Recombinant glucocorticoid induced tumor necrosis factor receptor (rGITR) induces NOS in murine macrophage

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Abstract   Glucocorticoid induced tumor necrosis factor receptor (GITR) is a new member of the tumor necrosis factor–nerve growth factor receptor superfamily of which the function has not been well studied. The extracellular domain of GITR was produced in *Escherichia coli* and purified as a single band of predicted M_r of 18.0 kDa. GITR and GITR ligand were expressed constitutively on the surface of Raw 264.7 macrophage cell line and murine peritoneal macrophages. An extracellular domain of GITR can activate murine macrophages to express inducible nitric oxide synthase and to generate nitric oxide in a dose- and time-dependent manner.   © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Glucocorticoid induced tumor necrosis factor receptor; Inducible nitric oxide synthase; Macrophage; Nitric oxide

1. Introduction

Glucocorticoid induced tumor necrosis factor receptor (GITR) is a member of the tumor necrosis factor receptor (TNFR) family induced by dexamethasone in murine T cells and may play a role in protecting T cells from activation induced cell death [1]. GITR shares a common motif with the TNFR family in the extracellular domain which has multiple cysteine-rich pseudorepeats [2] and shows close similarity in amino acid sequence with the cytoplasmic domains of 4-1BB, CD27, AITR [3–5]. This subfamily is implicated in diverse biological functions such as costimulation for T cell activation [6–8], and inhibition of activation induced cell death [9,10]. Recently signal transduction pathways for these molecules have been reported [11–13]. 4-1BB, AITR, and CD27 associate with TRAF2 to initiate a signal cascade for activation of nuclear factor κB (NF-κB). Little is known about the function of GITR.

Macrophages play key roles during immune response after activation [14]. Macrophages can kill bacteria, viruses, or parasites directly, secrete proinflammatory cytokines, act as anti-gen presenting cells, and finally participate in tissue remodeling. In the case of septic shock, they also release proinflammatory cytokines and nitric oxide (NO) and lead to severe systemic inflammation [15,16]. NO and TNF-α are considered primary mediators for pathogenesis of endotoxic shock related to development of circulatory failure, multiorgan failure, and ultimately death [17,18]. NO has been identified as an important signaling molecule involved in regulating a variety of biological activities. Macrophages release NO from the guanidino moiety of L-arginine via a reaction catalyzed by the inducible form of nitric oxide synthase (iNOS) by a variety of agents such as endotoxin, interleukin (IL)-1, TNF, and γ-interferon (IFN-γ) [19].

In this study, we report that macrophages express constitutively GITR and GITR ligand. Stimulation of macrophages with recombinant soluble GITR induced significant amounts of NOS with production of NO.

2. Materials and methods

2.1. Cells

Raw 264.7 cells, a murine macrophage cell line, were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin in six-well plates or in 10-cm dishes. Thioglycollate-elicited peritoneal macrophages were obtained from C57BL/6 mice which received 2 ml of 2% thioglycollate i.p. 4 days before. Cells were harvested by lavage of the peritoneal cavity with 6 ml of Hanks' solution.

2.2. Production of recombinant extracellular domain of GITR in *Escherichia coli*

The putative extracellular portion of GITR cDNA was amplified by the pfu polymerase chain reaction (PCR). An *Nhe*I site was generated at the 5′ end of the forward primer and an *Xho*I site and a stop codon were in the reverse primer. The sequence of the forward primer was 5′-ATCTAGCTAGCCAGCCGAGTGTAGTTGAG-3′, and that of the reverse primer was 5′-ATCCGCTCGAGGCCGTATTGCT-CAGTGGG-3′. The PCR product was digested by *Nhe*I and *Xho*I and the ∼0.4-kb fragment was purified. The *Nhe*I–*Xho*I fragment was inserted into the *Nhe*I/*Xho*I-digested pET28a vector, generating pET-GITR. The pET-GITR plasmid DNA was prepared and used to transform the *E. coli* BL21 expression strain (Novagen). The bacteria were grown at 37°C until the optical density at 600 nm reached 0.6–0.8. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM and the incubation continued at 37°C for an additional 6 h. To purify the GITR protein, bacteria were harvested by centrifugation, pellets were washed twice in buffer A containing 20 mM Tris–HCl, pH 7.5, 10 mM EDTA, and 2% Triton X-100, and homogenized using a sonicator for 3 min. Homogenate was dissolved in buffer A, stirred for 30 min at room temperature, and centrifuged. The pellet containing inclusion body was washed three times, and was stirred for 30 min in buffer B containing 50 mM Tris–

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Abbreviations: GITR, glucocorticoid induced tumor necrosis factor receptor; TNFR, tumor necrosis factor receptor; iNOS, inducible nitric oxide synthase; Ab, antibody; IPTG, isopropyl 1-thio-β-D-galactopyranoside

HCl, pH 8.5, 1% sodium dodecyl sulfate (SDS), and 1 mM 2-mercaptoethanol. The pellet was washed three times, dissolved in a buffer containing 10 mM Tris, pH 8.0, 6 M guanidine thiocyanate, 0.1% Triton X-100, and 5 mM 2-mercaptoethanol, and was homogenized for 3 min using a Branson sonifier 450 (Danbury, CT, USA). The supernatant was dialyzed sequentially against 2.4 M, 0.5 M, 0.1 M and 0 M of guanidine thiocyanate with 2-mercaptoethanol each for 3 h. Finally the renatured GITR protein was concentrated, treated by ultrafiltration with buffer exchange with phosphate based saline, and stored at $-70°C$.

2.3. Antibodies

The anti-GITR monoclonal antibody (mAb) mAH7 was produced by immunizing 7-week-old Sprague–Dawley rats with 50 μg of recombinant (r) soluble GITR emulsified in Freund's complete adjuvant. Rats received three consecutive i.v. injections at 2-week intervals. Four days after the last i.v. injection via the tail, the rat was killed and its spleen was removed. Spleen cells were fused with rat myeloma cells and the hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for GITR using purified histidine-tagged GITR protein. Twenty-four clones were isolated and subcloned. One clone, mAH7, was characterized and used in the present studies. Isotyping of mAb was performed by using the Immunopure Monoclonal Antibody Isotyping kit (Pierce, Rockford, IL, USA).

For polyclonal antiserum against GITR, rabbits were immunized with purified rGITR (100 μg/dose) emulsified in Freund's complete adjuvant. Rabbits received three consecutive s.c. injections in the back at 2-week intervals. The serum was obtained 2 weeks after the final injection. The titer was measured by ELISA and Western blot.

2.4. Immunoblot analysis

Cell lysate (50 μg) was separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes as described [20]. The blots were then washed in Tris–Tween buffered saline (TTBS, 20 mM Tris–HCl, pH 7.6 containing 137 mM NaCl and 0.05% (v/v) Tween 20), blocked overnight with 5% (w/v) bovine serum albumin (BSA), and probed with mAb for iNOS in 5% (w/v) BSA dissolved in TTBS. Using horseradish peroxidase conjugated secondary anti-mouse Abs (iNOS) were detected by enhanced chemiluminescence.

2.5. Flow cytometry

Cells (5×10^5/sample) were incubated in PFS buffer (phosphate based saline, 2.5% fetal bovine serum, and 0.1% sodium azide) with purified histidine-tagged GITR (none: isotype control) or anti-GITR polyclonal Ab (preimmune serum: isotype control) on ice for 30 min for detection of GITR ligand and GITR, respectively. Cells were washed three times in PFS, and incubated on ice for 30 min with mouse anti-His Ab for detection of GITR ligand. Cells were then incubated on ice for 30 min with FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for detection of GITR ligand and GITR, respectively. The cells were washed again as above and flow cytometry was performed using a FACSCalibur (Becton Dickinson).

2.6. Determination of NO_2^- concentration

NO_2^- production in culture supernatants was assayed by measuring nitrite, its stable degradation product, using Griess reagent. After stimulation, the supernatants were centrifuged and aliquots were mixed with 0.25 ml of Griess reagent to make a final volume of 0.5 ml and then incubated for 10 min at room temperature before measuring the absorbance at 540 nm. NaNO2 was used as a standard.

3. Results

3.1. Expression and purification of recombinant GITR from E. coli

Extracellular domain of GITR was produced in E. coli by cloning mouse GITR cDNA into the IPTG-inducible pET28a expression plasmid. The rGITR fusion protein with polyhistidine tag was found in the inclusion body. The rGITR was purified by extensive serial washings, denaturation, and renaturation. Approximately 14 mg of GITR per liter of E. coli culture was obtained. Purity of GITR was verified by the appearance of a single band of predicted M_r of 18.0 kDa by SDS–PAGE (Fig. 1). The M_r of the rGITR was 37 kDa in non-denaturing condition. This preparation was used for generation of polyclonal rabbit anti-GITR Ab and identification of GITR ligand by FACS. In some experiments the rGITR was purified by eluting the protein from 10% SDS–PAGE to avoid possible endotoxin contamination. This procedure enabled us to purify 1.3 mg of GITR per liter of E. coli culture. SDS gel-purified rGITR contained no more than a trace amount of endotoxin (< 0.4 EU/mg by the E-Toxate assay from Sigma Chemical).

3.2. Expression of GITR and GITR ligand in murine Raw 264.7 macrophages

We examined the presence of GITR ligand in macrophages to transmit the signal by soluble GITR. FACS analysis indicated that low levels of GITR and GITR ligand were expressed constitutively on the surface of the Raw 264.7 macrophage cell line and murine peritoneal macrophages as shown in Fig. 2A,B, respectively. For detection of GITR, mAH7 was also tried, but the peak shift was smaller than with polyclonal anti-GITR Ab, probably due to its low affinity (data not shown).

3.3. Production of NO and induction of NOS by GITR

Macrophages incubated with soluble GITR expressed a high level of iNOS and produced a significant amount of NO in a dose-dependent manner (measured as NO_2^-) (Fig. 3). Production of NO_2^- was detectable at 10 ng/ml of GITR after 24 h stimulation. Above 100 ng of GITR activation, the level of NO_2^- reached a plateau. The expression of iNOS was also time-dependent. It is detectable first at 15 h after GITR activation, peaks at 32 h, and is undetectable by 46 h (Fig. 4). However, released NO_2^- was detectable after 8 h stimulation with GITR and the level of NO_2^- remained at a plateau until 46 h. To clarify that NO_2^- production and expression of iNOS were due to GITR, the effect of GITR on expression of iNOS
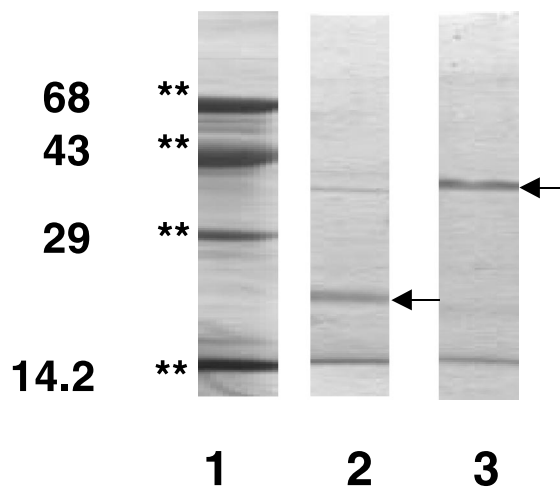

Fig. 1. SDS–PAGE of purified rGITR. The extracellular domain of rGITR was expressed in E. coli using plasmid pET28a. rGITR was purified to homogeneity and loaded on 10% SDS–PAGE under reducing (2) and non-denaturing conditions (3). Lane 1, molecular size markers; lane 2, purified rGITR with 2-mercaptoethanol; lane 3, rGITR without 2-mercaptoethanol.
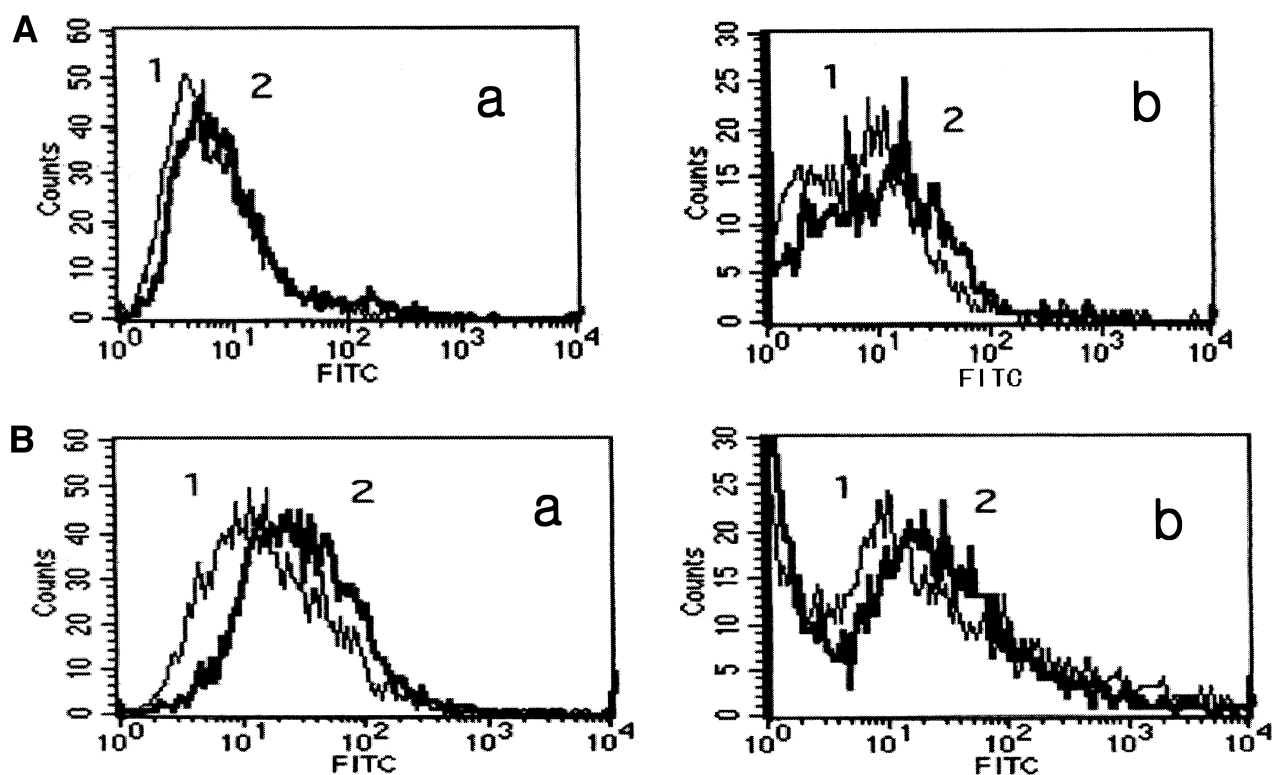
Fig. 2. A: FACS scan of GITR. Raw 264.7 cells (a) and mouse peritoneal macrophages (b) were incubated with rabbit preimmune serum or polyclonal rabbit anti-GITR Ab for 30 min on ice, washed three times, and finally incubated with FITC-conjugated goat anti-rabbit IgG (peak 2). Cells treated with preimmune serum were used as an isotype control (peak 1). B: FACS scan of GITR ligand. Raw 264.7 cells (a) and mouse peritoneal macrophages (b) were incubated without (peak 1) or with histidine-tagged GITR, monoclonal anti-histidine Ab (peak 2), and FITC-conjugated goat anti-mouse IgG each for 30 min on ice for GITR ligand detection. Cells were washed three times after each incubation. The results are representative of three experiments.

and NO_2^- production was measured with GITR after preincubation of anti-GITR polyclonal Ab. As shown in Fig. 5, iNOS induced by GITR was decreased after treatment with anti-GITR polyclonal Ab. The production of NO_2^- also decreased. Monoclonal anti-GITR Ab mAH7 also blocked the response induced by GITR to a lesser extent. To investigate whether the action of GITR was related to inflammation, the antiin-

flammatory agent dexamethasone was cotreated with GITR to macrophages. Simultaneous incubation of cells with GITR and dexamethasone for 24 h inhibits the formation of NO_2^- in culture supernatant (Fig. 6). The inhibition of NO_2^- production was dose-dependent. The iNOS induced by GITR also disappeared by adding dexamethasone to GITR, indicating that GITR is proinflammatory rather than antiinflammatory.
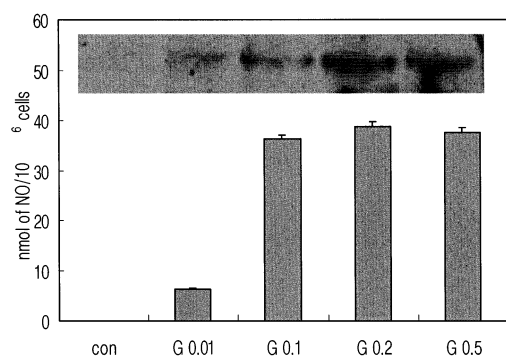

Fig. 3. Dose-dependent production of NO_2^- by GITR in mouse macrophage RAW 264.7 cells. Cells were treated with GITR at various concentrations (0.01, 0.1, 0.2, and 0.5 μg/ml) for 24 h, followed by the Griess reagent assay for NO_2^-. The data shown represent the mean ± S.D. of three independent assays. The cells used in the NO_2^- assay were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Section 2.
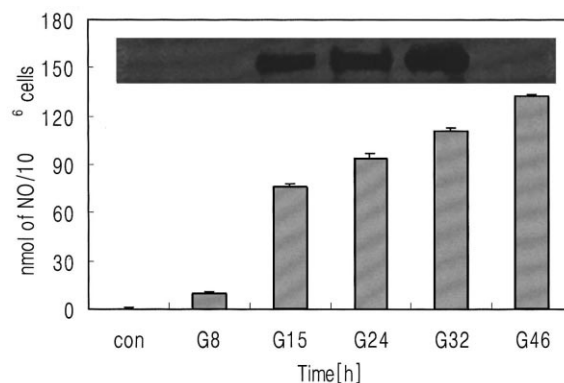
Fig. 4. Time-dependent production of NO_2^- by GITR in mouse macrophage RAW 264.7 cells. Cells were treated with 0.1 μg/ml of GITR for the indicated incubation times, followed by the Griess reagent assay for NO_2^-. The data shown represent the mean ± S.D. of three independent assays. The cells used in the NO_2^- assay were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Section 2.
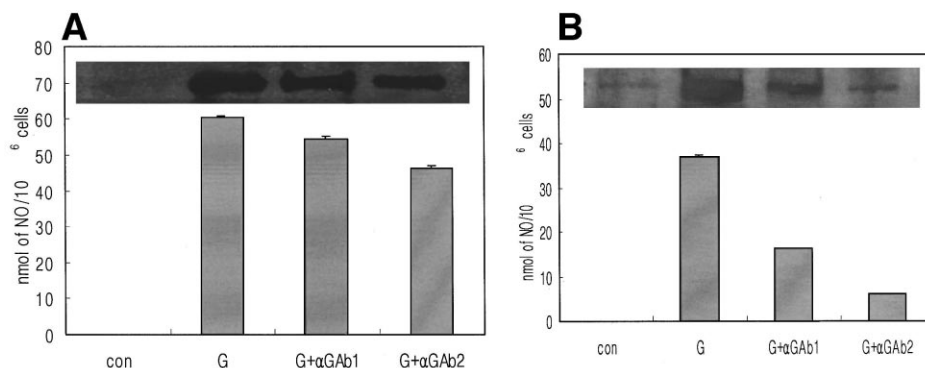
Fig. 5. Effect of anti-GITR Ab on NO_2^- production by GITR in mouse macrophage RAW 264.7 cells. GITR was preincubated with monoclonal anti-GITR Ab (mAH7) (Ab1, 20 μl; Ab2, 50 μl of hybridoma supernatants) (A) or with polyclonal anti-GITR Ab (Ab1, 30 μl; Ab2, 50 μl of serum) (B) for 1 h. Then cells were treated with GITR ± mAH7 or polyclonal anti-GITR Ab for 24 h, followed by the Griess reagent assay for NO_2^-. The data shown represent the mean ± S.D. of three independent assays. The cells used in the NO_2^- assay were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Section 2.

3.4. Verification of GITR activities

We wanted to verify that the observed effects of GITR on macrophages were genuine and not due to some non-specific activation or endotoxin contamination associated with the GITR protein preparation. Effects of GITR protein isolated from different sources were tested: GITR proteins produced by the baculovirus expression system as a fusion protein with a polyhistidine tag and those by HEK 293 cells as a fusion protein with Fc. They induced expression of iNOS and NO production as efficiently as did the *E. coli*-produced GITR protein (Fig. 7A). In addition, we tested the effects of GITR after coating the plates with GITR-Fc and GITR-histidine. No differences in NO production were found in either immobilized or soluble GITR stimulation (data not shown). We also examined whether GITR could induce iNOS and NO production in thioglycollate-elicited murine peritoneal macrophages. Peritoneal macrophages were also activated by baculovirus-produced GITR, expressed iNOS, and generated NO more efficiently than did Raw 264.7 cells (Fig. 7B).
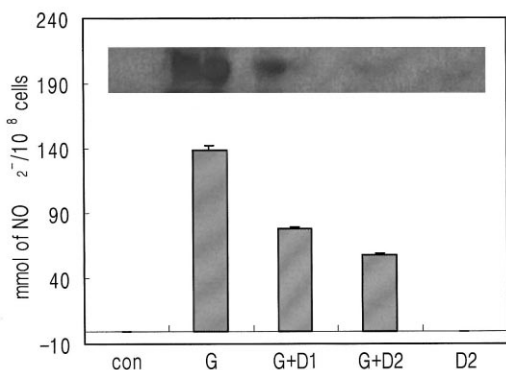
4. Discussion

GITR was described as a new member of the TNF–nerve growth factor receptor family and considered to have a T lymphocyte-related activity that control apoptosis in T cells [1]. Human TR11 (AITR), a full length cDNA from a human activated T cell cDNA library, has 55% identity with murine GITR at the amino acid level [5]. Although the high degree of
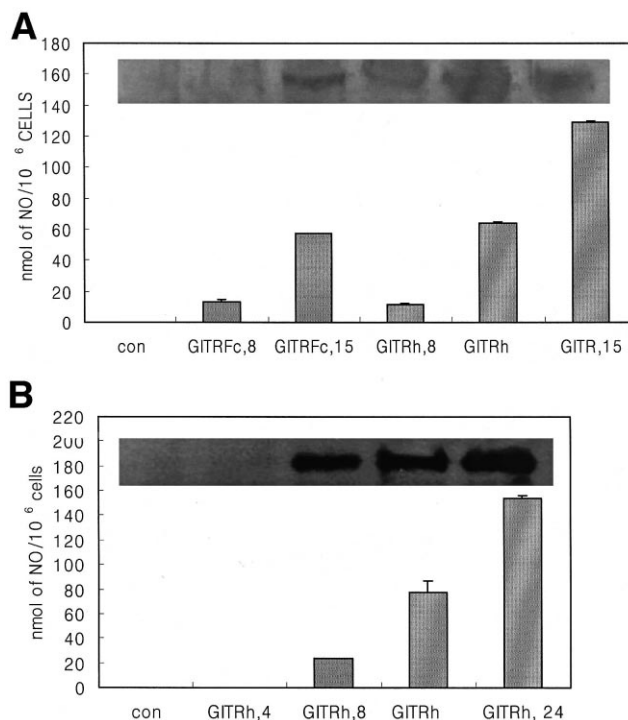

Fig. 7. Effects of other GITR preparations on NO_2^- production by mouse macrophage RAW 264.7 cells (A) or mouse peritoneal macrophages (B). Raw 264.7 cells were treated with GITR-Fc, GITR-His from baculovirus (GITRh), and GITR-His from *E. coli* (GITR) at 0.5 μg/ml for 8 and 15 h, followed by the Griess reagent assay for NO_2^-. Peritoneal macrophages were treated with 0.1 μg/ml of GITRh for 4, 8, 15, or 24 h. The data shown represent the mean ± S.D. of three independent assays. The cells used in the NO_2^- assay were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Section 2.

Fig. 6. Effect of dexamethasone on NO_2^- production by GITR in mouse macrophage RAW 264.7 cells. Cells were treated with GITR ± dexamethasone (D1, 10^{-7} M; D2, 10^{-6} M) for 24 h, followed by the Griess reagent assay for NO_2^-. The data shown represent the mean ± S.D. of three independent assays. The cells used in the NO_2^- assay were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Section 2.

sequence conservation between human and mouse suggests that AITR is likely to be the human homolog of murine GITR, they may display different biological activities, based on the following reasons. One is that there is a mismatch in the first cysteine-rich pseudorepeat between GITR and AITR and the other is that AITR is not inducible by dexamethasone. Although the function of AITR is not known clearly, AITR has been reported to mediate NF-κB activation through the TRAF2/NIK pathway like 4-1BB.

We have generated the extracellular domain of rGITR, a fusion protein with a polyhistidine tag, to investigate the cellular function of GITR. Since GITR ligand is required to transmit the signal for soluble GITR, the presence of GITR ligand in Raw 264.7 cells and murine peritoneal macrophages was demonstrated by FACS. GITR and GITR ligand were constitutively expressed at relatively low levels. We demonstrated that soluble GITR activates macrophages to express a high level of iNOS and generates large amounts of NO_2^- in a time- and dose-dependent manner. Production of NO_2^- was detectable at 10 ng/ml of GITR and reached a plateau above 100 ng of GITR activation after 24 h stimulation. The latter condition was used to follow NO_2^- production assays. Induction of iNOS and NO production by GITR were also observed in murine peritoneal macrophages.

Transduction of signal through the ligand has been also suggested for the OX40, CD40, CD30, and 4-1BB [21–23]. Soluble 4-1BB induces monocyte activation by expression of IL-6, IL-8, and TNF-α via bidirectional signaling [24]. Although GITR ligand has not been cloned yet, the presence was demonstrated by FACS. In this study, we showed that GITR ligand system induced macrophage activation by expression of iNOS, and this could be mediated by signaling through the ligand/receptor.

The expression level of iNOS was lowered by monoclonal anti-GITR Ab (mAH7) or polyclonal anti-GITR Ab, suggesting that the observed phenomena were due to GITR. The blocking effect by mAH7 was lower than that by polyclonal anti-GITR Ab, probably due to its low affinity. When detecting surface GITR in macrophages by FACS analysis, mAH7 showed a lower affinity than polyclonal anti-GITR Ab. We are now working to generate more mAbs of GITR with high affinities. It is not known whether the induction of NOS is caused directly by GITR or indirectly by early secreted cytokines, if any, after GITR stimulation. Further work is in progress to determine early events by GITR stimulation and to elucidate the role of GITR and GITR ligand in vivo.

The profile of iNOS expression is similar to that induced by migration inhibitory factor (MIF), which was found to influence a number of macrophage functions [25]. It had been reported that immune cells secreted MIF in response to physiological increases in glucocorticoid levels, and in turn, MIF can overcome the immunosuppressive effects of steroids on cytokine production and cellular activation [26]. It is generally accepted that immune cell responsiveness is controlled by an MIF/glucocorticoid dyad. It has been reported that besides GITR and MIF, lipopolysaccharide, TNF-α and IFN-γ can activate iNOS in in vitro macrophage systems [27,28]. Since the GITR preparation contained <0.4 EU/mg of endotoxin as determined by the E-Toxate assay, NO production could not be due to contaminated endotoxin of GITR.

Simultaneous incubation of cells with GITR and an antiinflammatory agent, dexamethasone, inhibits the formation of NO_2^- in culture supernatant and induction of iNOS in a dose-dependent manner. The induction of iNOS and NO production in macrophages by MIF and IFN-γ was also inhibited by adding dexamethasone [25]. It is generally known that glucocorticoid could affect the transcription of a number of nuclear proteins to inhibit the expression of iNOS. The activation of macrophages for iNOS expression by GITR could share a common transcriptional mechanism with MIF and IFN-γ. Among the widely used drugs in antiinflammatory therapy, glucocorticoids are effective in reducing inflammation, partly due to controlling NO generation. In contrast, soluble GITR resulted in the opposite effect.

In summary, we have shown that GITR activates macrophages to express a high level of iNOS and produce large amounts of NO in a time- and dose-dependent manner. GITR could serve as a physiological counterregulatory mediator and counteract the immunosuppressive effects by glucocorticoids.

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