Intraarticular gene delivery of CTLA4–FasL suppresses experimental arthritis

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Abstract

T lymphocytes are key inflammatory cells contributing significantly to the pathogenesis of Rheumatoid arthritis (RA). Biological treatments targeting T lymphocytes may provide an efficient approach for treatment of RA. CTLA4–FasL, a fusion product of extracellular domains of CTLA4 and FasL, integrating two inhibitory elements against T cells into one molecule, might be a desirable derivative of engineered soluble FasL or CTLA4 and have therapeutic potential in RA. The aim of this study was to investigate whether simultaneous induction of Fas-mediated apoptosis and blockade of co-stimulation signal by CTLA4–FasL gene delivery has a suppressive effect on adjuvant-induced arthritis (AIA) in Lewis rats. Recombinant adeno-associated virus (rAAV) vectors encoding rat CTLA4–FasL fusion gene (rAAV.CTLA4–FasL) or enhanced green fluorescent protein (rAAV.EGFP) were injected intraarticularly into both ankle joints after immunization. The ankles were monitored by measures of clinical, histological and inflammatory cytokines’ changes. Treatment using rAAV.CTLA4–FasL resulted in a significant suppression of AIA compared with rAAV.EGFP control, as reflected in the mainly clinical signs including articular index, ankle joint thickness and paw swelling and typically histological characters of arthritic joints including synovial hyperplasia, inflammatory cells infiltration and cartilage degradation. Treatment with rAAV.CTLA4–FasL also significantly decreased the levels of key proinflammatory cytokines in AIA joints. Moreover, local productions of transgene mRNA and protein of CTLA4–FasL were found in injected joints without systemic distribution. Our results indicate that rAAV.CTLA4–FasL profoundly suppressed experimental model of RA, implicating the potential therapeutic applications for suppression of RA by local joint delivery of CTLA4–FasL.

Keywords: adjuvant-induced arthritis, adeno-associated virus, gene therapy, rheumatoid arthritis, T lymphocytes

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease mainly affecting the joints, characterized by infiltration of inflammatory cells and synovial hyperplasia leading to the destruction of cartilage and bone. Although the trigger is still unidentified, significant progress has been made in the understanding of the pathogenesis of RA. Activated T lymphocytes are present in the inflamed synovium of RA patients and the important role of T lymphocytes has gained attention in the pathogenesis of RA, thus providing a rationale for the targeting of T cells with biological treatments (1, 2).

In addition to initial signal (antigen-specific signal), co-stimulatory signal is also necessary for T-cell activation. Competitive blockage of the interaction between co-stimulators (B7) anchored on antigen-presenting cells (APC) surfaces and their receptors (CD28) on T cells to inhibit T cell activation is a therapeutic strategy that has been explored in-depth for RA. CTLA4 is an attractive candidate for the treatment of RA, which binds to B7 with markedly higher affinity than does CD28 and thus blocks B7/CD28 co-stimulatory pathway. CTLA4-Ig, a soluble derivative of CTLA4, has even reached the clinical usage via induction of T-cell anergy (3–5). However, inhibiting B7/CD28 pathway by CTLA4-Ig is a way to suppress but not eliminate T-cell responsiveness (6, 7), and T-cell anergy induced by CTLA4-Ig is reversible (8, 9), highlighting the continuing need for other more effective therapeutic approaches.

The interaction between Fas and Fas ligand (FasL) plays a pivotal role in activation-induced cell death of T lymphocytes (10) and is responsible for the cytotoxicity of T lymphocytes (11). Consequently, Fas/FasL apoptosis pathway is crucial for homeostatic regulation of T lymphocytes. Though in both RA and animal models of arthritis, Fas is highly expressed
on various inflammatory cells in arthritic joints (12, 13), the expression levels of FasL are extremely low and as a result, most inflammatory cells survive. Through introducing mature FasL (mFasL) to up-regulate Fas-mediated apoptosis has been shown an efficient approach for treatment of experimental arthritis (14–16). However, FasL undergoes metalloproteinase cleavage in its extracellular domain, resulting in the release of soluble FasL (sFasL) (17). In general, sFasL is less potent at inducing apoptosis than membrane-bound mFasL, as shown in a variety of cell types (18–20). Moreover, the in vivo direct administration of sFasL renders severe side-effects, such as massive hepatocyte apoptosis which leads to the death of treated animals (21). Therefore, it is necessary to modify FasL as a soluble therapeutic molecule with less toxicity but remaining apoptotic activity.

The fusion protein of CTLA4 and FasL (extracellular domains of both), CTLA4–FasL, has been reported as an effective inhibitor for T cells without toxic effects. CTLA4–FasL not only inhibits T cell activation, but also benefits activated T cell death in vitro (22, 23) and in experimental models for prevention graft rejection in allografts (24–27). CTLA4–FasL fusion protein has been demonstrated significantly higher potency than either sFasL or CTLA4-Ig alone, or in combination, in counteracting T cells (23, 25). Consequently, CTLA4–FasL protein might be a desirable derivative of either soluble CTLA4 or sFasL, combining the capacities of co-stimulation blockade and apoptosis induction within a single recombinant protein.

Many acquired diseases including RA are now being considered for treatment by gene therapy. Adeno-associated virus serotype 2 (AAV2) is a suitable gene transfer vector and mostly used in animal studies of RA. In present study, by means of AAV2, we investigated whether CTLA4–FasL gene transfer has a suppressive effect on rat adjuvant-induced arthritis (AIA), an experimental model of RA dependent on T cells.

Methods

Cloning of recombinant CTLA4–FasL gene

cDNAs encoding the extracellular region of rat CTLA4 (residues 1–123 of mature protein, NCBI Genebank No. NM_031674) and rat FasL (residues 100–278 of mature protein, NCBI Genebank No. NM_012908) were extracted from rat splenocytes by PCR amplification using primers a and b and primers f and g (Table 1), respectively. Sequence-overlap primers c, d and e (Table 1) were designed according to oncostatin M signal sequence. After four rounds of PCR subsequently with pairs of a + b, c + c, d + b and e + e, oncostatin M signal and a Kozak sequence were finally introduced into the N-terminal of CTLA4. To construct the CTLA4–FasL fusion product, two pairs of primers, e with h and i with j (a Flag-tag sequence introduced in j, Table 1; primers h and i, contained overlap sequences), were used to re-amplify the genes of CTLA4 and FasL. After purification, the two PCR products were mixed and subjected to PCR for a couple of cycles. Then, primers e and j were added to produce the fusion gene. cDNA encoding rat full-length or mFasL was also extracted from rat splenocytes using reverse transcription (RT)–PCR. The genes of CTLA4–FasL, mFasL and enhanced green fluorescent protein (EGFP) were, respectively, cloned into pAAV2-neo plasmid by sites of EcoRI and BglII, downstream of Cytomegalovirus (CMV) major immediate-early gene promoter, followed by bovine growth hormone polyadenylation (polyA) signal, flanked at each end by the AAV2 145-bp inverted terminal repeats.

Purification of CTLA4–FasL fusion protein

C-terminal Flag-tagged CTLA4–FasL recombinant proteins were obtained from the supernatants of 293T cells transfected with pAAV2/CTLA4–FasL plasmid at 48 h. The proteins were purified at 4°C through affinity chromatography using anti-Flag M2 affinity gel beads (Sigma–Aldrich, St Louis, MO, USA), followed by competitive elution with a solution containing 100 μg ml⁻¹ 3×Flag peptide (Sigma–Aldrich) in 50 mM Tris–HCl, 150 mM NaCl (pH 7.4). Purified proteins were quantified using Bradford reagent according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA) using BSA as a standard, followed by filtration through 0.2-μm sterile syringe filters.

Flow cytometry

T cells and macrophages were obtained from AIA rat spleens, which were disseminated, disintegrated and removed of erythrocytes by osmotic shock. T lymphocytes were

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<sup>a</sup>Underlined and italic sequences are restriction sites for enzymes indicated in parenthesis.

<sup>b</sup>Boxed sequence is Flag-tag.
collected and purified through negative selection using MACS pan T cell sorting and purification kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The splenic adherent cells were cultured for 6–8 days after removing non-adherent lymphocytes, and FACS analysis showed that in this adherent fraction, the overwhelming majority (~90%) was macrophage with a small proportion of fibroblast (~10%). Cells were pre-incubated at 37°C for 45 min with purified CTLA4–FasL proteins (5 μg ml¯¹) and then were pelleted, washed twice with PBS and incubated on ice for 30 min with 10 μg ml¯¹ of rabbit monoclonal antibody (mAb) against C-terminus of rat FasL (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat mAb against N-terminus of rat CTLA4 (Santa Cruz Biotechnology), with rabbit IgG1 (Santa Cruz Biotechnology) or goat IgG1 (Santa Cruz Biotechnology) serving as negative controls. Cells were then incubated on ice for 30 min with FITC-conjugated goat anti-rabbit IgG (H + L) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or FITC-conjugated rabbit anti-goat IgG (H + L) (Jackson Immunoresearch Laboratories). Following washes, FITC-labeled cells were run and analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

Inhibition of the proliferation of T lymphocytes with CTLA4–FasL protein

T lymphocytes proliferation–inhibition assays in the presence of variable amounts of purified CTLA4–FasL proteins were performed using cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Tokyo, Japan). Briefly, T cells were stimulated with 3 μg ml¯¹ concanavalin A (ConA; Sigma–Aldrich) for 24 h and then dispensed in 96-well tissue culture plate with PBS and incubated on ice for 30 min with 10 μg ml¯¹ of rabbit monoclonal antibody (mAb) against C-terminus of rat FasL (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat mAb against N-terminus of rat CTLA4 (Santa Cruz Biotechnology), with rabbit IgG1 (Santa Cruz Biotechnology) or goat IgG1 (Santa Cruz Biotechnology) serving as negative controls. Cells were then incubated on ice for 30 min with FITC-conjugated goat anti-rabbit IgG (H + L) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or FITC-conjugated rabbit anti-goat IgG (H + L) (Jackson Immunoresearch Laboratories). Following washes, FITC-labeled cells were run and analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

Animals

Pathogen-free female Lewis rats (weight 100–120 g) were purchased from Vital River Laboratories (Beijing, China) and were maintained under pathogen-free conditions in the Animal Breeding Center of our institute. All animal studies and handling conformed to the Institutional Animal Care and Use Committee guidelines and approval of Beijing Institute of Basic Medical Sciences. All efforts were made to minimize animal suffering.

Production of recombinant adeno-associated virus vectors

Recombinant adeno-associated virus serotype 2 (rAAV2) vectors including rAAV.CTLA4–FasL and rAAV.EGFP (serving as the control virus) were produced as described previously (28). Briefly, recombinant adeno-associated virus (rAAV) plasmid DNA was transfected into BHK21 viral packaging cells using Lipofectamine 2000 (Invitrogen life technologies, Carlsbad, CA, USA). After G418 selection, the survival cells capable to express target gene were isolated and subsequently infected with recombinant herpes simplex virus 1 (rHSV1-rc/ΔUL2) (29) to package the rAAV vectors, followed by purification using a sequential process of nucleic isolation, density–gradient centrifugation and heparin–sulfate affinity column chromatography. Subsequently, viral titer of the purified viral vectors was determined by dot blot analysis with manipulative details same as reference (30), using a dilution series of corresponding vector plasmid DNA of known copy number as the standards.

Induction, treatment and evaluation of AIA

The AIA animal model was induced on day 0 by a single subcutaneous injection at the base of the tail of Lewis rats, with 220 μl (5 mg ml¯¹) of fresh heat-killed Mycobacterium tuberculosis (MT) H37Ra (Difco Laboratories, Detroit, MI, USA) in sterile mineral oil (Sigma–Aldrich). On the next day, rats were injected with 5 × 10¹¹ viral genomes of rAAV.CTLA4–FasL or rAAV.EGFP in both right and left ankle joints in a total volume of 50 μl saline. The severity of arthritis can be clinically assessed by direct observation of all four limbs in each animal and scored by grading each paw from 0 to 4, based on swelling of ankle and wrist joints and small interphalangeal joints: 0, no redness and swelling (normal joint); 1, redness and swelling in one digit or interphalangeal joint; 2, redness and swelling of more than one digit or interphalangeal joint or mild redness and swelling of ankle or wrist joint; 3, moderate redness and swelling of ankle or wrist joint but able to bend and walk; 4, severe redness and swelling and deformation of the ankle or wrist joint and inability to bend and walk. All four legs are scored, so the highest possible arthritic index is 16. Scoring was conducted in a blinded fashion. In addition, paw swelling was also quantified by measuring thickness of ankle diameter from the medial to the lateral malleolus with an electronic caliper and by measuring the increase in hind paw volume with water displacement plethysmometry. Ankle joints or organs were harvested on day 25 post-adjuvant after rats were sacrificed for histology, ELISA, western blot and mRNA studies.

Joint histology

Two 5-μm frozen sections of each specimen obtained from the arthritic joints of ankles on day 25 post-adjuvant were, respectively, stained with hematoxylin and eosin (H&E) or Safranin O-fast green. The H&E-stained sections were read, respectively, for the degrees of both synovial hyperplasia and inflammatory cells infiltration, which were graded as follows (0–3): 0, normal; 1, mild proliferation of synovium or minor inflammatory cells infiltration into joint space and synovium; 2, invasion of synovium into the joint space or moderate inflammatory cells infiltration; 3, extensive synovial hyperplasia with pannus erosion of the cartilage or extensive inflammatory cells infiltration in joint space and synovium.
The Safranin O-stained sections were read for cartilage depletion through the loss of proteoglycans, which was scored from 0 to 3, where 0 represents no loss of proteoglycans, while a score of 3 indicates complete loss of staining for proteoglycans (31).

**Detection of CTLA4–FasL mRNA by RT-PCR**

Ankle joints or organs were collected after rats were sacrificed and snap-frozen in liquid nitrogen, pulverized using a pestle and mortar and homogenized in Trizol Reagent (Invitrogen Life Technologies) using a tissue homogenizer. Total RNA was isolated from the aqueous phase and a total of 1–2 µg RNA was reverse transcribed according to the instruction manual. RT-PCR amplification mixture (50 µl) contained 25 ng template cDNA and 250 nM primers of a and j (Table 1) to obtain CTLA4–FasL fusion gene. Rat GAPDH was used as an internal reference gene control using primers of GAP5 and GAP3 (Table 1).

**Ankle homogenates**

The ankle homogenates were isolated from crushed joints by adding 2 ml of lysis buffer (20 mmol l\(^{-1}\) HEPES, 0.5 mol l\(^{-1}\) NaCl, 0.25% Triton X and protease inhibitors) to 200 mg pulverized ankle joint, mixed by rotations for 4 h at 4°C and then spun in a centrifuge. The supernatants were collected and used as ankle homogenates in western or ELISA analysis.

**Enzyme-linked immunosorbent assay**

Various amounts of purified proteins were coated onto 96-well ELISA plates overnight at 4°C with triplicate samples. After blocking with 3% BSA/PBS, the wells were incubated for 1 h at 37°C with primary mAb (2 µg ml\(^{-1}\)) of anti-rat CTLA4 (Santa Cruz Biotechnology) or anti-rat FasL (Santa Cruz Biotechnology) or anti-Flag (Sigma–Aldrich). HRP-conjugated secondary antibodies (0.4 µg ml\(^{-1}\)) were added and allowed binding for 40 min at 37°C. Finally, color was developed with 3,3′,5,5′-tetramethylbenzidine and the absorbance was measured at 450 nm. Cytokine levels in ankle homogenates were determined using some commercially available ELISA kits (R&D systems, Minneapolis, MN, USA) that specifically recognize rat cytokines tumour necrosis factor (TNF)-α, IL-1β, IFN-γ, IL-2, IL-4 and IL-10, according to the manufacturer's procedure.

**Western blot**

The purified fusion proteins or ankle homogenates were loaded on a 12% SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris buffered saline with tween (TBST) buffer for 60 min at room temperature. Blots analysis of purified proteins were incubated with anti-CTLA4 (Santa Cruz Biotechnology) or anti-FasL (Santa Cruz Biotechnology) or anti-Flag (Sigma–Aldrich), respectively, and that of ankle homogenates, with anti-Flag in TBST containing 5% nonfat milk. It was followed by incubation with respective HRP-conjugated secondary antibodies and the bands were developed with ECL substrate and exposed to X-ray film.

**Statistical analysis**

All data in this paper were analyzed by a professional statistician using SAS software version 9.1.3. Differences in clinical parameters, including articular index, ankle thickness, paw swelling and weight, were compared with repeated-measures, two-factor design of quantitative data two-way analysis of variance. Differences in histology, including H&E scores and Safranin O intensity scores, were analyzed by Kruskal–Wallis test. Differences in the cytokines’ levels in ankle homogenates were analyzed by Student’s t-test. Data are expressed as the mean ± SEM. P-values <0.05 were considered significant.

**Results**

**Construction and expression of rat CTLA4–FasL fusion gene**

The extracellular domain cDNAs of CTLA4 and FasL were cloned, respectively, and fused and then inserted into AAV2 vector to construct CMV-driven mammalian expression plasmid (Fig. 1A). All the primers used in cloning are listed in Table 1. The supernatants from 293T cells transduced with the recombinant plasmids were purified by anti-Flag M2 affinity gel. The molecular mass of purified CTLA4–FasL fusion protein is about 41–43 kD as shown in SDS–PAGE and western blot (Fig. 1B), consistent with that estimated from the amino acid sequence. The binding of anti-CTLA4, anti-FasL and anti-Flag to a series of concentrations of purified CTLA–FasL fusion protein, which possesses a Flag tag, coated onto plastic plates is shown in Fig. 1C. These findings showed that our rat CTLA4–FasL fusion gene product could be secretory expressed in AAV2 vector.

**The binding of CTLA4–FasL to B7 ligand and Fas receptor on cell surface**

The Fas+ activated T lymphocytes were incubated with increasing concentrations of purified CTLA4–FasL protein and detected by indirect immunofluorescence with anti-CTLA4 mAb. The desirable concentration of CTLA4–FasL protein for detection of binding activity with respect to T lymphocytes was 5 µg ml\(^{-1}\), with a high positive-staining ratio of 82.89% (Fig. 2A and B). In parallel, the binding activity to B7 ligand of CTLA4–FasL protein at a concentration of 5 µg ml\(^{-1}\) was also observed in B7+/Fas− Daudi cells (Fig. 2C) or rat splenic macrophages activated in vitro (Fig. 2D) with anti-FasL mAb. Furthermore, CTLA4–FasL protein exerted a significant proliferation-inhibition or apoptosis-induction activity to rat splenic T lymphocytes activated in vitro (Fig. 2E). These findings showed that CTLA4–FasL could bind to both Fas receptor and B7 ligand with respective domain.

**rAAV.CTLA4–FasL reduces clinical parameters of rat AIA**

The potential anti-arthritic effect of rAAV.CTLA4–FasL was tested through intraarticular (i.a.) administration of 5 × 10\(^{10}\) AAV vectors into the right and left ankle joints of rats respectively on the next day of immunization of AIA. Though slight redness and swelling also found in AIA ankle joints treated with rAAV.CTLA4–FasL, rats receiving rAAV.CTLA4–FasL exhibited a profound reduction in signs and symptoms of arthritis (Fig. 3A), and importantly, after administration of
rAAV.CTLA4–FasL, rats could stand on their hind limbs and walk almost normally, whereas rats treated with rAAV.EGFP could not stand on their hind limbs (Fig. 3B). Accordingly, there were statistically significant reductions in the articular index ($P < 0.001$) (Fig. 3C) and ankle joint thickness ($P < 0.001$) (Fig. 3D) from day 11 until day 25 in rAAV.CTLA4–FasL-treated rats. A pronounced reduction in paw swelling showed the sustained suppression of arthritis in ankle joints and hind paws by rAAV.CTLA4–FasL ($P < 0.01$ at days 11 and 13, $P < 0.001$ from days 15 to 25, Fig. 3E). Furthermore, the mean body weight of the animals treated with rAAV.CTLA4–FasL were significantly higher than those treated with rAAV.EGFP ($P < 0.01$ at days 11 and 13, $P < 0.001$ from days 15 to 25, Fig. 3F). In addition, the single i.a. injection of rAAV.CTLA4–FasL into normal joints did not cause any apparent adverse reaction at the site of injection, as evidenced by lack of inflammation or joint swelling (articular index = 0). In conclusion, these results demonstrated that rAAV.CTLA4–FasL but not rAAV.EGFP was effective at inhibiting the swelling and progression of rat AIA when administered in joints.

rAAV.CTLA4–FasL decreases joint inflammation and destruction

Histologic examination of ankle joints was performed to determine the treatment effects on inflammation and joint destruction. Consistent with the clinical data, the histological observations showed that the animals treated with rAAV.CTLA4–FasL demonstrated significantly ($P < 0.01$) reduced scores for synovial hyperplasia (score = 0–3) and inflammatory cells infiltration (score = 0–3) compared with the animals treated with rAAV.EGFP (Fig. 4). In addition to the effect on inflammation, we also investigated the effect on cartilage degradation by Safranin O staining, which showed joints from rAAV.CTLA4–FasL-treated animals exhibited significantly ($P < 0.001$) less proteoglycan loss and erosions than rAAV.EGFP-treated joints as determined by a semi-quantitative scoring system (score = 0–3, Fig. 5). In a word, only mild inflammations of synovial hyperplasia and inflammatory cells infiltration, with almost normal joint structure were found in rAAV.CTLA4–FasL-treated ankle joints. These results suggested the administration in joints of rAAV.CTLA4–FasL, but not rAAV.EGFP, resulted in significantly reduced inflammation and joint destruction in rat AIA.

rAAV.CTLA4–FasL down-regulates proinflammatory cytokines in AIA ankles

Because high expression of proinflammatory cytokines is characteristic of joint inflammation, the effect of therapy on joint TNF-$\alpha$, IL-1$\beta$, IFN-$\gamma$ and IL-2 expressions were examined. TNF-$\alpha$, IL-1$\beta$ and IFN-$\gamma$ were significantly ($P < 0.01$) reduced in the rat ankle joints treated with rAAV.CTLA4–FasL (Fig. 6). A marked reduction was also observed in IL-2 ($P < 0.05$), but no statistically significant changes were observed in anti-inflammatory cytokines, IL-4 and IL-10 (Fig. 6). These results indicate that treatment with rAAV.CTLA4–FasL decreases the levels of key proinflammatory cytokines including TNF-$\alpha$, IL-1$\beta$, IFN-$\gamma$ and IL-2 in the ankle joints of AIA rats but has no influence on anti-inflammatory cytokines.

Local expression in the joints of CTLA4–FasL

After i.a. injection of the rAAV vectors, rat CTLA4–FasL RNAs were found in the ankle joints treated with rAAV.CTLA4–FasL but not in control rAAV.EGFP-treated joints (Fig. 7A). To
confirm these findings, we also examined and identified the according expression of fusion protein by immunoblot analysis using joint lysates (Fig. 7B). At the end of experiments, organs including thymus, spleen, liver and kidney from the rats treated with i.a. administration of rAAV:CTLA4–FasL were collected to examine the spreading of recombinant virus using RT-PCR. Assessment of the distribution of the fusion construct after i.a. injection revealed that CTLA4–FasL DNAs were detectable in joint injection sites, whereas distant sites, such as thymus, spleen, liver and kidney, were negative (Fig. 7C). In addition, no CTLA4–FasL protein was found in plasma using a specific ELISA (data not shown).

Discussion

The present study demonstrates the clinical efficacy of CTLA4–FasL fusion product for inhibiting arthritis using rat AIA animal model. Administration in ankle joints of an AAV2 vector encoding the rat extracellular CTLA4–FasL fusion gene expressed from a CMV transcription promoter was highly effective at suppressing disease determined by clinical examination, histology observation and down-regulation of proinflammatory cytokines in the joint. Following local injection, the transgene products could be found locally in ankle joints, suggesting that tissue specific expression can be achieved. These findings implicate potential therapeutic applications for suppression of RA by local joint delivery of CTLA4–FasL.

T lymphocytes are key inflammatory cells in the initiation and progress of RA pathological process. A large amount of T lymphocytes infiltration in the joint space and synovium can be observed in RA joints and make their selective eradication an attractive therapeutic goal. Suppression of T-cell activation by blocking co-stimulation signal or inducing T-cell apoptosis through Fas-mediated pathway has been reported to suppress the development of experimental arthritis. However, simultaneous stimulation of Fas-mediated apoptotic pathway and blockade of the co-stimulatory pathway to inhibit RA has not been reported. The publishes of CTLA4–FasL fusion product, which has high potency to inhibit proliferation and induce apoptosis of T cells, bring forward a novel hint to us for treatment of RA. CTLA4–FasL fusion protein was once designed as a ‘trans signal converter protein’, in which the extracellular domain coding sequences for CTLA4 and FasL were linked in-frame (23). It was reported as a bifunctional inhibitory molecule to combine both co-stimulator blocking with CTLA4 domain and trans inhibitory signaling with FasL domain within a single protein (23, 25, 27). Therefore, CTLA4–FasL exerts dual inhibition in T cells via targeted both co-stimulatory and inhibitory pathways. Several studies have documented effectiveness of CTLA4–FasL both in vitro and in vivo. CTLA4–FasL inhibited primary mixed lymphocyte reaction and induced alloantigen-specific hyporesponsiveness ex vivo (25) and modulated the in vivo response of adoptively transferred allogeneic splenocytes (24). It was evidenced that CTLA4–FasL delayed rejection of cardiac grafts (26). It was appended that CTLA4–FasL induced apoptosis of activated T cells as early as 24 h after T cells’ activation by interfering with anti-apoptotic signals (22). In the present study, we demonstrate that an AAV mediated CTLA4–FasL gene transfer efficiently prevent the development of AIA in Lewis rats, an animal model of RA dependent on T cells, with evidence for significant reduction of inflammatory T lymphocytes infiltration. In our other studies performed by now, CTLA4–FasL is superior to mature FasL alone in blocking the progress of arthritis due to the adjunctive role of CTLA4 (unpublished data of our group).

In addition, in contrast to the in vivo lethality of sFasL administration, no evidence for such severe side-effect has been observed in animals treated with the CTLA4–FasL
fusion protein and also no adverse effect has been observed following administration of rAAV.CTLA4–FasL in this study. CTLA4–FasL is an alternative soluble form of FasL observed from our study.

Recombinant proteins including antibodies against TNF-α used in the clinic have a limited half-life, requiring repeated and expensive high dosages to achieve therapeutic concentrations in the joints and serious side-effects result from these high circulating levels of therapeutic proteins. An attractive alternative for systemic protein delivery is local gene therapy (32,33). In regards to viral vectors, AAV seems to be the most promising for future studies and usage in clinic (32–35). Several different AAV serotypes have been identified with AAV2 being the prototype. Recombinant AAV serotype 2 (rAAV2) is mostly used in animal studies of RA. No adverse effects have been found directly due to rAAV2 and long-term protein expression is desirable achieved with rAAV2 vector. Most cells in the joint are found to be transduced in vitro by rAAV2, including synovial fibroblasts (36) and chondrocytes (37). In vivo, stable expression also has been detected in synoviocytes and chondrocytes (38,39). Also desirable, rAAV preferentially transduces arthritic joint cells in vivo (36). These properties make AAV especially desirable for use in gene therapy of RA. i.e. gene transfer with AAV vectors, mainly used by rAAV2, has been proven to be a useful strategy for high expression of the therapeutic proteins within the local joint, preventing both systemic diffusion and side-effects (32,33). In present study, the lasting expression of CTLA4–FasL protein is detected in rAAV.CTLA4–FasL-treated rats, therefore obviating the necessity of repeated administration of costly recombinant fusion protein and rendering prolonged inhibition of arthritis.

Fig. 3. rAAV.CTLA4–FasL reduces the severity of joint inflammation in AIA. Representative gross appearances of hind paws and ankle joints of AIA rats treated with rAAV.CTLA4–FasL (A) and rAAVEGFP (B). In rAAV.CTLA4–FasL-treated rats, statistically significant reductions in the articular index (C), the sum of the ankle thickness on the right and left sides (D) and the sum of paw swelling on the right and left sides (E) over the 25-day AIA time course from day 11 (n = 10 observations each). Furthermore, the weights were markedly improved in rAAV.CTLA4–FasL-treated rats (F). Values are the mean ± SEM. **Represents P < 0.01; ***represents P < 0.001.
Tissue specific expression of target CTLA4–FasL protein has been achieved in local ankle joints following i.a. injection of the recombinant AAV vector. CTLA4–FasL genes used in all previous studies are all from human. To avoid immunological rejection of human products in rats, we cloned and constructed rat CTLA4–FasL fusion proteins.

Fig. 4. rAAV.CTLA4–FasL ameliorates the AIA pathology. A–C. representative H&E staining of normal ankle joints (A), AIA ankle joints treated with rAAV.CTLA4–FasL (B) and AIA ankle joints treated with rAAV.EGFP (C), from day 25 post-adjuvant immunization. (D) The histology of AIA ankle joints were, respectively, scored on a scale of 0–3 for joint inflammation parameters of synovial hyperplasia and inflammatory cells infiltration for two experimental groups on day 25 post-immunization. Scale bar = 200 μm. Values are the mean ± SEM. **Represents \( P < 0.01 \).

Fig. 5. Joint cartilage integrity of rAAV.CTLA4–FasL-treated and rAAV.EGFP-treated arthritic animals. (A) Joint from a normal animal with intense Safranin O staining, indicating normal intact cartilage; (B) a representative joint from an animal treated with AIA and i.a. injection of rAAV.CTLA4–FasL with relatively intact cartilage; (C) a representative joint from an animal treated with AIA and i.a. rAAV.EGFP with largely diminished Safranin O staining of the cartilage matrix and (D) stainings were semi-quantitatively scored on a scale from 0 to 3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. A significant reduction in cartilage destruction was seen in animals injected with rAAV.CTLA4–FasL compared with the control rAAV.EGFP. Scale bar = 200 μm. Values are the mean ± SEM. ***Represents \( P < 0.001 \).
gene, with a signal peptide at N-terminus to obtain secretory expression, and a Flag-tag at C-terminus used for detection of fusion protein expression in vivo to exclude inherent interferences. The fusion gene expression was identified in AAV2 plasmid with 293T cells, and the purified protein displayed the anticipated binding activities with its component parts. The AIA disease is an autoimmune arthritis mediated mainly by T cells, frequently used to study immunological aspects of RA and used as a model for developing and testing anti-inflammatory drugs. In our established rat arthritis model, success rate approaches almost 100% and clinical signs of inflammation including redness and swelling in paws, ankle or wrist joints are usually (>90%) observed by day 11 post-induction with MT in mineral oil. Malformation of joints, especially ankle joints in our rat model, usually starts around day 20 after immunization and remains irreversible if without effective intervention, therefore, it is desirable to observe the effects of therapeutic agents with this animal model for RA. Because it takes several days (about 8–10 days) to express target genes mediated by AAV gene transfer, we injected rAAV vectors in joints on the next day of immunization. Monitoring of disease development is done most easily by macroscopic examination. Although articular index may give a numerical score according to macroscopic observation on degree of inflammation based on the severity of joint swelling and redness, it provides a semi-quantitative and subjective assessment. Therefore, additional objectively monitoring the degree of inflammation and swelling are performed by measuring the volume of the paws using a water displacement method, the ankle thickness with an electronic thickness caliper and the weight. All of the results from different monitoring indexes reflect the same inhibitory trend of i.a. rAAV.CTLA4–FasL gene transfer. The main pathological manifestations in suffering joints of RA and animal arthritis are synovial hyperplasia, inflammatory cells infiltration and finally resulting in cartilage and bone destruction. Therefore, it is mainly observed in these changes to reflect the effects of therapeutic intervention. Only mild synovial hyperplasia and inflammatory cells infiltration, with almost normal joint structure was found in rAAV.CTLA4–FasL-treated ankle joints, suggesting i.a. rAAV.CTLA4–FasL gene transfer resulted in reduced pathology in rat AIA, consistent with the inhibitory effect in clinical observations. The clinical and histological examinations suggest that CTLA4–FasL may be a potential target for therapeutic intervention in RA.

Pro-inflammatory cytokines such as TNF-α and IL-1β have been proven to play critical roles in the inflammation and joint damage that occur in RA or arthritis animal model, and their expressions by inflammatory cells are strongly correlated to disease activity (40,41). The efficacy of CTLA4–FasL in down-regulation pro-inflammatory cytokines’ expressions including TNF-α and IL-1β as shown in present study may be attributed to the inhibition of inflammatory T cells because the inflammatory cytokines would be limited once the producing cells died or stayed anergy. CTLA4–FasL over-expression in the synovium and joint space may result in reduced numbers of cells responsible for producing proinflammatory cytokines.

Taken together, these data demonstrate a significant prophylactic effect of local delivery of CTLA4–FasL in inhibiting
CTL4–FasL gene transfer suppresses AIA

rat AIA. Although further detailed mechanisms needed to be investigated with CTL4–FasL's suppressive activities, the observations of this study implicate the potential therapeutic application for suppression of RA by local joint delivery of CTL4–FasL.

References