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Engagement of Glucocorticoid-Induced TNF Receptor Costimulates NKT Cell Activation In Vitro and In Vivo

Hyun Jung Kim,*† Hye Young Kim,*† Byoung Kwon Kim,* Sanghee Kim,§ and Doo Hyun Chung2*‡

Glucocorticoid-induced TNF receptor (GITR) is known to provide costimulatory signals to CD4+CD25− and CD4+CD25+ T cells during immune responses in vivo. However, the functional roles of GITR expressed on NKT cells have not been well characterized. In this study, we have explored the functions of GITR as a costimulatory factor on NKT cells. GITR was found to be constitutively expressed on NKT cells and its expression was enhanced by TCR signals. GITR engagement using DTA-1, an agonistic mAb against GITR, in the presence of TCR signals, augmented IL-2 production, the expression of activation markers, cell cycle progression, and the nuclear translocations of NF-κB p50 and p65. Furthermore, GITR engagement enhanced the production of IL-4, IL-10, IL-13, and IFN-γ by NKT cells and the expression level of phosphorylated p65 in NKT cells in the presence of TCR engagement, indicating that GITR provides costimulatory signals to NKT cells. The costimulatory effects of GITR on NKT cells were comparable to those of CD28 in terms of cytokine production. Moreover, the coinjection of DTA-1 and α-galactosylceramide into B6 mice induced more IL-4 and IFN-γ production than the coinjection of control mAbs and α-galactosylceramide. In addition, the adoptive transfer of DTA-1-pretreated NKT cells into CD1d−/− mice attenuated hypersensitivity pneumonitis more than control IgG pretreated NKT cells in these mice. These findings demonstrate that GITR engagement on NKT cells modulates immune responses in hypersensitivity pneumonitis in vivo. Taken together, our findings suggest that GITR engagement costimulates NKT cells and contributes to the regulation of immune-associated disease processes in vivo. The Journal of Immunology, 2006, 176: 3507–3515.

N atural killer T cells are a distinct subset of conventional αβ T cells and coexpress surface markers of both conventional αβ T cells and NK cells (1). NKT cells play critical roles in various immune responses in vivo, e.g., in the maintenance of self-tolerance, in autoimmune diseases (2–4), in tumor rejection (5), in pulmonary fibrosis (6), and in response to various infectious agents (7–9). Upon activation, NKT cells rapidly produce large amounts of IL-4 and IFN-γ (10), which play critical roles in the regulation of innate and adaptive immune responses (1). Moreover, it has been established that NKT cells regulate immune responses by modulating the Th1/Th2 balance in vivo. The NKT cells (Vα14 invariant NKT) of mice express a single invariant Vα14Jα281 TCR (Vα14i TCR) (11), which recognizes glycolipid Ags presented by the nonpolymorphic MHC class I-like protein CD1d (12). α-Galactosylceramide (α-GalCer)3 binds to CD1d molecules, and the complexes formed are recognized by the TCR of NKT cells (13, 14), resulting in NKT cell activation. In addition to TCR engagement, costimulatory signals are essential for many facets of T cell response, and without appropriate costimulatory signals, T cells may die or become anergic (15). It has been reported that NKT cells constitutively express CD28, ICOS, and CD40 on the cell surface and that these molecules play a role in regulating Th1/Th2 immune responses by NKT cells (16, 17). However, other costimulatory molecules that provide critical signals for NKT cell activation have not been well characterized. Glucocorticoid-induced TNF receptor (GITR), a member of the TNFR superfamily, has homology in its intracellular domain to TNF superfamily subgroup, e.g., 4-1BB, CD27, CD40, and OX40 (18–21). Moreover, GITR is highly expressed on conventional T cells and its expression is up-regulated during T cell activation (22). Recently, it was reported that GITR provides a potent signal to conventional CD4+CD25− T cells and CD4+CD25+ Treg cells, and that this interaction results in the activation and proliferation of these cells (22, 23). These reports suggested that GITR is a potent costimulator of the activation of T cell subpopulations. However, it has not been determined whether GITR is expressed on NKT cells, or whether it can exert functional roles as a costimulatory molecule on NKT cells.

In this study we explored the expression patterns of GITR on NKT cells and the functions of GITR as a costimulatory molecule during NKT cell activation. Our findings demonstrate that GITR is a potent costimulator of NKT cell activation in vitro and in vivo.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Orient Company. CD1d−/− (C57BL/6 background) mice were a gift from Dr. H. Gu (Columbia University, New York, NY) and RAG−/− Vα14i/Vβ8.2mice were a gift from Dr. M. Taniguchi (Chiba University, Chiba, Japan). Six- to 8-wk-old
mice were used in all experiments. Mice were bred and maintained under specific pathogen-free conditions in the Clinical Research Institute, Seoul National University Hospital (Seoul, Korea). All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee of the Clinical Research Institute (Seoul National University Hospital).

**Cell line and isolation of NKT cells from mouse**

DN32-D3 NKT cells (Vα14+ TCR mouse CD1d-specific NKT cell hybridoma) were a gift from Dr. A. Bendelac (University of Chicago, Chicago, IL) and were maintained in DMEM supplemented with 10% FBS and 5% of an antibiotic mix. After sacrifice, the livers of C57BL/6 (B6) mice were homogenized and resuspended in loading buffer (PBS containing 10% FBS and 1 mM EDTA), and overlayed onto Lympholyte-M (Cedarlane Laboratories). After centrifugation at 900 × g for 20 min at 25°C, liver mononuclear cells (MNC) were isolated at the interface and stained with PE-conjugated anti-NK1.1 (BD Pharmingen) and Cy-conjugated anti-TCR-β (clone H57-597; BD Pharmingen). NK1.1+ TCR-β+ NKT cells were then sorted using FACStar and CellQuest software (BD Biosciences). The splenocyte suspensions of RAG−/− Vα14+Vβ8.2+ mice were prepared and depleted of RBC using a RBC lysis buffer (Sigma-Aldrich). Vα14+Vβ8.2+ NKT cells were enriched from these splenocytes using magnetic beads (Miltenyi Biotec).

**Abs and flow cytometry analysis**

Hybridoma cells producing mAbs against GITR (DTA-1, rat IgG2a) were a gift from Dr. S. Sakaguchi (Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan) (19). Hybridoma cells producing mAbs against CD3 (2C11; hamster IgG), CD28 (37.51; hamster IgG), GITR (DTA-1, rat IgG2a), and CD25 (PC61, rat IgG) were purchased from the American Type Culture Collection (ATCC). Biotinylation of anti-GITR mAb was performed according to a standard method. Purified rat IgG2a, purified anti-CD28 (PV-1, hamster IgG), PE-conjugated anti-NK1.1 (45-2C11, hamster IgG), Cy-conjugated anti-TCR-β (clone H57-597), PE-conjugated anti-CD69 (H1.2F3, hamster IgG), and anti-CD25 (PC61, rat IgG) mAbs were purchased from BD Pharmingen. For biotinylation of mAbs, FITC-streptavidin (BD Pharmingen) was used as the second-step reagent. Cells were preincubated with 24G2 mAb (BD Pharmingen) to block FcγRII/III before incubating with mAbs. Flow cytometry was performed using FACSCalibur and CellQuest software (BD Biosciences).

**Measurement of cytokine production and cell proliferation**

Sorted NKT cells (2 × 10^5/wells) were stimulated with combinations of anti-CD3 mAb (0.25–1.0 μg/ml) plus anti-rat IgG, or α-Galectin (0.2–20 ng/ml) with soluble anti-GITR, anti-CD28 mAb, or control IgG (0.01–20 μg/ml) in flat-bottom 96-well plates for 48 h. After washing, cells up-regulate GITR expression, hepatic mononuclear cells isolated from normal B6 mice were treated with DTA-1 or control IgG for 30 min, washed with PBS, and then adoptively transferred into CD14−/− mice by i.v. injection 1 day before administering S. rectirufa Ag. For histological examination, the lungs were inflated with 1 ml of 10% neutral buffered formalin and fixed for 24 h, and routine histological techniques were used to paraffin embed entire lungs. The sections (4 μm) were cut, mounted on slides, and stained with H&E. Pathologic scores were defined as follows: 0, no lung abnormality; 1, the presence of inflammation and granulomas involving <10% of the lung; 2, lesions involving 10–30% of the lung; 3, lesions involving 30–50% of the lung; 4, lesions involving 50–80% of the lung; and 5, lesions involving >80% of the lung. Mean pathologic scores were determined for five mice per group.

**Real-time PCR analysis**

For quantitative real-time PCR, total RNA was isolated from whole lung homogenates using an RNaseasy kit (Qiagen) according to the manufacturer’s instructions. Contaminated genomic DNA was digested with DNase I (Qiagen), and RNA was reverse transcribed with MMLV-RT Taq polymerase (Koschen) before PCR. For real-time PCR, the following primers were synthesized by Applied Biosystems: GAPDH TGGACCACCAACT GCTTA forward, GGATGCGGATGATGTT reverse, and CAGAA GACTGCTGATGCCCCTC-VIC; IFN-γ AGCAAACAGCAAGC GAAA forward, CTGGACCTGTTGGTGGTTGA reverse, and CT CAAACTTTGGAATACCTAGTATGTCAT–TAMRA; IL-4 FAM-CTC CGT GCA TGG GGT CCC TTC TIC Black Hole Quencher (BioSource International); and TGF-β1 GCCACATGTGTTGACTCAGAAC–TAMRA. Reactions were performed in triplicate, with 1 μg of cDNA as a template. High Universal Master mix (Applied Biosystems), gene-specific TaqMan probe, the forward and reverse primers, and water. The GAPDH-specific TaqMan probe and forward and reverse primers were used as an endogenous control. Gene-specific PCR products were measured using an Applied Biosystems 7500 Sequence Detection System, and results for each cytokine were normalized vs GAPDH expression.

**Statistics**

Statistical significance was analyzed using the Prism 3.0 program. Student’s t test was run to determine the p-value when comparing two groups. Values of p < 0.05 were considered significant.

**Results**

**NKT cells constitutively express GITR on cell surface**

To explore the functional roles of GITR on NKT cells, we first examined whether or not GITR is expressed on the cell surface of these cells. Flow cytometric analysis revealed that GITR was substantially expressed on freshly isolated hepatic NKT cells, and on CD4+CD25+ and CD4+CD25− T cells (Fig. 1A). Expression levels, evaluated as mean fluorescent intensity, of GITR on NKT cells were similar to levels of GITR on CD4+CD25− T cells but lower than those on CD4+CD25+ Treg cells. In addition, NKT cell hybridoma cells (DN32-D3) also expressed surface GITR. These results indicate that NKT cells constitutively express surface GITR in the same manner as CD4+CD25+ and CD4+CD25− T cells. Next, to examine whether activating signals through TCR on NKT cells up-regulate GITR expression, hepatic mononuclear cells isolated from normal B6 mice were treated with α-Galectin in vitro. GITR surface expression of NKT cells was up-regulated by TCR engagement and peaked at 72 h after treatment with α-Galectin, whereas surface expression of conventional T cells was not after

**Induction of hypersensitivity pneumonitis (HP) and pathologic scoring**

HP was induced by intranasally instilling 150 μg of *S. rectirufa* (catalog no. 29034; obtained from ATCC) Ag in saline into mice under light anesthesia. The materials were applied at the nose tip and were inhaled voluntarily. This procedure was performed for 3 consecutive days per week for 3 wk. Mice were sacrificed with pentobarbital injection 4 days after the final treatment. To assess the effects of GITR on NKT cells in our HP model, sorted NKT cells from B6 mouse livers were incubated with DTA-1 or control IgG for 30 min, washed with PBS, and then adoptively transferred into CD14−/− mice by i.v. injection 1 day before administering *S. rectirufa* Ag. For histological examination, the lungs were inflated with 1 ml of 10% neutral buffered formalin and fixed for 24 h, and routine histological techniques were used to paraffin embed entire lungs. The sections (4 μm) were cut, mounted on slides, and stained with H&E. Pathologic scores were defined as follows: 0, no lung abnormality; 1, the presence of inflammation and granulomas involving <10% of the lung; 2, lesions involving 10–30% of the lung; 3, lesions involving 30–50% of the lung; 4, lesions involving 50–80% of the lung; and 5, lesions involving >80% of the lung. Mean pathologic scores were determined for five mice per group.
Hepatic MNC secreted more IL-2 when anti-CD3 mAb and concentrations of α-GalCer engagement (Fig. 1B). These findings suggest that the activating signals generated by cognate interactions between TCR and CD1d-α-GalCer complexes up-regulate GITR expression on NKT cells.

**Engagement of GITR by anti-GITR mAb costimulates NKT cells**

To investigate the costimulatory functions of GITR on NKT cells, hepatic MNC, including NKT cells, were stimulated with various concentrations of α-GalCer and soluble anti-GITR mAb (DTA-1). Hepatic MNC secreted more IL-2 when anti-CD3 mAb and DTA-1 were added to the cultures than when anti-CD3 mAb and control IgG were added (Fig. 2A). The amount of IL-2 secreted by hepatic NKT cells depended on the dose of DTA-1 applied. These findings suggest that GITR engagement activates NKT cells more in the presence of TCR engagement and that this results in greater IL-2 secretion in vitro. To address this activation in NKH hybridoma cells, DN32-D3 cells were cultured with either DTA-1 or control IgG in the presence of α-GalCer. Consistent with the results obtained in hepatic NKT cells, DN32-D3 cells were more activated using DTA-1 and α-GalCer in a dose-dependent manner than by control IgG and α-GalCer (Fig. 2B). DN32-D3 cells constitutively express surface CD1d molecules, and thus these cells stimulate themselves when α-GalCer is added to the culture medium (data not shown). Activation markers, such as CD69 and CD25, were up-regulated on NKT cells by GITR engagement but were not up-regulated by control IgG Ab engagement (Fig. 2C). These findings suggest that GITR engagement leads to NKT cell activation when TCR on NKT cells are simultaneously engaged. To assess whether activating signals to NKT cells through GITR induce cell cycle progression, we examined the division rates of NKT cells induced by GITR and TCR engagement using CFSE-labeled hepatic MNC. NKT cells stimulated with DTA-1 and α-GalCer showed a progressive increase in the number of divided cells between 48 and 72 h later, whereas NKT cells cultured with control IgG and α-GalCer did not (Fig. 2D). These results indicate that GITR engagement by DTA-1 provides signals for activation and proliferation of NKT cells. To confirm the costimulatory functions of DTA-1, we examined the nuclear translocation of NF-κB family molecules, e.g., p50 and p65, in DN32-D3 cells stimulated with α-GalCer alone or together with GITR engagement. The amounts of p50 and p65 in nuclear extracts were higher after stimulating with α-GalCer and DTA-1 than with α-GalCer alone (Fig. 3A). In contrast, the amounts of these molecules in the cytosolic fraction of DN32-D3 cells stimulated with α-GalCer and DTA-1 were similar to those in the cytosolic fraction of those stimulated with α-GalCer alone. Moreover, the expression of phosphorylated p65 was enhanced in NKT cells from RAG-14tgV18 mice when these cells were stimulated with anti-CD3 and DTA-1 compared with anti-CD3 and control rat IgG (Fig. 3B). In RAG-14tgV18 mice, NKT cells expressing Vα14Jα18 TCR in the absence of conventional T and B cells, resulting in a higher number of invariant NKT cells in the spleen than B6 mice (1). Therefore, these findings suggest that the signal transduction through NF-κB is also enhanced by GITR engagement on primary invariant NKT cells compared with control treatment. Combined, these results indicate that GITR engagement with α-GalCer stimulates NKT cells by inducing the translocation of NF-κB family molecules to the nucleus.

**GITR engagement induces cytokine production by NKT cells**

To further investigate the costimulatory functions of GITR on NKT cells, we measured the amounts of cytokines, i.e., IL-4, IL-10, IL-13, and IFN-γ secreted by NKT cells due to GITR engagement and α-GalCer stimulation. It has been reported that NKT cells rapidly secrete large amounts of IL-4, IL-10, IL-13, and IFN-γ after activation in vitro (10, 25–28). In DN32-D3 cells, GITR engagement by DTA-1 and the stimulation with α-GalCer induced larger amounts of IL-4, IL-10, IL-13, and IFN-γ than did control mAb and α-GalCer (Fig. 4, A–D). NKT cells isolated from normal B6 mice also secreted higher concentrations of IL-4, IL-10, IL-13, and IFN-γ after GITR and TCR engagement, and this occurred in a dose-dependent manner (Fig. 4, E–H). However, it is not clear whether the enhanced production of cytokines is due to increased numbers of NKT cells, or alternatively, due to increased cytokine...
secretion by individual cells. To address this issue, the evaluation of intracellular cytokines in these cells using flow cytometry was performed. GITR engagement by DTA-1 and the stimulation with α-GalCer induced higher levels of IL-4 and IFN-γ in NKT cells in B6 mouse liver MNC than did control mAb and α-GalCer (Fig. 4). These findings suggest that the enhanced production of cytokines is due to increased cytokine secretion by individual NKT cells rather than due to increased numbers of NKT cells. Taken together, these findings indicate that GITR engagement signals NKT cell activation, which increases cytokine production.

![Figure 2](image)

**Figure 2.** GITR engagement by anti-GITR mAb stimulates NKT cells. A, NKT cells from B6 livers were incubated with anti-CD3 mAb (1 μg/ml) plus anti-rat IgG/DTA-1 (0–20 μg/ml) or control rat IgG for 48 h. B, DN32-D3 cells were cultured with DTA-1 (0–20 μg/ml) plus anti-rat IgG or control rat IgG plus anti-rat IgG in the presence of α-GalCer (2 ng/ml) for 48 h. The concentration of IL-2 in culture supernatants was determined by ELISA. Statistical analysis was performed using the Prism 3.0 program. *, p < 0.001 and **, p < 0.05. C, The expression patterns of CD69 were analyzed on hepatic NKT cells 12 h after being stimulated by DTA-1 or by control rat IgG in the presence of α-GalCer (2 ng/ml). CD25 expression was also measured on hepatic NKT cells 24 h after stimulation. Values shown (inset) in flow cytometry diagrams represent the mean fluorescence intensity of the indicated molecules. Data are the mean ± SD of five mice in each group. The results shown are representative of three independent experiments.

![Figure 3](image)

**Figure 3.** DN32-D3 cells or NKT cells from RAG−/− Vα14βVβ8.2 mice were stimulated with DTA-1 (20 μg/ml) and TNF-α plus anti-CD3 mAb at a concentration of 0.25 μg/ml for 24 h. A, The cytosolic and nuclear fractions of DN32-D3 cells were then separated by SDS-PAGE and immunoblotted using anti-p50 and anti-p65 Abs. B, The total lysates of NKT cells from RAG−/− Vα14βVβ8.2 mice were separated by SDS-PAGE and immunoblotted using anti-phosphorylated p65 Ab. The results shown are representative of three independent experiments.

Comparison of the costimulatory effects of CD28 and GITR on NKT cells

To compare the costimulatory effects of GITR and CD28, a well characterized potent T cell costimulatory protein, we measured the amounts of IL-4 and IFN-γ secreted by DN32-D3 stimulated with anti-CD28 or anti-GITR mAb. Both anti-CD28 and anti-GITR mAb were able to stimulate DN32-D3 cells to produce IL-4 and IFN-γ in conjunction with α-GalCer. In DN32-D3 cells, the amounts of IL-4 and IFN-γ induced by anti-GITR mAb costimulation were comparable to those induced by anti-CD28 mAb in conjunction with α-GalCer, with the exception of IFN-γ at an α-GalCer concentration of 10 μg/ml (Fig. 5A). Moreover, sorted hepatic NKT cells (Fig. 5B) secreted larger amounts of IL-4 and IFN-γ due to CD28 engagement in conjunction with anti-CD3 mAb, and these were comparable to the amounts of cytokines secreted after GITR engagement, except for IL-4 in the presence of anti-CD3 mAb at a concentration of 0.25 μg/ml. These results indicate that GITR could be as potent a costimulator as CD28 during NKT cell activation.

Costimulatory effects of GITR on NKT cells in vivo

The treatment of α-GalCer in mice induces NKT cells to rapidly secrete IL-4 and IFN-γ in vivo (29). Therefore, to investigate the costimulatory effects of GITR on NKT cells in vivo, we injected DTA-1 and α-GalCer i.p. into B6 and CD1d−/− mice and measured IL-4 and IFN-γ amounts in serum. The injection of α-GalCer into B6 mice rapidly induced IL-4 and IFN-γ secretion in vivo, as reported previously (30). The levels of IL-4 and IFN-γ in serum of B6 mice were also enhanced by the administration of DTA-1 and α-GalCer vs α-GalCer and control rat IgG administration (Fig. 6). In contrast, α-GalCer administration did not induce IL-4 and IFN-γ secretion in serum in CD1d−/− mice. Likewise, the administration of DTA-1 did not alter the serum levels of IL-4 and IFN-γ.
in CD1d<sup>−/−</sup> mice even when it was coinfected with α-GalCer. These results suggest that GITR engagement on NKT cells provides costimulatory signals to NKT cells during α-GalCer activation in vivo. We then investigated whether GITR engagement on NKT cells affects clinical outcome in murine models of diseases. Recently, we found that CD1d<sup>−/−</sup> mice showed enhanced HP progression vs control B6 mice when these mice were nasally administered <i>S. rectivirgula</i> Ag. In addition, adoptive transfer of NKT cells (2 × 10<sup>5</sup> NKT cells/CD1d<sup>−/−</sup> mouse) attenuated HP in CD1d<sup>−/−</sup> mice much like B6 mice, which suggests that NKT cells attenuate murine HP in vivo (S. J. Hwang, unpublished observation). Therefore, we induced HP in B6 and CD1d<sup>−/−</sup> mice by administrating <i>S. rectivirgula</i> Ag as described in Materials and Methods. The severity of the disease process was monitored by measuring <i>S. rectivirgula</i>-specific IgG in serum and bronchoalveolar lavage (BAL) fluid and <i>S. rectivirgula</i>-specific T cell proliferation. In addition, we also evaluated the pathologic features in the lungs of these mice. To explore whether specific GITR engagement provides costimulatory signals to NKT cells that attenuate HP, sorted NKT cells were preincubated with either DTA-1 or control rat IgG for 30 min in vitro and then administered to CD1d<sup>−/−</sup> mice before inducing HP. Histological examinations revealed that the inflammatory responses, i.e., granuloma formation, peribronchial lymphoid hyperplasia, and pulmonary inflammation and fibrosis, were more severe in CD1d<sup>−/−</sup> mice adoptively transferred with preincubated NKT cells with control IgG than responses in CD1d<sup>−/−</sup> mice transferred with DTA-1-pretreated NKT cells (Fig. 7A). The adoptive transfer of NKT cells preincubated with DTA-1 into CD1d<sup>−/−</sup> mice induced lower amounts of <i>S. rectivirgula</i>-specific IgG in serum and BAL fluid and <i>S. rectivirgula</i>-specific T cell proliferation than the adoptive transfer of NKT cells preincubated with control rat IgG did (Fig. 7B). These findings indicate that GITR engagement on NKT cells contributes to HP attenuation in CD1d<sup>−/−</sup> mice. It is known that Th1 immune responses in vivo aggravate murine HP (31–36). Therefore, we explored cytokine profiles in HP models by measuring the amount of IL-4, TGF-β, and IFN-γ in the lung tissues of wild-type B6, CD1d<sup>−/−</sup> mice and CD1d<sup>−/−</sup> mice adoptively transferred with control or DTA-1 treated NKT cells. In real-time PCR assays, IFN-γ levels were higher in the lungs of CD1d<sup>−/−</sup> mice than in wild-type B6 mice, whereas IL-4 levels were lower in the lungs of...
the presence of CD3 engagement (0–1 h). Administration of DTA-1-pretreated NKT cells reduced IFN-γ and IL-4 levels in the lungs of CD1d<sup>−/−</sup> mice, whereas control rat IgG-pretreated NKT cells showed minimal IL-4 and IFN-γ level changes (Fig. 7C). These findings suggest that GITR engagement on NKT cells promotes Th2 cytokine profile in the lungs of mice, which protects against HP. Taken together, these results demonstrate that GITR engagement activates NKT cells in vivo by providing a costimulatory signal, and that this activation results in reducing <i>S. rectivirgula</i>-specific humoral and T cell responses and protects against HP.

**Discussion**

GITR is known to be highly expressed on CD25<sup>+</sup>CD4<sup>+</sup> Treg cells and to be expressed on CD25<sup>−</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells in low amounts (19, 22, 37). In this study, we demonstrate that GITR is constitutively expressed on the surface of NKT cells, and that its expression is similar to that found on the surface of CD25<sup>−</sup>CD4<sup>+</sup> T cells, but lower than that on CD25<sup>+</sup>CD4<sup>+</sup> Treg cells. TCR engagement by α-GalCer strongly enhanced GITR expression on NKT cells after 24 h of stimulation and this peaked within 72 h of activation, which is consistent with the NKT cell proliferation observed by CFSE labeling analysis (Figs. 1B and 2D). The constitutive expression of GITR on both CD25<sup>−</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells is up-regulated by activation signals using anti-CD3/anti-CD28 mAbs or rIL-2 (22, 37). A kinetic study demonstrated that GITR up-regulation on conventional T cells was induced rapidly after 6 h of stimulation and peaked within 24 h of activation (22). Therefore, it appears that signals transmitted through TCR on conventional T cells or NKT cells up-regulate surface GITR expression and that its up-regulation on NKT cells by α-GalCer stimulation is slower than on CD25<sup>−</sup>CD4<sup>+</sup> or CD8<sup>+</sup> T cells. The intrinsic characteristics of those cells may primarily contribute to the observed differences in the rates of GITR up-regulation. Alternatively, it is also possible that specific activating signals through TCR on conventional T cells or NKT cells may be different in terms of strength or signal pathway, which may contribute to the different rates of GITR up-regulation on these cells.

In this study, DTA-1 was used as an agonistic mAb against GITR to investigate the functions of GITR as a costimulatory factor on NKT cells. To assess the precise physiological functions of GITR on NKT cells, it is important to determine whether GITR engagement by DTA-1 is able to provide physiological signals to NKT cells. Several studies have demonstrated that anti-GITR mAbs engaged GITR in vitro as compared with GITR ligand-transfected cells or a soluble form of GITR ligand, and that this engagement resulted in the costimulation of CD4<sup>+</sup>CD25<sup>−</sup> T cells in the absence of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (19, 22, 37, 38). Moreover, it has been also reported that the administration of DTA-1 in CD1d<sup>−/−</sup> mice than in wild-type B6 mice. Moreover, the administration of DTA-1-pretreated NKT cells reduced IFN-γ production and increased IL-4 production in the lungs of CD1d<sup>−/−</sup> mice, whereas control rat IgG-pretreated NKT cells showed minimal IL-4 and IFN-γ level changes (Fig. 7C). These findings suggest that GITR engagement on NKT cells promotes Th2 cytokine profile in the lungs of mice, which protects against HP. Taken together, these results demonstrate that GITR engagement activates NKT cells in vivo by providing a costimulatory signal, and that this activation results in reducing <i>S. rectivirgula</i>-specific humoral and T cell responses and protects against HP.

**Figure 5.** The costimulatory signals induced by GITR engagement on NKT cells are comparable to those induced by CD28. A, DN32-D3 cells were stimulated using DTA-1 (●) or anti-CD28 mAbs (□) in the presence of α-GalCer (2 ng/ml). B, NKT cells were sorted from hepatic MNC freshly isolated from B6 mice according to NK1.1 and TCR-β expression. The sorted NKT cells were stimulated using DTA-1 (●) or control rat IgG (□) or anti-CD28 mAb (dotted bar) in the presence of CD3 engagement (0–1 μg/ml) plus anti-rat IgG. A and B, The concentrations of IL-4 and IFN-γ were measured in culture supernatants 48 h after stimulation. The results shown are representative of three independent experiments. Statistical analysis was performed using the Prism 3.0 program. ***, <i>p</i> < 0.001 and *<i>, p</i> < 0.05.

**Figure 6.** GITR engagement enhances IL-4 and IFN-γ production by NKT cells induced with α-GalCer in vivo. B6 and CD1d<sup>−/−</sup> mice were injected i.p. with α-GalCer (1 μg in 300 μl PBS) and simultaneously coinjected with DTA-1 (200 μg in 300 μl of PBS) or control rat IgG (200 μg in 300 μl PBS). Serum IFN-γ and IL-4 level changes in sera were monitored by ELISA. Data shown are the mean ± SD of three mice in each group. Similar results were obtained from three independent experiments. Statistical analysis was performed using the Prism 3.0 program. ***, <i>p</i> < 0.001 and *, <i>p</i> < 0.05.
vivo engages GITR on conventional T cells and Treg cells, and stimulates these cells (19, 22, 39). These findings and our results suggest that GITR engagement by DTA-1 stimulates target cells much like physiologic ligands. In addition, to understand the physiological roles of GITR in vivo, the cells capable of providing GITR ligand for the GITR engagement on NKT cells should be identified. GITR ligand is selectively expressed on the surface of B1 B cells at a high level, on conventional B2 B cells, macrophages, and B220<sup>+</sup> dendritic cells at an intermediate level, and on B220<sup>+</sup> dendritic cells at a low level (37, 38, 40). Endothelial cells and double negative thymocytes also express GITR ligand (41). Of these cells, GITR ligands on B cells, dendritic cells, and macrophages are possible in vivo candidates for interaction with the GITR of NKT cells because these cells express surface CD1d molecules for the interaction between NKT cells and APCs (42).

Agonistic mAbs against GITR have been reported to abrogate the suppressor function of CD25<sup>+</sup>CD4<sup>+</sup> Treg cells in vivo (19). Based on these observations, it was proposed that the reversal of suppression by anti-GITR mAbs is mediated by GITR engagement on CD25<sup>+</sup>CD4<sup>+</sup> Treg cells. However, GITR is also expressed on conventional αβ T cells at various levels, but the functions of GITR on these conventional T cells have not been completely explored. Recently, Stephens et al. (37) found that GITR engagement on CD25<sup>+</sup> responder T cells, but not on CD25<sup>+</sup> suppressor T cells, was required to abrogate suppression by a combination of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells from wild-type and GITR<sup>−/−</sup> mice in coculture experiments. Furthermore, GITR-mediated stimulation induced by DTA-1 or GITR ligand transfectants efficiently augmented proliferation, cell cycle progression, cytokine production, and the cytotoxicity of CD25<sup>+</sup>CD4<sup>+</sup> and CD25<sup>+</sup>CD4<sup>+</sup> T cells under the limited dose of anti-CD3 stimulation, indicating that GITR acts as a potent costimulator of CD25<sup>+</sup>CD4<sup>+</sup> conventional T cells and of CD25<sup>+</sup>CD4<sup>+</sup> Treg cells (22). These findings suggest that GITR expressed on T lineage cells acts as a potent costimulatory molecule with respect to cell activation. Therefore, it is expected that GITR expressed on NKT cells also provides costimulatory signals for NKT cell activation. GITR engagement induced more cytokine production in DN32-D3 and hepatic NKT cells only in the presence of TCR engagement. Costimulatory signals are provided by the ligation of membrane bound molecules that synergize with signals provided by TCR engagement (43, 44). Based on these characteristics of costimulatory factors, GITR molecules appear to function as potent costimulatory molecules for NKT cell activation.

CD28, which is constitutively expressed on T cells, has been reported to be the most potent costimulatory molecule in T cell
GITR provides costimulatory signals to NKT cells

activation (45, 46). CD27, CD134 (OX40), and CD137 (4-1BB), which are subgrouped in TNFR superfamily like CD28, have been reported to be costimulatory factors for T cell activation (20, 47). However, with the exception of CD28, NKT cells do not constitutively express these molecules on their cell surfaces (16). Hayakawa et al. (16) demonstrated that NKT cells constitutively express CD28 and CD40, and that the blockade of CD28-CD80/CD86 interactions inhibits α-GalCer-induced IFN-γ and IL-4 production by splenic MNC, whereas the blockade of CD40-CD154 interactions inhibits α-GalCer-induced IFN-γ production, but not IL-4 production. Consistent with these findings, CD28-deficient mice showed impaired IFN-γ and IL-4 production by splenic MNC in response to α-GalCer stimulation in vitro and in vivo, whereas the production of IFN-γ by splenic MNC, but not of IL-4, was impaired in CD40-deficient mice. Therefore, Hayakawa et al. (16) suggest that CD28-CD80/CD86 and CD40-CD154 provide costimulatory signals to NKT cells, and that these result in differential contributions to the regulation of Th1/Th2 immune responses by Vα14 invariant NKT cells in vivo. However, it is unclear whether NKT cells or other cell types in spleen cells produce these cytokines in their experimental systems. Recently, Matsuda et al. (48) determined that virtually all CD1d-α-GalCer tetramer+ cells in the liver produced both IFN-γ and IL-4 in CD40−/− and CD28−/− mice after α-GalCer administration using intracellular cytokine staining. The production of IFN-γ and IL-4 in activated Vα14i T cells from CD40−/− or CD28−/− mice was similar to the levels produced by these cells from wild-type mice. Therefore, they conclude that CD40−CD40L, and CD28−CD80/CD86 interactions are not required for the immediate IFN-γ production by Vα14i T cells, but that changes in ability of α-GalCer-primed NKT cells to induce IFN-γ secretion by NK cells may determine the extent to which they promote Th1 responses in vivo. In the present study, it was found that GITR engagement on NKT cells enhanced the production of IL-4 and IFN-γ in vitro and in vivo, and we compared the costimulatory effects of CD28 and GITR molecules on NKT cells in vitro system by measuring the production of IL-4 and IFN-γ. After activating NKT cells using α-GalCer or anti-CD3 mAbs, we found that the amounts of IL-4 and IFN-γ secreted by GITR engagement were comparable to those induced by CD28 engagement, indicating that the costimulatory effects of GITR on NKT cells are comparable to those of CD28.

We found that GITR engagement on NKT cells in the presence of α-GalCer augmented NKT cell activation in vivo. Moreover, coinjection of DTA-1 and α-GalCer into B6 mice enhanced IL-4 and IFN-γ levels in serum vs B6 mice coinjected with control rat IgG and α-GalCer. However, this result was not detected in CD1d−/− mice (Fig. 6). In addition, the disease processes of murine HP were more attenuated in CD1d−/− mice administered NKT cells preincubated with DTA-1 than in CD1d−/− mice administered NKT cells pretreated with control rat IgG (Fig. 7, A and B). Based on the cytokine profiles observed in the lung tissues of HP, the adoptive transfer of DTA-1-pretreated NKT cells induced an immune response in HP toward the Th2 type more than in control rat IgG-pretreated NKT cells (Fig. 7C). Thus, GITR engagement on NKT cells in the HP model contributed to the modulation of Th1/Th2 immune responses in vivo. These results suggested that GITR engagement on NKT cells induces costimulatory signals that augment NKT cell activation and that result in greater protection from the disease processes of HP in vivo. However, APC in CD1d−/− mice did not provide CD1d molecules for NKT cells during the interaction between NKT cells and APC in vivo. Therefore, signals through GITR on NKT cells appear to be able to activate NKT cells independently of TCR signals in the in vivo system, which is inconsistent with results obtained from in vitro assays. During the sorting of NKT cells from hepatic MNC by flow cytometry, TCR-β and NK1.1 on NKT cells were engaged by mAbs before pretreating with DTA-1 mAb in vitro. Therefore, it is likely that TCR-β engagement on NKT cells by mAb could provide enough TCR signal for activation, and consequently GITR engagement by DTA-1 on NKT cells provides costimulatory signals before adoptively transferring NKT cells into CD1d−/− mice. Alternatively, various membrane proteins expressed on NKT cells could be engaged by their ligands on APC in the absence of CD1d, and these molecules could sufficiently activate NKT cells rather than TCR. However, this possibility is less likely in vivo because no activating molecule is known to provide signals as potent as TCR with respect to NKT cell activation. Taken together, our results demonstrate that GITR engagement on NKT cells provides costimulation and modulates immune responses in vivo. Several reports have demonstrated that GITR engagement on T cells and CD4+CD25+ T cells regulates immune responses in autoimmune diseases like experimental autoimmune encephalomyelitis (39), autoimmune gastritis (19), and inflammatory bowel disease (49). Shimizu et al. (19) demonstrated that the removal of GITR-expressing T cells or the administration of a mAb to GITR produced autoimmune gastritis in normal mice. Kohm et al. (39) reported that anti-GITR mAb treatment in SJL mice with proteolipid protein 139–151-induced experimental autoimmune encephalomyelitis significantly exacerbated clinical disease severity and CNS inflammation, and that the prior depletion of CD4+CD25+ Treg cells failed to prevent experimental autoimmune encephalomyelitis exacerbation. These findings suggest a dual role for GITR, wherein GITR ligation both inactivates Treg cells and increases the activation and effector functions of CD4+CD25− T cells, thus resulting in enhanced T cell-mediated immunity. Furthermore, using a bone marrow transplantation model, Murglan et al. (50) demonstrated that recipients of an allograft containing CD8+CD25+ donor T cells showed increased graft-versus-host disease morbidity and mortality in the presence of GITR-activating mAb. Conversely, recipients of an allograft of CD4+CD25+ T cells showed a significant decrease in graft-versus-host disease after they were treated with GITR-activating mAb. These authors suggested that GITR has opposite effects on the regulation of alloreactive CD4+ and CD8+ T cells. Prior results and those of the present study indicate that GITR engagement on T cells, CD25+CD4+ Treg cells, or NKT cells contributes to the activation of these cells and that this modulates immune responses in vivo. Therefore, the agonistic anti-GITR mAbs and/or GITR ligand are potential therapeutic candidates for suppressing immune-related diseases that are regulated by NKT cell activation in vivo.

In summary, the engagement of GITR expressed on NKT cells enhances the cell activation and cytokine production by these cells in vitro and protects against HP in vivo. Therefore, we conclude that GITR provides costimulatory signals for NKT cell activation in vitro and in vivo and regulates immune-associated disease processes in vivo.

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Disclosures

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