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Soluble glucocorticoid-induced TNF receptor (sGITR) induces inflammation in mice

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Abbreviations: GITR, glucocorticoid-induced tumor necrosis factor receptor; HRP, horseradish peroxidase; TNFR/NGFR, TNF/nerve growth factor receptor

Abstract

Glucocorticoid-induced TNF receptor (GITR) was a new member of the TNF/nerve growth factor receptor (TNFR/ NGFR) family and induced in murine T cells by dexamathasone. Recombinant soluble GITR (sGITR) induced an inflammation in peritoneal membrane and changes in spleen after *i.p.* injection of 3 mg/kg in C57BL/6 mice. Spleen was enlarged and percentage of neutrophils and monocytes were increased. The area of red pulp in spleen was increased, while that of white pulp was decreased after GITR injection. The thickening of membrane and neutrophil infiltration was observed in peritoneal membrane with increased myeloperoxidase activity. At later time, neutrophil infiltration moved to inside the tissue with tissue damage. GITR ligand and GITR were expressed constitutively on the surface of spleen cells and cells from peritoneal fluid. In contrast, no significant change in the spleen and in peritoneal membrane was observed in mice treated with LPS. GITR may play a role in body's inflammatory processes.

Keywords: dexamathasone; glucocorticoids; inflammation; receptors, tumor necrosis factor

Introduction

Glucocorticoid-induced TNF receptor (GITR) was described as a new member of the TNF/nerve growth factor receptor (TNFR/NGFR) family and induced in murine T cell hybridoma 3D0 cells by dexamathasone (Nocentini *et al*., 1997). TR11 (AITR) isolated from a human activated T cell cDNA library has 55% identity with murine GITR at the amino acid level (Kwon, *et al*., 1999). The high sequence conservation between human and mouse suggests that TR11 is the human homologue of GITR. They may, however, have different biological activities because of following reasons; there is a mismatch in the first cysteine rich pseudorepeat between GITR and TR11, and TR11 is not inducible by dexamethasone. GITR shows a similarity in amino acid sequence of intracellular tails to those of 4-1BB, CD27, and TR11 (Kwon and Weismann, 1989; Jacquot *et al*., 1997; Heinisch *et al*., 2000; Takeda *et al*., 2000). It has been reported that TR11, 4-1BB, and CD27 transmit their signals through TRAF2 to NF-κB (Akiba *et al*., 1998; Arch and Thompson, 1998; Jang *et al*., 1998; Kwon, *et al*., 1999; Cannons *et al*., 2000).

Glucocorticoid hormones control the host immune responses to infection and tissue invasion and are generated in response to the systemic stress mediated by the hypothalamus-pituitary-adrenal axis. Glucocorticoids are known to be powerful anti-inflammatory agents which inhibit immune cell activation, proliferation, and cytokine productions (Goldstein *et al*., 1992).

In this study, we have shown that soluble GITR induces an inflammation in peritoneal membrane and changes in spleen after *i.p.* injection in C57BL/6 mice. GITR may play a role in body's inflammatory processes.

Material and Methods

Mateials and reagents

DMEM, FBS, penicillin, and streptomycin, LPS (from *Escherichia coli* serotype 055:B5), guanidine thiocyanate, and isopropyl β-D-1-thiogalactopyranoside (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). A mouse recombinant protein, GITR-Fc, was from Alexis Corporation (UK). Recombinant extracellular domain of GITR (sGITR) was prepared in *Escherichia coli*-pET 28a and purified as described previously (Shin *et al*., 2002). The sGITR was purified by eluting the protein from 10% SDS-PAGE to avoid possible endotoxin contamination. SDS gel-purified rGITR containd no more than trace amount of endotoxin (< 0.4 EU/mg by E-Toxate assay from Sigma Chemical). Polyclonal antiserum against GITR was obtained from rabbit as described before (Shin *et al*., 2002)

Mice and treatment

Seven-week-old male C57BL/6 mice (-20 g) were injected *i.p.* with 3 mg/kg of GITR or 50 mg/kg of LPS in saline. Control mice were injected with saline alone (n = 10). After 4 h (n = 5), 8 h (n = 6), 15 h (n = 7), 24 h (n = 7) of GITR or 3 h (n = 5), 15 h (n = 4) of LPS, the mice were sacrificed, blood was drawn, and peritoneal membrane, spleen, and peritoneal lipids were obtained. Part of the tissues was stored in 10% formalin, stained by hematoxylin and eosin, and photographed. Spleen was weighed and peritoneal membrane was homogenated, and cell

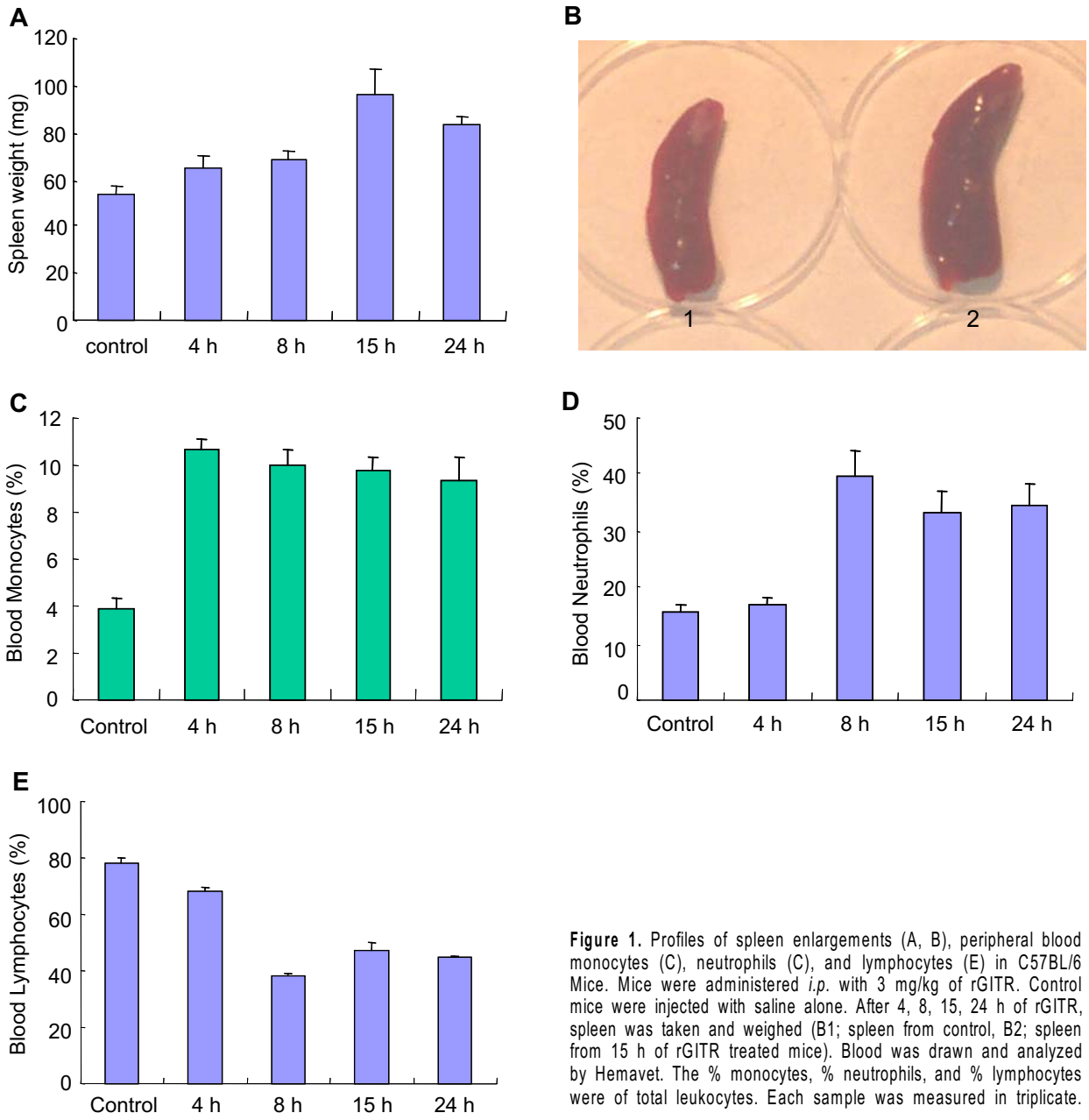

Figure 1. Profiles of spleen enlargements (A, B), peripheral blood monocytes (C), neutrophils (C), and lymphocytes (E) in C57BL/6 Mice. Mice were administered *i.p.* with 3 mg/kg of rGITR. Control mice were injected with saline alone. After 4, 8, 15, 24 h of rGITR, spleen was taken and weighed (B1; spleen from control, B2; spleen from 15 h of rGITR treated mice). Blood was drawn and analyzed by Hemavet. The % monocytes, % neutrophils, and % lymphocytes were of total leukocytes. Each sample was measured in triplicate.

lysate was obtained for myeloperoxidase assay.

Flow cytometry

Cells (5×10^5/sample) were incubated in incubation buffer (PBS, 10% human serum and 0.1% sodium azide) with purifed polyhistidine- tagging sGITR or rabbit anti-GITR polyclonal Ab on ice for 30 min for detection of GITR ligand and GITR, respectively. Same volume of PBS and preimmune serum were used as a isotype control for GITR ligand and GITR, respectively. Cells were washed three times in PFS (phosphate buffered saline, 2.5% fetal bovine serum and 0.1% sodium azide). The cells were incubated on ice for 30 min with mouse anti-His Ab in incubation buffer for detection of GITR ligand. Cells were washed three times. 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).

Results

Effects of GITR in mice

Previously extracellular domain of GITR activated macrophages to induce iNOS (Shin *et al*., 2002), COX-2 (Shin *et al*., 2002), and MMP-9 (Lee *et al*., 2003). We tested the effects of soluble GITR (sGITR) kinetically in C57BL/6 mice. When mice were treated intraperitoneally with sGITR at a dose of 3 mg/kg, spleen was enlarged (Figure 1A) and percentage of neutrophils and monocytes were increased in peripheral blood (Figure 1C and 1D) whereas control mice injected with PBS showed no significant changes. Spleen enlargement was eminent at 15 h after sGITR injection (Figure 1B). The percentage of neutrophil was maximum at 8 h and remained up to 24 h and that of monocyte reached maximum at 4 h and remained, while that of lymphocyte decreased (Figure 1E). Significant changes in spleen and peritoneal membrane was observed by histochemical analysis using hematoxylin and eosin staining. The area of red pulp in spleen was increased, while that of white pulp was decreased after sGITR injection (Figure 2A). The increase of red pulp in spleen was significant at 15 h and 24 h after sGITR injection. The thickening of membrane and neutrophil infiltration was observed in peritoneal membrane after 4 h, and was maximum at 15 h. The edema was decreased by 24 h after sGITR injection, but neutrophil infiltration moved to inside the tissue with tissue damage (Figure 3A). At the site of inflammation, neutrophils are among the first cells to arrive. Since activation of neutrophils results in myeloperoxidase into the extracellular matrix, myeloperoxidase level was determined in peritoneal membrane homoge-

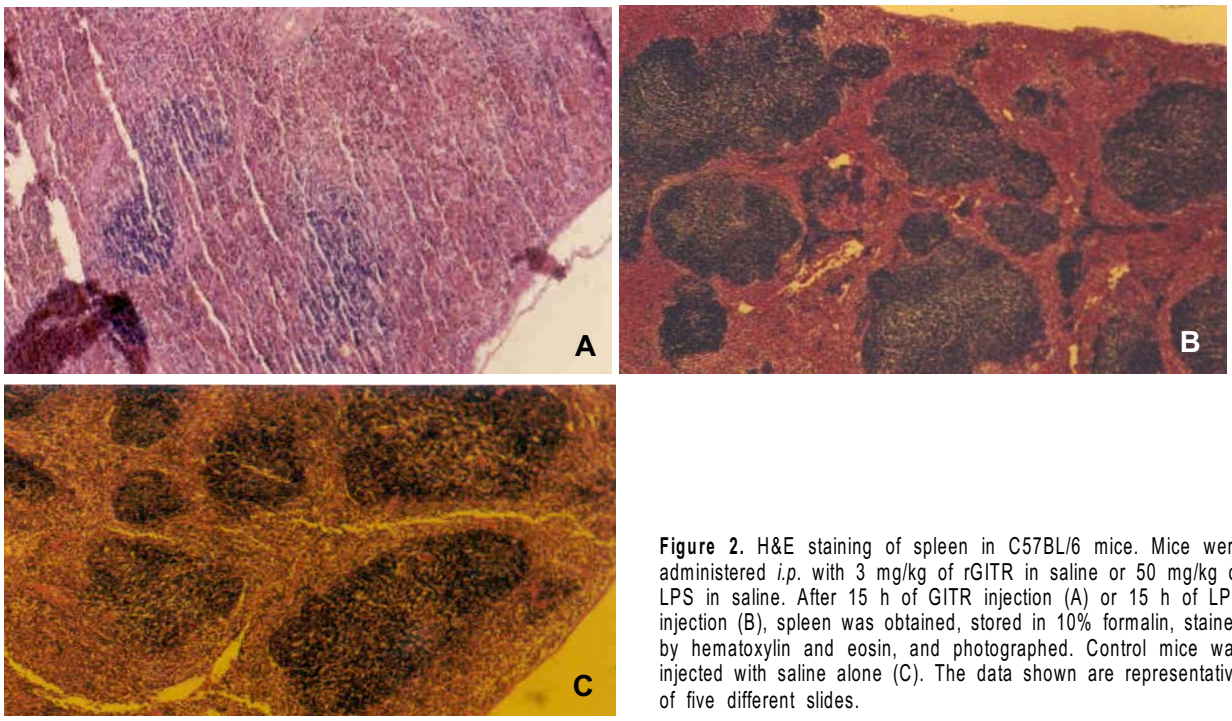

Figure 2. H&E staining of spleen in C57BL/6 mice. Mice were administered *i.p.* with 3 mg/kg of rGITR in saline or 50 mg/kg of LPS in saline. After 15 h of GITR injection (A) or 15 h of LPS injection (B), spleen was obtained, stored in 10% formalin, stained by hematoxylin and eosin, and photographed. Control mice was injected with saline alone (C). The data shown are representative of five different slides.

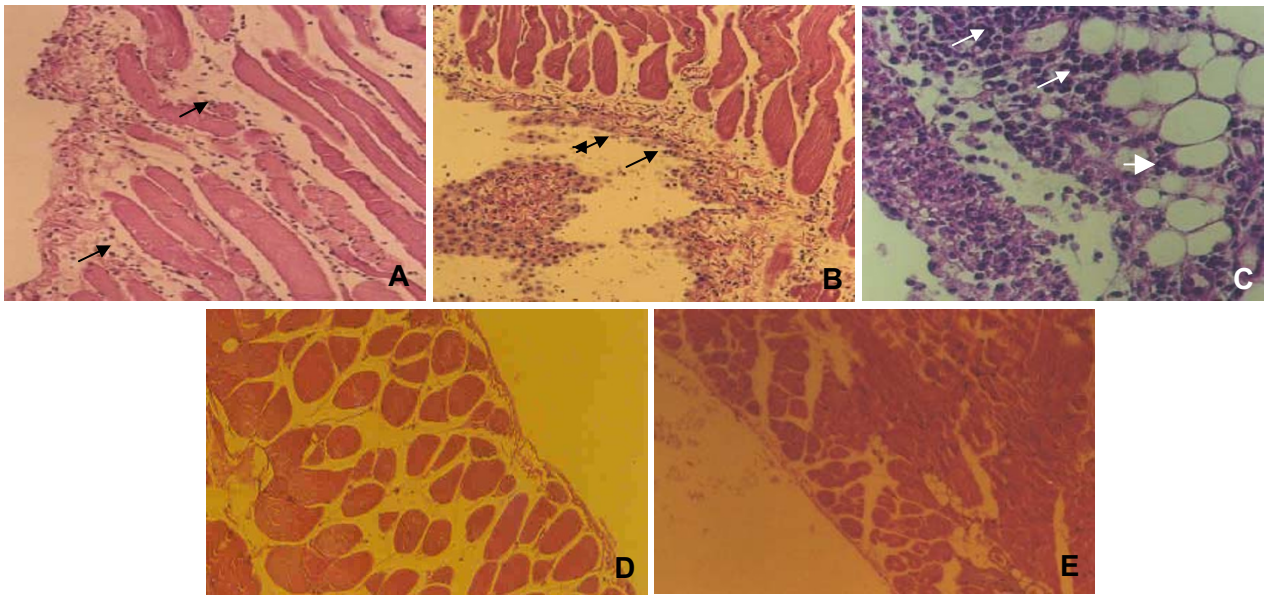

Figure 3. H&E staining of peritoneal membrane in C57BL/6 mice. Mice were admisistered *i.p.* with 3 mg/ kg of rGITR or 50 mg/ kg of LPS in saline. After 24 h (A) or 15 h of GITR injection (B-C) or 15 h of LPS injection (D), peritoneal membrane was obtained, stored in 10% formalin, stained by hematoxylin and eosin, and photographed. Infiltrated neutrophil (A), mesothelial cell growth (B), lymphocytes and neutrophil infiltration (C) were indicated by arrows. Control mice was injected with saline alone and peritoneal membrane was obtained after 15 h of injection (E). The data shown are representative of five different slides.

nates. Specific activity of myeloperoxidase increased about 4-fold after 8 h of sGITR stimulation, remained up to 24 h (Data not shown), supporting the above histochemical data as shown in Figure 3A. Mesothelial cell growth was observed at 15 h (Figure 3B). At 15 h lymphocyte and neutrophil infiltration were also observed in omental fat tissue while inflammation is under process (Figure 3C).

We wanted to verify that the observed effects of sGITR in mice were genuine and not due to some nonspecific 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 polyhistidine tag and those by HEK 293 cells as a fusion protein with Fc. GITR-Fc was injected in mice intraperitoneally at a dose of 0.3 mg/kg due to a limited supply. It also induced a mild inflammation in peritoneal membrane and increased proportion of red pulp in spleen after 15 h. In addition, infiltration of mononuclear cells such as lymphocyte, macrophage, and plasma cells was observed in omental fat tissue with a less extent (Data not shown). Similar pattern was observed with the GITR protein expressed from Baculovirus (Data not shown).

To exclude the possibility that inflammation is due to LPS-like contaminant from *E. coli*-produced GITR, the effects of LPS were observed. No significant change in the spleen (Figure 2B and 2C) and in peritoneal membrane was observed in mice treated with 50 mg/ kg of LPS or PBS alone, respectively (Figure 3D and 3E).

Generally TNFR family express its activity via receptor-ligand interaction. Since the change induced by sGITR was significant in the spleen and peritoneal membrane, the expressions of GITR and GITR ligand on the surface of spleen cells and cells from peritoneal fluid were examined by FACS analysis. GITR ligand and GITR were expressed constitutively on the surface of spleen cells (Figure 4A and 4B). Induction with LPS or PMA did not increase the expression levels of them (Data not shown). Large size cells from peritoneal fluid also expressed GITRL and GITR with much lower level than spleen cells did (Figure 4C and 4D).

Discussion

GITR was identified as a member of TNFR, and induced after dexamethasone stimulation. Although its function is not clear, GITR appears to protect T cells from apoptosis induced by anti-CD3 mAb. We generated extracellular domain of rGITR to test the effect of the sGITR in mice. When mice were injected with sGITR, spleen was enlarged and the ratio of red and white pulp in the spleen was increased. The actual areas of red and white pulp were not measured, but the area of red pulp appeared to be

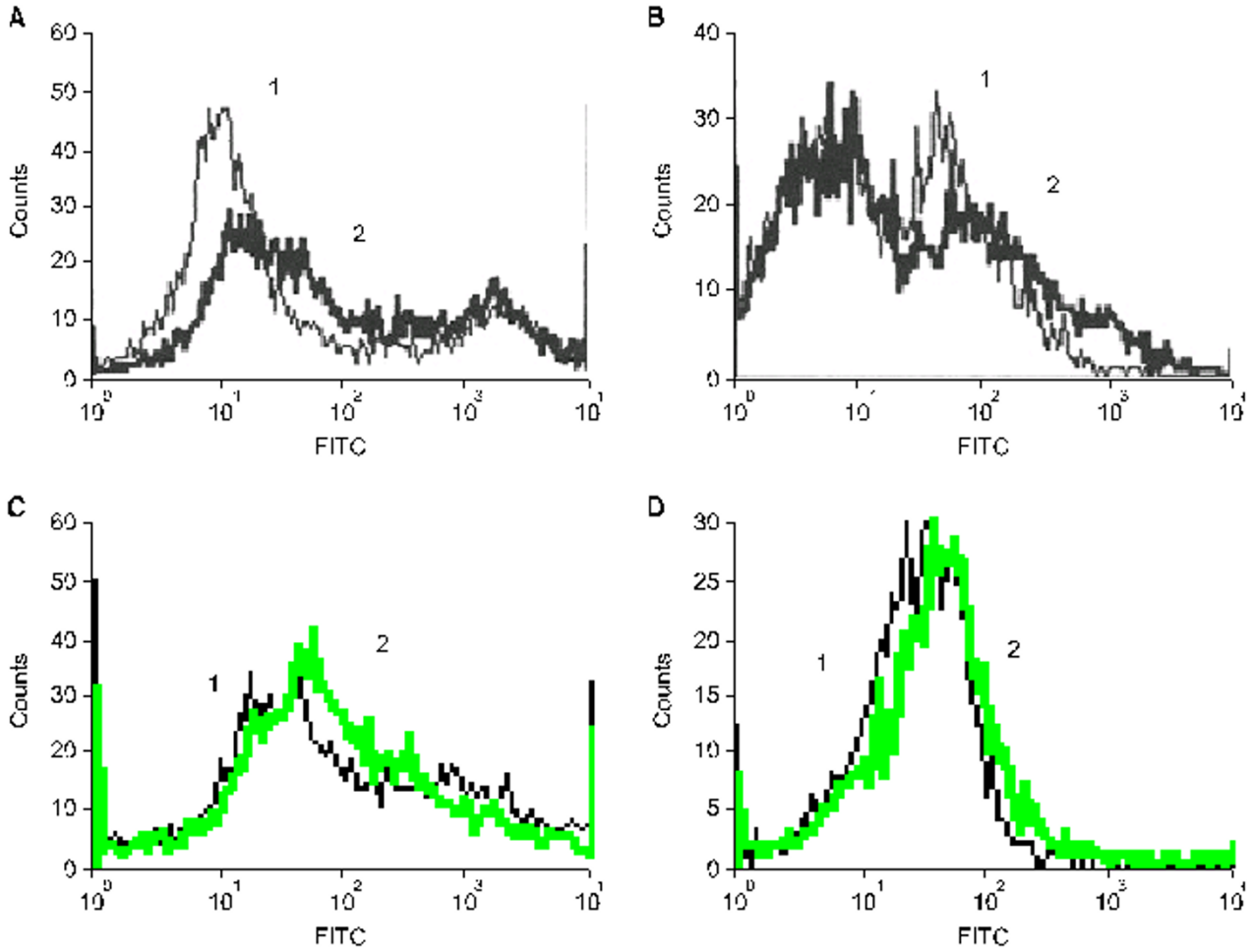

Figure 4. FACS scan of GITR ligand and GITR. For detection of GITR ligand (A), spleen cells were incubated with rGITR, monoclonal anti-histidine Ab, and FITC-conjugated goat anti mouse IgG (peak 2) each for 30 min on ice for GITR ligand detection. Cells were washed three times after each incubation. PBS instead of rGITR was used as an isotype control (peak 1). For GITR (B), spleen cells were incubated with 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). Cells from peritoneal fluids were prepared by washing peritoneum with PBS (C; GITR ligand, D; GITR).

enlarged due to spleen enlargement. Edema and neutrophil infiltration also observed in the peritoneal membranes. Percentage of peripheral blood neutrophils and monocytes were increased. Infiltrating neutrophils generate oxygen derivatives, serine proteases and zinc matrix metalloproteases (MMPs) to promote tissue injury related to inflammatory processes. Neutrophil infiltration into muscles and fat tissues were followed by muscle destruction. Previously, expressions of MMP-2 and MMP-9 were observed in peritoneal membrane homogenates after GITR injection (Lee *et al*., 2003). MMP-9 expression appeared to be related to the inflammatory response, whereas MMP-2 activation to be concomitant with the regeneration of new myofibers (Kherif *et al*., 1999). Growth of mesothelial cells was also observed in peritoneal membrane.

Since sGITR was produced from *E. coli* system, we need to verify the observed effect was from genuine GITR protein, not from contaminated endotoxin of sGITR protein preparation. The GITR preparation contained < 0.4 EU/mg of endotoxin as determined by E-Toxate assay and the level was quite low. However, we also tried other sources of sGITR protein from Baculovirus system and HEK 293 cells. They also showed similar effect in mice, although it was mild, suggesting that the observed effect was due to sGITR protein. Although sGITR produced inflammatory reaction similar to LPS, there are some differences. For example, when 50 mg/kg of LPS was injected, no significant changes were found up to 15 h. However, significant level of damage

was observed in skeletal muscles after 2 d and inflammation in peritoneum was after 5 d of LPS treatment (unpublished results).

Soluble forms of TNFR family protein have been demonstrated for TNF receptor family, CD27, CD30, and CD137 (Mohler *et al.*, 1993). These soluble receptor forms are generated *in vivo* by proteolytic cleavage or alternative splicing. In case of CD27 or CD137 which shared intracellular domains with GITR, their soluble forms were found in inflammatory states with a positive correlation. Soluble CD137 was demonstrated to be released by activated lymphocytes and be detectible in sera of patients of rheumatoid arthritis (Mitchel *et al.*, 1998). Soluble CD27 has been also demonstrated for stimulation of T lymphocytes in patients of multiple sclerosis (Hintsen *et al.*, 1999) and systemic lupus erythematosus (Font *et al.*, 1996).

In this study, presence of GITR and GITR ligand in the surface of mouse spleen cells and cells from peritoneal fluids were demonstrated by FACS. Although the expression levels were low, they were constitutively expressed. Taken together, these data indicate that injected sGITR induced inflammatory events in peritoneum and changes of spleen *in vivo*. It is possible that soluble receptor bind to the membrane bound ligand and gives a retrosignal, an inflammatory reaction. Or soluble GITR acts as an antagonist and blocks a tonic anti-inflammatory effect of GITR ligand. The answer could be waited until GITR ligand has been identified.

Our studies suggest that sGITR participates in the inflammatory process observed in peritoneal membranes. Inflammation is the body's reaction to invasion by infection or physical damage. It represents the consequence of capillary dilation with edema and the emigration of leukocytes. Once the initiating event has occurred, the prolongation of the inflammatory process resulted in a variety of immune responses such as activation of T cell and cytokine expression. In our studies sGITR triggered inflammatory processes in peritoneal membranes and neighboring fat tissues. GITR also change size and structure of the spleen with high ratio of red pulp to white pulp, although it is not clear what indicates those changes in the spleen. It remains to be determined whether the effect of GITR is mediated by GITR ligand upon binding to GITR.

Acknowledgement

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