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Soluble Glucocorticoid-Induced Tumor Necrosis Factor Receptor (sGITR) Increased MMP-9 Activity in Murine Macrophage

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Abstract Glucocorticoid induced tumor necrosis factor receptor (GITR), a new TNFR family, increased production of matrix matalloproteinase (MMP-9) in murine macrophages. Murine macrophages produced a band of gelatinolytic activity at 100 kDa when stimulated for 18 h with soluble GITR. MMP-9 was identified by gelatin zymography and Western blot. Previous results demonstrated that murine macrophages express GITR and GITR ligand constitutively. Induction of MMP-9 was synergistic with co-treatment of INF-γ. MMPs could play a critical role in progression and promotion of tissue injury after inflammation stimulated by GITR/ligand system. J. Cell. Biochem. 88: 1048–1056, 2003.

Key words: GITR; MMP-9; murine macrophages

Monocyte/macrophages are an integral part of the immune response in chronic inflammatory lesions associated with connective tissue destruction. The role of monocytes in the destruction of connective tissue is attributed, in part, to their production of matrix metalloproteinases (MMPs). MMPs are a family of extra-cellular matrix (ECM)-degrading enzymes that include the interstitial collagenases, gelatinases, or type IV basement membrane collagenases, stromelysins, matrilysin, metalloelastase, and membrane-type MMPs [Birkedal-Hansen, 1995]. The ECM plays a central role in maintaining the structural integrity of primitive multi-cellular organisms as well as that of highly complex mammals. MMPs cooperatively degrade all components of the ECM. The timely breakdown of ECM is essential for embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling [Kherif et al., 1999]. In addition, MMPs participate in many pathological processes such as arthritis, cancer, cardiovascular disease, and various kinds of ulcers [Parks and Mecham, 1998]. All MMPs are synthesized as prepro-enzyme and secreted as inactive pro-MMPs in most cases. Many of MMPs are inducible to growth factors, cytokines, physical stress, oncogenic cellular transformation, and chemical agents [Nagase and Woessner, 1999]. The enhanced MMP gene expression may be down-regulated by suppressive factors such as transforming growth factor β, retinoic acid, and glucocorticoid. The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibition by endogenous inhibitors, α-macroglobulin, and tissue inhibitor of MMPs (TIMPs).

Glucocorticoids are steroid hormones which prevent and suppress inflammation and activation of the immune response. Steroids produce anti-inflammatory effects by modulating the transcription of several genes related to inflammatory processes such as cytokines, cellular receptors, or adhesion molecules. The glucocorticoid-induced tumor necrosis factor receptor

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family-related gene (GITR) is a member of the tumor necrosis factor–nerve growth factor (TNF–NGF) receptor family and was induced in T cells by dexamethasone [Nocentini et al., 1997]. GITR is a 66–70 kDa homodimeric glycoprotein. Although little homology was found in the intracellular domain among TNFR members, GITR shares homologies for intracellular domain with 4-1BB, CD27, and TR11 [Kwon and Weismann, 1989; Arch and Thompson, 1998; Kwon et al., 1999]. Since the role of the membrane bound form of GITR has not been studied in macrophages, we cannot say that soluble GITR (sGITR) exactly mimics the membrane bound form. While cross-linking of 4-1BB activates T lymphocytes, soluble 4-1BB ligand has the opposite effect [Michel et al., 1998] Soluble Fas inhibits membrane bound Fas [Cascino et al., 1996]. Recombinant soluble TNF receptors can ameliorate arthritis in animal models by inhibiting the biological activities of TNF [Williams et al., 1995]. Several ligands of the TNF superfamily have been shown to transmit signals in both directions through the respective receptor and into the cells that express the ligand. Reverse signaling through 4-1BB ligand inhibited proliferation, induced apoptosis, and stimulated monocytes to generate IL-8 [Langstein et al., 1998]. The natural presence of soluble GITR has not been detected yet, but it is plausible due to the presence of alternatively spiced transcript without trans-membrane domain [Nocentini et al., 2000]. In fact, there is accumulating evidence for the natural occurrence of soluble forms of cell surface receptors. Such forms are produced either through the proteolytic cleavage of membrane bound receptors such as IL-2R [Josimovic-Alasevic et al., 1988] and TNFR [Heller et al., 1990], or by alternatively spliced transcripts encoding soluble forms of receptors such as Fas [Cascino et al., 1995] and 4-1BB [Michel and Schwarz, 2000]. Since GITR is predominantly expressed on $CD25^+$ $CD4^+$ regulatory T cells in the thymus and its periphery and its monoclonal antibodies neutralize $CD25^+$ $CD4^+$ T cell-mediated suppression in vitro, the function of GITR is implied to be related to the control of immunological self-tolerance [Shimizu et al., 2002].

In our studies, soluble GITR (sGITR) has induced MMP-9 in both in vivo and in vitro systems. Soluble GITR activates murine macrophages to secrete MMP-9. Secretion of MMP-9 was affected by addition of INF-γ and other metabolites which were generated from stimulation by sGITR.

MATERIALS AND METHODS

Cells

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

Production of Recombinant Extra-Cellular Domain of GITR

The putative extra-cellular portion of GITR cDNA was amplified by the pfu polymerase chain reaction (PCR). The pET-GITR plasmid DNA was prepared and used to transform the *Escherichia coli* (*E. coli*) BL21 strain (Novagen). Recombinant extracellular domain of GITR was produced in *E. coli* and purified as described before [Shin et al., 2002a]. SDS gel-purified rGITR contained no more than a trace amount of endotoxin (<0.4 EU/mg by E-Toxate assay from Sigma Chemical, MI).

To clone the GITR gene into a pAcHLT-A vector which tags histidine to the protein, an EcoR I site was generated at the 5′ end of the forward primer and a Kpn I site and a stop codon were in the reverse primer. The sequence of the forward primer was 5′-CCGGAATTCC-AGCCGAGTGTAGTTGAG-3′, and that of the reverse primer was 5′-GGGGTACCTCAAT-GGCCGTATTGCTCAGT-3′. The PCR product was digested by EcoR I and Kpn and the ~0.4-kb fragment was purified. The EcoR I-Kpn fragment was inserted into the EcoR I/Kpn-digested pAcHLT-A vector, generating pAcHLT-A-GITR. The pAcHLT-A-GITR plasmid DNA was prepared and used to transfect the Sf21 insect cells by the method of Pharmingen as recommended by the manufacturer. To purify the GITR protein, the Sf21 cells were harvested by centrifugation, pellets were washed twice in A buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 2% Triton X-100, and homogenized using a sonicator for 3 min. The inclusion body

was obtained and washed by the method as described before [Shin et al., 2002a]. Finally polyhistidine-tagging-GITR was prepared from 10% preparative SDS–PAGE and eluted. It was concentrated, treated by ultrafiltration with buffer exchange with saline, and stored at −70°C. This procedure enabled us to purify 0.2 mg of GITR per 100 ml of Sf21 cells. The molecular mass of the purified GITR was 20,000 in SDS–PAGE.

Mice and Treatment

Seven-week-old male C57BL/6 mice (~20 g) were injected i.p. with 3 mg/kg *E. coli*-produced GITR. Control mice were injected with saline alone (n = 10). After 4 (n = 5), 8 (n = 6), 15 (n = 7), 24 h (n = 7) of GITR, the mice were sacrificed, and peritoneal membrane was obtained. Peritoneal membrane was homogenated, and cell lysate was obtained for Western blot analysis.

Antibodies

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

Immunoblot Analysis

Ten micrograms of cell lysate were separated by SDS–PAGE in the absence of a reducing agent and transferred onto nitrocellulose membranes as described [Towbin et al., 1979]. 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 polyclonal Ab for MMP-9 and monoclonal Ab for MMP-2 in 5% (w/v) BSA dissolved in TTBS. Using HRP-conjugated secondary anti-rabbit Ab (MMP-9) and anti-mouse Ab (MMP-2) were detected by enhanced chemiluminescence.

Determination of MMP-9 Activity

Zymography was carried out according to the method of Fahime et al. [2000], using serum deprived culture supernatant. Ten microliters of each media was mixed with the same volume of SDS-sample buffer in the absence of a reducing agent and the sample was electrophoresed. After electrophoresis, gels were washed four times in 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 2.5% Triton X-100 for 1 h to remove SDS, rinsed briefly and incubated at 37°C for 18 h in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$). After staining with Coomassie Brilliant Blue R-250, gelatin-degrading enzymes were identified as clear zones of lysis against a blue background. The pattern of proteinase inhibition was investigated by adding one of the following to the incubation buffer: 2 mM phenylmethylsulfonyl fluoride (PMSF) as a serine proteinase inhibitor, 2 mM *N*-ethylmaleimide (NEM) as a cysteine proteinase inhibitor, or 10 mM ethlenediaminetetraacetic acid (EDTA) as a metalloproteinase inhibitor.

RESULTS

Expression of MMP-9 in Mouse Macrophages

In our initial studies [Shin et al., 2002a], an extra-cellular domain of GITR can activate murine macrophages to express inducible nitric oxide synthase (iNOS) and to generate nitric oxide (NO) in dose and time-dependent manners. GITR and GITR binding protein (GITR ligand) were expressed constitutively on the surface of the Raw 264.7 macrophage cell line and murine peritoneal macrophages, although the GITR ligand has not been cloned yet.

We have observed neutrophil infiltration in peritoneal membrane when sGITR was injected in mice intraperitoneally (unpublished). Infiltrating neutrophils generate oxygen derivatives, serine proteases and zinc MMPs to promote tissue injury related to inflammatory processes. The levels of MMPs were examined in peritoneal membrane homogenates. MMP-9 and MMP-2 were induced after GITR stimulation (Fig. 1A,B). MMP-9 (100 kDa) induction reached maximum level at 15 h; then, decreased after GITR stimulation. MMP-2 expression was constitutive in normal peritoneal membrane homogenate and was increased at 4–8 h with a less extent. MMP-9 is produced mainly by inflammatory cells such as macrophages.

To investigate whether the induced MMP-9 was due to infiltrated macrophages, induction of MMP-9 was determined in murine macrophages, RAW 264.7 cells stimulated by sGITR. The upper panel of Figure 2 shows that murine macrophages produced a blurred band of gelatinolytic activity at about 100 kDa when

Fig. 1. Western blot of MMP-9 (**A**) and MMP-2 (**B**) in peritoneal membrane homogenate of C57BL/6 mice after GITR stimulation. Mice were administered i.p. with 3 mg/kg GITR. Control mice were injected with saline alone. After 4, 8, 15, and 24 h of GITR, a sample of peritoneal membrane was taken. Ten microgram tissue homogenate was analyzed in SDS–PAGE by the method as described in Materials and Methods. The data shown are representative of five different ones. The blots were probed with polyclonal Ab for MMP-9 (A) or monoclonal Ab for MMP-2 (B) and detected by enhanced chemi-luminescence. The assay was performed in two different sets of animals.

stimulated for 18 h with 200 ng/ml sGITR (Fig. 2A). This activity was virtually undetectable in un-stimulated cells from the same preparation. It was time-dependent and remained up to 24 h of stimulation. The induction of 100 kDa product was detectable at 10 ng/ml GITR after 24 h stimulation (Fig. 2B). Above 100 ng GITR activation, the level of MMP-9 reached at the plateau. To confirm the tentative zymographic identification, anti-murine MMP-9 polyclonal Ab was used for immunologic identification of 100 kDa activity under our conditions. The antibody reacted with a protein from stimulated murine macrophages. The pattern of MMP-9 expression in Western blot was consistent with the gelatin zymographic evidence (Fig. 2C).

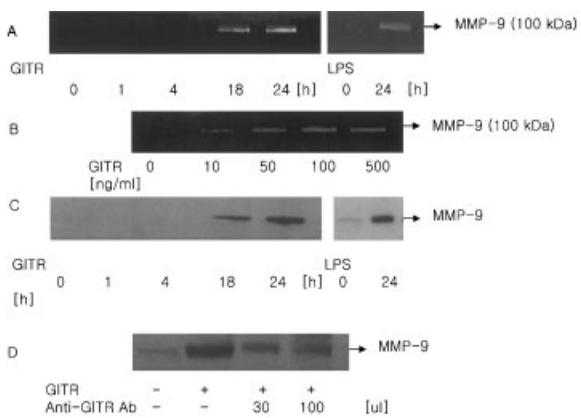

Fig. 2. Gelatin zymography of MMP-9 in murine macrophage, RAW 264.7 cells. Cells were treated with GITR at 200 ng/ml for 0, 4, 18, 24 h or LPS at 0.5 μg/ml for 0, 24 h without fetal bovine serum (**A**). MMP-9 was secreted after 18 h incubation of GITR with Raw cells. Cells were treated with GITR at various concentrations (A: 0, 10, 50, 100, and 500 ng/ml) without fetal bovine serum for 24 h (**B**). Supernatants were subjected to electrophoresis and Western blot analysis using MMP-9-specific Ab (**C**) as described in Materials and Methods. Cells were treated with 200 ng/ml GITR preincubated with polyclonal anti-GITR Ab for 1 h and were subjected to Western blot analysis (**D**). The arrow indicates the position of about 100 kDa gelatinase activities. The data shown are representative of three different sets.

The 100 kDa gelatinolytic activity was abrogated when exposed to EDTA before zymography, but all of the gelatinolytic activities were resistant to PMSF and *N*-ethylmaleimide (data not shown).

To clarify that induction of MMP-9 was due to sGITR, the effect of GITR on expression of MMP-9 was measured with sGITR after preincubation of anti-GITR polyclonal Ab. As shown in Figure 2D, substantial amounts of MMP-9 induced by sGITR were abrogated after two different concentrations of anti-GITR Ab in Western blot. (Fig. 2D)

Verification of GITR Activities

We wanted to verify that the above-observed effects of sGITR were genuine and were not due to some nonspecific activation or endotoxin contamination associated with the GITR protein preparation. To observe the induction of MMP-9 in macrophages, GITR proteins isolated from different sources were tested. GITR proteins produced in SF21 insect cells by the Baculovirus expression system as fusion protein with polyhistidine tag, and those by HEK 293 cells as fusion protein with Fc. GITRFc from HEK 293 cells or GITRh from SF 21 cells, were treated to induce MMP-9 in murine macrophage Raw 264.7 cells. Induction of MMP-9 was analyzed by Western blot. GITRFc induced MMP-9 after 18 h of GITR treatment in a dose-dependent manner, and GITRh also induced MMP-9 as efficiently as did the *E. coli*-produced GITR protein (Fig. 3A). In addition, we tested the effects of GITR after coating the plates with GITRFc and GITRh. No differences of MMP-9 induction were found in either immobilized or sGITR stimulation (data not shown). Next, we also examined whether sGITR could induce MMP-9 production in thioglycollate-elicited murine peritoneal macrophages. Peritoneal macrophages were also activated by GITRh, and expressed MMP-9 in a time-dependent manner. Expression of MMP-9 was detectable

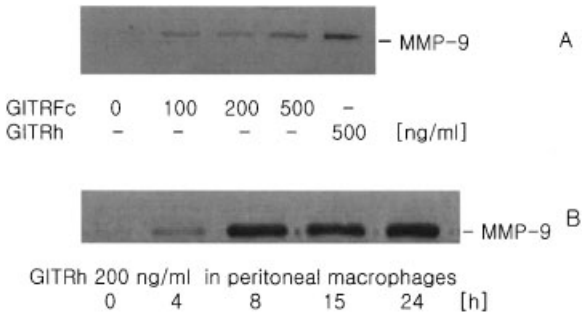

Fig. 3. Effects of other GITR preparations on MMP-9 expression by mouse macrophage, RAW 264.7 cell line (**A**) or mouse peritoneal macrophages (**B**). Raw 264.7 cells were treated with GITR-Fc at 100, 200, and 500 ng/ml and GITR-His from Baculovirus (GITRh) at 500 ng/ml for 24 h. Peritoneal macrophages were treated with 200 ng/ml of GITRh for 4, 8, 15, 24 h. The supernatants were subjected to electrophoresis and Western blot analysis using MMP-9-specific Ab as described in Materials and Methods. The data shown are representative of three different ones. The data shown are representative of three (**A**) and two (**B**) different sets.

at 8 h and reached at maximum level up to 24 h (Fig. 3B).

Effectors of MMP-9 Expression Stimulated by sGITR

To investigate whether the action of sGITR was related to inflammation, anti-inflammatory agent, dexamethasone, was co-treated with sGITR to macrophages. Simultaneous incubation of cells with sGITR and dexamethasone for 18 h inhibits the expression of MMP-9in culture supernatant, indicating that sGITR is pro-inflammatory (Fig. 4A).

Previously we have demonstrated that sGITR induced iNOS in macrophages [Shin et al., 2002a]. In order to know the relationship between NO production and MMP-9 induction stimulated by GITR, the effect of NO on MMP-9 induction was tested by adding the iNOS inhibitor S-methylisothiourea sulfate (SMT), or the NO donor, sodium nitroprusside (SNP) (Fig. 4B). In the presence of SMT, the sGITR-induced MMP-9 expression was decreased significantly. However, SNP that spontaneously produces NO after being added to the culture did not induce MMP-9 after 2 and 5 h of treatment. These results indicate that although exogenous NO did not induce MMP-9, MMP-9 induction stimulated by sGITR is related to the production of endogenous NO.

Next, we observed the effects of pro-inflammatory cytokines on MMP-9 induction stimulated by sGITR. Since the Th1 cytokine INF-γ is a necessary co-stimulus for induction of iNOS and NO expression of LPS [Chan and Riches, 1998] and for COX-2 expression induced by IL-1 [Barrios-Rodiles and Chadee, 1998], we have tested whether it has an effect on MMP-9 induction stimulated by sGITR. Macrophages were treated with INF-γ in the presence of sGITR and the level of MMP-9 was analyzed by Western blot in the culture supernatant. INF-γ alone did not induce MMP-9 (data not shown). A higher level of MMP-9 secretion was detected after 30 h in naïve macrophages whereas significantly increased levels were observed in primed cells after 15 h stimulated by 200 ng/ml

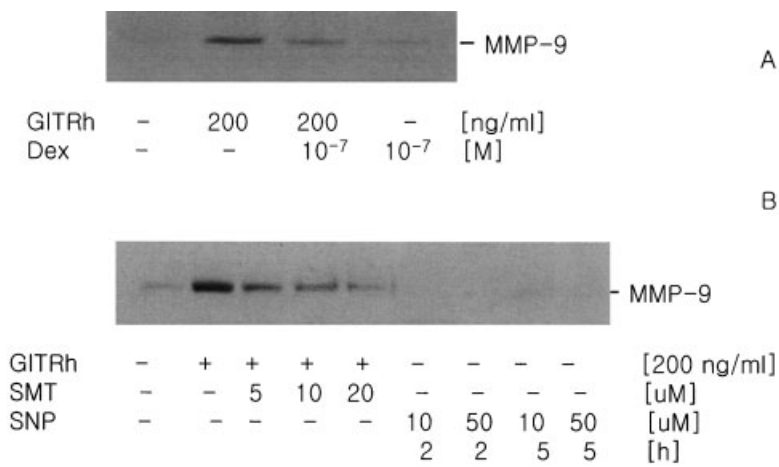

Fig. 4. Effect of dexamethasone, iNOS inhibitor, and NO donor on MMP-9 expression by GITR in mouse macrophage, RAW 264.7 cell line. Cells were treated with 200 ng/ml GITRh $\pm$ 10^{-7} M dexamethasone for 24 h (**A**). Cells were incubated with SMT (5, 10, and 20 μM) for 1 h and stimulated with 200 ng/ml GITRh for 24 h. SNP (10 and 50 μM) were added to cells for 2 and 5 h. The supernatants were subjected to electrophoresis and Western blot analysis using MMP-9-specific Ab as described in Materials and Methods. The data shown are representative of three different sets.

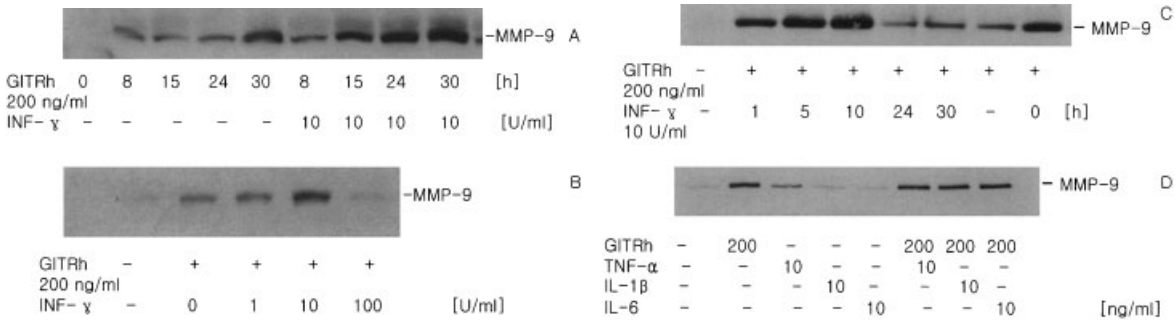

Fig. 5. Expression of MMP-9 protein in naïve and INF-γ-primed macrophages stimulated with sGITR. Raw 264. 7 cells were stimulated with 200 ng/ml GITRh in the presence or absence of INF-γ (10 U/ml) for 8, 15, 24, and 30 h (**A**). Cells were treated simultaneously with 200 ng/ml GITRh and different concentrations (0, 1, 10, 100 U/ml) of INF-γ for 24 h (**B**). Cells were pre-incubated with 10 U/ml INF-γ for indicated time points (0, 1, 5, 10, 24, and 30 h) and stimulated with 200 ng/ml of GITRh for 24 h (**C**). Cells were treated simultaneously with TNF-α (10 ng/ml), IL-1β (10 ng/ml), and IL-6 (10 ng/ml) in the absence or presence of 200 ng/ml GITRh for 24 h (**D**). The supernatants were subjected to electrophoresis and Western blot analysis using MMP-9-specific Ab as described in Materials and Methods. The data shown are representative of three different sets.

sGITR (Fig. 5A). As shown in Figure 5B, a higher level of MMP-9 induction was produced in INF-γ-primed macrophages as compared with naïve cells in a dose-dependently up to 10 U/ml, but above 100 U/ml INF-γ, MMP-9 production was lowered. We next determined whether the time of pre-exposure with INF-γ was critical for stimulating MMP-9 expression. Macrophages were pre-incubated with INF-γ at different times and then stimulated with sGITR for 24 h. Pre-exposure for 0 h up to 10 h with INF-γ increased MMP-9 expression level, but more than 24 h pre-exposure actually reduced MMP-9 secretion induced by sGITR (Fig. 5C). These data suggest that in a time- and dose-dependent manner, INF-γ can modulate the expression of MMP-9 stimulated by sGITR. However, some metabolites generated from stimulation by sGITR appear to affect the level of MMP-9 expression. Contrary to INF-γ, no synergism or antagonism was observed with co-treatment of sGITR with TNF-α, IL-1β, or IL-6 under the assay conditions (Fig. 5D).

DISCUSSION

We have demonstrated that sGITR activates macrophages to express a high level of MMP-9 in Raw 264.7 cells, as well as in thioglycollate-elicited murine peritoneal macrophages, both in a time-dependent manner. Treatment of sGITR resulted in significant changes related to an inflammatory action in peritoneal membrane (Unpublished results). The thickening of membrane and neutrophil infiltration were observed after sGITR treatment. Neutrophil infiltration moved to inside the tissue with tissue damage, suggesting that there could be an action of MMPs. There were infiltration of lymphocytes and macrophages in mesenteric fats in peritoneum of GITR-treated mice. At later stage, macrophage infiltration was observed in peritoneal membrane. We have demonstrated that both GITR and GITR ligand are expressed constitutively on the surface of murine macrophages [Shin et al., 2002a]. Since macrophage acts as a critical role in inflammation, we assumed that macrophage activation stimulated by sGITR could play a part in inflammatory actions induced by sGITR in vivo. The induction of cyclooxygenase-2 were also observed in homogenate of peritoneal membrane, supporting that there occurred inflammatory reactions [Shin et al., 2002b]. The expressions of MMP-2 and MMP-9 were observed in peritoneal membrane homogenates after GITR injection in mice. MMP-2 was up-regulated at early times after GITR stimulation, whereas MMP-9 was increased after MMP-2 induction. MMP-9 has been reported to be produced mainly by inflammatory cells such as macrophages [Xie et al., 1998]. Upon injury, MMP-2 was transiently increased, and MMP-9 was induced later and remained. MMP-9 expression appeared to be related to the inflammatory response, whereas MMP-2 activation seemed concomitant with the regeneration of new myofibers [Kherif et al., 1999]. In Raw 264.7 macrophage cells, induction of MMP-9 was detectable at 10 ng/ml sGITR and reached at the plateau above 100 ng GITR activation after 24 h stimulation. The length of time between sGITR treatment and increased

MMP-9 activity is 15–18 h. The upregulation of MMP-9 could be a direct effect of sGITR, an indirect one by secreted cytokines or chemokines, or their combinations after stimulation. We are investigating now to determine, if any, cytokines or chemokines induced at early time points by sGITR stimulation. The expression of *MMP-9* genes is often co-regulated and is enhanced by LPS, TNF-α, IL-1β, and the phorbol ester, phorbol 12-myristate 13-acetate (PMA). These inductions are mediated by the presence of NF-κB and SP-1 binding sites in synergistic cooperation with TPA responsive element [Kherif et al., 1999]. Induction of MMP-9 mRNA in PMA-stimulated cells was detectible at 5 h and maximal at 12 h [Lohi and Keski-Oja, 1995]. Similar pattern was observed in LPS-treated macrophages [Jin et al., 1999]. The activity profiles of MMP-9 induction were found after 24–48 h induction by LPS or the TNFRSF14 [Lee et al., 2001]. Since sGITR was produced from the *E. coli* expression system, the verification of observed phenomena was performed with different preparations of sGITR from HEK cells and SF21 cells by the Baculovirus expression system. Both preparations also activated macrophages to produce MMP-9.

A variety of powerful agents such as oxygen derivatives, serine proteases and zinc MMPs stored in granules may be released by transmigrating neutrophils. MMPs form a group of neutral proteinases which can degrade ECM components. MMP-9 is one of MMPs secreted from neutrophils. It usually cleaves native types IV and V collagens and fibronectin, laminin, entactin, and insoluble elastin to a less extent. MMP-9 is produced by polymorphonuclear leukocytes, macrophages, eosinophils, and lymphocytes [Lemjabbar et al., 1999]. MMP-9 is also a highly elevated gene transcript in LPS-stimulated human monocytes, implying that MMP-9 could be involved in orchestrating the inflammatory reactions with other LPS-inducible genes [Suzuki et al., 2000].

In murine macrophage RAW 264.7 cells, sGITR activates not only MMP-9 expression, but also iNOS previously shown [Shin et al., 2002a]. In our assay system, the addition of the iNOS inhibitor, SMT, significantly decreased the level of MMP-9 induced by sGITR. However, exogenously added NO donor, SNP, did not induce MMP-9. The failure of SNP to induce suggests that MMP-9 induction stimulated by sGITR is related to the production of endogenous NO, but could not be a result only of NO production generated by sGITR in our system. MMP-9 is demonstrated to be an inflammation-related molecule in the central nervous system with iNOS [Kyrkanides et al., 2001; Vegeto et al., 2001] However, the role of NO in regulating MMP synthesis and secretion has been contradictory and specific to certain cell types, and to the influence of other mediators in the microenvironment that may influence the production of NO and MMP-9. Trachtman et al. [1996] showed that iNOS induction by cytokines increased MMP activity in rat mesangial cells. Addition of the NO donor SNAP to the culture medium increased MMP-2 activity in a dose-dependent manner. In rabbit chondrocytes, NO also mediated IL-1β induced gene expression of MMP-9 and MMP-3 [Sasaki et al., 1998]. In contrast, *eNOS* gene transfer or treatment of cells with DETA NONOate has been demonstrated to inhibit MMP activity in smooth muscle cells [Gurjar et al., 1999; Eberhardt et al., 2000]. Eberhardt et al. [2000] also showed that endogenously generated NO inhibited MMP-9 mRNA level significantly in rat mesangial cells. Soluble GITR appears to mediate inflammation in vivo, raising the possibility that other effector molecules induced by sGITR participate in these reactions. The effects of candidate molecules such as INF-γ and pro-inflammatory cytokines on macrophage activation were determined in vitro system. Addition of pro-inflammatory cytokine, INF-γ, affected the expression of MMP-9 induced by sGITR. INF-γ showed synergism of MMP-9 expression stimulated by sGITR up to the concentration of 10 U/ml INF-γ; but above this concentration, the expression level was dropped rapidly. This finding could be related to the generation of NO and other metabolites produced by sGITR and INF-γ in macrophages. Other pro-inflammatory cytokines; such as TNF-α, IL-1β, and IL-6 did not affect the level of sGITR-induced MMP-9 expression. Although we have not determined whether sGITR induces pro-inflammatory cytokines in vivo, up-regulation of MMP-9 in peritoneal membrane homogenates could be due to combination activities, partly by a direct action of sGITR and partly by actions of metabolites stimulated by sGITR.

Glucocorticoid-induced tumor necrosis factor receptor (GITR) is a member of the tumor necrosis factor receptor (TNFR), induced by dexamethasone in murine T cells. Although

GITR is expressed on stimulation of glucocorticoid, the effects of sGITR have known to be pro-inflammatory rather than anti-inflammatory. This has also reported with macrophage migration inhibitory factor (MIF) [Bernhagen et al., 1998]. MIF production is induced by glucocorticoids, but it overrides the immunosuppressive effects of glucocorticoid. GITR could serve as a physiological counter-regulatory mediator that counter-reacts the immunosuppressive effects of glucocorticoids. GITR is a 228 amino acid type I trans-membrane protein with three cysteine pseudo-repeats in the extra-cellular domain and resembles CD27 and 4-1BB in the intracellular domain [Kwon and Weismann, 1989; Arch and Thompson, 1998; Kwon et al., 1999]. 4-1BB and CD27 associate with TRAF2 to initiate a signal cascade for activation of NF-κB and stimulate T cell proliferation [Vinay and Kwon, 1998; Takeda et al., 2000]. The cytoplasmic domain of one of GITR splicing variants shows homology with the cytoplasmic domain of CD4 and CD8 that interacts with tyrosine kinase Lck. 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 anti-GITR mAb can neutralize $CD4^+$ $CD25^+$ T cells-mediated suppression. These results suggest that GITR plays a functional role in regulating the $CD4^+$ $CD25^+$ cells The GITR ligand has not been identified yet, but its presence has been shown by FACS analysis in murine macrophage cells such as Raw 264.7 cells, and in peritoneal macrophages [Shin et al., 2002a]. The ligand for AITR (human GITR) was found to be a member of TNF family which is expressed in endothelial cells [Gurney et al., 1999; Kwon et al., 1999]. However, we cannot assume that this is also a case for an unidentified GITR ligand. It is possible that AITR and GITR may serve distinct functions from one another, despite the identity at the amino acid level, since AITR is not inducible by dexamethasone and there is a mismatch in cysteine pseudo-repeats. Since both of GITR and GITR ligand were found in murine macrophages, it is not clear whether sGITR-stimulated MMP-9 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 and expect to find some clues.

In summary, we have shown that sGITR activates macrophages to express a high level of MMP-9. MMP-9 induction was up-regulated by addition of INF-γ within certain ranges. The expression level of MMP-9 was related to other products which could be generated through stimulation of sGITR. MMP-9 could play an important role in progression and promotion of tissue injury after sGITR injection.

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