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Design, expression and characterization of a novel coexpression system of two antiarthritic molecules

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Abstract The complexity of rheumatoid arthritis (RA) pathogenesis makes combined blockade of multiple targets an attractive therapeutic strategy. The combination therapy with anti-TNF plus anti-T-cell has been mostly reported to provide greater efficacy than anti-TNF alone. TNFR (p75)-Fc fusion protein, which has been proven effective in clinics, is chosen as the TNF antagonist in this study. CTLA4-FasL fusion molecule, which has been well characterized in our previous studies for its suppressive effect in rat arthritis model, is chosen as the T-cell antagonist. In this study, furin cleavage site and 2A self-processing sequence were introduced to link upstream TNFR-Fc and downstream CTLA4-FasL and mediate separate coexpression of the two fusion proteins in a single recombinant adeno-associated virus (rAAV) vector. Using this expression system, we generated two fusion proteins with same size as their individual counterparts in vitro and in vivo, and the proteins desirably retained their parent biological activities. In vivo results demonstrated that furin-2A technology is able to regulate separate coexpression of these proteins under arthritic inflammatory conditions. This study describes a single rAAV vector for production of two antiarthritic molecules antagonizing both TNF and T cells, which may serve as an attractive expression system for RA gene therapy.

Keywords TNFR-Fc · CTLA4-FasL · Adeno-associated virus vector · Rheumatoid arthritis

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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. The pathogenesis of RA is complex and encompasses many factors including key proinflammatory cytokine TNFα and T cells, which are believed to play significant roles in the process of RA. TNFα blocking agents are effective in reducing clinical disease activity and suppressing the progression of structural damage of RA. Three of them are commercially available, including the p75 TNFα receptor (TNFR)-IgG1 Fc fusion protein, etanercept. Despite these desirable effects, approximately 30–50 % of RA patients do not respond to TNFα antagonists. Moreover, TNFα antagonists do not provide long-term effectiveness in most patients. 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 (Cope et al. 2007; Bennett 2008). The combination of anti-TNF therapy with anti-T cell therapy has been shown to produce synergically enhanced benefit in suppressing animal arthritis (Williams et al. 1994, 1995, 2000; Dépis et al. 2012).

CTLA4-FasL, a fusion product of extracellular domains of CTLA4 and FasL, which had been well characterized in our previous studies (Zhang et al. 2012a, b) for its antiarthritic effects in rat adjuvant-induced arthritis (AIA), an experimental model of RA dependent on T cells, was

chosen as an inhibitor of inflammatory T cells. CTLA4 blocks costimulatory signal for T-cell activation and FasL mediates apoptosis of T cells. CTLA4-FasL combines the capacities of costimulation blockade and apoptosis induction within a single recombinant protein, and exerts a more potent antiarthritic effect than FasL alone (Zhang et al. 2012b). We hypothesized that combining TNFR-Fc with CTLA4-FasL to simultaneously counteract TNFα and T cells could produce more powerful effect for treatment of RA.

Many acquired diseases including RA are now being considered for treatment by local gene delivery to minimize systemic distribution of gene product and the necessity of frequent readministration. Adeno-associated virus serotype 2 (AAV2) is a suitable gene transfer vector and mostly used in animal studies of RA; since it lacks viral genes, it is able to infect both dividing and quiescent cells, and can provide long-lasting gene expression in vivo (Adriaansen et al. 2006; Woods et al. 2008). In our previous studies (Zhang et al. 2012a, b), by means of AAV2, we showed that intra-articular (i.a.) gene transfer of CTLA4-FasL has an efficiently suppressive effect on rat AIA. The rat p75 TNFR-Fc gene delivery by i.a. administration in an AAV2 vector effectively suppressed rat arthritis, and importantly, i.a. administration resulted in a significantly lower systemic distribution of the gene product compared with intramuscular administration (Chan et al. 2002). Two clinical trials of an i.a. injection of a rAAV2 vector coding for a human p75 TNFR-Fc (tgAAC94), a protein identical to etanercept, provided encouraging data on safety and feasibility for further development of gene therapy for RA (Mease et al. 2009, 2010).

Equal and efficient expression of both vector DNAs within the same cell is hard to achieve as the host cell is usually unable to express both vectors with equal efficiency, which results in imbalanced expression of two molecules. The conventional method using internal ribosomal entry sites (IRES) to link dual molecules in a single vector leads to substantially low expression of the second gene (Mizuguchi et al. 2000). A regulated expression system has been previously reported for the constitutive expression of full-length two-chain antibodies (Fang et al. 2005; Jostock et al. 2010) or coagulation factor VIII (He et al. 2011) from a single open reading frame (ORF), in which heavy and light chains are linked by a furin cleavage site and a 2A self-processing sequence. With regard to translation and secretion, self-processing of 2A sequence occurs via an undefined mechanism as yet which separates the heavy and light chain peptides. Furin-catalyzed proteolysis and carboxypeptidase activity remove the remaining 2A-sequence peptide of 23 amino acids on the C terminus of upstream heavy chain. One of the potential advantages of this single furin/2A ORF approach is the forced equimolar expression of the upstream heavy chain and downstream light chain genes (Fang et al. 2005; Jostock et al. 2010; He et al. 2011). These studies provide a hint for us that a furin-2A mediated expression AAV system might regulate the simultaneous and separate expression of two antiarthritic molecules for enhancing therapeutic benefit of RA.

In this study, a regulated expression system was produced that allows coexpression of TNFR-Fc and CTLA4-FasL as separate proteins, following a single transfection or i.a. injection of a single recombinant AAV2 vector. This expression system of double fusion proteins described here may provide a more promising strategy for RA therapy due to antagonizing both TNFα and T cells.

Materials and methods

Materials and antibodies

Plasmids of CTFA (pAAV2/CTLA4-FasL) and PGFP (pAAV2/EGFP, the control plasmid) were generated in our previous study (Zhang et al. 2012a). Recombinant rat TNFα protein was purchased from Peprotech and actinomycin D (Act-D) was obtained from Sigma-Aldrich. Cell counting kit-8 (CCK-8) was purchased from Dojindo laboratories. Anti-TNFR mouse monoclonal antibody (mAb) for detection of the extracellular part of rat TNFRII, goat polyclonal antibody against N terminus (extracellular domain) of rat CTLA4, rabbit polyclonal antibody against amino acids 100–278 at the C terminus (extracellular domain) of rat FasL, rabbit IgG1 and goat IgG1, were all purchased from Santa Cruz Biotechnology. All of the secondary antibodies including peroxidase-conjugated goat anti-rat IgG (Fc fragment specific), peroxidase-conjugated rabbit anti-goat IgG (H+L), peroxidase-conjugated goat anti-rabbit IgG (H+L), FITC-conjugated goat anti-rabbit IgG (H+L) and FITC-conjugated rabbit anti-goat IgG (H+L) were all obtained from Jackson Immunoresearch Laboratories.

Cloning of recombinant fusion genes

cDNAs encoding the extracellular region of rat TNFR-II (NCBI Genebank No. NM_130426; 705 bp) and rat IgG1 Fc (NCBI Genebank No. M28670; 687 bp) were synthesized. The synthesized TNFR gene contains *Kpn*I site, a Kozak sequence (GCCGCCACC), start coden ATG, an original signal peptide for TNFR (as described in Genebank No. NM_130426, 63 bp), *Asc*I and *Pac*I sites at C terminus of TNFR, followed by stop coden TGA and *Eco*RI site. For the correct coden of the amino acid after introduction of the above *Asc*I and *Pac*I enzyme sites, an additional nucleotide A was introduced after *Asc*I site and nucleotide G after *Pac*I site (Zhang et al. 2007). This (*Kpn*I) Kozak-signal-TNFR(*Asc*I–*Pac*I–*Eco*RI) construct was cut from pGEM-T vector and ligated into pAAV2-neo plasmid

using *Kpn*I and *Eco*RI (Fig. 1). The synthesized IgG1 Fc fragment which contains an *Asc*I site with an additional nucleotide A and a *Pac*I site with an additional nucleotide G, was cut from pGEM-T vector and inserted into the recombinant pAAV2/TNFR vector by *Asc*I and *Pac*I (Fig. 1). The resulted recombinant expression plasmid pAAV2/TNFR-Fc was designated as TRFC.

The recombinant CTLA4-FasL fusion product was constructed as reported previously (Zhang et al. 2012a). Briefly, cDNAs encoding the extracellular region of rat CTLA4 and FasL were extracted from rat splenocytes, and a Kozak sequence and oncostatin M signal were subsequently introduced into the N terminus of CTLA4 by several rounds of PCR. The CTLA4 element was fused with FasL domain by overlap PCR and a Flag-tag was introduced into the C terminus of FasL in this process. The recombinant construct was inserted into the pAAV2-neo plasmid, and this recombinant vector was named CTFA (pAAV2/CTLA4-FasL) in the present study.

To generate the double fusion constructs of TNFR-Fc with CTLA4-FasL containing furin cleavage and 2A self-processing sequences between them, the sequence-overlap primers 1107P51, 1107P52, 1107P53 and 1107P54 were designed according to cDNA oligos for a 4-amino acid furin cleavage site (RAKR) and a 24-amino acid FMDV 2A peptide (APVKQTLNFDLLKLAGDVESNPGP). Using CTFA as the template, after four rounds of PCR subsequently with pairs of 1107P51+1101P3, 1107P52+1101P3, 1107P53+1101P3 and 1107P54+1101P3, furin-2A fusion sequence was finally introduced into the N terminus of CTLA4-FasL. The PCR product of furin-2A–CTLA4-FasL was digested by *Pac*I and *Eco*RI and inserted into TRFC vector at C terminus of TNFR-Fc. The resulting recombinant expression plasmid of pAAV2/TNFR-Fc–furin-2A–CTLA4-FasL was termed TFCF for abbreviation. The sequences of the primers used here are listed in Table 1.

Cell culture and transfection

Human embryonic kidney (HEK) 293 T cells and L929 mouse fibroblast cells were cultured in DMEM (high glucose; Hyclone) supplemented with 4 mM L-glutamine and 10 % fetal bovine serum (FBS). Daudi and Jurkat cells were cultured in RPMI-1640 (Hyclone) supplemented with 2 mM L-glutamine and 10 % FBS. Plasmid was purified using plasmid DNA purification kit (Qiagen) and 293 T cells were transfected in 6-well tissue culture plates with Entranster-H transfection reagent (Engreen Biosystem Company). Four hours after transfection, the cell culture medium was removed and the cells were fed fresh medium without 10 % FBS. At 48 h post-transfection, the supernatants were collected.

ELISA

For the detection of TNFR-Fc protein in the supernatants, recombinant rat TNFα protein was diluted in 0.05 M bicarbonate coating buffer (PH 9.6) to a final concentration of 2 μg/ml and coated onto 96-well ELISA plates (100 μl/well) at 4 °C overnight with triplicate samples. After blocking with 3 % BSA/PBS, the cell culture supernatants derived from different transfectants of TFCF, TRFC, CTFA and PGFP were added (100 μl/well) and incubated at 37 °C for 2 h. After washing with PBS, the wells were incubated for 1 h at 37 °C with 2 μg/ml of anti-rat TNFR followed by the incubation with peroxidase-conjugated secondary antibody (0.4 μg/ml) for 50 min at 37 °C, or the wells were incubated directly with peroxidase-conjugated anti-IgG Fc (0.25 μg/ml) at 37 °C for 50 min. Finally, color was developed with 3,3′,5,5′-Tetramethylbenzidine (TMB), and the absorbance was measured at 450 nm.

For detection of CTLA4-FasL protein in the supernatants, the plates were coated with anti-CTLA4 (5 μg/ml) or anti-FasL (5 μg/ml), respectively, at 4 °C overnight. After blocking, the cell culture supernatants were added and incubated at 37 °C for 2 h. After washing, the wells were incubated with primary mAb (2 μg/ml) of anti-FasL or anti-CTLA4, respectively, followed by incubation with corresponding peroxidase-conjugated secondary antibodies (0.4 μg/ml) of at 37 °C for 50 min. In addition, another sandwich ELISA assay was designed to exclude the possibility that TNFR-Fc and CTLA4-FasL would express as an integrated molecule without separation in the cell culture supernatants of TFCF-transfected HEK 293 T cells. The plates were coated with anti-CTLA4 (5 μg/ml) and then incubated with the supernatants of different transfectants, followed by incubation with peroxidase-conjugated anti-IgG Fc (0.25 μg/ml). The supernatants were replaced by PBS as the blank control in all experiments.

Western blot

The proteins in the supernatants were loaded on 8 % or 10 % SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5 % nonfat milk in TBST buffer for 2 h at room temperature. For blot analysis of target protein TNFR-Fc, the membranes were incubated with peroxidase-conjugated anti-IgG Fc. As for the analysis of CTLA4-FasL protein, the membranes were incubated with anti-CTLA4 or anti-FasL, respectively, followed by incubation with respective peroxidase-conjugated secondary antibodies. All antibodies were diluted in TBST containing 5 % nonfat milk. The bands were developed with ECL substrate and exposed to X-ray film.

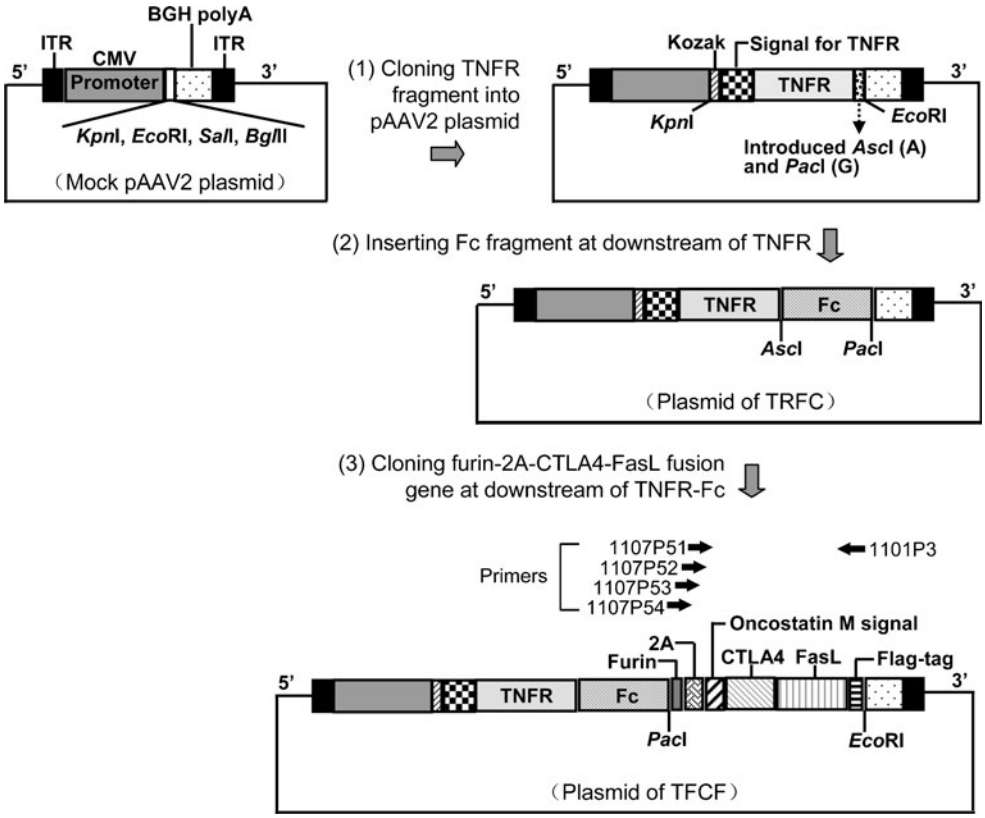

Fig. 1 Construction of recombinant adeno-associated virus (*AAV*) plasmids. Firstly, the synthesized construct of '*Kpn*I–Kozak sequence-start coden (ATG)-native signal peptide of TNFR–TNFR extracellular domain-*Asc*I with an appended nucleotide A-*Pac*I with an appended nucleotide G-stop coden (TGA)-*Eco*RI′ was cloned into pAAV2-neo plasmid by *Kpn*I and *Eco*RI. Subsequently, the synthesized rat IgG Fc fragment was inserted downstream of TNFR by sites of *Asc*I and *Pac*I. This expression vector of TNFR-Fc was termed TRFC. The furin and 2A sequences were introduced at N-terminal of oncostatin M signal peptide, by a couple of extension PCR reactions using CTFA (pAAV2/signal-CTLA4-FasL, which had been constructed previously) as the template, and the recombinant construct was inserted into TRFC at downstream of Fc fragment by *Pac*I and *Eco*RI. This expression vector of double fusion molecules of TNFR-Fc and CTLA4-FasL was named TFCF

Table 1 Primers used in PCR reaction for gene cloning and construction

Primers	Sequences (5′–3′)
1107P51	GACGTCGAGTCCAACCCTGGGCCCATGGGGGTACTG
1107P52	TTTGACCTTCTCAAGTTGGCGGGAGACGTCGAGTCC
1107P53	GCACCGGTGAAACAGACTTTGAATTTTGACCTTCTC
1107P54	CC*TTAATTAA*GAGGGCCAAGAGGGCACCGGTGAAA (*Pac*I)
1101P	CCG*GAATTC*TTATCACTTATCGTCGTCATC (*Eco*RI)
TR1	GGGTCGCGCTGGTCGTCGAAC
TR2	CATTGAACCAAGCATCACGGGT
Fc1	GTGCCCAGAAACTGTGGAGGT
Fc2	AGTCTCTCCCACTCTCCGGGT
F2ACF1	AGGGCCAAGAGGGCACCGGTG
F2ACF2	GGCTTATATAAGCTTGACTACAAG
Total1	ACCGCCGCCACCATGGCGCCC
Total2	GCTTGACTACAAGGATGACGA
GAP5	CGGTGTCAACGGATTTGGC
GAP3	CCATGCCAGTGAGCTTCCC

TNFα neutralizing activity in vitro

Mouse fibrosarcoma L929 cells, susceptible to TNFα cytotoxicity, were seeded in 96-well microplates at 2×10^4 cells/well in 100 μl DMEM containing 10 % FBS. After 24 h, recombinant rat TNFα was added at serial final concentrations (0.0625, 0.25, 1, 4 ng/ml) to 100-μl culture supernatants of HEK 293 T cells transfected with plasmids of TFCF, TRFC, CTFA and PGFP, respectively, with four repeats of each concentration. In parallel, TNFα was pre-incubated at fixed concentration (1 ng/ml) with serial different volumes (12.5, 25, 50, 100 μl) of culture supernatants from transfected 293 T cells, adding additional DMEM medium to make the total volume 100 μl. After pre-incubation at 37 °C for 1 h, Act-D (1 μg/ml) was added and the mixtures were added to each well to replace the original culture medium. After incubation for 24 h, CCK-8 solution (10 μl/well) was added to each well and incubated at 37 °C for 2 h. The absorbance at 450 nm (A_{450}) was measured using a microplate reader. The mean A_{450} was used to obtain the cell viability proliferation rate (%) according to the following formula: $[A_s-A_b]/[A_c-A_b]\times100$ %. As represents the mean A_{450} read from the wells with cells, CCK-8 solution and mixtures of TNFα, Act-D and the detected supernatants; A_c denotes the mean A_{450} read from the control wells (without cytotoxicity) with cells, CCK-8 solution, Act-D but without TNFα; A_b is the mean A_{450} read from the blank wells only with DMEM, Act-D, CCK-8 solution but without cells and TNFα.

Flow cytometry

Daudi or Jurkat cells (8×10^5) were collected and incubated with 1.8 ml of transfected 293 T cell supernatants in a 2-ml tube, rotating on a rotate device at 4 °C for 45 min with 20 rpm. The cells were pelleted, washed twice with PBS and incubated on ice for 30 min with 10 μg/ml of rabbit anti-FasL, or goat anti-CTLA4, using rabbit IgG1 or goat IgG1 as the isotype control. Cells were then incubated on ice for 30 min with FITC-conjugated goat anti-rabbit IgG (H+L) or FITC-conjugated rabbit anti-goat IgG (H+L). Following washes, FITC-labeled cells were run and analyzed on a BD FACSCalibur (BD Biosciences).

Preparation and identification of recombinant adeno-associated virus vectors

Recombinant adeno-associated virus serotype 2 (rAAV2) vectors including rAAV.CTLA4-FasL (designated as rAAV.CTFA in this study) and rAAV.EGFP were produced previously (Zhang et al. 2012a). To exclude possible inherent interferences, a flag tag was introduced at C terminus of CTLA4-FasL for detection of its expression in vivo (Zhang et al. 2012a). Here, to exclude inherent interferences of TNFR-Fc in vivo, the His-tagged TFCF and TRFC constructs with 6× histidine residues at the C terminus of Fc fragment were constructed for production of virus vectors. In this study, rAAV.TFCF and rAAV.TRFC were prepared according to the method as described previously (Zhang et al. 2012a). Viral titer of the purified viral vectors was determined by dot blot analysis with manipulative details same as Fang's report (Fang et al. 2005), using a dilution series of corresponding vector plasmid DNA of known copy number as the standards.

For further identification of the presence of recombinant TFCF cassette in the purified rAAV.TFCF, 5×10^5 viral genomes (vg) of virus vectors were treated with proteinase K (0.1 mg/ml) for 1 h in 55 °C water bath followed by boiling for 10 min to liberate the rAAV genomes. After centrifugation, 5 μl of supernatant was used as the template to amplify the target genes with a serial of primer pairs of TR1+TR2 (for TNFR fragment), Fc1+Fc2 (for Fc fragment), F2ACF1+F2ACF2 (for furin-2A–CTLA4-FasL fragment) and Total1+Total2 (for total TNFR-Fc–furin-2A–CTLA4-FasL). The sequences of those primers are listed in Table 1.

rAAV vector-mediated gene expression in vivo

Pathogen-free female Lewis rats (weight 100–120 g) were purchased from Vital River Laboratories. All rats were housed under pathogen-free conditions and treated according to the Institutional Animal Care and Use Committee guidelines and approval of Beijing Institute of Basic Medical Sciences. The AIA model was induced on day 0 by a single subcutaneous injection at the base of the tail of rats with 200 μl (5 mg/ml) of fresh heat-killed mycobacterium tuberculosis (MT) H37Ra (Difco Laboratories) in sterile mineral oil (Sigma-Aldrich). On the next day, rats were injected with 5×10^{10} vg of rAAV vector in right ankle joints in a total volume of 50 μl saline.

Ankle joints were collected after rats were sacrificed on day 25 post immunization 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 5 μg RNA was reverse transcribed according to the instruction manual. RT–PCR amplification mixtures (50 μl) contained 25 ng template cDNA and 250 nM corresponding primers listed above. GAPDH was used as an internal reference gene control using primers of GAP5 and GAP3 (Table 1).

The ankle homogenates were isolated from crushed joints by adding 2 ml of lysis buffer (20 mM HEPES, 0.5 M 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 subjected to Western blot analysis with anti-His (Sigma-Aldrich) or anti-

Flag (Sigma-Aldrich), respectively. β-Actin was used as an internal reference protein control.

Results

Construction of recombinant expression plasmids

As reported previously, CTLA4-FasL fusion gene was constructed using a series of primers by PCR and overlap PCR, and cloned into pAAV2-neo plasmid (the recombinant plasmid was named CTFA in this study). According to the analysis of sequences of pAAV2-neo vector and all inserted target molecules, CTLA4 cDNA contains *Kpn*I site and TNFR cDNA contains *Sal*I and *Bgl*II sites, making it desirable to insert TNFR gene firstly into pAAV2-neo plasmid. *Asc*I and *Pac*I sites were introduced at C terminus of TNFR for insertation of downstream fragments of Fc and CTLA4-FasL construct. After inserting the IgG Fc fragment into the C terminus of TNFR, the TNFR-Fc expression gene was completely cloned into pAAV2-neo plasmid downstream of CMV major immediate-early gene promoter, followed by bovine growth hormone (BGH) polyadenylation (poly A) signal, flanked at each end by the AAV2 145-bp inverted terminal repeats (ITRs) (Fig. 1). This recombinant expression plasmid of rat TNFR-Fc fusion molecule was designated as TRFC for convenient description. After introducing furin-2A fusion sequence at N terminus of CTLA4-FasL, the construct was cloned into TRFC at 3′ end of TNFR-Fc, and this final version of double fusion molecules in a single ORF was termed TFCF. Figure 1 summarizes the assembly of TNFR-Fc with CTLA4-FasL, along with furin-2A sequence between them and respective signals at their N termini. All recombinant sequences were confirmed by DNA sequencing.

Previous study (Zhang et al. 2012a) showed that CTLA4-FasL fusion protein could be secretorily expressed in the supernatants of 293 T cells transfected with CTFA. The secretory expression of TNFR-Fc is expected to be obtained in the supernatants of TFCF transfectants. To attain independent and simultaneous expression of TNFR-Fc and CTLA4-FasL from a same ORF, furin cleavage site and FMDV 2A self-processing sequence were introduced between the two fusion molecules. Since the 2A self-processing cleavage occurs between the last two amino acid residues at the C terminus of the 2A peptide, the upstream protein, TNFR-Fc, has 23 additional amino acid residues at its C terminus. To eliminate possible adverse effects caused by the remaining 2A residues, a furin cleavage sequence was introduced between the 2A sequence and TNFR-Fc, which may result in only two additional residues (RA) attached to Fc end. In this case, it might express TNFR-Fc and CTLA4-FasL independently from a single vector with only one additional residue (P) at N terminus of CTLA4-FasL and two residues (RA) at C terminus of TNFR-Fc, mediated by furin and 2A cleavage sites (Fig. 2).

Furin-2A-mediated TNFR-Fc and CTLA4-FasL expressions from a single ORF

All plasmids were transiently transfected into 293 T cells, and the expressions of target proteins TNFR-Fc or CTLA4-FasL, in the serum free media were determined by ELISA assay 48 h after transfection. The plates were coated with rat TNFα and subsequently incubated with transfected cell supernatants, followed by detection with anti-TNFR or anti-IgG Fc antibody respective. Samples from TFCF and TRFC transfectants were both obviously positive in above assays (Fig. 3a), suggesting that the upstream TNFR-Fc fusion molecule can be well expressed in TFCF plasmid. Double-antibody sandwich ELISA was used to detect the expression of target protein CTLA4-FasL. After coating with anti-CTLA4, the plates were incubated with transfected cell supernatants followed by detection with anti-FasL and the results showed that the samples from TFCF and CTFA transfectants were apparently positive (Fig. 3b, left panel). The parallel experiments using anti-FasL as the coated antibody and anti-CTLA4 as the detection antibody showed similarly positive results (Fig. 3b, right panel), suggesting that the downstream CTLA4-FasL fusion molecule can be well expressed in TFCF plasmid. These results suggest that the furin-2A fusion sequence efficiently facilitates TNFR-Fc and CTLA4-FasL expression from a single ORF. In addition, it was preliminarily determined whether TNFR-Fc and CTLA4-FasL proteins were separately expressed under mediation by furin-2A, using coated anti-CTLA4 as the capture antibody and anti-IgG Fc as the detection antibody. If the cleavages had not or insufficiently occurred with furin and 2A cleavage sites, TNFR-Fc and CTLA4-FasL would be expressed as a single community and the results would be positive in the sandwich ELISA assay using anti-CTLA4 in combination with anti-IgG Fc. The experimental results showed that the supernatant samples from TFCF transfectants were also negative as those from other transfectants (Fig. 3c), suggesting that TNFR-Fc and CTLA4-FasL proteins are supposed to be expressed separately but not as a combination.

The separated expressions of TNFR-Fc and CTLA4-FasL mediated by furin-2A sequence

To further characterize whether upstream TNFR-Fc and downstream CTLA4-FasL had been expressed separately as expected due to the usage of furin and 2A cleavage sites, the proteins in the serum-free supernatants of transiently transfected 293 T cells were separated on SDS-PAGE gels under both non-reducing and reducing conditions and subjected to Western blot analysis using a polyclonal goat

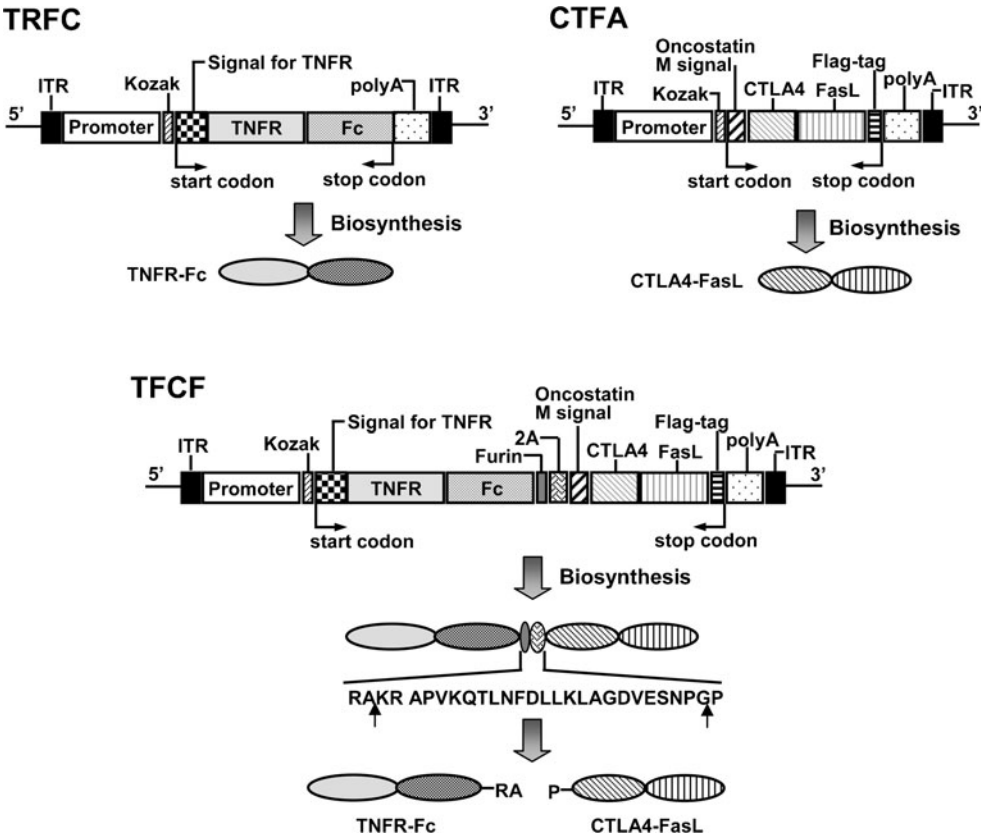

Fig. 2 Schematic illustration of the expected biosynthesis of TNFR-Fc or CTLA4-FasL alone or both by recombinant plasmids of TRFC, CTFA and TFCF, respectively. The native signals specific to TNFR and CTLA4 were used to attain the secretory expressions of TNFR-Fc and CTLA4-FasL. To obtain the separate expression of TNFR-Fc and CTLA4-FasL in a single cassette, a combination of furin cleavage site and 2A sequence was applied, which would produce the above two fusion proteins separately, without more residual amino acids derived from 2A peptide

anti-rat IgG Fc antibody. Under non-reducing condition, a prominent 130- to 140-kDa band, which is the expected size of a dimerized TNFR-Fc, along with a weaker one at 65–70 kDa corresponding to the monomer of TNFR-Fc, were detected in TFCF transfectant medium, and bands of same size were detected in the samples from parental TRFC transfectant medium (Fig. 4a, left panel). In contrast, in the presence of reducing agent, the prominent larger bands disappeared, leaving only the 65- to 70-kDa bands (Fig. 4a, right panel). As expected, no immunoreactive band was detected in medium from CTFA or PGFP transfectants (Fig. 4a). In parallel, the bands of 43–45 kDa, consistent with the molecular mass of CTLA4-FasL protein, were detected in TFCF- and CTFA-transfected supernatants with either anti-FasL (Fig. 4b, left panel) or anti-CTLA4 (Fig. 4b, right panel) under reducing condition, and no band was observed in the corresponding medium from TRFC or PGFP transfectants (Fig. 4b). In addition, if furin-2A does not efficiently work, additional protein band of 110–120 kDa (TNFR-Fc plus CTLA4-FasL and amino acid residues derived from furin and 2A sequences) is expected to be detected under reducing condition. In fact, no additional band like this was detected using anti-IgG Fc or anti-FasL or anti-CTLA4 (Fig. 4), suggesting that efficient and complete cleavages had happened at the furin and 2A cleavage sites.

Biological activities of TNFR-Fc and CTLA4-FasL expressed from furin-2A plasmid

An in vitro TNFα inhibition assay was performed using TNFα-sensitive mouse fibrosarcoma L929 cells to determine the neutralizing capacity of TNFR-Fc expressed by TFCF, using TRFC which expresses parent TNFR-Fc protein as the positive control, and CTFA (expressing parent CTLA4-FasL) and PGFP as the negative controls. Firstly, the inhibitory effect of 48-h transfected cell supernatants (100 μl) on rat TNFα cytotoxicity was observed with serial concentrations of TNFα. At each concentration, inhibition of TNF-mediated killing by soluble rat TNFR-Fc proteins expressed in the transfected supernatants of TFCF and TRFC, correlates with significantly increased viabilities as compared to those of CTFA and PGFP (Fig. 5a). Pre-incubation of TNFα, at concentration of 0.0625 ng/ml, with the transfected supernatants of TFCF and TRFC, increases the cell viability from about 40 % (CTFA, 39.95 %; PGFP, 38.02 %) to about 90 % (TFCF, 89.68 %; TRFC, 97.55 %) (Fig. 5a). The similar result was observed when the concentration of TNFα was set at 0.25 ng/ml (TFCF, 90.03 %; TRFC, 87.41 %; CTFA, 37.06 %; PGFP, 44.21 %) (Fig. 5a). There was an obvious increase in cell viability, from about 20 % to 70 % or more, when the TNFα concentration was fixed at 1 ng/ml (TFCF, 91.65 %; TRFC, 84.27 %; CTFA, 17.66 %; PGFP, 26.92 %)

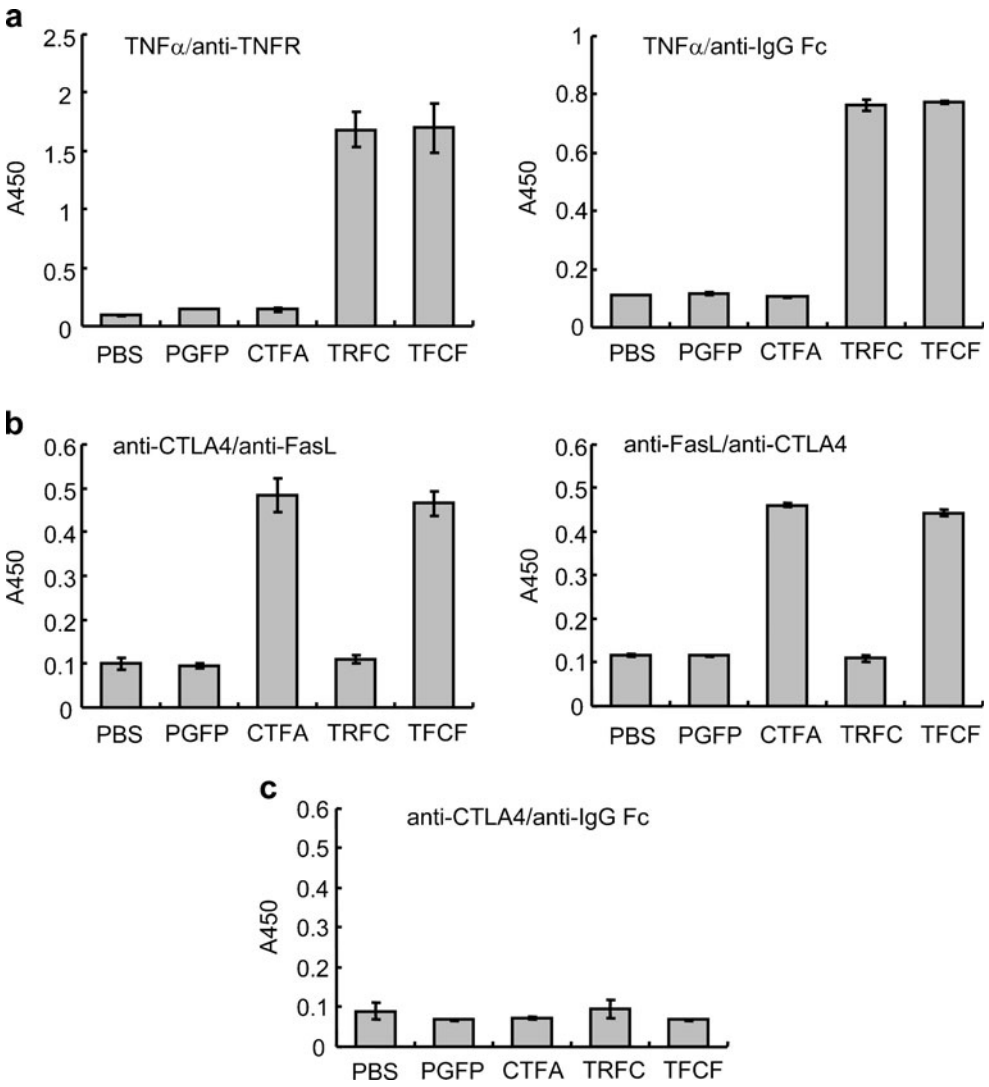

Fig. 3 ELISA assay of the in vitro expression of TNFR-Fc and CTLA4-FasL mediated by furin and 2A sequences in a single ORF. HEK 293 T cells were transiently transfected with recombinant plasmids and the supernatants were harvested at 48 h after transfection for protein analyses. **a** Indirect ELISA analysis of supernatants from 293 T cells transfected with TFCF, TRFC (positive control), CTFA or PGFP (negative control) plasmids by coating with rat TNFα protein and detecting with anti-TNFR and corresponding second antibody (*left panel*) or peroxidase-conjugated anti-IgG Fc (*right panel*). **b** Sandwich ELISA analysis of supernatants from 293 T cells transfected with TFCF, CTFA (positive control), TRFC, PGFP (negative control) plasmids by coating with anti-CTLA4 and detecting with anti-FasL and its second antibody (*left panel*) or by coating with anti-FasL and detecting with anti-CTLA4 and its second antibody (*right panel*). **c** Sandwich ELISA analysis by coating with anti-CTLA4 and detecting with anti-IgG Fc (peroxidase conjugate) to exclude the possibility of the expression of TNFR-Fc and CTLA4-FasL as a combination

or 4 ng/ml (TFCF, 69.06 %; TRFC, 70.28 %; CTFA, 16.43 %; PGFP, 22.73 %) (Fig. 5a). In parallel, pre-incubation of different volumes of TFCF- and TRFC-transfected supernatants with settled concentration of 1 ng/ml of TNFα, also achieved marked suppression of TNFα cytotoxicity to L929 cells compared with CTFA- and PGFP-transfected supernatants by increasing the cell viability from 20–30 % to 70–90 % (Fig. 5b). A dose-dependent manner was displayed in this assay that the higher cell viability was related with the more volume of TFCF- or TRFC-transfected supernatants (Fig. 5b), resulting from the neutralization of TNFR-Fc in the supernatants to TNFα cytotoxicity. These results suggest that the TNFR-Fc fusion product expressed from the furin-2A plasmid, TFCF, is biologically active as a TNFα antagonist similar to its parent control.

The binding activity to B7 ligand of CTLA4-FasL protein was observed in $B7^{+}/Fas^{-}$ Daudi cells. The cells were incubated with different transfected supernatants and detected by indirect immunofluorescence with anti-FasL antibody. The TFCF-transfected supernatant exhibited a high positive staining ratio of 58.89 %, similar to its parent sample from CTFA (Fig. 6a). In parallel, the binding activity to Fas receptor of CTLA4-FasL was observed in $Fas^{+}/B7^{-}$ Jurkat cells with anti-CTLA4 antibody, and the positive staining ratio of TFCF-transfected supernatant was almost same to its parent sample of CTFA (Fig. 6b). These findings showed that CTLA4-FasL fusion protein expressed from the furin-2A containing plasmid TFCF, could bind to both Fas receptor and B7 ligand with respective domain, similar to its parent protein.

The above observations indicate that the double fusion proteins of TNFR-Fc and CTLA4-FasL expressed from the single recombinant AAV vector, regulating by furin cleavage site and 2A self-processing sequence, have desirably retained their biological activities.

Furin-2A-mediated TNFR-Fc and CTLA4-FasL expressions in vivo

To determine that the obtained rAAV vectors harbor target genes, the vectors were treated with proteinase K followed

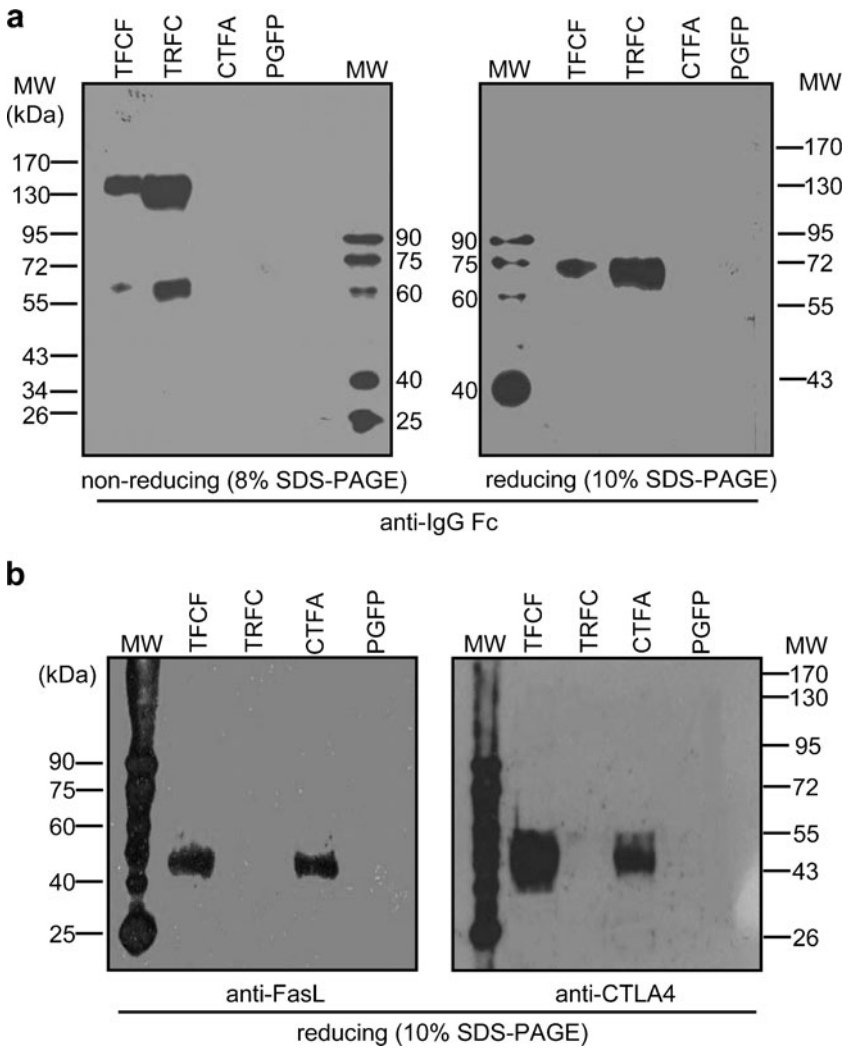

Fig. 4 In vitro expression of TNFR-Fc and CTLA4-FasL using furin-2A sequence-containing expression plasmid. HEK 293 T cells were transiently transfected with different plasmids and the supernatants were harvested for Western blot analyses. **a** Western blot analysis of TNFR-Fc containing supernatants under non-reducing (*left panel*) and reducing (*right panel*) conditions with anti-IgG Fc (peroxidase conjugate). **b** Western blot analysis of CTLA4-FasL containing supernatants under reducing conditions with anti-FasL (*left panel*) or anti-CTLA4 (*right panel*) and corresponding peroxidase-conjugated secondary antibodies

by boiling to remove viral coat proteins and liberate viral DNA. A series of group of primer pairs were used to amplify the corresponding fragments in TFCF cassette including TNFR, Fc and CTLA4-FasL. As shown in Fig. 7a, the bands with expected sizes were recovered from the gel and confirmed by sequencing. After the intra-articular injection of the rAAV2 vectors, TNFR-Fc–furin-2A–CTLA4-FasL RNAs were found in the ankle joints treated with rAAV.TFCF but not in the control rAAV.EGFP-treated joints (n=3 observations, Fig. 7b). The other rAAV vectors including rAAV.TRFC and rAAV.CTFA were also checked by means of these methods (data not shown). To measure that whether furin cleavage site and 2A sequence are able to regulate separate expressions of TNFR-Fc and CTLA4-FasL from a single rAAV vector in vivo under inflammatory conditions, we examined the according expressions of the two fusion proteins using joint lysates by immunoblot analysis, with anti-His for his-tagged TNFR-Fc and anti-Flag for flag-tagged CTLA4-FasL. The results showed that the detected band of his-tagged or flag-tagged protein derived from rAAV.TFCF-treated joint lysates was of same size with that from rAAV.TRFC- or rAAV.CTFA-treated joint lysates (Fig. 7c), suggesting that both of furin cleavage site and 2A sequence have mediated the separate expressions of upstream TNFR-Fc and downstream CTLA4-FasL, since if only one or neither of furin cleavage site and 2A sequence works, the molecule weight of the band from rAAV.TFCF-treated joint lysates is at least 2.5–3.0 kDa more than that from rAAV.TRFC- or rAAV.CTFA-treated samples.

Discussion

This study demonstrates that the combination of furin cleavage site and FMDV 2A self-processing peptide facilitates the efficient and independent expression of two fusion proteins of TNFR-Fc and CTLA4-FasL in vitro and in vivo from a single ORF, which both exert antiarthritic effects as reported in studies conducted by other groups and by our team. To our knowledge, no one else has shown that two

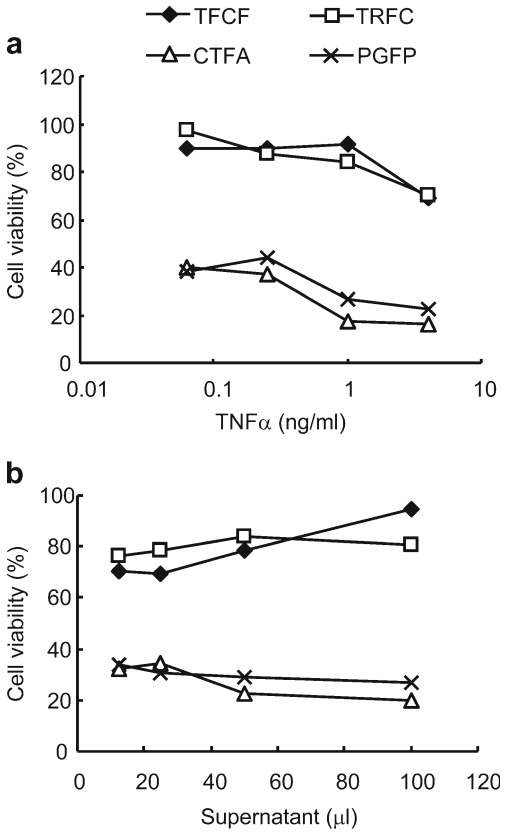

Fig. 5 The neutralization effect of TNFR-Fc, expressed under the mediation by furin-2A sequence, on rat TNFα cytotoxicity to L929 cells. The cells were seeded at 2×10^4 per well in 96-well cell culture plates. 24 h later, a serial of concentrations (0.0625, 0.25, 1, 4 ng/ml) of rat TNFα were pre-incubated with 100 μl of transfected supernatants at 37 °C for 1 h (**a**), or a serial of volumes of transfected supernatants (12.5, 25, 50, 100 μl) were pre-incubated with 1 ng/ml of TNFα at 37 °C for 1 h (**b**), followed by adding actinomycin D (*Act-D*) at 1 μg/ml and then adding the mixtures to the plates. After incubating the plates at 37 °C for 24 h, CCK-8 solution (10 μl/well) was added and incubated for 2 h. The absorbance at 450 nm was measured and the cell viability was obtained according to the formula described in Materials and methods. Assays were performed in four repeats of each condition in 96-well plate

functional molecules are fused in one gene transfer vector for RA treatment, providing biological activities as separate products similar to their parent parts.

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. CTLA4-FasL fusion protein was once designed as a 'trans signal converter protein', in which the extracellular domains of CTLA4 and FasL were linked in-frame (Huang and Tykocinski 2001). It was reported as a bifunctional inhibitory molecule to combine both costimulator blocking with CTLA4 domain and trans inhibitory signaling with FasL domain within a single protein (Huang and Tykocinski 2001; Elhalel et al. 2003; Shi et al. 2007). Therefore, CTLA4–FasL exerts dual inhibition in T cells via both costimulatory and inhibitory pathways. In our previous studies, we have firstly demonstrated that an AAV-mediated CTLA4-FasL gene transfer efficiently prevents 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 (Zhang et al. 2012a), and CTLA4-FasL is superior to mature FasL alone in blocking the progress of arthritis due to the adjunctive role of CTLA4 (Zhang et al. 2012b).

Biological therapies, especially those targeting TNFα have gained an undoubted success in RA treatment. However, a significant proportion of patients with RA do not respond to TNFα blockade, and therefore there is a compelling need to continue identification of alternative or additional therapeutic strategies for RA. Moreover, in view of the complexity of RA pathogenesis, combined blockade of functionally linked and relevant multiple targets has become an attractive therapeutic strategy and in particular, the combination treatment with T-cell targeting and TNFα inhibition is of potential interest (Williams et al. 1994, 1995, 2000; Dépis et al. 2012). Those previous reports have suggested that targeting T-cell- in combination with TNF-mediated inflammatory responses may provide greater efficacy than antagonizing TNFα alone in patients with RA. Hence, an approach combining TNFR-Fc, an agent for TNFα inhibition, with CTLA4-FasL, a potent inhibitor of T cells might be useful.

All previous studies on combination therapy with anti-TNF and anti-T-cell have used different antibodies against TNF and CD3 (Dépis et al. 2012) or CD4 (Williams et al. 1994, 1995, 2000). However, considering the side effects resulted from high circulating levels of therapeutic proteins produced in the human system, combination therapy using antibodies or proteins is not beneficial. For example, in a combination therapy in the treatment of RA patients, anti-TNF (etanercept) plus anti-IL-1 (anakinra) produced an increased safety risk of serious infections (Genovese et al. 2004). In addition, recombinant proteins including antibodies against TNFα used in the clinic have a limited half-life, requiring repeated and expensive high dosages to achieve therapeutic concentrations in the joints. Therefore, combination therapy with dual antibodies or proteins is not recommended for the treatment of patients with RA.

An attractive alternative for systemic protein delivery is local gene therapy (Adriaansen et al. 2006; Woods et al. 2008). In regard to viral vectors, AAV seems to be the most promising for future studies and use in clinics (Adriaansen et al. 2006; Woods et al. 2008; Evans et al. 2009). Several different AAV serotypes have been identified with AAV2 being the prototype. 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 desirably

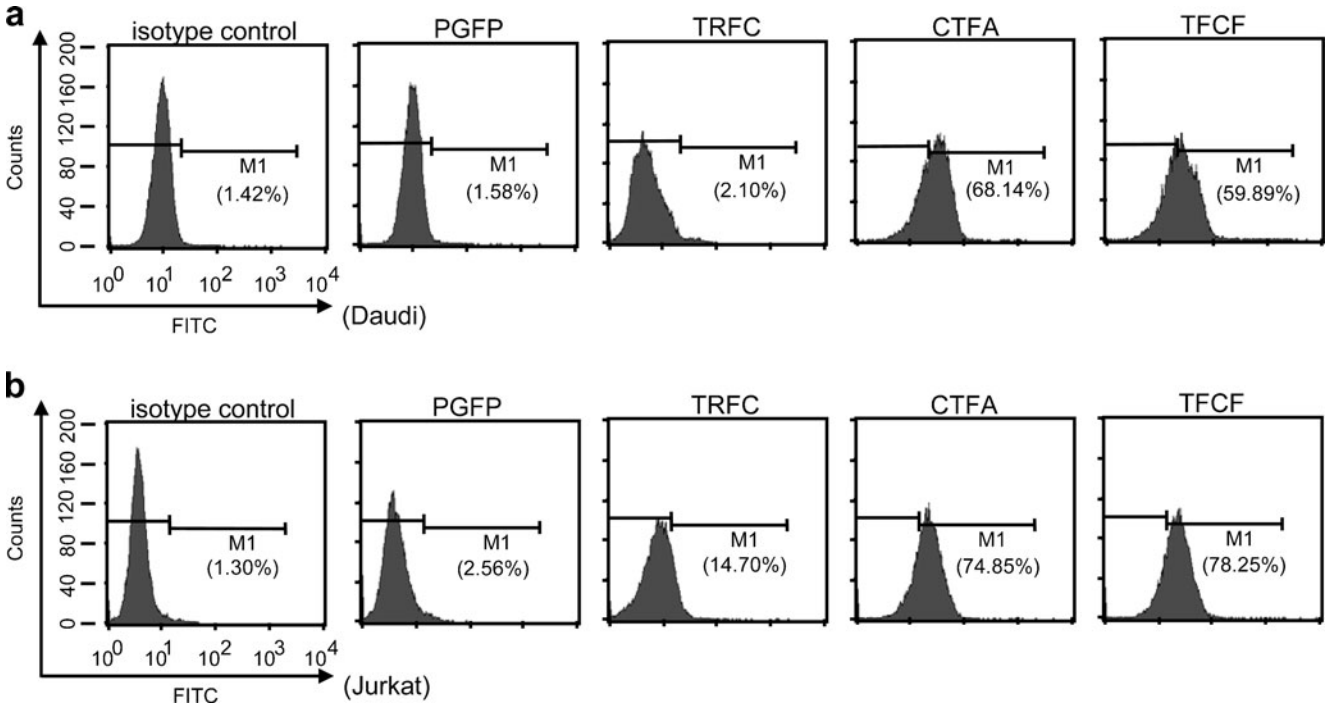

Fig. 6 The biological activities of CTLA4-FasL expressed under the mediation by furin-2A sequence for binding to $B7^+$ and Fas^+ cells. **a** $B7^+$ Daudi cells were incubated with the transfected-293 T cell supernatants and stained with 10 μg/ml of rabbit anti-FasL or isotype-matched control (rabbit IgG), and FITC-conjugated goat anti-rabbit IgG. **b** Fas^+ Jurkat cells were incubated with the transfected supernatants and stained with 10 μg/ml of goat anti-CTLA4 or isotype-matched control (goat IgG), and FITC-conjugated rabbit anti-goat IgG

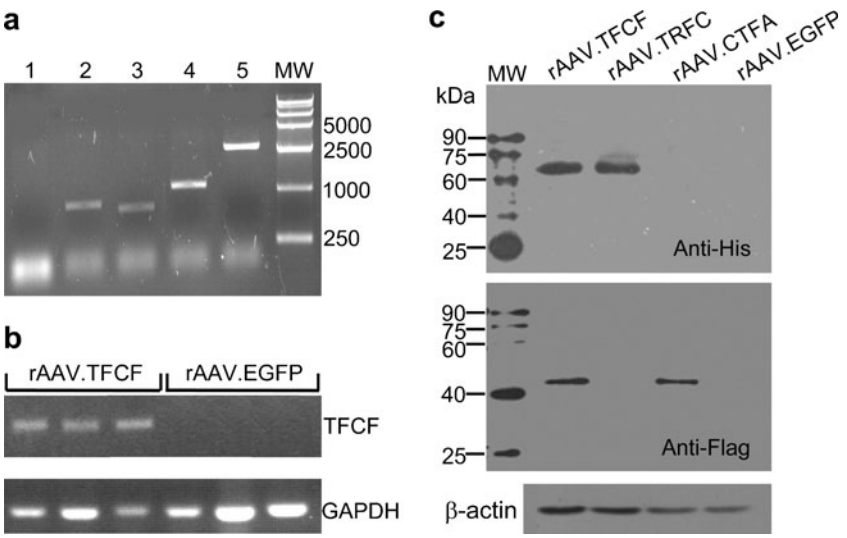

Fig. 7 Expressions of TNFR-Fc and CTLA4-FasL in vivo by rAAV vector-mediated gene transfer. **a** Identification of recombinant gene present in obtained rAAV vector. The vector of rAAV.TFCF was treated with proteinase K and boiling to liberate viral DNA, which was subjected to PCR amplification with a group of primers (in Materials and methods) for different fragments. *Lane 1* the negative control for lane 5 using rAAV.EGFP DNA as the template, *lane 2* TNFR fragment (750 bp), *lane 3* rat IgG Fc fragment (690 bp), *lane 4* furin-2A–CTLA4-FasL fragment (1,080 bp), *lane 5* TNFR-Fc–furin-2A–CTLA4-FasL fragment (2570 bp). **b** The mRNA expression of TFCF cassette in arthritic joints injected with rAAV.TFCF. Ankle joints injected with rAAV.TFCF were homogenized in Trizol reagent and subjected to RT-PCR reaction for amplification of TNFR-Fc–furin-2A–CTLA4-FasL full-length gene. **c** The protein expressions of TNFR-Fc and CTLA4-FasL in vivo. Ankle joints injected with rAAV.TFCF were homogenized in lysis buffer and subjected to Western blot analysis with anti-His or anti-Flag antibody under reducing conditions

achieved with rAAV2 vector. Most cells in the joint are found to be transduced by rAAV2, including synoviocytes and chondrocytes (Pan et al. 1999, 2000). Also desirable, rAAV preferentially transduces arthritic joint cells in vivo (Goater et al. 2000). These properties make AAV an especially desirable tool for use in gene therapy of RA. Intra-articular 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 (Adriaansen et al. 2006; Woods et al. 2008). In this study, we aim to establish a rAAV2 gene transfer system to simultaneously obtain separate expression of anti-TNF agent (TNFR-Fc) and anti-T-cell agent (CTLA4-FasL) in a single rAAV2 vector.

IRES is a traditional approach to express dual proteins from a single mRNA in a bi-cistronic setup. However, the translation initiation efficiency of IRES elements usually is significantly lower than that of the 5′-cap of the mRNA, frequently leading to an excess of translation product from the upstream cistron (Kaufman et al. 1991) and a substantially low expression of the downstream gene (Mizuguchi et al. 2000). Studies using mutant IRES elements with different translation initiation efficiencies identified that IRES element affects overall expression level of the downstream cistron (Li et al. 2007). Alternatively, a novel technology using furin cleavage site and 2A self-cleavage sequence has been described to allow balanced coexpression of antibody heavy and light chains from a single mRNA within a single ORF. 2A sequences are oligopeptides located between the P1 and P2 proteins in some members of the picornavirus family and can undergo self-cleavage to generate the mature viral proteins P1 and P2. Among various 2A or 2A-like sequences, FMDV 2A is particularly short and is able to cleave at its own C terminus between the last two amino acids through an enzyme-independent but undefined mechanism, probably by ribosomal skip, during protein translation (Ryan and Drew 1994; Donnelly et al. 1997; Donnelly et al. 2001; de Felipe et al. 2003; Szymczak et al. 2004; de Felipe and Ryan 2004). It has been described that use of 2A self-processing sequence allowed the construction of a single rAAV vector that contains the entire mAb expression cassette capable of efficient mAb expression at high levels with a balanced heavy (upstream) and light (downstream) chain ratio (Fang et al. 2005). And importantly, the 2A sequence peptide derived from foot and mouth disease virus did not elicit 2A-specific immune responses in immunocompetent animals following gene transfer of rAAV in vivo (Fang et al. 2005). Since the 2A self-processing cleavage occurs between the last two amino acid residues at the C terminus of 2A peptide, the upstream protein will retain 23 additional amino acid residues at its C terminus, which might affect the activity of the upstream protein. To eliminate possible adverse effects caused by the remaining residues derived from 2A peptide, a furin cleavage site sequence (RAKR) was designed and introduced upstream next to 2A sequence to facilitate the removal of 2A peptide-derived amino acids from the first protein during protein secretion. Furin is a ubiquitous transmembrane enzyme found in all vertebrate cells, known for its efficient processing of pre-prohormones or other pre-proteins. It functions as a catalyst in many cellular events and plays a role in many diseases and infections (Thomas 2002).

It is expected that the expression of our furin-2A construct would produce both upstream TNFR-Fc and downstream CTLA4-FasL proteins, with one amino acid residue in the 2A linker adding to the CTLA4-FasL protein by 2A self-processing, and two amino acids in the furin linker adding to the TNFR-Fc protein by furin cleavage. These residual amino acids hardly altered the molecular weights and activities of TNFR-Fc and CTLA4-FasL. In this study, by Western blot analysis for the size of target proteins expressed in vitro and in vivo, it was demonstrated that both 2A-mediated cleavage pathway to separate the upstream and downstream proteins, and furin-catalyzed proteolysis and carboxypeptidase activity to remove the remaining amino acids of 2A, are functioning in vitro and in vivo, and that both cleavages are complete. TNFα neutralization assay and cell-binding activity assay showed that TNFR-Fc and CTLA4-FasL proteins expressed from furin-2A construct displayed the same biological activities as their parent counterparts.

To avoid immunological rejection of human products in rats in future animal experiments, we cloned and constructed rat CTLA4-FasL and rat TNFR-Fc fusion genes with respective signal peptide at the N terminus to obtain secretory expression. It is controversial regarding whether the leading signal peptide is necessary for the second protein to enter into the endoplasmic reticulum (ER) for protein secretion. The lack of signal peptide at N terminus of the second protein resulted in cytosolic localization of the protein in yeast (de Felipe et al. 2003). In contrast, in mammalian cells, the second protein can enter into ER without a signal peptide (de Felipe and Ryan 2004). Considering the usage of the signal peptide for antibody light chain to mediate the secreted expression of the second (downstream) protein in other studies (Fang et al. 2005; Jostock et al. 2010) and since the signal peptide is cleaved during protein secretion, the downstream CTLA4-FasL gene in our TFCF construct retains the native signal sequence at its N terminus. The CTLA4-FasL protein secretorily expressed from this cassette is of the same molecular weight with that expressed from the parent construct, CTFA, suggesting that it is suitable to introduce the native signal peptide for the secretory expression of the downstream protein. Another consideration for use of rat TNFR-Fc gene was derived from our preliminary research which

demonstrated that human TNFR-Fc could not neutralize the cytotoxicity of rat TNFα (data not shown here), consistent with a previous report in which human TNFR-Fc protein did not inactivate rat TNFα, suggesting that it is not feasible to evaluate the effect of TNFR-Fc from humans in a future study with the rat arthritis model.

According to the analysis of sequences from AAV2 vector and all inserted elements, it was designed to clone the TNFR gene into the vector first and then simultaneously introduce two enzyme sequences, *Asc*I and *Pac*I, which were once used in a previous cloning method (Zhang et al. 2007), followed by cloning of IgG Fc fragment and CTLA4-FasL cassette as mentioned in Materials and methods and Results sections. Now that the furin-2A-regulated expression system has been identified to mediate the balanced expression of the first and second proteins, it should not matter which gene is placed on the upstream or downstream. This study provides a desirable tool and modality of a single gene transfer for the further study of the synergic effect of the combination treatment for RA with simultaneously counteracting TNF and T-cell in vivo.

In summary, we used the furin-2A fusion sequence to mediate TNFR-Fc and CTLA4-FasL expression from a single ORF, which enabled us to construct a single rAAV vector for the production of two desirable arthritis-inhibitory fusion molecules. This expression system presents a feasible gene therapy approach for long-term delivery of TNFα antagonist and T-cell antagonist in vivo. Furthermore, this strategy for expression of two desirably antiarthritic molecules in a single rAAV vector in vivo will make vector manufacturing possible for human use in the treatment of RA.

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Conflict of interest The authors declare that they have no conflict of interest.

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