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Glucocorticoid-Induced Tumor Necrosis Factor Receptor Family-Related Protein Exacerbates Collagen-Induced Arthritis by Enhancing the Expansion of Th17 Cells

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Rheumatoid arthritis (RA), a chronic autoimmune form of inflammatory joint disease, progressively affects multiple joints with pathological changes in the synovia, cartilage, and bone. Numerous studies have suggested a critical role for glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) in the pathogenesis of autoimmune arthritis by modulating both innate and adaptive immune reactions, but the underlying mechanisms by which GITR activation promotes arthritic progression remain largely unclear. In this study, we found that collagen-induced arthritis mice treated with the ligand of GITR (GITRL) displayed an earlier onset of arthritis with a markedly increased severity of arthritic symptoms and joint damage, in which significantly increased Th17 cells in both spleen and draining lymph nodes were observed. Notably, results showed that a marked expansion of Th17 cells with increased RORγt mRNA expression was induced from naïve CD4$^+$ T cells when cultured with GITRL. Consistently, normal mice that were treated with GITRL were found to display a substantial expansion of splenic Th17 cells. Furthermore, we detected elevated serum levels of GITRL in patients with RA, which were positively correlated with an increase in interleukin-17 production. Taken together, the results from this study have revealed a new function of GITRL in exacerbating autoimmune arthritis via the enhancement of the expansion of Th17 cells. ***(Am J Pathol 2012, 180:1059–1067; DOI: 10.1016/j.ajpath.2011.11.018)***

Glucocorticoid-induced tumor necrosis factor receptor (TNFR) family-related protein (GITR, also known as TNFRSF18), is a type I transmembrane protein mainly expressed on naïve and effector CD4$^+$ T cells.[1] Recent studies have demonstrated that activation of GITR, via interaction with its ligand (GITRL), is critically involved in modulating both innate and adaptive immune reactions.[2,3] GITRL is predominantly expressed by antigen-presenting cells including dendritic cells (DCs) and macrophages. Accumulating data have indicated that the functional interaction of GITR with its cognate ligand GITRL delivers a potent co-stimulatory signal to enhance T-cell activation and cytokine production with significant implications for cancer immunotherapy.[4,5] The GITRL/GITR pathway has been shown to modulate

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DC function and promote T-cell–mediated immunity.[6] We have recently reported that bone marrow–derived DCs transfected with viral vectors expressing GITRL show significantly increased capacity in driving T-cell proliferation and exerting enhanced anti-tumor immunity in a murine model of lung carcinoma.[7]

Rheumatoid arthritis (RA), a chronic autoimmune disease with increased morbidity and mortality, progressively affects multiple joints with pathological changes of synovial inflammation, cartilage damage, and bone erosion. Numerous studies have shown that both activation of T cells and production of pro-inflammatory cytokines are critically involved in RA pathogenesis.[8] GITR, a costimulatory molecule for $CD4^+$ effector T-cell activation, has been implicated in the development of autoimmune disease as revealed by studies on the murine model of collagen-induced arthritis (CIA).[9,10] Mice with a targeted deletion of the GITR gene show severely reduced collagen-induced arthritis, in which $GITR^{-/-}CD4^+CD25^+$ T regulatory cells display more potent suppressive function in inhibiting $CD4^+CD25^-$ effector T-cell proliferation.[10] In another elegant study by Patel et al, activation of GITR signaling by *in vivo* administration of anti-GITR antibody significantly exacerbates disease progression in CIA mice, demonstrating that GITR-mediated responses are closely involved in the development of autoimmune arthritis.[9] These findings provide strong evidence that GITR signaling stimulates $CD4^+$ effector T-cell proliferation but abrogates regulatory T-cell (Treg) suppressor activity. Current studies have revealed an important role of IL-17–producing $CD4^+$ T helper (Th17) cells in the development of autoimmune arthritis,[11] but it remains unclear whether GITRL signaling affects autoimmune progression via modulating the generation and function of Th17 cells. We have previously shown that IL-17 is a key effector cytokine in mediating the downstream effect of B-cell–activating factor–triggered exacerbation of arthritic progression in CIA mice.[12] Moreover, new evidence indicates that Th17 cells act as a potent B-cell helper in inducing B-cell differentiation, germinal center formation, and isotype class switching, which highlights a critical role of Th17 cells in the pathogenesis of inflammatory autoimmune diseases.[13]

In this study, we aimed to investigate whether GITRL expression is dysregulated during CIA development and how enhanced GITRL activation affects autoimmune pathogenesis *in vivo*. We found that GITRL expression was increased in both peripheral lymphoid organs and joint tissue in CIA mice, whereas primary Th17 cells expressed high levels of GITR. Moreover, administration of recombinant GITRL in collagen-immunized mice enhanced Th17 cell generation and exacerbated arthritis development. Furthermore, elevated levels of GITRL were detected in the sera of RA patients, which displayed a significant correlation with increased serum levels of IL-17 in these patients. Thus, our results have revealed a previously unappreciated role of GITRL in the pathogenesis of autoimmune arthritis.

Materials and Methods

Mice

DBA/1J mice (8- to 10-week-old, male) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and maintained in a specific pathogen–free animal facility at the Jiangsu University. All animal experiments performed in this study were approved by the Jiangsu University Animal Ethics and Experimentation Committee.

Recombinant GITRL Protein

Murine GITRL protein was obtained from R&D Systems for various *in vitro* experiments. For *in vivo* experiments, recombinant GITRL protein was produced and purified as described.[14] In addition, the control protein was prepared with the same protocol except with no mGITRL insert in the construct. Endotoxin was removed by ToxinEraser endotoxin removal resin (Genscript, Piscataway, NJ) and detected by E-TOXATE Kits (Sigma, St. Louis, MO). The endotoxin levels of both control and GITRL protein are <0.1 EU/mL. The eluted proteins were kept in sterile phosphate-buffered saline (PBS).

Induction and Assessment of Arthritis

The scheme of CIA induction and protein treatment was depicted in Figure 1A. Briefly, 100 μg of bovine type II collagen (CII; Chondrex, Redmond, WA) dissolved in 0.1mol/L acetic acid was emulsified with an equal volume of complete Freund's adjuvant (CFA, 2 mg/mL; Difco, Detroit, MI) and administered intradermally at the base of tail into DBA/1J mice. On day 21, a booster emulsion prepared with CII and incomplete Freund's adjuvant (Difco) was intradermally administered near the primary injection site. Following the same protocol, adjuvant-treated littermates that were given PBS in place of CII served as controls. Beginning on day 21, the mice were daily scored for arthritis severity, as previously described.[15] Briefly, the clinical severity of arthritis was graded as follows: 0, normal; 1, slightly swelling and/or erythema; 2, pronounced edematous swelling; 3, pronounced edematous swelling plus light joint rigidity; and 4, laxity.

Quantitative Real-Time PCR

Total RNA was extracted from the joint tissue of immunized mice or from cultured $CD4^+$ T cells, using TRIzol reagent (Invitrogen, Madison, WI). Samples of joint tissue were prepared from the front paw after removing skin tissue and bones under a dissecting microscope. The mRNA (mRNA) levels of selected genes were determined using the SYBR Green Two-Step qRT-PCR Kit with ROX (Invitrogen) according to the manufacturer's guidelines and as previously described.[12] The sequences of gene-specific primers spanning an intron are as follows: for GITRL, sense 5′-CTACGGCCAAGTGATTCCTGT-3′, antisense 5′-GATGATCCCCCAGTATGTGTT-3′; for RORγt,

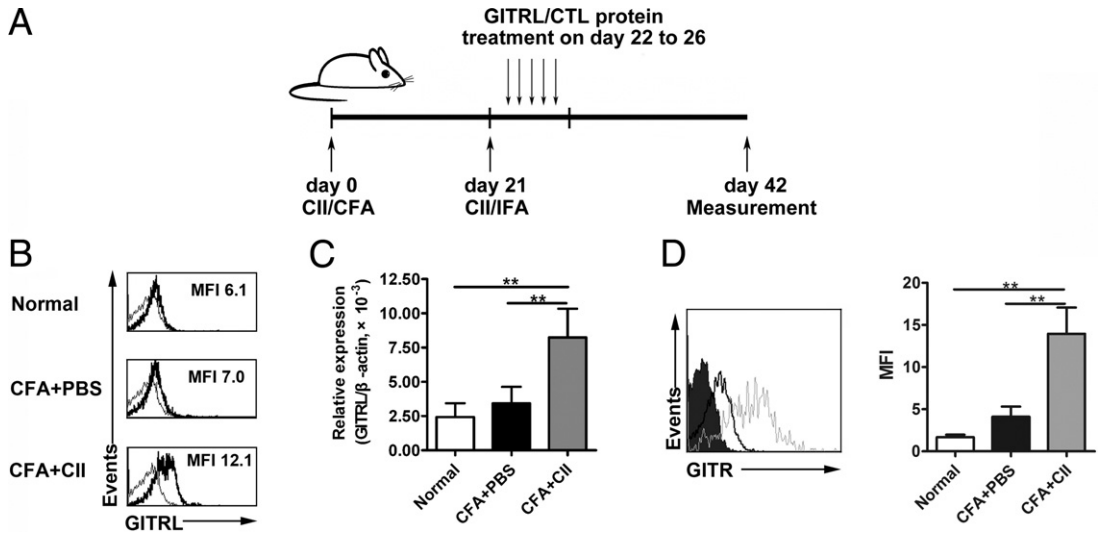

Figure 1. Up-regulated GITRL/GITR expression in CII- immunized mice. **A:** Scheme of CIA induction and GITRL or control protein administration. DBA/1J mice were immunized with CII/CFA on day 0 and boosted with CII/IFA on day 21. The treatment groups were intravenously injected with GITRL or control protein (20 μg per mouse) daily for consecutive 5 days (from day 22 to day 26). Mice were sacrificed on days 34 and 42 for various experiments. **B:** Up-regulated GITRL expression in CD11c$^+$DCs. DBA/1J mice were immunized with CFA plus PBS or CFA plus CII and sacrificed on day 42. Surface GITRL expression on DCs from inguinal lymph nodes was assessed by CD11c and GITRL double staining, followed by flow-cytometric analysis. CD11c$^+$ DCs were gated for the analysis of GITRL expression. The thin line shows isotype control staining and the thick line indicates the expression of GITRL. Representative results are from three independent experiments. **C:** Levels of GITRL mRNA expression in the joint tissue. RNA samples were prepared from skinless proximal interphalangeal joints of DBA/1J mice immunized with CII in CFA (CFA+CII) or CFA alone (CFA+PBS); DBA/1J mice without immunization (Normal) served as negative control. The mRNA levels of GITRL were quantified by quantitative RT-PCR, using β-actin as internal control. The relative expression of GITRL/β-actin is presented as mean ± SD (**$P < 0.01$). **D:** High levels of GITR expression in Th17 cells. DBA/1J mice were immunized with CFA plus PBS or CFA plus CII and sacrificed on day 42. Purified CD4$^+$ T cells from inguinal lymph nodes were stained for intracellular IL-17 and analyzed by flow cytometry. Black line shows staining for CD4$^+$ T cells; gray line is for IL-17$^+$ T-cell subset. Shaded area indicates isotype control staining. Results are representative of three independent experiments.

sense 5′-CCACCATATTCCAATACCTT-3′, antisense 5′-GCTGTCTGGACCCTGTTCT-3′; for IL-17, sense 5′-CTCCAGAAGGCCCTCAGACTAC-3′, antisense 5′-GGGTCTTCATTGCGGTGG-3′. The quantitative RT-PCR analysis was conducted with an Rotor Gene RG 6000 (Corbett, Australia), and the cycling parameters were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. Samples were run in triplicate, and their relative expression was determined by normalizing to the expression level of β-actin.

Cell Purification and Culture

CD4$^+$CD25$^-$CD62L$^+$ naive T cells were purified from inguinal draining lymph node or spleen cell suspensions by magnetic beads using a CD4$^+$CD62L$^+$ T Cell Isolation Kit II (Miltenyl Biotec, DE), with purity routinely over 95% (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). In addition, purified CD4$^+$CD62L$^+$ T cells were further examined for CD44 expression. CD4$^+$ T cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For Th17 cell differentiation, 5 × 10^5 naive CD4$^+$ T cells for each well were cultured in the presence of TGF-β (3 ng/mL) and IL-6 (20 ng/mL) in a 24-well plate precoated with anti-CD3 mAb (10 μg/mL) and anti-CD28 mAb (2 μg/mL) and treated with either GITRL protein (0.25 to 2 μg/mL) or control protein for 72 hours before further analysis.

Flow-Cytometric Analysis

Single-cell suspensions were prepared from spleen and inguinal lymph nodes of experimental mice. Surface or intracellular staining was performed using the following anti-mouse monoclonal antibodies from eBiosciences (San Diego, CA) and BioLegend (San Diego, CA): fluorescein isothiocyanate (FITC) anti-CD11c (clone N418) and anti–IL-17(clone eBio17B7), phycoerythrin (PE)–conjugated anti-GITRL (clone YGL 386) and anti-CD4 (clone GK1.5), PE-Cy5–conjugated anti-CD3 (clone 145-2C11). For detection of GITRL expression, CD11c$^+$ DCs from cell suspensions of inguinal lymph nodes were gated and the levels of surface GITRL expression were measured as mean fluorescence intensity (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). For IL-17 detection, cells were washed and stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of monensin (2 μmol/L) for 5 hours, and then stained with anti-CD4 mAb, fixed, and permeabilized using Intracellular Staining Kit (Invitrogen), followed by staining with anti–IL-17 mAb. Immunostained cells were analyzed using a FACS Calibur (Becton Dickinson, San Jose, CA).

ELISA

Serum levels of type II collagen–specific antibody were measured by a sandwich enzyme-linked immunosorbent assay (ELISA), as previously described.[16] Bovine type II collagen (Chondrex) were coated on 96-well MaxiSorp plates (Nunc, Rochester, NY) at 5 μg/mL in coating buffer

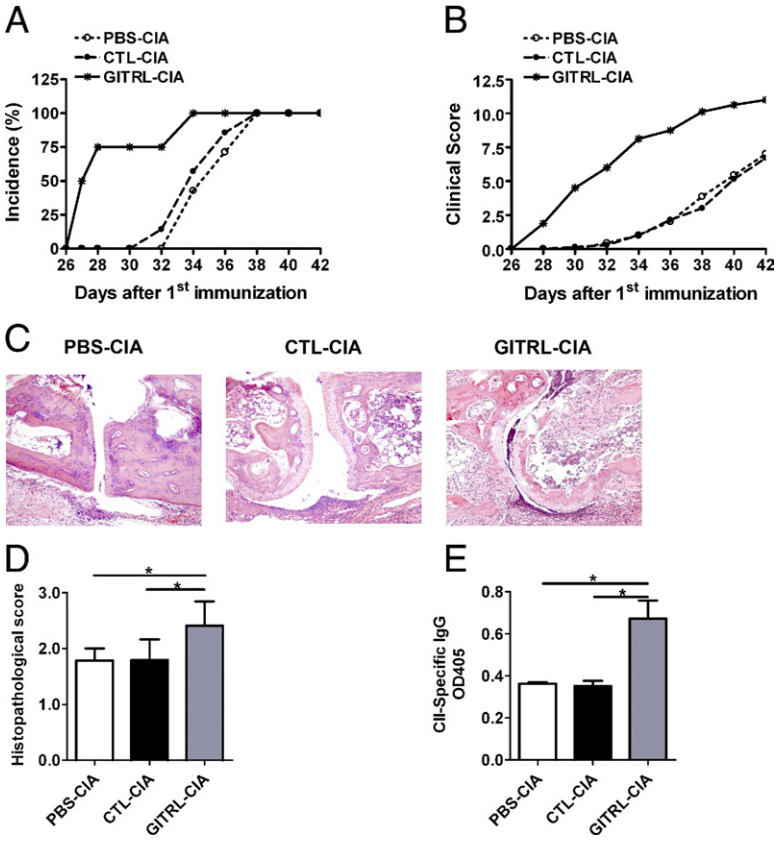

Figure 2. Exacerbated arthritis progression in CIA mice with GITRL treatment. **A:** Incidence of arthritis development among immunized mice treated with GITRL (GITRL-CIA), control protein (CTL-CIA), or PBS alone (PBS-CIA) was monitored every 2 days ($n = 12$ per group). **B:** Clinical scores of arthritis severity were assessed in PBS-CIA, CTL-CIA, and GITRL-CIA treatment groups ($n = 12$ for each group). **C:** Inflamed joints were sectioned for hematoxylin and eosin staining. Representative sections of joint tissue from each treatment group are shown (original magnification, ×100). **D:** Histopathological scores of joint tissue from PBS-CIA, CTL-CIA, and GITRL-CIA groups were assessed. Values are mean ± SD ($n = 6$ mice per group; $^*P < 0.05$). **E:** Serum levels of CII-specific autoantibodies from PBS-CIA, CTL-CIA, and GITRL-CIA groups were measured by ELISA. Values are mean ± SD ($n = 12$ mice per group; $^*P < 0.05$).

at 4°C overnight. Plates were washed before blocking with blocking buffer (0.5% gelatin, 0.5% bovine serum albumin, and 0.05% Tween 20 in PBS) at room temperature for 1 hour. Serially diluted serum samples (1:500) were added and incubated at room temperature for 1 hour. Plates were washed and incubated with HRP-conjugated goat anti-mouse IgG (H+L) (1:1000 dilution; KPL Inc.) at room temperature for 1 hour. 3,3′,5,5′-Tetramethylbenzidine substrate solution was then added to the plate and kept at room temperature for 15 to 30 minutes. A 50-μL quantity of H_2SO_4 (1 mol/L) was added to stop reaction, and absorbance at 450 nm was measured using a μQuant microplate reader (BIO-TEK Inc.).

Levels of IL-17 in culture supernatants or serum of murine samples were measured using an IL-17A ELISA Ready-SET-Go Kit (eBioscience), following the manufacturer's protocol. For detection of IL-17A and GITRL in the sera of human samples, ELISA kits were obtained from R&D Systems and eBioscience, respectively.

Histological Analysis

Murine joint tissue specimens were fixed in 10% buffered formalin for 3 days, followed by decalcification in 15% formic acid overnight before being embedded in paraffin. Tissue sections (4 μm thick) were prepared for hematoxylin and eosin staining. Histological analyses were performed using a widely used scoring system as previously described.[15]

Patients and Specimens

A total of 18 active RA patients (three men and 15 women) who met the American College of Rheumatology (ACR) 1987 revised criteria for the classification of RA were enrolled in this study.[17] The mean age of the patients was 57 ± 11 years (range, 28 to 71 years). Disease activity in RA patients was evaluated by DAS28.[18] Fifteen healthy control subjects were free of chronic pain, cardiovascular complaints, or other chronic inflammatory diseases and matched with patients for age and sex, with no statistically significant difference between the two groups. Serum samples were prepared from all patients and healthy controls. Briefly, 3 mL of venous blood was drawn by venipuncture from RA patients and healthy controls. Serum was obtained after centrifugation and stored at −70°C for the subsequent measurement of serum levels of sGITRL and IL-17. Ethics approval was obtained from Jiangsu University, and informed consent was obtained from all individuals.

Statistical Analysis

Data were presented as mean ± SD. Statistical differences were considered to be significant at $P < 0.05$ as determined by one-way analysis of variance or Student's *t*-test. The Mann Whitney *U* test was used for the statistical analysis of human samples, and a *P* value <0.05 was considered significant.

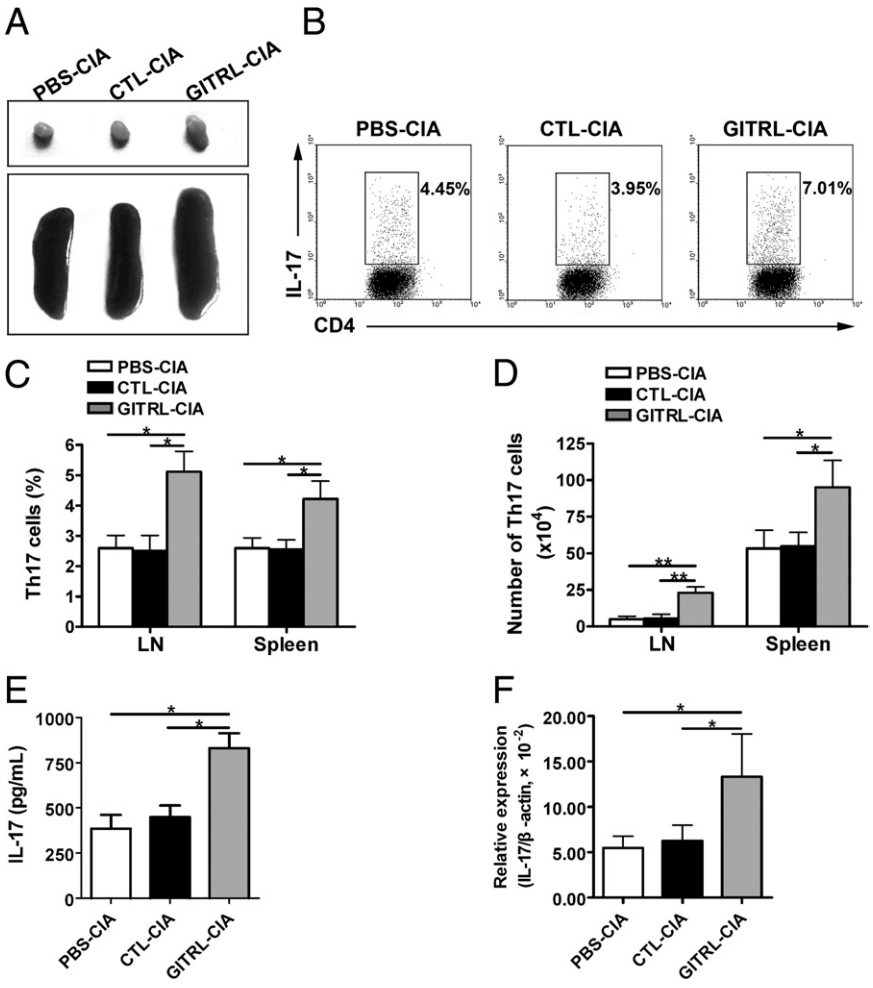

Figure 3. Expansion of Th17 cells in CIA mice treated with GITRL. All experimental mice were analyzed on the day 42 after the first CII-immunization. **A:** Representative images of inguinal lymph nodes and spleens from CIA mice with different treatments. **Upper panel** shows lymph nodes and **lower panel** shows spleens from CIA mice treated with PBS only (PBS-CIA), control protein (CTL-CIA), and GITRL protein (GITRL-CIA), respectively. **B:** Frequency of Th17 cells in draining lymph nodes from CIA mice with different treatments was analyzed by flow cytometry; percentages of Th17 cells aere indicated in representative flow-cytometric profiles. **C:** Percentages of Th17 cells in both lymph nodes and spleens from three treatment groups were shown ($n = 6$; $^*P < 0.05$). **D:** Total numbers of Th17 cells in inguinal lymph nodes and spleens from PBS-CIA, CTL-CIA, and GITRL-CIA mice ($n = 6$ per group) are shown ($^*P < 0.05$; $^{**}P < 0.01$). **E:** Serum levels of IL-17 from PBS-CIA, CTL-CIA, and GITRL-CIA mice ($n = 6$ per group) were determined by ELISA analysis. Values (mean ± SD) are shown ($^*P < 0.05$). **F:** Levels of IL-17 mRNA expression in inflamed joint tissue from PBS-CIA, CTL-CIA, and GITRL-CIA mice ($n = 6$ per group) were determined by qRT-PCR analysis. The relative expression levels of IL-17/β-actin are presented ($^*P < 0.05$).

Results

Increased GITRL Expression During CIA Development

Because previous studies have indicated a role for GITR activation in regulating immune responses and autoimmune pathogenesis, we examined whether GITRL expression was upregulated during the development of autoimmune arthritis using CIA murine model (Figure 1A). Flow-cytometric analysis revealed that $CD11c^+$ DCs purified from inguinal lymph nodes of CIA mice expressed elevated levels of GITRL as compared with those from adjuvant-treated control mice (Figure 1B). To determine whether GITRL expression is also increased in local joint tissue, we detected significantly upregulated GITRL mRNA expression in the joint tissue of CIA mice by quantitative PCR analysis (Figure 1C). Moreover, high levels of surface expression GITR expression were detected in $CD4^+$ T cells from inguinal lymph nodes, among which GITR expression was greatly increased in Th17 cell subset (Figure 1D). Together, these results suggested the possible involvement of enhanced GITRL-GITR interactions during the development of experimental arthritis.

Early Onset of Arthritis with Markedly Exacerbated Disease Severity in GITRL-Treated CIA Mice

To investigate a potential role of GITRL in the development of autoimmune arthritis, we treated CII-immunized mice with GITRL protein or control protein (20 μg per mouse) for five consecutive times starting from day 1 after the second immunization. On administration of GITRL protein *in vivo*, CII-immunized mice displayed an earlier onset of arthritis as compared with control protein- and PBS-treated mice. Almost 75% of mice in GITRL protein-treated group developed arthritis 7 days after the second immunization, whereas none of the mice in the control groups showed any clinical symptoms at this stage (Figure 2A). Moreover, the clinical scores for arthritis severity were significantly increased in GITRL protein–treated mice during a 42-day period of observation for CIA development (Figure 2B). Accordingly, histopathological analysis revealed more pronounced synovial inflammation, cartilage damage, and bone erosion in the joint tissues of GITRL protein–treated mice (Figure 2, C and D). Furthermore, serum levels of anti-CII autoantibodies were significantly higher in GITRL protein–treated

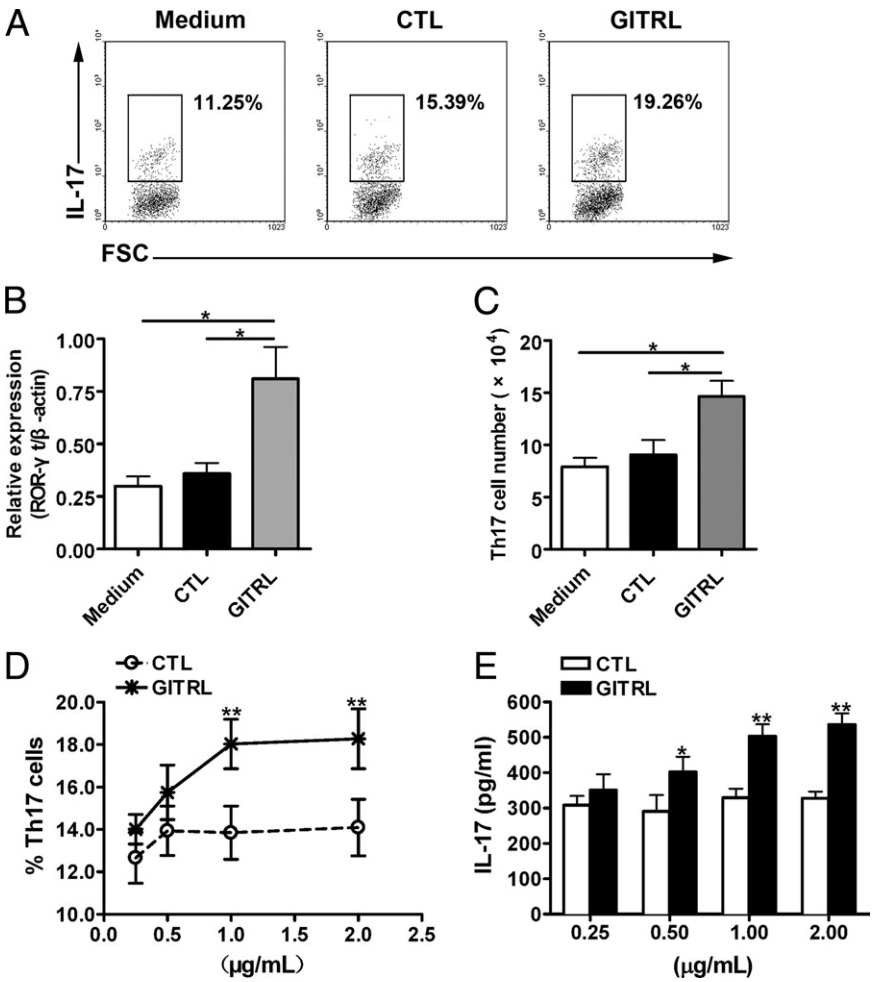

Figure 4. Expansion of Th17 cells in naïve $CD4^+$ T cell cultures treated with GITRL. **A:** Naive $CD4^+$ T cells from normal DBA mice were purified by MACS beads and cultured in Th17 cell polarization medium with GITRL or control protein treatment. After 72 hours, cells were stimulated with PMA, ionomycin, and monensin for 5 hours before performing intracellular staining for IL-17. Frequencies of Th17 cells in cultures with different treatments were indicated in the representative flow cytometric profiles. Results are representative of four independent experiments. **B:** Levels of RORγt expression, specific transcription factor of Th17 cells, in cultured $CD4^+$ T cells (as described in Figure 5A) were determined by qRT-PCR analysis. The relative mRNA expression levels of RORγt/β-actin from three independent experiments are presented ($^*P < 0.05$). **C:** Total numbers of Th17 cells generated from the culture of naive $CD4^+$ T cells (5×10^5) were enumerated by cell counting. Data (mean ± SD) are derived from three separate experiments ($^*P < 0.05$). **D:** Splenic $CD4^+$ T cells were cultured in Th17 cell polarized condition with treatment of various dosages of GITRL protein or control protein for 72 hours. The percentages of Th17 cells were analyzed by flow cytometry. Data (mean ± SD) are derived from three separate experiments ($^*P < 0.05$; $^{**}P < 0.01$). **E:** Concentrations of IL-17 in supernatant of T-cell cultures were determined by ELISA. Results are representative of three independent experiments. Values (mean ± SD) are derived from three separate experiments ($^*P < 0.05$; $^{**}P < 0.01$).

mice than those in control groups (Figure 2E). Together, these results indicated a role of GITRL in exacerbating CIA development.

Expansion of Th17 Cells in GITRL Protein–Treated CIA Mice

Because Th17 cells expressed high levels of GITR (Figure 1C), we sought to determine the possible effect of GITRL protein on Th17 expansion during CIA induction. After administration of GITRL protein *in vivo*, CIA mice showed much enlarged inguinal lymph nodes and spleens when compared with those of control protein-treated CIA mice (Figure 3A). As shown in Figure 3, B–D, GITRL treatment resulted in a approximately a twofold increase in both percentage and total number of Th17 cells in the spleen and inguinal lymph nodes from CIA mice, respectively. In addition, the levels of serum IL-17 concentration were elevated by approximately twofold in the GITRL-CIA group (Figure 3E). Consistently, quantitative real-time PCR analysis revealed significantly increased levels of IL-17 mRNA expression in the joint tissue from GITRL-CIA mice (Figure 3F). Interestingly, GITRL treatment did not significantly increase in the frequency of Treg cells in the spleen of CIA mice, but the total number of splenic of Treg cells was increased (see Supplemental Figure S3 at *http://ajp.amjpathol.org*). Thus, these findings provide strong evidence that GITRL protein plays a role in enhancing Th17 cell expansion and promoting IL-17 production during the development of experimental arthritis.

Enhanced Th17 Cell Generation in Normal Mice on GITRL Treatment in Vitro *and* in Vivo

To further confirm the function GLTRL protein in regulating Th17 cell differentiation, purified splenic $CD4^+$ T cells from normal mice were cultured under a polarized Th17 differentiation condition in the presence of GITRL protein or control protein for 72 hours. As shown in Figure 4A, flow-cytometric analysis detected a substantially increased frequency of $CD4^+IL\text{-}17^+$ T cells among naive $CD4^+$ T cells cultured in the presence of GITRL protein. Since it becomes clear that Th17 cell differentiation is dependent on the transcription factor RORγt, we also detected a 2.5-fold increase in RORγt mRNA expression in T cells treated with GITRL protein by quantitative real-time PCR analysis (Figure 4B). Notably, the total number of $CD4^+IL\text{-}17^+$ T cells was significantly increased in T-cell cultures on treatment with GITRL protein (Figure 4C). Moreover, both the percentage of Th17 cells and the con-

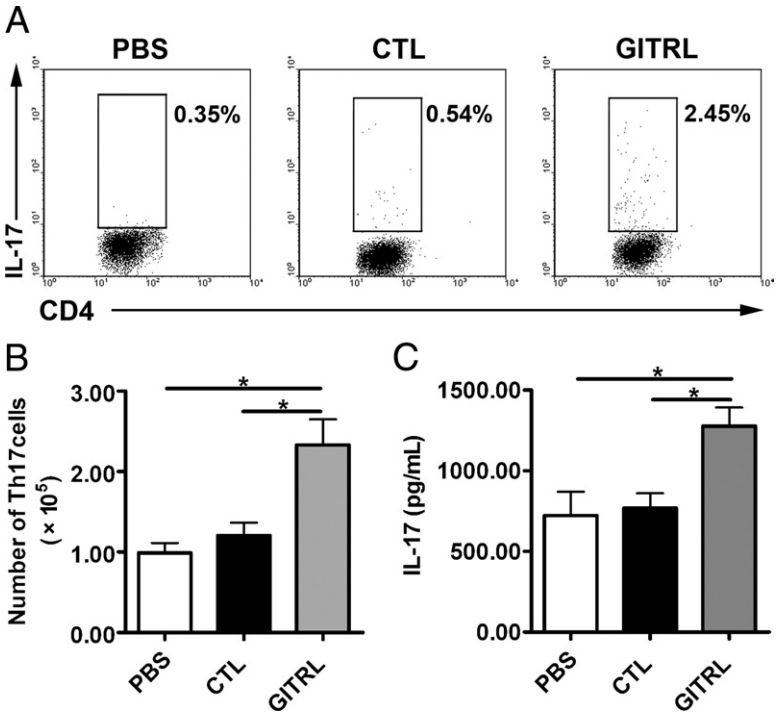

Figure 5. Expanded Th17 cells and increased IL-17 production in normal mice treated with GITRL. Normal DBA/1J mice were intravenously injected with GITRL protein (100 μg per mouse), control protein (100 μg per mouse), or the same volume of PBS, respectively. All mice were sacrificed on day 10 for analysis. **A:** Splenocytes from mice with different treatments were stimulated with PMA, ionomycin, and monensin for 5 hours, followed by IL-17 intracellular staining. The percentages of Th17 cells in the spleen were indicated in flow cytometric profiles. Results are representative of three independent experiments. **B:** Total number of Th17 cells in the spleens from different treatment groups was enumerated. Values (mean ± SD) are from three separate experiments (*$P < 0.05$). **C:** Concentrations of IL-17 in the supernatants of splenic $CD4^+$ T cells from mice with different treatments were determined by ELISA. Values (mean ± SD) are from three separate experiments (*$P < 0.05$).

centration of IL-17 in cultured supernatants were increased in a dose-dependent manner when T cells were cultured with various concentrations of GITRL protein (Figure 4, D and E). These results showed that GITRL protein promoted the expansion of $CD4^+IL\text{-}17^+$ T cells *in vivo*.

Next, we sought to examine whether the treatment with GITRL protein could enhance $CD4^+IL\text{-}17^+$ T-cell generation *in vivo*. Interestingly, both the frequency and total number of splenic Th17 cells were significantly increased in GITRL protein-treated DBA mice when compared with control protein–treated mice (Figure 5, A and B). In addition, purified splenic $CD4^+$ T cells from GITRL protein–treated mice secreted a significantly higher level of IL-17 than T cells from control protein–treated mice (Figure 5C).

Correlation of Elevated Serum GITRL Levels with Increased IL-17 in RA Patients

To explore whether GITRL expression is also dysregulated during RA pathogenesis in humans, we examined the serum levels of GITRL protein in RA patients. We detected significantly higher levels of GITRL protein in the serum samples of RA patients than in samples from healthy control subjects (Figure 6A). Further analysis revealed a positive correlation between elevated levels of GITRL protein and increased IL-17 production in RA patients (Figure 6B). Moreover, the increased levels of GITRL in RA patients were positively correlated with the DAS-28 scores of these patients (Figure 6C).

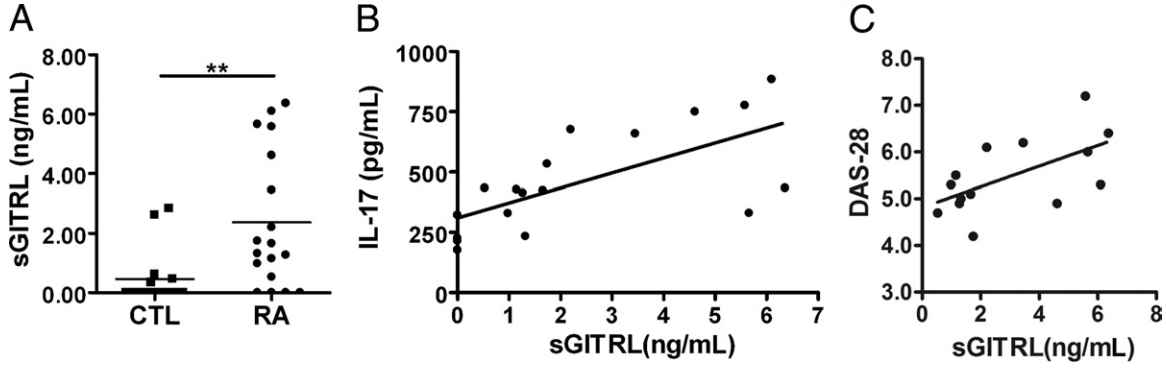

Figure 6. Increased serum GITRL level and its correlation with IL-17 in RA patients. **A:** Concentrations of serum GITRL in RA patients and healthy controls were determined by ELISA. Data were analyzed by Mann Whitney *U* test ($n = 18$ for RA patients, $n = 15$ for healthy controls; **$P < 0.01$). **B:** Correlation between serum GITRL and IL-17 levels in RA patients was evaluated on the data from RA patients by Pearson's correlation analysis ($r = 0.67$, $P < 0.01$). **C:** Correlation between serum GITRL and DAS28 in RA patients was evaluated on data from RA patients by Pearson's correlation analysis ($r = 0.59$, $P < 0.05$).

Discussion

In this study, we observed significantly increased GITRL expression in CD11c$^+$ DCs and high levels of GITR expression in Th17 cells from inguinal lymph nodes of CIA mice as compared with those from adjuvant-treated control mice. Moreover, CII-immunized mice treated with recombinant GITRL protein displayed an earlier onset of arthritis with markedly increased disease severity and joint damage. We further showed that GITRL could efficiently induce Th17 cell generation and IL-17 production both *in vitro* and *in vivo*. Thus, our results have identified a previously unappreciated function of GITRL in enhancing Th17 differentiation and exacerbating arthritis progression in CIA mice.

Extensive investigations have elucidated an important role of GITRL/GITR pathway in enhancing immune responses to tumors and viral infections.[1,3] Increasing evidence also indicates the involvement of GITR-mediated response in the pathogenesis of autoimmune arthritis, which is supported by recent studies using GITR-deficient mice and mice treated with anti-GITR antibodies.[9,10] In patients with RA, levels of GITR and GITRL expression were significantly elevated in the synovium as compared with levels in patients with osteoarthritis.[19] Moreover, primary macrophages from RA patients showed profoundly increased production of proinflammatory cytokines on stimulation with anti–GITR mAb.[19] In the current study, we detected elevated serum levels of GITRL in RA patients, which provide further evidence of the possible involvement of GITRL in RA pathogenesis. In addition, we detected upregulated GITRL protein expression in CD11c$^+$ DCs from inguinal lymph nodes and increased levels of GITRL transcripts in joint tissue of CIA mice. In a previous study, we also detected the increased production of B-cell–activating factor, a member of TNF cytokine family, in DCs at the acute stage of CIA development.[15] Thus, these results suggest that proinflammatory cytokines and co-stimulatory molecules produced by activated immune cells such as DCs are possibly involved in the initiation of autoimmune pathogenesis. To address the potential effect of increased GITRL expression on arthritis development, we observed an early onset of arthritis with markedly increased severity of clinical symptoms in CII-immunized mice treated with recombinant GITRL protein. As expected, increased levels of anti-CII antibodies were detected, correlated with substantially increased synovial inflammation, cartilage damage and bone erosion in joint tissue of GITRL-treated CIA mice. Together, our results provide direct evidence that GITRL-triggered signaling events led to the exacerbation of autoimmune progression in CIA mice. Of note, we observed significantly increased GITRL expression in DCs from RA patients as compared with healthy controls (Wang et al, unpublished data), despite the role of GITRL remaining unclear in RA pathogenesis. Thus, further studies are warranted to characterize the expression patterns of GITRL/GITR and their functional relevance in the pathogenesis of RA.

In mice, GITR is known to be mainly expressed by both naive T cells and activated T cells.[20] In addition, CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells are found to express high levels of GITR.[20] It has become clear that the functional interaction of GITRL with GITR drives T-cell activation through either costimulation of CD4$^+$ effector T cells or inhibition of Treg cell–suppressive function.[1–3] Here, we now provide new evidence that IL-17–producing Th17 cells express high levels of GITR expression as revealed by flow-cytometric analysis. Moreover, we show that GITRL can effectively induce Th17 cell differentiation from naive CD4$^+$ T cells under a Th17-polarized culture condition, which is further supported by *in vivo* findings that administration of GITRL protein into both normal mice and CII-immunized mice significantly enhances Th17 cell generation and IL-17 production. Thus, these results suggest a previously unrecognized role of GITRL in modulating Th17 cell generation. Interestingly, an elegant study by Van Olffen et al has shown that transgenic expression of GITRL on B cells induces a prominent increase of regulatory CD4$^+$_ T cells with delayed development of experimental autoimmune encephalomyelitis in GITRL transgenic mice.[21] It is plausible that either different dosages or differential signaling effects of GITRL between membrane-bound and soluble forms may account for the different effects of GITRL on Treg and Th17 cells. Although it is currently unclear whether, and to what extent, GITRL treatment inhibits Treg suppressive function or drives Treg cells to differentiate into proinflammatory Th17 cells in this study, our results from both *in vitro* and *in vivo* studies have indicated that the currently used dosage of GITRL appears to markedly enhance the generation of Th17 cells. Th17 cells have been well recognized for their critical role in the development of autoimmune diseases both in mice and in humans.[22–24] We have previously reported that a protective role played by NK cells in the development of experimental arthritis is possibly mediated by suppressing Th17 cell generation via interferon-γ production.[16] Studies by Mitsdoerffer et al have recently identified a new function of Th17 cells in regulating humoral immunity by promoting B cell differentiation and antibody isotype switching.[13] In this study, GITRL treatment has been shown to induce Th17 cell expansion and IL-17 secretion as well as increased anti-CII autoantibody production in CIA mice. Therefore, it is plausible to reason that GITRL-stimulated Th17 cells may enhance B-cell differentiation and autoantibody production during the development of autoimmune arthritis. Further studies are warranted to determine the effect of blocking GITR ligand on CIA development to confirm the therapeutic potential of this approach *in vivo*.

Consistent with our findings of upregulated GITRL expression in CIA mice, we have also detected significantly higher levels of serum GITRL protein in RA patients than in healthy control subjects. Moreover, the increased levels of GITRL in RA patients are positively correlated with increased IL-17 production and the DAS-28 scores indicates the functional implication of GITRL in RA pathogenesis.

Taken together, we show that GITRL treatment promotes Th17 cell expansion and exacerbates arthritis development in CIA mice. The current results have revealed a previously unrecognized function of GITRL in enhanc-

ing Th17 cell expansion and inflammatory arthritic progression. Our findings of a positive correlation between upregulated GITRL expression and increased IL-17 production in RA patients may facilitate the potential development of novel therapeutic targeting of GITRL/GITR pathway for the treatment of human RA.

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