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The soluble glucocorticoid-induced tumor necrosis factor receptor causes cell cycle arrest and apoptosis in murine macrophages

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Abstract

In order to clarify the mechanism by which soluble GITR (sGITR) inhibits the survival of murine macrophages we examined its effect on the macrophage cell cycle. Soluble GITR induced G1 phase arrest followed by apoptosis. It also reduced the expression of cyclins D2 and A, and of cdk4, resulting in reduced cdk2 and cdk4 activities. These findings suggest that sGITR arrests division of the macrophages in G1 by lowering the activities of cdk2 and cdk4, and that this leads to apoptosis.

Keywords: GITR; Cell cycle; Macrophages; G1 phase

Cell cycle progression is regulated by the periodic activation of a family of cyclin-dependent kinases (cdks). Cyclins and cdks are evolutionarily conserved proteins essential for cell cycle control in eukaryotes. The activation of cdks requires their association with specific cyclin regulatory sub-units [1]. Cdk activity is also regulated by cdk levels, phosphorylation of positive and negative regulatory sites, and interaction with stoichiometric inhibitors. The latter, in particular, mediate a wide range of antimitotic signals. However, their specific functions are not completely understood. In mammalian cells, the activities of three types of cyclins (A, D, and E) and their associated catalytic partners are necessary for progression through the first gap (G1) phase into the phase of DNA synthesis (S). Several D-type cyclins (D1, D2, and D3) are induced by mitogens in a lineage-dependent manner and combine with their catalytic subunits, cdk 4 and cdk6, as the cell cycle starts [1]. Cyclin D-cdk4 or -cdk6, cyclin E-cdk2, and cyclin A-cdk2 act sequentially during the G1 to S transition. The catalytic activities of cyclin D-dependent kinases increase throughout G1, and then, in late G1, the retinoblastoma protein (Rb) is phosphorylated to block its growth inhibitory action. Rb is the primary target of cyclin D-cdk4 or-cdk6, although cyclin E-cdk2 and cyclin A-cdk2 play secondary roles in phosphorylating Rb [2]. Cyclin E expression increases during G1 to a peak at the transition to S phase, and it forms complexes with cdk2 throughout this interval [3]. The expression of cyclin A late in G1 is also important for the G1 to S transition, because inhibition of cyclin A kinase prevents S phase entry [4].

Macrophages are widely distributed and contribute greatly to normal host defenses, as well as to various pathophysiological processes [5]. They respond to chemotactic signals, phagocytize pathogens and particulate matter, destroy pathogens, secrete a vast array of substances, and process and present antigens to T cells. They also display various biological activities ranging from enhancement of cell growth to cytotoxicity. Macrophage numbers can influence every aspect of the immune response.

The glucocorticoid-induced tumor necrosis factor receptor (GITR) is a member of the tumor necrosis factor receptor (TNFR) family; it was cloned after induction of murine T cells by dexamethasone [6]. There have been few studies of the expression and function of GITR. GITR is expressed in macrophages [7] and regulatory T cells [8] and there are indications that it is involved in the control of regulatory T cells [8,9]. GITR

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shares a common motif in its extracellular domain with the TNFR family [6] and the amino acid sequence of its cytoplasmic domain is similar to those of 4-1BB, CD27, and AITR [10,11]. This sub-family is implicated in diverse biological functions such as costimulation of T cell activation and inhibition of activation-induced cell death [12]. GITR and its ligand are expressed constitutively in macrophages, though at low levels [7], and the expression pattern of GITR is similar to that of 4-1BB [13].

In this report, we show that sGITR causes cell cycle arrest by decreasing cdk2 and cdk4 activities as a result of lowering the expression of cyclin D2, cyclin A, and cdk4. This is followed by apoptosis. Thus, inhibition of cdk2 and cdk4 activities can block G1 phase progression and inhibit the multiplication of murine macrophages.

Materials and methods

Cells and reagents. Cells of Raw264.7, a murine macrophage cell line, were obtained from the ATCC, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in 6-well plates or in 10-cm dishes. Bone marrow-derived macrophages (BMDM) were isolated from 4- to 6-week-old C57BL/6 mice as described [14], and prepared with modifications. The cells were cultured in dishes with 10% FBS and 30% L-cell-conditioned medium as a source of macrophage-colony stimulating factor (M-CSF). A homogeneous population of adherent macrophages was obtained after 11 days of culture. The BMDM were deprived of L-cell conditioned medium for 24 h to render them quiescent and then exposed to various concentrations of sGITR together with 1 ng/ml M-CSF (R&D Systems). Production of the recombinant extracellular domain of GITR in *Escherichia coli* has been previously described [7]. The soluble GITR was pre-incubated with 50 μg/ml polymyxin B for 2 h to avoid endotoxin contamination. The extracellular domain of TNFRH1 was also produced in *E. coli* as a fusion protein with a polyhistidine tag, and used as a negative control. The TNFRH1 was a generous gift from Dr. B. Kwon, of the IRC, UOU, Korea. Polyclonal anti-GITR Ab and anti-GITR ligand Abs were purchased from KOMED (Seoul, Korea).

Cell viability and cell cycle analysis. Cell viability was determined by measuring the exclusion of trypan blue. Cells (2×10^5) were incubated under the indicated conditions and viable cells were collected and counted with a hemacytometer. Cells were fixed in 70% ethanol after exposure to sGITR. In some cases the cells were stimulated with 0.5 μg/ml sGITR in the presence of the caspase inhibitors, Z-DEVD-FMK (10, 50 μM) (Sigma Chemical, St. Louis, MI, USA), and Z-VAD-FMK (10 μM) (Sigma Chemical), or control DMSO. Samples were treated with 50 μg/ml RNase A at room temperature for 30 min, and cellular DNA was stained with 20 μg/ml propidium iodide, and DNA content was analyzed with a FACS (Becton–Dickinson, San Jose, USA), based on the level of red fluorescence. The numbers of cell in each cell cycle phase were determined with DNA Modifit software (Becton–Dickinson, San Jose, USA).

Assay of apoptosis and DNA fragmentation. Fragmentation of DNA due to inter-nucleosomal cleavage was determined on samples of 3×10^6 cells treated with 1 μg/ml sGITR. The cells were harvested in ice-cold PBS and lysed by incubation in 0.5 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, and 1% SDS) for 16 h at 4 °C. Lysates were centrifuged at 13,000*g* to separate cleaved low molecular weight DNA, and the supernatants were phenol-extracted twice and precipitated. The pellets ware re-suspended in Tris–EDTA buffer containing 250 μg/ml RNase (Boehringer–Mannheim, Germany), heated at 65 °C for 10 min, and subjected to electrophoresis on 2% agarose gels containing ethidium bromide. TUNEL assays were performed using the "In Situ Cell Death Detection Kit, TMR red" (Roche, Mannheim, Germany), following the manufacturer's protocol. TUNEL-positive cells were analyzed by FACS.

Immunoblot analysis. Ten micrograms of each cell lysate was fractionated 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 polyclonal Abs against cyclin D2, cyclin E, cyclin A, and cdk4, or monoclonal Ab against cdk2, and Rb in 5% (w/v) BSA in TTBS. After addition of horseradish peroxidase (HRP)-conjugated secondary anti-rabbit Ab (cyclin D2, cyclin E, cyclin A, or cdk4) or mouse Ab (cdk2 or Rb), immunoreactivity was detected by enhanced chemiluminescence. Anti-cdk2 monoclonal and anti-cdk4, anti-cyclin D2, anti-cyclin E, and anti-cyclin A polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Rb polyclonal Ab came from B.D. Biosciences (San Diego, CA, USA).

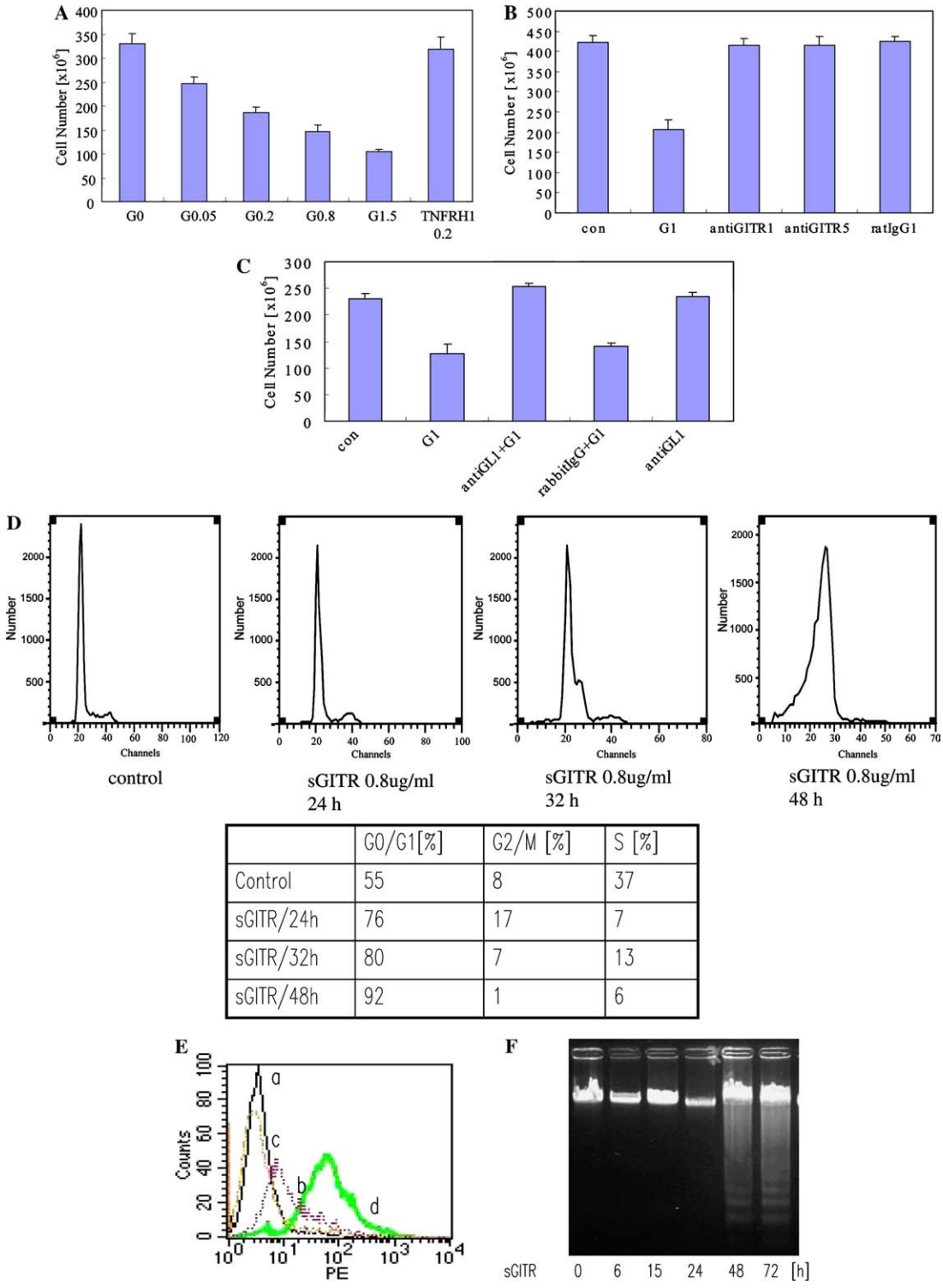
Immunoprecipitation and in vitro kinase assay. For immunoprecipitation, cells (5×10^5/sample) were lysed in lysis buffer (10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 0.1 mM Na_3VO_4, 1% Triton X-100, 0.5% NP-40, protease inhibitor cocktail, and 1 mM PMSF). One hundred micrograms of protein extract was reacted with 1 μg of anti-cdk2 Ab or anti-cdk4 Ab at 4 °C for 2 h and incubated with 20 μl protein G–Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, NJ). Immunoprecipitates were washed four times with lysis buffer and analyzed by immunoblotting. To assay cdks, complexes with anti-cdk4 or anti-cdk2 Ab were washed with lysis buffer and once with reaction buffer. Kinase activity was measured immediately in a reaction mixture containing 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg recombinant GST-Rb protein (Santa Cruz Biotechnology) or histone H1 (Sigma Chemical), 100 μM ATP, and 20 μCi [γ-^{32}P]ATP. After incubation at 30 °C for 15 min, the reaction was terminated by addition of 20 μl of 2× Laemmli sample buffer. Samples were electrophoresed on a 10% SDS–polyacrylamide gel, and, after staining with Coomassie brilliant blue and destaining with a 10% acetic acid and 30% methanol solution, the gel was dried and exposed to X-Omat film (Kodak, Rochester, NY).

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

Results

Growth inhibition and G1 arrest caused by sGITR

In previous studies, sGITR was found to activate macrophages and generated NO, PGE_2, and MMP-9 [7,17,18]. We also observed that stimulation with sGITR resulted in lowered cell numbers. Since murine macrophages express both GITR receptor and its ligand [7], we determined the effect of sGITR on the growth of murine macrophages. First, we examined the effect of sGITR on Raw264.7 macrophages incubated at 37 °C for 24 and 48 h (Fig. 1A). Increasing concentrations of sGITR caused a progressive reduction in the numbers of cells present after 24 h (data not shown) and 48 h of incubation. TNFRH1 as a negative control did not



	G0/G1[%]	G2/M [%]	S [%]
Control	55	8	37
sGITR/24h	76	17	7
sGITR/32h	80	7	13
sGITR/48h	92	1	6

Fig. 1. Soluble GITR inhibits growth of murine macrophages. (A–C) Numbers of viable Raw264.7 cells cultured in the indicated concentrations of sGITR (A), polyclonal anti-GITR Ab (1, 5 μg/ml) (B), or sGITR (1 μg/ml) in the presence of anti-GITR ligand Ab (1 μg/ml) or rabbit IgG (1 μg/ml) (C) for 48 h. Viable cells were counted by trypan blue exclusion. Each sample point was performed in triplicate and the results are represented as means ± SD. (D) Soluble GITR blocks the cell cycle in G1 phase. Cells (10^6) were cultured with 0.8 μg/ml sGITR for the indicated times. DNA content was determined by propidium iodide staining and FACS analysis. The cell cycle distribution was analyzed with the DNA Modifit program. (E) Detection of in situ cell death by the TUNEL reaction. Cells (1.5×10^6 cells) were incubated without (a) or with (b) 1 μg/ml sGITR for 48 h, and harvested cells were stained with the TUNEL reaction mixture, following the instruction manual of Roche (Mannheim, Germany). A negative control without terminal transferase is shown (c) and a positive control (d) was prepared by permeabilizing the cells with DNase I (3 U/ml). TUNEL positive cells are indicated by a curve shift in the FACS analysis. (F) DNA fragmentation by sGITR. Cells (3×10^6 cells) were treated with 1 μg/ml sGITR for the indicated times and apoptotic DNA was analyzed by agarose gel electrophoresis. Results are representative of three experiments.

affect NO production (data not shown) or cell numbers, demonstrating that growth inhibition was specific to sGITR.

In order to determine whether growth inhibition by sGITR was caused by signaling via the GITR ligand or by inhibiting an endogenous GITR signal, we neutralized the GITR or GITR ligand on the macrophage surface with the corresponding polyclonal Ab. Exposure to polyclonal anti-GITR Ab did not affect the growth of the murine macrophages (Fig. 1B), indicating that sGITR does not compete with an endogenous signal through the GITR. However, anti-GITR ligand Ab significantly reduced the growth inhibitory effect of sGITR, as shown in Fig. 1C. These results indicate that growth inhibition by sGITR involves signaling via the GITR ligand.

We investigated the effect of sGITR on cell cycle progression. The macrophages were incubated with sGITR and the cell cycle distribution was analyzed by flow cytometry. G1 phase arrest was detectible 24 h after exposure to sGITR and reached a maximum at 48 h (Fig. 1D). After synchronization of control cells, 55% were in G1, 37% in S, and 8% in G2/M. After 48 h continuous exposure to 0.8 μg/ml sGITR, 92% of the cells were in G1, 6% in G2/M, and 1% in S. The cells were also treated with various concentrations of sGITR for 32 h, because a hypo-diploid DNA fraction appeared at that time. As the concentration of sGITR increased, the proportion of G1 phase cell again expanded (Table 1).

The increased population of G1 phase cells in sGITR-treated cultures could represent G1-arrested cells or cells that were not G1-arrested but rather committed to apoptosis and subsequently detected as hypo-diploid cells. To explore these possibilities, we examined the effect of sGITR on the cell cycle of Raw264.7 cells pretreated with two caspase blockers, Z-DEVD-FMK and Z-VAD-FMK. Soluble GITR caused the accumulation of cells in G1 even in the presence of the caspase inhibitors (Table 2). Evidently the accumulation of G1 cells is due to G1 arrest, not to apoptosis of cells already committed to cell division.

Since a hypo-diploid DNA fraction appeared after prolonged sGITR stimulation, DNA breaks were examined using the TUNEL method (Fig. 1E) and an increase in TUNEL positive cells was observed 48 h after sGITR stimulation (Figs. 1E-a, b) Positive controls treated with DNase I also showed a shift due to TUNEL positive cells (Fig. 1E-d). The apoptosis-inducing potential of sGITR was confirmed by DNA ladder formation: agarose gel electrophoresis of the DNA of macrophages treated with sGITR showed the typical laddering observed after internucleosomal fragmentation (Fig. 1F). The above results demonstrate that the treatment of murine macrophage with sGITR induces apoptosis detectible 48 h after sGITR stimulation.

Table 1
Effect of cell cycle progression by sGITR in Raw264.7 cells

	G0/G1 (%)	G2/M (%)	S (%)
Control/32 h	47	28	25
0.05 μg/ml sGITR	58	12	30
0.2 μg/ml sGITR	70	8	22
0.8 μg/ml Sgitr	80	7	13
0.2 μg/ml TNFH1	42	35	23

Table 2
Effect of cell cycle progression by sGITR in the presence or absence of caspase blockers in Raw264.7 cells

	G0/G1 (%)	G2/M (%)	S (%)
Control/48 h	39	49	12
0.5 μg/ml sGITR	72	17	11
Z-DEVD-FMK, 10 μM	34	43	23
Z-DEVD-FMK, 50 μM	37	44	19
Z-VAD-FMK, 10 μM	37	51	13
sGITR + Z-DEVD-FMK, 10 μM	64	17	19
sGITR + Z-DEVD-FMK, 50 μM	65	17	18
sGITR + Z-VAD-FMK, 10 μM	70	20	10

Table 3
Effect of cell cycle progression by sGITR in BMDM

	G0/G1 (%)	G2/M (%)	S (%)
Control/48 h	69	16	15
0.2 μg/ml sGITR	78	14	7
2.0 μg/ml sGITR	82	13	6

We next examined whether cell cycle arrest in G1 phase also occurred in a homogeneous population of primary BMDM cultures responsive to physiological stimuli. After 11 days of culture we obtained a homogeneous population of adherent macrophages. These were incubated with sGITR for 48 h. As shown in Table 3, 69% of the control cells were in G1 phase, 16% in S phase, and 15% in G2/M phase 48 h after synchronization. After 48 h continuous exposure to 2 μg/ml sGITR, 82% of the cells were in G1 phase, 13% in G2/M, and 6% in S phase. As the concentration of sGITR was increased, the proportion of G1 phase cell also rose, though to a lesser extent than in the Raw264.7 cells. The viability of the BMDM cells was also reduced by sGITR (data not shown).

In summary, stimulation of macrophages with sGITR results in inhibition of cell growth via accumulation of cells in G1 phase and subsequent apoptosis. Growth inhibition appears to be due to signaling via the GITR ligand.

Decrease in cyclins D and, A, and cdk4 levels, and inhibition of cdk2 and cdk4 activities by sGITR

To investigate the mechanism by which sGITR causes G1 phase arrest, we examined cyclin expression and function. Since G1 phase progression depends on the

sustained expression of D-type cyclins and the action of the cdk [17], we first examined the effect of sGITR on the expression of cyclin D2 and cdk4. The level of cyclin D2 began to drop after 15 h exposure to sGITR, relative to control cells, and inhibition reached a maximum after 24 h (Fig. 2A) and was maintained up to 32 h of sGITR stimulation. Although sGITR stimulation reduced expression of cyclin D2 after 24 h relative to controls, the absolute level of cyclin D2 expression was not substantially lower than at earlier time points; rather cyclin D2 levels increased in the control cells over time whereas they failed to do so in the treated cells. The concentration of the cyclin D2-related kinase, cdk4, displayed a similar pattern. Soluble GITR treatment also reduced expression of cyclin A, but did not affect cyclin E or cdk2 (Fig. 2B). We next examined whether, in the sGITR-stimulated macrophages, cdk2 behaved as a cyclin A-dependent kinase and cdk4 as a cyclin D2-dependent kinase. Cell extracts were immunoprecipitated with anti-cdk2 Ab. Immunoprecipitates from untreated Raw264.7 cells contained cyclin A, and such cyclin A-containing immunoprecipitates decreased to zero 24 h after sGITR stimulation, with no change in the level of cdk2 as a loading control (Fig. 2C). Control cells yielded immunoprecipitates with anti-cdk4 containing cyclin D2 and cdk4, and the levels of both proteins dropped immediately after treatment with sGITR (Fig. 2C).

We next determined by Western blotting whether the decreased levels of cyclin D2, cyclin A, and cdk4 affected the phosphorylation of Rb. Exponentially growing cells contained both hyperphosphorylated and hypophosphorylated forms of Rb (Fig. 3A), and the level of hyperphosphorylated Rb started to decrease after 24 h of sGITR stimulation and was almost zero after 48 h (Fig. 3A). Thus, sGITR causes hypophosphorylation of Rb in Raw264.7 cells. In order to determine the influence of sGITR on cdk2 and cdk4 activities, we treated cells with sGITR for 32 h and measured cdk activities. Extracts were immuno-precipitated with anti-cdk2 and anti-cdk4 Ab, and the kinase activities of the immunopellets were determined from their ability to phosphorylate the exogenous substrates, histone H1 and GST-Rb, respectively. As shown in Fig. 3B, cdk2 activity was high in control cells, but dropped dramatically

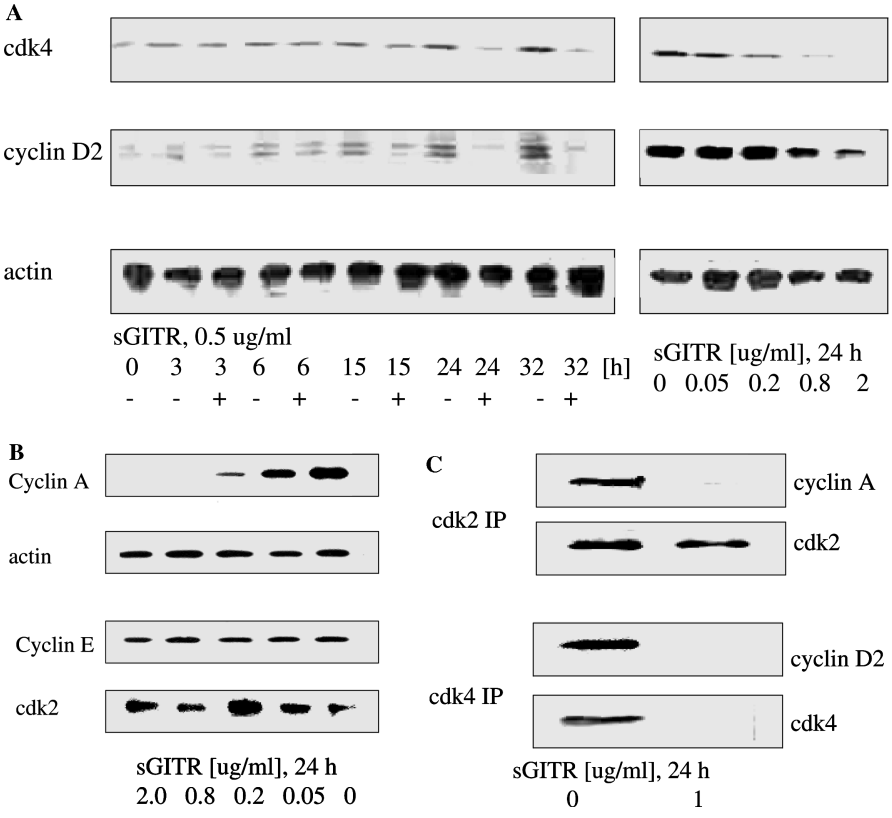


Fig. 2. Soluble GITR inhibits growth of Raw264.7 cells by reducing levels of cyclin D2, cdk4, and cyclin A expression. (A) Expression of cyclin D2 and cdk4 after treatment of sGITR was analyzed by Western blotting. Cells were cultured in 0.5 μg/ml sGITR for the indicated times or for 24 h at the indicated concentrations of sGITR. Expression of β-actin served as a control for sample loading and transfer efficiency. (B) Expression of cyclin A, cyclin E, and cdk2 after treatment with sGITR analyzed by Western blotting. Cells were cultured for 24 h with the indicated concentrations of sGITR. The expression of β-actin controlled for sample loading and transfer efficiency. (C) Anti-cdk2 and anti-cdk4 immunoprecipitates from untreated Raw264.7 cells (0 h), and cells cultured with 1 μg/ml sGITR for 24 h were analyzed by Western blotting using anti-cyclin A Ab and anti-cyclin D2 Ab. The blots were re-probed with anti-cdk2 and anti-cdk4 Ab. Results are representative of three experiments.

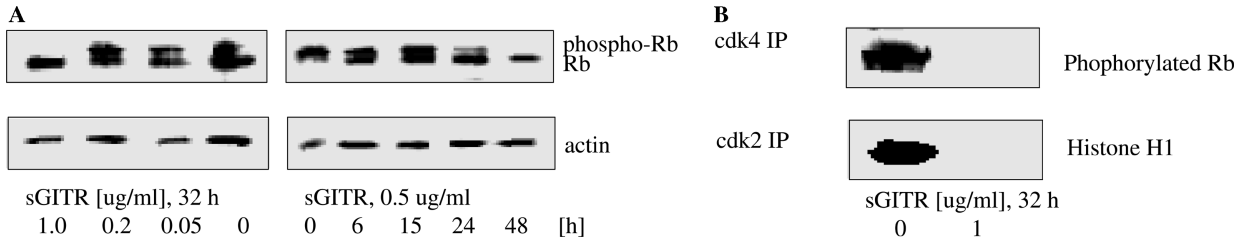


Fig. 3. Soluble GITR inhibits growth of Raw264.7 cells by lowering the activities of cdk2 and cdk4. (A) Levels of phosphorylated Rb and Rb after treatment with sGITR were analyzed by Western blotting. Cells were cultured in 0.5 μg/ml sGITR for the indicated times or for 32 h at the indicated concentrations of sGITR. The data are representative of three separate experiments. (B) Soluble GITR inhibits cdk2 and cdk4 activities. Cdk2 and cdk4 activities were assessed in vitro after 32 h stimulation with 1 μg/ml sGITR by measuring the phosphorylation of histone H1 and GST-Rb, respectively.

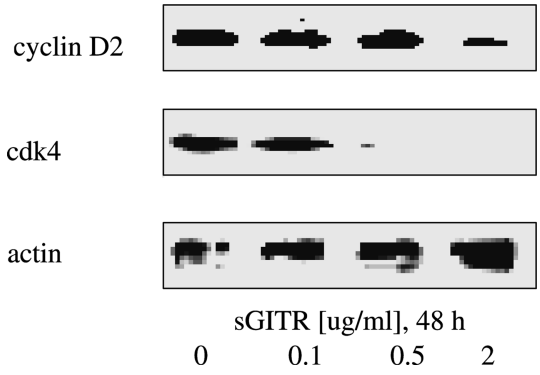


Fig. 4. Soluble GITR inhibits growth of BMDM by reducing levels of cyclin D2 and cdk4 expression. The expression of cyclin D2 and cdk4 after treatment of sGITR was analyzed by Western blotting. Cells were cultured for 48 h with the indicated concentrations of sGITR. Data are representative of three experiments.

following addition of sGITR. Cdk4 activity also fell after sGITR stimulation and phosphorylation of Rb decreased. These inhibitory effects on cdk2 and cdk4 activities are consistent with the cell cycle data demonstrating accumulation of the cells in G1. Similar effects of sGITR on cyclinD2 and cdk4 were observed in BMDM cells (Fig. 4).

The effects of nitric oxide on cell cycle arrest

Since sGITR generates nitric oxide (NO) in murine macrophages [7], and NO has been linked to cytotoxic effects in macrophages [18] and cell cycle arrest in smooth muscle cells [19,20], we blocked the sGITR-induced production of NO with the iNOS inhibitor *S*-methylisothiourea sulfate (SMT). Macrophages exposed to sGITR generated 51.7 ± 0.3 nmol of NO/10^6 cells, and SMT treated cells produced much reduced levels of NO (Table 4). SMT alone did not affect the cell cycle. SMT in combination with sGITR blocked NO production, but did not completely inhibit G1 arrest, as shown in Table 4. We also examined the effect of exogenous NO on the cell cycle. Addition of an NO donor, sodium nitroprusside (SNP), generated NO, but did not induce much G1 arrest. These results suggest that the observed G1 cell cycle arrest is induced by sGITR or its other metabolites rather than by NO.

Table 4
Effect of cell cycle progression and NO production by sGITR in the absence or presence of SMT

	G0/G1 (%)	NO level (nmol/10^6 cells)
Control	46	0
sGITR + SMT 5 μM	80	10.0 ± 1.2
sGITR + SMT 15 μM	77	7.3 ± 0.3
0.2 μg/ml sGITR	79	51.7 ± 0.3
SMT 15 μM	55	0
SNP 50 μM	50	77.3 ± 0.3

Discussion

Previous studies have demonstrated that interactions involving sGITR cause the macrophages to undergo biochemical, morphological, and functional modifications with the induction of nitric oxide synthase, cyclooxygenase-2, and MMP-9 [7,15,16]. The ability of sGITR to influence the cell cycle has not been previously investigated. In this report, we examined the ability of sGITR to affect the growth of murine Raw264.7 and BMDM macrophages, and investigated the factors that cause growth inhibition by means of cell cycle analyses. The presence of GITR and GITR ligand has been demonstrated in murine macrophages [7,21]. Soluble GITR blocked the proliferation of Raw264.7 cells and primary BMDM cultures by inducing G1 arrest. Interestingly, sGITR-mediated apoptosis of Raw264.7 cells took place quite slowly, requiring at least 2 days, indicating that sGITR induces apoptosis by an indirect route. Caspase blockers did not affect the sGITR-induced G1 arrest, which occurred much more rapidly than apoptosis. Cell cycle arrest followed by apoptosis is a common physiological response to stress. Thus for example, Zhang et al. [22] demonstrated that the novel retinoid CD437 induces S phase arrest of epithelial cells, followed by apoptosis.

Although NO inhibits the proliferation of a number of cell types, there have been few studies of the effects of NO donors on macrophage proliferation. Takagi et al. [23] reported that NO donors inhibited proliferation of the macrophage-like Mm1 cells, arresting them in G2/M, and NO donors induced G1 arrest of primary BMDM [24]. It is well known that NO has an antiproliferative effect on smooth muscle in vivo and in vitro [25]. This effect is associated with arrest in both G1 and S phases and is the result of changes in cdk2 activity correlated with altered cyclin A expression. Macrophages generate substantial amounts of NO in response to sGITR [7]. NO production is detectible 8 h after sGITR stimulation and persists up to 48 h. In the present experiments, sGITR decreased the level of cyclin D2, cyclin A, and cdk4 with resulting inactivation of cdk2 and cdk4 activities, and caused G1 phase arrest of the murine macrophages. It is unlikely that the G1 phase arrest caused by sGITR was due to the NO produced, since exposure to the NO donor, SNP, failed to induce G1 arrest even though we detected higher levels of NO in response to SNP than in response to sGITR. Moreover the addition, of an iNOS inhibitor, SMT, together with sGITR did not totally block G1 arrest although it caused a substantial decrease in the amount of NO generated.

Soluble GITR induced G1 phase arrest by lowering levels of cyclin D2, cyclin A, and cdk4, and caspase blockers did not prevent the G1 arrest induced by sGITR, indicating that sGITR-induced apoptosis is a later event. Cell cycle progression is regulated by the sequential action of cdks that are activated by associating with a number of cyclins. Anti-cdk4 and anti-cdk2 immunoprecipitates had reduced levels of cyclin D2 and cyclin A, respectively, after sGITR stimulation, confirming that cyclin D2 and cdk4 are associated, as are cyclin A and cdk2. This also suggests that cyclin A and cyclin D2 regulate cdk2 and cdk4 activity, respectively, during G1 phase progression. Expression of cdk4 was also lowered, but cdk2 levels did not change, after sGITR activation, and in vitro kinase assays revealed reduced levels of Rb kinase and histone H1 kinase activities after sGITR stimulation. Since G1 arrest preceded apoptosis in the sGITR-treated macrophages, the changes in cell cycle-related protein expression may be involved in the apoptosis.

The exact function of GITR is not clear, but several reports have suggested that GITR is involved in apoptosis [6,26]. Spincelli et al. [26] demonstrated that GITR induces apoptosis by interacting with the pro-apoptotic protein Siva, probably via GITR–GITR ligand binding. Since little about the function of the membrane-bound form of GITR is known in macrophages, we cannot be sure that sGITR exactly mimics the membrane-bound form. The natural presence of sGITR has not been detected, but alternatively spliced transcripts without a trans-membrane domain have been reported [27]. There are several reports of naturally occurring soluble TNFR or TNF family proteins in immunopathological conditions. Elevated soluble CD27 levels were found in the serum of systemic lupus erythematosus [28] and in Graves' hyperthyroidism [29]. Enhanced levels of the soluble forms of 4-1BB were also found in sera of patients with rheumatoid arthritis [30] and those of 4-1BB ligand in sera of patients with hematological malignancies [31]. Soluble forms of cell surface receptors can be produced either by proteolysis of membrane bound receptors such as TNFR [32], or by alternatively spliced transcripts encoding soluble forms of receptors such as 4-1BB [30]. Some ligands of the TNF superfamily have been shown to transmit signals in both directions through their respective receptors in cells that express the corresponding ligands. Reverse signaling through the 4-1BB ligand inhibits proliferation, induces apoptosis, and stimulates monocytes to generate IL-8 [33]. Since both GITR and GITR ligand were found in murine macrophages [7,21], it is not clear whether sGITR-stimulated cell growth inhibition in macrophages is caused by signal transduction through GITR ligand or by blocking the endogenous GITR signal. However, our neutralization experiments with anti-GITR or anti-GITR ligand Ab suggested that growth inhibition is due to signal transduction through GITR ligand.

Similar reciprocal expression of receptor and ligand has been found in other TNFR family members such as CD27 and CD70 [34], 4-1BB and 4-1BB ligand [13], and Fas and Fas ligand [35]. Monocytes express low but detectible levels of Fas and Fas ligand, and human monocytes cultured in vitro underwent spontaneous apoptosis mediated by Fas and Fas ligand on their cell surface, without requiring additional stimuli [35]. However, in the case of CD27 and CD70, a different phenomenon has been reported: engagement of CD27 induced apoptosis via interaction between CD27 and CD70 [34]. The B cell lines Ramos and Raji express on their surface relatively high levels of both CD27 and CD70 but do not undergo apoptosis under normal culture condition [34]. This may be explained by the relatively low cell density used for routine cell culture, which may not permit the interaction between CD27 and CD70 to reach the required threshold level [34]. The same phenomenon could occur in Raw264.7 cells, which have both GITR and GITR ligand on their surface.

The ultimate question is what roles sGITR-induced apoptosis play in the sGITR-mediated activation of macrophages. Apoptosis is a physiological cell death pathway that removes damaged or unwanted cells. The release of nuclear and cytoplasmic components of dying cells is potentially damaging and is related to the pathology of a wide range of inflammatory diseases. Cells of the immune system undergo death after activation as

a mechanism regulating their potentially destructive activity. This is the case of activation-induced apoptosis through CD3 in T cells [36] and of apoptosis induced by superantigen activation in lymphocytes [37]. Activation of macrophages also leads to their own death when the macrophages were exposed to cytokines such as INF-α [38]. Several disease studies have shown that macrophage apoptosis is common in atherosclerotic lesions [39] and in the synovial fluid of rheumatoid arthritis patients [40].

In summary, we have shown that sGITR inhibits the growth of murine macrophages via a signal through GITR ligand. This growth-inhibitory effect is due to sGITR-induced G1 phase arrest followed by apoptosis. Soluble GITR decreased levels of cyclin D2, cyclin A, and cdk4, resulting in reduced cdk4 and cdk2 activities.

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