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CD8 T Cell-Intrinsic GITR Is Required for T Cell Clonal Expansion and Mouse Survival following Severe Influenza Infection

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The regulation of T cell expansion by TNFR family members plays an important role in determining the magnitude of the immune response to pathogens. As several members of the TNFR family, including glucocorticoid-induced TNFR-related protein (GITR), are found on both regulatory and effector T cells, there is much interest in understanding how their effects on these opposing arms of the immune system affect disease outcome. Much work has focused on the role of GITR on regulatory T cells, little is known about its intrinsic role on effector T cells in an infectious disease context. In this study, we demonstrate that GITR signaling on CD8 T cells leads to TNFR-associated factor (TRAF) 2/5-dependent, TRAF1-independent NF-κB induction, resulting in increased Bel-2. In vivo, GITR on CD8 T cells has a profound effect on CD8 T cell expansion, via effects on T cell survival. Moreover, GITR is required on CD8 T cells for enhancement of influenza-specific CD8 T cell expansion upon administration of agonistic anti-GITR Ab, DTA-1. Remarkably, CD8 T cell-intrinsic GITR is essential for mouse survival during severe, but dispensable during mild respiratory influenza infection. These studies highlight the importance of GITR as a CD8 T cell costimulator during acute viral infection, and argue that despite the similarity among several TNFR family members in inducing T lymphocyte survival, they clearly have nonredundant functions in protection from severe infection.

The role of GITR in immune responses is complex and may be cell-type specific. Stimulation of macrophages with an anti-GITR Ab induces a proinflammatory response (4); however, recent reports have shown that GITR can play an inhibitory role for human NK cell activation (5, 6). GITR has also been extensively studied in the context of Tregs and generated much excitement when it was observed that agonistic anti-GITR Ab in cocultures of CD4+CD25+ effector T cells and CD4+CD25+ Tregs caused the abrogation of suppression by Tregs (1, 2). Interestingly, it was shown that GITR signaling on the effector T cells was necessary to render the cells less refractory to Treg-mediated suppression (7). A recent report showed that this phenomenon is not unique to CD4 T cells, and GITR signaling can induce resistance to CD4+ CD25+ Treg suppression in CD8 T cells as well (8).

In addition to enhancing T cell responses by raising the threshold for Treg suppression, GITR has also been shown to costimulate CD4 and CD8 T cells in vitro by enhancing proliferation, survival, and cytokine secretion (9, 10). GITR acts early in the immune response to lower the threshold for CD28 signaling in both CD4 and CD8 T cells (11). Whereas GITR’s costimulatory role for T cells is well documented in vitro, much remains to be elucidated as to its function in vivo, particularly on CD8 T cells. Notably lacking from the literature is any thorough assessment of GITR’s physiological role on CD8 T cells in an infectious disease context. Although a few studies have shown that agonistic anti-GITR Ab can enhance antiviral CD8 T cell responses in vivo (12–14), the mechanism whereby this occurs remains to be determined, as GITR is broadly expressed and its signaling on other immune cells may influence the CD8 T cell response indirectly. Clearly, more defined models are needed to study the intrinsic role of GITR on CD8 T cells in response to pathogens in vivo.

In this study, we sought to address the role of GITR on CD8 T cells during viral infection. By using an adoptive transfer model in which GITR is only absent on the responding CD8 T cells, we show that GITR is intrinsically required on CD8 T cells for maximal primary and secondary CD8 T cell responses to influenza. Moreover, we demonstrate that GITR-specific agonistic Abs enhance the response to influenza virus through direct effects of GITR on CD8 T cells. Although GITR does not influence cell division, it augments CD8 T cell survival by upregulating the prosurvival...
molecule Bcl-XL in a NF-κB–dependent manner. We found that GITR-induced NF-κB activation is positively regulated by both TNFR-associated factor (TRAF) 2 and 5, whereas TRAF1 is dispensable for this survival signaling. Although the absence of GITR on T cells did not affect disease outcome during mild influenza virus infection, during a severe and potentially lethal model of influenza infection we show that GITR on CD8 T cells augmented viral clearance and protected mice from death. These results highlight the importance of GITR as a CD8 T cell-intrinsic costimulatory receptor.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). CD45.1 and Thy1.1 congenic mice were crossed with OT-I mice (The Jackson Laboratory, Bar Harbor, ME). GITR−/− mice have been previously described (15). GITR−/− mice were backcrossed onto the C57BL/6 background for at least eight generations and then further crossed to generate CD45.1 GITR−/− OT-I mice. TRAF1−/− mice (16) were originally provided by E. Tisitsisok (Center for Blood Research, Boston, MA), and crossed to OT-I TRASC transgenic mice, as previously reported (17). Mice were maintained under specific pathogen-free conditions in sterile microisolator cages. Animal studies were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

Influenza virus infection

Six- to 10-wk-old C57BL/6 and GITR−/− mice were infected intranasally (i.n.) with 5 hemagglutinin units (HAU) influenza A/HK-X31 (H3N2). Thirty days postinfection, some mice were challenged with 5 HAU influenza A/PR8 (H1N1). For survival experiments using influenza A/PR8, mice were infected i.n. with 105 tissue culture infectious dose 50 (TCID50)/mouse. For immunization after adoptive transfer of wild-type (WT) GITR−/− OT-I T cells, mice were infected with 5 HAU and 6118 TCID50/mouse A/HK-X31-OVA (X31-OVA) or 105 TCID50/mouse A/PR8-OVA (PR8-OVA) (18), both generously provided by P. Doherty and P. Thomas (St. Jude Children’s Research Hospital, Memphis, TN). At the indicated times after X31-OVA infection, spleen, mediastinal lymph node, bone marrow, and lung were harvested for IFN-γ (BD Biosciences) and MHC class I tetramers were obtained from the National Institute for Allergy and Infectious Diseases tetramer facility (Emory University, Atlanta, GA). Influenza nucleoprotein (NP)356–374-specific CD8 T cells were surface stained with anti-CD8α, anti-CD45R0, and anti-CD44 (eBioscience, San Diego, CA), and D7/NP356–374 tetramers. For intracellular IFN-γ staining, splenocytes were restimulated with 1 μM NP356–374 for 6 h with Golgi Stop (BD Biosciences, San Jose, CA) at 37˚C. Cells were surface stained as above, fixed, and intracellularly stained for IFN-γ (BD Biosciences). Adoptively transferred OT-I cells were detected using anti-Thy1.1 and anti-CD45.1 (eBioscience) in conjunction with anti-CD8α. For intracellular IFN-γ staining, splenocytes and lymphocytes isolated from lung were restimulated at 37˚C with 1 μM OVA323–342 using Golgi Stop for 6 and 4 h, respectively. Cells were then surface stained for CD8, CD45.1, and/or Thy1.1, fixed, and intracellularly stained for IFN-γ (BD Biosciences). For detection of degranulation, 5 μg/ml anti-CD107a (BD Biosciences) was added at the beginning of the restimulation culture. Fluorescence minus one (FMO) controls or unstimulated samples (no peptide) were used as negative controls. Certain experiments also required staining with anti-GITR, anti-CD69, anti-CD44, anti-CD4, and anti-CD25 (eBioscience). Foxp3 staining was performed using a mouse Treg staining kit (eBioscience). Staining for apoptosis was carried out by adding annexin V (BD Biosciences) to surface-stained cells. Samples were analyzed using a FACSCalibur (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

T cell isolation, adoptive transfers, and agonistic Ab studies

WT and GITR−/− OT-I T cells were purified from lymph nodes and spleens of naive mice using a negative selection mouse CD8 T cell enrichment kit (StemCell Technologies, Vancouver, Canada). For memory experiments, memory-like WT and GITR−/− OT-I cells were generated, as previously described (19). In certain experiments, T cells were stained with 1 μM CFSE for 10 min at 37˚C, T cells were injected i.v. at 106 cells/mouse (for ratio experiments 5 ×104 of each cell type/mouse) with the exception of CFSE experiments, which used 105 cells/mouse, and PR8-OVA experiments, which used 103 cells/mouse. A day later, the mice were infected with X31-OVA or PR8-OVA, as described above. For experiments using agonistic Abs, X31-OVA was administered i.p. at a dose of 100 HAU/mouse with either 200 μg anti-GITR (DTA-1) hybridoma (2) or purified rat IgG (Sigma-Aldrich, St. Louis, MO).

Cell viability assay

WT and GITR−/− OT-I CD8 T cells were isolated from naive mice and cultured in vitro with irradiated splenocytes from C57BL/6 mice at a ratio of 1:4. Cultures were stimulated with a subdosepoint of SINFEKL (10–30 M), and in some cases T cells were CFSE labeled, as described above. CFSE analysis was performed on day 2, and annexin V staining of cultures was performed on days 0, 2, and 3.

Signaling studies: small interfering RNA knockdowns

OT-I T cells and, in some experiments, TRAF1−/− OT-I cells were stimulated in vitro at 0.1 μg/ml SINFEKL. Cells were subjected to lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) on day 3 of the culture, rested, and then stimulated with either 10 μg/ml DTA-1 or rat IgG. In some experiments, cells were preincubated with either 20 μg/ml NF-κB inhibitor 1-pyrrolidinecarboxi- dothioic acid (PDTC), ammonium salt (Calbiochem, San Diego, CA), or vehicle for 1 h prior to DTA-1 or rat IgG stimulation. For small interfering RNA (siRNA) knockdown experiments, cells were cultured with 20 ng/ml IL-15 (R&D Systems, Minneapolis, MN) for 72 h after lympholyzing. A total of 9 × 104 OT-I cells was then transfected with 1 μM siRNA targeting TRAF2, TRAF5, both TRAF2 and TRAF5, or a control scrambled duplex RNA (IDT Technologies, Coralville, IA). Transfections were performed using the Amaxa mouse T cell Nucleofector kit (Lonza, Cologne, Germany). Cells were rested for 20 h and then stimulated with 10 μg/ml of either DTA-1 or control rat IgG.

Western blots

Cells were lysed in 1% Nonidet P-40 with complete protease inhibitor mix (Roche, Basel, Switzerland). Lysates were quantified using a bichinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Lysates were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Membranes were probed with Abs specific for IκBα, Bcl-xL, TRAF2 (Cell Signaling Technology, Beverly, MA), TRAF5 (Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (Sigma-Aldrich), and incubated with HRP-conjugated anti-rabbit or anti-goat IgG (Sigma-Aldrich, Oakville, Ontario, Canada). Signals were revealed by chemiluminescence (GE Healthcare, Baie D’Urfé, Quebec, Canada) and visualized by autoradiography. Where indicated, quantitation was performed using Quantity One software (Bio-Rad, Hercules, CA).

Viral clearance

Lungs were excised from mice at various time points after X31-OVA and PR8-OVA infection and then homogenized in RPMI 1640 medium (1 g lung tissue/10 ml). Supernatant was obtained and stored at −70 ºC. Viral loads were determined by the Madin-Darby canine kidney assay with the Reed and Muench technique, as previously described (20).

Statistical analysis

Where indicated, p values were obtained using the Student t test, unpaired or paired for ratio experiments (two tailed, 95% confidence interval). The log-rank test was used to determine significance for mouse survival experiments. Statistically significant differences are indicated as *p < 0.05, **p < 0.01, or ***p < 0.001.

Results

GITR on the T cells is essential for maximal CD8 T cell responses to influenza virus

To examine the role of GITR on CD8 T cells during viral infection in vivo, we i.n. infected GITR−/− and WT C57BL/6 mice with influenza A/HK-X31 (H3N2) virus. Some mice were challenged with the serologically distinct influenza A/PR8 (H1N1) 30 d after infection.
the initial X31 infection to examine the secondary response (Supple-
mental Fig. 1 A). Ag-specific CD8 T cell responses to the
immunodominant NP366–374 epitope were assessed using fluo-
rescently labeled H-2Kd/3NP366–374 tetramers. Decreased influenza-
specific CD8 T cell numbers were observed in GITR−/− mice in
a subset of organs, but only at particular times of the immune
response (Supplemental Fig. 1B–F). As GITR can be expressed on
many different cell types during the course of an infection (3), it
was possible that the lack of GITR signaling on other cell types in
the local environments, such as Tregs, was indirectly affecting the
CD8 T cell response.

Therefore, to dissect the CD8 T cell-intrinsic effects of GITR
during viral infection in vivo, we crossed GITR−/− mice with
CD45.1 OT-I mice, whose CD8 T cells have transgenic TCRs
specific for the H-2Kd/3/OVA257–264 epitope. GITR−/− mice had
been backcrossed at least eight times before a further backcross to
generate the GITR−/− OT-I transgenic mice. Purified CD8 T cells
from CD45.1 GITR−/− OT-I and Thy1.1 WT OT-I mice were mixed
at a ratio of 1:1 and adoptively transferred into WT CD45.2 re-
pipients (Fig. 1A, 1B). One day later, mice were infected with
influenza A/HK-X31-OVA (X31-OVA), which carries the OVA257–264
epitope in its neuraminidase stalk (18). The ratio of WT to GITR−/−
OT-I T cells at the peak of the response was >1 in all organs
assayed, indicating significant defects in the recovery of GITR−/−
OT-I T cells in spleen, lung, mediastinal lymph node, and bone
marrow (Fig. 1C, 1D). Differences ranged from 2- to 4-fold, de-
dpending on the particular organ. A similar defect in GITR−/−
T cell recovery was observed at days 7, 9, and 12 postinfection (Fig.
1E). Thus, the defect in recovery of GITR-deficient CD8 T cells
could not be attributed to a difference in the kinetics of expansion
between WT and GITR−/− OT-I T cells. Therefore, these results
demonstrate that GITR is required on the CD8 T cells for their
maximal expansion during the primary response to influenza virus.

Competitive models can sometimes exacerbate differences be-
tween two cell populations, as each population is forced to compete
for the same pool of resources. As the previous experiments were
performed in a competitive fashion, we also investigated whether
GITR−/− OT-I cells would show a similar impairment when they
did not have to compete with OT-I cells for survival factors. When
CD45.1 OT-I or CD45.1 GITR−/− OT-I T cells were injected into
separate mice and infected with X31-OVA, similar defects were
observed, although slightly delayed, particularly in the lung (Sup-
plemental Fig. 2).

Although the GITR−/− OT-I mice had been extensively back-
crossed (n = 9), to rule out any differences in cell recovery due to
rejection, we injected a 1:1 mix of in vitro expanded (see Ma-
terials and Methods and below) memory-like WT and GITR−/−
OT-I T cells (3 million cells total) into mice and monitored their
perseverance without infection. At day 21 postinjection, both cell
types could still be detected, and the GITR−/− T cell numbers
were similar and, if anything, slightly higher in number than their
WT counterparts (data not shown), indicating that the defects
observed at day 7–12 of the primary response could not be at-
tributed to rejection of the knockout cells.

**GITR regulates CD8 T cell numbers, but not effector function**

Multiple costimulatory molecules in the TNFR superfamily have
been implicated in enhancing not only the magnitude of the T cell
response, but also its quality (21, 22). To assess whether GITR was
also regulating effector function of the responding CD8 T cells,
we examined IFN-γ production and the expression of CD107a,
a marker of degranulation, on WT and GITR−/− OT-I cells at the
peak of the primary response (Fig. 1F, 1G). Despite the fact that
there were fewer GITR−/− OT-I T cells overall, the proportion of
GITR−/− cells that was producing IFN-γ and expressing CD107a
was similar to the proportion of WT OT-I cells showing this
phenotype. Thus, whereas GITR is critical for maximal CD8 T
cell expansion in the primary response to influenza, it does not
influence effector function per T cell.

**GITR on the T cells is essential for their secondary expansion
in response to influenza virus**

Under some circumstances, memory T cells are less dependent on
costimulation than their naive counterparts (23). Therefore, we next
assessed whether GITR would play a role in the recall CD8 T cell
response to influenza virus. As there were already significant
defects in CD8 T cell numbers after the primary response, to avoid
the complications of these defects and focus on the effect of GITR
during the secondary response, we generated memory-like cells in
vitro, by culturing WT and GITR−/− OT-I splenocytes with Ag
followed by IL-15 treatment (19). At the end of the cultures, the WT
and GITR−/− OT-I cells showed a similar memory phenotype:
CD44high, CD69low, with GITR expression detected on the WT cells
(Fig. 2A). A 1:1 mixture of Thy1.1 OT-I and CD45.1 GITR−/− OT-I
memory-like cells was adoptively transferred into WT recipient
mice, and, a day later, the mice were challenged with X31-OVA (Fig.
2A). At several time points throughout the response, the number of
WT OT-I cells consistently outnumbered the number of GITR−/−
OT-I cells in all organs examined (Fig. 2C, 2D). As in the primary
response, the proportion of WT and GITR−/− cells producing IFN-
γ and expressing CD107a after ex vivo stimulation was approxi-
ately equal (Fig. 2E, 2F). GITR, therefore, is important for the
maximal expansion of CD8 T cells in both the primary and recall
response to influenza virus, and this effect is intrinsic to the
CD8 cells.

**Agonistic anti-GITR Ab enhances effector T cell expansion
during direct effects on the CD8 T cells**

The agonistic anti-GITR Ab DTA-1 enhances effector T cell responses in vivo (3). DTA-1 was originally thought to mediate its
effects by directly inhibiting Treg suppression (1, 2). However,
this has recently come under question (7, 24), as GITR can also
costimulate Tregs, causing them to
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FIGURE 1. Analysis of CD8 T cell-intrinsic effects of GITR during influenza infection. A, Thy1.1 OT-I and CD45.1 GITR$^{-/-}$ OT-I CD8 T cells ($5 \times 10^3$ of each) were injected as a 1:1 mixture into WT mice. One day later, the mice were infected i.n. with X31-OVA. On days 7, 9, and 12, the mice were sacrificed, and the ratio of WT/GITR$^{-/-}$ OT-I T cells was analyzed. Data are representative of two independent experiments, each using four to five mice. B, Representative FACS plot of original 1:1 Thy1.1 OT-I/CD45.1 GITR$^{-/-}$ OT-I ratio upon injection. Plot gated on CD8 T cells. C, Ratio of WT/GITR$^{-/-}$ OT-I T cells in various organs at day 9. D, Representative FACS plots of Thy1.1 and CD45.1 gating for ratio determination. E, Kinetics of WT and GITR$^{-/-}$ OT-I primary response. Graphs show percentage of transferred cells of total CD8 T cells at days 7, 9, and 12. At each time point, five mice were used. F and G, Percentage of IFN-$\gamma$-positive (F) and percentage of CD107a-positive cells (G) of transferred Thy1.1 OT-I or CD45.1 GITR$^{-/-}$ OT-I cells. Representative FACS plots of data in F and G are shown on the left. Plots are gated on CD8 T cells and then either Thy1.1 or CD45.1 cells. No Ag, unstimulated controls.
FIGURE 2. Role of CD8 T cell-intrinsic effects of GITR in recall responses of OT-I memory T cells in vivo. A, Thy1.1 OT-I and CD45.1 GITR−/− OT-I memory-like T cells were generated in vitro and were injected as a 1:1 mixture into WT mice as in Fig. 1. A day later, the mice were infected i.n. with X31-OVA. On days 6, 8, and 10, the mice were sacrificed, and the ratio of WT/GITR−/− OT-I T cells was analyzed. Data are representative of two independent experiments, each using four to five mice.

B, GITR, CD69, and CD44 expression on naive, activated, and memory T cells was analyzed at days 0, 2, and 9, respectively, of in vitro memory cell culture.

C, Ratio of WT/GITR−/− OT-I memory-like T cells in various organs at day 8. D, Kinetics of WT and GITR−/− OT-I secondary response. Graphs show percentage of transferred cells of total CD8 T cells at days 6, 8, and 10 in various organs. At each time point, four mice were used.

E, Percentage of IFN-γ-positive cells of transferred Thy1.1 OT-I or CD45.1 GITR−/− OT-I T cells in spleen and lung. F, Percentage of CD107a-positive cells of transferred Thy1.1 OT-I or CD45.1 GITR−/− OT-I T cells in spleen.
Whereas DTA-1 can costimulate both CD4 effectors and Tregs in our model, the increase in CD8 T cell expansion observed with DTA-1 treatment was found to be almost exclusively through CD8 T cell-intrinsic effects.

Role of GITR in the survival of CD8 T cells responding to influenza infection

Having demonstrated that GITR is essential for maximal CD8 T cell responses to influenza infection, we next sought to dissect the mechanism by which GITR was regulating cell recovery. The defect in numbers of GITR−/− OT-I cells in both the primary and recall responses of the i.n. influenza model could be attributable to differences in homing or a defect in either proliferation or survival. To study homing, the seeding of the cells was examined prior to influenza infection, 24 h after a 1:1 mixture of WT:GITR−/− OT-I cells was injected into mice. The ratio in all organs examined was close to 1 (data not shown), implying that the cells homed in an equal manner, at least before they encountered Ag. Moreover, the finding that defects in cell recovery of GITR−/− CD8 T cells were observed in all organs examined, and that ki-
nomic studies in the lung showed equal numbers of both WT and GITR−/− OT-I T cells early, with defects accumulating only later, argues against a trafficking defect.

To investigate whether the defect in recovery of GITR−/− OT-I T cells was due to a proliferative difference, WT mice were injected with CFSE-labeled WT or GITR−/− OT-I cells and infected with X31-OVA. We observed that both the number of divisions and the proportion of WT and GITR−/− OT-I cells that had undergone cell division were very similar (Fig. 4A). These results suggest that GITR is dispensable for Ag-dependent CD8 T cell division in vivo.

It is difficult to examine cell death in vivo because apoptotic cells are cleared very quickly by macrophages (26). Therefore, to test whether GITR−/− OT-I cells had a survival defect, we moved to an in vitro model. Equal numbers of either WT or GITR−/− OT-I CD8 T cells were cultured with irradiated B6 splenocytes and a suboptimal dose of peptide (10−10 M). At the beginning of the culture, the percentage of annexin V and propidium iodide-positive WT and GITR−/− OT-I cells was approximately equal (data not shown). By day 2, the cells had begun to proliferate and CFSE labeling indicated that approximately equal numbers of WT and GITR−/− OT-I cells had undergone division (Fig. 4B), consistent with the in vivo data. Whereas the proliferation of the two populations was similar, there was a marked difference in the survival of WT versus GITR−/− OT-I cells. On day 2, a higher proportion of GITR−/− OT-I cells compared with OT-I cells was annexin V positive and primed to undergo apoptosis (Fig. 4C, 4D). This trend became statistically significant by day 3, as the GITR−/− OT-I cells became increasingly annexin V positive. Interestingly, at a peptide dose that was too low to induce proliferation (10−11 M), both WT and GITR−/− OT-I cells had the same percentage of annexin V-positive cells (data not shown). This result implies that the difference in survival of WT and GITR−/− cells is TCR signal dependent and not due to some intrinsic homeostatic difference between the WT and GITR−/− OT-I cells. Based on these data, we infer that GITR signaling on CD8 T cells is required for their in vivo survival.

**GITR enables survival signaling via enhanced Bcl-xL expression downstream of NF-κB**

Activation of the NF-κB signaling pathway is essential for cell survival. It was previously reported that GITR can initiate NF-κB signaling in primary T cells (11, 25, 27). To confirm this in our model, OT-I CD8 T cells were activated with peptide and subsequently stimulated with anti-GITR (DTA-1) or rat IgG. Activating the cells first and then stimulating with DTA-1 allows GITR to be upregulated above basal levels before its triggering, mimicking the situation that would occur during an Ag-specific immune response in vivo. Stimulating activated CD8 T cells with DTA-1 led to NF-κB activation as observed by IκBα degradation (Fig. 5A). In contrast, stimulation with control rat IgG did not lead to significant IκBα degradation.

To further dissect the mechanism by which GITR enables survival signaling, we examined the expression of Bcl-xL, a prosurvival molecule downstream of NF-κB (28). When activated OT-I cells were stimulated with DTA-1, Bcl-xL expression was significantly enhanced compared with rat IgG stimulation, as measured at 3 h and as late as 20 h poststimulation (Fig. 5B). In contrast, when the OT-I cells were preincubated with an NF-κB inhibitor, PDTC, DTA-1 stimulation could no longer induce Bcl-xL to levels greater than in the rat IgG control, as measured at 3 h poststimulation (Fig. 5C). Similar results were seen at 20 h (data not shown); however, by this time the cells treated with the NF-κB inhibitor were beginning to die, presumably from the inhibition of this critical pathway. These results are consistent with the idea that GITR promotes survival of CD8 T cells through downstream NF-κB signaling, which in turn promotes the expression of Bcl-xL.

**FIGURE 4.** GITR on CD8 T cells is dispensable for proliferation, but required for CD8 T cell survival. A, A total of 105 CFSE-labeled CD45.1 OT-I or CD45.1 GITR−/− OT-I T cells was transferred into WT mice. One day later, mice were infected with X31-OVA and 3 d postinfection were sacrificed and CFSE analyzed via flow cytometry. Shown are representative FACS plots of CD45.1 gating and CFSE staining of transferred cells. Four to five mice were used per group in three independent experiments. B–D, Purified CFSE-labeled WT and GITR−/− OT-I T cells were cultured with irradiated splenocytes and 10−10 M SIINFEKL. B, CFSE analysis of WT and GITR−/− OT-I cells at day 2 of culture. C, Annexin V analysis on days 2 and 3. Plots are gated on CD8 CD45.1 T cells. D, Percentage of annexin V-positive cells of CD45.1 cells on days 2 and 3. Data in all panels are representative of two to four independent experiments.
results suggest that GITR regulates CD8 T cell survival, at least in part, by upregulating the expression of the prosurvival molecule Bcl-xL downstream of NF-κB signaling.

**TRAF2 and TRAF5, but not TRAF1, enhance GITR-induced NF-κB survival signaling**

Signaling downstream of TNFR family members is known to be mediated by the recruitment of TRAFs to the activated receptor (29). To further understand how GITR induces survival signaling, we investigated the role of several TRAF family members in mediating activation of NF-κB downstream of GITR. GITR has been shown to bind TRAFs 1, 2, and 3 via yeast-2-hybrid and overexpression studies (30, 31). In addition, TRAF5 has been shown to enhance NF-κB signaling downstream of GITR (32). The role of TRAF2 in GITR-mediated NF-κB signaling remains controversial (30, 31), and TRAF1, which is known to modulate TRAF2 signaling (33–36), has not been well investigated downstream of GITR. We sought, therefore, to determine the role of TRAFs 1, 2, and 5 in the activation of NF-κB downstream of GITR in CD8 T cells. To do this, we made use of siRNA to knock down TRAF2 and TRAF5, as well as use of TRAF1−/− OT-I mice. OT-I cells were activated in vitro and transfected with siRNA targeting T2, T5, both, or a scrambled control. Representative blots of TRAF2 and TRAF5 siRNA knockdowns. E, siRNA-treated cells were stimulated with either DTA-1 or rat IgG and analyzed for Bcl-xL expression. Representative IκBα blot at 30 min poststimulation. F, Graphs show DTA-1/rat IgG IκBα ratio at 15 and 30 min. Data are pooled from two independent experiments. KD, knockdowns; Scr, scrambled; T2, TNFR-associated factor 2; T5, TNFR-associated factor 5.
GITR protects against death following severe influenza infection

Although GITR was required for maximal T cell expansion during mild influenza infection (Fig. 1), it had no impact on mouse survival (data not shown). Therefore, to examine the potential role of GITR on influenza disease outcome, we turned to a more severe respiratory infection model using influenza A/PR8. Following i.n. infection with influenza A/PR8 (Fig. 6A), only 25% of the GITR<sup>−/−</sup> mice survived the infection under conditions in which 62% of the WT mice fully recovered (p < 0.05). These results indicated that GITR can protect mice from death in a lethal influenza model.

To further investigate whether GITR’s protective function was due to its intrinsic role on CD8 T cells, we transferred WT or GITR-deficient naive OT-I T cells into mice and then challenged them with X31-OVA or influenza A/PR8-OVA (PR8-OVA) (18). The X31-OVA induces a mild respiratory disease whereby mice lose ~5–10% of their body weight, but clear the virus by day 8 and completely recover. Under these circumstances, the absence of GITR on the T cells had no effect on initial weight loss or viral clearance and 100% of mice survived (Fig. 6B, 6C). In contrast, infection with PR8-OVA causes up to 30% weight loss and can be lethal in mice. Previous results from our laboratory had shown that transfer of just 1000 naive OT-I T cells to mice prior to infection with PR8-OVA can protect WT mice from death, whereas transfer of high numbers of T cells abrogates the protective effect due to immune pathology (37). Using this optimized T cell dose, we observed that 100% of the mice that received 1000 GITR<sup>−/−</sup> OT-I cells or no transferred cells succumbed to the infection. In contrast, 40% of the mice that received WT OT-I cells recovered and survived (Fig. 6D). Thus, the absence of GITR on CD8 T cell recapitulates the mouse survival defect after influenza infection seen when GITR is absent on all cells. Although the absence of GITR on the CD8 T cells had no effect on the initial weight loss (Fig. 6E) induced in response to the early innate response to the virus, the mice that received GITR<sup>−/−</sup> OT-I cells had a 5.6-fold (0.75 log) higher viral load by day 6 (Fig. 6F) and were visibly sicker at day 7–8 postinfection as compared with those that had received 1000 WT OT-I cells. Thus, CD8 T cell-intrinsic GITR can protect mice from death during severe respiratory influenza infection, but is dispensable during mild respiratory influenza infection.

Discussion

GITR is emerging as an important molecule for regulating CD8 T cell responses. Moreover, the use of agonistic anti-GITR Abs to
augment immune responses in vivo is of interest as a therapeutic tool. In administering GITR agonists in a therapeutic context, it will be important to understand GITR’s mechanisms of action and its cellular targets. Until now, the intrinsic role of GITR on CD8 T cells in vivo has been unclear. In this study, we make use of transgenic GITR−/− OT-I T cells to dissect the intrinsic role of GITR on CD8 T cells during influenza infection. We demonstrate that GITR is required on the CD8 T cells for maximal CD8 T cell expansion. Moreover, GITR on the CD8 T cells is also required for the enhancement of their responses following systemic administration of agonistic anti-GITR Ab. Whereas GITR did not enhance CD8 T cell proliferation, it played a key role in CD8 T cell survival. Under conditions of severe respiratory influenza infection, the presence of GITR on CD8 T cells led to augmented viral clearance and protection of the mice from death.

We first examined the NP366–374-specific CD8 T cell response when GITR−/− or WT C57BL/6 mice were infected with a mild strain of influenza. GITR−/− mice, however, showed defects only in certain organs at certain times. The lack of concordance between the GITR−/− OT-I adoptive transfer model, in which defects were seen in all organs examined, and the complete knockout, which showed only a subset of these defects, is most likely due to indirect effects of the lack of GITR on other cell types. Whereas GITR is present on naïve T cells with increased expression upon infection, GITR can also be expressed on dendritic cells, macrophages, NK cells, NKT cells, and B cells upon infection. Furthermore, it is well established that GITR is highly expressed on CD4+CD25+ Tregs and aids in their costimulation. It has been noted that immune responses are highly dependent on the ratio of effector T cells to Tregs in the local environment. Therefore, because GITR works to costimulate both effector and Tregs, it is possible that the defects seen in one organ and not the others are due in part to differences in the ratios of effector T cells to Tregs in the various organs. In addition, it has been shown that GITR can negatively regulate dendritic cell (DC) function. DCs from GITR−/− mice have been shown to more effectively stimulate IL-2 and IFN-γ production from CD4 T cells than GITR−/+ DCs (38). It is difficult, therefore, to interpret the role of GITR on CD8 T cells in a model in which GITR may be interacting on multiple cell types to influence the CD8 T cell response indirectly.

Using the GITR−/− OT-I adoptive transfer model, we showed profound recovery defects of GITR−/− CD8 T cells in both the primary and recall response. Having ruled out rejection, kinetic, proliferative, and homing differences between WT and GITR−/− OT-I T cells, we showed that GITR is essential for CD8 T cell survival. Consistent with this, we demonstrated that GITR signaling induces NF-κB, which in turn leads to induction of the prosurvival molecule Bcl-xL. Riccardi and colleagues (11) previously showed that GITR is required for CD28-mediated upregulation of Bcl-xL expression. In this study, we have observed that Bcl-xL expression is enhanced directly downstream of GITR-mediated NF-κB signaling, as early as 3 h after GITR ligation.

It is well established that GITR can activate NF-κB signaling (3). Recently, it was shown that several TNFR family members, including GITR, are capable of activating both the canonical and noncanonical NF-κB pathways (39, 40). In the current study, we show that the canonical NF-κB pathway is rapidly activated downstream of GITR signaling, requiring both TRAF2 and TRAF5. Our data are consistent with previous studies showing that TRAF5 is essential for CD8 T cell survival (41) and for maximal NF-κB activation downstream of GITR in CD4 T cells (32). There has been controversy, however, as to the role of TRAF2 in activating NF-κB downstream of GITR signaling. Kwon et al. (31) report TRAF2 to be a positive regulator of GITR-dependent NF-κB signaling, in agreement with our data and consistent with studies showing that TRAF2 and TRAF5 play redundant roles downstream of TNF signaling (42). Esparza and Arch (30), however, have reported that GITR uses TRAF2 in a novel fashion as an inhibitor of NF-κB activation. Both Kwon et al. and Esparza and Arch overexpressed TRAF2 and performed a NF-κB luciferase assay 24 h after GITR stimulation. This assay does not distinguish between activation of the canonical and noncanonical NF-κB pathways. As TRAF2 has been shown to be positive for activation of the canonical NF-κB pathway, but negative for activation of the noncanonical NF-κB pathway (43), the NF-κB luciferase assay readout would be a balance between these two roles and may depend on the levels of TRAF2 expression. Our study specifically tested the role of TRAF2 and TRAF5 in the canonical pathway of NF-κB activation downstream of GITR in primary T cells and showed that they have additive and positive effects.

TRAF1 has been shown to bind to GITR in a yeast–2-hybrid assay (30) and in overexpression studies (31); however, its role in GITR signaling in primary T cells has not been examined. We observed that TRAF1−/− OT-I CD8 T cells could initiate NF-κB signaling in a manner similar to WT cells, indicating that TRAF1 is dispensable for GITR-induced NF-κB activation. However, it is possible that TRAF1 has a redundant role in NF-κB signaling, which in its absence can be compensated for by TRAF2 and TRAF5.

Several studies have shown that the agonistic anti-GITR Ab, DTA-1, enhances both CD8 and CD4 T cell responses to viruses and tumors in vivo (3). It is not known, however, whether this is a direct consequence of GITR ligation on the T cells or whether it occurs indirectly through effects on other cell types. Earlier studies attributed the DTA–1–induced effector T cell expansion to an indirect mechanism whereby the Ab was targeting Tregs and attenuating their function (1, 2). Indeed, it has been shown that DTA-1 can cause the proliferation of Tregs, although whether this enhances or abrogates their suppression remains controversial (3, 24). DTA-1 can also costimulate effector T cells (9, 10) and cause them to become more refractory to Treg suppression (7). A few groups have attempted to use depletion studies to rule out the role of DTA-1 on various cellular subsets, such as Tregs, thereby suggesting a direct role for DTA-1 in T cell costimulation (44, 45). To our knowledge, however, no study has been able to directly address the question of whether GITR is required on CD8 T cells for DTA-1—induced expansion. By using GITR−/− OT-I T cells, we show that the CD8 T cells lacking GITR failed to expand in response to DTA-1, with responses similar to those of WT OT-I cells treated with rat IgG. Therefore, we conclusively show that DTA–1 ligation of GITR on CD8 T cells is essential for maximal CD8 T cell responses to influenza, and that in this model DTA–1 ligation on other cell types, such as Tregs, does not play a major role.

In apparent contrast to the current study, a recent paper demonstrated that transgenic expression of GITRL on B cells expands CD4 T cells, but not CD8 T cells in mice (46). This might reflect the failure of CD8 T cells and B cells to efficiently interact in vivo.

We demonstrate that GITR is essential for the survival of influenza-specific CD8 T cells, with little effect on proliferation or effector function per cell. In contrast, other studies have shown that administration of agonistic GITR Abs in viral or cancer models can enhance both the effector function and proliferation of CD8 T cells (3). It is likely that the administration of the anti-GITR Ab gives a supraphysiological signal, which is much stronger than that of the endogenous GITRL in the OT-I model. Therefore, it is possible that the signal from the agonistic Ab would be sufficient to drive enhancement of proliferation and effector function, whereas the endogenous signal is not. It should be noted that in our studies, the addition of agonistic Abs to in vitro cultures with suboptimal
peptide did not substantially enhance proliferation (data not shown) and therefore the effects of Ab may depend on the dosage and administration schedule used. Physiologically, however, our study shows that GTR’s main role on CD8 T cells in vivo is in promoting their survival.

A striking difference was observed in the requirement for GTR on CD8 T cells for disease outcome in mild versus severe influenza infection. Although expansion of CD8 T cells during infection with X31-OVA is impaired when the transfected T cells lack GTR, the mice still readily handle this infection and fully recover with complete viral clearance that is indistinguishable from mice receiving WT T cells. This is likely because in the milder influenza infection model, the combination of endogenous CD4, CD8, and Ab responses is able to readily control the virus (47). In contrast, in a more severe infection model with PR8-OVA, transfer of 1000 WT T cells protected 40% of the mice from death, whereas 100% of mice that received either no cells or 1000 GTR-deficient T cells succumbed to the infection. This decreased survival of mice that received GTR-deficient T cells as compared with WT T cells was associated with a 6-fold higher viral load at day 6 of the infection. As the only difference between the two groups is the presence of GTR on the transfected T cells, this argues that GTR on the CD8 T cells contributes to viral clearance and mouse survival. It is of interest to note that the mouse survival defect observed in the adoptive transfer model with GTR-deficient OT-I cells and PR8-OVA infection recapitulates the defect seen when the complete knockout GTR−/− mice are infected with unmodified PR8. This suggests that whereas GTR on other immune cell types may aid in the protection of mice from lethal influenza, GTR on CD8 T cells most likely plays a major protective role.

Our laboratory has previously shown that the TNF family ligand 4-1BB is also required for survival to severe influenza virus, but dispensable during mild flu infection (37). In that study, however, it was not determined whether this was a T cell-intrinsic effect. It has been suggested that the large number of TNF family receptors and ligands may have arisen by gene duplication, followed by diversification (48). The finding that GTR and 4-1BB costimulatory pathways are critical for mouse survival during severe influenza infection leads us to speculate that survival against severe viral infections may have contributed to the accumulation of TNF/TNFR costimulators during evolution. In contrast, there may also be circumstances in which such increased responses are pathological (49).

In summary, this study extensively describes the role of GTR on CD8 T cells during viral infection. We show that GTR is essential for maximal primary and secondary CD8 T cell responses to influenza. GTR is required for the survival of CD8 T cells, and GTR signaling induces TRAF2- and TRAF5-mediated NF-kB activation, leading to enhanced Bcl-x, protein expression. Moreover, during severe influenza infection, GTR on CD8 T cells can play a critical role in augmenting viral clearance and decreasing mouse mortality.

In addition, we demonstrate that GTR is required on CD8 T cells for agonistic anti-Ab-mediated CD8 T cell expansion in response to influenza virus, thus providing a mechanism for the potential immunotherapeutic use of anti-GTR agonistic Ab.

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Disclosures
The authors have no financial conflicts of interest.

References