observed in lymphoid organs of GITRL TG mice (Fig. 2), we did not find any sign of inflammation or cellular infiltration in either the thyroid gland, kidney, liver, stomach or intestines of these mice (Fig. 4C). This indicates that GITR stimulation does not impair the function of regulatory T cells in vivo and considering the increase of effector T cells, this suggests that the regulatory T cells in GITRL TG mice are rather competent in preserving the homeostasis within this more active immune system.

**GITR costimulation enhances CD4+ T cell proliferation and IL2 production**

To investigate whether the increase in effector 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). However, this comprehensive analysis did not reveal any significant differences in the apoptotic gene expression profile of naïve, effector and regulatory CD4 T cells isolated from GITRL TG mice compared to WT mice (data not shown).

Next, we set out to determine the effects of GITR triggering on T cell proliferation. CFSE labeled T cells from WT mice were stimulated with suboptimal 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+ T cell proliferation, but did not affect CD8+ T cell proliferation (Fig. 5A). The enhanced proliferation of CD4+ T cells via GITR engagement was no longer apparent when extra IL2 was added to these cultures, indicating that GITR engagement
Figure 5. GITR costimulation in vitro enhances CD4$^+$ T cell proliferation and IL2 production.

(A) Anti-CD3-induced proliferation of CFSE-labelled WT T cells cultured for 3 days in the presence of WT (filled graph) or GITRL TG (open graph) B cells with or without IL2. 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) IL2 concentration in supernatants (average of triplicate wells ± SD) after stimulating WT T cells as in (A) for 24 hours in the presence of a blocking antibody against CD25 to prevent IL2 consumption. Expression of CD25 (E) and CD69 (F) on CD4$^+$ T cells stimulated as in (A) after 24 hours. (G) Naïve WT CD4$^+$CD25$^-$ 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$^+$CD4$^+$ 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).
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affects the early proliferative capacity of CD4\(^+\) T cells. We found that increased GITR ligation raised the percentage of CD4\(^+\) T cells entering cell division (Fig. 5B), as well as the number of divisions that these cells underwent (Fig. 5C). Since the addition of IL2 enhanced T cell proliferation to a similar extent as the addition of GITRL TG B cells, we questioned whether GITR stimulation induced IL2 production. Indeed, when WT T cells were stimulated with anti-CD3, the addition of GITRL TG B cells induced almost 3-fold more IL2 than WT B cells (Fig. 5D). Moreover, GITR ligation increased the expression of CD25 and CD69 on CD4\(^+\) T cells, confirming the enhanced IL2 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\(^+\)CD25\(^-\) T cells from Ly5.1\(^+\) WT donor mice, labeled them with CFSE and transferred them into WT or GITRL TG (Ly5.2\(^+\)) 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 IL2 and increased proliferation of CD4\(^+\) T cells in vitro.

**GITR engagement in vivo increases the proliferation of effector and regulatory CD4\(^+\) T cells.**

As GITR engagement could directly and specifically enhance CD4\(^+\) T cell proliferation, we investigated the proliferative capacity of CD4\(^+\) 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^+FoxP3^-) 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^+FoxP3^+) that stained positive for Ki-67 was not significantly different between WT and GITRL TG mice, but the fraction of Ki-67^+ 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^+ T cell compartment, we found that GITRL TG mice contained more BrdU^+ cells than WT mice and this increase was most pronounced in the CD62L^- effector fraction (Fig. 6C-E). A GITR-mediated increase in proliferation was even more profound for regulatory T cells, as the percentage of CD4^+FoxP3^+ T cells that had incorporated BrdU had more than doubled compared with WT littermates (Fig 6C-D). In this case, it was the CD62L^+ CD103^- 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^+ 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^+ T cells in vivo by regulating their proliferation.
Figure 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+ T cells from WT and GITRL TG mice and (B) the percentage Ki-67+ cells of FoxP3− and FoxP3+ CD4+ T cells (average ± SD). (C) Representative intracellular staining for BrdU and FoxP3 on splenic CD4+ T cells from WT and GITRL TG mice, 16 hours after i.p. injection of 1 mg BrdU. (D) The percentage BrdU+ cells of FoxP3− and FoxP3+ CD4+ T cells (average ± SD). Characterization of proliferating FoxP3− and FoxP3+ CD4+ T cells based on CD62L (E) or CD103 (F) expression. The percentage of BrdU+ cells in each fraction is depicted for WT (white bar) and GITRL TG (black bar) mice (average ± 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).
Enhanced GITR ligation delays experimental autoimmune encephalomyelitis.

To examine the significance of the expansion of both regulatory and effector CD4+ T cells on a complex immune response in vivo, GITRL TG mice were subjected to EAE, an experimental model for multiple sclerosis. We selected this model as it induces autoimmunity by selective 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 36, 37. It is therefore conceivable that GITRL TG mice are protected from disease (i.e. limb paralysis), because they have more regulatory T cells, but it could also be that they display enhanced susceptibility to EAE compared to 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 obtain more insight in the mechanism of this difference in disease progression, we examined the CD4 T cell response in draining lymph nodes from WT and GITRL TG mice early after immunization. After 5 days, the impact of the immunization was readily visible in both mouse strains, as the draining (inguinal and lumbar), but not non-draining (axillary and brachial) lymph nodes, had considerably increased in size (data not shown). In contrast to what might be expected from the delay in disease progression, we found that the development of effector CD4 T cells was not perturbed in GITRL TG mice, as they contained even more effector CD4 T cells than WT mice (Figure 7C). Absolute numbers of regulatory CD4 T cells were also increased in GITRL TG mice (Figure 7C). Restimulation of lymph node cells with
Figure 7. Delayed EAE induction though 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). The absolute number of (C) CD4+ T cells and (D) IFNγ and IL17 producing CD4+ T cells on day 5 following EAE induction in the draining (inguinal and lumbar) and non-draining (axillary and brachial) lymph nodes from WT and GITRL TG mice. (E) Percentage regulatory (FoxP3+) and (F) non-regulatory effector (FoxP3–CD62L–) T cells within the CD4+ 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 ± 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 (• p<0.05, •• p<0.005). 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. (G) Size of cellular infiltrates in the spinal cord of WT and GITRL TG mice. Representative immunohistochemical staining and quantification of the spinal cord of WT and GITRL TG mice shows the presence of (H) CD68+ macrophages, (I) CD4+ T cells and (J) FoxP3+ regulatory T cells on a heamatoxylin background staining (20x magnification). Arrow indicates a single FoxP3+ cell. For quantification purposes, at least 6 infiltrates were analyzed per staining per mouse.
PMA-ionomycin corroborated an increase in effector T cells, as GITRL TG mice contained more IFNγ producing cells, whereas IL17 production was not affected (Figure 7D). Thus, GITR stimulation does not negatively affect the formation of autoreactive T cells following EAE induction, but, if anything, rather enhances it.

To further investigate the relative contribution of regulatory and effector CD4+ T cells in disease development in these mice, we examined the levels of both cell types in circulation following EAE induction. During the first 9 days, the fraction of regulatory CD4+ T cells decreased in both groups of mice, but GITRL TG mice continuously displayed more regulatory T cells in circulation than WT mice (Fig. 7E). Both GITRL TG and WT mice showed a disease-related increase of effector type CD4+ T cells in peripheral blood, but this occurred later in GITRL TG than in WT mice, which is interesting as it correlates with the observed delay in disease development (Fig. 7F). At 14 days after immunization, when both mouse strains had a comparable clinical score, there were no significant differences in effector or regulatory CD4 T cells in the blood of GITRL mice compared to WT mice.

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. The infiltrated areas were comparable in size between GITRL TG and WT mice (Fig. 7G). Moreover, these mice showed a comparable influx of macrophages (Fig. 7H) and CD4+ T cells (Fig. 7I) in the central nervous system and the influx of FoxP3+ cells was very low in both groups (Fig. 7J). Therefore, these data suggest that GITR engagement does not change the quality of this autoimmune response, but rather delays disease development. This is not due to a decrease in the formation of disease-related effector type CD4+ T cells, but might be related to an altered recirculation of these cells.
Discussion

Since its discovery in 1997, GITR has been the focus of many studies that address its biological function in cellular immunology\textsuperscript{5, 6, 9, 38, 39}. 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\textsuperscript{+} T cells. Detailed analysis revealed that this accumulation was a direct consequence of enhanced proliferation of both cell types in GITRL TG mice. The increase of effector and regulatory CD4\textsuperscript{+} T cells was not at the expense of the naïve CD4\textsuperscript{+} T cell pool. Together with the finding that transferred naïve WT T cells do not get activated in GITRL TG mice (Fig. 5G), this indicates that enhanced GITR triggering is not sufficient to activate naïve T cells, but that this process is still fully dependent on TCR activation. When TCR triggering is provided, GITR stimulation does enhance the expansion of newly activated CD4\textsuperscript{+} T cells, as can be concluded from the in vitro stimulation (Fig. 5A-C) and EAE immunization experiments (Fig. 7C). However, the BrdU incorporation experiments indicate that constitutive GITR triggering also enhances proliferation of effector and regulatory CD4\textsuperscript{+} T cells during the steady state situation (Figure 6E). To what extent the TCR is also required for this increased level of homeostatic proliferation in GITRL TG mice is not yet clear, as this expansion might also be driven by increased availability of cytokines like IL2. Transfer experiments with TCR-transgenic T cells could shed further light on this issue.

GITR ligation in vivo does not affect the anergic state of regulatory T cells in vitro, nor does it influence the suppressive function of regulatory T cells (Fig. 4A). The fact that GITRL TG mice do not develop any sign of organ inflammation or autoimmunity (Fig. 4C), despite the expansion of effector T cells, supports this notion and indicates that regulatory T cells are...
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fully functional in vivo in these mice and maintain homeostasis. The hypothesis that GITR regulates the size, but not the function of the regulatory T cell pool is supported by the observation that GITR−/− mice have normally functioning regulatory T cells, but fewer absolute numbers 14, 40. In vitro studies have shown that agonistic anti-GITR antibodies can induce proliferation of regulatory T cells in vitro in an IL2-dependent manner, also without affecting their suppressive activity 5, 14. We found that GITRL expression on B cells increased IL2 production by CD4+ 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 IL2 production through TRAF-5 mediated NF-κB activation 41. 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 IL2 for their proliferation 42, these findings indicate that GITR drives proliferation of both regulatory and effector T cells through the induction of IL2 from the latter. Second, it explains why GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells 14, since it was recently shown that regulatory CD4+ T cells exert their suppressive function through consumption of IL2 produced by activated T cells, leading to apoptosis of the latter 43. Since GITR triggering increases the production of IL2, non-regulatory T cells can thereby escape from or delay cytokine deprivation-induced apoptosis. These implications fit in a previously postulated model for GITR function 44, 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 IL2 receptor is
downregulated on regulatory T cells of GITRL TG mice, which is most likely a direct consequence of increased IL2 consumption driving enhanced proliferation. This is in agreement with recent findings that homeostatically proliferating regulatory CD4+ T cells in vivo express lower levels of the IL2 receptor than non-proliferating cells. 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). This is interesting, because we found that BrdU predominantly incorporated in the CD62L+ and CD103− population of regulatory T cells in GITRL TG mice (Fig. 6). This would thus indicate that GITR ligation induces proliferation of CD62L+CD103− regulatory T cells and that during this proliferation they become activated and accumulate as CD62L−CD103+ regulatory T cells. This would be in agreement with an earlier study, which described that regulatory T cells with a high turnover downmodulate CD62L after several cell divisions. Since CD62L is required for HEV-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−CD103+ 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+ T cells, as no effects on the proliferation or effector cell formation of CD8+ 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+ T cell responses was demonstrated, using agonistic GITR antibodies or GITR−/− mice. We found that both CD4+ and CD8+ T cells in GITRL TG mice had
downmodulated 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+ non-regulatory T cells than on CD8+ T cells (and data not shown), which could be the reason why GITRL expression has a stronger effect on CD4+ T cells than CD8+ 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+ T cells than in CD8+ T cells. Moreover, GITR upregulation following T cell activation is dependent on CD28 engagement in CD4+, but not CD8+ T cells. Thus, although GITR functions on both CD4+ and CD8+ T cells, it is differently regulated in these subsets. In our hands, deliberate triggering of GITR on CD8+ 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+ T cells.

The synchronized expansion of regulatory and effector CD4+ 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+ T cells upon HSV-2 infection or immunization with Freund’s complete adjuvants, 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 IL2 produced by effector T cells and our data suggest that GITR could play a role in this process. It is most likely that the simultaneous increase of regulatory and effector T cells is the reason why GITRL overexpression induces a mild phenotype compared to transgenic overexpression of other members of the TNF-superfamily, such as CD70, OX40L, 4-1BBL and LIGHT, which leads to severe immunopathology induced by effector T cells. The clinical consequence of an immune response might even depend on this ratio of effector vs. regulatory T cells, as the
GITR Enhances CD4⁺ T Cell Proliferation in vivo.

Experimental induction of both adjuvant arthritis and type 1 diabetes correlates with an increase of this ratio⁶³-⁶⁵. The same might apply for the EAE model, as depletion of regulatory T cells resulted in enhanced disease progression and severity⁶⁶. We found that the delay in disease induction observed in GITRL TG mice was not due to an inhibition in the formation of effector CD4⁺ T cells in the draining lymph nodes (Fig. 7C), but rather correlated with a delay in the increase of effector CD4⁺ T cells in circulation (Fig. 7E-F). As no differences were observed in final disease severity nor cellular infiltrates in the brain parenchyma, these observations suggest that GITR stimulation enhances both formation of effector and regulatory CD4⁺ T cells in lymph nodes and might delay autoimmunity by regulating emigration of effector CD4⁺ T cells from the lymph nodes. Whether GITR triggering has a direct effect on the egress of activated T cells from lymph nodes, or that this is an indirect effect mediated by regulatory T cells awaits further investigation.

In conclusion, we have shown that GITR serves as a costimulatory molecule in that it induces proliferation of regulatory as well as effector CD4⁺ 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 IL2 production and thereby the proliferation of cognate CD4⁺ 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 IL2 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.
Materials and 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 founders were identified by PCR analysis of tail or ear DNA, using the following PCR primers: pC3s1 (5’-GCAGTGACTCTC TTAAGGTAGCC-3’) and mGITRL4a (5’CTTGAGTGAAGTATAGATCAGTGTA-3’). Three GITRL TG founder lines (RW14, RW18, RW20) were propagated by mating with wild type (WT) C57BL/6 mice and offspring were 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. All experiments have been reviewed and approved by the AMC Animal Ethics Committee. 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 μm 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 x 10⁵ - 5 x 10⁶) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4°C with antibodies in the presence of anti-CD16-CD32 (clone 2.4G2). The following fluorescently or biotin-labelled monoclonal antibodies (and clone names) were obtained from Pharmingen: anti-B220 (RA3-6B2), anti-CD3ε (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 room temperature. Data were collected on a FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed with using FlowJo software (Treestar).

Intracellular cytokine staining

To determine direct ex vivo cytokine production, splenocytes were plated at 1 x 10⁶ cells/well in a 96-well round-bottom plate and stimulated with 1 ng/ml PMA and 1 μM ionomycin. After 2 hours incubation at 37°C, 1 ug/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 CD3 and CD4 or CD8, followed by fixation and permeabilization (Becton Dickinson). Cells were then incubated for 30 min with fluorescently labelled antibodies against IFNγ, IL2, IL10 or IL17 (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⁺ beads (Miltenyi Biotec). T cell enriched splenocytes were labeled with 0.25 μM carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37°C for 10 min 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⁺ 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 IL2 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 IL2 ELISA (Becton Dickinson) was performed according to instructions from the manufacturer.

**Regulatory T cell assay.**

Splenic CD4⁺CD25⁻ (responder cells) and CD4⁺CD25⁺ (regulatory cells) T cells were isolated by cell sorting using a FACS Aria (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 hrs with 1 μCi 3H-TdR ([Methyl-3H]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.

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

For adoptive transfers, naïve (CD25−) CD4+ T cells were purified from spleens and peripheral lymph nodes of Ly5.1 mice by negative selection using the CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec). Purified CD4+CD25− 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 (± 1x10^6 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 MOG35-55 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 TEC (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% H₂O₂ 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% NaN₃ for 1 h at 4°C. After washing in PBS, the sections were incubated with anti-rat HRP diluted in PBS/ 8% BSA/ 10% NMS/ 0.05% NaN₃/ 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 left out or substituted with an isotype control antibody. No immunoreactivity was seen for all negative controls.

For the visualization of cellular infiltrates in organs obtained from WT and GITRL TG mice, cryostat sections (7 μm) of the thyroid, kidney, liver, stomach, and small and large intestine were stained by Diff-Quick (Dade Behring) according to the manufactures instructions and analyzed by light microscopy.

**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.

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