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Induction of nitric oxide synthase (NOS) by soluble glucocorticoid induced tumor necrosis factor receptor (sGITR) is modulated by IFN-γ in murine macrophage

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Abbreviations: EMSA, electrophoretic mobility shift assay; GITR, glucocorticoid induced tumor necrosis factor receptor; HRP, horseradish peroxidase; iNOS, inducible nitric oxide synthase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LPS, lypopolysaccharide; PDTC, pyrolidine dithiocarbamate; TNFR, tumor necrosis factor receptor

Abstract

Earlier study showed that glucocorticoid induced tumor necrosis factor receptor (GITR), a new TNFR family, activated murine macrophages to express inducible nitric oxide synthase (iNOS) and to generate nitric oxide (NO). A possible involvement of pro-inflammatory cytokines on NO production by GITR was investigated *in vitro* systems and signaling molecules contributing to sGITR-induced iNOS production are determined in Raw 264.7 cells, a murine macrophage cell line. The result showed that the synergy was afforded by the combination of GITR with IFN-γ in a dose-dependent manner but IFN-γ alone was not able to induce NOS. No effects were observed with TNF-α, IL-1β, or IL-6 co-treated with GITR. To determine signaling molecules contributing to sGITR-induced iNOS production, a specific inhibitor for signal pathway proteins tested showed that PDTC (NF-κB) and genistein (tyrosine kinase) inhibited NOS induction significantly, while sodium orthovanadate (tyrosine phosphatase) potentiated NOS expression. These results suggest that activations of NF-κB were involved in induction of iNOS by GITR and IFN-γ priming caused earlier and stronger NF-κB activation.

Keywords: glucocorticoids; interferon type II; macrophages; nitric oxide; nitric-oxide synthase; receptors, tumor necrosis factor

Introduction

Nitric oxide (NO) has been identified as an important signaling molecule involved in regulating a variety of biological activities in immune systems. Activation of macrophages leads to release NO from guanidino moiety of L-arginine *via* reaction catalyzed by the inducible form of nitric oxide synthase (iNOS) (Chen *et al.*, 1999). A major part of iNOS regulation occurs at the transcriptional levels and changes in iNOS mRNA lead to different concentration of NO production in iNOS-expressing cells (Lowenstein *et al.*, 1993; Yang *et al.*, 2002). Although the signal transduction pathway for iNOS is not yet clear, many researches have been done for iNOS expression induced by proinflammatroy cytokines. The induction of iNOS is triggered and regulated by a series of signaling pathways including NF-κB transcription factor. The promoter region of the iNOS gene contains several transcription factor binding sites including that for NF-κB (Lowenstein *et al.*, 1993). The transcription factor NF-κB has been implicated as an essential part of pathogen- and stress-related responses of host organisms. Direct or indirect signals from pathogens or stress potently activated NF-κB that can induce many defense-related genes transcriptionally. The family of NF-κB protein was required for the enhanced iNOS gene expression when macrophages were exposed to LPS and other signals such as serum deprivation (Xie *et al.*, 1994; Liu *et al.*, 2001). In naive cells, NF-κB is associated with inhibitory protein, Iκ B-α in the cytoplasm, binding to NF-κB dimers and keeping its inactive state, but activation by appropriate signals leads to the phosphorylation of IκB-α by IκB-α kinase. Phosphorylated IκB-α is released from NF-κB complex, allowing the translocation of NF-κB into the nucleus, binding to DNA, and leading to activate the responsive genes (Lin Y *et al.*, 1995).

Macrophages are important elements in innate immunity. IFN-γ, initially produced by T cells during an infection, converts macrophages from a resting to an activated state, priming them for antimicrobial activity, increased killing of intracellular pathogens, and anti-

gen processing, and presentation to lymphocytes. These biological effects are mediated by increased proinflammatory cytokines, NO production and MHC class II expression after IFN-γ stimulation. IFN-γ synergizes with a second signal produced by LPS or TNF-α in the activation of macrophages through the activation of NF-κB which results in the transcriptional upregulation of a number of genes involved in a cell-mediated immune response.

Glucocorticoid induced tumor necrosis factor receptor (GITR) has been previously demonstrated to be an inflammatory mediator in mice (manuscript submitted) and NO production in Raw 264.7 cells (Shin *et al.*, 2002a). We have investigated whether pro-inflammatory cytokines show effects on NO production by GITR *in vitro* systems. And signaling molecules contributing to sGITR-induced iNOS production have been determined in murine macrophages.

Materials and Methods

Mateials and reagents

Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin, lipopolysaccharide (LPS) (from *Escherichia coli* serotype 055:B5), guanidine thiocyanate, pyrolidine dithiocarbamate (PDTC), SB202190, PD98059, genistein, and IPTG were obtained from Sigma Chemical Co. (St. Louis, MO). mAb for histidine and *E. coli* BL21 strain were from Novagen (Madison, WI). FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated goat IgG were from Southern Biotech. (Birmingham, AL). The NF-κB probe was from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant extracellular domain of GITR was prepared in *E. coli*-pET 28a (Shin *et al.*, 2002a) and in SF21 insect cell-pAcHLT-A (Shin *et al.*, 2002b) and purified as described previously.

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 units/ml penicillin, 100 μg/ml streptomycin in 6 well plates or in 10 cm dishes. Cells (10^6 cells) were incubated in medium in the absence or presence of indicated concentration of sGITR for indicated time period. The cells were lysed in hypotonic buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 10 mM NaF, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin), were centrifuged at 14,000 *g* for 30 min, and supernatant was obtained.

Immunoblot analysis

Ten μg of cell lysate were separated by SDS-PAGE and transferred onto nitrocellulose membranes. 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) BSA, and probed with anti-iNOS in 5% (w/v) BSA dissolved in TTBS. Using HRP-conjugated secondary anti-mouse Ab, the bands were detected by enhanced chemiluminescence.

Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA)

Control cells or IFN-γ-treated cells were stimulated with sGITR for different incubation time, and then nuclear extracts were prepared, following the method of Chen *et al.* (1998). A double-stranded oligonucleotide probe containing NF-κB binding sequences was purchased (5'-AGTTGAGGGGACTTTCCCAGGGC-3', Santa Cruz Biotechnology) and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Five μg of the nuclear extracts was incubated at 30°C for 20 min with 1 μg of ^{32}P-labeled NF-κB probe or mutant probe in 10 μl of binding buffer containing 1 μg of poly (dI·dC), 15 mM Hepes, pH 7.6, 80 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, and 10% glycerol. DNA-nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 5% polyacrylamide gel, and the gel was vacuum-dried and subjected to autoradiography using an intensifying screen at -80°C.

Determination of NO concentration

NO production in culture supernatants was assayed by measuring nitrite, its stable degradation product, using Griess reagent. After stimulation, the supernatants were centrifuged and its aliquots were mixed with an 0.25 ml of Griess reagent to make final volume of 0.5 ml and then incubated for 10 min at room temperature before measuring the absorbance at 540 nm. $NaNO_2$ was used as a standard.

Results

Effectors of iNOS stimulated by sGITR

GITR activates macrophages to express high level of iNOS and produces large amounts of NO in time- and dose-dependent manners (Shin *et al.*, 2002a) and induced inflammation in mice (unpublished results). Observed inflammatory response could be due to direct effects of GITR protein alone or combination of direct and indirect effects of GITR. GITR could induce other proinflammatory molecules that can faci-

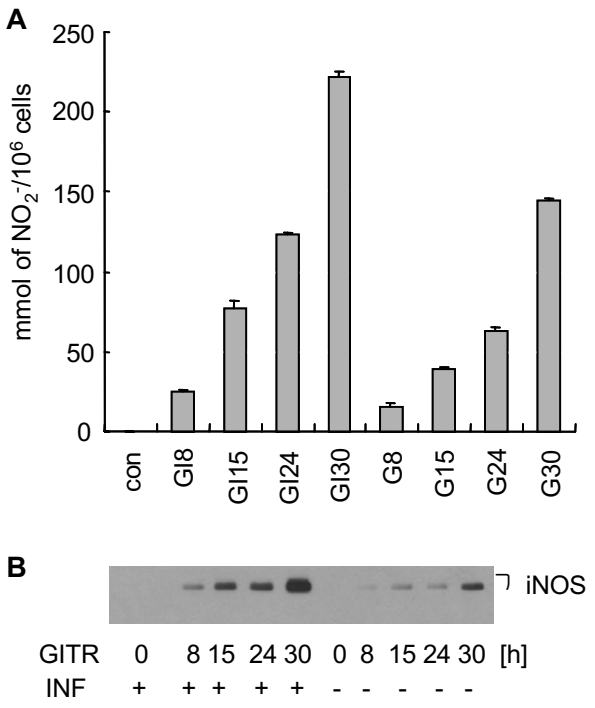


Figure 1. Time-dependent production of NO (A) and expression of iNOS (B) by sGITR in the presence or absence of IFN-γ (10 U/ml) in mouse macrophage, RAW 264.7 cells. Cells were treated with GITR at 0.2 μg/ml for 24 h, and followed by the Griess Reagent assay for NO. The data shown represent the mean±SD of three independent assays. The cells used in the NO assay were subjected to electrophoresed and Western blot analysis using iNOS-specific Ab as described in Materials and Methods.

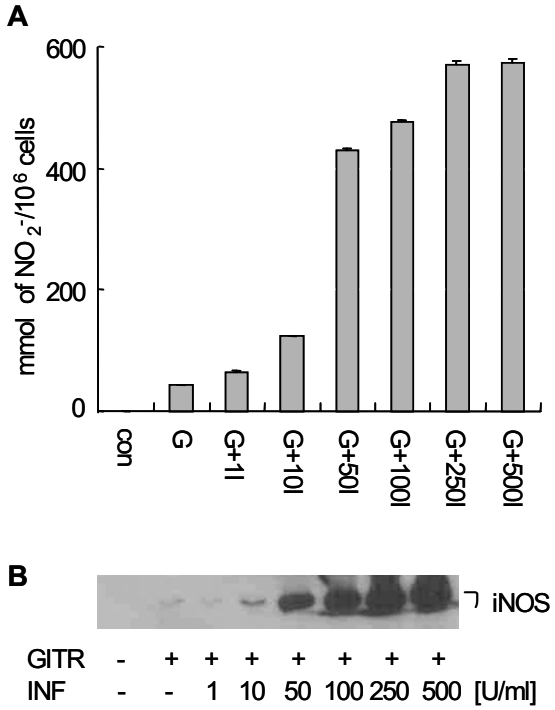


Figure 2. Dose-dependent production of NO (A) and expression of NOS (B) by sGITR with the different doses of IFN-γ (1, 10, 100, 250, and 500 U/ml) in mouse macrophage, RAW 264.7 cells. Cells were treated with GITR at 0.2 μg/ml for 24 h, and followed by determination of NO and Western blot analysis using iNOS-specific Ab. The data shown represent the mean±SD of three independent assays.

litate the whole inflammatory process *in vivo*. Since macrophages are activated by various cytokines *in vivo*, we observed the effects of pro-inflammatory cytokines on NOS induction stimulated by sGITR *in vitro*. Since the Th1 cytokine IFN-γ is a necessary co-stimulus for induction of iNOS and NO expression of LPS (Xie *et al.*, 1992), we have tested whether it has an effect on NOS induction stimulated by sGITR. Macrophages were treated with IFN-γ in the presence of sGITR, the level of NOS was analyzed by Western blot and the amounts of NO were determined in the culture supernatant using Griess reagent. IFN-γ alone was not able to induce NOS (data not shown). Higher amounts of NO were generated in primed macrophages than in naive one after stimulation of sGITR (Figure 1A). Pattern of NOS expression was similar to that of NO. NOS induced after 8 h and level was increased after 30 h in naive macrophages whereas significantly increased levels were observed in primed cells after 15 h stimulated by 0.2 μg/ml sGITR (Figure 1B). As shown in Figure 2A and 2B, a higher level of NO and NOS induction was produced in IFN-γ-primed macrophages as compared with naive cells in a dose-dependently from 10 to 500 U/ml IFN-γ. We next determined whether the time of pre-exposure with IFN-γ was critical for stimulating NO production and NOS induction. Macrophages were pre-incubated with IFN-γ for different times and then stimulated with sGITR for 24 h. Pre-exposure for 0 h up to 24 h with IFN-γ did not increase NO production and NOS expression significantly (Figure 3A and B). These data suggest that in a dose-dependent manner, IFN-γ can modulate the expression of NOS and NO production stimulated by sGITR. Contrary to IFN-γ, no synergism or antagonism was observed with co-treatment of sGITR with TNF-α, IL-1β, or IL-6 under the assay conditions (Data not shown).

NF-κB-specific DNA-protein complex formation in nuclei stimulated with sGITR

To determine signaling molecules contributing to sGITR-induced iNOS production, we tested the effects of inhibitors of MEK (PD98059), p38 (SB202190), tyrosine kinase (genistein), tyrosine phosphatase (sodium orthovanadate), and NF-κB (PDTC) on NOS induction of sGITR. SGITR-induced NOS production is not inhibited by the addition of PD98059 and SB202190,

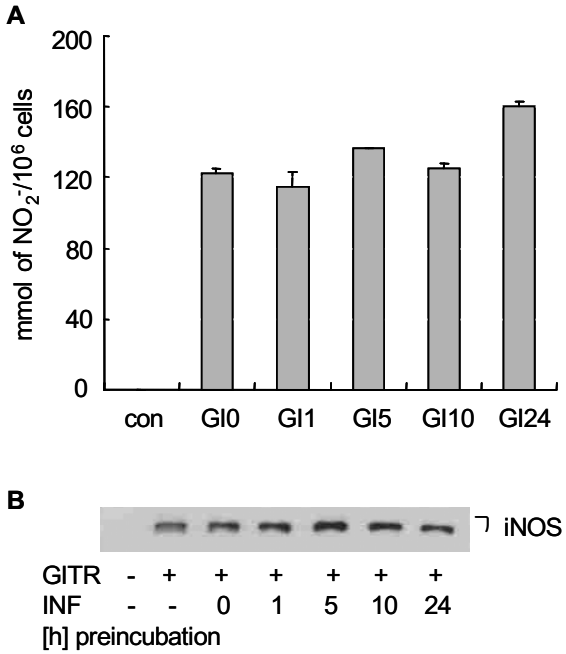
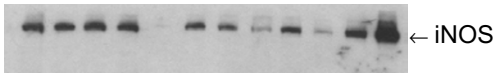


Figure 3. Effects of preincubation time with IFN-γ (10 U/ml) and sGITR on production of NO (A) and expression of NOS (B) in mouse macrophage, RAW 264.7 cells. Cells were pre-incubated with 10 U/ml IFN-γ for indicated time points (0, 1, 5, 10, and 24 h), stimulated with 0.2 μg/ml of sGITR for 24 h, and followed by determination of NO and Western blot analysis using iNOS-specific Ab. The data shown represent the mean±SD of three independent assays.



GITR	+	+	+	+	-	+	+	+	+	+	+	+	
SB202190	5	1	-	-	-	-	-	-	-	-	-	-	[uM]
PD98059	-	-	20	10	-	-	-	-	-	-	-	-	[uM]
PDTC	-	-	-	-	-	-	5	10	-	-	-	-	[uM]
Genistein	-	-	-	-	-	-	-	-	10	30	-	-	[uM]
SOV	-	-	-	-	-	-	-	-	-	-	10	30	[uM]

Figure 4. Effects of inhibitors on expression of NOS by sGITR in mouse macrophage, RAW 264.7 cells. Cells were pre-treated with each inhibitor for 1 h, stimulated with 0.2 μg/ml sGITR for 24 h, and followed by Western blot analysis using iNOS-specific Ab. SOV; sodium orthovanadate.

indicating that MEK and p38 MAPK is not involved in signal transduction pathway stimulated by sGITR (Figure 4). However, PDTC and genistein inhibited NOS induction significantly, while sodium orthovanadate potentiated NOS expression (Figure 4). These results suggested that NOS induction stimulated by sGITR is via activation of NF-κB and tyrosine kinase. Next, we have investigated the potential role of activation of NF-κB in the expression of iNOS by

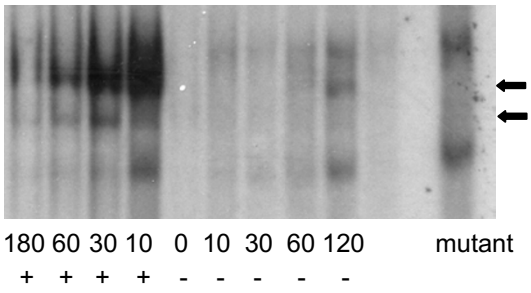


Figure 5. Kinetics of NF-κB-specific DNA-protein complex formation in nuclear extracts of RAW 264.7 macrophages stimulated with sGITR in the absence or presence of IFN-γ. Cells were treated with 0.2 μg/ml sGITR for 10, 30, 60, 120, and 180 min in the absence or presence of 10 U/ml IFN-γ. Nuclear extracts were prepared and NF-κB DNA-protein binding activity in the extracts were determined by EMSA as described Materials and Methods. Arrows indicated NF-κB-specific DNA compex formation.

sGITR in mouse macrophages. The time course of NF-κB activation after treatment with sGITR was studied. Nuclear extracts prepared from RAW cells were assayed for activated NF-κB in an EMSA. As shown in Figure 5, NF-κB-specific DNA-protein complex formation started to appear after 1 h, and reached maximum response after 2 h treatment with sGITR. After cells were co-treated with 10 U/ml IFN-γ and sGITR, the activation of NF-κB-specific DNA- protein complex formation appeared earlier than that induced by sGITR alone. Higher level of complex formation was observed at 10 min after sGITR stimulation and started to decrease. Co-treatment of IFN-γ with sGITR also induced another DNA-protein complex at lower level. Taking together, these results indicate that NF-κB-specific DNA-protein complex formation and tyrosine kinase regulate iNOS induction process stimulated by sGITR.

Discussion

We have observed that IFN-γ can increase the expression level of NOS and NO production stimulated by sGITR, whereas no effects were seen with co-treatment of sGITR with TNF-α, IL-1β, or IL-6. Activations of NF-κB and tyrosine kinase were involved in the induction process of NOS in murine macrophages stimulated by sGITR.

GITR was described as a new member of the tumor necrosis factor/nerve growth factor receptor (TNFR/NGFR) family. TNFR family shares common motif in extracellular domain, but not in cytoplasmic domain. However, GITR shows a similarity in amino acid sequence of intracellular tails to those of 4-1BB, CD27, and TR11 (Kwon and Weismann, 1989; Arch and Thompson, 1998; Kwon *et al.*, 1999; Kwon *et al.*,

2003). It has been reported that TR11, 4-1BB, and CD27 transmit their signals through TNFR associated factors (TRAF) to NF-κB (Vinay and Kwon, 1998; Takeda *et al.*, 2000). TRAF2 acts as adaptors to downstream signaling events which lead to NF-κB activation. The activation of the multisubunit inhibitor of IκB (IκB) kinase (IKK) complex, that induces IκBα and IκBβ phosphorylation. Phosphorylation of these inhibitory proteins leads to degradation and the release of Rel/NF-κB proteins from cytosol to the nucleus. iNOS is among the genes that are induced by NF-κB transcription factors and contains key NF-κB regulatory sites in its promoter. We demonstrated that sGITR activated macrophages to express high level of iNOS, which was mediated by activation of NF-κB and the level was synergized by IFN-γ. As shown, activation of NF-κB-specific DNA-protein complex formation was shown after 1-2 h treatment with sGITR. Since PDTC, a relatively specific inhibitor of the activation of NF-κB in macrophages, blocked the NOS induction, it appeared that NF-κB is involved in the induction of iNOS gene itself in sGITR-stimulated macrophages. However, we have not tested the effects of PDTC on binding of NF-κB proteins to the iNOS promoter.

Numerous cytokines and microbial products, often acting synergistically, stimulate expression of iNOS. The effective agents and combinations depend on cell types and species. The synergy afforded by the combination of TNF-α with any of the IFNs (Nathan, 1992) is particularly important because ingestion of most microbes elicits autocrine production and action of both TNF-α and IFNα/β (Green *et al.*, 1994). The physiological relevance of IFN-γ as an inducer of NOS is evident in the difficulty with which NOS is expressed in macrophages of mice rendered deficient in IFN-γ (Dalton *et al.*, 1993) or its receptor (Hwang *et al.*, 1993). Several readily encountered, noncytokine, nonmicrobial agents of diverse composition, such as ozone and asbestos, induce NOS or augment its induction (Nathan and Xie, 1994). It is not known whether these molecules work indirectly by eliciting cytokines. Soluble GITR could induce macrophages to make TNF-α and IFNα/β or work directly to show synergistic effect. Those possibilities are now under the investigation.

IFN-γ primes macrophages through the activation of the latent transcription factor, STAT-1. STAT-1 cooperates with NF-κB induced by the costimulatory signal and results in the transcriptional activation of IRF-1 (Ohmori *et al.*, 1997). The IRF family of transcriptional regulator has demonstrated recently to control a number of IFN-r responsive genes. IRF-1 plays a central role in the transcriptional regulation of iNOS (Salkowski *et al.*, 1996). Spink and Evans (1997) have demonstrated the presence of two adjacent IRF-1 responsive elements in the iNOS promoter.

GITR was initially cloned in the efforts of finding genes induced in murine T cell by dexamathasone (Nocentini *et al.*, 1997). Although GITR is expressed on stimulation of dexamethasone, the effects of sGITR have appeared to induce macrophages to activate and to show inflammatory response rather than anti-inflammatory one. This has also reported with macrophage migration inhibitory factor (MIF) (Bernhagen *et al.*, 1998). Glucocorticoids induce macrophage to generate MIF, but MIF overrides the immunosuppressive effects of glucocorticoid. GITR could serve as a physiological counter-regulatory mediator that counter-reacts the immunosuppressive effects of glucocorticoids. Initially GITR has been reported to protect cells from activation-induced cell death. Recently, the expression of GITR has been found to increase in the $CD4^+CD25^+$ cells using DNA microarray (Mchugh *et al.*, 2002). Shimizu *et al.* (2002) also has demonstrated that stimulation of GITR by anti-GITR mAb can block $CD4^+CD25^+$ T cells-mediated suppression. These results suggest that GITR plays an important role in controlling the regulatory T cells. The GITR ligand has not been identified yet, but its presence has been shown in murine macrophage cells (Shin *et al*-a., 2002). The ligand for AITR (human GITR) was found to be a member of TNF family which is expressed in endothelial cells (Kwon *et al.*, 1999). Since both of GITR and GITR ligand are found in murine macrophages, it is not clear whether sGITR-stimulated NOS induction was caused by signal transduction through GITR ligand or by blocking the endogenous GITR signal. We are currently investigating identification of the GITR ligand to find more inflormation. AITR (human GITR) has been demonstrated to transmit the signal via NF-κB activation like other TNFR family proteins. In our system sGITR also activate NF-κB to transmit the signal to induce NOS.

In summary, we have shown that sGITR activates macrophages to express NOS via NF-κB and tyrosine kinase activation. NOS induction and NO production stimulated by sGITR were up-regulated by addition of IFN-γ.

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