Effect of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand on the Reduction of Joint Inflammation in Experimental Rheumatoid Arthritis

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ABSTRACT

This study focused on the potential therapeutic effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on collagen-induced arthritis (CIA) and on the elucidation of the mechanisms involved. DBA/1J mice with established CIA were treated with various amount of recombinant soluble human TRAIL. The effects of TRAIL on the development and severity of CIA in this DBA/1J mouse model were assessed clinically and histologically, and a detailed investigation was conducted on proinflammatory cytokine and anticollagen-specific antibody levels. Cellular immunity was evaluated by investigating the proliferative responses and cytokine release profiles of splenocytes after TRAIL treatment. TRAIL treatment significantly reduced the severity and incidence of CIA, joint swelling, erythema, and edema. Histologic evaluations revealed that inflammatory cell infiltration, cartilage destruction, and bone erosion were significantly reduced in joints of TRAIL-treated mice with dose-dependent manner. TRAIL treatment also strongly decreased and/or normalized the productions of proinflammatory cytokines and of anti-collagen-specific antibodies in the sera of CIA mice. Furthermore, in vitro studies with primary splenocytes showed the cytotoxic effect of TRAIL on activated lymphocytes, with reduction of inflammatory cytokine release. These findings show that TRAIL administration is an effective anti-inflammatory treatment that prevents the development and progression of CIA in DBA/1J mice, and they suggest that TRAIL might be considered a potential treatment for human RA.

ABBRREVATIONS: RA, rheumatoid arthritis; TNF, tumor necrosis factor; IL, interleukin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; CIA, collagen-induced arthritis; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; CI, collagen immunization; CII, type II collagen; LPS, lipopolysaccharide; Con A, concanavalin A; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PHA, phytohemagglutinin; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; Th, T helper.
ration of immune homeostasis. Thus, the prevention of T-cell activation and the active induction of arthritogenic T-cell apoptosis may constitute an effective therapeutic strategy for the treatment of autoimmune arthritis.

TRAIL-induced apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein and a member of the TNF cytokine superfamily (Wiley et al., 1995). TRAIL potentially interact with five receptors, namely, two cell surface death receptors (death receptor (DR), TRAIL-R1 and DR5, TRAIL-R2; Chaudhary et al., 1997; Pan et al., 1997a; Walczak et al., 1997), two membrane-anchored decoy receptors (decoy receptor 1, TRAIL-R3 and decoy receptor 2, TRAIL-R4; Marsters et al., 1997; Pan et al., 1997b), and the soluble receptor osteoprotegerin (Emery et al., 1998). TRAIL can selectively induce the apoptosis of many tumor cells by binding to DR4 and DR5. This results in the recruitment of the Fas-associated death domain, which initiates caspase cascades that lead to apoptosis (Cohen, 1997; Ashkenazi and Dixit, 1998, 1999; Griffith and Lynch, 1998). However, TRAIL does not adversely affect normal cells (Gura, 1997). Recently, several reports have suggested that TRAIL and its death receptors may have therapeutic roles in RA (Mackay and Kalled, 2002; Tsokos and Tsokos, 2003; Evans, 2004). In vivo, a TRAIL blockade led to a profound hyperproliferation of arthritogenic lymphocytes and increased the productions of cytokines and autoantibodies. Furthermore, in vitro, TRAIL inhibited DNA synthesis and prevented lymphocyte cell cycle progression. Thus, unlike other members of the TNF superfamily, TRAIL is a prototype inhibitory cytokine that prevents autoimmune inflammation by inducing apoptosis and blocking cell cycle progression (Song et al., 2000; Liu et al., 2003; Lamahamedi-Cherradi et al., 2003; Yao et al., 2006; Martínez-Lorenzo et al., 2007). These findings suggest that systemically administered TRAIL represents a potential therapeutic strategy for the treatment of RA.

In the present study, we investigated the effect of TRAIL administered intraperitoneally on a collagen-induced arthritis (CIA) DBA/1J mouse model, which is the most commonly used RA model to investigate the pathogenic mechanisms and to explore the therapeutic effects of anti-inflammatory agents in RA. Furthermore, this model is pathologically, histologically, and immunologically similar to human RA (Luross and Williams, 2001; Brand et al., 2003). Thus, by using this experimental model of RA, we evaluated the biologic and therapeutic potentials of TRAIL treatment on autoimmune arthritis. More specifically, we examined the effects of TRAIL on clinical scores, incidence, joint histopathology, production of serum levels of proinflammatory cytokines and autoantibodies, and lymphocyte immune responses.

Materials and Methods

Mice. Male DBA/1J mice (7–8 weeks old, 20–22 g; SLC Inc., Shizuoka, Japan) were used for CIA induction and splenocyte isolation. Animals were housed under specific pathogen-free conditions and provided with standard food and water. All animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23, revised 1985). All animal experiments were performed in accordance with the ethical guidelines issued by the Animal Care and Use Committee of the College of Medicine, Seoul National University (Seoul, Korea).

Induction and Assessment of Arthritis. Bovine type II collagen (CII) (2 mg/ml; Chondrex, Inc., Redmond, WA) was emulsified in an equal volume of Complete Freund’s adjuvant (Chondrex, Inc.) in an ice-cold water bath. Male DBA/1J mice were first immunized subcutaneously at the base of the tail with 0.1 ml of this emulsion. On day 21, mice were given booster injection of 0.1 ml of emulsion but with incomplete Freund’s adjuvant (Chondrex, Inc.) in the same manner. Clinical signs of arthritis in the wrist and ankle joints were assessed visually under blinded conditions every other day. Clinical severities of arthritis were scored on a scale of 0 to 4 as follows: 0, normal; 1, slight swelling and edema; 2, moderate swelling and edema; 3, severe swelling and pronounced edema; and 4, joint deformity or ankylosis. Each limb was graded, yielding a maximum possible score of 16 per mouse.

TRAIL and Administration. The recombinant human TRAIL was prepared, purified, and characterized as described previously (Youn et al., 2007). The freshly purified TRAIL was diluted with phosphate-buffered saline (PBS) to allow doses of 30, 150, and 300 μg/300 μl of PBS/mouse. Mice were injected intraperitoneally with PBS or these various doses of TRAIL starting 1 day after the booster immunization [day 22 after collagen immunization (CI)], and these injections were continued daily (approximately days 22–40).

Histologic Evaluations. Mice were killed by cervical dislocation on day 41 post-CI, and knee joints were randomly collected; fixed in 10% neutral buffered formalin for 24 h; decalcified in PBS containing 20% EDTA, pH 7.4; and embedded in paraffin. Joint sections (5 μm) were then prepared, deparaffinized in xylene, and rehydrated through a graded alcohol series. Routine histology was performed by hematoxylin and eosin (H&E) staining.

Cell Culture. Jurkat cells (a human leukemia T-cell line) were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum containing 1% penicillin and streptomycin. TRAIL was prepared, purified, and characterized as described previously (Youn et al., 2007). The freshly purified TRAIL was diluted with PBS to allow doses of 30, 150, and 300 μg/ml PBS/mouse. Mice were injected intraperitoneally with PBS or these various doses of TRAIL starting 1 day after the booster immunization [day 22 after collagen immunization (CI)], and these injections were continued daily (approximately days 22–40).

Proliferation and Cytotoxicity Assay. To determine the effects of TRAIL on splenocyte proliferation, purified mouse splenocytes were cultured at 4 × 10⁶ cells/ml (100 μl/well) in 96-well plates containing heat-denatured CII (50 μg/ml), lipopolysaccharide (LPS; 20 μg/ml), or concanavalin A (Con A; 1 μg/ml) in the presence of 100 ng/ml TRAIL, respectively. Culture supernatants were collected 48 h later, and TRAIL cytotoxicities were determined using MTS assays. Cell viabilities (percentages) were calculated by expressing absorbance of treated samples as percentages of those of untreated controls. To investigate the cytotoxic effect of TRAIL on T cells, human leukemia T-cell line Jurkat cells were cultured at 4 × 10⁶ cells/ml (100 μl/well) in 96-well plates and stimulated with 50 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich, St. Louis, MO), 100 ng/ml LPS (Sigma-Aldrich), or 10 ng/ml Con A (Sigma-Aldrich) for 12 h, respectively. Unlike the splenocyte activations, the different mitogen of PHA was replaced to the CII for the Jurkat cell activation because of the weak immune reaction of the Jurkat cells on CII. After stimulation, predetermined amounts of TRAIL were added to final concentrations of 0 to 10,000 ng/ml, and samples were incubated for 24 h. MTS assays were performed on collected culture supernatants, as described above.

Cytokine Determinations. Serum samples were obtained from mice by aspirating retro-orbital blood at day 41 post-CI. All samples were stored at −80°C until used. Serum levels of TNF-α, IL-1β, IFN-γ, and IL-2 were determined using Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s instructions. To determine cytokine levels in vitro, mouse splenocytes (4 × 10⁶ cells/ml) were stimulated with 50 μg/ml heat-denatured CII, 20 μg/ml LPS, or 5 μg/ml concanavalin A for 48 h in
24-well plates in the presence of 100 ng/ml TRAIL. Supernatants were collected from each well, and the levels of IFN-γ and IL-2 were determined using enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA).

**Anticollagen Antibody Detection.** To determine collagen-specific autoantibody levels in vivo, serum samples were analyzed using ELISA kits (Chondrex, Inc.) for CII-specific IgG1 and IgG2a antibody levels, according to the manufacturer’s instructions.

**Annexin-V Staining Assay.** To examine the apoptotic effect of TRAIL on Jurkat cells, we used Annexin-V-FLUOS staining kits (Roche Diagnostics, Mannheim, Germany), as described previously (Youn et al., 2007). In brief, Jurkat cells were treated with Con A and TRAIL as described above, and then they were washed with PBS and stained with 100 µl of an Annexin-V-propidium iodide mixture for 15 min. Finally, apoptotic and necrotic cells levels were analyzed by fluorescence microscopy and counted.

**Western Blotting.** Equal amounts of protein (1 µg) were separated by SDS-polyacrylamide gel electrophoresis (12%) using the Mini-Protein II system (Bio-Rad Laboratories). Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), blocked with 5% milk in Tris-buffered saline buffer, and treated with primary antibodies for rabbit polyclonal active caspase-3 and β-actin (Abcam Inc., Cambridge, MA) and secondary antibody for horseradish peroxidase-conjugated goat polyclonal to rabbit IgG antibodies (Abcam Inc.). Protein bands were visualized using a chemiluminescence detection system (LumiLight; Roche Diagnostics), and membranes were exposed to photographic film (Carestream Health, Rochester, NY). β-Actin was used as an internal control.

**Statistical Analysis.** Data are expressed as mean ± S.E.M. Differences between groups were tested for statistical significance using the Student’s t test, and p values of <0.05 were considered significant.

**Results**

**TRAIL Reduced the Severity and Incidence in CIA Mice.** To evaluate the therapeutic effect of TRAIL on the development and pathogenesis of RA, mice were immunized with collagen. TRAIL was injected intraperitoneally at 30, 150, and 300 µg/mouse/day from day 22 post-CI (day 1 after the booster injection). CIA developed rapidly in mice immunized with CII, and clinical signs of the disease (periarticular erythema and edema) first appeared in hind paws at approximately 23 days post-CI, and all vehicle-treated mice were affected on day 25 (Fig. 1A). Hind paw erythema and swelling increased in frequency and severity in a time-dependent manner, and a mean maximum clinical score of 11.3 was reached between 31 and 35 days post-CI by vehicle-treated mice (Fig. 1B). In addition, the TRAIL dose-dependently reduced joint inflammation. Especially, high-dose TRAIL (300 µg/mouse) treatment significantly decreases clinical score (approximately 78.5% of control group; p < 0.01) and prevents the development of joint inflammation in 44.0% of the animals (Fig. 1, A and B). Furthermore, no significant change in body weight was observed for TRAIL-treated mice at any of the dosages administered (data not shown).

**TRAIL Reduced the Joint Inflammation in CIA Mice.** To investigate the effects of TRAIL on pathologic changes of inflamed joints, H&E staining was performed. Histologic evaluations on day 41 of vehicle-treated mice revealed signs of severe arthritis (infiltration of inflammatory cells (lymphocytes, macrophages, neutrophils, and plasma cells) into joint cavities and periarticular soft tissue), pannus formation, cartilage destruction, and bone erosion, which are characteristic of CIA (Fig. 2). In contrast, TRAIL treatment significantly abrogated synovial tissue inflammation and significantly reduced inflammatory cell infiltration and joint destruction with dose-dependent manner compared with vehicle-treated controls (Fig. 2).

**TRAIL Reduced the Production of Proinflammatory Cytokines in CIA Mice.** To investigate whether TRAIL modulates the inflammatory process by regulating the secretions of cytokines, we measured the serum levels of proinflammatory cytokines. As illustrated in Fig. 3A, substantial increases in proinflammatory cytokine levels were found in the serum samples of vehicle-treated mice on day 41 post-CI. In contrast, TNF-α levels were significantly and dose-dependently lower in TRAIL-treated groups than in vehicle-treated controls (p < 0.05). In particular, at the highest dose (300 µg/mouse) TRAIL dramatically reduced serum levels of TNF-α to levels that were similar to those of normal mice;
Fig. 2. Histopathologic investigations in knee joints of normal mice and CIA mice with and without TRAIL treatments. Mice were killed day 41 post-CI and their knee joints were analyzed for histology after H&E staining. Original magnification, 200×. Scale bar, 500 μm.

Fig. 3. Effect of TRAIL on the productions of the proinflammatory cytokines TNF-α (A), IL-1β (B), IFN-γ (C), and IL-2 (D) in sera of CIA mice. Serum samples were collected from vehicle-treated controls or mice treated with different doses of TRAIL (30, 150, and 300 μg/mouse/day) at 41 days post-CI. Concentrations of cytokines were measured by ELISA. Data are expressed as mean ± S.E.M. (n = 9–10/group). *, p < 0.05; **, p < 0.01 versus vehicle-treated controls.
furthermore, similar results were observed for IL-1β, IFN-γ, and IL-2 (Fig. 3, B–D; p < 0.05).

**TRAIL Reduced Humoral Immune Responses in CIA Mice.** High levels of circulating anti-CII antibodies in serum invariably accompany the development of CIA and RA, and their production is a major determinant of susceptibility to RA (Luross and Williams, 2001). To assess the effects of TRAIL on humoral immune response against CII, we examined the concentrations of serum anti-CII antibodies. Compared with the normal mice, the circulating serum antibody levels (IgG1 and IgG2a) were markedly elevated in CIA mice. As shown in Fig. 4, the productions of anti-CII IgG1 and IgG2a were inhibited dose-dependently by TRAIL (p < 0.01). Specifically, the level of anti-CII IgG2a was significantly more reduced than that of anti-CII IgG1 by TRAIL (at 30, 150, and 300 μg/mouse), which induced 55.8, 83.7, and 93.1% reductions in IgG2a levels and 9.4, 28.2, and 55.4% reductions in IgG1 levels, respectively. These results indicate that TRAIL inhibited humoral immune responses in our murine model of RA.

**TRAIL Reduced Cellular Immune Responses in CIA Mice.** CIA is initiated by collagen-specific lymphocytes. Thus, to test the effect of TRAIL on cellular immune responses, proliferative responses and cytokine production were examined in splenocytes stimulated with various autoantigens. As shown in Fig. 5, the proliferative responses of splenocytes cultured with CII (50 μg/ml), LPS (20 μg/ml), or Con A (5 μg/ml) in the presence of TRAIL at 100 ng/ml were 17.2, 32.6, and 30.1% reduced versus vehicle-treated controls (Fig. 5A; p < 0.01). Splenocytes produced large amounts of IFN-γ and IL-2 when stimulated with CII (50 μg/ml), LPS (20 μg/ml), or Con A (5 μg/ml), but levels were lower in TRAIL-treated cells than in vehicle-treated controls (Fig. 5, B and C; p < 0.01). These results indicate that TRAIL inhibited cellular immune responses in our model.

**TRAIL Had a Cytotoxic Effect and Induced Apoptosis in Activated T Lymphocytes.** To examine the cytotoxic effect of TRAIL on Jurkat cells, cell viabilities were determined using MTS assays. Cells were stimulated with various immune activators (PHA, LPS, or Con A) for 12 h and then treated with different concentration list of TRAIL for 24 h. As illustrated in Fig. 6A, TRAIL had a significant dose-dependent cytotoxic effect.

Next, to determine the apoptotic effect of TRAIL on Jurkat cells, cell death was investigated by Annexin-V-FLUOS staining. Cells were stimulated with Con A (10 ng/ml) for 24 h, and then they were treated with 100 ng/ml TRAIL for 0, 3, 6, 12, and 24 h. As was expected, TRAIL was found to induce apoptosis in a time-dependent manner (Fig. 6B). In particular, after 24 h of treatment with 100 ng/ml TRAIL, 76.7 ± 2.91% of cells had undergone apoptosis (Fig. 6B; p < 0.01).

To determine whether TRAIL-induced apoptosis was mediated by caspase activation, Western blotting was performed. As illustrated in Fig. 6C, TRAIL increased the activation of caspase-3 levels in Jurkat cells in a time-dependent manner; maximal enhancement was observed after approximately 12 h of exposure. These results suggest that the cytotoxic effects of TRAIL are attributable to caspase activation.

**Discussion**

RA is a typical chronic and systemic autoimmune disorder that severely affects motility because of the damage caused by inflammation and joint destruction. However, its precise origin and pathogenesis remain still unclear. Numerous disease-modifying drugs and biopharmaceuticals have been examined in the context of RA, and these studies resulted in the identification of TNF receptor antagonists (Lipsky et al., 2000; Genovese et al., 2002). However, there remains a need to increase the efficacy and safety of agents with superior therapeutic potentials. Recently, the role of TRAIL on RA was studied by investigating its apoptotic effect on activated T cells and synoviocytes and its local anti-inflammatory effect on RA-affected joints (Song et al., 2000; Liu et al., 2003; Lamhamedi-Cherradi et al., 2003; Yao et al., 2006; Martínez-Lorenzo et al., 2007). However, the systemic effects of exter-

Fig. 4. Effect of TRAIL treatment on the production of CII-specific antibodies IgG1 (A) and IgG2a (B) in CIA mouse serum. Serum samples were collected from vehicle-treated controls or TRAIL-treated mice (30, 150, and 300 μg/mouse) at 41 days post-CI. Concentrations of CII-specific antibodies were measured by specific ELISA. Data are expressed as mean ± S.E.M. (n = 10/group). *, p < 0.05; **, p < 0.01 versus vehicle-treated controls.
nally administered TRAIL on the pathology of RA and its pharmacodynamic effects are substantially unknown. The present study demonstrates the potential efficacy of systemically administered TRAIL on the pathogenesis of RA in a murine CIA model. The CIA mouse model used in the present study has been widely used for investigating the pathogenesis of autoimmune arthritis and the therapeutic efficacies of potential anti-inflammatory drugs. In the present study, our examinations revealed that the systemic administration of TRAIL ameliorated the clinical (severity and incidence) and histopathological (infiltration of inflammatory cells into joints, formation of pannus, hyperplasia of the synovial lining, cartilage destruction, and bone erosion) manifestations of CIA and that it had no noticeable toxic side effects (Figs. 1 and 2). Furthermore, the observed dose-dependent therapeutic effects of TRAIL (clinical scores and incidences) demonstrated that the observed anti-inflammatory effect was due to the exogenous TRAIL administration. These findings strongly indicate that systemically treated TRAIL attenuates the progression of arthritis and joint injury in CIA mice, presumably due to the unique anti-inflammatory potentials.

During RA development, proinflammatory cytokines, such as TNF-α, IL-1β, INF-γ, and IL-2, play important roles in chronic joint inflammation and the acceleration of pannus formation and in the mediation of cartilage and bone destruction (Smolen et al., 1996; Lümemann et al., 2002). Therefore, the regulations and/or normalizations of these cytokine levels are probably important from the therapeutic standpoint. As illustrated in Fig. 3, the induction of CIA (the vehicle-treated control group) significantly elevated proinflammatory cytokine levels in sera. However, TRAIL administrations strongly reduced and/or normalized inflammatory response during arthritis development by down-regulating proinflammatory cytokines levels (e.g., TNF-α, IL-1β, INF-γ, and IL-2) in the sera of CIA mice. In line with its macroscopic therapeutic effects, TRAIL dose-dependently reduced proinflammatory cytokine levels, which demonstrates its anti-inflammatory effects. Moreover, unlike the clinical investigations (Fig. 1) where the
high-dose TRAIL treatment (300 μg/mouse) showed mild symptoms of RA development, the almost normalized serum proinflammatory cytokine levels measured in the TRAIL-treated group strongly suggested the effective systemic anti-inflammatory functions of TRAIL. Furthermore, TRAIL was found to have a negligible effect on blockage of inflammatory cytokine responses caused by a severe immunosuppression. This implies that TRAIL-based RA therapy is probably free of immunosuppression-associated side effects, which have been associated with other immunosuppressants examined in the context of RA (Schnabel and Gross, 1994; Borchers et al., 2004; Li et al., 2004).

The onset of CIA is often accompanied by high levels of circulating autoantibodies, especially IgG2a subclass antibodies, which initiate joint inflammation (Luross and Williams, 2001). Thus, the inhibition of IgG2a antibody production is at least partly responsible for attenuating CIA. TRAIL was found to reduce IgG1 and IgG2a levels, although its effect was greater on IgG2a levels (Fig. 4). Because IFN-γ mediates the switching of antibody production to IgG2a, it is possible that TRAIL reduced IgG2a levels by inhibiting IFN-γ production. Furthermore, because the productions of IgG2a and IgG1 are driven by Th1- and Th2-associated responses, respectively, these findings suggest that TRAIL affects Th1 response more so that Th2 immune response against CIA and that this leads to the suppression of humoral immune responses in CIA.

The effective treatment of autoimmune arthritis requires the elimination and/or inactivation of arthritogenic lymphocytes, including activated T cells. Thus, potential therapeutic strategies in RA should address the regulation of T-cell activation, the induction of activated T-cell apoptosis, the prevention of autoaggressive lymphocyte expansion, and the reduction of inflammatory cytokines or autoreactive antibodies release by activated lymphocytes. To test the effect of TRAIL on these features, we evaluated cellular immune responses in CIA. Although TRAIL weakly inhibited splenocytes proliferation, it was found to block splenocyte activation by inhibiting cytokine production (INF-γ and IL-2). Actually, it has been reported previously that TRAIL can inhibit T-cell activation, subsequent cell cycle progression, and cytokine production in mice and human autoreactive and foreign antigen-specific T cells (Song et al., 2000). Furthermore, in the present study, TRAIL was also found to have a potent dose-dependent cytotoxic effect on Jurkat cells (a typical T-cell line) stimulated with various immune-stimulators (Fig. 6). This cytotoxic effect of TRAIL on the Jurkat cells was found to be associated with apoptosis induced by caspase activation (Fig. 6C). Moreover, the apoptosis of activated T-cells impairs T helper functioning in B cells, which would influence the productions of CII-specific antibodies of different isotypes.

In conclusion, this study demonstrates that the TRAIL treatment significantly suppresses the progression of CIA and suggests that this is primarily due to the anti-inflammatory and/or immunomodulatory activities of TRAIL. This inhibitory effect on the pathogenesis of RA may be associated with the modulation of lymphocyte activations, the elimination of activated lymphocytes by apoptotic pathways, and the reduction of inflammatory cytokines and anti-CII specific antibody release. These findings suggest that systemic TRAIL offers a novel therapeutic approach to RA and to other chronic autoimmune diseases.

References
TRAIL Ameliorates Inflammation in Rheumatoid Arthritis


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