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Anti-GITR Agonist Therapy Intrinsically Enhances CD8 T Cell Responses to Chronic Lymphocytic Choriomeningitis Virus (LCMV), Thereby Circumventing LCMV-Induced Downregulation of Costimulatory GITR Ligand on APC

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The costimulatory TNFR family member GITR can provide important survival signals for CD8 T cells. However, little is known about the regulation of this pathway during a chronic infection. In this study, we show that GITR ligand (GITRL) is maximally induced on APCs at day 2 post-lymphocytic choriomeningitis virus (LCMV) clone 13 infection, but is downregulated to below baseline levels by day 8 postinfection (p.i.), and remains so at the chronic stage of infection. At its peak, GITRL expression is highest on macrophages, with lower expression on conventional and plasmacytoid dendritic cells. GITR expression was highest on T regulatory cells but was also detected on Th1 and LCMV-specific CD8 T cells at day 8 p.i. and was maintained at low, but above baseline levels at the chronic stage of LCMV infection. As GITRL was limiting at the chronic stage of infection, we investigated the potential of therapeutic stimulation of GITR at this stage using agonistic anti-GITR Ab. Anti-GITR treatment at day 21 p.i. increased the frequency and number of LCMV-specific CD8 T cells, resulting in increased in vivo CTL activity and a concomitant decrease in viral load, despite the persistence of PD-1 expression. These effects of anti-GITR were CD8 T cell intrinsic, with no detectable effects on Th1 or T regulatory cells. In contrast to other TNFR agonists, such as anti–4-1BB, which can cause immune pathology, a single therapeutic dose of anti-GITR did not induce splenomegaly or increase serum alanine transaminase.

These studies identify GITR as a promising therapeutic target for chronic infection. The Journal of Immunology, 2014, 193: 5033–5043.
mechanism and efficacy of the anti-GITR agonist mAb DTA-1 in a chronic infection with LCMV cl 13 (22), a model that has successfully predicted key immunological factors and therapeutic mechanisms that are relevant to human chronic viral infections, such as HIV (23). To this end, we evaluated GITR and GITR ligand (GITRL) expression kinetics during LCMV cl 13 infection, and we administered agonist anti-GITR at day 21 postinfection (p.i.), a therapeutically relevant time point at which T cell responses to LCMV have become functionally exhausted. Despite low-level sustained expression of GITR on Treg, Th1, and LCMV-specific CD8 T cells, we found that, at the chronic stages of infection, macrophages and dendritic cells (DC) had lost expression of GITR to below baseline levels. Promisingly, we could compensate for the loss of GITR by administering a single dose of agonist anti-GITR Ab (clone DTA-1). DTA-1 resulted in a durable, cell-intrinsic effect on CD8 T cell responses and a 10-fold reduction in spleen and lung viral load. Importantly, CD8 T cell expansion was limited to the virus-specific cells and did not produce splenomegaly or liver inflammation, in contrast to the reported effects of anti-4-1BB agonist therapy, which can have pathological effects, including cytokine storm, splenomegaly, and hepatitis (16–18). Our findings are of particular importance given the recent interest in GITR as a therapeutic target in multiple diseases (20). We find that a single dose of anti-GITR is effective as a stand-alone therapy that has significant effects on viral control, showing durable effects up to 2 wk posttreatment, without overt immune pathology.

Materials and Methods
Reagents and Abs
Biotinylated H-2D<sup>+</sup>/GP<sub>33–41</sub>, KAVYNFATM, H-2D<sup>+</sup>/GP<sub>276–286</sub>, SGVENPG-GYCL, and H-2D<sup>+</sup>/nuclear protein (NP)<sub>396–404</sub>, FQPQNGQFI monomers were obtained from the National Institute for Allergy and Infectious Disease tetramer facility (Emory University, Atlanta, GA) and tetherizer and tetramerization with streptavidin allophycocyanin (Molecular Probes). Anti-CD8 (clone 53-6.7), anti-CD107a (clone 15A7), anti-CXCR5 (clone 2G8), and anti-CD25 (clone PC61.5) were purchased from BioLegend. FITC IgG isotype (clone 17A2), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD19 (clone eBio1D3), anti-CD44 (Bcl)-6 (clone 7D1) were obtained from the National Institute for Allergy and Infectious Disease and anti-GITR (clone DTA-1; obtained from S. Sakaguchi, Osaka University) with streptavidin allophycocyanin (Molecular Probes). Anti-CD8 (clone 53-6.7), anti-CD107a (clone 15A7), anti-CXCR5 (clone 2G8), and anti-CD44 (Bcl)-6 (clone 7D1) were purchased from the National Institute for Allergy and Infectious Disease.

LCMV focus-forming assay
Organs were harvested and immediately placed on dry ice. Organs were later thawed and homogenized, and supernatant dilutions (range: 10<sup>–10</sup> to 10<sup>6</sup>) were used to infect a MC57 cell monolayer under a 2% methylcellulose-MEM overlay. Forty-eight hours later, monolayers were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with rat anti-LCMV mAb (clone VL-4). Following secondary goat–anti-rat-HRP, a colorimetric reaction with o-phenylenediamine (Sigma-Aldrich) was used to quantify LCMV-infected foci.

Bone marrow chimeras
GITR<sup>+</sup>/GITR<sup>+</sup> mixed bone marrow chimeras were generated by i.v. reconstituting lethally irradiated age-matched female CD90.1 mice (The Jackson Laboratory) with a 1:1 mixture of GITR<sup>+</sup>/CD45.1.1 GITR<sup>+</sup>/CD45.2 bone marrow cells for a total of 5 × 10<sup>6</sup> cells. Following irradiation and reconstitution, mice were given 2 mg/ml neomycin sulfate (Bio-Shop, Burlington, ON) for consecutive 2 wk, after which mice were rested for an additional 90 d before use.

Data analysis and statistics
Samples were acquired with an LSR II or LSR Fortessa (BD Biosciences) with FACSDiva software. Flow cytometry data were analyzed with FlowJo (TreeStar, Ashland, OR). All statistical analyses were performed using GraphPad Prism v6 (La Jolla, CA). Unpaired Student t tests were used to compare two groups, with p values indicated on figures, as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 for all data, except bone marrow chimeras in Fig. 6D, in which a paired t test was used.

Results
GITR is rapidly induced on macrophages and DC following LCMV cl 13 infection
To assess the expression of GITR during the course of a chronic infection, we analyzed GITR expression on multiple immune subsets at different points p.i. as compared with a naive control (for gating strategy, see Supplemental Fig. 1). Previous reports have shown that resting APC from naive mice have low constitutive expression of GITR, that is rapidly upregulated following TLR
stimulation in vitro or at the acute stage of HSV-1 infection in vivo (24, 25). However, the expression of GITRL during a persistent viral infection has yet to be reported. Surface GITRL increased to a maximum at day 2 p.i., followed by a steady decrease to day 8 p.i. to below baseline (naive), after which GITRL remained below baseline levels up to day 21 p.i. The kinetics of GITRL upregulation and downregulation were similar on CD11b<sup>high</sup> F4/80<sup>+</sup> macrophages, CD11c<sup>high</sup> DC, and plasmacytoid DC (Fig. 1A, 1B); however, the CD11b<sup>high</sup> F4/80<sup>+</sup> macrophages expressed almost 10-fold higher levels of GITRL than the conventional or plasmacytoid DC.

Given that TNFR superfamily ligands on B cells have been shown to play important roles in chronic LCMV infection, and constitutive GITRL overexpression on B cells affects disease outcome in autoimmune models (26, 27), we also evaluated endogenous GITRL expression on B cells and Fox<sup>+</sup> GL-7<sup>+</sup> germinal center (GC) B cells. On total B cells, GITRL was not detectable at any point following infection; however, at day 8 p.i., GITRL was expressed on GC B cells, albeit at low levels (Fig. 1C).

Additionally, we noted low-level GITRL expression on Th1, Treg, LCMV-specific CD8 T cells, and NK cells, with similar expression kinetics as APC populations. The levels of GITRL on activated CD4 T cells were <10% the level observed on activated macrophages, although comparable to that on activated DC (Supplemental Fig. 2A–C).

As it was conceivable that the increased expression on macrophages relative to DC was due to increased infection by virus, we assessed the expression of GITRL on directly infected APC, using an LCMV-specific Ab to detect LCMV-infected cells (Fig. 1D). The number of detectable LCMV<sup>+</sup> cells was too low to evaluate GITRL expression at days 2 and 5 p.i.; therefore, we investigated GITRL expression on LCMV<sup>+</sup> and LCMV<sup>−</sup> APC at day 8 p.i. At this time, the level of expression was too low to measure on DC, but could be detected on macrophages (Fig. 1E, left). Unexpectedly, the level of GITRL was lower on the LCMV<sup>−</sup> compared with LCMV<sup>+</sup> macrophages, whereas the opposite was true for the inhibitory ligand PD-L1, which has been previously reported to be upregulated on LCMV-infected relative to LCMV-uninfected APC (Fig. 1E, right) (28, 29). Taken together, these data demonstrate that most APC populations maximally express GITRL at day 2 p.i., followed by rapid downregulation to below prestimulation levels. Additionally, LCMV-infected cells appear to specifically downregulate GITRL with concomitant increases in PD-L1 expression, perhaps serving as a second layer of immune regulation of T cell cosignaling.

Following acute infection with the same dose of LCMV Armstrong, there was only a modest increase in GITRL on macrophages at day 2 p.i. compared with the dramatic increase observed with LCMV cl 13. However, we also observed a decrease in GITRL to below baseline by day 8 p.i. (Fig. 1F). GITRL induction on other APC populations was largely comparable between LCMV Armstrong and cl 13 at all time points (data not shown).

**GITR is highly expressed on Treg, Th1, and LCMV-specific CD8 T cells**

We next examined the expression of GITR on CD4 T cell subsets, CD8 T cells, and NK cells throughout the course of LCMV cl 13 infection. Consistent with previous reports (30, 31), GITR was expressed at the highest levels on Foxp3<sup>+</sup> Tregs in naive mice, and this level of expression increased following LCMV cl 13 infection, reaching highest levels at day 8 p.i. and persisting at high levels to day 21 p.i. (Fig. 2A; for gating strategy, see Supplemental Fig. 1). GITR is present at low levels on NK cells and naive and memory T cells and increases on these populations upon activation (20). Consistently, the level of GITR increased on T-bet<sup>+</sup> Th1 cells, reaching a maximum at day 8 p.i., coinciding with peak LCMV-specific CD8 T cell responses, and returning to baseline by day 21 p.i. (Fig. 2A). Similarly, GITR was upregulated maximally on D<sup>β</sup>H2/3<sub>NP396-404</sub> and D<sup>β</sup>H2/3<sub>GP33-41</sub>-specific CD8 T cells relative to total CD8 T cells, before returning to low, but above baseline levels at day 21 p.i. (Fig. 2B). Too few Ag-specific CD8 T cells were available to analyze at day 2 p.i.; however, at day 5 p.i., LCMV-specific CD8 T cells had a lower mean fluorescence intensity for GITR than at day 8 p.i. Thus, GITR is present on resting T cells, with maximal expression showing delayed kinetics relative to the induction of GITRL.

We observed similar upregulation of GITR on NK cells, with maximal expression at day 8 p.i., followed by downregulation to low, but above baseline levels (Fig. 2C). GITR expression was also detected on macrophages, conventional and plasmacytoid DC, and both total and GC B cells (Supplemental Fig. 2D–F). We also found that GITR was similarly upregulated on T and NK cells during LCMV Armstrong infection (data not shown).

In sum, effector CD4 T, CD8 T, and NK cells maximally express GITR at day 8 p.i., after which these cells downregulate GITR to low levels, although still detectable and above baseline. This is in contrast to GITRL expression, which falls to below baseline, and to GITR expression on Tregs, which is maintained at high levels throughout the response. The finding that GITR expression was sustained during chronic LCMV infection, albeit at low levels, whereas GITRL levels were reduced to below baseline, suggests that GITRL is limiting at the chronic stage of infection and that GITR agonists may be of value for therapy of chronic LCMV infection.

**Agonistic anti-GITR improves control of chronic LCMV infection with no detectable immune pathology**

To evaluate the therapeutic potential of GITR in chronic infection, we took advantage of the widely used anti-GITR agonist mAb, DTA-1 (31) (Fig. 3A). Mice were treated with a single dose of 200 ng anti-GITR at the chronic stage (day 21 p.i.) of infection and evaluated for viral load 2 wk post–DTA-1 treatment to evaluate both efficacy and durability of the response (Fig. 3A). Treatment with DTA-1 improved viral control 10-fold in the spleen and lung and had a slight (2-fold), but significant improvement in viral control in the kidney over rat IgG control-treated animals (Fig. 3B).

Both LCMV infection as well as anti–4-1BB agonist therapy can cause liver inflammation (16, 17, 32). Moreover, anti–4-1BB agonists cause splenomegaly (16, 17). In contrast, although DTA-1 agonist improved viral control, we did not observe any splenomegaly or increases in alanine aminotransferase, an indicator of liver pathology, in the DTA-1–treated infected mice (Fig. 3C, 3D). Anti–4-1BB agonists have also been shown to cause nonspecific expansion of CD4<sup>+</sup>CD44<sup>high</sup> CD4 and CD8 memory T cells (16–18). To address whether DTA-1 had similar effects, we took advantage of the widely used anti-GITR agonist mAb, DTA-1 (31) (Fig. 3A). Mice were treated with a single dose of 200 ng anti-GITR at the chronic stage (day 21 p.i.) of infection and evaluated for viral load 2 wk post–DTA-1 treatment to evaluate both efficacy and durability of the response (Fig. 3A). Treatment with DTA-1 improved viral control 10-fold in the spleen and lung and had a slight (2-fold), but significant improvement in viral control in the kidney over rat IgG control-treated animals (Fig. 3B).

Both LCMV infection as well as anti–4-1BB agonist therapy can cause liver inflammation (16, 17, 32). Moreover, anti–4-1BB agonists cause splenomegaly (16, 17). In contrast, although DTA-1 agonist improved viral control, we did not observe any splenomegaly or increases in alanine aminotransferase, an indicator of liver pathology, in the DTA-1–treated infected mice (Fig. 3C, 3D). Anti–4-1BB agonists have also been shown to cause nonspecific expansion of CD4<sup>+</sup>CD44<sup>high</sup> CD4 and CD8 memory T cells (16–18). To address whether DTA-1 had similar effects, we compared the effects of anti–4-1BB (3H3) with DTA-1 on spleen size and memory T cell numbers in uninfected mice (Fig. 3E, 3F). In contrast to anti–4-1BB agonist, which clearly caused splenomegaly and expanded CD4<sup>+</sup>CD4 and CD8 memory T cells (16–18). To address whether DTA-1 had similar effects, we compared the effects of anti–4-1BB (3H3) with DTA-1 on spleen size and memory T cell numbers in uninfected mice (Fig. 3E, 3F). In contrast to anti–4-1BB agonist, which clearly caused splenomegaly and expanded CD4<sup>+</sup>CD4 and CD8 memory T cells in uninfected mice, we observed no effect of anti-GITR on the number of CD8 CD4<sup>+</sup>CD44<sup>high</sup> cells in uninfected mice and only a marginal effect on CD4 CD44<sup>high</sup> T cell numbers.

In sum, a single dose of agonist anti-GITR provided at a therapeutically relevant, chronic time point results in a durable increase in viral control without causing overt immune pathology. We also treated mice with agonist anti-GITR at day 8 p.i., but observed no effect on viral load or T cell responses at day 28 p.i. (data not shown); thus, the more clinically relevant chronic time point was
FIGURE 1. GITRL is rapidly upregulated following LCMV cl 13 and Armstrong infection, but later downregulated to below-preactivation levels in LCMV-infected macrophages and DC. (A–C) Expression kinetics of GITRL between days −1 and 21 p.i. on CD11bhigh F4/80+ macrophages (A), plasmacytoid and CD11clow DC (B), and total and Fas+ GL-7+ GC B cells (C) following LCMV cl 13 infection. For full gating strategy, see Supplemental Fig. 1. (D) Representative FACS plots of LCMV-infected cells from LCMV cl 13-infected mice at day 8 p.i. (top) or naive mice (bottom) to show representative gating strategy for LCMV-infected cells. (E) Expression of GITRL and PD-L1 on LCMV-infected CD11bhigh F4/80+ macrophages and LCMV-uninfected macrophages at day 8 p.i. (F) Kinetics and representative FACS plots of GITRL expression on CD11bhigh F4/80+ macrophages between days −1 and 21 p.i. following LCMV Armstrong infection. Numbers adjacent to histograms indicate delta mean fluorescent intensity (dMFI) relative to fluorescence minus one (open histograms). Dashed lines represent baseline/naive expression level of GITRL. Representative histograms show median GITRL expression on indicated subsets, with day indicating time p.i. Data are representative of two to three mice per group in a single kinetics experiment. Data points indicate mean ± SEM.
chosen for further study. We next aimed to understand the immunological mechanisms underlying the effect of agonist anti-GITR.

**Agonistic anti-GITR augments CD8 T cell responses to LCMV with no apparent effects on CD4 T cell subsets**

Given the high level expression of GITR on CD4 and CD8 T cells, we evaluated the ability of anti-GITR (DTA-1) to improve T cell immunity to chronic LCMV using the same experimental design as in Fig. 3A. Despite expression of GITR on Treg and Th1 (Fig. 2A), we found that DTA-1 treatment had only a marginal effect on total numbers of CD4 T cells (Fig. 4A). Moreover, anti-GITR had no effect on the proportion of CD4 T cells that were Treg, Th1, or T follicular helper cell (Th1), nor did DTA-1 affect the absolute numbers of these cells relative to rat IgG control (Fig. 4B–D). Additionally, DTA-1 did not improve functionality of CD4 cells following peptide restimulation ex vivo with I-A<sup>b</sup>-restricted LCMV GP<sub>66-81</sub> peptide. The proportions of IFN-γ<sup>+</sup> and IFN-γ<sup>+</sup> IL-2<sup>+</sup> were similar between rat IgG- and DTA-1–treated mice (Fig. 4E, 4F). DTA-1 also had no effect on the total numbers of B cells or Fas<sup>+</sup> GC B cells (Fig. 4G; for gating strategy, see Supplemental Fig. 1).

Given that previous work has demonstrated a CD8 T cell–intrinsic role for GITR in promoting the response to acute influenza A infection (19), we evaluated the effect of DTA-1 on CD8 T cells during chronic LCMV cl 13 infection. We found that DTA-1 slightly increased total, but not CD44<sup>high</sup>, CD8 T cells in terms of frequency, but not absolute number relative to rat IgG control (Fig. 5A and data not shown). Importantly, the frequency as well as total number of LCMV Db/GP33–41– and GP276–286-specific CD8 T cells increased ~2-fold in response to agonist anti-GITR relative to rat IgG control (Fig. 5B, 5C). There was, however, no effect on Db/NP396–404-specific T cells, which were scarce at day 35 p.i. Expression of Tim-3 and PD-1, both of which have been shown to negatively impact CD8 T cell responses in the context of...
chronic LCMV infection (5, 6), was unchanged in response to DTA-1 (Fig. 5D). DTA-1 also did not improve per-cell production of the cytotoxic molecule granzyme B (Fig. 5E). Consistent with unchanged expression of markers of T cell exhaustion, DTA-1 did not improve the frequency of cells that produce IFN-\(\gamma\), the amount of IFN-\(\gamma\) produced per cell, nor the coexpression of CD107a or TNF (Fig. 5F, 5G and data not shown). The total number of IFN-\(\gamma\)/CD107a coproducers following ex vivo GP33–41 peptide restimulation was consistently, but not significantly higher, in DTA-1–treated animals (Fig. 5F).

The above findings demonstrate that DTA-1 treatment at the chronic stage of infection increases the frequency and number of LCMV-specific CD8 T cells, without increasing the level of effector function per cell. To test whether this translates into an increase in overall CTL activity, we conducted an in vivo CTL assay and found that, indeed, DTA-1 significantly enhanced specific lysis of GP33–41-pulsed target splenocytes relative to rat IgG control (Fig. 5H). Thus, increasing the number, but not the per-cell function, of Ag-specific CD8 T cells results in increased killing and is consistent with the observed increase in viral control following DTA-1 treatment.

We attempted to further enhance the CD8 T cell response with additional doses of DTA-1, but found that two doses of DTA-1 (one at day 21 p.i. and another at day 23 p.i.) in fact negated the effects on LCMV-specific CD8 T cell expansion by a single dose of DTA-1 (Fig. 5I). Unlike agonist anti–4-1BB that synergized with anti–PD-L1 blockade (33), we found that a combination of DTA-1 with anti–PD-L1 or anti-IFNAR did not synergize (data not shown). Taken together, DTA-1 effects are optimal as a single 200 \(\mu\)g dose delivered at day 21 p.i., and DTA-1 treatment expands...
Ag-specific CD8, but not CD4 T cell responses to improve control of LCMV cl 13 without causing overt immune pathology.

**Agonistic anti-GITR intrinsically enhances CD8 T cell responses to LCMV cl 13**

The wide expression of GITR on T cells, NK cells, and APC [Fig. 2 and reviewed in (20)] made it possible that the effects of DTA-1 on CD8 T cell expansion were indirect due to effects on APC or other immune cells. To evaluate whether DTA-1 was acting intrinsically on the CD8 T cells in this model, we generated CD45.2

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**FIGURE 4.** Agonistic anti-GITR Ab has little or no effect on CD4 T cell or B cell responses to LCMV cl 13 infection. Mice were infected and treated as in Fig. 3A. (A) The frequency and absolute number of total CD4 T cells in the spleen were measured 2 wk following rat IgG or DTA-1 treatment. (B) Representative gating strategy for Th1, Treg, and Thf (for a more in-depth gating strategy, see Supplemental Fig. 1, which also confirms that the PD-1<sup>high</sup>
CXCR5<sup>high</sup> Tfh are also Bcl-6<sup>+</sup> and ICOS<sup>+</sup>). The frequency (C) and absolute number (D) of Foxp3<sup>+</sup> Treg, CD44<sup>high</sup> Th1, or Thf cells 2 wk following control rat IgG or DTA-1 treatment. (E) Freshly isolated splenocytes from rat IgG- or DTA-1–treated mice were restimulated ex vivo with I-A<sup>b</sup>–restricted LCMV GP<sub>13-41</sub> peptide, after which intracellular IFN-γ and IL-2 were evaluated at day 35 p.i., with representative staining shown (F). (G) The absolute numbers of total B cells and Fas<sup>+</sup>/GL-7<sup>+</sup> GC B cells from rat IgG- or DTA-1–treated mice. Each symbol represents an individual mouse, with bars indicating mean ± SEM. All parameters were measured in at least three independent repeats, with a pooled total of at least nine mice per group for (A)–(F), two independent repeats with seven to nine mice per group for (G). *p < 0.05.
the ratio of GITR+/+ : GITR−−/− D5/GP33−41−specific CD8 T cells was 2:1 (Fig. 6B, 6C, right), consistent with an intrinsic role for GITR in CD8 T cell survival (19). After DTA-1 treatment, the ratio of GITR+/+ : GITR−−/− DB/GP33–41−specific CD8 T cells increased from ~2:1 to 5:1 (Fig. 6B–D), suggesting that DTA-1 is indeed acting directly on the GITR-sufficient CD8 T cells, allowing them to outcompete the GITR−−/− T cells, rather than due to indirect effects on other cells. This ~2-fold increase in LCMV-specific CD8 T cells is reminiscent of the effects of DTA-1 that we observed in wild-type C57BL/6 mice (Fig. 5B, 5C). Importantly, we found no significant change in Foxp3+ Treg ratios or Th1 ratios between pre- and post–DTA-1 treatment (Fig. 6C, left and middle). However, we did find that the ratio of CD45.2 GITR+/+ : CD45.1 GITR−− was 2.5:1 for Th1 at day 8 p.i., implying that GITR may have an additional role in the early CD4 T cell response (Fig. 6C). In sum, we find that the expansion of LCMV-specific CD8 T cells by DTA-1 is due to direct CD8 T cell–intrinsic effects.

Discussion
Costimulatory members of the TNFR superfamily can have potent and sometimes pathological effects. For example, constitutive

FIGURE 5. Agonist anti-GITR results in a durable Ag-specific enhancement in CD8 T cell immunity to chronic LCMV infection. (A–I) Mice were treated as in Fig. 3A. (A) The frequency and absolute number of total CD8 T cells 2 wk following rat IgG or DTA-1 treatment, with effect on DNP396–404−, GP33–41−, and GP276–286−specific CD8 T cell responses and representative tetramer staining shown in (B) and (C). (D) Expression of PD-1 and Tim-3 was measured on DNP396–404−specific CD8 T cells 2 wk following rat IgG or DTA-1 treatment, with representative FACS shown. (E) LCMV GP33–41−specific CD8 T cell expression of granzyme B with representative FACS shown. (F) Splenocytes from rat IgG- or DTA-1–treated mice were restimulated with DNP-restricted LCMV GP33–41 peptide, after which intracellular IFN-γ and TNF and surface CD107a were measured by flow cytometry, with representative flow cytometry plots shown (G). (H) In vivo CTL assay of GP33–41−pulsed target cells in DTA-1 or rat IgG-treated mice, with representative staining shown for treatment groups and controls. (I) Mice were treated as in Fig. 3A with the following modification: treated with a single (1×) or two (2×) doses of 200 μg rat IgG or 200 μg DTA-1 at days 21 and 23 p.i., and the effects on DNP396–404−specific CD8 T cell responses were evaluated 2 wk later in the spleen (day 35 p.i.). Each symbol represents an individual mouse, with bars indicating mean ± SEM. Data in (A)–(D) are pooled from four independent experiments with at least 14 mice per group. Data in (E) are pooled from two independent experiments with at least seven mice per group. Data in (F) and (G) are pooled from three independent experiments with at least 11 mice per group. Data in (H) are pooled from two independent experiments with seven mice per group. Data in (I) are pooled from two independent experiments, with five to six mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001.
B cell–specific CD70 expression resulted in IFN-γ–dependent B cell depletion as well as accumulation of effector T cells and early lethality (34, 35). During chronic LCMV infection, CD27–CD70 and type I IFN signaling are pathological and disrupt splenic architecture, and, in fact, blockade of these pathways helped to restore functional immunity and viral control (10, 28, 29). It is clear that, during chronic LCMV infection in mouse or HIV infection in humans, a balance must be struck by therapeutic candidates; they must be effective, yet not so effective that the overzealous immune response is in fact pathological and/or self-limiting (36–38). In addition to self-limiting inflammation, there are also examples of ineffective therapies targeting T cell costimulatory molecules. During chronic LCMV infection in mouse or HIV infection in humans, TRAF1 is lost in virus-specific CD8 T cells so 4-1BB cannot signal, and therefore anti–4-1BB is ineffective as a therapy (12). Even when 4-1BB signaling was restored by IL-7 treatment to upregulate TRAF1, these effects were not ideal in that they resulted in an increase in the total number, but not the proportion of LCMV-specific T cells (12). This may be because anti–4-1BB agonist treatment can expand bystander 4-1BB+ memory T cells (16–18). Additionally, exogenous OX40 costimulation decreased Tfh responses and failed to lower LCMV cl 13 viral load (11). It is clear that TNFRs have varied therapeutic potential in the context of chronic infection, and that immunostimulatory potency must be tempered in the therapeutic targeting of costimulatory or coinhibitory molecules.

In this study, we find that the costimulatory TNFR family member GITR, although not desensitized like 4-1BB, is not engaged endogenously because, by the chronic stage of infection, GITRL is lost to below prestimulation levels (Fig. 1). Others have also shown that GITRL on B cells and DC is reduced to below-prestimulation levels following in vitro TLR-3, TLR-4, and TLR-9 stimulation (24), with similar findings in vivo following HSV-1 infection (25). NF-1 is a key transcription factor that binds to the Gitrl promoter, and NF-1 levels in nuclei are modulated by TLR stimulation (39).

In addition to TLR signaling, it has been reported that type I IFN can induce GITRL on several cell types, and during LCMV cl 13...
infection, a massive type I IFN response is detectable as soon as 18 h p.i. (28, 40). Taken together, it is not unreasonable to speculate that APC also upregulate GITRL in response to activation by type I IFN, explaining the peak in GITRL expression at days 2–5 p.i. Interestingly, type II IFN (IFN-γ) has been shown to impair GITR expression (40). Taken together, it is tempting to consider a model for a coordinated type II IFN regulation of GITRL expression on APC: in the early response to LCMV, pre-T cell priming, type I IFN predominates, resulting in GITRL upregulation, whereas following T cell activation, large amounts of IFN-γ are produced and may serve to downregulate GITR expression on APC as a negative feedback loop to prevent sustained and potentially otherwise pathological T cell costimulation.

GITRL is expressed at highest levels on macrophages, with ∼10-fold higher expression than the maximum level detected on conventional and plasmacytoid DC. The upregulation of GITR on macrophages was particular to LCMV cl 13 infection, as, during LCMV Armstrong infection, there is only a marginal upregulation of GITRL on macrophages, reaching only 4% of the level of cl 13 infection (Fig. 1D). It was conceivable that this was due to preferential infection of the macrophages leading to higher expression of GITRL. However, examination of the infected versus uninfected macrophages at day 8 p.i., when sufficient numbers of LCMV-infected cells had accumulated, showed that, if anything, GITRL expression was specifically reduced on LCMV-infected cells (Fig. 1). Although the staining by this time point was rather weak, it appears that LCMV-infected cells have lower levels of GITRL, although concomitantly upregulating PD-L1, the latter finding having previously been reported (28, 29). LCMV infection results in inhibition of IFN regulatory factor 3 by the LCMV NP (41), which may impair type I IFN-mediated GITRL expression, as discussed above. Despite the downregulation of GITRL on APC at the chronic stage of infection, we could circumvent this regulatory mechanism by administering an agonistic anti-GITR (DTA-1) to safely and durably enhance CD8 T cell responses and viral control.

In HSV-1 (25), acute and chronic FV (21, 42), influenza (19), and LCMV cl 13 infection, treatment with anti-GITR enhances CD8 T cell responses. In HSV and acute FV infection, activated CD8 T cells are more functional; in HSV and acute FV infection, activated CD8 T cell responses. In HSV and acute FV infection, activated CD8 T cells are more functional; in acute influenza infection, which noted a CD8 T cell–intrinsic effect of anti-GITR with no apparent influence on the CD4 T cell response (19). During chronic FV infection, Dittmer et al. (21) found that treatment with DTA-1 improved CD8 T cell responses, ostensibly via effects on Tregs, although they did not actually test this. In fact, DTA-1 only had a therapeutic effect in this model when coadministered with a supraphysiological number of FV-specific CD8 T cells. In this study, we use bone marrow chimeric mice to demonstrate a CD8 T cell–intrinsic effect of anti-GITR with no effects on Th1 or Foxp3+ Tregs, similar to previous studies using acute influenza infection, which noted a CD8 T cell–intrinsic effect of GTR with a slight, but significant increase in CD44high CD4 T cells also (19). Others have also demonstrated a CD8 T cell–intrinsic effect of DTA-1 in the poorly immunogenic B16 melanoma model (43). We find it surprising that, despite high-level GITR expression on CD4 T cells, particularly Foxp3+ Tregs, there is no obvious effect on Th1 or Treg cells following treatment with DTA-1. Additionally, it is somewhat puzzling that DTA-1 does not have a therapeutic effect on CD8 T cells at day 8 p.i. when these cells express GITR maximally. One possibility is that at day 8 p.i. there are multiple costimulatory receptors and ligands present, and the addition of exogenous GITR costimulation is redundant and therefore does not further enhance the response. However, at later time points when co-inhibitory molecules predominate and costimulatory 4-1BB is desensitized (12, 44), exogenous GITR costimulation is efficacious.

It is interesting in our model that DTA-1 results in a quantitative increase in DP'/GP34−1 and GP276–286–specific CD8 T cells through direct cell-intrinsic effects, concomitant with reductions in viral load (Figs. 3, 5). However, we do not find an increase in IFN-γ–producing CD8 T cells in response to GP33–41 peptide restimulation ex vivo. It is possible that these cells expand, but are dysfunctional, and have therefore lost the ability to produce IFN-γ (3). Thus, DTA-1 may expand CD8 T cells that have lost cytokine production but retain the ability to control viral load through their cytotoxic function. Consistently, the DTA-1–expanded CD8 T cells at the chronic stage of LCMV cl 13 infection retain a high level of granyme B, as do the LCMV-specific CD8 T cells from control rat IgG-treated mice (Fig. 5E) (44). In keeping with this, it has been demonstrated that PD-1high cells that are functionally exhausted retain cytotoxic function (45). It is worth noting that we observed a consistent although nonsignificant increase in the absolute number of IFN-γ/CD107a coproducer CD8 T cells following ex vivo LCMV D/5/GP34−1 peptide restimulation (Fig. 5F). The enhanced killing of GP34−1–pulsed targets in DTA-1–treated mice affirms that the increased accumulation, but not per-cell functional enhancement, of LCMV-specific CD8 T cells underscores the enhanced viral control in this model. The ability of GITR to expand CD8 T cells and control viral load without increasing inflammatory cytokines makes it an attractive candidate for therapy of chronic HIV infection, in which chronic inflammation contributes to disease progression (46).

Previous studies have shown that GITR induces the prosurvival Bcl-2 family member Bcl-xL in a NF-κB–dependent manner (19). Moreover, during acute infection, GITR was shown to intrinsically increase Ag-specific CD8 T cell numbers without affecting the rate of division (19). Thus, in the present model, it is likely that the major effect of anti-GITR in inducing greater numbers of CD8 T cells is mediated through effects on T cell survival.

The therapeutic use of GITR for chronic infection in humans is of particular interest. Similar to our findings in the LCMV cl 13 model, in which GITR expression is sustained at the chronic phase of LCMV cl 13 infection (Fig. 2), others have reported that GITR expression is also sustained in HIV-infected patient PBMC samples (47). Anti-GITR increased the CD4 T cell response, but not the CD8 T cell response to HIV p55 protein Ag. It is difficult to evaluate the lack of effect on CD8 T cells because these cells respond poorly to intact protein Ag. Further study on the therapeutically potential of GITR for human chronic viral infection will be of interest.

In conclusion, we demonstrate that the GITR costimulatory pathway is rendered nonfunctional at the chronic stage of infection through loss of expression of its ligand, GITRL. However, agonist anti-GITR Ab can overcome this deficit and directly expand CD8 T cells and lower viral load. Agonist anti-GITR may represent an ideal therapeutic candidate for chronic infection; although the effects are modest relative to PD-L1 blockade (5), a single dose of anti-GITR resulted in durable effects without overt immune pathology.

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Disclosures

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